# Side-specific odor representation in alpha-lobe extrinsic neurons

Martin Fritz Strube-Bloss, Martin Paul Nawrot and Randolf Menzel

## Abstract

Side-specific conditioning experiments were performed. During the 5-hour experimental procedure, we extra-cellularly recorded the activity of single mushroom body-extrinsic neurons [ENs]. During the pre- and the post-conditioning phases, two odors were presented 10 times, separately, on each antenna. The differential conditioning of the odors, in which one odor was presented reinforced (CS+) and the other non-reinforced (CS-), was always performed on the side contralateral to the recording position. The PE1 neuron was identified on the basis of its firing pattern and excluded from our analysis in order to focus on non-PE1 neurons. Already during acquisition we found activity changes in the contralateral ENs, which resulted - after a three-hour rest - in a stable and reliable representation of the different side-specific stimuli. We were able to show that after contralateral conditioning the same odor tested on the different antenna evoked different activity patterns in the recorded ENs, which shows that the laterality of the odor stimulus is represented after learning on the level of the Mushroom body [MB]-extrinsic neurons which form the output of the MBs.

## Introduction

Bilateral symmetric organization of sensory systems is a widespread phenomenon that allows better integration of information from the surroundings. In principal, the information received at the two input sides is slightly different. This difference can be used by the brain to add an accessorial dimension, meaning that the two brain sides have to collaborate to construct the environmental representation. The

honeybee brain is organized in a bilateral, symmetric manner up to the higher-order integration centers, the mushroom bodies [MBs] (Mobbs et al., 1982). They are involved in the regulation of motor actions like walking behavior (Martin and Heisenberg, 1998), and since they were discovered by Dujardin they have been described as learning and memory centers or centers for intelligent actions (Dujardin, 1850; for review, Strausfeld, 1998). 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 antenna (Menzel et al. 1974). After a retention period this association is also retrievable from the other brain side (Sandoz and Menzel, 2001). Thus, in bees both phenomena exist: side-specific and bilateral learning. As the integration of the olfactory information of both hemispheres seems to be time-dependent it may depend on consolidation phenomena. Consolidation time seemed to be not the only prerequisite for integrating the information from both antennae; the complexity of the learning task may also play an important role. This is the case, for instance, in non-elemental learning tasks like negative patterning (A+B+AB-), where two single olfactory components are reinforced if they appear alone, but the compound of both is non-reinforced, a task solved by honeybees (Deisig et al., 2001). Negative patterning was shown to need the input to both brain sides (Komischke et al., 2003), whereas for more elemental tasks like positive patterning (A-B-AB+) the processing into one hemisphere seems to be sufficient (Komischke et al., 2003). Furthermore, bees with an ablated MB are not able to solve side-spanning learning tasks, but they learn to solve differential conditioning (Komischke et al., 2005). In addition, the phenomenon of blocking between odorants in binary mixtures after pre-exposure to one element of the mixture (Smith and Cobey, 1994) is dependent on input from both antennae. Removing the input from one antenna eliminates the blocking of one odor by the other. (Thorn and Smith, 1997)

The neuronal correlates of interhemispheric processing involved at the different stages of the olfactory pathway are unknown. There is, however, considerable evidence that the alpha lobe-extrinsic neurons [ENs] that read out the MB information (Rybak and Menzel, 1993) show learning-dependent plasticity. Thus electrical stimulation of the Kenyon cells [KC], which are the main component of the MBs (Heisenberg, 2003), leads to the formation of associative long-term potentiation (LTP) in the pedunculus-

extrinsic neuron one [PE1] (Menzel and Manz, 2005), which is the most prominent and intensively-investigated cell extrinsic to the MB. It is also known that this neuron changed its response during classical conditioning (Mauelshagen, 1993). Extra-cellular long-term recordings also document that the PE1 shows a reduction in the response to the rewarded stimulus after a bee has associated an odor with a reward (Okada et al., 2007). Other ENs, which exit the MB via the ventral alpha lobe, were completely recruited after the honeybee had built an association between a conditioned stimulus [CS] and an unconditioned stimulus [US] (Strube-Bloss et al., 2008a, chapter 2). The projection fields of most ENs leaving the MB at the ventral part of the alpha lobe are restricted to only one protocerebral hemisphere, 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 connect both brain sides (Rybak and Menzel, 1993). Studying these neurons during side-specific learning tasks should be promising for understanding inter-hemispheric integration of olfactory information. Here we adapted a behavioral experiment by Sandoz and Menzel (2001) in which these authors could show that the unilateral differential conditioned information (A+B- on one antenna) is transferred after only 3 hours to the contralateral brain side. On this side, the bees had no experience from the previous trials; we measured the activity of single ENs during that task. We are able to show that already during the unilateral conditioning, ENs of the contralateral MB are involved in the computation of the side-specific information which leads to a stable side-specific representation of this stimulus after a 3-hour rest (consolidation) time at the level of ENs. Although the activity changes in single units after contralateral conditioning were manifold, the dominating stimulus was the side-specific CS+.

## Material and Methods

### Laboratory animals

Foraging honeybees (*Apis mellifera*) were trapped in a transparent box at the entrance of an outdoor hive 2 hours prior to training and were anesthetized on ice. The immobile bees were harnessed in metal tubes such that only the mandibles, proboscis and antennae could freely move (Bitterman et al., 1983). Two hours before starting the experiment the heads were fixed with wax onto the metal tube and the scapi of the antennae were fixed with low-melting-point wax on the head capsule. Afterwards the bees' antennae were spatially separated with a piece of transparent plastic (3x3 cm; 0.2 mm thick). The silhouette of each bee's head was cut out and gaps between the cuticle and the plastic were filled with wax. This wax was also used to fasten the plastic wall onto the head of the subject.

### Odor stimulation

A 12-channel- olfactometer was adapted from Galizia et al. (1997) and fitted with 5ml syringes (odor chambers). A constant air stream (1.5 m/s speed) was delivered through two Teflon tubes (6 mm in diameter). The needles of five syringes were inserted into each tube, so that side-specific odor stimulations could be applied. Odors were diluted in paraffin oil to a 0.01 concentration. Filter papers (2 $cm^2$) were soaked with 10 µl of odor solution and placed in the syringes. During the odor stimulation, which lasted 3 seconds, only 2.5ml of the air volume of the chambers were injected into the constant air stream to avoid concentration gradients. A Visual Basic Script (VBA 6.0, Microsoft, USA) written by Frank Schaupp was used to control the 12 fast magnetic valves (Lee, Westbrook, Connecticut) of the odor-supplying device as well as the data acquisition (timing of odor stimulation). It also provided the experimenter with auditory cues, so that he recognized the on- and the offset of the reward stimulation to assure consistent stimulation during trials and experiments.

## Experimental paradigms

### *Side-specific odor stimulation*

To investigate the involvement of alpha-lobe-extrinsic neurons in side-specific odor computation we used four different odors (eugenol, heptanal, octanal, limonene) [Sigma-Aldrich Chemie GmbH]. The outlets of the two tubes (see: *Odor stimulation)* with the constant air streams were placed 1cm from each antenna. During the 3-sec stimulation the odors were injected as described into the constant air stream of the respective tube. An exhauster hood (tube with 10 cm diameter) was placed behind the bee to remove all odor molecules. The odors were presented contralaterally and ipsilaterally to the recorded EN. Differential conditioning was always performed at the antenna contralateral to the electrode position.

### *Contralateral differential conditioning*

In each experiment two of the four odors were chosen by chance and presented separately at the ipsi- and the contralateral antenna - relative to the recording position - in a pseudorandomized way 10 times each with an inter-trial interval (ITI) of 1 min. This pre-acquisition test phase [PreAcq] was followed after 20 minutes by the acquisition phase [Acq] which was a differential conditioning procedure with the two odors (conditioned stimuli [CS]) at only one antenna with an ITI of 1 min. One of the odors was chosen to be the reinforced stimulus (CS+). This odor was presented for 3 seconds followed by an unconditioned stimulus (US, 30% sucrose solution, for 3 seconds). CS and US were overlapping by 1 second. The other odor was presented non-reinforced (CS-). CS+ and CS- were presented in a pseudorandomized way, 10 times each. After 3 hours, the post-acquisition test [PostAcq] was carried out: the two odors were again presented 10 times each again at the ipsi- and the contralateral antenna related to the recorded EN in the same way as in the PreAcq.

**Electrophysiology**

Before inserting the electrodes a small window (1.5 x1.5 mm) was cut unilaterally between the compound eye and the glued-on plastic wall. Head glands and trachea sacks above the alpha-lobe were removed and the electrode was positioned at the ventral part of the alpha-lobe at a depth between 100 and 250 µm. Following insertion the whole gap was filled with silicon (KWIK-SIL Sarasota, FL, USA) in order to prevent the brain from drying out and to solidly anchor the electrode within the brain and the head capsule. Thus the recordings could last for hours.

To monitor single-unit EN activity we inserted an electrode consisting of three closely-spaced wires (polyurethane-coated copper wire, 14 µm in diameter [Electrisola, Escholzmatt, Switzerland]) into the alpha-lobe of the brain of a honeybee. Electrodes were manufactured as described previously (Mizunami et al., 1998; Okada et al., 1999). The wires were glued together with wax onto a tungsten wire (100 µm in diameter and 1-2 cm long) that was attached to a glass capillary. The glass capillary was fixed onto an adapter that allowed us to connect the electrode with the Headstage (Headstage-27 Amplifier Neuralynx, Tucson, AZ, USA). Signals used for spike detection were measured differentially from all three electrode pair combinations using the Patch Panel (ERP-27, Neuralynx, Tucson, AZ, USA). A silver wire with a diameter of 25 µm (Nilaco, Tokyo, Japan) inserted into the right compound eye served as a ground electrode. The electric signals were amplified by a Lynx-8 Amplifier (Neuralynx, Tucson, AZ, USA) with a 1-9 kHz band-pass filter. After importing the files into Spike2 format with a sampling frequency of 20 kHz this software (Cambridge Electronic Design, Cambridge, UK) was used to apply a high-pass filter (300-10 kHz) and semi-automatic spike sorting techniques (template-matching) which allowed us to separate up to 5 individual neurons per recording.

## Response detection

### *Response detection in each single trial*

To decide **trial-by-trial** whether or not an odor presentation led to a response in the recorded unit, we focused on the frequency changes of the events of the extracted units before and directly after stimulus onset. We compared the inter-spike-interval (ISI) distribution 3 seconds before odor onset with the ISI between the first 40-450 milliseconds immediately after odor onset, as suggested by Hollander and Wolfe (1973) using the Wilcoxon rank-sum test (for details cp. methods Chapter 2). We calculated the reliability index (*RI = odor presentations that evoked a detectable response divided by the total number of odor repetitions)* from the 10 presentations of each single odor.

### *Response detection for the pooled trials of each odor*

All of the tested odors in any of the experimental phases were presented 10 times. Thus it is also possible to detect an averaged response per odor for each single unit in each experimental phase. We applied two methods for detecing a response. (1) The ten trials per odor were pooled and Peri Stimulus Histograms (PSTHs) with 50 ms bin size were produced. A response to an odor is detected if the rate response PSTH in the phasic observation window 40-450 ms after stimulus onset crossed the spontaneous PSTH at a level that was +/- 3 times above/lower than the SD of the spontaneous PSTH activity rate. (2) We pooled the ISIs from all trials in a 3 sec. window before stimulus onset and in the phasic observation window 40-450 ms after stimulus onset. We then again proceeded as in the case of single trial response detection applying a Wilcoxon rank sum test ($p<0.1$) to the two distributions. To be conservative we used both tests in parallel. An odor evoked a response if one (1) **or** the other (2) test was significant.

**Monitoring Behavior**

Besides the activity of single ENs, we also observed the behavior of the bee via an electrophysiological access. We monitored the proboscis extension response (PER), which is mediated by the muscle M17 of the bee (Rehder, 1987). During the acquisition phase a behavioral response was detected if the activity of the M17 muscle started right after the odor onset before the reward (US) was presented (Fig.1). For the CS-, and also in the test phase where no US was presented, M17 activity during the odor presentation was interpreted as a behavioral response. The differences between CS+ and CS- in the respective trials were tested with a G-test for contingency tables (log-likelihood ratio for contingency tables) for each trial. Differences were considered to be significant if $p < 0.05$.

## Results

### Animal Behavior

In order to observe the bees' behavior simultaneously with the single neuronal activity of mushroom body-extrinsic neurons, we recorded the M17 muscle (Rehder, 1987). A behavioral response was detected during the acquisition phase if the activity of the M17 muscle started right after odor onset, before the reward (US) was presented (Fig. 1B). The bee's antennae are specially separated and a differential conditioning was carried out on the contralateral antenna with regard to the EN recording position (Fig. 1A). The treated animals learned already at the second trial [$G=6.4$; $p<0.05$; $df=1$] to differentiate between the reinforced odor (CS+) and the non-reinforced odor (CS-) (Fig. 1C) presented only at the contralateral antenna. During the retention test after three hours about 80% of the subjects remembered the side-specific learned association correctly, responding much more to the CS+ odor than to the CS- odor (15.4%) presented at the contralateral side, where it was also presented during the acquisition phase (Fig. 1D). The differentiation between the CS+ and the CS- at this side was highly significant [$G=10.7$; $p<0.001$; $df=1$] although the last trial of the acquisition phase was only near-significant (Fig. 1C; trial 10 [$G=3.4$; $p = 0.06$; $df=1$, n.s.]). Presenting the same odors on the ipsilateral antenna relative to the recording position of the ENs (Fig. 1A) showed only a tendency toward more CS+ (30.7%) than CS- responses (7.7%), with no difference between stimuli [$G = 2.36$; $p = 0.12$; $df=1$, n.s.]). We conclude that in this experiment bees did not transfer or generalize the learned information between the brain sides (Fig. 1D). The side-specific information about the CS+ thus seems to be stable and is not intermixed between brain sides. In the following paragraphs we will show that the neuronal representation after three hours at the level of the ENs is also side-specific.

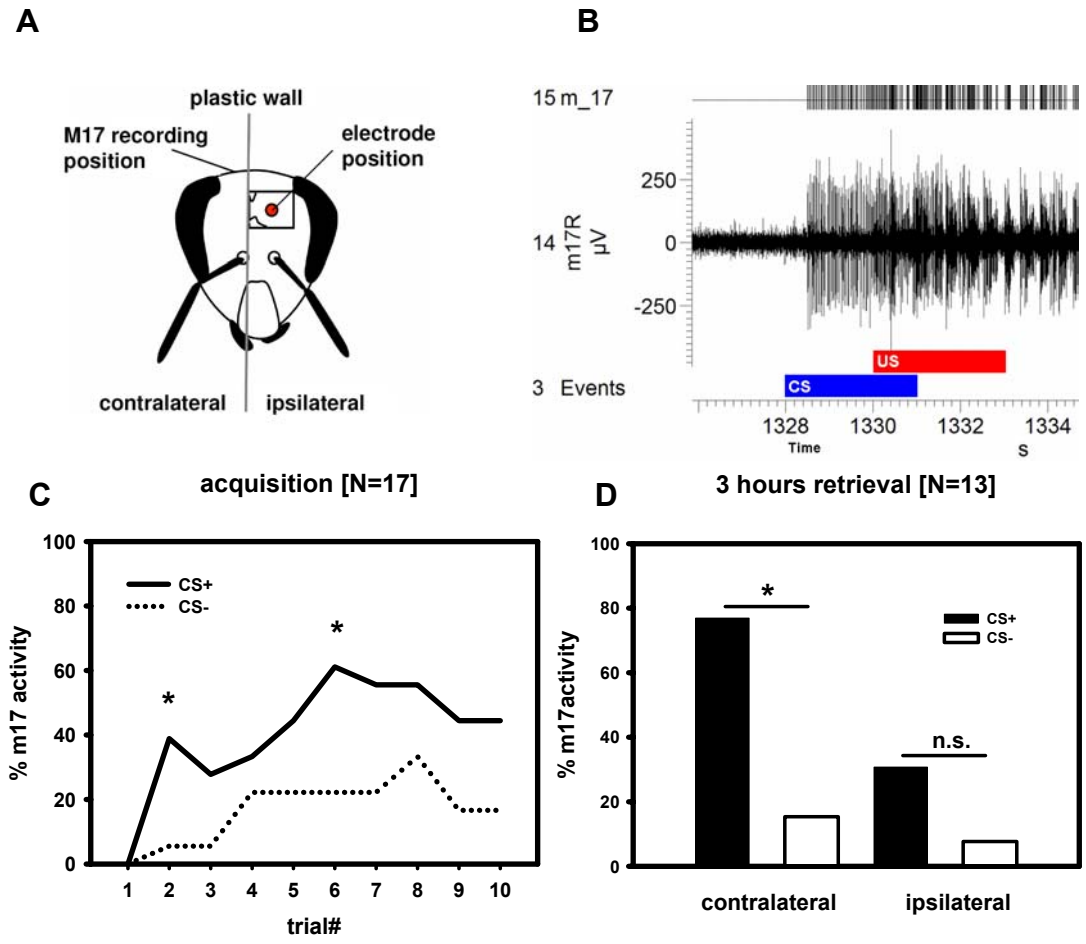


***Figure 1. Side-specific differential conditioning.*** ***A:*** *Scheme of the experimental procedure. The bee antennae are spatially separated and the electrode is inserted on only one side of the bee brain into the ventral part of the alpha-lobe (red dot). The contra- or the ipsilateral antenna- relative to the electrode position - is stimulated, depending on the experimental phase.* ***B:*** *The activity of the M17 muscle is used to observe the behavior of the bee simultaneously with the recording of the mushroom bodie- extrinsic neurons [ENs]. A behavioral response is detected if the activity of the muscle M17 started right after the odor (CS, blue) onset, before the reward (US, red) was presented.* ***C:*** *Acquisition curve of the 17 subjects whose neuronal activity was recorded during a differential odor conditioning in which one odor is presented reinforced (CS+) and another odor is presented non-reinforced (CS-). Note that the differential conditioning is always done on the contralateral antenna, as determined by the recording position. The subjects are able to discriminate the two stimuli (trial 2 [G=6.4; p<0.05; df=1]; trial 6 [G=5.8; p<0.05; df=1]; trial 10 [G=3.4; p=0.06; df=1, n.s.].* ***D:*** *Retrieval test after 3 hours shows that nearly 80% of the subjects remember the CS+ correctly. The difference between the CS+ and the CS- is significant [G=10.7; p<0.001; df=1]. Note: the 13 tested bees did not generalize or transfer the association to the ipsilateral side [G=2.3; p=0.12; df=1, n.s.].*

**Neuronal responses**

***Recruitment of ENs after contralateral differential conditioning***

Extra-cellular long-term recordings were performed to record the activity of single ENs during all three experimental phases of a classical conditioning experiment. During the PreAcq two odors were presented 10 times, separately, on each antenna with an ITI of 1 min. After 20 min, the two odors were differentially conditioned, but only at one side also with an ITI of 1 min (Acq). Note that the conditioning is always presented on the contralateral antenna, relative to the recording position of ENs. After the three-hour rest, during the PreAcq, the two odors were again presented separately on each antenna. The ITI in that phase was also 1 min. The experimental procedure lasted 5 hours in total. Figure 2 shows the responses of two simultaneously-recorded ENs. The two neurons behave slightly differently, but their contribution to the neuronal network that computes the side-specific information of the CS+, let the subject discriminate between the information on the different antennae as confirmed by its behavior (Fig. 2C). Only the presentation of the CS+ on the contralateral antenna, when the related odor was presented during the acquisition, resulted in M17 activity in the PostAcq (red squares, Fig. 2C), whereas the same odor (CS+) presented on the ipsilateral antenna evoked no M17 activity (grey squares, Fig. 2C). The information about the reinforced odor is learned and stored in a side-specific configuration. This side-specific configuration is reflected in the responses of the two simultaneously-recorded example units. During the contralateral differential conditioning both units are already involved in the computation of the reinforced stimulus. Unit ID 11 responded during the overlap of CS and US with a decrease in spiking activity. The other unit (ID 12) seems to be excited during the overlapping occurrence of CS and US. Both units shift this activity to the odor onset (Fig. 2 [Acquisition]). After 3 hours of resting time in the PostAcq the responses in both example units to the different side-specific odor combination are reliably established (for RIs cp. Fig. 5). Interestingly, the side-specific CS+ presented on the contralateral side evoked excitation, whereas the same odor presented to the ipsilateral side leads to inhibition of unit 11. Unit 12 showed in the PostAcq a clear response to the side-specific CS+. The same odor presented at the ipsilateral side evoked only a much smaller response in that unit. The CS- seems to have no effect.

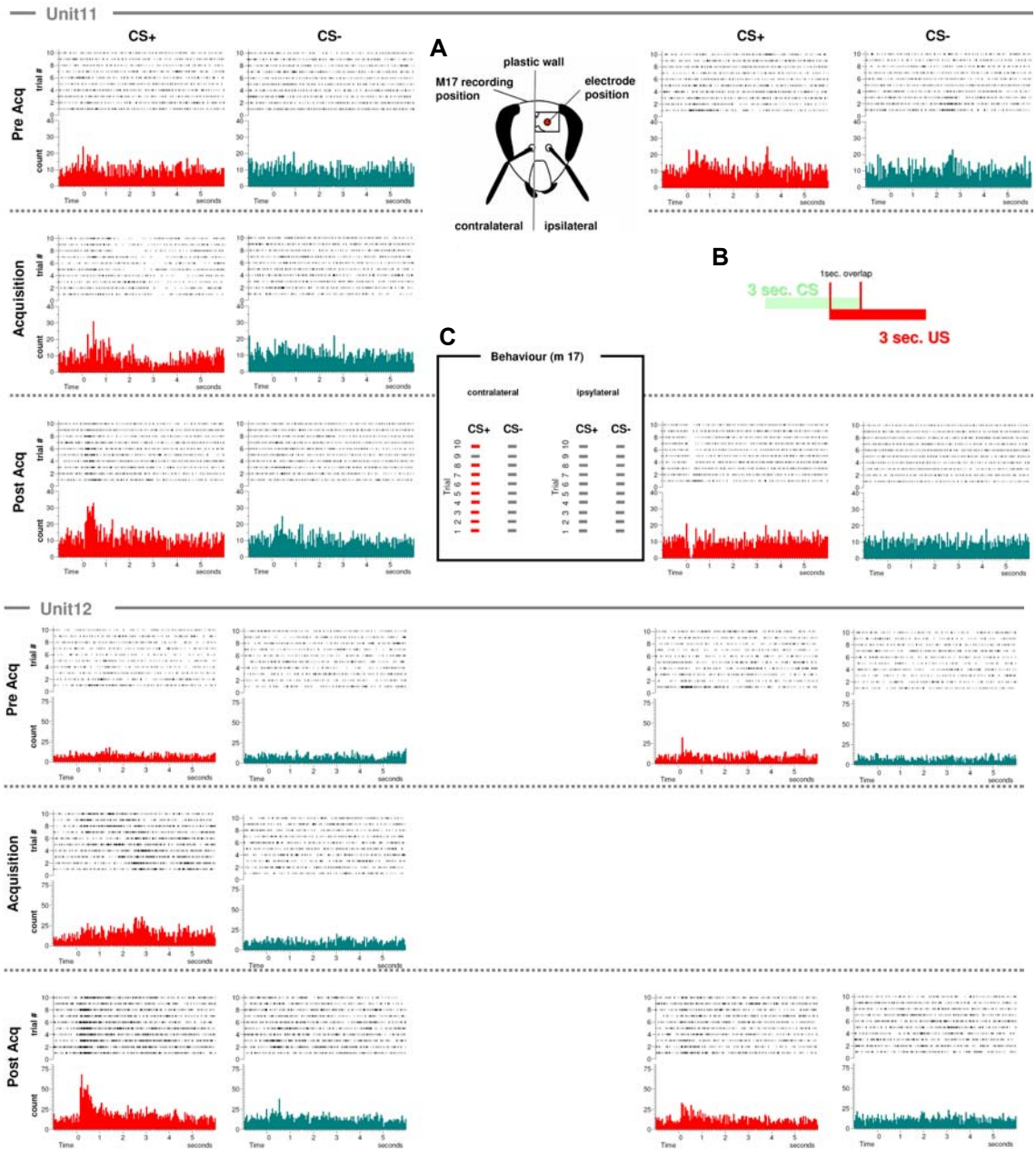


***Figure 2. Examples of two simultaneously-recorded units.*** *The experimental scheme is shown in Inset* ***A*** *(see also Fig. 1A). Responses to the presented odors in the respective experimental phases on the contralateral antenna, relative to the recording position, are shown on the left and responses to the odors for the ipsilateral stimulation on the right. Under each dot display - which includes the spike trains to the 10 repetitions of each odor - the peri stimulus histograms (PSTH) are drawn with a bin size of 50ms. The odor onset is marked by zero and lasted 3 seconds. During the acquisition phase* ***[Acquisition]*** *one odor is presented with a reward (sucrose)* ***[US]*** *which also lasted 3 seconds (see inset* ***B****). Note that during the acquisition the differential conditioning takes place only at the antenna contralateral to the recording position. The PSTHs for the CSs+ (red) and CSs-(green) are shown for the three experimental phases*

*delimited by the dashed grey lines. Unit 11 (upper example) responded in the pre-acquisition phase* ***[Pre Acq]*** *to the odor that will be used during the acquisition phase as the CS+ (red PSTH) quite less independent of the presentation side. A response is detectable in only a few trials. In addition, the odor that will be used as the non-reinforced stimulus during differential conditioning (CS-) evoked no responses in the PreAcq. During the Acquisition the overlap of CS and US [CS+] (see inset* ***B****) seems to decrease the spiking activity of this neuron. Note that during that time information about the ipsilateral received reward can be only conveyed by the VUMmx1 neuron, because the ipsilateral antenna received neither an odor nor a reward. This is not the case fore the CS-. In the post-acquisition phase* ***[PostAcq]****, after the bee was allowed to rest for 3 hours, a clear increase in the PSTH for the contralateral CS+ is visible. The presentation of this odor at the ipsilateral antenna evoked a decrease in the firing rate of that neuron. In this example the CS- seems to not influence the spontaneous rate of this neuron. Unit 12 (lower example), which is recorded simultaneously, does not respond in the PreAcq, as well. Only the ipsilateral presentation of the future CS+ evoked a small response in the corresponding PSTH. Unlike Unit 11, the overlap between CS and US in the acquisition resulted in an increase in the activity of this neuron, which seems to be shifted to the odor onset in the last conditioning trials. The CS- is not affected during the differential conditioning. In the PostAcq a clear response appears to the side-specific CS+. The same odor presented on the ipsilateral side evoked only a much smaller response in that unit. The CS- seems to have no effect. The subject whose neurons we present in that figure is able to discriminate the side-specific CS+ from the CS+ odor presented on the ipsilateral antenna (see inset* ***C****). Red squares indicate a behavioral response; grey squares show cases of no response in the bee's behavior.*

***Representation of the side-specific CS+ at the level of ENs***

Focusing on the response activity in Pre-Acq, we found very few units that already responded to the contralateral odor presentation of the CS+ or the CS- (unit ID 2, 5, 9, 13, 16, 24, 29). But many of them responded to the ipsilateral stimulation for both presented odors (CS+/CS-; Fig. 3). That demonstrated, at least, that the bees' antennae really were spatially separated (with regard to olfactory stimulation). To detect whether an odor evoked a response we pooled the data across all 10 trials (c.p. Strube-Bloss et al., 2008b; Chapter 2, response detection). In the Post-Acq there are many recruited units that now respond to the contralateral CS+ (e.g. unit ID 1, 2, 3, 4, 6, 7,…, Fig. 3). After calculating the difference between the pre- and the post-acq we found that not only does the contralateral odor presentation lead to a response change in the recorded units, but that the response spectrum to the ipsilateral stimulation is also affected. Looking at the single units that changed in response to the ipsilateral stimulation shows that this change is mostly a drop-out, meaning the unit is no longer responding (e.g. unit ID 4, 16; Fig. 3) or that it changed from being excited before the conditioning to being inhibited after acquisition (e.g. unit ID 11, 15; Fig. 3). However, the recorded units show the most change and recruitment to respond to the contralateral side-specific stimuli that were also presented during differential conditioning (Fig. 3). The dominating stimulus for this side-specific recruitment is the "real" side-specific CS+ which was presented reinforced during the acquisition (Fig. 4). The same odor presented ipsilaterally ("wrong" CS+) could cause an inhibition (unit ID 11; Figs. 2 and 3).

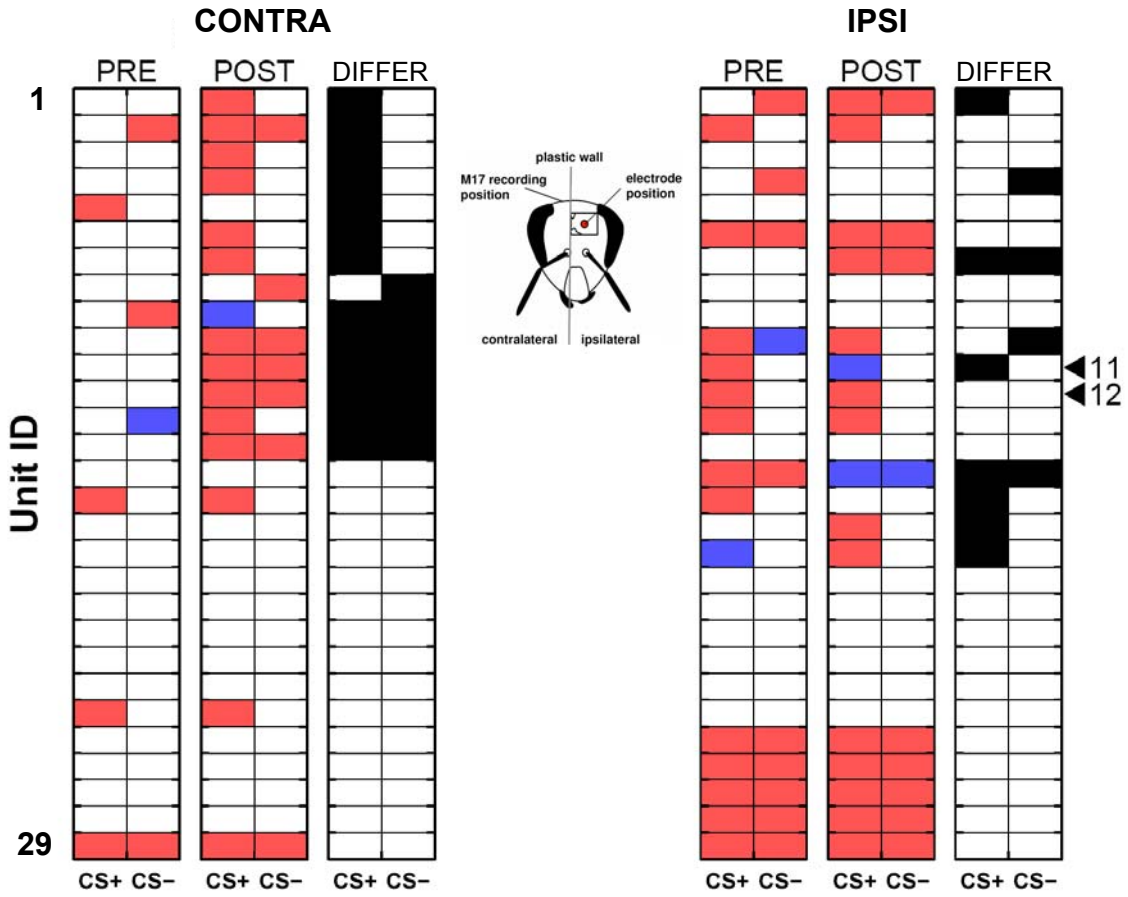


***Figure 3. Contralateral effected units.*** *Overview of the 29 units recorded from the 13 subjects that received site-specific odor stimulations. In the pre-acquisition phase [PRE-ACQ] two odors were presented (CS+ and CS-) separately on each antenna. Note that during that phase the CS+ was not reinforced. After the acquisition phase, during which the CS+ odor (reinforced) and the CS- odor (non-reinforced) were presented only at the contralateral antenna, with regard to the recording position, both odors were tested again, separately, on each antenna [POST-ACQ]. In both phases detectable responses of the pooled 10 trials for each odor-side combination were marked in red for excitatory responses, blue for inhibitory responses and white for those with no detectable response. Note that some units were recruited to respond in the POST-ACQ to the side-specific CS+ (e.g. unit ID 1, 2, 3, 4, 6, 7,…). In the "DIFFER"- column the differences between pre- and post-acquisition phases for the different units were calculated for each odor. Units that show a difference (learning-dependent plasticity) were marked in black. For the other units (white) no difference was detected between the experimental phases. Such plasticity is observed on both sides, albeit more strongly on the conditioning side.*

***Odor learning drives ENs to respond more reliably to the side-specific CS+***

The bee olfactory system is organized in a bilateral way up to the higher-level integration center MB (Mobbs et al., 1982). That is also mirrored by the responses to the contra-lateral odor presentation during the pre-test phases of the present experiments. The presentation of an odor to the contralateral antenna related to the recording position of the ENs leads in most cases to an undetectable response, although we averaged across all 10 trials of each side-specific odor presentation (Fig. 3), whereas presentations of the same odors to the ipsilateral brain side already evoked responses in many units. Also, the reliability indices **[RIs]** are much smaller when presenting the odor to the contralateral side during the Pre-Acq (Fig. 5, light green).

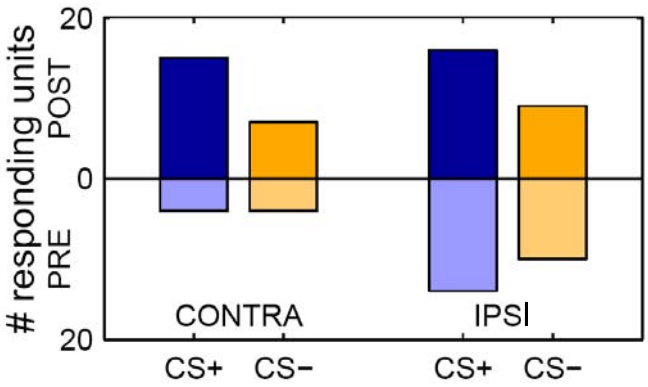


***Figure 4. Contralateral affected units.*** *Units that show learning-dependent plasticity after the bees were treated with contralateral differential conditioning (cp. Fig. 3; black matrix elements) were taken and analyzed regarding their side-specific odor responses before conditioning (PRE) and after (POST). By presenting the odor ipsilateral to the recording position neither the number of units that respond to the CS+ nor the number of units that respond to the CS- differed between the PRE and the POST test phases. Interestingly, the number of units that responded to the contralateral odor presentation is increased for both the CS+ and the CS- in the pre-acquisition phase. These units are recruited to respond to the contralateral odor presentations (for individual details cp. Fig. 3).*

The same odors presented during the pre-test phase to the ipsilateral antenna evoked much more reliable responses in different units. Focusing on the difference between the RIs of the pre- and the post-acquisition phases on the individual-unit level reveals manifold changes after the contralateral differential conditioning (Fig. 5). Note that some units were recruited to respond highly reliably in the POST-ACQ to the side-specific CS+ (e.g. unit ID 2, 6, 10, 11, 12,…), but other units lost their capacity to

respond to the ipsilateral odor presentation (ID 19). Besides such individual changes, the dominant influence is produced by the contralateral presentation of the side-specific conditioned odors, namely by both CS+ and CS-.

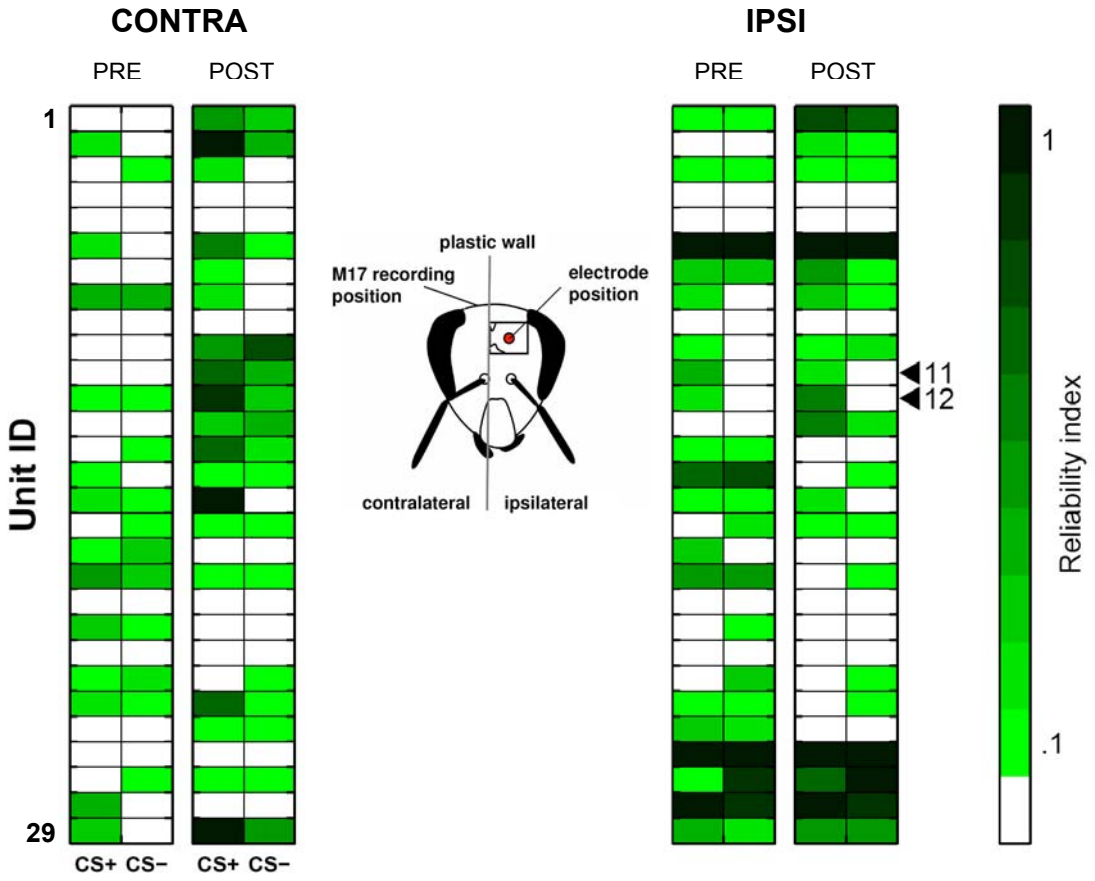


***Figure 5. Reliability indices of all units.*** *Overview of the reliability indices* ***[RIs]*** *of the 29 units that were recorded from the 13 subjects that received side-specific odor stimulations. Each square indicates one color-coded reliability index* ***[RI]*** *which is the number of presentations of one particular odor that evoked a significant response divided by the total number of presentations of that odor. In the pre-acquisition phase* ***[PRE-ACQ]*** *two odors were presented (CS+ and CS-) separately on each antenna. Note that during that phase the CS+ was not reinforced. After the acquisition phase, during which the CS+ odor (reinforced) and the CS- odor (non-reinforced) were presented only at the contralateral antenna related to the recording position, both odors were tested again 10 times separately on each antenna* ***[POST-ACQ]****. Highly reliable RIs are marked in dark green to black, lower RIs in green to light green, white matrix elements designate instances with no detectable responses in any of the single trials. Note that some units were recruited to respond highly reliably in the POST-ACQ to the side-specific CS+ (e.g. unit ID 2, 6, 10, 11, 12,…). The examples shown in detail in Figure 2 are marked by arrows. Unit ID 1, 10, 11 did not respond in any of the PRE- trials for contralateral stimulation (white), but recruited to respond relatively reliably after the acquisition phase to the side-specific CS+ (RI>0.5).*

A comparison of the mean RI of the contralateral affected units of the pre- and the post-conditioning phases reveals that the dominance of the side-specific conditioned odors appears quite clearly (Fig. 6). Before the acquisition the mean RI of both odors is 0.1, meaning that one out of ten odor presentations evoked a detectable response. During the post-test phase the mean RI for the side-specific CS+ is four times higher (Fig. 6).

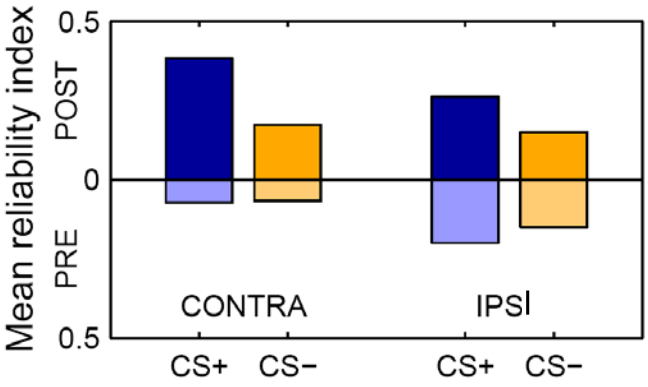


***Figure 6. Mean RI change of contralateral affected units.*** *Units that show learning-dependent plasticity after the bees were treated with contralateral differential conditioning (cp. Fig. 3; black matrix elements) were taken and analyzed as to changes in their RIs for the side-specific odor presentation before conditioning (PRE) and after (POST). By presenting the odor ipsilaterally in regard to the recording position neither the mean RI of units that respond to the CS+ nor the mean RI of units that respond to the CS- showed large differences between the PRE and the POST test phases. The mean reliability of units to the contralateral odor presentation is increased for both the CS+ and the CS- in the pre-acquisition phase. The related units are recruited to respond to the contralateral odor presentations more reliably than before the contralateral conditioning (for individual details cp. Fig. 5).*

**Conclusion**

The results presented reflect the complexity involved in integrating side-specific stimuli in the network of ENs. Note that in the present paper significant changes in the single units were evoked by contralateral differential odor conditioning. An integration of the contralateral stimulus was already observable during the overlap of CS and US (Fig. 2). After conditioning, in the post-acquisition phase as well the averaged response changes (Figs. 3 and 4), as the changes in the RIs (Figs. 5 and 6) document the side-specific integration and representation of the different CSs+ (contra = “real” CS+; ipsi = “wrong” CS+) and CSs- (contra = “real” CS-; ipsi = “wrong” CS-), because the different test odors evoke different response patterns in the recorded units. In extreme instances the “real” CS+ evoked an excitation, whereas the “wrong” CS+ resulted in an inhibition of the same unit (Fig. 2). However, the dominating stimulus that recruited initially non-responding units or increased the reliability of initially-responding units is the side-specific CS+, as illustrated by Figures 4 and 6.

## Discussion

### The neuronal correlate for side-specific odor learning

In the present study, we are able to show that after a side-specific differential odor conditioning the side-specific CS+ is represented in the activity pattern of the alpha-lobe-extrinsic neurons. Therefore units were recruited to differentiate between the CSs+ presented on that antenna, where it was also given during the conditioning ("real" CS+) from the ipsilateral tested CS+ ("wrong"). So far it had been known that this neuronal type could be recruited after a differential learning task (Strube-Bloss et al., 2008b; Chapter 2). Recruited units can be related to naively odor non-responding units (cp. Strube-Bloss et al., 2008a; Chapter 1). Other units that already responded to the ipsilateral odor presentation dropped out after learning, changed their initially excitatory response to an inhibition or did not change their activity to the presented odors. These units can be related to the initially-responding units. Their response characteristics are described by Strube-Bloss (2008a; Chapter1). The contributions of the recruited, the inverted, the dropping out or the superficially unaffected units to the neuronal network that computes the side-specific odors may be different, but the result, the animals' behavior, shows the network's success. The tested bees learn to discriminate the side-specific "real" CS+ from the "wrong" CS+ presented on the untrained antenna and both CS- combinations. In our experiment a tendency toward more response to the CS+ than to the CS- was found only on the untrained brain side (Fig. 1D). It therefore seems that information was not transferred to the untrained brain side, as described by Sandoz and Menzel (2001).

### Transfer = loss of spatial information

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 spatial memory is the independence of the two antennae regarding their 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 from both antennae is necessary for building a compound, therefore both sides must be 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 antenna and proboscis) induced bilateral sensitization. Note that one antenna is enough. We always presented the US to the contralateral antenna and the proboscis. Both brain sides should be sensitized to integrate the separately-received input from both antennae, which during the acquisition is one odor contralateral and no odor ipsilateral to the recording position. The learning performance and the test after acquisition showed that the subjects learned this task (Fig. 1). They also remember the side-specific information correctly. The CS+ presented at the "wrong" side resulted in the bees not responding with PER. This is contradicts the finding that bees transfer olfactory information according to a similar paradigm to the other brain side within a time window of between 10 minutes and 3 hours (Sandoz and Menzel, 2001). The fact that the bees in the present study did not transfer the information - even though we let the bees rest for 3 hours - could be caused by the slightly different paradigm. We trained the bees with 10 acquisition trials instead of 4, and our inter-trial intervals, at 1 minute are relatively short. That could lead to a stronger, more frequent sensitization, resulting in a more stable memory. The behavior that is interpreted as transfer (Sandoz and Menzel, 2001) could also be interpreted as a loss of discriminating the CS+ presented at the correct antenna, and that would indicate that the bees were generalizing between side-specific stimuli. One other possibility could be that the bees had a different internal state (motivation). During the 10 CS+ trials of the acquisition in the present paradigm the bees had received more than twice as much sugar solution than during the 4 acquisition trials in the experiments by Sandoz and Menzel (2001). After three hours, they are not as hungry as the bees in their experimental procedure. That could be the cause of different strategies, like generalizing between similar odors on the respective antenna. The observation of the animal's internal state or the computing network, respectively, seems to be essential for the correct interpretation of the behavioral data, and was included in the present study. We were able to observe single units of the computing network, where we found a side-specific representation of the CS+ presented on the correct antenna, where it was also

presented during the acquisition. A simple transfer of the information between the brain sides would add up to a loss of the spatial information, and was not observable in our experiments. The stable side-specific representation of odor stimuli could also be the reason why removing the input from one antenna eliminates the blocking of one odor by another, as demonstrated by Thorn and Smith (1997). Single odorants in binary mixtures can be blocked after pre-exposure to one element of the mixture (Smith and Cobey, 1994). Initially, the odors were presented simultaneously to both antennae. After covering one antenna, as by Thorn and Smith (1997) did, the bee is exposed to a completely different and new stimulus that is now side-specific. Blocking would only occure if the initial odor element is presented like initially on both antennae.

**Contralateral recruitment of previous non-responding units**

General response properties like odor specificity and reliability (Strube-Bloss et al., 2008a; Chapter 1) of alpha lobe-extrinsic neurons were changed after the bees learned to discriminate two odors. This means that plastic units became recruited and/or more reliable and changed their odor spectrum (Strube-Bloss et al., 2008b; Chapter 2). The honeybee brain is organized in a bilateral, symmetric manner up to the higher-order integration centers: the mushroom bodies [MBs] (Mobbs et al., 1982). In this study we inserted the recording electrode only into the output region of one MB to record EN activity. If both sides are really separated we should not be able to detect a response evoked by an odor presented at the contralateral side. This is, however, what we observed in the Pre-Acq conditioning phase. Contralateral odor presentation evoked no or very few and unreliable responses (Fig. 3 and 5).

The aim of this study was to investigate the phenomenon of transfer between both MBs (cp. Sandoz and Menzel, 2001) at the EN level. This phenomenon occurs 3 hours after contralateral differential conditioning. Therefore the bees in the present study were allowed to rest for three hours after contralateral training. In the post-acquisition test we found units that were recruited to respond to the contralateral odor presentation. But if the same odor is presented on the ipsilateral antenna the unit does not respond, although the information should be transferred to this side. Some units responded with excitation to the CS+ presented at the “real” side, and were inhibited by presenting the CS+ odor

on the ipsilateral, the "wrong" side (cp. Unit ID 11). This suggests that the two different side-specific CS+ combinations were computed differently by the underlying network. Units that responded reliably in the Pre-Acq phase to the ipsilateral CS+ odor stopped responding or responded unreliably to that odor in the Post-Acq step. Other units were unaffected by the contralateral differential conditioning. These kinds of stereotypic units were also described previously by Strube-Bloss et al. (2008b; Chapter 2). However, the two side-specific CSs+ ("real" contralateral; "wrong" ipsilateral) evoke different activity patterns in the Ens, which let the bees discriminate both stimuli; this was also supported by the behavior (Fig. 1).

The present experiments confirm that both MBs work closely together at least three hours after conditioning. Not only time, but the complexity of the task also seemed to be an important factor. Komischke et al. (2003) showed that the unrestricted activity of both MBs is the prerequisite for solving non-elemental learning tasks like negative patterning, whereas elemental paradigms can be solved by using the input from only one antenna. Furthermore, bees with one ablated MB are not able to solve side-spanning learning tasks, but they learn to solve differential conditioning (Komischke et al., 2005). This is reflected in the present study as well. We presented the differential conditioning to only the contralateral antenna, so that initially only one brain side gets olfactory input (Fig. 1). The integration of the olfactory information from the ipsilateral antenna may start as early as during acquisition, as illustrated by the increased activity triggered by the US (reward). It should also be noted that during that time, information about the ipsilaterally-received reward can only be conveyed by the $VUM_{mx1}$ neuron, which responds weakly to olfactory and visual stimuli, but shows a massive response to sucrose stimulation (Hammer, 1993), because the ipsilateral antenna received neither an odor nor sucrose. The increased activity during the US presentation measured in ENs may be governed indirectly, meaning that there is no direct synapse between ENs and the $VUM_{mx1}$. The sucrose stimulation alone must evoke a KC activity pattern which - on the next neuronal level (ENs) - leads to an increase or decrease of activity (Fig. 2). The clawed Kenyon cells [cKC] respond to the contiguity of odor and sucrose as the pairing of the odor and sucrose lead to prolonged and/or increased odor responses (Szyszka et al., 2005). The presence of the sucrose alone may possibly be sufficient to induce a separate sucrose-evoked cKC pattern which is read out by the ipsilateral EN. These

reward-triggered activity change in the ipsilateral MB may be the prerequisite for integrating this activity in the compound of both MBs. However, after 3 hours the response to the side-specific odor stimuli were established and evoked different activity patterns in the ENs depending on which side was stimulated.

**The role of inhibition**

The side-specific odor representation after contralateral odor conditioning at the EN level is rather complex (cp. Figs. 3 and 5). The same odor presented to the contralateral antenna evoked a different response as presented to the ipsilateral antenna. That means that the laterality as an additional feature of the stimulus is represented after learning at the output of the MB (ENs). In extreme cases the odor presented to the contralateral antenna leads to excitation the same odor presented to the ipsilateral antenna, resulting in an inhibition in the same unit (Figs. 2 and 3). Other units were initially excited and after the learning task they were inhibited (cp. Fig. 3). The cause of that inhibition could be related to the alpha-lobe-extrinsic neurons of the protocerebral calycal tract (PCT) which are GABA-immunoreactive (ir) inhibitory neurons. These neurons could be effective twice: first, locally, by sending their collaterals down the peduncle and reaching the dendrites of ENs; the second inhibitory action would be recurrent: 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). Intra-cellular recordings from PCT neurons showed that they are indeed affected by presenting an odor/sucrose pairing which resulted in a response reduction (Grünewald, 1999). This is only a hint that the pairing of an odor with sugar can influence the inhibition mediated by the PCT neurons within a short time window. It is absolutely possible that other PCTs increased their response after odor learning. These could be the ones which are effective locally and which inhibit the ENs if the CS+ is presented at the “wrong” side. Thus the representation of an associated odor stimulus in the honeybee brain includes both the activity change of excitation and the activity change of inhibition, which are then involved in the network that computes the consolidated side-specific stimulus as illustrated by the activity changes of the units recorded here (Figs. 1 and 2). This inhibition of single ENs after establishing an association between an odor and a reward

could also be the cause of single odors that were blocked after they were presented in a binary mixture, as demonstrated by Smith and Cobey (1994).

**Acknowledgements**

This work was part of a cooperation hosted by the Bernstein Center for Computational Neuroscience Berlin (BCCN Berlin) and supported by grants from the Bundesministerium für Bildung und Forschung (BMBF; grant 01GQ0413). We thank Farzad Farkhooi for extracting the PE1, Bernhard Komischke and Jean-Christophe Sandoz for fruitful comments on an early version of this manuscript. We also thank Juergen Rybak for discussions regarding the anatomy and recording position.

## References

**Bitterman, M.E.**, Menzel, R., Fietz, A., and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J Comp Psychol* **97**, 107-119.

**Braun, G. and Bicker, G.** (1992). Habituation of an appetitive reflex in the honeybee. *J Neurophysiol* **67,** 588–598.

**Deisig, N.**, Lachnit, H., Giurfa, M. and Hellstern, F. (2001). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learn & Mem* **8**, 70–78.

**Dujardin, F.** (1850) Memoire sur le systeme nerveux des insects. *Ann Sci Nat Zool* **14**, 195-206.

**Erber, J.**, Masuhr, T. H., and Menzel, R. (1980). Localization of short-term memory in the brain of the bee, *Apis mellifera*. *Physiol Entomol* **5**, 343-358.

**Galizia, C.G**., Joerges, J., Kuttner, A., Faber, T. and Menzel, R. (1997). A semi-in-vivo preparation for optical recording of the insect brain. *J Neurosci Methods* **76**, 61-69.

**Grünewald, B.** (1999). Physiological properties and response modulations of mushroom body feedback neurons during olfactory learning in the honeybee, *Apis mellifera*. *J Comp Physiol* **185**, 565-576.

**Hammer, M.** (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* **366**, 59-63.

**Heisenberg, M.** (2003). Mushroom body memoir: from maps to models (Review). *Nat Rev Neurosci* **4**, 266-275.

**Hollander, M. and Wolfe, D.** (1973). Nonparametric Statistical Methods. *Wiley & Sons,* New York.

**Komischke, B.**, Sandoz, J.C., Lachnit, H. and Giurfa, M. (2003). Non-elemental processing in olfactory discrimination tasks needs bilateral input in honeybees. *Behav Brain Res* **145**, 135–143.

**Komischke, B.**, Sandoz, J.C., Malun, D. and Giurfa, M. (2005). Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L. *Eur J Neurosci* **21,** 477–485.

**Martin, J-R.**, Ernst, R. and Heisenberg, M. (1998). Mushroom bodies suppress locomotor activity in Drosophila melanogaster. *Learn & Mem* **5**, 179-191.

**Mauelshagen, J.** (1993). Neural correlates of olfactory learning in an identified neuron in the honey bee brain. *J Neurophysiol* **69**, 609-625.

**Menzel, R. and Manz, G.** (2005). Neural Plasticity of Mushroom Body-Extrinsic Neurons in the Honeybee Brain. *J Exp Biol* **208**, 4317-4332.

**Menzel, R.**, Erber, J. and Masuhr, T. H. (1974). Learning and memory in the honeybee. In: *Experimental analysis of insect behaviour* (ed. L. Barton-Browne), **pp**. 195–217. Springer, Berlin, Germany.

**Mizunami, M.**, Okada, R., Li, Y.S. and Strausfeld, N.J. (1998). Mushroom bodies of the cockroach: Activity and identities of neurons recorded in freely moving animals. *J Comp Neurol* **402,** 501-519.

**Mobbs, P.G.** (1982). The brain of the honeybee Apis mellifera I. The connections and spatial organization of the mushroom bodies. *Phil Trans R Soc Lond B* **298**, 309-354.

**Okada, R.**, Rybak, J., Manz, G. and Menzel, R. (2007). Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain. *J Neurosci* **27(43)**, 11736 –11747.

**Okada, R.**, Ikeda, J. and Mizunami, M. (1999). Sensory responses and movement-related activities in extrinsic neurons of the cockroach mushroom bodies. *J Comp Physiol* [A] **185**, 115-129.

**Rehder, V.** (1987). Quantification of the honeybee's proboscis reflex by electromyographic recordings. *J Insect Physiol* **33**, 501–507.

**Rybak, J**. and Menzel, R. (1993). Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. *J Comp Neurol* **334**, 444-465.

**Sandoz, J.C. and Menzel, R.** (2001). Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. *Learn & Mem* **8**, 286–294.

**Sandoz, J.C.,** Hammer and M., Menzel, R. (2002). Side-specificity of olfactory learning in the honeybee: US input side. *Learn & Mem* **9**, 337–48.

**Smith, B.H. and Cobey, S.** (1994). The olfactory memory of the honeybee *Apis mellifera* II. Blocking between odorants in binary mixtures. *J Exp Bio* **195**, 91–108.

**Strausfeld, N.J.** (1998) *review*. Crustacean-insect relationships: the use of brain characters to derive phylogeny amongst segmented invertebrates. *Brain Behav Evol* **52**(4-5), 186-206.

**Strube, M.F.** (2005). Honigbienen (*Apis mellifera carnica* L.) bilden seitenübergreifende Konfigurationen aus den olfaktorischen Erregungen beider Pilzkörper. Diplomarbeit; Institut für Neurobiologie der Freien Universität Berlin.

**Strube-Bloss, M.F.**; Farkhooi, F., Nawrot, M.P. and Menzel, R. (2008a, chapter 1). Odor specificity and response reliability of mushroom body extrinsic neurons in the honeybee

**Strube-Bloss, M.F.**; Nawrot, M.P. and Menzel, R. (2008b, chapter 2). Recruitment and learning induced plasticity in alpha-lobe extrinsic neurons of the honeybee

**Szyszka, P.**, Ditzen, M., Galkin, A., Galizia, C.G. and Menzel, R. (2005). Sparsening and temporal sharpening of olfactory representations in the honeybee mushroom bodies. *J Neurophysiol* **94**, 3303-3313.

**Thorn, R.S. and Smith, B.H.** (1997). The olfactory memory of the honeybee *Apis mellifera*, III. Bilateral sensory input is necessary for induction and expression of olfactory blocking. *J Exp Biol* **200,** 2045–55.