Recruitment and learning induced plasticity in alpha-lobe extrinsic neurons of the honeybee

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Abstract

Mushroom bodies (MBs) in the insect brain are higher-order centers involved in integration of olfactory, visual and mechano-sensory information. They are also known to be involved in neural plasticity underlying associative olfactory learning. Extrinsic neurons (ENs) of the alpha lobe provide an output of the MB. These were investigated by extracellular long term recordings. Single-unit activity of typically 2-5 ENs was measured.

In initially untrained animals we found ENs which were non-responding and units which were already initially responding to odor stimulation. To set a basis for the investigation of learning induced changes we first characterized the neurons response profiles regarding their odor specificity and response reliability across repetitions of ten times ten odor stimuli.

In a differential conditioning experiment the animals were exposed to two odors, the forward-paired CS+ and the unpaired CS-. Before conditioning the CSs and three additional odors were presented 10 times each to characterize the odor specificity to 5 different odors, and to estimate the reliability of the responses to each of the odors. In a post test phase (3 hours after the conditioning) all five odors were again tested 10 times, to compare the reliability indices before and after conditioning, and to determine any changes of odor specificity. The differential conditionings led appear two types of units. One group exhibited clear learning dependent change and were therefore called “plastic”. A second group of “stereotypic” units, did not change their response after reward association. These units showed also a very broad response spectrum, which makes them rather odor unspecific. Plastic units could be classified into two different types. The first type had shown initial a unreliable responses to few odors before the

subjects had built an association. After the animals had built an association about 30% of this group were recruited to respond reliably to the rewarded odor (CS+). The second group of plastic units initially did not respond to any of the odors but showed reliable responses to either the CS+ or CS- or both after the conditioning experiment.

Introduction

The olfactory system in vertebrates as well as in invertebrates offers many advantages to study learning and memory formation by addressing olfactory learning paradigms (Davis, 2004; Wilson and Mainen, 2006). Learning and memory formation are parallel processes during which the behavior of a subject changes step by step to adapt to the actual relevant environmental situation. These processes are going along with the modification of neuronal excitability and synaptic strength between neurons (Milner et al., 1998). The measurement of the activity of single neurons before, during and after learning and memory formation is essential to characterize the underling mechanisms at the cellular level. In vertebrates, extracellular recordings have been successfully used to monitor neural processes during learning and memory retrieval at the single-neuron level (Sutherland and McNaughton, 2000; Goldman-Rakic, 1995; Rolls et al., 1996; Schultz, 1998). Also in insects extracellular long term recordings are 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 honeybees extra-cellular long term recording were established to record the activity of one identified MB extrinsic neuron, the pedunculus extrinsic neuron 1 [PE1] in a behaving animal during a classical conditioning experiment (Okada et al., 2007).
Classical conditioning in honeybees is a robust and well-studied type of learning which is based on the proboscis extension reflex [PER]: if sucrose solution (unconditioned stimulus; US) is delivered to the antennae or proboscis, bees respond with a reflectory extension of their proboscis (Kuwabara, 1957; Menzel et al., 1974; Vareschi, 1971). This reflex is usually paired with olfactory cues (conditioned stimulus; CS) such that conditioning leads to learned behavior of the CS. After three such absolute conditioning

trials, a long-lasting stable memory is formed (Menzel et al., 1991). Also in a differential conditioning procedure, it has been shown that bees can learn to discriminate two odors, usually after two to three learning trials (Bitterman et al., 1983). Mushroom bodies within the insect brain are higher-order centers performing integration of olfactory, visual and mechano-sensory information. They are involved in the regulation of motor actions like walking behavior (Martin and Heisenberg, 1998) and have been described long time ago as centers for intelligent actions (Dujardin, 1850; Strausfeld, 1998, review). Amnestic treatments support the theory that the MB is strongly involved in memory consolidation. In honeybees, the conditioned response probability was greatly reduced when the MBs had been treated shortly after a single learning trial (Erber et al., 1980; Menzel et al., 1974). In Drosophila a branch specific memory trace is formed between 3 to 9 hours after conditioning only in the alpha-branch of the MB (Yu et al., 2006).

Extrinsic neurons [ENs] form the output of the MB via the alpha-lobe in the honeybee. They receive their input from the MB intrinsic neurons, the Kenyon cells. The PE1 is one of the most prominent cells extrinsic to the alpha lobe with large branches collecting its information from the Kenyon cells [KC] and it changes its response during classical odor conditioning (Mauelshagen et al., 1993). Extracellular long term recordings document also that the PE1 shows a reduction in the response to the rewarded stimulus after learning (Okada et al., 2007).

In the present study we focused on a different group of ENs which connect the MB with the neuropils around the alpha lobe and with the lateral protocerebral lobe [LPL] and partially with the contralateral MB (Rybak and Menzel, 1993). We combined differential conditioning experiments with extracellular long-term recordings and investigated learning-induced changes of single unit response properties, in particular we analyzed odor specificity and trial-to-trial response reliability as described by Strube-Bloss et al. 2008a (Chapter 1).

Material and Methods

Laboratory animals

Forager honeybees were caught at the entrance to the hive at the afternoon one day before the experiment. They were anesthetized on ice, and harnessed in a metal tube, so that the bees could freely move their proboscis and the antennae. Before keeping the bees over night at room temperature they were fed with a 30% sucrose solution.

Electrophysiology

The heads of the animals were fixed with wax on the metal tube and the scapi of both antennae were fixed with low-melting-point wax onto the head capsule. A small unilateral window (1.5 x1.5 mm) was made between the compound eye and the midline of the antennae of the bee. Head glands and trachea sacks above the alpha- lobe were removed and the electrode was positioned in 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 fix the electrode un- movable with the brain. With this treatment, the recordings could last for hours.

To monitor single-unit 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 (*Apis mellifera*). Electrodes were manufactured as described previously (Mizunami et al., 1998; Okada et al., 1999). The wires were glued together with wax onto a 1-2 cm long tungsten wire (100 µm in diameter) that was attached to a glass capillary. The glass capillary was fixed to 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 pair wise combinations of the three electrode wires using the Patch Panel (ERP-27, Neuralynx, Tucson, AZ, USA). A silver wire with a diameter of 25 µm (Nilaco, Tokyo, Japan) was inserted into the right compound eye and 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 these software (Cambridge Electronic Design, Cambridge, UK) were used to applying a high-pass filter (300-10 kHz) and semi-automatic spike sorting techniques (template-matching) with which we could separate up to 5 individual neurons per recording.

Recording Position

All recordings were done, by inserting the electrode at the ventral region of the alpha lobe (cp. Strube-Bloss et al., 2008a; chapter1). ENs which can be 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 neuropils around the alpha lobe and with the lateral protocerebral lobe [LPL]. Only the type A7 connects the ipsilateral hemisphere with the contralateral MB (Rybak and Menzel, 1993). Before starting the respective experimental paradigm we recorded 3min of the spontaneous activity of the different ENs and analyzed that activity with the view to separate the PE1 neuron (Tab.1). This was possible because of the typical double or triplet spike pattern of the PE1 neuron (Mauelshagen, 1993) which is characteristic for only this neuron in the alpha lobe exit (Menzel and Manz, 2005) and could also be used to identify it extracellularly (Okada et al. 2007).

Monitoring the Behavior

Besides the activity of single ENs we also observed the behavior of the animal through electrophysiology by monitoring the proboscis extension response (PER) that is mediated by the muscle M17 (Rehder, 1987). During the acquisition phase a behavioral response was detected if the activity of the muscle M17 started right after the odor onset before the reward (US) was presented (Fig. 2). For the CS- and also in the test phase were no US was presented activity of the M17 during the odor presentation leads to a behavioral response. The differences between CS+ and CS- in the respective trials were

tested by the application of a G-test for contingency tables (log likelihood ratio for contingency tables) for each trial. Differences were considered to be significant if $p < 0.05$.

Odor stimulation

A 12-channel- olfactometer was adapted from Galizia et al. 1997 and appareled with 5ml syringes (odor chambers). A constant air stream (1.5 m/s speed) was delivered through a teflon tube (6 mm in diameter). The needles of the syringes were inserted into this air stream. Odors were diluted in paraffin oil to a 0.1 volume concentration. Filter papers ($2cm^2$) were soaked with 10 µl of odor solution and placed in the syringes. During the three-second odor stimulation, 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 knows the on- and the off-set for the reward stimulation to assure a consistent stimulation over trials and experiments.

Experimental paradigms

10x10 odor repetitions

To investigate the general initial response properties of the alpha lobe extrinsic neurons we started the experimental procedure only if we found responses to at least one odor after electrode insertion. This way we mostly recorded neurons that did show initial responses in the naive animal. We recorded 25 different ENs in 10 animals (Fig.3) and stimulated the subject with 10 different odors (2-octanol, octanal, 2-nonanone, 1-nonanol, cineole, linalool, limonene, eugenol, 1-heptanal, hexanal [Sigma-Aldrich Chemie GmbH] 100 times diluted in paraffin oil). During the 3 second lasting

stimulation the odor or the control was injected as described into the constant air stream. The odors were presented with an inter trial interval (ITI) of 1 min. The sequence of the 10 odors was always the same and was repeated 10 times. The outlet of the tube with the constant air stream was placed 1 cm away from the bee´s head. An exhauster hood (tube with 10 cm diameter) was placed behind the bee to remove all presented odor molecules.

Differential conditioning

In a second experiment we addressed the question if the defined general response properties of the single units could be influenced by a differential conditioning experiment. Therefore we recorded 38 units out of 17 bees by inserting the electrode into the ventral part of the MB without any bias which means, that we started the experimental procedure also if we saw no responses to any odor. Out of this unbiased electrode insertion we calculated the ratio between initial non responding ENs and initial responding ENs (Fig. 3.). The main differential conditioning experiment was divided into three phases.

1. *Pre-acquisition phase [PreAcq]:* five different odors and the control were presented for 3 seconds 10 times each in a pseudo-randomized manner (ITI 1s).
2. *Conditioning phase [Acq]:* two out of the five odors were chosen to be the conditioned stimuli (CS). One of them was selected to be the rewarded 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 unrewarded (CS-). Each, CS+ and CS- were presented pseudo randomized with an ITI of 1 minute, 10 times.
3. *Post-acquisition phase [PostAcq]:* like in the pre-test phase all 5 odors are presented again 10 times without any kind of reward.

The subject was allowed to rest between the different phases. The resting time between phase 1 and 2 lasted 15-20 minutes whereas the PostAcq phase followed the Acq phase after 3 hours. Additionally we recorded the spontaneous activity for 3 minutes before

the pre-, and the post-test phase. For analyzing the acquisition phase behaviorally we added the data of experiments, were we did not test five different odors in the pre- and post-acquisition phase. In these experiments only the two odors which we used during the differential conditioning were presented 10 times each in the different experimental phases. In total, we successfully recorded 36 animals during the acquisition phase. Of those, we could test 29 animals for discrimination between CS+ and CS- in the post-acquisition phase, and 17 animals that received CS+, CS- and all three different control odors also in the post-acquisition phase. These different groups of animals were used by analyzing the behavior of the subjects during the different experimental phases and explaining the different numbers in figure 2.

Response detection

Response detection in each single trial

To decide in each trial if the presentation of an odor leads to a response in the recorded unit or not, we focused on the frequency changes of the events of the extracted units before and directly after the stimulus onset. These observable changes could occur in two directions: the odor presentation either leads to an increase or to a decrease in the spiking frequency. One valid measure for the frequency change is a change in the Inter-Spike-Interval (ISI) distribution. We focused on two observation windows. One was the 3 second lasting recording window before every stimulus onset. The other was the first 40 – 450 ms after the odor onset in which the most dramatically change in the ISI distribution occurred (Fig.1A). We then pooled intervals in the spontaneous observation window from all trials. To detect a significant ($P<0.1$) response in a single trial we used a Wilcoxon ranksum test as suggested by Hollander and Wolfe (1973), testing the nullhypothesis that the ISIs in the single trial response observation window are from the same distribution as the pooled ISIs in the spontaneous observation window. With this test we are able to detect both, excitatory responses (polarity 1) and inhibitory responses (polarity -1). The polarity of the occurring rate change indicated the comparison of the mean ISI distribution. ($P=1$; if the mean ISI of the spontaneous observation window was greater than the mean ISI of the phasic observation window; -1; if the mean ISI of the

spontaneous observation window was smaller then the mean ISI of the phasic observation window and 0 if the mean ISI of both windows are equal). Out of the 10 presentations of each single odor we calculated the reliability index RI as the odor presentations that evoked a detectable response divided by the total number of odor repetitions [see Fig 1B]).

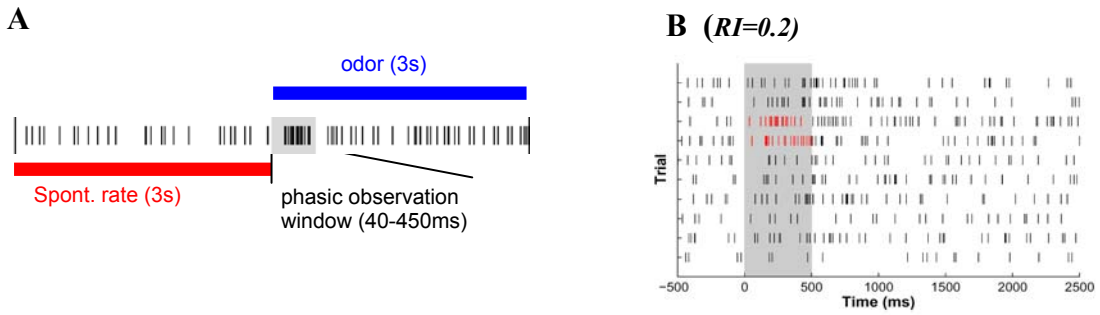

Figure 1: Reliability analysis. A; *The example spike train shows the spontaneous observation window (red) and the phasic observation window (grey). To decide if a response occurred or not the Inter- Spike-Interval (ISI) distribution in this windows were tested in the sense of significant differences via the Wilcoxon rank sum test (p<0.01). The blue bar marked the odor stimulus.* ***Polarity:*** *P=1 if the mean ISI of the spontaneous observation window > mean ISI of the phasic observation window (excitation); -1 if the mean ISI of the spontaneous observation window < mean ISI of the phasic observation window (inhibition) and 0 if the mean ISI of both windows are equal.* **B;** *The example illustrated a* ***R****eliability* ***I****ndex* ***(RI)*** *of 0.2 (RI=odor presentations that evoked a response divided by the total odor repetitions of one odor). Ten odor repetitions (from 1 lowest to the 10st top) are shown. The odor onset is market by 0 and last for 3 sec. The grey bar marked the 500 ms response detection window. Only in two trials a response is detected (red).*

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 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 3s window before stimulus onset and in the phasic observation window after stimulus onset (Fig. 1A). 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.

Results

Animal behavior

We observed the activity of single ENs using extra cellular recording technique before, during, and after differential conditioning. We were able to observe learning induced plasticity at this neuronal level that is related to the output of the MB. Besides the activity of ENs we also monitored electrophysiologically the proboscis extension response (PER) which was mediated by the muscle M17of the bee (Rehder, 1987 [see Fig.2]). Thus we were able to compare the behavior of the single bee with their internal neuronal activity. The stress produced by using electrophysiological methods in the present treatment did not influence the learning performance of the bees (Fig.2). The discimination rate for the CS+ and the CS- was significant after the 5^{th} trial (G-test: $G=7.2$; $p<0.01$; $df=1$[Fig.2B]). The developed association is significantly retrievable after the 3hours resting time (Fig.2C trial 1[$G=13.3$; $p<0.001$; $df=1$]). This association lasted for all of the presented tests and is also significant for the last trial (Fig.2C trial 10 [$G=5.7$; $p<0.05$; $df=1$]). Note, that there is no extinction observable. The 17 animals, that also received 5x10 odors in the preAcq and in the postAcq (see methods), were also able to discriminate all the control odors from the CS+ in the first trials of the preAcq test phase (Fig.2D trial 1 [$G=5.37$; $p<0.05$; $df=1$]). They did not generalize between the three control odors. Compared with the larger group of animals in figure 2C the smaller group of this subjects might extinct there behavior up to the 10^{th} trial (Fig.2D).

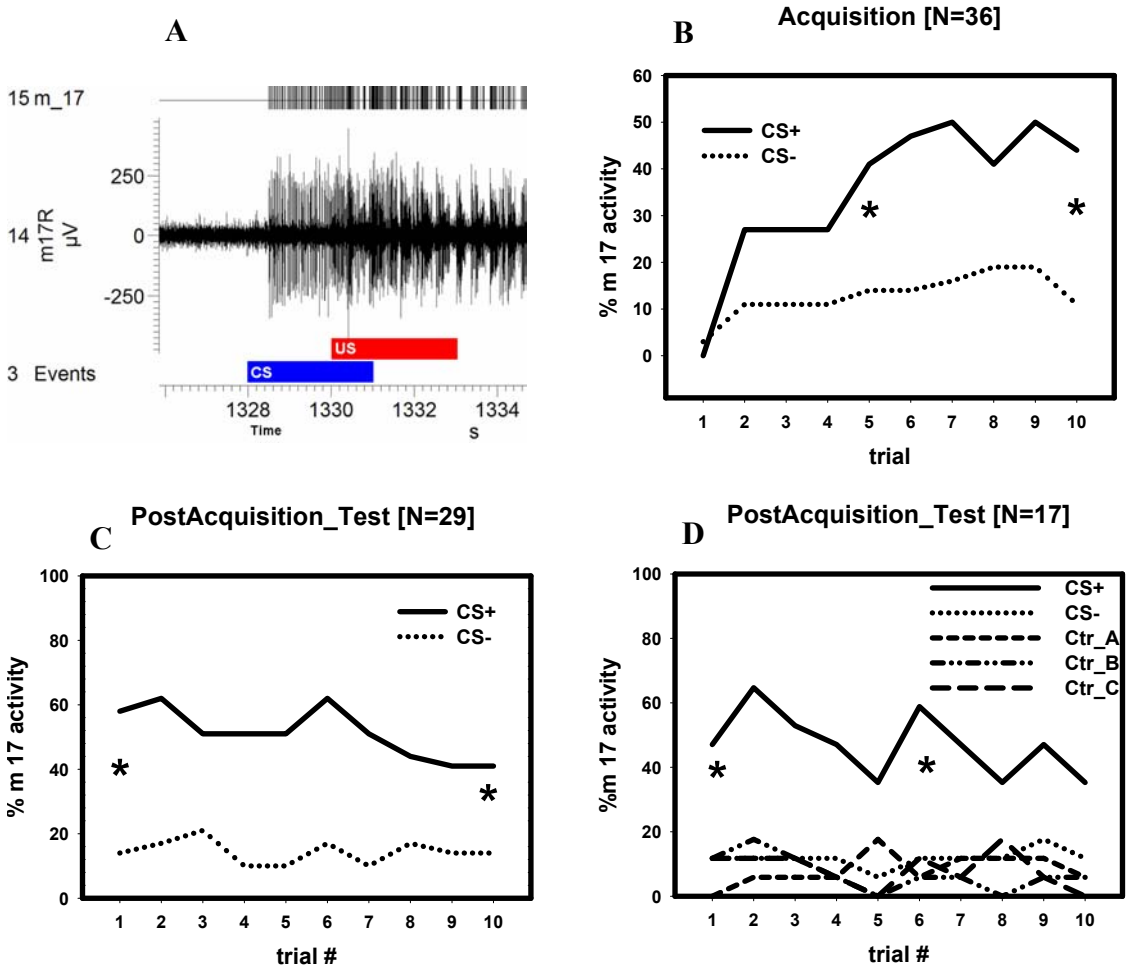

Figure 2: Monitoring of the Behavior. A; *The activity of the muscle M17 is used to observe simultaneously with the recording of the mushroom body extrinsic neurons [ENs] the behavior of the bee. The percentage of bees in which we were able to detect a response in the M17 canal in the related trial is drawn on the y-Axis. 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.* ***B:*** *Acquisition curve of the subjects. The bees learn to discriminate between CS+ (odor+reward) and CS- (different odor without reward) significantly ($p<0.05$) marked by asterisk (trial 5 [$G=7.2$; $p<0.01$; $df=1$] trial 10 [$G=10.5$; $p<0.01$; $df=1$]).* ***C:*** *In the post test phase after 3 hours the discrimination capability is stable for al 10 test trials. Note: In our experimental paradigm extinction doesn't occur. In all test trials the discrimination between CS+ and CS- is significant (trial 1[$G=13.3$; $p<0.001$; $df=1$], trial 9 and 10 [$G=5.7$; $p<0.05$; $df=1$]).* ***D:*** *Generalization test 3 hours after the acquisition for the 17 subject that received 5x10 odor stimulations in the pre- and the post- acquisition phase. The bees did not generalize between the odors. The CS+ is significantly differentiated from all other odors (trial 1 [$G=5.37$; $p<0.05$; $df=1$]; trial 6 [$G=8.79$; $p<0.05$; $df=1$]. Note; in these experimental group the last trials showing no differences between the CS+ and any of the other tested odors [trial 8-10 n.s.].*

Neuronal Response Characterization

Before investigating the learning induced changes in the ENs after the animals had made an association we defined the steady state of the ENs by characterising there initial, general response properties.

ENs were recorded extracellularly by measuring differentially from three electrode pair combinations of the inserted wire electrode (see Methods). After inserting the electrode without bias (see methods) we observed that some of the spontaneously active units did not respond to any of the odor presentations. These units we called “initially non responding” (24%). Another large group of units already responded to at least one of the presented odors; we call these 'initially responding' units (76%; Fig.3C). We used both the ISI distribution and the mean rate to detect an odor response in the trial-averaged activity (see methods and Fig.3 B).With both methods we were able to detect units that showing inhibitory responses and excitatory responses (Fig.3D). Note, that during the stimulation with air in all 10 trials there is no response detectable, indicating an uncontaminated odor supplying device.

Odor specificity

To characterize the properties of EN responses we focused on the initial responding units. To address the question of odor specificity we pooled all 10 repetitions of an odor and analyzed how many of the presented odors are effective in eliciting a response that can be detected with at least on of the two response detection methods (see Methods; Fig. 3B). 24 out of the 25 initially responding units responded to five or more odors either with excitation or inhibition. (Fig. 3D). In 16 putative ENs all ten odors evoked responses. Only one out of 25 units responded to only one odor. The number of effective odors shows that, in general, the ENs are odor unspecific.

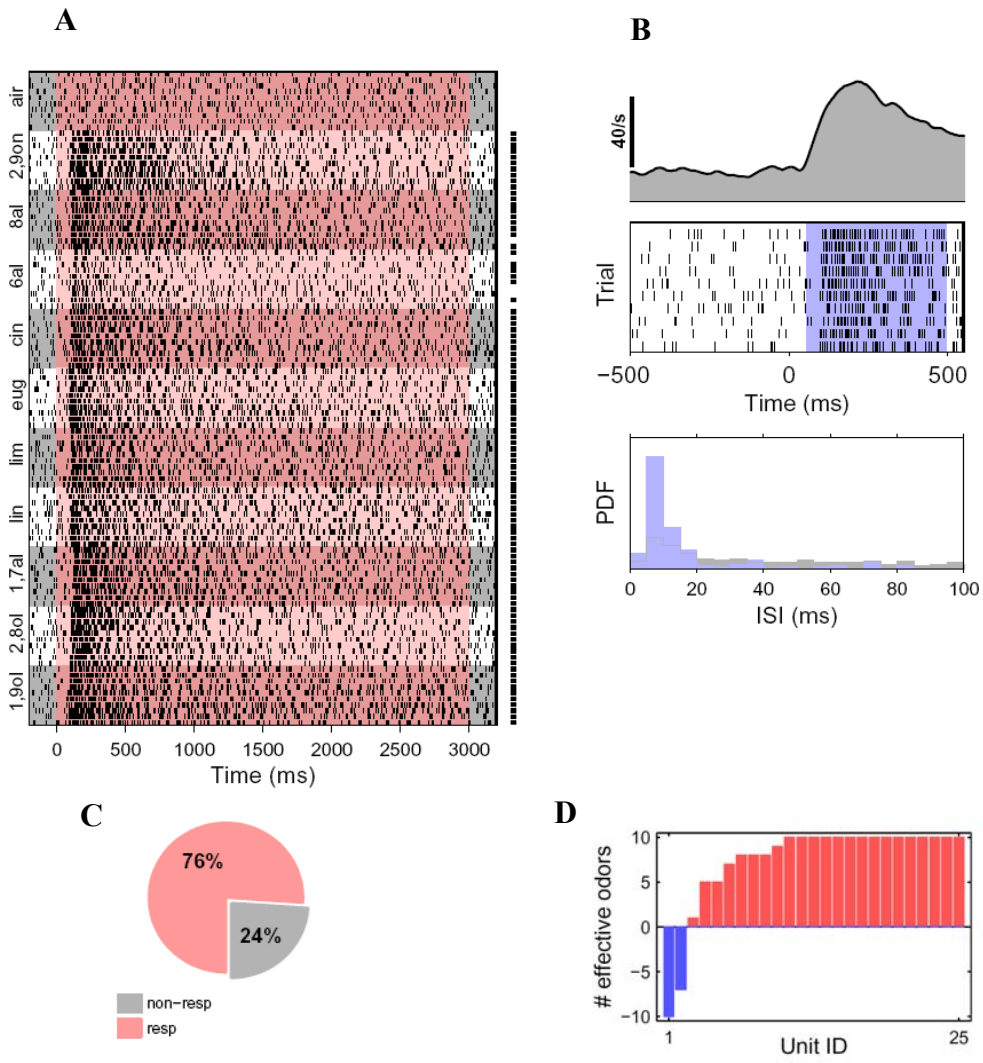

Figure 3. Odor response detection: ***A:*** *Raster plot of odor responses from one unit, with ten trials per odor. 1,9ol: 1-nonanol, 2,8ol: 2-octanol, 1,7al: 1-heptanal, lin: linalool, lim: limonene, eug: eugenol, cin: cineole, 6al: hexanal, 8al: octanal, 2,9on: 2-nonanon, air: paraffin oil only.* ***B:*** *zoom in into the ten repetitions of cineole (cin). Upper panel: pooled rate response. Middle panel: all ten trials, the response detection window is highlighted in blue. Lower panel: ISI distribution before (gray) and within (blue) the response detection window.* ***C:*** *Pie-chart of the percentages of initially non-responding units (gray), and initially responding units (red) that were detected by at least one of the detection methods.* ***D:*** *number of odors that evoked an detectable response in the related Units (red: excitatory; blue: inhibitory responding units)*

Reliability of Initial responding Units

By analyzing the trial by trial variability of the responses of the units to the 10 repetitions of one identical odor we found that although the initial responding units were excited or inhibited by many different odors (Fig. 3D) they did not respond in every trial. Focusing on the reliability indices [RI] of the different recorded initial responding units two groups can be described: reliable units (RIs> 0.8; black to dark green) and unreliable units (RIs< 0.4; green to light green) to the 10 repetitions to the different tested odors (Fig. 4A). In general, units that show smaller RIs also responded to a fewer odors (Fig. 4B). That gets obvious by comparing the RI matrix (Fig. 4A) with the binary response detection matrix (Fig. 4B). Focusing on the unit ID1 (Fig. 4) illustrates, that our binary response detection criterion for the pooled trials of one odor is very sensitive. Using the single trial response detection only 1 response is detected (RI=0.1 for lin) but in the pooled data for each odor we were able to detect a response in al of 10 odors.

Thus, there are two different behaving units. One group (~50%) responding very reliable these units responding also to al of the 10 odors. The other group of unreliable responding units, responded also more odor specific. Note, that these neurons became prominent when we analyzed in the following break the learning induced changes of the "plastic" units.

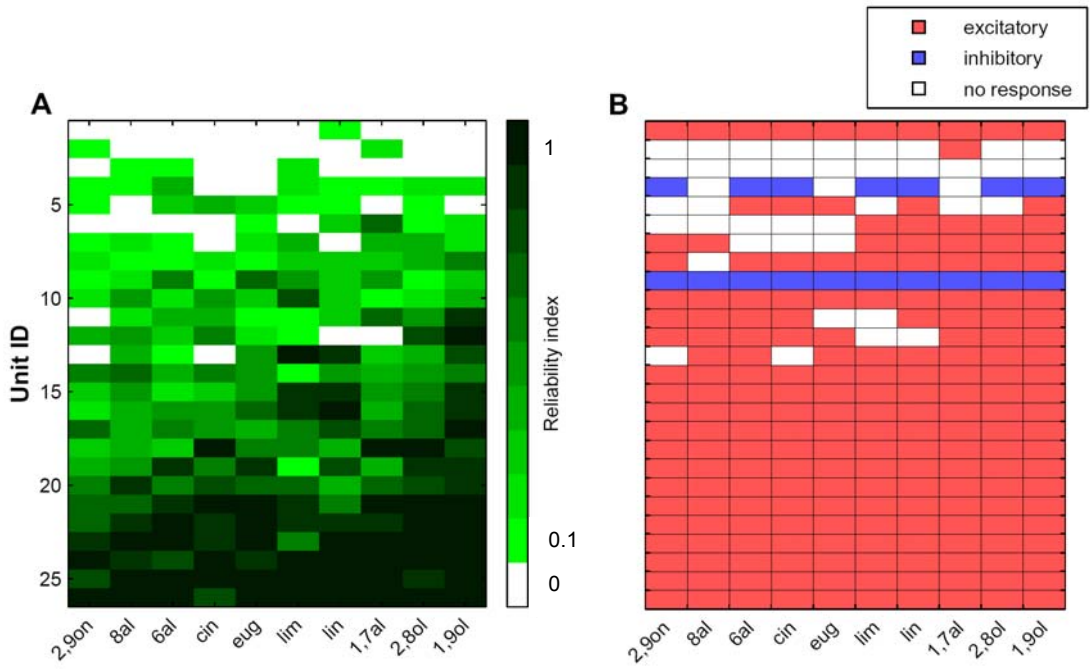

Figure 4: Trial by trial reliability and averaged binary response of 26 units. A: *False color coded Reliability Indices [RI] of the units that could have responded to 10 trials of 10 different odors. The lower units (20-26) responding very reliable (RIs> 0.8; black to dark green) to all of the 10 different odors (2,9on=2-nonanon; 8al=octanal; 6al=hexanal; cin=cineole; eug=eugenol; lim=limonene; lin=linalool; 1,7al=1-heptanal; 2,8ol=2-octanol; 1,9ol=1-nonanol). The upper units responded more unreliable (RIs< 0.4; green to light green). Note that the alpha-lobe extrinsic neurons show a broad response spectrum with very different reliability indices. White matrix elements refer to units that showed no response to any of the 10 repetitions of 10 odors.* ***B:*** *Binary responses for the 26 units. A response to an odor is detected if the pooled rate response in the response detection window is +/- 3 times above/lower than the SD of the pooled spontaneous activity rate* ***or*** *if the pooled ISI distribution between the spontaneous phase and the response detection window is significant different (Wilcoxon ranksum test; p<0.01). Note that this kind of responds detection is highly sensitive. Note that this response detection in rather sensitive as illustrated by unit ID1, were the RI matrix (A) shows that only in one single trial we are able to detect a response whereas in the pooled trials for each odor a response is detected.*

Learning induced changes

Initial non-responding units became recruited after differential conditioning

Focusing on the steady state of the initial untreated units we found 76% that are already responding unspecific to different odors (Fig. 3C). Although these units are odor unspecific (Fig. 3D) two groups appear. One group responded very reliably to various odors, whereas the other group of units responded more unreliably, in that not every presentation of one identical odor evoked a response in the related units (Fig. 4A). Besides these units which already initially responded to the presented odors, we also found 24% of units that were not responding initially (Fig. 3C). In the following learning experiment we addressed the question if building an association between an odor (CS) and a sucrose reward (US) could lead to a change in any of the described initial response properties, meaning that a initially non responding unit starts to respond, or unspecific and unreliable responding units become specific and/or reliable after conditioning. We used five different odors and presented them 10 times each before and after classical conditioning. Two of the odors were randomly chosen to be the rewarded odor (CS+) or the unrewarded odor (CS-) during a differential conditioning (Fig. 5). In principle the bees that were conditioned in our experimental procedure learned to discriminate between the CS+ and the CS- during the conditioning phase and remind that information after the 3h consolidation test (cp. Fig. 2). The two examples in Fig. 5 show two initially non responding units that were recorded in two different animals. None of the five different odors evoked a response in both units in the pre acquisition phase [PreAcq]. During the acquisition phase of differential conditioning the upper unit starts to respond during the one second overlap between CS and US. Note that the spontaneous rate of this unit started to decrease after the first two CS/US stimulations (Fig. 5C, trial number 61 and 62). This decrease did not occur in the lower example unit. In the post-acquisition phase [PostAcq] after the subjects were allowed to rest for 3 hours a clear response appears for the CS+ and the CS- presentations. The mean rates in the right column (pre acquisition [light grey]; post-acquisition [dark grey]) illustrating, that only the CS+ (highly) and the CS- (smaller) evoked a response in the upper unit the other three odors let the units be unaffected. The other unit seems to work in an opposite way, meaning that not the CS odors evoked a response, but rather the three “novelty”

test odors let the rate increase. Thus, the recruited putative ENs are responding odor specifically in a complex network.

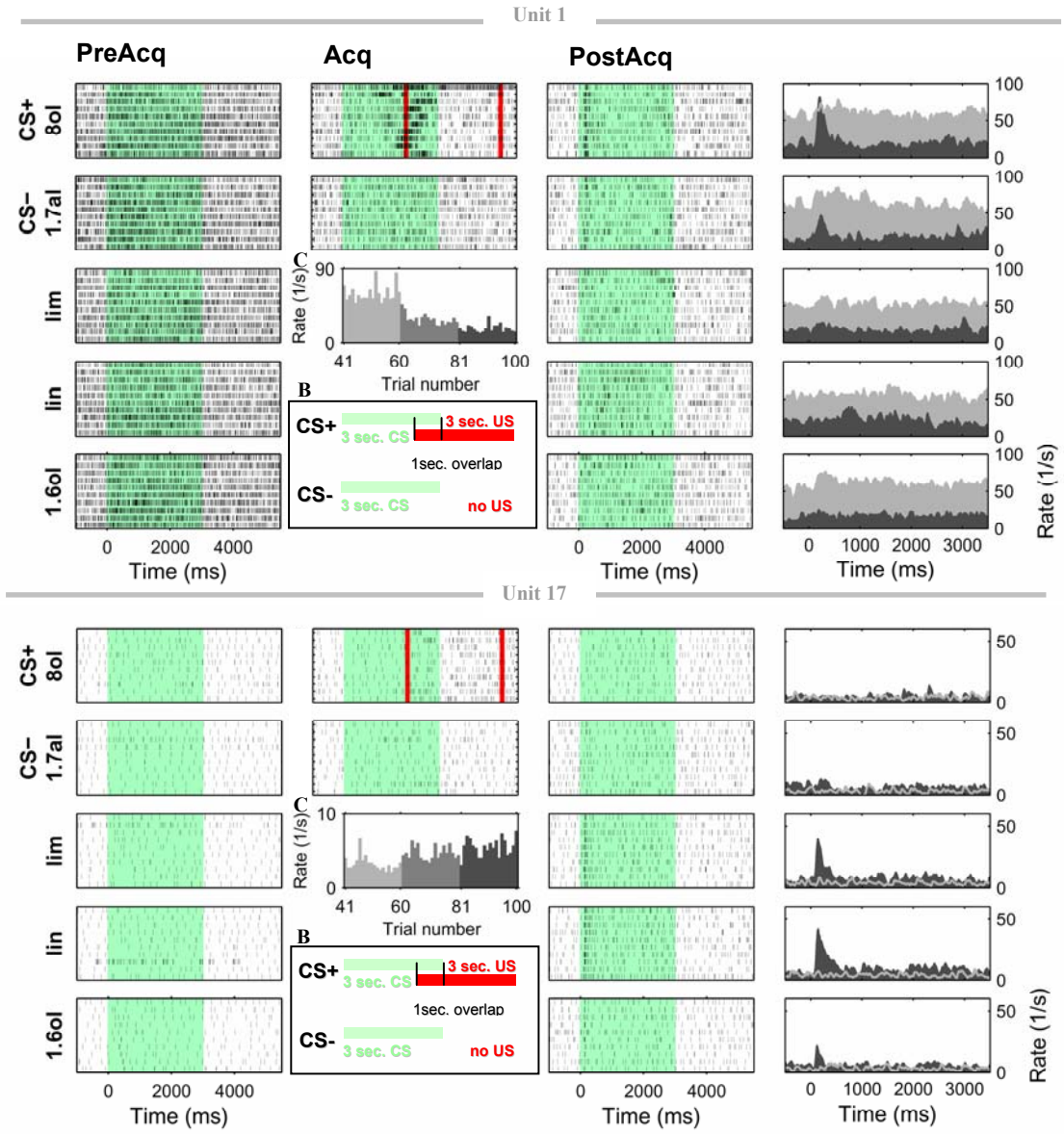

Figure 5. Responses before, during and after differential odor conditioning of two example units (ID1 and 17). *In the Pre-acquisition Phase* ***[PreAcq]*** *five different odors (1.6ol=1-hexanol, lin=linalool, lim=limonene, 1.7al=1-heptanal, 8ol=octanol) were presented in a pseudorandom manner 10 times each, the spike trains are ordered by there odor identity. During the acquisition phase* ***[Acq]*** *two of them were used by chance to be presented as rewarded odor* ***[CS+]*** *or as unrewarded odor* ***[CS-]****; for details look at inset* ***B****. The three seconds of odor stimulation are marked in green, the 3 sec. of US are marked in red. After the subject had rested for three hours all five odors were again tested 10 times each in the Post-acquisition Phase* ***[PostAcq]****. The comparison between the averaged rates of the PreAcq (light grey) and the PostAcq (dark grey) is shown in the right column (20ms bin size). Note, that both example units*

seem to behave in an opposite way. Both units were recruited to respond in the PostAcq within the first 500ms (phasic). Unit 1 responded for the CS+ and showed also a small response for the CS- but not for the three other test odors. Unit17 responded to the three tests, but not for the conditioned odors. Inset ***C:*** *rate histograms illustrating the spontaneous rate 3 seconds before the onset of the CS odors averaged over 20 trials (10 trials CS+ and 10 trials CS-). The different experimental phases are separated by grey color (light grey=PreAcq; middle grey=Acq and dark grey=PostAcq). The recruitment of the upper example unit is going along with a decrease in the spontaneous firing rate starting directly after the first two CS+ pairings during the Acq. (trial 61 and 62). In the lower example the spontaneous rate seems to be not effected.*

Stereotypic and plastic units in a learning neuronal network

After calculating the difference between the pre-acquisition phase and the post-acquisition phase we found two main categories of recorded units, namely units that did exibit differences in their pooled responses to the different odors between the pre- and the post- acquisition phase (~50%) and those that did not. Those units which show differences are therefore learning dependent plastic (Fig. 6, black squares). Both groups are including units which were initially non-responding. Note that in the group of the plastic units the initially non responding units are completely recruited after the subjects had built an association (Unit ID2, 16). Other plastic units that are already initially responding to a few odors are changing there response profiles to the different odors after differential conditioning. Thirty percent of them (unit ID1, 2, 3, 9, 10 and 11) did not exhibit a response to the CS+ in the pre-conditioning phase, but were recruited to respond to the CS+ odors in the post acquisition phase. Initially non responding units belonging to the stereotypic group are completely unaffected by the stimulation with the different odors; neither in the pre-acquisition phase nor in the post-acquisition phase any response to the 10 repetitions of the five used odors is detectable. Other stereotypic units were responding in the pre- and the post-acquisition phase to the same odor spectra and seemed to be not effected by differential conditioning (Fig. 6). Comparing the number of effective odors between stereotypic and plastic units during the test phases shows a clear difference between both groups (Fig. 6, right panel). The plastic units responded to a much smaller odor spectrum, whereas the stereotypic units responded to nearly all of the odors (Fig. 6, right panel).

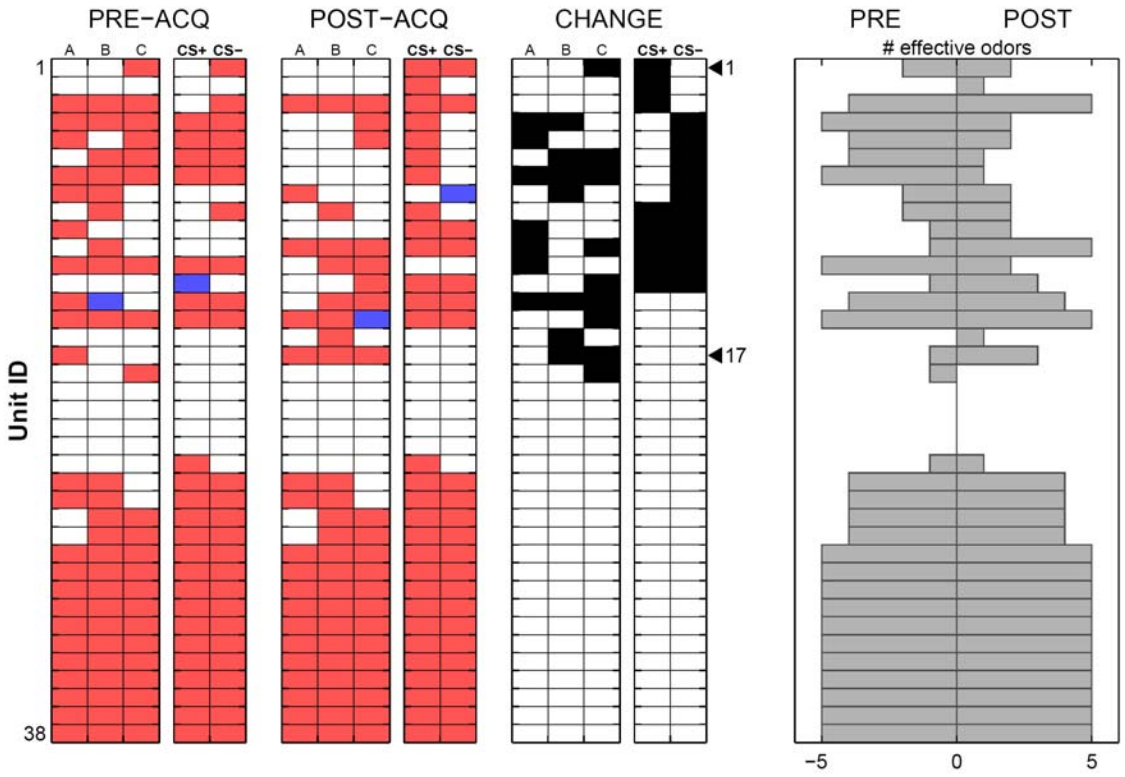

Figure 6. Stereotypic and plastic units. *Overview of the 38 units that were recorded from the 17 subject that received 5x10 odor stimulations in the pre- and the post-acquisition phase (for comparing the behavior of the subjects see Fig2D). In the pre-acquisition phase [PRE-ACQ] and in the post-acquisition phase [POST-ACQ] detectable responses of the pooled trials for the three control odors (A,B,C) and the CS odors (CS+ and CS-) were marked in red for excitatory responses, blue for inhibitory responses and white marking no detectable response (for response detection compare Fig.2B). Note that 30% were recruited to respond in the PRE-ACQ to the CS+ (unit ID 1, 2, 3, 9, 10, 11). In the" CHANGE"- column the difference between pre and post acquisition phase 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 between both experimental phases was detected. These units are called stereotypic. Note that there are also units related to this group which were not responding to any of the 5 different odors in both experimental phases. The example units shown in fig 5 are marked with arrows (unit1 and unit 17). The right column illustrates the number of odors that evoked a detectable response in the PRE and the POST-ACQ in the plastic units (top) and the stereotypic units (down). Note that the stereotypic units responded in both phases to nearly al of the 5 different odors, meaning they are odor unspecific. The plastic units responded in both phases to only a few odors, which make them rather odor specific.*

Non-conditioned and conditioned odors were represented by the activity of plastic units

By comparing the RI between the PRE-ACQ and the POST-ACQ of the plastic units it becomes visible that the changes are not random. The units rather changed their odor spectrum to be dominated by only a few odors. On average across all units, the respondes are more reliably to the CS+ odors (illustrated by figure 7B and 7C). Focusing on individual single units we found various different behaviors. For example, unit ID10 responded in the PRE-ACQ to odor A[RI=0.2], C[RI=0.1] and the CS-[RI=0.2]. After the conditioning, in the POST-ACQ the unit responded to odor B[RI=0.1] and the CS+[RI=0.7. Other plastic units changed their reliability spectrum more in the direction to the three different untrained odors than to the conditioned ones and are recruited to respond to them (cp. Unit ID17, Fig.5 and 7). Note that also highly reliable units were affected to decrease there reliability after conditioning (Fig. 7 unit ID 3, 4, 7, 12). Thus the learning induced change in the reliability indices on single unit level for the different odors can be manifold but may contribute to the associative network. However the animals made the right decisions as illustrated in figure 2.

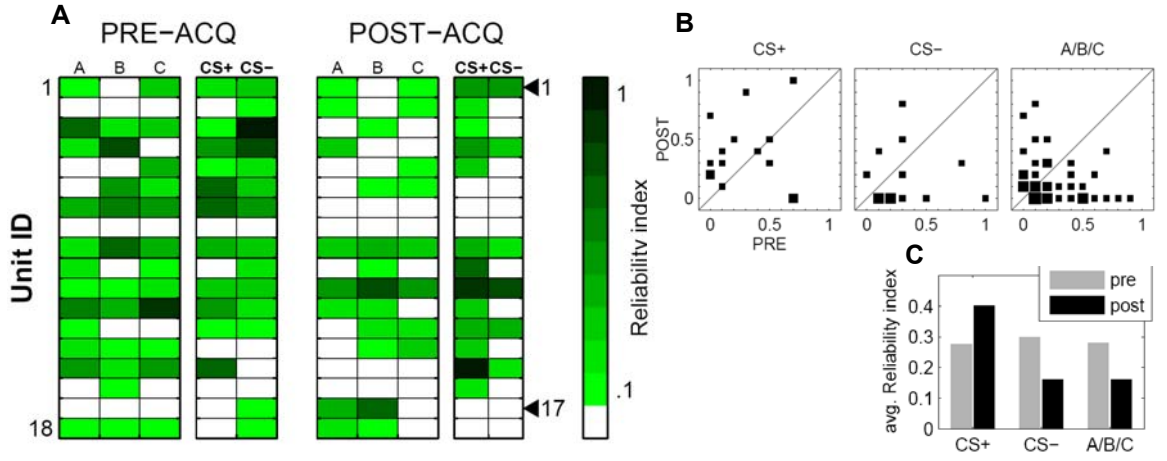

Figure 7. Reliability before and after differential odor conditioning of the plastic units (ID 1-18; cp. Fig. 8): A: *Each square marked one color coded reliability index* ***[RI]*** *which is the number of presentations of one particular odor that evoked a significant response divided by the total number of presentations of that odor (see also Fig.4A). Comparing both experimental phases, it gets visual that the response spectra of the plastic units are changing. E.g. unit ID 10 responded in the pre-acquisition phase* ***[PRE-ACQ]*** *to odor A [RI=0.2], C[RI=0.1] and the CS-[RI=0.2]. After the conditioning, in the* ***[POST-ACQ]*** *the unit responded to odor B[RI=0.1] and the CS+[RI=0.7] for which it is recruited. The example units shown in figure 5 are marked by arrows (unit1 and 17). Inset* ***B and C*** *The comparisons between the RIs of the PRE-ACQ and the POST-ACQ for the CS+, the CS- and the three additional test odors (A,B,C) together were illustrated in* ***B*** *for the single units and in* ***C*** *averaged across units. RIs that were 0 in both phases (PRE- and POST-ACQ) were excluded. In* ***C*** *the averaged RIs separately for both phases are shown excluding similar RIs ratios (diagonal of* ***B****). Note, that the averaged RIs of the PRE-ACQ are nearly the same for al three odor types, only for the CS+ the averaged RIs increasing after conditioning. The other RIs are decreasing in the POST-ACQ. However, the individual RI changes of the single units are manifold (cp. A).*

Discussion

In the present study we were able to extend the characterization of initially responding and non-responding ENs (Strube-Bloss et al., 2008a; chapter 1), by characterizing the learning induced changes of single-unit response which occurred during and after differential odor conditioning.

Stereotypic and plastic units

We were able to show that not all units were affected by the presented learning paradigm. Two classes of such *stereotype* units were found. One group did never respond during the pre- and the post-acquisition phase. The other group is responding very reliably to the different odor presentations. Units belonging to this group are also rather odor unspecific and can be related to the initially reliably responding units (Strube-Bloss et al., 2008a; chapter 1).
Other units were clearly affected by the presented learning paradigm. These *plastic* units consisting of initially non-responding units (*pre* acquisition) which were recruited to specifically respond to one or more of the presented odors in the post-acq-test phase (Fig. 6). The other group of plastic units is responding initially rather unreliable to only very few odors. The odor spectrum and the reliability of these units were affected by differential conditioning. The contributions to the neuronal network that processes the conditioned (CS+ and CS-) and the untrained odors (A, B, C) may be different for these different neuron types. However, the tested bees learned to discriminate the CS+ and the CS- (acquisition), and remembered that information after 3 hours of resting time without generalizing between the untrained odors (Fig. 2).

Recruitment of initially non responding units

We found the most dramatic difference in the comparison between the pre-acquisition phase and the post-acquisition phase in initially non-responding units that were completely recruited to respond to the different presented odors after the animals had build their associations. Typically, units belonging to that group were not only

recruited to respond to the CS+ or the CS- odors, but we could also observe recruitment to various combinations of responses of the three test odor. This is not surprising considering the fact that the subjects had to discriminate between odors to make the decision if they should extend their proboscis or not. To make this decision, the underlying network had to realize and categorize all occurring odors. After the conditioning and the three hours of resting time the units at the output stage of the MB appear to reflect the values of the different odors used during the present learning paradigm.

The main input into the ENs comes from the MB intrinsic neurons, the Kenyon Cells [KC]. Yu et al. (2006) demonstrated that in *Drosophila* a protein-synthesis-dependent, delayed memory trace is formed in the axons of the KCs 3-9 hours after a spaced training. In the present study the post-acquisition test took place 3 hours after the bee was allowed to rest. After that time we observed units that were completely recruited and changed their odor response spectrum (Fig. 6 and 7). The plasticity change observed in the KC axons by Yu et al. (2006) may drive the related output neurons which were now excited by the related KC input. However, the question is which synapse had changed, the one between PNs and KC or the one between KC and ENs? Or are the ENs in themselves plastic? Possibly both mechanisms are in effect. The best studied EN in the honeybee brain is the PE1. It is known, that electrical stimulation of the KCs leads to a formation of associative long-term potentiation [LTP] in that neuron (Menzel and Manz, 2005). However, the PE1 can be not related to initially non-responding ENs and is maybe involved in other parallel memory processing as the completely recruited ENs. Also in other insect species there is much evidence that the ENs themselves are plastic, e.g. in locusts where spike timing dependent plasticity [STDP] occurs between KCs and β-lobe neurons (Cassenaer and Laurent, 2007). Another example comes from Drosophila, where a delayed memory trace is formed after 30 minutes only in the vertical (α-lobe) branch of the dorsal paired medial neuron [DPM] which is an odor generalist (Yu et al., 2005) like the most ENs in the honeybee (Strube-Bloss et al., 2008a; chapter 1). In this paper we found plastic units that were initially non-responding and recruited to respond after a three hours delayed memory test. We were not able to resolve the issue of the synaptic level which changed but in the following we will discuss the prerequisites of the observed recruitment. Following

the idea that KCs are supposed to respond highly odor selective and sparse in *Drosophila* (Turner et al., 2007; Wang et al., 2004), locusts (Jortner et al., 2007; Perez-Orive et al., 2002; Stopfer et al., 2003) and honeybees (Szyszka et al., 2005) would mean that the respective odor and the sucrose stimulation alone evoked a very special KC pattern. Both stimuli occurring together would produce a separate KC pattern. Szyszka (2005b) discussed coincidence detection between CS and US at the level of clawed Kenyon Cells [cKC]. The coincident occurrence of odor and reward prolonged and/or increased the odor responses in the cKCs. The unit1 shown in the example in figure 5 starts already to respond during the acquisition phase, but only if both stimuli (CS and US) are overlapping. That may be driven by the increased activity of the cKCs (Szyszka, 2005b). This coincidence between the odor and the reinforcer seems to be detected by the recorded unit and seems to be one character and the prerequisite for an EN to be recruited. However, after the three hours resting time the unit showed a clear response to the CS+.

Odor learning drives ENs to be more reliable

The plastic units which we reported on seem to be very special and obviously involved in memory formation. The responses of these units in the pre-acquisition phase are rather unreliable to the odors which were effective in these neurons. After the acquisition and the three hours resting time the units changed their reliability for different odors whereby this change was dominated by the CS+ (Fig .7). What mechanism should drive these neurons to respond initially unreliably, but more reliably after consolidation?

Taking into account that KCs respond in a reliable odor selective and sparse manner (cp. previous paragraph), each occurrence of an odor (each trial) would evoke a specific activity pattern in the KCs and would drive the ENs to respond. But this is not the case. Accordingly, there must exist an additional mechanism upstream of the ENs that influenced the transmission between the odor activated KCs and the ENs. There is large evidence that the alpha-lobe-extrinsic neurons of the protocerebral calycal tract (PCT) which are GABA immunoreactive (ir) inhibitory neurons are involved in that mechanism. These neurons could 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). The inhibitory activity of these neurons on strategically important synapses should drive the ENs of the ventral part of the MB to be sometimes silent and sometimes active to the actual relevant stimulus. It is still unclear what drives the GABA ir PCT neurons to be some times active and sometimes not. Possibly these neuron's are involved in an attention-related process and convey the internal state of the animal to realize only sometimes the presented odor. To approximate to the answer of these question it would be necessary to record both types of alpha lobe extrinsic neurons simultaneously and investigate the correlations between there respective activity.

However, the reliability to respond to an odor is increased after differential odor conditioning, meaning that nearly every trial of the respective odor leads to a response in the recorded unit. If the cause of unreliability before learning was the inhibition by the PCTs and the units are now much more often excited by the odor, the inhibition by the PCTs should be gone. Grünewald (1999) recorded intracellular from PCT neurons and found a reduction of the response by presenting an odor/sucrose pairing. This is only a hint in the direction, that the pairing of an odor with sugar can reduce the inhibition mediated by the PCT neurons, but it would explain the increased reliability after learning.

Spontaneous rate change

One observation that was made in several units was that the spontaneous rate changed, mostly after the first CS/US pairing (cp. Unit1 Fig. 5). Because of the occurrence during the spontaneous activity this should be a network effect, which may be driven by an inhibition which down-regulates the overall activity in the corresponding MB network, possibly to increase the contrast for the associated odor. Interestingly this effect does not show up in any of the recorded units that were recruited. Also simultaneous recorded units show different effects regarding the down regulation of their spontaneous rate. I.e. one unit could be down regulated whereas the spontaneous rate of a second unit remained constant. If the down regulation is activated

by inhibition the cause could be related to the GABA-immunoreactive (ir) inhibitory feedback neurons (Bicker et al., 1985; Gruenewald, 1999) of the protocerebro-calycal-tract (PCTs). One idea of how to test for the influence of the PCT neurons would be the local blocking of GABA during the acquisition phase. This should disconnect the inhibitory component from the computing network and the state of the pre conditioning phase should again be observable. However, the different spontaneous rate changes in simultaneously recorded units could be also a hint for parallel processing of the association, and should be tested in further experiments.

Different types of memory require different ENs

We demonstrated that there are different behaving units collecting their information from the KC of the MB. These neurons connecting the MB with other neuropiles of the bee brain. Thereby the information from around 170000 KCs converged to only around 400 ENs. Obviously the decoding of the received stimuli is already done, because a reduction to only 400 neurons would imply a loss of lots of features of the strongly decoded stimulus. Since the MBs are thought to be the centers for memory formation (Dujardin, 1850; Strausfeld, 1998, review) the neurons transmitting their output should include and reflect memory formation by changing their response properties. 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), the neural basis of the memory trace is a hot topic in research. In the honeybee 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 AL, the calyces of the MB and the LPL. During the reward presentation, the VUM mx1 activates all of these neuropiles simultaneously. Meaning that directly after the first CS/US pairing a possible memory trace exists, that includes all mentioned neuropiles. We don't know yet, If the whole network represents the steady state of memory, or if the different memory are divided into different brain regions. 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 honey bees 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. Possibly early processing stages of the olfactory system showing plastic effects but this does not necessarily mean that they are involved in memory formation.

Rather should one higher order level into the brain represent the different memory phases. The ENs of the MB showing different 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). That could mean that early long term memory and stable long term memory can be represented at the same neuronal level. Following the categorization of Strube-Bloss (2008a; Chapter 1) the PE1 would be an initially responding neuron. Other units related to this group also increased or decreased there rate response to the conditioned odor after few pairings with the reward. These neurons reflecting already short term memory related changes. Other ENs like the initially non responding units, that were recruited to respond after the tested three hour time window seem to highly reflect long term memory related changes. The neuronal network is the same but the mission and the contribution to the different memory traces is different for the single extrinsic neuron.

Conclusion

The general response properties like odor specificity and reliability to the repetition of one and the same stimulus of alpha lobe extrinsic neurons are features that can be influenced by learning. At this neuronal level we found units that became recruited after learning, these units starting already during the acquisition to detect the coincidence between CS and US. Other plastic units changed there odor spectrum to which they respond. These changes are going along with increasing reliability, which we found is dominated by the CS+, but at the single neuronal level also by different odors (Fig. 7). The significant changes in the different directions may support the idea, that the mushroom body is a centre memory formation, because the prerequisite for the formation of different memory traces is parallel processing in the sense of consolidation, reconsolidation, formation of the different memory traces (short, midterm, long-term memory).

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References

Bicker, G., S. Schafer and T. G. Kingan (1985). Mushroom body feedback inter neurons in the honeybee show GABA-like immunoreactivity. *Brain Res* **360**, 394-397.

Bicker, G., Kreissl, S. and Hofbauer, A. (1993). Monoclonal antibody labels olfactory and visual pathways in Drosophila and Apis brains. *J Comp Neurol* **335**, 413-424.

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.

Cassenaer, S. and Laurent, G. (2007). Hebbian STDP in mushroom bodies facilitates the synchronous flow of olfactory information in locusts. *Nature* **448(7154)**, 709-713.

Davis, R.L. (2004). Olfactory learning. *Neuron* **44**, 31-48.

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.

Farkhooi, F., Strube-Bloss, M.F. and Nawrot, M.P. (submitted). A non-renewal point process model for neuronal spiking.

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.

Goldman-Rakic, P.S. (1995) Cellular basis of working memory. *Neuron* **14**, 477-485.

Grünewald, B. (1999). Morphology of feedback neurons in the mushroom body of the honeybee, *Apis mellifera*. *J Comp Neurol* **404**, 114-126.

Grünewald, B. (1999b). 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.

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

Jortner, R.J., Farivar, S.S. and Laurent, G. (2007). A simple connectivity scheme for sparse coding in an olfactory system. *J Neurosci* **27(7)**, 1659–1669.

Kuwabara, M. (1957), Bildung des bedingten Reflexes von Pavlovs Typus bei der Honigbiene *Apis mellifica*. *J Fac Sci Hokkaido Univ Ser VI Zool*. **13**, 458-464.

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. (1999). Memory dynamics in the honeybee. *J Comp Physiol **185***, 323-340.

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. and Müller, U. (1996). Learning and memory in honeybees: From behaviour to neural substrates. *Ann Rev Neurosci* **19**, 379–404.

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.

Menzel, R., Hammer, M., Braun, G., Mauelshagen, J. and Sugawa, M. (1991). The Behaviour and Physiology of Bees. *CAB International Wallingford, CN.* 323-351.

Milner, B. Squire, L.R. and Kandel, E.R. (1998). Cognitive neuroscience and the study of memory. *Neuron* **20**, 445-468.

Mizunami, M., Weibrecht, J.M. and Strausfeld, N.J. (1993). A new role for the insect mushroom bodies: Place memory and motor control. In: *Biological Neural Networks in Invertebrate Neuroethology and Robotics*. (eds. R. D. Beer, R. Ritzmann, and T. McKenna) **pp.** 199–225. Academic Press, Cambridge, MA.

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.

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.

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.

Peele, P., Ditzen, M., Menzel, R. and Galizia, C. G. (2006). Appetitive odor learning does not change olfactory coding in a subpopulation of honeybee antennal lobe neurons. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **192**, 1083-103.

Perez-Orive, J., Mazor, O., Turner, G.C., Cassenaer, S., Wilson, R.I., and Laurent, G. (2002). Oscillations and sparsening of odor representations in the mushroom body. *Science* **297**, 359-365.

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

Rescorla, R.A. (1988). Pavlovian conditioning: It's not what you think it is. *Am Psychol* **43**, 151-160.

Rolls, E.T., Critchley, H.D., Mason, R., Wakeman, E.A. (1996). Orbitofrontal cortex neurons: Role in olfactory and visual association learning. *J Neurophysiol* **75**, 1970-1981.

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.

Schultz, W. (1998). Predictive reward signal of dopamine neurons. *J Neurophysiol* **80**, 1-27.

Stopfer, M., Jayaraman, V. and Laurent, G. (2003). Intensity versus identity coding in an olfactory system. *Neuron* **39**, 991-1004.

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-Bloss, M.F., Farkhooi, F., Nawrot, M.P. and Menzel, R. (2008a; chapter1) Odor specificity and reliability of alpha-lobe extrinsic neurons in the honeybee.

Sutherland, G.R. and McNaughton, B. (2000). Memory trace reactivation in hippocampal and neocortical neuronal ensembles. *Curr Opin Neurobiol* **10**, 180-186.

Szyszka, P. (2005a). Odor coding and neural plasticity in the mushroom body of the honeybee. *Dissertation*, Institute for neurobiology Freie Universität Berlin

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

Turner, G.C., Bazhenov, M. and Laurent, G. (2008). Olfactory representations by *Drosophila* mushroom body neurons. *J Neurophysiol* **99**, 734-746.

Vareschi, E. (1971), Duftunterscheidung bei der Honigbiene: Einzelableitungen und Verhaltensreaktionen. *Z Vgl Physiol* **75**, 143-173.

Wang, Y., Guo, H.-F., Pologruto, T.A., Hannan, F., Hakker, I., Svoboda, K. and Zhong, Y. (2004). Stereotyped odor-evoked activity in the mushroom body of *Drosophila* reavealed by green fluorescent protein-based Ca2+ imaging. *J Neurosci* **24,** 6507-6514.

Wilson, R.I. and Mainen, Z.F. (2006) Early Events in Olfactory Processing. *Ann Rev Neurosci* **29**, 163–201.

Yu, D., Ponomarev, A. and Davis, R. L. (2004). Altered representation of the spatial code for odors after olfactory classical conditioning: Memory trace formation by synaptic recruitment. *Neuron* **42**, 437–449.

Yu, D., Keene, A.C., Srivatsan, A., Waddell, S. and Davis, R.L. (2005). *Drosophila* DPM neurons form a delayed and branch-specific memory trace after olfactory classical conditioning. *Cell* **123**, 945–957.

Yu, D., Akalal, D.B. and Davis, R.L. (2006). Drosophila alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. *Neuron* **52**:845-855.