Neural reward systems in the honeybee: Characterizing the involvement of the mushroom body (MB) extrinsic neurons in reward processing

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Reversal learning in honeybees

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by

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CONTENT

SUMMARY

This thesis is twofold; neuronal reward systems of the honeybee (*Apis mellifera*) were characterized using extracellular recording techniques aimed at recording from the mushroom bodies' extrinsic neurons (ENs), and memory formation of the honeybee was studied using a pharmacological approach. To this end different learning paradigms were used: olfactory reversal learning and a simple form of learning - habituation of the proboscis extension reflex. Both paradigms were studied while recording from the ENs. Applying protein synthesis inhibitor (emetine) the formation of long term memory was studied using reversal learning paradigm.

Chapter I - the effect of a protein synthesis inhibitor (emetine) on the memory formed after reversal learning was investigated. Summer bees and winter bees were studied, each yielded different results. In summer bees emetine was found to inhibit the consolidation of the excitatory learning following reversal, and in winter bees emetine was found to block the consolidation of the inhibitory learning.

Chapter II – here the effect of emetine on the memory consolidation formed after differential learning in winter bees was studied, again using reversal learning paradigm. Emetine was shown to block the spontaneous recovery following reversal learning.

Chapter III- extracellular recordings from mushroom bodies' extrinsic neurons (ENs) were performed while bees were exposed to an olfactory reversal learning paradigm. It was found that a sub-population of the ENs developed a learning-related neuronal response to the unrewarded odor during acquisition of differential learning. No learning-related changes were observed in the ENs following and during reversal learning.

Chapter IV – a non-associative form of learning, habituation of the proboscis extension response, was studied whilst conducting extracellular recordings from the ENs. Only a small sub-population of the ENs was found to respond to the solely presentation of reward to the antenna, out of which one cell habituated its response resembling one neuronal basis for behavioral habituation.

ZUSAMMENFASSUNG

Die vorliegende Dissertation umfasst zwei Themengebiete; neuronale Belohnungssysteme der Honigbiene (*Apis mellifera*) wurden mit Hilfe extrazellulärer Ableitungen Pilzkörper extrinsischer Nervenzellen (EN) charakterisiert. Darüber hinaus wurden pharmakologische Methoden angewandt, um die Ausbildung von Gedächtnisspuren, ebenfalls anhand der Honigbiene, zu untersuchen. Zu diesem Zweck wurden verschiedene Lernparadigmen verwandt: Olfaktorisches Umkehrlernen und eine einfache Form des Lernens – Habituation des Rüsselreflexes (PER). Beide Paradigmen wurden während der Ableitung der EN durchgeführt. Unter Anwendung des Hemmstoffes der Proteinbiosynthese, Emetine, und unter gleichzeitiger Durchführung des Umkehr-Lernparadigmas wurde die Bildung von Lernen und Langzeitgedächtnis studiert.

Kapitel I - untersucht wurde der Effekt des Proteinbiosynthese Hemmstoffes (Emetine) auf die Gedächtnisbildung nach dem Umkehrlernen. Sommer- und Winterbienen wurden mit unterschiedlichen Resultaten untersucht. In Sommerbienen blockierte Emetine die Konsolidierung des exzitatorischen Lernens nach der Umkehrung, in Winterbienen wurde hingegen die Konsolidierung des inhibtorischen Lernens durch den Hemmstoff verhindert.

Kapitel II – hier wurde der Effekt von Emetine auf die Gedächtniskonsolidierung nach differentiellem Lernen in Winterbienen exploriert, abermals unter Anwendung des Umkehr-Lernparadigmas. Hier konnte herausgefunden werden, dass Emetine das spontane Wiederauftreten der Verhaltensantwort (PER) nach dem Umkehrlernen hemmt.

Kapitel III – während die Bienen dem olfaktorischen Umkehr-Lernparadigma ausgesetzt waren, wurde erneut extrazellulär von Pilzkörper extrinsischen Neuronen abgeleitet. Es wurde herausgefunden, dass eine Subpopulation dieser Zellen eine lernrelatierte neuronale Antwort auf den unbelohnten Duft während der Akquisition der differentiellen Konditionierung etablierten. Keine lernrelatierten Veränderrungen dieser Neurone wurde während oder nach dem Umkehrlernen festgestellt.

Kapitel IV – eine non-assoziative Form des Lernen – Habituation des Rüsselreflexes (PER) - wurde während der extrazellulären Aufnahme der Pilzkörper extrinsischen Neurone untersucht. Eine kleine Subpopulation dieser Neurone antworteten ausschließlich auf die Zuckergabe auf die Antenne, eine dieser Zellen habituierte ihre neuronale Zuckerantwort, welches die neuronale Grundlage für die Verhaltenshabituation widerspiegelt.

GENERAL INTRODUCTION

ABSTRACT

Learning is considered to be a biological process that facilitates an organism's adaptation to its environment. Each life form relies on an effective accomplishment of basic biological functions, to which the adequate physiological systems have evolved. Nevertheless, physiological processes can be improved and finely tuned in order to adapt to a given environment or a social context, this is achieved by learning. Prior experience which result in lasting changes in behavior can be characterized as learning and memory processes. In the past generation, the study of neuroscience challenges with the investigation of cellular and molecular mechanisms and the identification of cellular circuits and networks underlying learning and memory formation.

The objectives of this thesis were to investigate neuronal reward processing in both associative and non-associative learning, and to elucidate the effects of protein synthesis on the consolidation of excitatory and inhibitory associations formed into long term memory after a complex form of associative learning. To this end the honeybee (*Apis mellifera*) was studied using two different learning paradigms, reversal learning and habituation. General concepts of non-associative and associative learning, memory consolidation, excitatory and inhibitory learning will be first introduced, followed with the rational to study those processes using reversal learning paradigm and habituation paradigm. The honeybee as a model system will be then shortly presented followed by the main objectives of this work.

Non-associative and Associative learning

Animals have to learn to adjust their behavior to a constantly changing and unpredictable environment; this requires either acquisition of new behavior or the inhibition of a previously common response and is achieved through one of the two basic categories of learning: non-associative and associative learning. Non-associative learning results from an organism's experience with a single stimulus or event, and associative learning contracts causal relations

between two or more stimuli in an organism's environment (Domjan, 2010). In both types of learning the behavioral response following learning could be either increased or decreased. For example habituation and sensitization are two forms of non-associative learning where the former results in a decrease of response to repeated stimulation and the latter in an increase of response. In associative learning, pairing a stimulus (CS) with reinforcement (US) might result in the emergence of a behavioral response to the CS but learned responses can also be reduced and inhibited if the CS is presented in the absence of the US, i.e. extinction learning (Domjan, 2010).

Memory consolidation

Memories are internal representations of the world which serve to guide behavior (Dudai, 2002). Animals' experience with environmental stimuli should be efficiently stored and retrieved to allow prediction of the immediate future, this holds true for both forms of learning. Consolidation is the stabilization process of a memory following acquisition, and this process requires a cascade of intracellular events (McGaugh, 2000; Dudai, 2002). Already in 1885 Ebbinghaus was able to demonstrate that newly acquired associations are labile to interference shortly after acquisition but become resistant over time. It was then suggested that during this sensitive time window following learning, memories become stabilized, a process called consolidation (Müller & Pilzecker, 1900). Many studies have since confirmed this view and found that the formation of long-term memories (LTM) can be disturbed upon administration of amnestic agents during this discrete time window following learning (McGaugh, 1966). The effects of protein synthesis inhibitors on LTM have been thoroughly investigated in a wide range of paradigms and it seems that protein synthesis is crucial during or immediately after learning (Davis and Squire, 1984).

Excitatory and inhibitory learning

Pavlov (1927) was the first to demonstrate that new behaviors could be established through mechanisms of association. Pairing a neutral stimulus (CS) with reinforcement (US) might result in the emergence of a behavioral response to the CS, termed excitatory learning. An association between the CS and the US is formed such that the presentation of the CS leads to behavioral

response formerly elicited by the US. But learned responses can also be reduced and inhibited if the CS is presented in the absence of the US, i.e. extinction learning, here a new association is formed where the CS predicts the absence of reinforcement. Different behavioral phenomena such as renewal, reinstatement, rapid acquisition and spontaneous recovery suggest that extinction learning does not destroy the CS-US association, but rather result in a new association: CS-no US (Myers and Davis, 2002; Bouton, 2004). Extinction learning is a process which also depends on protein synthesis, hence supporting the hypothesis that a new association between the CS and no US is formed rather than a mere abolishment of the old CS-US association (Pedreira & Maldonado, 2003; Berman & Dudai, 2001).

Reversal learning paradigm

At its mundane form, reversal learning paradigm begins with the pairing of a stimulus (CS+) with a US; once the animal learned this association, the reversal phase begins, in which the formerly associated stimulus is no longer presented in the presence of the US, but rather a different stimulus predicts the appearance of the US. For example, reversal learning in the Morris water maze starts with a training phase in which animals learn to locate a hidden platform (CS+), subsequently the reversal phase begins with the platform located in an opposite quadrant of the maze and animals learn to reverse their responses to the new location. However, reversal learning can be designed such that two opposing stimuli will be introduced in both learning phases, as in the reversal of differential conditioning. In such a design, learning begins with alternate presentations of one stimulus (CS+) paired with a US while another stimulus is paired with non-reinforcement (CS-), in the reversal phase the contingencies of the stimuli are inversed. Designed such, reversal learning paradigm allows the study of both excitatory and inhibitory learning in each phase of the paradigm, i.e.in the differential and in the reversal phase.

The honeybee as a model system

A useful research approach for investigating learning processes is using a model system: focusing on an organism that exhibits the desired form of learning and has an accessible nervous system.

Models permit investigation of desired aspects under more simple and controlled conditions. One such valid model in the domain of neuroscience is the honeybee (Apis mellifera) which was profoundly investigated in various learning and memory paradigms (Menzel and Erber, 1978; Menzel et al., 1993; Menzel, 1985). The honeybee's proboscis extension reflex is an appetitive component of its feeding behavior and was found to be successfully conditioned with olfactory stimuli in a classical form of conditioning (Takeda, 1961; Bitterman et al., 1983). If the antenna of the honeybee is stimulated with a sugar solution (US) the mandibles open and the proboscis is extended. When an olfactory stimulus (CS) is paired with a sugar presentation to the antenna, bees quickly develop a proboscis extension response (PER) to the mere odor presentation. PER conditioning is an associative form of learning entailing features of classical conditioning as observed in vertebrates, such as differential conditioning, extinction, blocking, second-order learning and reversal learning (Bitterman et al, 1983; Komischke et al., 2002; Hussaini et al., 2007; Guez and Miller, 2008, to name just few). Utilizing the proboscis appetite reflex in response to reward also enabled the investigation of habituation and sensitization, the two main forms of nonassociative learning (Braun and Bicker, 1992; Hammer et al., 1994).

In the honeybee, memory formation consists of distinctive phases and each is dependent upon different molecular pathways: short-term and mid-term memories depend on existing proteins, and two forms of long-term memory (LTM) are controlled by different signaling cascades (Menzel and Müller, 1996; Menzel, 1999; Müssig et al., 2010). Early LTM and late LTM were identified in the honeybee; the former depends on translation processes and the latter on transcription processes (Wüstenberg et al., 1998; Friedrich et al., 2004). The effects of transcription and translation inhibitors on the memory formation of the honeybee have been profoundly studied in simple forward pairing paradigms (Wüstenberg et al., 1998; Menzel et al., 2001; Friedrich et al., 2004) and in extinction paradigms (Stollhoff et al., 2005; Stollhoff and Eisenhardt, 2009).

Taken together, it is clear that the honeybee is a suitable model organism for the study of both associative and non-associative forms of learning, and for the investigation of memory

consolidation of different learning phases. The experiments conducted for this thesis were done with restrained honeybees while operating its appetitive learning behavior.

Objectives

Neuronal reward processing in both associative and non-associative learning were studied along with the effects of protein synthesis on the consolidation of long term memory formed after excitatory and inhibitory associations. To this end two different learning paradigms were utilized: habituation of the proboscis reflex – a non-associative form of learning, and olfactory reversal learning - a complex form of associative learning. Furthermore, two investigating approaches were used: extracellular recordings from the mushroom bodies' extrinsic neurons, a neuronal population assumed to be critically involved in learning related plasticity, and the administration of pharmacological agent (emetine) which inhibits protein synthesis.

This thesis aimed to elucidate the effects of emetine on early long term memory formed after olfactory reversal learning. Specifically it was inquired in two different groups of bees, summer and winter bees, in order to investigate whether the requirements for protein synthesis are seasonally dependent.

Focusing on winter bees the effect of emetine on the memory formed following olfactory differential conditioning and its effect on the acquisition and expression of reversal learning was studied.

Since reversal learning was found to be dependent on intact MBs, the ENs of the MB were characterized when bees were trained with olfactory reversal learning. This was done using extracellular recordings techniques.

Finally, ENs were studied during a simple form of learning, the habituation of the proboscis extension reflex.

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MEMORY FORMATION IN REVERSAL LEARNING OF THE HONEYBEE

ABSTRACT

In reversal learning animals are first trained with a differential learning protocol, where they learn to respond to a reinforced odor (CS+) and not to respond to a nonreinforced odor (CS-). Once they respond correctly to this rule, the contingencies of the conditioned stimuli are reversed, and animals learn to adjust their response to the new rule. This study investigated the effect of a protein synthesis inhibitor (emetine) on the memory formed after reversal learning in the honeybee *Apis mellifera.* Two groups of bees were studied: summer bees and winter bees, each yielded different results. Blocking protein synthesis in summer bees inhibits consolidation of the excitatory learning following reversal learning whereas it blocked the consolidation of the inhibitory learning in winter bees. These findings suggest that excitatory and inhibitory learning may involve different molecular processes in bees, which are seasonally dependent.

INTRODUCTION

In classical conditioning, animals learn to associate an originally neutral stimulus (CS) with a biologically significant stimulus (US) if the CS is followed by the US (forward pairing). Animals are also capable of acquiring an opposite contingency for a given CS, i.e. the absence of the US. Following Pavlov's (1927) terminology, differential conditioning consists of two such contingencies, where the stimulus which precedes the appearance of the US (CS+) retains an excitatory valence, and the one which predicts the absence of the US (CS-) retains an inhibitory one. In reversal learning the animal is first introduced to differential conditioning and once such discrimination has been learned, the stimuli's contingencies are reversed and the animal learns to adapt its response to the new rule. Following Pavlov (1927), forward pairing of CS with

reinforcement generates excitatory learning whereas extinction leads to inhibitory learning. Thus reversal learning is a paradigm entailing rather more complex learning than a simple acquisition and extinction, as the animal has to form such new associations on the background of inverted contingencies. The molecular underpinnings of acquisition and extinction learning are believed to differ, particularly in regard to the requirement of protein synthesis. In a wide range of experimental preparations, protein synthesis inhibition was found to block memory formation of acquisition learning (e.g. Davis and Squire, 1984; Abel, et al. 1997; Lattal and Abel, 2001). In extinction on the other hand, the administration of protein synthesis inhibitors yielded conflicting results which probably depend on the experimental protocol used (e.g. Flood, et al., 1977; Berman and Dudai, 2001; Stollhoff, et al., 2005; Duvarci, et al., 2006). Altogether, reversal learning provides an adequate paradigm to study both acquisition and extinction learning and memory.

The honeybee (*Apis mellifera*) serves as a valid model for the study of the underlying mechanisms of learning and memory (Menzel, et al., 2006) for which many paradigms of conditioning were tested. It was found that the results follow the rules of classical conditioning as known from laboratory mammals (Bittermann, et al., 1983; Menzel & Bittermann, 1983; Menzel, 1990). Odors are used as CSs, and sucrose solution as US for hungry bees. Several forms of memory developing in series and in parallel have been described leading to lifelong memory under appropriate conditions (Menzel, 1990).

Memory formation has been shown to consist of distinctive phases; each depends on different molecular pathways: short-term and mid-term memories depend on existing proteins, and two forms of long-term memory (LTM) are controlled by different signaling cascades (Menzel & Müller, 1996; Müssig, et al. 2010). Notably, early LTM (eLTM) depends on translation and late LTM (lLTM) depends on transcription processes (Friedrich, et al., 2004; Wüstenberg, et al.,

1998). When applied shortly prior to acquisition, emetine, a protein synthesis blocker which inhibits translation processes, is known to inhibit eLTM consolidation in the honeybee. The effects of transcription and translation inhibitors have been studied so far only in simple forward pairing paradigms (Friedrich, et al., 2004; Menzel, et al., 2001; Wüstenberg, et al., 1998) and in extinction paradigms (Stollhoff, et al., 2005, Stollhoff and Eisenhardt, 2009).

In the honeybee, reversal learning was found to have a heritable component which is manifested in the rapidity to reverse from the former CS- to the new CS+ association (Ferguson, et al., 2001). However, Ben-Shahar et al. (2000) found differences in the extinction rate of the former CS+ during the reversal phase, which were derived from the bees' behavioral state: nurses showed faster rates of extinction than foragers. Taken together, these finding suggest that two dissociable processes constitute the reversal learning, i.e. excitatory learning and inhibitory learning.

Using local anaesthetics to block the main output region of the mushroom body (MB), Devaud et al. (2007) were able to demonstrate that the acquisition of reversal learning requires an intact MB activity, whereas simple differential learning (the first phase in reversal paradigm) was spared. It was also shown that experiencing olfactory reversal learning improves the bee's future performance in solving further discrimination reversals (Komischke, et al., 2002), a feature that might serve to optimize bee's foraging efficiency when food-source profitability changes. However, those studies were designed so, that the temporal spacing of each phase from the next allowed only the formation of short-term and mid-term memories in this paradigm.

Here the effect of emetine on the eLTM formed after reversal learning was investigated in order to elucidate the consolidation of excitatory and inhibitory associations formed after reversal learning, into eLTM. To this end, each learning phase took place on a different day, when translation dependent memories are formed. Two groups of honeybees were used: summer bees

and winter bees, because it was observed in earlier experiments (Menzel, et al., 2001) that inhibiting transcription factors yields different results in summer and winter bees, specifically, winter bees did not develop long-lasting memory following spaced conditioning. We found that blocking protein synthesis during consolidation of reversal learning inhibits the consolidation of the excitatory learning in summer bees whereas consolidation of inhibitory learning was blocked in winter bees.

MATERIALS AND METHODS

General procedures related to behavior

The experiments were conducted in Berlin, Germany using honeybees (*Apis mellifera carnica*) from the colonies of the laboratory. Experiments were carried out in summer time (July/August 2009), using bees raised in outdoor hives, and in winter time (November/December 2009), using bees kept in small flight cages (1 m^3) in a glasshouse. One day prior to the experimental procedure, foraging bees were caught at the hive entrance when leaving the hive; they were then immobilized by cooling and harnessed in small metal tubes. In the evening bees were fed to satiation with a 1 M sucrose solution. On each experimental day, bees were fed in the afternoon to satiation and then kept in a dark and humid box at room temperature (~22°C, ~70% humidity).

Conditioning of the PER

All acquisition and retrieval trials shared a standardized protocol; each acquisition trial began by positioning a test bee in front of an exhaust fan. Odor stimuli (CS) were applied after 10 s (duration 4 s) and were delivered through 5ml syringe, each containing a filter paper soaked with 4 µL of pure odorant, 2-octanone and 1-hexanol (Sigma-Aldrich Chemie GmbH). Computer-controlled magnetic valves were used for the delivery of the odorants, allowing constant air flow. The presentation of the US started 3 s after odor onset by touching the antennae with a toothpick soaked in sucrose solution to induce proboscis extension. US delivery lasted for 4 s during which animals were allowed to lick sucrose solution with the proboscis (hence 1 s overlap between CS and US).

On unrewarded trials (CS-) all conditions remained the same, except there was no presentation of the US (sucrose). A positive response was scored if the proboscis was extended during the CS and before the US.

Reversal learning protocol

On the first day animals were subjected to a differential conditioning protocol with two odorants A and B (2-octanone and 1-hexanol), one forward paired with the US (sucrose solution), the other unrewarded (day one: A+ vs. B-). Each odorant was presented 6 times in a pseudo-randomized order and the sequence of odor presentation was identical for all subjects (ABBABAABABBA). Odor identities were counter balanced across subjects.

The intertrial interval was 10 minutes. On the following day the reinforcement pattern was reversed (day 2: A- vs. B+) whereas all other conditions remained constant. Retention tests were carried out on the third day, where both odorants were presented in the absence of reward.

Acquisition curves are presented as percentages of bees showing conditioned PER for each pair of CS+ and CS- presentations, which constitute one block trial.

Emetine treatment

Emetine (catalog #45160; Fluka, Buchs, Switzerland) was dissolved in PBS (in mM: 137 NaCl, 2.7 KCl, 10.1 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.2). One microliter of emetine (10 mM) was injected manually into the flight muscle using a calibrated glass capillary. Animals were injected 30 min before the reversal conditioning. Control bees were injected with 1 µl of PBS.

Data analysis

Only animals that survived until the retention test and then showed an unconditioned response to sucrose were included. The ordinates give the probability of PER responses. The McNemar χ^2 test (Zar, 1997) was used (SigmaStat) for within-group comparison of the CR to the different odors. The G-test for contingency tables (log likelihood ratio) was used when testing the differences in CR for each odor for between group comparisons.

Control experiments

Control experiments were designed in order to rule out a general effect of emetine on performance. On the first day bees were subjected to a differential conditioning protocol as described above. On the following day, bees were assigned randomly to two groups and were injected with either emetine or PBS, and after 30 minutes a retention test for both odors (in the absence of a reward) was carried out. On the third day bees underwent another retention test for both odors.

RESULTS

Summer experiments

Emetine inhibits the new excitatory learning when applied in summer

On the first experimental day, summer bees were trained to differentiate between two odorants, one being rewarded (A+) whereas the other was presented alone (B-). Each odor was presented 6 times; by the last differential learning trials the proportions of CRs to the A+ and B- were 76% and 2%, respectively (McNemar's Test: χ^2= 112.00, P <0.001, df = 1) (**Fig. 1 A**).

On the following day, 30 minutes prior to reversal learning training, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS (phosphate buffer used as saline for emetine). All bees were then trained to the reversed rule (A- vs. B+). By the last differential learning trials the proportions of CRs to A- and B+ were 4% and 49% in the PBS group (McNemar's Test: χ^2 = 29.03, P = <0.001, df = 1) and 8% and 39% in the emetine group, respectively (McNemar's Test: χ^2 = 21.04, P = <0.001, df=1) (**Fig. 1 B**). Emetine injections had no effect on acquisition during this phase.

On the third day, 24 hours after the reversal learning, all bees were subjected to a retention test for both odorants (**Fig. 1 C**). The group injected with PBS scored significantly higher for odor B than for odor A (McNemar's Test: $\chi^2 = 4.267$, $p < 0.05$, df = 1) indicating that the reversal rule had been learned and was remembered. In contrast, the emetine injected group scored the same for both odors (McNemar's Test: $\chi^2 = 0.050$, NS, df = 1), indicating that this group did not remember the reversed rule. Moreover, the emetine injected group scored significantly lower for odor B in comparison with the PBS injected group (G test: G=4.254, $p< 0.05$, df = 1), which indicates an emetine-treatment induced interference with consolidation of excitatory learning.

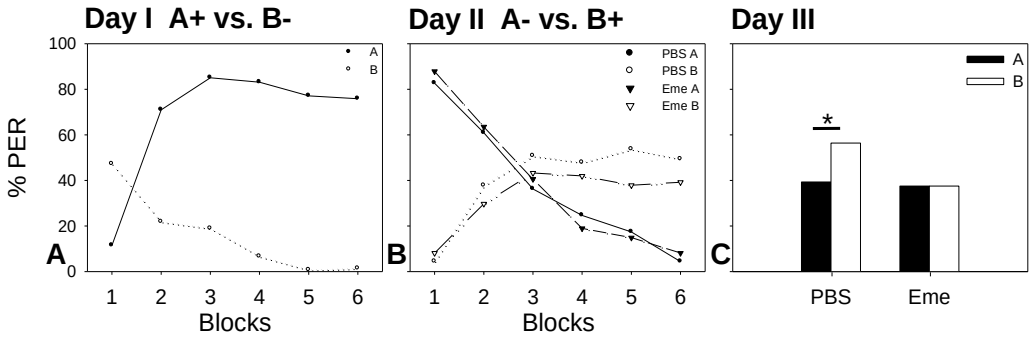

Figure 1. In summer the systemic application of emetine 30 min before reversal learning inhibits consolidation of the new reversal learning. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by either of the two odorants A (filled shapes & bars) and B (open shapes & bars). **A**: On day one bees were untreated and trained to the

differential conditioning rule (A+ vs. B-), each stimulus was presented 6 times, shown here in 6 blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B-). At the last trials a significant difference in CRs between odors was observed (McNemar's Test, $p < 0.005$). **B**: On day two, 30 min after emetine (triangles) or PBS (circles) injections, a reversal protocol was applied (A- vs. B+), each stimulus was presented 6 times, shown here in 6 blocks, (solid lines for A+ vs. dashed lines for B-) at the last trials both PBS and emetine groups exhibited a reversed preference (McNemar's Test, $p < 0.005$ for both groups). **C**: On day three, both groups were subjected to a retention test for both odorants in the absence of a reward. The PBS treated control group showed a significant preference for odor B (McNemar's Test, N=56, $p < 0.05$), the emetine treated experimental group showed no preference (McNemar's Test, N=64, NS) and bees responded significantly less often to odor B than in the PBS group (G test, $p < 0.05$).

Control experiments: When applied 24 h after differential learning, emetine has no effect on memory retrieval

On the first experimental day, summer bees underwent a differential conditioning protocol, as described above. By the last differential learning trials the proportions of bees exhibiting the CR to the A+ and B- were 74% and 5%, respectively (McNemar's Test: $\chi^2 = 53.01$, $p < 0.001$, $df = 1$) (**Fig. 2 A**). On the following day, 30 minutes prior to a retention test, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS. At the retention test both groups scored significantly higher to odor A than to odor B, as shown by their CRs; PBS group 73% and 39%, respectively (McNemar's Test: $\chi^2 = 8.47$, $p < 0.005$, $df = 1$), emetine group 75% and 27%, respectively (McNemar's Test: $\chi^2 = 13.13$, $p < 0.001$, $df = 1$) (**Fig. 2 B**). There was no significant difference in the proportions of bees exhibiting a CR between the two experimental groups (G test odor A: G PBS vs. G Eme = 0.039, NS, df = 1; G test odor B: G PBS vs. G Eme = 1.31, NS, df = 1). Another retention test was carried out on the third day. Again both groups scored significantly higher for odor A than for odor B, PBS group 64% and 37%, respectively (McNemar's Test: $\chi^2 = 5.78$, $p < 0.05$, $df = 1$) emetine group 71% and 31%,

respectively (McNemar's Test: $\chi^2 = 7.68$, $p < 0.01$, df = 1) (**Fig. 2 C**). Again both groups did not differ in their proportion of bees exhibiting a CR to both odorants (G test odor A: G PBS vs. G Eme = 0.035, NS, df = 1; G test odor B: G PBS vs. G Eme = 0.032, NS, df = 1). A general non specific effect of emetine on learning and memory can thus be ruled out.

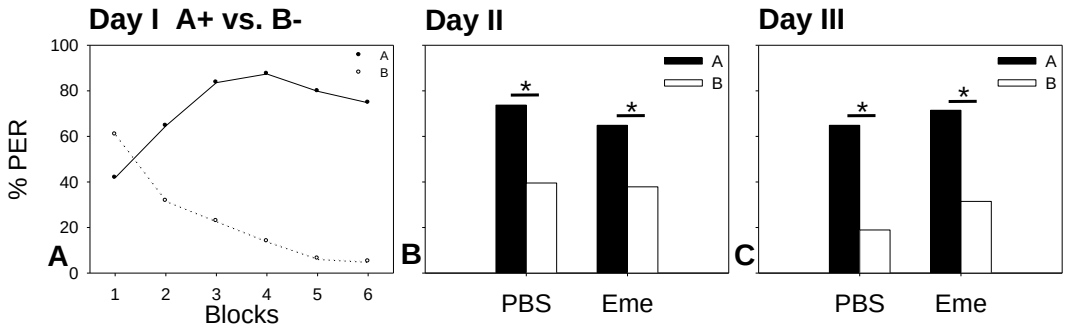

Figure 2. The systemic application of emetine 30 minutes before a retention test has no effect on memory retrieval after 24 hours. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by the two odorants A (filled shapes & bars) and B (open shapes & bars). **A**: On day one bees were untreated and trained to differential conditioning (A+ vs. B-) each stimulus was presented 6 times, shown here in 6 blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B-). For the last trial a significant difference in the percentage of bees exhibiting the CR between odors was observed (McNemar's Test, $p < 0.001$). **B**: On day two, 30 min after emetine or PBS injections, a retention test was carried out in the absence of reward. Both groups exhibited a significant preference for odor A (McNemar's Test - PBS group: $p < 0.05$; Eme group: $p < 0.01$). **C**: On day three, all groups were subjected to

another retention test for both odorants. Both groups scored significantly higher for odor A than for odor B (McNemar's Test - PBS group: N=37,$p < 0.05$; Eme group: N=35, $p < 0.01$).

Winter experiments

Emetine inhibits the new inhibitory learning when applied in winter

The same protocol was applied to winter bees (**Fig. 3**). By the last differential learning trials on day 1 the proportions of bees exhibiting the CR to the A+ and B- were 63% and 7%, respectively (McNemar's Test: $\chi^2 = 68.01$, P = <0.001, df =) (**Fig. 3 A**). On the following day, 30 minutes prior to reversal learning training, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS (phosphate buffer used as saline for emetine). All bees were then trained the reversed rule (A- vs. B+). By the last differential learning trials the proportions of bees exhibiting the CR to the A- and B+ were 22% and 57% in the PBS group (McNemar's Test: $\chi^2 = 14.06$, P = <0.001, df=1), respectively, and 25% and 61% in the emetine group (McNemar's Test: $\chi^2 = 17.05$, P = <0.001, df=1), respectively (**Fig. 3 B**). As in the summer experiments, emetine injections had no effect on the acquisition curves during this phase. On the third day a retention test for both odorants was carried out (**Fig. 3 C**). As in summer bees, the group injected with PBS scored significantly higher for odor B than for odor A (McNemaer's Test: $\chi^2 = 9.091$, $p < 0.05$, df=1) indicating that bees learned to associate odor B with reward. As in summer, no significant difference between the two odorants was observed in the emetine injected group (McNemar's Test: $\chi^2 = 0.571$, NS, df=1). However, as opposed to the results achieved in the summer where emetine inhibited the excitatory association, here the emetine injected group scored significantly higher for odor A, when compared with the PBS group (G test: G=4.0422, p <0.05, df=1).

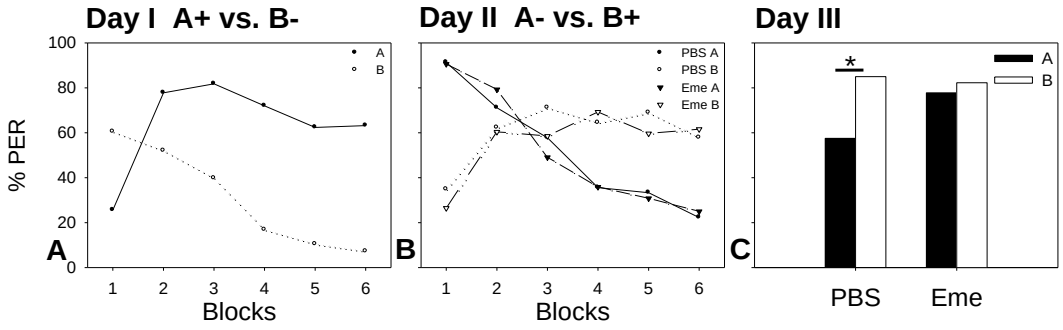

Figure 3. In winter the systemic application of emetine 30 min before reversal learning inhibits consolidation of the new inhibitory learning. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by the two odorants A (filled shapes & bars) and B (open shapes & bars). **A**: On day one bees were untreated and differentially conditioned (A+ vs. B-) each stimulus was presented 6 times, shown here in 6 blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B-). At the last trials a significant difference in CRs between odors was observed (McNemar's Test, $p < 0.005$). **B**: On day two, 30 min after emetine (triangles) or PBS (circles) injections, a reversal protocol was applied (A- vs. B+), each stimulus was presented 6 times, shown here in 6 blocks, (solid lines for A+ vs. dashed lines for B-), at the last trials both PBS and emetine groups exhibited a reversed preference (McNemar's Test, $p<0.05$ for both groups). **C**: On day three, all groups were subjected to retention tests for both odorants in the absence of a reward. The PBS group showed a significant preference for odor B (McNemar's Test, N=40, $p < 0.05$), the emetine group showed no preference (McNemar's Test, NS, N=45) and scored significantly higher for odor A than PBS group (G test, $p < 0.05$).

Control experiments: When applied 24 h after differential conditioning, emetine has no effect on memory retrieval

On the first experimental day, winter bees underwent a differential conditioning protocol, as described above. By the last differential learning trials the proportions of bees exhibiting CRs to the A+ and B- were 65% and 15%, respectively (McNemar's Test: $\chi^2 = 27.57$, $p < 0.001$, df = 1) (**Fig. 4 A**). On the following day, 30 minutes prior to a retention test, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS. At the retention test both groups scored significantly higher to odor A than to odor B; PBS group 64% and 38%, respectively (McNemar's Test: $\chi^2 = 4.26$, $p < 0.05$, df = 1), emetine group 74% and 42%, respectively (McNemar's Test: $\chi^2 = 4.76$, $p < 0.05$, df = 1) (**Fig. 4 B**). There was no significant difference in the proportions of bee exhibiting the CR between the two experimental groups (G test odor A: PBS vs. Eme = 0.74, NS, df = 1; G test odor B: PBS vs. Eme = 0.15, NS, df = 1). Another retention test was carried out on the third day, again, both groups scored significantly higher for odor A than for odor B, PBS group 70% and 41%, respectively (McNemar's Test: χ^2= 5.06; $p < 0.05$; df = 1) emetine group 73% and 38%, respectively (McNemar's Test: $\chi^2 = 6.05$, $p <$ 0.05, df = 1) (**Fig. 4 C**). Again both groups did not differ in the proportion of bees exhibiting the CR to both odorants (G test odor A: G PBS vs. G Eme = 0.05, NS, df = 1; G test odor B: G PBS vs. G Eme = 0.09, NS, df = 1). As in summer, a general non specific effect of emetine on learning and memory can thus be ruled out.

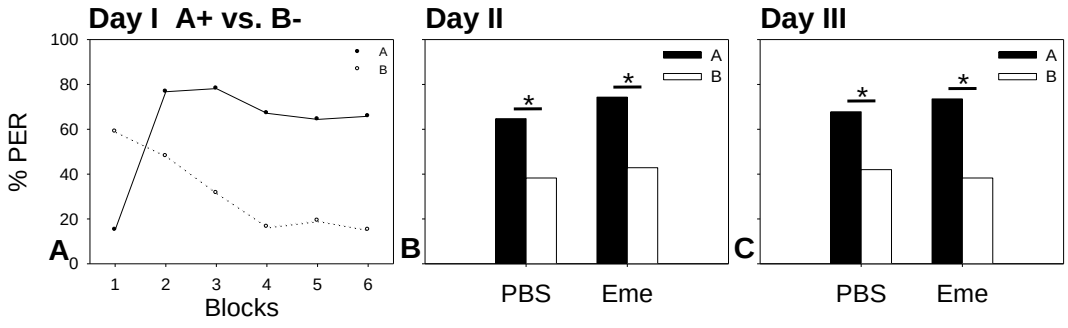

Figure 4. The systemic application of emetine 30 minutes before a retention test has no effect on memory retrieval after 24 hours. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by the two odorants A (filled shapes & bars) and B (open shapes & bars). **A**: On day one bees were untreated and differentially conditioned (A+ vs. B-) each stimulus was presented 6 times, shown here in 6 blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B-). At the last trials a significant difference in CRs between odors was observed (McNemar's Test, $p < 0.001$). **B**: On day two, 30 min after emetine or PBS injections, a retention test was carried out in the absence of reward; both groups exhibited a significant preference for odor A (McNemar's Test: PBS group: N=31, $p < 0.05$; Eme group N=34, $p < 0.01$). **C**: On day three, all groups were subjected to another retention test for both odorants. Both groups scored significantly higher for odor A than for odor B (McNemar's Test: PBS group: $p < 0.05$; Eme group: $p < 0.01$).

DISCUSSION

Two learning processes take place while an animal experiences a reversed CS-US contingency: a new excitatory learning and a new extinction learning of the original memory. Unlike a regular extinction, reversal learning involves the continued delivery of a reinforcer and a manifestation of a new preference is hence formed. It has been long known that new memories must be stabilized if they are to persist; this process is called consolidation and requires a cascade of intracellular events (McGaugh, 2000; Dudai, 2004).

The administration of amnestic agents during a discrete time window following learning can disturb the formation of long term memories. In the honeybee, the systemic administration of emetine, a translation inhibitor, shortly before an absolute appetitive conditioning yields no effect on the learning process but blocks consolidation of long term memory when tested at 24 h after acquisition (Stollhoff et al., 2005).

The effect of protein synthesis inhibitors on memory formation has been investigated so far in honeybees in either simple forward conditioning, or in regular extinction paradigms (Stollhoff et al., 2005; Friedrich et al., 2004; Wüstenberg et al., 1998). In addition, eLTM is affected by actinomycin D, a transcription-inhibitor, under spaced conditioning but not under massed conditioning (Menzel et al., 2001). Under a regular extinction paradigm, the emetine effect depends on the number of retrieval trials presented. When applied systematically 30 minutes before the presentation of two retrieval trials (non rewarded CS presentations), it blocks the extinction learning at a 24 hours retention test, whereas for five retrieval trials the spontaneous recovery at 24 hours retention test is blocked (Stollhoff et al., 2005).

The present study tested the different effects of protein synthesis inhibitor on reversal learning in two groups of honeybees, summer and winter bees.

The main findings from these experiments are that the requirements for protein synthesis in winter bees and summer bees appear to differ with respect to the kind of memory consolidation. In general, emetine did not fully block reversal learning in either summer bees, or in winter bees. In summer bees emetine injected shortly before reversal learning impaired the manifestation of the new CS-US relation but did not affect the extinction of the original preference, when tested 24 hours later. In the winter bees however, emetine yielded an inverse effect: the manifestation of the new CS-US relation remained intact, whereas the extinction of the original preference was blocked, when tested 24 hours later. These results suggest a double dissociation with respect to the protein synthesis requirements in reversal learning: emetine targets different memories (excitatory memory vs. inhibitory memory), and this effect is different with respect to the line of bees used (summer vs. winter).

It has already been suggested that seasonal variations in honeybees might result in a range of changes from behavior over neurotransmitter and pheromones levels to protein metabolism (Crailsheim 1986; Currie & Jay 1988; Harris & Woodring 1992; Balderrama et al., 1996). Winter

bees used in this study were kept under rather artificial conditions. They were housed in small flight cages under circadian illumination, humidity and temperature conditions that mimicked summer. The bees were foraging for sucrose and pollen, and the colony did not form a winter cluster. The queen continued or started to lay eggs at a low rate. It was observed that in contrast to summer bees, these bees did not form transcription-dependent lLTM after multiple spaced conditioning trials (Menzel et al, 2001). Thus the hormonal status of winter bees that are exposed to simulated summer conditions must be different from real summer bees. So far it has been believed that these differences affect consolidation of lLTM but our study shows that they also affect consolidation of translation-dependent eLTM in a learning-dependent way. In the future it would be interesting to investigate the effects of transcription inhibitors on the lLTM of reversal learning.

Translation-dependent memory consolidation requires existing mRNA and a mechanism that targets the synthesized proteins to the respective synaptic sites. Our findings suggest that the excitatory and inhibitory memory traces after olfactory reversal conditioning are differently dependent on cellular mechanisms that express the seasonal hormonal changes. In *Drosophila* the short-term memory trace of excitatory aversive conditioning and that of extinction learning of such excitatory learning (thus a form of inhibitory learning) appear to depend on different molecular mechanisms of the same neurons, the gamma lobe Kenyon cells of the mushroom body (Schwärzel et al. 2002). It is also known in *Drosophila* that the transition from short-term to long-term olfactory aversive memory is accompanied by a shift from gamma lobe related Kenyon cells of the mushroom body to vertical lobe related cells (Pascual and Preat, 2001). It is not known, however, whether the consolidation of such excitatory and inhibitory memory traces in *Drosophila* involves only translation or both transcription and translation. We also do not know yet for the bee whether the effects we see may also require transcription. Recently, using a series of single-gene *Drosophila* mutants, Qin and Dubnau (2010) found that extinction of olfactory aversive one-day memory depends on different molecular mechanisms than those involved in

associative learning. Other supporting evidence for the dissociation between classical learning and extinction learning arise from vertebrate studies in which pharmacological and genetics disruptions were shown to affect extinction but not classical conditioning (e.g. Cain et al., 2002; Marsicano et al., 2002). Taken together, we interpret our data as supporting the concept developed for *Drosophila* with respect to the mechanistic and molecular separations between excitatory and inhibitory memory traces. If the transition from short to long-term memory would lead to a separation at the network level, a specific control by hormonal factors expressing the differences between summer and winter bees may be more easily understood. In such a scenario the transfer of excitatory and inhibitory memory traces to the specific networks for long-term memory store would be differently controlled by these hormonal factors.

In this study, differences between summer and winter bees are also evident in the acquisition curves and the retention tests of the reversal learning, irrespective of the experimental groups. Summer bees display general lower levels of proboscis extension response during the acquisition of the reversal learning, compared to winter bees. This also holds true for the retention test on the third day. Such a disparity might result from different brain levels of the biogenic amine octopamine, which is known to influence response threshold to sucrose (Page and Erber, 2002), and its brain levels are correlated both with age and behavioral specialization of bees (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). In addition, injections of octopamine to specific brain regions served as a substitute for sucrose in an associative learning (Hammer and Menzel, 1998), again pointing to its involvement in the processing of sucrose reward.

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USING REVERSAL LEARNING TO STUDY MEMORY FORMATION IN THE HONEYBEE

ABSTRACT

In the course of differential conditioning animals learn to discriminate between two opposing stimuli, one predicts the appearance of reinforcement (CS+) and the other predicts its absence (CS-). If the contingencies of the stimuli are then reversed, animals learn to redirect their responses in accordance with the new rule and this type of learning is termed reversal learning. Both differential and reversal phases entail an excitatory association between a CS and a US, and an inhibitory association generated by the lack of reinforcement. In the honeybee *Apis mellifera*, inhibition of protein synthesis following reversal learning phase, was shown to affect memory consolidation of the excitatory learning in summer bees, and the inhibitory learning in winter bees. This study tested the effect of emetine on the memory consolidation formed after differential learning in winter bees, using reversal learning paradigm. The consolidation of the differential learning was found to be intact in the emetine group. However, Emetine was shown to block the spontaneous recovery following reversal learning.

INTRODUCTION

In differential conditioning, animals learn to distinguish between two stimuli with opposing outcomes, one precedes the appearance of a biologically significant stimulus (US) and the other predicts its absence (hence, CS+ and CS-, respectively). Once animals learn to discriminate between the stimuli, the contingencies of the CSs can be reversed and animals learn to redirect their responses according to the new contingency. This type of learning can be viewed as reversal learning, during which animals are confronted with two different forms of learning: acquisition

learning and extinction learning. Pavlov (1927) was the first to suggest that acquisition takes place due to an excitatory association between the CS and US which is generated by the reinforcement, whereas the lack of reinforcement superimposes an inhibitory learning process, leading to extinction learning.

The honeybee (*Apis mellifera*) serves as a valuable and valid model system for the study of the underlying mechanisms of learning and memory (Menzel, et. al., 2006). Different paradigms of conditioning were successfully studied in the honeybee, with results following the rules of classical conditioning achieved with laboratory mammals (Bittermann, et al., 1983; Menzel & Bittermann, 1983; Menzel, 1990). Using the proboscis extension reflex (PER), honeybees can be conditioned to olfactory stimuli, where odors serve as CSs and sucrose solution as US (Takeda, 1961; Bittermann, et. al., 1983). This effect is associative by nature and constitutes a classical conditioning learning, and can lead to several forms of memory which develop either in series or in parallel (Menzel, 1990).

Memory formation has been shown to consist of several distinctive phases; a short-term memory (STM) and a mid-term memory (MTM) both depend on existing proteins, and a long-term memory (LTM) which requires translation and transcription factors (Tully et al., 1994; DeZazzo and Tully, 1995; Huang, 1998). In the honeybee two forms of LTM are recognised: early LTM (eLTM) and late LTM (lLTM), which are controlled by different signalling cascades (Menzel & Müller, 1996; Müssig, et al. 2010), notably, eLTM depends on translation and lLTM depends on transcription processes (Friedrich, et al., 2004; Wüstenberg, et al., 1998). In the honeybee, the administration of emetine, a protein synthesis blocker which inhibits translation processes, was shown to hinder the consolidation of both eLTM and lLTM (Friedrich, et al., 2004; Stollhoff, et. al., 2005). It is worth mentioning that as for today the effects of translation and transcription inhibitors have been usually studied in simple forward pairing paradigms (Friedrich, et al., 2004;

Menzel, et al., 2001; Wüstenberg, et al., 1998) and in extinction paradigms (Stollhoff, et al., 2005, Stollhoff and Eisenhardt, 2009). Recent studies conducted in our lab (Hadar and Menzel, 2010) examined the effects of emetine on the memory formed after a rather more complex form of learning, i.e. reversal learning. In these series of experiments emetine was shown to yield different results which were dependent on the group of bees used: summer bees or winter bees. More specifically, the systemic injection of emetine shortly before the reversal learning phase was shown to inhibit the consolidation of excitatory learning in summer bees, whereas it was the consolidation of the inhibitory learning that was blocked in winter bees.

The molecular underpinnings of acquisition and extinction learning are believed to differ, especially in regard to the requirements of protein synthesis. In a wide range of experimental preparations, inhibition of protein synthesis was found to block memory formation of acquisition learning (e.g. Davis and Squire, 1984; Abel, et al. 1997; Lattal and Abel, 2001). On the other hand, in extinction learning the administration of protein synthesis inhibitors yielded conflicting results which might stem from the different experimental protocols used (e.g. Flood, et al., 1977; Berman and Dudai, 2001; Stollhoff, et al., 2005; Duvarci, et al., 2006). Seen from this angle, reversal learning paradigm offers a parallel study of the mechanisms underlying both acquisition and extinction learning.

As mentioned, in winter bees, the injection of emetine shortly before the reversal learning phase interferes with the consolidation of the inhibitory memory formed during this phase (Hadar and Menzel, 2010), whereas the new excitatory memory remained intact. This, together with the results achieved with summer bees, might suggest that emetine, when injected before two opposing learning acquisitions, hinders the consolidation of only one of the learning, while sparing the other. Nonetheless, it might well be that this effect is specific to the reversal phase, where memory traces from the first differential learning phase interfere with the new acquisition.

In order to study the above, emetine was injected shortly before the differential learning phase in a reversal paradigm, using winter bees. Under such conditions, one of two possible outcomes was expected: either an emetine-induced blockage of the consolidation of the inhibitory learning, or an emetine-induced inhibition of the consolidation of both the inhibitory and excitatory learning.

Unfortunately the results achieved in this study do not allow drawing any concrete conclusions regarding emetine's effect on the consolidation of differential learning, as there was no significant difference between the emetine group and the PBS group for the memory formed after the differential conditioning. Moreover, in contrary to the expected time course of the effect of emetine, in this study a significant difference between the PBS and emetine group was observed during the acquisition phase of the differential conditioning. This is rather surprising as emetine, being a translation inhibitor agent, is known to affect behavioural expression starting from one day after application. Furthermore, when tested 24 hours after the reversal learning phase, the group injected with PBS exhibited high CR's to both odorants, i.e. failed to respond to the reversal learning contingency. Plausible explanations are addressed in the discussion chapter.

MATERIALS AND METHODS

General procedures related to behaviour

The experiments were conducted in Berlin, Germany using honeybees (Apis mellifera carnica) from the colonies of the laboratory. Experiments were carried out in winter time (October/November 2009) using bees kept in small flight cages (1 m3) in a glasshouse. One day prior to the experimental procedure, foraging bees were caught at the hive entrance when leaving the hive; they were then immobilized by cooling and harnessed in small metal tubes. In the

evening bees were fed to satiation with a 1 M sucrose solution. On each experimental day, bees were fed in the afternoon to satiation and then kept in a dark and humid box at room temperature.

Conditioning of the PER

All acquisition and retrieval trials shared a standardized protocol; each acquisition trial began by positioning a test bee in front of an exhaust fan. Odor stimuli (CS) were applied after 10 s (duration 4 s) and were delivered through 5ml syringe, each containing a filter paper soaked with 4 µL of pure odorant, 2-octanone and 1-hexanol (Sigma-Aldrich Chemie GmbH). Computer-controlled magnetic valves were used for the delivery of the odorants, allowing constant air flow. The presentation of the US started 3 s after odor onset by touching the antennae with a toothpick soaked in sucrose solution to induce proboscis extension. US delivery lasted for 4 s during which animals were allowed to lick sucrose solution with the proboscis (hence 1 s overlap between CS and US).

On unrewarded trials (CS-) all conditions remained the same, except there was no presentation of the US (sucrose). A positive response was scored if the proboscis was extended during the CS and before the US.

Reversal learning protocol

On the first day animals were subjected to a differential conditioning protocol with two odorants A and B (2-octanone and 1-hexanol), one forward paired with the US (sucrose solution), the other unrewarded (day one: A+ vs. B-). Each odorant was presented 6 times in a pseudo-randomized order and the sequence of odor presentation was identical for all subjects (ABBABAABABBA). Odor identities were counter balanced across subjects.

The intertrial interval was 10 minutes. On the following day the reinforcement pattern was reversed (day 2: A- vs. B+) whereas all other conditions remained constant. Retention tests were carried out on the third day, where both odorants were presented in the absence of reward.

Emetine treatment

Emetine (catalog #45160; Fluka, Buchs, Switzerland) was dissolved in PBS (in mM: 137 NaCl, 2.7 KCl, 10.1 Na2HPO4, 1.8 KH2PO4, pH 7.2). One microliter of emetine (10 mM) was injected manually into the flight muscle using a calibrated glass capillary. Animals were injected 30 min before the differential conditioning. Control bees were injected with 1 µl of PBS.

Data analysis

Only animals that survived until the retention test and then showed an unconditioned response to sucrose were included. Shown are the percentages of bees which exhibited proboscis extension responses to the applied odorants. The McNemar $\chi 2$ test (Zar, 1997) was used (SigmaStat) for within-group comparison of the CR to the different odors. The G-test for contingency tables (log likelihood ratio) was used when testing the differences in CR for each odor for between group comparisons.

RESULTS

On the first experimental day, 30 minutes after PBS or emetine injections, bees were trained to differentiate between two odorants, one being rewarded (A+) where the other was presented alone (B-) (Figure 1A). Each odor was presented 6 times; already by the second block (first trial of odor B) a significant difference to the proportions of CRs between PBS and emetine group was found, 80% and 47%, respectively (G Test: $G=8.47$, $p<0.001$, $df=1$). This significant difference

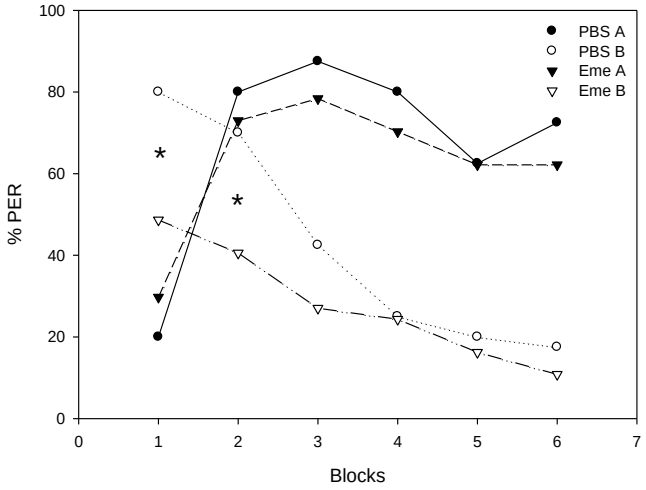
was seen again in the third block (second trial of odor B, with 70% of CRs in the PBS group and 40% in the emetine group (G Test: G=6.86, p=0.005, df=1). On the third trial of odor B no significant difference between PBS and emetine group was observed (42% PBS, 27% emetine, G Test: G=2.03; NS, df=1). By the last differential trials, the proportions of CRs to the A+ and B- in the PBS group were 72.5% and 17.5%, respectively (McNemar's Test: $\chi 2$= 20.45, P = <0.001, df = 1) and in the emetine group 62% and 10%, respectively (McNemar's Test: $\chi 2$= 17.05, p<0.001, df=1).

On the following day, bees were trained with a reversal learning protocol (A- vs. B+) (Figure 1B). In the first two blocks of the reversal learning, both PBS and emetine groups scored significantly higher for odor A than for odor B, with 87.5% and 20%, respectively in the PBS group and 81% and 19% respectively in the emetine group (McNemar's Test: PBS group: $\chi 2$= 25.03, p<0.001, df=1; McNemar's Test: emetine group: $\chi 2$= 21.04, p<0.001, df=1). Moreover, there was no significant difference between the two groups in their CRs scores for both odorants (G Test odor A: G=0.67, NS, df=1; G Test odor B: G=0.01, NS, df=1). During the course of the reversal learning, a significant difference between the two experimental groups was seen in the forth block of odor A, where the PBS group scored significantly higher than the emetine group, with 57.5% and 35%, respectively (G Test: G=3.89, p<0.05, df=1). By the end of the reversal learning, both groups scored significantly higher to odor B than to odor A: PBS group 75% and 32% (McNemar's Test $\chi 2$=15.05, p<0.01, df=1) and emetine group 67% and 24%, respectively (McNemar's Test $\chi 2$=12.5, p<0.01, df=1). There was no significant difference between the two experimental groups.

On the third day, 24 hours after the reversal learning, all bees were subjected to a retention test for both odorants. (Figure 1C). For both groups, no significant difference of the CRs between the two odorants was observed. The PBS group scored 80% for odor B and 82% for odor A (McNemar's Test: NS, df=1) and the emetine group scored 65% for odor B and 57% for odor A (McNemar's Test: NS, df=1). However, there was a significant difference between the PBS group and the emetine group with respect to odor A (G Test: G=6.18, p<0.05, df=1).

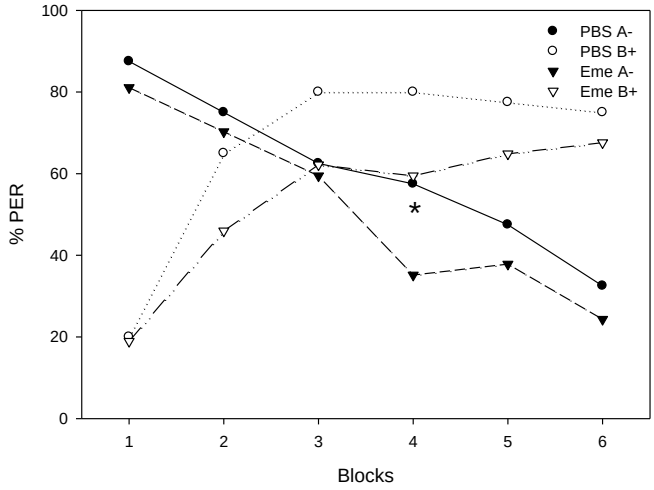
A

Day I A+ vs. B-



B

Day II A- vs. B+



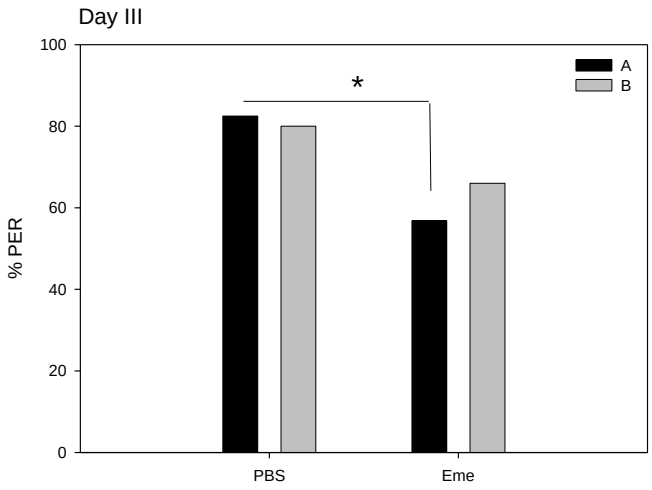


Figure 1. Reversal learning in winter bees, injected systematically with either PBS or emetine 30 min before differential learning. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by either of the two odorants A (filled bars) and B (open bars). A: On day one, 30 minutes after emetine (triangles) or PBS (circles) injections, bees were trained to the differential conditioning rule (A+ vs. B-), each stimulus was presented 6 times, shown here in 6 blocks, (solid line and filled symbols for A+ vs. dashed line and open symbols for B-). A significant difference between PBS and emetine group was observed in the first two trials for B (G Test: $p<0.001$, for both trials). At the last trials a significant difference in CRs between odors was observed (McNemar's Test: PBS group $p< 0.001$; emetine group: $p< 0.001$). B: On day two, a reversal protocol was applied (A- vs. B+), each stimulus was presented 6 times, shown here in 6 blocks, (solid lines for A+ vs. dashed lines for B-). In the fourth block of odor A the PBS group scored significantly higher than the emetine group (G Test: $p<0.05$). At the last trials both PBS and emetine groups exhibited a reversed preference (McNemar's Test: PBS group $p < 0.01$; emetine group $p< 0.01$). C: On the third day, both groups were subjected to a retention test for the two odorants in the absence of a reward. Both PBS treated control group and emetine treated group showed no significance preference for either odor A or B (McNemar's Test: PBS group,

NS, N=40; emetine group, NS, N=37), the emetine treated experimental group scored significantly lower for odor A than PBS group (G test, $p < 0.05$).

DISCUSSION

It has been demonstrated that the administration of amnestic agents during a discrete time window following learning can disturb the formation of long term memories. In the honeybee, the systemic administration of emetine, shortly before an absolute appetitive conditioning which consists of three CS-US trials, yields no effect on the learning process but blocks consolidation of long term memory when tested at 24 h after acquisition (Friedrich et al., 2004; Stollhoff et al., 2005). On the other hand, the memory induced by single trial conditioning is not affected by emetine (Friedrich et al., 2004). It seems that emetine's effect on memory is dependent on the strength of the association which is formed; exclusively targeting strong learning. Based on this data, bees were injected with emetine shortly before undergoing olfactory differential conditioning, comprised of 6 learning trials for each odor (CS+, CS-). On the following day bees were trained with a reversed learning protocol, and tested under extinction conditions on the third day. In contrast to the expected effect of emetine, a significant difference between the emetine and PBS group was seen during the first two trials of the inhibitory learning (CS-), i.e. already in the first hour after injections. Assuming these results reflect a genuine phenomenon, there are two possible explanations for this emetine-derived effect, which are not necessarily mutually exclusive. The high CR's to the first presentations of the unrewarded odor (CS-) observed in the PBS group are due to olfactory generalization, it hence might be that emetine blocks this form of process. Another explanation is that emetine hampered the behavioural expression of the generalization learning, i.e., the ability to respond with PER. The latter explanation implies a short term mechanism for such an effect, as PER scores return to normal as time from injections

elapsed. Since such an effect was not observed when emetine was injected prior to reversal learning phase (Hadar and Menzel, 2010), if those explanations are to be accepted, they must hold true for the effect of emetine on differential conditioning solely. This is not totally unexpected as animals which undergo reversal phase are not novel to the concept of differential learning and should not resort to a strategy most suitable for 'naïve' subjects.

On the following day there was no significant difference between the PBS and the emetine group in the CR's to both odors in the first two trials, which serve as retention tests for the learning formed in the first day. During the reversal learning acquisition, the emetine group scored lower CRs for odor B than did the PBS group, though only by the forth trial this effect was statistically significant. It thus seems that emetine, when injected before a differential conditioning, has a minor effect on additional learning, if all. These findings are surprising, as emetine was expected to hinder the memory formation of the differential phase. However, although no prominent effect was seen on this day, at the following day upon retention test a difference emerged between the PBS and emetine treated groups. The PBS group scored highly for both odors, hence failed to differentiate between the two stimuli. More specifically although maintaining a high responsiveness to the stimulus last associated with reward, they also kept exhibiting high responsiveness to the lately extinguished stimulus. This could be interpreted as the phenomenon of spontaneous recovery. More interestingly, this effect was not seen in the emetine group, which was statistically different from the PBS group, thus it can be suggested that spontaneous recovery was blocked by emetine. This is in accordance with previous results obtained by Stollhoff et al. (2005) where following five extinction trials emetine was found to block spontaneous recovery.

According to Bouton (1993, 2004), extinction is only one example of temporal interference with performance derived from a new learning, whereas the initially trained association remains intact. Two phenomena in which the conditioned response recovers following extinction learning are

spontaneous recovery and rapid acquisition. It is assumed that responding in accordance with the CS-US association learning results from a failure to retrieve the extinction memory. As winter bees were shown to display general high levels of proboscis extension responses during both acquisition and retention test (Hadar and Menzel, 2010). In the current study this was clearly observed in the retention test, where responses in the PBS group were as high as 80%. This effect might stem from the high brain levels of the biogenic amine octopamine which in bees known to influence response threshold to sucrose (Page and Erber, 2002). It was found that octopamine's brain levels are correlated with both the age and the behavioural specialization of bees (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). Though speculative, following Bouton's (1993) interference hypothesis, one might suggest that winter bees fail to retrieve the extinction memory and hence are more amenable to spontaneous recovery, as a result of their high octopamine levels which yield them more sensitive to reward related associations.

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STUDYING THE INVOLVEMENT OF THE ENS OF THE HONEYBEE IN OLFACTORY REVERSAL LEARNING

ABSTRACT

The honeybee (*Apis mellifera*) serves as a valid model for the study of the underlying mechanisms of learning and memory. The mushroom bodies (MBs) are its higher-order centers dedicated to integration of various modalities, and involved in neuronal plasticity following associative olfactory learning. Here, extracellular recordings from MB extrinsic neurons (EN) were performed while bees were exposed to an olfactory reversal learning paradigm. Following reversal learning, ENs changed their response pattern to odor presentation. A sub-population of the ENs developed a learning-related neuronal response to the unrewarded odor during acquisition of differential learning. No learning-related changes were observed in the ENs following and during reversal learning.

INTRODUCTION

Learning results in the modification of neuronal excitability and the synaptic strength between neurons (Milner et al., 1998 to add ref). It is believed that these changes consequently lead to an adaptive behaviour. The olfactory system in both vertebrates and invertebrates has been proven to be adequate for the study of learning and memory processes (Davis, 2004; Wilson and Stevenson, 2003; Wilson and Mainen, 2006, Okada et al., 2007). Hence, the combination of cellular recording techniques whilst exposing an organism to a learning paradigm allows monitoring the neuronal changes underlying learning and memory formation. In the honeybee, extracellular long term recordings were successfully employed to record neuronal activity in a behaving animal

during olfactory conditioning (Okada et al., 2007; Strube-Bloss et al., in press). Those studies focused on a defined group of neurons in the honeybee brain, the mushroom body (MB) extrinsic neurons of the alpha lobe (ENs).

The MB is a neuronal structure known to be involved in learning and memory capabilities (Menzel et al., 1974; Erber et al., 1980; Menzel, 1999, 2001) and assumed to play a major role during complex learning (Menzel & Giurfa, 2001; Giurfa, 2003, 2007). The input and output regions of the MB are segregated spatially. The calyces are the main input region and the lobes constitute the output region (Mobbs, 1982). A convergence of odor pathways from the antennal lobe (conditioned stimulus) and reward information from the VUMmx1 neuron (unconditioned stimulus) take place within the lip region of the calyx onto the Kenyon cells (KC). Szyszka et al. (2008) reported an associative as well as non-associative plasticity in the KC, following appetitive odor conditioning. The ENs of the MB receive their input from the Kenyon cells and constitute one of the output pathways of the MB. One identified MB neuron, the pedunculus extrinsic neuron 1 (PE1) has been extensively studied using extracellular recording technique in a behaving animal undergoing olfactory classical conditioning (Maulshagen, 1993; Menzel and Manz, 2005; Okada et al., 2007). This neuron responds to olfactory, mechanosensory and visual stimuli, and exhibits a plasticity following olfactory learning. Recently, while recording from a different population of EN, Strube-Bloss et al. (in press) found them to encode odor-reward associations following classical conditioning. Using local anaesthetics to block the main output region of the MB, Devaud et al. (2007) demonstrated that the acquisition of olfactory reversal learning was hampered whereas differential learning was spared.

Taken together, it seems that ENs encode the past experience of an animal with learned stimuli and that complex olfactory learning are dependent on an intact MB.

This study combined extracellular recordings from the ENs of the alpha lobe during olfactory reversal learning. In such a paradigm, honeybees first learn to differentiate between two odorants, one predicts the presentation of reward (CS+) and the other predicts the absence of reward (CS-). In the following phase, the odorants contingencies are reversed and animals learn to divert their behavioral response in accordance with the new contingency. At the end of the reversal learning, animals are tested for their behavioral responses for both odorants, in the absence of reward. Data recorded from ENs suggest that following reversal learning those neurons change their response pattern to odor presentation, though no tendency towards a conditioned odor was found. It was also found that a sub-population of the ENs develop a learning-related response to the unrewarded odor during acquisition of differential learning. No learning-related changes were observed in the ENs following reversal learning.

MATERIAL AND METHODS

Subjects

Foraging honeybees (Apis mellifera carnica) were caught at the entrance of the hive during the afternoon, one day prior to the electrophysiological experiments. Bees were anesthetized using ice and then harnessed by stripes of tape in little metal tubes which allowed the free movements of the antennae, mandibles and proboscis (Bitterman et al. 1983). The scapes of the antennae were fixed onto the head using eicosane (Sigma) allowing only the movement of the flagellum. Bees were fed to satiation with 30% sucrose solution and kept overnight at a dark humid chamber at room temperature.

Dissection

Dissections for electrophysiology procedure were made after bees were restrained and placed in a magnetic stand adapted for the electrophysiological setup. Dissections were made under a compound electrode. A 25 μm-diameter silver wire (Nilaco, Tokyo, Japan) served as the reference electrode and was inserted into the upper occellus. Another 25 μm-diameter silver wire (Nilaco, Tokyo, Japan) was used to record the activity of the M17 muscle which is involved in the proboscis extension response (PER) (Rehder, 1987), and was hence inserted between the right occellus and the compound eye. For the recording of the ENs, a tiny unilateral square was cut between the midline of the bee head and the antenna. The area above the alpha-lobe was removed from head glands and trachea sacks. The electrode was inserted in the ventral part of the alpha-lobe at a depth between 100 and 250 μm. Upon signals detection, the area surrounding the electrode was filled with two-component silicon (KWIKSIL Sarasota, FL, USA) in order to prevent a the drying out of the brain and the movement of the electrode.

Odor stimulation and experimental paradigm

General handling – upon signals' activity detection, and after sealing the area surrounding the electrode, bees were left untouched for about half an hour, in order to gain a reliable signal recording and to avoid tissue's movement.

Odor-supplying device

A multi-channel olfactometer was adapted from Galizia et al. (1997), each channel was equipped with a 5ml syringe serving as an odor chamber. A constant air stream (1.5 m/s) was delivered from a 6 mm in diameter Teflon tube and the needles of all syringes were inserted into this tube. Odors were diluted in paraffin oil at a 10-2 concentration. Filter papers (3cm2) containing 10 μl of the experimental odor solutions were placed in the syringes. Odor stimulation lasted 3 seconds,

during which only 2.5 ml of the air volume of the 5 ml syringes were injected into the constant air stream in order to avoid concentration gradients. Bees were placed 1 cm away from the Teflon tube's outlet and an exhaust hood (tube with 10 cm diameter) was located behind the bee to suck the remains of the odors. The magnetic valves (Lee, Westbrook, Connecticut) of the odor-supplying device were computer-controlled using a Visual Basic Script (VBA 6.0, Microsoft, USA) written by Frank Schaupp. Time stamps of valve opening and closing were marked with the data acquisition system.

PER Conditioning

Only bees that responded with proboscis extension response (PER) following antenna stimulation with sucrose solution were chosen to be trained. The conditioning of the PER begun with a 3 seconds odor stimulus (CS+). The presentation of the US started 2 seconds after odor onset (hence, there was a 1 second overlap between CS and US) and lasted 3 seconds. First, the antennae were touched with a toothpick soaked in 1.2 M sucrose solution to induce proboscis extension and then the animals were allowed to lick the sucrose solution.

On unrewarded trials (CS-), all conditions remained the same, except there was no presentation of the US (sucrose).

Reversal paradigm

In each experiment two different odors served as CSs (odors A and B) and another odor as a control (odor C). Odor identities were counter balanced across subjects. All acquisition and retrieval trials shared a standardized protocol.

Pre-acquisition phase – each of the three odors was randomly presented for 3 times over 3 second duration and with an inter-trial interval (ITI) of 1 minute.

Differential conditioning phase – 20 minutes after the pre-acquisition phase animals were subjected to a differential conditioning protocol with two odorants A and B, one forward paired with the US (sucrose solution), the other unrewarded (A+ vs. B-). Each odorant was presented 6 times in a pseudo-randomized order and the sequence of odor presentation was identical for all subjects (ABBABAABABBA). The intertrial interval was 3 minutes.

Reversal conditioning phase – 1.5 hour later the reinforcement pattern was reversed (A- vs. B+) whereas all other conditions remained constant.

Test phase - retention tests were carried out 1.5 hour after the reversal conditioning, where all odorants were randomly presented in the absence of reward.

Electrophysiology

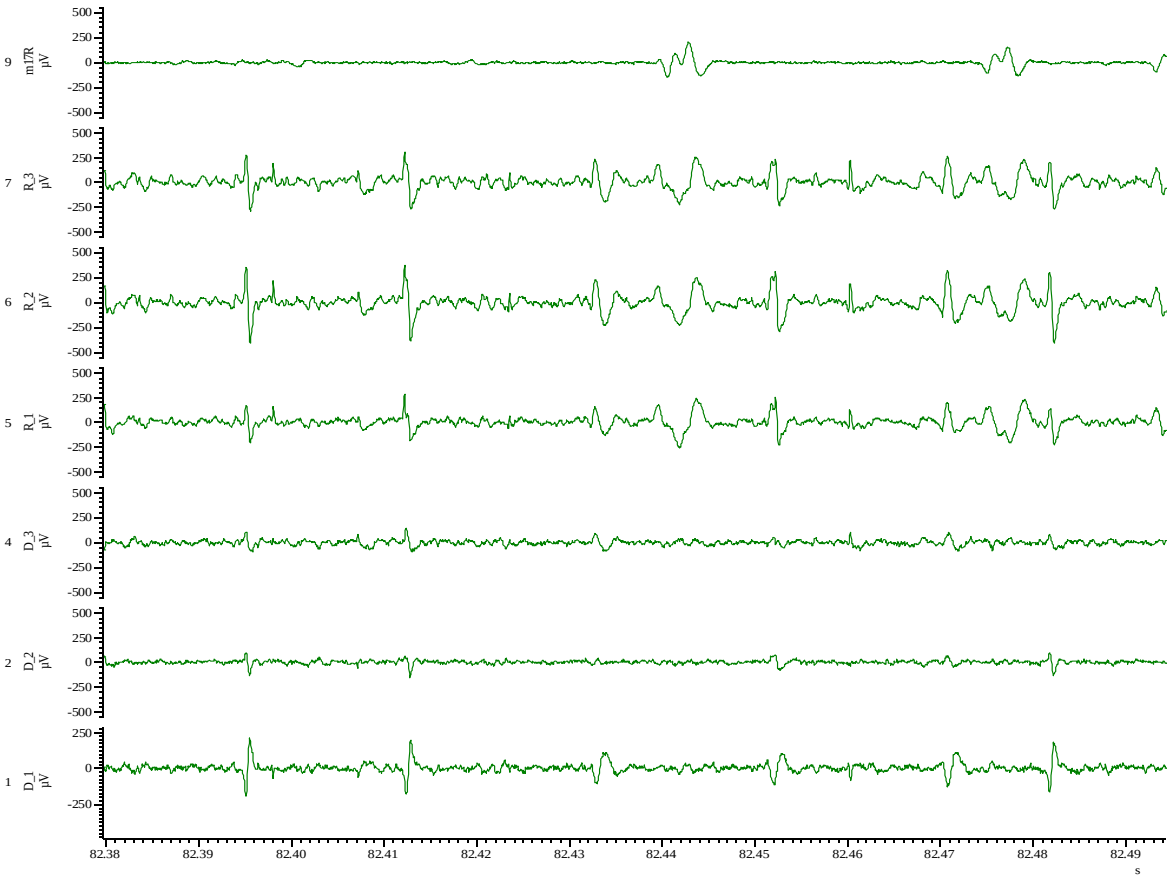
Electrode

Electrodes consisted of three polyurethane-coated copper wires, 14 μm in diameter (Electrisola, Escholzmatt, Switzerland). The electrode preparation was adopted from Ryuichi Okada (Mizunami et al., 1998; Okada et al., 1999) and was modified to obtain three recording channels. The three wires were waxed together onto a tungsten wire (1-2 cm long and 100 μm in diameter) and then attached to a glass capillary. A custom-built adapter held the glass capillary and was connected to the headstage amplifier (Headstage-27 Amplifier Neuralynx, Tucson, AZ, USA).

Differential Recording

The three different wires allowed a differential recording which consisted of the three possible combinations arising from the subtraction of each raw channel with a different one. Figure 1 illustrates the three raw channels with its three differential combinations. Channels R1, R2 and R3 correspond to the three wires measured only against the reference electrode. Channels D1, D2 and D3 represent the three differential recording combinations. The activity of the M17 muscle

which is involved in the movement of the bee's proboscis is also depicted in Fig. 1, and is shown in the upper channel (m17R). One can clearly observe that activity from this channel lead to strong voltage modifications in the three raw channels, measured only against the reference electrode. To avoid such misinterpretations, signals were analysed from the differential channels which are not affected from the motor activity of the M17 and are thought to reflect the neuronal activity in the close vicinity to the tip of the electrode. Utilizing this data allows to record and identify from up to five different neuronal units simultaneously. Electrical signals were amplified by a Lynx-8 Amplifier (Neuralynx, Tucson, AZ, USA). A 1-9000 Hz band-pass filter was used and data was digitized at a 20 kHz sampling frequency. The recording files were then imported into Spike2 (Cambridge Electronic Design, Cambridge, UK) data format with a high-pass filter (600 Hz).



Time in seconds

Figure 1: recordings of the neuronal and muscle activity. The muscle which mediates the proboscis extension response (upper channel, m17R) was recorded and hence allowed a simultaneous behavioural assessment. For the neuronal activity a combinational recording WAS used: a three-wire recording electrode (channels R1, R2, R3) yields 3 differential recording combinations where noise and muscle activity are filtered out (channels D1, D2, D3)

Spike Sorting

A semi-automatic spike sorting technique was used (template-matching, see Fig. 2).

This process of spike sorting is based on two differentiating features: i.e. spike shape and spike amplitude. Each neuron has its intrinsic electrochemical dynamic which generate an action potential with an individual characteristic shape. The amplitude of the neuron's spike as depicted in the electrode is derived from the spatial distance of the neuron from the tip of the electrode. Hence those two features allow the classification of action potentials into distinct neuronal units. As mentioned, for the analysis of the alpha lobe-extrinsic neurons, only the differentially recorded channels were used. Spike sorting was made using Spike2 template sorter, each channel was sorted separately. Upon sorting a principal component analysis (PCA) was used to classify the different spike events into separate units.

Visualization of the recording site

In order to confirm that recording were attained from the alpha-lobe extrinsic neurons, a visualization technique was applied. To this aim, the tip of the electrode was dipped into Micro Ruby shortly before the insertion of the electrode. At the end of the experimental procedure, the electrode was moved and the brain was dissected and fixed in 4% formaldehyde diluted in 50% methanol for 24 hours at 4 C°. Preparations were then rinsed for 20 minutes in phosphate-buffered saline (PBS; pH 6.7), diluted 1:4 in distilled water, dehydrated in an increasing ethanol series (30 %, 50 %, 70 %, 90 %, 99 %, 100 %) each step lasted 10 minutes, then cleared in a mixture of 50% methyl-salicylate (MS) and 100% ethanol and embedded as whole mounts in MS in double-sided custom slides. A confocal laser scanning microscope (Leica TCS SP2) with a Leica HC PL APO CS 10.0×0.40 UV dry lens objective was used to scan the preparations. Results confirm that the recorded signals can be related to the ventral part of the alpha-lobe (Fig. 2 to add). As the depth of the electrode (between 100-250 μm) was also controlled, one might

conclude that the recorded ENs were probably types A1, A2, A4, A5 or A7 (Rybak and Menzel, 1993).

Data analysis

Statistics

The McNemar $\chi 2$ test (Zar, 1997) was used (SigmaStat) for within-group comparison of the neuronal response to the different odors. The Kruskal-Wallis One Way Analysis of Variance on Ranks was used when testing the differences in the neuronal responses to one odor across learning trials (SigmaStat).

Response detection

For the detection of a neuronal response evoked by an odor, spike trains were converted to Peri-Stimulus-Time-Histograms (PSTH) using a bin width of either 50ms or 200ms. A bin width of 200ms was chosen as some units exhibited a low firing rate (7.4 -12.5 Hz). For pre-acquisition and test phase the mean (m) and standard deviation (SD) across all bins preceding a specific odor were estimated. To this end duration of 3s was chosen, starting 3.5s prior to odor onset (hence, terminating 0.5s before odor onset). For an excitatory response, again spike trains across trials were pooled and a rate bin which crossed a threshold of m+2.5*SD during 600ms after odor onset was tested (starting 50ms after odor onset). For an inhibitory response the baseline m was first subtracted and then a negative threshold (-2.5*SD) was applied. For the detection of a neuronal response during differential learning phase and reversal learning phase, the mean m and standard deviation SD were calculated separately for each spike train preceding an odor onset, and were calculated as specified for each trial alone.

General properties of odor representation of the mushroom-body extrinsic neurons

Extracellular recordings were performed using a three wire electrode inserted into the ventral part of the alpha-lobe, at a depth of 100-250 µm. Based on the insertion site and the results achieved from visualization of the recording position, recorded neurons are considered to be types A1, A2, A4, A5 or A7 (Rybak and Menzel, 1993). A total of 18 single units (in 6 animals) were isolated and analysed. The spontaneous activity in all units varied between 7.4 and 43 Hz. Three different odors were tested before and after the two learning phases. As can be shown in Figure 3.A, not all units were odor-sensitive under naïve conditions. Only 44.4% exhibited an odor response to at least one odor whereas the remaining 55.5% were initially odor-insensitive. Interestingly, none of the 18 recorded units were found to respond to all three odors, 27.7% of the units responded to one odor and the rest 16.6% to two odors (Fig. 3B). Disparate from the results of Strube et al. (2010), here, two units were found to respond once in excitation and once in inhibition to two different odors, prior to the learning phase (Fig. 4, units 16 & 18, black and pink cells, respectively).

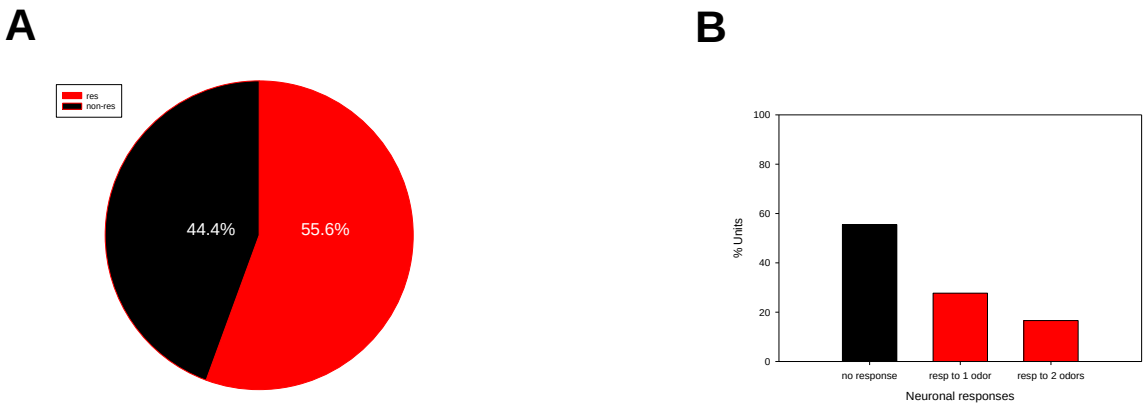


Figure 3: Odor responses prior to conditioning in EN-neurons. A) Percentage of odor sensitive (red) and odor non-sensitive units (black). B) As in A), percentage of odor sensitive (red) and odor non-sensitive units (black) in relation to the number of odors which evoked responses.

Odor representation of the mushroom-body extrinsic neurons after reversal learning

One and a half hour after reversal learning phase, bees underwent a test phase where both conditioned odors and the control odor were introduced in the absence of reward. Figure 4.A depicts the neuronal responses evoked by the presented odors following conditioning in all 18 recorded units. After conditioning an increased proportion of the ENs respond to at least one odor (72%), and this difference is significant compared to the proportions of odor responses before conditioning (McNemar's Test: $\chi 2=4.16$, $P<0.05$, df = 1). Interestingly, this difference is not specific to the learned odors, but reflects a general tendency of response across all odors (pre-acquisition phase vs. post-acquisition phase, McNemar's Test: All units together: Odor A:: $\chi 2=0.16$, NS, df = 1; Odor B: $\chi 2=0.12$, NS, df = 1; Control odor: $\chi 2=0.25$, NS, df = 1; Recruited units: Odor A: McNemar's Test: $\chi 2=0.0$, NS, df = 1; Odor B $\chi 2=0.57$, NS, df = 1; Control odor: $\chi 2=0.25$, NS, df = 1: Non-recruited units: Odor A: McNemar's Test: $\chi 2=0.0$, NS, df = 1; Odor B: $\chi 2=0.0$, NS, df = 1; Control odor: $\chi 2=0.0$, NS, df = 1). As prior to conditioning, in the test phase none of the units responded to all three odors, and across all units only 11% responded to two of the three presented odors (Fig. 4.B). It might be concluded that in these recorded units no odor reward associations were observed following reversal learning.

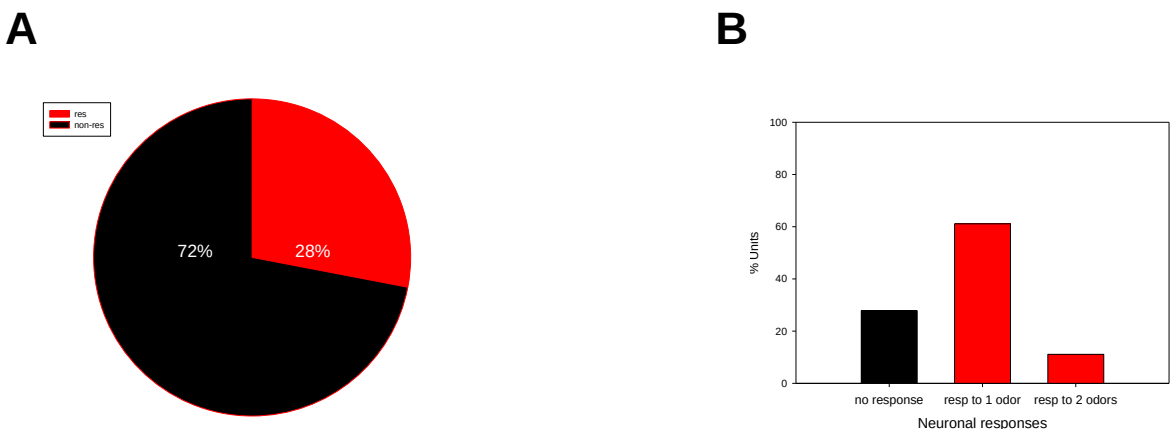
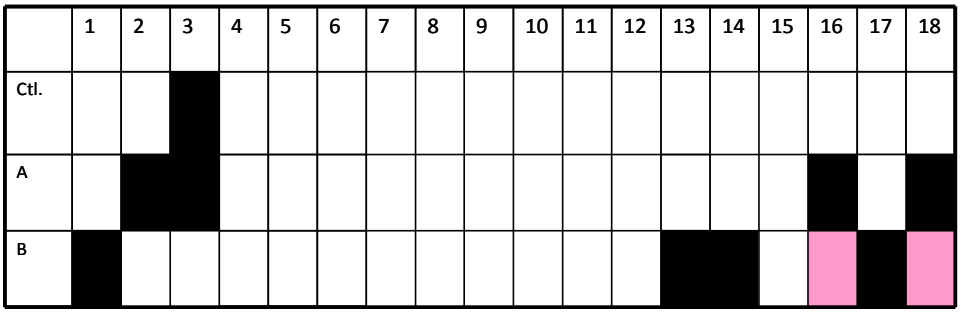


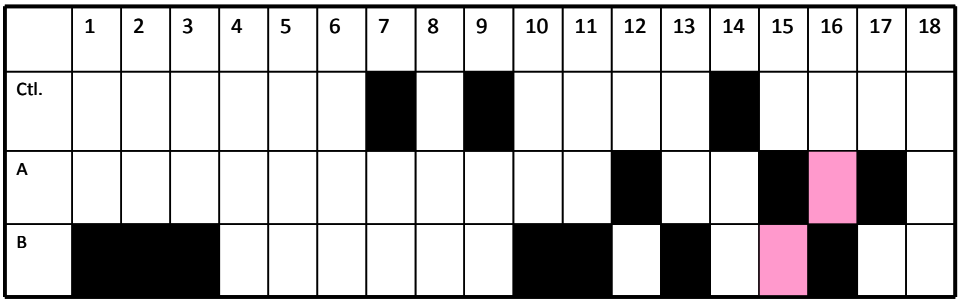
Figure 4: Odor responses following conditioning in EN-neurons. A) Percentage of odor sensitive (red) and odor non-sensitive units (black). B) As in A), percentage of odor sensitive (red) and odor non-sensitive units (black) in relation to the number of odors which evoked responses.

The single unit responses distribution for all odors in the pre-acquisition and test phase is depicted in figure 5.A and B, respectively. A change matrix (Fig. 5.C) illustrates the changes in the neuronal responses between pre conditioning and post conditioning phases (after both differential and reversal learning). As can be seen in Fig. 5.C, 10 out of 18 units exhibited a newly recruited excitatory response, but this phenomenon was not odor specific. Some units ceased responding to a given odor ('dropped' units), but interestingly, aside from one unit who stopped responding to both conditioned odors (unit 18), all other dropped units showed also a recruited response to one of the other odors. Figure 5.C provides a detailed depiction of all of the observed neuronal changes following conditioning.

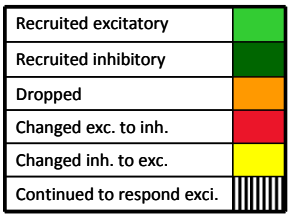
A PRE



B POST



C **Change Matrix**



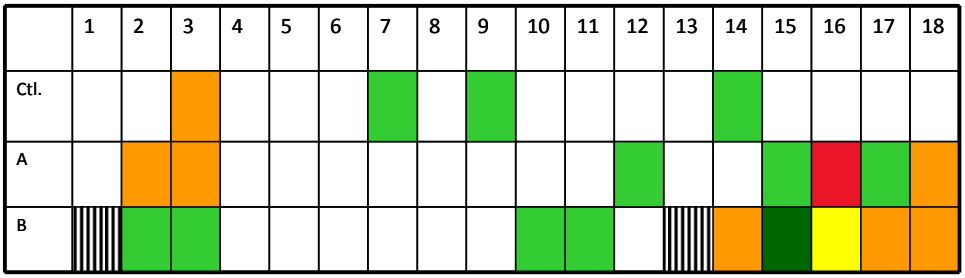


Figure 5: Distribution of odor responses prior and post reversal learning. A) Single unit responses to the control odor (Ctl.) and to the two conditioned odors (A and B) in the pre-acquisition phase (PRE). B) Single unit responses to the control odor (Ctl.) and to the two conditioned odors (A and B) in the post-acquisition phase (POST). Each column illustrates one unit, and each box indicates its response (black: excitatory, pink: inhibitory, white: no response) to a single odor as specified at the beginning of each row. C) **Response differences between pre and post conditioning.** A change matrix illustrates the changes in the neuronal responses between pre conditioning and post conditioning (both differential and reversal phases). Green indicates newly recruited excitatory response, dark green indicates newly recruited inhibitory response, orange indicates dropped units, red indicates a change from excitatory to inhibitory response, yellow indicates a change from inhibitory to excitatory response and black vertical stripes indicate units which continued to response in excitation to a given odor.

A sub-population of the ENs develop a learning-related neuronal response to the unrewarded odor during acquisition of differential learning

In order to investigate changes in neural response in the EN after complex form of learning, reversal learning paradigm was used (cf. Material and Methods). Reversal learning consists of two phases; first a differential learning phase is carried out, followed by the reversal of the CSs contingencies (reversal phase). At the behavioural level, in the last trial of the differential phase 4

out of the 6 bees showed a PER when presented with the rewarded odor (henceforth, odor A) and showed no PER to the unrewarded odor (henceforth, odor B). These bees were called “learners”. Across all bees, there was no significant difference in the neuronal response to odor A when pre-acquisition phase was compared with the last trial of the differential learning phase (McNemar's Test: $\chi 2= 0.9$, NS, df = 1). Again, no significant difference in the neuronal response to odor B between pre-acquisition phase and the last trial of the differential learning phase was observed (McNemar's Test: $\chi 2= 0.1$, NS, df = 1).

Based on the behavioral responses, again, no significant difference was observed when the comparison was done only among the learners group (Odor A: McNemar's Test: $\chi 2= 1.12$, NS, df = 1; Odor B: McNemar's Test: $\chi 2= 1.12$, NS, df = 1).

During the differential phase, no significant differences were observed in the neuronal response of the different units across trials, to both odors (Friedman Repeated Measures Analysis of Variance on Ranks: Odor A: H = 5.83, NS, df=5; Odor B: H = 8.96, NS, df=5). When divided to learners and non-learners, among the learners group by the end of the differential phase more units begun to respond to odor A, though this was not significant (P=0.06) (Friedman Repeated Measures Analysis of Variance on Ranks $\chi 2= 10.58$, NS, df = 5) and no difference was found in the responses to odor B (Friedman Repeated Measures Analysis of Variance on Ranks $\chi 2= 9.32$, NS, df = 5). Among the non-learners no difference was found to both odors (Friedman Repeated Measures Analysis of Variance on Ranks; odor A: $\chi 2= 1.47$, NS, df = 5; odor B: $\chi 2= 2.85$, NS, df = 5).

Interestingly, when only the units that showed a recruited excitatory response at the test phase were considered in the analysis (Fig.6.A), an increase in the neuronal response to the unrewarded odor, but not to the rewarded odor was observed during the differential phase (Friedman Repeated Measures Analysis of Variance on Ranks, odor B: $\chi 2 = 16.29$, $P<0.01$, df=5; odor A: $\chi 2 = 9.84$, NS, df=5). This effect was due to a difference between the first and the last trial of the

differential conditioning (Dunn's comparisons, P<0.01). Across all other units (non-recruited units) during differential conditioning trials no significant difference was observed (Friedman Repeated Measures Analysis of Variance on Ranks, odor A: $\chi 2 = 4.61$, NS, df=5; Odor B: H = 0.92, NS, df=5).

A **B**

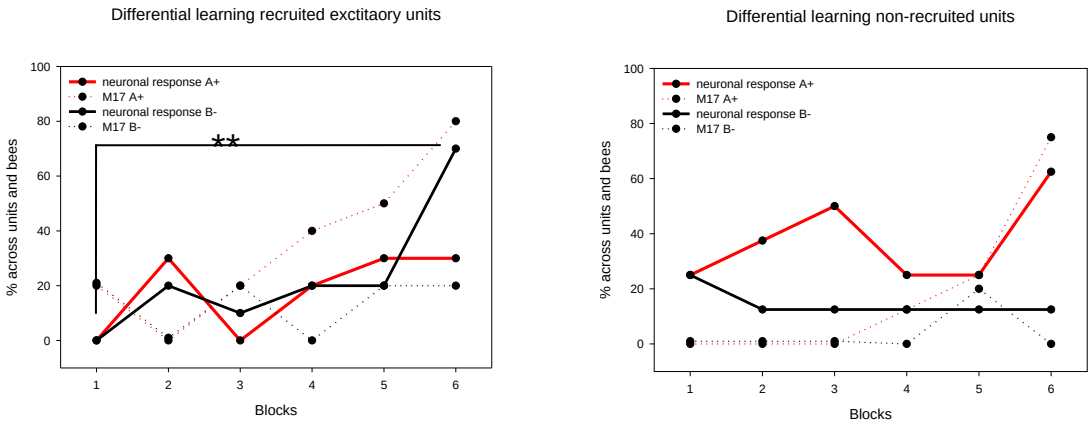


Figure 6: Neuronal and muscle activity for recruited and non-recruited units during differential learning. Shown are percentages of responding units (solid lines) and their correlative muscle activity as was recorded from the M17 (dashed lines) evoked by either of the two odorants A (red) and B (black). Each pair of odor A and odor B presentations constitute one block trial. A) Recruited units during differential learning. A significantly larger number of units responded to the presentation of odor B in the last learning trial, compared to the first learning trial (Friedman Test, N=10, P<0.001). B) Non-recruited units during differential learning. By the last trial a larger number of units increased their response to odor A, but not significantly (Friedman Test, N=8, NS).

When comparing figure 6.A with 6.B it becomes apparent that recruited and non-recruited units differ in the direction of their neuronal response during the differential phase; a larger proportion of the recruited neurons start respond to odor B at the last trial, whereby in the non-recruited group this trend is reversed. In both groups the difference in the neuronal response between the two odors becomes most apparent in the last trial, but not significant (McNemar's Test; non-recruited P=0.13, recruited P=0.13).

A mid-term memory effect might take place in the ENs following differential conditioning

One and a half hour after the end of the differential learning phase the reversal phase has begun; hence responses in the first trial of this phase reflect the mid-term memory consolidation of the differential phase. Here, only 3 bees continued to respond to odor A. Again, comparisons of the neuronal responses between pre-acquisition phase and the first trial of reversal phase to both odors were made. Also here, across all bees no significant difference was observed, for both odors (Odor A: McNemar's Test: $\chi2= 0.0$, NS, df = 1; Odor B: McNemar's Test: $\chi2= 0.25$, NS, df = 1). When divided to recruited and non-recruited groups, again no difference between the pre-acquisition phase and the first trial of reversal phase to both odors was found (McNemar's Test, Non-recruited: Odor A: $\chi2= 0.06$, NS, df = 1; Odor B: $\chi2= 0.25$, NS, df = 1; Recruited: Odor A: $\chi2= 0.5$, NS, df = 1; Odor B: $\chi2= 0.67$, NS, df = 1).

At the neuronal level, when the last trial of differential learning was compared with the first trial of reversal learning, across all units no significant difference was observed (McNemar's Test: odor A: $\chi2= 0.44$, NS, df = 1; odor B: $\chi2= 1.5$, NS, df = 1). Again, same results were seen when the comparisons were made separately for the recruited and non-recruited units, though a strong trend (P=0.07) in the reduction of the number of responding neurons was found in the recruited group to odor B (McNemar's Test; Recruited: odor A: $\chi2= 0.0$, NS, df = 1; Odor B: $\chi2= 3.2$, NS, df = 1; Non-recruited: odor A: $\chi2= 0.16$, NS, df = 1; Odor B: $\chi2= 0.0$, NS, df = 1). This finding, together with the neuronal change observed during differential learning in the recruited units,

might point to a short-term memory effect for an unrewarded stimulus which is represented in the ENs (see Discussion).

Learning-related changes in the ENs were not observed during acquisition of reversal learning

In the course of reversal learning no differences were found among the percentage of neuronal responses across trials to both odors (Friedman Repeated Measures Analysis of Variance on Ranks: All units together: Odor A: $\chi 2 = 5.83$, NS, df=5; Odor B: $\chi 2 = 8.96$, NS, df=5; only recruited: Odor A: $\chi 2 = 1.28$, NS, df=5; Odor B: $\chi 2 = 0.93$, NS, df=5; non-recruited: Odor A: $\chi 2 = 0.73$, NS, df=5; Odor B: $\chi 2 = 1.75$, NS, df=5).

Figure 7 illustrates both the neuronal and behavioural responses to odors A and B across all reversal learning trials, presented for recruited and non-recruited units separately (Fig.7.A and 7.B, respectively). Observing the two figures, it is clear that reversal learning is reflected in neither the behavioural nor the neuronal level, for both groups of units (see Discussion).

A **B**

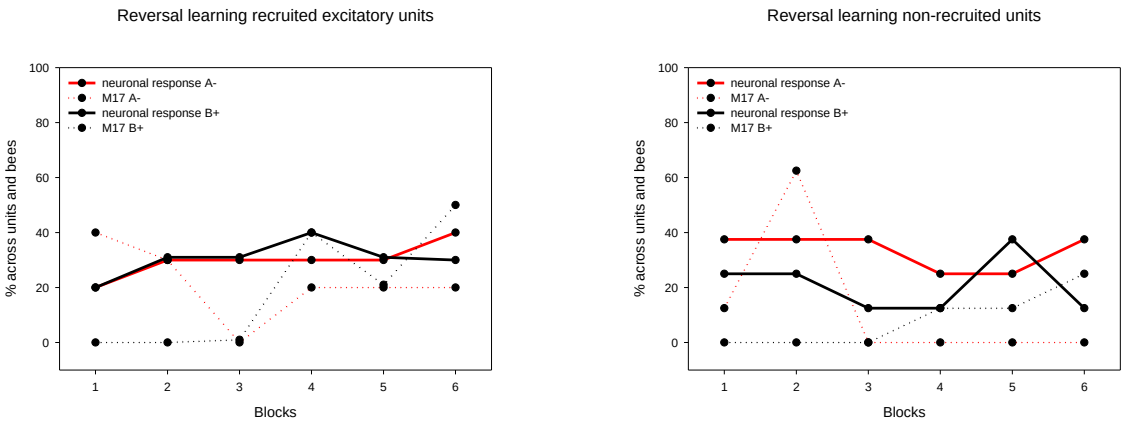


Figure 7: Neuronal and muscle activity for recruited and non-recruited units during reversal learning. Shown are percentages of responding units (solid lines) and their correlative behavioral response as was recorded from the M17 (dashed lines) evoked by either of the two odorants A (red) and B (black). Each pair of odor A and odor B presentations constitute one block trial. A) Recruited units during reversal learning. No significant difference in the course of reversal learning was observed, to both odors (Friedman Test, N=10, NS). B) Non-recruited units during reversal learning. Also here, no difference during reversal learning was observed, to both odors (Friedman Test, N=8, NS).

DISCUSSION

In the search for neuronal correlates of olfactory learning and memory, extracellular activity of putative mushroom body extrinsic neurons (ENs) was extracted while bees were presented with a reversal learning protocol. The MBs of the insect brain are important centers for learning and memory (e.g. Menzel, 2001; Heisenberg, 2003). The MB calyx is a major convergence site of second order olfactory neurons as well as gustatory sensory neuropils (Mobbs, 1982; Hammer, 1993). It has been postulated that during classical conditioning associations between an odorant and a sugar reward are formed within the MB of the honeybee (Menzel, 2001) and following appetitive odor learning, the intrinsic neurons of the MB, the Kenyon cells (KC), undergo associative plasticity (Szyszka et al., 2008). However, in contrary to flies (Heisenberg et al., 1985; deBelle & Heisenberg, 1994; Dubnau et al., 2001), some simple forms of learning can be successfully learned by bees even in the absence of intact MB (Malun et al., 2002; Komischke et al., 2005). The ENs gather information from KC and further convey this information to various parts of the honeybee brain and hence constitute the MB output region (Rybak and Menzel, 1993). Upon blockade of the main output region of the MB, the alpha-lobe, olfactory differential learning was found to be intact, whereas the acquisition of the reversed contingencies was impaired (Devaud et al., 2007). It was hence concluded that output from the MB is a requirement

for the acquisition of reversal learning. The exact neuronal processing during complex forms of learning within the MB, and specifically in its output region, still awaits a thorough exploration. The present study was designed to investigate neuronal activity of the ENs during a one form of complex learning- reversal learning.

An increased number of ENs respond to odor presentation following reversal learning

Here, following reversal learning an increased number of EN units were found to respond to odor presentations, though this response was not specific to the odor identity. This finding is in disagreement with results previously found in our lab (Strube-Bloss et al., paper in press), where ENs were recruited to respond solely to the CS+ odor, and therefore considered to encode for the rewarded stimulus. Such a discrepancy might result from a different ENs' population being recorded. Although an attempt was made to target the same neuronal population by inserting the electrode to the ventral part of the alpha-lobe between a depth of 100-250 μm, such a possibility exists. Another difference between the current study and previous results relates to the fewer number of odor evoked responses observed here. None of the recorded units exhibited neuronal responses to all three used odors, neither in the Pre-acquisition phase, nor in the Test-phase. Again, this might be explained due to different neuronal population being recorded from. Nevertheless, almost third of the units developed new responses to one or two of the presented odors, indicating an experience-derived neuronal plasticity.

A sub-population of the ENs develop a learning-related neuronal response to the unrewarded odor during acquisition of differential learning

During differential conditioning, across all neurons no significant differences were observed in the number of responding units to both odors. A surprising finding arises when focusing only on the units that in the Test-phase exhibited a newly recruited response to any of the three presented

odors; during the differential phase, this sub-population developed a neuronal response only to the unrewarded odor. The responses to the rewarded odor remained unchanged during all conditioning trials, whereas only by the last presentation of the CS- odor a significant increment of responding neurons was observed. It could well be that those neurons encode the short-term memory for an unrewarded odor in the context of a rewarded one, i.e. in the course of differential conditioning. To recall, 170,000 KCs receive second-order sensory input from different modalities (Gronenberg, 1986; Mobbs, 1982; Schröter and Menzel, 2003) and their axons converge to about merely 400 ENs in the vertical (α)-lobe and in the medial (β)-lobe (Kenyon, 1896; Mobbs, 1982; Rybak and Menzel, 1993). It is therefore believed that these neurons are involved in learning-induced plasticity and this supposition is supported by studies done in different insect species, including the honeybee (Cassenaer and Laurent, 2007; Yu et al., 2005; Mauelshagen, 1993; Menzel and Manz, 2005). As the KCs were shown to change their responses to both rewarded and unrewarded odors (Szyszka et al., 2005), a plausible explanation for this finding would be that in the context of disparity conditions (i.e. differential conditions) some of the ENs encode exclusively the information for the coming absence of a rewrad, but only once stimuli acquire reliable prediction features. Interestingly this learning-related plasticity took place in the course of training; this could be as a result of the relative high numbers of trials presented for each stimulus (6 for each CS), leading to a rather lengthy training phase (over 30 min.). It is worth pointing out, that in the non-recruited group an opposite trend was observed, where an increase in the neuronal responses to the rewarded odor over the course of learning occurred; however, this trend was not statistically significant (P=0.13). Since the number of the non-recruited units in this study was rather small, it might well be that this effect would be significant upon increasing the N number. It seems that recruited and non-recruited units represent two different populations which differ in their preference for stimuli's identity encoding.

A mid-term memory effect might take place in the ENs following differential conditioning

At the first trial of the Reversal-phase, the number of both recruited and non-recruited units did not differ in regard to the two odors. Though statistically not significant (P=0.07), a decrease in the number of responding units to odor B (formerly unrewarded) in the recruited group was observed (from 7 responding units in the last trial of differential phase to 2 in the first trial of reversal phase). Following differential conditioning, KC responses were found to be subjected to associative plasticity (Szyszka et al., 2008). Specifically, a recovery from repetition-induced decrease in the neuronal responses following pre-exposure was observed solely to the rewarded odor whereas a further decrease to the non-rewarded odor was seen. Moreover, Szyszka et al. (2008) found a considerable alteration in the spatial pattern of activated KCs in regard to the CS-, which also included a dropout of initially CS- responding KCs. One possibility is that like observed in the KC, the ENs also decrease responses to an unrewarded odor, and due to the relative low number of recorded units in this study, such an effect has not reached statistical significant. If this holds true, it might be that in this study, the population of ENs which exhibit a recruited excitatory response, code the new mid-term memory for unrewarded stimuli, and as time elapses that memory becomes independent of the ENs and consolidation is found elsewhere. In mammals for example, compelling evidence point to the involvement of new circuits following memory consolidation, specifically the Hippocampal-Neocortical Interactions where information is initially processed in the Hippocampus and upon consolidation stored in specific neocortical regions (for review see Wiltgen et al., 2004).

Learning-related changes in the ENs were not observed during acquisition of reversal learning

Learning-related changes were not observed during reversal learning, in neither recruited nor non-recruited units. In both groups not more than 40% of the units responded to either one of the presented odors. Moreover, no significant difference in the neuronal responses between the two

conditioned odors was observed (Fig.7), in none of the learning trials. Looking at the behavioral performance during this phase it is clear that this new learning rule was poorly learned. Assuming these results reflect a genuine phenomenon, the straightforward explanation would be that those recorded neurons do not encode complex form of olfactory learning. However the poor learning curves together with the relative small number of recorded units suggest a careful examination of the results and call for a future replication.

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STUDYING THE INVOLVEMENT OF THE ENS OF THE HONEYBEE IN HABITUATION OF THE PROBOSCIS EXTENSION REFLEX

ABSTRACT

The mushroom bodies of the honeybee's brain are paired central structures involved in the processing of stimuli of different sensory modalities and play a prominent role in memory formation and higher order learning. The extrinsic neurons (ENs) of the alpha-lobe compose one of the output regions of the mushroom bodies and were shown to modulate their responses toward a reward predicting stimuli (CS+). However, little is known about their properties upon simple reward administration. To study that, extracellular recordings of single ENs were performed while bees endured repetitive sugar reward stimulations to the antenna, a well-defined paradigm which results in a behavioral habituation of the proboscis extension reflex. A sub-population of the ENs was found to respond to mere reward administration out of which one cell habituated its response resembling one neuronal basis for behavioral habituation.

INTRODUCTION

The mushroom bodies (MB) in the honeybee brain are multisensory integration centers. The calyces of the MB are the main input region and the lobes serve as the output region (Mobbs, 1982). The MB extrinsic neurons (ENs) of the alpha lobe are a well defined morphological class of neurons, they receive their input from the Kenyon cells (KC) of the calyces. ENs were shown to exhibit neuronal plasticity in an olfactory conditioning paradigm (Mauleshagen, 1993; Okada, 2007; Strube-Bloss et al.), More specifically, these neurons were found to modulate their tuning strength which was mainly dominated by increased responses to reward predicting stimuli (CS+) (Strube-Bloss et al., in press). One of the most prominent neuron of the alpha lobe ENs is the PE1

which posses large branches collecting information from KCs. This neuron shows a reduction in its neuronal response towards a rewarded stimulus (Okada, 2007). Little is known about the properties of these neurons upon plain reward administration.

The mere administration of sucrose solution to the antenna of the honeybee elicits a reflexive extension of the proboscis (PER) (Kuwabara, 1957). Repetitive stimulation of one antenna with a low concentration of sucrose leads to a decrement and finally to the disappearance of this response, which can be restored by stimulating the contra lateral antenna with a high sucrose concentration (Braun & Bicker, 1992). This behavioral plasticity conforms to essential parametric characteristics for habituation. Habituation is a ubiquitous phenomenon which is defined as a behavioral response decrement as a result of repeated stimulation. Habituation is a non-associative form of learning, hence considered to be the most basic form of learning and neuronal plasticity (Harris, 1943; Humphrey, 1933; Thompson & Spencer, 1966). The underlying neural mechanism have been studied in varies systems. The most prominent include the gill and siphon withdrawal reflexes of the mollusk, Aplysia californica (Castellucci et al., 1970; Byrne, 1982), the hindlimb flexor reflex of the spinal cat (Groves & Thompson, 1973), and the tail flip escape response of the crayfish (Krasne, 1969; Zucker 1972). These investigations account for activity changes upon behavioral habituation in the intrinisc properties and synpatic connections of sensory-, motor- and interneurons, as well as changes in muscle properties. In addition, changes in central neurons upon behavioral habituation have been described. One mechanism underlying habituation is homosynaptic depression at excitatory synapses from sensory neurons to inter- and motor neurons, studied in the Aplysia gill-withdrawal reflex circuit (Bailey & Chan, 1988; Byrne, 1982; Castelucci et al., 1970; Castelucci & Kandel, 1974; Edmonds et al., 1990; Klein et al., 1980). Wood et al. (1994) depicted decreased firing rate in the ipsilateral proleg motor nerve as a consequence of habituating the proleg withdrawal reflex in isolated proleg preparations of hawk moth larvae, manduca sexta. However, little is known about higher-order neurons, that receive information about a stimulus applied repetetively from upstream neurons which are also involved

in learning and might carry additional information necessary for goal-directed behavior, for example the state of motivation as a consequence of the saturation level of the animal. Neural correlates underlying habituation might therefore be mediated by a distributed network with multiple plastic changes in sensory and central microcircuits.

This study combined extracellular recordings from the ENs of the alpha lobe during the repetitive stimulation of one antenna with a droplet of sugar solution. The proboscis extension response (PER) is elicited by this appetitive component of the bee's feeding behavior. As the release of the PER depends on the bee's degree of satiation (Menzel et al., 1991; Braun & Bicker, 1992), and in order to investigate whether the satiation level during habituation is differentially encoded in MB ENs, bees were tested under different satiation conditions, either hungry or upon feeding. All together ten bees were dissected of which 36 different units were sorted and identified. Only seven units were found to respond to sugar administration on the antenna. Sugar responses were not observed in all experimental trials, as neuronal responses fluctuated over experimental trials. However, neuronal responses of only one unit were correlated with the stimulation sequence and exhibited a significant lower firing rate during behavioral habituation in comparison with their activity before habituation evolved. No such correlations or significant differences were detected in the remainder sugar responsive units.

MATERIAL AND METHODS

Subjects

Honeybees (Apis mellifera carnica) were caught at the hive entrance during the afternoon, one day prior to the experiments. Bees were anesthetized using ice and then harnessed by stripes of tape in little metal tubes which allowed the free movements of the antennae, mandibles and proboscis (Bitterman et al. 1983). The scapes of the antennae were fixed onto the head using

eicosane (Sigma) allowing only the movement of the flagellum. Bees were fed to satiation with 30% sucrose solution and kept overnight at a dark humid chamber with room temperature.

Experimental paradigm

General handling - Once a signal activity was detected and the area surrounding the electrode was sealed, bees were left untouched for half an hour, to achieve a reliable signal activity and to avoid tissue's movement.

Habituation of the appetitive reflex

Droplets of 5% sucrose solutions were used as habituating stimuli (Braun and Bicker, 1992) and were applied to the tip of the antenna using a toothpick with a 10 sec. inter trial interval (ITI). The habituation protocol was applied to hungry bees, and then again to the same bees when they were satiated (upon feeding). The number of trials varied between bees and conditions as a result of the individual time needed to habituate (see figure 1 for the visualization of the experimental design). If no visible movement of the proboscis occurred in five subsequent trials, it was considered as a full habituation of the proboscis reflex. The habituated antenna was either the antenna stemming from the recorded hemisphere (ipsi) or from the contralateral hemisphere (contra). As the satiation level of the bee affects the number of trials needed to achieve an habituation of the PER, bees were tested under two conditions, when hungry (fed 12 hours before experiment started) and when satiated (fed shortly before experiment started). Habituation was not achieved in all of the tested conditions. Table 1. summarizes all experimental conditions across a total of seven sugar responsive units recorded in three different bees.

The stimulation of the antenna was computer-controlled using a Visual Basic Script (VBA 6.0, Microsoft, USA) written by Frank Schaupp. Time stamps of stimuli were marked with the data acquisition system.

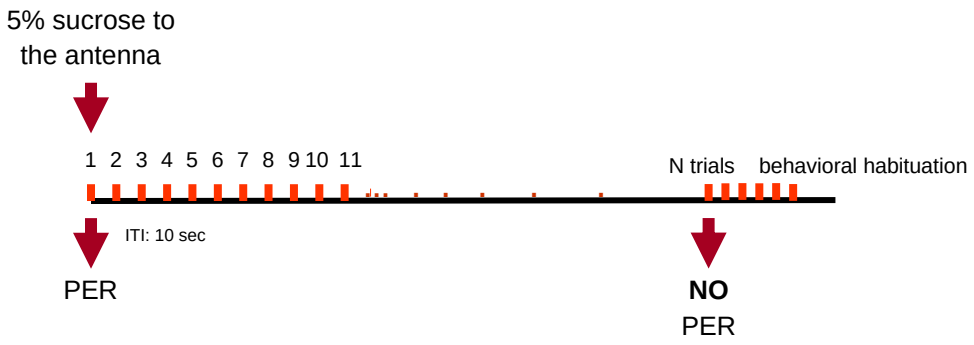


Figure 1. Experimental paradigm. Bees were fed with a 5% sucrose solution to one antenna with an ITI of 10 sec as long as the bee were not responding with a PER in five consecutive trials, which was interpreted as behavioral habituation (see text).

Dissection

Restrained bees were placed in a magnetic stand adapted for the electrophysiological setup and dissections were made under a compound electrode. A 25 µm-diameter silver wire (Nilaco, Tokyo, Japan) served as the reference electrode and was inserted into the median occellus. Another 25 µm-diameter silver wire (Nilaco, Tokyo, Japan) was used to record the proboscis extension response (PER) which is mediated by the M17 muscle (Rehder, 1987) and was hence inserted between the right lateral occellus and the compound eye. For the recording of the ENs neurons, a tiny unilateral square was cut between the midline of the bee head and the antenna. The area above the alpha-lobe was removed from head glands and trachea sacks. The electrode was inserted in the ventral part of the alpha-lobe at a depth between 100 and 250 µm. Once signals were detected, the area surrounding the electrode was filled with two-component silicon (KWIKSIL Sarasota, FL, USA) in order to prevent the drying out of the brain and the movement of the electrode.

Electrophysiology

Electrode

Electrodes consisted of three polyurethane-coated copper wires, 14 μm in diameter (Electrisola, Escholzmatt, Switzerland). The electrode preparation was adopted from Ryuichi Okada (Mizunami et al., 1998; Okada et al., 1999) and was modified to obtain three recording channels. The three wires were waxed together onto a tungsten wire (1-2 cm long and 100 μm in diameter) and then attached to a glass capillary. A custom-built adapter held the glass capillary and was connected to the headstage amplifier (Headstage-27 Amplifier Neuralynx, Tucson, AZ, USA).

Differential Recording

The three different wires allowed a differential recording which consisted of the three possible combinations arising from the subtraction of each raw channel with a different one (for an illustration see Figure 1 Chapter 3). Signals were only analysed from the differential channels which are not affected from motor activity of the M17 and are thought to reflect the neuronal activity in the close vicinity to the tip of the electrode. Utilizing this data allowed to record and identify from up to five different neuronal units simultaneously. Electrical signals were amplified by a Lynx-8 Amplifier (Neuralynx, Tucson, AZ, USA). A 1-9000 Hz band-pass filter was used and data was digitized at a 20 kHz sampling frequency. The recording files were then imported into Spike2 (Cambridge Electronic Design, Cambridge, UK) data format and a high-pass filter (600 Hz) was applied.

Spike Sorting

The process of spike sorting is based on two differentiating features: spike shape and spike amplitude. Each neuron has its intrinsic electrochemical dynamic which generates an action potential with an individual characteristic shape. The amplitude of the neuron's spike as depicted in the electrode is derived from the spatial distance of the neuron from the tip of the electrode.

Hence those two features allow the classification of action potentials into distinct neuronal units. As mentioned, for the analysis of the alpha lobe-extrinsic neurons, only the differentially recorded channels were used. Spike sorting was made using Spike2 template sorter, each channel was sorted separately. Upon sorting a principal component analysis (PCA) was used to classify the different spike events into separate units.

Visualization of the recording site

In order to confirm that recording were attained from the alpha-lobe extrinsic neurons, a visualization technique was applied. To this aim, the tip of the electrode was dipped into Micro Ruby shortly before the insertion of the electrode. At the end of the experimental procedure, the electrode was moved and the brain was dissected and fixed in 4% formaldehyde diluted in 50% methanol for 24 hours at 4 C°. Preparations were then rinsed for 20 minutes in phosphate-buffered saline (PBS; pH 6.7), diluted 1:4 in distilled water, dehydrated in an increasing ethanol series (30 %, 50 %, 70 %, 90 %, 99 %, 100 %) each step lasted 10 minutes, then cleared in a mixture of 50% methyl-salicylate (MS) and 100% ethanol and embedded as whole mounts in MS in double-sided custom slides. A confocal laser scanning microscope (Leica TCS SP2) with a Leica HC PL APO CS 10.0×0.40 UV dry lens objective was used to scan the preparations. Results confirm that the recorded signals can be related to the ventral part of the alpha-lobe (Figure 2). Taken together with the controlled depth of the electrode (between 100-250 ìm), one can conclude that the recorded ENs were probably type A1, A2, A4, A5 or A7 (Rybak and Menzel, 1993).

Data Analysis

Response detection

All neuronal responses (spike trains) for each experimental condition were converted to Peri-Stimulus-Time-Histogram (PSTH) using a bin width of 50ms.

For the detection of neuronal responses derived from a specific stimulus, inter-spike intervals (ISIs) were calculated separately for each experimental trial in two separate time windows: 3 seconds preceding a delivered stimulus and during 1-2 seconds following stimulation, when a robust response was seen. A Wilcoxon rank-sum test was then applied to test for a significant ($P< 0.05$) difference in the medians for the two ISI distributions of each trial separately. This approach was chosen since most of the neuronal responses lasted over several hundred milliseconds and response detection based on the ISI distribution was described to be powerful for the detection of slow and longer lasting response changes (Strube-Bloss & Nawrot, in press).

Normalization of neuronal responses

For the normalization of neuronal responses, the number of spikes for each trial was calculated during 1 sec. preceding a delivered stimulus (spontaneous activity) and 1 sec. following a delivered stimulus (sugar response), again, when a robust response was visible). The change in spike rate (Δ) was then calculated by subtracting the later value from the former, and this difference was divided by the total sum of both values. Hence for each trial a normalized value was computed, ranging from -1 to +1; reflecting a decrease of neuronal response in response to sugar stimulation or an increase, respectively. When no change in spike frequency was observed, this value was 0. For each experimental condition, the mean of the normalized responses was calculated across trials before a behavioral habituation was achieved, and for trials in which a behavioral habituation was reached. For the comparisons of neuronal responses between non-habituated and habituated trials, one-way ANOVA was employed. A linear regression of normalized neuronal responses over trials was computed for each experimental condition. One-way ANOVA and in one case Kruskal-Wallis one-way analysis of variance (due to the lack of normal distribution) was calculated between the first five trials before behavioral habituation and the five trials in which habituation occured to determine rate changes underlying behavioral habituation. We confined our analysis to the first five trials in order to keep the sample sizes symmetrical. We visualized, however, the mean of the normalized firing rate for all trials needed

until behavioral habituation (five consecutive trials without a PER) occured (figure 4). Therefore, statistics and mean values of the figures have to be interpreted separately.

Statistical and programming software

For response detection data analysis was carried out with Matlab. For the normalization of neuronal responses Spike2 software (Cambridge Electronic Design, Cambridge, UK) script was used. Statistics were carried out using SigmaStat software; plots and figures were done using SigmaPlot software.

RESULTS

Visualization of the recording position at the output region of the mushroom body

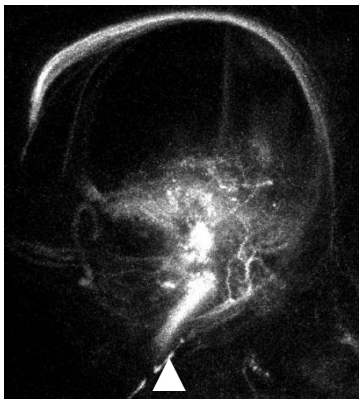


Figure 2. Visualization of the recording site.

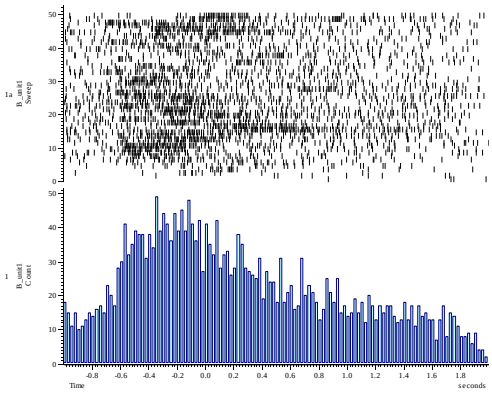
To confirm that recordings were made from the alpha-lobe-extrinsic neurons, the tip of the recording electrode was dipped into a fluorescent dye (micro-ruby) before inserting it to the brain. Upon ending the experimental procedure, the brain was dissected and scanned with a

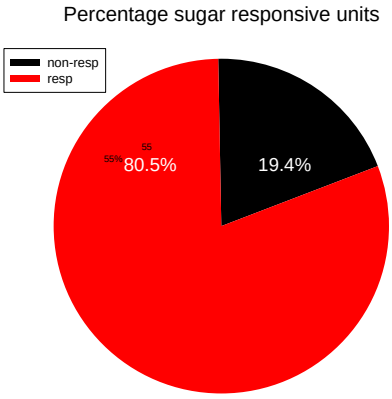
confocal laser scanning microscope. Shown in figure 2 is the left alpha-lobe of the MB, with fluorescent areas distributed in its ventral part. The electrode has been inserted at the outer ventral border of the lobe (see white arrow), where the dye was transported along the MB EN arborizations inside of the lobe. Results thus confirm that the recorded signals can be related to the ventral part of the alpha-lobe. As the depth of the electrode (between 100-250 µm) was also controlled, one might conclude that the recorded ENs were probably types A1, A2, A4, A5 or A7 (Rybak and Menzel, 1993).

General properties of sugar responsiveness of the mushroom-body extrinsic neurons

A total of 36 single units (in 10 animals) were isolated and sorted. The spontaneous activity in all units varied between 8.18 and 33.26 Hz. As can be shown in Figure 3, only about 20% (resp.) of the recorded ENs were sensitive to sucrose stimulation of the antenna. The other 80% (non-resp.) were sugar-insensitive, as could be seen by converting their spike trains into Peri-Stimulus-Time-Histograms (PSTH), and hence were not further analyzed.

A **B**





▲

Figure 3: Sucrose responses in EN-neurons. A) Represantative example of one unit firing in response to sugar application in multiple trials. The arrow indicates the onset of sugar administration. B) Percentage of sucrose sensitive (red) and sucrose non-sensitive units (black).

Table 1 summarizes the percentage of trials that clearly showed a sugar response in the seven identified units, for both, the trials before and during behavioral habituation. Responses were calculated by comparing the statistical difference of the two medianes of the ISI-distriution before and after sugar stimulation (see Methods). The criterium for behavioral habituation was met as soon as the bee did not extend its proboscis in five consecutive trials. The amount of trials needed until behavioral habituation evolved varied from animal to animal. For each of the seven units and each of the four conditions (condition 1: Ipsilateral hungry, condition 2: Ipsilateral saturated, condition 3: Contralateral hungry, condition 4: Contralateral saturated) the percentage of "Before behavioral habituation" and "During behavioral habituation" are depicted. Note that some animals did not meet the criterium of behavioral habituation in some conditions. Specifically, bee 1 (unit 1 & 2) did not exhibit behavioral habituation durig both hungry conditions (ipsi-, and contralateral). Bee 2 (units 3 & 4) did not habituate during the ipsilateral saturated condition, and bee 3 (unit 5-7) did not show habituation during the contralateral hungry condition. Not all conditions were tested in all animals. Missing conditions were marked with a dash in table 1. In bee 2 both hungry conditions are missing, while in bee three the ipsilateral hungry condition was not tested. As for both hungry conditions (condition 1 and 3) either the animal did not meet the criterium for behavioral habituation or the conditions have not been tested, they were not included in the comparative analysis that aimed to elucidate rate changes underlying behavioral habituation. The minimum amount of responsive trials was 10% (of the total amount of trials needed until behavioral habituation has been fully established) for unit 3 in condition 2 (ipsilateral saturated), the maximum amount of responsive trials was 90.62% for unit 1 in the contralateral saturated condition. The mean of the percentage of responsive trials before and during behavioral habituation for the seperate conditions were as follows: In the ipsilateral

hungry condition (condition 1), 27.5% (SD = 2.5) of the trials before behavioral habituation were responsive, as mentioned above no data is available during habituation. In the ipsilateral saturated condition (condition 2), 40.14% (SD = 23.11) of the trials before and 43.39% (SD = 38.37) of the trials during behavioral habituation were counted as responsive. For the contralateral hungry condition (condition 3), 44 % (SD = 18.07) of the trials before behavioral habituation were responsive, again, no data wass available during behavioral habituation. For the fourth condition, contralateral saturated, 20.41% (SD = 20.41) of the trials were responsive before, while 38.37% (SD = 21.06) of the trials during behavioral habitutaion were responsive. Thus, the two comparable conditions did not feature a decline in responsiveness, at least concerning the overall frequency of responses before compared to during habituation. On the contrary they even show a slight increase in responsiveness during habituation. In order to analyze if the magnitude of responses declines, linear regression was calculated between the normalized firing rate of the single units and the sequence of trials. Only one unit, unit 5 in the ipsilateral saturated condition showed a linear decline with proceeding trials before behavioral habituation has been fully accomplished ($F = 11.45$, $p = .002$), see table 1. All other units did not exhibit any linear correlation with the sequence of trials, hence were stable in their response magnitudes throughout the trials before and during behavioral habituation, respectively. For the comparison of neuronal responses between non-habituated and habituated trials, one-way ANOVA was employed. The normalized rate of the first five trials before behavioral habituation and the five trials in which behavioral habituation was detected were compared. Only unit 5, the unit that exhibited a linear regression with trial sequence before behavioral conditioning also evidenced a significant difference, here. The rate was decreased during behavioral habituation in comparison with the first five trials before behavioral habituation ($F = 5.33$, $p = .005$), also in the ipsilateral saturated condition. No other significant differences were found, neither in this unit in other conditions, nor in the remainder units (see table 1).

In figure 4 the rates of the single units in the two saturated conditions were visualized. Note, that here all trials before full behavioral habituation was reached were considered, the average normalized firing rate of these trials was plotted against the mean rate during habituation.

Table 1. Percentage of trials, before and during behavioral habituation, in which units were sugar responsive & results from linear regression and one-way ANOVA analysis

		Responsive trials (%)		Linear Regression				ANOVA	
		Before	During	Before vs. Trials		During vs. Trials		Before vs. During	
Units	Condition	habituation	habituation	F	p	F	p	F (H)	p
1	1	25.00	no habituation	0.05	ns.				
	2	89.40	66.67	0.16	ns.	1.37	ns.	H=0.54	0.555
	3	75.00	no habituation	1.66	ns.				
	4	90.62	50.00	0.01	ns.			2.72	0.138
2	1	30.00	no habituation	0.54	ns.				
	2	26.30	16.67	0.58	ns.	0.46	ns.	0.18	0.687
	3	50.00	no habituation	0.02	ns.				
	4	40.00	12.50	0.09	ns.	0.13	ns.	1.66	0.234
3	1	-	-						
	2	10.00	no habituation	0.16	ns.				
	3	-	-						
	4	61.11	60.00	1.34	ns.	2.21	ns.	0.08	0.787
4	1	-	-						
	2	43.33	no habituation	1.08	ns.				
	3	-	-						
	4	25.00	0.00	1.94	ns.	1.38	ns.	0.08	0.153
5	1	-	-						
	2	46.42	63.63	11.45*	0.002	1.53	ns.	5.33*	0.005
	3	25.00	no habituation	0.07	ns.				
	4	32.00	46.15	0.32	ns.	0.01	ns.	0.16	0.703
6	1	-	-						
	2	27.58	30.00	2.32	ns.	0.51	ns.	2.43	0.159
	3	42.50	no habituation	0.08	ns.				
	4	40.00	53.80	0.54	ns.	1.03	ns.	0.42	0.533
7	1	-	-						
	2	37.93	40.00	0.03	ns.	0.04	ns.	0.74	0.115
	3	27.50	no habituation	0.01	ns.				
	4	44.00	46.15	0.01	ns.	0.86	ns.	0.24	1.643

** significant difference*

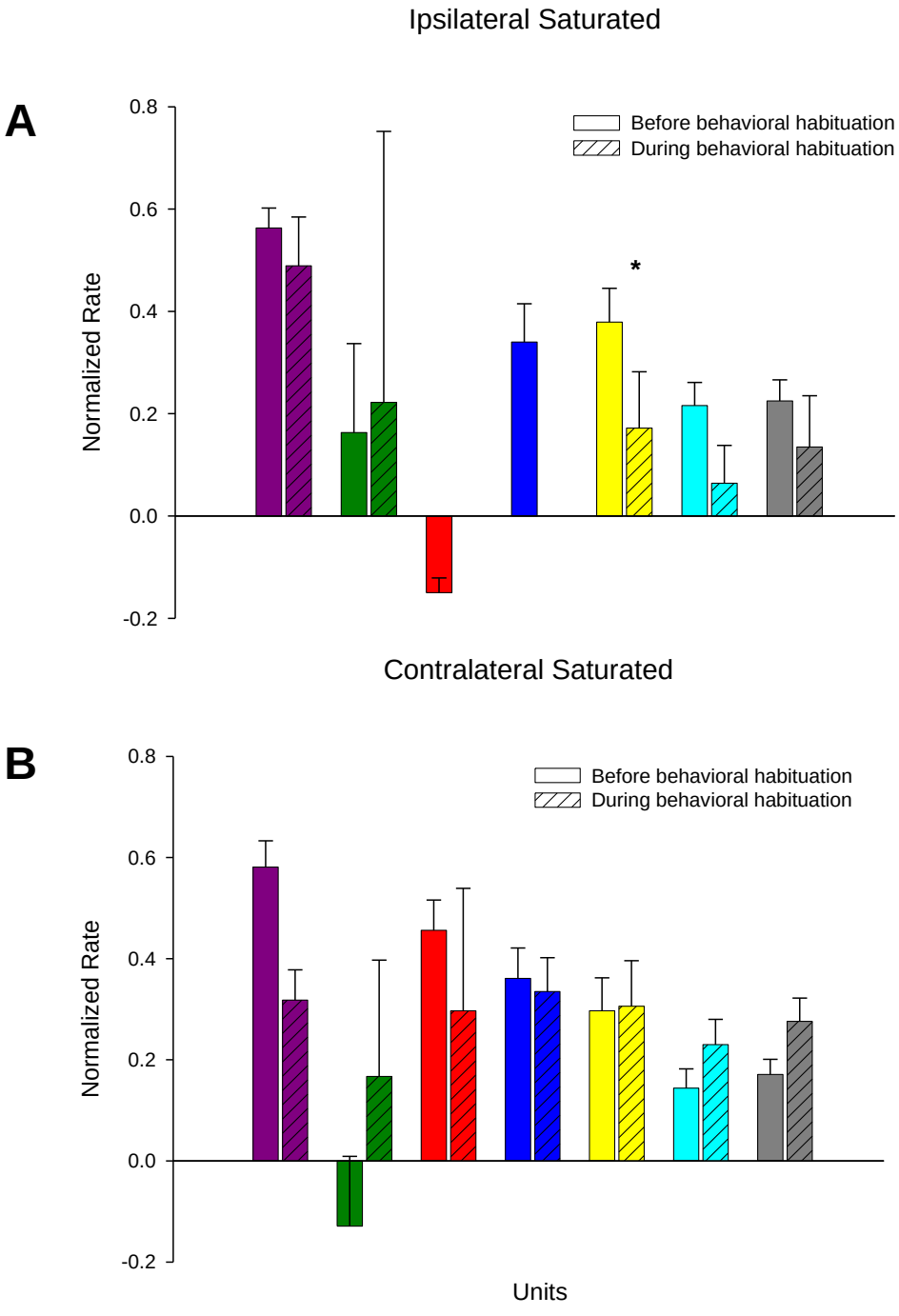

Ipsilateral Saturated
A
Before behavioral habituation
During behavioral habituation
Normalized Rate
0.8
0.6
0.4
0.2
0.0
-0.2
*
Contralateral Saturated
B
Before behavioral habituation
During behavioral habituation
Normalized Rate
0.8
0.6
0.4
0.2
0.0
-0.2
Units

Figure 4. The mean normalized firing rate from all trials before behavioral habituation was fully established (solid bars) was plotted against the averaged rate during habituation (hashed bars) for **A** the ipsilateral saturated and **B** the contralateral saturated condition, respectively. Shown are the units in the same order as described in table 1. From left to right: Unit 1 (purple), unit 2 (green), unit 3 (red), unit 4 (blue), unit 5 (yellow), unit 6 (cyan), unit 7 (grey). The significant difference between the rate during habituation in comparison with the rate before habituation, that was assessed with one-way ANOVA and calculated with the first five trials before behavioral habituation only is marked in A for unit 5 with an asterisk.

DISCUSSION

36 MB ENs were recorded during repetitive application of sugar water to the ipsi- or contralateral antenna while the bees were either hungry or satiated. Seven units (19.4%) were found to be sugar responsive while the rest did not respond upon sugar administration. Investigations of whether the activity of those sugar responsive units changed depending on the motivational state of the animal, and whether these rate changes are also seen in the contralateral hemisphere were conducted.

Bees did not develop behavioral habituation when they were hungry, irrespective of ipsi- or contralateral sugar administration. Sugar water was applied without five consecutive trials in which the bees did not show a PER. This is consistent for all animals investigated. This phenomenon was already described by Braun & Bicker (1992). The authors concluded that state dependence of habituation became manifested in a smaller response decrement and the need for more trials until disappearance of the response has evolved in hungry compared with satiated animals.

In the satiated condition behavioral habituation evolved, but the amount of trials needed for the disappearance of the response varied from animal to animal.

Neuronal activity before behavioral habituation was explored and compared with its rate during behavioral habituation.

Only one unit was found to show a significant decline in firing rate as a response to sucrose application when the bee has developped prominent behavioral habituation in comparison with the rate before habituation. This unit`s rate already declined with the proceeding of trials before behavioral habituation has been fully established. Thus, the more often the sugar was applied the less this unit fired. With this linear decline in rate this unit (amongst others with the same physiological characteristic) presumably promotes behavioral habituation until a certain threshold in decline in rate has been reached to allow for the reflection of habituation in the behavior of the animal. As already mentioned in the introduction central neural correlates have been found in motor neurons of hawk moth larvae that declined their firing rate upon habituation of the proleg withdrawal reflex. This unit was found to habituate only in the ipsilateral saturated and not in the contralateral saturated condition, in line with the finding of a stronger habituation effect in ipsilateral motor neurons in the moth on the first trial opposed to contralateral motor neurons (Wood et al., 1997). However, when they compared the standardized responses on both sides over 20 trials, they found the magnitude of decrement to be similar for the two sides. They therefore propose a shared neuronal pathways underlying behavioral habituation. Different explanations may exist for the fact that here no contralateral response decrement was observed. First, the neuronal pathways differ between honeybees and the hawk moth. Also, the neuronal habituation happened in motor neurons, which directly receive input from sensory neurons. Thus, beyond anatomical differences in the two organisms, here the MB output neurons were recorded (instead of motor neurons) and they presumably comprise the highest cognitive level in the honeybee brain. Based on earlier findings about the involvement of the ENs in learning and memory (Strube-Bloss et al., in press) and context-dependency tasks (Syed Abid Hussaini, PhD thesis), it seems clear that the functions of these neurons are more diverse than the function of

motor neurons. This is supported by the fact that only one out of seven sugar responsive units exhibited neuronal habituation while the remainder units did not significantly change their response rate upon behavioral habituation, although also in these units weak changes are reflected in the average firing rate of all trials before behavioral habituation has fully established (see figure 4), that did not reach significance when only the first five trials were compared with the five trials during habituation using one-way ANOVA (see Methods for explanation). For example, unit 1, displayed in purple in figure 4, showed a trend for response decrement during behavioral habituation when the contra- but not the ipsi-lateral antenna was stimulated. Unit 2, displayed in green in figure 4, even inversed its response polarity: Before behavioral habituation it responded with an inhibition while this response reversed to an excitation throughout behavioral habituation. Nonetheless, these differences in firing rate were not sufficiently high to reach statistical significance.

The averaged response of unit 3 (outlined in red) before behavioral habituation was inhibitory for the ipsi- and excitatory for the contralateral sugar administration. Again, there is a trend for a response reduction exhibited during behavioral habituation for the contralateral condition, although this difference did not reach significance when the five trials were compared, respectively. Unfortunately, data is lacking for this unit`s response during behavioral habituation, as this bee did not exhibit PER habituation in this condition. Similar to unit 5 (displayed in yellow) as the only significantly habituating unit also unit 6 and 7 (displayed in cyan and grey) showed a trend towards response decrement in the ipsilateral condition, while this pattern is inversed for the contralateral condition. As all these changes in firng rate were not detected as significant one possible assumption is that the initial responses during the first five trials were less strong and that they became more pronounced in the following trials until they decrease again upon behavioral habituation, or that the fluctuations in the responses of the six units are not related to neuronal habituation. Further analysis is needed for clarification.

What might be concluded by now is that only one out of seven sugar responsive units exhibited pronounced neuronal habituation along with the habituation of the proboscis extension response. The response decrement of this unit happened exclusively in the ipsilateral condition, leading to the assumption that for the units explored the pathway that transmits the reward information is distinct for the two hemispheres. MB ENs receive input from MB intrinsic neurons, the Kenyon Cells (KC). The VUMmx1 neuron, which arborizes in the dorsal suboesophageal ganglion and innervates odor processing brain neuropiles, the antennal lobe glomeruli, the lateral protocerebrum and the mushroom body calyces has been shown to mediate the unconditioned stimulus (US) like the sugar water used in these experiments (Hammer, 1993). Depolarizing this neuron could substitute the sugar reward during olfactory conditioning and was sufficient to establish a conditioned response after training if the correct temporal contingencies between the CS and VUMmx1 activity were met. When the association of the CS and US is established, the VUMmx1 neuron shifts its response towards the CS, similar to dopamine neurons originating in the ventral tegmental area (VTA) in vertebrates (eg. Schulz, 2000). The VUMmx1 neuron releases octopamine. Clawed KCs express a specific type of octopamine receptor (AmOA1) in their cell bodies, dendrites and axons (Sinakevitch et al., 2011). Some clawed KCs (also called KCs Type II) innervate all regions of the calyx (Farris et al., 2004), and are therefore believed to carry information from different sensory modalities, whereas others are locally restricted to the lip or collar (Rybak & Menzel, 1993). As KCs provide the main input to MB ENs, they could be the source of the US information reflected in the nearly 20 % of these recorded units. This sample could also serve for the neuronal integration of state dependency. For unit 1 and 2, sugar responses during the contralateral condition were 0.2 and 0.9 Z-scores higher, respectively when the bees were hungry compared to normalized firing during the contralateral saturated condition, both before behavioral saturation has been established (data not shown). It was also in the contralateral saturated condition where a trend for response decrement was observed during behavioral habituation, but not in the ipsilateral saturated condition. One can thus speculate that these MB ENs are either particularily involved in sugar responses deriving either from the ipsi- or from the contralateral antenna, but not both, and that then these responses are the ones that are

mediated by saturation levels with a higher firing rate in the hungry state opposed to the saturated state. Also, this saturation-level dependent firing has been only observed for these two units, suggesting a complex network of MB ENs serving different roles involved in the expression of behavioral habituation of the PER. The logic behind sugar responses that are stable although behavioral habituation takes place might be that a subpopulation of MB ENs resemble the convergent side of sugar available to the animal and context dependent motivation: in a highly motivated context some of the neurons might signal this state dependence to neighbouring neurons which in turn do not habituate as the bee is hungry while being in a saturated condition habituation in some of these neurons occurs. Still, the information that sugar is still available – irrespective of the animal's state dependence – can be useful for the animal in terms of learning rich food locations that need to be remembered during foraging. This hypothesis is assumable as these MB ENs are critically involved in learning related plasticity as described above.

More cells have to be recorded under similar conditions in future experiments in order to further elucidate the role of MB Ens in behavioral habituation.

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OUTLOOK AND CONCLUSIONS

In this thesis neuronal reward processing in both associative and non-associative learning were studied along with the effects of protein synthesis on the consolidation of long term memory formed after excitatory and inhibitory associations. The honeybee (*Apis mellifera*) served as a model organism and two different learning paradigms were utilized: habituation of the proboscis reflex – a non-associative form of learning, and olfactory reversal learning - a complex form of associative learning. At the neuronal level the honeybees' extrinsic neurons (ENs) were studied using extracellular recordings under the two mentioned paradigms. For the study of long term memory consolidation the reversal learning paradigm was used combined with pharmacological interference in protein synthesis processes. To this end emetine, a translation inhibitor was injected in two different stages of the learning paradigm, either shortly before olfactory differential conditioning or shortly before olfactory reversal conditioning. In the latter experiments two different groups of bees were studied, winter and summer bees, each yielded different results. All experiments were conducted with restrained bees operating its appetitive feature - the extension of the proboscis in response to the delivery of a reward, sugar solution.

Main findings

Chapter I - the effect of a protein synthesis inhibitor (emetine) on the memory formed after reversal learning was investigated. Summer bees and winter bees were studied, each yielded different results. In summer bees emetine was found to inhibit the consolidation of the excitatory learning following reversal, and in winter bees emetine was found to block the consolidation of the inhibitory learning.

Chapter II – here the effect of emetine on the memory consolidation formed after differential learning in winter bees was studied, again using reversal learning paradigm. Emetine was shown to block the spontaneous recovery following reversal learning.

Chapter III- extracellular recordings from mushroom bodies' extrinsic neurons (ENs) were performed while bees were exposed to an olfactory reversal learning paradigm. It was found that a sub-population of the ENs developed a learning-related neuronal response to the unrewarded odor during acquisition of differential learning. No learning-related changes were observed in the ENs following and during reversal learning.

Chapter IV – a non-associative form of learning, habituation of the proboscis extension response, was studied whilst conducting extracellular recordings from the ENs. Only a small sub-population of the ENs was found to respond to the solely presentation of reward to the antenna, out of which one cell habituated its response resembling one neuronal basis for behavioral habituation.

Taken together it seems that in the honeybee, seasonal variations result in different requirements for protein synthesis under complex form of olfactory learning. It also might be concluded that spontaneous recovery of the early long term memory formed after extinction learning in a reversal paradigm is blocked by emetine administration.

The ENs of the honeybee were found here to develop a learning related response to the unrewarded odor (CS-) over the acquisition of olfactory differential learning. Surprisingly no learning-related changes were observed following or during the reversal phase. However, following reversal learning these neurons were found to change their response pattern to odor presentation, suggesting an effect on the neuronal network as a result of reversal learning.

When studied under simple form of learning, habituation to a reward stimulus, a sub-population of the ENs was also found to respond to mere reward presentations onto the antenna, suggesting the ENs are sensitive to both forms of learning.