Associative plasticity and context modulation in GABAergic feedback neurons of the mushroom body output in the honeybee (*Apis mellifera*)

Inaugural - Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat)

Submitted to the Department of Biology, Chemistry, and Pharmacy

Freie Universität Berlin

by

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August 2011

1st Reviewer: Prof. Dr. Dr. h.c. Randolf Menzel

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Date of defense: September 29th 2011

Chapter 2 is based on the following manuscript:

Visual and olfactory associative plasticity in an inhibitory local and recurrent pathway in the honeybee (*Apis mellifera*)

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Ina Klinke and Randolf Menzel discussed the experimental design (adapted from Gerber & Smith, 1998). Ina Klinke performed the experiments, analyzed the data, and wrote the manuscript. Randolf Menzel provided suggestions, criticisms, and corrections.

Content

General summary

Using a combined visual and olfactory paradigm, adopted from Gerber & Smith (1998), honeybees (*Apis mellifera*) were trained to discriminate between a rewarded and a non-rewarded set of compounds. At the same time, multiple feedback neurons of the protocerebral-calycal tract (PCT) of the bee brain were recorded in the extracellular space in order to study associative plasticity, visual context modulation of olfactory processing, and attention-related stimulus selection processes.

Chapter I – Honeybees were trained to discriminate a rewarded compound stimulus, consisting of a color and an odor, from a non-rewarded compound. Recordings lasted over three days, on which training and retention tests were performed. During retention tests, subsets of the recorded PCT neurons established associative plasticity to the odor and color stimuli in discrete time windows, indicating consecutive consolidation processes of novel and familiar stimulus combination. Associative plasticity was expressed by an antagonistic rate code that correlated with the bees' ability to discriminate behaviorally between the stimuli. The functional implications of the findings for the inhibitory local and recurrent feedback loop of PCT neurons are discussed in the frame of experience-dependent memory storage and the preparation of appropriate motor responses.

Chapter II – Visual modulation of olfactory processing was studied using the same behavioral paradigm. Upon stimulus selection, when the bees extended their probosces during retention tests, strong inter-modal interaction effects of odor responses and reward expectancy were evident. Modulation was both facilitating and suppressing depending on the learned value of the visual context stimulus. These results provide a physiological basis for the context-specific behavioral effects described in the litertaure.

Chapter **III** – Enhanced local field potential (LFP) power in the frequency band between 1-25 Hz has been identified during training following both CS+ and CS- color onset. This signal might reflect global attention and sets the animal in a state ready to prepare and execute a motor response. Additionally, during compound training, phase-locking between

PCT spikes and the enhanced LFP signal was detected in learner bees only, and might thus be regarded as a prerequisite to successfully encode a visual context cue as reward predicting.

Zusammenfassung

Unter Verwendung eines kombinierten visuellen und olfaktorischen Paradigmas, adaptiert von Gerber & Smith (1998) wurden Honigbienen (*Apis mellifera*) trainiert, ein belohntes Stimuluspaar von einem unbelohnten zu unterscheiden. Zur gleichen Zeit wurden multiple Rückkopplungsneurone vom Protocerebral-calycal Trakt (PCT) aus dem Bienengehirn extrazellulär abgeleitet. Ziel war die Untersuchung assoziativer Plastizität, visueller Kontextmodulation olfaktorischer Prozessierung, sowie aufmerksamkeitsbezogener Stimulus Selektion.

Kapitel I – Honigbienen wurden darauf trainiert, ein belohntes Stimuluspaar, bestehend aus einer Farbe und einem Duft, von einem unbelohnten Paar zu unterscheiden. Extrazelluläre Ableitungen wurden über maximal drei Tage hinweg durchgeführt. An jedem Tag wurde trainiert und getestet. Während der Tests entwickelten Subgruppen der PCT Neurone assoziative Plastizität zu Duft und Farbe in spezifischen Zeitfenstern, was auf sequentielle Konsolidierungsprozesse von neuen und bereits bekannten Stimuluskombinationen schließen lässt. Assoziative Plastizität wurde durch antagonistische Ratenkodierung ausgedrückt, welche mit der Fähigkeit des Tieres, die Stimuli zu unterscheiden, korrelierte. Die funktionalen Implikationen der Befunde für den lokalen und rekurrenten Pfad der PCT Neurone werden im Rahmen erfahrungsabhängiger Gedächtnisspeicherung und der Vorbereitung einer geeigneten Verhaltensantwort diskutiert.

Kapitel II – Unter Verwendung desselben Paradigmas wurde visuelle Modulation olfaktorischer Prozessierung untersucht. Während einer Verhaltensantwort im Test wurden starke intermodale Interaktionseffekte der neuronalen Antwort auf den Duft und während

der Belohnungserwartung deutlich. Modulation drückte sich sowohl in einer Verstärkung, als auch in einer Abschwächung der neuronalen Duftantworten aus, abhängig von der gelernten Wertigkeit des visuellen Kontextstimulus. Diese Befunde stellen eine physiologische Basis für die in der Literatur beschriebenen Kontext spezifischen Verhaltenseffekte dar.

Kapitel III – Ein verstärktes lokales Feldpotential im Frequenzbereich zwischen 1-25 Hz wurde während des Trainings jeweils zu Beginn des belohnten und des nicht belohnten Farbreizes identifiziert. Dieses Signal bildet möglicherweise ein neuronales Korrelat zur visuellen Aufmerksamkeit, welches das Tier in einen Zustand versetzt, in dem es eine zielgerichtete Verhaltensantwort vorbereiten und ausführen kann. Zusätzlich wurde während des Kombinationslernens gefunden, dass die Aktionspotentiale jeweils in einer bestimmten Phase der Oszillation feuern. Dieser Befund bezieht sich ausschließlich auf Bienen, die die Stimuluskombinationen erfolgreich lernten. Dieser neuronale Befund wird daher als mögliche Bedingung für das erfolgreiche Erlernen von visuellen Kontextreizen diskutiert.

1. General introduction

Two basic functions of the brain are essential when studying learning and memory processes on the neuronal level. First, the brain is specialized in pattern recognition and pattern completion, in order to make predictions about future events. And secondly, pattern recognition happens on the basis of plastic changes in the brain that are derived from prior experiences. Thus, learning and memory is the basis to communicate with and adapt successfully to the constraints of the changing environment. Context stimuli might enhance or suppress an action or even stimulus retrieval of information, based on the context specific experience. For example, writing an exam in the same room in which classes took place might facilitate retrieval of prior encoded information in the class room. However, one prerequisite might not have been missing during classes: Attention.

Objectives

Honeybees (*Apis mellifera*) perceive their environment primarily through their olfactory and visual senses. The objectives of the present work were therefore to elucidate the neuronal basis for visual and olfactory learning and memory formation, the modulation of odor processing in a visual context as well as the role of attention during memory acquisition. In the following sections, classical conditioning as a way to study associative plasticity, the honeybee as a model organism, and its visual and olfactory processing pathways, including the inhibitory recurrent pathway from which was recorded will be outlined briefly. A short overview will be provided about the distributed memory traces in time and space, the importance of inhibitory feedback in neural networks, and cooperative neural coding regimes.

Classical conditioning

How memory works and if memory storage can be resolved at the level of individual nerve cells is an intruiging aspect in neuroscience, that excited researcher already in the late 19th century. In classical conditioning, a neutral stimulus (conditioned stimulus, CS) is

presented together with a biologically significant stimulus (unconditioned stimulus, US), which has the innate feature to elicit a particular response in the animal (conditioned response, CR). After having presented the CS with the US together in an adquate temporal contingency for a couple of times, the animal forms an association between the CS and US such, that the presentation of the CS alone elicits the CR, without the presence of the US. Classical conditiong traces back to the russian physiologist Ivan Pavlov (1849-1936), who discovered its properties around the beginning of the 20th century. This well defined learning procedure gave rise to study learning and memory formation systematically, and allowed the simultaneous investiagtion of physiological changes underlying behavioral adaptation.

The honeybee (Apis mellifera) as a model system

In order to understand the underlying neuronal changes upon learning and memory formation the investigation of sytems with reduced neuronal complexity but preserved behavioral richness is desirable. The honeybee (*Apis mellifera*) fulfills such prerequisites and has been proven to be a valid model for the study of learning and memory, both at the behavioral and neuronal level (Menzel at al., 2006). Honeybees are capable of associating various stimuli as colors and odors with sucrose presentation which represents the reinforcing stimulus in classical conditioning (Bitterman et al., 1983; Menzel, 1990; Menzel & Müller, 1996). The proboscis extension response (PER) of the honeybee is elicited upon sugar (US) presentation to the antennae of a hungry bee. When preceded by a neutral stimulus (CS) an association between the two stimuli is formed (Kuwabara, 1957).
On the neuronal level, important structures, the mushroom bodys (MBs), known to be involved in learning and memory formation as well as sensory integration, are accessible for both intra- and extracellular electrophysiological recordings of single neurons (eg. Okada et al., 2007; Strube-Bloss et al., 2011, Krofczik, et al., 2008), and calcium imaging of multiple neurons (eg. Hähnel & Menzel, 2011).

Visual and olfactory processing in insects

Olfactory processing shares striking similarities in vertebrates and invertebrates (Hildebrand & Sheperd, 1997), indicating that the olfactory systems in both species evolved facing fundamental similar sensory challenges, or similar evolutionary constraints (Eisthen, 2002). In honeybees, olfactory receptor neurons (ORN) project to the antennal lobe (AL), which serves as the first olfactory processing stage in the bee brain, and consists of discrete glomeruli. All ORN expressing the same olfactory receptor (OR) converge into the same glomerulus (Vosshall et al., 2000), which are connected by local neurons (LNs), most of them are GABAergic (Anton & Homberg, 1999). Both neuron types, projection neurons (PNs) and LNs receive excitatory input from ORNs, PNs excite LNs, while LNs can in turn synaptically inhibit PNs, thus forming reciprocal dendrodendritic connections (Wilson & Laurent, 2005). In the AL, odor identity and intensity are coded by spatio-temporal activity patterns (Galizia et al., 1999; Couto et al., 2005). Approximately 800 uniglomerular PN, the AL output cells, form two different axonal tracts (Mobbs, 1982), the lateral antenno-cerebral tract (l-ACT) and the medial antenno-cerebral tract (m-ACT). They target the lateral horn (LH) and the MB input region, the calyx.

The calyx is subdivided into three concentric regions, the lip, the collar, and the basal ring, each receiving terminals of sensory neuropils (Mobbs, 1982), including projections from the optic lobes transmitting visual information (Gronenberg, 1986).

The corpora pedunculata or MBs are higher-order integration centers (Mobbs, 1982; Strausfeld, 2002; Gronenberg, 1986), and known to be involved in learning and memory formation (Menzel et al. 1974, Erber et al. 1980, Heisenberg, 1989; DeBelle & Heisenberg, 1994). They consist of paired neuropils comprising approximately 170,000 densely packed intrinsic neurons per hemisphere, the Kenyon cells (KCs) (Abel et al., 2001). The dendritic arborizations of the KCs are the main postsynaptic elements of the cup-shaped calyces. They relay multisensory information onto the MB output neurons in the α- and β-lobes, and pedunculus (Schürmann, 1973). Information of each circular zone of the calyx is represented in the pedunculus and lobes as a stratum (Mobbs, 1982). While the calyx is regarded to be the main input region of the MB the lobes represent the main output region (Kenyon, 1896; Schürmann, 1974; Strausfeld, 1976).

PCT neurons connect the MB output with its input and provide both local and recurrent inhibition

Neurons having their somata outside of the MB constitute the class of MB extrinsic neurons (ENs). Approximately 400 ENs arborize within the MB output region, and have been classified according to the locations of their soma clusters A1-A7 (Rybak and Menzel, 1993). The A3 cluster forms the protocerebral calycal tract, the PCT. The primarily inhibitory recurrent pathway of PCT neurons form fine horizontal bands within the anterior-dorsal α-lobe in which they receive modality specific excitatory input from KCs and provide local feedback onto MB EN e.g. with dendrites of the pedunculus-extrinsic neuron number 1 (PE1), potentially providing learning dependent inhibitory input to the PE1 (Okada et al., 2007). PCT neurons further connect the MB output, the α- and β lobes and pedunculus, with all sensory subcompartments of the MB calyx predominantly connecting the same modality specific output and input layers (Schäfer and Bicker, 1986; Grünewald, 1999a). Since most PCT neurons are Gamma-aminobutyric acid (GABA) immunoreactive (ir) they appear to provide selective inhibitory input to the calyces (Bicker et al., 1985; Schäfer and Bicker, 1986). Electron microscopy revealed GABA-ir profiles in the calyx documenting reciprocal synaptic contacts with PNs und monosynaptic contacts with KCs (Ganeshina & Menzel, 2001). Since PNs are presynaptic to KCs and postsynaptic to PCT neurons their output will be indirectly mediated via feedforward inhibition by these GABA-ir neurons.

Distributed memory traces in time and space

Activity-dependent synaptic plasticity is generally considered a neural basis for learning and memory, (Hebb, 1949, Squire, 1987, Kandel & Schwart, 1982) leading to changes in network dynamics of neural circuits. In this frame, learning depends on induced plasticity that finally leads to a stable, consolidated memory. However, neurons undergoing cellular and molecular modifications such as longterm depression (LTD) and long term potentiation (LTP) do not necessarily subserve long-term memory storage.

The plasticity machinery in the brain is instead subserved by multiple memory systems that act either synergistically or on different time scales in distributed brain regions [in

mammals eg. Hippocampus: Squire (2004), Sutherland and McNaughton (2000) and connected medial temporal lobe (Suzuki, 2008), prefrontal- and orbitofrontal cortex (Rolls & Treves, 1998; Rolls & Kesner, 2006; Goldman-Rakic (1995); Histed et al. (2009); Wiltgen (2004), and ventral tegmental area with respect to reward encoding: Schultz (1998)].

Also in insects, memory traces were found to be distributed; In *Drosophila melanogaster*, to date, 6 distinct cellular memory traces following aversive olfactory conditioning have been identified in distributed brain regions which form and disappear across different time windows (Davis, 2011). Based on the bundling of axonal projections in the MB lobes, *Drosophila* KCs can be subdivided into three morphological subsets (Crittenden et al., 1998), that were associated with distinct memory traces: α`/β` MB neurons, with intermediate memory (Wang et al. 2008), alpha branch of the α/β MB neurons, with a first long term memory (LTM) trace (forming 9 to 24h after training, Yu et al., 2006), and the γ cells with a second LTM trace (established 18 to 48 hours after training, Akalal et al., 2010). Memory traces in *Drosophila* are thus consolidated at the MB output synapses in distributed cell clusters and spread over time.

In the honeybee brain, multiple forms of associative plasticity have been observed in MB intrinsic and extrinsic neurons (ENs): KCs, that follow a sparse coding regime, suitable for high dimensional memory storage (Perez-Orive et al., 2002) express plasticity at the postsynaptic sites in the lip region of the calyx (Szyszka et al, 2008). One class of MB ENs revealed reorganizations in their odor response spectra after associative learning in favor of the previously rewarded odor (Strube-Bloss et al, 2011). One morphologically identified MB output neuron, the PE1 neuron, was found to reduce its firing rate to the previously rewarded odor (Mauelshagen, 1993; Rybak & Menzel, 1998; Iwama & Shibuya, 1998; Menzel & Manz, 2005 Okada et al., 2007). The PE1 receives putative input from PCT neurons (Okada et al., 2007). Using intracellular recordings and calcium imaging, associative plasticity could be evidenced in PCT neurons in the minutes to hours range (Grünewald, 1999b, Hähnel & Menzel, 2010). PCT neurons respond to a variety of sensory modalities (Homberg & Erber, 1979; Gronenberg, 1987; Schildberger, 1981), and are therefore a potential candidate for multisensory learning and memory formation in the bee

brain. Their coding strategies during visual and olfactory learning and memory formation are the focus of the present work.

The importance of recurrent inhibition in learning and memory formation

The diversity of cortical function is provided by inhibition (Buzsáki, 2006). A neural assembly with exclusive excitatory connections can solely change the level of excitation, moving the system only in a forward direction. Conversely, the implementation of inhibitory connections into a neuronal ensemble enables self-organizing nonlinear processing which results in complex activity patterns that crucially depend on the connectivity between excitatory and inhibitory nodes.

Different forms of inhibition in neural assemblies have been described (after Buzsáki, 1984; 2006, Freund & Buzsáki, 1996). A recurrent inhibitory network provides negative (inhibitory) feedback when increased discharge rates of the (excitatory) principal cell elevate firing of the inhibitory interneuron, which in turn decreases the firing of the principal cell. Negative feedback thus provides stability in a network by restricting excitatory discharges. In a feedforward inhibitory configuration, increased firing of the inhibitory interneuron, as the primary event, results in a decreased output rate of the excitatory target cell, increasing temporal precision of the rate code. In this line, feedforward inhibition can narrow the temporal window of discharge probability of the excitatory target neuron with submillisecond precision of spike timing (Pouille & Scanziani, 2001).

Feedforward inhibition has been shown to effectively regulate the temporal integration of synchronous spikes from antennal lobe PNs onto KCs via delayed inhibition from lateral horn neurons in the insect calyx (Assisi et al., 2007). Via this mechanism the sparse firing regime of KCs might be governed, which is thought to be suited for high dimensional coding and memory storage (Perez-Olive et al. 2002).

Spike-timing plasticity observed in MB ENs of the locust, which leads to either an enhancement or a reduction of synaptic efficiency, has been shown to crucially depend on the precise timing of spikes from KCs (Cassenaer & Laurent, 2007).

A third form of neuronal feedback is lateral inhibition. When a principal cell recruits an inhibitory interneuron by increased excitation, the interneuron can silence the surrounding of the principal cell, thus enhancing activity segregation. Lateral inhibition has been described to effectively enhance odor discriminability in the insect olfactory system (Linster and Smith 1997; Perez-Orive et al. 2004; Wilson and Laurent 2005).

Recent studies ascribe a key role to GABAergic local and recurrent inhibitory circuits in learning and memroy formation itself, like controlling synaptic strength and plasticity (Maccafferi & Lacaille, 2003, Lledo & Lazarini, 2007), modulating LTP and LTD (Steele & Mauk, 1999), as well as a role in feature extracion as a basis for learning and memory, as pattern segregation (Abraham et al., 2010), temporal integration (Assisi et al., 2007), and oscillatory synchronization (Tanaka et al., 2009).

Specifically, Steele & Mauk (1999) studied recurrent inhibitory connections in the CA1 region of the hippocampus. The authors could evidence what has already been proposed by computational studies (LeMasson et al., 1993; Tsodyks et al., 1997): Spike activity of postsynaptic pyramidal cells recruits GABA-mediated recurrent inhibition and influences the induction of plasticity by promoting LTD or LTP depending on the pyramidal cell's spike activity. Increased inhibitory feedback onto pyramidal cells in case of high pyramidal firing favors the induction of LTD over LTP, while accordingly, the induction of LTP is enhanced when inhibitory synaptic transmission is blocked (Gustafsson & Wigström, 1990). Recurrent inhibtion may thus prevent runaway changes of LTP and LTD and therefore regulates training-induced patterns of synaptic strengths that encode memories.

Additionally, increased inhibition was associated with decreasing reaction times in decision making tasks and the appearance of theta oscillations (Smerieri et al., 2010). In the hippocampus, the phase of the theta rhythm itself has been shown to influence the timing of pyramidal neuron firing, the induction of LTP (theta peaks) and LTD (theta troughs) (Hölscher et al., 1997). In this frame, theta rhythm from inhibitory neurons may influence synaptic plasticty and the maintenance of memory.

Cooperative neuronal coding regimes

It is generally accepted that a neuron sends its message, in form of action potentials, down its axon to all neurons to which it is anatomically connected. In turn, receiving neurons

integrate inputs from all brain cells to which they are interconnected. However, anatomical conjunctions might decelerate information processing when distant brain areas need to communicate. This might be the case for information processes between the prefrontal cortex (PFC) and the hippocampus in mammals. Executive functions critically rely on interaction with information encoded in memory. For such large-scale functional integration, neural communication through field coherence or spike field coherence has been proposed (Fries, 2005). In order to analyze the local field potential (LFP), which is assumed to be composed of subthreshold synchronized synaptic activity in a broader area of the extracellular space, the low-pass filtered signal of the recorded activity needs to be extracted. Figure 1 illustrates recording of the LFP.

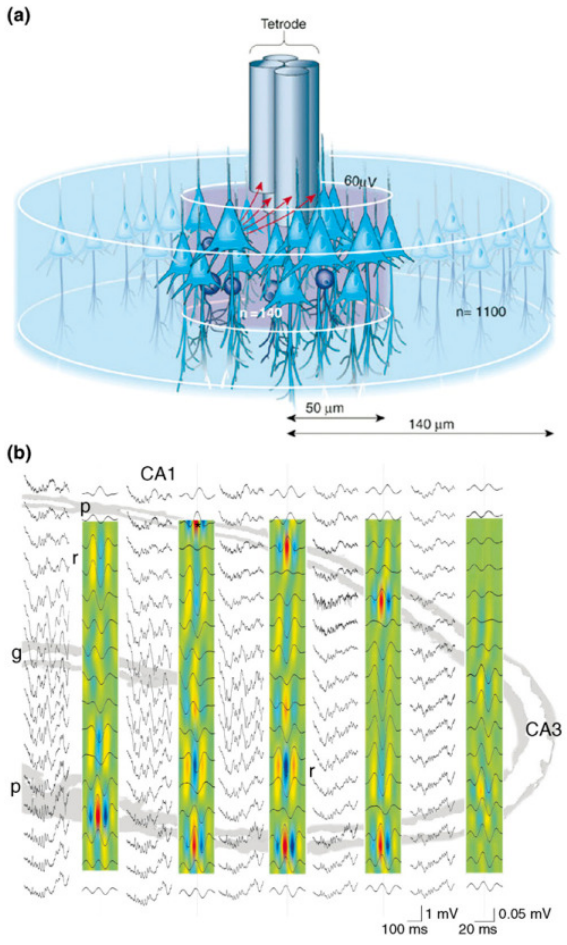

Figure 1. Multisite recording of field oscillations. (a) The electrode, the number of neurons and the volume of the brain tissue from which the activity could be recorded are depicted. (b) Gamma-wave field potentials (30-80 Hz) in the rat hippocampus recorded from five shanks with a distance of 300 µm between shanks. The color panels indicate

averaged gamma-wave field potentials. Red and blue correspond to sources and sinks, respectively. Figure adopted from Le Van Quyen & Bragin, (2007) Analysis of dynamic brain oscillations.

Having extracted the different frequencies of the LFP, the occurrence of single unit spikes or multiunit activity (MUA) can be analyzed with respect to the oscillatory cycle in a specific frequency range. Phase synchronization, that is when two LFPs oscillate with a constant phase shift, provides windows for successful communication between different brain areas. LFP oscillations reflect fluctuations in neuronal excitability, which affects neural output as well as the sensitivity to neural input (Volgushev et al., 1998; Elbert & Rockstroh, 1987; Fröhlich & McCormick, 2010). Inverse phases of an oscillation cycle (eg. 0° versus 180°) correlate with membrane potential depolarization (so-called up states), while shifted phases (eg. 90° versus 270°) corespond to membrane potential hyperpolarization (so-called down states). Action potentials emitted during up phases are thought to be transmitted from one brain region to another and are likely to trigger action potentials in the target area if they arrive again during the depolarized phase of an oscillation cycle. This mechanism would guarantee that pre- and postsynaptic spikes occur in close temporal relationship, a prerequisite for spike timing-dependent plasticity leading to long-term potentiation (LTP) between synapses (Makram et al., 1997; Abbott & Nelson, 2000; Caporale & Dan, 2008). If, conversely, no phase-locking or phase-locking with an inappropriate phase lag occurs, action potentials will arrive at a phase of an oscillation, in which no spikes are elicited in the target area, thus preventing LTP and hence, learning. Also, phase-locking within one brain area can time pre- and postsynaptic spikes such that STDP can induce both, LTP and LTD, depending on whether the postsynaptic neuron fires shortly after (LTP) or before (LTD) the presynaptic neuron.

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2. Visual and olfactory associative plasticity in an inhibitory local and recurrent pathway in the honeybee (*Apis mellifera*)

Abstract

In order to study visual and olfactory associative plasticity in a local and recurrent inhibitory neural network, we applied a combined visual and olfactory conditioning paradigm while recording extracellularly from inhibitory feedback neurons of the protocerebral-calycal tract (PCT) in the honeybee (*Apis mellifera*) brain. PCT neurons connecting the mushroom body (MB) output with its input developped associative plasticity to both sensory modalities. Associative plasticity was expressed in antagonistic rate codes towards the reinforced and non reinforced stimuli. These rate changes correlated with the behavioral performance of the animals, and peaked at discrete time windows for different neurons over a recording time of up to three days. Thus the MB input receives highly selective information about learned stimuli, and this information depends on consolidation of multisensory memory over the range of days.

Introduction

Learning and memory processes rely crucially on activity-dependent synaptic plasticity (Hebb, 1949, Squire, 1987, Kandel & Schwart, 1982) leading to changes in the network dynamics of neural circuits. In this context, learning depends on induced plasticity that ultimately leads to a stable, consolidated memory via an experience and a time-dependent self-organizing neural process. GABAergic local and recurrent inhibitory circuits are thought to play a key role in adaptive network organization (Maccafferi & Lacaille, 2003, Lledo & Lazarini, 2007, Steele & Mauk, 1999). GABA-ir recurrent neurons are also known from a central structure of the insect brain, the mushroom bodies (MB) of the honeybee (*Apis mellifera*). The rather small number of these recurrent neurons (appr. 100 neurons of the protocerebral calycal tract, PCT) and their well defined anatomy and connectivity

within the MB (Rybak & Menzel, 1993, Grünewald, 1998a, Okada et al., 2007) make them an attractive model for the study of inhibitory recurrent pathways in memory formation. PCT neurons project back from the major output region of the MB to its input, and thus serve as a putative inhibitory feedback loop in a structure known to be involved in learning and memory formation (Menzel et al. 1974, Erber et al. 1980; Heisenberg, 1989; DeBelle & Heisenberg, 1994). These neurons receive multimodal input from the MB intrinsic Kenyon cells (KCs), provide local feedback to other extrinsic neurons within the output region (Okada et al., 2007) as well as local feedforward feedback onto olfactory projection neurons (PN) and KCs at the MB input site, the calyces (Ganeshina & Menzel, 2001). PCT neurons respond to a variety of sensory modalities (Homberg & Erber, 1979; Gronenberg, 1987; Schildberger, 1981), and both intracellular recordings and calcium imaging revealed olfactory associative plasticity in the minutes to hours range (Grünewald, 1999b, Haehnel & Menzel, 2011). Neural correlates of associative learning in bees can be investigated with a robust paradigm, the olfactory reward conditioning of the proboscis extension response (PER) (Menzel at al., 2006, Menzel, 1990; Menzel & Müller, 1996). So far, all attempts to uncover neural correlates of associative learning in the honeybee brain have been restricted to elemental forms of learning, focusing on olfactory conditioning. Here, we apply a combined visual/olfactory learning paradigm first described in behavioral studies by Gerber & Smith (1998), and recorded from multiple units of the PCT for up to 3 days. Gerber and Smith found enhanced conditioned responses on testing an olfactory stimulus after visual pretraining (color) and subsequent compound training. Although the visual stimulus does not gain control over the conditioned behavior it appears to facilitate olfactory learning by arousing and focusing attention. We find that PCT neurons do in fact establish memory traces for both visual and olfactory stimuli as expressed in contrasting rate changes towards the reinforced and the specifically nonreinforced stimuli. These rate changes peak in discrete time windows for different neurons over the three days of recording. The combinatorics of associative plasticity in different PCT neurons indicate a multi-faceted experience-dependent and highly organized inhibitory feedback loop.

Material and Methods

Animal treatment

Foraging honeybees (*Apis mellifera carnica*) were caught at the hive entrance, anesthetized on ice, and harnessed in metal tubes, as described by Bittermann et al. (1983). To ensure that the bees were hungry and motivated they were not fed prior to the experiments. Only bees showing the proboscis extension response (PER) after touching one antenna with 30% sucrose solution were included in experiments.

Dissection

A small window (1.5 mm^2) was cut into the head capsule between the compound eyes along a saggital plane with the head fixed to the stage with dental wax. The first joints of the antennae were immobilized using low-temperature melting n-icosane. The head glands and trachea sacks located on the surface of the brain were carefully moved aside until the alpha-lobe could be clearly identified. A silver wire electrode was inserted between the right compound eye and the right lateral ocellus for electromyogram recordings of the M17 muscle that innervates the proboscis and whose activity reflects proboscis movement. The reference electrode was inserted either into the right compound eye or into the median ocellus. The bee was positioned such that the brain surface was exactly 90° horizontal to the set-up table. Microruby was attached to the tip of the multi-wire electrode in order to stain the electrode position inside the brain and to investigate the stained region by confocal microscopy after the experiment. The electrode for neuronal recordings was inserted at the medial-lateral border of the left alpha-lobe, and placed 60 – 250 µm below the anterior surface by means of a micromanipulator attached to the electrode holder. After successful placement of the electrode the window in the head capsule was covered with a drop of silicon (two components of KWIK-SIL Sarasota, FL, USA, mixture 1:1). The silicon kept the brain from drying out and additionally fixed the electrode in the brain, ensuring the stability of the electrode position over several days. The experiment started 30 minutes later when the silicon had bonded and the animal had recovered from the dissection.

Behavioral task

The behavioral paradigm of Gerber & Smith (1998) was adopted and modulated for our purpose to study visual and olfactory memory over a time span of up to three days. The baseline responses of the recorded units towards the stimuli were determined before training sequentially stimulating the animal with three odors and three colors each five times with an inter-stimulus interval (ISI) of one minute. After a break of 10 minutes color pretraining began. One color was chosen randomly to be rewarded during six training trials while another randomly chosen color was not rewarded. The bee was illuminated for 12 seconds. After eleven seconds the bee was fed for three seconds with a droplet of sugar water attached to the tip of a wooden stick. As a control for the movement and humidity levels, a wooden stick with a droplet of pure water was moved towards the bee in unrewarded trials in order to test whether the units would respond to the water vapour and/or the visual stimuli attached to the movement of the stick.

After another pause of 10 minutes, compound color-odor training began. During the rewarded condition the bee was again illuminated with the previously rewarded color, but this time an odor was additionally applied seven seconds after color onset, for 4 seconds in total. The reward was applied three seconds after odor onset. Thus there was one second of overlap between the three sensory modalities, before color and odor were turned off. During the unrewarded condition the previously unrewarded color was paired with another odor, here no reward was delivered. Both conditions were trained within six trials each. Thirty minutes later the bee was tested three times for all stimuli, both separately and in combination. Furthermore, color-odor combinations which had not been trained were also tested. It is important to note that this experimental procedure, including training and retention tests, was repeated on each consecutive day.

Electrophysiology

Electrodes

In the first series of experiments, the electrode for neuronal recordings consisted of two, in the second series of three polyurethane-coated copper wires (14 µm in diameter, Electrisola, Escholzmatt, Switzerland) glued together with wax and attached to a piece of glass capillary (~ 2 cm in length) for handling via the micromanipulator. The ends of the

wires were de-insulated and attached to the amplifier input connectors by means of conducting silverglue. Resistances of single wires were in the range of 1 to 2 MΩ. Silver wires (0.05 mm in diameter, Advent, Eynsham Oxon England) were used for the reference electrode as well as for the electromyogram recordings of M17 activity.

Amplifiers

Each electrode wire was connected to the head stage of a preamplifier (npi electronic). Filters were set to high pass of 10 Hz and low pass of 10 kHz. Hum noise (50 Hz) was eliminated by an additional filter (Hum Bug; Digitimer, Hertfordshire, UK). Neural activity was sampled at a rate of 20 kHz through an analog-to-digital converter (1401 micro MKII; Cambridge Electronic Design, Cambridge, UK) and initial data analysis was performed by Spike2 software (Cambridge Electronic Design) including signal storage, control of stimulation devices and preanalysis of the data. The amplifier used a band pass filter with cut-off frequencies between 10 Hz and 10 kHz.

Visualization of the recording position

After the experiment, the brains were dissected, fixed overnight in 4% formaldehyde with 1 µl Lucifer yellow for enhanced background staining, washed in PBS, dehydrated in rising concentrations of alcohol (20%, 50, 70%, 99%, and 100 %), and cleared with methylsalicylate. A Leica TCS SP2 confocal laser-scanning microscope (Wetzlar, Germany) was used for scanning with a 20x or 10x water objective. Two excitation wavelengths were applied, 428nm for the background (Lucifer yellow), and 560 nm for Microruby.

Odor and color stimulation

Odor stimulation was computer-controlled, using an olfactometer with separate channels for each odor, described elsewhere (Galizia et al., 1997). In each experiment, three different odors were used to which the bee did not show a spontaneous PER. Two of the odors were used for differential conditioning, the third as a control odor. The olfactomter was placed in front of the bee such that the end of the outlet was at a distance of approximately 5 cm to the bee's head. A constant airstream (speed 1.5 m/s) was sent

through a Teflon tube (6 mm in diameter). The control of magnetic valves via the Spike2 software allowed the addition of a particular odor to the airstream. An exhaust pipe behind the animal ensured that odor did not accumulate.

Color illumination was provided by a light guide connected to a lamp with filters for green, yellow, and blue light. The exit of the light guide was placed in front of the bee beside the odor pump. Light switches were conducted manually, the precise timing of on- and off-switches were announced by a sound coming from a loud speaker that was connected and controlled by the Spike2 software.

Odor delay

The time between the trigger as recorded by the Spike2 program and the actual opening of the magnetic valves was approximately 3 ms. After opening of the magnetic valves the odor arrived at the bee's antennae another 41.14 ms later, according to the speed of the airflow and the distance from odor injection to the bee. Thus, there is a constant odor delay of 44.14 ms. We considered this factor in our analysis.

Data analysis

Spike sorting

The differential channel or the three separate - single-ended - channels, and the channel that recorded from the M17 were high-pass filtered at 300 Hz using the Spike2 software (Cambridge Electronic Design, Cambridge, UK). The semi-automated template matching algorithm of Spike2 was used for spike sorting.

In the case of three neuronal channels, the two channels with the best quality were sorted together such that amplitude and waveform were constantly and simultaneously checked on both channels at the same time. The third channel was not used anymore to avoid false positives. Besides careful visual inspection, sorting quality was controlled by means of a principal component analysis of the first three components of each sorted unit. The dots of the single units (encoded in one particular color) needed to be clearly separated from each other in order to ensure good sorting quality and to avoid false positives and negatives in spike trains of a single unit. The recorded M17 activity was separated from noise by threshold sorting in Spike2.

Response detection for single unit activity

In order to identify rate changes of single units as a significant response to a stimulus, and to define their polarity, we applied a dual approach (Strube-Bloss et al., 2011). First, we compared the median of the pooled inter-spike interval (ISI) distributions of all trials within a test of two time windows, 2 sec before and 100-600 ms after stimulus onset ($p<0.05$).With this method subtle excitatory or inhibitory rate changes can be detected that disperse over a period of some hundred milliseconds. In order not to miss short but strong rate changes, we then pooled spike trains from all trials of one test, before and after stimulus onset in the same time windows as described above, and summed them in a Peri-Stimulus-Time-Histogram (PSTH) with a bin width of 50 ms. We then calculated the mean *m* and the standard deviation *SD* across all bins preceding the stimulus and then checked for a rate bin in the PSTH after stimulus onset that exceeded the threshold of three times the standard deviation plus the mean bin preceding the stimulus.

Response detection for M17 activity

Rate responses were estimated with a kernel convolution with a sliding window width of 50ms (Nawrot et al., 1999). Therefore, the spike trains of the muscle activity during training sessions were binned with one ms precision (1ms bins) before reward application in a two-second time window from stimulus onset. During test sessions, a four-second time window after stimulus onset was chosen for response detection. After convolution, a threshold was applied: As soon as more than three spikes emerged within the sliding window, a response was detected.

Normalization of firing rates

Absolute firing rates were normalized to the preceding ongoing activity of the same unit [(post-pre)/(post+pre)]. This normalization procedure produces values between 1 and -1 and resembles the change in neuronal activity due to a stimulus rather than the absolute rate.

Calculation of the neuronal learning score, delta (Δ)

In order to systematically investigate distinct time windows of strongest associative plasticity expressed by neuronal subpopulations of the recorded PCT neurons, we calculated a neuronal learning score, *delta* (Δ), as an index of the strength of the unit's associative plasticity during a given test, separately for odors and colors. First, the difference between the normalized rate responses of CS+ and CS- was calculated for pretraining, and tests during day 1 to 3. Then the calculated difference for pretraining was subtracted from each test difference investigating whether response differences between CS+ and CS- stimulus changed over the course of learning. To gain an absolute value, all negative scores were multiplied by -1. Thus, the absolute Δ scores resemble the change (in relation to pretraining) in the capacity of each unit to differentiate between the CS+ and CS- stimulus. The higher the Δ score the more the rate responses differed for the conditioned stimuli. Units were subsequently grouped according to the test in which they expressed their highest Δ, either on day 1, 2, or 3. Δ values of units that were assigned to one group were statistically compared with the Δ values of the same units in the other tests using a paired t-test, to assess whether Δ values differed significantly. As a control for the fact that we had already selected the highest Δ values (and assigned these to the group) we destroyed the relationship between the data points by shuffling data points 100 times and executed a second t-test with the shuffled scores closest to the median of the 100 permutations.

Statistics

In order to test significant rate changes between pretraining and tests, repeated measures MANOVA was applied to subpopulations of units. For behavioral changes in the percentage of M17 responses, the non-parametric McNemar test and the binomial test were used where appropriate. Δ scores of single units were compared using paired t-tests. Proportions of naïve responses were calculated with the binomial test. Regression analysis was used to explore whether pretraining response differences to the odor and color that were subsequently reinforced predicted the discrete time window of strongest associative plasticity for odors and colors together.

Results

Behavioral performance

Proboscis extension responses (PERs) reflect the conditioned response in the paradigm used. PERs were monitored with electromyogram recordings of the muscle (M17) that innervates the proboscis of the bee. Behavioral responses of 30 recorded bees were quantified during color pretraining, and subsequent compound training on day 1, and during tests on day 1-3. Acquisition was characterized by the percentage of behavioral responses to the rewarded and unrewarded stimuli of all bees per trial. Note that visual pretraining and compound training were applied not only on the first day but on each of the three days before testing. Retention tests were conducted 30 minutes after the last compound training trial on each of the three days and consisted of 3 extinction trials during which odors and colors were presented without reward. Responses were counted for all test trials for all bees. Not all bees survived the three days. Of the 30 bees recorded on the first day, 19 survived until the second, and 5 until the third day. During visual pretraining, the bees did not extend their probosces (fig. 1 *A*). During compound training odor responses were already significantly different for the rewarded and unrewarded compound after a single training trial [trial 2: $Chi^2 = 5.882$; $p = .015$; df=1. trial 6: $Chi^2 = 13{,}067$; $p = .001$; df=1], (see fig. 1 *B*). No behavioral responses to the colors could be detected during compound training. Retention tests for odors alone showed significantly more responses towards CS+ odor than to CS- and the control odor respectively in all tests (fig. 1 *C*), [Day1. CS+ vs. CS-: $Chi^2 = 22.132$; $p = .001$, CS+ vs control: $Chi^2 = 14.049$, $p = .001$, CS- vs. control: ns; Day2. CS+ vs CS-: $Chi^2 = 14.45$, $p = .001$, CS+ vs. control: $Chi^2 = 9.481$, $p = .002$, CS- vs. control: $Chi^2 = 4$, $p = .046$; Day3. CS+ vs control: $Chi^2 = 8.1$, $p = .004$, CS+ vs CS-: Binomial test: $p = .001$, Control vs. CS-: ns].

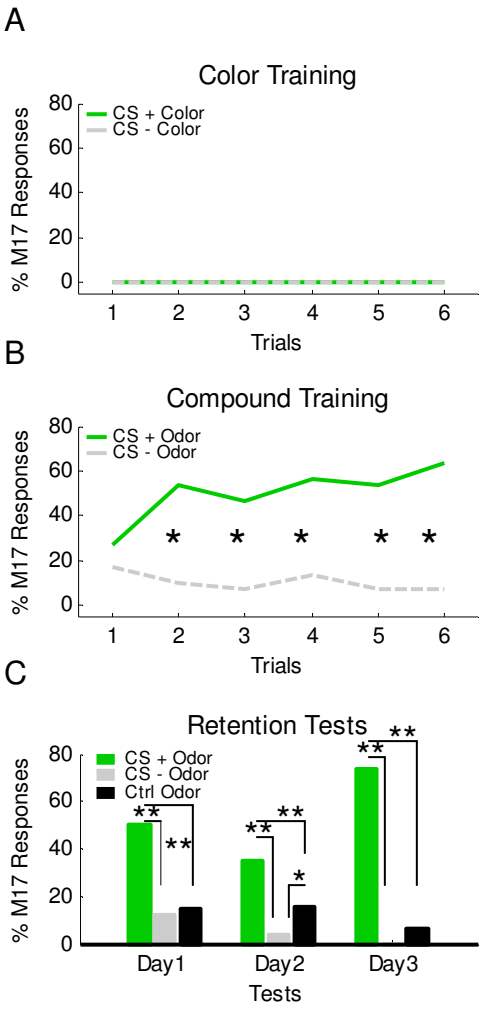


Figure 1. Behavioral Performance. ***A*** Acquisition curves, color pretraining on day 1. The bees (N=30) did not extend their probosces towards any of the trained colors. ***B*** Average acquisition curve for compound training on the first day. The bees extended their probosces significantly more towards the CS+ odor (before reward onset) than towards the non rewarded CS- odor, already after one pairing. ***C*** Retention tests on day 1-3. As colors did not evoke PERs, test outcomes for odors are shown. On each test day, the CS+ odor evoked significantly more responses than the CS- and the control odor. Additionally, on test day 2 control responses were significantly higher than CS- responses. No CS- responses were detected on day 3.

Neuronal responses towards odors and colors in naïve animals

During day 1, neuronal activity was recorded from 164 single PCT units of 30 bees. Before each bee was trained, three different odors and colors were applied, in order to get the naïve, baseline neuronal activity of the single units towards the respective stimuli. Responses were detected within a time window of 500 ms, beginning 100 ms after stimulus onset. Percentages of excitatory [in brackets: inhibitory] responding units were as follows for the three odors and three colors. Odors: 32 units (19.5%) [7 (4.3%)] responded only to one odor, 42 units (25.6%) [7 (4.3%)] to two odors, and 48 units (29.3%) [4 (2.4%)] responded to all three odors. Colors: 39 units (23.8%) [1 (0.6 %)] responded to one color, 24 units (14.6%) [8 (4.9%)] to two colors, and 38 units (23.2%) [7 (4.3 %)] responded to all three colors. The following proportions were found for odor-color combinations: 83 units (50.6%) [8 (4.9%)] responded to at least one odor and one color, 11 units (6.7%) were excited by at least one odor and inhibited by one or more colors, 7 units (4.3%) showed the opposite response pattern. 19 units (11.6%) responded naïvely to all odors and all colors, while 24 units (14.6%) exhibited no naïve responses to either odor or color. These results document the multimodal sensitivity of PCT neurons and the multitude of combinatory effects. It is particularly important that some neurons in naïve animals already responded to the stimuli used in the following training session as CS+ or CS-, others did not respond to CS+ or CS-. This applies to both odors and colors.

Associative plasticity of naïve responding and naïve nonresponding units

We next analyzed the unit responses to colors and odors and their dynamics of associatve plasticity throughout the course of learning and testing. Units were first grouped based on their naïve responses to the conditioned odors and colors. In order to capture neural dynamics over the maximum time span, we first addressed units recorded for the entire three days (N = 33).

Naïve nonresponding units were recruited to respond specifically to the CS+ stimulus to odors on day 3 and to colors on day 1, and for odors they exhibited a broader range of associative plasticity than naïve responding units by increasing their excitation rate towards the CS+ and increasing their inhibition rate to the CS- stimulus. Naïve responding units changed their excitation magnitude to the CS+ and CS- (for odors strongest on day 1, for

colors on day 2). The timescale on which associative plasticity was expressed differed for odors (naïve nonresponding units: day 3 [Within-subject effect: $F(11, 77) = 2.42$, $p = .012$]; naïve responding units: day 1 [$F(11, 132) = 5.539$, $p < .000$]), and colors (naïve responding units: day 2 [$F(11, 66) = 2.9$, $p = .004$]). Naïve nonresponding units increased their rate to the CS+ color during test day 1, though this increase is not statistically different in pairwise comparison from the CS- and control color responses during the same test [$F(11, 77) = 2.245$, $p = .02$].

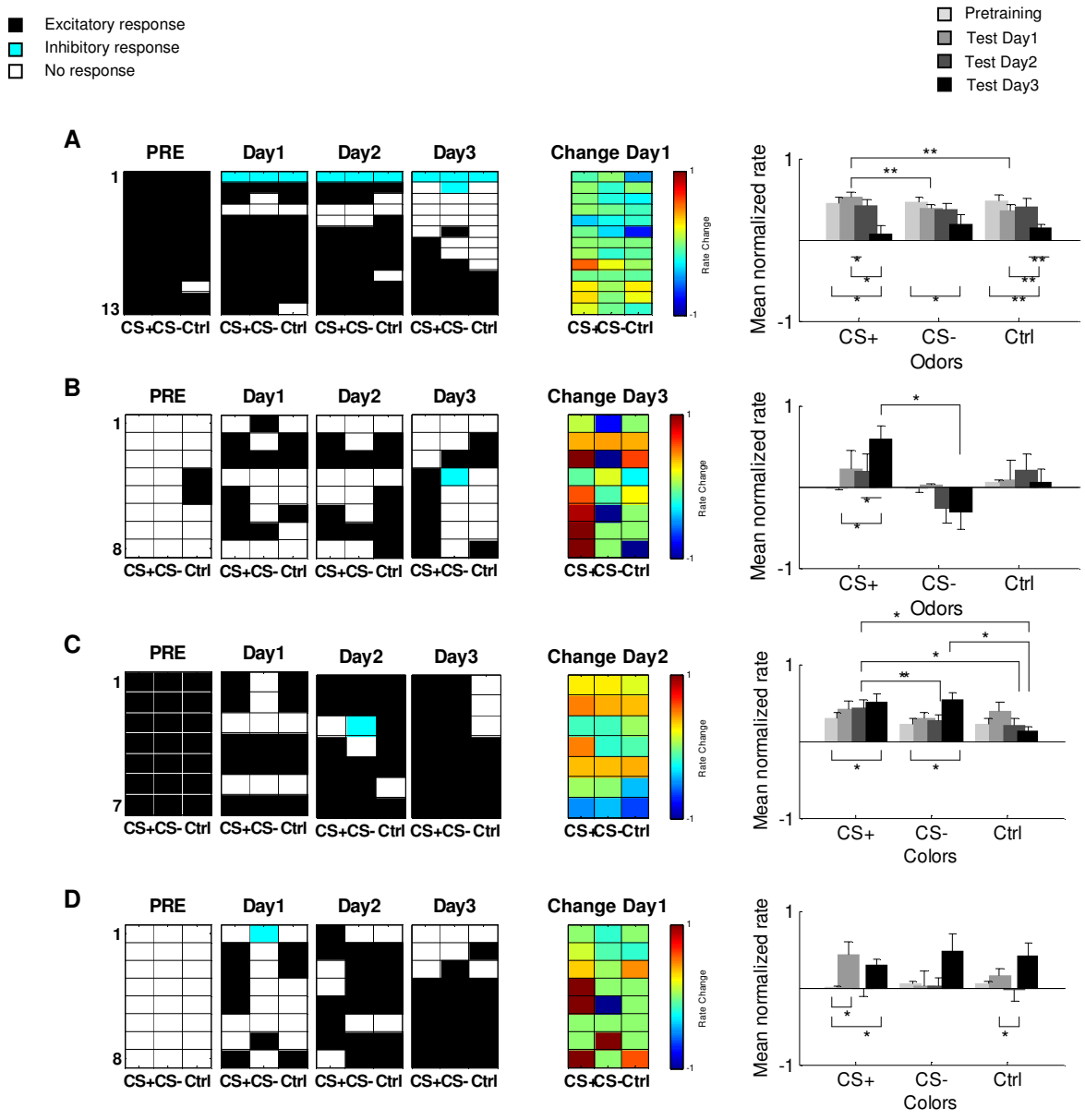


Figure 2. Odor and color responses of naïve responding and naïve nonresponding units at different times after conditioning. Responses before training (PRE) and during each consecutive retention test on day 1-3 are illustrated for naïve responding (***A, C***), and naïve-nonresponding units (***B, D***), first for odors, then for colors. Significant responses are visualized in the response matrices for the single units arranged horizontally in each row of the matrices (black: excitatory response, cyan: inhibitory response, white: no response). The change matrices illustrate the strongest change in comparison to pretraining responses. Alongside the response matrices, the normalized rate for the same units is plotted for CS+,

CS-, and control stimulus. While naïve nonresponding units became recruited to respond strongest to the CS+ odor, they additionally changed their rate anatgonistically to both odors (increased excitation to CS+, increased inhibition to CS-), naïve responding units only changed their level of excitation to the CS+, CS-, or control. The timescale on which associative plasticity was expressed differed for odors and colors and for naïve responding versus naïve nonresponding units (see text and change matrices).

We tested whether units that were recorded in an animal exhibited the same proportion of naïve responses and no naïve responses to the three odors and three colors before the training using the binomial test. Only three of 19 bees, recorded for two or three days, expressed a significantly higher proportion of naïve responses than no naïve responses to both odors and colors (p`s < .05). Three bees showed significantly more naïve responses to odors only, while units of three other bees expressed a higher proportion of responses to colors and units of yet another bee a significantly higher proportion of no responses to colors before training. In nine bees the units did not differ significantly in their amount of naïve responses and no naïve responses to odors and colors before the training.

Associative plasticity quantified by the neural learning score Δ

In order to systematically investigate distinct time windows of the strongest associative plasticty effect we calculated a neural learning score Δ, as an index of the plasticity strength, separately for odors and colors (see *Methods* for caluclation of Δ). For each unit and each odor and color test, Δ was calculated. Units were grouped separately for odors and colors, based on the test in which they expressed their highest Δ (test day 1, day 2, or day 3), and these values were compared to the Δ values of the same units in the other tests, in which these units exhibited lower Δ scores. The Δ values were significantly higher than the corresponding Δ values for the same units in the remainder tests for all assigned groups, for odors as well as for colors (paired t-test, all p`s < .05). To control for the fact that we selected the highest Δ values and compared these with the remainder values we destroyed the relationship between the data points as descibed in *Methods* and executed paired t-tests between the permutated data points. All paired t-tests with permutated data points were not significant (all p´s > .05). This indicates 1) that the single units featured significantly higher

associative plasticity in one discrete time window (the assigned Δ group), and 2) that these significantly higher plasticity levels were not due to the mere distribution of data points, but could instead be related to the changes of the single units over time.

Associative rate changes in groups of units classified by their Δ score

Units were grouped according to the highest Δ value in the 3 tests and according to the sign of the Δ score (positive or negative) leading to 6 subgroups. Figure 3 summarizes the rate profiles with respect to odor responses, and figure 4 with respect to color responses for the 6 subgroups. Note, that the two upper panels depict rates for pretraining, test day 1, and test day 2, and that only the lowest panel additionally illustrates the rate changes for test day 3 (for those units that were recorded for the entire three days and were assigned to the Δ groups on day 3). Main effects of MANOVA are noted first for odor and then for color responses. P-values of pairwise comparisons between one odor (eg. CS+ in different tests) and between different odors (CS+ vs CS- vs Ctrl) in the same test are notated in figures 3 and 4 with asterisk (* $p < .05$, ** $p < .01$, and *** $p < .001$).

Odor responses

For all 6 subgroups, units with positive Δ values significantly increased their rates to the CS+ odor, while units with negative Δ values significantly decreased their rates to the CS+ odor. With the exception of -Δ units day 2 and -Δ units day 3, all other subgroups additionally expressed the inverse rate change to the CS- odor, namely a significant decrease in +Δ units, and a significant increase in -Δ units. For five subgroups the strongest rate difference between CS+ and CS- odor were apparent during the particular test the units were assigned to (on day 1, 2, or three for Δ units day 1, 2, and 3, respectively). Only in units assigned to -Δ day 3, the rate difference between CS+ and CS- odor did just not reach significance, possibly due to the low sample size (N = 6). Instead, the rate response to the CS+ and control odor decreased significantly from pretraining to test day 3, while CS- odor responses did not change. For all other subgroups, rate responses to the control odor did not change. Pretraining responses to the CS+ and CS- odor were significantly different in both Δ units day 1, and in -Δ units day 2. [Statistical significance of pairwise comparisons: see fig. 3. Main within-subject effects: Test day 1, +Δ (N = 16): $F(8, 120) = 2.695$, $p = .009$;

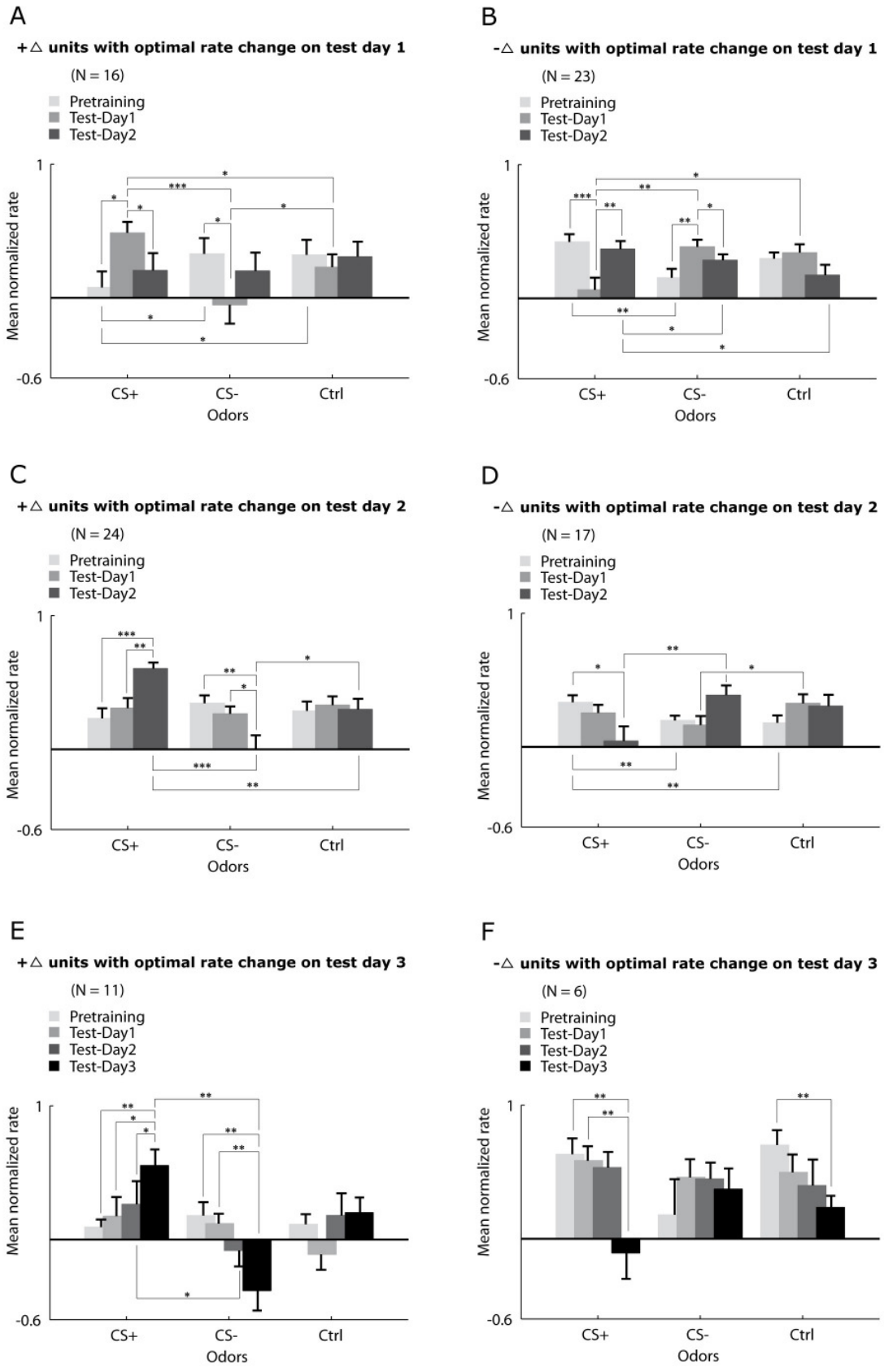
test day 1, -Δ (N = 24): F(8, 176) = 5.249, $p < .000$; test day 2, +Δ (N = 24): F(8, 184) = 5.86, $p < .000$; test day 2, -Δ (N = 17): F(8, 128) = 3.078, $p = .003$; test day 3, +Δ (N = 11): F(11, 110) = 5.026, $p < .000$; test day 3, -Δ (N = 6): F(11,55) = 3.52, $p = .001$]. One unit did not develop associative plasticity to odors. Its rate did not change during subsequent tests.

Color responses

As for odors, units with positive Δ values significantly increased their rates to the CS+ color, while units with negative Δ values significantly decreased their rates to the CS+ color in both Δ groups, day 1 and 2 (see fig. 4 *A-D*). In the same Δ groups, as for odor responses, units additionally expressed the inverse pattern, a decrease to the CS- color in +Δ groups, day 1 and 2, and an increase to the CS- color in -Δ group, day 1. Only two units exhibited optimal rate change on day 3 with positive Δ values. The low sample size did not allow for statistical evaluations. Nevertheless, comparable to the same Δ group for odor responses there is a trend towards a steady increase towards the CS+ color and a decrease towards the CS- color with its peak rate difference during test day 3, (compare fig. 3 *E* with fig. 4 *E*). For both odor and color responses, units with optimal rate change on test day 2 and with a negative Δ value only decreased their rates to the CS+ stimulus, but did not significantly increase their rate to the CS- stimulus. For four subgroups the strongest rate difference between CS+ and CS- color were apparent during the particular test the units were assigned to (on day1, and 2 for Δ units day1, and day2, respectively, fig.4 *A-D*). Again, the trend in +Δ units day3 also indicates rate peak differences on day 3. Units with optimal rate changes to colors on test day 3 did not change their rate to the CS+ color, but instead increased their rate to the CS- color strongest on test day 3. In the same group rates also increased to the control color from pretraining to test day 1 (fig. 4 *F*). Compare the same group for odor responses in which rates decreased to CS+ and control odor but did not change to the CS- odor (fig. 3 *F*). For odors and colors, the -Δ day 3 group is the only group with rate changes to the control stimulus.

In contrast to odor responses, pretraining responses to the CS+ and CS- color differed significantly only in +Δ units day 1 (and not additionally in -Δ units day 1 and -Δ units day 2). [Statistical significance of pairwise comparisons: see fig. 4. Main within-subject effects:

Test day 1, +Δ (N = 16): F(8, 120) = 4.463, $p < .000$; test day 1, -Δ (N = 26): F(8, 200) = 4.511, $p < .000$; test day 2, +Δ (N = 19): F(8, 144) = 3.66, $p = .001$; test day 2, -Δ (N = 20): F(8, 152) = 2.883, $p = .006$; test day 3, -Δ (N = 9): F(11, 88) = 4.641, $p < .000$]. Five units did not develop associative plasticity to colors. Their rates did not change during subsequent tests.


A
+△ units with optimal rate change on test day 1
(N = 16)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
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CS+
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-△ units with optimal rate change on test day 1
(N = 23)
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Test-Day1
Test-Day2
Mean normalized rate
1
-0.6
CS+
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+△ units with optimal rate change on test day 2
(N = 24)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
1
-0.6
CS+
CS-
Ctrl
Odors
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-△ units with optimal rate change on test day 2
(N = 17)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
1
-0.6
CS+
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Odors
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+△ units with optimal rate change on test day 3
(N = 11)
Pretraining
Test-Day1
Test-Day2
Test-Day3
Mean normalized rate
1
-0.6
CS+
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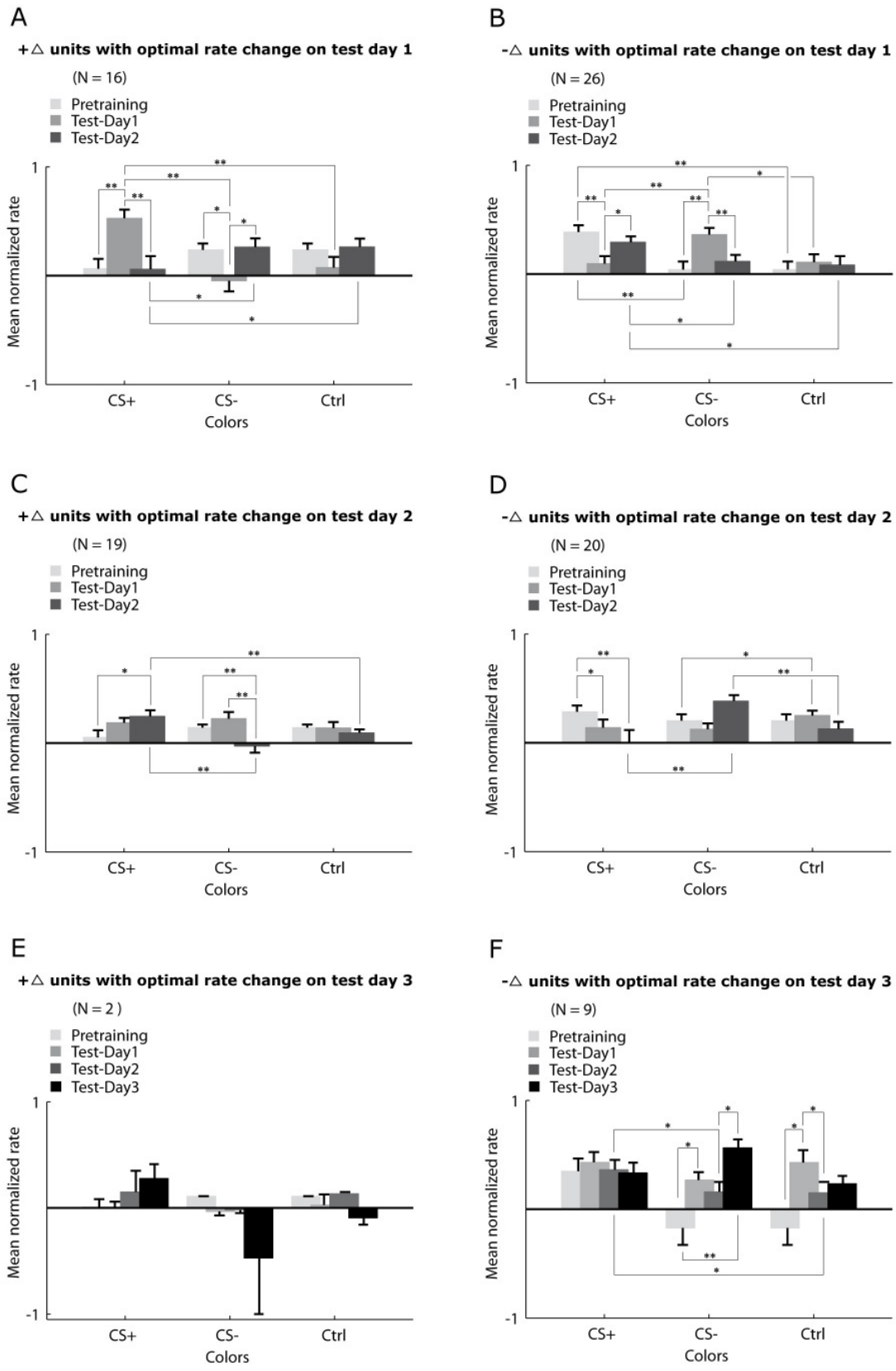
Ctrl
Odors
F
-△ units with optimal rate change on test day 3
(N = 6)
Pretraining
Test-Day1
Test-Day2
Test-Day3
Mean normalized rate
1
-0.6
CS+
CS-
Ctrl
Odors

Figure 3. Associative rate changes as expressed in positive and negative Δ for odors during tests 1-3. 6 distinct groups were identified based on the test in which they expressed their highest positive or negative Δ value to the conditioned odor (top panels: Δ units test day 1; middle panel: Δ units test day 2; bottom panel: Δ units test day 3). Positive (left columns) and negative Δ values (right column) were analyzed separately. Normalized rates are shown for pretraining, and the different tests, illustrated in graded gray scales. Typically, positive Δ units increased their rates towards the CS+ and decreased it towards the CS- odor, while negative Δ units expressed the opposite pattern. Asterisk depict p-value of pairwise comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).


A
+△ units with optimal rate change on test day 1
(N = 16)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
1
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Colors
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-△ units with optimal rate change on test day 1
(N = 26)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
1
-1
CS+
CS-
Ctrl
Colors
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+△ units with optimal rate change on test day 2
(N = 19)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
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CS+
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-△ units with optimal rate change on test day 2
(N = 20)
Pretraining
Test-Day1
Test-Day2
Mean normalized rate
1
-1
CS+
CS-
Ctrl
Colors
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+△ units with optimal rate change on test day 3
(N = 2)
Pretraining
Test-Day1
Test-Day2
Test-Day3
Mean normalized rate
1
-1
CS+
CS-
Ctrl
Colors
F
-△ units with optimal rate change on test day 3
(N = 9)
Pretraining
Test-Day1
Test-Day2
Test-Day3
Mean normalized rate
1
-1
CS+
CS-
Ctrl
Colors

Figure 4. Associative rate changes as expressed in positive and negative Δ for colors during tests 1-3. 6 distinct groups were identified based on the test in which they expressed their highest positive or negative Δ value to the conditioned color. Conventions are the same as in figure 5. Note that units assigned to -Δ test 3 did not change their rate towards the CS+ color, rather the rate increased towards the CS- color. In all other cases, both the rate towards the CS+ and the CS- color changed in opposite directions most strongly on the test day to which they were assigned.

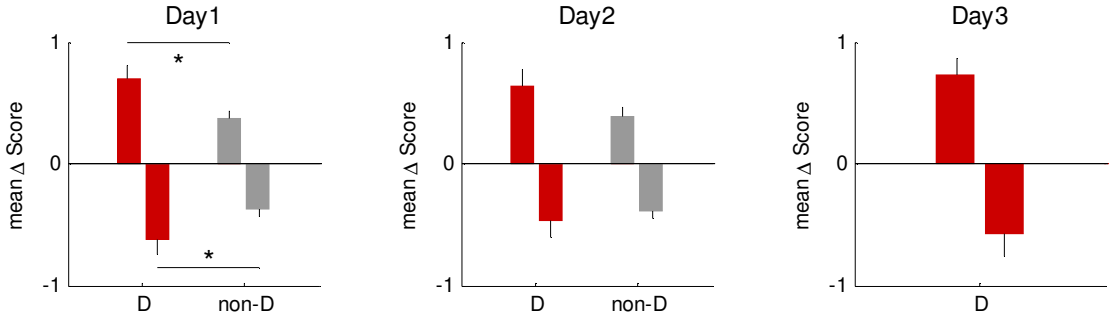
Temporal relationship between color and odor associative plasticity in the same units

We next examined the temporal relationship between the single units for odor and color associative plasticity. Out of 98 units recorded for two or three days, one unit did not develop associative plasticity to odors, and five units did not develop associative plasticity to colors. Of the 92 units that did develop plasticity to both odors and colors, the majority, 39 units (42.4%), exhibited plasticity to odors and colors during the same test, 34 (37%) units developed associative plasticity first to colors and in a subsequent test to odors, while the minority of 20 units (21.7%) developed associative plasticity first to odors and later to colors. Thus, most of the recorded PCT neurons represent the optimal rate code towards visual and olfactory cues that were combined during training either during the same discrete time window or first to the visual and only later to the olfactory cue.

Sixteen units developed plasticity to both modalities during test day 1, 19 units during test day 2, and 4 units during test day 3. We tested whether pretraining responses to the stimuli that later became the CS+ odor and CS+ color predicted the day of associative plasticity using regression analysis. The mean rate difference between CS+ odor and CS+ color for units that estalished associative plasticity to both modalities was 0.43, while units that did not establish associative plasticity before day 2 expressed a lower mean rate difference (0.21). Regression analysis and t-test revealed both a trend ($p = .082$) towards the observed phenomenon, namely that units responding more differently to the two stimuli before training were more likely to develop earlier associative plasticity than units that fired more similarly to the stimuli before training.

Correspondence between behavioral performance and neuronal delta scores

Electromyogram recording of the behavioral responses allowed us to address the question of whether Δ scores correspond to behavioral performance. Each bee was classified as a discriminator if it showed the conditioned response (PER) at least once during three test trials to the CS+ odor and no PER to the CS- odor for tests on each day. Δ values were grouped according to discriminators and non-discriminators (see fig. 5). Positive as well as negavite Δ scores were higher in magnitude in discriminators compared to non-discriminators on day 1, this difference was significant based on the significance level α = 0.1 (p = .06). All 5 bees discriminated correctly during testing on day 3.



p< 0 .1

Figure 5. Discriminator bees expressed higher +/- Δ values than non-discriminator bees. Positive and negative Δ scores were higher for discriminators (D), illustrated in red, than for non-discriminators (non-D), outlined in gray during test day 1. *p < 0.1. During day 3, all bees discriminated correctly.

Associative plasticity as expressed by the population response

Next we asked how fast the rate codes for the learned stimuli evolved during stimulus presentation on day 3 for all units that were recorded for three days. We estimated stimulus representation by calculating time-resolved Euclidean distances between the CS+ and the CS- stimulus for pretraining, on test day 1, day 2, and day 3 in the neuronal ensemble space. Figure 6 illustrates the results for odor responses and figure 7 for color responses together with example units that expressed their highest associative plasticity on day 1, day 2, or day 3 at the top of each figure. For odor responses, the average Euclidean distance

between CS+ and CS- was considerably higher during test day 3, compared with test days 1 and 2 (fig. 6 *B*) - a result that was expected since 17 of these 33 units belonged to the Δ group with optimal rate changes on day 3 (see figure 3). As an additional result, the latencies of the rate changes were shorter during test day 3 compared to test days 1 and 2. This indicates that either the optimal rate change between CS+ and CS- odor evolved with shorter latencies than suboptimal rate changes or that shorter latencies accompanied improved behavioral performances as animals were able to discriminate correctly during test day 3. In order to estimate the timing of reward association we contrasted the average Euclidean distance between the CS+ and CS- stimulus with the Euclidean distance between the CS- and control stimulus (fig. 6 *C*). For odor-reward associations, reward decoding starts ~118 ms after odor onset, reached its peak around 270 ms and lasted until ~660 ms after odor onset. For color responses the time evolution of Euclidean distances expressed two peaks, during test day 2 and test day 3 (fig. 7). However, latencies of these peaks differed considerably (~200 ms earlier on day 3). The neuronal ensemble consisted of equal proportions of 11 units that developed their optimal rate change to colors on days 1, 2, and 3. Hence, the proportion of units in different Δ groups cannot account for latency discrepancies. As on day 3, all animals had learned to discriminate the stimuli perfectly, the shorter latency of the neuronal ensemble on day 3 could be one possible neuronal basis for improved performance. Color-associated reward prediction began earlier than for odors (90 ms versus 118 ms), but here the distance was higher between CS- and control color. 180 ms after color onset the distance between the two neural representations reached its maximum. Reward prediction ended already 230 ms after stimulus onset, the total time of reward representation was thus shorter for color-associated cues (~140 ms) than for odor-associated cues (~545 ms).

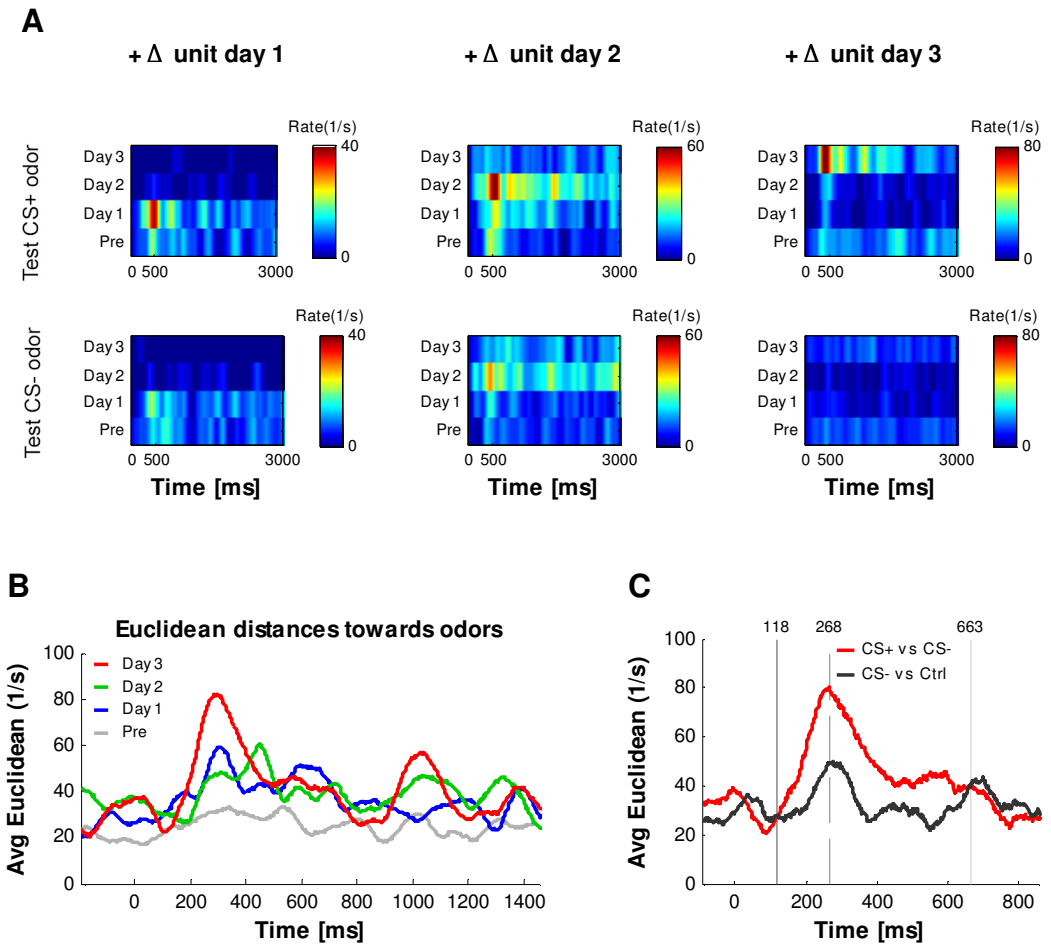


Figure 6. Population coding of learned odors. ***A*** Time-resolved firing rates during 3 sec of odor stimulation were estimated with a Gaussian kernel (σ= 50 ms) for three example units (+Δ units day 1, day 2, day 3) each illustrated for pretraining (Pre), test day 1, day 2, and day 3. The units exhibited increased rates towards the CS+ odor (upper panel) as opposed to the CS- odor (lower panel) during the respective retention test. ***B*** Time evolution of Euclidean distance of an ensemble of 33 units recorded for three days between the two conditioned odors (CS+ vs. CS-) during pretraining (gray), test day 1 (blue), test day 2 (green), and test day 3 (red). The first 1.4 seconds of odor stimulation are shown. A Gaussian kernel with w=200 and σ = 50 was used to estimate the time-resolved firing rates. The Euclidean distance is highest during test day 3 and rises earlier and higher in comparison with day 1 and day 2. The distance on day 3 peaks twice, the first peak around 270 ms after odor onset, the second ~1 second after odor onset. During pretraining, the neural assembly did not encode the two odors differently. ***C*** illustrates the time of reward

prediction on day 3, i.e. when the bees differentiated correctly between the odors. Population rate vectors are plotted for the same units as in ***A*** between the CS+ and CS- odor (red), and the CS- and control odor. The neuronal ensemble encodes the reward-associated odor versus the odors not associated with a reward differently from ~118 ms after odor onset. The conditioned odor reaches a distinct ensemble representation and peaks ~270 ms after odor onset and is kept differently until ~660 ms after odor onset, when the neural representations converged again.

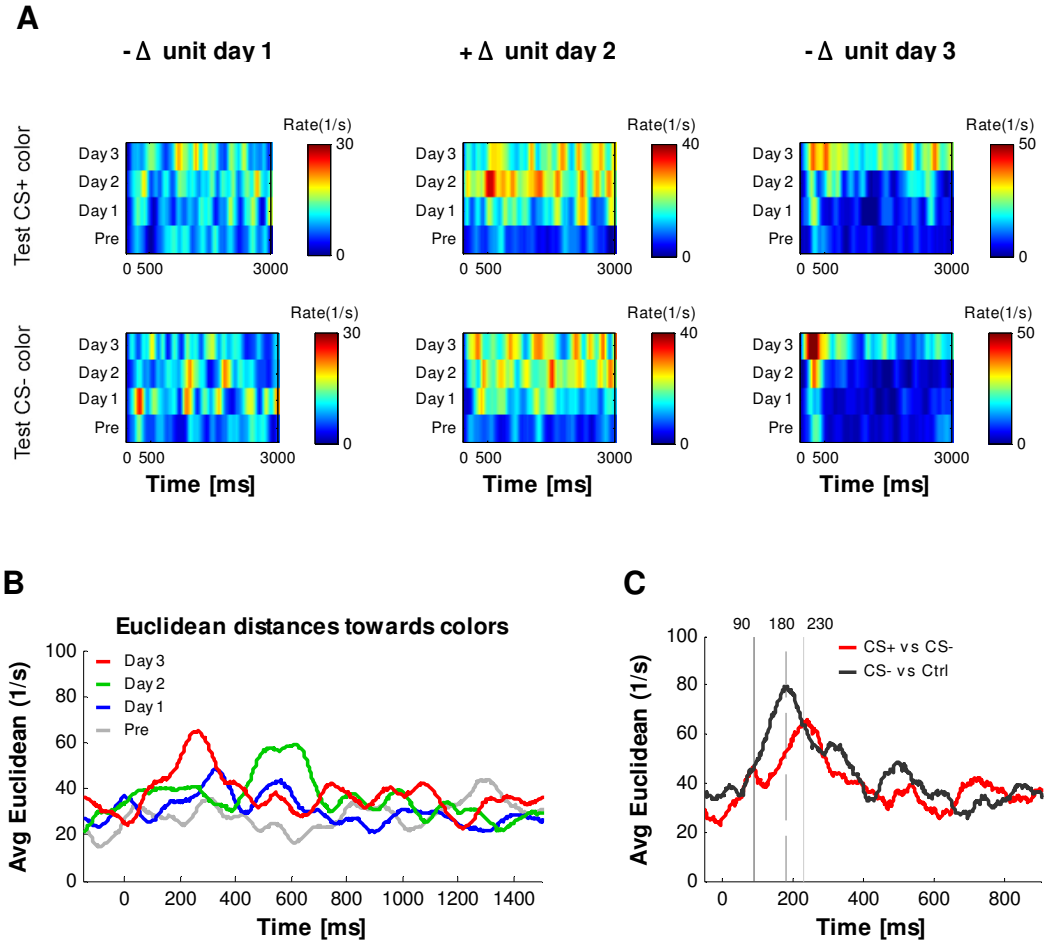


Figure 7. Population coding of learned colors. Time-resolved firing rates during 3 sec of color stimulation calculated in the same way as in figure 7 for odors. ***A*** Three example units are shown: -Δ unit day 1, +Δ unit day 2, and – Δ unit day 3. –Δ units are more strongly activated by the CS- color while the +Δ units are more strongly activated by the CS+ color, in the respective test. ***B*** The average Euclidean distances are plotted between

CS+ and CS- color during the different tests for the same 33 units as in figure ***7B***. As for odor decoding, the distance is highest during test day 3 (red), but the peak is lower in comparison with the peak of odor Euclidean distance (compare fig. 7), though the slope rises earlier for color decoding immediately after color onset. During test days 1 and 2, the latency of the evolution of Euclidean distance is much longer than that on test day 3, also visible in the example units in *A*. ***C*** Color-associated reward prediction begins earlier than for odors (90 ms versus 118 ms), but here the distance is higher between CS- and control color. ~180 ms after color onset, the distance between CS+ and CS- color reached its maximum. Reward prediction ends ~230 ms after stimulus onset.

Discussion

Recurrect GABAergic PCT neurons connecting the MB output with its input develop associative plasticity in a combined visual and olfactory conditioning paradigm to both sensory modalities apparent in long-lasting multi-unit extracellular recordings. Associative plasticity was expressed in changes in the spike rates towards the reinforced and non reinforced stimuli. These rate changes correlated with the behavioral performance of the animals, and peaked at discrete time windows for different neurons over a recording time of up to three days. Thus the input to the mushroom body receives highly selective information about learned stimuli, and this information depends on consolidation of multisensory memory over the range of days.

Behavioral performance

We adopted the behavioral paradigm used by Gerber & Smith (1998) to study visual and olfactory associative learning. Gerber & Smith found facilitated olfactory learning following color preconditioning and subsequent color-odor training. Since bees do not respond to trained colors under these conditions the facilitative component of color training indicates a hidden form of associative or attentional effect. In our study odor learning was as high in combined color-odor training as in single odor conditioning. The unrewarded odor elicited substantially lower responses.

Distributed associative plasticity in mushroom body extrinsic neurons

The PCT neurons recorded here belong to high-order interneurons of the insect brain that are extrinsic to the MB. In *Drosophila* and honeybees, multiple distributed forms of associative plasticity have been observed in both MB intrinsic KCs (Zars et al., 2000; Dubnau et al., 2001; Pascual & Préat, 2001; Yu et al., 2006; Krashes et al., 2007; Wang et al., 2008; Szyszka et al., 2008; Blum et al., 2009) as well as in MB extrinsic neurons (ENs) (Mauelshagen, 1993; Rybak & Menzel, 1998; Iwama & Shibuya, 1998; Menzel & Manz, 2005; Yu et al. 2006; Akalal et al., 2010). In honeybees, several kinds of MB ENs were found to change their response properties during olfactory conditioning, e.g. the morphologically identified pedunculus extrinsic neuron (PE1) reduces its response to the

learned odor (Mauelshagen, 1993; Okada et al., 2007); a group of MB ENs located at the ventral aspect of the α-lobe enhances or reduces their responses to the conditioned stimuli and develops their plasticity within hours following training (Strube-Bloss et al, 2011); PCT neurons (intracellular recordings: Grünwald, 1999b; calcium imaging: Hähnel & Menzel, 2011) specifically change their responses to the trained odor on the same day after learning. However, no continuous long-lasting recordings of MB ENs have been performed so far, and in no case were compounds of olfactory and visual stimuli used as conditioned stimuli.

The recurrent pathway of the mushroom body in the honeybee brain

We focused on recurrent neurons of the PCT since these are promising candidates for coding multisensory stimuli due to their multisensory response characteristics (Gronenberg, 1986). In accordance with Gronenberg's results, we found that about 50% of the recorded PCT neurons responded to at least one odor and one color before training.

Associative plasticity in PCT neurons leads to selective responses to trained olfactory and visual stimuli

We found antagonistic rate changes to the reinforced and nonreinforced color and odor stimuli in subsets of neurons. Thus, associative plasticity is expressed by a rate-dependent gradual de-correlation between rewarded and unrewarded stimuli, and these rate changes correlated with the animals' behavioral performance. Furthermore, these associative changes peaked in different time windows for different neurons, either 30 minutes, 24 or 48 hours after the initial training. As we trained the animals every day anew, different time windows of associative plasticity could reflect different stages of consolidation. During the first retention test, contrasting rate changes possibly constitute novel information while rate changes during tests 2 and 3 might represent repetition-related plasticity due to additional training.

Neuronal tuning profiles before training determined the direction of associative plasticity

We observed that naïve nonresponding neurons exhibited a broader range of plasticity than neurons that responded to odors or colors before training. The nonresponding neurons were recruited and developed predominantly an excitatory response to the CS+ and an inhibitory response to the CS- odor that peaked on the third day. Naïve responding neurons only changed their level of excitation to the CS+ and/or CS- stimulus. Further, naïve responses to one modality determined the direction of rate change. Low naïve responses determined an increase while high responses determined a decrease in response to one stimulus after learning. In particular, neurons with significant rate differences towards the conditioned odors before training were more likely to undergo plasticity already during the first day, while units that expressed plasticity on the third day exhibited no significant pretraining rate difference. We did not observe this phenomenon for color associative plasticity. Thus, neurons that were already firing differently to the conditioned odors might code new associations, while neurons already firing similarly to the conditioned odors changed more slowly and might therefore be more likely to participate in experience-dependent consolidation processes of familiar associations. Additionally, neurons that developed plasticity to both odors and colors on the first day as opposed to the second day exhibited a trend towards higher rate differences between the subsequently rewarded odor and color before training. Thus, pretraining rate differences might facilitate early unimodal and multimodal associative plasticity.

PCT neurons express associative plasticity to colors

This is the first time that visual associative plasticity is evident despite the fact that no motor output is elicited by learned visual stimuli in harnessed honeybees. Until now, learning studies in honeybees were restricted to olfactory elemental forms of learning. While the olfactory CS elicits the conditioned response effectively, training a visual CS requires the ablation of both antennae in order to elicit a PER, which in turn precludes olfactory learning (Hori et al., 2006; Niggebrügge et al., 2009). Gerber & Smith (1998) demonstrated that color pretraining facilitates subsequent olfactory learning. Here we show that the multimodal GABAir recurrent feedback neurons develop the same anatgonistic rate

changes to visual as to olfactory CSs, potentially reflecting the neural substrate for the facilitative effect of color pretraining during different discrete time windows after learning.

PCT neurons provide both local and recurrent inhibitory feedback

The primarily inhibitory recurrent pathway of PCT neurons form fine horizontal bands within the anterior-dorsal α-lobe in which they receive modality-specific excitatory input from KCs and provide local feedback onto MBs' EN e.g. with dendrites of the PE1 potentially providing learning-dependent inhibitory input to PE1 (Okada et al., 2007). PCT neurons further connect the MB output, the α- and β lobes and pedunculus, with all sensory subcompartments of the MB calyx predominantly connecting the same modality-specific output and input layers (Schäfer and Bicker, 1986; Grünewald, 1999a). Since most PCT neurons are GABA-ir they appear to provide selective inhibitory input to the calyces (Bicker et al., 1985; Schäfer and Bicker, 1986). Electron microscopy revealed GABA-ir profiles in the calyx documenting reciprocal synaptic contacts with PNs und monosynaptic contacts with KCs (Ganeshina & Menzel, 2001). Since PNs are presynaptic to KCs and postsynaptic to PCT neurons their output will be indirectly mediated via feedforward inhibition by these GABA-ir neurons.

Functional implications of MB GABA-ir recurrent neurons

Modality-specific KCs of the MB input predominantly receive sensory-specific input via PCT neurons that code former stimulus-dependent experience. This selective input might depress (in the case of enhanced CS+ responses) or strengthen (in the case of reduced CS- responses) PN-KC synapses in the calyx thereby regulating input to the KCs in an experience-dependent way. Convergence of the reward encoding neuron VUMmx1 (Hammer & Menzel, 1998) with KCs suggests that also the PN-KC synapse undergoes associative plasticity, which was found in Ca imaging experiments on the dendrites of MB intrinsic neurons (Szyszka et al., 2008). In the case of enhanced feedforward inhibition, PCT neurons could hypothetically suppress the value signal, thus nullifying the modulatory input. In this context, PCTs would shut down value modulation onto the PN-KC synapses in the calycal microcircuits, consequently modulating the correct synaptic weight that can be detected by olfactory PNs for incoming stimuli via the reciprocal PN-PCT synapse. A

similar mechanism was described by Steele & Mauk (1999) for recurrent inhibitory connections in the CA1 region of the hippocampus. The authors provided evidence in favor of computational studies (LeMasson et al. (1993); Tsodyks et al. 1997) proposing that the spike activity of postsynaptic pyramidal cells recruits GABA-mediated recurrent inhibition and influences the induction of plasticity by promoting LTD or LTP depending on the pyramidal cell's spike activity. Steele & Mauk demonstrated that increased inhibitory feedback onto pyramidal cells in case of high pyramidal firing favors the induction of LTD over LTP, while accordingly, the induction of LTP is enhanced when inhibitory synaptic transmission is blocked (Gustafsson & Wigström, 1990). GABAergic inhibitory neurotransmission onto PN-KC synapses in the MB calyx could likewise prevent runaway induction of LTP or LTD and therefore regulate training-induced patterns of synaptic strengths that encode memories. A computational model for associative plasticity specifically in the MB (Smith et al., 2008) ascribes the same regulatory role to synaptic weights in postsynaptic neurons in the calyx for recurrent inhibitory neurons.

We found two functional subgroups of PCT neurons. The positive Δ units increased their rate while negative Δ units decreased their rate to the CS+ stimulus. This mechanism was often accompanied by the inverse rate change for the CS- stimulus, enhancing the antagonistic rate code. While positive Δ units might prevent the PN-KC synapse in the calyx from over-strengthening through the induction of LTD, negative Δ units might enhance LTP, therefore promoting learning through decreased inhibitory feedback. Evidence in favor of this hypothesis arises from the GABAergic anterior paired lateral neuron (APL) of *Drosophila*, which has striking morphological similarities with the PCT neurons but was only found once in each hemisphere (Liu & Davis, 2009). The APL neuron suppresses and is suppressed by olfactory learning, suggesting that reduced inhibition promotes learning (Liu et al, 2009). PCT subgroups might both enhance or depress synaptic strength in specific postsynaptic neurons in the MB calyx, subserving experience-dependent memory storage in calycal microcircuits. This hypothesis is further supported by a recent study by Hourcade et al. (2011) who reported learning-dependent increases in the same functional calycal microcircuits in the honeybee.

General conclusion

The inhibitory recurrent pathway of the mushroom body informs the input and output regions of the MB about learned and consolidated stimulus combinations. The higher sensitivities of naïvely nonresponding neurons to associative change indicates the recruitment of a specific subset of PCT neurons to the combination of learned stimuli. The antagonistic associative plasticity further specifies this subset of neurons suggesting the transmission of both learning-related depression and facilitation of MB output neurons as well as stimulus coding at the MB input. Subdivisions of these PCT neurons are further characterized by their separation over time during which associative plasticity is expressed, possibly reflecting memory acquisition in the hour and day range.

Assuming specific connectivities of the subsets of these recurrent neurons with MB intrinsic and extrinsic neurons, a highly selective feedback about the learned value of stimulus combinations could be provided.

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3. Visual modulation of olfactory processing in inhibitory local and recurrent feedback neurons of the honeybee (*Apis mellifera*)

Abstract

In honeybees (*Apis mellifera*) preconditioning of a color stimulus and subsequent compound conditioning of a visual and an olfactory stimulus together facilitates olfactory learning (Gerber & Smith, 1998). In order to elucidate the neuronal basis for cross-modal interaction, the behavioral paradigm of Gerber & Smith (1998) was adopted, and extracellular recordings from multisensory inhibitory feedback neurons of the honeybee brain were applied. Enhanced response probabilities and shortened reaction times to the trained odor were observed when the odor was announced by the trained color. A possible neural substrate could be ascribed to a subset of neurons that exhibited enhanced firing after color onset, probably reflecting enhanced attentional levels, and reduced response latencies to the odor. The firing rate of the whole neuronal population towards the odors, when tested together with a color, perfectly matched the integrated sum of the acquired values of the color and the odor together. In the entire neuronal ensemble, this phenomenon was only present when the bee extended its proboscis after odor presentation. As the neurons also coded stimulus values of expected odor cues, in the absence of odor presentation, olfactory modulation might hence depend on the expectation of the odor cue based on the learned value of the color. Via local and recurrent feedback, the neurons might inform motor and sensory areas about the relevance of stimulus combinations for behavior.

Introduction

Learning enhances the sensitivity of neural assemblies to process incoming stimuli (Buzsáki, 2006), and facilitates action-selection processes for appropriate behavior (Roelfsema et al., 2010). Associative learning can be studied with classical conditioning.

Classical conditioning derives from an association between a neutral stimulus (also conditioned stimulus, CS) and a biologically significant stimulus (also unconditioned stimulus, US), such that in the course of learning the CS acquires the rewarding valence (or value) of the US, leading to a conditioned response (CR) evoked by the mere presentation of the CS. Animals learn to associate the CS with the US. However, the CS is certainly not the sole cue being associated with the US, rather multiple context cues in which learning takes place can act as a CS and might influence subsequent action-selection processes. Experimentally, context cues can be operationalized by pretraining the subject to one stimulus and subsequently train this stimulus together with a second cue, either from the same or another sensory modality.

Indeed, humans as well as animals use input from one sensory modality to either facilitate or attenuate processing of a stimulus from a dfiferent sensory modality, and adapt their behavior accordingly. Auditory speech perception, for example, can be modulated by lip reading, (Eimer & Schrögen, 1998).

In humans cross-modal visual and olfactory processing has been shown to enhance odor responses and reduce reaction times (RTs) when visual congruent cues preceded odor presentation in comparison to incongruent visual cues in the anterior hippocampus and rostromedial orbitofrontal cortex (OFC) (Gottfried & Dolan, 2003). The activtaion of the congruent pair exceeded the activation of the stimuli alone, as has been previously described in human and animal studies of multisensory integration (Stein & Meredith, 1993; Calvert et al., 2000).

Also insects use the information from one sensory modality to adapt their behavior to a cue from a second sensory modality (Matsumoto & Mizunami, 2004). In the honeybee (*Apis mellifera*) intra-modal compound conditioning, for either visual or olfactory cues, was shown to attenuate conditioned responses to both modalities (Smith & Cobey, 1994; Couvillon et al., 1997). However, no such blocking effect was found when using two stimuli of different modalities (visual and olfactory cues) (Gerber & Smith, 1998).

Olfactory learning in honeybees can be modulated by a color via an occasion-setting mechanism (Mota et al., 2011), and preconditioning of one color followed by compound conditioning with the color and an odor together has been shown to enhance response

probabilities, prolong response durations and shorten the RT when testing the odor after training (Gerber & Smith, 1998).

The critical question underlying these cognitive dissociations is how the brain accomplishes action-selection based on the learned value of the stimuli.

We aimed to elucidate the neuronal basis of cross-modal interaction by adopting the behavioral paradigm of Gerber & Smith (1998) while recording from a distinct neuronal population in the honeybee brain. The target neurons, GABA-ir local and recurrent feedback neurons of the protocerebral-calycal tract (PCT) had been shown to be multimodal (Homberg & Erber, 1979; Gronenberg, 1987; Schildberger, 1981), and are therefore regarded to be a suitable neuronal candidate for multisensory interaction effects. Since they provide recurrent feedback to the input of the MB, where they connect reciprocally with sensory neurons, as well as local feedback presumably onto premotor neurons, they might serve as a relay station between sensory and motor areas.

In the present study context cues were operationalized with colors that were differentially pretrained, one color was rewarded while the other was not, and both colors were trained together with a specific odor. The set of compounds with the CS+ color was rewarded while the set with the CS- color was not rewarded. Different to the behavioral paradigm of Gerber & Smith, we used differential color preconditioning, as well as differential compound conditioning, and we tested for odors and colors alone, the trained compounds and the reversed compounds, which have not been trained. On the behavioral level, we found enhanced response probablities to the trained CS+ compound (CS+ color & CS+ odor) opposed to the reversed CS+ compound (CS- color & CS+ odor), and reduced RTs to the trained CS+ compound in comparison with the CS+ odor presentation alone.

On the neuronal level, during retention tests, we found reduced response latencies and increased rates in a subset of neurons to the CS+ odor when the CS+ color preceded odor presentation in comparison to test trials in which the CS- color or no color preceded the CS+ odor, which might reflect the physiological basis underlying reduced RTs of behavioral PER`s.

Considering the entire neuronal ensemble only the CS+ color enhanced CS+ odor responses during retention tests. Interestingly, in trials in which the bees extended their

probosces, both colors modulated odor responses and reward expectancy to both odors. Visual modulation of olfactory responses in PCT neurons thus occurs more pronounced upon stimulus selection, which implies the necessity of attention (Rizzolatti, 1987; Allport, 1989). Upon attention, the firing rate towards the odors perfectly matched the integrated sum of the acquired values of both stimuli. Testing the CS- color alone led to a pronounced inhibition that was evident when the CS- odor was expected to be presented, again, only in trials in which a motor response was executed. Modulation thus depends on the expectation of the odor cue based on the learned value of the color.

Material and Methods

Animal treatment

20 foraging honeybees (*Apis mellifera*) were caught at the hive entrance, anesthetized on ice, and harnessed in metal tubes, as described by Bittermann et al. (1983). The bees were not fed before the experiment begun, in order to make them hungry and motivated. Only bees showing the proboscis extension response (PER) after touching one antenna with 30% sucrose solution were considered for experiments.

Dissection

A small window (1.5 mm^2) was cut into the head capsule between the compound eyes along a saggital plane with the head fixed to the stage with dental wax. The first joints of the antennae were immobilized using low temperature melting n-icosane. The head glands and trachea sacks laying on the surface of the brain were cautiously moved aside until the alpha-lobe could be clearly identified. A silver wire electrode was inserted between the right compound eye and the right lateral ocellus for electromyogram recordings of the M17 muscle that innervates the proboscis and whose activity reflects proboscis movement. The reference electrode was either inserted into the right compound eye or into the median ocellus.

The bee was positioned such that the brains surface was exactly 90° horizontally to the set-up table. Microruby was attached to the tip of the multi wire electrode in order to stain the electrode position inside of the brain and to investigate the stained region by means of confocal microscopy after the experiment. The electrode for neuronal recordings was inserted at the medial-lateral border of the left alpha-lobe, and placed 60 – 250 µm below the anterior surface by means of a micromanipulator attached to the electrode holder. After successful placement of the electrode the window in the head capsule was covered with a drop of silicon (two components of KWIK-SIL Sarasota, FL, USA, mixture 1:1). The silicon prevented the brain from drying-out and additionally fixed the electrode in the brain, ensuring the stability of the electrode position over several days. The experiment

started 30 minutes later when the silicon had bonded and the animal recovered from the dissection.

Behavioural Task

The behavioural paradigm of Gerber & Smith (1998) was adopted and modulated for our purpose to study visual inter-modal interaction effects. The baseline responses of the recorded units towards the stimuli were determined before training sequentially stimulating the animal with three odors and three colors each five times with an inter-stimulus interval (ISI) of one minute. After a brake of 10 minutes color pretraining began. One color was chosen randomly to be rewarded during six training trials while another randomly chosen color was not rewarded. The bee was illuminated for 12 seconds. After eleven seconds the bee was fed for three seconds with a droplet of sugar water attached to the tip of a wooden stick. As a control for the movement and humidity levels, a wooden stick with a droplet of pure water was moved towards the bee in unrewarded trials in order to control for humidity levels and hand movements.

After another pause of 10 minutes, compound color/odor training began. During the rewarded condition the bee was again illuminated with the previously rewarded color, but this time an odor was additionally applied seven seconds after color onset, for 4 seconds in total. The reward was applied three seconds after odor onset. Thus there was one second of overlap between the three sensory modalities, before color and odor were turned off. During the unrewarded condition the previously unrewarded color was paired with another odor, here no reward was delivered. Both conditions were trained within six trials each. Thirty minutes later the bee was tested for all stimuli seperately and in combination, three times, respectively. Furthermore, color/odor combinations not trained were also tested. Importantly, this experimental procedure, including training and retention tests, was repeated on each consecutive day. Note that Gerber & Smith (1998) manipulated color pretraining to explore its influence on odor retrieval while we always pretrained colors differentially additional to subsequent differential compound training but manipulated the test conditions. While assuming inter-modal facilitation in case of color pretraining we addressed the additional question whether the behavior and the neuronal activity to the odor cues was different during tests depending on the color that preceded the odor.

Electrophysiology

Electrodes

In a first series of experiments, the electrode for neuronal recordings consisted of two, in a second series of three polyurethane-coated copper wires (14 µm in diameter, Electrisola, Escholzmatt, Switzerland) that were glued together with wax and attached to a piece of glass cappillary (~ 2 cm in length) for handling via the micromanipulator. The ends of the wires were deinsulated and attached to the amplifier input connectors by means of conducting silverglue. Resistances of single wires were in the range of 1 and 2 MΩ. Silver wires (0.05 mm in diameter, Advent, Eynsham Oxon England) were used for the reference electrode as well as for the electromyogram recordings of M17 activity.

Amplifiers

Each wire of the electrode was connected to the head stage of a preamplifier (npi electronic). Filters were set to high pass of 10 Hz and low pass of 10 kHz. Hum noise (50 Hz) was eliminated by an additional filter (Hum Bug; Digitimer, Hertfordshire, UK). Neural activity was sampled with a rate of 20 kHz through an analog to digital converter (1401 micro MKII; Cambridge Electronic Design, Cambridge, UK) and initial data analysis was performed by Spike2 software (Cambridge Electronic Design) including signal storage, control of stimulation devices and preanalysis of the data. The amplifier used a band pass filter with cut-off frequencies between 10 Hz and 10 kHz.

Visualization of recording position

After the experiment, the brains were dissected, fixed over night in 4% formaldehyde with 1 µl Lucifer Yellow for enhanced backround staining, washed in PBS, dehydrated in rising concentrations of alcohol (20%, 50, 70%, 99%, and 100 %), and cleared with methylsalicylate. A Leica TCS SP2 confocal laser-scanning microscope (Wetzlar, Germany) was used for scanning with a 20x or 10x water objective. Two excitation wavelengths were applied, 428nm for the backround (Lucifer yellow), and 560 nm, for Microruby, were used.

Odor and color stimululation

Odor stimulation was computer-controlled, using an olfactometer with seperate channels for each odor, described elsewhere (Galizia et al., 1997). In each experiment, three different odors were used to which the bee did not show a spontaneous PER. Two of the odors were used for differential conditioning, the third one as a control odor. The olfactomter was placed in front of the bee such that the end of the outlet had a distance of approximately 5 cm to the bee's head. A constant air stream (1.5 m/s speed) was send through a teflon tube (6 mm in diameter). The control of magnetic valves via the Spike2 software allowed adding a particular odor to the airstream. An exhaustion pipe behind the animal ensured that odor did not accumulate.

Color illumination was provided by a light guide connected to a lamp with filters for green, yellow, and blue light. The exit of the light guide was placed in front of the bee beside the odor pump. Light switches were conducted manually, the precise timing of on- and off-switches were announced by a sound coming from a loud speaker that was connected and controlled by the Spike2 software.

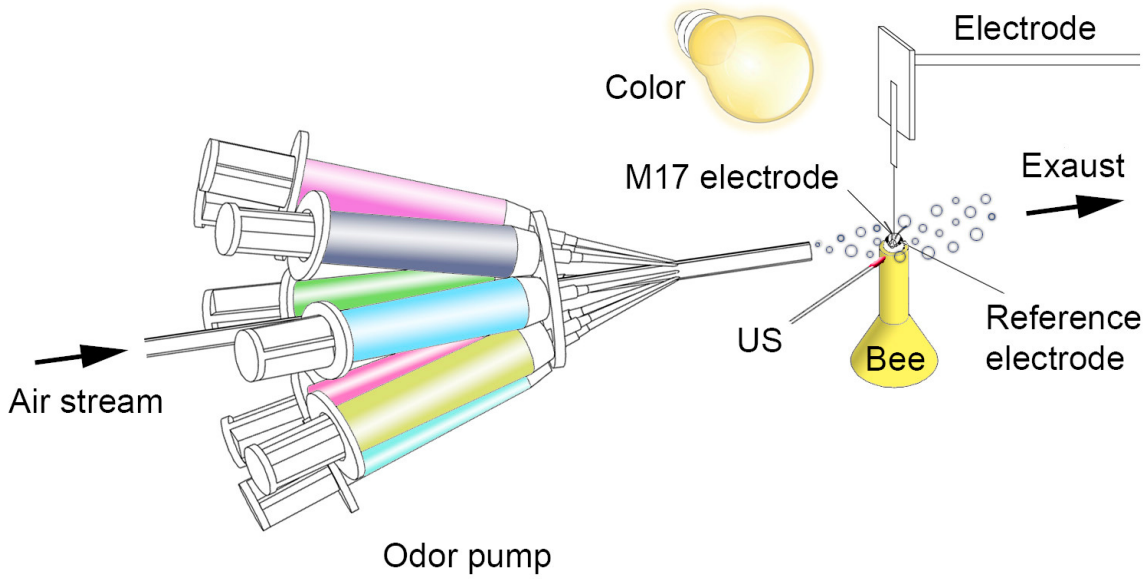

Figure 1. Three-dimensional illustration of the electrophysiological set-up used during recording sessions. Shown is the odor pump with its constant air stream, to which odors are added during stimulation. The exhaust prevented accumulation of odor molecules

around the bee. Besides odors, colors were used as conditioned stimuli throughout the experimental procedure.

Odor delay

The time between the trigger as recorded by the Spike2 program and the actual opening of the magnetic valves was approximately 3 ms. After opening of the magnetic valves the odor arrived at the bee`s antennae another 41.14 ms later, according to the speed of the airflow and the distance from odor injection to the bee. Thus, there is a constant odor delay of 44.14 ms. We considered this factor in our analysis.

Data analysis

Spike sorting

The differential channel or the three separately - single-ended - channels, and the channel that recorded from the M17 were high-pass filtered at 300 Hz using the Spike2 software (Cambridge Electronic Design, Cambridge, UK). The semi-automized template matching algorithm of Spike2 was used for spike sorting.

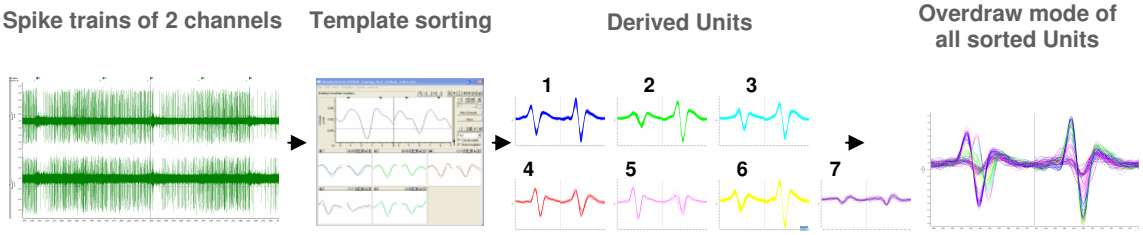

Figure 2. Spike sorting procedure *using the semi-automaized template matching algorithm provided by Spike2.*

In case of three neuronal channels, the two channels with the best quality were sorted together such that amplitude and waveform were constantly and simultaneously checked on both channels together. The third channel was not used anymore to avoid false positives. Sorting quality was, beside careful visual inspection, controlled by means of a principal component analysis of the first three components of each sorted unit. The dots of the single units (encoded in one particular color) needed to be clearly separated from each other in order to ensure good sorting quality and to avoid false positives and negatives in spike

trains of a single unit. The recorded M17 activity was seperated from noise by threshold sorting in Spike2.

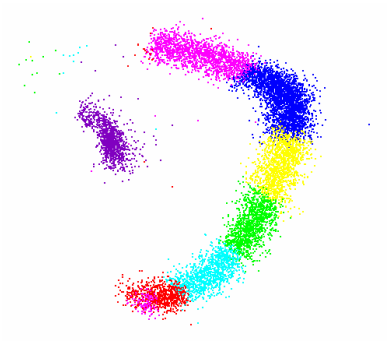

Figure 3. Principal component analysis *of single unit activity*

Response detection for M17 activity

Rate responses were estimated with a kernel convolution with a sliding window width of 50ms (Nawrot et al., 1999). Therefore, the spike trains of the muscle activity during training sessions were binned with one ms precision (1ms bins) before reward application in a two seconds time window from stimulus onset. During test sessions, a four seconds time window after stimulus onset was chosen for response detection. After convolution, a threshold was applied: As soon as more than three spikes within the sliding window emerged, a response was detected.

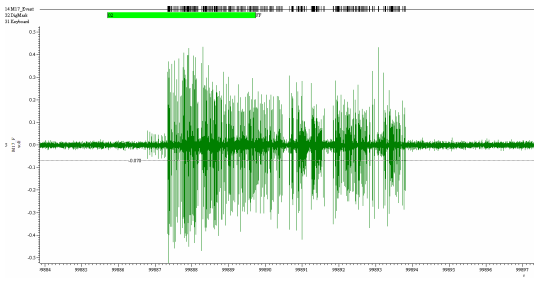

Figure 4. Threshold sorting of muscle activity

Analysis of neuronal data

Analysis of neuronal activity was performed by constructing Peri-Stimulus-Time-Histograms (PSTHs). The subsequent analysis was related to four time windows that related to four different behavioral events: W1: color onset (-8 to -6 seconds), W2:

preparatory period (-6 to 0 seconds), W3: odor onset (0 to 2 seconds), and W3: reward omission (2 to 4 seconds). The last time window is referred to as reward omission as no reward was applied during retention tests, when neuronal data was analyzed. Firing rates were normalized using the antecedent ongoing activity between 1 to 3 seconds before the first cue onset (color onset), on a trial-by-trial basis for each individual cell. Z-scores were calculated from an approximate Poisson distribution, as described in Totah et al. (2009), where Z is defined as the ((observed number of spikes per bin) – (expected number of spikes)) / √expected number of spikes, while the expected number of spikes = ((total number of spikes during baseline time period) / (baseline time duration)) * (time bin size). Using this equation, an excitatory unit was defined as significantly responding if the Z-score was above 2.36 (which corresponds to a p value < 0.01) in x consecutive time bins or below 1.28 for inhibitory responses ($p < 0.1$). The lower threshold for inhibitory responses was chosen in order to compensate for the opposite of the well known ceiling effect what we call the "bottom effect": There can only be zero spikes, but not a number of negative spikes. For the analysis of neuronal activity towards color onset, odor onset, and reward omission, a bin size of 50 ms was used. In order to define significant responses during the preparatory activity, a bin size of 200 ms was used, two consecutive bins had to exceed the defined response threshold. In order to assess differences in neuronal activity due to the different behaviour of the animals. First the grand average of all recorded neurons was assesed, then the units were grouped based on the subsequent behavior of the animal (PER vs no PER trials). Population activity of these two groups (PER vs no PER) was compared for each condition and each time window. Thereafter, population activity of PER trials only was assessed during the last two time windows, odor and reward omission responses (W3 & W4), again, for all tested conditions.

Statistics

First the grand average of all recorded units (N = 116) was calculated and each unit`s activity was compared in each time window (W1-W4) using Repeated Measures MANOVA with the within-subject factor "condition". When PER vs no PER trials were compared, MANOVA analysis were used in which time was defined as the within-subject factor and the behavior (PER vs no PER) as between-subject factor.

Results

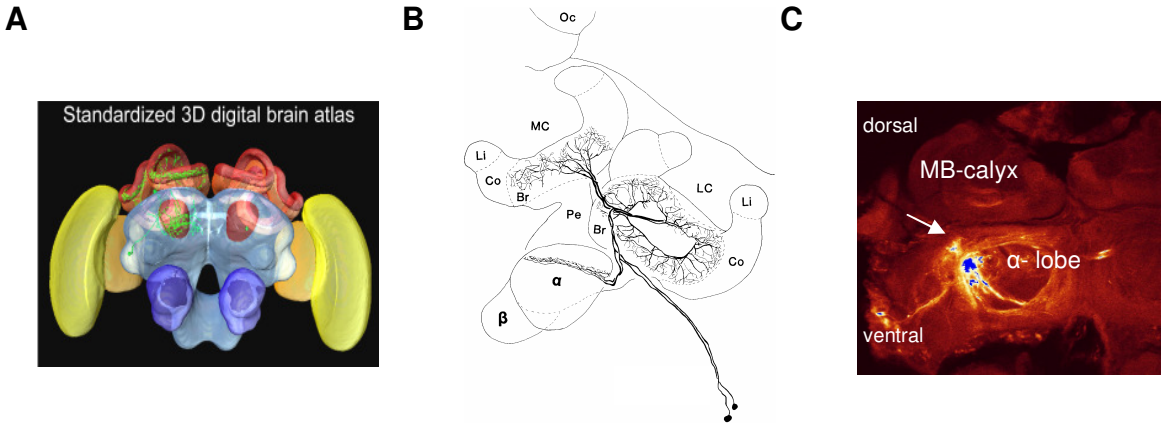

Figure 5. Morphological structure of the PCT and visualization of the recording position. ***A*** 3-D standardized digital atlas of the honeybee brain with reconstructed PCT neuron (green) by Ruth Bartels. ***B*** Schematic diagram of PCT neurons within the honeybee MB, adopted from Grünewald (1999). PCT neurons receive multimodal input from the KCs within modality specific bands of the α-lobe. Flow of information is toward all subcompartments of the calyces. (Abbreviations: *α*, α-lobe; *β*, β-lobe; *Pe*, pedunculus, *Co*, collar, *Br*, basal ring; *Li*, lip; *LC*, lateral calyx; *MC*, median calyx; *OC*, lateral ocellus). ***C*** The confocal image shows staining with mircroruby of somata of PCTs lying in the periphery of the lateral protocerebrum. One main neurite is stained that arborizes within the left α -lobe of the MB. Electrode position is indicated by the white arrow.

Behavioral performance

We wanted to test, whether the behavioral and neuronal responses towards the conditioned odors differed when the odors were presented alone or when they were preceded and accompanied by a color during retention test 30 minutes after color pretraining and color/odor compound training. During training, two colors were differentially pretrained. One was rewarded (CS+ color), the other not (CS- color). During the subsequent compound training, an odor (CS+ odor) was preceded and accompanied by the CS+ color, this set was rewarded. The CS- odor was preceded and accompanied by the CS- color. This compound was not rewarded. During the test, odors and colors were tested alone. We also tested the trained compounds (CS+ color & CS+ odor; CS- color & CS- odor), further called “trained

CS+ compound" and "trained CS- compound" and also the reversed pairs (CS- color & CS+ odor; CS+ color & CS- odor), further called "reversed CS+ compound" and "reversed CS- compound".

Harnessed bees do not extend their proboscis towards colors (Niggebrügge, et al., 2009). In this study, also no PER`s were observed towards colors. We therefore only illsutrated percentages of PER`s of all three test trials to odors and compounds for 20 bees on the first test day1, for 11 bees on the second test day, and for four bees on the third test day.

We calculated differences of PER responses between the 6 different test contingencies using the McNemar test, and illustrated them in figure 6 (CS+ odor, trained CS+ compound, reversed CS+ compound, CS-odor, trained CS- compound, reversed CS- compound, in this order).

To display the results, we kept one color coding scheme throughout this work. CS+ odor responses are always illustrated in black, and CS- odor responses in grey, while green indicates the CS+ color and orange the CS- color. Compounds are thus outlined with two colors, one for the color and one for the odor.

The CS+ odor elicited 33,3 % PER`s in 20 bees in 60 test trials (three trials per contingency). This were significantly more PER`s in comparison with the CS- odor (Chi^2 = 7.682, p = .006), the trained CS- compound (Chi^2 = 10.316, p = .001), and the reversed CS- compound (Chi^2 = 5.263, p = .02). The trained CS+ compound elicited a PER in 45% of all trials, significantly more than the reversed CS+ compound (Chi^2 = 9.091, p = .003), the CS- odor (Chi^2 = 13.793, p = .001), the trained (Chi^2 = 18.375, p = .001), and the reversed CS- compound (Chi^2 = 12.042, p = .001). Moreover, the reversed CS+ compound elicited a PER in 27% of all test trials and significantly more than the CS- odor (Chi^2 = 4.050, p = .04), and the trained CS- compound (Chi^2 = 7,692, p = .006).

During test day2, The CS+ odor evoked more responses than the CS- odor (Chi^2 = 5.818, p = .016), and than the reversed CS- compound (Chi^2 = 4.083, p= .043). The CS+ compound effected more responses than the reversed CS+ compound (Chi^2 = 4.167, p = .041).

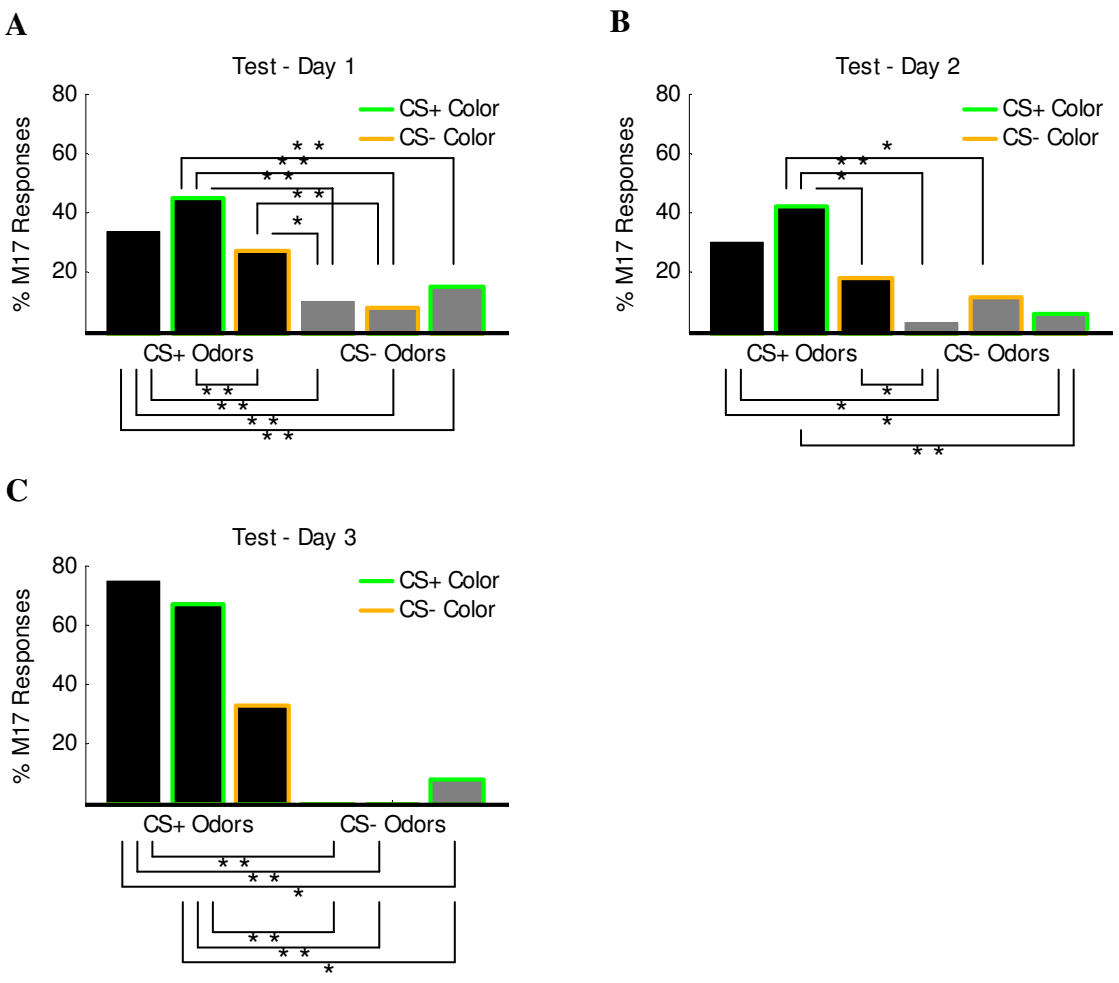

Figure 6. Bees learned to distinguish the CS+ from the CS- contingencies. ***A*** Percentage of PER`s of 20 bees, each during three test trials, day1 (from left to right: CS+ odor: 33.3%, trained CS+ compound: 45%, reversed CS+ compound: 27%, CS- odor: 10%, trained CS- compound: 8.3%, reversed CS- compound: 15%). Bees extended their probosces significantly more to the CS+ (black bars) than to the CS- (grey bars) contingencies. All comparisons between the CS+ and the CS- contingencies were significant with the exception that the reversed CS+ compound (black/orange bar) did not elicit more PER`s than the reversed CS- compound (grey/green bar). Also, more PER`s were observed towards the trained in comparison with the reversed CS+ compound. (See text for statistics). ***B*** Percentage of PER`s of 11 bees on test day2 (CS+ odor: 30%, trained CS+ compound: 42%, reversed CS+ compound: 18%, CS- odor: 3.03%, trained CS- compound: 12%, reversed CS- compound: 6%). While the trained CS+ compound elicited

significantly more PER`s on day2 than all other contingencies, except CS+ odor, the latter elicited more responses than the CS- odor and the reversed CS- compound and lost its behavioral dominance above the trained CS- compound. The reversed CS+ compound elicited more responses than the CS- odor. ***C*** By day3, animals responded to a great deal of CS+ contingencies (CS+ odor: 75%, trained CS+ compound: 67%, reversed CS+ compound: 33%), while not a single trial was responded for the CS- odor and CS- compound. However, when the CS+ color preceded the CS- odor (reversed CS- compound) 8.3% of the trials were responded.

Behavioral response latencies

In the following, we explored behavioral response latencies, to assess, whether a preceding color either fastens or attenuates the behavioral reaction time to the CS+ and CS- odors. For test day1, all trials in which the bees extended their probosces to the trained and reversed rewarded compound as well as to the CS+ odor were investigated. Behavioral reaction times (M17 Response latency) of the bees (N = 5) were pooled for each condition. Reaction times to the three different conditions were compared using Repeated Measures MANOVA. The bees exhibited significantly different reaction times to the conditions [$F(1, 10) = 13.397$, $p = .004$]. The bees reacted significantly faster to the CS+ odor when the odor was preceded by the CS+ color (trained CS+ compound: mean reaction time: 773.2 ms) in comparison with the presentation of the CS+ odor alone (mean reaction time: 1416.3 ms). The difference between the reversed CS+ compound (mean reaction time: 1250.8 ms) and the two other conditions was not significant (see fig. 7 ***A***).

Neuronal response latencies

We asked whether the neuronal activity in these trials mirrors the behavioral response latencies. To this end, we explored the activity of units that exhibited a significant response between color and odor onset (between -6 and 0 seconds) during the same trials that were analyzed for the behavioral latencies. We hypothesized that units with a significant response after the CS+ color onset and before the CS+ odor onset might anticipate the CS+ odor and might thus react faster in comparison with the condition in which only the CS+ odor was presented.

5 units from 3 bees matched this criterium and were selected and compared during the three different conditions (CS+ odor, illusrated in black, trained CS+ compound, illustrated in green & black, reversed CS+ compound, illustrated in orange & black). In figure 7 ***B***, the neuronal activity of these 5 units is illusrated throughout color onset (-8 sec), preparatory period (-6 till 0 sec), odor onset (0 sec), reward omission (3 sec), odor and color offset (4 sec). In fig 7 ***C***, a zoom in is shown for the first 500 ms after odor onset (grey area in 7 ***B***). A threshold for response detection was defined as 4 times the standard deviation of the mean ongoing activity before stimulus onset (grey horizontal line in 7 ***C***). Population latencies were estimated by means of linear interpolation. Latencies thus resemble the point in time when the population response became significantly deviant from the ongoing activity by exceeding the threshold. The population latency was shortest for the trained CS+ compound. The threshold was exceeded ~108 ms after odor onset, while the population response to the reversed CS+ compound became significant ~136 ms and to the CS+ odor only ~196 ms after odor onset. Thus, the CS+ color enabled a few units to exhibit significant preparatory responses after color and before odor onset while the same units responded faster to the CS+ odor after the onset of the CS+ color as opposed to the CS- color and no color. Additionally, the odor response was significantly increased in magnitude when the CS+ color preceded the CS+ odor in comparison to the CS- color and no color ($p < .05$). This was also true for the rate response before reward omission (3 sec). Here, the rate response was graded: It was strongest for the trained CS+ compound, medium for the CS+ odor only and lowest for the reversed CS+ compound, indicating that the CS+ color increased reward expectation while the CS- color decreased reward expectation in comparison with the CS+ odor only condition, respectively.

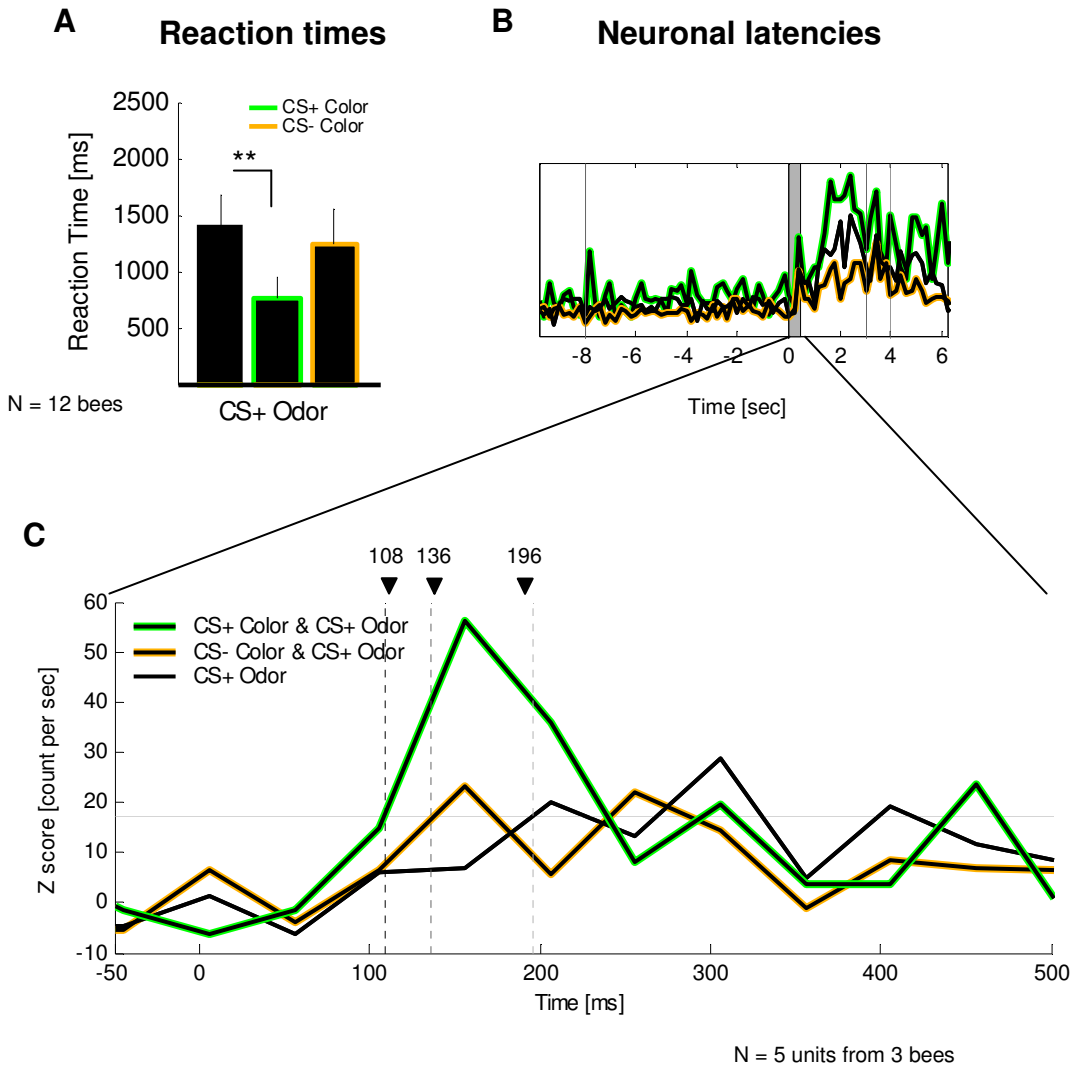

Figure 7. Behvaioral and neuronal response latencies towards CS+ odor, trained and reversed CS+ compounds. ***A*** PER reaction times to the CS+ odor were significantly reduced when the CS+ color preceded the CS+ odor in retention tests on day1. ***B, C*** Neuronal activity in the same trials as in ***A*** of units that exhibited a significant response between CS+ color onset and CS+ odor onset. Unit activity is plotted for all three conditions. The zoom in (***C***) depicts latencies of the population response to the CS+ odor, again, for all three conditions: CS+ color & CS+ odor (green & black line), CS- color & CS+ odor (orange & black line), and only the CS+ odor (black line). The neuronal ensemble exhibits decreased population response latencies and increased rate responses to the odor and before reward omission when the CS+ color preceded the CS+ odor in comparison with the CS- color and no color.

Analysis of the grand average

In order to capture the neuronal activity of all recorded units to the test conditions (odors and colors alone, CS+ and CS- trained and reversed compounds) the grand average was calculated for all conditions and analyzed in predefined time windows around the behavioral events (W1 = color responses, W2 = preparatory responses, W3 = odor responses, W4 = reward omission responses) during retention tests on day1. See fig. 8 for the grand average to odor and colors only (8 ***A***), and to the trained and reversed compounds (8 ***B***). In both graphics the four time windows that were analyzed separately for each condition are marked in grey shaded areas.

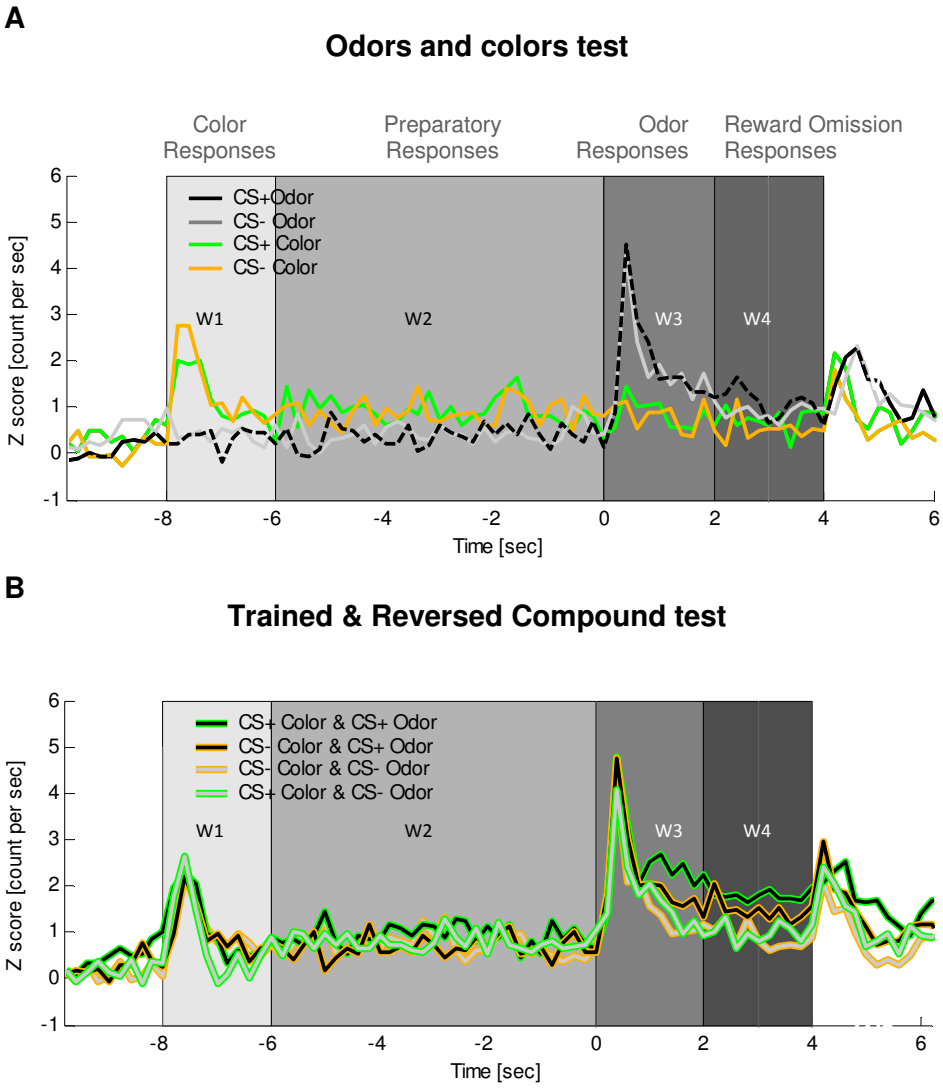

Figure 8. Grand average for all test conditions. In ***A***, the average population activity of all recorded units is outlined during color and odor tests. In ***B***, population activity to trained and reversed compounds is plotted. The population activities were devided into four time windows surrounding behavioral event (W1 = color responses, W2 = preparatory responses, W3 = odor responses, W4 = reward omission responses).

Neuronal response differences between conditions for all four time windows

The mean population activity of all recorded units was calculated for all conditions per time window surrounding the four behavioral events. The mean normalized rates were compared using Repeated Measures analyisis. The eight different conditions (CS+ color, green, CS+ odor, black, trained CS+Compound (green & black, reversed CS+Compound, orange & black, CS- color, orange, CS- odor, grey, trained CS- compound, orange & grey, and reversed CS-compound, green & grey) were defined as the within-subject factor. All units were considered for all conditions.

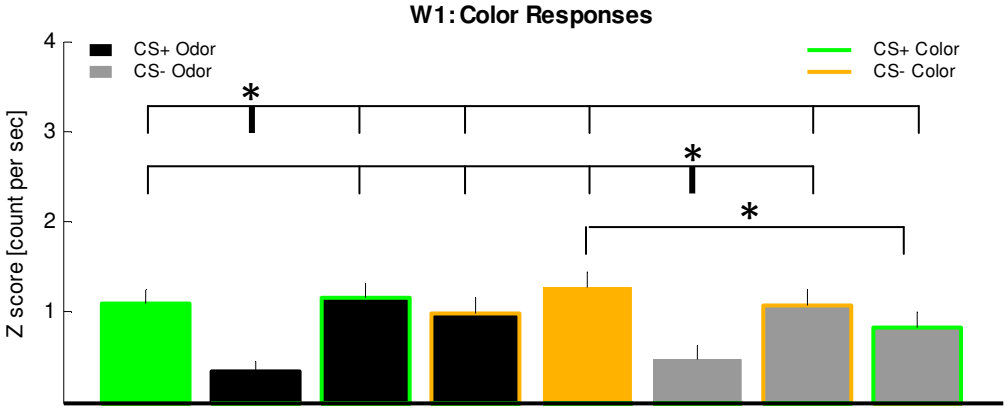

Figure 9. Color responses of grand average for all 8 test conditions. The mean population activity exhibited significantly different firing rates towards the tested conditions [$F(7, 805) = 6.599$, $p < .000$.]. The CS+ and the CS- odor elicited a significantly lower response during color presentation than all other conditions (p`s < .05), with the exception of the pairwise comparison between the CS- odor and the reversed CS-Compound ($p > .05$). The color response of the reversed CS- Compound is lower that the CS- color response when presented alone. This indicates that the CS+ color response

during the reversed compound reduces during the three repetitive test trials, probably as a feedback based of the following CS- odor.

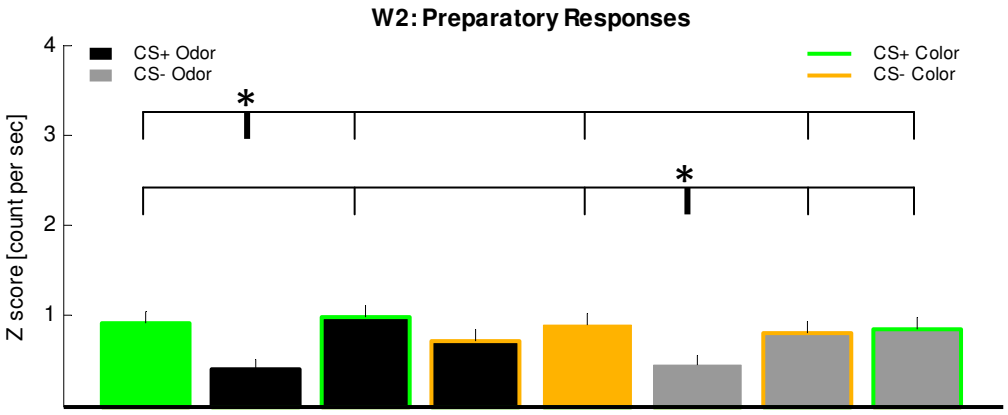

Figure 10. Preparatory responses of grand average for all 8 test conditions. The units also fired significantly different towards the conditions during the preparatory window: [F(7, 805) = 3.577, p = .001]. Both odor responses elicited significantly less responses than all other conditions with the exception of the reversed CS+ Compound, indicating that trial-by-trial feedback lowers the preparatory response after the units detected that in this condition the CS+ odor follows the CS- color.

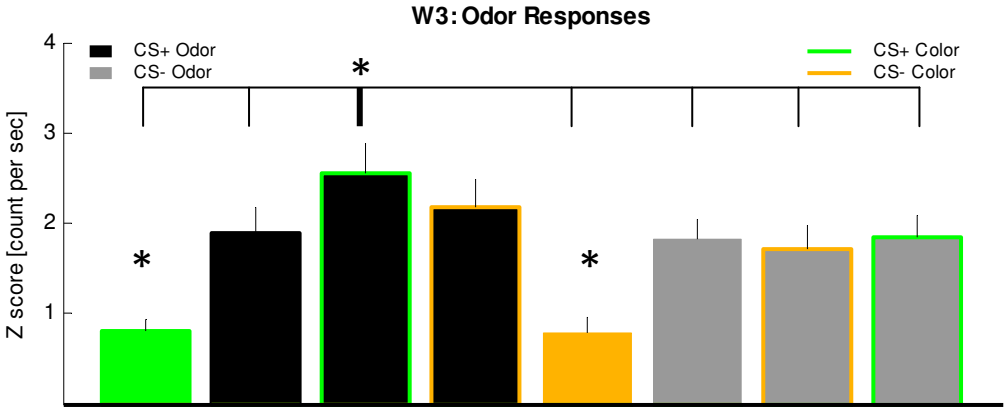

Figure 11. Odor responses of grand average for all 8 test conditions. Population activity was different for the 8 test conditions [F(7, 805) = 9.999, p < .000] The trained CS+ Compound is significantly higher than in all other conditions with the exception of the reversed CS+ compound. The color responses in the odor response window are lower than all other conditions.

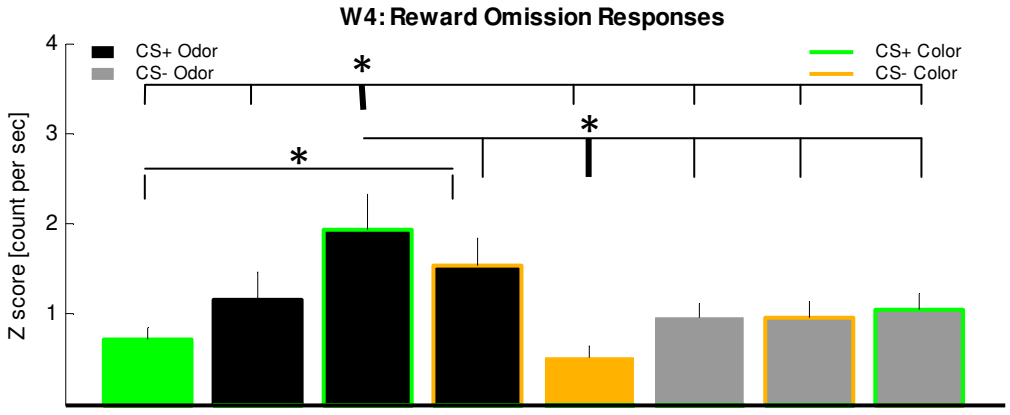

Figure 12. Reward omission response of grand average for all 8 test conditions. Units exhibited significantly different reward expectation to the 8 conditions [F(7, 805) = 4.661, p <.000]. As for odor responses, the response magnitude for the trained CS+ Compound around the time of US application during training is higher in the test in comparison to all other conditions with the exception of the reversed CS+ Compound. The response to the CS- color alone is significantly reduced opposed to all but two conditions, the CS+ Color and CS+ Odors.

Relation between rate response and behavior

In order to assess whether PCT neurons respond differently when a PER is executed in comparison with no execution of a PER we calculated interaction effects and main effects for each condition in each time window using MANOVA. We defined time (time bins) as the within-subject factor and the behavior (PER vs. no PER) as the between-subject factor. Significant interaction in one time window indicates different slopes of the firing of the units based on the behavior of the animal, a significant between-subject effect indicates whether the mean normalized firing rate differed significantly between the two groups

(PER vs no PER). Thus, here we compare, on a trial-by-trial basis, whether the population response differed during each time window. Note, that this analysis differs from the unit-wise analysis above. This time, units with no ongoing activity were excluded. Results are notated for each condition and within each condition for each time window. Table 1 summarizes rate differences during PER trials in comparison to no PER trials. Arrows indicate significant rate increases and decreases, respectively, during PER trials during the same stimulus condition and time window in comparison to no PER trials. Significant interaction effects, that indicate different slopes of the firing rate, are described as "different".

Table1. *Rate differences during PER trials in comparison to no PER trials*

	Color onset: W1		Preparatory period: W2		Odor onset: W3		Reward omission: W4	
	mean Rate	*Slope*	*mean Rate*	*Slope*	*mean Rate*	*Slope*	*mean Rate*	*Slope*
CS+ Color	↑	different	ns.	different	ns.	ns.	ns.	ns.
CS- Color	ns.	different	ns.	different	↓	ns.	ns.	ns.
CS+ Odor	-	-	-	-	ns.	ns.	↑	ns.
CS- Odor	-	-	-	-	↓	ns.	↓	ns.
Trained CS+ Compound	ns.	ns	ns.	ns.	↑	ns.	↑	ns.
Reversed CS+ Compound	ns.	ns.	ns	ns.	ns.	ns.	↑	ns.
Trained CS- Compound	ns.	ns.	ns.	ns.	↓	ns.	ns.	ns.
Reversed CS- Compound	ns.	ns.	ns.	ns.	ns.	different	ns.	ns.

↑ *significant rate increase*
↓ *significant rate decrease*
different *significant interaction*
ns. *not significant*

Condition: CS+ Color

The CS+ color response (W1) was significantly enhanced in PER trials in comparison with no PER trials [interaction effect: $F(10, 277) = 4.728$, $p < .000$; between-subject effect: $F(1, 286) = 7.367$, $p = .006$. (no PER: mean = 0.9, se = .17, PER: mean = 2.4, se = .05). During

the preparatory period (W2) the interaction effect between time x group was significant [$F(30, 257) = 1.665$, $p = .019$]. The groups did not differ significantly in their mean rate. During the time windows of odor response and reward omission response (W3 & W4), there were no rate differences depending of the behavior of the animals.

Condition: CS- Color

During the color response window (W1) [$F(10, 274) = 2.41$, $p = .009$] and preparatory period (W2) [$F(30, 254) = 2.542$, $p < .000$] significant interaction effects were evident Both between- subject effects were not significant. Around the time when the odor was presented during training (W3), responses to the CS- color were significantly inhibited in the PER group (mean: -0.9, se = .5) in comparison to the no PER group (mean = 0.8, se = .5), although no stimulus was presented at all [between-subject effect: $F(1, 283) = 10.295$]. No significant differences were observed around the omission of the reward (*W4*).

Condition: CS+ odor

The rate was significantly increased around the time of reward omission (*W4*) for the PER group (mean = 3.8, se = .6) in comparison to the no PER group (mean = 0.7, se = .4) [between-subject effect: $F(1, 294) = 17.89$, $p < .000$]. No differences were observed for the odor response.

Condition: CS- odor

Both, odor responses and responses around reward omission were reduced for the PER group in comparison to the no PER group. [between-subject effect, odor response: $F(1, 273) = 9.373$, $p = .002$; between-subject effect, reward omission response: $F(1, 273) = 5.382$, $p = .021$]. Mean odor responses: PER group: 1.2 (se = .3); no PER group: 2.5 (se = .2). Mean reward omission responses: PER group: 0.5 (se = .3); no PER group: 1.3 (se = .2).

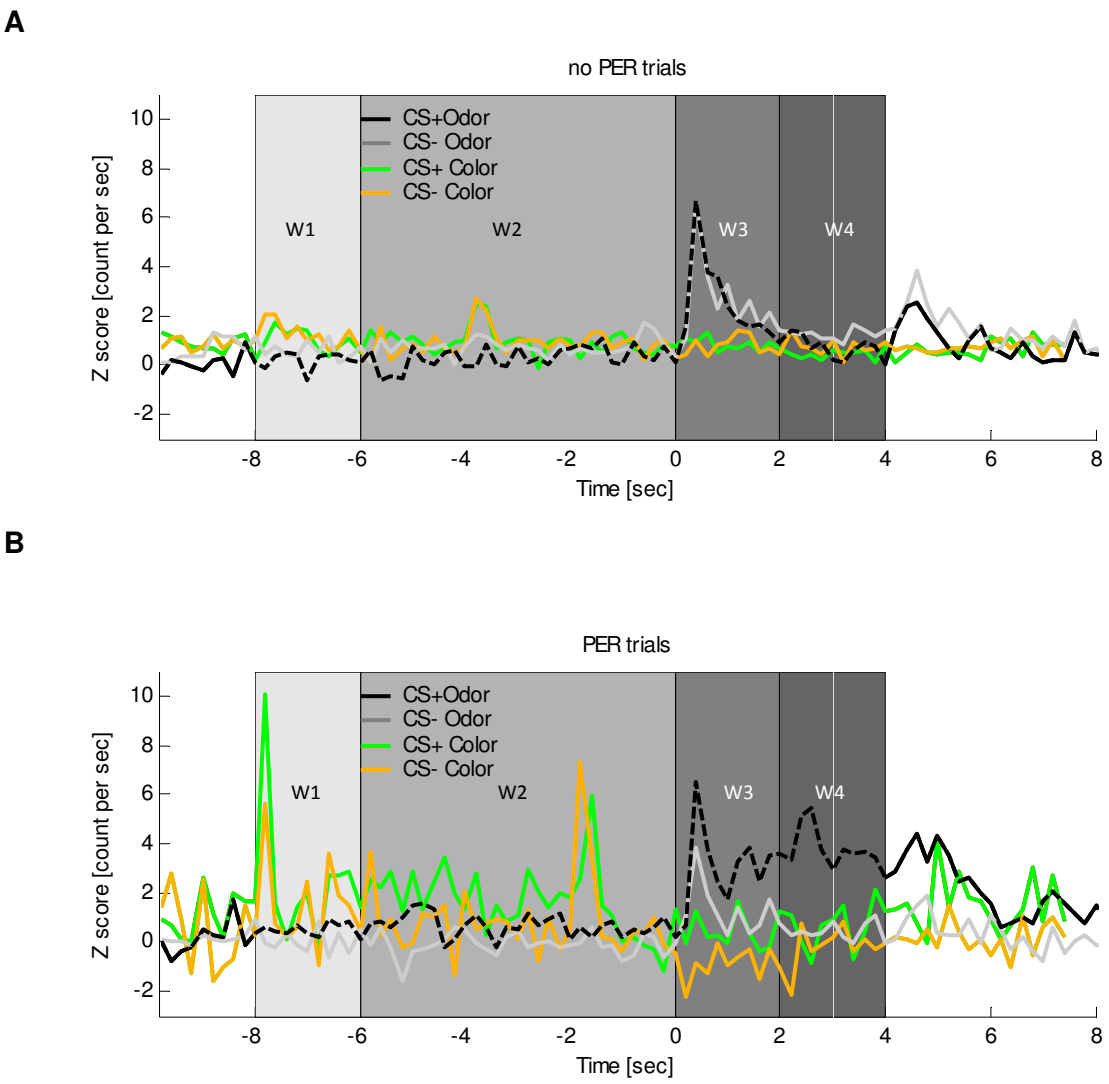

Figure 13. Rate differences between odor and color conditions increased during PER trials. ***A*** Normalized rates are similar for the CS+ odor (black line) and CS- odor (grey line) and also for the CS+ color (green line) and the CS- color (orange line) in all time windows (W1-W4). Differences between these conditions emerged during PER trials (***B***): The CS+ color response was enhanced during PER trials opposed to no PER trials and the slopes of both CS+ and CS- color are different during color response (W1) and preparatory period (W2) during both trials types. Additionally, the rate response to the CS- color during odor omission (W3) was significantly reduced during PER trials opposed to no PER trials. The CS- odor response was reduced in PER trials in comparison to no PER trials, while the rate response during reward omission (W4) was reduced for the CS- odor and enhanced for

the CS+ odor during PER trials. White vertical line indicates reward application during training. Note, that no reward was applied during test trials.

Condition: Trained CS+ Compound

During W1 and W2 (color and preparatory responses) there was no significant difference between the mean firing rate. The odor response (W3) was enhanced during PER trials (mean: 4.6, se: 0.5) in comparison with no PER trials (mean: 3.1, se: 0.4) [between-subject effect: $F(1, 300) = 6.096$, $p = .014$]. The rate response was also enhanced during reward omission (W4) during PER trails (mean: 4.7, se: 0.6) as opposed to no PER trials (mean: 1.9, se: 0.5) [between-subject effect: $F(1, 300) = 13.379$, $p < .000$].

Condition: Reversed CS+ Compound

There were no significant differences between PER and no PER trials in the first three time windows (W1-W3). However, during reward omission, the rate was significantly enhanced during PER trials (mean: 3, se: 0.6) in comparison with no PER trials (mean: 1.5, se: 0.3) [between-subject effect: $F(1, 286) = 5.149$, $p = .024$].

Condition: Trained CS- Compound

During the first two and the last time windows (W1, W2, W4) the mean firing rate during PER trials was the same as in no PER trials. But the mean odor response was reduced during PER trials (mean: 0.5, se: 0.8) as opposed to no PER trials (mean: 2.2, se: 0.2).

Condition: Reversed CS- Compound

Also for the reversed CS- compound, the first two and the last time window did not differ in their mean rate response for the two groups. But here, the time x group interaction was significant during the odor response (W3) [$F(10, 280) = 2.018$, $p = .032$], meaning that the slope of the firing rates during PER trials was different than during no PER trials. Although the between-subject effect did not yield significance, there was a trend for reduced odor responses during PER trials (mean: 1.5, se: 0.6) as opposed to no PER trials (mean: 2.5, se: 0.2).

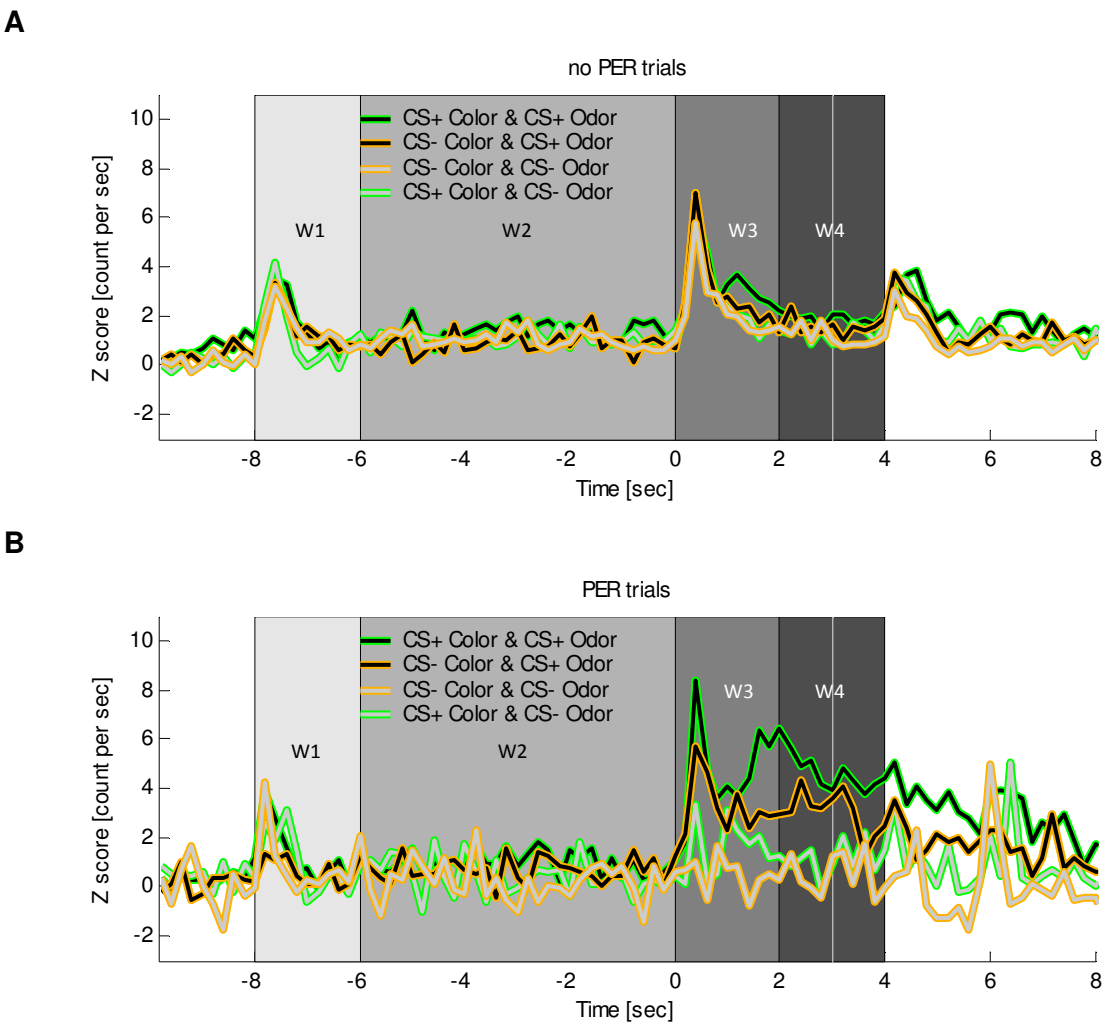

Figure 14. Rate differences between compound conditions increased during PER trials. ***A*** Normalized rates are similar for the trained CS+compound (green & black line), the reversed CS+compound (orange & black line), the trained CS-compound (orange & grey line), and the reversed CS-compound (green & grey line) in all time windows (W1-W4). Differences between these conditions emerged during PER trials (***B***): The CS+ odor response (W3) was enhanced during PER trials for the trained CS+compound, and the CS- odor response was reduced for the trained CS-compound during PER trials opposed to no PER trials. There was also a trend, though not significant, for a reduced CS- odor response in the reversed CS-compound condition. Note, that the odor responses in the four compound conditions are graded during PER trials, they are strongest when the CS+ color preceded the CS+ odor and nullified when the CS- color preceded the CS- odor. During

reward omission, responses were enhanced in both CS+ compound conditions (trained and reversed), indicating an enhanced reward expectation after the CS+ odor presentation. Around the sixth second, when the reward was retracted during training, both CS- compounds eleicited a strong off response, although no reward was applied during test trials, while no such off response was observed during the CS+compounds.

Color modulation of odor and reward omission responses during PER trials

In the following we assessed rate differences during PER trials between the different conditions during the odor response and the reward omission response window (W3 & W4), as during these time windows the strongest rate changes between PER trials and no PER trials occured. Figure 15 illistrates the mean normalized rate responses during both time windows for each condition. Both between-subject effects of MANOVA evidenced significant firing rate differences between conditions [Odor responses: $F(7, 70) = 32.735$, $p < .000$; Reward omission responses: $F(7, 70) = 62.142$, $p < .000$].

Pairwise comparison revealed significant different rate responses between both trained compounds opposed to reversed compounds. While the trained CS+compound elicited higher odor responses than the reversed CS+compound ($p = .009$), the trained CS- compound elicited reduced odor responses in comparison with its reversed counterpart ($p = .024$). However, only the trained CS+compound was significantly higher than the odor (CS+) alone ($p < .000$), indicating that the CS+ color was able to enhance the odor response. Additionally, the CS- color response was reduced in comparison with the CS+ color response ($p < .000$) around the time the odor was applied during training, but not during test trials.

During reward omission (W4), the CS- color response was still significantly lower than the CS+ color response ($p = .038$). The CS+ color enhanced both CS+ ($p = .01$) and CS- ($p = .01$) odor responses around reward omission in comparison with the CS+ and the CS- odor alone, respectively. The CS- color effectivey reduced the CS+ odor response ($p = .025$), but did not modulate the CS- odor response. The trained CS+ compound elicited still higher responses than the reversed CS+compound ($p < .000$), and the trained CS- compound yielded reduced responses than its reversed counterpart ($p = .005$).

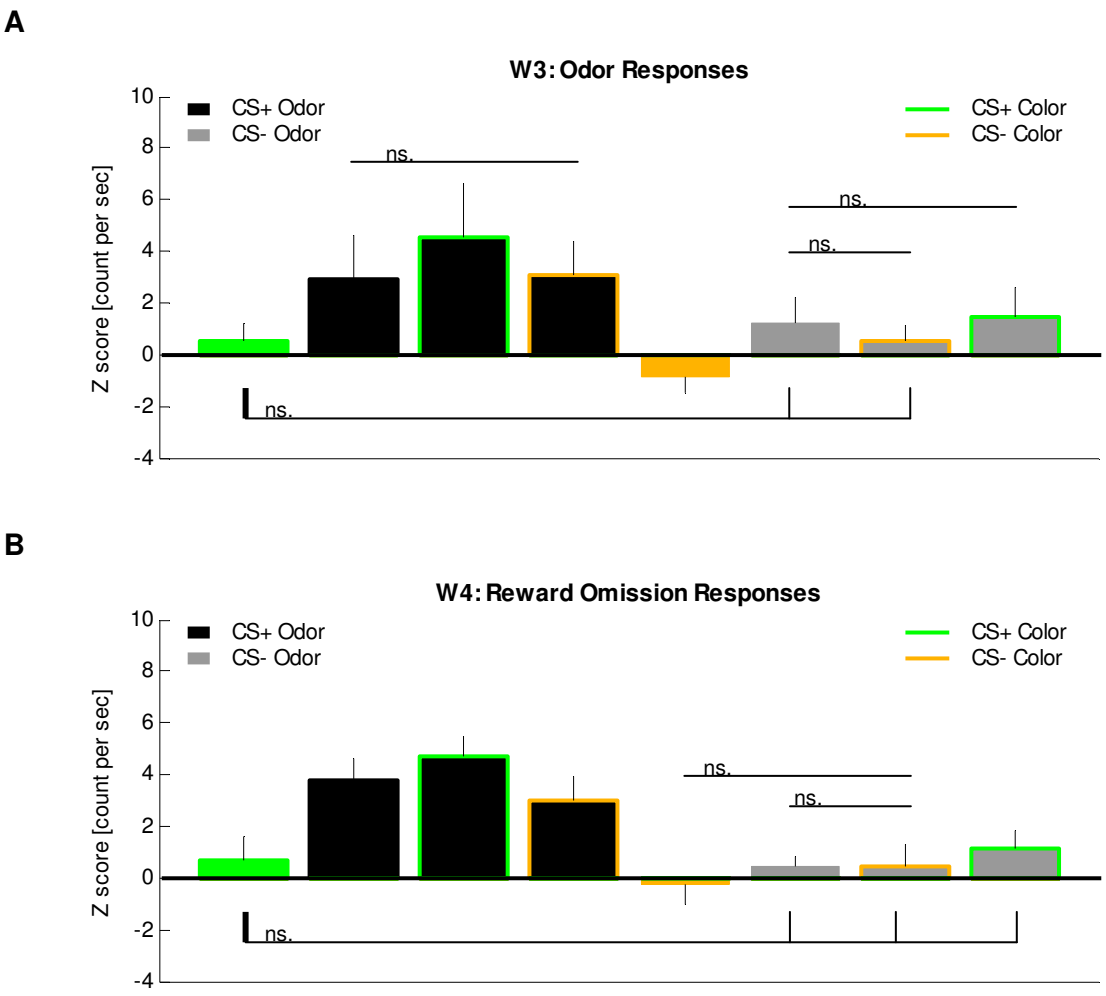

Figure 15. Color modulation of odor and reward omission responses during PER trials. ***A*** Odor responses were enhanced for the trained CS+compound (green & black bar) in comparison with the reversed counterpart (orange & black bar), while the trained CS- compound (orange & grey bar) was reduced opposed to its reversed counterpart (green & grey bar). However, only the CS+ color effectively enhanced the CS+ odor response in comparison with odor presentation alone (black bar). Both CS- compounds were not different from CS- odor presentation alone (grey bar). The CS- color (orange bar) was effectively reduced around odor omission (during color tests, no odor was presented) oppsed to the CS+ color presentation (green bar). ***B*** All rate differences during the odor response window shown in ***A*** outlasted throughout the reward omission window. Additionally, the CS+ color effectively enhanced CS- odor responses during the reversed CS-compound condition (green & grey bar), and the CS- color reduced CS+ odor responses

during the reversed CS+compound (orange & black bar) both in comparison with odor presentation alone.

Subgroup specific effects

So far, we have seen that only the CS+ color enhances CS+ odor responses, when all recorded units were pooled (grand average). The modulation of the CS- odor response was only evident during PER trials. In order to answer the question, whether specific subgroups of units modulate the CS- odor response, independent of the behavior of the animals, we grouped units together based on specific response features.

We found that units (N = 9), that responded significantly to both colors (CS+ and CS- color) during retention tests, modulated the CS- odor response independent from the behavior of the animal, when PER and no PER trials were pooled (see fig. 16 ***A***). During the odor response window (W3), these units exhibited significantly stronger rates to the CS- odor when the CS+ color (green) preceded the odor in comparison to the case the CS- color (orange) preceded the CS- odor (fig. 16 ***A i, iii***). This effect was enhanced when from this population only those units (N = 5) were considered that exhibited the antagonistic rate code to the conditioned colors strongest on day 1 (see chapter 2). Figure 16 ***B*** visualizes this effect. The rate response to the CS- odor is significantly enhanced when the CS+ color preceded odor presentation in comparison to CS- odor presentation alone (fig. 16 ***B iii***). Still, as in 16 ***A***, the CS- odor response is also enhanced when the CS+ color in comparison to the CS- color preceded odor onset. This result indicates, that PCT neurons can operate on different scales. The larger ensemble modulates odor responses primarily during PER trials, while units that exhibited associative plasticity during the same time are able to accomplish additional tasks, namely visual modulation of olfactory responses independent of the behavior of the animal.

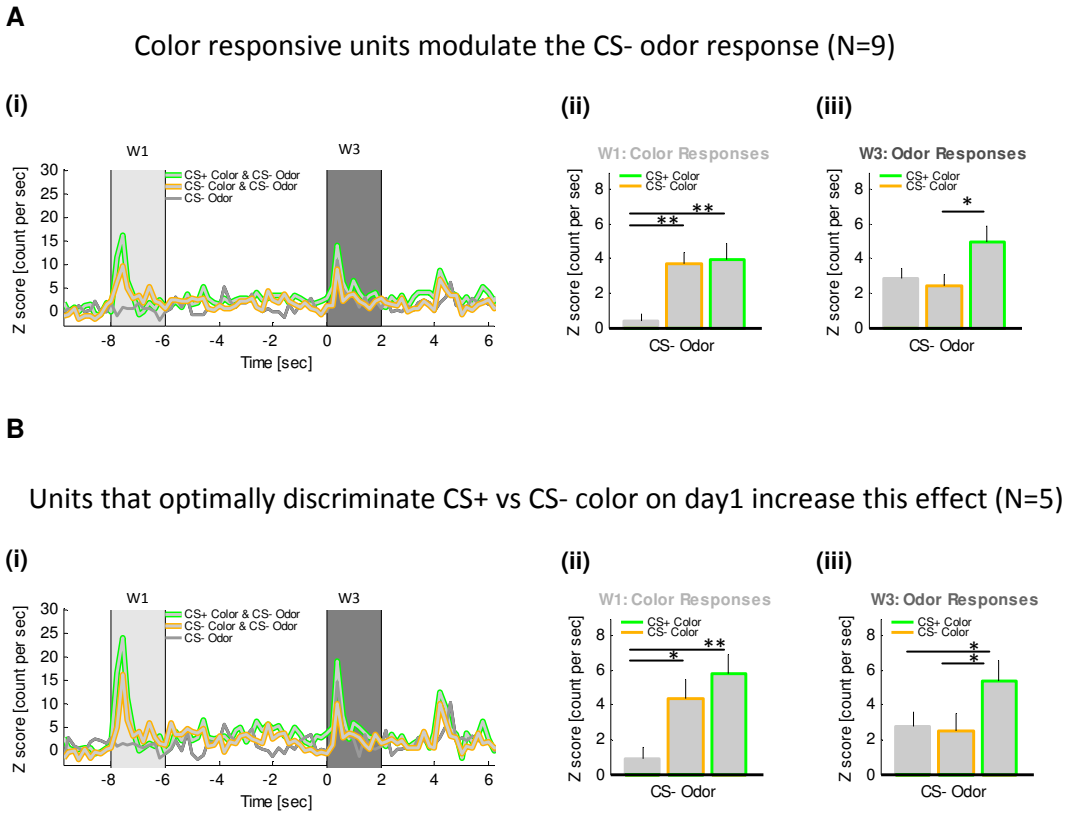

Figure 16. Units with optimal rate change on day1 towards colors modulate the CS- odor response independent of the behavior of the animal. Two subgrups of units were investigated concerning visual modulation of the CS- odor. First, all units that responded significantly to both CS+ and CS- color during retention tests (***A***). The first subplot shows time-resolved normalized firing rates (***i***) of this neuronal ensemble to the trained CS- compound (green), the reversed CS- compound (orange), and the CS- odor alone (grey). The two time windows that were analyzed (W1 – color responses & W3 – odor responses) are marked in grey. Analysis of these time windows are depicted on the right (W1: ***ii*** & W3: ***iii***). The CS- odor response was significntly enhanced when the CS+ in compariosn to the CS- color preceded the odor. ***B*** From the neuronal population above only those units were considered that could optimally discriminate between the colors on day1 (chapter 2). Here, additionally the CS+ color enhances CS- odor responses in comparison to the condition in which the CS- odor was applied alone.

Discussion

Behavioral inter-modal interaction effects

We studied behavioral as well as neuronal inter-modal interaction effects with a combined visual/olfactory compound paradigm first used by Gerber & Smith (1998). Previous studies described the phenomenon of blocking: When one stimulus was pretrained and subsequently conditioned in a compound, with a second stimulus from the same sensory modality, blocking to the second stimulus took place (Kamin, 1968; Smith & Cobey, 1994; Couvillon et al., 1997). However, in the honeybee (*Apis mellifera*) blocking was not found for two stimuli of different modalities (visual and olfactory cues) (Gerber & Smith, 1998). In case of inter-modal preconditioning to a color and subsequent compound conditioning to the color and an odor, Gerber & Smith (1998) found enhanced odor responses, reduced RTs, and longer response durations to the odor.

While the mentioned study manipulated color pretraining to explore its influence on odor retrieval we always conducted differential color pretraining but manipulated the test conditions. In addition to testing the odors and colors alone we also tested the trained and reversed compounds. Assuming inter-modal facilitation in case of color pretraining we addressed the additional question whether the behavior and the neuronal activity to the odor cues was different during tests depending on the acquired value of the color that preceded the odor. We indeed found enhanced response probablities when the CS+ color preceded the CS+ odor during retenion tests in comparison with CS+ odor presentation alone, while Gerber & Smith evidenced enhanced response probabilities to the CS+ odor alone following color preconditiong. We thus conclude that the response probability to the CS+ odor was further facilitated when the CS+ odor is presented with the CS+ color together during the test. Visual modulation of an olfactory stimulus has also been described using an occasion setting paradigm (Mota et al., 2011). The authors observed higher behavioral responses to an odor when this odor was preceded by a color that set the occasion for an upcoming reward in comparsion to another color that did not set the occasion for a reward. The bees were able to disambiguate the meaning of the same odor based on the preceding color. Both cited studies failed to find behavioral responses to the colors alone, as has been

reported before (Hori et al., 2006, Niggebrügge et al., 2009) in harnessed but intact honeybees. The present study also did not observe PER`s to color cues only. Nevertheless, the finding that visual stimuli do not elicit behavioural responses does not exclude the possibility that animals do perceive and use visual stimuli to focus their attention and to modulate odor responses based on the visual context. The cited studies and the present work stress this hypothesis.

We further found reduced behavioral RTs to the CS+ odor when the CS+ color preceded the odor in comparison with CS+ odor presentation alone in all comparable trials. As RTs were already shown to be reduced to the CS+ odor after color pretraining and subsequent compound training (Gerber & Smith, 1998) we could evidence further reduction of RTs when the CS+ color and odor were presented together during retention tests. This finding is a further evidence that bees learn the color cues and use them to initiate fast behavioral responses.

It is known that there is always a trade-off between the speed of reaction and the accuracy of the behavioral choice (Wickelgren, 1977), and that RTs are slowed down in an ambigious context (Hick, 1952; Vickers, 1970). Manipulating the difficulty to discriminate between oderants, it has been shown that olfactory discrimination in mice is high, independent of task difficulty, but that RTs increased by ~80 ms from the easiest to the most difficult condition (Abraham et al., 2004), indicating speed-accuracy tradeoff on a timescale of less than 100 ms. In studies using human subjects, the range of RTs ranged between tens of milliseconds to around one second in simple perceptual tasks (Luce, 1986; Usher & McClelland, 2001). In the present study we found reduced RTs of ~640 ms when bees anticipated the odor due to the preceding color presentation compared to the situation when the odor was not announced before. The strong reduction in RT underlines the importance of color cues for bees that might help to focus the attention and improve orientation toward the scent of flowers during foraging flights.

The current results stress context dependence in honeybees: An olfactory cue is perceived differently depending on the context. Also cockroaches are able to differentiate stimuli depending on the light context (Matsumoto & Mizunami, 2004). In contrast the fruitfly *Drosophila melanogaster* has been shown to rather generalize between visual context stimuli and that context generalization is processed in the MB (Liu et al., 1999). The ability

to discriminate between visual cues might thus depend on the biological significance for an organism.

The neural basis of cross-modal interaction effects

We aimed to elucidate the neuronal correlate of cross-modal interaction by recording from multiple neurons of the protocerebral calycal tract, PCT, throughout the experimental procedure. PCT neurons provide both local as well as recurrent inhibitory feedback in a prominent structure of the insect brain, the mushroom body (MB), that is known to be critical for learning, memory formation, and multisensory integration (Rybak & Menzel, 1993, Grünewald, 1999a, Okada et al., 2007). PCT neurons have been shown to be multimodal (Homberg & Erber, 1979; Gronenberg, 1987; Schildberger, 1981) and are additionally involved in olfactory associative learning in the minutes to hours range (intracellular recordings: Grünwald, 1999b; calcium imaging: Hähnel & Menzel, 2011). They were thus promising candidates to study cross-modal interaction and learning effects.

The reinforced color modulates response latencies and response rates in a subset of PCT neurons

During retention tests, we found reduced neuronal response latencies and increased rates in a subset of neurons to the CS+ odor when the CS+ color preceded the odor, mirroring the reduced RTs of behavioral PER`s. Response latencies in the same neurons were increased and response rates decreased when the CS- color or no color preceded the CS+ odor. Before and during reward omission the neurons exhibited ramp-like graded rates with strongest rates in case the CS+ color preceded the CS+ odor, intermediate rates, in case no color preceded the CS+ odor and low rates when the CS- color preceded the CS+ odor.

RTs have been studied in the framework of time integration in perception, memory and cognitive processes (Donders, 1969; Meyer et al., 1988). On the neuronal level, the accumulation of infomation in favor or against one choice of behavioral output has been intensively explored, as for example in prefrontal cortex and parietal neurons (Goldman-Rakic, 1995; Roitman & Shadlen, 2002).

In a combined visual discrimination reaction time task, in which monkeys had to report the direction of random dot motion by making a saccade to one of two peripheral choice targets, neurons from the lateral interparietal cortex (area LIP) exhibited ramp-like changes in their firing rate that predicted the decision of the animal (Roitman & Shadlen, 2002). The stronger (and thus clearer) the motion of the dots was, the shorter were neuronal response latencies and the steeper the rise in spike rate. LIP neurons are thus thought to integrate and accumulate evidence for or against a behavioral output.

In the study at hand, task difficulty was operationalized by testing the trained versus the reversed compounds. The latter resemble conflicting cues, in which a decision for or against a categorical behavioral output is more difficult and ambigious. While the CS+ color might set the animal into a preparatory mode in which the attention is focused and the behavior is planned, and the CS+ odor (trained CS+ compound) further strengthens this expectation for an upcoming reward, the CS- odor (reversed CS+ compound) acts conversely as a contradictory cue that might either stop or decelerate motor output.

In line, we found that the behavioral as well as the neuronal response of a subset of PCT neurons that exhibited already enhanced firing during the preparatory phase additionally developed a faster and a much stronger CS+ odor population response. The ramp-like change in firing rate around reward omission in PCT neurons, comparable to LIP neurons, might reflect the accumulation of evidence in the bee in favour of one specific behavior over another, and thus the level of reward expectation as the rate was highest for the condition in which the animal was already primed to expect a reward.

Attention-related preparatory signals have been found in neurons of the anterior cingulate cortex (ACC) in rats after a visual priming signal. Firing rates after the visual prime were highest before a correct choice was accomplished, and the high firing rate was associated with levels of attention (Totah et al. 2009). PCT neurons exhibited the strong preparatory signal exclusively after the CS+ color onset that might enhance the bees attention while the same neurons exhibited no preparatory signal after the CS- color onset. We thus conclude that the increased preparatory signal in a subset of PCT neurons reflects enhanced attentional levels elicited by the CS+ color that enables the animal to react faster to the following odor cue. Thus the underlying neuronal mechanism of RTs is in part governed by PCT neurons that are modulated in response latencies and response strength towards the

odor and during the expectation of a reward based on the learned value of the preceding color.

Rate difference between conditions are increased before behavioral perfomance

In order to compare all eight test conditions (CS+ and CS- colors and odors, CS+ and CS- trained and reversed compounds), the grand average of all recorded neurons was calculated for each condition. Differences were only apparent during the trained CS+ compound, that elicited stronger odor and reward omission responses than all other conditions with the exception of the reversed CS+ compound. Thus, also the CS- color enhanced the CS+ odor response (reversed CS+ compound), but this modulation was weaker than the modulation during the CS+ compound.

However, when the relation between behavior and firing rate was explored, highly pronounced rate differences between the conditions became evident, only in trials in which a motor response was made (PER trials), while rate responses to the different conditions were very similar during no PER trials.

Specifically, in PER trials in comparison with no PER trials, the CS- odor response was decreased, when the CS- odor alone or together with the CS- color (CS- compound) were tested. The rate during reward expectation was reduced testing the CS- odor alone.

Further, during PER trials in comparison to no PER trials, the rate to the CS+ odor and during subsequent reward expectation were enhanced testing the trained CS+ compound condition (CS+ color & CS+ odor), while testing the CS+ odor alone evoked higher rate responses during reward expectation, but not following odor onset.

Additionally, testing the CS- color alone, a prominent inhibition was observed during PER trials only at the time the CS- odor onset was expected. Thus, the presentation of the CS- color alone was sufficient to trigger a value-related memory of the expected CS- odor (see below). In contrast, testing the CS+ color alone, the response to color onset was enhanced in PER trials opposed to no PER trials. Testing the reversed compounds, rates during reward expectation were enhanced when the CS+ odor followed CS- color onset (reversed CS+ compound). The slope, but not the mean rate was different during CS- odor onset when the CS- odor followed CS+ color onset.

These rate changes during PER trials result in neuronal representations of the odor stimuli that perfectly matched the integrated sum of learned visual and olfactory stimulus values together (see below).

Different neuronal representation and visual modulation of olfactory responses of the whole neuronal PCT ensemble thus only occured upon stimulus selection, which implies the necessity of attention. According to the 'premotor theory of attention' (Rizzolatti, 1987; Allport, 1989), the relevant features of a specific stimulus, that is selected for a behavioral response, automatically receive attention. This theory is supported by eye movement experiments, in which attention is always directed to stimuli that are selected as targets for an eye movement (Hoffman & Subramaniam, 1995; Kowler et al., 1995; Deubel & Schneider, 1996).
We thus assume, that attentional levels are enhanced during or before the preparation of a motor response. In this line, attentional feedback signals from the PCT neurons might provide the link between sensory and motor areas, which is responsible for action selection. Such amplified representations of feature values have been observed in neurons of the primary visual cortex (V1), but also in higher cortical regions, like area LIP (Freedman & Assad 2006), and the prefrontal cortex (Freedman et al., 2001) during tests following perceptual learning. They are regarded as particularily useful, as small changes in the input can lead to categorically different behavioral responses (Oristaglio et al., 2006; Mirabella et al., 2007). Stimulus-value associations are regarded to induce plasticity particularly in higher visual areas (Law & Gold, 2008; Freedman & Assad, 2003; Li et al., 2009).

Thus, rate differences between PER and no PER trials evidence that PCT activity does not reflect mere sensory activity as the sensory input is the same during PER and no PER trials. Rather, in trials when a movement is planned the cues are evaluated on its acquired value for response selection, and the neuronal correlate of this value and the derived reward expectation is modulated by the preceding colors. The bees thus integrate both visual and olfactory sensory cues as indications for or against the possibility of receiving a reward. Reward expectations control goal-directed behavior, decision making and planning. Honeybees were shown to learn the sign and the magnitude of reward both during foraging

flight and under constraint laboratory conditions (Gil, 2010). In primates, reward expectations have been shown to be mediated by dopamine neurons of the prefrontal cortex and basal ganglia (Schultz, 1998, Watanabe, 1996). The observed reward modulation signals in PCT neurons may comparably reflect reward anticipation consisting of retrieving, retaining and anticipating the motivational value associated with visual, gustatory and olfactory images.

Neuronal error signals after incorrect performance

In vertebrates, cortical areas involved in response selection feed back to sensory areas, increasing the representation for objects relevant to behavior (Felleman & Van Essen, 1991; Desimone & Duncan, 1995). In this line, signalling an erroneous execution of a motor response is important in order to inform the sensory neurons about the conflict, the erroneous output. In this study, PERs following the presentation of the CS- odor were regarded as erroneous. We observed a phasic excitatory error signal during PER trials (see fig. 14 ***B***) testing both trained and reversed CS- compounds during the time the reward was terminated in CS+ training trials (6 sec after odor onset). We did not observe these error signals when the CS- odor or the CS- color were tested alone. The fact that the error signal also occured in the reversed CS- compound (CS+ color & CS- odor), suggests, that the last cue is taken to compare stimulus value with the selected action.

A general role in conflict monitoring has been ascribed to the anterior cingulate cortex (ACC) (for a review see Botvinick et al., 2004). Neuronal error signals in neurons of the ACC in rats were found following incorrect choices (Totah et al., 2009). These error signals occured in the same neurons that mediated attentional levels during the preparatory period (see above). Comparable to Totah`s results, the observed error signals in this study could be utilized by the neuronal network for conflict monitoring or serve as a reinforcement learning signal (Nieuwenhuis et al., 2004).

Memory traces for omitted cues during PER trials

What happens when a color cue is tested alone, without the subsequent presentation of the odor? Do PCT neurons only retrieve the value of a presented stimulus, or do they also retrieve the value of an expected but absent stimulus based on the trained temporal

relationship between color and odor cues? Indeed, PCT neurons exhibited a pronounced inhibition, during the time the CS- odor was expected after the onset of the CS- color alone. Thus, a neuronal representation of a stimulus value emerges upon its presentation as well as upon the expectation of this stimulus. The inhibition was not evident during trials, in which no PER was initiated. When tested alone, the CS+ color did not elicit a neuronal excitation at the time window when the CS+ odor was expected. Since on CS+ trials a strong response was already observed upon color onset, this signal might be sufficient for announcing the high value of the CS+ color. However, it remains unclear why only the CS- color triggers a value-related memory of the CS- odor and the CS+ color did not trigger a comparable value-related memory of the CS+ odor. Additionally, in comparison to the CS+ color the inhibition due to the CS- color lasted throughout reward omission, again only during PER trials. The CS- color thus not only triggers the memory for the CS- odor but further triggers the appropriate reduced reward expectation.

Comparison of the test conditions during PER trials only

Visual modulation of olfactory responses during PER trials

We further observed that both trained compounds elicited different odor responses – an enhanced odor response to the CS+ odor following the CS+ color and a reduced CS- odor response folowing the CS- color – but only the CS+ color gained the power to increase the CS+ odor response in comparison with the odor response during CS+ odor presentation alone. However, during reward omission, the modulation effect of the colors was present at a broader range: Here, the CS+ color enhanced odor responses in both conditions, the trained CS+ compound (CS+ color & CS+ odor) and the reversed CS- compound (CS+ color and CS- odor) in comparison with CS+ and CS- odor presentation alone. Also, the CS- color reduced responses to the CS+ odor around reward omission in comparison to CS+ odor presentation alone. However, the CS- color did not further reduce the CS- response when compared with CS- odor presentation alone.

Enhanced odor responses in the anterior hippocampus and rostromedial orbitofrontal cortex (OFC) as well as reduced reaction times have been observed when visual congruent cues preceded odor presentation (Gottfried & Dolan, 2003), indicating bimodal response

enhancement as a general feature of multisensory neurons (Stein & Meredith, 1993). Gottfried & Dolan additionally observed response decrements in bimodal incongruent patterns, comparable to our results.

Sensori-motor control in other MB ENs

Okada et al. (1999) recorded from MB output neurons in the cockroach whilst viusal, mechanosensory (tactile and air current) and olfactory stimuli were applied. Subgroups of neurons exhibited sensory-motor related activity. Some neurons were selective to the directions of turning behavior. The authors concluded that MB output neurons might integrate external sensory signals and internal motor-reporting signals to possibly enable the appropriate motor execution (Mizunami et al., 1998). Evidence in favor of this hypothesis arises from *Drosophila*. Martin et al. (1999) were able to show that the MB plays an important role in the termination of active walking phases.

We could now show that GABA-ir MB output neurons in the honeybee brain represent the learned value of single visual and olfactory stimuli, and additionally represent the value of a compound stimulus by the perfect integration of the sum of both stimulus values. As distinct value representations of the whole recorded neuron population only occured before an action was initiated (a PER), we stress the conclusion from Okada and coworkers that MB output neurons might represent a relay between sensory and motor areas, guiding the selection and initiation of motor patterns.

Subgroup-specific modulation effects

So far, we discussed the modulation effects during PER and no PER trials of the whole recorded neuronal ensemble. We could show that visual modulation of olfactory responses was primarily evident during PER trials, upon stimulus selection. Only the modulation of the CS+ odor by the CS+ color was evident independent of the behavior of the animal. We additionally investigated whether subgroups of neurons also exhibited modulation of the CS- odor, independent of the behavior. Indeed, CS- odor responses were enhanced by the CS+ color in comparison to CS- odor responses when the CS- color preceded odor onset in neurons that responded to both CS+ and CS- color during retention tests. From this neuronal population we analyzed separately units that optimally discriminated between the

conditioned colors (based on the analysis of the second chapter) during test on day1. Modulation effects were enhanced in this specific population. The CS- odor response was enhanced when the odor was preceded by the CS+ color not only in comparison with the trained CS- compound (CS- color & CS- odor), but also opposed to the condition when the CS- odor was presented alone. This result indicates, that PCT neurons can operate on different scales. The larger ensemble modulates odor responses primarily during PER trials, while units that exhibited associative plasticity during the same time are able to accomplish additional tasks, namely visual modulation of olfactory responses independent of the behavior of the animal.

Functional implications for the local and recurrent feedback loop

The PCT neurons receive multimodal input from presynaptic KCs which terminate in specific bands of the anterior-dorsal alpha-lobe. They presumably provide local feedback onto other MB ENs, e.g. onto the morphologically identified PE1 neurons, which is known to decrease its rate to a trained odor after learning and might hence receive learning related inhibition originating from the PCT (Okada et al., 2007). PCT neurons also provide recurrent feedback from the MB output to all input structures of the MB calyx, predominantly connecting the same sensory modality (Schäfer and Bicker, 1986; Grünewald, 1999a). Since most PCT neurons are GABA-ir they appear to provide selective inhibitory input to the calyces (Bicker et al., 1985; Schäfer and Bicker, 1986). In the calyx itself electron microscopy revealed GABA-ir profiles evidencing reciprocal synaptic contacts with PNs und monosynaptic contacts with KCs (Ganeshina & Menzel, 2001). As PNs are presynaptic to KCs and postsynaptic to PCT neurons their output will be indirectly mediated via feedforward inhibition by these GABA-ir neurons.

Link to motor areas via local inhibitory feedback

What kind of functional implications do the observed results imply for the local as well as for the recurrent feddback loop? Dendritic connections from the PCT to the PE1 neuron has been proposed (Okada et al., 2007), while the PE1 itself projects to the lateral horn (LH), where it synapses directly or via inhibitory interneurons onto decsending motor output neurons. Besides input from the MB, the LH activity is also mediated by olfactory

projection neurons (PN) originating in the antennal lobe (AL), the first neuronal processing stage in the honeybee brain. There are thus two independent pathways that converge in the LH onto descending neurons (Abel et al., 2001; Kirschner et al., 2006). While the AL might provide experience-indepent information about basal stimulus features the pathway via the MB might inform the LH about learned values of stimulus combinations. Comparable to the mammalian cortex the MB provides an overall inhibition onto behavior as ablating the MB have been shown to cause spontaneous uncontrolled motor output (in crickets: Huber, 1978, 1990; in *Drosophila*: Martin et al., 1998). PCT neurons might thus provide more inhibition to the PE1 neuron the more valuable a set of stimuli has been evaluated and consequently, decreased input from the PE1 to the LH would attenuate the level of inhibition that LH iterneurons provide for motor neurons, making a behavioral output more likely.

While integrating values of different context cues, here in form of colors, PCT neurons would therefore accumulate evidence for or against a behavioral output, thereby reflecting neuronal correlates that finally lead to a decision of the animal.

Link to sensory areas via recurrent inhibitory feedback

While a behavioral output might be more likely when PCT neurons signal a high stimulus value through increased firing rates - via the loacal feedback loop - the recurrent feedback might inform sensory neurons (PNs) which stimuli are relevant for behavior so that these relevant stimuli might be represented stronger in the MB input than irrelevant stimuli. Convergence of the reward encoding neuron VUMmx1 (Hammer & Menzel, 1998) with KCs suggests that the PN-KC synapse undergoes associative plasticity, which was found in calcium imaging experiments in dendrites of KCs (Szyszka et al., 2008).

During training, upon the action of octopamin that signals the reward, the recurrent feedback singnal back to the calyx could hypothetically potentiate active PN-KC synapses, thereby increasing the probability that the same choice to that particular stimulus will be executed in the future. In this line, learning would occur in the PN-KC synapses in the calyces and PCT neurons would regulate synaptic plasticity.

Since PNs are presynaptic to KCs and postsynaptic to PCT neurons their output will be indirectly mediated via feedforward inhibition by inhibitory PCT neurons. Feedforward

inhibition short after excitation has been shown to substantially increase the temporal precision of firing (Buzsáki, 2006). In this line, the temporal window of discharge probability of PNs could be narrowed by inhibition from the PCTs and would guarantee precise spike input from PNs onto KCs upon the modulatory action of octopamin released from the VUMmx1 neuron.

After learning, recurrent feedback to the input region of the MB might regulate long-term depression (LTD) and potentiation (LTP) in postsynaptic targets to prevent runaway changes of synaptic weigths, as has been suggested for inhibitory interneurons onto principal cells in the hippocampus (Steele & Mauk, 1999; Gustafsson & Wigström, 1990). Also here, precise spike-timing might induce either LTP, if the PN fires short before the KC, or LTD, if PN spiking occurs before KC discharge. Hebbian short-term dependent plasticity (STDP) has been observed between KCs and MB output neurons in the locust (Cassenaer & Laurent, 2007). STDP could therefore not only induce LTP or LTD between PN-KC synapses in the MB input, but also between KC-PCT synapses at the MB output.

While the whole neuronal ensemble might evaluate jointly stimulus values relevant for behavior, subgroups of PCT neurons establish antagonistc rate codes in the presence of correct behavioral choices (chapter 2). Neurons that established this antagonistic rate code for the conditioned colors on day1 were now shown to modulate CS- odor responses based on the visual context when both PER trials and no PER trials were pooled together. This indicates parallel processing operations. In this way, learned stimulus values independent of the behavior as well as signals evaluating the relevance for a behavioral response might be fed back to the input of the MB.

General conclusion

Honeybees react faster to a learned odor when odor presentation is announced by a learned color. Enhanced attentional levels after color onset might account for reduced reaction times. A subset of PCT neurons expressed enhanced rates during attention and reduced response latencies to the odor, and might thus reflect one possible neural basis for the behavioral findings. Upon attention, PCT's firing rate towards the odors perfectly match the integrated sum of the acquired values of a visual and an olfactory stimulus. Via local and recurrent feedback, they might inform motor (the MB output) and sensory areas (the MB input) about the relevance of stimulus combinations for behavior. Additionally,

subgroups of neurons that established the antagonistic rate code convey feedback signals of the actual learned stimulus values indepedent of the current behavioral response.

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4. Time-frequency estimation and phase locking during learning in GABAir feedback neurons of the honeybee (*Apis mellifera*)

Abstract

The insect mushroom bodies (MBs) are higher-order brain centers critical for multisensory integration, learning, and memory formation. We aimed to study combined visual and olfactory learning and memory formation in GABAergic feedback neurons at the MB output in honeybees (*Apis mellifera*). To this end we combined extracellular recordings with a visual and olfactory conditioning paradigm first used by Gerber & Smith (1998), and analyzed both, the oscillatory local field potential (LFP) and spike times of single units during learning and tests. We found increases in the 1-25 Hz frequency band after color onset during training in both rewarded and unrewarded trials, that were most pronounced before a behavioral response was initiated, suggesting attention-related stimulus selection during learning. Additional spike-locking to the enhanced oscillation was found only during the rewarded training trials in learner bees, which might increase the synaptic strength in KC-PCT synapses at the MB output during learning, and might thus reflect a prerequisite for succesful encoding of stimulus combinations. As also prolonged synchrony between single neurons was observed during the rewarded compound training, oscillatory and single neuron synchronization of MB output neurons might regulate attention-related learning and memory formation.

Introduction

Precise spike timing is one critical feature of successful communication between neuronal assemblies and has been shown to be facilitated by synchronized assemblies of neuronal input groups onto target neurons (Buszáki, 2006, Fries, 2005).

Oscillations of the extracellular local field potential (LFP) generally reflect the presence of synchronized synaptic activity of many neurons surrounding the recording electrode

(Adrian, 1942; Gelperin & Tank, 1990; Steriade, 1996). Stimulus-evoked oscillatory synchronization of neural assemblies have been found in the olfactory (Gelperin & Tank, 1990; Delaney et al., 1994, Laurent & Davidowitz, 1994), and visual system (Gray & Singer, 1989; Neuenschwander & Varela, 1993) of both vertebrates and invertebrates, and are associated with a broad range of neural plasticity and cognitive tasks, like arousal, perceptual integration, and attentional selection (Fries et al., 2001; Patel & Balaban, 2000). Oscillatory synchronization in the gamma range (>30 Hz) increases synaptic gain in postsynaptic target neurons, which lead to enhanced attention and behavioral responses to learned stimuli (Fries et al., 2001; Fries, 2005) and dynamic perceptual processing (Singer, 1993; Engel & Singer, 1993).

During olfactory processing, coherent firing of olfactory projection neurons (PNs) in the insect antennal lobe (AL), an analogue to the vertebrate`s olfactory bulb, was described to cause enhanced 20-35 Hz oscillations in the PN target area, the mushroom body (MB) calyx (Laurent & Davidowitz, 1994). The MB is a central neuropil structure in insects, critical for learning, memory and multisensory information processing (Menzel et al. 1974, Erber et al. 1980; Heisenberg, 1989; DeBelle & Heisenberg, 1994). Synchrony of PNs appears to be goverend by local interneuron inhibiton within the AL and may be related to fine odor discrimination (Stopfer et al., 1997). Synchronization thus appears to separate neuronal representations of similar olfactory inputs, which may be read out on a millisecond timescale by MB intrinsic cells (Kenyon cells, KCs) (Perez-Orive et al., 2002). Learning related power changes in the LFP following olfactory conditiong have been observed in the honeybee AL (50 Hz, Denker, et al., 2010), as well as in the MB of the fruitfly *Drosophila melanogaster* (70-80 Hz, Prieto-Godino & de Polavieja, 2010) suggesting that the LFP signal might be modulated upon adaptive value changes of the odor stimulus.

Concerning visual processing, an enhanced 20-30 Hz signal was observed upon stimulus selection after conditioning of a visual stimulus at the output of the MB in *Drosophila*, and this signal was enhanced with increased stimulus salience without learning (van Swinderen & Greenspan, 2003). Lower frequencies (>10Hz) during visual stimulation were not modulated by salience. Low frequency changes have been associtaed with the optomotor response, and its source was traced back to the optic lobes, however, as the strength of this

signal varies in short-term memory mutants, processing in central areas might be additionally invoved in its regulation (van Swinderen & Flores, 2007).

Attention-related stimulus selection and learning might thus be governed by changes in oscillatory power in the insect`s MB.

However, it is hard to locate the origin of enhanced LFP signals in the insect brain, and observed LFP oscillations might rather reflect a global signal instead of being ascribable to one specific brain region. Therefore, it would be desirable to find different LFP signals in different brain areas in the same insect, with identical recording electrodes. An additional caveat concerning the reliable interpretation of LFP signals is reflected by motor artefacts. Motor signals could intersperse from the periphery and cause enhanced power in some frequency bands. Therefore, it is important to record simultaneously at least from one motor source and compare the LFP signal from the motor area to the LFP signal recorded in central areas.

We low-pass filtered the recorded signal from the same electrodes, from which we derived single-unit spikes following high-pass filtering, in exactly the same way, Denker et al. (2010) received the LFP signal in the AL of the honeybee brain. We aimed to further elucidate oscillatory synchrony at the MB upon attention and learning. To this end, we recorded from multiple Gamma-aminobutyric acid (GABA) immunoreactive (ir) neurons of the protocerebral-calycal tract (PCT) at the ouput of the honeybees' MB. These neurons provide both local feedback within the MB output onto presumably premotor neurons (Okada, 2007), as well as recurrent feedback to the MB input, the calyx, forming functional microcircuits with olfactory PNs and KCs. (Ganeshina & Menzel, 2001). PCT neurons develop associatve plasticity in the minutes, hours, and day range (Grünewald, 1999; Haehnel & Menzel, 2011; chapter 2), are multisensory (Homberg & Erber, 1979; Gronenberg, 1987; Schildberger, 1981) and modulate olfactory processing based on the acquired value of a visual context stimulus (chapter 3). We adopted a combined visual and olfactory paradigm first used by Gerber & Smith (1998), that allowed us to study visual and olfactory attention-related stimulus selection and learning within the same behavioral sequence. The paradigm consisted of differential color preconditioning and subsequent compound training of color and odor cues together. A delay of 8 seconds between color and odor onset allowed us to study attention-related neuronal changes after color onset.

Single color and odor stimuli as well as the trained and the reversed compounds were tested 30 minutes after the last compound training trial.

We analyzed power changes of the LFP, phase locking of PCT neurons and the LFP, as well as cross-correlations among single neuron pairs throughout all phases of the experiment (pretraining, color preconditioning, compound conditioning, retention tests). We found increases in the 1-25 Hz frequency band after color onset during color preconditioning and compound training in both rewarded and unrewarded trials, that were most pronounced before a behavioral response was initiated, suggesting attention-related stimulus selection during learning. Additional spike-locking to the enhanced LFP signal was found only during the rewarded compound training trials in learner bees, which might increase the synaptic gain in KC-PCT synapses at the MB output and might thus reflect a prerequisite for succesful encoding of stimulus combinations. On the single neuron level, cross-correlations appeared after color onset in both compound training trials, while coherent spiking lasted throughout the entire behavioral sequence only during the CS+ compound condition. Oscillatory and single neuron synchronization of PCT neurons might thus regulate attention-related stimulus selection via local feedback within the MB output, and might selectively induce long-term memory storage upon attention.

Material and Methods

Animal treatment

Foraging honeybees (*Apis mellifera carnica*) were caught at the hive entrance, anesthetized on ice, and harnessed in metal tubes, as described by Bittermann et al. (1983). The bees were not fed before the experiment begun, in order to make them hungry and motivated. Only bees showing the proboscis extension response (PER) after touching one antenna with 30% sucrose solution were included in experiments.

Dissection

A small window (1.5 mm^2) was cut into the head capsule between the compound eyes along a saggital plane with the head fixed to the stage with dental wax. The first joints of the antennae were immobilized using low temperature melting n-icosane. The head glands and trachea sacks laying on the surface of the brain were cautiously moved aside until the alpha-lobe could be clearly identified. A silver wire electrode was inserted between the right compound eye and the right lateral ocellus for electromyogram recordings of the M17 muscle that innervates the proboscis and whose activity reflects proboscis movement. The reference electrode was either inserted into the right compound eye or into the median ocellus.

The bee was positioned such that the brains surface was exactly 90° horizontally to the set-up table. Microruby was attached to the tip of the multi wire electrode in order to stain the electrode position inside of the brain and to investigate the stained region by means of confocal microscopy after the experiment. The electrode for neuronal recordings was inserted at the medial-lateral border of the left alpha-lobe, and placed 60 – 250 µm below the anterior surface by means of a micromanipulator attached to the electrode holder. After successful placement of the electrode the window in the head capsule was covered with a drop of silicon (two components of KWIK-SIL Sarasota, FL, USA, mixture 1:1). The silicon prevented the brain from drying-out and additionally fixed the electrode in the brain, ensuring the stability of the electrode position over several days. The experiment

started 30 minutes later when the silicon had bonded and the animal recovered from the dissection.

Behavioural Task

The behavioural paradigm of Gerber & Smith (1998) was adopted and modulated for our purpose to study visual and olfactory memory over a time span of up to three days. The baseline responses of the recorded units towards the stimuli were determined before training sequentially stimulating the animal with three odors and three colors each five times with an inter-stimulus interval (ISI) of one minute. After a brake of 10 minutes color pretraining began. One color was chosen randomly to be rewarded during six training trials while another randomly chosen color was not rewarded. The bee was illuminated for 12 seconds. After eleven seconds the bee was fed for three seconds with a droplet of sugar water attached to the tip of a wooden stick. As a control for the movement and humidity levels, a wooden stick with a droplet of pure water was moved towards the bee in unrewarded trials in order to test whether the units may respond to the water vapour and/or the visual stimuli attached to the movement of the stick.

After another pause of 10 minutes, compound color/odor training began. During the rewarded condition the bee was again illuminated with the previously rewarded color, but this time an odor was additionally applied seven seconds after color onset, for 4 seconds in total. The reward was applied three seconds after odor onset. Thus there was one second of overlap between the three sensory modalities, before color and odor were turned off. During the unrewarded condition the previously unrewarded color was paired with another odor, here no reward was delivered. Both conditions were trained within six trials each. Thirty minutes later the bee was tested for all stimuli seperately and in combination, three times, respectively. Furthermore, color/odor combinations not trained were also tested. Importantly, this experimental procedure, including training and retention tests, was repeated on each consecutive day. Figure 1 illustrates the training precedure.

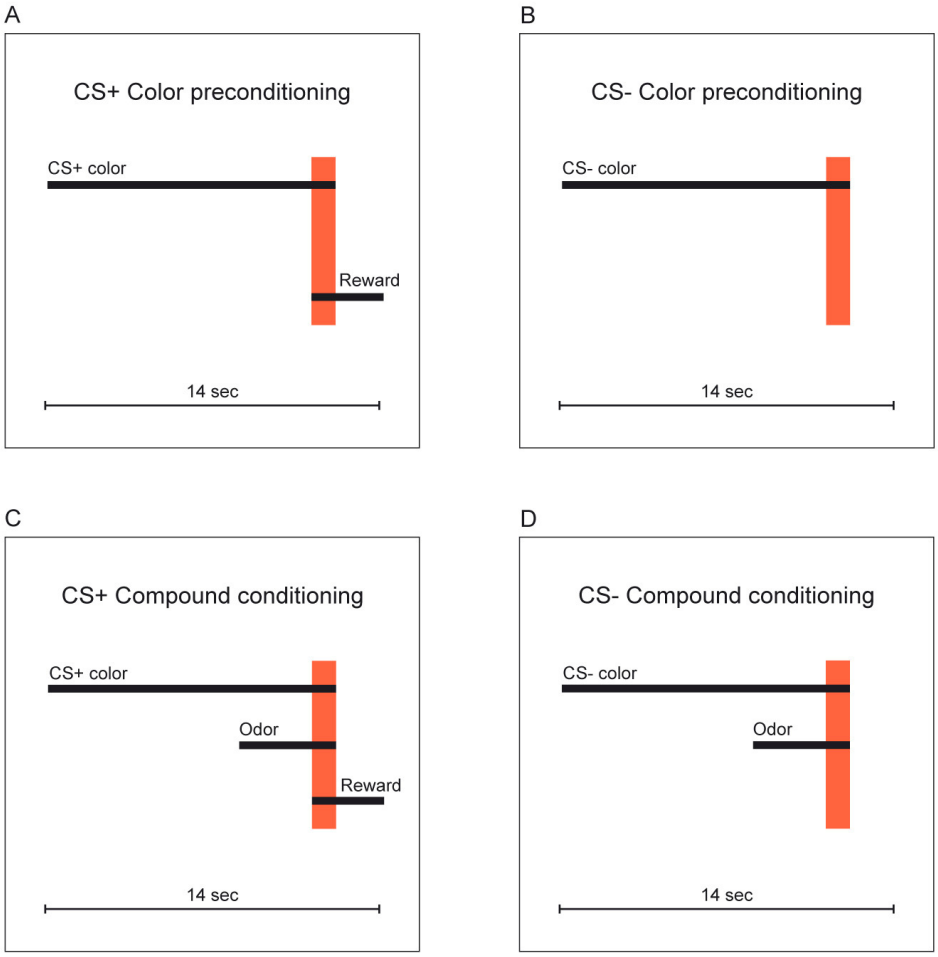


Figure 1. Experimental training procedure. During differential color preconditioning (***A, B***) one color (CS+ color) is combined with a sucrose reward (reward onset: 11 seconds after color onset) while another color (CS- color) is presented without a reward. During differential compound training (***C, D***) the previously rewarded color is trained with an odor (CS+ odor) and a reward, while the CS- color is presented with an odor (CS- odor) without additional application of the sucrose reward. Adapted from Gerber & Smith (1998). During retention tests, color and odor stimuli are tested separately, as well as in combination. Additional to the trained (CS+ and CS-) compounds, the reversed compounds (reversed CS+ compound: CS- color & CS+ odor; reversed CS- compound: CS+ color & CS- odor) are tested.

Electrophysiology

Electrodes

In a first series of experiments, the electrode for neuronal recordings consisted of two, in a second series of three polyurethane-coated copper wires (14 µm in diameter, Electrisola, Escholzmatt, Switzerland) that were glued together with wax and attached to a piece of glass cappillary (~ 2 cm in length) for handling via the micromanipulator. The ends of the wires were deinsulated and attached to the amplifier input connectors by means of conducting silverglue. Resistances of single wires were in the range of 1 and 2 MΩ. Silver wires (0.05 mm in diameter, Advent, Eynsham Oxon England) were used for the reference electrode as well as for the electromyogram recordings of M17 activity.

Amplifiers

Each wire of the electrode was connected to the head stage of a preamplifier (npi electronic). Filters were set to high pass of 10 Hz and low pass of 10 kHz. Hum noise (50 Hz) was eliminated by an additional filter (Hum Bug; Digitimer, Hertfordshire, UK). Neural activity was sampled with a rate of 20 kHz through an analog to digital converter (1401 micro MKII; Cambridge Electronic Design, Cambridge, UK) and initial data analysis was performed by Spike2 software (Cambridge Electronic Design) including signal storage, control of stimulation devices and preanalysis of the data. The amplifier used a band pass filter with cut-off frequencies between 10 Hz and 10 kHz.

Visualization of recording position

After the experiment, the brains were dissected, fixed over night in 4% formaldehyde with 1 µl Lucifer Yellow for enhanced backround staining, washed in PBS, dehydrated in rising concentrations of alcohol (20%, 50, 70%, 99%, and 100 %), and cleared with methylsalicylate. A Leica TCS SP2 confocal laser-scanning microscope (Wetzlar, Germany) was used for scanning with a 20x or 10x water objective. Two excitation wavelengths were applied, 428nm for the backround (Lucifer yellow), and 560 nm, for Microruby, were used.

Odor and color stimululation

Odor stimulation was computer-controlled, using an olfactometer with seperate channels for each odor, described elsewhere (Galizia et al., 1997). In each experiment, three different odors were used to which the bee did not show a spontaneous PER. Two of the odors were used for differential conditioning, the third one as a control odor. The olfactomter was placed in front of the bee such that the end of the outlet had a distance of approximately 5 cm to the bee's head. A constant air stream (1.5 m/s speed) was send through a teflon tube (6 mm in diameter). The control of magnetic valves via the Spike2 software allowed adding a particular odor to the airstream. An exhaustion pipe behind the animal ensured that odor did not accumulate.

Color illumination was provided by a light guide connected to a lamp with filters for green, yellow, and blue light. The exit of the light guide was placed in front of the bee beside the odor pump. Light switches were conducted manually, the precise timing of on- and off-switches were announced by a sound coming from a loud speaker that was connected and controlled by the Spike2 software.

Data Analysis

We applied three different analysis to our data. 1) Power changes in time-frequency spectra of the LFP, 2) Phase locking between multi-unit activity (MUA) and the LFP, and 3) pairwise cross-correlation of single unit activity. The different analysis steps are described in the following.

1) Analysis of time frequency spectra

The analysis of time frequency spectra was conducted by Johanna Derix, Intracranial EEG and brain imaging group, Albert-Ludwigs University, Freiburg.

Low-pass filtering

Signals from two electrodes recording neuronal activity from the MB output and another electrode recording from the M17 muscle (monitoring proboscis activity) were resampled to 1644Hz and low-pass filtered at 500 Hz in order to obtain the mass signal of the LFP, excluding the high frequency component of single-unit spiking activity.

Re-referencing

The LFP of one electrode was re-referenced against the LFP of the second one, yielding a bipolar configuration and thus minimizing the possible presence of artefacts, especially motor-related artefacts.

Calculation of time-frequency spectra

Time-resolved amplitude frequency spectra were calculated for each trial separately, applying the multi-taper method of Percival & Walden (1993), using a time window of 1/3s, time steps of 0.0195s, and three Slepian tapers. For the calculation of relative spectral power changes, the amplitude of each point in time and frequency was divided by the baseline activity. Baseline for each frequency bin was the median power of all trials comprised in one condition in the time interval of 12s to 9s before onset of the first stimulus presentation. For each bipolar electrode, averaged time-frequency spectra were determined by calculating the median power of each time-frequency bin over all trials, yielding one spectrum for each phase (pretraining, training, retention test). For the calculation of the group-averaged time-frequency characteristics, the trial-averaged time-frequency spectra of all bees were again median-averaged for each phase separately. In the time-frequency spectra, frequencies on the y-axis are given in Hz, while the color code depicts the changes in relative spectral power.

2) Analysis of Phase Locking

Analysis of neuronal data were conducted in six different time windows that related to the subsequent stimuli presentations during one behavioral event. One window served as the reference or base before cue onset: W0 (-10 to -8 sec). The other windows were defined as follows: Color onset: W1 (-8 to -6 sec), preparatory period: W2 (-6 to 0 sec), odor onset: W3 (0 to 2 sec), reward onset: W4 (2 to 4 sec), color/odor offset: W5 (4 to 6 sec).

Amplitude and phases of the LFP and spiketimes of multiunit activity (MUA) were extracted per bee for each stimulus condition and for each of the six different time windows. We conducted the analysis for all bees, and separately for learner and non-learner bees. Bees were charcterized as learner bees, if they extended their probosces at least once durnig three subsequent test trials to the CS+ odor in the CS+ compound condition and no

time to the CS- odor in the CS- compound condition. We additionally differentiated between PER and no PER trials. PER`s were monitored using electromyogram recordings of the muscle (M17) that innervates the proboscis. During PER trials the bees extended their probosces to the presented odor either before reward presentation during training or in the time of the entire odor stimulation during tests. During no PER trials no significant M17 responses were detected through the same time periods. For each condition and time window, during training and retention test phase locking was calculated between the MUA spike activity and the frequency band between 10-25 Hz, as we detected a significant increase in power in this frequency band. (see Results). We conducted analysis of non-uniformity testing the null-hypothesis that the neuronal population is uniformly distributed around the circle (the phase, illustrated in 360° using polar plots). We handeled an α-level of 0.05. All significant spike-locking events of the same condition and the same time window were averaged over bees and the mean phases were illustrated (see Results).

Analysis of cross-correlation between single unit pairs

Histograms of single unit activity were constructed again in all time windows and for all conditions during training and retention tests. All possible pairs of units recorded in a single animal were crosscorrelated pairwise. We used a binwith of 4 ms and a max lag of 200 ms. Results of one unit pair was illustrated using histograms that summed the coinciding spikes over time of spike train B in reference to its pair, spiketrain A.

Results

1) Power changes in time-frequency spectra of the LFP

Dissociation from muscular artefacts

Time-frequency spectra of LFP`s were calculated as described in *Methods*. In order to dissociate power changes of the neuronal LFP from muscular artefacts, we derived LFP`s from both the neuronal channels and the channel that comprised activity from the muscle, M17, which innervates the proboscis. Figure 2 outlines time-frequency spectra and the according spike activity of both neuronal and muscular activity of one learner bee during color preconditioning (***A & B***) and compound training (***C & D***). We observed a power increase in the 1-25 Hz frequency band in the neuronal LFP (see fig. 1 ***A-D***) beginning ~5 sec before odor onset, lasting for approximately 13 seconds in both color and compound conditiong and for CS+ and CS- training comparably. The first ~6 seconds of power enhancement occured in the absence of muscular activity. We thus conclude that power changes in this frequency band do not depend on muscular activity.

In the following, we will refer to this increased frequency band as the 'precursor signal', as it appeared before a reward was presented and a conditioned response was eventually elicited.

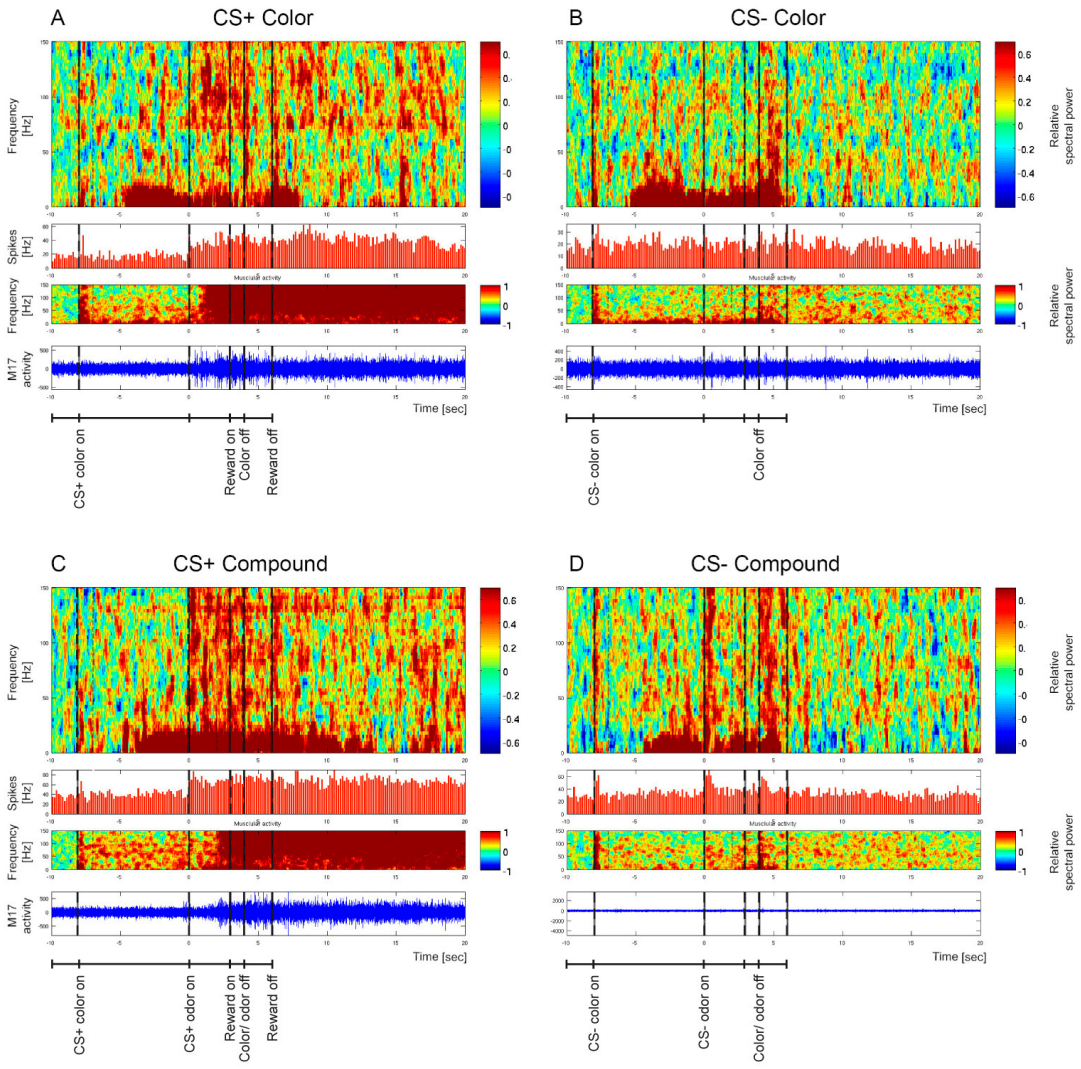


Figure 2. Power changes in LFP can be dissociated from muscular artefacts. Trial-averaged time-frequency spectra of neuronal and muscular activity were calculated for one example bee that succesfully learned stimulus combinations. Spectra of both, color preconditioning (***A***, to the CS+ color and ***B***, to the CS- color) and compound trainings (***C***, to the CS+ compound and ***B***, to the CS- compound) are shown. The upper panel of each subfigure (***A***-***D***) illustrates the relative spectral power of neuronal LFP, multi unit activity of PCT neurons is outlined in the second panel. Relative spectral power of muscular LFP and M17 activity are shown in the bottom two panels of each figure. The x-axis (time [sec]) is alligned to odor onset. Vertical dashed lines indicate behavioral events (color onset (-8s), odor onset (0s), reward application (3s), color/odor offset (4 sec), reward offset (6 sec)).

Strong power changes in the neuronal LFP were observed in the frequncy band from ~ 1-25 Hz in all 4 conditions. The increase in LFP power started already ~ 5 sec before odor onset, in the absence of power changes in muscular LFP.

Spectral characteristics of the precursor signal

Spectral analysis during color training revealed a pronounced precursor signal, beginning ~5 seconds after color onset. In order to analyze its spectral profile, we chose all 6 trials of the CS+ color training condition including all bees to calculate the median spectral power over a time window ranging from color onset (-8s) to oder onset (0s) for each frequency bin separately. Median-averaged power over all bees and trials is displayed as a blue line in fig. 3, the interquartile range (the distance between 25th and the 75th percentile) are indicated by the red tube. A total of 156 trials have been averaged.

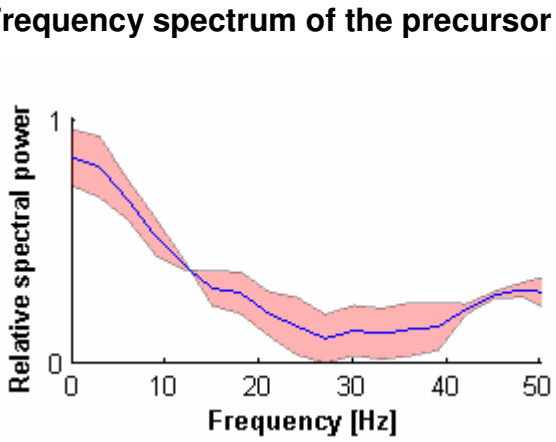


Figure 3. Power spectrum of the precursor signal. The precursor signal, calculated during the CS+ color training, between color onset and odor onset, features power enhancements in the frequency band between 1 and 25 Hz. Median-averaged power over all bees and trials is displayed as a blue line, the interquartile range (the distance between 25th and the 75th percentile) are indicated by the red tube.

In the following, time-frequency spectra averaged over all bees (learner- and non-learner bees) were calculated for pretraining, training and test phases of all conditions (CS+ and

CS- odor and color, trained CS+ and CS- compounds and reversed CS+ and CS- compounds). During pretraining, no compounds were tested.

Figure 4 gives the results for color and compound training trials, while figure 5 outlines time-frequency spectra for pretraining and tests. We found the precursor signal (as an enhamncement in power in the 1-25 Hz freuqncy domain) strongest during CS+ and CS- color training. The precursor signal was rather modest during the compound conditioning. While it stayed at a low level throughout all CS- compound training trials, it gradually increased from the first to the third trial during CS+ compound conditioning manifesting its maximum in the last CS+ compound trials. Additionally to the precursor signal in the 1-25 Hz frequency band, enhanced power in higher frequencies, including the 50 Hz frequency band, was observed only for the training trials that comprise the application of a reward (CS+ color training and CS+ compound training). However, no power changes were detected during pretraining and retention tests of all conditions (see fig. 4). In comparison, in case of the single learner bee (fig.2), the precursor signal was even strong during compound conditioning and during color conditioning, but it attenuated faster in the CS- compound training. Higher frequencies (50 Hz and higher) were enhanced and tightly locked to CS+ and CS- odor onset, but decreased much earlier for the CS- compound conditioning.

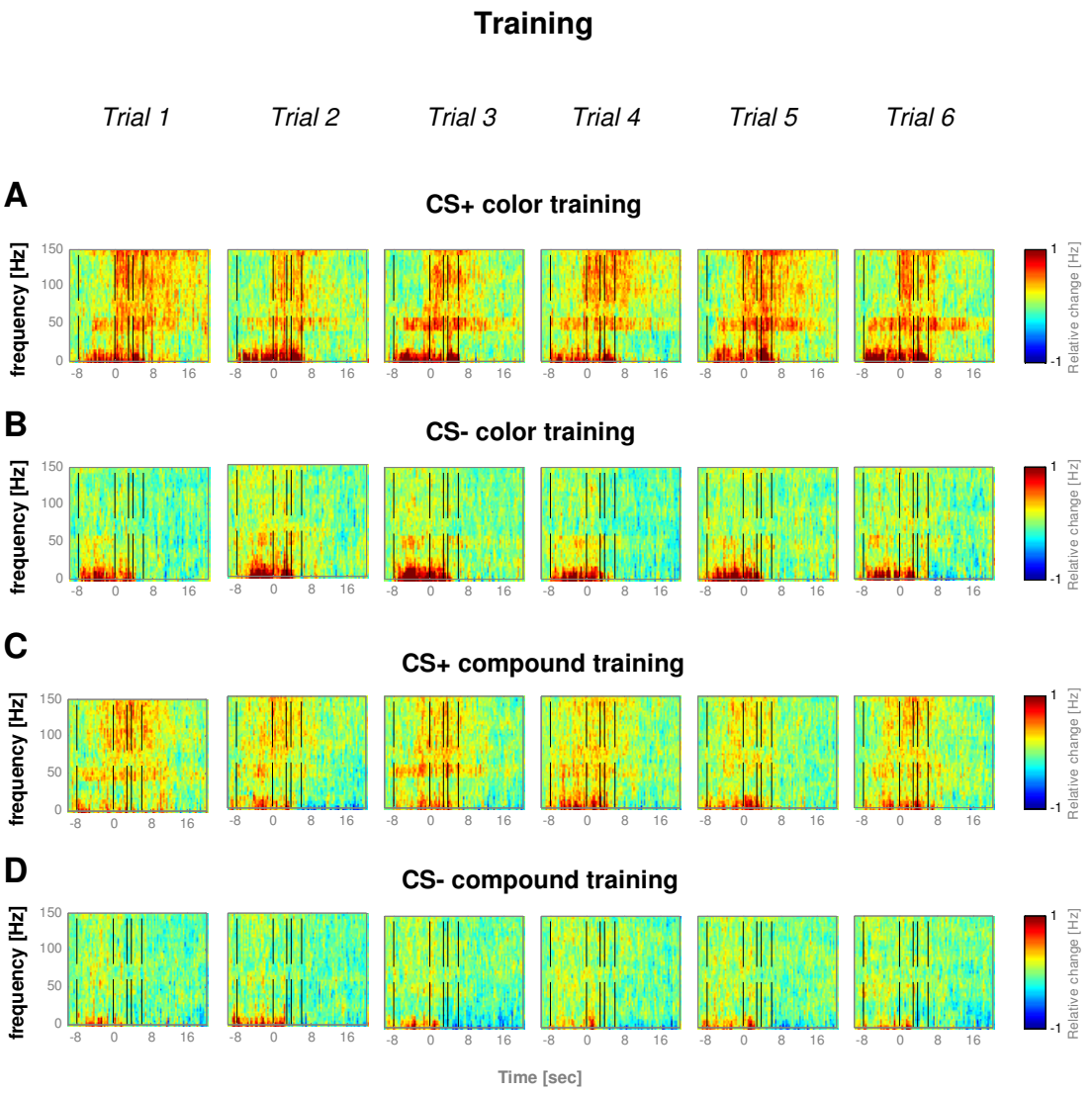


Figure 4. Time-frequency spectra of color training and subsequent compound training. Spectra are illustrated for all bees analyzed, for each of the six training trials separately. The y-axis depicts the frequency [Hz], while the colorcode depicts the change in relative spectral power. Vertical dashed black lines depict stimulus conditions (color onset, odor onset (only during compound conditiong), reward application, color and/or odor offset, reward offset). ***A*** CS+ color training, ***B*** CS- color training, ***C*** CS+ compound training, and ***D*** CS- compound training. From the first training trial, enhanced power in the frequency band of the described precursor signal (1-25 Hz) is visible during CS+ and CS- color training. Additionally, during the CS+ color training, power enhancement of higher frequencies are observed. Especially, the frequency band of 50 Hz was enhanced, beginning with the onset of the precursor signal. The precursor signal was also present,

albeit not as strong, during compound training (***C & D***). Again, enhancement of higher frequencies, including the frequency band of ~50 Hz, are only present during the CS+ compound condition.

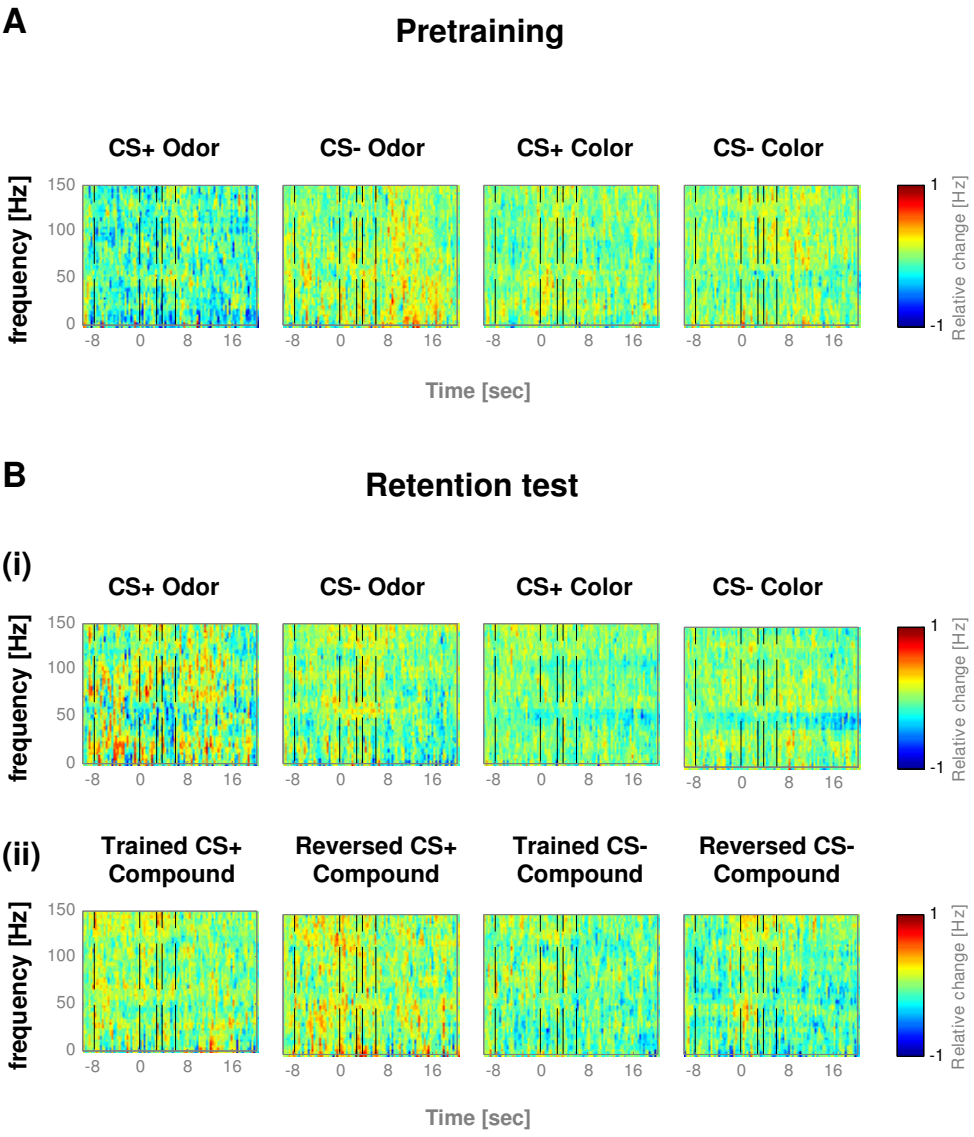
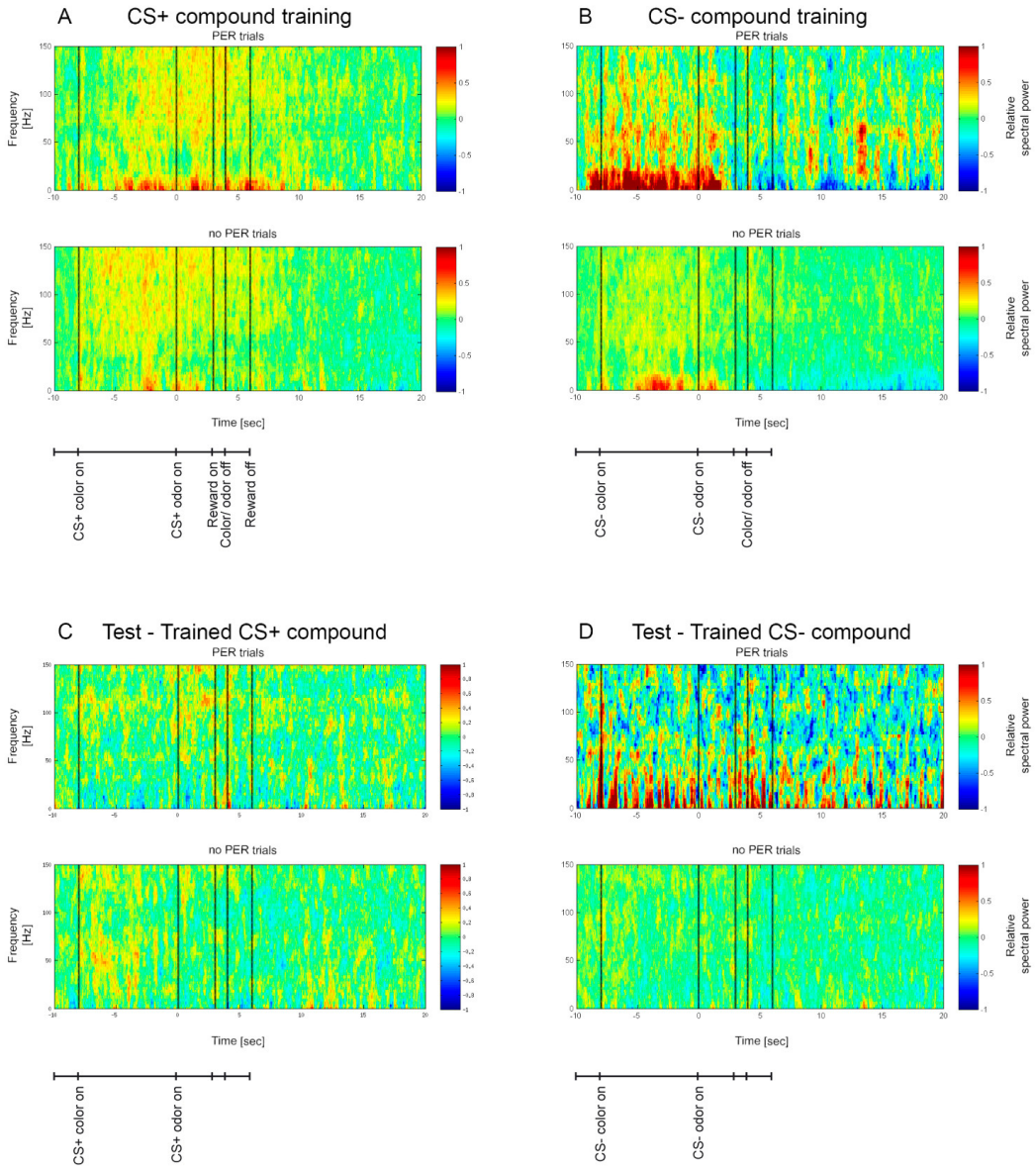


Figure 5. Time-frequency spectra during pretraining and retention tests. Conventions are the same as in figure 3. ***A*** illustrates time-frequency spectra for CS+ and CS- odors and colors before the traiing procedure started. No changes relative to baseline could be observed. During the retention tests of odors and colors alone (***B (i)***) as well as during trained and reversed compounds (***B(ii)***) no change in power could be detected.

Precursor signal is stronger before a PER is elicited during compound training

We calculated the same time-frequency spectra separately for trials in which the bee elicited a PER, the conditioned response, and plotted them against spectra during trials in which the bees did not elicit a PER (see fig. 6). We observed a stronger precursor signal during PER trials, during both CS+ and CS- compound trainings (fig. 6, ***A*** & ***B***, respectively). Here, the relative change in power was averaged over trials. The difference between PER / no PER trials is strongest during the CS- compound training. In retention tests, the precursor signal becomes evident during PER trials in the CS- compound. Testing the CS+ compound condition, the precursor signal was absent in both PER and no PER trials and a modest increase of higher frequencies (> 50 Hz) were detected during PER trials only. Testing the reversed CS+ compound (CS- color & CS+ odor), the precursor signal after CS- color onset was only present in trials in which the bees did not extend their probosces, while testing the reversed CS- compound (CS+ color & CS- odor) did not reveal strong differences in the time-frequency spectra between PER and no PER trials.


A
CS+ compound training
PER trials
no PER trials
Frequency [Hz]
Relative spectral power
Time [sec]
CS+ color on
CS+ odor on
Reward on
Color/ odor off
Reward off
B
CS- compound training
PER trials
no PER trials
Relative spectral power
Time [sec]
CS- color on
CS- odor on
Color/ odor off
C
Test - Trained CS+ compound
PER trials
no PER trials
Frequency [Hz]
Time [sec]
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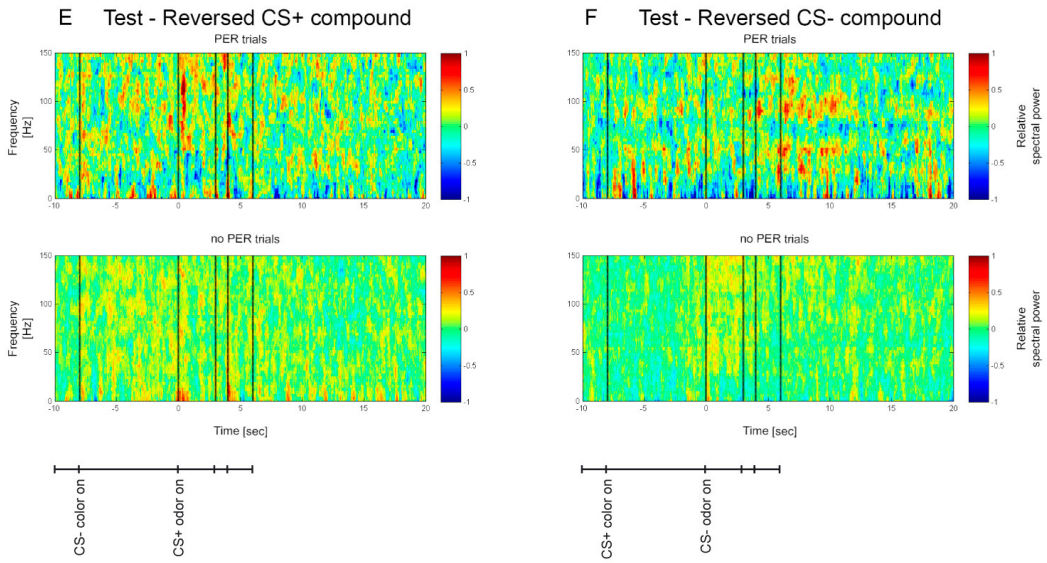

Figure 6. Comparison between PER and no PER trials in the time-frequency spectra during compound training and tests. ***A***, CS+ compound training, ***B***, CS- compound training, ***C***, test of trained CS+ compound, ***D***, test of trained CS- compound, ***E***, test of reversed CS+ compound, ***F***, test of reversed CS- compound. Upper panels illustrate PER trials while lower panels of each subfigure depict activity during no PER trials. During CS+ (***A***) and CS- compound training (***B***), the enhanced precursor signal is stronger present during PER trials (upper panels, respectively) than in no PER trials. Also, it is more pronounced during PER trials in the CS- compound training, when the bees erroneously extended their probosces. A possible surprise effect will be discussed below. During retention tests of the trained CS+ compound (***C***) the precursor signal is absent, during both, PER and no PER trials, while it is still present during PER trials in testing the trained CS- compound. Testing the reversed compound (reversed CS+ compound: CS- color & CS+ odor, ***E*** and reversed CS- compound: CS+ color & CS- odor, ***F***) revealed also the absent of the precursor signal. However, slight increases of higher frequencies were observed after the onset of the CS+ odor (***E***), and after the offset of the CS- odor (***F***).

2) **Phase locking between multi-unit activity (MUA) and the LFP**

After having extracted the frequency band in which power changes occured, we assessed phase locking between the filtered LFP in the frequency band of 10-25 Hz and MUA activity of each single bee during all test phases, and stimulus conditions. We ran the analysis for each time window separately. First, all significant trials, in which phase-locking occured ($p < .05$, non-uniformity test) were extracted. Subsequently, the mean phases of all significant trials per test phase, stimulus condition, and time window were calculated. We found differences in spike-locking during the preparatory period (between color and odor onset) when we compared learner versus non-learner bees.

The main result relates to enhanced phase-locking during the preparatory period in the CS+ compound training. Five bees that were identified as learner bees (12 learner bees in total, based on their performance during retention tests), exhibited significant phase-locking between MUA of PCT neurons and the LFP precursor signal, while only one non-learner bee (eight non-learner bees in total) exhibited significant phase locking during the same time window (see fig. 7). While phase-locking occured unspecifically, in different directions, quite frequently, also during the base window (before stimulus presentations), the enhanced spike-locking in the preparatory period during CS+ compound training occurred in one major direction in three of the 5 bees (in 15 units in total), namely 220°, and was highly significant in each bee (p range: from $p < .000$ to $p = .0002$). The one non-learner bee showed phase-locking ($p = .0006$) in the direction of 180° of the LFP.

Importantly, the same 5 bees that exhibited significant spike-locking during training also exhibited significant spike-locking during the test of the trained CS+ compound.

But during CS+ compound tests, only two bees featured spike-locking in the same direction (190°), while the other three locked to different phases of the LFP (p`s ranged from .000 to .004). More learner than non-learner bees exhibited significant spike-locking during the CS+ and the CS- conditions of color precoditioning and CS+ and CS- color tests, but during training and tests, phase-locking occured in different phases of the LFP. Only during the CS+ color test, MUA of two bees locked to the same phase of the LFP (200°, p`s = .0001 and .02, respectively). These are the same bees that exhibited significant spike-locking during CS+ compound training and test.

Spike-locking during training and test of the CS- compound condition was also more frequent in learner bees (training: 5 learner bees vs. 3 learner bees, test: 4 learner bees, no spike-locking among non-learner bees), however, phase-locking occured in different phases of the LFP in both training and test (p`s ranged from .000 to .04), and was therefore regarded as unspecific. As mentioned in *Methods*, additionally to the trained CS+ and CS- compounds, also the reversed compounds were tested.Testing the reversed CS+ compound (CS- color & CS+ odor), six learner bees showed significant spike-locking (p`s ranged from .000 to .04) during the preparatory period, however, only two bees locked to the same phase of the LFP (200°). Non-learner bees did not show phase-locking here. Further, no specific phase-locking was observed testing the reversed CS- compound (CS+ color & CS- odor).

Significant phase-locking during CS+ compound training

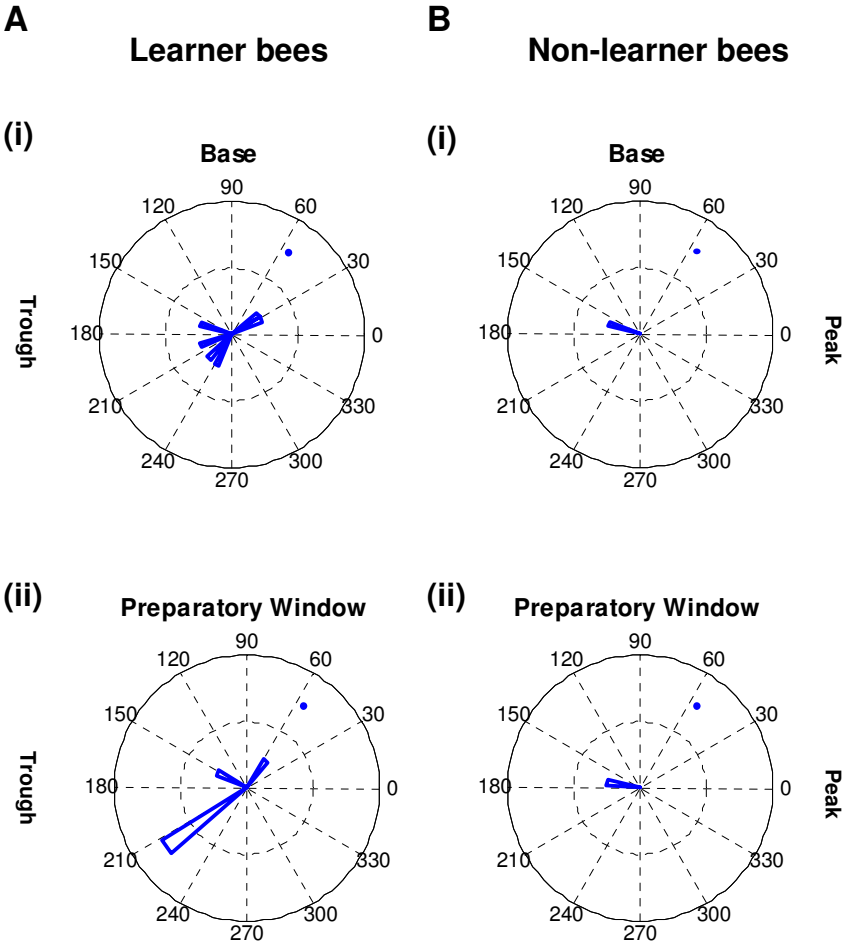

Figure 7. Increased phase-locking during the preparatory period of the CS+ compound training in learner bees. Polar plots illustrate significant phase-locking in learner bees (***A***) and non-learner bees (***B***) during CS+ compound training during base, before the presentation of the stimuli started (***i***), and during the preparatory period, after CS+ color onset and before CS+ odor onset (***ii***)**.** More bees that were able to discriminate the CS+ from the CS- compound during retention tests (learner bees) exhibited significant phase-locking during CS+ compound training (***A, ii***). MUA from PCT neurons locked at 220°, slightly after the trough of each oscillatory cycle of the LFP signal. Only one non-learner bee exhibited significant spike-locking during the preparatory period during CS+ compound training (***B, ii***)

Pairwise cross-correlation of single unit activity

In chapter 3, we described decreased reaction times of animals when the CS+ odor was announced by the CS+ color. We found a subset of PCT neurons that mirrored the decreased reaction times by decreased response latencies to the CS+ odor, specifically, only when the odor was previously announced by the correct color. The same units additionally exhibited enhanced firing rates during the preparatory period, the odor response window and during the expectation of the reward. These features were evident in PER trials, thus before the bee accomplished the correct behavioral response. Precise spike synchronization has been observed during movement preparation in the monkey motor cortex (Grammont & Riehle, 1999).

We therefore explored, whether these units also exhibited spike synchronization during one of the predefined time windows. We applied the method of cross-correlation as desribed in *Methods* for the pair of units that was recorded in the same bee (units # 56 and 60). We analyzed all defined time windows during CS+ and CS- compound training and during tests of trained and reversed compound conditions.

Figure 8 illustrates coincident spike occurrences after CS+ odor onset during the CS+ compound training (***B***) in comparison to a 2 second period before training started (base, ***A***). Figure 9 shows correlation histograms during training of the CS+ compound condition. We found an increased probability for spike synchrony of this unit pair with zero time lag during the preparatory window (after color and before odor onset) (fig. 9 ***C***), odor window (***D***), reward window (***E***), and during the offset of color and odor stimuli (***F***) in the CS+ compound training. Time windows for base and color onset did not exhibit conicident spike activity. During the CS- compound training (fig. 10), however, spike synchrony was only evident during the preparatory window (***C***).

Exploring the tests of trained and reversed compounds, cross correlations were almost lost. They were only, albei modest, evident during the preparatory periods of both trained CS+ and CS- compound tests (fig. 11, ***A*** & ***C***)

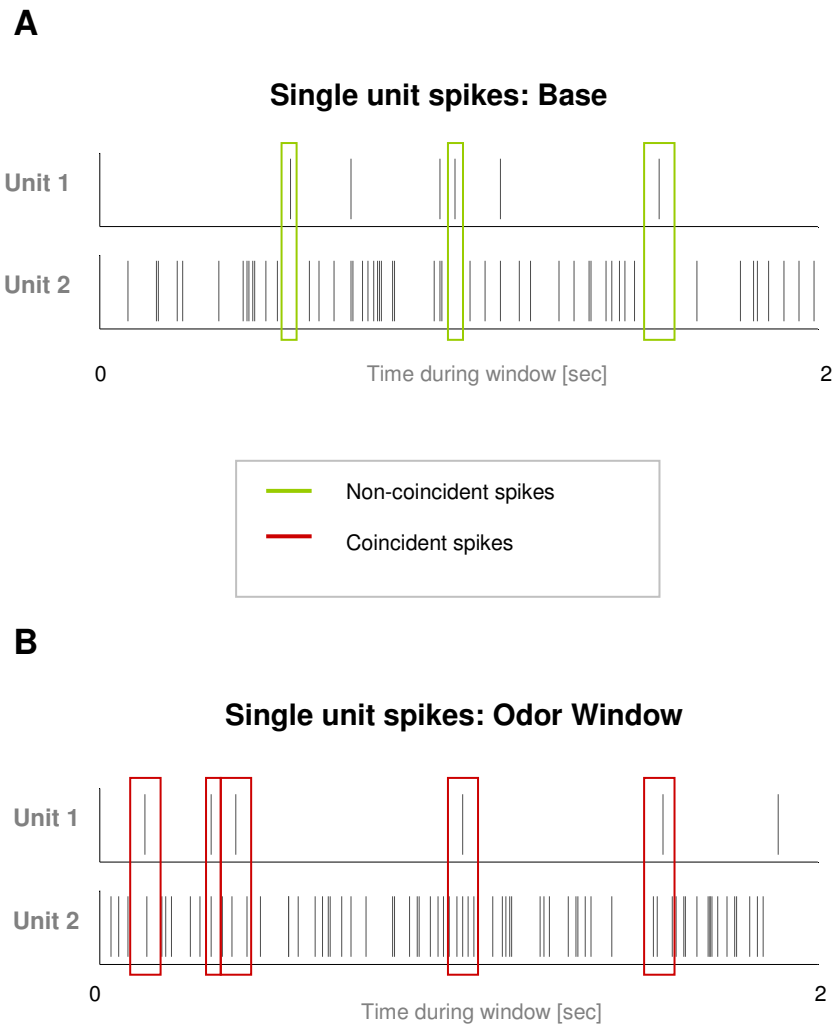

Figure 8. Single unit activity recorded within the same bee exhibit spike coincidences. Shown are 2 sec before CS+ compound training started (Base, ***A***), and 2 seconds following odor onset during CS+ compound training. Green boxes indicate no spike coincidences while red boxes mark coincident spike occurrence.

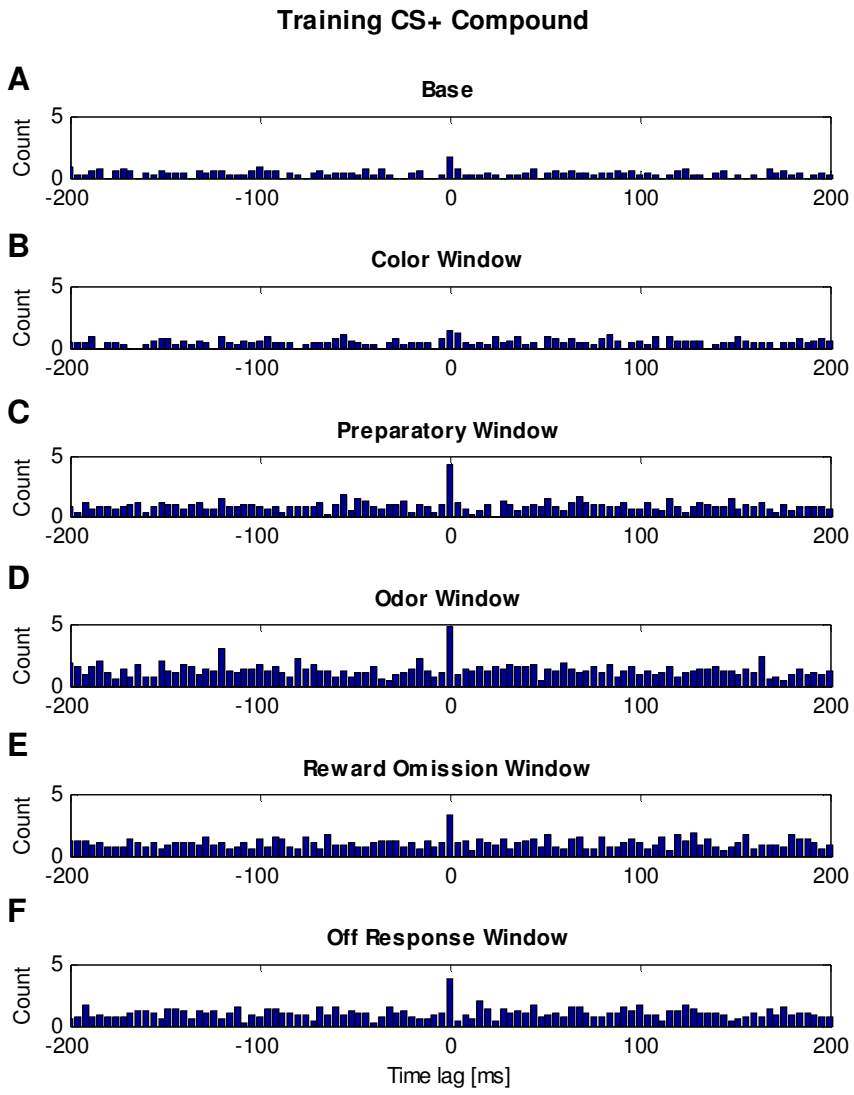

Figure 9. Increased spike cross correlation during the CS+ compound training. Spike synchrony is evident during the preparatory window (***C***), odor window (***D***), reward window (***E***), and during the offset of color and odor stimuli (***F***), and absent during base (***A***), two seconds before the training started, and the color window (***B***). Cross correlations were highly precise and occurred with a zero time lag.

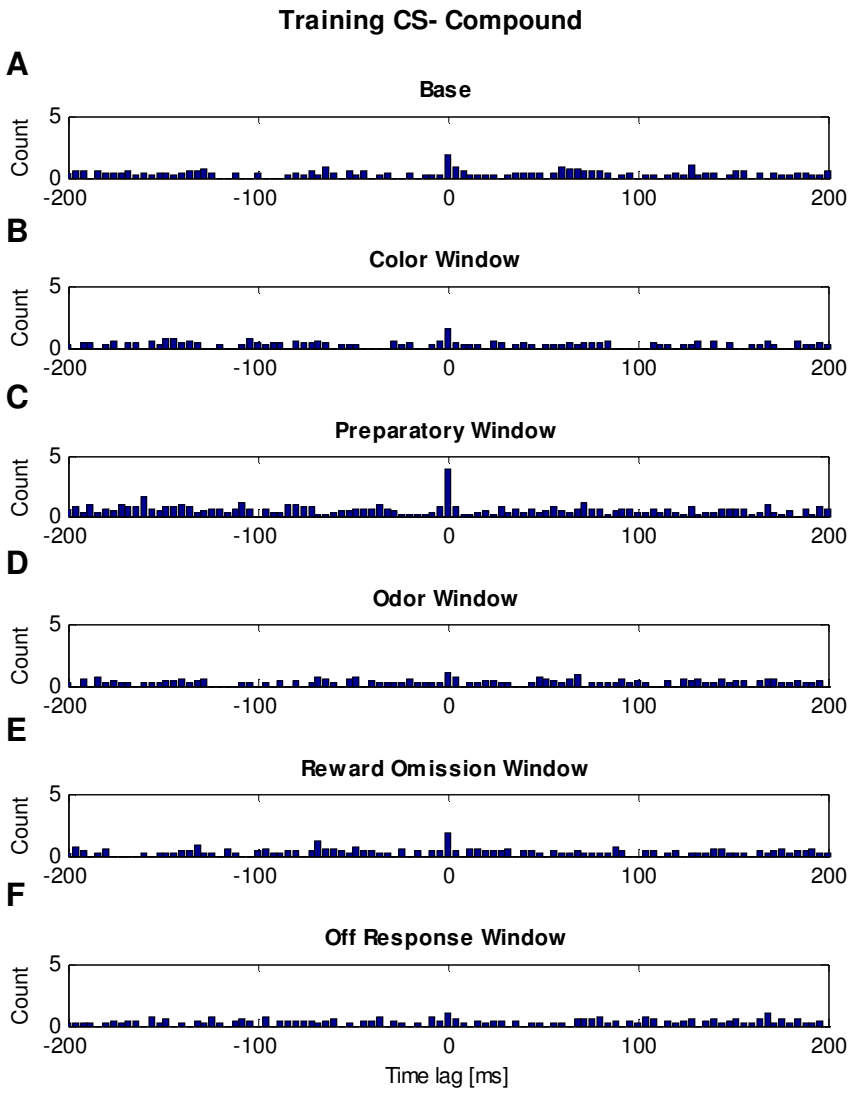

Figure 10. Increased spike cross correlation only during the preparatory window in the CS- compound training. Spike synchrony is evident during the preparatory window (***C***), and absent during all remainder windows (***A***, ***B***, ***D***, ***E***, ***F***). Although synchrony was only confined to one time window it was highly precise and occured with a time lag zero ms.

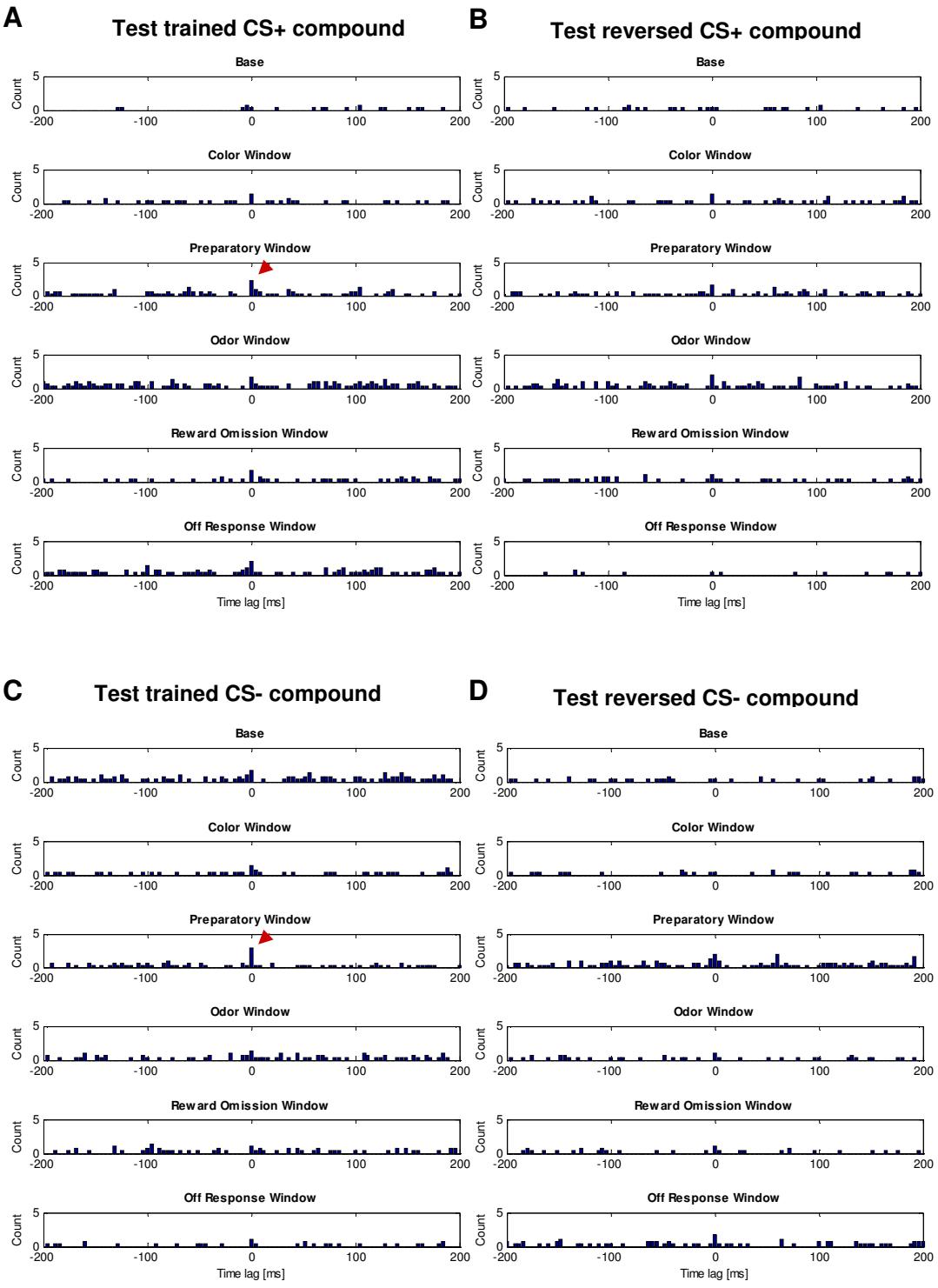

Figure 11. Cross-correlations during trained and reversed compound tests. While the unit pair did not fire in synchrony during the reversed CS+ (***B***) and CS- compound (***D***) tests, cross-correlations are evident, albeit to a low degree, during the preparatory windows

of the both trained copound (***A***, trained CS+ compound, ***B***, trained CS- compound), with zero time lag. All other time windows did not exhibit cross correlations.

Discussion

We recorded extracellularly from GABA-ir mushroombody (MB) interneurons of the protocerebral-calycal tract (PCT) that provides both local feedback within the alpha-lobe, the output structure of the MB, as well as recurrent feedback, connecting the major output region of the MB with its input. These neurons have been shown to exhibit olfactory and visual associative plasticity in the hours and day range (chapter 2).

We applied a combined visual and olfactory conditioning paradigm, in which we differentially pretrained two colors. One color was combined with a sugar reward 11 seconds after color onset. The same color was subsequently trained together with an odor that was applied 8 seconds after color onset. The delay between color and odor onset enabled us to investigate the preparatory period, after color onset and before odor onset, in which the animal might focus its attention and therefore prepare an adequate motor response. The paradigm was first used by Gerber & Smith (1998). The authors found enhanced, faster and prolonged behavioral responses to an olfactory stimulus during retention tests when compound training of a color and the odor was preceded by color preconditioning. We could verify the behavioral results of Gerber & Smith and found aditionally neuronal facilitating and suppressing modulation effects of odor responses upon stimulus selection based on the visual context (see chapter 3). The underlying mechanism of faster behavioral reactions to the CS+ odor in the rewarding visual context could be ascribed to reduced neuronal response latencies to the CS+ odor in a subset of PCT neurons, that showed already enhanced firing rates after the onset of the visual context cue. In the present chapter, we investigated power changes in the time-frequency spectra of the LFP, phase-locking between multi unit spikes and the LFP, as well as cross correlation between unit pairs.

Power changes in the LFP might reflect attentional processes during training to colors

We found enhanced power of the frequency band between 1-25 Hz, following both CS+ and CS- color onset during color preconditioning. We refer to this increased frequency

band as the 'precursor signal', as it appeared before a reward was presented and a conditioned response was eventually elicited.

Increase in power of low (below 10 Hz) and intermediate (20-30 Hz) frequency bands have been reported upon visual stimulation in the medial protocerebrum of *Drosophila melanogaster* (van Swinderen & Greenspan, 2003). While the slow potential response was very consistent, the 20-30 Hz response varied considerably with stimulus salience and was tentatively regarded as a neuronal correlate for selective attention.

In the present study, the precursor signal was evident during both CS+ and CS- training trials and might therefore not reflect selective but generalized attention upon visual stimulation. The precursor signal was already present in the first training trial. During CS+ training, a wooden stick with sucrose solution was approached to the bee before feeding. During CS- training, a wooden stick soaked in water was presented in the same way, in order to control for humidity levels. We can only speculate that eventually, the bees detected the humidity from the wooden stick already before feeding, and this might have enhanced attentional levels. Subtle changes in the environment might evoke such attentional changes. We are prone to exclude that the precursor signal is a mere effect upon humidity, as the precursor signal ceased in the course of training in non-learner bees, but persisted in learner bees throughout all training trials (see example bee). In contrast, humidity levels were kept at a constant level throughout training. Colors (blue and yellow) were balanced as serving for the CS+ or the CS- condition. Therefore, a color-specific effect can be excluded. Thus, the precursor signal from the first training trial onwards might be an attentional effect, caused by the color onset and additional subtle changes in the environment, like changes in humidity levels.

Power changes in the LFP might reflect attentional processes during training to compounds

The precursor signal was also present during compound training of colors and odors. In the example bee, the precursor signal was even strong during compound conditioning as during color conditioning, but it attenuated faster in the CS- compound training, possibly resembling reduced salience due to the lack of reward during the CS- condition. Beside the precursor signal of intermediate frequencies, also higher frequencies (50 Hz and higher)

were enhanced tightly locked to CS+ and CS- odor onset, but decreased much earlier for the CS- compound conditioning. The decrease during the CS- condition could possibly reflect a physiological signal of surprise as reward might have been expected but was not applied.

When all bees were pooled, however, the precursor signal and the increase in higher frequencies were much more moderate than in the selected example. Higher frequencies were restricted to the CS+ compound condition. As pooled time-frequency spectra were not seperated for learner versus non-learner bees, and as the example bee learned to discriminate between CS+ and CS- compounds correctly, we assume that non-learner bees decreased their attentional levels during compound training, reflected in the reduced precursor signal after color onset. Reduced attention might therefore be the cause of low learning scores in the subsequent test in non-learner bees. Learning requires attention (eg. Roelfsema et al., 2010). Learner bees might have held high attentional levels throughout compound conditioning, which might be reflected in pronounced precursor signals and an increase in higher frequencies strongest after CS+ odor onset.

Increase in higher frequencies (>50 Hz) revealed to be more specific to the CS+ odor. In the literature, increase in power in higher frequency bands upon odor stimulation have been described. In the MB of *Drosophila*, novel odor presentation led to increased power in the 70-80 Hz frequency band, while this response attenuated under repetition (Prieto-Godino & de Polavieja, 2010). Attenuation was rescued after an electric shock has been trained together with the odor. The authors suggest that the LFP signal is modulated upon a change in adaptive value of the odor stimulus and might thus reflect a simple neuronal correlate of higher-order olfactory processing in the medial protocerebrum of flies.

As we observed an increase in frequencies of 50 Hz stronger upon CS+ odor presentation during compound training trials, in which a reward was presented, in comparison with CS- compound training trails, enhanced power in higher frequencies could also reflect enhanced value representation of the rewarded odor. However, we found no power changes before or after training. Enhanced power in higher frequency bands might thus rely on the presence of a rewarding stimulus, and are therefore regarded as functioanlly different from the high frequencies enhancements in *Drosophila*.

Thus, while the precursor signal reflects general attention, power increases in higher frequencies might code the actual value of a stimulus that is being associated with the reward.

The precursor signal is stronger before the initiation of a behavioral response

We found no power changes in the LFP before or after the training when we averaged over all bees. However, when separately investigating trials in which a behavioral response (PER) followed, the precursor signal was more pronounced before stimulus selection (when a behavioral response was elicited) in comparison with trials in which no motor response was observed during both CS+ and CS- compound training trials.

By this separate analysis, the strong precursor signals during compound training could be predominantly ascribed to trials, in which stimulus selection occured.

According to the 'premotor theory of attention' (Rizzolatti, 1987; Allport, 1989), the relevant features of a particular stimulus, that is selected for a behavioral response, automatically receives attention. This theory is supported by eye movement experiments, in which attention is invariably directed to stimuli that are selected as targets for an eye movement (Hoffman & Subramaniam, 1995; Kowler et al., 1995; Deubel & Schneider, 1996). We may thus assume that attention during or before the preparation of a motor response might reflect enhanced attention prior to the motor output during training.

During retention tests, the precursor signal was still present during PER trials in the CS- compound condition, but was absent in both PER and no PER trials testing the CS+ compound.

Bees that extended their probosces during test to the CS+ compound revealed correct performance after training. Thus, the precursor signal might only occur when stimulus combinations are not yet learned but attention is directed to the compound stimulus. This fits to the observation that bees that extended their probosces during tests of the trained CS- compound condition, thus revealing incorrect behaviour, did not learn the stimulus combinations sufficiently, and might therefore exhibit the precursor signal as a correlate of enhanced attention.

The precursor signal as a neural correlate for surprise?

The precursor signal during PER trials in the CS- compound training was more pronounced compared to PER trials in the CS+ compound training (see 5***B***). Enhanced attentional levels might be caused by surprise. When the bees extend their probosces in the expectation of a reward but no reward is applied (during CS- compound training), the precursor signal might signal enhanced attention due to the deviation of an expectation. Dopamine neurons in vertebrates (Schultz, 1998), as well as the ventral unpaired median neuron number 1 of the maxillary neuromere (VUMmx1) in the honeybee brain (Hammer & Menzel, 1998), code deviations of reward expectation. Both neuron types respond to a reward when no reward was expected and through learning they begin to respond to stimuli that predict a reward. Neuromodulatory actions of the VUMmx1 neuron could hypothetically drive the attentional precursor signal, and promote learning when something unpredictable occurs. Although, the first surprise might rather occur during the time of reward omission, the detection of reward absence might be integrated in subsequent trials after color onset.

Spike-locking to the attentional precursor signal necessary for successful encoding of stimulus combinations

Spike-locking has been found to increase with selective attention (Niebur, 2002). While the precursor signal was evident in both CS+ and CS- color and compound conditioing trials, phase-locking to the precursor signal occured more often during the CS+ compound conditioning in those bees, that were later able to dissociate the compounds behaviorally (learner bees). Spike-locking in the preparatory period after color and before odor onset occured after the trough at 220° of the oscillatory cycle. While the power increase of the LFP in the 1-25 Hz frequency band might reflect attentional processes following color onset, additional spike-locking to the LFP oscillations might serve as a prerequisite for successful encoding of the correct training of stimulus combinations. Increased and more precise phase-locking during the encoding of information that is later remembered than during encoding of information that is later not well remembered has been documented in humans and animals (Fell et al., 2008, Miltner et al., 1999; Weiss & Rappelsberger, 2000; Fell et al., 2001; Babiloni, 2009).

Functional implication for the recurrent and local feedback loop

The primarily inhibitory recurrent pathway of PCT neurons forms fine horizontal bands within the anterior-dorsal α-lobe in which they receive modality specific excitatory input from KCs. PCT neurons connect the MB output, the α- and β lobes and pedunculus, with all sensory subcompartments of the MB calyx predominantly connecting the same modality specific output and input layers (Schäfer and Bicker, 1986; Grünewald, 1999a). Since most PCT neurons are GABAir they appear to provide selective inhibitory input to the calyces (Bicker et al., 1985; Schäfer and Bicker, 1986). In the calyx, PNs are presynaptic to KCs and postsynaptic to PCT neurons, therefore the output will be indirectly mediated via feedforward inhibition by GABA-ir PCT neurons. Additionally, convergence of the reward encoding neuron VUMmx1 (Hammer & Menzel, 1998) with KCs in the MB calyx suggests that the PN-KC synapse undergoes associative plasticity upon learning, which was found in calcium imaging experiments on the dendrites of KCs (Szyszka et al., 2008).

We found phase-locking of PCT neurons to an enhanced LFP signal of 1-25 Hz shortly after the trough of the oscillatory cycle, considerably more frequent in learner bees compared to non-learner bees following color onset during the rewarded (CS+) compound training. As this LFP signal is different from the observed 50 Hz signal in the AL that emerged specifically to the CS+ odor after learning (Denker et al., 2010), we assume that the LFP signal we measured is local to the recording position in the α-lobe at the MB output. We could also evidence that the precursor signal occured in the absence of motor activity. The enhanced signal is therefore regarded as independent from motor artefacts.

It was suggested that during training KCs transmit odor information as well as the gustatory information of the sucrose reward (Fröse A, 2009; Okada et al. 2007), and might provide coincident excitatory input onto PCT nerons. In the locust, hebbian spike-timing dependent plasticity (STDP) on a ~25ms timescale has been shown to occur between KCs and β-lobe neuron synapses, which are also located at the MB output, when presynaptic spikes preceded postsynaptic firing. (Cassenaer & Laurent, 2007). The authors observed that the values over which synaptic weights changed corresponded to the period of single odor-evoked 20 Hz oscillation cycles, and enhanced synchronization of β-lobe neurons. They further found that only within-cycle ‘coincidences’ modified the connection between KCs and its target neurons, when KC spikes occured just before the trough of the LFP and

shortly before β-lobe neuron spikes. In this line, the phase of the oscillation cycle times pre-and postsynaptic discharge, facilitating STDP.

We thus hypothesize, that the phase-locking found shortly after the trough of the LFP signal might indicate precise spike timing of PCT neurons, and might facilitate the occurrence of STDP between the KC, that might occur shortly before the trough, and the PCT synapses in learner bees during CS+ compound training. Attention might thus be a prerequisite for inducing long-term potentiation (LTP), and hence learning. Here, specifically the visual stimulus might be encoded, as spike-locking was observed during the preparatory period, leading to a memory of the visual stimulus that reliably predicts the occurrence of an odor and a reward. As we observed higher frequencies after the onset of the CS+ odor during the CS+ compound conditioning, precise spike-locking might occur to faster oscillations and might facilitate odor learning at the KC-PCT synapses in the same way. However, this mechanism still needs to be tested and can only be hypotheiszed by now.

Successful encoding generally leads to facilitated processing of learned stimulus combinations and potentiates responses to the learned stimuli (Fries et al., 2001; Fries, 2005). The read-out of facilitated stimulus processing is reflected in antagonistic rate codes of PCT neurons during retrieval as described in the second chapter. We could show that indeed the strength of the anatgonistic rate code correlates with correct behavioral responses of the animals. Local feedback of the PCTs onto premotor neurons might elicit the correct behavioral response. The recurrent feedback of PCT neurons back onto sensory (PNs) and KCs might selectively inform the MB input about leanred stimuli, consequently facilitating processing of these learned stimuli already in the MB input. The reciproke synapse in the calyx between PCTs and PNs might play a crucial role in this information process. Feedforward inhibition from PCTs onto the PN-KC synapse could precisely time spikes from PNs onto KCs and could therefore induce both, LTP and LTD, depending on the sequence of spikes from PNs and KCs. Faciliated processing of learned stimuli at the MB input and output (after the induction of LTP) might reflect a neuronal mechanism of attention, therefore substituting the precursor signal after learning.

However, as long as learning is suboptimal, the precursor signal is present, setting the system in a state in which learning can occur.

This is in line with our observation that the precursor signal was still present during the CS- compound test when an erroneous extension of the PER followed.

Spike coherence of neuron pairs might reflect enhanced attention and differentiates the CS+ from the CS- compound after odor onset during training

Spike coincidences of one neuron pair were evident during the preparatory period during the training of both CS+ and CS- compounds, probably reflecting the same neuronal process underlying attention that was already visible in the precursor signal of the LFP. While precise spike correlation continued throughout odor presentation, reward omission, and offset of the CS+ color and odor, spike coherence was not present anymore after the onset of the CS- odor during the CS- compound condition.

Neurons participating in one (synchronized) cell assembly, are regarded as dynamically coupled. Dynamic coupling occured during enhanced attentional states and was kept to behaviorally important events only. Neurons enganged in a synchronized cell assembly could thus functionally bind behvaiorally important stimuli, and could provide synchronized input to premotor neurons, fastening motor responses via local feedback.

PCT neurons have been suggested to innervate the Pedunculus extrinsic (PE1) neuron, that in turn inhibits lateral horn (LH) neurons (Okada, 2007), while LH neurons directly contact motor neurons. Coincident input to the PE1 might thus rapidly induce disinhibition onto LH and motor neurons, inducing fast motor responses.

However, we only observed spike synchrony to the CS+ odor during training, and not during subsequent tests, in which reduced neuronal response latencies and reduced reaction times have been described (chapter 2). In this particular case, the observed synchrony cannot account for reduced reaction times of the animal.

The literature provides many examples that motor cortical neurons change selectively their firing rate during the preparation and execution of goal-directed behavior (Tanji & Evarts, 1976; Georgopoulos et al., 1982; Riehle & Requin, 1989), and additionally, spike synchrony has been shown to increase upon motor preparation (Grammont & Riehle, 1999).

The probability of observing spike synchrony increases with firing rate of the neurons (Shadlen & Newsome, 1994). However, we showed precise spike synchrony also during the preparatory period of the CS- compound training, in which no increase in firing could be detected (chapter 2). We thus conclude, that the observed spike synchrony is an inherent feature of the neural assembly to focus the attention to a primed olfactory stimulus, and to ensure precise spike timing on a millisecond timescale (Riehle et al., 1997), that might lead to faster motor outputs. Moreover, neurons participating in one (synchronized) cell assembly, and are therefore regarded as dynamically coupled, do not necessarily exhibit the same firing patterns. This has also been observed by Grammont & Riehle, 1999).

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5. General discussion

Learning critically depends on synaptic plasticity and enables the nervous system to adapt to the environment (Hebb, 1949, Squire, 1987, Kandel & Schwartz, 1982). Learning enhances the sensitivity of neural assemblies to process incoming stimuli (Buzsáki, 2006) and facilitates action-selection processes for executing appropriate behavior (Roelfsma et al., 2010). In the present work, general neural mechanisms of a local and recurrent inhibitory network in the honeybee brain were investigated in the frame of learning and memory formation. Additionally, it was asked how attention-related processes might interfere with learning-related processes of inhibitory neurons. The bee has been proven to be a valid model system to study learning-related processes as it provides a broad behavioral repertoire, among which is the ability to associate visual and olfactory stimuli with a sugar reward during the classical conditioning of the proboscis extension response (PER) (Menzel at al., 2006, Menzel, 1990; Menzel & Müller, 1996). Moreover, its central nervous system is accessible for electrophysiological and imaging methods (Rybak & Menzel, 1993, Grünewald, 1999, Okada et al., 2007, Haehnel & Menzel, 2011).

We utilized two different analysis strategies: High-pass filtering of the extracellular recorded activity allowed us to study precise timing of locally generated action-potentials, while low-pass filtering of the same activity enabled the analysis of the mesoscopic population signal, the local field potential (LFP), that consists of synchronous subthreshold activities in the neuronal population surrounding the electrode.

The experimental paradigm

We recorded extracellularly from local and recurrent GABA-ir neurons of the protocerebral-calycal tract whilst applying a combined visual and olfactory conditioning paradigm, first used by Gerber & Smith (1998). We slightly changed the course of training and testing, such that differential color preconditioning were always applied as well as differential compound training, before testing the color and odors alone, the trained compounds (trained CS+ compound: CS+ color & CS+ odor; trained CS- compound: CS- color & CS- odor) as well as the reversed compounds (reversed CS+ compound: CS- color & CS+ odor; reversed CS- compound: CS+ color & CS- odor). A delay between color and

odor onset of 8 seconds allowed us to study attention-related processes after the bee was primed with one of the colors. The reward always followed 11 seconds after color, and 3 seconds after odor onset during the CS+ training trials.

Analysis of neuronal activity in chapter 3 and 4 was applied for defined time windows that related to stimulus presentations during one behavioral sequence. We separately analyzed color onset, the preparatory period as the time after color onset and before odor onset, odor onset, reward onset, and the offset of the color and the odor. The preparatory period lasted for 6 seconds, while all other time windows covered a period of two seconds.

Main features of the recorded neurons

Recordings were made from the primarily inhibitory recurrent pathway of PCT neurons. These neurons form fine horizontal bands within the anterior-dorsal α-lobe where they receive modality-specific excitatory input from KCs and provide local feedback onto MBs' EN. PCT neurons further connect the MB output, the α- and β lobes and pedunculus, with all sensory subcompartments of the MB calyx predominantly connecting the same modality-specific output and input layers (Schäfer and Bicker, 1986; Grünewald, 1999a). Since most PCT neurons are GABA-ir they appear to provide selective inhibitory input to the calyces (Bicker et al., 1985; Schäfer and Bicker, 1986). Electron microscopy study revealed GABA-ir profiles in the calyx documenting reciprocal synaptic contacts with PNs und monosynaptic contacts with KCs (Ganeshina & Menzel, 2001). Since PNs are presynaptic to KCs and postsynaptic to PCT neurons their output will be indirectly mediated via feedforward inhibition by these GABA-ir neurons.

Main findings

Chapter II - Testing the odors and colors alone, we found that subsets of the recorded PCT neurons established visual as well as olfactory associative plasticity in discrete time windows during a recording period of up to three days. Associative plasticity was expressed by an antagonistic rate code that correlated with the bee`s ability to discriminate behaviorally between the stimuli. Thus, the stronger the antagonistic rate code was the better the read-out of PCT neurons and the better the discrimination of the animals.

Chapter III - In the third chapter we specifically analyzed visual modulation of olfactory processing during the retention test of the first recording day. Here, we inspected the whole recorded neuronal population, during all trials, and additionally during trials in which the animals elicited a behavioral response (PER trials) versus trials in which no motor output was observed (no PER trials). Interestingly, we found that during PER trials PCT neurons perfectly integrated the sum of the learned values of both stimuli within one compound, which was expressed in the strength of the firing rate to the odor: The strongest odor response was visible during the trained CS+ compound (CS+ color & CS+ odor), an attenuated firing rate was observed when the CS- color preceded the CS+ odor (reversed CS+ compound). While the trained CS- compoud (CS- color & CS- odor) elicited nearly no neuronal response to the odor, the CS- odor response was enhanced, but still considerably lower than during the CS+ compounds, when the CS+ color preceded the CS- odor.

Chapter IV - In the fourth chapter an enhanced 1-25 Hz LFP signal was found after color onset during all training sessions (color preconditioning and compound conditioning for both CS+ and CS- conditions), which was predominatly present in trials in which a behavior was induced and might thus reflect global visual attention upon stimulus selection. Specifically during the CS+ compound training, phase-locking of PCT spikes and the enhanced LFP signal was observed after color onset and before odor onset considerably more often in learner-bees than non-learner bees, suggesting the facilitation of short-term dependent plasticity (STDP) between the KC and PCT synapse. Additionally, synchrony between single unit spikes was evident during compound training throughout the whole behavioral sequence, while during CS- compound conditioning, it was restricted to the preparatory phase.

Synthesis of findings

Circuit model of learning and memory processes in the mushroom body

Possible sites of associative plasticity in the MB input and output are depicted in the circuit model below (fig. 1). Conceivable roles of PCT neurons in mediating learning and memory processes, that were discussed throughout this work, are described.

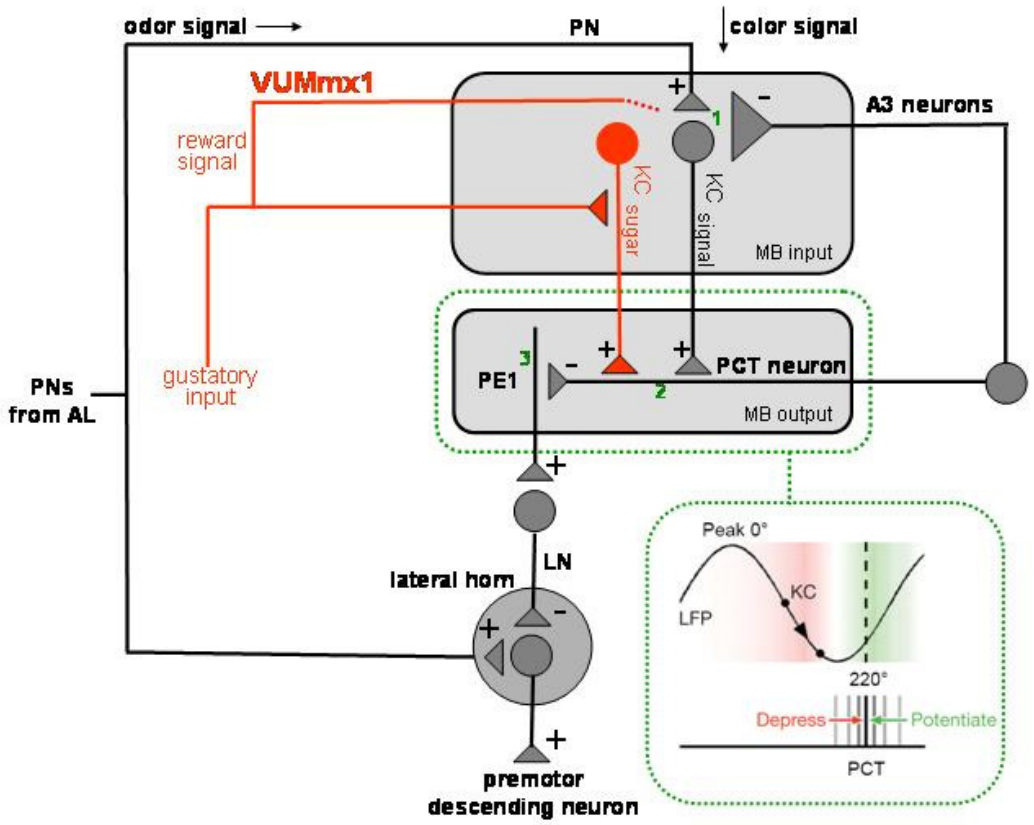

Figure 1. Circuit model of learning and memory processes in the mushroom body (MB). Pathway connectivities were adopted from Menzel et al. (2011), and Okada et al. (2007). An odor signal from the antennal lobe (AL) projection neurons (PNs) or a color signal is conveyed to the MB input, the calyx, upper grey box. Olfactory information arrives in the lip, visual information in the collar, mixed input in the basal ring of the calyx. For simplicity, the model depicts only the MB input as all subcompartments of the calyx. A3 neurons of the PCT neuron group connect the MB output (lower grey box) with the MB input. They reciprocally connect with PNs, and both PNs and PCTs contact KCs. These connections support both feedback and feedforward inhibition. Combination of these two types of regulatory mechanisms may provide stability and effective external control. Possible sites of associative plasticity are suggested: **1.** Synaptic plasticity was described between the PN-KC synapse (Szyszka et al., 2008). Different secenarios are conceiveable. a) Only PCT neurons that reduce their response to the learned stimuli could feedback into the calyx. Decreased inhibition could enhance the KC-PN synapse. b) Through feedforward

inhibition, PCTs could precisely time the occurrence of PN spikes onto KCs which could be both, in favor of LTP and LTD, depending on spike sequence from the neurons. Delayed inhibition of PN firing could also facilitate KC integration of synchronized PN input, as described (Assisi et al., 2007). **2.** During training the VUMmx1 innervates the calyx and signals the presence of reward. KCs convey visual, olfactory, and gustatory information onto PCT neurons at the MB output. Phase-locking of PCT neurons to the enhanced LFP oscillation, presumably reflecting attention, times spike occurrence of PCTs right after the trough of the oscillatory cycle (chapter 4, green box, graphic adopted and modified from Cassenaer & Laurent, 2007). Occurrence of KCs shortly before the trough would induce LTP by short-term dependent plasticity (STDP). The inverse spike sequence would promote LTD. After learning, subsets of PCT neurons read-out the antagonistc rate code from KC-PCT synapses und feedbback to the MB input (chapter 2). Runaway changes of synaptic strength could be prevented by the reciprocal feedback between the PN-PCT synapse after learning (Steele & Mauk, 1999). As both olfactory and visual stimulus values might be encoded between KC-PCT synapses, the PCTs integrate the sum of both color and odor value, in the odor response (chapter 3). **3.** High values are signalled by high discharge firing of the PCT ensemble, which in turn lowers the response of the PE1 neuron to the learned stimulus (Okada et al., 2007). As Menzel & Okada suggested, low discharge frequencies of the PE1 might decrease inhibition from local neurons (LNs) onto premotor descending neurons, such that the excitation from AL PNs could excite premotor neurons in the lateral horn and a behavioral response is executed. The direct sensory-motor pathway might thus be relaxed for high value stimuli (in line with Menzel et al., 2011).

As decribed in the genral introduction and as depicted by the circuit model above, memory traces in both vertebrate and invertebrate brains are distributed in time and space. In the honeybee brain, neural and cellular correlates of olfactory learning were described for the molecular, neural network and single neuron level, taking place in the AL, MB intrinsic and extrinsic neurons, as well as in the reward neuron VUMmx1.

Associative plasticity in the MB input could facilitate processing of learned stimulus combinations, when detected in the environment. Associative plasticty at the MB output

could initiate goal-directed behavior, and by means of recurrent inhibition adjust synaptic weights between PNs and KCs in correspondence with the environmental constraints.
Hence, through a complex inhibitory network, that links sensory input areas with motor output structures, memories might be regulated upon attention, and by the relevance of incoming stimuli for behavior, constantly updating distributed storage entities with the everchanging conditions of the environment.
These environmental conditions differ concerning their biological significance for an animal. In the present work, the visual modulation effects of olfactory processing and the strong reduction in reaction time to an odor after color onset strongly pleads for a high biological significance for color processing in honeybees. Colors might help to focus the attention and improve orientation toward the scent of flowers during foraging flights.
Thus, the value of incoming odor stimuli needs to be updated constantly in the context of visual cues.
Despite the fact that learned visual stimuli do not elicit a behavior in harnessed honeybees, this work evidences associative plasticity to colors through the same neuronal coding strategies also used for olfactory stimuli.

In sum, the PCTs inform the MB input and output about learned stimulus values in an experience-dependent way. Recurrent feedback to the MB input might selectively enhance PN-KC synapses upon attention, which might facilitate information processing to high value stimuli in the future. Local feedback to the MB output might select the appropriate motor pattern based on the combined value of single stimuli that emerge together in the environment.

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Acknowledgment

First of all I want to thank my supervisor, Prof. Menzel, for giving me the opportunity to accomplish this work in his laboratory. I learned a lot during fruitful and lively discussions, and in the course of writing the manuscript. Through his steady support I could develop my interest in many intriguing aspects in neurobiology.

I am deeply grateful to Johanna Derix and Nicole Cichon for the analysis and the support provided for the fourth chapter. Johanna calculated the time-frequency spectra, and Nicole helped me in programming the scripts for phase-locking and cross-correlations. Their support in programing traces back to Japan, when all three of us worked at the RIKEN Brain Science Institute.

Indeed, various people helped me with learning programming in matlab. I am deeply grateful to my friend Denise Berger. She encouraged me and provided endless support, especially during the nerve-stretching period at the beginning. I am also very thankful to Michael Schmuker, who also provided a lot of Matlab help. I also want to thank Saikat Ray. During his time as a student assistant in our lab, he helped me whenever I got stuck in the advanced period of programming. Without his help, many things would have taken much longer. I also want to thank Konstantin Lehmann, who provided various indispensable computer help. I thank Nora deCamp for introducing me into confocal microscopy, and for numerous discussions, ideas, comments, feedback, related to science and beyond. I am sure we will continue our “Ino” project some day. I am grateful to Martin Strube-Bloss who introduced me to the art of electrophysiology. He patiently taught me all essential details necessary for extracellular recordings in a minibrain. I want to thank Prof. Nawrot for his essential support concerning various details of my analysis. I am deeply grateful to my dear friend Ravit Hadar. That she was always by my side throughout my PhD. She read corrections for the most parts of the present work and provided essential criticisms and creative ideas. I thank my friend Marianna Gil. My work profitted from her thoughtful comments and remarks. I am also deeply grateful to my friend Émilia Pichenot, for always being there for me, for her curiosity about my project and useful comments.

I want to thank all members of the Menzel group, the Eisenhardt group, the Nawrot group, the Pflüger group, and the Brembs group for fruitful discussions inside and outside of the laboratory. I am indebted to Ryuichi Okada who arranged the set-up I was allowed to work on. Gisela Manz provided significant technical support especially in the beginning of my experiments. I am grateful to Sabine Funke, who always provided the administrative support throughout my PhD, and Peter Knoll for taking care of the bees.

Last but not least, I want to thank my family, my parents, my brother Stephan and Evelina for their endless support. And I want to thank Sebastian, who always believed in me and supported me in so many different ways. He creatively designed the 3D illustration of the elctrophysiological set-up, presented in this work.

Curriculum Vitae

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