Characterization of Mushroom Body Extrinsic Neurons in the Honeybee *Apis mellifera* and Their Role in Learning and Memory Formation: A Calcium Imaging Study

Dissertation

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Authors: Melanie Hähnel and Randolf Menzel

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Plasticity in Neurons of the Mushroom Body α-Lobe after Olfactory Conditioning

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Characterization of Morphologically Identified Mushroom Body Extrinsic Neurons

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Melanie Hähnel and Randolf Menzel designed and discussed the experiments. Randolf Menzel performed the intracellular electrophysiological recordings and iontophoretical stainings of mushroom body extrinsic neurons. Jürgen Rybak reconstructed the A5 extrinsic neuron and registered it into the Bee Standard Brain atlas. Melanie Hähnel performed the imaging experiments, analyzed the data and wrote the manuscript.

Summary

In this calcium imaging study properties of mushroom body extrinsic neurons were investigated. In a number of previous studies, the mushroom bodies, paired central structures of the arthropod brain, had been shown to process stimuli of different sensory modalities and to be necessary for memory formation.

For a further investigation of how neurons, involved in the mushroom body circuitry are subject to plasticity underlying learning processes, it is important to demonstrate how stimuli (e.g. odors) are represented by these neurons. In Chapter 2, it is described how honeybee foragers were exposed to a set of ten odors, to repeated stimulation with the same and different odors and to odor concentrations spanning several orders of magnitude. Also, responses to gustatory and visual stimuli were investigated. Activity in a subset of mushroom body extrinsic neurons of the α-lobe was observed during stimulus presentation employing calcium imaging. Activity in neurons of the mushroom body output region, in response to all tested odors was shown, exhibiting diverse temporal patterns. Extrinsic neurons also responded to all tested odor concentrations displaying a sigmoid dose-response relationship. In most cases, stimulation with one odor induced a diminished response to consecutive stimulations with the same or a different odor. It is concluded, the mushroom body extrinsic neurons measured in these experiments integrate information from their presynaptic partners,the Kenyon cells. Odors are represented in categories, however these categories are not related to the physical properties of the odor molecules. Furthermore, responses to visual and gustatory stimuli in extrinsic neurons were observed and characterized.

In Chapter 3 the focus was directed on extrinsic neurons with arborizations in the median region if the mushroom body α-lobe. Calcium imaging was employed to measure odor evoked activity in subsets of extrinsic neurons before and after subjects were trained in an appetitive odor learning paradigm (PER-conditioning). The behavioral response was monitored by recording the electro-myogram of the M17 muscle (protractor of the labium). It could be shown that bees form a robust memory in restrained conditions, while the imaging experiments were performed. Furthermore, learning related plasticity in mushroom body extrinsic neurons was found, manifested in an increased activity in response to the CS+ or in decreased activity in response to the CS– ten minutes after conditioning. This activity increase or decrease was correlated with the behavioral output. Previous findings, suggesting the mushroom body network is subject to associative and non-associative plasticity could be confirmed. The integrative properties of the extrinsic neurons may serve to relay learning specific information to downstream neuropiles.

In Chapter 4 it is described how extrinsic neurons of the mushroom body α-lobes belonging to different morphological subgroups were stained iontophoretically or via backfills. The question was addressed, whether extrinsic neurons belonging to different morphological subgroups have different physiological properties. Activity evoked by odor and sucrose stimulation of the antennae was measured in these neurons employing calcium imaging. Also, possible changes of activity induced by olfactory learning were investigated. Associative plasticity became immanent as a reduction of signal intensity in response to the rewarded odor after training and in a reduction of response variance. After the experiments the identity of the imaged structures was confirmed using confocal microscopy.

Zusammenfassung

In der vorliegenden Arbeit wurden optophysiologische Messungen, basierend auf Veränderungen in der intrazellulären Kalziumkonzentration von extrinsichen Pilzkörperneuronen durchgeführt. Aus einer Reihe von Studien geht hervor, dass die Pilzkörper, paarig vorliegende zentrale Strukturen des Arthropodengehirns, Reize verschiedener sensorischer Modalitäten verarbeiten und ein Rolle beim Lernen und der Gedächtnisbildung spielen.

Für ein weitergehendes Verständis der Beteiligung von Neuronen des Pilzkörpernetzwerkes an den Prozessen des Lernens und der Gedächtnisbildung und inwiefern diese Neurone lernbedingter Plastizität unterliegen, ist es wichtig, eine Charakterisierung der Representation von Reizen (z.B. Duftreizen) in diesen Neuronen vorzunehmen. In Kapitel 2 werden Experimente beschrieben in denen Honigbienensammlerinnen zehn verschiedenen Düften, Duftreizen von Konzentrationen verschiedener Größenordnungen und wiederholter Stimulierung mit gleichen und verschiedenen Düften ausgesetzt wurden. Darüberhinaus, wurden neuronale Antworten auf visuelle und gustatorische Stimulationen untersucht. Die Aktivität von Pilzkörper extrinsichen Neuronen während der Stimulierung wurde mittels optophysiologischer Methoden ("Calcium Imaging") untersucht. Es konnte Aktivität als Reaktion auf die Stimulierung mit zehn verschiedenen Düften gezeigt werden, die sich teilweise in ihren dynamischen Mustern unterschieden. Extrinsiche Neurone reagierten desweiteren auf Stimulierung mit allen verwendeten Konezentratio-

nen wobei sich ein sigmoides Verhältnis zwischen Konzentration und Reaktionsintensität abzeichnete. In den meisten Fällen bewirktke die Stimulierung mit einem Duft eine verminderte Reaktion auf eine folgende Stimulierung mit dem selben oder einem anderen Duft. Es wird daraus geschlossen, dass Pilzkörper extrinsiche Neurone in den vorliegenden Experimenten die Informationen ihrer presynaptischen Partner, der Kenyonzellen integrieren. Die Repräsentation verschiedener Düfte erfolgt in Kategorien, diese reflektieren jedoch nicht die physikalischen Eigenschaften der Duftmoleküle. Desweitereren wurden Reaktionen auf visuelle und gustatorische Reize beobachtet und charakterisiert.

In Kapitel 3 lag der Fokus auf extrinsichen Neuronen mit verzweigungen im medialen Teil des α-Lobus. Optophysiologische Messungen wurden genutzt um duftinduzierte Aktivtät in Pilzkörper extrinsischen Neuronen während der Konditionierung in einem appetetiven olfaktorischen Lernparadigma (Konditionierung der Rüsselstreckreaktion) zu messen. Die Verhaltensantwort wurde mittels elektromyografischer Messungen des M17 (Protraktormuskel des Labiums) überwacht. Es konnte gezeigt werden, dass Bienen während der optophysiologischen Messungen ein robustes Duftgedächtnis bilden. Ausserdem konnte Plastizität in extinsichen Neuronen im Zusammenhang mit Lernen gezeigt werden, die sich entweder in einer erhöhten Duftreaktion auf den CS+, oder einer verminderten Reaktion auf den CS– zehn Minuten nach der Duftkonditionierung manifestierte. Dieser Aktivitätsanstieg bzw. die Aktivitätsabnahme war korreliert mit dem beobachteten Verhalten. Die Ergebnisse vorangegangener Studien, die eine Beteiligung des Pilzkörpernetzwerkes an assoziativer und nicht-assoziativer lerninduzierter Plastizität vorschlagen, konnte bestätigt werden. Die integrativen Eigenschaften pilzköperextrinsischer Neurone könnten dazu dienen lernspezifische Informationen vom Pilzkörper an andere Gehrinregionen weiterzuleiten.

In Kapitel 3 wird beschrieben wie extrinsische Neurone verschiedener morphologischer Untergruppen iontophoretisch oder mittels aktiv transportierter Farbstoffe angefärbt wurden. Es wurde der Frage nachgegangen, ob extrinsische Neurone verschiedener Untergruppen Unterschiede in ihrem physiologischen Verhalten aufweisen. Durch Duft- oder Zuckerstimulation hervorgerufene Aktivität wurde mittels optophysiologischer Methoden gemessen. Darüber hinaus wurden mögliche Veränderungen in der Duftrepräsentation, hervorgerufen durch assoziative Duftkonditionierung, untersucht. Assoziative Plastizität wurde deutlich in Form von reduzierten Duftreaktionen und einer verminderten Reaktionvarianz in Bezug auf den belohnten Duft nach der Konditionierung. Im Anschluss an die physiologischen Experimente wurde die Identität der untersuchten Zellen mittels Konfokalmikroskopie überprüft.

Contents

1 General Introduction

Most animals possess the ability to modify their behavior in order to adapt to a given environmental or social context. Obviously, it is an advantage for any freely moving organism to possess the ability to interpret predictive properties from cues provided by the environment and to store such information. The identification of the structures involved in learning and memory formation and the unraveling of the mechanisms underlying the learning related plasticity of these structures, is still one of the main objectives of neuroscience.

In mammals it has been shown that various brain structures play a role in learning and memory formation. Associative memory is a fundamental form of plasticity, its neuronal correlates have been shown in a wide range of different brain areas in mammals. For example the hippocampus and the prefrontal cortex in monkeys are necessary to form associative memories, and the respective neurons of these structures exhibit learning related plasticity (Suzuki, 2007, review). In humans the activity of neurons in the amygdala and hippocampus contains information about the familiarity and retrieval of declaritive memories (Rutishauser et al., 2008).

In insects, the mushroom bodies (corpora pedunculata), paired neuropiles in the central brain, are involved in learning and memory formation. Inspired by the complexity of their structure and the fact that especially in social insects, the mushroom bodies are highly developed, Dujardin (1850) considered them as

the underlying structures of the insect's "intelligence". In honeybees it has been shown that impairment or lesioning of the mushroom bodies affects the ability for memory retrieval (Menzel et al., 1974; Erber et al., 1980). Also, for *Drosophila* it has been demonstrated that the mushroom body is involved in learning and memory formation (Heisenberg, 1989; de Belle and Heisenberg, 1994).

Studying Associative Learning in Insects

Studies on the involvement of the mushroom bodies in learning and memory formation have been primarily focused on odor learning. This approach has several advantages: Insects, such as the honeybee and, of course, *Drosophila* serve as model organisms, and from them scientists aim on gaining general insights of the principles involved in learning processes in the brain. Olfaction shares common principles in vertebrates and invertebrates (Hildebrand and Shepherd, 1997). From peripheral olfactory organs information about odors is relayed by receptor neurons to a second layer of neurons with glomerular organization, where odor identity and intensity is coded by spatio-temporal activity patterns (Galizia et al., 1999; Spors and Grinvald, 2002; Couto et al., 2005). The organization of the olfactory nervous system of insects and mammals shares fundamental similarities suggesting that the mechanisms for olfactory perception, discrimination and learning are shared as well (Davis, 2004). Further, associative odor conditioning, using the proboscis extension response (PER) is a well defined appetitive learning paradigm in the honeybee (Kuwabara, 1957; Bitterman et al., 1983). PER conditioning offers the advantages of a differential within-subject design and allows to relate effects specifically to the rewarded or unrewarded odor, respectively. Also, *Drosophila* is susceptible for associative odor learning, using classical aversive foot shock conditioning (Tully and Quinn, 1985). Numerous physiological studies have aimed on identifying and char-

acterizing neural correlates of learning, employing olfactory learning paradigms. In the honeybee Mauelshagen (1993) recorded intracellularly from an identified mushroom body extrinsic neuron (PE1) which reduces its responses to an odor presentation after pairing of the odor with sucrose reward. Hammer (1993) identified a neuron targeting the antennal lobe and the mushroom body calyces which mediates the unconditioned stimulus pathway (VUM$_{\mathrm{mx1}}$). Faber et al. (1999) and Faber and Menzel (2001) identified learning related plasticity in the antennal lobe and the mushroom bodies using calcium imaging. Grünewald (1999b) elecrophysiologically surveyed mushroom body extrinsic neurons of the protocerebral tract and found reduced odor responses after a single pairing of odor and sucrose. Recently, Szyszka et al. (2008) showed learning related plasticity in mushroom body Kenyon Cells employing calcium imaging and Okada et al. (2007) revisited the PE1-neuron and showed that in elecrophysiological recordings, it shows plasticity, while other recorded extrinsic neurons do not. In *Drosophila*, using aversive odor conditioning to induce memories, calcium imaging studies have revealed learning related plasticity in projection neurons (Yu and Davis, 2004), dopaminergic neurons (Riemensperger et al., 2005), in the mushroom body lobes (Yu et al., 2006; Wang et al., 2008) and in identified mushroom body extrinsic neurons (Yu et al., 2005; Liu and Davis, 2009). In *Drosophila*, short-term memory requires intact mushroom body γ-lobes (Zars et al., 2000) whereas long-term memory depends on the vertical lobes (Pascual and Preat, 2001). Also in the locust (Stopfer and Laurent, 1999), the cockroach (Watanabe et al., 2003) and in *Manduca* (Ito et al., 2008) efforts, focusing on learning related plasticity, have been made.

The Olfactory Pathway

In the honeybee as in other insects, olfactory information is perceived by olfactory sensory neurons located in the antennae. These neurons project into the antennal lobes, where neurons expressing the same receptor type converge onto sub-units called glomeruli (Kelber et al., 2006; Hallem and Carlson, 2006). The glomeruli are interconnected by a network excitatory and inhibitory of local neurons (Abel et al., 2001; Wilson et al., 2004). Calcium imaging studies have documented how olfactory information is initially coded by spatio-temporal patterns emerging in the antennal lobe (Joerges et al., 1997; Sachse et al., 1999; Galizia et al., 1999; Sachse and Galizia, 2002, 2003). Projection neurons from the antennal lobe ascend to higher order brain centers such as the lateral horn and the mushroom bodies (Schröter and Malun, 2000). In the mushroom body calyces these projection neurons synapse onto a high number of mushroom body intrinsic neurons called Kenyon cells (Kenyon, 1896), about 170.000 in the honeybee (Mobbs, 1982). Responses of individual Kenyon cells to odor stimulation have been shown to be highly selective and sparse, especially in the locust (Jortner et al., 2007; Perez-Orive et al., 2002; Stopfer et al., 2003), also in *Drosphila* (Wang et al., 2004; Turner et al., 2008) and in the honeybee (Szyszka et al., 2005). Moreover, input from the visual and somato-sensory system is provided to the mushroom body calyces (Mobbs, 1982; Gronenberg, 1986; Strausfeld, 2002). The functional aspects of the divergence has been associated with temporal decoding and filtering of synchronized projection neuron input (Laurent and Naraghi, 1994; Laurent et al., 1998; Szyszka et al., 2005; Ito et al., 2008) resulting in increased discrimination capacities (Smith et al., 2008) and playing an important role in learning related plasticity of the mushroom body network (Szyszka et al., 2008). In the lobes of the mushroom body, Kenyon cells converge onto a comparatively small number of mushroom

body extrinsic neurons (about 400 in the honeybee), which are mostly associated with integrating Kenyon cell input and conveying the output of the mushroom body to various brain regions (Mobbs, 1982; Rybak and Menzel, 1993). Extrinsic neurons have been shown to respond to different sensory modalities, namely olfactory, gustatory and visual stimuli (Homberg, 1984; Grünewald, 1999b), however the spatial segregation according to sensory modalities found in the mushroom body calyces seems to be preserved in the projection patterns of the Kenyon cells in the lobes (Strausfeld et al., 2000). It is therefore thought that extrinsic neurons sample different subsets of Kenyon cells associated with different sensory modalities and are involved in multi-sensory integration. Several subtypes of mushroom body extrinsic neurons have been described and classified according to the locations of their soma clusters A1-A7 (Rybak and Menzel, 1993). Functional aspects have been described in detail for neurons belonging to the A3 cluster (Grünewald, 1999b,a), also termed proto-cerebral-tract (PCT) neurons which have been shown to be GABA-immunoreactive (Bicker et al., 1985) . Dopamine-like immunoreactivity has been ascribed to neurons of the A1, A2 and A6 cluster (Schäfer and Rehder, 1989). One single identified neuron with ramifications within the lobes and pedunculus, the PE1-neuron, has been intensively studied and learning related plasticity has been described (Mauelshagen, 1993; Menzel and Manz, 2005; Okada et al., 2007). Among the functions discussed for the mushroom body is also sensory filtering (Strausfeld et al., 2000), as it is required for selective attention, choice behavior and context generalization (Liu et al., 1999; Tang and Guo, 2001; Xi et al., 2008) and the control of motor patterns (Huber, 1962; Martin et al., 1998).

Characterization of Mushroom Body Extrinsic Neurons Using Calcium Imaging

The objective of the current study is to characterize the role of the honeybee mushroom body extrinsic neurons in odor processing and to investigate possible learning related plasticity. In Chapter 2 odor representation at the level of mushroom body extrinsic neurons is characterized. Signals in response to different odors, odor concentrations and repeated odor stimulation are analyzed. Further, responses to visual and gustatory stimuli are investigated. In Chapter 3 three odors which reliably evoke excitatory responses in extrinsic neurons were selected to train bees in an appetitive associative odor learning paradigm (PER–conditioning). During the learning experiment calcium signals in extrinsic neurons are measured and the behavioral performance is monitored using electromyogram recordings from M17 (protractor of the labium). In Chapter 4 the responses of individual extrinsic neurons investigated and responses during odor and sucrose stimulation as well as during associative odor learning is analyzed in a defined subgroup of extrinsic neurons.

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2 Olfactory Representation in Mushroom Body Lobe-Neurons in the Honeybee

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2.1 Abstract/Summary

The mushroom bodies, paired central structures of the arthropod brain, have been shown to process stimuli of different sensory modalities and to be necessary for memory formation. For further investigation of how neurons, involved in the mushroom body circuitry are subject to plasticity underlying learning processes, it is important to demonstrate how stimuli (e.g. odors) are represented by these neurons. We therefore exposed honeybee foragers to a set of ten odors, to repeated stimulation with the same and different odors and to odor concentrations spanning several orders of magnitude. We also investigated responses to gustatory and visual stimuli. Activity in a subset of mushroom body extrinsic neurons of the α-lobe was investigated during stimulus presentation employing calcium imaging. We find activity, in neurons of the mushroom body output region, in response to all tested odors, exhibiting diverse temporal patterns. Extrinsic neurons also respond to all tested odor concentrations displaying a sigmoid dose-response relationship. In most cases, stimulation with one odor induces a diminished

response to consecutive stimulations with the same or a different odor. We conclude, the mushroom body extrinsic neurons measured in our experiments integrate information from the Kenyon cells. Odors are represented in categories, however these categories are not related to the physical properties of the odor molecules. Furthermore, we observed responses to visual and gustatory stimuli in extrinsic neurons.

Keywords

Insects, Mushroom Body, Neural Representation, Odor coding, Plasticity

Abbreviations

EN	Extrinsic Neuron
KC	Kenyon Cell
MB	Mushroom Body
PCT	Protocerebral Tract
PCA	Principal component analysis
PN	Projection Neuron
ROI	Region of Interest

2.2 Introduction

The mushroom bodies (MB) of insects are paired higher order multi-sensory integrating brain structures. They are involved in learning and memory formation (Menzel et al., 1974; Erber et al., 1980; Heisenberg, 1989; de Belle and Heisenberg, 1994), sensory filtering (Liu et al., 1999; Strausfeld et al., 2000) and the control of motor patterns (Huber, 1962; Martin et al., 1998). Recent studies have focused on the role of MB intrinsic and extrinsic neurons (EN) in the formation and retrieval of olfactory memory in the honeybee (Menzel and Manz, 2005; Szyszka et al., 2008; Okada et al., 2007) and in *Drosophila* (Riemensperger et al., 2005; Yu et al., 2006; Wang et al., 2008; Liu and Davis, 2009). However, so far little is known about the way different sensory modalities are represented at the level of the MB output, i.e. the ENs. Here, we investigate the representation of olfactory stimuli in MB main output region, we also provide examples of EN responses to visual and gustatory

stimulation.

For most animals, the ability to discriminate odors is important for the organization of feeding, mating and social behaviors, as well as for learning and the formation of memories associated with these behaviors. The processing of olfactory information in vertebrates and invertebrates shares common principles (Hildebrand and Shepherd, 1997): information about odors is relayed by receptor neurons from peripheral olfactory organs to a second layer of neurons with glomerular organization, where odor identity and intensity is coded by spatio-temporal activity patterns (Galizia et al., 1999; Spors and Grinvald, 2002; Couto et al., 2005). The overall similar organization of the olfactory nervous system of insects and mammals suggests that the mechanisms for olfactory perception, discrimination and learning also share common features (Davis, 2004).

To study sensory representation in a central structure of the insect brain, we focused on ENs in the MB-α-lobe. We injected the calcium sensitive dye Fura-2 into the Proto-Cerebral-Tract (PCT) approximately at the position referred to as α-exit (Mobbs, 1982). This way, we stained ENs belonging to the clusters A1/A2, A6 and A3 described by Rybak and Menzel (1993) (Figure 2.1). We imaged calcium signals in this subset of ENs of the α-lobe in response to different odors, repeated stimulation with the same odor and odor concentrations spanning several orders of magnitude. We further provide representative examples of responses to sucrose stimulation of the antennae and the representation of visual stimuli.

While the MB intrinsic neurons, the Kenyon cells (KC) respond with temporally sparse on-responses to odor stimulation and sometimes likewise sparse off-responses upon end of odor stimulation (Laurent and Naraghi, 1994; Szyszka et al., 2005; Ito et al., 2008) we observe a variety of temporal response patterns in response to different odors.
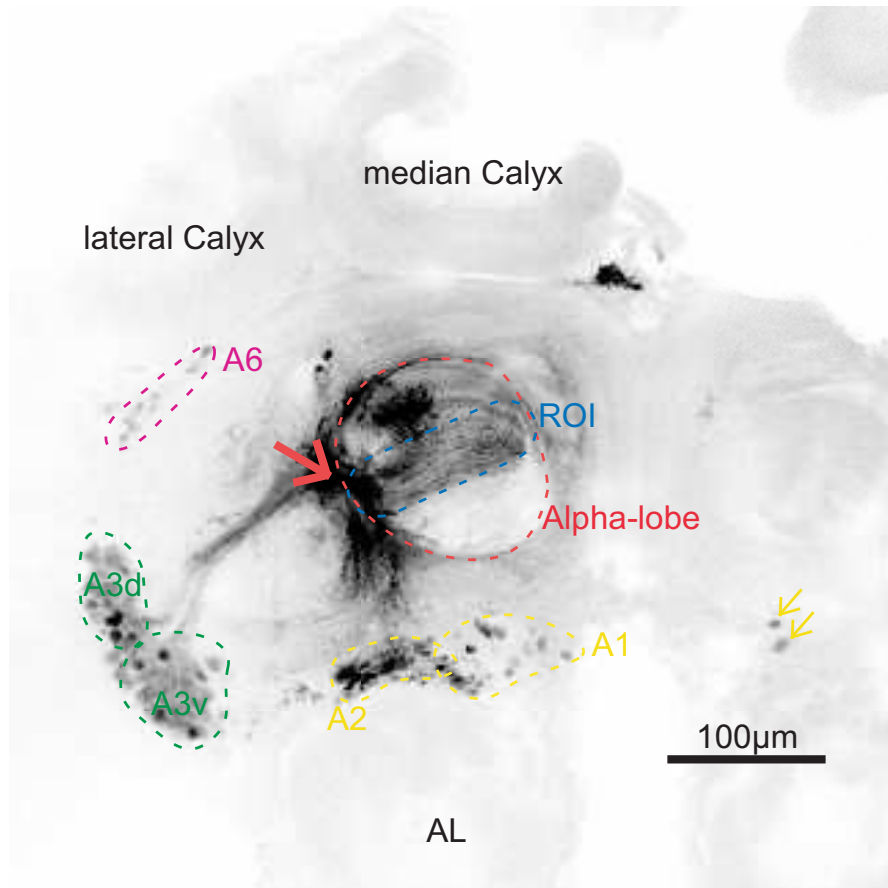
Figure 2.1: Confocal stack of one representative preparation. Red Arrow: Site of dye injection. Yellow Arrows: Somata on the contra-lateral site of dye injection, probably belonging to the A1/A2 cluster.

We also find odor concentration being represented in the responses of MB ENs. Repetitive stimulation with the same or a different odor evoked responses in ENs generally decreasing upon repeated stimulations. We identified the imaged neurons as EN dendrites in the median part of the MB α-lobe, overlapping with projection regions of KCs involved in olfactory processing (Mobbs, 1982; Strausfeld et al., 2000). We conclude that MB EN in our experiments integrate the information of KC populations. Response decline upon repeated stimulation with the same odor has been previously reported for KCs (Bazhenov et al., 2005; Szyszka et al., 2008) and suggested to be a neuronal correlate of non-associative learning processes. Coding of odor identity is transformed at the level of the MB output, into coding of categories which are not linked to functional group, chain length ore molecule class as it is for odor coding in the AL (Sachse et al., 1999).

2.3 Methods

Preparation and Dye Loading

Free flying honeybee foragers were caught at the hive entrance. They were chilled, and fixed in recording chambers as described by Szyszka et al. (2005), with some modifications. For the dye loading the head capsule was opened, and glands and trachea were moved to the side to expose the MB α-lobe surfaces. The tip of a glass capillary (Kwik-Fil, WPI, Sarasota, FL, USA) prepared with a micro pipette puller (P-97, Sutter Instruments, Novato, CA, USA) was broken at the tip at a diameter of about 10 μm, and was coated with a 10:1 mixture of Fura-2 dextran (10 000MW, Molecular Probes, Eugene, OR, USA) and lysine fixable tetramethyl-rhodamine-dextran (10 000 MW, Molecular Probes, Eugene, OR, USA) dissolved in distilled water. Dye was injected into the lateral part of the protocerebrum,

aiming at the PCT in a three/nine o'clock position of the α-lobe, referred to as α-exit (Mobbs, 1982) to stain MB extrinsic neurons which run along this tract (Figure 2.2, injection sight is indicated on standard bee brain, available for download at: `http://www.neurobiologie.fu-berlin.de/beebrain/`). After the staining procedure the head capsule was closed with the original cuticle piece. Bees were kept for 8–24 hours in a humid container at $17°$–$21°$C. Before the experiment, antennae were fixed with n-eicosan and legs and wings were cut. Part of the oesophagus, and its surrounding solid structures were gently pulled through an incision in the cuticle to prevent heavy movements of the brain (Mauelshagen, 1993). The cuticle piece on top of the brain was again removed, and the space in the head capsule above the brain was filled with a drop of two component silicone (Kwik-Sil, WPI, Sarasota, FL, USA). All gaps between the head and the recording chamber were sealed with Vaseline.

Stimulus Delivery and Imaging

We used a set of ten chemicals naturally occurring as plant odors. Odors were 1-hexanol (6ol), 1-octanole (8ol), 1-nonanole (9ol), heptanal (7al), linalool (lio), clove oil (clove), benzaldehyde (bal), cinneol (cin), citral (cit) (Sigma, Sigma-Aldrich Chemie GmbH, Munich, Germany) and rose oil (rose) (Nature, Teltau, Germany). All odors were diluted in paraffin oil (Sigma, Sigma-Aldrich Chemie GmbH, Munich, Germany).

Odor concentration was 10^{-2} (1%), except for rose oil which was used undiluted. For the generation of dose response curves we used pure octanol and octanol diluted in paraffin oil to 10%, 1%, 0.1%, 0.01% and 0.001%. Two ml of odor solution was applied to a strip of filter paper (1×3cm) of which two were placed in a plastic syrenge mounted on a custiom built, computer controlled olfactometer

as described by Galizia et al. (1997). Images were recorded at room temperature with a sampling rate of 5Hz using a TILL-Photonics imaging setup (Till Vision, Gräfelfing, Germany), mounted onto a fluorescence microscope (Zeiss, Germany). Measurements started 3s before stimulus onset and lasted for 10s. Neurons were recorded through a 60×x, 0.9W Olympus dip objective with an Imago CCD camera (640×480pixels, 4×x binned on chip to 160×120). The spatial resolution was $1.47\mu m \times 1.47\mu m$/pixel. Fura-2 was excited at 340 and 380nm for ratiometric measurements. Fluorescence was detected through a 410nm dichroic mirror and a 440nm long pass filter.

Stimulation Protocols

(I) Representation of 10 different odors: Each of the ten odors was presented to the subject 10 times in a pseudo-randomized way. Inter-trial interval was 1 minute.

(II) Representation of odor concentrations: Each odor concentration was presented to the subject three times. The experiment started with the lowest concentration followed by the second lowest and third lowest. This was repeated three times. Then the experiment continued with the three remaining concentrations in the same way. Inter-trial interval was 1 minute.

(III) Representation of repeated odor stimulation: Three odors were presented to each subject 10 times in a pseudo-randomized way. Inter-trial interval was 1 minute.

(IV) Representation of non-olfactory stimuli: Visual stimulation was a by-product of the excitation during imaging. Gustatory stimulation was delivered to the antennae as sucrose solution on the tip of a wooden toothpick.

Confocal Microscopy and Anatomy

After the experiment, bees were sacrificed and brains were removed and fixated in 4% formaldehyde in PBS overnight at 4°C and then rinsed in PBS, dehydrated in ethanol, and cleared in mehtyl salicylate. The stained brain structures were observed from the ventral surface with a confocal laser-scanning microscope (Leica TCS SP2, Leica, Wetzlar, Germany). The excitation wavelength was 543nm from a HeNe laser. The brain was scanned with an air HC PL APO objective lens of 10×/0.4NA and a 1.1-1.2 digital zoom at 4μm optical sections (Examples shown in Figure 2.1 and 2.2B).

Data Analysis

During the experiments images were inspected on a computer screen using the preview options of the recording software (TillVision, TILL-Photonics, Gräfelfing, Germany). Processing of imaging data was performed with custom written programs in IDL (RSI, Boulder, CO, USA). The ratio of Ca^{2+} signals from 340nm and 380nm measurements was calculated for each pixel. Background fluorescence (F) was determined by averaging over frames 4-13, and subtracted from the ratiometric signal (deltaF). For visual inspection, a mean of 15 frames during the stimulus (frame t15–t29) was calculated and a spatial low pass filter (3×3px) was applied. A Region Of Interest (ROI) was defined according to the observed signal (Figure 2.2 B). Figure 2.2C shows the ROIs for a total of nine bees used in the first set of experiments. Traces, reflecting the temporal pattern of the signal were calculated by averaging the pixels inside the ROIs without using any filtering. Signal intensity was calculated by averaging the values during the time window of the stimulus (t15–t29) for the on-responses (Figure 2.3). Quantitative data analysis and statistical analysis and was done in MATLAB (Mathworks, Cooperation,
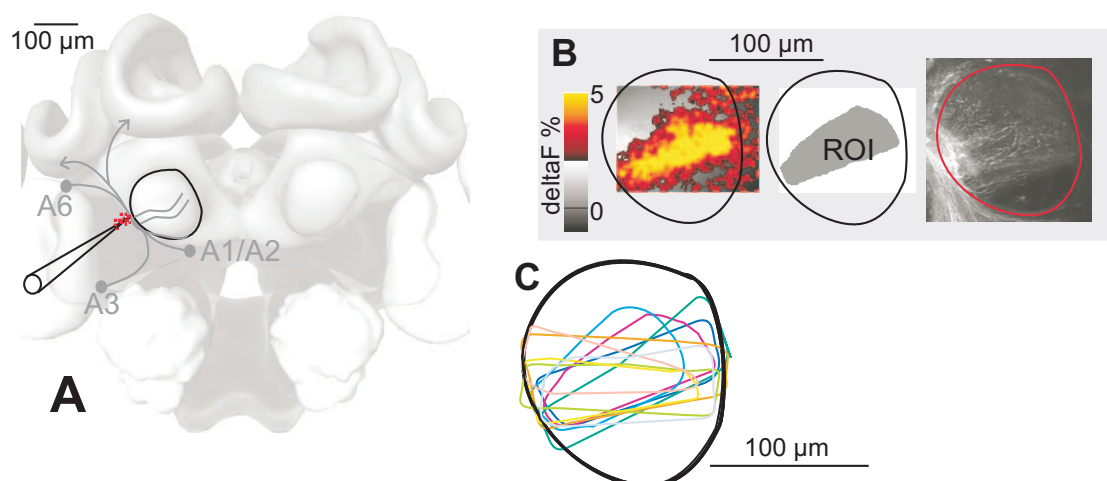
Figure 2.2: Injection site and calculation of imaging signal. **A:** Injection site at the protocerebral tract (PCT) at the α-exit of the α-lobe, outlined on a scheme of the Honeybee Standard Brain Atlas. **B:** False color image indicating the average signal during the stimulus, superimposed on raw fluorescent image of the α-lobe. Accordingly, an ROI was selected. After physiological experiments, the identity of imaged structures was confirmed using a confocal scanning microscope. **C:** Combined ROIs (n=9) overlap in median the region of the α-lobe. Images taken from the right α-lobe have been mirrored.

Natick, MA, USA).

2.4 Results

Representation of Different Odors

The dataset for this section is based on experiments with nine bees. Figure 2.1 shows a typical preparation with staining of the extrinsic neurons belonging to EN types A1/2, A3 and A6. Images were taken from the median part of the α-lobe (Figure 2.2). The selected ROIs are overlapping, suggesting overlapping subsets of EN have been imaged.

Ten different odors, naturally occurring in flowers were used to characterize odor representation in the MB lobes. Among these odors are primary alcohols of dif-
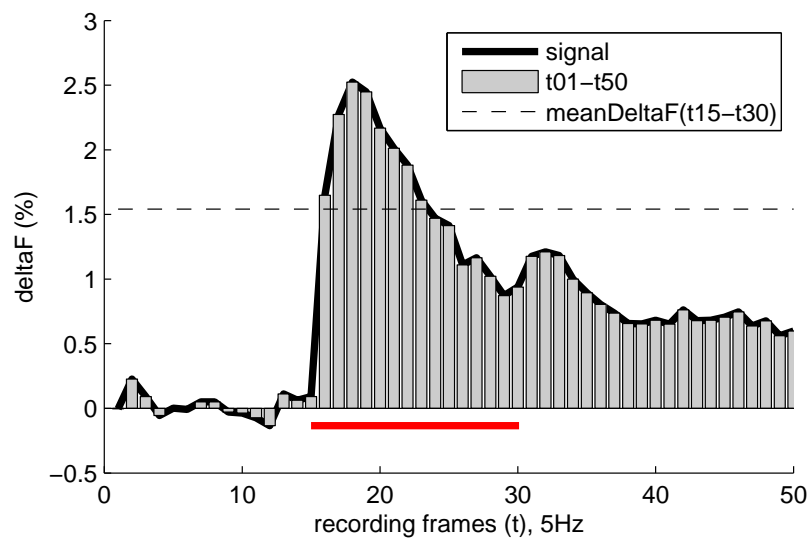
Figure 2.3: Quantification of imaging signal. The temporal dynamics of the signal visualized by connecting each value t1-t50. Signal intensity, can be calculated by averaging the values between t15 and t30. Intensity of the phasic part of the on-response was calculated by averaging across frame 15–20, intensity of the off-response was caclulated by averaging across frames 31–35 (red bar: stimulus duration, dashed line: mean signal during stimulus).
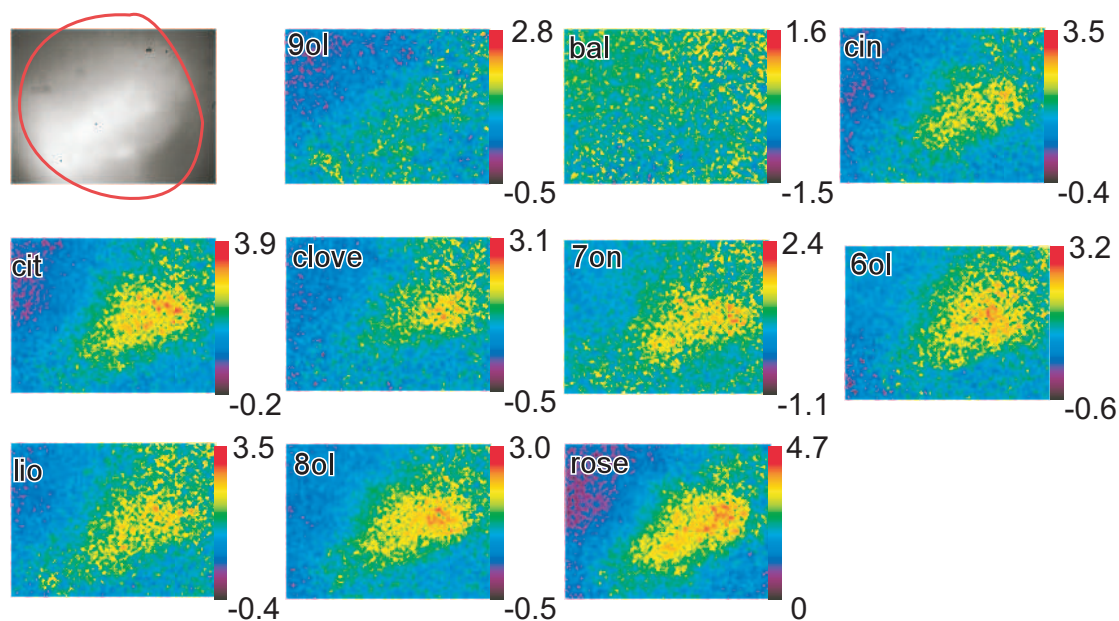
Figure 2.4: Spatial activation pattern of EN in the α-lobe. Images show mean signal (deltaF(340/380)) during stimulus (t15–t29). Scalebars give deltaF in percent for false color images. Each image is normalized to its mean during the stimulus. Upper left: grey-scale image shows raw fluorescent signal at 380nm excitation.

ferent chain length, aldehydes and aromats. Rose oil is a mixture of different molecules. The subset of extrinsic neurons we measured in our experiments, reliably responds to stimulation with all tested odors. An example for the spatial activation of EN in the α-lobe is shown in figure 2.4. The signal overlaps in the spatial domain in responses to the stimulation with different odors. The images represent mean activation patterns during the stimulus (t15–t29). For benzaldehyde, only a very small signal during the stimulus is observed.

Recordings from different animals show activity with similar temporal kinetics in response to the same odors. Eight of the tested odors elicit phasic-tonic activity patterns in ENs at odor onset. All odors also elicit off-responses upon the end of the three second stimulus, however the off-responses are not observed always in all
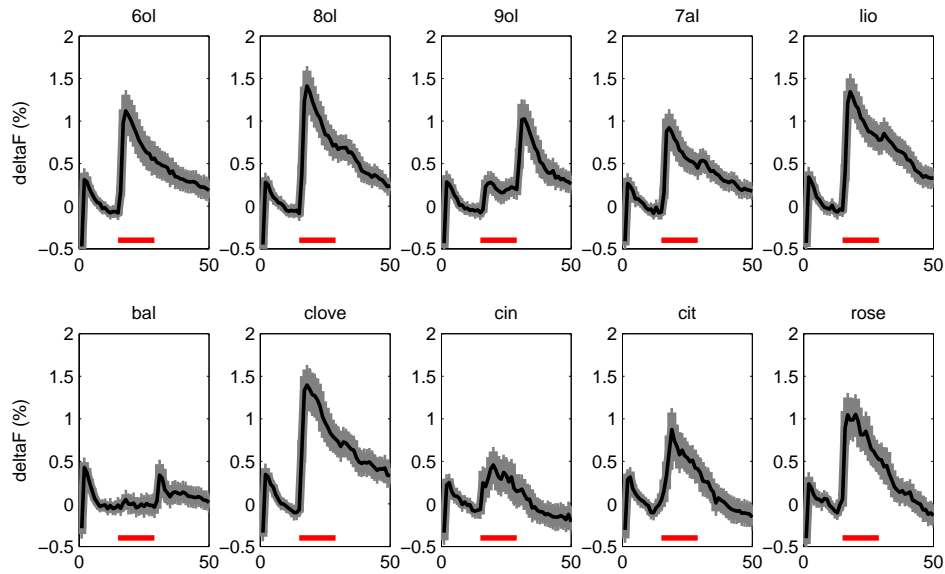
Figure 2.5: Mean signal traces in response to ten odors, naturally occurring in flowers. Red line indicates stimulus duration from t15–t29. Most odors exhibit phasic-tonic response profiles, however some odors evoke responses with different kinetic profiles. (n=30, 9 bees; error: sem)

animals. Figure 2.5 provides an overview of the different temporal kinetics of the responses to the ten tested odors.

Whereas the responses to most odors are dominated by a phasic-tonic temporal profile during the stimulus, the responses to nonanol and benzaldehyde are dominated by an off-response occurring after the end of the stimulus. As mentioned above, off-responses also occur in response to stimulations with all odors, but do not exceed the excitatory responses. Responses to 1-nonanol consist of a small tonic response during the stimulus, and a strong off-response at the end of the stimulus. For benzaldhyde, a strong off-response and almost no response during the stimulus is observed.

Each odor was presented three times to each subject in a pseudo-randomized sequence. Figure 2.6 shows that for most odors, the on-responses tend to decrease
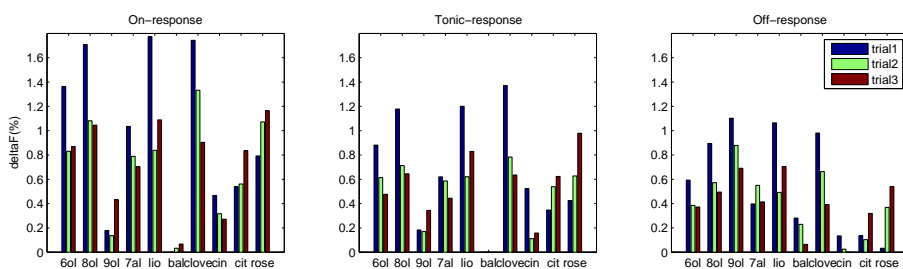
Figure 2.6: Intensities expressed as mean of on-responses (during the first third of the stimulus, t15–t20), tonic response parts (last two thirds of the stimulus, t21–t29) and off-responses (during one second after the stimulus, t30–35). The response intensity decreases upon repeated stimulation, except for odors citral and rose oil. (3 trials in n=9 bees)

upon repeated stimulation with the same odor. The first stimulation elicits in most cases the strongest responses. Also, for most odors the intensities during the phasic part of the responses are strongest during the first stimulations. Likewise, the off-responses decrease upon repeated stimulation with the same odor.

Rose oil and citral elicit excitatory phasic-tonic responses during the stimulus and small off-responses at the end of the stimulus, however there is no decrease in response intensity upon repeated stimulation with the same odor. Instead, the response intensity during all phases of the response increases upon repeated stimulation. For nonanol there is an increase in response intensity during the stimulus, but a decrease of the dominating off-response part. For benzaldehyde almost no response during the stimulus can be observed, however the off-response decreases upon repeated stimulation.

We ran a principal component analysis (PCA) on the ten-odor-dataset for the pooled responses across odors. With this method we obtain an image of the odor-space represented in the MB main output region. Plotting of the scores for the means of the different odors, gives no clustering according to functional group, or chain-length (Figure 2.7). In figure 2.7A the scores for the first and second principal components are plotted against each other. While in the first component the response strength seems to be represented, separating, for example, benzaldhyde, with almost no on-response, apart from linalool, which elicited the strongest responses during the stimulus. The second principal component accounts for the off-response assigning extreme values to nonanol and benzaldehyde. There is a tendency for cluster formation separating also hexanol and heptanal, which in the pooled curves have almost no off-responses (figure 2.5) from linalool, octanol and clove which have pronounced off-responses. Rose, cinneol and citral all load on the second component and nonanol and benzaldehyde which elicited dominating off-responses lie together in the fourth quadrant of the plot. In figure 2.7B the second principal component is plotted against the third principal component. The third principal component additionally accounts for the overall strength of the off-response and on-response. The odors with phasic-tonic on-responses cluster together around zero, while nonanol and benzaldehyde are separated. Cinneol and citral overlap and are separated from rose. However, the first two components already explain over 90% of the variance (figure 2.7C). Figure 2.7D shows the coeffcents calculated by the PCA. Note that the second component also catches an initial peak at the beginning of the measurment. We will discuss this initial peak in a following sections as a possible reperesentation of the optic stimulus induced by the excitation light during imaging.

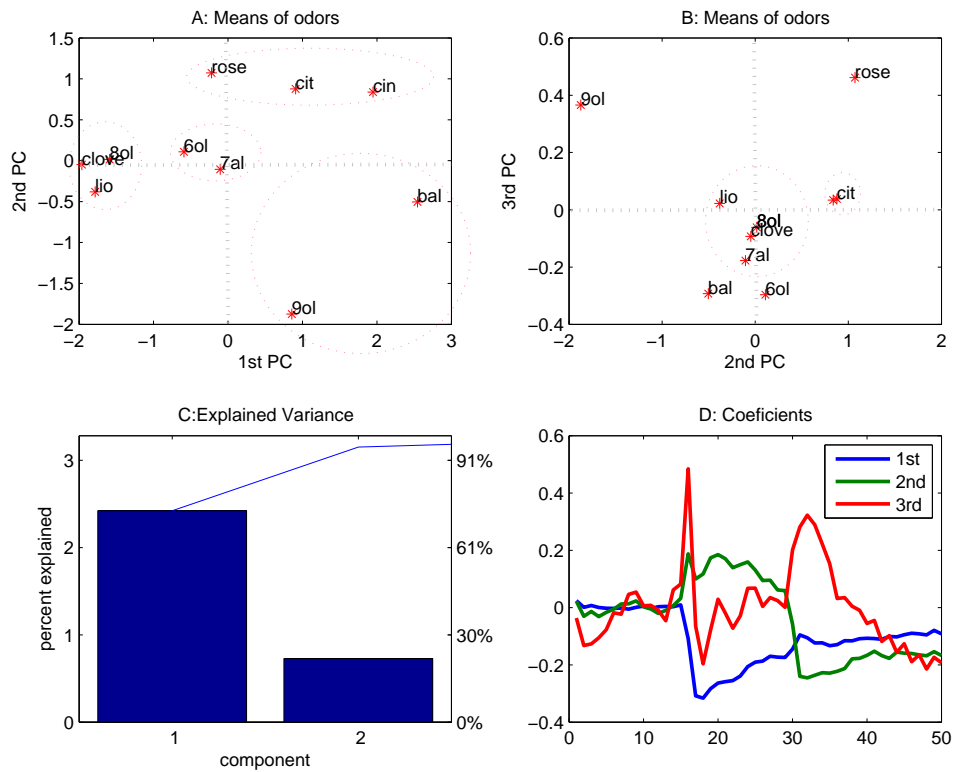We then ran a principal component analysis based on the complete original

Figure 2.7: Principal component Analysis for pooled data. **A:** Scores for the first and second principal component. **B:** Scores for the second and third principal component. **C:** Explained variance for the first and second component. **D:** Coefficients plotted for the first three principal components. (n=27, 9 bees)

10-odor-data set, comprising 270 observations, to investigate whether there is a clustering according to odors or possibly, according to the individual bees or trial number. The first three components explain about 90% of the total variance.

In figure 2.8A the scores for the first three components are plotted against each other. Different colors represent different bees. Each bee was exposed to three trials of ten different odors, thus each bee was tested 30 times. In figure 2.8A it becomes visible that the responses from different bees strongly overlap. There is no formation of clusters according to bee identity. Most scorings form a dense cloud with some outliers, for example for bee 3 which shows the highest response amplitudes.

In figure 2.8B we plotted the same data as in figure 2.8A, but assigned different colors and markers, respectively, to different trials regardless of odor. Also scores for the trial number strongly overlap considering the first three principal components. Since, as also shown in figure 2.6, the response strength increases for some odors and decreases for most others, the scores for the repeated trials do not move into one direction on the first component axis. The cloud of markers for the first trial, however, is more broadly spread than the cloud of the second trial. The third again, is more spread than the second.

In figure 2.8C we plotted the same data as in figure 2.8A and in figure 2.8B, however we assigned different colors and markers, respectively, to the different odors. Here, we observe cluster formation, e.g. for benzaldhyde and cinneol. In comparison with figure 2.5 these odors turn out to have quite different response kinetics from the majority of odor responses. Also citral, cinneol and rose cluster, separated from the main cluster comprising the responses with typical phasic-tonic patterns. This analysis confirms the results obtained from the PCA on the pooled data-set.
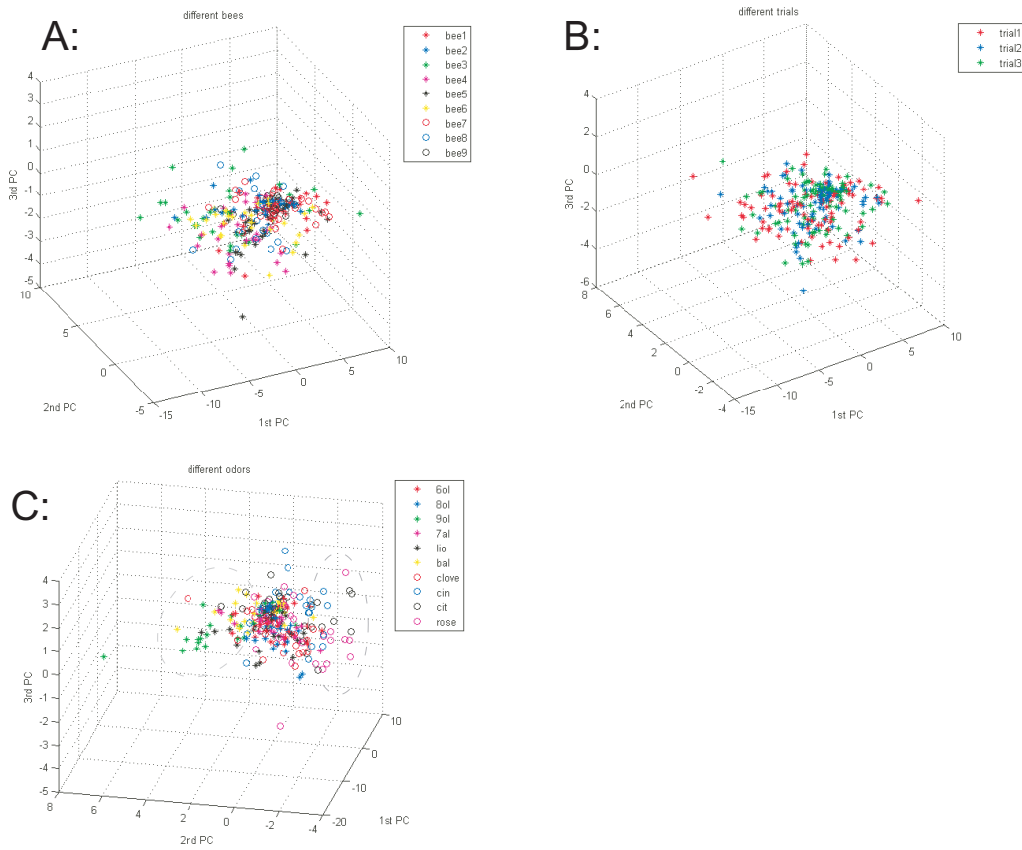
Figure 2.8: Scores for the first three components of the PCA based on the complete dataset (n=270, 9 bees). **A:** Different colors or markers, respectively, refer to different bees. There is no separation observed between individual bees. Although the scores for some bees are more widely spread than for others depending on the overall signal strength. **B:** Different colors or markers, respectively, refer to different trials. **C:** Different colors or markers, respectively, refer to different odors.

We then investigated the time trajectories obtained from a PCA on the dataset. The first three components explain about 90% of the total variance. In Figure 2.9A the time trajectories for the odor responses dominated by the on-response are plotted (first and second principal component). The trajectories set off at similar locations. The trajectories move around zero until the point of the response onset, at the beginning of the stimulus (t15). They move along the first two components until a turning point is reached at t18 (peak of response). The trajectories return to their resting position taking different courses which represent the different kinetics resulting from more or less strong off-responses. In figure 2.9B the time trajectories for the odor responses dominated by the off-response-part are plotted. For nonanol there is a loop starting at t15, representing the on-response-part, however the trajectory for benzaldehyde remains at resting position until the end of the odor stimulus at t30. At t30, both nonanol and benzaldehyde, set off until a turning point is reached at t31 (peak of the off-response) from which on the trajectories move back to resting position. The trajectories for odor responses differing from the phasic-tonic kinetics in figure 2.9A are plotted in figure 2.9C. The turning point for these responses is not reached at t18 but at t19, t23 and t25, respectively representing different response peaks.

Representation of Different Odor Concentrations

We tested six different odor concentrations of octanol in five ROIs in three bees. Octanol reliably elicits excitatory phasic-tonic odor responses in ENs (see previous section). The tested odor concentration ranges from pure odor to a 10^{-5} dilution of odor in paraffin oil. The odors were further diluted into the constant air stream. We secured, that responses to the lower concentrations are specific to the odor stimuli and not responses to the solvent, since the constant air stream is
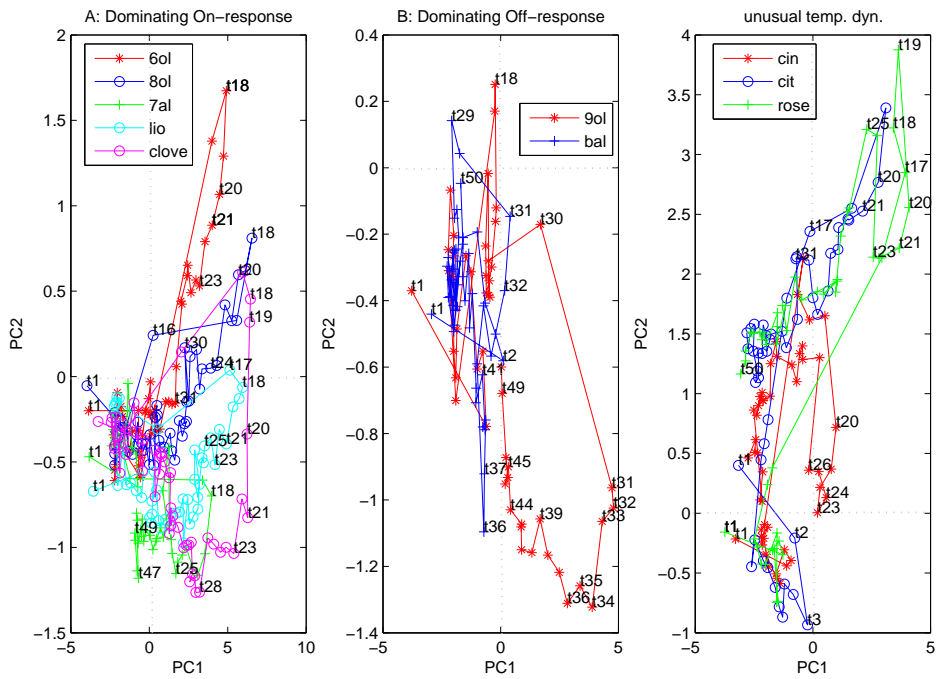
Figure 2.9: Time trajectories as revealed by a PCA on the dataset. **A:** Trajectories for responses with phasic-tonic kinetics dominated by on-response. **B:** Trajectories for responses dominated by the off-response. **C:** Trajectories for responses which differ from the clear phasic-tonic kinetics. (n = 270, 9 bees)
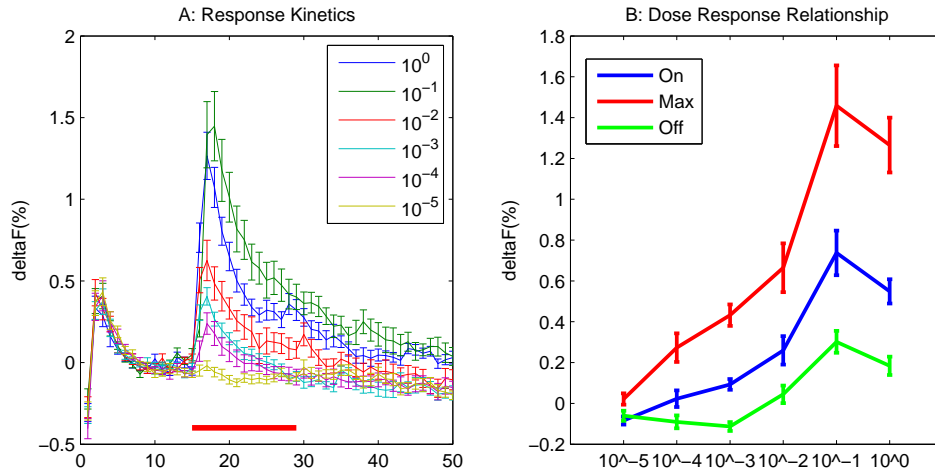
Figure 2.10: **A:** Mean responses to six different odor concentrations of octanol. Red Line indicates stimulus duration. **B:** Dose response relationship: Curves show relationship for "On" (mean response during the stimulus), "Max" (maximum of response during the stimulus), and "Off" (mean response one second after stimulus). (n=15, 3 bees, 5 ROIs, Error: sem)

directed through a vial containing a piece of filter paper with paraffin oil. Each odor concentration was presented three times to each subject and response intensity decreased upon repeated stimulation with the same concentration (data not shown). The experiments started with the lowest concentration; pure odor was presented last, to minimize sensory adaptation to the tested odor between concentrations. We find the response intensity of ENs (reflected by the signal amplitude) to be concentration dependent. The responses increase with higher concentrations, both in amplitude and duration. Figure 2.10A shows the temporal dynamics of the responses to the different concentrations. Almost no response is observed for the stimulation with the lowest odor concentration. The strongest response is elicited by the second highest concentration.

In figure 2.10B the dose-response relation ship is plotted for the mean on-response during the stimulus, for the maximum of the response amplitude and for

the mean off-response. The curves run in parallel, however there is no off-response observed for the three lowest concentrations. The second highest concentration represents the saturation level. 1% odor dilution, which was used for all odors except rose oil in all other experiments, elicits responses within the dynamic range of the dose-response relationship.

Representation of Repeated Odor Stimulation

To further investigate the repetition effect induced by repeated odor stimulation we selected three odors which reliably elicited excitatory responses (hexanol, octanol and linalool) and stimulated bees ten times with each odor. Odors were presented in a pseudo-randomized sequence to each bee. Results are plotted in figure 2.11. For hexanol there is a general tendency for a response decrease over all ten stimulations. To describe the dynamics of the repetition induced response we calculated a linear slope for each odor and each phase of the response (on-response, off-response and maximum of on-response). For the odors octanol and linalool there is no overall decrease in the slope of odor intensities over ten trials in the present data set. To quantify whether there is a significant increase or decrease in response strength we calculated the correlation between response intensity and number of measurement for the on-responses. Tabel 2.1 shows the calculated coefficients for the correlation and their p-values (Pearson's Correlation Coefficient). We find a significant correlation over ten trials for the repeated stimulation with hexanol.

We then looked at the repetition effect between stimulations with different odors. We used different sequences of odors delivered to the individual bees. The temporal dynamics of the cross-repetition effect for each bee is shown in figure 2.12 for the first three stimulations with different odors. Figure 2.13 shows the mean response intensities for the first three stimulations for each bee. For bee 2 and bee 3 the responses decrease over the three consecutive stimulations with different odors. For bee 1 the response decreases from the first to the second stimulation, however the third response exceeds the second. For bee 4 the third stimulation elicits the strongest response.
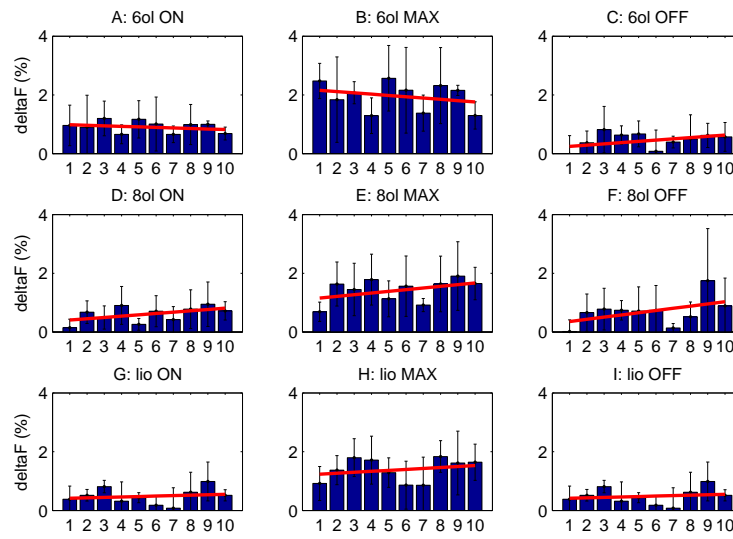
Figure 2.11: Repeated stimulation with three odors. A linear curve is fitted to the data representing response intensity (red graph). **A:** Mean on-response upon repeated stimulation with linalool. **B:** Maximum response amplitude for llinalool. **C:** Mean off-response upon repeated stimulation with linalool. **D, E, F:** Mean on-response, maximum response amplitude and mean off-response for octanol, respectively. **G, H I:** Mean on-response, maximum response amplitude and mean off-response for hexanol, respectively. (n=4 bees, error: standard deviation)

Table 2.1: Correlation between trial number and response strength

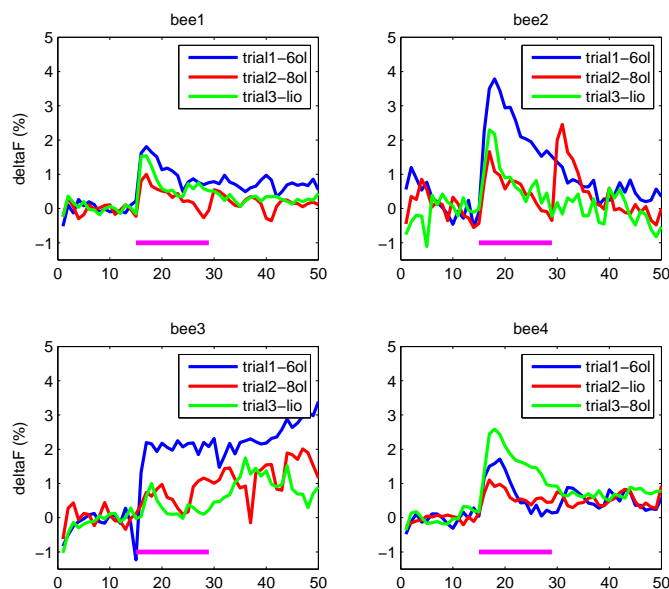|	odor	r^2 on	p on	r^2 max	p max	r^2 off	p off
bee 1	lio	.01	ns	.09	ns	.13	ns
	hex	.26	ns	.08	ns	.25	ns
	oct	.03	ns	.09	ns	.03	ns
bee 2	lio	.04	ns	.04	ns	.04	ns
	hex	.06	ns	.09	ns	.01	ns
	oct	.22	ns	.09	ns	.15	ns
bee 3	lio	.02	ns	.04	ns	.02	ns
	hex	.25	ns	.02	ns	.46	*
	oct	.02	ns	.04	ns	.02	ns
bee 4	lio	.13	ns	.09	ns	.30	ns
	hex	.00	ns	.00	ns	.30	ns
	oct	.19	ns	.26	ns	.02	ns
all	lio	.02	ns	.06	ns	.03	ns
	hex	.09	ns	.07	ns	.14	ns
	oct	.25	ns	.19	ns	.24	ns

Figure 2.12: Temporal dynamics of first three stimulations with different odors for bee 1 to bee 4 (magenta bar: stimulus duration).
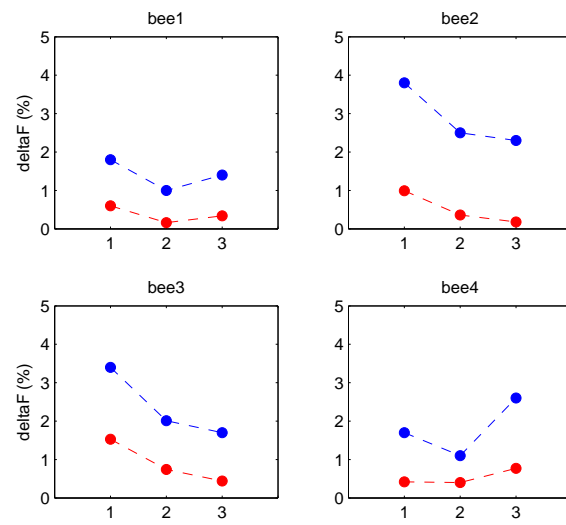
Figure 2.13: Mean response during stimulus to the first three stimulations with different odors for bee 1 to bee 4. Red graph: mean response during stimulus, blue graph: maximum response, odor sequence as in figure 2.12: 6ol, 8ol, lio; except bee 4: 6ol, lio, 8ol.
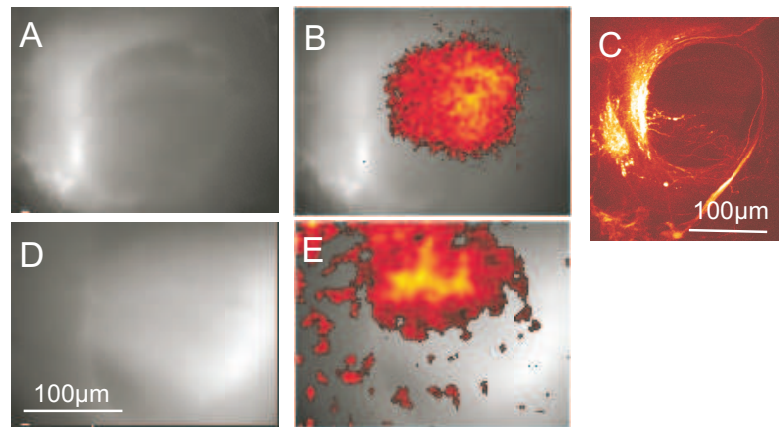
Figure 2.14: Spatial response patterns from two EN to visual stimuli induced by the excitation illumination of the mercury lamp during the calcium imaging. **A:** Raw fluorescence image for one bee. **B:** Superimposed visual signal in the upper third of the α-lobe. **C:** Stack from confocal scan of one of the EN showing the imaged neurons in greater detail. **D, E:** Raw fluorescence image and superimposed signal for another bee with a neuron responding to visual stimulation. (Signals are averaged images deltaF(340/380) of frames t1–t5)

Representation of Stimuli of non-Olfactory Modalities

In intracellular electro-physiological recordings individual EN have been already characterized as being multi sensory (Homberg, 1984; Grünewald, 1999b). We observed in a number of imaging recordings an initial peak during frames t1–t5. We interpret this initial peak as the response of ENs to visual stimulation, evoked by the light flashes used for the excitation of the calcium sensitive dye. In two bees, we found stained neurons exhibiting a light evoked activity peak at the beginning of the measurement as shown in figure 2.15. This response is very sparse in the temporal domain. The spatial signal of the odor evoked activity is shown in figure 2.14. For one preparation the confocal stack is available and shows neurons stained, overlapping with those from the previous experiments.

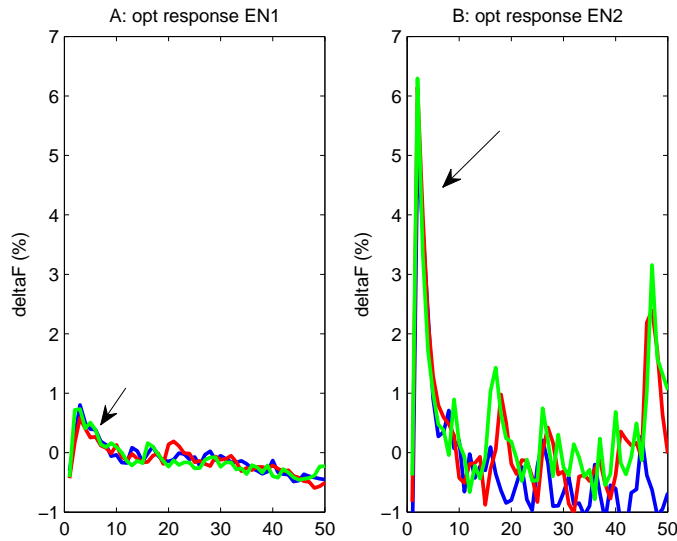We also tested gustatory responses, evoked by stimulation of the antennae with

Figure 2.15: **A, B:** Signals from two EN not responding to odor stimulation, however at the beginning of the measurement a response to visual stimulation can be observed, each preparation was measured three times. Arrows indicate response.

sucrose solution delivered on the tip of a toothpick. In figure 2.16 the spatial response patterns from EN evoked by odors and sucrose stimulation of the antennae is shown. The spatial patterns for odor and sucrose responses are overlapping, however the sucrose response exceeds the responses to the three tested odors in the shown preparation.

Figure 2.17 shows the temporal dynamics of the odor responses and the response to sucrose stimulation in one representative bee. The temporal dynamics of the sucrose response differ from those induced by odors, however this might be a by-product of the odors being precisely delivered via the computer controlled olfactometer, while for the sucrose stimulation we have no means of automatically delivering the stimulus, hence it was carried out manually, cued by an auditory signal for the experimenter.
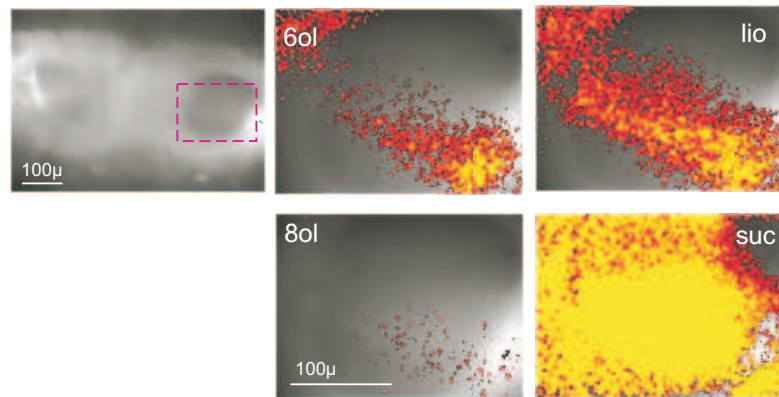
41

Figure 2.16: Spatial response patterns to odors (6ol, 8ol, lio) and sucrose stimulation of the antenna. Averaged images represent mean deltaF(340/380) during the stimulus (t15–t29) from repeated stimulations. (odors: n=10, sucrose: n=4, 1 bee)
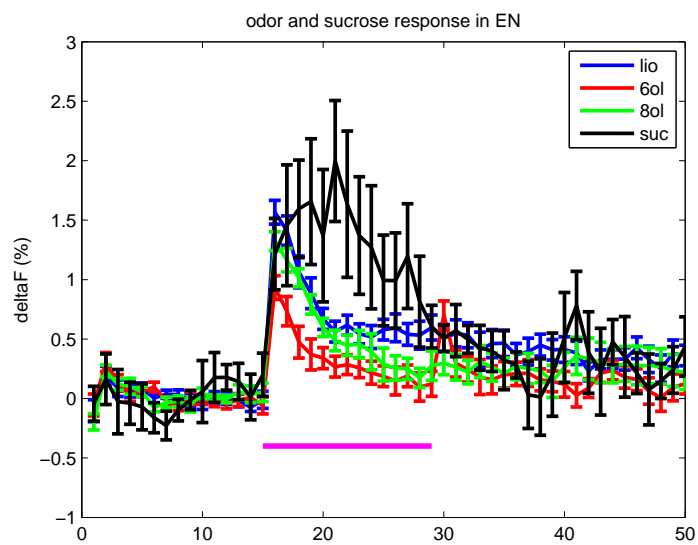

Figure 2.17: Temporal signal (traces) of calcium signal in response to sucrose and odor stimulation (odors: n=10, sucrose: n=4, 1 bee)

2.5 Discussion

We have imaged the responses to a panel of ten different plant odors, to odor concentrations spanning several orders of magnitude and to repeated stimulation with three odors in EN of the MB α-lobe. We further provide examples of responses in MB EN to visual and gustatory stimuli. To our knowledge this is the first account of odor representation at the level of the MB ENs using optical recordings. Our observations agree with previous results obtained from EN by means of electro-physiological recordings. Homberg (1984) found responses in individually recorded EN to stimulation of the antenna with rose-scent, sucrose, to mechanical stimulation of the antenna and to visual light stimuli. The mushroom body lobes probably represent the first stage at which antennal and visual information converges. However, also projection neurons have been found to respond to gustatory and olfactory stimuli (Homberg, 1984).

Further, the MB lobes are discussed to be involved in in the crosstalk between brain hemispheres and connections between the MB lobes from different hemispheres have been described (Rybak and Menzel, 1993). We did not test odors restricted to hemispheres, which in principal is possible (Sandoz et al., 2002). However, we observed MB EN in one hemisphere to respond to stimulation of the contra-lateral antenna with sucrose (data not shown). To investigate the cross talk between mushroom body lobes would be an interesting perspective for further investigations with optophysiological methods, a recent study in our lab focused on the memory transfer between hemispheres using electrophysiological recordings from MB EN, there it was shown that the lateralization of the side specific stimulus is represented at the level of the ENs up to several hours after association (Strube-Bloss, 2008).

Grünewald (1999b) described responses of A3ENs to sucrose and odor stimu-

lations consisting of phasic-tonic responses during the stimulus or on- and off-responses consisting of only one or a few spikes. The A3-neurons are very likely to contribute to a great proportion to the subsets of EN in our recordings, since we injected the dye into the tract in which their axons run (PCT). However, we observed also somata from different EN subgroups because they send dendrites around the MB lobes (compare figure 2.1 and Rybak and Menzel (1993)). The A3 or PCT-neurons are involved in an inhibitory feedback loop to the MB input (Bicker et al., 1985; Grünewald, 1999a,b).

Th MB EN receive input from a large number of KC (Mobbs, 1982; Rybak and Menzel, 1993) and the EN we have measured overlap with the projection region of KC receiving olfactory input from AL projection neurons (Strausfeld et al., 2000). Individual KCs respond very sparse to odor stimulation (Laurent et al., 1998; Szyszka et al., 2005) at the beginning and end of an odor stimulus and recently Ito et al. (2008) showed that in *Manduca sexta* on- and off-responses emerge from different subpopulations of KCs. Our data is consistent with the hypothesis that EN integrate the KC input and the responses we observe in EN may well represent these integrations. An alternative interpretations is the possibility of ENs being afferents to the MB lobe with a modulating function as suggested by Strausfeld (2002). However, so far there is no indication that MB ENs exist in the honeybee getting olfactory input from cells other than KC, which would account for the results of our physiological recordings and those of others, although bleb-like varicosities in the lateral protocerebrum, overlapping with the projection fields of PNs have been described (Rybak and Menzel, 1993).

Methodological Considerations

One main aspect of the staining procedure was to stain the same neurons across preparations. This, of course, is not fully feasible, due to the limited selectivity of the method. We nevertheless argue, to have measured signals from overlapping subsets of neurons, comparing the overlapping ROIs and because temporal activation patterns are consistent across preparations. The strength of the responses are in a close range between bees but the strength of the responses to different odors varies. In comparing the overall response strength between different odors one has to keep in mind that the vapor pressure differs among odors with e.g different chain length and that this plays a role in the way odors are perceived by the bee (Sachse et al., 1999). Calcium indicators, such as Fura-2 also act as a Ca^2+ buffer and may influence the dynamical aspects of Ca^2+-dynamics (Neher, 2008). Since, we did not analyze dynamics in detail and an intermediate temporal resolution for the measurements was chosen this is of limited importance for our questions.

Representation of Odor Categories in the Mushroom Body α-Lobe

In the response patterns of the AL molecules with similar chemical structure and carbon chain length tend to elicit similar glomerular activity patterns (Sachse et al., 1999); this is interpreted as representing the actual perceptual code of the honeybee (Guerrieri et al., 2005). We asked, whether odor coding of odor identity or chemical structure is preserved at the level of the MB main output region and manifested in different temporal kinetics. In recent experiments focusing on MB EN Strube-Bloss (2008) has characterized a subset of EN which responds unreliably to odors in general, and no distinguishable patterns in response to different odors are observed. In the subset of α-lobe ENs measured in our experiments we found odors being represented with unique time courses which allows a distinction between groups of

odors. The PCA confirms three main categories of odor responses:

1. Phasic-tonic responses dominated by an on-response

2. Responses dominated by the off-response

3. Responses with "unusual" temporal dynamics

A further particularity for two odors was that in contrary to the remaining tested odors, repeated stimulation with citral and rose odor did not induce diminished response amplitudes instead responses actually increased upon repeated stimulation. In the AL odor evoked spatial patterns are more similar for odors with the same functional group and similar chain length (Sachse et al., 1999). In the MB lobe we did not observe spatial patterns differing between odors. However, the spatial signals evoked by gustatory or visual stimulation overlaps with the odor-responses but is more broadly distributed. The visual stimulation elicits responses in the more dorsal part of the α-lobe consistent with the projection patterns of putative pre-synaptic KC, receiving input from the optical lobes (Mobbs, 1982; Strausfeld et al., 2000). The representation of odors in the MB does not reflect the precise identity of the odor or their physical properties our data rather suggests that odor categories are represented based on different properties of the odors possibly related to a higher level representation of relevant information.

Odor Concentration is Represented in the Mushroom Body Main Output Region

EN respond with increasing response intensities to increased odor concentrations up to a concentration of 10%. The response to pure odor is beyond the effective range of EN concentration sensitivity coding and the response amplitude is lower than the response to a 10% dilution. This may reflect a gain-control mechanism at some up-stream synapses influencing the dynamic range of EN responses and

consistent with the way of the different odor concentrations being represented at the level of the AL (Sachse and Galizia, 2003; Silbering et al., 2008). In a recent study Yamagata (2008) showed that information about odor concentration is coded differentially in the m- and l-ACT projection neuron tracts. The author suggests that the MBs might be involved in a behavioral switch mechanism, to distinguish between foraging phases involving high odor concentration and phases requiring the sensitivity for low concentrations. At the KC level responses are concentration dependent (Froese, personal communication), however coding of effective range may differ across KC subgroups. Possibly concentration dependency is reintegrated at the level of the MB output. Methodologically, it has to be considered that pure odors may behave different as a volatile, than in dilution with paraffin oil.

Initial Odor Stimulations Affect Response Strength in Following Stimulations

Szyszka et al. (2008) shows that KC respond to repeated odor stimulation with decreasing activity. This observation is referred to as repetition effect and suggested to be a correlate of non-associative learning processes. We observe a similar repetition effect in MB EN. During the first stimulations with different odors the response strength decreases. Also in the AL of the locust the decrease in spike frequency upon repeated odor stimulation is associated with learning processes (Stopfer and Laurent, 1999; Bazhenov et al., 2005). Ten repeated stimulation lead to a general decrease in response intensity but a gradual decrease from trial to trial could not be observed. We tried to quantify the repetition effect by correlating trial-number with response strength, however in most cases a significant correlation was not observed. Non-Associative learning, as habituation is considered to be a simple form of memory, yet its neurobiological mechanisms have not been fully understood (Wilson and Linster, 2008). In *Aplylsia* habituation of sensory

motor responses is mediated by synaptic depression (Kupfermann et al., 1970; ?). For a further investigation of whether the diminished response strength to odors in ENs represents a correlate of habituation processes, or if it is caused by sensory adaptation, one would have to find a behavior related to olfactory stimuli and habituation in the bee.

Implication for non-Odor Stimuli

Multi-sensory integration is an important task for the central nervous system and a prerequisite for associative learning and memory formation. We observed responses to gustatory stimulation and visual stimuli in MB EN. The initial peak at the beginning of the imaging measurement is observed in most preparations. We interpret this peak as response to the UV light of 340 and 380nm used to excite the Fura-2. Careful investigation of the signals shows that signals usually start with negative values, while frames 4–13 have been scaled to zero (calculation of deltaF). Indeed the response to the visual stimulation may last through the full recording and odor responses are imposed on top. In the honeybee the MB is necessary for the retrieval of olfactory memory. Therefore the information from the initially neutral stimulus (i.e. the odor) must converge with information about the reward (sucrose simulation). The VUM_{mx1} neuron, is an identified neuron in the honeybee brain shown to convey the response pathway to the MB input region (calyces) (Hammer, 1993). However, information about the reward may be relayed by parallel pathways and EN have been shown to respond to gustatory stimulation before.

Perspectives

A number of recent studies in different insect species have focused the attention on the involvement of MB EN in learning (in the honeybee: Strube-Bloss (2008); Okada et al. (2007), in *Drosophila*: Yu et al. (2005); Liu and Davis (2009) and in the locust Cassenaer and Laurent (2007)). As we have discussed here, the MB EN in the honeybee are probably not directly involved in decoding physical aspects of odors. To investigate whether the role of ENs in our experiments is in the MB network involved in attaching arbitrary meanings to odors we will further investigate EN responses during associative odor learning. Also we will focus on identifying and characterizing subtypes of ENs by means of optophysiological recordings.

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3 Plasticity in Neurons of the Mushroom Body α-Lobe after Olfactory Conditioning

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3.1 Abstract/Summary

The mushroom bodies, paired central structures of the arthropod brain, have been shown to be necessary for memory formation. Here we focus on neurons with arborizations in the mushroom body α-lobe, referred to as mushroom body extrinsic neurons. We used calcium imaging to measure odor evoked activity in subsets of extrinsic neurons before and after subjects were trained in an appetitive odor learning paradigm (PER-conditioning). The behavioral response was monitored by recording the electro-myogram of the M17 muscle (protractor of the labium). We show that bees form a robust memory in restrained conditions while we perform the imaging experiment. Further, we find learning related plasticity in mushroom body extrinsic neurons manifested in an increased activity in response to the CS+ or in decreased activity in response to the CS– ten minutes after conditioning. This activity increase or decrease is correlated with the behavioral output. We confirm previous findings suggesting the mushroom body network is subject to associative and non-associative plasticity. The integrative properties of the extrinsic neurons may serve to relay learning specific information to

downstream neuropiles.

Keywords

Associative Learning, Non-Associative Learning, Plasticity, Calcium Imaging, Insects, Mushroom Body

Abbreviations

AL	Antennal Lobe
CS+	Conditioned Stimulus (rewarded)
CS–	Conditioned Stimulus (unrewarded)
Ctrl	Control Odor (untrained)
EN	Extrinsic Neuron
KC	Kenyon Cell
ITI	Inter Trial Interval
lio	Linalool
M17	Protractor of the Labium
MB	Mushroom Body
PCT	Protocerebral Tract
PER	Proboscis Extension Response
PN	Projection Neuron
ROI	Region of Interest
US	Unconditioned Stimulus (reward)
6ol	Hexanol
8ol	Octanol

3.2 Introduction

Learning leads to behavioral changes and modified brain function and structure. The potential for such changes is usually referred to as plasticity. Insects are excellent models for physiological studies of brain function and plasticity, because their nervous system is easily accessible for electronic or optic recordings while, as in the case of olfactory learning in the honey bee, the relevant behavior may be observed. This makes the odor learning paradigm especially advantageous and olfactory processing shares common principles in vertebrates and invertebrates

(Hildebrand and Shepherd, 1997). Also, olfactory learning and neuronal plasticity has been suggested to follow common rules in insects and vertebrates (Davis, 2004).

In implicit learning we differentiate between associative forms of learning (e.g conditioning) and non-associative forms of learning (habituation, sensitization) (Kandel, 2001). Although, our experiments focus on the formation of associations between odors and reward, our experimental design allows us to observe non-associative learning, manifested in response changes to repetitive odor stimulation (repetition effect). Receptor neurons can adapt to a certain degree, to prolonged or repeated stimulation, however, behavioral habituation is regarded as a central phenomenon (Wilson and Linster, 2008, review).

In associative odor learning, a previously neutral odor stimulus is associated with an arbitrary meaning, e.g. a reward or an aversive stimulus. In the honeybee the formation of odor memories depends on parallel and sequential pathways, involving at least the first olfactory relay center, the antennal lobe (AL) and the mushroom bodies (MB)(Hammer and Menzel, 1998; Faber et al., 1999; Szyszka et al., 2008). Also in *Drosophila* the involvement of different neuronal populations in memory formation has been documented (Yu and Davis, 2004; Yu et al., 2005, 2006; Wang et al., 2008) . Here we focus on the honeybee MB, a central structure of the insect's brain. The MBs are involved in learning and memory formation (Menzel et al., 1974; Erber et al., 1980; Heisenberg, 1989; de Belle and Heisenberg, 1994), sensory filtering (Liu et al., 1999; Strausfeld et al., 2000) and the control of motor patterns (Huber, 1962; Martin et al., 1998). There have been several studies employing various techniques, such as Ca^{2+} Imaging (Szyszka et al., 2008) intracellular electrophysiological recordings (Grünewald, 1999b; Okada et al., 2007), as well as extracellular electrophysiological recordings (Strube-Bloss, 2008) and pharmacology (Komischke et al., 2005) aiming on revealing the role of the honeybee

MB neurons during memory formation and underlying mechanisms of associative plasticity. Associative and non-associative plasticity has been described at the input side of the MB in the calyces, in the Kenyon cell dendrites (Szyszka et al., 2008), and in a single identified MB extrinsic neuron, the PE1 (Okada et al., 2007; Menzel and Manz, 2005; Mauelshagen, 1993). Appetitive learning is mediated by the VUM_{mx1} neuron with its soma residing in the sub-oesophageal ganglion and octopaminergic terminals targeting the antennal lobes and MB calyces (Hammer, 1993).

In order to study learning related plasticity in the MB lobes, calcium imaging was used, which is well established in the honeybee to measure activity in the AL, the first olfactory relay station in insects (Joerges et al., 1997; Galizia et al., 1997, 1999; Sachse et al., 1999; Sachse and Galizia, 2002; Peele et al., 2006) and in the MB input region (Faber and Menzel, 2001; Szyszka et al., 2005). In this study we went one step further downstream of the olfactory pathway, focusing on neurons sending dendrites and axon collaterals into the MB lobes, the so called MB extrinsic neurons (EN) which mainly provide the output from the MB to other brain areas (Mobbs, 1982; Rybak and Menzel, 1993). The MB ENs have been extensively studied regarding their morphology (Mobbs, 1982; Rybak and Menzel, 1993; Strausfeld et al., 2000). EN have been shown to provide the main output from the MB, but also structures resembling pre-synaptic terminals belonging to EN in the lobes of the MB have been described (Strausfeld, 2002). We used repeated odor stimulations to investigate non-associative learning processes and an appetitive odor learning paradigm to study associative plasticity. We used a differential approach, to distinguish between excitatory and inhibitory learning effects. In the honeybee, we can take advantage of the proboscis-extension-response (PER) (Kuwabara, 1957) to condition subjects, i.e. to induce associative odor memo-

ries (Bitterman et al., 1983). Further, the behavioral output can be monitored and quantified by recording electro-myograms from the protractor of the labium (M17) (Rehder, 1987). We confirmed that bees form robust odor memories in restrained conditions while we perform calcium imaging. We further found plasticity related to non-associative learning in MB ENs, manifested in an overall decrease in response intensity upon repeated stimulation with the same odor, or repeated stimulation with sucrose solution. Associative plasticity is found in ENs, manifested in either, an increase of activity in response to the CS+ after conditioning, or a decrease of activity in response to the CS– after conditioning. We also find possible correlates of generalization in ENs. We comprised our findings, combined with findings from recent studies in a simple model of the MB circuitry to summarize our current view on olfactory processing and memory formation in the central brain of the honeybee.

3.3 Methods

In vivo Bee preparation and Dye Loading

Experiments were performed with pollen-foraging worker bees caught with small glass containers at the hive entrance upon their return from a foraging bout. They were brought into the laboratory and chilled for immobilization. After a few minutes they were mounted into special recording chambers (Galizia and Vetter, 2004). Eyes and thorax were fixed to the recording chamber with low melting point hard wax (Deiberit 502). After 20 minutes they were fed 20 μl of 30% sucrose solution and stored in a humid case. Staining procedure followed 1-4 hours later. The antennae were immobilized with n-iscosane. The head capsule above the brain was opened by removing a piece of cuticle and pushing glands

and trachea to the sides to allow access to the MB. A 10:1 mixture of FURA-2 dextran (potassium salt, 10 000 MW, Molecular Probes, Eugene, OR) and fixable tetramethylrhodamine dextran (10 000 MW, Molecular Probes, OR) was injected into the brain laterally to the MB α-lobe at an approximate 3/9 o'clock position (figure 3.1) using a glass capillary prepared with a puller (P-97, Sutter Instruments, Novato, CA) with a diameter of ca. 10μm. The staining probes were injected until the dye dissolved from the tip. Trachea and glands were put back into place and the cuticle piece was restored to its place in the head capsule. The antennae were losened. After about 20 minutes, bees were fed until satiation and put back into the humid case and stored over night at 20°C. Experiments started the next day. Therefore, the antennae were immobilized using n-eicosane. The abdomen and thorax were immobilized using either dental wax or a small piece of sponge that was pressed against the abdomen and fixed with a clip without damaging the bee. To prevent movements of the brain resulting from pumping from the oesophagus a small incision was cut into the cuticle above the labrum and part of the oesophagus and its surrounding solid structures were put under tension without damaging the oesophagus (Mauelshagen, 1993). The previously cut piece of cuticle above the brain was removed, and trachea and glands were partly removed, where they were covering the mushroom bodies. Heamolymph was sucked from the head capsule with a Kimwipe. All sites of surgery were covered with two component silicone (KWIKSIL, WPI). For the imaging experiment a drop of water was placed between the head and and dip objective such that it could be immersed into the droplet. Bees were placed on the microscope stage in front of an exhaust.
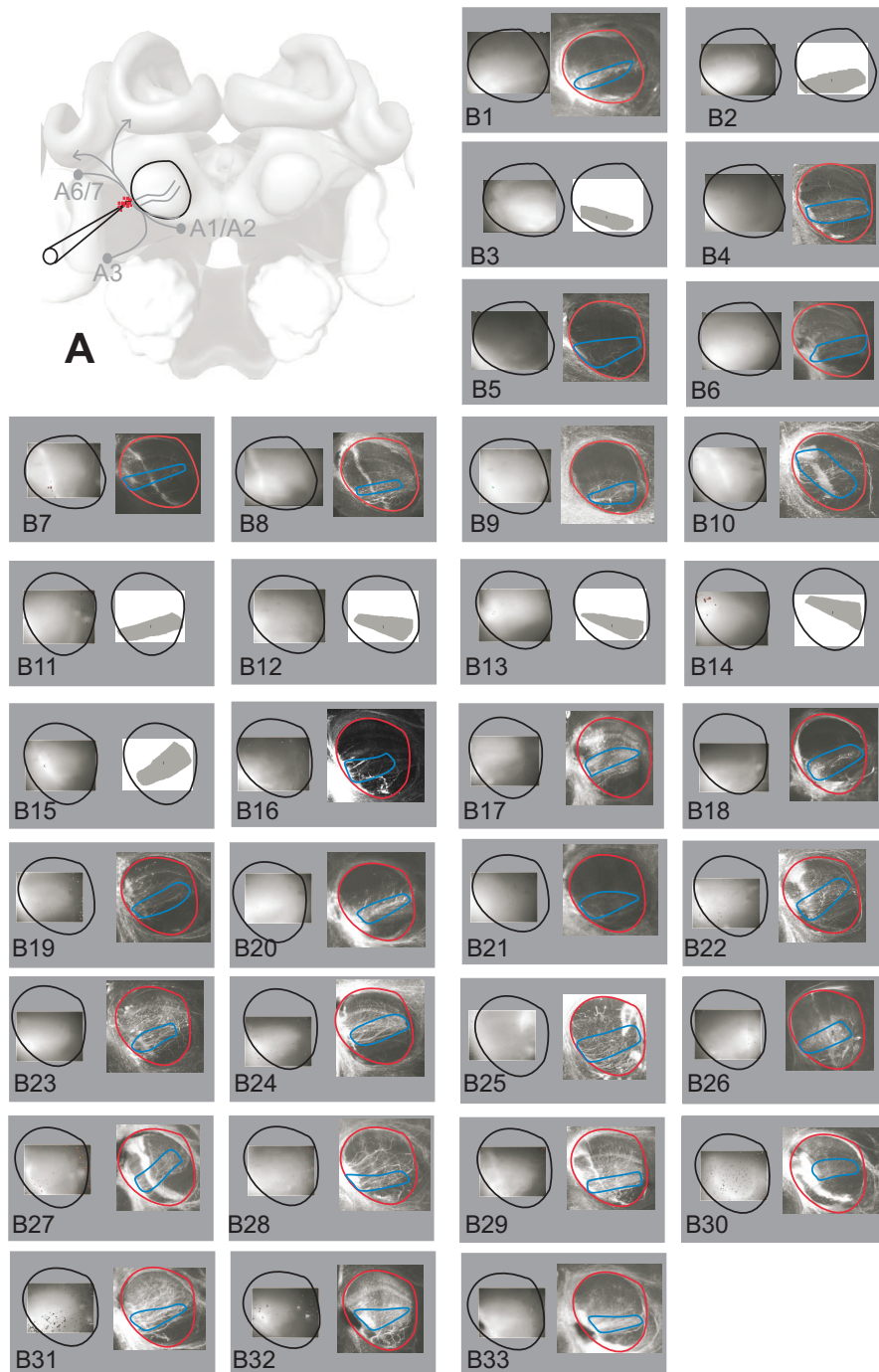
Figure 3.1: Injection site and confocal stacks **A:** Injection site into the protocerebral tract at the α-exit of the α-lobe, outlined on a scheme of the honeybee standard brain atlas. **B1-33:** Raw-flourescence images and confocal stacks of α-lobes from each bee. ROI as taken for the signal calculation is outlined in blue. Limits of α-lobe indicated in black and in red, respectively. Right α-lobes are mirrored.

Odor Stimulation and Imaging

Odors used were: hexanol (6ol), octanol (8ol) and linalool (lio) (all from Sigma). The odors were diluted in paraffin oil (Merck) at 1%. 20 μl of odor solution were placed on a piece of filter paper (3×1cm), of which two were placed in a 10ml plastic syringe. Using a computer controlled custom built olfactometer (Galizia et al., 1997), odors were diluted in a continous airstream which was directed towards the antennae. Odors were presented as 3 second pulses consisting of about 0.2ml of odor saturated air. Images were recorded at room temperature with a sampling rate of 5Hz using a TILL-Photonics imaging setup mounted on a fluorescence microscope (Axioskop, Zeiss, Germany). Measurements started 3 seconds before stimulus onset and lasted for 10 seconds. Neurons were recorded through a 60×, 0.9 W Olympus dip objective with an Imago CCD camera (640×480pixels, 4× binned on chip to 160×120). The spatial resolution was 1.47μm×1.47μm/pixel. Fura-2 was excited with illumination of 340 and 380nm for ratiometric measurements. Fluorescence was detected through a 410nm dichroic mirror and a 440nm long pass filter.

Experimental Protocol

The experimental protocol (Fig.1B 3.2) consisted of three phases: (i) pre-phase, (ii) training-phase and (iii) post-phase. In the pre-phase the three odors were each presented six times in a pseudo-randomized way with 1 minute Inter-Trial Interval (ITI). Training started 10 minutes after the pre-phase. During the training-phase one of the odors was paired with the delivery of 30% sucrose solution, on the tip of a toothpick delivered to the antennae and the proboscis. The sucrose stimulation was applied manually and was triggered by an accustic signal 1 second after odor onset. A second odor was presented during training without pairing. The two
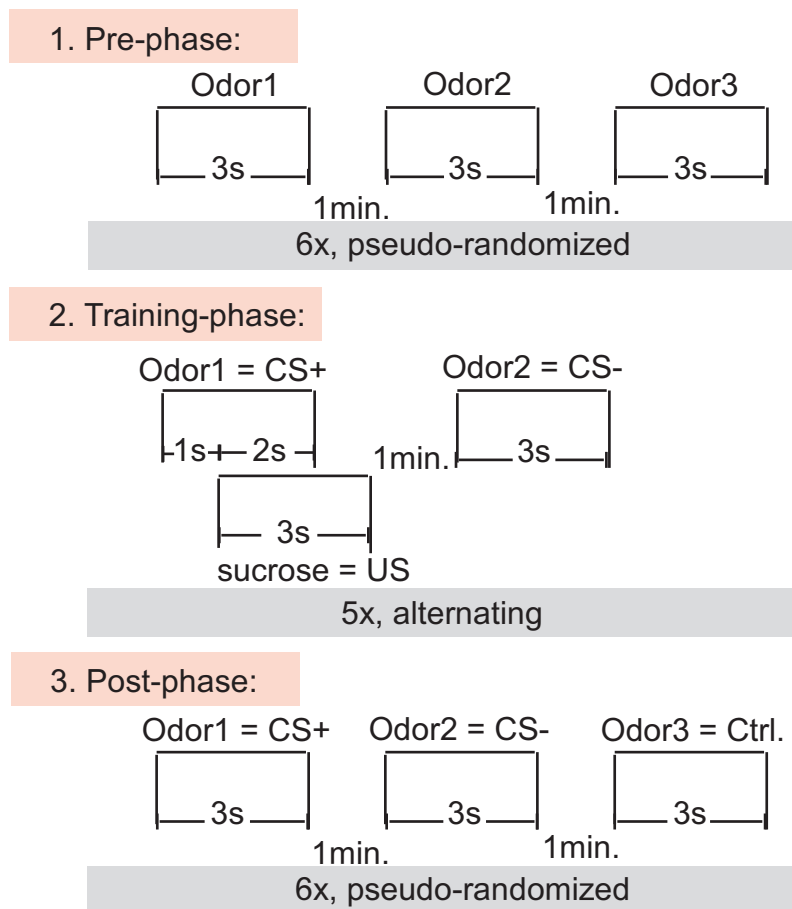
Figure 3.2: Stimulation Protocol: The experiment consisted of three experimental phases. Between each phase there was a break lasting 10 minutes.

odors presented during training would therefore be referred to as "CS+" and "CS–", respectivly. ITI during training was 1 minute. CS+ and CS– were presented alternately, each for 5 trials. Post-phase started 10 minutes after training. During the post-phase, all three odors were presented again for at least 6 trials each. The odor that was not presented during training would be referred to as "Control". ITI was 1 minute. CS+, CS– and Control were balanced for odors across animals (table 3.1). After the post-phase sucrose solution was presented alone for three trials.

M17 Recording

The protractor muscle of the labium (M17) was recorded extracellularly to monitor the behavioral responses related to learning i.e. the PER (Rehder, 1987). Therefore a copper wire was injected into the muscle close to the mouth parts, which is a modification of the original preparation described by Rehder (1987), to avoid interference with the recording by potentials originating in optic tract neurons evoked by the UV-light used to excite the calcium sensitive dye. A ground electrode was injected through the compound eye. The muscle potentials were amplified with a custom build pre-amplifier and recorded and digitalized with a CED system (Texas Instruments) and visualized on a computer screen using Spike2 (Texas Instruments). Responses were measured as number of spikes (above a certain threshold) per second during the three second stimulus subtracted by the number of spikes per second during three seconds before the stimulus. Normally there are no spikes detected when the muscle is at rest (figure 3.3).

Data Analysis

Processing of imaging data was performed with custom written programs in IDL (RSI, Boulder, CO). The ratio of Ca^{2+} signals from 340nm and 380nm measurements was calculated for each pixel. Background fluorescence (deltaF) was determined by averaging over frames t4–t13 and subtracted from the ratiometric signal (deF). For visual inspection a mean of 15 frames during the stimulus (frame t15–t29) was calculated and a spatial low pass filter (3×3px) was applied. The averaged image was displayed as a false color image.

Activity in neuronal structures was determined as activity spots in the false color images. Active regions were determined as Regions Of Interest (ROI) and the temporal dynamics of the activity was calculated as average of the pixels
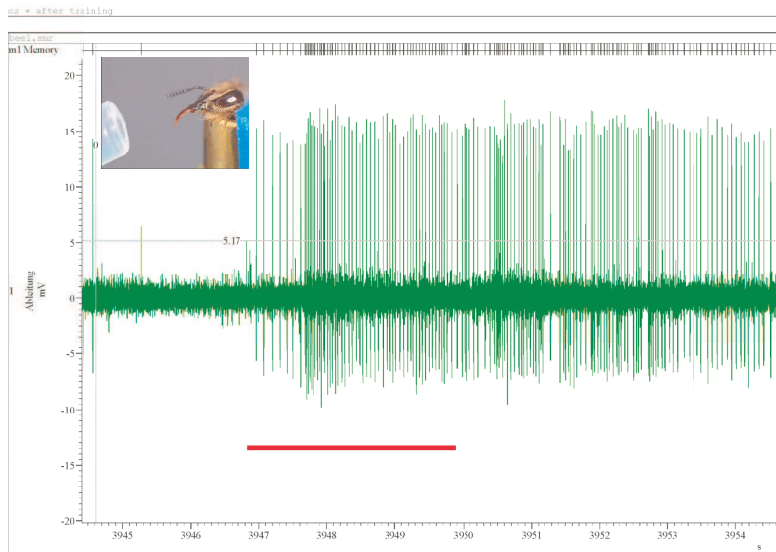
Figure 3.3: M17 response: After the pairing of an odor with a sucrose reward, the bee extends its proboscis in response to the odor alone. Red line indicates odor stimulus. Inset: The PER of the honey bee (Photo: B. Komischke).

in each ROI without any filtering or correction. The obtained time courses were further analyzed in Matlab (Mathworks Cooperation, Natick, MA, USA). Stimulus intensity was calculated as mean signal during the stimulus (frame 16–29) or during the initial stimulus (frame t16–t20) for the phasic component of the response and during frame 30–35 for "Off"- responses. Quantitative data analysis and statistical tests were performed in Matlab (Mathworks, Cooperation, Natick, MA, USA).

Morphological Analysis

After physiological measurements brains were dissected and fixated in 4% formaldehyde in PBS overnight at 4°C and then rinsed in PBS, dehydrated in ethanol, and cleared in mehtyl salicylate. The brain was placed onto an object slide under a coverslip and observed from the ventral surface with the use of a confocal laser-scanning microscope (Leica TCS SP2, Leica, Wetzlar, Germany). The excitation

wavelength was 543nm with a green HeNe laser. The brain was scanned with an air HC PL APO objective lens of $10\times/0.4$NA and a 1.1–1.2 digital zoom at 4μm optical sections. The mushroom bodies were scanned with an oil objective and 1.6–1.7 digital zoom at 2μm optical sections.

3.4 Results

We inspected odor responses to three selected odors (linalool, octanol and hexanol) during odor learning in MB ENs, in a total of 33 bees using calcium imaging. We backfilled MB ENs by injecting the membrane impermeable calcium indicator Fura-2 into the neurons' axons laterally to the MB lobes. We focused on the EN dendrites in the α-lobe of the MB. The lobes are thought to be the main output region of the MB where a high number of MB intrinsic neurons (KC) synapse onto ENs which connect the MB to other brain areas. The neurons imaged in our experiments arborize in the median part of the α-lobe which is known to be the projection site of KC, which receive input from olfactory projection neurons. Figure 3.1B1–B33 shows the Regions Of Interest (ROI) we selected according to the observed signal in stained ENs, superimposed onto confocal stacks of the stained structures. The pictures to the left of figure 3.1B1–B33 represent raw fluorescent images at 380nm illumination. The confocal images of the stained neurons represent stacks from confocal scans of the fixed and cleared brains after the imaging experiments. The confocal scans reveal overlapping sets of ENs were stained. We trained all 33 bees in an appetitive odor learning paradigm, according to the protocol described above (figure 3.2). M17 responses were recorded in a total of 29 bees. Table 3.1 provides an overview of the learning experiments. About 20 bees learned to extend the proboscis to the trained odor stimulus (CS+), however not all bees were able to discriminate the rewarded odor reliably from the

unrewarded and the control odor. In some cases bees generalized the PER to the control odor; in other cases the bees failed to discriminate between CS+ and CS–. Linalool, hexanol and octanol were used as CS+, CS– and control odor, respectively in a balanced design.

Bees Form Robust Odor Memories During Imaging Experiments

We trained bees in an appetitive odor learning paradigm. To confirm and quantify the conditioned proboscis extension response (PER), we successfully recorded the electro-myogram of the protractor muscle of the labium (M17) in 29 bees. This technique serves to make the evaluation of the PER more objective, since we are able to record stimulus on- and and offset together with the M17 related muscle potentials. The signals are digitized and stored on a computer, while imaging can be performed in the dark. After the experiment, the experimenter analyzes the responses, off-line, in "blind", hence not knowing which was the rewarded and which was the unrewarded odor. The spikes related to the PER are quantified and normalized to the maximum response of each bee, excluding the training responses to the US. Figure 3.4 shows the M17 responses to the CS+, the CS– and the control odor during the pre- and post-phase of the experiments. Responses to the CS+ observed during the post-phase have increased significantly. We observe a considerable fraction of bees already responding to one or more odors during the pre-phase. However, responses to CS– and control odor observed during the post-phase have significantly decreased. Figure 3.4D shows averaged responses to CS+, CS– and control odor before and after training, respectively. In most of the bees which extended the proboscis in response to the rewarded odor during the post-phase, the respective response intensity decreases rapidly across repeated trials.

Table 3.1: Experiments overview

Bee No.	CS+	CS–	Ctrl.	M17
1	6ol	lio	8ol	+
2	lio	6ol	8ol	+
3	lio	6ol	8ol	+
4	8ol	lio	6ol	+
5	8ol	lio	6ol	+
6	6ol	8ol	lio	−
7	6ol	8ol	lio	+
8	8ol	lio	6ol	+
9	lio	8ol	6ol	+
10	lio	8ol	6ol	+
11	6ol	lio	8ol	+
12	8ol	6ol	lio	+
13	6ol	8ol	lio	+
14	8ol	6ol	lio	−
15	lio	hex	oct	+
16	6ol	8ol	lio	−
17	8ol	6ol	lio	+
18	6ol	lio	8ol	+
19	8ol	lio	6ol	+
20	lio	8ol	6ol	+
21	8ol	lio	6ol	+
22	lio	8ol	6ol	+
23	8ol	lio	6ol	−
24	lio	6ol	8ol	+
25	lio	6ol	8ol	+
26	6ol	lio	8ol	+
27	6ol	8ol	lio	+
28	6ol	8ol	lio	+
29	8ol	lio	6ol	+
30	lio	8ol	6ol	+
31	6ol	8ol	lio	+
32	6ol	8ol	lio	+
33	8ol	6ol	lio	+
$\sum$(B)=33	$\sum$(6ol)=12	$\sum$(6ol)=9	$\sum$(6ol)=12	$\sum$(M17)=29
	$\sum$(8ol)=11	$\sum$(8ol)=13	$\sum$(8ol)=9	
	$\sum$(lio)=10	$\sum$(lio)=11	$\sum$(lio)=12	
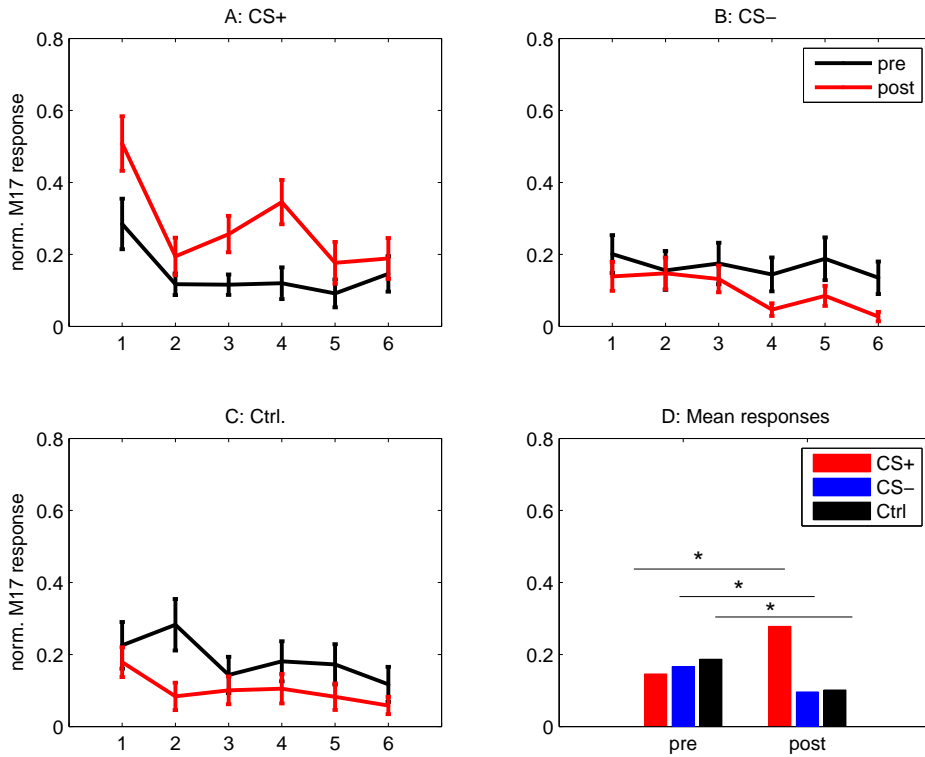
Figure 3.4: M17 responses during the imaging experiments. **A:** Responses to CS+, black graph: pre-phase, red graph post-phase, error: sem. **B:** Responses to CS–, black graph: pre-phase, red graph post-phase, error: sem. **C:** Responses to Ctrl., black graph: pre-phase, red graph post-phase, error: sem. The responses decrease acorss repeated stimulation in the pre- and post phase. CS+ responses have increased in the post test, while the responses to CS– and Ctrl. have decreased.(Threeway-ANOVA: $F(2,29)_{condition} = 9.52$, $F(5,29)_{trials} = 6.22$, $F(2,29)_{condition \times phase} = 18.74$, $p < 0.01$, $F(10,29)_{condition \times trial} = 2.06$, $p < 0.05$, Bonferroni-Post hoc test.) **D:** Averaged responses across trials, the response to CS+ has increased after training, while the responses to CS– and Ctrl. have decreased (Wilcoxon-signed-rank-test, * $p < 0.05$).

Figure 3.5 shows the different learning dynamics of the individual bees described above. Some bees respond to one or more odors already during the pre-phase. In some cases the preference is fully or partially unlearned (e.g bee5 and bee 28). In other cases the bee fails to unlearn the initially preferred odor. Some bees generalize between all presented odors (e.g. bee11). In few cases bees exclusively respond to CS+ in the post phase (e.g. bee9 and bee10). However across, the sample size the formation of robust odor memories can be detected, as revealed by the ANOVA.

These results confirm, bees can form a robust odor memory in restrained conditions during the imaging experiment. However, the learning dynamics are subject to considerable variability.
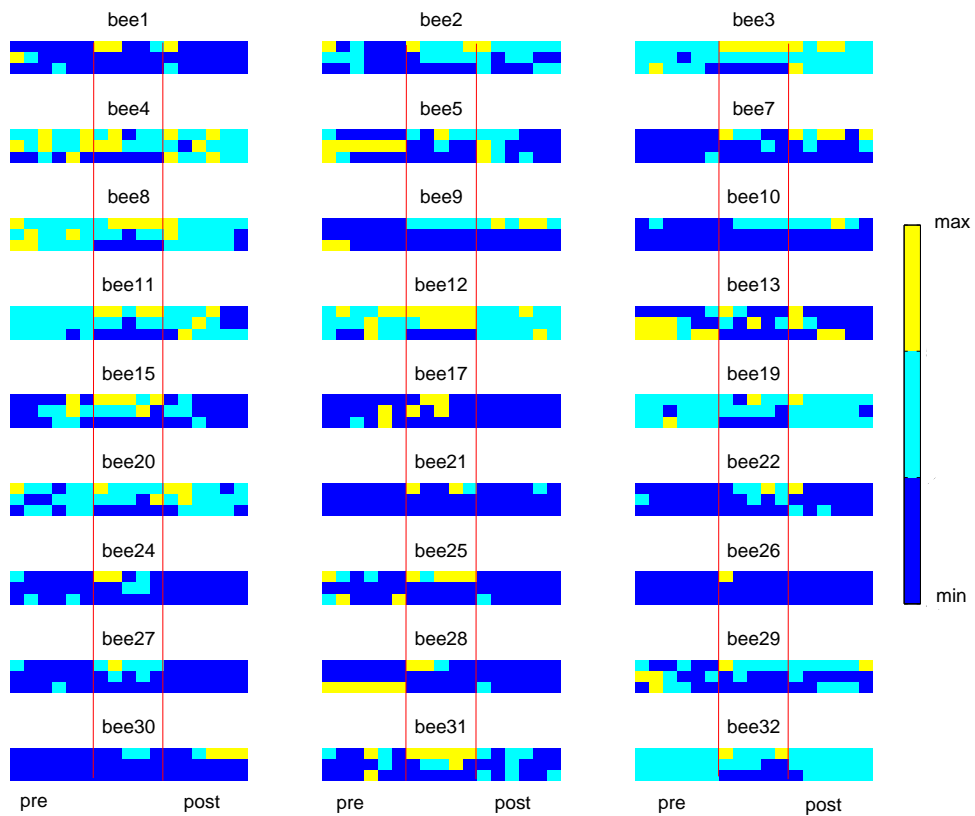
Figure 3.5: Learning performances of individual bees, M17 responses related to the PER represented in false colors, top row: CS+, middle row: CS–, bottom: control. Colorbar: performance index, blue: no response, cyan: moderate response, yellow: high response.

Responses Properties of Imaged Extrinsic Neurons

We analyzed EN responses to the stimuli serving as CS and US, respectively. We measured the activity induced by odor stimulation in the MB α-lobe. Figure 3.6 shows representative examples for odor evoked activity in false color scale superimposed on the raw-fluorescent images in eight bees. According to the spatial pattern of the activity an ROI was chosen. The three odors used as CS (hexanol, linalool and octanol), as well as the delivery of sucrose solution to the antennae, used as US, evoked reliable responses in EN dendrites in the α-lobe (figure 3.7). We did not observe any a priori difference between the odor responses. Figure 3.7A shows the temporal dynamics of the odor induced signal across bees. The odor responses usually consist of an on-response during the stimulus followed by an off-response upon stimulus-offset. Both, odor evoked on- and off-responses exhibit phasic-tonic response kinetics. In figure 3.7B averaged sucrose responses are shown. The kinetics of the sucrose response differs from the odor responses, however the manual delivery of sucrose to the antennae was less precise. As shown in figure 3.7, there is no difference in average response intensity for the three odors used as CS.
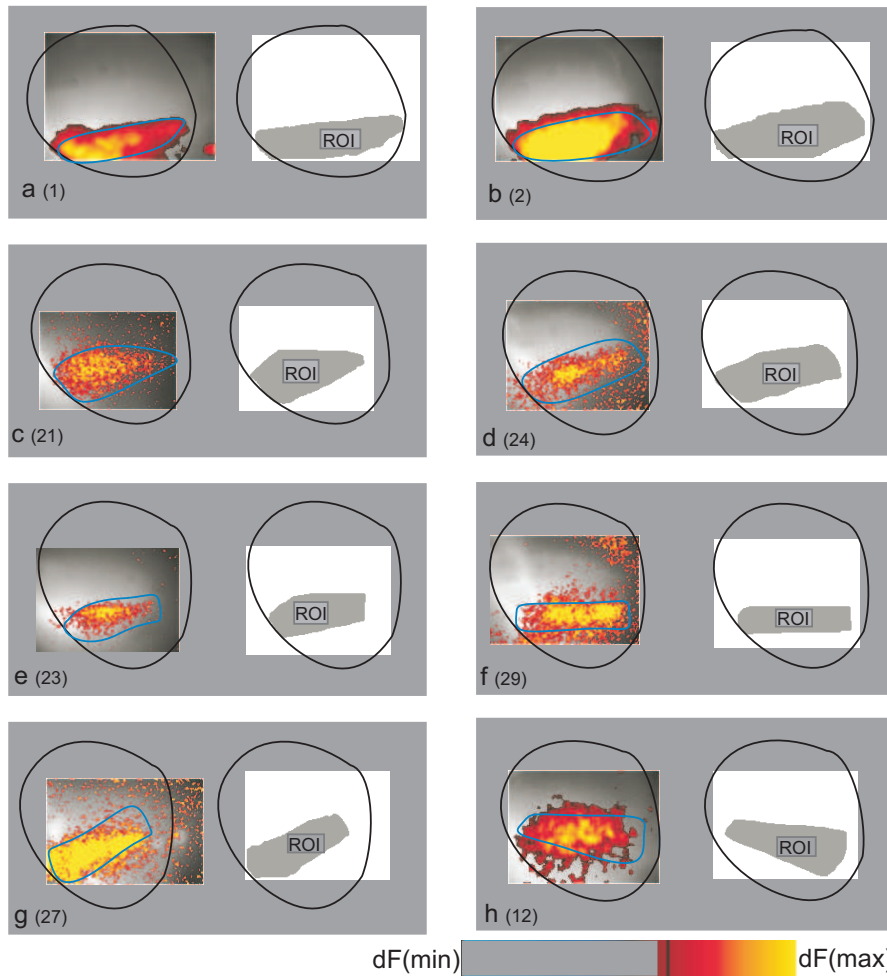
Figure 3.6: Spatial patterns of odor evoked calcium activity in 8 bees, activity patches represent the averaged signal during the stimulus superimposed onto the raw fluorescent images.
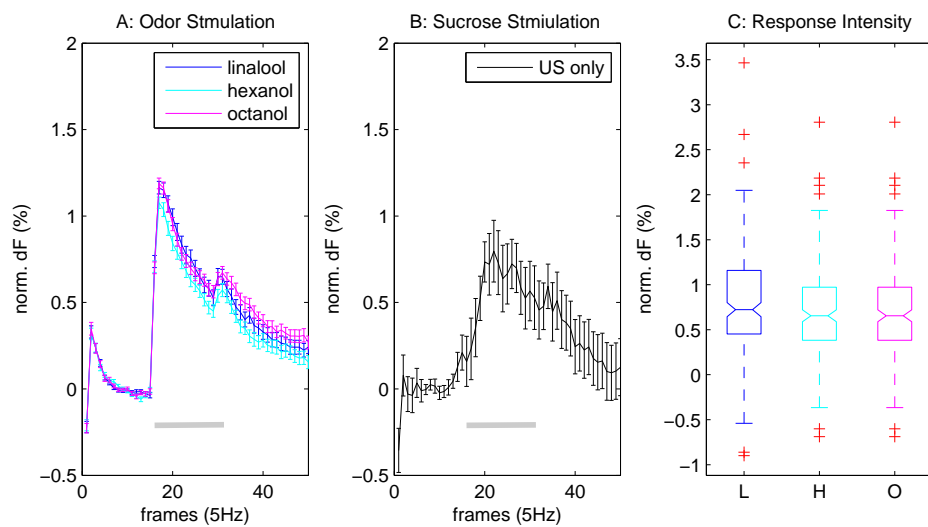
Figure 3.7: Odor and Sucrose Stimulation of the Antenna. **A:** Mean odor responses, traces represent averaged pixels across ROIs. All tested odors elicited activity in subsets of extrinsic neurons (n = 33, error: sem). **B:** Mean sucrose response. Sucrose solution delivered on a toothpick to the antenna elicited responses in subsets of EN (n=13, error: sem). **C:** There is no significant difference in mean response intensity (mean of response of frame 16–30 i.e. during the stimulus) between odors (Oneway-ANOVA, Bonferroni post hoc test, n.s.), L: linalool, O: octanol, H: hexanol.

Plasticity of Extrinsic Neuron Responses During odor Learning

We used calcium imaging to investigate putative plasticity in MB EN related to learning processes. Plasticity can be induced by associative and non-associative learning processes.

Non-Associative Plasticity

We analyzed the responses to odors upon repeated stimulation. Therefore, we presented three odors repeatedly in a pseudo-randomized fashion. The responses decrease significantly upon repeated odor stimulation (figure 3.8). The amplitude of the signal in response to a given stimulation is smaller than the responses to the preceding stimulation, excluding trial three. At least the first response amplitude is significantly higher than the subsequent response amplitudes (figure 3.8). We then looked at the effect of repeated sucrose stimulation. Sucrose was delivered to the antennae on a toothpick and elicited reliably the PER. Figure 3.9a and b show the decrease in response intensity upon repeated sucrose stimulation. Figure 3.9c shows that repeated sucrose stimulation also leads to habituation of the PER reflected in decreased M17 spike-rates. For the behavioral as well as for the neuronal response there is a significant decrease from the first to the third sucrose presentation.
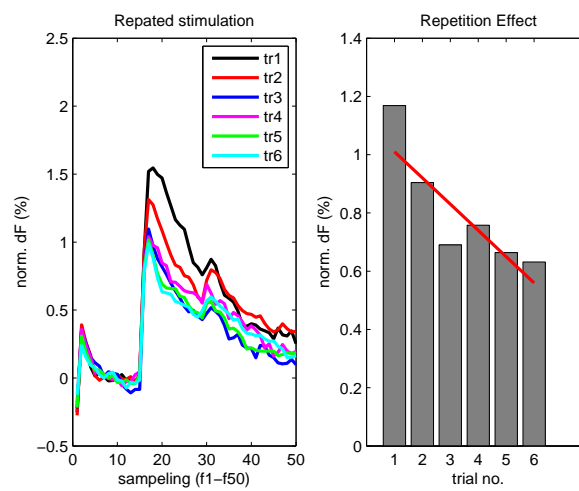
Figure 3.8: Response decrease upon repeated odor stimulation. A continuous trend is observed ($r^2_{\text{resp.intensity} \times \text{trial no}}$= 0.77), at least the response to the first odor stimulation is significantly different from the subsequent stimulations (Oneway-ANOVA, $F(5,32)$= 6.77, $p < 0.001$, Bonferroni-Post hoc test).
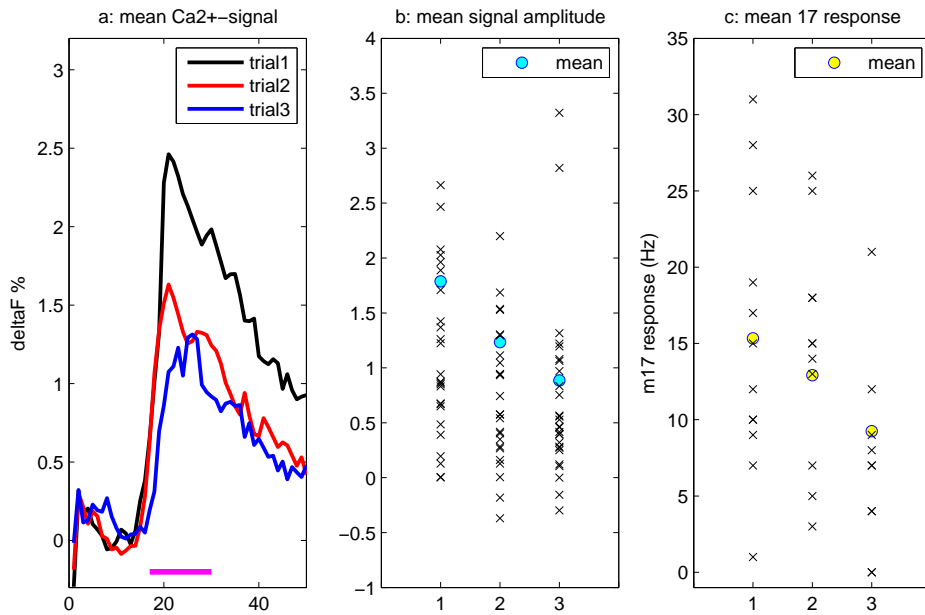
Figure 3.9: Representation of US. **a:** Response curves for repeated sucrose stimulation, inter-trial interval was 1 minute. **b:** Mean response amplitudes for repeated sucrose stimulation. There is a significant difference between first and third trial (Friedman-ANOVA, $\chi^2(2,28) = 11.21$, $p < 0.001$, Bonferroni-Post hoc test). **c:** Habituation of M17-response related to the PER, there is a significant difference between first and third trial (Friedman-ANOVA, $\chi^2(2,12) = 9.74$, $p < 0.001$, Bonferroni-Post hoc test).

Associative Plasticity

As observed in the behavior the effects of learning may be excitatory, i.e. related to the CS+, or inhibitory, i.e. related to the CS–. Plasticity can be the result of synaptic strengthening and increased neuronal excitability; in the opposite case it also may be the result of synaptic depression and decreased neuronal excitability.

Figure 3.10 shows the spatial calcium signal in one representative bee for which we observed an increased calcium signal in response to the CS+. The calcium signal in response to the control odor is also elevated after training.

Another example, in which the bee displays an increased calcium response to the CS+ after training is given in figure 3.11. Learning is also reflected in the behavior represented by the M17 responses. Thus, M17 responses and calcium signals in response to the CS+ are correlated. The intensities of the calcium signal in response to the control odor are significantly higher after training, than in the pre-phase, however for the control odor there is no generalization observed on a behavioral level. A correlation between M17 responses and calcium signals is not observed for the control odor.

We then asked whether there is a significant learning related effect across experiments. Figure 3.12 shows the calcium signals for each trial during the experiment pooled across all animals. We observe a general decrease in response strength upon repeated stimulation as already described in figure 3.8. While, for the CS– a continuous decrease in response strength is observed, for the CS+ and the control odor there is a tendency (although not statistically significant) for the responses in the first trial after training to exceed the response strength of the last trial before training.

To quantify this effect, we calculated a slope for each bee across the trials before and separately after training. Due to the repetition effect, response strength in one
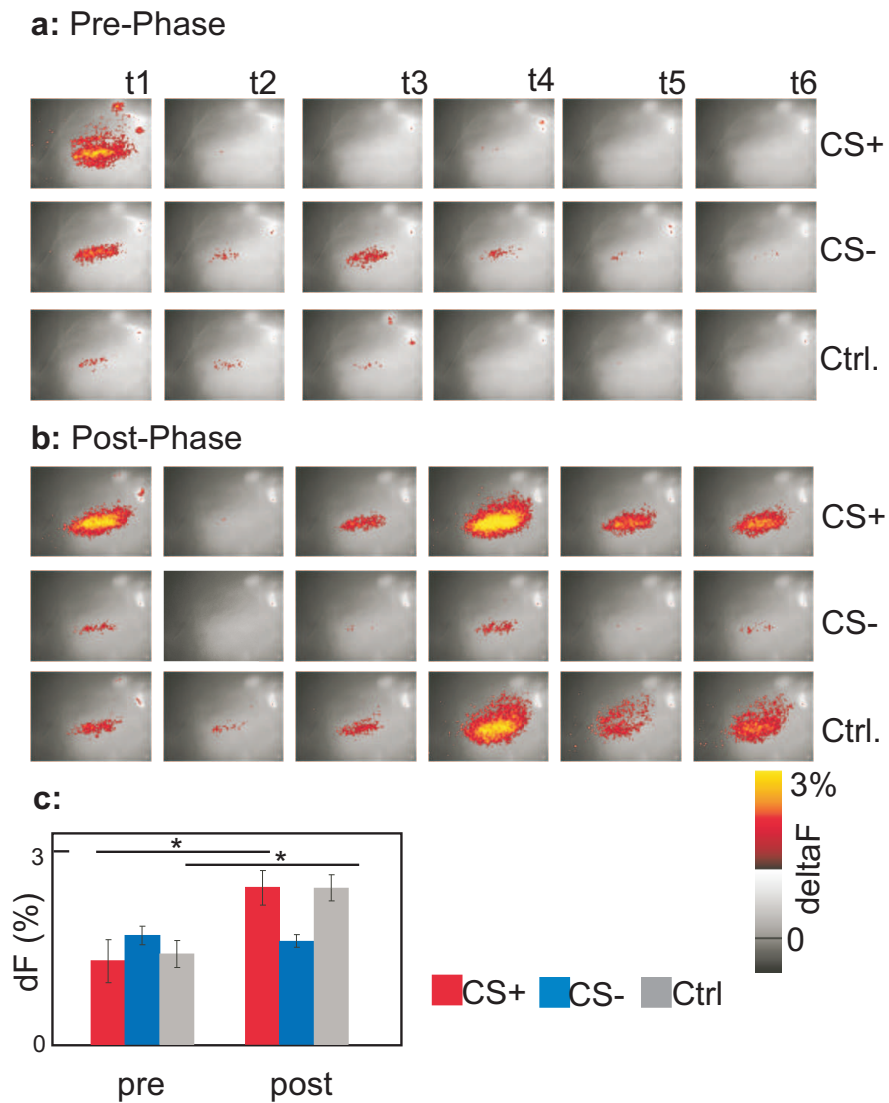
Figure 3.10: Calcium response during odor learning. **a:** Spatial calcium response to three odors during the pre-phase. **b:** Signal during the post-phase, signals above 1.5% deltaF are false color coded and superimposed on raw fluorescent image (380nm). **c:** Mean signal strength during stimulus for the pre- and post-phase across six trials, the response to the CS+ and the control odor exhibit a significant increase (Errors: sem, Wilcoxon test: $p < 0.05$)
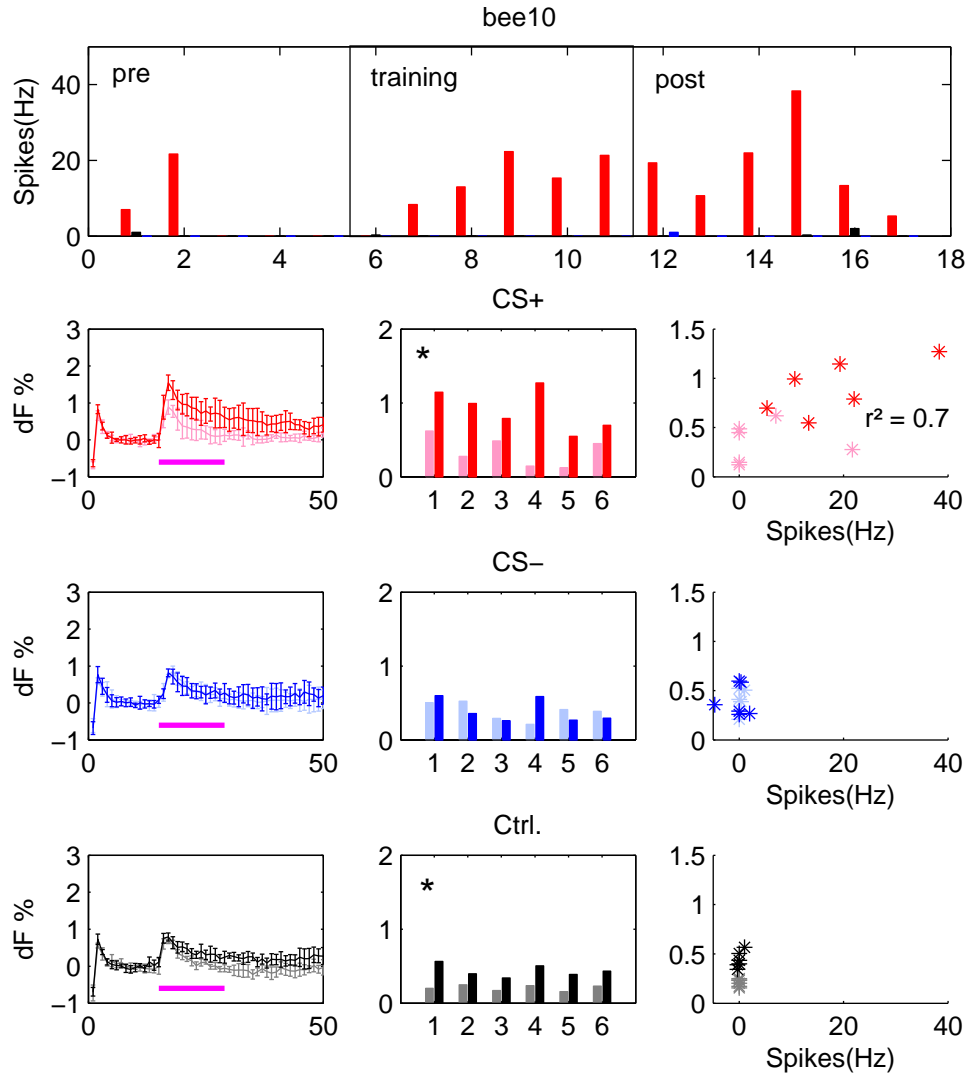
Figure 3.11: Calcium signals in EN in a bee that learned to specifically extend the pro-
boscis in response to the rewarded odor. Top: M17 Spikes as a direct
correlate of the PER. The bee extended the proboscis already in two trials
before the training. During training the bee extends the proboscis toward
the rewarded odor. After training the bee extends the proboscis toward the
previously rewarded odor. The calcium signal in response to the rewarded
odor after training is significantly enhanced compared to the response to
the odor before training. There is no significant difference between the cal-
cium signals before and after training in response to the unrewarded and
the control odor. For the CS+, there is a significant correlation between
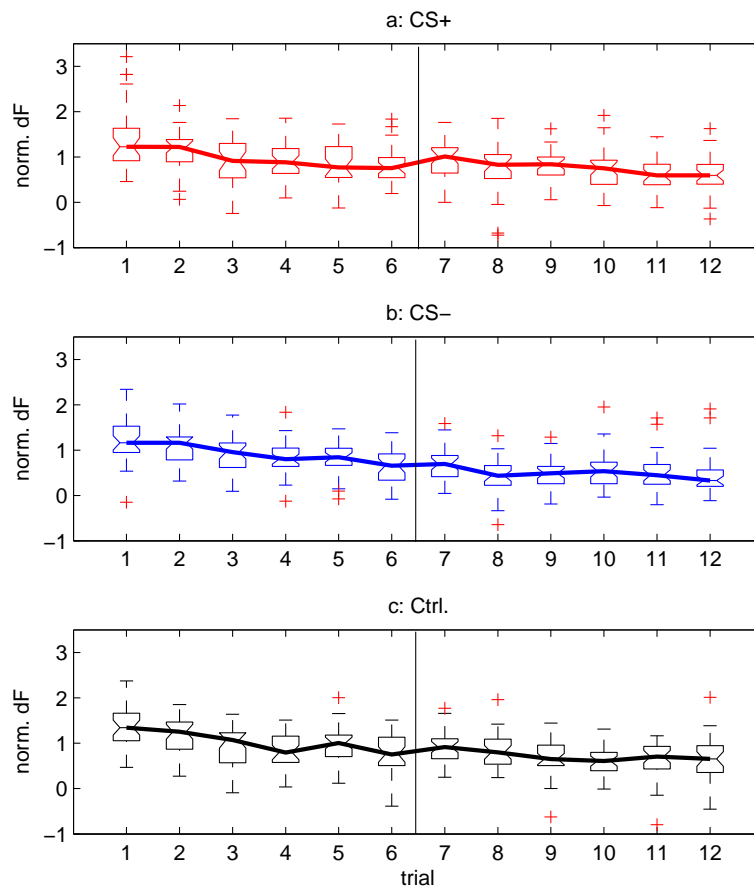M17 Spikes and calcium signal strength after training.

Figure 3.12: Median calcium signals for each trial. **a:** CS+ response intensities before and after training. There is a tendency toward an increased response in the first trial after training compared to the last trial before training. **b:** Response intensities for CS– continuously decrease during the course of the experiment. **c:** Response intensities for the control odor. There is also a tendency for an increase in response strength in the first trial after training compared to the last trial before training.
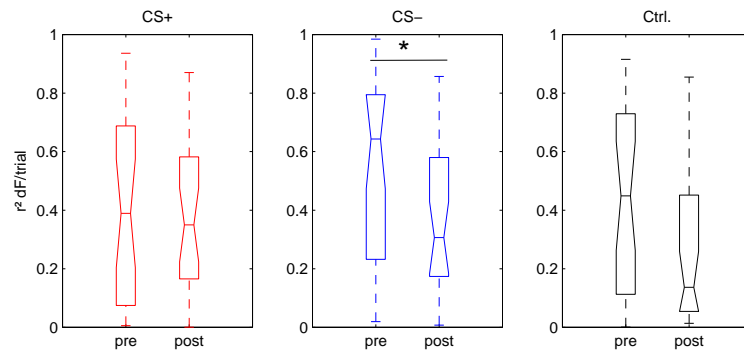
Figure 3.13: Coefficient of Determination (R^2) for the correlation of response strength and trial number across bees. R^2 for the CS– is significantly lower than for CS+ and Control (Wilcoxon signed rank test, n=33).

trial usually exceeds the strength of the subsequent response. However, there are exceptions. To take into account also the variance across trials, we determined the correlation between trial number and response intensity before and after training for each bee. We found there is a significantly smaller correlation between trial number and response strength after training for the CS–, while no difference in correlation for the CS+ and control odor is found. The results are shown in figure 3.13. This reflects the above described effect: for the CS– the decrease in response strength is continuous throughout the experiment resulting in a shallower slope after training. For CS+ and control the slope after training is as steep as before training.

To analyze the effects between pre- and post-phase and in between CS+, CS-and Control conditions we ran a threeway-ANOVA on the data set according to the analysis of the M17 data. The results are shown and described in figure 3.14. The ANOVA reveals a significant effect for the factors conditions (CS+, CS– and Control), the post hoc test reveals a significant difference between CS+ and CS-, as well as for control odor and CS– (figure 3.14d). There are also significant main effects observed for the factors "phase" (pre, post) and "trial" (1–6). There is also

a significant interaction between factors "phase" and "trial" (compare legend of figure 3.14 for details).

Finally, we analyzed for each individual bee whether a significant change in response intensity before and after training can be detected. Therefore, we performed a Wilcoxon-signed-rank test across the six trials before and after training for each bee and each condition. Figure 3.15 summarizes the results of the paired rank tests. As shown in figure 3.15a, in about 30% of the bees a significant change (increase or decrease) for the CS+ can be detected, for the CS– there is a significant change observed in about 50% percent of the bees and for the Control over 50% of the bees exhibit significantly changed responses. Figure 3.15b shows that the smallest fraction of bees exhibit an actual increase in response strength towards any of the conditions, however increased responses are only observed for the CS+ condition and to a lesser degree, for the control condition. Figure 3.15 shows the percentage of bees which exhibit a significant decrease in response strength after training. For most bees exhibiting a significant change between phases, this change is a decrease. All of the bees with a significant change for the CS– condition show a decrease.

Figure 3.16 gives an overview of the direction of response changes for each bee. Interestingly, many bees which do not show a change in response strength for the CS+ show a decrease for CS– or, for CS– and control condition. For the M17 responses, the data was coded in a binary way (response/no response). A paired comparison was not applicable for the behavioral data in this case, changes were calculated as number of responses in the pre-phase compared to number of responses in the post-phase.
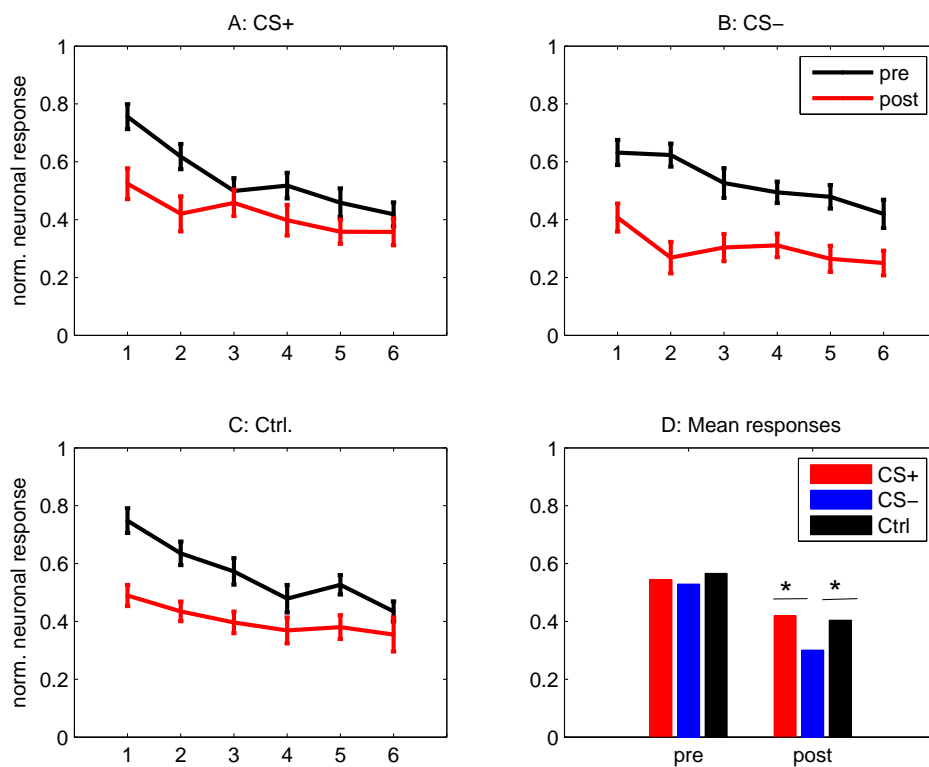
Figure 3.14: Changes in response strength before and after training.**a:** Mean response intensities for CS+ before and after training. **b:** Mean response intensities for CS– before and after training. **c:** Mean response intensities for control odor before and after training; errorbars: sem. **d:** Mean Response intensities across trials. A threeway-ANOVA we ran on the normalized data set confirms the repetition effect and reveals significant differences between CS+, CS– and control. ($F(2,33)_{condition} = 13.02$; $F(2,33)_{trial} = 19.73$; $F(1,33)_{phase} = 147.47$, $p < 0.01$). There is a significant difference between CS+ and CS–, as well as between control odor and CS– in the post-phase (Bonferroni post hoc test, * $p < 0.05$). The ANOVA also catches the differences in the repetition effect during the post-phase for the three conditions. There is a significant interaction between trial and phase ($F(5,33)_{trial \times phase} = 3.15$, $p < 0.01$) and a tendency, although not significant for an interaction between condition and phase $F(2,33)_{condition \times phase} = 2.74$, $p = 0.06$, n.s)
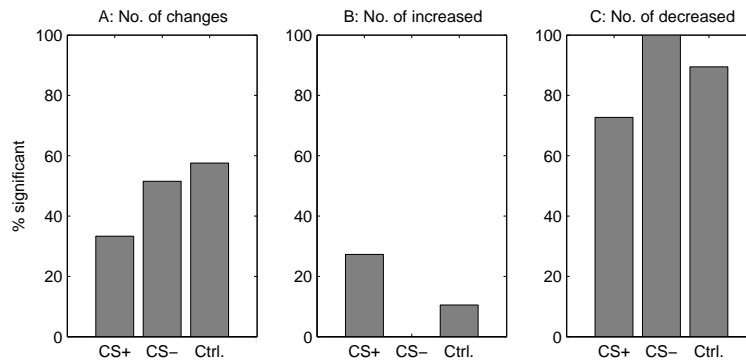
Figure 3.15: Number of changes in odor responses. **a:** Percentage of bees showing a significant response change between pre- and post-phase (Wilcoxon signed rank test, p < 0.5). **b:** Percentage of bees showing an increased response after training. **c:** Percentage of bees showing a decreased response after training.

3.5 Discussion

In the present study we imaged mushroom body extrinsic neurons in the α-lobe of the honeybee while the bees were repeatedly stimulated with odors and sucrose and after bees were trained in an appetitive odor learning paradigm. Simultaneously, we recorded electromyograms from the M17 muscle which controls the proboscis extension response. This way we were able to monitor and quantify odor learning relevant behavior, for the first time, during a calcium imaging study in the honeybee. The neurons we stained overlap as revealed by the analysis of confocal scans after the imaging experiments. The functional calcium indicator Fura-2 and the fixable dye ("Mini-ruby") are both coupled to dextran and have the same molecular weight, thus the staining from the fixable dye observed under the confocal microscope should reflect the staining with Fura-2. Therefore we can conclude with some certainty that overlapping sets of extrinsic neurons were imaged across experiments. Also, the observed responses show similar kinetics. However, in each preparation there is a slightly different subset of neurons stained. The exact iden-
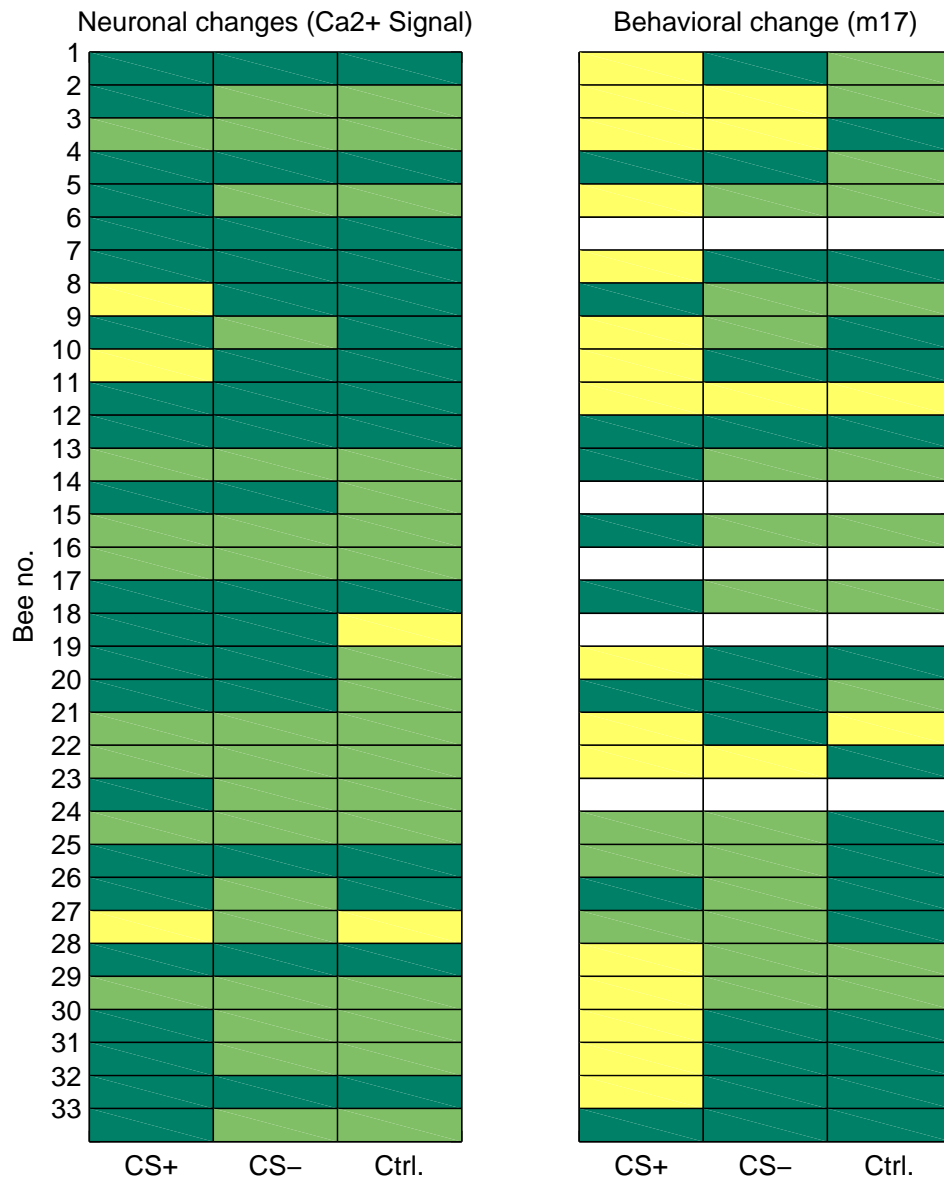
Figure 3.16: Significant changes before and after training in neurons (Wilcoxon signed rank test, p < 0.5) and bees showing a change in M17-responses (number of responses in post-phase > number of responses in pre-phase); dark green: no change, light green: decrease, yellow: increase, white: no recording.

tity of the imaged structures could not be verified, since the staining did not allow to trace the dendrites back to a particular soma or soma-cluster.

Monitoring and Quantification of Behavior

We modified the calcium imaging preparation described by Szyszka et al. (2005) such, that the bees could still freely move their mouth parts while restrained for the imaging experiment. This was mainly achieved by leaving muscles and oesophagus intact. Reduction of movement was achieved by the application of two component silicone which was filled into the head capsule. We could show that across sample size, bees form a robust odor memory during the imaging experiment. However, individual bees also display preferences to odors (possibly due to former experience in the field), they show generalization after training, or simply fail to form an association. This might especially be relevant for earlier imaging studies focusing on learning related plasticity in the honeybee brain, which lead to seemingly contradictory results (Faber et al., 1999; Peele et al., 2006, compare). The learning score we observed is comparatively low, which can be partly accounted for by the stress inducing and quite invasive preparation. The high number of odor exposures during the pre-phase is probably also sufficient to induce latent inhibition (Abramson and Bitterman, 1986).

Stimulus Repetition Induced Effects – A Neural Correlate of non-Associative Learning?

We observed a decrease in signal amplitude in response to odors and sucrose upon repeated stimulation. Szyszka et al. (2008) describes a similar repetition induced effect for KC in the honeybee. Since, for projection neurons emerging from the first order olfactory neuropile no repetition effect is observed in bees (Peele et al.,

2006), the decay in response strength is ascribed to non-associative odor learning, rather than to sensory adaptation to olfactory stimuli. In locusts a repetition induced decrease is described in olfactory projection neurons and also related to non-associative plasticity (Stopfer and Laurent, 1999; Bazhenov et al., 2005). Where previous studies could only speculate, that this response decrease is related to stimulus salience, since e.g. novel odors induce arousal etc. (Suzuki, 1974), we could directly show that the decrease in neuronal response upon repeated sucrose stimulation of the antennae coincides with habituation of the PER, induced by the same stimulations.

Associative Plasticity in Extrinsic Neurons

We investigated associative plasticity in extrinsic neurons employing a differential conditioning approach, which controls for non-associative effects, such as sensitization induced by sucrose stimulation. Also, in this approach, any associative effect can be related directly to the CS+ or CS–, respectively. Additionally, we introduced a third odor into the paradigm which was not presented during training (Control), to allow us to consider generalization effects. For individual bees we found associative plasticity manifested in a direct increase in response to the CS+ after training, accompanied by a decrease in response to the CS–. These results obtained from individual bees are robust and accessible for statistical analysis, since we chose an experimental design with repeated stimulus presentations to account for response variance. We document a case (figure 3.11) in which the response increase in ENs is directly correlated with the M17 response to the CS+ after learning. A response increase is also observed for the Control, possibly related to generalization. However a behavioral response to the Control odor is not observed. Across the sample size of 33 bees, plasticity is rather manifested in

the observation that responses to the CS+ decrease less after training, similar to what has been described as "recovery from repetition decrease" in Kenyon cells (Szyszka et al., 2008). We also tried to relate the neuronal changes induced by the learning experiment directly to behavioral changes observed in M17 recordings. The changes observed are not always fully consistent. Studies in mammals (Suzuki, 2007) and in humans (Rutishauser et al., 2008) provide implications for a correlation between the strength of the behavioral output with the immanence of neuronal plasticity, supporting a continuous memory model, which states: the stronger the neuronal response, the stronger the memory. In this model neuronal response and behavioral output are not necessarily related 1:1 but in a rather continuous fashion.

Mechanisms for Plasticity in Extrinsic Neurons and Implications for their Role in the Mushroom Body Network

ENs receive excitatory and possibly also inhibitory input from KCs. Modulatory input may be provided from different neurons. The identity of transmitters involved in the MB lobe network remains elusive. GABA-like immuno-reactivity (Bicker et al., 1985; Grünewald, 1999a), dopamine-like profiles (Schäfer and Rehder, 1989) and octopamine-like immuno-reactivity (Sinakevitch et al., 2005) have been documented in the MB α-lobe. Also profiles of two neuromodulatory peptides have been described (Strausfeld et al., 2000). The calcium signals observed in our experiments are likely to represent calcium influx through voltage gated calcium channels, as for PNs it has been shown that calcium imaging signals, reflecting the intracellular calcium concentration, are closely correlated to spiking activity (Galizia and Vetter, 2004) and more recently a similar approach in locusts has come to the same result (Moreaux and Laurent, 2007). Furthermore, the temporal dynam-

ics observed for our signals resemble those derived from spiking activity in ENs (Okada et al., 2007; Strube-Bloss, 2008). The region of the α-lobe where recorded signals from overlaps with the projection region of A3/PCT-EN dendrites (Mobbs, 1982; Rybak and Menzel, 1993). Also, the fact that we injected the dye into the region of the PCT, suggests that A3 neurons contribute to a large fraction of the observed calcium signal. However, Grünewald (1999b) reports an activity decrease of A3 neurons in response to an odor after a single pairing of this odor with sucrose which is interpreted in a straight forward manner: A3 neurons are involved in an inhibitory feedback loop, so it makes sense that there is a mechanism allowing the neurons to provide less inhibition in response to the rewarded odor. For the interpretation of our results this means we did either not primarily record from A3-neurons, or at least not from the same subset as Grünewald (1999b). There is also some indication that indeed not all A3 neurons exhibit GABAergic profiles and not all of them are involved in the inhibitory feedback-mechanism suggested by Bicker et al. (1985) and Grünewald (1999b) (Rybak, personal communication). Our results are largely consistent with Szyszka et al. (2008), which supports that EN integrate KC signals. Activity dependent presynaptic fascilitation at the KC-EN synapses may lead to an increased EN firing activity (Smith et al., 2008). The same authors in their modeling approach, also suggest modulatory feedback mechanism to EN synapses would be required to keep the neurons from "over-strengthening", i.e. inhibition occurs when there is no further reinforcing stimulus. This idea is consistent with the observed decrease in signal strength upon repeated odor presentation without reinforcement. For such a network, possibly further information about the reinforcing stimulus besides the VUM_{mx1}-pathway (Hammer, 1993) may be required. The octopaminergic profiles within the α-lobe could be an indication of the existence of value neurons besides the VUM_{mx1}. Figure 3.17 summarizes

our current view of the MB connections and network in a simplified scheme.

Perspectives

This study documents ENs of the α-lobe are subject to associative and non-associative plasticity. The degree and direction of neuronal changes differs across preparations as it has been recently also shown in extracellular electrophysiological recordings of EN subsets (Strube-Bloss, 2008). In future experiments we focus on directing the dye application to more defined subsets of extrinsic neurons and aim to address and individually identify imaged neurons with the perspective to reconstruct their morphology.
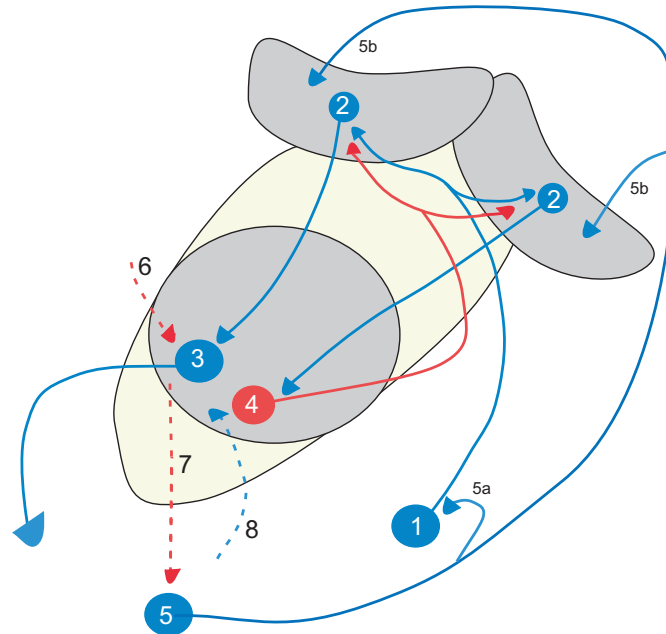
Figure 3.17: Simplified model of the MB network and Learning relevant connectivities. 1: Olfactory (and gustatory) input is provided to the MB by projection neurons. 2: Olfactory information diverges onto a large number of KC. 3: KC converge onto a relatively small number of ENs. 4: A subgroup of ENs forms an inhibitory feedback mechanism to the MB input, the exact targets remain unclear. 5: The VUM$_{mx1}$ neuron provides the US pathway to the first order olfactory neuropile (5a) and the MB input region (5b). 6: Putative inhibitory input to ENs suggested to explain non-associative plasticity. 7: Putative inhibitory feedback signal to value neuron to prevent over-strengthening. 8: Hypothetical reward pathway to MB output, if not mediated by the VUM$_{mx1}$. Blue connections: Excitatory input, red connections: Inhibitory input, solid lines: described connections, dashed lines: speculative connections.

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4 Characterization of Morphologically Identified Mushroom Body Extrinsic Neurons

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4.1 Abstract

The lobes are the main output regions of the mushroom bodies, which are paired central structures of the insect brain, involved in learning and memory formation. We stained extrinsic neurons of the mushroom body α-lobes belonging to different morphological subgroups iontophoretically or via backfills. We asked whether extrinsic neurons belonging to different morphological subgroups have different physiological properties. We measured activity evoked by odor and sucrose stimuli in these neurons employing calcium imaging. We also investigated possible changes of activity induced by olfactory learning. We find associative plasticity immanent as a reduction of signal intensity in response to the rewarded odor after training and in a reduction of response variance. After the functional characterization we confirmed the identity of the imaged structures using confocal microscopy.

Keywords

Associative Plasticity, Calcium Imaging, Electrophysiology, Honeybee, Mushroom Body

Abbreviations

CS+	Conditioned Stimulus (rewarded)
CS–	Conditioned Stimulus (unrewarded)
Ctrl	Control Odor (untrained)
EN	Extrinsic Neuron
GABA	γ-Amino-Buteryc-Acid
hep	Heptanal
KC	Kenyon Cell
lio	Linalool
mACT	median Antenno-Cerebral-Tract
M17	Protractor of the Labium
MB	Mushroom Body
PCT	Protocerebral Tract
PER	Proboscis extension response
PN	Projection Neuron
ROI	Region of Interest
US	Unconditioned Stimulus (reward)
6ol	Hexanol
8ol	Octanol

4.2 Introduction

Since their discovery more than a century ago, the mushroom bodies (MB) of insects have been regarded as centers for complex brain functions (Dujardin, 1850; Kenyon, 1896). These speculations have been strengthened by findings revealing that the formation of long term memory in honeybees depends on the functioning of the MB during the consolidation period following olfactory associative learning (Menzel et al., 1974; Erber et al., 1980; Komischke et al., 2005). The involvement of the MB network in learning and memory formation is a central topic in the neuroscience of insect model systems (for reviews see: Heisenberg (2003); Davis

(2004); Menzel (2008)). The structure and connections of the MB network in the honeybee has been investigated in some detail. Input from different sensory modalities diverges onto a high number of MB intrinsic neurons, the so called Kenyon Cells (KC)(Kenyon, 1896) which synapse in the MB lobes, the main output site of the MB, onto the MB Extrinsic Neurons (EN). The MB ENs connect to different areas of the brain and their somata are organized in clusters (Mobbs, 1982; Rybak and Menzel, 1993; Strausfeld, 2002; Strausfeld et al., 2009). Immuno-histochemical methods reveal band shaped substructures in the MB lobes, associated with functional subgroups of KC (Strausfeld et al., 2000). One particular subgroup of EN, the protocerebral tract (PCT) neurons have been investigated in greater detail and shown to be GABA immuno-reactive (Bicker et al., 1985; Grünewald, 1999a) and subject to associative plasticity (Grünewald, 1999b). Recently, also in *Drosophila* the involvement of GABAergic MB ENs in learning has been demonstrated (Liu and Davis, 2009). Dopamine-like immunoreactivity has been ascribed to neurons of the A1, A2 and A6 cluster (Schäfer and Rehder, 1989). One morphological subgroup, termed A4 (Rybak and Menzel, 1993) or type extrinsic (Strausfeld, 2002) have been described to innervate the ventral part of the α-lobe. One single identified neuron with arborzations in the MB lobe and pedunculus, the PE1-neuron, has been intensively studied and learning related plasticity has been described (Mauelshagen, 1993; Menzel and Manz, 2005; Okada et al., 2007).

In this study we combine physiological and morphological characterization techniques and apply them to MB ENs, arborizing in the α-lobe. Two methods were used to stain EN in the MB-α-lobe. We first measured responses to olfactory and gustatory stimuli in MB EN electrophysiologically, subsequently we iontophoretically injected a neuronal tracer and a calcium sensitive dye into the neuron and recorded calcium responses to olfactory and gustatory stimuli. In a separate group

of bees ENs were stained by backfilling the neurons with the calcium sensitive dye fura-2 and a fixable dye. These bees were exposed to an appetitive odor learning paradigm (Bitterman et al., 1983). After the physiological experiment we investigated the morphology of the stained structures under the confocal microscope. One neuron was reconstructed using 3D reconstruction tools.

4.3 Materials and methods

Experimental Design

Forager honeybees were caught at the hive entrance from hives kept in a flight room. Bees were chilled and fixed in plastic chambers as described by (Szyszka et al., 2005). For the iontophoretical filling of neurons, the overall experimental procedure followed Galizia and Kimmerle (2003). First electrophysiology was performed using peppermint, hexanole octanole and heptanone as odor stimuli (all obtained from SIGMA, except peppermint, obtained from a local drug store). After the odor stimulation two negatively charged dyes ALEXA-633 (Invitrogen, Eugene, Oregon, USA) or Lucifer Yellow (Invitrogen, Eugene, Oregon, USA) and FURA-2 (2.000 MW, Invitrogen, Eugene, Oregon, USA) were applied iontophoretically. Second, imaging experiments were performed using odor stimulation or bees were trained in an associative odor learning paradigm. In two bees the behavior (Proboscis Extension (PER) was monitored by recording electro-myograms from the m17 muscle as described in chapter 3 of this volume. For the backfilling of A4-extrinsic neurons dye (Fura-2 and Rhodamine dextran) was injected into the soma region of A4 neurons. Neurons were anterogradely stained over night. For the staining of A3 ENs dye was injected into the lateral rim of the MB-α-lobe, where their dendrites converge onto the axon. Calcium imaging was performed as

for iontophoretically filled neurons. Bees were exposed to odor stimuli or trained in an associative odor learning paradigm, according to the protocol described in chapter 3. After the physiological experiments the brains were dissected and fixated for a subsequent investigation of the measured structures under the confocal microscope.

Electrophysiology

Borosilicate glass-electrodes were pulled with a horizontal glass pipette puller (P-97, Sutter Instruments, Novato, CA). Electrodes were inserted into the ventral part of the alpha-lobe using a micromanipulator or Piezo-Stepper. The tips of the electrodes were filled with a mixture of 0.5 mg Fura-2 potassium salt (2.000 MW, Invitrogen, Eugene, Oregon, USA) and $5\mu l$ ALEXA-633 (Invitrogen, Eugene, Oregon, USA) or Lucifer Yellow (Invitrogen, Eugene, Oregon, USA) dissolved in HEPES (100mM/100ml, pH=7.3). The the back up solution of electrodes was KCl. After testing responses to odors, a hyperpolarizing current was applied for up to 20min. to inontophoretically fill the cell with dye. Bees were left to recover for about 1h, then they were brought to the imaging setup and imaging experiments followed as described below.

Backfilling of Extrinsic Neurons

Eyes and thorax of bees were fixed to the recording chamber with low melting point hard wax (Deiberit 502). Staining procedure followed 1-4 hours later. The antennae were immobilized with eiscosane (Sigma). The head capsule above the brain was opened by removing a piece of cuticle and pushing glands and trachea to the sides to allow access to the brain. A 10:1 mixture of FURA-2 dextran (potassium salt, 10 000 MW, Molecular Probes, Eugene, OR) and fixable tetram-

ethylrhodamine dextran (10 000 MW, Molecular Probes, OR) was injected into the neuronal tissue using a glass capillary pulled with a puller (P-97, Sutter Instruments, Novato, CA) with a diameter of ca. 10μ m. The staining probes were injected either into the somata region of A4-neuron soma-cluster ventro-laterally to the antennal lobe or into the lateral rim of the α-lobe to stain A3 neurons. Trachea and glands were put back into place and the cuticle piece was restored to its place in the head capsule. The antennae were loosened. After about 20 min. bees were fed until satiation and put back into the humid case and stored over night at 20°C. Experiments started the next day. Therefore the antennae were immobilized using n-eicosane. The abdomen and thorax were immobilized using either dental wax or a small piece of sponge that was pressed against the abdomen and fixed with a clip without damaging the bee. To prevent movements of the brain resulting from pumping from the oesophagus a small incision was cut into the cuticle above the labrum and part of the oesophagus and its surrounding solid structures were put under tension without damaging the oesophagus (Mauelshagen, 1993). The previously cut piece of cuticle above the brain was removed, and trachea and glands were partly removed were they were covering the mushroom bodies. Heamolymph was sucked from the head capsule with a Kimwipe. All sites of surgery were covered with two component silicone (KWIKSIL, WPI). For the imaging experiment a drop of water was placed between the head and and dip objective such that it could be immersed into the droplet. Bees were placed on the microscope stage in front of an exhaust.

Odor Stimulation and Imaging

Odors used were: hexanol, octanol, heptanal, and linalool (all from Sigma). Odors were diluted in paraffin oil (Merck) at 1%. 2μm of odor solution was placed

on a piece of filter paper (3×1cm) of which two were placed in a 10ml plastic syringe. Using a computer controlled custom built olfactometer (Galizia et al., 1997), odors were diluted in a continous airstream which was directed towards the antennae. Odors were presented as 3s pulses consisting of about 0.2ml of odor saturated air. Images were recorded at room temperature with a sampling rate of 5Hz using a TILL-Photonics imaging setup mounted on a fluorescence microscope (Zeiss, Germany). Measurements started 3 seconds before stimulus onset and lasted for 10 seconds. Neurons were recorded though a 60×, 0.9 W Olympus dip objective with an Imago CCD camera (640×480 pixels, 4× binned on chip to 160×120). The spatial resolution was 1.47 μm×1.47μm/pixel. Fura-2 was excited with illumination of 340 and 380nm for ratiometric measurements. Fluorescence was detected through a 410nm dichroic mirror and a 440nm long pass filter.

Morphological Analysis and Reconstruction

After physiological measurements brains were dissected and fixated in 4% formaldehyde in PBS overnight at 4°C and then rinsed in PBS, dehydrated in ethanol, and cleared in mehtyl salicylate. The brain was placed onto an object slide under a coverslip and viewed from the ventral surface with the use of a confocal laser-scanning microscope (Leica TCS SP2, Leica, Wetzlar, Germany). The excitation wavelength was 543nm using a green HeNe laser. The brain was scanned with an air HC PL APO objective lens of 10×/0.4NA and a 1.1-1.2 digital zoom at 4μm optical sections. The mushroom bodies were scanned with an oil objective and 1.6-1.7 digital zoom at 2μm optical sections. Morphological 3-D reconstruction was done in AMIRA 4.1 (Visage Imaging GmbH, Berlin, Germany).

4.4 Results

Selective Backfills of A4-Neurons

We stained a total of 8 A4-Neurons by injecting the calcium indicator Fura-2-dextran and fixable rhodamine-dextran into the somata-region of A4 neurons ventro-laterally to the antennal lobe neuropil. By applying this staining approach we stained a variety of structures projecting from the AL to the protocerebrum (e.g. projection neurons belonging to the median Antenno-Cerebral-Tract, mACT). However, within the MB-α-Lobe we found only arborizations of A4 neurons stained. As shown in figure 4.1c, A4 neurons have their somata residing ventro-laterally to the antennal lobe. Their axons ascend to the MB lobes. In figure 4.1a,b and d–i, arborizations of A4 neurons are shown. The axons of A4 neurons invade the alpha-lobe, where some of them arborize exclusively in the ventral part (also referred to as γ-lobe, Strausfeld (2002)). Others also invade the median part of the α-lobe (A4-2, compare Rybak and Menzel (1993)). All A4 neurons also have sparse arborizations within the lateral protocerebrum and around the α-lobe.

We imaged A4 neurons in the α-lobe while we stimulated the antennae with odors or sucrose solution. In figure 4.2 imaging of A4 neurons in one preparation during odor and sucrose stimulation is shown. Also responses in both MB calyces originating from projection neuron boutons belonging to the mACT are visible, as shown in figure 4.2b. Accordingly, three ROIs were chosen for the median and lateral calyx, as well as the MB-α-lobe (structures outlined in figure 4.2a). In figure 4.2c–e, the temporal dynamics of the responses to the stimulations with three odors and sucrose delivered to the ipsi-lateral antenna are shown for the three regions. The response kinetics in A4 ENs are more restricted to the initial
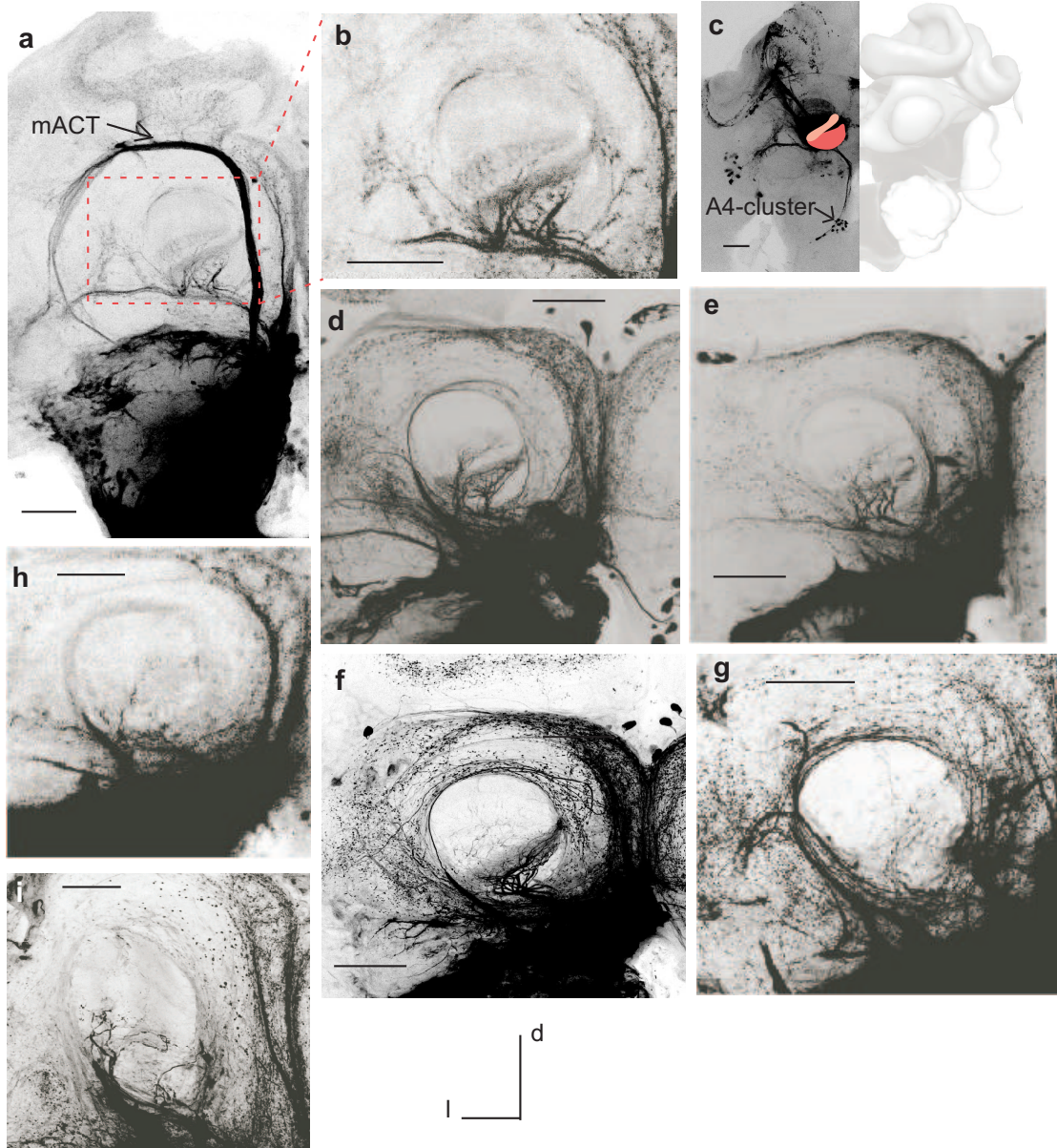
Figure 4.1: Confocal stacks of selectively stained A4 Neurons in the α-lobes. Across the brain a variety of structures are stained (e.g. AL projection neurons etc.), however in the α-lobe we only observe the arborizations of A4 neurons. **a:** Left brain hemisphere with injection site ventro-laterally to AL, with staining of AL projection neurons **b:** Arborizations of A4 neurons in the α-lobe **c:** Dye was injected into the α-lobe, Arrow: A4-soma cluster, red areas indicate projection fields of A4 neurons in the α-lobe. **d–i**: Arborizations of A4 neurons in the α-lobe. Scalebars: 100μm.
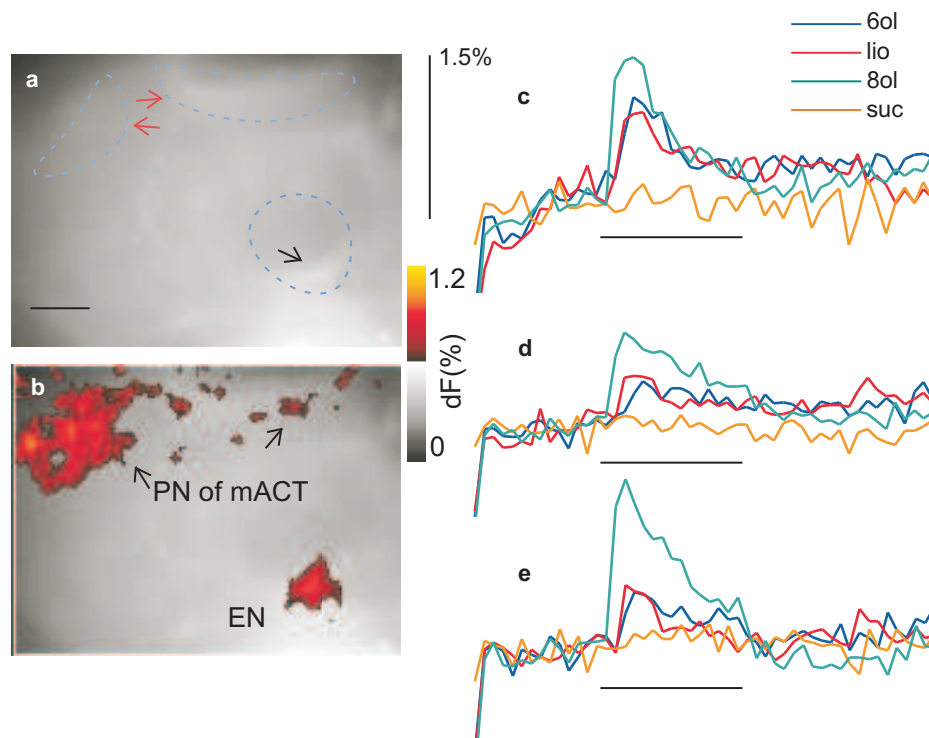
Figure 4.2: Responses of A4 neuron to odor and sucrose stimulation (preparation shown in figure 4.1a and b) . **a:** Raw fluorescent image of the right brain hemisphere, blue outlined structures: median and lateral calyces (red arrows), alpha-lobe (black arrow), scale bar: 100μm. **b:** Raw fluorescent image with superimposed spatial calcium signal, false color coded. **c:** Odor and sucrose stimulation evoked signals in the alpha-lobe. **d:** Odor evoked signals in the median MB calyx. **e:** Odor and evoked signals in the lateral calyx.

part of the response (figure 4.2c), while in the calyces the decay of the amplitude is slower. In all three ROIs octanol elicits the strongest responses, however the relation between the responses to octanol and the responses to linalool and hexanol differs across ROIs. No response to sucrose stimulation is observed in projection neurons or A4 ENs.

We trained a subset of six bees with stained A4 ENs in an appetitive odor learning paradigm. The learning experiments consisted of three phases: a prephase with six stimulations with three different odors, a training phase, in which
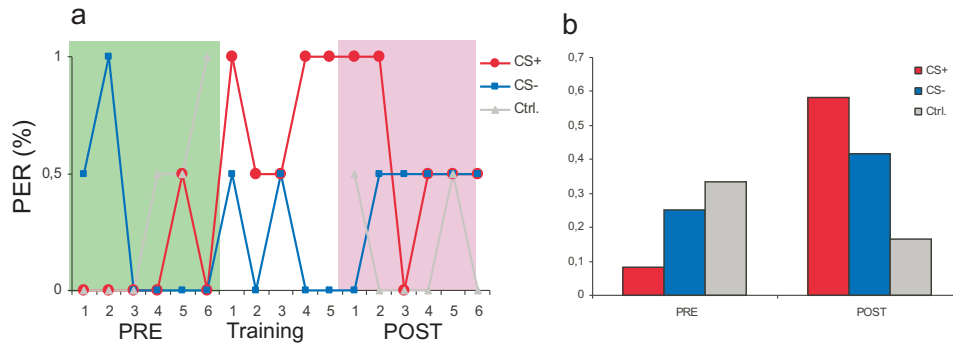
Figure 4.3: Learning Performance during Experiment **a:** PER during pre-phase, acquisition and post-phase. **b:** Mean PER across trials during pre- and post-phase (n = 2)

one of the odors (CS+) was paired with a sucrose reward (US) while another one of the odors from the pre-phase was presented alternatingly without reward (CS–), and finally a post phase with six presentation of all three odors (CS+, CS– and Ctrl.). A detailed description of the stimulation protocol for the learning experiments is given in Chapter 3. For two bees, M17-responses related to the PER were recorded. Figure 4.3 shows the behavioral performance of the two bees during the learning paradigm. Since normally the muscle is silent at rest, we counted a spike rate above base-line activity as response and a spike-rate below or equal to base-line as no response (a detailed description of the M17-recording procedure is also given in Chapter 3). The results obtained from two bees indicate that a robust odor memory is formed during the imaging experiment.

Figure 4.4 shows the temporal dynamics for the six bees trained in an odor learning paradigm. In the right column of figure 4.4 boxplots representing the response intensities before and after training are shown. Three bees show a significant decrease in response intensity to the CS+ after training. One bee also shows a significant reduction in response to the control odor. In the remaining bees there is no significant change in response intensity before and after training. However as

a general tendency, we observe a decrease in the variances of response intensities after training.

Figure 4.5 shows the mean variance across all six trials before and after training for CS+, CS– and Control. Exclusively for the CS+ condition there is a significant reduction in mean response variance after training.
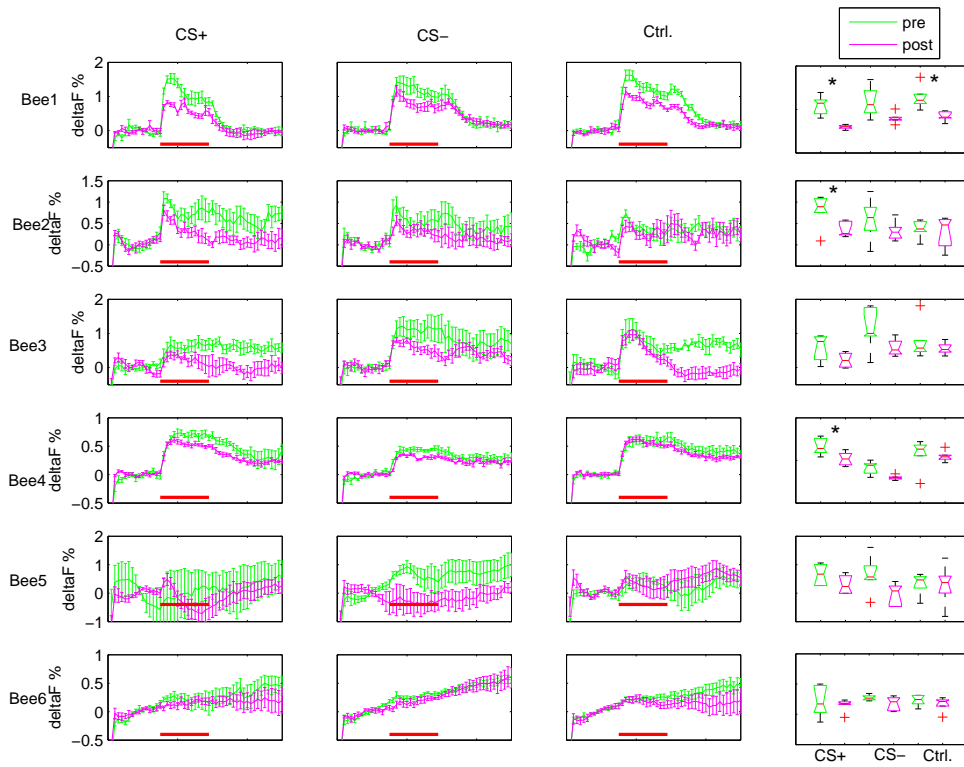
Figure 4.4: Calcium Response in A4-neurons before and after differential training. Temporal dynamics for the responses to CS+, CS– and Control before and after training. Left column: Response intensities: Three out of six bees show a significant decrease for the CS+; one bee also shows a significant decrease in response to the control odor after training. The remaining bees show no significant differences in response strength before and after training (Wilcoxon signed rank test, * p < 0.05).
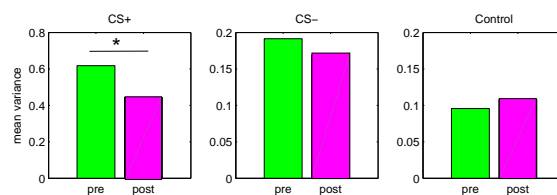

Figure 4.5: Mean variance of response intensities before and after training. Green: pre-phase, magenta: post-phase. For the CS+ there is a significant reduction of response intensity variance after training (Wilcoxon signed rank test, * p < 0.05).

Selective Backfill of A3-Neurons

We stained a subgroup of A3 neurons by injecting dye into the location of the α-lobe rim, where their dendrites converge to the axon (α-exit, according to Mobbs (1982)). In figure 4.6a a confocal stack of the left brain hemisphere with the stained of A3 neurons is shown. The somata in the lateral protocerebrum as well as the dendrites within the α-lobe and terminals within the MB calyces are visible. The signals in figure 4.6b are restricted to the dendritic region in the α-lobe and the dorsal somata-subcluster (A3–d neurons). Figure 4.6c shows temporal dynamics of the calcium signals observed in response to odor and sucrose stimulation in the dendritic and simultaneously in the somata region (figure 4.6d). The dynamics in the two regions are similar, however the responses in the somata display a slower response decay. Responses to sucrose stimulation of the ipsi- and contralateral antenna as well as to odors (heptanal and linalool) can be observed. Neither in the dendrites, nor in the somata can a response to hexanol be observed.
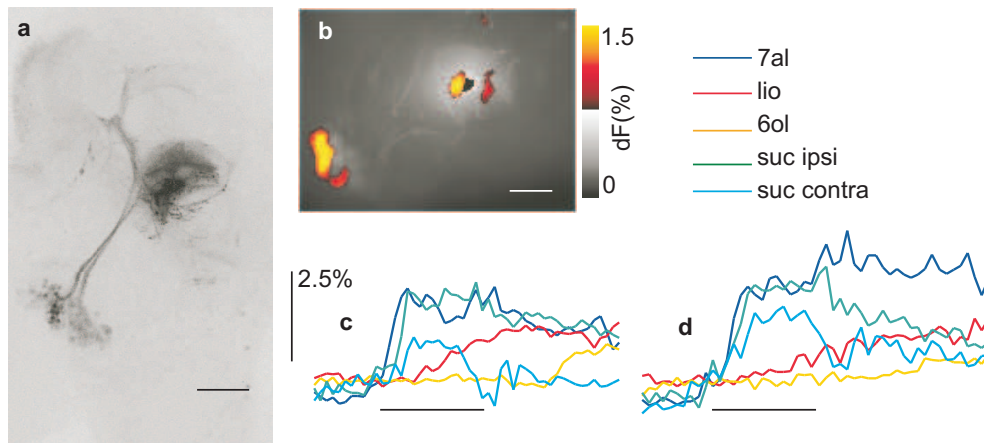
Figure 4.6: Backfill of A3 neurons. **a:** Confocal stack of left brain hemisphere. Visible are the somata, dendrites within the α-lobe and projections to the calyces of A3 neurons. **b:** Raw fluorescent image and super imposed calcium signals in false color scale of the same neuron. The odor evoked responses are restricted to the dendrites within the α-lobe and the dorsal subcluster of A3 somata (stimulation with heptanal). **c:** Temporal dynamics of calcium signal in dendritic region upon stimulation with heptanal, linalool, hexanol and sucrose stimulation of the ipsi- and contralateral antenna. **d:** Temporal dynamics of calcium signals upon odor and sucrose stimulation in the somata-region of A3 neurons. Scalebars: $100\mu m$

Iontophoretical Staining of Extrinsic Neurons in the α-lobe

In following the method established by Galizia and Kimmerle (2003) we recorded intracellularly from MB EN and subsequently injected a fixable dye (ALEXA-633 or Lucifer Yellow) together with the negatively charged potassium salt of the calcium indicator fura-2 via application of a positive current into these neurons. The electrode was inserted into the brain ventro-laterally to the MB α-lobe. When action potentials were recorded, the bees were stimulated with odors and sucrose. We successfully recorded and stained one A4 neuron, one A5 neuron and one A5-neuron or antennal lobe feedback neuron (ALf-1, compare Rybak and Menzel (1993)) via current injection (figure 4.7a–d). In figure 4.7a, the soma ventrally to the α-lobe and a projection to the AL is visible. Inside the α-lobe, as shown in figure 4.7b appears a band which is probably an A5, type-2 or type-3 (Rybak and Menzel, 1993). Figure 4.7e shows a 3D-reconstruction of the morphological structure of the A5-neuron shown in figure 4.7d.

Figure 4.8 shows the reconstruction of the A5 neuron after registration in tho the Bee StandardBrain. A detailed description of the registration procedure is given in Brandt et al. (2005). The arborizations within the optic tubercle were weakly stained and could not be reconstructed. The dendrites within the α-lobe could not bee individually reconstructed, however their projection area is indicated as a band.

When activity was measured intracellularly and responses to sucrose or odor stimulation were observed, a positive current was applied to the cell to iontophoretically stain the recorded structure. After 1h the preparation was brought to the imaging set-up and calcium signals were recorded. In figure 4.9 calcium signals observed in of the A5 neuron are shown. Figure 4.9a and b, show responses to heptanal observed in the A5 soma. In figure 4.9c and d the calcium signals in
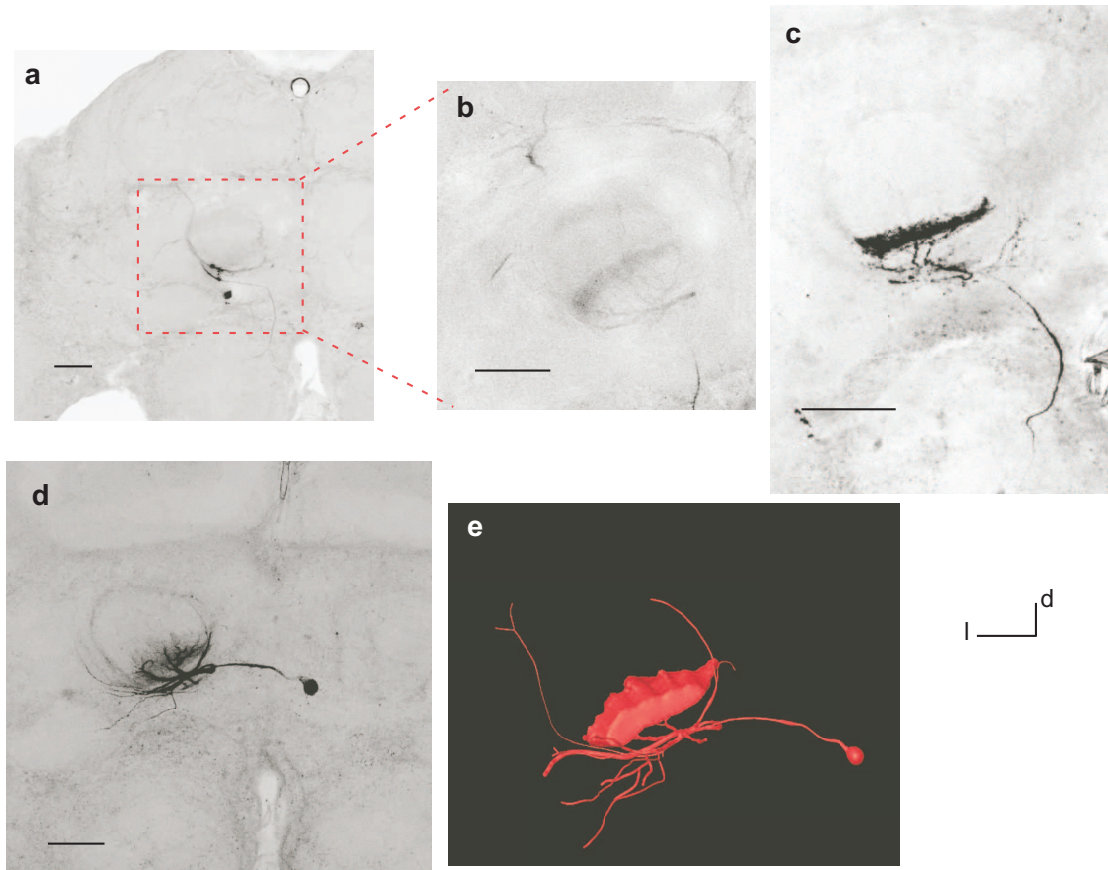
Figure 4.7: Intracellular inontophoretical staining of α-lobe extrinsic neurons, **a:** left brain hemisphere showing stained EN with a projection running medio-laterally to the AL, there is a soma stained ventrally to the α-lobe (probably ALf neuron). **b:** Arborizations in the α-lobe of the same preparation with, dendritic field resembles A5-neuron. **c:** A4 neuron stained with the same technique. **d:** A5 neuron stained iontophoretically, soma resides in the contra-lateral brain hemisphere. **e:** 3-D reconstruction of A5 neuron. Scalebar: 100μm

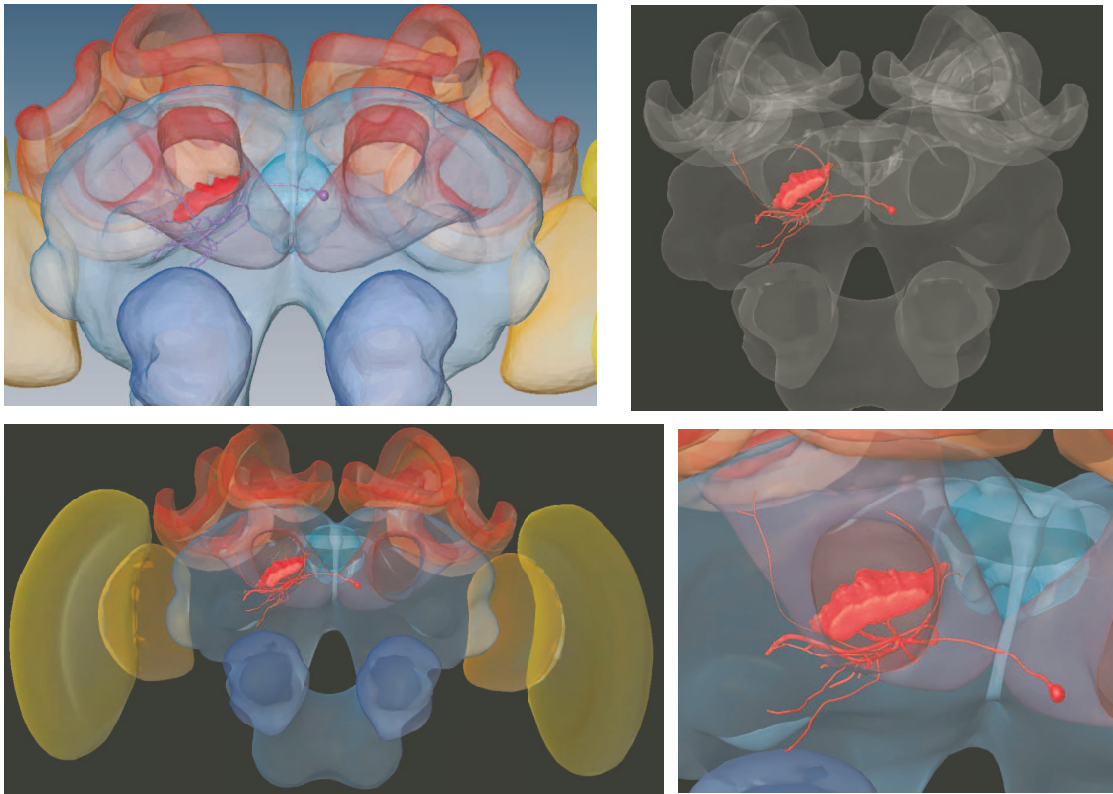Figure 4.8: Reconstruction of A5 neuron and registration into the standard brain. Axon, soma and arborizations around the α-lobe could be reconstructed. Dendritic arborizations inside the α-lobe are indicated as bandshaped structure. Arborizations in the optic tubercle were only weakly stained and could not be reconstructed. The standard brain atlas can be downloaded from http://www.neurobiologie.fu-berlin.de/beebrain/.
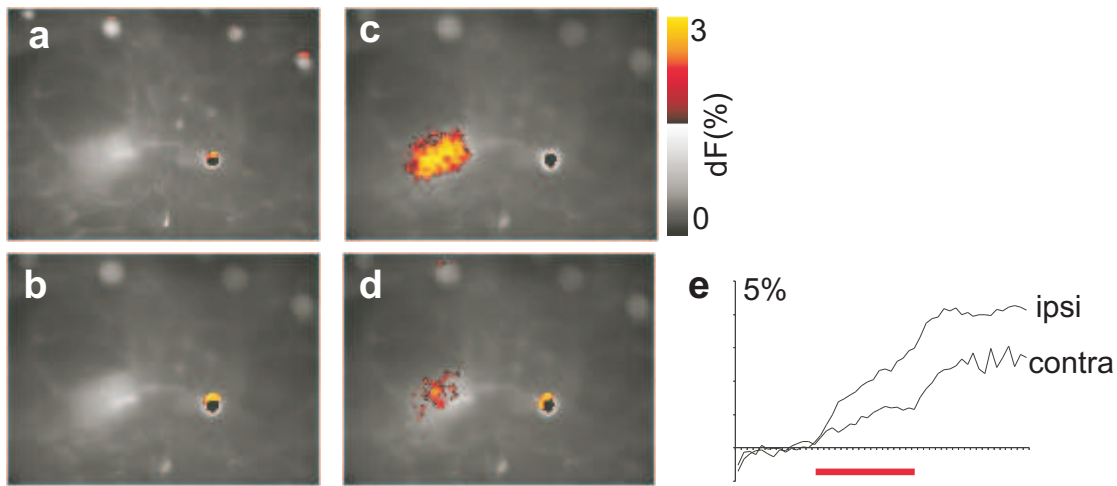
Figure 4.9: Iontophoretical staining of A5 neuron. **a,b:** In response to stimulation with heptanal, only a signal in the soma is observed. **c:** Calcium signals in soma- and dendritic region in response to sucrose stimulation of the ipsi-lateral antenna. **d:** Response to sucrose stimulation of the contra-lateral antenna. **e:** signal traces of sucrose responses for stimulation of the ipsi- and contra-lateral antenna.

responses to sucrose stimulation of the ipsi- and contra-lateral antenna are shown. Figure 4.9e shows the temporal dynamics of the sucrose responses observed in the dendritic region of the neuron within the ventral α-lobe.

Figure 4.10 shows the calcium signals recorded in the A4 neuron in response to heptanal, hexanol, octanol and sucrose stimulation of the ipsi- and contra-lateral antenna. While, as shown in figure 4.9, the recorded A5 neuron responds to sucrose stimulation of the ipsi- and contra-lateral antenna, the A4 neuron shown in figure 4.10 responds to sucrose stimulation of the ipsi-lateral antenna only. Odor evoked responses are observed for all three odors. Heptanal was presented four times. There is no repetition effect observed for repeated stimulation with the same odor, as shown in figure 4.10.
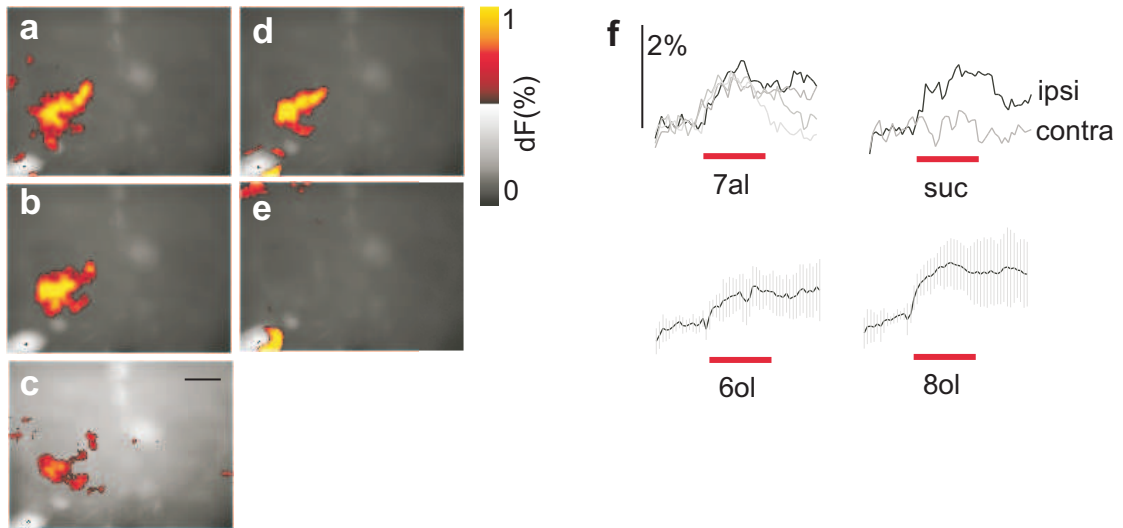
Figure 4.10: Iontophoretical staining of A4 neuron. **a:** Spatial signal in response to stimulation with heptanal. **b:** Spatial signal in response to stimulations with hexanol. **c:** Spatial signal in response to stimulation with octanol (scalebar 100μm, same imaging window in a–e). **d:** Spatial signal in response to sucrose stimulation of the ipsi-lateral antenna. **e:** Spatial signal in response to sucrose stimulation of the contra-lateral antenna. **f:** Temporal dynamics of odor and sucrose responses. Heptanal was presented four times. Sucrose was presented once to each antenna. Traces for hexanol and octanol represent the mean signal in response to ten presentations with each odor (errorbars: sem).

4.5 Discussion

In this study we have measured calcium signals from seven preparations of A4 neurons which were anterogradely stained via their somata, and from one preparation of A3-neurons stained via their axons, which run along the PCT in the lateral protocerebrum. Furthermore, we successfully measured calcium signals in two A4- and one A5-neuron, stained by injecting dye iontophoretically into their axons. We measured neuronal activity in response to odor and sucrose stimulation. In six A4-neurons we additionally measured neuronal activity before and after bees were trained in an appetitive odor learning paradigm. Electro-myographic recordings of the M17 muscle in two bees confirmed that robust odor memories are formed during odor conditioning in the imaging experiments. We found all neurons respond to odor stimulation and sucrose stimulation of the ipsi-lateral antenna. A5- and A3-neurons also respond to sucrose stimulation of the contra-lateral antenna, suggesting a connection between brain hemispheres. We found learning related plasticity in A4-neurons exhibited in reduction in intensity of responses to the CS+ after training in three neurons, and in a reduction in response variance between responses to the CS+ after training, across all six bees. This is to our knowledge the first account of a physiological study combined with the morphological identification of the measured structures of MB ENs in bees, other than the PE1-Neuron (Mauelshagen, 1993; Menzel and Manz, 2005; Okada et al., 2007).

A4 Neurons

Subsets of A4-neurons were stained by injecting dye into the somata-cluster located in the median rim of the antennal lobe. When dye was injected into this region, somata of A4-neurons could no longer be distinguished in confocal scans due to intensive staining of all AL structures. However, in the α-lobe only ar-

borizations of A4-neurons were observed. Rybak and Menzel (1993) describe two types of A4-neurons, termed A4-1, which ramify exclusively in the ventral part of the α-lobe (also referred to as γ-lobe) and A4-2, which send dendrites into the more median part of the α-lobe, however their dendrites lie probably still within the ventral compartment. Both project also to the lateral protocerebral lobe and into the ring-neuropil, were they form bleb-like varicosities. Confocal scans of our preparations reveal that in two cases both types of A4-neurons were stained. However, our imaging approach lacked resolution to distinguish between signals originating from one or the other type. On the other hand, we were able to distinguish between signals originating from structures distinct from MB ENs (e.g. signals originating from projection neurons), as shown in figure 4.2. We exposed six subjects with A4-neurons stained in the described fashion, to an appetitive odor learning paradigm. We chose an experimental design with repeated stimulations before and after training to make data obtained from individual subjects accessible for statistical analysis. We identified one case, with significantly reduced responses to CS+ and control odor after training. Two subjects exhibited reduced responses exclusively to the CS+ after training. We also investigated response variance for each odor before and after training. We found that response variance is significantly reduced after training only for the CS+. This is coherent with learning related plasticity in ENs recently described by Strube-Bloss (2008) who found an increased reliability in the firing of extracellularly recorded units after training. The author recorded from the ventral part of the α-lobe, and it is likely that A4-neurons were among the recorded units. The underlying mechanism may have its source in Kenyon Cell patterns becoming increasingly precise during the course of learning. This type of plasticity we observed in A4-neurons is different from plasticity observed in ENs we investigated earlier (see Chapter 3). We do not

observe a repetiztion effect in A4 neurons, giving some indication for a gain control mechanism at the Kenyon cell/A4 synapses, since clawed Kenyon cells which project to the ventral α-lobe show a response reduction upon repeated stimulation (Szyszka et al., 2008).

A3 Neurons

We stained neurons of the A3-cluster by injecting dye into the protocerebral tract (PCT) at the approximate location of the α-exit. These neurons have been shown to constitute a recurrent inhibitory feedback-loop from the lobes to the input region of the MB, the calyces (Mobbs, 1982; Bicker et al., 1985; Grünewald, 1999a,b). Recently an inhibitory feedback neuron connecting the MB output and input region has also been characterized in *Drosophila* showing learning related plasticity (Liu and Davis, 2009). Our confocal scan reveals stained somata of the ventral and dorsal A3-cluster (Rybak and Menzel, 1993), as well as their projections along the PCT, the dendritic ramifications within the α-lobe and weakly stained terminals in the MB calyces. We observed calcium signals in the somata- and dendritic-region in response to two odors and sucrose stimulation of both, the ipsi- and contra-lateral antenna. Responses were stronger, when applied to the ipsi-lateral antenna in our experiments, consistent with earlier observations described by Grünewald (1999b). The identity of the neurons mediating the cross-talk between brain hemispheres remains yet unclear. Response dynamics observed in the dendritic region resemble those observed in the somata region, with a tendency toward a slower response decay in the somata as described for signals from KC somata compared to their dendritic signals (Galizia and Kimmerle, 2003; Szyszka et al., 2008). No responses were observed in the terminals at the calyx-region.

Iontophoretical Stainings of A4 and A5 Neurons

We stained two A5-neurons and one A4-neuron iontophoretically as described by Galizia and Kimmerle (2003). Odor and sucrose evoked responses were observed during electrophysiological measurements preceeding the imaging experiments, however they were not recorded. A5-neurons have large somata and axons, their somata form a small cluster residing in the contralateral hemisphere and they send projections into the lateral protocerebrum and into the optic tubercle (Rybak and Menzel, 1993). For the A5-neuron we observed odor evoked responses (to heptanal) only in the soma. In the dendritic region we observed responses to sucrose stimulation of the antennae. The response to stimulation of the ipsi-lateral antenna was stronger than to stimulation of the contra-lateral antenna. Responses observed in iontophoretically stained A4-neurons are consistent with results obtained from backfills. Response dynamics observed for A4-neurons differ from those observed for A3-neurons and ENs we measured in an earlier study (Chapter 3). Whereas these other ENs respond with phasic-tonic dynamics, A4-neurons respond almost exclusively with a tonic response dynamic.

Perspectives

In future studies combining electrophysiological measurements and subsequent or simultaneous measurements with functional dyes in ENs (calcium imaging), it would be advantageous to record spike rates from ENs and compare them to the obtained calcium signals. Such an approach could elucidate the relationship between spike activity and calcium signals, and clarify whether spiking activity and calcium signaling is closely correlated, as it has been demonstrated for projection neurons in the honeybee (Galizia and Kimmerle, 2003) and in the locust (Moreaux and Laurent, 2007).

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5 General Conclusion

In this study the role of the honeybee mushroom body extrinsic neurons in odor processing and associative odor learning was investigated using calcium imaging. In Chapter 1 odor representation at the level of mushroom body extrinsic neurons was characterized. Signals in response to different odors, odor concentrations and repeated odor stimulation were analyzed. Further, responses to visual and gustatory stimuli were investigated. We found responses to all tested odors and to sucrose and visual stimulation. We also found odor concentrations being represented in extrinsic neurons exhibiting a sigmoid dose-response relationship, with strongest responses to a 10% dilution of odor in parafin oil. We observed a repetition effect which is most prominent during the first odor presentations. From these results we concluded, that Kenyon cell responses are integrated by extrinsic neurons and information about stimuli is relayed to other brain areas. In Chapter 2, three odors which reliably evoked excitatory responses in extrinsic neurons (as investigated in Chapter 2) were selected to train bees in an appetitive associative odor learning paradigm (PER–conditioning). A strong effort was made to actually monitor behavior during imaging experiments. Although, the conditioning of the PER is a strong and well defined paradigm, it is known that several factors influence the learning performance, as bee age, contingency of odor and reward stimulus and stimulation of the reward pathway restricted to the antennae or stimulating also the proboscis (Menzel and Bitterman, 1983; Wright et al., 2007). In this study

I have aimed on maximizing the probability for a good learning performance in the individual bee, by only using pollen foragers for the learning experiments and by delivering the reinforcing stimulus to the antennae as well as to the proboscis during training. This way it was possible to show that bees form a robust odor memory during imaging experiments and that neurons converging to the protocerebral tract exhibit learning related plasticity. In some cases a direct correlation between behavioral performance and calcium signals in extrinsic neurons can be observed. It was also possible to relate the repetition effect directly to habituation processes, by showing that a response reduction to the unconditioned stimulus (sucrose) coincides with PER-habituation. In Chapter 4 the responses of individual extrinsic neurons were investigated and responses during odor and sucrose stimulation, as well as during associative odor learning were analyzed in a defined subgroup of extrinsic neurons. We find learning related plasticity in A4-neurons manifested in a reduction of response varciance after training matching findings recently reported by Strube-Bloss (2008). By characterizing odor responses and responses to visual and gustatory stimuli in individul neurons it was attempted to gain insight to the representation of stimuli in mushroom body extrinsic neurons before changes induced by learning were investigated. One of the main advantages of calcium imaging is that a population of neurons can be simultaneously observed while an approximate knowledge of their identity is secured. However, in a population response individual differences between neurons may be overlooked or averaged out. This may be one of the reasons why previous studies in the honeybee employing calcium imaging to investigate learning induced changes in neuronal activity have lead to seemingly contradictory results (compare Faber et al. (1999) and Peele et al. (2006)). Among the limitations of the calcium imaging technique is also that measurements are restricted to a time window of approximately one

hour, hence only short term plasticity effects may be investigated.

The mushroom bodies have been shown to have generally inhibitory effect on behavioral output (Huber, 1962; Martin et al., 1998). Also the effects observed in this study on mushroom body extrinsic neurons are mostly inhibitory. The effects observed in Chapter 2 for the plasticity induced by associative learning compete with those induced by repeated stimulation. It is therefore concluded that the mushroom body network may provide the bee with the ability to attend to important stimuli (e.g. those related with reward) and ignore unimportant stimuli (which have no predictive property). For future experiments it would be extremely interesting to interfere with the mushroom body circuitry, such that these speculations can be addressed.

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