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RESEARCH ARTICLE

Long-term memory and response generalization in mushroom body extrinsic neurons in the honeybee *Apis mellifera*

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SUMMARY

Honeybees learn to associate an odor with sucrose reward under conditions that allow the monitoring of neural activity by imaging Ca^{2+} transients in morphologically identified neurons. Here we report such recordings from mushroom body extrinsic neurons – which belong to a recurrent tract connecting the output of the mushroom body with its input, potentially providing inhibitory feedback – and other extrinsic neurons. The neurons' responses to the learned odor and two novel control odors were measured 24 h after learning. We found that calcium responses to the learned odor and an odor that was strongly generalized with it were enhanced compared with responses to a weakly generalized control. Thus, the physiological responses measured in these extrinsic neurons accurately reflect what is observed in behavior. We conclude that the recorded recurrent neurons feed information back to the mushroom body about the features of learned odor stimuli. Other extrinsic neurons may signal information about learned odors to different brain regions.

Key words: honeybee, mushroom body, calcium imaging, olfactory learning, long-term memory, generalization.

INTRODUCTION

Behavioral, physiological and genetic approaches have identified the mushroom bodies (MBs) as essential structures in the insect brain that are involved in learning and memory formation, for example in *Drosophila* (de Belle and Heisenberg, 1994; Heisenberg, 2003), *Periplaneta* (Mizunami et al., 1998) and *Apis* (Menzel and Muller, 1996). The MBs comprise three types of neurons: projection neurons (PNs) ascending from sensory neuropiles; intrinsic neurons known as Kenyon cells (KCs); and extrinsic neurons (ENs), which connect the MBs to other brain regions. Previous calcium imaging studies on learning and memory using olfactory stimuli have focused on the antennal lobes and the KCs of the MBs (Faber et al., 1999; Faber and Menzel, 2001; Peele et al., 2006; Szyszka et al., 2008). Olfactory input reaches the MBs *via* olfactory PNs, which ascend from the antennal lobe. PNs have been characterized in detail in the honeybee (Abel et al., 2001; Szyszka et al., 2005; Krofczik et al., 2008; Yamagata, 2009). In the lip region of the MB calyces, the PNs synapse onto specified KCs (Mobbs, 1982). The KCs relay olfactory information to ENs in the ventral and medial partition of the MB lobes. The ENs are thought to provide mainly output from the MB to other brain areas, and have a highly organized structure consisting of horizontal layers in the MB lobes (Strausfeld et al., 2000; Strausfeld, 2002).

This calcium imaging study focuses on long-term memory effects in ENs. Previous studies have documented that ENs are subject to multiple associative plasticity phenomena, which were interpreted as neural correlates of learning and memory formation in the honeybee (Mauelshagen, 1993; Grünewald, 1999a; Okada et al., 2007; Haehnel and Menzel, 2010; Strube-Bloss et al., 2011). The intrinsic organization of the MB lobes and the location of the MB somata allow a classification of the ENs into anatomical subgroups (A1–A7) (Rybak and Menzel, 1993). One of these subgroups, the A3 or protocerebral-calycal tract (PCT) neurons, has been investigated in greater detail. The A3 are GABA-immunoreactive (Bicker et al., 1985; Grünewald, 1999b) and their responses to olfactory stimuli are modulated after learning (Grünewald, 1999a; Haehnel and Menzel, 2010). This is interesting because in both vertebrates (Freund and Buzsáki, 1996) and invertebrates (Perez-Orive et al., 2002; Liu et al., 2007; Groh and Rössler, 2008; Liu and Davis, 2009; Liu et al., 2009), GABA-transmitted inhibition seems to influence the pathways involved in learning and memory formation.

Since we identified a short-term memory trace in ENs directly after conditioning in a previous study (Haehnel and Menzel, 2010) we became interested in whether this associative effect persists over a larger time window and how it applies to generalization. Here, we labeled ENs of the A3 cluster and other ENs belonging to the A1/2 cluster with a calcium sensitive dye to investigate whether ensembles of these MB ENs change their responses after the formation of long-term memory induced by classical olfactory conditioning. We used three odors to test memory recall and generalization. Bees are known to generalize more strongly between odors with a more similar molecular structure than between odors belonging to different chemical classes (Laska et al., 1999; Guerrieri et al., 2005). It has also been shown that odor discrimination of structurally similar odors is affected by injections of the GABA antagonist picrotoxin into the antennal lobe, whereas odor discrimination of less similar odors is not (Stopfer et al., 1997). We found that odor responses to the previously trained odor were enhanced 24 h after conditioning. Furthermore, we show that when an odor is more strongly generalized, responses tend to be stronger than responses to a weakly generalized odor.

MATERIALS AND METHODS

Preparation

Honeybee (*Apis mellifera* Linnaeus 1758) foragers were caught at the entrance of outdoor hives during summer 2008. They were anesthetized on ice and mounted in Plexiglas® holders (Sachse et al., 1999). At least 2 h before conditioning they were fed a drop of 1 mol l^{-1} sucrose solution and stored in a humidified box.

Behavioral procedure

Approximately 50 bees were trained in a classical conditioning paradigm in which they learned to associate an odor with a sucrose reward (Bitterman et al., 1983). An odor stimulus [conditioned stimulus (CS)] was paired three times with a sucrose stimulus [unconditioned stimulus (US); 1 mol l^{-1} solution] delivered by hand to the antennae and then to the proboscis using a toothpick. The odor was presented through a computer-controlled olfactometer (Galizia et al., 1997) at a 1% dilution in paraffin oil. The odor stimulus lasted 3 s; the sucrose stimulus also lasted approximately 3 s and overlapped with the odor stimulus by 1 s. The inter-trial interval (ITI) was 10 min. Forty bees that extended their proboscis in response to the odor during the third conditioning trial were kept for calcium imaging on the following day. Of these, eight bees exhibited calcium signals in MB ENs and were therefore used for the retention protocol. During calcium imaging, five extinction trials of the previously trained odor (CS+) were presented. To test for generalization, an odor with a similar chemical structure and an odor with a different chemical structure were presented in alternation (Fig. 2A). The ITI was 1 min. The odors used were 1-hexanol (6ol), 1-octanol (8ol) and linalool (lio); 6ol and 8ol were balanced for use as the CS+.

Electromyograms and calcium imaging

For calcium imaging, the neurons were filled with a calcium indicator. Calcium signals were recorded using wide-field microscopy because this technique allows calcium imaging of neurons *in vivo* in the intact animal, which still responds to trained odor stimuli (Haehnel et al., 2009). We used a mixture of fura-2 dextran (10,000 MW, lysine fixable, Sigma-Aldrich, St Louis, MO, USA), which allows ratiometric measurements of intracellular calcium, and fixable tetramethyl rhodamine (10,000 MW, lysine fixable, Sigma-Aldrich) for later confocal imaging to confirm the identity of the labeled structures. Dye filling occurred shortly after the bees had been conditioned as described above. The head capsule was opened with a scalpel, and glands and trachea were partially removed. A thin glass capillary carrying dye paste at its tip was used to inject dye into the lateral rim of the MB alpha-lobe of both brain hemispheres. Subsequently, the piece of cuticle was restored and bees were kept until the next day. Bees were prepared for calcium imaging and electromyogram recordings 24 h after conditioning. Myograms of the M17, one of the muscles involved in proboscis extension, were recorded using a tungsten wire inserted into the head capsule at the M17 tendon, and a silver wire which was placed into the compound eye as a ground electrode (Rehder, 1987). Muscle potentials were amplified using a custom built pre-amplifier and digitalized with a CED 1401 interface (Cambridge Electronic Design Ltd, Cambridge, UK) to be visualized and stored on a computer using Spike2 software (Version 6, Cambridge Electronic Design Ltd). Muscle activity was continuously recorded during the complete protocol. For analysis, spikes were counted during a 10 s interval after odor onset.

Prior to calcium imaging, the piece of cuticle covering the brain and remaining glands and trachea were removed. To stabilize the brain, a drop of two-component silicone (Kwik Sil Adhesive, WPI, Sarasota, FL, USA) was filled into the head capsule. A small incision was made above the labrum, and the esophagus was pulled gently through the opening without damaging it (Mauelshagen, 1993). Fluorescence images were recorded at room temperature through a water immersion objective (×60, 0.9 NA, Olympus, Tokyo, Japan) with a sampling rate of 5 Hz using an imaging setup (Till Photonics, Munich, Germany) equipped with a CCD camera mounted onto a fluorescent microscope (Axioscope, Zeiss, Oberkochen, Germany). Images were recorded at 640×480 pixels and binned on chip to 160×120 pixels. Spatial resolution was 1.47×1.47 μm $pixel^{-1}$. Fura-2 was excited with illumination of 340 and 380 nm for ratiometric measurements. Fluorescence was detected through a 410 nm dichroic mirror and a 440 nm long-pass filter. All measurements began 3 s before stimulus onset and lasted for 10 s.

Confocal microscopy

Following *in vivo* Ca^{2+} imaging experiments, brains were dissected and fixated in 4% formaldehyde overnight, rinsed in phosphate buffered saline and dehydrated in rising ethanol steps. Brains were cleared in methyl salicylate and scanned with a confocal laser-scanning microscope (Leica TCS SP2, Wetzlar, Germany) using an oil immersion objective (×20, 0.4 NA). An excitation wavelength of 534 nm was used. Optical stacks were stored on a computer.

Data analysis and statistics

Custom-written programs were used (IDL Version 6.2, RSI, Boulder, CO, USA) for initial imaging data processing. The ratio of the 340 and 380 nm induced Ca^{2+} emission signals was calculated for each pixel. Background fluorescence, determined by averaging over frames 4–13, was subtracted from the ratiometric signal yielding deltaF (dF). For inspection of the spatial distribution of the signal, the mean of the 14 frames (16–29) during stimulation was calculated and a spatial Gaussian low-pass filter of 3×3 pixels was applied. Greyscale values of the Ca^{2+} fluorescence were transformed into false color scale. The active regions were determined as regions of interest (ROI), and temporal dynamics were calculated by averaging the greyscale values of all pixels inside the ROI without spatial filtering.

Muscle responses were quantified during an interval of 10 s after stimulus onset by counting M17 spikes in Spike2 software. A threshold was determined to avoid counting of smaller spikes derived from mandible or other muscles.

Statistical significance between responses was determined using one-way repeated-measures ANOVA or one-way ANOVA with a Bonferroni *post hoc* test using SYSTAT (V13, Systat Software Inc., Chicago, IL, USA). Graphs were prepared in MS Excel (Office 2007, Microsoft, Redmond, WA, USA).

RESULTS

Identification of neurons

ENs connect the lobes of the MB with different brain areas. A schematic of the bee brain based on a three-dimensional reconstruction (Brandt et al., 2005) is given in Fig. 1A. The co-labeling of the neurons targeted for calcium imaging with a fixable dye allowed us to identify the structures from which we obtained calcium signals during the behavioral experiment. Primarily, neurons of the A3 somata cluster were labeled along with some neurons of the A1/2 cluster (Fig. 1B). Labeled structures within the MB alpha-lobe resembled the band-like dendritic anatomy of the A3 neurons, which are mostly restricted to the median and dorsal parts of the alpha-lobe (Fig. 1C). Of these neurons, only the median layers have been reported to participate in olfactory processing, because they

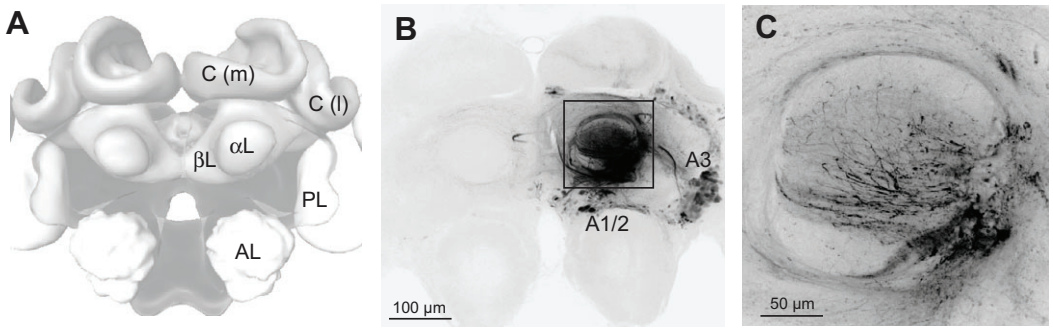

Fig. 1. Identification of structures with calcium activity in the honeybee *Apis mellifera*. (A) Model of the honeybee standard brain (http://www.neurobiologie.fu-berlin.de/beebrain/). AL, antennal lobe; C (l), lateral mushroom body calyx; C (m), median mushroom body calyx; PL, protocerebral lobe; αL, mushroom body alpha-lobe; βL, mushroom body beta-lobe. (B) Stack of confocal images showing the labeled structures investigated during calcium imaging in whole-mount of the honeybee brain. Labeled somata of mushroom body extrinsic neurons are visible in the lateral and ventral protocerebrum (A1/2, somata of A1 and A2 neurons; A3, somata of A3, also called PCT neurons). (C) Zoomed in picture of dendritic arborizations within the median region of the alpha-lobe where calcium signals were measured.

overlap with KCs receiving olfactory input (Grünewald, 1999b; Strausfeld et al., 2000). We conclude that calcium signals were mainly recorded from sub-populations of A3 ENs (PCT neurons) and possibly A1/2 neurons.

Behavior and electromyograms

Long-term memory retention and odor generalization was tested 24 h after training in eight bees. For training, an odor (8ol or 6ol) was paired three times with sucrose. During the retention test, the odor (8ol or 6ol) was presented five times, alternating with an odor with a more similar chemical structure (6ol or 8ol, respectively) and an odor with a more dissimilar chemical structure (linalool) (Fig. 2A). For the retention test, only bees were used that had learned the conditioning odor in the third training trial. The acquisition curve is shown in Fig. 2B.

To quantify the behavioral response during the retention and generalization test, we recorded electromyograms from M17 in all eight bees. We expected behavioral generalization to be stronger between the CS+ and the control odor with the more similar chemical structure (i.e. between the primary alcohols). However, this was not always the case. Consequently, we classified the control odors for each bee into a more strongly generalized (SG) odor and a more weakly generalized (WG) odor based on the strength of the behavioral response. An example of M17 recordings classified in this way is shown in Fig. 2C. We classified an odor as more strongly generalized when the number of M17 spikes during the proboscis extension response (PER) to the respective odor exceeded the number of spikes elicited in response to the other odor. Accordingly, the odor with fewer and weaker responses was considered more weakly generalized.

Overall, the generalization between odors was strong (Fig. 2D). When we took the repeated trials into consideration for each animal and tested for differences between the responses to CS+, SG and WG using a repeated-measures ANOVA, we found no significant difference (d.f.=2 odor categories, N=8 bees, F=2.960, P=0.074). There was a within-subject effect for trial number (d.f.=4 trials, N=8 bees, F=7.793, P<0.0001), as the M17 responses decreases with repeated stimulation because of behavioral habituation. When we pooled M17 responses for each stimulus category (CS+, SG and WG) across all trials and bees we found a significant difference between categories using a conventional one-way ANOVA (d.f.=2 odor categories, N=40 bees × trials, F=8.027, P=0.001). Applying a Bonferroni *post hoc* test for pairwise comparison, we found significant differences between CS+ and WG (P<0.0001) and SG and WG (P=0.037). Generalization was so strong that no statistical difference could be detected between CS+ and SG.

ENs exhibit enhanced responses to the trained and strongly generalized odor

During retention and generalization tests, the axon collaterals of ENs in the medio-lateral region of the MB alpha-lobes were imaged. Fig. 3A shows an example of the spatial distribution of the signals in response to the three odors presented during retention tests. We did not observe any obvious differences regarding the spatial distribution of the signals in response to the three different odors. However, the responses became weaker with increased number of stimulus repetitions. Fig. 3B presents the raw fluorescent image of the region shown in false colors in Fig. 3A. To investigate the temporal dynamics of the calcium signals we chose the region with increased calcium signal during odor stimulation (red and green in false color scale in Fig. 3A) as region of interest (ROI; red outlined area in Fig. 3B) and averaged the intensity of the pixels in this area. We observed some differences in the temporal dynamics of the calcium signal in response to the different odors, which mainly consisted of a more or less pronounced 'off-response', i.e. a second response peak upon stimulus offset (Fig. 3C). However, across animals we did not find a systematic relationship between the role of the odor as a CS+ and the temporal shape of the response and did not further analyze the off-response in this study.

To compare the response strength with the different stimuli we determined the mean signal during the stimulus (area under the response curve). We used a repeated-measures ANOVA to test for differences between the signals elicited by the three odors (6ol, 8ol and linalool) and found no significant differences (d.f.=2 odors, N=8 bees, F=2.348, P=0.120). However, we found a significant within-subject effect for trial number (d.f.=4 trials, N=8 bees, F=4.487, P=0.002), as the responses to the first odor stimulation for a particular odor during retention tests exceeded the responses in the subsequent trials.

Calcium signals in response to the CS+, SG and WG were different in strength (Fig. 3D). We used a repeated-measures ANOVA to compare the CS+ with the odors categorized as SG and WG based on the behavioral responses and found a significant difference between odor categories (d.f.=2 odor categories, N=8 bees, F=4.606, P=0.022). We also saw a significant within-subject effect for trial number (d.f.=4 trials, N=8 bees, F=4.754, P=0.002).

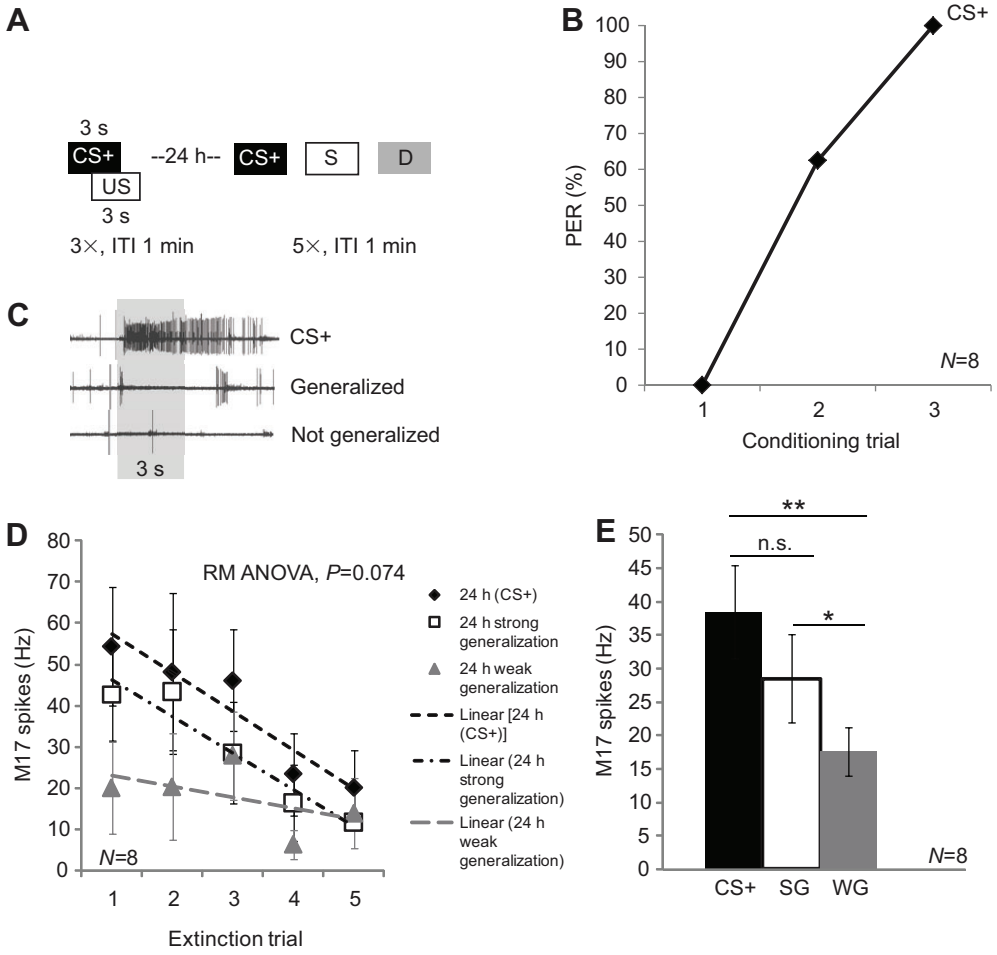

Fig. 2. Odor conditioning, retention and generalization in the honeybee *A. mellifera*. (A) Protocol: the odor used as conditioned stimulus (CS+) was forward paired three times with sucrose as the unconditioned stimulus (US). The inter-trial interval (ITI) was 1 min. On the following day (ca. 24 h later), the CS+ and two control odors were presented; one control odor was chemically more similar to the CS+ (S) and the other control odor was chemically different (D). The odors were presented five times each in an alternating fashion with an ITI of 1 min. (B) Ratio of bees that exhibited a proboscis extension response (PER) during conditioning trials (% PER, N=8). (C) Examples of electromyograms recorded from M17; the grey box represents the odor stimulus. (D) Rates of M17 spikes recorded during retention and generalization tests with the CS+ and control odors. We expected generalization to be stronger for the odors with structure more similar to that of the CS+. However, this was not always the case; therefore, control odors were divided into those with strong generalization (SG) and those with weak generalization (WG), regardless of odorant structure. Overall generalization was strong; a repeated-measures (RM) ANOVA failed to meet the 5% criterion for statistical significance (subjects: eight bees, between-subject factor: three odor categories, d.f.=2, F=2.960, P=0.074; within-subject factor: five trials, d.f.=4, F=7.793, P<0.0001). (E) Pooling the data for the M17 spike rates across trials yielded a significant difference between the CS+ trials and SG (one-way ANOVA, N=40 bees × trials, d.f.=2, F=8.027, P<0.0001; Bonferroni *post hoc* test, P<0.0001) and between SG and WG (Bonferroni *post hoc* test, P=0.037). No statistical difference was found between CS+ and SG.

There was a strong CS+ effect in the first trial, which attenuated during subsequent trials (Fig. 3D). When we pooled the responses across trials and bees and compared the categories using a one-way ANOVA with a Bonferroni *post hoc* test for pairwise comparison, we found a significant difference between CS+ and WG (d.f.=2 odor categories, N=40 bees × trials, F=4.875, P=0.009; *post hoc* test, P=0.008). There were no significant differences between CS+ and SG, or SG and WG. Thus, the calcium signals in response to the odors reflect what was observed in behavior, except that no difference between SG and WG was found.

DISCUSSION

The MB and olfactory memory

Earlier work in bees (Menzel et al., 1974; Menzel et al., 1996) and *Drosophila* (Heisenberg, 1989) has shown that the MB as a whole is involved in memory formation. In the calyces of the MBs, sensory (olfactory) information converges with modulatory input from the VUM_{mx1} neuron, which mediates the reward pathway in olfactory learning (Hammer, 1993). It has been shown that in the honeybee the MB intrinsic neurons – the KCs – alter their responses after olfactory conditioning (Szyszka et al., 2008). These authors showed that although the calcium signal in response to odors decreases with repetition, it decreased less for an odor that was previously associated with reward. This effect can also be observed in A3 ENs, which are postsynaptic to KCs, across the same time window (Haehnel and Menzel, 2010). Although the sparse representation of odors in the Kenyon cells is thought to be involved in the separation of overlapping and redundant representations in a structure associated with memory formation (Turner et al., 2008), the ENs may convey the readout of this representation to other brain areas and form feedback loops. It has also been demonstrated that different groups of MB ENs play different roles in learning and memory formation

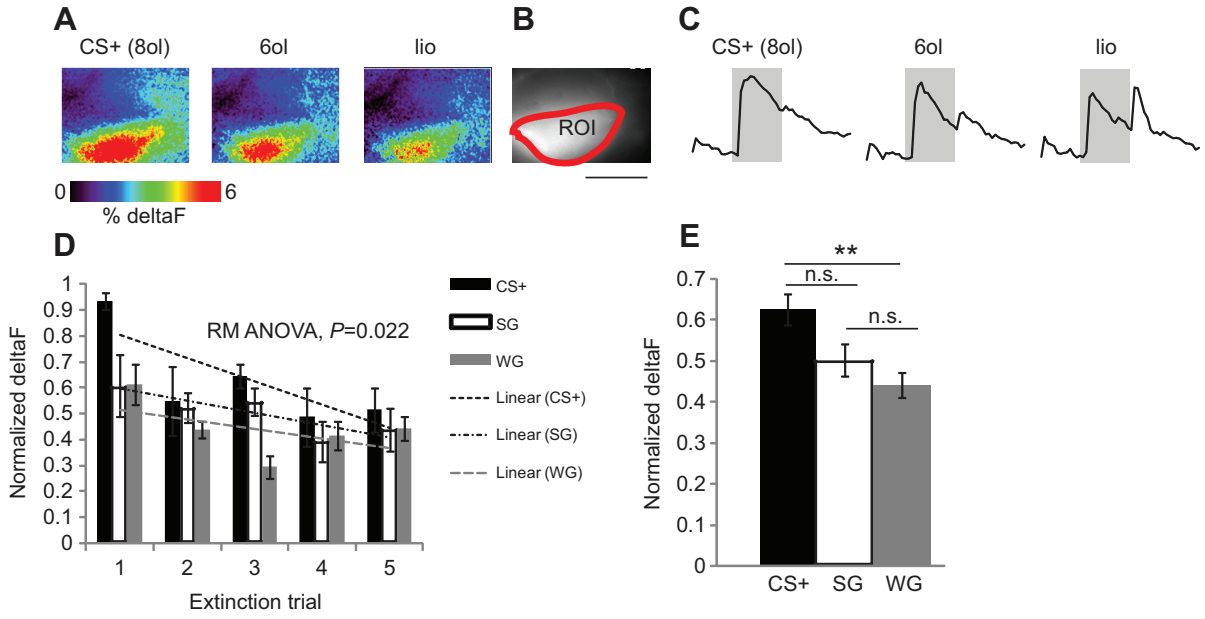

Fig. 3. Calcium imaging of axon collaterals in the median region of the alpha-lobe in the honeybee *A. mellifera*. (A) Spatial distribution of calcium signals in response to octanol (8ol; used as CS+), hexanol (6ol) and linalool (lio) in one representative bee. False color scale represents the mean deltaF (340/380 nm) percentage during the odor stimulus. The scale is the same as in B. (B) Raw fluorescent picture of the imaged region at 340 nm; the active region was chosen as the region of interest (ROI), indicated by the red frame. Scale bar, 100 μm. (C) Temporal dynamics of the signals were assessed by calculating the average intensity of deltaF across all pixels in the respective ROI. The gray box represents the odor stimulus. (D) Calcium signal strength during the five extinction trials for CS+, SG and WG as determined by the behavioral response. There was a significant difference between groups [repeated-measures ANOVA, subjects: eight bees, between-subject factor: three categories (CS+, SG and WG), d.f.=2, F=4.606, P=0.022; within-subject factor: five trials, d.f.=4, F=4.754, P=0.002]. (E) When data across trials and bees were pooled there was a significant difference between odor categories (one-way ANOVA, N=40 bees × trials, d.f.=2, F=4.875, P=0.009); pair-wise comparison yielded a significant difference between CS+ and WG (P<0.0001).

(Mauelshagen, 1993; Grünewald, 1999a; Okada et al., 2007; Strube-Bloss et al., 2011). It is likely that differences exist even between groups of A3 neurons, which would explain the differences between the results of our imaging studies and electrophysiological studies focusing on individual neurons (Grünewald, 1999a). Here we demonstrated that MB ENs belonging to the A3 somata cluster, also referred to as PCT neurons, and neurons of the A1/2 cluster exhibit changes in activity 24 h after classical olfactory conditioning. Calcium transients in response to the previously rewarded odor were stronger than responses to weakly generalized control odors, which were not presented during previous training. The difference in response strength was smaller when the control odor was strongly generalized in behavior (PER) than when the odor was weakly generalized. The difference between responses to the CS+ and the strongly generalized odor were not statistically significant for behavior and calcium imaging. This result underlines the importance of monitoring behavior during physiological studies on memory formation, because if no differences between physiological responses are detected it can be due to a failure to learn or generalization. We show that the associative effect found in the A3 neurons extends into long-term memory. In our earlier study (Haehnel and Menzel, 2010), a CS– was presented during training trials and, although a similar set of odors was used, generalization was difficult to interpret because odors were randomized for their use as CS+, CS– and control odor, thereby in some experiments the control was more similar to the CS+ and in others it was more similar to the CS–. Also, the labeling technique in both studies was slightly different and were, in general, coarser in the present study to increase the probability of a successful staining given the low survival rate, such that A1 and A2 ENs were also labeled, which may have contributed to the observed calcium signals. It will be a challenge for future studies to identify the neurons that mediate the repetition-induced depression in KCs (Szyszka et al., 2008) and in the groups of ENs described here and earlier (Haehnel and Menzel, 2010).

A substantial fraction of the neurons from which we recorded calcium signals belong to the A3 cluster (PCT neurons). These neurons have been shown to be GABA-immunoreactive (Bicker et al., 1985; Grünewald, 1999b). Earlier studies reported decreased activity in these neurons in response to odors after olfactory conditioning (Grünewald, 1999a; Okada et al., 2007); in addition, in *Drosophila*, GABAergic MB ENs (dorsal paired median neurons) decrease their activity after learning (Yu et al., 2005; Liu and Davis, 2009) whereas increased activity in these neurons inhibits memory formation (Liu et al., 2007; Liu et al., 2009).

However, the balance between excitation and inhibition is crucial for the functioning of neural circuits across the brain. For example, in vertebrates, mice with enhanced GABAergic innervations of the dentate gyrus show improved reversal learning and improved abilities to change their search strategy in the water maze (Morellini et al., 2010). In contrast, injections of GABA agonists into the brain cause amnesic effects in both vertebrates (Roth et al., 1984; Lister, 1985) and invertebrates (El Hassani et al., 2005; El Hassani et al., 2008; El Hassani et al., 2009). A recent study reported that ensembles of ENs are recruited after olfactory conditioning whereas others drop out of the odor response (Strube-Bloss et al., 2011). The enhanced calcium signals we found could therefore reflect newly recruited neurons after conditioning with the same spatial distribution as those neurons responding to the odor before training, as we did not observe any changes in the distribution of the calcium signal. An enhancement of calcium influx into neurons of the MB

body gamma-lobe related to long-term memory was also recently described in *Drosophila* (Akalal et al., 2010).

Generalization

During the retention test 24 h after classical conditioning, we presented the CS+ and two control odors. One of the control odors had a chemical structure more similar to the CS+ than the other. We expected generalization to be stronger between odors with a more similar chemical structure, because it was previously reported that bees generalize more between odors from the same chemical class (Guerrieri et al., 2005). However, when we looked at behavioral generalization, we found that some bees generalized more between the CS+ and the chemically similar odor and others generalized more between the CS+ and the chemically different odor, which could be due to our limited sample size. Therefore, we categorized the two odors (the chemically more similar and less similar odors) separately for each animal and ranked them according to the strength of behavioral response. When we compared the responses of MB ENs with the learned odor and with these two control odors we found greater differences in the neuronal responses between the CS+ and the control odor that was less likely to elicit a behavioral response than between the CS+ and the more strongly generalized odor. The olfactory system has to master discrimination and generalization, the latter leading to categorization. This categorization may depend on factors other than just the chemical similarity of odors. When bees forage, flower frequencies will vary to some extent, which affects odor components and their concentrations; however, bees are able to ignore these differences and generalize between small variations in composition (Wright et al., 2002; Wright and Smith, 2004). For our results, this could mean that, for example, a combination of odors may have been associated with a food source in the past, and bees in our experiments responded to the associated odor because olfactory memory is context insensitive (Gerber et al., 1996). In addition, studies modeling the perception of odors in insects and vertebrates predict that perceived odor similarity may depend on factors other than merely chemical structure (Schmuker et al., 2007; Haddad et al., 2008).

Conclusions

Our findings support the view that a MB's main output region, the potentially inhibitory recurrent pathway, undergoes stable and long-lasting associative plasticity indicative of a neural correlate of long-term memory. Findings in *Drosophila* suggest that different types of MB neurons are involved in different phases of memory formation and recall (Yu et al., 2005; Yu et al., 2006; Akalal et al., 2010). Therefore, in the honeybee as well as in *Drosophila*, it will be crucial in the future to elucidate the roles played by different neuron types in the MB body.

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