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SHORT COMMUNICATION

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Visualizing mushroom body response to a conditioned odor in honeybees

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Abstract Combining differential conditioning with optophysiological recordings of bee brain activity allows the investigation of learning-related changes in complex neural systems. In this study we focused on the mushroom bodies of the bee brain. Presenting different odors to the animal leads to significant activation of the mushroom body lips. After differential conditioning, the rewarded odor leads to stronger activation than it did before training. Activation by the unrewarded odor remains unchanged. These results resemble findings in the bee's antennal lobes, which are the first olfactory relay station in the insect brain. As an integrative neural network, enhanced activation of the mushroom body lip may carry additional information, i.e., for processing odor concentrations.

Introduction

Physiological correlates of memory traces are hard to come by because the neural traces left even after very simple forms of learning are distributed over different regions of the brain, making it very difficult to ascertain which of the traces are the important ones and how the different traces are related (Thompson et al. 1986; Squire 1987). Attempts at directly observing memory trace-related events in brain structures involved in memory formation (e.g. the hippocampus) have provided important insights into cellular synaptic plasticity events (e.g., Olds et al. 1989; Engert and Bonhoeffer 1999), but it is very difficult to relate these processes to the memory trace as it is relevant for behavioral control, because such studies can be carried out only in brain slices, not in the brain of an active animal.

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The honeybee brain has the advantage that its small size allows the visualization of neural activity by means of calcium imaging of brain structures involved in memory formation (Joerges et al. 1997; Galizia et al. 1999). The associative processes accompanying olfactory conditioning in harnessed bees are so robust that the animal shows normal acquisition and retention even when the brain is exposed to such optophysiological experiments (Faber et al. 1999). Combining the optophysiological recording technique with differential conditioning, we showed that the odor-induced patterns of neural activity in the primary olfactory neuropil, the antennal lobe (AL), are enhanced for the learned odor and that the respective activity patterns of the learned odor become less similar to those of non-learned odors (Faber et al. 1999). Our conclusion from these experiments was that the memory trace at the AL level expresses itself predominantly as a more intense activity pattern, and less as a qualitatively different pattern encoding the learned odor. Such a neural trace may not be unique for the learned odor because a more intense odor could also produce a more intense activity pattern. Additional signatures are necessary to provide the animal with a neural correlate of a learned odor, because bees are not confused in their learned response to odors if the odor intensity is changed over a wide range (Pelz et al. 1997). One possibility would be that other parts of the brain involved in processing the odor stimulus (e.g. the mushroom bodies) provide the necessary signature. We therefore imaged the odor-induced activity patterns in the lip region of the mushroom body (MB).

The lip region receives input from projection neurons conveying olfactory information from the AL to the MB. Odor stimuli elicit activity patterns in the lip region (Fig. 1). These patterns are different for different odors tested in the same animal.

Materials and methods

The optophysiological methods applied were the same as those described for AL imaging experiments (Faber et al. 1999). Prepara-

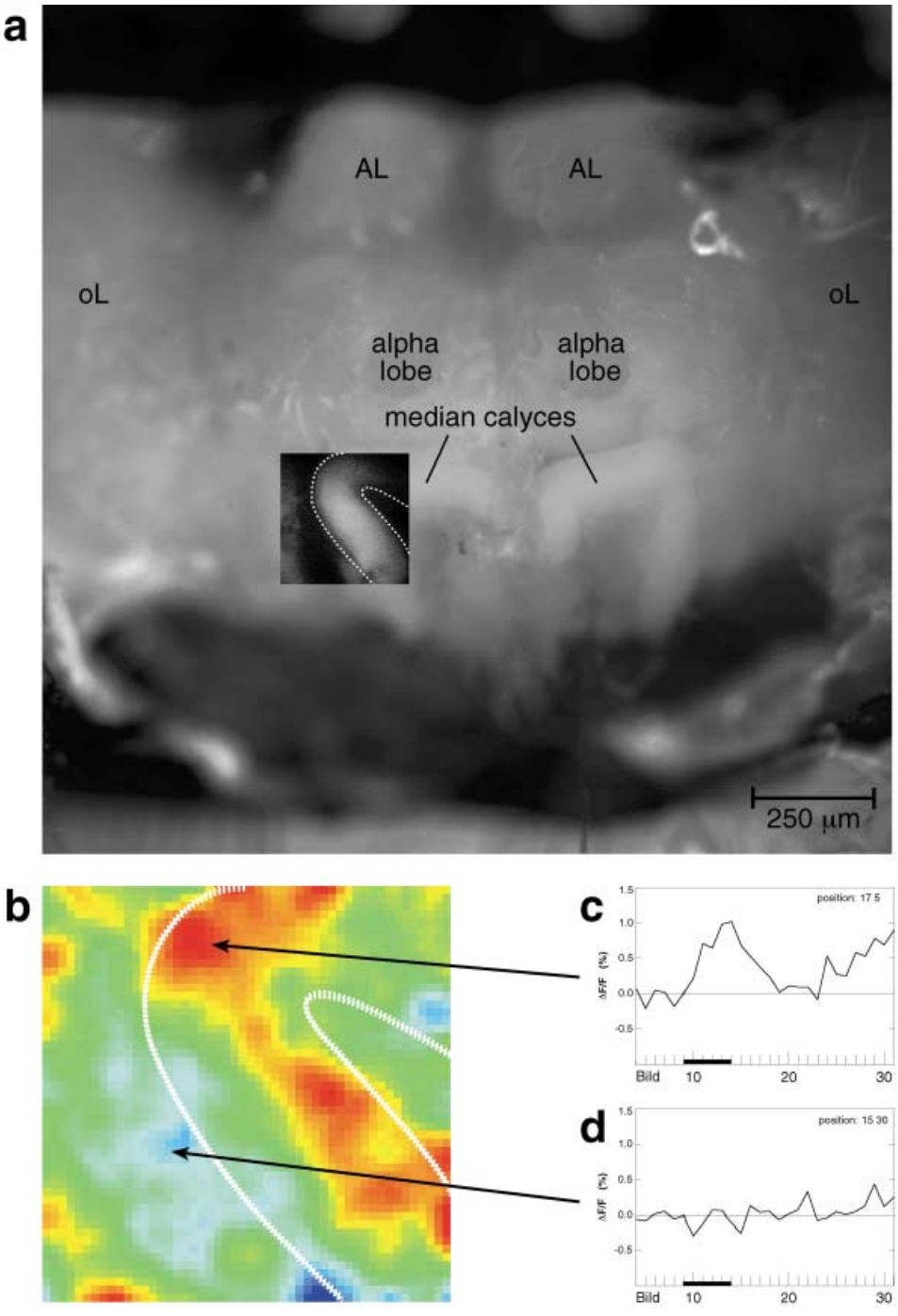
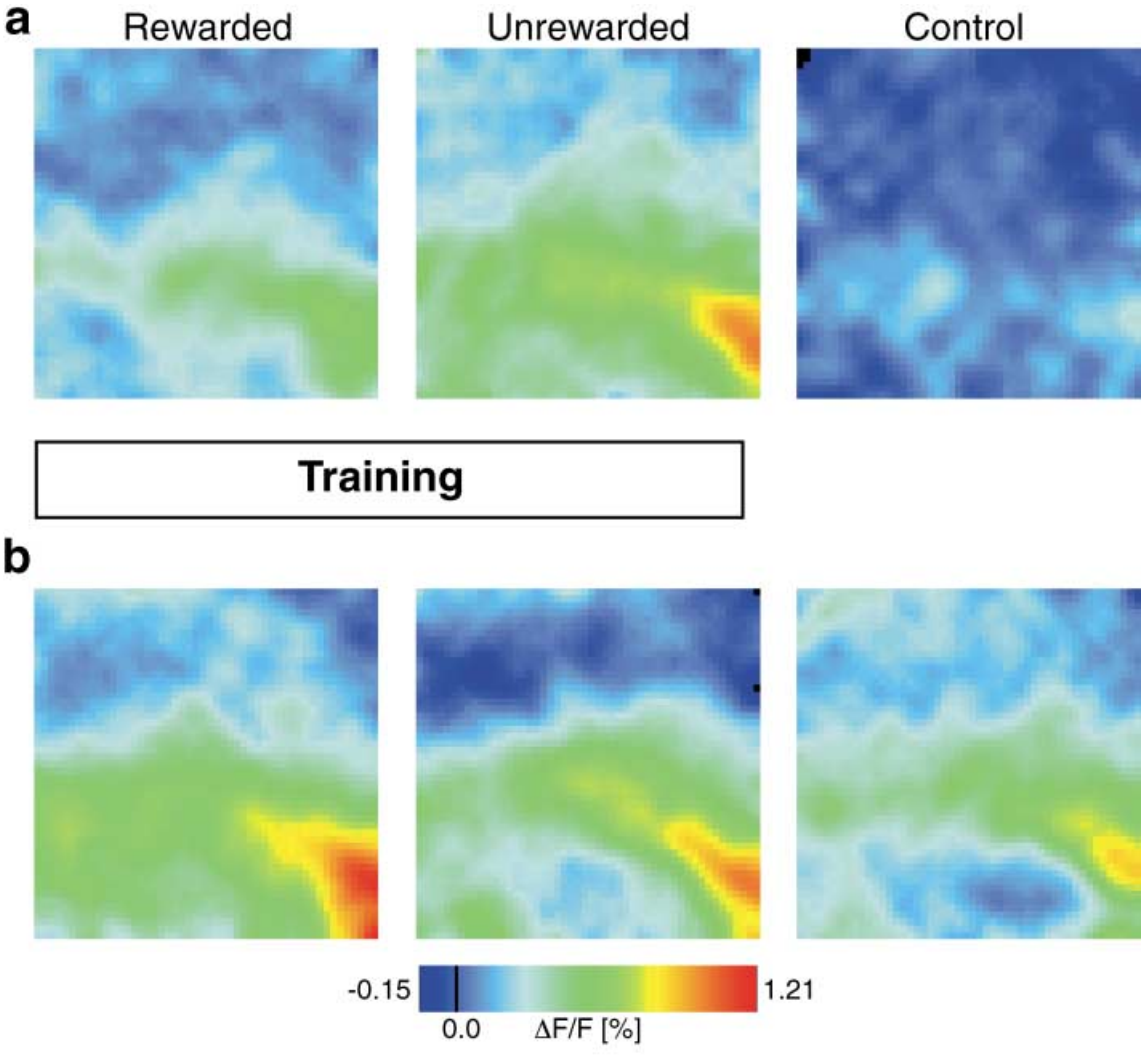


Fig. 1 **a** Overview of the bee brain (*AL* antennal lobe, *oL* optical lobe); the lips of the median calyces can be clearly seen. The *insert* shows a picture taken with 40× magnification. The lip is marked by a *dotted line*. Optical activity measures were taken of the same animal with the same lens. An example of odor stimulation is shown in **b**. The animal was stimulated with rose odor. As in **a**, the *dotted line* marks the borders of the lip. Odor-induced activity is apparent only in the lip region, as shown in **c** and **d**

tion and experimental design were similar to previously reported studies by Faber (Faber 1999; Faber et al. 1999). In short, adult worker honeybees were caught at the hive entrance, chilled and fixed in a recording chamber. A window was cut in the upper part of the head capsule to allow visual access to the lip region of the mushroom bodies. To prevent movement artefacts, the proboscis, mandibles and esophagus were carefully immobilized with small needles. The brain was stained with Calcium Green-2AM (Molecular Probes, Oregon) dissolved in 20% Pluronic F127 in DMSO (Molecular Probes) and diluted in bee saline (130 mM NaCl, 6 mM KCl, 4 mM $MagCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mosmol) for 45 min at 5°C. Before starting experiments, the brain was carefully rinsed with room-temperature (22°C) Ringer's solution. Optical recording on ten animals was performed with a custom-built imaging setup. The brain was perfused with gravity-driven, slightly cooled (19°C) air-saturated bee saline. A series of 40 frames (2 frames/s, exposure 240 ms) starting 4 s before stimulation, was recorded with a CCD camera (Photometrics CH250A). Odor stimulation was applied during frames 9–13 with a custom-built olfactometer. Background fluorescence (F) was determined by averaging frames 5–7. F was subtracted from all frames of a trial to give ΔF. Signals were expressed as relative change in fluorescence ($\Delta F/F$). Data were corrected for bleaching. For analysis of the imaging data, frames 9–17 were averaged to improve the signal-to-noise ratio. For presentation (Figs. 1b, 2a, b), pictures were spatially low-pass filtered (7×7 pixels). Activity was analyzed as integral of $\Delta F/F$ above threshold (top 50% of activity range). Statistical significance was


a
Rewarded
Unrewarded
Control
Training
b
-0.15
1.21
0.0
ΔF/F [%]


c
Integral above threshold
n = 10
Before training
After training
Rewarded
Unrewarded
Control
*
*
*

tested using the Wilcoxon signed-rank test. Three odors were used in a differential conditioning experiment. The odors were 1-hexanol, peppermint, and orange blossom. In a differential conditioning experiment the rewarded odor CS^+ is forward-paired with sucrose presented to the antennae and the proboscis, and one odor is presented alone in between the pairing trials (CS^-). In our experiment we added a third control odor to test for generalization. We applied three test trials, then five conditioning trials followed by three testing trials each, with an inter-trial interval of 10 min. The activity measures from each test were averaged and the two resulting data sets were compared for any changes in odor-induced activity. The experiment was balanced with respect to the three odors used; each one was used equally often as the rewarded odor, the unrewarded odor and the generalization control.

Results

Figure 2a, b shows an example of a differential conditioning experiment in which the rewarded odor was peppermint, the unrewarded odor was orange blossom, and the control odor was 1-hexanol. The three color-coded activity patterns for each of the three odors before conditioning are shown in Fig. 2a. The animal was then differentially conditioned by five CS^+ and CS^- trials presented in succession (not shown because no imaging could be performed during conditioning). Figure 2b shows the activity patterns for the same odors after conditioning.

The rewarded odor CS^+ induces significantly stronger activity patterns than the responses before conditioning (Fig. 2c). This is not the case for the specifically unrewarded odor CS^-, whose responses did not change. The control odor which we used to test for generalization also induced stronger responses after conditioning, but the change in response intensity is not significant (P=0.093), possibly due to the small sample size.

In earlier experiments concerning the AL, in which we conditioned the animals under the same conditions as during Ca^{2+} imaging, and also using the same set of odors, we found that animals associate the rewarded odor CS^+ with reward and respond to it more strongly afterwards, do not change their response to the unrewarded odor CS^-, and also respond more strongly to the control odor but significantly less so than to the rewarded odor (Faber et al. 1999; Fig. 2c). This indicates that the effects of olfactory conditioning are consistent between the different studies addressing different structures in the bee brain, and may reflect processes that are at work when animals are not prepared for optical recordings. Therefore we can assume that in the experiments reported here the animals would also have performed normally in olfactory conditioning after they had been prepared for optical measurements.

The imaging results from the lip region of the MB qualitatively resemble those collected from the AL (Faber et al. 1999). In both structures the learned odor induces stronger activities, and a strong overall change in the spatial distribution of the odor-induced activity patterns is not apparent. For the AL it was possible to assign foci of activity to particular structures, the glomeruli, and, indeed, there is a high level of reproducibility of odor-induced activity patterns on the level of identified glomeruli (Galizia et al. 1998, 1999). Such an analysis is not possible with the odor-induced activity patterns in the lip region, because activity foci cannot be related to particular structures, and thus a comparison between animals is not possible.

◀ **Fig. 2** **a** Quantitative changes in odor-evoked activity patterns in the lip region of the calyx of the mushroom body. An experiment was carried out using various odors: the rewarded odor CS^+, the unrewarded odor CS^- and the control odor. Activity patterns are shown for the rewarded (peppermint), the unrewarded (orange blossom) and the generalization test odor (control, 1-hexanol) before conditioning. Different odors are represented by different patterns of activity foci. **b** Patterns for the same odors recorded after conditioning. Activity increased for the rewarded odor CS^+, and did not change for the unrewarded odor CS^-. The control odor showed a small increase in activity. **c** Quantitative analysis of the data from ten animals. The odor-induced activity was quantified as described by Faber et al. (1999) and pooled for all CS^+, CS^- and the generalization control (S) odors. The odors used were 1-hexanol, peppermint and orange blossom. Each of the three odors was used as CS^+, CS^- and S odor in different animals. Conditioning leads to amplification of the odor-specific activity patterns for the rewarded odor (*P<0.05, Wilcoxon signed-rank test, n=10) in comparison to both the unconditioned and the generalization test odor

Discussion

Since the strength of the odor-induced Ca^{2+} activity in both the AL and the lip region of the MB correlates with the behavioral response measures after differential conditioning, one can conclude that neural events leading to stronger Ca^{2+} activities reflect some aspect of the memory trace. Since a stronger Ca^{2+} signal would also be induced by a stronger stimulus, stimulus intensity and learning effects are at least partially correlated at the level of these Ca^{2+} signals. At the behavior level, however, stimulus intensity and learning effects are not confounded, since bees are not confused by different intensities of the odors they have learned (Pelz et al. 1997). Therefore, the neural correlate of the learned odor needs an additional signature that specifies a learned odor. The increased Ca^{2+} signal may result from more synchronized neural activity, a component of neural odor coding that has been demonstrated both in the AL of the bee (Stopfer et al. 1997) and in both the AL (Laurent et al. 1996) and the MB of locust (Laurent and Naraghi 1994). Since the temporal resolution of our Ca^{2+} measurements (2 Hz) was far below the expected synchronization of spike activity (25–40 Hz) we could not test for such effects. It is conceivable, but needs further experimentation, that synchronization of neural activity as a consequence of learning might be a mechanism at the MB level, but not at the AL level, because learning appeared normal after interference with local field oscillations in the bee AL (Stopfer et al. 1997). It is also possible that widespread neuromodulatory neurons, like the octopamine-immunoreactive VUM_{mx1} (Hammer 1993) that are known to serve the AL and the lip, may become more active after learning. This has been shown for the VUM_{mx1} (Hammer 1997). Odors predicting a reward

may thus activate such modulatory neurons, and their Ca^{2+}-related activity may add to that induced by the odor. However, at the AL level, VUM_{mx1} activity does not enhance the Ca^{2+} signal as we measured it (Faber 1999). These possibilities can now be tested in the honeybee, because specific parts of the circuitry can be loaded with the Ca^{2+} dye (Sachse et al. 1999), and Ca^{2+} imaging will be combined in the future with multi-neuron and field potential recordings. These studies will help to understand which aspect of neural activity provides the signature in the brain for a learned stimulus.

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