Chapter II

Associative odor learning in honeybees strengthens Kenyon cell activity in the mushroom body

Summary

The mushroom bodies are higher-order brain centers and critical for odor learning in insects. We investigated the role of their intrinsic neurons, called Kenyon cells, in associative odor learning by combining differential conditioning with Ca^{2+} imaging. Odors evoked brief Ca^{2+} transients in sets of distributed Kenyon cells. Pairing an odor with sucrose induced a pronounced prolongation of odor responses without changing the ensemble of activated Kenyon cells. After training, the responses to the rewarded odor were enhanced, while the patterns of activated Kenyon cells remained unchanged. The results demonstrate that Kenyon cells act as coincidence detectors for odor and reward and are modulated by associative learning. Furthermore, odor learning does not change the Kenyon cells' combinatorial odor code.

Introduction

Learning leads to the modification of neuronal excitability and synaptic strength between neurons (Milner et al., 1998). These changes alter neural network activity and ultimately lead to adaptive behavior. Therefore, a mechanistic understanding of the neural processes underlying learning and memory formation requires the analysis of the spatial distribution of network activities. Olfactory learning paradigms are ideally suited for studying learning and memory in vertebrates and insects (Davis, 2004). In the latter, the relatively low complexity of the nervous system has allowed an integrative approach to odor learning which involves elucidating the molecular mechanism, identifying morphological structures, monitoring odor-induced network activity, and analyzing behavior (Faber et al., 1999; Menzel, 1999; Menzel, 2001; Dubnau et al., 2003; Yu et al., 2004). The formation of an olfactory memory depends on parallel and sequential processing at several stages, with the involvement of the

first two processing centers of the olfactory system, the antennal lobes (AL) and the mushroom bodies (MB) (Figure 2.1A) (Faber et al., 1999; Menzel, 2001; Yu et al., 2004; de Belle and Heisenberg, 1994; Tully et al., 1994; Connolly et al., 1996; Hammer and Menzel, 1998; Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002).

In the bee, as in other insects, the ALs are the primary olfactory processing centers that receive sensory input. Receptor neurons make synapses with local interneurons and projection neurons (PN) within the AL sub-compartments (about 160 glomeruli) (Galizia et al., 1999). PNs relay processed olfactory information from the AL to the lateral horn and the MBs (Abel et al., 2001; Müller et al., 2002). The MBs are higher-order integration centers which, in case of the bee, receive multisensory input in the calyces (mostly olfactory and visual). The MB output is spatially segregated from the calyces and occurs predominantly in the α- and β- lobes (Mobbs, 1982). MB intrinsic neurons, the Kenyon cells (KC), receiving input from different modalities, are arranged in separate concentric layers within the MB calyx, with olfactory PNs targeting the calyces' lip regions. Here, cholinergic PNs form excitatory synapses with KCs and GABAergic neurons, the latter providing local and recurrent inhibitory output onto PNs and KCs (Ganeshina and Menzel, 2001). Moreover, there are GABAergic feedback neurons which receive input from KCs in the lobes and send their axons back to the calyx (Grünewald, 1999). Thus, information flow from the PNs to KCs may involve both feedback and feedforward interactions. Any of these connections may be a possible site for plastic changes caused by olfactory learning. KCs also receive input from a putatively octopaminergic neuron (VUM_{mx1}) that has been identified as a neural substrate of the reinforcing function of the reward (US) (Hammer and Menzel, 1998; Hammer, 1993). The VUM_{mx1} neuron also innervates the olfactory pathway in the AL and the lateral horn. Thus, the MB lip region is one of three convergence sites for the olfactory CS and the rewarding US pathway. Therefore KCs could act as coincidence detectors for the CS and the US, and learning-related synaptic plasticity may occur at the PN/KC synapses.

Since the contents of memories are likely to be stored in the patterns of altered synaptic connections it is paramount to compare the spatial distribution of network activity before and after learning. Progress in this direction has been made for the bee AL by showing that after learning the glomerular responses to the rewarded odor (CS+) increased (Faber et al., 1999). Similarly, learning in a moth induced a net recruitment of neurons responding to the CS+ and a net loss of neurons responding to the CS- (Daly et al., 2004). Yu et al. (2004) demonstrated that olfactory learning leads to a short-term recruitment of presynaptic activities in PN dendrites in *Drosophila*. In the MB of the bee, Ca^{2+} responses in the MB lip increased for the CS+ after odor learning, though the cells involved were not identified (Faber and Menzel, 2001).

In *Drosophila* a subgroup of KCs, which constitute the γ-lobe, were found to be essential for short-term memory formation (Zars et al., 2000). Since the clawed KCs in bees (cKC, also termed class II or type 5 cells) (Rybak and Menzel, 1993) are thought to be homologues to the KCs of the γ-lobe in *Drosophila* (Strausfeld, 2002; Farris et al., 2004) one may assume that they are involved in olfactory memory formation.

Here we report Ca^{2+} imaging studies in which cKCs were recorded during classic odor conditioning. Honeybees learn to associate an odor stimulus (conditioned stimulus, CS) with an appetitive stimulus (unconditioned stimulus, US) under controlled laboratory conditions (Bitterman et al., 1983; Menzel, 1990) and in Ca^{2+} imaging experiments (Faber et al., 1999). We conditioned bees differentially by rewarding one odor (CS+) while another odor (CS-) was not rewarded. In naïve animals, odors evoked short responses in small sets of highly odor specific cKCs. We investigated how the coincident action of CS and US presentations is represented in the cKCs and whether their neural activity shows learning- and/or memory-related modifications. Pairing an odor with sucrose had two effects on the cKC activity: during training, cKCs exhibited prolonged responses only in those cKCs that were activated by the CS+. After training, these KCs' responses to the CS+ were enhanced. Our data are compiled in a circuit model of the MB, which suggests that odor representation in the MB is sparse, and odor memory is established by a combination of associative plasticity at the cKC input synapses and spike-rate dependent plasticity at their output synapses.

Material and Methods

Living bee preparation and dye loading

Experiments were performed with foraging honeybees, *Apis mellifera carnica*, which were prepared for the experiments as described earlier (Chapter I). Briefly, cKCs were selectively stained with the dextran-conjugated Ca^{2+} indicator Fura-2 dextran 10,000 MW (Molecular Probes) which was injected into cKC axons in the ventral part of the α-lobe. 8 to 24 hours later bees were prepared for experiments. To prevent movement artifacts, abdomen and legs were immobilized with dental wax, muscles which innervate the antennae were carefully removed, the mouthparts were truncated and the esophagus was taken out. Immediately afterwards, the head capsule was washed with bee Ringer (in mM: 130 NaCl, 7 $CaCl_2$, 6 KCl, 2 $MgCl_2$, 160 sucrose, 25 glucose, 10 HEPES, pH 6.7, 500 mOsmol). In order to stabilize the brain, a 1.5 % solution of low-melting agarose (Sigma, A2576) was injected into the head capsule. Experiments started 30 min after preparation.

Stimulation and imaging

cKCs were visible on the raw fluorescence images and could reliably be identified due to the position of their somata outside the calyx. Within the calyx lip region they exhibit columnarly arranged dendritic trees which measure 15 x 40 µm, with their somata lying outside the lip neuropil (Rybak and Menzel, 1993; Strausfeld, 2002). Experiments were performed on 14 bees. Bees were differentially conditioned during imaging experiments. In each experiment two out of three odors were presented (linalool, 2-octanol, and 1-hexanol, all from Sigma). Odors were diluted in mineral oil in order to reduce evaporation and to adjust for differences in vapor pressure (2-octanol, 60 %; linalool 46.2 %; 1-hexanol. 16.2 %). 4 µl of the odor solution were applied onto a 2 cm^2 piece of filter paper and placed in a 0.6 ml plastic syringe. Using a computer controlled olfactometer (Galizia et al., 1997), odors were injected into a continuous air stream that was directed at both antennae. Gentle air suction from the back cleared residual odors at all times. Each experiment lasted for ~ 1 hour and was divided in three parts: In a pretest, the two odors were alternately presented three

times with an inter-stimulus interval of 1 minute. Stimulus length was three seconds. The bees were trained 3 minutes after the pretest. Five training trials were performed in which one of the odors (CS+) was paired with a sucrose stimulus (30% w/w solution) at the antenna ipsilateral to the imaged MB, while the other odor was presented alone (CS-). Sucrose stimulation began 1 s after odor onset and lasted for 3 s, resulting in a 2-second overlap between odor and sucrose stimulus. The odors used for CS+ and CS- were balanced in order to exclude odor specific effects. 15 and 35 minutes after training CS+ and CS- were tested again 3 times.

Differential conditioning offers several advantages when performed together with physiological measurements. First, it induces a robust long-term memory in restrained bees (Menzel, 1990). Second, it allows separation of associative learning effects from nonassociative learning (e.g. sensitization) which may result from repeated sucrose presentations (Hammer et al., 1994). Third, the differential conditioning paradigm allows a within-animal comparison between CS+ and CS-, thus controlling for unrelated effects, such as variations due to bleaching or photo damage that would otherwise mask learning induced changes.

Images were acquired with a sampling rate of 5 Hz using a Till Photonics imaging set up mounted on a fluorescence microscope (Olympus BX-50WI). Measurements started 2 seconds before stimulus onset and lasted for 12 s. Fura-2 was excited at a single wavelength (390 nm) in order to reduce photodamage. Fluorescence was detected through a 60x, 0.9 W Olympus objective, a 410 nm dichroic mirror and a 440 nm long-pass filter with an Imago CCD camera (640 x 480 pixels, 4x binned on chip to 160 x 120 pixels). The imaged area covered 209 x 157 µm² of the median MB calyx.

Data analysis

Data were analyzed using custom-written programs in IDL (RSI). Background fluorescence (F) was determined by an average of 5 frames obtained before stimulation and was subtracted from every frame of a measurement to give ΔF. Signals were calculated as fluorescence change relative to background fluorescence ($\Delta F/F$). Bleaching was corrected by subtracting a logarithmic curve fitted to the mean brightness decay of the entire image frames, excluding frames during the stimulus (Galizia and Vetter, 2004). Signals were inverted, since Fura-2 decreases its

fluorescence at 390 nm excitation light in response to increasing Ca^{2+} concentrations. Activity patterns are shown as color-coded images, representing the averaged ΔF/F values of 15 frames (3 s) during the odor stimulus or odor/sucrose stimulus. For better visualization scattered light was reduced by applying an unsharp mask filter (50 µm kernel size) and a spatial low-pass filter (5 x 5 pixels). To analyze the response dynamics, pixels, corresponding to responsive cKC dendrites during the 3-second odor-pulse, were averaged without any filtering. Time courses were further analyzed in Excel (Microsoft). Signal magnitudes (Figure 2.4) were calculates as mean of 3 s odor stimulation or odor/sucrose stimulation, respectively. Correlation analysis of spatial activity patterns was performed by transposing the pixel values of two images (low-pass filtered) into vectors and computing the linear correlation between them. Only animals with stable focus were included in the correlation analysis. Statistics were performed with Statistica (StatSoft.).

Results

Learning the association between an odor (CS) and a reward (US) requires the convergence of the neural pathways and the initiation of lasting associative plasticity in the processing circuits. In the bee MB, PNs representing the CS and the reward pathway (VUM_{mx1} neuron) converge on cKCs in the lip region of the MB calyx. We imaged Ca^{2+} activity of cKCs in the lip to investigate how they respond to odor stimuli before, during and after odor learning.

Odor responses

An average of 163 ± 10 (mean ± SEM, range 106 - 226) individual somata of cKCs were visible in each preparation (Figure 2.1). Of these, up to 6 (2.4 ± 0.3, mean ± SEM) responded to one or the other of the two odors. Repeated stimulations with an odor reliably evoked Ca^{2+} transients in the same cKC somata and in their adjacent dendritic areas. The degree of overlap between the dendritic activity patterns evoked by different odors varied among preparations. However, the overlap between patterns of activated somata was always low, indicating the activation of different cKC populations with overlapping dendritic trees (Figure 2.1B). Since the somatic signal usually decreased during the experiments (presumably due to photodamage, see

Chapter III), we analyzed the responses in the dendritic region. There, odor stimuli evoked Ca^{2+} transients in 1 to 6 columnar or elongated activity patches in each preparation. These activity patches corresponded in size and shape to the dendritic arborizations of individual cKCs (Rybak and Menzel, 1993), indicating responses of individual cKCs. Most cKC were activated within 400 ms after odor onset and showed phasic response dynamics with incidental, smaller off-response at stimulus offset (Figure 2.2A).

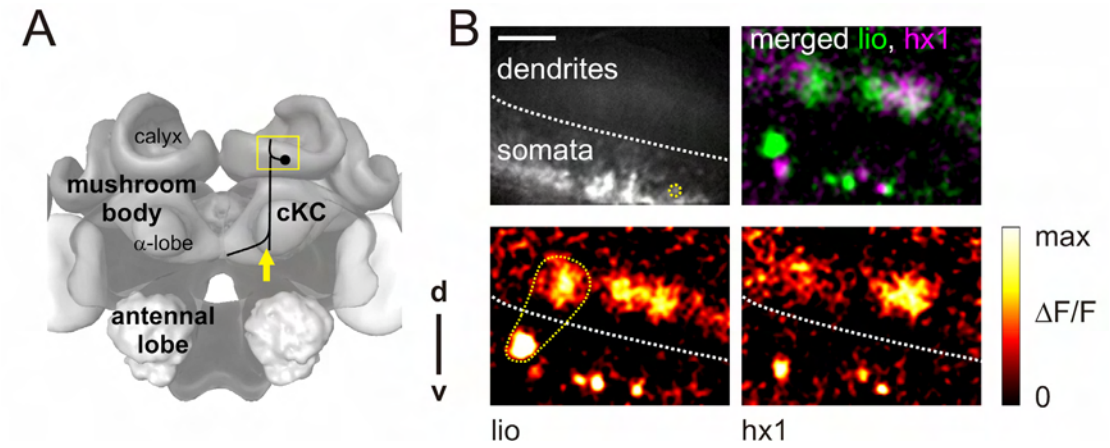

Figure 2.1 Imaging of clawed Kenyon cells

(A) Scheme of the bee brain. Clawed Kenyon cells (cKC) were stained via Fura-2 injection into the ventral α-lobe (arrow) and imaged in the mushroom body calyx (square). **(B)** The upper left panel shows a Fura-fluorescence image of cKCs in the lip region of the mushroom body calyx. The location of the somata (a single soma is outlined) ventral to the lip neuropil identifies them as clawed KCs. Below, color coded Ca^{2+} signals imaged in the same area show linalool (lio) and 1-hexanol (hx1) evoked responses in the somata and dendrites. Soma and dendritic activity patches often occurred in proximity (outlined), indicating that they belong to the same cKCs. The merged activity patterns of lio (green) and hx1 (magenta) show non-overlapping soma patterns (upper right panel). d = dorsal, v = ventral; scale bar 50 µm

Reward effects

Figure 2.2A shows the spatial distribution and time traces of Ca^{2+} signals during the 3 second odor stimulation and during the paired presentation with sucrose.

Ca^{2+} transients elicited by sucrose following odor stimulus occurred simultaneously in all odor-activated cKCs (Figure 2.2B), and often rose above the amplitude of the initial odor response outlasting the sucrose stimulus for several seconds (Figure 2.2A, B). Sucrose stimulation alone induced only minor Ca^{2+} increases as compared to odor stimulation in two control animals (data not shown).

As seen in the upper panels in Figure 2.2A, the amount of scattered light and the overlap of KC dendrites limit the segmentation of individual KCs. We therefore performed a pixel-based correlation analysis, which must be interpreted with care because it may not be sensitive to small changes in the activity pattern. However, the similarity (measured as correlation coefficient) between activity patterns evoked by repeated odor presentations (CS+ alone) did not differ from the similarity between activity patterns evoked by the odor alone and odor plus sucrose (Figure 2.2C). We therefore conclude that the pairing of an odor with a sucrose reward reactivates and thus prolongs cKC odor responses without recruitment or loss of activated KCs.

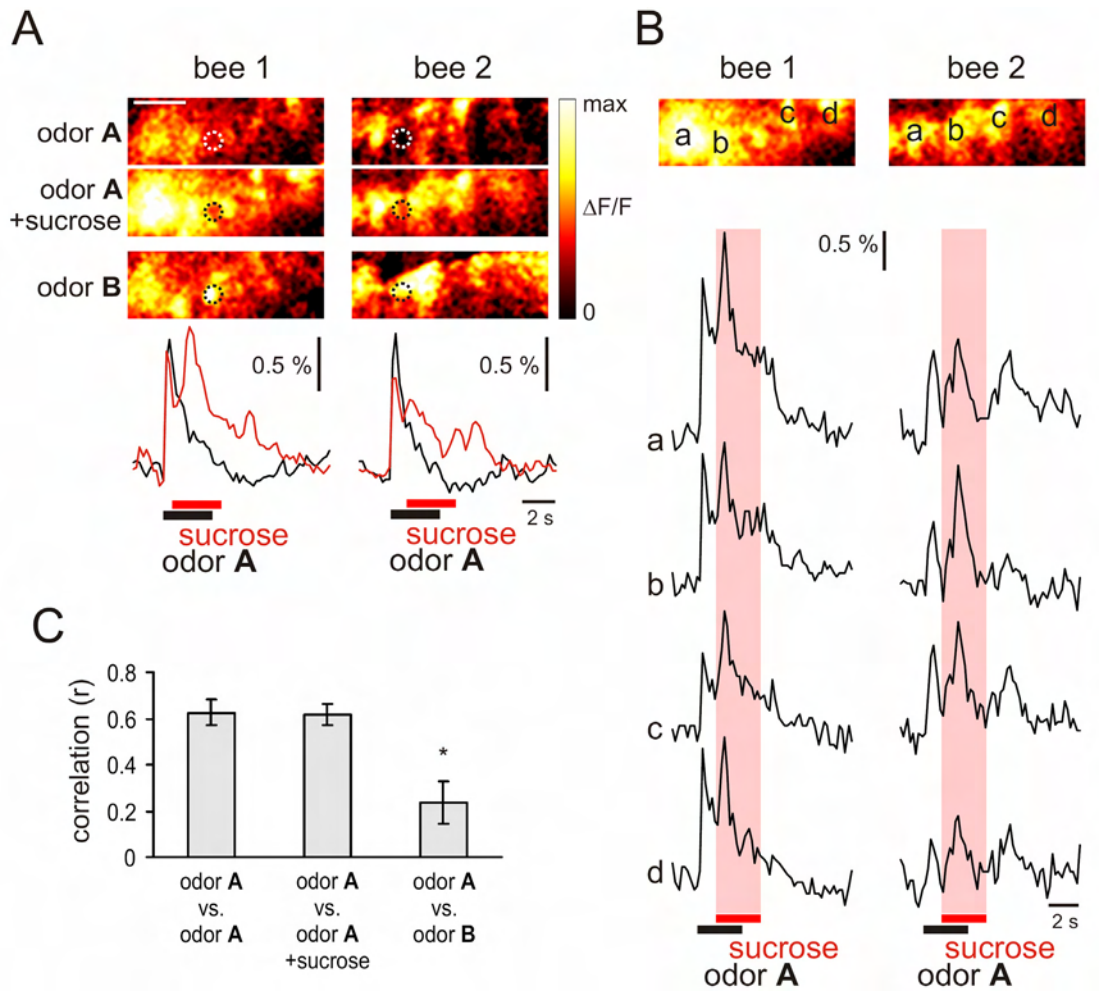

Figure 2.2 Clawed Kenyon cells are sensitive to the coincidence of odor and reward

(A) Dendritic cKC responses to an odor A alone, to the odor A paired with sucrose and to an odor B in two animals. The odors activated 4 to 6 dendritic regions. Pairing odor A with sucrose enhanced the Ca^{2+} signal in the regions activated by odor A alone. However, the patterns of activated cKCs did not change significantly (see (C)). Some of the regions (dotted circles) which were not activated by the combined odor A/sucrose-stimulus responded to odor B, showing that missing sucrose responses do not reflect incomplete cKC staining. Traces represent the average time courses of Ca^{2+} transients (3 odor and 5 odor/US stimulations) in the regions visible in the upper panels. Odor A alone (black curve) induced a phasic Ca^{2+} signal, while the presentation of sucrose (red curve) led to a second and prolonged response peak. **(B)** All cKCs activated by an odor responded simultaneously to the sucrose stimulus. Traces represent Ca^{2+} transients in distinct regions (circles) during a single measurement. The activated regions correspond in size and shape to the morphology of single cKCs and therefore reflect responses from different cKCs. **(C)** The population of odor activated cKCs remained unaffected by sucrose stimulation. No difference was found in the correlation coefficients between activity patterns evoked by two presentations of the odor alone, and those between odor alone and odor paired with sucrose. For comparison, the correlation between the patterns evoked by the two different odors A and B was significantly lower ($p = 0.001$, Friedman Repeated Measures Analysis of Variance on Ranks, $n = 8$). Traces ΔF/F; scale bar 50 µm

Learning effects on CS+ and CS- representations

Next we investigated whether odor learning induced changes in the cKC response properties. Figure 2.3 shows the spatial activity patterns and Ca^{2+} transients, evoked by the CS+ and CS- before and after training. The correlations between activity patterns evoked by repeated presentations of either CS+ or CS- before the training did not differ from the correlation between activity patterns evoked before and after training. We therefore conclude, that the set of cKCs responding to the CS+ or CS- remained stable (Figure 2.3B), and the changes visible in Figure 2.3A may reflect inherent variability. Since bees were trained in a differential paradigm, changes in odor representation may become apparent only when CS+ and CS- evoked patterns are directly compared. The correlation coefficient for the CS+ vs. CS- did not change (Figure 2.3C), confirming the lack of learning induced changes in the spatial activity patterns.

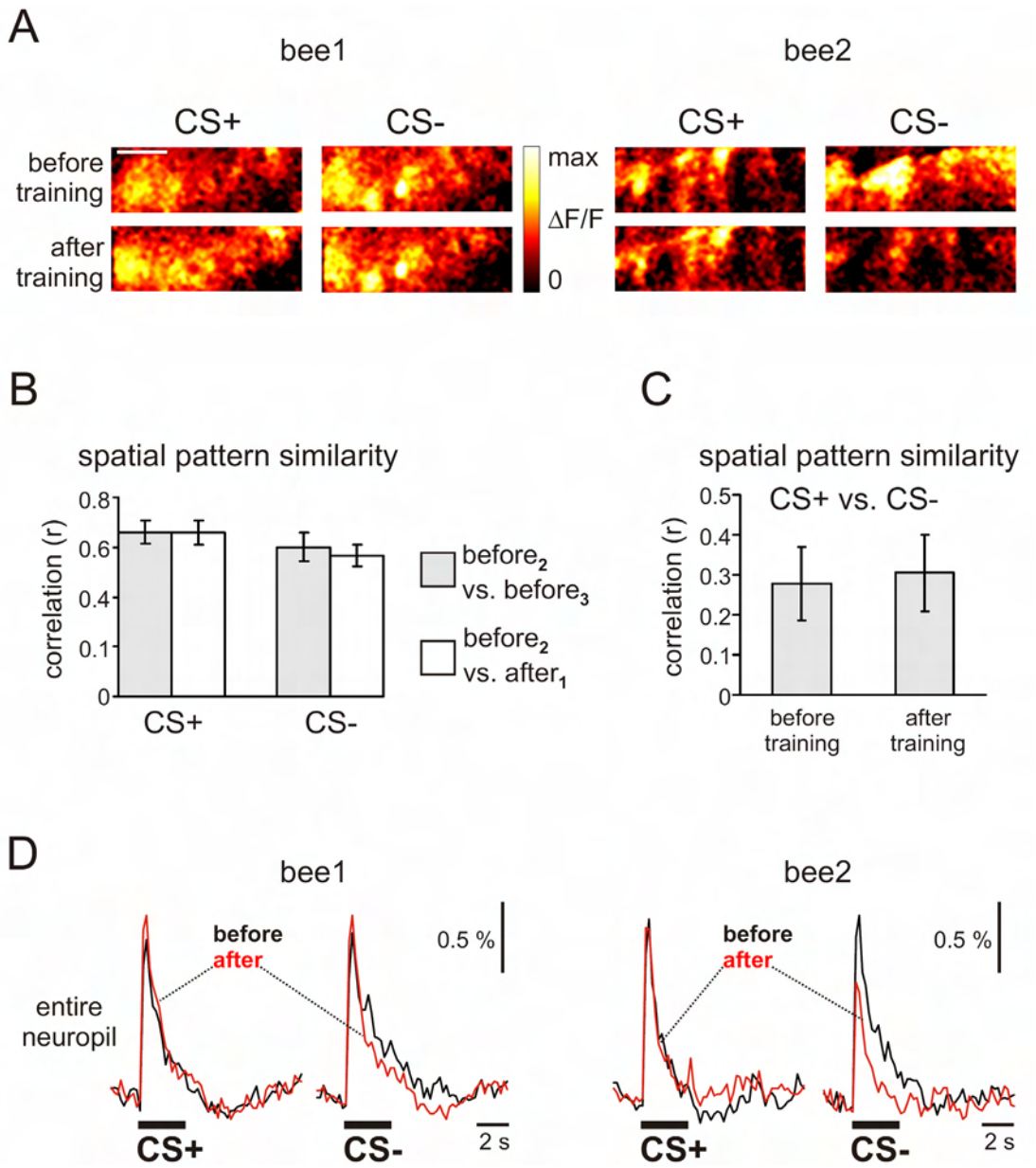

Figure 2.3 Clawed Kenyon cell responses before and after odor learning

(A) cKC responses to the rewarded (CS+) and the unrewarded odor (CS-) in two animals. The spatial pattern of activated dendrites showed only little change. **(B)** No difference was found in the correlation between activity patterns before training (2^{nd} vs. 3^{rd} trial) and after training (2^{nd} trial before vs. 1^{st} trial after training). (CS+, p = 0.84; CS-, p = 0.38, Wilcoxon Signed Rank Test, n = 8). **(C)** The similarity between CS+ and CS- induced activity patterns did not change after learning (p = 1, Wilcoxon Signed Rank Test, n = 9). **(D)** Average Ca^{2+} transients measured in the regions visible in (A) before (black traces) and 15 min after (red traces) training. After training the Ca^{2+} transients for CS+ and CS- were differentially changed. In bee 1 the CS+ response was slightly increased, while the CS- response was reduced. In bee 2 the signal strength remained unchanged for the CS+ and reduced for the CS-. Ca^{2+} signals show averaged responses to 3 stimulations. Traces ΔF/F; scale bar 50 µm

We then asked whether learning would induce changes in dynamic response properties of cKCs. Indeed, two main effects were observed (Figure 2.3D). In some animals the CS+ induced signals increased in amplitude and duration (6 out of 14), while the CS- induced signals were reduced. In other cases both the CS+ and CS- induced responses decreased, but the decrease in the CS- signal was stronger (5 out of 14). Thus, the CS+ induced signal increased relative to the CS- signal in 11 out of 14 animals ($p = 0.009$, paired t-test).

Figure 2.4A shows the evolution of the signal magnitude during the course of the experiments. The responses to both odors decreased by 30 % during the three stimulations in the pretest. The difference among stimulus repetitions was significant ($p = 0.001$) and no difference existed between CS+ and CS-. During training the CS+ responses were enhanced because the signal also contained the US-driven response. The response levels for the CS- did not decrease any further. 15 min after training the CS+ induced responses were enhanced relative to the CS- responses ($p = 0.015$). 35 minutes after the training the odors were presented again. Although signal quality was strongly degraded, the response difference between CS+ and CS- was still enhanced in 10 out of 14 (not significant).

The progressive degradation of the image quality during the experiment (due to dye bleaching) did not allow us to assess whether learning induces an absolute increase in the responses to the CS+, or only a relative change when compared to the CS-. In order to separate learning effects from changes due to loss of signal quality, we selected those experiments in which the overall strength of the summed CS+ and CS- responses changed less than 20 % (6 out of the 14 experiments). Figure 2.4B shows the mean signal amplitudes in all animals and the selected ones with stable signal quality. 15 min after the training the mean CS+ response increased in all selected 6 animals ($p = 0.02$; mean increase: 23 % ± 7 $\Delta F/F$), while the CS- response was reduced in 5 out of 6 ($p = 0.03$). Taken together, these results demonstrate that odor learning strengthens the cKC responses to the CS+ without changing the set of activated cKCs.

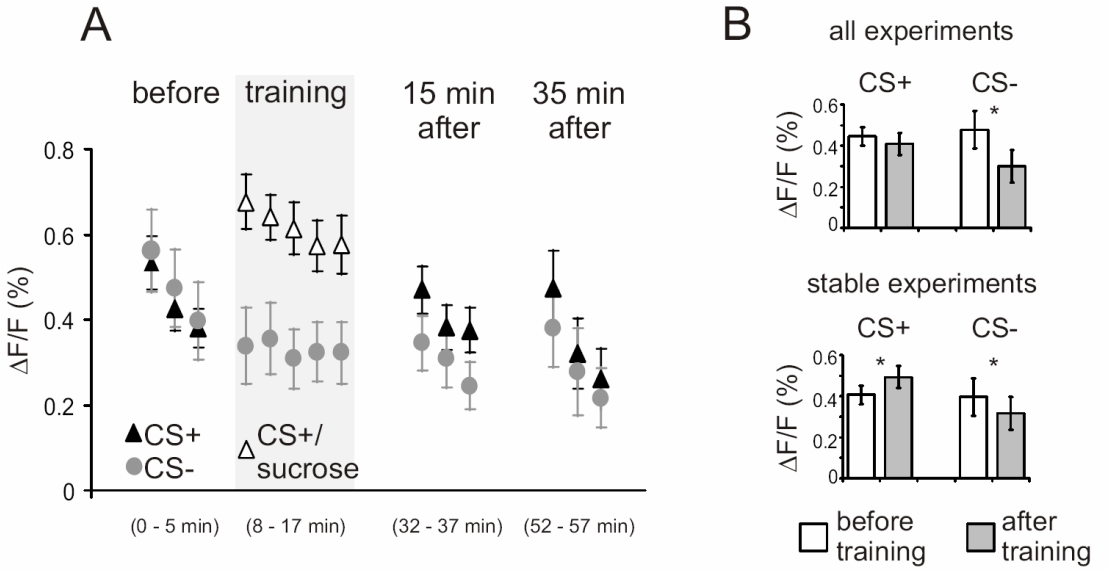

Figure 2.4 Response strength of clawed Kenyon cells shows learning induced changes

(A) Changes in response strength (mean signal of 3 s stimulation) during the course of the experiment. During the three stimulations in the pretest, the responses to both odors decreased by 30 %. The difference between stimulus repetitions, and not between CS+ and CS-, was significant ($p<0.001$, $F=10.5$, Two Way Repeated Measures ANOVA, $n = 14$). 15 min after training the differences between CS+ and CS- induced responses were significant ($p<0.015$, $F=7.9$, Two Way Repeated Measures ANOVA, $n = 14$), while they were not significant 35 after training. **(B)** To separate learning effects from changes due to loss in signal quality, animals were selected for which the summed signals for CS+ and CS- changed less than 20 % between pretest and the test after 15 min (6 out of the 14 animals). 15 min after the training the mean CS+ response increased in all animals ($p = 0.02$, Paired t-test, $t = -3.38$, $n = 6$), while the CS- response was reduced in 5 out of 6 ($p = 0.03$, Paired t-test, $t = 3.03$, $n = 6$).

Discussion

We conditioned honeybees and simultaneously imaged cKC dendrites in the lip region of the MB calyx. We found that cKCs are sensitive for the coincidence of CS (odor) and US (sucrose) induced activity, as their response to the CS is increased upon US presentation. After learning, the response to the CS+ alone was enhanced. Both effects were observed only in those cKCs that were activated by the CS+. Thus, the spatial activity patterns of activated cKC remained unchanged during the CS/US pairing as well as after learning. These results suggest that the formation of odor memory depends on the modulation of the spiking activity of cKCs, most probably through a heterosynaptic mechanism, involving the VUM_{mx1} neuron, as the neural correlate of the reinforcing function of the reward.

Properties of clawed Kenyon cell responses

We know from Ca^{2+} imaging studies in the AL (Galizia and Kimmerle, 2004) that the Ca^{2+} concentration is correlated with the spiking behavior. We therefore hypothesize that the Ca^{2+} signals recorded in cKCs are dominated by Ca^{2+} influx through voltage-gated Ca^{2+} and reflect spike activity, rather than Ca^{2+} release or regulation (see Discussion in Chapter I).

cKCs feature a sparse code in a two-fold way (Chapter I): First, a given odor activates a small proportion of highly odor specific cKCs. Second, they respond with brief and phasic Ca^{2+} transients, indicating the generation of only a few action potentials per odor stimulus. Theoretical analyses have shown that sparse codes help the formation and storage of associative memories (Olshausen and Field, 2004). Thus, the sparseness of cKCs responses makes them potentially good candidates for the substrate of associative learning (Heisenberg, 2003).

Clawed Kenyon cells are coincidence detectors for CS and US

cKCs respond to the contiguity of odor and sucrose as the pairing of the odor and sucrose lead to prolonged and/or increased odor responses. We found evidence that only those cKCs activated by the odor also respond to the reward. This specificity

indicates that the sucrose effect is mediated by a neuromodulator, rather than by an excitatory neurotransmitter which may recruit otherwise silent cKCs.

What causes the sucrose mediated increase in cKCs´ responses? KCs receive odor driven excitatory input from PNs and both local and global feedback inhibitory input (Ganeshina and Menzel, 2001; Grünewald, 1999; Laurent and Naraghi, 1994). Information about the reward is conveyed by the VUM_{mx1} neuron, which weakly responds to olfactory and visual stimuli, but shows a massive response to sucrose stimulation (Hammer, 1993). KC responses to PN activity are increased after octopamine application (Oleskevich, 1999). In bee KCs acetylcholine receptors are cation channels, while octopamine receptors are G-protein-coupled receptors (Grohmann et al., 2003; Wüstenberg and Grünewald, 2004). Octopamine may increase cKCs` excitability through fast phosphorylation of ion channels, thereby counteracting the inhibitory input from GABAergic neurons. Alternatively, octopamine might act presynaptically on GABAergic cells or PN terminals. In either case, release of octopamine would increase cKC sensitivity to the excitatory input from continuously active PNs. This suggests that odor specific activity patterns carried by PNs exist throughout odor stimulation and that cKCs are potentially able to read this continuously available odor code at any time during stimulation.

Clawed Kenyon cells show associative plasticity

The differential learning paradigm used in our experiments has been extensively studied in behavioral experiments using the proboscis extension reflex (PER) as a learning monitor (Bitterman et al., 1983; Menzel, 1990). 15 minutes after training, PER probability increases for the CS+ and decreases for the CS-. In our preparation we could not monitor behavioral responses. However, fixed bees, prepared for optophysiological measurements, show normal learning (Faber et al., 1999). Furthermore, the differential conditioning paradigm controls for non-associative effects. Therefore, we attribute the observed training and learning effects to neural correlates of learning processes. Odors were balanced, thus excluding the possibility of odor specific effects. Conditioning did not alter the set of activated cKCs. This conclusion was drawn from a pixel-based correlation analysis and must therefore be taken carefully. In order to clarify this point, one should perform these experiments with 2-photon laser scanning microscopy, which allows a higher spatial resolution

(see Chapter III). On the other hand, cKCs increased their response magnitude to the CS+ relative to the CS-. These changes correspond to the differential change in response probability typically observed in behavior. Since repeated odor stimulation alone reduces the cKC response strength, it cannot be excluded that the reduced CS- response may reflect a non-associative repetition effect. It is thus likely that the differential learning effect involves more than the input synapses to the cKC (see below).

What causes the enhancement in cKC response strength? In insects, associative odor learning depends on the cAMP/PKA signaling cascade within the MB, and adenylyl cyclase might be the molecular coincidence detector for the CS and US (Connolly et al., 1996; McGuire et al., 2001; Davis et al., 1995; Müller, 1997; Zars et al., 2000). Furthermore, octopamine increases the level of cAMP and activates PKA (Grohmann et al., 2003; Hildebrandt and Müller, 1995). Thus, the coincidence of depolarization and Ca^{2+} increase (acetylcholine - CS) with cAMP increase (octopamine - US) may activate PKA particularly strongly, which, in turn, may modulate receptor function and/or cell excitability through the phosphorylation of ion channels or gene expression, ultimately facilitating the synaptic transmission from PNs to cKC.

Alternatively, learning induced increase in cKC response strength could reflect an increase in PN activity. However, in contrast to cKC activity changes, PN activity changes previously reported were transient and lasted shorter than 15 minutes. Using a similar experimental protocol, Weidert (2003) found a differential learning effect in PN response strength only in honeybees tested 5 minutes after training, while no effect was present after 15 minutes. Similarly, learning induced changes in PN responsiveness in Drosophila were transient and lasted for less than 10 minutes (Yu et al., 2004).

A model of mushroom body function in odor learning

We compiled our results with anatomical and other physiological data in a model of memory formation in the MB (Figure 2.5). We propose that odor learning is based on two sequential steps and depends on heterosynaptic plasticity in the MB calyx (input area) and on monosynaptic, spike-rate dependent plasticity at the MB lobes (output area).

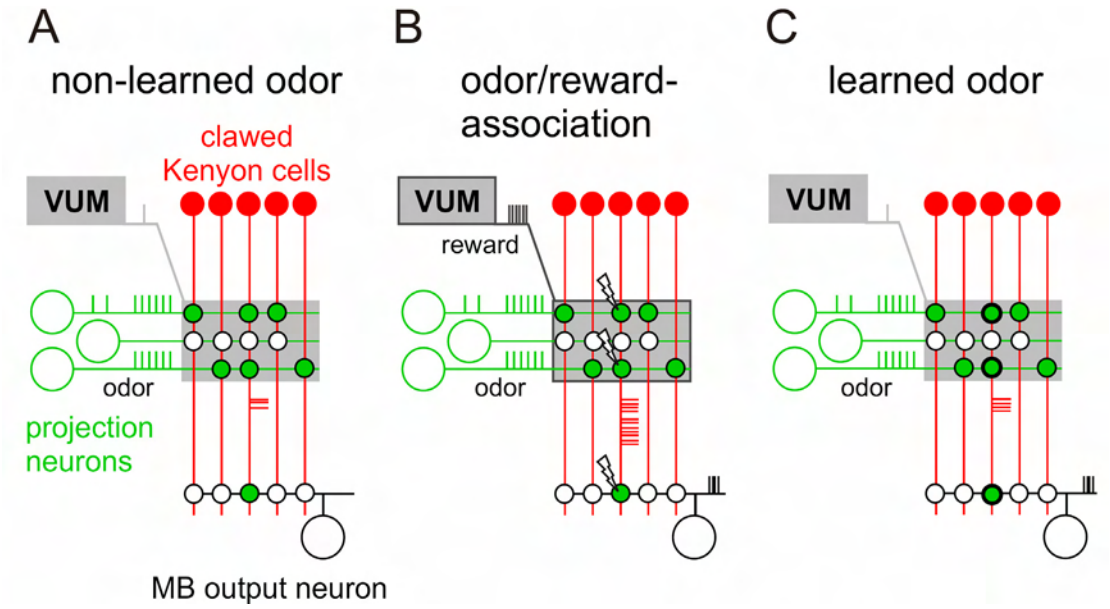

Figure 2.5 Model of mushroom body function in odor learning

(A) In the MB input (lip region of the calyx), cholinergic PNs make excitatory synapses (black circles) with cKCs. The octopaminergic VUM_{mx1} neuron, which represents the reward, targets PN/cKC synapses. In the MB output (α-lobe) a population of MB output neurons receives input from cKCs. Even though CS+ neurons receive input from many cKCs they are assumed to remain silent if an unlearned odor is perceived. **(B)** Memory formation depends on multiple plastic sites (flashes) in the MB. During training the VUM_{mx1} neuron releases octopamine in response to the reward. The increased octopamine concentration, together with the release of acetylcholine from PNs, increases cKC response strength and triggers associative facilitation of PN/cKC synapses. In addition, the increased spike-rate of cKCs may induce spike-rate dependent facilitation of cKC/MB output neuron synapses. **(C)** After odor learning, the population of responding cKCs remains unchanged, but their responses become stronger, leading to a reliable activation of the recruited MB output neuron by the rewarded odor. The MB output neuron encodes the new meaning of the learned odor as a predictor for reward.

At the input site, the coincident action of acetylcholine (CS) and octopamine (US) would first lead to an increased response followed by facilitation at PN/cKC synapses (see above). At the output site, the US evoked increase in the cKC spike-rate may induce an activity dependent facilitation and/or recruitment of cKC/output neuron synapses. Since cKC show no background activity and generate brief responses of only a few spikes (Perez-Orive et al., 2002) (Chapter I) a prolonged increase in spike frequency during CS/US presentation would reliably encode the contiguity of CS and US. If MB output neurons do not respond to low activity in KCs but are recruited by high activity, they would then only respond to the learned odor, and encode its significance as reward predictor. Such increased activity might lead to the release of the neuropeptide cholecystokinin (CCK) as a cotransmitter in cKCs. CCK

immunoreactivity has been found within the axon terminals of cKCs (Strausfeld et al., 2003), and Kloppenburg et al. (1990). found that CCK injection into the α-lobe of bees is followed by an increased probability of extending the proboscis in response to antennal stimuli. Interestingly, in vertebrates CCK acts as a co-transmitter released from pyramidal cell terminals only after prolonged depolarization (> 250 ms) (Ghijsen et al., 2001) and rats lacking CCK receptors are impaired in learning- and LTP (Nomoto et al., 1999).

One class of MB output neurons, the A4 neurons, feature properties that fit very well into our model. They have dendrites perpendicular to the axons of the cKCs, suggesting that they sample the output from many cKCs (Rybak and Menzel, 1993). Even though A4 neurons potentially receive massive olfactory input, preliminary experiments showed only rare odor responses in naive animals (R. Menzel, unpublished). Further experiments are required in order to test whether A4 neurons are indeed recruited after olfactory learning.

There might be additional mechanisms of odor memory involving other types of KCs and MB output neurons. One such neuron, PE1, shows associative plasticity in odor learning (Mauelshagen, 1993). It receives input from cKC and other KCs, and responds to olfactory, visual and tactile stimuli (Rybak and Menzel, 1998). Odors elicit excitatory responses that are enhanced for the CS+ and unchanged for the CS- after multi-trial differential conditioning. Intracellular recording from the PE1 neuron during electric stimulation of KCs paired with depolarization of the PE1 neuron showed associative LTP (R. M. and G. Manz, Proceeding of the 29th Göttingen Neurobiology Conference 2003).

A rather similar model has been proposed for *Drosophila* olfactory learning (Heisenberg, 2003). Based on the finding that in *Drosophila* synaptic transmission from KCs is not required during learning, the model assumes that short- to middle-term memories are stored within KC (Dubnau et al., 2001; Schwaerzel et al., 2002). In contrast to our model, the *Drosophila* model proposes that the recruitment of MB output neurons involves a hypothetical modulatory neuron that represents the US at the KCs' presynaptic terminals in the MB output region. In the bee at least, the VUM_{mx1} neuron does not innervate the MB output region. The model that we propose does not add hypothetical elements to the circuit and is consistent with the honeybee's anatomical and physiological data.

Conclusions

Taken together, our results show that odor learning does not modify the population of responding cKCs, but enhances their responses to the rewarded odor. Our data suggest that odor memories would be encoded by the recruitment of MB output neurons. Due to the sparse nature of the cKC code, overlaps between individual odor representations are unlikely and therefore a single MB output neuron could store many odor memories without interference. Since KCs of different sensory modalities project to the α-lobe, and extrinsic neurons receive input across the α-lobe, the suggested learning mechanism may not only lead to elementary odor learning but may also allow higher-order associations, in particular the embedding of olfactory memories into a multisensory context.

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