Multiple memory traces after associative learning in the honey bee antennal lobe

Lisa Rath, C. Giovanni Galizia and Paul Szyszka

Department of Biology, University of Konstanz, D-78457 Konstanz, Germany

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Abstract

We investigated the effect of associative learning on early sensory processing, by combining classical conditioning with *in vivo* calcium-imaging of secondary olfactory neurons, the projection neurons (PNs) in the honey bee antennal lobe (AL). We trained bees in a differential conditioning paradigm in which one odour (A+) was paired with a reward, while another odour (B–) was presented without a reward. Two to five hours after differential conditioning, the two odour–response patterns became more different in bees that learned to discriminate between A and B, but not in bees that did not discriminate. This learning-related change in neural odour representations can be traced back to glomerulus-specific neural plasticity, which depended on the response profile of the glomerulus before training. (i) Glomeruli responding to A but not to B generally increased in response strength. (ii) Glomeruli responding to B but not to A did not change in response strength. (iii) Glomeruli responding to A and B decreased in response strength. (iv) Glomeruli not responding to A or B increased in response strength. The data are consistent with a neural network model of the AL, which we based on two plastic synapse types and two well-known learning rules: associative, reinforcer-dependent Hebbian plasticity at synapses between olfactory receptor neurons (ORNs) and PNs; and reinforcer-independent Hebbian plasticity at synapses between local interneurons and ORNs. The observed changes strengthen the idea that odour learning optimizes odour representations, and facilitates the detection and discrimination of learned odours.

Introduction

Learning the simple association between two stimuli gives rise to multiple memory traces in the brain. These memory traces are expressed as altered synaptic connections and neural excitability, evolve over time, and are distributed across brain areas (Menzel, 2001; Thompson, 2005; Blum *et al.*, 2009). Here we ask how associative learning affects early sensory processing. We investigated the effect of associative odour–reward learning on odour processing in the honey bee primary olfactory area, the antennal lobe (AL), which is the insect analogue of the vertebrate olfactory bulb (Hildebrand & Shepherd, 1997). The insect olfactory system is a powerful model for studying mechanisms of learning and memory, because olfactory behaviour is highly plastic (Menzel & Bitterman, 1983; Giurfa, 2007), and the neural representations of olfactory and appetitive or aversive reinforcing stimuli are well characterized (Davis, 2004; Schwaerzel & Müller, 2006; Fiala, 2007). The ALs are subdivided into glomeruli with innate odour–response profiles (Galizia *et al.*, 1999b). The output of a glomerulus is carried via projection neurons (PNs) to higher brain centres (Menzel *et al.*, 2005). Within the AL a network of intra- and inter-glomerular inhibitory and excitatory local interneurons (LNs) is involved in odour processing (bee – Sachse & Galizia, 2002; fly – Shang *et al.*, 2007; Silbering *et al.*, 2008). Odour representations in

PNs are therefore the result of the interaction of many neurons in the AL. These interactions might sharpen or broaden odour tuning, alter concentration dependencies, change the synaptic gain, and generate or modify temporal patterns (bee – Sachse & Galizia, 2002; Linster *et al.*, 2005; Deisig *et al.*, 2010; fly – Wilson *et al.*, 2004; Shang *et al.*, 2007; Silbering & Galizia, 2007; Olsen & Wilson, 2008; locust – Perez-Orive *et al.*, 2002). The AL is not only involved in sensory odour processing, but also in associative odour–reward learning (bee – Hammer & Menzel, 1998; fly – Thum *et al.*, 2007). In this process octopaminergic neurons provide information about the reward (bee – Hammer, 1993; fly – Schwaerzel *et al.*, 2003). The coincidence of odour- and reward-induced activity leads to associative learning. Accordingly, associative odour learning affects odour representations in the AL (bee – Faber *et al.*, 1999; Sandoz *et al.*, 2003; Arenas *et al.*, 2009; Fernandez *et al.*, 2009; Denker *et al.*, 2010; fly – Yu *et al.*, 2004; moth – Daly *et al.*, 2004) and in downstream areas as the mushroom bodies (bee – Faber & Menzel, 2001; Szyszka *et al.*, 2008). It is thought that learning-induced changes further optimize the representation of learned odours that might improve the detection and discrimination of the learned odour (Faber *et al.*, 1999). However, the underlying mechanisms of neural plasticity in the AL are not well understood. We combined behavioural learning experiments with *in vivo* calcium-imaging, and investigated whether and how differential odour conditioning changes the neural representations in the uniglomerular PNs of the honey bee AL. We found associative plasticity of odour representations 2–5 h after discriminative odour

Correspondence: Dr P. Szyszka, as above.

E-mail: paul.szyszka@uni-konstanz.de

learning, which increased the distance between rewarded and unrewarded odours, and appeared as glomerulus-specific changes in PN response strength. These observations lead to a precise and testable prediction about which synaptic contacts are changed within the AL after appetitive conditioning.

Materials and methods

Animals

Experiments were performed with honeybee foragers (*Apis mellifera*).

Staining and preparation

The experiments took 2 days (Fig. 1A). The day before the experiments bees were caught from feeders near the hives, and the PNs of lateral and medial antenno-protocerebral tract (l- and m-APT) were stained with the calcium-sensitive dye Fura-2 dextran (Invitrogen, Molecular Probes, Eugene, OR, USA), as described in Szyszka *et al.* (2011; Fig. 1B). The next day (14–16 h after dye injection), bees were imaged, then trained and again imaged. Before imaging, the antennae were fixed temporarily with n-eicosan (Sigma Aldrich Chemie GmbH, Steinheim, Germany). The tracheae above the ALs were removed carefully. To reduce movements, the oesophagus and the surrounding muscles were lifted through a small opening in the clypeus, and fixed with two-component silicon (Kwik-Sil; World Precision Instruments, Sarasota, FL, USA), and the thorax and abdomen were gently pushed against the stage with a piece of foam (1 cm × 1 cm × 3 cm). The brain was covered with a thin layer of transparent Kwik-Sil (Okada *et al.*, 2007). Within 10–30 min after the preparation bees were put under the microscope and heated to 24–28 °C with an infrared lamp. The AL that showed better staining and stronger spontaneous activity was chosen for measuring.

Odour stimulation

We used 2-octanol, 1-hexanol and 2-nonanol for odour stimulation (all from Sigma Aldrich). The pure odorants were diluted to 10^{-2} in mineral oil (Sigma Aldrich). Odorant dilutions were prepared freshly every 4 weeks. Two-hundred microlitres odour solution was loaded onto a cellulose stripe (Sugi, REF 31003; Kettenbach GmbH KG, Eschenburg, Germany) located in a 3-mL syringe (Norm-Ject; Henke-Sass, Wolf GmbH, Tuttlingen, Germany). Syringes were prepared freshly every day. Odour stimuli were delivered as 4-s pulses with a custom-built computer-controlled six-channel olfactometer (Szyszka *et al.*, 2011). The air stream through each channel was 300 mL/min each controlled by a flowmeter (Analyt-MTC GmbH, Müllheim, Germany). Odours were injected into a continuous carrier air stream (1200 mL/min), which was directed to the bee via a glass tube (0.7 cm in diameter). The six channels added up to 1800 mL/min. Thus, there was a total air stream of 3000 mL/min directed at the bee positioned about 1 cm in front of the glass tube, corresponding to an air speed of 1.3 m/s. The binary mixture of 1-hexanol and 2-octanol was created by opening the valves of the two channels simultaneously. Continuous air suction behind the bee cleared residual odour.

Imaging

PNs in the AL were imaged through a water immersion objective (20×, NA 0.95; Olympus, Tokyo, Japan). The imaging system consisted of a fluorescence microscope (BX-50WI; Olympus), a light

source (Polychrome IV; Till Photonics, Gräfelfing, Germany) and a CCD camera (Imago QE; Till Photonics); 8×8 pixels of the camera were binned on-chip resulting in a resolution of 172×130 pixels ($441 \times 333 \mu\text{m}$). Each recording lasted 29 s and consisted of 232 double frames recorded with 340- and 380-nm excitation light at a rate of 8 Hz. Excitation and emission light were separated with a 420-nm dichroic mirror and a 490–530-nm emission filter. Bees were stimulated with 2-octanol and 1-hexanol (odours used as A+ and B– during training), their binary mixture, 2-nonanol and the solvent mineral oil as blank control. Odours were presented in a pseudo-randomized sequence (all permutations were used) with an inter-trial interval of 2 min. Odour stimulation was controlled by the ACQUISITION software of the imaging system (Till Vision; Till Photonics).

Differential conditioning

Bees were differentially conditioned with 2-octanol and 1-hexanol as either rewarded odour A+ or unrewarded odour B–. 2-Octanol and 1-hexanol were used equally often as A+. Bees received six training trials with A+ and six training trials with B–, presented alternately. In half of the bees training started with A+ in the other half with B–. The inter-trial interval between A+ and B– was 10 min. Bees were moved in front of the olfactometer 20 s before odour stimulation to habituate to the airflow. Odour pulses were 4 s long. For A+ the odour was paired with sucrose stimulus (1.25 M in water), which was applied to the antenna and proboscis with a metal preparation needle. Sucrose stimulation started 3 s after odour onset and lasted for 3 s. Thus, there was a 1-s overlap between odour and sucrose. A proboscis extension reflex (PER) was counted as response when the proboscis was extended horizontally. The training took 2 h. Memory was tested 0.5 h after the last training trial. During the test, bees were exposed to A, B, the binary mixture AB and 2-nonanol (N) as a new odour. The inter-trial interval was again 10 min, and the sequence of odour stimulation was pseudo-randomized (all permutations were used).

Experimental protocol

PNs were stained on the first day (Fig. 1A). On the second day, bees were differentially conditioned, and their learning and memory performances were measured behaviourally. One–nine hours after PN imaging bees were conditioned. Two–five hours after the last training trial PN responses were imaged again. Ninety bees were stained and conditioned. In 25 of these bees, PN responses could be measured until the end of the imaging experiment. Bees were classified as discriminators (more responses to A than to B in the 2nd–6th training and test trials) or non-discriminators (equal or fewer responses to A than to B; Roussel *et al.*, 2010). There were 12 discriminators (six received 1-hexanol, six received 2-octanol as A+) and 13 non-discriminators (nine received 1-hexanol, four received 2-octanol as A+).

Data analysis

Imaging data were analysed with custom-written programs in IDL (RSI, Boulder, CO, USA). First, measurements were movement corrected by aligning frames within and between measurements. Then, glomeruli were segmented with the help of an unsharp masked image of the raw fluorescence and a correlation image where the correlation of the signal traces between neighbouring pixels was calculated (Fig. 1D). Glomeruli were identified by comparing glomerulus position and size with the morphological atlas of the honeybee (Galizia *et al.*, 1999a)

FIG. 1. Bees learn after disrupting PN axons and imaging. (A) Scheme of the experimental protocol. (B) Frontal view of the honey bee central olfactory system. Different neurons are shown in the two hemispheres. Olfactory receptor neurons (ORN, black arrow) terminate in the antennal lobe (AL) glomeruli. Uniglomerular projection neurons (PN, green) transmit odour information from the AL to the mushroom body (MB) and to the lateral horn (LH). PN axons project along the lateral or median antenno-protocerebral tract (l-APT, m-APT) to the lateral and medial calyces (IC, mC). The MB intrinsic Kenyon cells (KC, magenta) send their axons into the MB lobes (α L, γ L, β L). In this study mostly l-APT PN dendrites that ramify in glomeruli in the frontal AL were imaged. The dotted square indicates the imaged area. The crosses indicate the sites of dye injection. Left – the bilaterally symmetrical VUM_{mx1} neuron (VUM, red) mediates the reinforcing function of the reward and connects the sub-oesophageal ganglion (SEG) with the AL, LH and MB calyces. (C) Odour-evoked PER during training and memory retrieval test 30 min after. (Ci) Learning and memory performance of bees treated equally to the imaging bees. Training – PER responses increased stronger to A+ than B– (two-way RM ANOVA, $F_{\text{trial}, 4,356} = 5.1$, $P < 0.001$; $F_{\text{odour}, 1,356} = 5.8$, $P = 0.02$; $N = 90$ bees; trials 2–6). Test – the response frequencies to the CS+ and the mix were higher than to the CS– and N (one-way RM ANOVA, $F_{\text{odour}, 3,267} = 5.4$, $P < 0.001$, Holm–Sidak *post hoc* tests). (Cii, Ciii) Learning and memory performance of bees that were imaged before and after conditioning (this is a subset of the bees in Ci). These bees were used to investigate learning-induced changes in PN responses. Bees were classified as discriminators (Cii; more A than B responses during the 2nd–6th training and test trials) or non-discriminators (Ciii; equal or less A than B responses). The test confirms the separation into learners and non-learners – in discriminators the response frequencies to the CS+ were higher than to the CS– (one-way RM ANOVA, $F_{\text{odour}, 3,33} = 4.1$, $P < 0.05$, Holm–Sidak *post hoc* tests; $N = 12$ bees), whereas in non-discriminators there was no difference between response frequencies (one-way RM ANOVA, $F_{\text{odour}, 3,36} = 1.4$, $P = 0.27$; $N = 13$ bees). Different letters mark a significant difference ($P < 0.05$). (D) Raw fluorescence and correlation images of the stained AL (left hemisphere) of a discriminator and colour-coded images of responses to A, B, the mixture AB, nonanol (N) and the blank control before and after training (mean of 4 s during stimulus). Scale bar – 100 μ m. For interpretation of color references in figure legend, please refer to the Web version of this article.

and the odour responses with the physiological atlas (Galizia *et al.*, 1999b; Sachse *et al.*, 1999). Signals were calculated as F_{340}/F_{380} . The baseline was shifted to 0 by subtracting the average signal before stimulation (frame 4–69 for odour stimulation). Activity patterns are

shown as colour-coded images of single measurements, which were filtered with a spatial low-pass filter of 5×5 pixels for better visualization (Fig. 1D). No filtering was used for quantitative analysis. Glomerulus responses were calculated from a 5×5 pixels square in

the glomerulus centre. Response strength was quantified as the mean signal during 4 s after stimulus onset (frames 72–103; colour-coded activity images and bar graphs in Figs 1–3). To calculate the global response strength across the entire AL, for each bee, the response strength of each glomerulus was calculated and averaged over all glomeruli (Fig. 2A). The distance between odour response patterns was quantified as follows: for each bee, the response strength of each glomerulus was calculated and the odour response pattern was represented as a vector of these values. The distance between two odour responses was quantified by calculating the Euclidean distance between the glomerular odour–response vectors (Fig. 2B). In Fig. 3 each glomerulus was grouped according to its responses to the odours A and B before training. For each glomerulus, odour-evoked signals (mean signal during 4 s after odour onset) were classified as responses when the signal was three times larger than the standard deviation of the background activity of the glomerulus. The standard deviation was calculated over 66 frames before stimulation and averaged over five measurements (A, B, AB, N and blank). Odour-evoked signals below three times standard deviation were classified as weak or no responses. Behavioural data were analysed with an ANOVA. Although a parametric ANOVA test is usually not applicable for dichotomous data, an empirical study has shown that ANOVA is appropriate under our conditions (Lunney, 1970). However, for the data shown in Fig. 2Cii and iii, the power of the ANOVA is low because of the small sample size. Statistical tests were performed with R (<http://www.r-project.org>), and Sigma Stat (SPSS, Chicago, IL, USA).

Results

We combined differential conditioning with *in vivo* calcium-imaging of PNs to identify learning-related changes in PN responses. We stained uniglomerular PNs that send their axons through either the m-APT or l-APT (Abel *et al.*, 2001; Kirschner *et al.*, 2006; Galizia &

Rössler, 2009; Fig. 1B). We measured the frontal part of the AL where l-APT innervated glomeruli are visible. Thus, even though we stained both tracts, we imaged predominantly l-APT glomeruli. We chose differential classical conditioning in order to identify excitatory and

FIG. 3. Associative plasticity in PN responses depends on the glomerulus response profiles. (A) PN response strength (mean of 4 s during stimulus $\pm$ SEM) before and after training in discriminators (Ai) and non-discriminators (Aii). Each glomerulus was divided into one of four groups according to its response profile before conditioning. (Ai) In discriminators ‘A glomeruli’ increased in response strength to A, B and the mixture AB [two-way RM ANOVA with factors odour and training (before vs. after training), $F_{\text{training}, 1,42} = 11.4$, $P < 0.01$; $F_{\text{training} \times \text{odours}, 2,42} = 1.7$, $P = 0.2$; $N = 22$ glomeruli]. ‘B glomeruli’ did not change in response strength ($F_{\text{training}, 1,18} = 0.3$, $P = 0.6$; $N = 10$ glomeruli). ‘A/B glomeruli’ decreased in response strength to all stimuli (two-way RM ANOVA, $F_{\text{training}, 1,72} = 5.5$, $P < 0.05$; $F_{\text{training} \times \text{odours}, 2,72} = 0.1$, $P = 0.9$; $N = 37$ glomeruli). ‘none glomeruli’ increased in response strength (two-way RM ANOVA, $F_{\text{training}, 1,128} = 21.9$, $P < 0.001$; $F_{\text{training} \times \text{odours}, 2,128} = 1.6$, $P = 0.2$; $N = 65$ glomeruli). (Aii) In non-discriminators neither ‘A glomeruli’ nor ‘B glomeruli’ changed in response strength [two-way RM ANOVA with factors odour and training (before vs. after training), ‘A glomeruli’ – $F_{\text{training}, 1,48} = 0.2$, $P = 0.7$; $N = 25$ glomeruli; ‘B glomeruli’ – $F_{\text{training}, 1,38} = 0.2$, $P = 0.7$; $N = 20$ glomeruli]. ‘A/B glomeruli’ decreased in response strength ($F_{\text{training}, 1,88} = 37.3$, $P < 0.001$; $F_{\text{training} \times \text{odours}, 2,88} = 7.6$, $P < 0.001$; $N = 45$ glomeruli), and ‘none glomeruli’ increased in response strength (two-way RM ANOVA, $F_{\text{training}, 1,148} = 6.3$, $P < 0.05$; $F_{\text{training} \times \text{odours}, 2,148} = 0.2$, $P = 0.8$; $N = 75$ glomeruli). Asterisks mark significant differences between before and after training (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (B) Mean PN response traces before (black) and after training (red) in discriminators (top) and non-discriminators (bottom). Because response changes were not odour-stimulus dependent, we averaged the traces for A, B and AB in order to increase the signal-to-noise ratio. Shaded areas indicate $\pm$ SEM. Stimulus bars indicate the 4-s odour pulse. For interpretation of color references in figure legend, please refer to the Web version of this article.

inhibitory learning effects. In this paradigm one odour is rewarded (A+) and associated with the reward (excitatory learning), whereas another odour is presented alone (B-) and associated with the absence of reward (inhibitory learning). During the memory retrieval test and during the two PN imaging sessions before and after training (Fig. 1A) we measured responses to A and B and additionally to their binary mixture and to nonanol (N), which was not presented during training in order to establish whether odour learning affects the representation of these odours. In this text, we refer to A and B as the rewarded and non-rewarded odours, respectively. In the context of acquisition, we refer to A+ (paired with reward) and B- (not paired with reward).

Associative odour learning is possible after disrupting the PN tracts by dye injection

Because we used an invasive staining procedure (Fig. 1B), which might interfere with the bees’ ability to learn or recognize odours, we

first ascertained whether bees were able to learn after PN staining and imaging (Fig. 1Ci). During training, the proportion of bees showing the conditioned response (PER) increased. However, PER frequency only differed slightly between A+ and B- ($P = 0.02$). In the memory retrieval test 30 min after training, bees had higher response rates to A and AB than to B and nonanol ($P < 0.001$). Thus, bees were still able to learn after disrupting their PN tracts by dye injection and after the imaging procedure. However, as compared with intact bees (data not shown), differentiation between A and B was poor.

Differential odour conditioning increases the distance between the rewarded and unrewarded odour

Having established that bees with stained PNs can still learn to associate an odour with a sucrose reward, we investigated whether differential conditioning alters odour representations in the AL. We therefore recorded dendritic PN responses in the AL glomeruli before

and after differential conditioning. In order to separate associative from non-associative effects of the training procedure, we divided bees into discriminators (Fig. 1Cii) and non-discriminators (Fig. 1Ciii). Separating bees into discriminators and non-discriminators considers the fact that associative learning performance differs across individual bees and does not reflect the mean population performance (Pamir *et al.*, 2010). Accordingly, such separation proved useful in previous studies that investigated the effect of odour learning on neural odour representations in honey bees (Okada *et al.*, 2007; Roussel *et al.*, 2010).

Odour stimuli evoked glomerular patterns of increased or decreased calcium signals (Fig. 1D). The global response showed a phasic-tonic time-course, and was strongest for the mixture AB (Fig. 2A). The stronger response to the mixture is most likely due to the fact that the mixture was presented as the sum of octanol and hexanol, and thus activates more glomeruli. The global response did not change significantly between before and after training in both discriminators (Fig. 2Ai) and non-discriminators (Fig. 2Aii). We next asked whether differential conditioning modulates glomerular response patterns, and analysed the distance between the response patterns of A, B and AB before and after the training. We quantified the distance between response patterns as Euclidean distance (Fig. 2B). In discriminators the distance between A and B increased after training, while the distance change between AB and either A or B was not significant (Fig. 2Bi). No significant change in inter-odour distances was detectable in non-discriminators (Fig. 2Bii). Thus, only in discriminators there was a differential change in PN response patterns, which made the A and B representation more distinctive after training.

Associative plasticity in PN responses depends on glomerulus response profiles

The differential changes in PN response patterns imply unequal changes across glomeruli. Are these changes predictable across animals based on the identity of glomeruli? In 11 of the 12 discriminators we could identify glomeruli according to the morphological and physiological AL atlas (Galizia *et al.*, 1999a; Sachse *et al.*, 1999). We did not find consistent training-induced changes in response strength (Fig. 2C), confirming previously published data (Peele *et al.*, 2006; Fernandez *et al.*, 2009). However, in addition to stereotypical glomerular responses, there is also variability across animals. We therefore analysed glomerular response changes on the basis of their individual response to the odours used. For each individual bee, we classified glomeruli into four types according to their responses to A and B before training, and compared the responses with the two odours and their mixture before and after differential conditioning (Fig. 3). 'A glomeruli' – glomeruli with a response to A and no or weak response to B; 'B glomeruli' – glomeruli with a response to B and no or weak response to A; 'A/B glomeruli' – glomeruli with responses to both A and B; 'none glomeruli' – glomeruli with no or weak response to both A and B. In discriminators, these glomerulus types showed differential changes in their response strength after the training (Fig. 3Ai). 'A glomeruli' increased in response strength equally to all test odours (ANOVA revealed no interaction between the factors 'training' and 'odours'). This increased response persisted for the entire stimulus length (see time-trace in Fig. 3B). 'B glomeruli' did not change in response strength. 'A/B glomeruli' decreased in response strength to all odours. This decrease persisted during the entire response time. 'None glomeruli' increased in response strength to all odours. This increase was strongest towards the end of the odour response, suggesting that it

is caused by a different mechanism than the previous effects, thus implying at least two synaptic processes in the network. In non-discriminators, neither 'A glomeruli' nor 'B glomeruli' changed in response strength; however, 'A/B glomeruli' and 'none glomeruli' showed the same training-induced changes as in discriminators and decreased and increased in response strength, respectively (Fig. 3Aii). These data show that there were differential changes in response strength across glomeruli after differential conditioning, and that these changes depended on the glomerulus response profiles and on whether the bee learned to discriminate between the rewarded and unrewarded odour.

Discussion

Odour representations in the primary olfactory area of insects and mammals are subject to experience-dependent plasticity. Various studies reported learning-induced changes in odour representations in the insect AL (bee – Faber *et al.*, 1999; Sandoz *et al.*, 2003; Arenas *et al.*, 2009; Fernandez *et al.*, 2009; Denker *et al.*, 2010; fly – Yu *et al.*, 2004; moth – Daly *et al.*, 2004) and the mammalian olfactory bulb (Kay & Laurent, 1999; Doucette & Restrepo, 2008). However, a mechanistic understanding of associative plasticity of odour representations is still missing. To shed light on the rules under which associative learning changes the functional AL circuitry, we combined behavioural learning experiments with *in vivo* calcium-imaging of selectively stained PNs in honey bees. We found associative plasticity of odour representations after differential conditioning. In bees that learned to discriminate between the rewarded odour A and unrewarded odour B, plasticity differentially affected A and B responses, and was apparent as glomerulus-specific changes in PN response strength. Moreover, the learning-induced changes in a glomerulus could be predicted from its response profile before training.

Methodological considerations

We stained PNs by injecting a calcium indicator into the PN axon tracts between the mushroom body calyces of both hemispheres (Fig. 1B). This staining procedure disrupts PN axons and disturbs their connection to the mushroom bodies. Because the mushroom bodies play an important role in learning and memory (Menzel, 2001), our staining procedure might interfere with odour-reward learning. Indeed, the reduced odour discrimination of the imaged bees (Fig. 1) compared with intact animals is in line with mushroom bodies' suggested role in discriminative odour learning (Heisenberg, 2003). Moreover, PN output to the mushroom bodies appears to be required for memory retrieval, as cutting PN axons upstream to the mushroom body calyces blocks memory retrieval (Peele *et al.*, 2006). Nevertheless, our bees were still able to learn and form an associative memory during differential conditioning (Fig. 1C). We assume that this ability derives from the fact that our staining procedure disrupted only a part of the connections between the ALs and mushroom bodies. It did not damage connections between the m-APT PNs and medial calyx, and between the l-APT PNs and lateral calyx.

We used a training protocol that induces multiple, dynamic memory phases (Hammer & Menzel, 1995). We recorded PN responses during a period of 2–5 h after conditioning, corresponding to mid-term memory, which requires the formation of a constitutively active protein kinase C within the AL (Grünbaum & Müller, 1998). Before the second imaging session we performed one memory retrieval test. This presentation of the learned odour without a reward may induce additional learning processes like extinction and reconsolidation of the

FIG. 4. Model of associative plasticity in the AL after differential conditioning. (A) Summary of changes in glomerulus response strength after differential conditioning (data from Fig. 3Ai). Equal signs indicate no change in response strength, arrows indicate significant changes and their direction. (B) Synaptic learning rules are as follows. (i) LTP at the excitatory ORN-to-PN synapse under the control of the unconditioned stimulus (US, reward) as a positive reinforcer. Coincident activity (red) at the ORN-to-PN synapse will strengthen synapses (arrow up) only if the US is present. (ii) Reinforcer-independent Hebbian LTP/LTD at the inhibitory LN-to-ORN synapse. Coincident pre- and postsynaptic activity (red) leads to LTP. No activity (blue) in the postsynaptic ORN and activity (red) in the presynaptic LN leads to LTD. (C) Model of learning-induced plasticity in the AL. First row – ‘A glomerulus’. The ORN-to-PN synapses are strengthened during the pairing of A+ with the US during training (rule 1). The LN-to-ORN synapses are strengthened during A+ presentation and weakened during B- presentation (rule 2), leading to no net change. Together, this results in an increased ORN-to-PN transmission (right column). Second row – ‘B glomerulus’. The ORN-to-PN synapses do not change because there is no coincidence of ORN activity and the US. The LN-to-ORN synapses are weakened during A+ presentation and strengthened during B- presentation (rule 2), summing up to no change in ORN-to-PN transmission. Third row – ‘A/B glomerulus’. The ORN-to-PN synapses are strengthened during A+ presentation (rule 1). The LN-to-ORN synapses are strengthened during A+ and B- presentation, and the increased inhibitory presynaptic output onto ORNs outweighs the increased strength of the ORN-to-PN synapse (rule 2). This sums up to a decreased ORN-to-PN transmission. Last row – ‘none glomerulus’. The ORN-to-PN synapses do not change because there is no coincidence of ORN activity and the US (rule 1). The LN-to-ORN synapses are weakened because there is no postsynaptic activity (rule 2). This will lead to an increased ORN-to-PN transmission. For interpretation of color references in figure legend, please refer to the Web version of this article.

acquisition memory. Therefore, the observed training effects in PN responses might stem from combined effects of memory acquisition, extinction and reconsolidation. In the present study we cannot unravel the potential contribution of these learning processes to the observed

effects. However, we assume that they are dominated by the associative memory acquisition because, in honey bees, both extinction and reconsolidation are absent or only weakly induced after a single retrieval trial (Stollhoff *et al.*, 2005, 2008).

Differential odour conditioning improves separability of neural odour representations

In discriminator bees, the neural representations of the rewarded odour A and the unrewarded odour B became more distinct after training (Fig. 2B). This finding is consistent with previous calcium-imaging studies in honey bees that found increased distances between PN responses to the rewarded and unrewarded odour after appetitive differential conditioning (Faber *et al.*, 1999; Fernandez *et al.*, 2009). Different to the findings of Faber *et al.*, we did not observe increased global responses towards the rewarded odour. Because Faber and colleagues performed neuron-type unspecific bulk-staining, the learning-induced increase in response strength might reflect activity in other neurons than PNs. Our data contradict a previous study by Peele *et al.* (2006) who did not find changes in PN responses after differential conditioning. There are some differences between ours and Peele's study, which could explain this discrepancy, as follows. (i) Peele and colleagues measured PN responses 5–13 min after training, whereas we measured 2–5 h after training. Thus, the learning effects that we found might require consolidation time of more than 13 min. (ii) Peele's staining procedure disrupted the axons of I-APT PNs and the reward-mediating VUM_{mx1} neuron axons in the lateral protocerebrum, and thus cut their connection to the mushroom body (Hammer, 1993). This lesion might prevent memory acquisition. (iii) Peele *et al.* conducted their experiments at room temperature, while we heated the bees to 24–28 °C. The higher temperature might strengthen the effects of learning-induced changes in synaptic weights.

Glomerular response changes can be predicted from their response profile before training

We were not able to relate the observed changes to identified glomeruli (Fig. 2C). This may indicate that associative odour learning affects different glomeruli in different animals. Indeed, given the finding that the neural odour representations are plastic, it is likely that different previous experience of the individual experimental animals may already have shaped their olfactory networks. Likewise, previous studies in honey bees failed to attribute PN response changes to the identity of glomeruli (Peele *et al.*, 2006; Fernandez *et al.*, 2009). However, we found associative plasticity in PNs that depended on the response profile of the innervated glomerulus before training (summarized in Fig. 4A). Where in the AL network do the glomerulus-type dependent changes occur? Glomeruli are interconnected by a dense network of LNs in bees (Galizia, 2008), some of which are global and some selective in their connectivity. Increasingly more LN subpopulations are being described (Kreissl *et al.*, 2010). Synaptic contacts abound between olfactory receptor neurons (ORNs), PNs and LNs, and the octopaminergic VUM_{mx1} neuron that signals the reward in appetitive odour learning (Hammer, 1993). Given the large number of possibilities, our experiments are not suitable to identify the plastic synapse within the network. We can, however, propose a minimal network that is sufficient to explain the observed changes, as a working hypothesis for future research. We started by observing a differential effect early in the response as compared with late in the response (compare 'A glomeruli' with 'none glomeruli' in Fig. 3B). Furthermore, we found that some changes were common to discriminators and non-discriminators, while others were not. This prompted us to postulate two synaptic processes, one affecting the ORN-to-PN synapses directly and related to associative memory traces, the other affecting inhibitory LN-to-ORN synapses related to non-associative memory. These two mechanisms follow two distinct learning rules (Fig. 4B). (i) Long-term potentiation (LTP) at excitatory ORN-to-PN synapses under control of the VUM_{mx1}. ORN-to-PN synapses undergo

LTP if the ORNs and PNs are active at the same time (Hebb, 1949) and the VUM_{mx1} signals the presence of a reward (Fig. 4C, 'A glomerulus' and 'A/B glomerulus' during A+ learning). A similar mechanism mediates associative LTP in sensory neurons in *Aplysia* with serotonin as reinforcer for synaptic plasticity (Carew *et al.*, 1984). (ii) Reinforcer-independent Hebbian LTP (coincident pre- and postsynaptic activity) and long-term depression (LTD; presynaptic activity that does not coincide with postsynaptic activity; Stent, 1973) at inhibitory LN-to-ORN synapses. Such bidirectional plasticity at inhibitory synapses has been found in the cerebellum (Aizenman *et al.*, 1998) – coincident pre- and postsynaptic activity leads to LTP, presynaptic and no postsynaptic activity leads to LTD. We propose the same bidirectional plasticity for LN-to-ORN synapses. This mechanism would modify the AL network based on odour-driven activity statistics, and would therefore function as an adaptive gain-control filter. ORN-to-PN transmission is amplified in glomeruli with weak input whereas it is weakened in glomeruli with strong input. After learning, the two postulated synaptic changes would sum up and result in an increased ORN-to-PN transmission in 'A glomeruli' and 'none glomeruli', no change in 'B glomeruli', and decreased transmission in 'A/B glomeruli' (Fig. 4C).

What might be the adaptive value of glomerulus-specific response changes? The increase in 'A glomeruli' might facilitate the detection of the learned odour A and chemically similar odours that activate the same ORNs. The 'A/B glomeruli' carry ambiguous information as they respond to both the rewarded A and unrewarded B. The decrease in responses after training may thus reduce ambiguity and may facilitate discrimination between A and B. In line with this hypothesis, we found a training-induced increase in the distance between the A and B patterns in discriminators but not in non-discriminators (Fig. 2B). The elevation of odour responses in 'none glomeruli' might reflect an unspecific increased sensitivity to odours in general. Taken together our results support the idea that learning modifies the functional AL circuitry, which ameliorates odour representations and thereby improves the bees' ability to detect and discriminate learned odours. Given the high functional similarity in olfactory networks across species it is tempting to speculate that similar mechanisms also occur in the mammalian olfactory bulb.

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Abbreviations

A+, odour A paired with the unconditioned stimulus; AL, antennal lobe; B–, unpaired odour B; I-APT, lateral antenna-protocerebral tract; LN, local interneuron; LTD, long-term depression; LTP, long-term potentiation; m-APT, medial antenna-protocerebral tract; ORN, olfactory receptor neuron; PER, proboscis extension reflex; PN, projection neuron.

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