

## SIDE-SPECIFIC OLFACTORY CONDITIONING LEADS TO MORE SPECIFIC ODOR REPRESENTATION BETWEEN SIDES BUT NOT WITHIN SIDES IN THE HONEYBEE ANTENNAL LOBES

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**Abstract**—Honeybees can be trained to associate odors to sucrose reward by conditioning the proboscis extension response. Using this paradigm, we have recently shown that bees can solve a side-specific task: they learn simultaneously to discriminate a reinforced odor A from a non-reinforced odor B at one antenna (A+B−) and the reversed problem at the other antenna (A−B+). Side-specific (A+B−/B+A−) conditioning is an interesting tool to measure neurophysiological changes due to olfactory learning because the same odorant is excitatory (CS+) on one brain side and inhibitory (CS−) on the opposite side. In the bee brain, the antennal lobe (AL) is the first olfactory relay where the olfactory memory is established. Using calcium imaging, we compared odor-evoked activity in the functional units, the glomeruli, of the two ALs, both in naive and conditioned individuals. Each odor evoked a different pattern of glomerular activity, which was symmetrical between sides and highly conserved among naive animals. In conditioned bees, response patterns were overall symmetrical but showed more active glomeruli and topical differences between sides. By representing odor vectors in a virtual olfactory space whose dimensions are the responses of 23 identified glomeruli, we found that distances between odor representations on each brain side were significantly higher in conditioned than in naive bees, but only for CS+ and CS−. However, the distance between CS+ and CS− representations was equal to that of naive individuals. Our work suggests that side-specific conditioning decorrelates odor representations between AL sides but not between CS+ and CS− within one AL. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** PER conditioning, olfactory learning, optical imaging, side-specificity, calcium.

A general question in the study of associative learning and memory is how stimulus-specific and outcome-related in-

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**Abbreviations:** AL, antennal lobe; CS+, reinforced conditioned stimulus; CS−, explicitly non-reinforced conditioned stimulus; LN, local interneuron; LPL, lateral protocerebral lobe; LTM, long-term memory; MB, mushroom body; MTM, mid-term memory; PCA, principal component analysis; PER, proboscis extension response; US, unconditioned stimulus; VUMmx1, ventral unpaired median neuron 1 of the maxillary neuromere.

formation is stored by the nervous system. Appropriate learning paradigms, a good description of the nervous system, and physiological techniques allowing to record the activity of neural networks are all necessary for understanding learning and memory processes. The honeybee *Apis mellifera* L. is an ideal model for the study of the mechanisms of learning and memory since (i) it shows clear learning abilities, (ii) its neural pathways have been extensively described, and (iii) it is used routinely in physiological experiments (Menzel, 1999, 2001).

Restrained honeybees can learn to associate odor stimuli (reinforced conditioned stimulus [CS]) with a sucrose reward (unconditioned stimulus [US]), building an association which can last a lifetime, and depends on the parallel and/or sequential involvement of at least five memory stages (Menzel, 1999, 2001). The identified neuron VUM-mx1 (ventral unpaired median neuron 1 of the maxillary neuromere) represents the neural substrate for the US-pathway (Hammer, 1993). Electrical stimulation of this neuron in temporal association with an odor stimulus can fully replace the US and induce an associative olfactory memory. The olfactory (CS) pathway is also well described: axons of the chemoreceptors on each antenna project to the 160 glomeruli of each antennal lobe (AL) where they synapse with about 4000 local interneurons (LN) and about 800 projection neurons. The projection neurons further convey the information via three tracts to higher brain centers, the mushroom body (MB) calices and the lateral protocerebral lobe (LPL). The VUM-mx1 neuron converges with the olfactory pathway at three sites, the ALs, the MBs and the LPLs. However, localized cooling experiments (Menzel et al., 1974; Erber et al., 1980) and octopamine injections (Hammer and Menzel, 1998) showed that only the ALs and the MBs are necessary for the establishment of appetitive olfactory memory. However, how the nervous system can establish stimulus-specific olfactory memories, i.e. memories which are specific to a CS, is still largely ignored. A critical procedure for showing stimulus-specific memories is that of differential conditioning, where one odor (CS+) is presented to the bee in association with a sucrose reward, while another odor (CS−) is explicitly presented without reward (Bitterman et al., 1983). After such a procedure, only the CS+ will elicit a behavioral response. In the present work, we aimed to understand what makes an odor a “CS+” or a “CS−,” by comparing *within the same animal* the neural substrates of the same odor both as CS+ and as CS−. We recently showed that honeybees can be trained to respond to odorants when presented on one side of the brain but not on the other side (Sandoz and Menzel, 2001). In the so-called A+B−/B+A− task, bees are alter-

nately presented with an odor A associated to a reward on one side, and to the same odor without reward on the opposite side. With an odor B, the opposite pattern is presented. In this situation, bees learn to respond to each odor on one side only, inducing a side-specific memory lasting at least 24 h. Thus, each odor is a CS+ on one brain side and a CS− on the opposite side.

Optical imaging techniques allow to measure the activity of numerous neurons at the same time, and may thus be used to follow changes in neural networks due to experience. In the honeybee, calcium imaging was successfully applied to record neural activity both from the ALs (Joerges et al., 1997) and the MBs (Faber and Menzel, 2001). In the ALs, odors elicit glomerular response patterns (Joerges et al., 1997) based on a code which is conserved between individuals (Galizia et al., 1999b; Sachse et al., 1999). Shortly after differential conditioning, calcium activity induced by the CS+ was found to be increased, while activity to the CS− remained unchanged (Faber et al., 1999). Moreover, based on a pixel-based correlation analysis, the authors suggested that odor response patterns were decorrelated as a result of conditioning, suggesting that CS+ and CS− could be differentiated better after conditioning. However, all analyses were performed on the whole AL, so that learning-induced changes could not be precisely related to individual glomeruli. Also, to what extent olfactory learning modifies the olfactory code at a longer timescale is as yet unknown, and we addressed this question in the present study.

The anatomical layout of the glomeruli of the ALs is conserved between individuals so that according to their form and size, they can be recognized from one bee to the next (Flanagan and Mercer, 1989a), which allowed to develop an anatomical atlas of the bee AL (Galizia et al., 1999a). Moreover, the arrangement of the glomeruli on each side of the brain is symmetrical, and odor-evoked response patterns on one side of the brain correspond to the mirror image of the other side, indicating that the olfactory code must be symmetrical (Galizia et al., 1998). Taking advantage of this anatomical and functional property of the bee's AL, we developed an *in vivo* preparation of the honeybee brain in which both ALs can be simultaneously imaged. Mapping odor-evoked responses to morphologically identified glomeruli, we compared the physiology of the two brain sides at the glomerular level. This was done for naive bees and for bees conditioned 24 h earlier in a side-specific discrimination (A+B−/B+A−).

## EXPERIMENTAL PROCEDURES

### Side-specific conditioning of the proboscis extension response (PER)

Worker bees were collected in the morning at the entrance to an outdoor hive, immobilized by short cooling, and fixed in metal harnesses with strips of tape placed behind the head and between the thorax and abdomen. To separate the olfactory input space of the bee into two independent zones, we used thin plastic walls placed between the antennae (Sandoz and Menzel, 2001). The walls were made of a 40 mm×50 mm piece of overhead transparency plastic, in which the shapes of the bee holder and of the

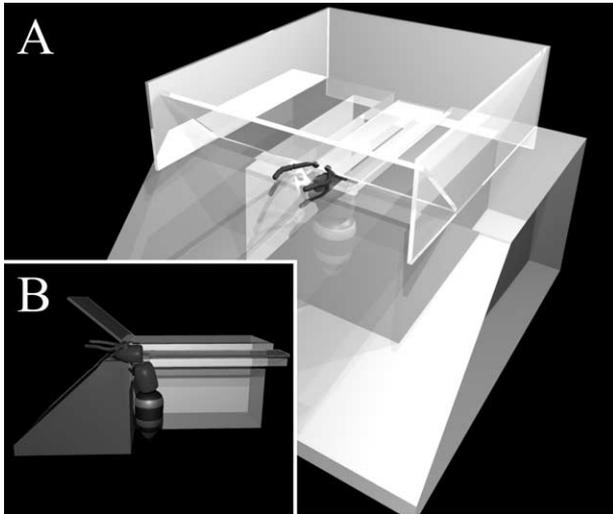
bee's head were precisely cut so as to fit snugly. Each wall was attached with low-temperature melting wax in order to close any remaining spaces and thus to prevent any leakage between sides. The wall was placed obliquely, allowing the proboscis to move freely. In all conditioning experiments, and on every experimental day, as many bees had the wall placed to the left of the proboscis and to the right. Bees were then left for 2 h before conditioning began so that they could habituate to the presence of the walls.

To produce side-specifically conditioned bees, we used a procedure in which two odors A and B were presented to bees. Each odor was presented together with a reward on one brain side, but without reward on the opposite side (A+B−/B+A− training; Sandoz and Menzel, 2001). Thus, on one side, bees were subjected to four rewarded (CS+) trials with odor A and four unrewarded trials (CS−) with odor B. On the opposite side, they received four rewarded trials with odor B and four unrewarded trials with odor A. Every second stimulation was performed on one given side. On each side, stimulations with odors A and B were provided in a pseudo-randomized order ABBABAAB or BAABABBA. Bees thus received a total of 16 trials with 5 min inter-trial intervals (10 min between stimulations on the same side). The role of the sides was balanced between animals, so that every day as many bees received a given stimulation pattern on the left side as on the right side. For each trial, a bee was placed facing a holder to which two syringes could be attached. During odor stimulations, one syringe at a time was placed on the holder, with its outlet on the side of the separation wall which was to be stimulated. The odorant from the syringe was released parallel to the wall and directed toward the bee's antenna. An exhaust vent 10 cm behind the bee ensured that all released odors were vented out of the experimental room. During rewarded (CS+) trials, bees were placed into the apparatus and had 15 s familiarization to the overall experimental context before the odor CS was presented, for 6 s on one side. During the first 3 s of odor presentation, the occurrence of a proboscis extension was noted by the experimenter. Three seconds after onset of the CS, the antenna placed on the same side was stimulated with the US, leading to a proboscis extension. The bee was then rewarded for 3 s by food uptake at the proboscis (compound US). During unrewarded (CS−) trials, the odor was presented on one side without any stimulation with the US. Two odors, limonene and 1-hexanol (Sigma-Aldrich, Deisenhofen, Germany) were used as CS+ and CS−. They were chosen because they induce very clear side-specific conditioning in a previous study (Sandoz and Menzel, 2001). Five microliters of pure compound were soaked on a 1 cm<sup>2</sup> piece of filter paper which was inserted into a 20 ml syringe. As US, a 30% w/w sucrose solution was used. Bees were used in optical imaging experiments 24 h after conditioning.

### Simultaneous optical recordings of the two ALs

#### Honeybee preparation

Naive or conditioned worker bees were immobilized by short cooling, and fixed in a Plexiglas recording chamber using low-temperature melting wax so that the head could not move. The antennae were fixed to the front of the chamber using two-component silicon (Kwik-Sil; World Precision Instruments, Sarasota, FL, USA). Small pieces of plastic foil (0.5 mm thick) were then waxed at an angle to the front, and vertically to the sides of the chamber, to create a small pool around the head (Fig. 1). Thus, the antennae remained in the air, while the brain could be kept under saline. A window was then cut in the cuticle of the head. Glands and trachea were removed to expose both ALs, and the esophagus was removed. The brain was then washed with saline (in mM: NaCl, 130; KCl, 6; MgCl<sub>2</sub>, 5; sucrose, 160; glucose, 25; HEPES, 10; pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich) to remove any enzyme released during the preparation. Then the saline was gently removed, and the brain was bathed with 50 μl of dye solution. The dye consisted in Calcium-Green-2AM (50 μg) dissolved with 50 μl Pluronic F-127 (20% in dimethylsulfoxide) in 800 μl saline (Calcium-Green and Pluronic from



**Fig. 1.** Optical imaging preparation. (A) Recording chamber allowing to keep the brain of bees under physiological fluid, while the antennae remain in an air stream directed to the front of the chamber. The microscope's objective is placed about 3 mm above the bee's head. (B) Cut view of a side representation of the bee in the recording chamber. Odor stimulations would be directed to the bees' antennae from the Left side of the figure.

Molecular Probes, OR, USA). The piece of head cuticle was then replaced on the opening and the bee was left for 1 h on an ice bed. After staining, the brain was thoroughly washed with saline, and the final preparation for optical recordings was performed. In order to avoid background fluorescence from mouthpart muscles, a small insect needle (diameter: 100  $\mu\text{m}$ ; length: 10 mm) was used as a mask, and was inserted between the ALs and fixed outside of the head capsule with wax. Finally, the abdomen was removed to prevent movement artifacts.

### Optical recordings of odor-evoked activity

*In vivo* calcium imaging recording were carried out as described elsewhere (Galizia et al., 1998). Stained bees were placed under an epifluorescent microscope (10 $\times$ , NA 0.3, water immersion), and the head region was immersed in Ringer. A goniometer allowed to incline the preparation slightly to the left or to the right, so that the surfaces of both ALs were in focus. Each measurement consisted in 40 frames at a rate of 1.43 frames/s. Integration time for each frame was 500 ms. Odorant stimuli were given at the 9th frame for a duration of 2 s. The spatial resolution was of 170 $\times$ 170 pixels (binned on the chip of a cooled 12-bit CCD camera; Photometrics CH250A, AZ, USA). Each binned pixel corresponded to 6  $\mu\text{m}$  $\times$ 6  $\mu\text{m}$ . The light source was a halogen lamp (150 W) driven by a stabilized power supply (68830; Lot Oriel, Darmstadt, Germany). The filter set was composed of a BP 448–495 for excitation, a 505 dichroic, and a BP 517–580 for emission.

Under the microscope, a constant air-stream in which odor stimuli could be injected was directed to the antennae of the bee. Odor sources were prepared by applying 5  $\mu\text{l}$  of pure substance

onto a 1  $\text{cm}^2$  piece of filter paper in a 1-ml plastic syringe. For odor stimulations, syringes were inserted in a Teflon tubing inlet and 0.8 ml of odor-saturated air was injected manually into the permanent air-stream stimulating the bee.

As odors we used the two odorants which were used for conditioning (1-hexanol and limonene), plus three odorants which would allow us to see the effect of conditioning on novel odorants. These odors were 1-nonanol (Sigma-Aldrich), clove and orange essential oils obtained from a local drugstore. A syringe containing only a piece of filter paper was used as air control. An experimental run consisted in 3 series of six presentations with 1–2 min inter-trial intervals. Each time, odors were presented in the same fixed order: air, 1-nonanol, limonene, 1-hexanol, clove-oil, orange.

### Mapping of glomeruli

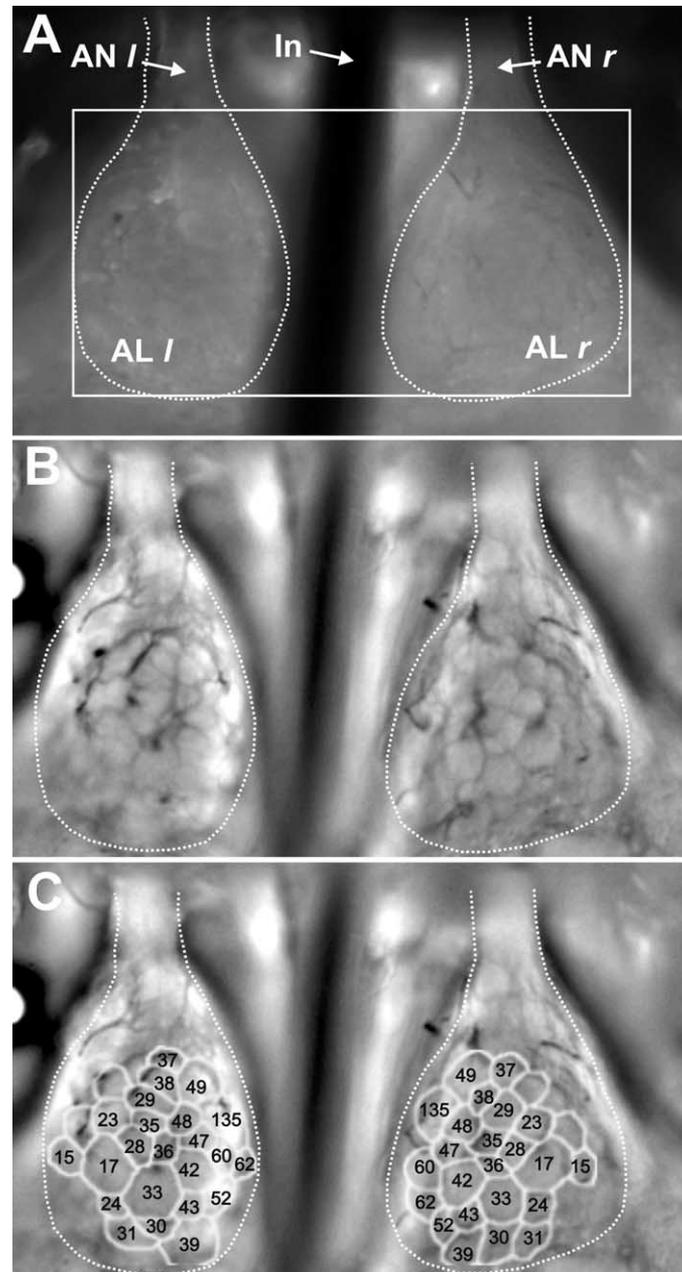
During optical imaging, the glomerular structure of the ALs is not visible (Fig. 2A). To reveal it, the brain was bathed with the mixture of a protease (from *Bacillus licheniformis* in propylene glycol; Sigma-Aldrich) which digested the brain sheath and of the dye RH795 which stained cell membranes (Molecular Probes). After 1 h, the brain was washed with Ringer and fluorescent photographs were taken at 5–10 different focal planes (Fig. 2B). Photographs were then contrast-enhanced and subjected to an unsharp filter, using Adobe Photoshop, and the borderlines of single glomeruli were reconstructed for each AL from the different focal depths. Individual glomeruli were then identified using the standardized AL atlas (Galizia et al., 1999a and at <http://www.neurobiologie.fu-berlin.de/honeybeeALatlas/>). For each AL, the reconstructed glomerular mask allowed to determine the coordinates of the different glomeruli in the calcium-imaging data. A total of 23 glomeruli, 22 innervated by the T1 tract, and one by the T3 tract (number 135, corresponding to glomerulus T3-45), could be identified in all AL preparations (Fig. 2C). Six glomeruli were recognized in only a few preparations and were thus not included in the analysis.

### Signal calculation

Calcium-imaging data were analyzed using custom-made software written in IDL (Research Systems Inc., CO, USA). First, possible irregularities of the camera system were corrected by subtracting a dark frame (average of three measures with shutter closed) from the data, to obtain the raw fluorescence data. The background fluorescence of the preparation,  $F$ , was calculated before the odor stimulation as the average of frames 5–7. The signals were then calculated at each frame as  $\Delta F/F$ . Finally, data were corrected for dye bleaching using a spatial unsharp filter. This filter calculated from each frame an unsharp picture, using the smooth function of IDL with a width of 35 pixels, i.e. 210  $\mu\text{m}$ , which was subtracted from the  $\Delta F$  data. The width of the unsharp filter represented the size of more than four glomeruli in each direction which ensured that only global bleaching, and no glomerular signal, was subtracted from the data. Excitation of single glomeruli in response to each odor was calculated by averaging 25 pixels (5 $\times$ 5, corresponding to 30  $\mu\text{m}$  $\times$ 30  $\mu\text{m}$ ) at the center of the glomerulus, using the coordinates determined from the reconstruction of the AL glomerular structure (Fig. 3). The amplitude of the odor response was calculated using a non-linear model fit as developed by Stetter et al. (2001).

### Abbreviations used in the figures

AIR	air control	/L	left side
AL	antennal lobe	LIM	limonene
AN	antennal nerve	LIM+	side where limonene was reinforced
CLV	clove oil	NON	1-nonanol
HEX	1-hexanol	ORA	orange
HEX+	side where 1-hexanol was reinforced	r/R	right side
In	insect needle		



**Fig. 2.** Bilateral preparation of honeybee antennal lobes. In all images, ventral is up, and the bee axis is central. (A) Fluorescent view of the two ALs stained with Calcium-Green. The white frame around the two antennal lobes shows the window used for imaging recordings (see Fig. 3). (B) View of the two same ALs after digestion and additional staining with RH795, showing the glomerular borderlines. The image was contrast-enhanced and subjected to a digital unsharp filter to allow a better view of glomerular structure. (C) Reconstruction of the glomerular structure of each lobe. Note the symmetrical arrangement of similarly-shaped glomeruli, e.g. the V-arrangement of glomeruli T1-17, T1-33 and T1-42 on each side. All glomeruli are innervated by T1 and identified by their number only (i.e. T1-17 is marked as 17), with exception of T3-45, which is marked as 135.

## Statistics

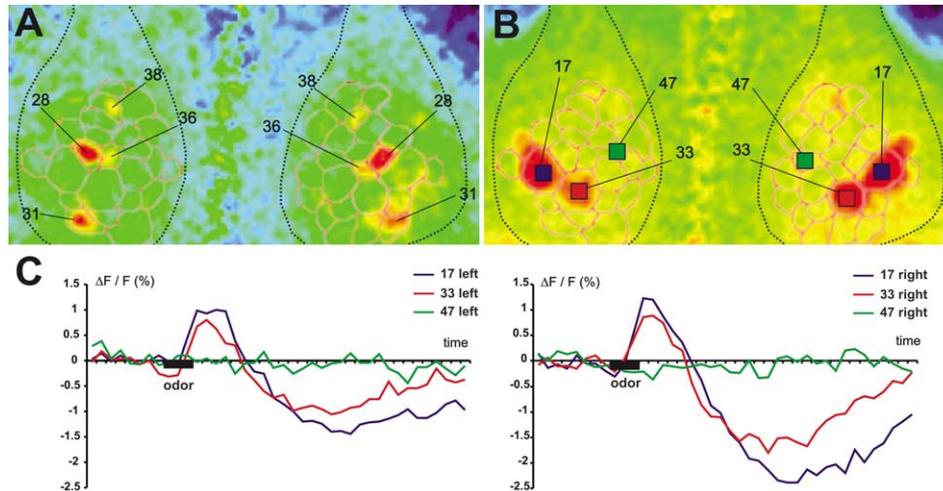
### Side-specific olfactory conditioning

The comparison of responses to the CS+ and to the CS– during training was made using a log-linear analysis performed on the frequencies of responses at each trial. Interactions of the design variables (successive trials, conditioning side) with the development of odor-evoked PER was considered significant

only if both partial and marginal association  $\chi^2$  were significant ( $P < 0.05$ ).

### Optical imaging

Forty-seven honeybees were used in calcium imaging experiments (26 naive and 21 conditioned). Out of these, six naive and six conditioned bees provided both bilateral activity in the ALs and showed good bilateral staining which allowed to re-



**Fig. 3.** False-color-coded activity maps in the ALs of a naive bee. (A) Glomerular structure superimposed on the false-color-coded map of this bee's response to 1-hexanol, showing the symmetrical odor-evoked signal (color code: dark blue, blue, green, yellow and red indicating increasing calcium signals). On each side, glomeruli T1-28, T1-31, T1-36 and T1-38 were active. (B) Odor-evoked response to 1-nonanol. Glomeruli T1-17 and T1-33 responded on both sides, while glomerulus T1-47 remained silent. (C) Time courses ( $\Delta F/F$  against time) of calcium signals are shown for both sides and the three glomeruli indicated in B. These data are from the same naive bee as that shown in Fig. 2.

construct the glomerular anatomy with confidence. Between 12 and 18 odor measurements were performed per animal, which gave a total of between 15 and 18 measurements for each odor stimulus in each group of animals. In the statistical analysis, odor-evoked activity was compared between sides. In naive bees, we compared the right and left sides of the brain to check the symmetry of the neural representation of odors. In bees conditioned in an A+B−/B+A− training, we compared the side where 1-hexanol was rewarded (and limonene unrewarded, called the HEX+ side) to the side where limonene was rewarded (and 1-hexanol unrewarded, called the LIM+ side). The activity was compared between sides in two ways:

*I) at the glomerular level.* To compare the number of active glomeruli in naive and in conditioned individuals, we used *t*-tests, with  $\alpha=0.05$ . To compare the activity of individual glomeruli between sides, we used paired *t*-tests. To reduce the risk of type I errors due to a high number of comparisons between sides, we included a correction of the significance threshold using the Dunn-Sidak correction:  $\alpha'=1-(1-\alpha)^{1/k}$ , with  $\alpha=0.05$  and  $k=23$  (number of tested glomeruli). Thus our significance threshold was  $\alpha'=0.0022$ . Note that this correction is highly dependent on the number of glomeruli observed in the study, and may hide biologically relevant differences. We therefore considered as near-significant, and possibly biologically relevant, differences in the range between  $\alpha=0.05$  and 0.0022.

*II) at the AL level.* The neural representation of an odor can be regarded as a vector in a multidimensional space, where each dimension is represented by a particular glomerulus. Since it is not possible to visualize vectors in a 23-dimensional space, we used principal component analysis (PCA) which identifies orthogonal axes (factors) of maximum variance in the data, and thus projects the data into a lower-dimensionality space formed of a subset of the highest-variance components. We calculated the three factors which explain most of the observed variance, using a normalized Varimax rotation. Such rotational strategy maximizes the variance of the calculated factors, while minimizing the variance around them.

To compare the distance of odor vectors between sides, we calculated their Euclidian distance in the 23-dimensional space, as:

$$d_{ij} = \sqrt{\sum_{k=1}^p (X_{ik} - X_{jk})^2}$$

with *i* and *j* indicating odors, *p* the number of dimensions, i.e. glomeruli, and  $X_{ik}$  the response in glomerulus *k* to odor *i*.

Distances between sides were calculated for each odor stimulus, and were then compared using a paired *t*-test, with  $\alpha=0.05$ . We also calculated the Euclidian distance between limonene and 1-hexanol odor vectors in naive and in conditioned bees, and compared them between groups using a *t*-test with  $\alpha=0.05$ .

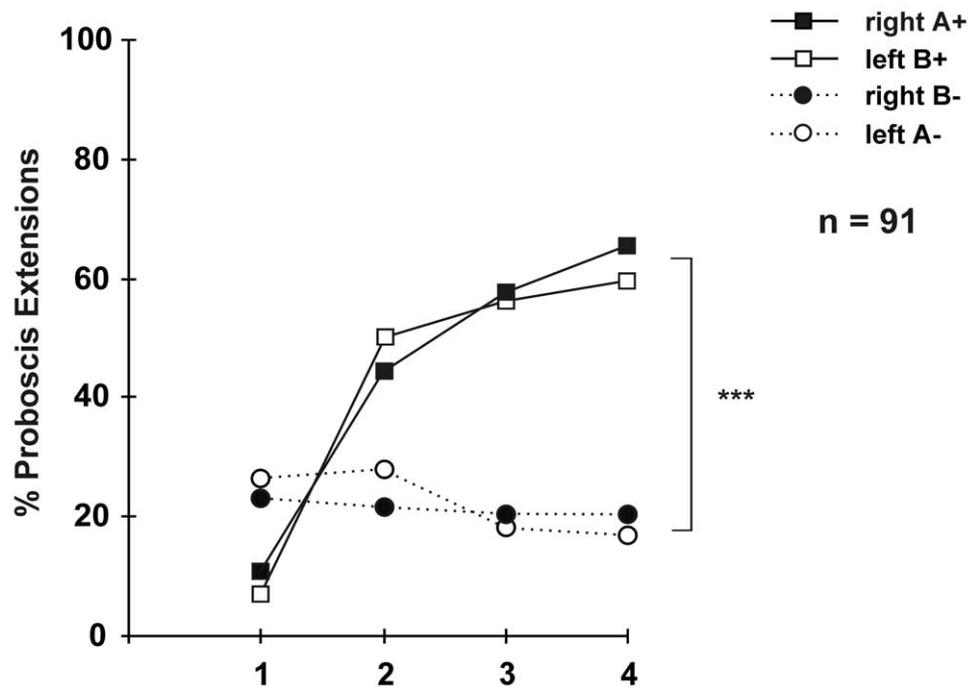
## RESULTS

### Side-specific olfactory conditioning

Bees conditioned in a side-specific conditioning procedure (A+B−/B+A− training) learned to respond to each odor on one side only (Fig. 4). Responses to the CS+ started at a level of 7–11% and reached 59–65% at the last trial ( $n=91$ ). In contrast, responses to the CS− decreased from 23 to 26% to 18–21%. Both sides learned equally well (log linear analysis, partial and marginal association  $\chi^2=0.01$ , NS). The difference between responses to CS+ and CS− was highly significant (log linear analysis, partial and marginal association  $\chi^2>75.8$ ,  $P<0.001$ ). In the last block of training, the proportion of bees which did not make any errors, i.e. which had learned the side-specific task with both odors was 25.3%. Only bees which had efficiently learned were used for optical imaging experiments ( $n=21$ ). From these, six bees allowed to record both bilateral calcium imaging signals and showed a good bilateral glomerular staining allowing to reconstruct the glomerular structure.

### Optical imaging

*Naive bees.* The anatomical reconstruction of the ALs on each side of the brain, showed that each lobe contains a symmetrical arrangement of glomeruli with sim-

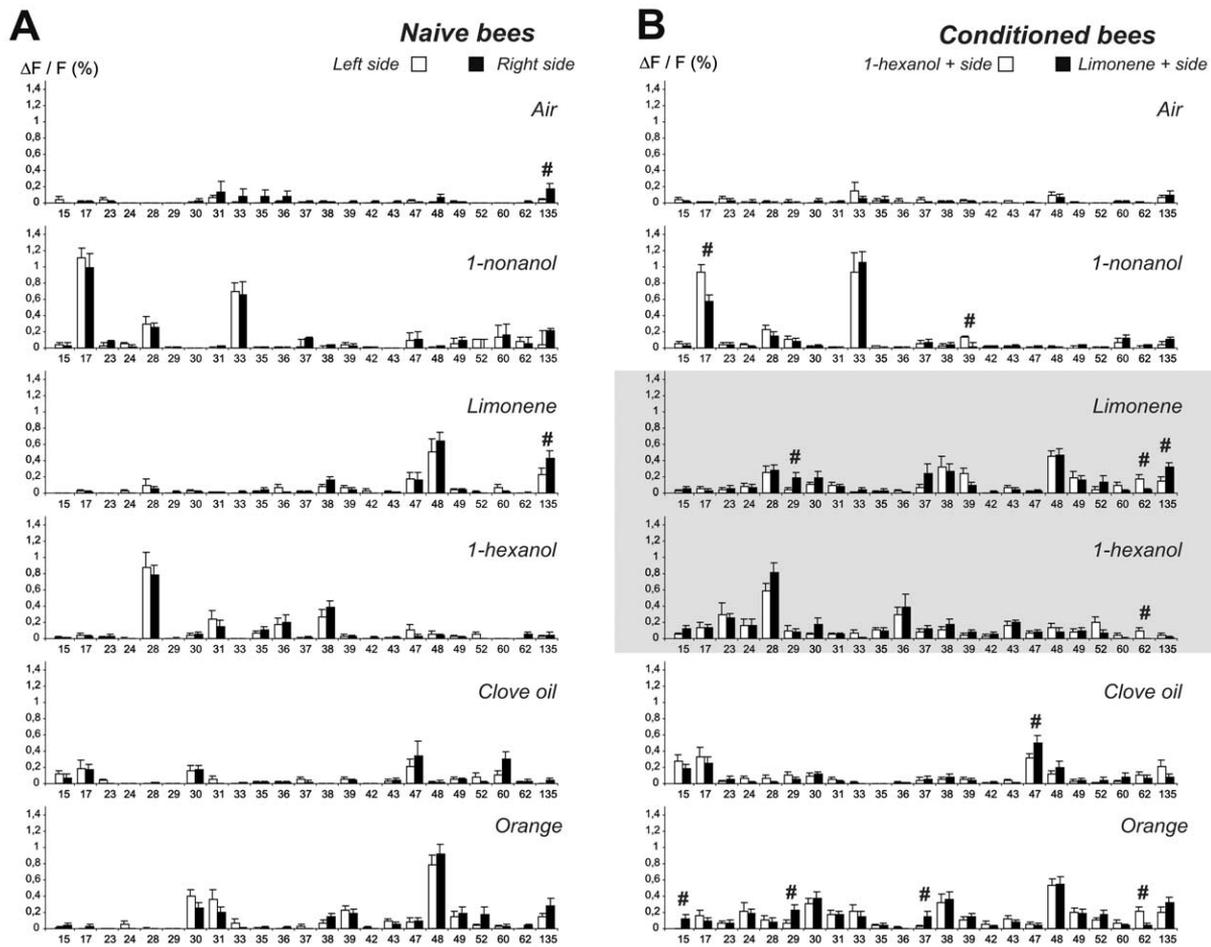


**Fig. 4.** Performances of bees trained in a side-specific conditioning procedure (A+B−/B+A− training). Responses to each odor increased on the side where it was rewarded, and decreased or remained stable on the side where it was explicitly unrewarded, showing a clear side-specific pattern of responses at the end of the procedure (\*\*\*:  $P < 0.001$ , log-linear analysis comparing responses to CS+ and CS−).

ilar size, shape and relative position (e.g. Fig. 2C). This suggested that such symmetrical glomeruli could have the same function on each side of the brain. Indeed, optical imaging measurements allowed us to visualize odor-evoked activity in both ALs simultaneously. Activity patterns were symmetrical, so that each given odor showed activity in symmetrically arranged glomeruli (Fig. 3A and B). We compared the amplitudes of the recorded signals for five odorants and an air control in 23 glomeruli identified on the left and the right brain sides (Fig. 5A). Different odors induced different activity spectra (see for instance in Fig. 3A, B and Fig. 5A: 1-nonanol triggered activity mainly in glomeruli 17 and 33, while 1-hexanol elicited activity mainly in glomeruli 28, 31, 36 and 38) which were consistent with those found in previous studies (Galizia et al., 1999b; Sachse et al., 1999). Such activity patterns were found to be fully symmetrical between sides: symmetrically arranged glomeruli presented signals of equal amplitude on the two sides, statistical comparison showing near-significant differences in only two cases (both in glomerulus T3-45, paired  $t$ -test,  $0.05 > P > 0.0022$ ). Odor-evoked activity patterns can be represented in a multidimensional space where the activity of each glomerulus represents one dimension. To visualize the representations of odorants in a reduced version of this 23-dimension olfactory space, we used PCA. The first three axes represented 39% of the whole variance. When representing the data according to the first three axes, we found that different odors were efficiently separated in this subspace, and that the measurements to each odor on the left and right brain sides were grouped together (Fig. 6A).

**Conditioned bees.** Calcium imaging measurements in honeybees trained in a side-specific conditioning procedure (A+B−/B+A−) also showed generally symmetrical patterns of activity in the ALs. Fig. 5B presents the response amplitudes of individual glomeruli on the side on which 1-hexanol was reinforced (and limonene was non-reinforced) and the side on which limonene was reinforced (and 1-hexanol non-reinforced). A precise analysis of the amplitude of the signals in the 23 glomeruli identified in each AL showed that signals of symmetrically arranged glomeruli were generally equal, although in a number of cases, near-significant differences were found (for instance, glomerulus 17 for 1-nonanol, or glomeruli 15, 29 and 37 for orange,  $0.05 > P > 0.0022$ ). The training odors, limonene and 1-hexanol which were each CS+ on one brain side and CS− on the other side, showed similar patterns of responses on the two sides. When visualizing odor representations using a PCA, we found a similar organization of odorants as in naive individuals, with different odors being separated but measurements on the two sides being grouped together (Fig. 6B). Also, the relative arrangement of odors was similar as in naive bees (see the 1-nonanol, orange, 1-hexanol triangle), although limonene seemed much closer to the representation of orange than in naive individuals.

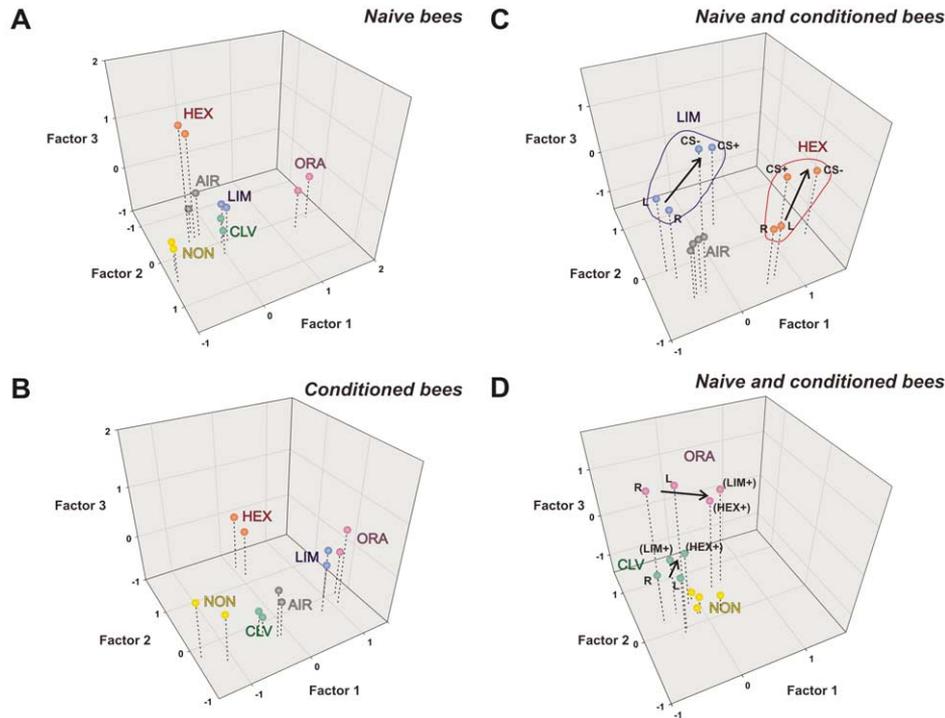
**Comparison between naive and conditioned bees.** The observation of response profiles obtained for naive and conditioned individuals shows that generally more glomeruli were active in conditioned bees (Fig. 5A and B). Indeed, the comparison of the number of active glomer-



**Fig. 5.** Glomerular responses in the two antennal lobes. (A) Responses of 23 glomeruli identified on each side to five odors and the air control in naive bees ( $n=6$ ), and (B) in conditioned bees ( $n=6$ ). Response amplitudes are shown in % fluorescence change ( $\Delta F/F$ )  $\pm$  S.E. In naive individuals, responses are compared between the left and the right brain side. In conditioned individuals, responses are compared between the side where 1-hexanol was reinforced and the side where limonene was reinforced. The zone in shaded gray corresponds to the profiles obtained for the two CSs. '#' signs correspond to glomeruli for which significant differences at the  $\alpha=0.05$  threshold were found ( $t$ -test). Note the higher number of active glomeruli and the increased occurrence of between-side differences in conditioned bees as compared with naive bees.

uli (amplitude above 0) between conditioned and naive bees showed a significant outcome in the case of four of five odors ( $t$ -test,  $P < 0.011$ ), air control and 1-nonanol stimulations inducing the same activity in both groups ( $t$ -test,  $P > 0.21$ ). A direct comparison of the activity of each glomerulus (averaging both sides) showed that usually silent glomeruli presented the most important increase in activity when conditioned (number 15, 23, 24, 29 and 43,  $P < 0.0022$ ), other silent glomeruli showing only near-significant difference (number 37, 42 and 62). All other glomeruli did not show any difference between naive and conditioned individuals. To allow a better understanding of how odor representations could be modified by side-specific conditioning, we performed a combined PCA with the data from both naive and conditioned bees. This visualization of odor vectors shows that odors have somewhat different representations in naive and in conditioned individuals (Fig. 6C, D). In particular, the representations of the two odors used for conditioning, 1-hexanol and limonene, show a clear

shift in the multidimensional odor space (Fig. 6C, see arrows). This shift appears for odor vectors of both training sides, although each of these two odors had been reinforced on one side and non-reinforced on the other side. Interestingly, the same tendency was observed for orange (Fig. 6D, see arrows). In order to quantify such effects, we computed a measure of odor similarity in the multidimensional odor space, by calculating the Euclidian distance between odor vectors (note that this measure takes all the 23 dimensions into account; for details see Experimental Procedures). First, we wanted to know how conditioning modifies the similarity between odor vectors on the two brain sides. We thus compared Euclidian distances between sides in naive and in conditioned individuals for each odor (Fig. 7A). Distances between sides were found to be similar for all odor stimuli except for the two odors used for conditioning, which showed higher distances between sides in conditioned bees than in naive bees ( $t$ -test,  $P < 0.05$  for 1-hexanol,  $P < 0.01$  for limonene). This indi-



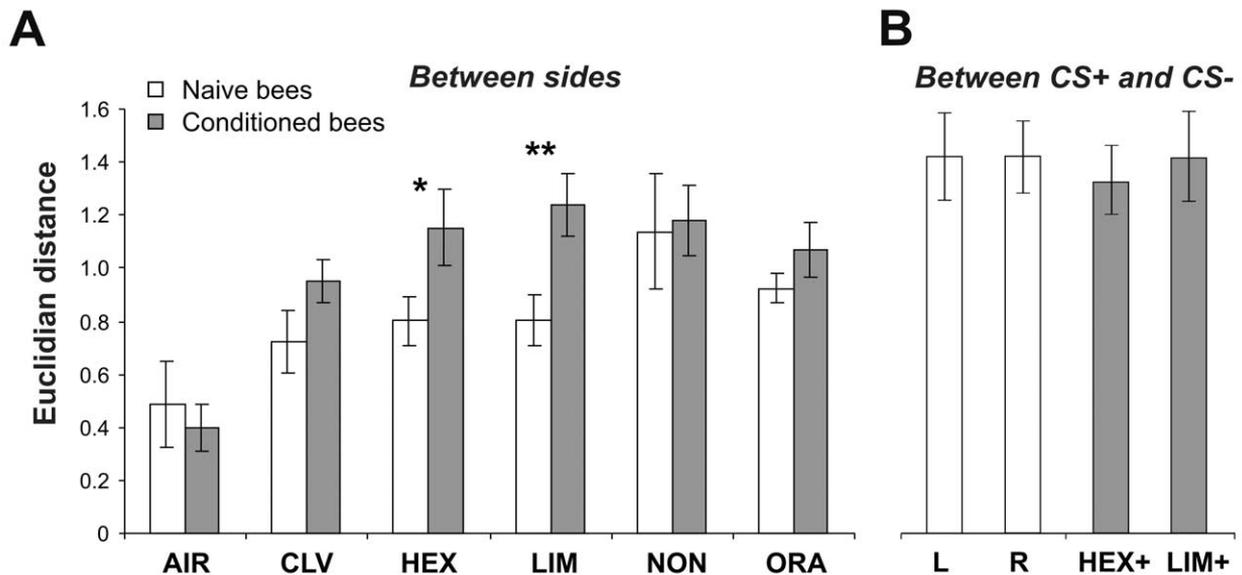
**Fig. 6.** Neuronal representation of odors in a virtual olfactory space. (A and B) PCA of odor-evoked activity patterns on each side of the brain for all measurements of A) naive bees and B) conditioned bees. The response of each glomerulus was used as a dimension for the analysis. For each odor, the barycenter of odor stimulations is shown in a coordinate composed of the first three variance factors (AIR: air control, NON: 1-nonanol, LIM: limonene, HEX: 1-hexanol, CLV: clove oil, ORA: orange). Note that although the analysis was run separately for naive and for conditioned individuals, similar primary factors were obtained so that the respective representations of odorants were similar in both cases. (C and D) PCA of odor-evoked activity patterns on each side of the brain including both naive and conditioned bees. The relative positions of odor representations between the two groups indicate the modification of odor representation through conditioning C) for the two CSs (LIM and HEX) and the air control (AIR). The arrows show the migration of odor representations between naive (L: left side, R: right side) and conditioned bees (CS+: side where the odor was reinforced, CS-: side where the odor was nonreinforced). D) same analysis showing the three other odorants (CLV, ORA, NON). The arrows show the migration of clove oil and orange representations between naive (L: left side, R: right side) and conditioned bees (HEX+: side where 1-hexanol was reinforced, LIM+: side where limonene was reinforced). Air and 1-nonanol representations were grouped together in both naive and conditioned bees.

icates that side-specific conditioning decorrelated the representations of conditioning odors between brain sides. We then asked whether conditioning had an effect on the amount of similarity between the representations of the CS+ and the CS- in each antennal lobe. We thus calculated, on each side, Euclidian distances between limonene vectors and 1-hexanol vectors (limonene was CS+ on one brain side and CS- on the contralateral side; vice versa for 1-hexanol). We found no change in the distances between these two odors in conditioned individuals as compared with naive bees (Fig. 7B, *t*-test, NS). Thus, side-specific conditioning did not decorrelate the odor representations of the two training odors within each side, but decorrelated the representations of these odors between sides.

## DISCUSSION

A previous study showed that when bees are trained to differentially associate two odors as CS+ and CS-, the intensity of odor-evoked calcium response in the antennal lobe is increased for the trained odor (CS+) and for a control odor, but not for the unrewarded odor (CS-). This was observed 10–30 min after conditioning (Faber et al.,

1999), i.e. in a time-range corresponding to mid-term memory (MTM, Menzel, 1999). Are these effects limited to MTM or do they carry over to long-term memory (LTM)? Since it is not possible to carry out calcium imaging measurements on honeybees for 24 h, and thus compare odor representation before and after LTM, we employed another strategy: by differentially conditioning the left and right side separately, we created an experimental assay that gave us a within-animal control. Thus, a single optical recording session 24 h after training was sufficient to reveal learning-induced long-term changes. We compared odor-evoked responses recorded on the side where an odor was a CS+ to those recorded simultaneously on the side where the same odor was a CS-. We found: (i) no differences in the responses of major glomeruli (i.e. most active glomeruli in the pattern) between CS+ and CS-; (ii) increased activity in many but not all minor glomeruli (i.e. usually silent glomeruli in the pattern); (iii) side-specific asymmetries in the responses of minor glomeruli; (iv) calculated distances between odor representations were significantly higher in conditioned bees than in naive bees for the CS+ and the CS- between the two brain sides, but not within each side.



**Fig. 7.** Learning decorrelated odor activity between sides but not within sides. (A) Euclidian distances between odor representations on the two brain sides for naive and conditioned bees. For the two odors used for conditioning (1-hexanol and limonene), odor representations were more distant between sides in conditioned bees than in naive bees. (Abbreviations as in Figure 6). There was no difference for the other odors (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ,  $t$ -test). (B) Euclidian distances between limonene and 1-hexanol on each brain side, for naive and conditioned bees. The same distance was found in all cases, without any significance between naive and conditioned individuals. (L: left side, R: right side, LIM+: side where limonene was reinforced, HEX+: side where 1-hexanol was reinforced).

### Symmetry of olfactory maps

Since we wanted to compare changes in olfactory representation after side-specific learning by comparing the two sides, we first had to show that in naive animals odor representation is symmetrical. Indeed, a number of studies have shown that odor maps are symmetrical in the olfactory bulb of vertebrates and in the ALs of insects. First, the topographical arrangement of glomeruli is generally conserved between individuals and symmetrical between brain sides (fish: Baier and Korsching, 1994; honeybee: Flanagan and Mercer, 1989). Second, olfactory receptor neurons expressing the same receptor gene terminate in symmetrically placed glomeruli (rodents: Vassar et al., 1994; Mombaerts et al., 1996; *Drosophila*: Gao et al., 2000; Vosshall et al., 2000). Third, radioactive 2-deoxyglucose studies in *Drosophila* (Rodrigues, 1988) and in rats (Johnson and Leon, 1996) and optical imaging recordings in bees (Galizia et al., 1998) and in rats (Rubin and Katz, 1999; Belluscio and Katz, 2001), showed symmetrical activity patterns. In the honeybee, pixel-based correlation analyses showed that the mirror image of odor-evoked activity maps recorded in one AL matched the activity in the contralateral AL (Galizia et al., 1998). Here we imaged the ALs on the two brain sides simultaneously and morphologically identified individual glomeruli, showing that symmetrically arranged glomeruli have indeed identical response spectra to odors. This is the first within-specimen demonstration on a glomerular basis of the bilateral symmetry of odor representation. For now, this demonstration is limited to the upper side of the ALs and to about 23 glomeruli which could be recognized there. However each AL is composed of about 160 glomeruli organized in four

subgroups T1 to T4, which represent input from different bundles of receptor neurons from the antenna (Mobbs, 1982). All the glomeruli identified in this study (apart from one) get their input from the T1 bundle, thus forming a distinct input region in the ALs. It is conceivable that the rules underlying odor coding in this region are different from those in other regions, since it appears that neurons projecting from such glomeruli to higher brain centers (MBs and LPL) show an anatomical (they project in several different tracts; Mobbs, 1982; Abel et al., 2001) and physiological dichotomy (they show different response properties to odors; Abel et al., 2001; Müller et al., 2002). Future work should thus explore these other regions of the ALs, in particular glomeruli of the T3 bundle, which are the most numerous but still difficult to access to using calcium imaging.

### Learning-induced changes in major glomeruli

In the AL, each glomerulus receives the axons of olfactory receptor neurons, which synapse onto projection neurons, which in turn relay the information to higher brain centers, like the MBs and the LPL (Mobbs, 1982). Between glomeruli, networks of inhibitory LNs filter the information in a lateral way and are thought to sharpen odor representation at this stage of the olfactory pathway (Christensen et al., 1993; Sachse and Galizia, 2002). In the ALs also, CS and US pathways converge, with the VUM-mx1 (neural substrate of the reinforcing function of the US) innervating AL glomeruli (Hammer, 1993, 1997). It is as yet unknown exactly which synaptic contacts exist between the CS and US pathways within the AL. It is however thought that the VUMmx1 neuron converges with the CS pathway both at

the pre- and at the post-synaptic side of the sensory-projection synapse (Menzel, 1999). Indeed, in a number of animal preparations, associative learning was shown to rely on plasticity at both the pre-synaptic and the post-synaptic side of modifiable synapses (chick: Salinska et al., 1999; *Aplysia*: Bao et al., 1998; Lechner and Byrne, 1998; rats: Zhuo et al., 1993). At both sites also, plasticity depends very strongly on a transient increase in calcium concentration (Bao et al., 1998). Therefore, increased calcium signals observed by Faber et al. (1999) shortly after training could fit with two possible mechanisms taking place on each side of these synapses: (i) associative facilitation at the presynaptic level (Hawkins et al., 1983, 1993) and (ii) hebbian amplification of postsynaptic calcium levels (Yuste and Denk, 1995). We found that after 24 h no difference appeared in major glomeruli between calcium signals obtained for an odor being a CS+ or a CS-. This suggests that the learning-related increases of odor-evoked calcium signals, described by Faber et al. (1999) have a limited duration after conditioning. This would indicate that these increased calcium signals actually reflect phenomena at the *induction* of learning-related synaptic plasticity, and are not by-products of the strengthening of synaptic contacts within the glomeruli. The calcium increase observed by Faber et al. (1999) would thus be part of early cellular processes which lead to the formation of more stable memories. In particular, the activation of cAMP-dependent protein kinase A, which is critical for the formation of LTM in the bee AL (Müller, 2000), is highly dependent on a cooperative action of intracellular calcium (reflecting odor-driven activity) and of octopamine-regulated adenylyl cyclase (mediated by the VUMmx1 neuron). Future work will have to describe the time course of increased calcium signals in detail to relate it to intracellular processes and to address the exact source (receptor and/or projection neurons) of these signals.

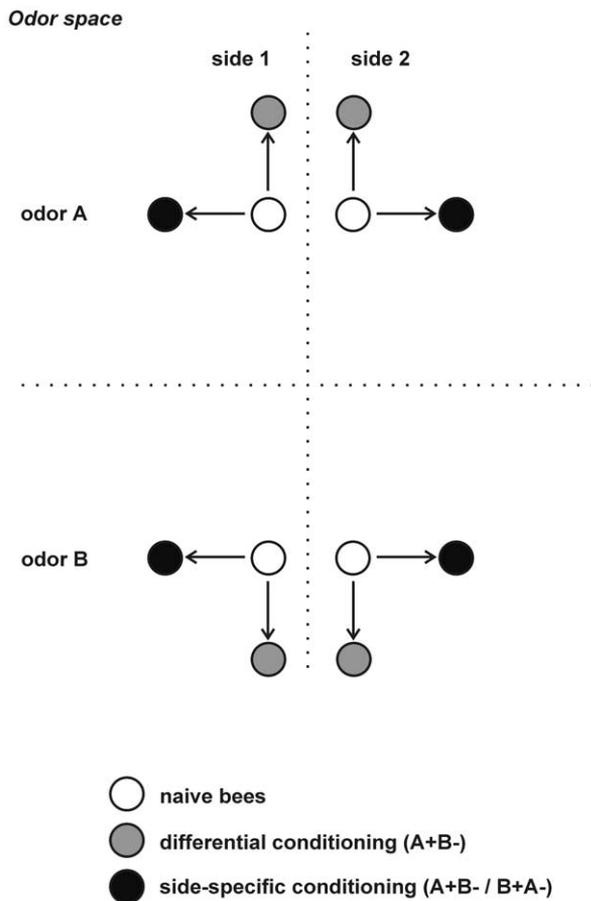
#### Learning-induced changes in minor glomeruli

While major glomeruli did not show modified responses to the CS+ or CS-, overall odor-evoked activity was modified. First, conditioned individuals showed more active glomeruli than naive individuals. This was found not only for the CS+ and CS-, but also for two odorant mixtures (orange and clove oil, which probably contain these individual components). This effect was limited to a number of usually "silent" glomeruli (such as glomeruli T1-23, T1-24, T1-29, T1-37 and T1-62; Sachse et al., 1999; Galizia et al., 1999) which were weakly active after conditioning, while others (such as glomerulus T1-35) remained silent. Second, in conditioned bees, we found an increased number of differences between sides. With two exceptions (glomerulus T1-17 for 1-nonanol, and glomerulus T1-47 for clove oil), these were in minor glomeruli. As discussed above, intracellular calcium dynamics seem unlikely to be still modified 24 h after conditioning. Alternatively, the increased signals in minor glomeruli may be due to the activity from additional cells, in particular from LNs. In our experiments the dye Calcium-Green 2AM was bath-applied so that it had access to all cells of the ALs: receptor

neurons (about 60,000 per lobe), LNs (about 4,000 per lobe), projection neurons (about 800) and glial cells. Receptor neurons contribute most to the signal (Galizia et al., 1998), but a proportion of the signals originates from the other cells. In particular, LN activity may become visible when receptor neuron activity is low, i.e. in minor glomeruli. In the bee AL, two major types of LNs are present: 13% diffuse homogeneously in the AL (homo-LNs), while the rest show a high branching density into one particular glomerulus (hetero-LNs; Flanagan and Mercer, 1989b; Fonta et al., 1993). The observed effects could reflect training-specific increases in the activity of hetero-LNs (possibly through reinforcement from the VUMmx1 neuron as proposed by Linster and Smith, 1997). During training, each brain side was subjected to opposite information. While one side learned that odor A was reinforced (and B not), the contralateral side learned that B was reinforced (and A not). If reinforcing an odor A induced the reinforcement of lateral inhibitory connections between particular glomeruli, and reinforcing an odor B induced the reinforcement of another pattern of lateral inhibitory connections, then between-side differences in glomerular responses would appear, as we found. Furthermore, since hetero-LNs branch in several glomeruli, we would expect previously inactive glomeruli to show weak responses. This associative plasticity would provide the bee with an "interpreted" odor representation, possibly allowing it to recognize meaningful odors more easily. It is possible to test this hypothesis by selectively staining the projection neurons with calcium-sensitive dyes, and evaluate their responses after an A+B-/B+A- training as in the present study. We anticipate that the representation of the learned odors should differ clearly between sides.

#### Changes in odor representation

When representing odor representations in a virtual olfactory space where the response of each glomerulus represents a dimension, we found that the distances of odor vectors between brain sides was modified by learning, as indicated by the selective effect on the two CSs: the representations of 1-hexanol and of limonene were more distant between sides after side-specific conditioning than in naive individuals. We also calculated the distances between CS+ and CS- representations within each side and found no difference between naive and conditioned individuals. Therefore the patterns evoked by the training odors were decorrelated between sides, but within each AL CS+/CS- patterns were not. To represent this result in a schematic way, Fig. 8 presents in an imaginary two-dimensional odor space the representations of two odors A and B on the two brain sides (white circles). In naive bees, the representations of each odor on the two brain sides are very close together, while the representations of odors A and B are wider apart. As shown previously (Faber et al. 1999), simple differential conditioning A+B- decorrelates the representations of A and B (gray circles). Odor representations are thus farther apart after such conditioning than in naive bees. In the present study, we found that after side-specific conditioning, the distance of the representa-



**Fig. 8.** Schematic view of changes in odor representations after differential and side-specific conditioning. This figure presents odor representations in an imaginary two-dimensional odor space. The representations of two odors A and B on the two brain sides are represented by circles, for bees with three different experiences (different shades of gray). The similarity between odor representations is represented by the direct distance between circles. In naive bees, the representations of two odors A and B on the two brain sides are very close together, while the representations of two different odors A and B are further apart. As shown previously (Faber et al. 1999), simple differential conditioning  $A+B-$  decorrelates the representations of A and B, so that A and B representations are farther apart after such conditioning than in naive bees. Since differential conditioning is carried out with bilateral olfactory stimulations, the same process takes place on both brain sides. During side-specific conditioning, honeybees have to give each odor a different value on each side. As shown in the present study, the distance of the representations of each odor A and B increases between sides, but the distance between the two training odors within sides does not change (black circles). These effects could represent two different kinds of processes in the bee brain which are necessary for solving these two different learning tasks.

tions of each odor increased between sides, but no difference appeared on each side in the distance between the two training odors (black circles). We think that the two different learning tasks have different constraints on the brain, and have thus different impacts on odor representations. In differential conditioning, bees have to differentiate between two odors, but there is no ambiguity regarding the meaning of each odor: one is rewarded, the other not. In side-specific conditioning, the situation is different:

this task implies an important ambiguity because each odor has a different meaning on the two sides: it is rewarded when presented on one side, but not on the other. To solve such a task, bees have to rely on the association of each particular odor *with* the input side. We thus think that our result that odor patterns were decorrelated between sides but not within sides reflect the fact that  $A+B-$ / $B+A-$  learning actually relies on the acquisition of side-specific rules of the type  $A+/A-$ . One could argue that this task also implies the differentiation of odors A and B on each side, but since we used odors which are already dissimilar, it is possible that brain processing probably concentrated on side-specific rules. It would be extremely interesting to see whether side-specific conditioning with more similar odors would then induce a decorrelation between the representations of the two odors.

### Role of the mushroom bodies

In the honeybee brain, higher order associations, in particular the organization of olfactory memories into their multisensory context, are thought to take place in the MBs, because it receives processed input from the olfactory, visual and mechanosensory modalities (Menzel, 1999, 2001; Menzel and Giurfa, 2001). Side-odor associations are probably also processed in the MBs: honeybees with a partial MB ablation after hydroxyurea treatment show a deficit in  $A+B-/B+A-$  conditioning (Malun et al., 2002). Such bees can learn a unilateral differential conditioning problem  $A+B-/0$  on the side where the median calyx is missing, but in a  $A+B-/B+A-$  problem, they only learn on the intact side. This would suggest that the learning deficit observed on the ablated side is induced by the presentation of the opposite problem on the contralateral (intact) side. We therefore think that side-specific information which allows bees to respond properly in our  $A+B-/B+A-$  problem is processed at least partially in the calices of the MBs. A feedback loop from the MB to the AL is accomplished by the AL-1 neurons, which project from the MBs and the LPL to the AL, innervating many glomeruli (Rybak and Menzel, 1993), and for which a modulatory function was already proposed (Abel et al., 2001). It is conceivable that synaptic changes in the ALs are under the control of the MBs. Side-specific differences in glomerular responses, as we recorded them, could then reflect asymmetrical descending information from the MBs. Further work is needed to evaluate the interesting possibility that feedback neurons play a role in modifying odor representations during appetitive olfactory conditioning in honey bees.

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