

Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L.

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Abstract

The mushroom bodies (MBs) are central structures in the insect brain that have been associated with olfactory learning and memory. Here we used hydroxyurea (HU) to treat honeybee larvae and induce partial MB ablations at the adult stage. We studied olfactory learning in honeybees with unilateral loss of the median calyces of their MBs and compared their ability to solve different forms of olfactory discrimination. When odorants were delivered in a side-specific manner, ablated bees could not solve either discrimination of the unambiguous problem (Paradigm 1: A+, B– on one antenna, C+, D– on the other; A+B–/C+D–) whereas they could solve at least one of both discriminations of the ambiguous problem (Paradigm 2: A+B–/A–B+), namely that proposed to their intact brain side. Non-ablated bees could learn side-specific discriminations on both brain sides. When odorants were delivered simultaneously to both antennae (Paradigm 3: A+B–C+D–), HU-ablated bees learned slower than HU-normal bees. Thus, in all three paradigms, the unilateral loss of a median calyx affected olfactory learning. We propose that the MBs are required for solving elemental olfactory tasks whose complexity is increased by the number of stimuli involved and that MB ablations could have an effect on the inhibition of information exchange between brain hemispheres.

Introduction

The mushroom bodies (MBs) are prominent structures in the insect brain (Strausfeld *et al.*, 1998) that have been associated with tasks requiring a certain level of behavioral plasticity (Menzel, 1999, 2001; Giurfa, 2003; Heisenberg, 2003). Studies on insect olfactory processing and learning have played a key role in our current thinking regarding MB function (Menzel, 1999, 2001; Heisenberg, 2003).

In honeybees (*Apis mellifera*), the MBs are believed to be centers of multimodal integration as they receive input from olfactory, visual, gustatory and probably mechanosensory pathways (Mobbs, 1982; Gronenberg *et al.*, 1996; Strausfeld *et al.*, 1998; Schröter & Menzel, 2003). They have been traditionally associated with olfactory learning and memory following studies principally using the olfactory conditioning of the proboscis extension reflex (PER; Erber *et al.*, 1980; Hammer & Menzel, 1998; Menzel, 1999, 2001; Menzel & Giurfa, 2001; Giurfa, 2003). In this classical conditioning paradigm (Takeda, 1961; Bitterman *et al.*, 1983), restrained bees learn to associate an odorant (conditioned stimulus or CS) with a reward of sucrose solution delivered to the antennae and the proboscis (unconditioned stimulus or US) such that, after successful conditioning, they extend their proboscis to the mere presentation of the odorant. In this way, a link between CS and US is established. Bees also learn to respond to a rewarded odor (CS+) but not to a non-rewarded odor (CS–) in a differential conditioning, which is based on associations that link one CS with a US and the other CS with the absence of US.

Bees can also learn non-linear, olfactory discriminations (Hellstern *et al.*, 1995; Chandra & Smith, 1998; Deisig *et al.*, 2001, 2002, 2003) in which each odorant appears rewarded as often as non-rewarded, thus creating ambiguity (e.g. negative patterning: A+, B+, AB–, where A and B stand for different odorants). Such forms of learning are termed ‘non-elemental’ in contrast to linear or ‘elemental’ forms in which the outcome of stimuli is non-ambiguous. It has been suggested that the MBs could be of fundamental importance for solving the more complex, non-linear learning problems (Giurfa, 2003; Komischke *et al.*, 2003). In fact, partial, unilateral lesions of the MBs have been found to be without effect on linear forms of learning (Malun *et al.*, 2002a) as bees with such lesions can learn a simple differential conditioning (CS+ vs. CS–) both in the olfactory (Malun *et al.*, 2002a) and in the tactile modality (Scheiner *et al.*, 2001). In olfactory learning, elemental associations can be established between odorants and reward of sucrose at the level of the antennal lobe (Hammer & Menzel, 1998; Farooqui *et al.*, 2003). The explicit role of the MBs for non-linear forms of olfactory learning has not yet been tested directly.

Here, we asked whether partial, unilateral lesions of the MBs affect different forms of olfactory learning in honeybees. We used a treatment in which first-instar larvae were fed with a solution containing hydroxyurea (HU) (Malun, 1998; Malun *et al.*, 2002a,b). HU kills dividing neuroblasts that originate development of the MBs, thus leading to a partial, but not complete, loss of the MBs in adult bees (Malun, 1998). We trained HU-ablated and non-ablated bees with different forms of olfactory discriminations, ambiguous or non-ambiguous. Odorants were delivered simultaneously to both antennae or in a side-specific manner by separating the antennal input with plastic walls between the antennae (Sandoz & Menzel, 2001). Three discrimination problems were given to the bees, one using bilateral odor stimulation of the antennae, and two using side-specific

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stimulations. In the former case, we conditioned bees with an elemental double discrimination (A+, B-, C+, D-). This discrimination was unambiguous with respect to the outcome of each stimulus. In the latter case, we conditioned bees either with a side-specific, elemental double discrimination (A+B-/C+D-, where / stands for the separation between the two antennae) or with a side-specific, non-elemental double discrimination (A+B-/A-B+) (Sandoz & Menzel, 2001). The former double discrimination was unambiguous whereas the latter double discrimination was ambiguous with respect to the outcome of each stimulus (A+A-, B+B-).

If the MBs do indeed play an important role specifically in non-elemental forms of learning, we expect ablated bees to show deficiencies in the non-linear discrimination (side-specific conditioning A+B-/A-B+) but not in the linear discriminations (side-specific A+B-/C+D- and bilateral A+B-C+D-). With respect to the use of olfactory information coming from one or both antennae, we expect that problems involving side-specificity (side-specific conditionings A+B-/C+D- and A+B-/A-B+) will be solved less efficiently than those involving both antennae (bilateral conditioning A+B-C+D-), as we found that honeybees were not able to learn another non-elemental olfactory discrimination, the negative patterning (see above) using only one antenna (Komischke *et al.*, 2003).

Materials and methods

Hydroxyurea treatment

The procedure for generating MB-ablated bees after HU application at the larval stage follows that of Malun (1998) and Malun *et al.* (2002a, b). First-instar larvae were taken out of their combs and placed on a food solution containing 0.5 μ L HU per 100 mL royal jelly. After 4–5 h, the larvae were rinsed in water and placed back into their combs. The combs were then placed back in the hive to ensure the further development of the larvae. One day before the adult animals emerged, the combs were again removed from the hive and placed in an

incubator. On the next day, freshly emerged bees were put into small wire cages (20 cm \times 5 cm \times 1 cm), which were then brought back to the hive. These cages allowed us to isolate HU-treated bees, thus facilitating later recovery. They keep bees within the natural environment of the hive and therefore permit interactions with hive mates. The cages were placed between adjacent combs for 11 days to allow full behavioral development of the bees, which ensures good odor-learning abilities (Laloi *et al.*, 2001). On day 11 of the adult stage, the cages were removed from the comb and placed on ice for 3 min to immobilize the bees. The bees were then fixed in their individual harnesses for experiments using the olfactory conditioning of the PER.

Experimental groups

After finishing experiments we opened the head capsules of the conditioned bees and determined under a stereo microscope the presence or absence of MB lesions. Assessment of the lesions was done blindly with respect to conditioning success. The HU-treated bees were divided into two groups a posteriori of the conditioning experiments: 'HU-normal bees', which despite the HU treatment did not show any MB ablation (Fig. 1B), and 'HU-ablated bees', which presented an ablation of one of the median calyces of the MBs (this is the predominant MB lesion that can be found in 11-day-old bees; see Fig. 1B). In our experiments, bilateral ablations of the two median calyces, which were obtained more often in previous work, appeared only in four cases ($n = 308$). These animals could thus not be taken into account in our analysis (such animals never responded to any CS+ or CS- during conditioning).

Olfactory conditioning experiments

Subjects

Bees were individually harnessed in metal holders so that they could only move their antennae and mouthparts, including the proboscis (Takeda, 1961; Bitterman *et al.*, 1983). They were kept in the dark and

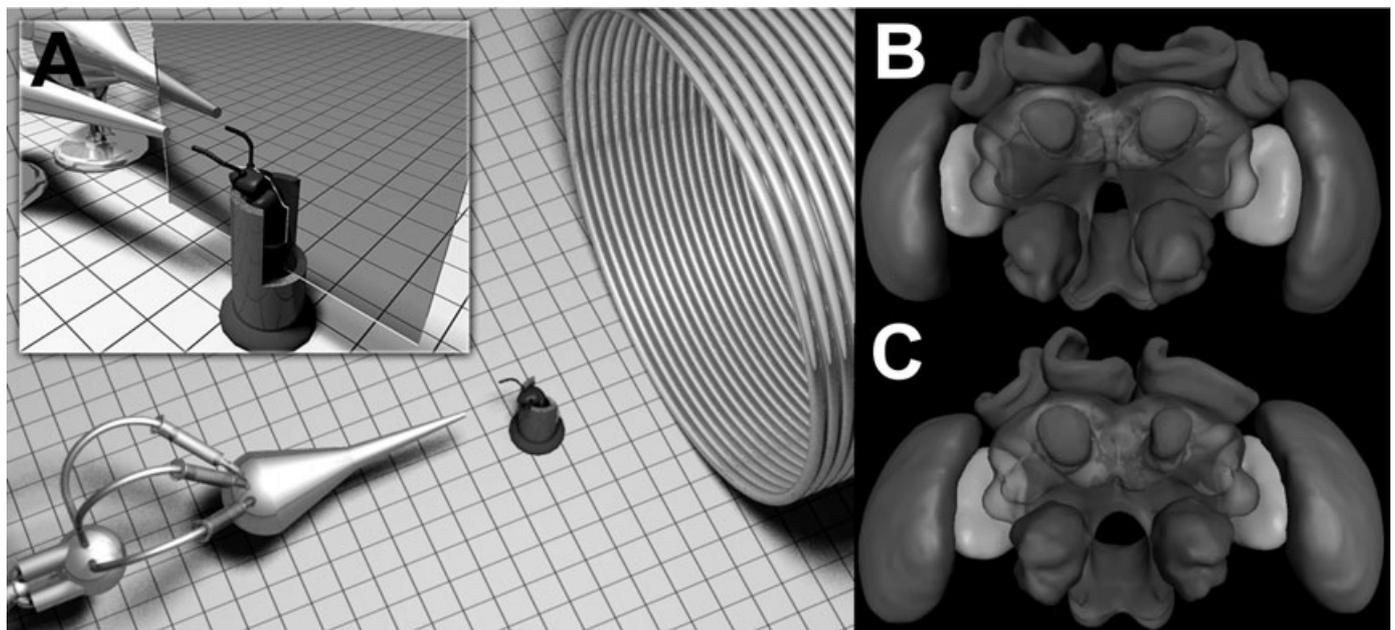


FIG. 1. (A) Bilateral and side-specific odorant delivery to a honeybee. A harnessed bee is placed in front of an odor-supplying device. In case of side-specific conditioning, a separating wall is glued onto the bee head and two devices stimulate both antennae separately (see inset picture). In case of bilateral conditioning, one device delivers the odorants to both antennae simultaneously (see large picture). (B) Three-dimensional reconstruction of an intact honeybee brain. (C) Three-dimensional reconstruction of a honeybee brain showing unilateral loss of the right median calyx after HU treatment (B and C adapted from Malun *et al.*, 2002a).

at high humidity for 3 h. Fifteen minutes before starting the experiments, each subject was checked for intact PER by lightly touching one antenna with a toothpick soaked with 30% sucrose solution without subsequent feeding (US). Extension of the proboscis beyond a virtual line between the open mandibles was counted as PER (UR). Bees that were subjected to side-specific olfactory conditioning and that therefore required separate olfactory inputs to the antennae had a plastic wall (40 × 50 mm) glued exactly between the antennae using low-temperature melting wax (Sandoz & Menzel, 2001) (Fig. 1A).

Unconditioned and conditioned stimuli

The US was 30% (w/w) sucrose solution. As CSs we used the odorants limonene, 2-octanol, heptanal and 2-nonanone (Sigma, Deisenhofen, Germany). These odors can be easily learned and differentiated by bees (Deisig *et al.*, 2001; Komischke *et al.*, 2002). On each experimental day, 4 µL of pure odorant was applied onto a fresh strip of filter paper. The paper strips were then inserted into a 1-mL plastic syringe and mounted in an odor-supplying device delivering a constant flow of clean air provided by a standard aquarium pump. Computer-driven solenoid valves (Lee Company, Essex, CT, USA) controlled airflow delivery. For bilateral odor stimulations, the bee was placed in front of the device and received such a flow. During periods of odorant delivery, the airflow was shunted through a syringe containing the odorant. For side-specific odor stimulations, we used a bilateral stimulation device producing two distinct airflows directed to the bees' antennae on the two sides, and which could provide odor stimulations independently of each other (Komischke *et al.*, 2003). An exhaust system was arranged behind the bees to remove odor-laden air.

Conditioning trials

Each trial lasted 28 s. At the beginning of each trial the subject was placed in front of the odor-supplying device for 15 s to allow familiarization with the training situation. Thereafter the CS was presented for 4 s. In reinforced trials, the US onset occurred 3 s after CS onset. Both antennae were lightly touched with a toothpick soaked with sucrose solution and after proboscis extension the bee was allowed to feed for 3 s. Thus, the interstimulus interval was 3 s and the overlap between CS and US was 1 s. The bee remained in front of the odor-supplying device until completing the 28 s of the trial and then returned to its resting position. Another bee was then placed in the experimental set-up. Such short trials have been used in previous works and did not impair learning of elemental or non-elemental olfactory discriminations (Deisig *et al.*, 2001, 2002, 2003).

Differential conditioning was used in all experiments. Bees had to learn to respond to rewarded odors (henceforth CS+) but not to non-rewarded odors (henceforth CS-). The inter-trial interval (measured between successive CS presentations) was 10 min. CS+ and CS- trials were alternated. In all experiments (see below), bees had four different CSs and received six trials per CS, making a total of 24 trials. Each experiment lasted 240 min.

Response measurement

We recorded whether a bee extended its proboscis after onset of the odor (CS) and before presentation of the sucrose solution (US) in the case of reinforced trials, such that the anticipatory response recorded could only have been evoked by the CS. Multiple responses during a CS were counted as a single PER. After completing each experiment, all animals were again checked for

proboscis extension reflex. Bees that did not show the PER at the end of the experiments were not included in the analyses (7.2%, $n = 302$).

Delivering the CSs separately to each antenna

In these two paradigms, the bees had a separating wall between the two antennae (see above) such that odorants reached each antenna separately.

In the first paradigm (Paradigm 1), bees were trained with four CSs, A, B, C and D, two of which were rewarded (A+, C+) and two were non-rewarded (B-, D-). Side-specificity was established by applying trials with one CS+ and one CS- on each side (A+B- on one side, C+D- on the other side). This means that bees trained in this paradigm had to learn two distinct differential conditionings, one on each side. Each CS was unambiguously associated with a US or with the absence of a US such that the discriminations underlying this problem could be solved elementally.

In the second paradigm (Paradigm 2) bees were trained with two odors A and B. Side-specificity was established by delivering a differential conditioning of opposed contingencies on each side (A+B- on one side, A-B+ on the other side). In this discrimination problem, the reversed contingencies for the same two odorants A and B generated ambiguity at the elemental level.

Delivering the CS to both antennae simultaneously

In this paradigm (Paradigm 3), the bees carried no separating wall between the antennae, so that odorants reached both antennae simultaneously. Bees were trained with four CSs, A, B, C and D, two of which were rewarded (A+, C+) and two were non-rewarded (B-, D-). Bees trained in this paradigm had to learn to respond to A and C but not to B and D. Each CS was unambiguously associated with a US or with the absence of US such that the discriminations underlying this problem could be solved elementally.

Statistical analysis

We measured the percentage of conditioned responses (%PER) in CS+ and CS- trials throughout the experiment. Group performance is reflected in the acquisition curves depicting the percentage of PER along six blocks of trials. Each block consisted of the four possible CS trials, the two CS+ and the two CS- trials. Four types of statistical comparisons were performed on the performances of bees in the four last blocks of trials. The first two blocks of trials were excluded from this analysis as high generalization between CSs occurs at the beginning of differential conditioning and therefore renders these two blocks inadequate for showing successful differentiation.

Comparisons between CS+ and CS- responses

To check whether bees significantly differentiated between CS+ and CS-, we compared the number of responses given by each bee to the two types of stimuli using a Wilcoxon matched-pair test. This test was performed within each antennal side for side-specific conditioning (Paradigms 1 and 2), or over all four stimuli for bilateral conditioning (Paradigm 3).

Comparisons between sides

To test whether bees showed different performances on the two antennal sides (Paradigms 1 and 2), we calculated for each bee, on each side, a differentiation index defined as:

$$I = (R_{CS+} - R_{CS-})/N_{CS+}$$

where R_{CS+} and R_{CS-} were the numbers of responses to the CS+ and to the CS-, respectively, and N_{CS+} was the number of CS+ trials (which is equal to the number of CS- trials). Thus, the index calculated for each pair of CS+/CS- trials how much more (or less) bees responded to the CS+ than to the CS-. The index could take values between -1 (if bees responded all the time to the CS- and never to the CS+, which never happened) and 1 (if bees responded all the time to the CS+ and never to the CS-; best differentiation possible). A value of 0 would be obtained if bees responded as much or as little to the CS+ as to the CS- showing thereby no differentiation. Almost all values obtained were between 0 and 1. We compared this index between antennal sides using a Wilcoxon matched-pair test.

Comparisons between groups

Comparing performance between groups was also done with the differentiation index. When between-side comparisons were not significant (Paradigm 1), we calculated the index over both sides (i.e. eight CS+ and eight CS- trials). When between-side comparisons were significant (Paradigm 2), we used the indices calculated for each side for between-group comparisons. In Paradigm 3, the index was calculated globally for all stimuli (i.e. eight CS+ and eight CS- trials). All comparisons between groups used a Mann-Whitney test.

Comparisons between paradigms

When necessary, we compared the differentiation index of bees in each paradigm by means of a Mann-Whitney test.

Results

Delivering the CSs to each antenna separately

Paradigm 1: elemental side-specific conditioning (A+B-/C+D-)

Figure 2 presents the results of the two groups of bees trained with this paradigm (HU-ablated, HU-normal). The two graphs on the left show

the performance of the HU-ablated bees. On both sides, bees generally responded at a low level (< 20%) to both CS+ and CS-. On the intact side, bees did not discriminate the odors because the responses to CS+ and CS- were not significantly different (Wilcoxon matched-pair test, $Z = 1.83$, n.s.). On the ablated side, bees could also not learn the discrimination (Wilcoxon matched-pair test, $Z = 1.46$, n.s.). There was no statistical difference in performance between sides (Wilcoxon matched-pair test, $Z = 0.63$, n.s.). HU-ablated bees were thus unable to learn any of the two discriminations.

The two graphs on the right (Fig. 2) show the performance of the HU-normal bees. On both sides, responses to the CS+ increased, whereas responses to the CS- remained very low. On the left side, bees discriminated the odors because the responses to CS+ and CS- were significantly different (Wilcoxon matched-pair test, $Z = 4.36$, $P < 0.001$). On the right side, bees also discriminated the odors significantly (Wilcoxon matched-pair test, $Z = 4.72$, $P < 0.001$). Both sides learned with equal success and no statistical differences were found between sides (Wilcoxon matched-pair test, $Z = 0.80$, n.s.). Comparison of performances in the HU-normal and HU-ablated bees yielded a significant outcome (Mann-Whitney test, $Z = 2.01$, $P < 0.05$).

Thus, HU-ablated bees did not learn either of the two differential conditioning tasks whereas HU-normal bees were able to learn. MB lesions impaired learning of an unambiguous, side-specific olfactory discrimination. MB-ablated bees were impaired in this elemental task even on their intact brain side.

Paradigm 2: non-elemental side-specific conditioning (A+B-/A-B+)

Figure 3 presents the results of the two groups of bees trained with this paradigm (HU-ablated, HU-normal). The two graphs on the left show the performance of the HU-ablated bees. HU-ablated bees solved the problem on their intact brain side but not on their ablated side, as shown by the acquisition curves. On the intact side, bees learned to discriminate the odors, responding significantly more to the CS+ than to the CS- (Wilcoxon matched-pair test, $Z = 2.93$, $P < 0.01$), whereas discrimination was not possible on the ablated side (Wilcoxon matched-pair test, $Z = 0.80$, n.s.). As a result, we

Paradigm 1: A+B-/C+D-

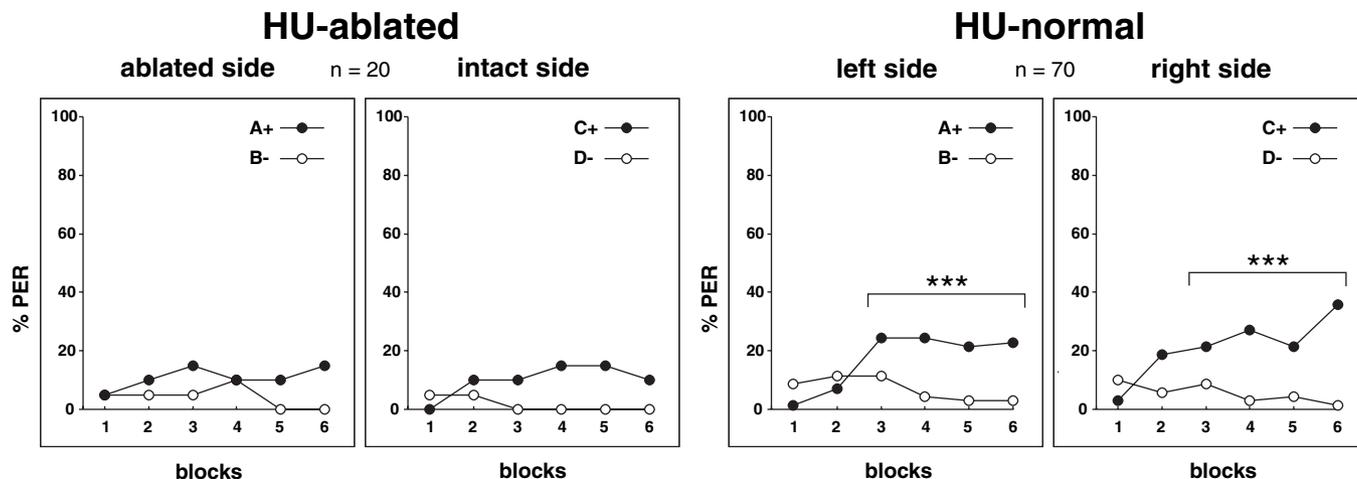


FIG. 2. Results of Paradigm 1 (elemental side-specific conditioning, A+B- on one side, C+D- on the other). Acquisition curves are presented for each brain hemisphere in two experimental groups, HU-ablated and HU-normal. HU-ablated bees learned the discriminations neither on the ablated nor on the intact side. HU-normal bees learned the double discrimination with equal success on both sides. Significantly higher responses to the rewarded odorant (CS+) than to the unrewarded odorant (CS-) are shown on each side by asterisks (Wilcoxon matched-pair test, $***P < 0.001$).

Paradigm 2: A+B-/B+A-

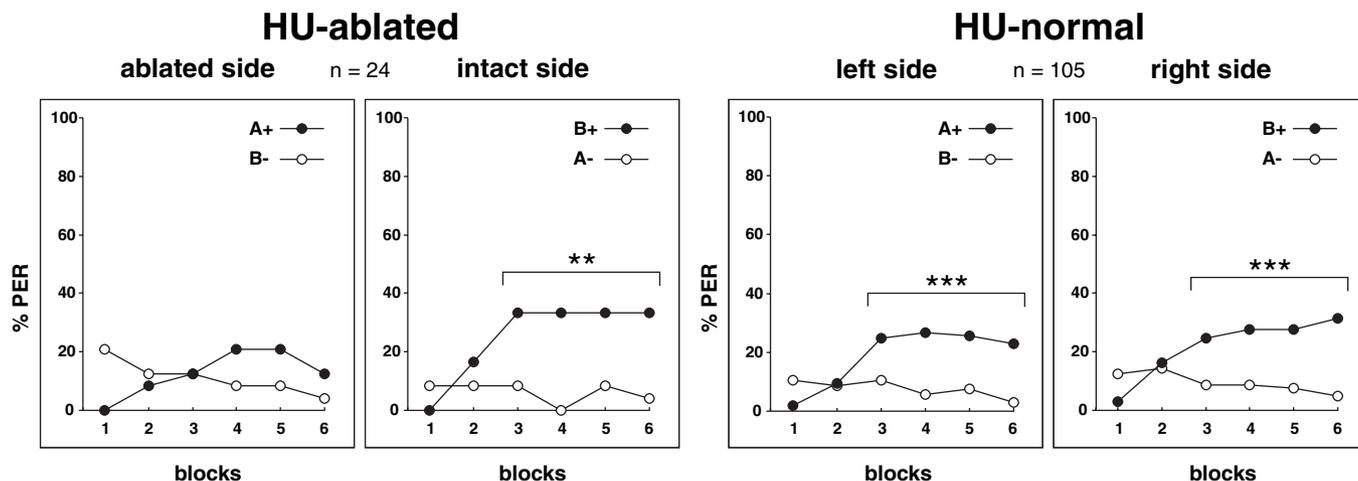


FIG. 3. Results of Paradigm 2 (non-elemental side-specific conditioning, A+B- on one side, B+A- on the other). Acquisition curves are presented for each brain hemisphere in two experimental groups, HU-ablated and HU-normal. HU-ablated bees did not learn the discrimination on their ablated side, but did learn it on their intact side. HU-normal bees learned both discriminations with equal success on both sides. Significantly higher responses to the rewarded odorant (CS+) than to the unrewarded odorant (CS-) are shown on each side by asterisks (Wilcoxon matched-pair test, $**P < 0.01$, $***P < 0.001$).

found statistical differences in learning performance between sides (ablated vs. intact side; Wilcoxon matched-pair test, $Z = 3.04$, $P < 0.01$).

The two graphs on the right (Fig. 3) show the performance of the HU-normal bees. On both sides, responses to the CS+ increased, whereas responses to the CS- remained low. On the left side, bees learned to discriminate the CS+ from the CS- (Wilcoxon matched-pair test, $Z = 5.01$, $P < 0.001$). On the right side, a significant discrimination was also found (Wilcoxon matched-pair test, $Z = 4.94$, $P < 0.001$). Learning success was equal on both sides such that no statistical difference was found between them (left vs. right: Wilcoxon matched-pair test, $Z = 0.52$, n.s.).

Overall comparison of differentiation success between groups (over both brain sides) could not be performed because of the heterogeneity in HU-ablated bees. However, comparing each side of HU-normal bees to the ablated side of HU-ablated bees yielded a significant outcome (HU-normal, left side vs. HU-ablated, ablated side: Mann-Whitney test, $Z = 2.55$, $P < 0.05$; HU-normal, right side vs. HU-ablated, ablated side: Mann-Whitney test, $Z = 2.61$, $P < 0.01$), whereas both comparisons with the intact side of HU-ablated bees were non-significant (HU-normal, left side vs. HU-ablated, intact side: Mann-Whitney test, $Z = 0.98$, n.s.; HU-normal, right side vs. HU-ablated, intact side: Mann-Whitney test, $Z = 1.14$, n.s.). This paradigm therefore shows a clear-cut effect of unilateral MB lesions,

Paradigm 3: A+B-C+D-

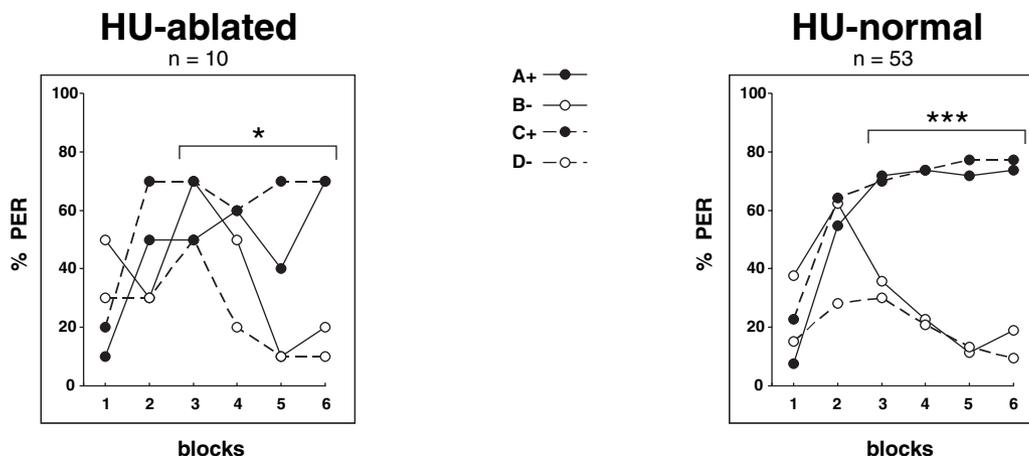


FIG. 4. Results of Paradigm 3 (elemental differential conditioning with four odors A+, B-, C+, D-). Acquisition curves are presented in two experimental groups, HU-ablated and HU-normal. Both groups learned to discriminate the olfactory stimuli at the end of the training procedure but HU-ablated bees were slower than HU-normal bees. Significantly higher responses to the rewarded odorant (CS+) than to the unrewarded odorant (CS-) are shown on each side by asterisks (Wilcoxon matched-pair test, $*P < 0.05$, $***P < 0.001$).

TABLE 1. Discrimination index calculated from the data of each experimental group

Paradigm	Discrimination index HU-ablated				Discrimination index HU-normal			
	Ablated side	Intact side	BS comparison	Bilateral conditioning	Right side	Left side	BS comparison	Bilateral conditioning
1	0.087 ± 0.061	0.125 ± 0.069	n.s.	–	0.221 ± 0.038	0.179 ± 0.036	n.s.	–
2	–0.021 ± 0.060	0.281 ± 0.077	<i>P</i> < 0.05	–	0.205 ± 0.036	0.183 ± 0.032	n.s.	–
3	–	–	–	0.312 ± 0.077	–	–	–	0.533 ± 0.041

Discrimination index presented with ± SEM For Paradigms 1 and 2 involving side-specific conditioning, an index value was calculated for each side (ablated and intact side, or right and left side). The 'BS' column after each group indicates between-side comparisons with a Wilcoxon matched-pair test (see text for details). For Paradigm 3, only one index value appears because this paradigm used bilateral conditioning. No between-side comparison could be performed.

namely that the ablated side performs worse in learning an A+ vs. B– discrimination than the intact side, which receives reciprocal training (A– vs. B+).

In summary, the comparison of the results of Paradigms 1 and 2 suggests that an ambiguous side-specific conditioning with two odors (Paradigm 2) may be easier to solve than an elemental one with four odors (Paradigm 1; compare Figs 2 and 3), which appears counter-intuitive on first inspection. This suggestion is based on the fact that HU-ablated bees were successful on the intact side in the ambiguous problem (Fig. 3) whereas they were unsuccessful on the same side in the non-ambiguous problem (Fig. 2). No difference, however, was found between Paradigms 1 and 2 in the case of HU-normal bees (Mann–Whitney test, *Z* = 0.63, n.s.), thus restricting the conclusion on the difference in the difficulty of Paradigms 1 and 2 to the case of HU-ablated bees.

Delivering the CS simultaneously to both antennae

Paradigm 3: elemental differential conditioning with four odors (A+B–C+D–)

Figure 4 presents the results of the two groups of bees trained with this paradigm (HU-ablated, HU-normal). The left graph shows the performance of the HU-ablated bees. Ablated bees solved the problem and learned to respond to A+ and C+ but not to B– and D– (Wilcoxon matched-pair test, *Z* = 2.49, *P* < 0.05). The right graph (Fig. 4) shows the performance of the HU-normal bees. These bees also learned to discriminate the two CSs+ from the two CSs– (Wilcoxon matched-pair test, *Z* = 5.90, *P* < 0.001). The comparison of the differentiation index between the HU-normal and HU-ablated bees was significant (Mann–Whitney test, *Z* = 2.34, *P* < 0.05; Table 1). Therefore, despite the fact that both groups achieved the discrimination at the end of training, HU-ablated bees achieved differentiation more slowly than HU-normal bees (see performance in the third and fourth blocks of trials in the two groups).

Discussion

Our results show that in all three paradigms, the unilateral loss of a median calyx affects olfactory learning. When odorants were delivered in a side-specific manner, ablated bees could not solve either discrimination of the unambiguous problem (Paradigm 1: A+, B– on one antenna, C+, D– on the other; A+B–/C+D–) whereas they could solve at least one of the two discriminations of the ambiguous problem (Paradigm 2: A+B–/A–B+), namely that proposed to their intact brain side. When odorants were delivered simultaneously to both antennae (Paradigm 3: A+B–C+D–), all bees learned, but HU-ablated bees learned more slowly than HU-normal bees.

The fact that MB-ablated bees trained in a side-specific manner with an elemental double discrimination (Paradigm 1: A+B–/C+D–) could not solve either discrimination was surprising as our previous work (Malun *et al.*, 2002a) showed that unilateral MB lesions did not affect the capacity to solve an elemental discrimination A+ vs. B– presented to the ablated side. In contrast to the present study, Malun *et al.* (2002a) did not use a separating wall but cut one antenna to deliver the odors in a side-specific manner to the remaining antenna. Owing to this procedural difference the work of Malun *et al.* (2002a) and our work cannot be directly compared. Nevertheless, the apparent contradiction between the results of Malun *et al.* (2002a) and our work could be explained if one assumes that gluing a wall on the head of the bee to separate the antennae is a stressful procedure and that the effect of MB lesions only shows up under such a stressful condition. However, this argument is improbable as foraging bees – not treated with HU – bearing such separating walls exhibit higher acquisition rates in double discriminations (A+B–/B+A–; Sandoz & Menzel, 2001; Sandoz *et al.*, 2003) than in the present study with bees that were not allowed to leave the hive. Moreover, the effect of HU lesions could also be detected in bees without separating walls and also trained with an elemental discrimination (Paradigm 3: A+B–C+D–).

Thus additional factors have to be found to account fully for the effect of HU ablations on elemental and non-elemental learning. In fact, our results can be analysed in the light of four main factors that could have influenced the bees' performance: (1) the number of conditioned odorants, (2) elemental vs. non-elemental discriminations, (3) crosstalk and information exchange between brain hemispheres and (4) side-specific vs. bilateral olfactory stimulation.

Number of conditioned odorants

Differences in performance between paradigms might arise from the amount of differential information that bees had to process. In other words, discriminations involving two odorants could be easier than those involving four odorants. This argument could explain why bees with unilateral MB lesions could solve the elemental discrimination A+ vs. B– (two odorants) presented to the ablated side (Malun *et al.*, 2002a) but could not solve an elemental double discrimination (Paradigm 1: A+B–/C+D–; four odorants).

Comparing Paradigms 1 (A+B–/C+D–: four odorants) and 2 (A+B–/A–B+: two odorants) seems to support this hypothesis as discrimination was better in Paradigm 2 than in Paradigm 1 (see performance of HU-ablated bees). However, it is not clear that in Paradigm 2 (A+B–/A–B+), the bees processed A and B only as two odors. As these odors had different contingencies on each side, we think that bees have to suppress information transfer between sides (see below) and to build separate representations of these odorants on each side, resulting in a situation in which the brain would have to

cope in fact with four different representations, i.e. four different stimuli. Thirdly, in Paradigm 3 (A+B–C+D–), in which there was no separating wall between antennae, ablated bees were trained with four odorants and could solve the task, although they were significantly slower than normal bees and were thus affected by the loss of one MB calyx. This not only indicates that the number of stimuli involved in the discrimination may determine whether MBs are solicited, but also indicates the necessity of achieving separate, unambiguous stimulus representations on each brain hemisphere. As indicated above, this effect may appear more clearly when bees carry a separating wall between the antennae.

Further experiments should be performed to compare the performance of different groups of bees conditioned with increasing numbers of odorants. To this end, stimuli should be delivered bilaterally to avoid the co-factor of side-specificity. In doing this, generalization between odorants should be kept to a minimum, which is possible by using perceptually distinct odorants. We thus propose that MBs may be required for solving elemental olfactory tasks whose complexity is enhanced by virtue of the number of stimuli involved.

Elemental vs. non-elemental discriminations

We assumed that the ambiguity underlying non-elemental discriminations could be an important factor determining the involvement of the MBs (Giurfa, 2003). We conjectured that ablated bees should show deficiencies in the non-elemental discrimination (Paradigm 2: A+B–/A–B+), which generated ambiguity at the level of the elements, but not in the elemental discriminations (Paradigm 1: A+B–/C+ D– and Paradigm 3: A+B–C+D–), which did not generate such ambiguity. Comparing Paradigms 1 and 3 (elemental) vs. 2 (non-elemental) introduces, however, a confounding factor that should be eliminated to appreciate the impact of the linearity of the discrimination task under study. This factor is the side-specificity of stimulus delivery, which is present in Paradigms 1 and 2 but not in Paradigm 3. In order to focus on the effect of linearity, we thus focus on a comparison between Paradigms 1 and 2, which are both side-specific.

In Paradigm 1 (A+B–/C+D–), bees had to learn an elemental discrimination whereas in Paradigm 2 (A+B–/A–B+), they had to learn a non-elemental, side-specific discrimination in which each odorant was rewarded as often as non-rewarded. We found an MB-lesion-specific effect in Paradigm 2, given that the performance of the HU-ablated bees was different between the intact and ablated side, the ablated side being deficient for solving the discrimination. Regardless, HU-ablated bees in Paradigm 2 solved at least one of the two discriminations. This was not the case in Paradigm 1, in which HU-ablated bees could not learn any of the side-specific discriminations. These results were counterintuitive to the suggestion that the MBs may contribute essentially to non-elemental forms of learning (Giurfa, 2003; Komischke *et al.*, 2003). From this perspective, the HU-ablated bees should have been more impaired in Paradigm 2 than in Paradigm 1. The opposite was the case. As mentioned above, the number of stimuli involved in these two paradigms (Paradigm 1: four odorants; Paradigm 2: two odorants) may have been a determining factor underlying this difference.

Crosstalk and information exchange between brain hemispheres

Malun *et al.* (2002a) showed that a single A+ vs. B– olfactory discrimination can be learned in a side-specific manner, even on the ablated side. Thus, the fact that bees were incapable of learning an A+

vs. B– olfactory discrimination on the ablated side both in Paradigms 1 and 2 could be related to the presence of an additional discrimination on the other side (C+ vs. D– in Paradigm 1 and A– vs. B+ in Paradigm 2). Crosstalk and information exchange occurs between brain sides and MBs may play a prominent role in this communication (Mobbs, 1982). Bees trained in a side-specific manner transfer the learned olfactory information to the contralateral brain hemisphere and react to the conditioned odor when presented to the contralateral antenna at least 3 h after conditioning (Erber *et al.*, 1980; Sandoz & Menzel, 2001). In addition, to be able to solve particular olfactory learning tasks, bees need input to the two brain sides (Thorn & Smith, 1997; Komischke *et al.*, 2003). We suggest that this transfer of stored information between the brain sides, which may or may not occur depending on the kind of information stored, was disturbed by MB lesions.

In the non-elemental, side-specific olfactory conditioning (Paradigm 2: A+B–/A–B+), normal, adult bees learn to respond appropriately to each odorant on its correct side (Sandoz & Menzel, 2001; Sandoz *et al.*, 2003). Bees trained in this paradigm and tested 24 h later still respond appropriately, thus showing that they have access to a mechanism that allows avoiding confusion between sides and keeping the representations of the odorants distinct and separate. This can be achieved if the transfer mechanism between brain sides mentioned above is actively inhibited during this task. In Paradigm 2, transferring information between sides may impair appropriate odor discrimination, as each side would end up with four representations A+ A– B+ B–. We therefore suggest that whenever contradictory information between the two sides is detected (i.e. A+_{left} A–_{right}; B–_{left} B+_{right}), the transfer between sides is inhibited. As a result, each side would end up with two unconnected representations: A+ B–, in one case, and A– B+ in the other case. The inhibition of crosstalk between sides would force the bees to build four separate representations, which would facilitate discrimination on both sides. MB ablations could have an effect on this inhibition process such that the ablated side cannot block the transfer from the intact side. As a result, HU-ablated bees would be left with a single discrimination on the intact side (say, A+ B–) but with a double, ambiguous discrimination on the ablated side (A+ A– B+ B–). Under these circumstances, bees should achieve the discrimination on the intact side but not on the ablated side. This is exactly what we found in Paradigm 2.

Another explanation would argue that transfer between sides is not the critical factor to consider but that the representations on the intact side (say, A+ B–) are more salient than those in the ablated side (say, A– B+). If in Paradigm 2 transfer between sides occurs despite contradictory information, bees would be left on each side with representations of the type A+ A– B– B+. They would therefore focus on the more salient discrimination A+ B–. This possibility implies that bees would always respond correctly on the intact side and would always respond wrongly on the ablated side. But in both cases, bees would respond differentially to odorants A and B. This was, however, not the case as bees in Paradigm 2 did not discriminate the odorants on the ablated side. Thus, we conclude that the lesions at the level of the MBs essentially affected the normal transfer of stored information between MBs and that such transfer may or may not occur in normal circumstances depending on the information stored in each brain side.

In Paradigm 1, bees were trained with A+B– on one side and C+D– on the other side. We have proposed that transfer between sides is impaired or inhibited when the side-specific information is contradictory, which is not the case in Paradigm 1. Accordingly, transfer should not be affected and each brain side would end up with four representations A+ B– C+ D–, similar to what happens in Paradigm 3 where the same four odorants with the same contingencies were conditioned bilaterally. Thus, the

impossibility of learning both side-specific discriminations in HU-ablated bees trained in Paradigm 1 may be related to the number of stimuli involved in the double discrimination (see above).

More experiments are necessary to test the mechanistic basis of information transfer between hemispheres in the bee brain. One possible way to test it is the use of a reversible blocking procedure of MB activity in a side-specific manner. In contrast to lesions, selective, reversible blocking has the advantage of showing whether distortion of the transfer between brain sides can be restored when the effect of blocking is no longer present. Reversible blocking of MB function can be achieved using local anesthetics (Müller *et al.*, 2003) or TTX (Kilpatrick & Cahill, 2003) in a side-specific manner. The experiments mentioned, which are currently in progress, will allow us to clarify the hypotheses mentioned above.

Side-specific vs. bilateral olfactory stimulation

In order to focus on the effect of side-specific vs. bilateral stimulation, we focus on the comparison between Paradigms 1 (A+B-/C+D-) and 3 (A+B-C+D-), which involved the same odorants A, B, C and D, with the same contingencies but with different spatial distribution (and the fact that in Paradigm 1 bees carried separating walls). HU-ablated bees could solve Paradigm 3 (although more slowly than normal bees) but not Paradigm 1 despite the common factors mentioned above. Consistent with previous results (Thorn & Smith, 1997; Komischke *et al.*, 2003), bilateral delivery of olfactory stimulation allowed here better discrimination performances. Our previous work (Komischke *et al.*, 2003) led us to the conclusion that bilateral olfactory input was necessary to solve a non-elemental discrimination such as the negative patterning (A+, B+, AB-; see Introduction). The present results allow us to extend this conclusion to elemental discriminations such as those involved in Paradigms 1 and 3.

Neural correlate of information exchange between MBs

In the honeybee brain, odor processing involves different stages and is symmetrical between sides. Axons of the chemoreceptors on each antenna project to the 160 glomeruli of each antennal lobe, the primary olfactory center, where they synapse with about 4000 local interneurons and about 800 projection neurons (Mobbs, 1982; Arnold *et al.*, 1985; Abel *et al.*, 2001). Projection neurons convey information to higher brain centers, the MBs and the lateral protocerebral lobes (Abel *et al.*, 2001; Mobbs, 1982). At the anatomical level, the olfactory pathways of the two brain sides are mainly connected at the level of the output of the MBs, the α -lobes and the ring neuropils around them, although a few connecting neurons between antennal lobes have also been reported (Mobbs, 1982). Placed at an intensive information crossway in the bee brain, each α -lobe and the surrounding ring neuropil receive information directly from the antennal lobe (ml-ACT tract, Abel *et al.*, 2001), as well as processed information from the MB calyces ipsilaterally and indirectly from the contralateral α -lobe (Rybak & Menzel, 1993; Abel *et al.*, 2001). The tracts connecting both MBs could therefore allow the transfer of information between brain sides and allow the joint activity of the two brain sides. The effect of MB ablations on such connecting tracts is still unknown, although we believe that, as the medial calyces are usually missing in ablated bees and the volume of the α -lobes is strongly reduced on the ablated side (Malun *et al.*, 2002b), developmental deficiencies may appear at this level. Indeed, previous work evaluated the anatomical impact of median calyx ablations (Malun *et al.*, 2002a,b) and made two important observations. First, ablation of such a central brain structure

strongly influences overall brain wiring. Although no volumetric or odor-evoked activity differences have been found at the antennal lobe level (Malun *et al.*, 2002a), the wiring pattern of projection neurons leaving the antennal lobe to relay information to the MBs was clearly modified. Such wiring changes are to be expected in the case of connecting neurons. Indeed, in some bilateral ablations of median calyces, the β -lobes on both sides are fused together, with Kenyon cells projecting to the contralateral brain side (Malun *et al.*, 2002b). We thus believe that inter-hemispheric neuronal wiring is disturbed in ablated bees. Secondly, determination of the amount of proteins involved in neural plasticity, learning and memory on the two brain sides of bees with a median calyx missing showed increased protein levels on the intact MB side, protein levels on the ablated side being similar to those of control animals (Malun *et al.*, 2002b). This means that during development, compensation processes appear to take place, which can have important processing consequences not only on the ablated side, but also on the intact side (Malun *et al.*, 2002b). Therefore, we believe that learning deficits, which can be either limited to the ablated side (Paradigm 2), or can affect both brain sides (Paradigm 1), can be explained on the basis of such unilateral and bilateral anatomical deficiency. A more detailed analysis of possible modifications in the wiring of MB extrinsic neurons, in particular of neurons connecting the two α -lobes, would be required to understand such effects better.

In summary, our data show that both MBs of the bee brain seem to work as a functional unit with respect to olfactory learning. A unilateral impairment caused by MB lesions affected all learning paradigms assayed, non-elemental and elemental. Elemental paradigms whose complexity is enhanced by increasing the number of stimuli involved in the discriminations may also solicit the MBs. Separate processing between brain sides can only be achieved in special cases in which the spatial separation of olfactory information has to be achieved as a part of the learning problem. In this case each brain hemisphere forms its own, exclusive memory by using only information from its ipsilateral antenna and by blocking information transfer from the contralateral brain hemisphere.

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Abbreviations

CS, conditioned stimulus; HU, hydroxyurea; MB, mushroom body; PER, proboscis extension reflex; US, unconditioned stimulus.

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