Learning in honey bees with brain lesions: how partial mushroom-body ablations affect sucrose responsiveness and tactile antennal learning

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Abstract Honey bees are ideal organisms for studying associative learning, because they can rapidly learn different sensory cues, even under laboratory conditions. Classical olfactory learning experiments have shown that the mushroom bodies (MBs), a prominent neuropil of the central nervous system of the bee, are involved in olfactory learning and memory formation. We tested whether the MBs are also involved in tactile antennal learning. As in olfactory learning, bees use the antennae during tactile learning to sense tactile cues. We produced specific MB ablations by applying the mitotic blocker hydroxyurea (HU). In Drosophila, HU-induced brain lesions of the MBs strongly impaired olfactory memory formation. As treatment with HU might also interfere with the processing of the reward stimulus, sucrose, we measured the responsiveness to sucrose stimuli in these bees. Treatment with HU led to partial ablations of the median MB subunits on one or both sides of the brain. We analysed side-specific effects in double-blind tests, testing sucrose responsiveness separately for each antenna, and conditioning first one antenna and then the other in a reversal learning assay. HU-treated bees without detectable ablations were less responsive to sucrose and had a poorer learning performance than untreated controls. Partial MB ablation did not additionally affect responsiveness to sucrose or tactile antennal learning. Interestingly, bees with bilateral MB ablations did not differ from untreated controls in their learning performance during the first learning phase. During reversal learning, acquisition in these bees was significantly lower than that in untreated controls. It is concluded that HU treatment affects sucrose responsiveness and tactile learning even without detectable ablation of neuropils. The effects of MB ablations on tactile learning are not side-specific and not correlated with the volume of the ablated neuropil.

Key words Apis mellifera · Mushroom bodies · Chemical ablation · Sucrose reward · Learning

Introduction

Honey bees (Apis mellifera) are particularly useful animals for the study of learning and memory formation, because they can learn different stimuli very fast. For many bees, a single association of an odour with a sucrose reward will lead to a memory lasting for days (Menzel 1999). Three pairings of an odour and a reward lead to a lifelong memory (Menzel 1999). Vertebrates need many more associations of an unconditioned stimulus with a conditioned stimulus to form a reliable memory (Pearce 1997). In addition, the brain structure of honey bees is less complex than that of a vertebrate brain. Important brain structures of the honey bees are the mushroom bodies (MBs) or corpora pedunculata, which were first described in detail by Dujardin (1850). The MBs are important centres for the processing of olfactory signals and for the formation of olfactory memory (Erber et al. 1987; Menzel et al. 1994). In honey bees, surgical removal of MBs after classical conditioning to odours results in a complete loss of conditioned responses to these stimuli (Voskresenskaya 1957). Reversible local cooling of MB sub-units impairs olfactory memory formation (Menzel et al. 1974; Erber et al. 1980). Injection of octopamine into the MBs facilitates memory storage and retrieval (Menzel et al. 1994). In addition, octopamine injections into the MBs can substitute for the sucrose reward in olfactory conditioning (Hammer and Menzel 1998). In the fruitfly, Drosophila melanogaster, mutants with defective MBs or flies whose MBs were chemically ablated show disruption of associative olfactory learning (Heisenberg et al. 1985;
de Belle and Heisenberg 1994; Heisenberg 1998). Although it is known that different sub-compartments of the MBs receive inputs from different sensory systems (Mobs 1985), the specific function of these sub-units in learning and memory is not yet understood.

We studied the effects of ablations of entire MB sub-units on associative tactile antennal learning in honey bees. The advantages of ablations are many and diverse. Individuals can be tested over an extended period and various behavioural assays can be used even in the same individuals. As ablations of MBs are not reversible, there is no time dependence of the treatment. This is a great advantage compared to the pharmacological application of substances that interfere with signal processing in the MBs, which are only effective for a certain period of time. The irreversibility of ablations, on the other hand, makes it necessary to compare different groups of treated and untreated animals and bees with different forms of ablations. In addition, chemical ablation performed early during development may lead to compensatory effects in the structure and function of the adult brain.

In contrast to side-specific olfactory learning, which usually involves damage to either antenna, tactile learning offers the opportunity to test the two antennae separately without damaging the insect. Bees can learn to associate an object within the range of one antenna with a reward. After acquisition, the bees discriminate an object presented to the conditioned side from an object presented to the opposite side (Erber et al. 1998).

Tactile learning depends on the operant activity of the bee during antennal scanning of an object. This is an important difference from classical conditioning using olfactory stimuli. As the MBs are probably involved in the generation of complex motor activities, operant tactile learning is a particularly sensitive assay to test whether there are asymmetries between the intact side and the side with partial MB ablations.

Appetitive learning in bees depends on responsiveness to sucrose (Scheiner et al. 1999, in press), which can be measured using the proboscis extension response (PER). Side-specific effects of treatment with the mitotic blocker hydroxyurea (HU) on the bees' responsiveness to sucrose can hence influence side-specific learning on either antenna. We therefore tested all bees for their responses to different sucrose concentrations side-specifically prior to conditioning. 1

Methods

Preparation of the bees

Treatment with HU was performed according to Malun (1998). First-instar larvae were removed from the hive and placed for 3-5 h in microwells containing 10 μl of HU solution (0.5 mg HU in 1 ml of a solution of royal jelly and water). Control animals were incubated in microwells containing a mixture of royal jelly and distilled water. The microwells were kept in a humid chamber at 33°C. The larvae were then rinsed with distilled water and returned to the hive for further development. Shortly before eclosion, pupae were removed from the frames and kept in small cages until they emerged. Adult bees were placed in small grated cages and remained in the hive for 5-7 days until the behavioural experiments started.

Behavioural assays

The experiments were designed as a double-blind test. Individual bees were coded, and the person doing the behavioural assays was ignorant of whether a bee had been treated with HU or not. The occurrence of ablations was determined after the learning assays by another person who did not know the results of the behavioural experiments.

For the behavioural assays bees were placed individually in small glass vials and cooled in a refrigerator maintained at 4°C until they showed the first signs of immobility. Then they were placed in small metal holders, with strips of adhesive tape attached between head and thorax and over the abdomen. The compound eyes of each bee were occluded with black acrylic paint (Lukas) to eliminate visual feedback (Erber et al. 1997). Bees were fed with c. 3 μl of 30% sucrose solution. Experiments started 1 h later.

Responsive to sucrose was measured by applying sucrose solutions in concentrations of 0.1%, 1%, 10% and 30% (weight/volume) separately to each antenna in ascending order. Prior to presentation of sucrose solutions of each concentration, we offered pure water to the antennae to test for sensitization effects. For the learning experiments we selected HU-treated bees and untreated controls which displayed a similar responsiveness to sucrose, thereby excluding learning differences based on differences in responsiveness to sucrose (Scheiner et al. 1999, in press). For conditioning, each bee was placed between two micromanipulators, which were selectively used to position a small rectangular metal plate (3 x 4 mm, smooth surface) into the range of one antenna. The few bees responding spontaneously to the tactile stimulus were discarded. During conditioning trials, the plate was brought into the range of one antenna, and the bee was allowed to scan it for 3 s before proboscis extension was elicited by applying sucrose solution to the same antenna. While scanning the plate, the bees were allowed to suck about 2 μl of the 30% sucrose solution. If a drop of sucrose solution accidentally touched the plate, it was cleaned with 70% propanol and water to exclude olfactory or gustatory cues. We first conditioned all bees using the left antenna eight times. Directly after the acquisition phase we measured extinction of conditioned responses and responses on the contralateral antenna in five unrewarded tests for each position in quasi-random order. Subsequently, bees were conditioned on the right antenna eight times. Extinction of conditioned responses on the right antenna and responses on the left antenna were tested as before. The inter-trial interval was 5 min throughout the experiment. Only full extension of the proboscis was counted as a positive response.

Histological examination

After the learning experiments, the brains of tested bees were dissected from the head capsule and checked for MB ablations in whole-mount preparations. To analyse HU-induced morphological changes in detail, neuropil areas of some of the brains were stained with synapsin antibodies (Reichmuth et al. 1995; Klagges et al. 1996). We used this method to detect the various types of MB ablations and to document these ablations in whole-mount preparations. Moreover, this method yields quantifiable results on volume changes of neuropils. Brains were fixed in 4% formalin in phosphate-buffered saline (0.1M PBS) overnight at 4°C, rinsed several times in PBST (0.1M PBS plus 1% Triton) for 6 h, pre-incubated in PBST plus 10% normal goat serum for 1 h and transferred into primary antiserum (antibodies against the synapsin proteins SYNPORF1 and ne46 were kindly provided by A. Hofbauer, Regens-

1 Some of the data have been published in abstract form (Malun et al. 2000; Scheiner et al. 2000).
burg, Germany and E. Buchner, Würzburg, Germany). A cocktail of both antisera with a final dilution of 1:30 was used. Incubation in primary antibody at room temperature lasted for 2 days. Subsequently, brain was thoroughly rinsed in PBST and incubated in secondary antibody overnight at room temperature (goat anti-mouse serum conjugated with Cy3, Dianova, final dilution 1:400). After rinsing in PBS, the brains were dehydrated in a graded series of ethanol concentrations, cleared in methyl salicylate and embedded in Permount (Fisher Scientific). Specimens were examined as whole-mount preparations with a laser scanning microscope (Leica TCS-4D) equipped with a Leitz microscope (DM RBE) and a krypton/argon laser light source. To visualise the fluorochrome Cy3 the rhodamine filter (excitation wavelength 568 nm) was used. Optical sections were taken every 4 μm throughout the brain. For documentation of the two types of MB ablation (one or both median MB sub-units ablated) and for comparison with the MB morphology of control animals, one optical section per brain was taken at the approximate depth of 250 μm as seen from the anterior surface of the brain (Fig. 1).

**Statistics**

**Gustatory response scores**

Responsiveness to water and sucrose stimuli was quantified by counting how many times each bee responded to the first water stimulation plus the different sucrose concentrations offered to each antenna. The number of responses represents the gustatory response score for one antenna of a single bee. The response scores ranged from 0 to 5 for each antenna and were compared between groups using a two-tailed Mann-Whitney U-test.

**Acquisition and extinction score.**

To analyse the learning performance of different experimental groups, we counted for each bee how many times it responded with the conditioned PER in the trials of the conditioning phases, in the unrewarded test trials involving the conditioned side, and in the tests involving the contralateral antenna. These values represent the acquisition scores and the extinction scores, respectively. Acquisition scores of tactile antennal learning in the first acquisition phase using the left antenna and those in the second acquisition phase using the right antenna ranged from 0 to 8. Extinction scores using either antenna ranged from 0 to 5. The frequency distributions of acquisition and extinction scores were statistically compared between different groups using a two-tailed Kolmogorov-Smirnov test. Acquisition scores of the two learning phases were compared within groups using a two-tailed Wilcoxon test.

**Results**

**Effects of HU treatment**

In our experiments, treatment of first-instar larvae with HU mainly resulted in three types of ablations: left median MB sub-unit ablated (HU\textsubscript{left median} n=47, Fig. 1B), right median MB sub-unit ablated (HU\textsubscript{right median} n=70), and both median MB sub-units ablated (HU\textsubscript{both median} n=41, Fig. 1C). In 267 bees, HU treatment did not lead to ablation of MB sub-units (HU\textsubscript{no ablation}). Untreated control bees (n=385, Fig. 1A) were used for comparisons.

Interestingly, in groups with one median MB sub-unit ablated, the lateral calyx on the ablated brain side had increased in volume. The entire MB on the contralateral hemisphere appeared to be unchanged. In bees with both
median MB sub-units ablated, only the lateral MB sub-units were present.

Responsiveness to sucrose

The mean gustatory response scores of controls and the different HU-treated groups are shown for both antennae separately in Fig. 2. Treatment with HU reduced responsiveness to sucrose irrespective of ablation effects. HU-treated bees without any ablations had significantly lower gustatory response scores than untreated control bees (right antenna: \( r = 5.360, P < 0.001 \); left antenna: \( r = 4.181, P < 0.001 \); \( n_{\text{control}} = 385 \), \( n_{\text{HU no ablation}} = 267 \); two-tailed Mann–Whitney \( U \)-test). Partial MB ablation had no additional effect on gustatory response scores. Regardless of whether one median sub-unit was ablated or both median sub-units were ablated, the groups did not differ in their gustatory response scores from HU-treated bees without ablations or from each other.

Tactile acquisition involving left antenna

We conditioned HU-treated bees and untreated controls with similar gustatory response scores to eliminate possible learning differences due to differences in responsiveness to sucrose (Scheiner et al. 1999, in press). The frequency distributions of acquisition scores in the first conditioning phase involving the left antenna (Fig. 3) were significantly different between the control group and most HU-treated groups (Table 1). Learning performance in most HU-treated groups was poorer than that in untreated controls. However, bees with both median MB sub-units ablated did not differ from controls in the frequency distribution of their acquisition scores. We classified bees that responded only once or not at all during the eight acquisition trials as non-learners. Bees with two or three conditioned responses were grouped as poor learners. Individuals responding with proboscis extension in at least 50% of the eight conditioning trials were classified as good learners (acquisition scores \( > 4 \)). In the group of non-learners, HU-treated bees without ablation or with unilateral ablation were found in equal proportions. Interestingly, there were many fewer non-learners among bees with bilateral ablations. The control group is clearly different from all HU-treated groups in that it only had about 20% of non-learners. It is interesting that more than 30% of the bees with ablation of the left median sub-unit were among the poor learners, whereas bees with ablations of both median sub-units were absent in this group. The most interesting result was found in the group of good learners: whereas none of the bees with ablation of the left median sub-unit is among the good learners, the group of bees lacking both median sub-units was nearly as common as in the control group. These results demonstrate that in the first acquisition phase (1) HU treatment alone impaired learning, (2) bilateral ablation, surprisingly, did not affect acquisition in comparison with that of untreated controls, and (3) unilateral MB ablation did not have side-specific effects on tactile learning.

Extinction after learning using left antenna

Extinction on the left side basically reveals the same picture as acquisition on the same side. The frequency distributions of extinction scores on this side (Fig. 4) were significantly different between the controls and the HU-treated groups without ablations or with unilateral ablations (Table 1). The group with ablation of both median calyces did not differ from the untreated control group. There are no significant differences in extinction between
the various HU-treated groups. We grouped the bees according to their scores as showing strong extinction (extinction score≤1), medium extinction (2 or 3 conditioned responses) or weak extinction (extinction score≥4). Whereas about 60% of control bees only showed weak extinction, HU-treated bees showed much stronger extinction. The group of bees lacking the left median MB sub-unit, which did not include a single good learner, did not respond at all during the extinction tests.

Reversal tactile learning involving right antenna

During the second conditioning phase using the right antenna, which started approximately 140 min after the first conditioning trial on the left antenna, learning performance was overall poorer. HU-treated bees without any ablations, those with ablation of both median calyces, and controls had significantly lower acquisition scores during the second acquisition phase than during the first acquisition phase (HU_left median: $z=2.059$, $P<0.05$; HU_right median: $z=1.997$, $P<0.05$; controls: $z=3.443$, $P<0.01$; two-tailed Wilcoxon test). In the other two groups with unilateral ablations, which had already shown poor acquisition during the first learning phase, no differences between the two acquisition phases were found. The frequency distributions of acquisition scores (Fig. 3) differ significantly between the controls and all HU-treated groups (Table 1), but not among the various HU-treated groups. Interestingly, bees whose MB sub-units were ablated in both hemispheres did not include a single good learner during reversal learning, although they had performed as well as untreated controls during the first conditioning phase.

These data show that during reversal learning using the right antenna (1) acquisition performance was overall poorer than during the first conditioning phase involving the left antenna, (2) none of the bees with bilateral ablations of MB sub-units showed more than two conditioned responses, although these bees had previously learned as well as untreated controls, and (3) no side-specific effects on tactile reversal learning were found in bees with unilateral ablations.
Extinction after reversal learning using right antenna

The frequency distributions of extinction scores (Fig. 4) are different between untreated controls and all HU-treated groups (Table 1). The various HU-treated groups did not differ from each other. Most bees of the HU-treated groups did not respond in the extinction tests after conditioning involving the right antenna.

Responses on the contralateral, non-conditioned antenna

After the acquisition using the left antenna, no bee showed proboscis extension more than once when the plate was presented to the contralateral antenna. This shows that the bees could discriminate very well between the two antennae in our learning paradigm. After acquisition involving the right antenna, very few bees responded two or more times on the contralateral left side, and they thus showed retention, because they were earlier trained on this side. Differences between the groups were not statistically significant.

Discussion

One approach to elucidating the role of different brain structures which are necessary for learning and the formation of memory is experimental ablation of these brain regions. This has been a standard technique for the last six decades (Lashley 1950). Using the genetic “knock-out” technique, this approach has now also been applied to gene products which are involved in learning (Takahashi et al. 1994). These methods can clearly help to identify neural elements and signal molecules which are necessary for specific forms of learning. On the other hand, ablations of neuropils can have severe side-effects which concern different behaviours in complex ways. We therefore analysed not only side-specific learning but also side-specific processing of the reward signal, sucrose, in order to differentiate between reward-specific and learning-specific deficits in animals with different forms of ablations.

Partial mushroom-body ablations

HU treatment of first-instar larvae stops the proliferation of MB neuroblasts, which results in the loss of complete MB sub-units (Malun 1998). In our experiments, we only generated bees exhibiting partial MB ablations (ablation of one or two median MB sub-units). It was always the median MB sub-units that were ablated and never the lateral ones. This is due to the time of HU incubation. HU treatment was performed on first-instar larvae, when the MB neuroblasts of the median MB sub-units start to proliferate and are thus susceptible to the mitotic blocker HU (Malun 1998). As shown by volumetric measurements (Malun and Moseleit 1999), the volume of the remaining
HU treatment impairs responsiveness to sucrose

HU treatment decreased responsiveness to sucrose. HU-treated bees without ablations were significantly less responsive to the different sucrose concentrations than untreated control bees. The cause for this behavioural modification is unclear. One hypothesis is that HU treatment affects neural structures different from the Kenyon cell neuroblasts. In Drosophila, for example, HU treatment reduces the volume of the antennal lobes by 30% in addition to the effect on MB cells (de Belle and Heisenberg 1994). In the honey bee, the antennal lobes appear to be unaffected by HU treatment (Malun 1998; Malun and Moseleit 1999), but the proliferation of neurons involved in the mediation of sensory antennal input and proboscis motor output or modulatory neurons might be impaired. We also do not know whether motoneurons are affected by HU treatment. Ablations on the single-cell level are difficult to detect, because they would not become apparent in volumetric measurements of neuropils. The effects of HU treatment therefore need further investigation, particularly with regard to sensory neurons, interneurons and neurosecretory cells involved in sucrose-elicited proboscis extension.

Responsiveness to sucrose is not affected by partial MB ablation

Unilateral or bilateral partial MB ablation did not increase the effects of HU treatment on the responsiveness to sucrose stimuli in honey bees. In our experiments, none of...
the groups with partial MB ablations differed in the distribution of gustatory response scores from the HU-treated group without ablations. This result suggests that MBs do not have a pivotal role in the mediation of sucrose stimuli. Other experiments support this assumption. When the monoamines dopamine and serotonin were injected into the MBs of honey bees, they drastically changed responsiveness to water vapour but had little effect on sucrose-induced proboscis extension (Blennau and Erber 1998). Braun and Bicker (1992) also suggest that MBs are not directly involved in the mediation of the sucrose-induced PER between antennal lobe and suboesophageal ganglion. They showed that injection of α-bungarotoxin into the MB calyces, which blocks cholinergic inputs via the median antennocerebral tracts, did not interfere with the elicitation of the PER. This experiment demonstrates that the projection areas of the median antennocerebral tract into the MB neuropil are not necessary for the PER. In contrast, injection of α-bungarotoxin into either antennal lobe completely blocked the PER. The authors therefore suggest that the PER is mediated by direct pathways between antennal lobe and suboesophageal ganglion. Our results from ablations of entire MB sub-units support this interpretation.

Tactile antennal learning is reduced by HU treatment

HU treatment reduced tactile antennal learning. HU-treated bees without any ablations had significantly lower acquisition and extinction scores than untreated controls. These learning differences are not a result of the observed differences in responsiveness to sucrose, as has been shown earlier to be the case in pollen and nectar foragers (Scheiner et al. 1999) and in foragers of two genetic strains (Scheiner et al. in press), because we selected HU-treated bees and untreated controls with similar sucrose responses for conditioning. HU treatment apparently damaged structures or neuronal mechanisms involved in tactile antennal learning. HU might affect the processing of tactile stimuli by acting on the mechanoreceptors or on neurons of the mechanosensory pathway. But as proliferation of antennal receptors presumably takes place later than that of Kenyon cells (Eichmüller and Schäfer 1995), it is unlikely that mechanoreception of tactile stimuli is different in HU treated bees. At present, nothing is known about possible effects of HU treatment on higher-order mechanosensory neurons in the brain. HU treatment might also affect the processing of rewards during learning. Another alternative is the action of HU on general signalling mechanisms in central neurons. However, in classical olfactory conditioning, HU treatment does not seem to affect acquisition (Malun et al. 2000).

The effect of HU treatment seems to account for all learning defects, because the HU-treated groups with ablations did not differ in their frequency distributions of acquisition scores from HU treated bees without ablations. However, it is very interesting that bees with bilateral ablations learned as well as untreated control bees during the first learning phase but did not show reversal learning, whereas animals with unilateral MB ablation on the conditioned side learned poorly during both acquisition phases. One hypothesis for this complex finding is that the learning defects in bees with unilateral MB ablation are due to the asymmetry of MB ablations, as bees with bilateral MB ablations showed normal acquisition in the first learning phase. In these bees, the MB neuropil is symmetrically distributed in the brain. In addition, in honey bees with bilateral ablations, the MBs occasionally fuse at the level of the β lobes, leading to direct connections between the MBs of the two hemispheres (Malun and Moseleit 1999).

The absolute MB volume does not seem to be a decisive factor in the tactile learning performance of honey bees. Bilateral ablation of MB sub-units leads to a loss of MB volume compared to untreated controls (Malun 1998). Nevertheless, the learning performance during the first acquisition phase did not differ from that of untreated controls. Olfactory learning experiments with bees are necessary to establish whether this effect is specific to tactile learning, because de Belle and Heisenberg (1994) demonstrated in Drosophila that olfactory learning was poor in animals with smaller MB calyx volume.

Bilateral ablations clearly affect reversal learning. In contrast to the first learning phase, these bees showed very poor acquisition during reversal learning and differed significantly from untreated controls. Apparently, bilateral ablations reduce the ability of these animals to acquire new tactile information shortly after a number of conditioning trials using another tactile cue. Further experiments are necessary to analyse whether this effect is caused by deficits in acquisition or due to modifications of tactile memory.

Our experiments demonstrate that the role of MBs in tactile antennal learning is not simple. The hypothesis that the ablation of MB sub-units on one brain side leads to side-specific deficits in learning has been falsified. Apparently, HU treatment also affects the processing of the reward signal. It is unclear whether this side-effect of chemical ablation has had similar consequences in previous experiments with HU ablations in insects. Future experiments are necessary to establish whether these HU effects occur in all forms of learning in insects or whether they are only apparent during operant conditioning, such as tactile antennal learning.

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