2 Chapter I

The Reconsolidation process in the honeybee

2.1 Introduction

In a classical conditioning paradigm an animal learns that a formerly neutral stimulus (conditioned stimulus, CS) predicts the appearance of an unconditioned stimulus (US). An association is thus formed between both stimuli; afterwards, the animals react to the CS with a conditioned response (CR) (Pavlov, 1927). Freshly acquired information is initially sensitive to interference and becomes resistant over time (Ebbinghaus, 1885). Interfering treatment, such as controversial information, shock treatment or cooling, can induce retrograde amnesia. Consolidation is a process by which the initially sensitive association becomes enduring (Müller & Pilzecker, 1900). There is strong evidence that de novo transcription of mRNAs and translation of proteins is necessary for the formation of long-term memories (LTMs) (Davis & Squire, 1984). Protein synthesis is thought to underlie morphological changes of synapses necessary for the association to become persistent (Kandel, 2001; Engert & Bonhoeffer, 1999). In fact, a long-term memory can persist from days to years.

Studies in the 1960's reported that memories are not only labile shortly after acquisition, but return to a labile state after retrieval of the acquisition memory. A repeated consolidation process is needed to maintain the memory. This phenomenon is referred as reconsolidation (Spear, 1973). One of the first reports comes from Misanin and colleagues, who observed that the application of electroconvulsive shock after retrieving a consolidated memory results in amnesia for the original learning. Amnesia was not induced if the retrieval was omitted (Misanin et al., 1968). Most of these early studies used systemically-applied treatment, like electroconvulsive shock or cooling to interfere with the reconsolidation process. Reconsolidation has recently become a renewed source of puzzling questions. Nader et al. (2000) demonstrated that the application of a translation inhibitor in a specific brain region, the lateral and basal nuclei of the amygdala, in conjunction with the retrieval of the acquisition memory, results in amnesia

in cued fear conditioning in rats (Nader et al., 2000). Since then many research groups examined the reconsolidation phenomenon in a wide range of species and behavioral paradigms. It has been observed in different aversive learning paradigms in vertebrate studies (Inda et al., 2005; Eisenberg & Dudai, 2004a; Lee et al., 2004; Anokhin et al., 2002; Vianna et al., 2001; Berman & Dudai, 2001; Nader et al., 2000), and in invertebrates, the crab *Chasmagnathus* (Pedreira et al., 2002), the slugs *Limex* (Yamada et al., 1992; Sekiguchi et al., 1997) and *Hermissenda* (Child et al., 2003) and the snail Lymnaea (Sangha et al., 2003a). There are only few studies on reconsolidation that use an appetitive paradigm. The involvement of protein synthesis in reconsolidation has been demonstrated for an instrumental incentive learning paradigm in rats (Wang et al., 2005) and impairment of reconsolidation after systemic manipulation of the N-methyl-D-aspartate (NMDA)-receptor function has been reported (Przybyslawski & Sara, 1997; Torras-Garcia et al., 2005). But there are also contrasting reports about failure to demonstrate reconsolidation. Protein synthesis seems not to be involved in maintenance of the memory following retrieval in an appetitive instrumental conditioning paradigm (Hernandez & Kelley, 2004) and in an inhibitory avoidance task (Cammarota et al., 2004) in rats.

The goal of this study was to test whether retrieval of a previously established association leads to a protein synthesis-dependent process (reconsolidation) in an appetitive paradigm in honeybees.

An olfactory conditioning paradigm, the proboscis extension response (PER) developed by Kuwabara (1957) was used to study the phenomenon (Kuwabara, 1957). Bees reflexively extend their proboscis if the antennae or the proboscis are touched with sucrose. Odors do not release this response in naive bees. This reflex-like behavior is used to train harnessed animals to associate an odor (CS) with sucrose reward (US) in a classical conditioning manner (Bitterman et al., 1983). Furthermore, the number of conditioning trials used to train a bee induces different memories. A single trial results in a memory whose induction is protein synthesis independent, and which decays over days, whereas three CS-US pairings induce the formation of a long-term memory (LTM)(Menzel, 1999; Müller, 2002). LTM is protein synthesis-dependent, as demonstrated by systemic application of anisomycin, a translation inhibitor, and actinomycin D, a transcription inhibitor (Wüstenberg et al., 1998; Menzel et al., 2001). If the consolidation process is disturbed by anisomycin an impaired retention of memory

is observed 3 and 4 days after acquisition (Wüstenberg et al., 1998). Recently it has been reported that the application of emetine, another translation inhibitor, leads to an impaired memory already one day after acquisition. The impairment lasted during the successive three days of the experiment (Friedrich et al., 2004).

In the experiments described here, reconsolidation of the acquisition memory will be disrupted by the use of two protein synthesis inhibitors, anisomycin and emetine. Memory retention was tested every day in order to study the time course of impairment following retrieval.

2.2 Methods

Animals

Honeybees were reared in the bee garden near the institute. All experiments were carried out from 2002 - 2005.

On the afternoon before the experiment started foraging bees leaving the hive were caught at the entrance of the hive using a plastic pyramid. Afterwards they were transferred into small glasses and anesthetized by chilling on ice. Once anesthetized, each bee was harnessed in a metal tube so that that it could freely move its antennae and mouthpart. One drop of 1 M sucrose was delivered immediately afterwards. The bees were fed to satiation in the evening. Experiments started the next day. During the rest of the experiment bees were fed every evening with four droplets of 1 M sucrose (~15 µl). The animals were fed at least 1 h after the last presentation of a CS trial. Between the test sessions bees were kept in a dark and humid box at 18-20° C.

Experimental schedule

Honeybees were trained with three CS-US pairings on day 1. On day 2 they were divided into two groups (Figure 2-1). Animals in the first group (retrieved group) were subjected to one CS-only trial, the other group acted as the non-retrieved control group. Both groups were further divided into two groups. One of each was injected with protein synthesis inhibitor; animals in the second group were injected with PBS. On the following days all four groups were subjected to one CS-only presentation per day to test the retention until day 6.

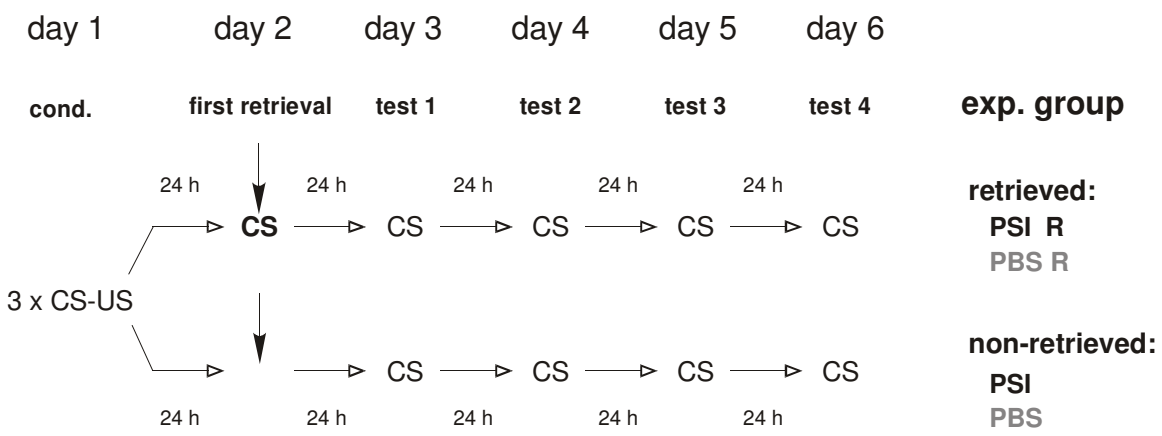

Figure 2-1 Experimental schedule for the experiments. On day 1 the animals received three CS-US parings with an ITI of 2 min (conditioning). 24 hours later bees in the retrieved group were subjected to the injection (black arrow) of protein synthesis inhibitor (PSI) or saline (PBS) and one CS-only presentation (first retrieval). Animals in the non-retrieved groups got only the injection. On days 3-6 the animals in all groups received one CS-only trial per day (test 1-4).

Conditioning of the proboscis extension response (PER)

An acquisition trial started with moving a bee from its resting position to the inlet of an exhaust fan. The odor (CS) was delivered after 10 s of accommodation time. Carnation oil from the pharmacy was used as CS, and was delivered through a 20 ml syringe (Omnifix, B.Braun Melsungen AG, Germany) that was loaded daily with 4 µl of carnation oil. The odor was presented for 5 s. Three seconds after the odor onset, sucrose (US) was applied by touching the antennae with a sucrose-moistened wooden toothpick; the bees were allowed to lick sucrose for 4 s. Acquisition consisted of three such pairings with an intertrial-interval (ITI) of 2 min. A bee scored positive when it extended its proboscis between the onset of the CS and the presentation of the US.

Retention test / CS-only trial

A CS-only trial consisted of 10 s accommodation period in front of an exhauster, followed by 5 s CS presentation without reinforcement of the US. The ITI, unless stated otherwise, was 24 h.

Injection

Anisomycin (Sigma Aldrich, Deisenhof, Germany, Cat No. A9789) and emetine (Fluka Chemie, Switzerland; Cat No. 45160) were dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.2) in different concentrations. One µl of protein synthesis inhibitor or PBS was injected manually into

the thorax using a calibrated glass capillary (capilettor tips and sticks, Selzer GmbH, Waghäusel, Germany; Cat No. 224804 and 224847). In the first experiment animals were injected 1 h before the first retrieval. For all other experiments the animals were injected 1 h after the first retrieval. Control groups were injected at the same time with protein synthesis inhibitor or PBS, but without being subjected to the CS-only trial.

Data analysis

For the first experiment only bees that survived the whole experiment were included in the analyses. For all other experiments only animals which survived the whole experiment and showed a response to sucrose after the last CS-only presentation were included. Bees which showed a spontaneous proboscis extension to the first odor presentation during the conditioning were excluded from all experiments. For the statistical analysis differences were considered to be significant if $p < 0.05$.

Acquisition

The critical tests were the comparisons between the different groups in the retention tests; therefore it was important to ensure that acquisition was similar for the different groups. To test this, a heterogeneity χ^2-test was used (Zar, 1997). If the data were determined to be heterogeneous, animals from the larger group were randomly removed from the analyses by means of a program kindly provided by Uwe Greggers. This was done 10 times for each data set and the effect on the statistics of the retention test were analyzed to ensure that the reported results are not an artifact of the selection process. In all cases the selection process did not change results of retention tests from significant to non-significant or vice-versa.

Retention test

A 2 x 2 G-test for contingency tables (=log likelihood ratio for contingency tables) was used to compare the CR at the retention test on one day (Zar, 1997). To compare the CR within one group a McNemar test implemented in Statistica (StatSoft, Tulsa, USA) was used.

2.3 Results

In rodents retrieval of a consolidated acquisition memory by presentation of the CS-only could lead to a new protein synthesis-dependent consolidation process referred to as reconsolidation. This reconsolidation process is only visible in behavior if it is blocked by an amnestic agent. The criterion to decide if animals undergo reconsolidation after retrieval is that 1) the conditioned response (CR) of the amnestic treated and retrieved animals is reduced in comparison to non-amnestic treated and retrieved animals and 2) the omission of the retrieval leads to no difference in CR between amnestic and non-amnestic treated animals (Misanin et al., 1968).

First experiment: Application of the mixture of anisomycin and emetine 1 h before the first retrieval does not inhibit reconsolidation

Friedrich et al. (2004) showed that a mixture of two protein synthesis inhibitors, anisomycin (10 mM) and emetine (10 mM), applied before the conditioning leads to a reduced CR 24 h later. In a first approach this mixture was used to test whether a CS-only trial triggers reconsolidation of the acquisition memory.

The experiment took place in the period from May to June 2002. On the first day harnessed bees received three CS-US pairings; 24 h later animals were divided into two groups and received a systemic injection of either phosphate-buffered saline (PBS) or the mixture of anisomycin + emetine (A+E). Each group was further divided into two groups. One has received a CS-only trial to retrieve the acquisition memory one hour after the injection (retrieved groups: PBS R and A+E R), the other was handled in the same way but was not subjected to the CS-only trial (non-retrieved groups: PBS and A+E). On the following four days memory was retrieved once a day by a CS-only presentation to test retention (Figure 2-2). In order to differentiate these CS-only presentations from those which retrieved the memory in conjunction with the injection on day 1, the latter is hereafter referred to as "first retrieval" (FR). Approximately 60 % of the 550 conditioned animals died during the experiment. But no effect of protein synthesis inhibitor on mortality has been observed; PBS- and A+ E-treated animals died at the same rate.

For the retrieved groups (Figure 2-2A) the CR of the PBS R group is below that of the A+E R group in the second acquisition trial, but this difference is not significant

(χ^2_{A2} = 3.00, df = 1, p = 0.08). At the last acquisition trial ~65 % of the animals in both groups responded to the CS with an extension of the proboscis. On day 2 the A+E R group reach a non-significantly better performance than the PBS R group (χ^2_{24h} = 3.43, df = 1, p = 0.06) at the first retrieval. There is no significant difference in CRs of both groups in the retention tests on the following four days. Therefore the injection of the translation inhibitor one hour before the first retrieval reveals no reconsolidation process. But during the multiple retention tests the CRs decrease significantly from 85 % on day 2 to 32 % on day 6 for the A+E R group, and from 74 % to 32 % for the PBS T group (McNemar: $\chi^2_{A+E\ R}$ = 25.29, df = 1, p > 0.001; $\chi^2_{PBS\ R}$ = 15.04, df = 1 , p > 0.001). Hence it follows that an extinction process took place, due to the multiple testing. Extinction refers to the behavioral phenomenon in which an animal responds less or less frequently to the CS because it experienced that the US is no longer predicted by the CS (Pavlov, 1927).

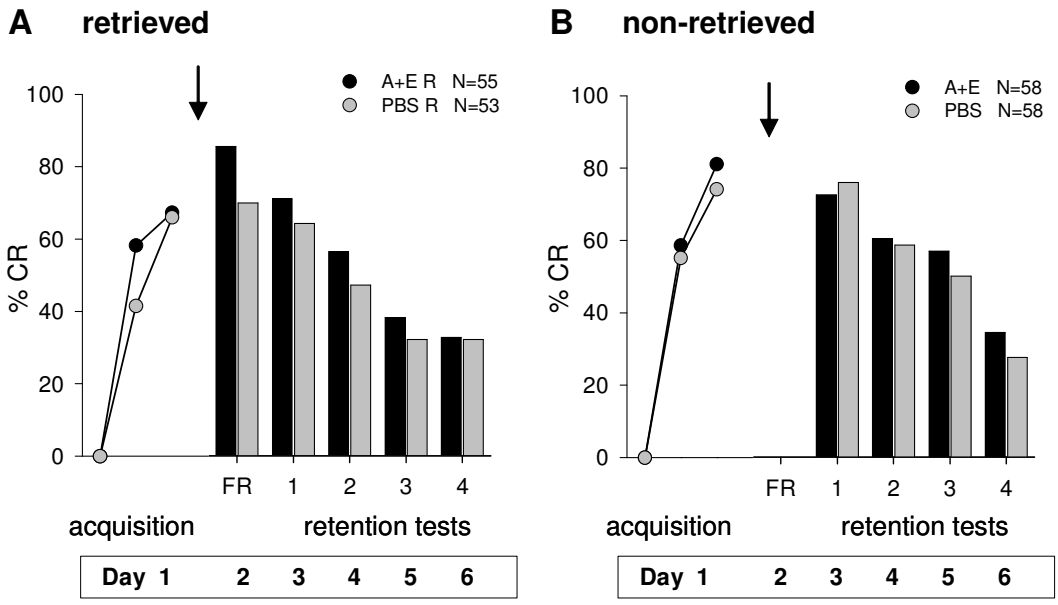

Figure 2-2: Application of the mixture of anisomycin and emetine (A+E) 1 h before the first retrieval does not inhibit reconsolidation. On day 1 animals were subjected to three CS-US pairings with an ITI of 2 min (= acquisition). After 24 h, on day 2, A+ E or PBS was injected followed by the first retrieval (FR) one hour later for the retrieved group (**A**). For the non-retrieved group (**B**) the CS-only presentation (FR) was omitted. On day 3 - 6 memory was tested (= retention test), Arrow = time of injection, grey = PBS injected control group, black = A+E injected group. % CR = Percentage of animals that showed a proboscis extension response during CS presentation.

For the non-retrieved animals (Figure 2-2B) the response probability of both groups (A+E, PBS) is similar during the course of acquisition. The animals received the injection on day 2, but no CS-only trial. There is no significant difference in response

probability in the following retention tests. This demonstrates that the acquisition memory after 24 h is no longer susceptible to protein synthesis inhibition. Additionally, the decrease in CR over time is also detectable in the non-retrieved groups. 73 % of the animals showed a response to the CS-only presentation on day 3, but on day 6 only 30 % of the animal reacted. A McNemar test revealed the decrease of around 40 % in response level to be significant for both groups. (McNemar: $\chi^2_{A+E} = 14.70$, df = 1, $p > 0.001$; $\chi^2_{PBS} = 24.30$, df = 1 $p > 0.001$).

Second experiment: Application of the mixture of anisomycin and emetine 1h after the first retrieval inhibits reconsolidation of the acquisition memory

Wüstenberg et al. (1998) showed that an injection of anisomycin (10 mM) one hour after conditioning inhibits the consolidation process of the acquisition memory, which leads to a reduced CR three days later. Since there is no detectable effect of the mixture of protein synthesis inhibitors injected before the first retrieval, the time of application was changed to a time point after the first retrieval of the acquisition memory. The experimental schedule was similar to the previous one, except that on day 2 the injection was given one hour after the first retrieval instead of one hour before. The experiment was conducted in July and August 2002.

Figure 2-3 shows the percentage of bees responding to the odor with a proboscis extension at various tested time points. On the last acquisition trial CR was between 50-65 % for all four subgroups. There are no significant differences in CRs during the acquisition trials. The retrieved groups (A+E R, PBS R) were tested 24 h later on day 2. 96 and 94 % of the bees showed a CR to the first retrieval and one hour later they received the PBS or A+E injection (Figure 2-3A). On the next three days (day 3 - 5) the response level of the PBS R group stayed at 80 % and decreased on day 6 to 63 %. The A+E R group shows a continuous reduction of the CRs from 79 % on day 3 to 45 % on day 6. By comparing the retention level of both retrieved groups on each day, a G-test revealed significantly reduced CRs of the A+E R group on day 5 and day 6 in comparison to CRs of the PBS R group ($G_{D5\_retrieved} = 5.13$, $p = 0.02$; $G_{D6\_retrieved} = 5.14$, $p = 0.02$, df = 1). In the non-retrieved groups (Figure 2-3B) CRs of the A+E group increase from day 3 to day 4 around 6 %. On the following days the response level

decreases to 66 % on day 6. For the PBS group a decrease from 80 % on day 3 to 61 % on day 6 is observed. The CRs of both groups are not significantly different in all retention tests.

Therefore applying a CS-only trial to a consolidated memory leads to a reconsolidation of the acquisition memory, which is expressed behaviorally 3 and 4 days later. The manifestation of this memory is inhibited if a protein synthesis inhibitor disrupts the reconsolidation process. Both non-retrieved groups showed no significant differences between CRs on all tested days. This finding rules out the possibility that the observed deficit in the amnestic-treated and retrieved group is due to a disruption of late waves of protein synthesis 24 h after training.

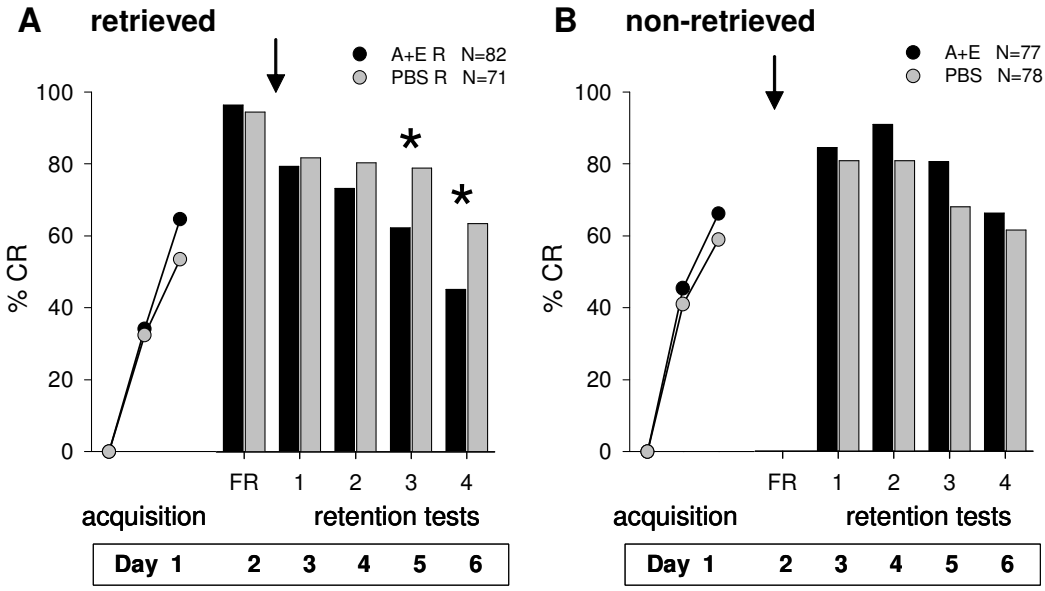

Figure 2-3: Application of the mixture of anisomycin and emetine (A+E) 1 h after the first retrieval inhibits reconsolidation of the acquisition memory. On day 1 animals were subjected to three CS-US pairings with an ITI of 2 min (= acquisition). After 24 h, on day 2, the retrieved group (**A**) received the first retrieval (FR); the CS-only presentation was omitted for the non-retrieved group (**B**). One hour later A+ E or PBS was injected. On day 3 - 6 memory was tested (= retention test); asterisks indicate significant differences between the CRs ($p < 0.05$). Arrow = time of injection, grey = PBS injected control group, black = A+E injected group. % CR = Percentage of animals that showed a proboscis extension response during CS presentation.

Third Experiment: Application of anisomycin or emetine 1 h after the first retrieval does not inhibit reconsolidation

Since the reconsolidation effect could be shown by an injection of the mixture of two protein synthesis inhibitors one hour after the first retrieval, the next question was to ask which of them is responsible for the inhibition of the reconsolidation process. To do so, experiments were performed with the individual use of protein synthesis inhibitors. The same amount of protein synthesis inhibitor was used as in the mixture. First the effect of anisomycin was studied. There were no changes in experimental schedule, but instead of the mixture, anisomycin (10 mM) was injected on day 2. There was no difference in mortality among the anisomycin- and the PBS-treated bees; in both groups ~60% of the animals died during the experiment. Figure 2-4 shows the performance of the four groups during acquisition and retention tests. Around 80 % of the bees respond on the last acquisition trial. One day later 95% of the retrieved animals (Ani R, PBS R) show a CR to the odor at the first retrieval (Figure 2-4A). A similar percentage of bees react in the non-retrieved groups (Ani, PBS) on day 3 (Figure 2-4B). In all groups response probability decreased significantly over time (McNemar: $\chi^2_{\text{Ani R}} = 29.03$, $p > 0.001$; $\chi^2_{\text{PBS R}} = 21.04$, $p > 0.001$; $\chi^2_{\text{Ani}} = 22.32$, $p > 0.001$; $\chi^2_{\text{PBS}} = 17.05$, $p > 0.001$, $df = 1$). But there are no differences in CRs between the retrieved groups, nor between the non-retrieved groups. Hence, with 10 mM anisomycin the reconsolidation process, visible with an injection of the mixture of anisomycin + emetine, cannot be disrupted.

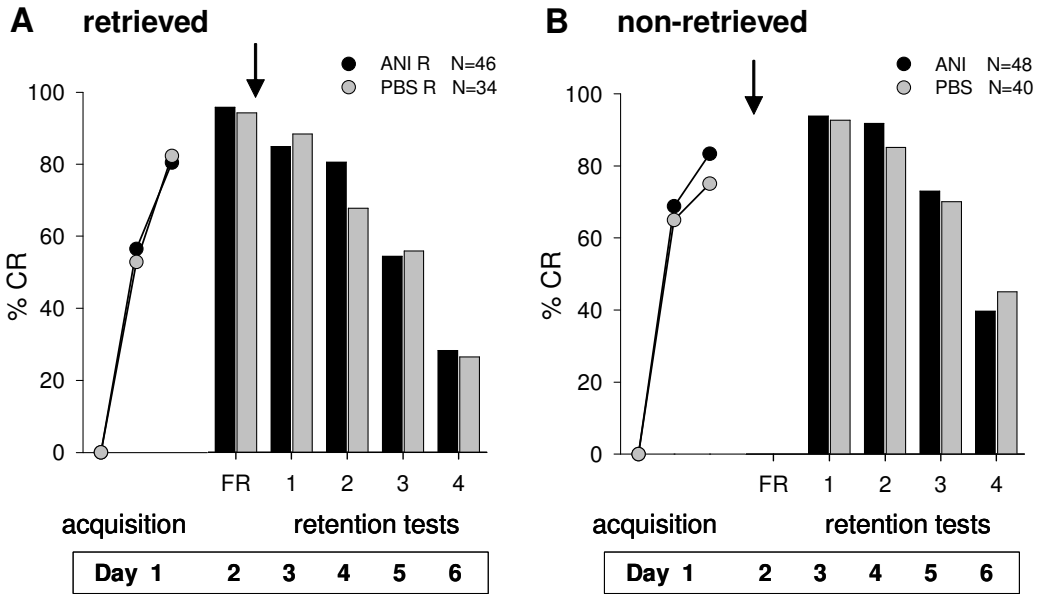

Figure 2-4: Application of the anisomycin (Ani) 1 h after the first retrieval does not inhibit reconsolidation. On day 1 animals were subjected to three CS-US pairings with an ITI of 2 min (= acquisition). After 24 h, on day 2, the retrieved group (**A**) received the first retrieval (FR), for the non-retrieved group (**B**) the CS-only presentation was omitted. One hour later Ani or PBS was injected. On day 3 - 6 memory was tested (= retention test). Arrow = time of injection, grey = PBS injected control group, black = Ani injected group. % CR = Percentage of animals that showed a proboscis extension response during CS presentation.

The following study tested if emetine has an effect on reconsolidation. After three CS-US pairings all experimental groups reach around 90 % response level (Figure 2-5). Injection of emetine (10 mM) (Eme) or PBS did not lead to a different mortality. In both groups around 55% of the animals survived the whole experiment. In the retrieved groups (Eme R, PBS R) nearly all bees reacted to the first retrieval on day 2 one hour before the injection (Figure 2-5A). The CRs of both groups decrease significantly to 47 % and 37 % on the last day of the experiment (McNemar: $\chi^2_{\text{Eme R}} = 18.38$, $p < 0.01$; $\chi^2_{\text{PBS R}} = 17.39$, $p < 0.01$, $df = 1$). But there were no differences in CR between the two groups on different days. For the non-retrieved groups (Figure 2-5B) CRs decreased significantly from 95% and 100% on day 3 to 44% and 57% on day 6 for the Eme and PBS group, respectively (McNemar: $\chi^2_{\text{Eme}} = 16.06$, $p < 0.01$; $\chi^2_{\text{PBS}} = 6.75$, $p < 0.01$, $df = 1$). On day 5 a difference of 19% in CRs is detectable, but this difference is not significant ($G_{\text{D5\_non-retrieved}} = 2.50$, $df = 1$, $p = 0.11$). There is also no difference in response level in the other retention tests. Thus application of the emetine part of the mixture does not inhibit the reconsolidation process.

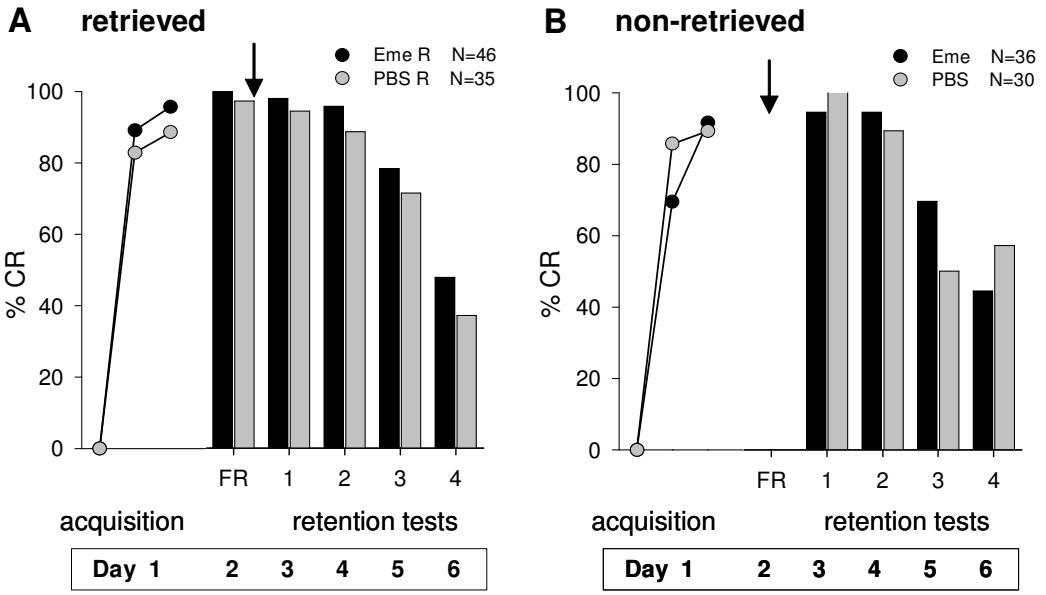

Figure 2-5: Application of emetine (Eme) 1 h after the first retrieval does not inhibit reconsolidation. On day 1 animals were subjected to three CS-US pairings with an ITI of 2 min (= acquisition). After 24 h, on day 2, the retrieved group (**A**) received the first retrieval (FR), for the non-retrieved group (**B**) the CS-only presentation was omitted. One hour later Eme or PBS was injected. On day 3 - 6 memory was tested (= retention test). Arrow = time of injection, grey = PBS injected control group, black = Eme injected group. % CR = Percentage of animals that showed a proboscis extension response during CS presentation.

Fourth Experiment: Application of the doubled amount of emetine 1h after the first retrieval inhibits reconsolidation of the acquisition memory

The observed disruption of the reconsolidation process with the mixture (A+E) is not due to a single component of the mixture. Individual use of emetine and anisomycin in a concentration of 10 mM cannot interfere with the reconsolidation process. Thus it is possible that the mixture of both inhibitors are necessary or that the total amount of protein synthesis inhibitor applied in the single component experiments is not sufficient to disrupt the reconsolidation process. In the following experiment the amount of protein synthesis inhibitors was therefore doubled, but the experimental schedule remained the same. The solubility of anisomycin in water or PBS is insufficient to produce a 20 mM solution. Another possibility would be to solve anisomycin in DMSO, but this was not done because DMSO is supposed to lead to an increase of the amount of phosphorylated cAMP responsive element binding protein (CREB) in the bee brain (Froese, 2005) and CREB is believed to be involved in the formation of LTM. Therefore, only emetine was used in a concentration of 20 mM (Eme2). The experiment was performed in June and July 2004 and May 2005. The increased dose of emetine has

no effect on the mortality; Eme2- and PBS-treated animals survived the experiment equally well.

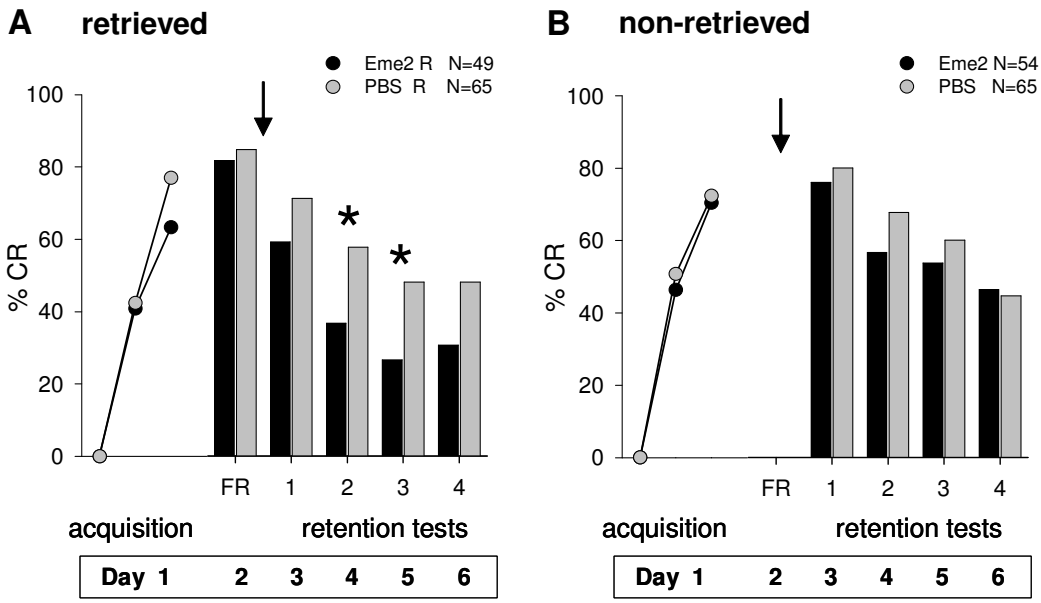

Figure 2-6: Application of a double amount of emetine (Eme2) 1 h after the first retrieval inhibits reconsolidation of the acquisition memory. On day 1 animals were subjected to three CS-US pairings with an ITI of 2 min (= acquisition). After 24 h, on day 2, the retrieved group (**A**) received the first retrieval (FR), for the non-retrieved group (**B**) the CS-only presentation was omitted. One hour later Eme2 or PBS was injected.. On day 3 - 6 memory was tested (= retention test), asterisks indicate significant difference between the CRs ($p < 0.05$). Arrow = time of injection, grey = PBS injected control group, black = Eme2 injected group. % CR = Percentage of animals that showed a proboscis extension response during CS presentation.

In Figure 2-6 the response to the CS during the whole experiment is shown for all tested time points. In the retrieved group (Figure 2-6A) fewer animals of the Eme2 R group reacted to the CS on the last acquisition trial than in the PBS R group, but this difference is not significant ($\chi^2_{A3\_retrieved} = 2.25$, $df = 1$, $p = 0.13$). The difference is diminished the next day at the first retrieval; in both groups around 80 % of the animals extended the proboscis. One hour later they were injected with Eme2 or PBS. CRs of the Eme2 R group decreased over the next days to 26 % on day 5 and then increased slightly on day 6. The increase of 4 % from test 3 to test 4 is not significant. CRs of the PBS R group also decrease over the multiple retention tests but not as steeply. They reach a response level of 48 % on day 5 and remained stable afterwards. This results in an increasing difference between both groups, which become significant on day 4 and day 5 ($G_{D4\_retrieved} = 4.48$, $p = 0.03$; $G_{D5\_retrieved} = 5.06$, $p = 0.03$, $df = 1$), but the difference on day 6 is not significant ($G_{D6\_retrieved} = 3.24$, $p = 0.07$, $df = 1$).

Animals in the non-retrieved group (Figure 2-6B) showed no difference during acquisition on day 1. On day 2 they received the injection (Eme2, PBS) but memory was not retrieved. The animals in both groups showed a CR of 75 and 80 % on day 3 and decreased uniformly to 45 % on day 6. There was no significant difference in CRs of both groups on the retention tests.

Hence, the double amount of emetine applied one hour after the first retrieval leads to memory impairment in comparison to the saline treated and retrieved group. Furthermore, there are no significant differences in CRs of the non-retrieved groups, demonstrating that the acquisition memory is consolidated 24 h after training and that the observed impairment is not due to an unspecific effect of the emetine treatment. Therefore, injection of 20 mM emetine one hour after the first retrieval disturbs the reconsolidation process and leads to an impaired retention 2 days afterwards.

Fifth Experiment: Repeated unreinforced CS-only presentation leads to extinction

In all presented experiments CRs decrease in all groups from the first CS-only presentation to the last one on day 6. This is probably due to the multiple presentations of the CS-only to test for retention of the acquisition memory. Therefore, beside the reconsolidation process a second process was induced. In all experiments an extinction process took place. Extinction occurs if a CS is presented repeatedly without US (Pavlov 1927). The extinction phenomenon is thought to be a form of new learning about the CS-noUS association which suppresses the CS-US association of the acquisition memory (Bouton, 2004).

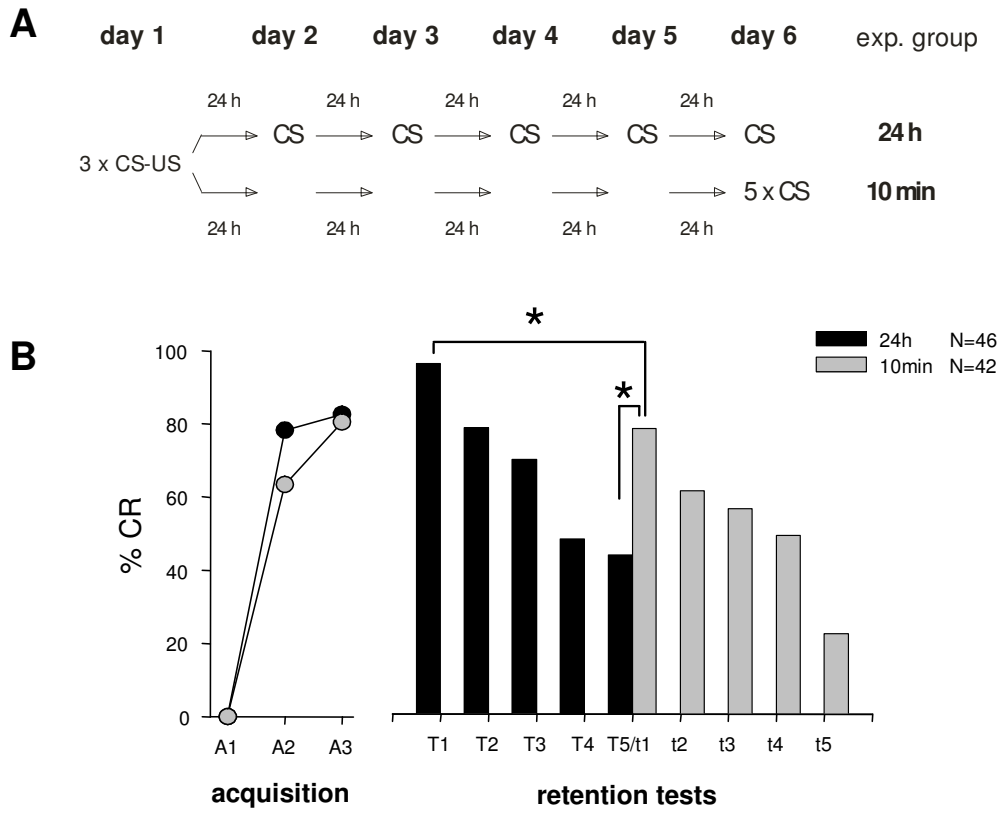

Figure 2-7: Five CS-only presentations lead to extinction. **A**. Experimental schedule. On day 1 animals were subjected to three CS-US pairings with an ITI of 2 min (=acquisition), on day 2 -6 the 24h group received one CS-only presentation per day. The 10 min group received all 5 CS-only trials on day 6 with an ITI of 10 min. **B**. Percentage of animals that extended the proboscis during CS presentation, asterisks indicate significant difference between the CRs ($p < 0.05$). black = 24 h group, A1-A3 = three acquisition trials, T1-T5 = retention tests of the 24h group, t1 - t5 retention tests of 10 min group on day 6.

An alternative explanation is that the memory decays over time, which is referred to as forgetting (Ebbinghaus, 1885; McGeoch, 1932). The course of forgetting has been studied for several paradigms (Wixted, 2004). To investigate if the observed decrease in CR during the multiple tests is due to forgetting or extinction an additional experiment was conducted. Bees were trained with three CS-US pairings and were then divided into two subgroups. One group (24h) received a CS-only trial once a day, as in the experiments described above. Bees in the other group (10min) were subjected to the same number of CS-only trials, but instead of a daily CS-only trial, the animals received 5 x CS-only trials on day 6 with an ITI of 10 min (Figure 2-7A).

The CRs in the 24h group decrease significantly from 96 % on day 1 to 43 % on day 6 (McNemar: $\chi^2_{24h} = 19.36$, df = 1, $p < 0.001$). On day 6 at the first retention test of the 10min group 78 % of the bees reacted to the CS with the extension of the proboscis. The retention level of the 10min group also decreased significantly over the multiple test

to 22% at the last test (McNemar: $\chi^2_{10min} = 19.36$, df = 1, $p < 0.001$). This shows that extinction is induced in both groups. If the decreased response in the experiments described above is due to forgetting, then there should be no difference between the CR on the last retention test of the 24h group (T5) and the first retention test of the 10min group (t1). As can be seen in Figure 2-7B, this is not the case, rather, significantly fewer animals in the 24h group reacted with a CR at the last CS-only trial than in the 10min group at the first presentation of the CS ($G_{24\_T5 \text{ vs. } 10\_t1} = 55.73$, df = 1, $p < 0.001$). Thus there is a significant difference of 18% in CRs between the first CS-only presentations in the 24h and 10min groups ($G_{24\_T1 \text{ vs } 10\_t1} = 6.39$, df = 1, $p = 0.01$), which could be interpreted as forgetting. This demonstrates that the decrease in CR in the former experiments is mainly due to an extinction process initiated by the multiple presentations of the CS-only.

2.4 Discussion

The presented results confirm that in harnessed honeybees three CS-US pairings lead to a long-term memory that, if not retrieved in the meantime, persists for five days at a high response level. Furthermore, memory retrieval 24 hours after conditioning initiates a protein synthesis-dependent reconsolidation of the acquisition memory. On the other hand, if memory is retrieved daily or repeatedly with a short inter-trial interval, an extinction process is initiated and the CR extinguishes over time.

Long-term memory in the honeybee

Repeated learning trials lead to a long-term memory which in free-flying bees lasts for a lifetime (Menzel, 1968) and for olfactory PER conditioning for at least 4 days (Menzel et al., 2001b; Grünbaum & Müller, 1998; Friedrich et al., 2004) (Fiala et al., 1998) (Wüstenberg et al., 1998). Here we confirm that three CS-US pairings lead to a stable long-term memory lasting five days. One group of animals received the first retention test after 1 day, the second group after five days. In both conditions the response level is high. The observed decrease in CR is, according to the traditional psychological view, due to forgetting (Ebbinghaus, 1885) and it is assumed that most memories dissipate over time (Klein, 2002). Psychologists assume that the engram, the physical representation of an association, fades with disuse. Retrieval may prevent decay or strengthen a decaying memory (McGeoch, 1932). According to the results presented here the number of retrieval trials determines reconsolidation or extinction

Retrieval of a consolidated memory leads to reconsolidation of the acquisition memory

As demonstrated here, retrieval of a consolidated memory leads to protein synthesis-dependent consolidation of the acquisition memory. This consolidation can be inhibited either by the application of a mixture of anisomycin + emetine or by emetine in double the amount as used in the mixture. If retrieval is omitted the acquisition memory at this time point is not susceptible to protein synthesis inhibitors. According to the definition by Misanin et al. (Misanin et al., 1968), this fulfilled the requirement to refer to this phenomenon as reconsolidation of the acquisition memory.

Injection of the mixture of anisomycin + emetine followed by the retrieval one hour later does not affect the reconsolidation process. This demonstrates that there is a specific time window for the inhibitor to interfere with the reconsolidation process.

Reconsolidation in the bee is less sensitive to disruption than consolidation

To disrupt consolidation of the acquisition memory a dose of 10 mM of anisomycin (Wüstenberg et al., 1998) or emetine (Friedrich et al., 2004) is sufficient. But as shown here, the translation inhibitors in the individual use have no effect on reconsolidation. In this concentration animals received 2 μg of anisomycin or 5 μg of emetine. If the mixture was applied a bee received both 2 μg of anisomycin and 5 μg of emetine and only then is an impaired memory - in comparison to PBS injected animals - detectable afterwards. With 10 μg of emetine the reconsolidation process is also disrupted. Therefore, the double amount of protein synthesis inhibitor disturbs the reconsolidation process but not the starting amount, which inhibits the consolidation of the acquisition memory. This indicates that reconsolidation in an appetitive olfactory paradigm in honeybees is less vulnerable to disruption than consolidation. A similar result is reported by Debiec and Nader (Debiec et al., 2002); they inhibit protein synthesis via local infusions into the hippocampus in order to interfere with the reconsolidation of fear memory in rats. On the other hand, the same amount of protein synthesis inhibitor, applied systemically, was used to disturb the consolidation and reconsolidation process in the crab Chasmagnatus (Pedreira et al., 1995; Pedreira et al., 2002). In other studies reconsolidation is more sensitive to disruption than consolidation. For instance, a smaller amount of protein synthesis inhibitor injected directly into the brain is sufficient

to inhibit reconsolidation in an avoidance task paradigm in chicks than the amount required for consolidation (Anokhin et al., 2002). Similarly, in shock avoidance in rats lower temperatures are necessary to inhibit consolidation than required for reconsolidation (Mactutus et al., 1982). Systemic and local applications of the consolidation blocker were used in different studies, but it seems that there is no simple general rule behind the sensitivity of the reconsolidation and consolidation processes. However, This differences support hypothesis that consolidation and reconsolidation are distinct processes.

Is the effect of the inhibitors only a temporal inhibition of the acquisition memory?

Disturbing reconsolidation by the mixture of anisomycin + emetine results in an impaired memory retention that is visible in behavior 3 and 4 days after the first retrieval (Figure 2-3). In contrast, the application of a double dose of emetine (20 mM) results in a reduced CR already 2 and 3 days after the first retrieval of the memory, but the CR increased 4% on the last day of the experiment (Figure 2-6), which results in a non-significant difference ($p = 0.07$) between the CR of the saline-treated and Eme2-treated groups. Most probably this is due to the extinction process induced by the multiple testing (as discussed below). But the question if the inhibition of the reconsolidated acquisition memory is only a transient effect of the inhibitors which decays over time cannot be excluded.

In the literature the reappearance of CR after interference with the consolidation process is referred to as 'spontaneous recovery' of acquisition memory (Lewis et al., 1968; Miller et al., 1974). It is assumed that the 'temporal inhibition' of the memory is due to a failure to retrieve the memory. The mechanisms for this are unknown. In addition, temporal inhibition of the acquisition memory is also reported after disruption of the reconsolidation process in rodents. In contextual fear conditioning the retrieval of the memory in conjunction with a systemic injection of protein synthesis inhibitor leads to an impaired memory retention 24 h later, but spontaneously recovers if tested 21 days later (Lattal & Abel, 2004). The authors speculate that during retrieval animals learn about the absence of the US in the trained context. If retention is tested 24 h after retrieval the extinction memory may be easier to retrieve than the acquisition memory. But as time passes, extinction memory becomes harder to retrieve, resulting in

spontaneous recovery. Lattal and Abel (2004) argue that protein synthesis inhibition facilitates the formation of an extinction process (Lattal & Abel, 2004). In other studies the opposite is demonstrated (Pedreira & Maldonado, 2003; Sangha et al., 2003b); (Berman & Dudai, 2001); if an extinction process is initiated by retrieval trials, protein synthesis inhibition results in impaired learning of the CS-noUS association. In honeybees two CS-only trials applied 24 h after acquisition lead to an extinction process, which could be inhibited by interference with emetine. This results in the maintenance of the memory retention (Chapter 2). Since the conditions in the experiments differ according to their injection times and amounts of inhibitors, the argument for facilitated extinction cannot be excluded, but seems unlikely. It would mean that the same drug inhibits extinction learning at one moment and enhances it a moment later. The presented data provide no concrete answer to the question whether experimentally-induced amnesia after retrieval is transient or long-lasting in bees. There is no recovery of CR in the experiment using the mixture of anisomycin + emetine and only minor recovery by the doubled amount of emetine. It is conceivable that spontaneous recovery occurs after a period longer than the observed 4 days. In order to test for this, the experiments would need to be prolonged.

When disturbing the reconsolidation process with the double amount of emetine, it could be argued that the impaired memory spontaneously recovered, since there is no significant difference between the retrieved groups on the last day of the experiment. Beside the fact that the recovery of CR is small and not significant, there are two additional points that should be considered: the possibility of reinstatement and the induced extinction process.

Reinstatement

One reason for the recovery of the CR might be reinstatement of the CR. In reinstatement the presentation of the US alone results in a reappearance of the CR, but only if the US is presented within in the context of the retention test (Rescorla & Heth, 1975). In the presented experiments bees were fed with sucrose every evening. Since sucrose is used as the US during conditioning, it can be argued that feeding in the evening initiates reinstatement. To circumvent this problem, different sucrose concentrations were used as US (1.25 M) and for feeding (1 M). Nevertheless, in rats extinguished CRs in a conditioned taste aversion paradigm can reappear by the

presentation of a weak US, which in itself is an ineffective US for conditioning in naive rats. Throughout this study rats remained in their home box; hence the context is the same during US presentation and the retention test (Berman et al., 2003). In the experiments presented here, feeding of the bees never took place at the experimental set-up. Hence the context during conditioning and feeding is different. Until now it has not been shown that reinstatement exists in PER conditioning in bees. It seems very unlikely that reinstatement is responsible for the "recovery" of the acquisition memory.

Extinction

Extinction is a well-known behavioral phenomenon in many paradigms (Myers & Davis, 2002; Delamater, 2004). Here I demonstrated that the repeated presentation of the CS-only with an ITI of 24 h results in an extinction process, which is observable in all presented experiments. This confirms data from an earlier study by Sandoz et al. (Sandoz et al., 1995). They compare the CR of bees tested in a cumulative fashion with bees with a single retention tests at the different time points. Multiple testing of the memory results in a decrease in the response, while the CRs in a single test remain at a level of ~90 % over 4 days. Extinction is thought to be new learning of the CS-noUS association which suppresses the original CS-US association (Pavlov, 1927; Bouton, 1993)}. Comparing the time course of CRs of the saline-treated groups in the presented experiments reveals a high variability in the decrease of CR. Figure 2-8 illustrates the absolute change of CR in all PBS R groups from one day to the next. Prominent is the high variability in change of CR from day 5 to day 6, the last day of the experiment. The range included no changes up to 34% of decrease. Why is the variability highest at the end of the experiment? One explanation might be that the different processes induced by retrieval trials (e.g. reconsolidation, extinction) compete here for behavioral control. By running the experiments at various times of the year, in different years and with animals from different colonies additional uncontrollable parameters are introduced that could influence the response probability.

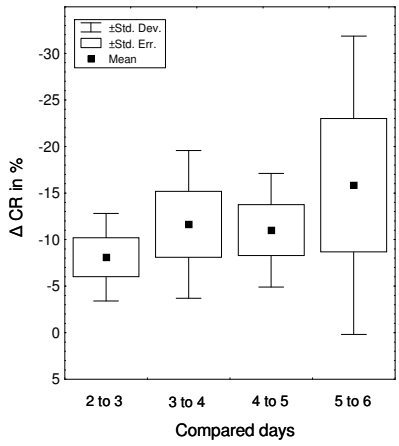

Figure 2-8 Decrease of CR of the saline-injected and retrieved groups (PBS R) of experiments 1-5. Illustrated are the means (black squares) of change in CR in % from one day to the next as indicated at the abscissa. Boxes represent the standard error and whiskers the standard deviation.

Disturbing the reconsolidation process with the double amount of emetine results in an impaired memory on test 2 and 3 after the first retrieval, but on test 4 the difference with the retrieved and non-amnestic treated group is not significant (Figure 2-6). This is most probably due to the induced extinction process. Extinction counteracts behaviorally with the reconsolidation process. A reconsolidation process strengthens the CR, whereas an extinction process diminishes it. In the experiments presented here the reconsolidation process is disrupted in retrieved and amnestic treated animals, which leads to a decrease of CR. In addition, the extinction process induced by multiple testing also decreases the response level in non-amnestic treated groups. Reconsolidation is defined by the differences in CRs between both groups. This becomes more difficult if an additional decrease of CRs due to extinction is involved. Furthermore, in all presented experiments the CR never decreased below 20%. Also in experiments with 1-trial PER conditioning it is demonstrated that after 4 days of daily retrieval around 20% of the animals still react (Sandoz et al., 1995). This residual 'memory' is not blockable with translation or transcription inhibitors (Grünbaum & Müller, 1998; Friedrich et al., 2004). It is conceivable that the CR in the retrieved and emetine-treated group could not decline further, due to some kind of 'ceiling effect'. Therefore the phenomenon of temporal inhibition of the acquisition memory observed with the double amount of emetine is probably due to the induced extinction process. For a final conclusion whether the impaired memory recovers spontaneously, an experiment should be conducted in which the animals receive single trials instead of multiple trials. This would exclude the extinction phenomenon.

Anisomycin vs. Emetine

With the double dose of emetine an impaired memory is seen two days after the first retrieval, one day earlier than with application of the mixture of anisomycin + emetine. A different time course for both inhibitors is also observed in discrimination learning in gerbils, but with an opposite time course. Injection of anisomycin results in a memory impairment on the successive two days, whereas emetine decrease the CR three days after application (Kraus, 2002). Therefore, one should consider the different actions of both protein synthesis inhibitors. Anisomycin, a protein synthesis inhibitor which is used in many reconsolidation experiments, binds at the 50S ribosomal subunit and inhibits the elongation of peptides (Grollman & With the technical assistance of Maria Walsh, 1967). In addition, it also activates two members of the mitogen-activated protein (MAP) kinase family, p38 and a stress-activated protein (SAP) kinase (Barros et al., 1997)directly. Emetine is supposed to act at the 40S subunit of the ribosome and blocks the translocation of the peptidyl-t-RNA (Carrasco et al., 1976; Jimenez et al., 1977). Through this arrest two other member of MAP kinase family are activated, the extracellular signal regulated protein kinases 1 and 2 (ERK 1/2).

The activation of ERK 1/2 in memory consolidation has been demonstrated for many paradigms and model systems. To my knowledge there are only two studies investigating the role of p38. In one study associative learning in rabbits leads to activation of p38 and of ERK 1/2. Furthermore, inhibition of p38 and ERK 1/2 results in impaired acquisition (Zhen et al., 2001). Recently it was shown that in honeybees three CS-US pairings increases the phosphorylation level of ERK 1/2. Moreover, inhibition of ERK 1/2 results in impaired memory retention two days after training. However, a change in phosphorylation level of p38 due to CS-US pairings could not be shown (Plekhanova, 2005). The involvement of ERK 1/2 in reconsolidation is shown in an object recognition task (Kelly et al., 2003) and in fear conditioning (Duvarci et al., 2005) in rats. Is it possible that the observed memory impairment in honeybees is due to the activation of ERK and/or p38? Since there is no data at all available about the involvement of p38 in memory formation in bees and in reconsolidation, the focus will be on ERK 1/2. In the studies mentioned the time course of consolidation and reconsolidation were similar in rodents. If one speculates that application of emetine leads to an activation of ERK 1/2, one should assume that this results in an improved memory. A retrieval trial can induce two processes, formation of a CS-noUS association,

which leads to consolidation of an extinction memory or reactivation of the CS-US association, which leads to reconsolidation of the acquisition memory. Thus during the retrieval bees can learn about the CS-noUS association and an extinction memory would be formed. The decreased response two days later could then be due to facilitated extinction learning. But as mentioned above, emetine inhibits the formation of extinction memory if two CS-only trials are applied 24 hours after conditioning (Chapter II). However, in this case emetine is applied in a low dose (10 mM). On the other hand, if one assumes that retrieval leads to reactivation of the CS-US association, the activation of ERK 1/2 could enhance the memory. It is unknown whether this could lead to a faster reconsolidation deficit. But in both cases the translational machinery that is thought to stabilize the extinction memory or the acquisition memory is inhibited.

Studies on the formation of LTM in honeybees used anisomycin, emetine or the mixture of anisomycin + emetine to interfere with the consolidation process. If anisomycin was systemically applied one hour after conditioning, memory impairment could be demonstrated 3 and 4 days afterwards (Wüstenberg et al., 1998). Emetine or the mixture of anisomycin + emetine were injected 30 min before conditioning and in both cases CR decreases significantly the next day. This impairment lasted for the observed period of four days (Friedrich et al., 2004).). If one assumes that emetine activates ERK 1/2, an improvement in memory should be seen on the following days, because during conditioning animals learn about the CS-US association. This is not the case.

Therefore, conclusions about the different action of both inhibitors cannot be made by exclusively interpreting the behavioral data. Further investigations are necessary to clarify this point. It would already help to inject animals with emetine and measure the phosphorylation level of ERK 1/2 at different time points.