SHORT COMMUNICATION

Long- but not medium-term retention of olfactory memories in honeybees is impaired by actinomycin D and anisomycin

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Abstract

Although work in a wide variety of species and paradigms has demonstrated that long-term memory is sensitive to the blocking of protein synthesis, previous studies have suggested that the honeybee might represent an exception to this rule. Retention tested one day after training was not impaired by the inhibition of translation by cycloheximide. Using blockers of either transcription (actinomycin D) or translation (anisomycin), we present experiments that reconcile this unusual finding by testing over longer retention periods. Honeybees were conditioned to associate an odourant with a sucrose reward. Typically, this leads to stable retention over days. However, injection of either drug led to lower retention after 4 days, whereas retention after 2 or sometimes even 3 days was unaffected. This dissociates two forms of memory: a protein synthesis-independent, medium-term memory (up to 3 days) and a protein synthesis-dependent, long-term memory lasting for at least 4 days.

Introduction

Long-term memory formation is generally found to be dependent on protein synthesis. This has been confirmed in animals ranging from insects and molluscs to mammals (Davis & Squire, 1984). The honeybee seemed to be an exception to this rule, as Wittstock et al. (1993) and later Menzel et al. (1993) and Wittstock & Menzel (1994) reported that its ‘long’-term memory is unimpaired by the inhibition of protein synthesis with cycloheximide. These studies typically tested for retention up to 1 day after training, because effects of protein-synthesis impairment are commonly detectable within that period (Davis & Squire, 1984). However, testing only over 24 hours may not be sufficient, for two reasons. First, retention of classical conditioning in honeybees after 4 days is better following spaced training (20 min inter-trial-interval, ITI) than massed training (30 s ITI), whereas no ITI effect could be detected on 1-day retention (Gerber et al., 1998). Because in Drosophila spaced but not massed training induces a protein-synthesis-dependent long-term memory (DeZazzo & Tully, 1995), in the honeybee (see Menzel, 1997) 4-day but not 1-day retention might require protein synthesis. Second, just as in the honeybee (Menzel et al., 1993; Wittstock et al., 1993; Wittstock & Menzel, 1994), a protein-synthesis-independent memory lasting for 1 or 2 days after training was also shown in Drosophila, whereas retention over longer periods required protein synthesis (DeZazzo & Tully, 1995). Thus, memory underlying 1-day retention might be regarded as a form of medium-term memory dissociable from ‘true’ long-term memory by its independence from protein synthesis (Bailey et al., 1996). We investigated whether retention in the honeybee 4 days after training might be impaired by either actinomycin D (ACT-D) or anisomycin (ANI). Both drugs impair protein production (meant to include transcription and translation) but do so by different mechanisms (see discussion; Hurwitz et al., 1962; Grollmann, 1967). We report that they both impair long-term but not medium-term retention, reconciling findings in honeybees and other model systems. Correspondingly, an analysis of how protein synthesis is regulated and, ultimately, which proteins are needed (Rose, 1995) might become possible also in honeybees (Eisenhardt et al., 1997; Grunbaum & Muller, 1998; ).

Materials and methods

Honeybees (Apis mellifera carnica) were caught as they departed from the hive, cooled and fixed in harnesses that allow free movement of antennae and mouthparts (Bitterman et al., 1983). Approximately 15 min after recovery, the bees were fed with three droplets of 1.25 M sucrose solution, a concentration used throughout the experiments. Over the subsequent 5 days, the bees were fed daily, about 1 h after the last acquisition or retention trial, respectively. They were kept overnight in a cool (18–20 °C), dark and humid box. One hour prior to acquisition or retention trials, respectively, the bees were fed with an additional droplet. This should equilibrate for motivational state. Feeding also served to test reflexes: extension of the proboscis after one antenna was touched with sucrose solution. A reflex (and later a conditioned response) was scored whenever the proboscis crossed a virtual line between the opened mandibles. Only honeybees showing intact reflexes (approx. 90%) were used.

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Carnation oil from a pharmacy served as conditioned stimulus, delivered through a 1-mL syringe daily loaded with a filter paper soaked with 5 µL odourant. A computer-controlled valve shunted air from an aquarium pump through the syringe to provide a 4-s odourant puff; odourant-loaded air was removed by an exhaust. The olfactory stimulus dominates associative memory, because it has a substantially higher salience than the mechanosensory (air puff) component (Menzel, 1990).

Two seconds after odourant onset, the unconditioned, rewarding stimulus was applied by touching both antennae with a sucrose-moistened toothpick. The bees were then allowed to lick sucrose, leading to a total reward duration of 4 s.

At the beginning of acquisition and retention tests, the bees were moved from their resting positions to the experimental site in front of the exhaust. After a 30-s accommodation period, odourant and reward were delivered. Acquisition consisted of three such pairings with an ITI of 2 min. Thus, animals remained at the experimental site for 4 min plus an additional 30 s each before the first trial and after the third. During retention tests, reward was omitted.

After acquisition, 1 µL ACT-D (Sigma, Deisenhofen, Germany; 1.5 × 10⁻³ M) or ANI (Sigma; 10⁻² M) dissolved in bee ringer, or ringer alone (CONTROL), respectively, was injected manually by means of a calibrated glass capillary inserted through a hole pricked into the thoracic tergite. The time of injection was varied between experiments: either 1 h (expts 1 and 2), 6 h (expt 3) or 24 h (expt 4) after acquisition. In expt 1, two groups were injected with either ACT-D or ringer. Experiments 2, 3, and 4 included additional groups injected with ANI. Retention was assessed once on each of the 4 days following acquisition; thus, each bee underwent four retention tests. Because in experiment 4 injection was performed 1 h after the first retention test, extinction was extended over an additional day. Only bees that survived the whole experiment and showed intact reflexes after the last retention test (approx. 90% of survivors) were considered. Survival rates were determined for all experimental groups to screen for harmful drug effects.

Data are presented as percentage of honeybees showing proboscis extension (% PE) on any given trial. Kruskal–Wallis tests were used for multiple-group comparisons of acquisition or retention phases, respectively. Corresponding two-group comparisons were carried out by Mann–Whitney U-tests. In case of significance, daywise χ²-tests were performed.

Results

As Fig. 1 shows for expt 1, retention levels are significantly lower in the ACT-D injected group when compared to the CONTROL. (U = 3422.5, P < 0.05). The ACT-D injected group shows impaired retention only on day 4 (χ² = 13.88, P < 0.001, d.f. = 1) but on none of the earlier days (for days 1, 2 and 3: χ² = 2.46, χ² = 0.07, χ² = 2.57, P > 0.05, d.f. = 1 in all cases). These effects were not due to spurious effects of variable acquisition levels, because these were statistically indistinguishable in CONTROL and ACT-D injected groups (U = 3764.0, P > 0.05). In addition, comparison of the survival rates shows a significantly better survival of the ACT-D group (no Fig.; U = 15225.5, P < 0.001). Thus, applied 1 h after conditioning, ACT-D impairs long-term retention but not medium-term retention.

In experiment 2, which also uses a postconditioning injection time of 1 h (Fig. 2A), no differences in the acquisition levels (H = 0.12, P > 0.05, d.f. = 2) become apparent, arguing against spurious differences in group composition. Retention levels do, however, show differences: Both the ACT-D group, which is a replicate of experiment 1, and the ANI group show reduced retention levels (H = 9.27, P < 0.05, d.f. = 2; ACT-D vs. CONTROL: U = 1074.5, P < 0.001; ANI vs. CONTROL: U = 2462.5, P < 0.05). Specifically, retention levels are different from CONTROL on day 3 and day 4 (ACT-D: χ² = 14.87, P < 0.001 for day 3 and χ² = 11.76, P < 0.001 for day 4; ANI: χ² = 3.97, P < 0.05 for day 3 and χ² = 8.35, P < 0.05, d.f. = 1 for day 4), but not on days 1 and 2 (ACT-D vs. CONTROL: χ² = 0.89 and χ² = 3.31, ANI vs. CONTROL: χ² = 1.3 and χ² = 0.74, P > 0.05, d.f. = 1 in all cases).

No difference was found in the survival rates of the different groups (no Fig.; H = 3.78, P > 0.05, d.f. = 2). Thus, applied 1 h after conditioning, both ACT-D and ANI impair long-term retention but not medium-term retention.

Figure 2B shows for experiment 3 that ACT-D or ANI injections 6 h after acquisition lead to significant differences in long-term retention between groups (H = 12.74, P < 0.05, d.f. = 2). In pairwise comparisons, however, only the ACT-D group (U = 545.0, P < 0.05) not the ANI group (U = 585.5, P > 0.05) shows significantly reduced retention levels when compared to the CONTROL. A comparison of daily retention levels shows significantly lower retention of the ACT-D when compared with the CONTROL group on day 4 (χ² = 4.62, P < 0.05), but not on earlier days (days 1, 2 and 3: χ² = 0.39, χ² = 0.06 and χ² = 1.92, P > 0.05, d.f. = 1 in all cases). As in all previous experiments, no differences are revealed for the acquisition phase (H = 3.05, P > 0.05, d.f. = 2). If tested across groups, mortality rates differ significantly (no Fig.; H = 15225.5, P < 0.05, d.f. = 2).

In pairwise comparisons, ACT-D does not influence mortality (U = 3135.0, P > 0.05) but ANI leads to higher mortality rates (U = 2544.5, P < 0.05) compared to the CONTROL. Thus, applied six hours after conditioning, ACT-D impairs long-term retention but not medium-term, whereas ANI does not have a significant effect at this postconditioning injection time.

Figure 2C1 shows for experiment 4 that neither ACT-D nor ANI injections lead to significant differences of retention levels (H = 0.06, P > 0.05, d.f. = 2) when injected 24 h after conditioning. As in all previous experiments, no difference was observed in the acquisition phase (H = 1.91, P > 0.05, d.f. = 2). This analysis refers to retention on days 1–4; this is reasonable, since these comparisons match the bees of all experiments with respect to the total duration of the
Fig. 2. Acquisition and retention for experiments 2–4. The figure presents the percentage of honeybees extending the proboscis on a given trial (% PE). Either ACT-D (N from A to C2: 58, 35, 88, 55), ANI (N from A to C2: 110, 27, 75, 50) or ringer (CONTROL; N from top to bottom 64, 47, 98, 66) were injected either 1 (A), 6 (B) or 24 h (C1, C2) after acquisition. C2 presents a subset of the animals from C1, but includes only those animals which survived until day 5 after acquisition. For multiple group comparisons of acquisition and retention, data were analysed with Kruskal–Wallis tests. All other details as in Fig. 1.

Discussion

We show that although neither ACT-D nor ANI impairs medium-term (1 or 2 day) retention, both impair long-term (4 day) retention. The question arises of which mechanisms underlie this impairment. Two points argue for protein production as the critical factor. First, ACT-D impairs transcription by intercalating into nuclear DNA (Hurwitz et al., 1962), and ANI impairs translation by blocking the peptidyl transferase reaction on the ribosome (Grollmann, 1967). In honeybees, these drugs most likely act in this way: Wittstock (1991) has shown for the honeybee that 45 min after injection of 300 nL $10^{-2}$ M ANI, protein synthesis is inhibited by 90%. Concerning ACT-D, preliminary experiments indicate a 60% decrease of mRNA nucleotide insertion when measured 2 h after injection of 1 µL $1.5 \times 10^{-3}$ molar ACT-D (L. Gruenberg, personal communication). Second, ACT-D and ANI interrupt the way from gene to protein at different points and by different mechanisms; what they have in common is their effect on protein production. Thus, the behavioural effect that ACT-D and ANI have in common can most plausibly be explained by their like effect on protein production. Furthermore, reduced vitality cannot explain our results: Although both drugs impair long-term retention, ACT-D was in one case shown to improve survival (expt 1), whereas ANI had in one case a detrimental effect (expt 3), which did not translate into reduced retention in that case (Fig. 2B).

ACT-D and ANI might act on retrieval rather than on memory formation. If this were the case, one would predict that retrieval should always be impaired when tested 4 days after injection. In experiment 4, however, injections 24 h after training leave retention intact on the fourth day after injection (Fig. 2 C2). Therefore, the impairment of long-term retention is likely due to an impairment of memory formation rather than of retrieval.

Our demonstration of a protein-synthesis-dependent form of long-term memory in the honeybee might be surprising: Wittstock et al. (1993) and Menzel et al. (1993) have reported that long-term retention is not impaired by inhibition of translation with cycloheximide. Retention tests in Wittstock et al. (1993) were performed up to 24 hs after training and thus remained in the range of medium-term retention. Our finding that medium-term retention is unaffected by ACT-D and ANI thus confirms these results of Wittstock et al. (1993). The study by Menzel et al. (1993) did test for retention of up to 3 days, but the lack of an effect might have been due to seasonal variation (see below; compare Fig. 1 with Fig. 2A). Additionally, sample size in that study might have been too low (about $n = 20$) for the results to reach significance. In our case, such a low sample size might have been the reason for a lack of a significant effect of ANI when injected 6 h after training.

Protein synthesis necessary for long-term memory generally seems to be restricted to one or two hours after training (Davis & Squire, 1984). However, results in chicks (Rose, 1995) and rats (Grecksch & Matties, 1980) indicate two windows of susceptibility to protein levels on day 4 represent performance three days after injection. Given these considerations, we prolonged testing in experiment 4 for one additional day and restricted the analysis of retention to days 2–5. As Fig. 2C2 shows, neither retention levels ($H = 3.88, P > 0.05$, d.f. = 2) nor acquisition levels ($H = 1.31, P > 0.05$, d.f. = 2) differ between groups. No differences in the mortality of the experimental groups were detectable (no Fig.: 4-day retention: $H = 8.54$, 5-day retention: $H = 0.15$, $P > 0.05$ and d.f. = 2 for both). Thus, applied 24 h after conditioning, neither ACT-D nor ANI impairs long-term retention.

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synthesis inhibition: one immediately after training and another beginning several hours after training. Interestingly, amnestic effects of antibodies to cell adhesion molecules presumably synthesized during the second window become apparent as late as 48 h after training (Alexinsky et al., 1997). Our results show a retention impairment when ACT-D is applied either 1 or 6 h after training. This could represent one long window of susceptibility to interference with protein production. If, however, a two-window organization of protein synthesis exists in honeybees (as it does in chicks and rats) this may represent the second window. Because no amnestic effects of ANI or ACT-D were detectable until as late as 3 days after training, this would roughly correspond to the above-mentioned late amnestic effects reported by Alexinsky et al. (1997). However, because we did not employ pretraining injection times or injection times between 1 and 6 h, it must remain unresolved for now how the time dependence of protein synthesis in honeybees relates to the findings in vertebrates.

Thus, our results suggest the existence of a medium-term memory that is independent from de novo protein production. Medium-term retention is stable for at least 2 days (Fig. 2A,B), in some cases even three (Fig. 1). The reasons for this variation remain unclear: the molecular machinery may vary seasonally, but direct evidence for such phenomena is lacking. Long-term memory (4 days), however, can be impaired by inhibition of protein production. Correspondingly, Gerber et al. (1998) have found that medium-term retention is equal after massed training (30 s ITI) and after spaced training (20 min ITI), whereas long-term retention is reduced for massed training. This might suggest that spaced rather than massed training leads to protein-synthesis-dependent long-term memory (Menzel, 1997), as demonstrated by DeZazzo & Tully (1995) for Drosophila. Also, as is the case in Drosophila, retention in the honeybees’ medium-term range might be heterogeneous. Müller (1996) demonstrated that at least two forms of memory underlie retention 24 h after conditioning: One is inducible by one-trial learning and insensitive to both cooling amnesia and inhibition of nitric oxide synthase (which might correspond to the anaesthesia-resistant medium-term memory in Drosophila), and the other is sensitive to such inhibition but not to cooling amnesia and is inducible only by at least three trials.

Thus, bees form at least two types of memories: medium-term memory independent of protein synthesis and long-term memory that is protein synthesis dependent. Further work must now concentrate on which proteins need to be produced for long-term memory and how their synthesis is regulated. Candidate enzymes involved in long-term memory formation might include protein kinases A (Eisenhardt et al., 1997) and C (Grünbaum & Müller, 1998).

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Abbreviations

ACT-D actinomycin D
ANI anisomycin
ITI Inter Trial Interval
PE Proboscis Extension

References