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Pharmacology, Biochemistry and Behavior 75 (2003) 141–146
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Time-dependent effects of cycloheximide on long-term memory in the cuttlefish

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Received 20 December 2002; received in revised form 28 February 2003; accepted 28 February 2003

Abstract

When shown prawns in a glass tube, cuttlefish promptly learn to inhibit their predatory behavior and retain this ability for a long time. The cellular and molecular mechanisms of this long-term memory (LTM) are not yet known. In this study, we analyzed the dependency of LTM on de novo brain protein synthesis. Cycloheximide (CXM), a protein synthesis inhibitor, is injected intravenously immediately, 1 h, 3 h, 4 h or 6 h after the training. Retention is tested 24 h posttraining. The injections of CXM revealed one period of memory sensitivity to pharmacological intervention. CXM administered immediately or 6 h after training has no effect on LTM. Conversely, injections given between 1 and 4 h posttraining resulted in amnesia. Taken together, findings of this study establish for the first time in *Sepia officinalis* that de novo protein synthesis is an essential and time-dependent event for LTM formation of this form of associative learning.

Keywords: Cephalopod; Cuttlefish; *Sepia officinalis*; Associative learning; Passive avoidance training; Long-term memory; Cycloheximide; Protein synthesis inhibition

1. Introduction

Cephalopods show complex behavioral abilities implying the emergence of elaborate cognitive capacities that are remarkable for an invertebrate. This agrees with the fact that they possess a well-developed nervous system. Cephalopods have the largest brains of all invertebrates and their behavior is comparable in many respects with that of the lower vertebrates (Packard, 1972). For many years, studies have been carried out on instrumental learning and memory abilities in *Octopus vulgaris* (Boal et al., 2000; Fiorito and Scotto, 1992; Moriyama and Gunji, 1997; for review, see Sanders, 1975) and *Sepia officinalis* (Agin et al., 1998; Chichery and Chichery, 1992; Dickel et al., 1997, 1998, 2000, 2001; Messenger, 1971, 1973, 1977). Surprisingly, cellular and molecular studies of behavioral plasticity are scarce in cephalopods (Agin et al., 2001; Fiorito et al., 1998).

During the last decades, a number of studies have revealed that long-term memory (LTM) formation requires a de novo brain protein synthesis. Indeed, pharmacological experiments have shown that administration of inhibitors of protein synthesis around the time of training impairs LTM. This technique was successfully used for a variety of tasks in vertebrates and more recently in invertebrates (for review, see Davis and Squire, 1984; Stork and Welzl, 1999). In contrast, short-term memory is based on transient changes in synaptic morphology (for review, see Stork and Welzl, 1999).

Cuttlefish, which capture prawns by a rapid strike with the paired tentacles, can learn not to strike at prawns visible behind glass over a 20 min period. They will not strike when the prawns are presented again a few minutes afterwards, and they show no recovery of the predatory response 24 h later. This learning appears to be associative: the striking of the tentacles against the glass is supposed to be painful. Furthermore, it was clearly demonstrated that the rate of waning can be influenced by changes in the level, or type, of reinforcement and that the decrease in number of strikes is not the result of motor fatigue or a temporary incapacity to make a tentacular ejection (Messenger, 1973). When using this training procedure, with various retention

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0091-3057/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0091-3057(03)00041-8

times (between 2 min and 2 days), a biphasic retention curve was obtained. Messenger (1971, 1973) has considered this curve as a product of two memory stores as in other animals: a labile short-term memory lasting minutes and a LTM lasting at least 2 days.

In the current study, we investigated the role of protein synthesis in long-term retention of training experience in the cuttlefish (*S. officinalis*). We used cycloheximide (CXM) that inhibits translation of mRNA (initiation, translocation and steps of elongation processes) (Gale et al., 1981).

2. Materials and methods

The life cycle of the cuttlefish in the English Channel is characterized by a succession of relatively homogeneous population cohorts (Chichery and Chichery, 1992). The adults aged between 21 and 22 months migrate towards coastal waters from the beginning of April to the beginning of May for reproduction. These animals then have a very rapid phase of senescence, which leads to their death at the end of June or the beginning of July. The maximum peak of egg laying is in May and the maximum peak of hatching is at the end of July or the beginning of August. The young cuttlefish then grow quickly and leave the cold coastal waters at the end of October. On the following spring (at the end of May or the beginning of June), these cuttlefish return to the coastal waters and undergo a new phase of extremely rapid growth. Then, the animals once again leave the coastal waters before the winter. At this stage, they average 16 months in age. Thus, according to the size of the animal and the date of its capture, an age can easily be assigned to it. Because of practical difficulties of transportation and housing, cuttlefish used in this study came from five batches. Animals (body weight between 700 and 1200 g) of both sexes were collected by a trawler several miles off Ouistreham (France) coast in August–October 2001 ($\approx$12–14 months old) for the first three experiments and

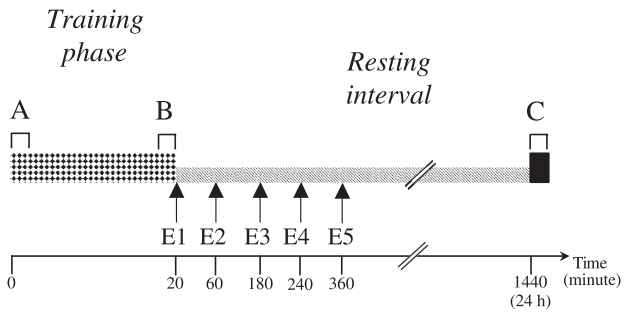

Fig. 1. Schematic representation of the experimental procedure. The figure shows the training phase (20 min duration), the resting interval (24 h duration) and the testing phase (3 min duration). Arrows indicate administration of either saline or CXM immediately (E1), 1 h (E2), 3 h (E3), 4 h (E4) or 6 h (E5) after training. (A and B) First and last 3 min of the training phase, respectively. (C) Three minutes of the testing phase.

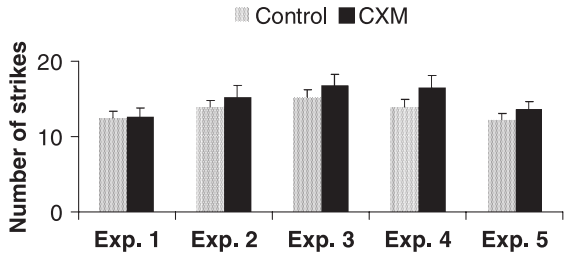

Fig. 2. Feeding motivation. The figure shows the number of tentacle strikes during the first 3 min of the training phase. Saline or CXM was injected either immediately (Experiment 1), 1 h (Experiment 2), 3 h (Experiment 3), 4 h (Experiment 4) or 6 h (Experiment 5) after training. Vertical bars indicate S.E.M.

in August–September 2002 ($\approx$12–13 months old) for the last two experiments. They were, thereafter, housed in individual tanks with circulating seawater at 15 °C. They were daily fed ad libitum with live shrimps. Animals showing external scars or not eating regularly were discarded. After acclimatization (3 days), animals from each batch were randomly assigned to two treatment groups: saline (CONTROL) and CXM (Sigma, St. Louis, USA). Each tested animal was naive at the outset and was used only once.

Cuttlefish were injected, for CXM groups, with 10 mg/kg body weight of CXM dissolved in physiological saline (10 mg/ml) or, for CONTROL groups, with an equivalent volume of physiological saline. This dose of CXM produced no incidence of obviously abnormal behavior up to 1 week following injection. Conversely, higher doses were left out because of their lethal or toxic effects. For instance, injections between 13 and 15 mg/kg caused a positive buoyancy, which impaired the animal's behavior (prey catching, body patterns and general motor activity). The injections were made through the side of the neck. Earlier experiments indeed have shown that the substances administered into this area with dense vasculature diffuse rapidly into the blood circulation (Chichery and Chanelet, 1972).

A schematic view of the experimental procedure is given in Fig. 1. A transparent glass tube filled with seawater and containing five shrimps was placed in the experimental tank. The glass tube was introduced into the tank 12 h prior to the start of the experiment, during which time the prey were concealed behind an opaque plastic cylinder placed around the tube. The glass tube containing the prey was opened to the view of the cuttlefish for a single session of 20 min (training phase). During this time, the number of strikes that a cuttlefish exhibited was counted. The exact time at which training was considered to have commenced was the moment of the first tentacle strike on the glass. Cuttlefish that did not make any attempt to capture a shrimp within the first minute were eliminated. To insure that feeding motivation was high, we only considered animals that made six or more capture attempts during the first 3 min of the training phase. At the

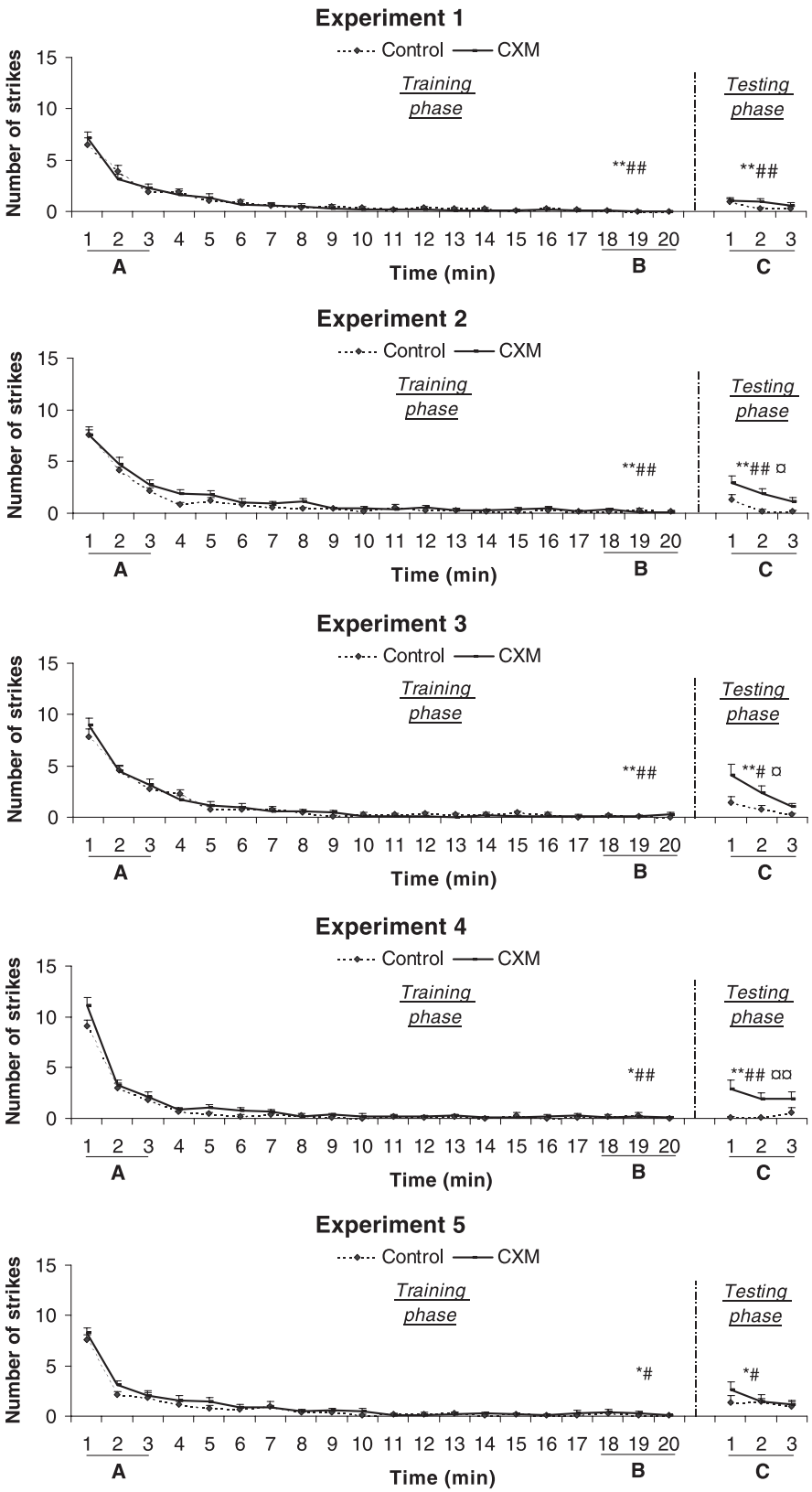

Fig. 3. Acquisition and retention curves. The figure shows the number of tentacle strikes during training and testing phases. Saline or CXM was injected either immediately (Experiment 1), 1 h (Experiment 2), 3 h (Experiment 4) or 6 h (Experiment 5) after training. (A and B) First and last 3 min of the training phase, respectively. (C) Three minutes of the testing phase. *Indicates significant difference between (B) and (A) or between (C) and (A) for CONTROL animals (Wilcoxon test for matched paired data; **P<.001, *P<.005). $^{\#}$Indicates significant difference between (B) and (A) or between (C) and (A) for CXM animals (Wilcoxon test for matched paired data; $^{\#\#}P$<.001, $^{\#}P$<.005). ¤ Indicates significant difference between CONTROL and CXM groups (Mann–Whitney U Test; ¤¤ P<.001, ¤ P<.05). Vertical bars indicate S.E.M.

end of the training phase, the opaque cylinder was again placed around the glass tube (in 3–4 s) and left for 24 h, during which time the cuttlefish were not fed. The injections of saline or CXM were made immediately [Experiment 1: $n=25$ (25)], 1 h [Experiment 2: $n=20$ (23)], 3 h [Experiment 3: $n=16$ (17)], 4 h [Experiment 4: $n=18$ (18)] or 6 h (Experiment 5: $n=14$ (15)] after training. At the end of the resting interval (24 h), the opaque cylinder was removed again and the number of strikes during a single 3 min session (testing phase) was counted to determine the level of memory recall of the animals for the inhibition of their predatory behavior. The injections and the testing phase were conducted using blind procedure.

To evaluate the feeding motivation between groups, the number of tentacle strikes observed during the first 3 min of the training phase was counted (Fig. 1A). The statistical significance of differences between CONTROL and CXM groups was evaluated using multiple comparisons based on Mann–Whitney *U* Tests (Siegel and Castellan, 1988).

To evaluate the acquisition performances within groups, the number of tentacle strikes observed during training phase was compared with the number observed during the last 3 min of the training phase (Fig. 1B). To evaluate 24 h retention performances within groups, the number of tentacle strikes observed during training phase was compared with the number observed during the 3 min of the testing phase (Fig. 1C). The statistical significance of differences between the two time periods was evaluated using a Wilcoxon Signed Ranks Test for matched samples (Siegel and Castellan, 1988).

To compare acquisition and retention performances between groups, the number of tentacle strikes during acquisition (Fig. 1B) or retention (Fig. 1B) was compared between CONTROL and CXM groups. The statistical significance of differences between the two groups was evaluated using multiple comparisons based on Mann–Whitney *U* Tests (Siegel and Castellan, 1988).

3. Results

In order to verify the possible effects of the individual experience of animals collected directly in the sea and aged between 12 and 14 months, the feeding motivation levels and acquisition performances were first evaluated.

Cuttlefish from all five experiments showed a high number of capture attempts during the first 3 min of the training phase and no statistical difference resulted between CONTROL and CXM-injected groups (Experiment 1: $U=313$, $P>.05$; Experiment 2: $U=228.5$, $P>.05$; Experiment 3: $U=115.5$, $P>.05$; Experiment 4: $U=123.5$, $P>.05$; Experiment 5: $U=78.5$, $P>.05$; Fig. 2). This means that the motivational state in groups is homogeneous.

CONTROL and CXM-injected cuttlefish from all five experiments made significantly less tentacle strikes during the last 3 min of the training phase when compared to their initial scores (Experiment 1: CONTROL $Z=-4.376$, $P>.001$, CXM $Z=-4.377$, $P>.001$; Experiment 2: CONTROL $Z=-3.924$, $P>.001$, CXM $Z=-4.203$, $P>.001$; Experiment 3: CONTROL $Z=-3.519$, $P>.001$, CXM $Z=-3.623$, $P>.001$; Experiment 4: CONTROL $Z=-3.73$, $P>.005$, CXM $Z=-3.725$, $P>.001$; Experiment 5: CONTROL $Z=-3.303$, $P>.005$, CXM $Z=-3.417$, $P>.005$; Fig. 3). Thus, the waning of the strikes during the training clearly shows that the animals inhibit their predatory behavior towards the shrimps enclosed in the glass tube. Moreover, acquisition did not differ significantly between CONTROL and CXM-injected groups (Experiment 1: $U=323.5$, $P>.05$; Experiment 2: $U=255$, $P>.05$; Experiment 3: $U=120.5$, $P>.05$; Experiment 4: $U=153$, $P>.05$; Experiment 5: $U=78.5$, $P>.05$; Fig. 3). Taken together, these results argue against spurious differences in group composition and allow us to study the retention performances.

In Experiments 1 and 5, CONTROL and CXM groups made significantly less tentacle strikes during the retention test when compared to their initial scores (Experiment 1: CONTROL $Z=-4.376$, $P>.001$, CXM $Z=-4.307$, $P>.001$; Experiment 5: CONTROL $Z=-2.991$, $P>.005$, CXM $Z=-3.070$, $P>.005$; Fig. 3). These results show a good retention of the learning task at 24 h. Moreover, retention did not differ significantly between CONTROL and CXM-injected groups (Experiment 1: $U=217.5$, $P>.05$; Experiment 5: $U=82.5$, $P>.05$; Fig. 3). Thus, applied immediately or 6 h after conditioning, CXM does not impair long-term retention.

In the other three experiments (Experiments 2–4), retention at 24 h was significant for all cuttlefish (Experiment 2: CONTROL $Z=-3.924$, $P>.001$, CXM $Z=-4.08$, $P>.001$; Experiment 3: CONTROL $Z=-3.518$, $P>.001$, CXM $Z=-3.437$, $P>.005$; Experiment 4: CONTROL $Z=-3.726$, $P>.001$, CXM $Z=-3.578$, $P>.001$; Fig. 3). However, retention was significantly better for CONTROL group than for CXM-injected group (Experiment 2: $U=129$, $P>.05$; Experiment 3: $U=73.5$, $P>.05$; Experiment 4: $U=61.5$, $P>.001$; Fig. 3). Thus, applied 1, 3 or 4 h after conditioning, CXM impairs long-term retention.

4. Discussion

Cuttlefish given an injection of CXM 1, 3 or 4 h (Experiments 2–4) after a single training phase (which normally leads to a significant LTM) showed impaired long-term retention performances compared to CONTROL animals when tested 24 h later. This deficit cannot be explained in terms other than drug-induced amnesia because no sign of discomfort (such as increase/decrease of motor activity level or physiological abnormalities) was recognizable in treated animals (e.g., Flood et al., 1973); in other words, they were indistinguishable from controls. Therefore, the deficit cannot be due to sickness induced by the

inhibitor. Furthermore, no significant difference in acquisition and feeding motivation levels could be found between CONTROL and CXM-injected groups. Gale et al. (1981) reported that CXM inhibits translation of mRNA (initiation, translocation and steps of elongation processes). This study establishes for the first time in *S. officinalis* that de novo protein synthesis between 1 and 4 h posttraining is an essential event for LTM formation of this form of associative learning. Our results are consistent with earlier research in various species in which it has been shown that LTM formation requires the production of new gene products that are normally expressed in a time-dependent manner after training (Grecksch and Matthies, 1980; Hermitte et al., 1999; Rose, 1995; Tiunova et al., 1998a,b; Wüstenberg et al., 1998).

Treated cuttlefish in Experiments 2–4 showed that CXM did not completely abolish the conditioned passive avoidance. This may be due to some memory consolidation occurring despite protein synthesis inhibition as has been suggested by Rosenblum et al. (1993) in rats or more likely to a moderate degree of cerebral protein synthesis inhibition. Indeed, we chose to inject a weak dose of CXM to avoid any toxic and side effects of the drug. Therefore, the intensity and duration of protein synthesis inhibition was likely insufficient for a complete blockage of LTM in our experimental conditions.

We show that CXM injected immediately after training was not able to disrupt a memory trace after 24 h. This result might be related to immediate early genes (IEG) (Guzowski, 2002). In fact, there are numerous reports indicating that the IEG is the first group of genes to be expressed following a synaptic activation. IEG is operationally defined as those RNA expressed in the presence of protein synthesis inhibitors and therefore do not require de novo protein synthesis for expression. Their induction is rapid, transient and protein synthesis independent (Anokhin et al., 1991; Cochran et al., 1984; Greenberg et al., 1986; Guzowski et al., 1999, 2000, 2001; Herdegen and Leah, 1998). Such an early expression of IEG can be easily considered given the duration of the training phase used in our study (20 min) and the high level of inhibition of the predatory behavior acquired by cuttlefish as early as 10 min after the training phase. Thus, after this initial protein synthesis-independent step of memory consolidation, the protein products of the IEG would act as transcriptional regulators by mediating the downstream expression of "late" genes sensitive to protein synthesis inhibitors and involved in long-term and stable changes occurring in neurons in response to learning (Guzowski, 2002; Sheng and Greenberg, 1990). When CXM is injected immediately after training, it is possible that the effective concentration of the drug becomes insufficient to disrupt this later protein synthesis-dependent step of memory consolidation.

Experiments performed in rats and chicks suggested the existence of two or three waves of protein synthesis during memory consolidation (Grecksch and Matthies, 1980; Rose, 1995; Tiunova et al., 1998a,b). In our study, injections of CXM between 1 and 4 h posttraining resulted in amnesia for the task, but injection at 6 h after training did not. This delay could represent the end of a time window of susceptibility to interference with protein production. It remains to determine whether other waves of protein synthesis after 6 h could be crucial in this passive avoidance task for LTM formation in cuttlefish.

In cephalopods, two important structures seem to be involved in learning and memory: the vertical lobe complex and the optic lobes (for review, see Sanders, 1975). We provided metabolic evidence in cuttlefish for the involvement of the superior frontal lobe (a structure of the vertical lobe complex) in these processes (Agin et al., 2001). Injections made directly into these regions at different times could give us more information in terms of specific location of LTM processes in this learning task. Such experiments have been done in mammals and have shown (e.g., for avoidance learning) that the amygdala is one of the primary sites of memory blocking effects of CXM (Bailey et al., 1999; Berman et al., 1978; Kesner et al., 1981).

In conclusion, the findings of this study establish for the first time in *S. officinalis* that de novo protein synthesis is an essential and time-dependent event for LTM of this form of associative learning. This process appears to be highly conserved in the course of evolution throughout the animal kingdom. We are now performing differential display polymerase chain reaction (PCR) as originally described by Liang and Pardee (1992) in order to determine the nature of proteins involved in the establishment of LTM in *Sepia*.

Acknowledgements

This research was supported by the "Ministère de la Recherche et de la Technologie" and the "Société de Secours des Amis des Sciences." We are grateful to Mrs. Chantal Marais for her technical assistance and to Dr. Sigurd von Boletzky for helping to correct the English translation.

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