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Cycloheximide Inhibits Context Memory and Long-Term Habituation in the Crab *Chasmagnathus*

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PEDREIRA, M. E., B. DIMANT, D. TOMSIC, L. A. QUESADA-ALLUE AND H. MALDONADO. *Cycloheximide inhibits context memory and long-term habituation in the crab* Chasmagnathus. PHARMACOL BIOCHEM BEHAV **52**(2) 385-395, 1995.—A shadow moving over head elicits an escape response in the crab *Chasmagnathus* that habituates promptly and for a long period. The effect of the protein synthesis inhibitor cycloheximide (CY) on this long-term memory was analyzed. Two hours after injection, 10 μg CY inhibited [^{14}C]-amino acid incorporation into cerebral plus thoracic ganglia by 88% and 20 μg by 92%, but no inhibition was found at 24 h. A single injection of 10–20 μg CY given 30 min before training, failed to affect the short-term habituation. Similar doses impaired both context memory (CM) and long-term habituation (LTH) when tested at 72 and 120 h but only CM at 24 h. Such a disparity was explained by an unspecific depressing effect upon the response, attributed to an interaction between CY and training. The hypothesis was confirmed, because CY injected immediately after training disclosed amnestic effect at 24 h on both CM and LTH. A similar effect was proven when animals were injected at 2 h but not at 6 h after training. Results from experiments with pretraining and pretesting injections put aside a state-dependence or retrieval deficit effects of the drug. Taken together, findings of this article argue strongly for de novo protein synthesis as a mechanism of LTH and for the close relation between CM and LTH.

Cycloheximide Crab Habituation Memory Arthropoda

A CONSIDERABLE body of evidence has been garnered during the last 2 decades showing that protein synthesis inhibitors produce impairment in the formation of long-term memory when applied shortly before or after training [e.g., (18,20, 21,34,43)].

Recent studies, mainly performed on invertebrates, and based on a cellular and molecular approach, strongly suggest the relationship of long-term memory to protein synthesis. Thus, it has been demonstrated, in *Aplysia,* that inhibitors of protein synthesis block the long-term facilitation induced by in vitro applications of 5-HT (30) and the endocytosis of cell adhesion molecules, an early step of learning-related synaptic growth (6). Likewise, in *Hermissenda,* they impair Ca^{2+}-mediated reduction of K^+ currents, which is a major component of the conditioned response (3), as well as the long-term enhancement of generator potentials in B photoreceptors (16). Lastly, in crayfish, they hinder the expression of long-term adaptation of synaptic transmission (32). However, in the honeybee, recent results failed to show an amnestic effect on long-term olfactory memory (29).

Concurrently, experiments with vertebrates concerning the involvement of gene expression in the consolidation of new experience, are in keeping with the idea that acquisition of long-term memory depends on protein synthesis (4,5,15).

The crab *Chasmagnathus granulatus* reacts to a shadow passing overhead with an escape response that habituates quickly and for several days (13,26,46). Ongoing research on this long-term habituation, aimed at analyzing mechanistic and theoretical aspects, has been underway for the last few years. Instances are studies on its specificity, showing that the habituation is stimulus specific (26,37), frequency specific (27), and context specific (46); on its adaptive value (46); on

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its relation with age (47) and circadian circle (35); and on the probable role that several neurotransmitters or neuromodulators play in the acquisition and/or retention of the habituated response (24,28,39,40,44–46,49). Such upsurge of interest for this long-term memory model is justified, not only by the reasons usually argued in favor of a "simple model" in studying cellular and molecular correlates of learning, but also by several useful experimental advantages. Thus, the habituated response is acquired after a brief training period, as short as 5 min when a high-frequency stimulation is used (27), through a fully automated device that allows working simultaneously with 40 subjects; the long-term memory is robust, repeatedly demonstrated throughout all the experiments at our laboratory; pharmacological experiments with *Chasmagnathus* show that the effective systemic doses are remarkably lower than those administered to other animals; a large number of *Chasmagnathus* can be collected in a single capture effort; and experiments may be performed during the whole year.

In the present work, the long-term memory model of *Chasmagnathus* is used to assess the effect of cycloheximide (CY), an inhibitor of protein synthesis, on long-term habituation. Should the hypothesis that memory depends on de novo protein synthesis be confirmed, such conclusion would fit well with preliminary results suggesting a correlation between *Chasmagnathus* learning and changes in gene expression (Romano, personal communication). In addition, the putative amnestic effect of CY could be used as a tool for exploring a controversial item of the habituation theory, namely, the relation between context memory and long-term habituation. In fact, previous experiments show that habituation in *Chasmagnathus* is context specific and that context memory could be demonstrated independently of the habituation process (46). Therefore, it should be expected that long-term habituation would be blocked whenever context memory were blocked by the amnestic agent.

METHOD

Animals

Animals were adult male *Chasmagnathus* crabs 2.6–2.9 cm across the carapace, collected from water less than 1 m deep in the rias (narrow coastal inlets) of San Clemente del Tuyú, Argentina, and transported to the laboratory, where they were lodged in plastic tanks (35 × 48 × 27 cm) filled to 2 cm depth with water, at a density of 35 crabs per tank. Water used in tanks and other containers during experiments was prepared with hw-Marinex (Winex-Germany) (salinity 10–14‰, pH 7.4–7.6). The holding room was maintained on a 12 L : 12 D cycle (lights on 0700–1900 h). Animals were fed rabbit pellets (Nutrientes SA, Argentina) every 3 days, and after feeding the water was changed. Temperature of both holding and experimental rooms as well as the alley between them was maintained within a range of 19–24°C.

Experiments were carried out within the first week after the animal's arrival and between November and June (i.e., late spring, summer, and fall). Each crab was used only in one experiment.

Apparatus

The apparatus is described in detail elsewhere (39). Briefly, the experimental unit was the actometer: a bowl-shaped plastic container with a steep concave wall and a circular central flat floor 10 cm diameter, covered to a depth of 0.5 cm with water. The crab was lodged in the container that was suspended by three strings from an upper wooden framework (23 × 23 × 30 cm) and illuminated by a 10 W lamp placed 30 cm above the animal. An opaque rectangle screen (25 × 7.5 cm) could be moved horizontally across the upper border of the framework by a motor at an angular speed that allowed it to cover the entire opening in 2.3 s. Screen displacements provoked a crab's running response and, consequently, container oscillations. A stylus was centrally cemented to the bottom of the container and connected to a piezoelectric transducer. Container oscillations induced, through the transducer, electrical signals proportional to the velocity of the oscillations (14). Such signals were amplified, integrated during the recording time (9 s), and translated into numerical units ranging from zero to 1530 before being processed by computer. Thus, the scores were correlated proportionally to the velocity and number of the container oscillations recorded during 9 s. The amplification of the voltage changes was kept at such a gain that scores remained below 1530. The experimental room had 40 actometers, isolated from each other by partitions.

A computer was employed to program trial sequences, trial duration, and intertrial intervals, as well as to monitor experimental events.

Description of the Escape Response in the Actometer

The escape response in the actometer consists in the crab starting to run in an attempt to move away from the passing screen. However, because the steep concavity of the circular wall prevents the animal from climbing up, each running effort is confined to the flat center of the container, in such a way that the escape response during a single trial resembles a series of flights from the center toward the base of the wall.

Experimental Procedure

A stimulation session had a fixed number of trials given with 180-s intertrial intervals and preceded by 30 min of adaptation in the actometer. Each trial lasted 9 s and consisted of passing the screen four times over the actometer, recording the crab's activity during the entire trial time. Two sessions per experiment were run, i.e., the training session (5, 15, or 30 trials) and the testing session (2 trials), separated by different intersession intervals. Crabs were individually housed during the entire intersession interval in plastic containers covered to a depth of 0.5 cm with water and kept inside drawers dimly lighted.

The usual experimental design throughout this article included four groups having equal number of crabs (n = 35–45). Two groups were water injected (WA-groups) and two were CY injected (CY-groups), and in turn, one group of each pair was trained (TR-group) and another untrained (control group, CT-group).

Before animals were placed in the actometers to start an experiment, they underwent a selection test: each crab was turned on its back and only animals that immediately returned to their normal position were used. The rationale behind this selection is that crabs with a slow righting reaction show a low responsiveness to a large diversity of stimuli, and at a later time, they usually present unhealthy symptoms. No more than 10% of tested crabs were eliminated.

The crab's responsiveness to the passing screen proved remarkably consistent up to 10 days after arrival, but on occasion, animals coming from different capture efforts presented differences in response level. Therefore, only crabs belonging to the same capture were used in each experiment.

No pretraining was performed to estimate response base-

line, because to obtain a reliable measurement, two trials after a 30-min adaptation time were required, which sufficed to induce, in the present experimental conditions, a noticeable and far-reaching fall in reactivity. However, an acceptance criterion aimed at ensuring a minimum level of responsiveness was employed. If a WA-TR group showed during the two first training trials a mean response of accumulated score lower than 600, results of the experiment were disregarded.

Although response levels varied widely within a given group, each individual crab kept roughly the same reactivity rank at training and testing (2,24). In other terms, the relative level of response of a crab within its own group seems to represent an individual behavioral feature.

Because the number of actometers was insufficient to run all groups of each experiment simultaneously, replications during the same day were necessary. An equal number of crabs per group was used in each replication, but animals of a same group were placed in a different set of actometers each time. Thus, any potential effect of time of day and/or between-actometer differences was offset.

Injections

Glass distilled water (dw) (50 μl) or cycloheximide (CY) solution were given through the right side of the cephalothoracic-abdominal membrane by means of a syringe fitted with a sleeve to control depth of penetration to 4 mm, thus ensuring that the injected solution was released roughly at the center of the pericardial sac. Cycloheximide was purchased from Sigma Chemicals, St. Louis, MO.

Data Analysis and Evaluation of the Amnestic Effect

When groups of 30 or more crabs each were used and the criterion of responsiveness was fulfilled, there was invariably a significant difference (t-test, alpha = 0.05) between mean testing scores of control and trained groups (15 training trials and 24 h of intersession interval; or 30 trials and 72 or 120 h interval) throughout previous experiments at our laboratory. On this account, a treatment was considered indicative of amnestic effect when a Duncan multiple range test, performed on testing data of the four groups, showed no significant difference both between CY-groups (CY-CT vs. CY-TR) and between control groups (WA-CT vs. CY-CT).

An additional method was used to illustrate the difference between WA- and CY-group differences, based on the expressed view that the relative level of response of a crab within its own group represents an individual behavioral feature. Animals were ranked considering their response level during their first confrontation with the iterative stimulus. Namely, trained crabs (WA-TR and CY-TR) were ranked according to their scores during their first two trials, while control crabs (WA-CT and CY-CT) according to their two-trial scores at testing. The testing score of each ranked trained crab was matched with that of the corresponding control crab and the percentage difference for each couple of data was estimated. A t-test (alpha = 0.01, two-tailed) was performed on the percentage differences corresponding to WA- and CY-groups, respectively.

Thus, the putative amnestic effect of a drug was assessed by focusing the data analysis on testing scores. Rescorla (38) argued convincingly in favor of using this sort of analysis instead of a paired training-testing comparison, stressing the need to distinguish clearly between time of input (training session) and time of assessment (testing session).

Definitions

Throughout this article the following expressions are used with the meaning here defined. Short-term habituation refers to the response decrement within the training session; long-term habituation to a retention of the response decrement demonstrated in the testing session.

Biochemical Methods

To determine the extent of protein synthesis inhibition in the cerebral and thoracic ganglia by CY, crabs were injected with dw or CY at a dosage of 10 μg or 20 μg per animal. One or 23 h later they were injected (2 μCi/crab) with a [^{14}C (U)]-aminoacid (0.25 mCi) mixture from Dupont-NEN (Boston, MA) in 50 μl of dw. The crabs were individually housed in small containers whose bottoms were lined with filter paper, killed after 1 h, and their ganglia removed.

Ganglia of the same group (eight ganglia corresponding to four crabs) were pooled, so that each group furnished a single sample. Hepatopancreas were extracted from animals with a 2-h CY (or dw) injection-sacrifice interval and treated as ganglia.

Immediately after extraction, ganglia were immersed into liquid nitrogen. Samples were homogenized in 0.5 ml of ice-cold chloroform : methanol 3 : 2 in a hand-driven homogenizer and the suspended material was transferred to centrifuge tubes. The homogenizer was washed with 0.5 ml of ice-cold chloroform : methanol 3 : 2 that was added to the homogenate. The tubes were then spun in a centrifuge at 5000 rpm for 10 min. The supernatant was carefully separated to be further processed following a modified Folch's method (36). The pellet was washed with 1 ml of chloroform : methanol : water 1 : 16 : 16 and then centrifugated at 5000 rpm for 10 min. The supernatant was discarded and the remaining pellet resuspended in 200 μl chloroform : methanol : water 1 : 16 : 16, vortexed, and a sample of 10 μl taken for protein determination by a modified version of Bradford method (11,12). Ice-cold 10% trichloroacetic was added to the suspension and filtered through a GF/C glass filter (Whatman) in a vacuum pump. The filter was dried under a IR lamp, transferred to scintillation vials with 3 ml of toluene-based scintillation, and counted; filter papers lining the bottom of crab containers were placed in 20 ml vials and counted in the same way.

The percentage of inhibition by CY was calculated by comparing relative specific radioactivity in drug-injected crabs with that of control animals.

RESULTS

Inhibition of [^{14}C]-Amino Acid Incorporation

Two hours after injection, 10 μg of CY inhibited incorporation of precursor into ganglia protein by 88% and 20 μg inhibited by 92%, but for both dosages, no inhibition was found at 24 h postinjection. Filter paper counts were similar to blank values, thus suggesting that there was no apparent excretion of radioactive material during the 1-h injection-sacrifice interval. Incorporation of precursor into hepatopancreas proteins was inhibited by 98% when assessed 2 h after 20 μg CY administration.

This extent and duration of protein synthesis inhibition fit well with the "amnestic threshold" generally reported for CY and anisomycin (33). However, it is noteworthy that the minimum CY dose necessary to produce in *Chasmagnathus* over 80% inhibition in 2 h (circa 0.60 μg/g), is remarkably lower than that required to obtain the same effect by systemic injec-

tion in intact vertebrates [e.g., 640 to 3200 μg/g in mice, (19)]. Indeed, the effective doses of CY in intact *Chasmagnathus* are equivalent to those used for intracerebral injections in rats [e.g., 10–20 μg into the amygdala, (10)]. Such a difference in CY effect is attributable to the peculiar nature of the crab blood-brain barrier when compared to that of most vertebrates (1).

Pretraining CY Does Not Affect Short-Term Habituation

Two experiments, one aimed at assessing the exploratory activity and another one the escape response performance, were simultaneously conducted, each including two groups of crabs: one preinjected with 20 μg of CY and another one with dw (WA). During the exploration experiment, crabs were injected immediately before placed in the actometer where they remained for 2 h without receiving shadow stimulation. Their spontaneous exploratory activity was recorded nine times for 20 s each, with a 15 min interval. During the training experiments, crabs underwent a 30-trial training session after a 30-min adaptation time and their reactivity for each trial was recorded. Figure 1a shows the performances of CY- and WA-groups during the exploration session, and Fig. 1b those of corresponding groups during the 30 training trials. The close resemblance of the curves for each experiment is manifest, thus suggesting that the preinjection of CY has no effect either on the exploratory behavior or on the short-term habituation to the iterated stimulus. No overt symptoms of sickness appeared during the entire training session (2 h) with a wide range of different doses, namely, 10, 20, 30, 50, 80, and 100 μg per animal. However, when a dose equal to or higher than 30 μg was used, a slight waning in the response at the last training trials was occasionally observed, whereas 24 h after injection appendage losses, a phenomenon termed autotomy (22), as well as lowering of reactivity and a growing number of deaths correlating with increasing dose were detected.

The finding that CY has no effect on the short-term habituation is in keeping with a consistent body of results, obtained from experiments with diverse animal species (7,8,23).

Effect of Pretraining CY on Long-Term Habituation

Seventy-two hours after injection. As it is currently argued that the degree of CY-induced amnesia depends, among other variables, on the training to testing interval (17), we started the present study by testing the CY effect at long intersession interval, namely, at 72 h after training. Results from previous experiments at our laboratory indicated that for ensuring such long-term habituation, a 30-trial training is necessary instead of the usual 15 trials required for a 24-h retention.

One group of crabs (WA-group) was injected with dw and another one (CY-group) with 10 μg of CY, both 30 min before training. Half of the animals in each group underwent a 30-trial training session and the other half remained in the actometers during the time corresponding to the training session (2 h), but without being stimulated by the passing shadow. Thus, four groups were obtained and termed: WA-CT, WA-TR, CY-CT, and CY-TR.

After a 72-h training to testing interval, all animals underwent the two-trial testing session. Two reasons account for choosing this number of testing trials. First, experiments of our laboratory with dw-injected *Chasmagnathus* have shown throughout a similar relationship between testing curves of trained and control groups. Invariably, the largest between-group difference took place at the beginning of the session (at the first two trials) followed by a decrease over trials and a convergency at the asymptotic portion, namely, after 8 or 10 testing trials (46). Second,the testing session was really a retraining session so that it seemed convenient to focus the statistical analysis on the initial trials because the putative disrupting effect of CY might be masked if results corresponding to a long series of trials were considered (17).

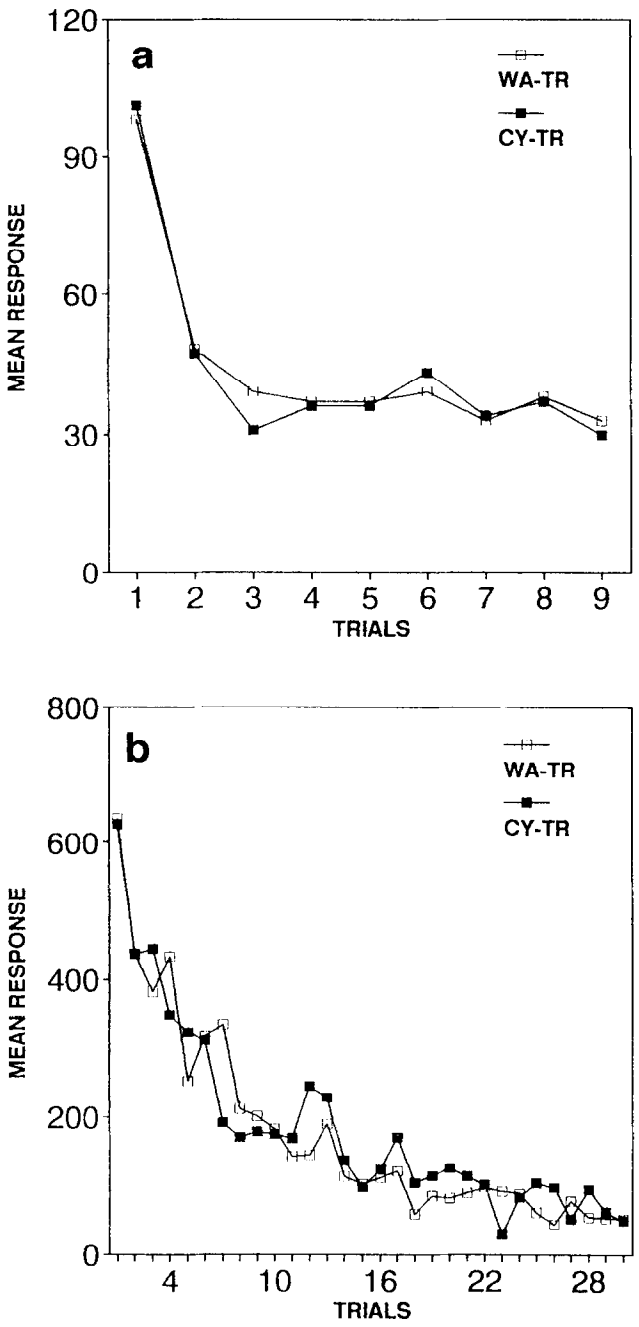

FIG. 1. Effect of CY on the exploratory activity (a) and on short-term habituation (b). White squares stand for distilled water-injected groups (WA-TR); black squares for 20 μg of cycloheximide-injected groups (CY-TR). Ordinates: (a) mean of spontaneous exploratory activity; (b) mean of escape response scores. Abscissae: (a) trials of 20 s each, 15 min intertrial; (b) trials of 9 s each, 3 min intertrial.

A 2 × 30 ANOVA of repeated measures performed on training data of WA-TR and CY-TR showed neither a significant between-group difference nor a group × trial interaction ($F = 0.25$ and $F = 0.6$, respectively). On the other hand, a significant correlation between response ranking at training and at testing was shown by a Spearman test for both WA- and CY-TR ($r = 0.75$, $p < 0.001$ and $r = 0.68$, $p < 0.001$, respectively). Thus, treated and untreated animals responded during training at equivalent levels and the reactivity ranking for each trained group was not dissimilar at training and testing. Parallel results (data not shown) were obtained throughout the following experiments.

A Duncan multiple range test performed on data corresponding to the two-trial testing block (Fig. 2a, left panel), disclosed no significant difference either between CY-groups or control groups. In addition, the mean of WA-TR proved to be significantly lower than that of CY-TR. A *t*-test applied to the percentage differences (Fig. 2a, right panel) disclosed a significant difference between WA and CY groups.

Thus, a 10 μg CY pretraining injection seems to produce a disrupting effect on the 72-h habituation.

To test whether CY impairs retention in a dose-dependent manner, two further experiments were carried out, one with the dosage increased to 20 μg per animal and one lowered to 5 μg. Except for such dose differences, the design and procedure of these experiments were as above.

Figure 2b presents data corresponding to the experiment with 20 μg. Results at testing were similar to those obtained with 10 μg CY.

Figure 2c displays scores corresponding to the experiment with the 5 μg dose. A Duncan test showed significant difference for between CY-groups but no difference between either control or trained groups (left panel). Unlike precedent experiments, no significant difference was found between mean percentage differences of WA- and CY-groups (right panel).

A replication of this series of experiments confirmed these results in all respects (data not shown).

Foregoing results are summarized in the following two points. 1) Pretraining injection with 10 μg of CY per animal produces deficit in the retention of the habituated response at 72 h. This outcome cannot be explained by an unspecific depressing or enhancing effect of the drug on both CY-groups, because no significant difference between control groups (CY-CT vs. WA-CT) was found. 2) A preinjected 5 μg dose fails to induce impairment in retention at 72 h, whereas the amnesia induced by 20 μg appears similar to that described for 10 μg. Thus, the amnestic threshold of CY may be estimated as 10 μg per animal, over which a dose-dependent effect cannot be reliably described mainly because an increasing untoward side effects becomes manifest. In other terms, the amnestic window of CY doses proves very narrow, a finding consistent with results reported for the action of this drug in other species (9).

Twenty-four hours after injection. To study the effect of a CY pretraining injection when tested after 24 h, experiments were done using the same doses that had amnestic effect at 72 h (i.e., 10 and 20 μg). Except for the intersession interval and number of training trials, design and procedure of these experiments were as above.

No retention impairment was found in CY-administered animals that underwent 15 training trials, either with 10 or 20 μg of preinjected CY (Fig. 3a,b). In fact, a Duncan test disclosed a significant difference both between CY-groups but no difference between either control or trained groups (left panel). No significant difference between percentage differ-

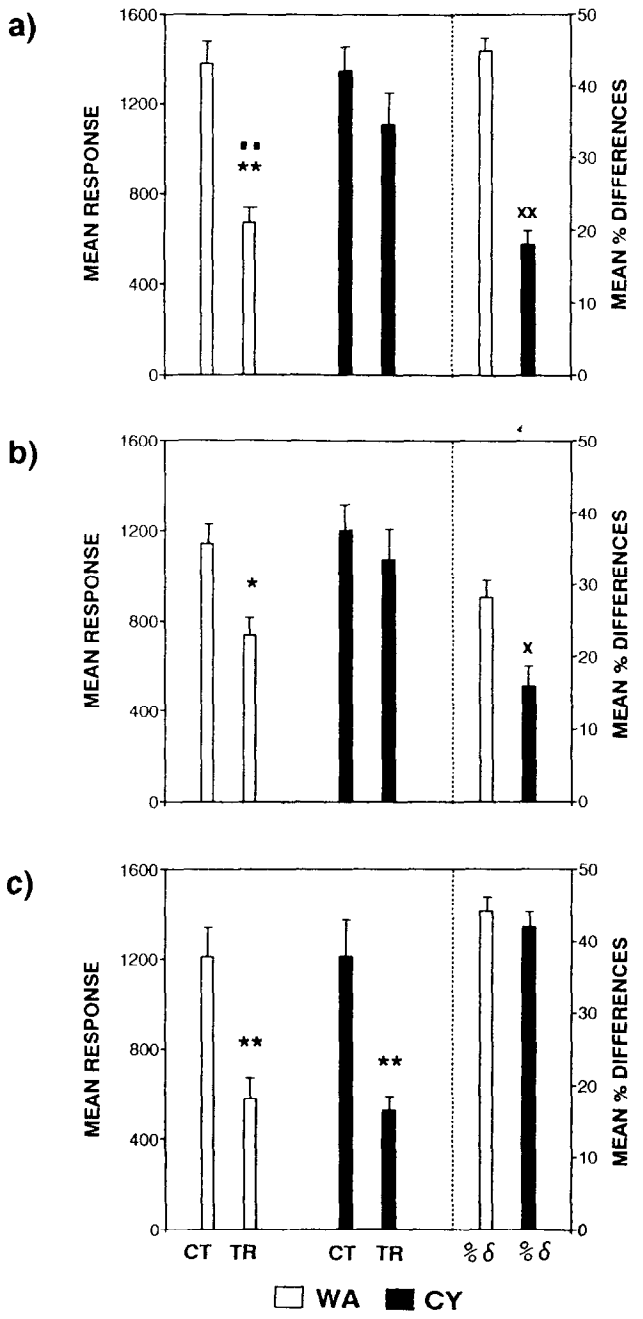

FIG. 2. Effect of 10, 20, or 5 μg CY pretraining injection on long-term habituation tested 72 h after 30 training trials. (a) CY-groups injected with 10 μg CY ($n = 40$); (b) 20 μg CY ($n = 35$); (c) 5 μg CY ($n = 35$). White bars stand for groups water injected 30 min before training (WA-groups); black bars for cycloheximide-injected groups (CY-groups). The height of each bar is the mean ± SEM. Left panels. Ordinates: mean of accumulative scores for two testing trials per group. Abscissae: CT, a control group (WA-CT or CY-CT); TR, a trained group (30 training trials, WA-TR or CY-TR). Duncan test: *Stands for $p < 0.05$ and ** for $p < 0.01$, in comparisons between WA-groups or between CY-groups; ■ for $p < 0.05$ and ■■ for $p < 0.01$ in comparisons between-trained groups (WA-TR vs. CY- TR). Right panel. Right ordinates: mean of percentage differences between scores matched by response ranking. Abscissae: %δ stands for percentage differences corresponding to WA-CT vs. WA-TR or to CY-CT vs. CY-TR. *t*-Test: × stand for $p < 0.01$, ×× for $p < 0.005$.

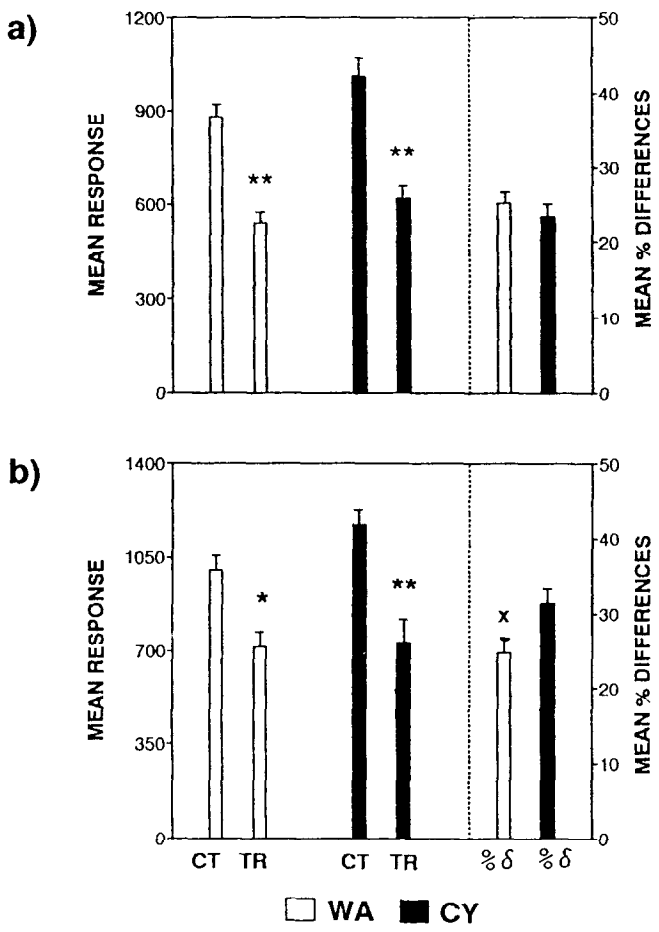

FIG. 3. Effect of 10 or 20 μg CY pretraining injection on long-term habituation tested 24 h after 15 training trials. (a) CY-groups injected with 10 μg CY (n = 45); (b) 20 μg CY (n = 45). Symbols as in Fig. 2. Trained-groups (WA-TR and CY-TR) had 15 training trials.

ences was shown in the 10 μg experiment (Fig. 3a, right panel), but interestingly, the mean of the CY percentage differences was statistically higher than that of the WA differences in the 20 μg experiment (Fig. 3b, right panel).

Before discussing the possibility that the amnestic effect is dependent on intersession interval (72 h vs. 24 h), it would be necessary to test different training intensities. In fact, retention impairment by protein synthesis inhibitors has been reported to depend on an interaction of factors, as extent of training and retention interval (19,48). Therefore, an additional series of experiments with only one dosage (20 μg) but two different numbers of training trials (30 and 5) were conducted.

Results of the experiment with 30 training trials, displayed in Fig. 4a, are in all respects similar to those obtained with 15 training trials (Fig. 3b).

Regarding the experiment with five training trials (Fig. 4b), the difference between WA-groups at testing barely reached statistical significance. This result is hardly surprising, because the number of training trials was remarkably lower than that usually employed for ensuring 24-h habituation (15 trials) and very close to that reported as not inducing long-lasting retention (3 trials) (26). On the other hand, a significant difference between CY-groups was found (left panel) and the t-test applied to the difference between percentage differences again showed a mean of CY percentage differences significantly higher than that of WA differences (right panel).

Results of this section may be summarized in two main points. 1) No CY-induced amnesia is shown by animals tested at 24 h, either with 10 or 20 μg of drug and 15 training trials, or with 5, 15, or 30 trials and 20μg of drug. 2) Most of the CY-groups exhibit a difference between control and trained groups larger than that corresponding to the WA-groups, as shown by the analysis of percentage differences.

Concerning point 1, results stand in marked contrast to those obtained at 72 h. A first attempt to explain such a difference might be done in terms of the graded effect hypothesis put forward regarding the amnestic action of protein synthesis inhibitors, namely, the CY-induced memory impairment would be shown only at 72 h because the amnestic effect increases with the training-to-testing interval (19).

However, the paradoxical result summarized in point 2 remains unexplained. In fact, trained crabs preinjected with CY not only fail to show loss in retention at 24 h but, instead, exhibited apparently a retention greater than that presented by

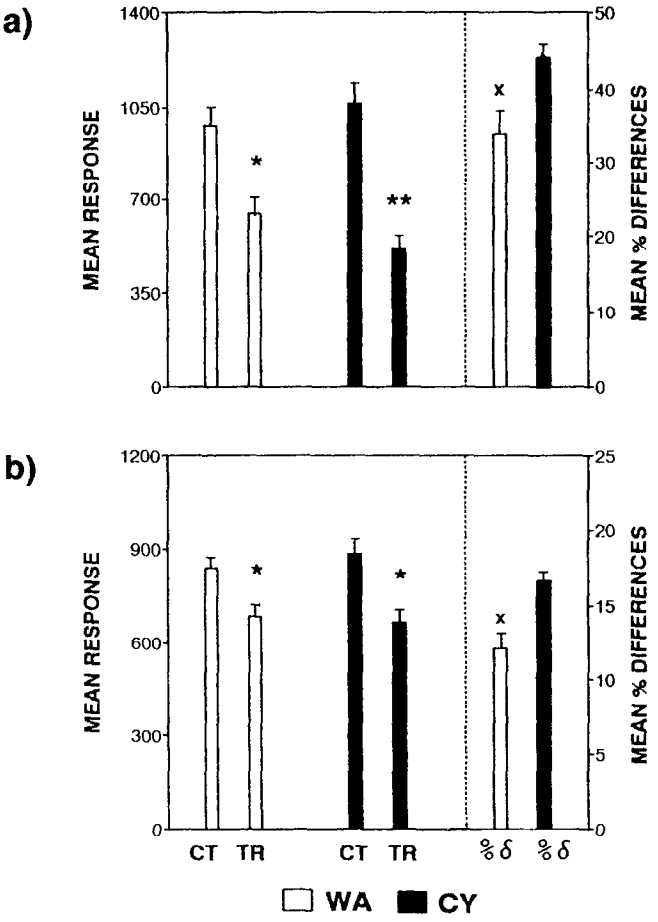

FIG. 4. Effect of 20 μg CY pretraining injection on long-term habituation tested 24 h after 30 or 5 training trials. (a) Trained groups (WA-TR and CY-TR) had 30 training trials (n = 35). (b) Five training trials (n = 40). Symbols as in Fig. 2.

the WA-trained group; in other words, CY seems to have a "hypermnestic" effect at 24 h.

One hundred twenty hours after injection. The aforementioned hypothesis that CY-induced amnesia could develop gradually after training was grounded on results from experiments with rats that included a series of retention intervals [e.g., 24 h, 1, 2, and 3 weeks; (19,42)]. However, only two intersession intervals were used in experiments above, so that it cannot be concluded whether the partial amnesia shown in *Chasmagnathus* at 72 h was actually the peak of retention impairment or a step in an increasing trend, or whether, at odds with the assumption that CY impairs memory consolidation, the amnesia disappears at longer intervals. To address this question, a further experiment was required aimed at estimating the amnesia level at a training to testing interval longer than 72 h.

For this purpose, a period of 120 h was chosen, in such a manner that the 72-h interval proved separated by the same time length (48 h) from either the shortest or the longest retention interval.

An experimental design closely similar to those of previous sections was used, with a 20-μg dose drug for CY-groups, 30 training trials and 120-h intersession interval. During the entire interval, crabs remained as usual, separately in the rest containers without being disturbed but changing the water after 3 days.

Results corresponding to the two-trial testing block (Fig. 5) were similar to those obtained at 72 h with the same dose (Fig. 2b). These results stand in marked contrast to those shown by crabs also receiving 20 μg CY and 30 training trials but tested at 24 h (Fig. 4a).

Therefore, the CY-induced partial amnesia shown at 72 h seems to persist without further change at least for a retention interval of 120 h, thus suggesting that the disrupting effect of the drug might be permanent, as should be expected if CY actually disrupts memory.

Effect of pretraining CY on context memory. A previous report (46) indicates that crabs seem to memorize the training environment after a 24-h intersession interval. In fact, animals that received prior exposure to the actometer without presentation of the passing screen exhibited at testing a lower level of escape response to the danger stimulus than those preexposed to a wholly dissimilar context.

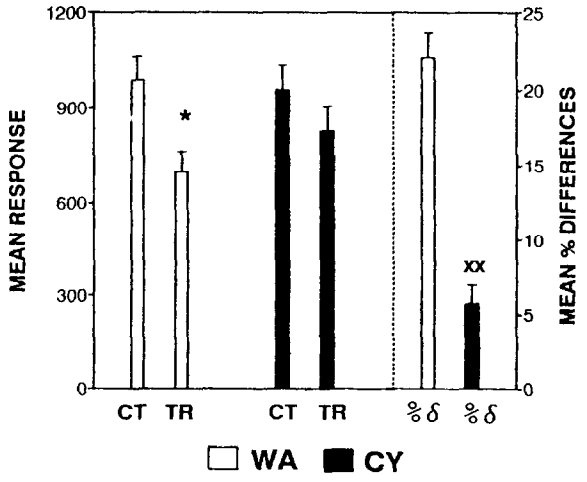

FIG. 5. Effect of 20 μg CY pretraining injection on long-term habituation tested 120 h after 30 training trials (n = 35). Symbols as in Fig. 2.

Therefore, the purpose of the following experiments was to investigate the effect of CY injected prior to training on the context memory. The procedure and experimental design were as follows.

One group of crabs (the same context-group, SAM) were kept in the actometers for a 2-h session, namely, for the total time of a 30-trial training session but without being stimulated with the passing screen. Another group (the different context-group, DIF) received a similar treatment but was kept in a transparent cylinder, whose floor was covered with a thin layer of sand, placed in a dimly lighted box. Half of the animals in each group were injected 30 min before being placed in the receptacles with dw and the other half with 20 μg of CY. Thus, four groups were formed and named WA-SAM, CY-SAM, WA-DIF, and CY-DIF. After an intersession interval, all the crabs underwent in the usual actometers a two-trial testing session with the passing screen, so that WA-SAM and CY-SAM had the same context in both sessions (actometer-actometer), while WA-DIF and CY-DIF had different contexts (cylinder-actometer).

Two experiments were conducted, one with 24-h and the other with 72-h intersession interval.

Figure 6a,b exhibits the analyses of testing results corresponding to experiments with 24 and 72 h of intersession interval. In both experiments, an impairment of context memory was manifest because no significant difference was disclosed between CY-DIF and CY-SAM (left panels) and the mean of WA percentage differences was statistically higher than that of CY percentage differences (right panels). An alternative explanation in terms of a possible sensitizing effect of the drug is untenable, as no difference between control groups (WA-DIF vs. CY-DIF) was disclosed. On the other hand, the similar reactivity of control groups could not be accounted for by a ceiling effect, due either to saturation of the experimental device or to the crab's escape response, because scores two to four times higher are obtained routinely in our laboratory using stimuli other than an opaque screen moved horizontally.

A further experiment was conducted to address the possibility that the above effect of CY might be an instance of state dependence [e.g., (25,31,34)]. If this were the case, retention should not be impaired when CY is given in association with both training and testing.

Four groups of animals as those of the precedent context experiments were made up but giving WA or CY both 30 min before training and 30 min before testing so that they were here named WA.WA-SAM, WA.WA-DIF, CY.CY-SAM, and CY.CY-DIF (Fig. 7). A retention interval of 24 h was used.

Results were closely similar to those obtained with animals that were given a single water or CY injection before training. Figure 7 displays results corresponding to the two-trial testing block. A Duncan test on these data showed no significant difference either between CY.CY-DIF and CY.CY-SAM or between controls (WA.WA-DIF vs. CY.CY-DIF) (left panel). In addition, according to the t-test analysis, the mean of WA percentage differences was significantly higher than that of CY percentage differences (right panel).

Therefore, the impairment of context memory induced by CY pretraining injection seems due to true amnesia (failure to form memory) but not to state dependence, because recall was not reinstated despite a return to the same drug state as that of training.

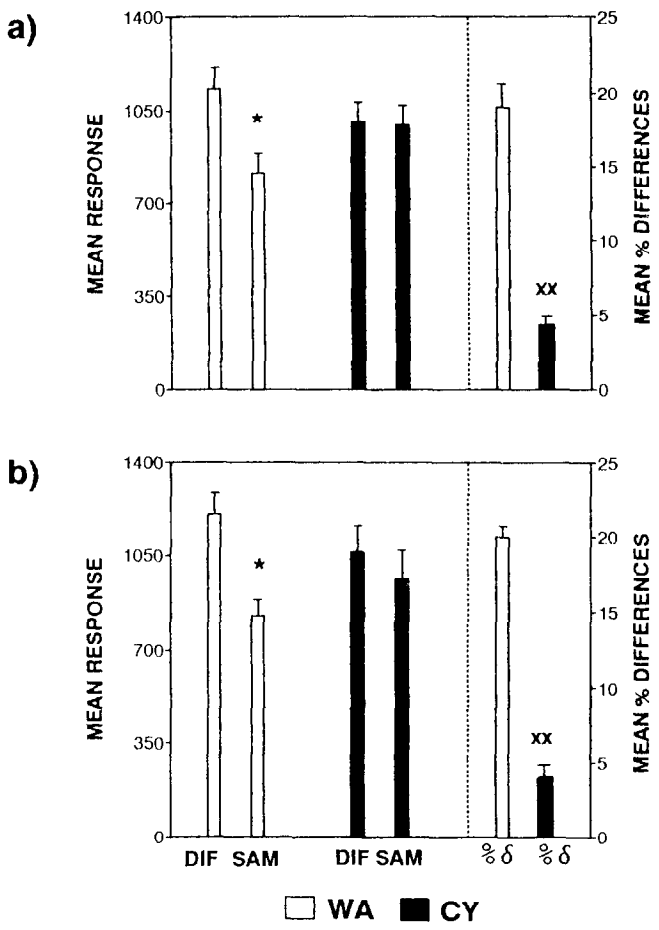

FIG. 6. Context memory. Effect of 20 μg CY pretraining injection when tested 24 and 72 h after training. (a) Testing at 24 h (n = 45); (b) at 72 h (n = 45). White bars stand for groups water injected 30 min before training (WA-groups); black bars for groups cycloheximide injected (CY-groups). The height of each bar is the mean ± SEM. Left panels. Ordinates: mean of accumulative scores for two testing trials per group. Abscissae: DIF, a group kept in cylinders during training and tested with two trials in actometers (WA-DIF or CY-DIF); SAM, a group in actometers at both training and testing (WA-SAM or CY-SAM). Duncan test: *Stands for $p < 0.05$ and ** for $p < 0.01$ in comparisons between WA-groups or between CY-groups. Right panel. Right ordinates: mean of percentage differences between scores matched by response ranking. Abscissae: %δ stands for percentage differences corresponding to WA-DIF vs. WA-SAM or to CY-DIF vs. CY-SAM. t-Test: × × for $p < 0.005$.

Effect of Posttraining CY on Long-Term Habituation and Context Memory

According to the foregoing results, the amnestic effect of CY on context memory and on memory of the habituated response seems to display different time constants. Whereas disruption of the context memory is shown 24 h after CY administration, an impairment of the long-term habituation is manifest only after longer time intervals, that is, the retention of the habituated stimulus at 24 h would be possible without memory of the environment which the animal was preexposed to.

Such a conclusion is at odds with previous reports from our laboratory (46), which show that crab's habituation is context specific, namely, that long-term retention of the habituated response depends on the memory of the environmental cues, all in keeping with the associative theory of habituation (50). However, this contradiction between results might be overcome by explaining the low reactivity of CY-TR groups at 24 h in terms other than long-term habituation. In fact, it might be hypothesized that CY administration followed by repeated presentation of a danger stimulus would produce a transient depressing effect on the response that fades with the intersession interval. In other words, the decrease in response level by CY-TR at 24 h would not result from the habituation process, but rather from an interaction between the CY-induced internal state and the iterated stimulation.

Should such assumption be correct, CY given immediately after training would be expected to induce an amnestic effect at 24 h, because no drug-training interaction takes place. Accordingly, the following two experiments were carried out with CY injected posttraining.

The experimental designs were as above, except that in the long-term habituation experiment water or CY (20 μg) was given immediately after the last training trial, whereas in the context memory experiment, after 2 h of crabs staying in the actometers or in the cylinders.

Figure 8a show testing results of the long-term habituation experiment. A Duncan performed on these data (left panel) disclosed no significant difference between either CY-groups or control groups. A t-test applied to the percentage difference

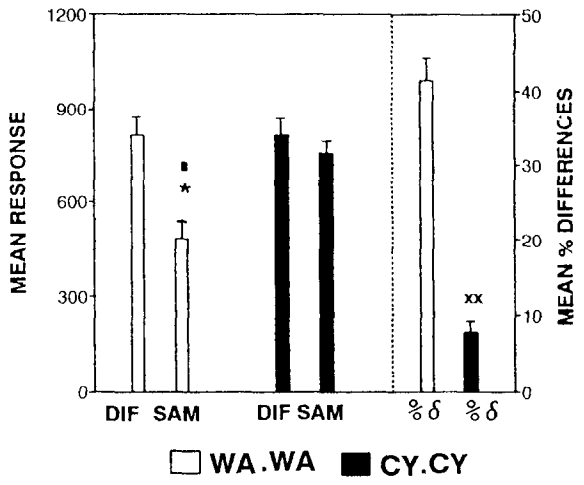

FIG. 7. Context memory. Effect of 20 μg CY pretraining and CY pretesting injections when tested 24 h after training (n = 40). White bars stand for groups water injected 30 min before training and 30 min before testing (WA.WA-groups); black bars for groups cycloheximide injected (CY.CY-groups). The height of each bar is the mean ± SEM. Left panels. Ordinates: mean of accumulative scores for two testing trials per group. Abscissae: DIF, a group kept in cylinders during training and tested with two trials in actometers (WA.WA-DIF or CY.CY-DIF); SAM, a group in actometers at both training and testing (WA.WA-SAM or CY.CY-SAM). Duncan test: ** for $p <$ 0.01 in comparisons between WA.WA-groups; *for $p < 0.05$ in comparisons between 0500 h groups (WA.WA-SAM vs. CY.CY-SAM). Right panel. Right ordinates: mean of percentage differences between scores matched by response ranking. Abscissae: %δ stands for percentage differences corresponding to WA.WA-DIF vs. WA.WA-SAM or to CY.CY-DIF vs. CY.CY-SAM. t-Test: × × for $p <$ 0.005.

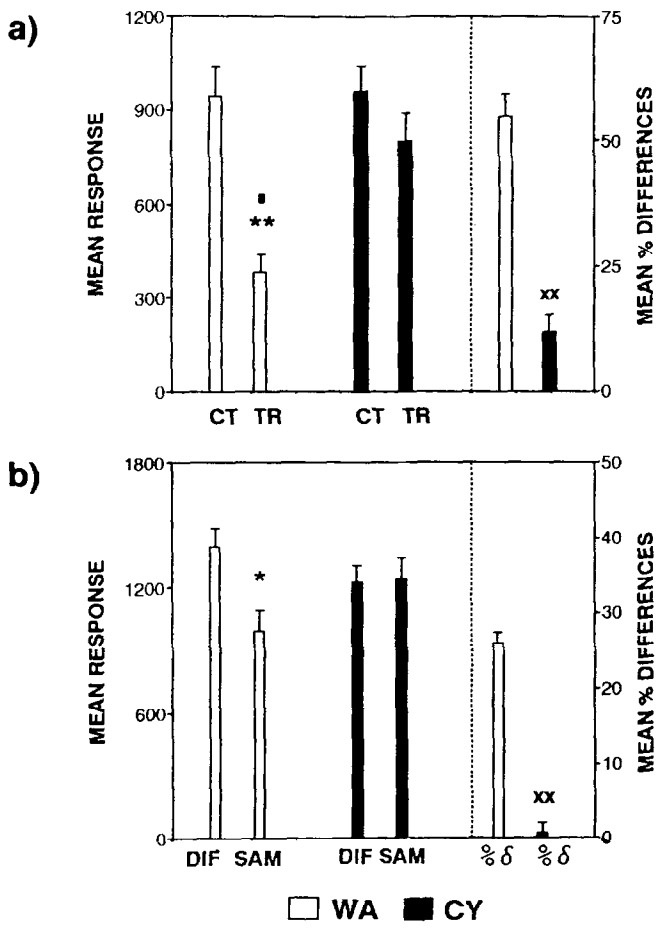

FIG. 8. Effect of 20 μg CY posttraining injection on long-term habituation and context memory tested 24 h after training. (a) Long-term habituation experiment (n = 35). Symbols as in Fig. 2. (b) Context experiment (n = 35). Symbols as in Fig. 6.

(right panel) revealed a significant difference between WA and CY percentage differences.

Figure 8b displays results corresponding to the context memory experiment. An impairment of context memory was manifest because no significant difference was found either for CY-DIF vs. CY-SAM or for WA-DIF vs. CY-DIF (left panel) and the mean of WA percentage differences was statistically higher than that of CY percentage differences (right panels).

Thus, a CY amnestic effect on both long-term habituation and memory of the context is found also at 24 h when the drug is administered immediately after training.

With the purpose of pointing out the time window of the CY effect, two further experiments on long-term habituation were carried out with CY (20 μg) administered 2 and 6 h after training. Results showed amnesia when the drug was injected at 2 h (Fig. 9a) but not at 6 h (Fig. 9b).

DISCUSSION

A single pre- or posttraining injection of CY induced a behavioral change that could not be explained in terms other than an amnestic effect of the drug. First, no significant difference could be found throughout between control groups (WA-DIF vs. CY-DIF), so that retention impairment is not attributable to a generalized enhancing effect of the drug. Second, amnesia is expressed by a positive act on the part of the trained crabs (an increase in reactivity of the trained CY-group), so that the CY effect cannot be accounted for by an association between a drug-induced sickness and the experimental situation [e.g. (31)]. Third, no alterations in spontaneous exploratory activity nor in reactivity were shown when CY was injected either immediately before training or before testing, a finding at odds with the view that CY acts on an intact animal by changing the level of motivation (30). Lastly, the memory impairment was shown when the drug was given 2 but not 6 hours after training, a result hardly compatible with any explanation of the retention deficit as unspecific sequelae of the CY injection.

In addition, the exclusion of "state dependent" learning as alternative explanation (Fig. 7) suggests that the amnestic effect involves a failure to form memory but not a deficit in the ability to retrieve information.

Together with the amnestic effect, CY produces in *Chasmagnathus* an inhibition of the protein synthesis that disappears 24 h after injection. The forward limit of the time window

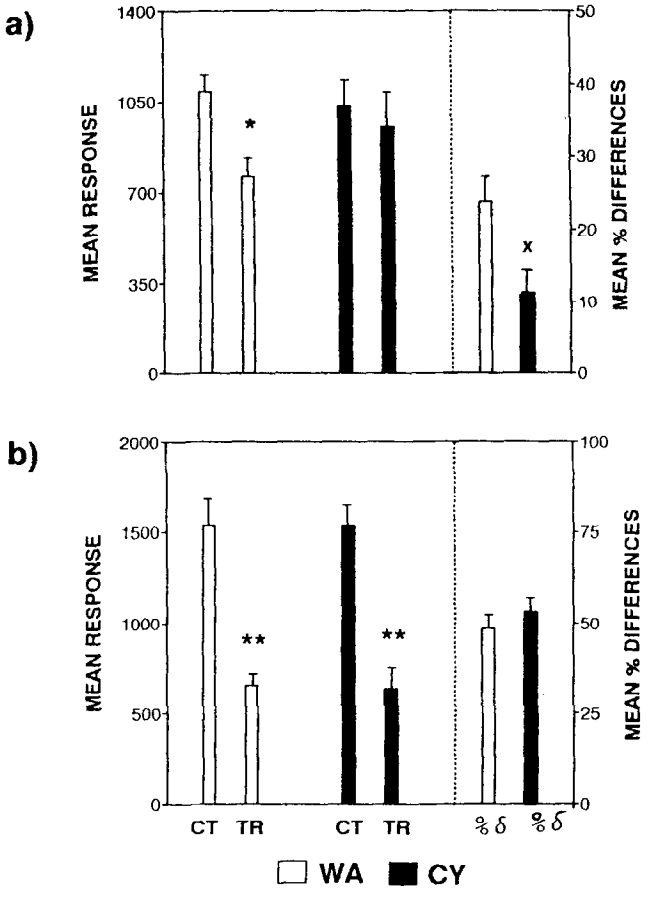

FIG. 9. Effect of 20 μg CY posttraining injection on long-term habituation tested 24 h after training. (a) Long-term habituation experiment (n = 40); injection 2 h after training. Symbols as in Fig. 2. (b) Long-term habituation experiment (n = 40); injection 6 h after training. Symbols as in Fig. 2.

during which the inhibitor has to be applied seems to be between 2 and 6 h after training.

These results argue strongly for de novo protein synthesis as a mechanism of long-term habituation and may, thus, help to gain insight into the cellular and molecular pathways underlying the consolidation of long-lasting memory.

Cycloheximide injected pretraining induced disruption of both context memory and long-term habituation 72 h after training. However, when treated crabs were tested at 24 h, the context memory was shown disrupted, though long-term habituation remained apparently unaffected. The disparity could be accounted for the graded effect hypothesis (19), but an alternative explanation was offered. Namely, the putative failure of pretraining CY to impair long-term habituation at 24 h would be due to an unspecific depressing effect upon the escape response, caused by interaction during training between the stressing effect of the iterative stimulation and the CY-induced internal state. In support, there was no retention at 24 h when the drug was injected posttraining, that is, in the absence of interaction between training and drug-induced internal state. A similar set of results was obtained in experiments with *Chasmagnathus* on amnesia acutely induced by ethanol (41) and in preliminary experiments on amnesia induced by actinomycin-D (Pedreira, personal communication).

The dual effect of CY-injected pretraining, namely, a long-lasting amnesic effect and a transient depressing effect, would explain the so-called "hypermnesia" described in experiments with testing at 24 h. In fact, the amnestic effect induces an increase in responding in the CY-CT group, whereas the depressing effect induces a reduction in responding in the CY-TR group.

To sum up, context amnesia inevitably implies long-term habituation amnesia, a result consistent with the context specificity reported for the habituation in *Chasmagnathus* (46) and with a tenet of the associative theory (50).

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REFERENCES

1. Abbott, N. J. Comparative physiology of the blood-brain barrier. In: Bradbury, M. W. B., ed. Physiology and pharmacology of the blood-brain barrier. Berlin: Springer Verlag; 1992:371–396.
2. Aggio, J. F.; Rakitin, A.; Maldonado, H. Short- and long-term sensitization by serotonin of the escape response in the crab *Chasmagnathus*. Pharmacol. Biochem. Behav. (in press).
3. Alkon, D. L.; Bank, B.; Naito, S.; Chung, C.; Ram, J.; Rasmussen, H. Protein synthesis inhibition prolongs Ca^{2+} mediated reduction of K^+ currents. Proc. Natl. Acad. Sci. USA 84:6949–6952; 1987.
4. Anokhin, K. V.; Mileusnic, R.; Shamakina, I. Y.; Rose, S. P. R. Effects of early experience on c-*fos* gene expression in the chick forebrain. Brain Res. 544:101–107; 1991.
5. Anokhin, K. V.; Rose, S. P. R. Learning-induced increase of immediate early gene messenger RNA in the chick forebrain. Eur. J. Neurosci. 3:162–167; 1991.
6. Bailey, C. H.; Chen, M.; Keller, F.; Kandel, E. R. Serotonin mediated endocytosis of ApCam: An early step of learning related synaptic growth in *Aplysia*. Science 256:645–649; 1992.
7. Barraco, D. A.; Lovell, K. L.; Eisenstein, E. M. Effects of cycloheximide and puromycin on learning and retention in the cockroach *Periplaneta americana*. Pharmacol. Biochem. Behav. 15: 489–494; 1981.
8. Bennett, E. L.; Flood, J. F.; Orme, A.; Rosenzweig, M. R.; Jarvik, M. Minimun duration of protein synthesis needed to establish long-term memory. Int. Soc. Neurochem. Abstr. 5:476; 1975.
9. Bennett, E. L.; Rosenzweig, M. R.; Flood, J. F. Protein synthesis and memory studied with anisomycin. In: Roberts, S.; Lajtha A.; Gispen, W. H., eds. Mechanisms regulation special function of protein synthesis in the brain. Amsterdam: Elsevier; 1977:319–330.
10. Berman, R. F.; Kesner, R. P.; Partlow, L. M. Passive avoidance impairment in rats following cycloheximide injection into amygdala. Brain Res. 158:177–188; 1978.
11. Boccaccio, G. L.; Quesada-Allue, L. A. Interference of sodium dodecyl sulfate in the Bradford assay for protein quantification. Anal. Asoc. Quimica Argentina 77:79–88; 1989.
12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Ann. Biochem. 72:248–254; 1976.
13. Brunner, D.; Maldonado, H. Habituation in the crab *Chasmagnathus granulatus*: Effect of morphine and naloxone. J. Comp. Physiol. [A] 162:687–694; 1988.
14. Cady, W. G. Piezoelectricity. New York: Dover; 1964.
15. Campeau, S.; Hayward, M. D.; Hope, B. T.; Rosen, J. B.; Nestler, E. J.; Davis, M. Induction of the c-*fos* proto-oncogene in rat amygdala during unconditioned and conditioned fear. Brain Res. 565:349–352; 1991.
16. Crow, T.; Forrester, J. Inhibition of protein synthesis blocks Long-term enhancement of generator potentials produced by one-trial in vivo conditioning in *Hermissenda*. Proc. Natl. Acad. Sci. USA 87:4490–4494; 1990.
17. Davis, H. P.; Squire, L. R. Protein synthesis and memory: A review. Psychol. Bull. 3:518–559; 1984.
18. Eisenstein, E. M.; Altman, H. J.; Barraco, D. A.; Barraco, R. A.; Lovell, K. L. Brain protein synthesis and memory: The use of antibiotic probes. Fed. Proc. 42:3080–3085; 1983.
19. Flood, J. F.; Bennett, E. L.; Rosenzweig, M. R.; Orme, A. E. Influence of training strength on amnesia induced by pretraining injections of cycloheximide. Physiol. Behav. 9:589–600; 1972.
20. Flood, J. F.; Bennett, E. L.; Orme, A. E.; Rosenzweig, M. R. Effects of protein synthesis inhibition on memory for active avoidance training. Physiol. Behav. 14:177–184; 1975a.
21. Flood, J. F.; Bennett, E. L.; Orme, A. E.; Rosenzweig, M. R. Relation of memory formation to controlled amounts of brain protein synthesis. Physiol. Behav. 15:97–105; 1975.
22. Fredericq, L. Sur l'autotomie ou mutilation par voie reflexe comme moyen de defense chez les animaux. Arch. Zool. Exp. Gen. 1:413–426; 1883.
23. Gibbs, M. E.; Ng, K. T. Psychobiology of memory: Towards a model of memory. Neurosci. Biobehav. Rev. 1:113–136; 1977.
24. Godoy, A.; Maldonado, H. Modulation of the scape response by D(Ala^2)Met-enkephalin in the crab *Chasmagnathus*. Pharmacol. Biochem. Behav. 50(3):445–451; 1995.
25. Lee, D. W.; Perlmutter, A. M.; Beniston, D. S.; Bennett, E. L.; Rosenzweig, M. R. Is anisomycin-induced amnesia for a passive avoidance task in chicks the result of state-dependent learning?. Dev. Brain Res. 49:179–184; 1989.
26. Lozada, M.; Romano, A.; Maldonado, H. Long term habituation to a danger stimulus in the crab *Chasmagnathus granulatus*. Physiol. Behav. 47:35–41; 1990.
27. Lozada, M. Habituación a largo término y especificidad del estímulo en el cangrejo Chasmagnathus granulatus. Doctoral Thesis, Unversidad de Buenos Aires; 1994.
28. Maldonado, H.; Romano, A.; Lozada, M. Opioid action on response level to a danger stimulus in the crab *Chasmagnathus granulatus*. Behav. Neurosci. 103:1139–1143. 1989.

29. Menzel, R.; Gaio, U. C.; Gerberding, M.; Nemrava, E. A.; Wittstock, M. Formation of long term olfactory memory in honeybees does not require protein synthesis. Naturwissenschaften 80:380–382; 1993.
30. Montarolo, P. G.; Goelet, P.; Castellucci, V. F.; Morgan, J.; Kandel, E. R.; Scharer, S. A critical period for macromolecular synthesis in Long-term heterosynaptic facilitation in Aplysia. Science 234:1249–1253; 1986.
31. Nakajima, S. Conditioned aversion of water produced by cycloheximide injection. Physiol. Psychol. 2:484–486; 1974.
32. Nguyen, P. V.; Atwood, H. L. Expression of long-term adaptation of synaptic transmission requires a critical period of protein synthesis. J. Neurosci. 10(4):1099–1109; 1990.
33. Patel, S. N.; Rose, S. P. R.; Stewart, M. G. Training induced dendritic spine density changes are specifically related to memory formation processes in chick *Gallus domesticus*. Brain Res. 463: 168–173; 1988.
34. Patterson, T. A.; Rose, S. P. R.; Bradley, P. M. Anisomycin and amnesia in the chick: state dependent-effects are not present with intracranial injections. Dev. Brain Res. 49:173–178; 1989.
35. Pereyra, P.; de la Iglesia, H.; Maldonado, H. Training to testing intervals different from 24 hours impair long term-habituation in the crab *Chasmagnathus granulatus*. Physiol. Behav. (in press).
36. Quesada-Allue, L. A.; Belocopitow, E. Eur. J. Biochem. 88:529–541; 1978.
37. Rakitin, A.; Tomsic, D.; Maldonado, H. Habituation and sensitization to an electrical shock in the crab *Chasmagnathus*, effect of background illumination. Physiol. Behav. 50:477–487; 1991.
38. Rescorla, R. A. Behavioral studies of pavlovian conditioning. Annu. Rev. Neurosci. 1:329–352; 1988.
39. Romano, A.; Lozada, M.; Maldonado, H. Effect of naloxone pretreatment on habituation in the crab *Chasmagnathus granulatus*. Behav. Neural Biol. 53:113-122; 1990.
40. Romano, A.; Lozada, M.; Maldonado, H. Non habituation processes affect stimulus specifity of the response-habituation in the crab *Chasmagnathus* granulatus. Behav. Neurosci. 105:542–552; 1991.
41. Saraco, M.; Maldonado, H. Ethanol affects context memory and long term habituation in the crab *Chasmagnathus*. Pharmacol. Biochem. Behav. 51(2/3):223–229; 1995.
42. Squire, L. R.; Barondes, S. H. Variable decay of memory and its recovery in cycloheximide-treated mice. Proc. Natl. Acad. Sci. USA 69:1416–1420; 1972.
43. Squire, L. R.; Barondes, S. H. Anisomycin, like others inhibitors of cerebral protein synthesis, impairs "long-term" memory of a discrimination task. Brain Res. 66:301–308; 1974.
44. Tomsic, D; Maldonado, H. Central effect of morphine pretreatment on short and long-term habituation to a danger stimulus in the crab *Chasmagnathus*. Pharmacol. Biochem. Behav. 36:787–793; 1990.
45. Tomsic, D.; Maldonado, H.; Rakitin, A. Morphine and Gaba: Effects on perception, scape response and long-term habituation to a danger stimulus in the crab *Chasmagnathus*. Brain Res. Bull. 26:699–706; 1991.
46. Tomsic, D.; Massoni, V.; Maldonado, H. Habituation to a danger estimulus in two semiterrestrial crabs: ontogenic, ecological and opioid modulation correlates. J. Comp. Physiol. [A] 173: 621–633; 1993.
47. Tomsic, D.; Dimant, B.; Maldonado, H. Age related deficits of long-term memory in the crab *Chasmagnathus*. (submitted).
48. Ungerer, A.; Chapouthier, G. Shuttle box learning in mice after puromycin injections. Folia Biol. (Krakow) 23(1):73–79; 1975.
49. Valeggia, C.; Fernandez-Duque, E.; Maldonado, H. Danger stimulus-induced analgesia in the crab *Chasmagnathus granulatus*. Brain Res. 481:304–308; 1989.
50. Wagner, A. R. Memory and habituation. In: Peeke, H. V. S.; Petrinovich, L., eds. Habituation, sensitization and behavior. London: Academic Press; 1984:103–153.