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Inhibitors of Protein and RNA Synthesis Block Context Memory and Long-Term Habituation in the Crab *Chasmagnathus*

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PEDREIRA, M. E., B. DIMANT AND H. MALDONADO. *Inhibitors of protein and RNA synthesis block context memory and long-term habituation in the crab* Chasmagnathus. PHARMACOL BIOCHEM BEHAV **54**(3) 611-617, 1996. — The crab *Chasmagnathus granulatus* reacts to a shadow passing overhead (a danger stimulus) with an escape response that habituates quickly and for at least 5 days. Recently, it has been reported that cycloheximide (CY) disrupts this long-term habituation and the corresponding context memory. In the present article, experiments with CY and an inhibitor of RNA synthesis, actinomycin-D (ACT), were parallelly conducted. An injection of CY (20 μg) or ACT (0.62 μg) reduced the incorporation of [^{14}C]-aminoacid into cerebral plus thoracic ganglia by 80% for 2 h and 59.7% for 1 h, respectively, but no inhibition was found at 24 h. Both ACT (0.62 μg) and CY (20 μg) administered immediately after training (15 trials with the danger stimulus) impaired either long-term habituation or context memory when tested at 24 h. Because ACT and CY have in common only their direct or indirect inhibitory effect on protein synthesis, this finding is considered as an additional evidence that long-term memory in *Chasmagnathus* requires de novo protein synthesis. However, pretraining ACT or CY impaired context memory at 24 h but not long-term habituation. Such a disparity is explained by an unspecific attenuating effect upon the response, attributed to drug × training interaction. Neither ACT nor CY affected short-term habituation.

Actinomycin-D Cycloheximide Antibiotics Crab Habituation Memory Arthropoda

THE CRAB *Chasmagnathus granulatus* reacts to a shadow passing overhead with an escape response that habituates quickly and for at least 5 days (13,22,26). Mechanistic and theoretical aspects of this robust long-term habituation have been extensively explored. Thus studies has been performed on its specificity (22,30,36), adaptive value (36), relation to age (37), circadian cycle (27), dependence on PKA activity (31), and modulation by opioids (19,24,29,30,34,35,38). Recently, disruption of both long-term habituation and context memory has been found in crabs injected cycloheximide (CY), a protein synthesis inhibitor (26).

Demonstration that memory storage requires de novo protein synthesis represents a pivotal finding in molecular studies on long-term memory [e.g., (4-6,15,25)]. The abovementioned result about CY-induced memory impairment in *Chasmagnathus* could be interpreted as another example of such a requirement. However, several investigators have warned about the possibility that amnesia induced by an antibiotic substance might be due to an action unrelated to protein inhibition, for instance, impairment of tyrosine hydroxylase activity in vertebrates (7,20,23). Therefore, additional evidence is required to ensure that the amnestic effect can be unambiguously attributed to protein synthesis inhibition. Amnesia should also be found with other chemicals that directly or indirectly inhibit protein synthesis but that differ from CY in structure and mechanisms of action (11).

Consequently, the main purpose of the present article is to conduct parallel experiments using CY and the total RNA synthesis inhibitor actinomycin-D to compare their amnestic effects on crab's memory.

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METHOD

Animals

Animals were adult male *Chasmagnathus* crabs 2.6–2.9 cm across the carapace, weighing 15–17 g, 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 was maintained within a range of 19–24°C as well as the alley between them.

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

Apparatus

The apparatus is described in detail elsewhere (29). 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 housed in the container, which 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 rectangular screen (25 × 7.5 cm) could be moved horizontally by a motor over the animal and across the upper surface of the framework in 2.3 s. Screen displacements provoked a crab's running response and consequent 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 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.

Drugs and Injection Procedure

Cycloheximide (CY) or actinomycin-D Mannitol (ACT) were diluted in distilled water (WA). Drugs were purchased from Sigma Chemicals. Fifty microliters of WA or drug solution (ACT or CY) 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. We currently use distilled water as a vehicle because 50 μl of dw proved not to affect crab's responsiveness (19).

Experimental Procedure

Two types of experiment were conducted: one aimed at testing CY and ACT effects on long-term habituation and the other one, at testing drugs' action on context memory.

A *training* session in a long-term habituation experiment included of 15 trials separated by 180 s intertrial interval and preceded by a 30 min adaptation time in the actometer. Each trial lasted 9 s and consisted of the screen passing four times over the actometer, recording the crab's activity during the entire trial time. After 24 h a *testing* session of a single trial was run, preceded by a 15 min adaptation time. During the intersession interval, crabs were individually housed in plastic containers covered to a depth of 0.5 cm with water and kept inside dimly lighted drawers. Each long-term habituation experiment consisted of four groups of animals. Two groups were water injected (WA-groups) and the other two were drug injected with either actinomycin-D (ACT-groups) or cycloheximide (CY-groups). In turn, one drug-injected and one WA-group were trained (TR-groups) while the other two groups remained untrained (control groups, CT-groups). These groups were named WA-CT, WA-TR, CY-CT (or ACT-CT), and CY-TR (or ACT-TR).

In a context memory experiment, one group of crabs (the same context-group, SAM) was kept in the actometer's container during 120 min without being confronted with the passing shadow. Another group (the different context-group, DIF) received a similar treatment, although was kept in a transparent cylinder with floor covered by a thin layer of wet sand and placed in a dimly lighted box. Half of the animals in each group were injected water (WA); the other half was given CY or ACT. Thus, each experiment consisted of four groups named WA-DIF, WA-SAM, CY-DIF (or ACT-DIF), and CY-SAM (or ACT-SAM). After the 24-h intersession interval, as above, all crabs underwent a testing trial session with the passing shadow in the usual actometers, so that SAM was exposed to the same context in both experimental sessions (actometer-actometer) while DIF experienced different contexts (cylinder-actometer).

We considered control groups to WA-CT and CY-CT (or ACT-CT) in long-term habituation experiments while to WA-DIF and CY-DIF (or ACT-DIF) in context memory experiments; and trained groups to WA-TR and CY-TR (or ACT-TR) in the former experiments while to WA-SAM and CY-SAM (or ACT-SAM) in the latter ones. Thirty to 40 crabs per group were employed. Each group in one experiment was ascribed the same number of individuals. Crabs' responsiveness to the passing screen proves remarkably consistent up to 10 days after arrival, but on occasion animals coming from different capture efforts present differences in response level. Therefore, parallel experiments with CY and ACT were performed with crabs belonging to the same capture.

In a series of experiments, injections were given immediately before placing crabs in the actometers for the first session, that is, 30 min before the training session started; in another series, crabs were injected immediately after the end of this session.

Data Analysis and Evaluation of the Amnestic Effect

Long-term memory was assessed by focusing data analysis on testing scores. Rescorla (28) convincingly argued in favor of using this sort of analysis instead of a paired training-testing comparison, stressing the need to clearly distinguish between time of input (training session) and time of assessment (testing trial).

In all previous experiments at our laboratory, without exception, a significant difference (t-test, alpha = 0.05) between mean testing scores from WA-CT and WA-TR was disclosed 24 h after training, provided that groups consisting

of 30 or more crabs were used and that they were given a proper amount of training (15 training trials with a 180-s intertrial interval). Coincidently, a significant difference was invariably found between mean testing scores from water injected crabs that had been (120 min) preexposed to the actometer (WA-SAM) and from those preexposed to a wholly dissimilar context (WA-DIF). Therefore, results from the present article were considered indicative of amnestic effect if a *t*-test performed on testing data failed to show a significant difference (alpha = 0.05, two-tailed) between a trained group and its respective control group.

ANOVA of repeated measures was performed on training scores of groups injected before this session.

Definitions

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 secondary protein synthesis inhibition in cerebral and thoracic ganglia by ACT, animals were injected WA or 0.62 μg/crab ACT. 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. After this later injection crabs were individually placed in small containers whose bottoms were lined with filter paper. One hour later animals were killed, their ganglia removed, and immersed into liquid nitrogen.

Ganglia from animals in the same group (eight ganglia corresponding to four crabs) were pooled, so that each group furnished a single sample that was homogenized in 0.5 ml ice-cold chloroform : methanol 3 : 2 by means of a hand-driven homogenizer and transferred to centrifuge tubes. The homogenizer was washed with 0.5 ml ice-cold chloroform : methanol 3 : 2 which were added to the homogenate. Samples were then centrifugated at 5000 rpm for 10 min. The pellet was washed with 1 ml chloroform : methanol : water 1 : 16 : 16 and 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 and vortexed. A 10 μl sample was taken for protein determination by a modified

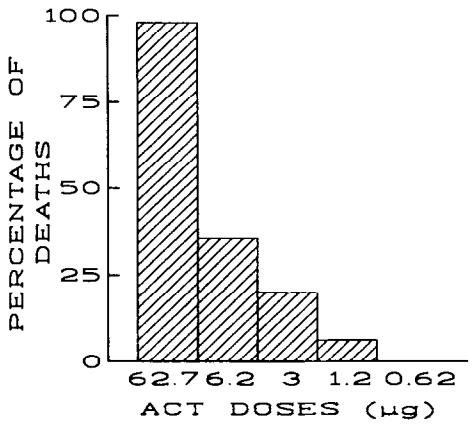

FIG. 1. Percentage of deaths produced by different doses of ACT. Ordinate: percentage of deaths. Abscissa: doses of ACT.

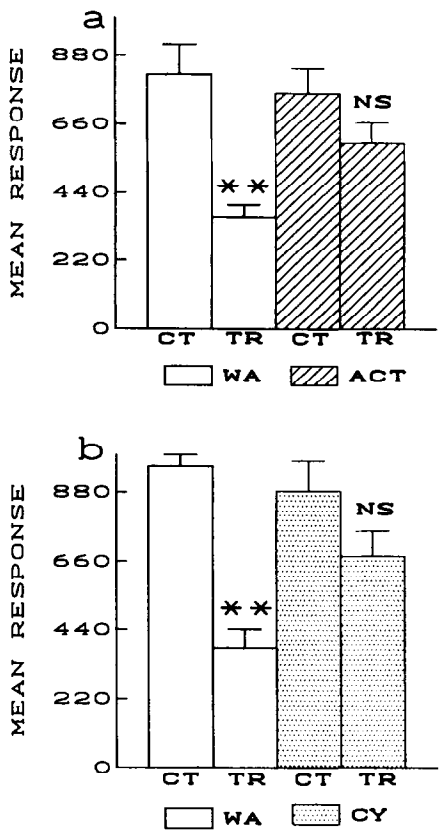

FIG. 2. Effect of posttraining injection on long-term habituation. (a) 0.62 μg ACT (n = 30). White bars stand for water injected groups (WA-groups); stripped bars for actinomycin-D injected groups (ACT-groups). The height of each bar is the means ± SEM. Ordinate: mean of testing score per group. Abscissa: CT, a control group (WA-CT or ACT-CT); TR, a trained group (15 training trials, WA-TR or ACT-TR). *t*-Test: **stands for $p < 0.01$, comparison between WA-groups; NS: no significant difference between ACT-groups. (b) 20 μg CY (n = 35). White bars stand for water injected groups (WA-groups); speckled bars for cycloheximide injected groups (CY-groups). The height of each bar is the means ± SEM. Ordinate: mean of testing score per group. Abscissa: CT, a control group (WA-CT or CY-CT); TR, a trained group (15 training trials, WA-TR or CY-TR). *t*-Test: **stands for $p < 0.01$, comparison between WA-groups; NS: no significant difference between CY-groups.

version of Bradford method (10,12). Ice-cold 10% trichloroacetic was added to the remaining 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 along with 3 ml toluene-based scintillation liquid 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 ACT was calculated by comparing relative specific radioactivity in drug-injected crabs with that of control animals.

RESULTS

Decrement of [^{14}C]-Aminoacid Incorporation Induced by ACT

One hour after ACT injection, precursor incorporation into ganglia proteins was reduced by a 59.7%. No decrement

was found 24 h postinjection. Filter paper counts were similar to blank values, thus suggesting there was no apparent excretion of radioactive material during the 1 h injection-sacrifice interval.

A similar biochemical analysis had revealed that 10–20 μg of CY produces over 80% protein synthesis inhibition after 2 h in *Chasmagnathus* (26).

Sickness Symptoms After Different ACT Doses

When crabs were given a 10–20 μg CY injection in 50 μl solution (circa 10^{-3} M), no overt symptoms of sickness appeared (27). A similar molarity ACT dose (62.75 μg per animal) did not produce immediate overt symptoms of sickness but proved lethal for 98% of the crabs 24 h after injection. The number of deaths decreased along with the dose and no lethal effect was found for 0.62 μg (Fig. 1). Animals surviving a dose higher than 0.62 μg often showed appendage losses, a phenomenon termed autotomy (16), as well as a decrement in reactivity to the passing screen.

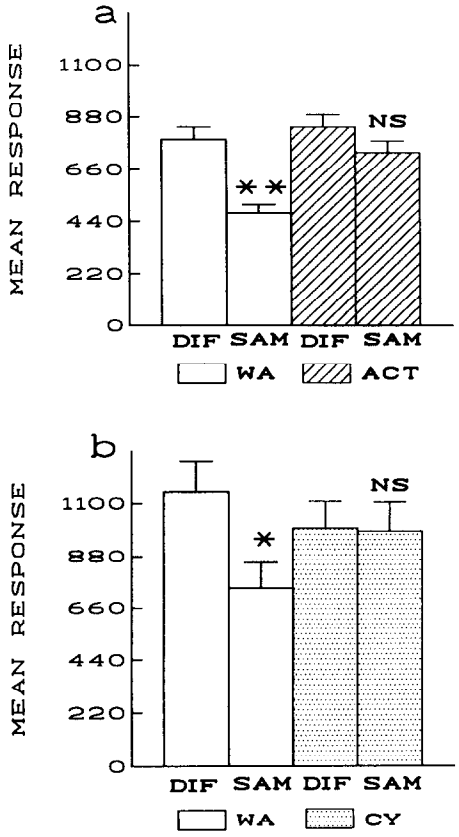

FIG. 3. Effect of posttraining injection on context memory. (a) 0.62 μg ACT (n = 40). White bars stand for water injected groups (WA-groups); stripped bars for actinomycin-D injected groups (ACT-groups). The height of each bar is the means ± SEM. Ordinate: mean of testing score per group. Abscissa: DIF, a different-context group: a group that was exposed to the cylinder at training and to the actometer at testing (WA-DIF or ACT-DIF); SAM, a same-context group: a group that was exposed to the actometer both at training and at testing (WA-SAM or ACT-SAM). t-Test: **stands for $p < 0.01$, comparison between WA-groups; NS: no significant difference between ACT-groups. (b) 20 μg CY (n = 33). White bars stand for water injected groups (WA-groups); speckled bars for cycloheximide injected groups (CY-groups). The height of each bar is the means ± SEM. Ordinate: mean of testing score per group. Abscissa: DIF, a different-context group (WA-DIF or CY-DIF); SAM, a same-context group (WA-SAM or CY-SAM). t-Test: *stands for $p < 0.05$, comparison between WA-groups; NS: no significant difference between CY-groups.

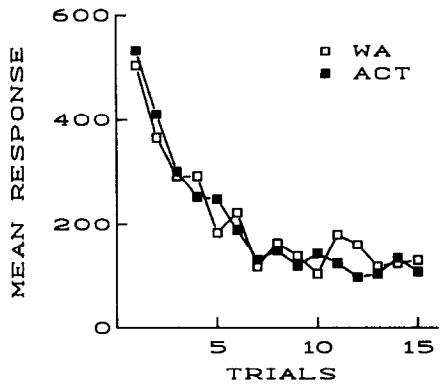

FIG. 4. Effect of ACT on short-term habituation (n = 31). White squares stand for water injected groups (WA-TR); black squares for 0.62 μg of actinomycin-D injected groups (ACT-TR). Ordinate: mean of escape response scores. Abscissa: trials of 9 s each, 3-min intertrial interval.

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

The purpose of the following four experiments was to compare the effect of ACT (0.62 μg) with that of CY (20 μg), both posttraining injected, on either long-term habituation or context memory.

Figure 2 presents testing results from long-term habituation experiments: the first experiment including WA- and ACT-groups (Fig. 2a) and the second one, WA- and CY-groups (Fig. 2b). A t-test showed the expected significant difference between WA-groups in both experiments, $t(58) = 4.3$, $p < 0.01$ (Fig. 2a), and $t(68) = 5.7$, $p < 0.01$ (Fig. 2b), respectively, but no significant difference either between both ACT- or CY-groups, $t(58) = 1.53$ (Fig. 2a), and $t(68) = 1.64$ (Fig. 2b), respectively, thus suggesting amnestic effects on long-term habituation.

Figure 3 illustrates data corresponding to the testing trial in context memory experiments: the first experiment including WA- and ACT-groups (Fig. 3a) and the second one, WA- and CY-groups (Fig. 3b). A t-test performed on these data disclosed the expected significant difference for WA-DIF vs. WA-SAM in both experiments, $t(78) = 4.3$, $p < 0.01$ (Fig. 3a), and $t(64) = 2.4$, $p < 0.05$ (Fig. 3b), respectively, but not for either ACT-DIF vs. ACT-SAM or CY-DIF vs. CY-SAM, $t(78) = 1.56$ (Fig. 3a), and $t(64) = 0.5$ (Fig. 3b), respectively, thus indicating amnestic effects on context memory.

Pretraining ACT Does Not Affect Short-Term Habituation

A water preinjected group (WA-group) displays a training curve closely similar to that of a 0.62 μg ACT preinjected group (ACT-group) (Fig. 4). A 2 × 15 ANOVA of repeated measures performed on these scores disclosed a significant trial effect, $F(14, 1092) = 26.5$, $p < 0.05$, but neither a sig-

nificant between groups difference, $F(1, 78) = 3.5$, nor a group × trial interaction, $F(14, 1092) = 0.68$. Therefore, no effect on short-term habituation is found by preinjection of 0.62 μg ACT, nor 20 μg CY (26), an outcome in keeping with a consistent body of results from experiments performed on diverse animal species (8,9,17,26).

Effect of Pretraining CY or ACT on Long-Term Habituation and Context Memory

Previous experiments with pretraining CY showed unexpected results because amnestic effect on context memory but not on long-term habituation was disclosed 24 h after training (26). The purpose of the following two experiments is to test whether similar results are obtained with a pretraining ACT injection. The design, as well as data analysis, were in all aspects equal to those in the previous section though crabs were injected immediately before training session.

Training performances of trained-groups after drug injections were very similar in both long-term experiments (data not shown). Results from the corresponding testing trial are displayed in Fig. 5. *t*-Test revealed the expected significant differences for WA-CT vs. WA-TR in both experiments, $t(60) = 2.4$, $p < 0.05$ (Fig. 5a), and $t(62) = 4.6$, $p < 0.01$ (Fig. 5b), respectively, as well as for ACT-CT vs. ACT-TR and for CY-CT vs. CY-TR, $t(60) = 2.5$, $p < 0.05$ (Fig. 5a), and $t(62) = 4.5$, $p < 0.01$ (Fig. 5b), respectively. Thus, pretraining administration of ACT or CY showed no amnestic effect on long-term habituation.

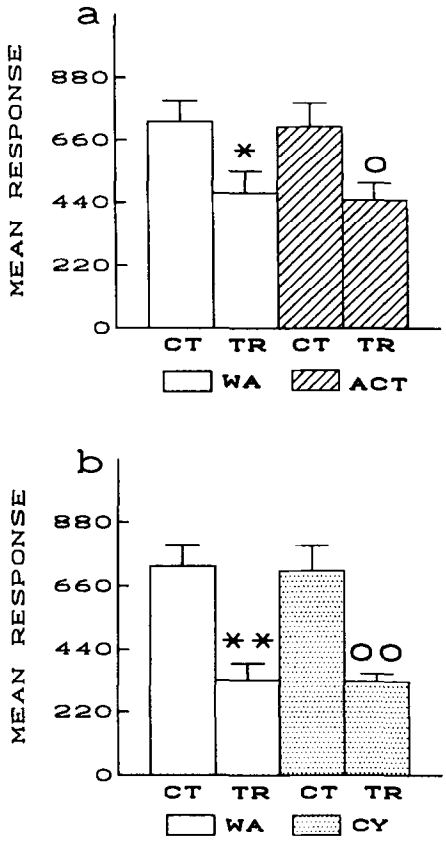

FIG. 5. Effect of pretraining injection on long-term habituation. (a) 0.62 μg ACT ($n = 31$). Symbols as in Fig. 2a. *t*-Test: *for $p < 0.05$, comparison between WA-groups; ○ for $p < 0.05$, comparison between ACT-groups. (b) 20 μg CY ($n = 32$). Symbols as in Fig. 2b. *t*-Test: **for $p < 0.01$, comparison between WA-groups; ○○ for $p < 0.01$, comparison between CY-groups.

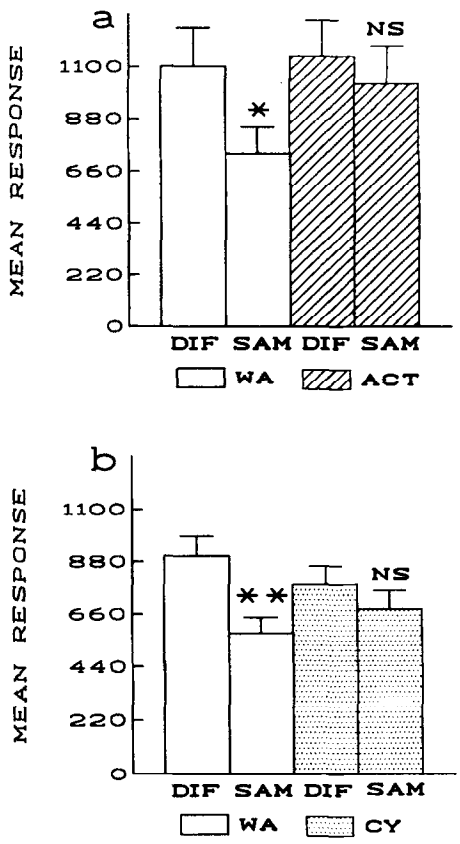

FIG. 6. Effect of pretraining injection on context memory. (a) 0.62 μg ACT ($n = 30$). Symbols as in Fig. 3a. *t*-Test: *for $p < 0.05$, comparison between WA-groups; NS for no significant difference between ACT-groups. (b) 20 μg CY ($n = 35$). Symbols as in Fig. 3b. *t*-Test: **for $p < 0.01$, comparison between WA-groups; NS for no significant difference between CY-groups.

By contrast, results from context experiments (Fig. 6a and b) showed amnestic effect by pretraining ACT or CY. *t*-Test disclosed the expected significant differences for WA-DIF vs. WA-SAM in both experiments, $t(58) = 2.1$, $p < 0.05$ (Fig. 6a), and $t(68) = 2.9$, $p < 0.01$ (Fig. 6b), respectively, but not for ACT-DIF vs. ACT-SAM or for CY-DIF vs. CY-SAM, $t(58) = 0.5$ (Fig. 6a), and $t(68) = 0.9$ (Fig. 6b), respectively.

Therefore, pretraining injection of 0.62 μg ACT or 20 μg CY per animal seems to impair context memory at 24 h but fails to produce a deficit in retention of the habituated response.

DISCUSSION

The impairing effects of posttraining CY or ACT on retention in both context and habituation experiments were closely similar and could not be explained in terms other than drug-induced amnesia. No significant difference (*t*-test) was found between control groups, namely, WA-CT vs. CY-CT (or vs.

ACT-CT) in long-term habituation experiments and WA-DIF vs. CY-DIF (or vs. ACT-DIF) in context experiments, so that retention impairment is not attributable either to a depressing effect or to a generalized enhancing effect, respectively. Noteworthy, no alterations in reactivity were found when drug was injected immediately before training, a finding at odds with the view that CY or ACT could act by changing the motivation level (25).

Apart from their amnestic effect, CY and ACT only have their inhibitory effect on protein synthesis in common; CY acts directly on the translation step while ACT, indirectly by blocking total RNA synthesis. Therefore, present results support the view that long-term memory in *Chasmagnathus* requires de novo protein synthesis.

Pretraining injection of CY or ACT induced disruption of context memory but not of habituation at 24 h. A similar result was obtained in previous experiments with pretraining CY (26) or pretraining ethanol (33), thus suggesting that retention of the habituated response at 24 h would be possible without memory of the environment to which the animal was preexposed. Such a conclusion is at odds with reports from our laboratory (36), showing that context memory plays a critical role in long-term habituation of *Chasmagnathus* and advancing an interpretation close to the associative theory of habituation (30). However, the low reactivity displayed by pretreated trained crabs at 24 h might be explained in terms other than long-term habituation. We hypothesized (26) that drug injection followed by repeated presentation of a danger stimulus would produce a transient depressing effect on the response, so that the response decrement at 24 h would not result from the habituation process but rather from an interaction between a drug-induced internal state and the iterated stimulation.

Two lines of evidence support the foregoing hypothesis. First, crabs exhibit disruption of both memories when tested 72 h after pretraining injection of CY (26), a result in keeping with the idea that the depressant effect induced by interaction is a transient phenomenon; second, posttraining administration of ethanol, CY or ACT impairs both memories at 24 h, an expected finding because no interaction between training and drug-induced internal state occurs when animals are posttreated.

No effect on the 24 h long-term habituation was found with the smallest concentration of ACT, 0.2 μg (data not shown), so the minimal effective dose was 0.62 μg/50 μl per animal (*circa* 0.04 μg/g). Such a dose is a noticeably low one, taking into account that systemic administration results in a concentration of roughly 10^{-7} M in hemolymph because its volume is estimated as 30% of the body weight (18). Previous experiments with *Chasmagnathus* have also shown that effective doses of drugs given by systemic injection were manifestly lower in this crab than in other animals, and equivalent to doses administered by intracranial injection in vertebrates or to those employed in bath solutions of in vitro experiments, for example, CPT-cAMP (31), cycloheximide (26), enkephalin (19), and serotonin (3). The lack of an endothelial blood-brain barrier in crabs (2), along with the fact that hemolymph is distributed through an extensive capillary system in various areas of the brain (1,2), could account for such a low threshold of drug action.

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