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Two Related Forms of Long-Term Habituation in the Crab *Chasmagnathus* Are Differentially Affected by Scopolamine

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BERÓN DE ASTRADA, M. AND H. MALDONADO. *Two related forms of long-term habituation in the crab* Chasmagnathus *are differentially affected by scopolamine.* PHARMACOL BIOCHEM BEHAV **63**(1) 109–118, 1999.—An opaque screen moving overhead elicits an escape response in the crab *Chasmagnathus*, which, after a few presentations, habituates for a long period (long-term habituation, LTH). Previous results distinguished two types of LTH: the (context-signal)-LTH yielded by spaced training, determined by an association between context and habituating stimulus, and cycloheximide sensitive; and the (signal)-LTH produced by massed training, context independent, and cycloheximide insensitive. Present experiments were aimed at studying the possible involvement of cholinergic mechanisms in one or both types of LTH, using the muscarinic antagonist, scopolamine (SCP). Results indicate that LTH acquired by spaced training (30 trials separated by 85 s) is blocked in a dose-dependent manner by posttraining SCP. Amnesia is shown with 100 ng SCP/g injected immediately before or after spaced training but not delayed 1-h posttraining. No effect of SCP on LTH acquired by massed training (300 trials separated by 4 s) is detected. Pretraining SCP induces a decrease in the response level at the initial trials of either a spaced or a massed training. It is concluded that the storage of (context-signal)-LTH may be selectively regulated by a muscarinic-cholinergic mechanism. However, the possibility that other cholinergic receptors would be involved in the consolidation of the (signal)-LTH is discussed. © 1999 Elsevier Science Inc.

Learning Massed and spaced training Habituation Scopolamine Acetylcholine Crustacea

AN opaque screen moving overhead elicits an escape repose in the crab *Chasmagnathus*, which, after a few presentations habituates for a long period (long-term habituation, LTH). Mechanistic and theoretical aspects of this robust long-term memory have been extensively explored (24). At first, LTH was considered an instance of simple habituation, because the response decrement fulfilled most of the parametrical conditions of such nonassociative learning (8). However, further results showed that two different types of LTH can be elicited by the iterative presentation of the same habituating stimulus, depending on the number of stimulation trials and mainly on the interval between them. When a crab is given spaced training (i.e., 30 trials separated by 85 s of intertrial interval, ITI) LTH is mediated by an association between the environmental features of the training place (the context) and the features of the screen moving overhead (the signal), thus being called the (context-signal)-LTH (44,45). In contrast, when a crab is given massed training (i.e., 300 trials separated by 4 s of ITI), LTH is not yielded by an associative learning between context and signal, depending only on the signal invariance, thus being called (signal)-LTH (32). Insofar as the research on these two instances of crab's LTH improves, their nature becomes more clearly different, both from behavioral and mechanistic view points: the (context-signal)-LTH is expressed by a reduction in the level of escape response at every trial of a six-trial testing session performed 24 h after training, while the (signal)-LTH is only expressed at the last five trials of the testing session (i.e., the retraining phase of testing) (32). The (context-signal)-LTH is cycloheximide sensitive and long lasting, and entails the building up of a strong and persistent freezing; in contrast, the (signal)-LTH is insensitive to cycloheximide and shorter lasting, and consists of a simple vanishing of the

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escape response without building up a different defensive response (18,30,31,33). Coincidently, the (context-signal)-LTH, but not the (signal)-LTH, seems to be mediated by the cAMP signal pathway (35,36), is positively modulated by angiotensins (11), and entails a κ-B like DNA binding activity (12). On the other hand, pretraining inhibition of glycoprotein synthesis by injecting 2-deoxygalactose seems to be the only interference that appears to affect the acquisition of both types of memory (40).

At this stage, we intend to explore if cholinergic mechanisms are involved in one or both crab LTHs, because muscarinic cholinergic receptors have been implicated in learning and memory processes in either vertebrates [e.g., (3)] or invertebrates [e.g., (13,28)], and such receptors have been found throughout the animal kingdom [e.g., (27,37,38,46,47,48]). As the starting point of this study, we examine in the present article the effect on each type of LTH of a highly specific muscarinc receptor ligand, the antagonist scopolamine. The presence of classical muscarinic receptors in the crab nervous system, as well as the potent antagonism of scopolamine, have been demonstrated by pharmacological binding studies, using the tritiated form of quinuclidinyl benzilate ([3H]QNB) (6).

METHODS

Animals

Animals were adults male Chasmagnathus crabs 2.6–2.9 cm across the carapace, weighing around 17.0 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 a 2-cm depth with diluted marine water, to a density of 20 crabs per tank. Water used in tanks and other containers during experiments was prepared using 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 S.A., Argentina) every 3 days, and after feeding the water was changed. Temperature of both holding and experimental rooms was maintained within a range of 22–24°C. Experiments were carried out within the first week after the animal's arrival from January to August. Each crab was used only in one experiment.

Apparatus

The experimental unit was the actometer (Fig. 1a)—a bowl-shaped plastic container (C) with a steep concave wall and a circular central flat floor 10 cm in diameter, covered to a depth of 0.5 cm with marine water. The crab was lodged 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 (L) placed 30 cm above the animal. A motor (M) moved horizontally an opaque rectangular screen (R) (a stripe of 25 × 7.5 cm) over the animal and along the upper surface of the framework, cyclically from 1 to 2, and vice versa (Fig. 1b). A cycle of movement lasted nearly 2.5 s. Screen displacements provoked a crab's running response and consequent container vibrations. A stylus was centrally cemented to the bottom of the container and connected to a piezoelectric transducer. Container vibrations induced electrical signals proportional to the velocity of the oscillations through the transducer. Such signals were amplified, integrated during the recording time (5 s), and translated into numerical units ranging from 0 to 3060, before being processed by a computer.

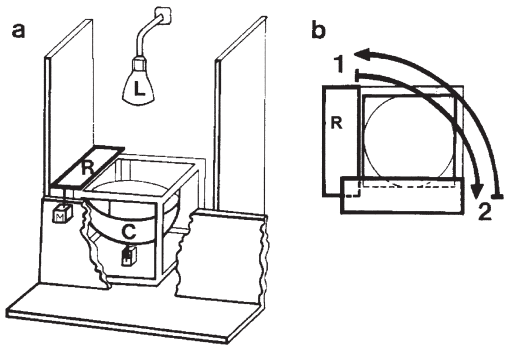

FIG 1. (a) The actometer, one of the 40 units of the apparatus. C: plastic container; R: rectangular screen; M: motor; L: 10 W lamp. (b) Movement of the screen during a cycle (from 1 to 2 and vice versa) of a trial. A cycle lasted circa 2.5 s; a trial (two cycles) circa 5 s.

Thus, the scores were proportionally correlated to the velocity and number of oscillations recorded during the 5 s. Scores lower than 150 per trial are hardly considered as corresponding to an escape response, according to results obtained by video analysis of the crab's activity (33). 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 (ITIs), as well as to monitor experimental events.

Experimental Procedure and Design

Each crab was moved from the holding room to one actometer in the experimental room. All the experiments included a training session and a testing session, separated by a 24-h interval. 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 dimly lit drawers. Each trial consisted of two successive cycles of screen movement without rest interval between cycles (Fig. 1b). Because each cycle lasted nearly 2.5 s, the total trial time was around 5 s. The trial was constituted in this way for two purposes: 1) to obtain a more conspicuous response for each trial, thus ensuring strong vibrations of the container per trial; and 2) to make certain that the passing screen enters the crab's visual field twice from two opposite sides during each trial, thus ensuring animals are similarly stimulated regardless of their positions inside the container. The activity of every crab was recorded during the entire trial time, both at training and the testing session.

Training session. Each experiment included one or more untrained group (U) that stayed in the actometers during the training session but without being trained, and one or more trained groups (T). All animals underwent a pretraining phase consisting of two trials with a 4-s intertrial interval (ITI). The crabs were assigned to each group, taking into account their response levels at pretraining, in such a way that a similar mean baseline of reactivity was obtained for the different groups of the same experiment. Two types of training with the same iterative stimulus were given—namely, spaced training and massed training. Crabs that underwent spaced training received 30 trials with an ITI of 85 s; whereas, those that were given a massed training, received 300 trials with an ITI of 4 s. The total period of training was the same for both types of training (i.e., 45 min). Rationale for choosing 30 trials of

spaced training vs. 300 of massed training is that such a number of training trials proves to be for each case the sufficient minimum number that yields a robust memory retention at testing (i.e., 24 h after training). Namely, failures in memory retention are shown at testing if such a number of trials or such a length of intertrial intervals are reduced (11,32).

It is worth noticing that the activity of the untrained crabs was also recorded during the period of time corresponding to each trial, though the screen was not moved at training for these animals.

Testing session. It consisted of either six trials with an ITI of 85 s, when a spaced training had been given, or six trials separated by 4 s, when a massed training had been used, for both untrained and trained groups. Throughout this study each experimental group consisted of 30 animals. A 10-min adaptation time preceded either the first training or the first testing trial.

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 5% of tested crabs were eliminated.

Crab's baseline of responsiveness to the passing screen, based on data from the two-trial pretraining, proved to be remarkably consistent up to 10 days after arrival; however, on occasion, the animals coming from different capture efforts presented differences in response level. Therefore, only the crabs belonging to the same capture were used in each experiment. The groups of each experiment were run simultaneously.

Drugs and Injection Procedure

Crustacean saline solution (19) was used as a vehicle. Fifty microliters of saline or scopolamine solution were given through the right side of the dorsal 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 in the pericardial sac. Scopolamine was purchased from Sigma Chemical Co.

Data Analysis

Long-term habituation was assessed by focusing the data analysis on testing scores, i.e., by estimating the difference at testing between the escape response level of the trained group (T) and that of the respective untrained group (U). Rescorla (34) 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 session). This view is plenty justified in the present case, because it has been demonstrated that long-term habituation in the crab is independent of the escape response level at training (43), a result consistent with similar findings in other animals (5,33).

According to the distinction previously proposed (32), the comparison between the performance at testing of a trained (T) group and its respective untrained (U) group was accomplished separately on each phase of testing, that is, on the mean response score corresponding to the first testing trial and on the mean response score corresponding to the block of the following five trials (retraining). It is worth noticing two main features that distinguish these two phases of testing: first, the first testing trial, unlike retraining, is not preceded during the 10-min of adaptation time by any event that retrieves the training stimulus. Second, retraining, unlike the first testing trial, involves an iterative presentation of the phasic stimulus. This brief repetition of the initial training procedure is a remarkable feature of this testing phase because LTH at retraining appears to be trial spacing specific (32).

In all previous experiments at our laboratory, without exception, a significant difference (t-test, $\alpha = 0.05$) between T and U was disclosed 24 h after training at both testing phases, when crabs (30 or more ones) received 30 trials with an ITI of 85 s, and a significant difference at retraining but not at first trial, when crabs were given 300 trials with an ITI of 4 s. Therefore, two pairs of U-T groups were used in experiments of this paper—i.e., one saline and one SCP pair—and results were analyzed using a priori planned comparisons, weighed ANOVA with $\alpha = 0.05$ (20,38). The planned comparisons included two types of contrast: a first contrast between untrained groups, whose performances are expected to be similar; and two other contrasts, one for each trained group vs. its respective untrained one. In addition, analyses on a trial-by-trial basis were performed on retraining data, and results with the respective level of significance were shown in the figures and their captions.

A trained group is considered that shows memory retention at testing when its response level is significantly lower than that of the respective untrained group, due to the fact that the former received a training session 24 h before.

Definitions

Short-term habituation refers to the response decrement within the training session; long-term habituation (LTH) to a retention of the response decrement demonstrated in the testing session (at least 24 h after training). Intertrial interval (ITI) refers to the rest interval between trials. Spaced training designates an experimental protocol of 30 training trials separated by a 85-s ITI; massed training, one of 300 trials separated by an ITI of 4 s. Context refers to the environmental features of the training place; signal refers to the screen repeatedly passed overhead. (Context-signal)-LTH is a long-term habituation yielded by spaced training, determined by a context-signal association, and expressed at both phases of testing, i.e., first testing trial and retention phase. (Signal)-LTH is a long-term habituation yielded by massed training, determined only by signal invariance, and expressed only at retention phase of testing.

RESULTS

Posttraining Scopolamine Disrupts the (Context-Signal)-LTH in Dose-Dependent Manner

A first experiment was aimed at testing the effect of increasing doses of scopolamine (SCP), given at the end of the training session, on the (context-signal)-LTH tested at 24 h. Six groups of crabs were formed. One untrained group (saline injected) included 60 animals that remained in the actometers during the entire training session without being stimulated. Five trained groups of 30 crabs each were given a spaced training (30 trials, ITI = 85 s), and injected with saline or different solutions of SCP (0.01, 0.1, 1.0, or 10.0 ng/g). A six-trial testing session was given at 24 h.

A 5×30 ANOVA (mixed repeated measures) computed on the values of the escape response corresponding to the five trained groups during the training session—i.e., prior to the

postraining injections (data not shown)—revealed no group differences and no significant group × trial interaction but a significant trial effect, $F(29, 4205) = 2.01$, $p < 0.01$. A similar result with trained groups was obtained in all experiments in which crabs were posttraining injected.

Figure 2 presents the mean escape response scores of the first testing trial (Fig. 2A) and that of the block of the following five trials (retraining) (Fig. 2B) corresponding to each group . A cursory inspection of these histograms suggests a dose-dependent amnestic effect of SCP on the LTH produced by the spaced training, either at the first testing trial or retraining. A Dunnet test was performed on these data to contrast the saline untrained group vs. each of the trained groups. The first trial (Fig. 2A) showed a significant difference ($p <$ 0.05) for all the contrasts except for saline untrained group vs. 10.0 ng/g SCP trained group. Concerning data from retraining (Fig. 2B), the analysis failed to show significant differences between untrained saline group and either 1.0 or 10.0 ng/g SCP trained groups.

Thus, doses equal to 10.0 ng/g SCP seems to disrupt the (context-signal)-LTH at both testing phases. The fact that the trained groups showed the same responsiveness during training discards the possibility of accounting for these results in terms of differential rates of task acquisition. However, because no untrained group injected with SCP was included in the experiment, an alternative explanation might be offered. Namely, it might be argued that the putative amnestic effect of the drug was due to an enhancing effect of the highest dose of SCP on the crab's responsiveness of the trained groups during testing. This argument was addressed in the following experiment, where an untrained group injected with SCP was included and a higher dose of SCP was tested to have a stronger amnestic effect.

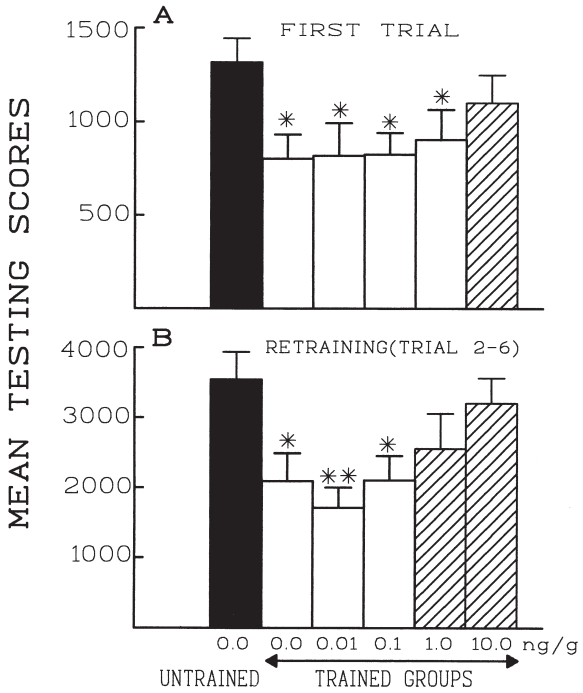

FIG 2. Effect of different doses of scopolamine injected after spaced training (30 trials; ITI = 85 s). A: Results corresponding to the first testing trial. Ordinate: mean testing scores (i.e., average of the escape response scores for the first trial of the testing session). Abscissa: different doses of scopolamine: ng per g body weight. Black bar: mean score (±SEM) of the untrained saline group; white bar: trained group significantly different from untrained group (Dunnet test); stripped bar: trained group no significantly different from the untrained group. (*) stands for $p < 0.05$. (**) stands for $p < 0.01$. B: Results corresponding to retraining. Ordinate: mean testing scores for the block of trials 2–6. Other symbols as in A.

Two pairs of untrained and trained groups were run. Crabs of one U-T pair were injected immediately after training with saline solution, and those of the other pair with 100 ng SCP/g, i.e., a dose higher than the largest dose tested above. Trained groups received spaced training (30 trials spaced by 85 s); while untrained groups remained in the actometers without stimulation. All animals were given six testing trials (also separated by 85 s) at 24 h.

Results corresponding to the testing session are exhibited in Fig. 3. The posttraining injection of SCP showed no effect on responding behavior at testing, because scores of saline and SCP untrained groups were similar. Trial–response curves of the saline groups (Fig. 3, left panel) reveal the pattern of U-T differences usually found after spaced training—namely, a conspicuous between-group difference at either the first trial or retraining. In contrast, SCP abates U-T differences at both testing phases (Fig. 3, right panel). Planned comparisons performed on testing data (Fig. 3, insets) confirmed these observations. No significant difference was found between untrained groups, but significant differences were disclosed between saline groups at both first trial and retraining, $F(1, 116) = 4.3$, $p < 0.05$, and $F(1, 116) = 10.3$, $p < 0.005$; respectively. On the contrary, the analysis showed no significant differences between SCP groups. Coincidently, a trial by trial analysis of retraining showed a significant difference between saline groups for each contrast, but not between SCP groups.

Therefore, LTH acquired by spaced training is spared following SCP injection. The lack of difference between untrained groups (saline vs. 100 ng/g of SCP) lays aside an alternative explanation in terms of an unspecific enhancing effect of SCP on responsiveness. Then, if the scopolamine-induced impairment of (context-signal)-LTH is true, it should be expected that the amnestic effect vanishes when the injection was delayed some time posttraining [e.g., (9)]. For testing this prediction, the following experiment was performed.

The experimental design was as above, except that animals were injected around 1 h after the end of the training session. Results corresponding to the six-testing trial session 24 h after training are illustrated in Fig. 4. A conspicuous difference between trained and untrained groups is exhibited in either saline or 100 ng SCP/g-injected crabs. Planned comparisons showed no significant difference in any contrast between untrained groups. On the contrary, a significant difference was found for U vs. T at the first testing trial for both saline and SCP groups, $F(1, 116) = 10.45$, $p < 0.005$; and $F(1, 116) = 21.5$, $p < 0.005$, respectively; and also at retraining, $F(1, 116) = 11.0$, $p < 0.005$; and $F(1, 116) = 19.1$, $p < 0.005$, respectively. Results from the trial-by-trial analysis of retraining were consistent with those obtained from the above retraining block analysis.

Thus, the memory impairment due to posttraining SCP is shown when the drug is given immediately but not 1 h after training, a result in keeping with the idea of a consolidation process with a defined time window. Besides, this result hardly makes tenable any explanation of the retention deficit of posttraining SCP in terms of an unspecific sequelae of the drug injection.

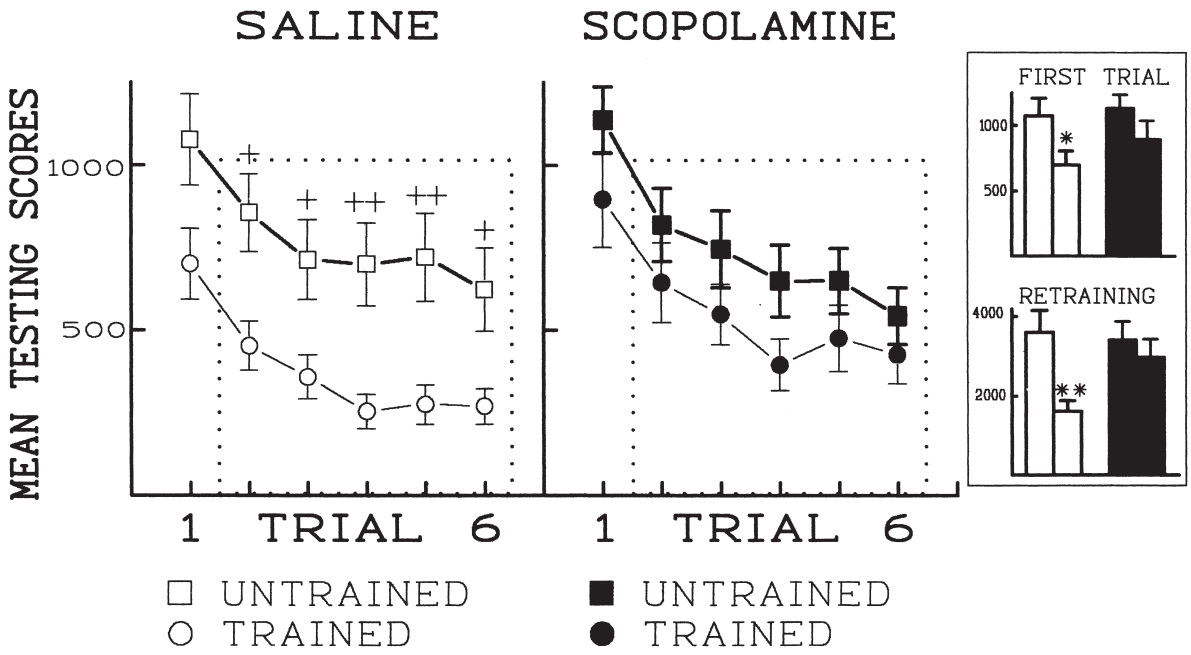

FIG 3. Effect of scopolamine (100 ng/g) injected after spaced training (30 trials; ITI = 85 s). Left panel: Testing results corresponding to saline groups. Ordinate: mean testing scores (±SEM) (i.e., average of the escape response scores for each trial of the six-trial testing session). Abscissa: trials 1 to 6. Open squares stand for the untrained saline group; open circles for the trained saline group. Results corresponding to retraining are enclosed by a pointed line; (+): $p < 0.05$; (++). $p < 0.01$ (analysis on trial by trial basis). Right panel: Testing results corresponding to scopolamine groups. Closed squares stand for the untrained scopolamine group; closed circles for the trained scopolamine group. Other symbols as in left panel. Insets: Planned comparisons: top—comparison between mean escape response scores of first testing trial; bottom—comparison between retraining scores (i.e., average corresponding to the block of the last five trials of the testing session); white bars stand for saline groups; black bars for scopolamine groups. First bar of each pair corresponds to the untrained group, second bar to the trained group. (*) $p < 0.05$. (**) $p < 0.01$.

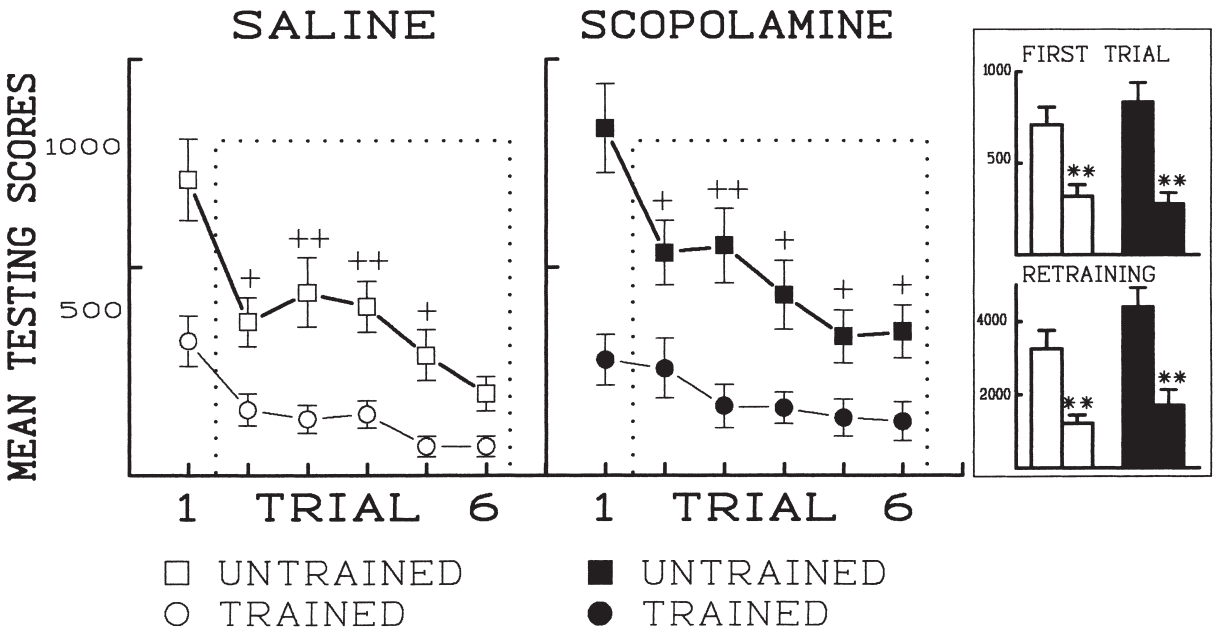

FIG 4. Effect of scopolamine (100 ng/g) injected 1 h after spaced training (30 trials; ITI = 85 s). Symbols as in Fig. 3.

Pretraining Scopolamine Affects the Training Performance and Impairs (Context-Signal)-LTH

The purpose of this section was to test whether SCP given prior to the spaced training also reduces the (context-signal)-LTH at 24 h. As above, two pairs of untrained and trained groups were included. Immediately before training—i.e., after the two-trial pretraining phase—crabs of one U-T pair were injected with saline solution and those of the other pair with a dose of 100 ng SCP/g. Trained groups received spaced training (30 trials spaced by 85 s), and all animals underwent a six-trial retention test session at 24 h.

Results at training are shown in Fig. 5A and B. A decreasing effect of SCP on responsiveness of both trained groups during the nonasymptotic portion of the training curve is apparent (i.e., trials 1–10) (Fig. 5A). As usual, the asymptotic portion starts after training trial 10. A 2 × 10 ANOVA (mixed repeated measures) performed on scores of the first 10 trials, disclosed significant group differences, $F(1, 58) = 8.0$, $p < 0.01$, and significant trial effect, $F(9, 522) = 14.7$, $p < 0.01$, but not significant group × trial interaction. A 2 × 10 ANOVA on the asymptotic portion of the curve (trials 11–30) gave no significant differences. Figure 5B shows the mean scores per trial corresponding to both saline and SCP untrained groups (i.e., animals for whom the screen was not moved during the entire training session). Such scores are lower than 150, and reflect no defensive activities (33). A 2 × 30 ANOVA on these data disclosed no significant differences between groups, thus suggesting that SCP does not alter the activity level corresponding to nondefensive strategies [i.e., exploring, walking, or resting (32)].

Performances during the six-testing trial session at 24 h (Fig. 6) were similar to those shown when SCP was posttraining injected (Fig. 3). Trial–response curves of the saline groups (Fig. 6, left panel) show at first trial and retraining the conspicuous U-T difference usually found after spaced training. In contrast, SCP decreases the U-T differences at both testing phases (Fig. 6, right panel). Planned comparisons performed on testing data (Fig. 6, insets) confirmed these observations. No significant difference was found between untrained groups, but significant differences were disclosed between saline groups at both first trial and retraining, $F(1, 116) = 6.5$, $p < 0.025$, and $F(1, 116) = 6.9$, $p < 0.01$; respectively. On the contrary, the analysis revealed no significant differences between the SCP groups. Coincidently, a trial-by-trial analysis of retraining showed a significant difference between saline groups for each contrast, but not between SCP groups.

Thus, in pretraining, like postraining, injection of 100 ng SCP/g impairs retention of the (context-signal)-LTH at 24 h. However, the present results, unlike those obtained with posttraining SCP, may not be unambiguously interpreted as supporting a modulatory effect of SCP on memory storage. Actually, training was not performed in a drug-free state, and SCP seems to alter the crab's performance at training.

Scopolamine Acts Selectively on the Type of Long-Term Memory

In this section we intended to test whether the amnestic effect of SCP on the (context-signal)-LTH is also observed on the (signal)-LTH.

The usual experimental design of four groups was employed. Crabs of one U-T pair were injected immediately after training with saline solution, and those of the other pair with 100 ng SCP/g. Unlike previous experiments, trained groups received massed training (300 trials with an ITI of 4 s).

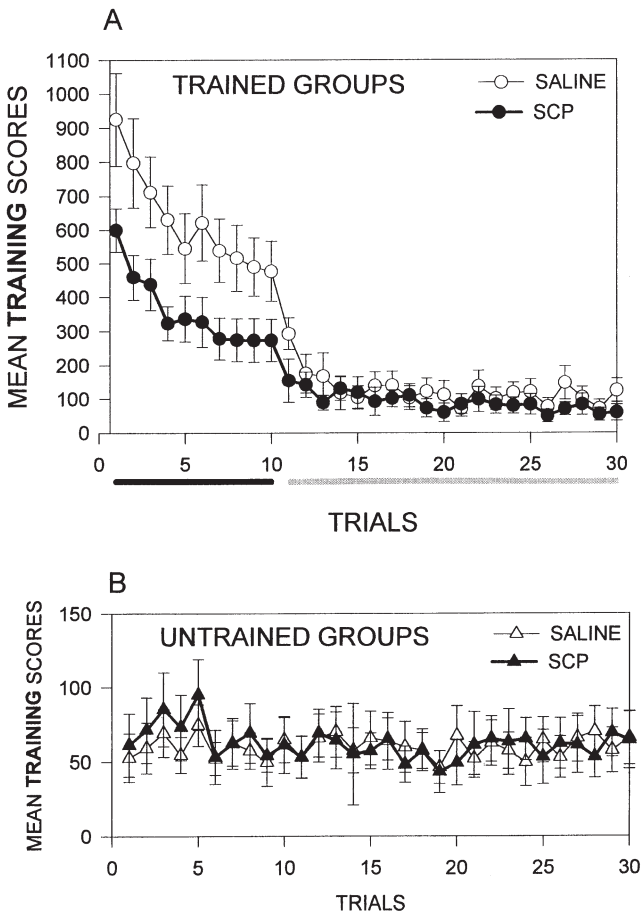

FIG 5. Effect of preinjected scopolamine (100 ng/g) on the performance during a 30-trial training session (ITI = 85 s). (A) Results corresponding to the trained groups. Ordinate: mean training scores (±SEM) (i.e., average of scores of escape response for each trial of the training session). Abscissa: first row—trials; second row—a horizontal black bar signalizes the preasymptotic portion of the curve (Trials 1–10); a horizontal gray bar indicates the asymptotic portion (Trials 11–30). Open circles correspond to the trained saline group; closed circles to the trained scopolamine group. (B) Results corresponding to the untrained groups during the same trials of (A) but without stimulation with the passing screen. White triangles stand for the saline untrained saline group; black triangles for the SCP untrained group.

All animals were given the six-testing trials at 24 h, with the same ITI of 4 s.

Results at testing are depicted in Fig. 7. Scores of saline and SCP untrained groups were similar, confirming that the posttraining injection of SCP has no effect on responding behavior at testing. Trial–response curves of either the saline groups (Fig. 7, left panel) or the SCP groups (Fig. 7, right panel) reveal the pattern of U-T differences usually found after massed training, namely, a conspicuous difference confined to the retraining phase. Planned comparisons performed on testing data (Fig. 7, insets) are in keeping with these observations. No significant difference was found between untrained groups, but a significant difference was disclosed between both saline and SCP groups at retraining, $F(1, 116) = 8.3$, $p < 0.005$, and $F(1, 116) = 13.3$, $p < 0.005$; respectively.

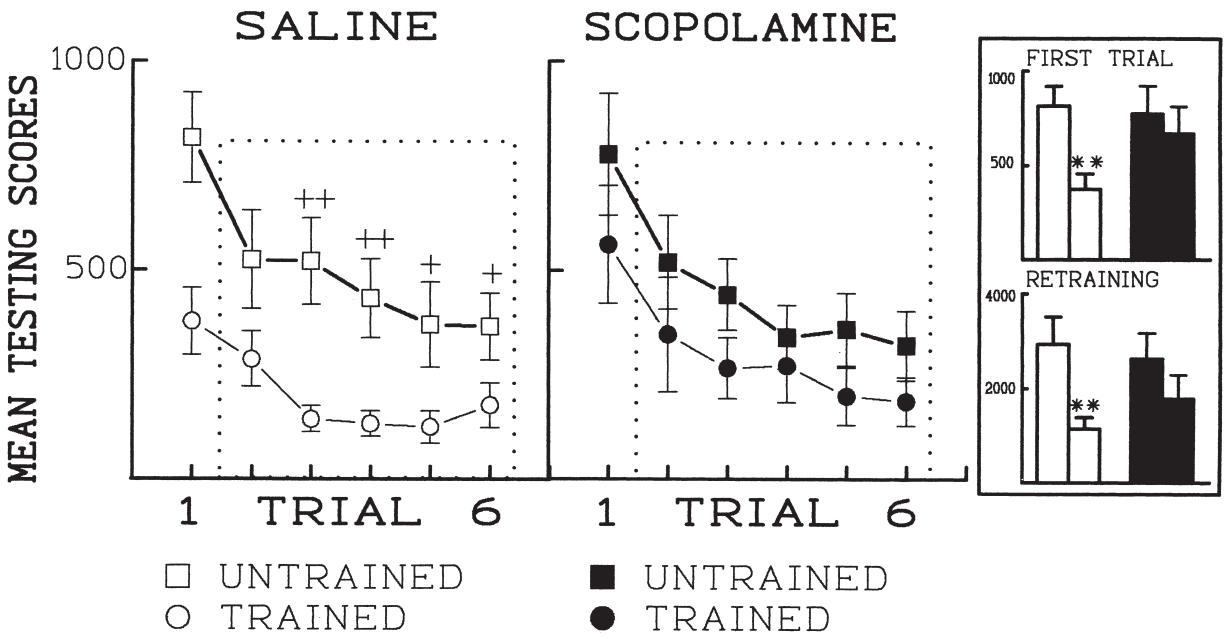

FIG 6. Effect of scopolamine (100 ng/g) injected before spaced training (30 trials; ITI = 85 s). Symbols as in Fig. 3.

Results from the trial-by-trial analysis of retraining were consistent with those obtained from the above retraining block analysis.

A further experiment was conducted to explore the effect of SCP administered before massive training—i.e., immediately after the two-trial pretraining phase. The experimental design was as above, except for the moment when crabs were injected. However, the response level of the SCP trained group was lower than that of the saline-trained group during the three initial trials of the massive training (data not shown). Like in the previous SCP experiments with spaced training, a 2×10 ANOVA (mixed repeated measures) was performed here on scores of the first 10 training trials. The analysis disclosed no significant group differences but a significant trial effect, $F(9, 522) = 30.3$, $p < 0.01$, and a significant group 3 trial interaction, $F(9, 522) = 2.9$, $p < 0.01$. Tests of simple main effect revealed a significant group effect on training trial 1, $F(1, 58) = 8.9$, $p < 0.01$, 2, $F(1, 58) = 6.3$, $p < 0.025$, and 3,

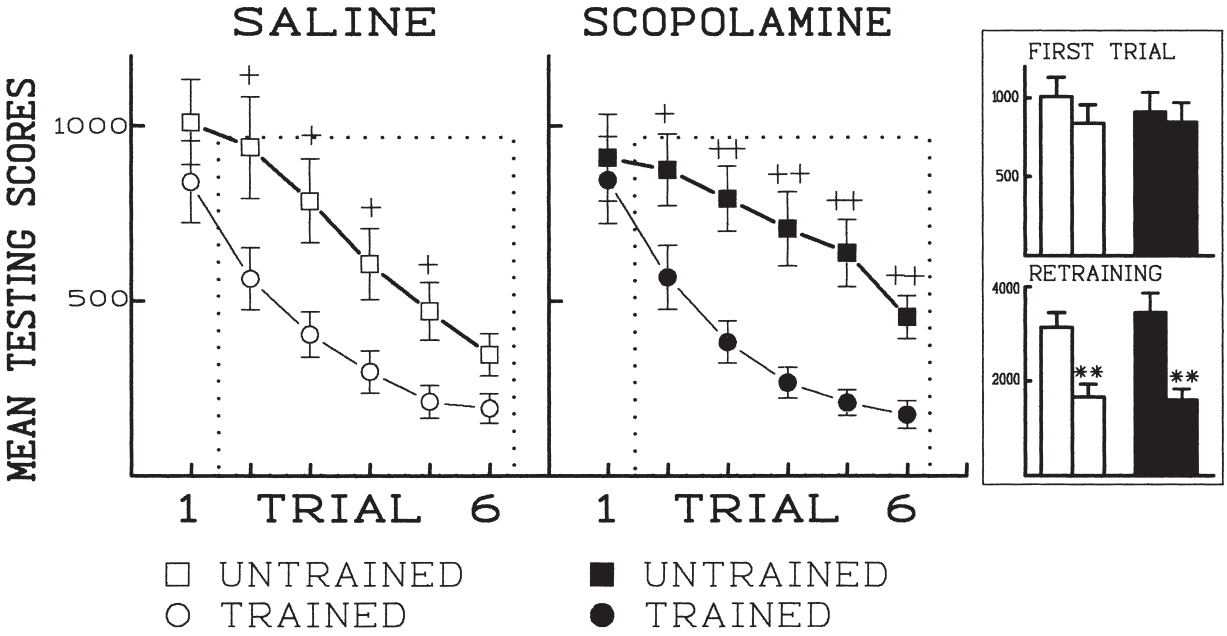

FIG 7. Effect of scopolamine (100 ng/g) injected after massed training (300 trials; ITI = 4 s). Symbols as in Fig. 3.

$F(1, 58) = 4.03$, $p < 0.05$; a result in keeping with those obtained on spaced training, though here the effect is confined to the first three trials. No significant differences in level of activity between saline and SCP untrained groups was found during the training session.

The pattern of results at testing (Fig. 8) was like that obtained when SCP was injected after massed training (Fig. 7)—namely, a noticeable difference between either saline or SCP groups confined at the retraining phase. Planned comparisons (Fig. 8, insets) disclosed significant differences only between saline groups or between SCP groups, both during retraining, $F(1, 116) = 4.1$, $p < 0.05$, and $F(1,116) = 7.2$, $p < 0.01$, respectively. Results from the trial-by-trial analysis of retraining were consistent with those obtained from the retraining block analysis.

This experimental outcome indicates that the crab's memory acquired after massed training seems to be impervious to the muscarinic–cholinergic antagonism—i.e., SCP seems to have different effects on each type of LTH.

DISCUSSION

Results from this article showed that posttraining injection of scopolamine, a muscarinic antagonist in the crab's brain (6), induces amnesia only after spaced training, suggesting a selective muscarinic–cholinergic involvement in the (context-signal)-LTH. Alternative interpretations, as those some times forwarded concerning the effect of scopolamine in experiments with vertebrates (7), are in this case hardly tenable. The possibility that the SCP effect on crab's LTH could be explained in terms of a direct effect on performance (49), or on sensory (17,21) or attention/arousal processes (10,29), has to be excluded. Actually, both training and testing of the posttraining SCP experiments in the crab were performed in a drug-free state (26). No difference at testing was detected between untrained groups, and no effect was shown by the delayed posttraining injections, thus discarding the possible proactive effects of SCP on nonmnemonic factors that may interfere with retention test performance.

A selective effect of SCP on (context-signal)-LTH is also shown when the drug is injected before the spaced training. An alternative interpretation of this result in terms of a selective effect of SCP on sensory processing or on the attentional function during spaced training is unlikely, because pretraining SCP has a similar decremental effect on the initial trials of both spaced and massed training. Besides, both types of training share salient features: the same eliciting stimulus, the same training time (45 min), and the same ability to induce short-term habituation. In addition, the level of activity of both the saline and SCP untrained group (i.e., when the screen was not moving) was similar during the training session.

However, it might be argued that the putative different sensitivity of spaced and massed crabs to scopolamine is only determined by the different "level of practice" during training (i.e., 30 vs. 300 trials). A consistent line of evidence (12,32) has shown that in the case under study the "level of practice"is not only determined by the number of training trials but, mainly, by the length of the rest interval between trials. According to our previous results, the level of practice of 30 training trials separated by 85 s and is tantamount to 300 trials separated by 4 s. In other words, such combinations of the number of trials and length of intertrial intervals yield memories of similar strength, although they are acquired through different mechanisms. Similar memory strength is a condition to test the effect of a drug on two different mnemonic processes.

When the injection of SCP was delayed 1 h posttraining, no effect on retention was observed, indicating a time-dependent effect of the SCP on memory storage processes. Remarkably, this effective time of posttraining injection is very similar to that obtained in this crab for the facilitatory or amnestic effect of PKA activation or PKA inhibition, respectively (23, 35,36), or to that reported for the facilitatory or amnestic effect of angiotensin II or saralasin, respectively (11). On the

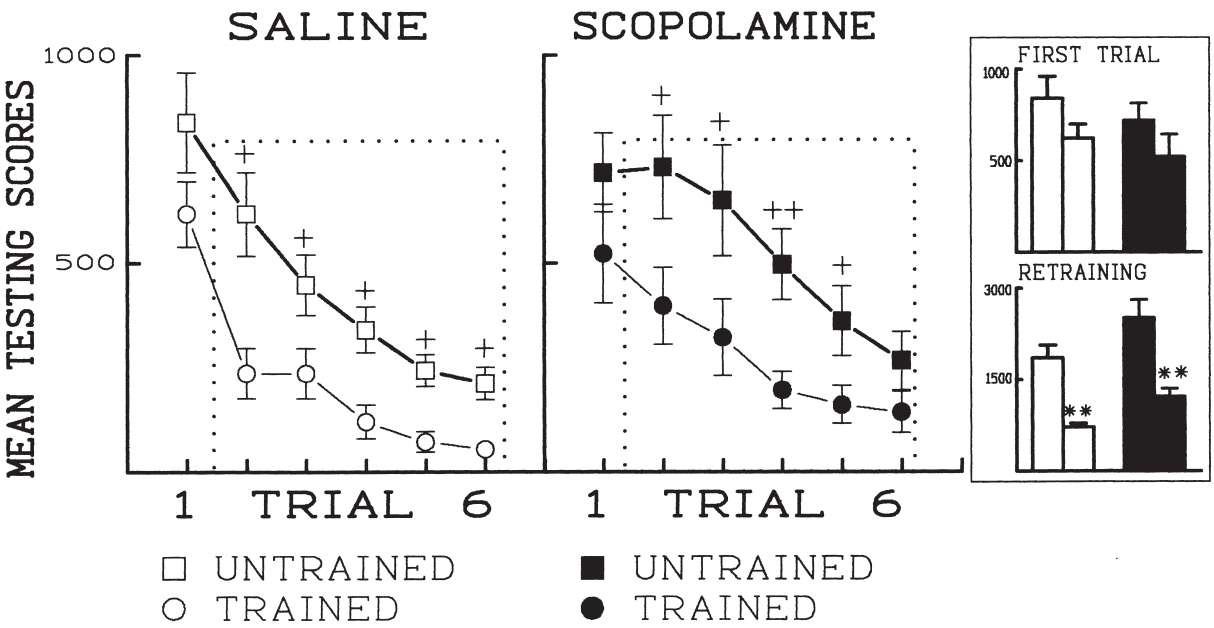

FIG 8. Effect of scopolamine (100 ng/g) injected before massed training (300 trials; ITI = 4 s). Symbols as in Fig. 3.

other hand, the protein synthesis inhibitor cycloheximide had a clear-cut amnestic effect when given 2 h after training but not at 6 h (30). Such a disparity in the forward limit of the effective time window would be accounted for by the well-known "time-locked" hypothesis, which considers the process of memory consolidation as a series of sequentially dependent stages (9,14). Accordingly, scopolamine (as well as the activator and the inhibitor of PKA, and saralasin and angiotensin II) would act upon the immediate aftermath of a training experience, that is, at an early stage of memory consolidation, while the action of cycloheximide would occur downstream of the event (41).

Results of the present study, suggesting that the mAchRs are selectively involved in the (context-signal)-LTH, do not discard the possibility that other modality of the cholinergic system may be implicated in the (signal)-LTH. Actually, it has been demonstrated in the lateral giant neuron escape circuit of the crayfish, which seems to underlie simple forms of learning (27), the presence of postsynaptic cholinergic receptors similar to a nicotinic type of receptor that has been extensively characterized in crustacean neurons and muscle (22,25).

The lowest effective dose used here was 10^{-5} M solution injected in 50 μl volume, which corresponds to 10^{-7} M in hemolymph, because the hemolymph volume is roughly 5 ml—30% of the body weight (15)—provided that the drug diffuses evenly throughout the crab's body. This effective dose is actually a very low one, but compatible either with the dose that inhibits the L-[^{3}H]QNB binding in the crab's brain [2×10^{-9} M (6)], or with the dose that blocked both sEPSP and the associated change of the spike threshold in the proleg retractor motoneurons of the tobacco hornworm [2×10^{-7} M (47)].

Previous experiments using *Chasmagnathus* have also shown that effective drug doses given by systemic administration were manifestly low, that is, equivalent or even lower than doses administered by intracraneal injections in vertebrates—e.g., cycloheximide (30), actinomycin-D (31), angiotensin II (11), enkephalin (16), or serotonin (4). The relative simplicity of the brain organization and the lack of an endothelial brain–blood barrier in crabs (1), together with the fact that blood is distributed throughout an extensive capillary system in various neuropil areas of brain (2,39), could account for the low threshold found for the drug action. The possibility of working with such low doses given by systemic injection makes this a suitable approach to study mechanistic aspects of the mnemonic processes. Pharmacological manipulation in behaving organisms allows testing for the relevance of the purported molecular processes on long-term memory, a necessary step to bring the search for the molecular basis of neuronal plasticity back to the behavior that it was designed to explain (42).

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