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Two related forms of memory in the crab *Chasmagnathus* are differentially affected by NMDA receptor antagonists

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Abstract

A visual danger stimulus (VDS) elicits an escape response in the crab *Chasmagnathus* that declines after a few iterative presentations. Long-lasting retention of such decrement, termed context-signal memory (CSM), is mediated by an association between danger stimulus and environmental cues, cycloheximide sensitive, correlated with PKA activity and NFκ-B activation, positively modulated by angiotensins, and selectively regulated by a muscarinic–cholinergic mechanism. The present research was aimed at studying the possible involvement of NMDA-like receptors in CSM, given the role attributed to these receptors in vertebrate memory and their occurrence in invertebrates including crustaceans. Vertebrate antagonists (±)-2-amino-5-phosphonopentanoic acid (AP5) and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801) were used. Memory retention impairment was shown with MK-801 10^{-3} M (1 μg/g) injected immediately before training or after training, or delayed 1 or 4 h, but not 6 h, posttraining. An AP5 10^{-3} M dose (0.6 μg/g) impairs retention when given before but not after training. Neither antagonist produced retrieval deficit. A memory process similar to CSM but nonassociative in nature and induced by massed training (termed signal memory, SM), proved entirely insensitive to AP5 or MK-801, confirming the view that distinct mechanisms subserve these different types of memory in the crab. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Learning; Memory; NMDA; AP5: MK-801; Crab; Crustacea

1. Introduction

An extensive series of experiments support the fact that NMDA receptors are implicated as mediators in several memory processes, e.g., spatial learning in the water or radial maze (Morris et al., 1986; Caramanos and Shapiro, 1994), inhibitory avoidance memory, (Jerusalinsky et al., 1992; Rickard et al., 1994; Burchuladze and Rose, 1992), early olfactory learning (Lincoln et al., 1988; Weldon et al., 1997), fear conditioning, (Miserendino et al., 1990), delayed conditional discrimination (Tan et al., 1989), brightness discrimination (Tang and Ho, 1988) and conditioned taste aversion (Gutiérrez et al., 1999). Such enhanced research on the role of NMDA receptors in neural learning mechanisms has resulted more from the discovery that these receptors are involved in induction of hippocampus LTP than for any other reason (Cain, 1997). Although grounded on very disparate learning paradigms, this research has been confined to vertebrate species alone. However, the occurrence of NMDA type of receptors in invertebrates has been often reported since their early description in the visual interneurons of crayfish by Pfeiffer-Lynn and Glantz (1991). In decapod crustaceans, several reports identify the presence of NMDA-related receptors by biochemical, immunostaining, Western blot analysis and electrophysiological techniques (Parnas et al., 1994, 1996; Feinstein et al., 1998; Schramm and Dudel, 1997; Burgess and Derby, 1997). Besides, the occurrence of such type of glutamate receptors has also been documented in mollusks, namely, in isolated ganglia of gasteropods by electrophysiological techniques (Moroz et al., 1993; Kavaliers et al., 1997) and in tissues of bivalves by high-performance liquid chromatography (Todoroki et al., 1999). On the other hand, cDNA isolated from *Drosophila* has been described, which encodes a putative NMDA receptor protein that displays 46% amino acid identity to the rat NMDAR1 polypeptide (Ultsch et al., 1993). Nevertheless, the role of NMDA in learning and memory processes

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in an invertebrate has not been studied, apart from a report on a cellular conditioning analog in *Aplysia* (Murphy and Glanzman, 1999). Therefore, the purpose of the present paper is to investigate such a function in a widely studied invertebrate memory model, namely, in the crab *Chasmagnathus*.

Upon sudden presentation of a rectangular screen passing overhead, the crab *Chasmagnathus granulatus* responds with a running reaction in an attempt to escape. The response declines over stimulation trials separated by rest intervals and the decrement persists for at least 5 days (Lozada et al., 1990; Pedreira et al., 1995). At first, this memory was considered an instance of simple habituation, because the response decrement fulfilled most of the parametrical conditions of such nonassociative learning (Brunner and Maldonado, 1988). However, further results showed that two different types of memory can be elicited by the iterative presentation of the same stimulus, depending on the number of stimulation trials but more critically on the interval between them. When a crab is given spaced training (i.e., 15 or more trials separated by 171 s of intertrial interval), long-term memory is mediated by a conditioned association between the environmental features of the training site (the context) and the features of the screen moving overhead (the signal) (Tomsic et al., 1998a,b), so that such long-term memory is termed context-signal memory (CSM). In contrast, when a crab is given massed training (i.e., 300 or more trials with a 2-s interval between trials), memory is nonassociative depending solely on the signal invariance, so that is termed signal memory (SM). Insofar as research develops on these two types of crab memory, their nature becomes more clearly discriminated, both from behavioral and mechanistic viewpoints: CSM is expressed by a reduction in the level of escape response from the first test trial of a six-trial testing session performed 24 h after training, whereas SM is only expressed from the second test trial (Pedreira et al., 1998). CSM is cycloheximide sensitive and long lasting, and entails the building up of a strong and persistent freezing; in contrast, SM is insensitive to cycloheximide and shorter lasting, and consists merely of the escape response tapering off without building up a specific defensive response (Hermitte et al., 1999; Pedreira et al., 1995, 1996). Furthermore, CSM, but not SM, is mediated by the cAMP signal pathway (Romano et al., 1996a,b; Locatelli et al., 2001), positively modulated by angiotensins (Delorenzi et al., 1996, 2000), selectively regulated by a muscarinic cholinergic mechanism (Beróon de Astrada and Maldonado, 1999), and mediated by the NFκ-B transcription factor (Freudenthal and Romano, 2000; Freudenthal et al., 1998).

Such correlation between memory modality and trial spacing, is also found in a powerful memory system of arthropods, namely the fruit fly (*Drosophila melanogaster*) (Tully et al., 1994). However, the peculiar feature of the crab's learning paradigm is that the diverse memory modalities are actually distinct types of memory, since CSM and SM involve two distinct learned responses, i.e., freezing response and nondefensive response, respectively (Pereyra et al., 2000). In contrast, although memory modalities of the fruit fly are also trial spacing dependent, they are temporal phases of the same type of memory, i.e., odor avoidance conditioning.

Hence, the aim of this work was to initiate a research on the role of NMDA type receptors in crab memory, exploring the possibility that these receptors are differentially involved in the associative and nonassociative crab's memory, namely, in CSM and SM. To this end, two selective and potent antagonists of mammalian NMDA receptors were used, namely: (±)-2-amino-5-phosphonopentanoic acid (AP5) and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801).

2. Materials and methods

2.1. Animals

Animals were adult male *Chasmagnathus* crabs 2.7–3.0 cm across the carapace, weighing around 17.0 g, collected from water less than 1 m depth in the *rias* (narrow coastal inlets) of San Clemente del Tuyú, Buenos Aires province, Argentina, and transported to the laboratory, where they were lodged in plastic tanks ($35 \times 48 \times 27$ cm) filled to 2-cm depth with diluted sea water, 20 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. Holding (tank) and experimental rooms were kept on a 12-h light–dark cycle (light on 07:00–19:00 h) at 22–24 °C. Animals were fed rabbit pellets (Nutrientes, Argentina) every 3 days and after feeding the water was changed. Experiments were carried out within the first week after the animal's arrival, from January to August, and between 08:00 and 18:00 h. Each crab was used only in one experiment. Experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Apparatus

The apparatus is described in detail elsewhere (Maldonado, 1997). 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 in diameter, covered to a depth of 0.5 cm with prepared water. The crab was lodged in the container suspended by three strings from an upper wooden framework ($23 \times 23 \times 30$ cm) and illuminated with a 10-W lamp placed 30 cm above the animal. A motor-operated opaque screen (a 25×7.5-cm rectangle) was moved horizontally over the animal's head, cyclically from left to right and vice versa. Screen displacements provoked a running response of the crab and resulting 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

their amplitude and frequency through the transducer. These signals were amplified, integrated during each 9-s trial and translated into numerical units ranging from 0 to 3000, before being processed by computer. The experimental room had 40 actometers, separated 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. A second apparatus (Equipment 2) with identical features but threefold more mechanically sensitive was used mainly to record levels of spontaneous crab activity in the actometers. Rationale for using equipments adjusted to diverse amplification is that small differences in quantity of movement can only be detected by the more sensitive setup that, in turn may become saturated when crabs display intense responses to the visual danger stimulus (VDS) represented by the moving screen.

2.3. Experimental procedure and design

Each crab was moved from the holding room to one actometer in the experimental room. 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 lasted 9 s and consisted of two successive cycles of screen movement. The activity of every crab was recorded during the entire trial time, both at training and testing session.

To test the effect of either NMDA receptor antagonist on both the associative and nonassociative crab memory (CSM and SM, respectively), two types of experiments were carried out, namely, spaced-training and massed-training experiments.

2.3.1. Spaced-training experiment

It included four groups of crabs: one untrained group (control, CT) that was kept in the actometers during the 1-h training session but without being trained, i.e., without being presented the VDS; and three (or two) trained groups that received, after a 15-m adaptation time, 15 trials with the VDS separated by an intertrial interval (ITI) of 171 s. Twenty four hours later, all the groups had an identical testing session: 15-m adaptation time followed by six trials with the VDS separated by an ITI of 171 s During the experiment, each crab was given an injection at a predetermined time. The untrained group and one of the three trained groups were injected with the vehicle and thus termed saline control (SAL-CT) and saline trained group (SAL-TR), respectively; and each of the two remaining trained groups was given a different dose of the same antagonist.

2.3.2. Massed-training experiment

The experimental design was as above including the untrained group, but the three trained groups received 300 trials with 2 s of intertrial interval (ITI = 2 s) during the training session and six trials with ITI = 2 s during the testing session.

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 discarded.

2.3.3. Drugs and injection procedure

Crustacean saline solution (Hoeger and Florey, 1989) was used as a vehicle. Fifty microliters of saline or drug solution was 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. The crab's circulatory system is a semiopen system in which the hemolymph (blood) flows freely throughout the hemocoelic cavity. From the pericardial sac, blood enters the heart through three pairs of valved, slit-like openings; and from the heart, blood is channeled to the brain through the anterior aorta.

The NMDA receptor antagonists AP5 and MK-801 were purchased from Sigma.

2.4. Data analysis

Memory retention, either CSM or SM retention, was assessed by focusing data analysis on testing scores, i.e., by estimating the difference at testing between the response level of the trained group (TR) and that of the respective untrained group (control group, CT). A TR is said to show *memory retention* when its mean response level at testing is statistically lower than that of the respective untrained group. However, if the difference between CT and TR fails to prove statistically significant when the former is saline injected and the latter drug injected, the failure is initially attributed to an amnesic effect of the drug. Rescorla (1988) 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 amply justified in the present case since it has been demonstrated that CSM retention in the crab is independent of the escape response level at training (Tomsic et al., 1991), a result consistent with similar findings in other animals (Applewhite et al., 1969; Peeke and Veno,1976).

Following a distinction previously proposed (Pedreira et al., 1998), the comparison between CT and TR at testing was accomplished separately on each phase of testing, that is, between the mean response scores corresponding to the first trial of the testing session (*first test trial*) and between the mean response scores corresponding to the block of the following five trials (*retraining* phase). Diverse reasons

support the pertinence of distinguishing these two contrasting phases at testing. Retention at first test trial is exclusive of CSM and retrieved by exposure to the context for 15 min before testing; instead, retention at retraining is an expression of SM and retrieved by the first presentation of the VDS (Pedreira et al., 1998). Several results have shown that administration of diverse amnesic or facilitatory drugs differentially affect the two test phases (e.g., Hermitte et al., 1999).

In all previous experiments at our laboratory, a significant difference (*t* test, $\alpha = 0.05$) between trained (TR) and untrained (CT) groups was invariably disclosed 24 h after training at both testing phases (CT > TR) when 15 or more training trials (ITI = 171 s) were given (spaced training), and on the other hand, a significant difference at retraining but not at first trial, when training consists of 300 or more trials (ITI = 2 s) (massed training). Such significant differences were also found when crabs were saline injected at diverse pre- or posttraining intervals.

In the analysis of data corresponding to experiments aimed at testing the effect of an antagonist administered before or at different times after training, the saline untrained group (SAL-CT) was contrasted with each of the TRs, i.e., with the saline trained group (SAL-TR) and with each of the TRs that received a different dose of the antagonist. For assessing between-group differences, planned comparisons on data obtained at each phase of the testing session were performed following a significant main effect in one-way analysis of variance (ANOVA) ($\alpha = 0.05$). On the other hand, in experiments to assess possible drug performance effects, two-way ANOVAs of repeated measures were used to evaluate differences between saline- and drug-injected groups ($\alpha = 0.05$). All response scores are represented as means ± S.E.M. We analyzed the data using STATISTICA'99 Edition. Windows 6.1 software package.

2.5. Definitions

Intertrial interval (ITI) refers to the rest interval between trials. *Spaced training* designates a training of 15 trials separated by a 171-s ITI; *massed training*, a training of 300 trials with 2-s rest interval between trials. *Context* refers to the environmental features of the training site (i.e., visual, chemical and texture features of the actometer recipient and the liquid medium); *signal* (i.e., VDS) refers to the screen repeatedly passed overhead. *Memory retention* is operationally defined as the statistically significant difference between response level of control and TRs at either testing phase.

3. Results

3.1. Effect of pretraining AP5 on the induction of CSM

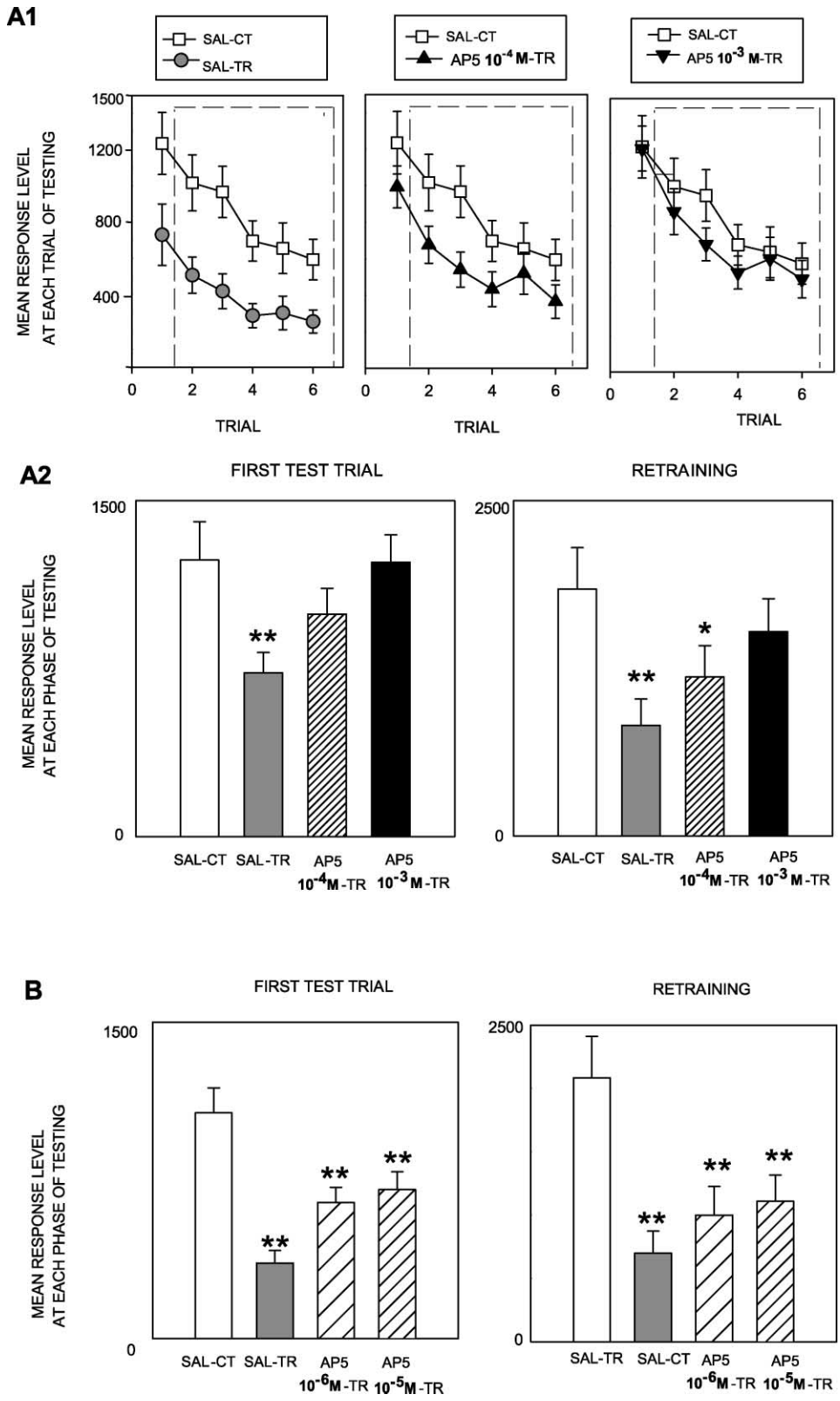
In the first experiment of this section, four groups of crabs ($n = 40$) were formed according to the four-group design: one SAL-CT that remained in the actometers during the entire training session without being presented the VDS; and three TRs preinjected with either saline (SAL-TR) or the smaller dose of AP5 (10^{-4} M TR) or the larger dose (10^{-3} M TR), and all given spaced training (15 trials, ITI = 171 s). Injections were given immediately before training. All groups received a six-trial testing session at 24 h.

Fig. 1A1 presents the trial–response curve at testing of the SAL-CT against that of each TR: Comparison with the SAL-TR (left panel) reveals the pattern of CT-TR differences usually found after spaced training, namely, a conspicuous disparity at both first test trial and retraining trials. By contrast, memory retention (CT-TR difference) was reduced for 10^{-4} and 10^{-3} M TR groups (middle and right panels). Planned comparisons performed on first test trial data (Fig. 1A2, left panel), following significant main effect in the ANOVA [$F(3,156) = 3.53$, $P < .025$], confirmed a significant CT-TR difference (memory retention) for SAL-CT versus SAL-TR ($P < .01$), but not vs. 10^{-4} or vs. 10^{-3} M TR. On retraining data (i.e., the average of accumulated scores of the last five test trials) (Fig. 1A2, right panel), planned comparison after ANOVA ($F = 4.32$, $P < .01$) showed significant difference for SAL-CT vs. SAL-TR ($P < .01$) or vs. 10^{-4} M TR ($P < .05$) but not vs. 10^{-3} M TR. Thus, these doses of AP5 exert a disrupting effect on memory retention, and such effect seems to be stronger at first test trial than at retraining. In order to test the reproducibility of such disparity between test phases, two further replications of this experiment were performed. Results proved (data not shown) memory impairment at first test trial for both doses in both replications, i.e., no difference for either SAL-CT vs. 10^{-4} or vs. 10^{-3} M; but at retraining, only the larger dose and only in one replication showed impairing effect on retention.

Fig. 1. Effect of AP5 injected before spaced training (15 trials; ITI = 171 s), on response level at test. (A) 10^{-4} or 10^{-3} M. A1: Trial–response curves at testing. Left panel: saline control group (SAL-CT, white squares) versus saline trained group (SAL-TR, gray circles) Middle panel: SAL-CT (as above) versus AP5 10^{-4} M trained group (upward black triangles). Right panel: SAL-CT (as above) versus AP5 10^{-3} M trained group (downward black triangles). Ordinate, mean test response (± S.E.M.) (i.e., average of the escape response scores for each trial of the six-trial testing session). Abscissa, Trials 1 to 6. Dashed lines encompass the retraining phase. A2: Planned comparisons: response scores of each trained group contrasted with those corresponding to data from SAL-CT (white bar). Ordinate, mean test response (± S.E.M.). Comparisons were focused on the first testing trial (left panel) and on the accumulated scores of the last five testing trials (retraining phase, right panel). Gray bar stands for SAL-TR; fine striped bar, for AP5 10^{-4} M trained group; black bar, for AP5 10^{-3} M trained group. * $P < .05$, ** $P < .01$. (B) 10^{-6} or 10^{-5} M. Planned comparisons: response scores of each trained group contrasted with those corresponding to data from SAL-CT (white bar). Comparisons were focused on the first testing trial (left panel) and on retraining (right panel). Gray bar stands for saline trained group (TR-CT); coarse striped bar, for AP5 10^{-6} M trained group; medium striped bar, AP5 10^{-5} M trained group. Other symbols as in A1.

Smaller doses of AP5 (10^{-6} or 10^{-5} M) injected pretraining, lacked effect on memory retention (Fig. 1B), i.e., planned comparisons showed CT-TR significant differences ($P<.01$) for all the three contrasts, either at first test trial (left panel) [ANOVA $F(3,156)=11.38$, $P<.001$] or at retraining (right panel) (ANOVA $F=9.0$, $P<.001$).

Therefore, AP5 administered immediately before spaced training seems to disrupt memory retention at 24 h in a dose-



dependent manner, apparently more consistent at first test trial than at retraining. The following series of experiments was aimed at exploring whether such absence of significant CT-TR difference at test could be explained in terms other than a specific effect on learning.

3.2. Analysis of possible performance effects induced by AP5

No overt symptoms of sickness or functional impairment could be detected in crabs either immediately after AP5 10^{-3} M injection or for periods of observation as long as 24 h. The righting reaction shown by a healthy crab when turned on its back was displayed with the same speed and strength by either AP5-injected or untreated animals. Instances of autotomy, i.e., appendage losses induced by self mutilation as defensive reaction (Fredericq, 1883), have often been shown by crabs in response to the injection of diverse drugs (e.g., Pedreira et al., 1995), but none were recorded after AP5 administration.

The effects of AP5 10^{-3} M dose on the response level to the VDS in groups that had not been previously trained was estimated in three different conditions (Fig. 2, Experimental protocols): Protocol A_1: 15 trials given immediately after injection; Protocol A_2: six trials given 24 h after an injection followed by a 1-h period of crab stay in the actometer; and Protocol A_3: six trials given 23 h after injection preceded by a 1-h period of crab stay in the actometer. A series of three experiments was conducted, adopting Protocol A_1, A_2 and A_3, respectively, and including two untrained groups per experiment ($n=35$): one untrained group injected with saline and the other with the same AP5 dose. Results are summarized in the three graphs of Fig. 2A. No difference whatsoever between performances of saline and AP5 was detected in any of the three experiments (ANOVA of repeated measures only showed significant trial effect, $P<.01$, for the three experiments). Therefore, when an AP5-injected group is not previously trained, the drug injection fails to affect per se crab defensive response level, regardless of the extension of the interval between injection and the VDS presentation or the relation between injection and context exposure.

A further experiment was performed for estimating AP5 effect on spontaneous crab activity, i.e., basic activity displayed without screen presentation (Pereyra et al., 1999). To detect such activity threefold more sensitive actometers were used (Equipment 2, Materials and methods). Two groups of 35 animals each were formed: a SAL group was injected with the vehicle and the other with the drug (10^{-3} M). After injection, both groups stayed in the actometers during 15 min, without VDS presentation. The activity was recorded in one hundred 9-s periods without interval between records. The level of spontaneous activity was closely similar for both groups during the entire recording period (Fig. 2C). The ANOVA of repeated measures disclosed no significant drug nor trial effect, suggesting that spontaneous activity shown by crabs when confined to the actometers is not affected by an injection of AP5.

Such absence of AP5 effects either on response level to the danger stimulus or on spontaneous activity of untrained groups, seems to rule out an explanation of AP5 effects on trained groups (Fig. 1A) in terms other than memory impairment.

3.3. Effect of posttraining and pretest AP5 on CSM

A first experiment of this series was aimed at exploring the effect of the highest dose of AP5 (10^{-3} M) on CSM, when given immediately after training. Three groups of crabs ($n=30$) were formed according to the three-group design: one SAL-CT that remained in the actometers during the entire training session without being presented the VDS; and two TRs injected with either saline (SAL-TR) or AP5 (10^{-3} M TR), and all given spaced training (15 trials, ITI = 171 s). All groups received a six-trial testing session at 24 h (ITI = 171 s).

Results are presented in Fig. 3A, where a clear-cut retention for both saline and AP5 TRs is shown. Planned comparisons following the ANOVA [$F(2, 87)=5.82$, $P<.01$] disclosed at first test trial a significant CT-TR difference (memory retention) for SAL-CT vs. SAL-TR or vs. 10^{-3} M TR ($P<.01$); and after ANOVA ($F=3.3$, $P<.05$), at retraining, a significant CT-TR difference for both contrasts ($P<.05$). Thus, administration of the drug immediately after spaced training fails to produce CSM blockade, at odds with the effects obtained by AP5 10^{-3} M injected pretraining.

A further experiment ($n=40$) was carried out aimed at testing the effect of AP5 administration on CSM retrieval. A three-group design as above was used, but the injections were given 20 min before the six-trial test session. Data corresponding to the testing session are shown in Fig. 3B. At first test trial, planned comparisons following the ANOVA [$F(2, 117)=7.7$, $P<.001$] disclosed a significant CT-TR difference for SAL-CT vs. SAL-TR or vs. 10^{-3} M TR ($P<.01$); and at retraining, after ANOVA [$F=4.6$, $P<.001$), a significant CT-TR difference for both contrasts ($P<.01$), suggesting that AP5 administration 20 min before testing fails to impair memory retrieval.

3.4. Effect of pretraining MK-801 on CSM

To further investigate the effect of mammalian NMDA receptor antagonists on crab CSM, the noncompetitive NMDA receptor antagonist MK-801 was used. In the first experiment of this series, four groups of crabs ($n=35$) were formed according to the four-group design: one SAL-CT that remained in the actometers during the entire training session without being presented the VDS; and three TRs injected with either saline or 10^{-5} or 10^{-3} M of MK-801 (groups termed SAL-TR, 10^{-5} and 10^{-3} M TR, respectively), all given spaced training (15 trials, ITI = 171 s). All groups received a six-trial testing session at 24 h (ITI = 171 s).

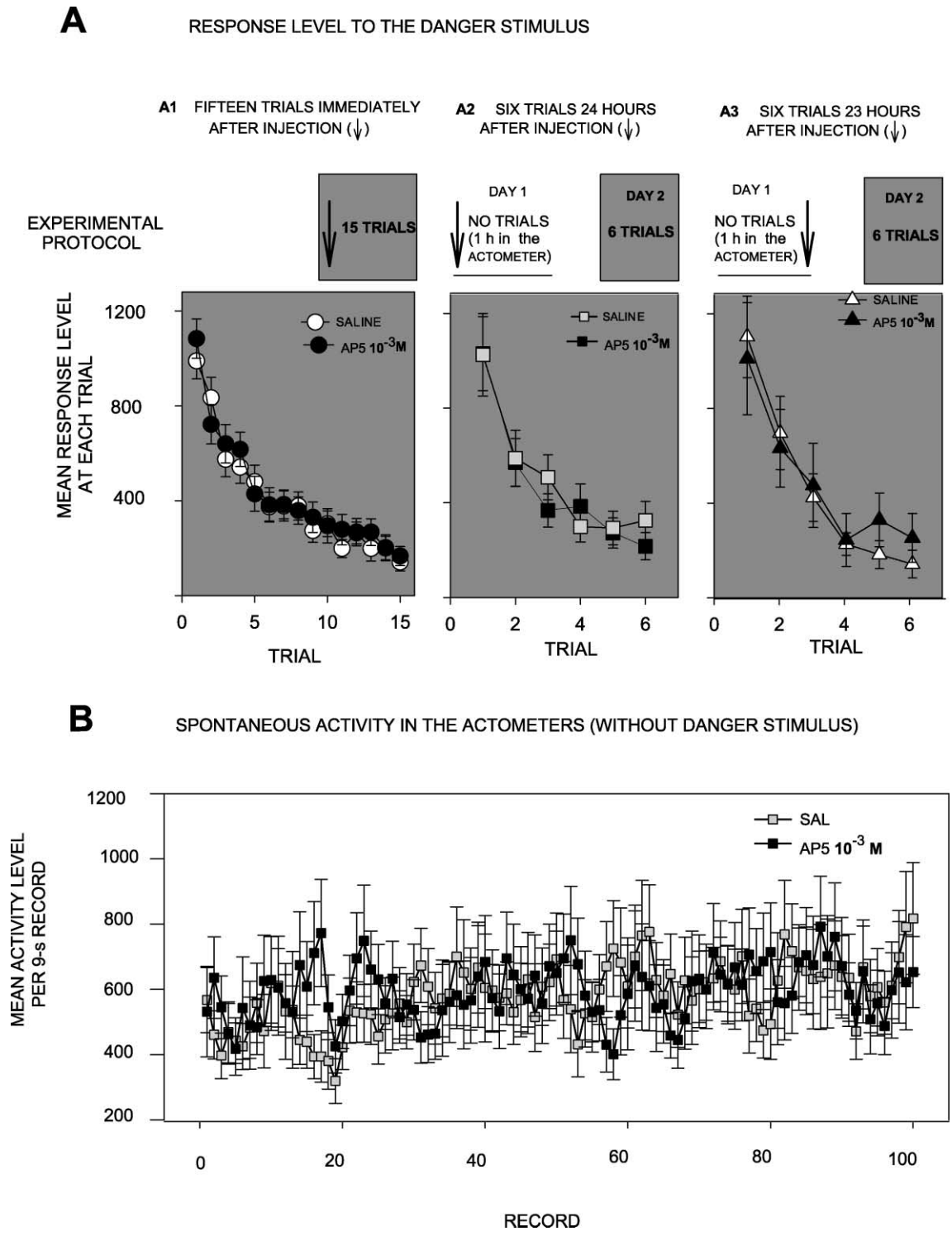


Fig. 2. Analysis of possible performance effects induced by AP5 10^{-3} M (0.6 μg/g). (A) Response level to the danger stimulus. First row: Experimental protocols: A1, A2 and A3 (see text). An arrow stands for injection time point relative to trial session and to the period of actometer exposure. Second row: Graphs corresponding to each protocol. Ordinate: mean of response scores at each trial (±S.E.M.). Abscissa: trials with danger stimulus presentation (ITI = 171s). White symbols stand for saline groups and black symbols for AP5 10^{-3} M; circle, square and triangle symbols for Protocol A1, A2 and A3, respectively. (B) Spontaneous activity in the actometers (without danger stimulus). Ordinate: mean of activity level per 9-s record (±S.E.M.). Abscissa: 100 successive 9.0-s records.

Fig. 4A presents the trial–response curve at testing of the SAL-CT against that of each TR: Comparison with the SAL-TR (left panel) showed the pattern of CT-TR differences usually found after spaced training, namely, a conspicuous

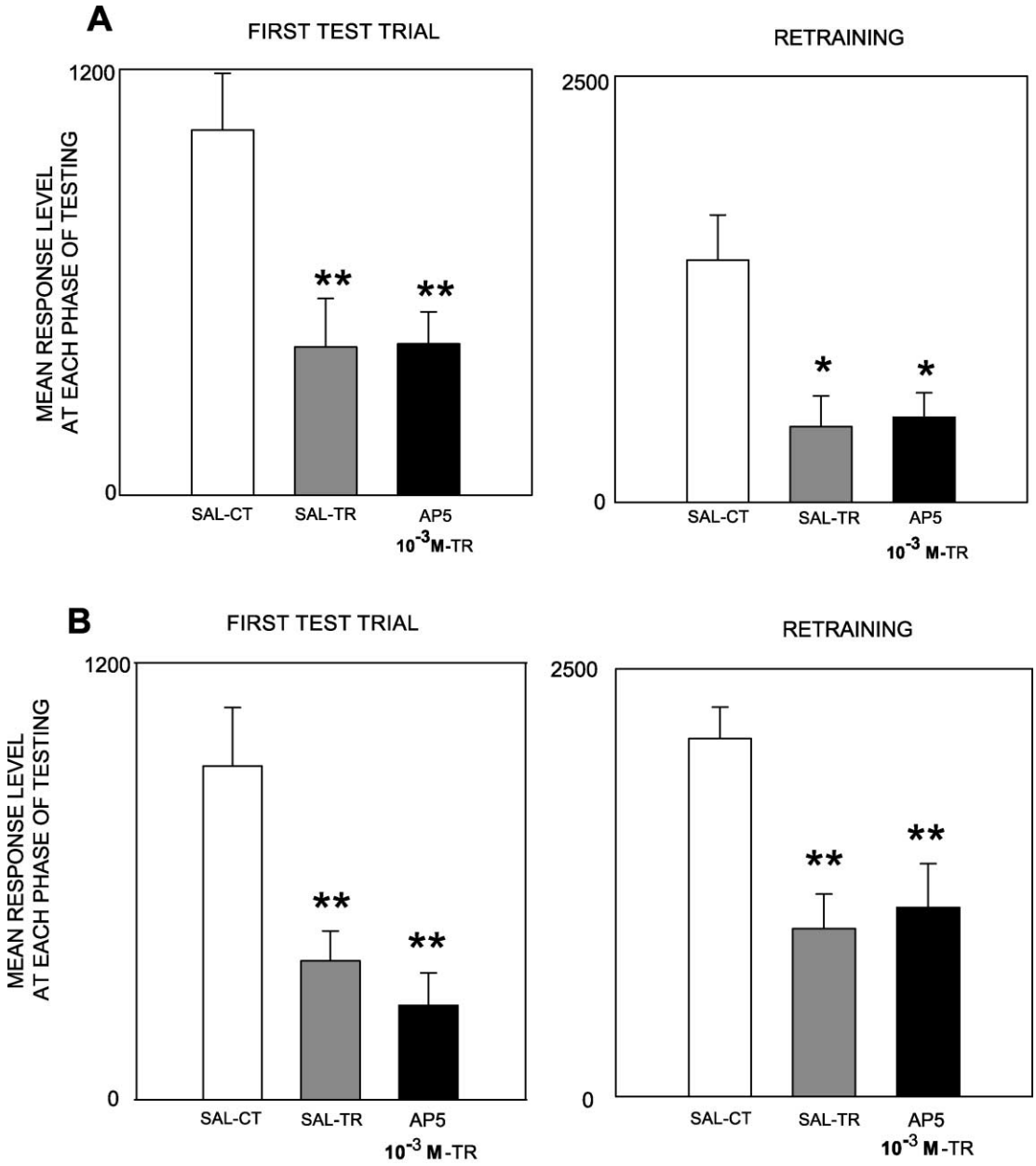


Fig. 3. (A) Effect of AP5 10^{-3} M injected posttraining, on response level at test. Left panel: first test trial. Right panel: retraining (accumulated scores of the last five testing trials). White bar stands for saline control group (SAL-CT), gray bar stands for saline trained group (SAL-TR); and black bar for AP5 10^{-3} M group. Planned comparisons: response scores of each trained group contrasted with those corresponding to data from SAL-CT (gray bars). * $P<.05$, ** $P<.01$. (B) Effect of AP5 10^{-3} M injected pretest, on response level at test. Symbols as in (A).

memory retention at both first trial and retraining phase. By contrast, CT-TR differences appear clearly reduced when SAL-CT was compared with MK-801 TRs (middle and right panels). At first test trial (Fig. 4B, left panel), planned comparisons, after ANOVA [$F(3,136)=3.82$, $P<.01$], disclosed a significant CT-TR difference (memory retention) for SAL-CT vs. SAL-TR ($P<.01$) but not vs. 10^{-5} or vs. 10^{-3} M TR. Concerning retraining data (Fig. 4B, right panel), planned comparisons after ANOVA ($F=3.2$, $P<.05$), disclosed a significant CT-TR difference for SAL-CT vs. SAL-TR ($P<.01$) but not vs. 10^{-5} or vs. 10^{-3} M TR.

Smaller doses of MK-801 (10^{-7} or 10^{-6} M) injected pretraining, lacked effect on memory retention (data not shown), i.e., significant differences for all the three contrasts were disclosed, either at first test trial or at retraining.

Therefore, MK-801 administered immediately before spaced training seems to impair CSM in a dose-dependent manner. The following series of experiments, similar to that above conducted with AP5, was aimed at exploring whether such absence of significant CT-TR difference at test could be explained in terms other than the impairing effect of MK-801 on memory retention.

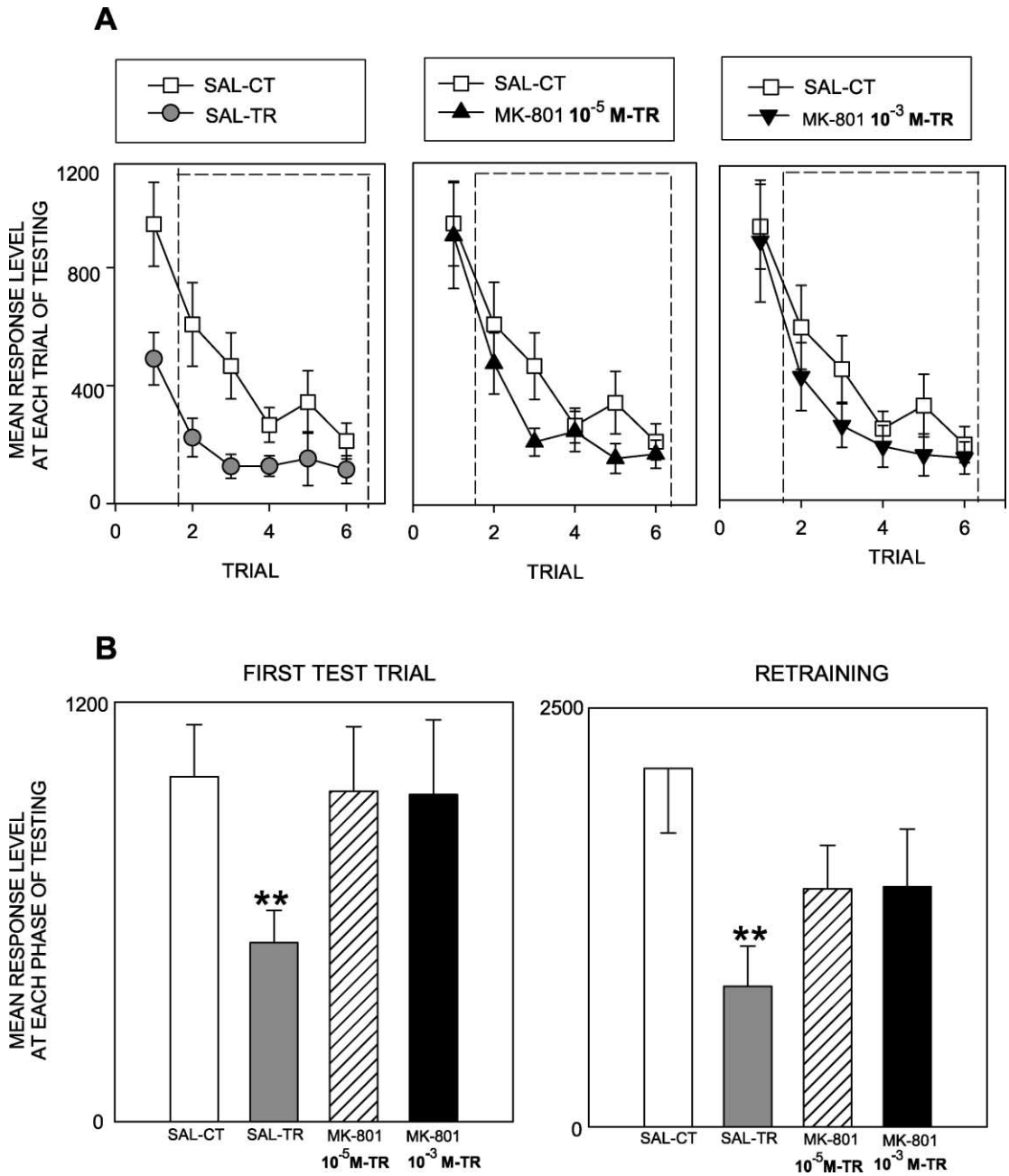


Fig. 4. Effect of MK-801 10^{-5} or 10^{-3} M injected pretraining, on response level at test. (A) Trial–response curves at testing. Left panel: saline control group (SAL-CT, white square) versus saline trained group (SAL-TR, gray circles) Middle panel: SAL-CT (as above) versus MK-801 10^{-5} M trained group (upward black triangles). Right panel: SAL-CT (as above) versus MK-801 10^{-3} M trained group (downward black triangles). Other symbols as in Fig. 1A. (B) White bar stands for SAL-CT; gray bar for saline trained group (TR-CT); fine striped bar for MK-801 10^{-5} M trained group; black bar, for MK-801 10^{-3} trained group. Other symbols as in Fig. 1B.

3.5. Analysis of possible performance effects induced by MK-801

No overt symptoms of sickness or functional impairment, as weakening of the righting reaction or instances of autotomy, were observed after MK-801 10^{-3} M injection for periods of observation up to 24 h.

An exact replica of previous four experiments with AP5 aimed at assessing performance effects (Fig. 2), were repeated with 10^{-3} M of MK-801. Results (data not shown) were closely similar to those obtained for AP-5. No difference whatsoever between performances of saline and MK-801 groups was detected at any trial in any of the four experiments, namely, neither the basic activity displayed without screen presentation nor the response elicited by the VDS was altered by MK-801 injection.

The conclusion from this analysis is similar to that drawn from experiments with AP5. The absence of MK-801

effects on untrained groups, seems to rule out an explanation of AP5 effects on TRs (Fig. 4A) in terms other than memory impairment.

3.6. Effect of posttraining MK-801 on CSM

This series of experiments was carried out to test the effect of MK-801 administered at different times after a 15-trial training session (ITI = 171). In all cases the four-groups design was used, i.e., one SAL-CT that remained in the actometers during the entire training session without being presented the danger stimulus; and three TRs injected with either saline (SAL-TR) or 10^{-5} or 10^{-3} M dose of MK-801 (groups termed SAL-TR, 10^{-5} and 10^{-3} M TR, respectively). All groups received a six-trial testing session at 24 h (ITI = 171 s).

Fig. 5 (0 h) presents testing results corresponding to groups ($n = 35$) injected immediately after training. At first test trial (left panel), planned comparisons, after ANOVA [$F(3,136) = 3.8$, $P < .01$] disclosed a significant CT-TR difference for SAL-CT vs. SAL-TR ($P < .01$) but not vs. 10^{-5} M TR or SAL-CT vs. 10^{-3} M TR. Concerning retraining data (right panel), planned comparisons after ANOVA ($F = 3.4$, $P < .025$), disclosed significant differences for the three contrasts ($P < .01$). Thus, either MK-801 dose, given immediately after spaced training, impairs CSM only at first test trial.

The following experiment ($n = 40$) was as above except that animals were injected 1 h after the end of the training session (Fig. 5, 1 h). At first test trial (left panel) planned comparisons [ANOVA, $F(3,156) = 4.1$, $P < .001$] disclosed a significant CT-TR difference for SAL-CT vs. SAL-TR or vs. 10^{-5} M TR ($P < .01$), but not vs. 10^{-3} M TR. Concerning retraining (right panel), comparisons (ANOVA $F = 3.5$, $P < .01$) showed significant differences for the same contrasts but again, no difference for SAL-CT vs. 10^{-3} M TR. Therefore, at first test trial, the 10^{-5} M dose showed memory impairment when given immediately but not 1 h after training, a result in keeping with the idea of a consolidation process with a defined time window, whereas the 10^{-3} M dose had impairing effect on CSM at both 0 and 1 h. At retraining phase, results are inconsistent as to 10^{-3} M dose, showing amnesic effect at 0 h but not at 1 h.

In a third experiment of this series, injections were given 4 h after training ($n = 40$) and results at testing session were as above with injections given 1 h after. At first test trial [ANOVA $F(3,156) = 5.4$, $P < .001$] a significant CT-TR difference was found for SAL-CT vs. SAL-TR or vs. 10^{-5} M TR ($P < .01$) but not vs. 10^{-3} M TR; and at retraining (ANOVA $F = 3.3$, $P < .01$), a significant difference for SAL-CT vs. SAL-TR or vs. 10^{-5} M TR ($P < .01$) but not vs. 10^{-3} M TR. Thus, again, only the 10^{-3} M dose impairs CSM retention at both testing phases.

When drug doses were administered 6 h after training, no effect with either dose was shown at test session (Fig. 5, 6 h), ($N = 35$): Planned comparisons revealed significant CT-TR differences for all the contrasts ($P < .001$), both at first test trial [ANOVA $F(3,136) = 7.1$, $P < .001$] and at retraining (ANOVA $F = 13.2$, $P < .001$). Thus, the disrupting effect of the higher dose disappears when given at 6 h after training.

To sum up, three conclusions may be drawn from results of this section: (1) Administration of MK-801 10^{-5} M immediately, but not 1 h, after training impairs CSM only at first test trial. (2) A dose of MK-801 10^{-3} M induces CSM impairment at first test trial when injected immediately, 1 or 4 h, but not at 6 h, after training. (3) Drug effect seems to be more consistent at the initial phase of test than at the second one, since both doses failed to show memory impairment at retraining in a case where they did at first test trial (Fig. 5, 0 h).

These results suggest that MK-801 disrupts memory consolidation with a defined time window. Besides, they represent additional support to the conclusion that memory impairment cannot be explained in terms of proactive drug effects on the performance at test.

A further experiment was carried out to assess the effect of MK-801 10^{-3} M on CSM when the drug was injected 20 m before test. The experimental design was as that used above for AP5. Results corresponding to the testing session (data not shown) revealed no impairment effect of the drug on CSM retention, i.e., there was a significant difference between the control saline group and the trained MK-801 group at both test phases, thus paralleling above results with AP5.

3.7. Effect of AP5 and MK-801 on SM

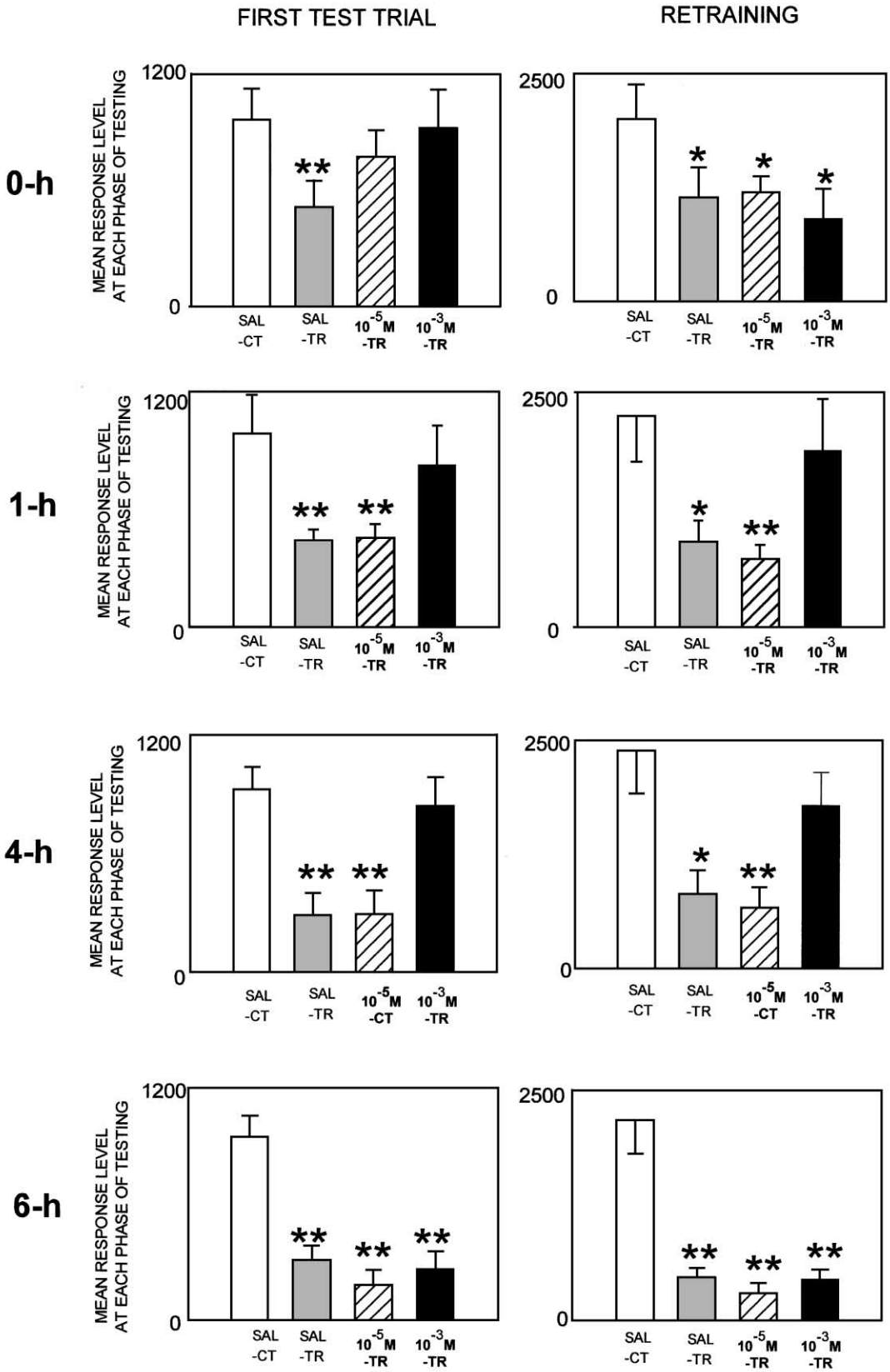
In this section, experiments were aimed at testing whether the impairing effect of AP5 and MK-801 on an associative memory (CSM), is also observed on a nonassociative memory (SM).

The usual dose design of four groups was employed ($n = 40$): Crabs of both SAL-CT and SAL-TR were injected with the vehicle and the other pair with MK-801 10^{-5} or 10^{-3} M. All injections were given immediately before

Fig. 5. Effect of MK-801 10^{-5} or 10^{-3}M injected after training, on response level at test. 0 h: Injections immediately after training. Testing session. Ordinate: mean of response scores at each trial (±S.E.M.). Left panel: first test trial. Right panel: retraining (accumulated scores of the last five testing trials). White bar stands for saline control group (SAL-CT), gray bar stands for saline trained group (SAL-TR); striped bar for MK-801 10^{-5} M TR; black bar for MK-801 10^{-3} M TR. Planned comparisons: response scores of each trained group contrasted with those corresponding to data from SAL-CT (white bar). * $P < .05$, ** $P < .01$. 1 h: injections 1 h after training. Testing session. Symbols as in 0 h. 4 h: injections 4 h after training. Testing session. Symbols as in 0 h. 6 h: injections 6 h after training. Testing session. Symbols as in 0 h.

the first training trial. Unlike previous experiments, TRs received massed training (300 trials with ITI = 2 s). All animals were given the six-test trial session at 24 h (ITI = 2 s).

At training (data not shown), trial–response curves corresponding to the three TRs (SAL-, 10^{-5} and 10^{-3} M TR) were very similar, and a 3×15 ANOVA of repeated meas-



ures showed no significant difference between groups nor interaction effect. Thus, the response level to the VDS is not altered by a previous injection of MK-801, either during spaced or massed training.

At testing, the pattern for each pair of curves (Fig. 6A) was the one routinely found after massed training with non-injected animals, namely, no significant difference at first test trial and a conspicuous difference at retraining. It is germane to recall here that spaced, but not massed training provides a CT-TR difference (memory retention) at first test trial (Pedreira et al., 1998). Planned comparisons (Fig. 6B) disclosed significant differences at retraining (ANOVA

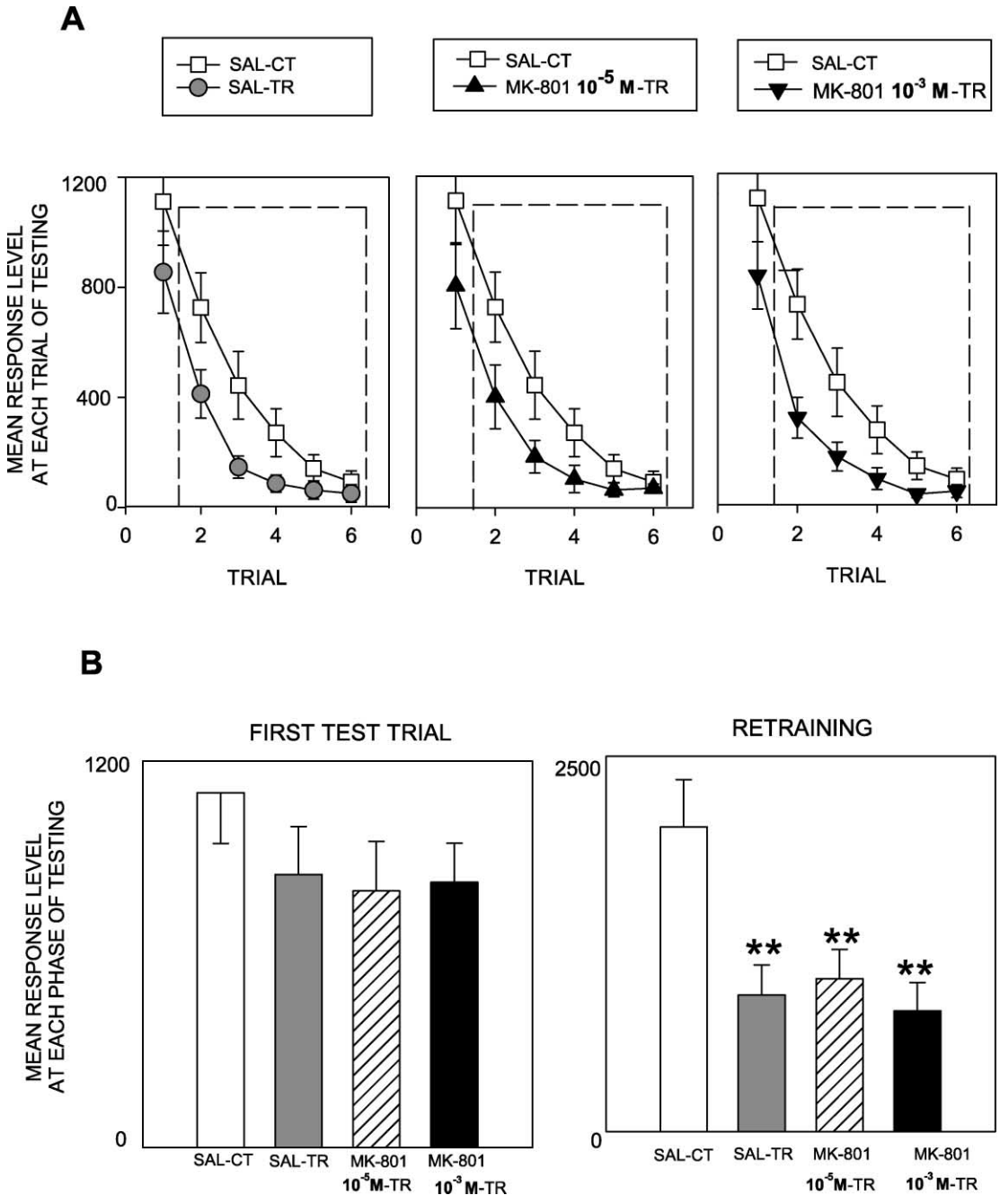


Fig. 6. Effect of MK-801 10^{-5} or 10^{-3} M injected before massed training (300 trials; ITI = 2s), on response level at test. (A) Trial–response curves at testing. Left panel: saline control group (SAL-CT, white squares) versus saline trained group (SAL-TR, gray circles). Middle panel: SAL-CT (as above) versus MK-801 10^{-4} M trained group (upward black triangles). Right panel: SAL-CT (as above) versus MK-801 10^{-3} M trained group (downward black triangles). Other symbols as in Fig. 1A. (B) White bars stands for SAL-CT; gray bar, for SAL-TR; striped bar, MK-801 10^{-5} M trained group; black bar, for AP5 10^{-3} M trained group. Other symbols as in Fig. 1B.

$F(3, 156) = 5.8$, $P < .001$) for the three contrasts, i.e., for SAL-CT vs. SAL-TR ($P < .05$), or vs. 10^{-4} M TR ($P < .05$), or vs. 10^{-3} M TR ($P < .01$).

A parallel experiment with AP5 was performed and similar training and testing results were obtained (data not shown).

Therefore, memory acquired after massed training, unlike after spaced training, seems to be impervious to either AP5 or MK-801, suggesting that these drugs exert contrasting effects on either crab memory type.

4. Discussion

Results from this paper showed that AP5 or MK-801 disrupts context signal, but not signal, memory in crabs, either when they are given immediately before training, or when MK-801 is injected up to 4 h after training. There are a number of reasons why this effect cannot be interpreted in terms of performance effects. Firstly, no overt symptoms of sickness or functional impairment (as instances of autotomy or sluggish righting reactions), were shown by crabs, whether immediately after drug injection or for observation periods up to 24 h. Secondly, neither arousal nor depression of spontaneous activity was detected when crabs were administered the highest dose of AP5 or MK-801 (10^{-3} M) (Fig. 2B). Thirdly, injections of such doses to untrained crabs lacked enhancing or depressing effect on the response level to the VDS, regardless of the injection–trial interval or the time point crabs were previously exposed to the actometer. The absence of drug-induced hyperactivity is especially relevant in the present study, considering that throughout the CSM impairment is evaluated as a reduction in the CT-TR difference at testing. Fourthly, the impairment of CSM retention at test (i.e., 24 h after training) was shown by MK-801 injections when given at 0, 1 or 4 h, but not at 6 h after training, a finding that seems incompatible with an explanation of test results in terms of proactive drug effects on test performance.

Both AP5 and MK-801 failed to impair retention at retraining in several cases where they did so at first test trial. Thus, the impairing effect of these drugs on CSM seems to be stronger at the initial test phase, a result similar to that obtained by injecting cycloheximide (Pedreira et al., 1995, 1996) or PKA inhibitors (Romano et al., 1996a,b); and consistent with the fact that some behavioral treatments as latent inhibition or extinction affect memory expression mainly at first test trial (Tomsic et al., 1998a,b). Rationale for this differential effect would be that these agents only impairs one of the two mechanisms underlying memory expression at test. According to a model that has been previously proposed (Maldonado, 1997, 2002; Pedreira et al., 1998; Tomsic et al., 1998a,b), CSM retention at first test trial is subserved by an associative mechanism, exclusive of this type of memory, based on the estimation of the training ITI extension. Instead, retention at retraining is subserved both by this CSM associative mechanism as well as by a nonassociative mechanism shared by CSM and SM, based on counting the number of discrete training trials. Therefore, if it is assumed that AP5 and MK-801 only affect the associative mechanism, it should be expected that their administration would produce consistent CSM impairment at first test trial but partial or inconsistent CSM at retraining, and no drug effect at all on SM. Present results accord with these predictions.

The forward limit of the time window for MK-801 (4–6 h) is within the range found for cycloheximide (Pedreira et al., 1995), but is twofold longer than that corresponding to the amnesic effect of scopolamine (Berón de Astrada and Maldonado, 1999) or than that corresponding to the facilitatory or amnesic effect of angiotensin II or saralasin, respectively (Delorenzi et al., 1996). Such a disparity would be accounted for by the well known ‘‘time-locked’’ hypothesis that regards the process of memory consolidation as a series of sequentially dependent stages (Davis and Squire, 1984; Gibbs and Ng, 1979). Accordingly, while scopolamine, as well as saralasin and angiotensin II-like peptides, would act upon the immediate aftermath of a spaced-training experience, that is, at an early stage of memory consolidation, the action of MK-801 and cycloheximide would occur downstream of the event (Scholey et al., 1993).

No memory impairment was disclosed by injecting AP5 immediately after training. However, this result does not rule out the possibility that AP5 exerts an impairing effect on CSM when given at longer time intervals after training–a caveat that has also to be posed concerning MK-801. In fact, several recent results (Locatelli et al., 2001; Merlo et al., 2001) indicate that either the PKA inhibitor (Rp-8-Cl-cAMPs) or the NFκ-B inhibitor sulfasalazine induces amnesia on crab CSM in two time windows, i.e., during training and 4–6 h after training, which roughly agrees with two phases of NFκ-B activation (Freudenthal and Romano, 2000). Therefore, it seems germane to perform further experiments aimed at exploring the effect of AP5 given at different time intervals after training.

The lowest effective dose was 10^{-4} M for AP5 and 10^{-5} M for MK-801, injected in 50-μl volume, which corresponds to 0.06 μg/g for AP5 and 0.01 μg/g for MK-801. Taking into account that hemolymph volume is roughly 5 ml, 30% of the body weight (Gleeson and Zubkoff, 1977), and assuming that drug diffuses evenly throughout the crab’s body, the actual concentration in tissues must be estimated on the basis of an at least 100-fold dilution. The minimal effective dose seems to be lower than that of experiments with vertebrates in which acute and systemic administration of these antagonists was also used (e.g., McLamb et al., 1990: Burchuladze and Rose, 1992). Previous experiments using *Chasmagnathus* have 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 (Pedreira et al., 1995), actinomycine-D (Pedreira et al., 1996), angiotensin II (Delorenzi et al.,

1996), enkephalin (Godoy and Maldonado, 1995), serotonin (Aggio et al., 1996), scopolamine (Berón de Astrada and Maldonado, 1999) and PKA inhibitors and activators (Romano et al., 1996a,b; Locatelli et al., 2001). The relative simplicity of the brain organization and the lack of endothelial brain–blood barrier in crabs (Abbott, 1970), together with the fact that blood is distributed throughout an extensive capillary system in various neuropil brain areas (Abbott, 1992; Sandeman, 1986), could account for the low threshold found for drug action.

Since NMDA-like receptors have been identified in Crustacea and on the other hand, AP5 and MK-801 are recognized as NMDA antagonists in mammals, the disrupting effect of these drugs on CSM may be interpreted as being mediated by this type of glutamate receptors. Therefore, these results would provide the first indication that NMDA-related receptors are involved in the storage of long-term memory in an invertebrate. However, a conclusive interpretation would require further research aimed at discerning how these drugs affect NMDA receptors in the crab's nervous system.

Acknowledgments

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