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Dissociation between memory reactivation and its behavioral expression: Scopolamine interferes with memory expression without disrupting long-term storage

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ABSTRACT

The reconsolidation hypothesis has challenged the traditional view of fixed memories after consolidation. Reconsolidation studies have disclosed that the mechanisms mediating memory retrieval and the mechanisms that underlie the behavioral expression of memory can be dissociated, offering a new prospect for understanding the nature of experimental amnesia. The muscarinic antagonist scopolamine has been used for decades to induce experimental amnesias The goal of the present study is to determine whether the amnesic effects of scopolamine are due to storage (or retrieval) deficits or, alternatively, to a decrease in the long-term memory expression of a consolidated long-term memory. In the crab *Chasmagnathus* memory model, we found that scopolamine-induced amnesia can be reverted by facilitation after reminder presentation. This recovery of memory expression was reconsolidation specific since a reminder that does not triggers reconsolidation process did not allow the recovery. A higher dose (5 μg/g) of scopolamine induced an amnesic effect that could not be reverted through reconsolidation, and thus it can be explained as an interference with memory storage and/or retrieval mechanisms. These results, showing that an effective amnesic dose of scopolamine (100 ng/g) negatively modulates long-term memory expression but not memory storage in the crab *Chasmagnathus*, are consistent with the concept that dissociable processes underlie the mechanisms mediating memory reactivation and the behavioral expression of memory.

1. Introduction

The reconsolidation hypothesis – which proposes that a previously consolidated memory can enter an unstable state when recalled, memory labilization, during which it is transiently sensitive to disruption – has challenged the traditional view of consolidation, including the notion that new memories, after the memory consolidation period is complete, are fixed and established. In this framework, reconsolidation studies have disclosed that the mechanisms mediating memory retrieval and the mechanisms that underlie the behavioral expression of memory can be dissociated (Ben, Gamache, & Nader, 2006; Coccoz, Maldonado, & Delorenzi, 2011; Finn, Roediger, & Rosenzweig, 2012; Frenkel, Maldonado, & Delorenzi, 2005a, 2005b, 2010; Sevenster, Beckers, & Kindt, 2012). These views, suggesting a more dynamic and plastic view of memory, have begun to take part in the discussion about the nature of experimental amnesia, i.e., storage *vs* retrieval views (Gold, 2006; Hardt, Wang, & Nader, 2009; Miller & Sweatt, 2006; Nader & Wang, 2006; Riccio, Millin, & Bogart, 2006; Sara & Hars, 2006).

A large number of studies have shown the recovery of memory after several amnesic treatments (Cahill, McGaugh, & Weinberger, 2001; Gold, Haycock, Marri, & McGaugh, 1973; Haycock, Gold, Macri, & McGaugh, 1973; Nader & Wang, 2006; Parvez, Stewart, Sangha, & Lukowiak, 2005; Philips, Tzvetkova, Marinesco, & Carew, 2006; Quartermain, Judge, & Leo, 1988; Rescorla, 1988; Riccio et al., 2006). These recoveries are the core of the ongoing debate on the nature of experimental amnesia: whether amnesic treatments interfere with memory consolidation or whether these treatments interfere with the retrieval process of memories that were effectively stored (de Hoz, Martin, & Morris, 2004; Hardt et al., 2009; Miller & Matzel, 2006; Nader & Wang, 2006; Rescorla, 1988; Squire, 2006; Tulving, 1983) (see special section The Neurobiology of Amnesia in Learn Mem, vol. 13, issue 5, 2006). On account of this, reconsolidation would offer a new prospect for understanding the nature of experimental amnesia (Gold, 2006).

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Our working hypothesis is that some neuromodulators can, during consolidation and reconsolidation, determine the ability of a memory to guide behavior, to increase or decrease its long-term expression, without affecting its long-term storage (Coccoz et al., 2011; Frenkel et al., 2005a, 2010). We have previously shown that saralasin, an angiotensin II antagonist, interferes with long-term memory expression; while protein synthesis inhibitors interfere with memory storage (or alternatively the retrieval process, Frenkel et al., 2005a, 2010). Here, we utilize this experimental approach -which makes use of the positive modulation of memory expression during reconsolidation- to distinguish whether scopolamine-induced amnesia reflects one of these two possible scenarios: (a) scopolamine does not interfere with the storage of a long-term memory, although it blocks memory behavioral expression or, on the other hand, (b) scopolamine interferes with storage mechanisms and, consequently, a memory trace is not stored long term or, alternatively, the stored memory trace cannot be retrieved. In both scenarios, memory is not expressed at a testing session; however, the main difference is that in the first scenario, memory is retrieved first and thus it can be labilized and reconsolidated.

Scopolamine is a muscarinic receptor antagonist with amnesic properties that has been used for decades in animals to induce impairment in learning and memory processes in both vertebrates and invertebrates (Baratti, Boccia, & Blake, 2009; Beron de Astrada & Maldonado, 1999; Buccafusco, 2009; da Silva et al., 2009; Deiana, Harrington, Wischik, & Riedel, 2009; Quirarte et al., 1994; Roldan, Bolanos-Badillo, Gonzalez-Sanchez, Quirarte, & Prado-Alcala, 1997; Terazima & Yoshino, 2010; Weinberger, 2006). It has been consistently found that scopolamine administration results in acquisition impairments (Blake, Boccia, Krawczyk, & Baratti, 2011; Decker & McGaugh, 1989; Decker, Tran, & McGaugh, 1990; Hasselmo, 2006; Hasselmo & Sarter, 2011; Ohno & Watanabe, 1996; Ohno, Yamamoto, & Watanabe, 1994). Retrograde amnesia in various animal species is consequently produced if cholinergic activity is reduced after training by scopolamine (Beron de Astrada & Maldonado, 1999; Blake et al., 2011; Boccia, Acosta, Blake, & Baratti, 2004; Quirarte et al., 1994; Roldan et al., 1997). Canonical views of the impairing effect of scopolamine on human memory (Frumin, Herekar, & Jarvik, 1976; Ghoneim & Mewaldt, 1975; Richardson et al., 1985) imply that it appears to be exerted on acquisition processes but not on retrieval processes (Ghoneim & Mewaldt, 1975, 1977). Although the blockade of cholinergic receptors results in acquisition and consolidation impairments in several memory models, both effects are different. While minimal doses of scopolamine are sufficient to impair acquisition, higher doses (above 4 mg/kg) are necessary to impair consolidation, suggesting a differential role of the cholinergic system in acquisition and consolidation (Blake et al., 2011; Elrod & Buccafusco, 1991; Rush, 1988).

The aim of the present work is to stress that one of the roles of the cholinergic system in modulating mnesic process is to determine whether the consolidating memory is going to take control of behavior at retrieval (i.e., is going to be expressed). Here, we evaluated whether experimental amnesia induced by scopolamine is due to a storage or retrieval deficit, or to a decrease in the long-term memory expression of a consolidated memory.

In the crab *Chasmagnathus*, a powerful memory paradigm based on a change in its defensive strategy against a visual danger stimulus (VDS) has been extensively studied. This aversive memory is termed context-signal memory, as it is a consequence of an association between the training context and the VDS (Maldonado, 2002; Romano et al., 2006; Tomsic, de Astrada, Sztarker, & Maldonado, 2009; Tomsic, Pedreira, Romano, Hermitte, & Maldonado, 1998). In this model, pre- or post-training injections of scopolamine produce an amnesic affect that was interpreted in terms of impairment of consolidation (Beron de Astrada & Maldonado, 1999). The working hypothesis of the present paper is that the amnesic effect of scopolamine is due to a disruption of long-term memory expression rather than to a disruption of long-term storage or to a failure of the mechanisms of retrieval. In light of this, we expect that after scopolamine administration there would be a memory trace that would not be expressed, but could be reactivated and labilized by the appropriate reminder. Consequently, we predict that we should be able to recover a fully expressible long-term memory if reconsolidation of the reactivated memory is improved. Changes in performance after a reminder might be due not to enhancement of the expression of a reconsolidating memory trace, but to other processes such as summation of a residual memory trace with additional learning produced during reminder presentation (Gold et al., 1973). If, as we propose, recovery after the amnesic effect of scopolamine is due to reactivation of an intact but non-expressible memory, then new learning and summation is not a minimum and sufficient explanation for this recovery, since retrieval of the old trace is required for labilization and reconsolidation. Consequently, it should be demonstrated that the recovery of memory performance is reconsolidation specific. This issue can easily be addressed in the context-signal memory of *Chasmagnathus*, since it was demonstrated that memory labilization and reconsolidation are not triggered by the reminder presentation *per se*, but when the reminder fulfils certain parametric conditions. In particular, a reminder of short duration that does not include reinforcement is able to trigger labilization and reconsolidation of the reactivated memory trace. On the other hand, a short but reinforced reminder is not able to do this (Frenkel et al., 2005a; Pedreira, Perez-Cuesta, & Maldonado, 2004). Therefore, if the expression of memory can be enhanced after a reminder by modulation of its reconsolidation then, unlike summation predicts, this enhancement should not occur when a reinforcement is presented contingent on the reminder. We expected that effective amnesic doses of scopolamine would impede the physiological modulatory function of cholinergic system -the endogenous systems that influence memory storage processes but do not serve as the neural bases of memory storage (Cahill & McGaugh, 1996)-; for instance, modulating the level of activation of multiple memory systems (Gold, 2008). However, given that acetylcholine is critical for a multitude of functions in the central nervous system, and not only for memory processes, it is expected that deeper interference of the cholinergic system would encompass an irreversible amnesic effect on memory due to interferences with several physiological process that, consequently, would affect encoding, the neural bases of memory storage or the formation of retrieval mechanisms. If after this vast pharmacological injury of the brain, there is not a memory trace to reactivate (or alternative retrieval mechanisms that are not normally established), then those treatments that can enhance memory during reconsolidation will not have enhancing effect.

2. Experimental procedures

2.1. Animals

Intermolt adult male crabs of the species *Chasmagnathus (Neohelice) granulatus* between 2.7 and 3.0 across carapace were collected from the narrow coastal inlets of San Clemente del Tuyú, Argentina. In the laboratory, crabs were kept on a 12:12 h light–dark cycle, in collective tanks (20 animals each) filled up to 2 cm deep with 12‰ seawater prepared with hw-Marinex (Winex, Germany) salt, pH 7.4–7.6. The holding and experimental rooms were kept at 22–24 °C and 80 ± 10% relative humidity. Experiments were done in the daytime within the first week after the arrival of animals. Each crab was used in one experiment only. Experimental procedures were in compliance with the policies on the use of

Animals and Humans in Neuroscience Research. All efforts were made to minimize the number of animals used and their suffering.

2.2. The experimental device

The experimental device, the actometer (Maldonado, 2002), referred to as the training context, consisted of a bowl-shaped opaque container, illuminated by a bottom light (5 W bulb), with a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter) covered to a depth of 0.5 cm with artificial seawater, where the crab was lodged before each experimental session. Eighteen seconds before each trial the bottom light went off and the top light (5 W bulb) came on. Then, a 9 s trial was given, in which an opaque rectangular screen (25–7.5 cm), termed visual danger stimulus (VDS), was moved twice horizontally over the animal, cyclically from left to right and vice versa, at a constant speed. During this 9 s trial, the top light was kept on. The VDS provoked an escape response in crabs and consequent container vibrations, which were converted into electrical signals through a piezoelectric, transducer placed on the external wall of the container. These signals were amplified, integrated during each 9-s trial, and translated into numerical units ranging from 0 to 16,000, before being processed by computer. The activity of every crab was recorded during each entire trial time. The experimental room had 40 devices, separated from each other by partitions. In experiment of Fig. 2B, a cylindrical (15 cm in diameter and 15 cm in height) plastic container with black and white striped walls, covered to a depth of 0.5 cm with artificial seawater, was used as a different training context during the *Reminder Session* on the second day. Between *Training*, *Reminder* and *Testing Sessions*, crabs stayed in resting containers (plastic cylindrical containers, 15 cm in diameter and 15 cm in height) covered to a depth of 0.5 cm with brackish water and kept inside dimly lit drawers.

2.3. Escape response and freezing

The intensity of container vibrations during a VDS presentation (a trial) depends on the magnitude of the defensive responses that each crab displays, when faced with an impending threat. Two types of defensive responses are distinguished, namely, escape and freezing responses (Pereyra, Gonzalez, & Maldonado, 2000). The escape response is a directional run of the animal in an attempt to move away from the VDS, while the freezing response consists of a rigid motionless display in which the crab lies flat on the substratum. During repeated VDS presentations (training), the escape response decreases in intensity and is progressively replaced by a freezing response. Throughout this study, data were recorded during a trial time, i.e., during the 9-s VDS presentation. In the untrained groups, response was recorded in intervals of 9-s coincident with VDS presentation in the respective trained group.

2.4. Training protocol

A *training protocol* consists of fifteen trials (9 s, two VDS presentations), with 3 min intertrial intervals (total training duration; 42 min), after a 10 min adaptation period in which animals do not receive VDS (Fig. 1). Since the memory under study arises as a consequence of an association between the context and the VDS. it was termed context-signal memory. Context-signal memory expression is revealed at testing session as a significant decrease in the activity of the animals when the VDS is presented. This decrease in activity is due to an increase in the number of animals displaying the freezing response. Here, we used a variation of the training protocol. In this case, 18 s before each trial the bottom light went off and the top light came on. Next, during the 9 s trial, the VDS was presented with the top lights on. This training protocol also induces an association between the iterated VDS presentation and the contextual features of the training context, where a robust freezing is also acquired throughout the 15-trial training

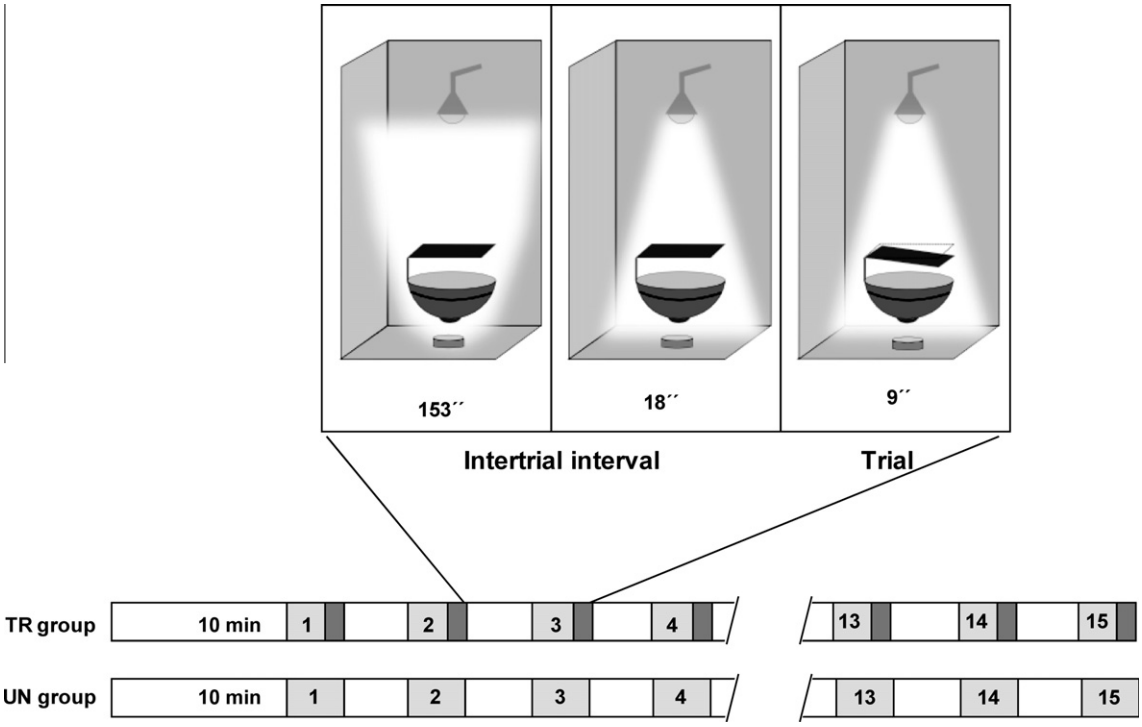

Fig. 1. *Schematic representation of the training protocol*. Bottom bars: protocols of the trained group (TR group) and the untrained group (UN group). Each protocol begins with 10′ adaptation period where there is no VDS and the bottom light is turned on. 18 s before each trial the bottom light went off and the top light came on (gray space). Next, during the 9 s trial, the VDS was presented with the top lights on (dark space). Times are indicated in seconds and minutes. Each trial is indicated with a number. A *training protocol* consists of 15 trials (9 s, two VDS presentations), with 3 min intertrial intervals (total training duration; 42 min).

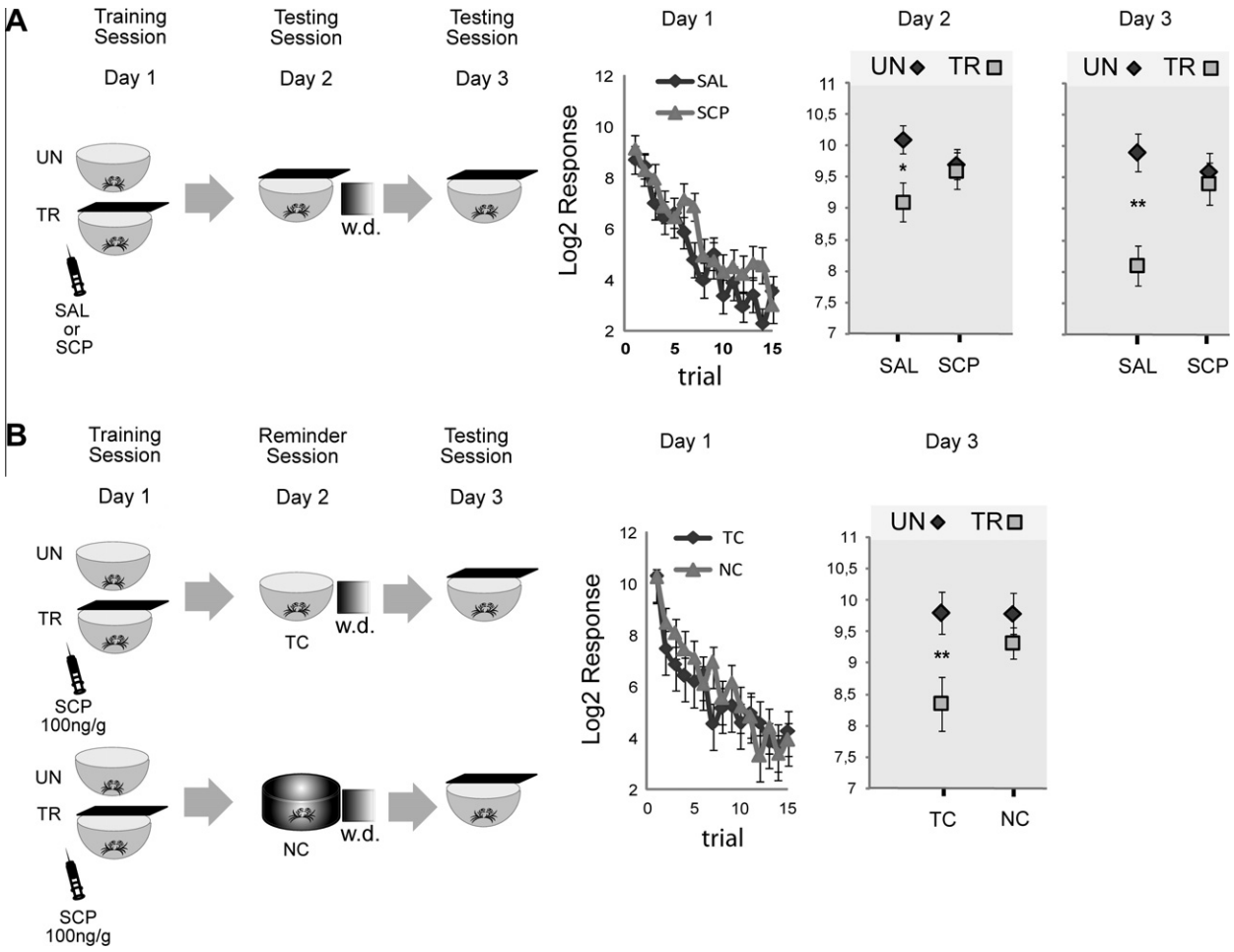

Fig. 2. *The amnesic effect induced by pre-training administration of scopolamine can be reversed after memory reactivation.* Left panel: experimental designs (black bar above boxes indicates visual danger stimulus, VDS). Right panel: *Reminder* and *Testing Session* results. Graphs ordinates: log2 trial scores during VDS presentation (means ± SE); open symbols (□): UT, untrained groups; filled symbols (■): TR, trained groups; w.d. indicates water deprivation. (A) *Scopolamine administration before training generates amnesia on Day 2 and Day 3*. On Day 1 TR groups received a 15-spaced trial training, while UT groups remained in the actometer during the same time without stimulation. Before *Training,* pair SAL groups were injected with saline solution and pair SCP groups were administrated with scopolamine. On Day 2, memory was tested with a single VDS presentation. Then all animals were returned to their resting containers and water deprived for 2 h. On Day 3, memory was tested with a single VDS presentation. For all groups, $N = 27$. (B) On Day 1, all groups were injected with SCP 100 ng/g before *Training.* On Day 2, Pair TC groups were re-exposed to the training context and Pair NC groups were exposed to a novel context for 5 min. Then all animals were returned to their resting containers and water deprived for 2 h. On Day 3 (*Testing Session*), memory was tested with a single VDS presentation. SCP-TR-TC, $N = 28$; SCP-UN-TC, $N = 25$; SCP-UN-NC, $N = 29$; SCP-TR-NC, $N = 30$.

and a clear retention is found when tested with the VDS presentation short term (2 and 4 h) and long term (1–5 days) (Maldonado, 2002; Pereyra et al., 2000; Romano et al., 2006; Tomsic et al., 1998, 2009; Fustiñana, Carbó Tano, Romano, Pedreiira, personal communications).

2.5. Experimental procedure and design

Experiments included three sessions: *Training Session* (Day 1), *Reminder Session* (Day 2) 24 h later and *Testing Session* (Day 3) another 24 h later. The experimental protocols involve pairs of crab groups, where each pair consists of a trained group (TR) and an untrained group (UN). Each group has between 25 and 40 crabs.

2.5.1. Day 1 – Training session

TR group: This group underwent a *training protocol.* First, each crab was placed in an individual training context for 10 min, without being stimulated with VDS. After this adaptation period, animals received 15 training trials (2.4.), separated by inter-trial intervals of 3 min. Finally, animals were immediately placed in individual resting containers until the following day.

UN group: Each crab of this untrained group was placed in an individual training context and remained there for the same time and in the same conditions as trained animals (2.2.), but without receiving the VDS. Then, animals were immediately placed in individual resting containers until the following day.

2.5.2. Day 2 – Reminder session

Three situations may arise in this session according to the design of the experiment in question (Maldonado, 2002; Pedreira et al., 2004):

Crabs were re-exposed to the training context for 5 min, in which the bottom light went off and a top light came on for the last 27 s without the VDS presentation. Then, animals were returned to their respective resting containers until the following day. This procedure reactivates and turns memory into a labile state, initiating the reconsolidation process.

Crabs were re-exposed to the training context for 5 min, in which the bottom light went off and a top light came on for the last 27 s, with the VDS being presented/added for the last 9 s. Animals were then returned to their respective resting containers until the

following day. This procedure prevents memory labilization, and reconsolidation is not initiated.

Crabs were exposed to a cylindrical and striped context and the VDS was not presented. This context is perceived as a novel context, and thus it does not trigger memory labilization and reconsolidation.

After the presentation of each reminder, crabs were returned to their resting containers and were immediately water deprived for 2 h in order to enhance memory expression. It was shown that, during reconsolidation, this real-life experience can reinstate memory expression after weak training protocols or after saralasin-induced amnesia. This effect has been shown to depend on the unreinforced presentation of the reminder. It is observable only after the termination of the reconsolidation time window, the temporal concurrence is a key requirement for reconsolidation facilitation and is mediated by activation of the transcription factor Rel/NF-kappaB (Frenkel et al., 2002, 2005a, 2005b, 2010).

2.5.3. Day 3 – Testing session

On the third day, crabs were re-exposed to the training context for 10 min and at the end memory expression was tested with a single VDS presentation as in 2.2.

2.6. Drug administration

Crustacean physiological saline solution (Delorenzi et al., 1996) was used as a vehicle (SAL). Fifty microliters of SAL or scopolamine (SCP) dissolved in physiological saline solution was given through the right side of the dorsal cephalothoraxic-abdominal membrane, by means of a syringe fitted with a sleeve to control the depth of penetration to 4 mm, thus ensuring that the injected solution was released in the pericardial sac (Maldonado, 2002). Two doses were used: 100 ng/g, the highest amnesic dose tested in a previous study (Beron de Astrada & Maldonado, 1999) and 5 μg/g. All drugs were purchased from Sigma Chemical Co. Saint Louis, Missouri, USA.

2.7. Memory retention criterion and data analysis

LTM expression was assessed on Day 3 by focusing data analysis on test trial scores, i.e., by estimating the difference between the response level of the trained group (TR) and that of the respective untrained group (UN). 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 approach is amply justified in this present case since it has been demonstrated that memory expression in crabs is independent of the escape response level at training (Maldonado, 2002; Romano et al., 2006; Tomsic et al., 2009). A TR group was said to show memory when its mean response level at the test trial was statistically lower than the respective UN group. Data were analyzed using analysis of variance (ANOVA) and a priori planned comparisons. As the variance of activity scores increases with the mean, thus violating the homogeneity of variance assumption of ANOVA, the data were log 2 transformed (Suárez, personal communication). The parameter under measure (container vibrations as a result of complex animals motor activities) is reflecting qualitatively different responses (as for example freezing and escape), therefore the variance of this parameter would be different when different responses are prevailing. All experiments described in this paper used two untrained–trained pairs (UN–TR). Three types of contrast per experiment were used: the first, between the two untrained groups of each pair; the second, between UN and TR of one pair; and the third, between UN and TR of the other pair. When only one pair of groups was compared, a *t*-test was used. The values resulting from the integration during 9 s of the vibrations measured by the four microphones were transformed to their log 2 and this value was used as a measure of crab response (log 2 response). Predictions about memory expression were based on whether there was significant difference in response at testing in each UN–TR comparison. We analyzed data using STATISTICA (StatSoft 6.0).

3. Results

3.1. Pre-training administration of scopolamine induces amnesia

In the *Chasmagnathus* associative memory model, animals associate the training context with the VDS passing overhead. After the iterative presentation of the VDS, a strong freezing-to-VDS response replaces the initial escape response. Thus, memory is revealed as a reduction in the escape response of the *trained group* (TR), compared with that of the paired *untrained* (UN) group; (UN > TR).

The amnesic effects on the context-signal memory of scopolamine (SCP) administrated both pre- or post-training have already been described in *Chasmagnathus* (Beron de Astrada & Maldonado, 1999). In the first experiment, we confirmed that SCP-administered animals before training do not show memory at *Testing Session* (Fig. 2A).

On Day 1, a pair of UN–TR groups of crabs was administered with physiological saline solution (SAL-TR and SAL-UN groups) and then underwent the *Training Session*, next the animals were removed to the resting containers until the next day. Simultaneously, on Day 1, another pair of UN–TR groups of crabs was administered with 100 ng/g of scopolamine before training (SCP-TR and SCP-UN groups). On Day 2 all animals underwent the *Testing Session*, in which crabs were re-exposed to the training context for 10 min and memory was tested by a single VDS presentation and then were returned to their resting containers and water deprived for 2 h. Finally, on Day 3, all animals underwent a second *Testing Session* (Fig. 2A).

Results at training are shown in Fig. 2A; no differences were disclosed between SCP and SAL groups during *Training Session*. ANOVA (repeated measures), performed on the scores of each trial, disclosed no significant group differences [F_{group} $(1,63) = 1.98$; $p = 0.164$] and, as expected, a significant trial effect [$F_{trial}(1,14) = 27.54$; $p < 0.001$].

Planned comparisons on activity scores on Day 2 [ANOVA: $F(3,104) = 3.30$, $p < 0.05$] and Day 3 [ANOVA: $F(3,104) = 5.98$, $p < 0.001$] disclosed significant differences in escape response between Pair SAL groups: on Day 2 (SAL-UN > SAL-TR, $^{*}p < 0.01$) and on Day 3 (SAL-UN > SAL-TR, $^{**}p < 0.001$); that is, SAL-TR group revealed memory at both *Testing Sessions*. On the other hand, there were no significant differences between SCP groups on Day 2 (SCP-UN ≈ SCP-TR, $p = 0.85$) and Day 3 (SCP-UN ≈ SCP-TR, $p = 0.79$). That is, pre-training scopolamine administration (100 ng/g) induces amnesia on Day 2, as expected by previous studies (Beron de Astrada & Maldonado, 1999), and on Day 3. On Day 2 and Day 3, there were no differences between untrained groups (SAL-UN ≈ SCP-UN, $p = 0.95$, Day 2; $p = 0.89$ Day 3). As has previously been shown (Beron de Astrada & Maldonado, 1999), we confirmed that SCP administered animals before training do not show memory 24 h later. In addition, we showed in the present study that this SCP induced amnesia persists up to 48 after training, even after a previous one-trial *Testing Session* (Fig. 2A).

In the previous experiment, the *Reminder Session* was performed as a regular *Testing Session* in the *Chasmagnathus* memory model: the VDS was presented, and therefore the change in response could be observed. However, this *Testing Session* does not

initiate reconsolidation (Frenkel et al., 2005a; Pedreira et al., 2004; Perez-Cuesta & Maldonado, 2009). If memory is allowed to reconsolidate, a water deprivation episode contingent on memory reconsolidation positively modulates memory expression, thus allowing us to distinguish between memories that can be reactivated, labilized and are not expressed, from those that are not stored long term, obliterated or altered in other retrieval mechanisms (Frenkel et al., 2005a, 2010). In the next experiment, we used this protocol to test if the recovery of long-term memory expression of the amnesic retrograde effect induced by pre-training scopolamine administration can be reversed by improving memory during reconsolidation.

3.2. The amnesic effect induced by pre-training administration of scopolamine can be reversed after memory reactivation

The amnesic effect of pre- and post-training SCP administration (100 ng/g) was interpreted, like the cycloheximide effect, as a disruption of LTM storage (Beron de Astrada & Maldonado, 1999; Frenkel et al., 2010; Pedreira, Dimant, & Maldonado, 1996; Romano et al., 2006). If SCP (100 ng/g) is an amnesic agent that impedes long-term memory formation, then there should be no memory to be reactivated, labilized and then susceptible to be enhanced 24 h after training. Thus, animals should not show memory retention at testing on the third day. However, if the amnesic effect of scopolamine implied a negative modulation of the long-term memory expression, the consolidated memory would maintain the capability to be reactivated by a reminder despite scopolamine treatment. The following experiment was focused on this question (Fig. 2B).

On Day 1, a pair of UN–TR groups of crabs was administered with SCP (100 ng/g) and then underwent the *Training Session*, next animals were removed to the resting containers until the next day. On Day 2 these animals were re-exposed to the original training context (TC) for 5 min (this procedure reactivates and initiates memory labilization during reconsolidation), then were returned to their resting containers and water deprived for 2 h, this treatment is known to improve long-term memory expression during reconsolidation (SCP-TR-TC and SCP-UN-TC groups). Simultaneously, another pair of UN–TR groups of crabs was administered with SCP (100 ng/g) and then underwent the *Training Session*, afterwards animals were moved to the resting containers until the next day. On Day 2, these animals were exposed to a novel context (NC) for 5 min and then were returned to their resting containers and were water deprived for 2 h (SCP-TR-NC and SCP-UN-NC groups). On Day 3, all animals were tested.

Results at training are shown in Fig. 2B; no differences were disclosed between training groups. ANOVA (repeated measures) performed on the scores of each trial, disclosed no significant group differences [$F_{group}(1,54) = 0.45$; $p = 0.51$] and, as expected, a significant trial effect [$F_{trial}(1,14) = 19{,}20$; $p < 0{,}001$].

At the *Testing Session*, planned comparisons on activity scores [ANOVA: $F(3,108) = 4.68$, $p < 0.01$] showed memory retention for animals re-exposed to the original training context (SCP-UN-TC > SCP-TR-TC, $p > 0.01$) despite scopolamine administration, but there were no differences between groups that were exposed to a novel context (SCP-UN-NC ≈ SCP-TR-NC, $p = 0.26$), nor between untrained groups (SCP-UN-TC ≈ SCP-UN-NC, $p = 0.89$). In sum, in this section, results showed that amnesia induced by pre-training scopolamine (100 ng/g) administration was reversed by a treatment that enhances memory during reconsolidation, after the *Reminder Session* that triggers reconsolidation. This recovery of the SCP amnesic effects, by exposing the animals to the conditioning context can be explained in terms of a recovery from retrieval impairment, additional learning, residual traces or summation effects (see special section The Neurobiology of Amnesia in Learn Mem, Vol. 13, Issue 5, 2006). However, those explanations should be discarded if recovery of amnesia depends upon reconsolidation. We tested this possibility hypothesis in the next experiments (Fig. 3A).

3.3. The recovery of memory expression after the amnesic effect of scopolamine is reconsolidation specific pre-training-scopolamine administration

To test this hypothesis, the first pair of groups of the previous experiment was replicated (SCP-TR-TC and SCP-UN-TC groups). Simultaneously, another pair of UN–TR groups of crabs was also administered with SCP (100 ng/g) and then underwent the *Training Session*, next animals were moved to the resting containers until the next day. On Day 2, these animals were re-exposed to the training context for 5 min and at the end received a single VDS presentation (Training context + VDS, TC + VDS), this is the reminder that does not trigger the reconsolidation process. Then, animals were returned to their resting containers and were water deprived for 2 h. On Day 3, all animals were tested (SCP-TR-TC + VDS and SCP-UN-TC + VDS groups).

Results at training are shown in Fig. 3A; no differences were disclosed between groups during *Training Session*. ANOVA (repeated measures) performed on the scores of each trial, disclosed no significant group differences [$F_{group}(1,55) = 0.30$; $p = 0.587$] and a significant trial effect [$F_{trial}(1,14) = 25.66$; $p < 0.001$].

On Day 2, the amnesic effect of scopolamine was disclosed. No significant differences between SCP-TR-TC + VDS and SCP-UN-TC + VDS groups ($t(52) = 1.39$, $p = 0.17$; Fig. 3A). During *Testing* on Day 3, planned comparisons on activity scores [ANOVA: $F(3,108) = 5.252$, $p < 0.01$] showed no differences between groups of the SCP-TR-TC + VDS and SCP-UN-TC + VDS groups ($p = 0.20$) despite crabs receiving another trial after being re-exposed to the training context on Day 2. Indeed, memory expression was again disclosed for the pair SCP-TC (SCP-UN-TC > SCP-TR-TC, $p < 0.001$) when memory was reactivated-labilized and improved during reconsolidation on Day 2. There were no differences between untrained groups (SCP-UN-TC ≈ SCP-UN-TC + VDS, $p = 0.57$) on Day 3. That is, the amnesia induced by pre-training-SCP (100 ng/g) administration was reversed by a *Reminder Session* that initiated reconsolidation followed by a treatment that enhances memory during reconsolidation.

3.3.1. Post-training scopolamine administration

An identical protocol as in the previous experiments was designed except that now the SCP (100 ng/g) was injected immediately post-*Training* (Fig. 3B). Consequently, this experiment included two pairs of groups: the TR-SCP-TC and UN-SCP-TC pair, with the TR-SCP-TC + VDS and UN-SCP-TC + VDS pair.

Results at training are shown in Fig. 3B; no differences were disclosed between groups during *Training*. ANOVA (repeated measures) performed on the scores of each trial, disclosed no significant group differences [$F_{group}(1,51) = 0.010$; $p = 0.92$] and a significant trial effect [$F_{trial}(1,14) = 24.09$; $p < 0.001$].

On Day 2, as was previously shown (Beron de Astrada & Maldonado, 1999), the groups injected with SCP prostrating did not show memory retention: there were no significant differences between groups that received the VDS (TR-SCP-TC + VDS and UN-SCP-TC + VD, $t(48) = 0.98$, $p = 0.33$). Accordingly, planned comparisons on activity scores at *Testing* on Day 3, [ANOVA: $F(3,96) = 6.68$, $p < 0.0001$], also showed no differences between TR-SCP-TC + VDS and UN-SCP-TC + VD ($p = 0.56$). Conversely, memory retention for TR-SCP-TC and UN-SCP-TC groups ($p < 0.001$) was disclosed in spite of the post-training SCP administration on Day 1 (Fig. 3B). There were no differences between untrained groups (UN-SCP-TC ≈ UN-SCP-TC + VD, $p = 0.26$) on Day 3. That is, the amnesic

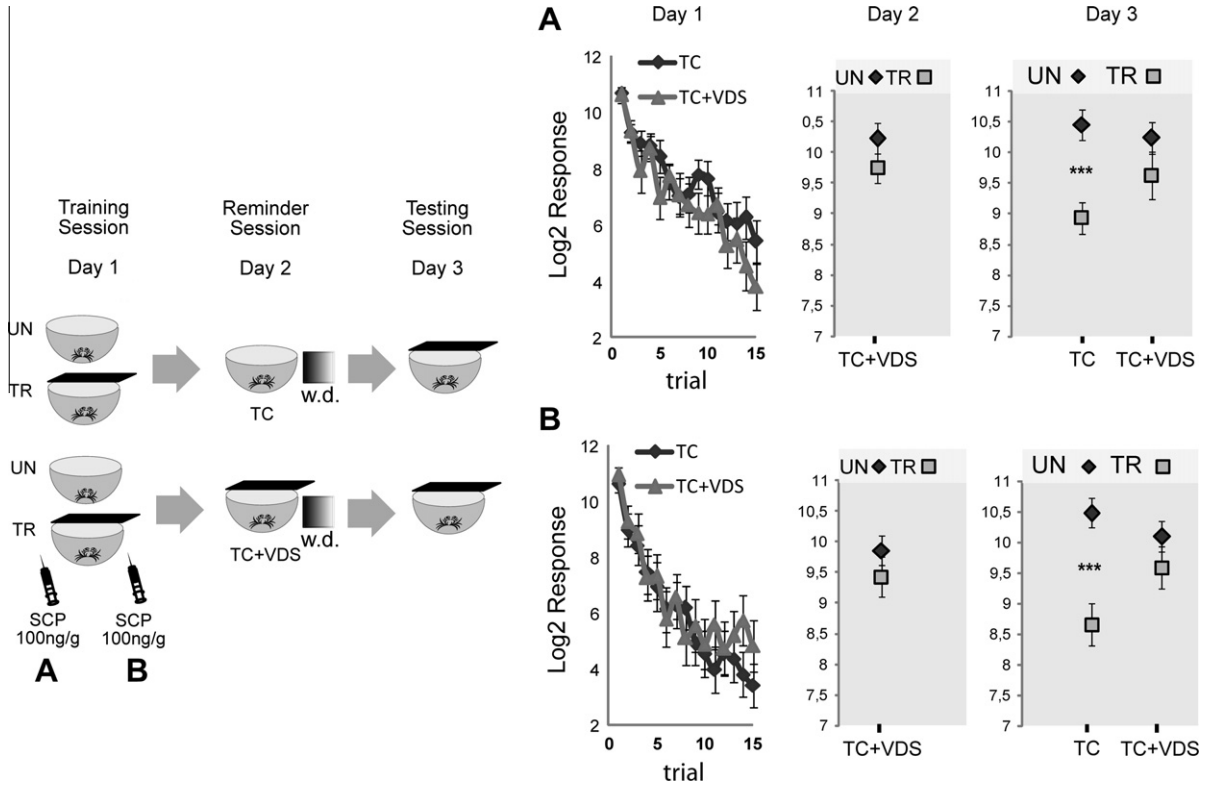

Fig. 3. *The recovery of memory expression after the amnesic effect of scopolamine is reconsolidation specific.*. Immediately before (A) or after (B) *Training*, all animals were injected with scopolamine (SCP) (100 ng/g). *Remainder Session*: 24 h later, all groups of animals were re-exposed for 5 min to the training context. At the end of this period, TC + VDS groups received a single VDS presentation and, conversely, TC groups were re-exposed to the training context without VDS presentations. Next, all animals were water deprived for 2 h in their individual resting containers. *Testing Session* (Day 3): memory expression is disclosed for animals of TC groups (TR-SCP-TC and SCP-TR-TC groups) but not when animals were exposed to the reminder without the VDS (TR-SCP-TC + VDS and SCP-TR-TC + VDS groups). SCP-UN-TC, $N = 29$; SCP-UN-TC, $N = 29$; SCP-UN-TC + VDS, $N = 27$; SCP-TR-TC + VDS, $N = 27$; UN-SCP-TC, $N = 25$; TR-SCP-TC, $N = 25$; UN-SCP-TC + VDS, $N = 25$; TR-SCP-TC + VDS, $N = 25$. Symbols as in Fig. 2.

effect induced by post-training-SCP (100 ng/g) administration was recovered by a treatment that enhances memory during reconsolidation, after a *Reminder Session* that initiated reconsolidation.

The experiment 3.2., Fig. 2B, showed that the re-exposure to the training context (a reminder) was necessary under conditions that trigger reconsolidation to improve memory expression. As in several memory models, a mismatch between what is expected and what actually occurs needs to take place to initiate reconsolidation. Specifically, in the *Chasmagnathus* memory model, reconsolidation does not take place after a reinforced presentation of the training context (i.e., a single VDS) is presented at the end of the re-exposure (Pedreira et al., 2004). Moreover, after weak training protocols, even when memory expression cannot be observed in the long term, reconsolidation can be triggered on the condition that the presentation of the training context is non-reinforced at reminder sessions (Frenkel et al., 2005a). Thus, even when animals do not express the freezing behavior in the long term, the association between the training context and the VDS can persist and is able to be reactivated under the adequate parametric conditions of the reminder. The important issue is that there is no re-training during the reminder, which is a necessary condition to reinstate the freezing response in subsequent testing sessions. Based on this property, we showed in the last two previous experiments that, after a reminder, a positive change in memory expression is induced at the *Testing Session* only in conditions in which the reminder initiates reconsolidation (training context exposure without VDS presentation). The reinforced reminder (re-exposure to the training context plus VDS presentation) impeded the improving effect of water deprivation that modifies memory expression, even when this reminder included more training cues than those presented during the *Reminder Session* that actually triggers the reconsolidation process. Consequently, the increase in memory expression showed in the previous experiments depends upon reconsolidation of the engram, and thus it is hard to explain in terms of the recovery of amnesia as a consequence of additional learning, residual traces, retrieval deficits or summation effects (Gold, 2006; Gold et al., 1973). In the following experiments, we tested whether scopolamine amnesia induced with a higher dose (5 μg/g) can also be reversed by improving memory during reconsolidation.

3.4. A higher dose of scopolamine induces an amnesic effect that cannot be reversed under the present parametric conditions

3.4.1. Post-training scopolamine administration

In this experiment, we tested whether it was possible to recover the SCP amnesic effect after the post-training administration of scopolamine in a higher dose, 5 μg/g (Fig. 4A). Two pairs of UN–TR groups of crabs were used in this experiment. On Day 1, a pair of UN–TR groups of crabs underwent the *Training Session,* and then animals were injected with saline. Next, crabs were moved to the resting containers until the next day (the TR-SAL-TC and UN-SAL-TC pair). Simultaneously, another pair of UN–TR groups of crabs underwent the *Training Session* and then was administered with SCP (5 μg/g), next animals were moved to the resting containers until the next day (the TR-SCP-TC and UN-SCP-TC pair). On Day 2, all animals were re-exposed to the training context for 5 min (*Reminder Session*). Then, crabs were returned to the resting containers and water deprived for 2 h. On Day 3, all animals were tested.

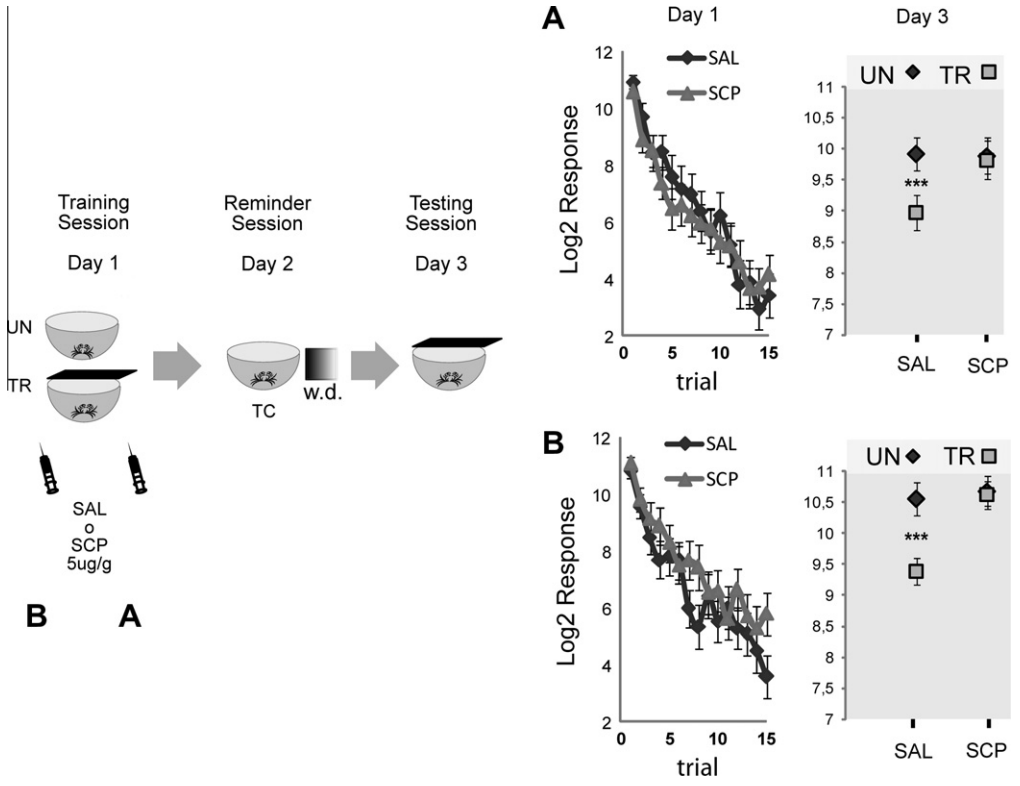

Fig. 4. *A higher dose of scopolamine, before or after training, induced an amnesic effect that cannot be reversed.* Immediately after (A) or before (B) *Training*, animals were injected with scopolamine (5 μg/g, Pair SCP) or with saline (Pair SAL). *Reminder Session*: 24 h later, all groups of animals were re-exposed for 5 min to the training context. Next, all animals were water deprived for 2 h in their individual resting containers. *Testing Session* (Day 3): memory expression is disclosed for the trained animals of SAL groups but not for SCP groups. UN-SAL-TC, $N = 25$; TR-SAL-TC. $N = 28$; UN-SCP-TC, $N = 31$; TR-SCP-TC. $N = 31$; SAL-UN-TC, $N = 22$; SAL-TR-TC. $N = 22$; SCP-UN-TC, $N = 23$; SCP-TR-TC. $N = 23$. Symbols as in Fig. 2.

Results at training are shown in Fig. 4A; no differences were disclosed between groups during *Training*. ANOVA (repeated measures) performed on the scores of each trial, disclosed no significant group differences [F_{group} (1,62) = 1.56; $p = 0.22$] and a significant trial effect [F_{trial}(1.14) = 25.33; $p < 0.001$].

Planned comparisons on activity scores during the *Testing Session* [ANOVA: $F(3,111) = 4.40$, $p < 0.001$] showed memory retention for the TR-SAL-TC and UN-SAL-TC groups ($p < 0.05$). However, there were no significant differences between TR-SCP-TC and UN-SCP-TC groups ($p = 0.56$). There were no differences between untrained groups (UN-SAL group ≈ N-SCP-TC; $p = 0.55$) (Fig. 4A). Unlike a dose of 100 ng/g administered in previous experiments, the amnesia induced by a high dose of SCP (5 μg/g) administered post-training could not be reversed by a treatment that enhances memory during reconsolidation, after the *Reminder Session* that trigger reconsolidation.

3.4.2. Pre-training scopolamine administration

In this experiment, we tested whether it was possible to recover the SCP amnesic effect injected immediately before training in a higher dose (5 μg/g) (Fig. 4B).

An identical protocol as the previous experiments was designed except that now the SCP (5 μg/g) was injected immediately before the *Training Session* (Fig. 4). Consequently, this experiment included two pairs of groups: the SAL-TR-TC and SAL-UN-TC pair, and the SCP-TR-TC and SCP-UN-TC pair (Fig. 4B).

Results at training are shown in Fig. 4B; no differences were disclosed between groups during *Training*. ANOVA (repeated measures) performed on the scores of each trial, disclosed no significant group differences [F_{group} (1,45) = 0.83; $p = 0.37$] and a significant trial effect [F_{trial}(1,14) = 11.36; $p < 0.001$].

Planned comparisons on activity scores during the *Testing Session* on Day 3 ANOVA: [$F(3,90) = 3.178$, $p < 0.05$] showed memory retention for SAL-TR-TC and SAL-UN-TC pair ($p < 0.01$). However, there were no significant differences between SCP-TR-TC and SCP-UN-TC groups ($p = 0.88$). There were no differences between untrained groups (SAL-UN-TC ≈ SCP-UN-TC $p = 0.60$) (Fig. 4B). Unlike low doses of SCP (100 ng/g) administered in previous experiments, the amnesia induced by a high dose of SCP (5 μg/g), administered pre-training,was not reversed by water deprivation after a *Reminder Session*. In sum, these results showed that amnesia induced by scopolamine (5 μg/g) administration pre- or post-training was not reversed by a treatment that enhances memory during reconsolidation, after the *Reminder Session* that triggers reconsolidation, under the present parametric conditions.

4. Discussion

The key finding of this study is that the experimental amnesia induced by pre- or post-training scopolamine administration does not imply the storage over time of the learned information, nor the capability to retrieve it by specific contextual cues. On the contrary, the amnesic effect implies a negative modulation of long-term memory expression, i.e., the process of gaining appreciable control over behavior when retrieved (Dudai, 2002; Eisenberg, Kobilo, Berman, & Dudai, 2003). The crabs trained and treated with scopolamine are able to construct a long-term memory that is not expressed but that can be reactivated during the *Reminder Session*. The reminder can reactivate and labilize the unexpressed memory; this was proved through the enhancement of long-term memory expression during reconsolidation. Both the storage of memory over time and its capability to be reactivated in the long term were

not affected in scopolamine-treated animals (100 ng/g). On the other hand, a higher dose (5 μg/g) of scopolamine, before or after training, induced an amnesic effect that we could not reverse under the present parametric conditions.

A vast number of studies have shown that a treatment or condition can reveal a memory that otherwise remains hidden. For instance, the phenomenon of latent memory is a common feature of learning and memory that was widely demonstrated across the animal kingdom, as well as in many studies that showed recovery of experimental amnesias (Cahill et al., 2001; Gold, 2006; Gold et al., 1973; Haycock et al., 1973; Lewis, 1976; Misanin, Miller, & Lewis, 1968; Nader & Wang, 2006; Parvez et al., 2005; Philips et al., 2006; Rescorla, 1988). In this context, reconsolidation studies may have much to offer regarding the nature of experimental amnesias (Gold, 2006). Key features in the present approach would lead to a different interpretation about scopolamine experimental amnesia because the enhancing mnesic effects specifically belong to the reconsolidation process.

Showing the necessity of the reminder (the novel context control in present study, Fig. 2B), and showing that the post-retrieval treatments should be given during a temporal window after memory reactivation to become effective are classic controls for reconsolidation (Dudai, 2009; Dudai & Eisenberg, 2004). Since the reminder that can induce reconsolidation is also a part of the cues presented at testing, other processes emerge as candidates for explaining the recovery of memory expression after post-retrieval treatments showed here, new learning for instance. Those classical controls cannot fully discard that a new learning process is occurring, which could explain the recovery of memory expression observed at testing. For example, a new learning could also be associative (it would depend on the contingence between the reminder and the post-reminder treatment) and observable only in the long term (Izquierdo et al., 2002). Several studies are concerned with these recoveries, remarking that the complete erasure of memory is not yet demonstrable and alternative explanations should not be discarded (Cahill et al., 2001; Hardt et al., 2009; Lewis, 1976).

Furthermore, the problem gets worse in the case of post-retrieval improvement of memory effects. Sub-threshold memories, which might remain after the administration of amnesic agents during consolidation, could be added to new memories formed during either the *Reminder Session* or subsequent treatments. These memories, which separately cannot be operationally noticed in the performance at testing, can be synergistically added (“summation”) and thus induce the expected change in behavior at testing (Gold et al., 1973). Several approaches that were able to recover memory from experimental amnesias might be considered as new learning added onto a residual memory trace (Squire, 2006). The *Chasmagnathus* memory paradigm allows us to hold memory in order to enter the reconsolidation stage by means of a full reminder presentation (i.e., by presenting the VDS at the end of the *Reminder Session*, here Day 2, Frenkel et al., 2005a; Pedreira et al., 2004). Even when the reminder is expected to be a strengthening factor in this situation (because more cues, i.e., the VDS, are being presented), the process of reconsolidation is not initiated when no new information seems to be available (Carbo Tano, Molina, Maldonado, & Pedreira, 2009; Coccoz et al., 2011; Forcato, Rodriguez, Pedreira, & Maldonado, 2010; Frenkel et al., 2005a; Morris et al., 2006; Pedreira et al., 2004; Perez-Cuesta & Maldonado, 2009; Rodriguez-Ortiz, Garcia-DeLaTorre, Benavidez, Ballesteros, & Bermudez-Rattoni, 2008). In our experiments, recovery after the reminder presentation is only observable when reactivation occurs under those parameters that trigger reconsolidation. In conclusion, it does not seem plausible to explain this case as an addition of sub-threshold memory traces, non-associative memories formed during both sessions, or other putative associative memories not related to the VDS-context association formed during *Training Session* (Squire, 2006). Even when new learning could happen under this situation, reconsolidation, and thus reactivation, of the first memory is a necessary condition for the recovery of performance after scopolamine treatment. Therefore, scopolamine-treated animals do build a long-term memory that potentially can be reactivated but not expressed, which would be comparable to the unexpressed long-term memory generated by weak training protocols (Frenkel et al., 2005a., 2010). Results strongly suggest that the neural representation of a VDS-context association persists after the scopolamine administration, since this representation can be specifically reactivated by a reminder and can enter a new labile phase. Remarkably, these results are in line with studies that show a dissociation between the mechanisms mediating memory labilization and the mechanisms that underlie the behavioral expression of memory (Ben et al., 2006; Coccoz et al., 2011; Frenkel et al., 2010; Sevenster et al., 2012; Blake, Boccia, Krawczyk, Delorenzi & Baratti, 2012).

On the other hand, an alternative explanation is the retrieval block hypothesis, amnesia results when memory of an event is stored but is rendered inaccessible by an amnesic treatment (Gold & King, 1974; Hardt et al., 2009; Miller & Matzel, 2006; Nader & Wang, 2006). This theoretical framework proposes that during consolidation retrieval links are formed and that pharmacological injury of these links, as well as incomplete consolidation processes after weak trainings, result in dysfunctional retrieval links that can however become functional under certain situations (like noncontingent treatments, reminders or modulation of retrieval by drugs or stressors). However, the main point of the results presented here is not that this experimental amnesia was reversed, but that reversion depends upon reconsolidation. That point has an obvious implication: reconsolidation has indeed occurred. The memory trace, which does not take control of behavior in the long-term as a result of scopolamine administration, is however accessed, reactivated by the reminder, evaluated (whether its prediction is accomplished or not), and can enter the labile phase. In other words, retrieval occurs even when the trace is not behaviorally expressed (Fig. 5).

The approach currently and previously presented (Frenkel et al., 2005a, 2010), i.e., a positive modulation of memory expression during reconsolidation, could be a useful experimental approach for distinguishing between reactivated but unexpressed and obliterated memories after amnesic treatments. The positive modification in memory expression after the reminder presentation is specifically attributable to the reconsolidation process, a modification that is hard to explain as a result of, for instance, adding or summation effects, partial retraining or retrieval deficits. What is evaluated at the testing session is not whether a memory survived or not, but whether it had been previously reactivated and become labile by the presentation of a reminder under conditions that trigger the reconsolidation process. This approach would provide the retrieval and storage views of amnesias with a new prediction in the case of positive results. In the case of negative results, as with cycloheximide (Frenkel et al., 2010) and the high dose of scopolamine, it is not possible to dissect whether the interference affected the consolidation process or, alternatively, obstructed the retrieval process (Miller & Matzel, 2006).

The highest dose of scopolamine (5 μg/g) showed an amnesic effect. However, we cannot find evidence that there is a memory trace that can be reactivated-labilized by the *Reminder Session* that triggers reconsolidation in this case. Acetylcholine is involved in a multitude of processes involving the central and peripheral nervous system. It should be expected that high doses of scopolamine would not only affect the normal modulation of long-term memory expression –as an endogenous system that influences memory storage processes but does not serve as the neural bases of memory storage (Cahill & McGaugh, 1996), but also they would affect perception and the whole physiological state of the animal. Under

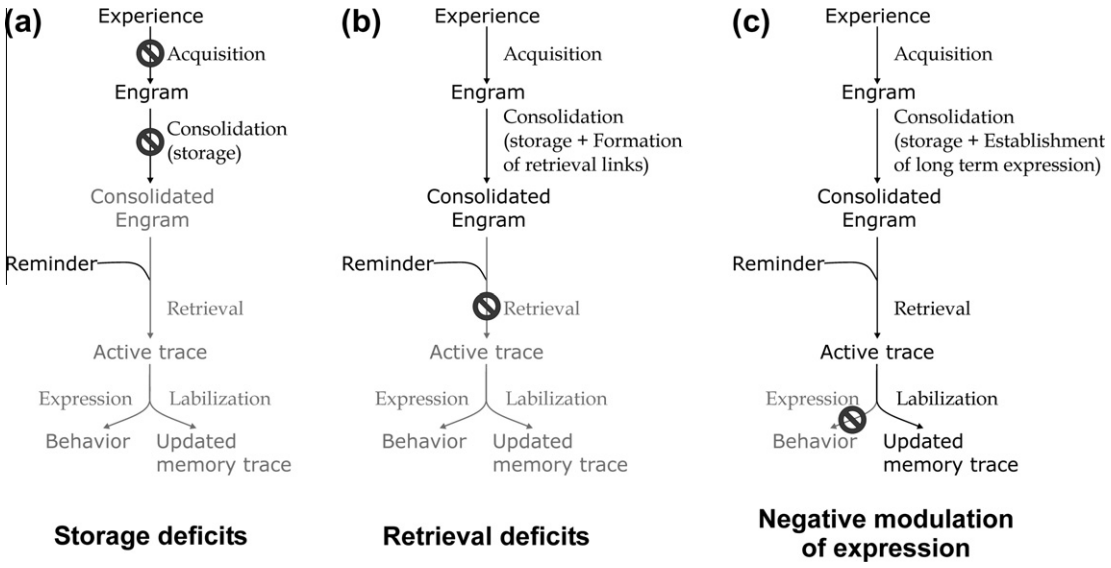

Fig. 5. Alternative explanations for experimental amnesia. Diagrams show some stages of memory. Pre- or post-training amnesic treatments interfere during acquisition or consolidation with the development of processes indicated with ⃠. When memory is not revealed in a long-term test, lack of retention can be explained as one of these possible situations: (a) Storage deficits: Enduring memories are not formed after interference by amnesic treatments or insufficient training. There is not memory reactivation during a testing session because there is not a consolidated long-term engram. Recovery can occur because residual molecular traces can survive and be added to new experiences (as during retraining). (b) Retrieval deficits: During consolidation retrieval links are formed that allow a reminder to reactivate the engram. Mnemonic information after amnesic treatments is intact, but cannot be accessed. Retraining or other reminder treatments can reinstate these links. (c) Negative modulation of expression: During retrieval the active trace (in the same sense of "ecphoric information" (Tulving, 1983)) is evaluated and the system should take the decision of whether express it or not. The decision is taken as a result of an interaction between retrieval cues and properties of the engram that were modulated during consolidation. After amnesic treatments that affect this modulation or after weak trainings, there is a long-term stored memory trace that can be reactivated but not behaviorally expressed. However, the unexpressed memory can enter in a labile state and its long-term expression be modified during reconsolidation.

those high doses, state dependence effects as well as deficits in storage or expression can explain the lack of reversion of performance after reminder presentation. Although we cannot find unspecific effects in control groups, whether this lack of reversal is specifically due to the interference of the memory process by scopolamine remains to be determined. It should be noted that, if reversion of the amnesic effects depends upon reconsolidation, it is fundamental to find conditions in which amnesia can be induced and not recovered by the reminder treatment, which would allow discarding other possible reminder-testing cue-related effects that could be abolished by the presentation of reinforcement.

It has been proposed that the cholinergic system would play a differential role in acquisition and consolidation (Blake et al., 2011; Elrod & Buccafusco, 1991; Rush, 1988). We found that both pre- and post-training injections of scopolamine do affect long-term memory expression. It could be proposed that memory expression could be affected by cholinergic activity during acquisition, for example, by cholinergic effects in the internal state of the animal during learning or in perceptual function. However, the differential role was proposed on the basis of the different effective doses that can induce anterograde or retrograde amnesia. It should be taken in account that differences in doses can also be explained by the degree of activation of cholinergic activity as a modulatory system during consolidation at the time of the effect of the drug.

From these results, it could also be expected that in vertebrates, mild -not strong- cholinergic dysfunction still allows the formation of long-term memories, but the traces of the stored memories do not take control of behavior at *Testing*. However, these memories would be in fact retrieved, labilized and enhanced during the reconsolidation process, allowing them to be expressed long term, *i.e.,*. to take control of behavior in subsequent evaluations (Blake et al., 2012). Thus, memory impairment observed in the scopolamine-induced experimental amnesia, and conceivably in Alzheimer's disease patients, caused by cholinergic dysfunction, might actually be a symptom of expression impairment rather than memory loss. Memories could still be there but remain unexpressed. Moreover, if these unexpressed memories could be reactivated-labilized and hence susceptible to being enhanced during reconsolidation, it could be possible to achieve behavioral expression of the stored information. This would offer different approaches to reducing memory impairment in some age and Alzeheimer's desease-related cognitive déficits which are associated with cholinergic disorders.

Acknowledgments

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