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Updating contextual information during consolidation as result of a new memory trace

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ABSTRACT

Reconsolidation studies have led to the hypothesis that memory, when labile, would be modified in order to incorporate new information. This view has reinstated original propositions suggesting that short-term memory provides the organism with an opportunity to evaluate and rearrange information before storing it, since it is concurrent with the labile state of consolidation. The *Chasmagnathus* associative memory model is used here to test whether during consolidation it is possible to change some attribute of recently acquired memories. In addition, it is tested whether these changes in behavioral memory features can be explained as modifications on the consolidating memory trace or as a consequence of a new memory trace. We show that short-term memory is, unlike long-term memory, not context specific. During this short period after learning, behavioral memory can be updated in order to incorporate new contextual information. We found that, during this period, the cycloheximide retrograde amnesic effect can be reverted by a single trial in a new context. Finally, by means of memory sensitivity to cycloheximide during consolidation and reconsolidation, we show that the learning of a new context (CS) during this short-term memory period builds up a new memory trace that sustains the behavioral memory update.

1. Introduction

Understanding the role of labile periods of memory is an open challenge. Nevertheless, most of the discussion about the functional value of the labile states, strengthening or rearranging memory, is nowadays principally restricted to the reconsolidation process (Alberini, 2007; Dudai, 2009; Sara, 2000). After learning, long-term memory formation must undergo a stabilization period (consolidation) at which memory can be interfered or strengthened by several treatments, including hormonal changes and experiences (McGaugh, 1966; McGaugh, 2000; Sara & Hars, 2006). During this time, behavior relies on short-term memory, a process that is believed to be parallel to long-term memory formation (Izquierdo et al., 2002; Sherff & Carew, 2004; Shirahata, Tsunoda, Santa, Kirino, & Watanabe, 2006). It has been proposed that the transient short-term memory phase, a period that is concurrent with consolidation, when memory is still labile, provides the organism with a better opportunity to evaluate, classify and rearrange information before long-term memory is stored (Dudai, 2002a; Gerber & Menzel, 2000; McGaugh, 2000; Menzel, 1999). For instance, it has been proposed that during this period it is possible to integrate new information into one single experience (Izquierdo & Chaves, 1988; Loftus & Palmer, 1974). Accordingly, there is a lot of evidence across phylum that a weak memory undergoing consolidation can be strengthened by a weak learning experience or by retrieval (Parvez, Stewart, Sangha, & Lukowiak, 2005; Summers, Crowe, & Ng, 2000). In this sense, a training procedure that induces short- but not long-term memory, may be enhanced by presenting a reminder trial during both consolidation and the period of short-term memory expression (Summers et al., 2000; Smal, Suárez and Delorenzi, unpublished results).

Here, the *Chasmagnathus* associative memory model is used to investigate whether during consolidation it is possible to update some attributes of a recently acquired memory. The associative learning paradigm is based on the escape response elicited by the presentation of a visual danger stimulus (US), an opaque rectangle passing above the animal. Upon the iterative presentation of US, the crab's escape response declines and a strong freezing response is built up (Pereyra, González, & Maldonado, 2000). The response decrement lasts for at least 5 days (Lozada, Romano, & Maldonado, 1990; Pedreira, Dimant, Tomsic, Quesada-Allue, & Maldonado, 1995). The memory formed using this paradigm is based on the association between the environmental features of the training context (CS) and the features of the screen moving above the animal (the signal, US) (Tomsic, Pedreira, Romano, Hermitte, & Maldonado, 1998); such memory was termed Context-Signal Memory (CSM). Studies performed on the mechanisms underlying different memory phases have shown that CSM consolidation, extinction and reconsolidation are blocked by protein synthesis inhibitors (Hermitte, Pedreira, Tomsic, & Maldonado, 1999; Pedreira, Dimant, & Maldonado, 1996; Pedreira, Perez-Cuesta, &

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Maldonado, 2002; Pedreira et al., 1995). Findings from studies done to investigate the mechanisms underlying reconsolidation showed a reliable CSM sensitivity to amnesic agents by re-exposing the animals for 5 min to the learning context, without US presentation, 24 h after training (Maldonado, 2002; Pereyra et al., 2000). This labile memory is sensitive to cycloheximide and other pharmacological agents (Pedreira & Maldonado, 2003; Pedreira et al., 2002). Nevertheless, memory does not become labile again if the reminder (the learning context) is reinforced with a US presentation (Frenkel, Maldonado, & Delorenzi, 2005; Pedreira, Pérez-Cuesta, & Maldonado, 2004).

Here, we explore whether during consolidation it is possible to update some attributes of a recently acquired memory, and whether this change in a memory's feature can be explained as modifications of the consolidating memory trace, or as result of the formation of a new memory trace. Specifically, we tested whether memory can be modified during this period in order to update information (i.e. to incorporate new context information). Firstly, we showed that strong training protocols generate a short-term memory that, in contrast to long-term memory, is not context specific. Secondly, in order to test whether this change in behavioral memory features can be explained as modifications of the consolidating memory trace, we found a reversion phenomenon of the cycloheximide-induced amnesia during consolidation by a single trial presentation in a new context. Finally, we used an experimental approach involving reconsolidation to show that causing the updated memory to become transiently labile again in the new context does not imply that the original memory trace will become transiently labile as well. Using this approach, we present evidence supporting the view that context memory update is due to a new memory trace, generated when memory is still being consolidated, i.e. labile.

2. Materials and methods

2.1. Animals

Intermolt adult male crabs of the species *Chasmagnathus granulatus* between 2.7 and 3.0 cm 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 carried out at daytime within the first week after the arrival of animals. Each crab was used in one experiment only. Experimental procedures are 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), consists of a container covered to a depth of 0.5 cm with artificial sea water, where the crab remains during each experimental session. The actometer is illuminated from above with a 5 W bulb. During each trial (9 s), an opaque rectangular screen (25–7.5 cm), termed the visual danger stimulus (US), was moved horizontally over the animal, cyclically from left to right and vice versa, at a constant speed (one trial = two passages of the visual danger stimulus). The US provokes in the crab an escape response and consequent container vibrations, which are converted into electrical signals through four piezoelectric transducers (microphones) placed on the external wall of the container. These signals are amplified, integrated during each 9-s trial, and translated by computer into numerical units (activity scores). In all experiments on this work, a single trial consists in two passages of the visual danger stimulus over the animal. The experimental room has 40 actometers, separated from each other by panels.

2.3. Escape response and freezing

The magnitude of container vibrations during the 9 s of US presentation (a trial) depends on the type and magnitude of the defensive responses the displayed by the crab when faced with an impending threat. Two types of defensive responses are distinguished: namely, escape response and freezing response (Pereyra et al., 2000). The escape response is a directional run of the animal in an attempt to move away from the passing screen (US), while the freezing response consists of a rigid motionless display in which the crab lies flat on the substratum. During repeated US presentations (training), the escape response decreases in intensity and is replaced by the progressive building up of a strong and long-lasting freezing (Pereyra et al., 2000; Romano et al., 2006; Tomsic et al., 1998). During context exposure without US presentation the crabs show no defensive responses, instead they are observed exploring or wandering. Throughout this article, data was only recorded during trials, i.e. during the 9-s US.

2.4. Training protocol

A strong *Training Protocol* consists of fifteen trials (9 s, two visual danger stimulus presentations), with 3 min intertrial intervals (total training duration: 42 min), after a 10 min adaptation period. Animals are kept in training context without receiving any stimulation from the US during 10 min before training (adaptation period). This protocol builds up a long-term memory that persists for at least 5 days (Maldonado, 2002).

2.5. Experimental context

Three kinds of containers were used during these experiments. Context A: the classical actometer: an orange plastic container with a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter). Context B: a cylindrical (15 cm diameter and 15 cm height) plastic container with black and white striped walls. Context C: a brown hexagonal plastic container (16 cm top length, 13 cm floor length and 15.5 cm height) with white spots. All containers were covered to a depth of 0.5 cm with artificial seawater.

As described in Section 2.2, the experimental room has 40 actometers (context A), each one with four microphones firmly attached to its base. Context B an C are arranged to fit inside the actometer, thus vibrations provoked by the motor activity of the animal can not be registered properly. Consequently, Context A is the only one in which the activity of the crab can be measured. Thus, experiments were designed in such a way that the *Testing Session* occurs in Context A. For the other sessions, any of the three contexts may be used. This imposes a limitation in experimental designs, since context presentation can not be counterbalanced. However, these contexts have been used as reactivation controls and as context-dependence controls in a number of works, proving that animals recognize them as different contexts (Frenkel et al., 2005; Pedreira & Maldonado, 2003).

2.6. Experimental procedure and design

Experiments included three or four sessions: a *Training Session* (Day 1), a *Single Trial Session* (Day 1; 0.5, 4 or 8 h after the *Training Session*), and a *Testing Session* (Day 2 or 3). Reconsolidation experiments included an additional *Reactivation Session* (Day 2). Experi-

mental design involved one or two pairs of crab groups, where each pair had a trained group (TR) and an untrained group (UN). TR and UN groups differed only in the *Training Session*; during this session, TR groups received US presentations, while UN groups remained the same time in training context, without any US presentation. Throughout the rest of the experiment, both groups underwent the same treatments. Thus, UN groups serve as retention control for their respective TR group as described in *Data Analysis*. Each UN or TR group comprises 30–40 crabs. Before animals were assigned to 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. For each experiment, experimental procedures were applied simultaneously for all groups.

2.7. Training Session (Day 1)

Trained animals (TR group) spent 10 min in the container (adaptation time), and then they received a strong training protocol: 15 training trial. Untrained animals (UN group) were kept in the training context during the entire training session as controls, i.e. without being presented the visual danger stimulus (US). Immediately after the training session, both UN and TR crabs were moved from the training context to be housed individually in the resting containers, i.e. plastic boxes covered to a depth of 0.5 cm with brackish water and were kept inside dimly lit drawers.

2.8. Single Trial Session (Day 1)

On Day 1, 0.5 h, 4 h or 8 h after Training Session, all groups spent 10 min in the *Single Trial Session* context (adaptation time), and then received one US presentation (a single trial). Immediately after, crabs were moved from the container to be housed individually in the resting containers.

2.9. Reactivation Session (Day 2)

On Day 2, in experiments involving a reactivation session, crabs were exposed to the *Reactivation Session* context for 5 min and then they were returned to their individual resting containers until next day. This procedure turns memory into a labile state (Frenkel et al., 2005; Pedreira et al., 2004) and does not produce extinction of the reactivated memory (Pedreira & Maldonado, 2003). For inducing extinction, it is necessary more than 1 h of unreinforced CS re-exposition (Pedreira et al., 2004; Pérez-Cuesta, Hepp, Pedreira, & Maldonado, 2007). A US can be presented during the last 9 s of the *Reactivation Session*. This procedure prevents memory from turning into a labile state (Frenkel et al., 2005; Pedreira et al., 2004).

2.10. Testing Session (Day 2 or Day 3)

On Day 2 or 3, depending on the experiment, crabs spent 10 min in the testing context (always context A, as described in Section 2.5) and then they were tested for memory expression with a single US presentation (a trial = two visual danger stimulus presentations.

2.11. Drug administration

Crustacean physiological saline solution was used as vehicle. Fifty microliters of saline or Cycloheximide solutions were given through the right side of the dorsal cephalothoraxic-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 (Maldonado, 2002).

Cycloheximide (Chx), purchased from Sigma, was dissolved in physiological saline. Doses were the same as those that have previously shown amnesic effects (Frenkel et al., 2005; Hermitte et al., 1999; Maldonado, 2002; Pedreira et al., 1995; Pedreira et al., 1996; Pedreira et al., 2004).

2.12. Memory retention criterion and data analysis

Memory retention was assessed by focusing data analysis on test trial scores, i.e. by estimating the difference between response level of trained group (TR) and that of the respective untrained group (UN) at the *Testing Session* (long-term memory) or at the *Single Trial Session* (short-term memory). Rescorla convincingly argued in favor of using this sort of analysis instead of a paired training–testing comparison, emphasizing the differences between time of input (*Training Session*) and time of assessment (*Testing Session*). This approach is amply justified in the present case since it has been demonstrated that Context-Signal Memory expression in crabs is independent of the escape response level at training (Tomsic, Maldonado, & Rakitin, 1991). A TR group is said to show memory retention when its mean response level at the test trial is statistically lower than the respective UN group. 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. For this, 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). In experiments that involved two pairs of groups, results were analyzed using analysis of variance (ANOVA) and a priori planned comparisons. Three types of contrasts per experiment were carried out: 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. In experiments that involved only one pair of groups, comparisons between TR and UN were statistically analyzed using a *t*-test. All response scores are represented as mean ± standard error. We analyzed data using STATISTICA (StatSoft, version 6.0).

3. Results

3.1. A single trial in a novel context updates the contextual information of long-term memory, if given 0.5 or 4 but not 8 h after training

The *Chasmagnathus* Context-Signal Memory (CSM) model is based on the crab's escape response elicited by the presentation of a screen passing overhead (visual danger stimulus, US). Through repeated presentations of the US, the escape response declines and is replaced by a freezing-to-US response that persists over time (Maldonado, 2002; Pereyra et al., 2000). Such long-term memory implies an association between the environmental features of the training context (CS) and the features of the screen moving overhead (the signal, US) (Tomsic et al., 1998) which thus is termed Context-Signal Memory (CSM). After a strong training protocol (STP, 15 spaced trials, total time = 42 min) animals show memory retention 24–120 h later and it can be revealed by testing the animals with a single US presentation. Crabs fail to exhibit memory when contextual cues are changed from training to testing (Hermitte et al., 1999; Tomsic et al., 1998).

Here, we studied whether memory can be updated during the consolidation period in order to incorporate contextual information.

A single trial is largely insufficient to build a long-term association between the conditioned stimulus and the training context. In the first experiments, we tested whether after strong training, it is possible to update memory information by presenting a single stimulus contingent on a novel context.

On Day 1, one pair of UN–TR groups underwent the *Training Session* (as described in Methods) in context B. Thirty minutes after training, both groups received a single US presentation in context A (*Single Trial Session*). On Day 2 both groups were tested for memory retention in context A (*Testing Session*) (Fig. 1A, left panel). Activity scores at the *Single Trial Session* (Fig. 1A, right panel) differed between trained and untrained animals (*t*-test, UN-0.5 h > TR-0.5 h; $p < 0.011$), revealing memory retention even when animals were tested in a novel context. This result was unexpected, since memory after fifteen trials in *Chasmagnathus* has been previously shown to be context specific when tested long-term (Tomsic et al., 1998). Thus, context specificity is the first evidence of a behavioral difference between short- and long- term memories induced in *Chasmagnathus* by strong training.

At the *Testing Session* (Day 2, Fig. 1A, right panel), *t*-test also revealed memory retention (UN-0.5 h > TR-0.5 h; $p < 0.002$). Therefore, a single trial in a novel context given 30 min after strong training is sufficient to update the contextual information of long-term memory.

In order to test whether this memory update is restricted to the consolidation period, the same procedure as above was performed but the delay of the *Single Trial Session* was varied relative to the time of the *Training Session*. The consolidation period in *Chasmagnathus*, as defined by the ability of both protein synthesis and RNA synthesis inhibitors to interfere with long-term memory formation, is up to 4 h after training (Pedreira et al., 1995; Pedreira et al., 1996). Two pairs of UN–TR groups underwent the *Training Session* in context B as before, but one pair received the *Single Trial Session* in context A 4 h after training (pair 4 h), while the other received it 8 h after training ending (pair 8 h). Both pairs were tested on Day 2 in context A (Fig. 1A, left panel). Activity scores at the *Single Trial Session* (Fig. 1A, right panel) revealed memory retention for the 4 h-pair (*t*-test, UN-4 h > TR-4 h; $p < 0.01$) but not for the 8 h-pair ($p = 0.38$). Thus, memory is expressed without being context specific at least up to 4 h, but not at 8 h. In other words, at least for the times tested in this work, a context-unspecific response is expressed during the long-term memory consolidation period.

At the *Testing Session* (Fig. 1A, right panel) planned comparison [ANOVA, $F(3,128) = 4.884$, $p < 0.003$] disclosed a significant difference in activity scores (i.e. memory retention) between UN and TR groups for the 4 h-pair (UN-4 h > TR-4 h; $p < 0.0004$), but not between those of the 8 h-pair ($p = 0.43$), nor between UN groups ($p = 0.28$). Therefore, long-term memory can be updated by a single trial in a novel context during a short period after training, at least up to 4 h, but not at 8 h.

To study whether memory can be expressed in the *Single Trial Session* context if the single trial precedes the strong training protocol, a pair of UN–TR groups was trained as before, but this time the *Single Trial Session* in context A took place 30 min before the *Training Session* in context B. A *t*-test on activity scores at Day 2 did not show memory retention in context A ($p = 0.62$, data not shown). However, since we only attempted this one time period for the Single Trial Session, it cannot be discarded that, under other parameters, a single trial before a strong training could be enough in order to build a CSM for the single trial context.

Memory expression in the second context on Day 2 can be explained by a loss of context specificity (i.e. context generalization (Dudai, 2002a)) or by the incorporation of a new association between the US and the novel context. To discard a generalization process due to retraining during the *Single Trial Session*, memory update was tested in animals that were retrained without exposure to the novel context (Fig. 1B). Two pairs of UN–TR groups underwent the *Training Session* in context B. Four hours after training, one pair was subjected to the *Single Trial Session* in a novel context (context A, BAA pair), as in the previous experiment. At the same time, the other pair was retrained with a single stimulus presentation in the same context as training (context B, BBA pair). Both pairs of groups were tested the next day in context A. A *t*-test on activity scores for the BAA on Day 1 showed memory retention (UN-BAA > TR-BAA; $p < 0.001$) at *Single Trial Session*, as expected from the previous results shown in Fig. 1A. At the *Testing Session*, planned comparison [ANOVA, $F(3,139) = 7.342$; $p < 0.0002$] showed memory retention (i.e. memory update occurs) for the BAA pair (UN-BAA > TR-BAA; $p < 0.0005$), but they were no differences between groups of the BBA pair ($p = 0.59$), nor between UN groups ($p = 0.53$). Thus, just a single retraining trial cannot modify memory in order to lose its context specificity. To test whether the sole exposure to the context A could be sufficient for memory update, a pair of UN–TR groups underwent the *Training Session* in context B as before, but at 4 h was exposed during 10 min to context A without the presentation of the visual danger stimulus (Fig. 1B, left panel). A *t*-test on activity scores at Day 2 did not show memory retention in context A (Fig. 1B, right panel; $p = 0.86$). Thus, a single presentation of the visual danger stimulus in the novel context is necessary to incorporate this new context into CSM.

It is possible that crabs can generalize the acquired response to context A as result of being retrained in any novel context. To test this hypothesis, the previous experiment was repeated, but this time animals underwent the *Single Trial Session* in context C, a context that is different from those of the *Training Session* and *Testing Session* (Fig. 1C). Two pairs of UN–TR groups were trained in context B and then re-exposed to a single US presentation in a novel context 4 h afterwards. The BAA pair underwent the *Single Trial Session* in the novel context A, the same context in which animals were tested later, on Day 2. The BCA pair underwent the *Single Trial Session* in context C and were tested 24 h later in context A. On Day 1, *t*-test on activity scores during the *Single Trial Session* (Fig. 1C, right panel) showed memory retention for the BAA pair (UN-BAA > TR-BAA; $p < 0.05$) as expected from the previous result. At the *Testing Session*, planned comparison [ANOVA, $F(3,139) = 2.833$; $p < 0.041$] disclosed memory for the BAA pair (UN-BAA > TR-BAA; $p < 0.006$), but not for the BCA pair ($p = 0.67$). There was no significant difference between UN groups ($p = 0.49$). Therefore, contextual information update by a single trial is unlikely to be due to a generalization process triggered by novelty presentation during the *Single Trial Session*. However, since it is not possible to evaluate how different the experimental arenas are for the animal, in both their perceptual properties and their interactions with the training procedure, this result must be interpreted with maximum rigor: 15 trials (strong training) in any given context, followed by a single trial in any second context do not build a response that is expressed in any different context at testing. It cannot be discarded that a certain combination of contexts *Training Session-Single Trial Session* could not build some degree of generalization. In the same way, it cannot be discarded that the particular combination used in this work (contexts A and B) could generate a certain degree of loss of context specificity.

In contrast to what is observed at 24 h or more after training, we found that crabs express (at least 4 h but under 8 h) a short-term memory in a context different from that of the *Training Session*. In addition, we showed that after a strong training protocol, a single trial in a novel context can update contextual memory information only if it is given during that time window. The period in which behavioral memory can be modified to include the new context (CS) coincides with the consolidation time window in *Chasmagnathus*: up to 4 h (but not 6 h) after training, systemic cycloheximide (a protein synthesis inhibitor) administration can block long-term memory formation (Maldonado, 2002; Pedreira et al., 1995; Pedreira et al., 1996; Frenkel et al. unpublished results).

The following experiments were aimed to study the protein synthesis requirements for memory update. In particular, we focused our studies on two critical moments: after the *Training Ses-*

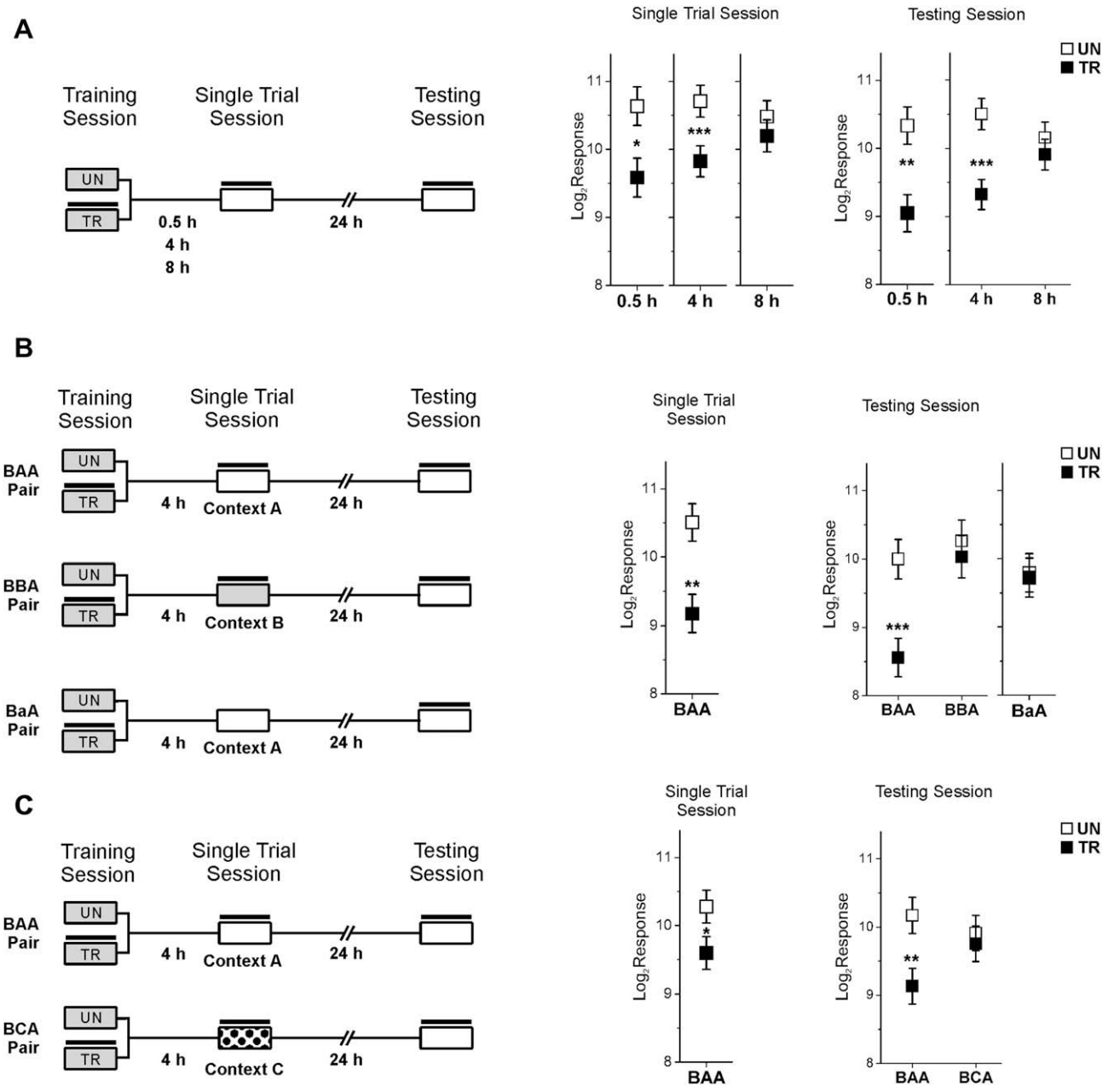

Fig. 1. Context-Signal Memory update by a single trial in a novel context up to 4 h, but not 8 h, after training. Left panel: experimental designs: white boxes represent context A, grey boxes represent context B, dotted box represent context C. A line above a box represents US presentation/s. In B and C Single Trial Sessions were performed 4 h after STP (15 trials) training. Testing Sessions were performed 24 h after training. Right panel: results: graph ordinates: log 2 response to stimulus presentation (means ± SE); open symbols (□): UN groups, filled symbols (■): TR groups. Significant differences between TR groups and their correspondent UN group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (A) *A single trial in a novel context 0.5 h or 4 h —but not 8 h— after training updates contextual information:* Short-term memory is revealed at 0.5 h ($^{*}p < 0.05$) and at 4 h ($^{***}p < 0.001$) in a novel context, while crabs does not show memory if tested in a different context 8 h after training. Twenty for 24 h after training, memory can be revealed in the novel context if crabs underwent the Single Trial Session 0.5 h ($^{**}p < 0.01$) or 4 h ($^{***}p < 0.001$) after training, but not if a single stimulus was presented 8 h after training. (B) *Novel context information and US presentation during Single Trial Session are necessary for memory update:* Stimulus presentation in the same training context during Single Trial Session is not sufficient for memory being revealed in a novel context. A novel context must be presented during Single Trial Session ($^{***}p < 0.001$). The presentation of the novel context alone, without stimulation with the US, is not sufficient for memory update. (C) *Novelty during the Single Trial Session is not sufficient for memory update; a new association between stimulus and the new context is built up during memory update:* Four hours after strong training in context B, a single trial in context A updates memory to be long-term revealed in this novel context ($^{**}p < 0.01$). But if Single Trial Session occurs in a third novel context, different of those of training and testing, then memory is not revealed at Testing Session in context A.

sion and after the *Single Trial Session*. Protocols in which the *Single Trial Session* occurs 4 h after the *Training Session* were used to ensure that both sessions were as separate as possible. We chose to test memory retention 48 h after training in experiments involving cycloheximide in order to extend our knowledge of how long-memory update persists, and therefore to establish the basis to perform further experiments involving a reconsolidation procedure on the second day.

3.2. Protein synthesis after the Single Trial Session is necessary for this memory update

We tested whether contextual information update depends on a protein synthesis-dependent consolidation process after the *Single Trial Session* and whether the original memory of the *Training Session* context regain their sensitivity to amnesic agents after reactivation by the *Single Trial Session*. Previous works (Maldonado,

2002; Pedreira et al., 1995; Pedreira et al., 1996) have shown that a cycloheximide injection has amnesic effects on CSM if given four but not 6 h after training. To ensure that a treatment that could affect *post-Single Trial Session* protein synthesis would not interfere with *post-Training Session* memory consolidation, cycloheximide was administrated 2 h after the *Single Trial Session* (i.e. cycloheximide is injected 6 h after the *Training Session*). A pair of UN–TR groups trained in context B and re-exposed to a single US presentation in context A 4 h later as in the previous experiments. Two hours after the *Single Trial Session*, animals were injected with cycloheximide (20 μg/crab). Testing in context A was performed at 48 h. Simultaneously, another pair of UN–TR groups underwent a similar procedure, except that the *Training Session* was in context A, and *Single Trial Session* occurs in context B (Fig. 2A, left panel). Thus, while both pairs had a cycloheximide injection after the *Single Trial Session*, the first pair (termed BAA) was tested for memory in the *Single Trial Session* context, and the second pair (termed (BAA) was tested for memory in the *Training Session* context.

At the *Single Trial Session* (Fig. 2A, right panel), *t*-test on activity scores for the BAA pair revealed memory retention (UN-BAA > TR-BAA; $p < 0.002$) as expected from previous results shown in this article. At the *Testing Session*, planned comparisons [ANOVA, $F(3,111) = 8.779$; $p < 0.00003$] disclosed memory retention for the ABA pair (UN-ABA > TR-ABA; $p < 0.00005$), thus showing that memory of the original training context was not affected by the drug. No differences were found between groups from the BAA pair ($p = 0.63$), nor between UN groups ($p = 0.93$). A control experiment was carried out in which a pair of UN–TR groups underwent the BAA procedure, but were injected with saline solution instead of cycloheximide (Veh pair). Predictably, *t*-test on activity scores revealed short- and long-term memory retention (Fig 2A, right panel, Day 1, at *Single Trial Session*: UN-Veh > TR-Veh; $p < 0.02$; Day 3, at *Testing Session*: UN-Veh > TR-Veh; $p < 0.02$), thus discarding unspecific effects of the injection procedure on long-term memory retention. Therefore, protein synthesis inhibition after the *Single Trial Session* in the novel context impedes the incorporation of information about the new context. Memory for the *Training Session* context remained insensitive to protein synthesis inhibition at this time, showing that *Training Session* memory did not become labile again. Neither is memory consolidation extended in time as result of the US presentation at *Single Trial Session*.

3.3. The cycloheximide-amnesic effect is reversed by the single non-contingent US presentation

A pre-training cycloheximide injection has amnesic effects on CSM if given 2 h but not 4 h before training ends (Maldonado, 2002; Pedreira et al., 1995; Pedreira et al., 1996). Therefore, an immediate *post-Training Session* cycloheximide injection should not have amnesic effects on a *Single Trial Session* that occurs 4 h after training.

To test *post-Training Session* cycloheximide effects on memory update (Fig. 2B), two pairs of UN–TR groups underwent the *Training Session* in context B and were post-training injected with cycloheximide (20 μg/crab) (Chx pair) or vehicle solution (Veh pair). Both pairs of groups received a single trial in context A and were tested on Day 3 in the same context (Fig. 2B, left panel). At the *Single Trial Session* (Fig. 3B, right panel) planned comparisons [ANOVA, $F(3,147) = 11.446$; $p = 0.000001$] showed significant differences between UN groups (UN-Veh > UN-Chx; $p < 0.05$), thus revealing short-term effects of the drug on the performance of untrained animals. In fact, crabs seem to be numbed after a cycloheximide injection, an effect that disappears after a few hours. However, the experimental device was sensitive enough to detect differences between groups of the Chx pair (UN-Chx > TR-Chx; $p < 0.00053$), thus showing that the context-unspecific memory expression at 4 h does not depend on post-training protein synthesis (a diagnostic feature of short-term memory (Alberini, 2009; Davis & Squire, 1984; Stough, Shobe, & Carew, 2006) neither does it depend on differences induced by cycloheximide on the internal state of the animal from training to testing. As expected, Veh pair also showed memory retention at *Single Trial Session* (UN-Veh > TR-Veh; $p < 0.05$). At the *Testing Session* on Day 3, planned comparisons [ANOVA, $F(3,139) = 4.975$; $p < 0.003$] disclosed memory retention for both pairs of groups (UN-Veh > TR-Veh; $p < 0.05$, UN-Chx > TR-Chx; $p < 0.005$), and there was no significant difference between UN groups ($p = 0.35$). This data shows that behavioral memory update was not impeded by protein synthesis inhibition immediately after the *Training Session*.

It could be suggested that long-term memory of the *Training Session* context is not necessary for memory update. However, since cycloheximide was largely proved to be an effective amnesic agent after a strong training protocol (Pedreira et al., 1995; Pedreira et al., 1996), but not before a single trial presentation, it should be demonstrated that cycloheximide is effectively blocking memory built up during the *Training Session* in this particular training procedure.

In order to achieve this a pair of UN–TR groups were trained in context A, immediately injected with cycloheximide (25 μg/crab) and exposed to a single trial in context B 4 h after training. Animals were tested in context A on Day 3 (pair ABA). Simultaneously, another pair of UN–TR groups underwent the same experimental procedure, except that these groups did not undergo the *Single Trial Session*. Instead, animals remained in their resting containers until testing on Day 3 (pair A_A) (Fig. 2C, left panel). At the *Testing Session*, planned comparisons [ANOVA, $F(3,97) = 6.823$; $p < 0.00035$] disclosed memory retention for the ABA pair (UN-ABA > TR-ABA, $p < 0.00003$) but not between groups of the A_A pair ($p = 0.97$), nor between UN groups ($p = 0.2$). Thus, cycloheximide-induced retrograde amnesia in crabs (as previously descript in Pedreira et al. (1995, 1996) and reproduced here in Fig. 2C, pair A_A) could be reversed by a non-contingent single trial, 4 h after training (Fig. 2C, right panel). In our attempt to block consolidation immediately after the *Training Session* we found that even after inhibition of mRNA translation, inhibition of more than 90% of amino acid incorporation (Pedreira et al., 1995), neither memory update nor memory of the *Training Session* context was impaired.

3.4. Contextual memory update during consolidation is generated by a differentially reactivable memory trace

Context information could be updated by a single trial during the consolidation time window. It is possible that this memory rearrangement was the result of modifications in the long-term memory trace undergoing consolidation. Another possibility is that a new long-term memory for the novel context was generated after the *Single Trial Session*. An experimental procedure to answer this question consists of affecting consolidated memories for each context by selectively reactivating them in the correspondent context (Barnes & Thomas, 2008; Debiec, Doyere, Nader, & Ledoux, 2006; Tronel, Milekic, & Alberini, 2005). If information about both contexts —the *Training Session* context and the *Single Trial Session* context— is encoded in the same updated memory trace, then a treatment that induces amnesic effects during reconsolidation of the memory reactivated in one context should induce amnesia to both contexts.

First, we tested whether memory for the second acquired context can be blocked by an amnesic treatment during reconsolidation. Two pairs of UN–TR groups were trained in context B and received a single trial 4 h later in context A. On Day 2, animals were placed in context A for 5 min (*Reactivation Session*). One pair of UN–TR groups underwent the *Reactivation Session* without being stim-

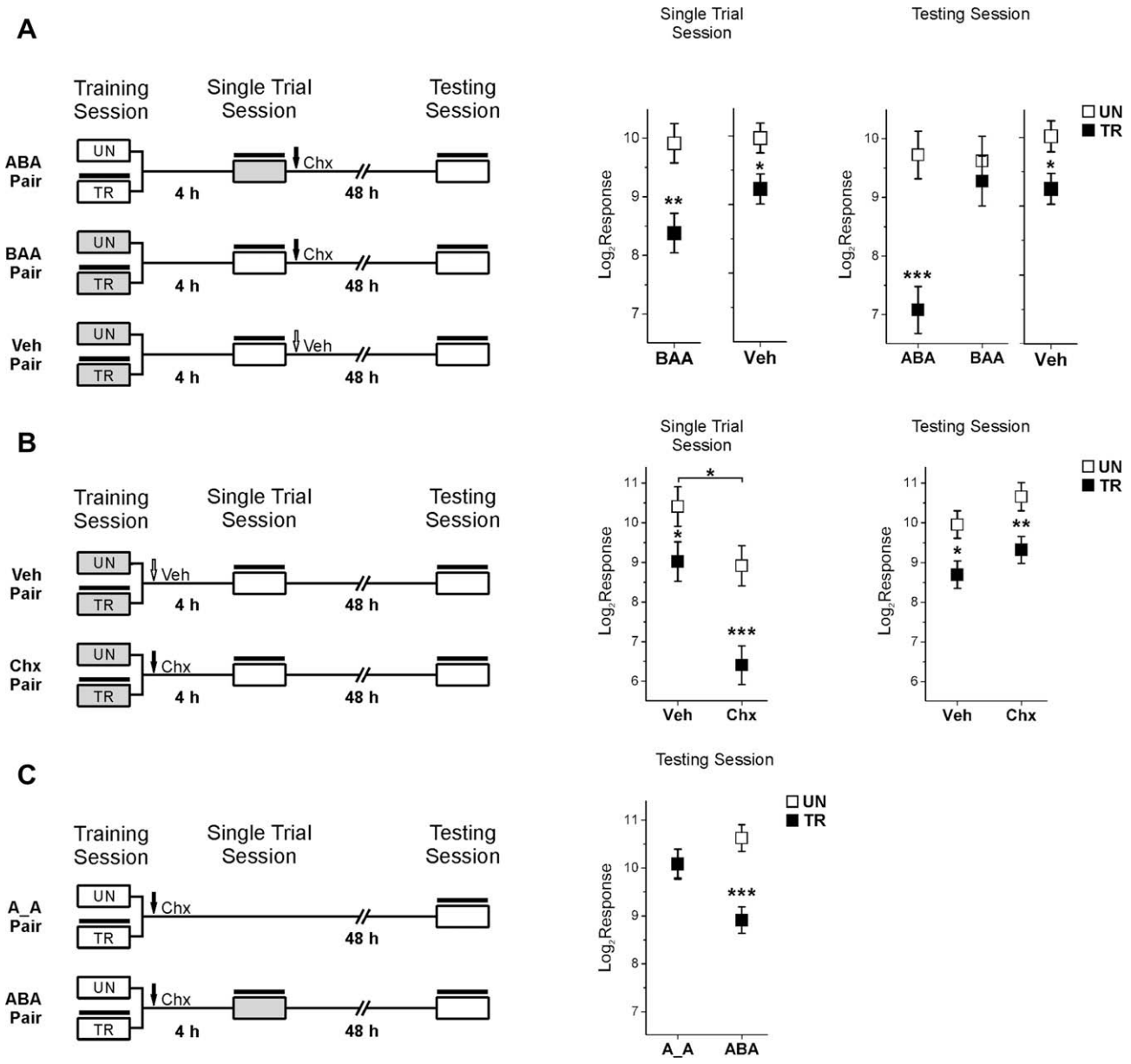

Fig. 2. Effects of close-to-training cycloheximide on memory update. Left panel: experimental designs: white boxes represent context A, grey boxes represent context B. A line above a box represents US presentation/s. Single Trial Sessions were performed 4 h after STP (15 trials) training. Testing Sessions were performed 48 h after training. Arrows indicate drug administration: open arrows, saline injection; filled arrows, cycloheximide injection. Right panel: results: graph ordinates: log 2 response to stimulus presentation (means ± SE); open symbols (□): UN groups, filled symbols (■): TR groups. Significant differences between TR groups and their correspondent UN group, unless particular comparisons were specified: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (A) *Post-Single trial session cycloheximide administration blocks memory update without affecting STP memory:* cycloheximide (20 μg/crab) injection 2 h after Single Trial Session only blocks memory of the second context but it does not affect memory of the first context ($^{***}p < 0.001$). (B) *Post-Training session cycloheximide injection does not block memory update:* Memory of the new context remains unaffected by post-strong training cycloheximide (20 μg/crab) injection ($^{**}p < 0.01$). At Single Trial Session short-term memory expression is neither affected by post-training injection ($^{***}p < 0.001$). (C) *Post-training cycloheximide-induced amnesia is reversed by a single non-contingent US presentation:* As known after previous experiments post-training cycloheximide (25 μg/crab) injection blocks LTM. Nevertheless, the Single Trial Session in a novel context reverses the cycloheximide retrograde amnesic effect ($^{***}p < 0.001$).

ulated with the US (BAaA pair); the other pair of groups were also exposed to context A, but received a single US presentation at the end of the 5 min of re-exposition (BAAA pair). One hour later, all groups were injected with cycloheximide (20 μg/crab). Animals were tested for memory retention in context A on Day 3 (Fig. 3A, upper panel). Reconsolidation does not take place when crabs are stimulated with the US during the reminder session (Frenkel et al., 2005; Pedreira et al., 2004).

At the *Single Trial Session*, planned comparisons [ANOVA, $F(3,146) = 5.791$; $p < 0.001$] disclosed memory retention for both pairs, as expected from previous results in this work (Fig. 3A, lower panel; UN-BAaA > TR-BAaA; $p < 0.05$, UN-BAAA > TR-BAAA; $p < 0.001$). There was no significant difference between UN groups ($p = 0.95$). At the *Reactivation Session* animals from the BAAA pair showed memory retention (*t*-test; $p < 0.05$), revealing that memory update occurred successfully, as shown in the previous experiment. At the *Testing Session*, planned comparisons [ANOVA, $F(3,116) = 2.831$] revealed memory retention for the BAAA pair (UN-BAAA > TR-BAAA; $p < 0.05$), but not for the BAaA pair ($p = 0.28$). There was no significant difference between UN groups ($p = 0.54$). We designed the experiment with this control pair because it is accurate to control specificity of cycloheximide-amnesic actions on reconsolidation. Another control experiment was carried out in which a pair of UN–TR groups underwent the BAaA procedure, but

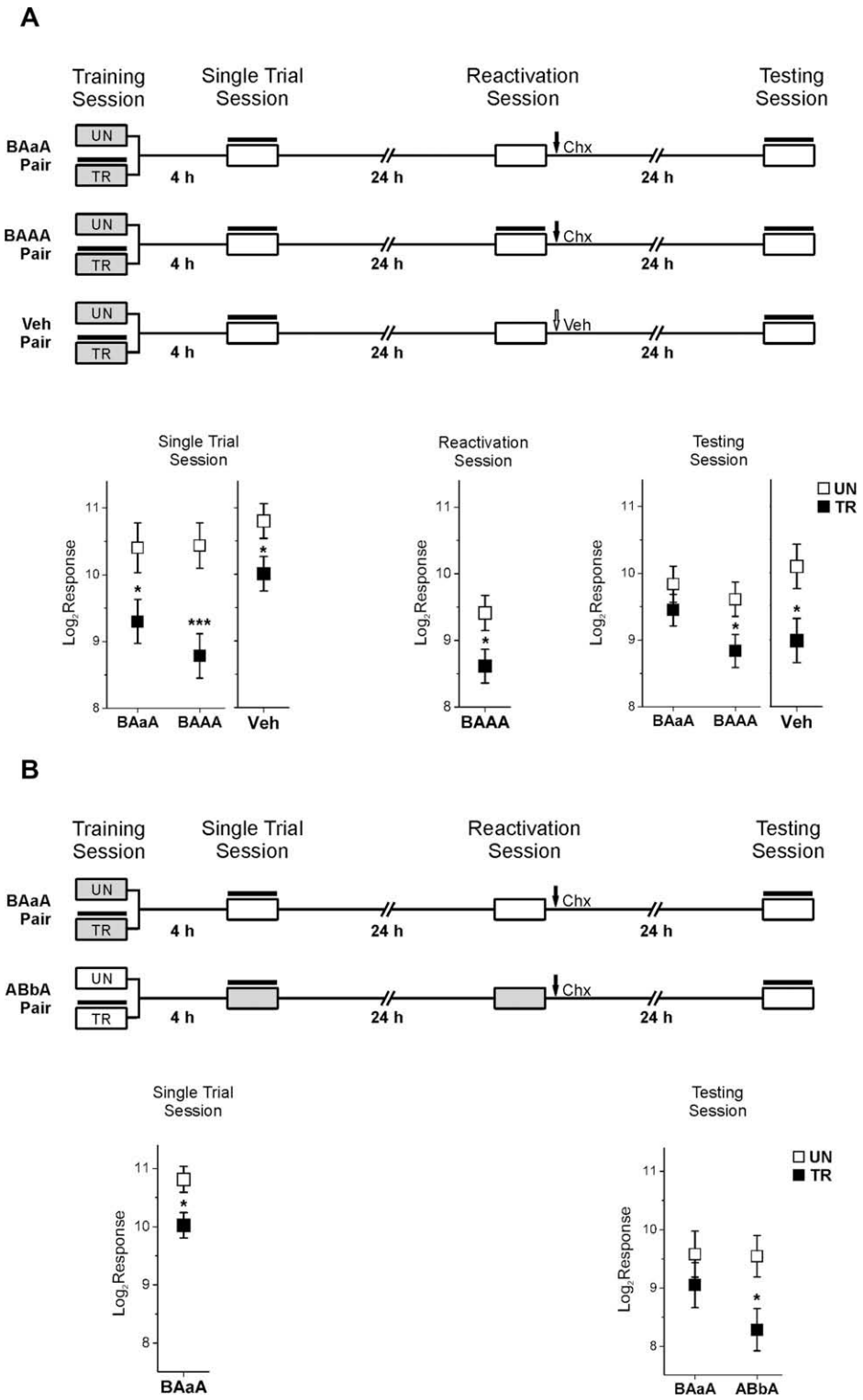

Fig. 3. Post-reactivation cycloheximide injection induces amnesia for reactivated context, but it does not block memory of non-reactivated context. Upper panels: experimental designs: white boxes represent context A, grey boxes represent context B. A line above a box represents US presentation/s. Single Trial Sessions were performed 4 h after STP (15 trials) training. Reactivation Sessions were performed 24 h after training. Testing Sessions were performed 48 h after training. Arrows indicate drug administration: open arrows, saline injection; filled arrows, cycloheximide injection. Lower panels: results: graph ordinates: log 2 response to stimulus presentation (means ± SE); open symbols (□): UN groups, filled symbols (■): TR groups. Significant differences between TR groups and their correspondent UN group: $^{*}p < 0.05$, $^{***}p < 0.001$. (A) *Memory of single trial session context is blocked by a cycloheximide injection* (20 μg/crab) *after reactivation in that context.* A single stimulus presentation at Reactivation Session, a process well known to impede memory for reconsolidate, prevents post-reactivation induced amnesia ($^{*}p < 0.05$). (B) *Cycloheximide injection after Single Trial Session context blocks memory of that context, but not to strong training context:* crabs injected with cycloheximide (25 μg/crab) an hour after memory reactivation in the Single Trial Session context do not reveal memory if tested in that context, but they do show memory when tested in the Training Session context ($^{*}p < 0.05$).

were injected with saline solution instead of cycloheximide (Veh pair). A *t*-test on activity scores revealed short- and long-term memory retention (Fig 3A, lower panel, Day 1, at *Single Trial Session*: UN-Veh > TR-Veh; $p < 0.05$; Day 3, at *Testing Session*: UN-Veh > TR-Veh; $p < 0.05$), thus discarding unspecific effects of the injection procedure on reconsolidation after the BA procedure on Day 1. Thus, memory of the *Single Trial Session* context can be disrupted after its reactivation, except when retrieval occurs under conditions in which memory do not become transiently labile again (i.e. a reinforced reminder presentation (Frenkel et al., 2005; Pedreira et al., 2004)). Previous works in reconsolidation and extinction of Context-Signal Memory in crabs have shown that 5 min of context re-exposure are insufficient to induce extinction of the reactivated memory (Pedreira & Maldonado, 2003; Pedreira et al., 2002; Pedreira et al., 2004). This is consistent with the present result, since the expression of the memory for the *Single Trial Session* was impeded by the cycloheximide injection, which can block extinction, after presentation of the unreinforced reminder.

Once proved that memory of the *Single Trial Session* context can be disrupted after reactivation, we tested whether the memory built up in the *Training Session* context is disrupted when the reactivated memory of the *Single Trial Session* context is interfered with during reconsolidation, or whether it remains intact. In other words, we tested whether memory of the first training become transiently labile by a reminder of the second learning experience.

Crabs were arranged in two pairs of UN–TR groups (Fig. 3B, left panel). One pair of groups underwent the BAaA procedure, as in the previous experiment (pair BAaA), and were injected with cycloheximide (25 μg/crab) 1 h after *Reactivation Session*. The other pair was trained in context A, and exposed to a single trial in context B. On Day 2, animals were re-exposed to context B for 5 min, without any US presentation, and, 1 h later, they were injected with the same dose of cycloheximide. They were tested on Day 3 in context A (pair ABbA).

Analysis of data from the BAaA pair (Fig. 3B, right panel) showed memory retention on Day 1 (*t*-test; $p < 0.014$) but not on Day 3 [ANOVA, $F(3,108) = 2.741$; $p < 0.05$] during the *Testing Session* ($p = 0.33$). This result is as expected from the preceding experiment. However, data analysis of activity scores at the *Testing Session* for the ABbA pair disclosed memory retention (UN-ABbA > TR-ABbA; $p < 0.02$). Thus, memory of the initial *Training Session* context remains even if memory of the second *Single Trial Session* context was disrupted after reactivation in this context.

4. Discussion

Using the *Chasmagnathus* Context-Signal Memory (CSM) model, we have shown a memory modification that can be driven by experience during a short period after learning, more precisely during the consolidation period, when behavior is guided by short-term memory. At 0.5 or 4 h after training, memory can be updated in order to incorporate new contextual information. During this time window, a short-term memory, which is protein synthesis independent and context-unspecific, is expressed (Figs. 1A and 2B). Memory update does not occur if modification attempts take place 8 h after training, when long-term memory is no longer susceptible to interference by protein and mRNA synthesis inhibitors (Pedreira et al., 1995; Pedreira et al., 1996; and Fig. 2B) and short-term memory is no longer expressed. Also, we could not update information when the single trial was performed a short time before the strong training. Finally, we have shown that this behavioral memory update, which depends on protein synthesis after the *Single Trial Session* (Fig. 2A), is not a rearrangement of the previous memory trace which is still consolidating. Instead, a different memory trace is formed, which can be reactivated and become transiently labile again without becoming transiently labile the original memory trace (Fig. 3).

It was proposed that short- and long-term memories differ not only in physiological characteristics, but also in certain behavioral properties (McGaugh, 1966). Nevertheless, to the best of our knowledge, few works have shown behavioral differences between these phases (Izquierdo et al., 2002; Medina, Schroder, & Izquierdo, 1999; Menzel, 1999). In *Chasmagnathus*, animals trained with a strong protocol failed to exhibit memory when tested 24 h after training if contextual cues are changed from training to testing (Tomsic et al., 1998). However, when they are tested during a short period after strong training (up to 4 h but not 8 h), crabs exhibit memory in a context different from that of training (Fig. 1A). Associative memories share associative and non-associative components (Kamprath & Wotjak, 2004). Consequently, non-associative components may be present, but not significantly represented in the long-term CSM expression (Hermitte et al., 1999; Tomsic et al., 1998). On the other hand, associative components may also be present at short-term, even when they would be masked by the expression of the no-associative ones. We have shown here that in CSM, in the short-term, context-independent components are much more heavily represented in this behavior than in the long-term. This is the first evidence in *Chasmagnathus* of a behavioral difference between short- and long-term memories. In *Chasmagnathus*, the ability of memory to control behavior after a weak training protocol also differs between short- and long-term memories (Smal, Suárez and Delorenzi, unpublished work). Early results showed (Brunner & Maldonado, 1988; Romano, Lozada, & Maldonado, 1991) that the decrease in the escape response of trained crabs, a short time after training, is not due to motor impairment caused by motor fatigue after training procedures. Because context A is the only one in which running response of the animals can be measured (see Materials and Methods for details), experiments were designed in such a way that context A was always used for testing. Therefore, whether what was learned in a context can be easier generalized to the other at short-term, or whether a particular order of context presentations is better to induce the long-term memory update, was not evaluated in this work. However, the fact that memory is expressed at short-term after training in context B, shows that at least acquisition of the response occurs in that context. In addition, these contexts have been used as reactivation controls and as context-dependence controls in a number of works, proving that animals recognize them as different contexts (Frenkel et al., 2005; Maldonado, 2002; Pedreira & Maldonado, 2003; Pérez-Cuesta et al., 2007). Context-unspecific memory expression shortly after training does not depend on protein synthesis (Fig. 2B), a diagnostic attribute of short-term memory (Davis & Squire, 1984; Dudai, 2002b; Izquierdo et al., 2002; Parvez et al., 2005; Stough et al., 2006). These context-unspecific behavioral changes during short-term memory expression would be essential to determine that during this period, a period that is concurrent with consolidation, the animals can evaluate, classify and rearrange information (Dudai, 2002a; Gerber & Menzel, 2000; McGaugh, 2000; Menzel, 1999). In this sense, memory generated by a strong training protocol can be updated in such a way that, in the long-term, crabs show memory retention when tested in the new context, different from the initial training context. This behavioral memory update could be obtained by presenting just a single trial in the novel context at 0.5 h or 4 h after training, when long-term memory is under consolidation and short-term memory is expressed. However, the attempt to update memory fails if it is carried out after 8 h, when long-term memory is consolidated and short-term memory is no longer guiding behavior (Fig. 1A). Contextual information update by a single trial cannot be explained as a generalization process due either to an increase in training intensity (Fig. 1B) or to the novelty presentation during the *Single*

Trial Session (Fig. 1C). The fact that the associative memory trace built by the *Training Session* did not become labile jointly with the new context after the *Reactivation Session* (Fig. 3) shows that a new association between the US and the novel context should be formed in order to produce contextual information update. In addition, if a non-associative component, like long-term habituation, were incorporated during the *Single Trial Session*, a decrease in escape response should have been observed in the *Testing Session* despite the amnesic treatment during reconsolidation. However, a certain degree of non-associative components can never be discarded (Kamprath & Wotjak, 2004), but it is not sufficient to account for this behavioral memory update.

In the framework of the present work, the focus question is whether memory modifications, like the incorporation of a new context into behavioral memory, could be due to a rearrangement of the consolidating memory trace, or whether it could be the result of the formation of a new memory trace during the development of short-term memory. A pharmacological approach to this question was to impede memory update by blocking memory formation after the *Training Session* or after the *Single Trial Session*. In our attempt to block consolidation immediately after the *Training Session*, we found that neither memory update nor memory of the *Training Session* context was impaired. Although the post-training-injected cycloheximide can block long-term memory formation after a strong training protocol (Hermitte et al., 1999; Pedreira et al., 1995; Pedreira et al., 1996; and Fig. 2C), the retrograde amnesia was rescued by a single stimulus presentation in the new context, 4 h after injection (Fig. 2B and C). Manipulations involving reminders have been typically used to see whether performance can be recuperated after an amnesic treatment (Nader & Wang, 2006). Classical works show that retrograde amnesias may be rescued by non-contingent unconditioned stimulus presentation (McGaugh, 1973). Possible explanations of the nature of this rescue may be conjectured. A possible reason is that the memory improvement would occur because the non-contingent learning may result in a sub-threshold engram for behavioral expression which adds to a sub-threshold engram that can survive the retrograde amnesic treatment to a level that can be recalled (Gold, Haycock, Marri, & McGaugh, 1973). Reversion of the amnesic effect of cycloheximide by a behavioral procedure may also be in concordance with the tagging hypothesis: after the single trial, *de novo* proteins provided by this event would be captured by tagged synapses, stabilizing the memory trace that otherwise should be disrupted (Moncada & Viola, 2007; Frey & Frey, 2008). In addition, activation of the internal representation of the strong training experience when short-term memory is expressed could induce the stabilization of the wounded trace or even the formation of a new memory of the retrieved experience.

Here we show the incorporation of a new CS–US association into behavioral repertory of the animal. Does this memory modification occur because of the formation of a new memory trace during the development of short-term memory? Memory dynamics involve different processes with different times and different neurobiological characteristics that could reflect possible biological functions. Short-term memory and long-term memory seem to be parallel processes that share some mechanisms (Izquierdo et al., 2002; Stough et al., 2006). A possible function for the transient short-term memory phase is to provide the organism with an opportunity to evaluate, classify and rearrange information before storing it (Menzel, 1999; Gerber & Menzel, 2000; Dudai, 2002a). Here, we have shown that a protein synthesis inhibitor administrated after the *Single Trial Session* can block memory updating, although the original memory build up during the *Training Session* remains unaffected by cycloheximide administration (Fig. 2A). Therefore, the long-term memory trace built up by the *Training Session* did not become labile after the *Single Trial Session*. This fact might be the first evidence contrary to the hypothesis of a modification in the original memory trace during behavioral memory update. That is, if the updating process returns the memory built up during the *Training Session* to a labile state, in order to incorporate new contextual information into the consolidating memory trace, the protein synthesis inhibition should cause an amnesic effect on both memories. Subsequently, we tested whether memory update can be explained as a modification of the original memory trace, or whether there are two differentially reactivable memory traces. In order to so, we used an experimental approach that was previously employed to isolate memory traces involved in complex learning (Tronel et al., 2005; Debiec et al., 2006; Barnes & Thomas, 2008). If the incorporation of the novel context-US association were a modification of the original memory trace, this memory trace would become reactivable by the new context when used as a reminder. Thus, if the blockade of reconsolidation triggered by the presentation of the updated context produced amnesia to the original training context too, it would indicate that there was only a single trace that was modified during memory update, or at least two traces intimately related. In other words, turning one of the traces into a transiently labile state should induce the other to become labile as well. On the other hand, if a new memory trace were built up during memory update, while the original one remained unchanged, then it would be possible to make labile the memory of the updated context after reactivation, without causing the original memory trace to become labile. Here, we have shown that memory of the novel context can become labile again after reactivation by presenting a reminder without reinforcement (Fig. 3A), a condition to trigger reconsolidation in *Chasmagnathus* (Pedreira et al., 2004; Frenkel et al., 2005) and several memory models (Dudai, 2006; Morris et al., 2006; Alberini, 2007). However, memory of the strong training context remains expressible even after the blockade of reconsolidation, triggered by the presentation of the updated context as a reminder (Fig. 3B). Interference with memory after reactivation affects only the memory of the reactivated context and depends on the reconsolidation process, not exclusively in the presentation of the CS (Fig. 3A). Therefore, the amnesic action of cycloheximide on memory after its reactivation depends on the lability of the reactivated memory trace, but it does not depend merely on the activation of processes related with the presentation of the reminder stimulus. In this work, amnesia induced during reconsolidation is interpreted as an impairment of the memory trace. Whether post-retrieval induced amnesia could be due to memory deficits or to retrieval deficits is a topic under discussion (Miller & Matzel, 2006; Miller & Sweatt, 2006). In this framework, contextual memory update during consolidation is mediated by a differentially reactivable memory trace. This result is in concordance with the experiment of Fig. 2A, which shows that the incorporation of the novel CS is not due to the rearrangement of the consolidating memory trace as a consequence of a memory updating mechanism inherent to this memory phase. In other words, behavioral memory update is due to a new memory trace generated during the consolidation period, at the same time as short-term memory is being expressed. The synaptic tagging would be one testable updating mechanism. The single trial would establish transient local synaptic tags that specifically capture the plasticity-related proteins generated during the previous strong training session. Since behavioral tagging has been reported only in rodents (Moncada & Viola, 2007), a large number of experiments remain to establish the experimental parameters and appropriate controls for testing this hypothesis.

In this work, we have shown that the contextual information of a behavioral memory can be updated to incorporate a new association during consolidation. A single trial in a new context, which is largely insufficient in order to generate a long-lasting freezing-to-

US response, can update behavioral memory. The modifications occur during the short-term memory expression period and a new memory trace is involved in this behavioral memory update. Evidently, this result does not discard the possibility that physiological processes engaged during memory consolidation were used to update behavioral memory. Whether updating behavioral memory engages the running consolidation processes to generate the new memory trace, or whether this updating is dependent on short-term memory expression, or both, needs additional study.

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