Research

# Memory reconsolidation and extinction in the crab: Mutual exclusion or coexistence?

Luis María Pérez-Cuesta[1,2] and Héctor Maldonado[1,2,3]

[1]*Laboratorio de Neurobiología de la Memoria, Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. II, C1428EHA Buenos Aires, Argentina;* [2]*IFIBYNE, CONICET, Ciudad Universitaria, Pab. II, C1428EHA Buenos Aires, Argentina*

**A conditioned stimulus (CS) exposure has the ability to induce two qualitatively different mnesic processes: memory reconsolidation and memory extinction. Previous work from our laboratory has shown that upon a single CS presentation the triggering of one or the other process depends on CS duration (short CS exposure triggers reconsolidation, whereas a long CS exposure triggers extinction), both being mutually exclusive processes. Here we show that either process is triggered only after CS offset, ruling out an interaction as the mechanism of this mutual exclusion. Also, we show here for the first time that reconsolidation and extinction can occur simultaneously without interfering with each other if they are serially triggered by respective short and long CS exposures. Thus, we conclude that (1) one single CS presentation triggers one single process, after CS offset, and (2) whether memory reconsolidation and extinction mutually exclude each other or whether they coexist depends only on whether they are triggered by single or multiple CS presentations.**

A conditioned stimulus (CS) exposure has the ability to induce two qualitatively different mnesic processes: memory reconsolidation and memory extinction (Eisenberg et al. 2003; Pedreira and Maldonado 2003; Suzuki et al. 2004). Reconsolidation involves a process of destabilization (labilization) and restabilization of the original memory trace (Nader et al. 2000). Extinction, in turn, is believed to involve the formation of a new memory trace whose information (CS–no US) has an opposite meaning to that of the original memory (CS–US) (Brooks and Bouton 1994).

It has been proposed that, while mechanistically different, these two processes share an important functional feature: They would both be involved in the acquisition of new information related to previous learning. While extinction transiently replaces the expression of the old memory with the newly formed one, it has been suggested that reconsolidation opens the old memory for updating (Nader et al. 2000; Sara 2000), and recent experiments have brought support to this hypothesis (Morris et al. 2006). Given this functional relationship between memory reconsolidation and extinction, and given that either process can be triggered by a CS exposure, the study of the mechanistic relationship between these two processes is of particular interest.

It has previously been established that upon a single CS presentation the triggering of one or the other process depends on certain parametric features of the CS, such as duration. Namely, a short CS exposure triggers memory labilization and subsequent reconsolidation, whereas a long CS exposure triggers memory extinction, both being mutually exclusive processes (Pedreira and Maldonado 2003; Suzuki et al. 2004).

An important question in order to understand this relationship between reconsolidation and extinction is whether the mutual exclusion upon a single CS presentation actually results from an interaction between these two processes (Mamiya et al. 2009) or, on the contrary, whether it implies the lack of such interaction (Pedreira et al. 2004). If the mutual exclusion does depend on an interaction (e.g., one process inhibits the other), this would assume that both processes must be triggered at some point, and then, upon the putative interaction, one process develops, while the other does not. On the other hand, if either process is triggered only after the CS offset, a point where the conditions are irreversibly met for only one process and not for the other, the possibility of an interaction should be ruled out. In this respect, our previous work with crabs shows that memory extinction is not triggered until the CS is terminated, and strongly suggests similar dynamics for reconsolidation (Pedreira et al. 2004; Pérez-Cuesta et al. 2007). Here we present conclusive evidence that CS–US memory is not labilized *during* a short CS exposure, thus demonstrating that labilization and reconsolidation as well are triggered after the CS offset. This conclusion thus rules out the possibility of an interaction between reconsolidation and extinction upon a single CS presentation and supports our hypothesis that only after the CS is terminated a switch mechanism operates driving memory to one fate or the other.

Given this mutual exclusion, a second important issue is whether either process intrinsically constitutes a constraint on the other or whether they can, under any circumstances, develop in parallel. Although this mutual exclusion mechanism is found after a single CS presentation, it is possible that different CS exposures presented serially are able to trigger both reconsolidation and extinction, one process after each CS. To address this issue we exposed trained crabs to a series of two unreinforced CS exposures, a first reconsolidation-inducing short CS exposure followed 15 min later by a second extinction-inducing long CS exposure, and we investigated the occurrence of reconsolidation and extinction. Here we show that upon this behavioral protocol CS–US memory undergoes both reconsolidation and extinction. Moreover, we show here for the first time that these two processes develop in parallel, i.e., they overlap in time with each other. Hence, we conclude that whether reconsolidation and extinction are mutually exclusive or whether they coexist depends only on behavioral experience (i.e., single vs. multiple CS presentation).

## Definitions

As previously pointed out (Myers and Davis 2002), extinction and related terms have been used in literature with different meanings. Therefore, it seems pertinent to define the meaning they are given here. Throughout this article, *memory extinction* (or simply *extinction*) refers to the process of formation (acquisition) of the new memory (CS–no US), i.e., the *extinction memory*, leading to

**[3]Corresponding author.**
**E-mail hector@fbmc.fcen.uba.ar; fax 54-11-4576-3384.**
Article is online at http://www.learnmem.org/cgi/doi/10.1101/lm.1544609.

the transient loss of CS–US memory expression. In turn, the experimental protocol leading to extinction is termed *extinction training*. On the other hand, we will refer to *extinction* (*memory*) *consolidation* as the process by which the newly formed extinction memory is stabilized, yielding a long-term extinction memory. The term *labilization* will only refer to the destabilization of the original memory, the CS–US memory.

## Results

Our previous extensive work with this contextual memory model in crabs has shown that a short exposure (<1 h) of crabs to the training context (CS) induces CS–US memory labilization and subsequent reconsolidation. This reconsolidation process is sensitive to the protein synthesis inhibitor cycloheximide (CHX) during a time window of 4–6 h after CS presentation, which produces a persistent amnesia when animals are tested 24 or 48 h later (Pedreira et al. 2002, 2004; Pedreira and Maldonado 2003).

On the other hand, a long exposure (>1 h) to the training context induces extinction. When crabs are tested shortly after the end of the long exposure to the context, a short-term protein synthesis-independent extinction memory is disclosed (Pérez-Cuesta et al. 2007). In turn, at a 24-h test, a protein synthesis-dependent long-term extinction memory is found (Pedreira and Maldonado 2003; Pedreira et al. 2004; Pérez-Cuesta et al. 2007). Finally, CS–US memory recovers spontaneously at a 48-h test, or upon reinstatement (Merlo et al. 2008).

In a first series of experiments (Exps. 1–4) we investigated whether CS–US memory could undergo reconsolidation and extinction simultaneously or whether either of these processes is a constraint on the other. To address this issue we trained crabs (Day 1) using the context-signal memory paradigm (Maldonado 2002) and 24 h later (Day 2) we exposed them to the training context for 15 min (short exposure inducing labilization and reconsolidation), and 15 min later we re-exposed them to the same context for an additional 2 h (long exposure inducing extinction). In different experiments, memory was challenged with an injection of CHX at different time points to evaluate whether CS–US memory was labilized or not. Finally, the crabs were tested for CS–US memory (Day 3) and, if no memory was found, they were tested for CS–US memory recovery (Day 4) to distinguish amnesia from extinction. At this point, we predicted that if the short CS exposure triggered CS–US memory labilization and reconsolidation, despite the subsequent extinction-inducing long CS exposure, CHX-injected crabs should show a persistent amnesia when tested on Day 3 and also on Day 4 due to the blockade of reconsolidation. On the other hand, saline (SAL)-injected crabs should show extinction on Day 3, but CS–US memory recovery on Day 4, due to the exposure to the 2-h extinction-inducing CS. Instead, if no CS–US memory labilization occurred, CHX should only disrupt extinction memory consolidation, leaving an intact CS–US memory expression both on Day 3 and on Day 4.

In a final experiment (Exp. 5), we addressed the question of whether CS–US memory is labilized during the CS exposure or whether, on the contrary, it remains consolidated until the CS offset.

Throughout this work, every experimental group of trained crabs was run simultaneously with a control group of untrained crabs to which it was compared in the test session.

### A short CS exposure induces memory reconsolidation despite subsequent extinction training

#### *Experiment 1*

To assay whether a short exposure to the training context was capable of inducing memory labilization in spite of subsequent extinction training, trained (TR) and untrained control (CT) crabs were injected on Day 2 with SAL or CHX, and 1 h later they were exposed to the training context (CS) with no reinforcement, either for 15 min (REC), for 2 h (EXT), or for 15 min + 2 h, 15-min apart (REC + EXT). All crabs were tested on Day 3 and again on Day 4 (Fig. 1). Consistent with our previous work, SAL-injected crabs receiving a single 2-h extinction-inducing context exposure (SAL–EXT group) showed extinction on Day 3 and CS–US memory recovery on Day 4, while the corresponding CHX-injected crabs (CHX–EXT group) showed CS–US memory retention at both tests as result of extinction memory consolidation blockade (Pedreira and Maldonado 2003) (Day 3: ANOVA main effect, $F_{(3,129)} = 2.83$, $P < 0.05$; SAL, $P = 0.365$; CHX, $P < 0.02$. Day 4: ANOVA main effect, $F_{(3,128)} = 4.79$, $P < 0.05$; SAL, $P < 0.01$; CHX, $P < 0.03$). On the other hand, SAL-injected crabs receiving a single 15-min reconsolidation-inducing context exposure (SAL–REC group) showed CS–US memory retention at both tests, while those injected with CHX (CHX–REC group) showed on Day 3 an amnesia that persisted on Day 4. (Day 3: ANOVA main effect, $F_{(3,121)} = 3.21$, $P < 0.05$; SAL, $P < 0.01$; CHX, $P = 0.749$. Day 4: ANOVA main effect, $F_{(3,121)} = 2.84$, $P < 0.05$; SAL, $P < 0.01$; CHX, $P = 0.591$). This is consistent with a reconsolidation blockade as previously shown (Pedreira et al. 2002; Pedreira and Maldonado 2003). In turn, while the twice-exposed crabs receiving SAL (SAL–REC + EXT) behaved as the former (i.e., showed extinction on Day 3 and CS–US memory recovery on Day 4), the corresponding CHX-injected crabs (CHX–REC + EXT) behaved as the latter (i.e., showed a persistent amnesia at both tests) (Day 3: ANOVA main effect, $F_{(3,153)} = 0.69$, $P = 0.5593$; SAL, $P = 0.3777$; CHX, $P = 0.4181$. Day 4: ANOVA main effect, $F_{(3,123)} = 2.73$, $P < 0.05$; SAL, $P < 0.04$; CHX, $P = 0.5415$).

Two sets of conclusions can be drawn from these results. First, (1) when a single 2-h extinction-inducing exposure is given, CS–US memory does not return to the labile state (CHX–EXT group still shows long-term CS–US retention despite CHX injection, which only blocked extinction consolidation; see also Pedreira and Maldonado [2003]), and (2) the 24-h-old consolidated memory was labilized in all groups receiving a 15-min context exposure; thus, a short CS exposure is capable of inducing CS–US memory labilization regardless of subsequent extinction training (CHX causes a persistent amnesia both in REC and REC + EXT groups). Hence, not only is the extinction-inducing long CS exposure unable to induce CS–US memory labilization (EXT groups), but it is also unable to prevent, impair, or revert in any way CS–US memory labilization upon the earlier short CS exposure (REC + EXT groups). Second, (1) in the REC + EXT groups, in spite of labilization being induced by the initial 15-min exposure, CS–US memory undergoes extinction and extinction memory is consolidated (SAL–REC + EXT group shows a long-term loss of conditioned response on Day 3), and (2) in addition to (and in spite of) extinction induction and ongoing extinction consolidation, CS–US memory reconsolidation is effectively taking place at some point after labilization (SAL–REC + EXT group shows intact long-term CS–US memory upon recovery on Day 4). Therefore, we can conclude that both mnesic processes, reconsolidation and extinction, are being serially triggered as a result of the respective short and long CS exposures.

### CS–US memory reconsolidates during and even after extinction

#### *Experiment 2*

We next addressed the central question of whether CS–US memory is in fact reconsolidating along with extinction or whether memory extinction could in some way be taking place once reconsolidation is over. In previous work at our laboratory, reconsolidation has shown a time window of 4–6-h sensitivity to

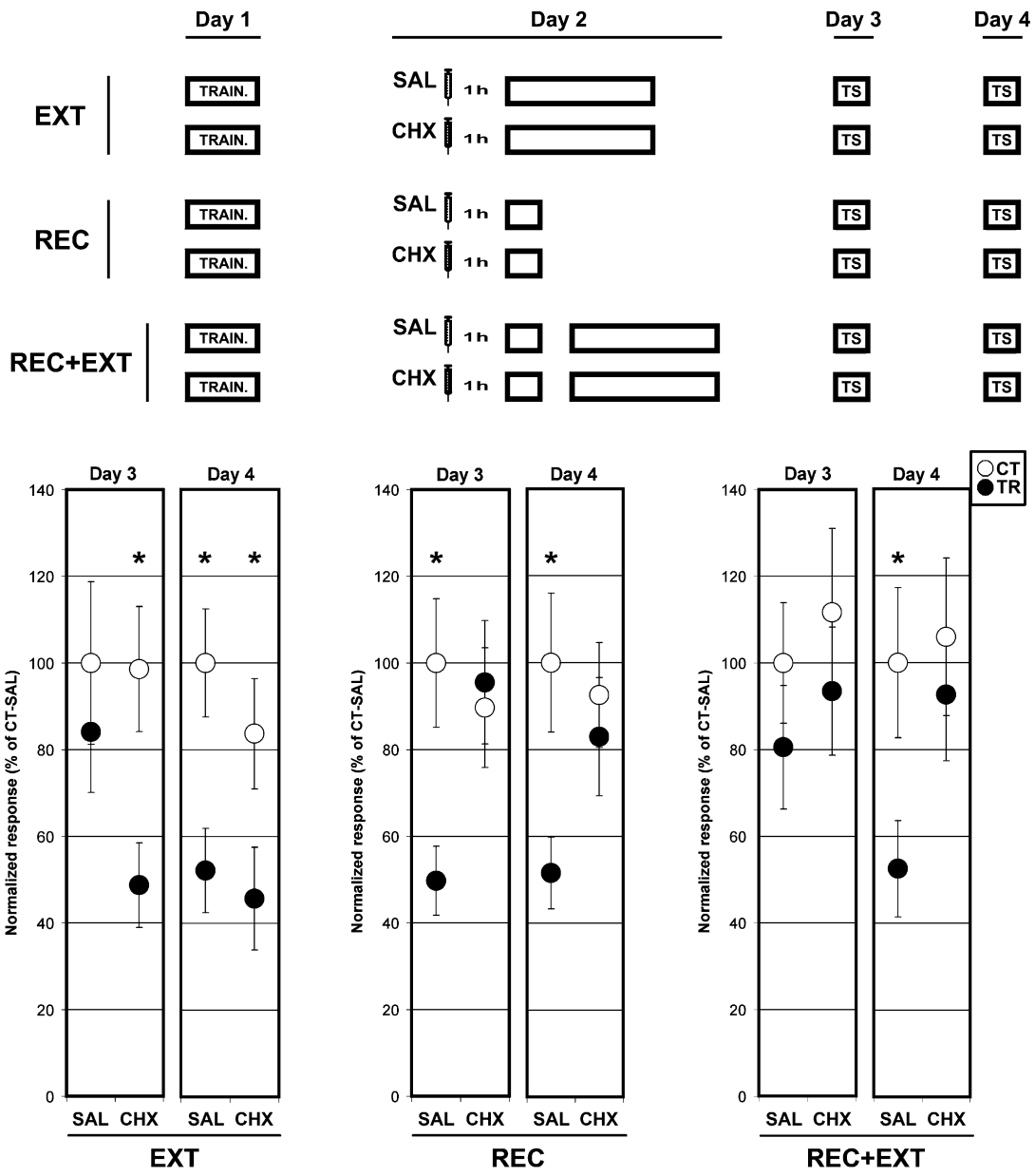


**Figure 1.** A short CS exposure induces memory reconsolidation despite subsequent extinction training. (*Top*) Experimental protocols. Day 1, training session (TRAIN.). Trained crabs received 15 spaced trials, while untrained control crabs remained in the training context for an equal amount of time. Day 2, SAL or CHX injection, followed 1 h later by unreinforced context exposure for 2 h (EXT), 15 min (REC), or 15 min and then 2 h, 15 min apart (REC + EXT). Days 3 and 4, test session (TS). Groups of crabs with different exposure protocols (Day 2) were run separately. (*Bottom*) Mean response ± SEM of untrained control crabs (○) and trained crabs (●) at test sessions of Days 3 and 4. Open boxes stand for the time crabs spent in the training context. (*) $P < 0.05$.

CHX (Pedreira et al. 2002). Therefore, CS–US memory is expected here to remain labile well after extinction training is over, unless there is an effect of extinction training on reconsolidation kinetics. To experimentally test this, we repeated the REC + EXT design of Experiment 1, but this time giving the injections 1 h after the two context exposures, that is, 3 h 15 min after the reconsolidation-inducing exposure, a point in time where CS–US memory is expected to be labile according to single-exposure experiments (Pedreira et al. 2002) (Fig. 2). The pattern of results obtained on Days 3 and 4 fully mimicked those from the REC + EXT groups in Experiment 1, evincing that CS–US memory reconsolidation was still in progress at the time of injections (Day 3: ANOVA main effect, $F_{(3,155)} = 0.98$, $P = 0.4041$; SAL, $P = 0.2152$; CHX, $P = 0.3878$. Day 4: ANOVA main effect, $F_{(3,123)} = 3.73$, $P < 0.05$; SAL, $P < 0.02$; CHX, $P = 0.0937$). Therefore, these results show conclusive evidence that the time course of memory reconsolidation overlaps with extinction training.

### *Experiment 3*

Although we have shown that this two-exposure experimental protocol induces long-term extinction memory (Fig. 1, SAL–REC + EXT), to conclude that reconsolidation and extinction are taking place simultaneously it should be shown that short-term extinction memory is already present 1 h after the end of extinction

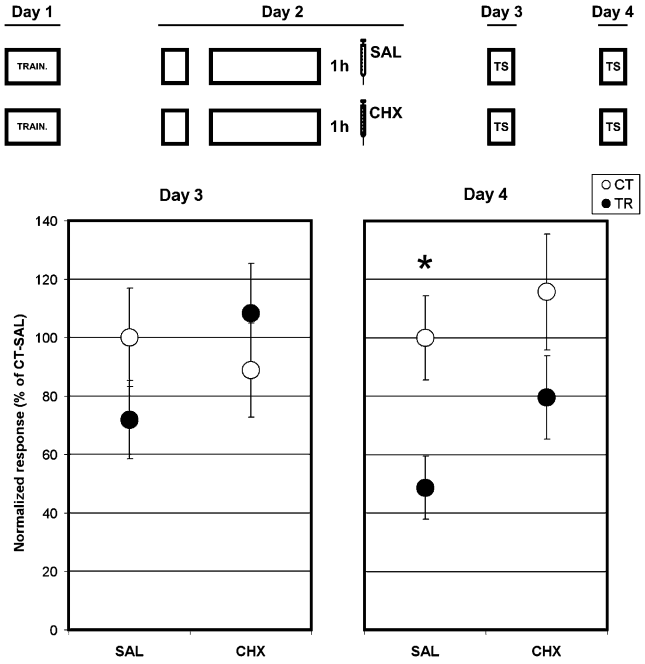


**Figure 2.** CS–US memory reconsolidates along and still after extinction training. (*Top*) Experimental protocol. Day 1, training session (TRAIN.). Day 2, unreinforced context exposures for 15 min and then 2 h, 15 min apart, followed 1 h later by SAL or CHX injection. Days 3 and 4, test session (TS). (*Bottom*) Mean response ± SEM of untrained control crabs (○) and trained crabs (●) at test sessions of Days 3 and 4. Open boxes stand for the time crabs spent in the training context. (*) $P < 0.05$.

training, a time point where CS–US memory is still reconsolidating (Fig. 2). In previous work (Pérez-Cuesta et al. 2007), we have found that CS–US memory is extinguished shortly after the end of a single long exposure. That is, crabs still express CS–US memory if they are tested at any time *during* the long CS exposure; but if they are tested shortly after the end of the exposure, extinction memory is disclosed. Therefore, if extinction developed in a similar way after a two-exposure protocol, we would expect to find CS–US memory expression when testing before the long CS offset, but to find that CS–US memory is extinguished when testing shortly after the long CS offset. To test this, we performed an experiment in which on Day 2 crabs were exposed to the training context for 15 min and then re-exposed again for 2 h, as in the previous experiments, but they were tested either immediately before or 5 min after the end of the long exposure (Fig. 3). The results show that the crabs still disclose CS–US memory when tested before the end of the long CS exposure, but this memory appears extinguished when tested a few minutes afterward (ANOVA main effect, $F_{(3,155)} = 2.99$, $P < 0.05$; BEF, $P < 0.01$; AFT, $P = 0.3547$). These findings confirm and extend previous work showing that CS–US memory is expressed during the course of a long context exposure, but extinction memory is expressed instead as early as a few seconds after the end of the exposure (Pérez-Cuesta et al. 2007). Briefly, these results show that CS–US memory is extinguished while it is still labile and reconsolidating. That is, memory reconsolidation and memory extinction actually occur in parallel.

### *Experiment 4*

The time windows of reconsolidation and extinction consolidation have shown in crabs to follow similar time courses of 4–6-h sensitivity to CHX after the end of the respective single CS exposure (Pedreira et al. 2002; Pedreira and Maldonado 2003). It has been suggested that memory reconsolidation and extinction processes could compete for the same molecular machinery and that this could represent some kind of constraint on their coexistence. Although we have shown that both memory processes are able to develop in parallel, we next asked whether the time course of reconsolidation could be delayed as a consequence of simultaneous extinction and/or extinction consolidation. To test this we repeated the design of Experiment 2, but delayed the injections to 3-h postexposures, a time point where reconsolidation is expected to be over (Pedreira et al. 2002) and extinction memory is expected to be still consolidating (Pedreira and Maldonado 2003) (Fig. 4). Accordingly, if reconsolidation kinetics were unchanged, we would expect CHX to impair extinction memory consolidation but not CS–US memory reconsolidation. The results disclosed that, while SAL crabs showed extinction and later CS–US memory recovery at test sessions, CHX crabs always showed CS–US memory retention (Day 3: ANOVA main effect, $F_{(3,105)} = 2.70$, $P < 0.05$; SAL, $P = 0.3059$; CHX, $P < 0.01$. Day 4: ANOVA main effect, $F_{(3,105)} = 4.87$, $P < 0.05$; SAL, $P < 0.01$; CHX, $P < 0.05$). The results were consistent with a blockade of extinction memory consolidation by CHX, evincing that (1) extinction memory is still consolidating 3-h postexposure, and (2) CS–US memory reconsolidation is terminated 3 h after the exposures (i.e., ~5 h 15 min after the reconsolidation-inducing short exposure). Thus, while these results do not rule out the possibility of an interaction between these two memory processes, they support

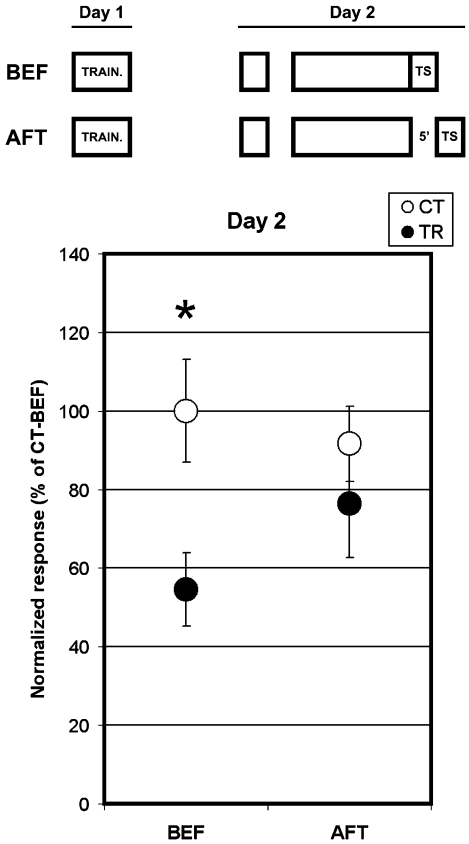


**Figure 3.** CS–US memory is extinguished while still labile and reconsolidating. (*Top*) Experimental protocol. Day 1, training session (TRAIN.). Day 2, unreinforced context exposures for 15 min and then 2 h, 15 min apart. Test session (TS) was performed immediately before the end of the 2-h exposure or 5 min after the 2-h exposure. (*Bottom*) Mean response ± SEM of untrained control crabs (○) and trained crabs (●) at test sessions of Day 2. Open boxes stand for the time crabs spent in the training context. (*) $P < 0.05$.

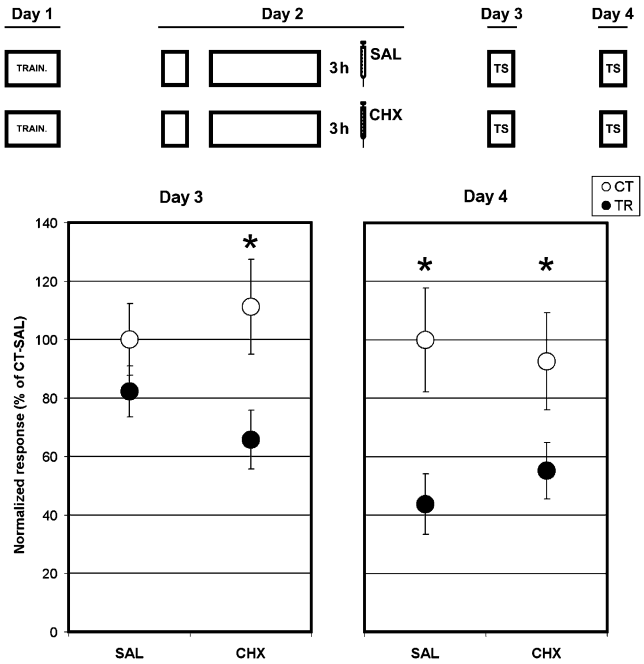


**Figure 4.** Reconsolidation time window duration is not affected by extinction. (*Top*) Experimental protocol. Day 1, training session (TRAIN.). Day 2, unreinforced context exposures for 15 min and then 2 h, 15 min apart, followed 3 h later by SAL or CHX injection. Days 3 and 4, test session (TS). (*Bottom*) Mean response ± SEM of untrained control crabs (○) and trained crabs (●) at test sessions of Days 3 and 4. Open boxes stand for the time crabs spent in the training context. (*) $P < 0.05$.

conserved kinetics. Moreover, the finding that extinction consolidation can be individually targeted after reconsolidation is over shows that not only CS–US memory reconsolidation and extinction memory consolidation occur in parallel, but they also still emerge as two distinct processes.

## CS–US memory is labilized after the CS offset

### *Experiment 5*

In this final experiment we address the question of when CS–US memory labilization is triggered. In previous work with crabs we have obtained several results strongly suggesting that CS–US memory remains consolidated upon the CS onset and throughout the CS exposure, with labilization occurring only once the CS is terminated (Pedreira et al. 2004). We next searched to provide more direct evidence supporting this hypothesis. In this memory paradigm, the CS exposure must meet two parametric conditions in order to trigger CS–US memory reconsolidation: short duration (<1 h) and nonreinforcement (Pedreira and Maldonado 2003; Pedreira et al. 2004). The inclusion of a reinforcement before CS offset prevents CS–US memory from undergoing labilization and reconsolidation, and thus memory remains unsensitive to later injections of CHX (Pedreira et al. 2004). This nonreinforcement condition strongly supports the hypothesis that CS–US memory labilization is triggered after CS offset. We reason that if CS–US memory labilization occurred upon CS onset, i.e., during CS exposure, then it would be unlikely that the occurrence of a reinforcement at a later point (before CS offset) is able to prevent memory labilization (i.e., an event that has already been triggered). So far this hypothesis has been tested using post-CS exposure injections of CHX. Here we searched to provide more direct evidence by injecting crabs with CHX *before* CS exposure. Crabs were trained (Day 1), and 24 h later (Day 2) they received a CHX injection 1 h before being exposed to a 15-min CS, including or not a reinforcement coterminating with the CS exposure (Fig. 5). As CHX was administered before the CS exposure, we predicted that if memory were to be labilized before the CS offset, and thus before the occurrence of the reinforcement, crabs would be amnestic at a long-term test regardless of including a reinforcement. Instead, the test on Day 3 shows that only crabs receiving an unreinforced CS exposure were amnestic, while those being reinforced at the last moment disclosed intact CS–US long-term memory (ANOVA main effect, $F_{(3,119)} = 3.72$, $P < 0.05$; CS group, $P = 0.0997$; CS–US group, $P < 0.05$). These results revealed that CS–US memory remained consolidated throughout CS exposure. Therefore, we conclude that labilization and reconsolidation are triggered once the CS exposure is terminated.

## Discussion

The major findings of the present work are that (1) memory labilization and reconsolidation, like extinction, are triggered after the CS is terminated, and (2) reconsolidation and extinction processes are mutually exclusive when they are triggered by a single CS exposure, but they can coexist and develop in parallel when they are serially triggered by respective short and long CS exposures.

### Memory labilization and reconsolidation, like extinction, are triggered after the CS is terminated

Here we demonstrate that upon a single CS presentation memory remains consolidated throughout the CS exposure, regardless of

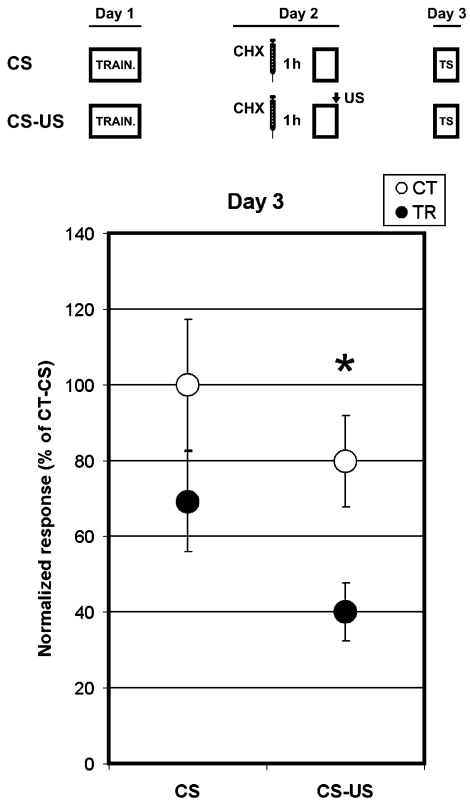


**Figure 5.** CS–US memory labilization is triggered after CS offset. (*Top*) Experimental protocol. Day 1, training session (TRAIN.). Day 2, CHX injection, followed 1 h later by context exposure for 15 min, either unreinforced or reinforced immediately before the end of exposure. Day 3, test session (TS). (*Bottom*) Mean response ± SEM of untrained control crabs (○) and trained crabs (●) at test sessions of Day 3. Open boxes stand for the time crabs spent in the training context. (*) $P < 0.05$.

whether it is a short or a long exposure. Therefore, we can conclude that CS–US memory is labilized and subsequently reconsolidated only after a short CS exposure is terminated. Two results directly support this conclusion. If memory returned to the labile state upon the CS onset, or during the short CS exposure, we predict that any reconsolidation-blocking agent already present in the system would disrupt CS–US memory, regardless of what happened next. Instead, we have found that both (1) the inclusion of a reinforcement at the last moment (Exp. 5), and (2) the protraction of the CS exposure for an additional 1 hr 45 min (Exp. 1, EXT groups; also Pedreira and Maldonado [2003]) prevent CS–US memory labilization. It could be argued that memory is labilized at the CS onset, or shortly after, and then this labilization process is reverted by either reinforcement or CS protraction, in a way that memory is summarily restabilized through a process independent of protein synthesis. Although this is a logical possibility, such a restabilization process has never been described.

In addition to this finding, we previously showed that memory extinction follows similar dynamics: i.e., CS–US memory is disclosed whenever it is tested within the long single CS exposure, but appears extinguished when tested as early as a few seconds after the CS offset (Pérez-Cuesta et al. 2007).

Altogether, these conclusions imply that one single CS elicits the triggering of one single process, which takes place only once the CS exposure is terminated. These conclusions in turn support the hypothesis that no interaction occurs between memory reconsolidation and extinction when only one CS is presented. Considering that the CS offset is a key point where the conditions are set only for one of the two processes, and that the triggering occurs after the CS offset, then an interaction between reconsolidation and extinction when only one CS is presented seems rather untenable, as an interaction would require both processes to be triggered.

In an interesting recent work using a contextual fear memory paradigm in mice (Mamiya et al. 2009), it has been found that the pattern of CREB activation and Arc expression in the hippocampus elicited by a reconsolidation-inducing 3-min CS exposure appears "reverted" when the CS exposure is protracted to 30 min, which induces extinction. These findings are interpreted in terms of an interaction, namely, extinction training inhibiting reconsolidation-related activation of CREB and Arc expression in the hippocampus. Although those results could indeed be a product of an interaction in that model, they are still compatible with our interpretation (single-process triggering after single CS offset).

The finding of the CS offset requirement also offers an insight into the conditions for memory labilization, which in turn broadens our understanding of the functional significance of such a puzzling process. It has been proposed that reconsolidation would serve memory updating by opening memory to the integration of new information in the background of the old memory (Nader et al. 2000; Sara 2000). The results of this study show that the agent of memory labilization is not the mere memory retrieval, but instead a certain reminder structure is required. Our previous and present results show that the reminder structure must meet two conditions in the crab's reconsolidation model: short duration (<1 h) and nonreinforcement. The nonreinforcement requirement has led us to propose the mismatch hypothesis (Pedreira et al. 2004), stating that memory is labilized only when there is a mismatch between the animal's prediction and what actually occurs (i.e., reinforcement vs. nonreinforcement). Interestingly, this hypothesis has been recently confirmed in a declarative memory paradigm in humans (Forcato et al. 2009). Also, this is in agreement with an updating hypothesis, supported by results from a recent work (Morris et al. 2006). Nevertheless, in other models it has been shown that a reinforced CS can induce memory labilization (Eisenberg and Dudai 2004; Duvarci et al. 2006; Lee 2008).

## Mutual exclusion or coexistence of reconsolidation and extinction depends on triggering by single or multiple CS presentations

We also show here that whether memory reconsolidation and memory extinction are mutually exclusive processes or whether they coexist in time depends only on behavioral experience, that is, on whether they are triggered by a single CS exposure or by successive CS exposures, respectively. This finding demonstrates in turn that when they do exclude each other (single CS exposure) this is not due to an intrinsic feature of either process representing a constraint on the other, but rather an outcome of a mnesic mechanism driving memory to one or the other fate.

In the present case, we show that reconsolidation is triggered after the first CS offset (Exp. 5), and then extinction is triggered after the next CS offset (Exp. 3). This conclusion supports our hypothesis that when several successive CSs are presented, a similar reconsolidation-extinction switch mechanism could occur after each CS offset (Pedreira et al. 2004). Here, since the time elapsed between offsets is shorter than the time window of reconsolidation (2 h 15 min vs. >4 h), CS–US memory reconsolidation overlaps with memory extinction (Fig. 6).

Extended to a behavioral protocol where several equal CSs are presented, as is the case in many multi-trial extinction protocols, these findings could imply that the first (or first few) CS offset(s) would trigger reconsolidation, while after a certain point (when conditions for extinction are met) further CS offsets would come to trigger extinction, regardless of ongoing reconsolidation. This hypothesis is also in agreement with the results of Duvarci et al. (2006).

Finally, these findings disclose an important qualitative difference between single-trial and multi-trial extinction. Upon single-trial extinction the expression of CS–US memory is transiently suppressed, but CS–US memory always remains consolidated. As no labilization occurs, we could hypothesize that in this

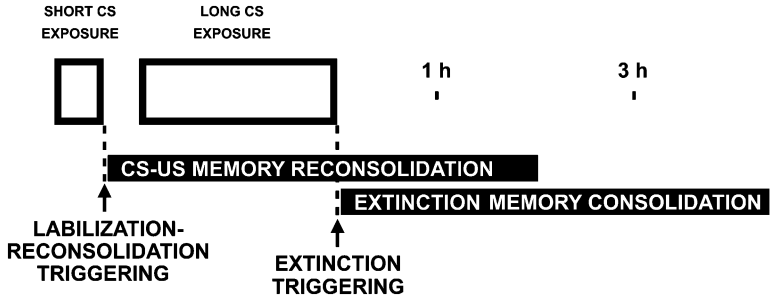


**Figure 6.** Interpretative model of the present results. Open boxes stand for CS exposure time intervals (short exposure, 15 min; long exposure, 2 h). Dotted lines indicate the approximate putative moment of labilization and reconsolidation triggering, after the short exposure and extinction triggering; after the long exposure, followed by extinction memory consolidation. Black bars stand for the approximate time course duration of reconsolidation of CS–US memory and consolidation of extinction memory, indicating the time points where CS–US memory (1 h) and extinction memory (3 h) were targeted.

case CS–US memory not only remains unexpressed but also unchanged. On the other hand, upon multi-trial extinction CS–US memory can undergo labilization and reconsolidation, thus becoming susceptible to modification by the current behavioral experience, i.e., extinction training, an experience with an opposite meaning to that of the labilized memory. This interpretation is in agreement with results obtained in rats in a very recent report (Monfils et al. 2009).

## Materials and Methods

### Animals

Animals were adult male crabs (*Chasmagnathus granulatus*) 2.7–3.0 cm across the carapace, weighing around 17.0 g, collected from water <1-m deep in the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina and transported to the laboratory, where they were lodged in plastic tanks (35 × 48 × 27 cm) filled to a 2-cm depth with diluted marine water, to a density of 20 crabs per tank. Water used in tanks and other containers during experiments was prepared using hw-Marinex (Winex), salinity 10%–14%, pH 7.4–7.6, and maintained within a range of 22°C to 24°C. The holding and experimental rooms were maintained on a 12-h light-dark cycle (lights on 07:00–19:00 h). Animals were fed rabbit pellets (Nutrientes S.A.) every 3 d, and after feeding the water was changed. Experiments were carried out within 10 d after the animal's arrival, between 08:00 and 18:00 h, from January to August. Each crab was used in only one experiment. Each experiment was performed with animals from the same capture event, excepting Experiment 1, in which required animals outnumbered captured animals. For this reason, EXT groups, REC groups, and REC + EXT groups were run separately, and the statistical analysis of this experiment was conducted accordingly. Nonetheless, they were plotted together for comparison purposes. All animals underwent a selection test: Each crab was turned on its back and only those that immediately returned to their normal position were used. The rationale behind this selection is that crabs with a slow correction reaction show a low responsiveness to a large diversity of stimuli and, at a later time, they usually present unhealthy symptoms. Experimental procedures are in compliance with the policies on the use of Animals and Humans in Neuroscience Research.

### The context-signal memory paradigm

The memory paradigm is based on a contextual learning, where crabs associate the training context (CS) with a visual danger stimulus (VDS, US) consisting of an opaque rectangular screen passing overhead (Maldonado 2002; Pedreira et al. 2002; Pedreira and Maldonado 2003). Two types of defensive responses to the VDS are distinguished, namely, escape and freezing response (Pereyra et al. 1999, 2000). The escape response is a directional run of the animal that attempts to move away from the passing screen, while the freezing response consists of a rigid motionless display in which the crab lies flattened on the substratum. During repeated VDS presentations (training), the escape response decreases in intensity and is replaced by the progressive building up of a strong and long-lasting freezing. This training procedure yields a long-term memory lasting for at least 5 d. At the test session, trained crabs (TR) and untrained control crabs (CT) responses are compared. All crabs (TR and CT) are placed in the training context and after a 5-min period they receive a VDS trial, during which the crabs' response is measured as container vibrations. A CT > TR significant difference in the response to the VDS is invariably found at test, and this difference is the operative definition of memory retention.

### The experimental device

This device (Maldonado 2002) referred to as the training context consists of a bowl-shaped opaque container 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 sea water, where each crab was lodged before each experiment. During each trial of 9 sec, an opaque rectangular screen (25 × 7.5 cm), the VDS, was moved horizontally over the animal, cyclically from left to right and vice versa, at a constant speed. The VDS provoked an escape response of the crab and consequent container vibrations, converted into electrical signals through a piezoelectric transducer placed on the external wall of the container. These signals were amplified, integrated during each 9-sec trial, and translated into numerical units 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.

### Experimental protocols and design

Each experiment lasted 2, 3, or 4 d and included three sessions, namely, training session (on Day 1), treatment session (on day 2), and test session (either on Day 2, after treatment [Exp. 3], or either on Day 3 and on Day 4 [Exp. 1, 2, 4, 5]). Untrained (CT) or trained (TR) groups of 30–40 crabs each were formed in each experiment.

#### *Day 1, training session*

Untrained animals (CT) were kept in the training context (CS) during the entire training session (circa 50 min) as controls, i.e., without being presented the VDS, and trained animals (TR), after being 5 min in the container, received 15 training trials, each consisting of a 9-sec VDS presentation (US), separated by intertrial intervals of 3 min. Immediately after the training session, both CT and TR crabs were removed from the training context to be housed individually in resting containers, i.e., plastic boxes covered to a depth of 0.5 cm with water, and kept inside dimly lit drawers.

#### *Day 2, treatment session*

Crabs were exposed to the training context (CS) either for 15 min (REC groups), for 2 h (EXT groups), or for 15 min, and 15 min later again for 2 h (REC + EXT groups). The VDS (US) was never presented during context exposures on Day 2 except where indicated (Exp. 5). An injection with SAL or CHX was given in some experiments, either 1 h before the beginning of context exposures (Exps. 1, 5), or 1 h after (Exp. 2), or 3 h after (Exp. 4) the end of context exposures. After treatment, crabs were returned to the resting containers.

#### *Test session*

At the test session, crabs were exposed to the training context for 5 min, and then received one 9-sec VDS trial. The test session was given either on Day 2, before or after the end of the long CS exposure (Exp. 3), or on Days 3 and 4 (Exps. 1, 2, 4, 5). After the test on Day 3, crabs to be retested on Day 4 were returned to the resting containers.

Repetitive handling of crabs in short time periods (such as 15 min) may produce nonspecific effects affecting memory to be tested. So on Day 2, instead of removing the crabs from the training context between both exposures, a virtual change in the context was produced by changing the illumination from above to below. This procedure provides a completely different environment to the crabs and it has previously shown (1) to effectively signal the end of exposure to the training context (Pérez-Cuesta et al. 2007) and (2) to be taken for a different context in contextual specificity experiments (Y Hepp, pers. comm.). Throughout this work, training sessions, CS exposures on Day 2, and test sessions occurred in the training context illuminated from above.

### Drugs and injection procedure

Crustacean saline solution (Hoeger and Florey 1989) was used as vehicle. Fifty microliters of SAL or CHX (40 μg/crab) solution 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. CHX was purchased from Sigma.

## Data analysis

Data analysis of this study is aimed at testing a basic prediction drawn from our extensive work on the crab's context-signal memory. Animals given 15 or more training trials with 3 min of intertrial interval (trained crabs, TR) show, at a test trial, a level of escape response noticeably lower than that of animals that remained in the training-context but without being trained (untrained control crabs, CT). A statistically significant CT > TR difference ($P < 0.05$) is invariably found, provided that the TR group received at least 15 training trials, every experimental group has not less than 25 animals, and crabs are tested in the training context at the same time of the day of training (Pereyra et al. 2000). Therefore, a trained group is said to show memory retention when the basic assumption is confirmed. Based on the CT > TR prediction, data are analyzed using a priori planned comparisons following a significant main effect in one-way ANOVA ($\alpha < 0.05$) (Rosenthal and Rosnow 1985; Howell 1987). Throughout this work, comparison of responses between CT groups in the same experiment never showed significant differences.

## Acknowledgments

This work was supported by FONCYT (grant PICT 2006-02261) and by CONICET (grant PIP2004/5466). We thank María Eugenia Pedreira and Silvia Pedetta for reading the manuscript and for helpful criticisms.

## References

Brooks DC, Bouton ME. 1994. A retrieval cue for extinction attenuates response recovery (renewal) caused by return to the conditioning context. *J Exp Psychol Anim Behav Process* **20:** 366–379.

Duvarci S, Mamou CB, Nader K. 2006. Extinction is not a sufficient condition to prevent fear memories from undergoing reconsolidation in the basolateral amygdala. *Eur J Neurosci* **24:** 249–260.

Eisenberg M, Dudai Y. 2004. Reconsolidation of fresh, remote, and extinguished fear memory in Medaka: Old fears don't die. *Eur J Neurosci* **20:** 3397–3403.

Eisenberg M, Kobilo T, Berman DE, Dudai Y. 2003. Stability of retrieved memory: Inverse correlation with trace dominance. *Science* **301:** 1102–1104.

Forcato C, Argibay PF, Pedreira ME, Maldonado H. 2009. Human reconsolidation does not always occur when a memory is retrieved: The relevance of the reminder structure. *Neurobiol Learn Mem* **91:** 50–57.

Hoeger R, Florey E. 1989. Catecholamine degradation in the hemolymph of the Chinese crab, *Eriocheir sinesis*. *Comp Biochem Physiol* **92C:** 323–327.

Howell DC. 1987. *Statistical methods for psychology*. Duxbury, Boston, MA.

Lee JL. 2008. Memory reconsolidation mediates the strengthening of memories by additional learning. *Nat Neurosci* **11:** 1264–1266.

Maldonado H. 2002. Crustaceans as models to investigate memory illustrated by extensive behavioral and physiological studies in *Chasmagnathus*. In *The crustacean nervous system* (ed. K Wiese), pp. 314–327. Springer, Berlin, Germany.

Mamiya N, Fukushima H, Suzuki A, Matsuyama Z, Homma S, Frankland PW, Kida S. 2009. Brain region-specific gene expression activation required for reconsolidation and extinction of contextual fear memory. *J Neurosci* **29:** 402–413.

Merlo E, Romano A. 2008. Memory extinction entails the inhibition of the transcription factor NF-κB. *PLoS One* **3:** e3687. doi: 10.1371/journal.pone.0003687.

Monfils MH, Cowansage KK, Klann E, Ledoux JE. 2009. Extinction-reconsolidation boundaries: Key to persistent attenuation of fear memories. *Science* **324:** 951–955.

Morris RG, Inglis J, Ainge JA, Olverman HJ, Tulloch J, Dudai Y, Kelly PA. 2006. Memory reconsolidation: Sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron* **50:** 479–489.

Myers KM, Davis M. 2002. Behavioral and neural analysis of extinction. *Neuron* **14:** 567–584.

Nader K, Schafe GE, LeDoux JE. 2000. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* **406:** 722–726.

Pedreira ME, Maldonado H. 2003. Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron* **38:** 863–869.

Pedreira ME, Pérez-Cuesta LM, Maldonado H. 2002. Reactivation and reconsolidation of long-term memory in the crab *Chasmagnathus*: Protein synthesis requirement and mediation by NMDA-type glutamatergic receptors. *J Neurosci* **22:** 8305–8311.

Pedreira ME, Pérez-Cuesta LM, Maldonado H. 2004. Mismatch between what is expected and what actually occurs triggers memory reconsolidation or extinction. *Learn Mem* **11:** 579–585.

Pereyra P, Saraco M, Maldonado H. 1999. Decreased response or alternative defensive strategy in escape: Two novel types of long-term memory in the crab *Chasmagnathus*. *J Comp Physiol* **184:** 301–310.

Pereyra P, González Portino E, Maldonado H. 2000. Conditioned defensive freezing in the crab is context-specific but not triggered by the context. *Neurobiol Learn Mem* **74:** 119–134.

Pérez-Cuesta LM, Hepp Y, Pedreira ME, Maldonado H. 2007. Memory is not extinguished along with CS presentation but within a few seconds after CS-offset. *Learn Mem* **14:** 101–108.

Rosenthal R, Rosnow RL. 1985. *Contrasts analysis focused comparisons in the analysis of variance*. Cambridge University Press, Cambridge, UK.

Sara SJ. 2000. Retrieval and reconsolidation: Toward a neurobiology of remembering. *Learn Mem* **7:** 73–84.

Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S. 2004. Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* **24:** 4787–4795.

*Received July 15, 2009; accepted in revised form August 18, 2009.*

# Memory reconsolidation and extinction in the crab: Mutual exclusion or coexistence?

Luis María Pérez-Cuesta and Héctor Maldonado

*Learn. Mem.* 2009 16: 714-721
Access the most recent version at doi:10.1101/lm.1544609