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Different dimensions of the prediction error as a decisive factor for the triggering of the reconsolidation process

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ABSTRACT

The reconsolidation process is the mechanism by which strength and/or content of consolidated memories are updated. Prediction error (PE) is the difference between the prediction made and current events. It is proposed as a necessary condition to trigger the reconsolidation process. Here we analyzed deeply the role of the PE in the associative memory reconsolidation in the crab *Neohelice granulata*. An incongruence between the learned temporal relationship between conditioned and unconditioned stimuli (CS-US) was enough to trigger the reconsolidation process. Moreover, after a partial reinforced training, a PE of 50% opened the possibility to labilize the consolidated memory with a reminder which included or not the US. Further, during an extinction training a small PE in the first interval between CSs was enough to trigger reconsolidation. Overall, we highlighted the relation between training history and different reactivation possibilities to recruit the process responsible of memory updating.

1. Introduction

Memory storage implies a passage from a fragile state to a stable form, a process called memory consolidation (McGaugh, 2000). However, following the presentation of a memory cue (reminder), consolidated memories become reactivated (labilized), followed by a process of re-stabilization, which is referred to as reconsolidation (Dudai, 2012; Lee, 2009; Nader, Schafe, & Le Doux, 2000; Sara, 2000). The reconsolidation process is crucial for the modification of existing memories and is the mechanism by which the strength and/or content of consolidated memories are updated (De Oliveira Alvares et al., 2012, 2013; Fernández, Boccia, & Pedreira, 2016; Forcato, Fernandez, & Pedreira, 2013, 2014; Forcato, Rodríguez, & Pedreira, 2011; Forcato et al., 2016; Inda, Muravieva, & Alberini, 2011).

The ability to make predictions and learn from errors based on stored information is a general coding strategy (Bar, 2009; Den Ouden, Kok, & De Lange, 2012). In their habitat, animals have to be sensitive to changes in the environment, either to addition or omission of important events, their timing or magnitude. The Prediction Error (PE) is defined as the difference between the prediction made and current events and implies the detection of a mismatch between past and actual experiences. It represents how surprising or certain was the outcome of the prediction made by the animal. In the experimental psychology field, general associative learning models argue that PE may be determined by the discrepancy between learning history and what can be learned on a given trial (Mackintosh, 1975; Pearce & Hall, 1980; Rescorla, Wagner, et al., 1972). Therefore, PE is proposed both as the driving force guiding memory acquisition and as a necessary condition during the reactivation of a consolidated memory reconsolidation (Exton-McGuinness, Lee, & Reichelt, 2015; Sevenster, Beckers, & Kindt, 2014). Consequently, in different reports it has been demonstrated that PE promotes the updating of consolidated memories and is prompted as a boundary condition for the reconsolidation process (Exton-McGuinness et al., 2015; Pedreira, Pérez-Cuesta, & Maldonado, 2004; Sevenster, Beckers, & Kindt, 2013). In associative learning paradigms, animals learn not only the association between different stimuli but also the temporal relation between them. In rodents, a change in the temporal relationship between the conditioned stimulus (CS) and unconditioned stimulus (US), called temporal PE, during retrieval is sufficient to trigger synaptic plasticity and reconsolidation of an aversive memory in the lateral

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amygdala (Díaz-Mataix, Martinez, Schafe, LeDoux, & Doyère, 2013). Moreover, Alfei, Ferrer Monti, Molina, Bueno, and Urcelay (2015) demonstrated that not only the earlier presentation of the US (30 s before training during retrieval) induced a PE but also the duration of the CS used during training determined which duration was necessary during retrieval to provoke a PE. Under this experimental condition, combining both temporal-specific memories (generated by different CS durations) with different CS-reactivation lengths, they demonstrated that the necessary time to reveal reconsolidation or extinction memory from CS presentation is highly dependent on the conditions established during training. Interestingly, as it has been demonstrated in other report (Merlo, Milton, Goozée, Theobald, & Everitt, 2014), there is also CS lengths whereas neither reconsolidation nor extinction was recruited (e.g. insensitive transitional or limbo period). In humans, PE is a necessary condition for reconsolidation of associative fear memory and it is determined by the interaction between the certainty of the original learning and features of the reminder. Thus, after an asymptotic level of learning, if memory retrieval follows a fully reinforced training, omission of a predicted reinforcement during reactivation destabilizes a consolidated memory (negative PE), whereas a reinforced reactivation trial would leave the memory intact (no PE). This kind of analysis was performed in our initial reports (Alberini, 2013). In contrast, if memory retrieval follows a partially reinforced training insufficient to reach the asymptotic level of learning, a similar reinforced reminder trial generates additional learning and consequently triggers the reconsolidation process (Positive PE) (Duvarci & Nader, 2004; Morris et al., 2006; Rodriguez-Ortiz, De la Cruz, Gutiérrez, & Bermudez-Rattoni, 2005; Sevenster et al., 2013).

In spite of the important contributions made to understand the reconsolidation process using invertebrate models (Alberini, 2013; Eisenhardt & Menzel, 2007), there are scarce systematic studies focused on the relevance of PE for the reconsolidation process in such models (Pedreira et al., 2004). The aim of the present study was to analyze the role of both types of prediction errors (i.e. temporal relation between stimuli and interaction between original learning history and retrieval) in the associative learning of the crab *Neohelice granulata*.

The crab's associative learning paradigm is based on its escape response, which is elicited by the presentation of a visual danger stimulus (VDS; an opaque rectangle passing over the animal). The iterative presentation of the VDS provoked a change in the defensive strategy from the escape to a freezing response (Pereyra, Saraco, & Maldonado, 1999). The acquired memory consists on the association between the environmental features of the training place, the context, and the VDS (CS and US respectively). In a recent study we described a new protocol (Fustiñana, Tano, Romano, & Pedreira, 2013) in which the features of the context were changed in a way that was contingent with the aversive stimulus to create a predictor value for the US. Thus, for each training trial, the context (CS) is discretely presented and finished together with the VDS (US). In addition, many reports have shown the ability of these animals to detect temporal differences, such as stimuli frequency or CS duration (Pedreira & Maldonado, 2003; Pedreira, Romano, Tomsic, Lozada, & Maldonado, 1998; Perez-Cuesta, Hepp, Pedreira, & Maldonado, 2007). Moreover, for the reconsolidation process we demonstrated the relevance of the mismatch during memory reactivation when the US is absent during the CS presentation, being the mismatch a case of negative PE (Frenkel, Maldonado, & Delorenzi, 2005; Fustiñana et al., 2013; Pedreira et al., 2004). We also established that upon a single CS presentation the triggering of reconsolidation or extinction memory depends on CS duration, both being mutually exclusive processes (Pedreira & Maldonado, 2003). Furthermore, including both processes in the same experimental design, Pérez-Cuesta and Maldonado (2009) demonstrated that reconsolidation and extinction can occur simultaneously, without interfering with each other, if they are serially triggered by respective short and long CS exposures. They concluded that memory reconsolidation and extinction may exclude each other or coexist, depending on whether they are triggered by a single or multiple CS presentations. However, the relation between both processes using the new protocol is still unexplored.

Given that this model system has proven valuable in the past for the study of reconsolidation and extinction (Alberini, 2013), the aim of the present study was to perform a systematic research of the putative role of PE regulating the beginning of the reconsolidation process. First, we focused on temporal factors, varying the temporal presentation of the CS and US. Then, we explicitly manipulate experimental parameters likely to generate both negative and positive summative PE's. The pattern of results was consistent with the hypothesis that PE might be critical triggering the reconsolidation process.

2. Materials and methods

2.1. Subjects

Adult male *Neohelice granulata* (formerly known as *Chasmagnathus granulatus*, Crustacea, Grapsidae) intertidal crabs, 2.6–2.9 cm across the carapace, weight 17 ± 0.2 g (n = 60), were collected from water <1 m deep in the estuarine coasts of San Clemente del Tuyu, Argentina, and transported to the laboratory where they were lodged in plastic tanks (30 × 45 × 20 cm) filled to 0.5 cm depth with diluted (12‰, pH 8.2–8.4) marine water (prepared from Red Sea Salt, USA), to a density of 20 crabs per tank. The holding room was maintained on a 12 h light–dark cycle (lights on 07:00–19:00 h). The temperature of both holding and experimental rooms was maintained within a range of 22–24 °C. Experiments were carried out between the third and the 10th day after the arrival of the animals. Each crab was used in only one experiment. Furthermore, all the groups included the same number of animals in each experiment, 30 crabs per group. Thus, in a standard experimental design that included two pairs of groups, 120 animals were used. Experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (USA) and the Argentinean guidelines on the ethical use of animals. This work was approved by our research institution.

2.2. Experimental device

The experimental device has been described in detail elsewhere (Fustiñana et al., 2013; Hepp, Pérez-Cuesta, Maldonado, & Pedreira, 2010; Maldonado, 2002; Perez-Cuesta et al., 2007). Briefly, the experimental unit was a bowl shaped opaque container surrounded by a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter). The container was filled with marine water to a depth of 0.5 cm. The crab was placed in the container, which was suspended from an upper wooden framework (23 × 23 × 30 cm) by three strings. A motor operated screen (US, an opaque rectangular strip of 25.0 × 7.5 cm) was moved horizontally over the animal from left to right, and vice versa. The screen's movements were cyclical. The screen displacements provoked the escape response of the crab and subsequent container vibrations. Each trial lasted 9 s and consisted of two successive cycles of movement. Four microphones were attached to the center of the outside base of the container. The microphones recorded the vibrations that were produced by the animal's response. These signals were amplified, integrated during the entire trial (9 s) and translated into arbitrary numerical units ranging from 0 to 8000. During

the experiment, the crabs were illuminated using a 5-W bulb placed either above or below the container. A computer was employed to program the trial sequences, trial illumination, trial duration and inter-trial intervals, and to monitor the experimental events. The experimental room contained 40 experimental devices that were separated from each other by partitions.

2.3. General experimental protocols

The training and other treatment sessions were preceded by 10 min of adaptation to the experimental device, which was illuminated from below. A typical training trial lasted 27 s with above illumination (CS), and the US was presented during the last 9 s. Thus, the US presentation coincided with the end of the CS presentation (CS-US_E). In an untypical training trial, the entire trail also lasted 27 s with above illumination (CS), but in this case the US was presented in the middle of the CS (between the 10th and 18ths second). Consequently, the CS was presented alone for 9 s after the US presentation (CS-US_M). The inter trial interval (ITI) between US presentations was 171 s, and the ITI between CS presentations was 144 s. A weak training protocol (WTP) consisted of 4 trials; and a strong training protocol (STP) included 15 trials presentation. During the ITI between CSs, the experimental unit was illuminated from below, which provoked a virtual change in the environmental features. The untrained (control group, CT) animals were kept in the experimental unit during the entire training session. These animals were not presented with the US, but were presented with the same pattern of light shift. Immediately after each session, the crabs were moved from the experimental unit to individual resting containers, which were plastic boxes that were filled with water to a depth of 0.5 cm. The resting containers were kept inside dimly lit drawers. One trial of the US was presented before the training to measure the responsiveness of each animal. No differences were found between groups in this pre-training trial for any of the experiments. A typical extinction training protocol consisted of 15 CS presentations of 8 min each, with an ITI of 20 s, presented in a way that ensures a total of 120 min CS re-exposition.

The treatment session implied the presentation of different type of reminders and pharmacological intervention. The objective was to modify the reminder structure to create different conditions to trigger the reconsolidation process. The testing sessions included the evaluation of the response to the US presented at the end of the testing session. To summarize the experimental protocols used throughout this report see Table 1 (Supplementary Table 1), describing the manipulations performed in each experiment.

2.4. Drugs and injection procedure

Crustacean saline solution (Hoeger & Florey, 1989) or dimethyl sulfoxide was used as the drug vehicle (VHC), depending on which drug was used. VHC or drug solution was injected through the right side of the dorsal cephalothoracic–abdominal membrane via a syringe that was fitted with a sleeve to control the depth of penetration to 4 mm, thus ensuring that the injected solution was side released into the pericardial sac. Bicuculline (Fluka Analytical), a competitive antagonist of $GABA_A$ receptor, was administered at a final dose of 2.69 μg/g diluted in DMSO; cycloheximide (CHX; Sigma Aldrich), which is a protein synthesis inhibitor, was administered at a final dose of 2.35 μg/g diluted in crustacean saline solution. Doses are expressed as μg of drug per gram weight of crab. The volume injected depended on the vehicle-type: When the vehicle was DMSO we administered 10 μl and when it was crustacean saline solution the volume used was 50 μl per crab.

2.5. Data analysis

From a functional perspective, this type of associative learning involves knowledge about CSs that have a pre-existing relation to an US. The animal performed two different behaviors after learning when it is confronted with each stimulus (anticipatory response when it is confronted to the CS and escape response facing the US; Fustiñana et al., 2013). Because the US is the more biologically relevant stimulus, the most important product of learning involves changes in how it modifies the response to the US (Domjan, 2005). Thus, our analysis was focused on the modification of the escape response when the US was presented (Fustiñana, de la Fuente, Federman, Freudenthal, & Romano, 2014). In nature, *Neohelice granulata* is chased by gulls. Thus, the escape response elicited by this type of stimuli is critical for survival. In the laboratory, sudden presentation of a rectangular screen passing overhead mimics the stimuli that are present in the field (VDS, US). The US elicits an escape response, which declines with repeated presentations (Tomsic, Massoni, & Maldonado, 1993; Tomsic, Pedreira, Romano, Hermitte, & Maldonado, 1998), and a strong freezing response is built up (Pereyra et al., 1999). The acquired memory is based on the association between the environmental features of the training place (the context, CS) and the VDS (US). In this framework, retention of the acquired learning during training was considered to have occurred when a significantly lower level of response for the escape response to the VDS at the testing session was found for the TR compared with its CT (i.e. both groups were injected with the same solution or treated with the same behavioral manipulation). The rationale for this criterion is based on previous experiments performed in our laboratory. In these experiments, a significant difference (t-test, $\alpha = 0.05$) between the TR and CT groups was invariably identified at testing sessions that took place 24 h or more after training. The experiments demonstrating this difference included 15 or more training trials with an ITI of 171 s. Accordingly, for the current experiments, a significant difference was predicted at testing between the CT and TR groups for the escape response after a STP. It was also demonstrated the absence of difference between groups after a WTP (Carbó Tano, Molina, & Pedreira, 2013). Therefore, throughout the current paper, the results were analyzed using *a priori* planned comparisons via a weighted means ANOVA with α (per comparison error rate) = 0.05, according to the standard method (Howell, 1987). A lack of difference between the CT and TR groups was assumed to indicate a lack of memory retention. For the case in which the extinction protocol was presented, a lack of retention was considered as extinction memory. A comparison between the CTs that received different treatments was necessary to determine the possible drug or behavioral manipulation side-effects that may have affected the response level at testing in a manner that was unrelated to training experience. In general, the statistical analysis of the test data included a set of three *a priori* planned comparisons (LSD-Fisher), namely, each pair of CT–TR groups and the comparison between the CT groups, using planned comparisons of least squares means with α (per comparison error rate) < 0.05 (Howell, 1987; Rosenthal & Rosnow, 1985). All of the values were represented as the normalized mean ± the standard error with respect to the main CT (100%, e.g. CT VHC). Data were analyzed using Statistica 8 (software package 3; StatSoft Inc., Tulsa).

2.6. Experimental procedure

The experiments were performed in three days, each separated by 24 h. On day 1, crabs passed through the training protocol. On day 2 during the treatment session, each pair of CT-TR groups received a different treatment (type of reminder and or drug injected). We designed three different types of reminder: (a) **no**

US: animals were exposed only to the context illuminated from above for 27 s; (b) **US_E**: animals were exposed to the context illuminated from above for 27 s and the US was presented from the 19th to the 27th sec, both stimuli ending at the same time; (c) **US_M**: animals were exposed to the context illuminated from above for 27 s and the US was presented from the 10th to the 18th sec, the CS stayed on 9 s after the US ended. On day 3, all groups were tested during the testing session with one CS-US trial and only the animal response to the US was evaluated.

Extinction training: 24 h after a STP, typical extinction training was performed. It consisted of 15 CS presentations of 8 min each, with an ITI of 20 s, presented in a way that ensures a total of 120 min CS re-exposition (Carbó Tano et al., 2013). To evaluate the spontaneous recovery effect a testing session (1 CS-US trial) was presented on Day 5.

3. Results

3.1. Temporal PE between inter-stimuli interval triggers the reconsolidation process

The goal of Experiment 1 was to determine if a temporal PE was a sufficient condition to trigger the reconsolidation process of a strong associative memory. Thus, to show that the reconsolidation process was at play we used cycloheximide (CHX) to impair the memory re-stabilization (Pedreira & Maldonado, 2003). In previous reports, we demonstrated that a full/complete learning trial used as a reminder (a CS-US pairing maintaining the temporal relationship) leaves the memory intact after the amnesic treatment (no PE; Carbó Tano, Molina, Maldonado, & Pedreira, 2009; Pedreira et al., 2004).

The experiment 1 included three pairs of CT-TR groups. During the training session, the TR groups received a strong training protocol (STP) of 15 CS-US_E presentations. In the treatment session, two pairs of CT-TR groups were exposed to the CS-US_E reminder and then were injected with CHX or VHC (US_E/CHX and US_E/VHC respectively). The last pair was exposed to the CS-US_M reminder and injected with CHX (US_M/CHX). All the animals were tested on Day 3 with 1 CS-US_E trial (Fig. 1A). On Day 2, the three pairs of groups showed memory retention [Fig. 1B left panel, ANOVA: $F_{5,187} = 2.55$; $p < 0.03$; Fisher LSD (CT vs. TR): US_M/CHX, $p < 0.05$ US_E/VHC, $p < 0.05$ and US_E/CHX, $p < 0.05$]. However during the testing session, only the US_M/CHX reminder, which included the temporal PE (the US was presented sooner than expected), showed memory impairment, and in spite of the drug treatment the pairs that received the reminder without temporal PE expressed memory retention [Fig. 1B right panel; ANOVA: $F_{5,187} = 1.83$; $p = 0.12$; Fisher LSD (CT vs. TR): US_M/CHX $p = 0.59$ US_E/VHC, $p < 0.05$ and US_E/CHX, $p < 0.05$].

The next series of experiments were performed to further explore our hypothesis that temporal PE between inter-stimuli interval triggers the reconsolidation process. We used a weak training protocol (WTP) and bicuculline (BIC) to facilitate memory reconsolidation (Carbó Tano et al., 2013; Fustiñana et al., 2013). As we mentioned before, we demonstrated that the absence of reinforcement during the reminder presentation triggers the reconsolidation process (Carbó Tano et al., 2009; Pedreira et al., 2004). Here, the comparison was performed between the reminder that included the temporal PE and as control, a reminder with no PE, to impair memory facilitation by the administration of BIC (Fustiñana et al., 2013). Thus, Experiment 2 included two pairs of CT-TR groups. During the training session the animals received a WTP. In the treatment session, one pair was exposed to the reminder with the temporal matching and the other pair was exposed to the reminder with temporal PE, both were injected with BIC (US_E/BIC, US_M/BIC). All the animals were tested on Day 3 (Fig. 2A). On Day 2 both pairs showed no memory retention [Fig. 2B left panel, ANOVA, $F_{3,113} = 1.15$; $p = 0.33$; Fisher LSD (CT vs TR): US_EBIC $p = 0.96$; US_M/BIC $p = 0.76$]; and only the US_M/BIC pair exhibited memory retention, while the US_E/BIC pair failed to display memory retention at testing session [Fig. 2B right panel, ANOVA, $F_{3,113} = 2.28$; $p = 0.08$; Fisher LSD (CT vs TR): US_E/BIC $p = 0.97$; US_M/BIC $p < 0.05$].

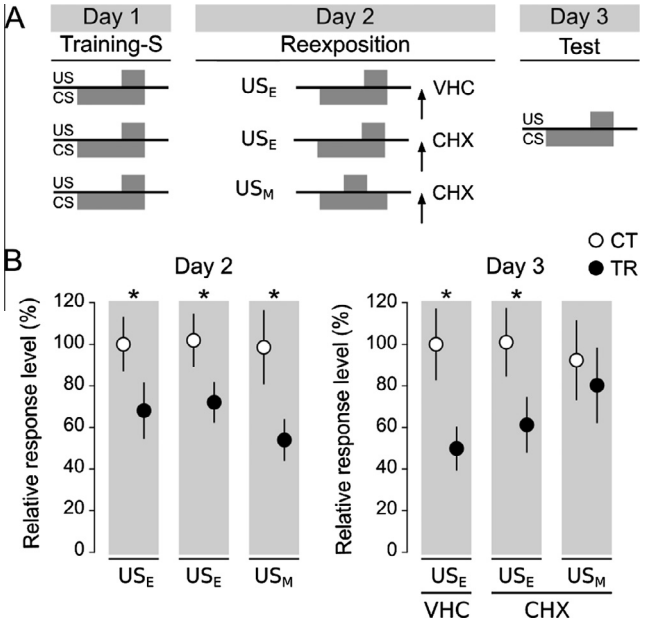

Fig. 1. Experiment 1. A. Experimental protocols. Day 1, strong training. Day 2, re-exposition session. US_E: the US was presented at the end of the CS; US_M: the US appeared at the middle of the CS presentation. Immediately after VHC or CHX were administrated. Arrow stands for the time of injection. Day 3, test session. B. Mean response to the US are represented in grey boxes. White circles stands for the control groups (CT) and black circles stands for trained groups (TR). Left panel response at Day 2. Right panel response level at Day 3. Data are expressed as mean response level ± S.E. normalized with respect to the CT group of the VHC pair. Planned comparisons (LSD): ∗ stands for $P < 0.05$ (TR < CT, memory retention).

To confirm such results we designed Experiment 3 whereas we compared the reminder with a temporal PE with another kind of mismatch, the reminder without reinforcement (Fustiñana et al., 2013). Experiment 3 included three pairs of CT-TR groups. During the training session, the TR groups received a WTP. In the treatment session, two pairs were exposed to the CS reminder (no-US) and then they were injected with BIC or VHC (no-US/BIC and no-US/VHC respectively). The last pair was exposed to the reminder with the temporal PE and treated with BIC (US_M/BIC). All the animals were tested on Day 3 (Fig. 3A). As we expected, after a WTP the pair CS-US_M/BIC failed to show memory retention on Day 2 (Fig. 3B left panel, $p = 0.76$). But as in Experiment 2, the same pair of groups exhibited memory retention on Day 3. In line with previous results, when the reminder was formed by the CS alone due to the facilitation effect of BIC on memory re-stabilization, the pair injected with the drug showed memory retention, while the other pair treated with VHC showed the expected absence of memory [Fig. 3B right panel; ANOVA, $F_{5,198} = 2.49$; $p < 0.05$; Fisher LSD (CT vs. TR): no-US/VHC, $p = 0.89$; no-US/BIC $p < 0.05$; US_M/BIC $p < 0.05$].

Finally, we analyzed if the effect of the temporal PE depends on the temporal relation between the stimuli in the training trial. First we explored memory retention obtained after a STP using a training-trial whereas the US was presented from 9th to 18th (Supplementary Fig. 1). We showed that this training trial structure did

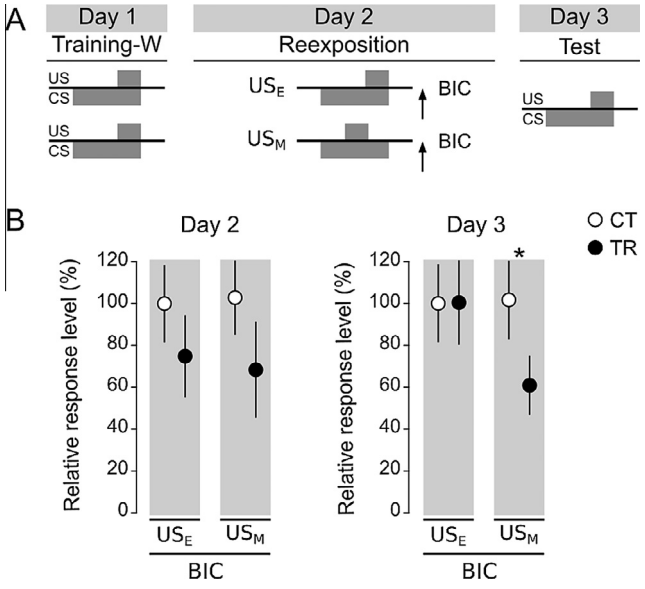

Fig. 2. Experiment 2. A. Experimental protocols. Day 1, weak training. Day 2, reexposition session. US_E: the US was presented at the end of the CS, US_M: the US appeared at the middle of the CS presentation. Immediately BIC was administrated. Day 3, test session. B. Mean relative response level to the US are represented in grey boxes. Left panel response at Day 2. Right panel response level at Day 3. Symbols as in Fig. 1.

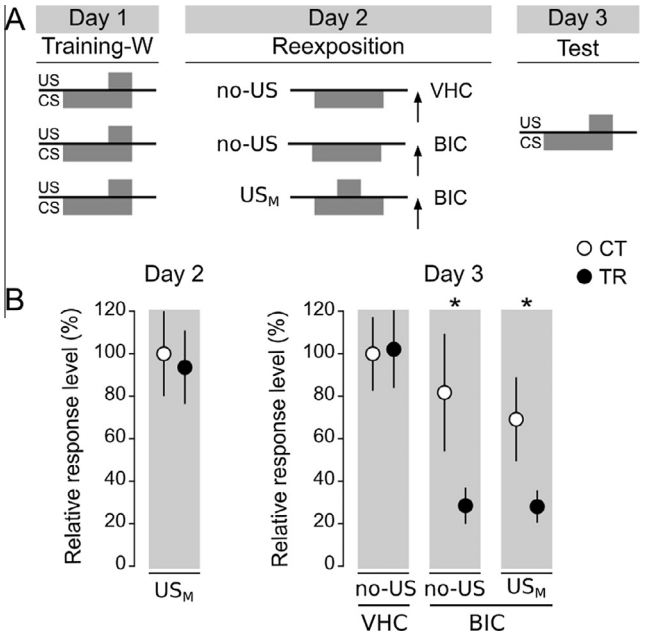

Fig. 3. Experiment 3. A. Experimental protocols. Day 1, weak training. Day 2, reexposition session. no-US: only the CS was presented; US_M: the US appeared at the middle of the CS presentation. Immediately after VHC or BIC were administrated. Day 3, test session. B. Mean relative response level to the US are represented in grey boxes. Left panel response at Day 2. Only reminders that included a US were analyzed. Right panel response level at Day 3. Symbols as in Fig. 1.

not affect memory retention. Then in Experiment 4 we included three pairs of CT-TR groups. During the training session, two TR groups received a WTP of 4 CS-US_E presentations and the other TR group received the same number of trials but with a CS-US_M structure. During the treatment session, both pairs of CS-US_E received a CS-US_M reminder. Then, one pair received an injection of BIC ($_EUS_M$/BIC) and the other pair received an injection of VHC ($_EUS_M$/VHC). The last pair trained with CS-US_M was exposed to the reminder with the temporal PE and treated with BIC ($_MUS_E$/BIC). All animals were tested on Day 3 (Fig. 4A). Due to the WTP the three pairs failed to display memory retention on Day 2 [Fig. 4B left panel, ANOVA, $F_{5,174} = 0.24$; $p = 0.94$; Fisher LSD (CT vs. TR): $_EUS_M$/VHC $p = 0.68$; $_EUS_M$/BIC $P = 0.76$; $_MUS_E$/BIC $p = 0.69$]. The results showed that in spite of the training trial structure, the temporal PE triggered the reconsolidation process given that BIC was capable to improve memory retention. As we expected, the pair that received VHC failed to express the associative memory at testing [Fig. 4B right panel, ANOVA, $F_{5,174} = 1.54$; $p = 0.18$; Fisher LSD (CT vs. TR): $_EUS_M$/VHC $p = 0.68$; $_EUS_M$/BIC $P < 0.05$; $_MUS_E$/BIC $p < 0.05$].

The next experiment was performed to be able to argue that the memory modification depends on the combination of the temporal PE condition and the pharmacological tool's used. We only included the treatment with VHC (crustacean solution or DMSO) to demonstrate that under these experimental conditions the retention was determined strictly by the training strength. Experiment 5 included three pairs of CT-TR groups. The TR group of one pair received a STP; one of the other TR groups received the WTP of 4 training trials with the US_E and the other with the US_M. On Day 2 each pair received a reminder which included a temporal PE in relation with the training history (US_M if the WTP included US_E and US_M in both WTP and STP when the training was performed with US_E). After the reminder presentation the pairs trained with the WTP were injected with DMSO and the remained pair which received the STP with crustacean solution. All the animals were tested on Day 3 with the trial structure used at training (Fig. 5 A). The results showed absence of memory retention on Day 2 or at testing session for the groups trained with WTP, and memory retention for the pair that received a STP on Day 2 and at testing session [Fig. 5B, ANOVA, $F_{5,\ 169} = 1.61$; $p = 0.15$: planned comparisons (CT vs. TR): STP-US_M/VHC $p < 0.05$; WTP-US_M/VHC $p = 0.92$; WTP-US_E/VHC $p = 0.68$].

As a whole, these experiments (1–5) support the role of the temporal PE as a central condition to initiate the reconsolidation process of an associative memory in an invertebrate model.

3.2. Temporal PE between inter-trial interval triggers the reconsolidation process

The comparison between the CS ITI during training of the original memory and extinction training shows that is possible to acquire the extinction memory leaving the reconsolidation process offstage when the ITI was very different from the ITI training (20 instead of 144 s) defined as a large PE (Carbó Tano et al., 2013). In the present experiment our proposal was that during the training session, crabs acquire not only the information of the relationship between the CS and the US, but also the data of the frequency of presentations of the CSs. We expected that the switch mechanism which guides memory to reconsolidation or extinction (Pedreira & Maldonado, 2003) might trigger the reconsolidation process instead of extinction memory, when a small discordance between the frequencies of presentations of the CS is detected (small PE). Thus, in our protocol if the interval between CS is higher during extinction training (>144 s), the labilization-reconsolidation mechanisms would be initiated. To test this hypothesis we performed Experiment 6. On Day 1, three pairs of CT-TR groups were trained with a STP. On Day 2 all the crabs went through extinction training. Two pairs received a standard extinction protocol (CS ITI = 20 s), and for the other pair, we modified the interval between the first and second CS re-exposure from 20 to 171 s. Finally, animals of one pair trained with 20 s and animals that received a 171 s in the first ITI of the extinction training were injected with CHX. The remained pair received a VHC injection. Animals were

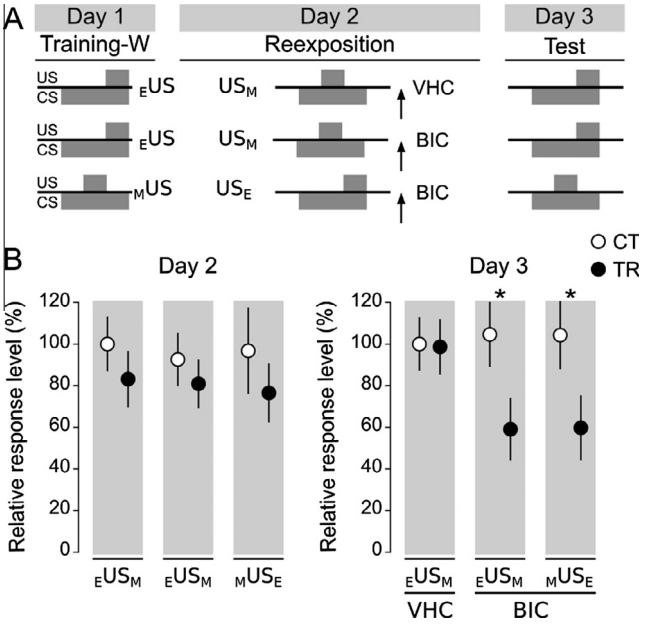

Fig. 4. Experiment 4. A. Experimental protocols. Day 1, weak training. $_{E}$US: during the 15 trails of training the US was presented concomitant with the end of the CS. $_{M}$US: the US was presented at the middle of the CS exposure. Day 2, re-exposition session. US_E: the US was presented at the end of the CS; US_M: the US appeared at the middle of the CS presentation. Immediately after VHC or BIC were administrated. Day 3, test session. Animals were tested using the same stimulus presentation scheme as in training. B. Mean relative response level to the US are represented in grey boxes. Left panel response at Day 2. Right panel response level at Day 3. Symbols as in Fig. 1.

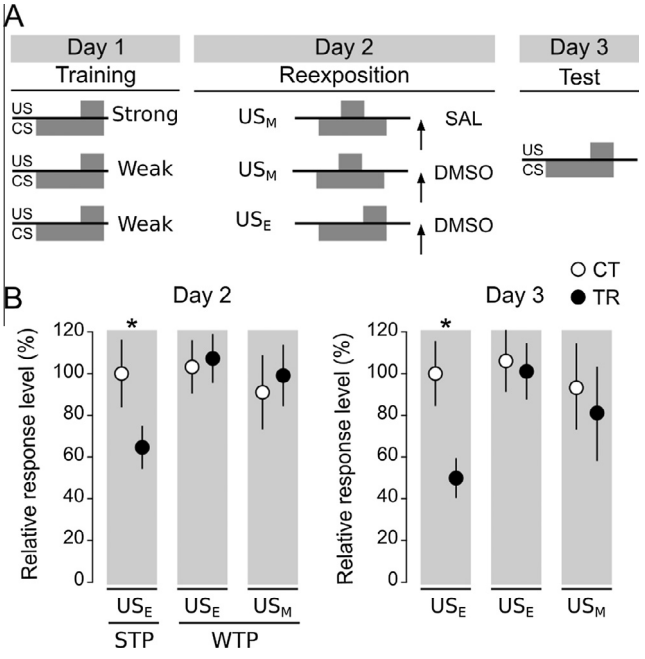

Fig. 5. Experiment 5 A. Experimental protocols. Day 1, strong or weak training. Day 2, re-exposition session. US_E: the US was presented at the end of the CS; US_M: the US appeared at the middle of the CS presentation. Immediately after SAL (crustacean saline solution) or DMSO were administrated. Arrow stands for the time of injection. Day 3, test session. B. Mean response to the US are represented in grey boxes. Left panel response at Day 2. Right panel response level at Day 3. Symbols as in Fig. 1.

tested on Day 3 in order to evaluate the extinction memory and then on Day 5 as part of the spontaneous recovery protocol (Fig. 6A). The results showed original memory retention for the pair injected with CHX in which the extinction CS ITI remained below than that of the original training (20 s) and no significant difference for the control pair (first ITI of 20 s injected with VHC). No significant differences were found for the pair with the interval of 171 s in the first ITI extinction training treated with CHX [Fig. 6B left panel, ANOVA, $F_{7,232} = 1.69$; $p = 0.11$: planned comparisons (CT vs. TR): 20 s/VHC $p < 0.81$ 20 s/CHX $p < 0.05$; 171 s/VHC $p < 0,05$; 171 s/CHX $p = 0.77$]. The spontaneous recovery evaluated on Day 5 showed the same profile of results for the pairs treated with CHX (no memory retention) and the recovery of the original memory for the pair which received the VHC, suggesting that the lack of significant differences in the groups with a first interval of 171 s CSs was due to an amnesiac effect on the original memory [Fig. 6B right panel, ANOVA, $F_{7,232} = 3.55$; $p < 0.05$: planned comparisons (CT vs. TR): 20 s/VHC $p < 0.05$ 20 s/CHX $p < 0.05$; 171 s/VHC $p < 0,05$; 171 s/CHX $p = 0.78$]. To confirm such suggestion we decided to perform a control experiment to show the effect of the large or small PE during the extinction training without drug treatment. Experiment 7 included two pairs of CT-TR groups were trained with a STP on Day 1. On Day 2, the crabs went through extinction training. One pair received a standard extinction protocol, and for the other pair, we modified again the interval between the first and second CS re-exposure from 20 to 171 s. Finally, animals received a VHC injection. Animals were tested on Day 3 (Fig. 7A). The results showed no significant differences between CT-TR pair after the typical extinction training, and memory retention for the pair with the small PE (171 s) [Fig. 6D left panel, ANOVA, $F_{3,128} = 3.18$ $p < 0.05$: planned comparisons (CT vs. TR): 20 s/VHC $p = 0.38$; 171 s/VHC $p < 0.05$]. The spontaneous recovery evaluated on Day 5 showed the recovery of the original memory for the pair trained with 20 s ITI, and the maintenance of memory retention for the other pair [Fig. 6D right panel, ANOVA, $F_{3,128} = 6.01$ $p < 0.05$: planned comparisons (CT vs. TR): 20 s/VHC $p < 0.05$; 171 s/VHC $p < 0.05$].

We concluded that the interval between CSs during extinction training is critical to decide the fate of the memory. Thus, surpassed the CS interval learned during training generated an incongruence enough to misestimated the first CS as part of the extinction training guiding the memory to reconsolidation.

3.3. Partially reinforced training generates a positive PE which always triggered the reconsolidation process

The goal of this series of experiments was to evaluate if a partially reinforced training (50% of reinforced training trials), could change the boundary condition of reconsolidation. Previous results showed that the reminder which triggers the reconsolidation process consisted in the presentation of the CS in absence of the US. On the other hand, the inclusion of the US, after a full reinforced training reaching an asymptotic level of learning, failed to trigger the process (Alberini, 2013). To this aim, first we demonstrated that a 50% partially reinforced training (8/16 training trials) produced a similar response level at the last training trail in comparison with a fully reinforced protocol, and it also induced the formation of a long term memory (Supplementary Fig. 2).The consolidation of this long term memory depended on protein synthesis (instead of 15 trials, Fustiñana et al., 2013; Supplementary Fig. 3). With this background, we performed the first experiment of this last series. Experiment 8 included two pairs of CT-TR groups. One group was trained with a STP of 100% and the other with 50% of reinforcement. Both pairs were exposed to the reminder with the US (no PE for STP 100% US_E and positive PE for STP 50% US_E) and after that, CHX was administered to all the animals. Memory retention was evaluated on Day 3 (Fig. 7A). Here, both pairs exhibited memory retention when the US of the reminder was presented on Day 2

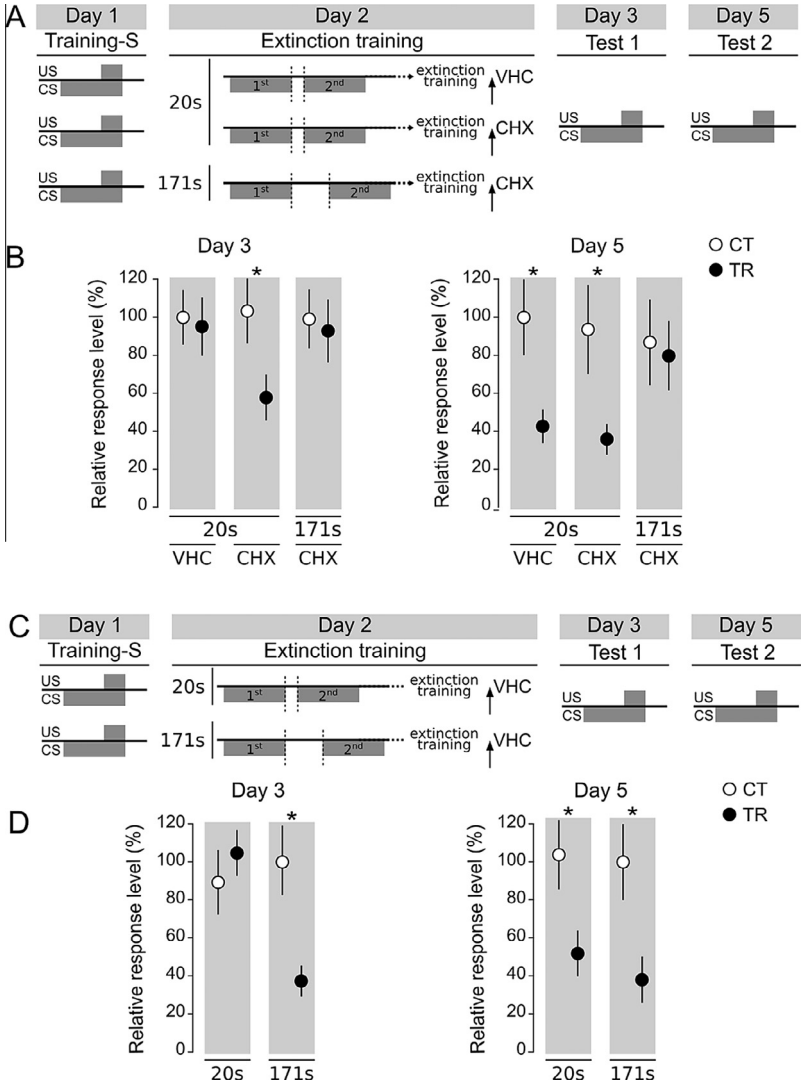

Fig. 6. Experiment 6. A. Experimental protocols. Day 1, strong training. The CS ITI presentation during training was of 144 s. Day 2, extinction training. 20 s: 20 s ITI between the first and the second presentation of the CS. From the second to the fifteenth CS presentation the ITI was 144 s. 171 s: 171 s ITI between the first and the second presentation of the CS. From the second to the fifteenth CS presentation the ITI was 144 s. Immediately after extinction training VHC or CHX was administrated. Day 3, test session. Day 5, spontaneous recovery test. B. Mean relative response level to the US are represented in grey boxes. Left panel response at Day 3. Right panel response level at Day 5. C. Experiment 7. Same as A. D. Same as B. Symbols as in Fig. 1.

[Fig. 7B left panel, ANOVA, $F_{3,132} = 6.49$; $p < 0.01$; Fisher LSD (CT vs. TR): STP 100% US_E $p < 0.05$; STP 50% US_E $p < 0.05$]. Again on Day 3, STP 100% pair which was exposed to a reminder with no PE expressed memory retention. Surprisingly, the STP 50% pair showed memory impairment reflecting that the memory was labilized by a reminder with a positive PE which included a US [Fig. 7B right panel, ANOVA, $F_{3,132} = 4.20$; $p < 0.05$; planned comparisons (CT vs. TR): STP 100% US_E $p < 0.05$; STP 50% US_E $p = 0.57$].

In the last experiment (Experiment 9), the STP 100% received a reinforced reminder (inclusion of a US) (100% US_E). The STP 50% pair received a reminder without reinforcement (negative PE) (50% no-US). All animals were injected with CHX immediately after the reminder presentation and tested on Day 3 (Fig. 8A). As expected, the US included in the reminder showed memory retention on Day 2 (Fig. 8B left panel $p < 0.01$). On Day 3, in spite of the drug treatment, the pair that received the reminder with the US (noPE) expressed memory retention; and the other pair that received the CHX after the negative PE (without US) showed memory impairment [Fig. 8B right panel, ANOVA, $F_{3,117} = 3.02$; $p = 0.03$; Fisher LSD (CT vs. TR): 100% US_E $p < 0.05$; 50% no-US $p = 0.52$].

Until now, we have demonstrated that a negative PE (absence of US) during treatment session with a full-reinforced training triggers the reconsolidation process. On the contrary, the inclusion of the US (no PE) results a boundary condition being the consolidated memory only retrieved. In this report, we showed that memory destabilization occurred not only in the absence of US-reinforcement (negative PE) but was also induced by a reinforced reminder (positive PE) when the training session involved a partial reinforcement schedule. In this sense, we demonstrated that the certainty of the original learning combining with the reminder features might open new scenarios to update the stored information.

4. Discussion

The interaction between the reactivation session and consolidated memory features determines whether memory retrieval could induce memory expression, labilization-reconsolidation and/or a new learning (Alberini, 2013; Alfei et al., 2015; Exton-McGuinness et al., 2015; Fernández, Bavassi, Forcato, & Pedreira, 2016; Piñeyro, Monti, Alfei, Bueno, & Urcelay, 2014). In this con-

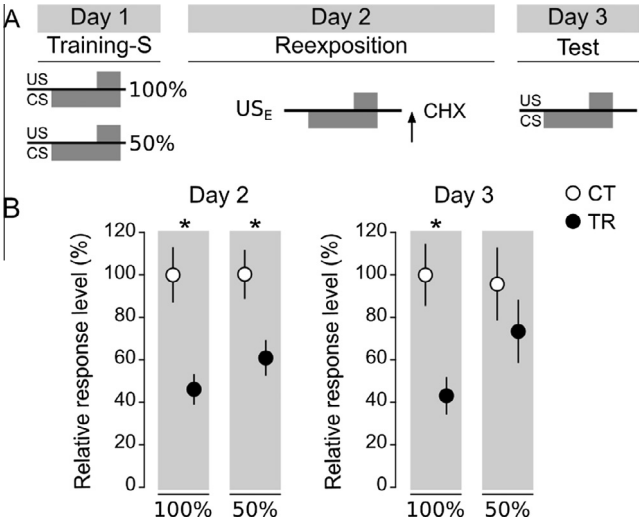

Fig. 7. Experiment 8. A. Experimental protocols. Day 1, strong training. As in experiment 6. Day 2, re-exposition session. US_E: the US was presented at the end of the CS. Day 3, test session. B. Mean relative response level to the US are represented in grey boxes. Left panel response at Day 2. Right panel response level at Day 3. Symbols as in Fig. 1.

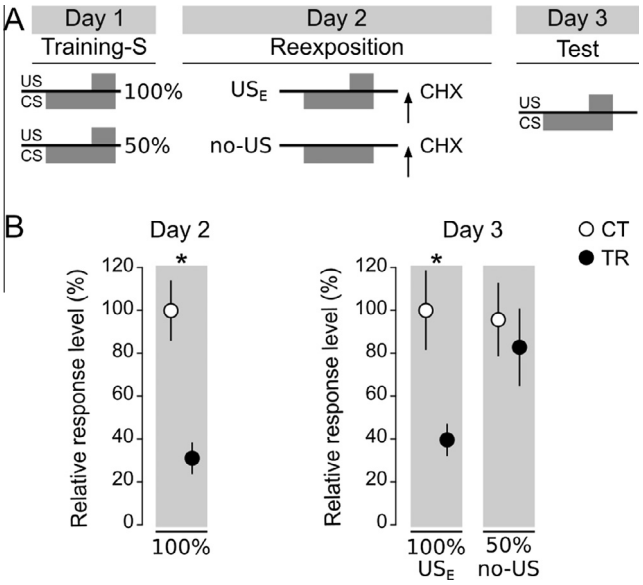

Fig. 8. Experiment 9. A. Experimental protocols. Day 1, strong training. 100%: the US was presented every one of the CS exposures. 50%: only half of the CS exposures were paired with the US. Day 2, re-exposition session. US_E: the US was presented at the end of the CS; no-US: only the CS was presented. Immediately after CHX were administrated. Day 3, test session. B. Mean relative response level to the US are represented in grey boxes. Left panel response at Day 2. Right panel response level at Day 3. Symbols as in Fig. 1.

text, PE is considered a mandatory boundary condition for memory acquisition and reconsolidation to occur (Exton-McGuinness et al., 2015; Fernández, Boccia, et al., 2016). The detection of a mismatch between past and actual events offers the opportunity to update the stored information. This relationship shows the tightly relation between the reconsolidation functions and PE role. As in other reports the current data are limited to a more correlative analysis. Thus, reconsolidation occurs when operationally there should be PE. In this sense, PE signals during memory acquisition in different brain areas were widely explored (Schultz, Dayan, & Montague, 1997; Schultz & Dickinson, 2000). Considering the role of PE and the similarity between new memories and reactivated ones (Spear, 1973, 1981) we could speculate the involvement of similar neurophysiological PE.

Previous work from different laboratories, reported individually different types of PE inducing the reconsolidation process (Alfei et al., 2015; Díaz-Mataix et al., 2013). Although, to the best of our knowledge, this constitutes the first report to systematically study in the same model and paradigm the role of PE in this memory phase. Here, we faced this challenge trying to demonstrate that the post-retrieval plasticity depends on the presence of PE during memory reactivation. To do so, two different series of experiments were designed to generate PE. First, the PE was induced by the violation of the temporal relationship between stimuli after a fully reinforcement schedule of training (Temporal PE). Secondly, we presented different types of reminders with positive or negative PE after a partial reinforcement schedule of training.

Learning an association between cues/events and their timing may be a tightly intertwined. Then, time is a critical element of the US expectation (Díaz-Mataix, Tallot, & Doyère, 2014; Díaz-Mataix et al., 2013; Gallistel & Balsam, 2014). In the first series of experiments, we changed the expected time for the US presentation, being this condition enough to trigger the reconsolidation process (Figs. 1 and 2). Varying the temporal relationship between CS and US generate a temporal PE, that in turn elicit an update of temporal expectancy rules. Here, we use pharmacological tools to impair (CHX) or facilitate (BIC) the re-stabilization of reactivated memory. This report is in line with the study of Díaz-Mataix et al. (2013). They demonstrated that when the rats freezing level reach its maximum during training, because the CS-US association is fully learned, the additional CS-US trial with the acquired temporal structure during reactivation is not sufficient to trigger reconsolidation. However, under the same training condition, the change in the timing between stimuli is enough to trigger the process. Similar results were found in other report where dopamine neurons from the ventral tegmental area in rats reflect reward prediction errors, changing with a delayed reward (Roesch, Calu, & Schoenbaum, 2007). Further, PE detection increases fMRI signals in the amygdala and hippocampus in humans (Metereau & Dreher, 2013) and also decreases the signals when the US is fully predicted (Dunsmoor, Bandettini, & Knight, 2008; Wood, Ver Hoef, & Knight, 2012).

In the experimental series using a partial reinforcement training schedule (50% reinforced trials), we show that both type of reminder types negative PE (CS presentation only) or positive PE (CS-US) triggers the reconsolidation process leaving the memory sensitive to amnesic treatments such as protein synthesis inhibitor like cycloheximide (Figs. 7 and 8).

The inclusion of a negative PE in the reminder is the most typical way to induce reconsolidation (Fernández, Boccia, et al., 2016). In this report, using a partially reinforced training we confirm our previous results using a fully reinforced one (Pedreira et al., 2004). With this training schedule the inclusion of the US (a fully reinforced reminder) implies no-PE is unable to trigger the process. Using a declarative memory paradigm in humans (Forcato, Argibay, Pedreira, & Maldonado, 2009), we also demonstrated the role of the negative PE in reconsolidation triggering and the reactivation failure when the PE is omitted. In this sense, Sevenster et al. (2013) using a fear conditioning in humans also showed that propranolol (β-adrenergic antagonist) only fails to impair memory reconsolidation when there is nothing to be learned during reactivation session (no PE). Smartly, in another study they find that when the shock electrodes, which delivered the US during training are not attached, the fear memory is not labilized (Sevenster, Beckers, & Kindt, 2012).

In comparison with the amount of reports that include negative PE in the reminder structure, there are few studies where a learning trial (positive PE) is used. This occurs when the conditioned

response does not reach the maximum level. In this case, a reinforced reminder (positive PE) is sufficient to trigger the reconsolidation process (Duvarci & Nader, 2004). Similar results were found in other type of memories (Eisenberg & Dudai, 2004; Milekic, Brown, Castellini, & Alberini, 2006). Here, using a similar design (partial reinforcement training schedule), we confirm the results obtained in humans by Sevenster et al. (2013) whereas the reconsolidation process is triggered with both types of PE.

Memory is guided to reconsolidation when incongruence between actual and past events is detected (Exton-McGuinness et al., 2015). Here, in spite of the repeated and prolonged presentations of CSs (extinction training) the incongruence provoked in the first CS ITI (Fig. 6) guided the memory to the reconsolidation process. This result also supported the notion that animals acquired the temporal relationship between the CS and the US, during training and the frequency of presentations of the CSs, in this case both frequencies are maintained fixed. Futures experiments may analyze if this sensitivity to the stimuli-timing is maintained with these frequencies presented in a variable manner. Thus, animals learn a model of the environment that is richer than simple associations between cues and aversive stimuli. In line with this assumption different reports propose that animals create a new memory for extinction because they have discovered a new "state (cause) of the world" different from the world represented in the original conditioning (Gershman & Niv, 2012). Gershman, Jones, Norman, Monfils, and Niv (2013) offered an adequate framework to analyze our results. They suggest that extinction training implies a persistent large PE (during the entire extinction training) justifying the formation of a new memory. However, if during extinction training the PE is reduced enough to prevent the formation of a new memory, the old fear memory is modified. Going back to our results, when the CSs frequency is lower than expected, the PE is computed as large and this new information required the formation of a new memory. However, when in the first ITI the frequency is larger but near to the timing expected, this represent a small PE and the original fear is modified trough the reconsolidation process. These results may also being interpreted under the trace dominance model (Eisenberg, Kobilo, Berman, & Dudai, 2003) .That is, the small PE is enough to subtract the first CS re-exposition from the extinction training; consequently the remained seven CS trials were insufficient to induce the extinction memory formation being the dominant trace the original memory.

Altogether, these results highlight the importance of the training history, which determine the different reactivation possibilities. Thereby, there is no universally-effective reactivation session to induce memory reconsolidation. The dynamic of this relationship depends on memory and reminder features. In this report, we demonstrated that a PE capable of inducing the reconsolidation process may be different from a simple omission of reinforcement (negative PE). Prediction error has several dimensions and could be generated by altering key elements such as time or reinforcement acquired during previous learning experience. All in all, the PE as a mandatory condition for reconsolidation process supports its functions as the main mechanism associated with memory updating. Even more, the results highlight the importance of the training history and different reactivation possibilities showing the tightly back and forth dependence between memory features and reminder components. In the case of the associative learning in crabs, they are capable of detect subtle differences between training and reactivation. Accordingly, it might reformulate the old one granting memory with extraordinary malleability in everyday life.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2016.10.016.

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