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The involvement of the GABAergic system in the formation and expression of the extinction memory in the crab *Neohelice granulata*

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Abstract

There is growing interest in the neurobiological mechanisms involved in the extinction of aversive memory. This cognitive process usually occurs after repeated or prolonged presentation of a conditioned stimulus that was previously associated with an unconditioned stimulus. If extinction is considered to be a new memory, the role of the γ-aminobutyric acid system (GABAergic system) during extinction memory consolidation should be similar to that described for the original trace. It is also accepted that negative modulation of the GABAergic system before testing can impair extinction memory expression. However, it seems possible to speculate that inhibitory mechanisms may be required in order to acquire a memory that is inhibitory in nature. Using a combination of behavioral protocols, such as weak and robust extinction training procedures, and pharmacological treatments, such as the systemic administration of $GABA_A$ agonist (muscimol) and antagonist (bicuculline), we investigated the role of the GABAergic system in the different phases of the extinction memory in the crab *Neohelice granulata*. We show that the stimulation of the GABAergic system impairs and its inactivation facilitates the extinction memory consolidation. Moreover, fine variations in the GABAergic tone affect its expression at testing. Finally, an active GABAergic system is necessary for the acquisition of the extinction memory. This detailed description may contribute to the understanding of the role of the GABAergic system in diverse aspects of the extinction memory.

Introduction

There is growing interest in the neurobiological mechanisms involved in the extinction of aversive memory. This cognitive process usually occurs after repeated or prolonged presentation of a conditioned stimulus (CS) that was previously associated with an unconditioned stimulus (US; Pávlov, 1927; Bouton, 2004; Rescorla, 2004; Davis & Myers, 2007). It is widely accepted that fear extinction involves a new association between the CS and the absence of the US rather than the erasure of the CS–US association. This view is supported by substantial behavioral evidence showing spontaneous recovery, renewal and reinstatement of a fear memory after extinction (Bouton, 2004; Rescorla, 2004; Hepp *et al.*, 2010). Following extinction learning, an inhibitory association seems to be formed that is behaviorally opposite to the original excitatory association (Myers & Davis, 2007; Radulovic & Tronson, 2010).

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter both in the CNS and in the peripheral nervous system (Erdö *et al.*, 1986). It is generally accepted that $GABA_A$ receptor agonists that are administered after extinction training disrupt memory retention, whereas antagonists facilitate extinction. Thus, the activation of GABAergic transmission impedes the consolidation of extinction memories. With respect to drug administration before extinction training, the pattern of findings suggests that increasing GABAergic transmission before extinction training disrupts the acquisition of extinction. Finally, the role of GABA in extinction retrieval and expression has not been clearly established (Berlau & McGaugh, 2006; Makkar *et al.*, 2010).

Our laboratory has been focused on the study of learning and memory in the grapsid crab *Neohelice granulata*. This 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 acquired memory is based on the association between the environmental features of the training place, the context and the VDS. Pharmacological experiments have revealed the mechanisms, neurotransmitter and modulators involved in the different memory phases (Maldonado, 2002). Specifically, we have demonstrated that an agonist of mammalian $GABA_A$ receptors impaired memory consolidation and reconsolidation, whereas an antagonist improved both processes (Carbó Tano *et al.*, 2009).

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Considering extinction as a new memory, the role of the GABAergic system during its consolidation would be similar to that described for the original trace. In spite of some differences, it is also accepted that negative modulation of the GABAergic system before testing can impair the expression of the extinction memory. Thus, it seems possible to speculate that inhibitory mechanisms may be required in order to acquire a memory that is inhibitory in nature (Konorski, 1948). The positive modulation of the GABAergic system should facilitate, whereas its inhibition by $GABA_A$ antagonists should impair extinction memory acquisition. Here, the goal is to characterize the role of the GABAergic system, in each phase of the extinction memory extending the particular features for each step associated with this memory. This detailed description might contribute to understand the selective involvement of the GABAergic system in different aspects of the extinction memory.

Materials and methods

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 Tuyú, 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 7.4–7.6) marine water (prepared from Cristalsea Marinemix salts, 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), the Argentinean guidelines on the ethical use of animals. This work is approved by our research institution.

Experimental device

The experimental device has been described in detail elsewhere (Maldonado, 2002; Pérez-Cuesta *et al.*, 2007; Hepp *et al.*, 2010; Fustiñana *et al.*, 2013). 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 (ITIs), and to monitor the experimental events. The experimental room contained 40 experimental devices that were separated from each other by partitions.

The training and other treatment sessions were preceded by 10 min of adaptation to the experimental device, which was illuminated from below throughout. A strong contextual Pavlovian conditioning (CPC) training session consisted of 15 trials. Each trial lasted 27 s with above illumination (CS), and the US was presented during the last 9 s. The ITI between US presentations was 171 s, and the ITI between CS presentations was 144 s. During the ITI between CS, 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 strong extinction training consisted of 15 CS presentations of 8 min each, with an ITI of 20 seg, presented in a way that ensures a total of 120 min CS re-exposition. The testing sessions included the evaluation of the anticipatory response when the CS associated with the aversive stimuli appeared for the first time, and the response to the US presented at the end of the testing session. In previous studies with this animal model it was shown that short-term retention of the response to the VDS presentation was attributed to a non-associative memory component. Such a process induces a general reduction in the response that is not specific to the stimulus or the context characteristics (Romano *et al.*, 1991). The associative component emerges as the time passes (Suárez *et al.*, 2010). In line with these results, we have recently demonstrated that with the CPC protocol the anticipatory response could be measured at least 4 h after CPC training (Fustiñana *et al.*, 2013). Thus, the associative memory could be evaluated only some hours after training. Consequently in this report on special occasions, such as the short-term test (3 min), only the US was evaluated. Further, based on the characteristic of the anticipatory response, that is a difference in the exploratory activity between CT and trained groups (TRs), this activity is dampened by the passage of time, specifically by the isolation between sessions. Thus, this response could not be evaluated in a long-term test at 96 h after extinction training. In summary, it has been established that the anticipatory response could not be measured at these time points (Fustiñana *et al.*, 2013). However, this limitation does not reduce the anticipatory response value because it might be considered as the expression of the Pavlovian conditioning. All in all, both responses to the US and CS clearly represent what the animal learned about each stimuli and the relation between them.

To summarize the experimental protocols used throughout this report see Table 1, describing the manipulations performed in each experiment.

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

TABLE 1. Summary of all the experiments performed in this work

Drug	Day 1	Day 2	Day 3	Day 5	Figures
		Extinction memory acquisition			
BIC_H	*$CS–US^w$ → Test				Fig. 1A
BIC_H	*$CS–US^w$	Test			Fig. 1B
BIC_H	$CS–US^s$	*CS^s	Test		Fig. 1C
BIC_H	$CS–US^s$	*CS^s → Test			Fig. 1D
BIC_H/CHX	$CS–US^s$	BIC_H *CS^s *CHX	Test		Fig. 1E
MUS_L	$CS–US^s$	*CS^w	Test	Test	Fig. 2A
MUS_L	$CS–US^s$	*CS^w → Test			Fig. 2B
MUS_L	$CS–US^s$	*CS^w	Test		Fig. 2C
MUS_L	$CS–US^s$	*CS^w	*Test		Fig. 2D
		Extinction memory consolidation			
BIC_H	$CS–US^s$	*CS^w	Test	Test	Fig. 3A
MUS_H	$CS–US^s$	*CS^s	Test		Fig. 3B
		Extinction memory expression			
BIC_L/BIC_H	$CS–US^w$	*Test			Fig. 4A
BIC_L	$CS–US^s$	CS^s	*Test		Fig. 4B

BIC, bicuculline, BIC_L: 1.34 μg/g, BIC_H: 2.68 μg/g; CHX, cycloheximide, 2.35 μg/g; CS, conditioned stimulus; CS^s, strong extinction training protocol; CS^w, weak extinction training protocol; $CS–US^s$, strong CPC training protocol; $CS–US^w$, weak CPC training protocol; MUS, muscimol, MUS_L: 0.6 μg/g, MUS_H: 1.8 μg/g; US, unconditioned stimulus.
Asterisk stands for the time point of drug injections.

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 released into the pericardial sac. The following drugs were used: (+)-bicuculline (BIC; Fluka Analytical), a competitive antagonist of $GABA_A$ receptor, was administered at a final dose of 2.69 μg/g (BIC_H) and 1.34 μg/g (BIC_L); muscimol (MUS; Sigma Aldrich), an agonist of $GABA_A$ receptor, was administered at a final dose of 1.8 μg/g (MUS_H) and 0.6 μg/g (MUS_L); cycloheximide (CHX; Sigma Aldrich), which is a protein synthesis inhibitor, was administered at a final dose of 2.35 μg/g. Doses are express as μg of drug per gram weight of crab.

Data analysis

From a functional perspective, Pavlovian conditioning involves learning about CSs that have a pre-existing relation to an US. Because the US is the more biologically relevant stimulus, the most important product of learning involves changes not only in how the organism responds to the CS (anticipatory response), but also in how it modifies the response to the US (Domjan, 2005). In nature, *Neohelice* 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 *et al.*, 1993, 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 learning acquired during training was considered to have occurred when a significantly lower level of response both for the anticipatory and escape response (to the training context, CS; and the VDS, US, respectively) 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 experiment, a significant difference was predicted at testing between the CT and TR groups for each measured response. Therefore, throughout the current paper, the results for the anticipatory response and the escape response were analysed using *a priori* planned comparisons via a weighted means ANOVA with α (per comparison error rate) = 0.05, according to the standard method (Howell, 2009). 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), 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 (Rosenthal & Rosnow, 1985; Howell, 2009). 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 analysed using R: A Language and Environment for Statistical Computing (R: R Core Team, 2012) and Rstudio (2012).

Results

The role of the GABAergic system in extinction memory acquisition

Within the theoretical framework of treating extinction as a form of new learning, it would be reasonable to predict that drugs that facilitate GABA transmission should also block the acquisition of extinction memories when such drugs are administered prior to the extinction training (Myers & Davis, 2007). Here, and in contrast with the current generally accepted idea, we offered an alternative view of such a process. In fact, we suggest that during acquisition, the role of the GABA system may be quite different. Indeed, we have speculated that the GABAergic system plays an opposite role, given the inhibitory nature of the new learning (Pávlov, 1927; Konorski, 1948; Rescorla, 2004; Davis & Myers, 2007). To test this hypothesis, a series of experiments was conducted using the $GABA_A$ antagonist BIC to block the endogenous GABA tone elicited during the acquisition of the extinction memory.

But firstly, it was necessary to explore the effect of BIC on the acquisition of the original CPC memory for the sake of comparison with its effect on the extinction memory. The first experiment included two pairs of CT–TR groups. Each pair received an injection of VHC or BIC_H and, immediately after the treatment, both pairs underwent a weak CPC training. The CPC memory was evaluated shortly after the training (10 min; Fig. 1A, upper panel; Table 1). Previous results demonstrated that only the response to the US could be evaluated under this condition because the anticipatory response emerged at least 4 h after CPC training (Fustiñana *et al.*, 2013; and see Materials and methods). The results showed that only the CT–TR pair that received the BIC_H before the weak training showed CPC memory retention at the short-term testing, while the VHC groups showed the expected absence of CPC retention (Fig. 1A, lower panel; ANOVA US TEST: $F_{3,116} = 2.47$, $P = 0.06$; CT VHC vs. TR VHC $P = 0.69$; CT BIC_H vs. TR BIC_H $P < 0.05$). In the next experiment, using a similar experimental design, we analysed the effect of the same dose. In this case, the testing session was performed 24 h

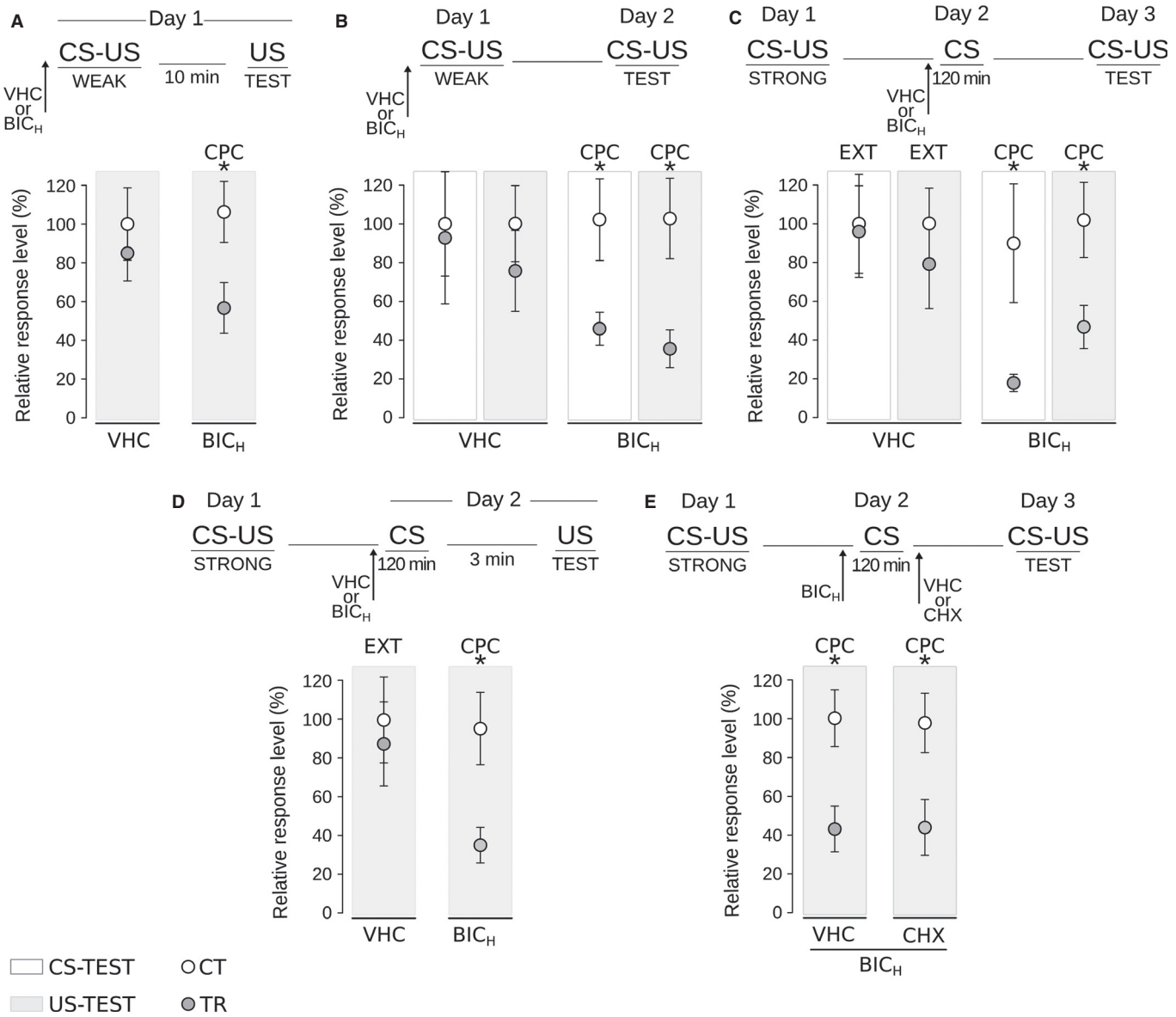

FIG. 1. (A) Upper panel: experimental protocol. Day 1, weak training. Immediately before vehicle (VHC) or drug (BIC_H, 2.68 μg/g) were administrated. Ten minutes after re-exposition, one test trial was presented. Only the response to the US was analysed. Lower panel: test session. (B) Upper panel: experimental protocol. Day 1, weak training. Immediately before VHC or BIC_H were administrated. The arrow stands for the time of injection. Day 2, test session. Both responses to the CS and the US were analysed. Lower panel: test session. (C) Upper panel: experimental protocol. Day 1, strong training. Day 2, 120 min re-exposition to the CS. Immediately before re-exposition VHC or BIC_H were administrated. Day 3, test session. Both responses to the CS and the US were analysed. Lower panel: test session. (D) Upper panel: experimental protocol. Day 1, strong training. Day 2, 120 min re-exposition to the CS. Immediately before re-exposition VHC or BIC_H were administrated. Three minutes after re-exposition one test trial was presented. Only the response to the US was analysed. Lower panel: test session. (E) Upper panel: experimental protocol. Day 1, strong training. Day 2, 120 min re-exposition to the CS. Immediately after re-exposition BIC_H was administrated, and immediately before VHC or cycloheximide (CHX) were administrated. Day 3, test session. Both responses to the CS and the US were analysed. Lower panel: test session. The mean anticipatory responses to the CS are represented in white boxes, and mean responses to the US presentation in gray boxes. At the top of the boxes, EXT, extinction memory retention; CPC, contextual Pavlovian conditioning memory retention. White circles = control groups (CT); black circles = trained groups (TR). Data are expressed as mean response level ± SE normalized with respect to the CT group of the VHC pair. Planned comparisons (LSD): $*P < 0.05$ (TR < CT, memory retention).

after the weak training (Fig. 1B, upper panel). A facilitating effect of BIC_H was demonstrated, as the BIC_H pair showed an anticipatory response and US memory retention at the long-term testing compared with the pair that received VHC before the weak training (Fig. 1B, lower panel; ANOVA CS TEST: $F_{3,116} = 1.63$, $P < 0.05$; CT VHC vs. TR VHC $P = 0.87$; CT BIC_H vs. TR BIC_H $P < 0.05$; ANOVA US TEST: $F_{3,116} = 2.11$, $P = 0.1$; CT VHC vs. TR VHC $P = 0.633$; CT BIC_H vs. TR BIC_H $P < 0.05$). It is important to note that the anticipatory response was expressed as a diminution of the exploratory activity and a reduction of the escape response level when the animals were confronted with the US. Collectively, these results demonstrate that the attenuation of the GABAergic tone before a weak training procedure facilitates CPC acquisition.

Next, we evaluated the effect of BIC on extinction memory acquisition. Based on the working hypothesis described above, the next experiment combined a strong extinction training (120 min of

total re-exposition; 15 trials of 8 min each of the above light) and BIC_H administration prior to the training (Fig. 1C, upper panel; Table 1). Two pairs of groups were trained with a strong CPC training on Day 1. After 24 h, one pair received an injection of VHC, while the other pair received BIC_H, immediately after both pairs received a robust extinction training. At the evaluation on Day 3, the results revealed that the VHC pair showed the presence of an extinction memory, but the pair treated with BIC_H displayed CPC memory retention (Fig. 1C, lower panel; ANOVA CS TEST: $F_{3,116} = 1.55$, $P = 0.2$; CT VHC vs. TR VHC $P = 0.87$; CT BIC_H vs. TR BIC_H $P < 0.05$; ANOVA US TEST: $F_{3,116} = 5.49$, $P < 0.05$; CT VHC vs. TR VHC $P = 0.18$; CT BIC_H vs. TR BIC_H $P < 0.05$).

Because the test was performed at the end of the acquisition and the consolidation phase, a first approach to distinguish which of these processes is affected, it was necessary to evaluate the effect of the treatment using short-term testing. Therefore, we designed a similar experiment, but with the test session performed immediately after the extinction training. In this case, only the US presentation was evaluated 3 min after the extinction training (Fig. 1D, upper panel; Table 1). The same pattern of results was obtained for the US when the test was conducted soon after the extinction training (Fig. 1D, lower panel). That is, the VHC groups show extinction memory retention, while the BIC_H pair showed impaired extinction memory acquisition; consequently, the CPC memory appeared at testing (ANOVA US TEST: $F_{3,116} = 3.06$, $P = 0.1$; CT VHC vs. TR VHC $P = 0.55$; CT BIC_H vs. TR BIC_H $P < 0.05$).

In summary, we concluded that the attenuation of the GABAergic tone by BIC played an opposite role on memory acquisition depending on whether the original CPC memory or the extinction memory was affected. The $GABA_A$ antagonist facilitated the acquisition of the CPC memory but might impair the acquisition of the extinction memory.

Previous research has demonstrated that re-exposure to the training context can trigger reconsolidation or extinction depending on the reminder duration (short and long re-exposition to the CS, respectively; Pedreira & Maldonado, 2003). Further, both processes can occur in parallel without competition between them when a short plus a long re-exposition to the CS was presented (Pérez-Cuesta & Maldonado, 2009). Here, with the CPC protocol, we used multiple presentations of the CS, which could simultaneously trigger both processes. The next experiment was designed to evaluate whether this protocol might induce or not both processes simultaneously.

To evaluate this hypothesis, we combined two treatments. First, we administered BIC_H prior to the extinction training to impair extinction memory acquisition. Second, we injected a protein synthesis inhibitor, CHX, which in turn could impair the CPC reconsolidation if this process was triggered under this experimental condition of repeated short re-expositions to the CS. Two pairs of groups were trained with a strong CPC training on Day 1. After 24 h, both pairs received an injection of BIC_H before the 120 min re-exposure to the training context (Fig. 1E, upper panel; Table 1) and another injection at the end of this session. One pair received an injection of VHC (BIC_H–VHC groups), and the other pair received an injection of CHX (BIC_H–CHX groups). All the animals were evaluated on Day 3. The evaluation revealed that the BIC_H–VHC and BIC_H–CHX pairs showed CPC memory retention (Fig. 1E, lower panel; ANOVA US TEST: $F_{3,116} = 5.01$, $P < 0.05$; CT BIC_H–VHC vs. TR BIC_H–VHC $P < 0.05$; CT BIC_H–CHX vs. TR BIC_H–CHX $P < 0.05$). The absence of effect of an amnesic agent such as CHX supports the view that the labilization-reconsolidation is not triggered after repeated re-exposures to the CS, suggesting that the CPC memory is not reactivated and remains stable and, consequently, impervious to the amnesic treatment.

To further confirm the role of the GABAergic system in the extinction memory acquisition, a series of experiments was designed to evaluate the effects of the $GABA_A$ agonist MUS on this phase. Previous research showed that a dose of about 0.8 μg/g had no effect on context signal memory (CSM) consolidation (Carbó Tano *et al.*, 2009). In this series of experiments, we used a dose of 0.6 μg/g (low dose of muscimol, MUS_L). The first experiment included three pairs of groups; each pair received a strong training protocol for the CPC on Day 1. On Day 2, after the injection of VHC for one pair and MUS_L for the other pair, these two pairs underwent a weak extinction training procedure (15 trials of 6 min each of the above light), with a total re-exposition time of 90 min; this amount of time is less than the minimal time necessary to induce extinction memory formation (Hepp *et al.*, 2010; Fustiñana *et al.*, 2013). The remaining pair underwent a reactivation session of 27 s after the MUS_L administration. This last pair was included to evaluate the possible effect of this MUS_L dose on CPC memory reconsolidation. The three groups were evaluated on Day 3 (Fig. 2A, upper panel). As expected, the VHC-90 and MUS_L-27 pairs showed CPC memory retention for both responses, ruling out a possible amnesic effect of MUS_L on the reactivated CPC memory (ANOVA CS TEST: $F_{5,174} = 3.33$, $P < 0.05$; CT VHC-90 vs. TR VHC-90 $P < 0.05$; CT MUS_L-27 vs. TR MUS_L-27 $P < 0.05$; ANOVA US TEST: $F_{5,174} = 3.75$, $P < 0.05$; CT VHC-90 vs. TR VHC-90 $P < 0.05$; CT MUS_L-27 vs. TR MUS_L-27 $P < 0.05$). In contrast, the MUS_L-90 min pair showed an absence of CPC retention, which we interpreted to be extinction memory retention according to the dose selected (Fig. 2A, lower panel; ANOVA CS TEST, CT MUS_L-90 vs. TR MUS_L-90 $P = 0.417$; ANOVA US TEST, CT MUS_L-90 vs. TR MUS_L-90 $P = 0.36$). In spite of the absence of effect on the MUS_L-27 when the CPC memory was reactivated (Fustiñana *et al.*, 2013), another test was performed. Thus, to further confirm if this result is due to an impairment of the original memory or due to the development of an extinction process, as both have a similar behavioral outcome, we use an extinction recovery protocol (Rescorla, 2004; Hepp *et al.*, 2010). After these protocols, the original memory re-emerges, and the extinction memory is no longer expressed (Myers & Davis, 2007). In the present study, we choose to use a spontaneous recovery protocol, which requires an additional testing trial on Day 5 (spontaneous recovery; Hepp *et al.*, 2010), to determine whether the results obtained on Day 3 represented the retention of the extinction memory (Fig. 2A upper panel; Table 1). We predicted the reappearance of the original memory and the absence of the extinction memory as a consequence of the passage of time. The results confirmed that the extinction memory was acquired (Day 3) and that it vanished as time passed on Day 5, while the VHC pair maintained CPC retention on Day 5 (Fig. 2A, lower panel; ANOVA US TEST: $F_{3,116} = 4.19$, $P < 0.05$; CT VHC-1.30 vs. TR VHC-90 $P < 0.05$; CT MUS_L-90 vs. TR MUS_L-90 $P < 0.05$).

Finally, to determine the acute effects of the treatment, we evaluated the effect of the drug in a short-term test. The experiment included two pairs of groups. The study procedure for the pairs was the same for Day 1 and Day 2, but the test was performed 3 min after extinction training (Fig. 2B, upper panel; Table 1). Here again, the facilitating effect was revealed to result from the combination of a weak extinction training and the MUS_L injection, suggesting that this acute drug effect might be due to the intervention on the acquisition of the extinction memory (Fig. 2B, lower panel; ANOVA CS TEST: $F_{3,116} = 1.96$, $P = 0.12$; CT VHC-90 vs. TR VHC-90 $P < 0.05$; CT MUS_L-90 vs. TR MUS_L-90 $P = 0.27$).

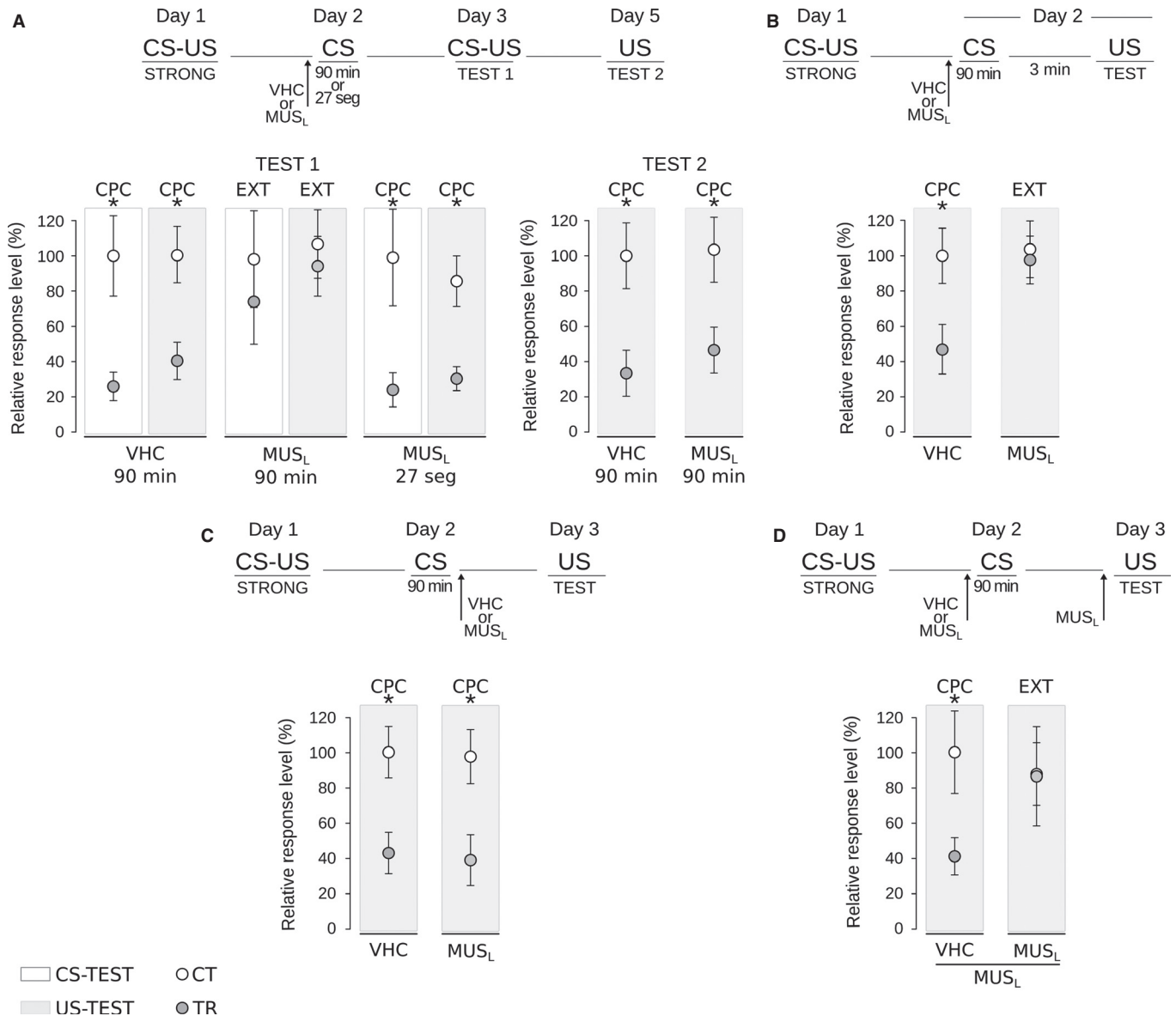

FIG. 2. (A) Upper panel: experimental protocol. Day 1, strong training. Day 2, 90 min or 27 seg re-exposition to the CS. Immediately before re-exposition VHC or MUS_L (0.6 μg/g) was administrated. Day 3, first test session. Both responses to the CS and the US were analysed. Day 5, second test session. Only the response to the US was analysed. Lower panel: test sessions. (B) Upper panel: experimental protocol. Day 1, strong training. Day 2, 90 min re-exposition to the CS. Immediately before re-exposition VHC or MUS_L was administrated. Three minutes after re-exposition one test trial was presented. Only the response to the US was analysed. Lower panel: test session. (C) Upper panel: experimental protocol. Day 1, strong training. Day 2, 90 min re-exposition to the CS. Immediately after re-exposition VHC or MUS_L was administrated. Day 3, test session. Only the response to the US was analysed. (D) Upper panel: experimental protocol. Day 1, strong training. Day 2, 90 min re-exposition to the CS. Immediately after re-exposition VHC or MUS_L was administrated. Day 3, test session. Immediately before the test, MUS_L was administrated. Only the response to the US was analysed. In all experiments data are expressed as mean response level ± SE normalized with respect to the CT group of the VHC-90 min pair. Symbols as in Fig. 1.

At this point, two different experiments were performed to sustain our vision about the role of the GABAergic system on the acquisition of the extinction memory. Therefore to demonstrate that MUS_L was necessary during the acquisition of extinction memory and not after the extinction training, we evaluated the effect of the drug administered immediately after weak extinction training procedure (Fig. 2C upper panel; Table 1). The experiment included two pairs of groups; each pair received a strong training protocol for the CPC on Day 1. Both pairs underwent a weak extinction training procedure 24 h before the injection of VHC for one pair and MUS_L for the other pair. The animals were tested on Day 3. The results revealed that the VHC-90 and MUS_L-90 pairs showed CPC memory retention for both responses, ruling out a possible facilitating effect of this dose on the extinction memory consolidation (Fig. 2C, lower panel; ANOVA US TEST: $F_{3,116} = 4.71$, $P < 0.05$; CT VHC-90 vs. TR VHC-90 $P < 0.05$; CT MUS_L-90 vs. TR MUS_L-90 $P < 0.05$). The absence of effect in contrast with that obtained when this dose was injected previous to a weak extinction training strongly suggests that a higher GABAergic tone facilitates the acquisition of this special type of memory.

Finally, a well-described effect in the literature is the state dependency. That is, the acquisition that occurs under the effects

of a drug (generally administered pre-training) may not transfer to the undrugged condition. This view is interpreted as a failure to retrieve learned information in different internal states (Estes, 1973; Spear, 1973; Spear & Gordon, 1981). In spite of the fact that state-dependent retention has been widely documented for the original learning, less research has specifically addressed this issue of the extinction memory (Bouton *et al.*, 1990). The last experiment was designed to reveal if the effect observed with the MUS_L depended or not on the similar internal state induced by the drug. Two pairs of groups received a strong training protocol for the CPC on Day 1. Both pairs underwent a weak extinction training procedure 24 h after the injection of VHC for one pair and MUS_L for the other pair. On Day 3, 10 min before testing, both pairs of groups received an injection of MUS (Fig. 2D, upper panel; Table 1). The VHC–MUS_L and MUS_L–MUS_L groups showed the same profile of results that was obtained when the injection was performed only before the extinction training. The VHC–MUS_L-90 pair showed CPC memory retention for the US response; on the contrary, the MUS_L–MUS_L-90 pair showed an absence of CPC retention, which we interpreted to be extinction memory retention according to the dose selected (Fig 2D, lower panel; ANOVA US TEST: $F_{3,116} = 2.2$, $P = 0.09$; CT MUS_L–VHC vs. TR MUS_L–VHC $P < 0.05$; CT MUS_L–MUS_L vs. TR MUS_L–MUS_L $P = 0.28$). Two main conclusions emerge from these results. First, MUS_L injected immediately before the testing session does not affect the expression of the CPC memory; second, the congruence in the internal state (both under the drug effect) does not modify the expression of the extinction memory, acquired when the weak training is combined with MUS_L.

Overall, these results reveal that during extinction memory acquisition, GABA plays a different role from that demonstrated in other models. Based on these results, it appears to be necessary to maintain an active GABAergic tone during the acquisition of the extinction memory.

The involvement of the GABAergic system in extinction memory consolidation

Considering extinction as a new memory (Myers & Davis, 2007), the consolidation of the extinction memory would engage the same neurotransmitters as the consolidation of the original memory, possibly with similar functions. As a result, $GABA_A$ antagonists should facilitate extinction memory consolidation, whereas $GABA_A$ agonists should impair extinction memory consolidation (Makkar *et al.*, 2010).

In a previous report, we have demonstrated that both memory consolidation and reconsolidation are dependent on the GABAergic system (Carbó Tano *et al.*, 2009). In line with this view, the systemic administration of MUS (1.8 μg/g) impaired memory consolidation and reconsolidation, whereas BIC administration (2.68 μg/g) improved both processes. Using the CPC protocol, we have also demonstrated that 2.68 μg/g of BIC administered after a weak training enhanced CPC consolidation (Fustiñana *et al.*, 2013).

Accordingly, we analysed the role of the GABAergic system during the consolidation of extinction memory. We performed an experiment in which we reduced GABAergic activity after extinction training by blocking $GABA_A$ sites with BIC. The experiment included three pairs of CT–TR groups. On Day 1, all the groups received a strong CPC. After the weak extinction training, one pair received an injection of VHC (VHC-90 groups), and the other pair was injected with 2.68 μg/g of BIC (BIC_H-90 groups).

The other CT–TR pair had the same treatment during Day 1 but, on Day 2, they received a strong extinction training and a post-training injection of vehicle (VHC-120 groups). Twenty-four hours later, the testing (Test 1) included the evaluation of both the anticipatory response when the CS associated with the aversive stimulus appeared for the first time and the response to the US at the end of the testing session on Day 3 (Fig. 3A, upper panel; Table 1). The results from Test 1 (Fig. 3A, lower panel) demonstrated the presence of an extinction memory when VHC-injected animals received the standard

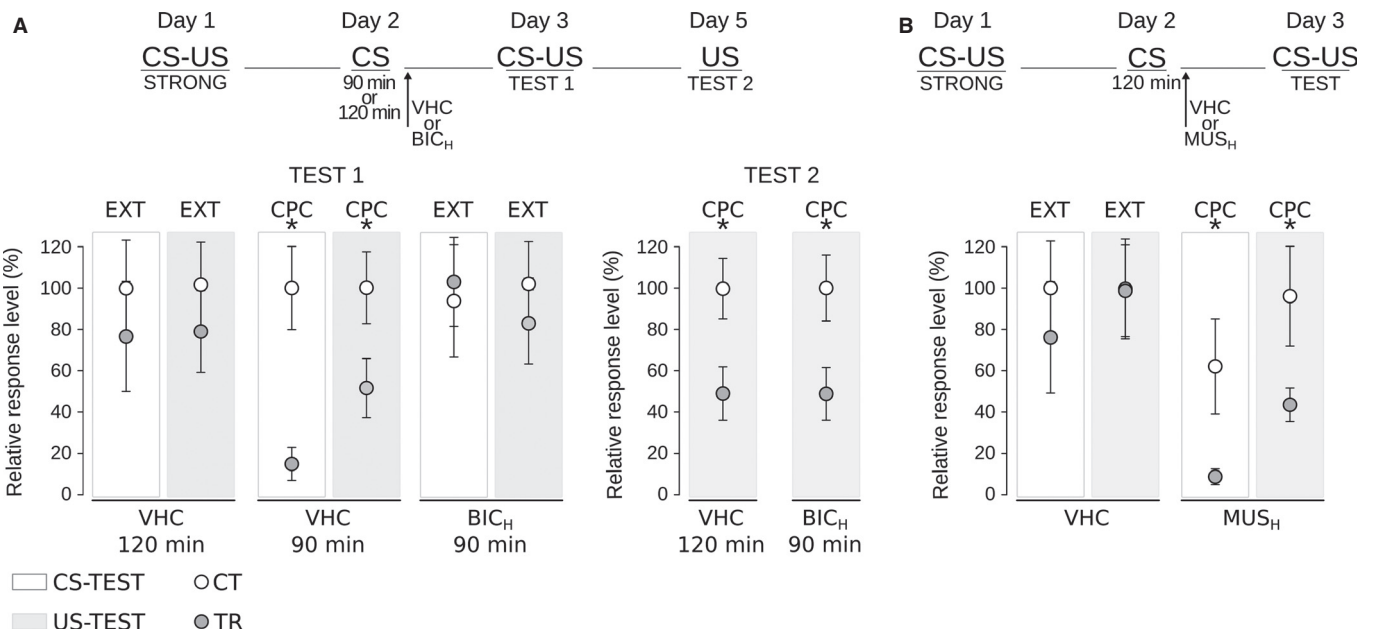

FIG. 3. (A) Upper panel: experimental protocol. Day 1, strong training. Day 2, 90 min or 120 min re-exposition to the CS. Immediately after re-exposition VHC or BIC_H was administrated. Day 3, first test session. Both responses to the CS and the US were analysed. Day 5, second test session. Only the response to the US was analysed. Lower panel: test sessions. (B) Upper panel: experimental protocol. Day 1, strong training. Day 2, 120 min re-exposition to the CS. Immediately after re-exposition VHC or MUS_H (1.8 μg/g) was administrated. Day 3, test session. Both responses to the CS and the US were analysed. Lower panel: test session. Symbols as in Fig. 1.

extinction training (ANOVA CS TEST: $F_{5,174} = 3.33$, $P < 0.05$; CT VHC-120 vs. TR VHC-2 $P = 0.73$; ANOVA US TEST: $F_{5,174} = 3.75$, $P < 0.05$; CT VHC-120 vs. TR VHC-2 $P = 0.16$). In contrast, and as previously reported (Hepp *et al.*, 2010), 90 min of CS re-exposure was not sufficient to induce extinction memory formation (CS TEST: CT VHC-90 vs. TR VHC-90 $P < 0.05$; US TEST: CT VHC-90 vs. TR VHC-90 $P < 0.05$). However, extinction memory formation was evident when the antagonist BIC_H was administered after the weak extinction training (BIC_H-90). These findings suggest that the consolidation of the extinction memory is facilitated after $GABA_A$ receptor blockade (CS TEST: CT BIC_H-90 vs. TR BIC_H-90 $P = 0.965$; US TEST: CT BIC_H-90 vs. TR BIC_H-90 $P = 0.413$).

To determine if this result is due to an impairment of the original memory or due to the development of an extinction process, as both have a similar behavioral outcome, we use a recovery protocol (Rescorla, 2004; Hepp *et al.*, 2010). To evaluate if the original memory re-emerged, and the extinction memory was no longer expressed, in the present experiment we used a spontaneous recovery protocol, which required an additional testing trial on Day 5 (Hepp *et al.*, 2010; Fig. 3A, upper panel, Test 2). It has been established that long-term testing (96 h after training) is not appropriate for measuring the anticipatory response (see Materials and methods), as only the response to the US is evaluated in this testing paradigm. The response to the US on Day 5 revealed that the original memory was expressed despite the prior formation of the extinction memory (Fig. 3A, lower panel; ANOVA US TEST: $F_{5,174} = 4.19$, $P < 0.05$; CT VHC-120 vs. TR VHC-120 $P < 0.05$; CT BIC_H-90 vs. TR BIC_H-90 $P < 0.05$).

Considering all the experiments performed until now using BIC_H, we obtained opposite effects (see Table 1; Figs 1C and D, and 3A). These effects depend on the type of extinction training, and the injection time point. Specifically, the facilitating effect on memory consolidation may support the previous suggestion that BIC_H impairs extinction memory acquisition, a role also supported by the acute observed effect.

To further confirm the role of the GABAergic system in the extinction consolidation process, the next experiment evaluated the effect of MUS on this particular memory phase. A 3-day experiment (Fig. 3B, upper panel; Table 1) included two pairs of CT–TR groups. On Day 1, all the groups received a strong CPC training. A strong extinction training was performed 24 h later. Immediately afterward, a CT–TR pair was injected with VHC, and the other pair was injected with MUS (1.8 μg/g, MUS_H groups). At testing, the results showed the presence of an extinction memory for the CS as well for the US only in the VHC group, and the results also showed the absence of an extinction memory and the presence of the CPC memory for the MUS_H pair (Fig. 3B, lower panel; ANOVA CS TEST: $F_{3,116} = 3.52$, $P < 0.05$; CT VHC-120 vs. TR VHC-120 $P = 0.43$; CT MUS_H-120 vs. TR MUS_H-120 $P < 0.05$; ANOVA US TEST: $F_{3,116} = 3.39$, $P < 0.05$; CT VHC-120 vs. TR VHC-120 $P = 0.84$; CT MUS_H-120 vs. TR MUS_H-120 $P < 0.05$). These findings confirmed that the 120-min session effectively induced extinction, and suggest that stimulating $GABA_A$ sites after extinction training impairs the consolidation of the extinction memory.

Collectively, these results support the view that the consolidation of the extinction memory involves the GABAergic system in the same way that it is involved in the consolidation of the original memory.

The involvement of the GABAergic system in extinction memory retrieval

From experiments using $GABA_A$ receptor agonists and paradigms of fear memories, there is some evidence that GABA can actually facilitate extinction memory retrieval (Makkar *et al.*, 2010). In this study, we analysed the role of the GABAergic system in extinction memory retrieval using the antagonist BIC. The use of an antagonist ensures that these receptors play a physiological role in this memory phase (Carbó Tano *et al.*, 2009).

Previous results with this memory paradigm support the hypothesis that extinction represents the formation of a new memory that coexists with the original one (Fustiñana *et al.*, 2013). Therefore, if the treatment is administered before testing, both memories are intact and could potentially be expressed. Considering the inhibitory nature of the extinction memory proposed above, we can hypothesize that fine variations in the GABAergic tone could inhibit or facilitate extinction memory expression at testing. Consequently, before performing the experiment, it was necessary to select a lower dose of BIC, different from that used in the previous experiments, to distinguish the effect of the drug between both memory traces (excitatory or inhibitory). The selected dose should be ineffective at facilitating CPC expression at testing but capable of blocking the expression of the extinction memory. The following experiment included three pairs of CT–TR groups (Fig. 4A, upper panel; Table 1). The TR groups received a weak CPC training on Day 1, while the CT groups remained in the context without stimulation. After 24 h, one pair was injected with VEH, while one of the other pairs received a high-dose injection of 2.68 μg/g of BIC (BIC_H), and the remaining pair received the low dose of 1.34 μg/g (BIC_L). Immediately after the treatment, the animals' responses were evaluated for the CS and US. The VHC pair demonstrated the absence of the CPC memory, as expected, given that they had received a weak training the day before. The same results were obtained for the pair treated with the BIC_L dose. In contrast, the pair that received the high dose (BIC_H) expressed the CPC memory for both testing stimuli (Fig. 4A, lower panel; ANOVA CS TEST: $F_{5,174} = 1.62$, $P = 0.15$; CT VHC vs. TR VHC $P = 0.78$; CT BIC_L vs. TR BIC_L $P = 0.74$; CT BIC_H vs. TR BIC_H $P < 0.05$; ANOVA US TEST: $F_{5,174} = 2.09$, $P = 0.06$; CT VHC vs. TR VHC $P = 0.93$; CT BIC_L vs. TR BIC_L $P = 0.82$; CT BIC_H vs. TR BIC_H $P < 0.05$).

In summary, a high dose of BIC was able to facilitate the expression of the CPC memory at testing. However, a low dose of BIC did not affect the expression of the CPC memory. These results suggest that the low dose of BIC had no effect on the animals' escape responses evoked by the VDS and that the locomotor activity of the animals was not affected by the treatment as was shown in previous reports with higher doses (Carbó Tano *et al.*, 2009; Fustiñana *et al.*, 2013).

At this point, it was possible to evaluate the effect of this BIC_L on long-term extinction memory retrieval. The following experiment included two pairs of CT–TR groups. Each pair received a strong training protocol for the CPC on Day 1. On Day 2, all animals received a strong extinction training (2 h), and the acquisition of the extinction memory was evaluated with a short-term test that was performed 3 min after extinction training (Fig. 4B, upper panel; Table 1). At Test 1, both pairs of groups showed extinction memory retention (Fig. 4B, lower panel; ANOVA US TEST: $F_{3,116} = 0.08$, $P = 0.96$; CT VHC vs. TR VHC $P = 0.71$; CT BIC_L vs. TR BIC_L $P = 0.73$). Before Test 2, 24 h after the extinction training, one pair was administered VHC while the other pair was injected with BIC_L. The evaluations of the anticipatory response and the VDS response were performed immediately after drug administration (Fig. 4B, upper panel). In this case, the BIC_L groups demonstrated the presence of the CPC memory at testing, while the VHC groups demonstrated long-term extinction memory retrieval (Fig. 2B, lower panel; ANOVA CS TEST: $F_{3,116} = 2.36$, $P = 0.07$; CT VHC vs. TR VHC

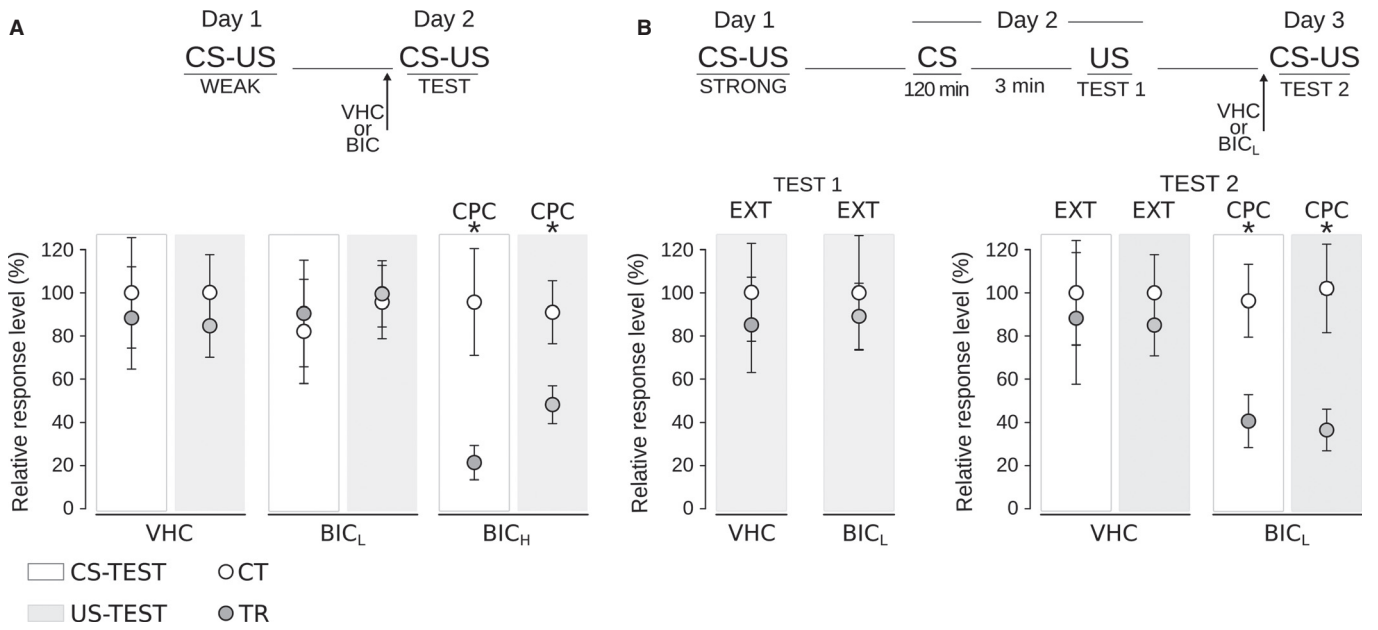

FIG. 4. (A) Upper panel: experimental protocol. Day 1, weak training. Day 2, test session. Both responses to the CS and the US were analysed. Immediately before the test, VHC or drug (BIC_L, 1.34 μg/g or BIC_H, 2.68 μg/g) was administrated. Lower panel: test session. (B) Upper panel: experimental protocol. Day 1, strong training. Day 2, 120 min re-exposition to the CS, 3 min after one test trial was presented. Only the response to the US was analysed. Day 3, second test session. Both responses to the CS and the US were analysed. Immediately before the test, VHC or BIC_L was administrated. Data are expressed as mean response level ± SE normalized with respect to the CT group of the VHC pair. Symbols as in Fig. 1.

$P = 0.74$; CT BIC_L vs. TR BIC_L $P < 0.05$; ANOVA US TEST: $F_{3,116} = 2.62$, $P < 0.05$; CT VHC vs. TR VHC $P = 0.34$; CT BIC_L vs. TR BIC_L $P < 0.05$). Collectively, the results showed that it is possible to facilitate the expression of the CPC memory acquired with a weak training following the high dose of BIC, but the low dose of BIC failed to affect the CPC expression after a weak training. Contrary to the effect demonstrated for long-term CPC memory retrieval after weak training, the low dose was capable of impairing the expression of long-term extinction memory retrieval. Together, these results show that fine variations of the GABAergic tone affect the appearance of an inhibitory memory at testing. Therefore, the attenuation of the GABAergic system impairs the expression of the extinction memory.

Discussion

Using a combination of behavioral protocols, such as weak and robust extinction training procedures, and pharmacological treatments, such as the administration of agonists and antagonists of $GABA_A$ receptors, we investigated the role of the GABAergic system in the different phases of extinction memory in an invertebrate model.

The primary difference in our research from previous studies emerged in the characterization of the role of GABA during the acquisition of the extinction memory. We evaluated the effects of modulating the GABAergic tone during extinction memory acquisition. The results were conclusive in demonstrating that during the acquisition of the extinction memory, an increased GABAergic tone facilitated extinction memory acquisition, while a reduction in such tone blocked extinction memory acquisition.

We demonstrated that when the $GABA_A$ receptor was modulated immediately after extinction training (extinction consolidation), the effect was similar to that observed when the modulation occurred after the original memory training. In both cases, the time of the drug administration ensured that the treatment affected the storage of the original memory or, as demonstrated in the current study, the storage of the extinction memory. The agonist impaired and the antagonist facilitated long-term extinction memory retention. Therefore, the consolidation of the extinction memory involves GABA in the same way that it is involved in the consolidation of the original memory (see the Discussion below).

Finally, we analysed the effect of the manipulation of the GABAergic system before testing. We studied the effect of the antagonist administered in different doses before the original memory retrieval or the extinction memory retrieval. BIC administered in a low dose (one without an effect on CPC memory retrieval) applied before the extinction memory evaluation impaired extinction memory expression.

A different role of GABA during the acquisition of an extinction memory

The acquisition of a conditioned fear response and of extinction is disrupted by similar pharmacological manipulations (Schafe *et al.*, 1999; Lin *et al.*, 2003), and both processes promote the activation of the same transcription factor as well (Josselyn *et al.*, 2001; Lin *et al.*, 2003). However, it is also obvious that these two forms of learning have opposite behavioral consequences (Pávlov, 1927). In this framework, it seems reasonable to anticipate differences in their neural basis, possibly in the form of differential plastic alterations at the level of neurotransmission. Considering the GABAergic transmission, it is possible to find opposite results. These contrary outcomes should sustain the last proposal or should reject it. Therefore, it is generally accepted that the activation of the $GABA_A$ receptors during acquisition seems to impair extinction memory formation (Kamano, 1972; Goldman, 1977; Corcoran & Maren, 2001; Hart *et al.*, 2009). However, some findings contradict this current scheme of fear learning and its relation to extinction memory (Maren &

Quirk, 2004). Using auditory fear conditioning, Akirav *et al.* (2006) demonstrated that the administration of MUS into the infralimbic region of the medial prefrontal cortex before an extinction training facilitated extinction, and the effect was maintained following extinction sessions performed 24 and 48 h after the drug administration. These authors concluded that increasing the GABAergic tone before extinction training facilitates, rather than impairs, the retention of extinction. They also used a lower dose in comparison with other reports; however, this dose was still sufficient to modify the GABAergic tone (Salinas & McGaugh, 1996; Wilensky *et al.*, 2000) without inactivating the infralimbic area, which occurs with higher doses (Helmstetter & Bellgowan, 1994; Wilensky *et al.*, 2000). However, it is not possible to reject a more parsimonious explanation between these studies using mammals as animal model. Some differences in these findings may be due to the specific site of drug infusion, in diverse regions of the brain. Moreover, those differences could also be related to systemic vs. local infusion of the drug (Makkar *et al.*, 2010). In this study the working hypothesis implied a more general description of the GABAergic role in diverse aspects of the extinction memory. We performed a systematic study of each extinction phase, using the same drugs and the same route of administration. The lack of hematoencefalic blood barrier (Abbot, 1970) in the crab ensures that the drugs reach every area of the CNS of these animals. This method permits to perform the general description of the role of the GABAergic system in each extinction memory phase. However, opposite effects seem to be disclosed when we compare the effect of the treatment before or after the extinction training. Systemic administration of MUS_L before weak extinction training facilitated extinction memory acquisition immediately after or at a long-term test. This dose had no effect when it was administered immediately after weak extinction training, being the original memory that was expressed at a long-term test. But, when strong extinction training was combined with MUS_H, an amnesic effect on extinction memory was revealed. We interpreted these differential effects as a consequence of the combination of different training with different doses, which in turn affected distinct extinction memory phases as acquisition and consolidation.

Using different approaches, several reports revealed either that mRNA coding for gephryin, a protein that contributes to the regulation of GABAergic neurotransmission by clustering $GABA_A$ receptors at the synapse, is upregulated in the basolateral amygdala after extinction training (Chhatwal *et al.*, 2005); or described an upregulation of GABAergic markers (mRNA levels of GABA-related genes) related to increased GABAergic transmission after the acquisition of the extinction memory in the amygdala (Heldt & Ressler, 2007).

Adding new evidence to the link between $GABA_A$ receptors and extinction, Lin *et al.* (2003) demonstrated that increased expression of $GABA_A$ receptors in the amygdala is required for the extinction of a cue fear-conditioning paradigm. These results demonstrate that $GABA_A$ receptor insertion in the amygdala contributes significantly to the formation of memory extinction. Studying memory processes at a more circuital level in target areas such as the amygdala, there is evidence that supports the role of GABA in the local inhibitory circuits in the amygdala that are associated with the different memory phases at different levels (Herry *et al.*, 2010). There are also results supporting the view of the critical role of a GABAergic microcircuit for the acquisition of extinction memory (Pan *et al.*, 2013).

In summary, these results strongly support an opposite role of the GABAergic system for the original memory and extinction memory, which results from a substantial modification of the availability or composition of $GABA_A$ receptors in different brain areas that are associated with emotional memories.

In line with this interpretation, this research with a classical behavioral-pharmacological approach adds new evidence supporting the inhibitory nature of the extinction memory.

More recently, Hart *et al.* (2009, 2010) showed that when rats were subjected to one or two cycles of contextual fear conditioning and extinction, administered the benzodiazepine, midazolam, before the first or second extinction, and then the freezing response was evaluated 24 h after the last session, midazolam impaired extinction learning but not its relearning. They hypothesized that rats in the second extinction training under the influence of the drug would recall the initial extinction, and that this recall would reactivate and strengthen the original inhibitory memory. Our results show facilitation in the acquisition of the initial inhibitory response. This difference could result from the difference in the animal model paradigm used and drug selected. Future experiments would analyse if this effect would be maintained or vary when a second extinction training session is included.

Dissecting the processes during extinction memory acquisition

Previous research using the classical paradigm CSM showed that memory extinction is not triggered until the CS is terminated, strongly suggesting similar dynamics for reconsolidation (Pedreira *et al.*, 2004; Pérez-Cuesta *et al.*, 2007). It was demonstrated that the CS–US memory is not labilized during a short CS exposure. In fact, both labilization and reconsolidation are triggered after the CS offset. This condition rules out the possibility of an interaction between reconsolidation and extinction under the single CS presentation. However, each process may intrinsically constitute a constraint on the other, or both processes may develop in parallel. Different CS exposures presented serially are able to trigger both reconsolidation and extinction, one process after each CS. To test this hypothesis, Pérez-Cuesta & Maldonado (2009) exposed CSM-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. Using this behavioral protocol, they showed that the CS–US memory underwent both reconsolidation and extinction, with these two processes developing in parallel.

Here, with the CPC protocol, we used multiple presentations of the CS, which could be able to trigger both processes in parallel. Specifically, we evaluated this working hypothesis in the experiment designed to evaluate whether the administration of BIC before extinction training might allow the reactivation of the original memory. The experiment design also assumes that the acquisition of the extinction memory is impaired by BIC. The combination of BIC_H previous to the extinction training to impair the extinction memory acquisition, and the administration of a protein synthesis inhibitor CHX, would be able to impair the original memory restabilization if the reconsolidation process is triggered under this experimental condition. The CPC memory was expressed at testing when the acquisition of the extinction memory was impaired by BIC_H. The appearance of the CPC memory at testing revealed that the CHX did not affect the stability of this memory and, consequently, the repeated presentations of the CS did not reactivate the original memory. Moreover, the animals could estimate correctly the context exposition time as a longer one because the reconsolidation was not triggered. Finally, the role of the GABAergic system is specific to the extinction memory acquisition, with the activity necessary to gain the information required for the new memory. Despite the

repeated presentation of the CS in this experiment, the processes were not triggered in parallel, as was demonstrated in other reports with longer intervals between the first and second CS presentations (Monfils *et al.*, 2009; Pérez-Cuesta & Maldonado, 2009; Schiller *et al.*, 2012). In this study, given the proximity between each CS re-exposition of 20 s, the separate experiences might be included in the same unique event. Moreover, the results suggest that during the extinction training, two processes occur simultaneously. First, the temporal relation used to present the CS guides the memory to extinction, leaving the original memory in an inactive state and, consequently, insensitive to the amnesic treatment. Second, the acquisition of the extinction memory depends on the active participation of the GABAergic system and its inhibition prevents the extinction memory acquisition. As a result, the original memory emerges intact at testing.

The role of GABA during the consolidation and retrieval of an extinction memory

The results obtained in this report are consistent with the findings of previous research over the last 30 years. The GABAergic system seems to play a decisive role in the consolidation and expression of extinction memories. Among the important antecedents for this study is the research conducted by Harris & Westbrook (1998), who demonstrated that FG-7142, an inverse agonist of the $GABA_A$ receptor that functionally decreases GABA transmission, dose-dependently impaired within-session extinction. Additionally, when FG-7142 was administered before testing, it blocked extinction retention in the context of extinction training but had no effect on performance in a novel context. Thus, it appears that GABA is involved in the expression of extinction in a context-dependent manner. With respect to the stabilization of the information after acquisition, GABA has been implicated in the consolidation of extinction. McGaugh *et al.* (1990) reported that the systemic administration of the GABA antagonist picrotoxin post-extinction training enhanced extinction retention in a test conducted 24 h after an active avoidance training. Using a contextual fear-conditioning paradigm, Berlau & McGaugh (2006) reported facilitated extinction by unilateral (right) intra-basolateral amygdala infusions of the GABA receptor antagonist BIC immediately but not 3 h after extinction training.

Regarding the role of GABA during extinction memory retrieval, the current results suggest that fine variations of the GABAergic tone affected the expression of the extinction memory. In fact, extinction memory has been considered to be an inhibitory memory; therefore, a principal role of this inhibitory neurotransmitter would be predicted.

Conclusions

Extinction memory formation is a process that shares several neurotransmitter systems with the formation of the initial memory. However, at the same time, some components are exclusively associated with the formation of the extinction memory. Thus, as Pavlov and Konorski had previously remarked, the most broadly distinguished difference between learning the initial memory and learning the extinction memory is that the initial learning process certainly entails excitatory synaptic connections, whereas extinction likely involves inhibitory synaptic connections (Konorski, 1948). Within this context, it seems likely that inhibitory neurotransmitters may play a crucial role in the different extinction memory phases. Taking this idea as a central topic, we investigated the role of GABAergic neurotransmission in the acquisition, consolidation and retrieval of extinction memories. Our results suggest that the inhibitory transmission that is mediated by GABA is necessary for extinction memory formation. It is also clear that GABA transmission is necessary for extinction memory expression at testing.

However, a different role has been demonstrated for extinction memory acquisition. Different theories provide divergent explanations for the fact that after the original memory formation and its extinction, opposing information about the same stimulus exists. In the majority of these theories, it is accepted that the original memory formation implies the establishment of an association between the stimuli representation. Moreover, each of these proposals has received experimental support (Tronson *et al.*, 2012). In one of these hypotheses, the extinction memory implies a new direct inhibition. Consequently, the inhibitory transmission is a mandatory component of this memory. For the other hypothesis, the inhibitory neurotransmitter for the new inhibitory circuits also plays an essential role. In this framework, our results shed light on the differential function of GABA transmission, revealing a new role during acquisition that is consistent with the role proposed in extinction models (Chhatwal *et al.*, 2005; Herry *et al.*, 2010; Pan *et al.*, 2013). Future studies using different tools may add new evidence to the understanding of the molecular processes associated with the need for a high inhibitory tone during extinction memory acquisition.

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Abbreviations

BIC, bicuculline; CHX, cycloheximide; CPC, contextual Pavlovian conditioning; CS, conditioned stimulus; CSM, context signal memory; CT, control group; GABA, γ-aminobutyric acid; ITI, inter-trial interval; MUS, muscimol; TR, trained group; US, unconditioned stimulus; VDS, visual danger stimulus; VHC, vehicle.

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