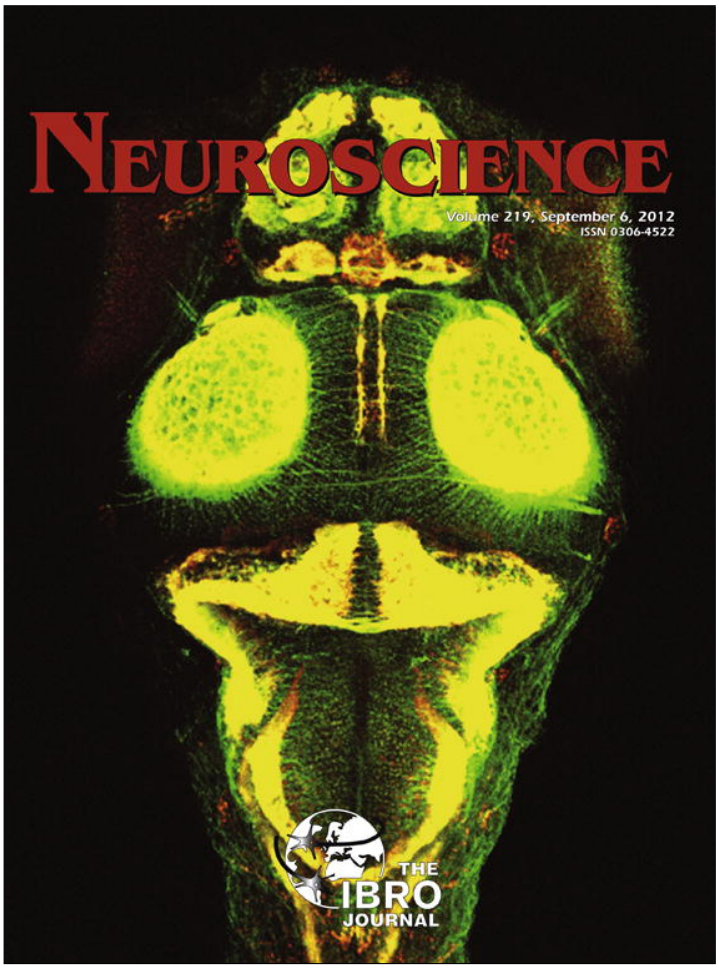

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RECONSOLIDATION INVOLVES HISTONE ACETYLATION DEPENDING ON THE STRENGTH OF THE MEMORY

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Abstract—Gene expression is a necessary step for memory re-stabilization after retrieval, a process known as reconsolidation. Histone acetylation is a fundamental mechanism involved in epigenetic regulation of gene expression and has been implicated in memory consolidation. However, few studies are available in reconsolidation, all of them in vertebrate models. Additionally, the recruitment of histone acetylation as a function of different memory strengths has not been systematically analyzed before. Here we studied the role of histone acetylation in reconsolidation using a well-characterized memory model in invertebrate, the context-signal memory in the crab *Chasmagnathus*. Firstly, we found an increase in histone H3 acetylation 1 h after memory reactivation returning to basal levels at 3 h. Strikingly, this increment was only detected during reconsolidation of a long-term memory induced by a strong training of 30 trials, but not for a short-term memory formed by a weak training of five trials or for a long-term memory induced by a standard training of 15 trials. Furthermore, we showed that a weak memory which was enhanced during consolidation by histone deacetylases inhibition, also recruited histone H3 acetylation in reconsolidation as the strong training does. Accordingly, we found the first evidence that the administration of a histone acetyl transferase inhibitor during memory reconsolidation impairs long-term memory re-stabilization. Finally, we found that strong training memory, at variance with the standard training memory, was resistant to extinction, indicating that such strong training induced in fact a stronger memory. In conclusion, the results presented here support that the participation of histone acetylation during reconsolidation is an evolutionary conserved feature and constitutes a specific molecular characteristic of strong memories.

Key words: long-term memory, histone acetylation, HDAC inhibitor, HAT inhibitor, reconsolidation.

INTRODUCTION

The classical hypothesis of long-term memory (LTM) formation formalized more than 50 years ago (McGaugh, 1966) states that once a memory is stored it can last in a stable form for long periods. It was postulated that memory formation involves an irreversible passage from a labile state to a stable form, a process termed *consolidation*. This phase was defined as a unitary process through which a newly formed memory initially sensitive to disruption becomes stable over time. Disrupting events include brain trauma, seizure, electroconvulsive shock, hypothermia, new learning and administration of drugs such as inhibitors of transcription and translation. Consequently, it was proposed that memory consolidation requires de novo mRNA and protein synthesis (Alberini, 2008).

More recently, this linear model of memory consolidation was challenged. An increasing number of studies in different species have shown that after reaching a stable form, memory can become transiently labile again if it is reactivated (Sara, 2000; Dudai, 2004). Treatments that interfere with consolidation, such as protein synthesis inhibitors, were effective in disrupting a reactivated memory (Nader et al., 2000; Pedreira and Maldonado, 2003). The reactivation of a memory can be achieved by presentation of a reminder of the learning event and, in such a way, becomes transiently sensitive to disruption, needing a process of re-stabilization in order to be maintained. This process is termed *reconsolidation*. Several studies in the last few years have demonstrated that reconsolidation partially recapitulates the anatomical, pharmacological and molecular substrates of consolidation (Nader, 2003; Alberini, 2005; von Hertzen and Giese, 2005). Regarding its functional value, it was proposed that reconsolidation provides an opportunity for memory updating and/or reinforcement (Dudai, 2006; Lee, 2009; Forcato et al., 2011; Inda et al., 2011).

It has been shown that regulation of gene expression is a necessary step for memory consolidation and reconsolidation (Agranoff et al., 1967; Goelet et al., 1986; Merlo et al., 2005; Mamiya et al., 2009). Nevertheless, the nature and the role of the mechanisms which regulate transcription during these memory phases required further investigation. Histone acetylation is an important chromatin regulatory mechanism involved in the gene expression

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Abbreviations: ANOVA, analysis of variance; CSM, context-signal memory; CT, control group; HAT, histone acetyl transferase; HDAC, histone deacetylases; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ITI, inter-trial interval; LTM, long-term memory; NaB, sodium butyrate; ROD, relative optical density; sLTM, strong long-term memory; SSZ, sulfasalazine; TR, trained group; VDS, visual danger stimulus.

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required for memory formation (Levenson and Sweatt, 2005; Wood et al., 2006). Chromatin modifying enzymes, which carry out histone acetylation and deacetylation, have already been described (Sterner and Berger, 2000): acetyl transferases (HAT) and deacetylases (HDAC), respectively. Histone acetylation induces transcription activation by increasing DNA accessibility to the transcription machinery (Norton et al., 1989; Vettese-Dadey et al., 1996). To our knowledge, there are few studies showing histone acetylation increments in memory reconsolidation, all of them in rodent memory models (Lubin and Sweatt, 2007; Maddox and Schafe, 2011). In addition, it was shown memory enhancement during reconsolidation in cued fear conditioning in mice using valproic acid (VPA) as inhibitor of HDAC (Bredy and Barad, 2008). Two other reports using different memory models in rats (Lubin and Sweatt, 2007; Yang et al., 2011) have shown that the disruptive effect of NF-κB inhibition on reconsolidation is reversed by HDAC inhibition. However, in these last studies, no memory enhancement effect of HDAC inhibitors during reconsolidation was found.

The memory model used in the present work, the context-signal memory (CSM) of *Chasmagnathus*, is based on the crab escape response elicited by the presentation of a visual danger stimulus (VDS), which consists in an opaque rectangle figure passing over the animal. The repeated presentation of the VDS provokes the fading of the initial escape response (Lozada et al., 1990) that is actively replaced by a robust freezing behavior, which persists over time (Pereyra et al., 2000). This long-term memory is mediated by the association between environmental characteristics of the training site (the context) and the features of the VDS (the signal). The CSM entails mRNA and de novo protein synthesis (Pedreira et al., 1995, 1996; Freudenthal and Romano, 2000). In this memory model, the activation of the transcription factor NF-κB plays a critical role in consolidation and reconsolidation (Freudenthal and Romano, 2000; Merlo et al., 2002).

In the CSM model, a training protocol of 15 trials (standard training) or 30 trials (strong training) produces LTM that lasts at least 5 days (Pedreira et al., 1998). Memory retention of learning acquired during training is defined as a significantly lower mean response level at the testing session of the trained group vs a control group that was not stimulated with the VDS during the training session. The memory retention at the testing session is evident in animals trained either with the standard or the strong protocol (Freudenthal and Romano, 2000). In contrast, weak protocols between 5 and 10 trials are unable to induce LTM formation (Romano et al., 1996). A weak training produces a short-term memory (STM) that lasts between 4 and 8 h (Suárez et al., 2010). These different training protocols have been useful for pharmacological experiments in order to assess the amnesic (standard and strong protocol) or enhancement effects (weak protocol) of various agents on memory (e.g., Hermitte et al., 1999; Frenkel et al., 2005a,b; Federman et al., 2009).

Once the CSM is consolidated and stable, retrieval of learned associations by presentation of the context can initiate one of two mutually exclusive processes (Pedreira and Maldonado, 2003). On the one hand, a brief context re-exposure without VDS presentation induces memory reactivation and reconsolidation. On the other hand, a prolonged context re-exposure induces memory extinction, which is evidenced as a temporary lack of retention. In fact, it has been shown that duration of the re-exposure to the training context acts as a switch guiding the memory course either toward reconsolidation or extinction, and that both processes are protein synthesis dependent (Pedreira and Maldonado, 2003). At a molecular level, reconsolidation requires NF-κB regulation of gene expression (Merlo et al., 2005), while extinction entails NF-κB inhibition (Merlo and Romano, 2008).

In previous work (Federman et al., 2009), we have shown that no changes in histone 3 (H3) acetylation occur during consolidation of a standard training protocol. However, strong training induced a significant increase in H3 acetylation 1 h post-training, returning to basal levels afterward. Accordingly, we have shown that the administration of two different HDAC inhibitors, sodium butyrate (NaB) and trichostatin A, allowed a weak training to induce long-term memory. All these findings support that H3 acetylation is involved in LTM consolidation in invertebrates, only revealed after strong training conditions. Therefore, here we ask whether histone H3 acetylation is induced by the re-activation of memories, during reconsolidation phase. Then we tested whether histone acetylation is necessary for memory reconsolidation by means of HAT inhibition and we tested whether a memory enhancement takes place during reconsolidation by means of HDAC inhibition. Finally, we inquire if histone acetylation is differentially induced by diverse training protocols. In the present work, we found an induction of histone acetylation during LTM reconsolidation, and an association between the level of histone acetylation during reconsolidation and the memory strength.

EXPERIMENTAL PROCEDURES

Animals

Adult male *Chasmagnathus* granulatus intertidal crabs, 2.6–2.9 cm across the carapace, weighing 17 ± 0.2 g (average from $n = 60$), were collected from water less than 1 m deep in the estuarine coasts of San Clemente del Tuyú, Argentina, and transported to the laboratory. 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 (light on 07:00–19:00 h). Temperature of both holding and experimental rooms was maintained within a range of 22–24 °C. Experiments were carried out between the 3rd and the 10th days after the arrival of the animals. Each crab was used in only one experiment. Experiments were carried out in accordance with the local regulations (CONICET, Argentina) for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Training–testing apparatus

The experimental unit called actometer was described in detail elsewhere (Romano et al., 1990). Briefly, it consists of a bowl-shaped plastic container, as part of the context, where the crab is lodged, and an opaque rectangular screen, the VDS, which moves horizontally above the animal. Each trial consists of

screen displacements which evoke the crab's running response and, as a consequence, container vibrations. In turn, these vibrations induce electrical signals through four piezoelectric transducers attached to the external surface of the container. Signals recorded during each trial were translated into numerical units ranging from 0 to 8000. The experimental room had 40 actometers, separated from each other by partitions. A computer was employed to program trial sequences, trial duration and inter-trial intervals (ITIs), as well as to monitor the locomotor activity of the animals during each trial.

Drugs and injection procedure

HDAC inhibitor NaB (Sigma, USA) was dissolved in crustacean saline (Hoeger and Florey, 1989). Saline solution was injected as vehicle (Veh). Fifty microliters of vehicle or 15 mM of NaB solution (83 μg per crab, 4.8 μg/g) was given through the right side of the dorsal cephalothoracic–abdominal membrane by means of a syringe fitted with a sleeve to control depth of penetration to 4 mm, thus ensuring that the injected solution was released roughly at the center of the pericardial sac. The total volume of hemolymph was estimated at 5 ml (30% of the body weight) (Gleeson and Paul, 1977) resulting in an approximate 100-fold dilution of the drug in hemolymph. HAT inhibitor C646 (Sigma) was dissolved in dimethyl sulfoxide. Ten microliters of dimethyl sulfoxide as vehicle or different doses of C646 solution (2 μg/μl, 4 μg/μl or 6 μg/μl) were administered to each animal in the same systemic way.

Procedure in memory evaluation experiments

Each experiment lasted 3 days and included three sessions: training (day 1), re-exposure (day 2) and testing (day 3) (except for the experiment of Fig. 3c, described in the text). Crabs were individually housed during the inter-session interval in plastic containers, covered to a depth of 0.5 cm with marine water and kept inside dimly lit drawers. In order to evaluate a potential enhancing effect of the HDAC inhibitor on memory reconsolidation, we used a weak training protocol which does not induce LTM. In this case, the training session consisted of five trials with an ITI of 171 s. In order to evaluate a potential amnesic effect of the HAT inhibitor or NF-κB inhibitor, we used a standard protocol or strong training protocol, both of which result in long-term memory. The standard protocol consisted in 15 trials, ITI = 171 s and the strong training consisted of 30 trials with the same ITI. In all cases the drugs were administered during reconsolidation phase (pre or post re-exposure). As previous reports showed deleterious effects on memory caused by the manipulation of control groups close to the reconsolidation phase of CSM (Pedreira and Maldonado, 2003; Merlo et al., 2005), in the pharmacological experiments we avoided injection times shorter than 20 min before or 30 min after re-exposure in all the experiments. In order to evaluate the resistance to extinction, a group of animals was trained with the standard and another group with the strong protocol. In all the experiments, each trial lasted 9 s and consisted of two cycles of presentation of the screen over the actometer. Each cycle lasted 2.5 s with 2 s of interval between cycles. The crab's activity was recorded during the entire trial time. The re-exposure session consisted of exposing the animals to the training context for 5 min (reconsolidation experiments) or 2 h (extinction experiments), without the presentation of the VDS. The testing session consisted of one trial. Both the training and testing sessions were preceded by 10 min of adaptation in the apparatus. The unit used during training session is referred to as the training context.

Behavioral data analysis

Retention of learning acquired during training was defined as a statistically lower level of response at the testing session for the trained (TR) group relative to the control (CT) group. The results of behavioral experiments were analyzed using a one way analysis of variance (ANOVA) with α (per comparison error rate) = 0.05, and post hoc comparison by Duncan test with the same α.

Training procedure in acetyl-H3 evaluation from brain histone-enriched extracts

Crabs were trained using a weak training (five trials, 15 min of duration) plus drug or vehicle administration, standard (15 trials, 45 min) or strong training protocol (30 trials, 90 min), depending on the experiments. In each experiment, a 5 min re-exposure session to the training context was carried out 24 h after training. Crabs were sacrificed at 1 or 2 h after the re-exposure session and the central brain (supraesophageal ganglion) was then dissected. Animals were lodged in individual containers with dim light during the time interval between training, re-exposure sessions and their sacrifice. For each experiment, non re-exposed but trained animals were used as control groups. In all the experiments acetyl-H3 levels were determined and the values were normalized to their respective control group performed simultaneously.

Histone-enriched extracts and Western blot assay

Animals were anesthetized by immersion in ice-cold water for 2 min. The central brain was then dissected. Twenty ganglions per sample were pooled in 1-ml buffered crab saline solution (pH 7.6). Histone-enriched nuclear protein extracts were obtained as follows. All the procedures were performed at 4 °C. Dissected supraesophageal ganglions were homogenized in buffer A (Hepes 10 mM, pH 7.8, $MgCl_2$ 1.5 mM, KCl 10 mM, DTT 1 mM, NaB 5 mM) using Teflon-glass dounce. Tissue homogenates were centrifuged at 7800*g* for 1 min. The pellet (nuclear fraction) was resuspended in buffer A and H_2SO_4 was added to reach a 0.4 N concentration. Histones were acid-extracted from nuclear fractions for 30 min at 4 °C. Acid extracts were centrifuged 10 min at 15,000*g*. The supernatants were transferred to a fresh tube and proteins were precipitated with 10 times volume of acetone at −20 °C, overnight. Precipitated proteins were collected by centrifugation at 15,000*g* for 15 min. Pellets were resuspended in distilled water. For Western blot assay, loading buffer was added and samples were incubated at 100 °C for 5 min, and immediately after placed on ice. Ten micrograms of each protein sample was run on SDS–PAGE with 15% acrylamide in the resolving gel. Proteins were then electro-transferred to polyvinyledene difluoride (PVDF) for immunoblotting. Western blot assay was performed with acetyl-specific H3 antibody (that recognizes acetylated H3 in both K9 and K14) and total H3 (CT, pan) antibody (Upstate), following the manufacturer protocol. Detection was made with luminol chemiluminescence kit (Santa Cruz Biotechnology Santa Cruz, CA, USA) as described by the manufacturer and the signals were digitalized by FUJI FILM-Intelligent Dark Box II apparatus with image reader LAS-1000 software. The relative optical density (ROD) was estimated using ImageJ 1.44p software. Values of ROD for acetyl-specific H3 band were relative to ROD for total H3 in each sample.

RESULTS

Strong memory involves a transient increase in histone H3 acetylation during reconsolidation

The following series of experiments were aimed at determining the level of histone H3 acetylation in the central brain (supraesophageal ganglion) of *Chasmagnathus* during the CSM reconsolidation phase. We had previously found changes in histone acetylation during consolidation 1 h after a strong but not after a standard training

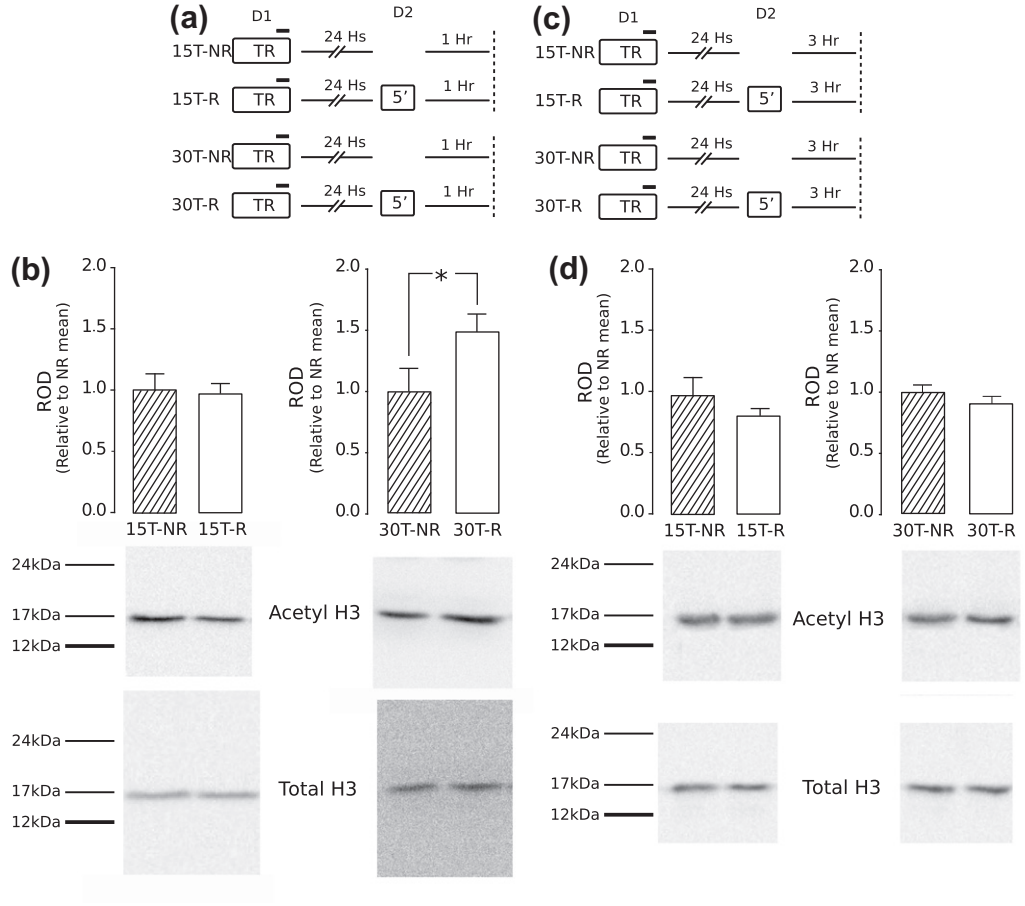

Fig. 1. Histone acetylation is increased during reconsolidation of strong training memory. (a, c) Experimental design diagram. Dashed lines indicate the time point (1 h or 3 h) at which the animals were sacrificed. 15T-NR: not re-exposed; 15T-R: re-exposed; 30T-NR: not re-exposed; 30T-R: re-exposed. (b, d) Mean ± SEM of the specific acetylated H3 histone bands, which have been normalized to the respective values of total H3 and the average value of 15T-NR or 30T-NR group. The result of each experiment comes from four independent experiments. $^{**}p < 0.01$. Representatives Western blots performed with acetyl-H3 specific antibody (top) and anti-total H3 (bottom) are shown below.

protocol (Federman et al., 2009). Here, we tested whether these training protocols induce histone acetylation after memory reactivation session. In the first experiment, the protocol was as follows: on the first day, 40 animals were trained with 15 trials of VDS presentation. Twenty-four hours later, 20 crabs were re-exposed for 5 min to the training context (15T-R group) while the remaining 20 crabs were not re-exposed (15T-NR group) (Fig. 1a). In the second experiment, another pair of groups was equally treated, with the exception that 30 trials were used instead of 15 (30T-R and 30T-NR groups) (Fig. 1a). One hour after the re-exposure session, all the crabs were sacrificed in order to obtain histone-enriched nuclear protein extracts from the central brain. The levels of histone H3 acetylation were evaluated by Western blot assay using an acetyl-specific H3 antibody and a H3 total antibody. Four independent experiments were carried out for each pair of groups. Fig. 1b (left panel) shows that there were no significant differences in the level of histone H3 acetylation between 15T-R and 15T-NR groups (Student t test NS, $p = 0.996$). On the contrary, we found a significant increase in histone H3 acetylation between 30T-R and 30T-NR groups (Student t test, $t = 7.187$, $p < 0.01$) (Fig. 1b, right panel).

In the previous work studying H3 acetylation in memory consolidation, we found that the acetylation increment after a strong training is transient, showing a peak 1 h after training, which returns then to basal levels. In addition, it is possible that standard training protocol may induce histone acetylation changes during reconsolidation but with a different kinetics. Therefore, we performed a third experiment in which we studied the level of brain histone H3 acetylation 3 h after re-exposure. Three independent experiments were carried out for each pair of groups. As Fig. 1d shows (left panel), there were no significant differences in the level of histone H3 acetylation between 15T-R and 15T-NR groups (Student t test NS, $p = 0.281$), 3 h after re-exposure. Also, we did not find differences between 30T-R and 30T-NR groups (Student t test NS, $p = 0.226$) at this time point during reconsolidation.

Thus, these results suggest that histone acetylation is increased during reconsolidation phase. Strikingly, this finding shows that histone acetylation is transient and only revealed during reconsolidation of a strong training memory, as occurs in the consolidation phase (Federman et al., 2009).

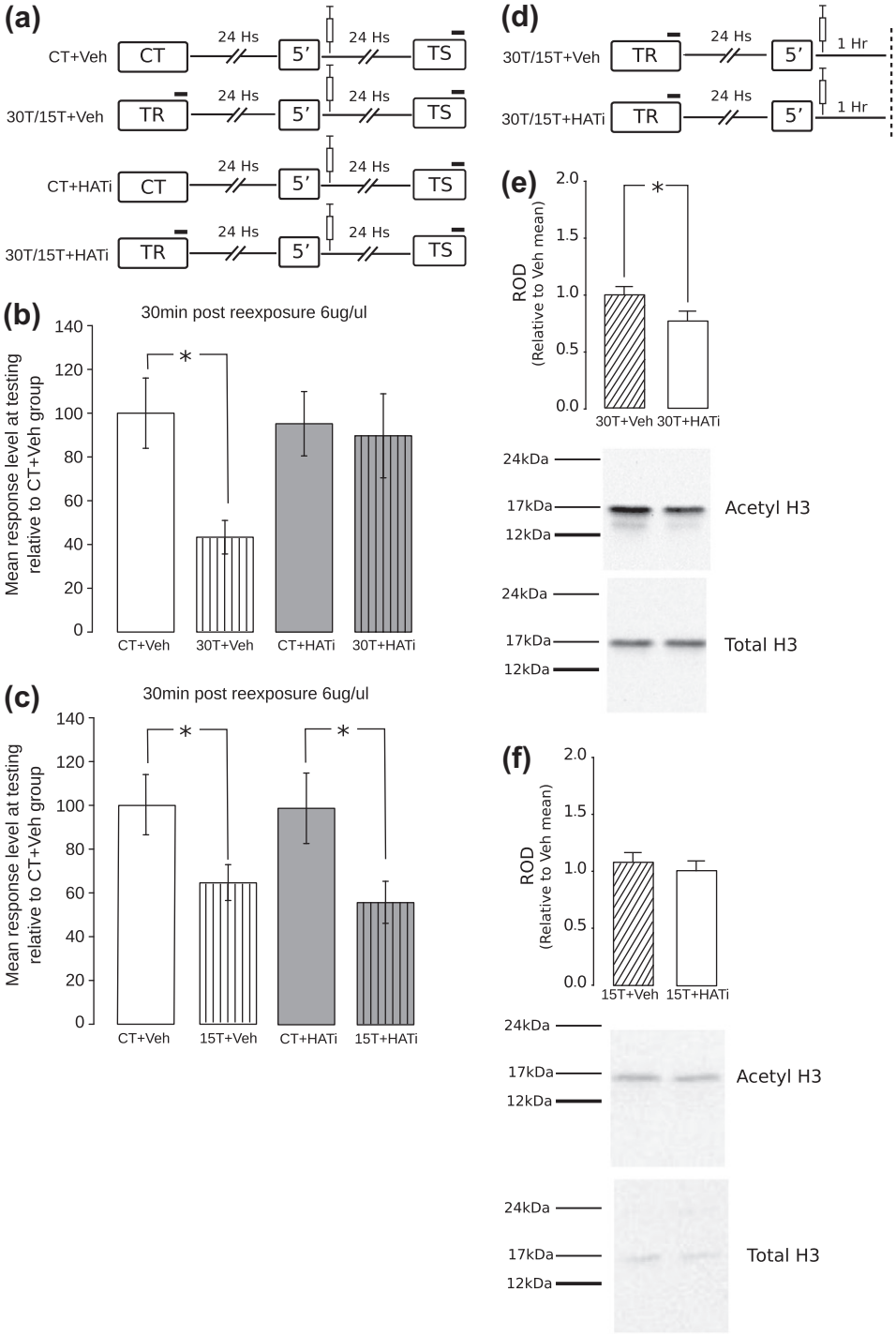

Fig. 2. Strong memory (but not standard memory) reconsolidation is impaired by HAT inhibition. Effect of the HAT inhibitor c646 during strong and standard memory reconsolidation. (a) Experimental design diagram. CT + Veh: untrained + vehicle injection; 15T/30T + Veh: standard/strong trained + vehicle injection; CT + HATi: untrained + c646 injection; 15T/30T + HATi: standard/strong trained + c646 injection. Animals were injected 30 min after context re-exposure. (b, c) Mean response level at the testing session during the VDS stimulation. (b) Animals were trained with 30 trials; 24 h later, they were re-exposed to the context and 30 min after re-exposure they were injected with 6 μg/μl doses of c646 HAT inhibitor. (c) Animals were trained with 15 trials; 24 h later, they were re-exposed to the context and 30 min after re-exposure they were injected with 6 μg/μl doses of c646 HAT inhibitor. Mean response level ± SEM. (e) Experimental design diagram. Animals were injected 30 min after context re-exposure. Dashed lines indicate the time point at which the animals were sacrificed. 15T/30T + Veh: standard/strong trained + vehicle injection; 15T/30T + HATi: standard/strong trained + c646 injection. (f) Mean ± SEM of the specific acetylated H3 histone bands, which have been normalized to the respective values of total H3 and the average value of 15T-Veh or 30T-Veh group. The result of each experiment comes from three independent experiments. $^{**}p < 0.01$. Representatives Western blots performed with acetyl-H3 specific antibody (top) and anti-total H3 (bottom) are shown below.

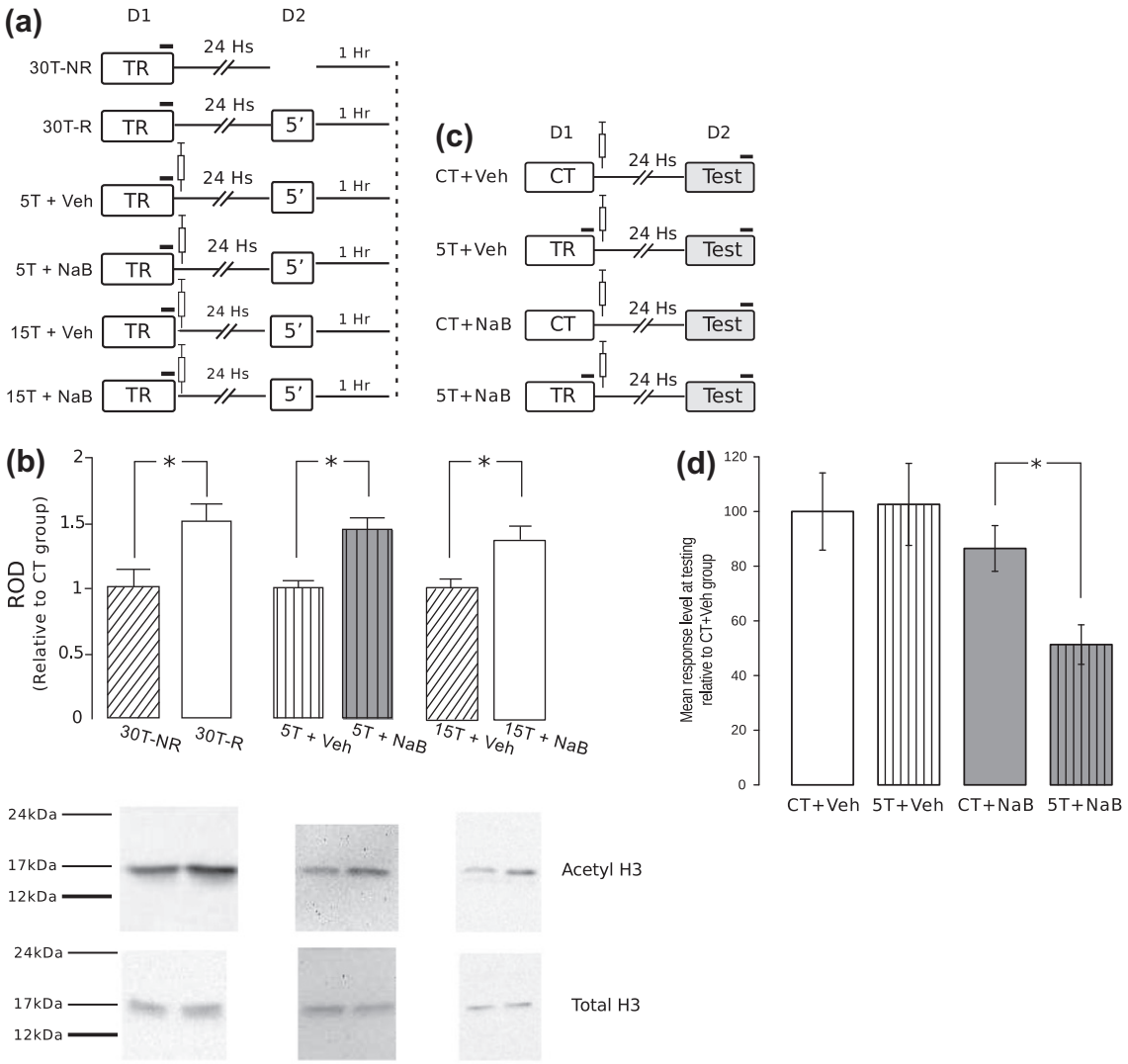

Fig. 3. Comparison of histone acetylation levels in reconsolidation between strong memory and weak memory enhanced by NaB. (a) Experimental design diagram. 30T-R: strong trained and re-exposed; 30T-NR: strong trained and not re-exposed. 15T + NaB: standard trained + sodium butyrate injection; 15T + Veh: standard trained + vehicle injection 5T + NaB: weak trained + sodium butyrate injection; 5T + Veh: weak trained + vehicle injection. Dashed lines indicate the time point at which the animals were sacrificed. (b) Mean ± SEM of the specific acetylated histone H3 bands, which have been normalized to the respective values of total H3 and the average value of 30T + NR group 15T + Veh and 5T + Veh, respectively. These results come from four independent experiments. $^{*}p < 0.05$. Representative Western blot performed with antibodies specific to acetyl-H3 (top) and anti-total H3 (bottom) are shown below. (c) Effect of HDAC inhibitor sodium butyrate injection during memory consolidation. Left panel: Experimental design diagram. Animals were injected immediately after weak training. CT + Veh: untrained + vehicle injection; 5T + Veh: weak trained + vehicle injection; CT + NaB: untrained + NaB injection; 5T + NaB: weak trained + sodium butyrate injection. Right panel: Mean response level at the testing session during the VDS stimulation. Mean response level ± SEM. $^{*}p < 0.05$.

Strong memory reconsolidation is impaired by HAT inhibition

The activity of the HAT p300 was shown as critical for memory in vertebrate models of learning (Oliveira et al., 2007, 2011). Given the important role of HAT enzymes in regulating gene expression associated with memory formation (Marek et al., 2011), we further investigate the role of HAT activity in reconsolidation phase in this invertebrate memory model. We evaluated if different doses of the p300 HAT inhibitor c646 could be effective to induce memory impairment. On day 1, half of the animals were trained with 30 trials of VDS presentations (30T), and the other half were placed in the training context but were not trained (CT). On day 2, crabs were re-exposed for 5 min to the same context and 30 min after re-exposure half of the crabs were injected with dimethylsulfoxide (DMSO) as vehicle (CT + Veh, 30T-Veh) and half of the crabs were injected with c646 (CT + HATi, 30T + HATi). Thus, four groups of 40 crabs were formed: CT + Veh, 30T + Veh, CT + HATi and 30T + HATi. On day 3, animals were tested for memory retention (Fig. 2a). There were significant differences at the testing session between both pairs of TR and CT groups (CT + Veh vs 30T-Veh and CT + HATi vs 30T + HATi) when the concentration of c646 drug was 2 μg/μl or 4 μg/μl after re-exposure (data

not shown) (ANOVA $F_{3,152} = 2.686$, $p < 0.05$; Duncan test, $p < 0.05$; and ANOVA $F_{3,110} = 7.634$, $p < 0.01$; Duncan test, $p < 0.01$; respectively). Therefore, no amnesic effect was found when these drug concentrations were used. We further increase the concentration of c646 up to 6 μg/μl. As Fig. 2b shows, there were significant differences between the groups CT + Veh and 30T + Veh (ANOVA $F_{3,87} = 3.010$, $p < 0.05$; Duncan test, $p < 0.05$). In contrast, no differences were found when the mean response level at test between CT + HATi and the 30T + HATi groups were compared (Duncan test NS, $p = 0.795$), demonstrating that the reconsolidation of the strong memory was impaired with the HAT inhibitor administration. This experiment presents the first evidence that HAT activity is necessary for memory reconsolidation.

In the following experiment, we evaluated whether the memory induced by the standard training of 15 trials is affected by the administration of the HAT inhibitor c646 30 min after re-exposure (Fig. 2a). As shown in Fig. 2c, no memory impairment was found (ANOVA $F_{3,134} = 3.4304$, $p < 0.05$; Duncan test, $p < 0.05$ for both comparisons).

In order to verify the effect of HAT inhibition on histone H3 acetylation levels during reconsolidation, we carried out the next experiment. We trained all the animals with the strong training protocol. One day after training, all the animals were re-exposed to the same context. Thirty minutes after re-exposure, the animals were divided into two groups: animals injected with HAT inhibitor at the dose of 6 μg/μl (30T + HATi) and animals injected with vehicle (30T + Veh). One hour after re-exposure, both groups were sacrificed to assess histone H3 acetylation levels in the central brain (Fig. 2d). As it is shown in Fig. 2e, significant decrement of H3 acetylation levels is observed 1 h after re-exposure in the animals injected with HAT inhibitor relative to the animals injected with vehicle (Student t test, $t = 14.22$, $p < 0.01$). Then, we performed a similar experiment using 15 trials training instead of 30 trails (Fig. 2f). In this case we did not find any effect of the drug (Student t test, $t = 0614$; NS).

Reconsolidation of a weak memory enhanced by a HDAC inhibitor during consolidation involves an increase in histone H3 acetylation

In Sections 'Strong memory involves a transient increase in histone H3 acetylation during reconsolidation' and 'Strong memory reconsolidation is impaired by HAT inhibition', we obtained evidence supporting that reconsolidation of a memory induced by a strong training is correlated with an increase in the level of acetylated H3, 1 h post re-exposure. As previous reports have shown that a weak memory is enhanced by the HDAC inhibitor NaB (Levenson et al., 2004; Yeh et al., 2004; Fischer et al., 2007; Vecsey et al., 2007; Federman et al., 2009; Stefanko et al., 2009), we set out to explore whether reconsolidation recruits similar epigenetic mechanisms in both strong training memory and enhanced memory. Thus, we conducted the following experiment: animals were divided into six groups (Fig. 3a). Two groups were trained with a strong training protocol (30T-R and 30T-NR) ($n = 20$ for each group). Other two groups were trained with a standard and other two with weak training protocol and, immediately post-training, were injected with saline (15T + Veh and 5T + Veh) or NaB (15T + NaB and 5T + NaB) ($n = 20$ for each group). Twenty-four hours post training, all groups underwent a brief re-exposure session, except 30T-NR group. One hour after re-exposure, the six groups were sacrificed to assess central brain nuclear protein extracts and histone H3 acetylation levels. The histone extraction of 30T-NR group was simultaneously run with that of the other groups. The results are shown in Fig. 3b and indicate a similar increase in histone H3 acetylation level at 1hpost re-exposure in the 30T-R, 15T + NaB and 5T + NaB groups (Student t test NS, $p = 0.382$), which were significantly higher than the 30T-NR, 15T + Veh and 5T + Veh groups, respectively (30T-R vs 30T NR, Student t test, $t = 2.245$, $p < 0.05$; 15T + Veh vs 15T + NaB, $t = 3.0564$, $p < 0.05$; 5T + Veh vs 5T + NaB, $t = 3.216$, $p < 0.05$). At the behavioral level, we repeated a previous experiment administering NaB immediately after a weak training of five trials (Fig. 3c). Half of the animals were trained with five trials of VDS presentation (5T) and the other half remained untrained (without VDS presentation) as controls (CT). Immediately after training session, crabs were injected with NaB or Veh (saline solution). Thus, four groups were formed: CT + Veh, 5T-Veh, CT + NaB, 5T + NaB, of 40 crabs each. The testing session was performed 72 h after training. This time point of the testing session was chosen to avoid unspecific behavioral effects of the drug. As Fig. 3c shows, there were no differences between the mean response levels at test for CT + Veh and 5T + Veh groups. However, there were significant differences between CT + NaB vs 5T + NaB mean response levels (ANOVA $F_{3,158} = 3852$, $p < 0.01$; Duncan test, $p < 0.05$). It was also found that the response level of 5T + NaB group was significantly lower than that of the 5T + Veh group (Duncan test, $p < 0.05$), confirming a memory enhancement effect of the HDAC inhibitor during consolidation. A similar experiment was performed using 15 trials of training instead of five observing, as was expected, memory retention (data not shown).

Accordingly, 5T + Veh group which was not able to induce LTM formation (Fig. 3c) also failed to increase H3 acetylation levels after re-exposure (Fig. 3b). These results confirm that the increment of H3 acetylation post re-exposure is specifically associated to reconsolidation and it is not due to unspecific effects of manipulation and/or simple exposure to the training context. As NaB administration during consolidation facilitates memory (Fig. 3c), the present experiments suggest that a weak memory, induced by a weak training, was probably converted to strong training memory-type by administration of the HDAC inhibitor.

In a previous work, we have already shown that NaB administration, at the same dose than we used here, induced histone acetylation increment in naïve crabs' brain, reaching a peak 15 min and decreasing 1 h after injection (Federman et al., 2009). Thus, this transient drug effect rules out that the alteration in histone H3 acetylation

produced by NaB during the consolidation of the weak training persists 24 h later for the duration of memory recall. Therefore, the increment in H3 acetylation found in the reconsolidation phase of 5T + NaB memory group in Fig. 3b is specific to memory retrieval and reconsolidation.

HDAC inhibition does not enhance CSM during the reconsolidation phase

Previous reports indicate that inhibition of HDAC induces memory facilitation in memory consolidation (Levenson et al., 2004; Yeh et al., 2004; Fischer et al., 2007; Vecsey et al., 2007; Federman et al., 2009; Stefanko et al., 2009; also see Fig. 4c). However, a memory enhancement effect of HDAC inhibitors during reconsolidation was only found using valproic acid in vertebrate models (Bredy and Barad, 2008), but not after NaB administration (Lubin and Sweatt, 2007; Yang et al., 2011). As we found here that histone acetylation is induced during reconsolidation in this invertebrate model (Figs. 1 and 3), we investigated whether a weak memory could be facilitated by the administration of the HDAC inhibitor NaB during reconsolidation, 24 h after training. Previous reports in the crab model indicate that weak memories can be strengthened during the reconsolidation phase by manipulations of neuromodulator angiotensin II levels (Frenkel et al., 2005a) and by administration of the GABAergic antagonist bicuculline (Carbo Tano et al., 2009). Thus, we performed here three independent experiments administering NaB at different times pre- and post-memory reactivation.

In the first experiment, a group of animals received a weak training protocol (5T) while another group of crabs were placed in the training context but remained untrained (CT). Twenty-four hours later, half of the trained animals were injected with NaB (5T + NaB) and the other half with saline solution (5T + Veh), and 20 min later both groups were re-exposed to the training context for 5 min. The testing session was performed 72 h after re-exposure (Fig. 4a). As shown in Fig. 4b, there were no significant differences between CT and 5T groups, demonstrating that there was no retention in any of the 5T groups (ANOVA $F_{3,150} = 0.283$, NS). In the second experiment, animals were injected with NaB or Veh 45 min after re-exposure session. As in the previous experiment, there were no differences at the testing session between any of the groups (Fig. 4c, ANOVA $F_{3,140} = 0.262$, NS). In a third experiment, animals were injected 1 h after the re-exposure session. As shown in Fig. 4d, no significant differences were found between groups at testing (ANOVA $F_{3,156} = 0.48$, NS).

There are two alternative explanations for these results: (1) the training protocol of five trials induces the formation of a weak memory that does not persist in a long-term form and, therefore, cannot be facilitated by the inhibition of HDAC after a re-exposure session. However, previous evidences from studies in this memory

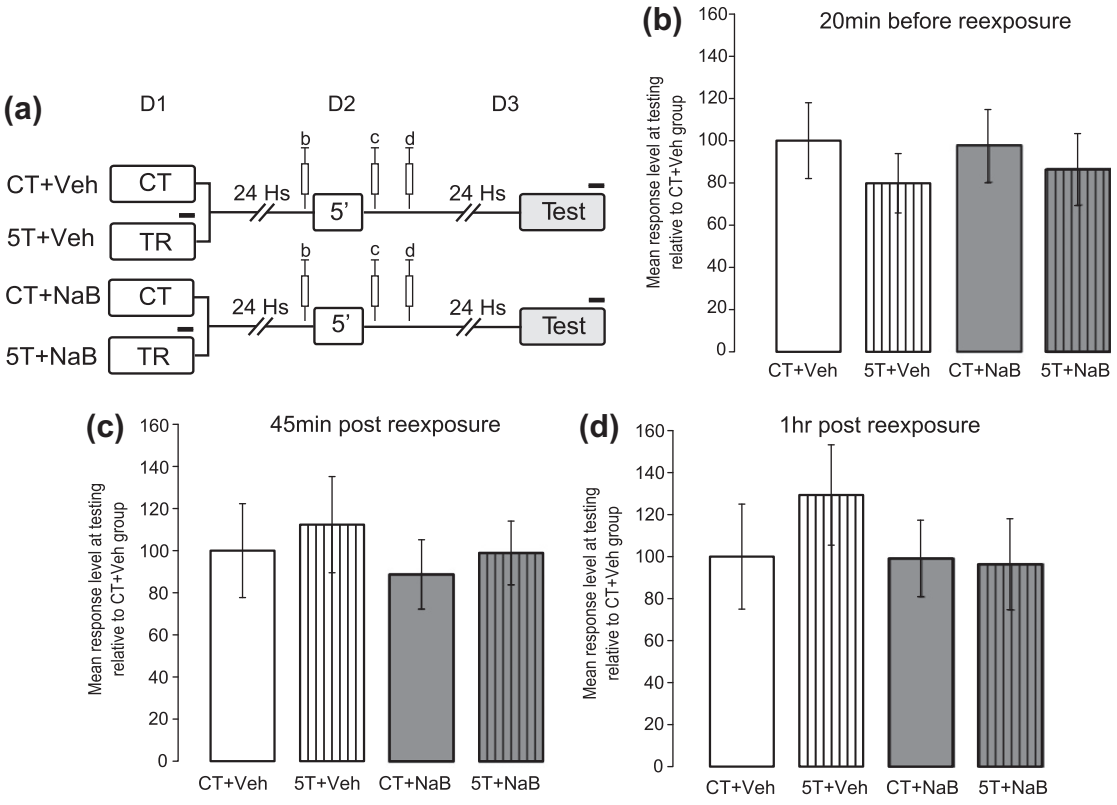

Fig. 4. Effect of HDAC inhibitor sodium butyrate injection during memory reconsolidation. (a) Experimental design diagram. CT + Veh: untrained + vehicle injection; 5T + Veh: weak trained + vehicle injection; CT + NaB: untrained + NaB injection; 5T + NaB: weak trained + sodium butyrate injection. (b) Mean response level at the testing session during the VDS stimulation. Animals injected 20 min before context re-exposure. (c) Animals injected 45 min post context re-exposure. (d) Animals injected 1 h after context re-exposure. Mean response level ± SEM.

model have shown that a weak memory can be facilitated by the GABAergic antagonist bicuculline (Carbo Tano et al., 2009) and by water deprivation during reconsolidation (Frenkel et al., 2005a,b). Therefore, a weak and non expressed memory trace is in fact present at the re-exposure session and it can be reactivated 24 h after weak training. This fact rules out this first explanation. (2) It is possible to induce the reconsolidation phase of a weak memory but, unlike what happens during the consolidation phase, it cannot be improved at this phase through the administration of HDAC inhibitors. During or shortly after weak training, mechanisms upstream of chromatin acetylation (i.e., transcription factor activation) are still available, at least at a minimum level, and for this reason NaB administration would be effective. On the contrary, the upstream processes might not to be reactivated in this weak memory during context re-exposure and the acetylation increments induced by the drug are not effective.

Therefore, in accordance with previous reports in vertebrates (Lubin and Sweatt, 2007; Yang et al., 2011), our experiments suggest that it is not possible to facilitate a weak memory by administering HDAC inhibitors during reconsolidation.

Strong memory is resistant to extinction

As we found a positive correlation between the level of histone acetylation and the strength of training during consolidation (Federman et al., 2009) and reconsolidation (Figs. 1 and 3), we tested if this correlation is reflected at the behavioral level. Therefore, we investigate if different training conditions induce in fact memories of different strength. Although both 15 and 30 trials-induced LTM are similarly expressed at the testing session, we postulate that they are different in terms of their strength. In order to obtain behavioral evidence supporting such hypothesis, we analyzed whether the strong training memory would be resistant to undergoing extinction (Annau and Kamin, 1961; Tully and Quinn, 1985). In the crab model, CSM extinction is induced by 2 h of context re-exposure 24 h after standard training. As a consequence of this prolonged non reinforced re-exposure, the conditioned response (induced by training) is temporarily inhibited (Merlo and Romano, 2008; Pérez-Cuesta and Maldonado, 2009; Hepp et al., 2009). In order to test if the memory induced by 30 trials of training is able to extinguish as does the one induced by 15 trials, we performed the following experiment. On day 1, two groups of crabs were trained with 15 trials, one group with 30 trials and a control group remained in the training context during the same time as the 30 trials group but without VDS presentations. On day 2, all groups received a session of extinction, consisting in 2 h of re-exposure to the training context, with the exception of one of the 15 trials groups which was not re-exposed. Thus, four groups were formed: CT-EXT, untrained control group with extinction session; 15T-NR, 15 trials training group not re-exposed; 15T-EXT, 15 trials training group with extinction session; and 30T-EXT, 30 trials training group with extinction session. In the present experiment, we have used 15T-NR group as a control of memory retention, but, as we have shown in the experiment of Fig. 1, memory retention is predictable for a 30T-NR group as well. Twenty-four hours after extinction session, all groups were tested with one trial of VDS presentation (Fig. 5a). As expected, the 15T-NR group showed a significantly lower level of response than CT-EXT group, indicating memory retention (Fig. 5b) (general ANOVA $F_{3,119} = 3.2923$, $p < 0.05$; Duncan test, $p < 0.05$). In contrast, 15T-EXT group showed similar levels of response than CT-EXT group as a result of the extinction session (Duncan test NS, $p = 0.81$). However, 30T-EXT group showed a significantly lower level of response than CT-EXT group (Duncan test, $p < 0.05$). These results indicate that 2 h of extinction session were unable to induce memory extinction after a strong training. This finding is in keeping with the idea that stronger memories are resistant to extinction, or require a more prolonged extinction session in order to extinguish. This behavioral difference between memories generated by standard and strong training protocols correlates with the difference at the molecular level in H3 acetylation induction that we found during consolidation and reconsolidation (Federman et al., 2009; Fig. 1). Accordingly, the amnesic effect of HAT inhibition was only found for strong training memory (Fig. 2). At this point, we propose a differentiation between LTM and strong LTM (sLTM).

DISCUSSION

In the present report, we show findings providing insight into the role of histone acetylation during memory reconsolidation. On the one hand, our present study in an invertebrate memory model, together with previous work in rodents (Lubin and Sweatt, 2007; Bredy and Barad, 2008; Maddox and Schafe, 2011; Yang et al., 2011), supports the involvement of histone acetylation in memory reconsolidation. Although we found no memory enhancing effect of HDAC inhibitor NaB when injected before or after the induction of reconsolidation by context re-exposure (Fig. 4), the requirement of histone acetylation activity for memory reconsolidation is supported by the fact that the HAT inhibitor c646 impaired memory when administered after retrieval (Fig. 2). This result constitutes the first evidence that HAT activity is necessary for memory reconsolidation. Further experiments are required to evaluate if this epigenetic mechanism is also involved in other processes that take place after retrieval, as memory extinction. The involvement of chromatin acetylation in extinction memory should be expected in invertebrates on the basis of recently found results in mammals (Bredy et al., 2007; Malvaez et al., 2010; Marek et al., 2011).

On the other hand, our results support that the recruitment of this epigenetic mechanism in reconsolidation is a molecular feature of strong memories. The scheme in Fig. 6 summarizes the present findings together with previous results in consolidation (Federman et al., 2009). Our experiments showed an increase in histone H3 acetylation 1 h after sLTM reactivation (Figs. 1 and 3). These results are consistent with our previous study in consolidation, in which we found that chromatin acetylation depends on the type of training used, occurring only after a strong training of 30 trials. In addition, we have found that if a weak memory is enhanced by administration of

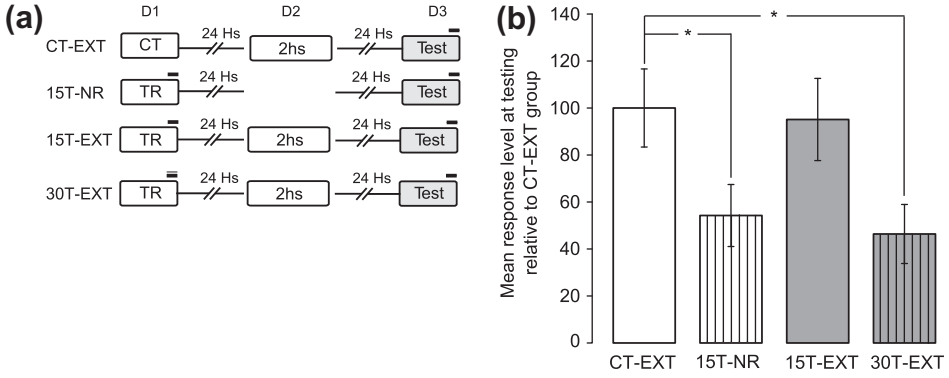

Fig. 5. Strong memory is resistant to extinction. (a) Experimental design diagram. CT-EXT: untrained animals and re-exposed; 15T-NR: standard trained and not re-exposed; 15T-EXT: standard trained and re-exposed; 30T-EXT: strong trained and re-exposed. (b) Mean response level at the testing session during the VDS stimulation. Mean response level ± SEM. $^{*}p < 0.05$.

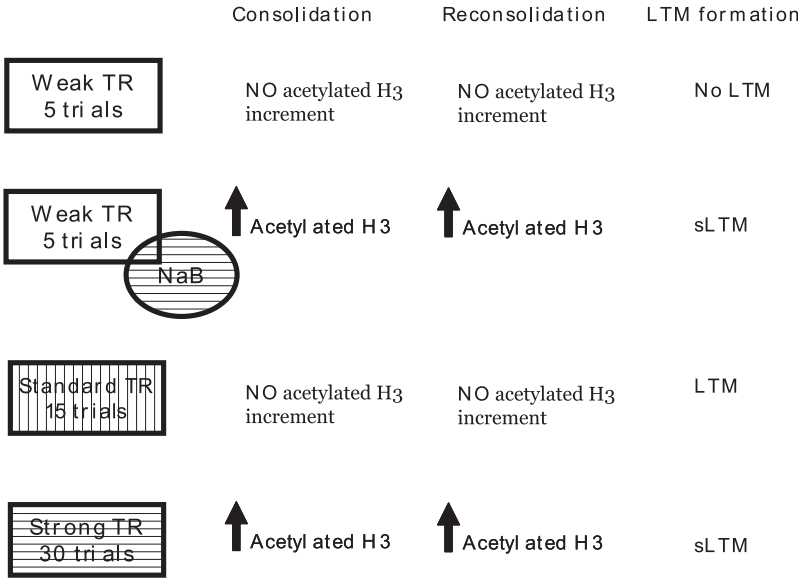

Fig. 6. Outline of histone H3 acetylation involvement in consolidation and reconsolidation in function of the strength of the memory. Schematic representation of present and previous (Federman et al., 2009) results were included.

HDAC inhibitor during consolidation, reactivation of this memory induces an increment in histone H3 acetylation during reconsolidation (Fig. 3), as it was found for strong memories. This finding suggests that when histone acetylation is increased during consolidation, the resultant memory involves histone acetylation again during reconsolidation. Moreover, the kinetics and the levels of acetylation during consolidation and reconsolidation (Figs. 1 and 3) are similar for both strong and pharmacologically improved memories. Taking into consideration that chromatin acetylation positively regulates transcription, our results suggest that gene transcription enhancement induced by the HDAC inhibitor during consolidation may enable enough amount of mRNA synthesis required to transform a weak memory trace in a sLTM. However, we cannot rule out that the increased acetylation induced by HDAC inhibition of other nuclear proteins different from histones could contribute to the memory improvement effect observed here.

Studies using contextual fear conditioning in mice demonstrated that memory resulting from strong training shows greater resistance to reconsolidation induction than that generated by weak training (Suzuki et al., 2004). Similarly, a recent study showed that a stressful experience prior to a learning session is able to reinforce the memory which, in such circumstances, becomes resistant to reconsolidation (Bustos et al., 2010). In contrast, we found here that sLTM reconsolidation can be induced by the same conditions (5 min of re-exposure) than a standard LTM (Fig. 1). Furthermore, both LTM and sLTM are similarly expressed at the testing session. However, we found that they are different in terms of their strength. The strength of a memory can be characterized by its persistence, its capability to be reactivated and updated under certain parametric conditions at the time of recall, and its resistance to extinction. In the crab memory model, it is not possible to test memory persistence for more than 5 days after training because the control groups' response level at that time point goes down. At 5 days after training both LTM and sLTM still persist. Resistance to extinction is often a sensitive measure of the strength of acquisition (Annau and Kamin, 1961; Tully

and Quinn, 1985). We demonstrated here that sLTM can be differentiated from LTM by their ability to resist extinction (Fig. 5) and, thus, memories generated by 15 or by 30 trials differ at the behavioral level. Furthermore, we distinguished LTM and sLTM at a molecular level through differences in histone acetylation induction. Accordingly, we found that sLTM requires histone acetylation. Effectively, the inhibition of HAT activity by means of the drug c646 resulted in memory impairment for sLTM but not for LTM (Fig. 2). The covalent modification of histones can be reflected in differences in the gene expression pattern, in either quantity or quality during consolidation and reconsolidation, allowing the difference between LTM and sLTM. Given that in the present work we only analyzed the general chromatin acetylation levels, further analysis is necessary to understand the involvement of this mechanism affecting particular genes in relation with specific memory processes.

In summary, taking together our present study and previous results, we postulate the existence of three types of CSM: (1) a weak memory, which is able to be enhanced by means of treatments like HDAC inhibitors administration during consolidation, (2) a LTM, which is reactivated by retrieval and is expressed in a long-term form, and (3) a sLTM which is reactivated by retrieval, expressed in a long-term form, resistant to extinction (and probably more persistent) and mechanistically characterized by a transient histone acetylation increment during the consolidation and reconsolidation phases. Our present results show that a weak memory can also be transformed into a sLTM by increasing the level of histones acetylation during consolidation (Fig. 3), but not by increasing this mechanism during reconsolidation (Fig. 4). Ongoing studies are aimed at determining whether this molecular signature of strong memories is present in mammalian memory models, in order to assess if such mechanism is evolutionarily conserved and plays a role affecting main characteristics of the memory traces such as persistence, reactivation susceptibility and extinction sensitivity.

CONCLUSIONS

The results present here support that histone acetylation is an evolutionary-conserved mechanism involved in memory reconsolidation. The present work shows evidence that genome marking through histone acetylation is a molecular feature of the gene transcription required for reconsolidation of strong memories.

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