Research

Histone acetylation is recruited in consolidation as a molecular feature of stronger memories

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Gene expression is a key process for memory consolidation. Recently, the participation of epigenetic mechanisms like histone acetylation was evidenced in long-term memories. However, until now the training strength required and the persistence of the chromatin acetylation recruited are not well characterized. Here we studied whether histone acetylation is involved in consolidation in invertebrates, whether it depends on the training strength, and whether it is a permanent or transient mechanism. We used a well-characterized memory model in invertebrates, the context-signal memory in crabs. Our results show no changes in histone 3 (H3) acetylation 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, the administration of histone deacetylase inhibitors sodium butyrate (NaB) and trichostatin A allowed a weak training to induce long-term memory. NaB enhanced memory in two phases during consolidation. These findings support that H3 acetylation (1) is involved in consolidation, (2) occurs only after strong training, (3) is a transient process, and (4) memory is enhanced in two phases. The coincidence of these phases with other mechanisms of gene expression is discussed.

Regulation of gene expression is postulated as a critical process for long-lasting storage of memories (Agranoff et al. 1967; Goelet et al. 1986). In order to persist, memory storage involves changes in the gene expression pattern. Involvement of epigenetic mechanisms was postulated to provide potentially stable marks in the genome for continuous regulation of specific gene expression (Tsankova et al. 2004; Kumar et al. 2005) (for review, see Colvis et al. 2005; Hsieh and Gage 2005; Levenson and Sweatt 2006; Barret and Wood 2008). Chromatin structure and function can be affected by various post-translational modifications of the amino-terminal tails of nucleosomal histones (Strahl and Allis 2000; Kouzarides 2007). Lysine acetylation is one of the best-characterized histone modifications. Chromatin modifying enzymes, which carry out protein acetylation and deacetylation, have already been described (Sterner and Berger 2000): histone acetyl transferases (HATs), such as CREB-binding protein (CBP/p300) and histone deacetylases (HDACs), respectively. Histone acetylation induces transcription activation by increasing DNA accessibility to the transcription machinery (Norton et al. 1989; Vettese-Dadey et al. 1996). Recruitment of epigenetic mechanisms, such as histone acetylation, methylation, and phosphorylation, has already been described for long-term plasticity and for vertebrate memory consolidation (Guan et al. 2002; Alarcon et al. 2004; Korzus et al. 2004; Wood et al. 2005, 2006a). In agreement with such findings, HDAC inhibitors' administration improved long-term memory in rats and mice (Levenson et al. 2004; Yeh et al. 2004; Fischer et al. 2007; Vecsey et al. 2007) and reverted memory deficits induced by genetic manipulation of the CBP gene (Alarcon et al. 2004; Korzus et al. 2004).

The interaction between transcription factors and chromatin is regulated by means of histone acetylation–deacetylation. In such a way, stable changes in gene expression are achieved by this regulation, which could be an important mechanism in the

consolidation process for the stability of long-term memory. However, to our knowledge, the steady state of the chromatin has not been evaluated, either during or after memory consolidation. In addition, although the first evidence of histone acetylation in neural plasticity was obtained in the mollusk *Aplysia*'s long-term facilitation of the sensory-motor neurons synapses (Guan et al. 2002), until now no direct evidence has been reported for chromatin modifications in long-term memory in invertebrates. Here we evaluate whether acetylation of histones is involved in memory consolidation and whether memory can be improved by HDAC inhibition in an invertebrate model. For such purposes, we made use of a well-characterized memory model, the context-signal memory (CSM), in the crab *Chasmagnathus*. In CSM, repeated presentation of a visual danger stimulus (an opaque screen that moves above the animal) provokes the fading of the initial escape response, which is actively replaced by a freezing response (Lozada et al. 1990). Fifteen or more spaced danger stimulus presentations induce an association between the iterated stimulus and contextual features. A long-term memory is formed, which lasts at least for a week and entails de novo protein synthesis and mRNA synthesis (Pedreira et al. 1996), activation of camp-dependent protein kinase (PKA) (Locatelli et al. 2000, 2002), activation of extracellular-signal-regulated kinase (ERK) (Feld et al. 2005), and activation of the NF- κ B transcription factor (Freudenthal and Romano 2000; Merlo et al. 2002).

The aim of the present work is to define the necessary training parameters to induce histone acetylation and to describe the time course of this process during memory formation. Using the crab memory paradigm we found that histone 3 (H3) is acetylated 1 h after training in the central brain during CSM consolidation. This change was induced only after strong training. In agreement with these findings, administration of the HDAC inhibitor sodium butyrate (NaB) produced memory enhancement, allowing a weak training protocol of five trials to induce long-term memory. Our results suggest that histone acetylation is recruited in consolidation as one of the molecular features of stronger memories.

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Results

Effect of the HDAC inhibitor sodium butyrate (NaB) on long-term memory

Sodium butyrate (NaB) is a widely used HDAC inhibitor. Previous reports indicated that inhibition of HDACs induces memory facilitation in mammalian memory models (Levenson et al. 2004; Yeh et al. 2004; Fischer et al. 2007; Vecsey et al. 2007). In order to evaluate whether the inhibition of HDACs induces memory enhancement in the crab CSM, we administered NaB at different time points pre- and post-training.

In the first experiment, a group of crabs was injected with NaB, while another group was injected with saline solution (SS). Immediately after the injection, all were placed in the actometers. Half of the animals of each group were trained (TR) 10 min later and the other half remained untrained as controls (CT), producing four groups, CT-SS, TR-SS, CT-NaB, and TR-NaB, of 40 crabs each. Figure 1A shows the results of the training session, indicating that drugs injected before training did not affect the acquisition phase (repeated-measures ANOVA, nonsignificant). Twenty-four hours later, all groups were tested. As shown in Figure 1B, nonsignificant differences were found between CT-SS and TR-SS, as expected for a weak training. On the contrary, comparison of CT-NaB and TR-NaB disclosed significant differences ($F_{(1,140)} = 4.2$; $P < 0.05$). These last statistical differences suggest that HDAC inhibitor caused memory enhancement by allowing a weak training to induce long-term retention. However, the comparison between TR-SS and TR-NaB showed no significant differences, suggesting that the memory facilitatory effect of NaB would be partial at this time point.

In the following experiment with the same groups' design, crabs were injected with NaB or SS immediately after training. As shown in Figure 2A there are no differences between CT-SS and TR-SS, but significant differences between CT-NaB vs. TR-NaB were found ($F_{(1,108)} = 4.0$; $P < 0.05$), indicating a memory enhancement effect of the drug. Unlike the previous experiment, there were also significant differences between TR-SS and TR-NaB ($F_{(1,108)} = 3.8$; $P < 0.05$), supporting a strong memory-enhancement effect of the HDAC inhibitor injection at this time point.

In the third experiment, animals were injected 3 h after training and, in this case, no significant differences were found for any pair of CT-TR group comparisons (Fig. 2B). The result of this experiment suggests that the time window for NaB facilitation effect in consolidation is shorter than 3 h. However, in previous studies in the crab CSM, a second phase of PKA and NF- κ B activity in consolidation was found (Freudenthal and Romano 2000; Locatelli and Romano 2005), and experiments with drugs that

inhibit PKA and NF- κ B activity showed a second phase of drug action during consolidation, around 6 h after training. Thus, we evaluated a possible second phase of memory facilitation by NaB in a fourth experiment in which the animals were injected 6 h after training. In agreement with previous findings, we found no differences between CT-SS and TR-SS, but significant differences between CT-NaB vs. TR-NaB ($F_{(1,106)} = 9.3$; $P < 0.01$), and between TR-SS and TR-NaB ($F_{(1,106)} = 4.8$; $P < 0.05$; Fig. 2C) supporting, once again, a strong memory enhancement effect of the HDAC inhibitor injection.

Finally, we performed an experiment with the same groups' design, but injecting animals at 12 h after training. In this experiment no significant differences were found in any group comparisons (Fig. 2D), suggesting that at 12 h the time window of HDAC inhibitor effect is closed, and thus it is not possible to induce a memory enhancement by injecting NaB.

In the five experiments presented in this first section, no differences were found in the CT-Sal vs. CT-NaB comparisons, indicating that the drug did not alter normal levels of response of untrained animals, demonstrating nonspecific effects.

Effect of trichostatin A on long-term memory

While NaB inhibits HDACs, it is also a putative inhibitor of chloride channels and phosphodiesterases (Resta-Lenert et al. 2001). Therefore, we evaluated the effect of an HDAC inhibitor structurally unrelated with NaB, Trichostatin A. We made the experiment with the same groups' design as in the case of NaB injections, but the crabs were injected with doses of TSA or DMSO as vehicle immediately after training. In agreement with the findings in the experiments with NaB injection immediately post-training (Fig. 2A), we found no differences between CT-DMSO and TR-DMSO groups, but significant differences between CT-TSA vs. TR-TSA ($F_{(1,122)} = 7.12$; $P < 0.01$; Fig. 3). Similar to the pretraining injection of NaB (Fig. 1), the comparison between TR-DMSO and TR-TSA shows no significant differences, suggesting that at this time point the facilitatory effect is partial. Nevertheless, this result supports that the memory-enhancement effect of NaB is due to inhibition of HDACs.

Effect of NaB injection on the level of histone acetylation in the crab's central brain

Previous experiments suggest that HDAC inhibition by NaB administration induced an increment in chromatin acetylation. As a consequence of the increased level of histone acetylation, long-term memory is improved, probably due to an enhancement of gene expression. In order to obtain evidence that histone acetylation is increased in the crab brain after NaB injection, we performed the following experiment: Three groups of 20 animals were injected with NaB at the same dose used in all of the experiments of the first two sections. At 15, 30, or 60 min after injection, animals were sacrificed and the central brain was dissected. A noninjected naïve group (NV) and a SS-injected group sacrificed at 15 min were included as controls. Histone-enriched protein extracts were obtained from a pool of 20 central brains for each group and Western blots were performed using acetyl-specific H3 antibody. The intensity of the specific band obtained with this antibody was estimated by densitometry, relative to the densitometric values obtained with an H3 total antibody. Figure 4A shows mean $\pm$ SE of the relative optic density (ROD) values with respect to

Figure 1. Effect of pretraining injection of the HDAC inhibitor sodium butyrate (NaB) during memory consolidation. (A) Training session after pretraining drug injection. (B) Escape response at testing session with pretraining injection. Mean response level $\pm$ SEM. (*) $P < 0.05$.

Figure 2. Effect of immediate, 3-, 6-, and 12-h post-training injections of sodium butyrate (NaB) during memory consolidation. (A) Escape response at testing session with immediate post-training injection. (B) Escape response at testing session of the 3-h post-training injection experiment. (C) Escape response at testing session of the 6-h post-training injection experiment. (D) Escape response at testing session of the 12-h post-training injection experiment. Mean response level $\pm$ SEM at testing. (*) $P < 0.05$; (**) $P < 0.01$.

the NV group from five independent experiments. A significant increase of H3 acetylation was found 15 min (general ANOVA $F_{(3,14)} = 4.9$, $P < 0.01$; Duncan test, $P < 0.01$) and 30 min after NaB injection (Duncan test, $P < 0.05$) in comparison with SS-15 min, decreasing at 60 min after injection (Duncan test, NS).

Time course of histone acetylation during memory consolidation

The following experiments were aimed at evaluating the level of H3 acetylation during memory consolidation. In the first series of experiments, crabs were trained (TR group) with the standard 15 trials of training protocol that induces long-term memory (Pedreira et al. 1998) or remained in the actometers without stimulation (CT group). A naïve (NV) group was added, as described in the Materials and Methods section. Twenty crabs per group were used. These three groups were repeated for each time point after training: 0, 1, 6, and 24 h. Four independent experiment repetitions were carried out for each time point. As showed in Figure 4B, no changes were found in the mean level of H3 acetylation with respect to the mean basal level of NV at any time after standard training.

In the second series of experiments 30 trials of strong training protocol were used. In these experiments the procedure used was the same as in the first series. Figure 5 shows the results in which a significant increase in H3 acetylation level was only found 1 h after training in the TR group compared with the NV and CT groups (general ANOVA, $F_{(2,6)} = 7.7$, $P < 0.05$; Duncan test, $P < 0.01$ and $P < 0.05$, respectively). Comparison between NV and CT showed no significant differences.

Thus, the level of H3 acetylation was increased in the crab brain 1 h after a strong training protocol, but not after a standard

training, and this increment was transient, returning to basal levels 6 h after training.

Levels of histone acetylation during memory facilitation by HDAC inhibition

In order to gain insight into the modulation of memory formation by HDAC inhibition, we studied whether a weak training protocol plus a HDAC inhibitor injection creates an increase of histone H3 acetylation as strong training does. A noninjected naïve group (NV) and a weak training protocol plus a SS injection (weakTR+SS) group were used as controls. Since we have seen significant differences during the consolidation of strong memory at 1-h post-injection (Fig. 5), we measured histone H3 acetylation at this time point after weak training. Four independent experiment repetitions were carried out. Figure 6 shows the results in which a significant increment of H3 acetylation level was found 1 h after weak training in the NaB (weakTR+NaB) group compared with both control groups (Student's t -test, $P < 0.05$). Thus, HDAC inhibition facilitates memory by means of increasing the histone H3 acetylation level 1 h after a weak training protocol.

Discussion

In the present work two independent lines of evidence support that histone acetylation is required in consolidation for stronger memories. The first line of evidence demonstrates that the HDAC inhibitor NaB, which increases histone acetylation (Fig. 4A), enhanced long-term memory during the consolidation phase. Systemic delivery of NaB prior to or after a weak training allowed for retention 72 h later (Figs. 1, 2A). On the contrary, animals administered saline solution showed no retention, as expected for the weak training used. Furthermore, we found that a weak training did not, in fact, induce histone H3 acetylation in the saline-injected group, but the injection of NaB increased H3 acetylation 1 h after weak training (Fig. 6), similar to the increment found after strong training without injection (Fig. 5).

Figure 3. Effect of immediate post-training injection of the HDAC inhibitor TSA during memory consolidation. Escape response at testing session with immediate post-training injection. Mean response level $\pm$ SEM at testing. (**) $P < 0.01$.

Figure 4. Levels of histone H3 acetylation in the crab brain. (A) Effect of sodium butyrate (NaB) injection on the level of histone acetylation. Mean $\pm$ SEM of ROD values of the specific acetyl histone H3 band normalized to NV group mean value. Results obtained from five independent experiments. Duncan test, (*) $P < 0.05$, (**) $P < 0.01$. (B) Time course of histone acetylation during memory consolidation after standard training. Mean $\pm$ SEM of ROD values of the specific acetyl histone H3 band normalized to NV group mean value. Results obtained from four independent experiments; Duncan test.

It is known that NaB inhibits Class I (nuclear localization) and Class II (shuttle between nucleus and cytoplasm) HDACs, resulting in increased histone acetylation and, therefore, facilitating gene transcription (Marks et al. 2004; Dokmanovic and Marks 2005). Gene expression is a critical requirement for the conversion of a labile short-term memory into a consolidated long-term memory. In a previous work, we found that a weak training protocol of five trials is unable to recruit sufficient activation of a transcription factor, NF- κ B (Freudenthal and Romano 2000), in order to induce long-term memory. Our present results suggest that gene-transcription facilitation induced by the HDAC inhibitor may enable the amount of mRNA synthesis required to transform a weak memory trace in a stronger long-term memory. Furthermore, NaB showed two phases of facilitation after training, the first one when administered immediately after training and the other 6 h after training. This effect was not found at 3 or 12 h after training. These two time windows for drug effect fit well with two phases of PKA activity after training and two time windows for PKA inhibitor effect found in this memory model (Locatelli et al. 2002; Locatelli and Romano 2005), suggesting that camp-dependent gene expression is involved in these two phases. Similarly, two phases of NF- κ B activation after training (Freudenthal and Romano 2000) and two time windows of NF- κ B inhibitor effect (Merlo et al. 2002) were found within the same time periods. Our results suggest that an increased H3 histone acetylation level by NaB injection during these two phases could compensate the lack of

NF- κ B-dependent transcriptional induction after a weak training (Freudenthal and Romano 2000). This increment allows for the basal transcriptional activity of this transcription factor, as well as others, to induce long-term memory formation.

In the second line of evidence, we found increased levels of H3 acetylation during CSM consolidation only after the strong training protocol. The antibody used for acetylated H3 detection was built to recognize acetylated lysines 9 and 14, both modifications previously associated with transcription activation in different taxa. There are two remarkable characteristics of this acetylation increment in consolidation: (1) Such an increment occurs only after 30 trials of strong training, indicating that not all long-term memory processes recruit this epigenetic mechanism, but only those that could induce a more persistent memory. This finding has an important interpretative consequence because all long-term memory is considered to require gene expression, and chromatin acetylation is indeed involved in transcription. Why then should there be differences between memory mechanisms induced by standard and strong training? We consider that there are genes normally accessible for the transcriptional machinery, while others require chromatin modifications to make them accessible to transcriptional functions (Saccani et al. 2001). Additionally, it has recently been shown that some genes, such as neural cell adhesion molecules (NCAM), are basally accessible in regulatory regions but undergo intragenic region histone modifications in

order to change their transcription and splicing (Schor et al. 2009). Therefore, our findings support the idea that some genes or genic regions are made accessible only after a great induction, and this change of expression of such genes contributes to a more persistent memory formation. Due to the fact that we performed a bulk analysis, we could not determine whether there is any change in the level of histone acetylation in specific promoters after the standard protocol of training. Further experiments using the examination of the acetylation process in particular promoters will be necessary to fully understand this chromatin modification mechanism during memory formation and to evaluate the differences obtained between standard and strong protocols used; (2) the H3 acetylation increment is transient, decreasing to basal levels 6 h after training. This is the first study of a time course for histone acetylation in memory consolidation, showing the temporary nature of the regulation of this molecular process during memory formation. Memory strength is associated with different gene-expression patterns under the influence of modifications in the chromatin structure of those genes. Our results do not rule out that a mechanism of acetylation of other residues in H3 histone or of other nucleosomal histones can account for the transcription during consolidation of weaker memories, as was the case in 15 trials of training. There is evidence that suggests the existence of an epigenetic code that regulates specific gene-expression profiles for different memory formation processes (Wood et al. 2006b).

Figure 5. Time course of histone acetylation during memory consolidation after strong training. (A) Mean $\pm$ SEM of ROD values of the specific acetyl histone H3 band normalized to total H3 antibody ROD values and to NV group mean value. Results obtained from four independent experiments. Duncan test, (*) $P < 0.05$, (**) $P < 0.01$. (B) Representative Western blot of protein extracts from 1-h post-training animals performed with acetyl-specific H3 (top) and total H3 (bottom) antibodies.

Although NaB is a widely used drug to study the effect of HDAC inhibition on memory, it has been found in other effects not related to histone acetylation (Resta-Lenert et al. 2001). The fact that TSA injection also induced memory enhancement (Fig. 3) supports the action that NaB on memory is due to HDAC inhibition. Moreover, here we found increasing H3 acetylation at 1 h after weak training in the group injected with NaB (Fig. 6) in marked correlation with the increment induced by strong training (Fig. 5). These results suggest that the facilitatory effect of the drug is explained, at least in part, by increasing chromatin acetylation. Nevertheless, it is predictable that the increased acetylation induced by HDACs inhibition of other nuclear proteins different from histones could contribute to the memory facilitation effect observed. Although we did not explore this in the present study, we propose that the transcription factor NF- κ B, a putative substrate of this enzyme (Sterner and Berger 2000; Yeh et al. 2004), could be another transcriptional regulation point that explains part of the effect that was found.

Our finding of an enhancing effect of HDAC inhibitors is consistent with previous results obtained in rodent memory models (Levenson et al. 2004; Yeh et al. 2004; Fischer et al. 2007; Vecsey et al. 2007). The results presented here and previous evidence in synaptic plasticity models in invertebrates, together with the previous evidence in vertebrates, support that histone acetylation is an evolutionarily conserved mechanism for gene expression regulation in consolidation of long-lasting memories.

Materials and Methods

Animals

Adult male *Chasmagnathus granulatus* intertidal crabs, 2.6–2.9 cm across the carapace, weighing 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 ($30 \times 45 \times 20$ cm) filled to 0.5-cm depth with diluted (12%, pH 7.4–7.6) marine water (prepared from Cristalsea Marinemix salts), 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). The temperature of both holding and experimental rooms was maintained within a range of from 22°C to 24°C. Experiments were carried out between the third and the tenth day after the animal's arrival. Each crab was used in only one experiment. Experiments were carried out in accordance with the local regulations 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 is described in detail elsewhere (Romano et al. 1990). Briefly, it consists of a bowl-shaped plastic container in which the crab is lodged and an opaque rectangular screen that moves horizontally above the animal. Screen displacements evoke the crab's running response, and, as a consequence, container vibrations that induce electrical signals through four piezoelectric transducers attached to the external surface of the container. Signals recorded during a trial were translated into numerical units ranging from 0 to 8000. The experimental room had 40 units, separated from each other by partitions. A computer was used to program trial sequences, trial duration, and intertrial intervals, as well as to monitor experimental events.

Figure 6. Levels of histone acetylation during memory facilitation by HDAC inhibitor injection. (A) Mean $\pm$ SEM of ROD values of the specific acetyl histone H3 band normalized to total H3 antibody ROD values and to NV group mean value. Results obtained from four independent experiments. Duncan test, (*) $P < 0.05$. (B) Representative Western blots performed with acetyl-specific H3 (top) and total H3 (bottom) antibodies.

Drugs and injection procedure

Fresh sodium butyrate (NaB) (Sigma) was dissolved in crustacean saline solution (SS) (Hoeger and Florey 1989). Fifty microliters of vehicle or 15 mM of drug solution (83 μ g per crab, 4.8 μ g/g) were 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 Zubkoff 1977) resulting in an approximate 100-fold dilution of the drug in hemolymph. Trichostatin A (TSA) (Sigma) was dissolved in dimethylsulfoxide (DMSO). Ten microliters of vehicle or 6 μ g/ μ L of drug solution (60 μ g per crab, 3.5 μ g/g) were given.

Procedure in memory evaluation experiments

Each experiment lasted about 4 d and included two phases: training session (day 1) and testing session (day 4). Crabs were individually housed during the intersession 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 drug on memory we used a weak training protocol that does not induce long-term memory. The training session consisted of five trials with an intertrial interval (ITI) of 171 sec. Each trial lasted 9 sec and consisted of two cycles of presentation of the screen over the actometer. Each cycle lasted 2.5 sec, with 2 sec of interval between cycles. The crab's activity was recorded during the entire trial time. 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 the training session is referred to as the training context. In all experiments, one group was trained (TR group), while the other was located in the device but remained untrained (control group, CT group). Each experiment consisted of a pair from the CT-TR group injected with the vehicle (SS pair or DMSO pair) and another pair injected with the drug (NaB pair or TSA pair), $n = 30$ –40 for each group.

Data analysis in drug effect evaluation

Retention of learning acquired during training was considered when a statistically lower level of response in the testing session was found for the TR group relative to the CT group injected with the same solution (drug or vehicle). This criterion came from previous experiments performed in our laboratory in which a significant difference (t -test, $\alpha = 0.05$) between trained (TR) and untrained groups (CT) was invariably disclosed at testing session 24 h or more, after 15 or more trial training protocols (ITI = 171 sec). Such significant differences were also found when crabs were injected with vehicle at various pre- and post-training intervals. Accordingly, predictions were for a significant difference between CT and TR groups in the testing session. Therefore, throughout this study results of the behavioral study were analyzed with a priori-planned comparisons using a weighted-means ANOVA with α (per comparison error rate) = 0.05, according to the standard method (Howell 1987). The lack of difference between CT and TR groups is thus assumed as no memory retention. A comparison between CT groups injected with drug or vehicle was necessary in order to determine incidental drug effects affecting response levels at testing in a way not related to the training experience. In general, the statistical analysis of testing data included a set of three a priori-planned comparisons, namely, CT-Vehicle vs. TR-Vehicle, CT-Drug vs. TR-Drug, and TR-Vehicle vs. TR-Drug, using a weighted-means ANOVA with α (per comparison error rate) < 0.05 (Howell 1987). In the first comparison, no difference between the CT-Vehicle vs. TR-Vehicle groups was expected due to the use of a weak training protocol in the last group. On the contrary, if the drug enhanced memory, differences in the comparisons between CT-Drug and TR-Drug groups and between TR-Vehicle vs. TR-Drug groups were expected.

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

Crabs were trained either using the standard training protocol of 15 trials (45-min duration) or the strong training protocol of 30

trials (90-min duration), both of which induce long-term memory, but the change in behavior is more pronounced at testing using the strong protocol (Freudenthal et al. 1998; Pedreira et al. 1998). In the consolidation-phase experiments, crabs were sacrificed at different time points after training (0, 1, 6, and 24 h) and the central brain (supraesophageal ganglion) was then dissected. Animals were lodged in individual containers with dim light during the time interval between training and their sacrifice. For each time point and training condition, a CT group was used. In the HDAC inhibitor effect experiments, animals were injected with 15 mM NaB or SS and were sacrificed 15, 30, or 60 min after injection. In the experiments for HDAC inhibitor effect after weak training protocol, crabs were trained using five trials of training and injected with 15 mM NaB or SS immediately after that. Animals were sacrificed 1 h after injection. In all of the experiments the acetyl-H3 level was determined and values were normalized to an untreated naïve group (NV) performed simultaneously with the other groups.

Histone-enriched extracts obtaining and Western blot assay

Animals were anaesthetized by immersion in ice-cold water for two min. The central brain was then dissected. Twenty ganglions per sample were pooled in 1 mL of buffered crab saline solution (pH 7.6). Nuclear protein extracts enriched in histones were obtained as follows: All of the procedures were performed at 4°C. Dissected supraesophageal ganglions were homogenized in buffer A (HEPES 10 mM pH 7.8 Cl₂Mg 1.5 mM KCl 10mM DTT 1mM, 5mM sodium butyrate). Tissue homogenates were centrifuged at 1000g for 15 min. The pellet was resuspended in buffer A and H₂SO₄ 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 5 min at 15,000 rpm. The supernatants were transferred to a fresh tube and proteins were precipitated with a 10 $\times$ vol of acetone at -20°C , overnight. Precipitated proteins were collected by centrifugation at 15,000 rpm for 15 min. Pellets were resuspended in distilled H₂O. For Western blot assay, loading buffer was added and samples were incubated at 100°C for 5 min and immediately placed on ice. Ten micrograms of each protein sample were run on PAGE with 15% acrylamide in the resolving gel. Proteins were then electro-transferred to PVDF for immunoblotting. Western blot assay was performed with acetyl-specific H3 (recognizes acetylated K9 and K14) and total H3 (CT, pan) antibodies (Upstate) following the manufacturer's protocol. The antibodies were built using a peptide corresponding to amino acids 1–20 of *Tetrahymena* histone H3 as immunogen, both of them having known cross-reactivity with human, mouse, rat, yeast, and chicken. Broad species cross-reactivity was expected due to high sequence homology. Detection was made with a Luminol chemiluminescence kit (SCB) 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 1.29 $\times$ software. Values of ROD for the acetyl-specific H3 band were relative to ROD for a total H3 in each sample.

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