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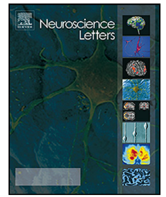

Research article

Enhancement of memory consolidation by the histone deacetylase inhibitor sodium butyrate in aged rats

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HIGHLIGHTS

- Sodium butyrate produced memory enhancement and persistence in aged rats.
- Memory formation in younger rats was not affected by sodium butyrate.
- Aged rats with normal memory might be particularly sensitive to sodium butyrate.

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ABSTRACT

Here we show that a systemic injection of the histone deacetylase inhibitor (HDACi) sodium butyrate (NaB) immediately after training in a step-down inhibitory avoidance task produced an enhancement of memory consolidation that persisted across consecutive retention tests during 14 days in aged rats, while it did not significantly affect memory in young adults. Control aged and young adult rats showed comparable basal levels of memory retention. Our results suggest that HDACis can display memory-enhancing effects specific for aged animals, even in the absence of age-related memory impairment.

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1. Introduction

Over the last decade, several studies have demonstrated that transcriptional regulation involved in the formation of long-term memories (LTM) needs the synchronized interaction of several transcription factors and transcriptional co-activators in the chromatin structure [1,2]. It is well established that epigenetic mechanisms, such as histone acetylation, orchestrate molecular events during LTM formation by relaxing or condensing the chromatin structure altering gene transcription [3]. Proteins named histone acetyltransferases (HATs) add acetyl groups to lysine residues of histones and are responsible for the modulation of the histone-DNA interactions. The chromatin structure relaxation leads to enhanced transcription which is a reversible process by the action of histone deacetylases (HDACs). HDACs acts by removing the acetyl group from lysine residues of histones and non-histone proteins favoring the closed repressive state of chromatin [4].

Pharmacological treatment with histone deacetylase inhibitors (HDACis), such as trichostatin A and sodium butyrate (NaB), induce a histone hyperacetylated state regulating the accessibility of chromatin to the transcription machinery, affecting gene expression [5] and the essential mechanisms acting in neurological diseases as well as those underlying memory formation [6–8].

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HDACis were first designed as anticancer agents [5] and the treatment with HDACis have been shown to enhance memory and ameliorate deficits in aged rats and experimental models of memory dysfunction [8,9,10,11]. Recent evidence has demonstrated epigenetic alterations in specific brain areas of aged animals [9,12] that may play a crucial role in aging being correlated to diseases, such as diabetes, cancer, neurodegenerative and psychiatric disorders [13]. In the present study we sought to establish whether HDAC inhibition by an acute systemic treatment with NaB affects LTM formation for a one-trial inhibitory avoidance (IA) task in aged rats.

2. Materials and methods

Young adult (3 months) or aged (20–24 months) male Wistar rats were obtained from our institutional certified breeding colony (CREAL-UFRGS). Animals were housed three per cage in plastic cages with sawdust bedding and maintained on a 12 h light/dark cycle at a room temperature of 22 ± 1 °C. The rats were allowed *ad libitum* access food and water. Experiments using aged and young rats were carried out separately and took place between 8 AM and 4 PM. All experimental procedures were performed in accordance with the Brazilian Guideline for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI) and were approved by the institutional animal care committee under protocol number 12-0424.

Animals were allowed to acclimate to the laboratory for 2 h before any experimental manipulation. One week before experimental manipulation animals were handled once a day every 2 days during home-cage cleaning. We used the single-trial step-down IA conditioning as an established model of fear-motivated memory. In step-down IA training, animals learn to associate a location in the training apparatus (a grid floor) with an aversive stimulus (foot-shock). The general procedures for IA training and retention test were described in previous report [14]. On training trials, rats were gently placed on the platform facing the left rear corner of the apparatus box and their latency to step down on the grid with all four paws was measured. Immediately after stepping down on the grid, rats received a 0.4-mA, 3.0-s foot shock and were removed from the apparatus. Retention test trials took place at different intervals after training. No foot shock was presented during retention test trials. No cut-off value of step-down latencies for the training session was assigned. A ceiling of 300 s was imposed on retention test measures. Step-down latencies on the retention test trials were used as a measure of IA memory retention. At the 21 day, rats were given a mild reminder shock (0.3-mA, 3 s), followed by a retention test 24 h later. Immediately after training rats received a single intraperitoneal (i.p.) injection of saline (NaCl 0.9%) or NaB (1.2 g/kg; Sigma, St. Louis, MO, USA) dissolved in saline in a 1.0 ml/kg injection volume. The dose of NaB was chosen on the basis of previous studies [10,11,15]. Rats were tested for memory retention 1 day after training and subsequently they were submitted to test sessions daily until 7 days. Animals were tested again at 14 days and 21 days after training. Additional test and reminder shock session were performed at 23 and 24 days after training.

Western blot analysis was performed as previously described [11,16]. Histones were extracted from hippocampal brain region of aged rats that were systemically treated with SAL or NaB immediately after training and euthanized 1 h after injections. Rats were trained in the IA learning task (TRAIN group), exposed to the context alone (1 min habituation in task chamber, HAB group) or exposed to aversive stimulus alone (04-mA 3-s shock, SHOCK group). The tissue was stored at −80 °C. The samples were homogenized ($n = 4$ per group) in 400 μl of 50 mM Tris, pH 7.0, 1 mM EDTA, 100 mM NaF, 0.1 mM PMSF, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail (P2714; Sigma–Aldrich). After 20 min on ice, samples were centrifuged at 12,000 rpm for 1 min. The supernatant was collected and the same volume of 0.2-N HCl was added. Acid extraction of histones was carried out over night at 4 °C then samples were centrifuged at 6500 g for 10 min at 4 °C. The supernatants were diluted 1/1 (v/v) in 100 mM Tris, pH 6.8, 4 mM EDTA, and 8% SDS and were then boiled for 5 min. The protein content was determined by the method of Lowry modified [17]. Thereafter, the loading buffer (40% glycerol, 100 mM Tris, bromopehnol blue, pH 6.8, 8% β-mercaptoethanol) was added to the sample. Twenty-five μg total protein was separated on a 10% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 (TBS-T) and were incubated overnight with the following antibodies: β-actin at 1:3000, H3 at 1:3000, acetyl-H3K14 at 1:1000; acetyl-H3K9 at 1:500 (ab34731, ab1791, ab52946, ab10812; Abcam, San Francisco, CA, USA). Thereafter, the membranes were incubated with goat anti-rabbit (ab6721, HRP) radish-conjugated secondary antibodies and reactions were developed by chemiluminescent substrate (LumiGlo). All steps were followed by three washes with TBS-T. The bands were quantified using the Scion Image® software, which is a derivative of NIH Image (Frederick, MD, USA). Total protein levels in the blotting were normalized according to each sample's β-actin protein levels and the results were expressed as a ratio of acetylated H3 residues to total H3.

Data are expressed as mean ± SEM. Comparisons between groups were performed using a Kruskal–Wallis analysis of variance followed by Mann–Whitney *U*-tests. Comparisons between trials within the same group were performed by Wilcoxon signed-rank test. Western blotting data were analyzed using an ANOVA followed by a Tukey's multiple comparison test. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

3. Results

The effects of intraperitoneal administration of NaB immediately after training on the retention and persistence of IA memory for aged rats are shown in Fig. 1. There was no significant difference between rats given SAL and NaB in training performances ($P > 0.05$; U 33.000). All rats were tested for retention 1 (Test 1d), 2 (Test 2d), 3 (Test 3d), 4 (Test 4d), 5 (Test 5d), 6 (Test 6d), 7 (Test 7d), 14 (Test 14d), 21 (Test 21d) and 23 (Test 23d) days after training. Immediately after Test 23d, rats were given a reminder foot shock and tested again 1 day later. Statistical comparison using Wilcoxon signed-rank test showed that animals in both groups displayed significant memory retention on Test 1d compared to training ($P < 0.001$ for SAL group and $P < 0.01$ for NaB group). Further analysis with Mann–Whitney *U*-tests showed that there were significant differences between SAL-treated rats and rats given NaB in Test 1d ($P < 0.01$; U 103.000), Test 2d ($P < 0.05$; U 94.000), Test 3d ($P < 0.01$; U 101.000), Test 4d ($P < 0.05$; U 90.000), Test 5d ($P < 0.05$; U 95.000), Test 6d ($P < 0.05$; U 92.000), Test 7d ($P < 0.05$; U 96.000) and Test 14d ($P < 0.05$; U 94.500), but not in the other behavioral trials. Both groups demonstrated significant retention levels at Reminder test when compared to training by Wilcoxon signed-rank test ($P < 0.001$ for SAL and $P < 0.01$ for NaB), additionally the retention level of NaB-treated aged rats in the Reminder test was significantly greater then SAL-treated aged rats as showed by Mann–Whitney U test ($P < 0.01$; U 95.000). The results indicate that NaB administration in aged rats resulted in significant enhancement of IA memory retention that lasted for 14 days compared to SAL treated rats.

On the other hand, younger animals treated with NaB did not demonstrate enhancement of IA retention (Fig. 2). Young adult rats were treated with NaB intraperitoneally immediately after train-

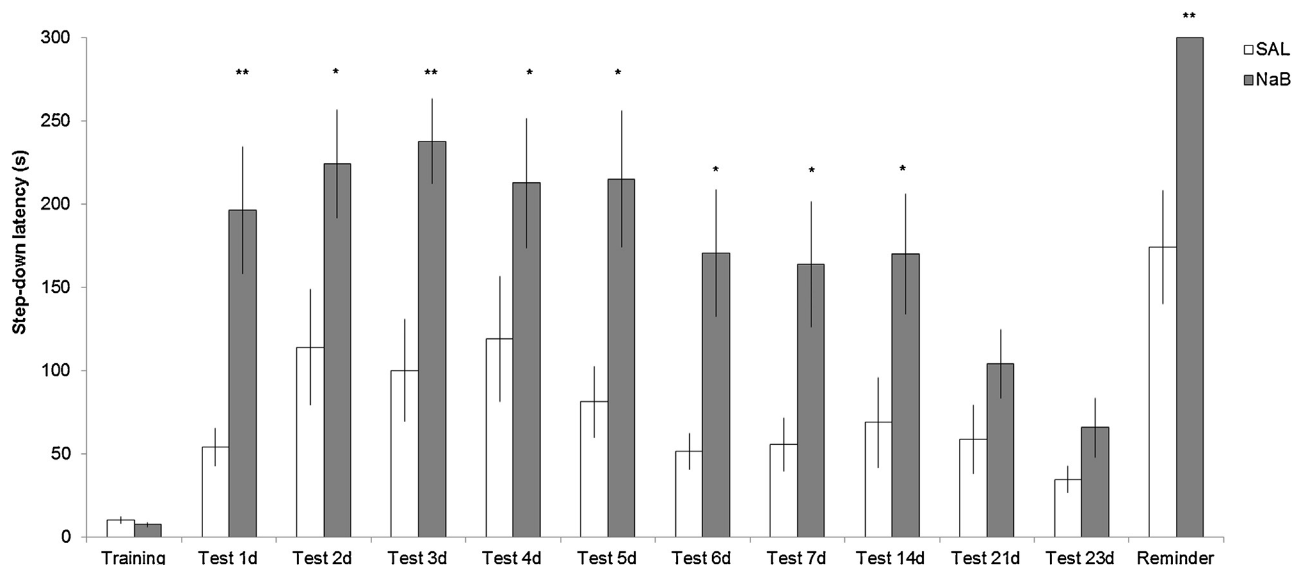

Fig. 1. Administration of NaB enhances long-term retention of IA memory in aged rats. Rats were trained and given an acute systemic intraperitoneal injection of SAL ($N = 12$) or NaB (1,2 g/kg, $N = 10$) immediately after training. Data are mean + SEM retention test latencies to step-down (s); $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to SAL-treated rats.

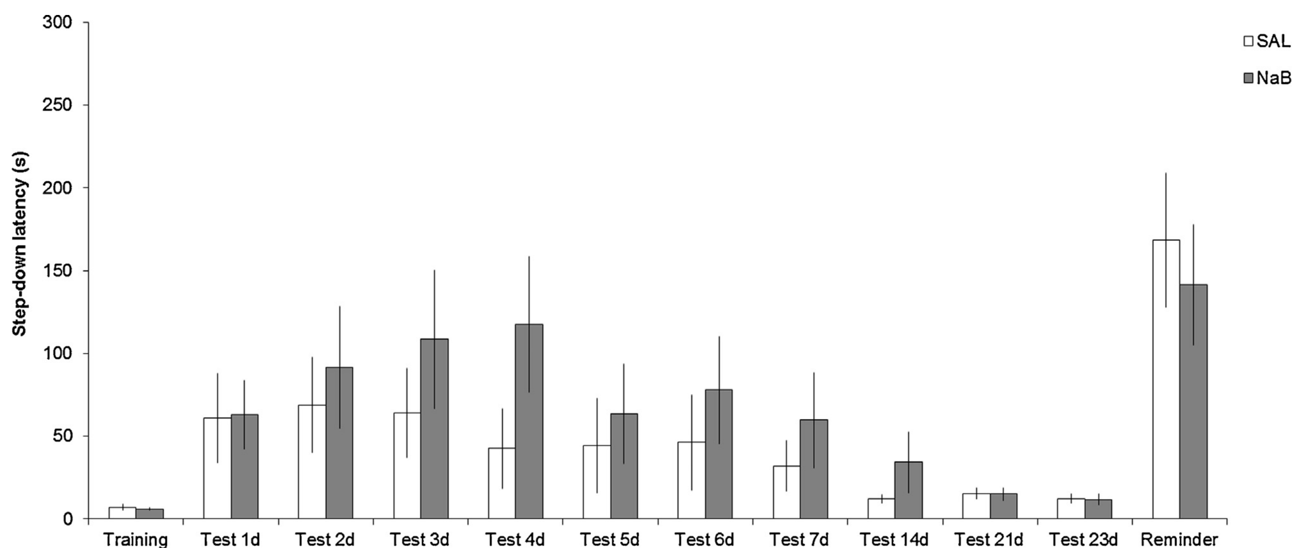

Fig. 2. Administration of NaB had no effect on long-term retention of IA memory in young rats. Rats were trained and given an acute systemic intraperitoneal injection of SAL ($N = 10$) or NaB (1,2 g/kg, $N = 10$) immediately after training. Data are mean + SEM retention test latencies to step-down (s).

ing and tested for retention 1 (Test 1d), 2 (Test 2d), 3 (Test 3d), 4 (Test 4d), 5 (Test 5d), 6 (Test 6d), 7 (Test 7d), 14 (Test 14d), 21 (Test 21d) and 23 (Test 23d) days after training. Immediately after Test 23d, rats were given a reminder footshock and tested again 1 day later. Although both groups demonstrated memory retention on Test 1d compared to training by Wilcoxon signed-rank test ($P < 0.01$ for both groups), no memory enhancement was observed in the group treated with NaB in comparison to SAL-treated group by further Mann–Whitney U-tests. In addition, no significant differences between SAL-treated rats and rats given NaB were observed in any other trial. There was a decline in retention levels across test trials, and both groups displayed a high retention level when tested after a reminder shock ($P < 0.01$ compared to training for both groups). The results indicate that NaB had no effect on learning in young adult rats.

In order to investigate if the pharmacological effects of NaB observed in aged rats during learning reflected biochemical alterations in specific histones modifications, we examined the acetylation level of histone H3 in both residues of lysine 9 and 14 in the hippocampus of aged rats. We chose those residues because histone acetylation of H3 lysine residues has been linked to memory formation and consolidation [15,32]. In addition, Peleg et al. [9] had found a transient increase of H3K9 and H3K14 acetylation 60 min in the hippocampus after fear conditioning in 3 and 16-month-old mice and Silva et al. [11] demonstrated that memory impairment induced by brain iron overload is accompanied by a reduction in H3K9 acetylation in the hippocampus that is rescued after treatment with NaB. Fig. 3A and B shows Histone H3 acetylation at lysine 9 and 14 in the hippocampus of aged rats treated with SAL or NaB. Systemic NaB treatment immediately after training did not affect H3K9 and H3K14 acetylation in aged rats measured 1 hour post-training. Although there was an apparent NaB-induced increase in H3K9 acetylation in trained rats compared to SAL group, this comparison did not reach statistical significance ($P > 0.05$).

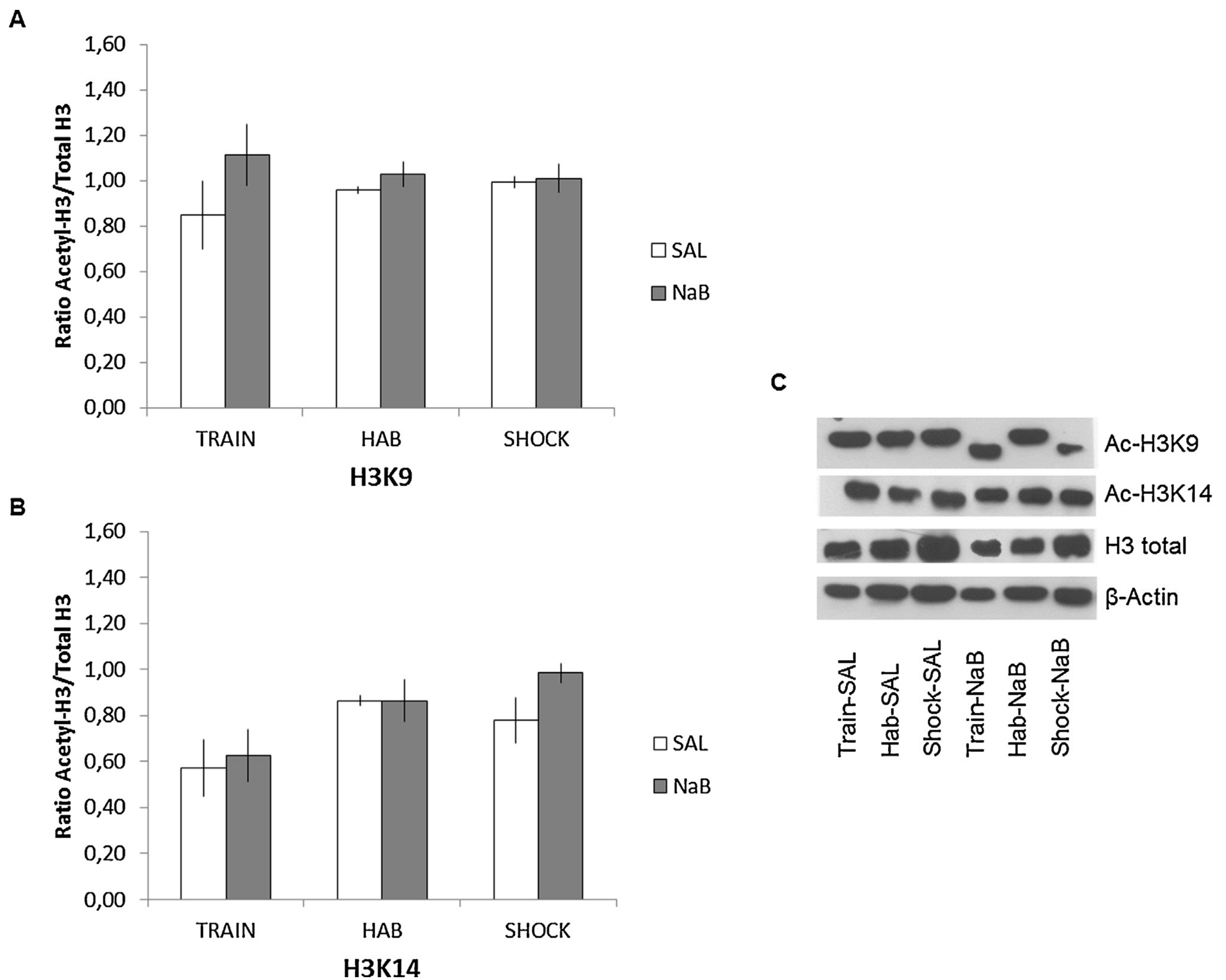

Fig. 3. Histone H3 acetylation in the hippocampus of aged rats treated with SAL or NaB. Rats were either trained in the IA learning task (TRAIN), exposed to the context alone (HAB) or exposed to aversive stimulus alone (SHOCK). Data are shown as means ± SEM ratio of acetylated H3K9 (A) or H3K14 (B) to total histone H3. (C) Representative Western blots for acetylated histones, total H3, and β-actin in the hippocampus; $N = 4$ animals per group.

4. Discussion

Recent evidence indicates that epigenetic mechanisms can orchestrate molecular events during long-term memory formation and affect learning in aged rats [2,6,7,8,9,10,12,15,18]. Our data indicate that treatment with NaB can enhance memory consolidation for aged rats but not young adult rats. In addition, it supports the view of learning being critically modulated by the activity of HDACs [3,19]. Since NaB injections were given after training, the NaB-induced improvement of memory observed in aged rats cannot be explained by drug-induced alterations in sensorial or motor parameters.

Kilgore et al. [8] showed that beyond rescuing memory deficits in a mouse model of Alzheimer's disease, treatment with HDACis resulted in a type of memory resistant to weakening in response to time or reactivation. Consistently, we found that the enhanced memory was more persistent lasting longer even with daily re-exposure to the IA apparatus when compared to control aged animals. Therefore, HDACis might be regulating mechanisms of plasticity generating stability and improving the efficiency of original memory consolidation when administered at specific time points after learning. This is in accordance with our previous results [14] and with evidence indicating that increased acetylation induced by HDACis is a molecular feature of stronger and more persistent memories [20]. Further studies are necessary to understand which mechanisms are being modulated by NaB in aged rats and additional tests evaluating memory in different time points after training and NaB administration are important to clarify this memory-enhancing effect.

Previously, our group has demonstrated that a posttraining injection of NaB did not affect memory in younger rats showing normal memory retention, but ameliorated aging-related memory deficits in an object recognition task [10,11]. Our findings extend that previous evidence by indicating that NaB can display a memory-enhancing effect that is specific for aged animals, even when they do not show age-related memory impairment. Many studies showed memory-enhancing effects of HDAC inhibitors in young rodents [7,8,15,18,21] but, in our study no effect of NaB was observed in young rats. Perhaps the null effect of NaB observed in young rats could be explained by differences in behavioral protocols and route of administration used that may reflect a complex action of NaB since there are variations of NaB effects across research laboratories. The hypothesis that animals with different genetic backgrounds can present different behavioral responses as well as diverse patterns of epigenetic modifications should also be taken into account when comparing different studies [8].

In our study, aged animals with no learning deficits were selectively affected by NaB administration. This result is in accordance

with studies demonstrating that aging is not always accompanied by memory impairment as nearly half of aged rats demonstrate normal learning scores [23,24,25,26,27]. Furthermore, different patterns of chromatin modifications occur in the hippocampus of young and aged rats after training and the use of HDACis might alter chromatin resulting in divergent gene transcription and behavioral responses [23,26]. Haberman and co-workers [26,27] demonstrated that although some gene profiles are the same between young and aged rats with no impaired memory, many other genes are differentially expressed. Thus, perhaps treatment with NaB could be altering gene expression by acting on those genes promoters. Our finding of specific memory-enhancing effects of NaB in aged rats could also be partly explained by the fact that epigenetic markers at histones may not be deregulated in these animals and possibly HDACs inhibition by NaB is affecting non-histone substrates [31].

The lack of alterations in H3 acetylation for aged rats in our work might be explained by transient variations in acetylation on numerous histones, not only in histone H3, triggered by memory formation [9,28]. Studies with altered H3 acetylation used object recognition training, contextual fear conditioning, spatial memory, food aversion training among others that differ from our IA training which could result in a different pattern of histone acetylation [29]. Peleg et al. [9] showed that age-related memory impairment was associated with a reduction in acetylation of H4K12, but not H3K9. In addition, Castellano et al. [22] demonstrated that increases in H3 acetylation after systemic administration of NaB occurred 30 min after injection but not at 60 min. NaB also act on HDACs with ubiquitous expression probably inducing acetylation in other substrates aside from nuclear histones [30]. Recently, enzymatic cleavage of histone H3 tail was reported *in vivo* [33] and it could interfere with histone acetylation biochemical measures since most studied modifications in histone H3 are located downstream of known H3 cleavage sites. Future experiments investigating acetylation in other lysine residues, comparing histone acetylation in aged and young rats as well as in habituated rats with naïve and non-habituated control rats might shed light on this issue.

5. Conclusion

In summary, our results indicate that aged animals, even with the absence of alterations in normal memory formation, might be particularly sensitive to the enhancing effects of a single systemic administration of an HDACi on the consolidation of aversively-motivated memory.

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