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Article

DNA Methylation and Histone Acetylation Contribute to the Maintenance of LTP in the Withdrawal Behavior Interneurons in Terrestrial Snails

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Abstract: Accumulated data indicate that epigenetic regulations, including histone modifications and DNA methylation, are important means for adjusting genes' expression in response to various stimuli. In contrast to the success in studying the role of DNA methylation in laboratory rodents, the role of DNA methylation in terrestrial snail *Helix lucorum* has been studied only in behavioral experiments. This prompted us to further investigate the role of DNA methylation and the interaction between DNA methylation and histone acetylation in the mechanisms of neuroplasticity in terrestrial snails using in vitro experiments. Dysregulation of DNA methylation by DNMT inhibitor RG108 suppressed significantly the long-term potentiation (LTP) of synaptic inputs in identified neurons. We then tested whether the RG108-induced weakening of potentiation will be rescued under co-application of histone deacetylase inhibitors sodium butyrate or trichostatin A. RG108-induced LTP deficiency was significantly compensated by increased histone acetylation. These data bring important insight to the functional role of DNA methylation as an important regulatory mechanism and a necessary condition for the development and maintenance of long-term synaptic changes in withdrawal interneurons of terrestrial snails. Moreover, these results support the idea of the interaction of DNA methylation and histone acetylation in the epigenetic regulation of synaptic plasticity.

Keywords: epigenetics; DNA methylation/demethylation; histone acetylation; synaptic plasticity; gastropods

1. Introduction

DNA methylation is known to play important roles in the regulation of long-term synaptic plasticity, learning and memory [1–4]. DNA methylation is carried out by a family of enzymes known as DNA methyltransferases (DNMT) and, as a rule, involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA. The presence of the DNMT, their encoding genes and all the necessary machinery in mollusks is beyond doubt [5–10]. It is known that the DNA methylation is primarily associated with the suppression of gene expression [11–13]. Previous studies demonstrated that DNMT is involved in long-term potentiation (LTP) not only in vertebrates (in medial prefrontal cortex [4] and hippocampus [3]) but also in mollusks (in *Aplysia californica* [14,15]). However, the role of DNA methylation on LTP in terrestrial snails remains unexplored.

Histone acetylation is one of the best studied histone modifications. Histone deacetylases (HDACs) and histone acetylases control chromatin structure through deacetylation and acetylation, respectively, of the N-terminal histone lysine residues. The more acetylated chromatin is, the more relaxed it is, which in turn leads to an increase in gene transcription [16–18]. According to the modern point of view, histone acetylation is an essential regulatory mechanism of synaptic plasticity. Sodium butyrate (NaB) and trichostatin A (TSA) are widely used histone deacetylase inhibitors (HDACis); TSA is supposed to inhibit both class I and class II HDAC enzymes [19] and NaB only class I HDACs

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without affecting class IIa, Iib, or III HDACs [20]. Moreover, several studies showed that HDACi can ameliorate LTP deficit [21–24].

Terrestrial snail *Helix lucorum* provides researchers with a relatively simple nervous system that tolerates laboratory conditions well, with large neurons with strictly defined functions [25]. At the same time, terrestrial snails exhibit quite complex behaviors, which makes them suitable candidates for the study of neuroplasticity processes. Withdrawal interneurons in terrestrial snails (also known as command neurons for avoidance behavior) are involved in synaptic plasticity underlying avoidance behavior. The cellular mechanisms for this behavior and plasticity are well characterized [26]. However, the molecular pathways underlying them are not well understood. Recently, there were found evidences that epigenetic modulation is a necessary regulatory component of the long-term synaptic plasticity and long-term memory in terrestrial snails [22,27–32]. Several recent reports indicated that such histone posttranslational modifications as serotonylation and acetylation contribute to LTP formation and maintenance [22,27]. The contribution of DNA methylation was only studied in in vivo experiments using two models of conditioned reflex learning (food aversion and contextual avoidance memory) [28,31]. Disruption of DNA methylation by the DNA methyltransferases inhibitor (DNMTi) RG108 has been shown to impair maintenance of both types of memory. This prompted us to further investigate the role of DNA methylation and the interaction between DNA methylation and histone acetylation in the mechanisms of neuroplasticity in terrestrial snails using in vitro models and intracellular recording of synaptic events in functionally identified neurons.

In the present study, we tested our hypothesis that DNA methylation in cooperation with histone acetylation regulate synaptic plasticity, by determining the effects of DNMTis and HDACis on LTP in withdrawal interneurons in terrestrial snail. We demonstrated that a session of five tetanizations associated with serotonin (5-HT) applications led to sustained long-lasting potentiation, and dysregulation of DNA methylation by DNMTi RG108 suppressed this LTP significantly. We then tested if co-application either of HDACis NaB or TSA with RG108 prevented the weakening of potentiation. We showed that RG108-induced LTP deficiency was significantly compensated by increased histone acetylation. These data bring important insight to the functional role of DNA methylation as an important regulatory mechanism and a necessary condition for the development and maintenance of long-term synaptic changes in withdrawal interneurons in terrestrial snails.

2. Materials and Methods

2.1. Animals

The work was performed in adult *Helix lucorum taurica L.* All animals were kept in terrariums under laboratory conditions. The terrariums were provided with high humidity, a sufficient amount of food, water, and a low concentration of animals, which contributed to the normal active existence of animals. In all experiments animals similar in weight and size (15g±5) were used. Before the experiments, the snails were in an active state for at least two to three weeks. Experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and the protocol was approved by the Ethical Committee of the Institute of Higher Nervous Activity and Neurophysiology of Russian Academy of Sciences.

2.2. Electrophysiological Experiments

The work was carried out on the isolated central nervous system of terrestrial snails. Details of preparation has been reviewed and will not be detailed here [25]. Using sharp glass microelectrodes filled with 2 M potassium acetate (tip resistance, 15–20 MOhm), the activity of visually identified giant withdrawal premotor (command) interneurons (parietal #3 and parietal #2) of the parietal ganglia was recorded. Excitatory postsynaptic potentials (EPSPs) evoked by electrical stimulation (duration 3 ms) of the second cutaneal (glutamatergic-like synapses [33]) or intestinal (acetylcholinergic-like synapses [34]) nerve were recorded. Intracellular signals were recorded with

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preamplifiers (Axoclamp 2B, Axon Instruments, CA, USA), digitized, and stored on a computer (Digidata 1400A A/D converter and Axoscope 10.0 software, both from Axon Instruments, CA, USA).

In each experiment, stimulus intensity was adjusted to evoke EPSPs of 4–10 mV amplitude from each stimulated nerve in withdrawal interneurons. In control experiments aimed at studying basic synaptic plasticity, test stimulation of the second cutaneal or intestinal nerves was carried out throughout the recording session with an interval between stimuli 10 minutes. To study the long-term plasticity, a homosynaptic potentiation protocol was used. At the beginning of the recording, five test stimulations of the second cutaneal or intestinal nerves were performed with an interval between stimuli 10 minutes, then tetanization of the second cutaneal or intestinal nerve was carried out (a burst of stimuli with a frequency of 10 Hz, a burst duration of 10 s, a 10-fold increase in the amplitude of the test stimulus). In total, tetanization trains were delivered five times with an interval of five minutes (Figure 1). Before each tetanization, 5-HT was added to the experimental bath, which was washed off two minutes after the tetanization. After the fifth tetanization, test stimulation of the second cutaneal or intestinal nerve was continued at the original stimulus amplitude every 10 minutes for at least 4 hours.

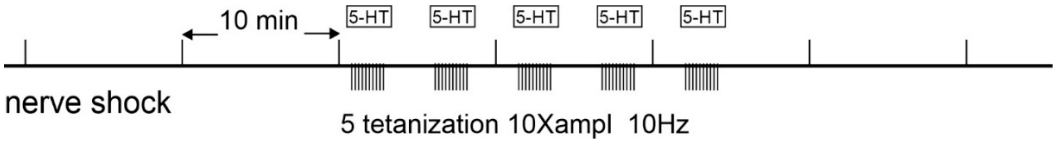

Figure 1. Schematic representations of protocol.

2.3. Drugs

HDACi sodium butyrate (NaB) (Sigma, St. Louis, USA) and serotonin (5-HT) (Tocris, Bristol, UK) were dissolved in sterile physiological Ringer solution (in mM: 100 NaCl, 4 KCl, 7 CaCl2, 5 MgCl2, and 10 Tris-HCl buffer (pH 7.8)). The DNMT inhibitors N-phthalyl-L-tryptophan (RG108) (Sigma, St. Louis, USA) and trichostatin A (TSA) were dissolved in DMSO (dimethyl sulfoxide) as stock solution. The final concentration of the substances in the experimental bath was as follows: DMSO – less than 0.1% (shown to be inactive in our preparations), NaB - $6x10^{-5}$M. 5-HT - 10^{-5}M, RG108 - $20X10^{-5}$M, and TSA - $0.5x10^{-6}$M (these concentrations were selected based on literature data [22,35]). The drugs were added to the experimental bath using a pipette.

Depending on the experimental design, either RG108 or RG108+NaB or RG108+TSA were applied to the bath solution 40 minutes before the first tetanization. During tetanization session an intensive "washing out" of the drugs was carried out.

The experiments included the following groups. "Control" groups – the groups in which only test stimulation was carried out. Group "control+RG108" – RG108 was in the experimental bath for the first 40 minutes of recording. "LTP" groups – groups in which a homosynaptic protocol was used to produce LTP. "LTP+RG108" group – LTP protocol was used and RG108 was in the experimental bath for the first 40 minutes of recording. "LTP+NaB" and "LTP+TSA" – groups were characterized by LTP and simultaneous application of the DNMTi RG108 and HDACi (NaB or TSA).

2.4. Data Analysis

The significant differences in the amplitudes of EPSPs were assessed using the Mann–Whitney test. The standard program STATISTICA 10.0 was used to process the results. All data are presented as mean ± S.E.M. Differences were considered significant at $p < 0.05$, and were denoted in figures by *#@%.

3. Results

In an effort to investigate whether DNA methylation contributes to long-term potentiation (LTP) in withdrawal interneurons of the snail nervous system, we used DNMTi RG108. In the first series of experiments, test stimulation of second cutaneal nerve was performed. In all control recordings

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without tetanization (groups: control, n=10; control+RG108, n=9) test stimulation led to a gradual decrease in EPSPs amplitude (here and further 100% of the initial EPSP amplitude is shown at time point "-40" min; control: 50.2±7.7% - at time point 120 min, 34.1±6.4% - at time point 250 min; control+RG108: 58.2±8.4% - at the time point 120 min, 42.2±5.8% - at time point 250 min). No significant differences were found between the EPSPs amplitude in the control and control+RG108 groups (Figure 2A). Thus, our findings suggest that RG108 did not affect the amplitudes of nonpotentiated glutamatergic EPSPs in withdrawal interneurons.

Next, we determined whether the RG108 administration affected the LTP of synaptic inputs in withdrawal interneurons. During the first five test stimuli, the EPSPs amplitude gradually decreased in both LTP groups (LTP, n=10; LTP+RG108, n=8). No differences were observed between EPSPs amplitudes in control, control+RG108, LTP and LTP+RG108 groups at 0 min time point (Figure 2A,D). Five tetanizations combined with 5-HT applications caused a pronounced increase in EPSPs amplitude in both LTP groups. Thus, immediately after the last tetanization combined with the application of 5-HT (30 min time point), the EPSP amplitude in LTP group was 226.0 ± 32.1%, and in LTP+RG108 group – 274.8 ± 28.0%. There were no significant differences between LTP and LTP+RG108 groups at 80 min time point while between LTP and control groups significant differences was found (Figure 2A,E). The decrease of the EPSPs amplitudes in LTP+RG108 group compared to the LTP group began 90 min after the completion of tetanization. Thus, at the time point 120 min, the EPSPs amplitude in LTP group was 172.3 ± 16.3%, while in LTP+RG108 group it was only 105.4 ± 33.1% ($p = 0.043089$). 4 hours after tetanization (time point 250 min), the EPSPs amplitude in LTP group significantly exceeded the amplitude of responses in the LTP+RG108 group (LTP – 120.2 ± 14.4%; LTP+RG108 – 25.6 ± 4.4%, $p = 0.000250$). Moreover, no differences were observed between EPSPs amplitudes in control, control+RG108 and LTP+RG108 groups at 250 min time point (Figure 2A,F). It should be noted that from 120 min time point to 250 min time point of the recordings, a significant difference was observed between LTP and LTP+RG108 groups ($p < 0.05$, Figure 2A) while the EPSPs amplitudes of LTP+RG108 group didn't differ significantly from those of the control group during the last 100 minutes of recordings (Figure 2A). Thus, the RG108 administration 40 min before LTP induction did not block the initial phase of LTP in glutamatergic synapses, but led to a significant weakening of the late phase of LTP in withdrawal parietal interneurons.

To address possible involvement of histone acetylation and its interaction with DNA methylation in LTP of withdrawal interneurons, we investigated the role of HDAC by adding HDACis (NaB or TSA) to the bath solution together with RG108 (Figure 2, groups LTP+RG108+NaB, n=8, LTP+RG108+TSA, n=8). Test stimulation before start of the potentiation protocol led to a gradual slight decrease in EPSPs amplitude in LTP+RG108+NaB and LTP+RG108+TSA groups, as in the groups described earlier (control, control+RG108, LTP, LTP+RG108). No differences were observed between those groups at 0 min time point (Figure 2B,D). Five tetanic stimulations combined with 5-HT applications caused a significant increase in the EPSPs amplitude: LTP of synaptic responses was observed in LTP+RG108+NaB and LTP+RG108+TSA groups (Figure 2B). Notably, the EPSPs amplitude in LTP+RG108+NaB and LTP+RG108+TSA were not affected by RG108 administration: EPSPs in LTP+RG108+NaB and LTP+RG108+TSA didn't differ from those in LTP group during the recording (Figure 2B, D, E, F). In addition, EPSPs amplitude in LTP+RG108+NaB and LTP+RG108+TSA significantly exceeded the EPSPs in LTP+RG108 in 210–250 min and 200-250 min time windows, respectively (Figure 2B). Thus, as HDACis (NaB or TSA) applications led to increased glutamatergic EPSPs amplitude in the late phase of LTP, it can be assumed that the increased level of histone acetylation can compensate the RG108-induced LTP deficits.

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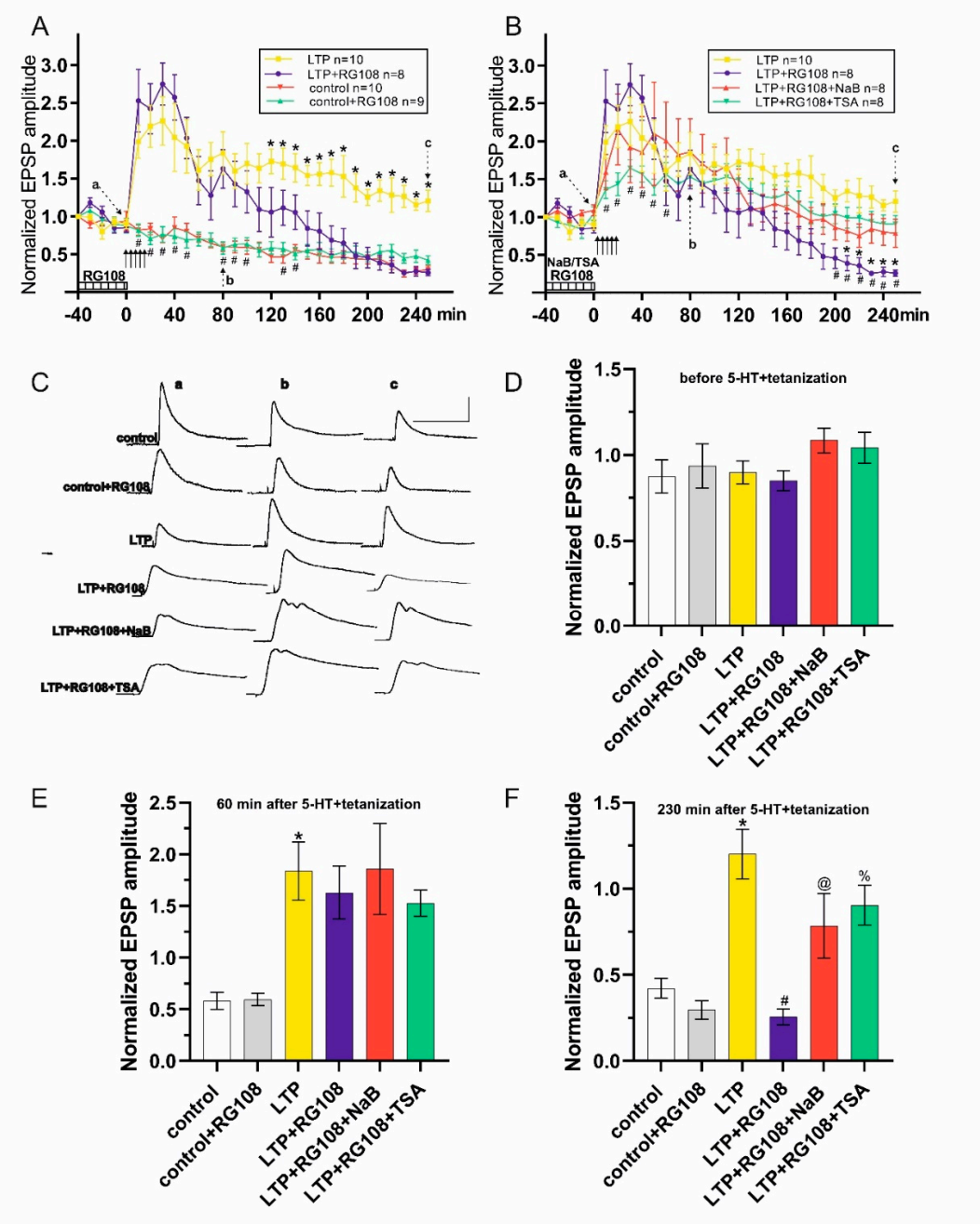

Figure 2. The effects of RG108, NaB and TSA on EPSPs amplitudes in control, under LTP induction and LTP maintenance in withdrawal interneurons with test stimulations of the second cutaneal nerve. (A) Effects of the bath applications of RG108 on the EPSPs: a time course plot of pooled data for the EPSPs. RG108 reduces LTP duration. * denotes $p < 0.05$ LTP+RG108 vs LTP; # denotes $p < 0.05$ LTP+RG108 v vs control. (B) Effect of the combined bath application of RG108 with either NaB or TSA on the EPSPs: a time course plot of pooled data for the EPSPs. Histone deacetylase inhibitors (NaB or TSA) prevented the weakening of potentiation. * denotes $p < 0.05$ LTP+RG108+NaB vs LTP+RG108; # denotes $p < 0.05$ LTP+RG108+TSA vs LTP+RG108. The data are presented as mean ± standard error of the mean. The duration of drugs' presence is shown as a striped bar at the bottom in (A) and (B). Arrows indicate the time of tetanization+5-HT applications in (A) and (B). (C) Example traces the EPSPs (a, b and c from plots A, B) in control, control+RG108, LTP, LTP+RG108, LTP+RG108+NaB and LTP+RG108+TSA groups. Scale bars – 5 mV, 500 ms. (D) Comparison of the effects of RG108, NaB and TSA before the tetanization+5-HT. No differences were observed between EPSPs amplitudes in groups before 5-HT+tetanization (time point 0 min) (E) Comparison of the effects of RG108, NaB and TSA on LTP induction: EPSPs amplitudes 60 min post 5-HT+tetanization (time point 80 min). EPSPs

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amplitude of LTP group were significantly higher than those in control; * denotes $p < 0.05$ LTP vs. control. Moreover, there were no significant differences between the groups with LTP induction. (F) Comparison of the effects of RG108, NaB and TSA on LTP maintenance: EPSPs amplitudes 230 min post 5-HT+tetanization (time point 250 min). Application of RG108 caused a significant decrease in EPSPs amplitude, while co-administration of RG108+NaB or RG108+TSA was shown to enhance LTP. * denotes $p < 0.05$ LTP vs control; # denotes $p < 0.05$ LTP vs LTP+RG108; @ denotes $p < 0.05$ LTP+RG108 vs LTP+RG108+NaB; % denotes $p < 0.05$ LTP+RG108 vs LTP+RG108+TSA.

In the second series of experiments, test stimulation of intestinal nerve (putative acetylcholinergic synaptic inputs) was performed. In all control recordings without tetanization (groups: control, n=10; control+RG108, n=10) test stimulation led to a gradual decrease in EPSPs amplitude (control: 58.0±7.0% - time point 120 min; 32.3±5.2% - time point 250 min; control+RG108: 45.4±4.4% - the time point 120 min; 38.3±6.0% - time point 250 min). No significant differences were found between the EPSPs amplitudes in the control and control+RG108 groups (Figure 3A). Thus, the results suggest that RG108 applications do not affect the amplitude of nonpotentiated acetylcholinergic EPSPs in withdrawal interneurons.

Next, we investigated whether the RG108 affect the LTP induction and maintenance in acetylcholinergic synapses in terrestrial snails. During the first five test stimuli, the EPSPs amplitude gradually decreased in both LTP groups (LTP, n=8; LTP+RG108, n=13). No differences were observed between EPSPs amplitudes in control, control+RG108, LTP and LTP+RG108 groups at 0 min time point (Figure 3A,D). Five tetanizations combined with 5-HT applications caused a pronounced increase in EPSPs amplitude in both LTP groups. Thus, immediately after the last tetanization combined with the application of 5-HT (30 min time point), the EPSP amplitudes in LTP group were 187.0 ± 19.7%, and in LTP+RG108 group – 168.1 ± 19.6%. However, despite the fact that the EPSPs amplitudes in the LTP+RG108 group were not affected by RG108 for the first 50 min after the tetanization protocol start, they became significantly reduced by the 80 min in comparison to the EPSPs amplitudes in the LTP group (Figure 3A,C,E). Weakening of the EPSPs in LTP+RG108 group relative the LTP group continued during the rest of recording. Thus, at the time point 120 min, the mean EPSPs amplitude in LTP group was 178.5 ± 24.2%, while in the LTP+RG108 group it was only 105.4 ± 15.8% ($p = 0.043089$). 4 hours after tetanization (time point 250 min), the mean EPSPs amplitude in LTP group still significantly exceeded the amplitude of responses in the LTP+RG108 group (LTP – 152.3 ± 15.3%; LTP+RG108 – 47.1 ± 17.5%, $p = 0.000250$). Moreover, no differences were observed between EPSPs amplitudes in control, control+RG108 and LTP+RG108 groups at 250 min time point (Figure 3A, F). It should be noted that from the 110 min time point to the 250 min time point of the recordings, a significant difference was observed between values in LTP and LTP+RG108 groups ($p < 0.05$, Figure 3A) while the EPSPs amplitudes in LTP+RG108 group didn't differ significantly from those of the control group during the last 130 minutes of recording (Figure 3A). Thus, the RG108 administration 40 min before LTP induction weakened the initial phase of LTP, and also led to a significant decrease of the late phase of LTP in acetylcholinergic synaptic inputs.

As a next step, we tested whether co-application of either NaB or TSA with RG108 was able to prevent the RG108-induced LTP weakening in acetylcholinergic synapses (Figure 3B, groups LTP+RG108+NaB, n=8, LTP+RG108+TSA, n=8). Test stimulation before applying the potentiation protocol led to a gradual slight decrease in EPSPs amplitude in LTP+RG108+NaB and LTP+RG108+TSA similar to the groups described earlier (control, control+RG108, LTP, LTP+RG108); no significant differences were observed between those groups at 0 min time point (Figure 3B, D). Five tetanic stimulations combined with 5-HT applications caused a significant increase in the EPSPs amplitude: LTP of synaptic responses was observed in LTP+RG108+NaB and LTP+RG108+TSA groups (Figure 3B). According to the data obtained, the EPSPs amplitudes in LTP+RG108+NaB were significantly higher than those in LTP+RG108 in the time window 80-250 min (Figure 3B,C,E,F), while at the early stage of potentiation differences in amplitudes were found only at time points 20 and 40 min. However, despite the preservation of EPSPs amplitudes in LTP+RG108+NaB at potentiated levels throughout the experiment, they were significantly different from those in LTP group (Figure 3B). Thus, at the time point 250 min the EPSPs amplitudes in LTP+RG108+NaB group were 98.0 ±

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5.3%. Notably, the EPSPs amplitudes in the LTP+RG108+TSA were not affected by RG108 administration: the EPSPs in LTP+RG108+TSA didn't differ from those in LTP group throughout the recording (Figure 3B-F). Thus, co-application of NaB or TSA with RG108 kept the EPSPs stably elevated. Therefore, it is possible to speculate that HDACis (NaB or TSA) can rescue the RG108-induced LTP deficits in acetylcholinergic synaptic inputs.

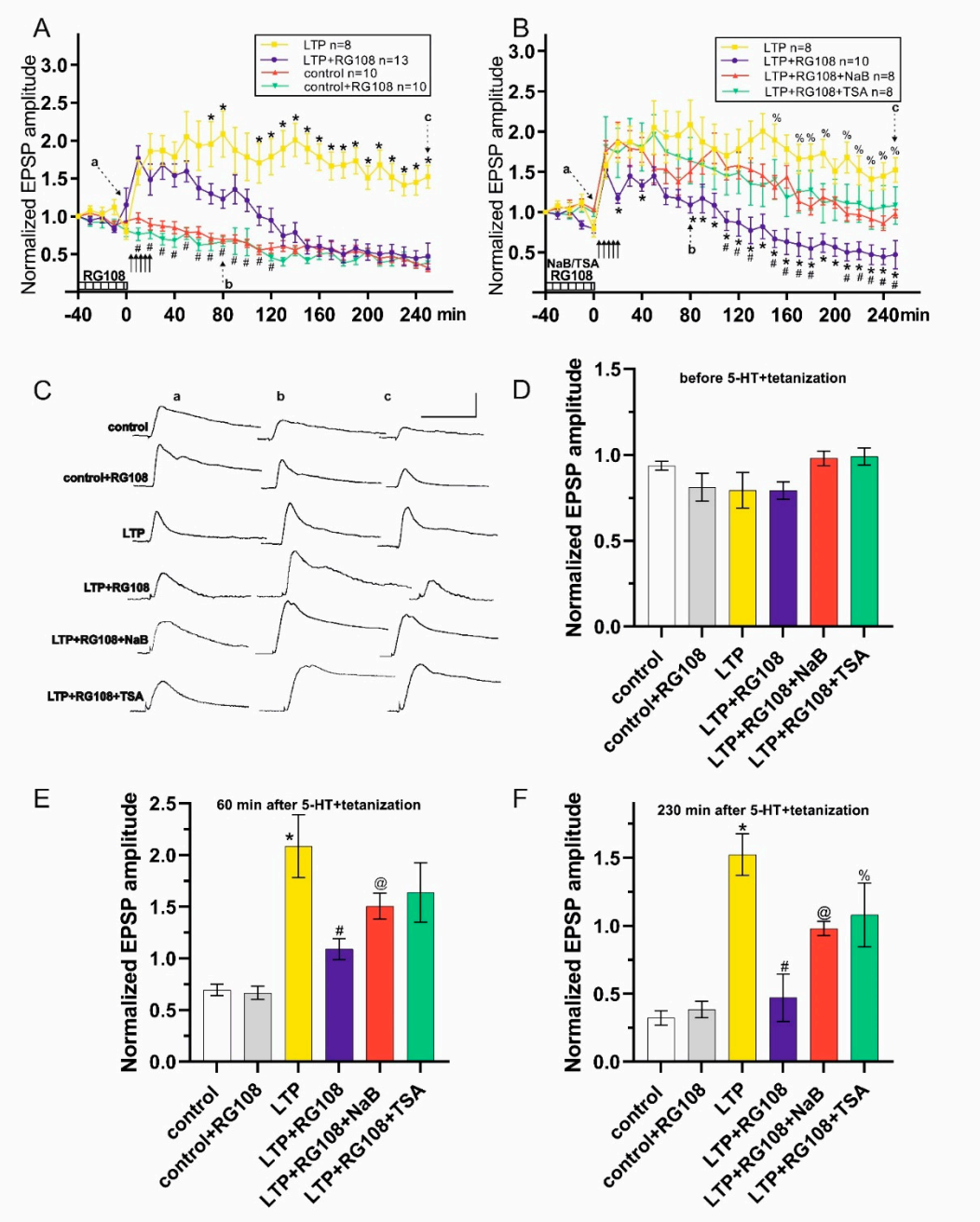

Figure 3. The effects of RG108, NaB and TSA on EPSPs amplitudes in control, under LTP induction and LTP maintenance in withdrawal interneurons with test stimulation of intestinal nerve. (A) Effects of the bath application of RG108 on the EPSPs: a time course plot of averaged data for the EPSPs. RG108 reduces the LTP maintenance. * denotes $p < 0.05$ in groups LTP+RG108 vs LTP; # denotes $p < 0.05$ LTP+RG108 vs control. (B) Effect of the combined bath application of RG108 with either NaB or TSA on the EPSPs: a time course plot of averaged data for the EPSPs. Histone deacetylase inhibitors (NaB or TSA) prevented the weakening of potentiation. * denotes $p < 0.05$ in groups LTP+RG108+NaB vs LTP+RG108; # denotes $p < 0.05$ LTP+RG108+TSA vs LTP+RG108. % denotes $p < 0.05$ LTP+RG108+NaB vs LTP. The data are presented as mean ± SEM. The duration of drugs presence is shown as a striped bar at the bottom in (A) and (B). Arrows indicate the time of tetanization+5-HT in

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(A) and (B). (C) Examples of the EPSPs (a, b and c from plots (A), (B)) in control, control+RG108, LTP, LTP+RG108, LTP+RG108+NaB and LTP+RG108+TSA groups. Scale bars – 5 mV, 500 ms. (D) Comparison of the effects of RG108, NaB and TSA before the tetanization+5-HT. No differences were observed between EPSPs amplitudes in groups before the 5-HT+tetanizations (time point 0 min) (E) Comparison of the effects of RG108, NaB and TSA on LTP induction: EPSPs amplitudes 60 min post 5-HT+tetanization (time point 80 min). EPSPs amplitudes of LTP group were significantly higher than those in control and LTP+RG108 groups; * denotes $p < 0.05$ LTP vs control; # denotes $p < 0.05$ LTP vs LTP+RG108. (F) Comparison of the effect of RG108, NaB and TSA on LTP maintenance: EPSPs amplitudes 230 min post 5-HT+tetanization (time point 250 min). Application of RG108 caused a significant decrease in EPSPs amplitude while co-administration of RG108+NaB or RG108+TSA was shown to enhance LTP. * denotes $p < 0.05$ LTP vs control; # denotes $p < 0.05$ LTP vs LTP+RG108; @ denotes $p < 0.05$ LTP+RG108 vs LTP+RG108+NaB; % denotes $p < 0.05$ LTP+RG108 vs LTP+RG108+TSA.

4. Discussion

We have examined the involvement of the DNMT activity in LTP in withdrawal interneurons. RG108 applications did not significantly affect the EPSPs amplitudes in control and control+RG108 groups when differing in released transmitters synaptic inputs were activated via second cutaneal or intestinal nerves (Figures 2 and 3, control and control+RG108 groups). This suggested that the DNMT inhibitor used did not affect the synaptic potentials by itself. Similar results were obtained in the study of the effect of DNMTis on basic synaptic transmission in the acute slices of hippocampus [3]. Next, we examined the effects of RG108 on LTP induction and maintenance. Following the induction protocol using the glutamatergic afferents, the potentiation of synaptic potentials in LTP+RG108 group was induced and maintained within 60 min after induction, but after 120 min time point the synaptic potentials gradually decreased to the nonpotentiated EPSPs amplitudes (Figure 2, LTP+RG108 vs LTP, LTP+RG108 vs control). Thus, the data obtained showed dependency of maintenance of LTP induced by the second cutaneal nerve tetanizations on the DNA methylation. Notably, the RG108 didn't completely prevented the LTP, affecting only the late LTP (maintenance), while the early LTP remained untouched. Moreover, the results obtained couldn't be attributed to a simple decrease in EPSPs amplitude by RG108 administration as we didn't observe the EPSPs amplitudes changes in recordings under the RG108 (Figures 2 and 3, control+RG108 group).

Regarding induction and maintenance of LTP induced by intestinal nerve tetanizations (putative acetylcholinergic synaptic inputs), we demonstrated that RG108 didn't prevent the induction of acetylcholinergic LTP but disrupted significantly its early and late phases. It appeared that RG108 significantly downregulated acetylcholine-mediated EPSPs starting from time point 70 min (Figure 3, LTP+RG108 vs. LTP). After the 120 min time point, the EPSPs amplitudes in LTP+RG108 and control groups were not significantly different from each other (Figure 3, LTP+RG108 vs. control). In summary, DNMT inhibition had a profound deteriorating effect on facilitated EPSPs evoked both by glutamatergic and cholinergic afferents. Thus, we can speculate that DNA methylation is required for development and maintenance of LTP in synaptic inputs of withdrawal interneurons in terrestrial snails.

These data are consistent with the previous studies showing that mechanisms of synaptic plasticity are dependent on dynamic changes in DNA methylation/demethylation [36]. Similar data on the role of DNMT in the regulation of synaptic plasticity were obtained in experiments in vertebrates. For example, J. Levenson and colleagues demonstrated that under the action of DNMTis the LTP was blocked [3]. In another study, it was shown that DNMTis disrupted the maintenance of LTP mediated by norepinephrine [37]. The attenuation of late LTP in the withdrawal interneurons found in our experiments (in other words, a violation of the transition from a short-term form of plasticity to a long-term one) is consistent with experiments where DNMTis were used to study memory mechanisms in vivo. It has been repeatedly shown that inhibition of DNMT disrupted both consolidation [14,38–41], as well as reconsolidation of memory [31]. It should be added that in our work, the effect of RG108 on DNA methylation has not been directly studied using molecular approaches. However, there are multiple proofs in the literature that the inhibition of DNMT by

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RG108 leads to DNA demethylation [42–45]. Based on these results, we assume that the effects of the DNMT inhibitor RG108 found in our work are associated with changes in DNA methylation.

However, we can only speculate about signaling molecules upstream of DNMT. In our experiments the LTP was induced by repeated exposure to 5-HT with stimulation of afferents, which corresponds to long-term memory formation through 5-HT release from modulatory serotonergic neurons of pedal ganglia [26]. Further, 5-HT might bind to different G protein-coupled receptors [46]. Those receptors might activate diverse molecular signaling pathways. The downstream signaling pathway after receptors' activation is extremely vague. However, it is well established that one of the 5-HT targets is protein kinase C (PKC) activation [47–51]. PKC is considered to be one of the most important enzymes in synaptic plasticity [47,52,53]. According to literature, different PKC isoforms might require different activator (for example, DAG and/or an increase in the intracellular Ca2+ [54–56]) and lead to different downstream activities. Levenson with colleagues demonstrated that PKC signaling pathway regulated the expression of DNMT genes [3]. To sum up, 5-HT through G protein-coupled receptors might lead to an increase in the intracellular Ca2+ via activation of IP3 receptors and RyRs [57] and DAG synthesis, PKC activation and changes in DNMT genes expression. Alterations of DNMT genes' expression in turn may cause changes in the methylation status of various plasticity-related genes [15,58–60]. The described model is hypothetical and does not exclude the contribution of other possible signaling pathways.

In our study, we also showed that LTP deficiency caused by the DNMTi RG108 was eliminated by pharmacologically increased level of histone acetylation (Figures 2 and 3, groups LTP+RG108+NaB, LTP+RG108+TSA). These data are consistent with a number of studies that demonstrated that an increased level of histone acetylation improved long-term synaptic plasticity [23,61–63] and rescued its deficiency [21,22].

Monsey et al [24] and Miller et al [23] also observed that DNMT inhibition resulted in significant LTP impairment, while co-application of DNMTi with HDACi rescued the deficit in LTP. Besides the fact that the data obtained emphasize the role of DNA methylation and histone acetylation as important epigenetic mechanisms for regulating synaptic plasticity in withdrawal interneurons, they also support the idea that DNA methylation and histone acetylation may affect each other to regulate the LTP [3]. Speaking of this, it should be noted that according to the results of several studies the DNMT inhibition may prevent histone acetylation [23,24,64,65]. The possible model through which HDAC might affect synaptic plasticity in withdrawal interneurons is described elsewhere (see [22]).

5. Conclusions

Despite the fact that terrestrial snail *Helix lucorum* is a representative of the largest class of gastropods, at the moment, the question of the role of DNA methylation in its synaptic plasticity is only beginning to be investigated. In this work, it was shown for the first time that the blockade of DNMT disrupts the maintenance of LTP in withdrawal interneurons caused by afferent stimulation (cutaneal or intestinal nerves) and 5-HT applications. This fact allows us to consider the activity of DNMT, and, consequently, of DNA methylation, as an important regulatory mechanism and a necessary condition for the development and maintenance of long-lasting synaptic changes in terrestrial snail.

In addition, the experiments conducted suggest that LTP, impaired by the DNMT inhibition, can be rescued by blocking the histone deacetylation. These results confirm the idea of the interaction of DNA methylation and histone acetylation in the epigenetic regulation of synaptic plasticity.

It should be noted that our study has some limitations. The first concerns the lack of molecular evidence of the action of the pharmacological drugs used (however, numerous literature data on the effects of these drugs were used to interpret the results). The second concerns the fact that we used two distinct HDACis, but only one DNMTi. We should note, however, that no harmful effects (from RG108 administration) were observed in any measurement of control parameters for synaptic transmission.

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