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The Role of Intracellular Calcium in Changing of Electrical Characteristics of Premotor Interneurons in Intact Snails and Snails During Various Forms of Plasticity

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Abstract
It was previously shown that both associative learning and the formation of long-term sensitization led to the increase in excitability of premotor interneurons of the defensive behavior of terrestrial snail *Helix lucorum*. In the present study, we analyzed the role of intracellular calcium ions in the maintenance of increased excitability in premotor interneurons of terrestrial snail after the formation of a conditioned defensive reflex. It was shown that the increase of the intracellular Ca^{2+} concentration after adding caffeine to the solution washing the nervous system of the mollusk led to a decrease of the threshold of action potential and to an increase of the critical level of depolarization without a change of the membrane potential of premotor interneurons in both intact and trained snails. The decrease of the intracellular Ca^{2+} concentration in premotor interneurons by the intracellular injection of (ethylene glycol-bis (2-aminoethylether)-N, N, N, N-tetraacetic acid) (EGTA) resulted in a significant increase of the threshold of generation of the action potential in intact snails. But the values of threshold of generation of the action potential in trained snails after injection of EGTA did not significantly differ from the values of studied parameters before injection. After application of the membrane-penetrating chelator, BAPTA-AM, the changes in the membrane and threshold potentials of premotor interneurons of intact and trained snails were not observed. Our results demonstrated that both the increase and decrease of intracellular Ca^{2+} concentration were not involved in maintaining the changes of membrane characteristics of premotor interneurons observed after associative learning.

Keywords Calcium ions · Associative learning · Identified neurons · Membrane potential · Threshold potential · Snail

1 Introduction

It is known that calcium ions play an important role in the formation of conditioned reflexes and various forms of facilitation and potentiation [1–4]. They participate in the regulation of various neuronal processes, due to their specific physicochemical characteristics; thereby, they are the most universal intracellular mediators [5, 6]. Calcium ions entering into the cell during its excitation, on the one hand, leads to changes of the properties of the ion channels of the membrane, and on the other hand, serves as signals to activate various biochemical processes [7, 8], such as initiating of transmitter release or activation of the intracellular signaling systems. The initial increase of intracellular calcium concentration when entering through the ion channels of the nerve cell membrane leads to a further increase in its concentration from the endoplasmic reticulum and mitochondria, which plays an important role not only in regulating short-term forms of plasticity but also in initiating its long-term forms [9–14]. Thus, calcium ions, carrying out the connection between electrical phenomena occurring in the cell membrane and reactions occurring inside the neuron, are directly involved in the integrative activity of the nerve cell [5, 6, 15, 16].

In previous works, it was shown that in the premotor interneurons of the defensive reflex, the membrane potential and the

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threshold of generation of action potential decreased after the development of the conditioned defensive reflex, as well as after the formation of long-term sensitization, which indicated an increase in their excitability [17–20]. Since it is known that the excitability of nerve cells depends on the Ca^{2+} ions [21], it seems necessary and desirable to continue the analysis of the role of Ca^{2+} ions in the maintenance of the long-term effects of associative learning [22]. Previously, we studied the role of extracellular calcium in the mechanisms of learning in a snail at the level of the parameters of the neuronal membrane. It was found that the increase in extracellular concentration of Ca^{2+} ions led to the increase in threshold potential and shifted the critical depolarization level towards positive values in intact snails, but this effect of membrane stabilization by the high level of extracellular Ca^{2+} was abolished in trained snails [23]. In the present study, we analyzed the role of intracellular calcium ions in the maintenance of changes of the membrane characteristics of premotor interneurons after the formation of a conditioned defensive reflex in terrestrial snail *Helix lucorum*.

2 Methods

Terrestrial snails *Helix lucorum* were used in this study. Before the experiments, the snails were kept for at least 2 weeks in the glass terrariums in a humid atmosphere at room temperature, with excess of food. In preparation for the main work, the defensive conditioned reflex for tapping on the shell was developed in 26 snails. The tapping on the shell was a conditioned stimulus that normally did not cause a defensive reaction of the animal. The air blow into the pulmonary cavity was used as an unconditioned stimulus, which caused an unconditioned defensive reaction of closure of the pneumostome. The combinations of stimuli presented with an interval of 2–4 min. The reflex was developed within 3 days as a result of the presentation of approximately 180–200 combinations of conditioned and unconditioned stimuli. The result of this training was the complete closure of the pneumostome in response to a conditioned stimulus, which was noted as a positive reaction [18, 23].

Analysis of the electrical characteristics was carried out on premotor interneurons of the defensive reflex LPa3 and RPa3 [24]. The registration of electrical activity was made using intracellular glass microelectrodes filled with 2.5 M KCl with input resistance of 5–10 MΩ. The resting membrane potential (Vm) and the threshold of generation of action potential (Vt) were recorded. The saline solution for the terrestrial snail contained NaCl 80 mM/l, KCl 4 mM/l, CaCl2 10 mM/l, MgCl2 5 mM/l, NaHCO 35 mM/l, and pH was 7.6–7.8. The increase in intracellular calcium concentration was achieved by adding 2 mM/l of caffeine (1,3,7-Trimethylxanthine) (Sigma) in the saline solution washing isolated nervous system of snail. It is known that caffeine causes calcium release from intracellular depots, mainly from the endoplasmic reticulum [5, 7, 22]. The injection of calcium chelator—EGTA (ethylene glycol-bis (2-aminoethylether)-N, N, N, N-tetraacetic acid) (Sigma) was used to reduce the calcium content inside the cell [25, 26]. The injection was made during 5 min with a negative current of 1 nA through a recording microelectrode, which was filled with solution containing 0.5 M EGTA. In this case, the input resistance of microelectrode was 10–20 MΩ. Then, the electrical characteristics of premotor interneurons were recorded every 5 min for 30 min. In addition, the experiments with the use of selective membrane-penetrated chelator of Ca^{2+} BAPTA-AM were carried out in intact and trained snails. BAPTA-AM was used at the concentration of 10^{-4} M.

The results were shown as mean ± SEM. The one-way repeated measurements ANOVA test was used to compare the values of potentials in control (saline solution) and after changing the concentration of Ca^{2+} within one group of animals. It used the statistical software SigmaPlot11. The statistical significance criterion was $p < 0.05$.

3 Results and Discussion

In the first series of experiments, we studied the effects of increasing the calcium concentration on the electrical characteristics of premotor interneurons of intact and trained snails, caused by release calcium from intracellular depots under the action of caffeine [5, 7]. It was found that the membrane potential Vm did not change after adding the caffeine at a concentration of 2 mM in bathing saline solution either in intact or in trained snails (Fig. 1A). The value of threshold potential Vt significantly decreased after adding caffeine from 19.8 ± 0.7 to 15.5 ± 0.9 mV ($p < 0.001$; $n = 10$) in intact snails and significantly decreased from 16.8 ± 0.5 to 12.8 ± 0.7 mV ($p < 0{,}001$; $n = 6$) in trained snails (Fig. 1B). This increase in the excitability of premotor interneurons followed by the increase of the calcium ion concentration without any changes of the membrane potentials depended on intrinsic mechanisms and did not connect with changes of the membrane excitability after training.

In the second series of experiments, we investigated the effects of the reducing of the calcium content in the neuron on the electrical characteristics of premotor interneurons of intact and trained snails by adding calcium chelators EGTA and BAPTA-AM [25, 26]. It was found that the membrane potential Vm did not change for 30 min after the decrease of intracellular calcium concentration by injection of EGTA in neuron either in intact snails or trained snails. In the group of intact snails, the threshold potential Vt significantly increased up to 22.5 ± 0.4 mV 30 min after the injection of EGTA compared with values before injection 19.2 ± 0.6 mV ($p < 0{,}001$; $n = 10$). In the group of trained snails, Vt tended to increase

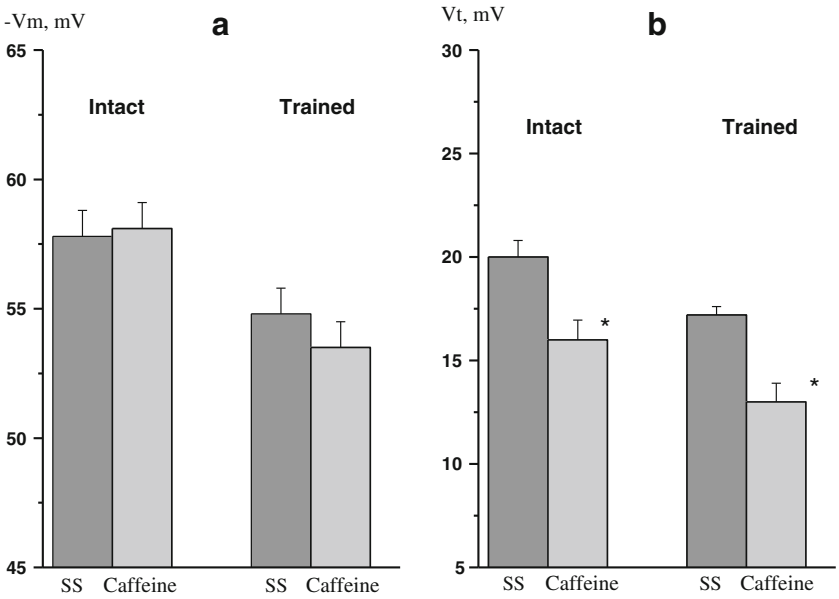

Fig. 1 The values of the electrical parameters of the premotor interneurons of the defensive reflex in intact and trained snails in saline solution and after adding caffeine. (Intact)—intact snails, (Trained)—trained snails, (SS)—saline solution, (Caffeine)—saline solution with caffeine. The vertical axis shows value of potential, in mV, (A)—the membrane potential (Vm); (B)—the threshold potential (Vt); Asterisks (*) indicate significant difference ($p < 0.05$, paired t test)

from 14.9 ± 1.2 mV before injection to 16.8 ± 0.8 mV ($n = 8$) 30 min after injection, but this difference was not significant (Fig. 2). Apparently, the contribution of intracellular calcium chelated by EGTA injection to the development of excitation of the premotor interneuron decreases after the training of the animals.

In continuation of this study, we carried out the experiments in which the decrease of intracellular Ca^{2+} concentration was achieved by adding the membrane-penetrating calcium ion chelator (BAPTA-AM) to the saline solution washing nervous system of mollusk. The buffer BAPTA-AM has the similar affinity to Ca^{2+} as EGTA but possesses the faster calcium-binding kinetics that allows it rapidly bind Ca^{2+} ions entering through the channels [27]. The registration of electrical characteristics of the premotor neurons showed that the membrane potential Vm and the threshold potential Vt did not significantly change after adding BAPTA-AM for all groups of animals (Fig. 3).

It is known that calcium ions play an important role in long-term forms of neuroplasticity. First of all, it is true for the induction of presynaptic facilitation [8, 28]. However, it was shown later that during the formation of a conditioned reflex, the changes at the level of postsynaptic neurons were observed [29]. It was also found that the intracellular injection of the calcium chelator EGTA blocked the induction of long-term depression [30], and the injection of $CaCl_2$ into the postsynaptic neuron produced the changes similar to synaptic facilitation [31]. In addition, it was found that the release of Ca^{2+} ions from intracellular storage played a decisive role in this process, while the entry of Ca^{2+} through potential dependent channels was needed only for initiating the release of Ca^{2+} ions from intracellular depots [7, 10, 32]. The main reservoirs

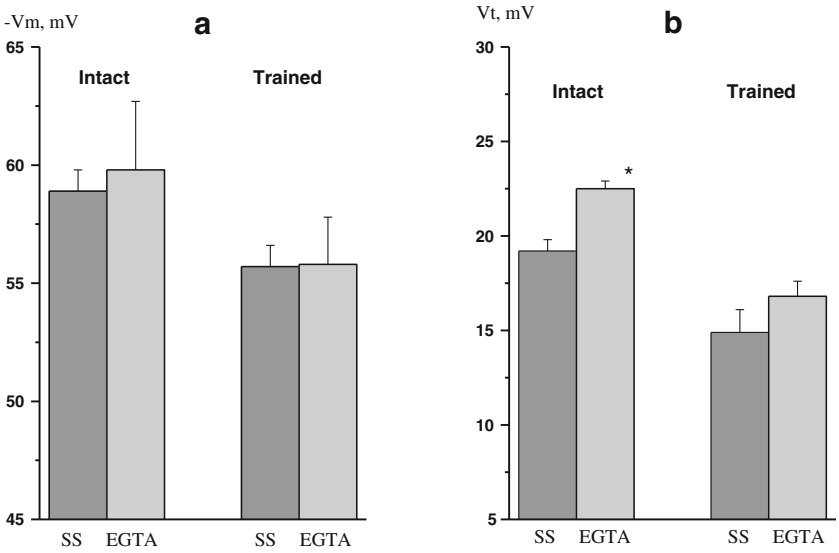

Fig. 2 The values of the electrical parameters of the premotor interneurons of the defensive reflex in intact and trained snails in saline solution and after injection of EGTA in premotor interneurons. (Intact)—intact snails, (Trained)—trained snails, (SS)—saline solution, (EGTA)—intracellular EGTA injection. The vertical axis shows value of potential, in mV, (A)—the membrane potential (Vm); (B)—the threshold potential (Vt); Asterisks (*) indicate significant difference ($p < 0.05$)

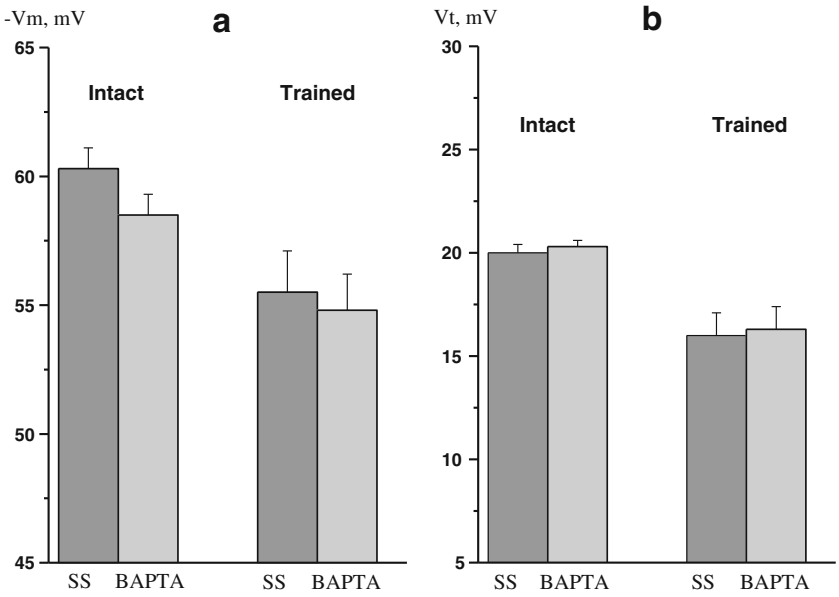

Fig. 3 The values of the electrical parameters of the premotor interneurons of the defensive reflex in intact and trained snails in saline solution and after adding of BAPTA-AM. (Intact)—intact snails, (Trained)—trained snails, (SS)—saline solution, (BAPTA)—saline solution with BAPTA-AM. The vertical axis shows value of potential, in mV. (A)—the membrane potential (Vm); (B)—the threshold potential (Vt)

of intracellular Ca^{2+} storage are the endoplasmatic reticulum and mitochondria [7, 33]. Recently, it becomes evident that not only maintaining a high concentration of cytosolic calcium is a key condition for the memory consolidation. In experiments on the formation of olfactory memory in Drosophila, it is shown that this process requires the transport of cytosolic calcium to mitochondria in the mushroom body neurons [34].

Earlier, it was found that the repetitive combination of conditioned and unconditioned stimuli elicited cumulative membrane depolarization of type B photoreceptors in *Hermissenda crassicornis* [35]. Similar depolarization after learning was observed on premotor interneurons of defensive behavior of terrestrial snail [17, 36]. Long-term depolarization of the membrane and the increase of the excitability were also accompanied by an increase of intracellular Ca^{2+} concentration, which can be recorded by visualizing intracellular Ca^{2+} using the fura-2 [37]. In further experiments, intracellular injection of the Ca^{2+} chelator EGTA and the antagonist of the release of intracellular Ca^{2+} heparin effectively prevented increase of the excitability caused by the light in photoreceptors of *Hermissenda* [29]. The investigation of the high-amplitude EPSP in the premotor interneurons in terrestrial snails showed that high-amplitude EPSP, like action potentials, was accompanied by the increases of intracellular calcium ion concentrations, which entered through the voltage-dependent Ca^{2+} channels [11]. Thus, the increase of intracellular Ca^{2+} concentration on the initial stage of the conditioning plays an important role in the formation of associative learning.

Our results showed that the increase of the calcium concentration by release of Ca^{2+} from the intracellular store led to the increase in the excitability of premotor interneurons in both intact and trained snails and did not affect the changes of the membrane characteristics after conditioning. The decrease of intracellular Ca^{2+} concentration in premotor interneurons by injection of EGTA significantly influenced the threshold potential only in the group of intact snails but not in the group of trained snails. The decrease of intracellular Ca^{2+} concentration in premotor interneurons by adding BAPTA-AM did not lead to specific changes in the electrical characteristics of these neurons in both intact and trained snails. That difference in effects of chelators depended on the speed of their ability to bind Ca^{2+} ions. Thus, we suggested that increasing or decreasing of intracellular calcium after training was not involved in the maintaining of the changes of the membrane characteristics of premotor interneurons observed after conditioning. The increase of intracellular calcium was largely needed at the stage of initiation of formation of learning.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Research Involving Humans and Animals Statement All experimental procedures were carried out according with requirements of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and according with guidelines of Kazan Federal University. The terrestrial snails Helix lucorum were used for present experiments. Capture of animals in the wild nature was carried out by competent persons without any pain and distress (Article 9 of Directive 2010/63/EU). Snails were transported asleep and then stored

asleep (Article 33 of Directive 2010/63/EU). Prior to the experiments snails were kept for no less than 2 weeks in a glass terrarium in a humid atmosphere at room temperature (Article 33 of Directive 2010/63/EU).

Informed Consent None.

References

1. Hawkins, R. D., Kandel, E. R., & Bailey, C. H. (2006). Molecular mechanisms of memory storage in aplysia. *Biology Bulletin, 210*(3), 174–191.
2. Schaffhausen, J. H., Fischer, T. M., & Carew, T. J. (2001). Contribution of postsynaptic Ca2+ to the induction of posttetanic potentiation in the neural circuit for siphon withdrawal in Aplysia. *The Journal of Neuroscience, 21*(5), 1739–1749.
3. Catterall, W. A., & Few, A. P. (2008). Calcium channel regulation and presynaptic plasticity. *Neuron., 59*(6), 882–901. https://doi.org/10.1016/j.neuron.2008.09.005.
4. Balaban, P. M., Korshunova, T. A., & Bravarenko, N. I. (2004). Postsynaptic calcium contributes to reinforcement in a three-neuron network exhibiting associative plasticity. *The European Journal of Neuroscience, 19*, 227–233.
5. Berridge, M. J. (1998). Neuronal calcium signaling. *Neuron., 2*, 13–26.
6. Brini, M., & Carafoli, E. (2000). Calcium signaling: a historical account, recent developments and future perspectives. *Cellular and Molecular Life Sciences, 57*, 354–370.
7. Rizzuto, R., & Pozzan, T. (2006). Microdomains of intracellular Ca2+: molecular determinants and functional consequences. *Physiological Reviews, 86*, 369–408.
8. Rusakov, D. A. (2006). Ca2+−dependent mechanisms of presynaptic control at central synapses. *The Neuroscientist, 12*(4), 317–326.
9. Burnashev, N., & Rozov, A. (2005). Presynaptic Ca2+ dynamics, Ca2+ buffers and synaptic efficacy. *Cell Calcium, 37*(5), 489–495.
10. Neher, E., & Sakaba, T. (2008). Multiple roles of calcium ions in the regulation of neurotransmitter release. *Neuron., 59*(6), 861–872. https://doi.org/10.1016/j.neuron.2008.08.019.
11. Malyshev, A. Y., & Balaban, P. M. (2014). Changes in intracellular calcium ion concentrations during generation of high-amplitude EPSP in neurons in the common snail. *Neuroscience and Behavioral Physiology., 44*, 681–686. https://doi.org/10.1007/s11055-014-9969-3.
12. Demuro, A., & Parker, I. (2013). Cytotoxicity of intracellullar Aβ42 amyloid oligomers involves Ca2+ release from the ER by stimulated production of inositol trisphosphate. *The Journal of Neuroscience, 33*(9), 3824–3833.
13. Blackwell, K. T., & Alkon, D. L. (1999). Ryanodine receptor modulation of in vitro associative learning in Hermissenda crassicornis. *Brain Research, 822*(1–2), 114–125.
14. Glancy, B., & Balaban, R. S. (2012). Role of mitochondrial Ca2+ in the regulation of cellular energetics. *Biochemistry., 51*(14), 2959–2973. https://doi.org/10.1021/bi2018909.
15. Kostyuk, P. G., Shmigal, A. V., Voitenko, N. V., Svichar, N. V., & Kostyuk, E. P. (1998). Endoplasmatic reticulum and mitochondria as elements of mechanism of intracellular signalization in nerve cell. *Rossiiskii fiziologicheskii zurnal (Russian)., 84*(10), 979–985.
16. Verkhratsky, A. (2005). Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiological Reviews, 85*(1), 201–279.
17. Gainutdinov, K. L., Gainutdinova, T. H., & Chekmarev, L. Y. (1996). Changes of electrical characteristics of command neurons during defensive reflex conditioning in terrestrial snail. *Zhurnal vysshei nervnoi deiatelnosti imeni I. P. Pavlova (Russian)., 46*, 614–616.
18. Andrianov, V. V., Bogodvid, T. K., Deryabina, I. B., Golovchenko, A. N., Muranova, L. N., Tagirova, R. R., Vinarskaya, A. K., & Gainutdinov, K. L. (2015). Modulation of withdrawal reflex conditioning in snails by serotonin. *Frontiers in Behavioral Neuroscience, 9*(279), 1–12. https://doi.org/10.3389/fnbeh.2015.00279.
19. Gainutdinov, K. L., & Beregovoi, N. A. (1994). Long-term sensitization in snail: electrophysiological correlations in command neurons of avoidance behavior. *Zhurnal vysshei nervnoi deiatelnosti imeni I. P. Pavlova (Russian), 44*(2), 307–315.
20. Gainutdinov, K. L., Andrianov, V. V., Gainutdinova, T. K., & Tarasova, E. A. (2000). The electrical characteristics of command and motor neurons during acquisition of a conditioned withdrawal reflex and formation of long-term sensitization in snails. *Neuroscience and Behavioral Physiology, 30*(1), 81–88.
21. Khodorov, B. I. (1974). *The problem of excitability. Electrical excitability and ionic permeability of the nerve membrane* (p. 301). New York: Plenum Press.
22. Kostyuk, P. G. (1992). *Calcium ions in nerve cell function* (p. 228). Oxford Neuroscience Series.
23. Silant'eva, D. I., Andrianov, V. V., Gainutdinova, T. K., Gainutdinov, K. L., & Pleshchinskii, I. N. (2006). The effects of changes in extracellular calcium concentrations on the electrical properties of command neurons after acquisition of a defensive conditioned reflex in snails. *Neuroscience and Behavioral Physiology., 36*(3), 209–212.
24. Balaban, P. M., Zakharov, I. S. (1992). Learning and development – bases of two phenomena. M.: Nauka (Russian). 151.
25. Pagala, M. K. D., & Taylor, S. R. (1998). Imaging caffeine-induced Ca2+−transients in individual fast-twitch and slow-twitch rat skeletal muscle fibers. *American Journal of Physiology. Cell Physiology, 274*, 623–632.
26. Jong, D. S., Pape, P. C., Baylor, S. M., & Chandler, W. K. (1995). Calcium inactivation of calcium release in frog cut muscle fibers that contain millimolar EGTA or Fura-2. *The Journal of General Physiology, 106*, 337–338.
27. Adler, E. M., Augustine, G. J., Duffy, S. N., & Charlton, M. P. (1991). Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *The Journal of Neuroscience, I*(6), 1496–1507.
28. Eliot, L. S., Kandel, E. R., Siegelbaum, S. A., & Blumenfeld, H. (1993). Imaging terminals of Aplysia sensory neurons demonstrates role of enhanced Ca2+ influx in presynaptic facilitation. *Nature., 361*, 634–637.
29. Murphy, G. G., & Glanzman, D. L. (1996). Enhancement of sensorimotor connections by conditioning-related stimulation in Aplysia depends upon postsynaptic Ca2+. *Proceedings of the National Academy of Sciences of the United States of America, 93*(18), 9931–9936.
30. Malyshev, A. Y., & Balaban, P. M. (1999). Synaptic facilitation in Helix neurons depends upon postsynaptic calcium and nitric oxide. *Neuroscience Letters, 261*(1–2), 65–68.
31. Lin, X. Y., & Glanzman, D. L. (1994). Hebbian induction of long-term potentiation of Aplysia sensorimotor synapses: partial requirement for activation of an NMDA-related receptor. *Proceedings of the Royal Society of London - Series B: Biological Sciences, 255*(1344), 215–221.
32. Talk, A., & Matzel, L. (1996). Calcium influx and release from intracellular stores contribute differentially to activity-dependent neuronal facilitation Hermissenda photoreceptors. *Neurobiology of Learning and Memory, 66*(2), 183–197.

33. Pathak, T., & Trbak, M. (2018). Mitochondrial Ca2+ signaling. *Pharmacology and Therapeutics, 192*, 112–123.
34. Drago, I., & Davis, R. L. (2016). Inhibition of mitochondrial calcium uniporter during development impairs memory in adult Drosophila. *Cell Reports, 16*(10), 2763–2776.
35. Alkon, D. L. (1984). Changes of membrane currents during learning. *The Journal of Experimental Biology, 112*, 95–112.
36. Gainutdinov, K. L., Andrianov, V. V., & Gainutdinova, T. K. (2011). Changes of the neuronal membrane excitability as cellular mechanisms of learning and memory. *Uspekhi Physiologicheskikh Nauk (Russian)., 42*, 33–52.
37. Muzzio, I. A., Ramirez, R. R., Talk, A. C., & Matzel, L. D. (1999). Interactive contributions of intracellular calcium and protein phosphatases to massed-trials learning deficits in Hermissenda. *Behavioral Neuroscience, 113*(1), 103–117.