5-Bromo-2'-Deoxyuridine Impairs Long-Term Food Aversion Memory in Edible Snail

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We studied the involvement of DNA synthesis into molecular mechanisms of long-term memory. Nucleoside analogue 5-bromo-2'-deoxyuridine (BrdU) is known to incorporate into synthesizing DNA and prevent subsequent DNA replication from this region. To investigate the effect of BrdU administration on long-term memory, terrestrial gastropods edible snails *Helix lucorum* were trained in the food aversion paradigm. Single-session training (carrot presentation combined with application of 10% quinine solution, three carrot presentations with 10-min intervals) resulted in the formation of long-term memory that persisted for at least 45 days. BrdU administration (250 mg/kg) 30 min before training impaired long-term memory tested 24 h later. Immunohistochemical study revealed BrdU incorporation in the nuclei of identified neurons of defensive behavior.

Key Words: *nucleoside analogues; DNA synthesis; neurons; memory; gastropods*

In modern neurobiology, structural modification of synapses and increase in synaptic transmission efficiency in neurons involved are considered to be the main mechanisms of long-term memory formation [5]. According to the theory, long-term fixation of these changes requires enhanced protein synthesis [6]. However, some findings can hardly be explained using this theory, *e.g.* the possibility of recovery of memory impaired by protein synthesis blockade during training in birds and mammals [3,8] and replacement of neuronal substrates necessary for memory retrieval following different times after training [4,7].

Amnestic effects of DNA synthesis inhibitors and its antimetabolites administered during the learning phase has been shown [2,9]. These data suggests that additional mechanisms of long-term memory formation and maintenance that involve DNA synthesis may exist in the nervous system.

Mollusks, that have well described circuit of identified neurons, appear to be a useful model in investigations focused on cellular neurobiology of learning [5]. Morphological, electrophysiological and cytochemical properties of neuronal circuits of defensive and feeding behavior in edible snail had been extensively investigated [1]. However, the possible role of DNA synthesis in long-term memory mechanisms was never studied on simple nervous system of mollusks.

The aim of this study was to investigate possible involvement of DNA synthesis in mechanisms of long-term memory in the mollusk. Research tasks included: development of aversive training model in edible snail ensuring long-term memory formation persisting for at least 14 days; evaluation of the effects of 5-bromo-2'-deoxyuridine (BrdU) administration on long-term memory in edible snail 24 h after training; immunohistochemical detection of BrdU incorporation into DNA in edible snail nervous system following food aversion training.

MATERIALS AND METHODS

The study employed 60 edible snails *Helix lucorum*, Crimea population weighting 30-35 g. Before training,

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the animals were deprived from food for 5-7 days. The experiments were conducted in accordance with the Order No. 267 Ministry of Health of Russian Federation (19.06.2003) and "Rules of Studies on Experimental Animals" (approved by the Ethics Committee of the P. K. Anokhin Institute of Normal Physiology; protocol No. 1, 3.09.2005). Training was conducted in the familiar environment. During training and testing, the latencies of approach to the food (2-3 g) the first consumatory movements were registered. The food was placed at the distance of 1.5 cm from the head of the free-moving animal. Food aversion training consisted in presentation of novel food (carrot) to the animal and application of 10% quinine hydrochloride solution on the food following the first consumatory movements. The following training protocols were used: single combination of conditioned stimulus (carrot) and 10% quinine solution; triple combination of the carrot and 10% quinine solution with 10-min intervals; triple presentation of the carrot with application of 10% quinine solution. During the triple presentation (with 10-min intervals) the snails under conditions of free behavior received quinine solution application only during the first carrot presentation; during the second and third presentations, quinine was applied only for the snails that did not avoid the carrot and tasted it again (10-15% of all experimental animals). Long-term memory was tested 4, 8, 24, 48 h, 14 and 45 days after training by presenting the conditioned (carrot) and differentiating (cabbage) stimuli. If the animals did not start to consume the food in 120 sec, the test was stopped and the reaction was considered aversive. Nucleoside analogue BrdU was administered in a dose of 250 mg/kg intracoelomicaly in saline 0.2 ml 30 min before training. The control animals received 0.2 ml saline.

Mapping of the neuronal substrates of long-term memory in edible snail was carried out using fluorescent immunohistochemistry. The patterns of BrdU incorporations in 24 h after the administration were comrpared in two groups: BrdU+training and BrdU+ active control. Paraharyngeal ganglion ring was removed and frozen in nitrogen vapors. Cryostat sections (20 μ) were stained according to the standard protocol including DNA denaturation with 5 N HCl at 55°C for 1 h. Primary sheep anti-BrdU antibodies (1:500; Exalpha) and secondary donkey anti-sheep AlexaFluor488 antibodies (1:500; Molecular Probes, Invitrogen) were used. For morphological DNA staining, the sections were stained with fluorescent dye Hoechst 33342 (1:1000) for 30 min at room temperature. The data were statistically processed using Statistica 8.0 software and nonparametric Mann–Whitney test and were presented as $M \pm SD$.

RESULTS

Comparison of different protocols of training in food aversion task in aversionedible snails revealed that the possibility of memory formation depends on the number of exposures to conditioned stimulus. Edible snails are unable to form long-term memory following single combination of carrot and 10% quinine solution. During training, the time to reach the food was 59.6±11.5 sec, whereas during the test 24 h later it was 85.6±30.9 sec (p=0.12; Fig. 1, *a*). When the animals were exposed to the combination of carrot and quinine three times with 10-min intervals, the results

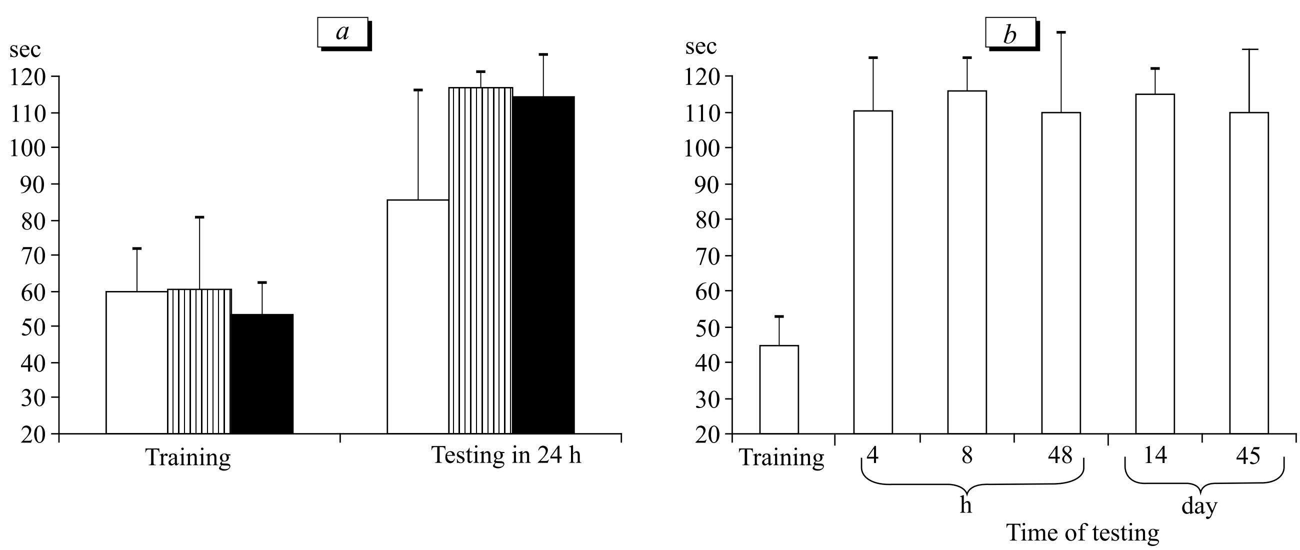


Fig. 1. Latent period for food capture in edible snails during food aversion training. *a*) effects of training protocol on possibility for long-term memory formation in edible snail: single combination (light bars), triple combination (shaded bars), and triple presentation (dark bars); *b*) test for the duration of food aversion following triple presentation of carrot and 10% quinine solution.

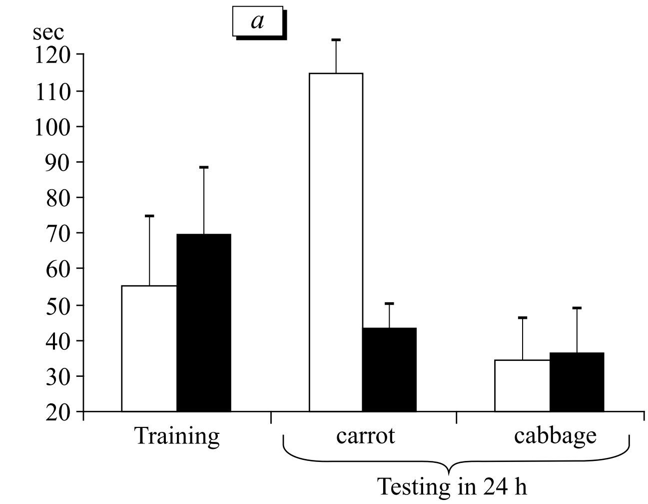
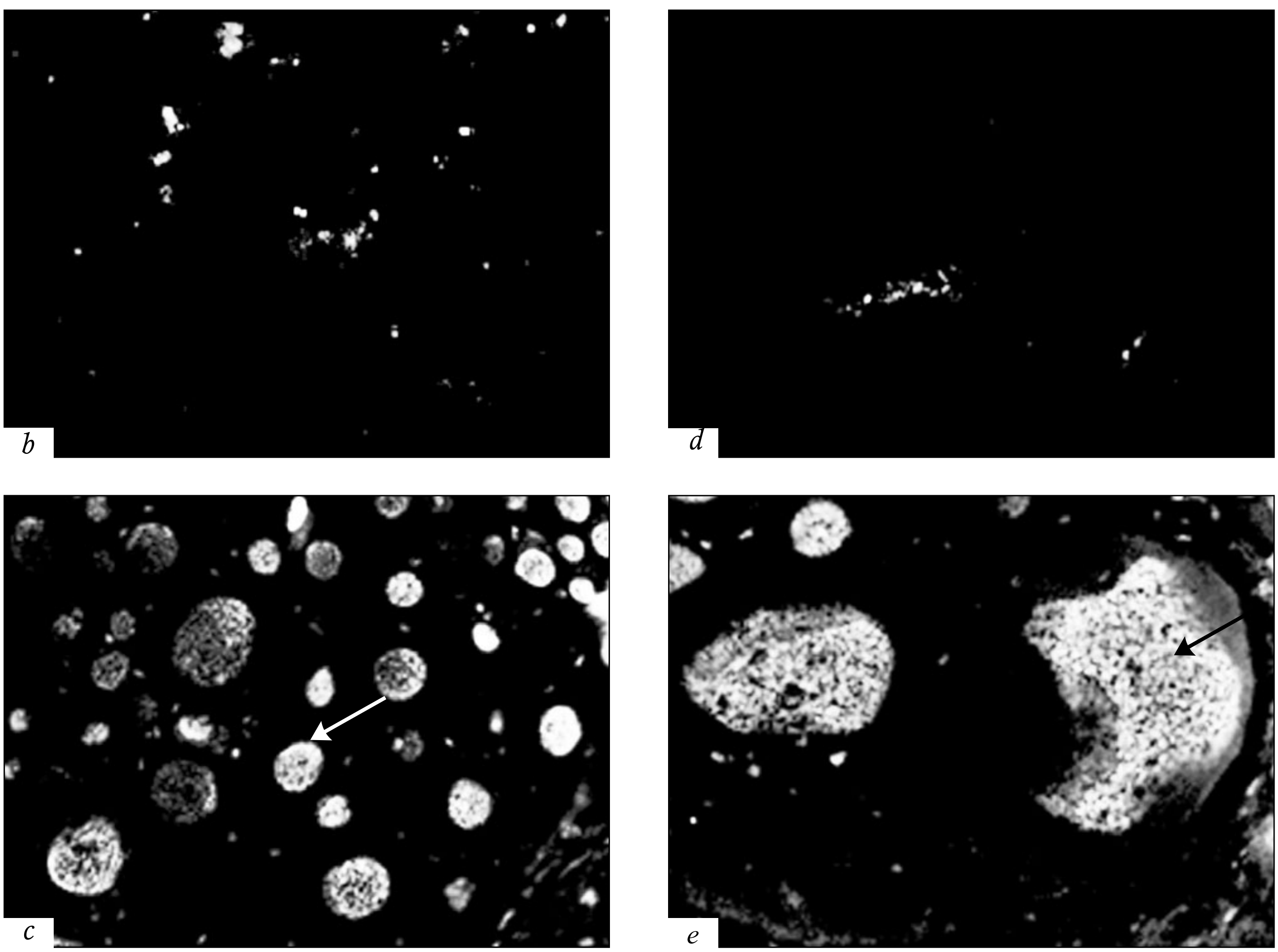


Fig. 2. Effect of administration of nucleoside analogue BrdU on edible snail training in food aversion paradigm. *a*) latent period for food capture before and after aversion formation in the animals injected with saline (light bars) or BrdU (dark bars) 30 min before training, as tested 24 h after for conditioned stimulus (carrot) and differentiating stimulus (cabbage); *b-e*) immunohistochemical labeling of BrdU incorporation in edible snail neurons: *b*, *d*) in nervous system of control animals; *c*, *e*) in snail nervous system 24 h after training, *b*, *c*) neurons in rostromedial area of pedal ganglion; *d*, *e*) identified Pa2 neurons of parietal ganglion. Arrows: immunohistochemically detected BrdU incorporations in neuronal nuclei after training.



were 60.2±20.4 and 116.8±4.6 sec during training and testing 24 h after it, respectively (Fig. 1, *a*). Triple presentation also ensured the formation of long-term memory: training – 53.2±8.9 sec, test 24 h after – 114.6±12.1 sec (Fig. 1, *a*).

Preservation of the memory, formed during the edible snail training in the food aversion paradigm with triple presentation was tested 4, 8, 48 h, 14 and 45 days after training (5 animals per each time point). Short-term memory test 4 h and 8 h after training demonstrated that time to capture the food in first trial during training was short – 44.8±8.2 sec and significantly increased 4 h (110.2±15.0 sec) and 8 h (115.8±9.4 sec) after training (Fig. 1, *b*).

Food aversion training resulted in the formation of long-term memory as tested 48 h (109.60±22.15 sec), 14 days (115.0±7.1 c), and 45 days later (110.0±17.3 sec; Fig. 1, *b*).

The administration of nucleoside analogue BrdU 30 min before training impaired memory during testing 24 h after training (69.4±18.9 sec in training, 43.4±6.8 sec when tested for conditioned stimulus) in comparison with saline administration (55.2±20.2 sec in training, 114.6±12.1 sec when tested for conditioned stimulus; Fig. 2, *a*). Presentation of the differentiating stimulus confirmed specificity of the aversion reaction in animals administered with saline (saline – 34.4±11.1 sec, BrdU – 36.2±12.7 sec; Fig. 2, *a*).

Immunohistochemical BrdU labeling in edible snail neurons detected BrdU incorporations in the nervous system 24 h after training, but not in control animals (Fig. 2, *b*). Immunoreactivity was observed in the neurons crucial for the formation of defensive behavior in edible snail (neurons in the rostromedial area of pedal ganglion, Pa2 and Pa3 neurons of parietal ganglion) and was insignificant in visceral ganglion neurons.

Thus, the obtained results suggest that DNA synthesis is necessary for the formation of long-term memory in models of aversive training in edible snails.

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