PHYSIOLOGY

Intravital Investigation of the Effects of Serotonin and Glutamate on the Dynamics of DNA Activity in L-RPl1 Neurons of Edible Snail

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Microloci of varying size with increased fluorescence were observed in L-RPl1 neuronal nuclei of snails using DNA-selective dye SYTO16. Application of serotonin and glutamate increased the number of small (<1 μ in diameter) microloci, while the number of medium and large (1-3 μ) loci decreased. Combined application of neurotransmitters produced more pronounced changes in the number of microloci compared to individual administration. RNA synthesis inhibitors abolished the effects of the transmitters. We hypothesized that the revealed small microloci of fluorescence are "active" DNA zones, where transcription of new genes are initiated.

Key Words: *serotonin; glutamate; neuron; SYTO16; DNA*

Ample data suggest that the formation of long-term memory and long-tern neural plasticity depends on activity of neuronal genetic apparatus and RNA and protein synthesis [6,9,14]. However, only few genes from hundreds and thousands of those activated in individual neurons during acquisition of new skills are now investigated. Spatiotemporal design of processes occurring in nerve cell nucleus during learning is virtually not studied.

In this context, the use of conceptually new methods allowing, apart from "standard" registration of electric activity of nerve cells, intravital comparative investigation of the dynamics of activity of their genetic apparatus, gains special attention. We previously developed methods for intravital study of the dynamics and spatial distribution of DNA and RNA in snail neuronal nuclei using fluorescent microscopy and image analysis system [3,4]. Since the mentioned issue is evidently complex, "reduction" of the learning process and modeling of its components on individual cells can be a promising approach for its experimental investigation. Using this approach we showed, that some neurotransmitter compounds, including serotonin and glutamate, induce plastic changes in neurons simulating the effects of learning [5].

Using intravital fluorescent dye SYTO16 we studied changes in DNA activity in the nuclei of defensive behavior command neurons L-RPl1 in intact snails and after serotonin and glutamate applications.

MATERIALS AND METHODS

Experiments were carried out on 28 LPl1 and RPl1 neurons of isolated CNS of edible snails *Helix lucorum* [1,7]. Defensive behavior command neurons

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L-RPl1 were visualized using an Olympus BX51 fluorescent microscope (Olympus) with an UMPlanFI 20×0.50 lens. Optical signal passed through relevant interference filters (excitation wavelength 490 nm, fluorescence wavelength 520 nm) was recorded by a DP50 camera (Olympus). The data were processed by a computer equipped with AnalySIS Doku (SIS) and ImageProPlus (Media Cybernetics) software kindly provided by Prof. N. N. Zolotov (Research Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences). After registration of the initial optical signal from unstained neuron, physiological saline containing membrane-permeable analog SYTO16 AM (100 μM; Invitrogen) was applied to the neuron for1 h followed by perfusion of the neuronal ganglia with physiological solution without dye.

Serotonin (100 μM, Sigma) and glutamate (5 μM, Sigma) were dissolved in physiological saline immediately before the experiment. Transmitter compounds were applied into the medium simultaneously or separately 5 times 2 min each with 15 min intervals. Application of RNA synthesis inhibitor actinomycin D (20 μM) or α-amanitin (20 μg/ml; Sigma) was started 60 min before application of neurotransmitters and was continued until the end of the experiment. In the control, application of RNA synthesis inhibitors was not followed by neurotransmitter application.

The experiments were started 3-4 h after completions of staining. By this time, fluorescent signal attained a relatively stable level. Fluorescent microimages were obtained with 15 min intervals from 1 h before to 3-5 h after the start of mediator application. L-RPl1 neuronal nuclei were stained nonuniformly; microloci of varying size with high fluorescence intensity were observed. For detection of microloci and evaluation of their number and size we used a special algorithm containing consecutive steps: after background correction (Feature width 51) and local equalization (Window 30, Step 5, Option Log) the median filtering was applied (Option 3×3, Passes 1, Strength 3) and binarization in neuronal nucleus area was carried out (70-255). Then, the microloci were counted and their size was determined (mean diameter averaged by two perpendicular axes passing through the center of the object). The data were normalized for each experiment (parameter before neurotransmitter application was set as 100%), averaged, and expressed in terms of percents of baseline values. Significance of differences was evaluated using Student's *t* test.

RESULTS

Incubation of snail ganglia in physiological saline containing SYTO16 AM resulted in staining of nucleus, but not cytoplasm of LPl1 neurons. Cell nucleus was stained nonuniformly: dot microloci with higher fluorescence intensity compared to surrounding areas (by 5-7%) were observed. These microloci formed a polygonal net-like structure. The inner parts of these polygonal structures remained dark and unstained (Fig. 1).

Statistical analysis showed that the mean diameter of microloci was 0.86±0.02 μ (n=54,321), and their number on the surface of neuron nucleus was 9573±562. All microloci were divided by the mean diameter into 4 groups: 8042±483 (84%) very small microloci (mean diameter 0.1-0.5 μ), 981±63 (10%)

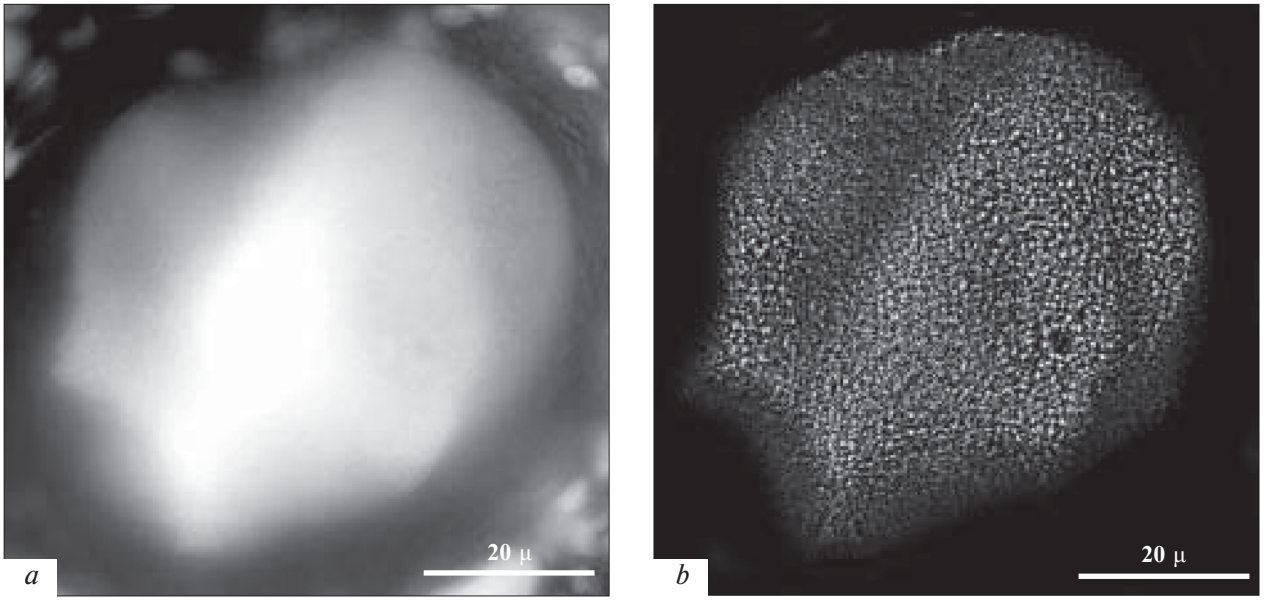

Fig. 1. Intravital imaging of RPl1 neuron of edible snail stained with DNA-selective dye SYTO16 before (*a*) and after (*b*) contrasting of microloci with high fluorescence level, ×20.

small (0.5-1 μ), 404±30 (4%) medium (1-1.5 μ), and 147±42 (2%) large (1.5-3 μ) microloci.

Serotonin significantly increased the number of small and very small microloci by 20-27% ($p<0.05$; $n=6$) 45-60 min after the first application, whereas the number of large microloci during this period decreased by 47-50% ($p<0.01$). By the end of the experiment, a trend to restitution of the baseline values was noted (120-150 min after the start of serotonin application). No significant changes in the number of medium microloci were noted (Fig. 2, *a*).

Significant increase in the number of very small microloci was observed 15-30 min after the first application of glutamate (by10-12%, $p<0.05$; $n=6$) accompanied by decrease in the number of medium (by 18-19%) and large (by 47-52%) microloci ($p<0.01$). A trend towards restitution of baseline parameter was noted 60-75 minutes after the start of glutamate application. No significant changes in the number of small microloci were observed (Fig. 2, *b*).

Simultaneous serotonin and glutamate application to CNS significantly increased the number of small and very small microloci (by 17-36%; $p<0.01$; $n=7$) 15-30 minutes after the start of neurotransmitter application and decreased the number of medium and large microloci (by 33-60%; $p<0.01$). These changes in the number of microloci persisted until the end of the experiment (Fig. 2, *c*).

Thus, these results indicate that specific changes in the number of certain microloci with specific for each exposure intensity, duration, and latency of the effects were noted after both isolated and combined application of serotonin and glutamate.

Since SYTO16 selectively binds with DNA and reflects changes in its activity [15], we hypothesized that changes in the number and size of microloci are induced by activation of transcription of new genes accompanied by DNA despiralization and reorganization in distinct chromosome loci. To check this hypothesis, we studied the influence of transcription inhibitors

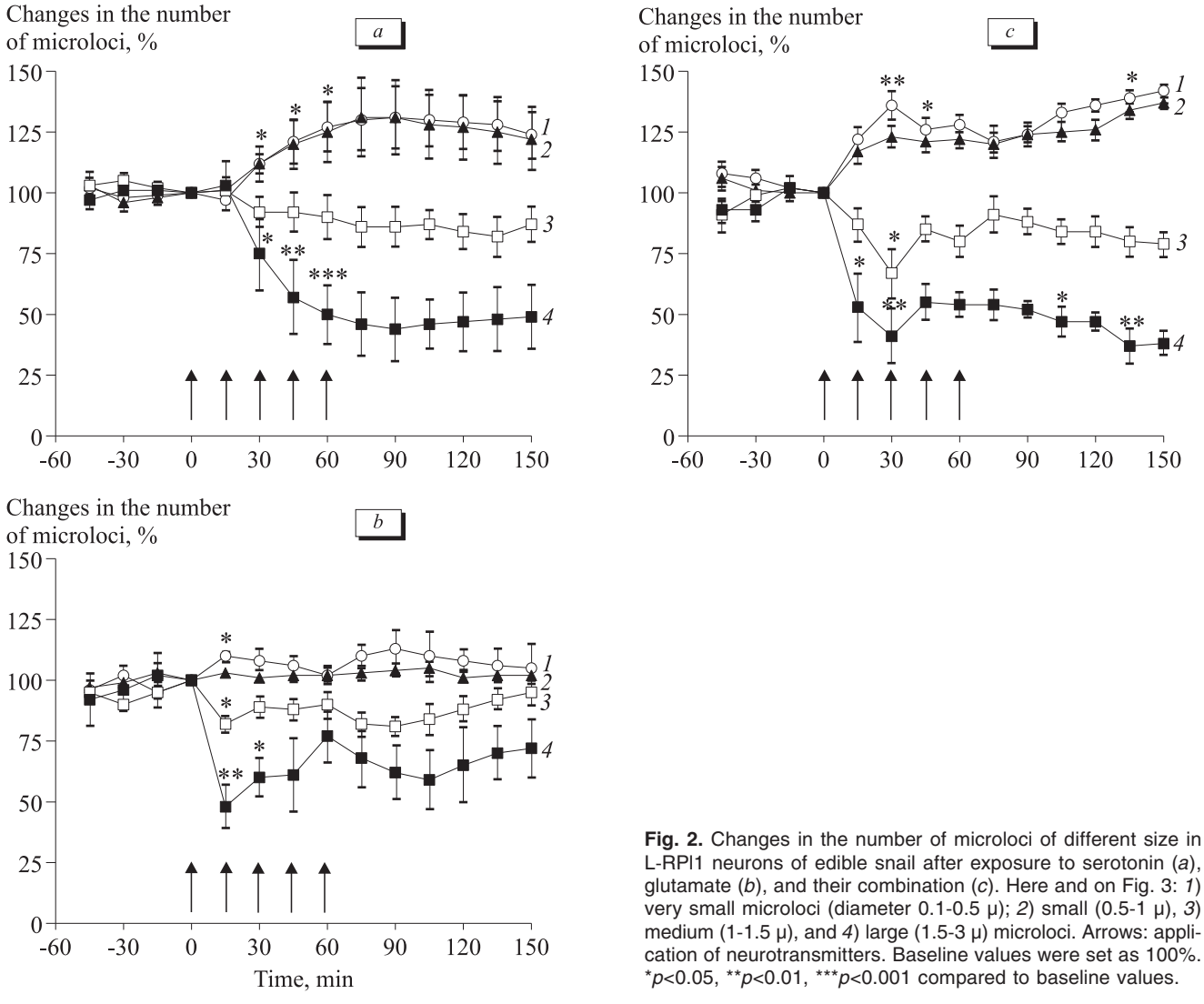

Fig. 2. Changes in the number of microloci of different size in L-RPl1 neurons of edible snail after exposure to serotonin (*a*), glutamate (*b*), and their combination (*c*). Here and on Fig. 3: *1*) very small microloci (diameter 0.1-0.5 μ); *2*) small (0.5-1 μ), *3*) medium (1-1.5 μ), and *4*) large (1.5-3 μ) microloci. Arrows: application of neurotransmitters. Baseline values were set as 100%. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to baseline values.

α-amanitin and actinomycin D on the effects induced by simultaneous application of neurotransmitters. α-Amanitin did not change the number of microloci of different size over 60 min before neurotransmitter application. Subsequent application of serotonin and glutamate against the background of inhibitor action by minutes 30 significantly increased the number of medium (by 15-20%; $p<0.05$; $n=5$) and large (by 30-46%, $p<0.05$) microloci. The trend toward restitution of the baseline parameter was noted 120-150 min after the start of neurotransmitter application. No significant changes in the number of small and very small microloci were observed (Fig. 3, *a*).

Actinomycin D produced similar effects. Application of actinomycin D for 60 min before neurotransmitter application did not change the number of microloci, while subsequent simultaneous administration of serotonin and glutamate significantly increased the number of large microloci (by 40-63%; $p<0.05$; $n=4$) by minutes 30-45. Restitution of the baseline parameters was observed by minutes 80-105 after neurotransmitter application. No significant changes in the number of medium, small and very small microloci were found (Fig. 3, *b*).

Thus, these findings indicate that transcription inhibitors suppress the effects of serotonin and glutamate in nuclei of LPl1 and RPl1 defensive behavior command neurons of edible snail.

Molecular genetic studies [10] showed that one part of the genome in mature neurons is in compact and another part is in "unwound" decondensed condition characterized by high DNA transcription level. Genome decondensation and activation are regulated by numerous extra- and intracellular factors, including neurotransmitters. One may assume that the effect of microlocus "dispersal" (increase in the number of small microloci and simultaneous decrease in the number of medium and large ones) observed in our experiments corresponds to DNA transition from supercoiled condition to decondensed one, when DNA sites are activated and form new gene transcription sites. This assumption was confirmed in experiments with transcription inhibiting agents, which are known to intercalate DNA strands and disturb transcription site formation [12].

Our experiments revealed high fluorescent microloci of varying size (0.1-3 μ). The size of free DNA (double strand) is known to be 30 nm, in interphase nucleus DNA molecule is folded densely and form "loops" 50-2500 nm in size [8,11,13]. These facts suggest that microloci observed in our studies correspond to loop-like structures with different condensation degree.

We also demonstrated qualitatively and quantitatively different dynamics of changes in the number of microloci of different size after exposure to the test neurotransmitters. Thus, changes after serotonin administration began only 15 min after the first application, whereas glutamate produced its effects immediately after the first application. Moreover, each group of microloci expressed specific changes after exposure to a certain neurotransmitter. Specificity of these effects suggests that each neurotransmitter has specific pattern of activated genes in L-RPl1 neurons and also specific intracellular molecular mechanisms mediating neurotransmitter effects. It was confirmed by our previous findings on activation of different se-

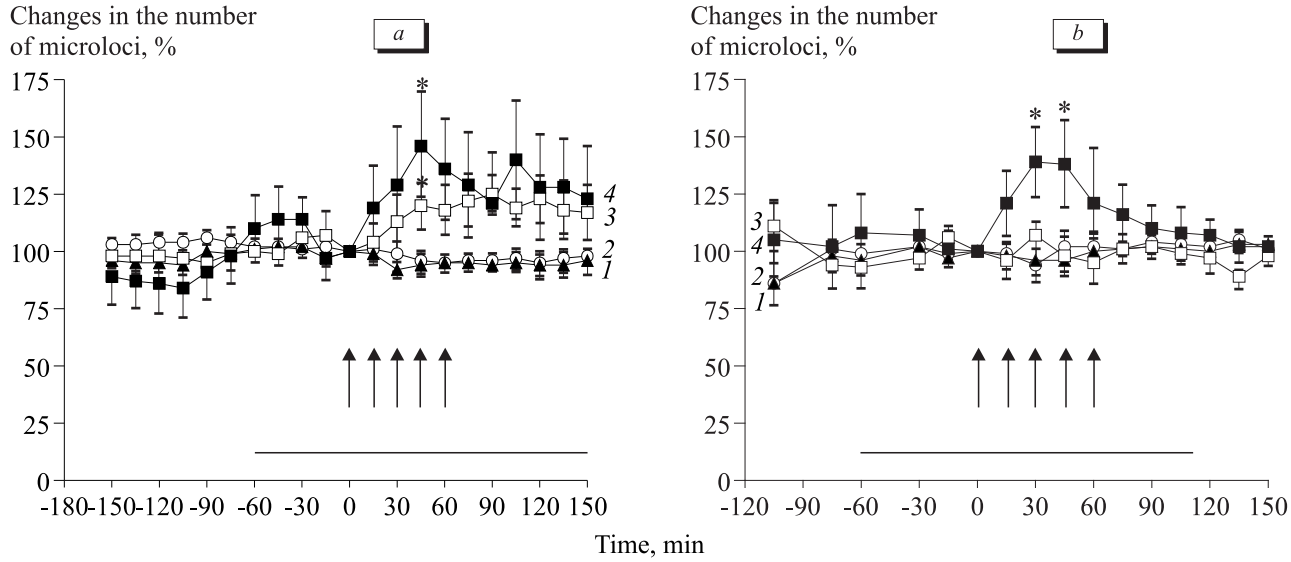

Fig. 3. Changes in the number of microloci of different size in L-RPl1 neurons of edible snail during combined exposure to serotonin and glutamate against the background of transcription inhibitors α-amanitin (*a*) or actinomycin D (*b*). Line under the curves: application of transcription inhibitor.

cond messengers and early genes by different serotonin and glutamate receptors [2].

Thus, our findings demonstrate specific intravital spatiotemporal dynamic changes in chromatin activity in neuronal nucleus possibly associated with transcription of new genes under the effects of certain neurotransmitters simulating the effects of learning.

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