GENETICS

Specific Changes in *c-fos* Expression and Colocalization with DNA in Identified Neuronal Nuclei of Edible Snail Following Neurotransmitter Application

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The effects of serotonin and glutamate on *c-fos* expression and c-Fos colocalization with DNA were immunohistochemically studied in defense behavior command neurons R-LPa2-3 in snail *Helix lucorum*. Simultaneous neurotransmitter application resulted in increased c-Fos-immunoreactivity and colocalization with DNA-specific stain Hoechst 33342 in LPa2 and RPa2 neurons with specific dynamics for each identified cell. In the nuclei of LPa3 and RPa3 neurons, neurotransmitter application did not significant change the c-Fos level. These findings are indicative of specific spatiotemporal changes in *c-fos* expression and c-Fos colocalization with DNA in investigated neurons of edible snail under the influence of neurotransmitters.

Key Words: *c-Fos; serotonin; glutamate; defensive behavior command neuron of the snail; plasticity*

Nerve cells are characterized by unique genetically-determined morphofunctional properties which may underlie their specific functions, particularly, plasticity which is involved in learning and memory processes [9]. Studies on mammal and mollusk neurons showed that changes in plasticity in cells during learning are induced by various neurotransmitters, including serotonin and glutamate [4,12]. Realization of the neurotransmitter effects involves intracellular regulatory systems that activate expression of certain immediate early genes, such as *CREB* (cAMP-responsive element protein), *C/EBP*, *c-fos*, *SRF*, *zif268 etc.* [1,13,15]. Synthesized mRNAs and proteins are believed to induce functional and morphological changes in neuronal synaptic connections that underlie the maintenance of their long-term potentiation or depression [9,10].

We have previously shown that serotonin and NMDA receptors as well as C/EBP, SRF, and zif268 transcriptional factors are specifically involved in the regulation of long-term synaptic plasticity of defensive behavior command neurons of snails induced by simple form of learning, nociceptive sensitization [6]. Using intravital fluorescent dye SYTO16 that selectively binds to DNA and reflects dynamics of its activity [7], we demonstrated that nociceptive sensitization of the snail or the serotonin and glutamate application result in genome activation characterized by specific changes in the number of DNA "microloci" in various command neurons stained with SYTO16 [8]. It is important to note that combined application of serotonin and glutamate produced more pronounced changes in

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DNA activity than isolated applications of neurotransmitters. Specificity of neurotransmitter effects suggests that they exert selective influences on activated genes in different identified snail neurons. At the same time, the peculiarities of transcriptional factor participation in functioning of identified neurons, their dynamics within nuclei and spatiotemporal relationships with DNA are virtually uninvestigated.

This study was aimed at investigation of peculiarities of immediate early gene *c-fos* expression and its spatiotemporal interactions with DNA in the nuclei of defensive behavior command neurons L-RPa2-3 of edible snail *Helix lucorum* against the background of serotonin and glutamate application.

MATERIALS AND METHODS

Experiments were carried out on isolated CNS preparations of edible snail *Helix lucorum* according to the method described previously [2,5]. Isolated CNS preparations were immobilized using microneedles in Petri microdishes half-filed with silicone-like Seal Guard rubber (Seal Guard Inc.) and perfused with Ringer solution for mollusks [2,3]. Serotonin and glutamate (Sigma) were dissolved in saline to concentrations of 100 μM and 5 mM, respectively. Neurotransmitters were administered simultaneously 5 times for 2 min each with 15-min intervals. CNS preparations was frozen in liquid nitrogen vapors 30 min and 1, 3, or 7 h after the first application. CNS preparations from intact snails not incubated with neurotransmitters served as the control.

Cryostat sections (20 μ) were obtained using HM 505E Microm (Carl Zeiss) and placed on slides treated with poly-L-lysine (Sigma). Following 10-min fixation in fresh 4% paraformaldehyde solution and washout in phosphate buffer (0.1 M, pH 7.4), the slides with sections were placed into immunohistochemistry assembling Sequenza Immunostaining Center (Shandon). Incubation with primary rabbit anti-c-Fos antibodies (H-125) (sc-7202; Santa Cruz Biotechnology) in 1:1000 dilution was carried out for 12-18 h at 4°C. Following incubation with primary antibodies, the sections were washed with phosphate buffer with 0.5% Triton X-100 and, to reduce nonspecific binding, slides were incubated for 30 min at room temperature in Image-iT Signal enhancer (Molecular Probes, Invitrogen), and then with secondary donkey Alexa Fluor 488 anti-rabbit antibodies (A-21206), Molecular Probes, Invitrogen) in 1:400 dilution. After washout, the sections were stained with DNA-specific dye Hoechst 33342 (1 mg/ml, Sigma) for 30 min at room temperature and embedded under coverslips with water base mounting GelMount Aqueous (Sigma). Negative control for specificity of immunohistochemical binding of primary antibodies was provided by skipping primary antibodies.

To visualize the neurons, we used fluorescent Olympus B×51 microscope (Olympus) with UMPlanFl 20×0.50 lens. The obtained images were processed using AnalySIS Doku (Soft Imaging System) or ImageJ (Wayne Rasband, National Institute of Health) software. The nuclei of defensive behavior command neurons L-RPa2-3 were identified on Hoechst 33342 stained sections and imaged twice using color-filters with excitation and emission wavelengths for Hoechst and c-Fos markers. Exposure for Hoechst fluorescence was determined automatically for each neuron. Manual exposure was used for immunostained c-Fos, it was experimentally selected, saved and used for c-Fos images of each neuron. In order to analyze the level of *c-fos* expression using Hoechst-labeled images, we constructed the "mask" (edge) of the nuclei, superimposed on c-Fos image of the neuron, and then measured the mean level of fluorescence (density mean, optical units, rel. units).

Colocalization of Hoechst label and c-Fos-immunoreactivity was examined using Huygens essential software (Scientific Volume Imaging B.V.). Preliminary deconvolution of the images was carried out to eliminate background and noise fluorescence, improve signal-to-noise ratio, increase sharpness and contrast. Overlap of two labels was measured as a degree of coincidence of two stains in one image. Measurements were conducted in rectangular area, inscribed into nucleus contour.

Obtained data was normalized for each experiment (parameter value before the neurotransmitter application was taken as 100%), averaged, and expressed as a percent to baseline level. Standard error of the mean ($M \pm SEM$) was calculated. Student's t test was used to assess significance level of the differences.

RESULTS

No significant differences were detected in *c-fos* expression intensity in the nuclei of defensive behavior command neurons in intact snail CNSs (n=6). Thus, mean fluorescence intensities in the nuclei were: for LPa2 242±28 CU (n=6), for LPa3 227±24 CU (n=6), for RPa2 204±12 CU (n=6), and for RPa3 236±29 CU (n=6). Dynamics of c-Fos-immunoreactivity had differences in each of mentioned identified neurons when serotonin and glutamate were applied together. Thus, in RPa2 nucleus, c-Fos immunoreactivity increased by 89±16% in comparison to baseline level (up to 385±33 CU; p=0.006; n=6) at 1 h following onset of neurotransmitter application. At 3 h, it was decreased down to CU (27±11%) and at 7 h it increased again up to 318±25 CU (56±12%; p=0.013; n=6). In LPa2

nuclei, c-Fos increased by 48±10% (up to 357±25 CU; p=0.014; n=6) at 1 h after onset of neurotransmitter application with subsequent additional increase up to 384±28 CU (59±12%; p=0.0048; n=6) at 3 h, and up to 452±10 CU (87±4%; p=0.0007; n=6) at 7 h. No significant changes in c-Fos levels were induced within 7 h by neurotransmitter application in nuclei of LPa3 and RPa3 neurons (Fig. 1). In control CNS neurons where no serotonin and glutamate were applied, no significant changes in c-Fos expression were detected within 7 h (data not shown).

In control snails, no significant differences were found in colocolizations of c-Fos-immunoreactivity

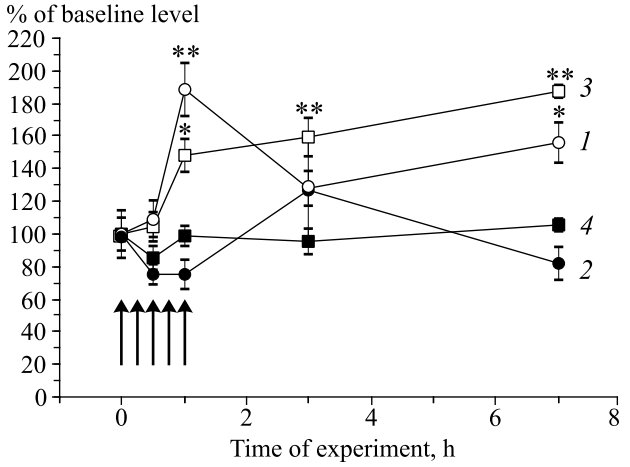

Fig. 1. Changes in c-Fos levels in the nuclei of defensive behavior command neurons RPa2 (*1*), RPa3 (*2*), LPa2 (*3*), and LPa3 (*4*) following simultaneous application of serotonin and glutamate on CNS. Ordinate: changes in c-Fos immunoreactivity (baseline levels were taken as 100%). Here and in Fig. 2: arrows show the time of neurontransmitter application. *p<0.05, **p<0.01 in comparison with baseline levels.

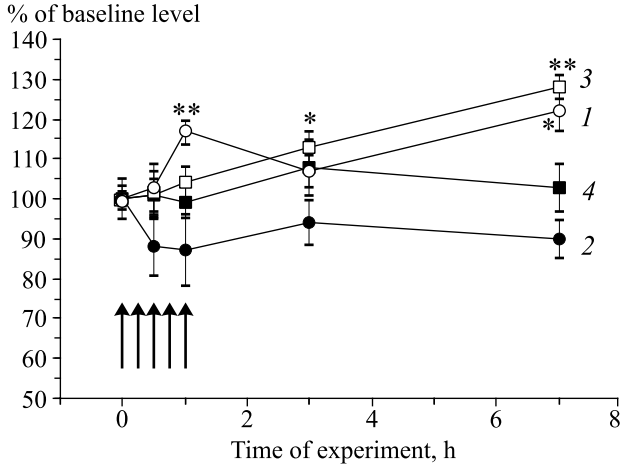

Fig. 2. Changes in level of colocalization of DNA-specific dye Hoechst 33342 and c-Fos in the nuclei of defensive behavior command neurons RPa2 (*1*), RPa3 (*2*), LPa2 (*3*), and LPa3 (*4*) following simultaneous application of serotonin and glutamate on CNS.

and Hoechst 33342 in neurons nuclei (n=6): LPa2 0.37±0.02 (n=6), LPa3 0.41±0.02 (n=6), RPa2 0.36± 0.01 (n=6), and RPa3 0.41±0.02 (n=6).

In RPa2 neuron nuclei, colocalization increased by 17±1% (up to 0.42±0.02; p=0.003; n=6) by 1 h after neurotransmitter application, with subsequent restoration of the baseline value at 3 h and increase up to 0.44±0.02 (by 22±2% in comparison to baseline; p=0.017; n=6) at 7 h after the onset of neurotransmitter application (Fig. 2). In LPa2 neurons, significant changes in colocalization values were detected at 3 h (13±5%, up to 0.41±0.01; p=0.049; n=6) and by 7 h (28±4%, up to 0.47±0.05; p=0.001; n=6). In RPa3 neurons, the decrease in colocalization level down to 0.36±0.01 (-12±1%, p=0.002, n=6) was detected at 30 min after the onset of neurotransmitter application and remained at that level for at least 7 h after the onset of neurotransmitter application. No significant changes in colocalization levels were observed in LPa3 neuron nuclei (p>0.05; n=6).

Our experiments revealed no differences in c-Fos level in intact neurons. Neurotransmitters triggered activation of *c-fos* expression in L-RPa2 neurons persisting for more than 7 h. In L-RPa3 neurons, no significant changes in c-Fos level were found following neurotransmitter application. Analysis of changes in c-Fos and Hoechst colocalizations demonstrated that colocalization increased in L-RPa2 neurons and remained unchanged or even was decreased in L-RPa3 neurons. Changes in c-Fos levels in snail neurons generally coincide with c-Fos dynamics in brain neural cells: various physiological exposures are followed by rapid, in 1-2 h, increase in c-Fos level with subsequent slow decrease for several hours [1,4].

Observed differences in c-Fos levels and dynamics, as well as its colocalization with DNA in investigated neurons following exposure to neurotransmitters, suggest presence of peculiarities in intracellular realization of their functions. Number of investigations demonstrated that defensive behavior command neurons, including L-RPa2-3, have serotoninergic and glutamatergic inputs; the serotoninergic cells that converge to the command neurons have been identified [2]. Stimulation or serotonin application on these neurons resulted in second messenger activation and in long-term changes in synaptic connection efficiency of the command neurons, as well as in transcription activation for a number of immediate early genes [4,6]. Body of mentioned facts indicates that serotonin is one of the key integration function controllers in defensive behavior command neurons of the snail, including transcription. In this regard, lack of the transmitter effects on c-Fos level in LPa3 and RPa3 was unexpected and appeared as an indicative of selective involvement of this immediate early gene in plasticity mechanisms,

mainly in LPa2 and RPa2 neurons. This assumption is also supported by the fact of neurotransmitter effects on degree of c-Fos and Hoechst colocalization in LPa2 and RPa2 neurons, and by the lack of changes or by decrease of its value in LPa3 and RPa3 neurons.

A number of peculiarities of genome functioning in L-RPa2-3 have been described in [11], where two genes were identified, *HCS1* and *HCS2*. *HCS2* was expressed in all four neurons, whereas *HCS1* – only in L-RPa3. In addition, *HCS1* and *HCS2* expression was induced by number of stimuli, particularly by serotonin application and sensitizing stimuli [11]. Findings concerning selective serotonin-dependent expression of *HCS1* only in L-RPa3 neurons, but not in L-RPa2, and *c-fos* in L-RPa2 neurons, but not in L-RPa3 neurons are indicative of substantial differences in gene expression pattern in these neurons and also raise the question concerning specific physiological significance of gene activation and synthesized proteins in command neurons.

Substantial differences in regulatory system activity in defensive behavior command neurons in edible snail were revealed in [14]. Serotonin application on the ganglions for 3.5 h resulted in significant increase in phosphorylated MAPK/ERK kinases in RPa2-3 command neurons. At the same time, no changes in the levels of phosphorylated kinase forms were detected under the conditions of exposure to serotonin in LPa2-3 neurons. Food aversion training in the snails also was revealed to increase MAPK/ERK levels in right parietal neurons, but not in left parietal neurons. Since investigated kinases possess regulatory effects on the transcription of a number of immediate early genes, including *c-fos*, the certain controversy in our results and findings in [14] should be noted. However, direct comparison of these findings is incorrect due to differences in the methods of investigation and in investigated factors. Apparently, further integrated studies are needed to analyze the problem under discussion and to solve the contradictions emerged.

Presented experimental results are indicative of specificity of spatiotemporal pattern of *c-fos* transcription and its colocalization with DNA in the nuclei of defensive behavior command neurons L-RPa2-3 in edible snail under the effects of neurotransmitters. Differential transcription of certain genes in command neurons can be associated with the peculiarities of their functional properties and integrative functions, which underlie adaptive reactions in the animals.

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