



Immediate-Early Genes Detection in the CNS of Terrestrial Snail

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Abstract

In the present work, using *in situ* hybridization, we studied the expression patterns of three molluscan homologs of vertebrate immediate-early genes *C/EBP*, *c-Fos*, and *c-Jun* in the central nervous system (CNS) of terrestrial gastropod snail *Helix*. The molluscan *C/EBP* gene was described in literature, while *c-Fos* and *c-Jun* were studied in terrestrial snails for the first time. Localization of the expression was traced in normal conditions, and in preparations physiologically activated using stimulation of suboesophageal ganglia nerves. No expression was detected constitutively. In stimulated preparations, all three genes had individual expression patterns in *Helix* CNS, and the level of expression was stimulus-dependent. The number of cells expressing the gene of interest was different from the number of cells projecting to the stimulated nerve, and thus activated retrogradely. This difference depended on the ganglia studied. At the subcellular level, the labeled RNA was observed as dots (probably small clusters of RNA molecules) and shapeless mass of RNA, often seen as a circle at the internal border of the cell nuclei. The data provide a basis for further study of behavioral role of these putative immediate-early genes in snail behavior and learning.

Keywords *c-Fos* · *c-Jun* · *C/EBP* · Gastropods · Terrestrial snail · Neurons

Introduction

Immediate-early genes (IEGs) constitute the population of effector genes which are rapidly upregulated by the sensory stimuli, and most of them are involved in the transcriptional regulation of other upregulated genes expressed with a delay (Gonzales et al. 2019; Kim et al. 2018; Pérez-Cadahía et al. 2011; Clayton 2000; Clayton et al. 2019). It is typical that, expression of the IEGs (i) is mostly absent in quiescent state and is induced by stimuli of many modalities, (ii) occurs within minutes, and (iii) takes place in the absence of *de novo* protein synthesis. A lot of studies devoted to the key role of the IEGs in development, learning and memory were made in vertebrates, and numerous sets of IEGs were identified (for rev., see Korzus 2003; Kaczmarek 2000; Okuno 2011; Clayton et al. 2019). In invertebrates, mollusks in particular, only several transcriptional factors homologous to the vertebrate IEGs were identified. Among them, CREB

was well studied due to its important role in the organization of behavior. It is known to increase transcription of other IEGs (Clayton et al. 2019). CREB1 was identified in *Aplysia* (Lewin and Walters 1999; Liu et al. 2008) and *Lymnaea* (Sadamoto et al; 2004a, b; 2010). But till now the role of molluscan IEGs in memory formation processes is poorly known. The main questions concerning putative IEGs identification in mollusks are still open: whether they are expressed constitutively, to which degree their expression is changing under behavioral stimuli, and do their expression is dependent on the protein synthesis? Analysis of a pattern of gene expression in a context of learning is necessary for understanding the IEGs function.

The data on CREB1 are diverse in different papers. It was shown for CREB1 in marine mollusk *Aplysia* (Liu et al. 2008) that it participates in consolidation of long-term synaptic facilitation and induces long-term sensitization of nociceptive sensory neurons (Lewin and Walters 1999), acting as typical transcription factor. But the level of its RNA and protein was in fact increased only twofold and only after two hours after serotonin application, being still elevated even next day. These features are atypical for the immediate-early genes of vertebrates. In *Lymnaea*, CREB1 was reported to be normally detected either in a restricted number of neurons

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(Sadamoto et al. 2004a, b), or in some cases in many cells (Ribeiro et al. 2003). Contradicting to this, other studies point out that the level of CREB1 in identified giant cell of *Lymnaea* was constitutively below the level of detection (Wagatsuma et al. 2005)—less than 10 copies of RNA per cell, and de novo synthesis is required for the memory consolidation, the process lasting at least one hour (Wagatsuma et al. 2006). Conditioned taste aversion learning significantly increased the LymCREB1 gene expression (Sadamoto et al. 2010). These data are typical for expression of the conventional IEGs in vertebrates.

Transcription factor CCAAT/enhancer binding protein (C/EBP) was shown to play an important role in the long-term memory consolidation in *Lymnaea* (Hatakeyama et al. 2004, 2006). The turnover of C/EBP is thought to be fast (Hatakeyama et al. 2006). Training with inedible food led in *Aplysia* (Levitan et al. 2008) to the fast, relatively short (1–2 h) and noticeable (ten times) increase in the C/EBP RNA expression. This gene participates as well in the development of a long-term facilitation in the defensive system of *Aplysia* (Alberini 2009; Yamamoto et al. 1999) where it was identified as typical immediate-early gene. C/EBP was cloned in the slug *Limax* (Fukunaga et al. 2006). It was shown to be upregulated in a protein synthesis-independent manner by various noxious stimuli applied to the slug's body.

Mammalian transcription factor AP-1 is a dimer of various proteins belonging to the Jun and Fos families (Shaulian and Karin 2002). The AP1 complex in *Aplysia* is a c-Jun homodimer (Sung et al. 2006). It was shown that after crushing the peripheral nerves, the AP1 complex level was elevated for weeks, which is unusual for the IEGs. Unfortunately, we found no information on *c-Jun* and *c-Fos* expression in the identified neurons of the molluscan CNS.

In our previous work (Xu et al. 2019), it was shown that chemical activation of the CNS of the snail *Helix* led, in conditions of protein synthesis blockade, to the significant increase of expression of numerous genes. Some of them were identified as homologs of vertebrate IEGs (transcription factors): *c-Fos*, *C/EBP*, *c-Jun*. The rapid and transient increase of expression under blockade of the protein synthesis conforms to the criteria of belonging to the group of immediate-early genes. Continuing this study, in the present work, we used the in situ hybridization method to trace the populations of neurons, where the identified genes expression occurs in stimulated CNS. The snail CNS is especially suitable for this aim, as it contains a lot of giant identified neurons with known roles in behavior (Balaban 2002). Our hypothesis was as follows: individual early genes should be expressed in distinct neuronal populations and pattern of expression should depend on the type of applied stimulus. Besides, we were interested in the question, whether the expression of these identified genes can be traced without

CNS stimulation. The last question was how the IEGs expression looks like at the subcellular level.

Methods

Animals

The work was performed in whole mounts (in situ hybridization series) and in 10 µm Paraplast sections (RNAscope method) of adult terrestrial snail *Helix lucorum* CNS.

Early Genes Sequencing and Data Analysis

Sampling of the nervous tissue and RNA-sequencing was described earlier in detail (Xu et al. 2019). Shortly, data from two types of preparations were collected and compared to reveal candidates for early genes in the snail. Control brains were treated in high-Ca²⁺, high-Mg²⁺ Ringer saline for 24 h at 4 °C to minimize the endogenous electrical activity (it was established by Balaban and Chase 1989). Comparison was made with the preparations of the second type, the activated ones. For this aim, the brains were treated with a saline containing caffeine (100 µM) and 5-HT (5 µM) for 20–25 min in presence of 20 µM of anisomycin (blockade of protein synthesis). After sequencing and quantification of contigs expression levels, the differential expression analysis and contig annotation were performed. For each putative protein-encoding RNA was calculated, the percentage of contigs which were upregulated/downregulated. The level of consideration for up- or downregulation was not less than 80% of initial level in case of consistent direction of the expression changes.

Among analyzed sequences, three were attributed as possibly encoding the early genes. The criteria were (a) fast and noticeable increase in quantity of specific RNA and (b) relative coincidence with previously established early genes sequences (for details, see Xu et al. 2019). Those were *c-Jun*, *C/EBP*, and *c-Fos*. The RNA-Seq data and the assembled snail neuronal transcriptome were deposited in the Gene Expression Omnibus (GEO) under the accession numbers, correspondingly, MN630086.1, MN630087, and MN630085.

In accordance with these data, short DNA probes were synthesized against sequences of contigs which had the highest probability to contain an alternative splicing junction, to make sure it only hybridize with RNA.

Probe's information

1. *C/EBP*: GGACACTATCATATATTTGCTACAAAC
TTTATAATAAGAAACGATTTTAAAGTGCAC

MM 18248

2. *c-Fos*: TCGACCATGCTTTGCTTTTGGCTCCCA
TTCTGCATCAGAATATTCCG

MM 15021

3. *c-Jun*: GAGGCTAAGACAGTTGCTAGTTTCTTC
TGTTTTGTTGCTGAGCTAGAA

MM 15573

Preparation and Hybridization

Using these probes, the hybridization experiments were performed on snail's CNS. Whole-mount ganglia were processed. Experiments in parallel were carried on the intact CNS, and CNS activated by electric stimulation of an appropriate nerve. A total of 63 animals were used. In previous work (Xu et al. 2019), chemical stimulation of the whole snail's brain was used. In this work, we selectively activated neuronal populations projecting in the selected for electrical stimulation nerve (retrograde stimulation). The nerves stimulated were (in separate experiments) as follows: anal nerve, left and right pallial nerves, intestinal nerve. The procedure was as follows: the central ganglion ring was dissected and maintained in saline in a Sylgard-based dish. Connective sheath was removed from the dorsal side of parietal ganglia. The activity of the putative command neurons for withdrawal behavior was recorded intracellularly (giant parietal ganglia neurons #2, and 3). A selected parietal ganglia nerve was repeatedly stimulated for 2 h via the polyethylene suction electrode: 10-stimulus trains (10 Hz) once per 30 s. The intensity of stimulation was chosen to elicit the burst of action potentials in the recorded parietal neurons at the beginning of the training. At the end of the procedure, the CNS was fixed with 4% paraformaldehyde, manually desheathed with fine forceps, washed and processed for the in situ hybridization procedure.

The hybridization in situ procedure was previously successfully performed in the *Helix* whole-mount brains with RNA probes and was reported in detail for RNA-to-RNA probes (Balaban et al. 2001). In this study, the procedure was slightly modified for DNA-to-RNA probes in that the hybridization proceeded at 37 °C instead of 50 °C, as the DNA probes were shorter than commonly used RNA probes.

Nerve Backfills

The retrograde labeling of the neurons projecting to the stimulated nerve (and thus activated via stimulation of the nerve) was done. For this aim, the cut end of the nerve was sucked into a pipette filled with 10% Neurobiotin in 0.1 M KCl. Total time of filling was chosen after pilot experiments and was about 24 h at 4 °C. The ganglia were fixed with 4% paraformaldehyde and processed as whole mounts with the Vectastain ABC kit. Each brain was used either for the retrograde staining or for the hybridization procedure.

RNAscope Method

The RNAscope method was used in 10 µm Paraplast sections of snail's brain to trace the pattern of RNA expression in cells. The RNAscope[®] 2.5 Assay (Advanced Cell Diagnostics, Inc.) was applied in accordance with manufacturer's instructions. This kit allowed observation of RNA-formed brown dots corresponding to the single RNA molecules at 20×, and 40× magnifications. Two probes were used: one against the *Helix c-Fos*, which was custom synthesized by manufacturer in accordance with sequence, established in our experiments (Xu et al. 2019). To prove that this new method is working in our hands, we used a commercially available (data based) probe against IGF2 (insulin-like growth factor of vertebrates) for control experiments in mice brain. Preparations of *Helix* brain were made in the same way as the ones used for in situ hybridization procedure.

Results

In Situ Hybridization Data

In control preparations, no staining was observed for all three IEGs studied (Fig. 1).

In preparations activated by means of electrical stimulation of the nerve, a rich and specific pattern of staining was detected. In Fig. 2, the examples of staining with the same probe (*c-Jun*) in conditions of different nerves stimulation are presented for the pleuro-parieto-visceral ganglia complex. In parallel, a location pattern of neurons projecting to the stimulated nerve (and, therefore, activated retrogradely) is presented. As it can be easily seen, two sets of cells: projecting neurons (= stimulated neurons), and *c-Jun* expressing neurons patterns appeared to be different, and only a few coincidences were detected. The pattern of *c-Jun* expression strongly varied, depending on the stimulated nerve. Interestingly, the number of neurons projecting to a specific nerve was greater than the number of neurons expressing the *c-Jun* RNA. Qualitatively, similar conclusions can be made for all three probes used: (a) gene expression pattern significantly varies, depending on which nerve was stimulated; (b) relatively low coincidence between neuronal population expressing the gene of interest and neuronal population projecting to the nerve was observed; (c) the population of projecting to a specific nerve neurons was much more numerous than the population of neurons expressing the gene of interest due to stimulation of the same nerve.

In other part of the snail brain (pedal ganglia), the results in part diverged with those observed in the pleuro-parieto-visceral ganglia complex. Figure 3 illustrates the data for *c-Jun* gene. All nerves chosen for stimulation originated from the pleuro-parieto-visceral ganglia, and, naturally, most

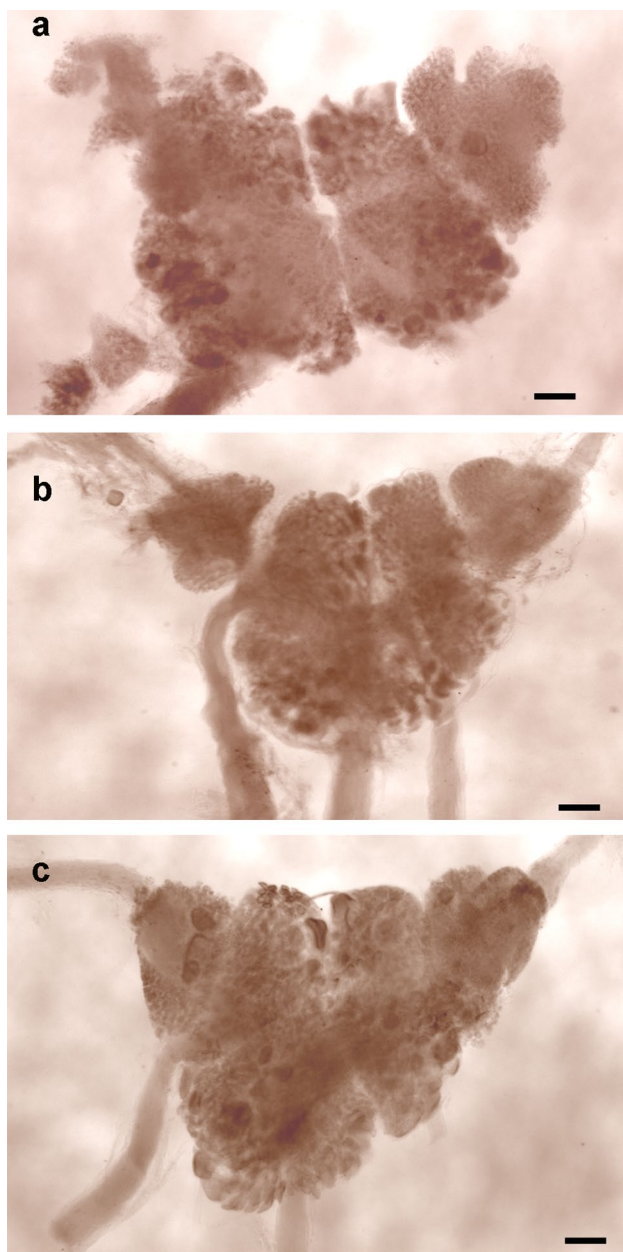


Fig. 1 No neurons in the pleuro-parieto-visceral ganglia complex are expressing *C/EBP* (a), *c-Fos* (b), or *c-Jun* (c) RNA in normal conditions. Scale bar: 200 μ m

of the neurons projecting to these nerves were located in these ganglia. Thus, the number of projecting (i.e., stimulated) neurons was not numerous (Fig. 3a, c, e). As a consequence, in pedal ganglia, the number of neurons expressing the gene of interest was bigger than the number of stimulated neurons (Fig. 3b, d, f) though less than it was observed in the pleuro-parieto-visceral ganglia. Partial coincidence between two populations of neurons (stimulated and the gene-expressing) and variability of pattern depending on which nerve was stimulated were common for pedal and

pleuro-parieto-visceral ganglia. Similar data were obtained for all three studied genes. In Fig. 4 data for *C/EBP* gene are presented, and the results are qualitatively the same, as in the case of *c-Jun*.

No neurons from cerebral ganglia are projecting to the pleuro-parieto-visceral ganglia nerves used for stimulation (not illustrated). Nevertheless, the restricted set of cells in the ganglia demonstrated the early genes expression after these nerves stimulation (Fig. 5). The set of cells was the same, no matter which nerve was stimulated, or which probe was used. It suggests existence of the expression of early genes in the strictly defined cellular population, probably stimulated indirectly (synaptically) due to existence of a functional interganglionic connections.

RNAscope Data

We were interested to answer the question, how the early genes expression looks at the subcellular level—what is the number of RNA molecules synthesized in a single cell. For that aim, we used the RNAscope method, which reveals single copies of RNA as separate dots (Advanced Cell Diagnostics, Inc), in sections of the snail's brain. As the control of the method, we have performed two series in parallel: with the RNA probe to IGF2 in mice brain sections, and with the snail *c-Fos* probe in snail's brain sections. While a typical pattern of staining was detected in mice neurons (Fig. 6h, i, random dots, which can be counted), the picture was quite different in the snail neurons. Instead of separate dots, we observed (Fig. 6a, c, e) clusters of dots (having different size), and a stained formless substance at the internal nuclei border. Double staining of the same sections with DAPI (Fig. 6b, d, f) demonstrated that these dots and substance were not stained by the DNA-specific stain, which identifies the brown dots and substance being the RNA material in its nature. In Fig. 6e, f one can see two closely located neurons: the left one does not express a large amount of RNA, so the DAPI staining is homogenous inside the nucleus, while in the right one a lot of RNA (present as dots and non-structured mass) masks the DNA content. Interestingly, the Click-iT method of RNA detection, which was shown (Ierusalimsky and Balaban 2018) to stain in *Helix* neurons total RNA population, stained the whole nuclei of neurons (Fig. 6g). Thus, the *c-Fos* definitely is located in the activated cell nuclei in an irregular manner.

Discussion

In our study, we for the first time traced the expression patterns of three molluscan immediate-early genes. While some data are present in literature about the gene *C/EBP*, no data, as far as we know, is published on molluscan

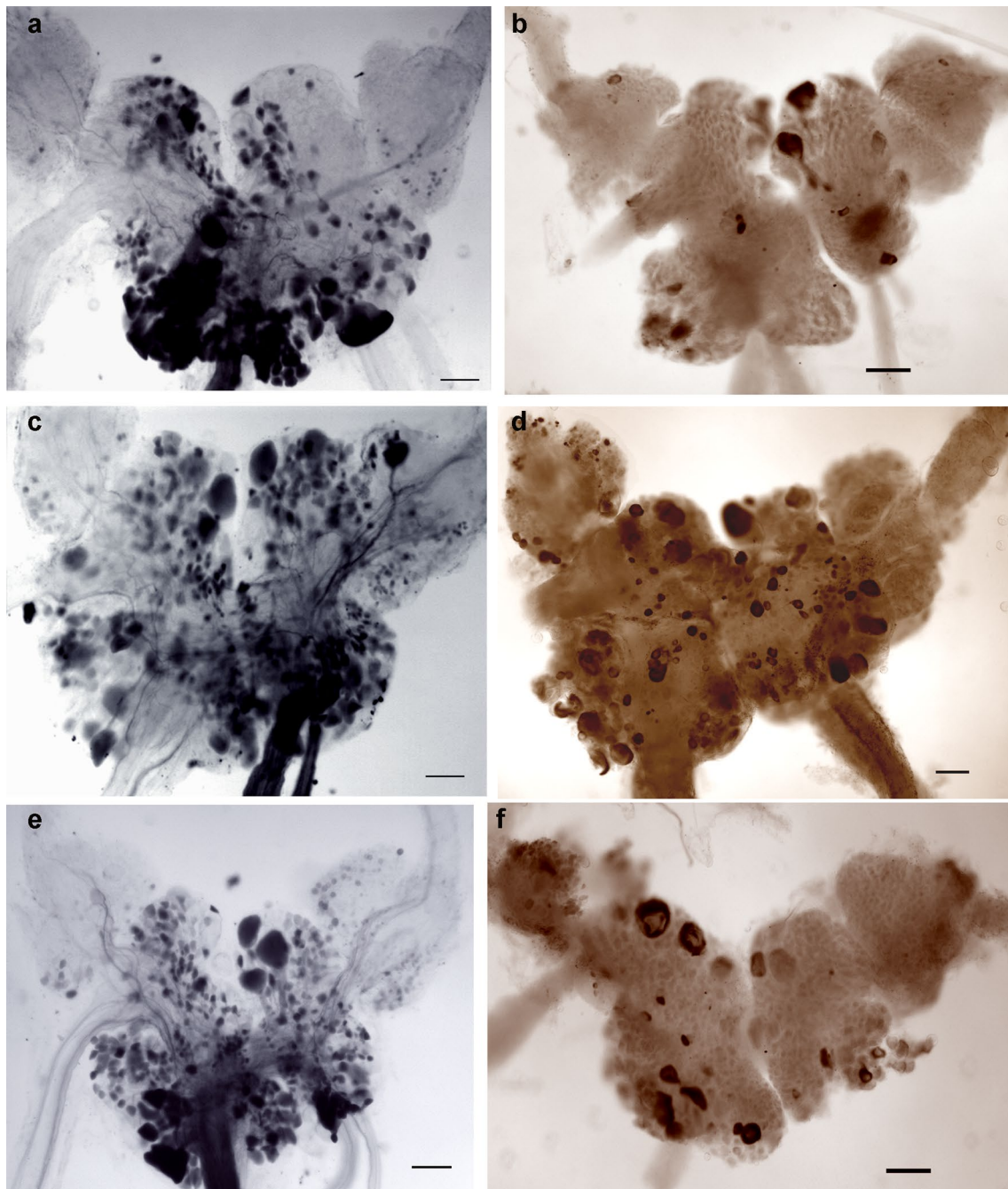


Fig. 2 Comparison of the *c-Jun* hybridization data in pleuro-parieto-visceral ganglia complex (after stimulation of the nerve) with actual morphology of neurons, projecting into the same nerve. Neurons projecting in the nerve were traced via backfill of the nerve with Neurobiotin. Left part (**a**, **c**, **e**) backfilled preparations. **a** Intestinal nerve was backfilled, **c** right pallial nerve was backfilled, **e** anal nerve was

backfilled. Right part (**b**, **d**, **f**) results of the in situ hybridization procedure in preparations stimulated via the corresponding nerves. Hybridization picture was different for each stimulated nerve. In all three cases the number of projecting (i.e., stimulated) neurons was bigger than the number of cells where *c-Jun* was expressed. Scale bar: 200 μm

c-Fos, and *c-Jun* genes. Two distinct features were revealed for all the three RNA probes tested: (a) pattern of the gene expression was specific for each stimulated nerve and (b) the quantity of the gene-expressing cells was not equal to the quantity of cells projecting to the stimulated nerve:

smaller in pleuro-parieto-visceral ganglia and bigger in pedal ganglia.

In neural network involved in *Lymnaea* feeding (Hatakeyama et al. 2006), and in withdrawal network of *Aplysia* (Yamamoto et al. 1999) were detected rapid synthesis and

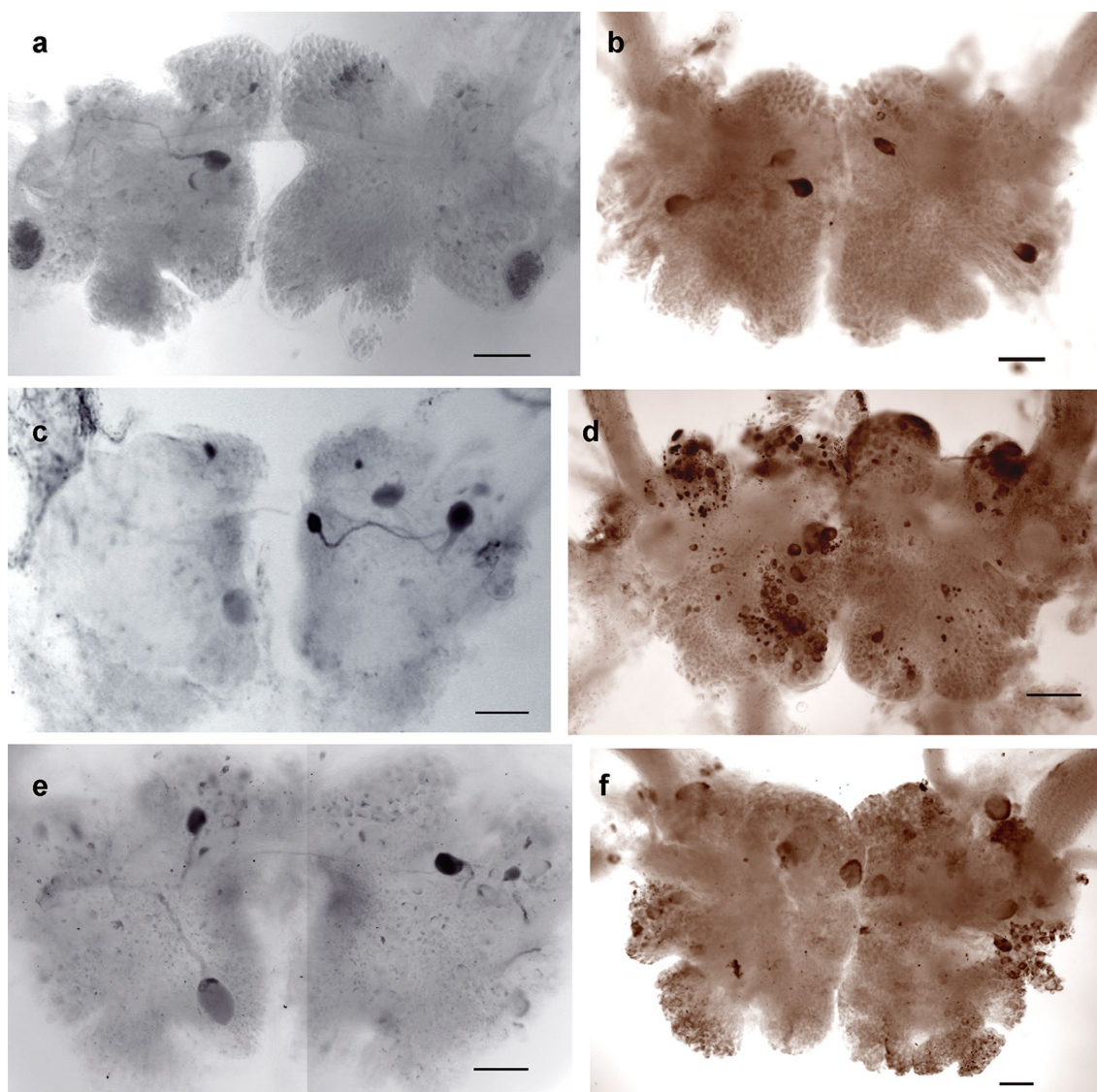


Fig. 3 Comparison of the *c-Jun* hybridization data in pedal ganglia (after stimulation of the nerve) with actual morphology of neurons, projecting into the same nerve. Left part (a, c, e) backfilled preparations. **a** Intestinal nerve was backfilled, **c** right pallial nerve was backfilled, **e** anal nerve was backfilled. Right part (b, d, f) results of the

in situ hybridization procedure. Hybridization picture was different for each stimulated nerve. In all three cases the number of projecting (i.e., stimulated) neurons was less than the number of cells expressing *c-Jun*. Scale bar: 200 μ m

phosphorylation of *C/EBP* and rapid breakdown of *C/EBP* mRNA, what is typical for the IEGs. Similar data were reported for *Aplysia* buccal ganglia neurons (Levitan et al. 2008), the expression being specifically restricted to the neurons involved in feeding behavior. In the slug *Limax* (Fukunaga et al. 2006), *C/EBP* was detected exceptionally in the procerebral lobes of the cerebral ganglia, the lobes playing the major role in the odors perception (Nikitin and Balaban 2001). Thus, the *C/EBP* gene shows a selective pattern of expression, the fact we also observed in our study, though the neuronal population differed from this reported for the other molluscan species.

In recent work on the dorsal striatum of mice (Gonzales et al. 2019), the spatial distribution of three immediate-early genes was traced in detail. Authors addressed several questions: 1. Are IEGs co-expressed across individual neurons? 2. Does the IEG response exhibit cell type specificity? 3. Are IEG-expressing neuronal assemblies clustered? Clear and precise answer was YES for all three questions: co-expressed, clustered, reflect cell specificity. In our work, the received data evidence to existence of clustering and cell specificity of expression for all three studied molluscan IEGs, while co-expression was not evident.

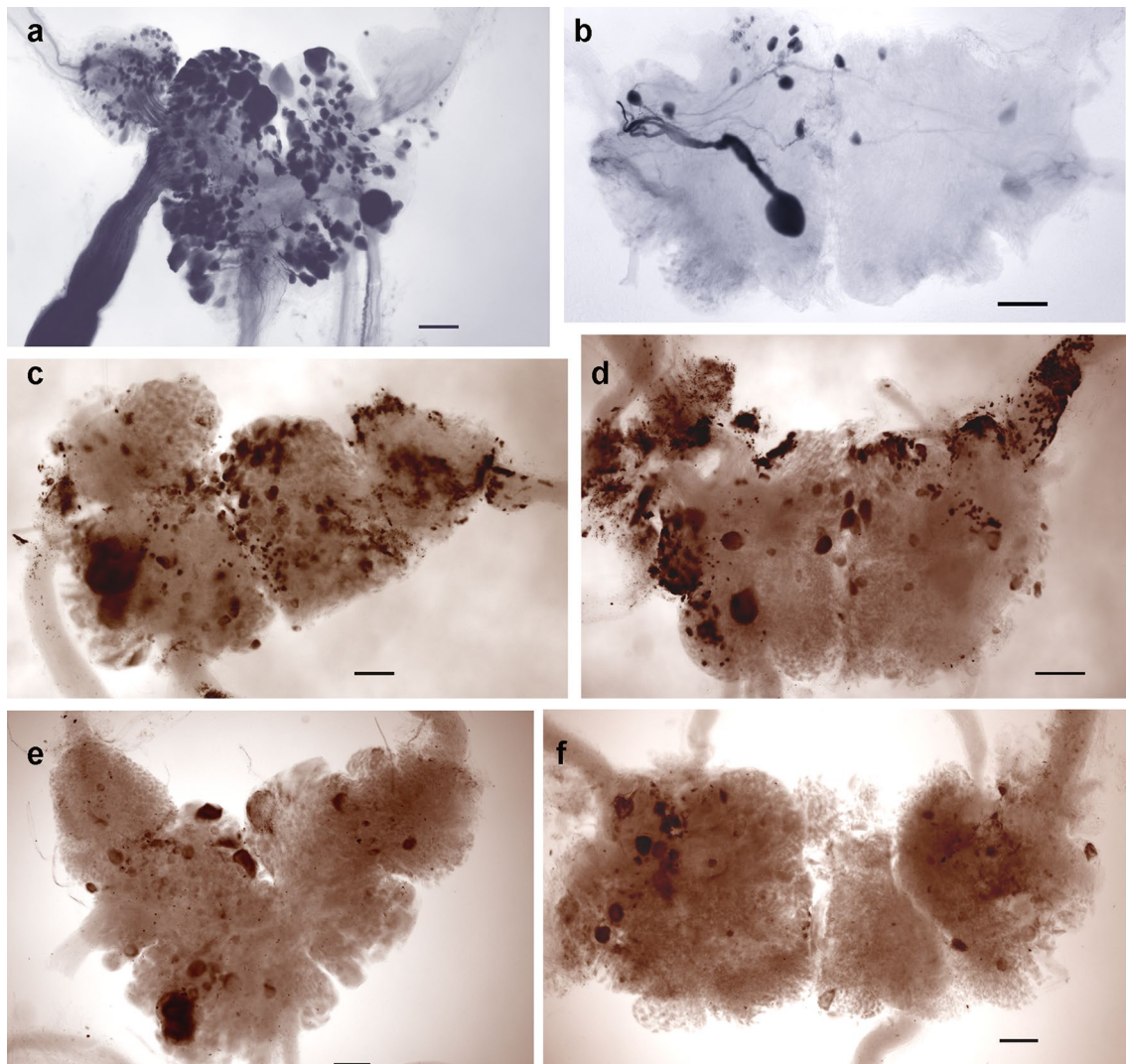


Fig. 4 Distribution of *C/EBP* expressing cells. **a, b** Neurons projecting to the left pallial nerve (**a** pleuro-parieto-visceral ganglia complex, **b** pedal ganglia). **c, d** hybridization data (*C/EBP* probe) in the same ganglia after stimulation of left pallial nerve. Note significant difference between the neuronal populations revealed by two meth-

ods. **e, f** Hybridization data (*C/EBP* probe) in the pleuro-parieto-visceral ganglia complex (**e**), and in the pedal ganglia (**f**) after the anal nerve stimulation. Neurons projecting to the anal nerve are illustrated in Figs. 2e and 3e. Scale bar: 200 μ m

Interestingly, no constitutive expression was observed in our work, and it is a “normal” feature for the immediate-early genes of vertebrates. The other important point of our study is a small coincidence between antidromically stimulated and candidate gene-expressing sets of neurons. This is an interesting observation, which probably has two sides. If the stimulated neuron does not express the gene of interest, it suggests that IEGs are expressed specifically, depending on the type of neuron. The probable identity of cells expressing different IEGs in the cerebral ganglia in our work testifies for this idea. In this area of cerebral ganglia, the clusters of molluscan insulin-containing cells are located (Ierusalimsky and Balaban 1997). This peptide

is playing important roles both in regulation of growth and in control of reproduction (Wijdenes et al. 1987; Kits et al. 1990). These broad functions of insulin-containing cells can explain the observed expression of different IEGs.

On the other hand, if IEGs expression is evident in the cell which was not directly stimulated, it clearly suggests the possibility of the gene activation via synaptic connections with other neuron (neurons) which were activated by the nerve stimulation. In vertebrates, it is known that *c-Fos* expression strictly correlates with neuronal excitation (Kaczmarek and Chaudhuri 1997), while the upregulation of AP-1 (dimer of variable content) does not always correlate with it.

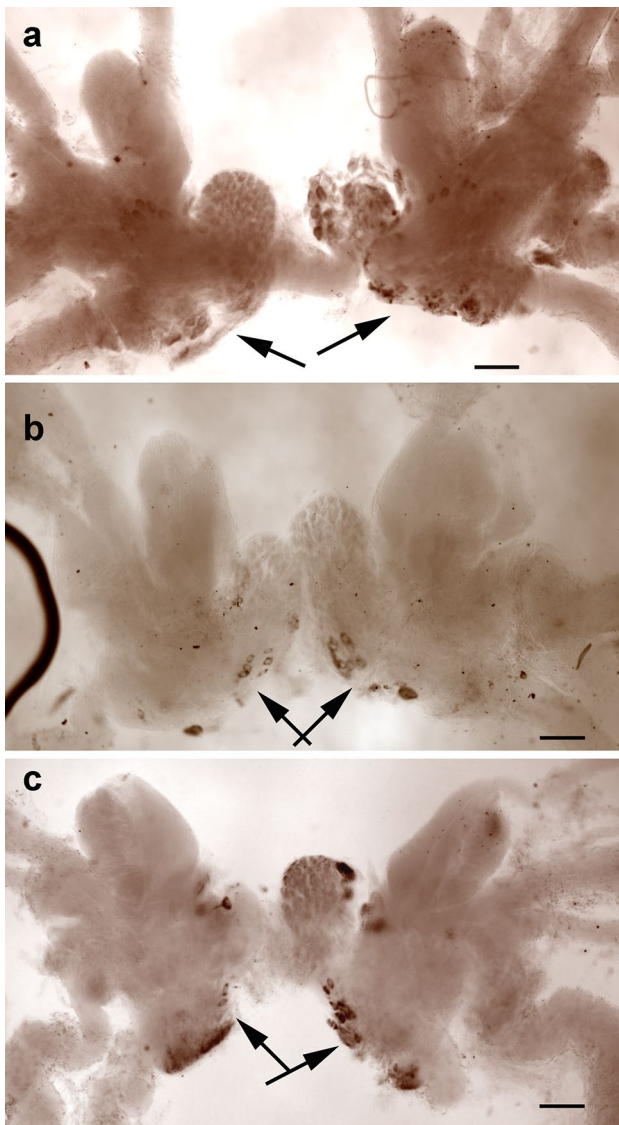


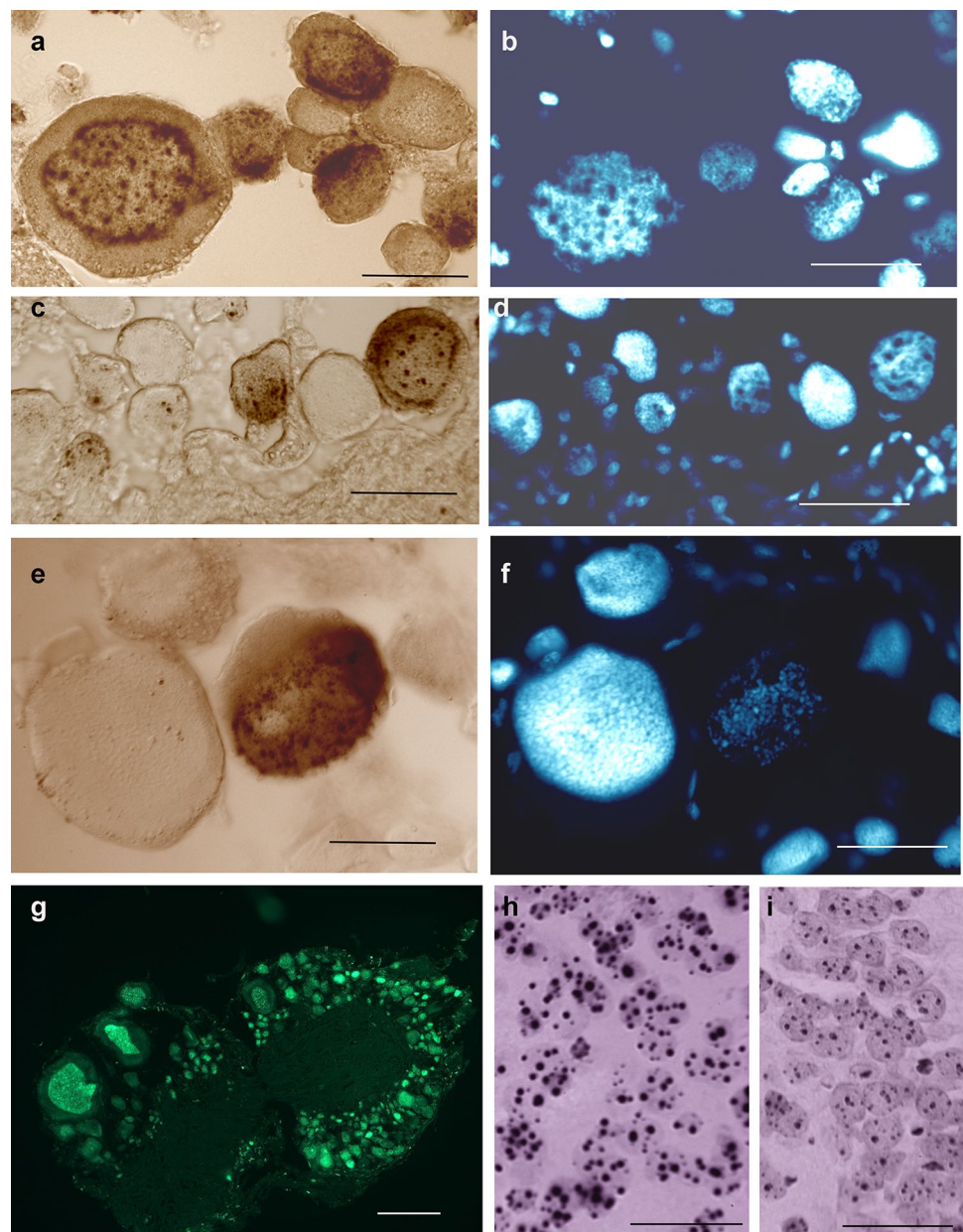
Fig. 5 IEG-expressing neurons in cerebral ganglia after stimulation of intestinal or right pallial nerve. No cerebral ganglia neurons are projecting to the pleuro-parieto-visceral ganglia nerves, while some of the cerebral neurons were revealed via the in situ hybridization. It seems that the same set of neurons was detected via hybridization (marked by arrows), no matter which nerve was stimulated or which RNA sense was used for hybridization. **a** *C/EBP* probe, intestinal nerve stimulation; **b** *c-Jun* probe, right pallial nerve stimulation; **c** *c-Jun* probe, intestinal nerve stimulation. Scale bar: 200 μ m

Most of the snail *Helix* neurons are giant ones and they are the polyploid cells (with exception of small pro-cerebral lobe neurons), whose DNA is enlarged by repeated endoreplication (Chase and Tolloczko 1987). This process was studied in detail in other terrestrial gastropod mollusk—*Limax* (Yamagishi et al. 2012). It was shown that the endoreplication entails whole genome, and irrespective of the transcriptional activities at different loci, the total number of DNA copies being as much as 10,000 in the biggest neurons. Interestingly, within the nucleus a uniform DNA synthesis take place. The transcription process normally is selectively located within the nucleus, and this was observed in our experiments with the RNAscope method. Our data suggest that stained dots represent the single RNA molecules, while un-formed substance at the internal border of nuclei represents the aggregated RNA molecules.

Literary data, though in part contradictory, altogether with our own data give no direct evidence for singling out the IEGs of mollusks as a separate class of IEGs due to their peculiarities of expression like low and small increase in their expression level or long-lasting expression. Anyway, too small is known about molluscan IEGs, and only a few of them are reliably identified. Clayton (2000) and Clayton et al. (2019) introduced the concept of the genomic action potential—a structured genomic response in the brain to acute experience. The main idea of this concept is the slow integration of afferent patterns of activity with long effects. The beginning of this integration is the activation of immediate-early genes.

Stimulation of different nerves in our experiments reveals diverse populations of neurons expressing the same immediate-early gene, which can be regarded as an analogue of in vivo situation when the brain receives stimuli of different modalities. Thus, the pallial nerves supply the mantle, mantle border, pneumostome, anal nerve supplies the mantle border and the diaphragm, and the intestinal nerve is the main nerve for internal organs (heart, aorta, kidney, liver, lungs, stomach, intestines)—Ierusalimsky et al. (1992). It suggests that more fine approach to the study of IEGs function can be used in the snail brain—comparison of IEGs expression patterns in distinct behavioral paradigms, including learning and formation of memory traces.

Fig. 6 Visualization of *c-Fos* RNA with the RNAscope method, **a–f** 10 μ m sections of parietal (**a–d**) and pedal (**e, f**) ganglia after application of *c-Fos* probe. In **a–f**, left parts represent the results of staining, while right parts represent the same preparations, DAPI-stained. **g** Example of total cellular RNA content (including immediate-early genes) in section of right parietal and right pleural ganglia, as revealed by Click-iT method. Note that conventionally all cells are stained, and staining is homogenous inside the cellular nuclei (**a, b**). **h** and **i** showing RNAscope image of the mouse brain (as a control for method) received with the IGF2 probe (**h** cortex, **i** CA1 hippocampal area). Scale bar: 100 μ m



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Author Contributions MVR made the electrophysiological experiments, VNI made the morphological experiments, and PMB participated in discussion and manuscript preparing.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All experiments were performed in accordance with the ethical principles stated in the EU Directive 2010/63/EU for animal experiments and were approved (#012 from 10.10.2014) by Ethical Committee of the Institute of Higher Nervous Activity and Neurophysiology of Russian Academy of Sciences.

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