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Local serotonin-immunoreactive plexus in the female reproductive system of hermaphroditic gastropod mollusc *Lymnaea stagnalis*

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ABSTRACT: Serotonin is known as general regulator of reproductive activity in molluscs. Here we presented a detailed description of morphological backgroung of 5-HT synthesis and reliase by local serotonin-immunoreactive (5-HT-IR) plexus in the female reproductive system of hermaphroditic gastropod *Lymnaea stagnalis*. Three distinct parts of 5-HT-IR local network can be distinguished: weak innervations of the oviduct by solitary fibers, rich innervations of oothecal gland muscular layer by varicose fibers, and dense plexus of 5-HT-IR cells and their processes along the epithelium and in the muscular layer of uterus (pars contorta). Fusiform and multipolar 5-HT-IR cells located intraepitheliarly and have big nuclei. The thick apical process entered the epithelium and contacted the inner lumen of the duct. Basal processes of numerous cells compacted into bundles which organized the basket-like 5-HT-IR plexus surrounding the epithelial lumen of the canal.
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KEY WORDS: Gastropoda, uterus, local network, serotonin, secretory cells.

Локальный серотонин-иммунореактивный плексус в женской части репродуктивной системы гермафродитного брюхоногого моллюска *Lymnaea stagnalis*

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РЕЗЮМЕ Известно, что серотонин регулирует репродуктивную активность у ряда моллюсков. В данной работе представлено детальное морфологическое описание локального серотонин-иммунореактивного (5-HT-IR) плексуса, который, по всей видимости, обеспечивает синтез и выброс серотонина в окружающие ткани женского отдела репродуктивной системы у гермафродитного брюхоногого моллюска

большого прудовика. Можно выделить три части локальной 5-HT-IR системы: иннервация овидукта одиночными волокнами, богатая иннервация оотеки волокнами с варикозами, мощный плексус, состоящий из 5-HT-IR клеток и их отростков в утерусе (извитая часть). Веретенообразные и мультиполярные 5-HT-IR клетки расположены интраэпителиально и имеют большое ядро. Толстый апикальный дендрит проходит сквозь эпителий и контактирует с внутренним просветом репродуктивного канала. Базальные отростки множества клеток объединяются в тяжи, формирующие плексус корзинчатой структуры вокруг эпителиального слоя канала.
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КЛЮЧЕВЫЕ СЛОВА: Gastropoda, утерус, локальная нервная сеть, серотонин, секреторные клетки.

Introduction

Reproduction is one of the most complex event in the molluscan life. Significant part of this process is driven by the neural cells, neurotransmitters and hormons released by neurons. Among other neurotransmitters, serotonin (5-hydroxytryptamine, 5-HT) play important role as an regulator of reproduction activity. Distribution of 5-HT-containing neurons within central ganglia as well as peripheral innervation was described for variety of molluscan species (Schmidt-Rhaesa et al., 2016). To the contrary, the data about local peripheral serotoninergic networks or plexuses containing both neuronal fibers and cell bodies are scarse.

Presence of 5-HT-immunoreactive structures has been shown in gonads and reproductive tract of several molluscan species (Matsutani, Nomura, 1986; Boyle, Yoshino, 2002; Delgado et al., 2012; Moroz et al., 1997). Expression of 5-HT receptors was found in the reproductive system of *L. stagnalis* (Gerhardt et al., 1996) and nudibranch *Aplysia californica* (Li et al., 1995). In many bivalves, application of 5-HT induces spawning (Fong et al., 1994) or stimulates parturition (Fong et al., 1998). In bivalve species 5-HT stimulates maturation of oocytes (Ram et al., 1996). 5-HT is involved into the afterspawning gonadal recovery of scallop *Argopecten purpuratus* (Martínez, Rivera, 1994). In freshwater snail *Biomphalaria glabrata* serotonin receptors antagonist methiothepin supress reproductive behavior and reduce egg laying (Muschamp, Fong, 2001) In *Lymnaea stagnalis* season-dependent or pharmacologically induced increase of serotonin level in female part of the reproductive system leads to significant activation of egg masses production (Ivashkin et al., 2015). While many facts indicate that 5-HT is involved in regulation of egg laying, only innervation of this part of the reproductive system was descibed.

The goal of the current study was to investigate in details disctribution of 5-HT-immunoreactive (5-HT-IR)elements in the female part of the reproductive system of the gastropod hermaphroditic mollusc *Lymnaea stagnalis*. The fact that 5-HT-IR organize network along the reproductive duct of *Lymnaea* has been found previously (Ivashkin et al., 2015). However, the morphological description was out of the main topic of that paper. Here we describe in detail the morphology of 5-HT-immunoreactive cells and their processes as well as relation of 5-HT-IR elements with epithelial layer and muscular fibers of oviduct, convoluted part of uterus and oothecal gland of *Lymnaea*.

Materials and Methods

Laboratory population of *Lymnaea stagnalis* L. was maintained at stable conditions (22–23°C, 16-8 h light-dark cycle) and fed by lettuce. Mature animals 15 mm length were anaes-

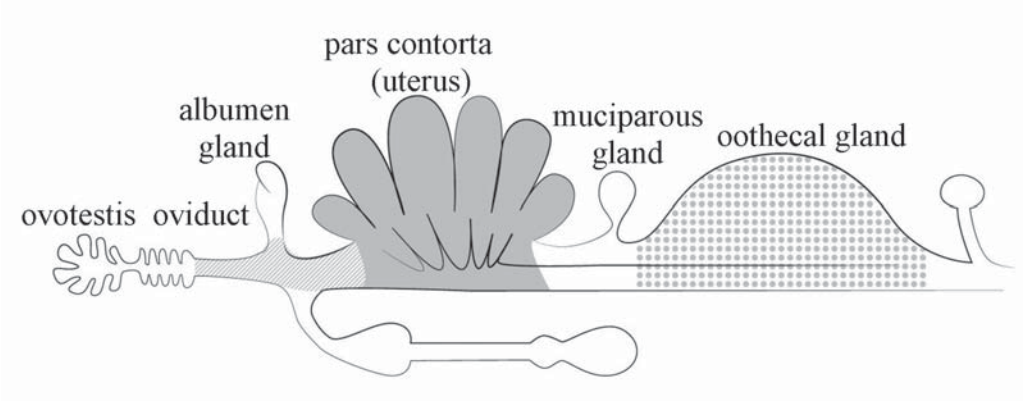

Fig. 1. Scheme of *Lymnaea stagnalis* reproductive system. The examined parts are marked gray.
Рис. 1. Схема репродуктивной системы *Lymnaea stagnalis*. Рассматриваемые в статье части помечены серым цветом.

thetized on ice and reproductive system was dissected. After fixation in 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.4) overnight at 10°C samples were washed in PBS and proceed for immunochemical detection of 5-HT as described earlier (Ivashkin et al., 2015). Briefly, whole-mount preparations as well as 25 μm cryostat sections were incubated in a-5-HT antibody (Immunostar) 1:2000 in PBS containing 5% Triton-X100 (PBS-TX) for 12 h at 10°C. After washing the preparations were incubated in a mixture of Goat anti rabbit Alexa 633 secondary antibodies and phalloidin-Alexa 488 (both Molecular Probes, 1:1000) overnight at 10°C. After washings nuclei were stained with DAPI, washed again and immersed in 70% glycerol. Preparations were examined under LCSM Leica SP5 (Leica, Germany) using appropriate wavelength-filters configuration settings, 0.5 μm thick optical sections were taken and processed with Leica software to obtain the whole image. HPLC High pressure liquid chromatography with electrochemical detector (HPLC-ED) was used for quantification of 5-HT as described earlier (Ivashkin et al., 2015). Tissues from three mature animals were used for one sample preparation, three samples were analyzed.

Results and Discussion

The female part of *Lymnaea stagnalis* reproductive system includes the oviduct proper, the uterus with its convoluted part (uterus), albumen gland and oothecal gland (oothec)(Fig. 1). We analyzed in detail the presence of 5-HT-IR elements in oviduct, uterus and oothec. Extensive network of fibers covers the external surface of the convoluted part of uterus (Fig. 2A). Sections through the folders demonstrate the basket-form 5-HT-IR fiber surrounding the epithelial layer of the duct (Fig. 2B). The positive cell bodies located intraepitheliarly (Fig. 2F, F-inset). Fusiform 5-HT-IR cells have one short basal process which is divided into two in a close proximity to the cell body (Fig. 2C-inset, E). Apical part of the cells contacted with inner lumen of the duct (Fig. 2C, H). The multipolar 5-HT-IR cell have thick apical process which located between epithelial cells and in some cases reaches the inner lumen of the duct (Fig. 2D-inset, F-inset). Cell bodies located close to the folder surface, the big nuclei of multipolar 5-HT-IR cell is clearly distinct from the surrounding nuclei of epithelial cells (Fig. 2D-inset, F-inset). Basal processes of both fusiform and multipolar 5-HT-IR cells were compacted into bundles which organized the dense 5-HT-IR plexus between the muscular fibers surrounding the epithelial lumen of the canal (Fig. 2E, F-inset). The solitary 5-HT-ir fibers leave the network and run into a deeper muscle layer. Solitary 5-HT-IR varicose fibers were observed among the muscular layer of the oothecal gland. Note that nerve bundles were preferentially

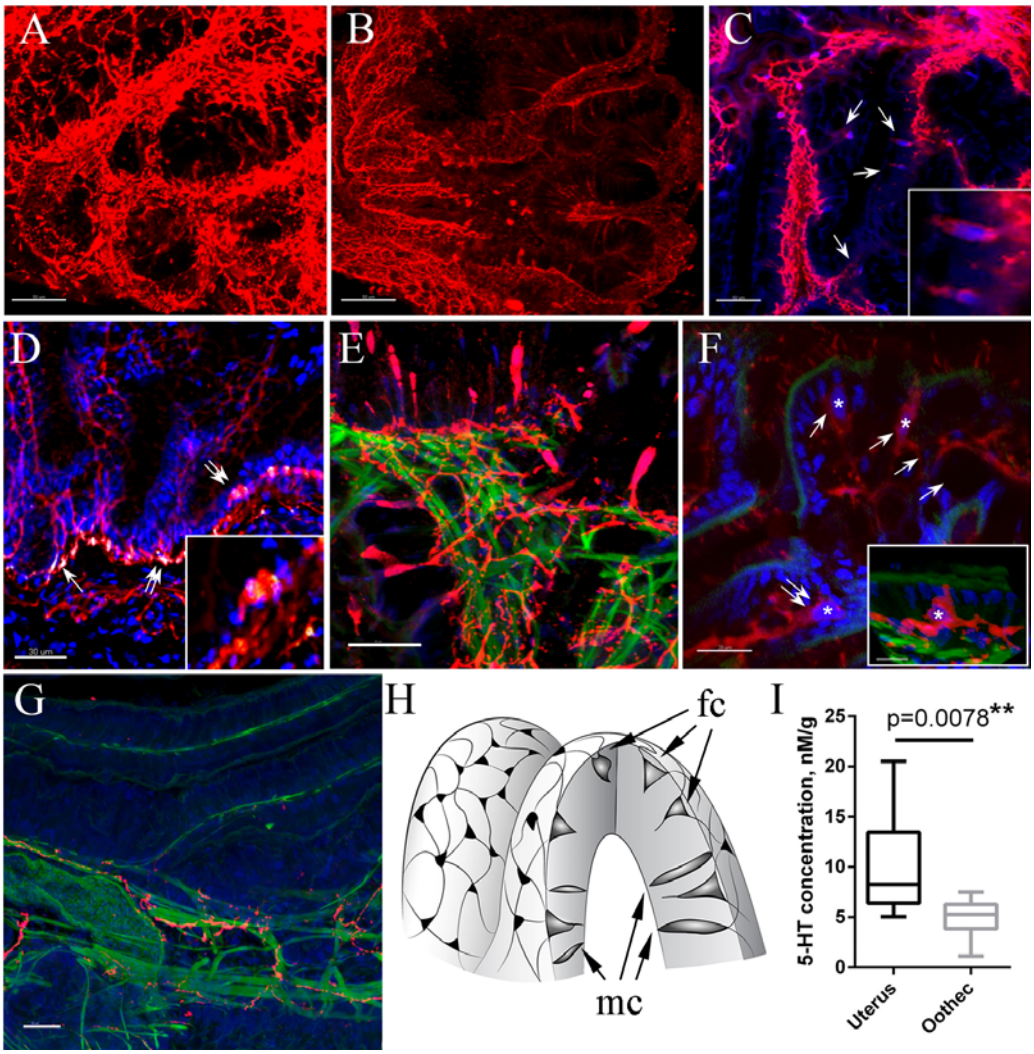

Fig. 2. Serotonin-immunoreactive (5-HT-IR, red) local network in *Lymnaea stagnalis* female part of the reproductive system. Muscles stained with phalloidin (green), cell nuclei marked with DAPI (blue). A — Whole mount preparation of uterus. Folders of the convoluted part are surrounded by basket-like 5-HT-IR fibers. B-E — Sections throught the lumen of the duct. Fusiform 5-HT-IR cell bodies located intraepitheliarly. Basal processes located around the muscle bundles. Insets demonstrate high magnification of immunopositive cells. While color on D and D-inset marks the colocalization channel of 5-HT-IR and DAPI. F — Hight magnification of uterus epitheliar layer. Asteriscs mark the big nuclea of 5-HT-IR cells. F-inset — Thick apical dendrite and two basal axons of multipolar 5-HT-IR intraepithelial cell. Arrow mark fusiform cells and double arrows mark multipolar cells. G — 5-HT-IR varicose fibers surrounding the muscle bundles in oothecal gland. H — Schematic representation of the 5-HT-IR network in the convoluted part (uterus) of the reproductive duct. Part of the folder are cut to demonstrate fusiform (fc) and multipolar (mc) cells position in the epithelial layer of the canal. Cell processes orginize basket-like net at the outer surface of the folder. I — HPLC measurements of 5-HT content in uterus and oothecal gland (Oothec). ** Statistical difference according to Mann-Whitney U-test. Scale bars: A, B, C, E, G — 50 μm; D, H — 30 μm; F — 20 μm; insets — 15 μm.
Рис. 2. Серотонин-иммунореактивная (5-HT-IR) локальная нервная сеть в женской части репродуктивной системы *Lymnaea stagnalis*. Мышцы окрашены фаллоидином (зеленый), клеточные ядра

located in close association with muscle fibers, running along solitary muscle or braided the muscle bundles (Fig. 2G). Solitary 5-HT-IR fibers also occurred along the oviduct (data not shown). HPLC measurements of 5-HT content within different parts of the *Lymnaea* female reproductive system demonstrate the high level of 5-HT within pars contorta compared with oothecal gland (Fig. 2I).

Thus three distinct parts of 5-HT-IR network can be distinguished in the female reproductive system of *Lymnaea stagnalis*: weak innervations of the oviduct by solitary fibers, rich innervations of oothecal gland muscular layer by varicose fibers, and dense plexus of 5-HT-IR cells and their processes along the epithelium and in the muscular fibers of uterus (Fig. 2H).

The most of features defined above are consistent with descriptions of 5-HT-IR structures availible from the literature for other species of molluscs. In the of gonad of the bivalvia *Patinopecten* serotonin immunoreactive fibers are distributed around the gonoduct and along the germinal epithelium (Matsutani, Nomura, 1986). In nudibranchs *Pleurobranchaea* and *Tritonia* all parts of the reproductive system are richly innervated by 5-HT-IR terminals (Moroz et al., 1997). In *Asperspina* (Opisthobranchia) serotonergic fibers were found in the proximal parts of female reproductive tract, including the spermathecum and the uterus. The fiber network has the highest density in uterus and is diminished abruptly near it's juncture with the nidamental gland while oviduct is not innervated at all (Delgado et al., 2012). In the albumen gland of *Biomphalaria glabrata* 5-HT was not found (Boyle, Yoshino, 2002).

In all mentioned above works authors described more or less extensive innervation of the female parts of the reproductive system and emphasize the central origin of this innervation. We also demonstrate numerous fibers along the female part of the reproductive duct. Rare 5-HT-IR fibers located in the wall of oviduct and oothecal part probably represents projections from the neurons located in the central ganglia. To the contrary, numerous local cell bodies were located in uterus and their fibers orginize the extensive dense network surrounding the epithelial layer and along the muscle fibers. Fusiform 5-HT-IR cell bodies which penetrate epithelium and contacted with inner lumen of the duct may suggest the primary receptor function for these cells. However, absence of sensory cilia at the end of the dendrite makes this suggestion doubtful. The multipolar 5-HT-IR cell resenble in the form, position and characteristic big nuclei size the exocrine cells in the reproductive tract of the *Lymnaea* which express egg-laying hormone (Van Minnen et al., 1989). The bulb-shape of apical dendrite of multipolar 5-HT-IR cells supports hypothesis about their possible exocrine function. Earlier we demonstrated that 5-HT level in uterus correlates with number of released eggs masses

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промаркированы DAPI (синий). A — Тотальный препарат утеруса. Складки извитой части утеруса окружены корзинчато-подобной сетью 5-HT-IR волокон. B-E — Срезы через внутреннюю часть канала. Веретенообразные тела 5-HT-IR клеток расположены интраэпителиально. Базальные отростки клеток окружают мышечные тяжи. На врезках представлены отдельные клетки на большем увеличении. Белым цветом на D и D-врезке показана колокализация 5-HT-IR и DAPI. F — Большое увеличение эпителиального слоя утеруса. Звездочки маркируют большие ядра 5-HT-IR клеток. F-врезка — Толстый апикальный дендрит и два базальных аксона мультиполярной 5-HT-IR интраэпителиальной клетки. Стрелки указывают на тела веретенообразных клеток, двойные стрелки указывают на тела мультиполярных клеток. G — 5-HT-IR волокна с варикозами, окружающие мышечные тяжи в оотеке. H — Схематическое представление организации 5-HT-IR сети в извитой части утеруса. Часть складки срезана, чтобы показать расположение веретенообразных (fc) и мультиполярных (mc) клеток в эпителиальном слое канала. Отростки клеток формируют корзинчатую структуру, оплетающую поверхность складки. I — HPLC измерения уровня 5-HT в утерусе и оотеке. ** Статистически достоверная разница по тесту Манна-Уитни. Масштабные линейки: A, B, C, E, G — 50 мкм; D, H — 30 мкм; F — 20 мкм; врезки — 15 мкм.

and serotonin-dependent behavior of embryos (Ivashkin et al., 2015). Thus, the intence local 5-HT-containing network of 5-HT-IR cells and fibers found in the uterus of *Lymnaea stagnalis* can serve as source of serotonin release into the lumen of reproductive truct.

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