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New data on innervation of the proboscis in *Bonellia viridis* females (Annelida: Thalassematidae: Bonelliinae)

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ABSTRACT: The proboscis of *Bonellia viridis* Rolando, 1822 females is the best studied among the echiurids and the rich data are extrapolated to other species of echiurids. However, very little is known about the fine structure and general organization of the nervous system of *B. viridis* and echiurids as a whole. The microscopic anatomy and ultrastructure of the nervous system of the proboscis of *B. viridis* are studied by the use of histology, micro-CT, SEM, TEM, and CLSM. The nerve cord, which forms a nerve loop, runs along the edges of the proboscis and gives rise to a number of peripheral nerves. For the first time, multiple radial neurite bundles innervating the margin of the terminal lobes and the frontal nerve passing along the margin are described. These nerves innervate the huge aggregation of glandular cells of the margin. The presence of additional nerve elements along the margin of the terminal lobes is determined by the specific function of the margin, which is used for the attachment of the proboscis to the substratum and for collection of food particles. The nerve loop of the proboscis has a typical structure: perikarya are located on the periphery of the nerve cord and the nerve projections extend into its centre. Among the nerve projections, the giant nerve fibre is defined. Neurons of four types, which differ from each other in the fine structure of soma, are described in the nerve loop. Some perikarya exhibit serotonin-like immunoreactivity. Specific cells, which contain intermediate filaments that are anchored to the basal lamina via hemidesmosomes are described for the first time and are identified as radial glia. We have suggested the origin of certain structure of echiurid nerve cord as a result of ingression of stratified neuroepithelium of annelid-like ancestor.
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KEY WORDS: nervous system, serotonin-like immunoreactivity, giant nerve fibre, radial glia, ultrastructure.

Новые данные об иннервации хобота у самок *Bonellia viridis* (Annelida: Thalassematidae: Bonelliidae)

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РЕЗЮМЕ: Хобот самок *Bonellia viridis* Rolando, 1822 изучен лучше всего среди эхиурид, а полученные обширные данные экстраполированы на другие виды эхиурид. Однако о тонкой структуре и общей организации нервной системы *B. viridis* и эхиурид в целом известно крайне мало. Микроскопическая анатомия и ультраструктура нервной системы хобота *B. viridis* была изучена с помощью гистологической техники, компьютерной микротомографии, СЭМ, ТЭМ и КЛСМ. Нервный ствол, который образует нервную петлю, проходит по краям хобота и дает начало ряду периферических нервов. Впервые описаны множественные радиальные пучки нейритов, иннервирующие край терминальных лопастей, и фронтальный нерв, проходящий вдоль края. Эти нервы иннервируют плотное скопление железистых клеток на краю. Наличие дополнительных нервных элементов по краям терминальных лопастей определяется специфической функцией переднего края лопастей, который используется для прикрепления хобота к субстрату и сбора частиц пищи. Нервная петля хобота имеет типичное строение: перикарии расположены на периферии нервного пучка, а нервные волокна проходят в его центре. Среди нервных волокон выделяется гигантское нервное волокно. В нервной петле описаны нейроны четырех типов, которые отличаются друг от друга тонкой структурой сомы. Некоторые перикарии проявляют иммунореактивность к серотонину. Специфические клетки, которые содержат промежуточные филаменты, прикрепленные к базальной пластинке через полудесмосомы, описаны впервые и идентифицированы как радиальная глия. Мы предположили, что определенная структура нервного ствола эхиурид возникла в результате погружения стратифицированного нейроэпителия у аннелидоподобного предка.

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КЛЮЧЕВЫЕ СЛОВА: нервная система, иммунореактивность к серотонину, гигантское нервное волокно, радиальная глия, ультраструктура.

Introduction

Echiurids are marine benthic invertebrates, which belong to annelid clade, but lack typical annelid characteristics such as segmentation and parapodia and have the aberrant body structure comprising proboscis and trunk (Pilger, 1993; Maiorova, Adrianov, 2020). The proboscis is a special organ of echiurids that is used to collect food particles around the burrow (Jaccarini, Schembri, 1977a). In most echiurids, the proboscis looks like a tongue-shaped outgrowth of the dorsal side of the body. Females of *Bonellia viridis* Rolando, 1822 have a long proboscis, which terminates with two lobes (Jaccarini, Schembri, 1977a), but their males demonstrate remarkable sexual dimorphism: they are much smaller and lack a proboscis at all (Schuchert, Rieger, 1990). The proboscis of *B. viridis* is the most studied among the echiurids. There are data on the mechanisms of its proboscis operation and movements (Jaccarini, Schembri, 1977b, Jaccarini, Schembri, 1979) and about proboscis structure: organization of musculature (Bosch, 1981), ultrastructure of integument (Bosch, Michel, 1979; Kuznetsov *et al*., 2021) and bonellin-producing cells (Bosch, 1977, 1979; Bosch, Michel, 1979; Kuznetsov *et al*., 2021), and blood vessels extended into the proboscis (Amor, 1973; Bosch, 1984; Kuznetsov, Temereva, 2022).

Little is known concerning the fine organization of the echiurid nervous system. In the literature, there are data on the general anatomy of the echiurid nervous system (Lawry, 1966a;

Pilger, 1993), its development (Hessling, 2002, 2003), and histology (Döhren, 2020). The fine structure of nerves has not been described before; there only fragmentary data on *Thalassema hartmani* (see Pilger, 1993) and male *B. viridis* (see Schuchert, Rieger, 1990).

In general, the central nervous system in echiurids consists of a ventral nerve cord that bifurcates anteriorly and extends into the proboscis as the nerve loop. The peripheral nervous system is composed of tiny bundles of nerves that branch from the loop to innervate the proboscis, and many pairs of nerves that branch from the ventral nerve cord in the trunk to innervate the body wall, the digestive tract, and the septal musculature (Lawry, 1966a). At the posterior end, the ventral nerve cord surrounds the rectum and innervates the body wall in that region (Pilger, 1993). According to histology data (Döhren, 2020), the ventral nerve cord of *Thalassema thalassema* (Pallas, 1774) and *Echiurus echiurus* (Pallas, 1766) contains giant nerve fibres and "glia fibres". However, the significance and appearance of these structures are not discussed in present papers. The development of the echiurid nervous system evidences the primary metameric nature of their unsegmented trunk (Hessling, 2002, 2003).

Because the organization of the nervous system is traditionally used to determine the relationships between different taxa at phyla level or higher (Helm *et al*., 2017; Beckers *et al*., 2019a, b; Borisanova *et al*., 2019; Kuzmina, Temereva, 2021), it seems important to know more about the nervous system structure in such unusual annelids as echiurids. Moreover, the detailed study of the echiurid proboscis nervous system will help to understand more in its operation, origination, and diversification within the group. The aim of this study is a detailed description of the innervation of the proboscis in *B. viridis* with the use of modern microscopic methods.

Material and methods

Four specimens of *Bonellia viridis* were collected at depth of 1–2 meters in the Vallon des Auffes, Marseille, France, 43°17′6″N, 5°20′59″E. The morphology of all specimens is studied with a Leica M165C stereomicroscope equipped with a Leica DFC420 digital camera (Leica Microsystems GmbH, Wetzlar, Germany). Proboscises were cut from the trunk and were then fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 420 mg NaCl for 8 h at + 4°C. Material was washed in the cacodylate buffer (3 times for 4 h) and then post-fixed in 1% osmium tetroxide in the same buffer. Four specimens have been studied by all methods listed below.

Computer micro tomography (micro-CT)

Fixed fragments of proboscis were additionally contrasted by exposure in 10% AgNO3 solution (SilverStar: 127775) for 12 h in complete darkness, washed and dehydrated in increasing concentrations of ethanol and acetone, and undergone critical point drying. The stacks of images were obtained using Bruker Skyscan 1272 tomography (Bruker, Billerica, USA) based on which a three-dimensional reconstruction was built using CTVox micro-CT Volume Rendering Software (Bruker, Billerica, USA).

Scanning electron microscopy (SEM)

To study the fine morphology of the proboscis, its fixed fragments were dehydrated in increasing concentrations of ethanol and acetone and underwent critical point drying in CO_2. Then material was mounted on stubs and sputter coated with platinum-palladium. Specimens were examined with a JEOL JSM-6380LA scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Histology and transmission electron microscopy (TEM)

Specimens were dehydrated in increasing concentrations of ethanol and isopropanol and then were embedded in Embed-812 resin (Electron Microscopy Science, USA). Semi-thin and ultra thin sections were prepared with a Leica UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Semi-thin sections were stained with methylene blue, viewed in an Olympus BX51 microscope (Olympus), and photographed with a Toupcam camera (ToupTek Photonics Co LTD). Ultrathin sections were stained with uranyl acetate (0.5%) and lead citrate (0.4%) and then examined with a JEOL JEM 100B electron microscope (JEOL Ltd., Tokyo, Japan).

Immunocytochemistry and confocal laser scanning microscopy (CLSM)

Fragments of the same four proboscises were fixed with 4% paraformaldehyde in 0.2 M phosphate-buffered saline (PBS) (pH 7.4) for 8 h at 4 °C. After fixation, specimens were washed in PBS with Triton X-100 (10%) (ThermoFischer) (PBT): 8 times for 20 min each. Nonspecific binding sites were blocked with 12% normal goat serum (Jackson ImmunoResearch, Newmarket, Suffolk, UK) in PBT overnight at 4°C. The specimens were incubated overnight in mixture of primary antibodies: rabbit against serotonin 5-HT (1:400) and mouse against acetylated-alfa-tubulin (1:700) in PBT at 4°C overnight. Material then was washed in PBT three times for 5 h each and exposed to the secondary antibody donkey anti-rabbit 488 (Life Technologies, A21206, 1:1000) and donkey anti-mouse 647 (TermoFisher Scientific, A-31571, 1:1000) in PBT for 24 h at 4°C. Then, the specimens were washed in PBS (four times for 60 min each), and embedded in glycerin. Specimens were examined with a Nikon Eclipse Ti confocal microscope (Nikon, Thermo Fisher Scientific, Waltham, MA, USA).

Image processing

Z-projections were generated using Image J version 1.43 software. Volume renderings were prepared using Amira version 5.2.2 software (ThermoFischer, Waltham MA, USA). Schemes, photographs, and Z-projections were processed in Adobe Photoshop CS3 (Adobe World Headquarters, San Jose, CA, USA).

Measurements

Measurements of some structures are done for two specimens. For each parameter, we have used four to five repetitions (n=4–5). The standard error is not calculated.

Results

General morphology of the body and organization of the proboscis

The body of *Bonellia viridis* females has a green colour and consists of a sac-like, non-segmented trunk and a long soft proboscis (Fig. 1A). The proboscis is a dorsoventrally flattened outgrowth, which consists of the proboscis trunk (Fig. 1B) and two long terminal lobes (Fig. 1C). The terminal lobes have a marginal edge, which is folded and has a specific colouration (Fig, 1C). The most terminal line of the margin is coloured in yellow and marks the place where a huge aggregation of gland cells is located (see below).

General anatomy of the nervous system

The central nervous system of the female *B. viridis* is represented by a ventral nerve cord in the body trunk and a nerve loop in the proboscis (Fig. 1D). The ventral nerve cord runs along the ventral side of the trunk from the base of the proboscis to the cloaca (Fig. 1D). Perpendicular collaterals (peripheral nerves) extend from the nerve cord to the body wall (Fig. 1 D). The nerve loop of the proboscis originates from the ventral nerve cord as two lateral nerve cords, which go around the mouth and extend along the lateral sides of the proboscis (Fig. 1D). Lateral nerve cords extend in the extracellular matrix dorsally in respect of the lateral coelomic canals containing lateral blood vessels (Fig. 1E, F). In the terminal lobes of the proboscis, the nerve cords merge together and form the nerve loop (Fig. 1D, G). The nerve loop gives rise to the radial neurite bundles, which extend mostly to the lateral side of the proboscis trunk (Fig. 1D). The nerve loop

ls — lateral side of the proboscis; ma — margin of the lobe; ml — marginal line; ne — neuropil; nl — nerve loop; p — proboscis; per — perikarya; t — trunk of the body; vs — ventral side; vnc — ventral nerve cord.

Рис. 1. Общая морфология тела и организация нервной системы хобота у самок *Bonellia viridis*. A — общий вид тела живого животного; B — ствол хобота у живого животного; C — терминальные лопасти с фронтальным концом и краем у живого животного; D — общая схема нервной системы, краевые пучки нейритов обозначены наконечниками стрелочек; E — поперечный разрез ствола хобота, СЭМ; F — нервная петля в поперечном срезе, СЭМ; G — нервная петля в хоботе, компьютерная микротомография; H — нервная петля по краю с радиальными пучками нейритов (наконечники) и фронтальным нервом, Z-проекции после иммуноокрашивания против ацетилированного альфа-тубулина; I — нервная петля по краю с радиальными пучками нейритов и фронтальным нервом, 3D-реконструкция.

Обозначения: abv — аксиальный кровеносный сосуд; cf — коллагеновые волокна; ds — дорсальная сторона; exm — внеклеточный матрикс; fn — фронтальный нерв; lbv — латеральный кровеносный сосуд; lc — латеральный целомический канал; lm — продольные мышцы; lo — лопасть хобота; ls — латеральная сторона хобота; ma — край лопасти; ml — краевая линия; ne — нейропиль; nl — петля нерва; p — хобот; per — перикарии; t — туловище; vs — вентральная сторона; vnc — вентральный нервный ствол.

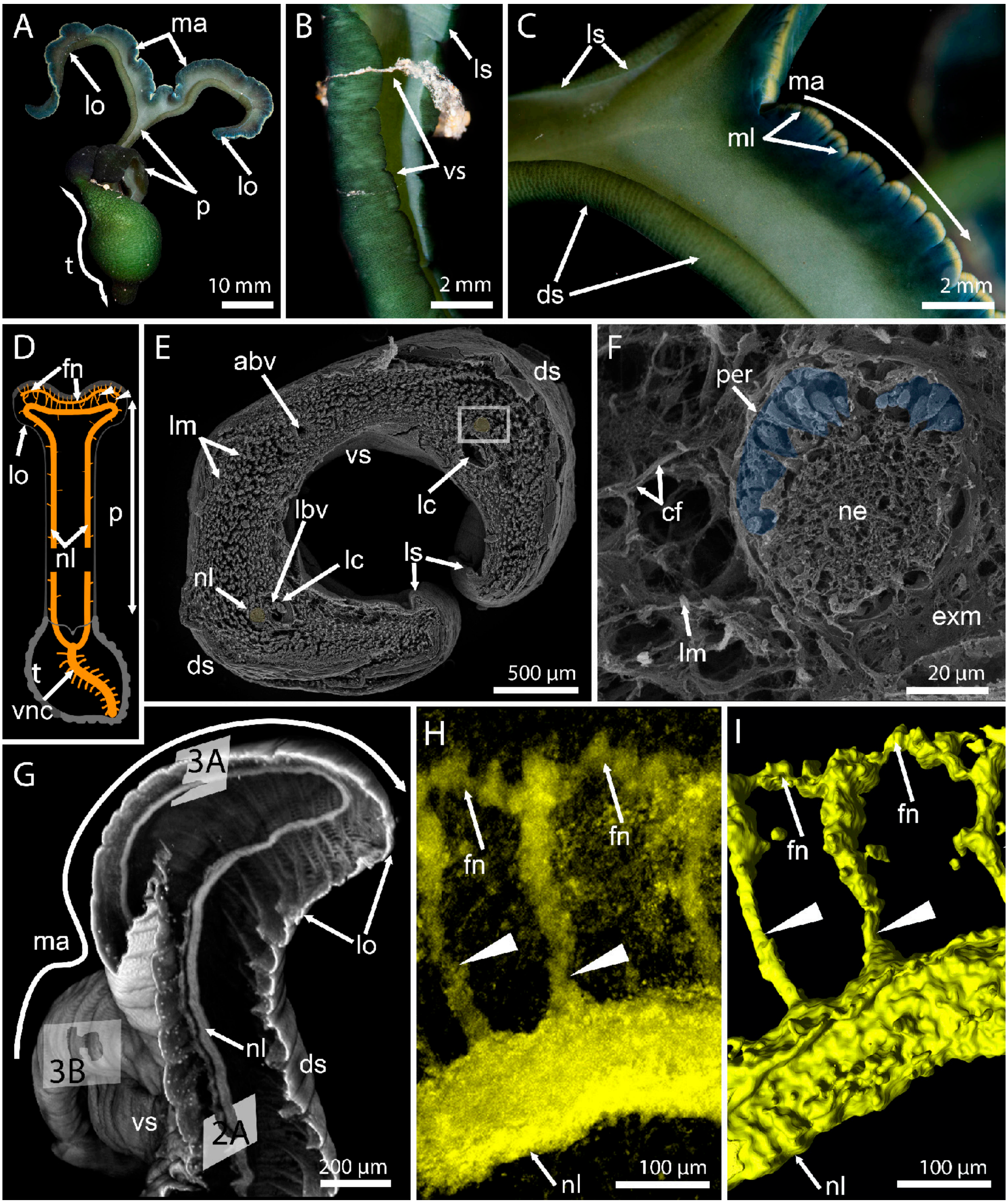

Fig. 1. General morphology of the body and the organization of the proboscis nervous system in females of *Bonellia viridis*. A — general view of the body of live animal; B — proboscis trunk in live animal; C — terminal lobes with the frontal edge and margin in live animal; D — the general scheme of the nervous system, margin radial neurite bundles are pointed by arrowheads; E — cross section of proboscis trunk, SEM; F — nerve loop in cross section, SEM; G — nerve loop in the proboscis, micro-CT; H — nerve loop on the margin with radial neurite bundles (arrowheads) and frontal nerve, Z-projections after immunostaining against acetylated alpha-tubulin; I — nerve loop on the margin with radial neurite bundles (arrowheads) and frontal nerve, 3D reconstruction.

Abbreviations: abv — axial blood vessel; cf — collagenous fibers; ds — dorsal side; exm — extracellular matrix; fn — frontal nerve; lbv — lateral blood vessel; lc — lateral coelomic canal; lm — longitudinal muscles; lo — lobe of the proboscis;

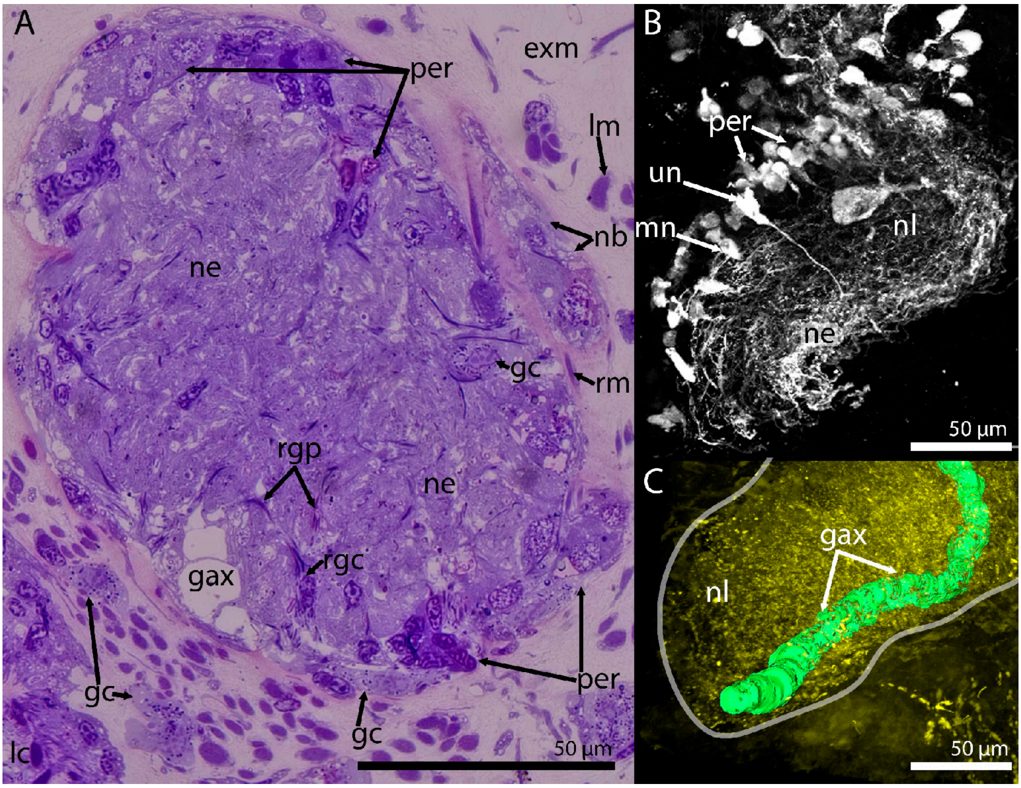

Fig. 2. Nerve loop of the proboscis of *Bonellia viridis* female in cross sections. A — semithin section of the nerve loop in the proboscis trunk; B — nerve loop, Z-projections after immunostaining against serotonin; C — nerve loop with 3D reconstruction of the giant axon, Z-projections after immunostaining against acetylated alpha-tubulin.

Abbreviations: bn — bipolar neuron; exm — extracellular matrix; gax — giant axon; gc — granulocyte; lc — lateral coelomic canal; lm — longitudinal muscles; mn — multipolar neuron; nb — neurite bundles; ne — neuropil; nl — nerve loop; per — perikarya; rm — ring musculature; rgc — radial glial cell; rgp — radial glial processes; un — unipolar neuron.

Рис. 2. Нервная петля хобота самки *Bonellia viridis* на поперечных срезах. A — полутонкий срез нервной петли в стволе хобота; B — нервная петля, Z-проекция иммуноокрашивания против серотонина; C — нервная петля с 3D-реконструкцией гигантского аксона, Z-проекции после иммуноокрашивания против ацетилированного альфа-тубулина.

Обозначения: bn — биполярный нейрон; exm — внеклеточный матрикс; gax — гигантский аксон; gc — гранулоцит; lc — латеральный целомический канал; lm — продольные мышцы; mn — мультиполярный нейрон; nb — пучки нейритов; ne — нейропиль; nl — нервная петля; per — перикарии; rm — кольцевая мускулатура; rgc — радиальная глиальная клетка; rgp — радиальные глиальные отростки; un — униполярный нейрон.

gives rise to the thick radial neurite bundles, which extend to the margin of the terminal lobes (Fig. 1H, I). In the margin, each radian neurite bundle forms two transverse branches. These branches, which originate from different radial neurite bundles, fuse each other and all together form the frontal nerve (Fig. 1D, H, I). Thus, the nerve loop runs parallel to the frontal nerve in terminal lobes and connects with it via numerous radial neurite bundles (Fig. 1H, I). In addition to the main neurite bundles, the nerve loop also gives rise to thinner neurite bundles that extend in various directions to the epidermis.

Histology and electron microscopy

In the cross-section, the nerve loop consists of peripherical perikarya that surround the central neuropil (fibre core) (Figs 1F; 2A). Perikarya do not form a continuous layer along the neuropil and are scattered as several groups or individual cells (Fig. 2A). Some perikarya are located within the neuropil (Fig. 2A, B). Immunostaining against serotonin reveals unipolar neurons oriented into the thickness of the nerve loop, bipolar neurons located along the nerve loop, and multipolar neurons (Fig. 2B). Serotonergic neurons have not been found in the frontal nerve or in other peripheral nerves (Fig. 2B). The neuropile occupies a greater part of the nerve loop in the cross-section, consists of many neurites, and contains a long cell projection with dense filaments (Fig. 2A). These projections belong to glial cells (see below). The giant nerve fibres (one or several) extend along the ventral side of the nerve loop (Fig. 2A, C). The nerve loop is surrounded by a fibrous extracellular matrix (Fig. 2A). Numerous mostly longitudinal muscles, secretary cells of different kinds, and neurite bundles are scattered in the extracellular matrix of the proboscis (Fig. 2A). In addition, amoeboid granulocytes are found both in the extracellular matrix and inside the nerve loop (Fig. 2A). At the margin of the terminal lobes, marginal neurite bundles innervate clusters of glandular cells and are interconnected by a frontal nerve (Fig. 3A). The frontal nerve contains single or clusters of several neuron somas on the periphery and a thick layer of the neuropil (Fig. 3B).

Perikarya

Based on the type of synaptic vesicles produced by nerve cells and other morphological and ultrastructural features, three different types of perikarya can be distinguished in the nerve loop (Fig. 4A, B, C) and another one only in the frontal nerve.

Perikarya of the first type (Fig. 4B, D) can be characterized as unipolar neurons with a spherical soma, in the centre of which there is a nucleus with a few amounts of heterochromatin and a large well-defined nucleolus. This type of soma is widespread in the nerve loop of the proboscis and is usually located externally. Their cytoplasm is filled with an extremely densely folded rough endoplasmic reticulum (RER), mitochondria, and numerous free ribosomes, which make the cytoplasm very dense (Fig. 4D). Part of the RER cisternae expands and transforms into vacuoles with granular contents (diameter about 0.5 μm) (Fig. 4D). The synaptic vesicles with electron-dense or lucent content (about 70 nm in diameter) are found on the periphery from the nucleus (Fig. 4D).

Perikarya of the second type (Fig. 4C, E) are elongated with an essentially oval nucleus containing significantly more heterochromatin compared to neurons of the other types. The processes of these neurons extend both radially to the centre of the nerve loop and embrace other types of neurons (Fig. 4C, E). The cytoplasm contains rare synaptic vesicles with a lucent content (70–100 nm in diameter). Due to the abundance of free ribosomes, the cytoplasm appears extremely dark. RER is poorly developed compared to other types of neurons (Fig. 4C, E). The cytoplasm contains the Golgi complex and numerous mitochondria. Dense granules up to 2 microns in size are found in some cells of this type (Fig. 4E).

Perikarya of the third type (Fig. 4B, C) are elongated or have an irregular shape, and have a rounded centrally located nucleus with a relatively small amount of heterochromatin. The processes of these neurons are oriented both radially going into the thickness of the nerve and along the nerve loop (Fig. 4B, C). The cytoplasm of these neurons contains rare synaptic vesicles or their dense clusters with electron-dense or lucent content (70–100 nm in diameter) (Fig. 5A). The RER form general expansions occupying not a small part of the soma and connects with the nuclear membrane forming a wide perinuclear space (Fig. 5A). The cytoplasm looks lucent and also contains vesicles with rare granular content (Fig. 5B). Dense granules up to 2 μm in size are found in some cells of this type.

Another type of perikarya was found only in the frontal nerve (Fig. 6A). The soma is located on the periphery of the frontal nerve, and the processes embrace along the neuropil (Fig. 6A) or sometimes go deeper (Fig. 6B). Their nucleus contains various amounts of heterochromatin (Fig. 6A). This type is distinguished by a large number of mitochondria, well-developed RER, a Golgi complex is localized in several places with the networks of tubules and vesicles surrounding it (trans Golgi network), and numerous free and associated ribosomes (Fig. 6C, D). The processes of these neurons form junctions with axons without any pronounced synaptic gap between two dense areas of the membranes (Fig. 6C).

Glial cells

Glial cells are well distinguished because of the presence of thick bundles of electron dense

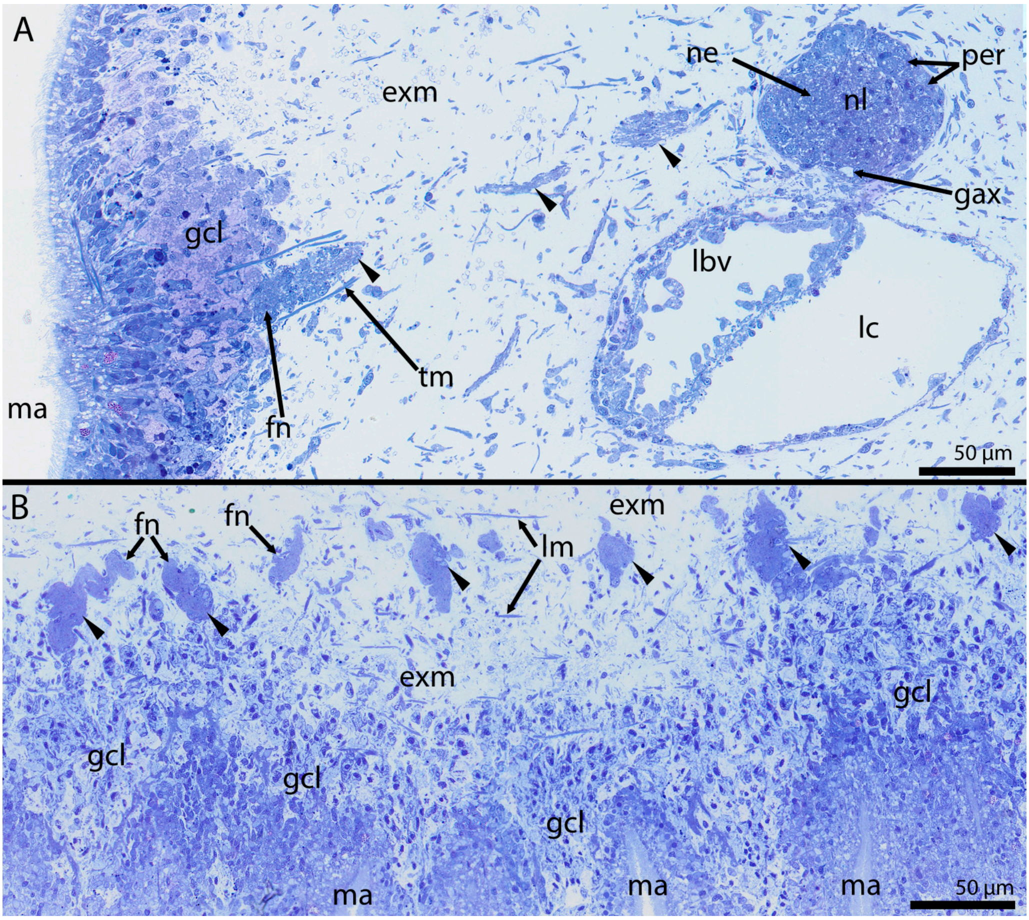

Fig. 3. Nerve loop in the margin of the terminal lobe with frontal nerve and neurite bundles in females of *Bonellia viridis*; semithin sections. Margin neurite bundles are pointed by arrowheads. A — the margin in the cross section; B — frontal section of the margin with frontal nerve and neurite bundles.

Abbreviations: exm — extracellular matrix; fn — frontal nerve; gax — giant axon; gcl — glandular claster; per — perikarya; lbv — lateral blood vessel; lc — lateral coelomic canal; lm — longitudinal muscles; ma — margin of the lobe; ne — neuropil; nl — nerve loop; tm — transverse musculature.

Рис. 3. Нервная петля на краю терминальной доли с фронтальным нервом и пучками нейритов у самок *Bonellia viridis*; полутонкие срезы. Краевые пучки нейритов обозначены наконечниками стрел. A — край на поперечном разрезе; B — передний разрез края с фронтальным нервом и пучками нейритов.

Обозначения: exm — внеклеточный матрикс; fn — фронтальный нерв; gax — гигантский аксон; gcl — железистый кластер; per — перикарии; lbv — латеральный кровеносный сосуд; lc — латеральный целомический канал; lm — продольные мышцы; ma — край лопасти; ne — нейропиль; nl — нервная петля; tm — поперечная мускулатура.

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ns I — neuron of the 1st type; ns II — neuron of the 2nd type; ns III — neuron soma of 3rd type; RER — rough endoplasmic reticulum; rgc — radial glial cell; tm — transverse musculature; vrgc — vesicle with granular content.

Рис. 4. Ультраструктура нейронов в нервной петле самок *Bonellia viridis* на поперечном срезе; ТЭМ. A, B, C — ультраструктура перикариона; D — перикарион первого типа, синаптические пузырьки обозначены наконечниками стрелок; E — перикарион второго типа.

Обозначения: cf — коллагеновые волокна; exm — внеклеточный матрикс; G — комплекс Гольджи; gc — гранулоцит; gcp — отростки глиальных клеток; gr — плотные гранулы; lm — продольные мышцы; mi — митохондрии; ne — нейропиль; nb — нервные пучки; ns I — нейрон 1-го типа; ns II — нейрон 2-го типа; ns III — сома нейрона 3-го типа; RER — гранулярный эндоплазматический ретикулум; rgc — радиальная глиальная клетка; tm — поперечная мускулатура; vrgc — везикула с зернистым содержимым.

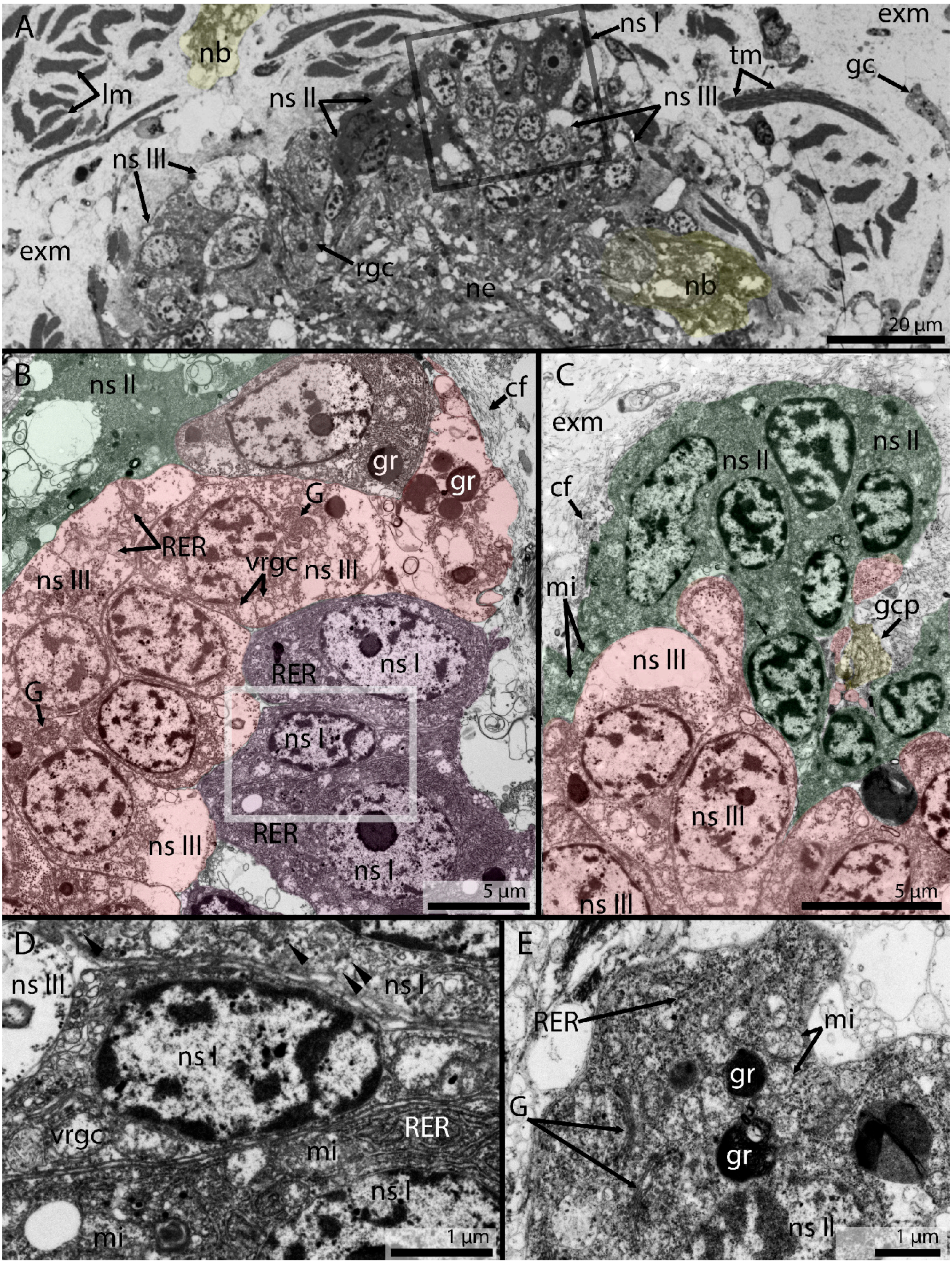

Fig. 4. Neuron ultrastructure in nerve loop of *Bonellia viridis* females on the cross-section; TEM. A, B, C — ultrastructure of the perikarya; D — perykaryon of first type, synaptic vesicles are pointed by arrowheads; E — perikaryon of second type.

Abbreviations: cf — collagenous fibers; exm — extracellular matrix; G — Golgi complex; gc — granulocyte; gcp — glial cell processes; gr — dense granules; lm — longitudinal muscles; mi — mitochondria; ne — neuropil; nb — nerve bunbles;

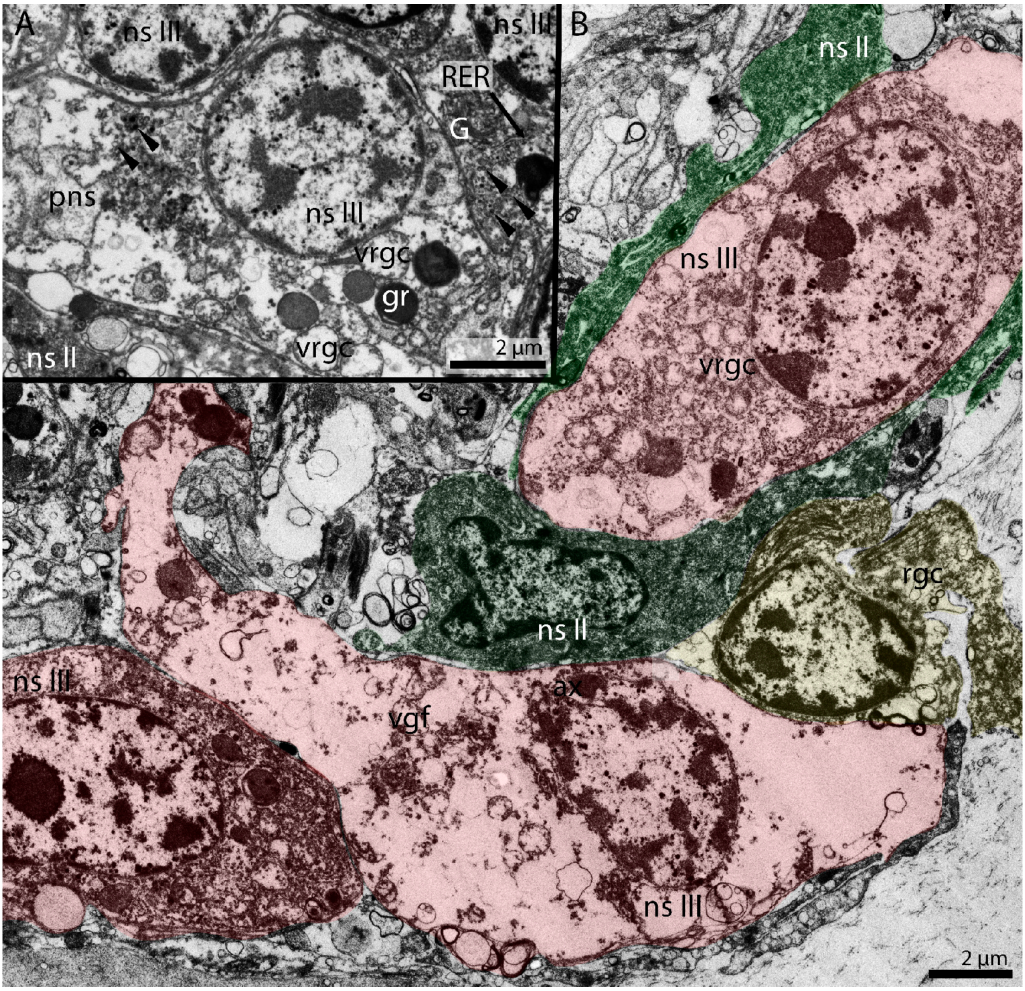

Fig. 5. Neuron ultrastructure in the nerve loop of *Bonellia viridis* females; TEM. A — neuron soma of the third type, neurotransmitter vesicles are pointed by arrowheads; B — perikarya of different types.

Abbreviations: G — Golgi complex; gr — granule; ns II — neuron of the 2nd type; ns III — neuron soma of 3rd type; pns — perinuclear space; RER — rough endoplasmic reticulum; rgc — radial glial cell; vrgc — vesicle with granular content.

Рис. 5. Ультраструктура нейронов в нервной петле самок *Bonellia viridis*; ТЭМ. A — сома нейронов третьего типа, везикулы нейромедиаторов обозначены наконечниками стрел. B — перикарии разных типов.

Обозначения: G — комплекс Гольджи; gr — гранула; ns II — нейрон 2—го типа; ns III — сома нейрона 3-го типа; pns — околоядерное пространство; RER — гранулярный эндоплазматический ретикулум; rgc — клетка радиальной глиалии; vrgc — везикула с зернистым содержимым.

filaments — these are intermediate filaments, which occupy numerous projections of cells and their somata as well (Fig. 7A–E). Soma of glial cells can be located both on the periphery near the extracellular matrix and in the neuropil (Fig. 7A, B). However, numerous projections of the glial cells always connect to the extracellular matrix, which surrounds the nerve (Fig. 7C). Bundles of intermediate filaments are anchored to extracellular matrix via hemidesmosomes (Fig. 7D). Numerous projections of glial cells extend in the neuropil in different directions and form an envelope around the perikarya and groups of nerve processes. However, the glial processes are extremely rare in the peripheral nerves.

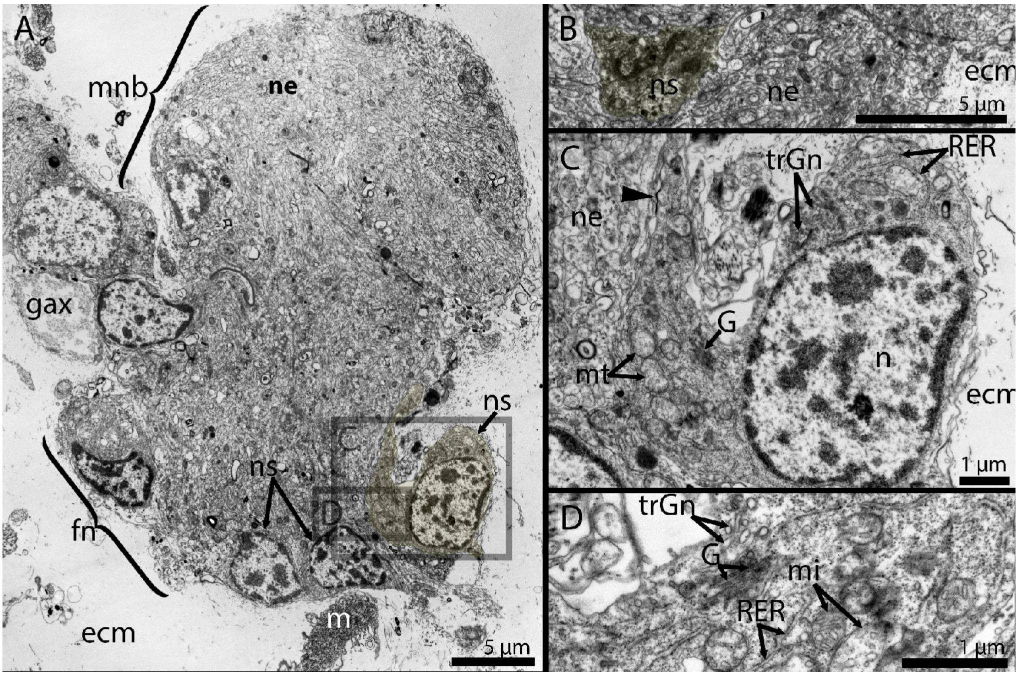

Fig. 6. Frontal nerve in the margin of the proboscis lobe in *Bonellia viridis* females; TEM. A — marginal neurite bundle gives rise to the frontal nerve; B — neuropil in the frontal nerve; C, D — soma in the frontal nerve, the gap junction is pointed by arrowhead.

Abbreviations: exm — extracellular matrix; fn — frontal nerve; G — Golgi complex; gax — giant axon; m — muscle cell; mi — mitochondria; mnb — margin neurite bundles; n — nucleus; ne — neuropil; ns — neuron soma; RER — rough endoplasmic reticulum; trGn — trans-Golgi network.

Рис. 6. Фронтальный нерв на краю лопасти хобота у самок *Bonellia viridis*; TEM. A — Краевой пучок нейритов дает начало фронтальному нерву; B — нейропиль в фронтальном нерве; C, D — сома в фронтальном нерве, щелевой контакт обозначен стрелкой.

Обозначения: exm — внеклеточный матрикс; fn — фронтальный нерв; G — комплекс Гольджи; gax — гигантский аксон; m — мышечная клетка; mi — митохондрии; mnb — краевые пучки нейритов; n — ядро; ne — нейропиль; ns — сома нейрона; RER — гранулярный эндоплазматический ретикулум; trGn — транс-Сеть Гольджи.

The nucleus has an irregular shape with folds and contains peripheral heterochromatin (Fig. 7A) and nucleolus (Fig. 7E). The RER is well developed, and forms an extension around the nucleus (Fig. 7A). The cytoplasm contains a Golgi complex and ovoid vesicles with a diameter of 0.2 to 0.8 µm with granular contents (Fig. 7A–E).

Neuropil

The fibre core (neuropil) is generally composed of densely interwoven neurites (axons and dendrites) and glial cell processes (Fig. 8A). Neurites differ from each other in diameter, cytoplasm density, and types of prevailing synaptic vesicle (Fig. 8A). In the neuropil, there are neurites of a large diameter with electron light cytoplasm and many synaptic vesicles with electron lucent content (Fig. 8B). Other neurites have dense cytoplasm with granular contents, mitochondria, and vesicles of different diameter with electron dense content (Fig. 8A).

Along the ventral edge of the nerve loop one or more giant axons extend (Figs. 8C). Giant axons have an irregular shape, they are flattened or rounded on the cross section and have about 20 µm in diameter (Fig. 8C). The giant axon forms short processes extending longitudinally or perpendicularly (Fig. 8C). Throughout their entire length the giant axons do not have a pronounced continuous envelope of auxiliary cells (Fig. 8C, D). Giant axons have electron-lucent cytoplasm containing rare granules, free ribosomes, vesicles of different diameter, and mitochondria (Fig. 8D).

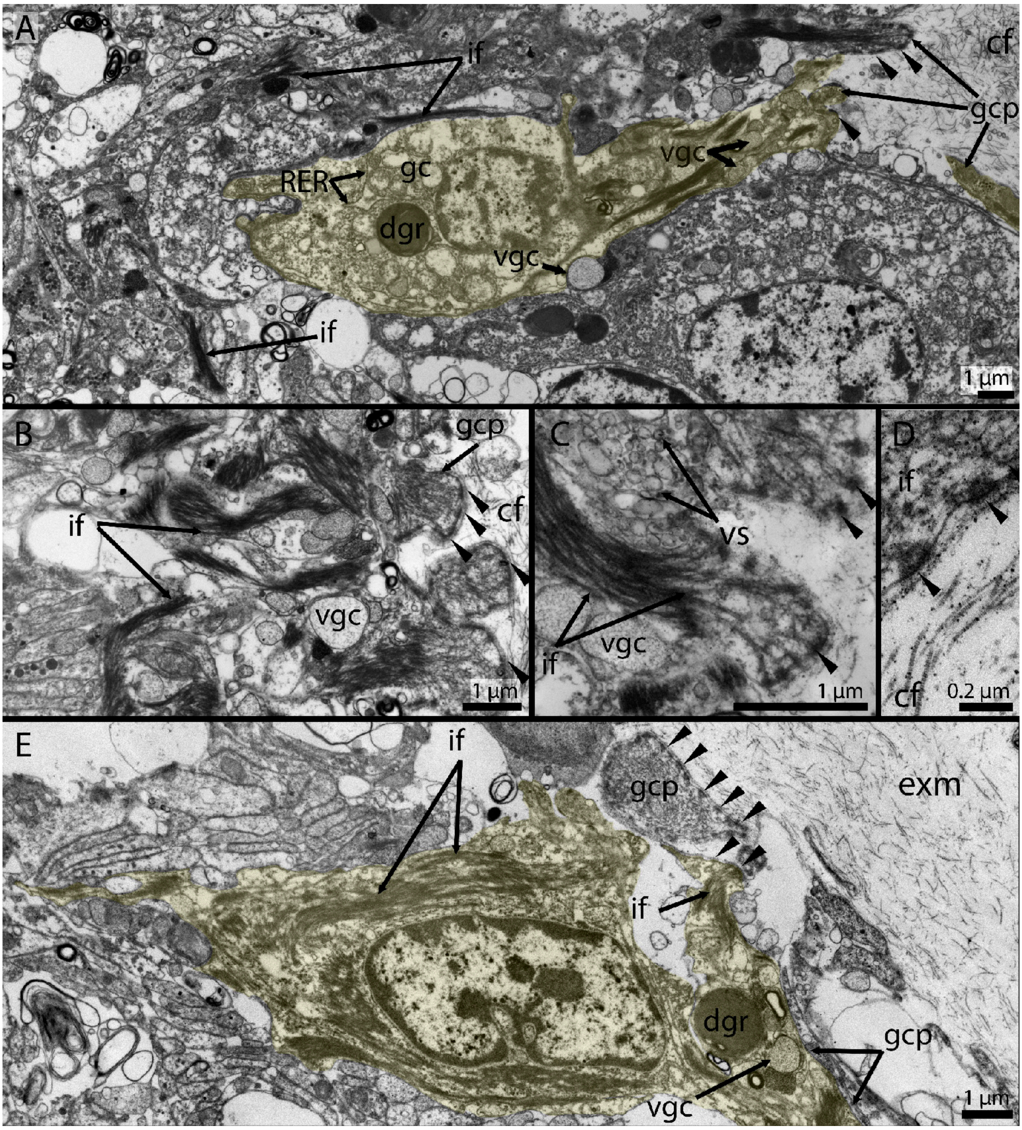

Fig. 7. Ultrastructure of radial glia of *Bonellia viridis* females; TEM. Hemidesmosomes are pointed by arrowheads. A — soma of radial glia cell; B, C — processes of radial glia on the basal part, near extracellular matrix; D — connecting radial glia cell to the extracellular matrix through hemidesmosomes, which are indicated by arrowheads; E — radial glia cell with processes.

Abbreviations: exm — extracellular matrix; cf — collagenous fibers; gcp — glial cell processes; gr —granules; if — intermediate filaments; ne — neuropil; RER — rough endoplasmic reticulum; rgc — radial glia cell; vgc — vesicle with granular content.

Рис. 7. Ультраструктура радиальной глии самок *Bonellia viridis*; ТЭМ. Полудесмосомы обозначены наконечниками стрелок. A — сома клетки радиальной глии; B, C — отростки радиальной глии в базальной части, вблизи внеклеточного матрикса; D — соединение клетки радиальной глии с внеклеточным матриксом через полудесмосомы, которые обозначены стрелками; E — радиальная глиальная клетка с отростками.

Обозначения: exm — внеклеточный матрикс; cf — коллагеновые волокна; gcp — отростки глиальных клеток; gr — гранулы; if — промежуточные филаменты; ne — нейропиль; RER — гранулярный эндоплазматический ретикулум; rgc — клетка радиальной глии; vgc — везикула с зернистым содержимым.

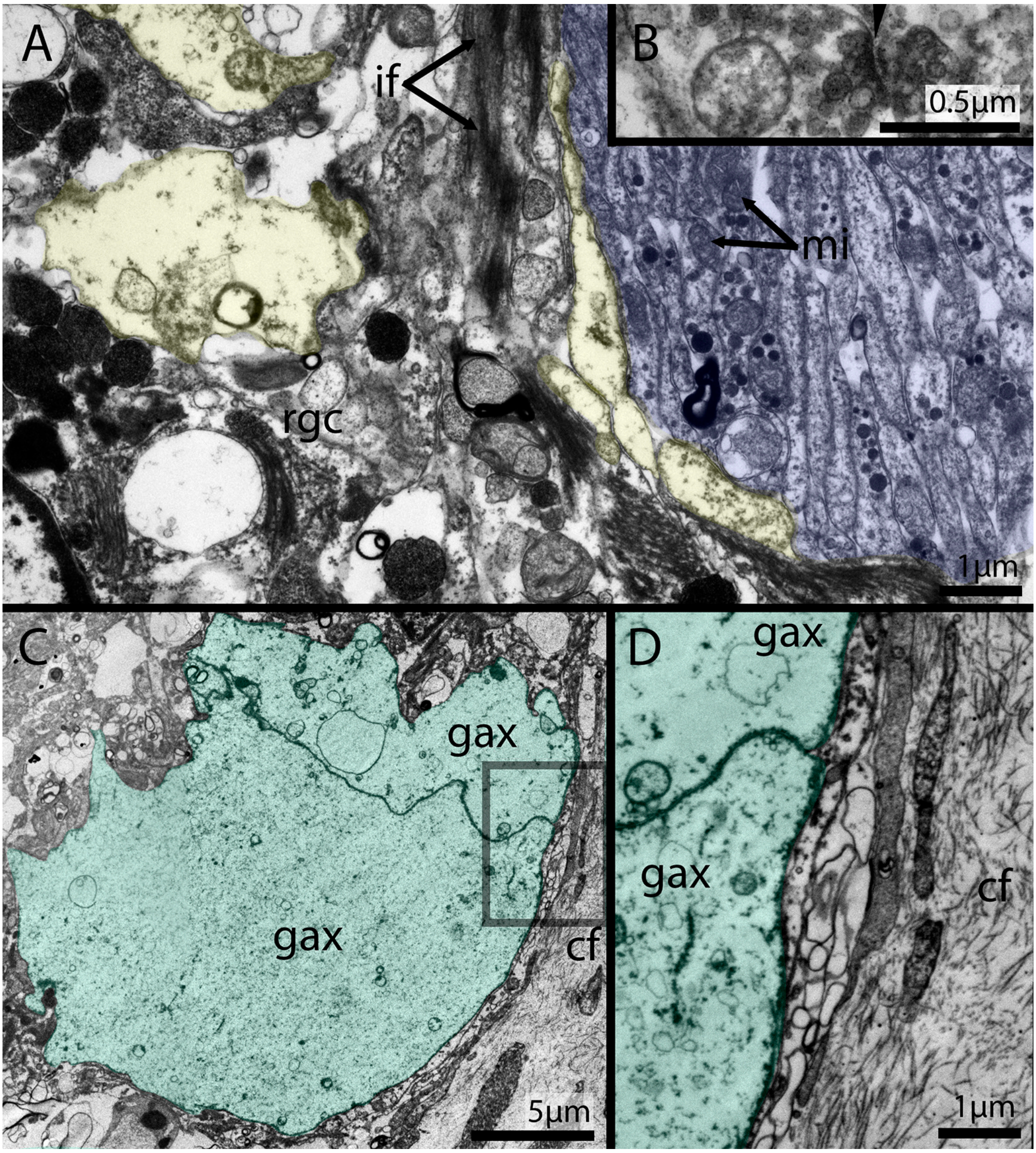

Fig. 8. Neuropil ultrastructure in nerve loop of *Bonellia viridis* female; TEM. A — neuropil of the nerve loop, neurites with electron light cytoplasm are in yellow, neurites with dense cytoplasm and granular contents are in blue; B — synapse is pointed by arrowhead; C, D — giant axon in the nerve loop of the proboscis trunk. Abbreviations: cf — collagenous fibers; gax — giant axon; if — intermediate filaments; mi — mitochondria; rgc — radial glial cell.

Рис. 8. Ультраструктура нейропиля в нервной петле самки *Bonellia viridis*; ТЭМ. A — нейропиль нервной петли, желтым выделены нейриты с электрон светлой цитоплазмой, синим – нейриты с плотной цитоплазмой и зернистым содержимым; B — синапс, обозначенный стрелкой; C, D — гигантский аксон в нервной петле ствола хобота.
Обозначения: cf — коллагеновые волокна; gax — гигантский аксон; if — промежуточные филаменты; mi — митохондрии; rgc — клетка радиальной глии.

The peripheral nerves are characterized by the same nerve fibres as in the nerve loop. However, the composition of peripheral nerves differs in that there are no glial cell soma in them. Giant nerve fibres were found only in the neurite bundles innervating the margin, except for the nerve loop. They are identical to the giant axon of the nerve loop in filling (see above), but they are distinguished by a smaller diameter (9–15 µm).

Discussion

Morphology of the nervous system

Previously, the nervous system of echiurids has been studied mainly at the level of general anatomy and histology (Pilger, 1993). Analysis of literature data allows us to conclude the unity of the general plan of the nervous system organization of all echiurids. The central nervous system is represented by the ventral nerve cord that branches in the mouth area into the nerve loop that runs into the proboscis (Lawry, 1966a; Pilger, 1993). There are no traces of metamery and pronounced ganglia in adult echiurids (Lawry, 1966a; Pilger, 1993; Hessling, 2002, 2003). However, signs of metamerism are found in the ventral nerve cord at larval stages of some echiurids (Hessling, 2002, 2003).

Although anatomical and histological data indicates a general plan of the nervous system structure for all echiurids, in *B. viridis* it has additional elements not previously described in echiurids. The strong innervation of the margin with peripheral nerves and the additional frontal nerve is correlated with a special structure and functions performed by the margin of proboscis terminal lobes in *B. viridis*. Specifically, along the margin line a massive secretion of mucus, which is produced by a large aggregation of subepidermal glandular cells, occurs (Kuznetsov *et al*., 2021). This mucus is used to collect food particles (Kuznetsov *et al*., 2021; Jaccarini, Schembri, 1977a). In addition, the margin contains a lot of multidirectional musculature that is used for fine motor skills in feeding behaviour (Kuznetsov *et al*., 2021; Jaccarini, Schembri, 1977b). Moreover, the terminal lobes of *B. viridis* proboscis are able to glue to the substrate (P. Kuznetsov, per observation). However, many other echiurids have a different morphology of the proboscis and its specific operation. Thus, in other bonnelid species, i.e. *Protobonellia zenkevitchi* Murina, 1976, the proboscis is subdivided into three zones and the terminal zone lacks the terminal lobes, but its connective tissue is armed by vacuolated cells, which probably supply the stability of the proboscis when it collects the food (Temereva *et al*., 2017). The involving of lateral edges of the proboscis into the collection of the food particles is suggested for *Lissomyema mellita* (Conn, 1886) (Kuznetsov *et al*., 2021) and *Ochetostoma erythrogrammon* Rüppel et Leuckart, 1828 (Chuang, 1962). It can be assumed that the frontal nerve can also be found in other species of Bonelliinae with a similar type of proboscis organization, with a pair of lobes. The neurite bundles, which go to the lateral side, and thin neurite bundles departing in different directions, are responsible for the glandular secretion (Kuznetsov *et al*., 2021).

Fine structure

There are no previous data on the fine structure of the nerve loop in echiurids, except for a partial description for *Thalassemia hartmani* Fisher, 1947. It only contains a mention of nerve fibres containing vesicles (Pilger, 1993). At the same time, glial cells were not found, and various types of perikarya were not identified (Pilger, 1993). Our study contains the first description of different types of perikarya and also provides some results concerning immunoreactivity of nerve cells in the nerve loop.

The combination of CLSM and TEM methods revealed the presence of at least four types of neurons in the nerve loop of *B. viridis*. The features in the cellular organization of neurons should be related to performing of various functions. However, it is difficult to assume a certain function without conducting a special neurophysiological study. Perikarya of the first and third types are characterized by numerous small vesicles with electron-dance and lucent contents. Thus, according to the totality of structural characteristics, these cells produce neurotransmitters and can correspond to serotonergic neurons marked by immunohistochemistry.

The abundance of granular endoplasmic reticulum in perikarya of the first type corresponds to a high level of synthetic processes and, in particular, protein synthesis. Another type (2nd) of perikarya in the nerve loop is characterized by an electron-dense cytoplasm with a large number of ribosomes. This indicates a high level of protein production and, accordingly, neurosecretion.

Perikarya of a special type are localized in the newly described frontal nerve. They are equipped with gap junctions. Probably the gap junctions are involved in the electrical transmission of a nerve impulse, however such contacts also provide the transfer of small signalling molecules. Probably these neurons are involved in the rapid operation of fine motor skills on the margin of the lobe.

Glial cells

Supportive cells, which perform defence, development, and nutrition nerve tissue, are described in many invertebrates (Mashanov *et al*., 2009, 2010; Richter *et al*., 2010; Helm *et al*., 2017). These supportive cells are traditionally named as glial cells. The term "radial glia" is mostly used for the description of development and regeneration of vertebrates (Miranda-Negrón, García-Arrarás, 2022; Mashanov *et al*., 2023). However, cells of radial glia have been described in key groups of Bilateria (Helm *et al*., 2017). These cells have specific ultrastructure and are characterized by the presence of electron dense bundles of intermediate filaments. These sells are discovered in intraepidermal nerve elements of annelids, priapulids, echinoderms, and hemichordates (Helm *et al*., 2017; Temereva *et al*., 2021). Glial cells in various taxa can perform similar functions (Bullock, Horridge, 1965; Barres, 2008). For the radial glia of the echiurid, one can assume a range of functions. Radial glial cells do not form a pronounced envelope around the neuron as described for some animals (for example Purschke, 2015; Borisanova *et al*., 2019). Thus, radial glial cells in the neural loop obviously do not carry a barrier function. At the same time, the presence of thick bundles of intermediate filaments and their connection with the extracellular matrix indicates the role of radial glia in maintaining the mechanical strength of the nerve. Perhaps radial glial cells give strength and resistance to stretching to the nerve loop as a whole, which suffers constant deformations (stretching, compression, twisting). However, it is believed that the key function attributed to the glia of both vertebrates and invertebrates, in particular insects, is to regulate the migration and growth of neurons (Edenfeld *et al*., 2005). Thus, one of the roles of the radial glia in the nerve loop may be the ontogenetic function.

Cells, which are structurally similar to radial glia, are described for the first time in the *B. viridis* nerve loop. Previously, glial cells have been established in the echiurid ventral nerve cord by method of paraffin histology (Döhren, 2020). Here, we suggest a scenario of evolutionary transformation of the echiurid neural loop of the proboscis, which is an isolated subepidermal nerve (Fig. 9A). A simple neuroepithelium (Fig. 9B) is considered to be a plesiomorphic state for the ancestor of Bilateria (Beckers *et al*., 2019b; Kuzmina, Temereva, 2020). Probably the next step of evolution is connected with its stratification and differentiation of functions (Fig. 9C). That is, by dividing the functions of the same type of cells in a simple neuroepithelium into radial glia and neurons (Fig. 9C). In this case, the neurons are isolated and fenced off from the external environment by glial cells. The next stage in the evolution of the central nervous system of bilateria is associated with its internalization (Fig. 9D) and subsequent isolation (Fig. 9E) (Beckers *et al*., 2019b; Kuzmina, Temereva 2020). Thus, a nerve with a perikarya surrounding the central neuropile (fibre core) appears from the multilayer neuroepithelium. A similar evolutionary path can be assumed for the nerve loop and for echiurids and annelids in general. Probably, the shift of the nervous system under the basal plate of the epidermis into the mesodermal tissue occurred repeatedly in Annelida as well as in other spiralians (Beckers *et al*., 2019b; Temereva *et al*., 2020).

Neuropil

The most interesting feature in the organization of echiurid neuropil is the presence of giant nerve fibres. The first mention about any giant axon we can find is in Spengel (1880) who thought that the 'neural canal' found in the ventral nerve cord of *Echiurus pallasii* Guérin-Méneville, 1831 might be a giant axon, and in Lawry (1966) who found it in the same case in *Urechis caupo* Fisher et MacGinitie, 1928. These fibres have also been described in the ventral nerve cord of *Thalassema thalassema*; and *Echiurus echiurus* by method of paraffin histology (Döhren, 2020). However, the presence of giant nerve fibres in the nerve loop of proboscis of *B. viridis* has never been reported before. Giant nerve fibres are typical structure of the nervous system in many other annelids and other phyla of invertebrates (Bullock, Horridge,

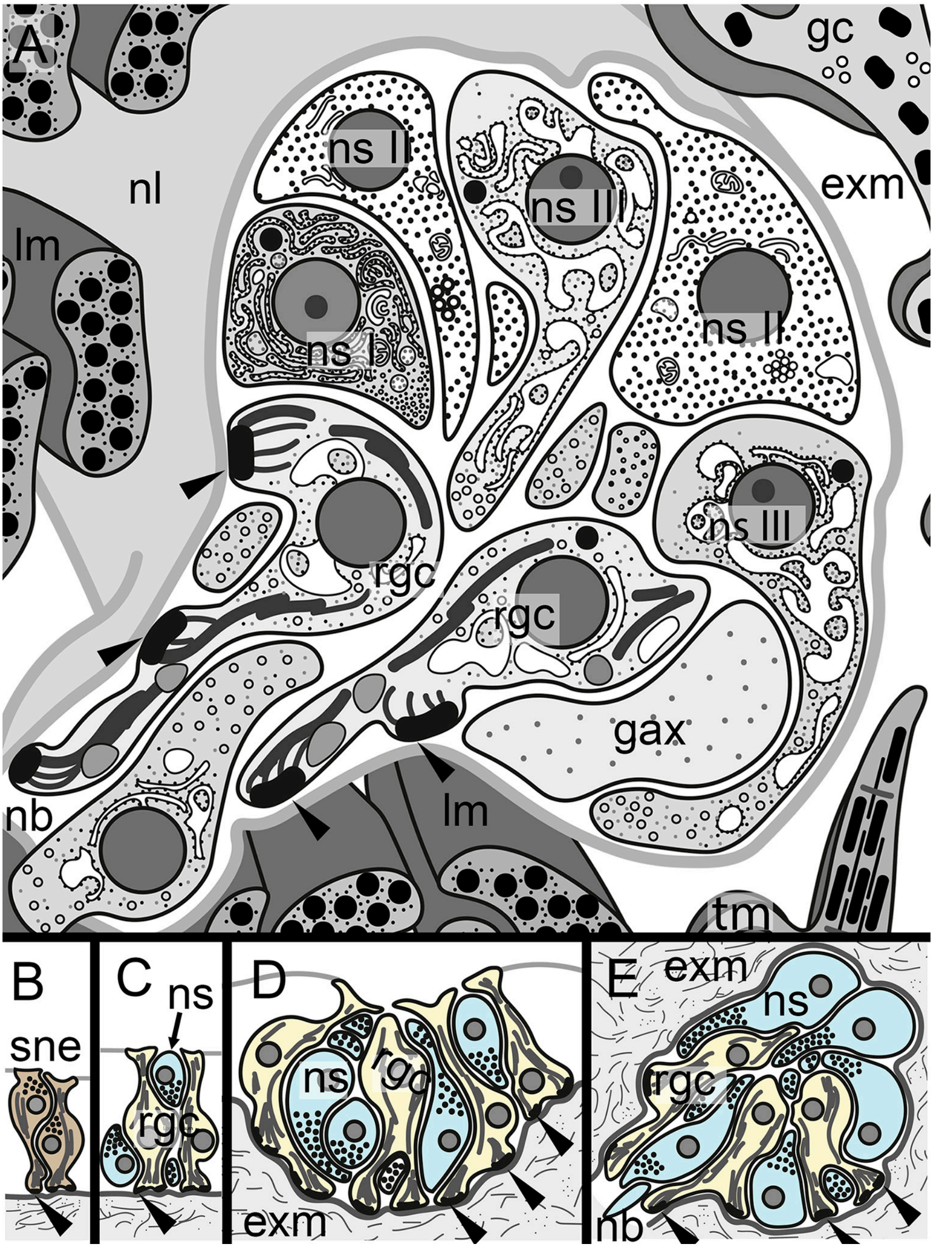

Fig. 9. Scheme of organization of nerve loop in cross section of proboscis of *Bonellia viridis* females (A) and hypothetical path of transformations leading to the formation of ehiuran nerve loop (B–E). B — simple neuroepithelium; C — stratified neuroepithelium; D — start of internalization in neuroepithelium; E — isolated subepidermal nerve, cells of radial glia are in yellow, perikarya are in cyan, hemidesmosomes are pointed by arrowheads.

Abbreviations: ep — epidermis; exm — extracellular matrix; gax — giant axon; gc — granular cell; lm — longitudinal muscles; nb — neurite bundles; nl — nerve loop; ns I — perikaryon of the 1st type; ns II — perikaryon of the 2nd type; ns III — perikaryon of 3rd type; rgc — radial glia cell; tm — transverse musculature.

Рис. 9. Схема организации нервной петли в поперечном срезе хобота самки *Bonellia viridis* (A) и гипотетический путь преобразований, приводящий к образованию нервной петли эхиурид (B–E).

1965; Fernandez *et al*., 1996; Malakhov, Galkin, 1998; Wollesen, 2015; Temereva, Malakhov, 2009; Beckers *et al*., 2019a, b). Accordingly to wide spread opinion, the giant nerve fibres provide swift transportation of the nerve signal from nerve centres to the musculature. The presence of giant nerve fibres in *B. viridis* proboscis can probably be explained by the enormous length of proboscis in this species. Such long proboscis needs quick innervation, which is probably supplied by giant nerve fibres.

Conclusion

The organization of the nerve loop in the proboscis of *Bonellia viridis* corresponds to the general plan of structure of the echiurids nervous system. However, it has a number of additional elements that were not noted earlier: enhanced innervation of the margin of the terminal lobes by a special frontal nerve and radial neurite bundles, which originate from the nerve loop. Such a strong innervation of the margin is associated with the special structure of the proboscis and the feeding mechanism. New data on the ultrastructure of the nerve loop and peripheral nerves in *B. viridis* expand our understanding of the functioning of the proboscis and the fine structure of the echiurids' nervous system as a whole.

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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B — простой нейроэпителий; C — многослойный нейроэпителий; D — начало интернализации в нейроэпителии; E — изолированный субэпидермальный нерв, клетки лучевой глии окрашены в желтый цвет, перикарии — в голубой, полудесмосомы обозначены наконечниками стрелок.

Обозначения: ep — эпидермис; exm — внеклеточный матрикс; gax — гигантский аксон; gc — гранулярная клетка; lm — продольные мышцы; nb — пучки нейритов; nl — нервная петля; ns I — перикарион 1-го типа; ns II — перикарион 2-го типа; ns III — перикарион 3-го типа. тип; rgc — клетка радиальной глии; tm — поперечная мускулатура.

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