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Sensory systems of *Lineus ruber* (Nemertea, Pilidiophora)

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Abstract

The aim of this study was to examine the organization of sensory systems in the heteronemertean *Lineus ruber* (Müller, 1774). Sensory systems of the head region (cerebral and frontal organs), body wall, proboscis and digestive tract of *L. ruber* were studied using Golgi–Colonnier silver impregnation, glyoxylic acid-induced fluorescence method for catecholamines (CAs) and immunochemical staining for serotonin (5-HT) and FMRFamide-related peptides (FaRPs). The study revealed a large number of intra- and subepithelial sensory cells in all of these structures and body regions. Only some of the sensory cells identified by silver impregnation were positive for CAs, 5-HT or FaRPs; the remaining cells appear to have different neurotransmitter modality. CA-containing (CA-C) intra- and subepithelial sensory cells were the most common cell types and were present in the epidermis and all the organs studied. All CA-C cells had a stiff cilium, which showed glyoxylic acid-induced fluorescence in the blue–green range indicative of CAs. In addition to CA-C cells, the cerebral organs had FaRP-immunoreactive sensory cells and argyrophilic cells containing an unidentified neurotransmitter and the frontal organs contained the neurites of 5-HT-immunoreactive, most likely efferent neurons. The presence of presumably mechanoreceptive CA-C cells in the frontal and cerebral organs indicates that these organs may perform not only a chemosensory, but also mechanosensory function.

Keywords Nemerteans · Sensory cells · Sense organs · Nervous system · Silver impregnation · Immunochemistry

Introduction

Nemerteans are a relatively small and little-known group of predatory unsegmented worms distinguished by a unique eversible proboscis used primarily for capturing prey. Molecular phylogenetic studies place nemerteans within Lophotrochozoa, but their phylogenetic affinities to other lophotrochozoan groups remain equivocal (Struck and Fisse 2008; Podsiadlowski et al. 2009; Kocot et al. 2017; Luo et al. 2018; Laumer et al. 2019; Marlétaz et al. 2019). The nervous and sensory systems show different levels of internalization and complexity in different nemertean groups and figure prominently in nemertean taxonomy (Gibson 1972; Chernyshev 2011; Beckers and von Döhren 2015). Many structural and functional aspects of these systems, however, are still poorly known, as the bulk of the literature on nemerteans are

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histological and electron-microscopical studies that provide information only on the general architecture of the nervous system and sense organs (see reviews in Bullock and Horridge 1965; Beckers and von Döhren 2015).

The nemerteans possess a variety of sense organs located mostly in the head region. In many species, there are two to many pairs of pigmented ocelli, some nemerteans have lateral organs of unknown function and species of the interstitial genus *Ototyphlonemertes* Diesing, 1863 possess statocysts (Gibson 1972; Chernyshev 2011; Beckers and von Döhren 2015). Two of the major sense organs commonly present in nemerteans are frontal and cerebral organs. Nemerteans are able to track and catch prey efficiently by following olfactory cues or sensing disturbances in the water (Reisinger 1926; Amerongen and Chia 1982; Chernyshev 2000; Kruse and Buhs 2000; Wang and Sun 2006) and both frontal and cerebral organs appear to be involved in this process, although their exact mode of functioning is still not fully understood.

Frontal organs have been described as small epithelial pits in the anterior part of the head region above the rhynchostome, the number of which can vary from one to three

in different nemertean species (Gibson 1972). The frontal organs are generally believed to function as chemoreceptors (e.g., Hyman 1951; Gibson 1972; Brusca et al. 2016) and their epithelial pits were observed to bulge out to the exterior presumably to expose their cilia for detection of olfactory cues (Reisinger 1926; Hyman 1951). Some evidence, however, indicates that frontal organs may act as mechanoreceptors (Reisinger 1926; Gibson 1972; Riser 1993). For instance, Gibson (1972) observed that in juveniles of *Lineus albocinctus* Verrill, 1900 the long cilia of the frontal organ are projected anteriorly during swimming, but retract, when the worm collides with an object. Behavioral experiments, in which nemerteans showed a decreased ability to track food 1 day after the excision of sensory pits (Wang and Sun 2006), appear to support the chemosensory function of the frontal organs. It cannot be ruled out, however, that behavioral changes observed in these experiments were caused by the severity of the injury inflicted to the animal or an insufficiently long time interval allowed for recovery, rather than by the loss of their frontal organs.

The cerebral organs are located laterally on each side of the head region. Each organ consists of a blind ciliated cerebral canal opening to the exterior, which is associated with sensory and gland cells. Most authors express the view that these organs function as chemoreceptors (Ling 1969, 1970; Ferraris 1985; Amerongen and Chia 1987) and some also suggest that they may perform a neuroendocrine (Ling 1969, 1970; Ferraris 1979, 1985) or an additional mechanoreceptive function (Ling 1969). The function of cerebral organs, like that of frontal organs, has been examined in behavioral experiments that showed that their removal weakens the ability of nemerteans to locate prey (Amerongen and Chia 1982). It remains unknown, however, which type of reception (chemo- or/and mechanoreception) is used by nemerteans in prey capture or defence and it is possible that the cerebral organs can perform not only a chemoreceptive, but also a mechanoreceptive function.

The anatomy and ultrastructure of the proboscis and the body wall have been studied in several nemertean species (Bürger 1891; Ling 1971; Oaks 1978; Norenburg 1985; Turbeville and Ruppert 1985; Turbeville 1991; Chernyshev 2010, 2011), but only a few studies have reported sensory elements within these structures (Bürger 1891; Ling 1971; Turbeville 1991). The organization and role of the peripheral nervous system (PNS) in relaying sensory information in nemerteans is also poorly explored. The nemertean PNS is usually described as consisting of several nerves and plexuses (subepidermal, commissural, stomatogastric, proboscidal, etc.) (Beckers et al. 2011; Beckers 2015), while the somata of neurons and sensory cells are often overlooked in descriptions. In some studies, however, numerous somata of sensory cells reactive to monoamines, neurotensin, choline acetyltransferase (a topographic marker of cholinergic cells) and NADPH-diaphorase (a topographic marker of nitroxidergic regulatory cells) have been revealed in the nemertean epidermis and enteric epithelium (Punin et al. 2003; Markosova et al. 2007; Zaitseva and Markosova 2008, 2009; Zaitseva and Petrov 2013) and in *Cerebratulus marginatus* Renier, 1804 nerve cells reactive to enzymes involved in the synthesis of hydrogen sulphide have been demonstrated in the body wall, digestive tract and cerebral organs (Chernyshev and Kotsyuba 2014).

The purpose of this study was to examine the architecture and cellular organization of sensory systems in *Lineus ruber* (Müller, 1774) (Pilidiophora: Lineidae) using the Golgi–Colonnier silver impregnation method and a combination of histochemical and immunochemical methods in conjunction with confocal microscopy. Since the mode of functioning of some sensory systems in nemerteans is not completely certain, a further goal of the study was to use the new information on morphology and distribution of sensory cells obtained by these different and complementary microscopy methods to gain additional insight into the functions of sense organs in nemerteans.

Materials and methods

Individuals of *Lineus ruber* were collected from the White Sea near the Kartesh Biological Station of the Zoological Institute RAS (66°20′22.3″ N 33°40′26.3″ E) and maintained in the laboratory at 5–10 °C.

For scanning electron microscopy, the worms were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) or 2% glutaraldehyde (Sigma-Aldrich) in 0.01 M PBS for 12 h. Mucus was removed from the body surface by immersing the worms in 16% glycerol for 12 h and then in 20% ethanol for 6–12 h (Zaiteva and Bocharova 1981). The worms were dehydrated in a series of ethanol and acetone solutions, then critical point-dried in carbon dioxide and mounted on stubs. The stubs were sputter coated with platinum and viewed at magnifications of 600–15,000 on a FEI Quanta 250 electron microscope.

For immunochemical studies of serotonin (5-HT) and FMRFamide-related peptides (FaRPs), the worms were fixed in 4% PFA (Sigma-Aldrich) in 0.01 M PBS for 2–4 h at room temperature, then rinsed and permeabilized 3 × 15 min in 0.25% Triton X-100 in 0.01 M PBS (Tr-PBS). All subsequent steps were performed at room temperature. Non-specific antibody binding was blocked with Image-iT FX Signal Enhancer (Molecular Probes) for 1 h. Blocked worms were rinsed in Tr-PBS (4 × 15 min), incubated for 12–24 h in anti-FMRFamide (1:600; Immunostar) or 5-HT (1:600; Sigma-Aldrich) antibodies, rinsed again in Tr-PBs (4 × 15 min) and incubated in Chromeo 488-conjugated secondary antibodies (1: 300) (Abcam) for 8–10 h. After

incubation, the worms were rinsed in Tr-PBS for 15 min and stained 8–10 h with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (1:150, Sigma-Aldrich), then rinsed once in 0.01 M PBS, and placed on slides as whole-mount preparations in 80% glycerol. To amplify the signal for FMRFamide, paraffin-embedded worms were sectioned at 5–6 μm, incubated with antibodies to FMRFamide (1:1000–1:2000; Immunostar) for 3 h and visualized using the streptavidin–biotin–peroxidase technique (Dako REAL™). Peroxidase activity was detected in a chromogenic mixture of 0.001% 3-3′-diaminobenzidine in an organic solvent (Dako REAL™) for 10 min. Staining specificity was checked by excluding primary antibodies.

Catecholamine-containing (CA-C) cells were revealed with the glyoxylic acid-induced fluorescence method (GIF) (Torre and Surgeon 1976) as modified by Zaitseva et al. (2015). This method is based on the ability of glyoxylic acid (a highly reactive aldehyde) to convert monoamines to strongly fluorescent molecules and has been used for detecting CAs in a wide variety of invertebrates (e.g., Hay-Schmidt 1990b; Gustafsson and Eriksson 1991; Schlawny et al. 1991; Kotikova 1995; Díaz-Balzac et al. 2010), including molluscs and nemerteans (e.g., Trimble et al. 1984; Hay-Schmidt 1990a; Croll et al. 1999; Kiehn et al. 2001; Zaitseva and Petrov 2013). For this method, intact animals were incubated for 1.5–2 h at 4 °C in a freshly prepared working solution of glyoxylic acid G10601 (Sigma-Aldrich), placed on slides, and dried first for 0.5–1 h at room temperature under a ventilator and then for 30 min in a thermostat at 60 °C. The dried objects were mounted in a CitiFluor AF87 mountant solution (EMS). The preparations were viewed on a Leica DM 4000B fluorescent microscope (Leica E4 filter cube) and on a Leica TCS SP5 confocal microscope (405 nm laser). Blue–green fluorescence under ultraviolet illumination was interpreted as indicative of catecholamines (CAs). Yellow fluorescence characteristic of serotonin was absent under wide-field fluorescence microscopy, which indicated that fluorescence revealed in the GIF preparations by confocal microscopy was also caused entirely by CAs.

To identify biogenic amines in the GIF preparations according to their emission spectra (Lindvall and Björklund 1974), series of images were captured from different regions of interest using the lambda-scan function of the confocal microscope. Lambda scan was performed under 405-nm excitation in 5-nm increments from 430 to 560 nm. The resulting emission spectra corresponded to those of catecholamines showing that the major part of fluorescence was likely produced by dopamine.

To provide a more complete picture of cellular architecture and spatial organization of the nervous and sensory systems, immunochemical and histochemical methods were supplemented with the Golgi–Colonnier staining, one of the classical silver impregnation methods used primarily on invertebrates. This method has the advantage of simultaneously revealing different types of nerve and sensory cells regardless of their neurotransmitter chemistry. For the Golgi–Colonnier method, the worms were relaxed in MgCl$_2$ and fixed for 3–7 days in a freshly made mixture of four parts of 2–3.5% potassium bichromate and one part of 25% glutaraldehyde (Sigma-Aldrich) in distilled water. In addition to fixation, this solution was used for impregnation of the worms by chromium salts, which is essential for the subsequent selective silver impregnation of the nerve elements. The fixed worms were rinsed in distilled water and impregnated in 0.75% silver nitrate in distilled water for 3–7 days. Time intervals for fixation and impregnation were chosen individually in each case, because the results of impregnation differ significantly depending on the size of the animals and because different intervals can result in differential impregnation of either superficial or more deeply lying nerve elements. After impregnation, the worms were embedded in celloidin, cut into sections at 40–80 μm, dehydrated, cleared and embedded in dammar gum. The preparations were viewed, drawn and photographed using NU-2 Carl Zeiss and Leica DMR IW light microscopes equipped with a Nikon Coolpix 4500 digital camera.

The images were edited using only the global image adjustment options in Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA). All fluorescent images of immunochemical and histochemical preparations are shown as maximum intensity projections of the Z-stacks.

Results

The major sense organs of *Lineus ruber* are located in the head region and comprise a pair of lateral rows of simple pigment cup eyes, three frontal organs, lateral cerebral slits and paired cerebral organs (Figs. 1, 2a). The frontal organs are represented by an unpaired medial and a pair of lateral protrusible ciliated pits opening to the exterior at the tip of the head above the rhynchostome (Figs. 1, 2a). Each cerebral organ consists of a ciliated cerebral canal, which is bent in three mutually perpendicular planes and is associated with sensory and gland cells, with the glandular portion of the organ fused with the dorsal cerebral ganglia. The canal begins with an opening in the posterior portion of the lateral cephalic slits, follows a complex path inside the head and ends blindly beneath and somewhat lateral to the posterior lobe of the dorsal cerebral ganglia (Figs. 1, 3g). The cerebral organs are connected to the dorsal ganglia via short wide nerves.

In the head region of *L. ruber*, especially in its frontal portion, silver impregnation reveals a large number of intra- and subepithelial primary sensory cells (Fig. 2d). Many of these cells show blue–green fluorescence indicative of CAs
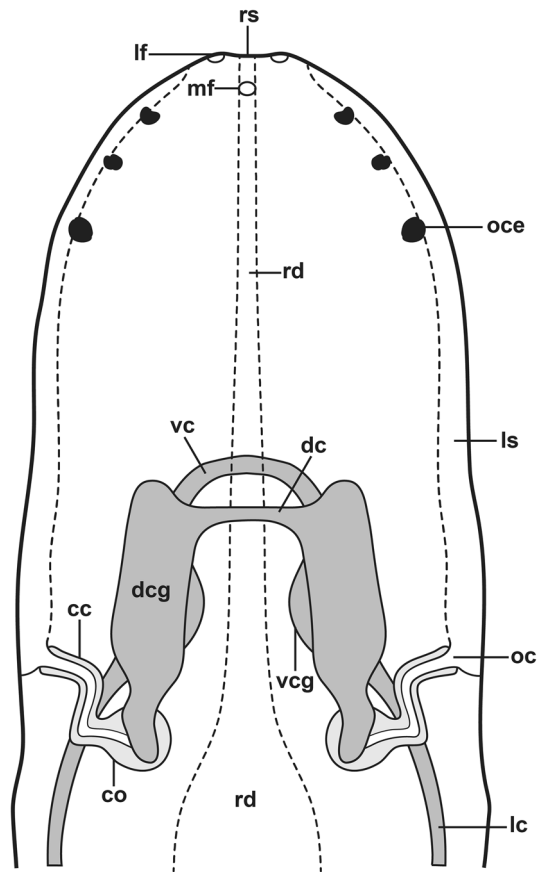
Fig. 1 Schematic drawing of the anterior end of the body of *Lineus ruber* showing the arrangement of the anterior sense organs and other major structures of the head region. *cc* cerebral canal, *co* cerebral organ, *dc* dorsal cerebral commissure, *dcg* dorsal cerebral ganglion, *lc* lateral nerve cord, *lf* epithelial pit of lateral frontal organ, *ls* lateral cephalic slit, *mf* epithelial pit of medial frontal organ, *oc* opening of cerebral canal, *oce* ocelli, *rd* rhynchodeum, *rs* rhynchostome, *vc* ventral cerebral commissure, *vcg* ventral cerebral ganglion

and at the apical ends of their dendrites bear straight cilia also fluorescent in the blue–green range (Fig. 2b, c, e–g). In these cells, the apical ends of dendrites are almost always highly fluorescent (Fig. 2b), but sensory cilia differ in fluorescence intensity. As the axons of these sensory cells proceed toward the brain, they join one another to form cephalic nerves that project into the dorsal cerebral ganglia (Fig. 2f, g). Among these cells, there are two pairs of cell groups located some distance from the epithelium of the frontal organs, each containing about 10 subepithelial somata of primary bipolar CA-C sensory cells. The dendrites of these cells reach into the epithelium of the frontal organs to form sensory endings with long cilia that show blue–green fluorescence indicative of CAs (Fig. 2c, e–g). Each lateral frontal organ is associated with a single group and the medial frontal organ with two groups of these cells. The axons of these cells converge into bundles to form cephalic nerves

that extend along the wall of the rhynchodeum into the dorsal cerebral ganglia (Fig. 2f). Axons of the cells of the left frontal organ and of the left group of the medial organ extend into the left cerebral ganglion and those of the corresponding right-side groups into the right cerebral ganglion. The double innervation of the medial frontal organ by the nerves originating from both dorsal cerebral ganglia can also be seen with anti-5-HT staining (Fig. 2h). No 5-HT- or FaRP-IR neurons are found associated with the frontal organs, but a large number of 5-HT- or FaRP-IR somata are seen in the cerebral ganglia and numerous FaRP-IR sensory cells are present in the epidermis of the head region (Fig. 3c, d, g). The frontal organs are innervated by 5-HT-IR neurites, with immunoreactivity being especially strong at the base of the epithelium of the ciliated pits (Fig. 2h).

In the cerebral organs, a significant number of intra- and subepithelial sensory cells of various shapes are revealed by silver impregnation in the cerebral canal; these cells have sensory endings at the epithelial surface of the canal and their axons form the subepithelial nerve plexus (Fig. 3a, b). FaRP-IR nerve elements are not found in the cerebral organs on whole mounts, but visualization with a streptavidin–biotin complex and the horseradish peroxidase technique on sectioned material reveals strong FaRP immunoreactivity in the neurites within the nerve of the cerebral organ and shows FaRP-IR sensory cells in the epithelium of the cerebral canal (Fig. 3c, d). A small number of CA-C (also presumably sensory) cells are present in the area of the canal (Fig. 3e, f). The lateral slits contain a large number of these cells, all of which, like those in the epidermis of the head region, bear straight, stiff cilia that fluoresce in the blue–green range. The axons of sensory cells of the cephalic slits form a basiepithelial plexus, which is densest at the openings of the cerebral canals and is highly fluorescent (Fig. 3e). The cerebral organs and their canals are innervated by 5-HT-IR neurites (Fig. 3g). These neurites are likely to project from the neurons of the dorsal cerebral ganglion within the nerves of the cerebral organs (Fig. 3g). The cerebral organs proper lack 5-HT-IR neurons.

We were unable to observe sensory cells in the ocelli and their connections to the dorsal cerebral ganglia. This can be explained by a masking effect of the dark ocellar pigment and by the presence of numerous CA-C sensory cells and their neurites in the adjacent epidermis, which cannot be readily distinguished from the nerve elements of the ocelli.

In the epithelium of the proboscis, silver impregnation reveals a large number of primary sensory cells unevenly distributed along the length of the proboscis (Fig. 4a). In the middle portion of the proboscis, which is normally everted during prey capture, these cells are positioned more closely. The axons of the sensory cells of the proboscis branch out at the base of the epithelium and appear to make synaptic contacts with one another. Some of these cells show blue–green
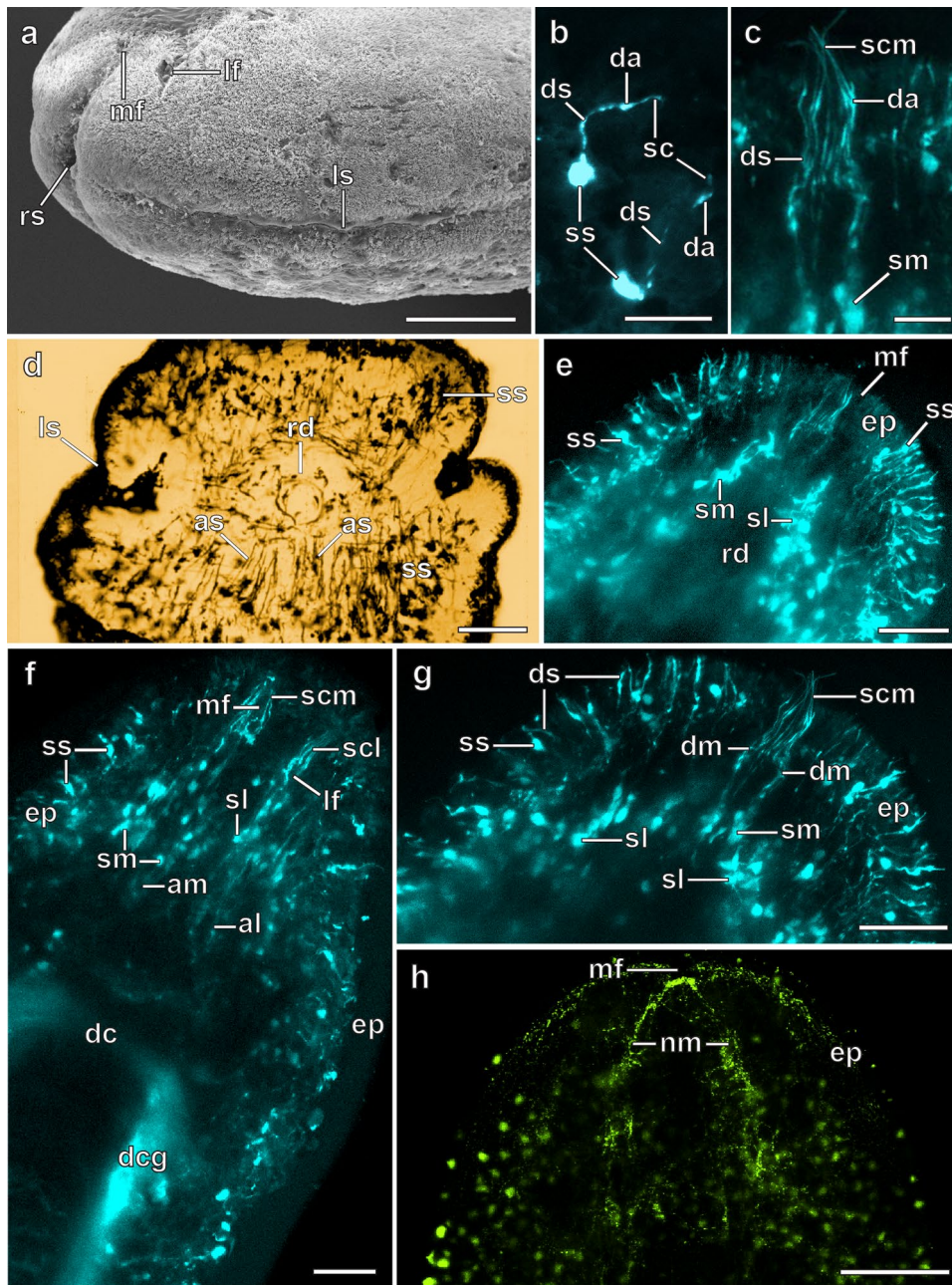
Fig. 2 Sensory systems in the anterior portion of the head region of *Lineus ruber*. **a** Anterolateral view of the head region (SEM) **b** Ciliated CA-C sensory cells in the dorsal epidermis (GIF). **c** Ciliated CA-C sensory cells in the medial frontal organ (GIF). **d** Transverse section through the frontal portion of the head region at the anterior end of the lateral cephalic slits (silver impregnation). Note that in this region the axons of sensory cells are still not grouped into nerves, but gradually converge toward the centre of the head in the direction of the cerebral ganglia. **e–g** Distribution of CAs in the dorsal portion of the head region (GIF). In **f** somata and neurites of only one lateral frontal organ are visible within the range of the maximum intensity projection. **h** Distribution of 5-HT in the dorsal portion of the head region (fluorescent immunochemistry). Scale bars: **a**, **d**, **h** 100 μm, **b**, **c** 10 μm, **e–g** 25 μm. *al* axons of sensory cells of lateral frontal organ, *am* axons of sensory cells of medial frontal organ, *as* axons of sensory cells, *da* dendrite apex, *dc* dorsal cerebral commissure, *dcg* dorsal cerebral ganglion, *dm* dendrite of sensory cells of medial frontal organ, *ds* dendrite of sensory cell, *ep* epidermis, *lf* epithelial pit of lateral frontal organ, *ls* lateral cephalic slit, *mf* epithelial pit of medial frontal organ, *nm* nerves of medial frontal organ, *rd* rhynchodeum, *rs* rhynchostome, *sc* sensory cilium, *scl* sensory cilia of lateral frontal organ, *scm* sensory cilia of medial frontal organ, *sl* somata of sensory cells of lateral frontal organ, *sm* somata of sensory cells of medial frontal organ, *ss* somata of intraepithelial sensory cells
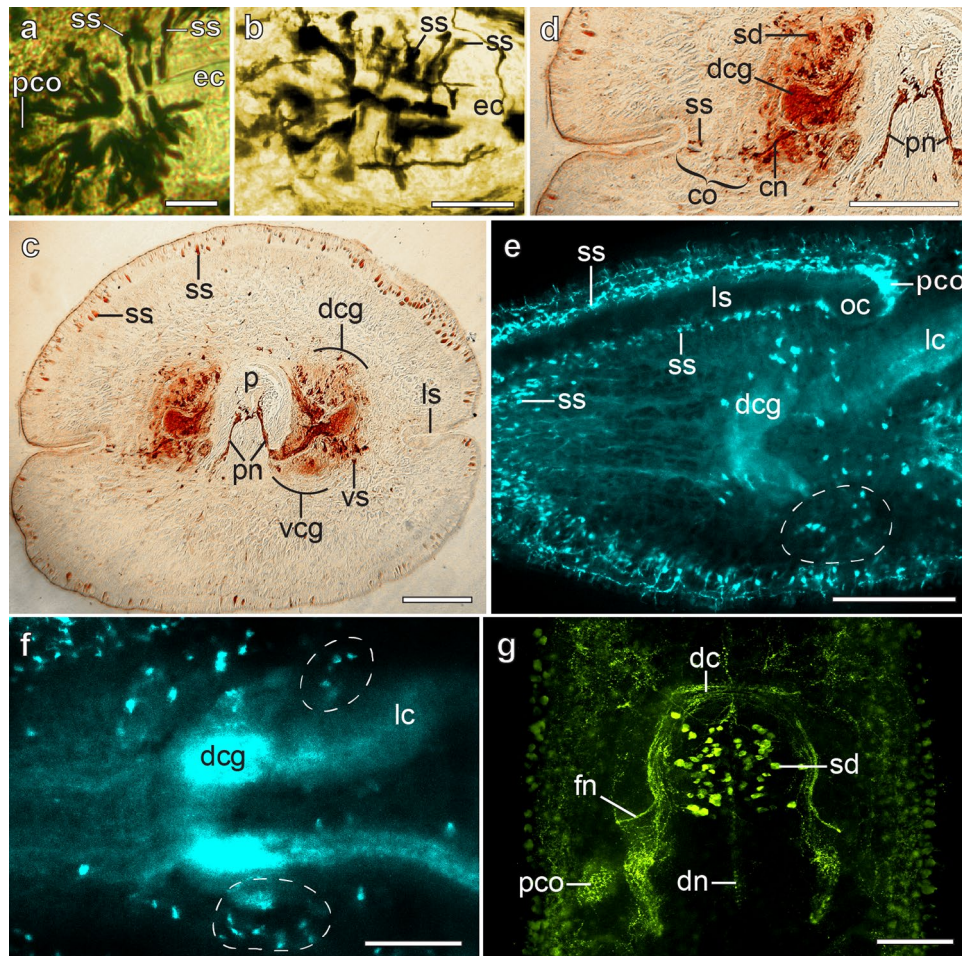
Fig. 3 Cerebral organs and lateral cephalic slits of *Lineus ruber*. **a, b** Intra- and subepithelial sensory cells of the cerebral organ (silver impregnation). **c, d** Transverse section through the brain and cerebral organs showing FaRP-IR intraepithelial sensory cells in the cerebral canals (streptavidin–peroxidase immunochemistry). **e, f** Numerous CA-C sensory cells in the lateral cephalic slits and a small number of CA-C cells in the cerebral canals (GIF). Dashed lines show the outlines of the cerebral organs. **g** Brain and cerebral organs showing the innervation of 5-HT-IR neurons of the dorsal cerebral ganglia by neurites of the sensory cells (fluorescent immunochemistry). Scale bars: **a, f** 50 µm, **b–e, g** 100 µm. *cn* cerebral organ nerve, *co* cerebral organ, *dc* dorsal cerebral commissure, *dcg* dorsal cerebral ganglion, *dn* dorsal nerve, *ec* epithelium of cerebral canal, *fn* nerve of fixator muscles, *lc* lateral nerve cord, *ls* lateral cerebral slit, *oc* opening of cerebral canal, *p* proboscis, *pco* plexus of cerebral organ, *pn* proboscidal nerves, *sd* somata of dorsal cerebral ganglion, *ss* somata of intraepithelial sensory cells, *vcg* ventral cerebral ganglion, *vs* somata of ventral cerebral ganglion

fluorescence characteristic of CAs or are immunoreactive to FaRPs. FaRP-IR neurites are present in the proboscidal nerves, the musculature of the proboscis and in the wall of the rhynchocoel (Figs. 3c, d, 4b).

Numerous primary intra- and subepithelial sensory cells of varying shapes were shown by silver impregnation to be located in the body wall throughout the body. These cells normally have a single dendrite that forms a sensory ending at the epithelial surface or branches out within the epithelium or beneath it without reaching its surface; these latter cells can be proprioceptive. A significant portion of the sensory cells in the body, like those in the head region, are also reactive to CAs (Fig. 5a, b). The CA-C cells are similar in morphology to the cells of the head: they are bipolar with a single dendrite that bears at its apical end a straight, stiff CA-C cilium showing blue–green fluorescence. In most cells, fluorescence is brightest at the enlarged apical part of the dendrite (Fig. 5b). The axons of these cells form basi- and subepithelial nerve plexuses and extend as small bundles into the adjacent regions of the lateral nerve cords where they become united into a single tract running from the ventral cerebral ganglia to the anus. In the area of the anus, the lateral nerve cords break down into individual CA-C sensory cells (Fig. 5a).

The lateral nerve cords are associated with FaRP-IR, 5-HT-IR and CA-C unipolar neurons that are located alongside the cords and decrease in number progressively from the anterior to the posterior end of the body. Judging from

Fig. 4 Transverse sections through the retracted proboscis of *Lineus ruber* showing intraepithelial sensory cells: silver impregnation (**a**) and FaRP-IR with streptavidin–peroxidase (**b**). Scale bars: 100 μm. *cm* circular musculature, *ie* internal epithelium of proboscis, *lm* longitudinal musculature, *pn* proboscidal nerves, *rhc* rhynchocoel, *ss* somata of intraepithelial sensory cells
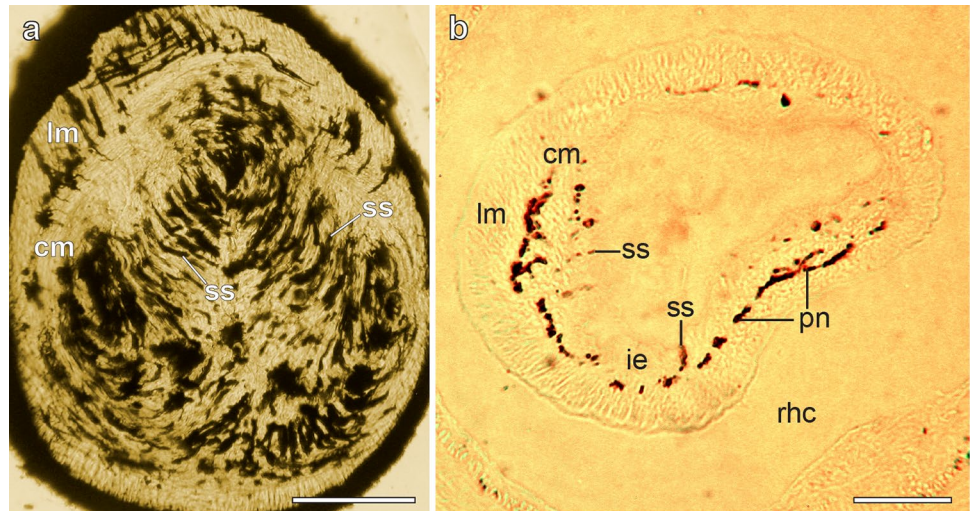

Fig. 5 Sensory systems of the body wall of *Lineus ruber:* GIF (**a, b, d**), silver impregnation (**c**), FaRP-IR (**e, f**), and 5-HT-IR (**g**). **a** Posterior end of the body showing a large number of CA-C sensory cells clustering around the anal opening. **b** Bipolar ciliated sensory cells in the epidermis with their axons forming the basiepithelial plexus. **c** Typical unipolar neuron of the lateral nerve cords. **d**–**g** Nerve cells in the plexus of the body-wall musculature sending neurites into the lateral nerve cords. Scale bars: **a**, **e**–**g** 100 μm, **b**, **c** 25 μm, **d** 50 μm. *ao* anal opening, *as* axons of sensory cells, *lc* lateral nerve cord, *ns* neuronal somata, *sc* sensory cilia, *ss* somata of intraepithelial sensory cells
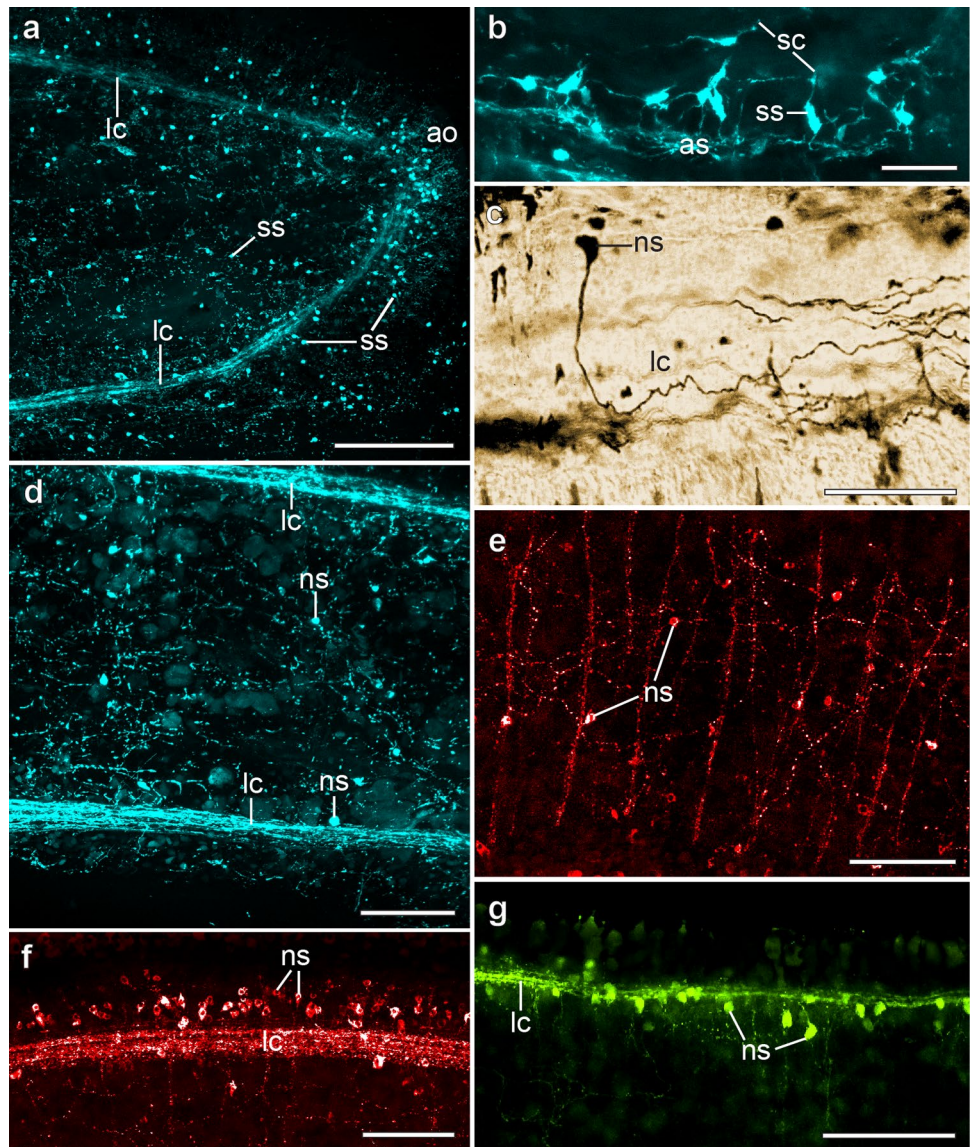
the branching patterns of their neurites among the axons of sensory cells in the tracts of the lateral nerve cords, these cells appear to be interneurons that mediate the primary processing of sensory information (Fig. 5). Most of the cells of the lateral nerve cords are immunoreactive to FaRPs, with the number of 5-HT-IR cells being much smaller; CA-C cells are very few in number and appear to be completely absent in the posterior portion of the nerve cords (Fig. 5a, d). Strong FaRP immunoreactivity is present in the body-wall nerve plexus, which is composed of bipolar neurons sending their neurites into the lateral nerve cords (Fig. 5e); these cells appear to innervate the body-wall musculature.

A significant number of intraepithelial primary sensory cells were found around the mouth and along the length of the digestive tract (Fig. 6). These cells vary in shape: some of them lack pronounced dendrites and are similar in morphology to the epithelial cells, others are bipolar with a well-developed sensory dendrite (Fig. 6a–f). Their axons branch out and form a basiepithelial plexus within the wall of the digestive tract. Some of these cells are FaRP- or 5-HT-immunoreactive (Fig. 6e). FaRP-IR sensory cells are found in small numbers along the length of the digestive tract, while the 5-HT-IR neurons are mostly present in the midgut. CA-C neurons are the most abundant type of sensory cell in the digestive tract. They form clusters around the mouth and in the epithelium of the anterior portion of the pharynx (Fig. 6g) and are evenly distributed throughout the digestive

tract. These cells resemble in morphology the intraepidermal CA-C sensory cells in the body and the head region.

Discussion

Our study of sensory systems of *Lineus ruber* has shown that nemerteans have a well-developed peripheral nervous system comprising a great number of different primary sensory cells. These cells are located in intra- and subepithelial positions in the head, body, proboscis and internal organs. The majority of sensory endings of these cells are evenly distributed throughout the epidermis forming concentrations in the canal of the cerebral organs, along the fronto-lateral surface of the head region and around the mouth. The axons of these cells form basi- and subepithelial nerve plexuses and join the adjacent ipsilateral nerves that lead toward the cerebral ganglia or the lateral nerve cords. Only some of the sensory cells revealed by Golgi–Colonnier silver impregnation in the present study correspond to CA-, FaRP- or 5-HT-reactive sensory cells; the remaining cells are likely to have different neurotransmitter modality, which is consistent with the results of the earlier studies that showed neurotensin-, choline-, and NO-ergic somata in the body wall, proboscis and digestive track of several nemertean species (Punin et al. 2003; Markosova et al. 2007; Zaitseva and Markosova 2008, 2009).

Fig. 6 Sensory cells around the mouth opening and in the digestive tract of *Lineus ruber*. **a–c, f** Sensory cells in the epithelium of the intestine (silver impregnation). **d** FaRP-IR sensory cell in the epithelium of the intestine (streptavidin–peroxidase immunochemistry). **e** 5HT-IR sensory cell in the epithelium of the intestine (streptavidin–peroxidase immunochemistry). **g** CA-C sensory cells and their neurites around the mouth opening and in the wall of the pharynx. Scale bars: **a–e** 25 μm, **f**, **g** 50 μm. *gd* gastrodermis, *m* mouth, *ov* ovaries between folds of intestine, *ph* pharynx, *ss* somata of intraepithelial sensory cells
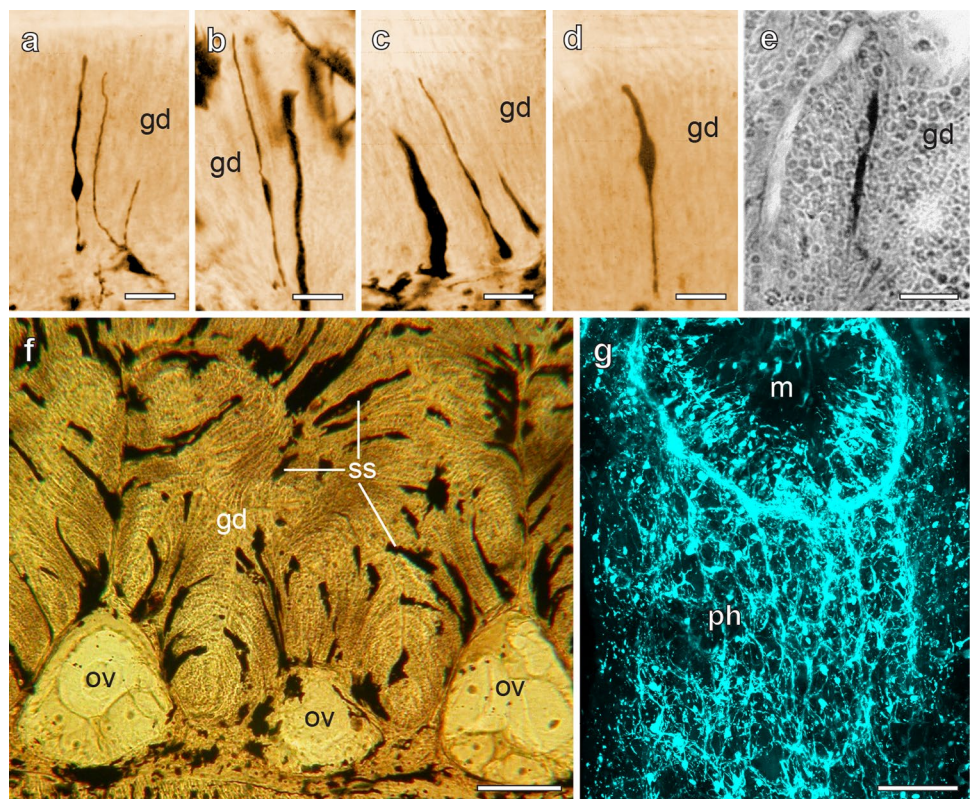
Several plexuses (subepidermal, commissural, stomato-gastric, proboscidal, etc.) have previously been identified in the PNS of heteronemerteans (Beckers et al. 2011; Beckers 2015). The results presented here and in our previous studies on several species on nemerteans (Markosova et al. 2007; Zaitseva et al. 2007; Zaitseva and Petrov 2013) have shown that these plexuses consist primarily of branching axons of numerous sensory cells located in the epidermis, digestive tract and proboscis that gradually converge into bundles and eventually project into the CNS. These plexuses also contain a few neuronal somata. Judging from the arrangement of the neurites arising from these somata, it can be presumed that together with axons of the CNS neurons, they participate in the efferent innervation of the musculature, epithelia of the body, proboscis, and intestine, and glandular structures (Markosova et al. 2007; Zaitseva et al. 2007; Zaitseva and Petrov 2013). It also cannot be excluded that some of these cells are proprioceptors.

In the nemertean proboscis, the sensory cells have first been identified by Bürger (1891) using supravital methylene blue staining. The innervation of the proboscis, its retractor muscle and the wall of the rhynchocoel by CA-C nerve elements and the presence of CA-ergic sensory cells have later been demonstrated in some nemertean species including *L. ruber* (Zaitseva and Petrov 2013). FaRP-IR sensory cells in the proboscis are described in the present study for the first time, although Fig. 9c, e in Beckers et al. (2013) shows similar FaRP-IR cell somata in the proboscis of *Procephalothrix filiformis* (Johnston, 1828).

The present study has revealed an abundance of sensory cells in the enteric epithelium of *L. ruber* and our earlier studies (Punin et al. 2003; Markosova et al. 2007; Zaitseva and Petrov 2013) have shown that this is also true of some other nemertean species. CA-C cells are distinguished from other enteric nerve elements by being evenly distributed throughout the enteric epithelium. These cells are morphologically similar to CA-C sensory cells located in the epithelium of the head region, body, and proboscis of *L. ruber* and have similar rigid, bristle-like cilia fluorescent in the blue–green range. The highest density of CA-C sensory endings in the epidermis of *L. ruber* was observed on the frontal and lateral surfaces of the head, including lateral slits, and around the oral opening. Our results suggests that epidermal CA-C cells are tactile mechanoreceptors that react to touch or water vibration allowing nemerteans to orient themselves in space and receive information on location, activity and size of prey, while enteric CA-C cells are mechanoreceptors that participate in the control of digestive peristalsis and movement of food through the intestine. The possible role of tactile mechanoreceptors is clearly seen in the feeding behavior of *L. ruber*. If an individual of *L. ruber* is touched by a piece of a polychaete or a mussel, the nemertean stretches its mouth to a size that allows it to swallow the food. If the food is too large, the nemertean crawls away leaving the food behind (O.V. Zaitseva and S.A. Petrov, unpublished observations).

CA-C sensory cells, similar to those of nemerteans, have been described previously in the digestive tract and the epithelia of the body and mantle in several species of gastropods (Zaitseva et al. 2019). Similar monociliated CA-C sensory cells were shown to be abundant in the entire epidermis, head and palps of polychaetes (Schlawny et al. 1991) and in the epidermis of the polypide and on the epistome and tentacles of bryozoans (Shunkina et al. 2015; Shunkina and Zaitseva 2017). These cells in lophotrochozoans are likely to act as mechanoreceptors, as they are abundantly present in the enteric epithelium and in the portions of the epidermis that are not specialized for chemoreception and are concentrated in places where increased mechanical sensitivity is to be expected. In contrast, at the places where mostly olfactory cells are concentrated in lophotrochozoans, for instance, at the tips of the tentacular cerebral organs of land snails and slugs, in the rhinophores of nudibranchs (Zaitseva 1994, 1999, 2016; Zaitseva et al. 2019) and in the nuchal organs of polychaetes (Schlawny et al. 1991 and our unpublished observations), CA-C sensory cells appear to be absent. The possibility of the mechanosensory function of CA-C cells is further strengthened by the presence in these cells of stiff, straight cilia, which are typical for mechanoreceptor cells in both vertebrate and invertebrate animals (Manley and Ladher 2008). In the CA-C sensory cells of polychaetes, the cilia are encircled by microvilli (Schlawny et al. 1991), which is a characteristic feature of mechanoreceptive cells in several animal groups. Ciliated chemoreceptor cells, by contrast, normally have mobile cilia and after chemical fixation often lie almost flat on the surface of the epithelium (Zaiteva and Bocharova 1981; Vinnikov 1982; Zaitseva 1994).

The frontal organs of nemerteans are usually described as epithelial sensory pits located near the rhynchostome. The results of the present study, however, have shown that the frontal organs do not have a distinct boundary and cannot be readily identified as proper organs. The somata of CA-C sensory cells that form sensory endings in the epithelial pits of the frontal organs are located far from these pits lying adjacent to the somata of other ciliated CA-C sensory cells that innervate the head region outside the frontal organs. The sensory cells of the frontal organs are situated bilaterally in two compact groups, each of which contains the same number of somata (Fig. 7a). In addition to CA-C cells, at the base of the pit of the frontal organ, there is a concentration of terminal branches of 5-HT-IR neurites (Fig. 7a) that run to the frontal organ within separate nerve bundles arising from the dorsal cerebral ganglia. We were unable to trace these processes to any specific neurons in the cerebral ganglia. A similar 5-HT-IR innervation of the frontal organs has also been described in the hoplonemerteans *Quasitetrastemma*
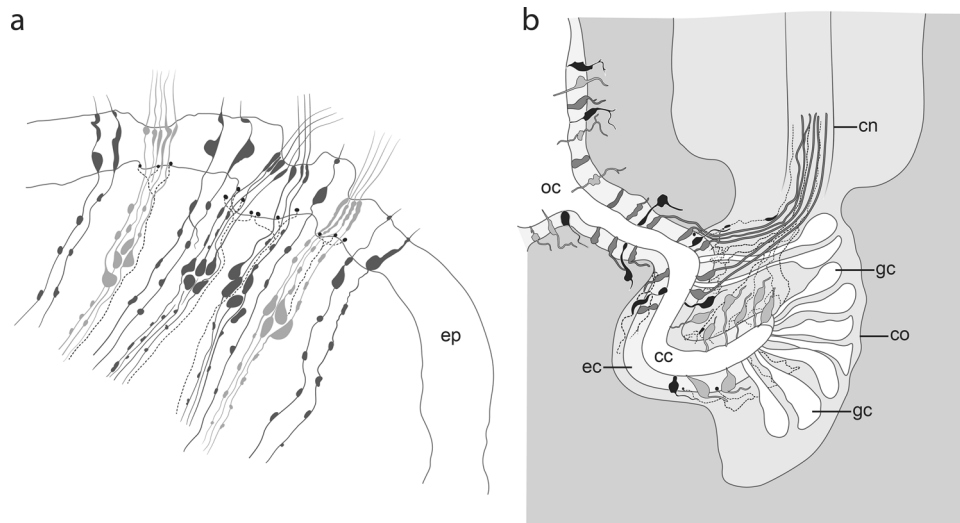
Fig. 7 Schematic drawings of the frontal and cerebral organs and associated sensory cells in *Lineus ruber*. **a** Sensory cells and their endings in the epithelial pit of the frontal organs: 5-HT-IR neurites terminating at the base of the epidermis in the medial and lateral frontal organs (dashed lines), CA-C cells located in the medial frontal organ and outside it in the head region (dark gray shading), and CA-C cells innervating the lateral frontal organs (light gray shading). **b** Sensory and gland cells in the cerebral organ: FaRP-IR cells and their dendrites and axons (dark gray shading), CA-C cells (light gray

shading), sensory cells revealed only by silver impregnation (black shading), and 5-HT-IR neurites (dashed lines). The location of the gland cells in the drawing is derived from immuno- and histochemical preparations, in which they are seen as unstained dark areas. Cilia of the CA-C sensory cells inside the cerebral canal are not shown, because they are usually concealed by the glandular secretion present inside the canal. *cc* cerebral canal, *cn* cerebral organ nerve, *co* cerebral organ, *ec* epithelium of cerebral canal, *ep* epidermis, *gc* gland cells, *oc* opening of cerebral canal

stimpsoni Chernyshev, 1992 and *Tetrastemma phaeobasisae* Kulikova, 1987 (see Chernyshev 2011; Magarlamov et al. 2020). Some cells at the base of the epithelium of the frontal organs in another *Lineus* species, *L. sanguineus* (Rathke, 1799), have been reported to also have high acetylcholinesterase activity (Reutter 1972). Serotonin and acetylcholine are likely to control the musculature that raises the bottom of the ciliated pit, when the frontal organ is in the active sensing state. The involvement of acetylcholine in contraction of smooth musculature is well known in both vertebrate and invertebrate animals and in nemerteans some indirect evidence of serotonin involvement in muscle contraction is provided by the innervation of fixator muscles in the head and wall of the rhynchodeum with 5-HT-IR neurites.

CA-C sensory cells of the frontal organs have long cilia, several times as long as those of the other CA-C sensory cells in the head region, but in both cases, the cilia are straight and rigid. We are inclined to view these cells, like other CA-C sensory cells, as mechanoreceptors. These putative mechanoreceptive cells are located very close to the rhynchostome, which is the opening through which the proboscis is everted during prey capture. It is likely that these sensory cells are needed to adjust the direction before thrusting the proboscis into the prey. It does not negate the possibility that some chemoreceptive cells of different neurotransmitter modality can also be associated with the epithelium of the frontal organs, but this remains to be

confirmed. The presence of long cilia associated with the frontal organs have previously been noted in nemerteans and these cilia were shown to retract when touched (Reisinger 1926; Gibson 1972; Riser 1993). Ultrastructural studies of the anterior portion of the head region in *L. ruber* have revealed two types of ciliated sensory cell with one or several cilia, although they were not localized specifically in the frontal organ (Storch and Moritz 1971). Most sensory cells had well-developed ciliary rootlets, which is generally typical of mechanosensory cells.

The nemerteans usually possess an odd number of sensory pits of the frontal organs (either 1 or 3), which is somewhat unusual given the fact that organs of spatial orientation in the head region of bilaterians are mostly paired. *Lineus ruber* and other lineids have three epithelial pits, but, judging from the number of separate groups of sensory cells and double efferent innervation of the medial frontal organ by bilaterally symmetrical nerves from both dorsal cerebral ganglia, it is likely that the lineids in fact possess two pairs of frontal organs, but the medial pair has a combined sensory field. The medial sensory pit in *L. ruber* is indeed somewhat larger than those of the lateral frontal organs. It might be expected that the same pattern of innervation is present in the other nemerteans with an odd number of sensory pits.

Previous electron microscopic, histological and histochemical studies have shown that cerebral organs of nemerteans contain specialized glandular cells and presumably

neuroendocrine cells as well as a significant number of ciliated bipolar cells that form sensory endings at the surface of the cerebral canal (Ling 1969, 1970; Ferraris 1985; Amerongen and Chia 1987). Ling (1969) has identified two types of ciliated sensory cells in the cerebral organs of *L. ruber* and presumed that they can respond to different types of sensory input: either chemical or mechanical stimuli. In the present study, numerous intra- and subepithelial sensory cells have been described around the canal of the cerebral organ in *L. ruber* using the silver impregnation method. A small portion of these cells located deep in the canal were shown to contain CAs and those positioned closer to the distal opening of the canal were FaRP-IR (Fig. 7b). The presence of two populations of sensory cells with different modality in the cerebral organs of *L. ruber* adds further support to Ling's hypothesis. FaRP-IR neurites and somata were also noted in the cerebral organs of lineids by Beckers et al. (2011) and Beckers (2015). In the present study, 5-HT-IR cell bodies have not been found in the cerebral organs, but 5-HT innervation was shown for the entire length of the cerebral canal. It is possible that in the cerebral organs of nemerteans, as in other Lophotrochozoa, serotonin participates in regulation of the ciliary apparatus and controls feeding behavior (see review in Gillette 2006). There is evidence that when potential prey is in close proximity, the cilia around the opening of the cerebral organs change their beating so that water is actively drawn into the organ (Reisinger 1926). A complex, regulated movement of water in the cerebral canals has also been observed by Amerongen and Chia (1987).

The shape of the epithelial canal of the cerebral organs may prove to have crucial importance in determining the function of these sensory organs. In some heteronemertean species, including *L. ruber,* this canal is bent in three different planes (Ferraris 1985), which is likely to impede the access of odorants to chemosensory cells located deep in the canal and may impair chemoreception. This raises the possibility that cerebral organs may have additional sensory functions other than chemoreception. Although almost all nemerteans, except the species of the genus *Ototyphlonemertes*, lack statocysts (Gibson 1972; Chernyshev 2011), the nemerteans are able to easily orient themselves in the gravitational field. The canal of the cerebral organ bears resemblance to the vestibular apparatus of vertebrates, which has morphologically similar canals arranged in three perpendicular planes (Smith 2008), and it is, therefore, possible that the cerebral organs may also function as a gravity-sensing system by detecting differential pressure on cilia of sensory cells in variously oriented segments of the cerebral canal.

The results of the present study revealed a diverse and rich system of intra- and subepithelial sensory cells associated with the body wall, digestive system, proboscis and specialized sensory organs of the head region in *L. ruber*. The complexity of these sensory structures in nemerteans is easily understandable in the context of the elaborate behavior of these animals associated with the predatory lifestyle. The cerebral organs may play a key role in detecting and tracking prey using both chemo- and mechanosensory input and the mechanoreceptors of the frontal organs may assist in aiming the proboscis toward the prey. The frontal organs are not clearly delineated anatomically and their sensory cells appear to form a common sensory unit with other sub- and intraepithelial sensory cells located along the anterior margin of the head region. It is reasonable to assume that the information coming from all these cells is summed up to improve the animal's ability to orient itself in space and catch prey. It is also likely that cerebral organs can receive polymodal information and their complex modality must be taken into account in designing future behavioral and physiological experiments.

Compliance with ethical standards

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