Zoomorphology (2015) 134:1–24
DOI 10.1007/s00435-014-0246-3

ORIGINAL PAPER

The nervous systems of Pilidiophora (Nemertea)

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Received: 4 April 2014 / Revised: 25 September 2014 / Accepted: 29 September 2014 / Published online: 31 October 2014

Abstract Today molecular data recover three higher taxa in nemerteans, Palaeonemertea, Pilidiophora and Hoplonemertea. *Hubrechtella dubia*, a former palaeonemertean taxon was excluded from palaeonemerteans and is now supported as the sister group to heteronemerteans. This taxon is called Pilidiophora, because *H. dubia* and Heteronemertea share a pilidium larva. To find additional morphological evidence for Pilidiophora, the nervous system of 13 species of this taxon were investigated using different histological techniques. The central or medullated nervous system of nemerteans consists of a ring-shaped brain and lateral branching medullary cords. While the brain of basally branching nemerteans shows no or only slightly posterior enlargement, the brain of Pilidiophora expands caudally. Pilidiophora possess a conspicuous sensory organ, the cerebral organ. Although this organ is also found in hoplonemerteans and certain palaeonemertean species, only in Pilidiophora this organ is directly connected to the dorsal lobe of the brain. Additionally, this organ terminates in a layer of neurons close to the blood vessel in all pilidiophoran species. The analysis indicates that although in *H. dubia* some characters of the nervous system show the plesiomorphic state, the morphology of the cerebral organ provides an apomorphic character for a taxon Pilidiophora.

Communicated by Andreas Schmidt-Rhaesa.

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Keywords Lophotrochozoa · Spiralia · Neuroanatomy · Heteronemertea · Pilidiophora · *Hubrechtella dubia* · 3D reconstruction

Introduction

In the last decade, the nervous systems of trochozoan taxa such as annelids and molluscs have intensely been studied using immunohistochemistry in order to reconstruct the phylogenetic relationships among these taxa (e.g., Heuer and Loesel 2008; Faller and Loesel 2008; Rothe and Schmidt-Rhaesa 2008; Wollesen et al. 2008; Heuer and Loesel 2009; Richter et al. 2010). For nemerteans, immunohistological data of the nervous system are still rare and restricted to a single heteronemertean and several palaeonemertean species Beckers et al. (2011, 2013). Palaeonemerteans are basally branching, while the remaining taxa, Hetero-, Hoplo- and Bdellonemertea show derived characters (Bürger 1895; Gibson 1972; Andrade et al. 2012, 2014; Kvist et al. 2014).

The central (or medullated) nervous system of nemerteans basically consists of a ring-shaped brain surrounding the proboscis and two laterally situated medullary cords that originate in the ventral part of the brain and are interconnected caudally (Bürger 1895; Gibson 1972). In few palaeonemertean species as well as in all heteronemerteans and some hoplonemerteans, the brain ring is posteriorly enlarged and forms a dorsal and a ventral pair of lobes (Bürger 1895; Gibson 1972; Beckers et al. 2011, 2013).

The central nervous system comprises a central neuropil, that is composed of the neurites of the neurons. The somata of the neurons surround the neuropil, which is regarded as typical for spiralians (Bullock and Horridge

1965; Schmidt-Rhaesa 2007). Several minor nerves, such as a dorsal nerve, proboscidial nerves, esophageal nerves and nerve plexus in different parts of the body, may be present (Bürger 1895; Gibson 1972; Turbeville 1991; Beckers et al. 2011, 2013; Beckers 2012). Nemerteans possess several sensory organs at the tip of their head. Photoreception is perceived by pigmented eyes of which up to hundred may be present in one animal (Gibson 1972). While frontal organs probably have a tactile function (Gibson 1972), cerebral organs most likely are chemoreceptors (Scharrer 1941; Ling 1969, 1970; Ferraris 1985; Amerongen and Chia 1982). The former comprise one dorsal and two lateral canals that are open to the exterior via small pits. The cerebral organs of heteronemertean species are attached to the dorsal lobe of the brain and gain contact to the environment by ducts, which are lined with sensory cells (Bürger 1895; Gibson 1972; Scharrer 1941; Beckers et al. 2011; Beckers 2012).

Some recent molecular studies do not support the monophyly of palaeonemerteans (Thollesson and Norenburg 2003; but see also Andrade et al. 2012). Most palaeonemertean groups actually form a basally branching grade, while the former palaeonemertean taxon Hubrechtidae is most closely related to Heteronemertea. Since species of both, Hubrechtidae and Heteronemertea, possess a pilidium larvae, Thollesson and Norenburg (2003) introduced the name Pilidiophora, indicating that a pilidium evolved in the common stem lineage of Heteronemertea and Hubrechtidae (Thollesson and Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014). Molecular data, however, do not unequivocally provide support for Pilidiophora. But the taxon Pilidiophora was also supported in a recent morphological analysis by the arrangement of the musculature of the proboscis (Chernyshev et al. 2013). In order to find additional morphological support for this taxon, the neuroanatomy of twelve heteronemertean species and *Hubrechtella dubia* was compared to palaeonemertean nervous systems that were recently studied in more detail (Beckers et al. 2013). To facilitate comparisons with previous studies, the same set of markers, terms and methods used in Beckers et al. 2013 were also applied here. Additionally, transmission electron microscopy was used to further characterize neurons, neuropil and glia. Classical Azan staining of histological sections was used to generate 3D reconstructions of the nervous systems.

Materials and methods

Animals

Lineus acutifrons Southern, 1913 (Fig. 1a) was collected from a sandy flat at Pouldohan (Britany, France). *Micrura purpurea* Dalyell, 1853 (Fig. 1b), *Riseriellus occultus* Rogers, Junoy, Gibson and Thorpe, 1993 (Fig. 1c), *Ramphogordius sanguineus* Rathke, 1799 (Fig. 1d), *Lineus longissimus* Gunnerus, 1770 (Fig. 1e) and *Ramphogordius lacteus* Rathke, 1843 (Fig. 1f) were found on the rocky shore of Le Cabellou close to Concarneau (Britany, France). *Lineus ruber* Müller, 1774 (Fig. 1g) and *Lineus bilineatus,* (Renier 1804) (Fig. 1h) were collected in Roscoff (Britany, France). *Lineus viridis* (Müller 1774) was collected at the Ile de Groix, Britany, France. All animals were collected in March 2011. *Notospermus geniculatus* (Delle Chiaje 1828) (Fig. 1i) and *Cerebratulus fuscus* Renier, 1804 (Fig. 1j) were sampled on the isle of Giglio/Italy in May 2013 in *Poseidonia* sea grass meadows by diving. *Hubrechtella dubia* Bergendal, 1902, was collected in the Gullmarsfjord, Sweden, in 2009. The structure of nervous system in *Apatronemertes albimaculosa* Wilfert and Gibson 1974 was recorded from the type series. *Baseodiscus hemprichii* (Ehrenberg 1831) was collected in Van Phong Bay/Vietnam in May 2005.

Immunohistochemistry and histology

Animals for immunohistochemical investigations were relaxed in a 7 % $MgCl_2$ solution and fixed afterward in 4 % paraformaldehyde in seawater. Animals were embedded into gelatine–albumen and cut into sections of 60 µm thickness. Afterward, animals were stained with the antibodies FMRF-amide (ImmunoStar, Hudson, WI, USA), Serotonin (Sigma-Aldrich, Saint Louis, MO, USA), conjugated α-Tubulin (Sigma-Aldrich, Saint Louis, MO, USA), Synapsin (Hybridoma Bank, IA, USA) and for nucleus staining Sytox (Invitrogen, Carlsbad, CA, USA). For a detailed description, see Beckers et al. (2013).

For histological studies, the animals were relaxed in a 7 % $MgCl_2$ solution and fixed afterward in Bouin's fluid (modified after Dubosq-Basil). Animals were dehydrated in an ascending ethanol series and embedded in Paraplast. The specimen were sectioned into slices of 5 µm thickness and stained with Azan. For a detailed description, see Beckers et al. (2013).

Ultrastructure

The head of *Lineus ruber* was fixed in a 2.5 % glutaraldehyde solution, buffered in 0.05 M phosphate 0.3 M saline (pH 7.2) at 4 °C for 2 h and kept in the same buffer. The specimen were postfixed in 1 % OsO_4 buffered in 0.05 M phosphate 0.3 M saline at 4 °C for 1 h, immediately afterward dehydrated in an ascending acetone series followed by propylenoxide and embedded in Araldit.

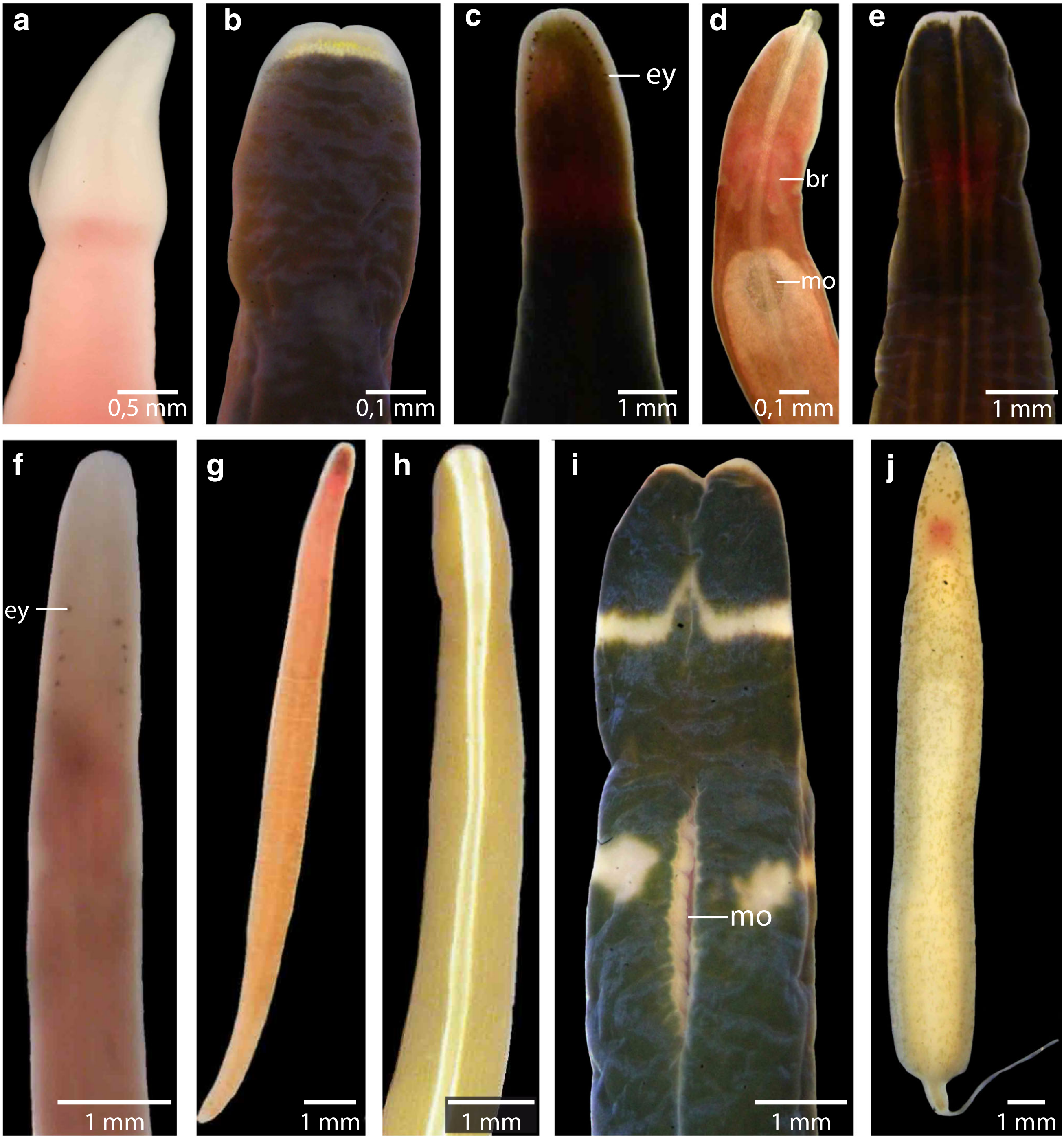


Fig. 1 Living specimen of **a** *Lineus acutifrons*. **b** *Micrura purpurea*. **c** *Riseriellus occultus*. **d** *Ramphogordius sanguineus* (ventral view). *br* brain, *mo* mouth opening. **e** *Lineus longissimus*. **f** *Ramphogordius lacteus*. *br* brain, *ey* eye. **g** *Lineus ruber*. **h** *Lineus bilineatus*. **i** *Notospermus geniculatus* (ventral view). *mo* Mouth opening. **j** *Cerebratulus fuscus*

Semithin section and ultrathin sections of 1 µm and 70 nm thickness, respectively, were cut on a LEICA UC6. Semithin sections were kept on glass slides and stained with Toluidine blue, while ultrathin sections were stained with uranyl acetate and lead citrate.

Analysis and 3D reconstruction

Azan-stained slices were analyzed with an Olympus microscope (BX-51). Every section was photographed with an Olympus camera (Olympus cc12) equipped with the dot slide system (2.2 Olympus, Hamburg) and aligned using imod (Kremer et al. 1996) and imod align (http://www.q-terra.de/biowelt/3drekon/guides/imod_first_aid.pdf). 3D reconstructions were performed with Fiji (1.45b) (Schindelin et al. 2012)/trakem (Cardona et al. 2012) and Amira (4.0) (see Fig. 3a). The schematic drawings were performed according to the 3D reconstructions with Illustrator and Photoshop (CS6).

Immunochemical investigations were performed with a Leica confocal laser scanning (Leica TCS SPE) microscope using the LAS AF 1.6.1 software. Stacks were loaded into Fiji (1.45b) (Schindelin et al. 2012) and further processed, using the "maximum projection" tool of the Image j software (Schneider et al. 2012). The images were finally processed with Fiji (1.45b) (Schindelin et al. 2012) and Photoshop (CS6). Ultrastructure sections were photographed with an Zeiss Lyra electron microscope using the analysis software and further processed using Fiji (1.45b) (Schindelin et al. 2012). Adobe Illustrator (CS6) was used to prepare all figures.

Statistical analysis

Neuron diameters were measured with Cell*D (3.2 Olympus) and statistically analyzed using the SPSS 15.0 statistical package. Data of each species and each class of neurons were tested for normal distribution with Shapiro–Wilk test. Since the data were normally distributed, parametric statistics (ANOVA) were used to test whether or not the size classes of each species differ significantly.

Data repository and voucher material

To allow data transparency, the aligned Azan-stained serial sections used for 3D modeling are freely available in MorphDBase: www.morphdbase.de (Grobe and Vogt 2008, 2014). Conspecific individuals of species studied (except *A. albimaculosa*, *H. dubia* and *B. hemprichii*) were collected at the same sampling site and deposited at the Institute of Evolutionary Biology and Zooecology of the University of Bonn as voucher material.

Hyperlinks

Lineus acutifrons: [https://www.morphdbase.de?P_Beckers_20140401-M-21.1]
Micrura purpurea: [https://www.morphdbase.de?P_Beckers_20140401-S-13.1]
Riseriellus occultus: [https://www.morphdbase.de?P_Beckers_20140401-S-7.1]
Ramphogordius sanguineus: [https://www.morphdbase.de?P_Beckers_20140401-S-8.1]
Lineus longissimus: [https://www.morphdbase.de?P_Beckers_20140401-S-6.1]
Ramphogordius lacteus: [https://www.morphdbase.de?P_Beckers_20140401-S-12.1]
Lineus ruber Müller: [https://www.morphdbase.de?P_Beckers_20140401-S-9.1]
Lineus bilineatus: [https://www.morphdbase.de?P_Beckers_20140401-S-4.1]
Notospermus geniculatus: [https://www.morphdbase.de?P_Beckers_20140401-S-14.1]
Cerebratulus fuscus: [https://www.morphdbase.de?P_Beckers_20140401-S-10.1]
Hubrechtella dubia: [https://www.morphdbase.de?P_Beckers_20140401-S-5.1]
Apatronemertes albimaculosa: [https://www.morphdbase.de?P_Beckers_20140401-S-11]
Baseodiscus hemprichii: [www.morphdbase.de/?P_Beckers_20140923-M-49.1]

Results

General remarks

The description of the morphological elements of the nemertean nervous system follows the definitions given by Beckers et al. (2013). All aligned slices of investigated specimen are freely available on Morph D Base (https://www.morphdbase.de). The respective hyperlinks are mentioned above. An overview of the elements present in the nervous system of Pilidiophora is given in Appendix Table 1.

The entire medullated nervous system may be surrounded by a prominent extracellular matrix (*ecm*) (outer neurilemma) (Fig. 2c). If the medullated nervous system or parts of it are located directly underneath the basal lamina of the epidermis, the outer neurilemma can basically not be discriminated from the latter. In these cases, the term outer neurilemma will only be used, if an *ecm* separates the outer face of the nervous system from the adjacent musculature, mesodermal tissue or blood vessel linings, in a way that the entire nervous system is surrounded by matrix. The brain of nemerteans consists of a central neuropil and peripheral layer of perikarya, called outer somata layer or outer layer of neuronal somata here. Both, central neuropil and peripheral layer of perikarya may be separated by an *ecm*, traditionally termed inner neurilemma (Figs. 2c, 5a, 6a, d).

Architecture of the nervous system

Heteronemertea

The central (medullated) nervous system (*cns*) (brain and lateral medullary cords) is located inside the musculature

(Figs. 2, 3b–e, 4). The central neuropil is partly or completely surrounded by a layer of perikarya (Figs. 3c, 5a, 6, 7f). All lineid species except *Apatronemerts albimaculosa* possess long lateral cephalic slits extending from the tip of the animal to the brain region. Posteriorly, these slits terminate in a duct that passes the subepidermal muscle layer and enters the posterior section of the brain. This duct is lined by ciliated sensory cells and known as cerebral organ (Gibson 1972). In *A. albimaculosa,* the cephalic slits are extremely short and known as cephalic pits (Gibson 1972).

Brain

On each body side, the anterior part of the brain consists of two laterally bulging neuropils that are commissurized by a dorsal and a more prominent ventral commissural tract (Figs. 2, 3a, c, 5a, 7a). The commissures are on the same transverse level, so that the brain is ring-shaped in all species studied (Figs. 2a, c, d, 5a), except for *Lineus acutifrons* where the dorsal commissure is located posterior to the ventral commissure (Fig. 2b) and *Apatronemertes albimaculosa* where the dorsal commissure is anterior to the ventral one. Posterior to the dorsal and ventral commissures, the brain is separated into a left and right section. Here, the neuropil shows infoldings and furrows as well as lateral tracts that connect the dorsal and ventral portions of the neuropil. On either side of the brain, the dorsal portion is located more laterally and partly overlaps the ventral portion (Figs. 3c, 5b, 7f). Further posteriorly, the neuropils of ventral and dorsal portions of the brain are clearly separated by an own inner neurilemma but are initially still surrounded by a common outer neurilemma (Fig. 4d). Here, the duct of the cerebral organ originates in the cephalic slits (Fig. 4d). Where it turns medially to pass the muscle layers, the neuropil and neurons of both brain parts are also separated by an outer neurilemma to form the paired dorsal and ventral lobes of the brain. In *Lineus bilineatus,* neurites of the dorsal lobe branch off from the neuropil, pass the inner neurilemma and prominently extend into the dorsal section of the outer layer of perikarya (Fig. 5b). In all lineid species studied, the ventral lobes taper caudally and are confluent with the lateral medullary cords (Figs. 2, 3a, 4f), while the dorsal lobes bifurcate to form two branches, of which the superior one is smaller than the inferior one (Figs. 2, 3a, d). Initially, both dorsal lobes share a common outer neurilemma (Fig. 3d). Further posteriorly, the two branches are divided by their own outer neurilemma. The cerebral organ is now located between the dorsal and the ventral lobe of the brain next to the inferior neuropil of the dorsal lobe. An inner neurilemma initially separates both, cerebral duct and inferior neuropil, but disappears some micrometers posteriorly (Figs. 2, 8a). Here, neurons of the inferior neuropil are apposed to those of the cerebral organ. On the level of the posterior most margin of the superior branch, the neurons of cerebral organ fuse with the neuropil of the inferior branch (Fig. 8a). This fusion also marks the posterior margin of the inferior branch neuropil. The cerebral organ turns slightly laterally and a large group of presumably neurosecretory cells is now visible in the ventromedial section of the inferior branch (Figs. 3e, 8a, b). The outer neurilemma is now surrounded by muscles and the lining of the cerebral blood vessels. The cerebral organ ends within a mass of putative neurosecretory cells that mark the posterior margin of the inferior branch of the dorsal lobe. Lining cells of the blood vessels rest on the outer neurilemma.

In summary, the brain of lineids is anteriorly ring-shaped, splits into a left and a right section, differentiates a dorsal and a ventral lobe and finally, at its posterior margin, consists of six portions, isolated from each other by an outer neurilemma (Figs. 2, 3a, 8a). These six are a pair of ventral lobes and paired inferior and superior branches of the dorsal lobe. The ventral lobe is continuous with the medullary cords; the inferior branch of the dorsal lobe merges with the terminal section of the cerebral organ. The size of the brain as well as the volume of its lobes differ between the specimen studied, but their general anatomy is largely uniform (Figs. 2, 3a).

The brain of *Apatronemertes albimaculosa* differs slightly from the remaining heteronemerteans. Each dorsal lobe gives rise to a neuropil branch that extends anteriorly and blindly ends within the neuronal layer of the anterior section of the brain. In addition, the dorsal lobe neuropil is crescent-shaped prior to its separation into the inferior and superior branch neuropil (Fig. 4a). In contrast to the remaining lineids, the entire dorsal lobe extends into the cephalic blood vessels.

In *Baseodiscus hemprichii,* the dorsal commissure of the brain is only weakly developed and is located anterior to the ventral one. The neuropil of the dorsal lobe is only slightly bifurcated and the superior dorsal lobe terminates soon. The neuropil of the inferior branch, however, is again bifurcated into a dorsal and ventral part; the ventral part is associated with the cerebral organ (Fig. 8e).

Medullary cords

The paired ventrolateral medullary cords run posteriorly between the longitudinal muscle layer and the inner circular muscle layer. The neuropil of the medullary cords is capped by a c-shaped layer of nuclei of type 1 neurons, so that the nuclei are located dorsally, laterally and ventrally. An inner neurilemma separates the layer of nuclei and the neuropil (Fig. 4f); the medullary cords are surrounded by an outer neurilemma.

Minor nerves and nerve plexus

Numerous cephalic nerves originate from the lateral parts of the anterior face of the brain, taper toward the anterior end and are apposed to the head lacuna (Figs. 2, 3a, b, 7a). A dorsal nerve arises from the mid-posterior face of the dorsal commissural tract (Figs. 2, 3a, 4e, f). Posteriorly, the nerve ascends dorsally to run posteriorly outside the inner circular muscle layer. In *Lineus longissimus*, *Lineus acutifrons* and *Riseriellus occultus,* an additional dorso-median cephalic nerve originates on the same level from the anterior margin of the dorsal commissure where the dorsal nerve originates from its posterior margin. Whether or not the anterior nerve is an anterior branch of the dorsal nerve could not be seen in terms of the course of its neurites (Figs. 2a, b, d, 3a). In these three species, the dorsal nerve is connected to the medullary cords by a prominent commissure that overarches the dorsal part of the rhynchocoel (Figs. 2a, b, d, 4e, f). The paired esophageal nerves originate from the ventral lobes, posterior to their separation from the dorsal lobes (Figs. 2, 3a, d, 4f). These nerves run in parallel toward the anterior margin of the mouth where they are connected by a small commissure. The nerves encircle the mouth (pharynx) and continue posteriorly ventrolaterally on either side of the esophagus. Along their entire length alongside the foregut, the esophageal nerves give rise to posteriorly directed branches that surround the gut. In *R. occultus, R. sanguineus and R. lacteus,* where the brain is located far anterior to the mouth opening, the esophageal nerves are elongated accordingly. In these three species, both esophageal nerves are connected by several commissures prior to surrounding the mouth (pharynx) (Fig. 2a, b, d). In *L. ruber* and *L. longissimus,* the esophageal nerves are also connected to the lateral medullary cords in front of the mouth opening (Fig. 2c, d). A ventral nerve is not present. The paired proboscidial nerves arise from the dorso-median portion of the ventral commissural tract (Figs. 2, 3b). The nerves are interconnected by a proboscidial plexus.

Several minor nerves originate in the ventral and dorsal lobes of the brain that run toward epidermis in all investigated heteronemerteans (Fig. 3d). These nerves pass the longitudinal muscle layer and innervate the dorsal or the ventral parts of the animal. Nerves running toward the dorsal side of the animal may originate in the ventral part of the brain and vice versa. While some of these nerves directly run to the respective face of the animal, some of them are branches of a single nerve that originates from one of the brain lobes (Fig. 3d).

Several nerve plexus are revealed by immunohistochemistry in *Lineus ruber* and *Lineus bilineatus* (Fig. 7b). Staining with the antibodies of FMRF, serotonin and tubulin reveals that the neurites of the intraepidermal nerve plexus are arranged in a regular, ladder-like manner. The neurites of the subepidermal, commissural plexus and the suprastomatogastric nerve plexus are horizontally arranged. The neurites of the intrastomatogastric nerve plexus are arranged in an irregular, net-like manner. The suprastomatogastric and the intrastomatogastric as well as the subepidermal nerve plexus are interconnected (Fig. 7b). There is a spherical concentration of neurites in the subepidermal plexus at the points where the connections to the suprastomatogastric plexus branch off (Fig. 7b).

Fig. 2 Schematic drawings after 3D reconstruction of the nervous system of some heteronemerteans (not in scale). **a** *Riseriellus occultus*. **b** *Lineus acutifrons*. **c** *Lineus ruber*. **d** *Lineus longissimus*. *gray*: neuropil; *bright blue* neurons (only drawn on *left side*); *dark blue* frontal organ; *green* cerebral organ; *red* cephalic slits, ducts of the cerebral organ; *yellow* proboscis nerves. *cn* cephalic nerves, *co* cerebral organ, *csi* cephalic slits, *dcp* dorsal commissural plexus, *dct* dorsal commissural tract, *dl* dorsal lobe of brain, *dn* dorsal nerve, *du* duct of the cerebral organ, *en* esophageal nerves, *ey* eye, *fo* frontal organ, *idl* inferior dorsal lobe of brain, *mc* medullary cord, *pn* proboscis nerves, *sdl* superior dorsal lobe of brain, *vl* ventral lobe of brain ▶

Sensory structures

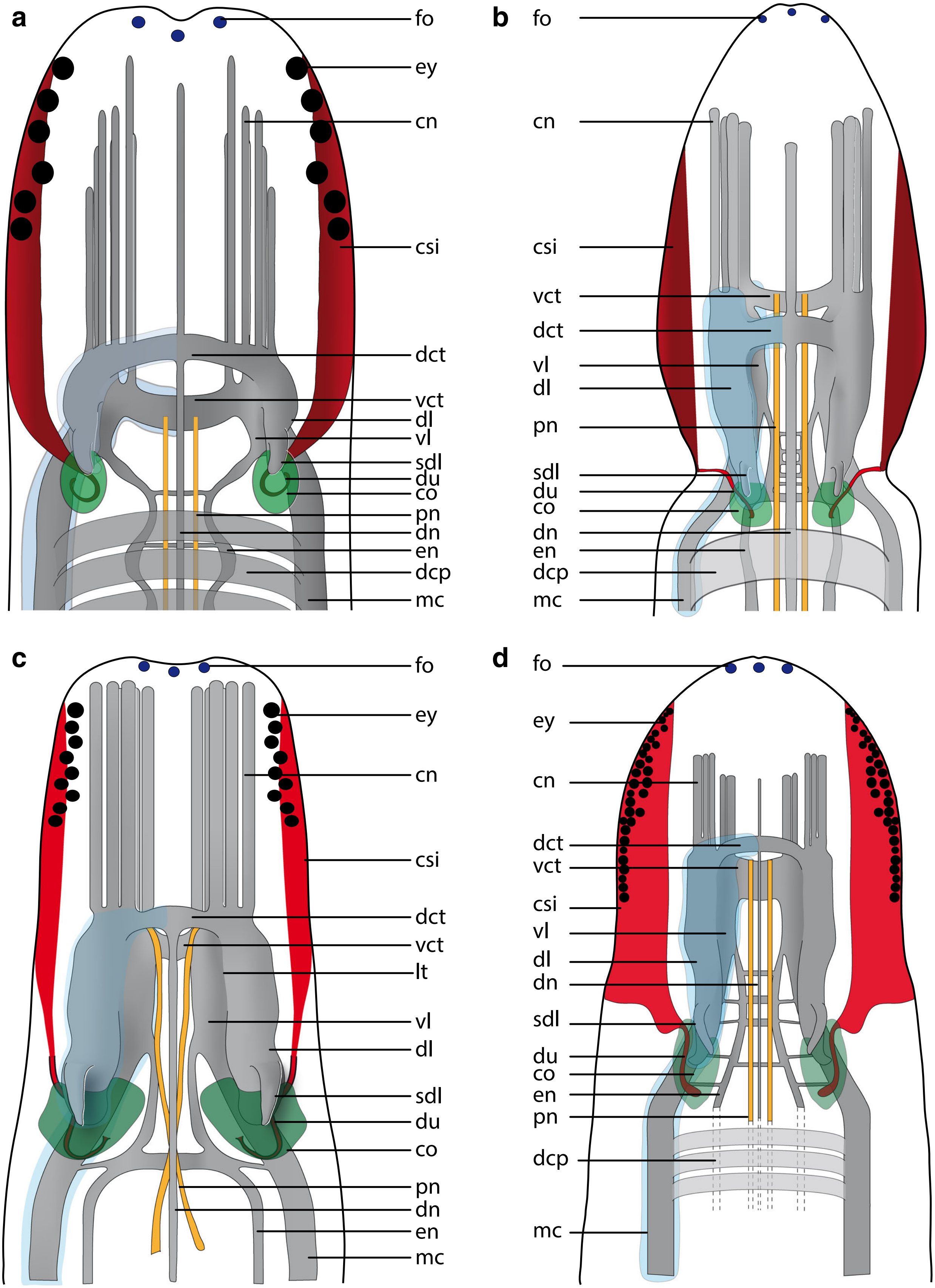
Frontal organ

The frontal organ of investigated lineid heteronemerteans is located at the very anterior tip of the animals. It consists of one dorsal and two lateral pits that are open to the exterior in front of the proboscis pore. Each pit tapers into a small, densely ciliated canal which runs underneath the epidermal basal lamina and terminates in a layer of neurons in front of the head lacuna (Fig. 8c). Neither the canals nor their lining cells are in contact; a connection of the lining cells to the brain was not found.

The frontal organ of *Baseodiscus hemprichii* is composed of one ventrally located pit at the tip of the head. This pit is located anterior to the proboscis pore and leads to a ciliated duct which terminates in a ciliated cavity. Posterior to the proboscis pore there are paired additional pits which lead into short ciliated canals.

Cerebral organ

The cerebral organ is an epidermal duct that originates at the posterior margin of the cephalic slits or the cephalic pits. This part of the cerebral organ will be called distal section of the cerebral organ in the following description. The duct pierces the basal lamina of the epidermis and runs posteriorly perpendicular to the longitudinal body axis toward the posterior part of the dorsal lobe of the brain (Figs. 2, 3a, 8a–d). This section of the cerebral organ will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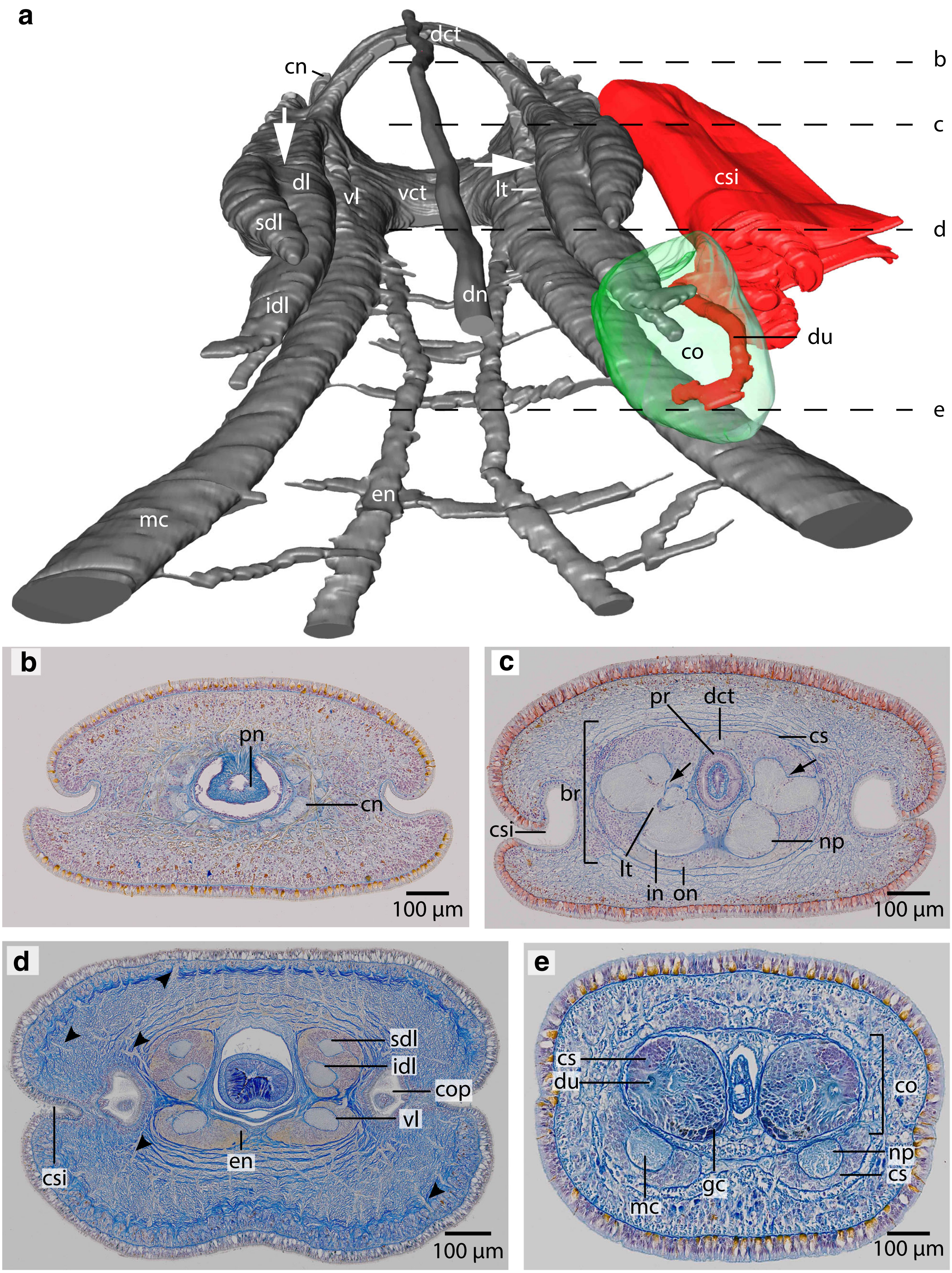
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◀**Fig. 3** **a** Snapshot of the 3D reconstruction of the nervous system of *Lineus longissimus*, dorsal view, **b–e** histological sections (5 µm), Azan staining, frontal (**b**) to caudal (**e**). **a** *cn* Cephalic nerves, *co* cerebral organ, *csi* cephalic slits, *dcp* dorsal commissural plexus, *dct* dorsal commissural tract, *dl* dorsal lobe of brain, *dn* dorsal nerve, *du* duct of the cerebral organ, *en* esophageal nerves, *idl* inferior dorsal lobe of brain, *mc* medullary cord, *sdl* superior dorsal lobe of brain, *vl* ventral lobe of brain. *Arrows* showing the furrows in the brain. *Note*: the cephalic slits and the cerebral organ were omitted on the left side. The proboscidial nerves and the neurons were also omitted. The letters on the right refer to the topological position of the slices shown below. **b** *Lineus ruber*. The cephalic nerves (*cn*) are laterally arranged around the rhynchocoel. Two proboscidial nerves (*pn*) are present. **c** *Micrura purpurea*. A lateral tract (*lt*) connects the dorsal and ventral part of the brain as well as a dorsal commissural tract (*dct*) connects the two dorsal lobes above the rhynchocoel. The brain shows in that part several furrows (*arrows*). The brain (*br*) is composed of an inner neuropil (*np*) and surrounding neuronal cell somata (*cs*). The neurons are separated by an inner neurilemma (*in*) to the neuropil. The whole brain is enclosed by an outer neurilemma (*on*). **d** *Lineus longissimus*. The cephalic slits (*csi*) lead into a cephalic organ pouch (*cop*). The brain is in that part divided into a ventral lobe (*vl*), a superior (*sdl*) and an inferior (*idl*) dorsal lobe on each side separated by an *ecm*. The esophageal nerves (*en*) originate in the ventral lobes of the brain. Several minor nerves (*arrowheads*) originate in the ventral lobe and lead into the epidermis. **e** *Ramphogordius sanguineus*. The duct (*du*) of the cerebral organ (*co*) runs through a huge layer of neurons (*cs*) and glandular cells (*gc*). The lateral medullary cords (*mc*) originate in the ventral or dorsal lobes of the brain

be termed proximal duct in the following description. The extension of the cephalic slits is species- or genus-specific, but most impressive in *Notospermus geniculatus*, where it causes a rectangular outline of transverse sections of the tip of the head (Fig. 4d). A distal, epidermal section of the duct can morphologically be discriminated from a subepidermal proximal section. A small pore connects the duct to the exterior. Underneath this pore, the distal duct widens to form a larger compartment with an intraepidermal dorsal and a ventral pouch (Figs. 3d, 8a, d). In *A. albimaculosa,* the duct directly leads to the cells of the cerebral organ; there is neither a compartment nor are there pouches.

In *Lineus ruber,* the distal section is densely lined with ciliated cells, most of them are sensory cells, since there is a direct connection to the inferior dorsal lobe, as revealed by immunostaining against alpha tubulin (Fig. 7c, d). The cilia of the part of the duct are longer than the cilia of the epidermis and can easily be discriminated from the latter (Figs. 4c, 8a). Toward the inner of the animal, the compartment tapers and gives rise to the proximal duct, which pierces the epidermal basal lamina and runs toward the brain. Within the inferior branch of the dorsal brain lobe, it runs posteriorly for several micrometers and is convoluted or laterally bending, depending on the species. Only in *Lineus acutifron*s this part of the proximal duct is straight (Fig. 2b).

The proximal duct terminates in a layer of neurons and voluminous glandular cells within the inferior branch of the dorsal lobe (Figs. 4d, 8a, 7c, d). The neurons and the glandular cells are in close contact to the blood vessels only separated from the latter by an *ecm* (Figs. 4e, 8b).

The neurons of the cerebral organ are of type 1 (*S1*) described for the brain (Fig. 8a, b) and show no immunoreactivity against FMRF in *Lineus ruber* (Fig. 7c, d). The cerebral organ of *N. geniculatus* is located ventrolateral of the dorsal lobe of the brain. It terminates close to the nephridial system.

The architecture of the cerebral organ of *Baseodiscus hemprichii* differs from that of the lineids. The proximal duct originates ventrolaterally from a pouch formed by a ventral epidermal fold (Fig. 8d). The course of the duct is straight dorsally (Fig. 8e), and first at its posterior end, it turns perpendicular toward the inside of the animal and terminates in a layer of neurons and glandular cells, close to the blood vessel. The glandular cell layer is not as developed as in lineids (Fig. 8e). The superior dorsal lobe is very short and does not rest on the cells of the cerebral organ (Fig. 8e). The neuropil of the inferior dorsal lobe is again bifurcated, and the ventral part is connected to the cells of the cerebral organ (Fig. 8e).

Eyes

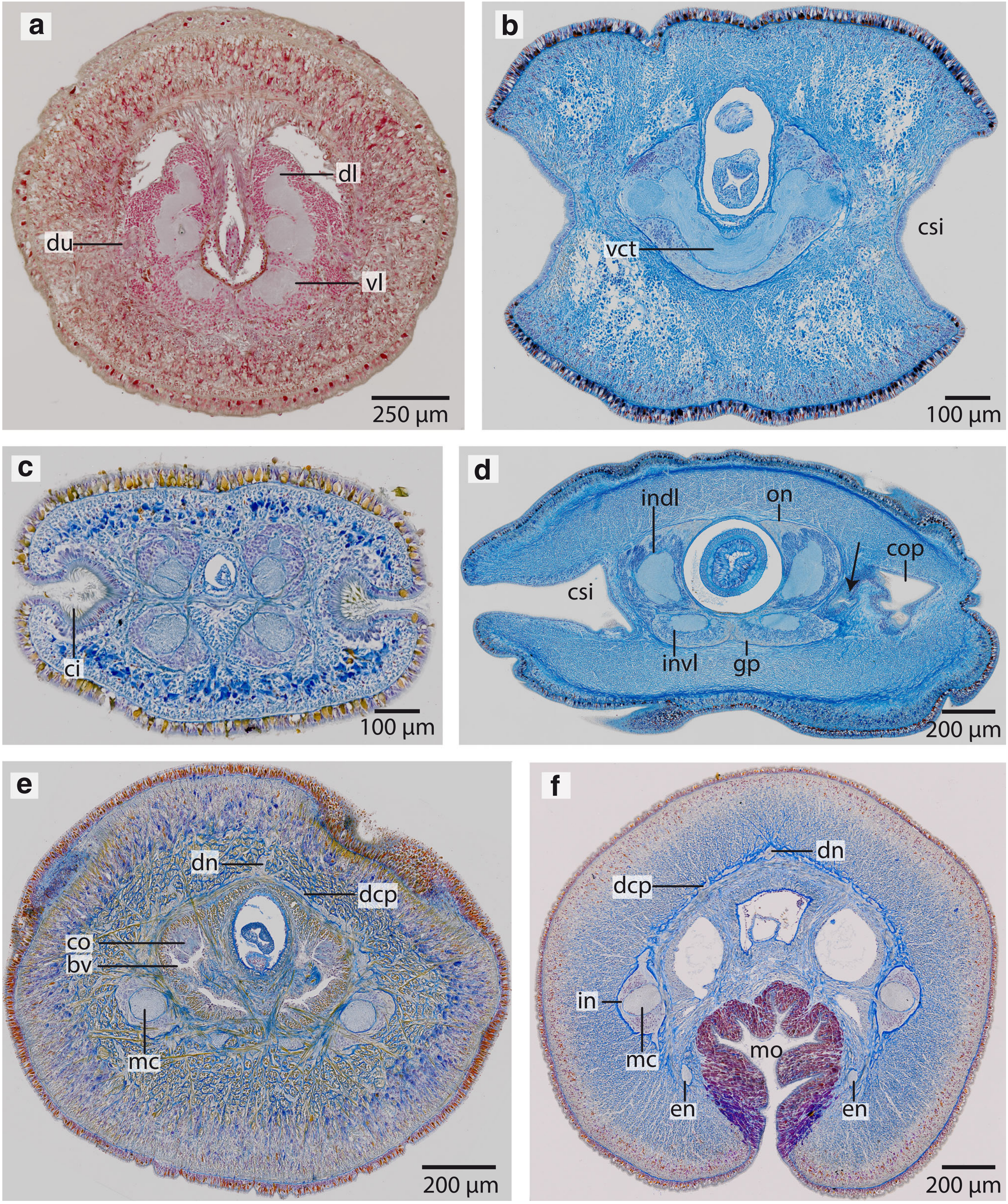
Numerous eyes are present in all investigated heteronemerteans except *L. acutifrons*. The pigment cup eyes are arranged on the margins of the head (Figs. 1f, 2). Prominent nervous connections between the eyes and the brain (optical nerves), as found in Hoplonemertea (pers. observation), were not detected.

Hubrechtella dubia (Hubrechtidae)

The central (medullated) nervous system (*cns*) *of H. dubia* is located directly below the epidermal basal lamina (subepidermally) (Fig. 9). An outer neurilemma as defined above is not visible (Fig. 9b).

Brain

The anterior most part of the brain consists of two crescent neuropils which are located dorsolateral of the rhynchocoel and which are connected by a dorsal commissure (Fig. 9a–c). The ventral commissure is located posteriorly and connects the ventral parts of the brain (Fig. 9c). Posterior to the ventral commissure, the neuropil consists of a pair of dorsal and ventral lobes. On either side of the brain, both are connected by a lateral tract which is smaller in diameter than the lobes. The dorsal lobes are located more laterally than the ventral ones; two additional dorsal commissural tracts connect the dorsal lobes of the brain neuropil (Fig. 9a). More posteriorly, where the cerebral



organ pierces the basal lamina of the epidermis, the lateral tracts are absent, so that the brain neuropil consist of four solitary lobes. On either side of the brain, however, dorsal and ventral lobes are still surrounded by a common layer of somata. The dorsal lobes are very short (Fig. 9a), turn inward, taper and are connected to the cells of the cerebral

◀**Fig. 4** Histological sections. (5 µm), Azan staining, frontal (**a**) to caudal (**f**). **a** *Apatronemertes albimaculosa*. The dorsal lobe (*dl*) of the brain extends into finger like protrusions. *du* duct of the cerebral organ, *vl* ventral lobe **b** *Cerebratulus fuscus*. The ventral parts of the brain are connected by a prominent ventral commissural tract (*vct*). **c** *Ramphogordius lacteus*. The cephalic slits are densely lined with cilia. d *Notospermus geniculatus*. The cephalic slits (*csi*) are very prominent and terminate in a cerebral organ pouch (*cop*). The duct (*arrow*) of the cerebral organ originates in the cerebral organ pouch (*cop*). The neuropils of the dorsal and ventral lobe are initially still separated by an own inner neurilemma (*indl*) and (*invl*), while neurons are still surrounded by a common outer neurilemma (*on*). The neuron layer is enwrapped by glia processes (*gp*). **e** *Riseriellus occultus*. The cells of the cerebral organ (*co*) terminate close to the blood vessels (*bv)*. A dorsal commissural plexus (*dcp*) connects the dorsal nerve (*dn)* with the lateral medullary cords (*mc*). **f** *Lineus acutifrons*. The esophageal nerves (*en*) run on both sides of the esophagus. The neurons of the medullary cords (*mc*) are separated by an inner neurilemma (*in*) to the neuropil. *dcp* Dorsal commissural plexus

organ. These are not separated into inferior and superior sections. The ventral lobes are confluent with the lateral medullary cords (Fig. 9d).

Medullary cords

In *H. dubia,* the medullary cords are situated between the basal lamina of the epidermis and the outer circular muscle layer (Fig. 9d). The neurons cover the neuropil dorsally and ventrally only. A ventral and a dorsal commissural plexus connect both medullary cords (Fig. 9d).

Minor nerves

The cephalic nerves are not separated from each other by a neurilemma and form a layer of neurites that extend as a hollow cylinder from the commissural tracts and the anterior face of the brain toward the anterior end. During its course, it tapers slightly. The cephalic nerve cylinder is covered by perikarya which characterizes it as a cephalic cord. The dorsal nerve originates in the median part of the first dorsal commissural tract (Fig. 9a). It runs to the posterior directly underneath the basal lamina of the epidermis. The esophageal nerves originate from the ventral lobes of the brain, encircle the mouth and run on both sides of the foregut (Fig. 9a). The paired proboscidial nerves originate in the dorso-median portion of the ventral commissural tract of the brain.

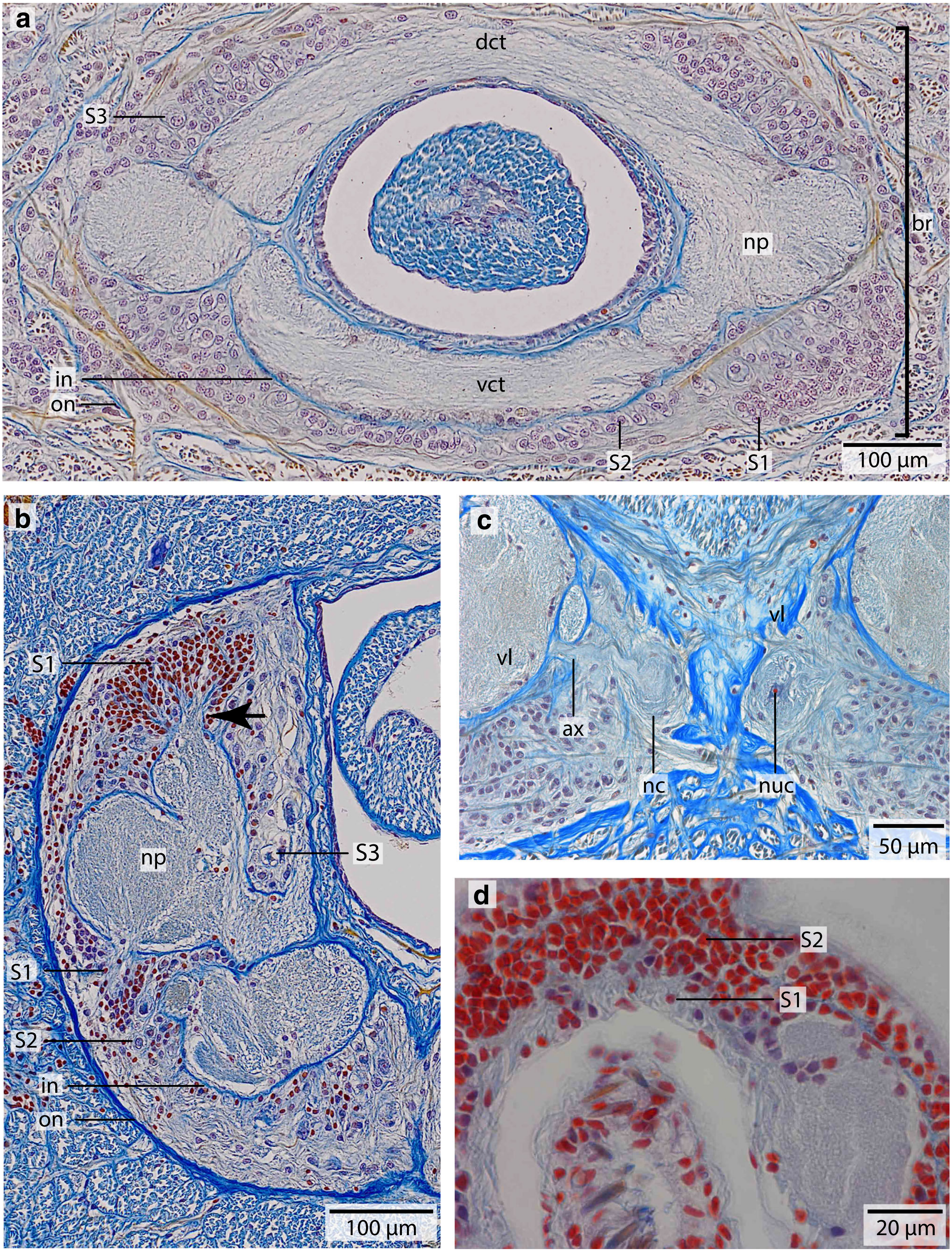
Sensory structures

Frontal organs and pigmented eyes are missing in *H. dubia*. The cerebral organ of *H. dubia* largely resembles that of the heteronemertean species (Fig. 9d, e). In *H. dubia*, however, the distal duct is short and opens into laterally situated pits. The proximal duct is not convoluted but runs straight into the dorsal lobe (Fig. 9a). As described for the lineid species, the proximal duct terminates in a layer of sensory and gland cells that rest retroperitoneally of the *ecm* of the blood vessel lining. Cells of the cerebral organ are directly connected to the dorsal lobe of the brain.

Microscopic anatomy of the pilidiophoran nervous system

Neuropil and glia The neuropil of the brain and medullary cords is composed of densely interwoven neurites that differ in diameter (Fig. 6d). Their arrangement differs inside the brain neuropil. Within tracts, they generally run in parallel, but are horizontally orientated in commissural tracts, while they are vertically arranged in the lateral tracts connecting the dorsal and ventral parts of the brain (Figs. 7a, 5a, b). The peripherally located somata of the brain and medullary cord nerve cells are enwrapped by several layers of glia causing a regularly multilayered envelope surrounding each soma (Fig. 6b). Each layer measures 35–85 nm in diameter, but it may be larger in certain regions and more voluminous where they seem to bulge into the nerve cell soma (Fig. 6c, e). The cytoplasm of the glia cells exhibits greater electron density than that of the nervous system and contains characteristic electron-dark, ovoid vesicles (Fig. 6b–e). The nucleus of the glia cells has higher electron density and contains more heterochromatin than of the nerve cells (Fig. 6b). Somata of glia cells are found in the periphery of the neuron layer next to the outer neurilemma of the *cns* (Fig. 6b), but only a few were seen next to the central neuropil (Fig. 6d). This glia layer in *L. longissimus* and *N. geniculatus* is very prominent and can be recognized by its yellowish color in Azan staining. (Figs. 3d, 4d). Glia envelopes, however, are largely restricted to somata; single neurites are not enwrapped by glia, although groups of neurites may be enwrapped by a glia envelope (Fig. 6d). Immunohistochemistry reveals that in *Lineus viridis,* a synapsin signal could be detected only in the neuropil layer (Fig. 7e), which underpins the strict isolation of somata by glia cells.

Neurons As proposed by Bürger (1895), neurons in Heteronemertea can be classified into three classes according to their size, with type 2 neurons having larger somata as type 1 neurons and type 3 neurons having larger somata as type 2 neurons (see Appendix Table 2). All neurons measured differ in structure, diameter and Azan stainability (Figs. 5a, b, d, 6a, d, e; Appendix Table 2). When stained with Azan, the color of the nuclei of the neurons ranges from orange to red to dark purple. The nucleus appears in bright purple and the cytoplasm is grayish. The cell bodies of type 1 neurons (globuli cells) are small and circular, and electron microscopy shows that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◀**Fig. 5** Histological Section (5 µm), Azan staining of different neurons in heteronemerteans. **a** *Lineus ruber*. The two halves of the brain are connected by a dorsal (*dct*) and ventral (*vct*) commissure above or below the rhynchocoel, respectively. The neurons are separated by an inner neurilemma (*in*) to the neuropil (*np*). Three different types of neurons can be discriminated, *S1–S3*. *S3* being twice as large as type two neurons (*S2*) and type two neurons being twice as large as type one neurons (*S1*). *br* Brain, *on* outer neurilemma. **b** *Lineus bilineatus*. Neurites (*arrow*) branch into the neuron layer. Here the *ecm* is interrupted. *in* inner neurilemma, *on* outer neurilemma, *S1–S3* neuron classes *1–3*. **c** *Lineus acutifrons*: The short axon (*ax*) of the neurochord cells (*nc*) originate in the ventral lobe (*vl*) of the brain. Nucleoli (*nuc*) dye red. **d** *Hubrechtella dubia*. Two types of neurons (*S1, S2*) can be discriminated in *H. dubia. S1* is small and circular, while *S2* is irregular formed and larger than type 1 neurons

their nucleus is surrounded by little cytoplasm (Figs. 5, 6a, d). The cells are distributed everywhere in the brain and are by far the most numerous (Figs. 5, 6a). The cell bodies of type 2 neurons appear more slender but more voluminous than type 1 neurons. Their outline is less circular, and they contain a higher amount of cytoplasm. Type 2 neurons primarily appear on the ventral lobe of the brain shortly behind the ventral commissural tract (Figs. 5a, b, d, 6a, e). The cell bodies of type 3 neurons are the most prominent (Figs. 5a, b, d, 6a). They are very numerous in the anterior part of the brain and are reduced in numbers in the posterior section where the brain neuropil is separated into a dorsal and ventral lobe.

The neurons of *H. dubia* fall into two classes only (Fig. 5d). Type 1 neurons are small and circular. Their nuclei are red when stained with Azan (Fig. 5d). Type 2 neurons are larger than class 1 neurons and their cell bodies have an irregular outline. In general, their nucleus is red or dark purple after the sections had been stained with Azan (Fig. 5d).

A fourth type of neurons that Bürger (1895) established, the neurochord cells, are found only in *Cerebratulus fuscus, Lineus acutifrons and Lineus bilineatus* (Fig. 5c). These cells are present on the ventral part of the brain, close to the roots of the esophageal nerves, as a pair of cells located in the right and left neuropil of the brain. Their huge perikarya are outside the neuropil and measure 63 µm in diameter in *Lineus acutifrons*. They house in this species a nucleus measuring 11 µm in diameter, which thus is at least three times larger than the biggest type 3 neuron found in this species. The axon of the cell is prominent; its diameter is 6 µm (Fig. 5c). Neurochords (fibers of the neurochord cells) in the neuropil of the lateral medullary cords were not found.

Discussion

Comparative neuroanatomy of Heteronemertea, Hubrechtidae and "Palaeonemertea"

Position

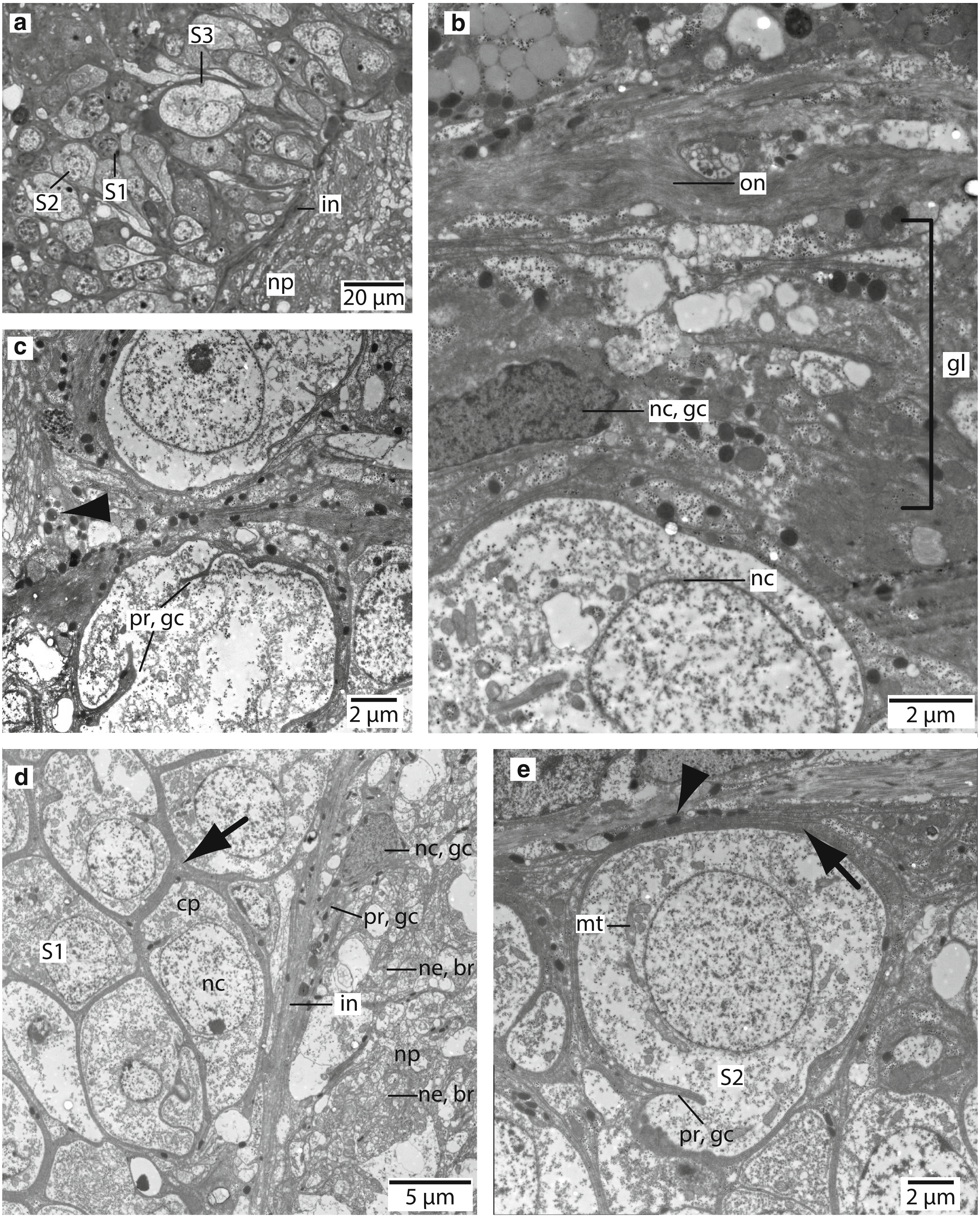
In all lineid heteronemertean species analyzed in this paper, the central nervous system (*cns*) is located inside the musculature. In Valenciniidae and heteronemerteans with a branched proboscis, the *cns* also is intramuscular. In contrast to lineids, the *cns* of *H. dubia* is located directly underneath the basal lamina, like in certain basally branching nemerteans, i.e., *Tubulanus* species (Beckers et al. 2013). The nervous system is identically located and organized in other *Hubrechtella* species and in *Hubrechtia desiderata* (Bürger 1895; Senz 2001).

Neurilemma

The central nervous system of the heteronemerteans studied is surrounded by an outer neurilemma, while an inner neurilemma separates the central neuropil from most of its perikarya. An inner and outer neurilemma are present in *Oligodendrorhynchus* species, which possess a branched proboscis, (Fernández-Álvarez and Anadón 2012), while an inner neurilemma is missing in some Valenciinidae (*Valencinia*, *Valencinura*) (Bürger 1895; Senz 2001). Since an inner neurilemma is also missing in Hubrechtidae (Senz 2001), a prominent matrix between neuropil and the outer layer of perikarya must have evolved within Heteronemertea. In palaeonemerteans, an inner neurilemma is only found in cephalothricid and carinomid species. According to the present interpretation, their inner neurilemma must have evolved convergently. The function of the extracellular matrix in the invertebrate nervous system, however, is largely unknown. The *ecm* is supposed to be involved in neurite formation, to maintain the structure and function and in repairing processes (Rutka et al. 1988; Harrel and Tanzer 1993).

Brain topology

The general anatomy of the central nervous system and the brain appears rather uniform in all investigated lineid heteronemerteans. Being located inside the body wall musculature, the brain of lineids is anteriorly ring-shaped. Along the anterior–posterior axis, it first splits into a left and a right crescent section, then differentiates a dorsal and a ventral lobe of which the dorsal lobe divides again into an inferior and a superior branch. At its posterior margin, the brain thus consists of six portions, isolated from each other by an own outer neurilemma. Both ventral lobes are confluent with the two lateral medullary cords and the inferior branches of the dorsal lobes are associated with the cerebral organ. Senz (2001) describes a tripartition of the brain neuropil in *Valencinia hubrechti*, and Bürger for *Valenicina longirostris,* but Bürger also describes that dorsal and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◀**Fig. 6** **a** Semithin section (1 μm), **b**, **c**, **d**, **e** ultrathin sections (70 nm) of the nervous system. **a** three different types of neurons can be discriminated in the brain of *Lineus ruber* (*S1–S3*). The circular type one neurons (*S1*) are the most numerous neurons. Type 3 neurons (*S3*) are the largest and the fewest in number of all neurons. The neurons are separated by an inner neurilemma (*in*) to the neuropil (*np*). **b** The glia layer (*gl*) is prominent between the neuron layer and the outer *ecm* (*on*). Nuclei of the glia cells (*nc, gc*) are situated in this layer. **c** Glia cell processes (*pr, gc*) draw into the somata of the neurons. **d** Type 1 neurons (S1) show the little cytoplasm (*cp*) surrounding the nucleus (*nc*). The neurites (*ne*) of the brain neuropil are different in diameter and are separated to the somata layer by an inner neurilemma (*in*). Processes of the glia cells enwrap each single neuron (*arrow*). **e** The neuron layer is enwrapped by glia processes (*arrow)* which contain electron dense ovoid vesicles (*arrowhead*). *nc* Nucleus, *mt* mitochondrion, *pr, gc* process of glia cell

ventral lobes are not sharply demarcated in *Valencinia blanca* (Bürger 1895). The overall anatomy of the *cns* in Heteronemertea with a branched proboscis is the same as in lineid heteronemerteans, but in *Gorgonorhynchidae* and *Dendrorhynchus,* only the neuropil of the dorsal lobe is divided into an inferior and a superior branch (Gibson 1977; Fernández-Álvarez and Anadón 2012), both remain surrounded by a common layer of somata.

Compared to the brain morphology of basally branching Nemertea, the brain of lineid heteronemerteans shows a pronounced compartmentalization in its posterior part along with a lobate outline of the neuropil. In palaeonemertean taxa Tubulanidae and *Carinina ochracea,* the brain ring shows only slightly compartmentalization, and in *Carinomidae* and *Cephalothricidae,* the neuropil of the dorsal and ventral lobes are only separated in their very posterior part (Beckers et al. 2013). Since the brains of basally branching nemerteans and Pilidiophora are ring-shaped in their anterior part, I suggest that the last common ancestor of nemerteans possessed a *cns* containing a circular brain with ventrolaterally branching medullary cords (Beckers et al. 2013). During its evolution, the posterior section of the pilidiophoran brain increased in size and distinct brain lobes evolved within heteronemerteans into such a complex topology as shown here for Lineidae. This was likely correlated with an increasing complexity of cerebral organ and its connection to the blood vessels.

Minor nerves

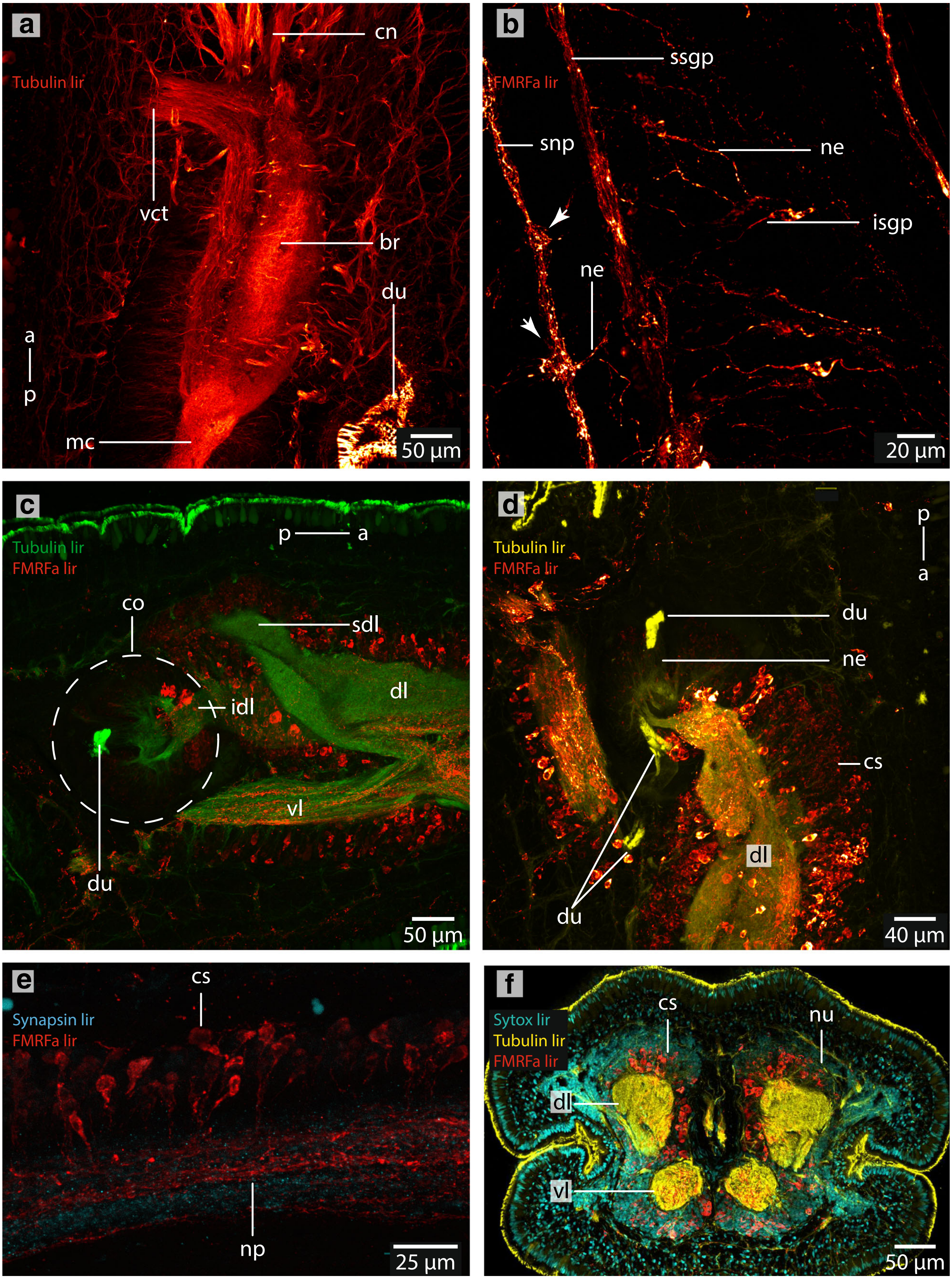
In all heteronemertean species studied, cephalic nerves follow the lateral parts of the rhynchocoel anteriorly. In *Valencinia* species, cephalic nerves originate in the ring-shaped brain but spread out toward the epidermis (Bürger 1895). In investigated lineids, the cephalic nerves do not show such a spreading. In *H. dubia,* cephalic nerve cords are present that form an anterior hollow cylinder. Although four cephalic cords are also described in cephalothricid species (Beckers et al. 2013), their specific arrangement is unique in *H. dubia*. If it is confirmed for all hubrechtid species, it most likely is an autapomorphy of this group. A dorsal nerve is present in all investigated taxa and originates in the medio-caudal portion of the dorsal commissural tract inside the musculature. The dorsal nerve migrates dorsally in its caudal extension and is eventually located dorsal to the inner circular musculature in heteronemerteans. Senz (2001) describes a second dorsal nerve in *Valencinia hubrechtii*. This nerve merges with the upper dorsal nerve posterior to the mouth opening. In *H. dubia,* the dorsal nerve remains subepidermal. A migration of the dorsal nerve into a dorsal region is also characteristic of those basally branching nemerteans where the brain is inside the musculature. In these species (*P. filiformis, C. mutabilis*), the dorsal nerve migrates toward the basal lamina of the epidermis and remains in this position (subepidermal) during its caudal extension. Esophageal nerves originate in the inner margins of the ventral lobes of the brain in heteronemertean species. While the origin of the esophageal nerves of *H. dubia* could not be detected, these nerves also originate in the ventral parts of the brain in palaeonemertean species, indicating that this state represents the plesiomorphic condition in nemerteans. The proboscidial nerves are always paired and originate in the dorsal part of the ventral commissural tract. The nerves are connected by a proboscidial plexus. Paired proboscidial nerves are also present in Valenciniidae (Bürger 1895; Senz 2001) and palaeonemerteans (Beckers et al. 2013). In heteronemertean species with a branched proboscis, only the roots of the proboscidial nerves are paired. The two nerves ramify posterior to the roots to form a plexus (Gibson 1977; Fernandez and Alvarez 2012).

Nerve plexus

In *Lineus ruber,* nerve plexus are present in the epidermis, below the epidermis, above the gut and inside the gut epithelium. Their neurites are differently arranged in different parts of the animals investigated. Plexus and a specific arrangement of their neurites were also described in palaeonemertean species and *Lineus viridis* (Heteronemertea) (Beckers et al. 2011, 2013), indicating that these plexus had already been present in a common nemertean ancestor. Distinct condensations occur in the subepidermal plexus in *Lineus ruber* where it is connected to the lateral medullary cords. These have also been reported from *Carinina ochracea* (Beckers et al. 2013) and may represent the plesiomorphic condition.

Neurons

Neurons can be homologized by morphology and their position in the brain in all investigated heteronemertean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◀**Fig. 7** CLSM, **a–d** *Lineus ruber,* **e** *Lineus viridis*, **f** *Lineus bilineatus;* (70 µm sections). **a** anti-α-Tubulin (*red*). Right brain half, ventral part. The neurites of the ventral commissural tract (*vct*) are horizontally arranged. The ventral part of the brain (*br)* is confluent with the lateral medullary cords *(mc)*. *a* Anterior, *cn* cephalic nerves, *du* duct of the cerebral organ, *p* posterior. **b** anti-FMRF (*red*). The subepidermal (*snp*), the suprastomatogastric (*ssgp*) and the intrastomatogastric plexus (*isgp*) are connected to each other by fine branches of neurites (*ne*). *Note:* the swellings in the subepidermal nerve plexus (*arrows*). **c** anti-α-Tubulin (*green*), anti-FMRF (*red*). The cerebral organ (*co*, dashed oval) is attached to the inferior dorsal lobe (*idl*) of the brain. *ca* Canal of cerebral organ, *cs* neuronal cell somata, *vl* ventral lobe, *sdl* superior dorsal lobe, d anti-α-Tubulin (*yellow*), anti-FMRF (*red*). The duct (*du*) of the cerebral organ is densely ciliated and convoluted. The sensory cells surrounding are connected to the brain by small neurites (*ne*). *cs* neuronal cell somata, *dl* dorsal lobe. **e** anti-FMRF (*red*), anti-synapsin (*blue*). The synapsin signal is restricted to the neuropil of the medullary cord. **f** anti-α-Tubulin (*yellow*), sytox (*blue*), anti-FMRF (*red*). Overview of the brain of *Lineus bilineatus* showing the more prominent dorsal lobe (*dl*) and the FMRF-positive neurons (*cs*) as well the nuclei (*nu*). *vl* ventral lobe

taxa. As already described by Bürger for *Cerebratulus marginatus* (1895) and further specified by Bianchi (1969a, b) four (Bürger 1895) or six Bianchi (1969a, b) types of neurons are discernable in lineid heteronemerteans. Only neurons of type 1–3 described by Bürger (1895) for *C. marginatus* were found in all lineid heteronemerteans in the present study. These cells are also found in *Lineus viridis* (Beckers et al. 2011). Type 4 neurons (neurochord cells) are found in *C. fuscus, L. bilineatus* and *L. acutifrons*. The cell bodies are very prominent and resemble large neurons described for molluscs (Bullock and Horridge 1965). The somata of the neurons are connected to the ventral part of the brain by a short but broad bundle of neurites (axon). Since histology does not reveal sufficient information about type 4 neurons, it has to be studied in more detail on the ultrastructural level. Senz (2000) describes neurochord cells for *Hubrechtella ehrenbergi* (Senz 2000). Whether or not they are homologous to the neurochord cells in described lineids remains to be shown.

In the present study, only two types of neurons were found in *H. dubia* and by Bürger (1895) in *Hubrechtella desiderata*. These neurons are homologous to the first and second type described for heteronemerteans. There is no information on neuron types in Valenciniidae. Only two types of neurons are described in *Oligodendrorhynchus* species (Fernández-Álvarez and Anadón 2012). Since three types of neurons are described for certain Palaeonemertea (Cephalothricidae, Beckers et al. 2013), the third type must have evolved convergently in lineids and Cepahlothricidae, or they were reduced in *H. dubia*.

Glia

Glia cells form a prominent outer layer in investigated taxa, peripheral to the neuronal somata and underneath the outer neurilemma. These cells form processes that extend into the layer of neuronal somata to surround them individually. Some processes extend even deeper and surround groups of neurites. Glia in the nervous system has not yet been investigated in palaeonemerteans. A prominent glia layer is also described for polychaetes (Bullock and Horridge 1965; Golding 1992) and molluscs, where it is called glio-interstitial system (Nicaise 1973). Glia must be studied in additional species to give any hypotheses on the evolution of this cell type.

Eyes

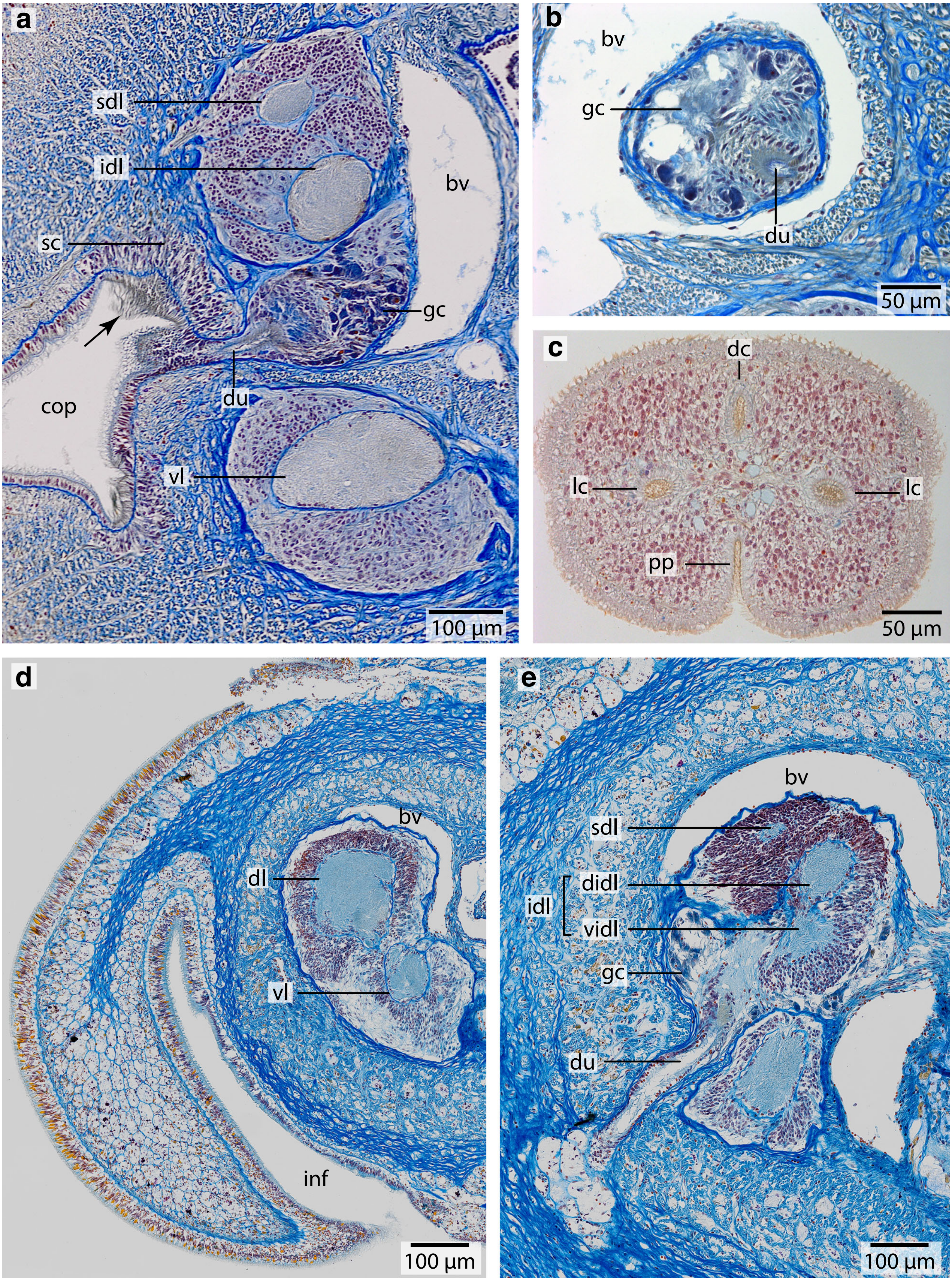
Investigated heteronemerteans, except *L. acutifrons*, possess several eyes on the margin of the head. Ultrastructural data indicate that they are rhabdomeric (Döhren and Bartolomaeus 2007). A connection of the eyes to the brain (optical nerve) could not be observed. Adult eyes are described for *H. desiderata* (Bürger 1895), but are missing in *H. dubia.*

Frontal organ

All investigated lineids except *H. dubia* posses a frontal organ. It consists of one dorsal and two lateral ciliated ducts which terminate in a layer of neurons. A connection of the cells lining the duct to the brain was not found in the present study, although it has been described in previous studies (Gibson 1972). The frontal organ of *Valenciniidae* consists of one dorsal pit (Bürger 1895; Senz 2001). Senz (2001) describes that the voluminous cerebral gland leads to the frontal organ in *Valencinia hubrechti*. This morphology is also present in certain hoplonemerteans (Bürger 1895, e.g., *Oerstedia dorsalis*, pers. observation). A frontal organ is not present in palaeonemerteans.

Cerebral organ

A close association of the cerebral organ to the blood vessels indicates a neurosecretory or osmoregulatory function (e.g., Scharrer 1941; Ling 1969, 1970; Ferraris 1985). The distal portions of the ducts originate from the posterior margin of deep longitudinal cephalic slits in investigated lineid heteronemertean species. Only in *A. albimaculosa* they originate from small pits. In *Baseodiscus hemprichii,* the ducts originate in pouches which are formed by ventrally located epidermal folds. These folds may be interpreted as ventrally located cephalic slits, but a final statement is not possible (see also Bürger 1895). In *Baseodiscus delineata,* the duct of the cerebral organ is connected to ventrally located pits (Bürger 1895; Senz 2001), and its cells do not terminate on the same level as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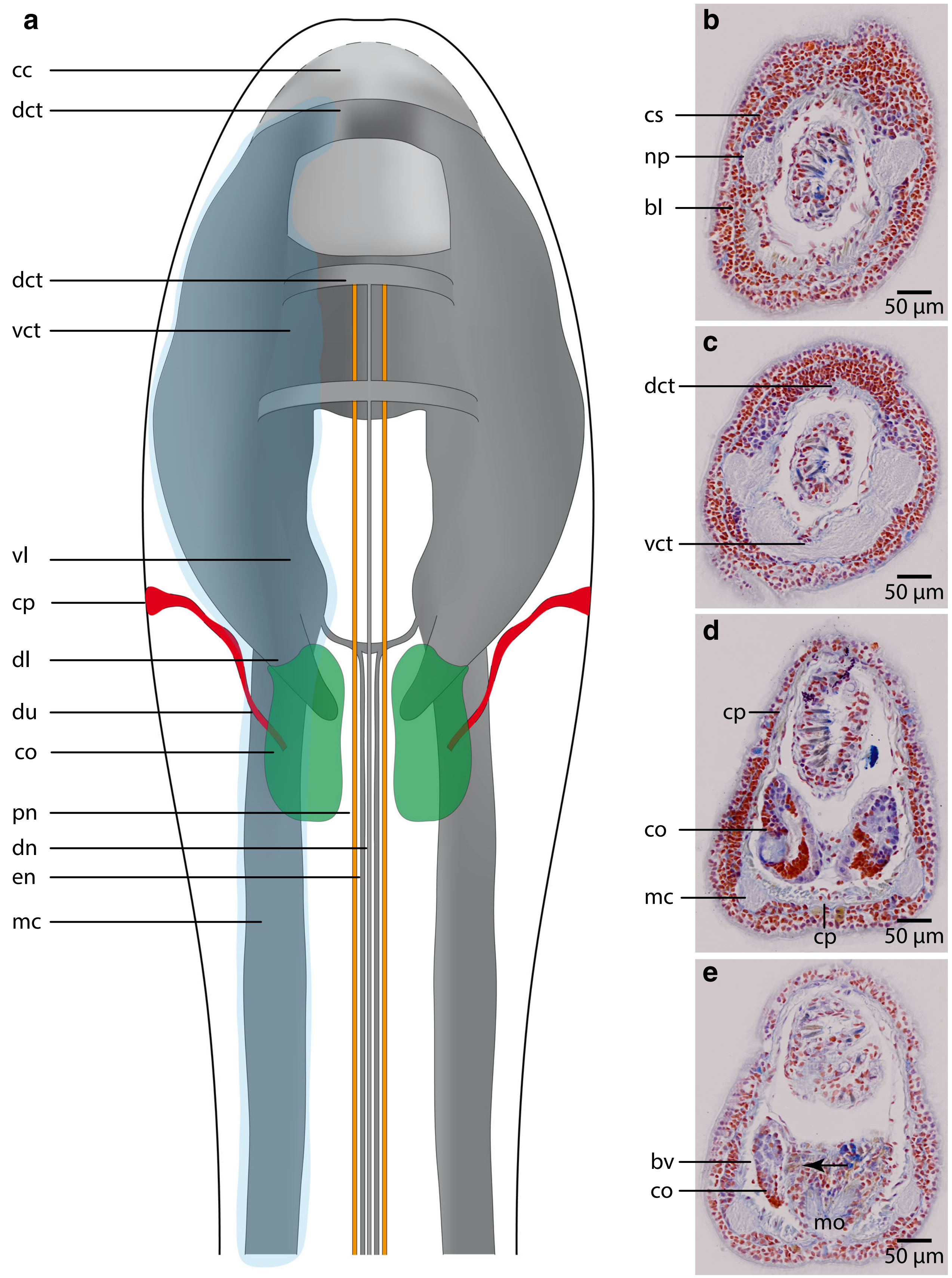
◀**Fig. 8** Histological Sections (5 µm) Azan staining. **a** *Lineus acutifrons.* The subepidermal duct (*du*) of the cerebral organ originates in the median portion of the cephalic organ pouch (*cop).* The cephalic slits as well as the cephalic organ pouch (*cop*) are lined with sensory cells (*sc*) which bear long cilia (*arrow*). The brain is in this part divided into three parts; the ventral lobe (*vl*) and the inferior dorsal lobe (*idl*) which is connected to the cerebral organ and a superior dorsal lobe (*sdl*) which rests on the cells of the cerebral organ. *bv* (blood vessel). **b** *Lineus acutifrons.* The subepidermal canal (ca) terminates in a layer of glandular cells (*gc*) close to the blood vessels (*bv*). **c** *Lineus ruber*. The frontal organ of heteronemerteans consists of one dorsal (*dc*) and two lateral canals (*lc*). *pp* proboscis pore. **d** *Baseodiscuss hemprichii*. The canal which leads to the cells of the cerebral organ originates in the median portion of the epidermal infold (*inf*). *bv* blood vessel, *dl* dorsal lobe, *vl* ventral lobe. **e** *Baseodiscuss hemprichii.* The proximal duct *(du)* runs straight dorsally, passes a layer of glandular cells (*gc*). The inferior dorsal lobe (*idl*) is again bifurcated into a dorsal inferior dorsal lobe (*didl*) and a ventrally located inferior dorsal lobe (*vidl*). Cells of the cerebral organ are conneted to the ventral inferior dorsal lobe (v*idl*). The superior dorsal lobe (*sdl*) is very short. *bv* blood vessel

the dorsal lobe, but are located laterally to the dorsal lobe. The cerebral organ is innervated by neurites which originate in the ventral part of the dorsal lobe. The inner end of the cerebral organ does not extend into blood vessels but is surrounded by small branches of blood vessels (Bürger 1895; Senz 2001). The cerebral organ of *Panorhynchidae, Oligodendrorhynchus* and *Gorgonorhynchidae* as well as that of *Polybrachiorhynchidae* resembles that of lineid heteronemerteans, but originates from small pits as in *H. dubia* (Gibson 1977; Fernández-Álvarez and Anadón 2012). Since the cerebral organ originates from cephalic pits in basally branching nemerteans (Beckers et al. 2013) and *H. dubia* as well as Valenciniidae (Bürger 1895; Senz 2001) and Hoplonemertea I interpret this morphology as the plesiomorphic condition in nemerteans. This suggests that deep cephalic slits evolved in the stem lineage of Lineidae and were secondarily reduced in *Apatronemertes*. The epithelia of the slits and pits are densely lined with sensory cells in investigated taxa. The cells bear cilia which line the inner part of the canals, and these cells are connected to the dorsal and ventral lobe of the brain by small branches of neurites. This arrangement is similar to the morphology of the distal portion of the ducts of the cerebral organ of palaeonemerteans (if present), but there the sensory cells around the canal are only connected to the dorsal lobe (Beckers et al. 2013). Additionally, their cerebral organ is intraepidermal and does not extend beyond the basal lamina of the epidermis.

Evidence for Pilidiophora

Bürger (1895) already stated that the anatomy of the central nervous system in Hubrechtidae shows an intermediate state between Heteronemerteans and palaeonemerteans. Being located subepidermally, the position of the *cns* in *H. dubia* actually is the same as in certain basally branching nemerteans (*Tubulanus* species) (Beckers et al. 2013). Additionally, the brain is less complex in terms of compartmentalization: It is divided into a dorsal and ventral lobe only in its very posterior part and a tripartition of the brain like in lineid heteronemerteans is not present. Like in *Carinina ochracea* (Palaeonemertea), only two types of neurons are present in *H. dubia*; the third type of neurons was not found. Neither an outer nor an inner neurilemma was discernable in *H. dubia*, which both are present in heteronemerteans. In certain palaeonemertean taxa, the outer (*C. ochracea*) or the inner neurilemma (*Tubulanidae*) are also missing (Beckers et al. 2013). The cephalic nerve cord of *H. dubia* is covered with neurons, which is also described for one palaeonemertean taxon (Cephalothricidae) (Beckers et al. 2013). The general problem, however, is that there are correspondences between Hubrechtidae and certain palaeonemertean species, while other palaeonemertean species show conditions that are found in heteronemertean species. There are two possible explanations for this: The correspondences in certain characters of central nervous system (*cns*) in heteronemerteans and certain palaeonemerteans evolved convergently. The Hubrechtidae would then have retained several plesiomorphies in the structure of their *cns*, like its position directly underneath the epidermis (subepidermal), the lack of an inner and an outer neurilemma, the cephalic cords, two types of neurons only, and unbranched dorsal lobes. The intramuscular position of the *cns,* an inner and outer neurilemma, cephalic cords, and a third type of neurons then evolved convergently in different nemertean lineages. The alternative hypothesis has to assume that several characters of the hubrechtid *cns* secondarily were simplified, so that inner and outer neurilemma got lost and the position of the *cns* changed from an intramuscular into a subepidermal position. The differing structure of the cephalic nerve cords in *Hubrechtella* species as exemplified here for *H. dubia* and those of Cephalothricidae than would have to be regarded as evidence for homoplasy.

However, the topology and special structure of the cerebral organ actually provides support for a taxon Pilidiophora. In all pilidiophoran species, its distal portion runs subepidermally and terminates in a layer of neurons and glandular cells close to the blood vessels, while the cerebral organ of palaeonemerteans terminates anterior to the basal lamina of the epidermis and has no contact to the blood vessels. Since in hoplonemerteans cerebral organs are situated anterior to the brain and are connected to the latter by a cerebral organ nerve (Bürger 1895; Gibson 1972; Beckers 2012), the strong topological relation between the cerebral organ and the dorsal lobe of the brain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◀**Fig. 9** *Hubrechtella dubia* **a** Schematic drawing after 3D reconstruction of the nervous system of *Hubrechtella dubia* and **b**–**e** histological sections (5 µm), Azan staining. *gray* neuropil; *bright blue* neurons (only drawn on left side); *green* cerebral organ; *red* cephalic pits, ducts of the cerebral organ; *yellow* proboscis nerves. **a** *co* Cerebral organ, cc cephalic cord, *cp* cephalic pit, *dct* dorsal commissural tract, *dl* dorsal lobe of brain, *dn* dorsal nerve, *du* duct of the cerebral organ, *en* esophageal nerves, *mc* medullary cord, *pn* proboscis nerves, *vl* ventral lobe of brain. *Note*: The commissural plexus as well as the dorsal part of the cephalic cord were omitted. **b**The central nervous system is composed of a central neuropil (*np*) and surrounding neuronal cell somata (*cs*). *bl* Basal lamina of epidermis. **c** Both lobes of the brain are connected by an dorsal commissural tract (*dct*) above the rhynchocoel and by a ventral commissural (*vct*) tract below the rhynchocoel. **d** The ventral lobe of the brain is confluent with the lateral medullary cords (*mc*). A commissural plexus (*cp*) connects both cords dorsally and ventrally. The duct of the cerebral organ (*co*) terminates in a layer of glandular cells and neurons. **e** The cells of the cerebral organ (*co*) terminate close to the blood vessel (*bv*). *Arrow* mouth lobe, *mo* mouth opening

and the blood vessels as well as the gland cells in Hubrechtidae and Heteronemertea support the monophyly of Pilidiophora in addition to the pilidium larva and evidence from recent molecular data (Andrade et al. 2012, 2014; Kvist et al. 2014) as well as from recent morphological studies (Chernyshev et al. 2013).

Appendix

See Tables 1, 2

Table 1 Elements of the nervous system of Pilidiophora

Taxon	Location of cns	Dorsal lobe bifurcated	Ventral lobe	Lateral tract	Dorsal commissure	Ventral commissure	Cephalic cords	Medullary cords	Cephalic nerves	Dorsal nerve	2nd dorsal nerve	Ventral nerve
Lineus ruber	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Lineus longissimus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Lineus bilineatus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Lineus acutifrons	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Riseriellus occultus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	several	1	Absent	Absent
Ramphogordius lacteus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Ramphogordius sanguineus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Notospermus geniculatus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Cerebratulus fuscus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Cerebratulus marginatus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Micrura purpurea	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Apatronemertes albimaculosa	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	several	1	Absent	Absent
Baseodiscus hemprichii	Intramuscular	?	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Hubrechtella dubia	Subepidermal	Absent	1 pair	1 pair	3	1	Present	1 pair	Absent	1	Absent	Absent
Hubrechtella desiderata	Subepidermal	Absent	1 pair	1 pair	1	1	Present	1 pair	Absent	1	Absent	Absent
Eupolia dilineata	Intramuscular	Absent	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Valencinia hubrechti	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	2	Present	Absent
Valencinia longirostris	Intramuscular	Absent	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Langia formosa	Intramuscular	Absent	1 pair	?	?	1	Absent	1 pair	Several	1	Absent	Absent
Gorgonorhynchus	Intramuscular	Present	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Panorhynchus	Intramuscular	Absent	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent
Polybrachiorhynchus	Intramuscular	Absent	1 pair	1 pair	1	1	Absent	1 pair	Several	1	Absent	Absent

Taxon	Esophag. nerve	Probscid. nerve	Cephalic slits	Frontal organ	Cerebral organ	Co in bv	Eyes	Neuron types	Neurochord cells	Outer neurilemma	Inner neurilemma	Author
Lineus ruber	2	2	1 pair	3 pits	1 pair	Present	Several	3	Absent	Present	Present	This study
Lineus longissimus	2	2	1 pair	3 pits	1 pair	Present	Several	3	Absent	Present	Present	This study
Lineus bilineatus	2	2	1 pair	3 pits	1 pair	Present	Several	3	Present	Present	Present	This study
Lineus acutifrons	2	2	1 pair	3 pits	1 pair	Present	Several	3	Present	Present	Present	This study
Riseriellus occultus	2	2	1 pair	3 pits	1 pair	Present	Several	3	Absent	Present	Present	This study
Ramphogordius lacteus	2	2	1 pair	3 pits	1 pair	Present	Several	3	Absent	Present	Present	This study
Ramphogordius sanguineus	2	2	1 pair	3 pits	1 pair	Present	Several	3	Absent	Present	Present	This study
Notospermus geniculatus	2	2	1 pair		1 pair	Present	Several	3	Absent	Present	Present	This study
Cerebratulus fuscus	2	2	1 pair		1 pair	Present	Several	4	Present	Present	Present	This study
Cerebratulus marginatus	2	2	1 pair		1 pair	Present	Several	4	Present	Present	Present	Bürger (1895)
Micrura purpurea	2	2	1 pair	3 pits	1 pair	Present	Several	3	Absent	Present	Present	This study
Apatronemertes albimaculosa	2	2	Absent		1 pair	Present	Several	3	Absent	Present	Present	This study
Baseodiscus hemprichii	2	2	Absent	?	1 pair	Present	Several	3	Absent	Present	Present	This study
Hubrechtella dubia	2	2	Absent		1 pair	Present	Absent	2	Absent	Absent	Absent	This study
Hubrechtella desiderata	2	2	absent		1 pair	present	Several	2	Absent	Absent	Absent	Bürger (1895)
Eupolia dilineata	2	2	Absent	1 pit?	1 pair	Absent	Several	?	Absent	Absent	Present	Bürger (1895)
Valencinia hubrechti	2	2	Absent	1 pit	1 pair	Absent	Absent	?	Absent	Absent	Present	Senz (2001)
Valencinia longirostris	2	2	Absent	1 pit?	1 pair	Absent	Absent	?	Absent	Absent	Present	Bürger (1895)
Langia formosa	2	2	Absent		1 pair	Absent		?	Absent	Present	Present	Bürger (1895)
Gorgonorhynchus	2	Root 2	Absent		1 pair		Absent	?	Absent	Present		Gibson (1977)
Panorhynchus	2	Root 2	Absent	3 pits	1 pair		Absent	?	Absent	Present	Present	Fernandez-Alvarez and Anadon (2012)
Polybrachiorhynchus	2	Root 2	Absent	3 pits	1 pair		Absent	?	Absent	Present		Gibson (1977)

Table 2 Average diameter of type 1–3 neurons

Neuron type	1	2	3
Species	剢 Diameter/µm		
L. ruber	4.45	8.24	16.04
L. longissimus	3.47	9.19	19.23
L. acutifrons	3.65	11.19	19.04
R. occultus	3.87	7.71	17.72
R. sanguineus	3.25	6.84	13.71
R. lacteus	3.31	7.05	16.90
L. bilineatus	3.34	10.01	17.49
M. purpurea	3.75	6.86	16.77
H. dubia	2.59	4.51	–

All differ significantly in size ($n = 10$)

Acknowledgements The author thanks two anonymous reviewers who helped to improve the manuscript. The author thanks Malin Strand for collecting *Hubrechtella dubia*, Daria Krämer for collecting *Cerebratulus fuscus* and *Notospermus geniculatus.* Alexej Chernyshev gently provided specimen of *Baseodiscus hemprichii*. Christiane Wallnisch for technical support and Thomas Bartolomaeus for discussions and suggestions on the manuscript.

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