First detailed description of the nervous system of the most complex lophophore and evolution of Brachiopoda

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Abstract

The lophophore is a tentacle organ unique to the lophophorates. Recent research has revealed that the organization of the nervous and muscular systems of the lophophore is similar in phoronids, brachiopods, and bryozoans. At the same time, the evolution of the lophophore in certain lophophorates is still being debated. Innervation of the lophophore has been studied for only two brachiopod species belonging to two subphyla: Linguliformea and Rhynchonelliformea. Species from both groups have the spirolophe, which is the most common type of the lophophore among brachiopods. In this study, we used transmission electron microscopy, immunocytochemistry, and confocal laser scanning microscopy to describe the innervation of the most complex lophophore (the plectolophe) of the rhynchonelliform species *Coptothyris grayi*.

The *C. grayi* lophophore (the plectolophe) is innervated by three brachial nerves: the main, second accessory, and lower. Thus, the plectolophe lacks the accessory brachial nerve, which is typically present in other studied brachiopods. All *C. grayi* brachial nerves contain two types of perikarya. Because the accessory nerve is absent, the cross nerves, which pass into the connective tissue, have a complex morphology and two ascending and one descending branches. The outer and inner tentacles are innervated by several groups of neurite bundles: one frontal, two lateral, two abfrontal, and two latero-abfrontal (the latter is present in only the outer tentacles). Tentacle nerves originate from the second accessory and lower brachial nerves. The inner and outer tentacles are also innervated by numerous peritoneal neurites, which exhibit acetylated alpha-tubulin immunoreactivity.

This result supports the following previously proposed hypothesis about the evolution of the lophophore in brachiopods: the morphology of the lophophore has evolved from simple to complex, whereas the innervation of the lophophore has evolved from complex to simple; the latter is indicated by a smaller number of lophophoral nerve tracts in species with complex lophophores. The reduction of the accessory brachial nerve and diminution of the main brachial nerve are associated with general reduction of the prosoma in brachiopods.

Introduction

Brachiopods are sessile benthic marine animals that have a bivalve shell. This phylum appeared in the early Cambrian and was dominant in many past marine communities [1–2]. Brachiopod species were very abundant in the past, but there are only about 400 species in recent fauna [3]. In bilaterian phylogeny, brachiopods together with phoronids and bryozoans are grouped into the Lophophorata clade. The lophophorates monophyly has been recently rebuilt according to morphological data [4–8] and molecular data [9–10].

Like all other lophophorates, brachiopods have a tentacular organ, the lophophore, which collects food particles from the water column [11, 12]. The lophophore consists of a brachial axis, which is a ribbon-like structure bearing a row of tentacles. Anteriorly, the brachial axis always forms an open loop, and the

rudiments of new tentacles form at its end [13]. The brachial fold stretches along the tentacles, and the brachial groove is located between the brachial fold and tentacles [14]. The mouth is located in the middle of the brachial axis and its position is always in the brachial groove between the row of tentacles and the brachial fold. In most brachiopods, the row of tentacles is double: the inner tentacles are located close to the brachial fold, and the outer tentacles are removed from the brachial fold. The brachial axis can twist in different directions and generally determines the morphology of the lophophore. Recent brachiopods exhibit nine types of lophophore morphology [15]. The morphologically simplest lophophores are the taxolophe (occurring only in ontogenesis) and the trocholophe, and the most complex is the plectolophe.

The evolution of brachiopods, which is still unclear, involves the development of the mineral skeleton, of the microstructure of the shell, and of the soft tissue of the lophophore. Traditionally, the evolution of the brachiopod lophophore is thought to have involved increasing complexity, i.e., the lophophore was thought to have evolved from the simple ring-like structure of the trocholophe to a very complex plectolophe with three arms [15, 16]. Detailed analyses of the literature including descriptions of extinct species revealed that the simple spirolophe rather than the trocholophe is the ancestral type of the lophophore in brachiopods [1, 13]. How the simple spirolophe can give rise to simpler forms, such as the trocholophe and schizolophe, or to more complex forms, such as the ptycholophe or plectolophe, is still unclear. Researchers recently assumed that paedomorphosis played a substantial role in the formation of different types of lophophores [13]. The same pathway, i.e., paedomorphosis, has recently been suggested to explain the evolution of the lophophore in phoronids. According to this view, the phoronid lophophore evolved in two different ways: simplification from an ancestral horseshoe-shed type to an oval lophophore via paedomorphosis and complication from ancestral horseshoe-shed type to spiral lophophore [17, 18].

The phylum Brachiopoda contains three subphyla: Linguliformea, Craniiformea, and Rhynchonelliformea. The relationship between these subphyla is also undefined. The brachiopod lophophore has been infrequently studied by immunocytochemistry and confocal laser scanning microscopy (CLSM). The nervous system of the lophophore of adult brachiopods was recently described for a species of Linguliformea [4] and a species of Rhynchonelliformea [19], i.e., *Lingula anatina* and *Hemithiris psittacea*, respectively. Both species have a spirolophe lophophore (the spirolophe). The lophophore nervous system has also been described in *Novocrania anomala* juveniles, which have morphologically simple lophophores, i.e., trocholophe and schizolophe [20].

Based on recent reports, researchers have noted that, for lophophorates in general and for brachiopods in particular, the organization of the lophophore nervous system is closely related to the morphology of the lophophore, i.e., morphologically simple lophophores have many nerve tracts, and morphologically complex lophophores have only a few nerve tracts [18, 19]. To help determine whether this pattern is true for other species, in the current research we studied the innervation of the lophophore of rhynchonelliform *Coptothyris grayi*, a species that has a plectolophe, i.e., a morphologically complex lophophore. After

describing the nervous system in *C. grayi*, we use the new data to make inferences on the relationships among the Brachiopoda and the evolution of the brachiopod lophophore.

Results

Morphology of the lophophore in C. grayi

C. grayi has the most complex type of lophophore among brachiopods – the plectolophe (Fig. 1A). The lophophore consists of three arms: two wing-like lateral arms and one spiral medial arm (Figs. 1A, B, 2A, 3A). The arms are formed by a strongly curved brachial axis that consists of a double row of tentacles and a brachial fold separated by a food groove. The mouth is located in the middle of the brachial axis in the food groove between the brachial fold and tentacle rows. On both sides of the mouth, in the lateral arms, the brachial axis runs along the lower part of each lateral arm, turns backward on its distal end, and passes the upper side of the lateral arm. Above the mouth, the left and right ends of the brachial axis extend into the middle arm, where they form a spiral, and end at the distal end of the middle arm (Fig. 2A). Thus, each arm consists of two brachial axes bearing two rows of tentacles, two brachial folds, and two food grooves (Figs. 1C, 3B). In the lateral arms, the brachial axes are close to each other, so that the epidermis between the two brachial folds forms a deep brachial gutter (Fig. 3B). Each row of tentacles, except for the area under the mouth, is double and consists alternating inner and outer tentacles: the inner tentacles are closer to the brachial fold than the outer tentacles (Fig. 1C).

Both the inner and outer tentacles have zones that differ in ciliation, in position relative to the other tentacles and the brachial fold, and in the histological structure of epithelium (Figs. 1D, 4A, B). Tentacles of both types have eight zones: one frontal, one abfrontal, two latero-frontal, two lateral, and two lateroabfrontal. Inner and outer tentacles differ from each other in form in transverse section and in the location of ciliated zones (Figs. 4A, B, 5A, B). In both inner and outer tentacles, the frontal zone faces the brachial fold. In outer tentacles, the frontal zone is concave and forms a deep frontal groove that is lined with cubic epithelium (Figs. 4A, 5A). In

inner tentacles, the frontal side protrudes and bears the ciliated ridge, which is formed by columnar epithelium (Figs. 4B, 5B). The abfrontal zone is opposite to the frontal zone. The abfrontal zone is wide in the outer tentacles but very narrow in the inner tentacles. Lateral zones form two densely ciliated ridges in tentacles of both types. The lateral ciliated ridges are located close to the frontal zone in the outer tentacles and are located close to the abfrontal zone in the inner tentacles. According to these locations of the lateral ridges, there are two latero-abfrontal zones in the outer tentacles, and two extensive latero-frontal zones in the inner tentacles (Fig. 4A, B).

General anatomy of the nervous system in C. grayi

Two ganglia, one subenteric and the other supraenteric, are the main elements of the nervous system in *C. grayi* (Figs. 2A-B, 3A). The subenteric ganglion is located under the mouth and gives rise to two thick lateral nerves, which innervate the body and muscles. The subenteric ganglion also gives rise to the lower

brachial nerves, which extend into both lateral brachial arms, skirt them, and penetrate into the middle brachial arm (Figs. 2A, B, 3A). The supraenteric ganglion is located above the mouth and consists of two lateral nerve centers, which are connected to each other via a thick nerve (Figs. 2A, 3A). The supraenteric ganglion gives rise to the pair of main brachial nerves, which extend at the base of the brachial fold along the middle line of each lateral brachial arm (Fig. 3B). At the end of each lateral arm, each main brachial nerve extends back to the mouth and then penetrates the middle brachial arm. The subenteric and supraenteric ganglia are connected to each other via thick circumoral connectives. Near the subenteric ganglion, each connective gives rise to a thick nerve, which extends into the middle brachial arm and joins the lower brachial nerve (Fig. 3A). The supraenteric ganglion also gives rise to nerves in the middle brachial arm; these nerves fuse with the main brachial nerves.

Innervation of the brachial arms in C. grayi

Each lateral arm is innervated by six brachial nerves: two main, two second accessory, and two lower (Figs. 3B, 6). Because each lateral arm is formed by the looped brachial axis, only

three nerves belong to each half of the brachial axis: one main, one second accessory, and one lower.

Two **main brachial nerves** extend along the middle line of each lateral arm and along the middle arm, at the base of the brachial folds (Figs. 2A, 3B, 6). In each lateral arm, two main brachial nerves are located near each other (Figs. 2B, 3B, 7A). According to CLSM, each main brachial nerve is formed by longitudinal neurite bundles, which exhibit acetylated alpha-tubulin immunoreactivity (Fig. 7B). Each main brachial nerve is completely located in the epithelium of the arm and resembles a compact neurite bundle that is 20–25 μm in diameter (Fig. 8A). Neurite bundles form a large neuropile, which is divided into several portions by basal projections of supportive cells (= radial glia cells) (Fig. 8A). Transmission electron microscopy (TEM) revealed that these projections contain electron-dense intermediate filaments, which extend from the apical to the basal part of the cells and which adhere to the basal lamina via hemidesmosomes (Fig. 8B, C). Perikarya of at least two types are associated with the neuropile (Fig. 8A). Perikarya of the first type have a compact soma with a small nucleus bearing a large nucleolus and electron-dense cytoplasm, which is filled with small mitochondria and an electron-dense matrix (Fig. 8B). The perikarya of the second type have a large soma, which contains a large nucleus with electron-light karyoplasm (Fig. 8C). The glial cells are located within the nerve projections (Fig. 8B). These cells have a compact soma and projections, which are filled with large electron-dense granules (Fig. 8D). These projections are numerous in the neuropile. The neuropil consists of two types of neurites (Fig. 8D). Neurites of the first type are the most abundant; they have electron-light cytoplasm, which contains many microtubules (Fig. 8D). Neurites of second type are rare; their cytoplasm is filled with numerous electron-dense small granules and dense-core vesicles (Fig. 8D); these neurites can form large varicoses, which contain numerous dense-core synaptic vesicles (Fig. 8E).

Each main brachial nerve gives rise to numerous **cross nerves** (Figs. 6, 7B-D). Each cross nerve penetrates the connective tissue of the brachial arm, extends to the epidermis of the food

groove, and then extends to the base of the tentacles (Fig. 9A). As a consequence, each cross nerve has two ascending parts and one descending part (Figs. 3B, 6, 7B). The cross nerve is formed by 40–50 neurites of different diameters (Fig. 9B). Some of these neurites contain electron-dense granules and dense-core synaptic vesicles (Fig. 9B). Each cross nerve is associated with several envelop cells, which surround the nerve and have flocculent electron-light cytoplasm with numerous mitochondria, vesicles, and canals of rough endoplasmic reticulum (Fig. 9C).

At the base of the tentacles, several cross nerves are grouped together in sites between the inner tentacles and give rise to two short, thick nerves, which extend between the bases of the inner tentacles to the frontal side of the outer tentacles (Fig. 7C). These short nerves are connected to the **second accessory brachial nerve** (Figs. 3B, 6, 9A, D). This nerve is formed by a group of perikarya and neurites, which are located at the base of frontal side of the outer tentacles and which skirt these tentacles laterally (Figs. 7D, E, 9A, D). These frontal semicircles are connected to each other by bridges, which extend along bases on the abfrontal sides of the inner tentacles (Fig. 7D, E). The ultrastructure of the accessory brachial nerve is similar to that of the main brachial nerve. TEM revealed two types of perikarya in the accessory brachial nerve. One type, which has a lot in common with perikarya of the main brachial nerve, is the most abundant (Fig. 9A, D). They are large, have electron-light cytoplasm, and contain a large nucleus that lacks a nucleolus (Fig. 10A). Their cytoplasm is filled with numerous Golgi apparatuses and vesicles with electron-lucent content (Fig. 10B). The perikarya of the other type (i.e., perikarya of the third type – *pkIII*) are located at the base of the epithelium and are not abundant (Fig. 9A). These perikarya have electron-dense cytoplasm and contain vesicles with electron-dense content (Fig. 10A). A specific feature of these perikarya is the presence of two centrioles (Fig. 10A).

The third large brachial nerve is the **lower brachial nerve** (Figs. 2B, 3A, 6). It extends along both outer sides of each brachial arm and extends about 350 μm from the tentacle base (Fig. 11A). The lower brachial nerve is represented by a thick aggregation of neurites and neurite

bundles, which abut each other and form a very thick compact nerve tract (Figs. 10C, 11C). The large basal neuropil is penetrated by long and thin basal projection of supportive cells (= radial glia cells) (Fig. 10C). The lower brachial nerve is associated with many perikarya, which form long projections that extend along the nerve (Fig. 11D). According to TEM, there are two types of perikarya, which are identical to the perikarya of the second accessory nerve. In the lower brachial nerve, the basal perikarya have the same peculiarities as perikarya from the second accessory brachial nerve: their cytoplasm contains numerous vesicles with electron-dense content and centrioles (Fig. 10D). These basal perikarya form the projections, which contain vesicles with electron-dense content, dense-core vesicles, and vesicles with electron-light content (Fig. 10E). The lower brachial nerve gives rise to the many radial nerves of the arm. These nerves form a thick nerve net along the outer surface of the brachial arm (Fig. 11A). A pair of radial nerves extend between the lower brachial nerve and the abfrontal side of each outer tentacle (Fig. 11A).

Innervation of tentacles

Corresponding to their difference in morphology, the outer and inner tentacles differ in innervation. The differences concern the connection of tentacle nerves and brachial nerves and the location of tentacles nerves in the outer and inner tentacles.

The **outer tentacles** contain seven groups of longitudinal nerves: one frontal, two lateral, two latero-abfrontal, and two abfrontal (Fig. 4C). The frontal nerve originates from the groups of perikarya of the second accessory brachial nerve (Figs. 6,7E). The frontal nerve is formed by many thin neurite bundles, each of which consists of 5–7 neurites of small diameter (Fig. 5C). These thin neurite bundles are scattered in the epithelium of the frontal grove, and the frontal nerve therefore has weak acetylated alpha-tubulin immunoreactivity (Fig. 4C). Lateral tentacle nerves arise from the second accessory nerve (Figs. 6, 7D, E). Each lateral nerve is formed by three thick neurite bundles, which extended into the epithelium of the lateral ciliated ridges (Fig. 5G). Each neurite bundle consists of 10–20 neurites, which have large diameters and are filled with electron-light cytoplasm (Fig. 5G). Latero-abfrontal nerves originate from the radial nerves of the lophophoral arm and arise from the lower brachial nerve (Figs. 6, 11A). Each latero-

abfrontal nerve is formed by one or two thick compact neurite bundles, which consist of > 50 neurites (Fig. 5H). The abfrontal zone is innervated by two abfrontal nerves, which originate from the radial nerves of the outer side of the lophophore arm (Figs. 6, 11B). According to immunocytochemistry, each abfrontal nerve consists of three neurite bundles (Fig. 4C), each of which is formed by 8–10 neurites (Fig. 5D).

In the inner tentacles, the frontal nerves originate directly from the cross nerves (Figs. 6, 7C). In one tentacle, the frontal nerve contains neurites from different cross nerves (Fig. 7C). According to immunocytochemistry, each frontal nerve consists of 3–5 separate neurite bundles (Fig. 4D). TEM revealed a continuous layer of neurites in the basal part of the frontal epithelium (Fig. 5F). All other nerves of the inner tentacles originate from the second accessory nerve (Figs. 6, 7D). In the inner tentacles, the lateral and abfrontal nerves are organized in the same way as in the outer tentacles except that the inner tentacles lack the latero-abfrontal nerve.

Both outer and inner tentacles are innervated by peritoneal neurites (Fig. 4E). These neurites originate from perikarya located at the base of the tentacles (Fig. 4E). Peritoneal neurites extend between the basal lamina and the cells of the coelomic lining (Fig. 5E). They have electron-light cytoplasm and contain bundles of microtubules (Fig. 5E).

Discussion

Innervation of the lophophore in brachiopods

The phylum Brachiopoda includes three subphyla: Linguliformea, Craniiformea, and Rhynchonelliformea [21]. Organization of the lophophore nervous system has been studied in specimens from all three groups, including the following species: *N. anomala* [20, 22], *Discinisca lamellosa* [23], *L. anatina* [4], *Gryphus vitreus* [24], and *H. psittacea* [19]. Innervation of the lophophore in brachiopods has mostly been studied via light microscopy [22–24], and only four species have been studied with TEM,

immunocytochemistry, and CLSM [4, 19, 20, 25]. According to all data, the central nervous system of brachiopods includes two ganglia, the subenteric and the supraenteric, which are located under and above the mouth, respectively. Adult brachiopods from the Linguliformea and Craniiformea lack the supraenteric ganglion

[22, 23]. Juveniles of *N. anomala* from the Craniiformea, however, have the supraenteric ganglion, which with development is transformed into the main brachial nerve [20].

The subenteric ganglion gives rise to the lower brachial nerve, whereas the supraenteric ganglion gives rise to the main brachial nerve. The main brachial nerve is connected to the accessory brachial nerve via numerous cross nerves, which extend into the connective tissue of the lophophore arms. These three brachial nerves, i.e., the main, accessory, and lower, are the major nerves of the lophophore in all brachiopods studied to date [4, 19–20, 22–24]. Two recent studies of lophophore innervation have revealed the presence of a second accessory nerve in the rhynchonelliform *H. psittacea* [19] and in the craniiform *N. anomala* [20]. Parts of this brachial nerve are represented by groups of FMRF-amide-like immunoreactive perikarya and have been previously described in *L. anatina* [4]. Brachiopods therefore have four major brachial nerves: the main, accessory, second accessory, and lower.

In *G. vitreus*, which has the plectolophe (the most complex type of lophophore among recent brachiopods), only the main and lower brachial nerves have been reported in the lateral arms (Bemmelen, 1883). The latter study also described the accessory brachial nerve in the medial arm of the *G. vitreus* lophophore [24].

According to our data, the plectolophe of *C. grayi* is innervated by three brachial nerves: the main, second accessory, and lower; the accessory brachial nerve is completely absent. In *H. psittacea*, the accessory brachial nerve is present but does not contribute to the innervation of the tentacles: the cross nerves do not merge with the accessory brachial nerve but skirt it and extend to the second accessory nerve. This state may be regarded as the first step in the reduction of the accessory nerve in the brachiopods from the Rhynchonelliformea, which is the most advanced

group of brachiopods. The next hypothetical step in the transformation of the lophophore nervous system is the reduction of the main brachial nerve, which is consistent with the finding that the brachia and tentacles in *G. vitreus* are innervated by accessory and lower nerves but not by the main nerve [24]. This pattern indicates that the organization of the nervous system associated with morphologically complex lophophores (like the plectolophe) is simpler than that associated with of morphologically simple lophophores (like the spirolophe). Thus, the complexity of the lophophore nervous system is inversely related to the morphological complexity of the lophophore.

In all brachiopods studied to date, the supraenteric ganglion (or the main brachial nerve if that ganglion is absent) and subenteric ganglion are connected to each other via circumenteric connectives. As shown in the current study, these connectives are present in *C. grayi*. In addition to circumenteric connectives, *C. grayi* has thick nerves that extend from both ganglia to the nerves of the median brachial arm. The

presence of these nerves may be explained by the complexity of the lophophore morphology: each arm must be innervated directly from the central nervous system to facilitate a rapid and proper response to stimuli. No other studied brachiopod has these additional nerves, probably because the lophophore morphology of other brachiopods is simpler than that of *C. grayi*, which has the plectolophe.

Innervation of tentacles

In brachiopods, all tentacles are highly specialized. The specialization is expressed by the presence of epithelial zones, which extend along different sides of the tentacle and which are associated with longitudinal nerves and muscle bundles [26]. All other lophophorates have a similar organization of tentacles [20, 27], but brachiopod tentacles are more specialized than phoronid tentacles. This specialization is also manifested by double row of tentacles and the

formation of prominent lateral epithelial ridges in brachiopod tentacles. The innervation differs in the inner and outer tentacles, i.e., the outer tentacles but not the inner tentacles have lateroabfrontal nerves. The lateroabfrontal tentacle nerves have also been documented in the outer tentacles of *H. psittacea* [19]. The presence of these nerves may be associated with the great extension of the lateroabfrontal zone in the outer tentacles. The lateroabfrontal zone also contains many gland cells, which produce the mucous that facilitates the "peeling off" of waste particles from the lophophore [15, 28]. The peeling off involves a reversal in the beating of cilia, which may also require the additional innervation provided by the lateroabfrontal tentacle nerves [28].

Ultrastructure of the lophophore nerves

In all brachiopods studied to date, the lophophoral nerves are located in the lophophore epithelium [4, 19, 25]. This epithelium is formed by columnar cells with long, thin basal projections, which contain electron-dense filaments and that are attached to the basal lamina via hemidesmosomes. This organization of epithelial cells, which are associated with the nerve tissues, suggests that these epithelial cells are "radial glia" [29, 30]. These epithelial cells protect the nerve cells and supply then with nutrients. In other brachiopods and some other lophophorates, typical glial cells have been described [4, 31]. These cells are located within the neuropil and form projections, which contain large electro-dense granules and surround neurite bundles and perikarya, and. Such glial cells were found in the main brachial nerve of *C. grayi* in the current study.

Two types of perikarya, i.e., large perikarya with electron-lucent cytoplasm and small perikarya with electron dense-cytoplasm, have been discovered in the lophophoral nerves of *C. grayi*. Both types of perikarya form projections. The same was recently described in the supraenteric and subenteric ganglia of *C. grayi* [25]. The main brachial nerve of *L. anatina* contains at least five types of perikarya [4]. The small number of perikarya types in *C. grayi* and in *H. psittacea* [19] may correlate with the general reduction of the main brachial nerve (see below).

Evolution brachiopods

Based on to the morphology of recent and extinct brachiopods, researchers have suggested that the simple the spirolophe is the ancestral type of lophophore for all brachiopods [13]. In ontogenesis, the simple spirolophe is gradually developed and undergoes transformation from a taxolophe into a trocholophe and a schizolophe. The simple spirolophe is known in linguliformes and rhynchonelliformes from the early Cambrian. It consisted of two shorts arms, which formed 1 or 2 coils and bore single row of tentacles [32−34]. This simple spirolophe gave rise to the more complex spirolophe with arms that form 5−7 coils and that is characteristic of recent Linguliformea, Craniiformea, and some recent and extinct Rhynchonelliformea. The plectolophe of terebratulids possibly originated from the schizolophe via paedomorphosis [13].

The organization of the lophophore nervous system in a brachiopod ancestor probably had much in common with that of a recent linguliform, *L. anatina.* Significantly, this brachiopod has a small prosoma with the protocoel [35]. A rudimentary prosoma can be also found in the planktonic stage of recent lingulids: it is the so-called unpaired apical tentacle [16, 36]. The brachiopod ancestor apparently had a large prosoma that contained the protocoel and the supraenteric ganglion with the main and accessory brachial nerves that play a major role in the innervation of tentacles (Fig. 12). In the brachiopod ancestor, the subenteric ganglion was apparently involved in the innervation of the body and mantle but did not contribute much to the innervation of the tentacles: it gave rise to a weak lower brachial nerve.

A sedentary life style and the presence of a two-valved shell, which completely covered the body and the lophophore, led to the reduction of the prosoma in brachiopods. The same we can see in bivalve mollusks, which lost their head. Because recent linguliformes retain the prosoma, their main and accessory nerves are still well developed and contribute substantially to the innervation of the tentacles (although the supraenteric ganglion is reduced in linguliformes). The main brachial nerve in recent linguliformes contains five types of perikarya and is formed by numerous neurite bundles including the giant nerve fibers [4]. In comparison with the main brachial nerve, the lower brachial nerve of *L. anatina* is weakly developed and is formed by several thin neurite bundles, which are not grouped together. In recent linguliformes, moreover, the complex lophophore bears double row of tentacles (instead of single row in the brachiopod ancestor). The innervation of adjacent alternating tentacles requires nerve nodules between tentacles: thus, the anlage of the second accessory brachial nerve appeared in recent linguliformes (Fig. 12). The relationship between the formation of double row of tentacles and the appearance of the second accessory brachial nerve has been shown in juveniles of *N. anomala* [20].

In rhynchonelliformes, the prosoma underwent reduction, and the role of the supraenteric ganglion was reduced. This led to a reduced contribution to tentacle innervation from the main and accessory brachial nerves. This reduction is also expressed in the number of types of perikarya: *H. psittacea* and *C. grayi* have only one or two types of perikarya in the main brachial nerve. The accessory brachial nerve also underwent reduction. Thus, in *H. psittacea*, the accessory brachial nerve is present but does not contribute to tentacle innervation (Temereva, Kuzmina, 2017), and in *C. grayi*, the accessory brachial nerve is absent. The diminution of the main brachial nerve and the reduction of the accessory brachial nerve probably

increased the importance of the subenteric ganglion and the lower brachial nerve, which is very thick in *C. grayi*. In addition, in both *H. psittacea* and *C. grayi*, the second accessory brachial nerve is well

developed and is represented by a true nerve tract (Fig. 12). The appearance of the second accessory nerve probably correlates with the extension of the double row of tentacles.

Conclusion

In brachiopods, the evolution of lophophore morphology generally involved either an initial complexity or an initial simplicity followed by increased complexity. For example, the ancestral simple spirolophe gave rise to the complex spirolophe, which is widespread in recent brachiopods from different groups [13]. A similar evolutionary path has been suggested for phoronids [17, 18].

The evolution of the lophophore nervous system in brachiopods, in contrast, appears to have involved the diminution of the supraenteric ganglion and the main brachial nerve and the gain of the subenteric ganglion and the lower nerve. This evolutionary path apparently correlates with the reduction of the prosoma, which is absent in the larvae and adults of most brachiopods. The reduction of the major nerve elements of the lophophore nervous system has been proposed for other lophophorates, i.e., for phoronids and bryozoans [7, 37].

Materials And Methods

Animals

Adults of *Coptothyris grayi (Davidson, 1852)* were collected in July 2015 in Vostok Bay, Sea of Japan. The collected specimens were relaxed in 7% $MgCl_2$ for 20 min and then photographed using a Leica M165C stereomicroscope equipped with a Leica DFC420 digital camera (Leica Microsystems GmbH, Wetzlar, Germany). Specimens were dissected to obtain the lophophore. Parts of the lophophores were fixed for semi-thin sectioning, scanning electron microscopy (SEM), TEM, immunocytochemistry, and CLSM.

Microscopy

For SEM and TEM, lophophores were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing NaCl and were then post-fixed in 1% osmium tetroxide in the same buffer. For SEM, parts of the lophophores were dehydrated in ethanol followed by an acetone series, critical point dried, and then sputter coated with platinum-palladium alloy. Specimens were examined with a JEOL JSM scanning electron microscope (JEOL Ltd., Tokyo, Japan).

For semi-thin sectioning and TEM, specimens were dehydrated in ethanol and embedded in Embed-812 resin (Electron Microscopy Science, USA). Semi-thin and thin sections were prepared with a Leica UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Semi-thin sections were stained with

methylene blue, observed with a Zeiss Axioplan2 microscope, and photographed with an AxioCam HRm camera (Carl Zeiss, Oberkochen, Germany). Ultrathin sections were stained with uranyl acetate (0.5%) and lead citrate (0.4%) and then examined with a JEOL JEM 100B electron microscope (JEOL Ltd., Tokyo, Japan).

For immunocytochemistry, lophophores of *C. grayi* were fixed in a 4% paraformaldehyde solution and washed in phosphate buffer (pH 7.4) (Fisher Scientific, Pittsburgh, PA, USA) with Triton X-100 (10%) (Fisher Scientific) (PBT). Non-specific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch, Newmarket, Suffolk, UK) in PBT. The specimens were incubated in primary antibody (anti-▯-Tubulin-mouse (1:700) (ImmunoStar, Hudson, WI, USA) in phosphate buffer with Triton X-100), washed in PBT, exposed to the secondary antibody (635-Alexa-Mouse (1:1000) (Invitrogen, Grand Island, NY, USA)), washed in phosphate buffer, embedded in Murray Clear, mounted on a glass slides covered with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA), and examined with a Nikon Eclipse Ti confocal microscope (Moscow State University, Moscow, Russia). Z-projections were prepared using Image J version 1.43 software. Volume renderings were prepared using Amira version 5.2.2 software (Thermo Fisher Scientific, MA, USA). TEM micrographs and Z-projections were processed in Adobe Photoshop CS3 (Adobe World Headquarters, San Jose, California, USA) to prepare panoramas and combinations of Z-projections.

Declarations

Ethics approval and consent to participate

The use of brachiopods in the laboratory does not raise any ethical issues, and therefore approval from regional and local research ethics committees was not required. The field sampling did not involve endangered or protected species. In accordance with local guidelines, permission for collection of material was not required.

Consent for publication

The authors have read the manuscript and consent to its publication.

Availability of data and material

The data sets analyzed during the current study are available from the corresponding author in response to reasonable requests.

Competing interests

The authors do not have competing interests.

Funding

Authors' contributions

ET has performed the research, analyzed the data, prepared all figures, and wrote the manuscript. TK has done most of the light and transmission electron microscopy studies, prepared all schemes, and wrote a part of discussion. Both authors have read and approved the final version of the manuscript.

Acknowledgements

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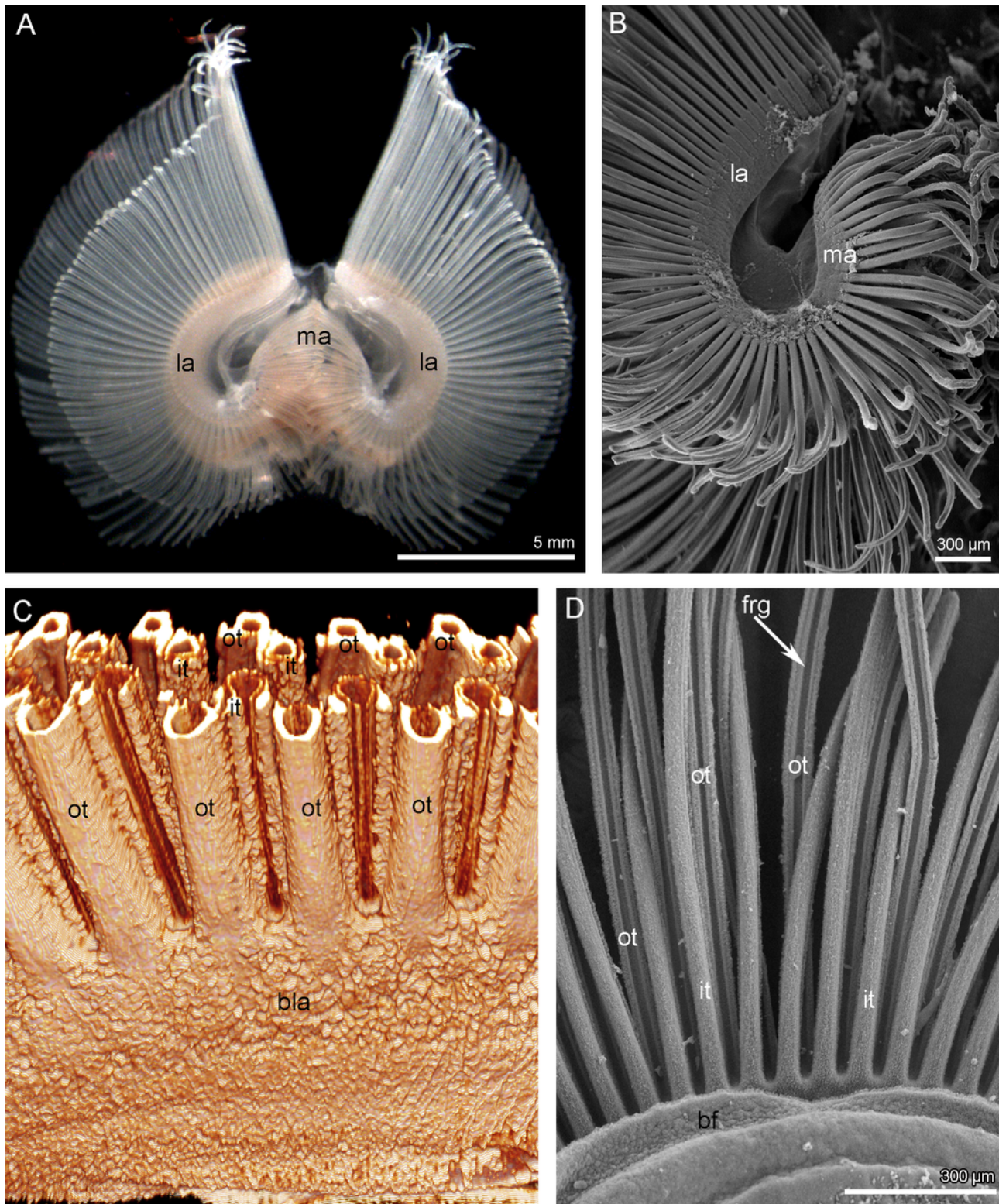
Figures

Figure 1

Organization of the lophophore in Coptothyris grayi. (A) Photograph of narcotized live lophophore. (B) A portion of lateral and median arms of the lophophore; SEM. (C) A portion of the lateral arm: two double rows of tentacles are visible; volume rendering after immunostaining against acetylated alpha-tubulin; CLSM. (D) Organization of the brachial axis, which bears double row of tentacle (inner and outer

tentacles) and the brachial fold; SEM. Abbreviations: bf – brachial fold; bla – base of the lateral arm; frg – frontal groove; it – inner tentacle; la – lateral arm; ma – median arm; ot – outer tentacle.
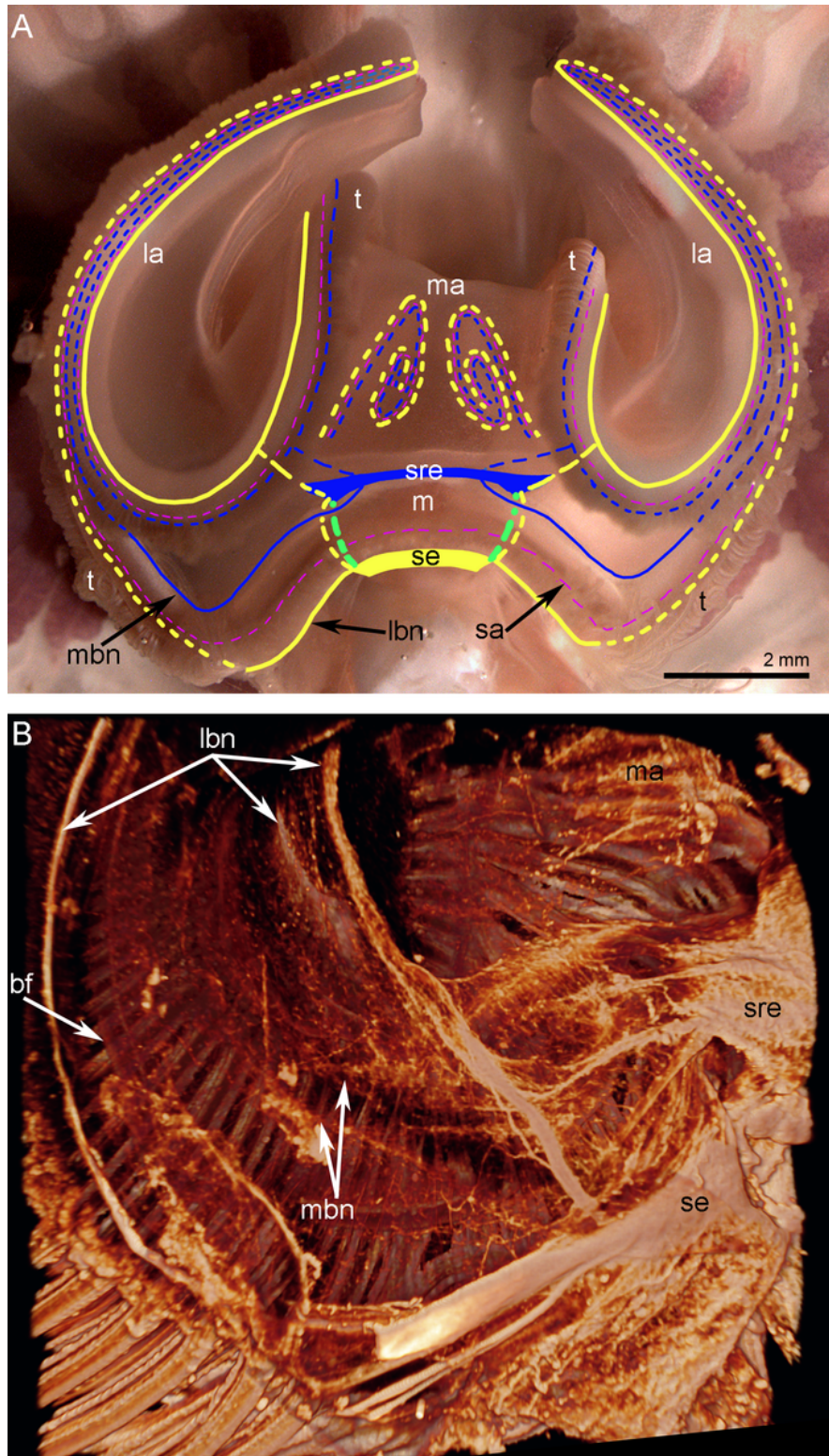
Figure 2

Organization of the central nerous system in Coptothyris grayi. (A) The photograph of live plectolophe with scheme of location of major nerve elements. (B) Central portion of the lophophore with parts of lateral and median arms; volume rendering after immunostaining against acetylated alpha-tubulin;

CLSM. Abbreviations: bf – brachial fold; lbn – lower brachial nerve; m – mouth; mbn – main brachial nerve; sa – second accessory brachial nerve; se – subenteric ganglion; sre – supraenteric ganglion; t – tentacles.
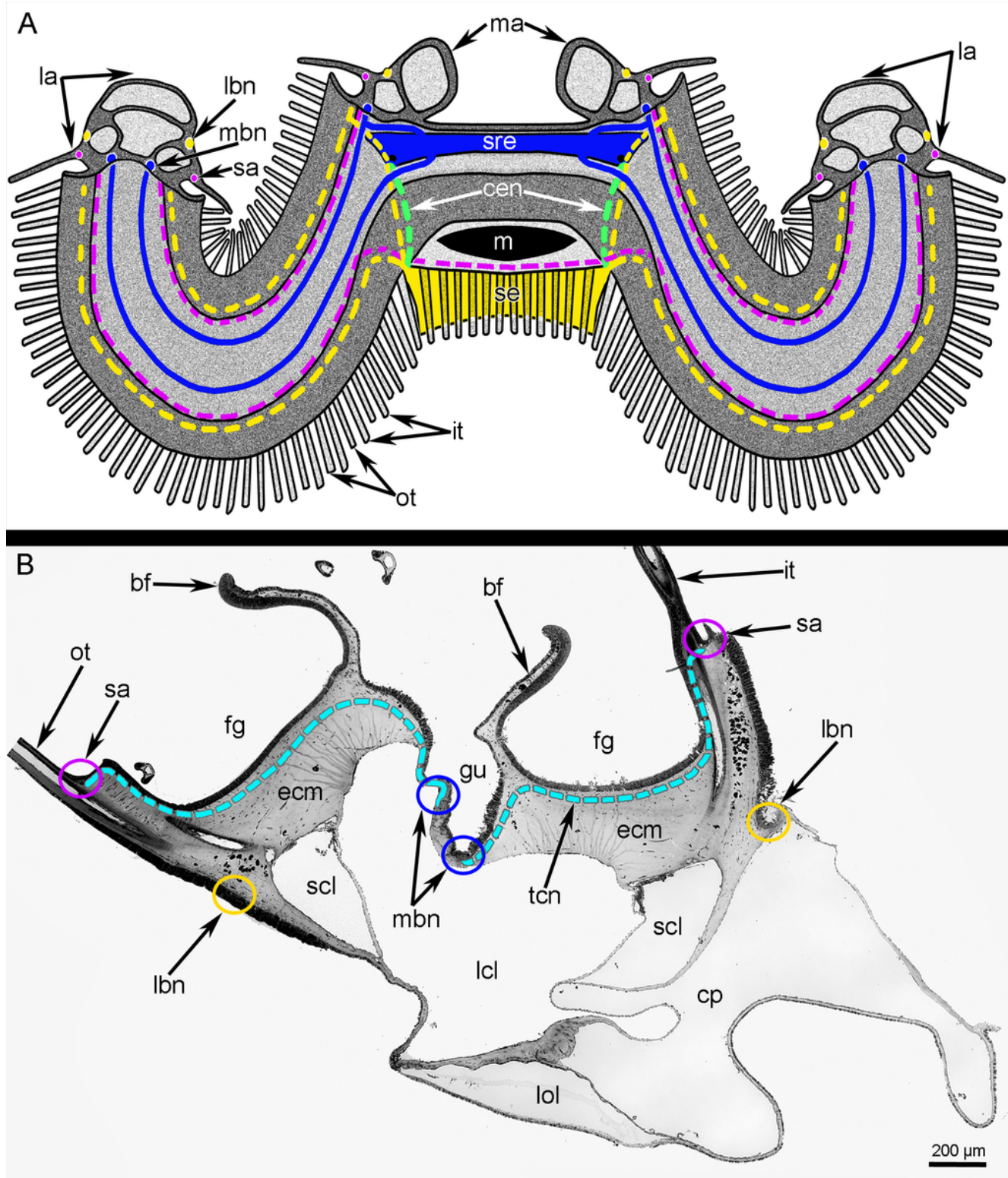
Figure 3

Location of ganglia and brachial nerves in the lophophore of Coptothyris grayi. (A) The scheme of central part of the lophophore; lateral and median arms are cut. (B) Semi-thin cross section of the lateral arm.

The location of different nerves is marked by circles of different colors, which correspond to the colors of nerves in Figures 3A and 6. Abbreviations: bf − brachial fold; cp − coelomic pouch; ecm − extracellular matrix; fg − food groove; gu − gutter; it − inner tentacle; lbn − lower brachial nerve; lcl − large canal of the lophophoral coelom; lol − lamella of loop of the lophophore brachidium; m − mouth; mbn − main brachial nerve; ot − outer tentacle; sa − second accessory brachial nerve; scl − small canal of the lophophoral coelom; se − subenteric ganglion; sre − supraenteric ganglion; tcn − trace of the cross nerves.
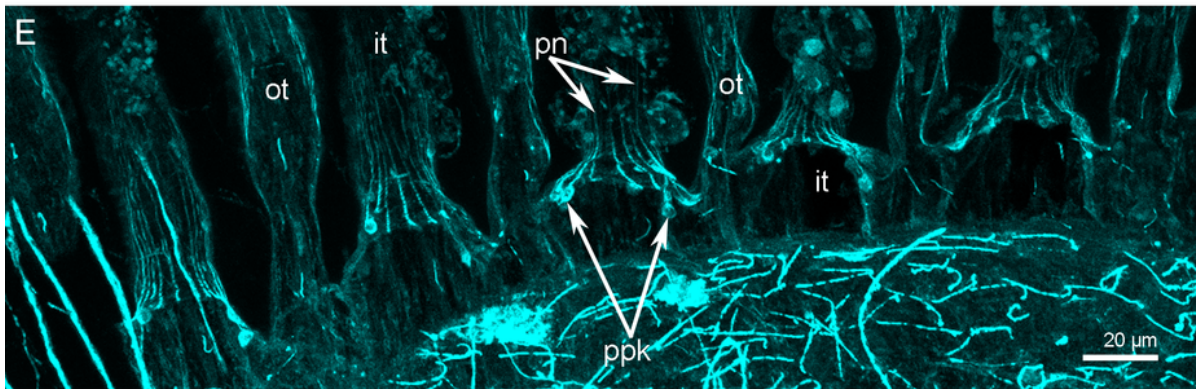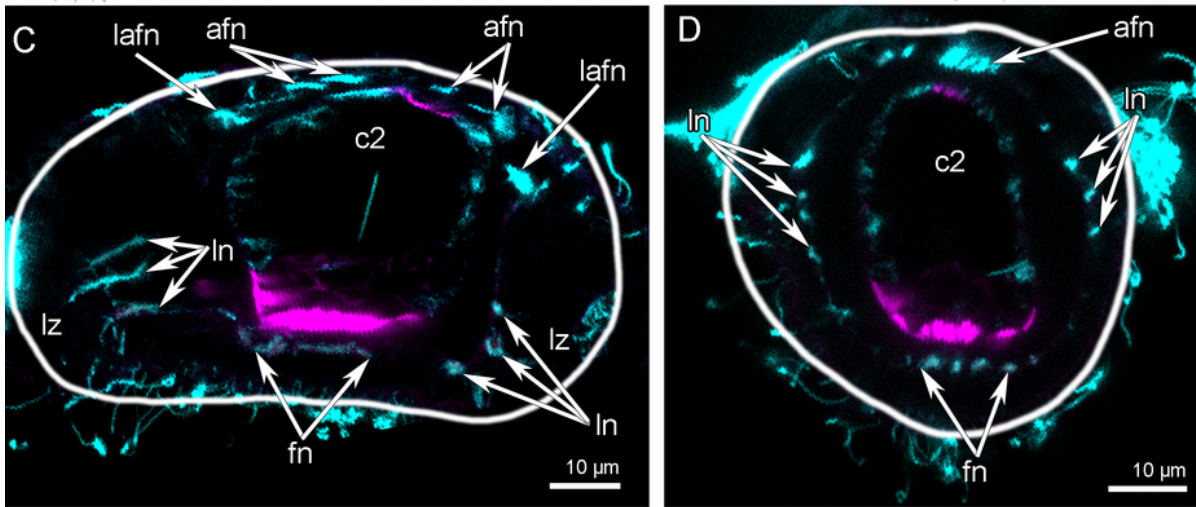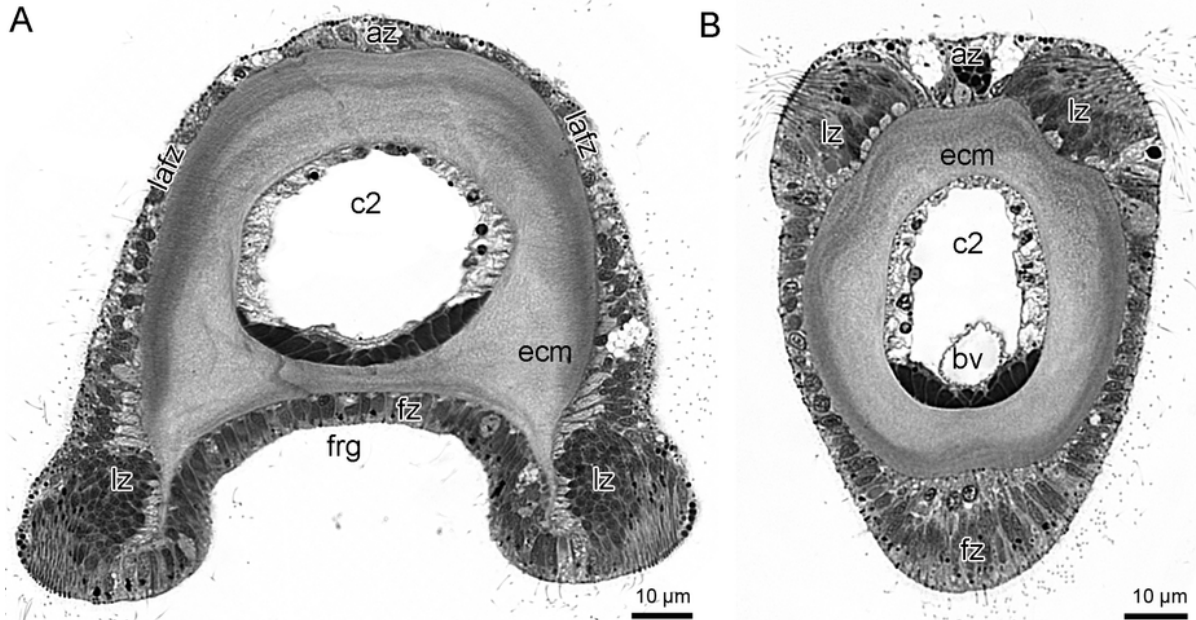
Figure 4

Organization of tentacles in Coptothyris grayi. Semi-thin transverse sections (A, B); Z-projections after immunostaining against acetylated alpha-tubulin (cyan) and staining with phalloidin (magenta) (C-E). (A) Outer tentacle. (B) Inner tentacle. (C) Z-projection of outer tentacle; the border of tentacle is shown by white line. (D) Z-projection of inner tentacle; the border of tentacle is shown by white line. (E) Z-projection peritoneal neurites in outer and inner tentacles. Abbreviations: af – abfrontal zone; afn – abfrontal tentacle nerve; bv – tentacle blood vessel; c2 – tentacle coelom (mesocoel); ecm – extracellular matrix; fz – frontal zone; fn – frontal tentacle nerve; frg – frontal groove; lafn – lateroabfrontal tentacle nerve; lafz – lateroabfrontal zone; ln – lateral tentacle nerve; lz – lateral zone; pn – peritoneal neurite; ppk – peritoneal perikarya.
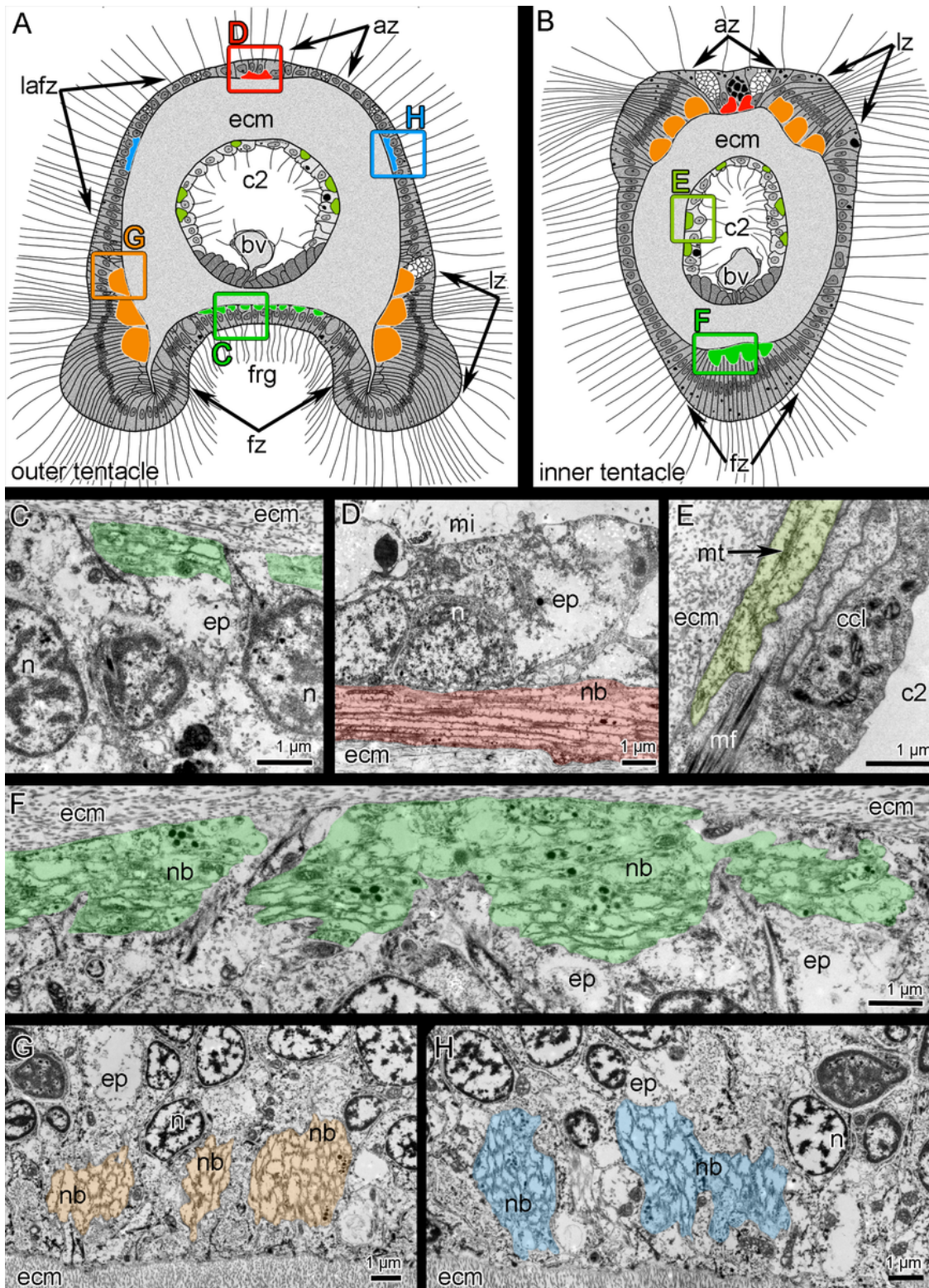
Figure 5

Innervation of tentacles of Coptothyris grayi. Schemes of cross section (A-B) – colored boxes and letters indicate places, which ultrastructure is given in the figure; TEM (C-H). (A) Organization of the outer tentacle. (B) Organization of the inner tentacle. (C) Frontal neurite bundle of outer tentacle. (D) Abfrontal neurite bundle of outer tentacle. (E) Peritoneal neurite of inner tentacle. (F) A portion of frontal nerve of inner tentacle. (G) Lateral tentacle nerve of outer tentacle. (H) Lateroabfrontal nerve of outer tentacle.

Abbreviations: af − abfrontal zone; bv − tentacle blood vessel; c2 − tentacle coelom (mesocoel); ccl − cells of coelomic lining; ecm − extracellular matrix; ep − epithelium; fgr − frontal groove; fz − frontal zone; lafz − lateroabfrontal zone; lz − lateral zone; mf − myofilaments; mi − microvilli; mt − microtubule; n − nucleus; nb − neurite bundle.
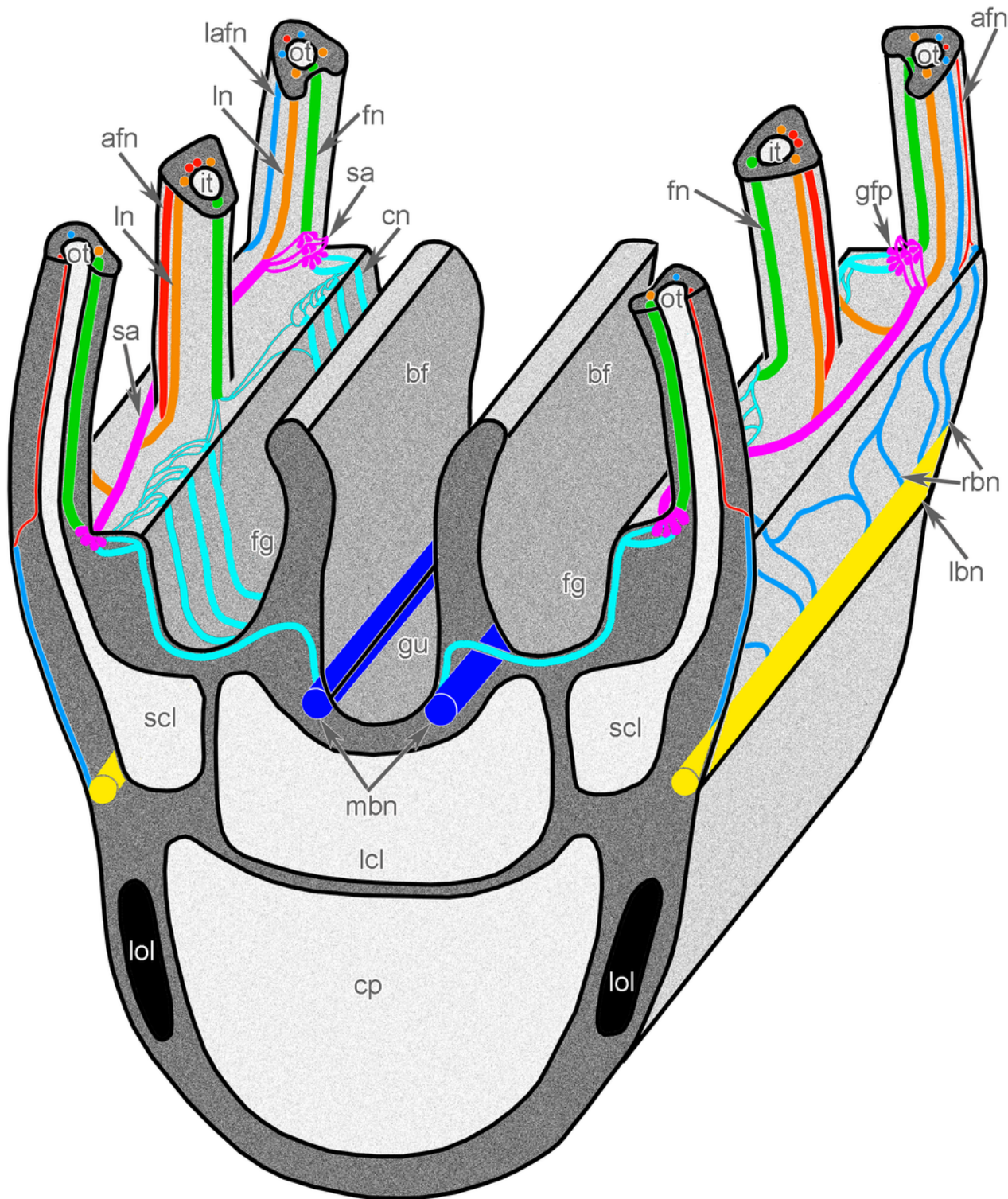

Figure 6

Scheme of innervation of lateral arm of the lophophore of Coptothyris grayi. Abbreviations: afn – abfrontal tentacle nerve; bf – brachial fold; cn – cross nerve; cp – coelomic pouch; fg – food groove; fn – frontal tentacle nerve; gfp – groups of frontal perikarya; gu – gutter; it – inner tentacle; lafn – lateroabfrontal tentacle nerve; lbn – lower brachial nerve; lcl – large canal of the lophophoral coelom; ln – lateral tentacle nerve; lol – lamella of loop of the lophophore brachidium; mbn – main brachial nerve; ot – outer tentacle; rbn – radial brachial nerves; sa – second accessory brachial nerve; scl – small canal of the lophophoral coelom.
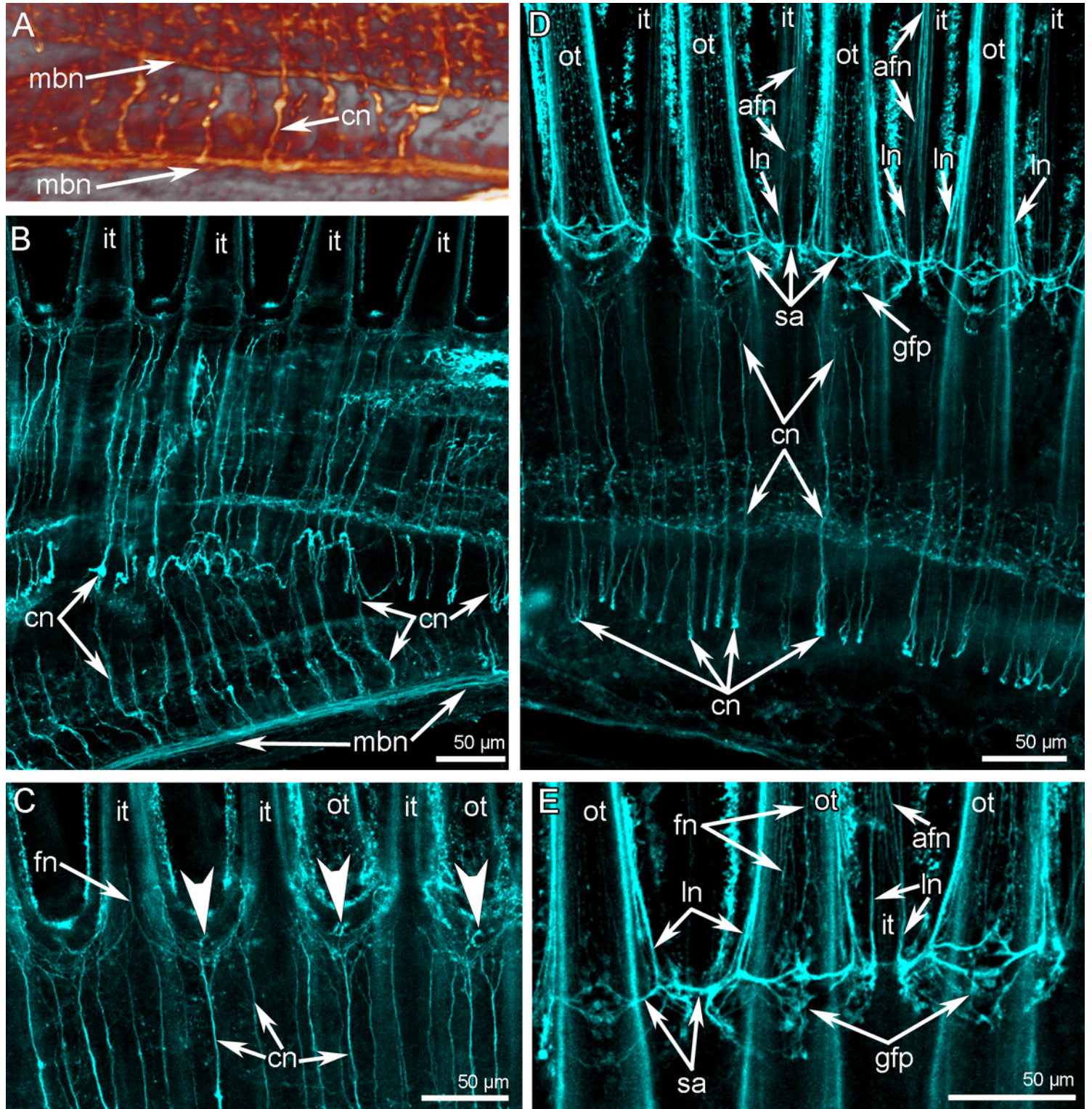
Figure 7

Details of innervation of the lateral arm in Coptothyris grayi. Volume rendering (A) and Z-projections (B-E) after immunostaining against acetylated alpha-tubulin (orange – in A and cyan in B-E). (A) Two main brachial nerves and cross nerves emanating from them. (B) Main brachial nerve and curved cross nerves. (C) The most frontal side of the inner tentacles: cross nerves extend to the places between inner tentacles and give rise to two thick short nerves (arrowheads), which extend to the frontal side of the outer tentacles. (D) The most frontal side of outer tentacles: groups of perikarya at the base of each tentacle are evident. (E) Groups of frontal perikarya and second accessory brachial nerve. Abbreviations: afn – abfrontal tentacle nerve; cn – cross nerve; fn – frontal tentacle nerve; gfp – groups of frontal perikarya; it – inner tentacle; ln – lateral tentacle nerve; mbn – main brachial nerve; ot – outer tentacle; sa – second accessory brachial nerve.
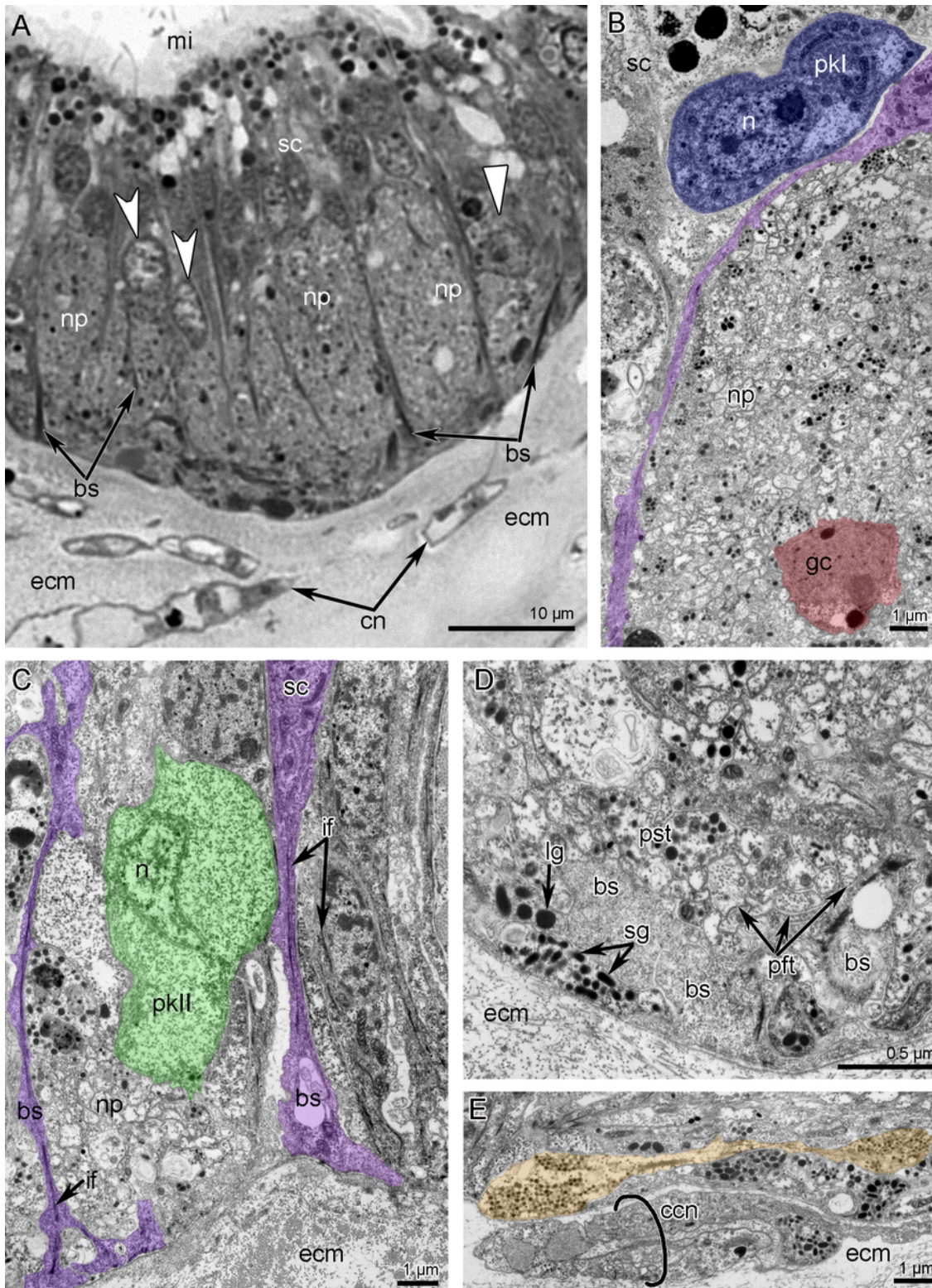
Figure 8

Organization of the main brachial nerve of Coptothyris grayi. Semithin (A) and ultrathin (B-E) transverse sections. (A) General view of the main brachial nerve: two types of pekikarya (perikaryon of first type is indicated by straight arrowhead; perikarya of second type are pointed by concaved arrowheads) and large neuropil are visible. (B) Perikaryon of first type (dark blue) and soma of glial cell (red) within neuropil. Basal projection of supportive cell is pink. (C) Perikaryon of second type (green) and numerous basal

projection of supportive sells (pink). (D) Neuropil: projections of different types are shown. (E) A portion of neuropil containing varicoses of nerve projection of second type (colored). Abbreviations: bs – basal projections of supportive sells; ccn – cells of cross nerve; cn – cross nerve; ecm – extracellular mayrix; gc – glial cells; if – intermediate filaments; lg – granules of large diameter; mi – microvilli; n – nucleus; np – neuropil; pft – projections of first type; pkI – perikarya of first type; pkII – perikarya of second type; pst – projections of second type; sc – supportive cells (= cells of radial glia); sg – granules of small diameter.
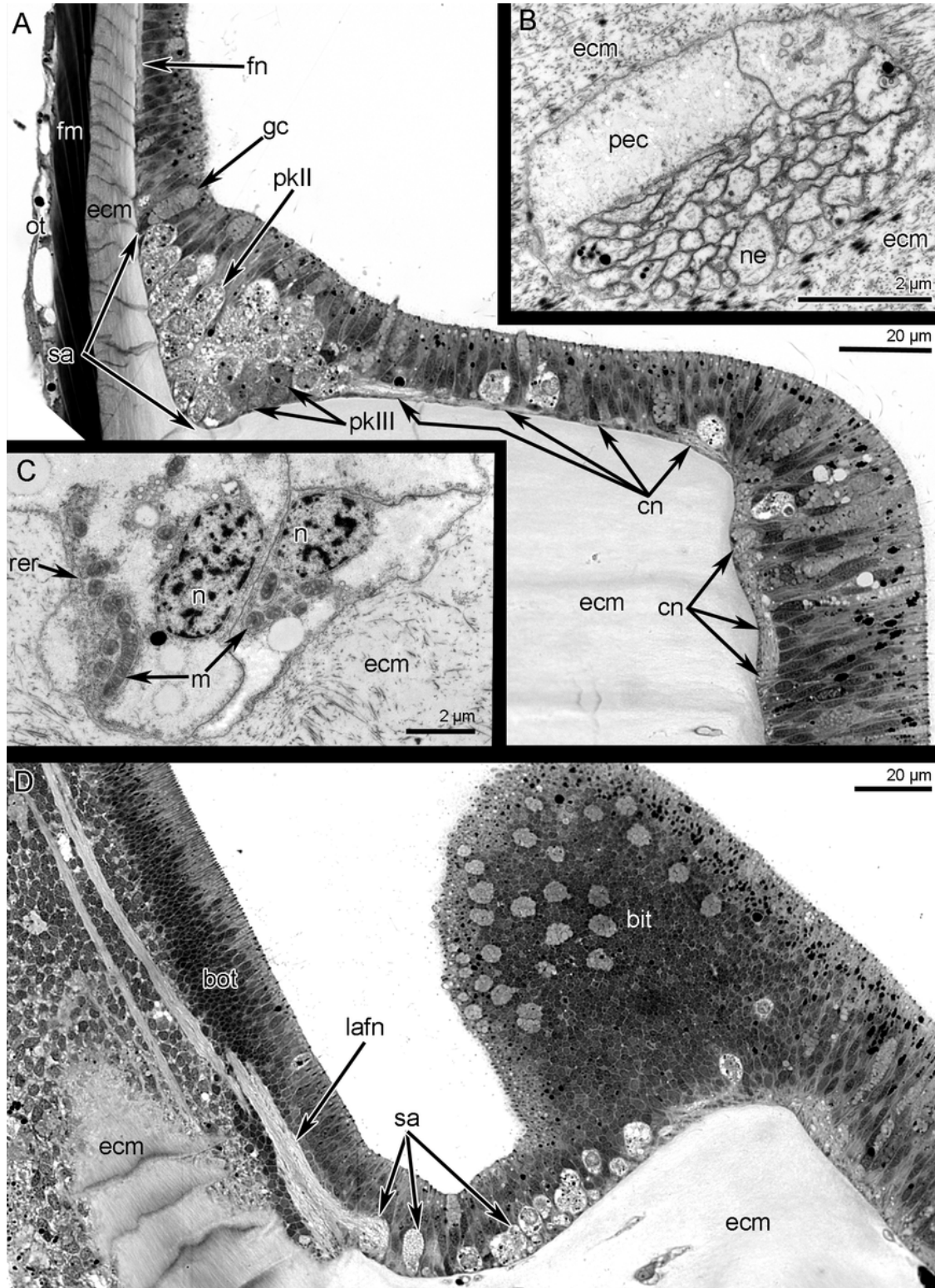

Figure 9

Organization of cross nerves and second accessory brachial nerve of Coptothyris grayi. Semithin (A, D) and ultrathin (B, C) sections. (A) Longitudinal mediofrontal section of the outer tentacle: groups of perikarya of second accessory brachial nerve and cross nerves are visible. (B) The transverse section of the cross nerve: neurites and projection of envelop cells are shown. (C) Ultrastructure of the envelope cells of cross nerve. (D) Longitudinal lateral section of the tentacles base: thick lateroabfrontal tentacle nerve is visible. Abbreviations: bit – base of inner tentacle; bot – base of outer tentacle; cn – cross nerve; ecm – extracellular matrix; fm – frontal tentacle muscle; fn – frontal nerve; gc – gland cell; lafn – lateroabfrontal tentacle nerve; m – mitochondria; n – nucleus; ne – neurite; ot – outer tentacle; pec – projection of envelop cell; pkII – perikarya of second type; pkIII – perikarya of third type; rer – rough endoplasmic reticulum; sa – second accessory brachial nerve.
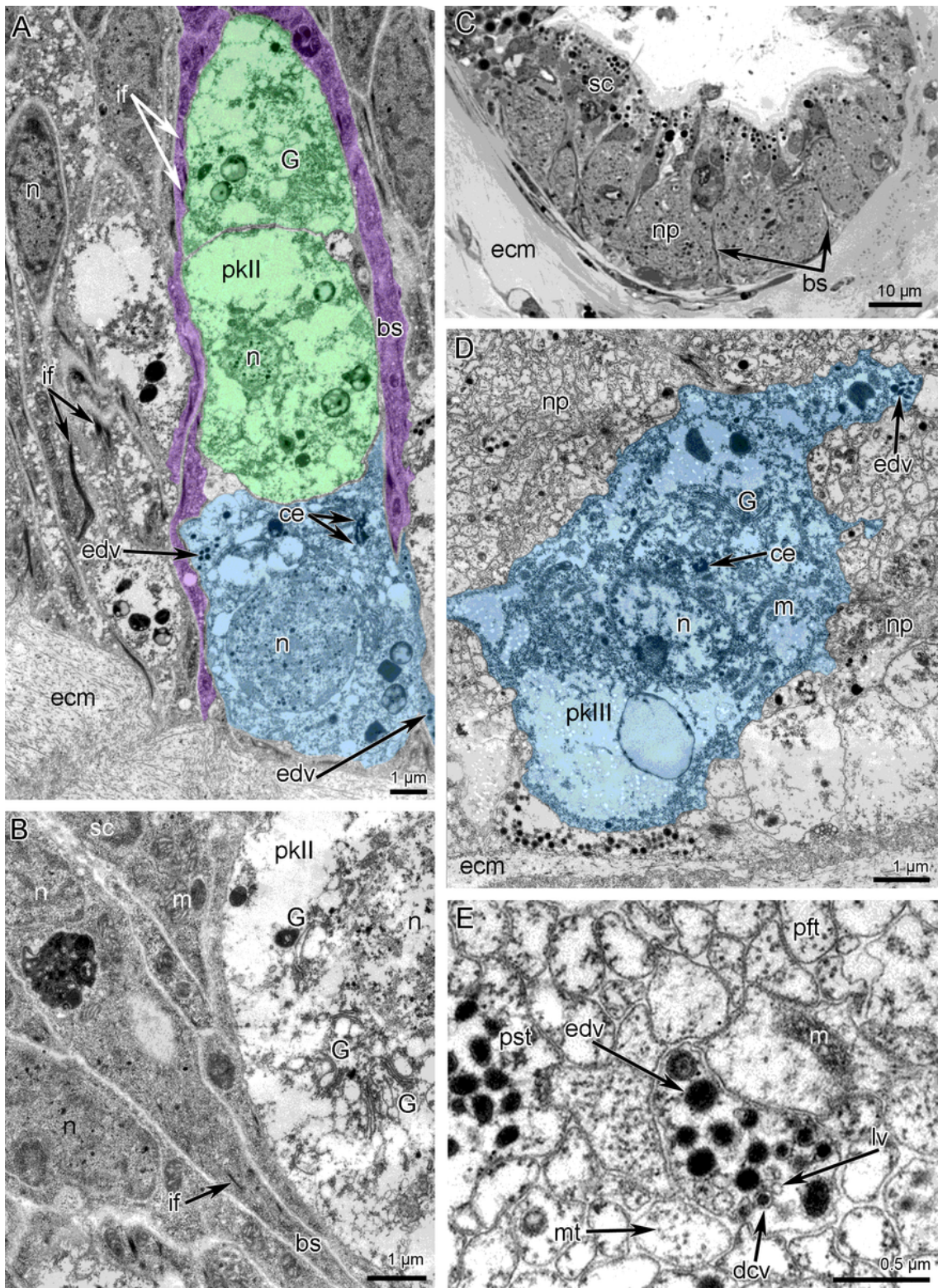
Figure 10

Details of ultrastructure of second accessory (A-B) and lower (C-E) brachial nerves of Coptothyris grayi. Ultrathin (A-B, D-E) and semithin (C) sections. (A) A portion of second accessory brachial nerve with perikarya of second type (green) and perikaryon of third type (blue), which are covered by basal projections of supportive cells (pink). (B) A portion of perikaryon of second type: many Golgi apparatuses are visible. (C) General view of the lower brachial nerve: large neuropil is crossed by long basal

projections of supportive cells (= cells of radial glia). (D) A portion of lower nerve with the perikaryoin of third type (blue). (E) A portion of neuropil: projections of different types are visible. Abbreviations: bs – basal projection of supportive cells; ce – centriole; dcv – dense-core vesicle; ecm – extracellular matrix; edv – vesicles with electron dense content; G – Golgi apparatus; if – intermediate filaments; m – mitochondria; mt – microtubules; n – nucleus, np – neuropil; pft – projections of first type; pkII – perikarya of second type; pkIII – perikarya of third type; pst – projections of second type; sc – supportive cells (= cells of radial glia); sg – granules of small diameter.
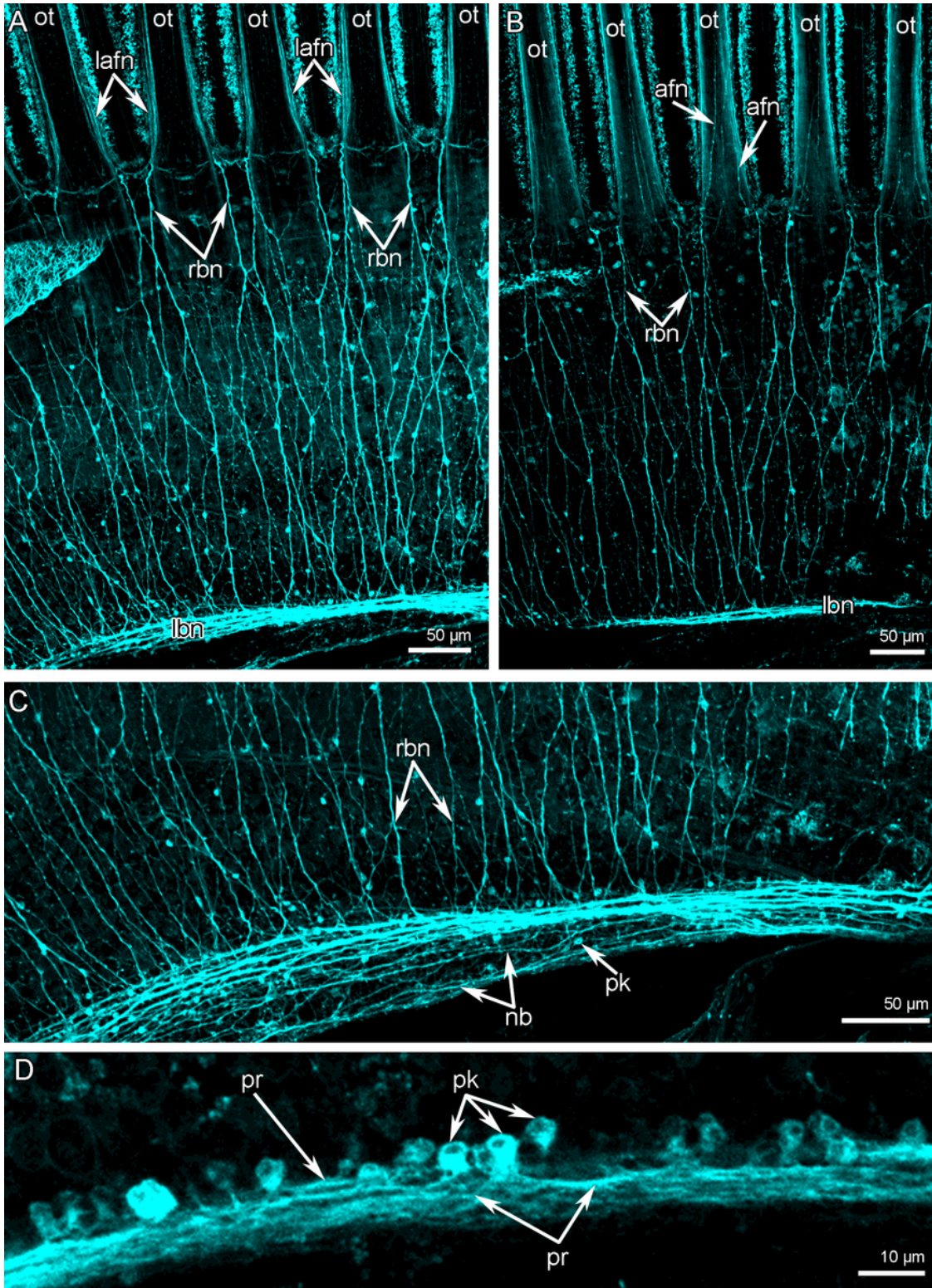
Figure 11

rganization of lower brachial nerve of Coptothyris grayi. Z-projections after immunostaining against acetylated alpha-tubulin (cyan). (A) A portion of the lateral arm viewe from the outer tentacles. (B) The most abfrontal side of outer tentacles. (C) A portion of the lower brachial nerve, which gives rise to the radial brachial nerves. (D) Magnified portion of the lower brachial nerve: perikarya and their projections are visible. Abbreviations: afn – abfrontal tentacle nerve; lafn – lateroabfrontal tentacle nerve; lbn – lower brachial nerve; nb – neurite bundle; ot – outer tentacle; pk – perikarya; pr – projection; rbn – radial brachial nerves.
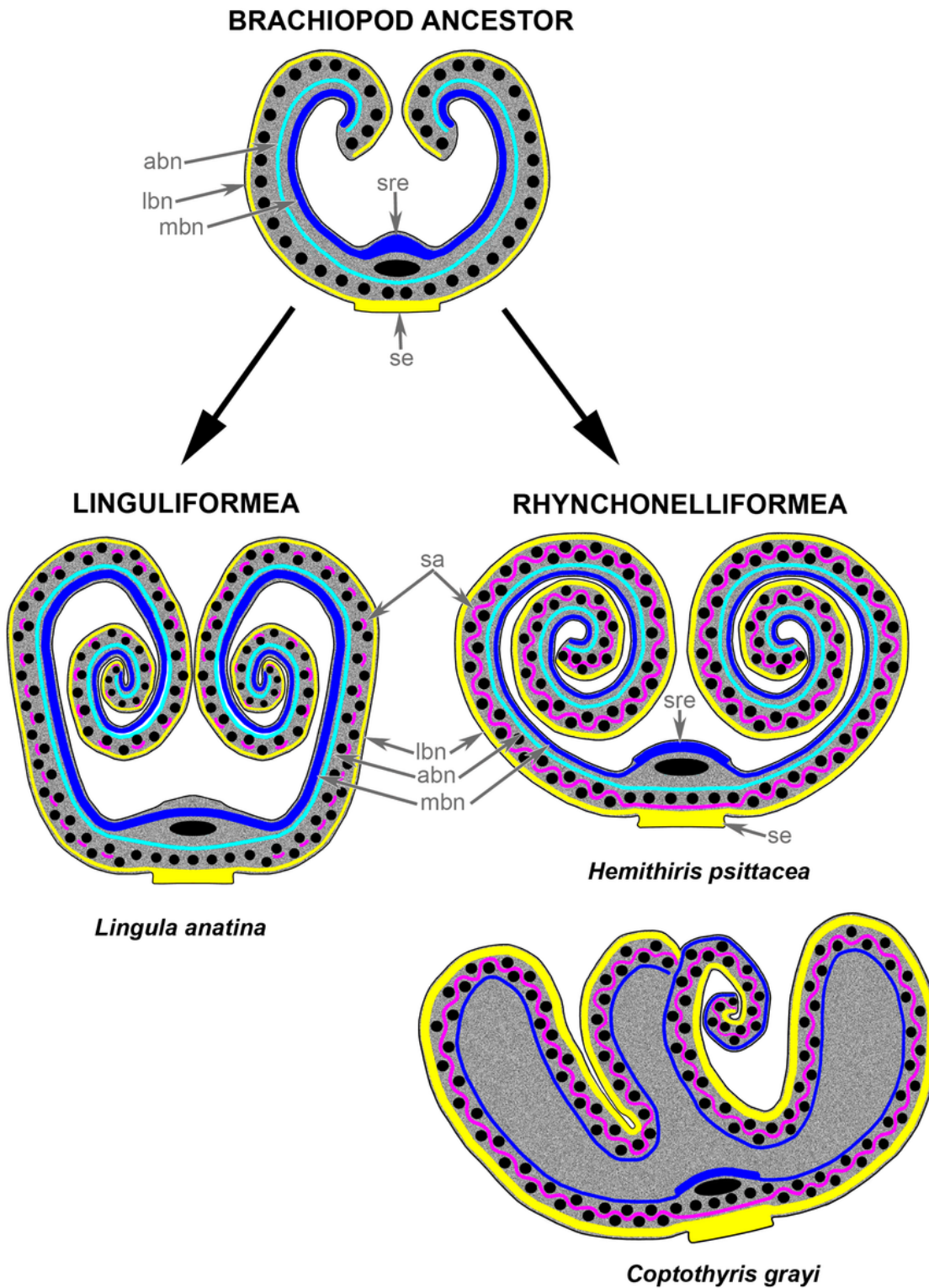
Figure 12

Schemes of evolution of the lophophore and its nervous system in barchiopods. The thickness of lines, which indicate different brachial nerves, makes sense and reflects the degree of intensity of certain nerve. Black circles indicate tentacles. Abbreviations: abn − accessory brachial nerve; lbn − lower brachial nerve; mbn − main brachial nerve; sa − second accessory brachial nerve; se − subenteric ganglion; sre − supraenteric ganglion.