

## Chapter 5

# **Serotonergic and FMRFamidergic nervous systems in gymnolaemate bryozoan larvae**

**Abstract** - A growing body of data from nervous systems of marine invertebrate larvae provides an ideal background for comparisons among higher taxa. The present data from Bryozoa, however, do not allow for a consistent hypothesis of an ancestral state hindering comparisons to other lophotrochozoan taxa. The larval nervous systems of the four gymnolaemate species *Flustrellidra hispida*, *Bugula fulva*, *Alcyonidium gelatinosum*, and *Bowerbankia gracilis* are examined by means of antibody staining against the neurotransmitters serotonin and FMRFamide, as well as against acetylated  $\alpha$ -tubulin. Despite considerable variation, a comparison reveals a common pattern of the distribution of serotonin. The neurotransmitter is found in at least two cells in the apical organ as well as in paired axial and lateral nerves emerging from a central nerve nodule. A ring nerve is present below the corona and at least two serotonergic cells are found between the corona cells. Nerve nodule and serotonergic coronal cells might represent unique bryozoan features, whereas the remaining elements show resemblance to the situation in most spiralian taxa. The data do not provide support for a closer relationship of Bryozoa to Kamptozoa, Phoronida or Brachiopoda.

## **Introduction**

In the last decade the increased utilization of immunohistochemistry in combination with confocal laser scanning microscopy has rendered detailed insights into the neural architecture of many marine invertebrate larvae. Besides important new findings in certain species the availability of these data from a broad range of taxa allows for comparisons between different species or groups in order to address evolutionary or phylogenetic questions. For example, Hay-Schmidt (2000) in his comparative study identified two major types of larval serotonergic nervous systems in the Bilateria. One is represented by Mollusca, Polychaeta, Entoprocta, Platyhelminthes, and Bryozoa whereas the other is found in Phoronida, Brachiopoda, and Deuterostomia.

The phylogenetic position of the Bryozoa is still unresolved. Based on morphology, they have traditionally been grouped together with Phoronida and Brachiopoda as Lophophorata

(Hyman 1959), which is either monophyletic and the sistergroup or a paraphyletic stem group to Deuterostomia (Ax 2001). Other data, especially from larval morphology, are suggestive of Bryozoa (Ectoprocta) as sister taxon to Kamptozoa (Entoprocta) (Nielsen 1971, 2001). Evidences from various molecular data sets consistently group all three lophophorate taxa together with spiralian as Lophotrochozoa (e.g., Halanych et al. 1995, Passamaneck and Halanych 2004, Philippe et al. 2005, Passamaneck and Halanych 2006, Waeschenbach et al. 2006, Helmkampf et al. 2008). In the different studies, however, the affinities of Bryozoa within Lophotrochozoa remain ambiguous.

A detailed analysis of the larval nervous systems adds important new characters to the known morphological data set and therefore might have the potential to provide arguments for sister-group hypotheses. However, with increasing number of examined species in each metazoan group it became obvious that nervous system characters are not always as conservative as earlier studies suggested. Instead, considerable variation concerning the distribution of neurotransmitters has been found between different species of several groups like e.g., within Mollusca (Page and Parries 2000), Kamptozoa (Wanninger et al. 2007), or Echinodermata (Byrne et al. 2007). Thus for comparisons of such characters across higher taxa it is especially important to cover a broad range of representatives in each taxon in order to get a consistent picture of putative ancestral character states.

For bryozoans, except from a brief report on *Bugula neritina* (Pires and Woollacott 1997), the only immunocytochemical data come from the cyphonautes larva of *Membranipora* sp. (Hay-Schmidt 2000) and the coronate larva of *Triphyllozoon mucronatum* (Wanninger et al. 2005b). The latter two studies have revealed crucial differences between the serotonergic nervous systems of these two larvae. However, bryozoans exhibit several more different larval types. Besides the planktotrophic cyphonautes, shelled lecithotrophic larvae and at least five different types of coronate larvae are known within the subtaxon Gymnolaemata (Zimmer and Woollacott 1977b). Taxonomic distribution as well as significant morphological differences argue for a convergent evolution of the various coronate larvae (Zimmer and Woollacott 1977b). It can therefore be expected, that their independent evolution has also resulted in apomorphic characters in their nervous systems.

In order to enhance the taxon-sampling for neural characters in Bryozoa, detailed descriptions of larval serotonergic and FMRFamidergic nervous systems in four gymnolaemate bryozoan species are given in this paper: *Flustrellidra hispida* has a shelled lecithotrophic larva, also referred to as pseudocyphonautes. The larvae of *Alcyonidium gelatinosum*, *Bugula fulva*, and *Bowerbankia gracilis* are classified as coronate larvae of types E, AEO/ps, and AEO/PS, respectively, according to Zimmer and Woollacott (1977b)

## Materials and methods

### Collection of larvae

Reproducing colonies of *Flustrellidra hispida* (Fabricius, 1780) and *Alcyonidium gelatinosum* (Linnaeus, 1761) were collected in the rocky intertidal of Concarneau (Brittany, France) between 2005 and 2006. Both species prefer thalli of *Fucus serratus*, whereas *F. hispida* occurs on the lower parts of the phylloids, and *A. gelatinosum* inhabits the tips. *Bugula fulva* Ryland, 1960 was collected in the harbor of Brest (Brittany, France) on the undersides of floating docks in May 2007. *Bowerbankia gracilis* Leidy, 1855 was collected in the harbor of Helgoland (Germany) in June 2006. Colonies of *F. hispida* and *A. gelatinosum* were kept in vessels with a constant current of fresh seawater entering the vessel at its base. The overflow at the top of the vessel was directed into a chamber with a 100- $\mu$ m plankton net. Shed larvae were obtained from the plankton net twice daily whereas larval release was highest during the morning hours. Colonies of *B. fulva* and *B. gracilis* were kept in 300-ml glass dishes. Emerging larvae are strongly phototactic and were attracted to the vessel wall by fiber-optics illumination. Clouds of larvae were then transferred into smaller dishes for fixation using a glass pipette. Live observations of larvae were accomplished with a Leica MZ 16A dissecting microscope and an Olympus BZ51 compound light microscope.

### Antibody staining

Specimens were fixed in 4% paraformaldehyde in 0.05 M PBS containing 0.33 M NaCl. Duration of fixation was from 30 min to 6 h at room temperature. Some larvae were relaxed using a 7% MgCl<sub>2</sub> solution prior to fixation. Blocking buffer (0.05 M PBS, 0.33 M NaCl, 0.1% Triton-X, 0.1% bovine serum albumine) was applied for two hours before incubating with primary antibodies (anti-serotonin Sigma S-5545, anti-ac- $\alpha$ -tubulin Sigma, anti-FMRamide abcam ab10352). Antibodies were used at a final dilution of 1:500 in blocking buffer. Incubation lasted approximately 12 h at room temperature. Specimens were washed in blocking several times during a period of 10 h to remove excess primary antibodies. Alexa-Fluor labeled secondary antibodies (Molecular Probes A-11008, A11004, A-21050) were applied in the same manner as the primary antibodies. In some cases subsequent counterstaining against f-actin with Alexa-Fluor-568 labeled phalloidin (1 U/500  $\mu$ l, 6-12 h) or against nuclei with propidium iodide (1/200, 20 min) was accomplished. Specimens were rinsed several times in PBS and mounted on glass slides in 90% glycerol, 10% PBS, 0.25% DABCO. Confocal image stacks were taken on a Zeiss LSM 410 and a Leica TCS SPE. 18 specimens of *F. hispida*, 8 of *A. gelatinosum*, 19 of *B. fulva*, and 11 of *B. gracilis* were examined. ImageJ with WCIF plugin bundle was used to process digital image stacks and

to generate projection views. Three-dimensional inspection was done by volume rendering using Voxx2.

### **Electron Microscopy and Histology**

Specimens were fixed either with 2.5% Glutaraldehyde in 0.05 M PBS containing 0.33 M NaCl (1h, 4°C) or, for SEM, with 1% OsO<sub>4</sub> in seawater. Fixative was removed by washing several times with PBS. Post-fixation using 2% osmium tetroxyde in PBS was done for 30-60 min at 4°C. Specimens were subsequently washed with PBS, dehydrated in a graded ethanol series, and embedded into araldite with propylene oxide as intermedium. Specimens were sectioned semithin (0.5µm) and ultrathin (60-70 nm) with a Leica UC6 microtome equipped with Diatome diamond knives. Ultrathin sections were picked with formvar-coated single-slot copper grids, stained automatically with uranyl acetate and lead citrate in a Nanofilm TEM STAINER, and examined on a Phillips CM 120 transmission electron microscope at 60 kV. Semithin sections were placed on glass slides and stained with toluidine blue (1% toluidine, 1% Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 20% sucrose) for 1 min at 60°C. An Olympus BZ51 compound microscope with SIS CCD camera was used for observation and documentation. For scanning electron microscopy, dehydrated larvae were critical point dried, mounted on specimen holders, and subsequently sputter-coated with gold. Specimens were examined using a FEI Quanta 200 SEM at 15 kV.

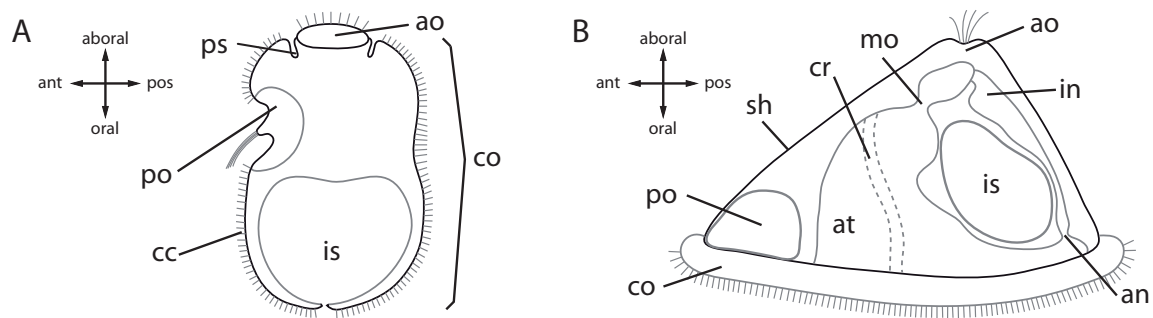
## **Results**

A general overview of the morphology of gymnolaemate larvae is given in Fig. 1. For further descriptions of larval morphology in the following species see Gruhl (2008) and references therein.

### ***Flustrellidra hispida***

*Serotonin immunoreactivity* (Fig. 2C-G, 3B, 9A) is found in the apical organ, the central larval nerve nodule, the corona, and in nerve processes connecting these elements. The corona bears nerves underlying the ring of ciliated cells as well as two cell bodies on each side of the larva that are located between these cells. A meshwork of delicate serotonergic fibers is found in both the oral and aboral epithelium.

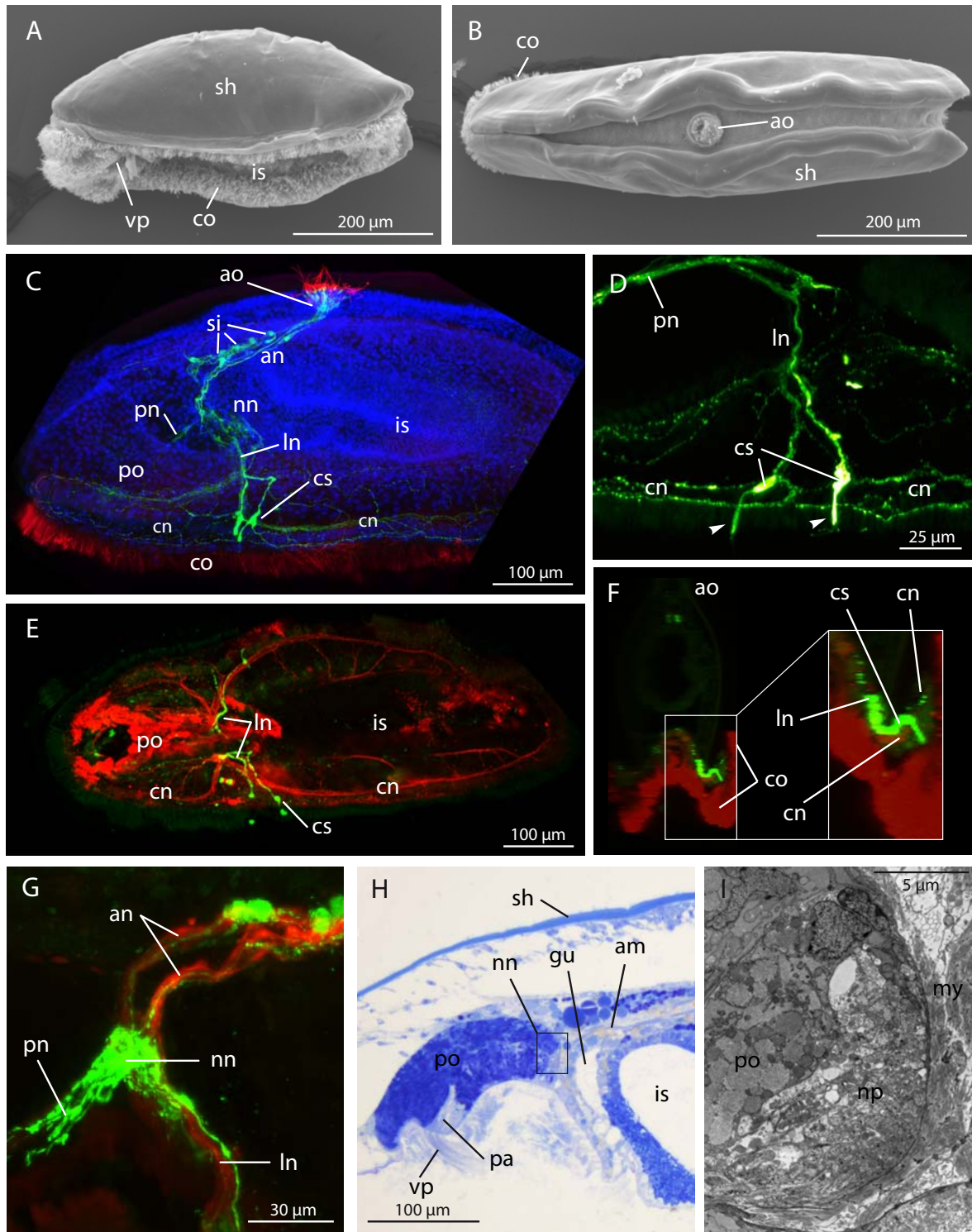
The nerve nodule (nn, Fig. 2C, G-I) is located in the anterior third of the larva. It is situated at the posterior side of the pyriform organ (po, Fig. 2H), that is oriented towards the larval rudimentary gut and internal sac. A dense serotonergic neuropil, but no immunopositive cell somata can be observed in this area. TEM analysis suggests that the neuropil is actually a part of the pyriform organ, because it is separated by a conspicuous extracellular matrix



**Fig. 1** General morphology of bryozoan larvae, here represented by two types. Because the terms dorsal and ventral can be misleading in bryozoan larvae, body axes are termed by convention aboral or apical for the former animal region of the embryo bearing the apical organ and oral for the opposite side, that is derived from the vegetal. The anterior (*ant*) region is defined by the presence of the pyriform organ, the opposite region is posterior (*pos*). A ciliary band, the corona (*co*), consisting of multiciliated cells is the main organ for swimming. All larvae bear an apical organ (*ao*), that can be either simple, i.e., consisting of a small number of cells, or complex. The pyriform organ (*po*), a complex of ciliated and glandular cells is situated anteriorly. At the posterior side, the internal sac (*is*), an epithelial invagination, functioning as adhesive organ during settlement, can be found. **A** Coronate larvae are lecithotrophic. The corona forms the largest part of the larval surface. The gut is either absent or vestigial. The aboral epithelium is invaginated in some cases, forming the so-called pallial sinus (*ps*). Between pyriform organ and internal sac a small ciliated cleft (*cc*) is located. **A** Cyphonautes larvae are planktotrophic and have a functional digestive tract (*in*). The aboral epithelium secretes the lateral shells (*sh*). The oral surface is densely ciliated and invaginated forming the atrium (*at*) with its ciliated ridges (*cr*), where plankton particles are captured. *an* anus, *mo* mouth.

from the interior of the larva, but not from the glandular epithelium of the pyriform organ (Fig. 2I). Five nerve processes emerge from the nodule. A compact unpaired nerve (pn, Fig. 2G) consisting of a large number of neurites penetrates the glandular region of the pyriform organ. It runs towards the so-called papilla, the part of the pyriform organ which bears the vibratile plume, a prominent ciliary tuft (Fig. 2H). A pair of axial nerves (an, Fig. 2C, G, 3B) each consisting of 2-3 serotonergic axons leave the nerve nodule in aboral-posterior direction and proceed towards the apical organ. The axons are connected to bipolar serotonergic interneurons (si, Fig. 2C, 3B) that are found beaded alongside the nerves.

The axial nerves terminate in the basal region of the apical organ (ao, Fig. 2C, 3B). TEM analysis shows a dense neuropil in that region (np, Fig. 3C). Several different types of cells can be differentiated in the apical organ (Fig. 3A, C). The outermost ring of cells resembles unciliated, relatively unspecialized epidermal cells with a large apical surface bearing microvilli. Between these occur three lateral pairs of small ciliated processes bearing each one cilium (inset, Fig. 3A). More ciliated cells are found more centrally. In the very center of the apical organ one large cell with vertically arranged myofilaments is situated (mc, Fig. 3C). Its contraction causes a depression in the center of the apical organ. Two types of cells show serotonin immunoreactivity (Fig. 3B): (1) Two flask-shaped cells (*as1*) are situated laterally



**Fig. 2** *Flustrellidra hispida* larva, unless stated otherwise anterior ends are oriented to the left. **A,B** SEM micrographs. **A** View from lateral/oral. **B** View from aboral, the apical organ is situated on the posterior midline between the two shells (*sh*) **C-G** Projections of three-dimensional confocal image stacks. Fluorescence staining with antibodies against serotonin (*green*) and  $\alpha$ -tubulin (*red*), and with the nuclear dye propidium iodide (*blue*). **C** Lateral view of larva, only elements from left half shown. Serotonergic nervous system comprising apical organ (*ao*), axial nerves (*an*) with serotonergic bipolar interneurons (*si*), central nerve nodule (*nn*), papilla nerve (*pn*), lateral nerves (*ln*), corona nerves (*cn*) and two serotonergic cell bodies (*cs*) within the corona. A network of delicate serotonergic

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of the central region. Their perikarya are located in a basiepithelial position. They are connected to the epithelial surface by a 1-2 $\mu$ m thick and 20  $\mu$ m long dendrite. The distal ends of the dendrites are broadened and bear on cilium. The cells represent bipolar, probably sensory neurons. Because of their shape and position it is likely that their dendrites are identical with two of the ciliated processes seen laterally in SEM micrograph (inset Fig. 3A). From each cell's basal perikaryon one axon emerges into the axial nerve and another one in opposite direction to the posterior part of the apical organ (Fig. 3B). The posterior axons unite to a single neurite that proceeds in apical direction between the epithelial cells located in the posterior portion of the organ. It terminates shortly underneath the apical epithelial surface.

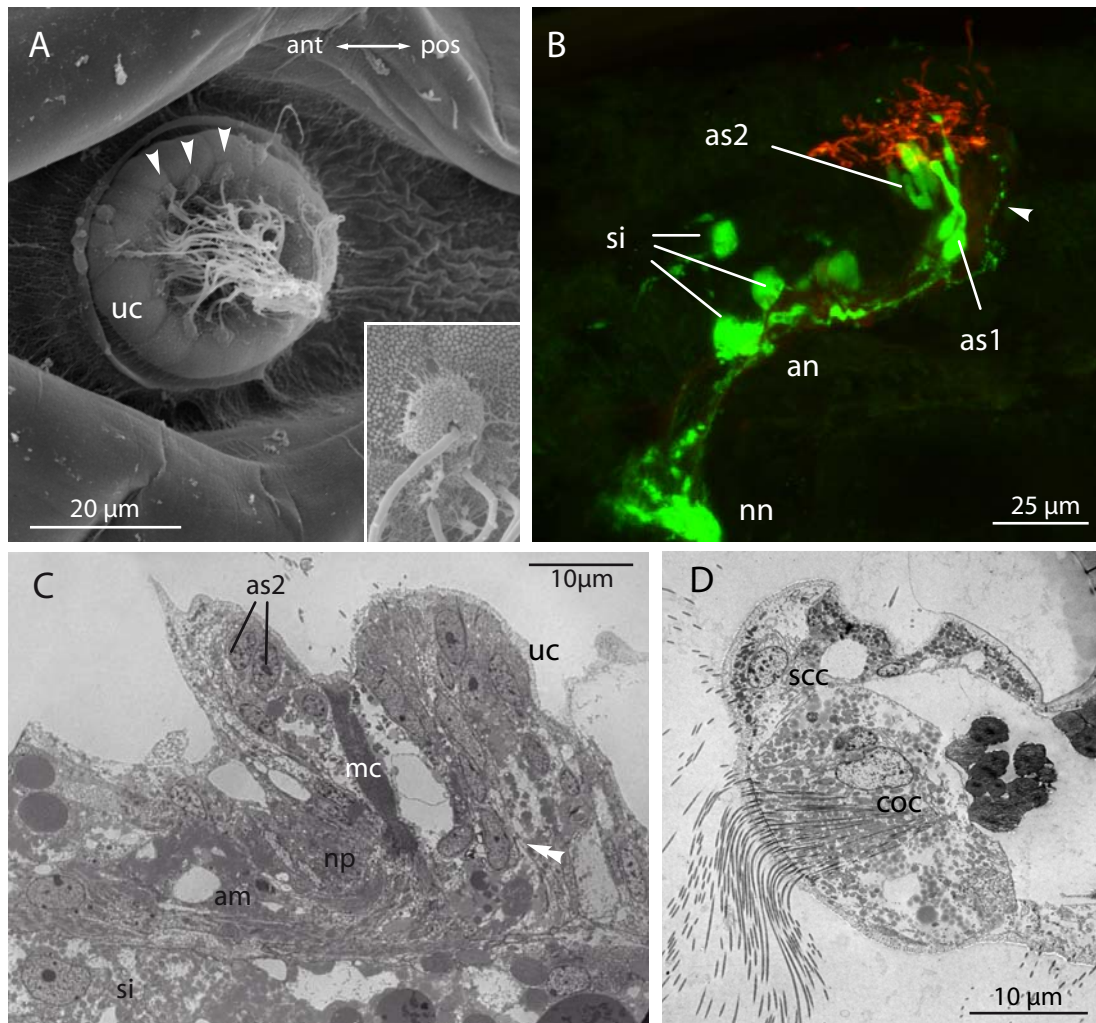
(2) The second type of serotonergic cells (as2, Fig. 3B) in the apical organ comprises four serotonergic neurons that are located in anterior position to the central depression of the apical organ. The cells are ciliated with their nuclei being located in their apical regions. Their basal regions taper into thin processes that seem to be somehow connected to cells of the first type. This group of cells can, by their shape and position, also be identified in TEM sections (Fig. 3C).

On each side of the nerve nodule, a lateral serotonergic nerve (*ln*, Fig. 2C, G) emerges proceeding in direction toward the corona.  $\alpha$ -tubulin immunoreactivity shows that these nerves contain much more fibers, which are not serotonergic (Fig. 2E, G). The nerves branch into three large tracts: The first one runs in anterior direction alongside the pyriform organ. The second and third curve in anterior and posterior direction respectively and run, as coronal nerves, parallel to the corona. In each of the tract at least one delicate serotonergic fiber can be found. The two brightest serotonergic axons in the lateral nerve (Fig. 2D) run parallel at first, then one runs in more anterior, one in more posterior direction. They terminate in two cell bodies (*cs*, Fig. 2D, F) that are located in close proximity to the corona cells.



fibers is found especially in the oral epithelium, between the corona and internal sac and pyriform organ, respectively. **D** Detail of lateral nerve (*ln*) and coronal serotonergic elements in lateral view. Serotonergic axons of the lateral nerves terminate in two serotonergic cells (*cs*). These are connected to corona nerves and project dendrites towards epidermal surface. Each dendrite (*arrowheads*) bears two cilia. **E** Whole mount, view from oral. For better visibility of  $\alpha$ -tubulin immunolabeling in neurites, large portions of the coronal cilia have been removed. The lateral nerves (*ln*) split into anterior and posterior nerves (*cn*) that run parallel to corona. Additional fibers are present around the opening of the internal sac (*is*) and the pyriform organ. **F** Projection of confocal cross sections of the region of the lateral nerves. The serotonergic cell bodies reside beneath the coronal cells and protude dendrites into ciliary band. **G** Lateral view. Detail of central nerve nodule (*nn*) in giving rise to paired axial nerves (*an*), lateral nerves (*ln*) and papilla nerve (*pn*). **H** Semithin sagittal section of anterior region of larva, light micrograph, toluidine blue staining. The central nerve nodule (*nn*) is adjacent to the posterior side of the glandular epithelium of the pyriform organ (*po*). **I** TEM micrograph of nerve nodule, representing region marked by rectangle in h. The nerve nodule consists of a dense neuropil (*np*). Muscle fibers (*my*) of anterior median muscles (*am*) attach to basal membrane surrounding pyriform organ and nerve nodule. *gu* rudimentary gut, *pa* papilla, *vp* vibratile plume





**Fig. 3** *Flustrellidra hispida*. **A-C** Apical organ of larva. Orientation as indicated in A. **A** SEM micrograph, apical view. The apical organ is contracted centrally. Small apical parts of monociliated flask-shaped cells (*arrowheads* and *inset*) occur between unciliated peripheral cells (*uc*). More ciliated cells are located in central region of the organ. **B** Projection of confocal image stack, lateral view, showing serotonin (*green*) and  $\alpha$ -tubulin (*red*) labeling in apical organ and underlying paired axial nerve (*an*). Two types of serotonergic cells reside laterally (*as1*) and anteriorly (*as2*) within the apical organ, serotonergic dendrites are present in the posterior region (*arrowhead*). Bipolar interneurons (*si*) are found alongside the axial nerve. **C** TEM micrograph of sagittal section showing central myoepithelial cell (*mc*), anterior median muscle (*am*) presumably serotonergic cells (*as2*), and interneurons (*si*). Nuclei in a basal position comparable to those of *as1* cells (*double arrowhead*). **D** TEM micrograph showing cross section of corona. Situated above (apically to) the row of corona cells (*coc*) is a row of less ciliated supra-coronal cells (*scc*). The oral epithelium bears clusters of ciliated cells. *ant* anterior, *nn*, nerve nodule, *np* neuropil, *pos* posterior

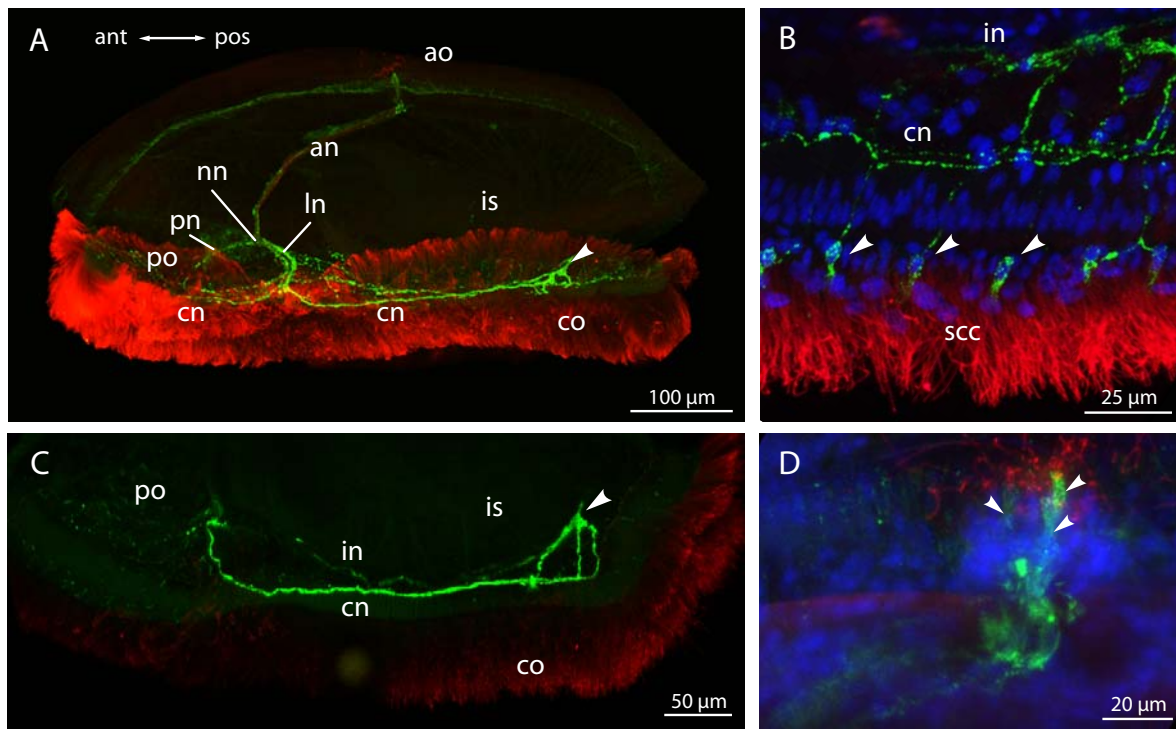
Their perikarya are situated underneath the corona cells; apical dendrites 10 $\mu$ m in length and 1-2 $\mu$ m in diameter protrude between the corona cells, in oral orientation, toward the epithelial surface, where they each bear two cilia. The cilia are much shorter than those of the multiciliated corona cells. Beneath the row of corona cells a few delicate serotonergic processes are found that are connected to the anterior and posterior coronal nerves on both sides of the serotonergic cells (Fig. 2D). The cells appear to form synapses with these processes.



A second nerve ring runs underneath the supracoronal ring of ciliated cells (Fig. 2D, F, 3D). Furthermore a delicate meshwork of serotonergic fibers is found in most of the oral surface. There are  $\alpha$ -tubulin-positive nerve strands that run parallel to the internal sac (Fig. 2E). They branch sequentially towards the posterior nerve. The last fiber branches at the posterior end of the internal sac.

The most prominent **FMRF immunoreactivity** (Fig. 4A-D, 10A) is found in the lateral nerves, and in the corona. It is also present, though much weaker, in the nerve nodule, the neuromuscular strand, and the apical organ.

The apical organ bears three centrally located flask-shaped cells (Fig. 4D), that possess long dendrites connecting to the apical surface of the organ. These cells are ciliated and resemble the type 1 serotonergic cells in their overall appearance. Furthermore a diffuse plexus



**Fig. 4** *Flustrellidra hispida*. Projections of confocal image stacks. Fluorescence staining with antibodies against FMRFamide (green) and  $\alpha$ -tubulin (red), and with the nuclear dye propidium iodide (blue). **A** Whole mount, lateral view. FMRF-immunoreactive elements of nervous system are in apical organ (ao), axial nerves (an), nerve nodule (nn), papilla nerve (pn), corona nerve (cn) and delicate fibers in oral epithelium. FMRFamidergic cell bodies are found within the apical organ, the corona, and, much brighter stained, on the oral side at the posterior end of the internal sac (arrowhead). The latter connect also to the posterior corona nerve. **B** Detail of corona, view from oral. Nerve fibers terminating in small FMRFamidergic cell bodies (arrowheads) branch at regular intervals from the anterior as well as the posterior corona nerve. The cells reside within the ring of supracoronal cells (scc) (see Fig. 3D). **C** Whole mount, view from oral side. **D** Detail of apical organ, lateral view. FMRF immunoreactivity is detectable in at least three flask-shaped cells (arrowheads) and within the neuropil situated basally to the organ. ant anterior, in internal sac nerve, is internal sac, po pyriform organ, pos posterior

is situated in the basal region of the organ. A number of nuclei can be located within this plexus, indicating that it might contain nerve cell somata or interneurons. However the signal is too weak to allow unambiguous localization.

Faint labeling is present in the axial nerve and the nerve nodule (Fig. 4A). In contrast to the pattern of serotonin immunoreactivity, only a few neurites are labeled within the nerve nodule. An unpaired process enters the pyriform organ. Additionally, a fine meshwork is found around the pyriform organ.

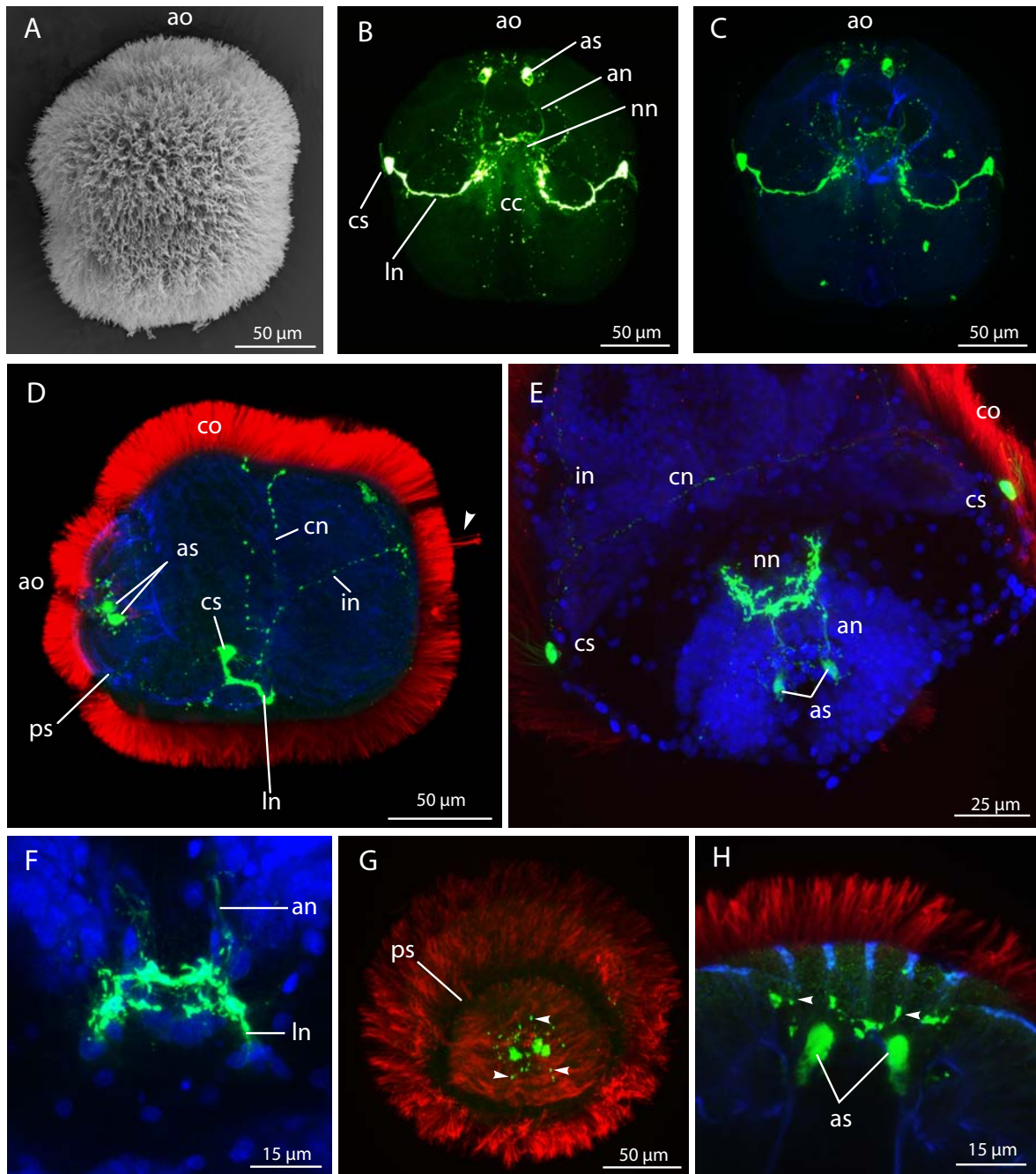
Brightly labeled lateral nerves, consisting of a few neurites, emerge from the nerve nodule heading toward the corona (ln, Fig. 4A, C). They branch into a weaker anterior and a brighter posterior nerve, both running parallel to the corona. At intervals of about 25  $\mu\text{m}$ , single processes branch off from these nerves in aboral direction (Fig. 4B). They connect to comparatively small neurons that are situated within the supracoronal band of ciliated cells. These neurons are 10-15  $\mu\text{m}$  in height, flask-shaped, with the nucleus situated basally. Their apical dendrite bears a few cilia. A further FMRFamideergic nerve (in, Fig. 4B, C) runs parallel to the opening of the internal sac. Between this and the outer corona nerve, as well as adjacent to the pyriform organ in the anterior part of the larva, a delicate meshwork of nerve fibers is present within the infracoronal epithelium. At the posterior end of the internal sac nerve a conspicuous FMRFamideergic neuron is found (Fig. 4A, C). Three brightly labeled fibers project outward from this cell, connecting to the coronal nerve.

As shown by labeling of  $\alpha$ -tubulin (Figs. 3E, 4), large portions of the larval nervous system are neither serotonergic nor FMRFamideergic. Especially the lateral nerves consist of a high number of neurites of which only a few became labeled with antibodies against one of the two neurotransmitters. Furthermore TEM analysis shows a high number of cell bodies within the apical organ, that are not immunoresponsive.

### *Bugula fulva*

**Serotonin immunoreactivity** (Figs. 5B-H, 6A-D, 9B) is found in the central nerve nodule located between pyriform organ and internal sac. Two axial nerves (an, Fig. 5B) connect to two cell bodies situated in the apical organ. Two lateral nerves run from the nerve nodule to the corona. A ciliated serotonergic cell is found in the corona. Furthermore delicate nerves are found in various regions of the larval surface.

The nerve nodule (nn, Fig. 5A, E, F) is located at the posterior side of the pyriform organ. It consists of a dense neuropil and resembles an arc with more or less axial elements in the lateral periphery and a transverse bridge connecting both these sides. Propidium iodide staining indicates several nuclei situated directly adjacent to the serotonergic neuropil (Fig. 5F). However, as no identifiable serotonergic perikarya occur, it is not clear whether these nuclei belong to nerve cells.



**Fig. 5** *Bugula fulva* larva. **A** SEM micrograph, view from posterior. **B-H** Projections of confocal image stacks. Fluorescence staining with antibodies against serotonin (green) and  $\alpha$ -tubulin (red). **B** Whole mount, view from oral side. Serotonergic nervous system comprises the apical organ (ao) with to cell bodies (as), paired axial nerves (an), central nerve nodule (nn), lateral nerves (ln). Ciliated serotonergic cells (cs) are found laterally in the corona. Delicate fibers are found around the ciliated cleft (cc) and around the pyriform organ in the aboral hemisphere. **C** Overlay of serotonin (green) and phalloidin-stained musculature (blue). **D** Whole mount, lateral view, left half of larva.  $\alpha$ -tubulin staining is only shown from central sagittal optical sections. Phalloidin-stained musculature (blue). Inner elements like nerve nodule are not visible because of opacity of larva. The lateral nerve branches of the posterior corona nerve (cn). The latter gives rise to a nerve (in) encompassing the internal sac to its oral opening. Here tufts of longer cilia are found (arrowhead). **E** Aboral hemisphere of larva, oblique view from aboral/posterior. blue = nuclei stained with propidium iodide. **F** Detail of central nerve nodule. blue = nuclei stained with propidium iodide. A serotonergic neuropil



Two serotonergic axons run from the lateral regions of the nodule in anterior direction toward the apical organ. They connect to two serotonergic cells (as, Fig. 5B, D, E, G, H) residing laterally in the apical organ, in proximity to its center. A considerable portion of the examined larvae (4 out of 19) showed three instead of two such cells in the apical organ (see Fig. 5G). The perikarya are found in a basiepithelial position. Several tiny dendrites emerge from the perikarya and run in apical direction towards the epithelial surface between the surrounding epithelial cells (Fig. 5G, H). It could not be determined whether the dendrites really reach the surface and whether they bear cilia. The surrounding cells in the central region are multiciliated and some bear vertically arranged bundles of myofilaments (Fig. 5H).

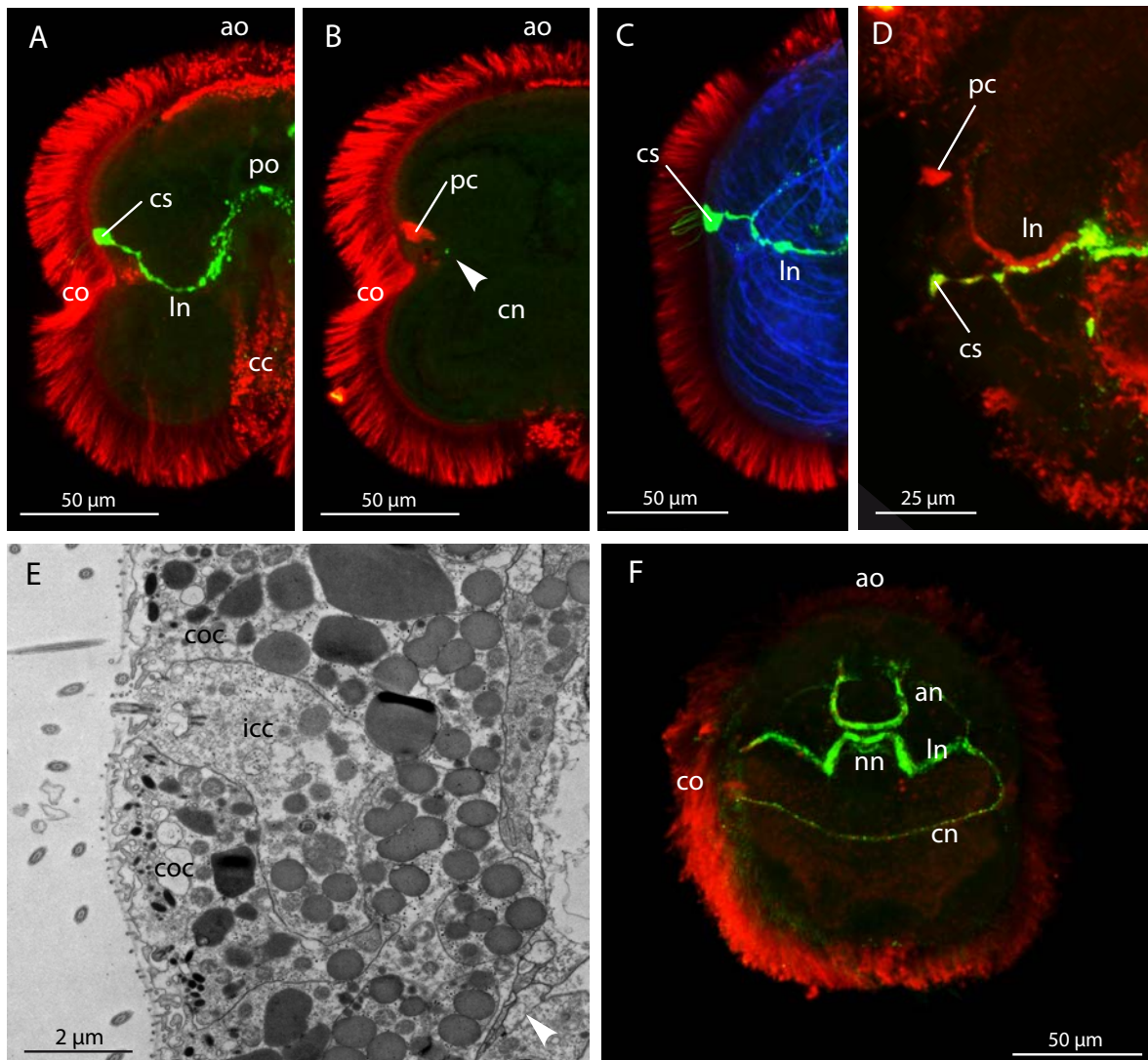
Two lateral serotonergic nerves (ln, Fig. 5B, D, 6A, C, D) run from the nerve nodule initially in oral direction, embracing the pyriform organ. They consist of a few serotonergic fibers. When reaching the anterior surface, the nerves curve in lateral direction towards the corona. Here each nerve branches and the brightest branch connects to an ovoidly shaped ciliated serotonergic cell that resides between the multiciliated corona cells. The cell bears 5-8 cilia at its apical surface (Fig. 6C). The cilia are short, measuring about 15  $\mu\text{m}$  in length in comparison to those of the surrounding corona cells that are 20-25  $\mu\text{m}$  long. The cells resemble the widely distributed intercoronal cells so-called in their ultrastructural appearance (Fig. 6E). Further delicate serotonergic fibers branch off from the lateral nerves (Fig. 5D). The first branch is the posterior coronal nerve that encircles the larva equatorially. On each side of the larva, one offshoot of the coronal nerve curves around the internal sac on its posterior side and terminates laterally to its opening at the oral side of the larva. At this location tufts of longer cilia are situated. The second branch of the lateral nerve runs in anterior/apical direction forming an arc around the pyriform organ (Fig. 5D, 6C). Diffuse labeling is also found around the pyriform organ.

**FMRFamide**-containing parts of the nervous system (Fig. 6F, 10B) comprise both the axial nerves running from the nerve nodule towards the apical organ and the lateral nerves that run towards the corona. These branch when reaching the corona, giving rise to one delicate anterior fiber, encompassing the pyriform organ and one posterior running parallel to the corona. FMRFamidergic cell bodies could not be observed.

**Other parts** of the nervous system that are neither serotonergic nor FMRFamidergic can be visualized by anti-acetylated- $\alpha$ -tubulin staining. The most prominent elements shown

← is present. Nuclei occur in close proximity to the neuropil, no serotonergic perikarya can be located. **G** Whole mount, view from aboral. Serotonergic cells are located centrally within the apical organ, a region referred to as the neural plate. This larva bears three instead of the usual two serotonergic cells. Several dendrites (*arrowheads*) radiate from the cells towards the surface. **H** Detail of neural plate, horizontal section. Phalloidin-stained musculature and cytoskeletal actin filaments (*blue*). The serotonergic perikarya (*as*) reside in a basiepithelial position. Several dendrites radiate (*arrowheads*) from the cells towards the surface. *co* corona, *ps* pallial sinus





**Fig. 6** *Bugula fulva* larva. **A-D** Projections of confocal image stacks. Fluorescence staining with antibodies against serotonin (green) and  $\alpha$ -tubulin (red), and phalloidin staining of musculature (blue). Details of lateral nerves, corona (co), and photoreceptors. **A** Projection of anterior horizontal optical sections showing lateral nerves (ln) and serotonergic cells (cs) residing in the corona. **B** Projection of posterior horizontal optical sections. Curled ciliary tuft of photoreceptor (pc) is visible in  $\alpha$ -tubulin staining. **C** Overlay of serotonin with f-actin signal. **D** Oblique view from aboral/anterior showing innervation of both photoreceptor and serotonergic coronal cell **E** TEM micrograph of intercoronal cell (icc), comparable in shape and position to those cells identified with serotonin staining. The cell resides between the multiciliated corona cells (coc). Subepithelial nervous processes are present (arrowhead). **F** Fluorescence staining with antibodies against FMRFamide (green) and  $\alpha$ -tubulin (red). Projection of confocal image stacks, whole mount, view from posterior. FMRFamide reactivity is found in axial nerves (an), nerve nodule (nn), lateral nerves (ln) and posterior coronal nerve (cn). ao apical organ, cc, ciliated cleft, co corona.

with this method are the lateral photoreceptor cells (pc, Fig. 6B, D). The cell bodies of the photoreceptor cells can not be visualized directly, but the putative receptive structure consisting of a bundle of heavily coiled cilia is very prominent. The photoreceptors are found in an intercoronal position, comparable to the coronal serotonergic cells but more posterior.

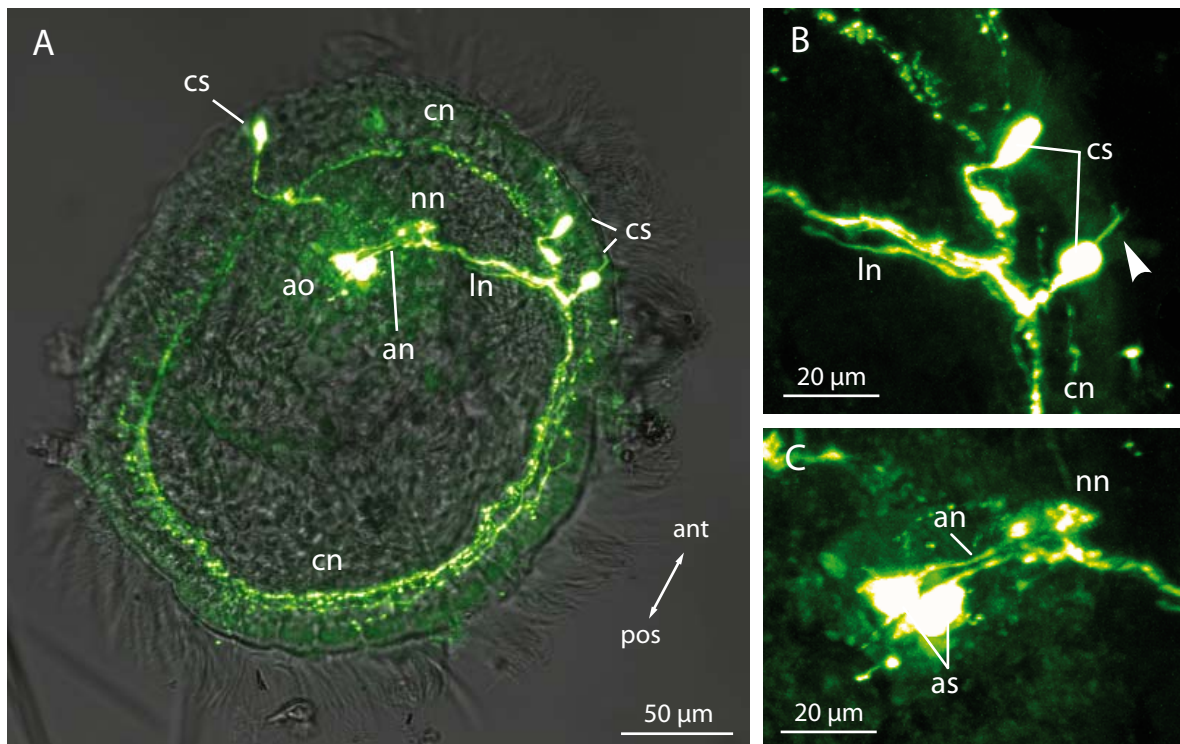


They are connected to the coronal nerve in the same pattern as the serotonergic coronal cell. As synapses are difficult to locate in  $\alpha$ -tubulin staining it can not be ascertained whether the nerves coming from the photoreceptors connect directly to the coronal nerve or just run parallel to it.

### *Alcyonidium gelatinosum*

**Serotonin immunoreactivity** is found in the central nerve nodule, two lateral nerves, the corona, the apical organ and, two axial nerves (Fig. 7, Fig. 9C).

The nerve nodule (nn, Fig. 7A, C) resides between pyriform organ and internal sac, in the height of the coronal ciliary band when viewed from lateral. It is composed of a neuropil; no serotonergic cell bodies are visible.



**Fig. 7** *Alcyonidium gelatinosum* larva. Projections of confocal image stacks. Fluorescence staining with antibodies against serotonin (green). **A** Whole mount, merged with transmitted light micrograph. Serotonin immunoreactivity is seen in apical organ (ao), axial nerves (an), nerve nodule (nn), lateral nerves (ln) and corona nerve (cn). The corona nerves comprise an anterior and posterior arc, each consisting of several neurites running concentrically at few  $\mu\text{m}$  distance. **B** Detail of corona. 1-2 serotonergic cells (cs) are found laterally at the junction of lateral and corona nerves. A dendrite (arrowhead) is emerging from the cell in apical direction. **C** Detail of apical organ, axial nerves, and nerve nodule. The apical organ contains ovoid serotonergic perikarya (as), each of which projects an axon towards the nerve nodule. ant anterior, pos posterior

Two ovaly shaped serotonergic cell bodies (as, Fig. 7C) are found centrally in the apical organ. From the bases of the perikarya of each of these cells one axon projects into the nerve nodule. The cells bear no apparent cilia or dendrites.

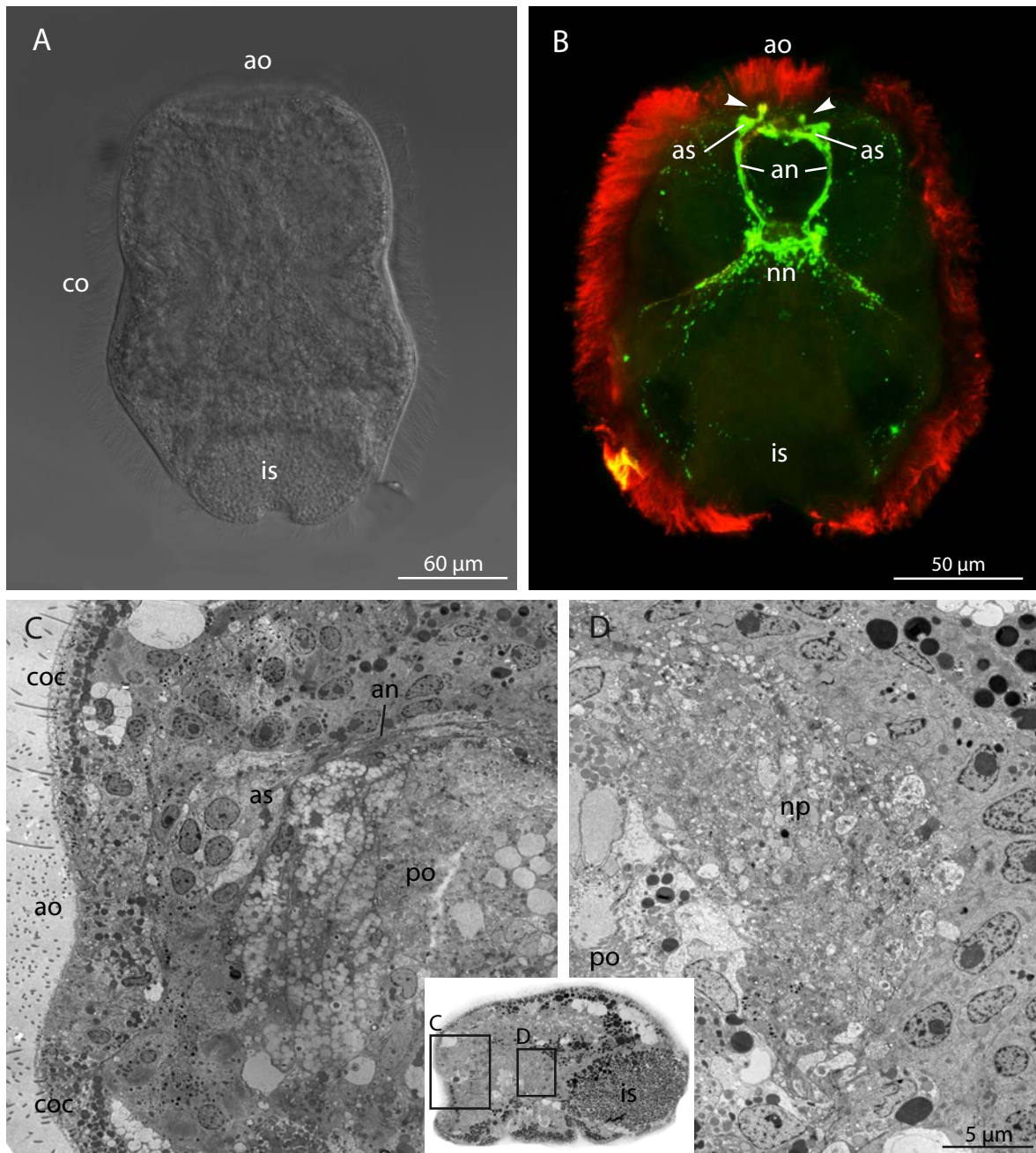
Two lateral nerves (la, Fig. 7A, B), consisting of at least three serotonergic nerve fibers run from the nerve nodule toward the corona on each side of the larva. When reaching the basal side of the coronal cells one neurite runs parallel to the corona in posterior direction. A second one curves anterior, proceeding in the same pattern on the anterior side of the larva. Anterior and posterior nerve constitute a complete ring. At discrete intervals, both of these nerves branch off delicate processes that run a few  $\mu\text{m}$  apically and orally respectively. They connect to delicate fibers running parallel to the main ring nerves. Near the region where the lateral nerves reach the corona one or two serotonergic cells (cs, Fig. 7A, B) are situated between the coronal cells. The oval cell bodies are found slightly aboral from the nerve ring. Each bears a few cilia that are much shorter than the coronal cilia. The cells are also connected to the nerve nodule. Each cell body sends out one axon that initially runs within the nerve ring and then curves into the lateral nerve running towards the nerve nodule. It could not be substantiated if synapses between these axons and the other serotonergic processes exist in the nerves. The number of the serotonergic coronal cells seems to vary individually. Of ten examined larvae two had two cells on each side, two had two cells on one and one on the other side, the remainder exhibited one cell on each side. Staining against FMRFamide gave negative results in *A. gelatinosum*.

### ***Bowerbankia gracilis***

**Serotonin immunoreactivity** (Fig. 8B, 9D) can be located in the apical organ, the central nerve nodule and two axial nerves connecting both elements. A fine meshwork of serotonergic processes is found beneath parts of the larval surface.

The nerve nodule (nn, Fig. 8B, D) is located on the posterior side of the pyriform organ. It comprises a dense neuropil with most of its serotonergic fibers oriented transversally. Cell bodies are not visible within the nodule. The majority of the fine epidermal fibers originate in the lateral regions of the nerve nodule. A bundle of fibers runs laterally on each anterior side. They branch and are found in the entire oral and posterior surface apart from the internal sac. A second group of fibers runs in apical direction, branching into a delicate meshwork around the pyriform organ.

The apical organ contains two basolaterally located clusters of serotonergic neurons (as, Fig. 8B, C). Each cluster consists of 3-5 perikarya. The latter are droplet-shaped with a slightly broadened apical side and a narrow basal region that terminates in an axon. The axons of each cluster form of an axial nerve that runs towards the central nerve nodule, passing the posterior side of the pyriform organ. On the apical side dendrites emerge from the cell bodies,



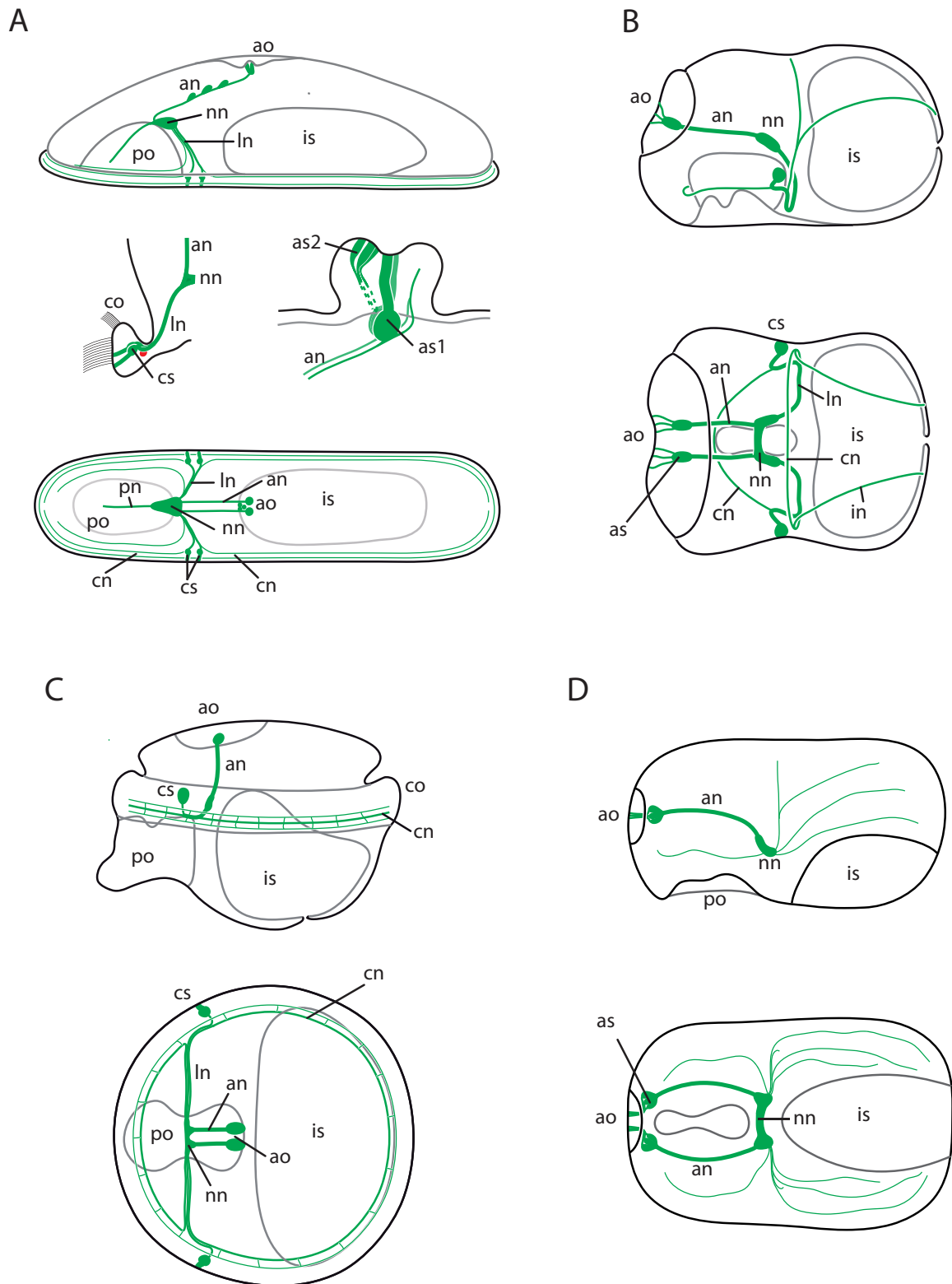
**Fig. 8** *Bowerbankia gracilis* larva. **A** Light micrograph, view from posterior. **B** Projection of confocal image stack. Fluorescence staining with antibodies against serotonin (green) and  $\alpha$ -tubulin (red), view from anterior. Serotonergic nervous system comprises apical organ (ao) axial nerves (an) and nerve nodule (nn). A fine meshwork of serotonergic fibers emanates from the lateral regions of the nerve nodule. Paired clusters of 3-5 serotonergic perikarya (as) are found basally to the apical organ. The cells project dendrites (arrowheads) towards the center of the neural plate and then apically to the surface. **C-D** TEM micrographs of sagittal section (inset) **C** Detail of apical region, as indicated in inset. The cluster of serotonergic cells (as), here showing four nuclei, is located underneath the apical organ. The anterior nerve terminates in the cluster. **D** Detail of central nerve nodule, as indicated in inset. The nodule, being directly adjacent to the glandular epithelium of the pyriform organ (po), consists of a dense neuropil (np). Several cell bodies are distributed around the nodule, but their identity as neurons remains unclear. co corona, coc corona cell, is internal sac

that project at first towards the larval midline and then curve towards the apical surface near the center of the apical organ.

All elements described so far also show  $\alpha$ -tubulin immunoreactivity. Additionally a much denser bundle of fibers emerging from the nerve nodule in oral direction can be detected. No conspicuous photoreceptor-like elements like in *Bugula fulva* were observed, though larvae showed strong positive phototaxis. FMRFamide positive elements are present in the larval nervous system of *B. gracilis*, but a detailed analysis of this was impossible, because primary antibodies showed high unspecific affinities to yolk-granules in epidermal and mesodermal cells causing a high amount of background fluorescence in the specimens.

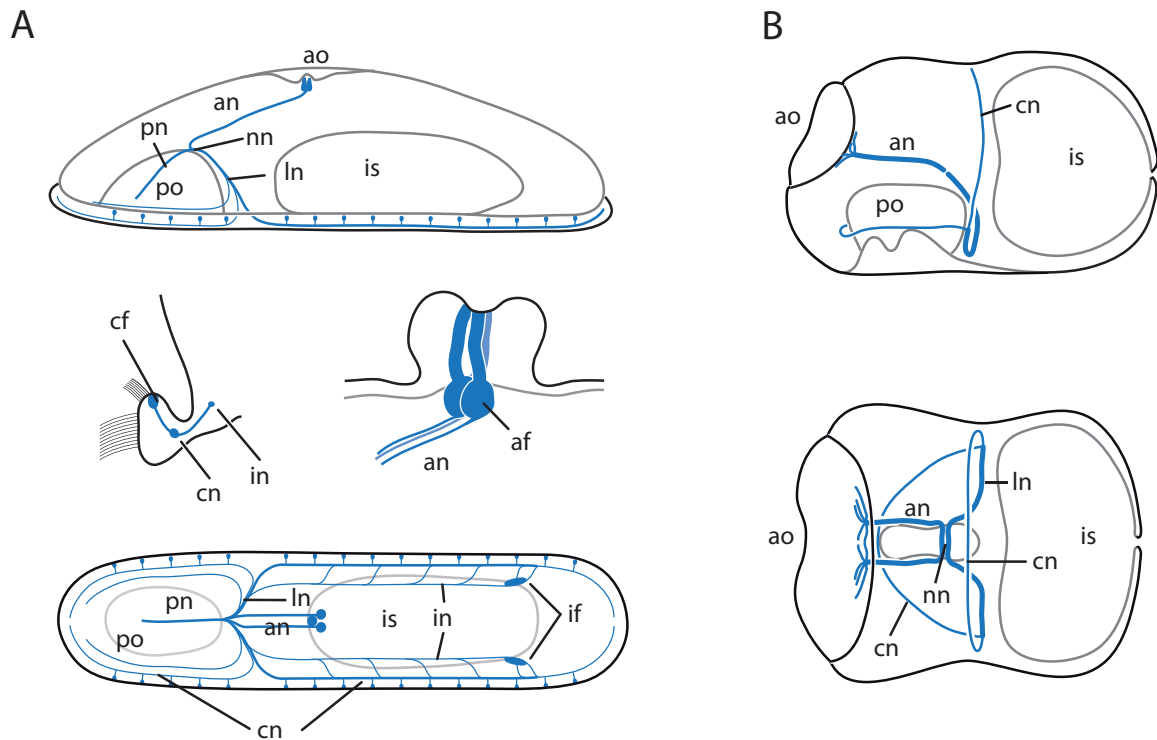
## Discussion

The general organization of the nervous system in gymnolaemate larvae is known since the early accounts of Prouho (1890, 1892), Calvet (1900) and Kupelwieser (1905). Several later ultrastructural studies on selected species have added more detailed information (d'Hondt 1973, 1977a, 1977b, Reed and Cloney 1982, Reed et al. 1988, Reed 1988, 1988a, Stricker et al. 1988b, Zimmer and Woollacott 1989a, 1989b, 1993, Zimmer and Reed 1994). Lecithotrophic and planktotrophic larvae exhibit a largely similar neural anatomy. This comprises a centrally located nerve nodule that gives rise to axial nerves connecting to the apical organ and lateral nerves connecting to a corona ring nerve. Sometimes short nerves occur that run from the nodule to the papilla of the pyriform organ. Serotonin immunoreactivity is found within all of the above mentioned elements in the species studied here. Only in *Bowerbankia gracilis* lateral nerves and coronal serotonergic cells are missing. However, the present results show some discrepancies to previous studies, which will be discussed in more detail below: Pires and Woollacott (1997) only differentiated between anterior and equatorial serotonergic elements. Since, however, the main focus of their study was the physiological/behavioral role of the neurotransmitter, the authors made no account to analyze the exact labeling with high resolution. Hay-Schmidt (2000) found no nerve nodule in *Membranipora* sp. and described serotonergic cell bodies in the pyriform organ and not in the corona. In *Triphyllozoon mucronatum* the nerve nodule is located underneath the otherwise immunonegative apical organ and axial nerves extend to two cell bodies in the pyriform organ (Wanninger et al. 2005b). The lateral nerves and the coronal serotonergic cell bodies resemble those found in the present study. A compilation of the currently available data on serotonergic systems has been made in Tab. 1. The results on FMRFamide immunoreactivity, however, appear patchy and allow only limited comparison and interpretation.



**Fig. 9** Comparison of larval serotonergic nervous systems (*green*) (see text) **A** *Flustrellidra hispida*, *top* view from lateral, *bottom* view from aboral **B** *Bugula fulva*, *top* view from lateral, *middle* details of corona and apical organ, *bottom* view from posterior **C** *Alcyonidium gelatinosum*, *top* view from lateral, *bottom* view from aboral **D** *Bowerbankia gracilis*, *top* view from lateral, *bottom* view from posterior. *ao* apical organ, *an* axial nerve, *as1* type 1 apical serotonergic neuron, *as2* type 2 apical serotonergic neuron, *co* corona, *cn* coronal nerve, *cs* coronal serotonergic neuron, *is* internal sac, *in* internal sac nerve, *ln* lateral nerve, *nn* nerve nodule, *pn* papilla nerve *po* pyriform organ





**Fig. 10** Comparison of larval FMRFamidergic nervous systems (*blue*) (see text) **A** *Flustrellidra hispida*, *top* view from lateral, *middle* details of corona and apical organ, *bottom* view from aboral **B** *Bugula fulva*, *top* view from lateral, *bottom* view from posterior. *af* apical FMRFamidergic neuron, *an* axial nerve, *ao* apical organ, *co* corona, *cn* coronal nerve, *cf* coronal FMRFamidergic neuron, *if* interneuron at internal sac, *is* internal sac, *in* internal sac nerve, *ln* lateral nerve, *nn* nerve nodule, *pn* posterior nerve *po* pyriform organ

### Central neural elements

A nerve nodule, sometimes also referred to as commissure or ganglion, located adjacent to the pyriform organ has been described in cyphonautes (Prouho 1892, Kupelwieser 1905), shelled lecithotrophic (Prouho 1890), as well as most coronate larvae (Calvet 1900, Reed and Cloney 1982, Reed and Woollacott 1982, Zimmer and Woollacott 1989b, 1993, Zimmer and Reed 1994), except in *Bowerbankia imbricata* (d'Hondt 1977b). However, Stricker et al. (1988b) did not explicitly describe such a structure for *Membranipora membranacea* and it was also not demonstrated by immunofluorescence in *Membranipora* sp. (Hay-Schmidt 2000). Wanninger et al. (2005b) described a commissure to reside beneath the apical organ in *Triphyllozoon mucronatum*. This might correspond to the nerve nodule, only situated in a different position. In coronate larvae the nerve nodule usually represents the largest concentration of nervous processes (Reed 1988, 1991). Most studies agree that the nerve nodule gives rise to paired axial and lateral nerves as well as to a single unpaired nerve running towards the papilla. The axial nerves run parallel to muscular tracts in many species. Both elements have therefore often been referred to as neuromuscular strand (Reed 1991). Although

**Tab. 1** Comparison of available data of serotonergic nervous system in Bryozoa. + present, - absent

Serotonin immunoreactivity in:	<i>Bugula neritina</i> (Pires and Woollacott 1997)	<i>Membranipora</i> sp. (Hay-Schmidt 2000)	<i>Triphylozoon mucronatum</i> (Wanninger 2005b)	<i>Flustrellidra hispida</i> (this study)	<i>Bugula fulva</i> (this study)	<i>Alcyonidium gelatinosum</i> (this study)	<i>Bowerbankia gracilis</i> (this study)
Central nerve nodule (neuropil)	?	-	-	+	+	+	+
Apical organ	2 cells ? (not described, but visible in their Fig. 3)	2 cells	neuropil (might correspond to nerve nodule)	2 + 4 cells	2 cells	2 cells	2 clusters of 3-5 cells
Pyriform organ	-	2 cells? (see Text)	2 cells	central unpaired nerve	-	-	-
Paired axial nerve (from nodule to apical organ)	+	-	-	+, interneurons	+	+	+
Lateral nerves (from nodule to corona)	?	(+) (from ao to corona)	(+) (from ao to corona)	+	+	+	diffuse fibers
Corona nerve ring	?	+	diffuse fibres	+	+	+	diffuse fibers
Serotonergic cells within corona	-	? (see Text)	2	4	2	2 (up to 4)	-

serotonin immunoreactivity was found in the nerve nodule in all species examined here, serotonergic elements in the papilla nerve were only present in *Flustrellidra hispida*.

In most of the studies mentioned above, the nodule is described as a neuropil or plexus, lacking neuronal cell bodies. Though some nuclei could be shown in close proximity to the nerve nodule of *Bugula fulva*, clear evidence for nerve cell bodies has not been provided in any study so far. Thus there is no evidence that the nerve nodule represents a ganglion. Moreover, interneurons in general have not been described in larvae of any bryozoan species so far except in *F. hispida*. Prouho (1890) described nerve cells along the neuromuscular strand. These most likely correspond to the bipolar serotonergic interneurons demonstrated in the present study, where also further FMRFamideergic interneurons were found situated at the posterior end of the internal sac with connection to the corona nerve. It remains uncertain if interneurons are generally absent in other larvae or just have not been found so far.

### Apical organ

In all species studied here, at least two serotonergic cells are found lateral to the center of the organ sending axons into the paired axial nerves. The cells share the basal location of their perikaryon. *Bowerbankia gracilis*, exhibits two basolaterally situated groups of cells whereas *F. hispida* shows a higher diversity of serotonergic cell types, comprising an additional cluster of easily distinguishable cells situated anteriorly in the apical organ, as well as a group of interneurons associated with the axial nerve. In contrast, FMRFamide could only be detected in *F. hispida* and *B. fulva*, whereas immunoreactive cell bodies occurred only in the former species. Therefore a comparison of FMRFamide immunoreactivity is difficult.

In *Bugula neritina*, Pires and Woollacott (1997) found serotonin immunoreactivity in axial nerves leading towards the apical organ. They did not explicitly mention the presence of cell

bodies in the apical organ, but their figure 4 strongly suggests a situation resembling what has been found in the congeneric species *B. fulva* in the present study. (Hay-Schmidt 2000) demonstrated the presence of two lateral serotonergic cells in the apical organ of the cyphonautes larva in *Membranipora* sp.. These cells bear basal axons and resemble the lateral type 1 serotonergic cells in *F. hispida* in shape and location. Only a single cross section of the organ is shown in Hay-Schmidt (2000: Fig. 1f), therefore it remains unclear, whether the second type of cells found in *F. hispida* also might occur in *Membranipora* sp.. According to Wanninger et al. (2005b) a basal serotonergic and FMRFamideergic plexus, but no serotonergic or FMRFamideergic cells are present in the apical organ of *T. mucronatum*. Instead, a pair of serotonergic cells, that appears quite similar to those found in the apical organ in *B. fulva*, is situated in the pyriform organ. This situation is without resemblance to any of the species examined so far. Thus further investigations are needed to elucidate whether this pattern is unique in *T. mucronatum* or can be found in further species.

Although the apical organ has been regarded as an important sensory structure from the very first descriptions to later detailed ultrastructural investigations (see Zimmer and Woollacott 1977b, Reed 1991, Mukai et al. 1997 for review), the modality of the received stimuli (chemical vs. mechanical) as well as the exact function of the different cells still remain unclear. The apical organ usually consists of different types of epithelial cells. While some cells show characteristics of primary sensory cells, others bear muscle fibers or motile cilia and seem to serve an effective function. Sensory and neural cells are usually concentrated in the center of the apical organ, often termed “neural plate”. In many larvae undifferentiated mesodermal cells are associated with the peripheral parts of the apical organ, forming together the annectula’s polypide anlagen (Reed 1991).

D’Hondt (1975, 1977a) differentiated between central and peripheral cells in the apical organ of *F. hispida*. The present results, however, show a higher diversity in the central cells with at least two different types of nerve cells. At least one type (as1) shows characteristics of sensory cells. Reed (1988) described three types of cells in the apical organ of *Bowerbankia gracilis*, central neural plate cells, cuneiform, and marginal cells. The serotonergic cells found in the present study closely resemble the first type in shape and position. In his detailed ultrastructural analysis, Reed found evidence for their putative nature as mechanoreceptive primary sensory cells. In the apical organ of *Bugula neritina* the central neural plate consists of bipolar oligociliated cells (Reed et al. 1988) clearly differing from the serotonergic cells found in *B. fulva* in the present study. Although the central region is ciliated in the latter species, the serotonergic cells are found basiepithelially with their apical dendrites lacking cilia. The highest diversity of cell types has been demonstrated in the apical organ of *M. membranacea* in an ultrastructural study by Stricker (1987).

In many other marine invertebrate larvae (see below) the apical organ is underlain by a ganglion, that is thought to represent the larval “brain”. Such a structure is apparently absent in bryozoan larvae. Only a nerve plexus underneath the apical organ is described from shelled larvae (for cyphonautes: Prouho 1892, Kupelwieser 1905, Stricker 1987) (for shelled lecithotrophic larvae: Prouho 1890, this study). The data from coronate forms are contradictory. D’Hondt described an apical ganglion in *Alcyonidium polyoum* (1973) and *Bowerbankia imbricata* (1977b). However, the author did not provide any ultrastructural documentation. (Wanninger et al. 2005b) found a serotonergic nerve plexus underneath the apical organ in *T. mucronatum*. Other ultrastructural studies found no apical neural concentration in coronate larvae (Reed and Cloney 1982, Reed and Woollacott 1982, Zimmer and Woollacott 1989b, 1993, Zimmer and Reed 1994). In the present study no plexus was revealed in *B. fulva*, *B. gracilis*, and *A. gelatinosum* using immunohistochemical, and additionally in the former two species ultrastructural techniques.

### Neural elements in the corona

All species examined in the present study possess serotonergic elements in the corona. Apart from *Bowerbankia gracilis*, where only delicate fibers occur, a nerve is situated basally to the coronal cells. In earlier histological and ultrastructural studies this has usually been described as ring nerve (Reed 1991). In the light of the data presented here the situation can be rendered a bit more precisely: As a common pattern, the lateral nerves, coming from the nerve nodule, always split into an anterior and posterior arc. In *F. hispida* and *A. gelatinosum* additional nerves run parallel to these arcs being connected by short processes to the former. This situation might be explained by the fact that in these species, in addition to the ring of corona cells, a further ring of so-called supracoronal ciliated cells is present (d’Hondt 1973, 1977a). In all species except *B. gracilis* additional tracts from the lateral nerves terminate in ciliated serotonergic cells situated in or near the corona. These cells have characteristics of sensory neurons. FMRFamide-like nerves were present in the corona of *B. fulva* and *F. hispida*, whereas immunoreactive cell bodies were only found in the latter species.

The serotonergic cells in the corona of *B. fulva*, apart from their ciliation, are very similar to those found in *T. mucronatum* (Wanninger et al. 2005b). They most likely represent intercoronal cells. This cell type was first discovered in *B. gracilis* (Reed 1980, Reed and Cloney 1982) and later described in several other coronate larvae (Zimmer and Woollacott 1989a, 1993, Zimmer and Reed 1994). Intercoronal cells occur in various shapes between the coronal cells. Most of them are thought to serve sensory functions (Zimmer and Woollacott 1989a). The cells in *B. fulva* closely resemble the oligociliated intercoronal cells described in *Bugula stolonifera* by Reed et al. (1988). The authors assumed a sensory function of these cells and found them to form both chemical synapses and gap junctions with the adjacent coronal cells. In contrast to the present study, Reed et al. (1988) found no neuronal

connections between the intercoronal cells and the remaining nervous system. Although the authors did not provide the exact number of intercoronal cells, it must be higher than two cells because they are situated between most of the corona cells, which are at least 32 in number. Thus the two serotonergic cells obviously represent only a fraction of the population of intercoronal cells. In *Bowerbankia gracilis* the intercoronal cells are monomorphic, very slender and elongate and bear kinocilia (Reed and Cloney 1982). The perikarya were found near the larval equator, in close contact to the equatorial nerve ring. I could not detect serotonin immunoreactivity in intercoronal cells in this species. This could be due to weak labeling, but as the serotonin signal was sufficient in other parts of the nervous system it is more likely that no serotonergic intercoronal cells are present in this species.

Intercoronal cells have so far been found neither in shelled (d'Hondt 1977a, Stricker et al. 1988a, Stricker et al. 1988b) nor in *Alcyonidium*-type larvae (d'Hondt 1973). The innervation of the serotonergic cells found in the coronae in *F. hispida* and *A. gelatinosum* is similar to that of the intercoronal cells described in other species in the present and in previous studies (Zimmer and Woollacott 1989a). Thus they can be regarded as homologous. They differ from those found in *B. fulva* in the cellular shape including basal nucleus and small apical process and in the lower number of cilia. Though situated very close to the ciliary band, it can not be proved if they actually reside between the coronal cells or just adjacent to the oral or aboral margin of the corona. However, even the latter case would not argue against homology, because Zimmer and Woollacott (1989a) already hypothesized that intercoronal cells might originate from infra- or supracoronal cells and gain their intercoronal position only in larvae with expanded coronas. Hay-Schmidt (2000) described a pair of serotonergic cells situated on each side of the pyriform organ. However, the provided image (2000: Fig. 1f) does not allow an exact localization, so that these cells could eventually also resemble intercoronal cells. In *F. hispida* numerous FMRFamide cells occur aboral to the corona within the supracoronal band of ciliated cells. A sensory function of these cells is also likely. No similar structures were found in the other larvae in the present study, but Wanninger et al. (2005b) demonstrated the presence of two FMRFamide, presumably intercoronal, cells situated peripherally in the larva of *Triphyllozoon mucronatum*.

As shown with simultaneous tubulin/serotonin staining, the serotonergic cells residing in the corona of *Bugula fulva* are obviously not identical with the photoreceptor cells, as carefully hypothesized by Wanninger et al. (2005b) for *Triphyllozoon mucronatum*, but instead represent two independent structures. Photoreceptors have previously been described ultrastructurally in five species of the genus *Bugula* as well as in *Tricellaria occidentalis* and *Scrupocellaria bertholetti* (all superfamily Cellularioidea) by Woollacott and Zimmer (1972) and Hughes and Woollacott (1978, 1980). The photoreceptors are always situated between the corona cells, contain a shading pigment and bear a bundle of cilia, that represent the receptive structure. In some cases the receptor cells are invaginated and the cilia are coiled to a



tightly packed mass. Although the form of the coil is different, the photoreceptors in *B. fulva* most closely resemble those described in *B. neritina* (Woollacott and Zimmer 1972).

Based on experimental results on phototactic behavior of different bryozoan larvae, summarized in Ryland (1977) it has been suggested that photoreceptor cells can be expected in many more species that show phototaxis, but lack distinct eyespots. The lack of eyespots might be simply due to the lack of pigment-containing cells and does not per se indicate the lack of receptor cells. Unpigmented photoreceptors are not uncommon among other invertebrate taxa, e.g., platyhelminths (Sopott-Ehlers 1991), nemertines (Vernet 1974), or polychaetes (Purschke et al. 2006). Ryland (1960) has found phototaxis in larvae of *Flustrellidra hispida*, Reed (1980) in *Bowerbankia gracilis*. However, though I have also observed this behavior, I could not find any structures that would indicate the presence of a ciliary photoreceptor cell using acetylated- $\alpha$ -tubulin stainings in these species. As the receptor cells in *Bugula fulva* resemble intercoronal cells in their location between corona cells, it could be possible that other, unobtrusive, intercoronal cells serve as photoreceptors in species lacking distinct eyespots. Evidence for a common origin of intercoronal and photoreceptor cells has been summarized by Zimmer and Woollacott (1989a) and Reed (1991).

### **Functional aspects of the nervous system**

As the serotonergic nervous system comprises both sensory elements (apical organ, intercoronal cells) and possible neuronal pathways to effector organs (ciliary band, musculature), it is likely to be involved in either locomotion or metamorphosis or both. To the contrary, feeding structures in planktotrophic cyphonautes larvae do not contain serotonergic elements as shown by Hay-Schmidt (2000) for *M. membranacea*. Pires and Woollacott (1997) showed for larvae of *Bugula neritina* that application of serotonin or its precursors induces a shift from photopositive to photonegative behavior. Serotonin is therefore likely to modulate the interaction between photoreceptors and effector organs. As the photoreceptors themselves do not express serotonin the modulation must take place either on the effector side or in interneurons. A switch from photopositive to photonegative behavior and thus from swimming to creeping locomotion marks the onset of settlement in most bryozoan larvae (Ryland 1977). To my knowledge, it has not been studied so far, whether this switch is exclusively endogenous or mediated by mechanical or chemical stimuli.

Metamorphosis starts with irreversible attachment to the substrate by eversion of the internal sac (Reed 1991) and is thought to be initiated by chemical stimuli, as many larvae exhibit distinct substrate preferences (Ryland 1974, 1976). The morphogenetic movements include muscular action as well as modulation or reversal of ciliary beat (Reed 1991). However, apart from the difficulty to substantiate a chemoreceptive nature of the serotonergic cells found in gymnolaemate larvae, these are only poorly exposed to the substrate during the

creeping phase of the larva and therefore unlikely to encounter chemical settlement cues. Sensory cells situated in the pyriform organ would be better candidates for reception of settlement cues. Induction of metamorphosis as well as modulation of ciliary beat by serotonin has been found in several other marine larvae (e.g., Couper and Leise 1996, McCauley 1997, Zega et al. 2005, 2007). Only in a few cases specific receptor structures for chemical settlement inducers could be identified, e.g. cells in the apical sensory organ of a gastropod larvae (Marois and Carew 1990, Hadfield et al. 2000).

### **Other bryozoan larvae**

Larvae of cyclostomes lack both apical organ and pyriform organ (Nielsen 1970). No sensory organs or nervous systems have been described in any species so far. Phylactolaemate larvae exhibit a concentration of nerve cells at the pole opposite to the primary polypide buds (Marcus 1926, Franzén and Sensenbaugh 1983). Nevertheless, homology to the apical organ of gymnolaemate larvae remains doubtful, as the polarity of the highly distinct phylactolaemate larva is unclear (see Reed 1991). No immunohistochemical data about the larval nervous system of phylactolaemates are present so far.

### **Comparison with other lophotrochozoans**

Bryozoans undergo a catastrophic metamorphosis involving a breakdown of the majority of larval tissues including the larval nervous system (Zimmer and Woollacott 1977a, Wanninger et al. 2005b), whereas the adult nervous system arises *de novo*. This is an important fact to keep in mind when comparing the larval nervous system of bryozoans to those of other lophotrochozoan taxa, which often show a mosaic of both strictly larval, i.e. transitory, and more or less continually developing adult components (Nielsen 2005a). The latter are unlikely to have counterparts in bryozoan larvae, therefore comparisons should focus on strictly larval components. Homology of the apical organ and the prototroch among spiralian taxa is hardly questionable and has found support in numerous studies in fields of comparative morphology and developmental biology. These structures are always lost at metamorphosis (see Nielsen 2004, 2005b, 2008 for review).

In the Mollusca a high diversity ranging from simple to more complex apical organs with a high number of serotonergic neurons is recognized. As a general and probably ancestral pattern, central flask shaped cells are flanked by bipolar peripheral cells, that usually connect to the prototroch. The number of flask-shaped serotonergic cells is, e.g., up to eight in polyplacophorans (Friedrich et al. 2002, Haszprunar et al. 2002, Voronezhskaya et al. 2002b), four in the scaphopod *Antalis entalis* (Wanninger and Haszprunar 2003), three in gastropods (Kempf et al. 1997, Page and Parries 2000, Page 2002, Dickinson and Croll 2003) and the bivalve *Mytilus trossulus* (Voronezhskaya et al. 2008). The flask-shaped cells can be ampullary cells, parampullary cells or tuft cells. 2-4 bipolar peripheral cells are present in most

species. Serotonergic innervation of the prototroch has been observed in several gastropods (Kempf et al. 1997), *M. trossulus* (Voronezhskaya et al. 2008), and the polyplacophoran *Mopalia mucosa* (Friedrich et al. 2002), but is missing in the polyplacophoran *Ischnochiton hakodadensis* and the scaphopod *A. entalis* (Wanninger and Haszprunar 2003). Interestingly, in the latter two species lateral projections terminating in flask-shaped cells situated close to the prototroch are found.

Hay-Schmidt (2000) found an apical organ containing two serotonergic cells in the swimming larva of the kamptozoan *Loxosoma pectinaricola*. A different situation has been recently described in the creeping larva of *Loxosomella murmanica* (Wanninger et al. 2007). Here 6-8 central flask-shaped cells and 8 bipolar peripheral cells are present in the apical. This pattern resembles the putative ancestral molluscan condition and, according to the authors, argues for a common ancestry of the two clades.

The serotonergic nervous systems in trochophore larvae of *Polygordius lacteus* (Hay-Schmidt 1995), *Phyllodoce maculata* (Voronezhskaya et al. 2002a), and *Pomatoceros lamarckii* (McDougall et al. 2006) approximately resemble each other, comprising an apical organ with 3 (*P. lacteus*) or 4-6 neurons (*P. maculata*), two lateral nerves, a prototroch nerve ring consisting of several fibers, a subesophageal ganglion and ventral nerve cords. However, several differences have to be pointed out. Two serotonergic cells are found dorsally beneath and associated with the prototroch ring nerve in *Phyllodoce maculata*, whereas it consists only of fibers in *Polygordius lacteus* and *Pomatoceros lamarckii*. In *Owenia fusiformis* serotonergic innervation of the ciliary band as well as serotonergic neurons in the apical organ are lacking completely (Hay-Schmidt 2000). In all species the cerebral ganglion of the adult nervous system develops underneath the apical organ, which loses serotonin immunoreactivity in later stages. FMRFamide is found in 20 mono- and bipolar neurons in the apical organ of *Polygordius lacteus*, as well as in the underlying neuropil. Two lateral nerves connect the apical organ to the two prototroch ring nerves and further to the subesophageal ganglion and ventral nerve cords. In *Pomatoceros lamarckii* no FMRFamidergic immunoreactivity is present in the prototroch. *Phyllodoce maculata* only seven FMRFamidergic cells are located in the apical organ. Two ventral cells project their axons around the prototroch.

Trochophore-like larvae of the sipunculan *Phascolion strombus* only exhibit two FMRFamidergic cells in the apical organ. Further FMRFamide and serotonin immunoreactivity arises only in anlagen of the adult nervous system (Wanninger et al. 2005a). In the larvae of the echiuran *Bonellia viridis* both serotonin and FMRFamide are found in prototroch and metatroch ring nerves as well as in an anterior loop pervading the apical hemisphere (Hessling 2002). FMRFamidergic cell bodies develop later in the apical organ whereas serotonergic cells are absent here. The apical organ in nemertean pilidium larvae is aneural

(Hay-Schmidt 1990a). The prototroch is innervated by a serotonergic ring nerve containing several cell bodies.

Phoronid actinotrocha larvae exhibit a much more complicated nervous system (Hay-Schmidt 1990b, 1990c, Santagata 2002). A high number of serotonergic cells is present in the apical organ and several nerve tracts connect to the ciliary bands as well as the larval tentacles. Both serotonergic and FMRFamide nerves are found along the main ciliary band.

Enteropneust exhibit of an apical organ with a large number of cells large number of serotonergic cells (Nezlin 2000, Nezlin and Yushin 2004, Nielsen and Hay-Schmidt 2007). The ciliary band is innervated by serotonergic nerves and, in *Ptychodera flava*, bears serotonergic cell bodies. A similar situation, is found in most echinoderm larvae (Byrne et al. 2007).

### Conclusion

The results of the present study complement the existing data to result in a more comprehensive view on the morphology of the nervous systems and the distribution of neurotransmitters in bryozoan larvae. Despite a considerable amount of both intra- and interspecific variation a common ancestral pattern for gymnolaemate Bryozoa can be postulated: The serotonergic nervous system comprises an apical organ containing at least two serotonergic cells. These give rise to nerves that connect to a central nerve nodule, which itself sends lateral nerves into the corona. At least two serotonergic neurons are present in the ciliary band. Less data are available for the FMRFamide immunoreactivity, but so far axial nerves, lateral nerves, corona nerves and eventually cells in the corona might constitute the FMRFamide nervous system.

The neuronal architecture with a simple apical organ resembles the common spiralian pattern. The general occurrence of apical serotonergic neurons innervating the ciliary band appears to be an even older character. So far the present data provide no further support for a sister-group relationship between Bryozoa and Kamptozoa, as the creeping-type larva is seen as ancestral in the latter taxon (Nielsen 1971). There is also no indication for affinities to Phoronida and Brachiopoda. The serotonergic cell bodies situated in the main ciliary band could be either unique bryozoan features, or, although more unlikely, be homologous to those found in hemichordates and echinoderms. A structure like the central larval nerve nodule has not been described from other lophotrochozoan taxa, however in these, other central nervous elements like apical and cerebral ganglia occur that might fulfill a similar function.

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