Chapter 2

Ganglion ultrastructure in phylactolaemate Bryozoa: evidence for a neuroepithelium

Abstract - In contrast to other Bryozoa, members of the subtaxon Phylactolaemata bear a subepithelial cerebral ganglion that resembles a hollow vesicle rather than being compact. In older studies this ganglion was said to originate by an invagination of the pharyngeal epithelium. Unfortunately, documentation for this is fragmentary. In chordates the central nervous system also arises by an invagination-like process, but this mode is uncommon among invertebrate phyla. As a first attempt to gather more data about this phenomenon, cerebral ganglia in two phylactolaemate species, *Fredericella sultana* and *Plumatella emarginata,* were examined on the ultrastructural level. In both species the ganglion bears a small central lumen. The ganglionic cells are organized in the form of a neuroepithelium. They are polarized and interconnected by adherens junctions on their apical sides and reside on a basal lamina. The nerve cell somata are directed towards the central lumen, whereas the majority of nervous processes are distributed basally. Orientation of the neuroepithelial cells can be best explained by the possibility that they develop by invagination. A comparison with potential outgroups reveals that a neuroepithelial ganglion is at least derived. Since, however, a reliable phylogenetic system of the Bryozoa is missing, a decision on whether such a ganglion is apomorphic for Bryozoa or evolved within this taxon can hardly be made.

Introduction

In the last 15 years phylogenetic analyses of gene sequences as well as gene expression data in many bilaterian taxa have led scientists to rethink traditional ideas about metazoan phylogeny (Halanych 2004) and to formulate new hypotheses about the evolution of nervous systems and especially the central nervous system (CNS) (Holland 2003). Unfortunately the amount of morphological data is not equally sufficient in all bilaterian taxa. One of the groups, in which the fine structure of the CNS is not well studied, is the Bryozoa. Although Bryozoa gained much interest of workers of the late 19th and early 20th century, some aspects of their neural architecture have remained unclear or sometimes simply were not included in relevant discussions.

Bryozoa or Ectoprocta are sessile suspension feeders forming encrusting or erect colonies. The majority of the approximately 5000 described extant species inhabits the oceans, whereas only a few occur in freshwater. Commonly the following subgroups are distinguished: the Phylactolaemata, which are exclusively limnetic, the marine Stenolaemata and Cheilostomata, and the Ctenostomata, which are chiefly marine with a few brackish-water and limnetic species (for introductory reviews see Ryland 1970, Mukai et al. 1997, Ryland 2005). Ctenostomata and Cheilostomata are usually united as Gymnolaemata. However, newer palaeontological data indicate that recent Stenolaemata and Cheilostomata are ingroups of Ctenostomata, the latter therefore being paraphyletic (Todd 2000, Ernst and Schäfer 2006).

Studies on the bryozoan nervous system have long revealed a crucial difference in the cerebral ganglion of phylactolaemates on the one side and of stenolaemates and gymnolaemates on the other. Wheras the ganglion appears as a rather compact assemblage of up to 25 neurons in the latter two groups (reviewed in Bullock and Horridge 1965), it is described as a hollow structure in phylactolaemates. The most detailed of the early studies are those of Kraepelin (1887, 1892), Gerwerzhagen (1913a, 1913b), Graupner (1930), and Marcus (1934). Most of these authors assume that the ontogenetic origin is by an invagination of the aboral pharyngeal epithelium. Actually they do not provide well documented observations. More detailed investigations on the nervous system were done from the 1960s onward, but these are exclusively on gymnolaemates (e.g., Lutaud 1973, Gordon 1974, Lutaud 1977, 1993).

To date the only ultrastructural data on the ganglion of a phylactolaemate species are provided by Mukai et al. (1997). Being sparsely documented and patchy, these data unfortunately do not provide a complete picture of the fine-structural organization of the ganglion. Formation of the central nervous system by invaginating ectodermally derived epithelia is an apomorphic feature of chordates and seldom occurs in invertebrate groups (Nielsen 2001, Ax 2001, but see Ruppert 1997a).

If the ganglion should also originate by invagination in phylactolaemates, the epithelial character of the nervous system would likely persist. Thus, an epithelial organization of the ganglion could reflect this neurulation process. Since apical adhaerens junctions unambiguously indicate epithelia, an ultrastructural analysis could provide evidence for a neuroepithelial organization of the ganglion in Phylactolaemata. To test this assumption, ganglia of two phylactolaemate species were studied at the ultrastructural level.

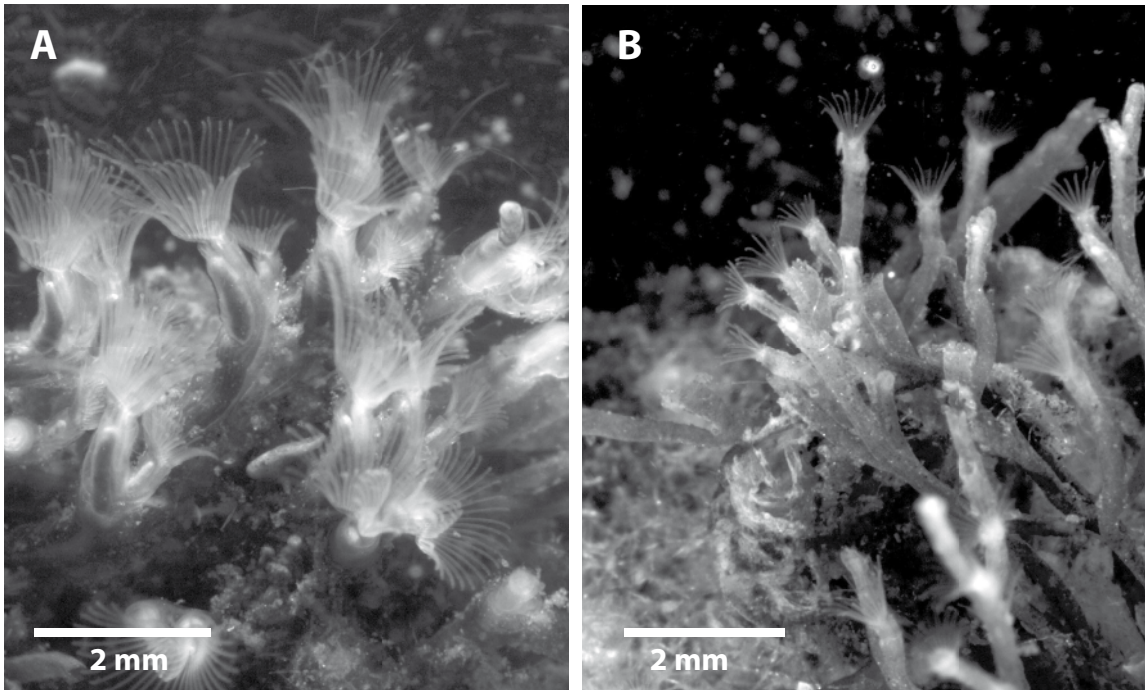


Fig. 1 Colonies of phylactolaemate bryozoans. **A** *Plumatella emarginata*. **B** *Fredericella sultana*

Materials and Methods

Colonies of *Fredericella sultana* (Blumenbach, 1777) were sampled in the summer months of 2004 and 2005 in the lake Obersee near Bielefeld, in the Teltow Canal, Berlin, and in the Salzgitter Canal near Braunschweig, all Germany. Colonies of *Plumatella emarginata* Allman, 1844 were collected in the lake Lehnitzsee, near Potsdam, Germany during October 2004.

One crucial aspect in the descriptions of phylactolaemate fine structure are artefacts produced during the fixation process. Phylactolaemata, like most other limnetic invertebrates have a very low osmotic value in their tissues, so that most commonly used fixatives are hypertonic in relation to the tissue. The resulting shrinkage of cells may cause large intercellular spaces that could erroneously indicate fluid-filled compartments inside the nervous system. Thus, for transmission electron microscopy colony parts or individual zooids were treated with different fixatives ranging from 1–2.5% glutaraldehyde in 0.01M phosphate buffer (ph 7.4; 24 mOsmol) or unbuffered in Milli-Q for 1h at 4° C.

Post-fixation was done with 2% osmium tetroxyde buffered in the same manner as the fixative for 30-60 min at 4°C. The specimens were dehydrated by a graded ethanol series and embedded in araldite with propylene oxyde as intermedium. Semithin and series of ultrathin sections were produced using a LEICA UC6 microtome and DIATOME diamond knives. Ultrathin sections were placed on formvar-coated single-slot copper grids and stained with uranyl acetate and lead citrate using an automatic TEM stainer (Nanofilm Technologie

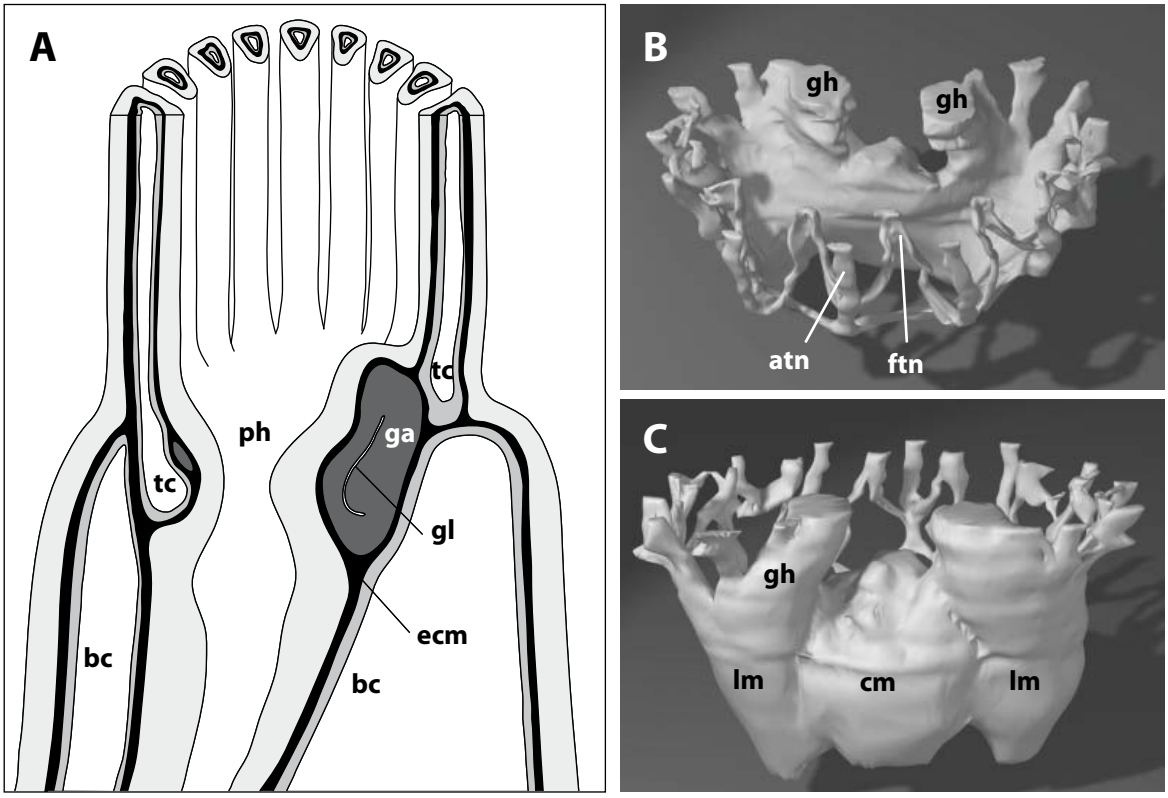


Fig. 2 A Schematic representation of a sagittal section through a zooid of *Fredericella sultana.* The ganglion is situated within the ECM (black) between the pharyngeal epithelium and the coelomic epithelium. **B-C** 3D reconstruction from serial sections of the ganglion of *Fredericella sultana.* **B** Frontal view. **C** Abfrontal view. *atn* abfrontal tentacle nerve, *bc* body coelom, *cm* central mass, *ecm* extracellular matrix, *ftn* frontal tentacle nerve, *ga* ganglion, *gh* ganglion horn, *gl* ganglion lumen, *lm* lateral mass, *ph* pharynx, *tc* tentacle coelom.

GmbH, Goettingen, Germany). For examination, a PHILIPS CM 120 was used at 60 kV. Images were taken using DITABIS erasable photoplates and processed using ANALYSIS and Adobe Photoshop software packages. 3D reconstructions were accomplished with BLENDER using MorphMesh python scripts.

Results

Phylactolaemate species differ in some morphological features from species of the Stenolaemata and Gymnolaemata. The most intriguing features are the horseshoe-shaped lophophore (Fig. 1A), which in *Fredericella sultana* is only slightly indented (Fig. 1B), and the epistome, an upper-lip-like organ, that traditionally is homologized with homonymous structures in Phoronida and Brachiopoda. The ectocyst (= cuticle) of phylactolaemate species is always uncalcified, in the shape of either a thin tube, consisting of chitin and tanned proteins or, in the case of *Cristatella* and *Pectinatella* species, of a gelatinous mass.

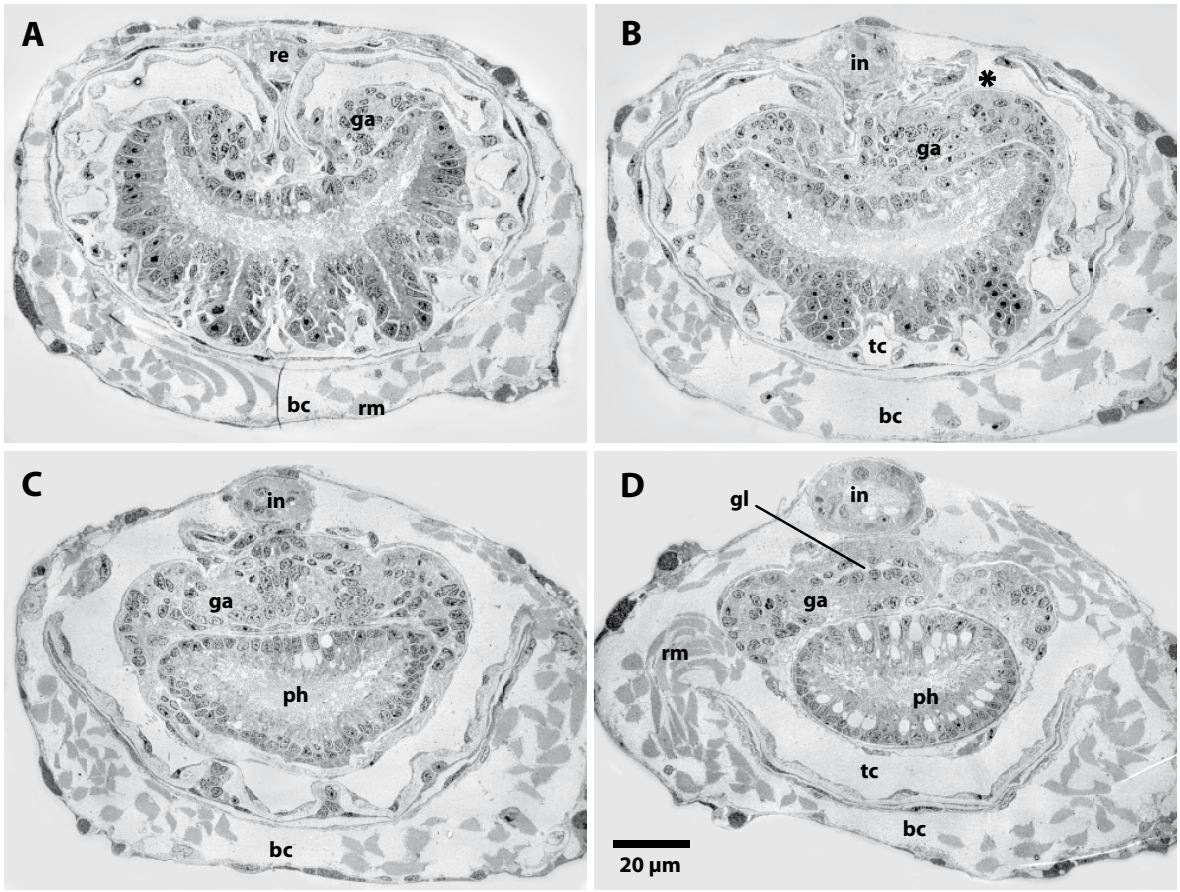


Fig. 3 A-D *Fredericella sultana.* TEM. Representative cross-sections of the ganglion from apical (**A**) to basal (**D**). The tentacle coelom opens into the body coeleom (*asterisk*). *bc* body coelom, *ga* ganglion, *gl* ganglion lumen, *in* intestine, *ph* pharynx, *re* rectum, *rm* retractor muscle, *tc* tentacle coelom.

Interzooidal walls are usually not present, so there is a colony-wide confluent secondary body cavity or coelom.

Gross morphology

Fredericella sultana is the phylactolaemate species with the smallest zooids. The colonies are antler-shaped, with their branches measuring 200-400 µm in diameter. The mouth is surrounded by the lophophore. The U-shaped gut ends with an anus, situated outside the ring of tentacles. The lophophore is nearly circular, bearing only a small concavity at its anal side. Usually 20-25 tentacles are present (Fig. 1B). Zooids of *Plumatella emarginata* measure about 500 µm in diameter. The lophophore is horseshoe-shaped and bears about 50 tentacles (Fig. 1A). The colonies are also of a branched type.

The ganglion basically is a nerve ring surrounding the pharynx. While the main portion forms a crescent-like structure partially encompassing the pharynx on its anal side (Figs. 2A, B, 3C), the ring is completed on the frontal side by a few small neurites only (Fig. 2B). The ganglion can be subdivided into three parts. A central mass parallels the longitudinal axis of the

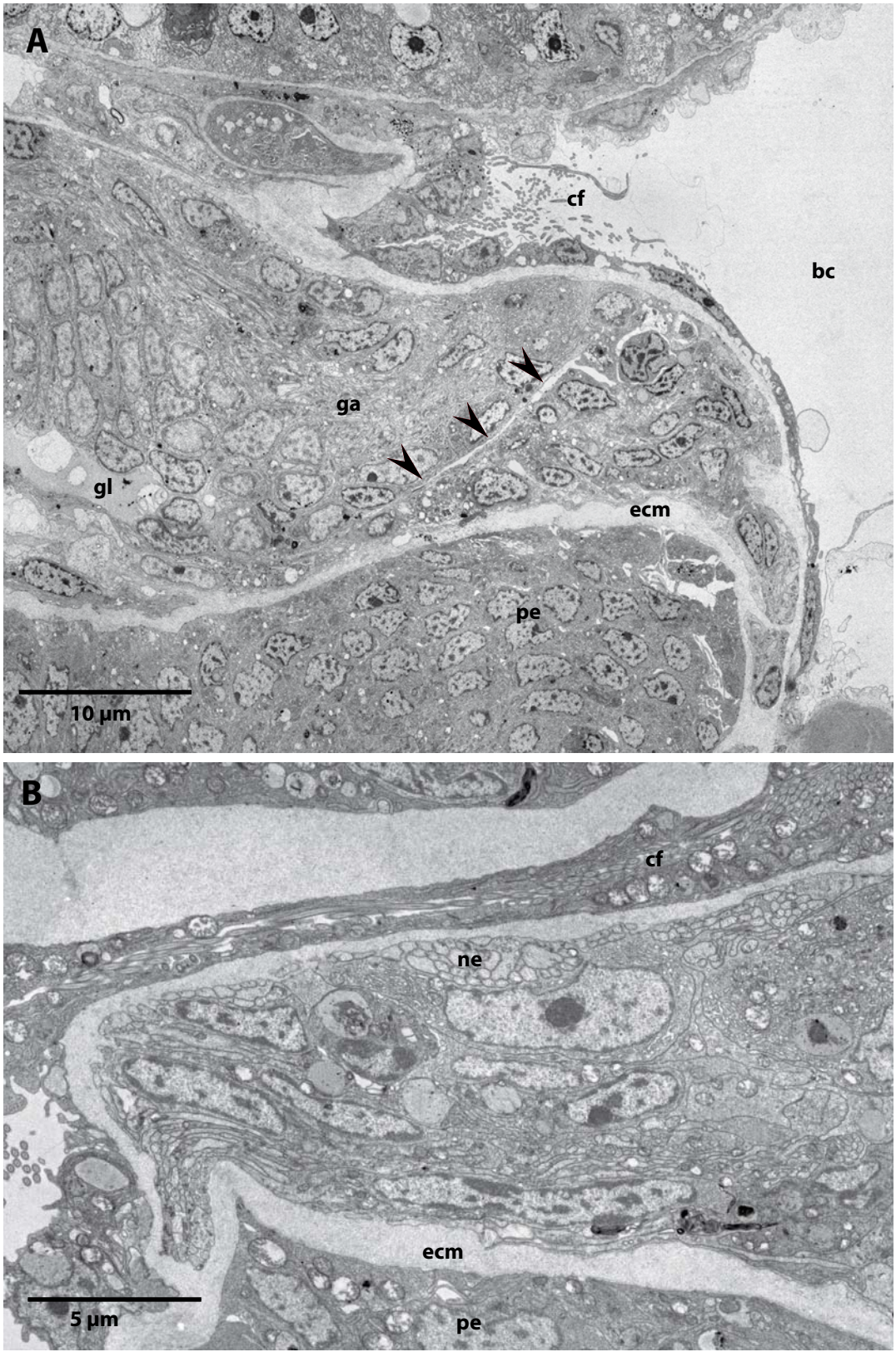


Fig. 4 *Plumatella emarginata.* TEM. **A** Right side of the ganglion, showing the ganglionic lumen in the central mass and the same compressed to a cleft in the lateral mass (*arrowheads*). **B** Lateral tip of the ganglion sending out processes around the pharynx. *bc* body coelom, *cf* ciliary field, *ecm* extracellular matrix, *ga* ganglion, *gl* ganglion lumen, *ne* neurites, *pe* pharynx epithelium.

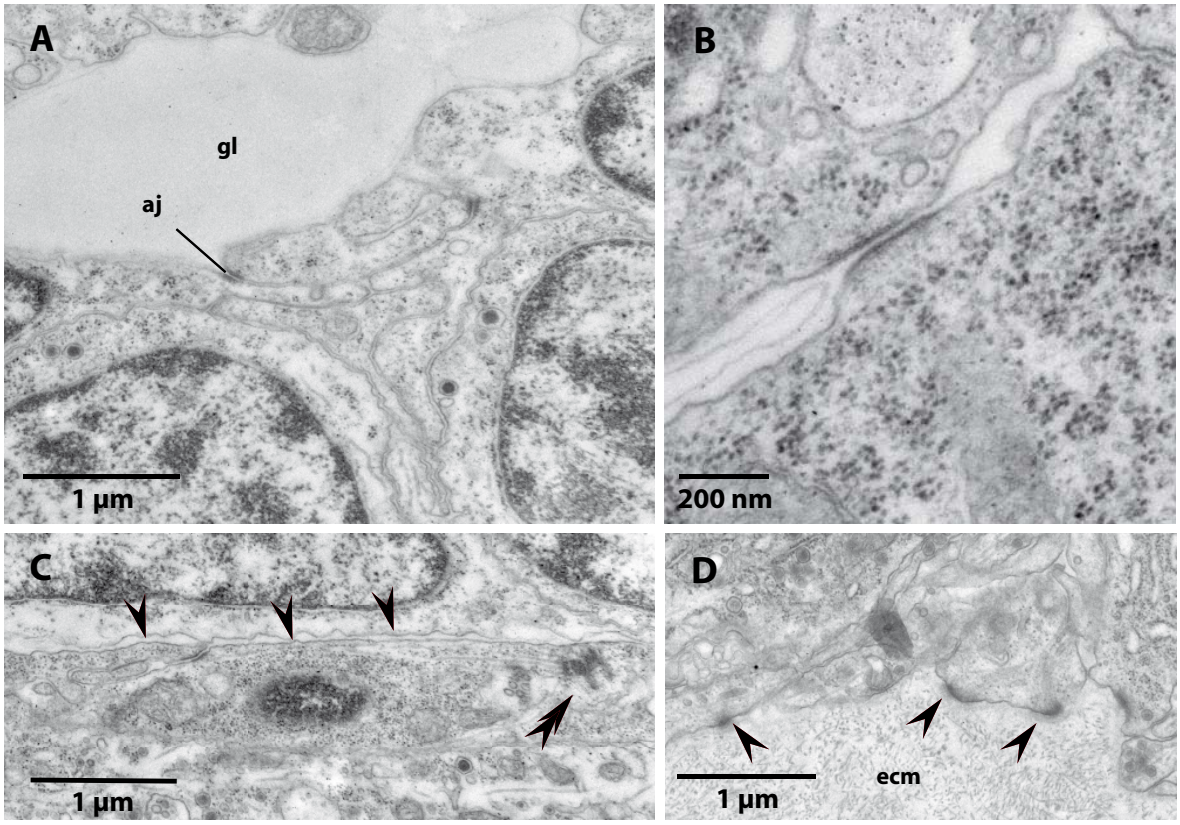


Fig. 5 *Plumatella emarginata.* TEM. Details of the ganglion's neurons. **A** Neurons facing the central lumen with their apical side, innterconnected by apical adherens junctions. **B** Adherens junction. **C** Lumen compressed to a cleft (*arrowheads*), cell bearing rudimentary ciliary rootlet (*double arrowhead*). **D** Neurons are connected to surrounding ECM through hemidesmosomes (*arrowheads*). *aj* apical adherens junction, *ecm* extracellular matrix, *gl* ganglion lumen.

pharynx (Fig. 2C), while the two lateral masses are frontally directed and embrace the pharynx. Anally directed protrusions of the lateral masses form the so-called "ganglion-horns" (Fig. 2C). These are much more pronounced in *Plumatella emarginata* than in *Fredericella sultana*, since they extend into the lophophore arms which are lacking in the latter species. The ganglion is a vesicle with a small lumen between its oral and anal wall (Figs. 3D, 4A) in both species. The wide, fluid-filled lumen described by earlier authors is likely to be artificial (see remarks on fixation procedures). All parts of the ganglion are completely subepithelial and underlie the basal matrix of the pharyngeal epithelium (Figs. 3, 4).

Ganglion structure

The cerebral ganglion is completely embedded in an ECM, ranging between 0.3 and 2 µm in thickness in *Plumatella emarginata* (Fig. 4), and 0.2 and 0.7 µm in *Fredericella sultana*. Its narrowest portion lies at the oral side of the ganglion that directly faces the pharyngeal epithelium. The ganglionic tissue is not homogenous; nervous processes and nerve cell somata are distinctly distributed within the ganglion. The central mass and thicker parts of the lateral masses are clearly bilaminate as described above. The cells show a distinct apical-

basal polarity characteristic for epithelia (Fig. 5). Apical adherens junctions connect the neighboring cells, but never occur between cells of opposed epithelial sheets (Fig. 5A-C). The central mass contains a small lumen (Figs. 3D, 4A, 5A). Here, the nuclei of the surrounding neuroepithelial cells are situated in the apical region of the cells, facing the interior lumen (Fig. 3D).

The lateral masses also show a central lumen (Fig. 4A), but as in the central mass it generally is a tiny cleft, being larger only in a few areas. The masses become narrower while encircling the pharynx and merely consist of a few nerve processes when meeting on the frontal side. We could not ascertain whether the nervous processes from both sides connect at the frontal side, or whether the processes are just interdigitating without being connected. Glial cells were not found.

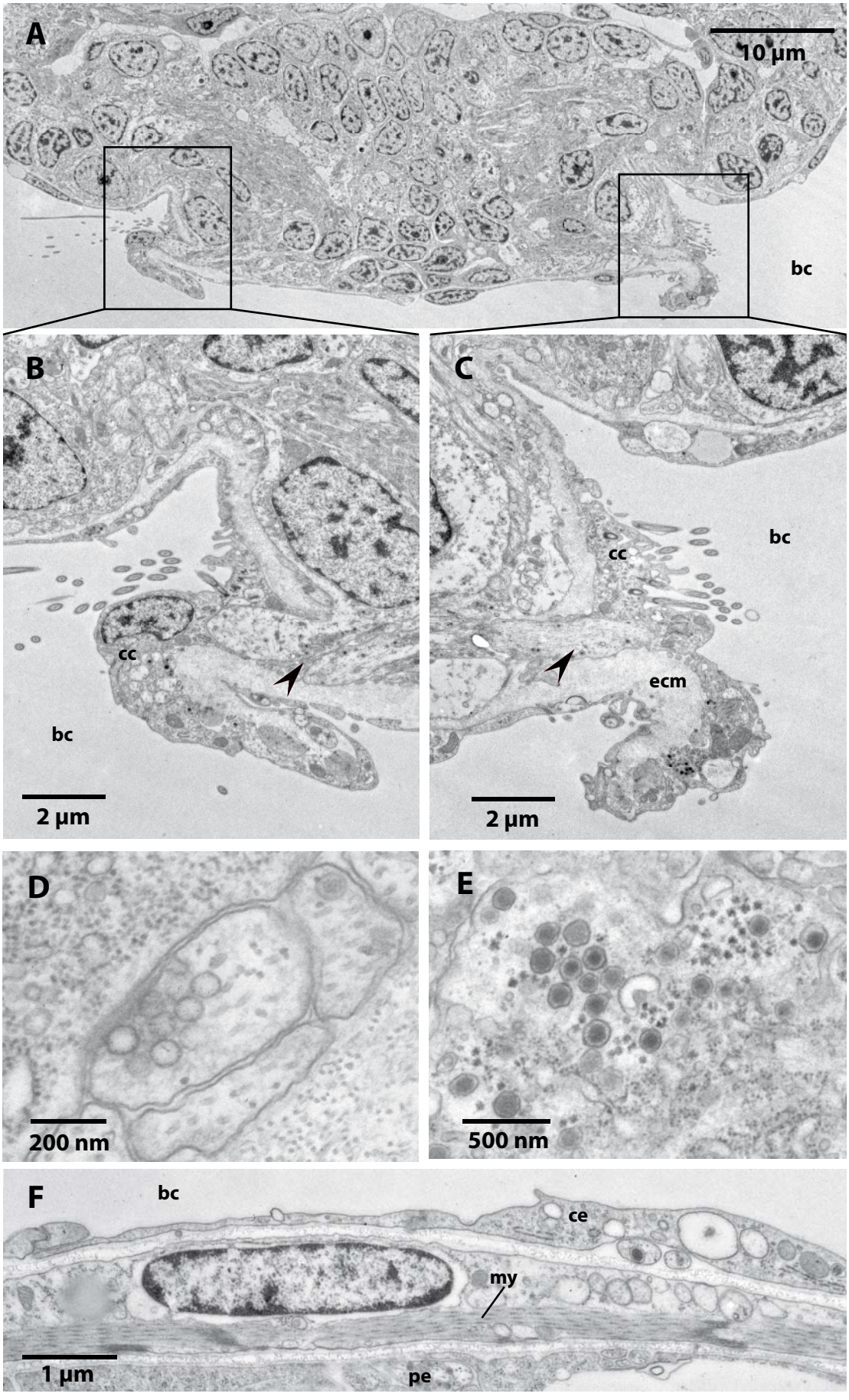
Neuroepithelial cells

The nerve cells of the ganglion are clearly neuroepithelial and overlie a layer of axons and dendrites, summarized here under the term neurites. These cells resemble each other in their subcellular composition, so that all of them seem to belong to the same type. Besides being interconnected by apical adherens junctions they bear rudimentary ciliary rootlets near their apices (Fig. 5C). Processes originating from the basal side of the neuroepithelial cells penetrate the basal layer of nervous processes and anchor the neuroepithelial cells to the ECM via hemidesmosomes (Fig. 5D). The neuroepithelial cells contain numerous vesicles, 80 – 130 nm in size, with a large electron-dense core-region surrounded by a lighter halo and a vesicular membrane (Fig. 6E). They occur in cell somata as well as in those neurites that have a larger diameter. Pronounced Golgi complexes and RER are found. The nuclei measure up to 3 µm and are roundish to oval in shape. Neurites vary in diameter from 100 to 700 nm and typically contain neurotubules. Additionally, electron-lucent (neuro)vesicles chiefly occur in neural processes (Fig. 6D). Axo-somatic as well as axo-dendritic synapses can be found (Fig. 6D).

Nervous connections and peripheral nervous system

The tentacle nerves branch off from the circumpharyngeal ring, protruding upward into the tentacles (Fig. 2B,C). The nerves leaving the ring are intertentacular, and proceed into the intertentacular membrane, situated at the tentacle bases. A few µm upward, they branch off

Fig. 6 *Fredericella sultana.* TEM. **A** Abfrontal side of the ganglion. **B-C** Details of nervous connections (*arrowheads*) to coelomic ciliary fields. **D** Synapse between axon and nerve cell soma. **E** Electron-dense neurovesicles. **F** Muscle cell in ECM between pharyngeal epithelium and coelomic epithelium. *bc*, body coelom, *cc* ciliated coelothelial cell, *ce* coelomic epithelium, *ecm* extracellular matrix, *my* myofilaments, *pe* pharynx epithelium.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oblique processes. These processes meet those originating from the neighbouring tentacles and together comprise the frontal tentacle nerves.

The ring musculature of the gut is composed of myocytes completely embedded in the surrounding ECM (Fig. 6F). Since the cirumpharyngeal section of the ganglion also lies in this ECM, nerve cells and muscle cells are often closely arranged, so they are likely to be connected. Large retractor muscles are found inside the trunk coelom. These are attached to the ECM of the lateral ganglionic masses (Fig. 3D).

On the anal side of the ganglion there is a direct cellular connection to ciliary fields (Fig. 6A-C), which reside in the forked canal. These consist of multiciliated cells of the coelomic epithelium. Such cells are evenly distributed in most areas of the secondary body cavity, but appear concentrated and pronounced here. There is an area of about 2x2 µm where cells of the ganglion contact the ciliated coelothelial cells.

The nerve cells contain numerous small electron-dense vesicles resembling dense-cored vesicles (Fig. 6E). They are noticeably concentrated e.g., at the ciliary fields on the anal side.

Discussion

Our results show that the cerebral ganglion of both phylactolaemate bryozoan species is neuroepithelial. This has been evidenced by apical adherens junctions, structures that are restricted to epithelial tissues. This strict epithelial organization also provides some hints on the still unknown origin of the ganglion, and indicates that the ganglion is most likely to arise from another epithelial tissue by invagination. A possible candidate is the pharyngeal epidermis lying close to the ganglion. The assumption is supported by observation that the ECM between the ganglion and the pharyngeal roof is very thin. It is also substantiated by a recent study by Wöss (personal communication) on germinating statoblasts. Another possible source for the neuroepithelium would be the coelomic lining. This assumption gains the only support by the observed contact between coelothelium and neuroepithelium passing the ECM on the anal side of the ganglion on the level of the ciliary fields. This evidence does not seem convincing.

The ganglion in gymnolaemate bryozoan species is situated in the same position as in phylactolaemates. As shown by Gordon (1974) for *Cryptosula pallasiana* the ganglion is clearly subepithelial, being situated beneath the basal lamina of the pharyngeal roof. Nevertheless, there are crucial differences from the phylactolaemate ganglion since it appears as a solid mass without any sign of an epithelial nature. It furthermore consists of only a limited number of cells. There also seems to be no clear distribution of nerve cell somata as found in parts of the ganglion in both phylactolaemate species studied here. The ontogenetic origin of

the ganglion in gymnolaemates is unknown. Virtually nothing is known about the structure of the ganglion in cyclostomes. Any information in this respect would be important for considering theories about the evolution of these characters within the Bryozoa.

Phylogenetic considerations

The position of the Bryozoa within the Bilateria is still unresolved. Generally it is assumed that Bryozoa, Phoronida, and Brachiopoda share a common ancestor and form the taxon Lophophorata, although even this is not unequivocally accepted (Lüter and Bartolomaeus 1997, Ax 2001, Nielsen 2002). Presently three different hypotheses exist that are supported by data from different sources.

1. Bryozoa are closely allied with Phoronida and Brachiopoda, with which they are either united as a monophyletic group Lophophorata (Hyman 1959), or at least form a paraphyletic assemblage at the base of Deuterostomia (Ax 2001). This view is based on the analysis of morphological features and has no support from molecular data.
2. Bryozoa are embedded in a taxon Lophotrochozoa, consisting of Brachiopoda, Phoronida, Entoprocta, Annelida, Mollusca and several other taxa with a trochophore-like larva. This view is strongly supported by analyses of gene sequence data such as 18S, 28S , EF1α as well as Hox gene arrangements and analysis of mitochondrial genomes (e.g., Halanych et al. 1995, Peterson and Eernisse 2001, Passamaneck and Halanych 2004, Philippe et al. 2005, Waeschenbach et al. 2006). However the exact position of Bryozoa within Lophotrochozoa can not be resolved with these data.
3. Bryozoa are the sister-group of Kamptozoa (Entoprocta), based somewhere in the Spiralia (Nielsen 1971, 2001). Arguments for this hypothesis come mainly from similarities in larval morphology in both groups.

This still unresolved position of the Bryozoa directly influences the interpretion of our results since the internal relationships among the Bryozoa are also affected by the question for their sister group. Traditionally Phylactolaemata are regarded as sister group of a taxon consisting of Stenolaemata and Gymnolaemata (see Woollacott and Harrison 1997, Todd 2000, Nielsen 2001, Ax 2001, Ernst and Schäfer 2006). This hypothesis is primarily substantiated by reduction of certain morphological characters (eg. the epistome or the horseshoe-shaped lophophore) in the common stem lineage of Stenolaemata and Gymnolaemata. This hypothesis, however, presupposes a common ancestry of Bryozoa, Brachiopoda and Phoronida, since both characters also are found in all species of the latter two taxa. Molecular data are too sparse at the moment so that no stable phylogenetic hypothesis can be obtained from the available SSU and LSU sequences. Improved taxon sampling could bring some light to this in the future. Taking fossil data also into account, ancestors of the stenolaemates and not

phylactolaemates left the oldest fossils (Boardman et al. 1983), but this may be due to the low potential of the latter for fossilization.

Irrespective of these unresolved problems we shortly want to compare the known data to those from taxa which are presently discussed to be closely related to the Bryozoa, namely the Phoronida, Brachiopoda, Entoprocta and Deuterostomia. The nervous system of the Phoronida is the best-studied of the three lophophorate groups. Numerous detailed studies are available (de Selys-Longchamps 1907, Silén 1954, Fernandez et al. 1996, Herrmann 1997). In adults a nerve ring is found at the base of the lophophore. This ring is situated basiepithelially, i.e., above the basal lamina, at the base of the epidermis. There is a concentration of nerve cells and processes at the anal side that is referred to as ganglion (Fernandez et al. 1996, own observations). In contrast to the situation in Bryozoa, the ring nerve and the ganglion are situated outside the ring of tentacles. Virtually nothing is known about the fine structure of the ganglion in adult brachiopods. Both articulates and inarticulates have a concentration in the form of a circumesophageal ring, whereas the former have a sub- and a supraesophageal ganglion, the latter only a subesophageal one. The most detailed studies are those of Blochmann (1892, 1900) and van Bemmelen (1883). Blochmann describes the ganglion and the main nerves to reside basiepithelially in the outer body epithelium in inarticulates. Van Bemmelen states the CNS to be embedded within the connective tissue (or ECM) in articulates. From these studies it is also not clear whether the ganglion lies within the ring of tentacles, as in bryozoans, or outside as found in phoronids. There are no hints suggesting a vesicular nature. There are also no data about the ontogenetic origin of the ganglion. In Entoprocta a pair of subepidermal ganglia, interconnected to each other by a commissure, lie between intestinal tract and atrial epidermis (Nielsen and Jespersen 1997). There is no evidence either for a lumen inside the ganglion or for an epithelial organization.

In some deuterostome taxa, parts of the neural epidermis become invaginated in a tube-like pattern. In certain echinoderms, i.e., species of the Ophiurida, Echinoida, Holothurioida, the ectoneural part of the nervous system becomes internalized via the formation of epineural channels, but this situation most likely evolves within this taxon (Smith 1984). In earlier studies the collar tract of enteropneust hemichordates, which gets internalized by invagination comparable to the pattern in chordates, was regarded to represent the CNS in this group and possibly be homologous to the chordate dorsal nerve cord. However recent studies showed this organ does not function as part of the central nervous system. The enteropneust CNS is instead represented chiefly by an intraepidermal net-like plexus (Lowe et al. 2003). This situation can be interpreted either as resembling the ancestral eumetazoan state or as a secondary simplification (Holland 2003). In chordates, the central nervous system is internalized during embryonic development via the well-known process of neurulation. A specific part of the embryonic ectoderm invaginates and forms the dorsal nerve cord as well as the brain vesicle. The internalized (formerly ectodermal) tissue remains a hollow structure

and is likely to maintain its epithelial nature in the adult at least in cephalochordates (Mewes 1973, Ruppert 1997, Wicht and Lacalli 2005).

Conclusions

Although there is no doubt that the nervous system in the phylactolaemate species studied is epithelial, this finding remains rather isolated within the Bryozoa. The neuroepithelium found in Chordates can hardly be brought into an evolutionary relation to the phylactolaemate Bryozoa, as there are no arguments for a sister group relationship between the two groups. Those taxa for which a closer relationship or sister group relationships can be substantiated, show a compact ganglion without a neuroepithelium. Provided that the ganglion of Stenolaemata and Gymnolaemata actually is compact, it is most parsimonious to assume that the neuroepithelial ganglion is an autapomorphy of the Phyloactolaemata. Thus, the neuroepithelial organization of the phylactolaemate central nervous system evolved convergently to that found within the Echinodermata and the Chordata. The reasons for the evolution of such a nervous system remain to be analyzed.

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