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Neuroanatomy of the tube feet and tentacles in *Holothuria glaberrima* (Holothuroidea, Echinodermata)

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Abstract Echinoderms are a key group in understanding the evolution of the nervous system in the Metazoa. Remarkably, little is known about echinoderm neurobiology. The echinoderm podia, which are unique echinoderm modifications and comprise structures responsible for locomotion and feeding, have been largely neglected in nervous system studies. Here, we have applied immunohistological approaches using different neuronal markers to describe the neuroanatomy of the holothurian podia and its relation to the muscular component. We show, using the sea cucumber *Holothuria glaberrima* (Selenka, 1867), the direct innervation of the podia by the ectoneural component of the nervous system, as well as the existence of a connection between the nervous system components in the main nerves, the muscle, and the connective tissue. These findings confirm the ectoneural origin of the tube feet's main nervous system and demonstrate its neuroanatomic complexity. We also show the presence of fibers and neurons within the tube feet mesothelium and connective tissue. The study of these simple structures will help us elucidate the echinoderms' neuromuscular circuit and their evolutionary relationships.

Keywords *Holothuria glaberrima* (Holothuroidea, Echinodermata) · Tube feet · Tentacles · Neuropeptides · Nervous system

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Introduction

Echinoderms comprise an important group of animals in the study of nervous system evolution. However, when compared to other deuterostomes and to some protostome groups, the echinoderm nervous system has been less studied. Furthermore, most studies of echinoderm neurobiology focus on the radial nerves, the main structure of the echinoderm nervous system. In recent years, there has been a growing interest in echinoderm neurobiology. We, and others, have taken advantage of new markers, namely antibodies against neuropeptides, to describe some of the components of the echinoderm nervous system (Moore and Thorndyke 1993; Birenheide et al. 1998; Inoue et al. 1999; Nakajima et al. 2004; Burke et al. 2006a; Tamori et al. 2007). Our group has focused on members of the Holothuroidea (Díaz-Miranda et al. 1995, 1996). We have previously described the enteric nervous system (García-Arrarás et al. 1999, 2001) and, more recently, the nervous component associated with the connective tissue (Díaz-Balzac et al. 2007). Here, we focus on the nervous component of body wall structures associated with the water-vascular canal, namely, the tube feet and tentacles. We focus on determining the extent of the nervous system innervation on the muscular component as well as within the connective tissue, since the echinoderms are well known for having mutable connective tissues, which are under nervous system control (Motokawa 1984; Birenheide and Motokawa 1996).

The tube feet are podia, which imply they are a type of body wall protrusion associated with the water-vascular system. The podia vary greatly among the different classes of echinoderms in their number, localization, and function. In holothurians, the tube feet are the main type of podia and are referred to as locomotor podia, in contrast to other types

of podia named non-locomotor or papillate podia (Hyman 1955). These are unique echinoderm modifications and represent the main structures by which many holothurians attach to the substratum, achieve locomotion, and undergo a large range of activities (Hyman 1955). Holothurian tentacles are also associated with the water-vascular system, making them similar, in some respects, to the tube feet. In fact, some controversy exists on whether the tentacles are modified podia (Bouland et al. 1982; McKenzie 1987; Smiley 1994; Mashanov and Dolmatov 2000).

Almost all of the current knowledge of the podial and tentacular nervous system is based on classical histological descriptions done early during the last century (see Hyman 1955) or on more recent studies done with electron microscopy (Wood and Cavey 1981; Cavey and Wood 1981; Rieger and Lombardi 1987; Flammang and Jangoux 1992; Pentreath and Cobb 1972; Smiley 1994; Cavey 2006). These studies reveal the detail of cells and fibers, but the overall view of the circuitry and anatomical structures is still lacking. Thus, our current view of the podial and tentacular nervous system is that of a neural plexus located between the longitudinal muscle fibers and the connective tissue layer (Flammang and Jangoux 1992; Smiley 1994).

Here, we describe the innervation of podia and tentacles and their subdivisions into muscular and connective tissue components in a representative of the Holothuroidea, the aspidochirote *Holothuria glaberrima* (Selenka, 1867). We also provide a detailed characterization of the distribution and morphology of cells and fibers within the nervous system of the tube feet, and propose a model circuitry. Using this new information, we compare it to available histological and neuroanatomical tube feet and tentacle data (Hyman 1955; Florey and Cahill 1977, 1982; Moore and Thorndyke 1993) from other echinoderm species that have been studied thoroughly, especially Asteroidea and Echinoidea species. This cellular and histological description, together with the recent publication of the purple sea urchin genome (Sea Urchin Genome Sequencing Consortium 2006) and the identification of many conserved vertebrate neural genes (Burke et al. 2006b), will surely enhance the growing interest in echinoderm neurobiology.

Materials and methods

Animals

Adult *Holothuria glaberrima* (Selenka, 1867) (Holothuroidea, Aspidochirotida) specimen (10–15 cm in length) were collected from the rocky shores of the north coast of Puerto Rico. The animals were kept in sea water aquaria at the University of Puerto Rico in Río Piedras.

Tissue sections

Specimens were anesthetized in 0.2% 1,1,1-trichloro-2-methyl-2-propanol (Sigma, St. Louis, MO) for 10 min and dissected by longitudinal section of the body wall. Samples from the anterior and ventral part of the body wall, where the tentacles and tube feet are located, respectively, were dissected and fixed in 4% paraformaldehyde at 4°C for approximately 24 h. Tissues were rinsed 3 times for 15 min with 0.1 M phosphate-buffered saline (PBS) and left in a 30% sucrose solution at 4°C for at least 24 h before proceeding to embed them in Tissue-Tek (Sakura Finetek, Torrance, CA). Cryostat tissue sections (8–20 μm) were cut and mounted on Poly-L-lysine-coated slides.

Histology

Cryostat tissue sections were stained with Milligan's Trichrome (Humason 1979). Shortly, the sections were cleared in xylene for 10 min, hydrated in a decreasing alcohol series (95–70%) and allowed to mordant in potassium dichromate solution (76.5 mM potassium dichromate-22% HCl-22% ethanol, 6 min). Sections were then stained using the following washes, dyes and times: water rinse, acid fuchsin (1.2 mM, 7 min) (J. T. Baker Chemical, Phillipsburg, NJ), water rinse, 1% phosphomolybdic acid (3 min) (Sigma, St. Louis, MO), orange G in 1% phosphomolybdic acid (44.2 mM, 8 min) (Allied Chemicals, Gujarat, India), 1% acetic acid (2 min), 2.4% aniline blue (8 min) (Sigma, St. Louis, MO), and 1% acetic acid (3 min). Once the protocol was completed, the sections were dehydrated through an increasing series of alcohol (from 70 to 95%), cleared in xylene (10 min), and cover glass mounted using Piccolyte mounting media (Ward's Natural Science Establishment, Rochester, NY). The stained sections were observed and photographs were taken on a Nikon Eclipse E600 microscope.

Immunohistochemistry

The indirect immunofluorescence method was followed (García-Arrarás 1993; Díaz-Balzac et al. 2007). In brief, tissues were incubated for 1 h in goat serum 1:50 (Invitrogen, Carlsbad, CA), 15 min in 1% Triton X, and two other rinses in 0.1 M PBS, followed by the overnight incubation at room temperature with the primary antibodies or FITC-labeled phalloidin (Sigma, St. Louis, MO) at a dilution of 1:2,000. The primary antibodies used include the RN1 monoclonal antibody (Díaz-Balzac et al. 2007) raised against a homogenate of the radial nerve of *H. glaberrima* and used at a dilution of 1:100,000; the rabbit antiserum αGFSKLYamide No. 23 2i2s (Second injection and second

bleeding) (Díaz-Miranda et al. 1995) prepared against a GFSKLYa synthetic peptide and used at a 1:1,000 dilution; the rabbit antiserum αgalanin-1 2i3s (Second injection and third bleeding) (Díaz-Miranda et al. 1996) prepared against galanin (Calbiochem Corp. San Diego, CA) and used at a 1:1,000 dilution; the rat antiserum αSp-SynB: (Nakajima et al. 2004; Burke et al. 2006a) prepared against the recombinant protein made up of the amino acids 177-420 of the predicted Sp-SynB protein and used at a 1:200 dilution; the monoclonal antibody αβ-tubulin, (Sigma T-4026 Lot. 024K4862) clone TUB 2.1 prepared against tubulin from rat brain and used at a 1:500 dilution; and the Hg-fCol monoclonal antibody (Quiñones et al. 2002) from the Hg-fCOL clone, prepared against fibrous collagen extracted from *H. glaberrima*'s large intestine and used directly from the hybridomas supernatant.

The secondary FITC antibodies, Goat anti-mouse (Biosource, Camarillo, CA, #AMI0408 Lot. 3501), Goat anti-rabbit (Biosource, Camarillo, CA, #ALI0408 Lot. 1402), Goat anti-rat (Biosource, Camarillo, CA, #ARI3408 Lot. 1601), were used at a 1:50 dilution for double-labeling indirect immunohistochemistry. Also, the Cy3-conjugated secondary antibodies, Goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, #115-165-068 Lot. 47814) and Goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, #111-165-144 Lot. 50694), were used at a 1:2,000 dilution for double-labeling indirect immunohistochemistry.

A 10-min rinse in 1 μM Hoechst dye (Sigma, St. Louis, MO) was done to stain the cell nuclei, and the slides were mounted in a buffered glycerol solution. In cases where double labeling was performed, the two primary antibodies were applied simultaneously, and later, the two secondary antibodies were added together (see García-Arrarás 1993). Tissues were examined and photomicrographs were taken on a Leitz Laborlux fluorescent microscope with N2, I2/3 and D filters or on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII and DAPI filters.

Results

Tube feet

The most conspicuous podia of *H. glaberrima* were its locomotory tube feet. These were more commonly localized on the ventral side of the body wall, differentiating the dorsal–ventral axis of their bilateral symmetry. Morphologically, these were tubular projections of the body wall that end with a terminal disk, and its lumen was a continuation of the water-vascular canal.

Classical dyes

Tube feet were localized within the ventral body wall, surrounded by the connective tissue of the dermis. Milligan's Trichrome (MT) staining of longitudinal (Fig. 1a) and cross sections (Fig. 1b) of the tube feet stem clearly showed the different tissue layers: the epidermis, connective tissue, podial nerve, and mesothelium. The mesothelium can be divided into the muscle cells whose fibers are oriented longitudinally and the adluminal cells (sometimes referred to as coelomic epithelial cells). The tube feet of *H. glaberrima* have a terminal disk. It is composed of an inner coelomic epithelium, a connective tissue layer enclosing the supporting ossicles, an epidermis and a cuticle (Fig. 1c). The disk was connected to the tube feet stem through longitudinal muscular fibers, which were better observed near the stem distal portion where they appeared to attach to the supporting ossicles. Two clusters of cells bodies were observed in the terminal disk. The inner cluster was composed of loosely packed cell bodies found adjacent to the ossicle layer. The outer cluster was made by the densely packed epidermal cell bodies found beneath the tip of the terminal disk. The clusters of cells were separated by a connective
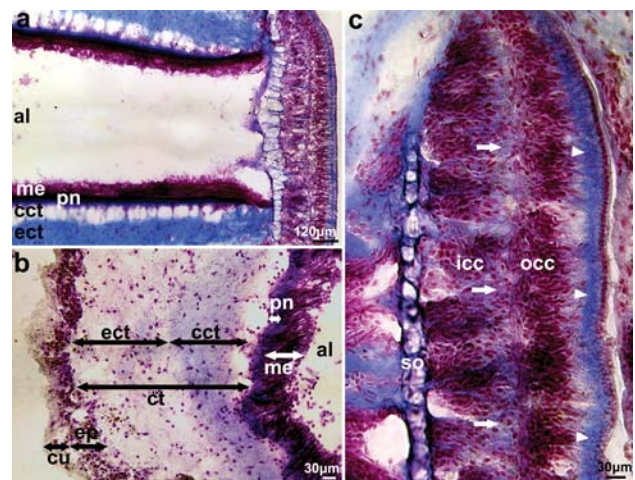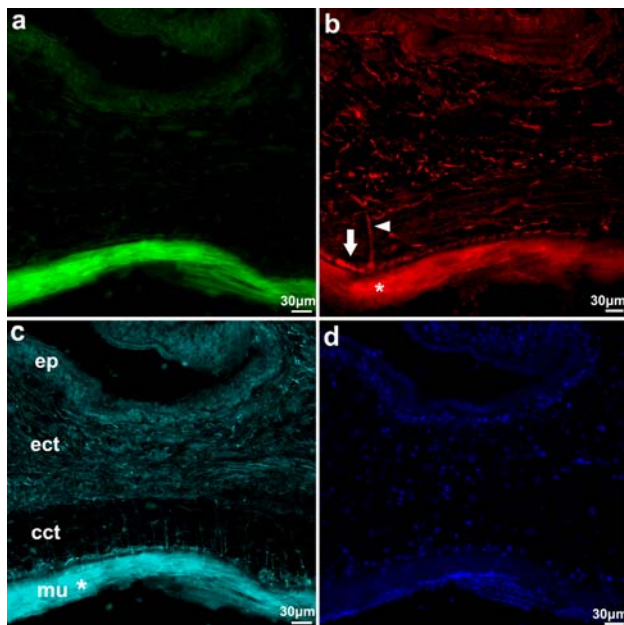

Fig. 1 *Holothuria glaberrima* (Holothuroidea). Milligan's Trichrome staining of the tube feet. The muscles are labeled in *red or magenta*, the connective tissue is in *blue* and the nervous system in a *light purple*. **a** Longitudinal section through the tube feet showing the mesothelium bordering the lumen, the connective tissue, and the distal disk (*right side*). **b** Transverse section through the tube feet stem showing the tissue layer subdivision: cuticle, epidermis, connective tissue (external connective tissue and central connective tissue), podial nerve, and mesothelium. **c** Longitudinal section through the disk, showing the two cell groups divided by a slightly stained layer (*arrows*), as well as the aniline blue-stained layer above the outer cell group or epidermal cells (*arrowheads*). *al* ambulacral lumen, *ct* connective tissue, *cu* cuticle, *de* dermis, *ect* external connective tissue, *ep* epidermis, *icc* inner cell cluster, *cct* central connective tissue, *me* mesothelium, *occ* outer cell cluster, *pn* podial nerve, *so* supporting ossicles

tissue layer, stained by aniline blue. Another stained aniline blue layer was found at the tip of the terminal disk, possibly corresponding to collagenous protrusions extending from the disk connective tissue layer.

Immunohistochemistry

Immunohistochemical experiments with the nervous system (RN1), connective tissue (Hg-fCol), muscle (phalloidin) and nuclear (Hoechst) markers, confirmed the layer subdivision observed with the Milligan's Trichrome stain (MT) and provided finer details of its anatomical organization (Fig. 2).
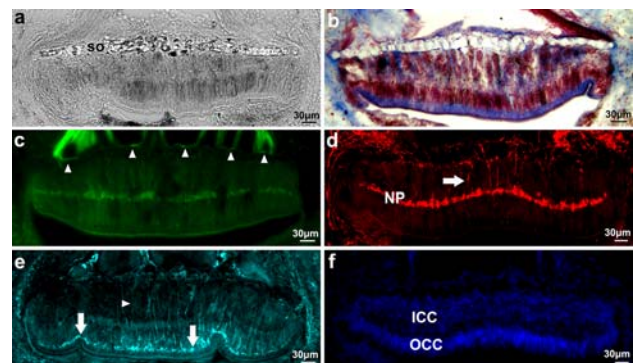
The only muscle-containing structure, as revealed by phalloidin labeling, was the longitudinal muscle of the mesothelium (Fig. 2a). The podial nerve was strongly labeled with RN1 immunostaining, and this marker also revealed the presence of other neural structures within the tube feet. A conspicuous neural plexus, consisting of RN1 immunopositive cells and fibers, was localized within tube feet connective tissue (Fig. 2b). In addition, Hg-fCol immunostaining revealed a directional distribution of collagen fibers within the connective tissue (Fig. 2c). When combining

these observations into consecutive slides treated with Hg-fCol and RN1, a differential distribution of collagen and nervous fibers was observed. The RN1-labeled connective tissue plexus was found in areas where collagen fibers were not abundant, and vice versa, collagen fibers were abundant in areas with little RN1 innervation. Also, the fibers labeled by each marker close to the podial nerve were opposed in direction; those labeled with RN1 were parallel to the longitudinal muscle fibers, while collagen fibers were perpendicular to it. Finally, Hoechst staining clearly confirmed the presence of many nuclei within the epidermis, while only scarcely spread nuclei were observed within the stem's connective tissue (Fig. 2d).

Triple-labeling experiments confirmed the observations made with the Milligan's Trichrome stain in *H. glaberrima* terminal disk. First, phase microscopy and MT staining clearly showed the localization of the supporting ossicles in the proximal part of the disk, just above to where the longitudinal muscle of the stem ends and probably serving as an anchor to the muscle fibers (Fig. 3a, b). Phalloidin labeling was only observed in the muscular attachment of the disk to the tube feet stem through muscle fibers located in the disk mesothelium; therefore, no muscle fibers or cells were labeled within the disk itself (Fig. 3c). RN1 immunoreactivity demonstrated the presence of a nerve plate consisting mostly of fibers, between the cell clusters (Fig. 3d). Fibers immunopositive to RN1 were observed connecting the

Fig. 2 *Holothuria glaberrima* (Holothuroidea). Longitudinal section through the stem of the tube feet. **a** Phalloidin-stained section showing the longitudinal muscle layer. **b** RN1 immunoreactivity showing the differential distribution of the nervous system within the *cct* and *ect.* The podial nerve (*arrow*) can be observed, as well as a projection (*arrowhead*) coming off from the podial nerve. **c** Hg-fCol immunoreactivity within the tube feet showing the differential distribution of collagen fibers within the *cct* and *ect*. **d** Hoechst-stained section showing the distribution of cells within the tube feet stem. *ect* external connective tissue, *ep* epidermis, *cct* central connective tissue, *mu* longitudinal muscle, * autofluorescence due to paraformaldehyde
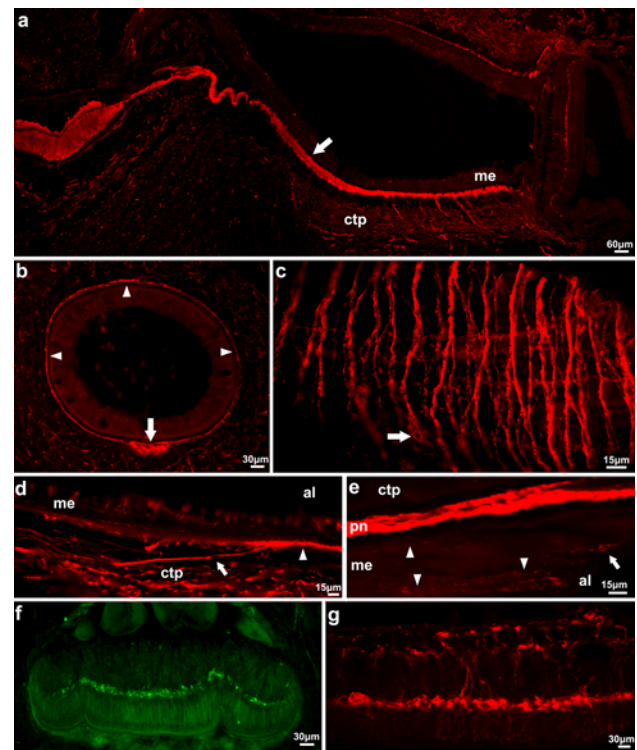

Fig. 3 *Holothuria glaberrima* (Holothuroidea). Longitudinal sections through the tube feet's disk. **a** The supporting ossicles (*arrows*) of the tube feet's disk are best observed in a phase-contrast section. **b** Milligan's Trichrome-stained section showing the inner and outer clusters of cells in magenta and the connective tissue layer in blue. **c** Phalloidin toxin-stained section revealing how the presence of muscle is limited to the attachment of the mesothelia to the base of the ossicles (*arrowheads*). **d** The nerve plate as revealed by RN1 immunoreactivity in addition to the individual fibers (*arrows*) arising from the apical nerves. **e** Collagen distribution within the disk as revealed by Hg-fCol immunostaining. The connective tissue layer can be observed (*arrows*), as well as the collagen fibers (*arrowhead*) extending up to the connective tissue layer. **f** Hoechst-stained section showing the loosely packed inner cluster of cells and the densely packed outer clusters of cells. *icc* inner cell cluster, *np* nerve plate, *occ* outer cell cluster, *so* supporting ossicles

nerve plate with the apical nerves, making the nerve plate a continuation of the podial nerve. Collagen fibers were observed extending up to the connective tissue layer, which was clearly labeled by Hg-fCol and partially devoid of cells (Fig. 3e). The Hoechst staining corroborated the presence of two clusters of cell bodies within the tube feet, separated by connective tissue and nerve fibers (Fig. 3f).

Tube feet nervous system innervations and subdivisions

Holothurians tube feet are associated with the ectoneural nervous system and immunolabeling with RN1 provided some striking visual confirmation of this innervation. A cross section of *H. glaberrima* body wall showed the podial nerve being formed by a prolongation of the lateral nerve extending from the ectoneural subdivision of the radial nerve (Fig. 4a). This nerve went unbranched through the body wall dermis to the tube foot's stem. Once the nerve reached the tube feet, its nervous system could be divided into four main parts: the podial nerve and podial cylindrical fenestrated sheath, the connective tissue plexus, the mesothelium nervous system, and the disk nervous system. The most intense labeling with RN1 occurred within the fibers that make up the podial nerve and the podial cylindrical fenestrated sheath (Fig 4a, b, c). Double-labeling experiments with Hoechst and RN1 showed that the former had a large congregation of cell bodies located in the periphery of the neuropile; while the latter is formed by continuous projections of fibers that extend from the podial nerve, circumscribing the tube feet stem (Fig. 4c). The podial cylindrical fenestrated sheath was localized within the connective tissue layer and adjacent to the mesothelium basement membrane. The projections that formed the podial cylindrical fenestrated sheath extended regularly at specific intervals, depending on the size of the tube feet, and bipolar cell bodies could sometimes be observed in them; reason why in longitudinal sections of the tube feet many parallel small nerves were observed between the mesothelium and the connective tissue, perpendicular to the longitudinal muscle fibers (Fig. 4c). Both the podial nerve and podial cylindrical fenestrated sheath were also strongly labeled by anti-β-tubulin, while subpopulations of fibers within them were labeled by other neural markers such as anti-Sp-SynB, anti-GFSKLYamide, and anti-galanin (results not shown).

Double labeling with RN1 and Hoechst showed the presence of RN1 immunoreactive cells and fibers within the connective tissue plexus (Fig. 4d). These cells measured approximately 10 μm in length and 5 μm in width and were also immunoreactive to anti-β-tubulin. A subpopulation of the RN1 immunopositive cells (approximately one-third) in the connective tissue plexus was also immunoreactive to anti-Sp-SynB, but none were immunopositive to anti-

Fig. 4 *Holothuria glaberrima* (Holothuroidea). Nervous system distribution in the tube feet, as revealed by RN1 immunoreactivity. **a** Longitudinal section through the body wall showing the connection of the tube feet by a lateral nerve, extending from the ectoneural component of the radial nerve and forming the podial nerve (*arrow*). **b** Transverse section through the tube feet, showing the bean-shaped podial nerve (*arrow*) and podial cylindrical fenestrated sheath (*arrowheads*). **c** Tangential section of the tube feet showing the projections perpendicular to the podial nerve that forms the cylindrical fenestrated sheath. RN1 immunopositive cells (*arrow*) can also be found in the projections. **d** RN1 immunopositive bipolar cell (*arrow*) connecting the cylindrical fenestrated sheath (*arrowhead*) and the connective tissue plexus. **e** RN1 immunopositive fibers (*arrowheads*) and cell (*arrow*) within the tube feet's mesothelium. **f** anti-GFSKLYamide immunoreactivity within the tube feet disk is only observed in a subpopulation of fibers within the nerve plate. **g** In contrast, RN1 immunoreactivity is observed in individual fibers (*arrows*) coming from the apical nerves, in addition to the nerve plate. *al* ambulacral lumen, *ctp* connective tissue plexus, *me* mesothelium, *pn* podial nerve

GFSKLYamide or anti-galanin. Triple-labeling experiments with RN1, phalloidin, and Hoechst showed that cells and fibers were also present within the mesothelium (Fig. 4e). The cells were only labeled with RN1 or β-tubulin (no immunoreactivity was observed with anti-Sp-SynB, anti-GFSKLYamide or anti-galanin) and were mostly bipolar or multipolar; the cell body measuring 7 μm in length and 4 μm in width. The most impressive observation was that of RN1 immunopositive fibers, some of them immunoreactive to anti-Sp-SynB, crossing or connecting nervous structures. Fibers could be followed within the tissue sections and appeared to be entering or going out of the cylindrical fenestrated sheath, into or out to the connective tissue

plexus and the mesothelium (Fig. 4d). The fibers connecting with the connective tissue plexus were more abundant and occasionally RN1 immunopositive cell bodies were found in both locations (Fig. 4d). In the mesothelium, fibers extending from cells lining the lumen were observed traversing the longitudinal muscle and could sometimes be followed into the podial cylindrical fenestrated sheath and podial nerve.

The tube feet's terminal disk nervous system was connected to the stem's nervous system by means of apical nerves, as was revealed by RN1, anti-GFSKLYamide, and Hoechst labeling (Fig. 4f, g). A strong labeling of fibers was observed in the neural plate with RN1, anti-Sp-SynB, anti-GFSKLYamide, and anti-galanin though no somas were identified. RN1 immunopositive fibers were observed connecting the podial nerve in the stem with the nerve plate in the terminal disk. These fibers corresponded to the apical nerves, from which individual RN1 immunopositive fibers were observed outlining the supporting ossicles of the disk, before reaching the nerve plate (Fig. 4g). Subpopulations of fibers within the apical nerves were also labeled by anti-Sp-SynB and anti-GFSKLYamide, but rarely by anti-galanin.

Tentacles

Most specimens of *H. glaberrima* had 20 tentacles (stems) localized at the anterior end of the animal, although specimens with 19 and 21 tentacles were also identified. The tentacles, similar to the tube feet, were outgrowth of the body wall and were associated with the water-vascular system and the ectoneural nervous component of the circumoral nerve ring. Morphologically, the tentacle was subdivided into two major structures, the stem, proximally, and the branches, distally. The stem was first subdivided into 5–10 secondary branches that gave rise distally to 3–5 tertiary branches. From the latter, 3–5 quaternary branches were observed extending distally and ending in a rounded papillae.

Classical dyes

Staining with MT revealed that the tentacles' stem and branches were extensions of the body wall, and their tissue layer histology was very similar to that of the tube feet stem (Fig. 5a, b), while the papillae resembled the disk's histology (Fig. 5c). The stem and branch layers as identified in a cross section of the tentacle's stem were the cuticle, epidermis, connective tissue, tentacular or buccal nerve, connective tissue, and the mesothelium (Fig. 5b). Some notable histological differences between the tube feet and the tentacles were observed with MT. First, a thicker aniline blue-stained layer was localized below the buccal nerve and the
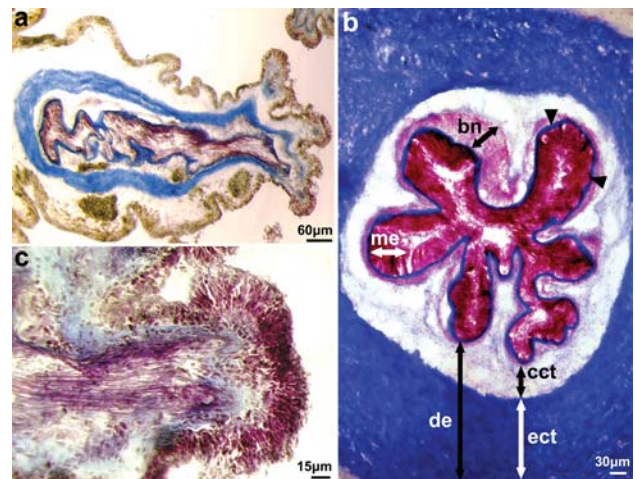
Fig. 5 *Holothuria glaberrima* (Holothuroidea). Milligan's Trichrome staining within the tentacle. The muscles are labeled in *red or magenta*, the connective tissue is in *blue* and the nervous system in a *light purple*. **a** Low magnification of a longitudinal section through the tentacle showing the tentacular stem and its irregular lumen, as well as branches originating from the main stem. **b** Transverse section through the stem showing the tissue layer subdivision: connective tissue (external connective tissue and central connective tissue), buccal nerve, inner connective tissue (*arrowheads*), and mesothelium. **c** Higher magnification of the tentacle showing the final branches of the tentacle with its rounded papillae and the absence of supporting ossicles. *bn* buccal nerve, *ct* connective tissue, *de* dermis, *ect* external connective tissue, *cct* central connective tissue, *me* mesothelium

buccal cylindrical fenestrated sheath. Second, the lumen of the tentacle had a lobular shape. Third, 2–5 buds were observed in the papillae of each quaternary branch, when compared to only one disk per tube foot stem. Fourth, no supporting ossicles were found within the tentacle buds (Fig. 5c).

Immunohistochemistry

The tissue layer distribution was verified by immunohistochemistry, and some differences were observed (Fig. 6a–d). The muscular component resembled that of the tube feet, being the longitudinal muscle the only muscle present within the tentacle. The connective tissue distribution was the same for the external connective tissue (*ECT*) and central connective tissue (*CCT*), but not for the internal connective tissue (*ICT*). The *ICT* was intensely stained by aniline blue, while no detectable immunoreactivity to Hg-fCol was observed.

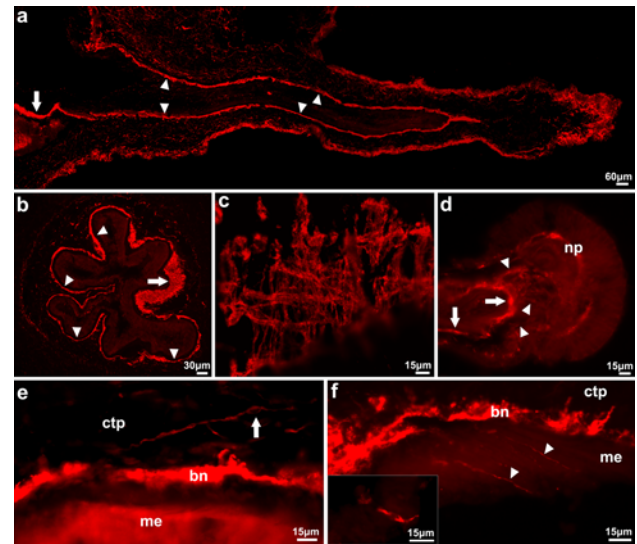*Tentacles nervous system innervations and subdivisions*

The holothurian tentacular nervous system is associated with the ectoneural component of the nervous system, as shown by the origin of the tentacular nerve from the circumoral nerve ring. The tentacular nerve was formed by
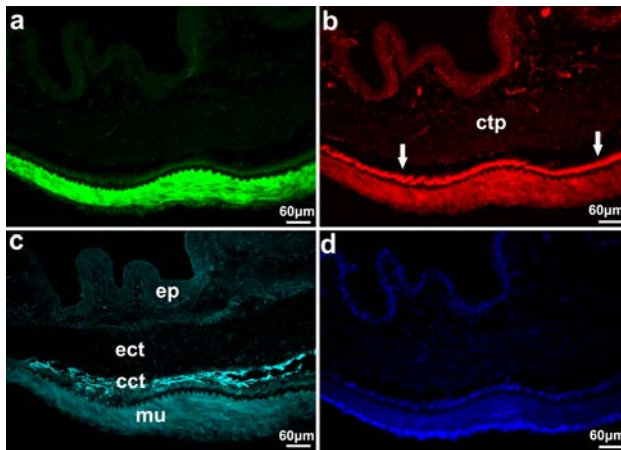
Fig. 6 *Holothuria glaberrima* (Holothuroidea). Longitudinal section through the stem of the tentacle. **a** Phalloidin-labeled section showing the longitudinal muscle layer. **b** The distribution of the nervous system within the tentacle as revealed by immunoreactivity to RN1. Immunolabeling was observed within the buccal nerve (*arrows*) and the connective tissue plexus. **c** Hg-fCol immunoreactivity in the tentacle showing the differential distribution of collagen fibers within the connective tissue. **d** Hoechst-stained section showing the distribution of cells within the tentacular stem. *ep* epidermis, *ctp* connective tissue plexus, *cct* central connective tissue, *mu* longitudinal muscle

Fig. 7 *Holothuria glaberrima* (Holothuroidea). Nervous system distribution in the tentacle, as revealed by RN1 immunoreactivity. **a** Longitudinal section through the tentacles showing the connection of the tentacle by a lateral nerve that originates from the circumoral nerve ring. This nerve forms the buccal nerve (*arrow*), which forms the buccal cylindrical fenestrated sheath (*arrowheads*). **b** Transverse section through the tentacle showing the bean-shaped buccal nerve (*arrow*) and buccal cylindrical fenestrated sheath (*arrowheads*). **c** The buccal cylindrical fenestrated sheath is formed by perpendicular projections of the buccal nerve at different levels. **d** Longitudinal section through the tentacle's papillae showing the nerve plate and the apical nerves (*arrowheads*), extending from the buccal nerve and buccal cylindrical fenestrated sheath (*arrows*). **e** RN1 immunopositive bipolar cell (*arrow*) connecting the buccal cylindrical fenestrated sheath and the connective tissue plexus. **f** RN1 immunopositive fibers (*arrowheads*) and cells (*insert*) within the mesothelium. *bn* buccal nerve, *ctp* connective tissue plexus, *me* mesothelium, *np* nerve plate

a single prolongation that entered the basal end of each tentacle (Fig. 7a). The tentacular nervous system was divided into four main components: the buccal nerve and buccal cylindrical fenestrated sheath, the connective tissue plexus, the mesothelium plexus, and the papillae plexus. RN1 immunoreactivity was very similar to that of the tube feet in all the four components. Briefly, it was more prominent in the buccal nerve, which is observed, in cross section, as a bean-shaped enlargement within the buccal cylindrical fenestrated sheath (Fig. 7b). As observed in the tube feet, this nerve was also labeled by anti-β-tubulin, while a small subpopulation of fibers within it was labeled by anti-Sp-SynB (not shown). Compared to the tube feet findings in which around 30% of the fibers were immunopositive to anti-GFSKLYamide, only a few fibers within the buccal nerve, around 5%, were immunoreactive to anti-GFSKLYFamide or anti-galanin. Once the unbranched buccal nerve entered the tentacle, projections were observed at regular intervals extending in an orthogonal direction to the longitudinal muscle fibers, forming the buccal cylindrical fenestrated sheath (Fig. 7b, c).

An extensive labeling of RN1 immunopositive fibers and cells was observed within the connective tissue plexus (Fig. 7a, b, e). The cells measured approximately 10 μm in length and 5 μm in width. Minor subpopulations of RN1 immunopositive fibers were also labeled within the connective tissue plexus by anti-GFSKLYamide or anti-Sp-SynB, but no immunoreactivity to anti-galanin was observed. On the other hand, very few fibers and cells were found in the

mesothelium, and these were only labeled by RN1 (Fig. 7f). The cells within the mesothelium were mostly unipolar, the cell body measuring approximately 7 μm in length and 4 μm in width. Double labeling with RN1 and Hoechst showed that the cells were localized among the cell bodies of the tentacular luminal epithelia (Fig. 7f). The distribution of fibers within the mesothelium was similar to that of the tube feets' in which projections from these unipolar cells were observed entering the mesothelium and extending to the buccal cylindrical fenestrated sheath and buccal nerve. As in the tube feet, RN1 immunopositive fibers were observed entering or going out of the buccal cylindrical fenestrated sheath from or to the connective tissue plexus and the mesothelium. The fibers connecting with the connective tissue plexus were more abundant and, occasionally, RN1 immunopositive cell bodies were associated with them (Fig. 7e). Immunoreactivity to anti-Sp-SynB was also observed in a subpopulation of the RN1 immunopositive fibers entering or going out of the buccal cylindrical fenestrated sheath from or to the connective tissue plexus.

The fourth component, the papillae nervous system, was analogous to that of the tube feet's terminal disk nervous system. Fibers within the nerve plate were strongly labeled by RN1 and anti-β-tubulin, while subpopulations of fibers immunoreactive to RN1 were labeled by anti-Sp-SynB and anti-GFSKLYamide. No fibers immunopositive to anti-galanin or somas immunoreactive to any of the markers were observed within this plexus. The papillae nervous system was connected to the stem's nervous system by means of apical nerves that extended from the buccal nerve and buccal cylindrical fenestrated sheath up to the papillae plexus and through fibers coming from the connective tissue plexus, as shown by RN1 immunoreactivity (Fig. 7d). The apical nerves were also immunoreactive to anti-β-tubulin, and a subpopulation of fibers within them to anti-Sp-SynB and anti-GFSKLYamide, but not to anti-galanin.

Discussion

Podial innervation by the ectoneural component of the radial nerve

Hyman (1955) established that the tube feet and tentacles are innervated by the ectoneural component of the radial nerve. Our results provide unambiguous evidence by showing sections in which the lateral nerves could be clearly followed from the radial nerve or circumoral nerve ring to the respective podia. The innervation of the tube feet by a lateral nerve derived from the ectoneural component of the radial nerve has also been reported for the sea urchins *Strongylocentrotus franciscanus* (A. Agassiz, 1863) (Echinoidea, Echinoida), *Arbacia lixula* (Gray, 1835) (Echinoidea, Arbacioida) and *Echinus esculentus* (Linnaeus, 1758) (Echinoidea, Echinoida) (Florey and Cahill 1977), the starfish *Asterias rubens* (Linnaeus, 1758) (Asteroidea, Forcipulatida) (Moore and Thorndyke 1993), and the sea cucumber *Apostichopus japonicus* (Selenka, 1867) (Holothuroidea, Aspidochirotida) (Inoue et al. 1999) using different approaches (transmission electron microscopy and immunohistochemistry). The same has not been true for the tentacular innervations, as these structures are not well developed or present in other echinoderm classes (Hyman 1955).

Using RN1, we confirmed that these nerves that enter these specialized organs do not branch until they are embedded in the organ. They only branch to give rise to the podial nervous system. Interestingly, the presence of a neural plexus underneath the epidermis has been reported in the sea cucumbers *Parastichopus californicus* (Stimpson, 1857) (Holothuroidea, Aspidochirotida) (Cavey 2006) and *Holothuria forskali* (Chiaje, 1841) (Holothuroidea, Aspidochirotida) (Flammang and Jangoux 1992) through TEM studies and named the basiepithelial plexus. Description of

this plexus using immunofluorescence in *H. glaberrima* shows that, although it is more conspicuous underneath the epidermis or in the *ECT*, the plexus, called the connective tissue plexus, is found throughout the dermis (Díaz-Balzac et al. 2007).

Podial nervous system

The podial nerves in the tube feet and tentacles are the component of the podia that have been best described in the literature (Hyman 1955; Flammang and Jangoux 1992; Bouland et al. 1982; Smiley 1994; Inoue et al. 1999; Cavey 2006), as they are conspicuous and easily identifiable. These are formed by extensions of the ectoneural component of the radial nerve known as lateral nerves. The same markers that label the radial nerve and lateral nerves (RN1, anti-β-tubulin, 1E11, anti-Sp-SynB, anti-GFSKLYamide, anti-galanin, anti-NGWIYa-L1), also label the podial nerve; supporting the idea that the podial nerve is an elongation of the ectoneural component of the radial nerve (Flammang and Jangoux 1992; Inoue et al. 1999; Cavey 2006; Tamori et al. 2007). Interestingly, very few RN1 immunopositive cells were observed in the main podial nerve, although neuronal bodies have been well documented in ultrastructural studies (Smiley 1994; Cavey 2006). This is somewhat troublesome, but it can be explained by the same phenomenon observed in the radial nerve that RN1 fails to label the neuronal soma (Díaz-Balzac et al. 2007).

The podial cylindrical fenestrated sheath conserves the same configuration as the podial nerve and radial nerve, a peripheral arrangement of cells and the neuropile in the center; though the numbers of cells diminish within the first. The small number of cells within the podial cylindrical fenestrated sheath might be the reason why many ultrastructural studies (Florey and Cahill 1977; Cavey 2006), and even immunohistological studies (Inoue et al. 1999), do not account for cells bodies within the projections. Another possible explanation is that these cells may not express the usual markers that are used to identify neurons in the echinoderm nervous system. This was observed by Tamori et al. (2007) in the tube feet of the sea cucumber *Apostichopus japonicus*, as some of these cells were immunoreactive to anti-stichopin, but not to 1E11 (a marker of a neuronal-specific SynaptotagminB).

Due to its localization, the podial nerve has been proposed to be part of the connective tissue layer (Florey and Cahill 1977; Wood and Cavey 1981; Cavey and Wood 1981; Rieger and Lombardi 1987; Cavey 2006), as it is embedded within the connective tissue, and depending on the echinoderm, it may be closer to the mesothelium or to the epidermis. Our findings in *H. glaberrima* are consonant with these previous findings. The main difference in the tissue layer arrangement between holothurian and other

echinoderms is the location of the podial nerve (longitudinal nerve) and its cylindrical fenestrated sheath. In the sea urchins *Strongylocentrotus franciscanus, Arbacia lixula* and *Echinus esculentus* (Florey and Cahill 1977) and starfish *Asterias rubens* (Moore and Thorndyke 1993), it is located underneath the epidermis, while in the holothurians, it is found near the mesothelium. This difference is probably due to the size of the corresponding connective tissue layer. The podial nervous system can be subdivided into three other components, which are the podial connective tissue plexus (including what has been described as the basiepithelial plexus), the podial mesothelium nervous system, and the disk nervous system (papillae nervous system in the tentacle). We will discuss them separately and then try to provide a cohesive view of their connections.

Our group (Díaz-Balzac et al. 2007) has previously described the presence of a connective tissue nerve plexus within the tube feet and tentacle, and has now found it to be connected to the podial nerve and cylindrical fenestrated sheath. We observed cell processes of bipolar cells connecting the podial cylindrical fenestrated sheath and the connective tissue plexus. We have previously proposed that the neural plexus within the connective tissue of the body wall is the entity responsible for controlling the tensility changes of the body wall (Díaz-Balzac et al. 2007), a characteristic of the echinoderm mutable connective tissue. This theory is also supported by the expression of the neuropeptide stichopin in fibers and cells present in the connective tissues of the sea cucumber *Apostichopus japonicus* and its role in the regulation of the stiffening of the body wall (Tamori et al. 2007). Mutable connective tissue has also been reported to be present in the tube feet of the sea urchin *Paracentrotus lividus* (Gray, 1825) (Echinoidea, Echinoida) and starfish *Marthasterias glacialis* (Gray, 1840) (Asteroidea, Forcipulatida) (Santos et al. 2005). Therefore, the neural plexus within the tube feet's connective tissue probably has the same function as that of the body wall in controlling tensility changes and giving them this particular characteristic.

The disk or papillae nervous component has been little studied. In general, it has been described as being similar to the epidermal plexus with connections to the main podial stem plexus. Our results agree with this description, however, it is slightly more complex, since the nerve plate fibers seem to come from two sources: by way of thin projections from the apical end of the podial nerve or directly from the connective tissue plexus. Abundant RN1, anti-β-tubulin, anti-Sp-SynB, and anti-GFSKLYamide immunopositive fibers, but no immunopositive cells, were found within the nerve plate, even though a large group of cell nuclei corresponding to the epidermis were identified. Some of these nuclei probably correspond to the neurons present within the neural plate of the disk of *Holothuria forskali* described by Flammang and Jangoux (1992) that are not labeled by

our markers. The nervous system of the papillae was referred by Bouland et al. 1982 as the epineural plexus, but as there are no major differences between the epineural and hyponeural plexus, we consider that a more appropriate name for it is the papillae plexus.

Finally, we describe for the first time, the presence of nervous system components within the mesothelium. The mesothelium has been described ultrastructurally as composed of mainly two types of cells, adluminal and muscle-retractor cells (Flammang and Jangoux 1992; Cavey 2006). Now, we have added a third less abundant type, which may play an important role in the podial physiology and deserves further attention and analysis. These cells could correspond to the neuron-like cells reported by Cobb (1987) to be present among the muscles cells of the podia, as well as in other organs. In the sea cucumber *Holothuria forskali*, Flammang and Jangoux (1992) observed the presence of thin cellular processes (but not cell bodies), characterized by electron-dense, membrane-bound granules within their cytoplasm. These cells, named granulocytes, have also been reported to be present in other the echinoderms (Florey and Cahill 1977; Wood and Cavey 1981; Cavey and Wood 1981; Rieger and Lombardi 1987). Therefore, it is very likely that the cells labeled by RN1 within the mesothelium are the granulocytes. The same type of cell has been found in other coelomic epithelia (Díaz-Balzac et al. 2007), and the possibility exists that these are sensory neurons that are sensing changes in the coelomic fluid environment or in the coelomic epithelium itself. It is difficult to describe these neuron-like cells found within the coelomic epithelia as a plexus, since they appear to be isolated and it remains unclear to what other cells they may be connecting. Nonetheless, they extend long fibers into the muscular component of the mesothelium and could be involved in modulating the contractile response.

Muscular layer innervation

The innervation of the muscle cells within the podial mesothelium has been one of the mysteries associated with echinoderm neurobiology. It has been proposed that it correspond to an indirect innervation, in which the control comes from nerve fibers of the podial nerve that end at (but do not cross) the basal lamina of the mesothelium (Bouland et al. 1982; Flammang and Jangoux 1992; Cavey 2006). This is based on the absence of fibers observed within the mesothelium and that no fibers are observed to be crossing the basal lamina. Our results show that there are fibers extending into or coming from the muscle, and even RN1 immunopositive cell bodies were identified within the mesothelium. However, it is important to highlight that the number of cells and fibers is still well below the numbers expected for muscle innervation. Particularly, if we compare
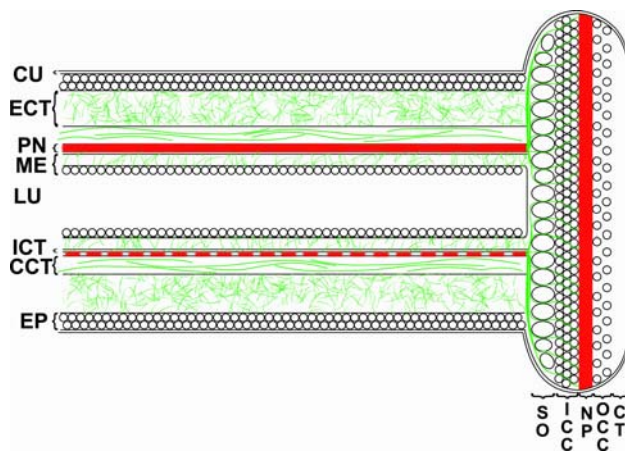
Fig. 8 *Holothuria glaberrima* (Holothuroidea). Model proposed for the neuroanatomy of the podia. In *red* are the neural fibers that correspond to the major nerves as identified by co-labeling with RN1 and anti-GFSKLYamide, while in *green* are the individual cells and fibers labeled by only RN1. *cu* cuticle, *ect* external connective tissue, *pn* podial nerve, *me* mesothelium, *lu* lumen, *ict* internal connective tissue, *cct* central connective tissue, *ep* epidermis, *so* supporting ossicles, *icc* inner cluster of cells, *np* nerve plate, *occ* outer cluster of cells

this labeling with that of RN1 labeling in the body wall longitudinal muscle (unpublished observations) where immunoreactive fibers are observed adjacent to every muscle fiber. Thus, the innervation of the podial muscle remains a mystery to be solved as well as the role of the RN1 cells and fibers within the coelomic epithelia.

Anatomical circuits

Our results using RN1 immunoreactivity data together with previous descriptions of the podial nervous system can be summarized in a proposed neuroanatomical circuit for the podia (Fig. 8). The circuit can be divided into five main sections or connections. First, the podia are connected to the main nervous system by a major lateral nerve that projects from the ectoneural component of the radial nerve or circumoral nerve ring. This explicitly suggests that the podial nervous system, especially the podial nerve, is derived mostly from the ectoneural component of the nervous system. Second, the podial nerve sends orthogonal projections every certain distance around the podial stem forming the podial cylindrical fenestrated sheath. Third, individual fibers from the podial cylindrical fenestrated sheath connect with either the connective tissue nerve plexus or the mesothelium. Fourth, there are cell bodies localized within the connective tissue plexus and mesothelium that send projections to the podial cylindrical fenestrated sheath. The only piece of information missing in the circuit is the localization of most neuronal cell bodies, since our markers only labeled a few cell bodies. The most probable place for their localization is the periphery of the

podial nerve, as seems to occur with the cell somas of the radial nerve (Díaz-Balzac et al. 2007). A fifth connection is observed in the distal end of the podia, which may be characterized by a papillae, a sucker, or a terminal disk. In these, individual apical nerves from the podial nerve and the connective tissue plexus connect with the neural plate of the tube feet disk. Even though here we propose a model of the circuitry for the connections of the nervous system, more experimentation is needed in order to understand the direction of the connections, as we do not know with great assurance which fiber correspond to motor, sensory, or interneurons.

It is our hope that this neuroanatomical description, together with the recent identification of many conserved vertebrate neural genes (Burke et al. 2006b) following the acquisition of the sea urchin genome (Sea Urchin Genome Sequencing Consortium 2006), enhance the growing interest in echinoderm neurobiology. This will also facilitate the coming together of echinoderms' cellular and molecular biology, an essential combination in order to answer complex questions and understand complicated processes such as the evolutionary relationships of the Metazoan nervous systems.

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