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ORIGINAL PAPER

The catecholaminergic nerve plexus of Holothuroidea

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Abstract Catecholamines have been extensively reported to be present in most animal groups, including members of Echinodermata. In this study, we investigated the presence and distribution of catecholaminergic nerves in two members of the Holothuroidea, *Holothuria glaberrima* (Selenka, 1867) (Aspidochirotida, Holothuroidea) and *Holothuria mexicana* (Ludwig, 1875) (Aspidochirotida, Holothuroidea), by using induced fluorescence for catecholamines on tissue sections and immunohistochemistry with an antibody that recognizes tyrosine hydroxylase. The presence of a catecholaminergic nerve plexus similar in distribution and extension to those previously reported in other members of Echinodermata was observed. This plexus, composed of cells and fibers, is found in the ectoneural component of the echinoderm nervous system and is continuous with the circumoral nerve ring and the radial nerves, tentacular nerves, and esophageal plexus. In addition, fluorescent nerves in the tube feet are continuous with the catecholaminergic components of the radial nerve cords. This is the first comprehensive report on the presence and distribution of catecholamines in the nervous system of Holothuroidea. The continuity and distribution of the catecholaminergic plexus strengthen the notion that the catecholaminergic cells are interneurons, since these do not form part of the known sensory or motor circuits and the fluorescence is confined to organized nervous tissue.

Keywords *Holothuria glaberrima* (Echinodermata) · *Holothuria mexicana* (Echinodermata) · Holothuroidea · Catecholamines · Neurotransmitter · Nervous system

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Introduction

The anatomy of the nervous system of echinoderms has been described by several investigators (Hyman 1955; Cobb 1969a, b, 1987; Cottrell and Pentreath 1970; Pentreath and Cobb 1972; Märkel and Röser 1991; Newman and Thorndyke 1994; Smiley 1994; Díaz-Miranda et al. 1995, 1996; García-Arrarás et al. 2001; Mashanov et al. 2006; Díaz-Balzac et al. 2007, 2010). Extensive information has been gathered in recent years on the nervous components of the larval stages (Buznikov et al. 2005; Nakano et al. 2006; Bishop and Burke 2007; Murabe et al. 2008; Elia et al. 2009). However, much less is known about the organization of the nervous system in adult organisms. Information concerning the different types of neurons is scarce, particularly on their biochemical specialization, e.g. the neurotransmitters, the cells use for communication. Catecholamines, one of the 'classical' neurotransmitters of the metazoans, have been extensively reported to be present in most animal groups (Sulston et al. 1975; Hay-Schmidt 1990, 1992; Gonzalez and Smeets 1991; Pierre et al. 1997; Pulver et al. 2003; Faller et al. 2008; Voronezhskaya et al. 2008; Martínez-Rubio et al. 2009), including members of Echinodermata. The occurrence of catecholaminergic cells and fibers has been demonstrated in the larvae of members

from three of the representative groups of Echinodermata: Echinoidea, Asteroidea, and Holothuroidea. Evidence also exists for the presence of catecholaminergic cells and fibers in tissues of adult Asteroidea, Ophiuriodea, and Echinoidea (Cottrell 1967; Cobb 1969a; Cottrell and Pentreath 1970). The only study looking into the presence of catecholamines in the Holothuroidea was done in *Apostichopus japonicus* (Selenka, 1867) (Aspidochirotida, Stichopodidae) (Inoue et al. 2002). In this study, catecholamines were only detected in the connective tissue of the dermis. We have now performed an extensive study on the presence of a catecholaminergic plexus in Holothuroidea and demonstrate convincing evidence for a more extensive catecholaminergic nerve plexus in various tissues and organs of members of Holothuroidea, in particular the species, *Holothuria glaberrima* (Selenka, 1867) (Aspidochirotida, Holothuroidea) and *Holothuria mexicana* (Ludwig, 1875) (Aspidochirotida, Holothuroidea). The results presented in this study will be useful for future comparative studies, as well as for a better understanding of the subdivisions and complexity of the nervous system of Echinodermata.

Materials and methods

Animals

Adult *Holothuria glaberrima* (Selenka, 1867) (Aspidochirotida, Holothuroidea) and adult *Holothuria mexicana* (Ludwig, 1875) (Aspidochirotida, Holothuroidea) specimens were collected from coastal areas of northeastern Puerto Rico. The specimens were either used immediately for histochemistry or were kept in an outdoor pool at the Institute of Neurobiology in San Juan (*H. mexicana*) or in 200 L aquaria (*H. glaberrima*). In some cases, animals were also kept in sea water aquaria at the University of Puerto Rico in Río Piedras.

Catecholamine histochemistry

Specimens were anesthetized by placing them in 6% $MgCl_2$ before dissection. The dissected tissues included the entire digestive tract, tentacles, circumoral nerve ring with adjacent structures, body wall (including the longitudinal muscle bands along with the radial nerves), cloaca, respiratory tree, hemal system, and the gonads. Two methods for detecting catecholaminergic cells and fibers were used. The first method was a modification of the sucrose phosphate glyoxylic acid (SPG) method described by De la Torre and Surgeon (1976). In brief, tissues were frozen at −30°C in Tissue-Tek (Sakura Finetek, Torrance, CA), immediately after removal. Sections (20–25 μm) were recovered on gelatin-coated slides and covered with the SPG solution (1% glyoxylic acid, 0.2 M sucrose in 0.236 M KH_2PO_4 buffer at pH 7.4) for 3–5 min. Excess solution was removed, and the slides were dried in cool air for 10 min and oven-heated for 5 min at 80°C. In some cases, the SPG method was applied to squashed whole mounts of tissues from the digestive tract. For these experiments, thin unfixed sections of tissues were obtained with a scalpel and immersed in the SPG solution as described for tissue sections. The tissues were sometimes stretched with forceps or 'squashed' by pressuring with a cover slip before air-dried.

The second catecholamine-detecting method was a modification of the formaldehyde–glutaraldehyde (FAGLU) method of Scholer and Armstrong (1982). In this method, the dissected tissues were fixed in a solution of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer for 1–2 days. The tissues were then transferred to the same fixative solution containing 20% sucrose for 24 h, frozen at −30°C, sectioned in a cryostat (12–30 μm), and mounted on gelatin-coated slides. The slides were then dried under a cold blower for 20 min and heated in an oven at approximately 80°C for 10 additional minutes to allow the fluorescence-inducing reaction to take place.

Slides obtained from each of the two methods were coverslipped using paraffin oil and examined in an epifluorescence Nikon microscope equipped with a D filter for CA-fluorescence detection. In order to observe whether the catecholaminergic structures were continuous with each other, sections were taken every 60–100 μm and analyzed as described earlier.

The specificity of the SPG- and FAGLU-induced fluorescence was corroborated by testing the method with a positive control, adrenal chromaffin cells of avian species (Ramírez-Ordóñez and García-Arrarás 1995). These cells are well-known catecholaminergic cells. In addition, the induced fluorescence was abolished if cells were not dried thoroughly or if water was added, prior to applying the paraffin oil. This hydration is known to disrupt the catecholamine-induced fluorescent reaction (De la Torre and Surgeon 1976).

Immunohistochemistry

Specimens for immunohistochemistry were anesthetized by placing them in 0.2% 1,1,1-trichloro-2-methyl-2-propanol (Sigma, St. Louis, MO) before dissection. The body wall (including the longitudinal muscle bands along with the radial nerves) was dissected and fixed in 4% paraformaldehyde at 4°C for approximately 1 h. Tissues were then rinsed 3 times for 15 min with 0.1 M phosphate-buffered saline (PBS), and left in a 30% sucrose solution at 4°C. Once the tissues had been in 30% sucrose solution for at least 24 h, they were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA). Cryostat tissue sections of 14 μm were cut and mounted on Poly-L-lysine-coated slides.

The indirect immunofluorescence method was followed (García-Arrarás 1993; Díaz-Balzac et al. 2007). In brief, tissues were rinsed for 5 min in 0.1 M PBS, followed by a 1-h incubation with goat serum 1:50 (Invitrogen, Carlsbad, CA). The incubation was followed by a 15-min rinse in 1% Triton X, and two other rinses in 0.1 M PBS. Subsequently, the sections were incubated overnight with the primary antibody anti-TH (used at a dilution of 1:100), which was raised against tyrosine hydroxylase of quail, *Coturnix coturnix Japonica* (Fauquet and Ziller 1989). The incubation was followed by three 15-min rinses with 0.1 M PBS, a 1-h incubation with the secondary antibody, and finalizing with three more 15-min rinses with 0.1 M PBS. The secondary CY3 antibody used was the Goat anti-mouse Cy3 (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, #111-165-144 Lot. 50694), which was used at a 1:2,000 dilution.

Cell nuclei were stained with 2 μM DAPI (Sigma, St. Louis, MO) in the buffered glycerol solution used to mount the slides. Tissues were examined, and photomicrographs were taken on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII and DAPI filters. Images were recorded using the Spot Basic software (version 4.7; Diagnostic Instruments, Sterling Heights, MI). These were cropped, brightness and contrast adjusted, using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

The anti-TH marker used in this study was thoroughly characterized previously by means of immunoblotting and immunohistochemistry (Fauquet and Ziller 1989). Immunoreactivity with the echinoderm tyrosine hydroxylase enzyme was expected due to the high sequence conservation observed in this gene throughout the metazoans. Interestingly, this antibody does not crossreact with the mammalian tyrosine hydroxylase, but it does react with the avian *Gallus gallus* (Linnaeus, 1758) (Galliformes, Aves) and the amphibian *Pleurodeles waltlii* (Michahelles, 1830) (Urodela, Amphibia) tyrosine hydroxylase. In addition, negative controls that consisted of adding goat serum to the sections during the incubation of the primary antibody, instead of anti-TH, and diluting the primary antibody, were analyzed to determine the specificity of the antibody. No immunoreactivity was observed in the negative controls. Additionally, diluting the anti-TH antibody until the labeling was too weak to be clearly observed diminished the immunoreactivity.

Results

The two fluorescent histochemical methods used for detecting catecholamines in tissues produced the characteristic blue-green fluorescence in cells and fibers that indicated the presence of catecholamines. The localization of catecholamines to specific structures and their distribution, as observed with both methods, were similar; however, the brightness obtained using the SPG method was substantially stronger than that obtained using the FAGLU method. The SPG method permitted a larger number of fibers and cells bodies to be seen, as wells as an easier and rapid examination of the tissues under low magnification. Thus, most of our data, and in particular all the micrographs presented in the figures, were obtained using the SPG method. Additionally, it was also verified through immunohistochemistry with an antibody made against tyrosine hydroxylase, the rate-limiting enzyme in the catecholamine synthesis pathway.

In both species of Holothuroidea, catecholamine fluorescence was limited to nervous tissue, specifically the nerve ring and radial nerves, and associated with nervous elements within the tentacles, tube feet, and the esophageal region of the digestive tract. The fluorescence was found in cells and fibers of the ectoneural nervous system. Fluorescent fibers were abundant and distributed throughout the nervous tissue; however, within the nervous tissue itself, they did not constitute the main bulk of the structure since many non-fluorescent areas were observed. The fibers had varicosities that were observed as pinpoints of fluorescence and, in fact, most of the fluorescent structures were distinguished by these fluorescent dots of variable sizes that in some instances could be seen to be connected. Cells were usually found within or adjacent to the fiber networks, where they were sometimes concealed by the strong fluorescence of the fibers. Uni, bi, and multipolar cells were detected, usually isolated, but never in ganglion-like structures; although, occasionally, two or three cells were observed together. Following is a brief description of the catecholaminergic structures in each tissue.

Circumoral nerve ring

The circumoral nerve ring, which lies close to the inner surface of the calcareous ring and slightly above its medial axis showed strong catecholaminergic fluorescence distributed through it (Fig. 1a). The fluorescence was mainly localized in fibers and, occasionally, small (5.0 ± 0.3 μm long, 3.0 ± 0.3 μm wide; mean ± SE) intense fluorescent cells were found within the fiber networks (Fig. 1b). In some of the sections examined, the junction with the radial nerve could be seen as five large branches emerging from the posterior part of the ring. In the anterior part of the nerve ring, a number of smaller branches, corresponding to the tentacular nerves were observed. The luminal part of the nerve ring had fluorescent structures that were continuous with a nerve plexus in the digestive tract. Catecholamine-induced fluorescence was continuous throughout the extensions of nervous tissue (nerve ring-tentacular nerve, nerve ring-radial nerve, and nerve ring-esophageal plexus).

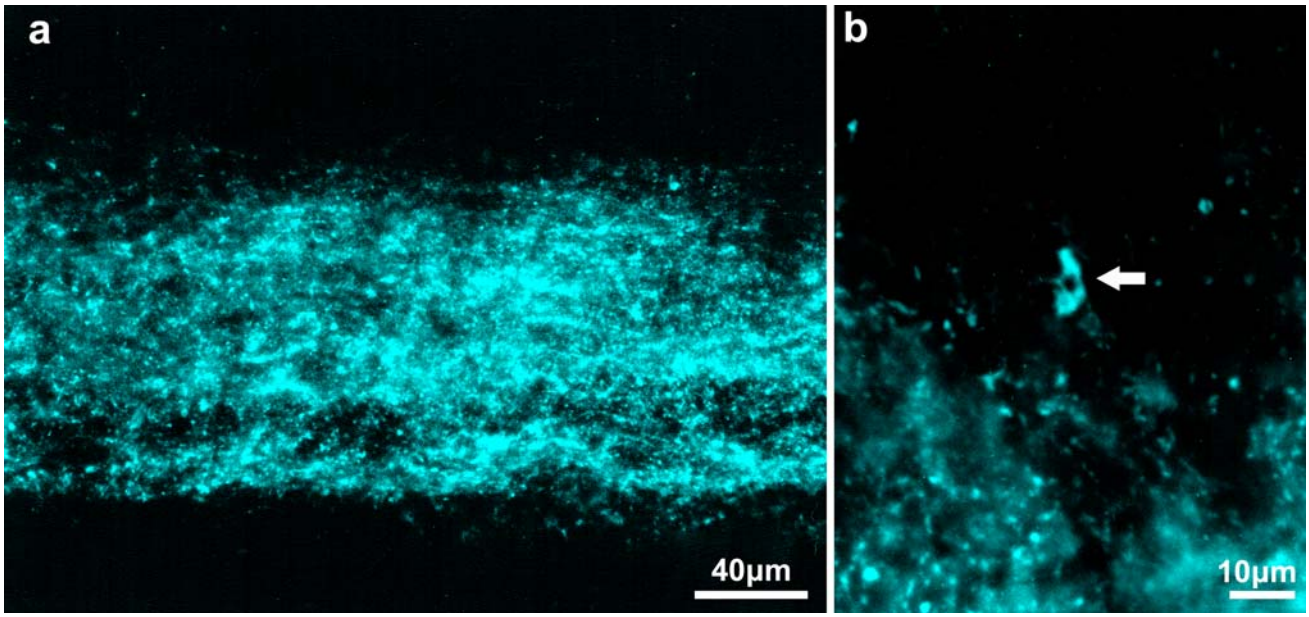


Fig. 1 *Holothuria glaberrima* (Holothuroidea). Catecholamine-induced fluorescence in the circumoral nerve ring. **a** Transverse section at the level of the circumoral nerve ring showing the network of catecholaminergic fibers. **b** Catecholaminergic cells (*arrow*) present within the fiber network, which are better distinguishable in its border

Tentacles

Varicose fibers and cells similar to those of the nerve ring were found in the tentacular nerve (Fig. 2a–c). The fluorescence could be followed from the nerve ring and was visualized in the tentacular nerve as it entered the base of the tentacle. As we moved toward the proximal part of the tentacle, the nerve plexus extended around the central lumen of the water vascular canal, forming the ectoneural nerve ring. As the tentacles branched, the tentacular nerve also branched and was present within each lobe of the upper portion of the tentacle, where it ended just below the tip. The plexus was always observed surrounding the lumen of the water vascular canal between two layers of connective tissue, a thin inner one that bordered the mesothelium and a wider outer layer that formed the bulk of the tentacle. Outside of the tentacular nerve and ectoneural nerve ring, no other tissue layer of the tentacle had fibers or cells that exhibited any induced fluorescence.

Radial nerve cord

The radial nerve cord of echinoderms has two main subdivisions: the ectoneural and hyponeural components. A plexus of fluorescent varicose fibers and cell bodies was found to be limited to the ectoneural component (Fig. 3a). This plexus encompassed the whole area of the nerve, but did not extend outside the nerve boundaries set by the connective tissue layers. Catecholaminergic cells were found in the ectoneural nerve portion, their somata usually lying in the periphery (Fig. 3b) from where they extended what appeared to be a single nerve fiber directed toward the central part of the nerve cord, where the catecholaminergic plexus is most abundant. No differences were found in radial nerves from the anterior, middle, or posterior sections of the organisms, suggesting that the five radial nerves have a continuous catecholaminergic ectoneural portion that extends from the origin of the radial nerve in the nerve ring, located in the anterior end of the organism, to its posterior end. In addition, extensions of the ectoneural component of the radial nerve that innervate the ambulacral tube feet in the ventral part of the organisms showed catecholaminergic fluorescence (Fig. 3c–e).

Catecholamine presence in the radial nerve cord was corroborated by immunoreactivity to anti-TH (Fig. 4a). There were approximately 4–7 anti-TH immunopositive cells per section, located on the periphery of the neuropile. These cells measured approximately 6 μm in length and 4 μm in width and had a unipolar or bipolar shape (Fig. 4b). The anti-TH immunoreactive fibers were almost exclusively observed in the center of the ectoneural component of the radial nerve. Additionally, the immunopositive fibers could be observed extending into the ectoneural lateral nerves that went into the body wall and formed the podial nerve. Immunopositive cells and fibers were observed as well in the tube feet's podial nerve and the nerve plate of the tube feet's disk (Fig. 4c, d).

Tube feet

In *H. glaberrima* ventral body wall, catecholaminergic branches originating from the radial nerves were observed to innervate the tube feet (Fig. 3a). These fibers formed a single bundle that entered the tube feet at its base and

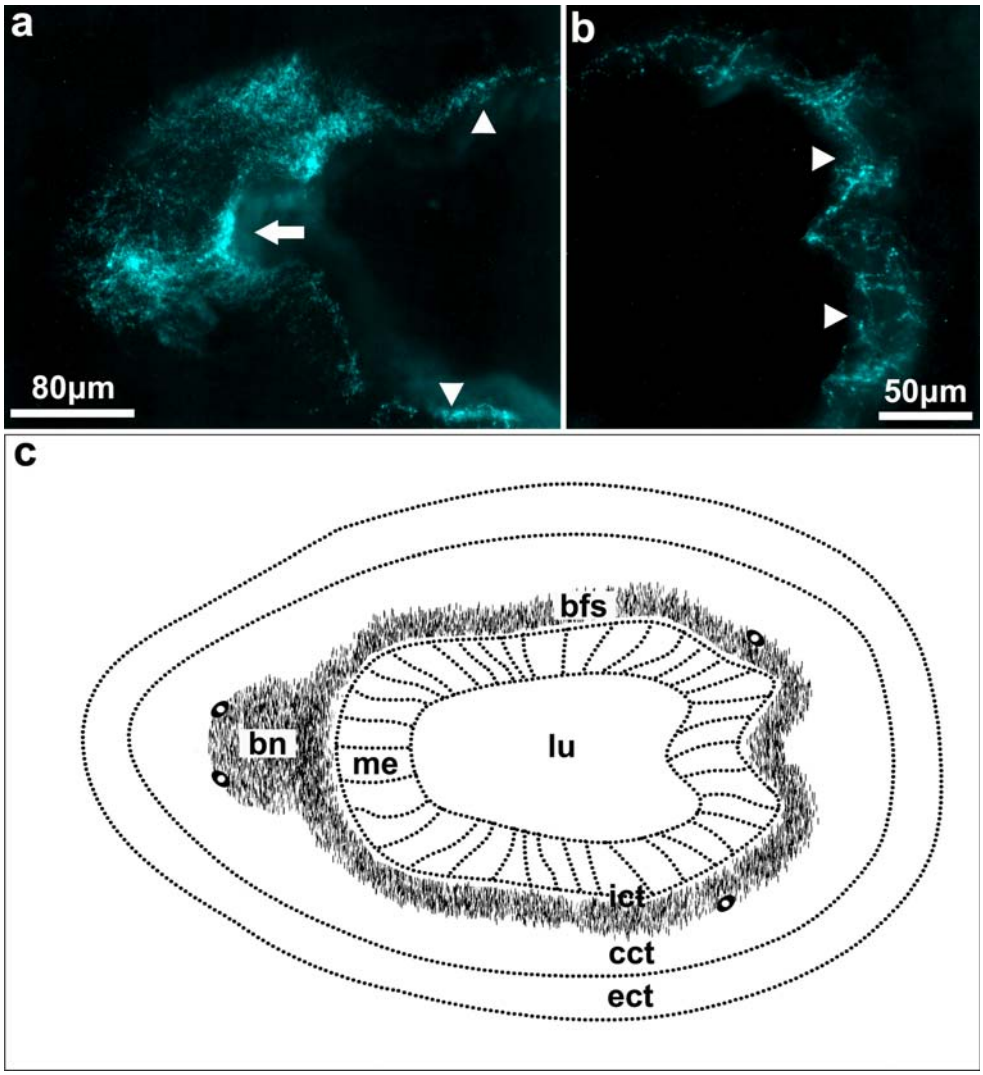


Fig. 2 *Holothuria glaberrima* (Holothuroidea). Catecholamine-induced fluorescence in the tentacles. **a**, **b** Transverse section through the tentacle showing catecholamine-induced fluorescence in the buccal nerve (*arrow*) and in the buccal cylindrical fenestrated sheath (*arrowheads*). **c** Diagram depicting the distribution of the catecholaminergic plexus within the tentacle of Holothuroidea. *bfs* buccal cylindrical fenestrated sheath, *bn* buccal nerve, *cct* central connective tissue, *ect* external connective tissue, *ict* inner connective tissue, *lu* lumen, *me* mesothelium

extended toward the tip (Figs. 3c, d, 4c). None of the fibers extended from the nerve trunk to the connective tissue plexus or the mesothelium plexus. The catecholaminergic fluorescence of the tube feet and its localization were similar to that observed for the tentacles.

Digestive tract

In the esophagus, a strongly fluorescent layer or plexus of cells and fibers was found in both species. In *H. glaberrima*, this layer measured approximately 20 µm in width (Fig. 5a). The plexus was localized within the inner connective tissue or submucosal layer on the luminal side of the muscle layer. Smaller nerve bundles were occasionally seen to diverge toward the inner layers of the tract, ending within the connective tissue layer, but were never observed to reach the mucosal layer. The esophageal catecholaminergic nerve layer was continuous with the circumoral nerve ring in the anterior part of the organism. At the posterior end of the esophagus, where it joined the small intestine, the nerve plexus transforms into a tract-like plexus (Fig. 5b). These tracts seem to disappear gradually, terminating approximately at a distance of about 1 cm after reaching the small intestine. Thus, no catecholaminergic fluorescence was observed in most of the digestive tract including most of the descending small intestine, the ascending intestine and the large intestine.

Whole mounts and transverse sections of the esophagus allowed a more detailed analysis of the catecholaminergic plexus (Fig. 5c–g). The fiber tracts were observed to vary in size from 2 to 16 µm with a mean width of 5.4 ± 0.6 µm (mean ± SE). These primarily ran in a longitudinal pattern following the length of the esophagus; however, the pattern was not entirely linear, so as to intertwined to create a network. Cells were found within the tracts, usually isolated at a mean distance of 300 µm from each other, although sometimes they were found in pairs. These cells were larger than those observed in the nerve ring or radial nerve, measuring 13 µm in length and 7 µm in width, and showing uni, bi, and multipolar ramifications. Their fibers could be

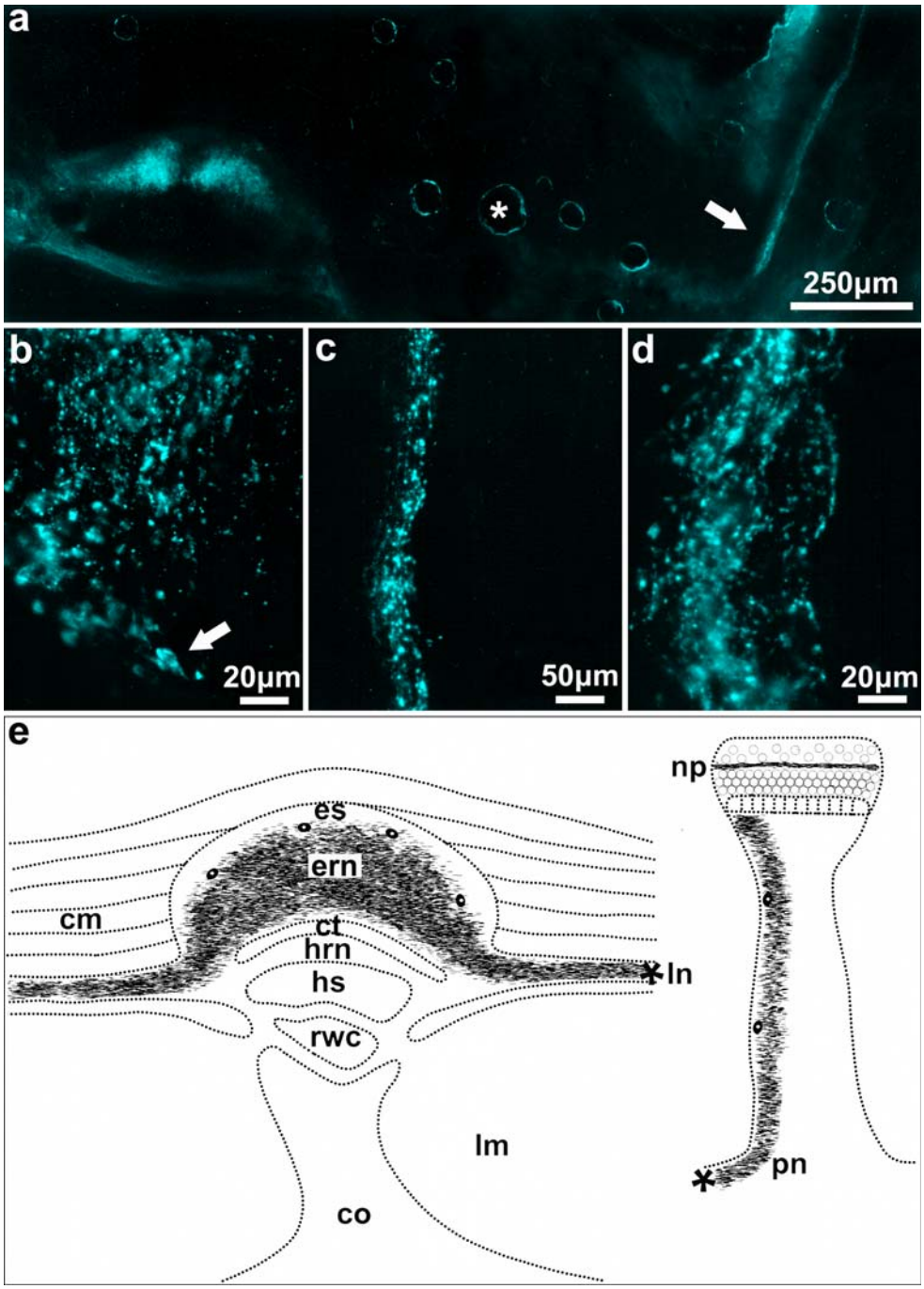


Fig. 3 *Holothuria glaberrima* (Holothuroidea). Catecholamine-induced fluorescence in the radial nerve cord and tube feet. **a** Transverse section of the body wall showing the induced fluorescence present in the ectoneural portion of the radial nerve cord and in the podial nerve (*arrow*). No fluorescence was present in the hyponeural component of the radial nerve cord. The circular structures (*) are artifacts produced by air bubbles introduced during the SPG procedure. **b** A section of the radial nerve at higher magnification showing details of the fiber network and a cell (*arrow*) present in the ectoneural component. **c** The podial nerve shows catecholamine-induced fluorescence, as can be observed in a longitudinal section. **d** A higher magnification of individual fluorescent fibers detected in the podial nerve. **e** Diagram showing the catecholaminergic containing structures within the radial nerve cord and tube feet. The anatomical continuity between the two nerves is also depicted as lateral nerves, branches from the radial nerve (*), which form the podial nerve as it enters the tube feet. *cm* circular muscle, *co* coelom, *ct* connective tissue, *ern* ectoneural component of the radial nerve, *es* epineural sinus, *hrn* hyponeural component of the radial nerve, *hs* hyponeural sinus, *lm* longitudinal muscle, *ln* lateral nerve, *np* nerve plate, *pn* podial nerve, *rwc* radial water vascular canal

followed for a short distance before they were lost among the other fluorescent fibers in the tract.

Non-catecholaminergic tissues

No catecholamine fluorescent or anti-TH immunopositive structures were observed in sections of the respiratory tree, the tubules of the hemal system, the cloaca and its suspensor ligaments, the gonads and its junction with the esophagus or in lantern-related structures such as the stone channel and the Polian vesicles. In sections of the body wall, which included the body wall muscles, no catecholaminergic cells or fibers other than those within the radial nerve or tube feet were observed.

Discussion

Catecholamines in the Echinodermata

Chemical studies, using the radial nerves of Asteroidea and Echinoidea, have shown that the catecholamines present in

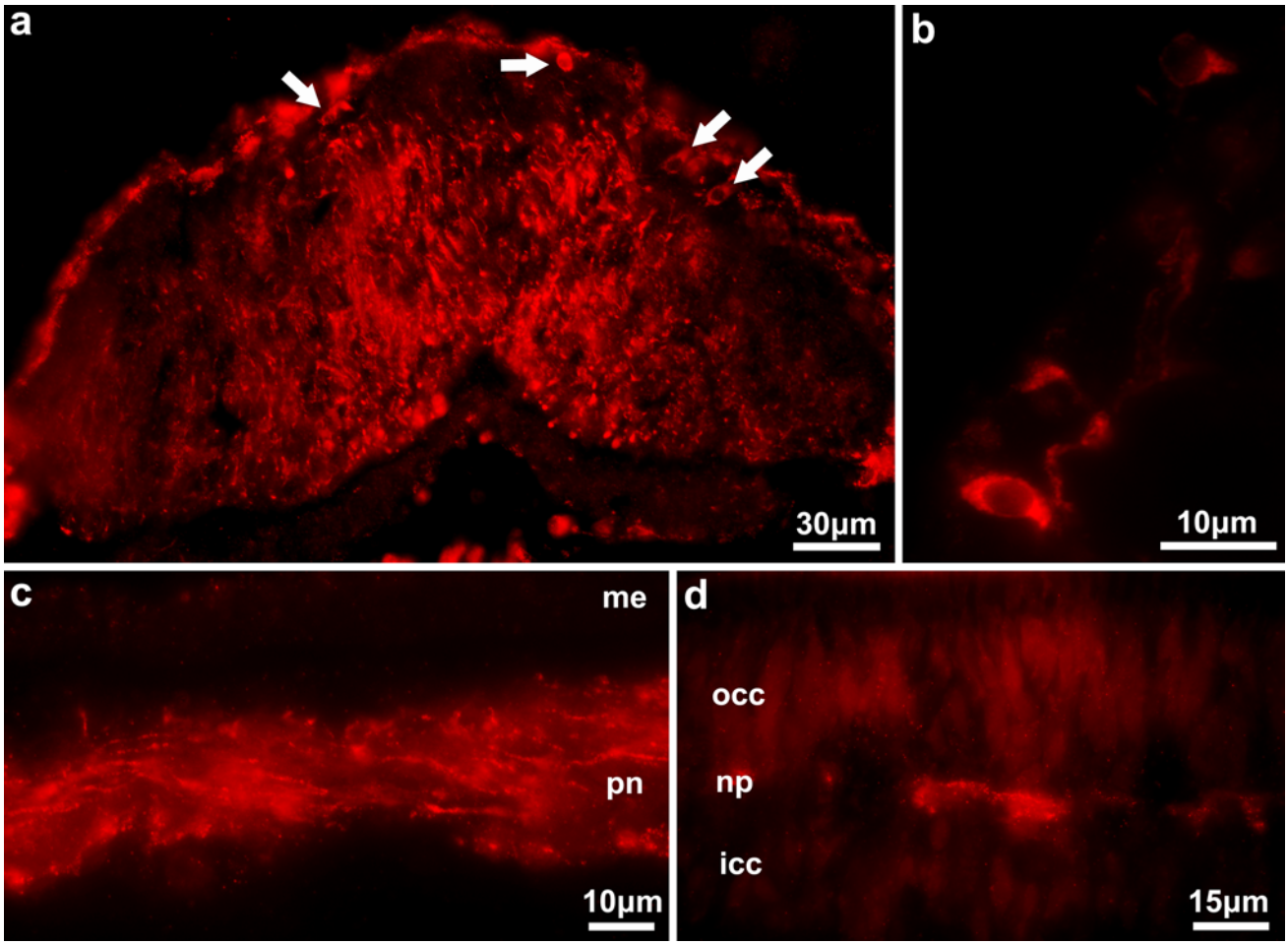
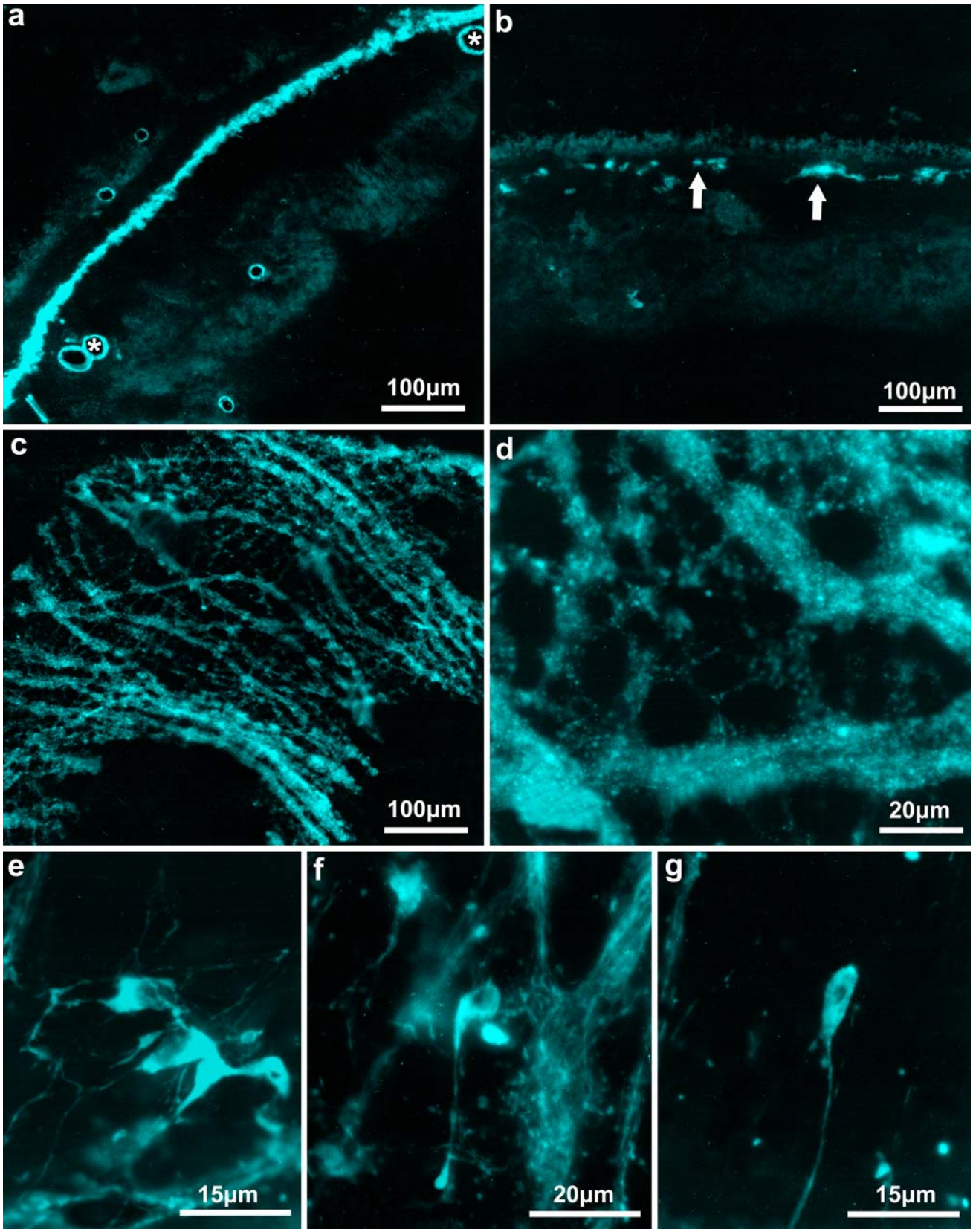


Fig. 4 *Holothuria glaberrima* (Holothuroidea). Anti-tyrosine hydroxylase immunoreactivity distribution within the radial nerve cord and tube feet. **a** Transverse section through the body wall showing anti-TH immunoreactivity in fibers and cells (*arrows*) of the ectoneural component of the radial nerve. **b** Anti-TH immunoreactive cells in the ectoneural component of the radial nerve. **c** Anti-TH immunopositive fibers present within the podial nerve. **d** Anti-TH immunopositive fibers located in the nerve plate of the tube feet's disk. *icc* inner cell cluter, *me* mesothelium, *np* nerve plate, *pn* podial nerve, *occ* outer cell cluster

echinoderms are mainly noradrenaline and dopamine (Cottrell 1967; Sloley and Juorio 1990). Additionally, catecholamines were also obtained from the stomach and tube feet of Asteroidea (Sloley and Juorio 1990). It is difficult, however, to reach any conclusions on the function of the plexus based solely on the type of catecholamine present. The presence of noradrenaline and dopamine is common to organisms closely related to the Chordata. Protostomes, on the other hand, appear to have higher levels of octopamine than of adrenaline (Vaughan 1988). In members of Chordata, noradrenaline and dopamine are present in interneurons of the brainstem, while noradrenaline is also found in motoneurons of the autonomic nervous system (Bradford 1986). These noradrenergic motoneurons are known to innervate muscle, glands, and other neurons. In this respect, the catecholaminergic cells of the Holothuroidea are most similar to the interneurons of the central nervous system than to the motoneurons of the autonomic nervous system. Our results strengthen the notion that the catecholaminergic cells are interneurons (Cobb 1969a), since these do not form part of the known sensory or motor circuits and the fluorescence is confined to organized nervous tissue, and in no case are fibers seen that could innervate muscle or non-nervous tissue.

Ectoneural catecholaminergic plexus

Our results are consistent to what has been previously reported in most adult echinoderms. Catecholaminergic nerves and fibers have been reported, using the formaldehyde-induced fluorescence method in members of three groups of echinoderms, the Asteroidea [*Patiriella calcar* (Lamarck, 1816) (Asterinidae, Asteroidea) and *Asterias rubens* (Linnaeus, 1758) (Asteriidae, Asteroidea)], the Ophiuroidea [*Ophiothrix fragilis* (Müller and Troschel, 1840) (Ophiothricidae, Ophiuroidea)], and the Echinoidea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◀ **Fig. 5** *Holothuria glaberrima* (Holothuroidea). Catecholamine-induced fluorescence in the esophagus. The network of fluorescent fibers forming the catecholaminergic esophageal plexus is shown in transverse sections (**a**) at the level of the esophagus as a continuous layer of fluorescence observed between the muscular and submucosal layers, and (**b**) at the level of the esophagus–small intestine connection as discontinuous bundles (*arrows*) that will eventually disappear at a more posterior location. **c** Tangential sections of the esophagus showing the catecholaminergic fiber bundles forming the plexus. **d** Higher magnification of the catecholaminergic fiber tract. **e–g** Examples of the different morphologies exhibited by cells of the esophageal plexus. The circular structures (*) are artifacts produced by air bubbles introduced during the SPG procedure

[*Heliocidaris erythrogramma* (Valenciennes, 1846) (Echinometridae, Echinoidea)] (Cobb 1969a; Cottrell and Pentreath 1970). A study undertaken in *Apostichopus japonicus*, concluded that catecholaminergic fluorescence was only present in the connective tissue of the dermis, while no fluorescence was detected in the radial nerve. Using the same method, the aqueous aldehyde (FAGLU) histofluorescence, we did observe catecholaminergic fluorescence in the radial nerve and confirmed this by immunoreactivity with an antibody made against tyrosine hydroxylase. Our results, clearly follow those reported for the radial nerve and tube feet of the Asteroidea, Ophiuroidea, and Echinoidea, in which catecholaminergic fluorescence were found to be limited to the ectoneural portion of the radial nerve cord and the podial nerve (Cobb 1969a; Cottrell and Pentreath 1970). Thus, we have now found a similar type of ectoneural catecholaminergic plexus to be present in a fourth echinoderm group, the Holothuroidea.

Contrary to previous observations on the neuroanatomy of the tube feet and tentacle (Bouland et al. 1982; Flammang and Jangoux 1992; Díaz-Balzac et al. 2010), the catecholaminergic plexus is limited to the main nerves of the podia, the podial nerve and the podial cylindrical fenestrated sheath. This limited distribution goes in accordance with the hypothesis that catecholaminergic cells are interneurons, as the catecholaminergic plexus does not directly innervates the mesothelium or connective tissue plexus. The only functional study of catecholamines in Echinodermata was undertaken in Asteroidea, showing that reserpine treatment causes unccordination of the podia; suggesting that the plexus plays a functional role in the tube feet movement and their coordination (Cottrell and Pentreath 1970). Although these experimental data are far from being conclusive, it correlates with the specific distribution of cells and fibers in the ectoneural nervous system, as we observed in our experiments by histochemistry and immunohistochemistry.

Esophageal catecholaminergic plexus

Catecholamine histofluorescence has also been described in the esophagus of the sea urchin *H. erythrogramma* (Cobb 1969a, b), and in the stomach of the starfish *A. rubens* (Cottrell and Pentreath 1970). Our group (García-Arrarás et al. 2001) has also briefly described the presence of catecholamines in the esophagus of *H. glaberrima* and *H. mexicana*. We now extend the description of its distribution in this report. The presence of catecholamines, as we found for Holothuroidea, appears to be limited to the inner nerve plexus, as defined by Cobb (1969a). In the representative of the Ophiuriodea, *O. fragilis*, no catecholamines were detected in the gut (Cottrell and Pentreath 1970). However, from the description provided by the authors, it is not possible to define exactly the areas of the digestive tract that were studied. Thus, it is still possible that, like for the Holothuroidea, a catecholaminergic plexus is present in the esophageal/stomach region, but not in the intestinal region. The description of the catecholaminergic plexus in the esophagus constitutes one of the most important findings of our work. Cobb (1969a, b) had already described the presence of catecholaminergic cells and fibers to this plexus, but did not record its extension. Our results show that the strongly fluorescent catecholaminergic plexus gradually disappears at the intestinal junction and is not found in the digestive system of the organisms, suggesting a hitherto complexity of the enteric nervous system and further overriding the notion that the echinoderm nervous systems are formed by a homogenous nerve net.

Continuity of the catecholaminergic plexus

The continuity of the catecholaminergic plexus is also a salient discovery. It identifies a component of the echinoderm nervous system, recognized by its neurotransmitter products, which is extensively distributed within various structures, but always surrounded by connective tissue. The catecholaminergic system is formed by a continuous plexus of cells and fibers that extends from the nerve ring to the tentacles and to the radial nerves, and from the latter to the tube feet. The other components of this plexus, the tentacular nerves and the inner plexus of the esophagus, also converge in the nerve ring. A diagram depicting the catecholaminergic plexus in the Holothuroidea is shown in Fig. 6. The hypothesis that this plexus is common to other echinoderms is strongly supported by the finding of catecholaminergic fiber and cells within homologous structures in representatives from different groups of the Echinodermata. Nonetheless, it must me modified according to the organism, and thus this explains the finding of an extensive catecholaminergic plexus in the stomach of Asteroidea (Cottrell and Pentreath 1970).

The catecholaminergic system provides for the possibility that a particular subdivision of the echinoderm nervous system acts as a functional unit. However, the function of the system itself remains undetermined and

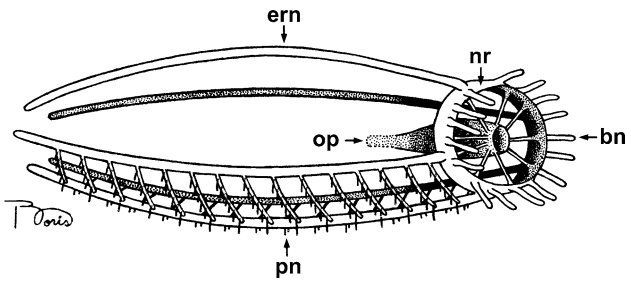

Fig. 6 Diagram depicting the catecholaminergic plexus in the Holothuroidea. The plexi in the circumoral nerve ring (*nr*), ectoneural component of the radial nerve (*ern*), buccal nerve (*bn*), podial nerve (*pn*), and esophagus (*op*) are continuous with each other, forming the structure shown in the diagram. An emphasis is done in the continuity and extension of the catecholaminergic plexus, and because of simplification we have omitted some details (such as ramification of the buccal nerve or the gradual disappearance of the esophageal plexus)

can only be speculated. It has been proposed that catecholaminergic cells in Echinodermata are interneurons (Cobb 1969a; Cottrell and Pentreath 1970). Our results strengthen this notion, since the cells are not found within the areas that form part of the known sensory or motor circuits of Holothuroidea. All the fluorescence is correlated with organized nervous tissue, and in no case are fibers seen that could innervate muscle or non-nervous tissue. In the esophagus, the plexus has been proposed to be associated with the control of secretory cells; however, the evidence is circumstantial (Cobb 1969a). We have found that during the process of evisceration, in both *H. glaberrima* and *H. mexicana*, the point of rupture between the eviscerated intestine and the esophagus lies at the level where the catecholaminergic plexus terminates (unpublished observations). Thus, it is tempting to speculate that the catecholaminergic plexus in Holothuroidea might play a role in the evisceration process.

In summary, our results show a distinct and physically continuous subdivision of the nervous system of Echinodermata. This subdivision is characterized by the presence of catecholaminergic cells and fibers, in which dopamine and noradrenaline are probably the main catecholamines expressed. The cells of this system are likely to be interneurons, most closely related to the catecholaminergic cells in the chordate central nervous system than to the peripheral motoneurons of deuterostomes or the catecholaminergic cells of the protostomes.

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