



The nervous system component of the mesentery of the sea cucumber *Holothuria glaberrima* in normal and regenerating animals

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Abstract

The mesenterial tissues play important roles in the interactions between the viscera and the rest of the organism. Among these roles, they serve as the physical substrate for nerves connecting the visceral nervous components to the central nervous system. Although the mesenterial nervous system component has been described in vertebrates, particularly in mammals, a description in other deuterostomes is lacking. Using immunohistochemistry in tissue sections and whole mounts, we describe here the nervous component of the intestinal mesentery in the sea cucumber *Holothuria glaberrima*. This echinoderm has the ability to regenerate its internal organs in a process that depends on the mesentery. Therefore, we have also explored changes in the mesenterial nervous component during intestinal regeneration. Extensive fiber bundles with associated neurons are found in the mesothelial layer, extending from the body wall to the intestine. Neuron-like cells are also found within a plexus in the connective tissue layer. We also show that most of the cells and nerve fibers within the mesentery remain during the regenerative process, with only minor changes: a general disorganization of the fiber bundles and a retraction of nerve fibers near the tip of the mesentery during the first days of regeneration. Our results provide a basic description of mesenterial nervous component that can be of importance for comparative studies as well as for the analyses of visceral regeneration.

Keywords Mesentery · Enteric · Regeneration · Echinoderm · *Holothuria*

Introduction

The mesentery, previously viewed as a sheet of tissue whose main role was to attach the viscera to the body wall, has recently been described as a novel organ (Coffey and O'Leary

2016). This new vision presents a contiguous organ that extends across most of the digestive tract and that partakes in functions that include circulatory, lymphatics, nervous, and endocrine.

The anatomy of the mesentery has been described in mammals, particularly humans (Byrnes et al. 2018), and in some other vertebrates, however, a comparative study in other animal groups is lacking. In members of the phylum Echinodermata, the mesentery has been characterized as being made up of three tissue layers: two mesothelia, and a connective tissue located between them (Hyman 1955. Smiley 1994). The mesothelium (formed by coelomic epithelia and myocytes) is continuous with the mesothelia of the attached organs at one end and with the mesothelium of the body wall at the other end. Thus, in the digestive tract, the mesentery mesothelium is continuous with that of the digestive organs. Similarly, the underlying connective tissue is also continuous with the connective tissue of the organs at one end and with the body wall connective tissue at the other end.

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As in other species, the echinoderm intestinal mesentery serves to attach the loops of the digestive tube to the body wall (García-Arrarás et al. 2018). However, in some echinoderms, the mesentery also plays an important role in the animal's ability to regenerate some of its internal organs (Hyman 1955, Mashanov and García-Arrarás 2011). This regenerative role has been extensively studied in the sea cucumber *Holothuria glaberrima* where, following evisceration of most of its digestive tract, a new intestine forms at the torn edges of the mesentery, close to where the eviscerated intestine was previously attached (García-Arrarás et al. 1998, 2011).

The mesentery is also known to contain nervous components. These have been studied in mammals (Sheehan 1933; Gillard and Read 1971; Amenta et al. 1981), but in echinoderms have been poorly characterized. In view that studies in different species indicate that the nervous system might play an important role in regenerative events (Singer 1974; Carlson 2007; Farkas and Monaghan 2017), it is necessary to characterize the mesenterial nervous system in *H. glaberrima* and the changes it might undergo during the intestinal regeneration process. In this context, it is possible that neuronal components of the mesentery might be crucial in the process of regeneration. Here, we describe the anatomy of *H. glaberrima* intestinal mesentery and its nervous system component. In addition, we explain the changes observed in the nervous system at different stages of the intestinal regeneration process.

Materials and methods

Animals

Sea cucumbers (*Holothuria glaberrima*) were collected in the rocky shores of northeast Puerto Rico. In the lab, the animals were placed in seawater aquaria with constant oxygenation using air pumps. Evisceration was induced in animals by injecting 2–4 mL of KCl 0.35 M into the coelom as previously explained (García-Arrarás et al. 1998). Uneviscerated animals were used to study the normal anatomy of the holothurian mesentery and as controls in intestinal regeneration experiments.

Eviscerated animals were left to regenerate for different time periods: 1, 4, 8, 10, and 15 days post-evisceration (dpe). Before sacrificed, animals were anesthetized in 10 mM 1,1,1-trichloro-2-methyl-2-propanol hydrate (98%) in seawater for 1 h. The tissues obtained were used for histological sections or whole mount techniques.

Animals were sacrificed using a blade to cut behind the animal's ring canal. Dissection was made by placing the animals sideways in the plate with the posterior end toward the experimenter and the ventral side with the tube feet toward the

experimenter's left. The body wall was then cut open longitudinally and pinned to the plate to facilitate the dissection.

Tissue sections

A portion of about 3 × 3 cm of the body wall containing the intestinal mesentery was carefully dissected with a razor, without damaging the attached mesentery and fixed in 4% paraformaldehyde overnight at 4 °C. The following day, tissues were washed 3 times in phosphate-buffered saline (PBS) (pH 7.4) for 15 min each. They were stored in 30% sucrose at 4 °C until use. Prior to sectioning, tissues were embedded in Tissue Tek OCT (Electron Microscopy Sciences, USA) compound and frozen. Sections (20 μm) were cut in a Leica CM 1850 Cryostat at –31.0 °C and picked up in poly-L-lysine-coated glass slides.

Whole mounts

In *H. glaberrima*, as in many other holothurians, the dorsal mesentery attaches to the esophagus and descending portion of the small intestine, the lateral mesentery attaches to the ascending portion of the small intestine while the ventral mesentery attaches to the descending large intestine (Hyman 1955; García-Arrarás et al. 1998). For whole mounts, the ventral mesentery was dissected by cutting it as close as possible to the body wall. Tissues to be used for whole mounts were fixed in Zamboni fixative (2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7–7.4, see García-Arrarás 1993). For this, the mesentery was pinned in sylgard-covered petri dish, keeping the tissue as extended as possible. Zamboni fixative was added, completely covering the tissue, and stored in the refrigerator (4 °C) overnight. The following day, the tissue was unpinned and transferred to glass vials. The fixative was removed through a series of three washes (15 min each) of the following substances in order: ethanol 80%, DMSO, and 0.1 M PBS. The mesentery was then stored in PBS with 15 mM NaN₃ at 4 °C until use.

Immunohistochemistry—tissue sections

Immunohistochemical techniques have been described previously (García-Arrarás et al. 2011; Díaz-Balzac et al. 2016). In brief, double-labeling was performed by adding the two primary antibodies of interest to the tissue sections and leaving them on a wet chamber overnight at room temperature. The following day, after completing three washes with PBS for 15 min each, the corresponding secondary antibodies were added for 1 h, followed by three more PBS washes. Coverslips were mounted using buffered glycerol solution containing DAPI D9542 (10 μg/mL) and sealed with nail polish (García-Arrarás 1993).

Primary antibodies used were as follows: anti GFSKLYFamide 1:1000 dilution, which labels a population of nerve fibers (Díaz-Miranda et al. 1995); monoclonal Meso1 undiluted monoclonal culture media, which labels the mesothelial tissues (García-Arrarás et al. 2011); RN1 1:50,000 ascites fluid, which labels a START domain found in lipid-binding proteins that appears to be specific to the holothurian nervous system (Díaz-Balzac et al. 2007; Rosado-Olivieri et al. 2017); and HgColI 1:10 diluted culture media, which labels holothurian collagen (Quiñones et al. 2002). Secondary antibodies used were goat anti-mouse GAM Cy2 (Jackson ImmunoResearch, PA) and goat anti-rabbit GAR Cy3 (Jackson ImmunoResearch, PA) at 1:400 and 1:1000 dilutions, respectively. Muscle F-actin marker Phalloidin TRITC (Jackson ImmunoResearch, PA) was used as well at a 1:6000 dilution following the same protocol as for secondary antibodies.

Immunohistochemistry—whole mount method

Normal or regenerating mesentery whole mounts were treated for 48 h with 2.0 mL of the primary antibody RN1 (1:1000). Afterwards, the primary antibody was removed and the mesenteries were washed three times with 0.1 M PBS. Then, the secondary antibody, GAM Cy3 (1:1000), was applied overnight. The following day, the antibody was removed and the samples were washed three times with 0.1 M PBS and mounted on a slide. For this, the mesentery was placed on a glass microscope slide, and using a pair of pins, it was completely extended, avoiding folds or wrinkles. Five hundred to 700 μL of DAPI D9542 (10 $\mu\text{g}/\text{mL}$) in 1:1 PBS/glycerol were carefully added to the tissue, so that the previous step of stretching was not disrupted. Then, a coverslip was laid on the tissue and the slide was sealed using nail polish.

Slides with tissue sections or whole mounts were observed with a fluorescence microscope (Olympus BX51) and with a confocal laser scanning and difference interference-contrast (DIC) microscope (Carl Zeiss LSM510, Leica TCS SP2 or Olympus Fluoview FV300).

Fiber quantification

Quantification of nerve fibers in the mesentery whole mounts was done using Nikon's NIS-Elements microscope imaging software. Photos of RN1-labeled mesenteries were taken using the $\times 40$ objective in a Nikon DS-Qi2 camera. A perpendicular line was traced on the image, and the fibers that intersected the line were quantified. To standardize the data, fibers were quantified in two different locations of the mesentery, each with an area of $85,284.54 \mu\text{m}^2$, and the average of these was used.

Fibers were quantified in three different areas of the mesentery: proximal (adjacent to the body wall), medial, and distal

(adjacent to the intestine). The distance of the nerve fibers to the edge of the mesentery was measured by tracing a line from the tip forming rudiment at the tip of the mesentery to the first group of nerve fibers in the mesentery. The distance was measured in three different areas of the same animal, and the average of the three values was used.

The Tukey method was used as statistical analysis comparing the control or uneviscerated animals and the experimental animals to find if changes in quantity of nerve fibers were significant.

Results

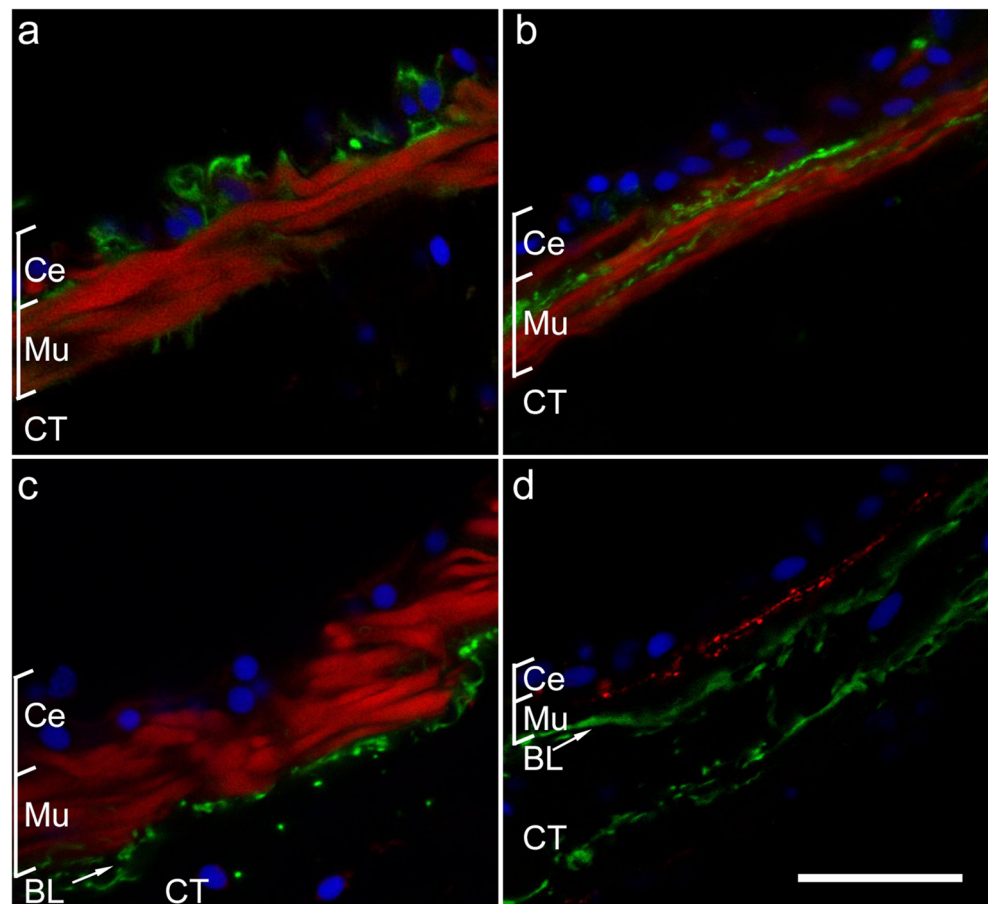
Mesentery nervous system anatomy

Tissue sections

Previous studies from our group have mentioned the presence of nerve fibers and nerve cells within the intestinal mesentery, however, most of these have been done in passing, as part of descriptions where the real focus was the intestine or the regenerating intestinal rudiment. To best characterize the localization and organization of the mesentery nervous components, we have now used five different markers in various combinations of double labeling: fluorescent phalloidin labeling of muscle cells, monoclonal Meso1 labeling of mesothelium, monoclonal IBA2 labeling of collagen fibers, monoclonal RN1 labeling of most nervous system components and polyclonal anti-GFSKLYamide labeling of a subpopulation of nervous system fibers.

Double labeling with Meso1 and fluorescently labeled phalloidin showed the histological relationship between the coelomic epithelia and the underlying muscle cells within the mesothelial layer (Fig. 1a). Meso1 labeled both coelomic epithelium and muscle cells while fluorescent phalloidin labeled the polymerized actin in the muscle contractile apparatus. The results clearly showed the organization of the coelomic epithelium forming the external layer of the mesentery with the muscle cells localized under the coelomic epithelia. To determine the relation of the connective tissue and the mesothelium, we performed double labeling with the IBA2 that recognizes the holothurian fibrous collagen and fluorescent phalloidin. The results showed the presence of collagen in the basal lamina that lies underneath the muscle layer and that separates the mesothelium from the connective tissue layer (Fig. 1b). To determine the location of the nervous tissue components in the mesentery, we used two nervous markers: antibody RN1 and anti-GFSKLYFamide. RN1 was used in double-labeling experiments with fluorescently labeled phalloidin. The results showed that the large majority of nervous fibers lie within the mesothelium, particularly as long fibers that run above and among the muscle cells (Fig. 1c).

Fig. 1 Organization of mesenterial tissue layers as determined by specific markers. Non-eviscerated animal's mesentery labeled with **a** mesothelium antibody Mesol (green) and muscle tissue marker phalloidin (red). **b** Nervous tissue antibody RN1 (green) and muscle marker phalloidin (red). **c** Collagen antibody IBA2 (green) showing basal lamina and muscle marker Phalloidin (red). **d** Anti-GFSKLYFamide (red), labeling a subpopulation of nerve fibers and collagen antibody IBA2 (green) showing basal lamina. These markers show the location and organization of the different tissue layers. The tissue layers boundaries are shown using brackets: (Ce) coelomic epithelium, (Mu) muscle, and (CT) connective tissue. The presence of the basement layer (BL) is shown with arrows. Nuclei are labeled with DAPI (blue). Bar = 25 μ m



In some cases, a few fibers were observed in the connective tissue (not shown). Anti-GFSKLYamide and IBA2 further demonstrated the nervous tissue association, showing that this subpopulation of nerve fibers was also found within the mesothelium associated with the muscle layer, and that they were separated from the connective tissue compartment by the basal lamina below the muscle layer (Fig. 1d).

In summary, the histological results provided a clear description of the tissue organization of the mesentery and the localization of its nervous system components.

Whole mounts

Tissue sections provide a limited view of the spatial distribution of nervous elements. Therefore, we decided to use whole mount techniques to better visualize the overall organization and spatial relation of the nervous components. Whole mounts demonstrated a large number of nerve fibers in the mesothelial layers. Although these fibers were observed as running almost in a straight line, parallel to each other, they contained ramifications along their entire length from the body wall to the intestine. These ramifications probably correspond to the fibers observed in the tissue sections that are associated with the muscle layer. The nerve fibers appeared to be distributed in a

converging pattern where they were homogeneously distributed as they entered the mesentery close to the body wall and eventually grouped together into bundle-like structures producing a striated pattern as one moves closer to the intestine (Fig. 2a–c). Thus, in the medial area of the mesentery, these bundles were large with an average width of 52.5 μ m and a mean distance between them of 47 μ m, while in the area closer to the intestine, the bundles were smaller (24.8 μ m) and showed a smaller average distance between them (26 μ m). The bundles resembled a nerve-like structure; however, the fibers appeared to be loosely associated with one another and did not form a solid or fasciculated structure. Quantification of nerve fibers in the mesothelial layers showed an average of 25 fibers (per field of view at $\times 40$ x) in the proximal area (adjacent to the body wall), 20.5 in the medial area and 17.2 in the distal area close to the intestinal tissue.

Individual nerve fibers were very thin, ranging in thickness from 1.75 to 7.11 μ m, with an average value of 3.71 μ m, although some of the thicker fibers might account for more than one fiber that were close together and difficult to separate individually. The nerve-like bundles were analyzed in terms of the number of fibers associated with them, resulting in 7.2 fibers per bundle in the medial areas compared to 4.3 fibers

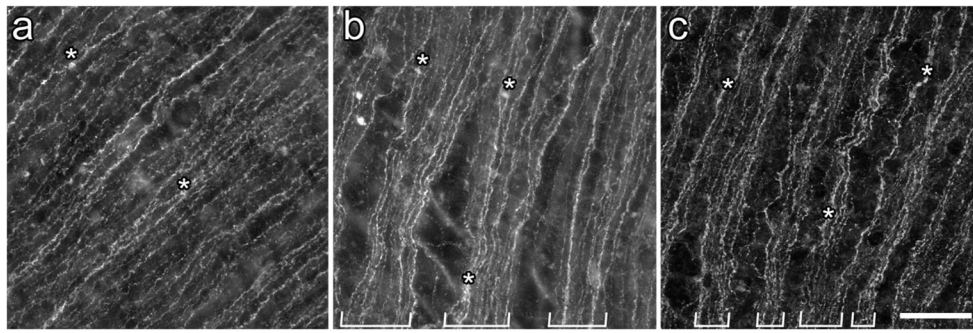


Fig. 2 Whole mounts of mesentery showing the organization of nerve bundles in normal non-eviscerated animals. Whole mounts were labeled with RN1 antibody that labels nervous components. **a** Labeling at the proximal area where the mesentery was attached to the body wall, shows a dispersed nerve fiber plexus. This plexus becomes organized in bundles

as we move to the medial area (**b**) of the mesentery and form distinct agrupations in the distal area (**c**) close to where the intestine is attached. Brackets show the presence of nerve bundles in the mid and distal regions of the mesentery. Asterisks show areas where neuronal cell bodies could be found. Bar = 100 μm

per bundle in the distal area. The organization and number of fibers in both mesothelial layers of the mesentery appeared to be similar; no evident difference could be observed between them.

Neuronal somata were also observed within the mesentery mesothelia. The somata were of approximately $21 \mu\text{m}^2$ in area ($\sim 5 \mu\text{m}$ diameter) with neurites that extended into the surrounding plexi. The neurons showed various morphologies, from circular to oval and different types of ramifications some being pseudounipolar, bipolar, or multipolar. These cells were found interspersed among the fibers of the mesothelial layers and most of the time were difficult to localize due to the large number of fibers surrounding them.

Cells and fibers were also present in the connective tissue between both mesothelia, but their abundance and organization differed greatly from those of the mesothelia. First, the number of fibers was much smaller and whenever observed, the fibers were isolated from each other; in many cases, the fibers could be followed to the neuronal soma from where they originated (Fig. 3c, d). Second, the fibers within the connective tissue were perpendicular to those of the mesothelia (Fig. 4). Third, the neuronal cell bodies were easily observed in the connective tissue, due in part to the few number of fibers that allowed for isolated cells to be identified. Their somata were also around $20 \mu\text{m}^2$ in area and were bipolar or multipolar with their fibers extended along the connective tissue. Many of these cells had an elongated morphology where the fibers seemed to arise from the cellular poles.

Changes in the mesenteric nervous components during regeneration

In holothurians, intestinal regeneration takes place from the free end of the mesentery beginning in the region that was adjacent to the original intestine. To determine the changes that might take place in the mesentery nervous component during the regenerative process, we did mesentery whole

mounts of animals at different stages of regeneration from 1 to 15 dpe. We found that the mesenteric nervous system undergoes little anatomical/morphological alterations during regeneration. The fiber network of the mesothelia, as well as the isolated nerve cells and fibers in the connective tissue, were still observed during the regenerative process. As discussed below, this is a dramatic difference from other tissue components, such as muscle tissues and extracellular matrix (ECM), which are significantly altered during regeneration. Nonetheless, upon closer examination, two changes were indeed observed: (1) a slight disorganization of the mesothelial plexus and (2) a retraction of fibers from the free end of the mesentery.

Nervous system disorganization

Although the fiber network of the mesothelium was clearly visible in regenerating animals, it appeared a bit more diffuse than in normal non-eviscerated animals. In particular, the delimitations of a single striation were harder to ascertain. We analyzed the nerve network in terms of number of fibers and their width. We observed an increase in the number of fibers in the mesothelial plexus. This increase was first observed in the area proximal to the body wall and eventually extended to the mid section of the mesentery (Fig. 5). At later stages of regeneration, it was also observed in the area near the regenerating rudiment (10–15 dpe). We also measured the width of the fibers and observed that their mean width ($3.71 \mu\text{m}$) in the non-regenerating mesothelium was significantly reduced to $2.17 \mu\text{m}$ in the 4-dpe mesentery and eventually to $1.32 \mu\text{m}$ in the 8 dpe mesentery.

Retraction and re-innervation

Immunohistochemical results in the mesentery of regenerating animals demonstrated that, at 1 dpe, mesothelial nerve fibers were found extending right up to the distal end of the

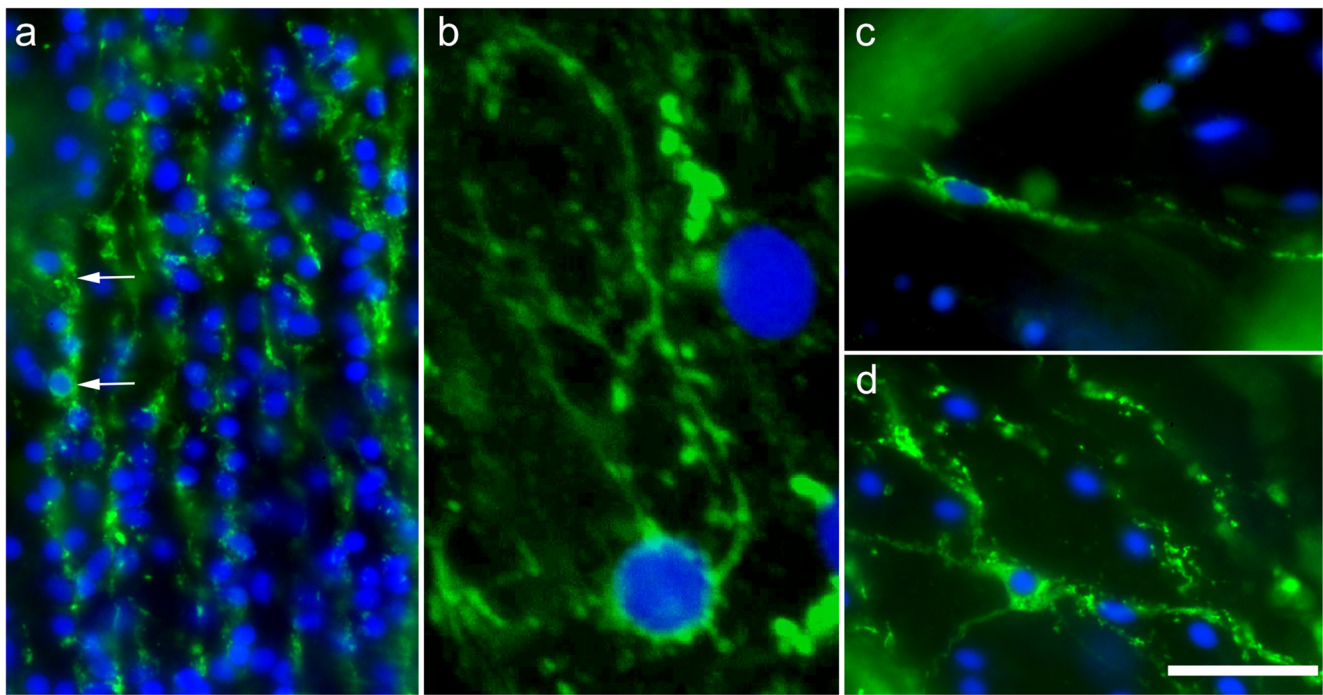


Fig. 3 Nerve cells in the mesenteric nerve plexus of normal non-visceralized animals. Labeling with the RN1 antibody labels nerve cells in the **a, b** mesothelial layer and the **c, d** connective tissue layer of the mesentery. As observed, the neurons in the mesothelial layer are found

within the large nerve fiber bundles (arrows). Most cell nuclei are not associated with the RN1 labeling and correspond to coelomic epithelial cells (**a**). Labeled cells in the connective tissue layer are isolated and usually show an extended morphology (**c, d**). Bar = **a, c, d** 25 μm , **b** 7 μm

mesentery near the area where the intestine had been attached (Fig. 6a). At 4 dpe and continuing up to 10 dpe, there is a progressive loss of fibers near the tip of the mesentery. This was documented in the decreased number of fibers from the distal region of the mesentery (Fig. 6 b and c). Fiber retraction peaked at 8 dpe where almost no fibers were identified in the terminal end of the mesentery. However, fibers were found to extend into the area adjacent to the regenerating intestine in 10 dpe animals. At 15 dpe, the prominent nerve fiber network could be observed throughout most of the mesentery, and at the distal end, individual nerve fibers could be seen crossing

the tip of the mesentery and entering the regenerating intestine (Fig. 6d). Eventually, fibers from the mesentery extended and re-innervated the tissues in the regenerating intestinal rudiment.

It is important to highlight that at all stages, the denervated region encompassed a length of only around 300–650 μm from the free end of the mesentery toward the body wall. This represents a rather small percentage of the mesentery (< 10%) that can extend from 5 to 25 mm from its attachment in the body wall to where the intestinal rudiment is forming (Figs. 7).

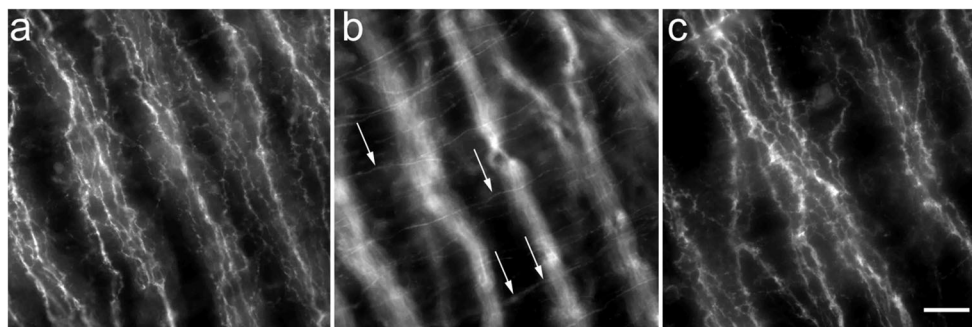
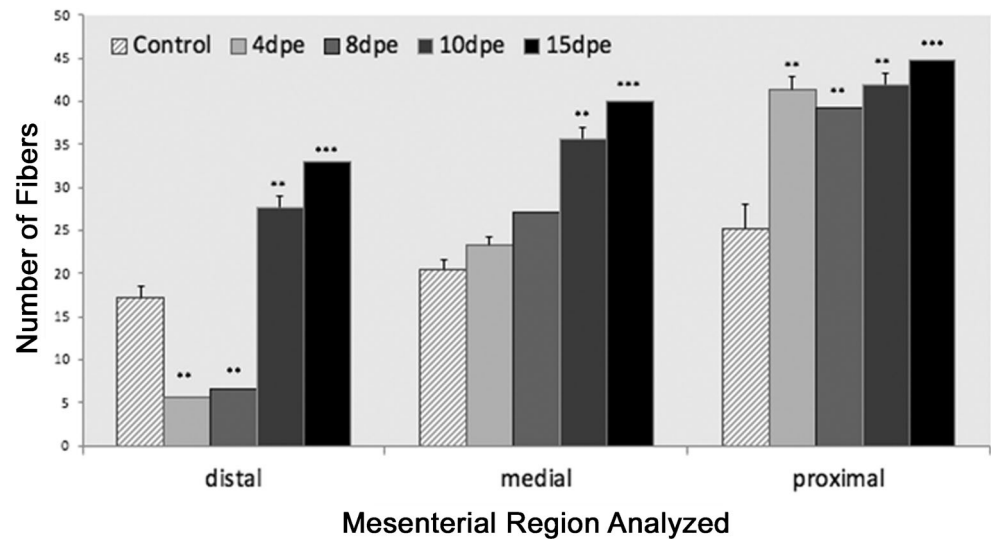


Fig. 4 Mesentery whole mounts showing the nervous component in different tissue layers. Whole mounts labeled with RN1 are observed at different focus levels. **a, c** Mesothelial layers that are separated by **b** the connective tissue layer. While the mesothelia has large fiber bundles that

run parallel from the body wall to the intestine, the fiber network in the connective tissue (arrows in **b**) is made up of fine fibers that run perpendicular to the mesothelial bundles (the mesothelial bundles are out of focus but still can be observe). Bar = 50 μm

Fig. 5 Quantification of nerve fiber abundance in different regions of the mesentery during the intestinal regeneration process. Nerve fibers were labeled in whole mounts of normal and regenerating animals with the RN1 antibody and counted under the fluorescent microscope. (Graphs represent the mean \pm SE, $n = 3$)



Discussion

Histological description of the holothurian mesentery

Our immunohistological analyses confirm previous descriptions of the holothurian intestinal mesentery anatomy. A diagram depicting the anatomical organization, including the described nervous components is shown on Fig 8. First, its two external mesothelial layers are formed by an external layer of coelomic epithelium (peritoneocytes) that lies over a muscle layer (myocytes) as is a common rule in holothurians (Smiley 1994). Second, the muscle layers are separated from the internal connective tissue layer by collagen-containing basement laminae. This has been described previously in a study focusing on the holothurian ECM (Quiñones et al. 2002) and is well known to be the general organization of echinoderm mesothelium (Smiley 1994; Rieger and Lombardi 1987; Dolmatov and Ginanonva 2001). Third, nervous elements are mainly found within the mesothelial layers and appear to be preferentially associated with the muscle tissue (García-Arrarás et al. 2001). Fourth, the connective tissue contains few mesenchymal cells, some of which are nerve cells (García-Arrarás et al. 1998, 1999; Díaz-Balzac et al. 2007). Finally, the mesothelia, as well as the connective tissue layers of the mesentery, are continuous with those of the intestine at one end and with the body wall coelomic cavity at the other (Díaz-Miranda et al. 1995; Tossas et al. 2014).

This tissue organization layout conforms to the basic plan of coelomate animals. The continuity of the mesenteric tissues with the peritoneum at the level of the body wall and with the intestinal tissues has also been described in mammals (Coffey and O'Leary 2016). However, in mammals and other organisms considered to be higher in the evolutionary tree of life, the mesentery contains other tissue types, mainly of hemal and lymphatic origin, that are absent from most of *H. glaberrima* digestive tract.

The nervous system component of the mesentery

The nervous system of the mesentery has been best described in mammals, where it has been proposed to contain three types of fibers: (1) intestinal nerves—those that merely cruise through the mesentery, without making any innervation, and whose function is to innervate the intestinal tissues; (2) vascular and lymphatic nerves—those that innervate vessel that run along the connective tissue of the mesentery; and (3) true mesentery nerves—those that innervate the mesentery tissues themselves (Sheehan 1933). Most of the nerve fibers are thought to originate in sympathetic or parasympathetic ganglia (Sheehan 1933). A large body of information has been acquired during the last four decades on the neuronal components that are associated with the blood and lymphatic vessels. These vessels have been shown to be innervated by catecholaminergic, cholinergic, and peptidergic nerves and in many cases, in addition to the histological description, functional analyses have been made (Furness 1973; Gillard and Read 1971; Khaisman and Borodulya 1978; Scott et al. 1989; Amenta et al. 1981; Guarna et al. 1991; D'Andrea et al. 2013). Similarly, some information is available on sensory nerves that originate in the intestinal tissues and transverse the mesentery to connect with the central nervous system (Kastelein et al. 2018). However, very few modern studies have been directed to the “true” mesenteric nerves.

It is, therefore, extremely challenging to compare the holothurian and vertebrate mesenteric nervous components. For one, the absence of blood or lymphatic vessels in most of the holothurian mesentery preempts the possibility that the holothurian nervous component in the connective tissue is part of the vascular or lymphatic vessel innervation. The prominent group of nerves that form the striations, which extend from the body wall to the intestine in *H. glaberrima*, probably corresponds to the intestinal nerves that innervate the intestine and

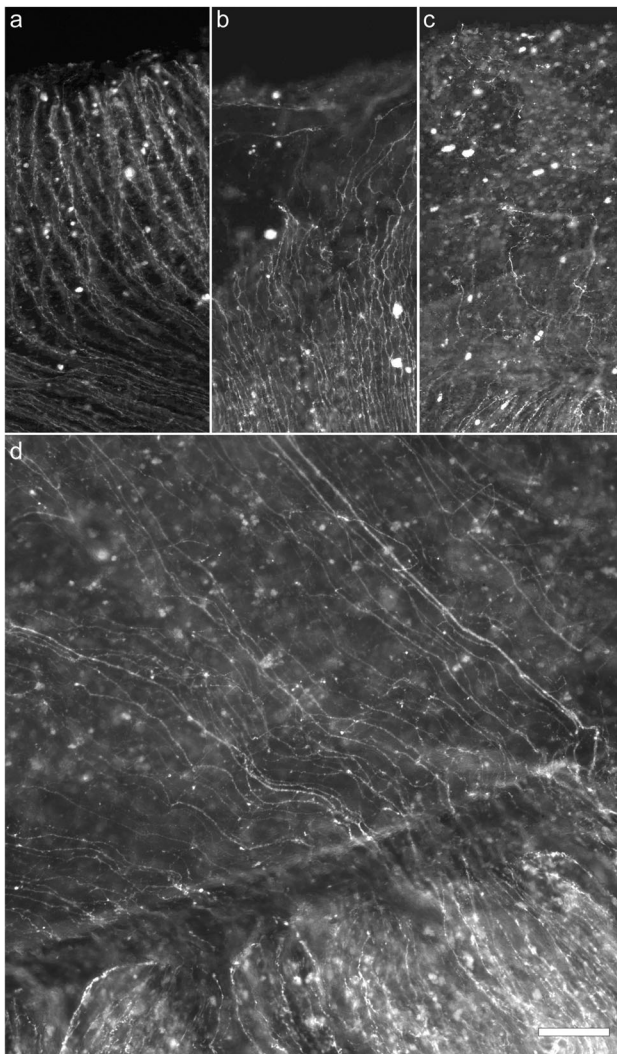


Fig. 6 Changes in fiber content at the distal end of the regenerating mesentery. Mesenteric nerves labeled with RN1 in the distal end of animals undergoing intestinal regeneration at **a** 1 dpe (days post-evisceration), **b** 4 dpe, **c** 10 dpe, and **d** 15 dpe. While in the 1 dpe animal (**a**) nerve fibers extend to the tip of the mesentery where it has been recently detached from the intestine, a retraction is observed in the following days (**b**, **c**). **d** At 15 dpe reinnervation of the regenerating intestinal rudiment can be observed as individual nerve fibers (arrows) originating from the mesenteric fiber bundles are observed crossing into the regenerating tissue. Bar = **a–c** 200 μm , **d** 140 μm

that most likely originate in the hyponeural component of the radial nerve cord (Hyman 1955). Although this is the most logical assumption, it cannot be firmly established since the overall echinoderm nervous system connectivity has been poorly described. Nonetheless, some of the nerves in these striations are probably also innervating the mesenterial tissues, or at least the mesenterial muscles. The fibers entering the muscle tissues have been documented here in transversal sections, as well as in previous publications (Candelaria et al. 2006, Tossas et al. 2014). That a specific fiber population innervates the mesenterial muscle can also be the reason why more fibers were observed near the body wall region of

the mesentery than close to the intestinal tissue; which suggests that fibers end at the place of mesenterial innervation, and do not extend throughout the complete mesentery. This innervation of the mesenterial muscle has not been well described in vertebrates where they comprise a fine plexus of unmyelinated nerves described by Sheehan (1933) as extending into the avascular portion of the mesentery.

Equally enigmatic are the small nerve ganglion cells that have been described to be associated with the mesenterial nerves (Sheehan 1933). The same type of cell was described in our study, thus suggesting that they are an important component of deuterostome mesenteries. Their possible function, however, remains undetermined. Are these the cells responsible for the innervation of the muscle tissue? Or are they modulating the response of the mesentery external innervation? Do they form a local network intrinsic to the mesentery? Or are they receiving information from the central and/or enteric nervous components? All these questions will need further experimentation in order to be answered.

The neuronal component in the connective tissue of the holothurian mesentery is possibly part of the connective tissue nervous subdivision that has been previously described by our group and that is not found in vertebrates (Díaz-Balzac et al. 2007). This nervous component has been associated with the modulation of the connective tissue mechanical properties, a property of echinoderm connective tissues (Wilkie 2005).

Involvement of the nervous system in intestinal regeneration

Our results show that the mesenterial nervous components remain almost unaltered through the regeneration process. The small retraction is an expected outcome of axotomy, as it has been shown that neurons that are injured in the terminal segments of their axons undergo a partial degeneration (named Wallerian degeneration in vertebrates) that extends retrogradely from the injured tip (Hilliard 2009). Thus, as the large majority of fibers in the mesentery are thought to originate from cells in the radial nerve cords or within the mesentery itself and are traveling toward the intestine, there will be a partial retrograde loss of fibers after the evisceration process. This retrograde axonal degeneration is followed by a regrowth of fibers from the proximal axonal segment that has been well documented in many vertebrate and invertebrate species (Muller and McGlade-McCulloh 1987; Hilliard 2009; Geuna et al. 2009). Fiber regrowth can lead to multiple sproutings that may account for the increase in fiber number observed during the regeneration process in our system. However, the increase in fiber number could also be explained by a process similar to the “compartmentation” described in vertebrate nerves (Morris et al. 1972) where fibers form smaller fascicles or bundles during regenerating stages. Thus, in the regenerating holothurian mesentery, individual axons

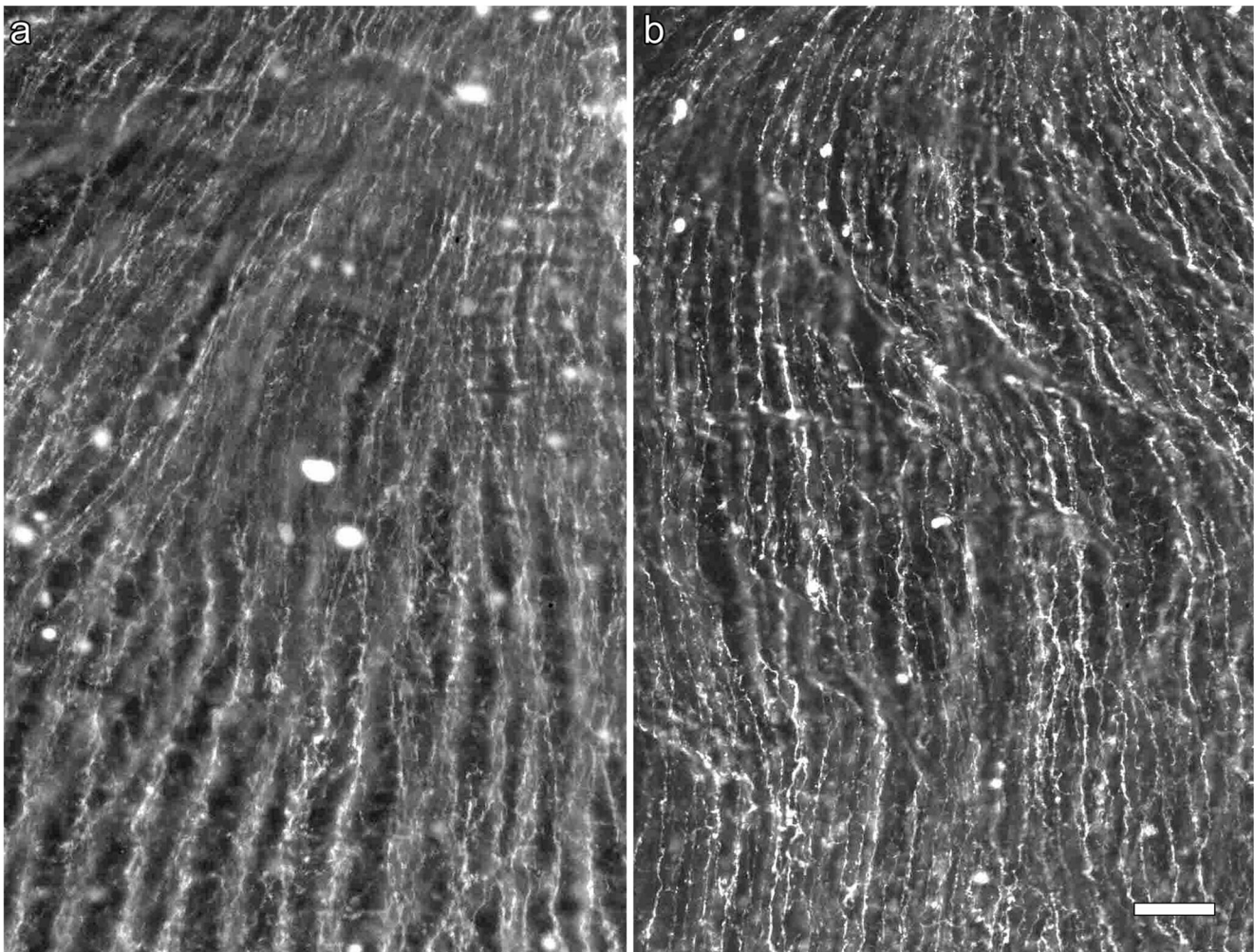


Fig. 7 Comparisons of normal and regenerating mesentery nervous components. Whole mounts of **a** normal, non-eviscerated and **b** regenerating mesenter whole mounts labeled with RN1 label the nervous component of the mesothelial layer. The overall organization of the

nervous components is maintained during regeneration although the fiber bundles in the regenerating intestine appear less organized than those in the normal mesentery. Bar = 100 μ m

would not adhere or group with others and a larger number of “axons” would be counted upon observation.

The retraction and eventual reinnervation observed also provide a perfect counterpart to previous observations from our lab (Tossas et al. 2014). In her experiments, describing the regeneration of the enteric nervous system, Tossas described how the initial step is the disappearance of fibers within the forming rudiment, and how, at \sim 10 dpe, the rudiment has grown in size, but few if any fibers are present within it. Subsequently, she describes that in the following days, external fibers from the mesentery enter the rudiment and innervate the newly formed muscle and other tissues. Thus, the time frame of the fiber retraction documented at the mesentery-intestinal border clearly coincides with the loss of fibers observed in the intestinal rudiment. At later stages in the regeneration process (10–15 dpe), the nerve fibers begin to reinnervate the mesentery. This usually starts at 10 dpe and proceeds during the following days. In

accordance with this study, we observed that at 15 dpe, the nerve fibers that ran along the mesentery began to innervate the forming intestine.

Ironically, our most striking result is the relative stability of the mesenterial nervous network during the regeneration process. Such stability is perplexing in view that the underlying muscle and connective tissue undergo dramatic changes. In previous works, we have shown that during the first 2 weeks following evisceration, the ECM is remodeled (Quiñones et al. 2002) and a large extent of the mesentery loses its muscle component as muscle cells dedifferentiate (Candelaria et al. 2006; García-Arrarás et al. 2011). Both ECM remodeling and muscle dedifferentiation occur in a gradient, beginning at the mesentery tip where the intestinal rudiment is forming and moving gradually toward the body wall. This implies that the mesenterial nervous system maintains its overall structure even when many other surrounding tissues are undergoing changes. It is possible that the small degree of disorganization

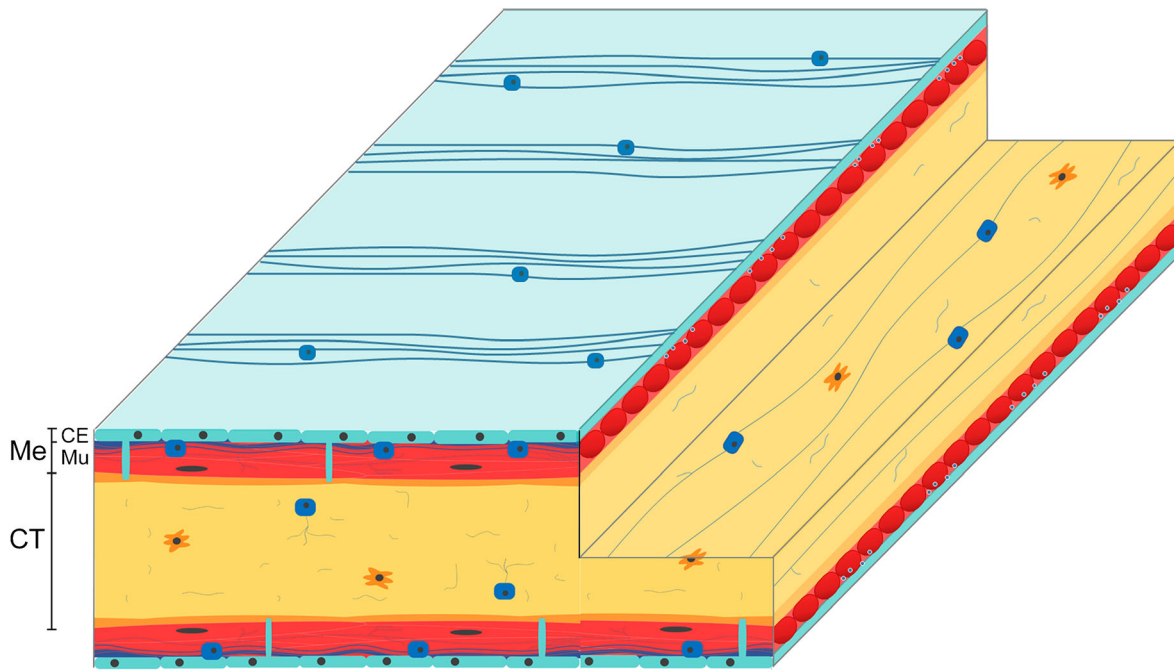


Fig. 8 Schematic model of the holothurian mesentery. Two mesothelial layers (M) surround a connective tissue layer (CT). The mesothelial layers are formed by an outer layer of coelomic epithelia or peritoneocytes (CE) and a muscle layer (Mu). Neuronal cell bodies are interspersed within the mesothelium and numerous neuronal fibers run along the layer, mainly in the area between the coelomic epithelium and the muscle. These fibers group in loose bundles that run from the body

wall to the intestine. Muscle cells and peritoneocytes are in contact with the basal lamina that separates the mesothelium from the connective tissue. Few cellular elements are found within the connective tissue, but some of them appear to be neuron-like cells (N) whose fibers are oriented perpendicular to those in the mesothelial layer, as can be observed in a longitudinal view of the mesentery depicted on the right side of the diagram

that we observed is due to the loss of muscle synapses and/or changes in the fiber extensions into the muscle layer.

On the other hand, the presence of fibers in the mesentery of regenerating animals, opens the door to the possibility that the nerves are influencing the regeneration process. Instances of nerve-dependent regeneration are well documented in both invertebrates and vertebrates (Carlson 2007; Stocum 2011). Amphibians provide some of the best-studied examples, where it has been demonstrated that regeneration of the limb is dependent on the presence of the nerve (Stocum 2011; Farkas and Monaghan 2017). In this system, molecules associated with the regeneration process have been isolated and characterized (Kumar et al. 2007). Thus, in the holothurian, a plausible scenario would be that the remaining fibers provide signals for the formation of the rudiment at the free end of the mesentery or otherwise that the reinnervating fibers direct some of the later events that take place in the formation of the new intestine.

Little is known of the changes that might occur in vertebrate mesenteric nervous components during injury or surgery. Nonetheless, recent findings are alerting medical personnel of the importance of the mesentery in surgical procedures (Seghal et al. 2018). Thus, our results open the door to the study of the possible role of the nervous system in basic science as well as in clinical settings and its relation with the regeneration or healing following injury or surgery of internal organs.

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Compliance with ethical standards

Ethical treatment of animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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