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Regeneration of the Enteric Nervous System in the Sea Cucumber *Holothuria glaberrima*

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ABSTRACT

Among higher metazoans, echinoderms exhibit the most impressive capacity for regeneration. Holothurians, or sea cucumbers, respond to adverse stimuli by autotomizing and ejecting their visceral organs, which are then regenerated. Neuronal fibers and cell bodies are present within the viscera, but previous regeneration studies have not accounted for the nervous component. We used light microscopic immunocytochemistry and ultrastructural studies to describe the regeneration of the enteric nervous system in the sea cucumber *Holothuria glaberrima*. This study provides evidence that the enteric nervous system of this echinoderm regenerates after evisceration and that in 3–5 weeks the regenerated system is virtually identical to that of noneviscerated animals. The regeneration of the enteric nervous system occurs parallel to the regeneration of other organ components. Nerve fibers and cells are observed within the mesenterial thickenings that give rise to the new intestine and within the internal connective tissue prior to lumen formation. We also used bromodeoxyuridine incorporation to show that proliferation of the neuronal population occurs in the regenerating intestine. The regeneration of the nervous system commands high interest because members of the closely related phylum Chordata either lack or have a very limited capacity to regenerate their nervous system. Thus, holothurians provide a model system to study enteric nervous system regeneration in deuterostomes. J. Comp. Neurol. 406:461–475, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: nerve regeneration; echinoderms; neuropeptides; neurogenesis; catecholamines; precursors

In the present decade, although great advances have been made in the understanding of the nervous system, its regeneration or lack of it still stands as one of the least known aspects of neurobiology. Molecules and cells that hinder or promote the repair, elongation, and reconnection of fibers or the production of new neurons have been identified and are being studied. Most studies on nervous system regeneration have focused on the central nervous system and on the autonomic and motor subdivisions of the peripheral nervous system.

Studies on the regeneration of the enteric component have lagged behind. Nearly or almost all of the in vivo studies on the regeneration of the enteric nervous system (ENS) have been done by following two types of injury-producing manipulations: intestinal transection and laser ablation (Kobayashi and Nishisaka, 1985; Kobayashi et al., 1989; Endo et al., 1986; Trudung et al., 1990; Saffrey and Burnstock, 1994; Tokui et al., 1994). These studies have documented some aspects of neuronal regeneration, namely the regeneration of nerve fibers and their growth across the injured region. Following transection and reanastomosis, functional recovery of intestinal motility and myoelectric activity coincides with the regeneration of peptidergic nerve fibers (Galligan et al., 1989). Although fiber regeneration has been shown to occur, replacement of lost enteric neurons has not been determined. Thus, all

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previous studies on the regeneration of the ENS have been limited to the regeneration of fibers and do not include the possibility of neurogenesis taking place.

We used an echinoderm, the sea cucumber *Holothuria glaberrima*, to study ENS regeneration. Among higher metazoans, echinoderms exhibit the most impressive capacity for regeneration (Hyman, 1955; Brndsted, 1969; Bode and David, 1978; Nicholls, 1987; Gremigni, 1988). This potential, particularly in members of the class Holothuroidea, is manifested by the process of evisceration (Dawbin, 1949; Hyman, 1955; Swan, 1966). When these organisms are exposed to adverse stimuli, they respond by ejecting most of their internal organs. Evisceration is followed by a period of regeneration, during which the ejected organs are replaced (Bertolini, 1930, 1932; Kille, 1935; Dawbin, 1949; Hyman, 1955; Mosher, 1956; Swan, 1966; Bai, 1971; Smith, 1971a,b).

In the present study, we show, by immunocytochemistry and ultrastructure analysis, that the ENS of *H. glaberrima* regenerates after evisceration. An ENS virtually identical to that of noneviscerated animals can be obtained 3–5 weeks after evisceration. Moreover, we show that at least some of the enteric neurons are generated by cell division. This amazing process of regeneration of the nervous system commands high interest because members of the closely related phylum Chordata either lack or have a very limited capacity to regenerate their nervous system component (Jessell, 1991; Hitchcock and Raymond, 1992). Thus, holothurians may provide an advantageous experimental model system to study ENS regeneration in deuterostomes.

MATERIALS AND METHODS

Animals

Specimens of *H. glaberrima* (10–15 cm) were collected from the northeast coast of Puerto Rico. Animals were eviscerated by injecting 2–4 ml of 0.35 M KCl into the coelom and placed in sea water aquaria at 20–24°C. Eviscerated animals were allowed to regenerate up to 35 days. Experimental and control animals were anesthetized in 6% $MgCl_2$ for 1–2 hours before being killed. At least three animals per stage (5, 7, 14, 21, 28, and 35 days postevisceration; PE) were used for the histological studies.

Immunocytochemistry

Noneviscerated and regenerating (5, 7, 14, 21, 28, and 35 PE) intestines were fixed in either 4% paraformaldehyde or picric acid–formaldehyde (Zamboni) fixative (see García-Arrarás, 1993). Fixation in paraformaldehyde was done for 1 hour at 4°C. Tissues were then rinsed in 0.1 M phosphate buffered saline (PBS; pH 7.4) and placed in 30% sucrose until sectioned. Fixation in Zamboni was performed for at least 12 hours at 4°C; the tissues were then dehydrated in a series of alcohols, cleared in xylene, and rehydrated before being placed in 30% sucrose–PBS. Tissues were mounted in embedding medium (OCT, Miles Inc., Elkhart, IN), and 10–12-μm sections were cut at −20°C in a cryostat (Leica CM 1900), picked up on polylysine-treated slides, and dried under air for 1–2 hours.

Tissue sections were treated with nonimmune goat or bovine serum at a dilution of 1:50 for 15 minutes, rinsed in PBS (three times, 10 minutes each), and incubated with the antisera overnight at room temperature in humid chambers. In some cases, the tissues were treated with PBS–0.1% Triton X-100 for 15 minutes before the addition of the antibody. Sections were then rinsed with PBS and treated with the secondary antibody, goat anti-rabbit or anti-mouse coupled to fluorescein isothiocyanate (GAR-FITC; BioSource International, Camarillo, CA) at a dilution of 1:50 for 1 hour at room temperature. The sections were rinsed with PBS, mounted with buffered glycerol (pH 8.6), and observed with a Leitz Laborlux fluorescence microscope equipped with an I2/3 filter.

Antibodies used include anti-galanin (Díaz-Miranda et al., 1996), GFSKLYFamide (Díaz-Miranda et al., 1992, 1995), acetylated α-tubulin (García-Arrarás and Viruet, 1993), cholecystokinin (CCK; García-Arrarás et al., 1991a), and monoclonal antibody F6 (see below).

Catecholamine histochemistry

A modification of the sucrose phosphate glyoxylic acid (SPG) method described by de la Torre and Surgeon (1976) was used. A similar technique was used to detect catecholaminergic cells and fibers in the holothurian larva (Burke et al., 1986). In brief, tissues were frozen at −30°C in embedding medium (O.C.T.) immediately after removal. Sections (20–25 μm) were recovered on polylysine-treated slides and incubated in SPG solution (1% glyoxylic acid, 0.2 M sucrose in 0.24 M KH_2PO_4 buffer at pH 7.4) for 3–5 minutes. Excess solution was removed, and the slides were dried in air for 10 minutes and oven heated for 5 minutes at 80°C. In some cases, the SPG solution was applied to wholemounts of the intestine previously stretched with forceps and flattened by pressuring with a coverslip before being air dried.

Slides were coverslipped using paraffin oil and examined in an epifluorescence Nikon microscope equipped with a D filter for catecholamine fluorescence detection.

Monoclonal antibodies

The procedure for monoclonal antibody production in *H. glaberrima* has been described previously (García-Arrarás et al., 1998). In brief, antibodies were made against intestinal tissue from which the luminal epithelium had been removed. The fusion was performed by the stirring method described by Harlow and Lane (1988) with a spleen:myeloma cell (SP20) ratio of 10:1. Wells exhibiting good hybridoma growth were assayed by using tissue culture supernatant as the source of primary antibodies. Those showing interesting immunoreactive patterns were subcloned by limiting dilution. The clone F6, used in the present study, represents one of the clones obtained.

Electron microscopy

Dissected noneviscerated and regenerated large intestine were fixed in Karnovsky's fixative for 1 hour at 4°C (Hayat, 1986). After fixation, tissues were rinsed three times in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. Postfixation was performed with 1% OsO_4 for 30 minutes at 4–6°C. Tissues were then rinsed with distilled water and then dehydrated with ascending ethanol concentrations until a final solution of 1:1 ethanol:propylene oxide was reached. The tissues were gradually changed to resin (Embed 812) in molds that were left at 60°C for 48 hours. Fine sections (60–90 nm) were made by using an LKB microtome, stained with 1% uranyl acetate and lead citrate as described by Reynolds (1963), and observed in a Zeiss 60-kV transmission electron microscope.

Incorporation and double labeling

Proliferating cells of the regenerating intestine were labeled as reported in a previous study by injecting the eviscerated animals with a single pulse (0.05 mg/g wet weight) of bromodeoxyuridine (BrdU; Sigma, St. Louis MO) diluted in sea water (García-Arrarás et al., 1998). Animals were kept for 48 hours in the aquaria before being killed, and the regenerating intestine was fixed in 4% paraformaldehyde. The stages studied were 8, 10, and 12 PE. Noneviscerated animals, kept in the aquaria for 2 weeks. were injected with BrdU and used as controls. At least three animals were used for each stage studied.

Tissues were prepared for immunocytochemistry as described above. Double-labeling immunocytochemistry was performed by using a monoclonal antibody against BrdU (Amersham, Arlington Heights, IL) at a 1:10 dilution and the rabbit polyclonal antibody against GFSKLY-Famide at a 1:1,000 dilution. Tissue sections were incubated with goat serum at a 1:50 dilution, treated with PBS–0.2% Triton X-100, and rinsed with PBS before an overnight incubation in a mixture of the primary antibodies at room temperature. The following day, tissue sections were rinsed again with PBS and incubated in a mixture of secondary antibodies (goat anti-mouse [GAM]-rhodamine and GAR-FITC, both diluted 1:50; BioSource International) for 1 hour. These were then rinsed once again with PBS, incubated with Hoechst nuclear dye (1 μM for 2 minutes in the dark; Sigma), and rinsed twice before mounted in buffered glycerol. Tissues were examined with a Leitz Laborflux fluorescence microscope equipped with I2/3, N2, and D filters.

RESULTS

Intestinal regeneration

Holothuria glaberrima specimens eviscerate most of their internal organs, including the small and large intestines, the hemal system, and the left respiratory tree (Fig. 1). The holothurian digestive tract has been well described (Dawbin, 1949; Hyman, 1955; Mosher, 1956; Bai, 1971; Smith, 1971a,b). In *H. glaberrima* (Díaz-Miranda et al., 1995), the digestive tract consists of a short esophagus, a descending and ascending small intestine, and a large intestine attached to the cloaca. There is no conspicuous stomach or rectum in this species. The small and large intestines have a similar tissue structure consisting of five tissue layers: coelomic epithelial lining, outer connective tissue, longitudinal and circular muscles, inner connective tissue, and luminal epithelium (Hyman 1955; García-Arrarás et al., 1998). The main differences between the small and large intestines consists of the thickness of the inner connective tissue layer, which is prominent in the large intestine (García-Arrarás et al., 1998).

Evisceration causes the intestine to detach from the mesenteries and to rupture at the esophageal–intestinal junction in the anterior end and at the intestinal–cloacal junction posteriorly. A full description of the regeneration process and the cellular mechanisms associated with it has been described elsewhere (García-Arrarás et al., 1998). In brief, the intestinal system is the first to regenerate from a thickening that forms at the edges of the torn mesentery. Within this structure, cells appear to migrate and divide to form the luminal epithelia of the new intestine. Three weeks after evisceration, the regenerated structure appears to be functional and contains the four main layers present in the original organ: the serosa or coelomic lining, the circular and longitudinal muscle layers, the submucosa or inner connective tissue layer, and the mucosa or luminal epithelium. Figure 2 shows a schematic diagram of these events.

Regeneration of nervous components

Immunocytochemical analysis. The use of neuronal markers allowed us to describe the ENS of *H. glaberrima* in noneviscerated specimens before the regeneration studies were done. We have put together a composite view of the ENS from our previous studies by using several markers for nervous components (see García-Arrarás et al., 1991a,b; García-Arrarás and Viruet, 1993; Díaz-Miranda et al., 1995, 1996; see Fig. 3). The ENS is composed of plexi of cells and fibers in the coelomic lining/muscular, inner connective tissue, and luminal epithelium layers. Except for the catecholamine plexus in the anterior end of the small intestine, the neuronal components of the ENS are found in both small and large intestines.

Because the intestinal tissue layers in the 21 PE regenerates are similar to those of noneviscerated animals, we compared their ENSs to determine whether regeneration of the nervous component occurs.

Coelomic lining/muscular. These two tissue layers have been referred to as the perivisceral mesothelium by Smiley (1994). Among the ENS components present in the coelomic lining are different populations of peptidergic cells, usually found isolated or in small groups (see García-Arrarás et al., 1991a,b; García-Arrarás and Viruet, 1993; Díaz-Miranda et al., 1995, 1996). These cells extend processes toward the muscular and inner connective tissue layers, thereby forming part of a dense fiber network that lies beneath the coelomic epithelium in close association with the muscle layer and of fiber tracts that communicate between the coelomic epithelium and the inner connective tissue layer. In the present study, we found all of these neuronal markers in the 21 PE regenerated intestine (Fig. 4). Cells expressing CCK-like immunoreactivity (LI), for example, were found isolated or in pairs at regular intervals of 224 ± 52 μm (mean ± S.E.) in noneviscerated organisms and 216 ± 48 μm in regenerated specimens. Similarly, cells expressing GFSKLYFamide and those expressing galanin-LI (GAL-LI) were found in the intestine of regenerated organisms. Their localization, distribution, and overall immunoreactivity were similar to those of noneviscerated organisms.

Neuronal fibers were also found in the regenerated organisms. These fibers, which express the neuropeptides GFSKLYFamide, GAL-LI, or CCK-LI and acetylated α-tubulin, show immunoreactive patterns similar to those of noneviscerated sea cucumbers, being concentrated between the coelomic epithelium and the muscle layer and surrounding the longitudinal muscle fibers (Fig. 4).

Inner connective tissue. This layer has been referred to as the connective tissue compartment by Smiley (1994). In the noneviscerated animal, the nervous component of the inner connective tissue layer is formed by fibers expressing immunoreactivity to acetylated α-tubulin, GFSKLYFamide, or CCK-LI that appear to originate from those neurons in the coelomic epithelium and are oriented perpendicular to the circular muscle. Such fibers transverse the inner connective tissue layer, extending from the muscular layer

Fig. 1. *Holothuria glaberrima* specimen shown before (top) and after (bottom) evisceration. The evisceration process is completed within 3 minutes after injection with 0.35 M KCl. The eviscerated organs include the intestine, hemal system, one respiratory tree, and gonads.

to the luminal epithelium, branching only sporadically (see Fig. 4).

No peptidergic cells were detected with our antibodies within the inner connective tissue layer of the noneviscerated intestine. Nevertheless, cellular elements of neuronal nature have been identified with the monoclonal antibody F6, which was generated in our laboratory *H. glaberrima* intestinal tissue. These F6-labeled cells are approximately 8–10 μm in size and have a large nucleus that occupies most of the soma. The immunoreactivity can be observed surrounding the nucleus and in fine but very long fibers that usually project from opposite ends of the cell. Each cell has two or three of these extensions. The fibers express immunoreactivity throughout their length, and varicosities with prominent immunoreaction are usually observed. These fibers form a network within the inner connective tissue layer, being more prominent than the cell bodies, such that we usually observed one or two cell bodies per intestinal section, but the very conspicuous fiber network is present throughout most of the layer. The F6-labeled cell type is present only within connective tissue, primarily in the inner connective tissue, but occasionally within the connective tissue beneath the coelomic lining and in the mesentery.

The presence of the F6-labeled cells (Fig. 5A,B) and their fiber network (Fig. 5C,D) is similar in both noneviscerated and regenerated specimens. No anomalous distribution of cell bodies or fibers was observed after regeneration.

Luminal epithelium. Cells expressing GFSKLYFamide are also found within the luminal epithelium. When compared

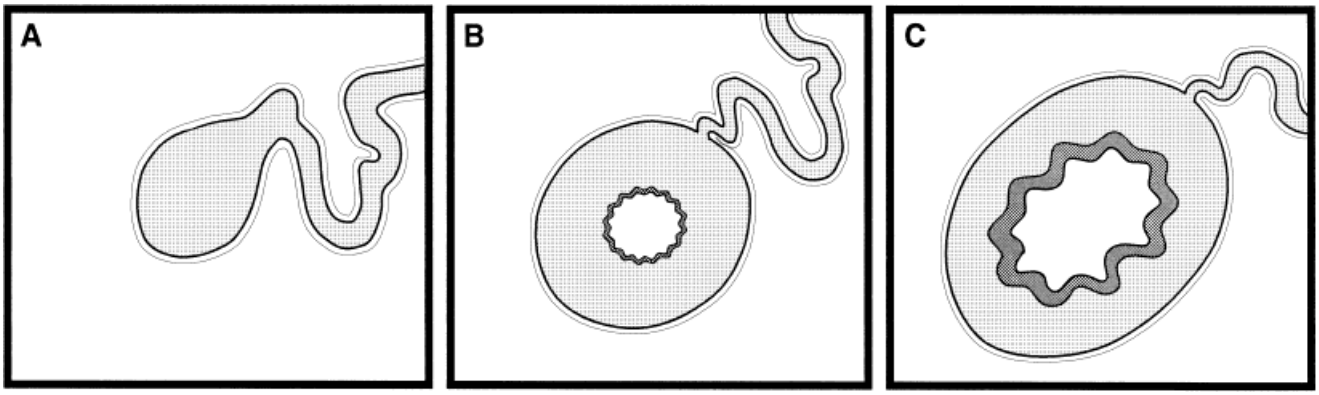

Fig. 2. Schematic diagram of the process of intestinal regeneration of *Holothuria glaberrima* as shown by transverse sections. **A:** First week (stages II–III). Regeneration of the intestine begins at the loose mesentery edge, where the separation with the intestine occured during evisceration. The mesentery hypertrophies and a blastema-like structure is formed. **B:** Second week (stages III–IV). Formation of the luminal epithelium and intestinal lumen by cellular proliferation. **C:** Third week (stage V). Enlargement of the luminal epithelium layer and reduction in the internal connective tissue layer to reach proportions similar to those of the noneviscerated intestine.

with the epithelial mucosal cells, the neuropeptide expressing cells are rare, accounting for fewer than 1% of the mucosal cell population. They resemble vertebrate neuroendocrine cells but show long extensions that sometimes extend toward the coelomic lining. The cells are found in the regenerated intestines in number, morphology, and distribution similar to those of noneviscerated animals (Fig. 6).

Catecholaminergic plexus. A catecholaminergic plexus is also present within the digestive tract of the noneviscerated animal. The plexus is composed of neurons and fibers in the inner connective tissue layer adjacent to the muscle layers and is probably analogous to the basiepithelial plexus described in the starfish by Cobb and Raymond (1979). In the sea cucumber, the plexus is primarily localized in the esophagus but extends to the oral end of the small intestine, becoming less conspicuous as one moves caudally and eventually disappearing, covering about one-third of the descending small intestine. Nevertheless, the posterior part of the plexus lies within tissue that is eviscerated (the esophagus is not eviscerated); hence, the catecholaminergic cells and fibers serve as additional markers of neuronal regeneration. The catecholaminergic plexus is present in the regenerated intestine (Fig. 7). The spatial distribution of catecholaminergic cells and fibers in the regenerated section is similar to that of noneviscerated specimens, namely the plexus is arranged in longitudinal tracts, with the neuronal cell bodies interspersed along the tracts. The plexus in the regenerated organisms is continuous from the esophagus into the regenerated small intestine, without any obvious disruption between what was not eviscerated and what was regenerated. The arrangement of the catecholaminergic tracts in the regenerated plexus tends to be highly organized, with tracts extending parallel to each other and with less fibers criss-crossing from one tract to the next. In addition, the cell somas in the regenerated area are smaller in size than those in the noneviscerated area (14 vs. 24 μm).

Ultrastructural analysis. The most prominent nervous structure observed with the electron microscope was the extensive nerve plexus that lies between the coelomic epithelium and the muscle layers. This plexus is made of fibers that differ in size, from 100 to 900 nm in width. The fibers themselves contain a heterogeneous population of vesicles that can be clear and small (25–50 nm), dense and small (60–100 nm), granular and large (100–200 nm), or clear and large (150–250 nm). As a matter of fact, a previous ultrastructural immunocytochemical analysis showed the presence of dense vesicles containing GFSKLY-Famide within cells and nerve fibers of the intestine of *H. glaberrima* (Díaz-Miranda et al., 1995). The fiber network is closely associated with the muscle layers, and in many cases the muscle cells extend cytoplasmic prolongations that appear to establish nonspecialized synaptic contacts. The ultrastructural analysis of the 21 PE regenerated intestines showed the same heterogeneous composition of nerve fibers and vesicles of the nerve plexus as those described for the noneviscerated organisms (Fig. 8).

In summary, immunohistochemical studies at the light microscope and studies at the electron microscopic level show that, after three weeks of regeneration, all of the nervous components known to form the ENS of these echinoderms are present in the regenerated intestine, and their distribution patterns are similar to those in noneviscerated organisms.

Temporal analysis. These results show that regeneration of the ENS occurs in holothurians after evisceration. The next step was to study the timing of the regeneration process. We divided the regeneration process in *H. glaberrima* into several stages based on the presence and proportional size of the different tissue layers (García-Arrarás et al., 1998). Soon after the evisceration process and wound repair (stage I), the new intestine begins to form as a thickening at the end of the torn mesenteries. This thickening grows in size, and within 5–7 PE becomes a blastema-like structure made of a single cell epithelium, similar to the coelomic epithelium surrounding a mass of cells within a connective tissue layer (stage II). The luminal epithelium appears 7–14 PE, forming the lumen within the connective tissue space (stages II and IV).

Within this scenario it was of interest to determine whether regeneration of the nervous system occurred simultaneously with the formation of the blastema-like structure and with the appearance of the different tissue layers or occurred later and was present only after most of

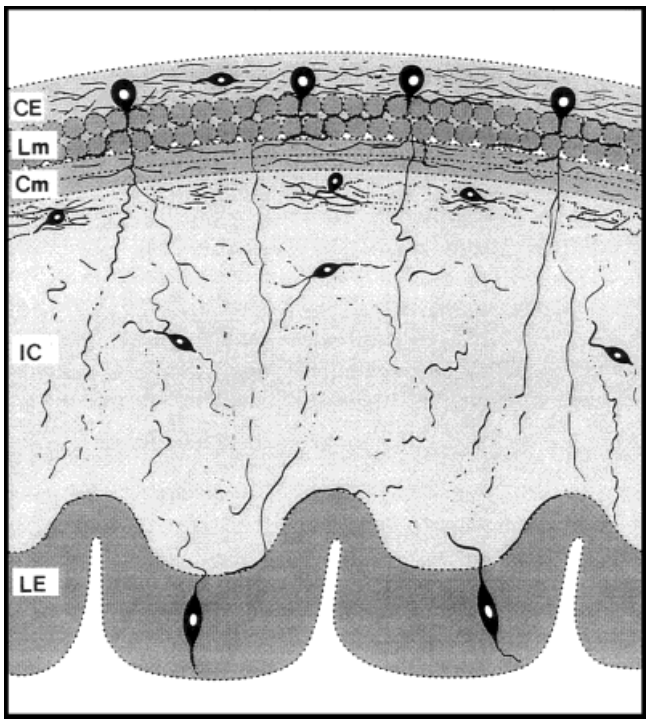

Fig. 3. Composite diagram of the holothurian enteric nervous system. The enteric nervous system in *Holothuria glaberrima* includes peptidergic neurons (10–12 μm) in the coelomic epithelium (CE) and a dense fiber plexus associated with the longitudinal (Lm) and circular (Cm) muscle layers. Neuronal fibers are also found within the inner connective tissue layer (IC), where they are mostly observed to be perpendicular to the circular muscle layer. The neurons in the coelomic epithelium and some of the fiber populations can be detected with antibodies to the neuropeptides cholecystokinin, GFSKLYFa, and galanin. The nerve plexus and the fibers in the inner connective tissue layer can also be recognized with the acetylated α-tubulin antibody. An additional component of the nervous system is recognized by the monoclonal antibody F6. This corresponds to isolated cells of about 8 μm in diameter present in the inner connective tissue and in the connective tissue associated with the coelomic epithelia, with processes that form a neural net within the connective tissue. Neuroendocrine cells expressing GFSKLYFa are found within the luminal epithelium (LE). In the anterior part of the intestine, a catecholaminergic nerve plexus is present in the inner connective tissue layer, adjacent to the muscle layer.

the regenerated organ was formed. To decide between these alternatives, specimens were dissected at 5, 7, 10, and 14 PE and treated for immunocytochemistry with the different neuronal markers.

Our results show that the nervous system regenerates in parallel with the formation of the mesenterial thickenings and with the appearance of the different tissue layers. Nerve fibers immunoreactive to GFSKLYFamide, galanin, F6, and acetylated α-tubulin were observed in the coelomic lining of the blastema-like structure at 5 PE and in the inner connective tissue layer within the next 2 days (Fig. 9). The F6-labeled cell bodies were also found within the blastema-like structure in the 5 and 7 PE regenerates, whereas peptidergic cells were not found until 10 and 14 PE. The peptidergic cells were found, with two exceptions, in the same tissue layers as in noneviscerated animals: the coelomic lining and in the case of GFSKLYFamide in the luminal epithelium. The two cases in which transient abnormal localization of peptidergic cell was observed were in 10 and 14 PE animals in which (1) round cells expressing GFSKLYFamide or galanin were found in the inner connective tissue layer of the intestine and adjacent mesentery, and (2) unipolar neurons expressing GFSKLYFamide were found in the coelomic lining of the mesentery. These cells showed large fibers with strongly fluorescent varicosities. Equally significant is the fact that many of the GFSKLYFamide- and F6-labeled cells within the regenerating intestine were found in the inner connective tissue near the mesentery, suggesting that neuronal precursors may be migrating from the mesentery.

Cell division. The enteric neurons that are present in the regenerated intestine could be arising by transdifferentiation and/or migration of cells already present in the organism or by cellular proliferation and the appearance of new neurons. To determine the origin of the neuronal population, we focused on the GFSKLYFamide-expressing neurons and examined whether these cells proliferate in the regenerating intestine as measured by the incorporation of BrdU. Our results show that these peptidergic cells develop from a group of actively dividing cells in the regenerating organ.

The general pattern of cell proliferation was similar to that described previously (García-Arrarás et al., 1998) in showing high levels of cell proliferation in the coelomic and luminal epithelia but low levels within the innerconnective tissue layer. Cells expressing both BrdU and GFSKLYFamide were detected as early as 10 PE. All these cells were observed in the inner connective tissue layer and in the coelomic epithelium of the regenerating intestine only at the level where the luminal epithelium was forming or had already formed. In general, these cells were few in number, approximately up to three cells per tissue section analyzed, and were sparsely distributed in the inner connective tissue and coelomic epithelium. They were characterized by a round morphology, with an approximate diameter of 8–10 μm and a conspicuous nucleus (Fig. 10). Fiber extensions were not seen in the GFSKLYFamide/BrdU-labeled cells, even though a profuse and conspicuous nerve fiber network immunoreactive to GFSKLYFamide was observed in the coelomic lining, in the inner connective tissue layers of the regenerating intestine, and in the mesentery. The double-labeled cells were intermingled with other GFSKLYFamide-expressing cells that were not BrdU labeled.

Double-labeled cells were also detected in the connective tissue of the mesentery adjacent to the regenerating intestine. In fact, of the total number of GFSKLYFamide/BrdU-immunoreactive cells, approximately half was found in the inner connective tissue of the mesentery, and the rest was dispersed throughout the inner connective tissue and coelomic epithelium of the intestine.

Preabsorption of the sera mixture, i.e., anti-GFSKLYFamide and anti-BrdU with 10 μg/ml of the synthetic peptide before its application to the tissue sections, completely abolished the GFSKLYFamide immunoreactivity.

DISCUSSION

Echinoderm regeneration capabilities

Echinoderms are famous for their spectacular regeneration of body parts. Sea and brittle stars can regenerate all arms, as long as the central disk remains undamaged

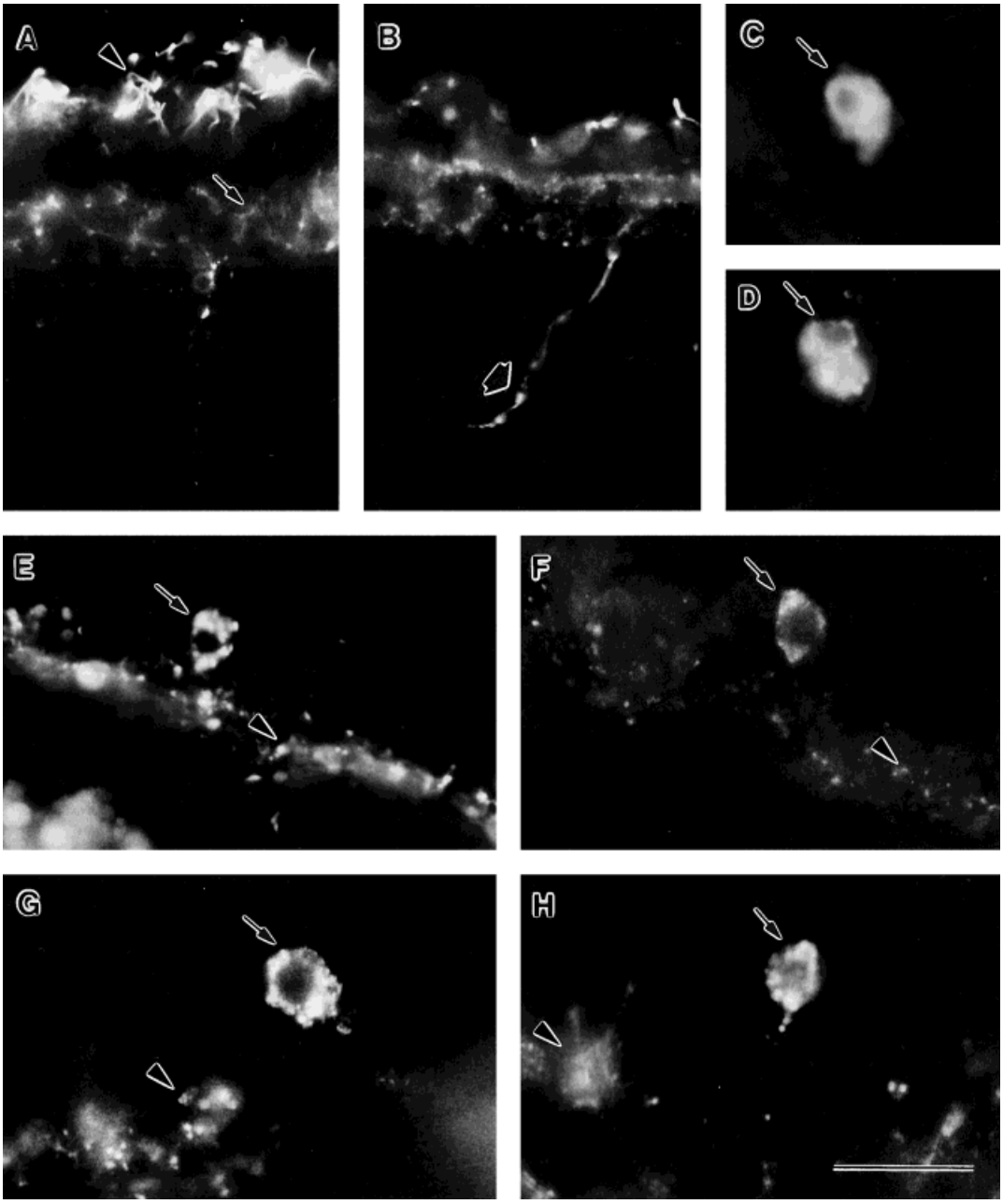

Fig. 4. Regeneration of the enteric nervous system component associated with the coelomic epithelium. Immunohistochemistry in transverse intestinal sections was done by using neuronal markers to compare the enteric nervous components of noneviscerated organisms (**A,C,E,G**) with those of organisms regenerated after 3–5 weeks (**B,D,F,H**). Immunoreactivity to acetylated α-tubulin detects the cilia in coelomic epithelia (arrowheads), a prominent nerve plexus associated with the muscle layer (small arrows), and fibers extending into the inner connective tissue layer (large arrows in A,B). Immunoreactivity to the neuropeptides CCK (C,D), galanin (E,F), and GFSKLYFa (G,H) detect cells (arrows) and nerve fibers (arrowheads) within the enteric nervous system. Scale bar = 26 μm in A,B, 20 μm in C–H.

(Hyman, 1955). Crinoids have been shown to regenerate their main disk (Amemiya and Oji, 1992). Holothurians can regenerate different body parts after not only evisceration but other forms of autotomy (Bertolini, 1930, 1932; Dawbin, 1949; Mosher, 1956; Swan, 1966; Bai, 1971). In particular, gut regeneration in holothurians can also occur after bisection (Gibson and Burke, 1983) and seasonal atrophy (Fankboner and Cameron, 1985).

Regeneration of the nervous component has been implied in general terms (Smith, 1971a,b; Amemiya and Oji, 1992; Candia-Carnevali et al., 1993, 1995); nevertheless, it has been little studied at the cellular level. In starfish, catecholaminergic fibers to the arm tips regenerate (Mladenov et al., 1989), and regeneration of a subpopulation of neurons and fibers has been studied recently (Moss et al., 1998). Nerve fibers and cell bodies are present in starfish

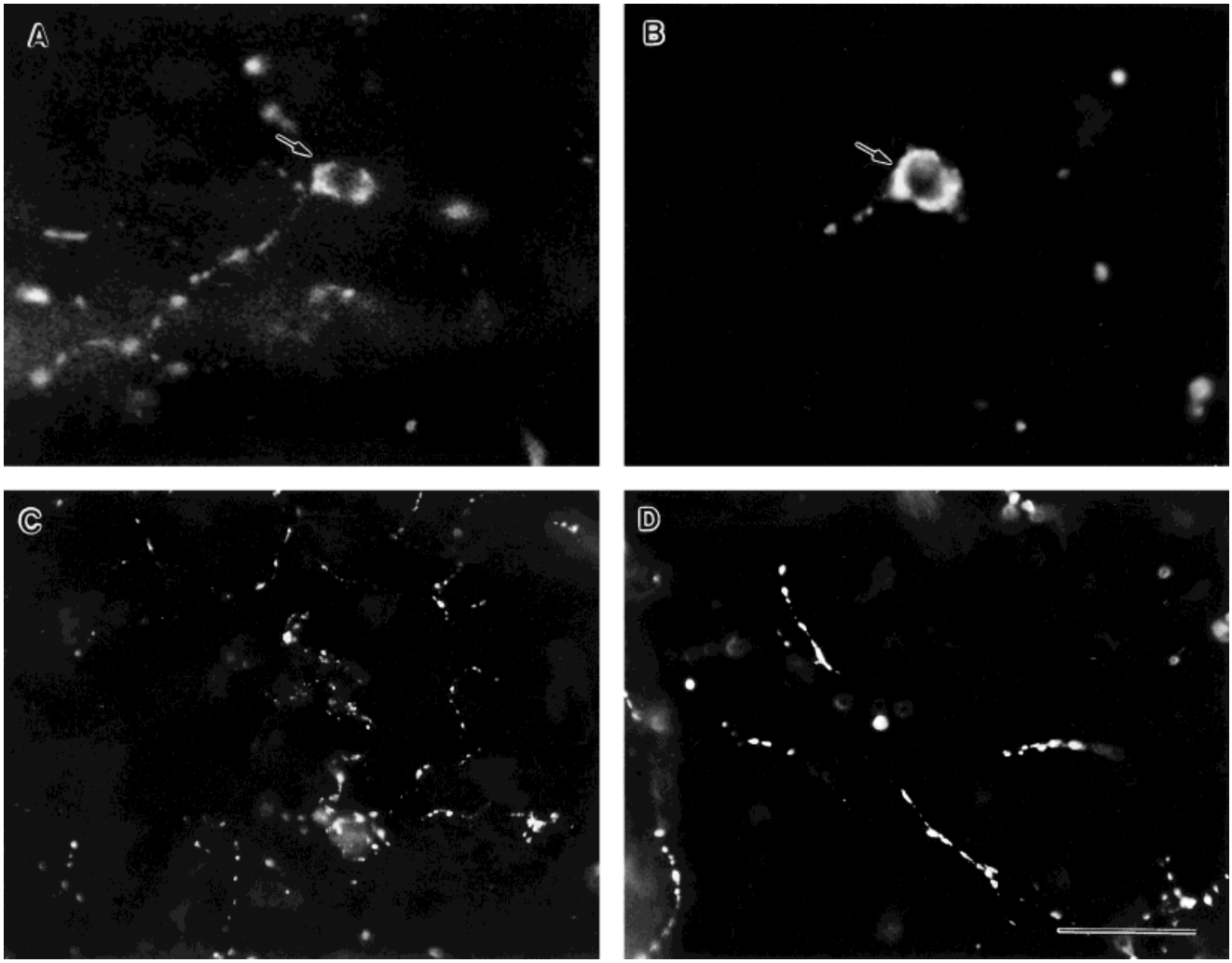

Fig. 5. Regeneration of the inner connective tissue layer component of the enteric nervous system. Transverse sections of the sea cucumber intestine were treated with the monoclonal antibody F6 by immunocytochemistry to compare the enteric nervous components of noneviscerated organisms (**A, C**) with those of organisms regenerated after 3–5 weeks (**B, D**). The F6 antibody labels cells (arrows, in A, B) and a fiber network in the inner connective layer of the intestine (C,D). Scale bar = 20 μm in A,B, 32 μm in C,D.

viscera (Cobb, 1986) and regeneration of their cardiac stomach has been described (Anderson, 1962, 1965a,b), but regeneration of the nervous component itself has not been studied.

The main hurdle in echinoderm studies stems from the fact that their neurons are small and not easily identifiable (Cobb, 1986). We have identified several nervous components of *H. glaberrima* by using the glyoxylic-acid-induced fluorescence to detect catecholamines and antibodies to the neuropeptides CCK (García-Arrarás et al., 1991a), GFSKLYFamide (Díaz-Miranda et al., 1992, 1995), and galanin (Díaz-Miranda et al., 1996). Two other antibodies were used: anti-acetylated α-tubulin (García-Arrarás and Viruet, 1993), which recognizes enteric nerve fibers, and monoclonal antibody F6, which recognizes a nerve cell population within the connective tissue.

Regeneration of enteric nervous components

Two forms of nervous regeneration may occur in the intestine. First, extrinsic fibers grow through the mesentery from neurons located outside the intestine. During evisceration, the fibers would be damaged, but the somas would remain intact. Regenerating fibers would then reinnervate the intestine once it is formed or during its formation. The presence of fibers immunoreactive to acetylated α-tubulin and to the neuropeptides in the regenerating structure suggests that fibers are indeed regenerating.

A second type of nervous regeneration would be the appearance of new neurons within the regenerated structure. The evidence for this type of regeneration is compelling. First, neurons expressing neuropeptides, catecholamines, and those labeled with F6 are present in the regenerated intestine. The morphology and distribution pattern of these cells in the 21 PE regenerates are identical to those in the noneviscerated controls. Second, peptide-expressing cells have undergone cell division, implying that new neurons are being produced. Thus, the ENS, similar to the muscular, epithelial, and other components of the intestine (García-Arrarás et al., 1998), has regenerated within a month after evisceration.

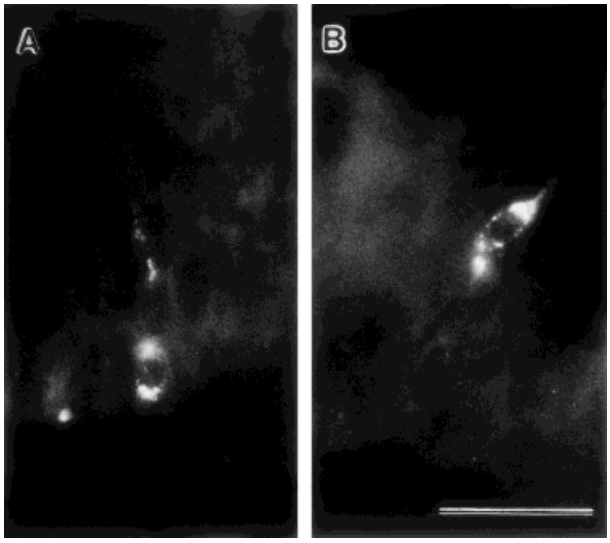

Fig. 6. Regeneration of the luminal epithelium component of the enteric nervous system. Neuroendocrine cells expressing the neuropeptide GFSKLYamide can be observed in transverse sections of the intestine from noneviscerated organisms (**A**) and from organisms after 3–5 weeks of regeneration (**B**). Scale bar = 18 μm.

Origin of neuronal cells

Based on our observations, a theory of how ENS regeneration occurs in *H. glaberrima* starts to emerge. We propose that neurons are generated by an epimorphic mechanism in which precursors present in the coelomic lining of the mesentery divide and differentiate into new neurons (Fig. 11). These cells, before fully differentiating, migrate into the adjacent regenerating structure. This theory, although based mostly on correlative evidence, is supported by several observations. First, proliferating sites are present in the coelomic epithelia of the intestine and in the adjacent mesentery during intestinal regeneration in *H. glaberrima* (García-Arrarás et al., 1998). If the neurons detected in the newly regenerated intestine originate from actively dividing cells, then these are the potential sites for them to arise and repopulate the new structure.

Second, galanin- and GFSKLYFamide-expressing cells appear transiently in the mesentery and within the internal connective tissue of the regenerating intestine. These cells are not found in noneviscerated organisms or in more advanced stages of regeneration.

Third, some peptidergic cells in the mesentery show BrdU incorporation, an indicator that cell division has occurred within the preceding 48 hours. About half of the dividing peptidergic cells are found in the connective tissue layer of the mesentery. This tissue layer is continuous with the submucosa of the regenerating intestine and may be a pathway for cell migration into the regenerating structure.

Fourth, in early regeneration stages, the F6-labeled cells are found in the region of the blastemalike structure that is adjacent to the mesentery, suggesting that these cells migrate from the mesentery.

In a study by Fankboner and Cameron (1985), where seasonal atrophy of the viscera was observed, the gut wastes away to a thin line of primordial tissue on the mesentery margin. It would be interesting to determine the outcome of the neurons during atrophy because regeneration of the intestine after atrophy follows a sequence of events similar to that after evisceration.

Our regeneration theory only applies to the peptidergic and F6 cells in the coelomic epithelium and inner connective tissue and does not account for the catecholaminergic cells in the anterior intestine or for the neuroendocrine cells that may have other origins. It is possible that different neuronal components differ in their regeneration process. This possibility is particularly important because recent experiments have indicated that the regeneration of peptidergic neurons in starfish ectoneural system seem to occur without cell division taking place (Moss et al., 1998).

Regeneration and embryogenesis mechanisms share some similarities. However, the development of the ENS has not been described in echinoderms. Nevertheless, in organisms that have been studied, the ENS originates by cell proliferation, migration, and differentiation. In birds and mammals, the cells of the vagal and sacral neural crest migrate and colonize the intestinal primordia, giving rise to the myenteric and submucous ganglia (Yntema and Hammond, 1954; Gershon, 1981; LeDouarin, 1982; Gershon et al., 1993). In *Xenopus*, enteric precursors from the neural crest migrate through the mesentery into the forming digestive tract (Epperlein et al., 1990). Similarly, in the moth *Manduca*, the ENS arises from cells in the foregut epithelium that migrate to their final location, undergoing limited mitotic activity prior to their differentiation (Copenhaver and Taghert, 1989a,b, 1991). Thus, embryonic studies in both protostomes and deuterostome organisms have shown that ENS forms from epithelial precursors that migrate and redistribute in the intestinal primordia during development.

Moreover, in the protochordate *Ciona intestinalis*, where regeneration of the nervous system has been studied, some new neurons have been shown to form from an epithelial tube early in regeneration (Bollner et al., 1992, 1995). Similarly to our observations in *H. glaberrima*, these investigators have shown that at least some of the new neurons incorporate BrdU and thus are originating by cell proliferation.

The coelomic epithelium of holothurians has been classified as a complex tissue composed of adluminal, neural, and myoepithelial cells (Smiley and Cloney, 1985; Rieger and Lombardi, 1987) and thus may be the source of new nerve cells. In fact, Dolmatov et al. (1996) provided some evidence showing that, during longitudinal muscle regeneration, muscle precursors may originate from the coelomic epithelia.

However, the alternative explanation that neuronal cells differentiate from migratory cells present in the coelomic space or hemal system cannot be ruled out. Such might be the case in the crinoid echinoderm *Antedon mediterranea*, where amoebocytes or coelomocytes have been proposed to be involved in the regeneration of the brachial nerve (Candia-Carnevali and Bonasoro, 1994; Candia-Carnevali et al., 1995). Similarly, in the regeneration of the neural ganglion of ascidians, a transdifferentiation mechanism of pluripotential haemoblasts to neurons has been proposed (Bollner et al., 1995).

Temporal sequence of events

All nervous elements detected by our probes appear before regeneration of the intestine is completed, suggest-

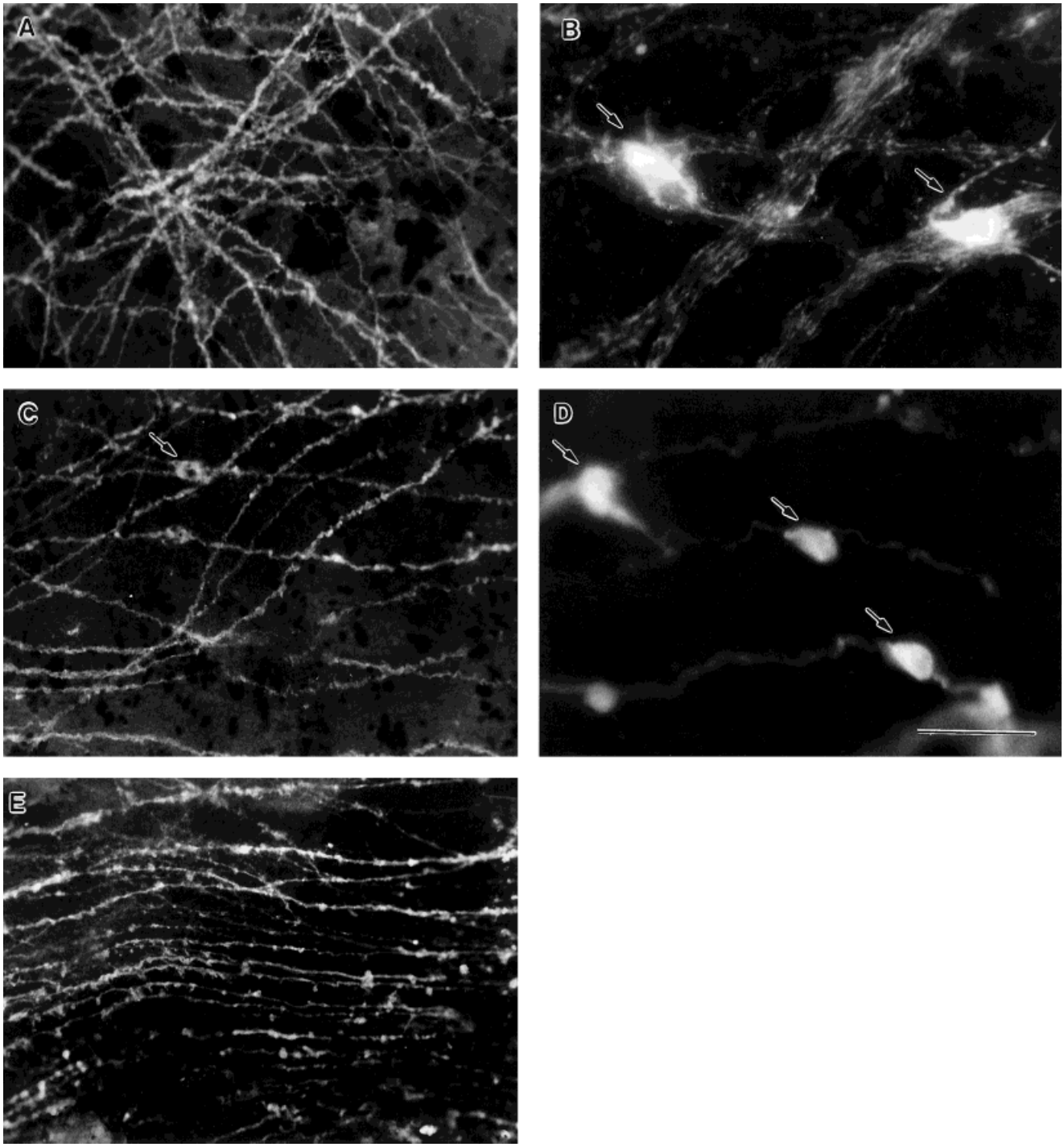

Fig. 7. Regeneration of the catecholaminergic nerve plexus in the esophageal–intestinal region. Glyoxylic-acid-induced fluorescence to detect the presence of catecholamines was used to localize nervous elements in wholemounts of the digestive tract of *H. glaberrima*. In wholemounts from the anterior intestine of noneviscerated specimens, the fiber plexus (**A**) and cells (**B,** arrows) can be clearly observed. Similarly, the plexus and cells (arrows) are found in wholemounts from eviscerated animals, extending from the esophagus (**C**), a segment that was not eviscerated, to the intestine (**D,E**), a regenerated region. Scale bar = 200 μm in A,C,E, 33 μm in B, 27 μm in D.

ing that the nervous system may be important in the regeneration of the intestine itself. It has been postulated that factors originating from the nervous tissue exert an important modulation of the regenerating process (Singer, 1952; Huet, 1975; Huet and Franquinet, 1981; Olsen and Tassava, 1984; Bonasoro et al., 1995). In both the salamander (Singer, 1952; Olsen and Tassava, 1984) and starfish (Huet, 1975), regeneration of the limb depends on the presence of nerves. Therefore, in the sea cucumber, processes that occur in late regeneration stages, such as

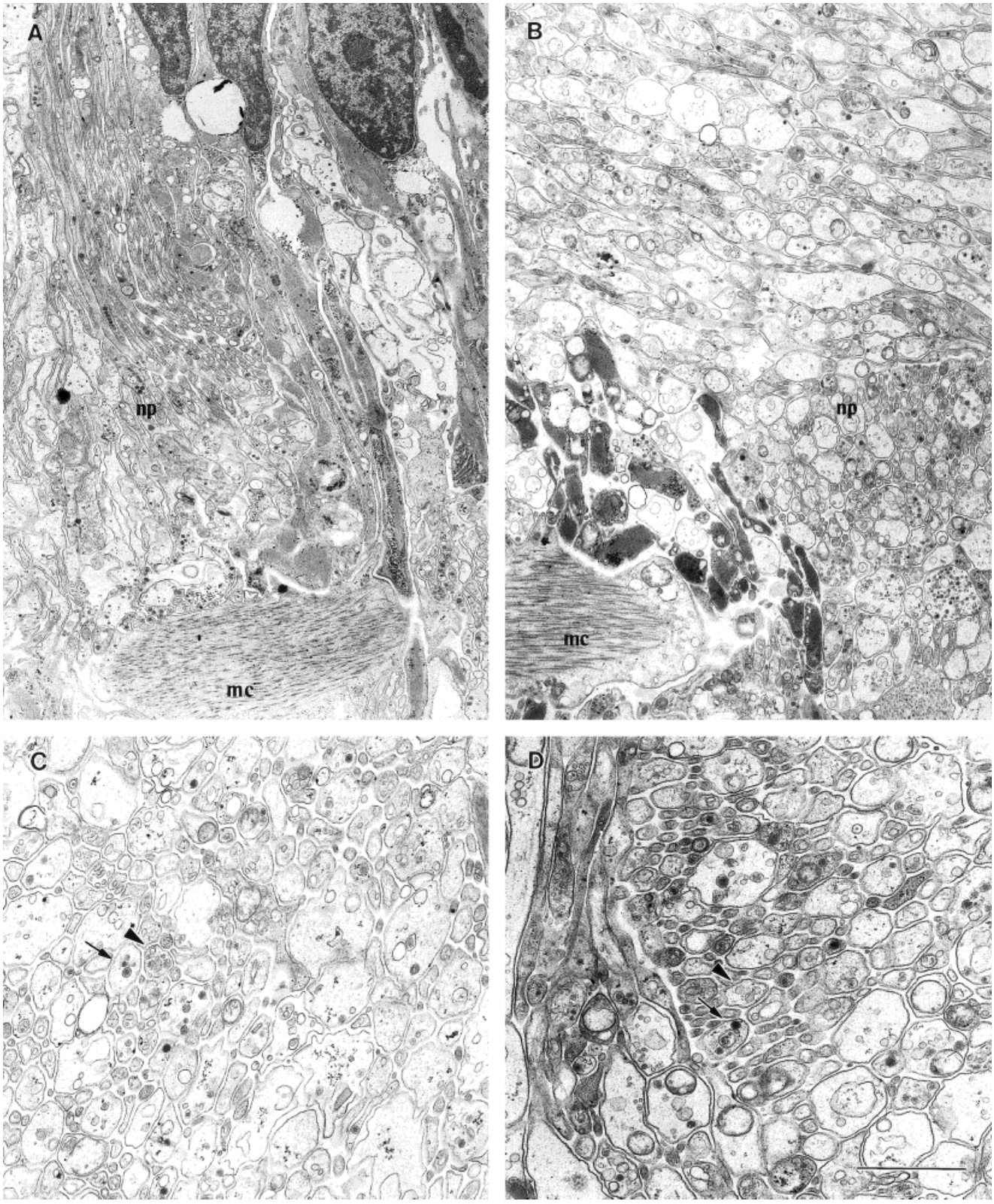

Fig. 8. Transmission electron micrographs of the nerve plexus loclated in the coelomic epithelium of noneviscerated intestines (**A, C**) and intestines after 3 weeks of regeneration (**B, D**). This nerve plexus is formed by a heterogeneous fiber population adjacent to the muscle layer. C,D: Detail of the ultrastructure of the nerve plexus. np, nerve processes; mc, muscle cell. Arrow points at a dense core vesicle containing process. Arrowhead points at a clear vesicle containing process. Scale bar = 3.4 µm in A,B), 1.3 µm in C,D.

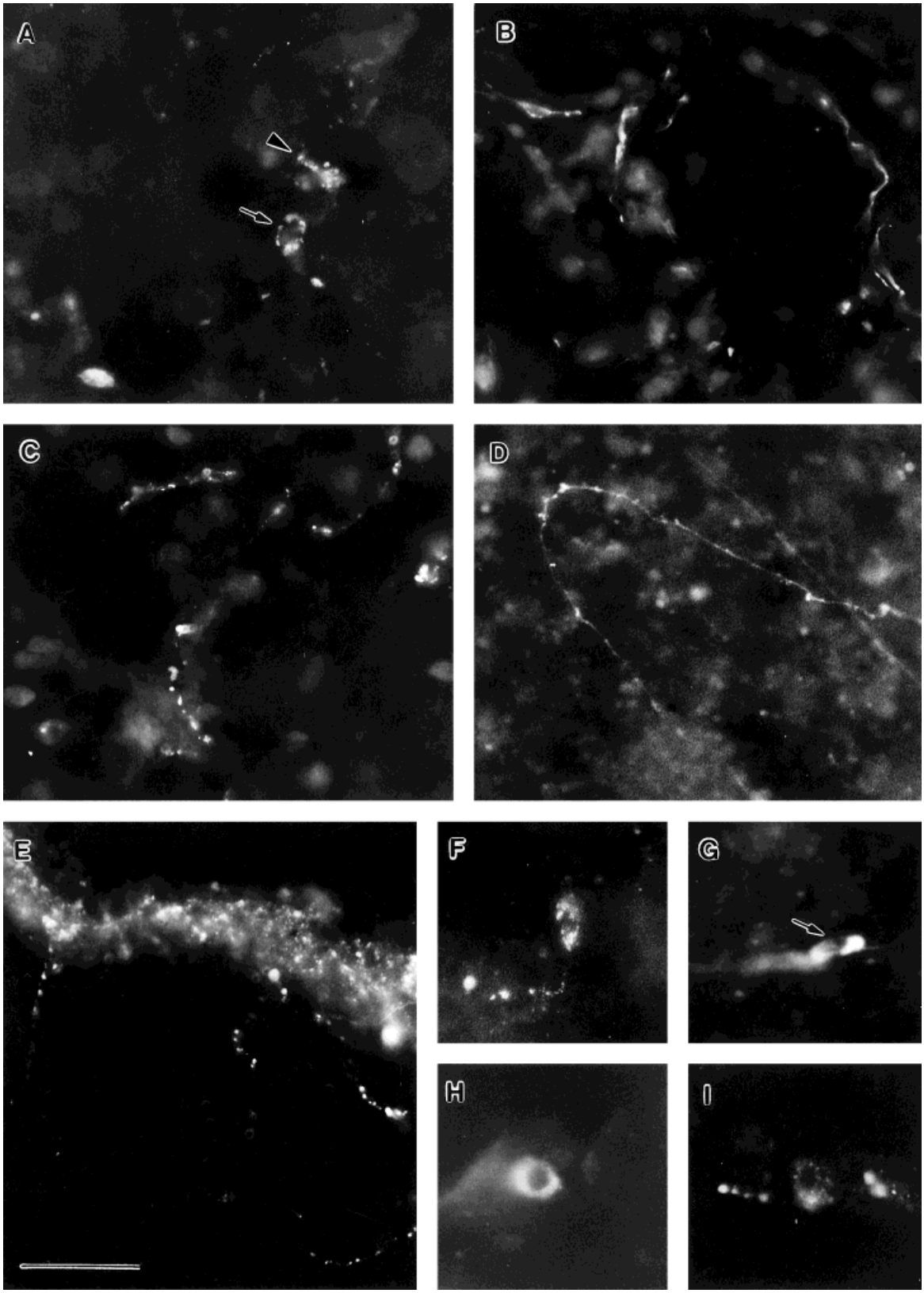

Fig. 9. Regeneration of the enteric nervous system occurs concomitant with the regeneration of the digestive tube. **A–D:** Neural elements can be detected in the blastemalike thickening that forms during the first week of regeneration. These include neuronal bodies (arrow) and fibers (arrowheads) detected with the monoclonal antibody F6 (A) and neuronal fibers immunoreactive to GFSKLYFa (B), acetylated α-tubulin (C), and those containing catecholamines as detected by glyoxylic-acid-induced fluorescence (D). In eviscerated organisms during the second week of intestinal regeneration, in which the lumen has already formed, neuropeptide (GFSKLYFa) immunoreactivity in the coelomic epithelium, inner connective tissue layer (**E**), and luminal epithelium (**F**) is similar to that found in the noneviscerated organisms. In the anterior part of the intestine, catecholaminergic cells (**G,** arrow) are already found in the plexus. Round cells expressing immunoreactivities to galanin (**H**) and GFSKLYFamide (**I**) can be found in the connective tissue layer of the mesentery adjacent to the regenerating intestine. Scale bar = 26 μm in A, 34 μm in B–D, 30 μm in E, 20 μm in F–I.

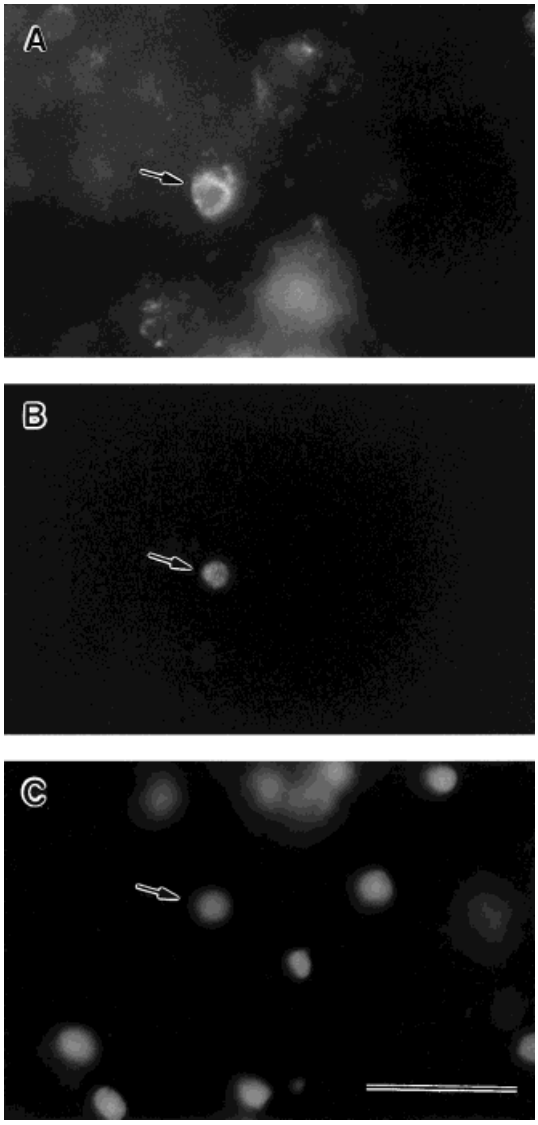

Fig. 10. Transverse sections of a 10 days postevisceration, regenerated intestine showing a cell expressing GFSKLYFamide (**A**) that has simultaneously incorporated the thymidine analogue bromodeoxyuridine in its nucleus (**B**). This cell is located in the inner connective tissue layer of the intestine. Total nuclei population within the tissue section is shown by Hoechst nuclear dye (**C**). Arrows indicate the double-labeled cell. Scale bar = 20 μm.

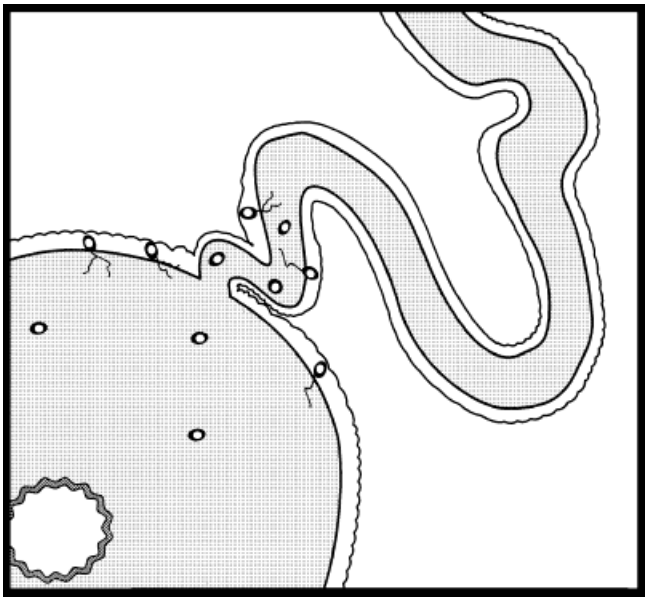

Fig. 11. Proposed model for the regeneration of the enteric nervous system in *Holothuria glaberrima*. Our results suggest that neurons in the regenerating intestine develop from an actively dividing cell population located in the mesentery adjacent to the regenerating organ. These cells migrate through the connective tissue of the mesentery into the inner connective tissue layer of the regenerating intestine. They appear transiently at the time when the luminal epithelium is also organizing, suggesting that the cells may be involved in the establishment of the new neuronal population in the regenerated organ. The diagram represents a cross section of the regenerating intestine, with a portion of the adjacent mesentery.

the appearance of the luminal epithelium or the organization of the muscular layer, may be under nervous influence.

Echinoderms as model systems

Most invertebrates used as models in regeneration studies, such as coelenterates, flatworms, or annelids (Hyman, 1955; Brndsted, 1969; Bode and David, 1978; Nicholls, 1987; Gremigni, 1988), are evolutionarily distant from the chordates. In contrast, echinoderms, being deuterostomes, are considered to be one of the closest relatives to members of the phylum Chordata. They have been used extensively in developmental studies, in particular those concerning the early steps of embryogenesis. The holothurians present several additional advantages as a model system. First, evisceration occurs in a fixed program, thereby reducing the individual variability inherent to surgical amputations, transections, or laser ablations. Second, the basis for the regeneration of the viscera appears to reside within a well-defined cell population. Third, regeneration occurs in a rather short time and can take place in the aquarium. Our model system could complement models that offer other advantages not available in echinoderms, such as the possibility of performing genetic studies.

The fact that the echinoderms exhibit such an amazing regeneration capacity opens the door to answer new experimental questions, such as: Which genes or gene products are important for ENS regeneration to occur? Does the ENS has some influence on intestinal organogenesis? Are there growth factors specific for enteric neurons? Future experiments to answer these questions will help in understanding and overcoming the limits to nervous regeneration found in chordate deuterostomes. Thus, we can study the factors that allow regeneration of the ENS in these organisms and the influences that the nervous system may exert on tissue or organ regeneration.

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