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Identification of Nerve Plexi in Connective Tissues of the Sea Cucumber *Holothuria glaberrima* by Using a Novel Nerve-Specific Antibody

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Abstract. The echinoderm nervous system is one of the least studied among invertebrates, partly because the tools available to study the neurobiology of this phylum are limited. We have now produced a monoclonal antibody (RN1) that labels a nervous system component of the sea cucumber *Holothuria glaberrima.* Western blots show that our antibody recognizes a major band of 66 kDa and a minor band of 53 kDa.

Immunohistological experiments show that, in *H. glaberrima,* the antibody distinctly labels most of the known nervous system structures and some components that were previously unknown or little studied. A surprising finding was the labeling of nervous plexi within the connective tissue compartments of all organs studied. Double labeling with holothurian neuropeptides and an echinoderm synaptotagmin showed that RN1 labeled most, if not all, of the fibers labeled by these neuronal markers, but also a larger component of cells and fibers. The presence of a distinct connective tissue plexus in holothurians is highly significant since these organisms possess mutable connective tissues that change viscosity under the control of the nervous system. Therefore, the cells and fibers recognized by our monoclonal antibodies may be involved in controlling tensility changes in echinoderm connective tissue.

Introduction

The neurobiology of species from the phylum Echinodermata has lagged behind that of other invertebrates. One impediment to study has been the lack of markers to identify nerve cells and fibers, which are usually small and sometimes surrounded by a calcite skeleton (Pentreath and Cobb, 1972; Cobb, 1987). The classical experiments aimed at describing the echinoderm nervous system mainly used methylene blue as a marker, but this does not identify the nervous components with sufficient specificity (Pentreath and Cobb, 1972). Some investigators have identified neuronal and fiber populations that express particular neurotransmitters, mainly catecholamines (Cobb, 1969; Cottrell and Pentreath, 1970) and neuropeptides (Elphick *et al.,* 1995; Newman *et al.,* 1995a, b; Díaz-Miranda *et al.,* 1995, 1996; Inoue *et al.,* 1999). In addition, isolated descriptions of cells or fibers have been obtained through ultrastructural studies using electron microscopy (Pentreath and Cobb, 1972; Cobb, 1987; Byrne, 1994, 2001; Cavey and Märkel, 1994; Chia and Koss, 1994; Heinzeller and Welsch, 1994; Smiley, 1994). However, a broader view of the echinoderm nervous components has been difficult to obtain. Only recently has some progress been made in describing embryonic echinoderm nervous systems with the use of a monoclonal antibody that appears to label synaptotagmin (Nakajima *et al.,* 2004; Burke *et al.*, 2006).

The principal echinoderm nervous system consists of a series of radial nerves connected anteriorly to a nerve ring. It has two main components: the ectoneural and the hyponeural (Hyman, 1955; Cobb, 1987). The ectoneural system makes up the nerve ring and the outer part of the radial nerve cords and has been ascribed a sensory and motor function. The hyponeural system is present in the inner portion of the radial nerve cord and is associated with motor control of the musculoskeletal system. There are other components of the nervous system, but they have been less

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studied. Examples of these are the entoneural, or aboral, nervous system found mainly in crinoids (Hyman, 1955) and the enteric nervous system described by our group (García-Arrarás *et al.,* 2001) in the sea cucumber. Ironically, the least known nervous component might be the most interesting: found within the connective tissue compartments, it has been associated with a unique echinoderm characteristic, the variable mechanical properties of the connective tissue (Serra-von Buddenbrock, 1963; Cobb, 1989). Because of this property, echinoderm connective tissues have been named catch connective tissue or mutable connective tissue (Takahashi, 1967a, b; Motokawa, 1988; Cobb, 1989; Wilkie, 2001; Motokawa and Tsuchi, 2003). Changes in tissue mechanical properties are evident as either a stiffening or a softening of the tissue. There is some controversy about the mechanism by which the connective tissue changes its stiffness (Koob *et al.,* 1999; Trotter *et al.,* 2000; Motokawa and Tsuchi, 2003), but it has been established that the phenomenon is under the control of the nervous system (Motokawa, 1988; Koob *et al.,* 1999; Wilkie, 2001). In fact, juxtaligamental cells, a cell type described in echinoderms, have been associated with connective tissue mutability (Wilkie, 1979).

We have now obtained a monoclonal antibody, RN1, which appears to label most, if not all, nervous components of the holothurian nervous system. We used this antibody to begin a precise description of the cells and fibers that form the echinoderm nervous system. In particular, we focused our attention on the nervous system components found within the connective tissues. Our results show an extensive nerve plexus within all connective tissue components. Here we describe the cells and fibers that form the connective tissue nervous plexi of the body wall, the tube feet, the digestive tract, and other major structures of the sea cucumber *Holothuria glaberrima.*

Materials and Methods

Animals

Adult specimens (10–15 cm in length) of the sea cucumber *Holothuria glaberrima* Selenka, 1867, were collected from the rocky shores of the north coast of Puerto Rico and kept in seawater aquaria.

Antibody preparation

One of the radial nerves from an *H. glaberrima* specimen was dissected and cleaned of surrounding tissue as much as possible. One half of this nerve was homogenized in 200 μl of 70% ethanol using a manual glass homogenizer. The homogenate was then centrifuged for 10 min at 3000 $\times$ *g,* the supernatant was discarded, and the pellet was resuspended in 100 μl of 0.1 mol l^{-1} phosphate-buffered saline (PBS). The pellet was broken by grinding it between two glass slides, diluted in 200 μl of 0.1 mol l^{-1} PBS, and frozen in 133-μl aliquots at -20°C until use.

Prior to immunization, 67 μl of PBS was added to one aliquot to make up a final volume of 200 μl. The sample was then mixed with 200 μl of Titer Max Gold adjuvant (Titer Max, Norcross, GA). Two male 6–8-week-old Balb/c mice were injected intraperitoneally with 100 μl of the emulsion. A booster, without the adjuvant, was administered after 7 weeks, and the fusion was made 3 days later.

The monoclonal antibody was prepared as described previously (García-Arrarás *et al.,* 1998). In brief, the fusion was performed by Harlow and Lane's (1988) stirring method previously used in our laboratory (García-Arrarás *et al.,* 1998; Quiñones *et al.,* 2002) with a spleen/myeloma (SP20) ratio of 10:1. Hybridoma supernatants were tested using immunohistochemistry on *H. glaberrima* radial nerve sections. The cells in one well were cloned and shown to produce a monoclonal antibody that specifically labeled the radial nerve. This antibody was named RN1.

Ascites fluid was obtained by injecting the RN1-producing-hybridomas into male Balb/c mice as described by Harlow and Lane (1988). In brief, 8-week-old mice were injected with 0.5 ml of incomplete Freund's adjuvant into the peritoneum; 10 days later, 0.5 ml of RN1-producing-hybridomas in PBS was injected intraperitoneally. The mice were sacrificed 11 days post-injection, and the ascitic fluid was obtained.

Immunohistochemistry

Adult specimens were sacrificed and samples from the body wall (including radial nerve, longitudinal muscle, and ambulacral tube feet), tentacles, respiratory tree, hemal system, gonads, and digestive tract (esophagus, small intestine, and large intestine) were dissected and fixed in 4% paraformaldehyde at 4 °C for about 24 h. Tissues were rinsed three times for 15 min with 0.1 mol l^{-1} PBS and then left in a sucrose 30% solution at 4 °C for at least 24 h before they were embedded in Tissue Tek. Cryostat tissue sections (7–30 μm) were cut and mounted in Poly-L-lysine-coated slides.

The indirect immunofluorescence method was followed (García-Arrarás, 1993). The primary antibodies used included the RN1 monoclonal (see above); the rabbit antiserum αGFSKLYamide No. 23 2i2s (second injection and second bleeding) (Díaz-Miranda *et al.,* 1995) prepared against a GFSKLYa synthetic peptide and used in a dilution of 1:1000; the rabbit antiserum αgalanin-1 2i3s (second injection and third bleeding) (Díaz-Miranda *et al.,* 1996) prepared against galanin (Calbiochem Corp., San Diego, CA) and used in a 1:1000 dilution; the rat antiserum αSp-SynB: (Nakajima *et al.,* 2004; Burke *et al.,* 2006) prepared against the recombinant protein made up of the amino acids 177–420 of the predicted Sp-SynB protein and used in a

1:200 dilution; and the monoclonal antibody anti-β-tubulin (Sigma T-4026 Lot 024K4862), clone TUB 2.1, prepared against tubulin from rat brain and used in a 1:500 dilution for double-labeling indirect immunohistochemistry. Negative controls included the use of the hybridoma cell culture medium (without cells grown on it) and the absence of any primary antibody. The secondary FITC antibodies were goat anti-mouse (Biosource, Camarillo, CA, #AMI0408 Lot 3501), goat anti-rabbit (Biosource, Camarillo, CA, #ALI0408 Lot 1402), and goat anti-rat (Biosource, Camarillo, CA, #ARI3408 Lot 1601), used in a 1:25 dilution for double-labeling indirect immunohistochemistry. Also, the Cy3 secondary antibodies goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, #115-165-068 Lot 47814) and goat anti-rabbit (Jackson ImmunoResearch Laboratories #111-165-144 Lot 50694), were used in a 1:1000 dilution for double-labeling indirect immunohistochemistry.

The cell nucleus was stained with a 10-min rinse with Hoechst dye, and the coverglasses were mounted in a buffered glycerol solution. When double labeling was performed, the two primary antibodies were added together first and the two secondary antibodies were added together later (see García-Arrarás, 1993). For the whole mounts, the same process described previously (García-Arrarás *et al.,* 1991) was followed. Tissues were then examined on a Leitz Laborlux fluorescence microscope with N2, I2/3, and D filters or on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII, and DAPI filters.

Western blot

Radial nerve samples were homogenized using a Polytron (Brinkmann Intruments, Westbury, NY) in three different buffers: (1) PBS solution containing protease inhibitors (Calbiochem Corp., San Diego, CA) at 4 °C; (2) lysate buffer for protein extraction (20 mmol l^{-1} Hepes pH 8.0, 150 mmol l^{-1} NaCl, 2 mmol l^{-1} DTT, 5 mmol l^{-1} EDTA, 1 mmol l^{-1} PMSF, and 1.0 DNAse I unit per ml) for 30 min under constant shaking; and (3) Tri Reagent Extraction Solution (Molecular Research Center, Inc., Cincinnati, OH) using the protocol recommended by the suppliers.

Protein concentration was determined using the Coomassie Plus protein assay (Pierce, Rockford, IL). Samples of 10–100 μg of protein were run at 200 V in 10% SDS-PAGE under denaturing conditions, using a BioRad Mini Protean Electrophoresis system (approximately 45 min). The gel was equilibrated in Towin buffer for 15 min. The transfer was performed at 200 V for 1 h on ice in a Mini Trans Blot Cell (BioRad, Hercules, CA).

The PVDF membrane was incubated overnight in 5% nonfat dry milk as a blocking solution, washed three times (Tris-buffered saline with 0.2% Tween 20), and incubated for 1 h in RN1 antibody diluted 1:10,000 with RPMI 1640 medium supplemented with 5% horse serum. A negative control was done with a radial nerve Tri Reagent extract, using RPMI 1640 medium supplemented with 5% horse serum during the primary antibody incubation period, while everything else was done as usual. After three washes of 20 min each, the membrane was incubated for 1 h in secondary antibody (sheep anti-mouse IgG peroxidase-linked) (Amersham Biosciences, Piscataway, NJ) diluted 1:5000 with the same RPMI 1640 supplemented medium. After three more washes of 20 min each, the membrane was incubated with ECL detection reagents (Amersham Biosciences, Piscataway, NJ) for 1 min and exposed to X-ray film.

Results

Antibody preparation and antigen characterization

Hybridoma culture media were screened for neural specific antibodies by using the immunofluorescent technique on radial nerve sections of *Holothuria glaberrima.* Cells from a positive well were cloned by limiting dilution, and the antibody was named RN1. Cloned hybridoma cells were used to produce ascites fluid, which showed great specificity for neural structures, labeling them at a dilution of 1:50,000 to 1:100,000 for immunohistochemistry.

In Western blots of radial nerve homogenates, the antibody could also be used at a high dilution (1:10,000–1:20,000), showing labeling of a principal band of about 66 kDa and a weaker band of about 53 kDa (Fig. 1). The main 66-kDa band was present in all tissue homogenates, while the 53-kDa band was seen weakly in homogenates using protein lysate buffer and more strongly in homogenates done with Tri Reagent. No band was observed in the negative control (Fig. 1).

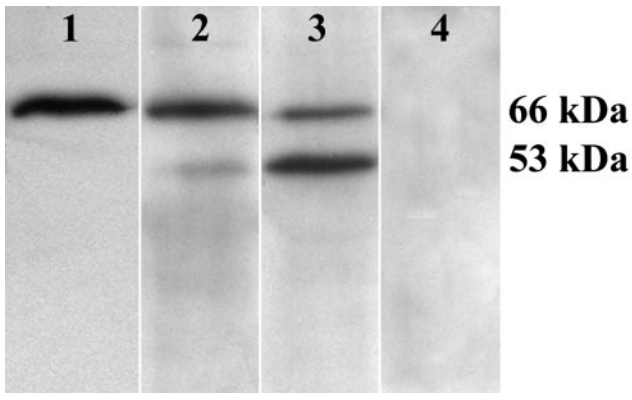

Figure 1. Western blot of holothurian radial nerve preparations using RN1. The antibody recognizes a major band of ~66 kDa in all nerve preparations, including homogenates in three solutions: phosphate-buffered saline (1), protein lysate buffer (2), and Tri-Reagent (3). A second band of ~53 kDa is observed in radial nerve homogenates made in lysate buffer and Tri-Reagent No band was observed in the negative control (4) made using Tri-Reagent nerve homogenate.

RN1 expression was compared with that of β-tubulin, a tubulin isoform that apparently recognizes a neuron-specific isoform in the holothurian (unpubl. data). In contrast to RN1, Western blots with β-tubulin showed only one band, of about 56 kDa (results not shown).

RN1 immunoreactivity in the radial nerve

Holothurian radial nerves are subdivided into two main regions: the ectoneural and the hyponeural components. The nerves are ganglionated, with cell bodies in the periphery of the central nerve region, or neuropile, and extend peripheral branches that communicate with other organ systems. RN1 produced an intense labeling of the radial nerve (Fig. 2a). Both ectoneural and hyponeural components were equally labeled, whereas the basement membrane that separates the two components was not labeled. Intense labeling was observed mainly in the neuropile, whereas the neuronal somata in the periphery of the radial nerve were either labeled weakly or not at all. The intense labeling precluded the possibility of observing individual fibers. In fact, the labeling was so dense that the only unlabeled areas were where the tissues had contracted (due to fixation artifact) or where spherule-type cells were found. RN1 also labeled the peripheral nerves that arise from the radial nerve.

To show the neuronal specificity of our antibody, we compared RN1 to the few available markers that are known

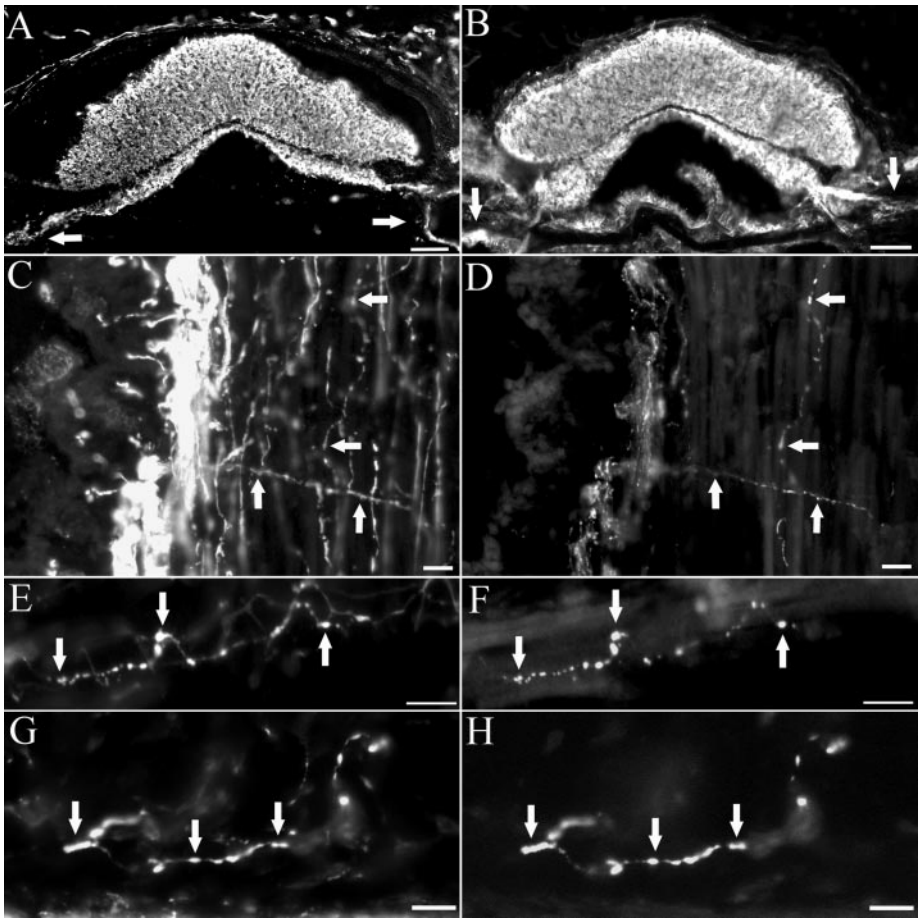

Figure 2. Transverse sections of the radial nerve cords and the peripheral nerve branches, showing RN1 immunoreactivity. (A, B) Section through the nerve cord showing RN1 (A) and β-tubulin (B) immunoreactivity in both the ectoneural and hyponeural nerve components. The basement membrane that separates the two nervous components is not labeled, but labeling is found within the peripheral nerves (arrows) that originate in the radial nerve. (C, D) Double labeling for RN1 (C) and GFSKLYFa (D) shows nerve fibers extending from the peripheral nerve branches to the circular muscle. A small percentage of the RN1 labeled nerve fibers (arrows) are co-labeled with the anti-GFSKLYFa. (E, F) Double labeling of RN1 (E) and anti-galanin (F) in fibers within the circular muscle. A few of the RN-labeled nerve fibers (arrows) are co-labeled with anti-galanin. (G, H) Double labeling of RN1 (G) and anti-Sp-SynB (H) in fibers within the connective tissue plexus of the intestine. A small percentage of the RN1-labeled nerve fibers (arrows) are co-labeled with anti-Sp-SynB. Scale bar = 30 μm in A and B; 15 μm in C, D, E, F, G, and H.

to label neuronal structures in echinoderms, namely two antibodies against neuropeptides (GFSKLYFamide and galanin; see Díaz-Miranda *et al.,* 1995, 1996), a monoclonal antibody against β-tubulin (Tossas *et al.,* 2004), and a recently described antibody against one of the echinoderm isoforms of synaptotagmin (Burke *et al.,* 2006).

Initial comparisons were between labeling with RN1 and with the β-tubulin monoclonal. Since both antibodies are mouse monoclonal, double labeling was not possible. Nevertheless, labeling of adjacent sections showed a strikingly similar pattern. This included intensive labeling of the radial nerve component and of fibers within the hyponeural and ectoneural nerve branches (Fig. 2a, b). However, there were some clear differences. First, β-tubulin labeled the cell bodies within the radial nerve cord, structures that were only weakly labeled (if at all) by RN1. Second, RN1 clearly labeled the fibers innervating the circular and longitudinal muscle, whereas these fibers were not labeled by the β-tubulin antibody (results not shown).

The results of double-labeling experiments using both neuropeptide antibodies were very similar. In short, all fibers that were labeled with the GFSKLYFamide and galanin antibodies were also labeled by RN1. However, the number of fibers labeled by RN1 greatly exceeded those labeled with the neuropeptide antibodies. For example, individual fibers could sometimes be detected originating within the lateral nerves projecting from the hyponeural nervous tissue, and some of these showed co-labeling with anti-GFSKLYFamide (Fig. 2c, d). However, the number of fibers in the nerve and within the circular muscle was much greater for RN1 than for the neuropeptide. Similarly, galanin fibers co-expressed RN1; however, other fibers expressing RN1 were not labeled with the peptide antibody (Fig. 2e, f)

Finally, double labeling with anti-Sp-SynB showed fibers to be co-labeled with RN1. However, once again, the number of fibers immunoreactive to RN1 was larger than those immunoreactive to synaptotagmin. This was clearly observed in the submucosal layer of the digestive tract, where large fibers were co-labeled with both antibodies but fine fibers were mainly labeled only with RN1 (Fig. 2g, h).

RN1 immunoreactivity in the body wall/dermis/epidermis

The holothurian body wall is formed mostly by a thick dermis that has few cells and varying amounts of connective tissue (see Hyman, 1955). At the outer edge the epidermis is thinner and there is a cuticle; toward the interior are radial nerves and layers of circular and longitudinal muscles. Immunoreactivity to RN1 was found throughout the dermis and epidermis in various types of fibers (Fig. 3). Large bundles of RN1-immunoreactive fibers were observed within the body wall, usually perpendicular to the epidermis (Fig. 3b). These bundles correspond to the podial nerve, a branch of the ectoneural nervous system that innervates the tube feet. However, the most extensive labeling corresponded to smaller fibers and fiber groups dispersed throughout the connective tissue (Fig. 3). In fact, the connective tissue of the body wall was characterized by having multiple fibers and occasional cell bodies labeled with RN1. Most of the nerve fibers appeared to be either isolated fibers or groups of few fibers (different from the fiber bundles that innervated the tube feet described above). Slight differences were observed in the amount, orientation, and thickness of the fibers associated with different dermis layers or connective tissue types.

Isolated fibers and small bundles of fibers could be found within the epidermis (Fig. 3b). The direction of the fibers was not well defined, although many of the small bundles ran parallel to the external edge, and many of the isolated fibers could be observed to reach apically to the cuticle-like structure. No cell bodies could be identified, suggesting that these fibers originate from perikarya found elsewhere.

The outer layer of the dermis, adjacent to the epidermis, was characterized by the presence of pigmented cells dispersed throughout the connective tissue. Pigment-containing cells were not labeled by the RN1 antibody. In this layer there were many individual fibers and small bundles of RN1-labeled fibers (Fig. 3c). The fibers did not appear to have a definite orientation, and many of them branched. Immunoreactive isolated cells 7.5 ± 0.8 μm in length and 4.3 ± 0.6 μm in width (mean ± SD, $n = 10$) were observed. The cells were dispersed throughout the connective tissue but not associated with particular structures or localizations. One to three fibers originated from each cell. Toward the epidermis, the number of immunoreactive cells and fibers appeared to decrease, and many fibers were seen oriented perpendicular to the epidermis and then bordering the dermis-epidermis boundary.

In the inner layer of the dermis, the orientation of fibers followed that of the circular muscle that lies beneath it (Fig. 3d). Both individual fibers and some small bundles were observed. As in the other areas of dermal connective tissue, isolated cells with fibers originating from them were also labeled.

In double-labeling experiments, GFSKLYFamide-containing fibers were rarely seen within the dermal tissues; when found, GFSKLYFa immunoreactivity was associated with the large fiber bundles that were also immunoreactive to RN1 (Fig. 4). GFSKLYFa immunoreactivity was never observed in the individual fibers within the connective tissue that were labeled by RN1. Similarly, galanin-like immunoreactivity was also restricted to the larger fiber bundles and was not observed in the small fiber or individual fibers (data not shown). Moreover, a previously identified group of cells that express galanin-like immunoreactivity in the body wall (see Díaz-Miranda *et al.,* 1996) was not labeled

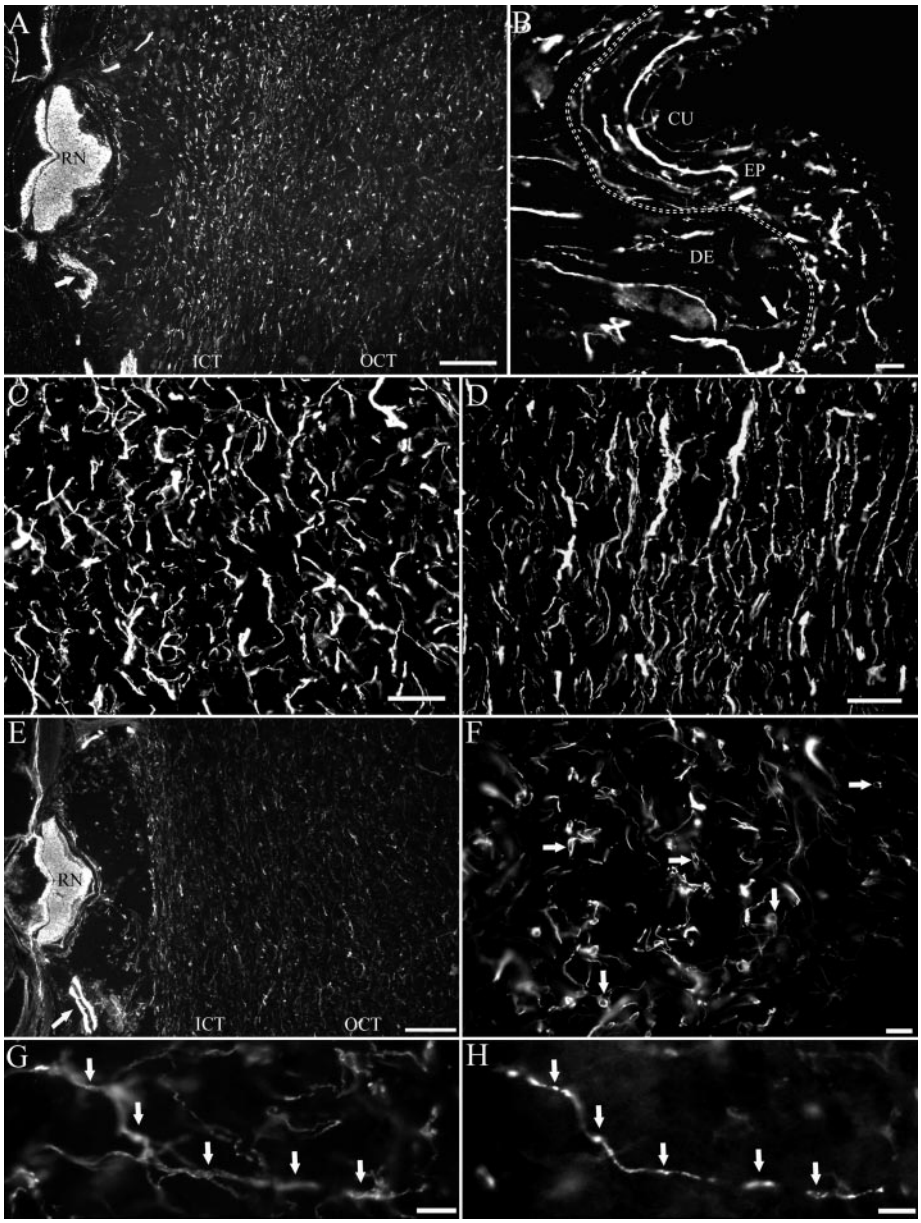

Figure 3. Transverse sections of the radial nerve cord and body wall of the sea cucumber. (A) Low magnification of the body wall, showing RN1 immunoreactivity in the radial nerve and the connective tissue plexus. Peripheral nerves (arrow) that arise from the radial nerve can be observed. (B) At a higher magnification, the distribution of RN1-immunopositive fibers and cells (arrow) in the dermis and epidermis is observed. (C) RN1-immunopositive fibers in the outer connective tissue layer of the dermis show a less organized orientation (D) The RN-immunopositive nerve fibers in the internal connective tissue of the dermis can be seen to have a parallel orientation. (E) Section similar to (A) of the body wall, showing anti-β-tubulin immunoreactivity in the radial nerve and the connective tissue plexus. Peripheral nerves (arrow) that arise from the radial nerve can be observed. (F) The distribution of anti-β-tubulin-immunopositive fibers and cells (arrows) in the outer dermis at a higher magnification. (G, H) Double labeling of RN1 (G) and Sp-SynB (H) in fibers within the outer dermis. A small percentage of the RN1-labeled nerve fibers (arrows) are co-labeled with the anti-Sp-SynB. Scale bar = 180 μm in A and E; 30 μm in C and D; and 15 μm in B, F, G, and H. CU, cuticle; DE, dermis; EP, epidermis; ICT, inner connective tissue; OCT, outer connective tissue; RN, radial nerve.

by RN1 (data not shown). The former is made up of neurosecretory-type cells with short, thick projections and is limited to the outer part of the dermis. In contrast, RN1 immunoreactivity was found in longer, thinner fibers and was present throughout the connective tissue of the dermis.

Other neuronal markers did label some of the fine fibers

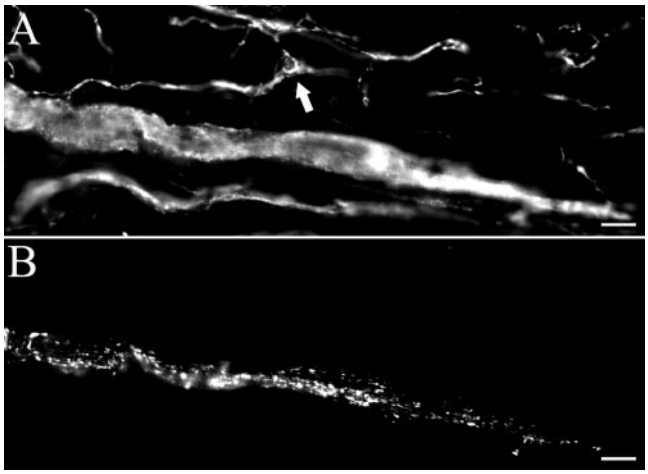

Figure 4. Transverse sections of the body wall of the sea cucumber showing double labeling of RN1 and GFSKLYFa immunoreactivity in nerve fibers within the dermal connective tissue. (A) RN1 labeling is found in large fiber bundles as well as in smaller isolated fibers that seem to connect to the large bundles. (B) In contrast, GFSKLYFa immunoreactivity is found in only a small percentage of the nerve fibers within the large fiber bundle, and none of the small individual fibers that lie outside the bundle are labeled. Similarly, the neuron-like cell within the connective tissue that is shown to be labeled by RN1 in A (arrow) does not express GFSKLYFa immunoreactivity. Scale bar = 15 μm in A and B.

within the dermal tissues of the body wall. This included the β-tubulin marker, which labeled cells and fibers whose localization and morphology are similar to those labeled by RN1 (Fig. 3e, f). In addition, the antibody against synaptotagmin labeled at least some of the RN1-labeled fibers (Fig. 3g, h). Double labeling with both antibodies showed that about one-third of the RN1 fiber population within the outer dermis was labeled by anti-Sp-SynB.

RN1 immunoreactivity in the connective tissues in other organs

The extensive labeling of RN1 in the connective tissue of the dermis and epidermis prompted us to investigate whether the connective tissues of other organs were similarly innervated by RN1-labeled fibers.

In the intestinal tissue, RN1 labeled previously known nervous structures (see García-Arrarás *et al.,* 2001), mainly a large fiber plexus in the mesothelium and a fiber plexus in the internal connective tissue, or submucosa (Fig. 5). In the internal connective tissue, immunolabeling was found in a large network of fibers and many small cells (Fig. 5a, b). The presence of fibers within the connective tissue diminished as one moved toward the luminal epithelium, but a smaller, though distinct, fiber layer was seen at the boundary between the connective tissue and the luminal epithelium (Fig. 5a, b). No RN1 immunoreactive fibers were observed within the luminal epithelium layer. The neurosecretory cells in the luminal epithelium that are recognized by anti-GFSKLYFa were not labeled by RN1 (Fig. 5b, c), as demonstrated by double labeling; however, most anti-GFSKLYFa immunopositive fibers in the connective tissue layer were also labeled by RN1 (Fig. 5d, e). It is in whole mounts that the connective tissue plexus is best observed (Fig. 6). Small cells of various morphologies (oval, rounded, and triangular), but all showing a large nucleus, could be observed to be interconnected in a fiber meshwork. Two or three thin varicose fibers projected from each neuronal body. Isolated immunoreactive cells were observed occasionally within the mesentery plexus (see Fig. 9g), and

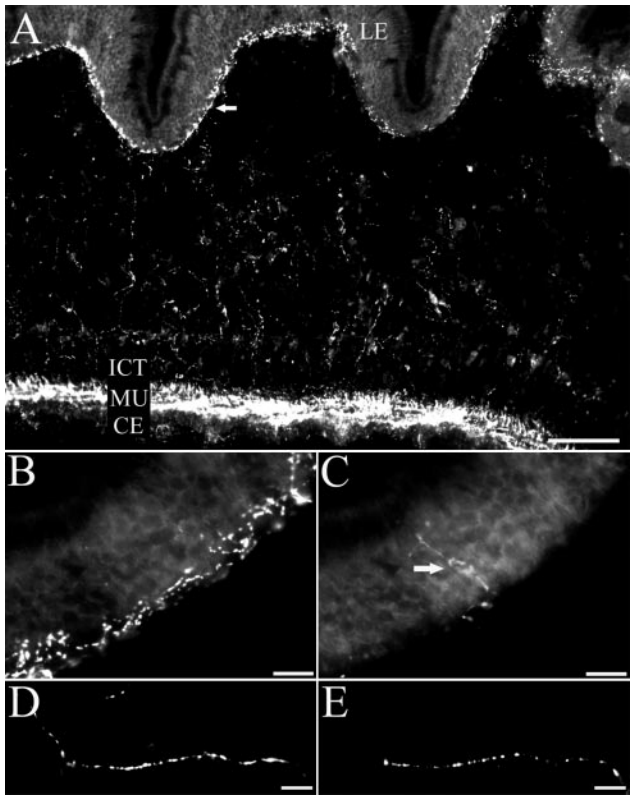

Figure 5. Transverse sections of the posterior, or large, intestine of the sea cucumber, showing the distribution of RN1 immunoreactivity in the digestive system. (A) At a low magnification, RN1 immunoreactivity can be seen associated with the coelomic epithelium and muscle layers and in the internal connective tissue plexus. Although most of the connective tissue plexus consists of a network of loosely interconnected fibers, a thin but neatly organized plexus adjacent to the luminal epithelium basement membrane can be observed (arrow). (B, C) Double labeling with RN1 (B) and anti-GFSKLYFa (C) shows that most RN1 fibers within the inner connective tissue do not show neuropeptide immunoreactivity. In particular, the nerve plexus that lies underneath the luminal epithelium is not labeled with the GFSKLYFa antibody. In contrast, the endocrine-like cells (arrow) within the luminal epithelium labeled show imunoreactivity to GFSKLYFa but not to RN1. (D, E) Double labeling with RN1 (D) and anti-GFSKLYFa (E) shows that some nerve fiber located in the connective tissue plexi express both markers. CE, coelomic epithelium; ICT, inner connective tissue; LE, luminal epithelium; MU, muscle. Scale bar = 30 μm in A; 15 μm in B, C, D, and E.

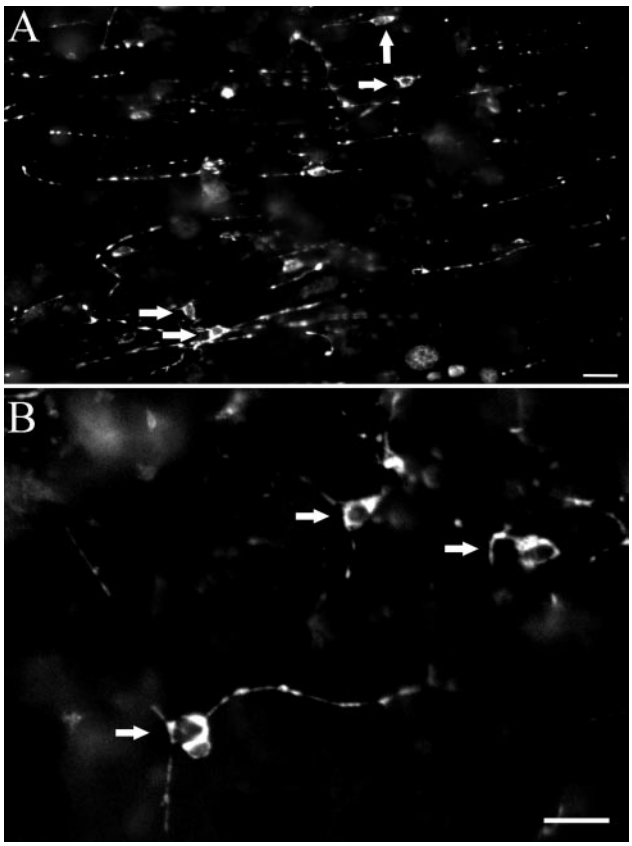

Figure 6. Whole mounts of the posterior intestine of the sea cucumber, showing immunoreactivity to RN1. (A) The RN1-immunoreactive nervous plexus within the internal connective tissue is shown to be made of small cells (arrows) interconnected by thin varicose fibers. (B) A different whole mount at a higher magnification shows three neuron-like cells (arrows) immunoreactive to RN1. Scale bar = 30 μm in A; 15 μm in B.

RN1-immunoreactive fibers were also observed within the mesentery connective tissue.

The podial nerve of the tube feet and the nerve plexus within the connective tissue, adjacent to the mesothelium, were labeled by RN1. In addition, an extensive RN1-labeled immunoreactive plexus was found within the connective tissue (Fig. 7a). This plexus formed a mesh-like structure in which neurons could be seen to be interconnected by a fiber meshwork. Most of these cells had a bipolar or pyramidal morphology and measured 10 ± 2 m in length and 5 ± 1 μm in width (mean ± SD, n = 10) (see Fig. 9c, d). Although some fibers in the main nerve were immunoreactive to GFSKLYFamide, neither the neurons nor fibers in the connective tissue plexus were immunoreactive to GFSKLYFamide or galanin (not shown). Nonetheless, other neuronal markers did label the fiber plexus within the connective tissue. As in the body wall dermis, β-tubulin labeling showed a pattern of cell and fiber distribution similar to that of RN1 (Fig. 7c). In addition, anti-Sp-SynB labeled a subpopulation of fibers within the connective tissue (Fig. 7e, f). RN1 labeling in the tentacles followed a pattern very similar to that found in the tube feet (Fig. 7b, d), although the fiber plexus in the outer connective tissue of the tentacle appeared to have fewer cells than in the tube foot.

In the respiratory tree, labeling with RN1 antibody showed a prominent nerve plexus in the connective tissue (Fig. 8a, b). Immunoreactive cells were also observed, and some fibers were apparently associated with the muscle layer below the coelomic epithelium. Cells were small and isolated, rounded, oval, or triangular, with one or a few fibers originating from them.

RN1 labeling of fibers within the small internal connective tissue layer of the hemal system was intense (Fig. 8c, d). A few small round cells were observed among the outer layer of epithelial cells and appeared to send projections into the connective tissue layer.

A strongly labeled fiber plexus was observed within the connective tissue layer of the female gonad (Fig. 8e, f). Large bundles of fibers appeared to run longitudinally, while narrower fibers were interspersed among the large bundles. Cells similar in morphology to those found in the respiratory tree were found within the connective tissue. Fibers from the connective tissue layer were sometimes observed to enter the germinal epithelium and surround some of the forming oocytes.

Plexus heterogeneity

When cell types were compared, it became obvious that different kinds of connective tissue plexi were present. A prominent type was the one found in the dermis. These cells were elongated, usually Y-shaped, and gave rise to rather thick projections that formed the plexus fibers (Fig. 9a). Their nuclei were medium in size, round, and usually within the center of the cell. The plexus fibers were usually thick fibers that labeled homogenously with the RN1 antibody. Similar types of fibers were found within the connective tissue plexus of the tube feet and tentacle (Fig. 9b–d). On the other hand, cells in the intestinal nerve plexus were smaller and rounder, and the nerve fibers were much thinner and varicose (Fig. 9f). The plexi found in other connective tissues such as the gonads and respiratory tree lay somewhere in between: the cells were larger than the ones in the dermis but not as elongated, and fibers were thinner but not as thin as those in the digestive tract (Fig. 9e).

Immunoreactive neuron-like somata were also observed in various tissues that are not necessarily associated with traditional connective tissue layers. These cell bodies were mainly observed in the coelomic epithelia, or mesothelium, of the body wall and viscera, including that of the longitudinal muscle, the tube feet lumen, and the intestinal mesentery (Fig. 9g–i). These cells were usually small, 7 ± 1 μm in length and 4 ± 1 μm in width (mean ± SD, n = 10), and few in number. They were either unipolar or bipolar and

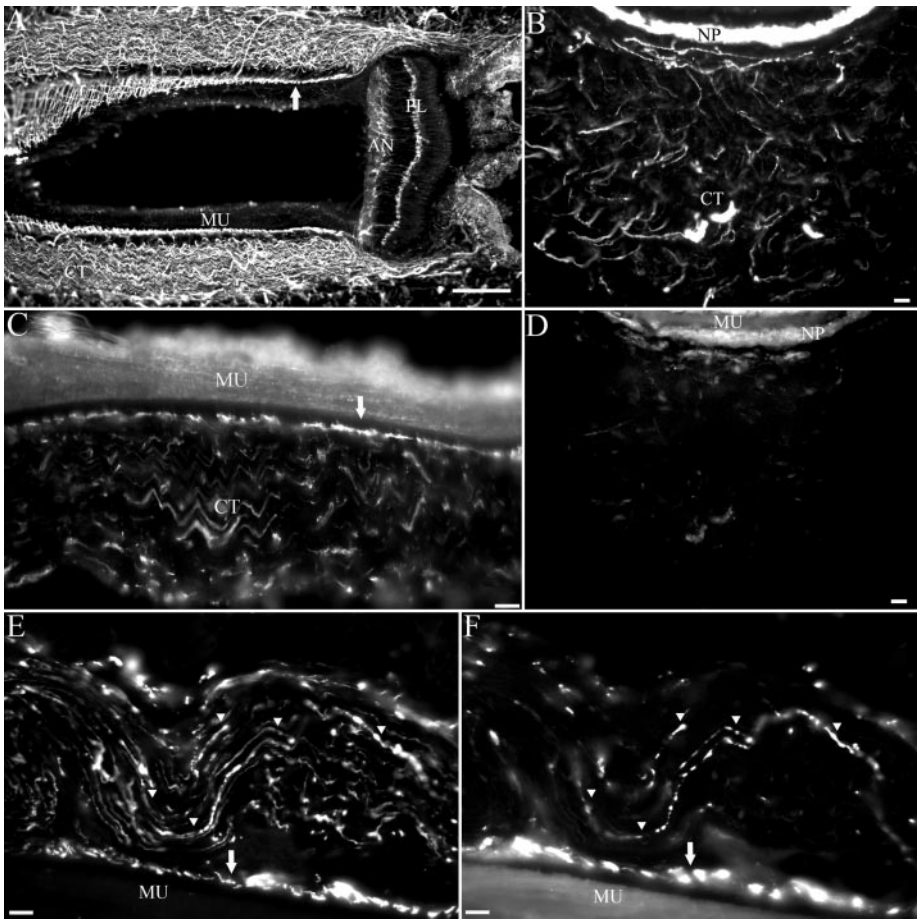

Figure 7. RN1 immunoreactivity in the tube feet and tentacles of the sea cucumber. (A) Longitudinal section of a tube foot shows the labeling of the nerve plexus (arrow) adjacent to the muscle and of the apical nerve and nerve plate at the tip of the tube foot. However, the most prominent labeling is found in the fiber plexus within the connective tissue of the tube foot. (C) A high magnification of a longitudinal section of the tube foot, showing the immunoreactivity of β-tubulin in the nerve plexus (arrow) adjacent to the muscle and in the connective tissue. (B, D) Similar findings are observed in transverse sections of the tentacle of the sea cucumber, where RN1 (B) labels fibers within the main nerve plexus adjacent to the muscle layer and fibers in the connective tissue plexus, but GFSKLYFa (D) shows immunoreactivity only in the former. (E, F) Double labeling of RN1 (E) and Sp-SynB (F) in the main podial nerve (arrow) and in fibers within the tube foot connective tissue (arrowhead). A small percentage of the RN1-labeled nerve fibers (arrowhead) are co-labeled with the anti-Sp-SynB. Scale bar = 180 μm in A; 30 μm in B and D; and 15 μm in C, E, and F. AN, apical nerve; CT, connective tissue; MU, muscle; NP, nerve plexus; PL, neural plate.

extended fibers that entered the underlying tissues that are mainly myoepithelia or muscle tissue.

Discussion

Neuronal specificity-antibody characterization

There is strong evidence to support the neuronal specificity of RN1 labeling. First, it labels all structures previously described as being components of the nervous system in holothurians. This includes the ectoneural and hyponeural components of the radial nerves, which are the major divisions of the echinoderm nervous system (Hyman, 1955; Cobb, 1987), as well as other well-known structures such as the tube feet and the tentacular nerves and major lateral nerves originating from the radial nerve cords and found within dermal and muscular structures. Moreover, RN1 also labels previously described nervous structures within the enteric nervous system, which is one of the best-described nervous systems in the holothuroids (García-Arrarás *et al.,* 1999, 2001). RN1 also labeled all neuronal structures previously shown to be labeled by GFSKLYFamide (Díaz-Miranda *et al.,* 1995) and galanin (Díaz-Miranda *et al.,* 1996) in the radial nerve of *H. glaberrima* or by antibodies against the neuropeptide NGIWYa in the radial nerve of *Apostichopus japonicus* (Inoue *et al.,* 1999). It remains troublesome that RN1 did not produce a strong labeling of

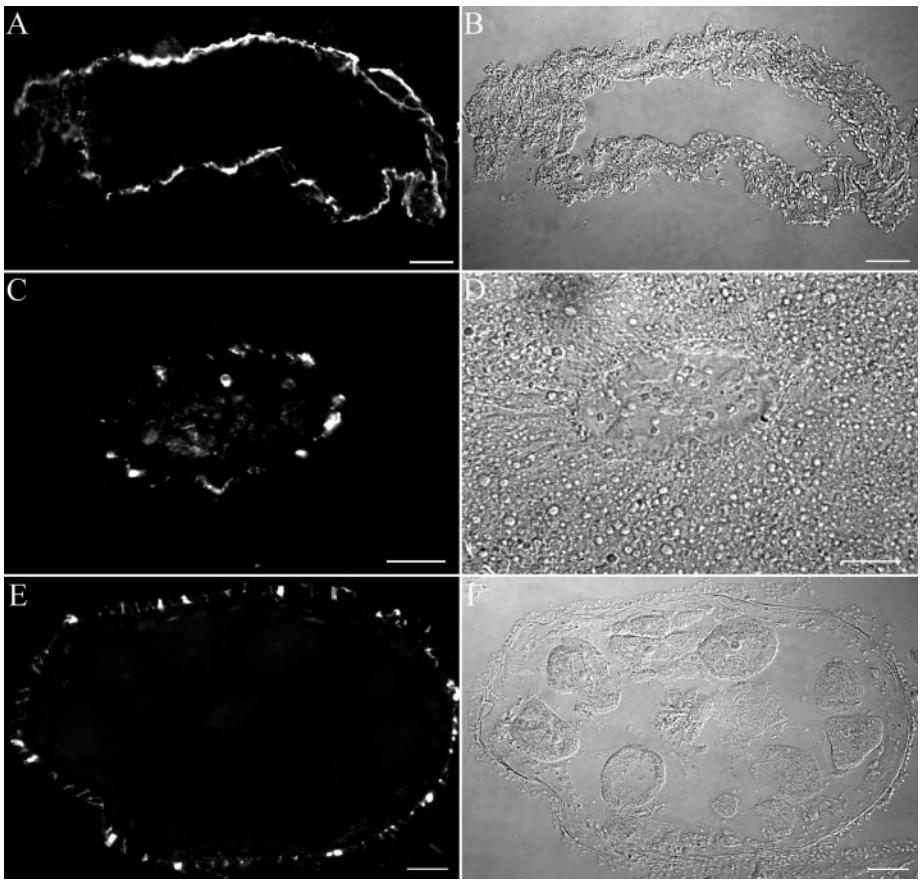

Figure 8. RN1 immunoreactivity in various organs of the sea cucumber. (A, C, E) Transverse sections of the respiratory tree (A), the hemal system (C), and a female gonad (E), showing immunoreactivity to RN1. The same tissue sections are shown in phase contrast in B, D, F. In all tissues, RN1 immunoreactivity is mainly associated with a nerve plexus in the connective tissue compartment. Scale bar = 30 μm in A, B, E, and F; 15 μm in C and D.

the neuronal soma in the periphery of the radial nerves nor in the intestinal coelomic epithelia, although it did label neuronal bodies within the peripheral tissues. In some cases the intense labeling of the plexus made it difficult or impossible to clearly identify nerve cells that were embedded within the plexus. In addition, a recent paper by Mashanov and colleagues (2006) describes as glial cells most of the cell bodies in the periphery of the radial nerves of *Eupentacta fraudatrix.* This could explain why these cells were not labeled by RN1. An alternative explanation could be that, as occurs with other markers, the RN1 may be distributed preferentially to the axon or dendrites but absent from the cell soma (Mandell and Banker, 1995; Peng *et al.,* 1986). A final possibility is that RN1 labels only a subdivision of the holothurian nervous system, albeit a large one in view of the dense labeling found in the radial nerves.

The second, and major, piece of evidence to show that RN1 is specifically labeling nervous tissues comes from the comparison with other neuronal markers. Comparisons to the labeling produced by a commercial β-tubulin antibody show extensive similarities between RN1 and β-tubulin labeling. The β-tubulin antibody was made against purified rat brain tubulin (Sigma product # T-4026) and has been shown to recognize a tubulin isoform (Gozes and Barnstable, 1982; Matthes *et al.,* 1988). Although double labeling with RN1 was not possible, the fact that the β-tubulin antibody also recognized extensive fiber plexi in connective tissue compartments strongly supports the neuronal nature of the RN1 epitope. Particularly strong evidence is the observation that cells and fibers similar to those observed with RN1 were found within the body wall dermis and the connective tissues of the tube feet.

Double labeling results also demonstrate the neuronal nature of RN1 labeling. All fibers labeled by antibodies to GFSKLYFamide, galanin, and synaptotagmin were labeled by RN1. The fact that the labeling by RN1 is much more extensive is to be expected. The neuropeptides are known to label only a subpopulation of cells and fibers (Díaz-Miranda

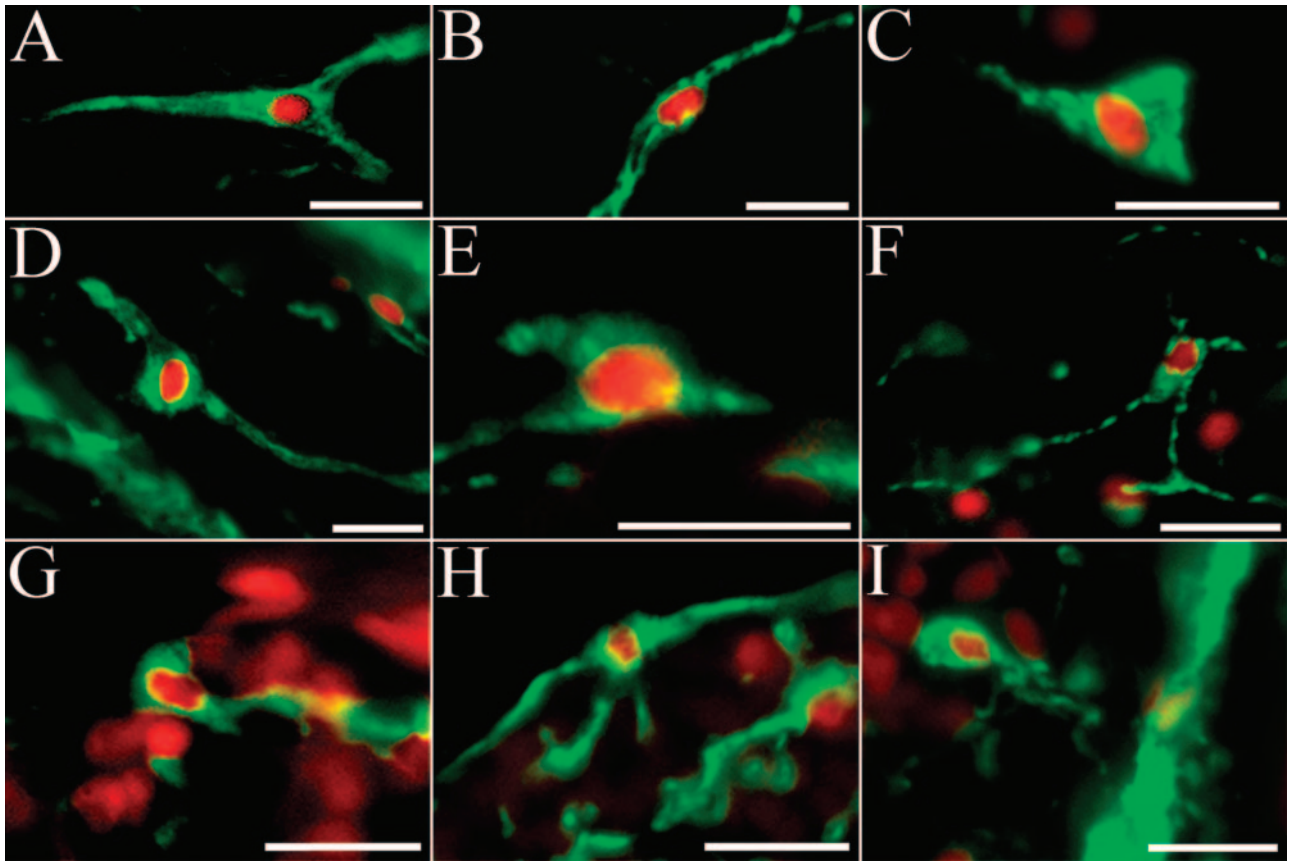

Figure 9. RN1 labeling of neuron-like cells. (A–F) Immunolabeling with RN1 (green) and nuclei labeling with Hoescht dye (red) was used to identify neuron-like cells in the connective tissue compartments of the body wall dermis (A), tentacle (B), tube feet (C, D), female gonad (E), and large intestine (F). (G–I) RN1-labeled isolated cells with neuron-like morphologies were also found in the coelomic epithelia, or mesothelia, of the intestinal mesentery (G), longitudinal body wall muscles (H), and body wall (I). Scale bar = 15 μm in A–I.

et al., 1995, 1996); thus we could expect that RN1 labels not only this population but also other fibers not labeled by the neuropeptide antibodies. A similar result is found with anti-Sp-SynB. In that case, the studies of Burke and colleagues (2006) found at least two synaptotagmin isoforms in the sea urchin genome. Thus different populations or divisions of the echinoderm nervous system may express one of the two synaptotagmin isoforms, whereas RN1 is labeling the neuronal fibers that express both forms.

Although RN1 appears to label a larger population of nerve fibers than other markers, its labeling does not extend to endocrine cells. Our results showed that RN1 did not label the neuroendocrine cells in the intestinal luminal epithelium that express GFSKLYFa immunoreactivity (Díaz-Miranda *et al.,* 1995), nor did it label a cell type within the body wall that is recognized by anti-galanin antisera and has been previously described as endocrine-like (Díaz-Miranda *et al.,* 1996). These results further suggest that the epitope labeled by RN1 is specific to neuronal fibers.

Western blots showed that the molecules recognized by RN1 have a molecular weight of ~66 kDa and ~53 kDa while that of β-tubulin is closer to 56 kDa. Nonetheless, the possibility that RN1 is labeling other tubulin isoforms cannot be dismissed. The molecular weight of the protein recognized by RN1 is similar to the mammalian low-molecular neurofilament subunit that ranges from 60 to 70 kDa in weight, as well as to neurofilament subunits found in squid (Szaro *et al.,* 1991; Grant *et al.,* 1995). In addition, the presence of specific neurofilament isoforms in different cellular compartments, such as has been found in the axon and cell bodies of *Aplysia* (Drake and Lasek, 1984), could be responsible for the lack of labeling in some of the neuronal somas.

The molecule recognized by RN1 could be a non-cytoskeletal protein associated with motor processes or vesicles. Evidence for this possibility comes from the punctuated staining pattern observed in some tissues (particularly the intestine connective tissue plexus) and from the fact that

the monoclonal antibody against synaptotagmin (a vesicle-associated protein) was obtained using similar extraction procedures (Burke *et al.,* 2006). Experiments are in progress to fully characterize the molecules identified by RN1.

Connective tissue nervous system

The connective tissue plexi are probably the least known and studied of the echinoderm nervous system. Hyman (1955) described the presence in the dermis of a general nerve plexus that is continuous with the radial nerves. However, the fibers she described probably correspond to the larger nerve bundles in which we observed immunoreactivity to the neuropeptides, and some of which might even correspond to the podial nerves. Similarly, Serra von Buddenbrock (1963) found nervous elements within the holothurian dermis connective tissue and proposed that they influence connective tissue stiffening. However, she provided only a scant description of the nervous system component, and no photographs or figures. More recently, Smiley (1994), in an extensive review on the microscopic anatomy of holothurians, provided little information on the cell and fiber types within the connective tissue compartments.

This lack of information is primarily due to the fact that the neuronal markers used to identify other nervous components do not recognize cells or fibers in the connective tissue plexi. For example, catecholaminegic neurons and fibers have been described within various tissues of the echinoderms, but mainly restricted to the ectoneural nervous system component (Cobb, 1969; Cottrell and Pentreath, 1970). Gamma aminobutyric acid (GABA) immunoreactivity has also been localized to components of the radial nerves and the tube feet nerves, and to the basiepithelial nerve plexus of the digestive tract, but no expression was observed in connective tissue compartments (Newman and Thorndyke, 1994). Similarly, antibodies to various neuropeptides—SALMFamides (Moore and Thorndyke, 1993), GFSKLYFa (Díaz-Miranda *et al.,* 1995), NGIWYamide (Inoue *et al.,* 1999), and galanin (Díaz-Miranda *et al.,* 1996)—label the radial nerves and the tube feet nerves extensively. However, other than fiber bundles traveling through the connective tissue compartments, they do not recognize the cells or fibers forming the connective tissue plexi. This is particularly surprising for NGIWYa, a peptide that was isolated from the dermal tissue of *Apostichopus japonicus* and shown to cause stiffening of the body wall dermis of *A. japonicus* and *Holothuria leucospilota* (Birenheide *et al.,* 1998). Immunoreactivity to this peptide was observed within the dermal connective tissues, but it is apparently restricted to some of the larger nerve bundles (Inoue *et al.,* 1999) and labels neither the smaller fibers nor the cells that we find to constitute the main connective tissue plexi. Similarly, within the digestive tract, most of the labeling associated with antibodies against GFSKLYF-amide, NGIWYa, or galanin is found within the mesothelium, and only occasional fibers are observed in the connective tissue.

In fact, the only description of the connective tissue plexi in the digestive tract was done by our group (García-Arrarás *et al.,* 1999, 2001), using another monoclonal antibody (F6) that labeled some very specific cell and fiber populations. These same small cells and fiber plexi are recognized by RN1; however, although F6 is specific to this plexi, RN1 appears to label a larger population of fibers within the digestive tract and elsewhere.

At present, the description of a plexus within the connective tissue compartments has been mainly limited to ultrastructural analyses, most of which make only passing mention of some nerve fiber or similar structure. For example, Kawaguti (1966) studied the body wall of the sea cucumber *Stichopus japonicus* and described some fine nerve fibers in the epidermis but claimed they were too few and too irregular to be called a nerve plexus. Similarly, Byrne (2001) described the presence of bundles of axons and neurosecretory-like processes within the introvert dermis of the sea cucumber *Eupentacta quinquesemita.* These structures contained many different types of vesicles, suggesting a large variation of fiber types. In addition, she described axons containing large dense vesicles in the intestinal connective tissue.

The best description of neuron-like cells within the connective tissue was done by Wilkie (1979), who identified what he called juxtaligamental cells within the connective tissue of brittle stars. In these organisms, cell bodies are found between ligaments and ossicles, and fibers extend and branch in the ligaments (Heinzeller and Welsch, 1994). Juxtaligamental cells have long, varicose, axon-like processes that lie within the collagen fibers. They appear to mediate the changes in stiffness of ligaments and tendons (Wilkie, 1979, 2002). Similar types of cells have been found to be associated with cirral ligaments in crinoids (Holland and Grimmer, 1981) and with changes in body wall stiffness in holothurians (Koob *et al.,* 1999). These cells contain different types of vesicles that can be characterized in terms of size, morphology, and electron density (Koob *et al.,* 1999; Wilkie, 2002).

For sea cucumbers, two reports describe a nerve plexus within the connective tissue of the tube feet—one report for *Holothuria forskali* (Flammang and Jangoux, 1992) and one for *Parastichopus californicus* (Cavey, 2006). Both reports show that this nerve plexus lies within the connective tissue compartment, runs adjacent to the basal membrane of the mesothelium, and sends multiple small lateral nerves that traverse the connective tissue toward the epidermis. These fibers that extend into the connective tissue have large electron-dense granules (Cavey, 2006) similar to those previously described for the juxtaligamental cells. The exten-

sive plexus found within the tube feet connective tissue certainly underscores the possibility that it plays an important role in holothurian physiology associated with changes in connective tissue mechanical properties. Therefore, the nervous plexi of the connective tissue compartments of echinoderms, although acknowledged by some authors, have been poorly described. In view of our results it is evident that the abundance of cells and fibers within these tissue plexi has been mostly understated.

Mutable connective tissue

The presence of juxtaligamental cells (and in particular of cells containing large electron-dense vesicles) has been closely associated with the mutable connective tissue of echinoderms (Cobb, 1988; Wilkie *et al.,* 2004). Examples of connective tissue mutability include the ability of a sea urchin to lock its spines in a particular position and the ability of a sea cucumber to stiffen its body wall (Cobb, 1989). More recently, mutable collagenous connective tissue has been described, together with the presence of juxtaligamental cells, in the tube feet of sea stars and sea urchins (Santos *et al.,* 2005). Since the changes in connective tissue viscosity are thought to be under nervous system control and RN1 labels neuronal cells and fibers within the connective tissue, we propose that RN1 is indeed recognizing the neuronal substrate responsible for mediating the changes in the mutable connective tissue. This conclusion is strengthened by the similarity in size and morphology between our cells and the previously reported juxtaligamental cells and by reports from Motokawa (1988) in which he clearly states that this cell type is the only one found abundantly and ubiquitously in the connective tissues of echinoderms. Moreover, juxtaligamental cells are thought to respond to cholinergic signals (Wilkie, 2002) that are known to modulate the connective tissue viscosity (Motokawa, 1987). Our results also show nerve fiber bundles of various sizes within the body wall connective tissue, the larger ones originating from the ectoneural compartment of the radial nerve. This suggests that these nerves branch within the connective tissue, providing the circuitry for connections to the connective tissue plexi. However, in the absence of direct evidence linking the RN1-labeled cells with the juxtaligamental cells, there are two possibilities: (1) the juxtaligamental cells, which have been identified as being neurosecretory, are labeled by RN1; or (2) only neuronal cells are labeled by RN1, and these are different from the juxtaligamental cells.

Finally, it is our hope that RN1 will serve to better characterize the nervous system of the Echinodermata, as well as to identify its previously unknown components. Moreover, in view of the phylogenetic location of echinoderms, we expect that the use of this antibody will aid in studies of nervous system development and evolution.

Acknowledgments

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