Stichopin-containing nerves and secretory cells specific to connective tissues of the sea cucumber

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Stichopin, a 17-amino acid peptide isolated from a sea cucumber, affects the stiffness change of the body-wall catch connective tissues and the contraction of the body-wall muscles. The localization of stichopin in sea cucumbers was studied by indirect immunohistochemistry using antiserum against stichopin. Double staining was performed with both stichopin antiserum and 1E11, the monoclonal antibody specific to echinoderm nerves. A stichopin-like immunoreactivity (stichopin-LI) was exclusively found in the connective tissues of various organs. Many fibres and cells with processes were stained by both the anti-stichopin antibody and 1E11. They were found in the body-wall dermis and the connective tissue layer of the cloacae and were suggested to be connective tissue-specific nerves. Oval cells with stichopin-LI (OCS) without processes were found in the body-wall dermis, the connective tissue sheath of the longitudinal body-wall muscles, the connective tissue layer of the tube feet and tentacles, and the connective tissue in the radial nerves separating the ectoneural part from the hyponeural part. Electron microscopic observations of the OCSs in the radial nerves showed that they were secretory cells. The OCSs were located either near the well-defined neural structures or near the water-filled cavities, such as the epineural sinus and the canals of the tube feet. The location near the water-filled cavities might suggest that stichopin was secreted into these cavities to function as a hormone.

Keywords: bioactive peptide; stichopin; catch connective tissue; sea cucumber; echinoderm

1. INTRODUCTION

Regulatory peptides are signal molecules that are widely distributed in the animal kingdom. In echinoderms, several bioactive peptides have been isolated (Elphick *et al.* 1991; Díaz-Miranda *et al.* 1992; Iwakoshi *et al.* 1995), and their presence has also been predicted from genomic sequencing data (Elphick & Thorndyke 2005; Burke *et al.* 2006b). However, there are only a few detailed studies on their action or localization. The peptides whose localization or action that have been well investigated are SALMFamide 1 and SALMFamide 2 from the starfish *Asterias rubens* (Elphick *et al.* 1991), GFSKLYFamide from the sea cucumber *Holothuria glaberrima* (Díaz-Miranda *et al.* 1992), and stichopin, the NGIYWamide, holokinin 1 and holokinin 2 from the sea cucumber *Apostichopus japonicus* (Iwakoshi *et al.* 1995).

This paper focuses on the distribution of peptides in the connective tissues of sea cucumbers. Echinoderms have a unique connective tissue, called the catch or mutable connective tissue, which changes its mechanical properties under nervous control (Wilkie 1996). A detailed pharmacological study by Motokawa (1987) strongly suggested the cholinergic control of the catch connective tissue of the

holothurian body-wall dermis. We could find no examples of the neuronal control of connective tissues outside the phylum Echinodermata, and thus the following questions are worth studying. (i) What kind of nerves are involved in the control? (ii) Is there any nerve specific to connective tissues? (iii) Are hormones also involved in the control?

The peptidergic systems, besides cholinergic systems, also seem to control the stiffness of catch connective tissues because peptides from *A. japonicus* affected the stiffness of its dermis (Birenheide *et al.* 1998). Holokinin 1 and holokinin 2 soften whereas NGIYWamide stiffens the dermis; stichopin neither stiffens nor softens, but it inhibits acetylcholine-induced stiffening. These peptides also affect the contraction of the longitudinal muscle of the body wall (LMBW; Iwakoshi *et al.* 1995; Inoue *et al.* 1999). Among these peptides, the structure of stichopin and NGIYWamide is unique and no related peptide is reported from other animal phyla (Iwakoshi *et al.* 1995). The localization of the NGIYWamide has been demonstrated by immunohistochemistry (Inoue *et al.* 1999). NGIYWamide-like immunoreactivities were found in the radial nerve cord and the podial nerve, as well as in the fibres in the body-wall dermis. These results strongly suggested the presence of peptidergic nerves controlling the connective tissues.

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In this study, we raised antiserum against stichopin, which is another unique bioactive peptide composed of 17 amino acids. With the use of this antiserum and 1E11, the monoclonal antibody specific to the neurons of echinoderms (Nakajima *et al.* 2004b), we could provide evidence for the presence of connective tissue-specific nerves controlling the mechanical properties of the connective tissues. Our results also suggest that stichopin is a paracrine/endocrine modulator as well as a neuropeptide.

2. MATERIAL AND METHODS

(a) *Animals*

Specimens of the sea cucumber *Apostichopus japonicus* were collected near the Noto Marine Biological Station of the University of Kanazawa. The animals were shipped to the Tokyo Institute of Technology and maintained in an aquarium of closed circulating seawater at 18°C.

(b) *Production of stichopin antiserum*

Polyclonal antibodies recognizing stichopin were produced in a rabbit according to standard procedures. Briefly, the synthetic peptide stichopin was conjugated via dimethyl suberimidate (DMS; Pierce) to bovine thyroglobulin (B.ThG; Sigma). DMS is a homobifunctional imidoester cross-linker, which reacts with amino residues of a peptide and a carrier protein (Hand & Jencks 1962). The conjugate (100 µg peptide per animal) containing complete Freund's adjuvant (Difco) was initially injected into two New Zealand white rabbits and, thereafter, the conjugate (75 µg peptide per animal) together with incomplete Freund's adjuvant (Difco) was injected five times. During immunization, the antibody titre was monitored by an enzyme-linked immunosorbent assay. After the final boost, blood was collected from the ear vein for serum preparation. The serum was incubated overnight with 1 mg ml⁻¹ of B.ThG at 4°C and then centrifuged. The supernatant was passed through a membrane filter (Millipore—sterile, low protein-binding type, 0.45 µm filter unit) and 0.1% sodium azide was added. Aliquots of the filter were stored at -80°C.

(c) *Immunohistochemistry*

For the immunohistochemistry, frozen sections were used. Various organs were dissected from the sea cucumber and fixed in 4% paraformaldehyde in artificial seawater (Jamarine Laboratory, Japan) for 1 h at room temperature. After fixation, the tissues were washed three times with phosphate-buffered saline (PBS) and then immersed in acetone for 2 min at -20°C. After washing with PBS, the samples were cryoprotected with a graded series of sucrose (10–20%), and then frozen into OCT compound (Sakura Finetek, USA) using dry ice–acetone mixture. The frozen tissues were cut into 6–8 µm sections in a cryostat (Leica CM 1850, Germany) and mounted on glass slides. The sections were washed with PBS, blocked with 1% normal goat serum in PBS for 30 min and treated with a primary antibody.

The primary antibodies used were the one against stichopin (used at 1 : 2000) and the monoclonal antibody 1E11 (Nakajima *et al.* 2004a,b). The culture supernatant of the 1E11 hybridoma cells was used as the primary antibody without dilution. The sections were incubated overnight at 4°C with either one of the antibodies for single staining or the mixture of both antibodies for double staining. After washing

with PBS, the sections were incubated with the secondary antibody diluted with PBS for 1 h at room temperature.

For the indirect immunofluorescent labelling, the secondary antibodies used were the Cy2-conjugated anti-rabbit IgG (used at 1 : 50) for the anti-stichopin antibody and the Cy3-conjugated anti-mouse IgG (used at 1 : 100) for the 1E11. For double staining, the mixture of the two secondary antibodies was used. The sections were mounted in Fluoromount-G medium (Electron Microscopy Sciences, USA) under a cover-slip after the unbound secondary antibody was washed with PBS.

Some sections treated with the anti-stichopin antibody were incubated with peroxidase-conjugated anti-rabbit IgG (used at 1 : 1000) for the enzyme immunohistochemistry. To visualize the immunostaining products, True Blue (Kirkegaard & Perry Laboratories, USA) was used as the substrate for the peroxidase. The developing time was 3–10 min. The tissues were then dehydrated in a series of ethanol and mounted under a cover-slip.

The sections were observed and photographed under a light microscope (Nikon Labophoto2, Japan) with epifluorescent equipment (Nikon EFD3, Japan). The secondary antibodies used in this study were purchased from Jackson ImmunoResearch Laboratories, Inc., USA.

The pre-absorption controls were carried out to determine the specificity of the primary antiserum. The stichopin antiserum (diluted 1 : 2000) was incubated with 10⁻³ M stichopin overnight at 4°C. In another negative control, the treatment with a primary antibody was omitted from the procedure.

To study the histological organization of the tissue, some sections were stained with Milligan's trichrome (Humason 1979).

(d) *Transmission electron microscopy*

For observation using a transmission electron microscope, samples were fixed according to Matsuno & Motokawa (1992). The specimens were pre-fixed in a solution containing 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 90 min at room temperature. After rinsing with the buffer, these specimens were post-fixed with 1% OsO₄ in 0.1 M phosphate buffer (pH 7.3) for 120 min. The specimens were then dehydrated in a graded series of ethanol (70, 80, 90, 95 and 98%, and absolute), treated with glycidyl *n*-butyl ether and embedded in epoxy resin. The thin sections were stained with uranyl acetate followed by lead citrate and examined using a transmission electron microscope (JEM-100C; JEOL, Japan).

Semi-thin sections (1–2 µm) were cut, stained with 1% toluidine blue and examined under the light microscope.

3. RESULTS

The outer surface of the sea cucumber body wall is covered with an epidermis under which there is a thick dermal connective tissue layer (figure 1). The inner surface of the body wall is covered with a circular muscle layer except in each ambulacral region where a radial nerve cord, a radial water canal and a longitudinal muscle are present.

In both the indirect immunofluorescent labelling method and enzyme immunohistochemical method, the antiserum against stichopin stained the same types of cells

Figure 1. Diagrams of (a) a sagittal section of sea cucumber and (b) a cross section of the body wall containing a radial nerve cord. C, cloaca; CM, circular muscle; CN, circumoral nerve ring; D, body-wall dermis; DT, digestive tract; EN, ectoneural part of the radial nerve; ES, epineural sinus; HN, hyponeural part of the radial nerve; HS, hyponeural sinus; LMBW, longitudinal muscle of the body wall; PC, podial water canal; PN, podial nerve; R, respiratory tree; RC, radial water canal; RN, radial nerve; T, tentacle; TF, tube foot; TN, tentacular nerve.

(figures 2 and 3). In the pre-absorption controls, stichopin at 10^{-3} M mostly eliminated the immunoreactivity (figure 3). In the other negative control in which the treatment of the primary antibody was omitted, some tissues were stained, but the signal was much weaker than that of the sections treated with the primary antibody.

(a) Radial nerve cord and circumoral nerve ring

The main nervous system of sea cucumbers is a circumoral nerve ring and five radial nerve cords (figure 1). The circumoral nerve ring consists of the ectoneural part only. It was strongly labelled with 1E11, but showed no stichopin-like immunoreactivity (stichopin-LI).

The radial nerve cord is composed of a thicker ectoneural part and a thinner hyponeural part (figures 1b and 2a). They are separated by a thin layer of connective tissue that connects to the body-wall dermis at the lateral sides of the radial nerve cord. In the semi-thin sections, apparent connections between the ectoneural and hyponeural parts were sometimes found, which seem to correspond to the short neural bridges reported for other sea cucumbers (Mashanov et al. 2006).

A cluster of cells stained with toluidine blue was found in the connective tissue in the lateral margins of the radial nerve cord (figure 4a). In the cross section of $1\ \mu\text{m}$ thickness, there were up to 15 cells in each cluster. The cells are oval, or sometimes round, in shape and are approximately $10\text{--}15\ \mu\text{m}$ in diameter. Each cell contains vesicles stained with toluidine blue in the semi-thin sections. With Milligan's trichrome, these vesicles were not stained well, but the nuclei of the cells were stained red. Other types of cells were scarcely found in this region of the connective tissue. These cells were immunoreactive to the anti-stichopin antibody (figure 2b). Stichopin-LI was found neither in the ectoneural nor in the hyponeural part of the radial nerve cord, although they were strongly stained by 1E11 (figure 2a,b). Double staining with anti-stichopin antibody and 1E11 showed that most cells with stichopin-LI were labelled with 1E11 (double-headed arrow in figure 2a-c), but some were unlabelled (arrow-heads in figure 2b,c).

In the transmission electron microscopy, the stichopin-containing cell had a nucleus with an irregular shape and was packed with spherules of $2\text{--}5\ \mu\text{m}$ in diameter (figure 4b). The spherule consists of a matrix of intermediate electron density with several core-like structures of $200\text{--}400\ \text{nm}$ in diameter with a high electron density. The cell has no processes such as axons. No basal lamina surrounds the stichopin-containing cells, while we sometimes observed cellular processes surrounded by a basal lamina in the connective tissue layer. These processes may correspond to a neural bridge connecting the ectoneural and hyponeural parts of the radial nerve cord.

(b) Tube foot and tentacle

The tube feet on the ventral side of the sea cucumber were studied. The tube foot wall consists of, from the luminal side to the outside, a mesothelium, an inner connective tissue layer, nervous tissue, an outer connective tissue layer and the epidermis (Flammang 1996). The nervous tissue consists of a podial nerve and a nerve plexus. The nerve plexus forms a cylindrical meshwork and a podial nerve runs longitudinally on one side of the cylinder. Both the nerve plexus and the podial nerve were stained strongly by 1E11 (figure 2d), but not by anti-stichopin antibody. The 1E11-positive fine fibres extend from the nerve plexus towards the outer connective tissue (figure 2d). Cells with stichopin-LI were found in the outer connective tissue very close to the nerve plexus (figure 2e). These cells were oval in shape and not labelled by 1E11 (figure 2e).

The tissue organization in the wall of the tentacles was the same as that of the tube foot. The nervous tissue of the tentacle was labelled with 1E11, but not with the anti-stichopin antibody. Oval cells with stichopin-LI (OCS), but negative to 1E11, were found in the inner connective tissue layer.

(c) Longitudinal body-wall muscle

In the LMBW, many fibrous structures were stained by 1E11, but very little stichopin-LI was found. LMBW is covered with a connective tissue sheath that is thicker in the region close to the mesentery connected to the body wall. This sheath, especially the thick region, contained many clusters of OCS, but without immunoreactivity to the 1E11 (figure 2f).

Figure 2. (Caption opposite.)

(d) *Body-wall dermis*

We observed the dorsal dermis. Many fibres and some cell bodies in the dermis were labelled with 1E11 (figure 2*g,j*). Double staining revealed that some of these fibres and cell bodies were also stained by the anti-stichopin antibody (figure 2*g-l*). Some cells immunoreactive to both antibodies have a process (double arrowhead in figure 2*i*).

The fibres and cell bodies showing stichopin-LI were always stained by 1E11 in the general dermis. However,

the cells near the water canal were exceptions. The dorsal dermis contains water canals of papillae. Some oval cells near the canals with stichopin-LI were not labelled by 1E11 (arrowheads in figure 2*k,l*). The podial nerve and the nerve plexus of the dorsal papillae were stained by 1E11, but not by the anti-stichopin antibody (arrow in figure 2*j,l*) as in the tube feet in the ventral dermis.

Oval or elongated oval cells were found in the dermis close to the epineural sinus (ES) of the radial nerve cord.

Figure 2. (*Opposite.*) Immunofluorescence against 1E11 (red) and anti-stichopin antibody (green). (*a–c*) Cross section of the radial nerve cord. HS, hyperneural sinus. Immunoreactivities to (*a*) 1E11 and (*b*) anti-stichopin antibody, and (*c*) a merged image of (*a*) and (*b*). Ectoneural (EN) and hyponeural (HN) parts showed no reactivity to the anti-stichopin antibody. The connective tissue (CT) separating EN and HN parts contained oval cells reactive to both the anti-stichopin antibody and 1E11 (double-headed arrow) and those reactive to the anti-stichopin antibody alone (single arrowhead). In the dermis near the epineural sinus (ES), oval cells reactive to both antibodies were observed (double arrowhead). Scale bar, 100 μ m. (*d,e*) Cross section of a tube foot stained by 1E11. CT, outer connective tissue; M, longitudinal muscle; NP, nerve plexus; PN, podial nerve. Scale bar, 100 μ m. (*e*) Enlarged view of the area marked in (*d*) showing the merged image of double staining. The arrowhead indicates an oval stichopin-containing cell. (*f*) Cross section of longitudinal muscle of body wall showing oval cells with stichopin-like immunoreactivity in the connective tissue sheath of the muscle. Scale bar, 100 μ m. (*g–i*) Body-wall dermis. Immunoreactivity to (*g*) 1E11 and (*h*) anti-stichopin antibody, and (*i*) a merged image of (*g*) and (*h*). Fibrous structures were labelled with 1E11 (single- and double-headed arrows), some of which were also labelled with the anti-stichopin antibody (double-headed arrows). A cell with a short process (double arrowheads) labelled with both antibodies was also observed. Scale bar, 50 μ m. (*j–l*) Body-wall dermis close to a water canal of a papilla whose border is marked with broken lines in (*j*). Immunoreactivity to (*j*) 1E11 and (*k*) anti-stichopin antibody, and (*l*) a merged image of (*j*) and (*k*). Arrow, podial nerve; double arrowhead, cell labelled with both 1E11 and anti-stichopin antibody; single arrowhead, oval cells stained by the anti-stichopin antibody but not with 1E11. Scale bar, 50 μ m. (*m–o*) Cross section of connective tissue of cloaca. Immunoreactivity to (*m*) 1E11 and (*n*) anti-stichopin antibody, and (*o*) a merged image of (*m*) and (*n*). Part of the fibrous structures labelled with 1E11 showed a stichopin-like immunoreactivity (arrows). A cell body labelled with both 1E11 and the anti-stichopin antibody was observed (arrowheads). Scale bar, 50 μ m.

Some of them were labelled by both the anti-stichopin antibody (figures 2*b* and 3) and 1E11 (double arrowhead in figure 2*a–c*).

(e) Digestive tract

The digestive tract of this species forms an s-shaped loop (Sang 1963). We examined the ascending part of its digestive tract. The wall of the digestive tract consists of, from the luminal side to the coelomic side, the luminal epithelium, connective tissue and the muscular coelomic epithelium. A strong 1E11 immunoreactivity was found in the nerve plexus located in the coelomic epithelium. No stichopin-LI was found in this organ.

(f) Cloaca

Histological organization of cloacae is similar to the other parts of the digestive tract. A strong 1E11 labelling was observed in the nerve plexus of the coelomic epithelium, but no label was found in the luminal epithelium. In the connective tissue layer, some fibres and cell bodies showed reactivity to the 1E11 (figure 2*m*). Some of these fibres and cells were also labelled by the anti-stichopin antibody (figure 2*n,o*).

4. DISCUSSION

(a) Stichopineric nerves specific to connective tissues

We found cells and fibres with stichopin-LI in the connective tissues of various organs, such as the body-wall dermis, LMBW, tube feet and cloacae. The stichopin-containing cells were even found in the connective tissue separating the ectoneural part from the hyponeural part of the radial nerve cord. No stichopin-LI was found in the well-defined nervous structures, such as the ectoneural and hyponeural parts of the radial nerve cords, the circumoral nerve ring and podial nerves. These nervous structures were, however, strongly stained by 1E11, the monoclonal antibody raised against the extract of the radial nerves of adult starfish. Synaptotagmin B, also termed synaptotagmin1-1, is the antigen for 1E11 (Burke et al. 2006a). Because this monoclonal antibody clearly identified neurons in the larvae of various echinoderms and some adults, this has been regarded as a good marker for nerves of the echinoderms (Nakajima et al. 2004b;

Figure 3. Stichopin-like immunoreactivity shown with peroxidase-based method in cross sections of the body wall containing the ES of a radial nerve cord. Scale bar, 100 μ m. (*a*) Immunoreactive oval cells (arrow) in the dermis. (*b*) A pre-absorbed control of (*a*).

Nakano et al. 2006; Saha et al. 2006). The present study showed that 1E11 is also a good marker for nerves in adult sea cucumbers. Many fibres and cells with processes involving the 1E11 reactivity were found in various connective tissues. Their shape and reactivity strongly suggested that they are neurons. A rich population of 1E11-positive fibres was found in the body-wall dermis. This is known from electron microscopy studies (Motokawa 1982) and by immunohistochemistry (Diaz-Miranda et al. 1995; Inoue et al. 1999) that the body-wall dermis contains neuronal processes.

The result that stichopin-LI was not observed outside connective tissues does not necessarily mean that stichopin-containing cells are not neurons. Some of the 1E11-positive fibres and cells with processes in the connective tissues were also immunoreactive to the anti-stichopin antibody, and thus are very likely stichopin-containing nerves. Stichopin very likely works as a neuropeptide in the connective tissues. The result that stichopin-LI was confined to the connective tissues suggests that the stichopineric nerves are the nerves specific to the connective tissues. Such an echinoderm-specific nervous system seems to have co-evolved with the catch connective tissues that are also unique to echinoderms. Because the externally applied stichopin inhibits stiffening of the dermis induced by acetylcholine (Birenheide et al. 1998), the stichopin-containing neurons in the dermis very likely regulate the stiffness of the body-wall dermis, the typical catch connective tissue.

In cloaca, we found stichopin-containing nerves in the connective tissue layer. Cloacae are the organs involved in digestion and respiration. During respiration, the pumping activity of this organ draws and expels seawater through

Figure 4. Stichopin-containing cells (arrow) in the connective tissue between the ectoneural (EN) and hyponeural (HN) parts of the radial nerve cord. (a) Semi-thin section stained with toluidine blue. D, body-wall dermis. Scale bar, 10 μ m. (b) Transmission electron micrograph. Arrowheads indicate two basal laminae separating the connective tissue from the nervous tissues. N, nucleus of the stichopin-containing cell. Scale bar, 5 μ m.

the anus to aerate the respiratory trees (Hyman 1955). The extremely thick connective tissue layer with the stichopin-containing neurons is exceptional in holothurian digestive tracts. This feature is thus very likely associated with the pumping activities, not with digestion. The pumping rate may be modulated through the modulation of the mechanical properties of both the cloacal and the body-wall connective tissues (Wolcott 1981).

(b) Stichopin-containing secretory cells

There are cells with stichopin-LI that were oval in shape and apparently lack processes. Such OCSs were found in the body-wall dermis close to the water vascular canals, the dermis close to the ES, the connective tissue sheath of LMBW, the connective tissue adjacent to the nerve plexus of the tube feet and the tentacle, and the connective tissue separating the hyponeural part from the ectoneural part of the radial nerve cord. An ultrastructural study of the OCS in the radial nerves showed that the OCS was packed with spherules, suggesting a secretory nature. OCSs other than those in the radial nerves may also secrete stichopin. OCSs were found either in the connective tissue close to fluid-filled body cavities or in the connective tissue close to the prominent nervous systems, such as the epineural and hyponeural parts of the radial nerves and nerve plexus of the tube feet. The function of OCS near the nervous systems is easy to imagine. It may secrete stichopin in response to nearby nerves or its secretion may modulate nerves nearby. In either case, stichopin is probably a paracrine factor affecting nerves or other cellular elements controlling the mechanical properties of connective tissues. The paracrine regulation is well known in other animals (Haas *et al.* 2005; Cain *et al.* 2006; Mukai *et al.* 2006). In echinoderms, the cholinergic system in tube feet has been suggested to work in a paracrine way (Florey & Chahill 1980).

Unlike the OCS near the nervous systems, the function of the OCS near the body cavities is rather difficult to predict. Paracrine regulation is not probable because there were few other cells around this type of OCS to be affected. OCSs faced the ES, the water-vascular canal of the papillae or the main coelom in the case of OCS in the connective tissue sheath of LMBW. It is tempting to imagine that OCS secretes stichopin into these body cavities and that stichopin is transferred in the cavities to work as a kind of hormone on

its targets. Thus, it is probable that OCSs are non-neural paracrine/endocrine cells.

OCSs other than those in the radial nerves were not stained by 1E11; among the OCSs in the radial nerves, some were negative, but most were positive to 1E11. This might be the difference in the isoforms of the synaptotagmins. It is highly probable that sea cucumbers have several isoforms because the sea urchin genome showed 14 synaptotagmin isoforms (Burke *et al.* 2006b). In vertebrates, several synaptotagmin isoforms are localized not only to neurons but also to some secretory cells, such as the chromaffin cells and pancreatic β -cells (Marquze *et al.* 1995; Gut *et al.* 2001; Sdhof 2002). Whether the OCS has a positive or negative immunoreactivity to 1E11 possibly depends on the types of synaptotagmins in the OCS.

Our results strongly suggested that stichopin was contained both in the nerves and secretory cells. Examples of bioactive peptides contained both in the nerves and secretory cells have been reported in vertebrates and invertebrates (King & Millar 1995; Winther *et al.* 1999). It will be an interesting question whether stichopin-containing nerves and secretory cells belong to the same cell lineage during development. Our results also suggested the presence of nerves specific to the connective tissues. To our knowledge, such nerves are the first reported in the animal kingdom.

Our experiments conformed to relevant local animal welfare laws, guidelines and policies.

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