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Authors: Inoue, Masahide, Tamori, Masaki, and Motokawa, Tatsuo

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Innervation of Holothurian Body Wall Muscle: Inhibitory Effects and Localization of 5-HT

Masahide Inoue, Masaki Tamori* and Tatsuo Motokawa

Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152-8551, Japan

ABSTRACT—We investigated innervation to body wall muscles as well as distribution of 5-HT (serotonin) and its effects on longitudinal muscles of body wall (LMBW) of the sea cucumber *Apostichopus japonicus*. With serial sections we found neural branches and fibers extending from hyponeural part of radial nerve towards LMBW and circular muscles of body wall. With the aqueous aldehyde (Faglu) method yellow fluorescence indicating indolamines was observed in LMBW and in the mesentery connecting LMBW to the body wall. With indirect immunohistochemistry 5-HT-like immunoreactivity was observed in LMBW and in mesentery. These results strongly suggested that both LMBW and mesentery contained 5-HT. The effects of monoamine neurotransmitters were studied in LMBW. Putative neurotransmitters tested were 5-HT, adrenaline, noradrenaline, dopamine, and DOPA at the concentration of 10^{-6} M. The application of 5-HT caused no contraction or relaxation, but it inhibited the contraction induced by 10^{-6}–10^{-5} M acetylcholine (ACh). Catecholamines were ineffective by themselves and had no effects on the contraction induced by ACh. The present histological, histochemical, and pharmacological studies strongly suggested that holothurian LMBW was innervated by inhibitory serotonergic neurons of the hyponeural nervous system.

Key words: 5-HT, echinoderm, sea cucumber, muscle contraction

INTRODUCTION

Holothurian nervous systems have been poorly investigated. Even signal transmission remains mysterious. This is due to the fact that holothurian nerve cells are very small, difficult to identify and often embedded in dense connective tissue (Cobb, 1987). To understand holothurian nervous systems, it is necessary to investigate neural pathways and to identify neurotransmitters and modulators.

The main components of holothurian nervous system are a circumoral nerve ring and five radial nerve cords. The former consists of ectoneural part only and the latter does ectoneural part and hyponeural part (Hyman, 1955). Both hyponeural and ectoneural nervous systems are involved in motor control. The details of neural pathways of these nervous systems, however, remain unknown. In our previous study we found that muscles in podia and tentacles were innervated by ectoneural nerves, not by hyponeural nerves, while the circular muscles of body wall received hyponeural innervation (Inoue *et al.*, 1999). Innervation of the longitudinal muscle of body wall (LMBW) was not studied, although this muscle has been a favored material in pharmacology, especially for bioassay of acetylcholine (Welsh, 1966).

Several substances are known to function as mediators in the nervous system of sea cucumbers. For example, it was reported that acetylcholine (ACh) induced contractions of LMBW (Welsh, 1966; Takahashi, 1974; Sugi *et al.*, 1982). ACh is believed to be the ubiquitous neurotransmitter in echinoderm muscles (Pentreath and Cobb, 1972; Cobb, 1987). Recently, several types of neuropeptides have been found in the nervous system of echinoderms (Thorndyke and Candia Carnevali, 2001). In sea cucumbers, it is suggested that some peptides work as neurotransmitters controlling muscles and catch connective tissues (Díaz-Miranda *et al.*, 1995; Birenheide *et al.*, 1998; Inoue *et al.*, 1999).

The presence of monoamines, such as dopamine, noradrenaline and 5-HT (serotonin) were also shown by histochemical methods in echinoderms (Cobb, 1969; Cottrell and Pentreath, 1971; Dolder, 1975; Burke *et al.*, 1986; Ghyoot and Cobb, 1994; Chen *et al.*, 1995; Candia Carnevali *et al.*, 1996; García- Arrarás *et al.*, 2001; Thorndyke and Candia Carnevali, 2001). In sea cucumbers, catecholamine distributions were shown in the nervous tissues of embryos (Chen *et al.*, 1995) and in the nerve plexus in the connective tissue layer of adult digestive tracts (García-Arrarás *et al.*, 2001). The functions of monoamines as well

* Corresponding author: Tel. +81-3-5734-2656;
FAX. +81-3-5734-2946.
E-mail: mtamori@bio.titech.ac.jp

as their distribution in the radial nerve cord remain unknown in sea cucumbers. Here we report the hyponeural innervation to LMBW and the distribution of monoamine and their effects in LMBW.

MATERIALS AND METHODS

Specimens of the sea cucumber *Apostichopus japonicus* were collected near Noto Marine Biological Station of the University of Kanazawa. The animals were kept in circulating seawater aquarium at *ca*. 18°C.

Serial sections

Animals were anesthetized in 1% menthol in artificial seawater (ASW, Jamarin Lab., Japan). Dermis samples including the radial nerve, the circular muscle and LMBW were dissected and immediately fixed with 10% formalin overnight. Samples were dehydrated in an ethanol series and embedded in Paraplast Plus (Sigma-Aldrich, Japan). Serial sections of 10 μm thickness were stained with Milligan trichrome (Humason, 1979).

Histochemical fluorescence

Aqueous aldehyde (Faglu) histofluorescence method was employed as described by Schöler and Armstrong (1982). Samples were dissected from dermis including the radial nerve and LMBW. We examined and photographed the sections with a fluorescence microscope (Nikon Labophoto-2). An excitation filter with a peak transmittance of 380-420 nm was used in conjunction with a dichroic mirror (DM 430) and a barrier filter (BA 450) which reflected light below 450 nm. Negative controls were done with no glutaraldehyde added to the fixative. Such preparations exhibited no fluorescence at all. The Faglu method allowed us to distinguish the endogenous blue fluorescence of catecholamines from the yellow fluorescence of indolamines (Furness *et al*., 1977).

Immunohistochemistry

Dermis samples including the radial nerve, the circular muscle and LMBW were dissected and fixed with 3% paraformaldehyde in ASW for 1 hr at room temperature. The samples were then cryoprotected by infiltrating with a graded series of sucrose (10–20%). Finally, the samples were frozen in O.C.T. compound (Sakura Finetek, USA) using dry ice. Thin sections of frozen tissue were cut at 10 μm on a Cryostat (Leica CM 1850, Germany) and were picked up on glass slides coated with γ-aminopropyltriethoxysilane. The sections were washed twice in phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (Acros Organics, USA) in PBS for 30 min. The sections were then incubated with a primary antibody against 5-HT (Sigma-Aldrich, Japan) for 1 hr at room temperature. The primary antibody raised in rabbit was diluted in PBS to 1:10000 before use. After three washes in PBS, the sections were incubated with a peroxidase-conjugated goat anti-rabbit Ig G (Jackson Immunoresearch Laboratories, Inc., USA) diluted in PBS (1:1000) for 1 hr at room temperature. After three washes in PBS, True Blue (Kirkegaad and Perry Laboratories, USA) was used as a substrate for the peroxidase to visualize the immunostaining product. The incubation time in True Blue was 10 min. Pre-absorption controls for immunohistochemistry were carried out using the primary antibody that had been incubated with 10^{-2} M 5-HT overnight at 4°C.

Pharmacological test

LMBW was dissected from *A. japonicus* and cut into strips of *ca*.15×3×3 mm. An end of the sample was fixed to a holder in an experimental trough filled with 5 ml ASW. The other end was connected *via* a silver chain to an isometric force transducer (LVS-20GA, Kyowa, Japan). Temperature was controlled *via* a water bath at *ca*. 18°C. Effects of monoamines were examined either singly or with acetylcholine (ACh; Nacalai Tesque, Japan). When applied singly monoamine was added to the trough filled with ASW to a final concentration of 10^{-6} M. Effects of monoamine on the contraction induced by ACh were studied as follows. At the start of the experiment, ACh was applied and the contraction thus induced was taken as a control. ACh was then washed out with a constant flow of ASW for *ca*. 15 min. Subsequently, the monoamine to be examined was added to a concentration of 10^{-6} M, and 30 seconds later ACh was applied again. After the second thorough rinse with ASW, ACh-induced contraction was recorded as a recovery. Monoamines used for this study were adrenaline (Merck, Germany), noradrenaline (Tokyo Kasei Kogyo, Japan), dopamine (Wako Chemical, Japan), DOPA (Nacalai Tesque, Japan) and 5-HT (Wako Chemical, Japan).

RESULTS

Neural pathways

Cross sections of the inner surface of the body wall of *A. japonicus* were observed after staining with Milligan trichrome. With Milligan trichrome collagen fibers were colored in green, nuclei and muscle cells were colored in red to red purple and nerve fibers were colored in purple (Fig. 1A–F). A cross section of the body wall shows that the radial nerve is located in the connective tissue layer (Fig. 1A). The coelomic lining of the body wall gives rise to a circular muscle layer. However, this layer does not form a complete circle: the circular muscle is interrupted by the LMBW and the radial nerve in each radius. The LMBW was connected to the body wall at both sides of the radial nerve by mesenteries. The radial nerve is composed of a thicker ectoneural and a thinner hyponeural part separated by a thin partition of connective tissue. These results confirmed the description of Hyman (1955).

Nerve fibers emerging from the edge of hyponeural nerve ran into the mesentery (Fig. 1B). Different levels of focus of the same preparation revealed that the fiber continued inside the mesentery extending towards the LMBW (Fig. 1C, D).

A branch extending from the hyponeural nerve towards the circular muscle layer could be seen (Fig. 1E). This branch gave rise to several smaller fibers that continue into the coelomic epithelium lining the circular muscle (Fig. 1F). Serial sections revealed that such branches emerge at varying intervals along the length of the radial nerve.

Histochemical fluorescence

We inspected the LMBW, the radial nerve, the mesentery connecting LMBW to the body wall and the connective tissue of the dermis about the presence of fluorescence in Faglu-treated sections. Yellow fluorescent spots, suggesting the presence of indolamines, were observed in the LMBW proper (Fig. 1G). Yellow fluorescent spots and fibers with varicosities could be seen in the mesentery (Fig. 1H). Blue fluorescent spots, suggesting the presence of catecholamines, were observed in the connective tissue of the dermis (data not shown). In the radial nerve no fluorescence could be seen.

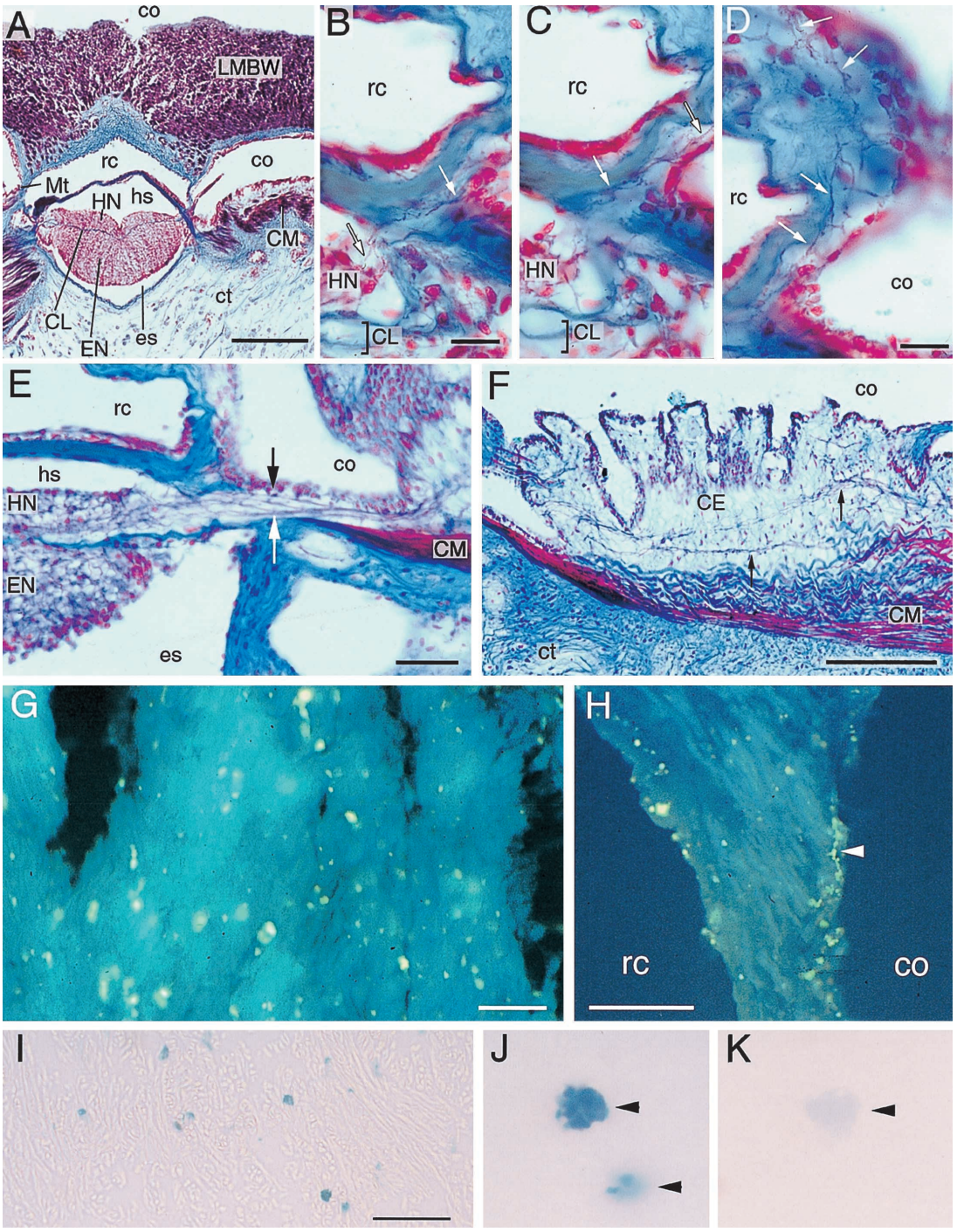

Fig. 1. Cross section of inner surface of holothurian body wall stained with the Milligan trichrome method (**A–F**), histochemical fluorescence indicating monoamines (**G, H**; Faglu method) and 5-HT-like immunoreactivities (**I, J, K**). (**A**) Radial nerve consisting of hyponeural (HN) and ectoneural parts (EN) separated by a thin connective tissue layer (CL). The radial nerve is located in connective tissue of the dermis (ct) and underneath the LMBW. Scale bar, 100 μm. (**B–D**) Mesentery connecting LMBW to the body wall. Fibers (arrows) extend from the hyponeural nerve (HN) towards LMBW. The direction of body wall is to the bottom, the direction of LMBW is to the top. **C** is a micrograph with a different level of focus from **B**, but the same area. **D** is a portion nearer to LMBW than **B, C**. Scale bar, 10 μm. (**E**) Branch (between white and black arrows) extending from the hyponeural nerve (HN) towards circular muscles (CM). Scale bar, 20 μm. (**F**) Fibers (arrows) located in the coelomic epithelium (CE) lining the circular muscles. Scale bar, 100 μm. (**G**) Cross section of LMBW. Numerous spots show yellow fluorescence specific to indolamines. Scale bar, 10 μm. (**H**) Cross section of mesentery. A yellow fluorescent fiber (arrowhead) including varicosities and several spots were observed. The direction of body wall is to the bottom, the direction of LMBW is to the top. Scale bar, 20 μm. (**I**) 5-HT-like immunoreactivities in the cross section of LMBW. Numerous spots among muscle fibers show the immunoreactivities. (**J**) A part of **I** at higher magnification. Oval stsructures which include the immunoreactive contents are indicated by arrowheads. (**K**) A preabsorbed control of **J**. The stain was reduced (arrowhead). **J** and **K** are from successive sections but the immunoreactive structures are not the same one. Scale bar in **I** is common to **I, J** and **K**. The bar corresponds to 100 μm for **I** and 25 μm for **J** and **K**. Abbreviations used are as follows: CE, coelomic epithelium lining the circular muscles; CL, connective tissue separating EN and HN; CM, circular muscle; co, somatocoel; ct, connective tissue layer in body wall; EN, ectoneural part of radial nerve; es, epineural sinus; HN, hyponeural part of radial nerve; hs hyponeural sinus; LMBW, longitudinal muscle of body wall; Mt, mesentery; rc, radial water canal.

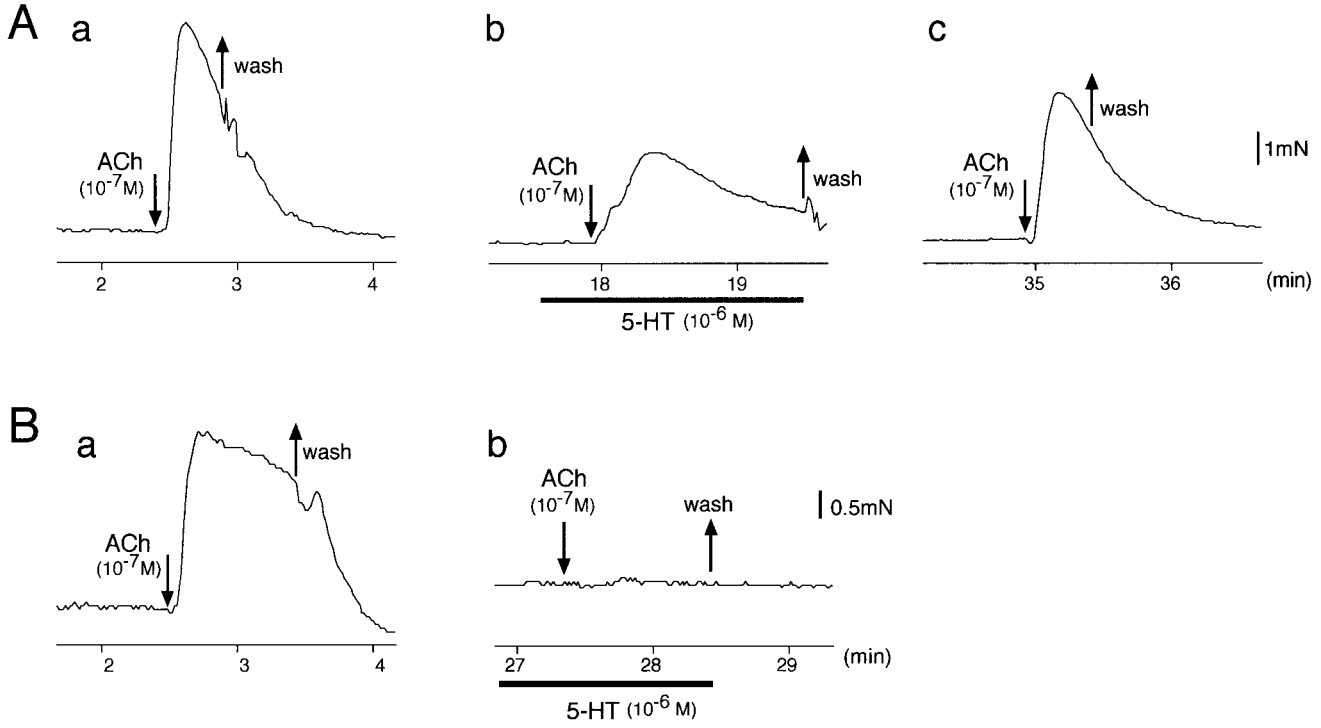

Fig. 2. Inhibitory effect of 5-HT on ACh-induced contraction of LMBW. (**A**) Example of partial inhibition. **a**: Control contraction induced by ACh. **b**: 5-HT reduced ACh-induced contraction. **c**: Contraction by ACh was restored after washing with ASW. (**B**) Example of complete inhibition. **a**: Control contraction induced by ACh. **b**: 5-HT completely inhibited ACh-induced contraction.

Immunohistochemistry

The yellow fluorescence suggested the presence of indolamines, possibly 5-HT, in LMBW, which was confirmed by immunostaining. The cross section of LMBW was stained with antibody to 5-HT. Labeled blue structures were observed among muscle fibers (Fig. 1I). These structures looked oval with immunoreactive contents (Fig. 1J). The labeled structures seem to correspond to the yellow fluorescent spots found with the Faglu method. Pre-absorption of the antibody against 5-HT with 10^{-2} M 5-HT reduced the immunoreactivity (Fig. 1K).

Blue deposits were also detected in the mesentery (data not shown). The pre-absorption control, however, showed blue deposits also.

Pharmacology

When applied singly, 5-HT, adrenaline, noradrenaline, dopamine and DOPA at the concentration of 10^{-6} M caused no contraction or relaxation of LMBW. ACh (10^{-7} M) caused contraction of the LMBW of *Apostichopus japonicus* (Fig. 2A a, 2B a) as reported earlier (Takahashi, 1974; Sugi *et al.*, 1982). The 5-HT (10^{-6} M) had inhibitory effects on the ACh (10^{-7} M)-induced contraction. In 13 out of 16 cases, 5-HT reduced the contraction (Fig. 2A). In the rest 3 cases complete inhibition was observed (Fig. 2B). The average of the peak tension in 5-HT was 31.2±25.4% (±S. D., N=16) of control. The peak tension in 5-HT was statistically different from that of control (paired t-test, $p<0.01$). In the case of partial inhibition, the effect of 5-HT was completely recovered by washing with ASW (Fig. 2A c). Statistical analysis showed that the peak tension recovered was not significantly different from that of control before application of 5-HT (paired t-test, N=14, $p>0.05$). When higher ACh concentration (10^{-6} M) was used, the inhibitory effect of 5-HT was much smaller: the mean tension in 5-HT was 81.4±9.2% of control (±S. D., N=20). The reduction in tension was statistically significant ($p<0.01$). Adrenaline, noradrenaline, dopamine and DOPA, all at the concentration of 10^{-6} M, had no effect on ACh-induced contraction.

DISCUSSION

We observed fibers and branches emerging from the hyponeural nerve towards the longitudinal muscles of the body wall (LMBW) and circular muscles. Hyman (1955) suggested that the hyponeural nervous system should be regarded as mainly or exclusively motor, supplying the muscle fibers of the body wall. Our present finding supports Hyman's speculation. Hyponeural innervations into muscles were frequently encountered also in other echinoderms (echinoid lantern retractor muscle, Cobb and Laverack, 1966; ophiuroid intervertebral muscles, Stubbs and Cobb, 1981; arm muscle of asteroid, Cobb, 1978). The direct ectoneural innervation into muscle has been shown, however, only in the outer ring muscles of echinoid spines (Peters, 1985). We thus concluded that holothurian LMBW was

innervated by hyponeural nerves not by ectoneural nerves.

With two different methods, indirect immunohistochemisty and Faglu method, we provided the strong evidence for the presence of 5-HT both in LMBW and in the mesentery connecting LMBW to radial nerve cord. In LMBW both methods showed oval structures. Faglu method suggests these structures to contain indolamines and the immunohistochemistry does to contain 5-HT. These results strongly suggest the presence of 5-HT in LMBW. In the mesentery 5-HT-like immunoreactive spots were observed. We were not sure whether they were specific to 5-HT because spots were also observed in the control. The double check by Faglu method, however, strongly suggested that some of the spots found in the mesentery were specific reaction to 5-HT. We thus confirmed by two different methods the presence of 5-HT in LMBW and mesentery connecting LMBW to the radial nerve cord of sea cucumbers. The 5-HT-like immunoreactivities in sea cucumbers have been reported only in larvae (Burke *et al*., 1986); in adults the presence of 5-HT was suggested in tube feet by dichromate staining method (Dolder, 1975).

In contrast to LMBW and mesenteries, neither 5-HT-like-immunoreactivities nor monoamine-like fluorescence were observed in the radial nerve. This does not simply mean the absence of monoaminergic neurons. The echinoderm nerves have some peculiar features (Cobb, 1989). Synapses are usually not found. The histochemical methods seem to detect neurotransmitters only in the places where they are concentrated. The absence of synapses may make such places rare in the radial nerve. Another feature is that the echinoderm nerve cells are usually quite small. The diameter of axons is about 0.3 μm and it rarely exceeds 1 μm (Pentreath and Cobb, 1972). The monoaminergic neurons in the radial nerve may be too small to be detected by the present methods. The small size of neurons of echinoderms has prevented us from following the whole neural pathway except in brittle stars that have large neurons.

Unlike sea cucumbers, 5-HT-like-immunoreactivities were found in main nerve trunks of some other echinoderms. In the brittle star *Ophiura ophiura*, 5-HT-like immunoreactivities were observed in two bundles of ectoneural fibers of the radial nerve, in a bundle of transverse fibers in interganglia and in two groups of 4 to 5 cell bodies in each ganglion (Ghyoot and Cobb, 1994). The brittle stars may be an exception among echinoderms because large nerve cells and synapses are encountered in this group. In the feather star *Antedon mediterranea*, Candia Carnevali *et al*. (1996) reported the distribution of dopamine and 5-HT in the brachial nerve, the main crinoid nervous system that does not correspond to the radial nerve found in other classes of echinoderms.

The present pharmacological study showed that 5-HT inhibited ACh-induced contraction of LMBW. This was the only effect we found among monoamines tested on LMBW. In the isolated cloaca of the sea cucumber *Stichopus moebii*, the application of 5-HT, adrenaline, and dopamine caused ambiguous responses (Hill, 1970). In asteroid tube feet low concentrations of both adrenaline and noradrenaline and octopamine caused contractions, whereas higher concentrations of adrenaline and noradrenaline relaxed tube feet; 5-HT was not effective (Protas and Muske, 1980). In sea urchins, noradrenaline and dopamine may cause relaxation of lantern muscles (Pentreath and Cobb, 1972) but monoamines were ineffective in tube feet (Florey and Cahill, 1980). In the larva of sea urchin dopamine and 5-HT had stimulatory effects on the esophageal muscle (Gustafson, 1991). In summary, the response of muscles of echinoderms to catecholamines is variable, probably due to differences between species and organs used. It could be noted that excitatory effects of 5-HT were not observed in adult echinoderm muscles. As the inhibitory effect of 5-HT on ACh-induced contraction has never been examined before, this might also be found in other echinoderm species.

Among other putative neurotransmitters γ-aminobutyric acid (GABA) may have inhibitory effects on LMBW of *Apostichopus japonicus*. The responses of LMBW to GABA are, however, species dependent. GABA inhibited the ACh-induced contraction in *Sclerodactyla briareus*, whereas in *Actinopyga* it had excitatory effects (Devlin, 2001). Muneoka and coworkers (Iwakoshi *et al*., 1995; Birenheide *et al*., 1998; Ohtani *et al*., 1999) isolated 25 peptides from the present sea cucumber. Some of them may well inhibit ACh-induced contraction, because about half of the peptides isolated showed inhibitory effects on the twitch contraction of LMBW invoked by electrical stimulation, and because stichopin, one of the 25 peptides, inhibits action of ACh in the body-wall catch connective tissue of the present sea cucumber (Birenheide *et al*, 1998). Thus several neuromediators seem to be involved in the control of the contractility of LMBW.

In the mesentery, through which LMBW was connected to the radial nerve, we observed a branch of hyponeural nerve ran through it towards LMBW by conventional staining. We also observed spots and fibers with varicosities with 5-HT-like reactivities by histochemical methods. The fibers with varicosities are no doubt nerve fibers. These results thus strongly suggest that some of the hyponeural fibers innervating LMBW are serotonergic. The spots in the mesentery may well be cell bodies of nerves containing 5-HT.

Inside LMBW we observed oval structures with 5-HT-like reactivities. This, together with the pharmacological results, strongly suggests that 5-HT was really working in LMBW. The oval structure with 5-HT-like reactivity in LMBW seems to be the serotonergic neuron although it did not have the conventional appearance of oval cell body with slender axons. The muscles of echinoderms have a peculiar feature: they extend slender projections or muscle tails to nerves to receive information. In LMBW several muscle cells group together to form a rosette-like bundle, in whose center an oval nerve cell is disposed (Motokawa, 1982; Sugi *et al*., 1982). Each muscle cell sends muscle tails towards the nerve cell. It is believed that neurotransmission occurs at the junction between the muscle tail and the nerve cell. With this

system of transmission, we could expect the most frequently encountered figures of nerve cells in cross section would be oval ones, not slender axons. This is the reason why we regard the oval structures in LMBW as 5-HT containing neurons. Preliminary measurements were made on the distance between the center of rosettes and on the distance between the oval structures. They agreed well: they were both around 10–20 μm.

In conclusion, the present histological, histochemical, and pharmacological studies strongly suggested that the holothurian LMBW was innervated by inhibitory serotonergic neurons of the hyponeural nervous system.

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