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Authors: Takahama, Hideki, Haibara, Kumi, Oumi, Tomoyuki, Ukena, Kazuyoshi, Morishita, Fumihiro, et al.

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Immunohistochemical Localization of Annetocin, an Earthworm Oxytocin-Related Peptide, and Identification and Ultrastructural Characteristics of the Annetocin-Secretory Cells in the Oligochaete Earthworm *Eisenia foetida*

Hideki Takahama[1]*, Kumi Haibara[1], Tomoyuki Oumi[2], Kazuyoshi Ukena[2], Fumihiro Morishita[2], Yasuo Furukawa[2], Osamu Matsushima[2], Hiroyuki Minakata[3] and Kyosuke Nomoto[3]

[1]*Department of Biology, Faculty of Education, Oita University, Dannoharu 700, Oita 870-1192, Japan*
[2]*Department of Biological Science, Faculty of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan*
[3]*Suntory Institute for Bioorganic Research, Simamoto, Mishima, Osaka 618-8503, Japan*

ABSTRACT—Annetocin is an egg-laying-inducing oxytocin-related peptide which we have previously isolated from the earthworm, *Eisenia foetida*. Here we report the results of immunohistochemical and ultrastructural studies on annetocin-secretory cells in the earthworm. Annetocin-immunoreactive (IR) cell-somata were located mainly at the ventro-lateral side of the subesophageal ganglion. Only four annetocin-IR cells were seen in the cerebral ganglion. Some annetocin-IR cells displayed unipolar-like structure with a process directing to the core region (the neuropile) of the ganglion. Annetocin-IR fibers were also observed in the neuropile of the ventral ganglia and the ventral nerve cord between the 4th and the 30th segments including the clitellum, but not in the posterior segments (31-55th). The number of annetocin-IR fibers decreased from the 4th to the 30th segment. The annetocin-secretory cells were identified by the immunogold staining, and filled with gold-labeled vesicles, 200-250 nm in diameter, which included moderately electron dense material. The annetocin-secretory cells possessed a euchromatic nucleus, well-developed rough endoplasmic reticulum and Golgi apparatus. Some of the annetocin-secretory cells were found to form a neurohemal-like structure, where somata or fibers with loose glial investment came in contact with the coelomic space at the ventral side of the subesophageal ganglion. The results suggest that annetocin is a neuropeptide produced and secreted by the neuron in the cerebral and subesophageal ganglia of the earthworm.

INTRODUCTION

Neurosecretion is a phenomenon that neuron produces and releases substances which act as hormones and/or transmitters. The magnocellular hypothalamo-neurohypophysial system in vertebrate is a representative of the neurosecretion. In mammals, oxytocin and vasopressin, nonapeptides structurally similar to each other, are produced and secreted in the magnocellular hypothalamo-neurohypophysial system. Two lineages related to oxytocin and vasopressin are present in other vertebrate classes except the cyclostomes which have only vasotocin (Acher, 1993). In invertebrates, some peptides belonging to the oxytocin-vasopressin superfamily have also been identified: Conopressin G and S isolated from the gastropod molluscs, *Conus geographus* and *C. striatus*, respectively (Cruz *et al*., 1987), Lys-conopressin (conopressin G) from *Aplysia kurodai* (McMaster *et al*., 1992), *Lymnaea stagnalis* (Van Kesteren *et al*., 1992) and the leech *Erpobdella octoculata* (Salzet *et al*., 1993a), cephalotocin from the cephalopod mollusc, *Octopus vulgaris* (Reich, 1992), and Arg-vasopressin-like diuretic hormone from the insect, *Locusta migratoria* (Proux *et al*., 1987).

As to oligochaete annelids, the existence of the oxytocin- or vasopressin-related peptide in the central nervous system has been suggested by immunohistochemical studies in *Tubifex tubifex* (Yahya *et al*., 1983) and in *Pheretima hilgendorfi* (Kinoshita and Kawashima, 1986). We have isolated and chemically identified an oxytocin-related peptide, annetocin, from the earthworm, *Eisenia foetida* (Oumi *et al*., 1994).

* Corresponding author: Tel. +81-975-54-7574; FAX. +81-975-54-7623.

Annetocin induces a series of egg-laying-related behaviors in *E. foetida* and in the leech, *Whitmania pigra* (Oumi *et al.*, 1996) and also stimulates the gut and nephridial movements in the earthworm (Oumi *et al.*, 1994; Ukena *et al.*, 1995). However, it remains to be investigated which type of cell or tissue contains this peptide in the earthworm.

In the present study, to determine whether annetocin is produced by neural or glandular tissue, we raised antibody in rabbits and investigated the localization of annetocin by immunohistochemistry. Furthermore, we identified the annetocin-secretory cells by immunogold labeling and observed their fine structural features by electron microscopy. The results show that the annetocin-secretory cells are neurons in the circumoesophageal ganglia.

MATERIALS AND METHODS

Specimens of the lumbricid earthworm, *Eisenia foetida*, were purchased from a fishing-bait store, maintained at room temperature in the wet compost and used for the following experiments.

Antibody preparation

Three mg of synthetic annetocin was added to bovine thyroglobulin aqueous solution (20 mg/1.5 ml, Sigma Chemical, St. Louis, MO, USA). Under constant stirring, 130 mg of carbodiimide was added to the solution and its pH was adjusted to 5.5-6.0 with 0.1 N HCl. The solution was incubated for 24 hr at 4°C, and then dialysed against distilled water. The dialysate containing annetocin coupled with thyroglobulin was lyophilized and stored at -20°C.

Two mg of the coupled annetocin was dissolved in 0.5 ml distilled water and mixed with 0.5 ml of Freund's complete adjuvant (incomplete adjuvant for secondary and subsequent immunization, Difco, Detroit, MI, USA) using a sonicator. Each female rabbit (New Zealand White) was subcutaneously injected with this emulsified mixture at 20 sites in the back (50 μl per site). The injections for immunization were made at two-week intervals. The titer for immunoreactivity of the serum was also checked every two weeks by a dot blot assay (Hodgson *et al.*, 1985) or an enzyme-linked immunosorbent assay (Salzet *et al.*, 1992). Two weeks after the fourth injection, the blood was drawn from the ear artery. The collected blood was incubated for 1 hr at 37°C and overnight at 4°C, before it was centrifuged for 10 min at 3,000 rpm to separate the serum from the blood-clot. Thimerosal (0.01%) was added to the serum, and it was stored at -80°C or 4°C until immunohistochemical use.

Unfortunately, we did not examine the cross-reactivity of the antibody with the mammalian related peptides, oxytocin and vasopressin.

Procedures for immunohistochemistry

Anterior end to 55th segment of *Eisenia foetida* including the clitellum (25th-30th segments) was cut into small pieces and fixed in 4% paraformaldehyde-0.1 M phosphate buffer (pH 7.4, 4°C) for 4 hr. After washing three times in PBS (0.9% NaCl - 10 mM phosphate buffer, pH 7.4) for 1 hr, they were dehydrated to ethanol and benzene, and embedded in paraffin. Serial sections were cut transversely at 5 μm in thickness. Deparaffinized sections were immunostained by the indirect immunofluorescence method and ABC method (Vector Lab., Burlingame, CA, USA). To reduce background staining, hydrated sections were preincubated in blocking solution (1% BSA, 1% normal goat serum, 0.05% Triton X-100 in PBS) for 30 min at room temperature. They were then incubated with anti-annetocin antiserum (diluted 1/500 in blocking solution for the indirect immunofluorescence method and 1/2000 for the ABC method) overnight at 4°C. Subsequently, they were washed three times in PBS for 10 min and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or biotinylated anti-rabbit IgG (diluted 1/100 in blocking solution, Organon Teknika, NC, USA) for 2 hr at room temperature. The sections were washed three times in PBS for 30 min and mounted in Gel/Mount (Biomed., Foster, CA, USA). Immunoreactive somata and fibers in the central nervous system were counted by examining the immunostained serial sections of five or six earthworms. Preparations were observed under an Olympus-BHA fluorescence micro-

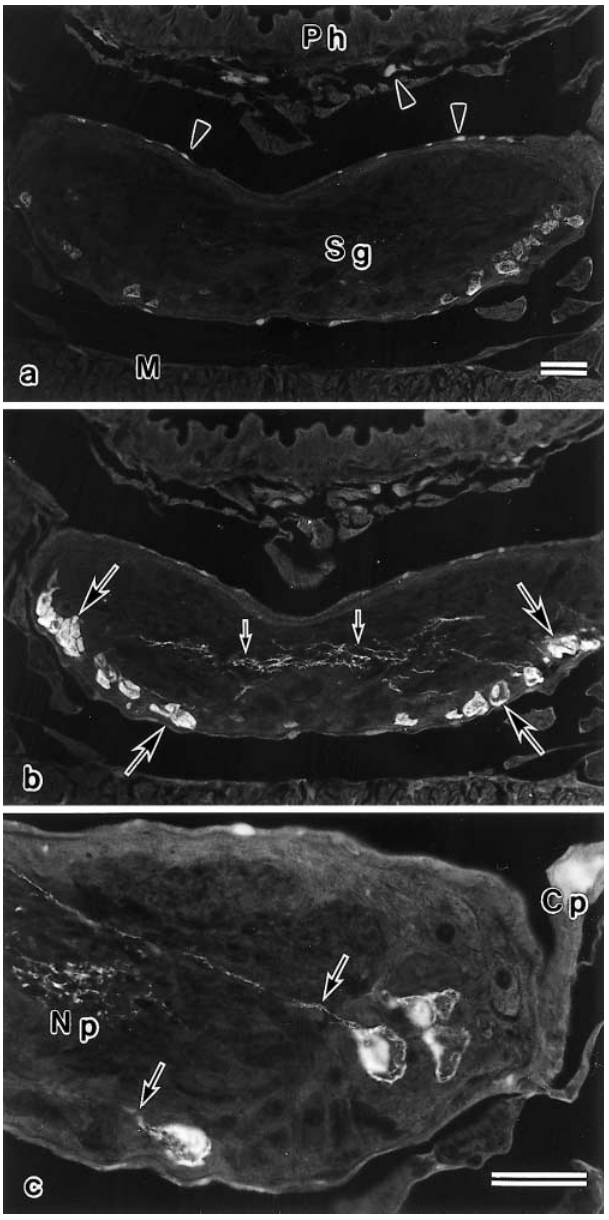

Fig. 1. Light micrographs of transverse sections through the subesophageal ganglion (Sg) immunohistochemically stained with anti-annetocin antiserum preabsorbed with an excess amount (1 mg/ml) of annetocin antigen (**a**) and with the antiserum without the antigen (**b** and **c**) by the indirect immunofluorescence method. (**a**) Nonspecific staining is seen in the capillaries (arrowheads). Ph, pharynx; M, muscle layer of body wall. (**b**) There are numerous somata (large arrows) of the annetocin-IR cells at the ventro-lateral side and numerous fibers (small arrows) of the annetocin-IR cells in the central region of the ganglion. (**c**) Each immunopositive cell sends an axon (arrows) to the core region, the neuropile (Np). Cp, capillary. Scale bars: 50 μm.

scope. Control experiments were run without anti-annetocin antiserum or using the antiserum preabsorbed for 24 hr with an excess amount of annetocin (1 mg/ml antiserum).

Electron microscopy

For immunogold staining, small tissue pieces of the anterior body were immersed in the phosphate-buffered 4% paraformaldehyde-0.05% glutaraldehyde solution for 4 hr at 4°C. After rinsing in the phosphate buffer, they were dehydrated in a graded ethanol series to 70% ethanol at 4°C, and embedded in LR White acryl resin (London Resin Co., Basingstoke, UK). Ultrathin sections of the specimens were stained by the indirect immunogold technique using anti-annetocin antiserum (diluted 1/500) and 10 nm gold-conjugated goat anti-rabbit IgG antibody (diluted 1/100, Zymed Lab., San Francisco, CA, USA).

For conventional electron microscopy, small pieces of the anterior part of earthworms were routinely fixed in the phosphate-buffered 2.5% glutaraldehyde fixative solution for 2 hr at 4°C, and postfixed in 1% osmic acid-0.1 M phosphate buffer (pH 7.4) for 1 hr at 4°C. After washing in the same buffer, they were dehydrated in a graded ethanol series and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Both the sections for immunogold staining and conventional electron microscopy were observed with a Hitachi HU-12 transmission electron microscope.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The significant differences were analysed by multiple *t*-test.

RESULTS

Immunohistochemistry

All tissues constituting the earthworm body anterior to the 55th segment were immunohistochemically examined on serial sections with an anti-annetocin antiserum, and annetocin-immunoreactive (IR) cells including their fibers were detected only in the central nervous system between the 3rd and 30th segments. In the both control experiments without the primary antiserum and with the antiserum preabsorbed with an excess amount of annetocin, the immunostaining was disappeared or diminished except the signal in capillaries (Fig. 1a). The positive signal observed in blood vessels with the preabsorbed antiserum (Fig. 1a) was reasonably assumed to be non-specific one, because it was also observed in preparations treated with only secondary antibody (without primary antibody) and because the signal in somata in the subesophageal ganglion (Fig. 1b) was scarcely detected with the preabsorbed antiserum (Fig. 1a). Thus, we decided that the specific immunoreactivity was only the signal that was largely decreased in intensity when treated with the preabsorbed antiserum.

In the subesophageal ganglion, numerous somata (34.3 ± 7.9, n = 6) of annetocin-IR cells were located mainly at the ventro-lateral side, and annetocin-IR fibers were observed in the core region (Fig. 1b). Some somata of annetocin-IR cells were situated in a row or formed clusters, and others were scattered. Some annetocin-IR cells possessed a process directing to the core region (the neuropile) of the subesophageal ganglion, displaying unipolar-like structure (Fig. 1c). In the cerebral ganglion two pairs of annetocin-IR cells were seen at each lateral side (data not shown). No annetocin-IR somata were detected in any ventral ganglion or ventral nerve cord (Fig. 2). In transverse sections of the ventral ganglia and the ventral nerve cords of different segments, a pair of clusters of annetocin-IR fibers was observed in their core region, neuropile, but not in the peripheral region (Fig. 2). As shown in Figs. 2 and 3, annetocin-IR fibers were significantly more numerous in the 4-6th segment (72.2 ± 11.9) than in the 12-15th

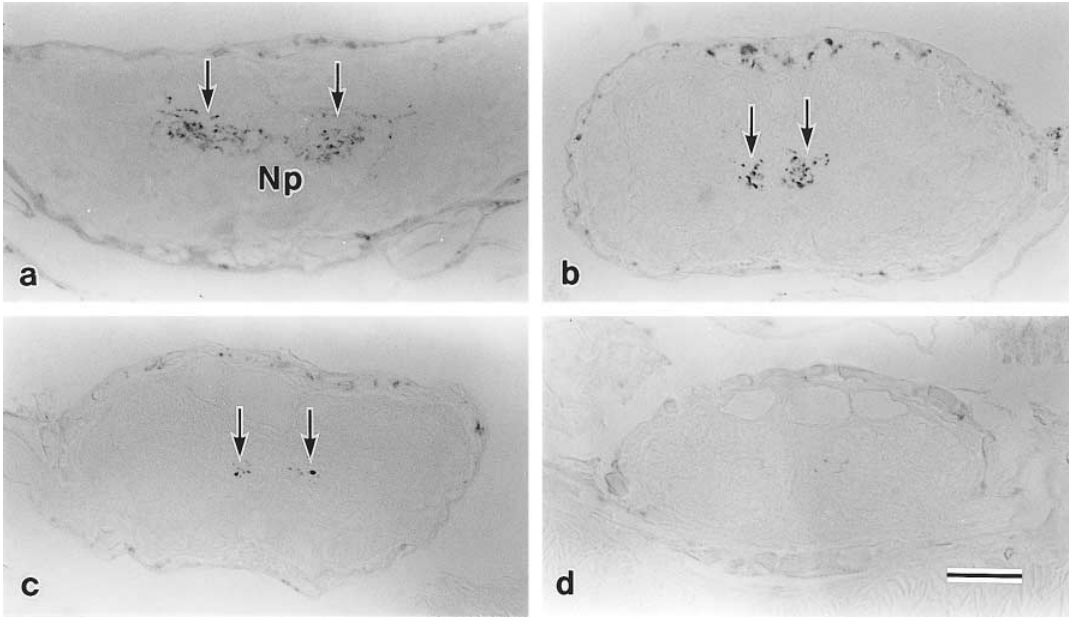

Fig. 2. Light micrographs of transverse sections through the ventral ganglion or ventral nerve cord in 4-6th segment (**a**), 12-15th segment (**b**), 25-30th segment (**c**) and 50-55th segment (**d**) immunohistochemically stained by the ABC method. A pair of clusters of annetocin-IR fibers (arrows) is seen in the core regions (the neuropile; Np) of the ventral ganglion or nerve cord in **a**, **b** and **c**, but not in **d**. Non-specific staining is seen in the capillaries. Scale bar: 50 μm.

segment (42.2 ± 12.6) ($P < 0.005$). Similarly, the number of the IR fibers of the 12-15th segment was larger than that of the 25-30th segment (22.6 ± 6.3) ($P < 0.05$). In the ventral ganglia and ventral nerve cord between 31st and 55th segments, annetocin-IR fibers completely lost (Fig. 2d).

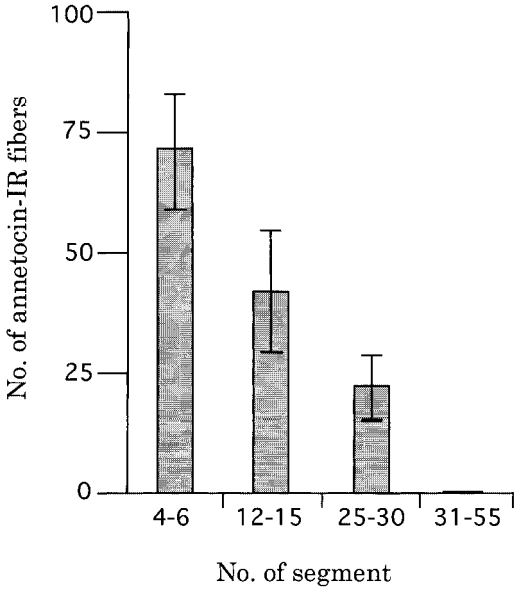

Fig. 3. Numbers of annetocin-IR fibers in the ventral ganglion or ventral nerve cord of 4-6th segment, 12-15th segment, 25-30th segment and 31-55th segment. Each bar represents the mean ± standard deviation measured in five animals.

Electron microscopy

The annetocin-secretory cells were identified by the immunogold staining with the anti-annetocin antiserum, and immunogold particles were detected on the pale vesicles in the somata (Fig. 4a) and fibers (Fig. 4b) of the immunopositive cells in the subesophageal ganglion. The immunopositive vesicles were 200-250 nm in diameter. In conventional electron microscopy, these vesicles were homogeneous and contained moderately electron dense material (Figs. 5, 6). The annetocin-secretory cells possessed a euchromatic nucleus, well-developed rough endoplasmic reticulum and well-developed Golgi apparatus, and were usually surrounded by several layers of glia cells (Fig. 6a). In some immunopositive cells, the positive signal was not observed on the intracellular structures such as nucleus and rough endoplasmic reticulum. An axonal area protruded from somata of the annetocin-secretory cells was rich in microtubules (Fig. 6b). The same type of the vesicles was detected in some fibers within the neuropile of the subesophageal ganglion (Fig. 6c). These fibers were also visible in the neuropile of the ventral ganglia and nerve cord between 4th and 30th segments. At the ventral side of the subesophageal ganglion, neurohemal-like structure was observed, in which the somata and fibers of the annetocin-secretory cells were partially surrounded by thin layer of glia

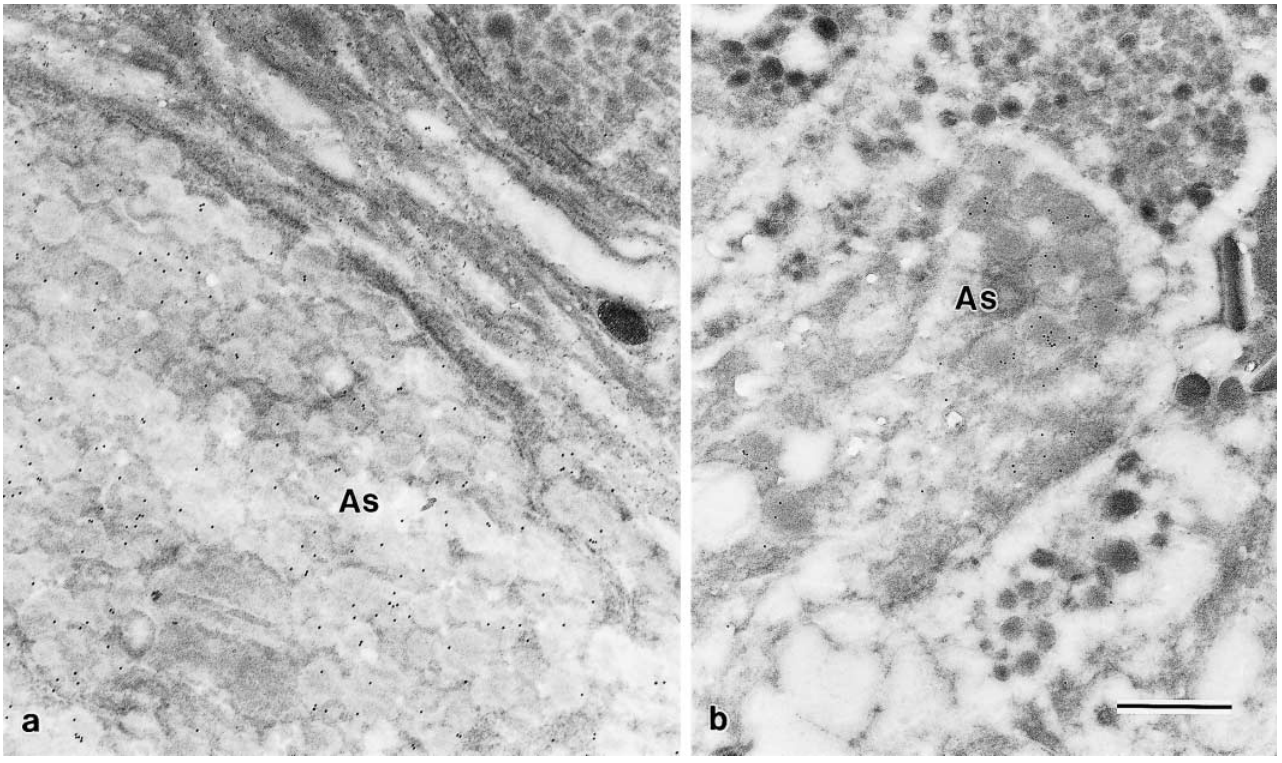

Fig. 4. Immunogold labeling for anti-annetocin antiserum of transverse sections through the somata (**a**) of the immunopositive cell at the ventral side and the immunoreactive fibers (**b**) in the neuropile of the subesophageal ganglion. Immunogold particles are detected on the pale vesicles in the somata and fibers of the annetocin-secretory cells (As) but not on the electron dense vesicles in other types of neurons or fibers. Scale bar: 0.5 μm.

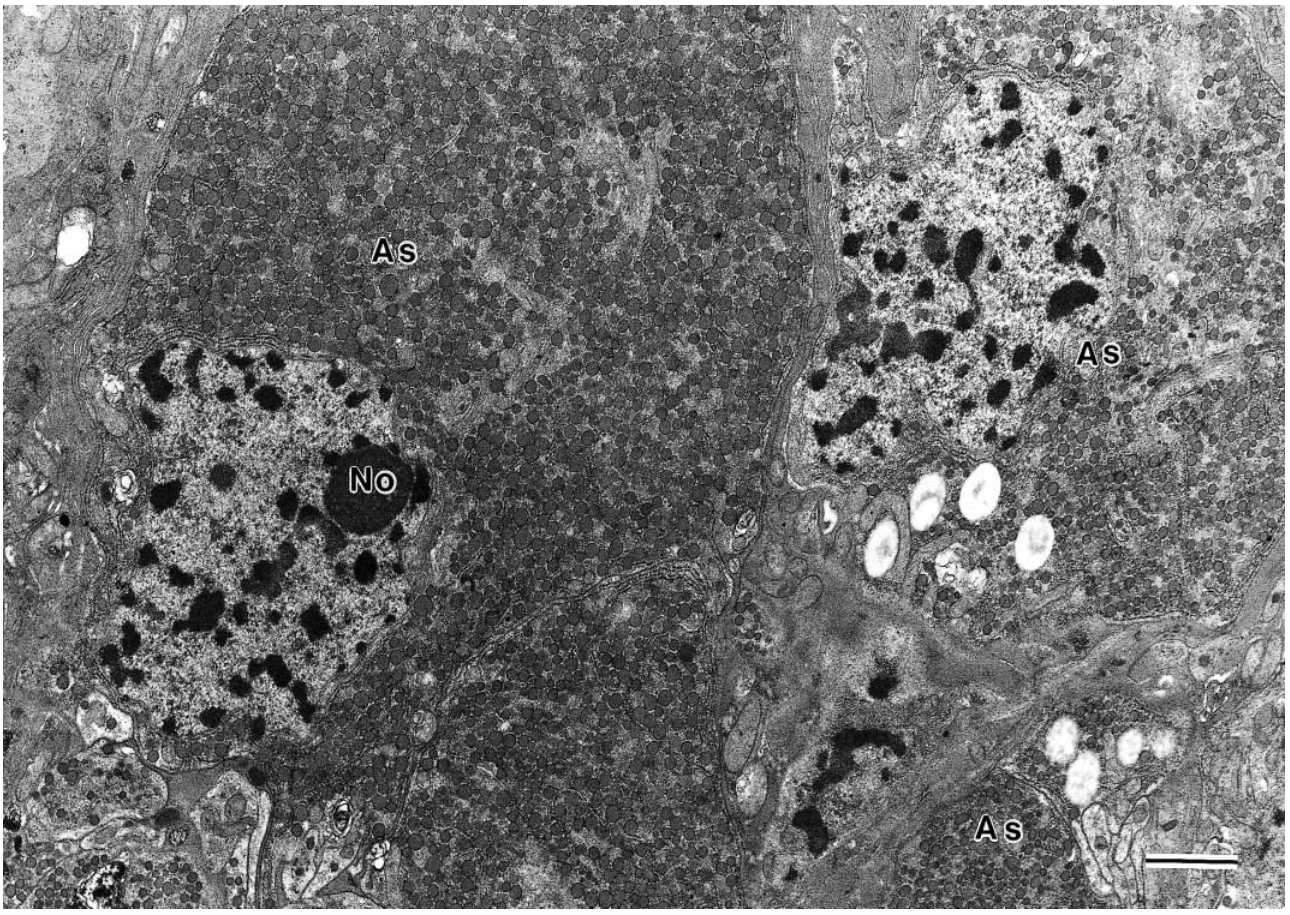

Fig. 5. Electron micrograph of a section through the somata of the annetocin-secretory cells (As) in the subesophageal ganglion. A euchromatic nucleus and a nucleolus (No) are seen in an annetocin-secretory cell. Scale bar: 1 μm.

cells or directly exposed to the coelomic space without any investment of glia cells (Fig. 6d). Muscle cells and capillaries were observed near this structure.

DISCUSSION

We have previously isolated annetocin, an oxytocin-related nonapeptide, from the extract of whole body of the earthworm *Eisenia foetida* (Oumi *et al*., 1994), and have demonstrated that this peptide exerts potent modulatory effects on the earthworm gut motility (Ukena *et al*., 1995) and also induces *in vivo* egg-laying behaviors in *E. foetida* (Oumi *et al*., 1996). The present study includes the results of immunohistochemical localization of annetocin-IR materials, identification of the annetocin-secretory cells by immunogold labeling, and morphological characterization by electron microscopy.

As to annelids, evidence on neurosecretion has been first reported by Scharrer and Scharrer (1937), and studied by many investigators (Schmid, 1947; Herlant-Meewis, 1956, 1957; Hubl, 1956; Takeuchi, 1965, 1967; Dogra, 1967; Fujii *et al*., 1989). Ultrastructural features of the neurosecretory cells have also been studied in the central nervous system of earthworms (Aros *et al*., 1977; Golding and Whittle, 1977; Golding and May, 1982; Golding and Pow, 1988; Al-Yousuf, 1988; Al-Yousuf *et al*., 1992). Recent immunohistochemical investigations have predicted that the central nervous system in some annelids contains peptidic substances belonging to the oxytocin-vasopressin superfamily (Yahya *et al*., 1983; Kinoshita and Kawashima, 1986; Malecha *et al*., 1986; Salzet *et al*., 1993b). Actually, two peptides of this superfamily, annetocin and Lys-conopressin, have been so far identified in annelids such as the earthworm (Oumi *et al*., 1994) and the leech (Salzet *et al*., 1993a). In the present study, annetocin-immunoreactivity was detected only in the neurons in the central nervous system of the earthworm, suggesting that annetocin is a neuropeptide in *E. foetida*. In a pulmonate mollusc *Lymnaea*, it has been reported that Lys-conopressin, a vasopressin-related peptide, is located in the central nervous system and in the periphery innervating male sexual organs (Van Kesteren *et al*., 1995).

Neurohemal sites have been known in some annelids, where neurosecretory cells are partially surrounded by thin layer of glia cell or are devoid of glial investment and where neurosecretory substances are released into the coelomic space (Hagadorn, 1958; Hagadorn and Nishioka, 1961; Hagadorn *et al*., 1963; Golding and Whittle, 1977; Webb, 1980). These structures are well-developed in the cerebral ganglion of polychaetes and leeches, and are named

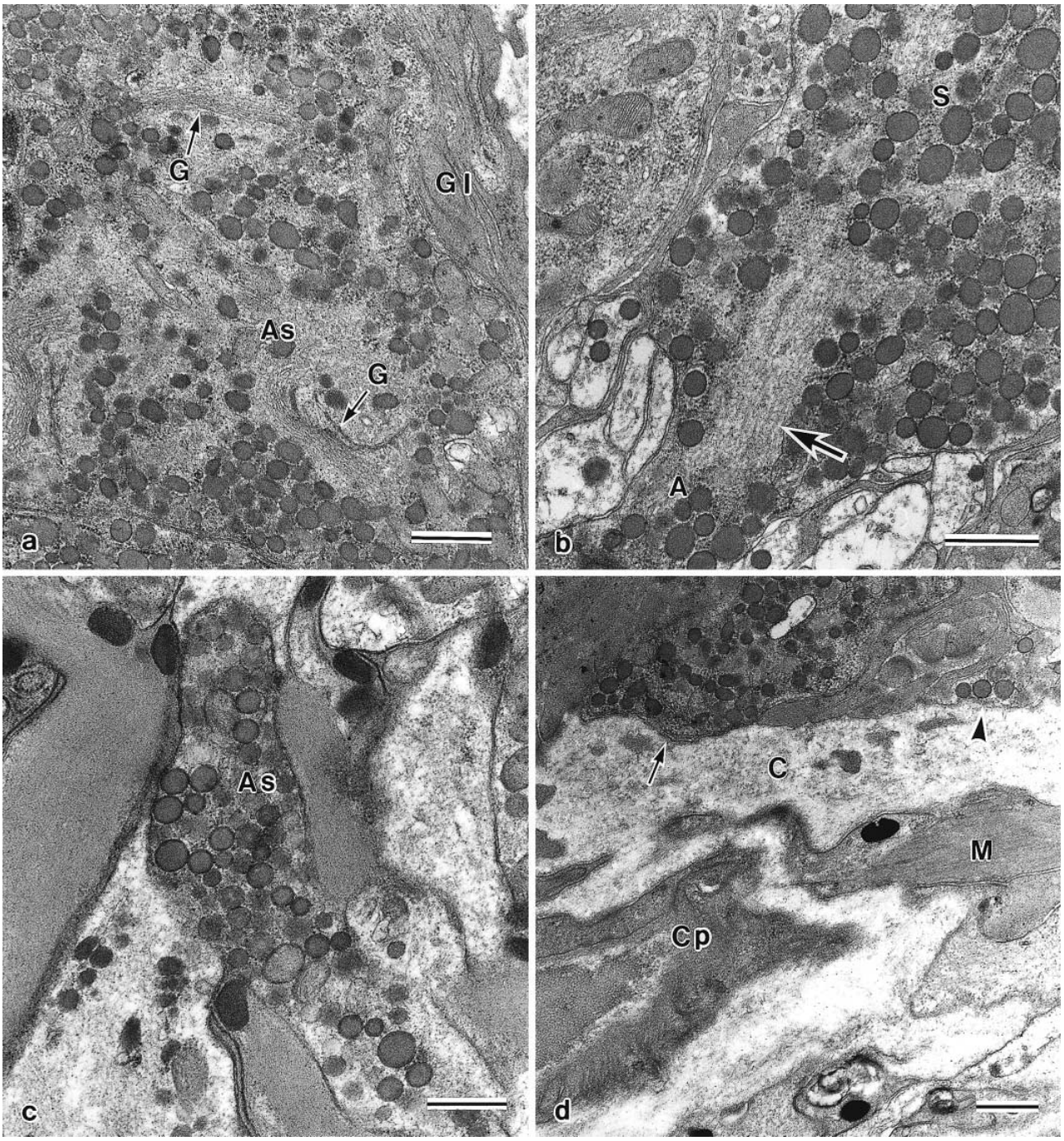

Fig. 6. Electron micrographs of sections through the somata and the fibers of the annetocin-secretory cells (As) in the subesophageal ganglion. (**a**) Golgi apparatus (G) are seen in the somata of the annetocin-secretory cell which is surrounded by processes of the glia cells (Gl). (**b**) An axon (A) is projected from the somata (S) of the annetocin-secretory cell. Numerous microtubules are seen in the axonal area (arrow). (**c**) In the neuropile a fiber of the annetocin-secretory cell comes in contact with fibers of other type of neurons. (**d**) Note that a part of the somata and fiber of the annetocin-secretory cells, which are directly faced to the coelomic space (C), is surrounded by thin layer of the glia cell (arrow) or devoid of glial investment (arrowhead). M, muscle cell; Cp, capillary. Scale bars: 0.5 μm.

"neurohemal complexes" (Golding and Whittle, 1977; Webb, 1980). In the subesophageal ganglion of the earthworm *E. foetida*, we have observed the somata and axons of the annetocin-secretory cells with a loose investment of glia, which come in direct contact with the coelomic space. This structure in the earthworm resembles the foregoing neurohemal complexes in the leeches and polychaetes. Thus, our ultrastructural data suggest that the annetocin-secretory cells secrete it to the coelomic space at the neurohemal site as a hormonal substance. This seems to be one of neurosecretions of the earthworm.

In other oligochaete annelids, ultrastructural investigations

have shown that the neuropile in the central nervous system consists of terminals of neurosecretory cells (Golding and Whittle, 1977; Al-Yousuf *et al*., 1992); within the neuropile, they observed possible features of exocytotic release of neurosecretory vesicles into extracellular space. We have demonstrated that some annetocin-IR cells send their axons to the neuropile. The number of somata found in the cerebral and subesophageal ganglia was much smaller than that of fibers in the ventral nerve cord at the 4-6th segment, suggesting the axonal arbolization of neurons in the ganglia (Telkes *et al*., 1996). In the present study, we have also clarified the localization of the annetocin-IR fibers, which are detected only in the neuropile but not in the peripheral region of the ventral ganglion or the ventral nerve cord. Further, the number of annetocin-IR fibers decreased from the rostral (4-6th) to the caudal segment (25-30th) of the earthworm. These results lead us to speculate that the annetocin-containing vesicles are axonally transported and discharged in the neuropile of each segment between 4th and 30th, although the axonal terminal of the annetocin-secretory cell was not identified ultrastructurally in this study. Thus, it is possible that annetocin functions as a neurotransmitter or modulator as well as a hormone in earthworms. In molluscs, some of neurosecretory materials are thought to act as neuromodulators (Levitan and Treistman, 1977; Branton *et al*., 1978; Fujisawa, 1996). Recently, Van Kesteren *et al*. (1995) have reported that Lys-conopressin plays a role as neuromodulator within the cerebral ganglion in *Lymnaea*, and Martínez-Padróon *et al*. (1992) have also demonstrated modulatory effects of conopressin G (Lys-conopressin) on gill behaviors in *Aplysia californica*.

Annetocin induces a series of behaviors related to egg-laying involving contraction of the body-wall muscles as well as mucous secretion from the clitellum in the earthworm *E. foetida* and the leech *Whitmania pigra* (Oumi *et al*., 1996). In the egg-laying behaviors, the anterior part of the body plays a more important role than the posterior one in these animals (Barnes, 1987). In the present study, the distribution of annetocin immunoreactive materials was limited to the anterior part of the earthworm body, confirming the physiological role of this peptide in the reproductive behavior as reported previously (Oumi *et al*., 1996). In *Lymnaea stagnalis*, it has been reported that conopressin-gene products are present in nerve fibers within the vas deferens where conopressin induces muscular contractions, suggesting an involvement of this peptide in the male reproductive behaviors (Van Kesteren *et al*., 1995). It is interesting to note that there is a functional conservation of the reproductive regulation between these invertebrate peptides and oxytocin in mammals.

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