Cell Tissue Res (1985) 240:255–260

Cell and Tissue Research

Immunocytochemical demonstration of neuropeptides and serotonin in the tapeworm *Diphyllobothrium dendriticum*

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Summary. The present immunocytochemical study concerns the distribution of four neuropeptides, FMRF-amide, vasotocin, leu-enkephalin and neurotensin, and of the bioamine serotonin in the plerocercoid larva of *Diphyllobothrium dendriticum*. Anti-FMRF-amide and vasotocin-reactivity occurs in perikarya and nerve fibres in the CNS and PNS of this worm. The peptide-containing fibres surround and seem to innervate the musculature and to terminate beneath the basal lamina of the tegument at the inner surface of the bothridia, suggesting a neurotransmitter function. Anti-leu-enkephalin reaction occurs in perikarya and fibres in the main nerve cords and in the PNS. Anti-neurotensin reactive fibres were observed in the neuropile of the nerve cords. Serotonin immunoreactivity was found in neurons in the ganglionic commissure of the brain and along the main nerve cords. This study is the first immunocytochemical identification of neuropeptides and serotonin in a parasitic flatworm and the information gained may be of importance for the development of new antihelminthics.

Key words: Neuropeptides – Serotonin – *Diphyllobothrium dendriticum* – Immunocytochemistry

Interest in the neurobiology of parasitic platyhelminths is growing (see Shaw 1981, 1982). Firstly, the parasitic platyhelminths, together with the free living turbellarians, constitute a group of lower invertebrates in which cephalisation has taken place and the neural elements have been condensed to become ganglia and large nerve cords. The worms thus represent a phylogenetically interesting group at the base of the evolutionary tree. Secondly, the development of antihelminthics is often dependent on knowledge of the nervous system (see Andrew et al. 1983). The platyhelminths lack an ordinary circulatory system and endocrine glands. Consequently the nervous system has to play an important integrative role.

The nervous system of the tapeworm *Diphyllobothrium dendriticum* consists mainly of peptidergic and aminergic neurons (Gustafsson and Wikgren 1981a, b, c). The peptidergic neurons occur at the surface of the lateral ganglia of the brain with processes extending into the connecting commissure. They furthermore occur along the main nerve cords and in the peripheral nerve bundles in the cortical parenchyma. The peptidergic neurons are rapidly activated during the transfer of the plerocercoid larvae from the poikilothermic intermediate fish host to the homeothermic final host (Gustafsson and Wikgren 1981b). Release of peptidergic neurosecretory material takes place beneath the basal lamina of the tegument in connection with this change of the host (Gustafsson and Wikgren 1981c). The aminergic neurons are located in the commissure between the two lateral ganglia of the brain and along the main nerve cords of the strobila. The perikarya send long processes to the peripheral nerve net in the cortical parenchyma.

Recently peptidergic neurons have been demonstrated in a number of invertebrates with antisera to vertebrate peptides. These results suggest that peptides have a wide distribution in the animal kingdom (Scharrer 1978; Boer et al. 1980). To further document this point the distribution of four neuropeptides, FMRF-amide, vasotocin, leu-enkephalin and neurotensin, in the plerocercoid larva of the tapeworm *Diphyllobothrium dendriticum* was studied. Furthermore, the distribution of serotonin was investigated.

Materials and methods

Plerocercoid larvae of *Diphyllobothrium dendriticum* (Cestoda, Pseudophyllidae) were obtained from whitefish (*Coregonus lavaretus*) from Lake Pyhäjärvi, SW Finland. The larvae were excised from the stomach wall and fixed over night at +4° C in either 4% paraformaldehyde (PF) or 0.5% glutaraldehyde (GA) in phosphate buffer at pH 7.6. The worms were embedded in histowax and 6-µm sections were cut. Pseudoperoxidase activity was inhibited by 0.3% H_2O_2 in H_2O (30 min). Unspecific background staining was inhibited by incubation in 1% Bovine Serum Albumin (BSA) in Tris buffered saline (30 min). Subsequently the sections were immunocytochemically stained with the unlabelled antibody enzyme peroxidase-antiperoxidase (PAP) method (Sternberger 1974). The following antisera were used (dilutions in parentheses): anti-FMRF-amide (1:500, 1:1000); anti-vasotocin (1:500); anti-leu-enkephalin (1:500, 1:1000); anti-neurotensin (1:500, 1:1000) and anti-serotonin (1:500, 1:2000).

Anti-leu-enkephalin was kindly donated by Dr. M. Pelto-Huikka, University of Tampere, Finland and anti-neurotensin by Dr. J.M. Polak, Royal Postgraduate Medical School, London. Furthermore, anti-leu-enkephalin and anti-serotonin were purchased from Immunonuclear. The

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incubation time was 24 h. The sections were then incubated with swine-anti-rabbit serum (1:10) (DAKOPATTS) and with PAP (1:80) (DAKOPATTS). The peroxidase was made visible with a solution of 0.03% 3,3-diaminobenzidine in 0.05 M Tris HCl at pH 7.5, containing 0.01% H_2O_2. About 10 worms were stained with each antiserum, except vasotocin, where only 2 worms were used. The controls for specificity included (1) ommitting primary antibody, (2) use of non-immune serum (NRS) and (3) in the case of anti-FMRF-amide and anti-vasotocin, solid-phase absorption with the homologous antigen.

Results

Controls

The various controls performed gave no positive immunoreaction. No differences in immunoreactivity were observed after fixation in PF and GA.

Anti-FMRF-amide

In the scolex of *D. dendriticum* a distinct pattern of anti-FMRF-amide positive fibres was observed. They surround the bothridial muscles and extend into the top of the scolex. At the level of the bottom of the frontal pit, where the two bothridia unite and the two lateral ganglia appear, a considerable amount of anti-FMRF-amide positive fibres occur. They surround the ganglia as thick bundles. Only few fibres extend into the ganglionic commissure (Fig. 1). In the more basal parts of the scolex the anti-FMRF-amide positive fibres form thick bundles around the main nerve cords with distinct extensions to the nerve net surrounding the bothridial muscles (Fig. 2). The longitudinal muscles, which run from the basal part of the scolex backwards, are surrounded by anti-FMRF-amide positive fibres of the peripheral nerve net (Fig. 3). The two main nerve cords and the peripheral nerve bundles are interconnected by way of distinct anti-FMRF-amide positive fibres.

Anti-FMRF-amide positive perikarya (size 10 × 5 µm) were observed at regular distances along the main nerve cords beginning with the basal parts of the scolex (Fig. 4). Positive perikarya were also observed in the peripheral nervous system (PNS) (Fig. 5). They are interconnected by way of fibres, thus forming a continuous ring-like nerve net.

Part of the anti-FMRF-amide positive fibres terminate immediately beneath the basal lamina of the surface tegument. The highest concentration of positive terminals occur at the inside of the bothridia in the most anterior part of the scolex (Fig. 6). In the basal parts of the scolex, on the outer surface of the bothridia (Fig. 2), and along the rest of the body only few positive terminals were observed. The FMRF-amide positive terminals do not penetrate the basal lamina of the tegument.

Anti-vasotocin

The anti-vasotocin positive fibres in the worm form a network similar to that of the anti-FMRF-amide positive fibres (Figs. 7, 8). The amount of positive fibres is, however, smaller. Only few positive perikarya were observed (Fig. 9).

Anti-leu-enkephalin

Anti-leu-enkephalin positive fibres were observed in the periphery of the two lateral ganglia and to a lesser extent in the connecting commissure. In the basal parts of the scolex the amount of positive fibres is higher. They are located in the two main nerve cords and in the peripheral nerve bundles (Fig. 10).

In the CNS anti-leu-enkephalin positive perikarya (size 10 × 5 µm) were observed in the basal parts of the scolex and from thereon throughout the body adjacent to the two main nerve cords of the worm (Fig. 11). Furthermore positive perikarya occur in the PNS associated with the longitudinal nerve bundles in the cortical parenchyma (Fig. 12). On an average in each section one to two perikarya per main nerve cord and five perikarya in the cortical parenchyma were observed.

A few anti-leu-enkephalin positive terminals were observed beneath the basal lamina of the tegument. Positive terminals are not restricted to the scolex region but occur along the surface of the worm.

Figs. 1–6. IC reactivity to anti-FMRF-amide in *Diphyllobothrium dendriticum* plerocercoid

Fig. 1. Cross section of scolex at level of ganglionic commissure (*gc*). Nerve fibres surround two ganglia (*g*) and bothridial muscles (*m*). PF fix. Bar = 60 µm. × 250

Fig. 2. Fibres extending from nerve cord (*c*) in basal part of scolex to peripheral nerve net (*pn*). Note axon terminal (*arrow*) beneath basal lamina of tegument (*t*). PF fix. Bar = 30 µm. × 625

Fig. 3. Cross section of plerocercoid midbody. Nerve fibres extend from main nerve cord (*c*) through main longitudinal muscle layer (*lm*) to peripheral nerve net (*pn*). Note peripheral nerve bundles (*arrows*). GA fix. Bar = 30 µm. × 625

Fig. 4. Perikarya in main nerve cord (*c*). GA fix. Bar = 15 µm. × 1300

Fig. 5. Perikaryon (*arrow*) and fibres in peripheral nerve net (*pn*); *lm* longitudinal muscle fibres. GA fix. Bar = 15 µm. × 1300

Fig. 6. Cross section of bothridium showing fibres surrounding bothridial muscles (*m*). Note positively stained axon terminals (*arrows*) beneath basal lamina of tegument (*t*) on inner side of bothridium. PF fix. Bar = 30 µm. × 625

Figs. 7–9. IC reactivity to anti-vasotocin in *D. dendriticum*. PF fix

Fig. 7. Fibres surrounding main nerve cords (*c*) and bothridial muscles (*m*) in basal part of scolex. Bar = 60 µm. × 250

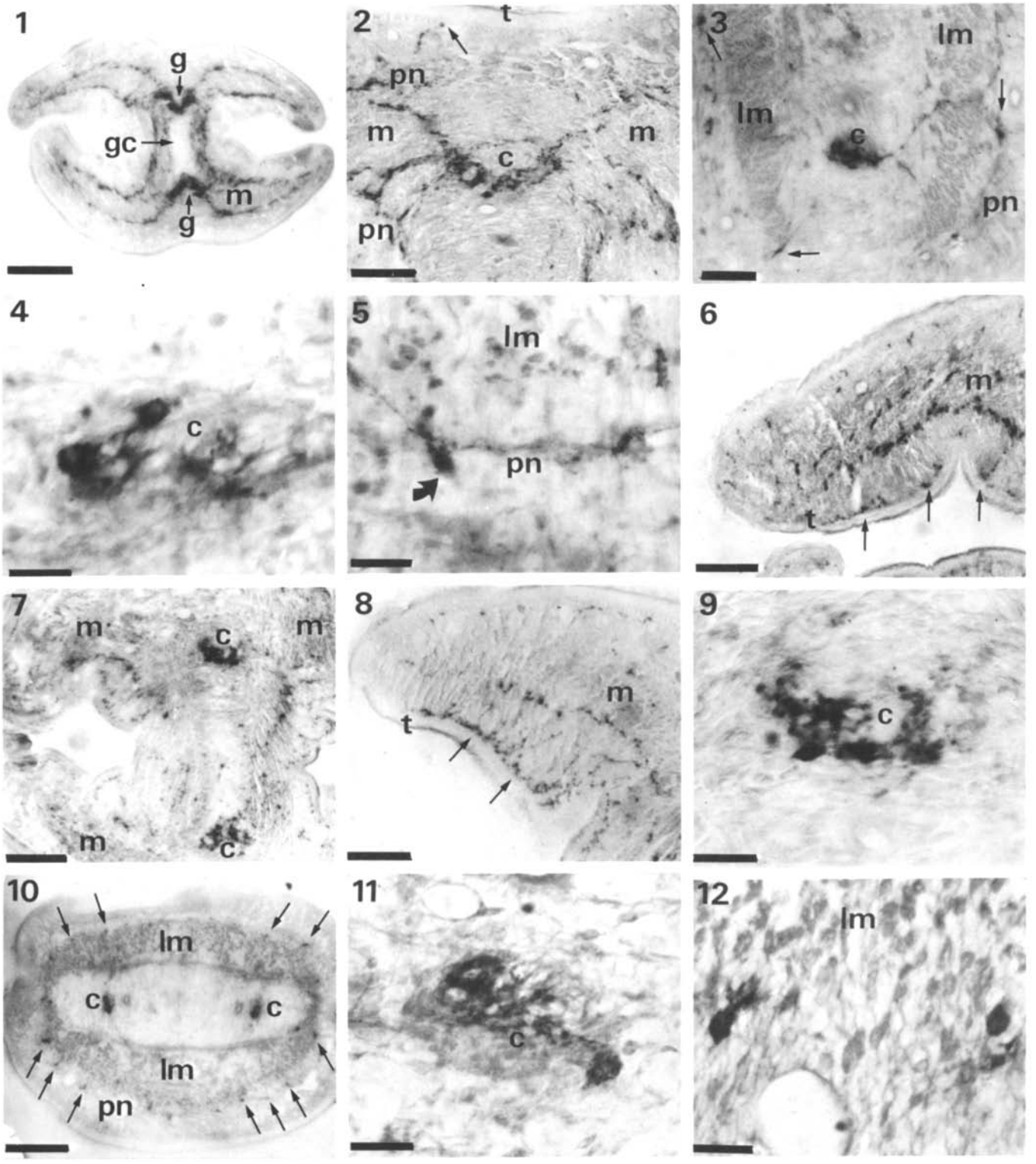


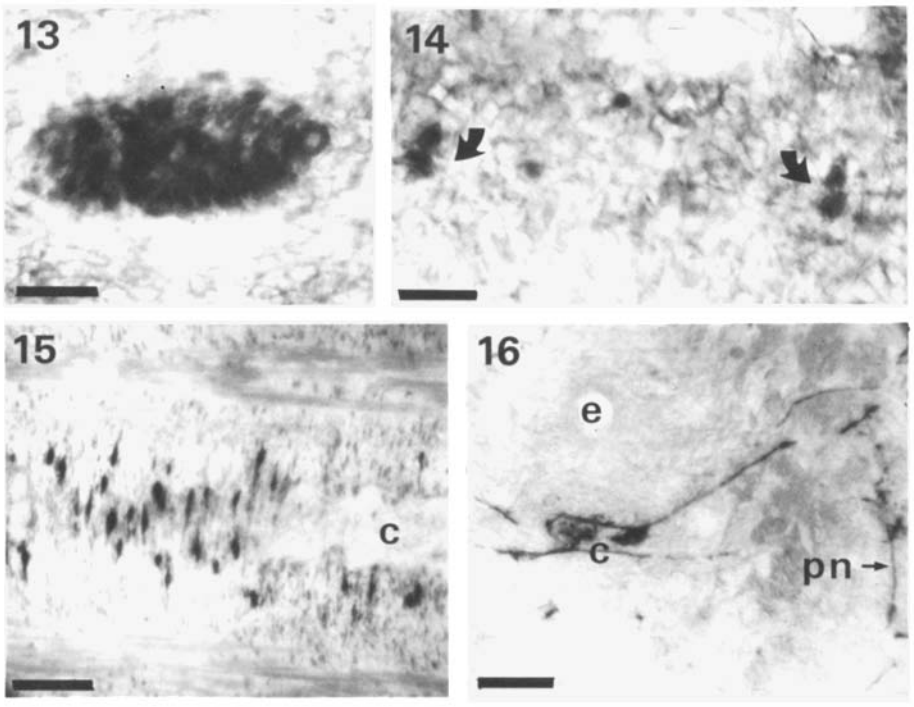
Fig. 8. Fibres and axon terminals (*arrows*) beneath basal lamina of tegument (*t*) in bothridium; *m* bothridial muscles. Bar = 30 μm. × 625

Fig. 9. Perikarya in main nerve cord (*c*). Bar = 15 μm. × 1300

Figs. 10–12. IC reactivity to anti-leu-enkephalin in *D. dendriticum*. GA fix

Fig. 10. Anti-leu-enkephalin positive fibres surround main nerve cords (*c*) and extend through longitudinal muscle layer (*lm*) to peripheral nerve net (*pn*), where they form peripheral nerve bundles (*arrows*). Bar = 150 μm. × 130

Figs. 11, 12. Anti-leu-enkephalin positive perikarya and fibres in main nerve cord (*c*) (**11**) and peripheral nerve net (**12**); *lm* longitudinal muscle fibres. Bar = 15 μm. × 1300



Figs. 13–14. IC reactivity to anti-neurotensin in main nerve cord (**13**) and peripheral nerve bundles (*arrows*) (**14**) of *D. dendriticum*. GA fix. Bar = 15 μm. × 1300

Figs. 15–16. IC reactivity to anti-serotonin in *D. dendriticum*. PF fix

Fig. 15. Longitudinal section showing main nerve cord (*c*) with perikarya. Bar = 60 μm. × 250

Fig. 16. Bipolar perikarya in main nerve cord (*c*) and nerve fibres in peripheral nerve net (*pn*); *e* excretory duct. Bar = 30 μm. × 625

Anti-neurotensin

Anti-neurotensin positive fibres were observed in the main nerve cords and in the peripheral nerve bundles (Figs. 13, 14). No positive perikarya were observed.

Anti-serotonin

Positive perikarya (size 14 × 7 μm) and fibres were found in abundance in the nervous system of *D. dendriticum*. The perikarya are located in the ganglionic commissure and along the main nerve cords (Fig. 15). They are most often bipolar but occasionally multipolar. Long fibres with varicosities extend from the perikarya through the longitudinal muscle layer to the peripheral nerve net (Fig. 16).

Discussion

The wide distribution of biologically active peptides (BAP) in invertebrates is by now well documented (see e.g. Greenberg and Price 1983) and supports the view that they are phylogenetically ancient substances (Scharrer 1978) acting as neurotransmitters, neuromodulators and neurohormones (Haynes 1980; Cottrell et al. 1983). Information on the occurrence of vertebrate neuropeptides in platyhelminths is limited to a few reports on planarians (Bautz et al. 1980; Schilt et al. 1981; Carraway et al. 1982; Venturini et al. 1983). This IC study is the first identification of vertebrate-like neuropeptides in a parasitic flatworm. Positive reactions were obtained with antisera to FMRF-amide, a peptide first isolated from the bivalve clam *Macrocallista nimbosa* (Price and Greenberg 1977), and furthermore with antisera to the vertebrate peptides vasotocin, leu-enkephalin and neurotensin. In addition, the distribution of serotonin was studied. Only part of the cells and nerve terminals previously stained with paraldehyde fuchsin (Gustafsson and Wikgren 1981a, b), appeared after IC staining with the four peptide antisera used in this study. A similar situation has been reported by Schot et al. (1981) who found that only part of the AB/AY and phloxin positive cell bodies in *Lymnaea stagnalis* appeared after IC staining with antisera to 15 BAPs.

FMRF-amide

FMRF-amide is a phylogenetically old neuropeptide that occurs already in Coelenterata (Grimmelikhuijzen 1983). It has also been localized in the microturbellarian *Microstomum lineare* (Reuter et al. 1984). In *D. dendriticum* positive perikarya occur from the basal parts of the scolex backwards along the central and peripheral nerve cords. The multitude of positive fibres, which extend to the top of the scolex, and the small amount of positive cells indicate that the FMRF-amide containing cells are multipolar with long branching fibres. The anti-FMRF-amide positive fibres are closely associated with the musculature of the worm. The large bothridial muscles, by which the adult worm attaches itself to the intestinal wall of the vertebrate host, are especially well innervated by FMRF-amide positive fibres. Also the body musculature, i.e., the thick longitudinal and the transverse muscle bundles, are innervated by FMRF-amide containing fibres. Anti-FMRF-amide containing terminals occur frequently in the subtegumental region on the inside of the bothridia. This surface is in intimate contact with the intestinal villi of the host and is supposedly important as a sensitive and absorptive surface. In molluscs FMRF-amide and related substances have been shown to act as muscular neurotransmitters and neuromodulators (Greenberg and Price 1980; Schot and Boer 1982; Cottrell et al. 1983; Schot et al. 1983; Geraerts et al. 1984). The close association between the anti-FMRF-amide containing fibres and the musculature suggests a similar function in *D. dendriticum*.

Vasotocin

Our results with anti-vasotocin show great similarities with those obtained with anti-FMRF-amide. Schot et al. (1983) found a similar situation in *Lymnaea stagnalis*, where part of the FMRF-amide positive perikarya and fibres also stained with anti-vasotocin. Whether this double staining is the result of cross reaction of the antisera or whether in these elements two antigenic determinants are present can not be concluded from these results.

Leu-enkephalin

Enkephalin immunoreactivity has been shown in annelids (Alumets et al. 1979; Zipser 1980), molluscs (Schot et al. 1981; Greenberg and Price 1983) and planarians (Venturini et al. 1983). In *D. dendriticum* both perikarya and fibres in the CNS and PNS stained clearly with antisera to leu-enkephalin. In molluscs and planarians a relationship between enkephalins and the dopaminergic system has been demonstrated (Stefano and Catapane 1979; Juel 1982; Venturini et al. 1983). In cestodes, including *D. dendriticum*, serotonin is the only occurring biogenic amine (see Gustafsson and Wikgren 1981a). Voigt et al. (1981) and Greenberg et al. (1981) have studied the close structural and functional relationship between FMRF-amide and the precursor peptides for enkephalins. The last mentioned authors suggest a coevolution of the two calsses of neuropeptides from an ancestral peptide. In the present study immunoreactivity for both peptides has been shown in the nervous system of *D. dendriticum*.

Neurotensin

Carraway et al. (1982) have studied the evolutionary distribution of neurotensin-like material and have found it in all vertebrate and invertebrate classes. They are of the opinion that neurotensin "has an ancient lineage and that it participates in processes basic to animal life". In *D. dendriticum* immunoreactivity for neurotensin was demonstrated in the neuropile of the CNS and PNS. The lack of positive perikarya indicates either that they contain the neuropeptide in too low concentration or that a transformation takes place in the neuronal fibres by which the active neuropeptide is split from a larger immunonegative propeptide molecule.

Serotonin

Within the Platyhelminthes the turbellarians and the trematodes contain both catecholamines and serotonin. The cestodes contain only serotonin (see Gustafsson and Wikgren 1981a). The results of this study confirm the earlier observations on the distribution of aminergic neurons in *D. dendriticum* (Gustafsson and Wikgren 1981a). Gustafsson (1984) has described synapses between aminergic fibres and a multitude of postsynaptic elements including muscle cells and peptidergic neurons. Based on the morphology of the synapses she suggests an excitatory nature for them. Ribeiro and Webb (1983) discuss the function of serotonin as a possible excitatory neurotransmittor in *Hymenolepis diminuta*. In molluscs serotonin and FMRF-amide are reported to have similar (excitatory) effects on heart musculature (Greenberg and Price 1980), although no clear relationship between the two substances has yet been demonstrated.

Acknowledgements. We want to thank Professor L. Pelliniemi for technical advice, Mr. J. Hindström for technical assistance, Stiftelsens för Åbo Akademi forskningsinstitut and Styrelsen för Åbo Akademi for financial support.

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Accepted November 15, 1984