The brain of the Nemertodermatida (Platyhelminthes) as revealed by anti-5HT and anti-FMRFamide immunostainings

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Abstract The taxa Nemertodermatida and Acoela have traditionally been considered closely related and classified as sister groups within the Acoelomorpha Ehlers 1984 (Platyhelminthes). Recent molecular investigations have questioned their respective position. In this study, the 5-HT and FMRFamide immunoreactivity (IR) in the nervous system of two nemertodermatids, *Nemertoderma westbladi* and *Meara stichopi*, is described. The 5-HT immunoreactive pattern differs in the two nemertodermatids studied. In *M. stichopi*, two loose longitudinal bundles of 5-HT-immunoreactive fibres and an basi-epidermal nerve net were observed. In *N. westbladi* the 5-HT-IR shows a ring-shaped commissural structure, different from the commissural brain of acoels. In both nemertodermatids, FMRFamide immunoreactive nerve fibres followed the 5-HT-immunoreactive fibres. It is demonstrated that the Nemertodermatida have neither a ‘commissural brain’ structure similar to that of the Acoela, nor a ‘true’, ganglionic brain and orthogon, typical for other Platyhelminthes. The question of the plesiomorphic or apomorphic nature of the nervous system in Nemertodermatida cannot yet be answered. The neuroanatomy of the studied worms provides no synapomorphy supporting the taxon Acoelomorpha. © 2000 Harcourt Publishers Ltd

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Introduction

The Nemertodermatida comprises a dozen species of marine turbellariomorph worms, free-living or symbiotic in holoturians. The phylogenetic position of the taxon has recently been discussed in detail by Lundin (1999). The Nemertodermatida was formerly included in the Acoela (Steinböck, 1930–31; Westblad 1937; 1949). Later, Karling (1940, 1974) classified them in the separate taxon Nemertodermatida, chiefly owing to the presence of a distinct gut lumen. The uniflagellar sperm structure, unusual for flatworms, further supported this view (Tyler & Rieger 1975, 1977; Rieger et al. 1991). The features of spermiation of a nemertodermatid species indicate that the uniflagellar sperm type of the Nemertodermatida is not a secondary reduction from an acoel-like sperm type (Lundin & Hendelberg 1998). These observation support the status of the Nemertodermatida as a separate taxon beside the Acoela. However, similarities between the two taxa in epidermal ciliation (Smith et al. 1986; Lundin 1997), glandular and sensory structures (Smith & Tyler 1986; Ehlers
Meara stichopi

Specimens of Westblad, 1949 were obtained

I. Animals

Materials and methods

I. Animals

Specimens of Meara stichopi Westblad, 1949 were obtained from the intestine of the holothurian Sichopus tremulus (Gunnerus, 1767) sampled at 400 m depth in the Raunefjord fiord near Bergen (Norway). Specimens of Nemertoderma westbladi Steinbock, 1938, were collected at the depth of 30–50 m from muddy bottoms in the vicinity of Kristineberg Marine Research Station at the Gullmar Fjord on the Swedish west coast.

II. Immunocytochemistry

Specimens of M. stichopi and N. westbladi were fixed in Stefanini’s fixative (2% paraformaldehyde and 15% picric acid in 0.1 M Na-phosphate buffer) at pH 7.6, stored for several weeks in fixative at +4°C, and rinsed for 24–48 h in 0.1 M Na-phosphate buffer (pH 7.6) containing 10–20% sucrose. The worms were either handled as whole mounts on poly-L-lysine coated glass slides or embedded in Tissue Tec and sectioned at 10–20 μm on a Bright cryostat. The sections were placed on gelatin coated glass slides, allowed to dry, and frozen at –70°C. Prior to staining the sections were thawed and immersed in Phosphate-buffer saline (PBS) containing 1% bovine serum albumin (BSA) and 0.2% Triton X-100 (PBS-T). Immunostaining was performed according to the indirect immunofluorescent method of Coons et al. (1955).

Staining with one primary antibody

The concentrations for the primary antibodies were 1:500 in all stainings. Incubations were performed with goat anti-5-HT (INCSTAR) antiserum and/or rabbit antibodies against the invertebrate neuropeptide FMRFamide (INCSTAR). The incubation time was 36–48 h. Thereafter, the preparations were rinsed 3 × 5 min in PBS-T and incubated for 1–2 h with the secondary antibody, either TRITC-labelled rabbit-anti-goat or FITC-labelled swine-anti-rabbit immunoserum (Tago) (dilution 1:40), washed 3 × 5 min in PBS-T and incubated for 1–2 h with the secondary antibody, either TRITC-labelled rabbit-anti-goat or FITC-labelled swine-anti-rabbit immunoserum (Tago) (dilution 1:40), washed 3 × 5 min in PBS, mounted in 50% glycerol in PBS and stored in the dark at –20°C.

Double-stainings

Double stainings were done by 36–48 h long incubation in a mixture of the two antibodies, followed by consecutive incubations with TRITC-labelled rabbit anti-goat and FITC-labelled swine anti-rabbit immunoserum. The controls for specificity included: (1) omitting the primary antibody, and (2) using non-immune serum.

III. Microscopy

The preparations were examined in a Leitz Orthoplan microscope combined with filter blocks I2 and N2. Photomicrographs were produced by an Olympus automatic photomicrograph system, model PM 10ADS (film: TMAX 400). A confocal scanning laser microscope (CSLM: LEICA TCS 4D) was used to better visualise the details of the nervous system. The max-projection option was used to obtain reconstructions from a series of optical sections.

IV. Computer Processing of Immunocytochemistry

Micrographs

Files obtained from confocal scanner microscopy were processed with Adobe Photoshop 4.0. Only the commands
Results

I Meara stichopi

a. 5-HT IR
The 5-HT immunoreactive nerve fibres are arranged at different depth (Figs 1, 2A-E, 3A–B). On a deeper level, below the muscles, two thick lateral bundles of fine beaded nerve fibres run in longitudinal direction on each side of the body. Two large 5-HT immunoreactive cells are symmetrically disposed at the beginning of the bundles (Figs 1, 2A). No other nerve cells could be detected at this level. In the frontal end of the body the fibres are loosely packed and form broad streams of numerous fibres in the middle part of the worm (Figs 1, 2A,E, 3B). In the posterior direction the number of fibres decreases and the two lateral bundles merge together just short of the posterior end of the body (Figs 1, 2C). In the anterior part of the body the longitudinal nerve bundles are interconnected by several commissures, consisting of a single nerve fibre and running across the centre of the worm (Figs 1, 2A). The main streams of fibres regularly send branches to the periphery. These branches start as small triangular bundles, but more peripherally they consist of only one fibre (Figs 1, 2A, C, E, 3B).

In addition to this parenchymal nervous system, the 5-HT IR reveals an basi-epidermal coarse meshed nerve net, forming a mantle close to the base of the epidermis (Figs 1, 2B, D, 3A). The net is composed of numerous multipolar cells, about 10 µm in diameter, connected by nerve fibres. Optical sections of the fore end of the body (Figs 2A–B, D–E) and those of the middle part of the body (Figs 3A,B) clearly demonstrate that the surface network and the parenchymal nerve bundles lie at different levels in the same body region. These two parts of Meara’s nervous system (basi-epidermal net and parenchymal nerve bundles) are interconnected by surface branches of the inner bundles.

b. FMRFamide IR
Two lateral bundles of FMRFamide immunoreactive fibres were detected below the muscles, in the parenchyma (Figs 2F, 3C). The optical sections of double-stained preparations (Figs 2D–F, 3A–C) demonstrate that the FMRFamide immunoreactive fibres are situated even deeper than the 5-HT immunoreactive parenchymal bundles, actually underlying the latter and closely following them. The FMRFamide bundles consist of numerous closely packed fibres. They are interconnected in the anterior end of the body by thin beaded fibres (Fig. 2F). No FMRFamide immunoreactive cells were visualised. The epidermis is devoid of FMRFamide IR (Fig 3C).

II. Nemertoderma westbladi

a. 5-HT IR
The 5-HT IR reveals an anterior subepidermal brain-like concentration composed uniquely of commissures (Figs 3D–G, 4, 5A,C). The commissures form two rings, stronger dorsally, one anterior to the statocyst and one posterior (Figs 3D,E, 4). Sometimes the rings seem incomplete, open...
Fig. 2  *Meara stichopi*, nervous system. **A.** 5-HT IR in the anterior end of the body. **A**—optical section at the parenchymal level. Note two lateral beaded fibre bundles (fb) interconnected by fine commissures (c) and sending branches to the periphery (pb). Two immunoreactive nerve cells (n) lie at the beginning of the bundles. **B**—optical section at basiepidermal level showing basi-epidermal coarse meshed nerve net, composed of numerous multipolar nerve cells (n) connected by nerve fibres. c—5-HT IR in the posterior end of the body. The parenchymal fibre bundles (fb) become thinner and merge together. **D-F.** Optical sections of the anterior end of the same animal, double stained with anti-5-HT (D, E) and anti-FMRFamide (F) antibodies. Closer to the surface (D) lies the nerve net of beaded fibres with neurons, while deeper (E) lie two lateral parenchymal bundles, sending branches (pb) toward the nerve net. Note the position of the statocyst (s), devoid of immunoreactive elements. Closely packed bundles of FMRFamide immunoreactive fibres (F) underlie the 5-HT immunoreactive parenchymal bundles, and closely follow them. Note the thin commissures (c), interconnecting the bundles.
Fig. 3  A–C. Meara stichopi. Optical sections of the mid-body region of the same animal, double stained with anti-5-HT (A,B) and anti-FMRFamide (C) antibodies. Note the extensively developed surface nerve net (A), the parenchymal nerve bundles (B) with numerous fibres branching both to the periphery and toward the centre of the worm and deeper lying closely packed FMRFamide immunoreactive fibres (C). D–G. 5-HT IR in Nemertoderma westbladi. D,E. Optical section of the brain. Note two nerve rings, one anterior (ar) and one posterior (pr) to the statocyst (s) with a curtain of thin fibres (f), more developed dorsally. Paired lateral fibres (lf) beginning from the lower ring, are the strongest. F,G. Dorsal side of the brain around the statocyst (s). Note several presumable neurons incorporated into the rings (arrows) and the abundance of thin nerve fibres (f) interconnecting the rings or running in the posterior direction.
**Fig. 4** *Nemertoderma westbladi*, schematic drawing of the organisation of 5-HT immunoreactive elements in the nervous system. View from the ventral side. Note two commissural rings, stronger dorsally, one anterior (ar) to the statocyst and one posterior (pr), a curtain of numerous thin fibres interconnecting the rings and surrounding the statocyst (s), two thin lateral nerve fibres (lf) with few associated neurons (n) and numerous fine fibres associated with the rings running in the anterior (af) and posterior (pf) directions.

**Fig. 5** *Nemertoderma westbladi*. Optical sections of the anterior end of the same animal, double stained with anti-5-HT (A,C) and anti-FMRFamide (B,D) antibodies. The dorsal parts of 5-HT immunoreactive nerve rings (A) are followed by two transversal strands of FMRFamide immunoreactive fibres (B). More dorsally (C), the 5HT immunoreactive brain rings look like a bow-tie around the statocyst (s), while a pair of thin dorsal FMRFamide immunoreactive fibres runs backward (D). Note several presumable neurons incorporated into the rings (arrows).
at the ventral side (Fig. 3F, G). At the dorsal side, near the statocyst, the upper and the lower rings close to each other, occasionally forming a bow-tie structure around the statocyst (Figs 3F, 5C). A curtain of numerous thin fibres interconnects the rings dorsally and surrounds the statocyst (Figs 3D, F, G). No distinct cell bodies associated with the rings were observed, however, some pictures suggest the presence of several small neurons incorporated into the rings themselves (Figs 3, F,G).

From the area of the nerve rings two thin treads of symmetrically located lateral nerve fibres run posteriorly in a longitudinal direction (Figs 3E, G, 4). It was possible to follow them only in the anterior body region. In addition, a drapery of numerous fine beaded nerve fibres run backwards from the periphery of the rings, especially on the dorsal side (Figs 3D,G,4,5A,C). A few cell bodies can be seen associated with the fibres (Fig. 5C). The anterior ring sends numerous branches towards the frontal end (Figs 3D, G, 4).

b. FMRFamide IR

Only few data are available on the FMRFamide IR pattern in *N. westbladi* (Figs 5B,D). Occasionally two strands of thin fibres were observed following the dorsal parts of 5-HT immunoreactive brain rings. Thin paired dorsally located nerve fibres originating from these fibre strands run backward (Fig 5D).

Discussion

The nervous structures as revealed by 5-HT and FMRFamide immunostainings in the head of both species of Nemertodermatida differ clearly from the brains of most flatworms (Catenulida and Rhabditophora). No ‘true’ ganglionic brain or neuropile were observed. Common for the nervous system of *M. stichopi* and most flatworms are the 5-HT immunoreactive surface nerve net and the parenchymal longitudinal cord-like nerve bundles connected by transverse commissures. The primitive features of *M. stichopi* are the loose structure of nerve fibre bundles and the presence of only two 5-HT immunoreactive cells at the frontal end.

The nervous system of *M. stichopi*, that lacks any anterior brain-like structure, seems more primitive than that of *N. westbladi*, but that may be a result of reduction caused by the endosymbiotic mode of life of *M. stichopi*. Alternatively, taking into account the submuscular position of the nervous system in *M. stichopi* in comparison with its subepidermal position in *N. westbladi*, the former could be considered more advanced.

As the two species of the Nemertodermatida display considerable differences in the structure of their nervous-system, the comparison with the Acoela proves difficult. The two commissural 5-HT immunoreactive rings in *N. westbladi* differ clearly from the rosette-shaped commissural brain of most acoels (see Raikova et al. 1998). In *N. westbladi* the numerous nerve fibres running in posterior direction are not concentrated in nerve cords as in the Acoela. The 5-HT immunoreactive pattern in *M. stichopi* has nothing in common with that of acoels. The FMRFamide immunoreactive pattern of the nemertodermatids differs considerably from that of acoels. The Acoela studied so far are characterised by two lateral clusters of large peptidergic cells, lying at the periphery of the 5-HT-immunoreactive commissural brain, thus the 5-HT immunoreactive pattern is totally different from the FMRFamide one (see Reuter et al. 1998). In nemertodermatids, the patterns of nerve fibres, immunoreactive respectively for 5-HT and FMRFamide are similar, like in most flatworms (for references see Reuter & Gustafsson 1995).

The questions of the plesiomorphic or apomorphic nature of the nervous system in Nemertodermatida and the monophyly of the Acoelomorpha cannot yet be answered. The subepidermal position and very simple construction of the neuronal centralisation in the frontal end in *N. westbladi* and the absence of anterior nerve centralisation in *M. stichopi* indicate a more primitive nature of the nervous system in the Nemertodermatida than in the Acoela. However, the nerve structures in *M. stichopi*, *N. westbladi* and the Acoela are so different between themselves that at present it is difficult to formulate a well corroborated hypotheses of homology/synapomorphy. Consequently, in the neuro-anatomy of the studied worms we have not found any synapomorphy supporting the taxon Acoelomorpha. The final conclusions concerning the phylogenetic implications of acelomorph neuroanatomy will need additional data from other nemertodermatid species as well as studies on the distribution of other neuroactive substances in nemertodermatids and acoels.

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