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Patterns in the nervous and muscle systems in lower flatworms

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ABSTRACT. In order to test the monophyly of the Plathelminthes and the phylogenetic relationships of Acoela and Nemertodermatida, studies on the neuroanatomy of these groups were performed using anti-serotonin (5-HT) and anti-FMRF related peptides (FaRPs) immunocytochemistry. The Catenulida + Rhabditophora seem to be monophyletic. Four synapomorphies are proposed for these taxa. The presence of: 1. a bilobed brain showing 5-HT- and FaRP immunoreactivity. 2. a distinct neuropile, showing 5-HT and FaRP immunoreactivity. 3. two main nerve cords (MC's) aligned by 5-HT-immunoreactive (IR) marker neurones, 4. FaRP-immunoreactivity in the stomatogastric nervous system (NS). No synapomorphies were detected between Acoela and other Plathelminthes. The IR patterns of Acoela are characterised by: 1. The presence of a 5-HT-IR commissural brain. 2. The presence of clusters of FaRP-IR cells not integrated into a brain of the flatworm type. 3. The absence of a regular orthogon. 4. The absence of serotoninergic marker neurones along the main nerve cords (MC's). 5. The absence of a stomatogastric FaRP positive nervous system (NS). No support was obtained for a taxon Acoelomorpha. Our data are compatible with the hypothesis that both the Acoela and the Nemertodermatida do not belong to the Plathelminthes.

KEY WORDS: neurons, muscles, phylogeny, Platyhelminthes.

INTRODUCTION

In the paper "Is the Turbellaria polyphyletic?" SMITH et al. (1986), were the first to question the monophyly of the Plathelminthes. They pointed out that no synapomorphies were actually known between Catenulida, Acoelomorpha and Rhabditophora. The question of the monophyly of the Plathelminthes still remains open. The Plathelminthes, and particularly the Acoela have been in focus in recent molecular studies (see RUIZ-TRILLO et al., 1999; LITTLEWOOD et al., 1999 BERNEY et al., 2000). Traditionally, the Nemertodermatida and the Acoela have been classified as sister groups within the Acoelomorpha (EHLERS, 1985). In recent molecular investigations this position has been questioned.

In the discussion of the phylogenetic position, the organisation of the nervous system (NS) has been used as one of the discriminating criteria (see REUTER et al., 2001). The application of immunocytochemical (ICC) techniques has proven reliable for studies of flatworm neuroanatomy (see REUTER & GUSTAFSSON, 1995). Particularly informative results have been obtained using antibodies raised against serotonin (5-HT) and FMRF related peptides (FaRPs). By combining phalloidin staining for F-actin and ICC staining of neuroactive substances, it is possible to study the spatial interrelationships between muscles and nerves.

Here data of the 5-HT and FaRP immunoreactivity patterns in the taxa Macrostomida and Catenulida are reviewed (WIKGREN & REUTER, 1985; REUTER & GUSTAFSSON, 1995). Thereafter, recent data on the 5-HT and FaRP immunoreactivity patterns in the taxa Acoela and Nemertodermatida are presented (RAIKOVA et al., 1998, 2000, 2001; REUTER et al., 1998, 2001). Furthermore, new data concerning the spatial relationship between muscles and nerves in all above-mentioned taxa are presented. Finally, the phylogenetic implications of the data are discussed.

MATERIAL AND METHODS

Species

Specimens of 1. *Stenostomum leucops* Dugès, 1828 (Catenulida) were collected from a stock culture main-

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tained in containers with tap water, 2. *Macrostomum lineare* Müller, 1774 (Macrostomida) were collected from brackish water at Stortervo, Pargas (SW Finland), 3. the Acoela: *Anaperus biaculeatus* Boguta, 1970, *Childia groenlandica* Levinsen, 1879, were collected in the vicinity of the White Sea Biological station at Cape Kartesh (Russia), *Faerlea glomerata* Westblad, 1945 and *Paraphanostomum crassum* Westblad, 1942 from the vicinity of Kristineberg Biological station (West coast of Sweden), and specimens of *Avagina incola* Leiper, 1902 were obtained from the gut of the sea urchin *Spatangus purpureus* O.F. Müller, 1776 in the vicinity of Bergen, (Norway). 4. the Nemertodermatida: *Nemertoderma westbladi* Steinbock, 1938 were collected in the vicinity of Kristineberg Marine Research Station (West coast of Sweden) and *Meara stichopi* Westblad, 1949 obtained from the intestine of the holothurian *Stichopus tremulus* Gunnerus, 1767 at Raunefjord near Bergen (Norway).

Immunocytochemistry

For details of the method see KRESHCHENKO et al. 1999 and REUTER et al. 2001.

The specimens 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 24-48h 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 10-20µm on a Bright cryostat. Immunostaining was performed according to the indirect immunofluorescence method. The concentrations for the primary antibodies were 1:500. Incubations were performed for 36-48h either with a mixture of goat anti-5-HT (INCSTAR) and rabbit anti-FMRF (INCSTAR) or with a mixture of rabbit anti-5-HT (INCSTAR) and guinea pig antiserum against the native flatworm neuropeptide GYIRF (Bdelloura candida) (Johnstone et al., 1995). Thereafter the incubations were rinsed 3x5 min in PBS-T, followed by consecutive incubations with TRITC- or FITC-labelled secondary antibodies (from DAKO and Cappel).

Phalloidin staining of musculature

Whole mounts or cryosections were stained with TRITC-conjugated phalloidin (Sigma) (1:2000) for 20 min to 2h at 4°C. The phalloidin staining was performed on the same whole mounts or cryosections that had been stained with a-5HT and a-FaRPs, studied in the confocal microscope and photographed.

Microscopy and computer processing of immunocytochemistry micrographs

The preparations were examined in a Leitz Orthoplan microscope combined with filter blocks I2 and N2. A confocal scanning laser microscope (CSLM: Leica TCS 4D) was used to visualise the details of the nervous system.

Files obtained from CSLM were processed with Adobe Photoshop 4.0. Only commands "mode RGB-Grayscale", level of "grey", "brightness" and "contrast" were used, to avoid any distortion of the information contents of the image.

RESULTS

Catenulida

In *Stenostomum leucops,* ICC staining with anti-5-HT and anti-FaRP reveals a bilobed brain with a fibrillar neuropile. Frontally, lateral lobes emerge from the brain *sensu stricto*. The lobes innervate sensory pits, which are connected by transverse muscles (Fig. 1A). 5-HT and FaRP immunoreactivity occur in separate sets of neurones (WIKGREN & REUTER 1985). FaRP immunoreactivity dominates in the ventral cords. 5-HT immunoreactivity occurs in all nerve cords and in the nerve plexus close to the body surface. The two main nerve cords (MC's) are aligned by serotoninergic marker neurons. Only FaRP positive cells and nerves are seen in the stomatogastric NS and they dominate in the ventral cords. By the use of confocal scanning laser microscopy (CSLM) and phalloidin staining of F-actin, we can show that FaRP positive nerves innervate the pharynx (Fig. 1 B) and extend to the muscles connecting the sensory pits (Fig. 1A).

Macrostomida

In *Microstomum lineare*, the 5-HT and FaRP immunoreactivity patterns are similar to those in other Rhabditophora. The peptidergic and aminergic cells surround the neuropile composed of a tangled mass of nerve fibres. Serotoninergic marker neurones are aligned along the MC's. An orthogon and nerve nets close to the body surface are observed (see REUTER & GUSTAFSSON, 1995). FaRP immunoreactivity characterises the stomatogastric NS. Combined staining for F-actin, FaRP and 5-HT immunoreactivity reveals the spatial relationship of neuronal substances and muscle layers. FaRP immunoreactivity occurs in the nerve net around the gut musculature (Figs 1 C-E). FaRP positive cells join the pharyngeal nerve ring and fibres cling to the pharyngeal muscles (Fig. 1 F). In contrast 5-HT immunoreactivity occurs in the subepidermal and submuscular nerve nets of the body wall (REUTER et al., 1995). The difference in distribution patterns is shown in a sagittal section (Fig. 1 G).

Acoela

The immunoreactivity patterns for 5-HT and FaRPs in eight species of Acoela have recently been studied (RAIKOVA et al., 1998; REUTER et al., 1998; 2001).

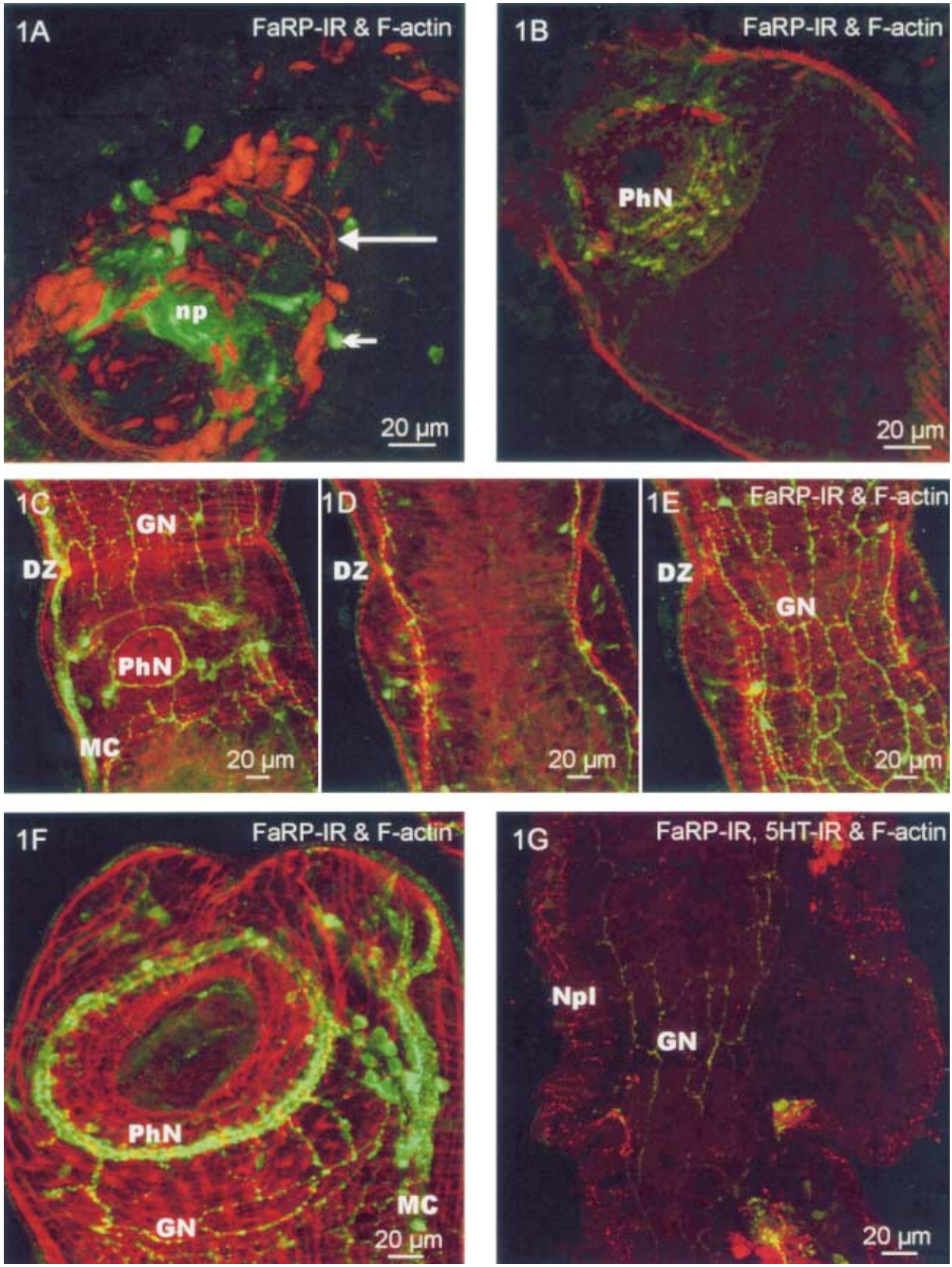
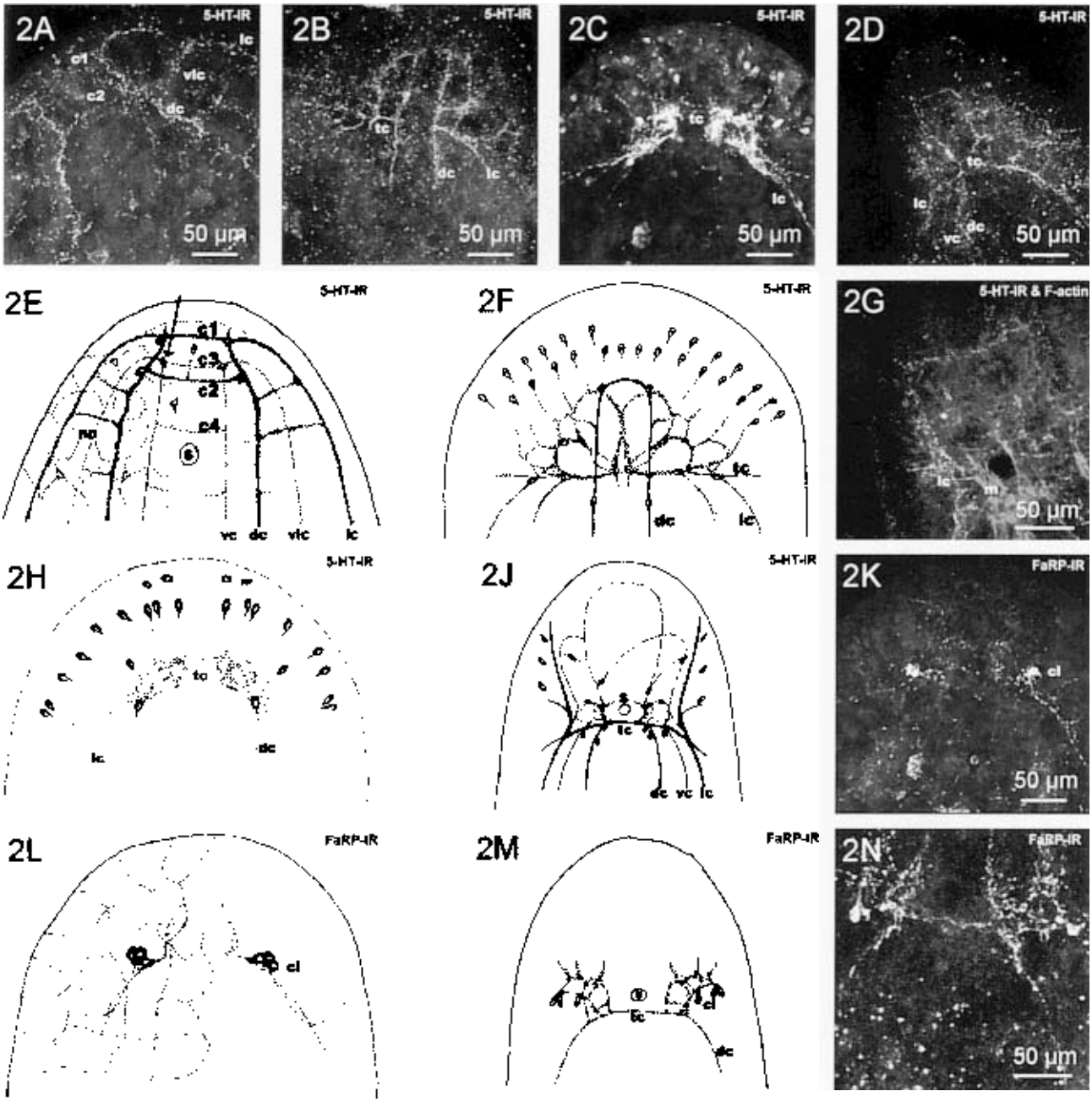


Fig. 1 A-B. – *Stenostomum leucops,* A. Brain and frontal lobes, FMRF-IR (green) in neuropile (np) and in sensory cells in sensory pit (short arrow), phalloidin stained F-actin (red) in transverse muscle fibres (long arrow) between frontal lobes B. FaRP-IR in pharyngeal nervous system (PhN) spatially related to phalloidin stained F-actin in pharyngeal muscles.

Fig. 1. C-G. – *Microstomum lineare.* C-E. Optical sections from three different body levels showing FaRP-IR nerve net (GN) adjoining phalloidin stained F-actin of gut musculature, main longitudinal nerve cord (MC), division zone (DZ). F. FaRP-IR in pharyngeal nervous system (PhN) and gut nerves (GN) in contact with phalloidin stained F-actin in muscles. G. Sagittal section showing FaRP-IR in gut nerve net (GN) and 5-HT-IR in nerve plexus (Npl) of body wall.

***5-HT-IR pattern*.** 4-5 pairs of serotoninergic longitudinal nerve cords form a symmetrical anterior structure composed of nerve fibres, associated with a few cell bodies - a commissural brain. The longitudinal nerves do not form a regular orthogon. 5-HT IR occurs in an irregular network of subepidermal fibres. In addition, submuscular fibres were observed innervating reproductive structures (RAIKOVA et al., 1998). The shape of the brain displays variations in organisation (see WESTBLAD, 1948). According to him a development from a ring-shaped brain to a bridge-shaped brain can be followed. Three terms – barrel-, rosette and bridge-shaped – are proposed for the acoelan brain by REUTER et al. (2000).

The barrel-shaped brain, represented by *Faerlea glomerata*, is characterised by 5-HT-IR longitudinal nerve cords, forming the ribs of the barrel, and transverse fibres, connecting the longitudinal cords and representing the barrel hoops (Figs 2 A, E). The dorsal fibres are stronger than those on the ventral side. All fibres are located close to the body surface. No contact between the 5-HT-IR fibres and the statocyst, lying deep in the parenchyma, was observed. This shape corresponds to the ring-shape of



Figs. 2 A-J. – Patterns of 5-HT-IR in commissural brain of four acoels; A, E. *Faerlea glomerata*, B., F. *Childia groenlandica*, C., H. *Avagina incola*, D., J. *Paraphanostoma crassum*. Transverse brain commissures (c1, c2, c3, c4, tc), dorsal nerve cords (dc), ventrolateral nerve cords (vlc), lateral nerve cords (lc), ventral nerve cords (vc), cell cluster (cl), statocyst (s). In G. phalloidin stained muscles (m) close to the cell cluster and 5-HT-IR in brain loops and lateral nerve cord (lc) in optical section of *P. crassum*.
Figs. 2. K-N. – FaRP-IR patterns in acoels. K., L. *Avagina incola*, M.,N. *Paraphanostoma crassum*. FaRP-IR cell clusters (cl).

WESTBLAD (1948). We prefer the term barrel-shape, because the transverse fibres are clearly weaker than the longitudinal (REUTER et al., 2000).

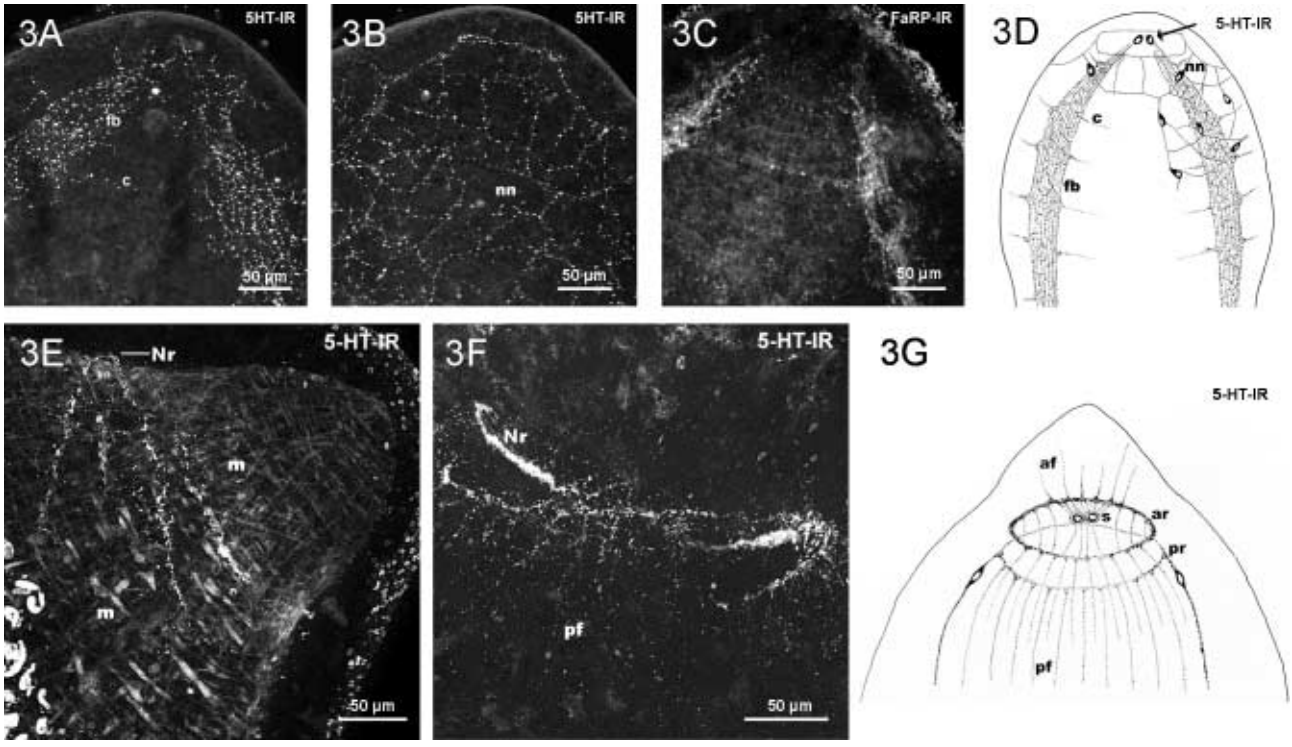
The rosette-shaped brain, represented by *Childia groenlandica,* is characterised by 5-HT-IR anterior loops, joining a common transverse commissure on the dorsal side. In *C. groenlandica*, the loops are large and loosely connected (Figs 2 B, F). When the loops are small and concentrated a bridge-like construction is formed as in *Avagina incola* (Figs 2 C, H).

In the bridge-shaped brain of *Paraphanostoma crassum* (Figs 2 D,G, J) a strongly stained dorsal transverse commissure gives rise to a semi-circle around the statocyst. The construction of the brain and the close association between the nerves and the statocyst indicate a distinct evolutionary development from a superficial centralisation of neurons close to the body surface leading to a true cephalisation.

***FaRP-IR pattern*.** In all the acoels studied the peptidergic patterns differ considerably from the serotoninergic patterns. Symmetrical clusters of cells located peripherally to the 5-HT-IR commissural brain were revealed in all acoels excluding *F. glomerata,* in which no reaction was obtained (see REUTER et al. 2000). By using antibodies against the native flatworm neuropeptide GYIRFamide, nerve fibres around the cell clusters in *A. incola* and *P. crassum* were visualised (Figs 2 K-N). Double-staining with 5-HT and FaRP antibodies show, that the FaRP-IR cell groups are located peripherally to the 5-HT-IR commissural brain.

Nemertodermatida

So far only two species of Nemertodermatida, *Meara stichopi* and *Nemertoderma westbladi*, have been studied with ICC technique in order to compare their neuroanatomy to that of the Acoela (RAIKOVA et al., 2001). The results revealed a surprisingly different pattern between the acoels and the two nemertodermatid species, but also between the last mentioned species themselves. In *M. stichopi* two 5-HT-IR cells occur close to the statocyst. From the cells, symmetrical parenchymal bundles of loosely packed nerve fibres extend posteriorly. A superficial nerve plexus also occurs (Figs 3 A-D). In *N. westbladi,* two basiepthelial 5-HT-IR fibre rings send numerous fine fibres in both posterior and anterior directions. Some of the fibres to the statocyst lie deeper in the parenchyma (Figs 3 E, G). Double-staining with phalloidin for F-actin, clearly shows the basiepithelial position of the nerve ring, outside the body muscles (Fig. 3 F). In *M. stichopi*, the peptidergic pattern corresponds in general to the serotoninergic pattern (Fig. 3 C). In *N. westbladi*, FaRP immunostaining occasionally revealed fibres at the same level as the serotoninergic nerve rings (Fig. 3 G).



Figs. 3. A-D. – *Meara stichopi.* A-B. 5-HT-IR patterns, C. FaRP-IR pattern, D. schematic drawing of 5-HT-IR pattern, bundle of nerve fibres (fb), transverse commissure (c). nerve net (nn), note two frontal nerve cells (arrow) in D.
Figs. 3. E-G. – *Nemertoderma westbladi.* E. 5-HT-IR showing basi-epithelial nerve ring (Nr) located peripherally to the phalloidin stained muscle layer (m). F. 5-HT-IR in two "brain rings" sending nerve fibres posteriorly (pf). G. Schematic drawing, anterior nerve ring (ar), posterior nerve ring (pr), nerve fibres running anteriorly (af) and posteriorly (pf), statocyst (s).

PHYLOGENETIC IMPLICATIONS

Stomatogastric NS

The peptidergic innervation of the stomatogastric NS in Rhabditophora has been described repeatedly (see REUTER & HALTON, 2001, MAIR et al., 1996). The presence of a peptidergic innervation of the stomatogastric NS in Catenulida (WIKGREN & REUTER, 1985, REUTER et al., 1995) and Rhabditophora, points to a synapomorphy for these taxa. The lack of FaRP immunoreactivity in the central parenchyma of Acoela can be explained either as a reduction of the NS in Acoela, lacking a gut, or alternatively as a plesiomorphy. The latter seems more likely, taking into account the basal position of the Acoela in the phylogenetic tree of the Bilateria (see RUIZ-TRILLO et al., 1999). In the nemertodermatid *N. westbladi*, which has an epithelial gut, the absence of FaRP immunoreactivity indicates that the lack of gut innervation represents a plesiomorphy. 5-HT immunoreactivity occurs in the pharynx in all flatworm taxa except Catenulida and Acoelomorpha and thus forms an apomorphy for the Rhabditophora.

Innervation of musculature

Regarding Macrostomida and all other Rhabditophora, as well as the Acoela and the Nemertodermatida, the fine meshed 5-HT-IR nerve plexuses at the body wall – the subepidermal close to the roots of the cilia and the submuscular close to the body wall musculature (REUTER et al., 1995; LADURNER et al., 1997) – may be plesiomorphic. In *Anaperus tvaerminensis* Luther, 1912, EHLERS (1994) described submuscular neurones enveloped by longitudinal muscle cells. A similar close association between 5-HT-IR nerves and longitudinal muscle fibres was observed in *A. biaculeatus*. A double nerve/muscle function of the muscles radiating from the wall of the statocyst in "Paraphanostoma Arten" was suggested by WESTBLAD (1948). In our studies, no 5-HT-IR fibres were observed reaching the strong muscles around the statocyst in *F. glomerata*. Thus some other neuronal signal substances probably innervate the strong muscles radiating from the statocyst wall. The functional aspect of the spatial relationship between muscles and nerves needs further research.

Synapomorphies for Catenulida and Rhabditophora

In the following respects the immunoreactivity patterns of Catenulida correspond to those observed in Rhabditophora.

1. The bilobed brain is composed of 5-HT- and FaRP-IR cells
2. The neuropile is distinct, showing both 5-HT and FaRP immunoreactivity
3. The MC's are aligned by 5-HT-IR marker neurones
4. FaRP-IR occurs in the stomatogastric NS

These features can be regarded as synapomorphies for Catenulida and Rhabditophora.

Acoela vs Plathelminthes; no synapomorphies

Acoela differs from other Plathelminthes by:

1. The presence of a structure named the commissural brain, showing anti-5-HT immunoreactivity, but showing no resemblance to the bilobed brain structure in Plathelminthes, with its nerve cells surrounding a neuropile.
2. The presence of clusters of peptidergic FaRP positive cells, that are not integrated into a brain of the common flatworm type.
3. The absence of a regular orthogon. Only longitudinal nerve cords, connected by irregular nerve fibres forming subepidermal and submuscular nerve plexuses, were observed.
4. The absence of serotoninergic marker neurones along the MC's.
5. The absence of a stomatogastric FaRP positive NS.

Thus no synapomorphies were found in the organisation of the NS of Acoela and Plathelminthes (RAIKOVA et al., 1998; REUTER et al., 2000).

No support for the taxon Acoelomorpha

As to the Nemertodermatida, the presumed sister taxon of the Acoela (see LUNDIN, 1997 for discussion), our studies concerning the organisation of the NS revealed no synapomorphies either with that of the Acoela (RAIKOVA et al., 2000 a) or with that of the Rhabditophora. The basiepithelial position of the NS, the very simple construction of the neuronal centralisation in the frontal end of *N. westbladi* and the absence of any anterior centralisation in *Meara stichopi,* indicate a more primitive nature of the NS in the Nemertodermatida than in the Acoela. However, here further research is needed.

CONCLUSIONS

SMITH et al. (1986) presented five possible cladograms (A-E) for the taxon Plathelminthes. The results of our studies support cladogram B, i.e. that the group Catenulida + Rhabditophora is monophyletic. As to the position of taxon Acoela, recent molecular studies place it separately from flatworms (see RUIZ-TRILLO et al., 1999; LITTLEWOOD et al., 1999). Our results support this view. However, according to BERNEY et al. (2000) the Acoela belong to the Plathelminthes. The position of the Nemertodermatida also seems basal, though no support was found for taxon Acoelomorpha

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