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The nervous system of *Bothriomolus balticus* (Proseriata) – a contribution to the knowledge of the orthogon in the Plathelminthes

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Summary. The nervous system (NS) of *Bothriomolus balticus* (Proseriata) was studied by the immunocytochemical (ICC) method with antisera to RFamide, SALMFamide and serotonin and with the histochemical GAIF method. The use of the ICC technique provided a much more precise morphological account of the nervous system than had previously been possible. The obtained data are discussed in connection with the comparative morphology of the nerve cords of the Plathelminthes. A similar position does not grant direct correspondence between nerve cords in the taxon Seriata. Marginal cords had probably an independent origin in the Monocelididae and Otoplanidae. The ventral (main) cords of *B. balticus* seem to correspond to the lateral (main) cords of the Monocelididae. It can be hypothesized that both: (1) a shift of the main cords accompanied by formation of new cords from the plexus and fusion of other cords and (2) a redistribution of nerve processes and perikarya between the cords, take part in the evolution of cords in the Plathelminthes. The first hypothesis seems to explain the difference in the position of main cords in proseriates, though, the second hypothesis might dominate, for example, in the Neorhabdocoela and the Neodermata. The correctness of the evolutionary analysis of the nerve cords in plathelminths can only be provided by neurons or neuron groups marking these structures.

A. Introduction

Until now there has been very little discussion of neurological data in connection with the theory of the evolution of the orthogon (see Reisinger 1972; Reuter 1990; Joffe 1990) and has been scarely used in the phylogenetic research of the Plathelminthes.

The nervous system (NS) of parasitic plathelminths has been studied using the immunocytochemical (ICC) method in the Pseudophyllidea (see Gustafsson et al. 1986; Gustafsson 1991; Wikgren et al. 1986), the Cyclophyllidea (see Fairweather et al. 1990a, b; McKay et al. 1991a; Maule et al. 1991), the Monogenea (see Reuter 1987, 1988; Maule et al. 1989a, b, 1990a, b; McKay et al. 1991b) and the Digenea (see Gustafsson 1987; Skuce et al. 1990a, b; Fairweather et al. 1987; Magee et al. 1989; McKay et al. 1990; McKay et al. 1991c). Of the free-living plathelminths, only representatives of the Catenulida (see Wikgren and Reuter 1985; Reuter 1988; Wikgren et al. 1990; Wikgren and Thorndyke 1990) and the Macrostomida (see Reuter et al. 1986; Reuter 1988; Reuter and Palmberg 1989) have been studied in detail on whole mounts. Only fragmentary data, mainly from sections, are available for the other taxa (Wikgren et al. 1986; Reuter et al. 1988; Kerschbaum et al. 1988; Eriksson et al. 1990; Kabotyanski et al. 1991). ICC studies of such important groups as the Seriata and the Neorhabdocoela are thus necessary. The distribution of putative catecholamines (CAs) has been studied by the cytochemical glyoxylic acid induced fluorescence (GAIF) method in about 40 species of plathelminths (reviewed by Joffe and Kotikova 1991; Shishov 1991), but only the Monocelididae have been studied among the Proseriata.

The neuroanatomical implications drawn from ICC results are few (Reuter 1990; Gustafsson and Reuter 1992; Johnston et al. 1990; Fairweather and Halton 1991; Halton et al. 1992). On the other hand, much attention has been paid to single neurons and groups of neurons identified in different specimens by the GAIF method (Hauser and Koopowitz 1987; Joffe and Kotikova 1988, 1991). Based on the concept of neuron homology (Sakharov 1974), several homologous groups of neurons have been indicated (Joffe and Kotikova 1988, 1991) and preliminary phylogenetic implications have been suggested (Joffe 1991).

We investigated distributions of CAs and IRs to 5-HT, RFamide, and SALMFamide in *Bothriomolus balticus* Meixner, 1938 (Proseriata Otoplanidae). In the practical part of the work, we paid special attention to identified neurons and neuron groups and aimed at as

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complete a description of each specific subpopulation of neurons as possible. These data are used for comparative discussions of the nerve cords in the orthogons of the Plathelminthes which are mainly based on a new approach using neurochemically characterized neurons as markers of the nerve cords. Though the available data are not yet sufficient for reliable phylogenetic conclusions, we demonstrate that this kind of neurological evidence may be instructive as to the phylogeny of the Plathelminthes.

B. Materials and methods

The material was collected from a sandy beach at Mustfinn in the vicinity of Åbo in the middle of October, after which the worms, lived for about a month in the laboratory at +4° C.

Immunocytochemistry. For the ICC demonstration of 5-HT and neuropeptides, the indirect immunofluorescence technique was used, mainly following the protocols by Wikgren and Reuter (1985). The worms were flat-fixed for 6–8 h in "Stefanini's fluid" at +4° C and washed overnight with 0.1 M Na-phosphate buffered saline (TBS) (pH = 7.4) containing 10% sucrose. They were then mounted on object glasses covered with poly-L-lysine, air-dried and stored at −70° C. Prior to immunostaining they were pretreated with Tris buffered saline containing 1% bovine serum albumin and 0.2% Triton X-100 (BSAT) for 2 h at +4° C. The preparations were then incubated for 48 h in primary antibody in BSAT, rinsed in TBS 3 × 5 min, incubated in secondary antibody in BSAT for 2 h at room temperature, rinsed in TBS 3 × 5 min and embedded in 50% glycerol in TBS. Preparations were stored at −20° C. The primary antisera used were anti-RFamide 146 III (kindly donated by Dr. C.J.P. Grimmelikhuijzen), anti-SALMFamide (kindly donated by Dr. M.C. Thorndyke) and anti-5-HT (Incstar). All of them were used in dilution 1:500. The secondary antiserum was anti-rabbit immunoglobulin swine serum conjugated with rhodamine (DAKO). It was used at a dilution of 1:20 in BSAT. Incubation in BSAT without primary antibody was used as the control.

Each immunopositive structure was considered registered when it had been recognized in at least three specimens. As a precaution against nonspecific recognition by primary antisera (see Stretton et al. 1991), the weakly stained structures were not regarded as "revealed" in a given specimen. When such structures that had been labelled repeatedly, but weakly, are described, the weak staining is always acknowledged. The results of immunostaining were variable even with the worms mounted on the same object glass, 2–3 glasses with 4–5 worms on each were used with each antiserum, and the whole series was repeated if necessary. In practice, this provided more than three observations of each described structure.

GAIF. The protocol used was similar to that used by Joffe (1991) and Reuter and Eriksson (1991). The worms were incubated for 40 min at room temperature in 0.1 M PBS with 1% glyoxylic acid (GA). After the incubation the preparations were air-dried, heated at +80° C for 15 min and embedded in liquid paraffin. Control procedures were incubation in the media without GA and quenching of the fluorescence with water. The reproducibility of the GAIF method is relatively low and, to provide at least 3–5 recognitions for each structure, about 60 worms were used.

C. Results

I. General antomy

The present description of the general topographic anatomy of the nervous system is based on the results from all the neuroactive substances studied. As far as this matter is concerned, all the substances rendered very similar results, though antisera to RFamide was the most illustrative one. Only a few CA-positive nerves lacked a-RFamide IR: the innervation of the ciliary pits, the connection between the lateral cords and the bases of the ventral cords, and the commissure between the ventral cords behind the brain (Fig. 1). The a-SALMFamide gave results which were nearly identical to a-RFamide; a-5-HT was found only in nerves which were also a-RFamide positive.

The majority of the revealed neurons were bipolar or unipolar with the process dividing near the perikaryon. They had an elongated or pear-like form with the size of the perikarya being mainly about 9–14 × 5–7 μm. Only a few perikarya were prominent on the preparations for larger or smaller size, which has in such cases been specifically acknowledged in the text.

The nervous system is of a pronounced orthogonal type with a brain, four pairs of longitudinal cords and numerous ring commissures (see Fig. 1 for a generalized scheme). The ventral cords (VN) are the most prominent ones (Fig. 3D, E). They run approximately at a distance of up to one-quarter of the body width from the body margins, and join near the posterior extremity of the body (Figs. 5F, 6C). Lateral cords (LN) lie on the dorsal side of the body at about half of this distance from the body margins or nearer to them (Fig. 3D, E). The dorsal cords (DN) are less wide than the lateral ones. A pair of very thin marginal cords (MN) runs along the lateral margins of the body (Figs. 2A, 3E, 4B).

1. Head (Figs. 1, 2)

Near the brain, each ventral cord gives off a thin anterior branch (AV) which proceeds forward under the brain. The anterior branches of the lateral cords (Fig. 1B, AL) branch off from the roots of the lateral cords not far from the brain (Fig. 1B, RLN; see also Fig. 2B: arrows mark perikarya associated with the RLN). The dorsal cords run forward over the brain. The dorsal cords and distal portions of the roots of the lateral cords are connected by a hemicircular commissure (Fig. 2C, C), which is much thicker than the ring commissures and nearly as wide as the dorsal cords themselves. The marginal cords do not have roots, that is, they do not have direct connection with the brain. Anteriorly to the brain, they bend in a medial direction along the ventral surface of the body, reach the anterior branches of the ventral cords and join each other. Anterior branches of the ventral and lateral cords, as well as the anterior protions of the dorsal cords, meet at the anterior pole of the body and form a small ring (Fig. 2A, arrow), which probably surrounds the ducts of the frontal glands. Several pairs of anterior nerves go off from the brain and unite with the other nerves of the anterior pole of the body. One pair of anterior nerves is especially prominent (Fig. 2B, arrowhead).

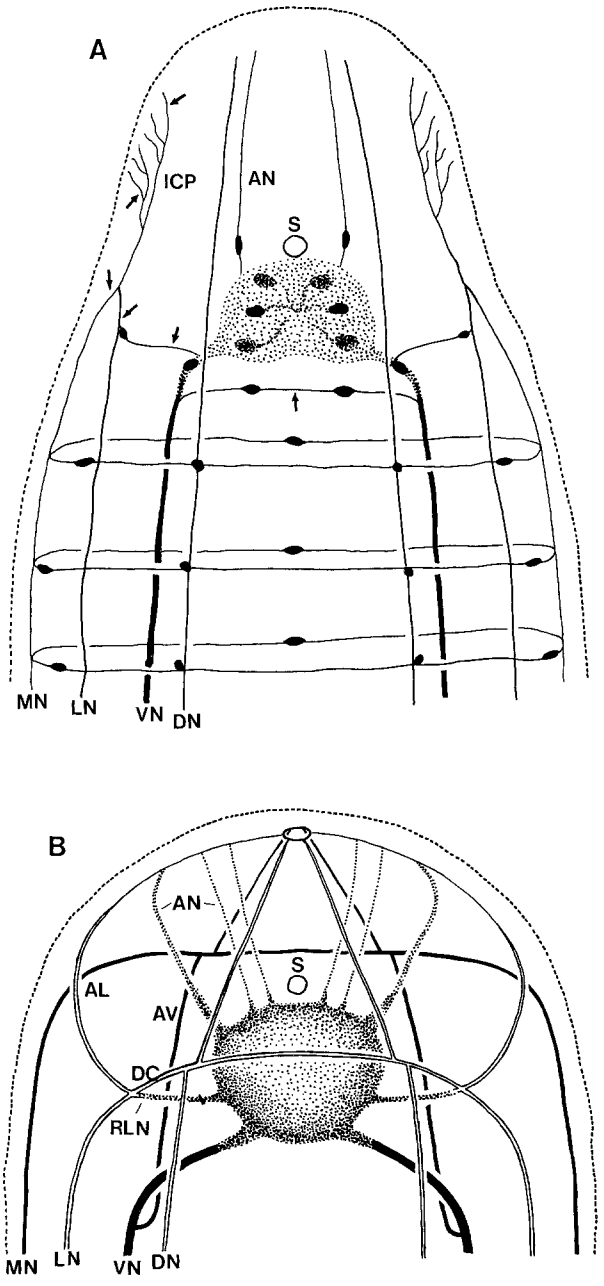


Fig. 1 A, B. Schematic representation of the nervous system of *B. balticus*. **A** CA-ergic innervation of the anterior portion of the body. Perikarya are *filled* or *densely dotted* (two pairs of neurons occupying ventral position in the brain). *AN* anterior nerves; *DN* dorsal nerve cords; *ICP* innervation of ciliary pits; *LN* lateral nerve cords; *MN* marginal nerve cords; *S* statocyst; *VN* ventral nerve cords; nerves lacking a-RFamide are marked with *thin arrows*. Note the absence of CAs in the roots of the lateral cords. **B** Nerves of the head region, a generalized scheme based on ICC (mainly a-RFamide) data (numerous perikarya are not shown). *AL* anterior branches of lateral cords; *AV* anterior branches of ventral cords; *DC* dorsal hemicircular commissure; *RLN* roots of the lateral cords; other designations as in **A**. Comparing the schemes, note that the worms fixed in Stefanini's fluid for ICC are contracted, while the worms subjected to glyoxylic acid for revealing CAs are not. Dorsal cords from a zig-zag in **B** and the hemicircular commissure is straight, while in **A** the dorsal cords are straight (and the hemicircular commissure would make a zig-zag if it contained CAs to be revealed by GAIF method). Similarly, V-like structure formed by the lateral cord and its anterior branch in **B** is straightened in **A**

2. Orthogon (Figs. 1, 3)

Numerous metameric ring commissures connect the cords in the area between the brain and the adhesive plate. Anteriorly to the pharynx, the commissures run via marginal cords. Behind the pharynx, the marginal cords do not differ much in diameter from the longitudinal plexal fibres. They mainly lose the appearance of uninterrupted fibres and, to receive the connection with ring commissures, they send a short nerve fibre to the lateral cords or locally fuse with them (Fig. 4B).

Two short nerves run from the united portion of the ventral cords to the posterior portion of the adhesive plate (Fig. 6C), while more laterally and anteriorly numerous short thin fibres run from the united portion of the ventral cords to the lateral margins of the adhesive plate. In vivo observations show that it is the posterior end of the adhesive plate which is most often used by the worms to stick to the substrate.

Plexal fibres, with the exception of those in the head region and adhesive plate, have an orthogonal orientation – longitudinal or transversal (Figs. 3B–E, 4). Longitudinal fibres often run without notable interruption along the whole length of the body (Fig. 3D, E, empty arrows). Plexal transverse fibres are poorly represented.

Innervation of the pharynx includes a nerve ring in its distal part and numerous meridional nerves (Fig. 5B, C, E). A delicate nerve net is present around the walls of the male copulatory organ (Fig. 6F).

II. a-RFamide

RFamide positive fibres were present in all parts of the nervous system.

I. Brain

The central part of the brain, i.e. the neuropile, gave strong immunoreaction. Up to 10–15 positive neurons, mainly unipolar, were observed on each side at the periphery of the brain (Fig. 2A, B). At least some cells localized in the anterior portion of the brain were bipolars sending their central process to the brain and directing the peripheral process to the anterior portion of the body (Fig. 2B). Particularly intense RF IR was observed in a pair of dorsocaudal brain cells (Fig. 2B, C). A group of several RFamide immunoreactive cells was observed near the root of each lateral cord (Fig. 2B).

2. Submuscular nerve net

One unipolar positive cell adjoined the anterior part of each ventral and lateral cord (Figs. 2C, 3A). The other RFamide immunopositive perikarya of the submuscular nerve net were related to ring commissures and were, as a rule, bipolar. They can be classified into two groups, midventral and lateral.

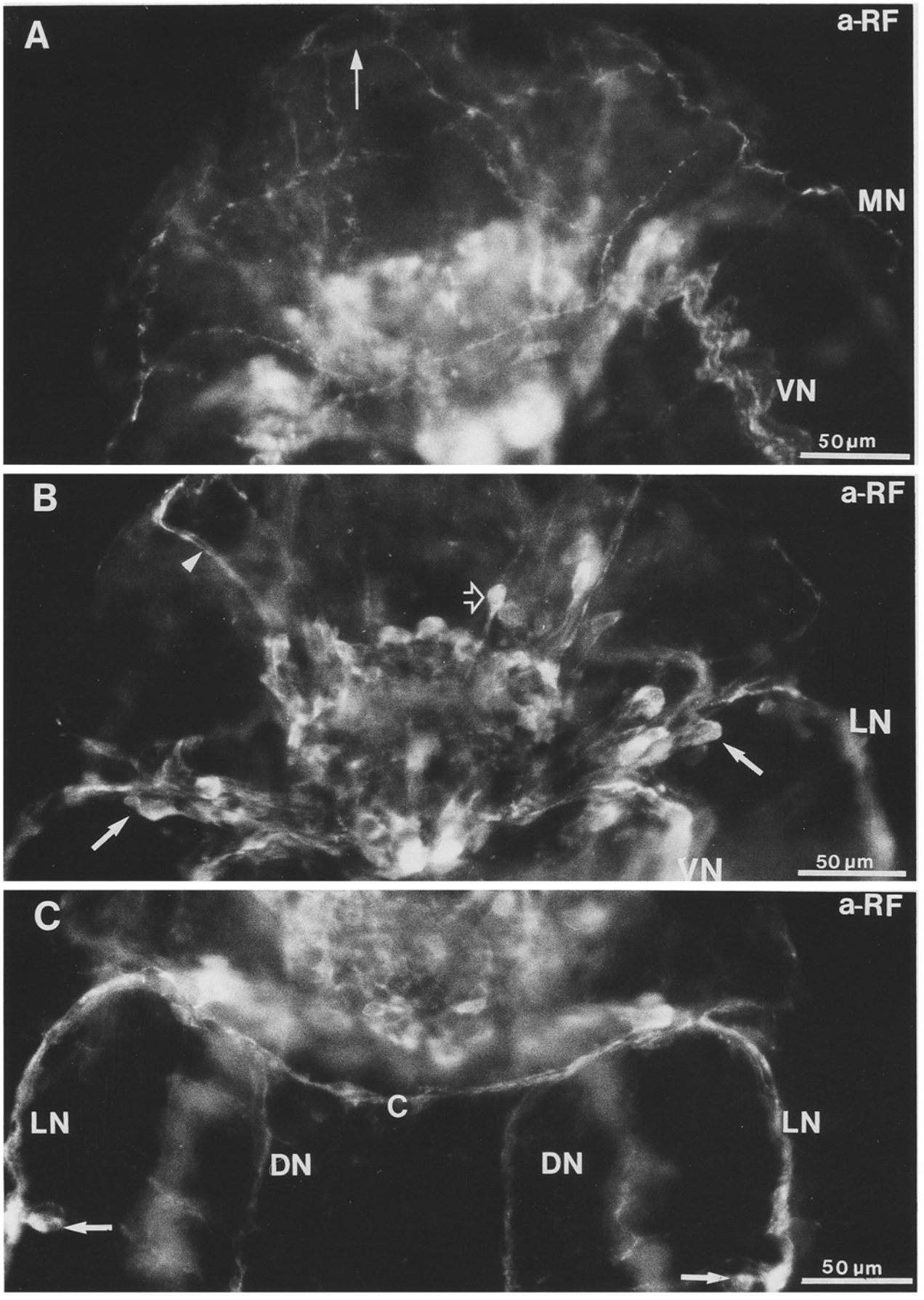


Fig. 2A–C. RFamide IR in head, whole mounts. Brain and nerve cords, ventral nerve cords (*VN*), lateral nerve cords (*LN*), dorsal nerve cords (*DN*), marginal nerve cords (*MN*). **A** Ventral view of nerve cords and anterior nerve fibres including frontal fibre ring (*arrow*). **B** Neurons in the roots of lateral cords (*arrows* mark unipolar cells) and in the brain (*empty arrow* marks perikaryon of a bipolar cell, its processes are weakly stained); anterior nerve of the most prominent pair (*arrowhead*). **C** Commissure uniting dorsal and lateral cords (*C*), unipolar neurons adjoining lateral cords (*arrows*)

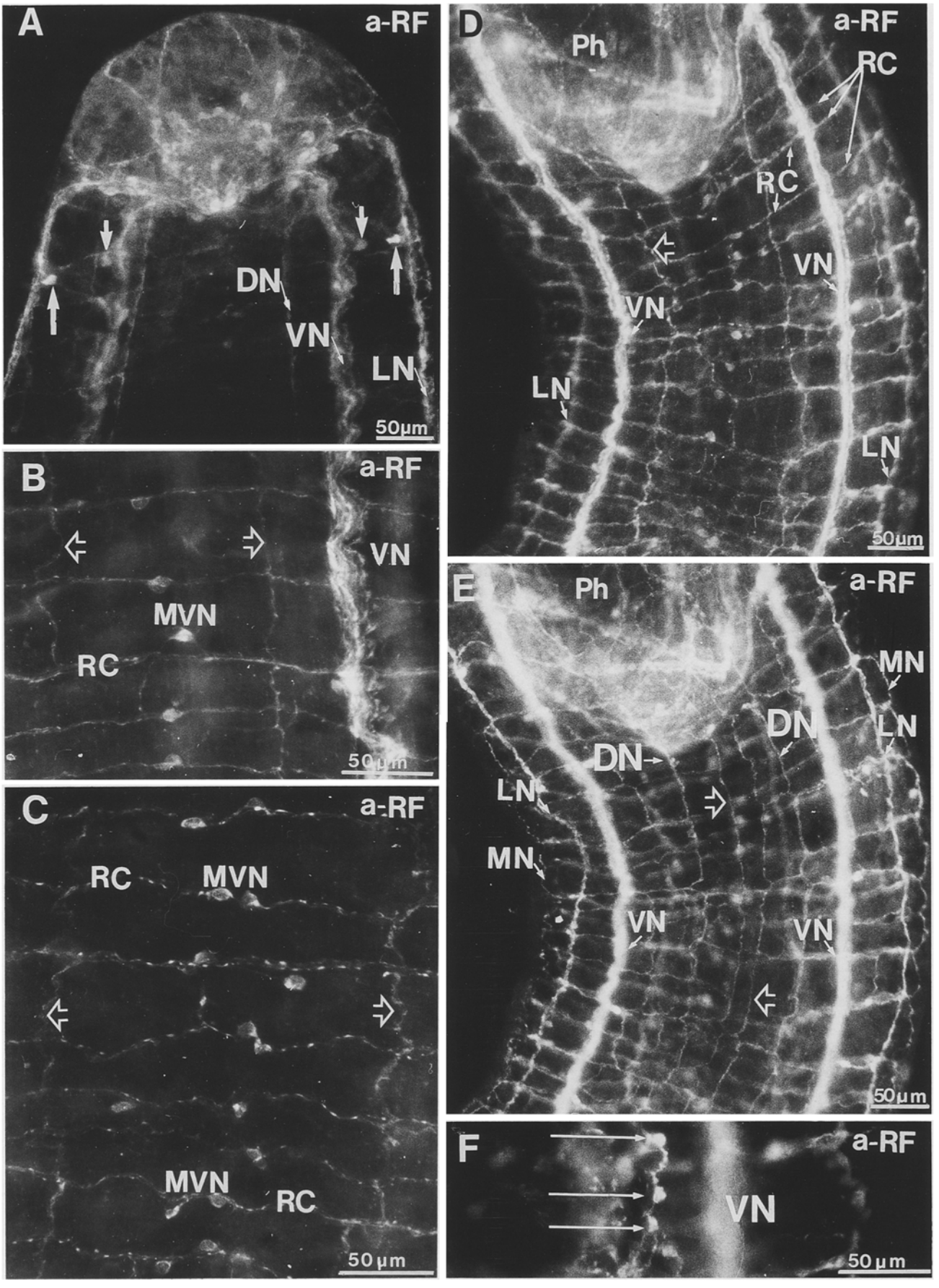


Fig. 3A–F. RFamide IR in orthogon, whole mounts. **A** Brain, ventral (*VN*), lateral (*LN*) and dorsal (*DN*) cords, unipolar neurons associated with lateral (*long arrows*) and ventral cords (*short arrows*). **B, C** Ring commissures (*RC*), plexal fibres (*empty arrows*); **B** single medioventral neurons (*MVN*); **C** paired medioventral neurons (*MVN*). **D, E** Orthogon behind pharynx; **D** ventral side; **E** dorsal side, pharynx (*Ph*), ring commissures (*RC*), plexal fibres (*empty arrows*), ventral cords (*VN*) with associated perikarya (*small arrows*), dorsal cords (*DN*), marginal cords (*MN*). **F** Neurons of genital plexus (*arrows*)

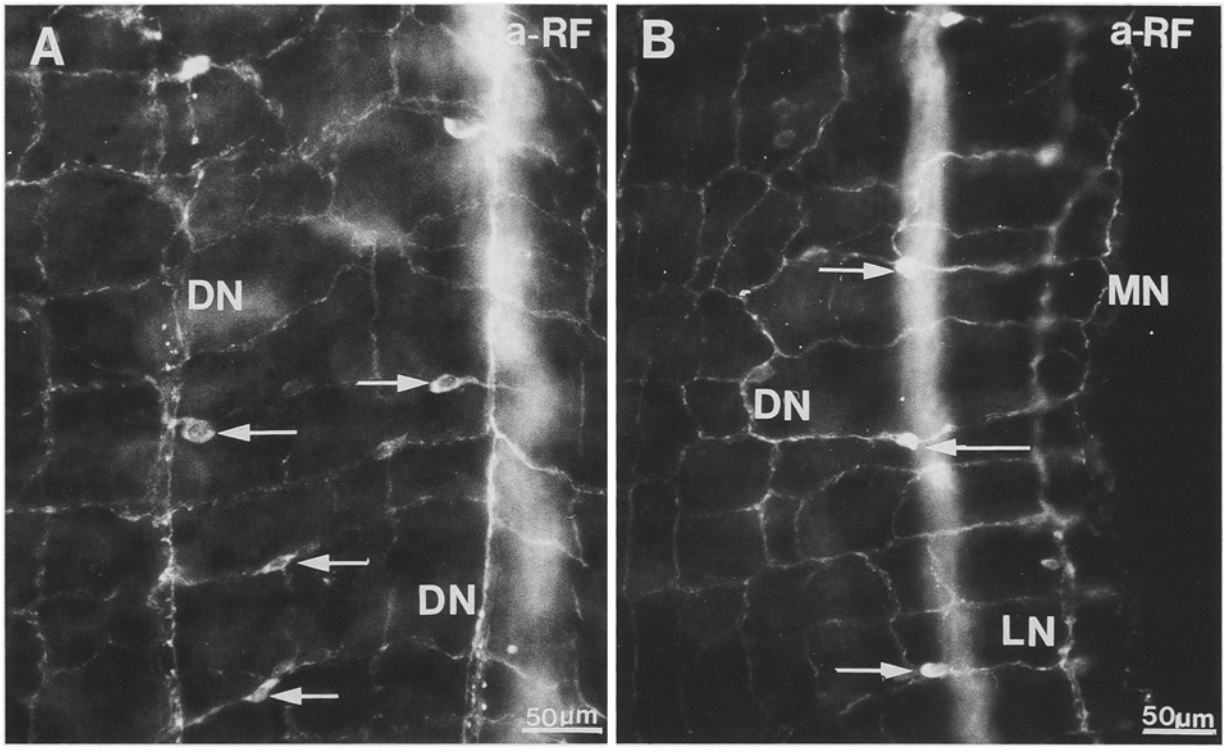


Fig. 4A, B. RFamide IR in L-group neurons, whole mounts. Neurons (*arrows*) between **A** dorsal (*DN*) cords and **B** lateral (*LN*) and dorsal (*DN*) cords. *MN* marginal cord

3. Midventral (VV) group

This group consists of cells with perikarya situated in the medioventral zone limited by the ventral cords (Fig. 3B–D). Each neuron is associated with a ventral portion of some ring commissure and sends its processes in lateral directions. More often there were one or two cells lying close to each other in the middle part of the commissure (Fig. 3B, MVN). If two perikarya were present, one of them often showed only weak IR. Less often there were two symmetrically located cells with similar intensity of immunoreaction (Fig. 3C, MVN). A condition intermediate between the two mentioned above was also readily observed. Even the neighbouring commissures in the same worm could differ in this respect. Nevertheless, in each worm one or the other of the two polar patterns seemed to prevail.

4. Lateral (L) group

The perikarya of this group were usually observed in the dorsolateral parts of the ring commissures. Sometimes they adjoined lateral, sometimes dorsal cords (Fig. 4A, DN), but more often they were situated between them (Fig. 4B, DN). Very rarely were they seen between lateral and marginal cords or between the dorsal cords (Fig. 4A, two cells at the bottom). The presence of one perikaryon on each side of the commissure seems to be a basic pattern. Nevertheless, in some commissures no neurons of the lateral group could be observed on one or both sides of the body, while some included three or four cells. At the same time, the position of the perikarya could be rather different even on the right and left sides of the same commissure. In each specimen, few cells of this group demonstrated a much more intense fluorescence than the other ones. These intensely reacting cells were distributed more or less evenly over the length of the body, but did not seem to occupy the same places in different specimens.

At the posterior part of the adhesive plate, an unpaired mediodorsal perikaryon was seen in many specimens (cf. Fig. 5F); in some cases it was accompanied by another, asymmetrically lying cell.

5. Pharyngeal innervation

Two prominent bipolar cells (PhV) were observed anteriorly to the pharynx, being submerged deeper in parenchyma than ring commissures. The size of their perikarya reached 12–15 × 12–15 µm or 10 × 17–20 µm. They sent one process to the ventral cord and the other one to the pharynx (Fig. 5A). Three other types of a-RFamide neurons were related to the pharynx. Cells of the first type, type I, adjoined the pharyngeal nerve ring (Fig. 5B). Two processes going into the pharyngeal nerve ring in opposite directions were seen in such cells if the perikarya were situated at a small distance from the nerve ring. The perikarya of cells of the second type, type II, often bipolar (Fig. 5B), sent their processes in meridional pharyngeal nerves and occupied a more

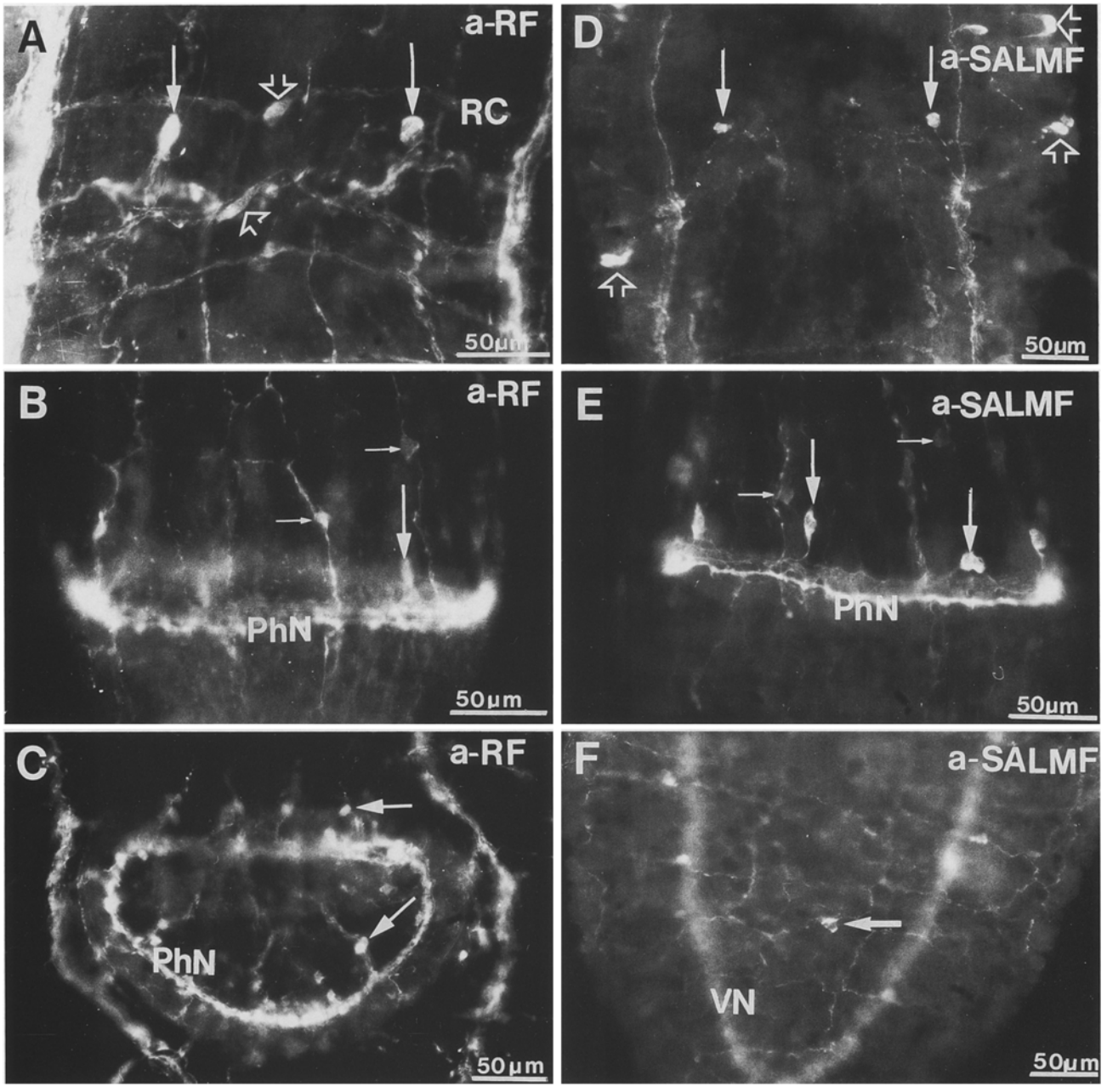


Fig. 5A–E. Pharynx, whole mounts. **A–C** RFamide IR; **D, E** SALMFamide IR. **A** RF positive PhV cells at deep focus level anteriorly to pharynx (*arrows*), ring commissures (*RC*) with associated neurons at focus level near the surface of the body (*empty arrows*). **B** Pharynx profile view, nerve ring (*PhN*) with associated neuron, type I (*arrow*), meridional pharynx nerves with bipolar cells, type II (*thin arrows*). **C** Pharynx ventral view, nerve ring (*PhN*) and cells (*arrows*). **D** SALMF positive PhV cells anteriorly to pharynx (*arrows*) and lateral pharynx neurons, type III (*empty arrows*) innervating pharynx. **E** Pharynx profile view, nerve ring (*PhN*) with adjoining neurons, type I (*arrows*), meridional nerves with cells, type II (*thin arrows*). **F** SALMFamide IR in tail, mediodorsal cell (*arrow*) in dorsal nerve plexus, ventral (*VN*) cords

proximal position (up to the middle of the pharynx). Each meridional nerve included one cell of this type. Cells of the third type, type III, were situated anteriorly to the pharynx near the lateral portions of the body and deeper in the parenchyme than the other nerve structures. The processes of these unipolar cells were sent to the meridional nerves of the pharynx. The number of pharyngeal neurons in adult worms was up to 6–8 for the first cell type (Fig. 5C), up to 10–12 for the second cell type and up to 8–10 on each side of the body for the third cell type.

6. Genital plexus

An immunopositive plexus surrounded the male copulatory organ. In its dorsal portion, relatively numerous small multipolar perikarya (mainly 6–8 µm in diameter, rarely up to 10 µm) were present (Fig. 3F).

III. a-SALMFamide

SALMFamide IR was revealed in all cords and commissures (Fig. 5D, F) as well as in the nerves of the head and adhesive plate. Its distribution over the perikarya was identical to that of a-RFamide. SALMFamide IR was found in the neurons of the brain. A pair of brightly fluorescing dorsocaudal cells had a location corresponding to that of a-RFamide positive cells in the posterior portion of the brain. In addition, SALMFamide IR was observed in the neurons adjoining the proximal parts of ventral and lateral cords, as well as in all cell types innervating the pharynx (Fig. 5D, E). A less intense IR characterized the neurons of the lateral group and medioventral neurons (a very weak reaction, if present). An unpaired cell mediodorsally near the connection of ventral cords was also readily registered (Fig. 5F).

IV. a-5-HT

5-HT IR was found in the brain, cords and commissures, and in the nerves of the head and adhesive plate, but it was much more poorly represented than RF- or SALMFamide IR.

1. Brain

The neuropile contained a loose net of positive fibres. There were seven positive perikarya, all of them being restricted in their position to the posterior portion of the brain (Fig. 6A). The larger cell (or a pair of very closely connected cells, up to 10×22–22 μm in size) occupied a medial position, three pairs of normal sized cells lay on the same level. Not less than five immunopositive cells lay in the root of each lateral cord (Fig. 6A). One of them sent the central process to the brain and the peripheral process to the lateral cord (Fig. 6A, empty arrow), three sent the only process to the brain (Fig. 6A, arrowhead), whereas the fifth possibly sent the peripheral process also to the anterior branch of the lateral cord.

2. Submuscular nerve net

Even in the ventral cords only a loose bunch of thin processes was stained with antisera, while the other cords and commissures had the appearance of very thin fibres (Fig. 6C). One row of immunopositive neurons lay along each ventral cord (Fig. 6B). They were pseudounipolar cells sending their two processes into the ventral cord in the opposite direction. The number of these cells was approximately equal to the number of circular commissures between the brain and pharynx, but no signs of a direct connection between a neuron and the nearest commissure were noted. Behind the level of the anterior part of the pharynx such cells were lacking (Fig. 6C).

3. Pharynx innervation

Both the meridional nerves and the nerve ring of the pharynx were immunopositive. Up to 6–8 unipolar perikarya were situated in the lateral portions of the body anteriorly to the pharynx (Fig. 6D). Their processes could be traced to join the meridional pharyngeal nerves. Few processes going off from the ventral cords also entered the meridional nerves of the pharynx.

4. Genital plexus

The proximal (anterior) part of the lyre-like copulatory organ and the tubiform sperm duct were surrounded by a delicate 5-HT immunoreactive nerve plexus with minute varicose swellings (Fig. 6F, arrow). The plexus was formed by the processes of 6–10 multipolar perikarya which were situated dorsally or dorsolaterally to the ventral cord (Fig. 6E). Processes of these cells also entered the ventral cord.

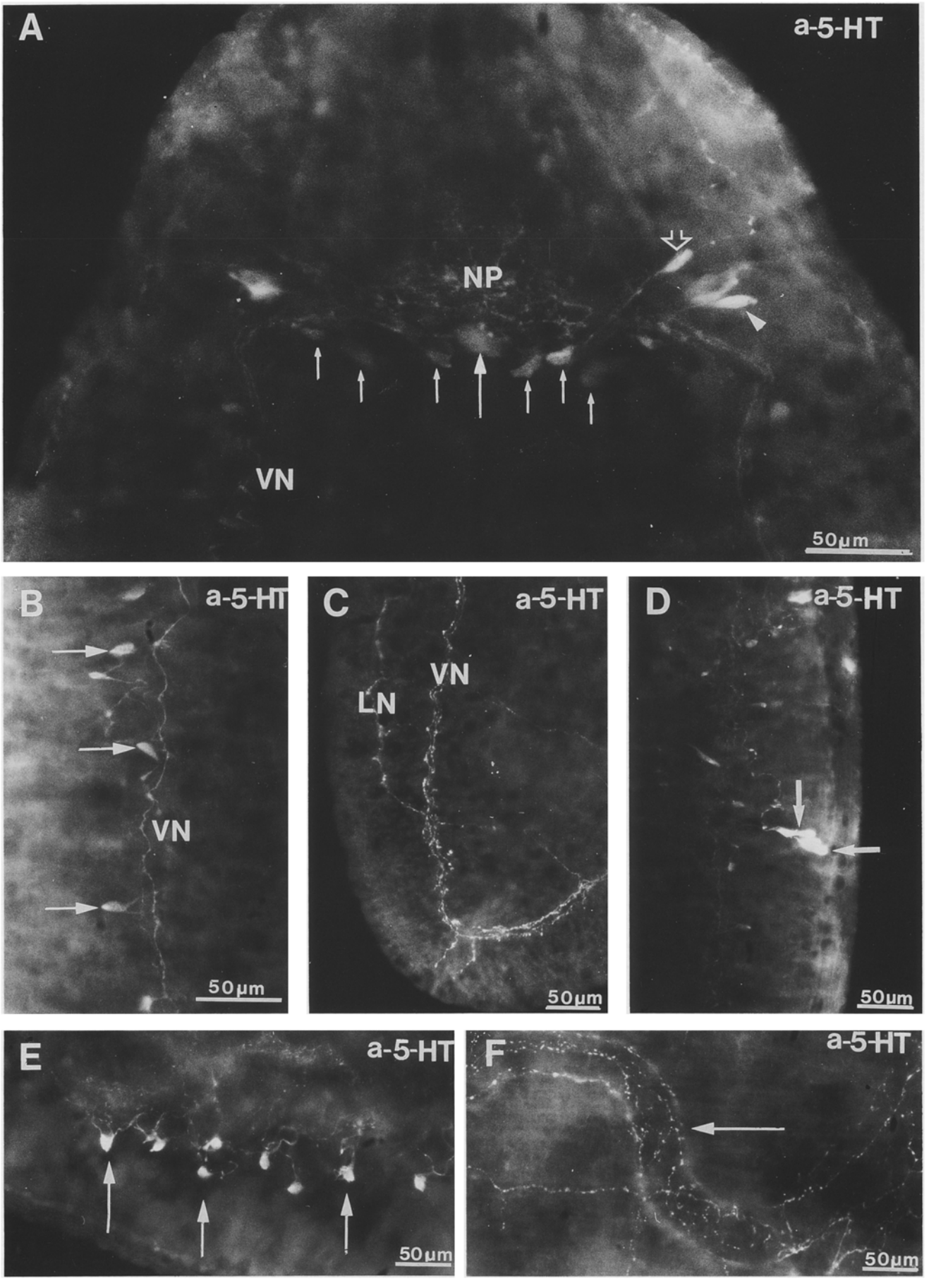
V. CAs (GAIF)

Green glyoxylic-acid-induced-fluorescence was observed in the brain, nerve cords and commissures, in the head region, pharynx and adhesive plate (Fig. 1A, Fig. 7).

1. Brain and head region

The neuropile contained a dense net of positive fibres. Three pairs of perikarya lay near the brain and sent their processes only to or via the brain (Fig. 7A; c1,c2,c3). The most anterior pair was situated behind the statocyst, the next one had a relatively dorsal position. The posterior pair lay on the same level as the first one, the distance between neurons being larger than between the cells in the first two pairs. Two neurons lay beside the statocyst (Fig. 7A; an). They sent one process to the brain and another one to the anterior pole of the body (it is out of the focus plane on Fig. 7A). At the level of the brain, the fibres from the lateral and marginal cords united to form a branching nerve which innervates ciliary pits (Fig. 7B).

Fig. 6A–F. 5-HT IR, whole mounts. **A** Brain: seven neurons caudally to neuropile (NP). Unpaired neuron, or two cells lying close to one another (*long arrow*) and three pairs of cells (*short arrows*). Group of neurons in the root of lateral cord: neuron with a central and peripheral process (*empty arrow*), one of the neurons with single central process (*arrowhead*), note also thin fibres in ventral cords (*VN*) and adjoining neurons. **B** Neurons (*arrows*) adjoining ventral cord (*VN*). **C** Ventral (*VN*) and lateral (*LN*) cords joining each other in tail end. **D** Neurons situated laterally ahead of the pharynx to which each of them sends a process (*arrows*). **E** Neurons of genital plexus (*arrows*). **F** Fibre plexus with minute varicose swellings (*arrow*) in the walls of sperm duct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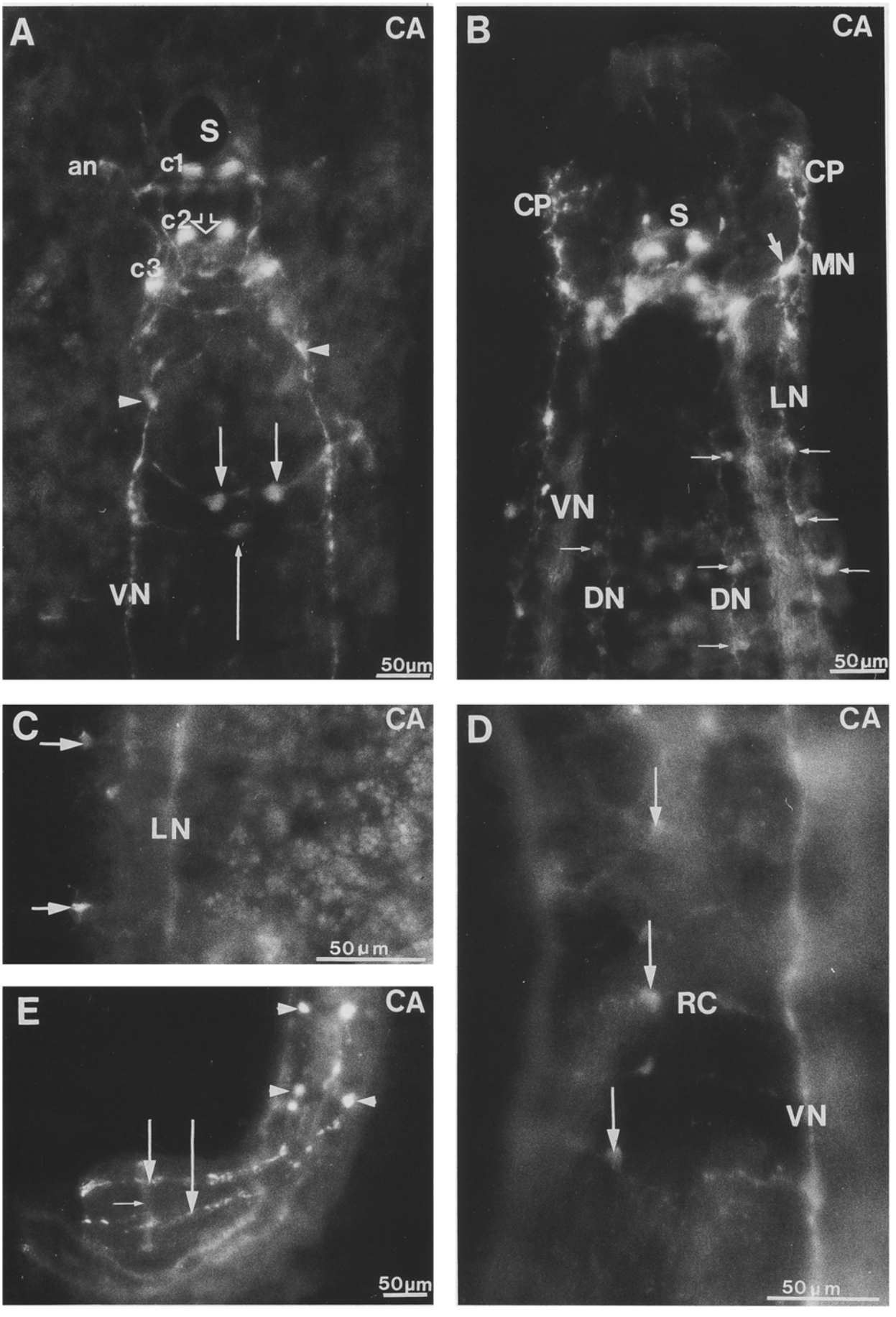
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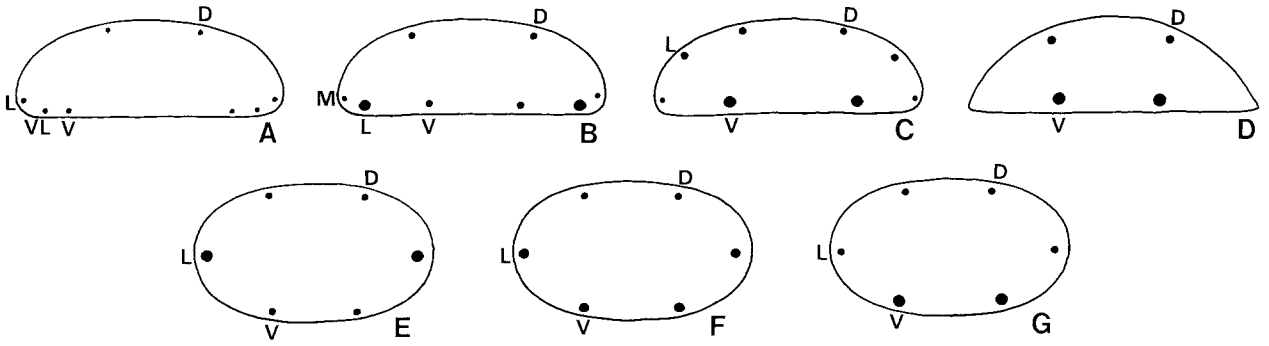

Fig. 8A–G. The number, position and relative width of longitudinal nerve cords in the Seriata (**A–D**) and some other Plathelminthes (**E–G**). **A** *Minona trigonopora*: relative width of cords not described, cords designations as in Reisinger 1972. **B** *Monocelis* and *Archilopsis*. **C** *Bothriomolus balticus*. **D** *Bdelloura candida*. **E** *Microstomum lineare*. **F** One of the variants found, e.g. in some Neorhabdocoela turbellarians. **G** The variant seemingly most common for the Prolecithophora, the Neorhabdocoela and the Neodermata, ventral cords dominate. Cords: D, dorsal; L, lateral; M, marginal; V, ventral; VL, ventro-lateral

2. Submuscular nerve net

The ventral cords were the richest in CAs. One positive neuron was situated in the anteriormost portion of each ventral cord (Fig. 7A, arrowheads). Behind it, a commissure which included two bipolar perikarya connected the ventral cords (Fig. 7A). At the level of the brain, one perikaryon adjoined each lateral cord (Fig. 7B, arrow). A short positive fibre went from this cell to the ventral cord (Fig. 7B). One positive neuron probably adjoined each dorsal cord at the level of the brain, though the presence of these cells cannot be doubtlessly postulated from our data. The first ring commissure was situated immediately behind the ventral commissure with two neurons. Each ring commissure contained five perikarya. One of them was an unpaired cell lying medially between the ventral cords (Figs. 7A, long arrow; 7D, arrows). One pair of cells (L cells) lay in any position between the lateral and marginal cords (Fig. 7B, C). The cells of the second pair (D cells) adjoined the dorsal cords (Fig. 7B). This pattern seemed to be more or less retained all along the body, though it was more pronounced in the anterior half of the body (Fig. 7B).

3. Pharynx innervation

Both the meridional nerves and the nerve ring of the pharynx contained positive fibres. Only one type of neurons sent their processes to the pharynx. They were unipolar cells lying in the lateral portions of the body anteriorly to the pharynx (Fig. 7E, arrowheads). Not more than four such cells were seen on either side of the body. The positive processes of the pharynx innervation did not reach the positive structures of the submuscular nerve net. Thus the positive innervation of the pharynx looked separated from the other parts of the GAIF-positive nervous system.

D. Discussion

Much attention has been paid to the phylogenetic significance of the orthogonal organization of the nervous system in the Plathelminthes (for review see Ehlers 1985). In the discussion it has been stressed that morphologically similar types of nervous systems may have arisen independently, demonstrating striking parallelism (Kotikova 1986) which is due to certain general trends in the evolution of the nervous system and the influence of the specialized forms of the body (Joffe 1990). The present study contributes to the discussion with new data on the insufficiently known neuroanatomy of the Proseriata.

I. Number of cords in the Monocelididae and the Otoplanidae

An orthogon with four pairs of longitudinal nerve cords and numerous ring commissures was first described by Reisinger (1925) in *Bothrioplana semperi* Braun, 1881. Then it has been found in the Monocelididae: *Minona trigonopora* Ax, 1956 (see Reisinger 1972) as well as in *Monocelis* and *Archilopsis* (see Kotikova 1976; Joffe and Kotikova 1991). However, in the representatives of the Otoplanidae, only three pairs of longitudinal cords have been described (Ax 1956; Kotikova 1976). The "miss-

Fig. 7A–E. Catecholaminergic innervation, GAIF whole mounts. **A** Head, ventral view (from specimen notably stretched longwise during preparation), statocyst (*S*), neuropile (*empty arrow*), cells near brain (*c1, c2, c3*), bipolar neuron (*an*) beside statocyst (its peripheral process is out of the focus plane), ventral cords (*VN*), neurons at beginning of ventral cords (*arrowheads*), paired bipolar neurons of connecting commisure (*arrows*), medial single neuron (*long arrow*) of ring commissure. **B** Head, dorsal view, statocyst (*S*), innervation of ciliary pits (*CP*) from lateral (*LN*) and marginal (*MN*) cords, neuron at the anteriormost portion of lateral cord (*arrow*), dorsal (*DN*) cord, neurons adjoining *DN* and *LN* (*thin arrows*, D and L cells from three successive ring commissures are seen on the right side of the worm). **C** L-group cells near head (*arrows*), lateral nerve cords (*LN*). **D** Ventromedial cells (*arrows*), ventral nerve cords (*VN*), ring commissure (*RC*). **E** Pharynx: cells anteriorly to pharynx (*arrowheads*) sending their processes to the meridional pharyngeal nerves (*arrows*), ring nerve (*thin arrow*)

ing" pair is a pair of thin cords running along the lateral margins of the body. In the present study two thin longitudinal marginal nerves were demonstrated in *Bothriomolus balticus* (Otoplanidae) by IR to RFamide and SALMFamide, as well as positive reactions for CAs.

II. Comparison of "main" cords

A difference in dominance and position of cord pairs exists between the studied representatives of the Monocelididae and the Otoplanidae (Fig. 8A–C). The most pronounced cords in the Monocelididae, *Archilopsis*, *Monocelis* (see Joffe and Kotikova 1991) and *Promonotus schultzei* (Meixner 1943) (see Reuter 1990; Reuter and Eriksson 1991), are the lateral ones, which are situated on the ventral side of the body, run ventrolaterally near the lateral margins of the body and join at the posterior end of the body. The marginal cords are situated between the lateral and dorsal ones (Fig. 8A, B). However, in *B. balticus* the ventral cords are the most pronounced ones and join at the posterior end. The lateral cords occur on the dorsal side of the body and the marginal cords are situated between the lateral and ventral ones (Fig. 8C). Thus, even though the ventral cords of *Bothriomolus* occupy a more medial position than the lateral cords of the studied species from the Monocelididae, these cord pairs look rather similar.

The majority of the Plathelminthes including the Monocelididae (Joffe 1991; Joffe and Kotikova 1991; Reuter and Eriksson 1991) has a group of CA-positive neurons (L group) situated in postcerebral lateral zones of the body between the ventral and dorsal cords. The lateral cords contain many CA-positive fibres and receive processes directly from the perikarya of these cells. In *B. balticus*, however, the ventral cords rather than lateral ones are rich in CAs.

The ventral cords of *B. balticus* are also rich in a-RFamide positive material and they are the only cords showing IR to substance P (Reuter unpublished data). In *P. schultzei* however, the strongest IR to RFamide is observed in the lateral cords (Reuter 1990) which also demonstrate IR to substance P (Reuter unpublished data).

All these facts imply that the ventral ("main") cords of *Bothriomolus* correspond to the lateral ("main") cords of the Monocelididae and not to the ventral cords. The term "main cords" can be defined as follows: (1) they include notably more nerve processes and are wider than the others and (2) more neurons and groups of neurons are associated with main cords than with the other cords.

As far as the distribution of the neuroactive substances is concerned, the main cords of various representatives of the Plathelminthes have much in common. Serotoninergic neurons are of special interest in this connection. Postcerebral 5-HT positive neurons associated with cords and commissures (neurons of, for example, the pharynx and genital system are not discussed here) seem to mark the main cords in nearly all the Plathelminthes. These cells either send their processes only to the main cord, so that they reach other cords only via the main one, or they direct one process (or processes) to the main cord and the other (or other) to another cord.

In *Stenostomum* (Catenulida), a pair of lateral cords with associated 5-HT positive cells has been observed (Wikgren and Reuter 1985). In *Microstomum* (Macrostomida), 5-HT IR neurons are associated with lateral main cords (Reuter et al. 1986; Palmberg and Reuter 1990). The observations on *B. balticus* and the preliminary data on *Archilopsis* sp. imply that the 5-HT positive cells are associated only with the main cords independently from the position of these cords. In the Tricladida *Paludicola*, 5-HT positive cells are mainly associated with the ventral cords. Additional positive cells occur scattered in the submuscular nerve net (Welsh and Williams 1970; Kerschbaum et al. 1988) and supposedly innervate ciliary cells (Sakharov et al. 1988). In the Cestoda, 5-HT immunoreactive cells are also associated with main cords (Gustafsson 1991; McKay et al. 1991a). The other Neodermata would need special discussion, but the available data are insufficient for this.

Preliminary data on a representative of the Acoela suggest that the a-5-HT immunopositive neurons are distributed more or less evenly, without a special relation to any pair of nerve cords (Reuter et al. 1988), which might be a plesiomorphic condition (cf. arguments about the position of the Acoelomorpha in the system of the Plathelminthes in Ehlers 1985 and Rieger et al. 1990; see also Joffe 1991 for the implication from data on the distribution of CAs). But as far as the distribution of the a-5-HT in Seriata is concerned, the available data confirm the correspondence of the lateral cords in the Monocelididae and the ventral cord in the Otoplanidae.

The topographic position of the main cords is different in various plathelminths. In the Macrostomida, the main cords are the lateral ones (Reuter et al. 1986; Reuter 1988; Joffe 1991; Joffe and Kotikova 1991; Reuter and Wikgren 1991). The only pair of cords demonstrated so far by ICC methods in *Stenostomum* (the Catenulida, see Wikgren and Reuter 1985; Reuter 1988) also occupies a lateral position.

Data on the distribution of AChE are available for several species from the Lecithoepitheliata and the Prolecithophora and for more than 70 species from the Neorhabdocoela and the Neodermata (reviewed in Joffe 1990). About 40 species have been studied by the GAIF method (reviewed by Joffe and Kotikova 1991; Joffe 1991; Shishov 1991; for the Lecithoepitheliata: Joffe, unpublished data). According to these data, in the Lecithoepitheliata, the ventral cords are the main ones. In the Prolecithophora and Neorhabdocoela, either the lateral cords slightly dominate, or the lateral and the ventral cords are equally well developed, or the ventral cords dominate. In the Proseriata, either the ventral or the lateral cords dominate. In the Tricladida, the ventral cords dominate pronouncedly. Unlike the majority of the other Plathelminthes, Even CA-positive cells are predominantly associated with ventral cords (Welsh and Williams 1970; Joffe unpublished data). In the Cestoda, the main cords have a lateral position and are submerged

deep in the parenchyme, but the majority of the Monogenea and the Digenea seems to have ventral cords as the main ones. This is particularly the case with all the species studied with ICC methods so far (see Fairweather and Halton 1991; Halton et al. 1992).

Thus one can note a trend towards the localization of the main cords in a ventral position (Fig. 8) in various phylogenetic lineages of the Neoophora ("the higher plathelminths"), while in the earliest groups to diverge the main cords occupy a lateral position (Catenulida and Macrostomida). The only histochemical study of the Acoela (Mamkaev and Kotikova 1972) demonstrates that the main cords in the Acoela may be pronounced or not pronounced, but discussion about this group has to wait until more data are available (see Reisinger 1972; Kotikova 1986; Joffe 1990 for contradicting views). The significance of the lateral position of main cords in the Monocelididae also remains open for discussion, but it may well be a plesiomorphic condition for the Seriata.

III. Plexus, cord-like structures and marginal cords

Transverse plexal fibres and commissures observed in the Proseriata are mainly very regular. Commissures can be distinguished from plexal fibres by the presence of associated perikarya (Reuter 1990). In *B. balticus*, transverse plexal fibres are practically lacking, but longitudinal fibres reminiscent of cords (because they are seen in the same position in different specimens) are present. Neurons marking the cords have to be identified to delimit objectively plexal fibres from thin nerve cords.

The regular plexus organization seems to be related to the presence of numerous densely situated ring commissures which, in turn, correlate with the elongated and flattened form of the body (see Joffe 1990 for discussion). A similar form of the body and nervous system with numerous ring commissures is also characteristic of the Lecithoepitheliata and the Cestoda. Correspondingly, in the represenatives of the Lecithoepitheliata two pairs of thin nerves, called either plexus strings or "minor" nerve cords, have been found (Kotikova and Timoshkin 1987; Timoshkin 1991). They run just above the ventral and dorsal cords at the level of the subepidermal plexus while the cords themselves lie deeper in the parenchyma. In some cestodes the number of longitudinal nerves with cord-like appearance varies even in different portions of the body (see, e.g. Gustafsson et al. 1986).

The presence of marginal cords also seems to correlate with the "proseriate-like" form of the body (see also Joffe 1990). No marginal cords have been observed in the Tricladida (Hanström 1926; Gelei 1909–12; Joffe unpublished data), which is the sister group of the Proseriata, but they are present in some (but not all) representatives of the Lecithoepitheliata (Reisinger 1968; Kotikova and Timoshkin 1987; Böckerman et al. 1994; Joffe unpublished data). Both in the Proseriata and the Lecithoepitheliata, the marginal cords have much in common with the "strengthened" plexal fibres. Direct connection of the marginal cords to the brain (roots) has never been found in either group. The marginal cords of the Monocelididae, *Monocelis* and *Archilopsis*, are closely associated with the lateral (main) cords, but not so in *Bothriomolus*. The different positions of marginal cords in the studied species from the Monocelididae and the Otoplanidae (Fig. 8A–C) imply that these cords have probably been formed independently even in these two taxa.

Thus, in the discussion of the evolution of the nervous system, three parallel phenomena have to be taken into account. In addition to a reduction in the number of longitudinal nerves and a strengthening of the remaining ones (Reisinger 1970, 1972), a concentration of plexal fibres to longitudinal cords may occur.

IV. Adhesive plate

The ICC methods revealed two horn-like nerves on the adhesive plate of *B. balticus*, but GAIF-positive cells are lacking, as well as on the posterior portion of the body in the Tricladida. In contrast, bipolar GAIF-positive neurons are present on the adhesive plate of the studied species of the Monocelididae (Joffe and Kotikova 1991). These marker neurons deserve a study in the other families of the Proseriata because the position of the Monocelididae in the system of the Proseriata remains questionable (cf. Sopott-Ehlers 1985; Martens and Schockaert 1988). More so, all the studied representatives of the Proseriata have unpaired medial CA-positive and a-RFamide positive neurons (this article, Reuter 1990; Joffe and Kotikova 1991). These cells are manifested neither in the Tricladida (see above) nor in the triclad-like *Bothrioplana semperi* (Joffe, unpublished data) and their presence might be a good synapomorphy for the Proseriata.

V. On the possible modes of the evolution of nerve cords

Traditionally, the nerve cords in the studies of the Plathelminthes have been designated according to their position in the body and this attitude seems to be justified, for example, for the Neorhabdocoela and the Neodermata. Yet our results demonstrate that a similar position does not always grant direct correspondence between cords. Figure 8 illustrates that one cannot just negate the problem by renaming, for example, the main cords of the Monocelididae as "ventral". In this case similar problems would arise with other pairs of cords.

The first assumption one could make is that the pair of main cords is the only constant and important structure and that it can be shifted ventrally or laterally, while the other longitudinal cords arise by concentration from the plexus or fuse with the main cords and each other when and where it is necessary. This postulation is obviously oversimplified. It implicitly assumes that the other cords, unlike the main ones, are somewhat like simple bunches of processes, which does not seem correct. Neurons of various transmitter specificity located in close association with cords other than the main ones

are known, for example, in the Neorhabdocoela and the Neodermata (Reuter 1987; Joffe and Kotikova 1991; Shishov 1991; McKay et al. 1991a). Furthermore, all the histochemical and immunocytochemical data agree that the Neorhabdocoela and the Neodermata have basically three pairs of longitudinal cords, but the degree of development of main cords is different in these taxa.

These facts suggest a second alternative assumption that in the course of evolution the processes of nerve cells and perikarya are redistributed between cords, while the cords themselves do not change their positions (see Fig. 8). Such a redistribution of cells and processes is not a new assumption in that it is always supposed as a mechanism for centralization, ganglionization, etc. Versatility typical for archaic groups of organisms (Mamkaev 1986) as well as parallelism (Kotikova 1986) characterize the morphological organization of the nervous system in the different taxa along the flatworm phylogenetic line. It is a prerequisite for a diversity brought forth by functional demands of adaption to different habitats and different life styles.

Neither of the two above-mentioned assumptions seems in itself to explain all the available data. Probably both a shift of the main cords accompanied by formation of new cords from the plexus and fusion of the other cords and a redistribution of nerve processes and perikarya between the cords, take part in the evolution of cords, and the relative contribution of each mode of evolution may differ in various phylogenetic lineages.

The first possibility seems to explain the difference in the position of main cords in the Seriata, while, for example, in Neorhabdocoela and Neodermata, the second possibility seems to dominate. Due to the lack of specific markers for cords and their derivatives it is difficult to determine the contribution of each possibility in each particular case. Homologous neurons and neuron groups (see Joffe and Kotikova 1991; Joffe 1991 for the special discussion on neuron homologies in the Plathelminthes) are the only structures which might serve as markers. The present discussion demonstrates that a correct analysis of the evolution of conductive paths (cords and commissures) depends on whether we know the neurons or neuron groups marking these structures.

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