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Neuropeptide F-immunoreactivity in the tetrathyridium of *Mesocestoides corti* (Cestoda: Cyclophyllidea)

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Abstract. The distribution pattern and subcellular localisation of neuropeptide F (NPF) immunoreactivity (IR) in the tetrathyridium stage of *Mesocestoides corti* were investigated by whole-mount immunocytochemistry in conjunction with confocal scanning laser microscopy (CSLM) and by immunoelectron microscopy using immunogold labeling. Using an antiserum directed to the C-terminal decapeptide amide (residues 30–39) of synthetic NPF (*Moniezia expansa*), CSLM revealed NPF-IR throughout the central and peripheral nervous systems of parental and dividing tetrathyridia. Ultrastructurally, gold labeling of NPF-IR was confined to the contents of the smaller of the two sizes of electron-dense neuronal vesicle identified.

The use of *Mesocestoides corti* as a research model for experimental studies in cestode biology has been recognised since Specht and Voge (1965) first reported that the second larval (metacestode) stage, or tetrathyridia, were capable of multiplying asexually in the body cavity of mice and that they could be maintained indefinitely by serial passage. In chemotherapy studies, the mouse-tetrathyridium system would seem to offer an excellent means of exploring the potential of drug-delivery systems for helminth parasites, such as the use of liposomes as carriers of antiparasitic drugs (Croft 1986; Hrckova and Velebny 1993; Hrckova et al. 1993). A novel target for liposomised anthelmintics could be the recently discovered neuropeptidergic systems of helminths, in particular the authentic invertebrate regulatory peptide, neuropeptide F (NPF; see review by Halton et al. 1992).

NPF was first isolated from *Moniezia expansa* by Maule et al. (1991) and has since been detected in all invertebrates examined thus far, including coelenterates, platyhelminths, annelids, molluscs and insects (Maule et al. 1993; Verhaert et al. 1992). Where examined physiologically [e.g. the NPY-related peptide (=NPF) from *Aplysia*], the action of NPF has been found to be inhibitory, prolonging hyperpolarisation and a reduction in the spike rate of neurones (Rajpara et al. 1992). This finding and the peptide's seemingly restricted occurrence in invertebrates means that the NPF family offers enormous potential in novel anthelmintic discovery.

The aim of the present investigation was to examine the asexually proliferative tetrathyridia of *M. corti* for the presence of NPF-immunoreactivity and to explore its distribution and subcellular localisation, using, respectively, confocal scanning laser microscopy and post-embedding immunogold labeling at the ultrastructural level.

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Materials and methods

Tetrathyridia of *Mesocestoides corti*, the strain originally isolated from a lizard population by Specht and Voge (1965), were maintained by intraperitoneal and peroral passage through ICR-strain laboratory mice. Specimens were recovered from the peritoneal cavity of infected mice in Hanks' balanced salt solution (HBSS), washed several times and kept at 37° C in HBSS prior to fixation as described below.

Immunocytochemistry

Immunostaining was performed using the indirect immunofluorescence technique of Coons et al. (1955). Flattened whole-mount preparations ($n=100$) were fixed at 4° C for 4 h in 4% (w/v) paraformaldehyde (PFA; Agar Aids, Cambridge UK) in phosphate-buffered saline (PBS: 0.145 *M* NaCl, 0.025 *M* $NaH_2PO_4 \cdot 2H_2O$, 0.075 *M* Na_2HPO_4; pH 7.2), followed by washing (three changes) in PBS containing 0.5% (v/v) Triton X-100, 0.1% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) sodium azide for 48 h. Specimens were incubated in primary antisera [either 790(1), which was raised to the intact NPF molecule, i.e. residues 1–39, or 792(1), which was raised to the C-terminal decapeptide amide of synthetic NPF, i.e. residues 30–39, both at a working dilution of 1:800] for 48 h at 4° C and were then washed for 24 h in PBS at 4° C before and after immersion in secondary antiserum (fluorescein isothiocyanate-labeled swine anti-rabbit IgG; Dako Ltd., High Wycombe, UK) for 24 h. The preparations were then washed for 24 h in PBS:glycerol (1:9, v/v) and viewed with an MRC 500 confocal

scanning laser microscope (CSLM; Bio-Rad Lasersharp, Abingdon, Oxfordshire, UK). Controls included (a) omission of primary antibody; (b) substitution of primary antisera with non-immune rabbit serum (Dako Ltd); and (c) liquid-phase preabsorption with NPF (C-terminal fragment 30–39), bovine pancreatic polypeptide (bPP), and FMRFamide (in a range of concentrations ranging from 200 to 1000 ng/ml diluted antiserum).

Immunogold electron microscopy

For electron microscopy, the tetrathyridia ($n=24$) were subjected to an indirect post-embedding method for immunolabeling as follows. The metacestodes were fixed at 4° C for 40 min in 2% double-distilled glutaraldehyde (GTA; Agar Scientific Ltd., Stansted, UK) in 0.1 *M* cacodylate buffer (pH 7.2) containing 0.1 *M* sucrose and were then buffer-washed prior to post-fixation in 0.5% osmium tetroxide for 30 min at 4° C. The specimens were buffer-washed, dehydrated rapidly in ethanol at 4° C, infiltrated in Epon 812 resin (Polaron Equipment Ltd., Watford, UK) and thermally cured for 40 h at 60° C. Thin sections (70 nm in thickness) were cut, mounted on bare 200-mesh nickel grids, etched with 10% H_2O_2 for 10 min at room temperature and rinsed with 20 m*M* TRIS-HCl buffer (pH 8.2) containing 0.1% (w/v) BSA and Tween 20 (1:40 dilution). The sections were exposed to normal goat antiserum (NGS) for 30 min at room temperature and then incubated overnight with primary antibody [NPF 792(1)] diluted to 1:10000 with 0.1% BSA/TRIS-HCl buffer for 18 h at room temperature. Sections were then washed in TRIS/BSA as described above and, after their transfer to a 25-µl droplet of 10-nm-size gold-conjugated goat anti-rabbit IgG (Biocell, Cardiff, UK) for 2 h, were lightly fixed with 2% double-distilled GTA (3 min), washed with buffer and rinsed with distilled water. Finally, sections were double-stained with alcoholic uranyl acetate (8 min) and aqueous lead citrate (12 min) and examined in a JEOL 100 CX electron microscope at 100 kV. Controls consisted of (a) using non-immune rabbit serum (Dako Ltd) in place of the primary antiserum, (b) using gold-labeled antiserum in the absence of primary antiserum and (c) liquid-phase preabsorption of the antiserum with NPF standard (100–400 ng/ml diluted antiserum).

Results

Immunocytochemistry

Preliminary microscopic observations of NPF-immunoreactivity in the tetrathyridia of *Mesocestoides corti* revealed that staining was evident only with the 792(1) antiserum to NPF (30–39), i.e. the one raised to the C-terminal decapeptide amide of NPF, and for this reason it was used throughout the study. All of the observed immunoreactivity was confined to the central and peripheral elements of the nervous system of the tetrathyridium (Fig. 1). Thus, staining revealed ganglionic masses of neurones in close association with the region medial to the posterior margin of each of the four suckers; no obvious cerebral ganglia, as distinct from the four sucker ganglia, were apparant from the staining. The absence of cerebral ganglia per se was confirmed by CSLM by examining composite images of the scolex derived from a series of optical sections through the forebody of the worm. Commissures connect the sucker ganglia and are interrelated by a dorsoventral commissure (Figs. 2, 3). Each sucker is served by a plexus-like array of fine, immunoreactive fibres derived from the sucker ganglia (Fig. 3).

Typically, there are five pairs of longitudinal nerve cords. These include the main lateral longitudinal nerve cords, which are the best developed and therefore stain the strongest; the dorso-lateral nerve cords; the ventro-lateral nerve cords; the dorso-median nerve cords; and the ventro-median nerve cords (Figs. 2, 4). All of these nerve cords extend posteriorly from the sucker ganglia through the body region to a common terminus, which itself is in the form of a nerve plexus surrounding the excretory pore. Just prior to joining the excretory plexus, the three lateral nerve trunks on each side of the worm fuse to form a common nerve cord (Fig. 5). Numerous circular commissures interconnect the longitudinal nerve cords and, in places, many of these give rise to a peripheral subsurface network of fine immunoreactive fibres (Fig. 4).

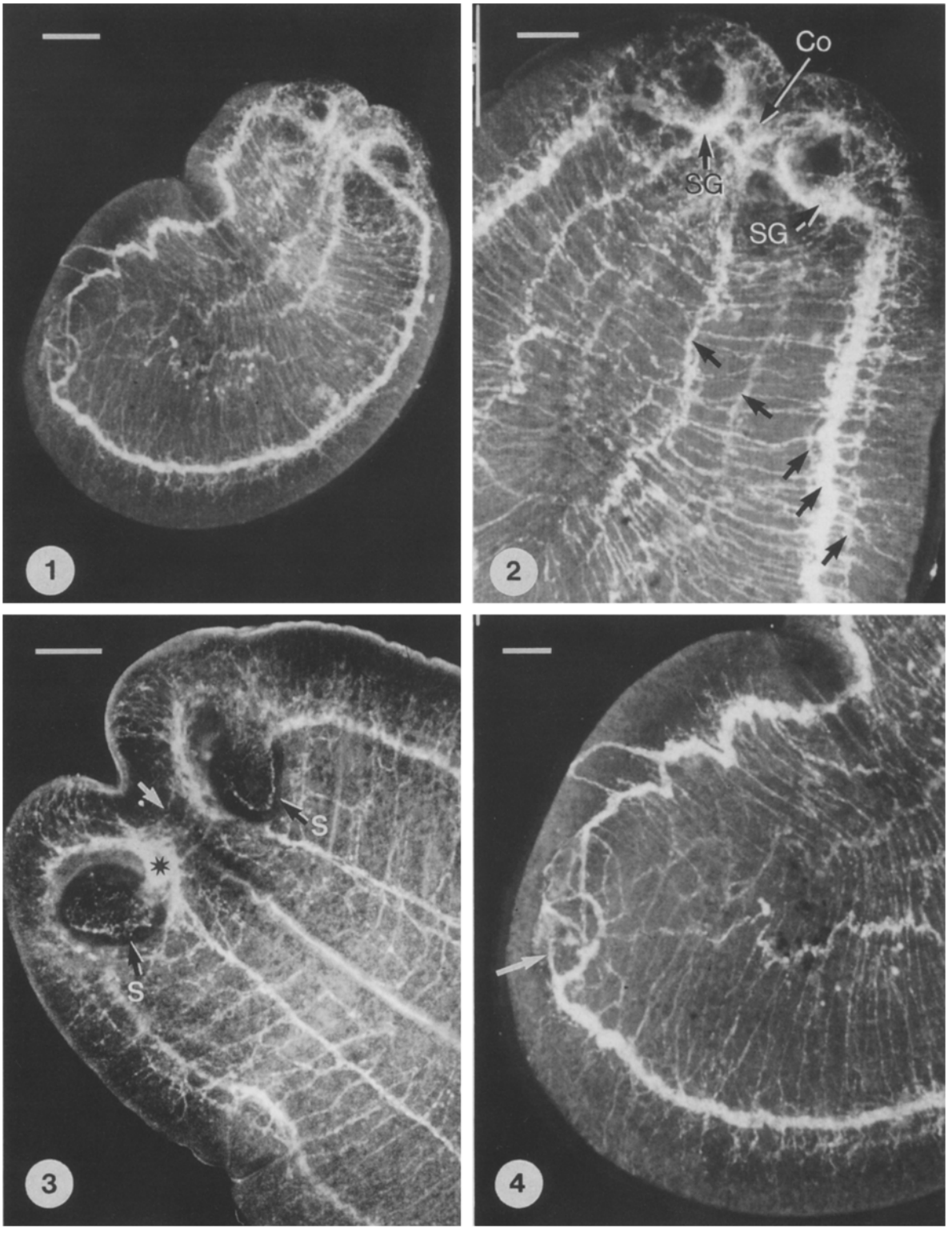
During longitudinal fission, the sucker ganglia and longitudinal cords of the parental organism are divided equally between the two resultant progeny. This process involves the development of a common longitudinal nerve trunk between the two organisms that, as development proceeds, differentiates into the main lateral and two median nerve cords (Fig. 6).

No immunoreactivity was obtained in the absence of primary antiserum or following the substitution of primary antiserum with non-immune serum. All of the immunoreactivity was quenched following preincubation with 200 ng NPF (30–39)/ml but was unaffected by the addition to the incubation medium of up to 1000 ng bPP or FMRFamide/ml.

Immunogold electron microscopy

The preparative protocol used in the present immunogold-labeling work involved secondary fixation in osmium tetroxide and embedding and sectioning of tissue in an epoxy plastic. These procedures produced ultrastructural preservation as good as that obtained in our previous studies of subcellular localisation of flatworm neuropeptides, where osmium was omitted and the use of low-temperature methacrylate resins, such as K4M or LR Gold, were essential to retain antigenicity (see Brennan et al. 1993a, b; Maule et al. 1992). However, it should be noted that although the method described herein preserved sufficient NPF-antigenic sites in *M. corti* tetrathyridia to produce good-quality gold labeling, this may not be the case for other peptide/antisera interactions or specimen types.

Ultrastructural examination of the sections of *M. corti* revealed profiles of duct-like axons delimited by a membrane and containing electron-dense vesicles, neurotubules and mitochondria in the longitudinal nerve cords (Figs. 7, 8). At least two distinct sizes of neuronal vesicle were identified, both with moderately electron-dense, homogeneous matrices: a larger form measuring approximately 160–170 nm in diameter and a smaller form of some 60- to 75-nm size (Fig. 7). Mixed populations of the two vesicle sizes were never apparent in any of the axons examined. Gold labeling of the NPF (30–39) antibody was found almost exclusively over the



Figs. 1–6. Whole-mount preparations of *Mesocestoides corti* tetrathyrida viewed by confocal scanning laser microscopy, showing immunoreactivity (IR) to NPF. **Fig. 1.** Survey image of a tetrathyridium, showing that the NPF-IR is distributed throughout the nervous system. Bar = 100 μm. **Fig. 2.** Anterior portion of a tetrathyridium, showing NPF-IR in the sucker ganglia (*SG*) and associated commissures (*Co*) and in the 5 pairs of longitudinal nerve cords (*unlabeled arrows*) and numerous cross-connectives. Bar = 50 μm. **Fig. 3.** Each sucker (*S*) is served by a plexus-like array of fine, NPF-immunoreactive fibres derived from the sucker ganglion (*). Note the transverse commissures that interconnect the sucker ganglia (*unlabeled arrow*). Bar = 50 μm. **Fig. 4.** Posterior portion of a tetrathyridium, showing that the longitudinal nerve trunks have a common terminus in the form of a nerve plexus (*arrow*) surrounding the excretory pore. Bar = 50 μm

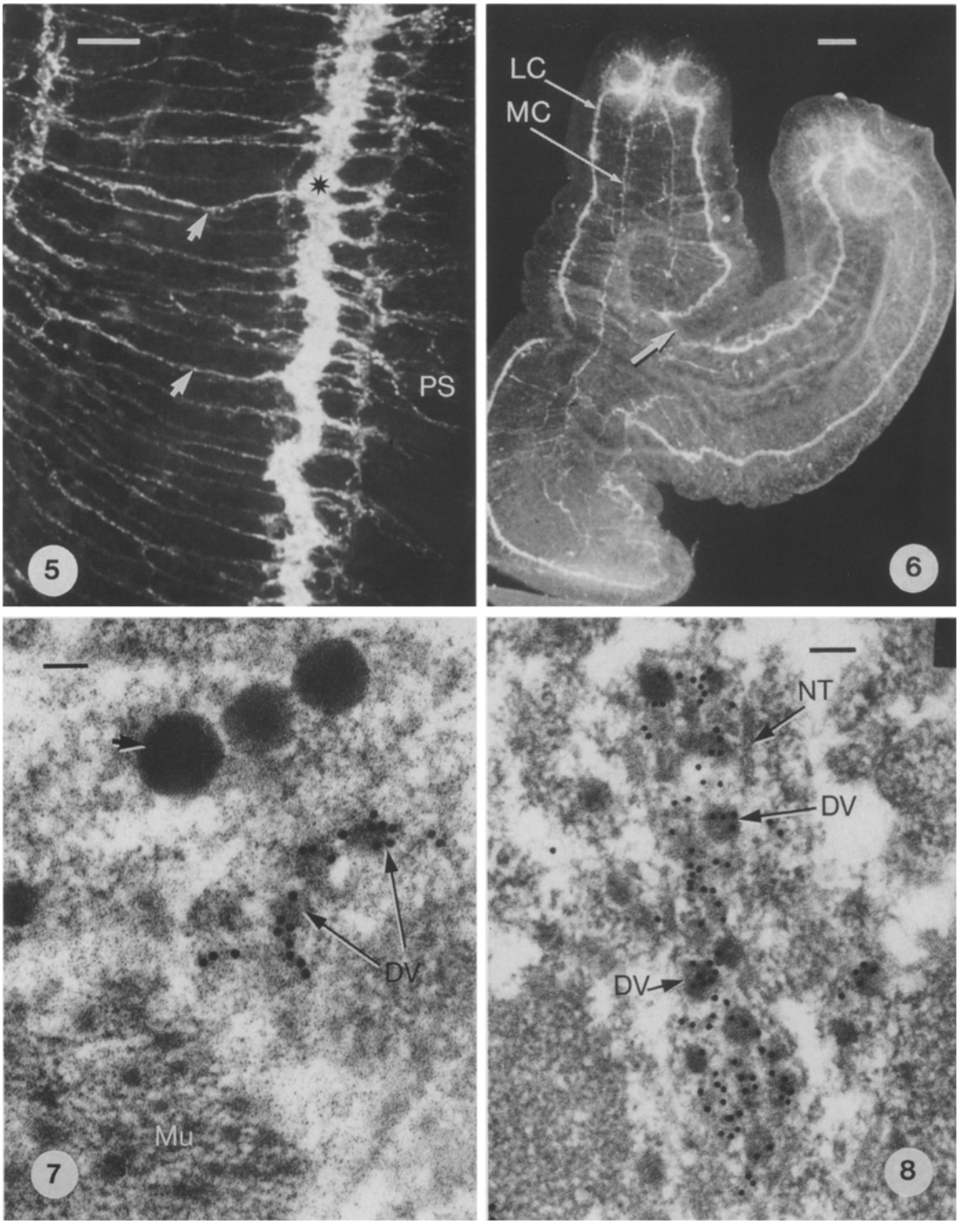


Fig. 5. A portion of the lateral margin of a tetrathyridium, showing details of the structural arrangement of the longitudinal nerve cords, all of which are interconnected by circular connectives (*arrows*). Note the prominent main lateral longitudinal nerve cord (*) and the numerous branches that give rise to subsurface fibres of the peripheral nervous system (*PS*). Bar = 25 μm. **Fig. 6.** A tetrathyridium undergoing longitudinal fission, during which the nervous system is divided equally between the two resultant progeny. The process involves the development of a common nerve trunk between the two organisms (*unlabeled arrow*) that, as development proceeds, differentiates into the median (*MC*) and lateral (*LC*) nerve cords. Bar = 100 μm. **Figs. 7, 8.** Immunogold labeling of neuronal vesicles in an *M. corti* tetrathyridium. **Fig. 7.** NPF-IR in dense vesicles (*DV*) in a nerve terminal in close apposition to a muscle fibre (*Mu*). Note the separate population of larger vesicles that are unreactive for NPF (*unlabeled arrow*). Bar = 0.1 μm. **Fig. 8.** A portion of a CNS axon showing gold probe (10-nm size) concentrated over the contents of electron-dense vesicles (*DV*). Note the neurotubules (*NT*). Bar = 0.1 μm

contents of the smaller vesicles, with little or none of the gold probe occurring over the surrounding cytoplasm and other axonal organelles (Fig. 7). Not all of the smaller vesicles observed were immunoreactive for NPF, nor was labeling found associated with any of the larger vesicles or in tissues outside of the axons. Immunoreactivity was blocked completely following preincubation of sections with 200 ng NPF (30–39)/ml, and incubations of tissue in which the primary antiserum was omitted or substituted by non-immune serum were at all times negative.

Discussion

The peptidergic system in *Mesocestoides corti* tetrathyridia, as visualised by immunostaining for NPF, is illustrated schematically in Fig. 9. In gross morphology, it resembles that described for the *M. corti* tetrathyridium by Hart (1967) following staining for cholinesterase (ChE) activity, but with one major difference: no cerebral ganglion as such was found in the metacestode following immunostaining with the NPF antiserum. This observation is in contrast to the description by Hart (1967) of there being a cholinergic "bipartite brain" in addition to sucker ganglia. Examination of the photographic figures of the whole-mount preparations of tetrathyridia published by Hart and stained for ChE activity reveals an intense staining of the scolex region, in which little or no detail of its innervation can be discerned. Hart describes this dense staining of the scolex as a "black cap region" containing an "apparently structureless, cholinesterase reaction", believing it to reflect the presence of a dense array of nervous elements. The use in the present immunocytochemistry study of CSLM to section the scolex optically, i.e. to collect internal images of its innervation at different levels in the z-dimension, clearly revealed the scolex innervation to consist essentially of a large ganglionic accumulation adjacent to each of the four suckers, together with associated commissures. No cerebral ganglion per se was evident, although, collectively, the sucker ganglia and their commissures may serve as a "brain".

Cerebral ganglia apart, the overlap in staining that has been observed in the peptidergic and cholinergic elements of the tetrathyridium nervous system, as seen through a comparison of the present findings with those of Hart (1967), points to the possibility of some co-localisation of neuroactive peptides and classic transmitter molecules. The co-existence of neuronal substances in the nervous systems of other flatworms has been alluded to in several accounts of platyhelminth neurochemistry (see Gustafsson and Wikgren 1989; Maule et al. 1990) and would seem to be consistent with the situation described for the nervous system of higher organisms (see Hökfelt et al. 1986), where it is known that the post-synaptic response can be modulated by a complex interaction of fast- and slow-acting messenger molecules.

The immunocytochemical data presented herein demonstrate the presence of intense NPF-immunoreactivity

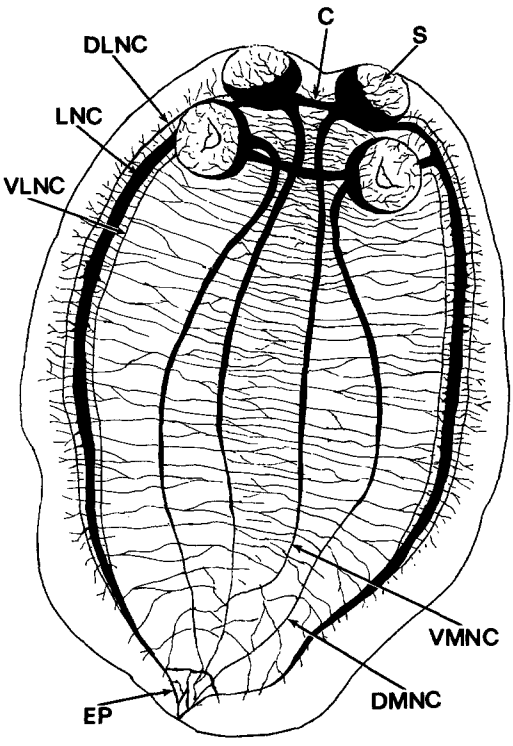


Fig. 9. Diagrammatic representation of the distribution of neuropeptide F-immunoreactive nerve elements in the tetrathyridium of *M. corti*. *C*, Commissure; *DLNC*, dorso-lateral nerve cord; *DMNC*, dorso-median nerve cord; *EP*, excretory plexus; *LNC*, main lateral nerve cord; *S*, sucker; *VLNC*, ventral lateral nerve cord; *VMNC*, ventral median nerve cord

in the nervous system of *M. corti* tetrathyridia. Results from the preabsorption experiments in which all of the demonstrable immunoreactivity was blocked completely by synthetic NPF (30–39) (*Moniezia expansa*) antigen but was unaffected by either bPP or FMRFamide confirm the staining as being due to the presence of an authentic NPF-related peptide. For an explanation of the necessity to eliminate the possibility that some of the staining may be due to bPP or FMRFamide immunoreactivity, see Maule et al. (1992). The failure of non-C-terminal NPF (*M. expansa*) antisera to immunostain the worm indicates that there are primary structural differences between NPF (*M. expansa*) and the putative NPF (*M. corti*). However, the finding that immunostaining was obtained only with the antiserum directed to the C-terminal hexapeptide amide of NPF (*M. expansa*) suggests a C-terminal homology between NPF (*M. expansa*) and the putative NPF (*M. corti*) that has likely been conserved evolutionarily. Such constraints on the C-terminal domain of NPF point to this portion of the molecule as serving an important biological function, mediated most likely through receptor interaction with key amino acid residues.

There are relatively few documented accounts of helminth neuropeptide immunoreactivity at the ultrastructural level. Where studies have been made, they have shown co-localisation of a number of putative peptide mediators in dense-cored vesicles throughout the central

and peripheral nervous systems. Thus, immunoreactivities for PP, FMRFamide and NPF have been demonstrated by triple labeling of neuronal vesicles in the monogenean *Diclidophora merlangi* and the cestode *M. expansa* (Brennan et al. 1993a, b), suggesting an apparent homogeneity of peptide antigenic sites in the flatworm nervous system. However, in both parasites, as in the present studies on *M. corti* tetrathyridia, all of the immunoreactivities were quenched by NPF; this finding and the observation that neither PP nor FMRFamide blocked NPF-immunoreactivity, support the view that most, if not all, of the PP/FMRFamide immunoreactivities that have been demonstrated thus far in parasitic flatworms are due to NPF-like neuropeptides.

NPF was first isolated from the ruminant tapeworm *M. expansa* by Maule et al. in 1991, since when C-terminally directed NPF-antisera have been generated and used to demonstrate the occurrence of NPF-immunoreactive neuropeptides in a wide range of helminth parasites (Maule et al. 1993). All of the evidence gathered to date suggests that NPF is the most abundant and widespread native neuropeptide in the nervous systems of helminths, and, as such, is a possible candidate molecule for therapeutic exploitation. However, full evaluation of the peptidergic nervous system in helminths as a novel target for rationally designed antiparasitic drugs will be possible only when structure/function information on NPF and its receptor becomes available. Work in this direction is in progress in our laboratories in that the putative NPF (*M. corti*) neuropeptide is being isolated for sequence analysis.

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