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Neuropeptide F-immunoreactivity in the monogenean parasite *Diclidophora merlangi*

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Abstract. The localisation and distribution of neuropeptide F (NPF)-immunoreactivity (IR) in the monogenean fish-gill parasite, *Diclidophora merlangi*, have been investigated by whole-mount immunocytochemistry interfaced with confocal scanning laser microscopy and, at the ultrastructural level, by indirect immunogold labeling. Using antisera directed to intact synthetic NPF (*Moniezia expansa*, residues 1–39) or to the C-terminal decapeptide (residues 30–39) of synthetic NPF (*M. expansa*), immunostaining was found throughout the central (CNS) and peripheral nervous systems (PNS), including the innervation of the reproductive system. Immunoreactivity was found to be more intense using the antiserum to the C-terminal decapeptide fragment of NPF. At the subcellular level, gold labeling of NPF-IR was found exclusively over the contents of dense-cored vesicles that occupied nerve axons of both the CNS and the PNS. The distribution pattern of immunostaining for NPF mirrored exactly that previously documented for the vertebrate pancreatic polypeptide (PP) family of peptides and for FMRFamide. This finding and the results of preabsorption experiments strongly suggest that NPF is the predominant native neuropeptide in *D. merlangi* and that it accounts for most of the immunostaining previously obtained with PP and FMRFamide antisera.

Studies conducted over the past 5 years have shown that regulatory peptides are an important component of the neurochemistry of all parasitic platyhelminths examined (Halton et al. 1992). There are documented accounts for numerous trematode and cestode species in which immunoreactivities (IR) to some 30 different vertebrate and invertebrate peptides have been demonstrated neuronally using well-characterised antisera (see Gustafsson 1990; Fairweather and Halton 1991). Although most of these immunocytochemical findings have yet to be confirmed either by radioimmunoassay or from structural information on the peptide in question, it is nevertheless clear that in common with other metazoans investigated, a diverse array of neuropeptides exist within the parasitic platyhelminths.

Of the peptide immunoreactivities (IR) thus far demonstrated in flatworm parasites, including the monogenean, *Diclidophora merlangi*, the most extensive staining has been obtained using antisera directed against members of the neuropeptide Y (NPY) superfamily, notably pancreatic polypeptide (PP), and to the native invertebrate tetrapeptide, FMRFamide (Halton et al. 1991; Maule et al. 1990a, b). In *D. merlangi*, a complete overlap of immunostaining has been demonstrated at both light and electron microscopic levels using antisera to the C-terminal hexapeptide amide of PP and to FMRFamide, suggesting a common identity for PP and FMRFamide antigenic sites within the same neurone (Brennan et al. 1992). However, antigen preabsorption experiments, including the use of the recently discovered native flatworm peptide, neuropeptide F (*Moniezia expansa*; Maule et al. 1991), have indicated that cross-reactivity of the two antisera with a single parasite neuropeptide is likely. Since Brennan et al. (1992) showed that the PP and FMRFamide immunoreactivities demonstrable in *D. merlangi* were quenched by preabsorption with NPF (*M. expansa*), it would seem that some, if not all, of the PP/FMRFamide immunostaining in the monogenean was due to an NPF-like peptide.

To explore this possibility, the present paper describes the presence and distribution of NPF-IR in *D. merlangi*, using both confocal scanning laser microscopy and post-embedding immunogold labeling at the ultrastructural level. This represents the first demonstration of NPF-IR in a trematode.

Materials and methods

Specimens of *Diclidophora merlangi* were recovered from the gills of whiting (*Merlangius merlangus*) freshly caught in the Irish Sea

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and landed at Portavogie, County Down, Northern Ireland. These were transferred to artificial sea water and maintained at 4° C.

Primary antisera

The primary antisera were raised in New Zealand White rabbits by immunisation with the intact synthetic neuropeptide F (*Moniezia expansa*, residues 1–39) and with the C-terminal decapeptide (residues 30–39) of synthetic NPF (*M. expansa*), coupled to bovine serum albumin (BSA) using glutaraldehyde (GTA). Suitable antisera, coded 791(1) raised to NPF(1–39) and 792(1) raised to NPF (30–39), were characterised and validated for use in both immunocytochemistry and radioimmunoassay by Maule et al. (1992).

Immunocytochemistry

For confocal scanning laser microscopy, immunostaining was obtained using the indirect immunofluorescence technique. Flattened whole-mount preparations 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) BSA and 0.1% (w/v) sodium azide for 48 h. Specimens were incubated in primary antisera [either 790(1) or 792(1)] 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. Finally, the preparations were mounted in PBS:glycerol and viewed using an MRC 500 confocal scanning laser microscope (CSLM; Bio-Rad Lasersharp, Abingdon, Oxfordshire, UK).

Controls included the omission of primary antiserum; the substitution of primary antiserum with non-immune serum (Dako Ltd., High Wycombe, UK); liquid-phase preabsorption with NPF (C-terminal fragment 30–39), bovine pancreatic polypeptide (bPP), and FMRFamide (in a range of concentrations from 200 to 1000 ng/ml diluted antiserum).

Immunogold electron microscopy

Post-embedding electron microscopical immunocytochemistry was performed using an indirect immunogold method. Slices (1 mm in thickness) of fresh worms were fixed at 4° C for 40 min in 2% double-distilled GTA (Agar Scientific Ltd., Stansted, UK) in 0.1 M cacodylate buffer (pH 7.2) containing 3% (w/v) sucrose and were then buffer-washed, dehydrated through ethanol at −20° C, and infiltrated overnight in LR Gold resin (Agar Scientific Ltd., Stansted, UK) for polymerisation under a quartz-halogen, visible light source for 28 h at −20° C.

Sections (70 nm in thickness) were collected on bare 200-mesh nickel grids and were incubated first with normal goat antiserum (NGS) for 30 min at room temperature and then with primary antibody [NPF 792(1)] diluted to 1:30,000 with 20 mM TRIS-HCl buffer (pH 8.2) containing 0.1% (w/v) BSA and Tween 20 (1:40 dilution) for 18 h at room temperature. Following this incubation, the sections were washed in TRIS/BSA before and after transfer to a 25-µl droplet of 15-nm-size gold-conjugated goat anti-rabbit IgG (Biocell, Cardiff, UK) for 1 h, lightly fixed with 2% double-distilled GTA (3 min) and then washed with buffer and distilled water. Immunogold labeling using more than one antiserum was carried out by the sequential addition of the antisera separated by incubation with goat anti-rabbit IgG labeled with three different-sized gold probes (i.e. 5-, 10- and 20-nm size). This triple-labeled material was then fixed and washed as described above. Finally, all sections were double-stained with alcoholic uranyl acetate (15 min) and aqueous lead citrate (8 min) and examined with a JEOL 100CX electron microscope (100 kV).

Controls were prepared using non-immune rabbit serum (Dako Ltd., High Wycombe, UK) in place of the primary antiserum, using gold-labeled antiserum in the absence of a primary antiserum, and by liquid-phase preabsorption of the antiserum with NPF standard (500–5000 ng/ml diluted antiserum).

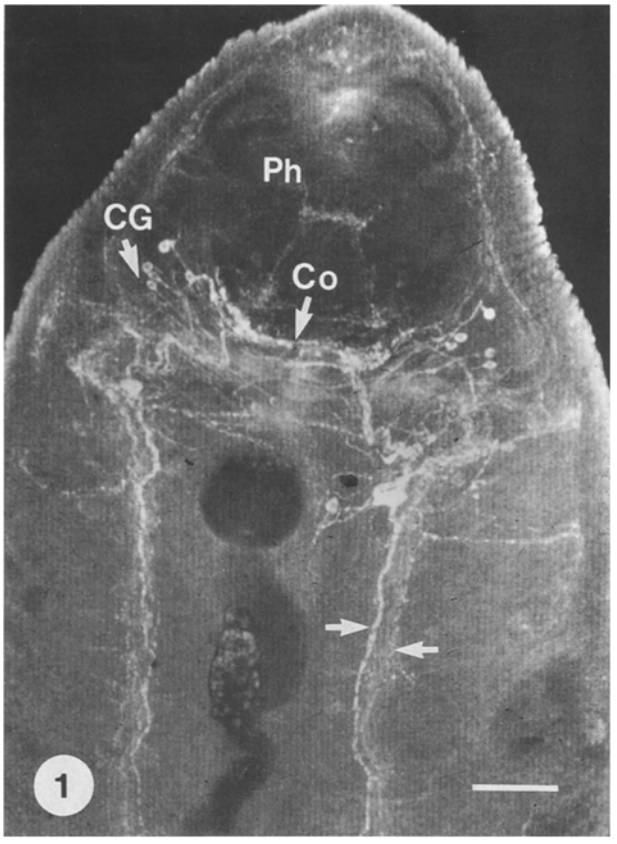
Results

Immunocytochemistry

Preliminary results obtained using CSLM showed that although the two antisera used produced identical staining patterns, the intensity of fluorescence was far greater for the antiserum directed to the C-terminal decapeptide of NPF [i.e. 792(1)], and for this reason it was employed throughout the study. The observed IR was confined to the central and peripheral nervous systems of *Diclidophora merlangi* and produced a distribution pattern and intensity of staining comparable with that previously described for C-terminally directed PP and FMRFamide antisera (Maule et al. 1990a, b). Thus, in the forebody of the worm, there was strong NPF-IR in the cerebral ganglia and commissure, in the paired longitudinal nerve cords, and in the rich innervation associated with the pharynx and mouth region (Figs. 1, 2). In the haptor, NPF staining of the ventral nerve cord and its junctions with the nerves supplying the peduncles and clamps revealed the same array of reactive fibres that connect with the PNS in this region, as has previously been shown using antisera to PP and FMRFamide (Fig. 3). Similarly, in the female reproductive system, NPF-IR was evident in the innervation of the walls of the oviduct, in the vitelline reservoir, in the common ovovitelline duct and in the plexus of fibres and cell bodies that encircle the ootype/Mehlis' gland complex (Fig. 4).

Negative results were obtained in the absence of primary antiserum and following the substitution of primary antiserum with non-immune serum. NPF-IR was abolished following preincubation with 500–1000 ng NPF (1–39)/ml and 200–1000 ng NPF (30–39)/ml but was unaffected by the addition to the incubation medium of bPP or FMRFamide over the same dilution range.

Figs. 1–4. Whole-mount preparations, viewed by confocal scanning laser microscopy, showing immunoreactivity (IR) to NPF. **Figs. 1, 2.** Forebody region showing NPF-IR in the cerebral ganglia (*CG*), commissure (*Co*), ventral and dorsal longitudinal nerve cords (*unlabeled arrows*) and innervation around the pharynx (*Ph*) and mouth (*Mo*). Bar, 100 µm. **Fig. 3.** Haptor region showing NPF-IR in the ventral nerve cord (*VNC*) and its junction with the transverse nerve cord (*TNC*) that provides nerves to the peduncles. Note the somata (*unlabeled arrows*). Bar, 100 µm. **Fig. 4.** Ootype/Mehlis' gland region showing NPF-IR in somata (*unlabeled arrows*) and fibres innervating the walls of the ootype (*Ot*) and oviduct (*OD*). Note the ovary (*Ov*). Bar, 100 µm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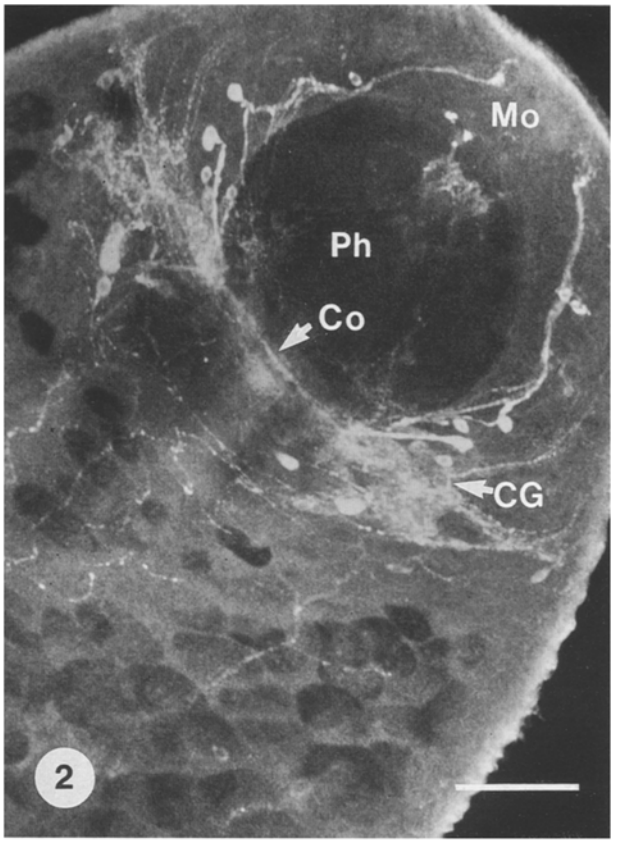
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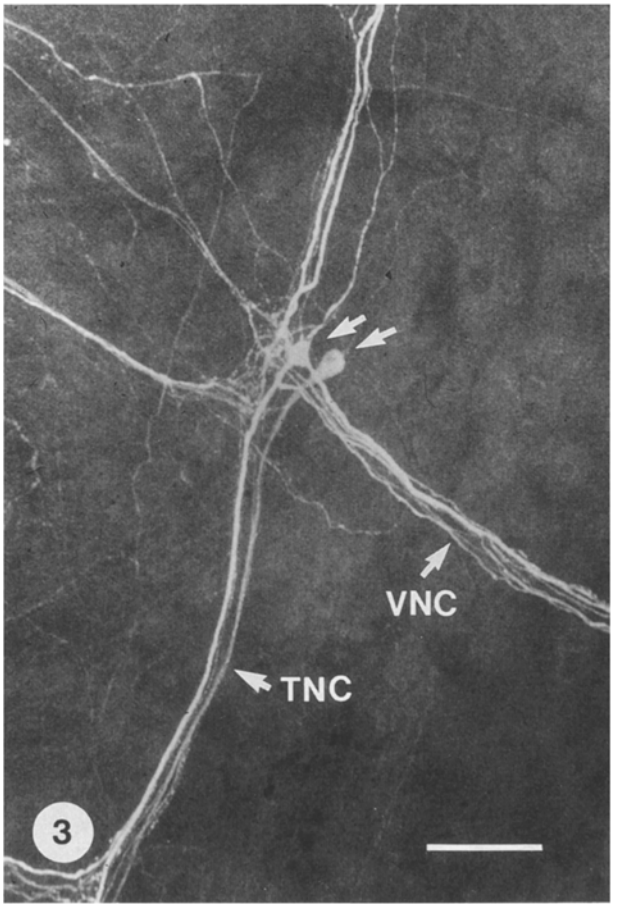
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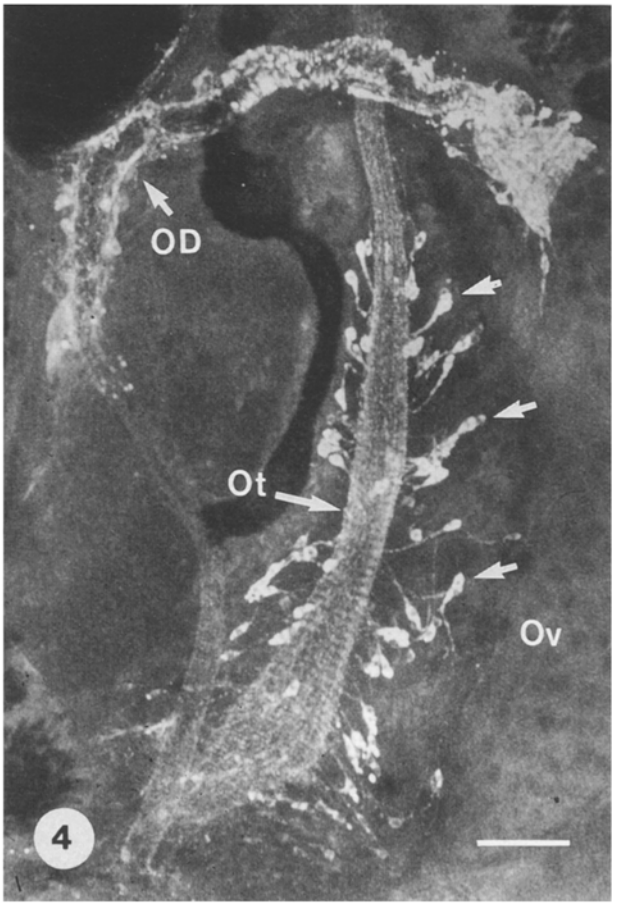
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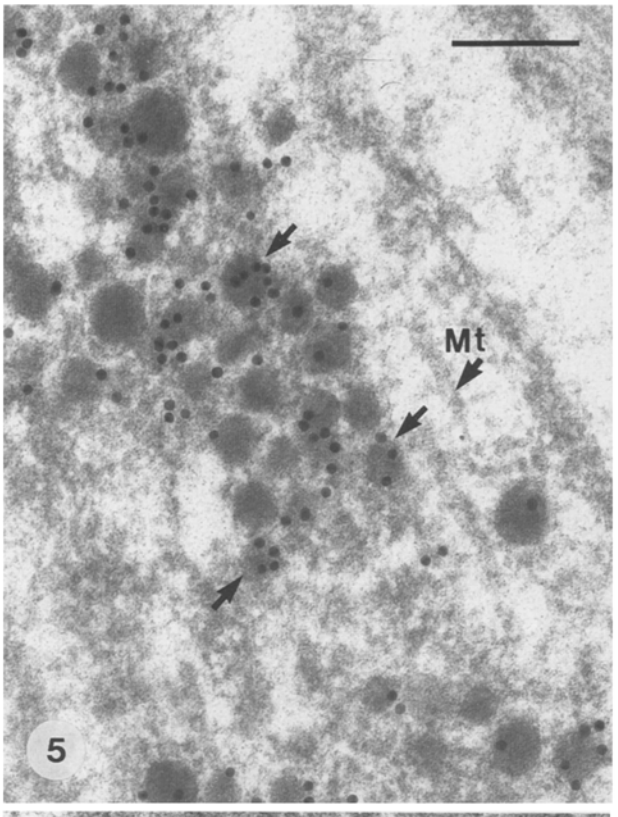
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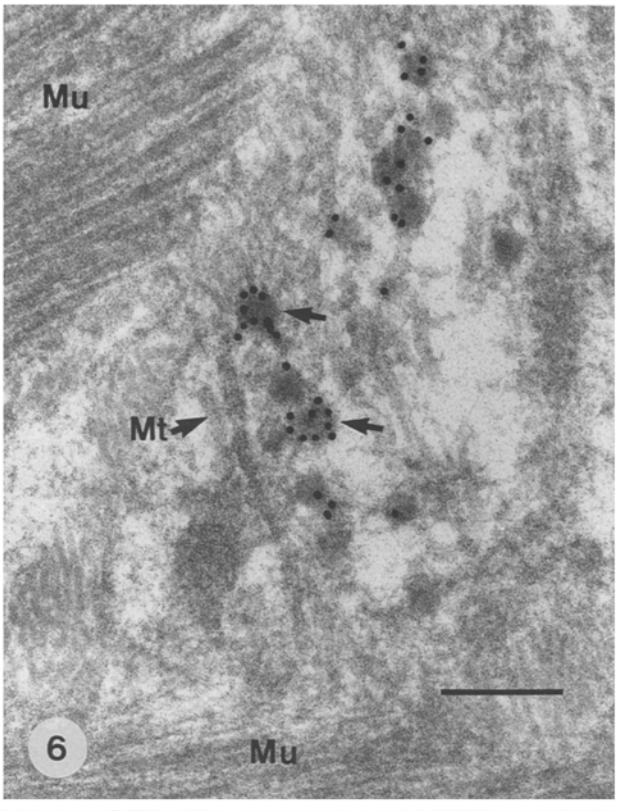
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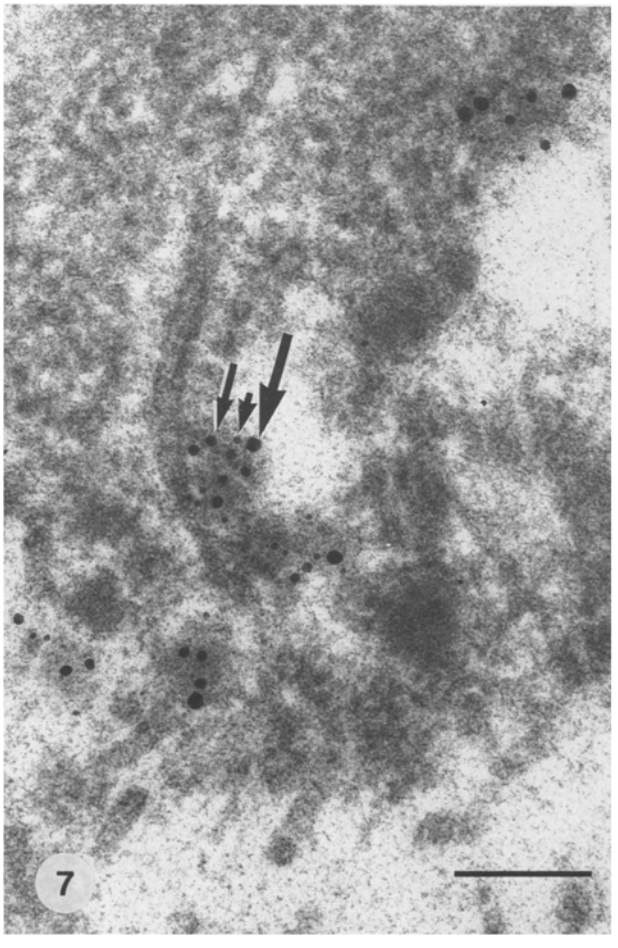
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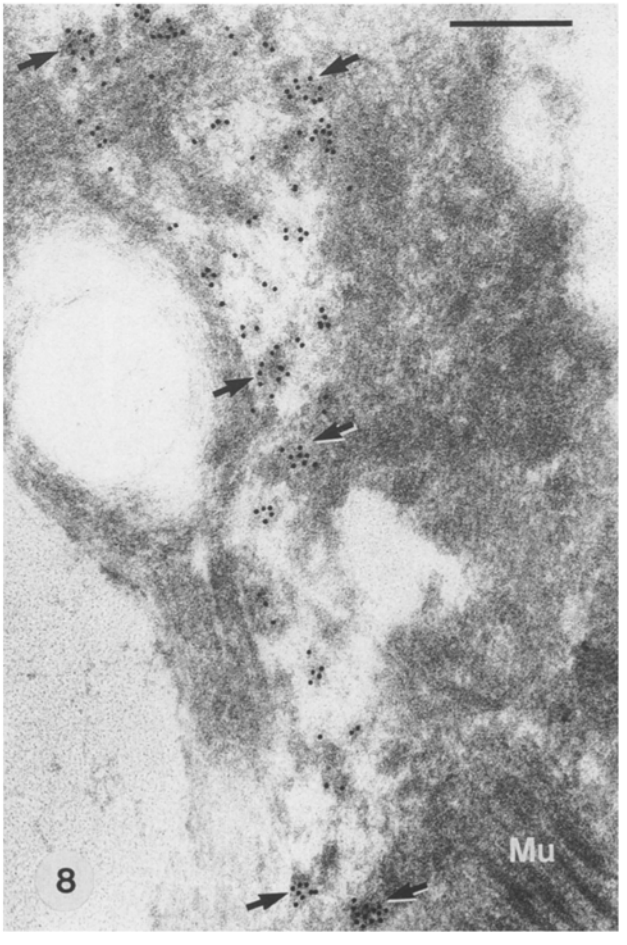
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Immunogold electron microscopy

Gold labeling of the NPF antibodies was associated almost exclusively with the numerous dense-cored vesicles (mean diameter, approximately 90 nm) that occupied nerve axons in both the central and the peripheral nervous systems of the worm (Figs. 5, 6). In general, gold particles were found only over the core material of the vesicle, with relatively few of them occurring over the surrounding cytoplasm or other organelles. A number of the dense-cored vesicles were unlabeled, and no labeling was observed outside the axon. The multiple labeling technique demonstrated the occurrence of NPF-, PP- and FMRFamide-IRs within the same dense-cored vesicles (Fig. 7). In the region of the Mehlis' gland cells, gold label was found over the accumulations of dense-cored vesicles that occupy the necks and over the axon-like ducts of the peptidergic cells innervating the ootype wall (Fig. 8). Immunoreactivity was completely blocked following preincubation of sections with NPF, and incubations in which the primary antiserum was omitted or replaced with non-immune serum were consistently negative.

Discussion

Previous immunocytochemical and radioimmunometrical studies have shown that in common with most other parasitic platyhelminths examined, the most predominant immunoreactivity identified in *Diclidophora merlangi* has been obtained using antisera to members of the NPY superfamily and, in particular, to the highly conserved C-terminal hexapeptide amide of PP (Maule et al. 1989, 1990a, b). Throughout the nervous system of the worm, immunostaining has revealed an apparent co-localisation of immunoreactivity to all three members of the superfamily (PP, NPY, PYY), suggesting cross-reactivity with a single endogenous parasite peptide. Using sequential reverse-phase HPLC fractionation and radioimmunoassay, Maule et al. (1992) isolated and characterised the PP-like peptide from *D. merlangi* and showed that it was not authentic PP. Unfortunately, it was not possible to purify sufficient peptide to permit structural analysis. However, all of the evidence indicated that the peptide had immunochemical characteristics analogous to those of the recently discovered native invertebrate peptide family, neuropeptide F.

Neuropeptide F was first isolated from *Moniezia expansa* by Maule et al. in 1991, since when analogous peptides have been isolated and sequenced from the turbellarian, *Artioposthia triangulata* (Curry et al. 1992), and from the gastropod mollusc, *Helix aspersa* (Leung et al. 1992). All three of these molecular species contain the sequence Arg-X-Arg-Phe-NH_2 (RXRFamide), where X is either Phe (F) or Thr (T), and all cross-react with antisera directed to the C-terminal hexapeptide amide of PP. The finding that the immunostaining observed for NPF in *D. merlangi* was stronger using the antibody directed to the C-terminal fragment than that produced by the whole NPF molecule points to a C-terminal homology between NPF (*M. expansa*) and the putative NPF (*D. merlangi*) that is evolutionarily conserved and perhaps critical for physiological function.

Under the high-dilution and competitive conditions of RIA, the whole-molecule NPF antiserum [790(1)] was found to cross-react only with NPF (1–39) (Maule et al. 1992). However, since immunostaining with antiserum 790(1) in *D. merlangi* could be abolished using the C-terminal decapeptide of NPF, it would appear that in common with antiserum 792(1), a C-terminal epitope was recognised. It would therefore appear that a small subpopulation of C-terminally directed NPF antibodies, which cross-react with the native *D. merlangi* neuropeptide under ICC conditions, is present in antiserum 790 (1). This would explain both the low intensity of immunostaining obtained using this antiserum and the NPF (1–39)-specific characteristics of 790(1) that are evident under RIA conditions (see Maule et al. 1992). This possibility was confirmed by the abolition of immunostaining by preincubation of antiserum 790(1) with 200–1000 ng of the C-terminal decapeptide of NPF/ml.

The discovery of the NPF family of peptides, which possesses an Arg-Phe-NH_2 (RFamide) at the C-terminus, likely accounts for previous descriptions of an overlap of immunostaining for PP and FMRFamide. Indeed, the failure of either PP or FMRFamide to block demonstrable NPF-immunoreactivity in *D. merlangi* in the present study supports the view that some, if not all, of the PP/FMRFamide immunostaining that has previously been described as being present in the nerves of the worm is attributable to an NPF-like peptide. This would also account for the co-localisation of immunoreactivity for NPF, PP and FMRFamide that was demonstrable in the same dense-cored vesicles by triple-gold-probe labeling.

The results presented herein represent the first description of NPF-immunoreactivity in a member of the class Trematoda. Unpublished studies conducted on other parasitic platyhelminths and invertebrate taxa in our laboratory have revealed extensive immunostaining for this neuropeptide in their nervous systems, showing a pattern of staining correspondingly similar to that previously documented for the PP family of peptides. Thus, it is becoming increasingly apparent that NPF (*M. expansa*) is a member of a family of peptides with a wide phylogenetic distribution among invertebrates; indeed,

Figs. 5–8. Immunogold labeling of dense-cored vesicles (DCV). **Fig. 5.** Section of CNS axon showing gold labeling (size 15 nm) of NPF antibody concentrated over DCV (*arrows*). *Mt*, Microtubule. Bar, 0.25 μm. **Fig. 6.** NPF-IR in DCV (*arrows*) in a nerve terminal in close apposition with muscle fibres (*Mu*). *Mt*, Microtubule. Bar, 0.25 μm. **Fig. 7.** A portion of CNS axon following triple-labeling to demonstrate in the one DCV IR to NPF (10-nm gold probe, *medium-sized arrow*), PP (5 nm, *small arrow*) and FMRFamide (15 nm, *large arrow*). Bar, 0.25 μm. **Fig. 8.** Portion of the axon-like duct of a peptidergic cell innervating muscle (*Mu*) in the ootype wall and containing gold-labeled DCV for NPF-IR (*arrows*). Bar, 1 μm

the cross-phyletic resemblance of NPF to vertebrate PP suggests that the NPF family may be the invertebrate equivalent of the NPY superfamily. Clearly, it is now important to investigate the physiological relevance of this abundant native invertebrate neuropeptide as a potential messenger molecule for drug targeting. To this end, work is currently in progress to isolate and characterise the NPF receptor.

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