Neuropeptide F-immunoreactivity in the monogenean parasite

**Dictidophora merlangi**

A.G. Maule¹, G.P. Brennan¹, D.W. Halton¹, C. Shaw², C.F. Johnston², and S. Moore³

Comparative Neuroendocrinology Research Group, Schools of ¹ Biology and Biochemistry and ² Clinical Medicine, The Queen's University of Belfast, Belfast BT7 1NN, UK
³ Peninsula Laboratories Europe Ltd., Merseyside WA9 3AJ, UK

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**Abstract.** The localisation and distribution of neuropeptide F (NPF)-immunoreactivity (IR) in the monogenean fish-gill parasite, *Dictidophora 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 was found throughout the central nervous system (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.

Of the peptide immunoreactivities (IR) thus far demonstrated in flatworm parasites, including the monogenean, *Dictidophora 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 *Dictidophora merlangi* were recovered from the gills of whiting (*Merlangius merlangus*) freshly caught in the Irish Sea.
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 Na2HPO4, 2H2O, 0.075 M NaH2PO4, 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 then were 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); liquid-phase preabsorption with NPF standard (30-39) and with the C-terminal decapeptide (residues 1-39) and with the C-terminal decapeptide (residues 30-39) of synthetic NPF, were characterised and validated for use in both immunocytochemistry and radioimmunoassay by Maule et al. (1992).

**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 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 antisera 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 *Dielidophora 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