# Comparative analysis of the distribution of bradykinin-, GYIRFamide- and neuropeptide F-like immunoreactivities in the monogenean, *Diclidophora merlangi*

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SUMMARY

An indirect immunocytochemical technique combined with confocal scanning laser microscopy has been used to demonstrate immunoreactivities to the nonapeptide, RPPGFSPFR (bradykinin, BK) and the endogenous flatworm regulatory peptide, GYIRFamide in the nervous system of the monogenean, *Diclidophora merlangi*. In addition, a simultaneous double-labelling technique was employed to examine possible co-localization of GYIRFamide- and neuropeptide F (NPF) immunoreactivities, using antisera to the C-terminal nonapeptide-amide of NPF (*Moniezia expansa*, FAIIGRPRF.$NH_2$). BK immunostaining was restricted to a small population of nerve cells and associated fibres within the ventral nerve cords and to 2 pairs of nerve cells innervating the cirrus and the pharynx, respectively. No immunopositive nerve cells and fibres were identified within the brain or in association with the female reproductive apparatus. In contrast, GYIRFamide staining was abundant throughout the central and peripheral nervous systems, and appeared similar to the staining pattern revealed using an FMRFamide antiserum. GYIRFamide immunoreactivity was localized to nerve cells and fibres within the paired cerebral ganglia and the longitudinal ventral, dorsal and lateral nerve cords and their numerous interconnecting transverse commissures. The plexuses of the buccal suckers, pharynx and clamps of the haptor were strongly immunopositive for GYIRFamide, as were nerve cells innervating the ootype, the oviduct and the vitelline reservoir of the reproductive apparatus. Double-labelling experiments indicated an apparent co-localization of GYIRFamide and NPF immunoreactivities.

Key words: FMRFamide-related peptide, FaRP, kinin, bradykinin, immunocytochemistry.

## INTRODUCTION

Recent immunocytochemical (ICC) and physiological studies have revealed that flatworm nervous systems not only utilize so-called classical neurotransmitters, such as acetylcholine (ACh), serotonin (5-hydroxytryptamine, 5-HT) and $\gamma$-aminobutyric acid (GABA), but also employ a variety of peptidic substances in neurotransmission (Halton *et al*. 1994; Reuter & Gustafsson, 1995). The fact that platyhelminths are acoelomate and lack true circulatory systems perhaps necessitates that their nervous systems exhibit a degree of versatility, combining features of neurons and endocrine cells. Of the peptides demonstrated in flatworms, the best known are the neuropeptide F (NPF) peptides and the FMRFamide-related peptides (FaRPs), several of which have been isolated and their primary structures determined (Maule *et al*. 1991, 1993, 1994; Curry *et al*. 1992; Johnston *et al*. 1995, 1996*a*). There is now strong evidence that neuropeptides are involved in specific physiological processes, including neuromuscular transmission. For example, when applied exogenously to whole worms or muscle cell-preparations, FaRPs have been shown to be myoactive in a number of flatworm species, suggesting a neurotransmitter or neuromodulatory effect on the musculature (Day *et al*. 1994; Johnston *et al*. 1996*a*; Marks *et al*. 1996). In the monogenean, *Polystoma nearcticum*, there is strong immunocytochemical evidence that FaRP immunoreactivities in neurons innervating the egg-forming apparatus vary seasonally, and that the neuropeptides involved are expressed only when the parasites are reproductively active (Armstrong *et al*. 1996). Otherwise, relatively little has been documented on FaRPs in flatworms, in contrast to the situation in nematodes, where some 22 native FaRPs have been structurally characterized, 8 of which have been tested physiologically exhibiting potent myo-excitatory and inhibitory properties (see Geary *et al*. 1995; Maule *et al*. 1995).

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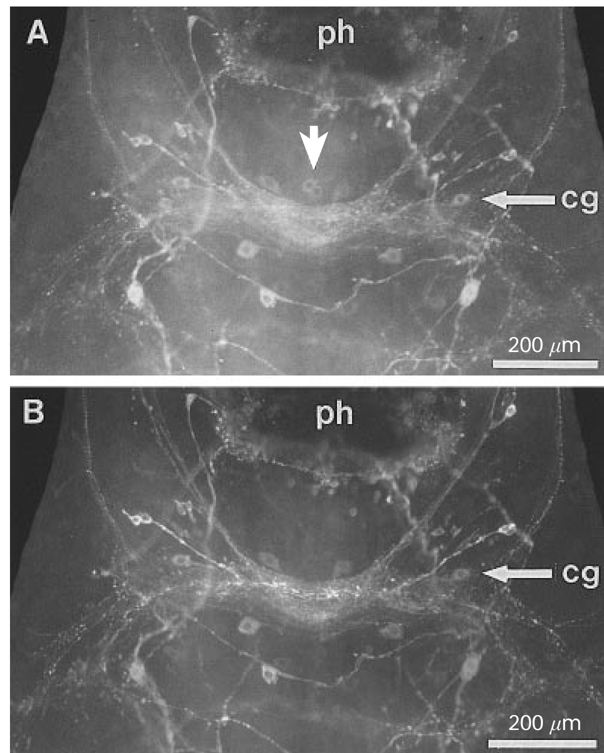


Fig. 1. Simultaneous double-labelling of GYIRFamide (A) and neuropeptide F (B) in the nervous system of *Diclidophora merlangi*. Neurons and fibres of the cerebral ganglia (cg) display almost identical localization of immunoreactivities to GYIRFamide and NPF. Note a pair of nerve cells (A, arrow) below the pharynx (ph), which display GYIRFamide immunoreactivity only.

Kinins are a family of low molecular weight peptides and have been identified in a number of vertebrates. However, they have not been reported previously from lower invertebrate groups, including platyhelminths. Known kinins include bradykinin (BK, RPPGFSPFR) and kallidin (KRPPGFSPFR), both of which are present in blood plasma and numerous other tissues and are believed to play an important role in many physiological processes, including vasodilation, smooth muscle contraction, blood pressure regulation, primary inflammatory responses as well as cell proliferation (see Bhoola, Figuero & Worthy, 1992). Despite their widespread distribution, there is only little evidence for kinins acting as neurotransmitters or neuromodulators. Nevertheless, central and peripheral neurons and neuroendocrine cells, such as those in the adrenal medulla, have been shown to contain all of the components of the kinin–kallikrein system: tissue kallikrein, high and low molecular weight kininogen, kinins, kininases and kinin receptors (Walker, Perkins & Dray, 1995; Hall & Geppetti, 1995).

As a preliminary immunocytochemical screen to studying a possible role for kinins in platyhelminths, the present work examines the distribution of BK-like immunoreactivity in the monogenean, *Diclidophora merlangi*, and compares the staining results to the immunoreactivity pattern for the native flatworm FaRP, GYIRFamide. In addition, simultaneous double-labelling experiments have been employed to determine whether there is co-localization of GYIRFamide with NPF.

## MATERIALS AND METHODS

### *Antibody production*

Synthetic Lys$^0$-bradykinin (kallidin; 0·5 mg; Peninsula Laboratories Europe Ltd, St Helens, UK) and 2 mg of ovalbumin were dissolved in 0·5 ml of 0·1 M PBS, and 0·5 ml of 0·5 % (w/v) glutaraldehyde was then added and the resulting conjugate emulsified with 3 ml of Freund's complete adjuvant. Equal volumes of the mixture were administered to 2 guinea-pigs, which were boosted after 1 month with 20 % of the original quantity made up with Freund's incomplete adjuvant. The protocols for the production of GYIRFamide and NPF antisera, which were raised in guinea-pig and rabbit hosts, respectively, have been published previously (Maule *et al*. 1993; Johnston *et al*. 1996*a*).

### *Immunocytochemistry*

*GYIRFamide and bradykinin*. Live specimens of *D. merlangi* were collected from the gills of freshly caught whiting, *Merlangius merlangus*, and maintained in artificial sea water (ASW) containing 0·01 % (w/v) glucose at 4 °C. The worms were stained separately for GYIRFamide and bradykinin as whole-mounts, according to the indirect immunocytochemical method of Coons, Leduc & Connolly (1955). Specimens were flat-fixed for 1 h in 4 % (w/v) paraformaldehyde in 0·1 M phosphate-buffered saline (PBS) and then transferred into fresh fixative for another 3 h. After a 48-h wash in antibody diluent (AbD, PBS pH 7·4, containing 0·1 % (v/v) Triton X–100, 0–1 % (w/v) bovine serum albumin and 0·1 % (w/v) $NaN_3$), specimens were incubated in primary antiserum (rabbit anti-BK or guinea-pig anti-GYIRFamide, respectively) for 96 h. Before and after exposure to fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or FITC-conjugated anti-guinea-pig IgG, worms were rinsed for 24 h in AbD. Finally, specimens were mounted on glass slides in glycerol/PBS (9:1), viewed and photographed using an MRC 500 confocal scanning laser microscope (Bio-Rad, Lasersharp, Abingdon, Oxfordshire, UK).

*Double-labelling of NPF and GYIRFamide*. Fixed worms were washed for 48 h and then simultaneously incubated with anti-GYIRFamide and anti-NPF for 96 h. After a 24-h wash in AbD, worms were first incubated with tetrarhodamine isothiocyanate-conjugated anti-rabbit IgG for 24 h, washed again, and, to minimize non-specific binding, re-incubated overnight with anti-NPF. Specimens

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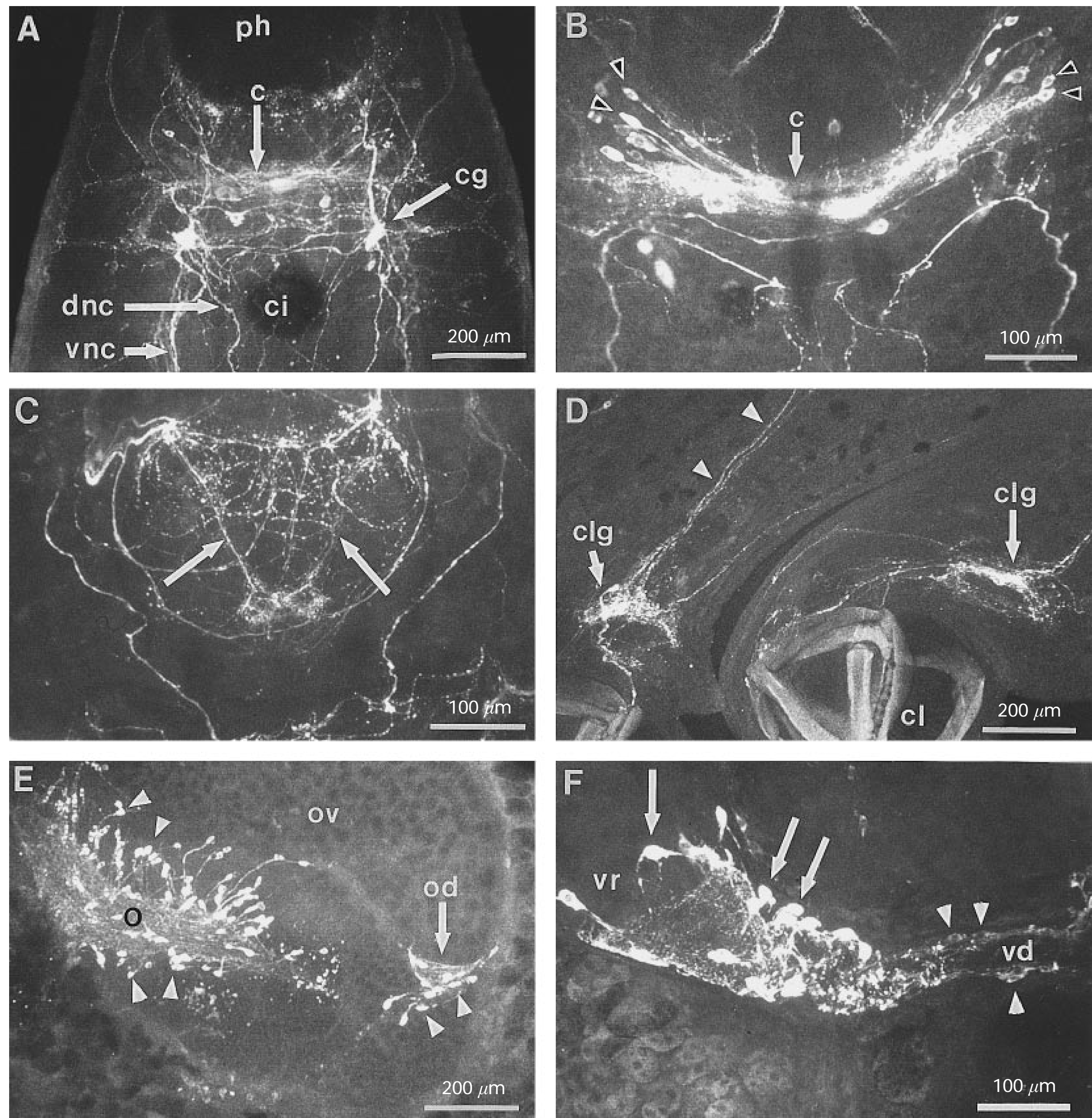


Fig. 2. Confocal scanning laser micrographs (CSLM) showing GYIRFamide immunoreactivity in the nervous system of *Diclidophora merlangi*. (A) Distribution of immunostaining in neural elements of the anterior forebody region between the pharynx (ph) and the cirrus (ci). Immunoreactive neurons appear mainly associated with the cerebral ganglia (cg). Fibres originating in the brain contribute to the commissure (c) which connects the 2 cerebral ganglia, as well as the ventral (vnc) and dorsal nerve cords (dnc), respectively. (B) Immunopositive neurons within the brain are predominantly unipolar (arrowheads). Nerve fibres emanate from these cells and contribute to the prominent commissure (c) which connects the cerebral ganglia. (C) Detail of the innervation of the pharynx musculature with nerve fibres organized into a dense plexus in the posterior end of the pharynx (arrows). Note the varicose appearance of the fibres. (D) Innervation of 2 adjacent pedunculate clamps (cl). Nerve fibres (arrowheads) connect ventral nerve cords with the clamp ganglia (clg) at the base of each holdfast. (E) GYIRFamide immunostaining in neurons (arrowheads) innervating the ootype (o) and the oviduct (od). The upper part of the image shows the ovary (ov). (F) Innervation of the vitelline reservoir (vr) by multipolar nerve cells (arrows). Less intensely stained neurons (arrowheads) line the emerging vitelline duct (vd).

were then immersed in FITC-conjugated anti-guinea-pig IgG for 24 h and, finally, washed for 24 h and mounted on glass slides in glycerol/PBS and viewed and photographed using an Olympus BH-2 fluorescence microscope.

*Controls*. Controls included (i) omission of primary antiserum, (ii) substitution of primary antiserum with non-immune serum, and (iii) liquid-phase pre-adsorption of primary antiserum with the appropriate antigen for 24 h (100–1000 ng BK–BSA-

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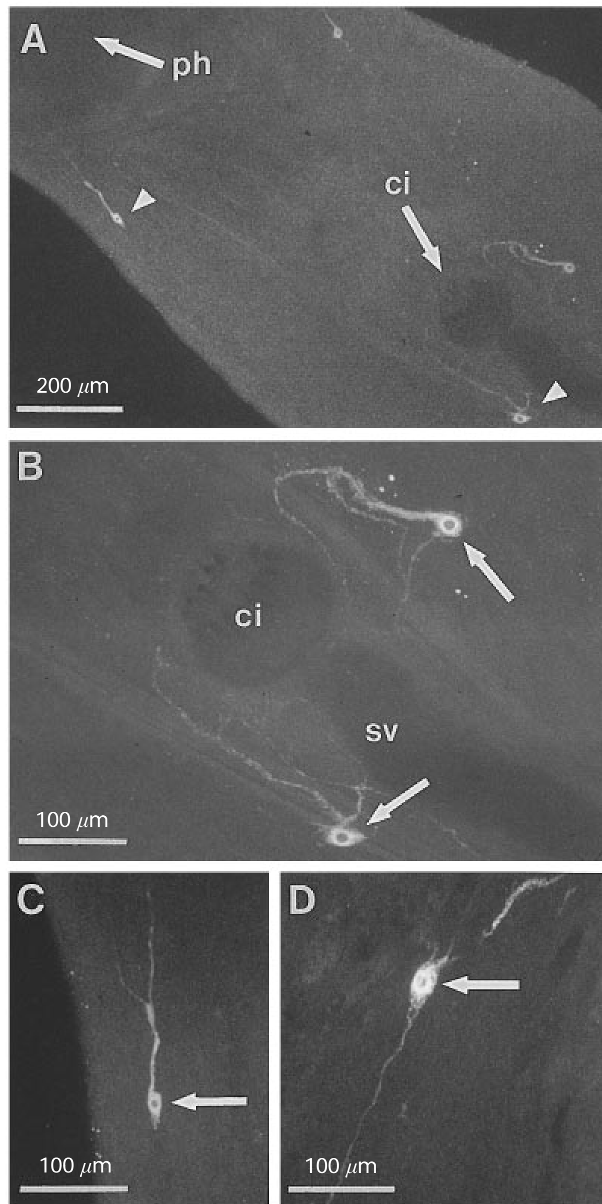


Fig. 3. Confocal scanning laser micrographs of bradykinin (BK) immunoreactivity in the nervous system of *Diclidophora merlangi*. (A) Distribution of BK immunostaining in the forebody region with bilaterally organized neurons (arrowheads) associated with the innervation of the cirrus (ci) and the pharynx (ph), respectively. (B) Detail of neurons innervating the cirrus (ci) and the terminal part of the seminal vesicle (sv). Note the large somata of the multipolar neurons (arrows). (C) Unipolar, pharynx-innervating neuron. Note the large somata (arrow). (D) Bipolar BK-positive neuron within one of the ventral nerve cords. Note the large somata (arrow).

conjugate/ml diluted antiserum and 200–1000 ng GYIRFamide/ml diluted antiserum, respectively). In addition, cross-reactivity of the employed GYIRFamide antibody with FMRFamide (250–1000 ng/ml diluted antiserum) was determined.

## RESULTS

GYIRFamide and neuropeptide F (NPF) immunoreactivities (IRs) were abundant throughout the central (CNS) and peripheral (PNS) nervous systems of *D. merlangi* and displayed an almost identical distribution (Fig. 1).

Within the CNS, nerve cells and fibres of the paired cerebral ganglia, together with the 3 pairs of longitudinal nerve cords, exhibited strong GYIRFamide- and NPF-IRs. PNS immunostaining was evident in neuronal plexuses innervating the musculature of the clamps in the haptor, the ducting of the female reproductive system, and the buccal suckers and pharynx of the feeding apparatus.

The cerebral ganglia contained some 10 immunopositive nerve cells, each with an average diameter of 15–20 μm; smaller nerve cells were scattered in the periphery of each cerebral ganglion (Fig. 2A). Neurons within the cerebral ganglia were predominantly of the unipolar type, with smaller numbers being bi- and multipolar. Both of the cerebral ganglia were joined by a well-developed dorsal cross-commissure (Fig. 2B); a minor single-fibred commissure was present in a ventral position. Also in the forebody region, fibres emanating from brain neurons innervated the musculature of the mouth and the 2 buccal suckers. The pharynx was innervated by fibres originating from neurons in the brain and by 2 large GYIRFamide-immunopositive neurons positioned at the posterior end of the pharynx. The bulk of the nerve fibres in the pharynx formed a basket-like plexus at the posterior end of the pharynx, with additional immunoreactive fibres extending ventrally and dorsally along its length (Fig. 2C).

Three pairs of longitudinal nerve cords emanate from the brain in ventral, lateral and dorsal array, respectively. At intervals, transverse commissures cross-link the lateral and ventral cords, with fewer connections evident between the lateral and dorsal cords. The ventral nerve cords are the most prominent and are fused at the level of the haptor. Here, a number of immunoreactive nerve cells were detected at the base of each of the pedunculate clamps, to which a ganglionic cluster of 3–5 nerve cells and associated fibres extended to anastomose as a plexus of fine fibres throughout the sucker-like musculature of the clamps (Fig. 2D).

Within the female reproductive system, GYIRFamide- and NPF-IRs were most intense in neurons (numbering *ca.* 100) innervating the ootype (Fig. 2E); immunostaining also occurred in nerve plexuses along the oviduct (Fig. 2E), the vitelline reservoir and the vitelline duct (Fig. 2F). Within the male system, immunopositive fibres originating from 2 large neurons innervated the muscle of the cirrus.

Compared to the GYIRFamide/NPF-IR pattern, bradykinin (BK) immunostaining was less abundant

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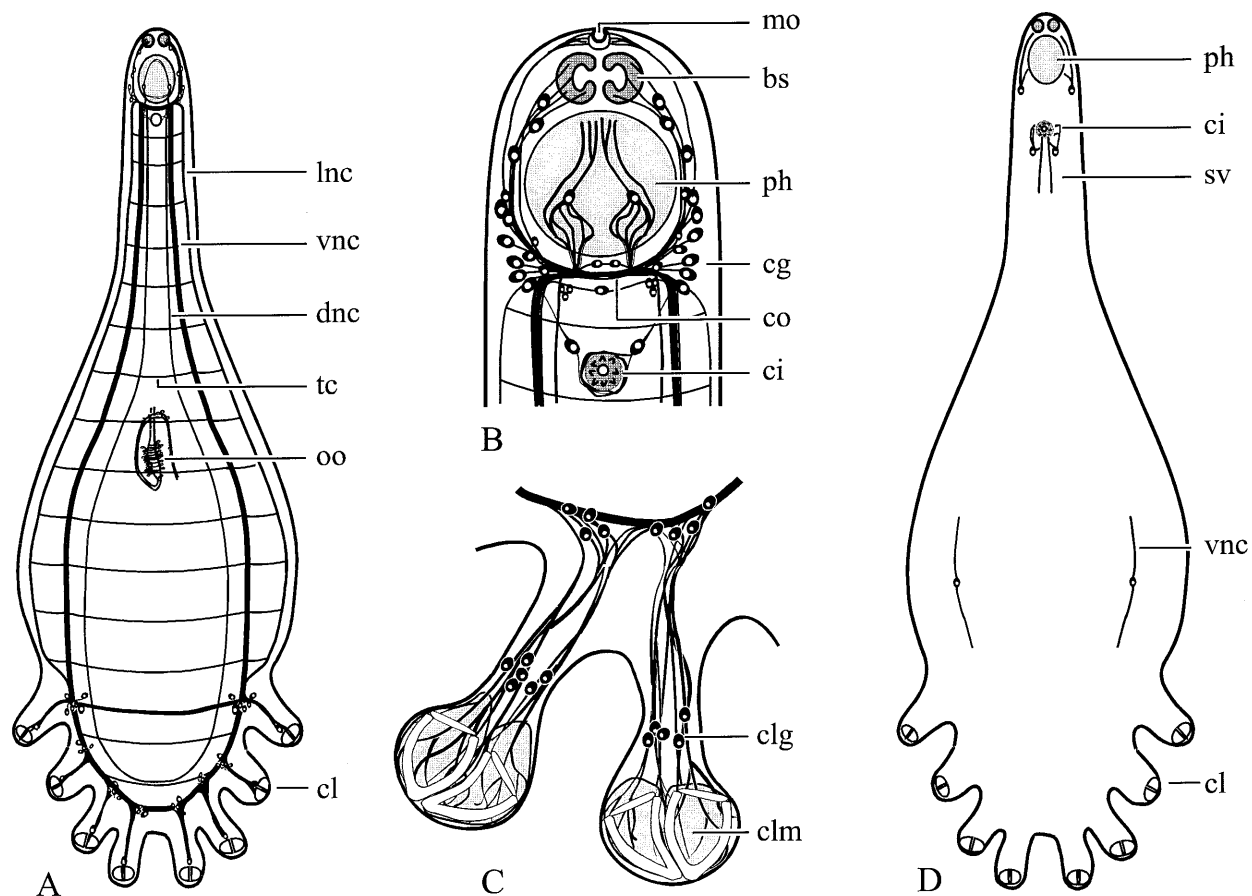


Fig. 4. Schematic diagram of GYIRFamide/neuropeptide F immunostaining (A) with details of the peptidergic innervation of the head region (B) and the pedunculate clamps (C), and bradykinin-immunostaining (D) in the nervous system of *Diclidophora merlangi*. bs, Buccal sucker; cg, cerebral ganglion; ci, cirrus; cl, clamp; clg, clamp ganglion; clm, clamp musculature; co, cross-commissure; dnc, dorsal nerve cord; lnc, lateral nerve cord; mo, mouth; oo, ootype; ph, pharynx; sv, seminal vesicle; tc, transverse commissure; vnc, ventral nerve cord.

in *D. merlangi*. BK-IR was localized to a pair of cells innervating the cirrus and the pharynx (Fig. 3A), and a pair of cells within the VNC (Fig. 3D). No immunostaining was apparent in the brain or in the innervation of the female reproductive apparatus. The nerve cells innervating the cirrus appeared multipolar (Fig. 3B), whereas those innervating the pharynx (Fig. 3C) and within the VNC were bipolar. BK-positive nerve cells were larger in size than GYIRFamide-positive neurons, with a diameter of *ca*. 20–25 $\mu$m.

### *Controls*

*Bradykinin*. No staining occurred following omission of the primary antiserum or after substitution of the primary antiserum with non-immune serum. Pre-incubation of primary antiserum with 500 ng/ml BK–BSA-conjugate for 1 h also blocked all immunostaining.

*GYIRFamide*. No immunostaining was detected after omission of primary antiserum or following the substitution of the primary antiserum with non-immune serum. Blocking of staining occurred after pre-adsorption of the primary antiserum with 200 ng/ml GYIRFamide for 1 h. Immunostaining was suppressed by some 50% of normal after pre-incubation of GYIRFamide antiserum with 1000 ng/ml FMRFamide for 1 h.

## DISCUSSION

This study documents for the first time bradykinin (BK) immunoreactivity (IR) within the nervous system of a flatworm, and GYIRFamide-IR within the nervous system of *D. merlangi*. While GYIRFamide-IR was widespread in the nervous system, BK-IR was restricted to a few nerve cells and fibres in the forebody of the worm (Fig. 4). The double-labelling experiment suggests the co-localization of IRs for GYIRFamide and neuropeptide F (NPF).

The distribution of GYIRFamide/NPF-positive nerve cells and fibres appeared identical to previously reported staining patterns resulting from the application of FMRFamide antisera (Maule *et al*. 1989, 1990). However, the isolation of endogenous FaRPs

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and the failure to detect authentic FMRFamide in extracts of flatworms so far, suggest that FMRFamide-IRs in a number of platyhelminth species, including *D. merlangi*, are due to cross-reactivity of FMRFamide antisera with native peptides that possess C-terminal RFamide motifs but which differ in the N-terminal amino acid sequences. The overlap of staining for GYIRFamide and NPF may, indeed, be due to the co-existence of FaRP and NPF-like peptides in the same neurons. However, due to the likelihood of cross-reactivity of FaRP-antisera with NPF, this study cannot demonstrate unequivocally the presence of either peptide. The isolation and biochemical characterization of endogenous FaRPs and an NPF-like peptide from *D. merlangi* and the subsequent production of specific antibodies would enable the relative distribution patterns of these peptides to be established.

GYIRFamide was first isolated from the triclad turbellarians, *Bdelloura candida* and *Dugesia tigrina* (Johnston *et al*. 1995, 1996*a*). It has previously been demonstrated immunocytochemically in *B. candida*, and results from physiological studies have shown it to exhibit strong myo-excitatory effects (Johnston *et al*. 1996*b*). Johnston *et al*. (1996*a*) suggested that *B. candida* muscle fibres express FaRP receptors and that these respond to ligands bearing C-terminal RFamide residues; moreover, the most potent compounds tested by these workers were those with an isoleucine or proline residue at position 3 from the C-terminus. In preliminary studies on *D. merlangi*, using muscle-strip preparations, GYIRFamide has been found to elicit a marked increase in muscle tension, contraction frequency and amplitude (C. Moneypenny, personal communication), and these data would support the view that trematode FaRPs more closely resemble turbellarian, rather than cestode, analogues (Johnston *et al*. 1995). The physiological data on the effects of GYIRFamide on the somatic muscle of *D. merlangi* and the widespread occurrence of GYIRFamide immunostaining in neural structures observed in the present study suggest an innervation of the musculature involving nerve cells and fibres that express a peptide with an XYIRFamide motif.

Little is known of the function(s) of NPF in helminths, but based on its ubiquitous distribution in parasitic flatworms, a role in neurotransmission/neuromodulation has been suggested (Maule *et al*. 1995). The structurally related peptide, *Aplysia* neuropeptide Y (apNPY) from the mollusc, *Aplysia californica*, is released from bag cell neurons at the initiation of egg-laying and appears to serve as a potent pre-synaptic inhibitor of visceromotor functions (Rajpara *et al*. 1992).

Compared to GYIRFamide immunostaining, BK-IR was highly restricted and localized to a small number of nerve cells in the vicinity of the cirrus and the pharynx. A number of physiological actions (see Introduction section) have been identified in mammals, with little evidence available for the presence of kinins in invertebrates. Where examined, the only invertebrate kinins so far reported are those from bee- and wasp-venoms (Yoshida, Geller & Pisano, 1976; Piek *et al*. 1987). The present results point to an earlier appearance of kinins in evolution. However, due to the restricted localization of BK-IR, the structure and biochemical characterization of a flatworm kinin will require the application of molecular biology methods. Also, more evidence will be needed to assign a biological function for kinins in platyhelminths and their nervous systems. On the other hand, GYIRFamide and structurally-related peptides, including the NPF-family, are well established as a major class of invertebrate peptide transmitters, and there is strong evidence that they are involved in neuromuscular transmission in platyhelminths.

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