Parasitol Res (1990) 76:409–419

Parasitology Research

A cytochemical study of the serotoninergic, cholinergic and peptidergic components of the reproductive system in the monogenean parasite, *Diclidophora merlangi*

A.G. Maule[1], D.W. Halton[1], C.F. Johnston[2], C. Shaw[2], and I. Fairweather[1]

[1] School of Biology and Biochemistry and [2] Department of Medicine, The Queen's University of Belfast, Belfast BT7 1NN, Northern Ireland, UK

Accepted February 2, 1990

Abstract. The reproductive system of the monogenean gill parasite, *Diclidophora merlangi*, was examined for the presence of cholinergic, serotoninergic and peptidergic innervation using cytochemical and immunocytochemical techniques. Cholinesterase activity and 5-hydroxytryptamine immunoreactivity (5-HT-IR) were confined to neural elements of the male reproductive system, being evident in the innervation of the cirrus, whereas only 5-HT was present in nerves and somata of the elongate seminal vesicle. Peptidergic innervation was localised to both the male and female reproductive systems of the worm. Within the female reproductive apparatus pancreatic polypeptide, peptide tyrosine tyrosine, neuropeptide Y, substance P, neurokinin A, eledoisin, FMRFamide and gastrin/cholecystokinin immunoreactive fibres and somata were observed in the oviduct, vitelline reservoir and ovovitelline duct. Intense peptide immunoreactivity was identified in fibres in the wall of the ootype and in a surrounding population (>100) of somata that were situated beyond Mehlis' gland cells and all of which were connected to the ootype wall by fine cytoplasmic connectives. The strategic location of this peptidergic cell population infers its involvement in the egg-forming sequence in this platyhelminth parasite.

Immunocytochemical and biochemical studies have provided substantial evidence that in addition to classical neurotransmitters, invertebrates contain an enormous molecular diversity of messenger molecules, most notably in the form of neuropeptides (Greenberg and Price 1983; Haynes 1980; Platt and Reynolds 1988; Walker and Holden-Dye 1989). Moreover, many of these peptides appear to be derived from ancestral molecules that are evident in unicellular eukaryotes and in certain prokaryotes (Le Roith et al. 1982, 1983; Roth et al. 1986). Of the invertebrate phyla, the platyhelminths represent a distinct stage in neural evolution, in that they are not only the earliest of organisms to display an aggregation of associative neurones in the anterior end to form a primitive "brain", but also the first in which there is consolidation of peripheral neurones and their processes into longitudinal nerve cords. To date, there are documented accounts of immunoreactivity to some 25 different regulatory peptides in a wide range of free-living and parasitic platyhelminths. These include turbellarians (Bautz et al. 1980; Carraway et al. 1982; Jennings et al. 1987; Reuter 1988; Reuter and Palmberg 1987, 1989; Reuter et al. 1984, 1986, 1988; Schilt et al. 1981; Venturini et al. 1983; Wikgren and Reuter 1985; Wikgren et al. 1986), monogeneans (Maule et al. 1989a, b; Reuter 1987, 1988), digeneans (Basch and Gupta 1988; Gupta and Basch 1989; Gustafsson 1987; Magee et al. 1989; Richard et al. 1989; Thorndyke and Whitfield 1987) and cestodes (Fairweather et al. 1988, 1990; Gustafsson 1985; Gustafsson and Wikgren 1989; Gustafsson et al. 1985, 1986; Kumazawa and Moriki 1986; Wikgren et al. 1986).

Although precise functions for these platyhelminth regulatory peptides have yet to be ascribed, many of them are believed to serve as neurotransmitters/neuromodulators in controlling mechanisms of neuromuscular function. However, several authors have described the occurrence of regulatory peptide immunoreactivities in the reproductive apparatus of parasitic flatworms (Basch and Gupta 1988; Fairweather et al. 1990; Gustafsson 1985; Magee et al. 1989; Maule et al. 1989a; Richard et al. 1989), raising the possibility that peptides may play key roles in influencing aspects (e.g. morphogenetic, physiological) of reproduction. Moreover, since the production of large numbers of eggs is essential for the survival of parasitic platyhelminths, regulatory peptides functioning in the reproductive system may provide interesting new targets for chemotherapeutic evaluation.

The present paper describes for the first time the results of applying confocal scanning laser microscopy (CSLM) to examine the distribution of regulatory peptide immunoreactivities in the innervation of the repro-

Reprint requests to: D.W. Halton

ductive apparatus of *Diclidophora merlangi*. Of the phylogenetically important peptides examined, pancreatic polypeptide (PP), peptide tyrosine tyrosine (PYY) and the molluscan peptide, FMRFamide, have previously been identified in the reproductive system using conventional immunofluorescence microscopy; the remaining molecules include the tachykinins and gastrin/cholecystokinin (CCK) and are new recordings in the worm. Results are compared with those of immunostaining for 5-hydroxytryptamine (5-HT) and with the cytochemical demonstration of cholinesterase (ChE) activity.

Materials and methods

D. merlangi were removed from the gill filaments of whiting (*Merlangius merlangus*) that had been landed (at Portavogie, County Down, Northern Ireland) within 8 h of being caught in the Irish Sea. The worms were washed thoroughly in aerated artificial sea water (ASW) (4° C), and any damaged specimens, as detected by staining with Evans' blue (Halton and Arme 1971), were discarded. In all cases, 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 (0.145 *M* NaCl; 0.025 *M* $NaH_2PO_4 \cdot 2H_2O$; 0.075 *M* Na_2HPO_4) (PBS) (pH 7.2), followed by washing (three changes) in PBS.

Cholinesterase (ChE) activity was visualised using the acetylthiocholine iodide (AThChI) technique of Koelle (1951), after Gomori (1952). Specimens were rinsed briefly in distilled water (to remove phosphate) prior to incubation. Controls included the omission of the substrate and the addition of the specific ChE inhibitor, eserine sulphate (1×10^{-5} *M*) (Sigma Chemical Company Ltd, Dorset, UK).

For the cytochemical demonstration of 5-HT and regulatory peptide immunoreactivities, the indirect immunofluorescence technique of Coons et al. (1955) was employed. Following fixation, specimens were washed for 24 h at 4° C in PBS containing 0.35% (v/v) Triton X-100 (to solubilise membranes) and 0.1% (w/v) sodium azide (PBS/TX/NaN_3) (Sigma Chemical Company Ltd, Dorset, UK). Primary antisera (for details of antisera and working dilutions, see Table 1) were diluted in PBS/TX/NaN_3 containing 1% (w/v) bovine serum albumin (BSA) fraction V (Miles Labs, Slough, UK) prior to their application to specimens for 48–72 h at 4° C. Specimens were then washed for 24 h in PBS at 4° C before and after the addition of either fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit or anti-guinea-pig IgG antiserum (Dako Ltd, High Wycombe, UK). Specimens were mounted in PBS:glycerol (1:9) containing 2.5% (w/v) 1,4-diazabicyclo-[2.2.2.]octane (Sigma Chemical Company Ltd, Dorset, UK).

Immunoreactivity (IR) was visualised by an Olympus BH-2 incident fluorescence microscope, fitted with a supplementary EY455 excitatory filter, or by an MRC 500 confocal scanning laser microscope (Bio-Rad Lasersharp, Abingdon, UK). Control incubations included: omission of primary antiserum; substitution of primary antiserum with non-immune rabbit serum (Dako Ltd, High Wycombe, UK); and addition of appropriate and inappropriate antigen (50–200 ng pure peptide/ml diluted antiserum; 200–500 ng 5-HT creatine sulphate/ml diluted antiserum).

Results

The structural organisation of the reproductive apparatus in *D. merlangi* follows the common trematode plan and consists of a male system comprising multiple testes, vas deferens, seminal vesicle and cirrus; and a female system comprising an ovary, oviduct, seminal receptacle, ootype and Mehlis' gland, and uterus, together with vitellaria, vitelline ducts and associated reservoir (Frankland 1955; Rennison 1953; Smyth 1976).

5-HT-IR in the reproductive system

Within the male reproductive system, the cirrus, seminal vesicle and vas deferens all displayed serotoninergic innervation. Two distinct, 5-HT-immunoreactive bipolar neurones encircle the posterior portion of the cirrus and give rise to fibres that branch and anastomose throughout the muscular walls of the elongate seminal vesicle (Figs. 1, 2). Also extending posteriorly from the cirrus and situated peripherally to the muscle layers of this duct, are broad immunoreactive nerve fibres and numerous associated bi- and multipolar somata (ca. 16 × 11 μm) (Fig. 2). A few fine, 5-HT-immunoreactive nerve fibres were identified in the walls of the vas deferens, but somata were not evident. The testes showed no evidence of 5-HT-IR. All control incubations were negative, and the addition of 500 ng/ml 5-HT in the liquid phase totally abolished immunostaining. No 5-HT-IR was detected in the female reproductive system and associated ducts of the worm.

Cholinesterase activity in the reproductive system

The only cholinesterase activity identified in the male reproductive system was that occurring in association with the male copulatory organ, where reactivity for the enzyme was apparent in an extensive system of fine nerve fibres that divided and anastomosed in the cirrus musculature (Figs. 3, 4). Nerve fibres that were reactive for cholinesterase also served to connect the paired cerebral ganglia and the cirrus of the worm (Fig. 3). Control incubations without substrate, as well as those containing 1×10^{-5} *M* eserine were at all times negative. No staining for cholinesterase activity was demonstrable in the female reproductive system of *D. merlangi*.

Regulatory peptides in the reproductive system

Pancreatic polypeptide family. Antisera to PP [221(6), 204(8)], PYY [301(3), 297(3)] and NPY [163(2), 8999(6)] all produced staining of exactly the same structures in both male and female systems; the results, therefore, are described concurrently.

The cirrus revealed a rich network of nerve fibres that were immunoreactive for PP, PYY and NPY; no peptidergic cell bodies were found associated with this innervation. PP-IR was evident in a band of fine nerve fibres situated at the opening of the cirrus (Fig. 5) and in nerve fibres that encircle it (Fig. 6). At intervals around the outer margin of the cirrus, the PP-immunoreactive nerves form small swellings that consist of collec-

Table 1. Details of the antisera used

Antiserum raised against	Regional specificity	Code number	Working dilution	Source[a]
5-Hydroxytryptamine (5-HT)	–	448 (1)	1:100	A
Synthetic hexapeptide of bovine pancreatic polypeptide (PP)	C-terminal	PP221 (6)	1:200	A
Bovine PP	Mid- to N-terminal	PP204 (8)	1:200	A
Synthetic peptide tyrosine tyrosine (PYY)	Non-C-terminal	PYY297 (3)	1:500	A
Synthetic PYY	Non-C-terminal	PYY301 (3)	1:250	A
Synthetic porcine neuropeptide Y (NPY)	–	RPN.1702	1:400	B
Synthetic NPY	C-terminal	8999 (6)	1:400	C
Synthetic NPY (26–36)	C-terminal	163 (2)	1:1,000	A
Porcine glucagon	N-terminal	YY234	1:250	A
Porcine glucagon	C-terminal	YY89	1:200	A
Porcine secretin	–	S10	1:50	A
Porcine vasoactive intestinal polypeptide (VIP)	–	341 (3)	1:100	A
Porcine VIP	–	39H2T	1:100	D
Synthetic porcine peptide histidine isoleucine (PHI)	–	426 (4)	1:500	A
Synthetic gastrin 1–17	Mid- to N-terminal	00156	1:200	A
Synthetic cholecystokinin-8 (CCK)	–	619 (2)	1:200	A
Synthetic CCK-8	–	621 (2)	1:200	A
Synthetic rat calcitonin-gene-related peptide (CGRP)	–	RPN.1842	1:300	B
Synthetic substance P (SP)	C-terminal (6–11)	152 (4)	1:1,000	A
Synthetic SP	C-terminal (7–11)	GSP10	1:500	A
Synthetic SP	–	17H2T	1:100	D
Synthetic SP	–	AB30	1:800	E
Synthetic SP	–	RPN.1572	1:200	B
Synthetic neurokinin A (NKA)	C-terminal	NKA (4)	1:200	F
Synthetic NKA	C-terminal	570 (3)	1:200	A
Synthetic eledoisin	C-terminal	E28307	1:400	G
Synthetic kassinin	Mid- to N-terminal	K78305	1:200	G
Porcine gastrin releasing peptide (GRP)	Mid-molecule	409 (3)	1:100	A
Porcine GRP	C-terminal	LR16 (10)	1:100	H
Synthetic bombesin	–	B43	1:100	I
Synthetic human calcitonin	–	260 (8)	1:100	A
Synthetic xenopsin	C-terminal	GXP5	1:200	F
Synthetic neuromedin-C	C-terminal	R402 (3)	1:250	A
Synthetic neurotensin (NT)	C-terminal	28H2T	1:100	D
Synthetic NT	C-terminal	NT6 (2)	1:200	A
Synthetic somatostatin (SRIF)	–	RPN.1612	1:100	B
Synthetic SRIF	–	3-4-84	1:100	J
Synthetic SRIF	–	OB1 (5)	1:100	A
Porcine adrenocorticotropic hormone (ACTH)	–	B13	1:100	I
Synthetic atrial natriuretic peptide (ANP) (4–28)	–	561 (4)	1:200	A
Motilin	–	PK2(p)q	1:1,000	K
Porcine insulin	–	GP11 (3)	1:500	A
Synthetic FMRFamide (FMRF)	C-terminal	494 (2)	1:500	A

[a] Source: *A* Department of Medicine, The Queen's University of Belfast, Northern Ireland; *B* Amersham International plc, Aylesbury, Buckinghamshire, England; *C* Dr. M.M.T. O'Hare, Rigshospitalet, Copenhagen, Denmark; *D* Immuno Nuclear Corporation, Stillwater, Minnesota, USA; *E* CRB Ltd., Harston, Cambridge, England; *F* Dr. J.M. Conlon, Max-Planck-Institut, University of Göttingen, FRG; *G* Dr. E. Theodorsson, Karolinska Hospital, Stockholm, Sweden; *H* Prof. T.J. McDonald, University Hospital London, Ontario, Canada; *I* MILAB, Malmo, Sweden; *J* Prof. Y.C. Patel, Royal Victoria Hospital, Montreal, Canada; *K* Dr. P. Kwasosowski, University of Surrey, Guildford, England

tions of fine fibres. Fine nerves emanate from these swellings and, in places, appear to provide connections with the peripheral nerve net, just beneath the tegument of the worm.

In the female reproductive system, no PP-IR was apparent in the ovary of the worm. However, PP-immunostaining was evident in the oviduct, vitelline reservoir, common ovovitelline duct and in the ootype/Mehlis' gland complex (Figs. 7–10). A well-developed plexus of peptidergic nerve fibres and cell bodies is present in the walls of the oviduct. The somata associated with this innervation are either bi- or multi-polar (measuring ca. 16×11 μm) and occur at regular intervals along the length of the duct. The mouth of the duct that leads to the seminal receptacle is also served by a rich innervation of PP-immunoreactive nerve fibres. The walls of the vitelline reservoir and the common ovovitelline duct have extensive peptidergic innervation, although there are only relatively few somata in their walls (Figs. 8, 10). Two PP-immunoreactive bipolar somata (ca. $12 \times$

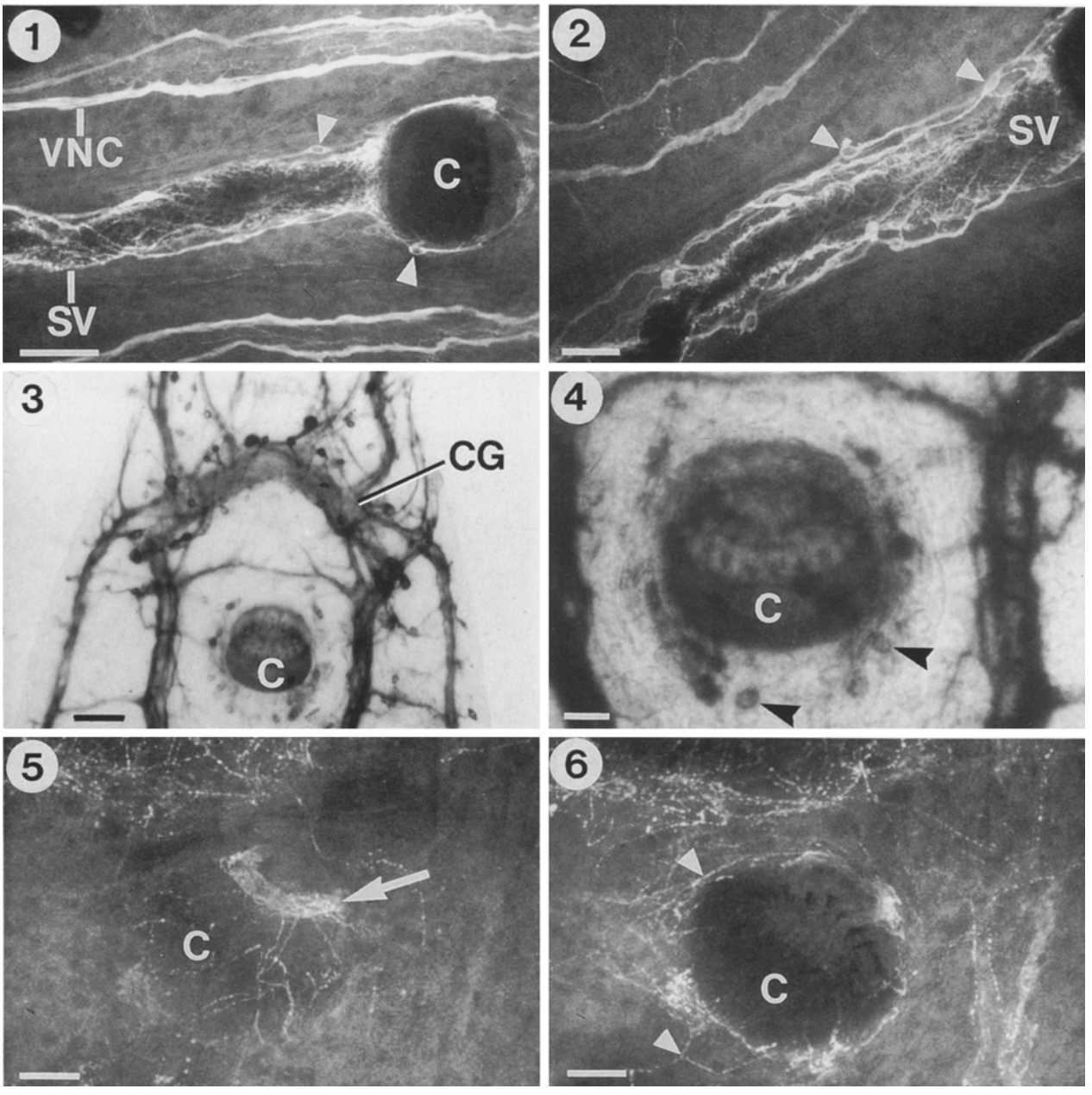
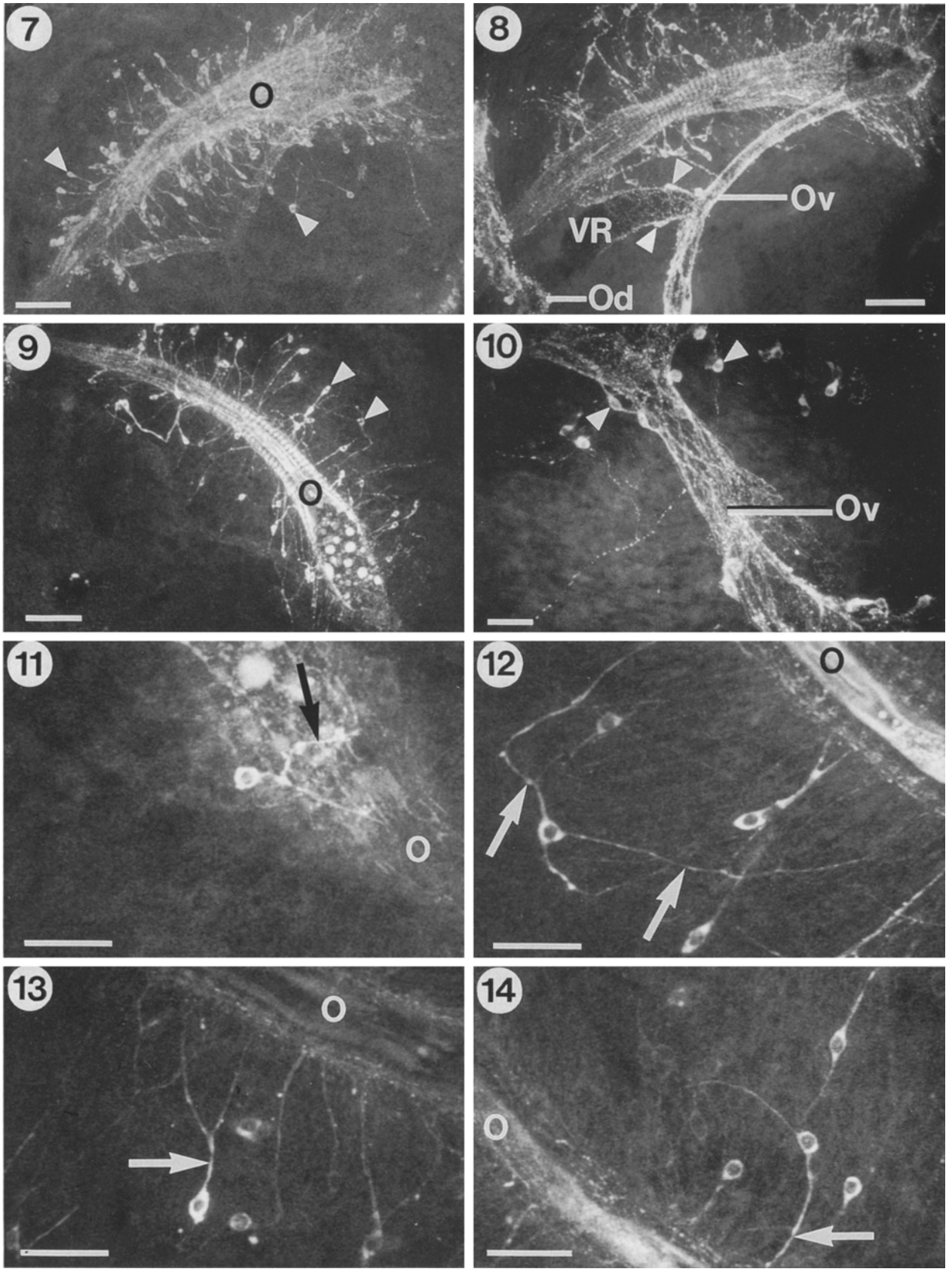


Fig. 1. CSLM fluorescence image of 5-HT-IR in nerve fibres and somata (*arrowheads*) that innervate the cirrus (*C*) and seminal vesicle (*SV*). *VNC*, ventral nerve cord. Bar, 100 μm. **Fig. 2.** CSLM fluorescence image of 5-HT-IR in the walls of the elongate seminal vesicle (*SV*). Note the numerous somata (*arrowheads*) associated with the innervation of this organ. Bar, 50 μm. **Fig. 3.** ChE activity in the forebody region, showing the position of the muscular cirrus (*C*) in relation to the cerebral ganglia (*CG*). Bar, 100 μm. **Fig. 4.** ChE activity in the musculature of the cirrus (*C*) and some surrounding somata (*arrowheads*). Note the intensity of reaction in the musculature of the cirrus. Bar, 50 μm. **Figs. 5, 6.** Micrograph images of two consecutive CSLM optical sections through the cirrus (*C*), showing PP-IR. **Fig. 5.** Peripheral nerve elements in the cirrus wall and in a band of fibres at its opening (*arrow*). **Fig. 6.** Surrounding nerve fibres that connect the innervation of this organ with the PNS (*arrowheads*). Note the genital hooklets in Fig. 6. Bar, 50 μm

Fig. 7. CSLM fluorescence image of PP-IR in nerve fibres innervating the walls of the ootype (*O*) and in a surrounding population of peptide cells (*arrowheads*). Bar, 100 μm. **Fig. 8.** CSLM fluorescence image of PP-IR in nerve fibres innervating the walls of the oviduct (*Od*), vitelline reservoir (*VR*) and ovovitelline duct (*Ov*). Note the somata at the junction of the oviduct and the vitelline reservoir (*arrowheads*). Bar, 100 μm. **Fig. 9.** CSLM fluorescence image of PYY-IR in the ootype duct (*O*) wall and in a population of surrounding somata (*arrowheads*). Note the banding pattern of staining in the ootype musculature. Bar, 100 μm. **Fig. 10.** CSLM fluorescence image of PYY-IR in nerve fibres and somata (*arrowheads*) innervating the wall of the ovovitelline duct (*Ov*). Bar, 50 μm



Figs. 11–14. CSLM fluorescence images of PYY-IR in the peptide cells surrounding the ootype (*O*). Note that all of the cells are connected to the duct wall by fine cytoplasmic connections (*arrows*) and that the cells are a mixed population of uni- (**Figs. 11, 13**), bi- (**Fig. 14**), and multi-polar (**Fig. 12**) somata. Bar, 50 µm

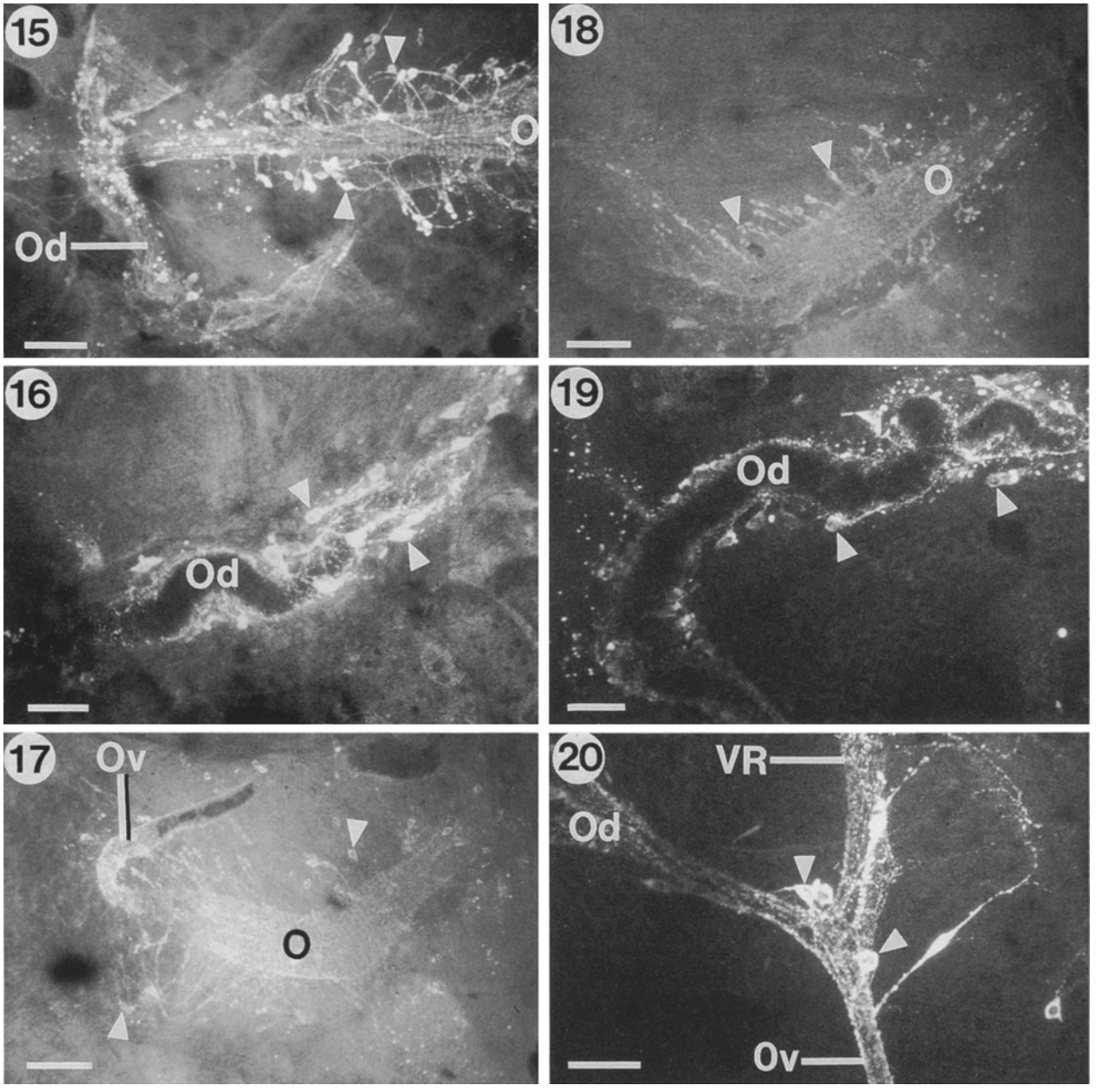


Fig. 15. CSLM fluorescence image of FMRFamide-IR in nerve fibres of the oviduct (*Od*) and ootype (*O*) walls and in a population of somata (*arrowheads*) surrounding the latter. Bar, 100 μm. **Fig. 16.** CSLM fluorescence image of FMRFamide-IR in nerve fibres and somata (*arrowheads*) innervating the walls of the oviduct (*Od*). Bar, 50 μm. **Fig. 17.** CSLM fluorescence image of gastrin/CCK-IR in nerve fibres innervating the common ovovitelline duct (*Ov*) and ootype duct (*O*) walls and in the population of somata (*arrowheads*) encircling the ootype. Bar, 100 μm. **Fig. 18.** CSLM fluorescence image of eledoisin-IR in the walls of the ootype duct (*O*) and in the surrounding population of somata (*arrowheads*). Bar, 100 μm. **Fig. 19.** CSLM fluorescence image of SP-IR in the walls of the oviduct (*Od*). Note the numerous immunoreactive somata (*arrowheads*) associated with the innervation of this duct. Bar, 50 μm. **Fig. 20.** CSLM fluorescence image of NKA-IR in neurones (*arrowheads*) at the junction of the oviduct (*Od*), vitelline reservoir (*VR*) and ovovitelline duct (*Ov*). Immunoreactive nerve fibres are present in the lining of these three ducts. Bar, 50 μm

18 μm) form part of the innervation of the vitelline reservoir and are positioned at a point close to that where it joins with the oviduct (Fig. 8). Peptidergic nerve fibres form an extensive component of the walls of the common ovovitelline duct as it extends posteriorly, before reflecting anteriorly and becoming distended to form the ootype duct. It was in this latter portion of the duct that the most intense PP-IR was recorded. An array of anastomosing nerve fibres runs through the wall of the ootype and, although many of the fibres appear varicose in nature, there is an apparent lack of somata (Figs. 7–9). In many instances, the distributional pattern

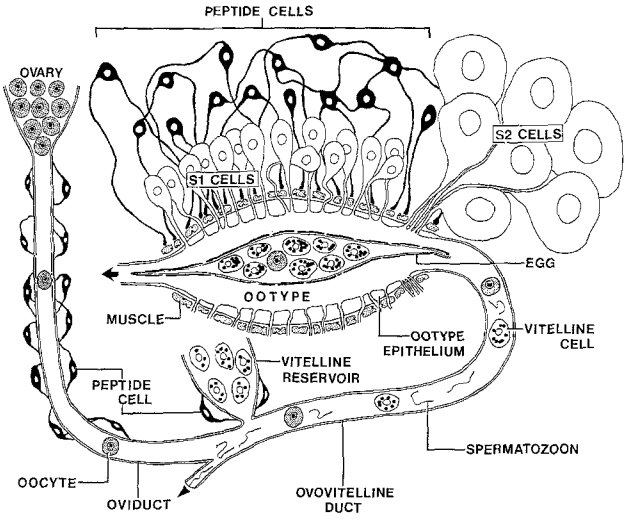


Fig. 21. Diagrammatic interpretation of the relationship of the regulatory peptide-immunoreactive somata with the female reproductive system of the monogenean parasite, *Diclidophora merlangi*. The S1 and S2 cells represent the two types of Mehlis' gland cell identified in the parasite. It should be noted that the walls of the oviduct, vitelline reservoir, ovovitelline duct and ootype are all well endowed with peptidergic nerve fibres. Positive immunostaining for PP, PYY, NPY, FMRFamide, gastrin/CCK, NKA and eledoisin was identified in nerve fibres innervating all of the ducts shown and in the peptide cells that surround the ootype and the oviduct. However, SP-IR was not localised in the lining of the ootype or in the population of peptide cells that encircle the ootype duct. Note that only the distal portion of the duct that extends from the seminal receptable to the oviduct exhibited peptidergic innervation. Peptidergic innervation was not found in the rest of this duct or in the seminal receptacle (*arrowhead*). Once formed, the egg passes anteriorly from the ootype into the uterus (*arrow*)

of immunostaining corresponded to that of the circular muscle bands of the ootype wall (Fig. 9). Surrounding the ootype and situated beyond the level of the S1 cells of Mehlis' gland is a large population (>100) of cells that display PP-IR (Figs. 7–9). These peptide cells constitute a heterogeneous population of uni-, bi- and multipolar somata (ca. 13 × 10 μm) (Figs. 11–14) and are connected either directly or indirectly to the ootype wall by fine immunoreactive cytoplasmic connectives. The cells converge on the walls of the ootype along its entire length to the junction with the uterus.

FMRFamide. The IR obtained to FMRFamide was similar to that recorded with antisera to members of the PP family, both in distribution and in intensity (Figs. 15, 16). To discount the possibility of cross-reactivity between the FMRFamide antiserum and the parasite PP-like peptide, preabsorbtion of the FMRFamide antiserum was attempted with 200–500 ng/ml bovine PP. In all cases, the antiserum failed to be preabsorbed.

Gastrin/cholecystokinin (CCK). Gastrin/CCK-immunoreactivity using antiserum 619(2) was not as intense as that obtained with the other regulatory peptide antisera. However, it was apparent in the peptide cell population surrounding Mehlis' gland and in nerve fibres innervating the ootype wall (Fig. 17). Weak immunostaining occurred in the walls of the oviduct and vitelline reservoir.

Tachykinin peptide family. Positive IR for tachykinin peptides was obtained with the SP antiserum 152(4), the NKA antiserum 570(3) and the eledoisin antiserum E28307, whereas the SP antiserum GSP10, the NKA antiserum NKA(4) and the kassinin antiserum K78305 failed to identify any parasite antigen. Immunoreactivities to the molluscan tachykinin, eledoisin, and to NKA were associated with the walls of the ootype, as well as with a few of the surrounding population of peptide cells (Fig. 18). The localisation and intensity of immunostaining with NKA antiserum 570(3) and eledoisin antiserum RE2 were identical. Although IR to SP, NKA and eledoisin was evident in the oviduct (Fig. 19), vitelline and common ovovitelline duct (Fig. 20), none was apparent with the SP antisera in the ootype/Mehlis' gland complex.

Negative results were obtained with all of the control incubations, and 200–500 ng/ml of the appropriate peptide antigen abolished all immunostaining. The distribution of peptidergic cells and their relationship with the proximal ducts of the female reproductive system in *D. merlangi* is shown schematically in Fig. 21.

Discussion

In general, platyhelminths are hermaphroditic, and they display a complexity of reproductive organs and accessory ducts that dominate the body. Typically, all parts of the male and female systems except the gonads are underlain by a muscular stratum of both circular and longitudinal fibres. The contraction and relaxation of this muscle would seem essential for the orderly process of egg formation, and regulation of this motility presumably resides in the associated nervous system. Where examined, the reproductive apparatus in turbellarians is especially rich in nerve terminals, with all of the associated organs, cavities and ducts being rich in subepithelial plexuses and nerves innervating the musculature (Baguñà and Ballester 1978). Nerves have also been identified in association with various reproductive ducts of monogeneans (Williams 1960). In *D. merlangi*, the central and peripheral nervous systems have been shown to contain cholinergic, serotoninergic and peptidergic components (Halton and Morris 1969; Halton et al. 1987; Maule et al. 1989a, b); the present results demonstrate that there is a similar complexity in the innervation of its reproductive system.

Of the classical messenger molecules, acetylcholine (ACh) is believed to function as an inhibitory neurotransmitter in parasitic platyhelminths (Fetterer et al. 1977; Holmes and Fairweather 1984). ChE activity, used in the present study to indicate the possible presence of ACh, was confined to the muscular cirrus in the male reproductive system. The cirrus is located antero-ventrally in the forebody, just posterior to the cerebral gan-

glia. Since the worm lacks a vagina, it is believed that the cirrus, which is armed with a ring of 16 genital hooklets, gaffs a hole in the tegument of a partner worm for purposes of hypodermic impregnation (Macdonald and Caley 1975). In this activity, it is possible that the cholinergic nerve fibres, which connect the cirrus directly with the paired cerebral ganglia, serve a central role in controlling the muscular activity of this organ. The absence of ChE activity from other parts of the reproductive system may mean that there is little or no involvement of ACh in the co-ordination of reproductive function. However, some caution should be exercised in interpreting the presence of ChE activity as being indicative of cholinergic nerves. The enzyme is not exclusive to cholinergic nerves and can be found in non-cholinergic neurones as well as in non-nervous tissue.

5-HT-IR occurs extensively in the male reproductive system, being evident in nerves innervating the cirrus, seminal vesicle and vas deferens. In parasitic flatworms, 5-HT is believed to function as an excitatory neurotransmitter (Mansour 1984). In *D. merlangi* it was found to have a stimulatory effect on muscular activity in vitro (Maule et al. 1989c). The finding of numerous 5-HT-immunopositive nerve fibres and somata in intimate association with the walls of the seminal vesicle suggests a strong serotoninergic influence over the activity of this organ. The demonstration of 5-HT-IR in the cirrus and seminal vesicle confirms previous findings of its occurrence in these organs (Halton et al. 1987); however, its presence in the vas deferens is a new finding. The absence of 5-HT-IR from the female reproductive system is in direct contrast to the finding in *Fasciola hepatica* of 5-HT immunostaining in the ootype/Mehlis' gland complex (Fairweather et al. 1987). In this digenean trematode, it was postulated that the indoleamine plays a regulatory role in egg production.

In contrast to classical neurotransmitters, neuropeptides were evident in both the male and female reproductive apparatus of *D. merlangi*. This finding, along with the absence of cholinergic and serotoninergic nerve fibres and somata from the female reproductive apparatus, suggest that neuroactive peptide molecules may have an important controlling influence on the muscular coordination of this system. Regulatory peptides have been localised in association with the testes in other parasitic worms. For example, PYY- and vasoactive intestinal polypeptide (VIP)-immunoreactivities were identified in nerve fibres innervating the testis follicles of *Trilocularia acanthiaevulgaris* (Fairweather et al. 1990), and peptide histidine isoleucine (PHI)-immunopositive nerve fibres have been shown to innervate the testicular follicles of *Diphyllobothrium dendriticum* (Gustafsson 1985). However, within the male system of *D. merlangi* there was an absence of regulatory peptide-IR from the testes, and no peptide IR was found in conjunction with the vitelline glands. In contrast, growth hormone releasing factor (GRF) and FMRFamide immunoreactive nerve fibres have been described as being present in the vitelline glands of *D. dendriticum* (Gustafsson 1985), and CCK-IR has been localised within the vitelline follicles in *Schistosoma* species (Basch and Gupta 1988).

Immunoreactivity to members of the PP family, i.e. PP, PYY and NPY, and the molluscan peptide FMRF-amide was prevalent in the muscular cirrus. The male copulatory organ of the free-living turbellarian *Microstomum lineare* was also found to have FMRFamide-IR associated with it (Reuter et al. 1984), as was the cirrus sac of *D. dendriticum* (Gustafsson 1985). Also, PYY-IR was localised in two cell bodies of the prostate gland in *F. hepatica* (Magee et al. 1989), and VIP-IR was observed in the nerve supply of the cirrus sac in *T. acanthiaevulgaris* (Fairweather et al. 1990). No SP-like IR was localised in the cirrus of *D. merlangi*, whereas this peptide has been found in the prostate gland and muscle layer underlying the cirrus in *Echinostoma caproni* (Richard et al. 1989). The presence in the cirrus of *D. merlangi* of cholinergic, serotoninergic and peptidergic nerve fibres points to the possible co-existence of classical transmitters and peptides within the same neurone, a phenomenon now widely recognised as occurring in vertebrates (Hökfelt et al. 1986, 1987; Lundberg and Hökfelt 1983; Scharrer 1982) and invertebrates (Osborne et al. 1982; O'Shea and Schaffer 1985).

The most dominant immunoreactivity identified in the nervous system of *D. merlangi* was that obtained with antisera to members of the PP peptide family (Maule et al. 1989a, b). This also proved to be the case in the female reproductive system of the worm. However, the present results are the first demonstration of NPY-, gastrin/CCK-, SP-, NKA- and eledoisin-immunostaining in the reproductive system of a monogenean. Immunoreactivity to PP, PYY, NPY and FMRFamide displayed identical distribution patterns in the oviduct, vitelline reservoir, ovovitelline duct and ootype/Mehlis' gland complex. The antisera to PP, PYY and NPY are known to be cross-reactive and would therefore seem to be binding the same parasite antigen. This interpretation is consistent with earlier chromatographic analyses that identified a single PP-like peptide in the worm (Maule et al. 1989d). It should be noted that the cross-reactivity of FMRFamide antisera with PP has previously been documented (Dockray and Williams 1983; Dockray et al. 1981). As the FMRFamide immunostaining in *D. merlangi* could not be blocked with PP (50–500 ng/ml), it would appear that this is not a product of the non-specific binding of the FMRFamide antiserum with the parasite PP-like peptide.

PP-, FMRFamide-, NKA- and SP-IRs were intense in nerve plexuses in the wall of the oviduct, extending from its junction with the ovary to that with the vitelline reservoir. The large number of peptidergic somata associated with this innervation suggests that peptides must not only be involved in the regulation of the muscular activity of this duct, but, through paracrine secretion, could also be involved in the maturation of developing ova en route to the ootype. Rich peptidergic innervation was also evident in a subepithelial plexus of the vitelline reservoir, with two prominent somata positioned at the junction of this duct with the oviduct. These cells may influence the muscular, valve-like structures that control the entry of vitelline cells into the oviduct at this point. A comparable role has been postulated for PP-immuno-

reactive cells in nerve plexus I in *F. hepatica*, which are situated at the entrance to the ootype, where they are believed to regulate the movement of ova and vitelline cells (Magee et al. 1989). Basch and Gupta (1988) have described a similar neuronal plexus of six perikarya in the region between the ovary and common vitelline duct in *Schistosoma mansoni*.

In the present study, there was no evidence of a genito-intestinal canal in *D. merlangi*. In this respect, Kearn (1986) has stated that in monogeneans that lack such an exit route, greater control is necessary in regulating the volume of vitelline cells released from the vitelline reservoir, the corollary being that the acquisition of a more sophisticated means of control may have led to the loss of the genito-intestinal canal. It may be that the peptidergic cells at the junction of the vitelline reservoir and oviduct reflect such sophistication in *D. merlangi*.

An extremely rich innervation of PP- and FMRF-amide-immunoreactive nerve fibres is present in the ovovitelline and ootype duct walls of *D. merlangi*. It is in the ootype that the egg-shell material is deposited and the egg is shaped prior to its exit into the uterus. The co-ordination of the processes that occur within the ootype has been thought to involve the S1 and S2 cells of Mehlis' gland (Hardcastle 1977). These cells are believed to be akin to the "mucous" and "serous" cells described for cestodes (Löser 1965) and for *F. hepatica* (Threadgold and Irwin 1970). The exact function of Mehlis' gland cells remains enigmatic, although it has been postulated that their secretions provide a template upon which the semi-liquid egg-shell protein is deposited during egg development (Smyth and Halton 1983).

A previous study by Maule et al. (1989a) identified a population of approximately 30 peptidergic somata situated around Mehlis' gland cells. However, with the aid of optical sectioning provided by CSLM, this number would seem to be nearer 100, all of the somata being immunoreactive for PP, FMRFamide, gastrin/CCK, NKA and eledoisin. The presence of PP-, gastrin/CCK-, FMRFamide-, NKA- and eledoisin-like peptides in this population of cells points to a possible co-existence of regulatory peptides, although no attempt has been made to verify this by multiple staining or by elution restaining techniques. Co-localisation of peptide molecules in mammals is well documented (Beauvillain et al. 1984; Hökfelt et al. 1986, 1987; O'Shea and Schaffer 1985; Tramu et al. 1977; Vanderhaeghen et al. 1983), and this phenomenon would appear to be common in lower species (Evans and Calabrese 1989; Grimmelikhuijzen 1983; Reuter and Palmberg 1989).

The peptide cell population surrounding Mehlis' gland is heterogeneous in that it consists of uni-, bi-, and multi-polar somata that are interconnected with each other and with the ootype wall, possibly an indication that they influence each other as well as the ootype itself (see Fig. 21). Many of the fine axonal connections of the peptide cells surrounding Mehlis' gland extend along the walls of the ootype duct, and since there is a distinct banding pattern of peptide IR in these walls that corresponds to the circular muscle layer of this duct, it is likely that the peptide cell products influence the muscle activity of this organ.

Typical of the polyopisthocotyleans, *D. merlangi* produces large, fusiform eggs with long, polar filaments. Kearn (1986) stated that "it is remarkable that an egg mould (ootype) made of soft tissue and capable of vigorous muscular deformation is able to reproduce repeatedly and faithfully an egg of relatively uniform shape and size". Clearly, the process involves a precise and well-co-ordinated mechanism of muscle control. The finding of a complex array of some 100 peptide-secreting cells with cytoplasmic connections terminating at the musculature of the egg chamber points to a regulatory role for peptides in egg assembly and reproductive function. Thus, it is possible that the peristaltic movement of the antagonistic muscle layers that invest the egg chamber and associated ducts is under the regulatory influence of one or more peptides. The nature of the peptide secretion may be such that a more long-term paracrine or almost hormonal effect is exerted; for example, the effect could be for the control and maturation of Mehlis' gland. Peptides may also influence the release of Mehlis' gland secretion by interacting with the ducts of these cells so as to synchronise release of secretion with the appearance of egg material in the ootype.

Although immunostaining for the tachykinins NKA and eledoisin occurred in the ootype/Mehlis' gland complex, IR for the mammalian tachykinin SP was restricted in its distribution to the oviduct, vitelline reservoir and common ovovitelline duct. Since the NKA antiserum 570(3) cross-reacts with eledoisin (Maule et al. 1989e), it is reasonable to assume that the tachykinin-IRs in the ootype represent a single parasite peptide. These differences in the distribution of SP-IR and that of NKA and eledoisin agrees with previous chromatographic analyses, which identified two tachykinins in extracts of *D. merlangi* (Maule et al. 1989e).

The present results add to what is a considerable body of information on the distribution and immunocytochemical localisation of regulatory peptides in trematodes and cestodes, and the way is now open for progress in biochemical and functional studies on their role in parasite reproductive biology.

References

Baguñà J, Ballester R (1978) The nervous system in planarians: peripheral and gastrodermal plexuses, pharynx innervation, and the relationship between central nervous system structure and the acoelomate organization. J Morphol 155:237–252

Basch PF, Gupta BC (1988) Immunocytochemical localization of regulatory peptides in six species of trematode parasites. Comp Biochem Physiol [C] 91:565–570

Bautz A, Schilt J, Richoux J-P, Dubois M-P (1980) Detection immunocytologique, denombrement et localisation des cellules a somatostatine (SRIF) chez deux especes de Planaires, *Dugesia lugubris* et *Dendrocoelum lacteum* (Turbellaries, Triclades). C R Seances Acad Sci 291:833–836

Beauvillain JC, Tramu G, Garaud J (1984) Coexistence of substances related to enkephalin and somatostatin in granules of the guinea-pig median eminence: demonstration by use of col-

loidal gold immunocytochemical methods. Brain Res 301:389–393

Carraway R, Ruane SE, Kim H-R (1982) Distribution and immunochemical character of neurotensin-like material in representative vertebrates and invertebrates: apparent conservation of the COOH-terminal region during evolution. Peptides 1:115–123

Coons AH, Leduc EH, Connolly JM (1955) Studies on antibody production: I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J Exp Med 102:49–60

Dockray GJ, Williams RG (1983) FMRFamide-like immunoreactivity in rat brain: development of a radioimmunoassay and its application in studies of distribution and chromatographic properties. Brain Res 266:295–303

Dockray GJ, Vaillant C, Williams RG (1981) New vertebrate brain-gut peptide related to a molluscan neuropeptide and an opioid peptide. Nature 293:656–657

Evans BD, Calabrese RL (1989) Small cardioactive peptide-like immunoreactivity and its colocalization with FMRFamide-like immunoreactivity in the central nervous system of the leech *Hirudo medicinalis*. Cell Tissue Res 257:187–199

Fairweather I, Maule AG, Mitchell SH, Johnston CF, Halton DW (1987) Immunocytochemical demonstration of 5-hydroxytryptamine (serotonin) in the nervous system of the liver fluke, *Fasciola hepatica* (Trematoda, Digenea). Parasitol Res 73:255–258

Fairweather I, Macartney GA, Johnston CF, Halton DW, Buchanan KD (1988) Immunocytochemical demonstration of 5-hydroxytryptamine (serotonin) and vertebrate neuropeptides in the nervous system of excysted cysticercoid larvae of the rat tapeworm, *Hymenolepis diminuta* (Cestoda, Cyclophyllidea). Parasitol Res 74:371–379

Fairweather I, Mahendrasingam S, Johnston CF, Halton DW, Shaw C (1990) Peptidergic nerve elements in three developmental stages of the tetraphyllidean tapeworm, *Trilocularia acanthiaevulgaris*: an immunocytochemical study. Parasitol Res (in press)

Fetterer RH, Pax RA, Bennett JL (1977) *Schistosoma mansoni*: direct method for simultaneous recording of electrical and motor activity. Exp Parasitol 43:286–294

Frankland HMT (1955) The life history and bionomics of *Diclidophora denticulata* (Trematoda, Monogenea). Parasitology 45:313–351

Gomori G (1952) Microscopic histochemistry, principles and practice. University of Chicago Press, Chicago

Greenberg MJ, Price DA (1983) Invertebrate neuropeptides: native and naturalized. Ann Rev Physiol 45:271–288

Grimmelikhuijzen CJP (1983) Coexistence of neuropeptides in hydra. Neuroscience 9:837–845

Gupta BC, Basch PF (1989) Human chorionic gonadotropin-like immunoreactivity in schistosomes and *Fasciola*. Parasitol Res 76:86–89

Gustafsson MKS (1985) Cestode neurotransmitters. Parasitol Today 1:72–75

Gustafsson MKS (1987) Immunocytochemical demonstration of neuropeptides and serotonin in the nervous system of adult *Schistosoma mansoni*. Parasitol Res 74:168–174

Gustafsson MKS, Wikgren MC (1989) Development of immunoreactivity to the invertebrate neuropeptide small cardiac peptide B in the tapeworm *Diphyllobothrium dendriticum*. Parasitol Res 75:396–400

Gustafsson MKS, Wikrgren MC, Karhi TJ, Schot LPC (1985) Immunocytochemical demonstration of neuropeptides and serotonin in the tapeworm *Diphyllobothrium dendriticum*. Cell Tissue Res 240:255–260

Gustafsson MKS, Lehtonen MAI, Sundler F (1986) Immunocytochemical evidence for the presence of "mammalian" neurohormonal peptides in neurones of the tapeworm *Diphyllobothrium dendriticum*. Cell Tissue Res 243:41–49

Halton DW, Arme C (1971) An in vitro technique for detecting tissue damage in *Diclidophora merlangi*: possible screening method for selection of undamaged tissues or organisms prior to physiological investigation. Exp Parasitol 30:54–57

Halton DW, Morris GP (1969) Occurrence of cholinesterase and ciliated sensory structures in a fish gill fluke, *Diclidophora merlangi* (Trematoda, Monogenea). Z Parasitenkd 33:21–30

Halton DW, Maule AG, Johnston CF, Fairweather I (1987) Occurrence of 5-hydroxytryptamine (serotonin) in the nervous system of a monogenean, *Diclidophora merlangi*. Parasitol Res 74:151–154

Hardcastle A (1977) Ultrastructural observations on the reproductive system of *Diclidophora merlangi* (Monogenoidea: Polyopisthocotylea). PhD Thesis, The Queen's University of Belfast, Northern Ireland

Haynes LW (1980) Peptide neuroregulators in invertebrates. Prog Neurobiol 15:205–245

Hökfelt T, Holets VR, Staines W, Meister B, Melander T, Schalling M, Schutlzberg M, Freedman J, Björklund H, Olson L, Lindh B, Elfvin L-G, Lundberg JM, Lindgren JA, Samuelsson B, Pernow B, Terenius L, Post C, Everitt B, Goldstein M (1986) Coexistence of neuronal messengers – an overview. Prog Brain Res 68:33–70

Hökfelt T, Millhorn D, Seroogy K, Tsuruo Y, Ceccatelli S, Lindh B, Meister B, Melander T, Schalling M, Bartfai T, Terenius L (1987) Coexistence of peptides with classical neurotransmitters. Experientia 43:768–780

Holmes SD, Fairweather I (1984) *Fasciola hepatica*: the effects of neuropharmacological agents upon in vitro motility. Exp Parasitol 58:194–208

Jennings JB, Davenport TRB, Varndell IM (1987) FMRFamide-like immunoreactivity and arylamidase activity in turbellarians and nemerteans – evidence for a novel neurovascular coordinating system in nemerteans. Comp Biochem Physiol [C] 86:425–430

Kearn GC (1986) The eggs of monogeneans. Adv Parasitol 25:175–273

Koelle GB (1951) Elimination of enzymatic diffusion artifacts in histochemical localization of cholinesterase and survey of their cellular distribution. J Pharm Exp Ther 103:153–171

Kumazawa H, Moriki T (1986) Immunoenzymatic demonstration of a presumptive prolactin-like substance in *Hymenolepis nana*. Z Parasitenkd 72:137–139

Le Roith D, Shiloach J, Roth J (1982) Is there an earlier phylogenetic precursor that is common to both the nervous and endocrine systems? Peptides 3:211–215

Le Roith D, Shiloach J, Berelowitz M, Frohman LA, Liotta AS, Krieger DT, Roth J (1983) Are messenger molecules in microbes the ancestors of the vertebrate hormones and tissue factors? Fed Proc 42:2602–2607

Löser E (1965) Der Feinbau des Oogenotop bei Cestoden. Z Parasitenkd 25:413–458

Lundberg JM, Hökfelt T (1983) Coexistence of peptides and classical neurotransmitters. Trends Neuropharmacol Sci 6:325–333

Macdonald S, Caley J (1975) Sexual reproduction in the monogenean *Diclidophora merlangi*: tissue penetration by sperms. Z Parasitenkd 45:323–334

Magee RM, Fairweather I, Johnston CF, Halton DW, Shaw C (1989) Immunocytochemical demonstration of neuropeptides in the nervous system of the liver fluke, *Fasciola hepatica* (Trematoda, Digenea). Parasitology 98:227–238

Mansour TE (1984) Serotonin receptors in parasitic worms. Adv Parasitol 23:1–36

Maule AG, Halton DW, Johnston CF, Fairweather I, Shaw C (1989a) Immunocytochemical demonstration of neuropeptides in the fish-gill parasite, *Diclidophora merlangi* (Monogenoidea). Int J Parasitol 19:307–316

Maule AG, Halton DW, Johnston CF, Shaw C, Fairweather I (1989b) The serotoninergic, cholinergic and peptidergic components of the nervous system in the monogenean parasite, *Diclidophora merlangi*: a cytochemical study. Parasitology (in press)

Maule AG, Halton DW, Allen JM, Fairweather I (1989c) Studies

on motility in vitro of an ectoparasitic monogenean, *Diclidophora merlangi*. Parasitology 98:85–93

Maule AG, Shaw C, Halton DW, Johnston CF, Fairweather I (1989d) Localization, quantification and characterization of pancreatic polypeptide immunoreactivity in the parasitic flatworm *Diclidophora merlangi* and its fish host (*Merlangius merlangus*). Gen Comp Endocrinol 74:50–56

Maule AG, Shaw C, Halton DW, Johnston CF, Fairweather I, Buchanan KD (1989e) Tachykinin immunoreactivity in the parasitic flatworm *Diclidophora merlangi* and its fish host the whiting (*Merlangius merlangus*): radioimmunoassay and chromatographic characterisation using region-specific substance P and neurokinin A antisera. Comp Biochem Physiol [C] 94:533–541

Osborne NN, Cuello AC, Dockray GJ (1982) Substance P and cholecystokinin-like peptides in *Helix* neurons and cholecystokinin and serotonin in a giant neuron. Science 216:409–411

O'Shea M, Schaffer M (1985) Neuropeptide function: the invertebrate contribution. Ann Rev Neurosci 8:171–198

Platt N, Reynolds SE (1988) Invertebrate neuropeptides. In: Lunt GG, Olsen RW (eds) Comparative invertebrate neurochemistry, ch 5. Croom Helm Ltd, Beckenham, pp 175–226

Rennison BD (1953) A morphological and histochemical study of egg shell formation in *Diclidophora merlangi* Kühn. Dissertation, Trinity College, Dublin

Reuter M (1987) Immunocytochemical demonstration of serotonin and neuropeptides in the nervous system of *Gyrodactylus salaris* (Monogenea). Acta Zool (Stockh) 68:187–193

Reuter M (1988) Development and organisation of nervous systems visualized by immunocytochemistry in three flatworm species. Prog Zool 36:181–184

Reuter M, Palmberg I (1987) An ultrastructural and immunocytochemical study of gastrodermal cell types in *Microstomum lineare* (Turbellaria, Macrostomida). Acta Zool (Stockh) 68:153–163

Reuter M, Palmberg I (1989) Development and differentiation of neuronal subsets in asexually reproducing *Microstomum lineare*. Histochem 91:123–131

Reuter M, Karlin T, Schot LPC (1984) Immunocytochemical demonstration of peptidergic neurons in the central and peripheral nervous system of the flatworm *Microstomum lineare* with antiserum to FMRF-amide. Cell Tissue Res 238:431–436

Reuter M, Wikgren M, Lehtonen M (1986) Immunocytochemical demonstration of 5-HT-like and FMRF-amide-like substances in whole mounts of *Microstomum lineare* (Turbellaria). Cell Tissue Res 246:7–12

Reuter M, Lehtonen M, Wikgren M (1988) Immunocytochemical evidence of neuroactive substances in flatworms of different taxa – a comparison. Acta Zool (Stockh) 69:29–37

Richard J, Klein MJ, Stoeckel ME (1989) Neural and glandular localization of substance P in *Echinostoma caproni* (Trematoda-Digenea). Parasitol Res 75:641–648

Roth J, Le Roith D, Lesniak MA, Pablo F de, Bassas L, Collier E (1986) Molecules of intercellular communication in vertebrates, invertebrates and microbes: do they share common origins? Prog Brain Res 68:71–79

Scharrer B (1982) Peptidergic neurons. Acta Morphol Neerl Scand 20:219–223

Schilt J, Richoux JP, Dubois MP (1981) Demonstration of peptides immunologically related to vertebrate neurohormones in *Dugesia lugubris* (Turbellaria, Tricladida). Gen Comp Endocrinol 43:331–335

Smyth JD (1976) Introduction to animal parasitology, 2nd edn. Hodder and Stoughton, London Sydney Auckland Toronto

Smyth JD, Halton DW (1983) The physiology of trematodes. Cambridge University Press, Cambridge

Thorndyke MC, Whitfield PJ (1987) Vasoactive intestinal polypeptide-like immunoreactive tegumental cells in the digenean helminth *Echinostoma liei*: possible role in host-parasite interactions. Gen Comp Endocrinol 68:202–207

Threadgold LT, Irwin SWB (1970) Electron microscope studies of *Fasciola hepatica:* IX. The fine structure of Mehlis' gland. Z Parasitenkd 35:16–30

Tramu G, Leonardelli J, Dubois MP (1977) Immunohistochemical evidence for an ACTH-like substance in hypothalamic LH-RH neurons. Neurosci Lett 6:305–309

Vanderhaeghen JJ, Lotstra F, Liston DR, Rossier J (1983) Proenkephalin, [met]enkephalin, and oxytocin immunoreactivities are localized in bovine hypothalamic magnocellular neurons. Proc Natl Acad Sci USA 80:5139–5143

Venturini G, Carolei A, Palladini G, Margotta V, Lauro MG (1983) Radioimmunological and immunocytochemical demonstration of met-enkephalin in planaria. Comp Biochem Physiol [C] 74:23–25

Walker RJ, Holden-Dye L (1989) Commentary on the evolution of transmitters, receptors and ion channels in invertebrates. Comp Biochem Physiol [A] 93:25–39

Wikgren M, Reuter M (1985) Neuropeptides in a microturbellarian – whole-mount immunocytochemistry. Peptides 6 [Suppl 3]:471–475

Wikgren M, Reuter M, Gustafsson M (1986) Neuropeptides in free-living and parasitic flatworms. An immunocytochemical study. Hydrobiologia 132:93–99

Williams JB (1960) The dimorphism of *Polystomum integerrimum* (Frolich) Rudolphi and its bearing on relationships within the Polystomatidae: part I. J Helminthol 34:151–192