J. exp. Biol. ***182****, 81–96 (1993)*
Printed in Great Britain © The Company of Biologists Limited 1993

SENSORY AND NEUROSECRETORY INNERVATION OF LEECH NEPHRIDIA IS ACCOMPLISHED BY A SINGLE NEURONE CONTAINING FMRFamide

A. WENNING*, M. A. CAHILL
Fakultät für Biologie, Universität Konstanz, W-7750 Konstanz, Germany
U. HOEGER
Institut für Zoologie, Johannes Gutenberg-Universität Mainz, W-6500 Mainz, Germany
AND R. L. CALABRESE
Department of Biology, Emory University, Atlanta, GA 30322, USA

Accepted 29 April 1993

Summary

The neural control of the excretory system of the medicinal leech *Hirudo medicinalis* has been characterized morphologically and chemically using light and electron microscopy, immunocytochemistry and biochemistry. Immunoreactivity against RFamide-like peptides revealed elaborate neuronal aborizations of a neurone in the nephridium, around the urinary bladder sphincter and in the central nervous system. The processes arose from the nephridial nerve cell (NNC), a previously identified receptor neurone. Using a combination of reverse-phase high pressure liquid chromatography, radioimmunoassay and subsequent Edman degradation and mass spectrometry, authentic FMRFamide has been identified as the major peptide of the NNC. Sensory and neurosecretory innervation of the nephridia is thus accomplished by a single neurone, which is thought to modulate nephridial performance.

Introduction

Following a blood meal, medicinal leeches clear large salt and water loads rapidly by means of their excretory organs, the nephridia. The mechanisms of ion and fluid transport of the urine-forming cells have been well characterized (Zerbst-Boroffka and Wenning, 1986), but the efferent control mechanisms are unknown. Neither blood-borne factors (i.e. diuretic hormones) nor intrinsic properties of the urine-forming cells are involved in the clearance of ions and water after a meal. The nephridia are densely innervated (Boroffka *et al.* 1970; Haupt, 1974; Wenning and Cahill, 1986). Axon profiles containing both large dense-cored vesicles and small clear vesicles, and lying near the urine-forming cells, were thought to originate from efferent neurones located in the central nervous

*To whom correspondence should be addressed.

Key words: Annelida, leech, *Hirudo medicinalis*, nephridium, HPLC, microsequence analysis, immunocytochemistry, ultrastructure, FMRFamide.

system (CNS) (Wenning and Cahill, 1986). The afferent innervation of the nephridium is accomplished by the nephridial nerve cell (NNC), a receptor neurone with its soma on the bladder (Wenning, 1983; Wenning and Cahill, 1986). The NNC is sensitive to changes of extracellular Cl^- concentration (Wenning, 1989; Wenning and Calabrese, 1991).

In this report, we show that the neurosecretory innervation of the urine-forming cells expresses FMRFamide-like immunoreactivity (FLI). Peptides of the RFamide family were first identified as neuromodulators in molluscs (Price and Greenberg, 1977). They are found in all animal phyla and exert diverse physiological effects on neurones and muscles (for references, see Evans *et al.* 1991), but they have not been associated with the modulation of ion transport. About 40 neurones in the leech CNS, mostly motoneurones, express FLI (Kuhlmann *et al.* 1985), and five different peptides of the RFamide family have been identified in the leech CNS (Evans *et al.* 1991). We show that the sensory neurone, the NNC, is the source of the neurosecretory innervation of the urine-forming cells and that FMRFamide is the major peptide present in the NNC. The data suggest that the NNC is bifunctional. In addition to monitoring blood Cl^-, the NNC may modulate ion transport rates and thus nephridial performance. Some of these results have appeared in abstract form (Wenning *et al.* 1992).

Materials and methods

Adult leeches (*Hirudo medicinalis*) were obtained from commercial suppliers. Breeding boxes were maintained from June to September at 25–27˚C (natural daylight). Cocoons were removed within a day of deposition and kept moist at 23±1˚C. Embryos were staged according to the number of days after cocoon deposition (E1=day 1, etc.). Numbering of segments follows the convention of S. M. Shankland, E. R. Macagno, D. A. Weisblat, K. J. Muller and W. B. Kristan (personal communication): R1–R4 for the segments forming the anterior sucker, M1–M21 for the unfused ganglia and their associated peripheral structures and C1–C7 for abdominal segments fused into the posterior sucker.

Embryos were removed from cocoons at various stages and kept in pasteurized artificial pond water (Muller *et al.* 1981) at 23˚C. At different stages, the yolk sac was opened on the side opposite to the embryo and pinned out in leech saline (Muller *et al.* 1981). The yolk was removed to expose the embryo, which was then processed for histology. Juveniles (length 1–2cm) were decapitated, opened dorsally and the NNCs of one or several midbody segments exposed. Adults were opened dorsally in leech saline, the chain of ganglia was severed posterior to the brain and several nephridial complexes (a nephridium with its urinary bladder) were removed and processed for histology.

Histology

Immunocytochemistry

To demonstrate FMRFamide-like immunoreactivity (FLI), we followed the protocol of Kuhlmann *et al.* (1985). Whole embryos or isolated nephridial complexes were fixed overnight in a mixture of 2% paraformaldehyde and 15% picric acid in 0.1mol l^{-1}

phosphate buffer. After being rinsed in buffer, they were left overnight at 6˚C in a solution of 0.4% Triton X-100 and 0.02% sodium azide in 0.1mol l^{-1} phosphate buffer (PBTA). To reduce non-specific labelling, specimens were incubated in 20% normal goat serum (NGS; Gibco Laboratories, Chagrin Falls, Ohio, USA) in PBTA in the cold for 2h. The first antibody (polyclonal; raised in rabbits against synthetic FMRFamide conjugated to succinylated bovine thyroglobulin; no. 671C; Marder *et al.* 1987) was diluted 1:600 in PBTA and applied in the cold for 18–24h. After being rinsed in PBTA, the specimens were incubated for 1h in the secondary antibody (goat anti-rabbit IgG, Sigma, Deisenhofen, Germany) at a dilution of 1:40, followed by several washes in phosphate-buffered saline (PBS1; 0.01mol l^{-1} phosphate buffer, 0.9% NaCl) and in PBTA. They were then exposed to a rabbit peroxidase-conjugated antibody (Dako, Hamburg, Germany) diluted 1:300 in PBTA for 7h, following the PAP technique of Sternberger (1979). As chromogen, 3,3′-diaminobenzidine tetrahydrochloride (10mg per 30ml PB; Sigma, Deisenhofen, Germany) was reacted with 0.015% H_2O_2. Specimens were then either dehydrated and mounted in Canada balsam (Merck, Darmstadt, Germany) or post-fixed in 2% OsO_4 in 0.1mol l^{-1} phosphate buffer and processed for electron microscopy as described below. For older embryos (from E20 onwards), fixation time was longer (36–48h) and incubation time in the primary antibody was for a minimum of 24h (see Dircksen *et al.* 1991). Control experiments in which the primary antibody was preincubated with FMRFamide did not stain and thus confirmed the specificity of the first antibody.

Double labelling

Nephridial nerve cell bodies of adults were ionophoretically injected with Lucifer Yellow (LY CH lithium salt, 5% in distilled water, Sigma Chemical Co.) using glass microelectrodes (80–150 MΩ, backfilled with 2mol l^{-1} LiCl) and hyperpolarizing current pulses (20–40min). In juveniles, cell bodies were pressure-injected (2–5min). Specimens were then processed for immunocytochemistry as described above, except that all steps were carried out in the dark and that a Texas-Red-conjugated secondary antibody was used (Jackson Immuno Research, West Grove, Pennsylvania, USA;1:40 in PBTA) for 1 h. Specimens were mounted in a mixture of glycerol (80%) and PBS1 (20%) and examined and photographed using a fluorescence microscope with the appropriate filters.

Electron microscopy

For demonstration of FLI, methods described by Dircksen *et al.* (1987), Bendayan (1984) and Childs *et al.* (1986) were employed. Isolated nephridial complexes from adults were immersed in 2% glutaraldehyde in 0.1mol l^{-1} phosphate buffer (pH7.4) for 1 h. They were post-fixed in 2% OsO_4 in 0.1mol l^{-1} phosphate buffer for 1h. After dehydration in alcohols, the specimens were embedded in Spurr's low-viscosity epoxy resin. Thin sections on nickel grids were treated as follows: etch, 4% periodic acid, 1h; rinse, three times in distilled water, once in PBS1; block, NGS 1:30 in PBS1, 15min; rinse, PBS1; first antibody, 1:1200 in PBS1, 16–18h, cold (4–8˚C); rinse, three times in PBS1; gold label, 10nm gold conjugated to goat anti-rabbit IgG (AuroProbe EM, Janssen, Belgium) 1:40 in NGS/PBS1 (1:30), 1–2h; rinse, PBS1, distilled water. Sections

were allowed to dry, stained with uranyl acetate and lead citrate, and examined in a Zeiss 900 electron microscope.

In some cases, pre-fixed tissue was embedded in gelatine and sectioned on a vibratome (Pelco 101). 30 μm sections were then processed by the PAP method. Sections in which label was found were post-fixed in osmium, embedded in Spurr's resin and processed for electron microscopy.

Determination of peptide amount (ELISA)

Nephridia and the part of the bladder wall bearing the soma of the NNC were isolated from adult leeches. Care was taken to remove any remnants of heart and secondary blood vessels. The nephridial complexes obtained from one animal (12–26) were combined and stored at either +8 or −10°C in 1.5ml reaction vials containing 500 μl of acidified methanol (90% methanol, 9% glacial acetic acid, 1mmol l^{-1} dithiothreitol, 1% water; Evans *et al.* 1991). For complete extraction of peptides, the reaction vials were briefly immersed in liquid nitrogen and the nephridia were broken up by ultrasonic disintegration. The homogenates were centrifuged at 4°C for 5min at 10000 ***g***, the supernatant was removed and the pellet was resuspended by sonication in 500 μl of acidified methanol. After centrifugation, the supernatants were combined and dried under a stream of air. Dried samples were suspended in 220 μl of phosphate-buffered saline (PBS2; 150mmol l^{-1} NaCl, 10mmol l^{-1} NaH_2PO_4, pH7.4) containing 1% NGS, and 50 μl samples were used for peptide assay by enzyme-linked immunosorbent assay (ELISA) (see below).

The FMRFamide content of the extracts was determined by competitive ELISA according to Kingan (1989), using synthetic FMRFamide coupled to porcine thyroglobulin (Tager, 1976) as competitive tracer. In brief, after coating the ELISA plates (Titertek, Flow Laboratories, Meckenheim, Germany) with the thyroglobulin–peptide conjugate, the wells were loaded with primary antibody (1:50000) followed by either sample or standard solution of synthetic FMRFamide.

After adsorption of the primary antibody, the amount of antibody bound to conjugate was quantified using peroxidase-labelled goat anti-rabbit IgG (1:2000 in PBS2) as secondary antibody and 2′,2-azino-bis(3-ethylbenzthiazoline-6-sulphonate) (ABTS; 2.75% in 10mmol l^{-1} phosphate buffer, pH6.0, containing 0.0075% H_2O_2) as substrate. The plates were read after 30–120min at 415nm in a Dynatech MR 5000 ELISA plate reader. All measurements were carried out in quadruplicate. A calibration curve was constructed using standard amounts (3–50pmol) of synthetic FMRFamide and the peptide content of the samples was calculated from regression lines obtained from semi-logarithmic fits of standard amounts of peptides with corresponding absorbances.

Peptide extraction, purification and sequencing

Three separate batches of approximately 1000 nephridia were isolated as described above and stored at −20°C. They were homogenized in acidified methanol using a straight-walled glass grinder and then sonicated over dry ice/acetone with a Branson model 250 sonicator. The resulting slurry was spun in an Eppendorf microcentrifuge (model 5414) for 10min, the supernatant reserved, and the pellet re-extracted with

methanol and recentrifuged. Supernatants were combined and concentrated to approximately 1ml under vacuum with a Speed Vac concentrator. The extract was then loaded on a C18 Sep-Pac cartridge (Waters), previously equilibrated with methanol, desalted with at least 10ml of water, and eluted with 5ml of methanol. The eluate was concentrated to dryness in a Speed Vac concentrator and reconstituted in 0.5–1ml of distilled water.

RFamide peptides were purified from the extract by four-step, reverse-phase high pressure liquid chromatography (rpHPLC), similar to that described by Evans *et al.* (1991). For steps 1–3, a Whatman Partisil 5 ODS-3 column (C18, 5mm particle size, 250mm×4.7mm) was used with a Rainin Rabbit HPLC machine. For step 4, an Aquapore OD-300 column (C18, 7mm particle size, 30nm pore size, 250mm×1 mm) was used with an Applied Biosystems micro separation system HPLC machine. Step 1: flow, $1 ml min^{-1}$; gradient, 10%–85% acetonitrile over 65min; fractions, 1ml; counterion, 0.1% trifluoroacetic acid (TFA). Step 2: flow, $1 ml min^{-1}$; gradient, 10%–40% acetonitrile over 60min; fractions, 1ml, counterion, 0.1% TFA. Samples were oxidized by incubation in 1.5% hydrogen peroxide for at least 4h prior to step 2. Step 3: flow, $1 ml min^{-1}$; gradient, 14%–38% acetonitrile over 58min; fractions, 1ml; counterion, 0.1% heptafluorobutyric acid (HFBA). Step 4: flow, $0.09 ml min^{-1}$; gradient, 0%–40% acetonitrile over 75min; fractions, volumes were collected corresponding to ultraviolet absorbance peaks (214nm); counterion, 0.1% TFA. Fractions containing RFamide immunoreactivity were detected by radioimmunoassay (RIA) with antibody 671c as described by Marder *et al.* (1987), combined as necessary (5ml samples, steps 1–3 and 2ml sample, step 4) and vacuum-concentrated (Speed Vac) to achieve the desired volume for the next step of purification or sequencing.

FMRFamide (Bachem) and FMRFamide (met sulphoxide form) were run as standards for each rpHPLC step and their retention time was determined by monitoring optical absorbance at 214nm. To produce the sulphoxide form, FMRFamide was oxidized by incubation in 1.5% hydrogen peroxide for at least 4h.

Sequencing of purified peptides was carried out at the Emory University (Microchemical Facility) by Dr Jan Pohl, using automated Edman degradation on an Applied Biosystems model 477A protein sequencer with an on-line phenyltiohydantoin (PTH) analyzer. In each sequence, contaminating amino acids detected by the PTH analyzer were at significantly lower levels than the designated amino acid in the sequence, and Dr Pohl approached each sequence unaware of the expected result.

Results

Anatomy

The use of a polyclonal antibody directed against FMRFamide-like peptides revealed FMRFamide-like immunoreactivity (FLI) in the nephridial nerve cell (NNC) of *Hirudo medicinalis*, demonstrating the complete architecture of the cell for the first time. The NNC is known to branch within the CNS and in the nephridium. The extent of these arborizations and the dense innervation of the outlet region of the urinary bladder (Figs 1, 2) became apparent. Projections of the NNC were identified among the sphincter muscles

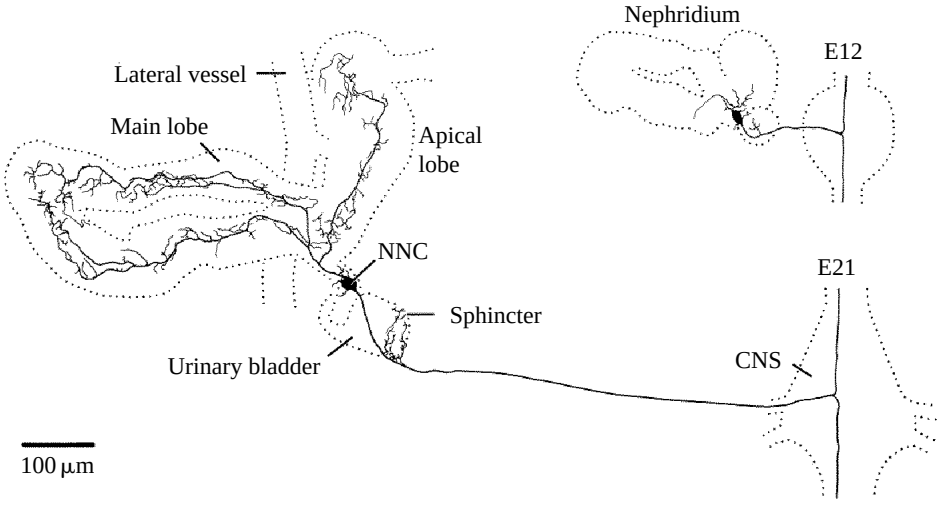

Fig. 1. *Camera lucida* drawings of nephridia and ganglia in left midbody segments of *Hirudo medicinalis* at embryonic stages E12 and E21 after immunostaining for FMRFamide. The nephridial nerve cell (NNC) is shown with its projections into the nephridium, bladder sphincter and central nervous system (CNS).

of the urethra (Wenning and Cahill, 1989) through intracellular injection of horseradish peroxidase (HRP) (Fig. 2B).

Earlier evidence which showed labelled and unlabelled axon profiles in the nephridium following intracellular injection of HRP into the NNC (Wenning and Cahill, 1986) suggested that at least one additional neurone innervates each leech nephridium. The existence of unlabelled profiles, however, could result from incomplete transport of HRP into some distal branches in adult leeches.

Successful application of topical markers used in immunocytochemistry can be hindered by overlying tissues. To obtain good penetration of solutions and antibodies, it is a great advantage to use smaller, thinner specimens. Therefore, embryos at various stages of development were used in this study to obtain a complete picture of FLI associated with the excretory complex. Leech embryos consist of only a few layers of tissue (Fernandez and Stent, 1982). By embryonic day 20 (E20; 60% of development), the nephridia are complete (Wenning *et al.* 1993) but are covered by only a thin layer of tissue. In embryos processed for FLI as whole mounts at stage E12 (36% of development), projections of the NNC are visible in the nephridium and in the segmental ganglion (Fig. 1). Later, processes from the NNC flank the lumen of the central canal (Fig. 3A,B), a hollow tube continuous throughout the nephridium in which the final urine is formed (for a detailed description of nephridial anatomy, see Boroffka *et al.* 1970; Zerbst-Boroffka and Wenning, 1986). In a given cross section, several profiles of the NNC are found around the central canal (Fig. 3A,B). By E21, the NNC has covered most areas of the nephridium and innervates the sphincter muscles of the urinary bladder (Figs 1, 2). At no stage in embryogenesis or later were branches from a second neurone expressing FLI found innervating the nephridium.

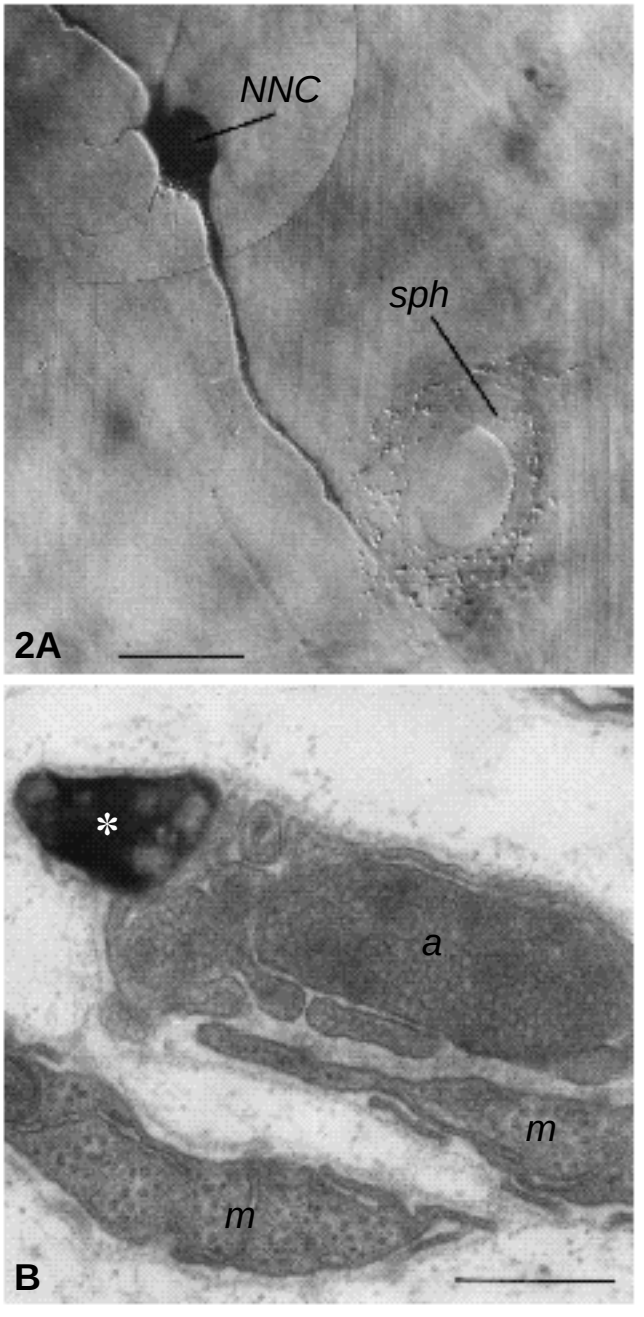

Fig. 2. (A) Whole mount of an embryo (E30) showing the nephridial nerve cell (*NNC*) and its arborization on the bladder sphincter (*sph*) stained to reveal FMRFamide-like immunoreactivity (FLI). Anterior is at the top. Nomarski differential interference contrast optics. Scale bar, 50 μm. (B) Electron micrograph of bladder sphincter muscles (*m*) and their dual innervation by a process of the NNC labelled intracellularly with horseradish peroxidase (asterisk) and a second axon (*a*). Scale bar, 0.5 μm.

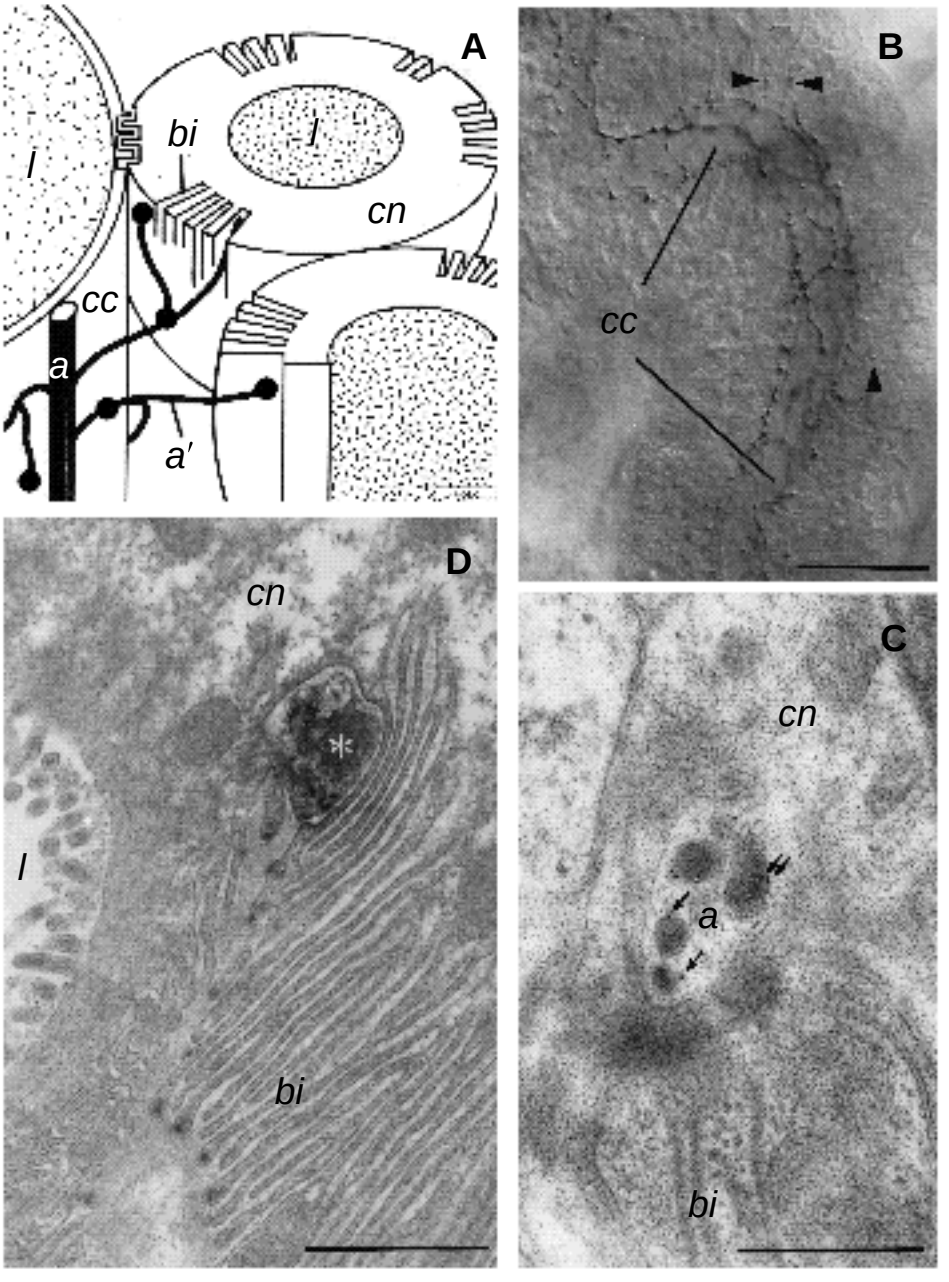

Fig. 3

Varicose axon branches of the NNC form a dense network around the urine-forming cells (Fig. 3). The ultrastructure of the nerve terminals in the nephridium has been described (Haupt, 1974; Wenning and Cahill, 1986). To determine whether they express FLI, sections reacted against FMRFamide using the PAP technique (Sternberger, 1979) were fixed and embedded for electron microscopy. Reaction product was found between

Fig. 3. Varicose axons expressing FLI are associated with the urine-forming cells of the nephridium. (A) A schematic drawing showing the pattern of neural processes on canalicular cells (*cn*). The axons are found on smooth surfaces of the cell as well as in the cleft of the basal infoldings (*bi*). *a*, main branch of NNC; *a′*, secondary branch of NNC; *cc*, central canal; *l*, lumen. (B) Part of a nephridium seen in a whole mount of an E30 embryo stained to reveal FLI. The main axons follow the central canal (*cc*) and occasionally branch (arrowheads) among the canalicular cells. Scale bar, 50 μm. (C) Electron micrograph showing an axon (asterisk) expressing FLI after treatment with the PAP technique. It is closely associated with a canalicular cell (*cn*). *l*, lumen of canalicular cell; *bi*, basal infoldings of canalicular cell. Scale bar, 1 μm. (D) Electron micrograph of an axon (*a*) on a canalicular cell (*cn*) after treatment with a gold-conjugated secondary antibody to visualize FLI. Gold particles (10nm, arrows) are seen on the neurosecretory granules. The ratio of label to background here is 59:1, measured on a total area of 90 μm^2. *bi*, basal infoldings of canalicular cells. Scale bar, 0.5 μm.

basal infoldings of the primary urine-forming canalicular cells (Fig. 3C). Use of the second antibody alone gave no reaction, indicating that no endogenous peroxidases interfere with the specificity of the labelling. When immunogold was conjugated to the secondary antibody, gold particles were found associated with dense-cored vesicles in axon profiles contacting the urine-forming cells (Fig. 3D).

To confirm that the FLI derives from the NNC and not from a different neurone, intracellular dye-injection was combined with immunocytochemistry. The labelled structures were identical using both methods (Fig. 4). Whereas earlier dye-injection experiments were carried out on adults (Wenning and Cahill, 1986), juveniles were used for the experiments reported here. The distance the injected dye must travel is thereby greatly reduced. The distribution of dye in juveniles was sufficient to label the thin varicose processes that leave the main branch along the central canal and contact the urine-forming tissue (Fig. 4).

The results demonstrate that the NNC is the only neurone innervating the nephridium to express FLI and is the source of the previously described neurosecretory innervation of the urine-forming cells.

Isolation and sequence of RFamide peptides from leech nephridia

A competitive ELISA was used to estimate the amount of FMRF-like peptide present in one nephridium. Nephridia were collected over a period of several months. We found 1.16±0.9pmol (N=9, ± S.D.) of peptide per nephridium in samples stored at −10°C (range 0.3–3.2pmol) and 1.42±0.6pmol (N=15, ± S.D.) of peptide in samples stored at +8°C (range 0.3–2.5pmol).

In separate experiments, we sought to determine the identity of the peptide(s) responsible for FLI in the nephridium. Evans *et al.* (1991) isolated and sequenced five peptides (FMRFamide, FLRFamide, YMRFamide, YLRFamide and GGKYMRFamide) from the CNS of the medicinal leech. We reasoned that one or more of these peptides might account for the FLI found in the NNC, and so developed a four-step rpHPLC purification procedure, similar to that employed by Evans *et al.* (1991) to isolate RFamide peptides from leech CNS extracts. Three batches of nephridia (approximately 1000 nephridia each) were extracted, desalted and processed separately with rpHPLC. Fractions from one rpHPLC step, which contained significant levels of immunoreactivity,

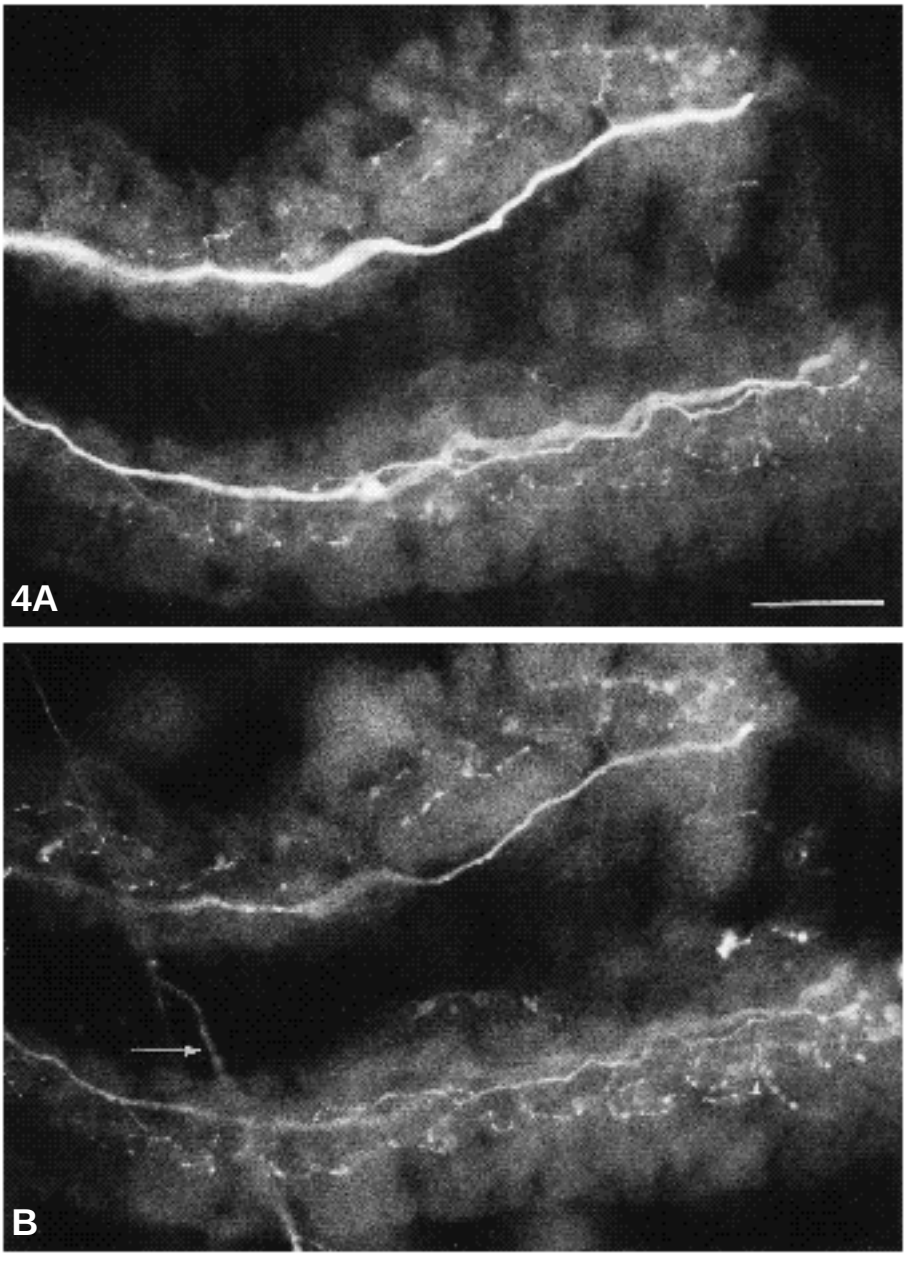

Fig. 4. Intracellular and topical double labelling of the NNC processes in the nephridium of a juvenile leech photographed in a fluorescence microscope. (A) The branching pattern seen after injection of the NNC with Lucifer Yellow. Note that even fine processes in the nephridium are filled with dye. Scale bar, 50 μm. (B) FLI was visualized in the same preparation using a Texas-Red-conjugated secondary antibody. The label completely coincides with that in A. FLI is also associated with the enteric network lying dorsally in a different plane of focus (arrow). Same magnification as A.

Table 1. *Sequence data from leech nephridial RFamide peptides*

	Cycle number							
	1	2	3	4	5	6	7	8
Purification batch 1								
Amino acid	F	M	R	F				
Quantity (pmol)	27	19	6.7	5.9	–	–	–	–
Purification batch 2 (illustrated in Fig. 5)								
Amino acid	F	M	R	F				
Quantity (pmol)	32	15	5.6	10	–	–		

Identity and amount of the predominant amino acid detected in each sequence cycle for two peptides sequenced. –, no detectable amino acids in sequence cycles.

as determined by an RFamide peptide radioimmunoassay (RIA) (Marder *et al.* 1987), were passed onto the subsequent rpHPLC step. Each batch yielded similar results and in two batches the end result was the isolation and sequencing of FMRFamide (Table 1); no RFamide peptide was successfully isolated from the third batch. Results for each of the rpHPLC purification steps from one of the successful batches are detailed below.

Step 1 (Fig. 5A) resulted in two bands of immunoreactivity, one of which migrated near oxidized FMRFamide (met-sulphoxide form, arrow) (fractions 15 and 16) and the other close to FMRFamide (arrowhead) (fractions 19–22). Fractions 19–22 were oxidized with H_2O_2 and passed on to step 2 (Fig. 5B), which yielded a single band of immunoreactivity with a peak (fraction 20) that co-migrated with oxidized FMRFamide (Fig. 5B, arrow). Fractions 20–22 were passed on to step 3 (Fig. 5C), yielding a large peak (fraction 34), which co-migrated with oxidized FMRFamide (Fig. 5C, arrow). Fraction 34 was passed on to step 4, yielding several peaks at 214nm, one of which (Fig. 5D, arrow) contained detectable levels of RFamide immunoreactivity. The fraction containing this peak was sequenced and yielded FMRF (Table 1). The other successful purification showed a similar pattern of migration through the purification steps and also yielded the sequence FMRF.

Because the purified peptides sequenced as FMRF co-migrated with oxidized FMRFamide in steps 2 and 3 and were detected by our RIA, which is specific for amidated peptides (Marder *et al.* 1987), we conclude that nephridia of *H. medicinalis* contain authentic FMRFamide. Moreover, because FLI in the nephridia is limited to the NNC, we conclude that the NNC contains authentic FMRFamide.

Discussion

The results presented here show that the NNC, a previously characterized Cl^- receptor neurone (Wenning, 1989; Wenning and Calabrese, 1991), is also the source of neurosecretory innervation of the urine-forming tissue of the leech and that one of its endogenous peptides is FMRFamide. It is unlikely that an additional, efferent neurone, one without FMRFamide, also innervates the nephridium. In numerous preparations in which the nephridial nerve was wick-filled in both directions using $NiCl_2$, cobalt

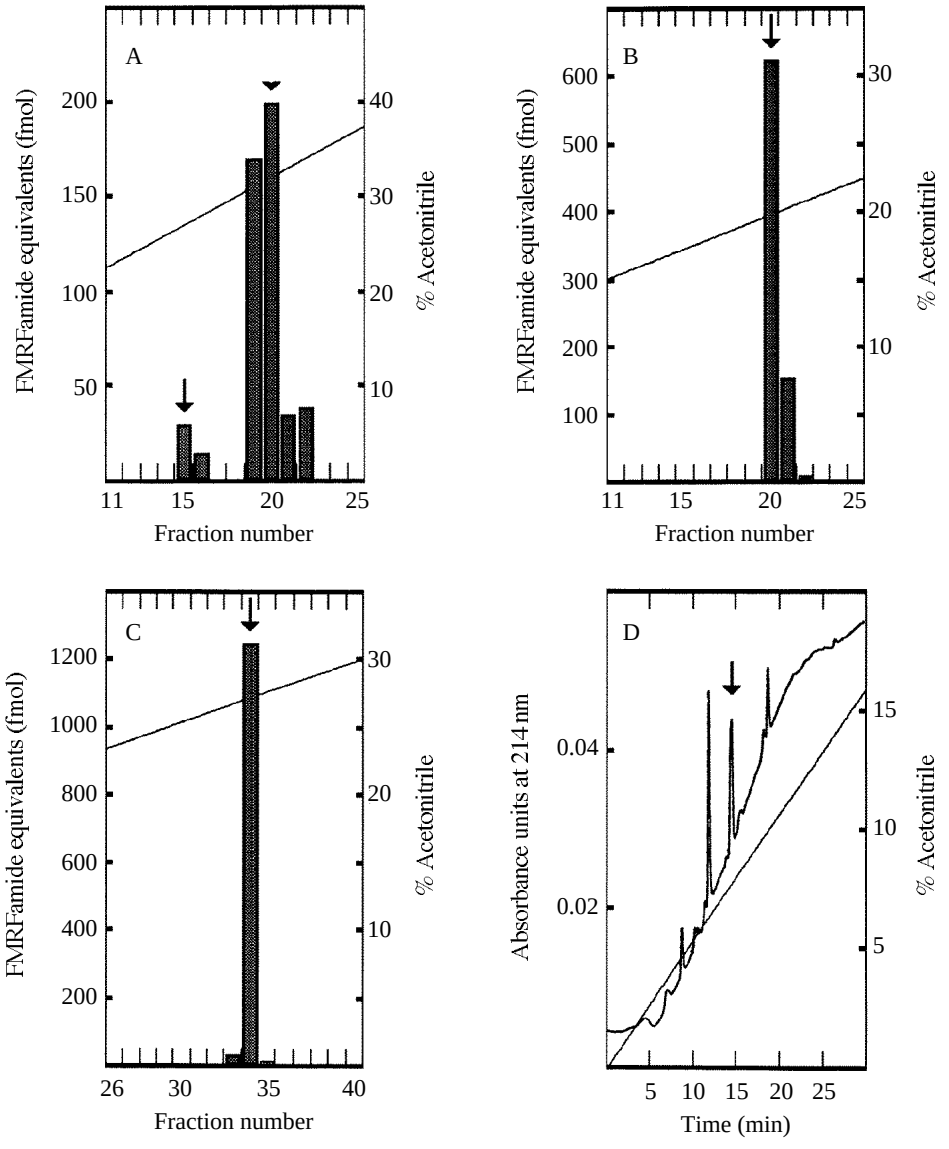

Fig. 5. Chromatograms of four steps in the purification process which led to the successful isolation and sequencing of FMRFamide. (A–C) In steps 1–3, peptides were detected by RFamide RIA and the amount of RFamide immunoreactivity in a 5ml sample. FMRFamide equivalents (left-hand vertical axis) are plotted for a range of fractions, where identified leech RFamide peptides are known to migrate (Evans *et al.* 1991). The arrowhead (in A) indicates the fraction in which the FMRFamide standard migrates and the arrows (in A–C) indicate the fraction in which oxidized FMRFamide standard migrates. (D) In step 4, peptides were detected optically. Absorbance at 214nm is plotted for the first 30min of the purification gradient (left-hand vertical axis). Fractions corresponding to each absorbance peak were collected. Only the fraction indicated by the arrow contained RFamide immunoreactivity, as determined by RFamide RIA on a 2ml sample. It was sequenced and yielded FMRF. In each chromatogram the acetonitrile/water gradient (right-hand vertical axis) is indicated by the diagonal line.

hexammine, HRP or Lucifer Yellow, no cell bodies were ever detected in the CNS or in the periphery, even though processes of the NNC were filled in adjacent segments (Wenning and Cahill, 1986, and unpublished observations). Five FMRFamide-related peptides present in similar amounts have been isolated from leech CNS (Evans *et al.* 1991). The fact that we detected only FMRFamide in the NNC does not exclude the possibility that related peptides are present at lower concentrations.

Examples of dual action (sensory and neurosecretory) by a single neurone, as described here, have recently been described in both vertebrates and invertebrates. In the carotid body of mammals, the arterial chemoreceptors release transmitters (González *et al.* 1992). The traffic between the transduction site (for P_{O_2}) and the release site (of catecholamines) is thought to be mediated by the inhibition of a K^+ current and subsequent activation of a Ca^{2+} current that permits transmitter release (González *et al.* 1992). In the oval organ of the second maxilla in decapod crustaceans, neurosecretory vesicles are present in the transducing terminals of sensory neurones and Ca^{2+}-dependent release of peptides from these terminals has been demonstrated (Pasztor and Bush, 1989; Pasztor, 1989). In both the NNC and the sensory cells of the oval organ, the effect of peptide release is unclear. Neurohumoral release can affect the surrounding tissue or provide feedback to the transduction sites and thereby alter their sensitivity (Pasztor and Bush, 1989). In the leech, the NNC supplies the transporting epithelial cells in the nephridium, the muscles of the bladder sphincter and other neurones in the CNS. All these arborizations show FLI, indicating that the NNC may have three separate targets for peptide release.The contact between the NNC and the urine-forming cells in the nephridium is particularly interesting, since reports on similar innervation patterns in transporting epithelia are sparse. Known examples are the supraorbital salt-secreting glands of marine birds (Kühnel, 1972) and the rectal epithelium of centipedes (Wenning, 1979). In those excretory organs where fluid formation is active, ion secretion rates depend on the extracellular fluid volume of the organ and/or on blood-borne factors (Malpighian tubules in insects, reviewed by Spring, 1990; salt glands in marine birds, Gerstberger, 1991; skin, colon and rectum of amphibians, reviewed by Clauss, 1989). Neural modulation has been demonstrated in salt glands (see Gerstberger, 1991). Both steps of urine formation in the nephridium of jawed leeches involve active transport (Zerbst-Boroffka, 1975) and the urine-forming cells are innervated by the neurosecretory NNC. Since leeches lack a centrally organized hormonal system or neurohaemal organs, such direct innervation is not surprising. It has been described for other transporting tissues (salivary glands, Marshall and Lent, 1988; Walz *et al.* 1988; Wuttke *et al.* 1989; and crop, Hogg *et al.* 1983; Leake *et al.* 1986).

Since FLI is associated with NNC terminals (Fig. 3D), it is probable that FMRFamide is released, but its effects and the underlying mechanisms are unknown. There are no reports of involvement of RFamide-like peptides in the modulation of active transport. FLI is, however, associated with the excretory system of a freshwater snail (*Helisoma duryi*). Neurosecretory cells expressing FLI contact muscles associated with the kidneys which might affect filtration pressure. An effective filtration pressure, however, could not be demonstrated (Saleuddin *et al.* 1992). Several peptides involved in salt and water regulation in vertebrates were investigated in rhynchobdellid leeches. One of these,

angiotensin, provokes water loss (20% above control) in *Theromyzon tessulatum* after the third blood meal (Salzet *et al.* 1992). Oxytocin, fragments of oxytocin and extracts of the supraoesophageal ganglion of *Erpobdella octoculata* have an antidiuretic effect when injected into *Theromyzon tessulatum* (Salzet *et al.* 1993). However, general conclusions about the factors involved are premature, since the mechanism of urine formation in the nephridia of these leeches are unknown and nephridial performance differs greatly even among jawed leeches (Wenning, 1987).

The identification of FMRFamide as the major peptide of the NNC permits us to test the effects of neurosecretion in the nephridium. We measured urine output in isolated nephridial complexes superfused with different concentrations of FMRFamide (Wenning *et al.* 1991), but the results of these experiments were ambiguous. There are several factors that could account for this. First, the primary function of FMRFamide may be to modulate the Cl^- sensitivity of the NNC. Pasztor and Bush (1989) showed that endogenous proctolin modulates the sensitivity of the stretch receptor neurones in the oval organ of crustaceans. Given the extent of peripheral arborization of the NNC and the close association of its varicose endings with the urine-secreting tissue, it is unlikely that sensitivity modulation is the only effect of FMRFamide. Second, although isolated preparations produce urine at what appears to be a normal basal rate (Zerbst-Boroffka and Wenning, 1986), they are deprived of their normal blood circulation and may not respond normally to superfused neuropeptides. Endogenous peptidases and physical barriers (i.e. profuse basal infolding) may prevent superfused peptides from reaching their receptors. Third, isolated nephridia may already be modulated to their maximal extent by endogenous transmitter release, as a result of dissection. Finally, the NNC's neurosecretion may be directed towards salt rather than volume output, which are regulated independently in leeches (Zerbst-Boroffka *et al.* 1982). The NNC is thought to act as an ion receptor through its sensitivity to the blood Cl^- level, an important factor for ion homeostasis in an animal with a low blood Cl^- concentration and a high-Cl^- diet (Wenning, 1989; Wenning and Calabrese, 1991). Simultaneous measurement of volume and salt output should elucidate the physiological role of FMRFamide in the leech nephridium.

Dr R. Elde generously donated the antibody against FMRFamide used in the immunocytochemistry and ELISAs. We thank Ms U. Greisinger and S. M. Daniel for excellent technical assistance, Dr J. Pohl for sequencing the peptide and Dr U. Homberg for patient advice on immunocytochemical techniques. Supported by the DFG (We 745/4-1 to A.W.; SFB 156 to M.A.C.) and NIH (NS24072 to R.L.C.).

References

BENDAYAN, M. (1984). Protein A-gold electron microscopic immunocytochemistry: methods, applications, limitations. *J. Electr. Micr. Tech.* **1**, 243–270.

BOROFFKA, I., ALTNER, H. AND HAUPT, J. (1970). Funktion und Ultrastruktur des Nephridiums von *Hirudo medicinalis*. I. Ort und Mechanismus der Primärharnbildung. *Z. vergl. Physiol.* **66**, 421–438.

CHILDS, G. V., UNABIA, G. AND ELLISON, D.(1986). Immunocytochemical studies of pituitary hormones

with PAP, ABC and immunogold techniques: evolution of technology to best fit the antigen. *Am. J. Anat.* **175**, 307–330.

CLAUSS, W. (1989). Hormonal regulation of ion transport. *Verh. dt. zool. Ges.* **82**, 27–44.

DIRCKSEN, H., MÜLLER, A. AND KELLER, R. (1991). Crustacean cardioactive peptide in the nervous system of the locust, *Locusta migratoria*: an immunocytochemical study on the ventral nerve cord and peripheral innervation. *Cell Tissue Res.* **263**, 439–457.

DIRCKSEN, H., ZAHNOW, C. A., GAUS, G., KELLER, R., RAO, K. R. AND RIEHM, J. P. (1987). The ultrastructure of nerve endings containing pigment dispersing hormone (PDH) in crustacean sinus glands: identification by an antiserum against synthetic PDH. *Cell Tissue Res.* **250**, 377–387.

EVANS, B. D., POHL, J., KARTSONIS, N. A. AND CALABRESE, R. L. (1991). Identification of RFamide neuropeptides in the medicinal leech. *Peptides* **12**, 897–908.

FERNANDEZ, J. AND STENT, G. S. (1982). Embryonic development of the hirudinid leech *Hirudo medicinalis*: structure, development and segmentation of the germinal plate. *J. Embryol. exp. Morph.* **72**, 71–96.

GERSTBERGER, R. (1991). Partial uncoupling of salt gland blood flow and secretion in the Pekin duck (*Anas platyrhynchos*). *J. Physiol., Lond.* **435**, 175–186.

GONZÁLEZ, C., ALMARAZ, L., OBESCO, A. AND RIGUAL, R. (1992). Oxygen and acid chemoreception in the carotid body chemoreceptors. *Trends Neurosci.* **15**, 146–153.

HAUPT, J.(1974). Function and ultrastructure of the nephridium of *Hirudo medicinalis.* II. Fine structure of the central canal and the urinary bladder. *Cell Tissue Res.* **152**, 385–410.

HOGG, N., FLASTER, M. AND ZIPSER, B. (1983). Cross-reactivities of monoclonal antibodies between selected leech neuronal and epithelial tissues. *J. Neurosci. Res.* **9**, 445–457.

KINGAN, T. G. (1989). A competitive enzyme-linked immunosorbent assay: applications in the assay of peptides, steroids and cyclic nucleotides. *Analyt.Biochem*. **183**, 283–289.

KUHLMANN, J. R., LI, C. AND CALABRESE, R. L. (1985). FMRFamide-like substances in the leech. I. Immuncytochemical localization. *J. Neurosci.* **5**, 2301–2309.

KÜHNEL, W. (1972). On the innervation of the salt gland. *Z. Zellforsch. mikrosk. Anat.* **134**, 455–438.

LEAKE, L. D., CROWE, R. AND BURNSTOCK, G. (1986). Localisation of substance P-, somatostatin-, vasoactive intestinal polypeptide- and met-enkephalin-immunoreactive nerves in the peripheral and central nervous system of the leech (*Hirudo medicinalis*). *Cell Tissue Res.* **243**, 345–351.

MARDER, E., CALABRESE, R. L., NUSBAUM, M. P. AND TRIMMER, B. (1987). Distribution and partial characterization of FMRFamide-like peptides in the stomatogastric nervous system of the rock crab, *Panulirus interruptus*. *J. comp. Neurol.* **259**, 150–163.

MARSHALL, C. G. AND LENT, C. M.(1988). Excitability and secretory activity in the salivary gland cells of jawed leeches (Hirudinea: Gnathobdellida). *J. exp. Biol.* **137**, 89–105.

MULLER, K. J., NICHOLLS, J. G. AND STENT, G. S. (1981). (eds) *Neurobiology of the Leech.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

PASZTOR, V. M. (1989). Modulation of sensitivity in invertebrate sensory receptors. *Sem. Neurosci.* **1**, 5–14.

PASZTOR, V. M. AND BUSH, B. M. (1989). Primary afferent responses of a crustacean mechanoreceptor are modulated by proctolin, octopamine and serotonin. *J. Neurobiol.* **20**, 234–254.

PRICE, D. A. AND GREENBERG, M. J. (1977). Structure of a molluscan cardioexcitatory peptide. *Science* **197**, 670–671.

SALEUDDIN, A. S. M., KHAN, H. R., ASHTON, M.-L. AND GRIFFOND, B. (1992). Immunocytochemical localization of FMRFamide in the central nervous system and the kidney of *Helisoma duryi* (Mollusca): its possible antidiuretic role. *Tissue & Cell* **24**, 179–189.

SALZET, M., VERGER-BOCQUET, M., WATTEZ, C. AND MALECHA, J. (1992). Evidence for angiotensin-like molecules in the central nervous system of the leech *Theromyzon tessulatum* (O.F.M.). A possible diuretic effect. *Comp. Biochem. Physiol.* **101**A, 83–90.

SALZET, M., WATTEZ, C., VERGER-BOCQUET, M., BEAUVILLAIN, J. C. AND MALECHA, J.(1993). Oxytocin-like peptide: a novel epitope colocalized with the FMRFamide-like peptide in the supernumerary neurons of the sex segmental ganglia of leeches – morphological and biochemical characterization; putative antidiuretic function. *Brain Res.* **601**, 173–184.

SPRING, J. H.(1990). Endocrine regulation of diuresis in insects. *J. Insect Physiol.* **36**, 13–22.

STERNBERGER, L. A.(1979). *Immunocytochemistry* (2nd edition). New York: Wiley.

TAGER, H. S. (1976). Coupling of peptides to albumin with difluor-dinitrobenzene. *Analyt. Biochem.* **71**, 367–375.

WALZ, B., SCHÄFFNER, K.-H. AND SAWYER, R. T. (1988). Ultrastructure of the anterior salivary gland cells of the giant leech, *Haementeria ghilianii* (Annelida, Hirudinea). *J. Morph.* **196**, 321–332.

WENNING, A. (1979). Structure and function of the hindgut of *Lithobius forficatus* (Myriapoda, Chilopoda). In *Myriapod Biology* (ed. M. Camatini), pp. 135–142. London, New York: Academic Press.

WENNING, A. (1983). A sensory neuron associated with the nephridia of the leech *Hirudo medicinalis* L. *J. comp. Physiol.* **152**, 455–458.

WENNING, A.(1987). Salt and water regulation in *Macrobdella decora* (Hirudinea: Gnathodbelliformes) under osmotic stress. *J. exp. Biol.* **131**, 337–349.

WENNING, A. (1989). Properties of a set of internal receptors in the medicinal leech, the nephridial nerve cells monitor extracellular chloride concentration. *J. exp. Biol.* **143**, 115–132.

WENNING, A. AND CAHILL, M. A.(1986). Nephridial innervation in the leech, *Hirudo medicinalis* L. *Cell Tissue Res.* **245**, 397–404.

WENNING, A. AND CAHILL, M. A. (1989). Structural and ultrastructural features of the inlet and outlet regions of the urinary bladders of the leech, *Hirudo medicinalis*. *J. Morph.* **201**, 285–291.

WENNING, A., CAHILL, M.A., GREISINGER, U. AND KALTENHÄUSER, U. (1993). Organogenesis in the leech: development of nephridia, bladders and their innervation. *WilhelmRoux Arch. EntwMech. Org.* (in press).

WENNING, A. AND CALABRESE, R. L. (1991). Mechanism of Cl^- sensitivity in internal ion receptors of the leech: an inward current gated off by Cl^- in the nephridial nerve cells. *J. comp. Physiol.* A **168**, 53–61.

WENNING, A., KALTENHÄUSER, U., GREISINGER, U. AND CAHILL, M. A. (1991). FMRF-NH_2 als diuretischer Faktor im Egel, *Hirudo medicinalis* L.: Immunhistochemie und Physiologie. *Verh. dt. zool. Ges.* **84**, 434 (abstract).

WENNING, A., KALTENHÄUSER, U.,GREISINGER, U. AND HOEGER, U. (1992). Afferent and efferent innervation of leech nephridia occurs by a single identified neuron expressing FMRF-NH_2-like immunoreactivity. *Soc. Neurosci. Abstr.* **18**, 267.

WUTTKE, W., SAWYER, R. T. AND BERRY, M. S. (1989). Extraglandular innervation of the salivary cells of the leech *Haementeria ghilianii*: neuronal stimulation elicits gland cell action potentials. *J. exp. Biol.* **143**, 389–410.

ZERBST-BOROFFKA, I.(1975). Function and ultrastructure of the nephridium in *Hirudo medicinalis* L. III. Mechanisms of the formation of primary and final urine. *J. comp. Physiol.* **100**, 307–315.

ZERBST-BOROFFKA, I. AND WENNING, A. (1986). Mechanisms of regulatory salt and water excretion in the leech, *Hirudo medicinalis* L. *Zool. Beitr.* **30**, 359–377.

ZERBST-BOROFFKA, I., WENNING, A. AND BAZIN, B. (1982). Primary urine formation during diuresis in the leech, *Hirudo medicinalis*. *J. comp. Physiol.* **146**, 75–79.