Cell Tissue Res (1993) 274:605–618

Cell & Tissue Research

Immunocytochemical mapping of the novel echinoderm neuropeptide SALMFamide 1 (S1) in the starfish *Asterias rubens*

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Received: 17 December 1992 / Accepted: 30 April 1993

Abstract. The recent isolation and characterization of the SALMFamide neuropeptides S1 and S2 from the starfish *Asterias rubens* has initiated a series of studies on their distribution. Specific antisera have been raised against S1 and used in light-microscopical immunocytochemistry. The results of this study reveal for the first time a possible hyponeural innervation of the visceral musculature of the gut and the widespread neuronal distribution of S1, (i) in axons and cell bodies of both ectoneural and hyponeural regions of the radial nerve cord and circumoral nerve ring, (ii) in the nerve ring and nerve plexus of the tube feet, (iii) in the apical muscle, (iv) in skin, and (v) extensively throughout the digestive system. These discoveries are of particular interest in terms of the possible functional roles for S1 in *Asterias rubens*.

Key words: SALMFamide – Neuropeptide – Immunocytochemistry – Mapping – *Asterias rubens* (Echinodermata)

Introduction

A considerable amount of information is currently available describing the presence of conventional neurotransmitters in echinoderms. Unger (1962) revealed the presence of acetylcholine (ACh) chromatographically in the radial nerve cord (RNC) of *Asterias glacialis*, whereas Pentreath and Cottrell (1968) showed that extracts of RNC from *A. rubens* contained large quantities of the same transmitter. The catecholamines dopamine and noradrenaline have been found in the ectoneural nerve plexus (Cottrell and Pentreath 1970; Huet and Fraquinet 1981) and are believed to be involved in tube foot movement and regeneration. Serotonin is, apparently, absent from the adult nervous system although there is a single histochemical report of 5-HT in the tube feet of a holothurian and a starfish (Dolder 1975). Serotonin is, however, an important component of the nervous system in larval echinoderms (Bisgrove and Burke 1986; Nakajima 1988). The RNC has also been found to contain significant amounts of the transmitter gamma aminobutyric acid (GABA) (Osborne 1971; Florey et al. 1975; Gyhoot and Cobb, personal communication).

Neuropeptide-like molecules have been reported from a number of echinoderm species including the mammalian neuropeptides vasoactive intestinal polypeptide, neuropeptide Y and neurotensin in crinoids (Welsch et al. 1989); cholecystokinin in the holothurian gut (García-Arrarás et al. 1991a); pancreatic polypeptide (PP), peptide YY (PYY), melanocyte-stimulating hormone (MSH) and somatostatin in the gut of the starfish *Marthasterias glacialis* (Martínez et al. 1991); an insulin-like substance from *Pisaster ochraceus* (Wilson and Falkmer 1965); and the molluscan cardioacceleratory peptide FMRFamide in *Asterias rubens* (Elphick et al. 1989) and *Holothuria glaberrima* (García-Arrarás et al. 1991b). In each case, the experiments were necessarily based on the use of heterologous antisera and therefore suffered from the usual problems associated with this method (Thorndyke 1986).

Until now, only two native echinoderm neuropeptides have attracted attention. One of these, an autotomy promoting factor, has been extracted from the coelomic fluid of sea stars but not sequenced (Mladenov et al. 1989). The other, gonad-stimulating substance (Shirai et al.1987) has been localized to a population of cells lining the perihaemal epithelium in *Pycnopodia* (Caine and Burke 1985) and only partially sequenced (Shirai et al. 1987). The SALMFamides are the first neuropeptides to be fully characterized and sequenced from any echinoderm. These peptides have been isolated from the RNC of *A. rubens* and *A. forbesii*; S1 is an octapeptide with the amino acid sequence GFNSALMFamide and S2 is a dodecapeptide with the sequence SGPYSFNSGLTFamide (Elphick et al. 1991a,b). They share approximately 67% sequence homology.

Part of this work has been previously reported as an abstract: (Moore et al.1990, 1991)

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To analyse the occurrence and distribution of S1, we have raised a specific antiserum to the C-terminal analogue of the peptide KYSALMFamide (Elphick et al. 1991b). The present paper reports the use of this antiserum to study the tissue distribution of S1 in the starfish *A. rubens* employing optical immunocytochemistry (ICC).

Materials and methods

Antisera production and characterization

Polyclonal antisera were raised in half lop-eared rabbits to the C-terminal pentapeptide analogue of S1, KYSALMFamide. This analogue was conjugated through the lysine residue to thyroglobulin using glutaraldehyde (Elphick et al. 1991b). The bleed following the fourth boost (BLIV) was characterized in a radioimmunoassay (RIA) by the two native peptides S1 and S2, the immunogen KYSALMFamide, as well as FMRFamide, FLRFamide and LPLRFamide. The RIA followed a protocol originally developed in this laboratory and is discussed in detail elsewhere (Elphick et al. 1991b).

Pre-absorption controls for immunocytochemistry were carried out using the native peptides and thyroglobulin, FMRFamide, FLRFamide and LPLRFamide at concentrations of 10 nmoles ml^{-1} of optimally diluted primary antiserum.

Immunocytochemistry

The presence of S1 in *A. rubens* was investigated by light-microscopical immunocytochemistry using the PAP method (Sternberger 1979). Tissues were dissected from the animal and fixed immediately in sea-water Bouin's fluid with 5% acetic acid for 1 h at 4°C and then transferred to the same fixative without acetic acid for a further 2–4 h. The tissue was subsequently dehydrated and embedded in paraffin wax. Sections (8 μm thick) were mounted on poly-L-lysine coated slides and immunostained with BLIV primary antiserum at a dilution of 1:500–1000, overnight at room temperature. Following washes in phosphate-buffered saline (PBS) the sections were incubated for 1 h in peroxidase-conjugated swine anti-rabbit serum (P217: DAKO, High Wycombe, Bucks.) and then in peroxidase anti-peroxidase (Z113: DAKO). The immunostaining was visualized with 50 mg diaminobenzidine and 100 μml hydrogen peroxide in 70 ml PBS, pH 7.4; development time was 3–5 min. Sections of whole arm and circumoral nerve ring (CONR) required decalcification; several methods were employed but any involving the use of EDTA significantly reduced the level of immunoreactivity. It was found that tissue left in Bouin's fluid without acetic acid for 8–10 days became sufficiently decalcified to allow sectioning with good preservation of antigenicity.

Whole-mounts

Pieces of cardiac stomach, pyloric stomach and oesophagus were removed and pinned out in Sylgard-coated dishes as whole-mounts. The tissues were fixed in sea-water Bouin's fluid without acetic acid for 2 h. The tissue permeability was improved by rapid dehydration in methanol followed by rehydration and a 1-h pre-incubation step in PBS with 0.1% Triton X-100 (T-6878: Sigma, Poole, Dorset). The tissue was subsequently incubated in primary antibody (1:200 BLIV) overnight at room temperature. S1 immunoreactivity was visualized with the fluorophore FITC (fluorescein isothiocyanate, Nordic 3752: Nordic, Slough, Berks.) diluted 1:70 for 1–2 h at room temperature. After washing, the tissue was mounted on glass slides with aqueous mountant containing 0.15% N-propyl gallate (Sigma P-3130) and viewed under a Zeiss Axioplan microscope.

Results

Antibody characterization

RIA characterization revealed BLIV to be slightly more cross-reactive than previous bleeds (Elphick et al. 1991b) with a 400-fold difference in affinity between the two native peptides (Fig. 1). However, pre-absorption tests on tissue sections confirmed that the antiserum was totally specific under ICC conditions. The slight discrepancy between RIA and ICC may reflect the different ways in which the antigen is presented to the antibody.

Immunocytochemistry

Circumoral nerve ring (CONR) and radial nerve cord (RNC) The CONR is situated in the disc region surrounding the mouth. It lies just beneath and is attached to the peristomal epidermis, which it innervates. Structurally, the CONR is identical, although flatter, than the five RNCs with which it connects (Fig. 2A,B; 3A,B). Both the CONR and RNC comprise two distinct regions, the ectoneural and hyponeural regions, separated by a continuous band of connective tissue of varying thickness (Cobb 1970). The ectoneural nerve plexus is believed to be ectodermal in origin and both sensory and motor in function, whereas the hyponeural plexus (Lange's nerve) is thought to be of mesodermal origin and purely motor in function (Smith 1965; von Hehn 1970; Cobb 1987).

S1 immunoreactivity is present in the ectoneural and hyponeural nerve plexuses of the CONR associated with axon profiles and bipolar epithelial cell bodies (Fig. 3A,B). Ganglion-like structures are believed to be present at the point where the RNCs join the ring (Smith 1965). However, no change in the staining pattern in that area has been observed with this S1 antibody to suggest a ganglionic-like concentration of cell bodies.

The RNC, situated between the ambulacral ossicles, is surrounded on each side by two rows of tube feet (Fig. 4). Proximally, it is connected to the CONR whereas distally, it terminates as the optic cushion, the only organized

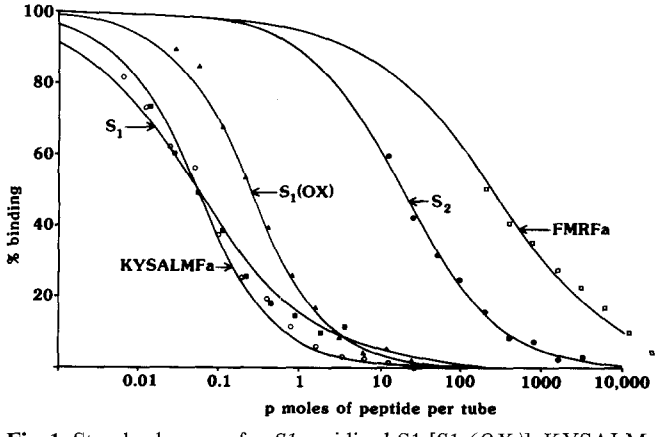
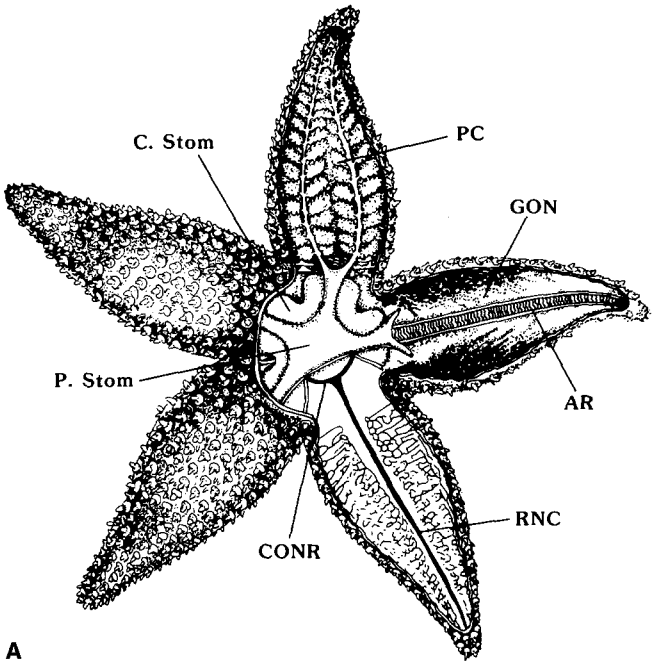


Fig. 1. Standard curves for *S1*, oxidised S1 [*S1 (OX)*], KYSALMFamide (*KYSALMFa*), S2 and FMRFamide (*FMRFa*) with BLIV antiserum



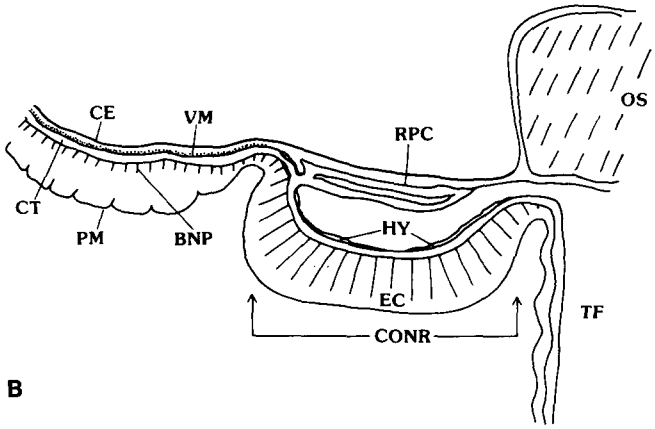
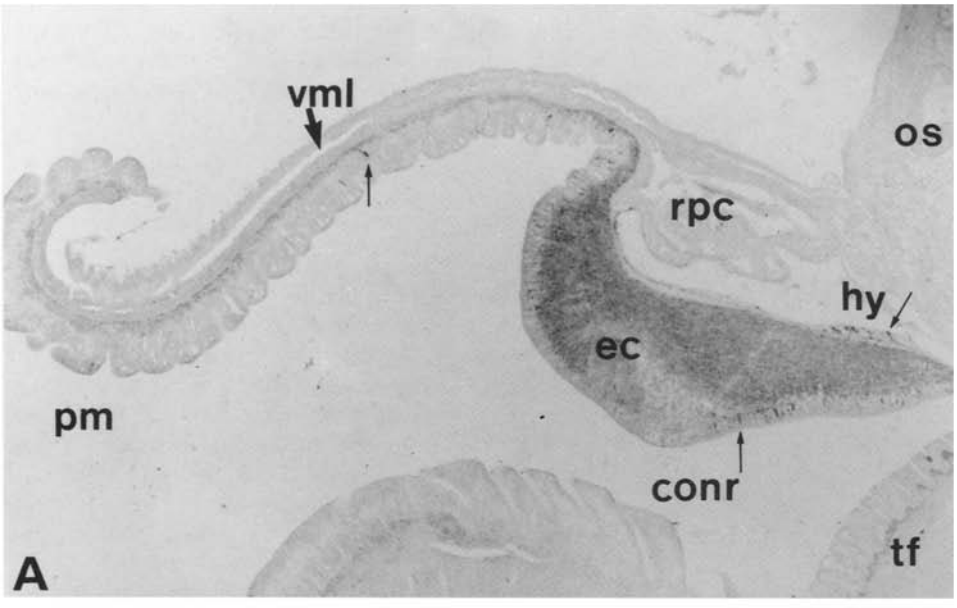


Fig. 2A,B. Schematic drawings of *Asterias rubens*. **A**. Position of the radial nerve cord (*RNC*); circumoral nerve ring (*CONR*); gonads (*GON*); ambulacral ridge (*AR*); digestive system: pyloric stomach (*P. Stom*); cardiac stomach (*C. Stom*); pyloric caeca (*PC*). **B** Diagrammatic representation of the *CONR* and peristomal membrane (*PM*). Connective tissue (*CT*); coelomic epithelium (*CE*); visceral muscle (*VM*); radial perihaemal canal (*RPC*); ossicle (*OS*); tube foot (*TF*); ectoneural nerve plexus (*EC*); hyponeural nerve plexus (*HY*); basiepithelial nerve plexus (*BNP*)



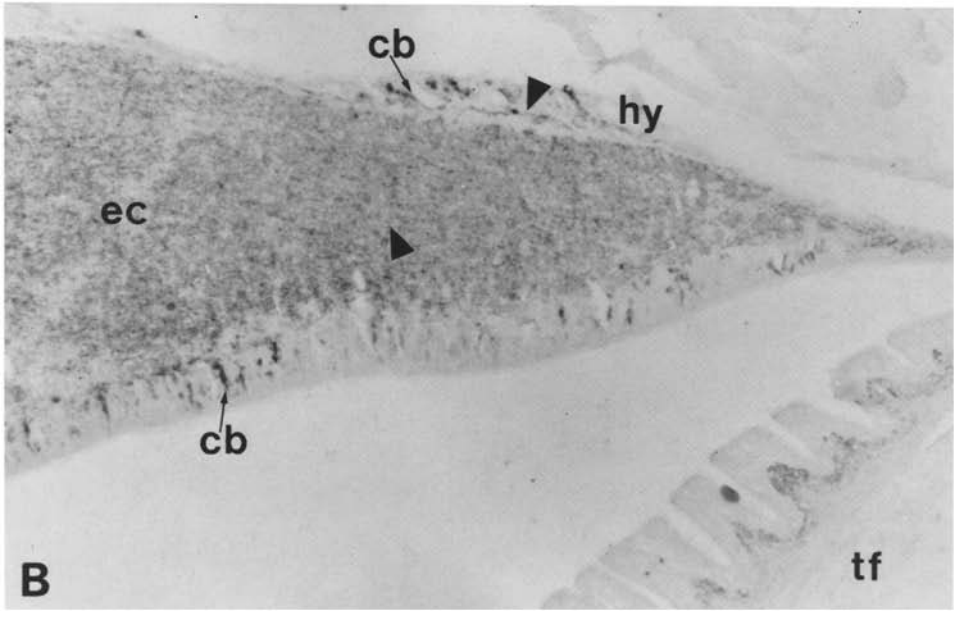


Fig. 3A,B. Circumoral nerve ring. **A** S1 immunoreactivity associated with ectoneural and hyponeural axons and cell bodies *(arrows)* in the CONR (*conr*) and peristomal membrane (*pm*). ×55. **B** Detail of immunoreactive hyponeural and ectoneural cell bodies and fibres (*arrowheads*) ×188. The level of immunostaining was reduced because of the decalcification procedure. Visceral muscle layers (*vml*); radial perihaemal canal (*rpc*); ectoneural nerve plexus (*ec*); ossicle (*os*); hyponeural nerve plexus (*hy*); tube foot (*tf*); cell body (*cb*)

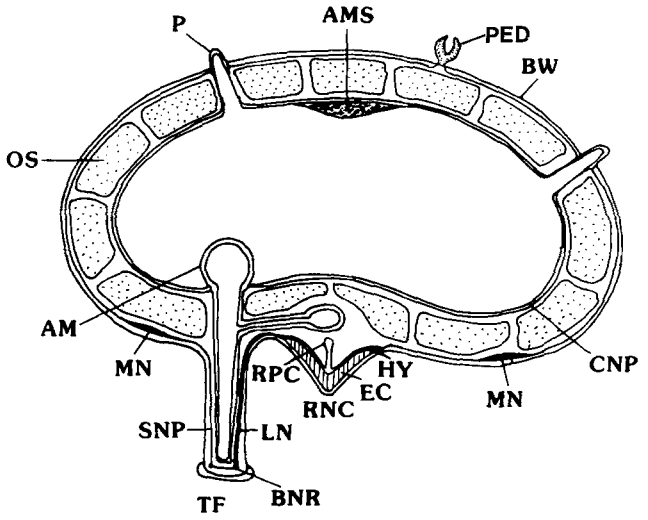


Fig. 4. Diagrammatic representation of a transverse section taken through the arm of *Asterias* showing the position of the radial nerve cord (*RNC*), tube feet (*TF*) and apical muscle (*AMS*); pedicellariae (*PED*); body wall (*BW*); coelomic nerve plexus (*CNP*); marginal nerve (*MN*); hyponeural nerve plexus (*HY*); ectoneural nerve plexus (*EC*); radial perihaemal canal (*RPC*); longitudinal nerve (*LN*); basal nerve ring (*BNR*); sub-epithelial nerve plexus (*SNP*); ampulla (*AM*); ossicle (*OS*); papulae (*P*)

sense organ (Smith 1937). The nerve cord is bounded orally by a layer of cuticle that separates it from the external environment, and aborally by the coelomic epithelium and perihaemal canal (Fig. 4).

ICC revealed the presence of S1 in both ectoneural and hyponeural elements of the nerve cord; it was associated with axons and epithelial cell bodies (Fig. 5A,B). The majority of the ectoneural immunoreactive cell bodies were bipolar, with fibres extending into the cuticle layer in one direction and ramifying through the neuropile in the other (Fig. 5C). The nerve fibres were particularly concentrated in the lateral borders of the cord mainly in a longitudinal orientation. Occasionally, however, laterally oriented axons were also observed, especially at the edges of the nerve cord supplying the innervation to the tube feet (Fig. 6A) and the marginal nerves (Fig. 6B).

S1 immunoreactivity associated with the hyponeural cell bodies revealed an interesting pattern of staining, with the cell bodies grouped at regular intervals in a ganglionated fashion, on alternate sides of the nerve cord (Figs. 5A, 6C). The majority of the nerve fibres in the hyponeural region emerged from monopolar cell bodies, (Fig. 5B) and extended laterally, possibly innervating the inter-ambulacral muscles (Fig. 6E).

S1 immunoreactivity in the optic cushion is confined to the ectoneural nerve plexus (Fig. 6F) and is associated with bipolar cell bodies and axons (Fig. 6D). The hyponeural nerve plexus is restricted to the nerve cord proper.

Tube feet and marginal nerves

The tube feet are situated in the ambulacral groove and are an integral part of the water vascular system, being functionally involved in a variety of behaviours including locomotion, feeding and monitoring environmental change. They are connected to the RNC by the outer cuticle layer and the underlying sub-epithelial ectoneural nerve plexus, which is continuous with the ectoneural nerve plexus of the radial nerve. The nerve plexus is situated amongst epithelial cells and supporting fibres, and is separated from the longitudinally oriented retractor muscle fibres by a region of connective tissue. Ciliated coelomic epithelial cells line the tube foot lumen (Fig. 4).

S1 immunoreactivity was present extensively in axons of the sub-epithelial nerve plexus and basal nerve ring (Fig. 7A) and was associated with the single longitudinal nerve that comprised a local thickening of the sub-epithelial plexus (Fig. 7A,B). Epithelial cell bodies were evident in the proximal region of the tube foot near to its connections with the RNC (Fig. 6E). The majority of the innervation was supplied from the ectoneural region of the RNC, with the marginal nerves believed to innervate the outer row of tube feet (Fig. 6B; Cuénot 1948).

The marginal nerves are continuous with the extensive skin plexus (Figs. 4, 7D) which supplies part of the papulae innervation (Fig. 7C), whilst providing the only innervation to the pedicellariae (Fig. 7D; Roberts and Campbell 1988).

Apical muscle

This structure comprises a muscular thickening located aborally on the inside of the dorsal body wall (Fig. 4). It is believed to be involved in arm flexion during locomotion and feeding (O'Neill 1989). At the point where the arm joins the disc, the apical muscle is straddled by a band of circular sub-epidermal muscle, viz. the aboral autotomy plane, a predetermined point at which the arm always breaks during autotomy (Wilkie et al. 1990).

The apical muscle is bounded orally by a single layer of ciliated coelomic epithelial cells and an overlying nerve plexus that appears to be continuous with the visceral innervation of the gut. Aboral to the nerve plexus is a region of longitudinal muscle separated from a deeper circular muscle layer by a band of connective tissue (Figs. 8; 9A).

S1 immunoreactivity is associated extensively with the coelomic nerve plexus supplied by bipolar epithelial cell bodies (Fig. 9B). Fibres from the nerve plexus ramify extensively throughout the overlying longitudinal muscle layer as far as the connective tissue boundary; no fibres have been found to cross this barrier (Fig. 9A). S1 immunoreactivity is also present in the deeper circular muscle layer (Fig. 9A). Some sections have revealed that S1 immunoreactive neurones from this circular muscle layer penetrate between the aboral body wall ossicles, with fibres occasionally being associated with the calcite tissue itself (Fig. 9C). The area between the ossicles comprises reticular muscle and supporting connective tissue that is believed to realign the ossicles (O'Neill 1989).

S1 immunostaining of the apical muscle has suggested for the first time that the coelomic nerve plexus is contin-

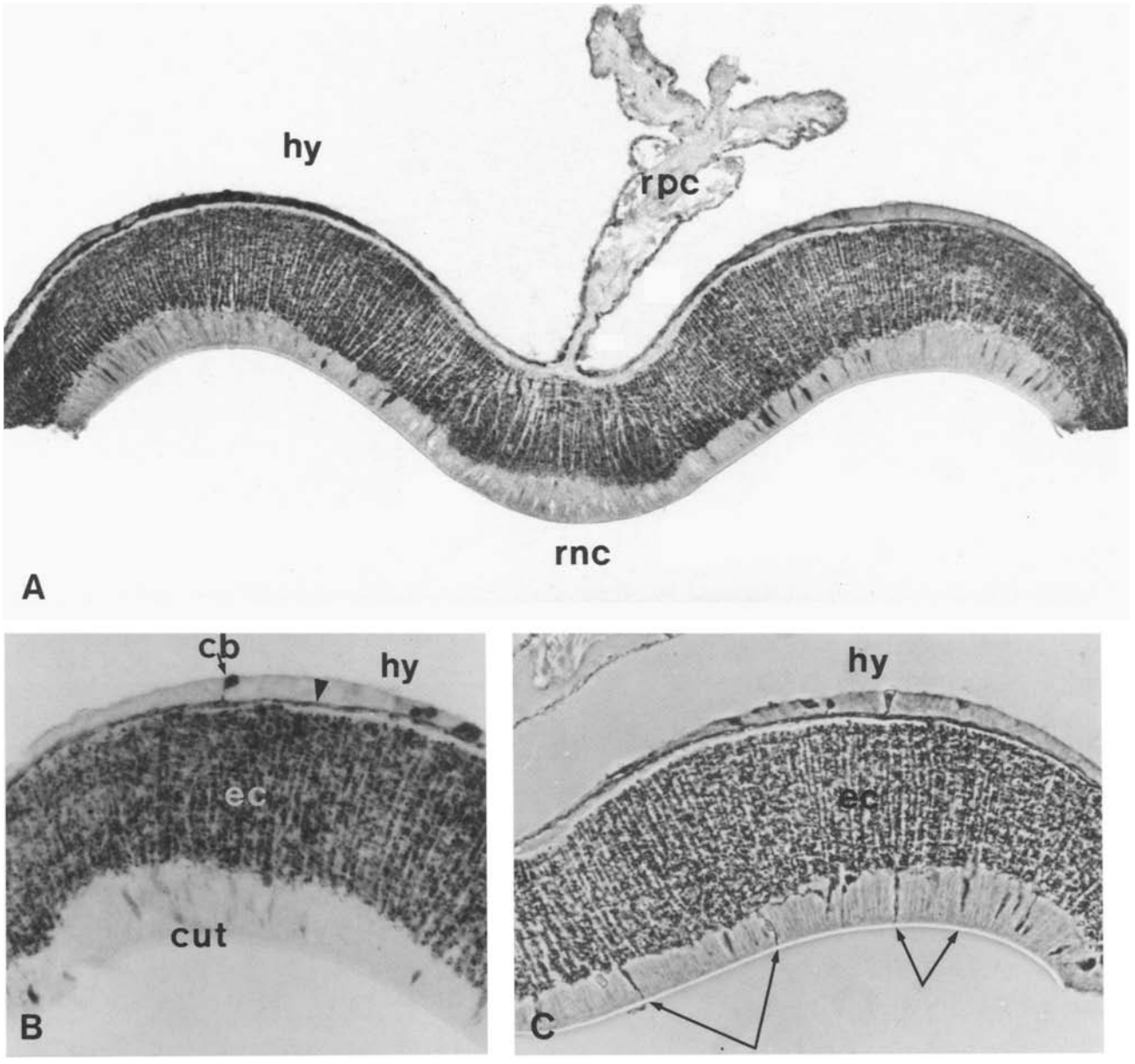


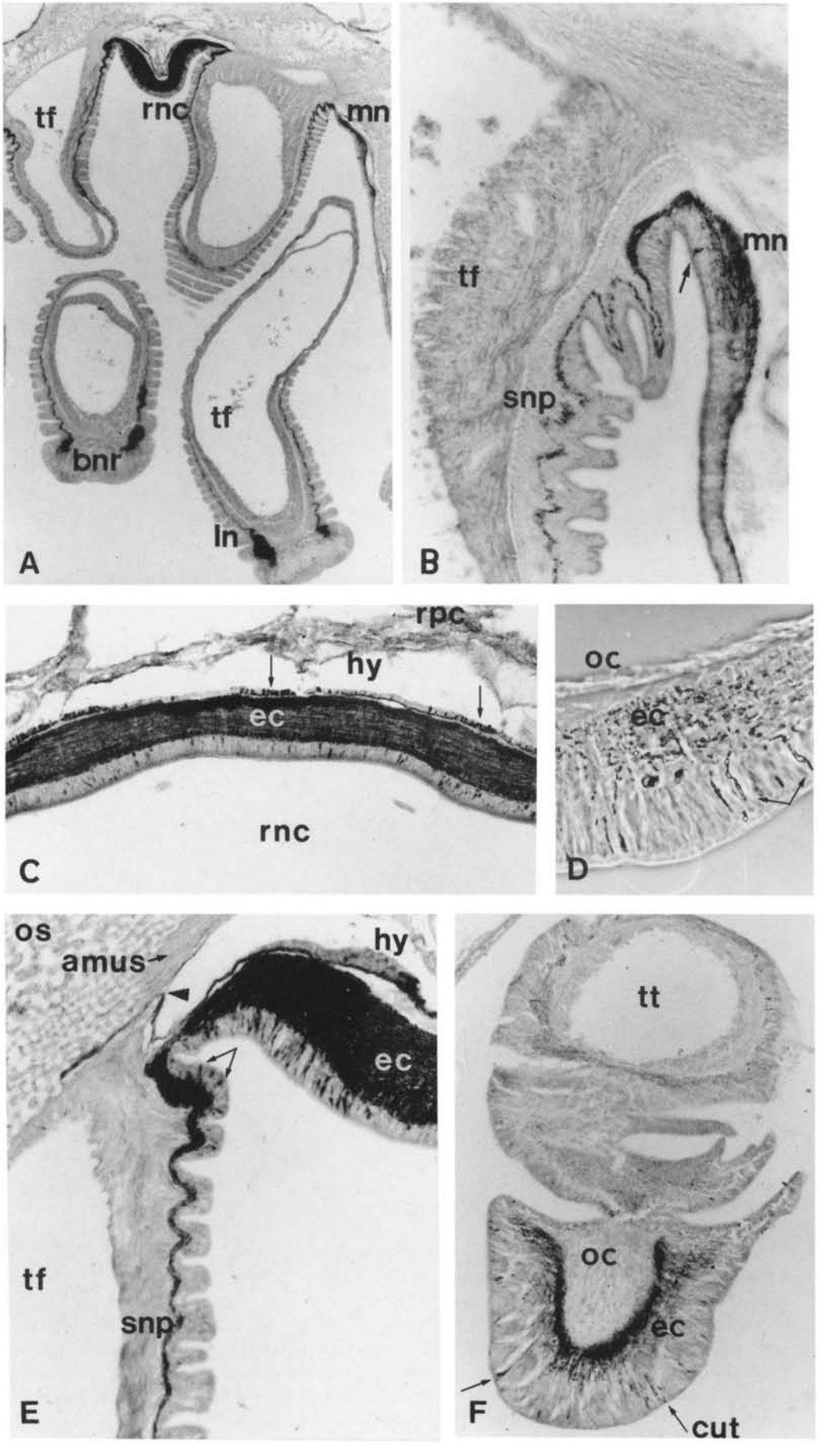
Fig. 5A–C. Radial nerve cord. **A** S1 associated with both ectoneural and hyponeural elements of the RNC (*rnc*) with "ganglionic-like" collections of S1 immunoreactive hyponeural cell bodies present (*arrow*). ×136. **B** An S1 immunoreactive monopolar hyponeural cell body (*arrow*) with fibres extending into the underlying plexus (*arrowhead*). ×300. **C** S1 immunoreactive bipolar ectoneural cell bodies (*arrows*) with fibres extending into the ectoneural neuropile. ×260. Radial perihaemal canal (*rpc*); hyponeural nerve plexus (*hy*); cuticle layer (*cut*)

uous with the innervation that supplies the visceral muscles of the pyloric caeca (Fig. 9D,E). This connection occurs via the mesenteries, which are present continuously along the inside of the arm suspending the caeca in the coelom (Fig. 9D,E).

On either side of the apical muscle, the circular and longitudinal muscle layers with their associated S1 immunoreactivity enter the papulae. The innervation of these structures has previously been described in detail by Cobb (1978).

Digestive system

There are at least four identifiable areas comprising the digestive system, each area having a similar cellular composition. The mouth of *A. rubens*, protected by the peristomal membrane, opens into a short oesophagus. Nutrients pass through this region into the stomach, which fills a large proportion of the disc area and which is divided by a horizontal constriction into a large oral chamber, the cardiac stomach, and a smaller aboral pyloric stom-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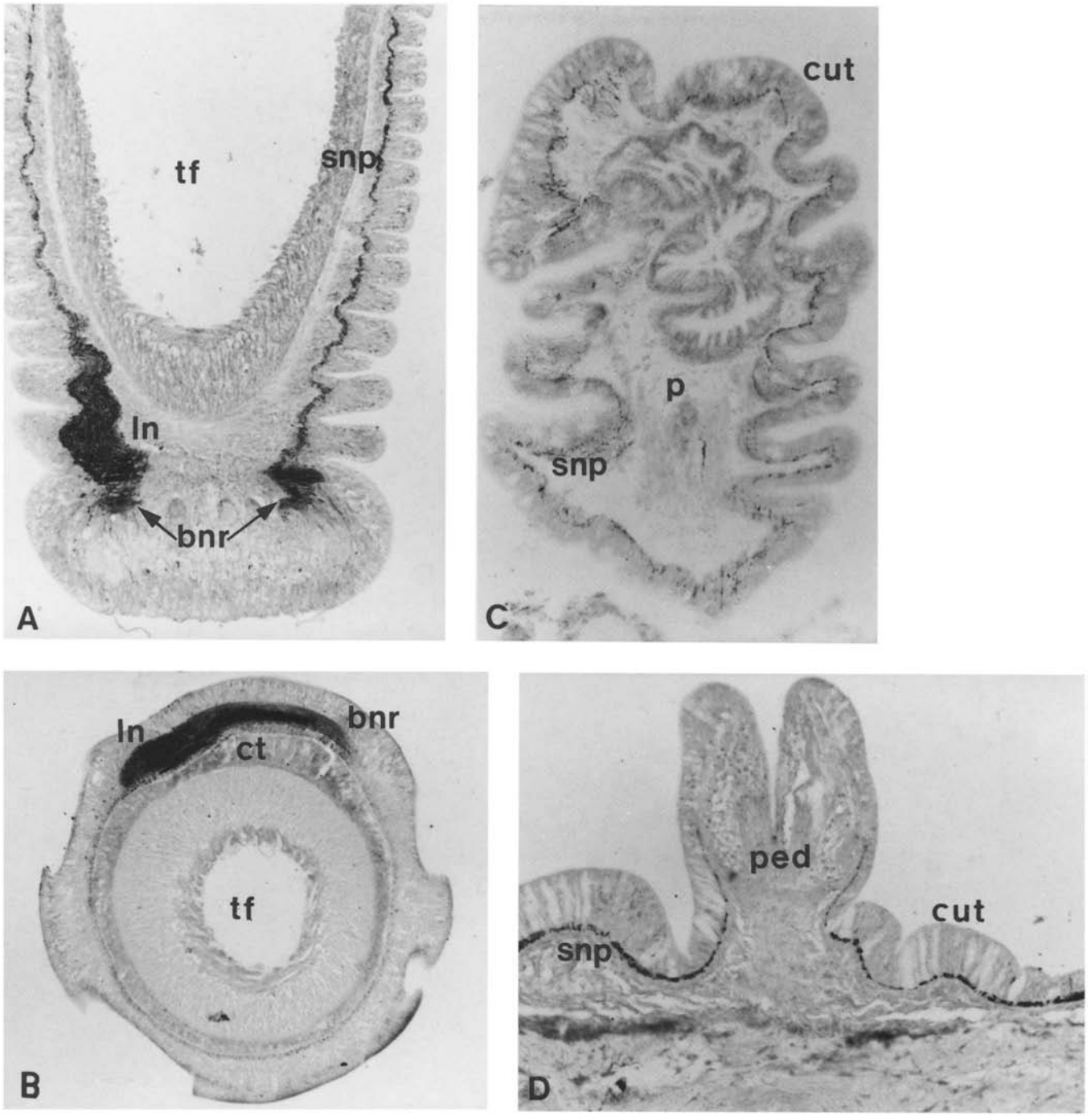

Fig. 7A–D. Tube feet (*tf*), papulae (*p*) and pedicellariae (*ped*). **A** Longitudinal section of a tube foot (*tf*), S1 immunoreactivity associated with the sub-epithelial nerve plexus (*snp*), longitudinal nerve (*ln*) and basal nerve ring (*bnr*). × 133. **B** Transverse section of the tube foot (*tf*), S1 immunoreactivity associated with the longitudinal nerve (*ln*) as it forms the basal nerve ring (*bnr*). × 90. **C,D** S1 immunoreactivity localised to nerve fibres associated with the sub-epithelial nerve plexus (*snp*) supplying the papulae (p) and pedicellariae (ped). **C** × 110; **D** × 171. Connective tissue (*ct*); cuticle layer (*cut*)

Fig. 6A–F. RNC, tube feet and optic cushion. **A** Ectoneural axons from the RNC (*rnc*) innervate the two rows of tube feet (*tf*) via the longitudinal nerve (*ln*) and basal nerve ring (*bnr*). × 50. **B** S1 immunoreactivity associated with the marginal nerve (*mn*), supplied by the RNC and bipolar epithelial cell bodies (*arrow*). × 283. **C** Longitudinal section of the RNC showing "ganglionic-like" collections of hyponeural (*hy*) cell bodies (*arrows*). × 120. **D** Bipolar ectoneural (*ec*) cell bodies (*arrows*) associated with the optic cushion (*oc*) × 333. **E** The tube foot (*tf*) innervation is derived from the RNC with cell bodies evident at the point where the tf and RNC connect (*arrows*). The hyponeural axons appear not to innervate the tf but extend (*arrowhead*) out towards the ambulacral muscles (*amus*) above. × 210. **F** S1 immunoreactivity was found to be associated only with the ectoneural neuropile (*ec*) and epithelial cell bodies (*arrow*) of the optic cushion (*oc*) and terminal tentacle (*tt*). × 133. Cuticle layer (*cut*); sub-epithelial nerve plexus (*snp*); radial perihemal canal (*rpc*); ossicle (*os*)

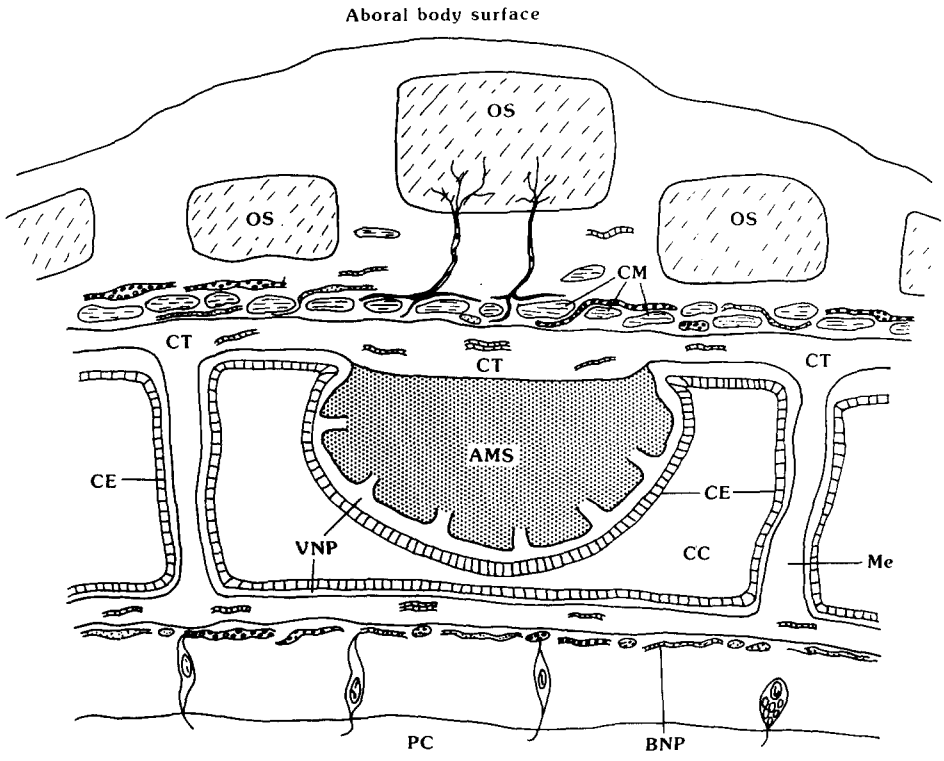

Fig. 8. Diagrammatic representation of the apical muscle (*AMS*) against the aboral body wall; the position of the pyloric caeca (*PC*) and mesenteries (*Me*) is also shown. Basiepithelial nerve plexus (*BNP*); coelomic epithelium (*CE*); coelomic cavity (*CC*); connective tissue (*CT*); ossicle (*OS*); visceral nerve plexus (*VNP*)

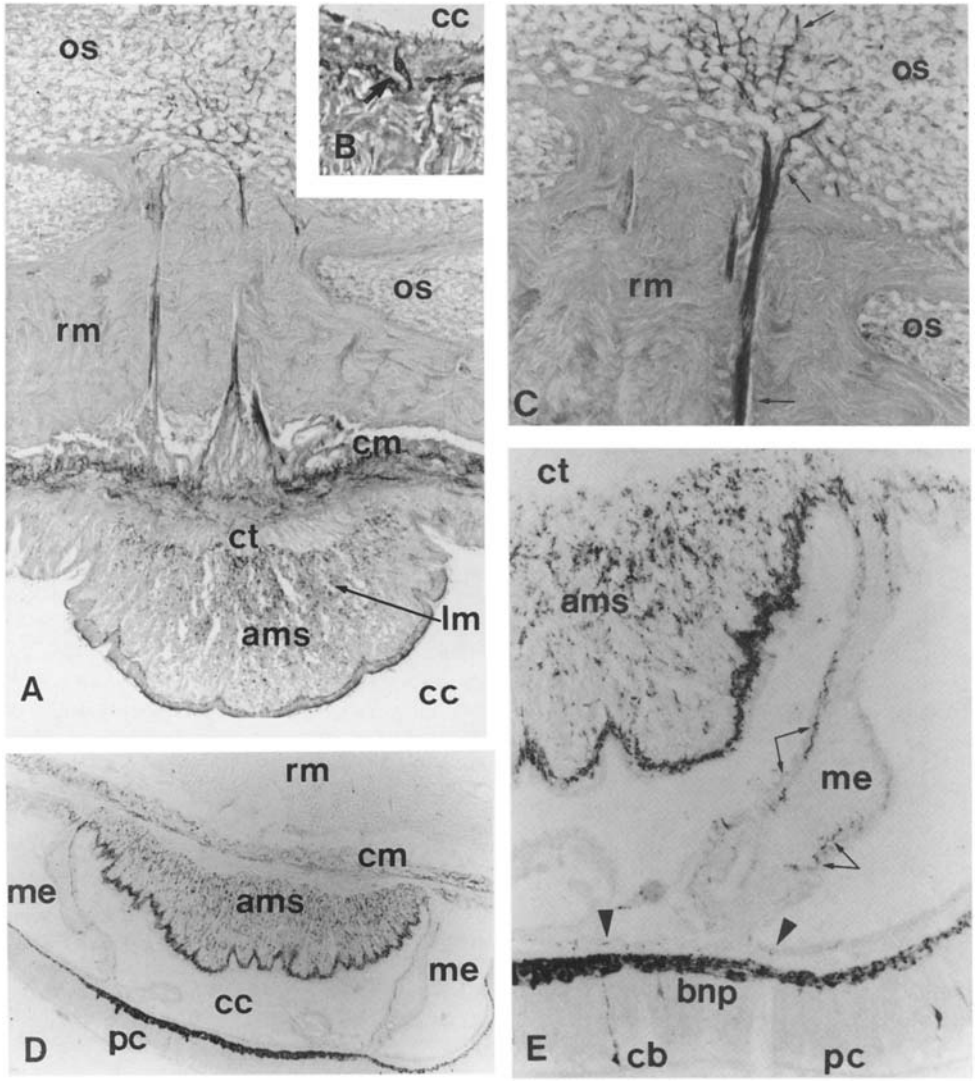

Fig. 9A–E. Apical muscle (*ams*). **A** S1 immunoreactive nerve fibres are associated with both the longitudinal (*lm*) and circular muscle (*cm*) layers, which are divided by a region of connective tissue (*ct*). ×112. **B** S1 immunoreactive bipolar epithelial cell body *(arrow)*. ×333. **C** S1 immunoreactive nerve fibres (*arrows*) associated with the circular muscle layer penetrate the reticular muscle (*rm*) to the aboral body wall ossicles (*os*). ×170. **D** Transverse section of *ams* showing part of the pyloric caeca (*pc*) suspended by two mesenteries (*me*). S1 immunoreactivity is associated with the longitudinal and circular muscle layers (*cm*) of the *ams* and the visceral and basiepithelial nerve plexuses of the caeca. ×110, see also Fig. 12**B**. **E** S1 immunoreactivity associated with the *ams* maybe continuous with the innervation associated with the mesenteries (*me; arrows*) and visceral muscle (*arrowheads*). ×200. Coelomic cavity (*cc*); connective tissue (*ct*); basiepithelial nerve plexus (*bnp*)

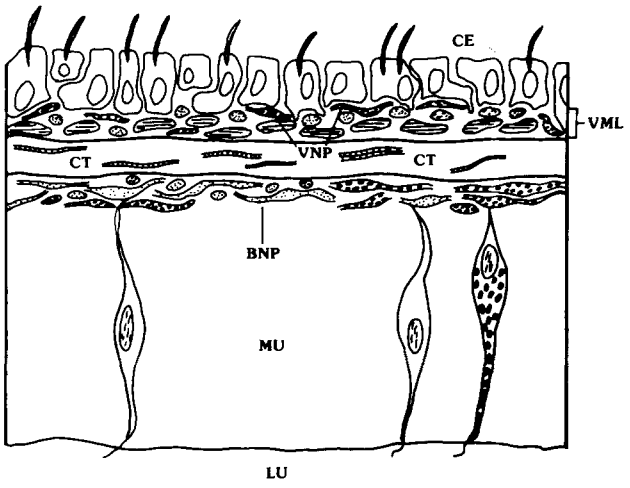

Fig. 10. Diagram showing the general anatomy of the gut of *Asterias rubens*. Lumen (*LU*); mucosa (*MU*); basiepithelial nerve plexus (*BNP*); connective tissue (*CT*); visceral nerve plexus (*VNP*); visceral muscle layer (*VML*); coelomic epithelium (*CE*)

ach. A short duct leads from the pyloric stomach into the pyloric caeca, where nutrients are stored and absorbed. A short intestine bearing two rectal caeca opens up into the anus from the aboral wall of the pyloric stomach.

The tissues are bounded by a single layer of ciliated peritoneal epithelial cells, beneath which are two thin layers of longitudinal and circular muscle with their associated innervation. A continuous layer of connective tissue separates the muscle from the main nerve plexus and adjacent tall ciliated epithelial cells that line the lumen of the gut. The majority of these cells are ciliated, producing the currents that propel food particles from the cardiac stomach through to the pyloric caeca. Other cell populations include mucous goblet cells and granular secretory cells (Fig. 10).

Whole-mounts of the digestive system revealed S1 immunostaining as a complex network of varicose fibres, together with numerous cell bodies (Fig. 11A). Many of the nerve fibres in the oesophagus were oriented perpendicular to the mouth opening (Fig. 11B), whereas in the other tissues, the pattern was less ordered, with the fibres forming a meshwork (Fig. 11C).

The oesophagus is attached to the peristomal membrane and is lightly grooved. S1 immunoreactivity in tissue sections is associated with the nerve plexus and numerous mucosal cell bodies (Fig. 11D).

Whilst feeding, *Asterias rubens* everts the cardiac stomach through the mouth, after which the stomach is retracted back into the disc by contraction of the intrinsic and extrinsic retractor muscles. Unlike the oesophagus, the stomach is extensively pouched and folded producing a large surface area for digestion. S1 immunoreactivity is evident both in the nerve plexus and mucosal cell bodies, with the nerve plexus becoming thickened at the point where the intrinsic retractor muscles insert (Fig. 11E; Anderson 1954). As with the cardiac stomach, S1 immunoreactivity in the pyloric stomach is also associated with axons in the nerve plexus and mucosal cell bodies (Fig. 11F).

The pyloric caeca (which receive partially digested material from the pyloric stomach) comprise absorptive, secretory and storage cells. Histologically, this tissue is similar to the other regions, although the pattern of S1 immunostaining is slightly different, with immunoreactive nerve fibres in amongst the visceral muscle layers and in association with the nerve plexus and mucosal cell bodies (Fig. 12A,B). A small amount of S1 immunoreactivity is also associated with the nerve plexus and cell bodies of the rectal caeca.

No S1 immunoreactivity was detected in either the male or female gonads. Pre-absorbed controls carried out with the native peptide resulted in S1 immunostaining being effectively abolished (Fig. 12C,D). Pre-absorption with thyroglobulin, FMRFamide and FLRFamide had no significant effect, whereas S2 and LPLRFamide reduced but did not abolish the immunostaining. The minimal reduction of immunostaining noticed with S2 preabsorption was evenly distributed throughout all the tissues and was considered to be attributable to the common C-terminal F-amide. Comparison of the results following the use of normal BLIV antiserum and that preabsorbed with S2 showed no detectable difference in the distribution of immunoreactivity. Thus, we were convinced that BLIV revealed authentic S1 localization.

Discussion

The Echinodermata are the only deuterostome invertebrate phylum, a feature that allies them to the Urochordata, which are invertebrate deuterostomian members of the phylum Chordata. This feature combined with their radial symmetry secondarily derived from bilateral ancestors, an internal skeleton of articulating calcite ossicles and possession of a true coelom make them a fascinating and evolutionary significant group of animals.

The SALMFamides (S1 and S2) are the first neuropeptides to be isolated and fully characterized from this phylum. They have been identified from extracts of RNC from *A. rubens* and *A. forbesii* (Elphick et al. 1991a,b). RIA has demonstrated an abundance of S1 in the nervous system of *A. rubens* (Moore et al. 1991). Here, specific antisera have been used to map the distribution of S1 at the light-microscopical level. This is the first time that a fully sequenced, native echinoderm neuropeptide has been demonstrated immunocytochemically.

The adult echinoderm nervous system comprises five RNCs and a central CONR. Both structures consist of an oral ectodermal motor and sensory nerve plexus (the ectoneural) and an aboral mesodermal motor plexus (the hyponeural). Initially, it was believed that the nerve ring acted as a "brain" controlling the arms via centres at the junction of each RNC with the nerve ring (Smith 1945; Kerkut 1955). This idea of cephalization has been largely discredited following the suggestion that these "centres", if present, are more functional than histological (Smith 1950). Instead, the nerve ring is believed to act as a link between the arms, all of which can control whole animal behaviour (Cobb and Moore 1989).

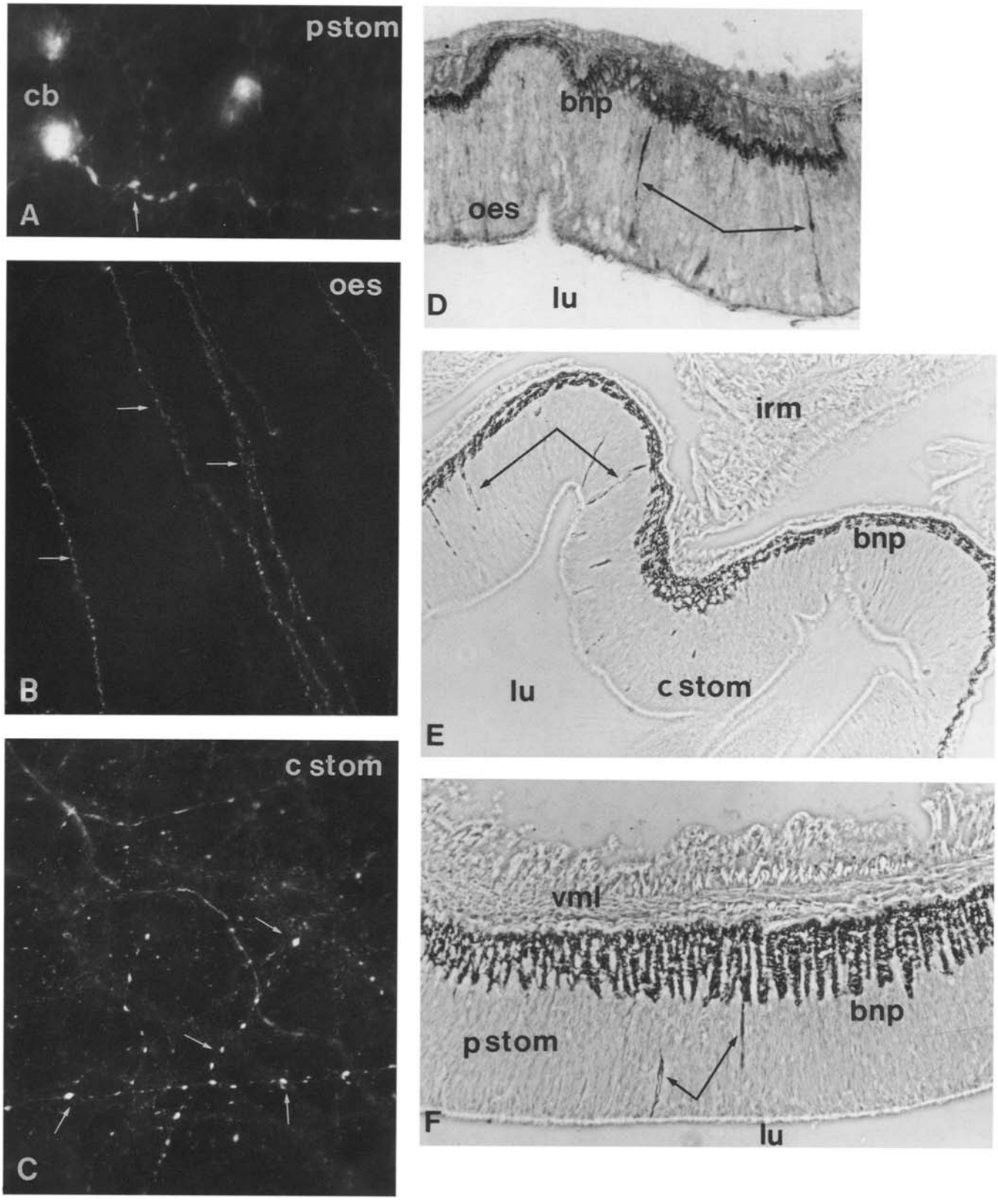

Fig. 11A–F. Whole-mounts and sections of the digestive system. **A** Pyloric stomach (*pstom*) showing an S1 immunoreactive monopolar cell body (*cb*) and axon (*arrow*). ×615. **B** Oesophagus (*oes*) showing S1 immunoreactive axons (*arrows*) oriented perpendicular to the mouth opening. ×230. **C** Cardiac stomach (*cstom*) showing a meshwork of varicose S1 immunoreactive neurones (*arrows*). ×615. **D** S1 immunoreactivity associated with the basiepithelial nerve plexus (*bnp*) and mucosal cell bodies (*arrows*) of the oesophagus (*oes*). ×267. **E** Cardiac stomach (*cstom*). S1 immunoreactivity associated with cell bodies (*arrows*) the nerve plexus (*bnp*) which thickens at the insertion of the intrinsic retractor muscles (*irm*). ×210. **F** Pyloric stomach (*pstom*), S1 immunoreactivity localised to the nerve plexus (*bnp*) and mucosal cell bodies (*arrows*). ×267. Visceral muscle layers (*vml*); lumen (*lu*)

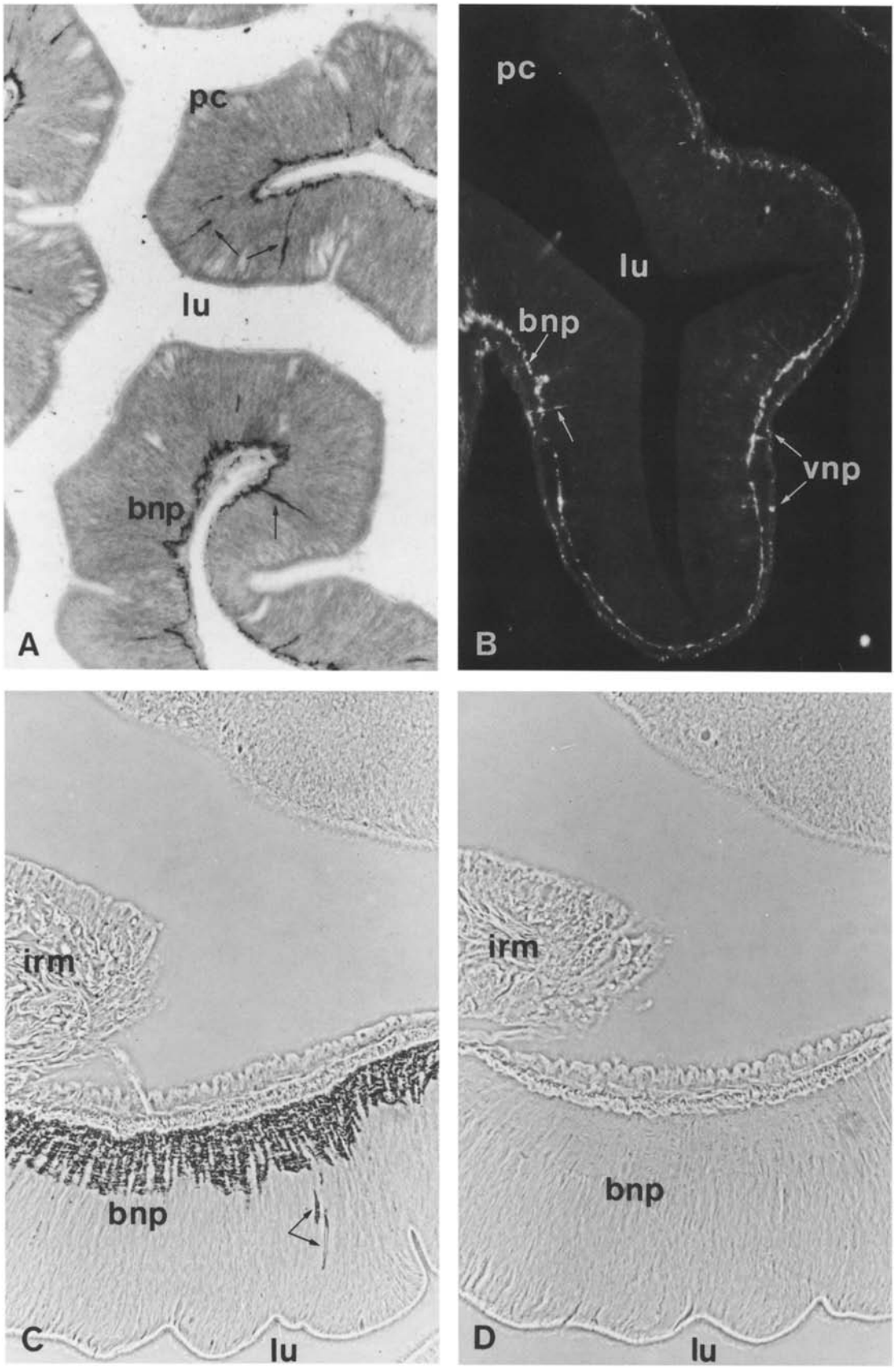

Fig. 12A–D. Digestive system. **A,B** Pyloric caeca (*pc*); S1 immunoreactivity associated with the basiepithelial (*bnp*) and visceral (*vnp*) nerve plexuses and mucosal cell bodies (*arrows*). × 210. **C** Test section of cardiac stomach showing S1 immunoreactivity associated with the basiepithelial nerve plexus (*bnp*) and cell bodies (*arrows*). × 210. **D** Adjacent control section of cardiac stomach pre-absorbed with native S1 peptide. × 210. Lumen (*lu*); intrinsic retractor muscle (*irm*)

The extensive neuronal distribution of S1 demonstrated in the present study has revealed a number of previously unrecognised aspects of the asteroid nervous system, aspects that may have important functional implications: (1) the possible hyponeural innervation of the apical and visceral gut muscles from the CONR, (2) the ganglion-like collections of hyponeural cell bodies situated on alternate sides of the RNC, (3) the innervation of the optic cushion, which is supplied by the ectoneural nerve plexus only, (4) the innervation of the ambulacral muscles by hyponeural nerve fibres, and (5) the distinctive band of staining in the lateral borders of the ectoneural nerve plexus suggesting a regional concentration of fibres, perhaps forming a specific tract on each side of the RNC.

The immunocytochemical distribution of S1 in the tube feet described here for *A. rubens* has confirmed the presence of the longitudinal nerve first described in *Astropecten irregularis* (Smith 1937, 1944), *Diadema setosum* (Kawaguti 1964) and *Echinus esculentus* (Florey and Cahill 1977). This appears simply as a local thickening of the ectoneurally derived plexus, which invests the connective tissue zone adjacent to the muscle layer. At the tip of each tube foot, this branches to form the basal nerve ring.

The co-ordinated activity of the tube feet is responsible for the characteristic locomotory behaviour of starfish. Their movement is complex and varied, including slow postural changes, sustained increases in tone associated with feeding and rapid changes in length in response to chemical and/or mechanical stimuli (Florey and Cahill 1980). To date, ACh, GABA and the catecholamines dopamine and noradrenaline have all been implicated in controlling this sophisticated array of behaviours (Florey and Cahill 1980). Clearly, our findings of extensive S1 immunoreactivity in association with the nerve plexus, longitudinal nerve and nerve ring lends strong support to the idea that this molecule is involved in the synchronization of tube foot motor activity. Unfortunately, preliminary isotonic bioassay tests designed to monitor the effect of S1 on tube foot muscle activity have failed to detect any response, either direct or indirect (Elphick et al. 1990). This however does not negate the possibility of a more subtle modulatory or sensory function. Clearly, the role of S1 in this system is more complex than one might predict.

The innervation of the digestive system was first referred to by Hamann (1885) who described it as originating from the visceral nerve plexus. Investigations into the innervation of this system have revealed that the basiepithelial nerve plexus is ectoneurally derived from the CONR (Jangoux 1982; Hyman 1955), whilst the presence of a visceral nerve plexus has been well documented (Cobb 1969; Cobb and Raymond 1979; Jangoux 1982), its origins remain obscure. The results of this report indicate for the first time that this plexus might be hyponeurally derived from the CONR. S1 immunostaining of the CONR has revealed that fibres from the hyponeural element may be involved in innervating the muscle layers beneath the coelomic epithelium. Connective tissue divides the muscle from the ectoneurally derived basiepithelial nerves, thereby maintaining the separation of the two plexuses, a fundamental feature of this nervous system. The innervation associated with the visceral muscle appears to be continuous throughout the digestive system to the apical muscle, which is connected to the gut through the mesenteries present on either side.

On present evidence, it is difficult to determine whether the gut mucosal cell bodies represent endocrine cells (Martínez et al. 1989) or whether they are genuinely neuronal perikarya serving the basiepithelial plexus. Ultrastructural investigation will be essential if this question is to be answered with any certainty.

In common with all other multicellular animals, digestive processes in starfish include a combination of secretory, absorptive and motor activity. A feature unique to asteroids, however, is their unusual extraoral feeding behaviour whereby the cardiac stomach is everted through the mouth and inserted between the shells of the bivalve molluscan prey (Jangoux 1982). Following feeding, the stomach is retracted into the central disc, a process that has been shown to involve cholinergic contractions of the stomach wall muscles and the extrinsic and intrinsic retractor strands (Basch 1956). In contrast, stomach eversion involves the relaxation of this system with ATP, adenosine and adrenaline being known to act as potent relaxants (Knight et al. 1990; Hoyle and Greenberg 1988). It has been suggested (Cobb and Raymond 1979) that the extensive neuronal network underlying the gut epithelium is involved in the innervation of various secretory cells, the presence of S1 in these nerve fibres and endocrine-like mucosal cells provides a firm basis for speculation on its role in the control of digestive activity. Clearly, S1 is a prime candidate for the role of a modulator of secreto-motor functions. Bioassay studies with S1 on isolated stomach preparations have however failed to elicit a motor response (Elphick et al. 1991a), so evidently this peptide has a more subtle role, possibly as a transmitter or modulator of sensory information through an endocrine or neuroendocrine route.

Previously, the apical muscle has been described as an apical nerve (Cuénot 1890, 1891), a simple longitudinal muscle (Hyman 1955) or a muscle with a nerve cord (O'Neill 1989). Our studies make it clear that this structure has a more complex organization. It comprises longitudinally arranged muscle with an innervation believed to stem from the pyloric caeca mesenteries. The primary role of the apical muscle involves generating postural changes in the arm during feeding, locomotion and reproductive activities. It also forms part of the extensive structural (skeletal) system found in the aboral body wall; this includes circular and reticular muscle associated with calcareous body wall ossicles and an extensive framework of tensile mutable connective tissue characteristic of this phylum (O'Neill 1989; Wilkie 1988). The interplay and balance of "tensions" between these muscle, ossicle and connective tissue systems is responsible for the co-ordination of overall body posture (O'Neill 1989). Subtle alterations in the dynamics of this system can bring about profound changes in body wall rigidity, including autotomy (Motokawa 1984; O'Neill 1989). Investigations into the control of this system are rare, although there are reports of peptide-like autotomy pro-

moting factors that may act on the connective tissue (Mladenov et al.1989). Reports that are available provide inconclusive data; ACh induces contraction of the apical muscle and longitudinal elements in the body wall, whereas the actions of catecholamines are equivocal. The presence of S1 closely associated with the innervation of the muscle layers and in amongst the aboral skeletal ossicles strongly implies a role for this neuropeptide in the control processes that must underlie the complex postural events recorded for these animals (O'Neill 1989; Motokawa 1988).

The extensive neuronal distribution of S1 revealed by this report has emphasised the value of neuroanatomy as a means of elucidating functional networks for newly identified signalling molecules and as a tool for revealing hitherto unknown aspects of nervous systems. A determination of function will rely on the identification of suitable preparations for bioassay although, in the past, echinoderms unfortunately have proved refractory in this respect, particularly because of the small size and relative inaccessibility of their nervous system (Cobb 1987). The recent localization of the SALMFamides in other echinoderm classes, including echinoids (Elphick et al. 1992), ophiuroids (J.L.S. Cobb and M. Gyhoot, personal communication) and holothurians (Díaz-Miranda et al. 1992) may provide alternative models for functional studies. The giant neurones in the RNC of ophiuroids offer an especially exciting prospect for electrophysiological investigations.

Acknowledgements. This work was carried out during the tenure of a Science and Engineering Research Council (UK) studentship to S.J.M. The authors would like to thank Lynne Etherington, Zyg Podhorodecki and Anton Page for assistance in preparation of the figures, and Lesley Brownlie for taking care of the rabbits.

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