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# Gastro-intestinal and neurohormonal peptides in the alimentary tract and cerebral complex of *Ciona intestinalis* (Ascidiaceae)

## Their relevance to the evolution of the diffuse neuroendocrine system

H.A.R. Fritsch*, S. Van Noorden**, and A.G.E. Pearse**

* Anatomisches Institut der Universität Kiel, Kiel, Bundesrepublik Deutschland;
** Department of Histochemistry, Hammersmith Hospital, RPMS, University of London, London, England

**Summary.** Polypeptide-hormone producing cells were localized in the alimentary tract and cerebral ganglion of *Ciona intestinalis* using cytochemical, immunocytochemical and electron-microscopical methods.

Antisera to the following peptides of vertebrate type were employed: bombesin, human prolactin (hPRL), bovine pancreatic polypeptide (PP), porcine secretin, motilin, vasoactive intestinal polypeptide (VIP), β-endorphin, leu-enkephalin, met-enkephalin, neurotensin, 5-hydroxytryptamin (5-HT), cholecystokinin (CCK), human growth hormone (GH), ACTH, corticotropin-like intermediate lobe peptide (CLIP) and gastric inhibitory peptide (GIP).

Immunoreactive cells were found both in the alimentary tract epithelium and in the cerebral ganglion for bombesin, PP, substance P, somatostatin, secretin and neurotensin. Additionally, in the cerebral ganglion only, there were cells immunoreactive for β-endorphin, VIP, motilin and human prolactin. 5-HT positive cells, however, were restricted to the alimentary tract.

No immunoreactivity was obtained either in the cerebral ganglion or in the alimentary tract with antibodies to leu-enkephalin, met-enkephalin, CCK, growth hormone, ACTH, CLIP and GIP. Prolactin-immunoreactive and pancreatic polypeptide-immunoreactive cells were argyrophilic with the Grimelius' stain and were found in neighbouring positions in the cerebral ganglion.

At the ultrastructural level five differently granulated cell types were distinguished in the cerebral ganglion. Granules were present in the perikarya as well as in axons. The possible functions of the peptides as neurohormones, neuroregulators and neuromodulators are discussed.

*Send offprint requests to:* Dr. H.A.R. Fritsch, Anatomisches Institut der Universität Kiel, Neue Universität, Haus N 10, 2300 Kiel, Federal Republic of Germany

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**Key words:** Peptide immunoreactivity – Cerebral ganglion – Alimentary tract – *Ciona intestinalis* (Ascidiaceae) – Diffuse neuroendocrine system

The endocrinology of the nervous system, and of the alimentary tract and pancreas, is a field of study receiving an increasing amount of attention. The isolation, characterization, sequencing, and finally synthesis of a series of biologically active peptides from mammalian gut and amphibian skin has provoked much research into the relevance of these substances in human pathophysiology.

At present there are at least 15 candidates for gut hormone status, ranging from well-established peptides such as gastrin to recent newcomers such as pancreatic polypeptide and bombesin.

Many observations, in a wide range of vertebrate species, have established the dual localization of some of these peptides in the nervous system and in the endocrine cells of the gut and pancreas. This raises the question not only of their significance in these two situations, but also of the mechanism by which the dual localization has been achieved.

Did all the gut peptides first arise in nerve cells as neurotransmitters or neurohormones and then in the course of evolution appear in endocrine cells in the gut and elsewhere? Or, alternatively, have the gut endocrine peptides evolved independently in specialised endodermal cells related to the enterocytes? In an attempt to answer these questions, many animals lower than mammals on the phylogenetic scale have been examined, and the occurrence of polypeptide hormone-producing cells in the alimentary tract and in the nervous system of lower vertebrates and even of some invertebrates in now well established (Wilson and Falkmer 1965; Mehrotra and Falkmer 1968; Falkmer 1972; Davidson et al. 1971; Falkmer et al. 1973; Van Noorden and Pearse 1974; Fritsch et al. 1976, 1978, 1979, 1980a, b; Fritsch and Sprang 1977; Falkmer et al. 1977; Sundler et al. 1977; Tunas 1977; Van Noorden and Patent 1978; Van Norden and Falkmer 1980; Thorndyke and Bevis 1978; Bevis and Thorndyke 1978, 1979; Thorndyke and Probert 1979; Duve and Thorpe 1980; Schot et al. 1981).

The comprehensive neuroendocrine control system of the vertebrates must have had its origins in organisms lower on the phylogenetic scale; this study was designed to investigate the gut peptide hormones from an evolutionary standpoint. To this end, the experimental animal chosen was *Ciona intestinalis*, the sea squirt, a representative of the protochordates which are thought to be a phylogenetic offshoot from the deuterostomian line leading from invertebrates to vertebrates.

We have already reported our findings on the immunocytochemical localization of calcitonin, somatostatin, substance P and gastrin-like substances in the alimentary tract and cerebral ganglion of this animal (Fritsch et al. 1978, 1979). The present work is a continuation of the investigation.

## Materials and methods

*Animal material.* Adult specimens of *Ciona intestinalis* were collected by a diver in the western Baltic Sea. Further animals were obtained from the Marine Biological Station, Plymouth, U.K. The animals (30–50 mm long, Baltic Sea, 50–70 mm long, English Channel) were aggregated in colonies, which were either

attached to large brown algae or secondary sediment. They were kept in glass aquaria at 5° C in aerated seawater (16‰/32‰) from their places of origin.

*Histological methods.* The entire neural complex, located between the two siphons, was removed, as was the alimentary tract with oesophagus, stomach, upper intestine and lower intestine.

*Light-microscopical methods.* The material was fixed for a short period (3 h) in Bouin's fluid, in methanol-free formaldehyde (MFF) (Polak et al. 1971a), or in 6% glutaraldehyde. Fixation was followed by standard dehydration through graded alcohols, embedding in Paraplast and serial sectioning. For the formaldehyde-induced fluorescence (FIF) method to demonstrate amines (Falck et al. 1962) further material was freeze-dried, fixed in formaldehyde vapour at 60° C for 3 h, and embedded in Paraplast for serial sectioning.

*Electron microscopical methods.* The tissues were fixed for 2 h in either 3.5% glutaraldehyde in 0.1 M phosphate buffer + 7.5% sucrose, pH 7.4, or 2.5% glutaraldehyde + 2% paraformaldehyde in 0.2 M phosphate buffer + 7.5% sucrose, pH 7.4 for 2 h.

Afterwards the material was osmicated in 1% $OsO_4$ solution in 0.1 M phosphate buffer, dehydrated, and embedded in Araldite. Sectioning was carried out on a Reichert Ultramicrotome $UOM_3$. The sections were stained by the combined uranyl acetate-lead citrate method (Watson 1958; Reynolds 1963).

The sections were examined either with a Zeiss EM 9A microscope or with a Siemens Elmiskop 101.

*Immunohistochemical methods.* Antibodies were available to synthetic amphibian bombesin, human prolactin (hPRL), bovine pancreatic polypeptide (PP), porcine secretin, motilin, vasoactive intestinal polypeptide (VIP), β-endorphin, leu-enkephalin, met-enkephalin, neurotensin, 5-hydroxytryptamine

**Table 1.**

| Antibody | Raised to | Source | Regional specificity | Cross-reactivity |
|---|---|---|---|---|
| Bombesin | synthetic amphibian | RPMS[a] | N-mid | |
| Human prolactin | | NIAMDD[b] | | |
| PP | nat. bovine | R. Chance, Lilly & Co, GB | | |
| Secretin | nat. porcine Secretin | RPMS | | |
| Motilin | nat. porcine Motilin | RPMS | C-term | |
| N-VIP | nat. porcine VIP | RPMS | N-term | |
| β-Endorphin | synthetic | RPMS | C-term | |
| leu-Enkephalin | synthetic | RPMS | C-term | |
| met-Enkephalin | synthetic | RPMS | C-term | |
| Neurotensin | synthetic bovine | RPMS | whole | |
| 5-HT | synthetic | I. deMey, Janssen Pharmazeutics, Belgium | whole | |
| CCK | synthetic | RPMS | 9–20 | |
| Human GH | | Wellcome, GB | | |
| ACTH | nat. | Wellcome, GB | C-term | β-End., CJP |
| CLIP | synthetic | Wellcome, GB | | ACTH |
| GIP | nat. porcine | RPMS | middle | Glucagon |

[a] NIAMDD: National Institute for Arthritis, Metabolism and Digestive Diseases, Los Angeles, USA
[b] RPMS: Royal Postgraduate Medical School, London, GB

(5-HT), cholecystokinin (CCK), human growth hormone (GH), ACTH, corticotropin-like intermediate lobe peptide (CLIP), and gastric- inhibitory peptide (GIP). They had been produced in rabbits and tested by radioimmunoassay (RIA) and by immunostaining in mammalian tissues (Polak et al. 1975). For details see Table 1.

The indirect immunofluorescence technique (Coons et al. 1955) and the peroxidase-anti-peroxidase (PAP) method (Sternberger 1974) were used. The primary antibodies were diluted in 0.01 M phosphate-buffered normal saline, pH 7.1 (PBS). The dilutions used ranged from 1/200–1/2,000, depending on the titre of the antibody.

The second layer for immunofluorescence was fluorescein-conjugated goat anti-rabbit globulin (Miles, diluted 1/100). For the PAP method, unlabelled goat anti-rabbit globulin (Miles, 1/200) was used, followed by rabbit PAP complex (UCB Bioproducts, diluted 1/300). The attached peroxidase was revealed by the diaminobenzidine reaction (Graham and Karnovsky 1966). Sections were mounted in buffered glycerine for immunofluorescence or were dehydrated and mounted in DEPEX for the PAP stain.

*Controls.* Specific immunostaining was quenched by pre-absorption of the diluted primary antibody with 10 nmol/ml of its specific antigen. Further control reactions were carried out by omitting the primary antibody or by using albumin or non-immune rabbit serum as the first layer.

*Argyrophilia.* Argyrophilic cells were revealed by the silver impregnation method of Grimelius (Grimelius 1968).

## Results

*Light-microscopical results.* The neural complex of the Ascidiaceae is composed of two parts, the cerebral ganglion, representing the central nervous system (Figs. 1, 2), and the neural gland which is an epithelial structure.

The neural gland has a central cavity connected with the ciliated funnel which lies next to the ganglion and terminates in the buccal cavity of the pharynx.

The cerebral ganglion, about 1–2 mm long and with a diameter of 1 mm, consists of nervous tissue with perikarya of various sizes and shapes, arranged at the periphery in several layers. The axons of these cells run towards the centre of the ganglion, forming a meshwork in the transitional zone before entering the medulla. The centre of the ganglion consists mainly of densely packed nerve fibres with single small perikarya between them. The nerve fibres are arranged longitudinally and run into the nerve trunks at both sides of the ganglion. In contrast, the nerve fibres in the transitional zone are not preferentially oriented along the longitudinal axis. The alimentary tract of the Ascidiaceae (Fig. 3) consists of pharynx, oesophagus, stomach, intestine and atrium (rectum); its morphology has been described (Millar 1953).

*Immunohistochemical results.* The cerebral ganglia contained granules in perikarya and nerve fibres which gave a positive antigen-antibody reaction with antibodies to hPRL, PP, secretin, bombesin, VIP, $\beta$-endorphin, neurotensin and motilin (Fig. 2). The strongest immunoreactivity was obtained after fixation for a short time (3 h) in Bouin's fluid. The number of cells immunoreactive with the different antibodies varied considerably. However, in each ganglion investigated, some cells immunoreactive for each antibody were present. No reaction was obtained with antibodies to leu-enkephalin, met-enkephalin, 5-HT, ACTH, CLIP, growth hormone, CCK and GIP. However, this does not exclude the possibility of false negative results,

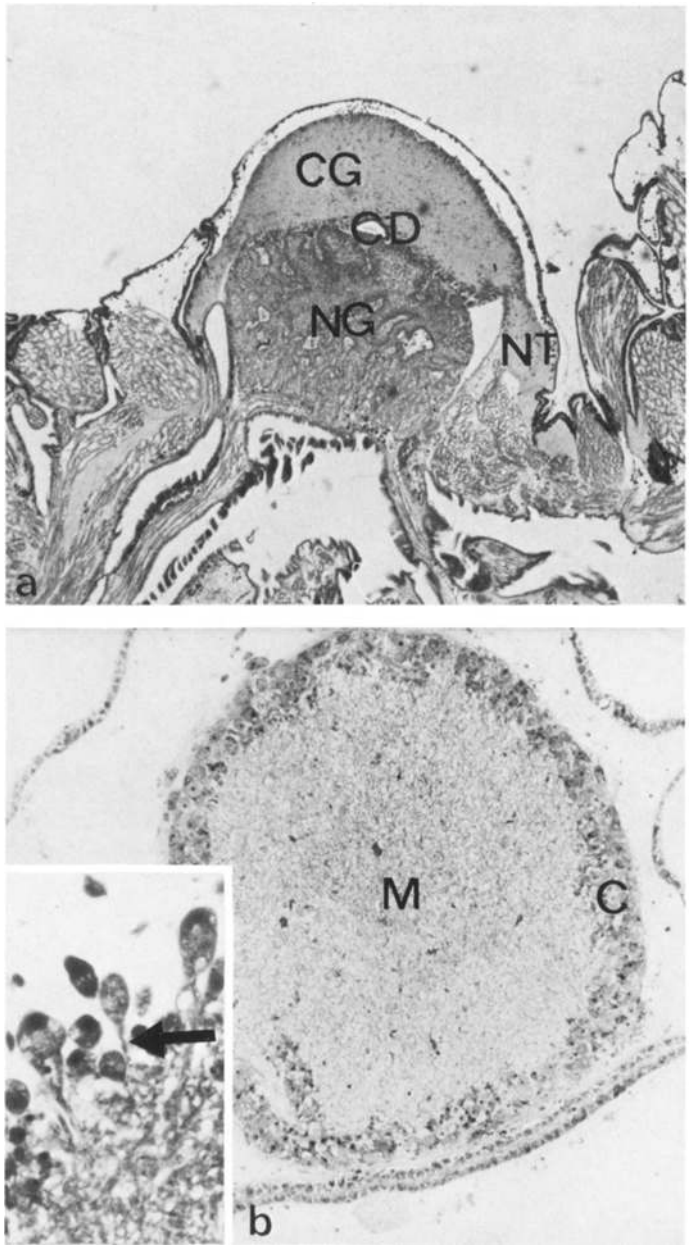


**Fig. 1 a, b.** *Ciona intestinalis.* Longitudinal section through the neural complex (**a**) and cross section through the cerebral ganglion (**b**). The perikarya are predominantly arranged in several layers in the cortex (**b**) sending axons into the transitional zone and the medulla (*Inset* in **b**, *arrow*). *CG* cerebral ganglion, *NG* neural gland, *NT* nerve trunk, *CD* central duct, *C* cortex, *M* medulla, Haematoxylin and eosin (**a**), toluidine blue (**b**, *Inset*). a ×120; b ×480; *Inset* ×750

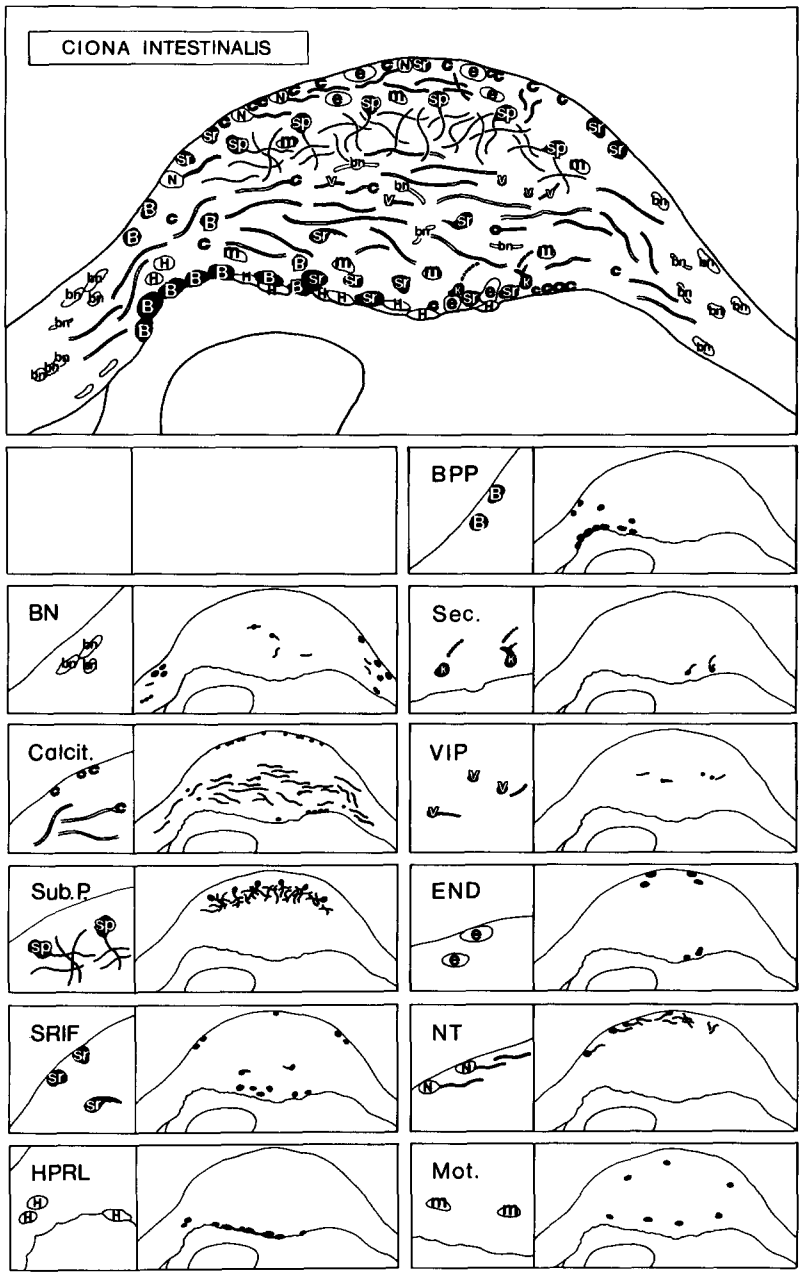


**Fig. 2.** *Ciona intestinalis.* Localization of peptides in the cerebral ganglion: *bn* bombesin; *c* calcitonin; *sp* substance P; *sr* somatostatin (SRIF); *H* (human) prolactin (HPRL); *B* (bovine) pancreatic polypeptide (BPP); *K* secretin; *V* vasoactive intestinal polypeptide (VIP); *e* endorphin; *N* neurotensin; *m* motilin

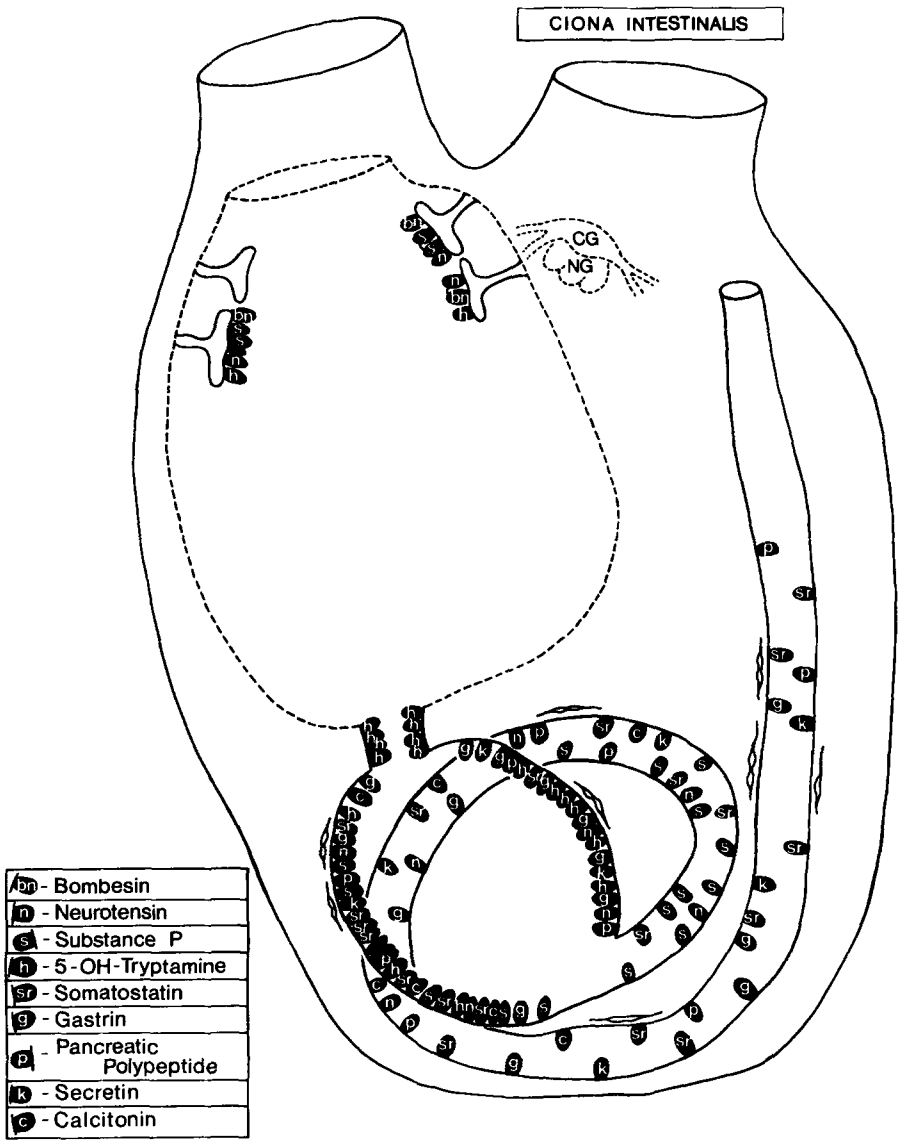


**Fig. 3.** *Ciona intestinalis.* Localization of polypeptides in the alimentary tract: *bn* bombesin; *s* substance P; *n* neurotensin; *g* gastrin; *k* secretin; *p* (bovine) pancreatic polypeptide (BPP); *c* calcitonin; *sr* somatostatin (SRIF); *h* 5-OH-tryptamine

since the molecular structure of peptides in *Ciona* may be different from that of the peptide antigens to which the antibody was raised. The attempt to demonstrate "intrinsic" amines with the FIF-method gave negative results in the ganglion, but was positive in some endocrine cells in the alimentary tract.

*Neurotensin-like immunoreactivity*

Cells immunoreactive for neurotensin were located in the outer layer of the cortex of the ganglion facing the bordering capsule (Fig. 4a, b). The cells are round or pear-shaped, of medium size in relation to the other perikarya, and they give rise to a single axon which is also positively immunostained. The nerve fibres run through the cortex diagonally into the transitional zone, where they form an irregular meshwork of beaded fibres (Fig. 4b). Approaching the medulla the neurotensin-immunoreactive fibres become sparse, being absent from the centre of the medulla itself. In the cortex the neurotensin-immunoreactive perikarya are irregularly scattered as single cells, and no specific distribution pattern could be discerned. In the alimentary tract neurotensin-immunoreactive cells of the "open" type were found in the stomach and throughout the entire intestine (Fig. 4c). Many more cells were present in the stomach and the upper intestine than in the lower intestine, where they were sparsely scattered among non-immunoreactive cells. In no area was a particular grouping observed. There was no immunoreaction after preabsorption of the neurotensin antibody with its antigen.

*5-HT-like immunoreactivity and FIF*

No biogenic amines were shown by the FIF method in the ganglion. In accordance with this result no neurons were immunoreactive with an antibody to 5-HT. In the alimentary tract, however, fluorescent cells could be demonstrated with the FIF-method in the oesophagus, stomach and upper intestine (Fig. 4d). The greatest number of fluorescent cells occurred in the oesophagus and the stomach. Corresponding results were obtained with an antibody to 5-HT (Fig. 5a, b). The serotonin-immunoreactive cells were observed to rest on the basal lamina with their apices extending to the epithelial surface.

*Secretin-like immunoreactivity*

Secretin-like immunoreactivity was present in some single, medium-sized perikarya, restricted to the outer layer of the cortex, which is in contact with the neural gland of the cerebral complex (Fig. 5c). Quite often two short-stemmed axons could be seen leaving the perikaryon of these immunoreactive cells in the direction of the transitional zone (Fig. 5c). In the transitional zone itself there were some secretin-immunoreactive beaded nerve fibres which seemed to be topographically related to the secretin-immunoreactive perikarya (Fig. 5c). These fibres were restricted to the transitional zone. In the stomach and in the upper and lower intestine sparsely scattered secretin-immunoreactive cells were found. Pre-absorption of the antibody with its antigen prevented the immunoreaction.

*β-Endorphin-like immunoreactivity*

With an antibody to β-endorphin immunoreactivity was found in large perikarya with eccentrically placed nuclei, situated in the outer layer of the cortex of the ganglion (Fig. 6a, b). Perikarya immunoreactive for β-endorphin were present in

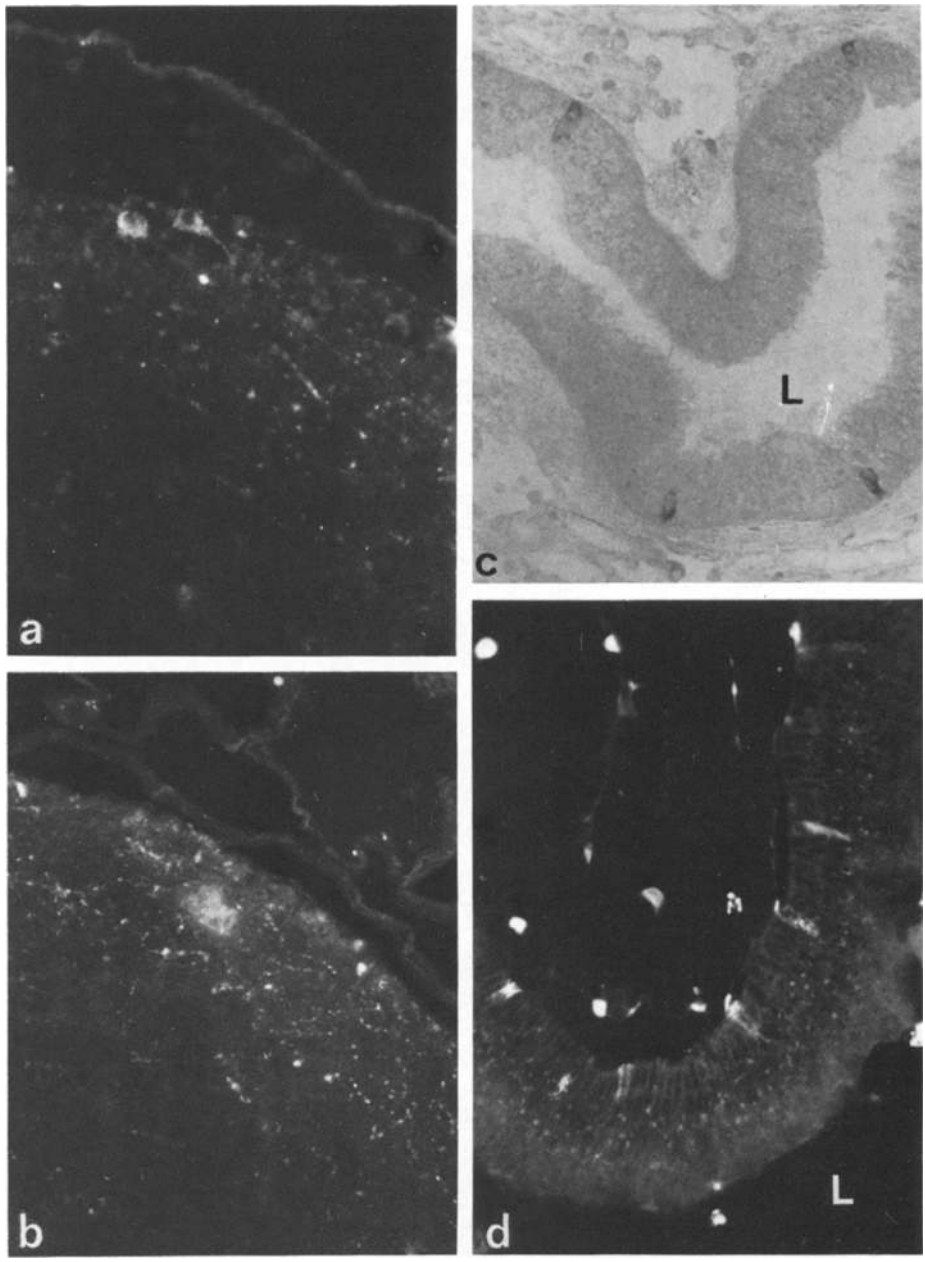


**Fig. 4a–d.** *Ciona intestinalis.* **a, b** Neurotensin-like immunoreactivity in perikarya and in fibres of the outer-layers of the cortex of the cerebral ganglion. Epithelial cells reacting with anti-neurotensin (PAP method) appear as scattered single elements in the alimentary tract (**c**). *L* lumen. **d** Paraformaldehyde-induced fluorescence in the alimentary tract (stomach). *L* lumen. a–b ×390; c ×300; d ×500

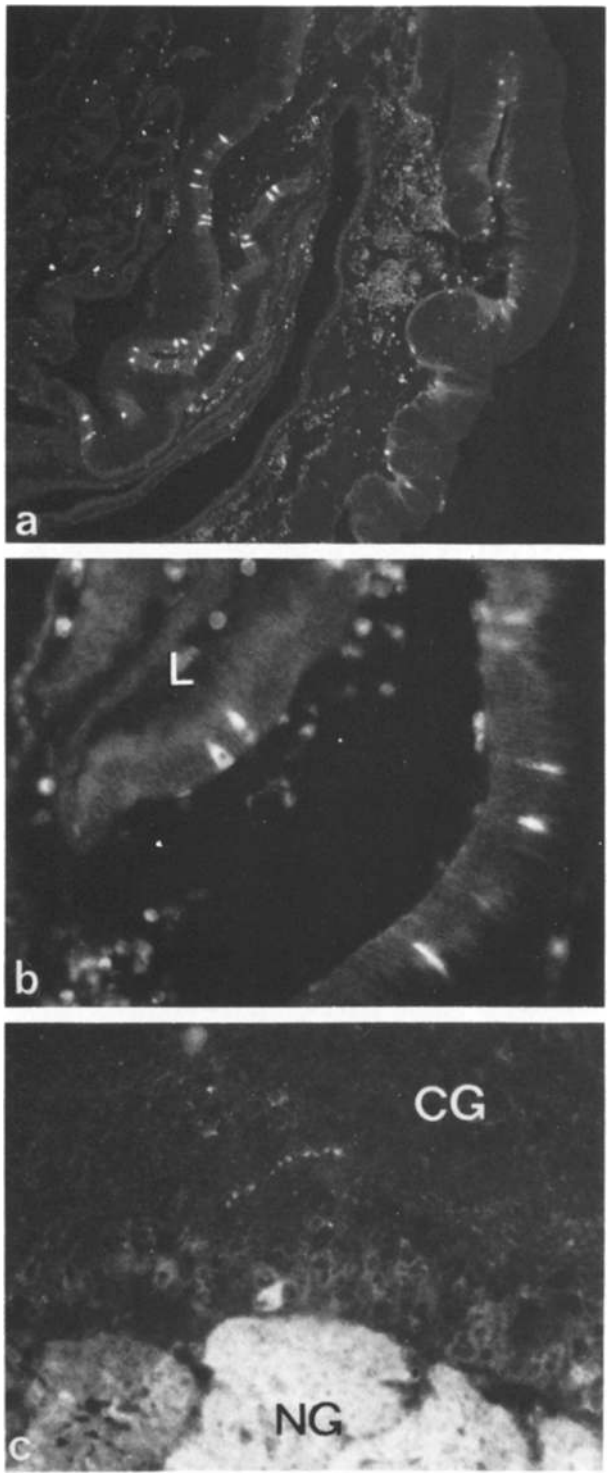


**Fig. 5a–c.** *Ciona intestinalis.* Immunofluorescence photomicrographs of epithelial cells of the oesophagus obtained with anti-5-OH-tryptamine (**a, b**). Note the density of 5-HT-immunoreactive cells (**a**). Immunofluorescence photomicrograph showing the cerebral ganglion after incubation with an antibody to secretin (**c**). Short processes emerge from the cells. *G* cerebral ganglion, *NG* neural gland. a ×250; b ×400; c ×390

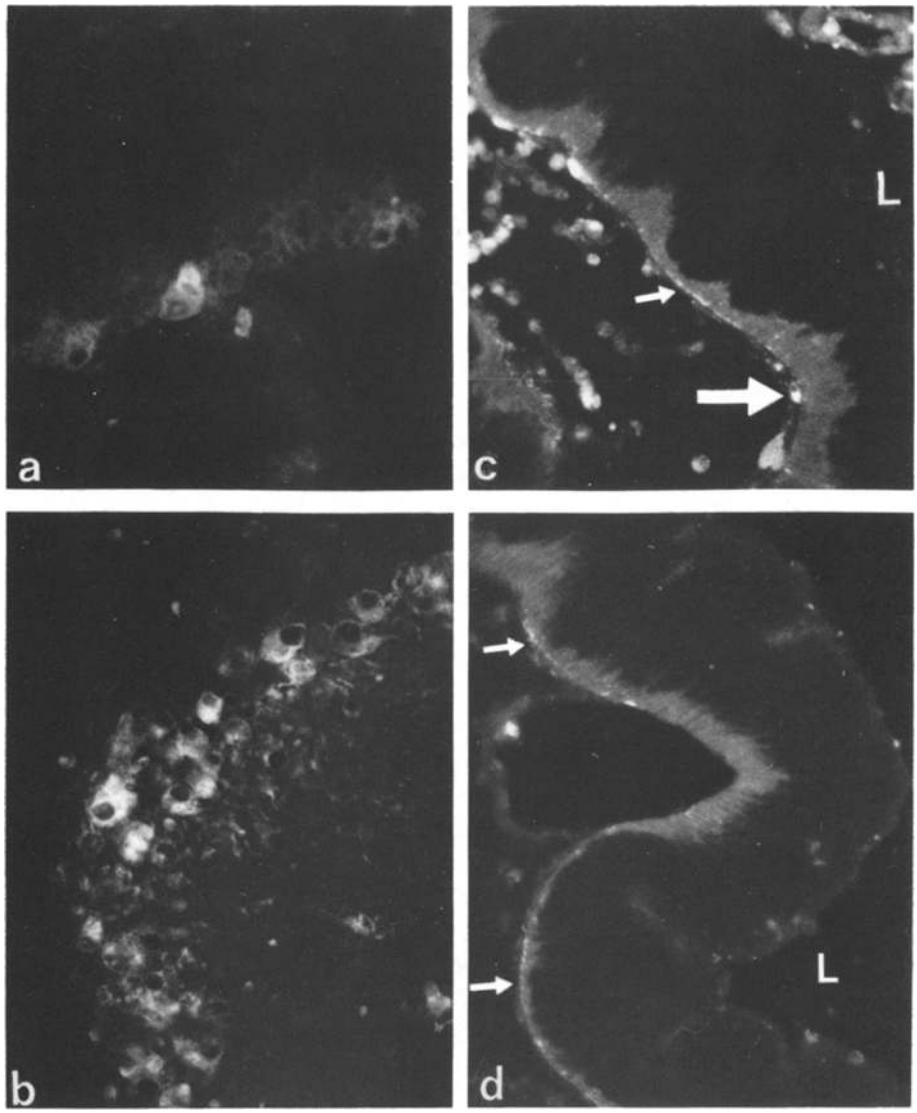


**Fig. 6a–d.** *Ciona intestinalis.* β-Endorphin-like immunoreactivity in the cortex of the cerebral ganglion (**a, b**) and in nerve fibers and small cells (*arrows*) underlying the epithelium of the oesophagus (**c, d**). *L* lumen. a–d × 390

the ventral and the dorsal areas of the ganglion. No axonal processes from these nerve cells could be localized by immunohistochemistry, and no β-endorphin immunoreactive perikarya or fibres were ever found in the inner layers of the cortex. In some immunoreactive perikarya, which apparently contained less antigen, there was a graded intensity of the fluorescence, which was highest at the basal part of the cell.

No epithelial cells of the alimentary tract were immunoreactive for β-endorphin. However, small β-endorphin-positive cells and thin fibres ran through the connective tissue underlying the epithelium of the oesophagus (Fig. 6a, d). Pre-absorption of anti-β-endorphin with its antigen abolished all immunoreactivity.

*Bombesin-like immunoreactivity*

A group of perikarya quite different from those described above reacted with an antibody to bombesin. In the cerebral ganglion itself only a few scattered, medium-sized nerve cells in the outer layers of the cortex showed bombesin-like activity. The majority of bombesin-immunoreactive nerve cells were found as single elements or in small groups at the beginning of the nerve trunks which leave the cerebral ganglion on both sides (Fig. 7a–c). In some cases they were arranged in a chain, with each cell giving rise to a short anti-bombesin-positive axon (Fig. 7a). Single, beaded nerve fibres reacting with anti-bombesin could be found in the medulla of the ganglion without immunoreactive perikarya in their immediate vicinity. These nerve fibres were preferentially arranged parallel to the longitudinal axis of the ganglion. Absorption of the antibody with bombesin abolished all immunoreactivity. In the alimentary tract bombesin-immunoreactive cells were seen only in the gill-bar epithelium of the pharynx. No immunoreactive nerves were seen (Fritsch et al. 1980b).

*Prolactin-like immunoreactivity*

An antiserum to hPRL demonstrated another population of perikarya in the cerebral ganglion entirely different from those already described in both number and position. The hPRL-positive cells mainly occupied the area of the cerebral ganglion facing towards the ciliated funnel and the neural gland (Fig. 8). They consisted of medium-sized to large, round to spindle-shaped nerve cells, distributed in several layers of the cortex. In no case were hPRL-immunoreactive perikarya observed either in the transitional zone or in the medulla. The positive cells tended to form groups (Figs. 8b, 9) and gave rise to hPRL-positive nerve fibres (Fig. 9). These beaded nerve-fibres seemed to form links between hPRL-immunoreactive perikarya of different sizes. In some cases immunoreactive fibres which run alongside the outer layer of the perikarya of the cortex can be seen to branch (Fig. 9). We have no evidence as to whether they form synapses with non-hPRL-immunoreactive cells and fibres. Small nerve cells reacting with anti-hPRL also appear in the area of the nerve trunks. Furthermore, beaded hPRL-positive nerve fibres ran in the connective tissue underlying the epithelium of the pharynx wall (Fig. 8a, arrows). Absorption of the antibody with hPRL abolished the staining. No positive staining was found in the alimentary tract.

*Pancreatic polypeptide-like immunoreactivity*

In the cerebral ganglion, next to the hPRL-immunoreactive perikarya, medium-sized to large perikarya reacted with an antibody to PP (Fig. 10a, b). Immunostaining for PP was limited to cell bodies and did not reveal immunoreactive processes. These PP-immunoreactive perikarya were mainly positioned in the outer layers of

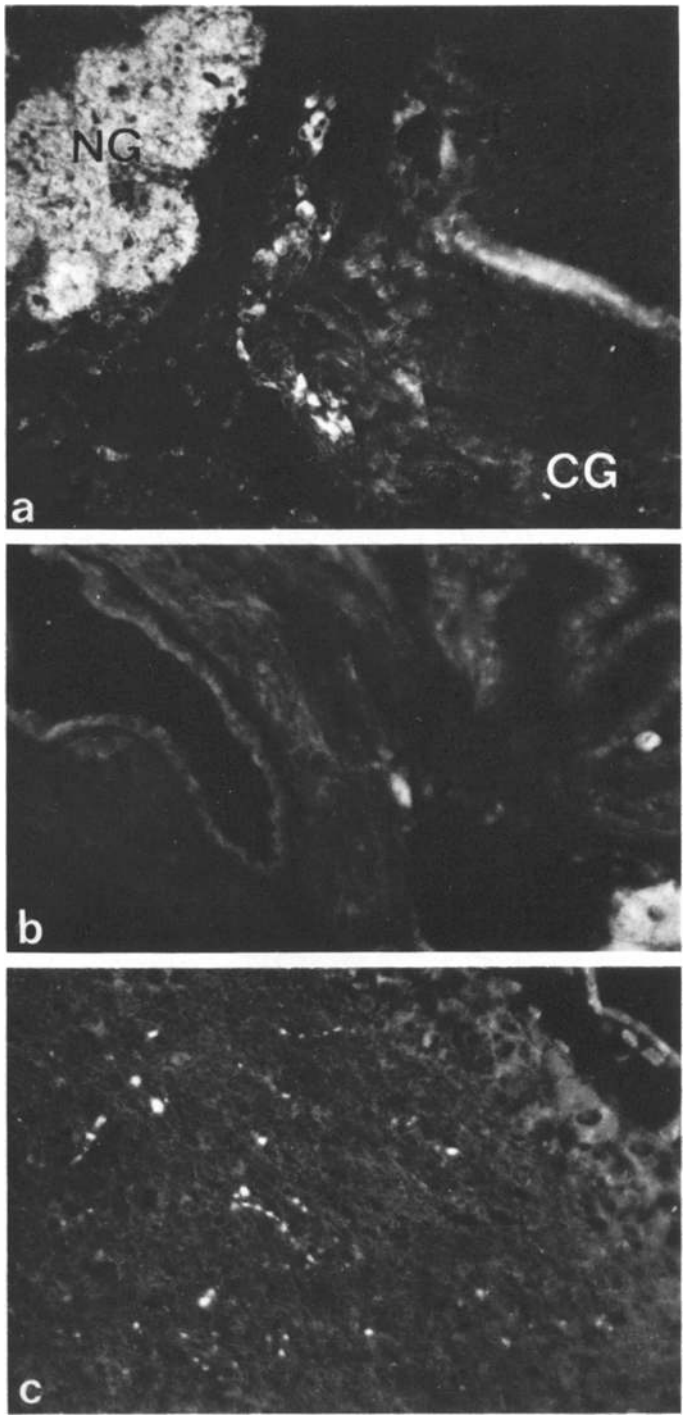


**Fig. 7a–c.** *Ciona intestinalis.* Immunofluorescence photomicrographs showing the cerebral ganglion. Bombesin-like immunoreactivity is seen in small to medium-sized perikarya in the proximal nerve trunks emerging from the ganglion (**a, b**). Short nerve fibers reacting with anti-bombesin can be observed in the medulla (**c**). *CG* cerebral ganglion, *NG* neural gland. a–c × 390

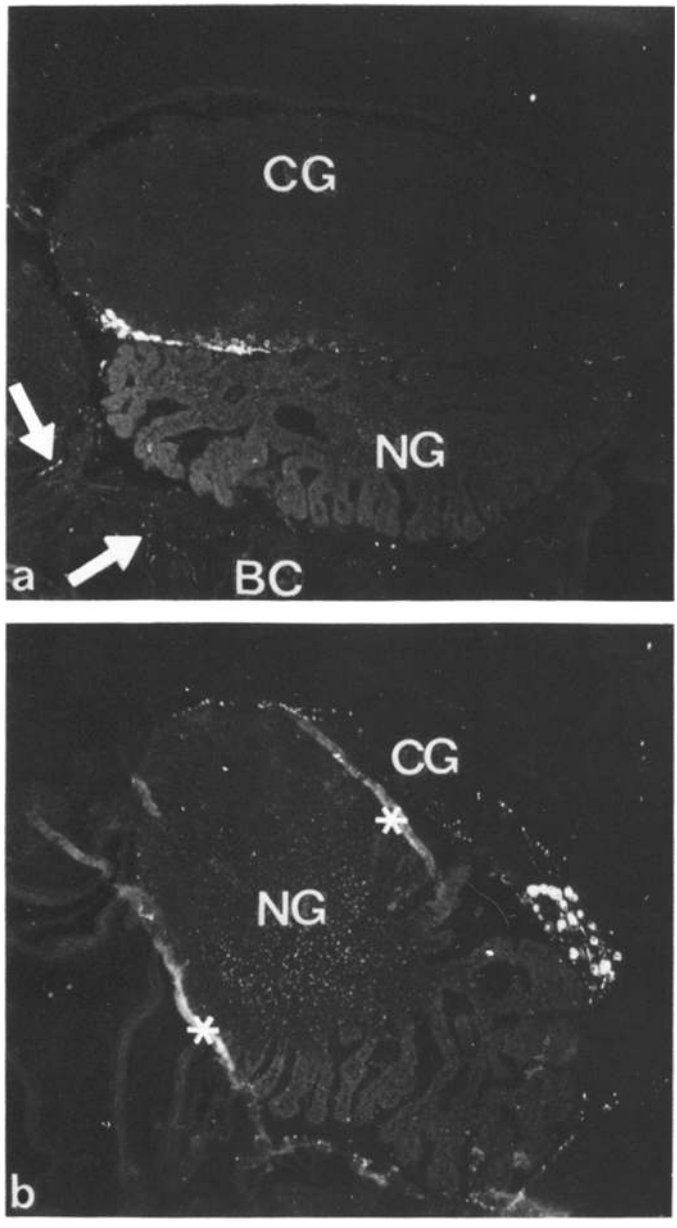


**Fig. 8a, b.** *Ciona intestinalis.* Immunofluorescence photomicrographs of the cerebral ganglion after incubation with an antibody to human prolactin (hPRL). Perikarya and nerve fibers in the outer layers of the cortex towards the neural gland (*NG*) react with anti-hPRL (**a, b**). Nerve fibres may appear underlying the epithelium of the buccal cavity (**a,** *arrows*). *CG* cerebral ganglion, *NG* neural gland, *BC* buccal cavity, * artefacts. a, b ×160

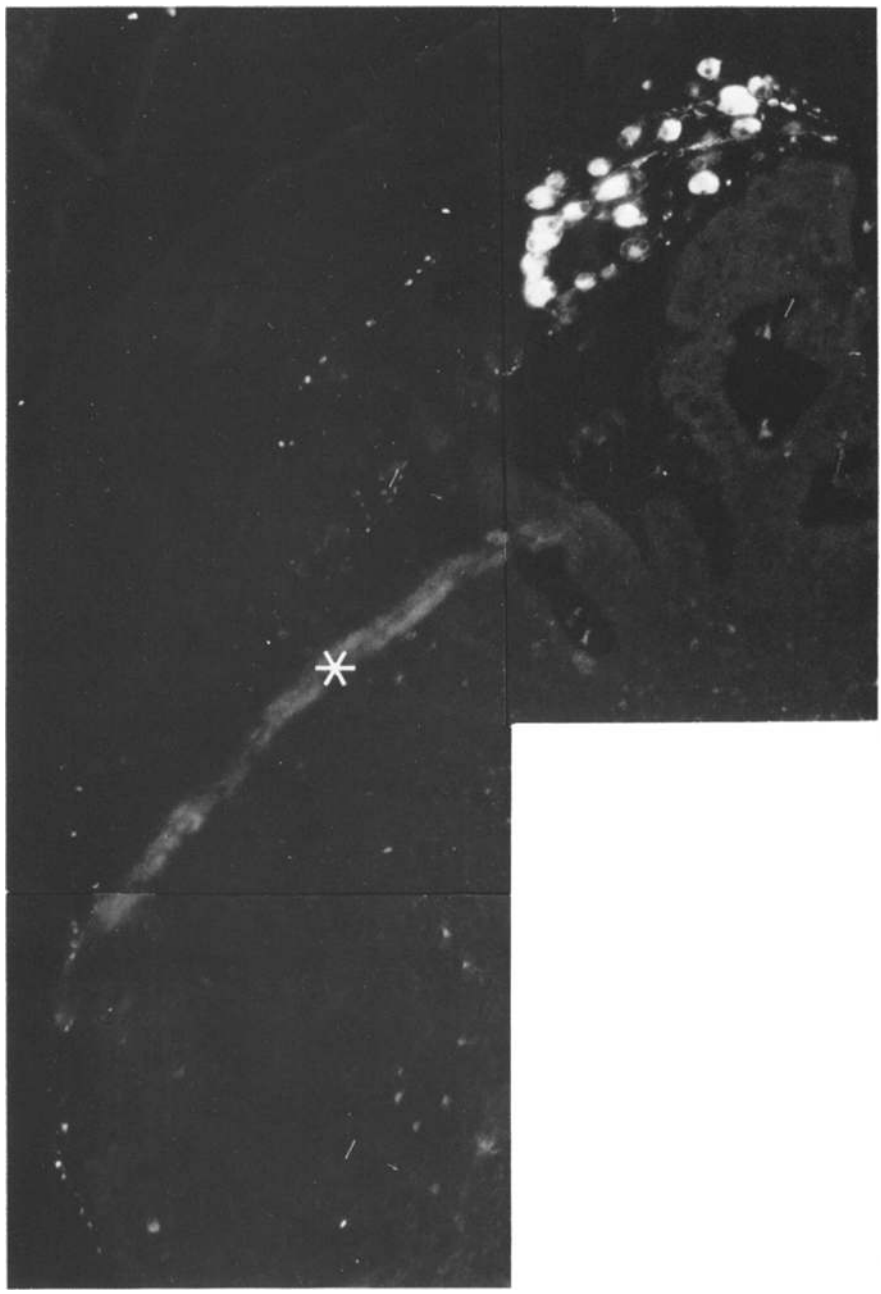

**Fig. 9.** *Ciona intestinalis.* Immunofluorescence photomicrograph showing a group of hPRL-immunoreactive perikarya and nerve fibers revealing a beaded structure in the cerebral ganglion. *NG* neural gland, * artefacts. × 390

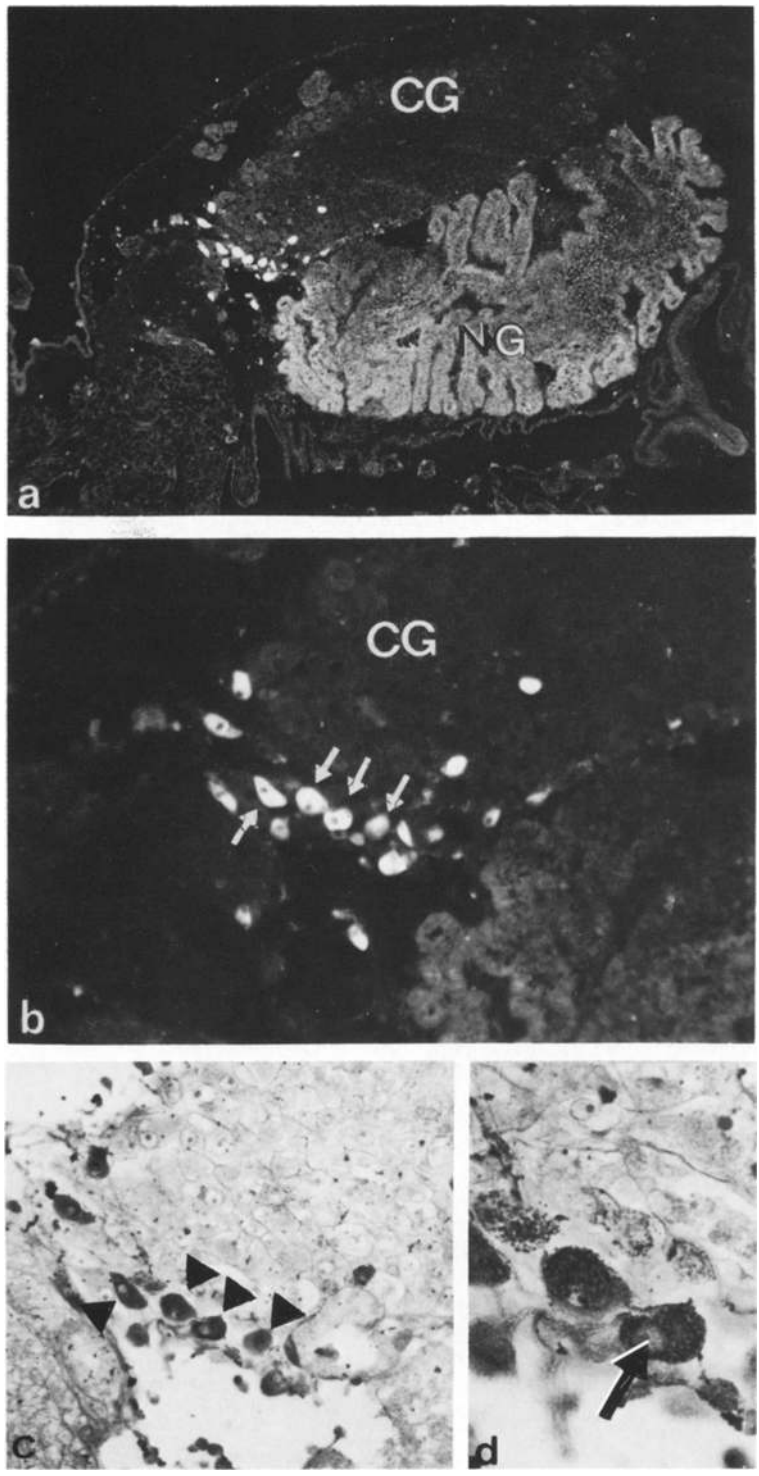


**Fig. 10a–d.** *Ciona intestinalis.* Immunofluorescence photomicrograph of the cerebral ganglion. PP-immunoreactive perikarya in the cortex form small cell groups in the area where the ciliated funnel emerges from the neural gland (**a, b**). The PP-immunoreactive perikarya are argyrophilic with the Grimelius stain (**c, d**). *CG* cerebral ganglion, *NG* neural gland. a ×160, b ×270, c ×300, d ×750 (Oil immersion)

the cortex of the ganglion, in contact with the neural gland duct. There was an accumulation of PP-like perikarya next to the ciliated funnel, and some more single, spindle-shaped PP-immunoreactive cells were present in the connective tissue layer underlying its epithelium (Fig. 11 b). It could be seen from serial sections that PP-immunoreactive and hPRL-immunoreactive cells lay next to each other (Fig. 11 b, c).

Anti-PP serum absorbed with hPRL reacted in the same way as anti-PP applied alone and positive immunoreactivity was obtained after absorption of anti-hPRL serum with PP. No immunoreactivity could be observed, however, after quenching anti-hPRL or anti-PP with their particular antigens.

In the alimentary tract anti-PP showed single, irregularly scattered cells in the epithelium, resting on the basal lamina (Fig. 11 d). Their filamentous apical processes reached the surface of the epithelium. These cells were observed mainly in the upper intestine, proximal to the stomach.

Absorption controls were the same as for the cerebral ganglion.

*VIP-like immunoreactivity*

With an antibody to N-terminal vasoactive intestinal polypeptide immunoreactivity was found in small perikarya of the cortex and in nerve fibres of the medulla and transitional zone of the cerebral ganglion. The fibres reveal a beaded structure without any preference in direction. They form an irregular meshwork in the medulla. Approaching the transitional zone the VIP-like fibres become sparse. No immunoreactive axons rising from the perikaryon could be observed.

In the alimentary tract no immunoreactivity to N-terminal VIP was found. Preabsorption of the antibody with its antigen prevented the immunoreaction in the cerebral ganglion.

*Motilin-like immunoreactivity*

Single scattered perikarya at the inner layer of the cortex reacted with an antibody to motilin. They were medium-sized cells with neither immunoreactive axons leaving the perikaryon nor any other immunoreactive fibres in one of the zones of the cerebral ganglion. A few small to medium sized cells immunoreactive to motilin were placed in the medulla. The alimentary tract did not reveal any immunoreactivity to motilin. Preabsorption of anti-motilin with its antigen abolished all immunoreactivity.

*Neural gland*

With some of the polypeptide antibodies a positive reaction occurred in the epithelium of the neural gland, particularly in regions adjacent to the cerebral ganglion. The results obtained with control reactions were, however, not sufficiently clear to describe the neural gland conclusively as a source of the relevant peptides. We think it unlikely that the epithelial neural gland is a source of any of the peptides studied. Further work is in progress on this part of the cerebral complex.

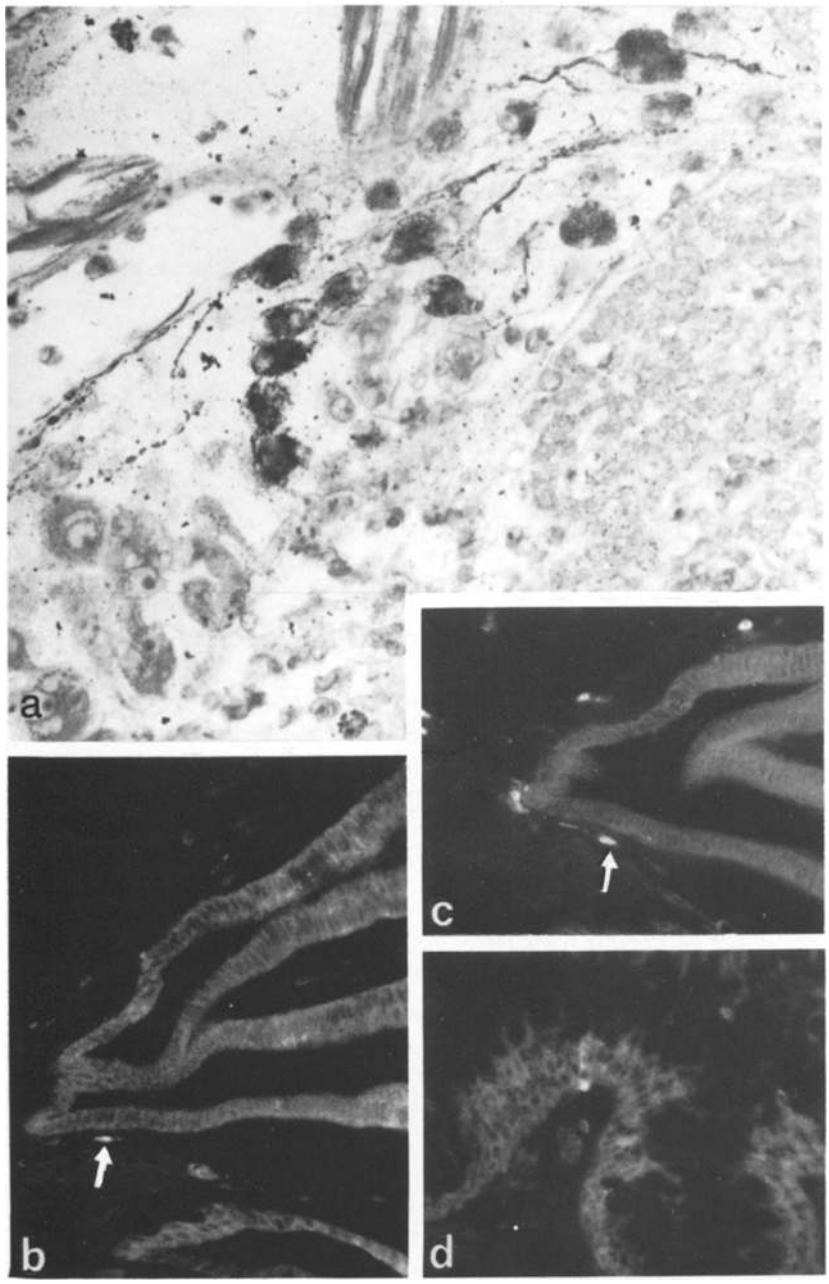


**Fig. 11a–d.** *Ciona intestinalis*. Cerebral ganglion (**a**). Argyrophilic perikarya and nerve fibres correspond to hPRL-like neurons as shown in Fig. 9. PP-like (**b**) and hPRL-like (**c**) immunoreactivity can be observed in small cells which are localized next to each other underlying the epithelium of the proximal ciliated funnel. **d** Single, scattered PP-immunoreactive epithelial cells occur in the upper intestine. a ×480, b–d ×390

*Histochemical methods*

After immunostaining and photography the sections were stained for argyrophilia by the Grimelius method. In Bouin-fixed material argyrophili perikarya and nerve fibres could always be demonstrated. If the tissues were fixed either in MFF or 6% glutaraldehyde, the sections had to be post-fixed with Bouin's fluid in order to obtain positive results.

In the cerebral ganglion there were argyrophil perikarya and nerve fibres (Fig. 11a) that were identical with the hPRL-immunoreactive nerve cells and fibres shown in Fig. 9. There was branching of some of the beaded nerve fibres which ran between the non-argyrophil perikarya. Perikarya reacting with anti-PP were also argyrophil (Fig. 10c, d). At higher magnifications fine granules could be seen in the cells which did not extend into the axons. The area of the nucleus was always free from granules (Fig. 10d, arrow). The presence of argyrophil and argentaffin endocrine cells in the alimentary tract of *Ciona intestinalis* has already been described (Fritsch 1976).

*Electron-microscopical results*

Ultrastructural examination confirmed the light-microscopical description of the ganglion as divided into cortex, transitional zone and medulla. In the cortex the perikarya are densely packed and interspersed with axons of variable diameter, which contain a number of organelles, including granules (Fig. 12). The perikarya can be distinguished morphologically by these characteristic secretory granules which vary greatly in number. Most obvious are medium-sized to large perikarya which contain electron-dense granules 150–200 nm in diameter (Fig. 12). The nucleus in the centre of the cell contains a large nucleolus and the secretory granules are evenly distributed throughout the cytoplasm. The granules are bounded by a membrane, separated from the content by a narrow halo. A considerable proportion of the cytoplasm is filled with glycogen. The endoplasmic reticulum forms vesicles or short irregular tubules.

There is a second population of perikarya, with a similar cytoplasmic appearance, but containing smaller electron-dense granules with a narrow halo and an average diameter of 100–150 nm.

These granules mainly occupy the periphery of the cell, while the centre of the perikaryon next to the nucleus is filled with vesicles of the endoplasmic reticulum. Another cell type, characterized by a large amount of laminated rough endoplasmic reticulum (Fig. 13a) contains highly electron-dense granules with a mean diameter of 200 nm. These perikarya are preferentially located in the outer layer of the cortex. Their granules, which also have a narrow halo, occupy the periphery of the cell.

A fourth type of small- to medium-sized perikarya, located in the outer layers of the cortex, contain larger electron-dense granules, of more then 200 nm in diameter, with a minimal halo. The shape of the granules varies from round to oval and from short to elongated (Fig. 13b). In general, these cells show a well developed, laminated, rough endoplasmic reticulum.

In the inner layers of the cortex a fifth type of perikaryon can be distinguished with medium electron-dense granules of 100–200 nm in diameter. The variably

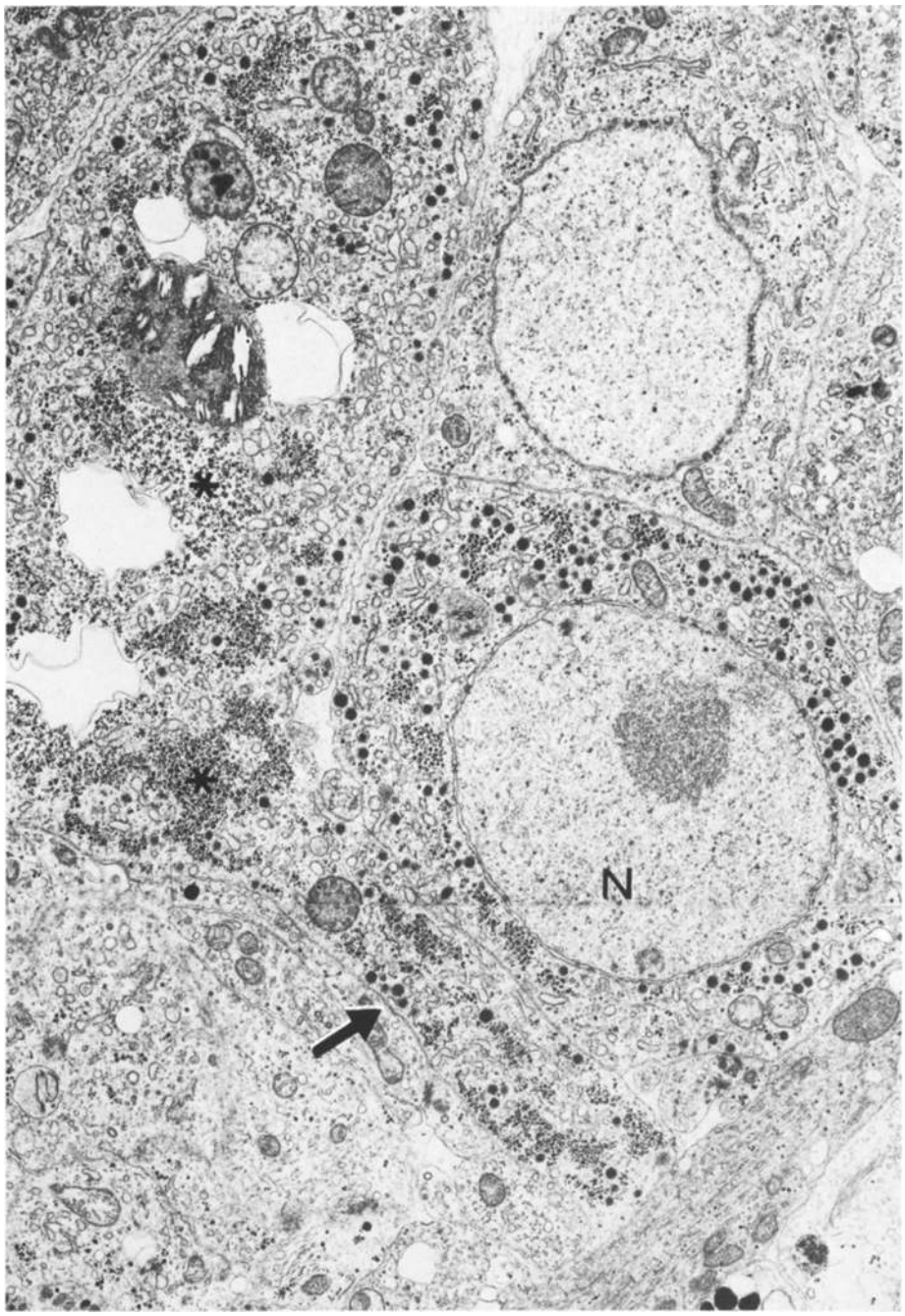


**Fig. 12.** *Ciona intestinalis.* Ultrastructure of perikarya in the cortex of the cerebral ganglion. The medium-sized perikarya contain electron-dense granules with a narrow halo and a mean diameter of 150–200 nm. *N* nucleus, * glycogen. ×9,000

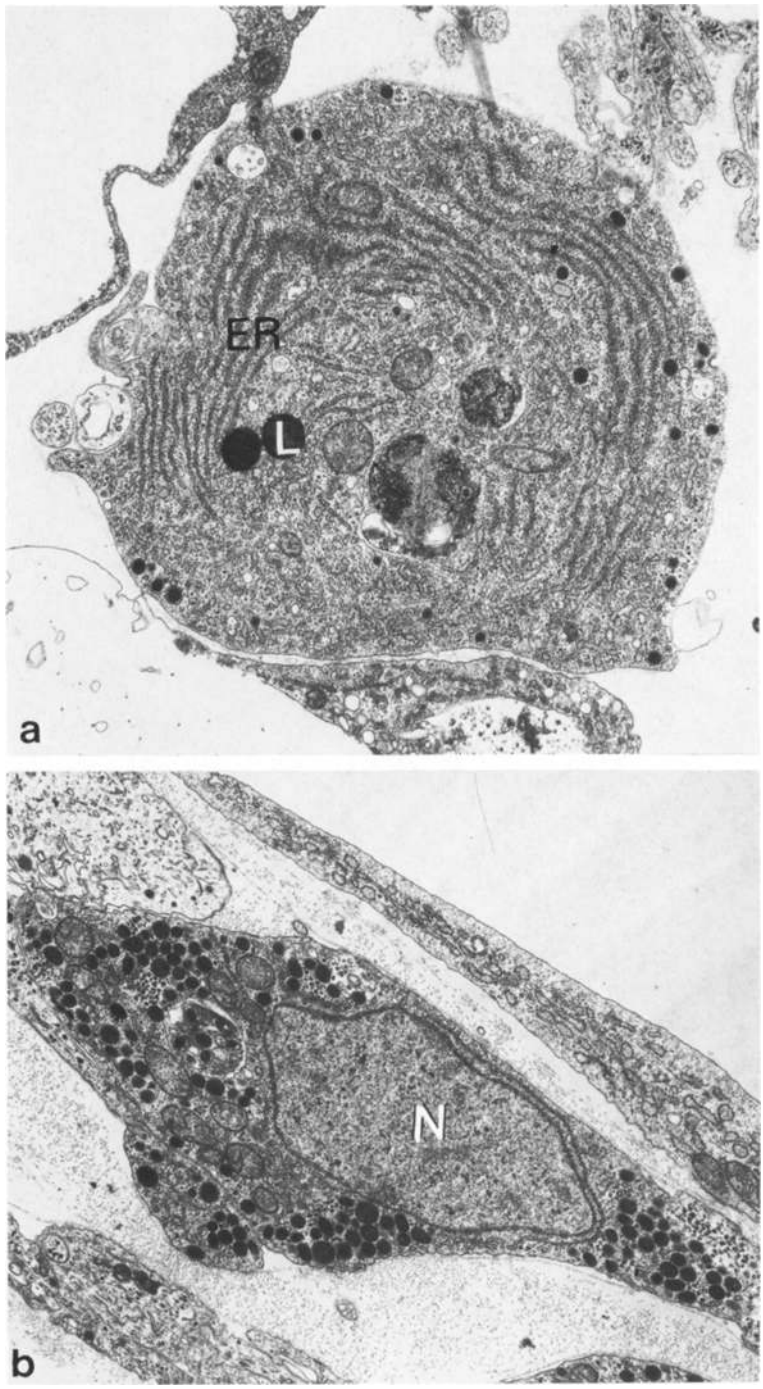


**Fig. 13a, b.** *Ciona intestinalis.* Ultrastructure of other neuron types in the outer layer of the cortex of the cerebral ganglion (**a, b**). Apart from electron-dense secretory granules of different sizes and shapes these cells are characterized by a laminary arranged RER. *L* lysosome. *ER* endoplasmic reticulum, *N* nucleus. × 8,000

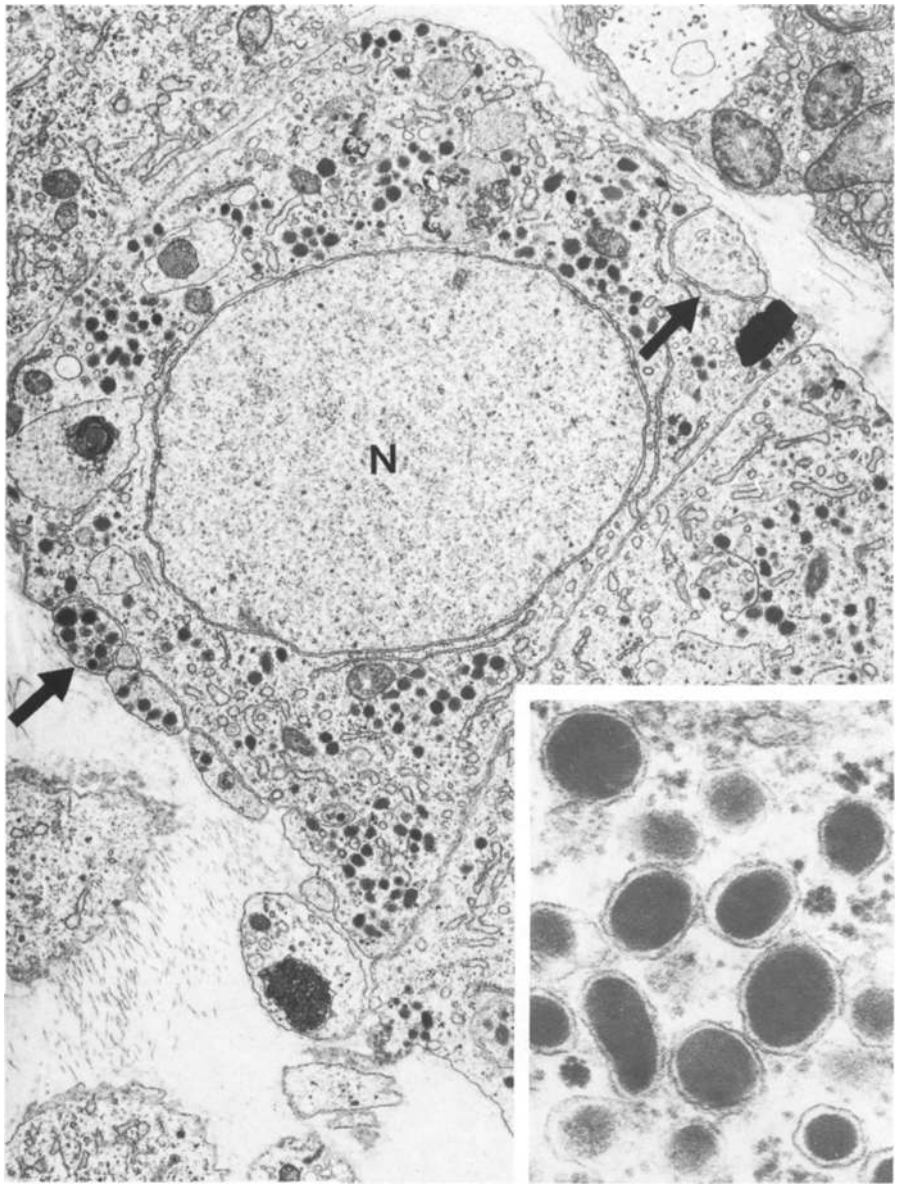


**Fig. 14.** *Ciona intestinalis.* Perikaryon from the cortex of the cerebral cortex containing secretory granules ( ⌀ 150–200 nm). Attached to the perikaryon nerve fibres can be observed (*arrow*). *Inset:* Electron-dense granules with a halo of the perikaryon. *N* nucleus. ×12,000. *Inset* ×28,000

narrow halo surrounding the granules is not very distinct, and may be filled with diffuse non-homogeneous granular material. The granule membrane is often irregular but sometimes completely smooth.

All the described types of secretory granule can be observed in the medullary and transitional zone nerve fibres. Their number and distribution varies according

to the thickness of the fibres. When these are thin, they are lined up in a row. The fibres seem to have "boutons terminaux" that contain the same type of granules, mixed with many small vesicles.

In the cortex, nerve fibres run between the perikarya and in membrane contact with them. As well as simple attachments between perikarya and nerve fibres there are contacts in which a nerve fibre is partly surrounded by the plasmalemma of the perikaryon (Fig. 14).

The ultrastructure of endocrine cells in the alimentary tract has been reported previously (Fritsch and Sprang 1977; Tunas 1977).

## Discussion

Invertebrates rely mainly on neurosecretory regulation for control of their bodily functions (Scharrer 1978), while vertebrates have developed "true" hormonal regulation from endocrine cells either scattered singly or aggregated into glands. They also have, or have retained, a newly discovered system of peptide-containing nerves.

The importance of *Ciona* as a test animal in this study is that the protochordates, of which it is a member, are generally considered to be the living representatives of ancestral animals common to the invertebrate and vertebrate lines of evolution. It is thus of great interest to discover (i) whether these animals have a variety of regulatory peptides such as are found in vertebrates, and apparently also in invertebrates (Schot et al. 1981; Duve and Thorpe 1980; Thorndyke and Bevis 1978; Thorndyke and Probert 1979), and (ii) whether these substances are disposed mainly in the nervous system in the invertebrate fashion or in endocrine cells or glands following the vertebrate trend.

The results of this survey indicate that *Ciona* may truly represent a vertebrate near-relation. Peptides are present in great variety and are intriguingly located both in nerves, particularly in the cerebral ganglion, and in what appear to be endocrine cells dispersed in varying concentrations along the alimentary tract. We have as yet no proof as to whether the function of these cells is endocrine, releasing secretion into the blood to act on a distant target, or paracrine, acting on neighbouring cells by diffusion. Similarly, we know little of the structure of the peptides we have identified with antibodies raised to vertebrate antigens. The most we can say at this stage is that different peptides containing antigenic sites identical with those in mammalian peptides are present; of their actions we can say nothing. Recent work suggests that gastric secretion in another protochordate *Styela clava,* is stimulated by a peptide (CCK) although the mechanism is not yet clear (Thorndyke and Bevis 1980). Even in mammals, however, the physiological roles and significance of most of the known peptides are still obscure.

Investigation of the similarities and differences between *Ciona* and its vertebrate relations, with respect to the localization of immunoreactivity to individual peptide antibodies, may help to define some evolutionary trends.

### *Neurotensin*

Neurotensin in higher vertebrates is present in nerve fibres in the central nervous system (Carraway and Leeman 1973, 1975, 1976), but in the gut it is found almost

exclusively in endocrine cells. The same localization seems to apply in fish and cyclostomes. Although in 11 species of teleost fishes Langer et al. (1979) found neurotensin only in immunoreactive nerves supplying the gut, Reinecke et al. (1980) have immunostained neurotensin cells in the gut of teleost and elasmobranch fishes, of a cyclostome and, in agreement with the present work, of *Ciona intestinalis.*

The possible biological actions of neurotensin in mammals include the inhibition of gastric secretion and motility (Blackburn and Bloom 1981), and the predominant localization of neurotensin in the lower intestine suggests a postdigestive role perhaps concerned with the absorption of fat.

Neurotensin-like substance occurs widely throughout the animal kingdom (Carraway 1981), differing in structure from species to species, but with marked conservation of the biologically active C-terminal portion of the molecule. Several different molecular forms may exist in one animal: thus an antibody to xenopsin, an amphibian skin peptide that is structurally similar to neurotensin, will identify an additional population of cells with neurotensin-like immunoreactivity in many animals (Reinecke et al. 1980).

In its nervous system location, in vertebrates, neurotensin presumably functions as a neurotransmitter, neuromodulator or releasing factor.

Evaluation of the molecular structure of "neurotensin" *Ciona* may provide some clues as to its origin and relationships. It seems certain, at least, that neurotensin-like molecules have existed from an early stage in the emergence of the vertebrates and that *Ciona* represents a link between the invertebrates and vertebrates in having its members of the neurotensin family in a dual localization.

### *5-Hydroxytryptamine*

The presence of 5-HT in ascidians was described biochemically by Erspamer (1946). Applying the FIF-method a distinct fluorescence can be obtained in certain cells in the alimentary tract of *Ciona intestinalis*, which is compatible with the production of biogenic amines. Results corresponding with this were obtained with an antibody to 5-HT (serotonin), and both correspond with the distribution of argyrophilic and argentaffin cells in the alimentary tract (Fritsch 1976).

In vertebrates, serotonin has been considered to be involved in acid secretion in the stomach (Black et al. 1958) as well as being a neurotransmitter. However, in this study it could not be demonstrated in the cerebral ganglion.

### *Secretin*

Until recently secretin was recorded only in endocrine cells, containing small secretory granules, the S cells, in the mucosa of the mammalian duodenum and upper jejunum (Polak et al. 1971 a, b; Bloom 1974). Latest indications, however, are that secretin-like substances are also present in the brain (Mutt et al. 1979). Thus the localization of secretin-like immunoreactivity in the alimentary tract and in the cerebral ganglion of *Ciona* correlates well with its distribution in mammals. Secretin-like activity was found by bioassay in intestinal extracts of cyclostomes (Barrington and Dockray 1970; Nilsson 1973), and Bevis and Thorndyke (1979) demonstrated secretin-like immunoreactivity in the epithelium of the stomach in

another protochordate, *Styela clava*, in cells that also showed other APUD-characteristics previously defined.

The observations of Larsson and Jørgensen (1978) on the differentiation of endocrine cells in the mammalian gastrointestinal tract seem to be of phylogenetic importance in this context. These authors described a developmental stage of secretin-producing cells, during which the cells could be shown with the FIF-method to synthesize and store a biogenic amine. Similar results have been obtained for entero-endocrine cells in larvae fish (Rombout et al. 1978).

### *β-Endorphin*

The demonstration of β-endorphin-like immunoreactivity in certain perikarya in the cerebral ganglion, and in nerve fibres of the oesophagus but not in endocrine cells suggests a neurotransmitter and/or neuromodulator function. The endorphins (ENDOgenous moRPHIN) are a group of opiate-like substances isolated from the CNS and the pituitary gland of different mammalian species (Guillemin et al. 1976; Guillemin 1977). Although β-endorphin contains the entire sequence of met-enkephalin in the N-terminal part of the molecule (Cox et al. 1975; Li and Chun 1976) and is itself a part of the precursor β-LPH-molecule (Guillemin 1977), we observed no cross-reactivity of endorphin-positive cells with antibodies to leu- or met-enkephalin. Endorphins in mammals are concentrated mainly in the anterior and intermediate lobes of the pituitary gland, where they are localized in corticotrophic cells (Polak et al. 1978a; Begeot et al. 1978); low concentrations only were found in the CNS (Guillemin 1977). The pituitary of the trout (*Salmo irrideus*) contains β-endorphin immunoreactivity in corticotrope (ACTH), and melanotrope (MSH) cells (Follénius and Dubois 1978). The results obtained by Mains et al. (1977) are of particular interest from a phylogenetic viewpoint, showing that in pituitary tumor-cell lines both ACTH and endorphin are parts of a large precursor molecule. These findings were confirmed by Guillemin et al. (1977). In *Ciona intestinalis*, however, ACTH-like immunoreactivity was not observed.

### *Bombesin*

Bombesin-like immunoreactivity was found in the cerebral ganglion of *Ciona* in a restricted localization in the medulla, and in the roots of the bilateral nerve trunks leaving the ganglion. No cortical perikarya reacted with anti-bombesin, using an antibody that did not cross-react with substance P, a peptide sharing a C-terminal amino acid sequence with bombesin. Fritsch et al. (1980b) reported the presence of bombesin-immunoreactive cells in the epithelium of the pharynx of *Ciona intestinalis*. Thus there is a dual localization of a bombesin-like substance at a low phylogenetic stage.

Bombesin is one of the peptides first isolated from amphibian skin (Erspamer and Melchiorri 1973). There is now sufficient evidene that bombesin, among other amphibian peptides, occurs in the gut and nervous system of other vertebrates (Polak et al. 1976, 1978b; Walsh and Holmquist 1976; Pearse et al. 1977; Polak and Bloom 1979; Lechago et al. 1978; Moody and Perk 1979; Dockray et al. 1979; Timson et al. 1979; Langer et al. 1979). Contrary to the general trend, the early

finding of bombesin-like immunoreactivity in mammalian gut cells has not yet been substantiated at least in the adult stage (Walsh et al. 1981), though it is present in nerves and is also found in epithelial cells in the foetal lung (Wharton et al. 1978). In birds and some lower vertebrates bombesin-like peptides are found in cells in the gastrointestinal tract as well as in the nervous system (Lechago et al. 1978; Walsh et al. 1981).

*Prolactin*

With an antibody to hPRL, immunoreactive perikarya and nerve fibres were found in a characteristic position in the cerebral ganglion. The single prolactin-immunoreactive cells are connected by nerve fibres which either run within the cortex, parallel to the surface, to still unknown target neurons, or leave the ganglion in order to seek contacts in the periphery.

In all vertebrate species prolactin is found in epithelial cells in the anterior pituitary, and it is responsible for a variety of physiological effects (cf. Bern 1967). An exclusive occurrence in the anterior pituitary was denied, however, by Fuxe et al. (1977a), who found prolactin-like immunoreactivity in nerve terminals of several hypothalamic areas. Prolactin thus joins other adenohypophyseal hormones such as $\beta$-endorphin, ACTH, (Krieger et al. 1977) and $\beta$-MSH (Schuster et al. 1977) as occurring also in the CNS.

The occurrence of a substance related to the anterior pituitary hormone, prolactin, in the cerebral ganglion, leads us to re-open the discussion on whether the cerebral complex of protochordates is a structure homologous to the vertebrate pituitary (Julin 1881). Despite several attempts to confirm this early proposal (Willey 1893; Elwyn 1937; Dodd 1955, 1975) there is as yet no support for it from embryological investigations. Nevertheless Lender and Bouchard-Madrelle (1964) were able to prove that the entire cerebral complex in *Ciona intestinalis* is of neural origin. Takor Takor and Pearse (1975) suggest that the anterior pituitary is similarly derived, and the finding of the brain-specific protein S-100 in the stellate cells of the pars distalis (Cocchia and Miani 1980; Nakajima et al. 1980) appears to support this contention. Thus the peptide-secreting cells described here and previously (Fritsch et al. 1979) in the cerebral ganglion and, by inference, in the alimentary tract as well can be considered to derive from neuroendocrine-programmed ectoblast, which includes neural crest and neuroectoderm (Pearse 1978).

*Pancreatic polypeptide (PP)*

Next to the prolactin-like neurons in the cerebral ganglion there are medium-sized to large perikarya immunoreactive with an antiserum to bovine PP. No immunoreactive nerve fibres can be observed. In a second localization, PP-immunoreactive cells were found in the epithelium of the alimentary tract. PP is a polypeptide composed of 36 amino acids (Kimmel et al. 1971, 1975; Lin and Chance 1974). It was first demonstrated in a specific endocrine fourth cell-type of the pancreas and in the gastrointestinal tract of mammals and birds (Larsson et al. 1974, 1975, 1976a; Sundler et al. 1977d; Alumets et al. 1978; Rawdon and Andrew

1979; Rahier et al. 1979) and pancreatic islets of teleosts (Klein and Van Noorden 1980; Langer et al. 1979; Van Noorden and Patent 1978). At present PP is still among the "candidate hormones" of unknown physiological relevance (Kimmel et al. 1978; Lin and Chance 1978; Floyd et al. 1978). Recent perfusing studies in volunteers revealed, however, that in man PP inhibits pancreatic and biliary secretion in both the fasting state and during low dose stimulation of the exocrine pancreas. These actions of PP on the exocrine pancreas and gallbladder are directly opposite to those of CCK (Adrian et al. 1981).

The probability that it has widespread actions is supported by the findings of Lorén et al. (1979), who demonstrated PP-immunoreactivity in the central and peripheral nervous system of mammals and birds. These authors were able to show immunoreactivity for PP in the cerebral and sub-oesophageal ganglia of the earthworm *Lumbricus terrestris* (Sundler et al. 1977a). In the water snail, *Lymnaea stagnalis,* PP-like immunoreactivity is present in the nervous system (Schot et al. 1981). We have noted small perikarya and nerve fibres with VIP-like immunoreactivity in the cerebral ganglion of *Ciona intestinalis*, but a particular association with PP-immunoreactive neurons has not been established.

The PP-immunoreactive neurons of *Ciona* are, however, closely associated with neurons containing hPRL-like immunoreactivity in the region of the ciliated funnel. In view of the present lack of knowledge of the physiological role of PP further considerations can only be speculative. The findings of Lorén et al. (1979), Sundler et al. (1977a, d) and Schot et al. (1981) in vertebrates and invertebrates, and the findings described here in a species representing a link between the two groups, make it probable that PP belongs to the growing list of peptides with an ancient history of neurotransmitter and/or neuromodulator functions.

*Vasoactive intestinal polypeptide (VIP)*

For several years VIP was considered to be a gut hormone, but in 1976 it was shown that VIP has a widespread distribution in the body localized in neurons (Bryant et al. 1976; Larsson et al. 1976b; Said and Rosenberg 1976), including perikarya and nerve fibres in the CNS (Fuxe et al. 1977b). In its molecular structure it is closely related to secretin, and to a lesser extent, to pancreatic glucagon and to gastric inhibitory polypeptide. Only secretin could be demonstrated as well in the cerebral ganglion of *Ciona*, but in a different cell population and only in a few nerve fibres. The wide distribution of VIP, its predominant presence in neurons, and its ability to influence many body functions make it unlikely that the peptide functions as a circulating hormone. It seems rather probable that it serves as a product of paracrine secretion, i.e. as a local hormone, a neurotransmitter, or a neuromodulator, both in the central and the peripheral nervous systems (Said 1981). It may be suggested that this could be true for the occurrence of VIP-immunoreactivity in the cerebral ganglion of *Ciona* as well.

*Motilin*

Availability of a specific anti-motilin antibody made it possible to study the distribution of motilin-immunoreactive perikarya in the cerebral ganglion, but not in the alimentary tract, where no immunoreactivity to motilin was observed.

It has been well recognized that motilin cells are populated in the duodenum and upper jejunum in man, monkey, dog and pig (Pearse et al. 1974; Forssmann et al. 1976; Tobe et al. 1976; Heitz et al. 1978; Helmstaedter et al. 1979). Most of these cells were found to be enterochromaffin. This could not be shown for the alimentary tract of *Ciona intestinalis*, however.

More interestingly, it has been found that motilin-containing neurons are present in the submucosa and muscle layers of the mammalian gut. A motilin-like immunoreactivity was found in brain tissue extract by Yanaihara et al. (1978) and indeed motilin-containing neurons were recently found in the central nervous system of mammalia (Chey and Lee 1980). It is interesting to notice that motilin, unlike other well-recognized gut hormones, does not share a molecular similarity with either the secretin or gastrin family.

*Other peptides*

We have already reported the presence of perikarya containing somatostatin, substance P, and calcitonin-like immunoreactivity (Fritsch et al. 1979).

*Argyrophilia*

In the cerebral ganglion argyrophil cells stained with the Grimelius' technique (Grimelius 1968) were found to be identical with the hPRL- and PP-immunoreactive neurons and argyrophil perikarya and nerve fibres were previously described in the cerebral ganglion of *Ciona intestinalis* (Fritsch et al. 1979), some of them identified with calcitonin cells. For *Ciona*, specific correlation with other immunoreactive cells has not so far been achieved, although in mammals and fishes pancreatic PP cells have been shown to be argyrophilic (Gepts et al. 1978; Klein and Van Noorden 1980).

*Ultrastructure*

Five cell types containing characteristic granules can be distinguished in electron-microscopic preparations of the neural ganglion. These granules are also present in the nerve fibres, and their structure, size and shape provide for characterization along the line of the "Lausanne classification" of gastro-entero-pancreatic endocrine cells (Solcia et al. 1978). By contrast, the immunohistochemical results reveal a considerably greater number of different cell types, so that correlation of a distinct polypeptide to a single granule type, on the basis of its ultrastructural appearance, is insufficient. This has already been indicated for the ultrastructure of endocrine cells in the alimentary tract of *Ciona intestinalis* (Fritsch and Sprang 1977) and for the pancreas of a teleost (Klein and Van Noorden 1980).

*Conclusion*

The finding of identical peptides in the central and peripheral nervous system and in the endocrine cells of the alimentary tract of a protochardate, supports the concept

of the unity of the Diffuse Neuroendocrine System, from its origins in invertebrates to the complex control system which it represents in vertebrate species. Conservation of molecular structure during evolution has been shown for calcitonin (Fritsch et al. 1980a; Girgis et al. 1980) and even further, to the limits of animal life, for insulin (Le Roith et al. 1980). In all Metazoa peptidergic neurons serve a variety of neurohormonal and local neuroregulatory functions. The multiplicity of possible mechanisms of control by neurohormones in invertebrates has been discussed by Scharrer and Weitzmann (1976) und Frontali and Gainer (1977), and the demonstration in *Ciona* of the "new" neuropeptides, substance P, bombesin, and $\beta$-endorphin, provides further evidence for the participation of this control mechanism in the neuroendocrine-enteric axis.

No conclusive statement on the function and interrelationships of invertebrate polypeptides can be made at the present stage of investigation. Yet it is clear that during the past few years a solid foundation has been created for further basic investigations concerning the increasing number of peptides found in dual endocrine and neural localizations.

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