Cell Tissue Res. 202, 263–274 (1979)

Cell and Tissue Research

Localization of Somatostatin-, Substance P- and Calcitonin-like Immunoreactivity in the Neural Ganglion of *Ciona intestinalis* L. (Ascidiaceae)

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

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

Summary. Indirect immunofluorescence studies using antisera to synthetic somatostatin, human calcitonin and substance P indicate, in the neural complex of the sea-squirt, *Ciona intestinalis* L., that these polypeptides are present in large perikarya situated at the periphery of the cerebral ganglion as well as in some smaller perikarya in the medulla. In the medullary and transitional zone, there are nerve fibres that cross-react positively with anti-calcitonin and anti-substance P.

Key words: Polypeptide-like immunoreactivity – Neurosecretory neuron – Cerebral ganglion – *Ciona intestinalis*.

In a discussion of endocrine mechanisms it is convenient to distinguish between neuroendocrine and epithelial endocrine structures. The neural complex of the tunicates has been one of the most thoroughly studied subjects in protochordate endocrinology (Berill, 1950; Millar, 1953; Barrington, 1964, 1965). It consists of two main parts: (i) the cerebral ganglion as a nervous structure, and (ii) the neural gland as an epithelial structure.

The latter possesses a cavity which communicates with the neural-gland duct running in close proximity to the ganglion. This opens as the so-called ciliated funnel in the roof of the buccal cavity (Fig. 1b). The cerebral ganglion, about 1–2 mm long and 1 mm in diameter, is a compact nervous structure with neurons of various shapes and sizes arranged peripherally in several layers. Their axonal filaments seem to enter the fibrous medulla to form a criss-cross pattern in the transitional zone, before merging with the fibres of the medulla. The fibrous core consists mainly of densely packed fibres with only a few, mainly smaller nerve cells.

Send offprint requests to: Dr. H. Fritsch, Anatomisches Institut der Universität Kiel, Neue Universität, Olshausenstr. 40–60, Haus N10, D-2300 Kiel, Federal Republic of Germany

Acknowledgement. Supported by a grant of the Deutsche Forschungsgemeinschaft (Fr 523/1). The authors are grateful to Mr. T. Schang, for supplying the material. The skillful technical assistance of Mrs. R. Sprang is gratefully acknowledged

0302-766X/79/0202/0263/$02.40

The orientation of the fibres reveals a longitudinal pattern in the central area of the core and in the neighbourhood of the emergence of the nerve trunks. In contrast, there is a rather open meshwork of fibres in the so-called transitional zone between cortex and medulla.

In early investigations Mazzi (1952, *Ciona*) and Scharrer (1953, *Molgula*) found no definite signs of the existence of cells stainable with Gomori's chrome-alum haematoxylin-phloxin in the cerebral ganglion. Dawson and Hisaw (1964), however, applied the PAF reaction and described two morphological types of neurosecretory cells. Electron microscopic studies on the neural ganglion of *Ciona* by Thiebold and Illoul (1965) and Chamboust (1966) showed granule-containing neurosecretory cells in the ganglion itself, whereas Lane (1971) did not observe such cells in the neural gland.

Examining the neural complex by fluorescence microscopy after treatment of the tissue with diethylpseudoisocyanine, Olsson (1969) described the presence of intracellular fluorescent material, but his interpretation of the fluorescing cells as neurosecretory was by no means conclusive. Dodd and Dodd (1966), looking for neurohypophyseal hormones in the neural complex of *Ascidiella* and *Ciona,* concluded that this structure does not elaborate any hormones comparable with the neurohypophyseal octapeptides. Nevertheless, it seemed likely that the granules described must represent storage of some specialised peptide or protein, and in the light of the abundance of new data concerning neurosecretory neurons, peptidergic neurons and their evolution (reviewed by Scharrer, 1978), it seemed to us pertinent to test the neural complex of *Ciona intestinalis* for possible immunoreactions with antisera to vertebrate peptide hormones.

Materials and Methods

Animal Material. Ciona intestinalis were collected by a diver in the western Baltic sea. The animals (30–50 mm long) were aggregated in colonies, which were either attached to brown algae or to secondary sediment. They were kept in aerated sea-water (16‰) in glass aquaria at 5°C.

Histological Methods. The entire neural complex, located between the two siphons, was dissected out. The material was fixed for a short period (3 h) either in Bouin's fluid, or in methanol-free formaldehyde (MFF) (Polak et al., 1971) or in 6% glutaraldehyde, followed by standard dehydration through graded alcohols, embedding in paraplast and serial sectioning. For the formaldehyde-induced fluorescence (FIF)-method further material was freeze-dried, fixed in formaldehyde vapour at 60°C for 3 h, and embedded in paraplast. Antibodies to synthetic cyclic somatostatin (SOM), synthetic calcitonin (Wellcome, 1973) and substance P were produced in rabbits, tested by radioimmunoassay (RIA) and by immunostaining in mammalian tissues (Polak et al., 1975). The indirect immunofluorescence technique (Coons et al., 1955) was applied to sections using rabbit anti-SOM (dilution 1/200) or anti-calcitonin (dilution 1/200) or anti-substance P (dilution 1/200) as the first layer, and fluorescein-conjugated goat-anti-rabbit globulin as the second layer. Control staining was carried out with all three antibodies, which had been preabsorbed with their appropriate antigen (10 nmol/ml diluted antiserum) or with one of the two other antigens investigated. Further control staining was carried out with the second layer only or with albumin as the first layer. Sections were mounted in buffered glycerine and examined in a Zeiss fluorescence microscope.

Staining for argyrophilia was carried out according to the Grimelius (1968) technique which is a standard method for the demonstration of some "endocrine" storage granules in vertebrate and invertebrate material.

Results

All methanol-free formaldehyde (MFF)-, Bouin-, and glutaraldeyhde-fixed material examined contained apparent granular cells immunoreactive with antisera to somatostatin, calcitonin and substance P. The strongest reactions followed short fixation with Bouin's fluid. Although the number of immunoreactive cells varied considerably from animal to animal, each cerebral ganglion always contained some positive cells. In the cerebral ganglion no intrinsic fluorescence could be observed after the FIF procedure.

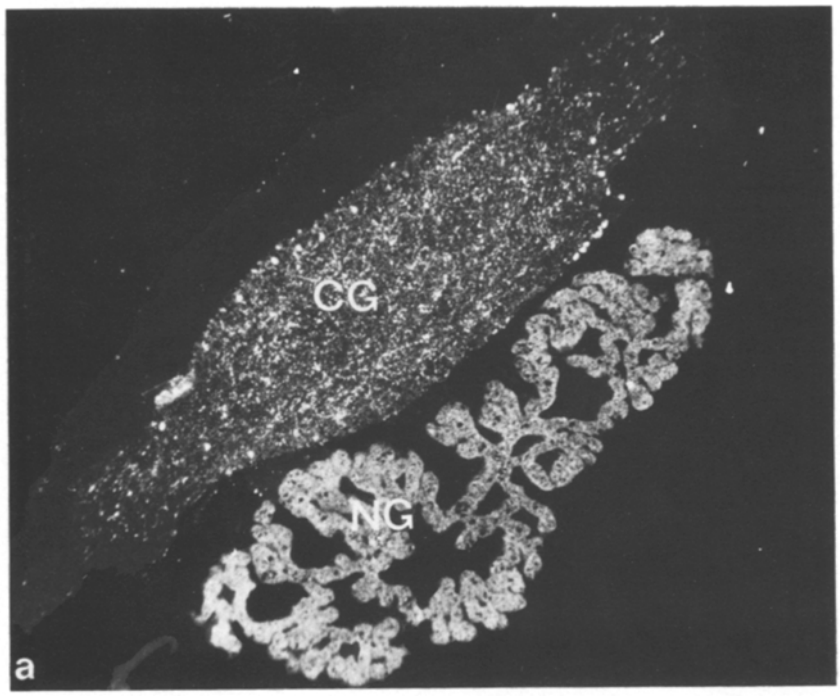
When the immunostained sections were stained for argyrophilia by the Grimelius technique, positive results were obtained. Cells containing argyrophilic granules were located in the outer layers of the cortex toward the capsule, and in small numbers in the medulla (Fig. 5a, c, d). Fibres running parallel to the longitudinal axis of the ganglion showed a beaded structure, just as observed after application of antisera to their contained peptides (Fig. 5b–d). Because of the irregular course of the argyrophilic fibres, however, they could be followed in a single section for a short distance only.

The cells immunoreactive with antiserum to SOM were found primarily as large perikarya in more peripheral positions, forming an outer layer of the cortex (Fig. 2a–c). Single smaller cells of various shapes could be found between them. Their axonal filaments did not reveal any cross-reactivity with anti-SOM. Comparing several cerebral ganglia from different animals, the SOM-like cells were seen to be more numerous at the border between the ganglion and the neural gland than on the opposite side of the ganglion. The core of the ganglion contained only scarce SOM-positive cells of various shapes and sizes. Some were very small round elements with a large nucleus, others medium sized and drumstick-shaped (Fig. 2c).

Anti-SOM serum, which had been absorbed with substance P, calcitonin or albumin, gave positive staining. No staining was seen, however, with the anti-SOM serum absorbed with SOM.

A very different cell population from that described above was observed to be immunoreactive with anti-human calcitonin. Positive staining was found predominantly in small cells forming the outer layer of the cortical zone toward the capsule (Fig. 3d). Single medium-sized cells reacting with anti-calcitonin were located in the transitional zone (Fig. 2b), where they exhibited a typical polygonal shape. The medulla of the cerebral ganglion was dominated by fibres reacting positively with anti-calcitonin (Fig. 3a, c). These were orientated parallel to the longitudinal axis of the ganglion and showed occasional branching. Higher magnification revealed their beaded structure. As their course could be followed through the different parts of the ganglion only in a 6 µm section, it was assumed that they were part of an interwoven meshwork containing fibres non-immunoreactive to anti-calcitonin. Sometimes, they originated/ended on small positive immunoreactive cells in the medulla. These cells were distinct from those stained by anti-SOM serum. No staining was observed after absorbing anti-calcitonin serum with calcitonin, whereas staining was positive after absorption with SOM, substance P or albumin.

The third antiserum tested on the cerebral ganglion, anti-substance P, gave a very different reaction from those with the two antisera described above. Cells



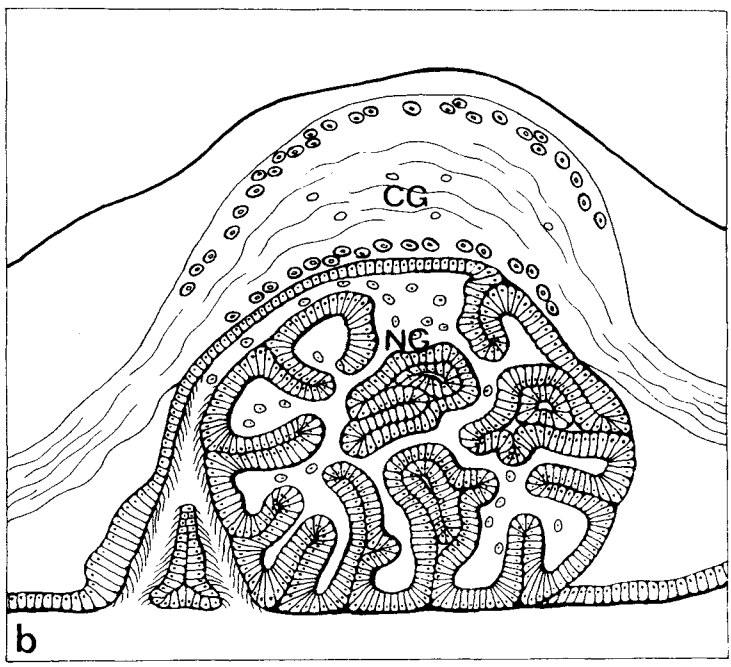


Fig. 1. a Immunofluorescence photomicrograph showing the neural complex of *Ciona intestinalis*. Calcitonin-like immunoreactivity is seen in cells and fibers of the neural ganglion, as well as in the neural gland. *CG* cerebral ganglion, *NG* neural gland. ×135. **b** Diagrammatic median section through the neural complex of *Ciona*

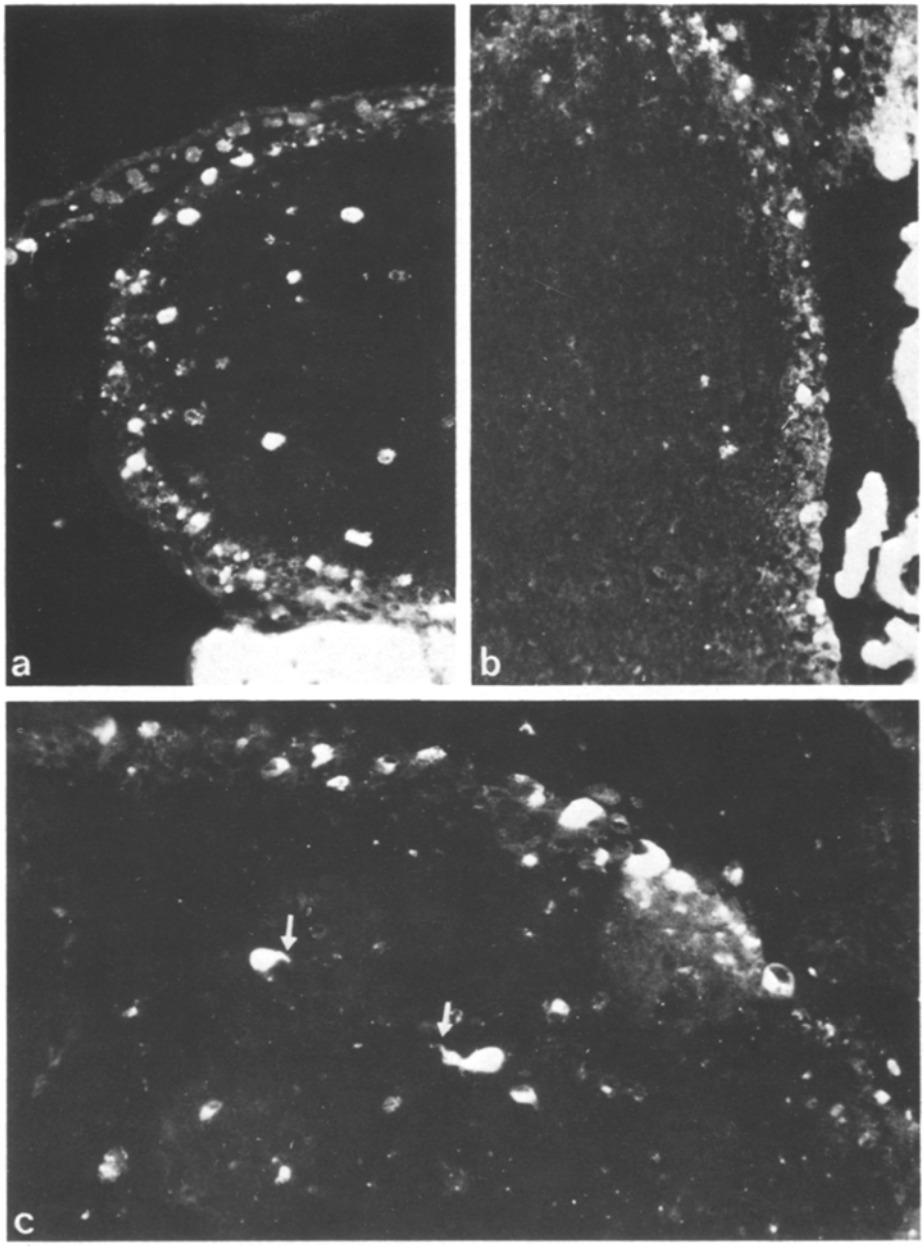


Fig. 2. a–c. Immunofluorescence photomicrographs showing the neural ganglion of *Ciona intestinalis*. Somatostatin-like immunoreactivity is seen mainly in cells of various sizes in the outer cortical layer of the neural ganglion. Short processes emerge from the cells situated in the medulla (*arrows*). × 515

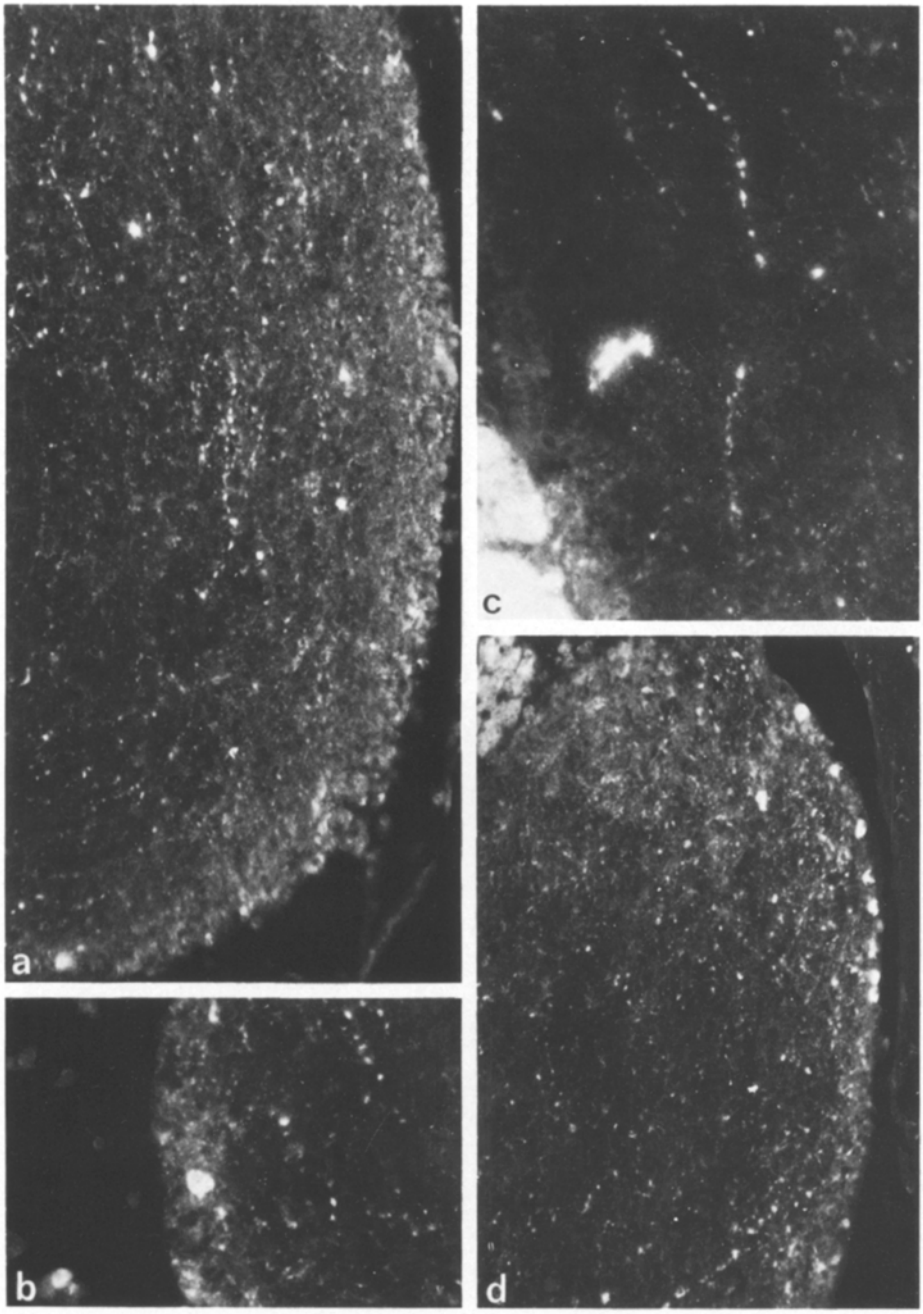


Fig. 3 a–d. Immunofluorescence photomicrographs showing the neural ganglion of *Ciona intestinalis*. Calcitonin-like immunoreactivity in small cells (**d**) in the outer subcapsular layer of the cortex; cells of larger size in another layer of the cortex (**b**); beaded fibers running longitudinally to the axis of the medulla. (**a–d**). a–c ×515; d ×708

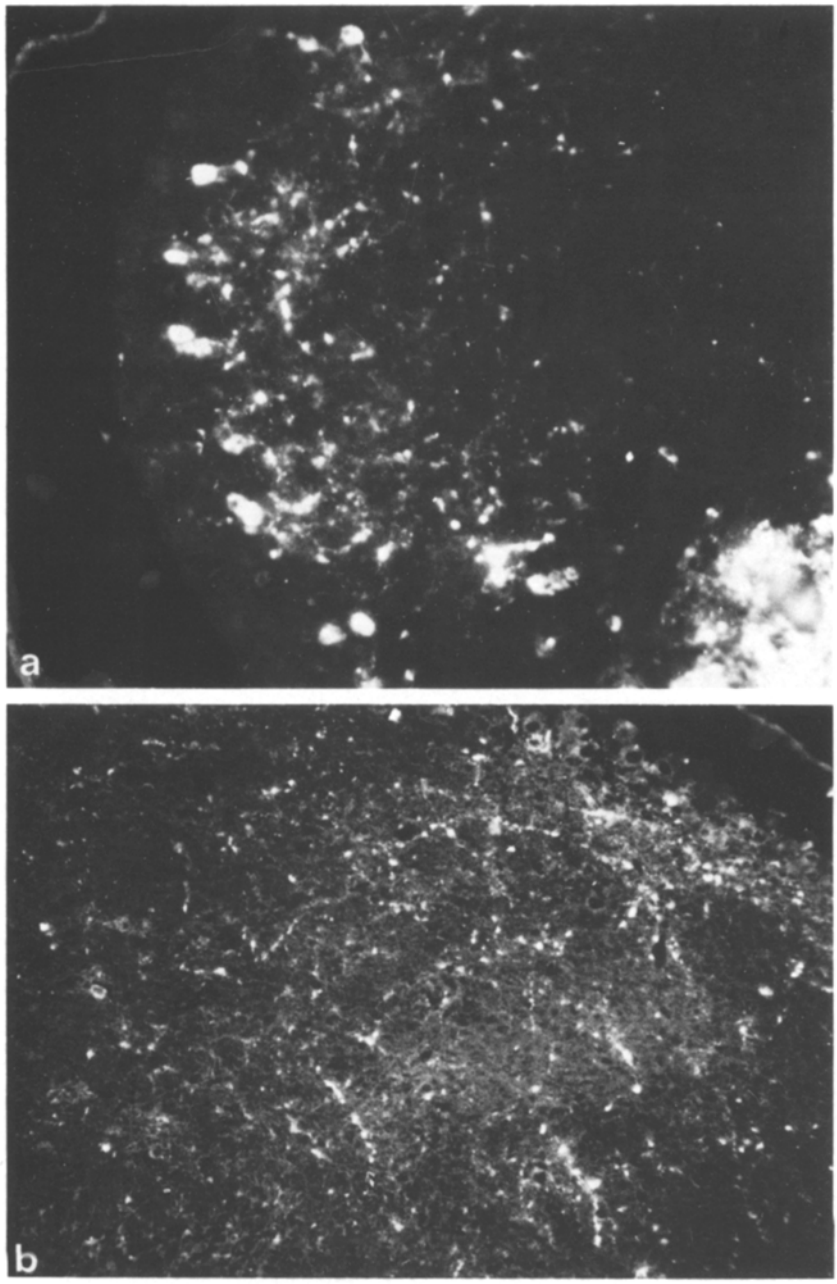


Fig. 4a and b. Immunofluorescence photomicrographs showing the neural ganglion of *Ciona intestinalis*. Substance P-like immunoreactivity in cells of one of the inner cortical layers **a**; beaded fibers forming a meshwork in the transitional zone **b**. × 540

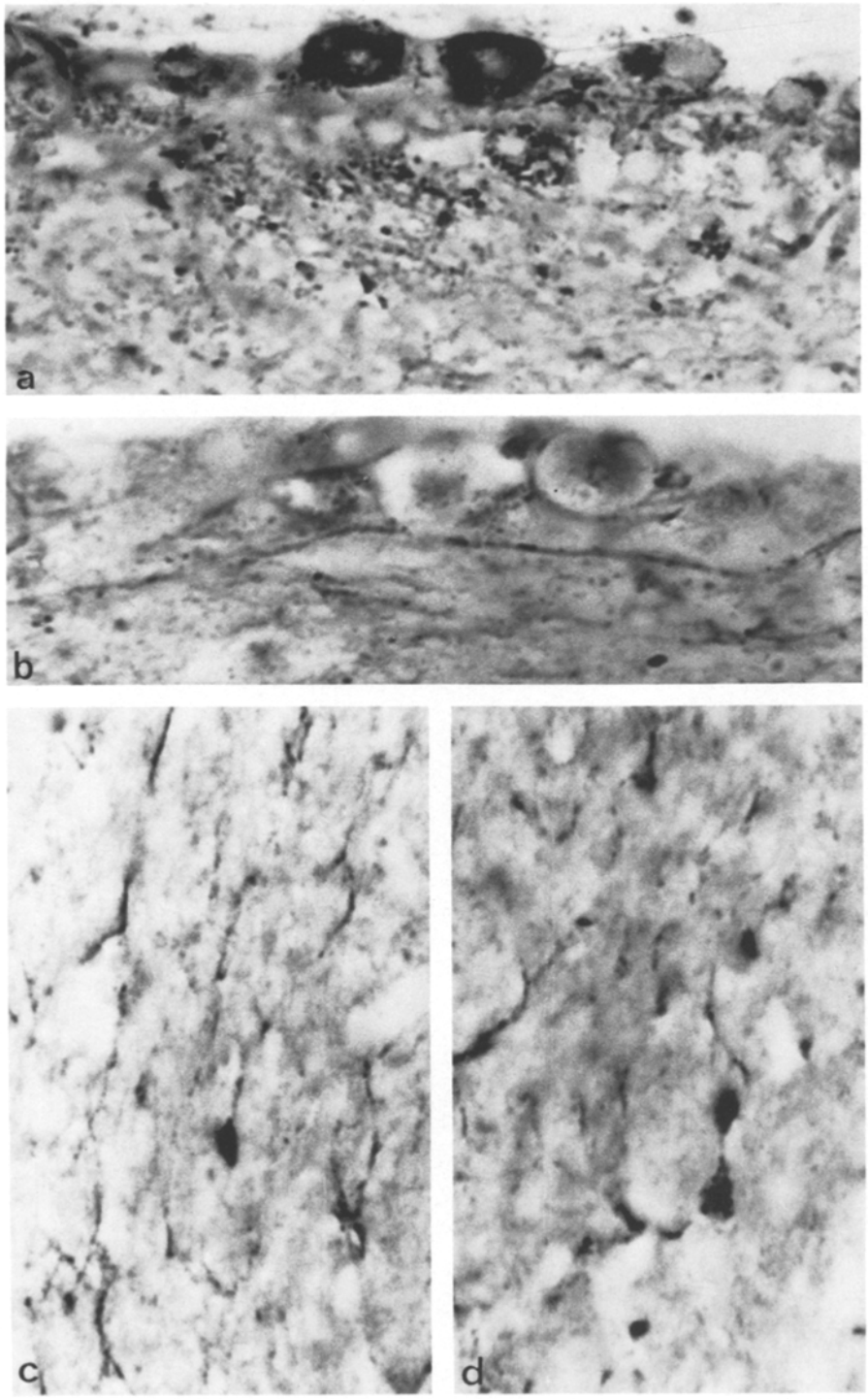


Fig. 5a–d. The neural ganglion of *Ciona intestinalis*. Argyrophilic cells in the cortex **a** and medulla **c, d,** as well as beaded fibers **b–d** in both regions. ×2520

immunoreactive for substance P were located in one of the inner cortical layers, facing the capsule (Fig. 4a). Forming a unique cell population in size and shape, they were found neither in the outer layer of the cortex toward the capsule nor in the cortex facing the neural gland. Bordering the transitional zone, they obviously send their fibres into this part of the ganglion. The course of the fibres did not reveal any dominant orientation, but they seemed to form a criss-cross pattern (Fig. 4b) making it difficult to decide whether inidividual fibres were merely overlapping, or actually branching. Like other peptidergic nerve fibres, these fibres were also beaded, but they did not enter the medulla. No positive reaction could be observed in the core of the ganglion with anti-substance P.

With each antiserum used in the investigation, some degree of positive staining was obtained in the epithelial neural gland component of the complex as well as in 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. Further work on this point is in progess.

Discussion

The "common peptides", i.e., peptides common to the central and peripheral divisions of the nervous system on the one hand, and to the gastroenteropancreatic and related endocrine systems in mammals on the other, are receiving an increasing amount of attention, although their physiological functions are still largely obscure (Pearse, 1976, 1977, 1978). Furthermore, several peptide-like activities, as part of a postulated diffuse endocrine system, can be demonstrated in the gastrointestinal tract of lower vertebrates and the alimentary tract of invertebrates (Wilson and Falkmer, 1965; Mehrotra and Falkmer, 1968; Falkmer, 1972; Davidson et al., 1971; Falkmer et al., 1973, 1977; Van Noorden and Pearse, 1974; Fritsch et al., 1976, 1978; Fritsch and Sprang, 1977; Van Noorden et al., 1977). These include calcitonin, which is present, in *Ciona,* not only in mucosal endocrine cells but also in the submucous plexus, in the form of ganglion cells and fibres (Fritsch et al., 1979).

Somatostatin-like immunoreactivity was demonstrated in endocrine cells in the alimentary tract of *Ciona intestinalis* (Fritsch et al., 1978), in combination with gastrin-like immunoreactivity in similar cells in the same area. Here, the somatostatin-like substances might act as a possible release inhibiting factor. In view of its widespread distribution in the median eminence, the arcuate nucleus, the ventromedial nucleus of the hypothalamus and the nucleus periventricularis in mammals (Hökfelt et al., 1975; Pelletier et al., 1974; Brownstein et al., 1975; Sétáló et al., 1975; Elde and Parsons, 1975; Palkovits et al., 1976; Baker and Yu, 1976), it does not seem unreasonable to find somatostatin-like immunoreactivity (SLI) in the cerebral ganglion of the invertebrate, *Ciona intestinalis* as well. Various other peptides or peptide-like substances have now been found in the "brains" of various invertebrates (Van Noorden et al., 1979; Van Noorden and Falkmer, 1979), e.g., gastrin and insulin in insects; PP and VIP in the earthworm; enkephalin, PP and substance P in the snail. As the SLI cells are situated predominantly in one area of the ganglion, it may be suggested that the various neurotrophic effects of SOM in the central nervous system which indicate that it serves as a kind of neurotransmitter, are also present in Ciona.

The SLI cells cannot be restained with the silver technique of Grimelius (1968) for argyrophilia, which indicates that the argyrophilic neurons of the cerebral ganglion must produce a peptide with different functions. This hypothesis can be confirmed by the demonstration of substance P-like immunoreactivity in the cells of the inner layer of the cortex, which send a criss-cross meshwork of fibres into the transitional zone, avoiding the medulla. Although it must be regarded as the doyen of the common peptides having first been described by von Euler and Gaddum (1931), the physiological role of substance P in mammals is still not entirely clear. It is likely to be the excitatory transmitter released by primary afferent terminals (Konishi and Otsuka, 1974). No indication can be given at present of its possible role in *Ciona intestinalis*. Nevertheless, the specifically arranged substance P-like cells, and the restriction of their immunoreactive fibres to the transitional zone, could indicate a specific role for the neural complex. It was impossible to establish whether these cells can communicate with each other via their fibres, or which other neuron is stimulated by them, if any. Being arranged as they are, they could play the role of interneurons, but evidence for this suggestion is still lacking.

The most surprising results were obtained with an anti-human calcitonin. With this sensitive antiserum giving positive staining in mammal material, not only was there a population of immunoreactive smaller cells in the outer layer of the cortex, but also a large number of positive-reacting fibres. The latter were situated in the medulla of the ganglion, lying parallel to its long axis. The beaded appearance of the fibres indicates that they are axons, and their content of immunoreactive peptide suggests a possible function in neurotransmission. Calcitonin may perhaps now be regarded as a new neurotransmitter, controlled by somatostatin. Somatostatin acts not only as a powerful carrier of inhibitory information, but also is able to suppress the release of calcitonin from the thyroid gland.

At least some of the cells and fibres which are argyrophilic with the Grimelius technique appear to be identical with those showing calcitonin-like immunoreactivity. The silver positive cells are of about the same size, lie in the same position, and their fibres also show a beaded structure.

The capacity of peptidergic neuronal elements to establish contact with a variety of effector cells, including neurons (Scharrer, 1978), makes it therefore at least probable that peptidergic neurotrophic mechanisms of the kind presumed to operate in vertebrates, i.e., signals of neurotransmitter- or neuromodulator-type, occur also amongst the invertebrates.

There is no doubt that several peptides are produced in the cells of the cerebral ganglion of *Ciona intestinalis*. Their function as well as their relationship to each other remains unclear, but the fact of their existence encourages further investigation into the evolutionary neuroendocrinology of invertebrates.

References

Baker, B.L., Yu, Y.-Y.: Distribution of growth hormone-release-inhibiting hormone (somatostatin) in the rat brain as observed with immunocytochemistry. Anat. Rec. **186,** 343–356 (1976)

Barrington, E.J.W.: An endocrinological approach to the problem of the origin of the vertebrates. Ann. Soc. Roy. Zool. Belg. **94,** 161–178 (1964)

Barrington, E.J.W.: The biology of Hemichordata and Protochordata, pp. 176, Edinburgh and London: Oliver and Boyd: 1965

Berill, N.J.: The Tunicata. Roy. Soc. Publ. London No 133, 1–354 (1950)

Brownstein, M., Arimura, A., Sato, H., Schally, A.V., Kizer, J.S.: The regional distribution of somatostatin in the rat brain. Endocrinology **96,** 1450–1461 (1975)

Chambost, D.: Le complexe neural de *Ciona intestinalis* (Tunicier, Ascidiacea) Etude asymétrique aux microscopes optique et électronique. Compt. Rend. Acad. Sci. **263,** 969–971 (1966)

Coons, A.H., Leduc, E.H., Connolly, J.M.: Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. **102,** 49–59 (1955)

Davidson, J.K., Falkmer, S., Mehrotra, B.K., Wilson, S.: Insulin assays and light microcopical studies of digestive organs in protostomian and deuterostomian species and in coelenterates. Gen. Comp. Endocrinol. **17,** 388–401 (1971)

Dawson, A.B., Hisaw, F.J. Jr.: The occurrence of neurosecretory cells in the neural ganglia of tunicates. J. Morphol. **114,** 411–424 (1964)

Dodd, J.M., Dodd, M.H.I.: An experimental investigation of the supposed pituitary affinities of the ascidian neural complex. In: Some Contemporary Studies in Marine Science. (H. Barnes, ed.). pp. 233–252, London: Allen & Unwin: 1966

Elde, R.P., Parsons, J.A.: Immunocytochemical localization of somatostatin in cell bodies of the rat hypothalamus. Am. J. Anat. **144,** 541–548 (1975)

Euler, U.S., v., Gaddum, J.H.: An unidentified depressor substance in certain tissue extracts. J. Physiol. (Lond.) **74,** 192 (1931)

Falkmer, S.: Insulin production in vertebrates and invertebrates. Gen. Comp. Endocrinol. Suppl. **3,** 184–191 (1972)

Falkmer, S., Emdin, S., Havu, N., Lindgren, G., Marques, M., Östberg, Y., Steiner, D.F., Thomas, N.W.: Insulin in invertebrates and cytostomes. Amer. Zool. **13,** 625–638 (1973)

Falkmer, S., Elde, R.P., Hellerström, C., Petersson, B., Efendić, S., Fohlmann, T., Siljevall, T.-B.: Some phylogenetical aspects on the occurrence of somatostatin in the gastro-entero-pancreatic endocrine system. A histological and immunocytochemical study, combined with quantitative radioimmunological assays of tissue extracts. Arch. Histol. Jpn. **40,** Suppl. 99–117 (1977)

Fritsch, H.A.R., Van Noorden, S., Pearse, A.G.E.: Cytochemical and immunofluorescence investigations on insulin-like producing cells in the intestine of *Mytilus edulis* L. (Bivalvia). Cell Tissue Res. **165,** 365–369 (1976)

Fritsch, H.A.R., Sprang, R.: On the ultrastructure of polypeptide-hormone-producing cells in the gut of the ascidian, *Ciona intestinalis* L. Cell Tissue Res. **177,** 407–413 (1977)

Fritsch, H.A.R., Van Noorden, S., Pearse, A.G.E.: Localization of somatostatin and gastrin-like immunoreactivity in the gastrointestinal tract of *Ciona intestinalis* L. Cell Tissue Res. **186,** 181–185 (1978)

Fritsch, H.A.R., S. Van Noorden, Pearse, A.G.E.: Immunocytochemical reactivity of a calcitonin-like peptide in the alimentary tract of *Ciona intestinalis*. (in preparation, 1979)

Grimelius, L.: A silver nitrate stain for cells in human pancreatic islets. Acta Soc. Med. Upsal. **73,** 243–270 (1968)

Hökfelt, T., Efendić, S., Hellerström, C., Johannson, O., Luft, R., Arimura, A.: Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special references to the A_1-cells of the pancreatic islets and to the hypothalamus. Acta Endocrinol. **80** (Suppl. 200), 1–41 (1975)

Konishi, S., Otsuka, M.: Excitatory action of hypothalamic substance P on spinal motorneurones of newborn rats. Nature **252,** 734–735 (1974)

Lane, N.J.: The neural gland in tunicates: Fine structure and intracellular distribution of phophatases. Z. Zellforsch. Mikrosk. Anat. **120,** 80–93 (1971)

Mazzi, V.: Esistono fenomeni neurosecretori nelle Ascidie? Boll. Zool. **19,** 161–163 (1952)

Mehrotra, B.K., Falkmer, S.: Occurrence of insulin producing cells in the gastrointestinal tract of invertebrates. Diabetologia **4,** 224 (1968)

Millar, R.H.: Ciona, L.M.B.C. Memoirs. 1–123, Liverpool: Colman 1953

Olsson, R.: General review of the endocrinology of the Prochordata and Myxinoidea. Gen. Comp. Endocrinol. Suppl. **2,** 485–499 (1969)

Palkovits, M., Brownstein, M.J., Arimura, A., Sato, H., Schally, A.V., Kizer, J.S.: Somatostatin content of the hypothalamic ventromedial and arcuate nuclei and the circumventricular organs in the rat. Brain Res. **109,** 430–434 (1976)

Pearse, A.G.E.: Peptides in brain and intestine. Nature **262,** 92–93 (1976)
Pearse, A.G.E.: The diffuse neuroendocrine system and the "common peptides". In: Molecular Endocrinology. ed. McIntyre and Szelke, 309–323, Amsterdam: Elsevier/North Holland 1977
Pearse, A.G.E.: Diffuse neuroendocrine system: peptides common to brain and intestine and their relationship to the APUD concept. Ed. J. Hughes pp. 49–57, London: Macmillan: 1978
Pelletier, G., Labrie, F., Arimura, A., Schally, A.V.: Electron microscopic immunohistochemical localization of growth hormone-release inhibiting hormone (somatostatin) in the rat median eminence. Am. J. Anat. **140,** 445–450 (1974)
Polak, J.M., Bussolati, G., Pearse, A.G.E.: Cytochemical, immunofluorescence and ultrastructural investigations on the antral G cells in hyperparathyroidism. Virchows. Arch. Abt. **9,** 187–197 (1971)
Polak, J.M., Grimelius, L.C., Pearse, A.G.E., Bloom, S.F., Arimura, A.: Growth-hormone-release inhibiting hormone in gastrointestinal and pancreatic D-cells. Lancet I: 1220–1222 (1975)
Scharrer, B.: Peptidergic neurons: facts and trends. Gen. Comp. Endocrinol. **34,** 50–62 (1978)
Scharrer, E.: Das Hypophysen-Zwischenhirnsystem der Wirbeltiere. Anat. Anz. Suppl. **100,** 5–29 (1953)
Sétáló, G., Vigh, S., Schally, A.V., Arimura, A., Flerkó, B.: GH-RIH-containing neural elements in the rat hypothalamus. Brain Res. **90,** 352–356 (1975)
Thiebold, J., Illoul, F.: Recherches sur la neurosécrétion chez une ascidie *Ciona intestinalis*. Bull. Soc. Hist. Nat. Afr. Nord **56,** 87–97 (1965)
Van Noorden, S., Pearse, A.G.E.: Immunoreactive polypeptide hormones in the pancreas and gut of the lamprey. Gen. Comp. Endocrinol. **23,** 311–324 (1974)
Van Noorden, S., Östberg, Y., Pearse, A.G.E.: Localization of somatostatin-like immunoreactivity in the pancreatic islets of the hagfish, *Myxine glutinosa* and the lamprey, *Lampetra fluviatilis*. Cell Tissue Res. **177,** 2, 281–285 (1977)
Van Noorden, S., Falkmer, S.: Gut-islet endocrinology. Some evolutionary aspects. Invest. Cell Path. 1979 (in press)
Van Noorden, S., Fritsch, H.A.R., Grillo, T.A.I., Polak, J.M., Pearse, A.G.E.: Immunocytochemical staining for vertebrate peptides in the nervous system of a gastropod mollusc. Gen. Comp. Endocrinol. (1979) (in press)
Wilson, S., Falkmer, S.: Starfish insulin. Can. J. Biochem. **43,** 1615–1624 (1965)

Accepted July 2, 1979