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Substance P- and cholecystokinin-like immunoreactivity during post-metamorphic development of the central nervous system in the ascidian *Ciona intestinalis*

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Abstract. Following metamorphosis, the neural ganglion of ascidians is thought to be formed via the proliferation of epithelial cells comprising the ciliated duct. In adults, neuronal cell bodies expressing substance P- and gastrin/cholecystokinin-like immunoreactivity exhibit clearly defined patterns of distribution. Previous work shows that these patterns are re-established during regeneration of the adult ganglion. We have used antisera against substance P and cholecystokinin to monitor the formation of these patterns during normal post metamorphic development in *Ciona intestinalis*. Substance P cells first appear in the ganglion in animals of 1 mm body length. Cholecystokinin antiserum was not used at this stage but revealed a clear adult-like pattern of cells in the anterior region at the 3 to 5-mm stage. Substance P cells do not exhibit an adult pattern until animals have a body length of more than 10 mm. Proliferation in the neural complex was studied using the bromodeoxyuridine/anti-bromodeoxyuridine technique. Results suggest a mechanism whereby cells are born in the ciliated duct and later migrate to the ganglion. Double-labelling experiments indicate that more than 11 days elapse between cell birthdates and the expression of either of the peptides. Data presented suggest that the distributional patterns for these peptides during normal development are similar to those seen during regeneration.

Key words: Neural development – Differentiation – Peptides – Proliferation – Bromodeoxyuridine – *Ciona intestinalis* (Urochordata, Tunicata)

Introduction

The development of ascidians has attracted considerable interest since their chordate features were first recognized by Kowalevsky (1866). The embryo and the larva have received most interest and much of the attention has focused on the development of the nervous system. This is formed, as in all chordates, through neurulation (see Venuti and Jeffery 1989 for a review) and much of the lineage for the larval central nervous system has been described (Nicol and Meinertzhagen 1988a, b, 1991).

The post metamorphic events that follow the settling of the non-feeding free-swimming larva and transform it to a sessile suspension feeder are less well known. The immediate gross morphological changes such as resorption of the tail and larval nervous system have been described and reviewed by Cloney (1977). The adult nervous system is derived from a primordial epithelial layer formed in the embryo from the anterior left side of the neural tube (Elwyn 1937). This joins anteriorly with the pharynx where it becomes the ciliated funnel and extends posteriorly as a tubular epithelium (the dorsal strand) towards the ovary. Close to the point where the ciliated funnel emerges into the pharynx the epithelium proliferates to form both the neural ganglion (brain) and the neural gland which together comprise the neural complex. The differentiation of cells into neurons and their expression of a specific phenotype is the culmination of neural development and is accompanied by morphological and behavioural specializations.

Immunocytochemistry has revealed a wide spectrum of neuropeptides in the neural complex of the adult ascidian (Thorndyke and Georges 1988) and although Georges (1985) has described serotonin-like immunoreactivity in juvenile *Ciona intestinalis*, no similar reports exist for neuropeptides during the early stages of development following metamorphosis. Here we describe the development of substance P-like (SP-li) and gastrin/cholecystokinin-like (CCK-li) immunoreactive neurons in the neural complex of juvenile *Ciona intestinalis*. These neuropeptides are amongst the best characterized from the adult *Ciona* brain (O'Neil et al. 1987; Johnsen and Rehfeld 1990).

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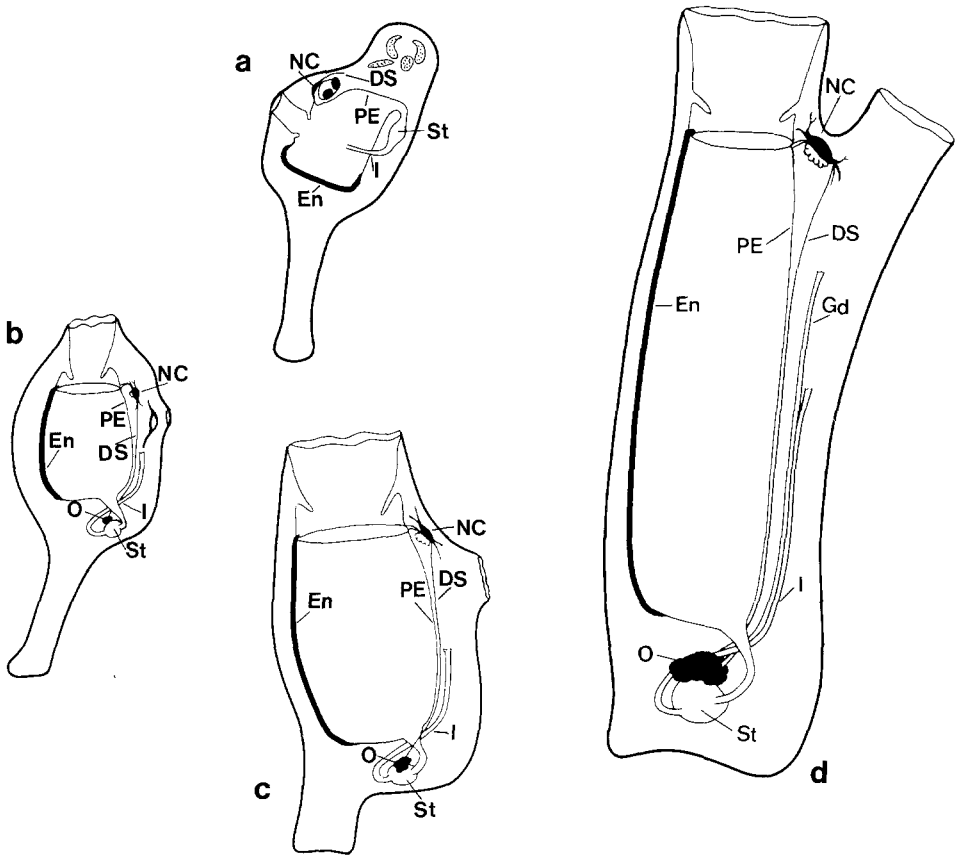

Fig. 1. Schematic representations of whole animals at the various developmental stages employed indicating the location of the neural complex: at settlement (**a**), the 1-mm stage (**b**), 3 to 5-mm stage (**c**), and a sexually fully mature adult (**d**). *DS* Dorsal strand; *En* endostyle; *Gd* gonoducts; *I* intestine; *NC* neural complex; *O* ovary; *PE* pharyngeal epithelium; *St* stomach

Materials and methods

Animals

Ciona intestinalis were collected by divers at Tjärnö Marine Biological Laboratory, Sweden, and maintained in a running sea-water system. Cross-fertilized embryos that were obtained by mixing gametes from two individuals were raised in plastic Petri dishes floating on a water bath at 16° C; they were allowed to settle and metamorphose. This took place 2 days after fertilization. Later stages were obtained from a wild population of naturally spawned larvae that had settled on larger specimens. This natural population was divided into the following classes: 1 mm, 3–5 mm, 6–10 mm and 11–15 mm in body length (Fig. 1).

Peptide immunocytochemistry

For immunocytochemistry, all animals were fixed in Bouin's fluid; the animals were anaesthetized in 0.03% MS222 (Sigma, St. Louis, USA) solution to prevent contraction when immersed in the fixative. Larger specimens (11–15 mm) were cut in half to facilitate penetration of the fixative. The preparations were dehydrated and embedded in paraffin wax. Serial sections were cut at 6 μm and mounted on poly-L-lysine coated glass slides, dewaxed in xylene and rehydrated in a graded ethanol series.

Two antisera were used: rabbit anti-SP (P4) and anti-CCK (L48), both of which were C-terminal specific. Following incubation in primary antiserum, preparations were transferred into 1:50 swine anti-rabbit serum (Dako, High Wycombe, UK) for 60 min prior to incubation with 1:100 rabbit peroxidase anti-peroxidase complex (PAP; Dako). Immuno-peroxidase reaction was visualized by 0.035% 3-3′-diaminobenzidine (DAB) and 0,01% H_2O_2.

Limited availability of the 1-mm stage restricted the analysis in this class to SP-li only.

Bromodeoxyuridine labelling of the developing nervous system

DNA synthesis was monitored using incorporation of the substituted nucleotide, 5-bromodeoxyuridine (BrdU) (Sigma) and revealed by a monoclonal antibody against BrdU (Becton Dickinson, Cowley, UK). Animals were immersed for 12 h in BrdU (250 μM) dissolved in filtered sea-water then sacrificed immediately, or 5, or 11 days after the 12 h pulse. This concentration has been found to produce strong immuno-labelling in regenerating neural complex (Bollner et al. 1991). Fixation and embedding followed the same protocol employed for peptide immunocytochemistry. Prior to incubation with the anti BrdU monoclonal antibodies, slides were

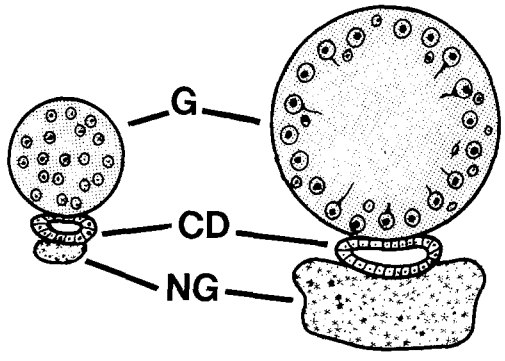

Fig. 2. Schematic representation of early (*left*) and later (*right*) transverse sections of the neural complex. *CD* Ciliated duct; *G* ganglion; *NG* neural gland

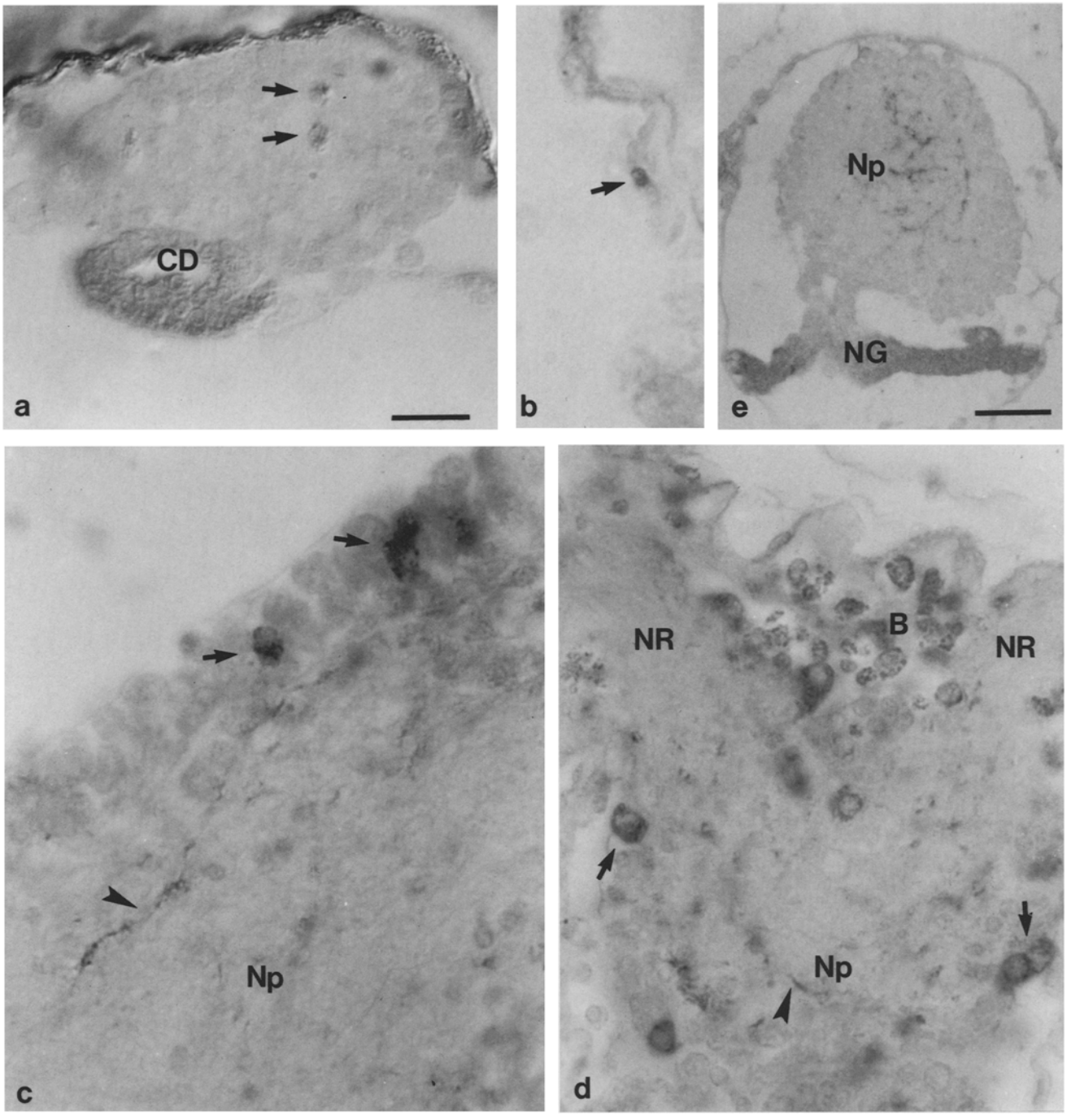

Fig. 3a–e. Immunoreactivity in the neural complex up to the 3 to 5-mm stage. *B* Blood cells; *CD* ciliated duct; *NG* neural gland; *Np* neuropile; *NR* nerve roots. **a** Substance P-like (SP-li) immunoreactivity in the neural complex of a 1-mm stage juvenile showing immunoreactivity in two small cells (*arrows*) in a central position; cells of the ciliated duct also display reaction to this antiserum. *Bar:* 10 μm; ×1265. **b** SP-li immunoreactive cells (*arrow*) in the epithelium of the ciliated funnel at the 1-mm stage. Magnification as in **a**. **c** SP-li immunoreactive cell bodies in the cortical layer (*arrows*) and fibres (*arrowhead*) extending towards the *Np* in the ganglion of a 3 to 5-mm juvenile. Magnification as in **a**. **d** CCK-li immunoreactivity in the anterior region near *NR* at the 3 to 5-mm stage with cell bodies in the cortex (*arrows*) and some fibres (*arrowhead*) in the *Np*. Magnification as in **a**. **e** Low magnification of a transverse section through the mid region of the ganglion at the 3 to 5-mm stage showing extensive CCK immunostaining of fibres in the *Np*. *Bar:* 25 μm; ×481

immersed in 2 N HCl for 20 min followed by washing in phosphate-buffered saline (PBS) and incubation with 1:50 rabbit anti-mouse IgG for 45 min and then 1:100 mouse PAP for 30 min. The reaction product was revealed by 0.035% DAB and 0.01% H_2O_2, with $NiCl_2$ added to a final concentration of 0.03% in 0.05 M TRIS-HCl buffer, pH 7.6.

BrdU and peptide double labelling

Three different techniques were used for double-labelling BrdU and peptide: (1) Following dewaxing and acid treatment as described above, simultaneous incubation with anti-BrdU (Becton Dickinson) and the peptide antiserum was carried out overnight

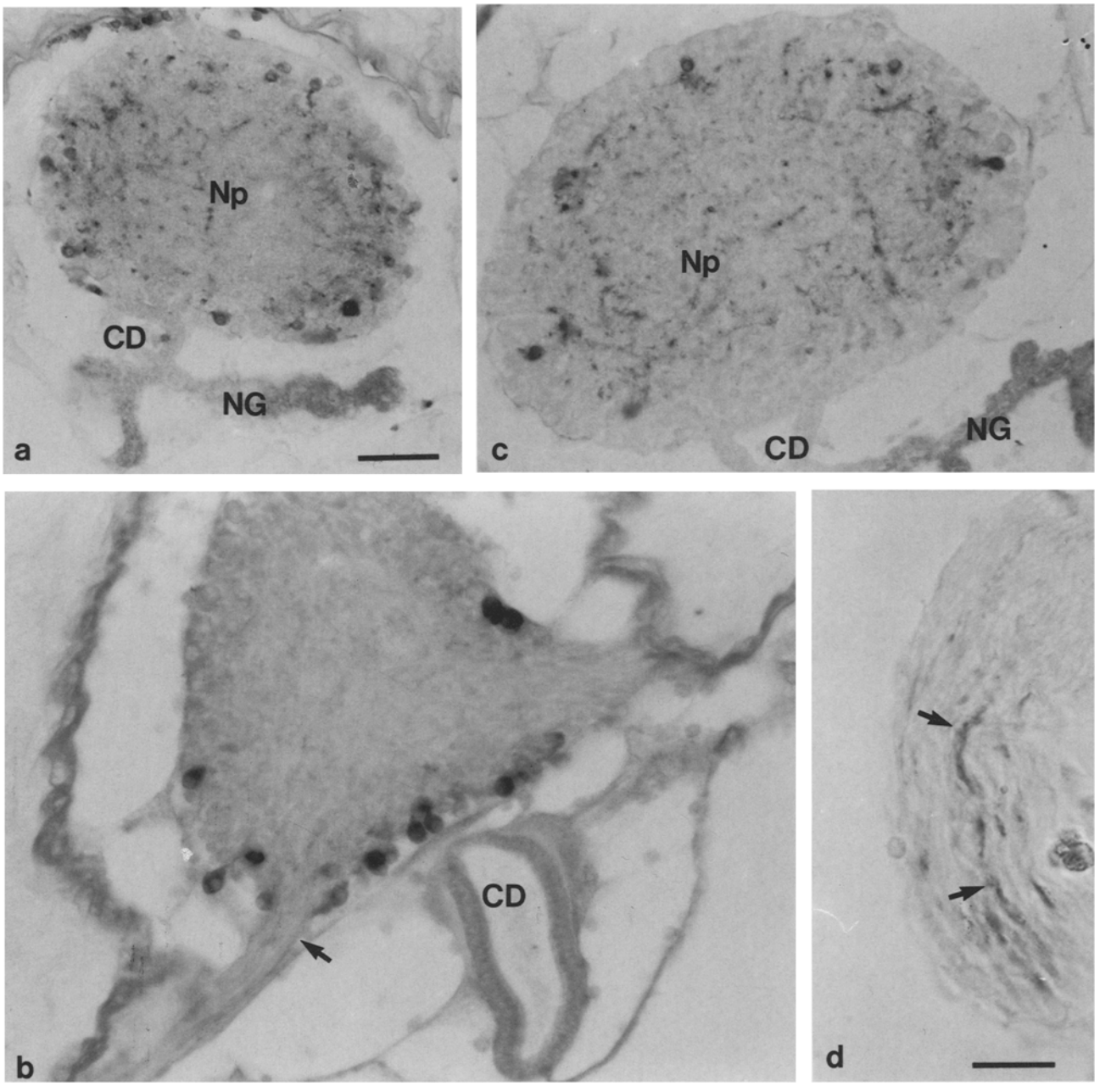

Fig. 4a–d. SP-li and CCK-li immunoreactive cells in the neural complex at the 6 to 10-mm and 11 to 15-mm stages. *CD* Ciliated duct; *NG* neural gland; *Np* neuropile. **a** Transverse section through the mid region of the ganglion at the 6 to 10 mm-stage showing abundant SP-li immunoreactivity in cortical cell bodies and in fibres in the neuropile. *Bar:* 25 μm; × 506. **b** CCK-li immunoreactive neurones near the anterior nerve exits at the 6 to 10-mm stage and a fibre in an anterior nerve root (*arrow*). Magnification as in **a**. **c** SP-li immunoreactivity at the 11 to 15-mm stage; transverse section through the mid region of the ganglion showing SP-li immunoreactive cells in the inner layer of the cortex and immunostained fibres extending towards the central *Np*. Magnification as in **a**. **d** SP-li immunoreactive fibres (*arrows*) in one of the posterior nerve roots at the 6 to 10-mm stage. *Bar:* 10 μm; × 1265

at room temperature. After being washed, sections were incubated with a mixture of rhodamine-conjugated rabbit anti-mouse IgG (1:30) (Vector Laboratories, Peterborough, UK) and fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (1:30) (Dako) for 60 min before further washing, mounting and viewing under a microscope equipped for epi-fluorescence. (2) The sections were developed as above for BrdU staining and were then treated with 3% H_2O_2 for 5 min prior to incubation with the peptide antiserum overnight. The second primary anti-serum was visualized using DAB according to the protocol described above for peptide immunocytochemistry. (3) Incubation overnight in peptide antiserum was followed by incubations in goat anti-rabbit biotinylated IgG (Vector) and FITC-conjugated Avidin D (Vector) both at a 1:200 dilution in PBS for 60 min each. All incubations were separated by washes in PBS. After treatment with 2 N HCl for 40 min and a brief rinse in 0.1 M borax followed by 2 × 5 min in PBS, sections were incubated with rat anti-BrdU supernatant (Sera-Lab, Crawley Down, UK) diluted 1:5 in PBS with 1% bovine serum albumin overnight at 4° C followed by incubation in rabbit anti-rat biotinylated IgG and Texas-red-conjugated avidin, both at a 1:200 dilution in PBS for 60 min each. Again, incubations were separated by washes in PBS.

Results

After settlement and during metamorphosis, the ciliated duct is seen as an evagination of the pharyngeal wall. The epithelium is thicker in this region and is clearly distinguishable from the ordinary pharyngeal epithelium. This evagination extends towards the epidermis running along it on the outside of the larval sensory vesicle and passing caudally towards the gut region, where it will later make contact with the ovary.

At the 1-mm stage, the neural complex consists of an epithelial structure, the ciliated duct, described above, which expands forming a cavity, dorsal to which is the early neural ganglion or brain (Figs. 1, 2). At this stage, the ganglion appears as a morphologically homogeneous population of predominantly small cells with little cytoplasm surrounding the nuclei, although occasionally a larger cell can be seen.

In the 3 to 5-mm group, the neural gland occurs as foldings of the ventral side of the ciliated duct with the ganglion on the dorsal side. The ganglion at this stage begins to become more structured with a central neuropile surrounded by a cortex of cell bodies. Smaller cells are visible in the neuropile.

At the next investigated stage (6–10 mm), the gross morphology of the neural complex has a form resembling that of the mature adult. The brain is arranged with a layered cortex from which larger cells in the periphery send processes towards the central neuropile through an inner layer of smaller cells (see Figs. 2, 4). At some point during this stage, the larval nervous system (previously seen in the form of the pigmented ocellus and the otolith) finally disappears.

Peptide immunocytochemistry

The earliest stage at which the developing neural complex was examined for peptide expression was at 1-mm body length. Here, some cells of the developing ganglion and cells of the ciliated duct showed weak SP-li immunoreactivity (Fig. 3a). SP-li immunoreactivity was also detected in epithelial cells that may have been part of the ciliated duct (Fig. 3b).

In animals of 3 to 5 mm body length, clear SP-li immunoreactivity was detected in the neural complex. Here, staining was evident in ganglionic cell bodies mainly in the cortex and in fibres extending from the cortex towards the neuropile (Fig. 3c). In some instances, SP-li immunoreactive cell bodies also exhibited very thin immuno-positive processes (not visible on photographs). CCK-li immunoreactive cells were also seen in the ganglion at this stage. The majority of these were located in the anterior portion of the ganglion near the nerve root exits. Some of these cells had processes interpreted as axons (Fig. 3d). An extensive network of CCK-li immunoreactive fibres was seen throughout the neuropile including areas without CCK-li immunoreactive cell bodies (Fig. 3e).

At the 6 to 10-mm stage, there was an increase in the number of cells immunoreactive to both the SP and CCK antisera. Furthermore, a larger proportion of these cells had axon-like processes (Fig. 4). This stage also revealed the first immunoreactive fibres detectable in the nerve roots of the ganglion. Both SP-li and CCK-li immunoreactivity was seen in a few fibres of the anterior and posterior nerve roots (Fig. 4b, d).

The 11 to 15-mm stage confirmed the patterns seen in earlier stages, the only difference being the finding that SP-li immunoreactive cells were more clearly located in the inner region of the cortical zone, with relatively little SP-li immunoreactivity still scattered in the neuropile (Fig. 4c).

BrdU incorporation

When BrdU was administered to animals as soon as they had settled on the substratum, incorporation was found in all identifiable adult tissues, including the pharynx, endostyle and alimentary tract. In the developing nervous system, labelled nuclei were abundant along the full visible length of the ciliated duct (Fig. 5). In addition, BrdU incorporation was also seen in the thickened epithelium at the border between the pharnygeal wall and the duct (Fig. 5).

In an attempt to determine the birth-dates of the brain cells, animals of 3 to 5-mm, 6 to 10-mm and 11 to 15-mm body length were exposed to BrdU for 12 h and sacrificed either immediately, or 5 or 11 days later. All three age-groups showed the same patterns of BrdU incorporation, except that in the 11 to 15-mm group, labelled nuclei were seen more caudally in the neural ganglion than in younger (smaller) animals.

None of the animals sacrificed immediately after the BrdU pulse showed any labelled nuclei in the brain, although at this time immunostained nuclei were found in the neural gland, the ciliated duct and in the thick epithelium of the ciliated funnel (Fig. 6a). Although a

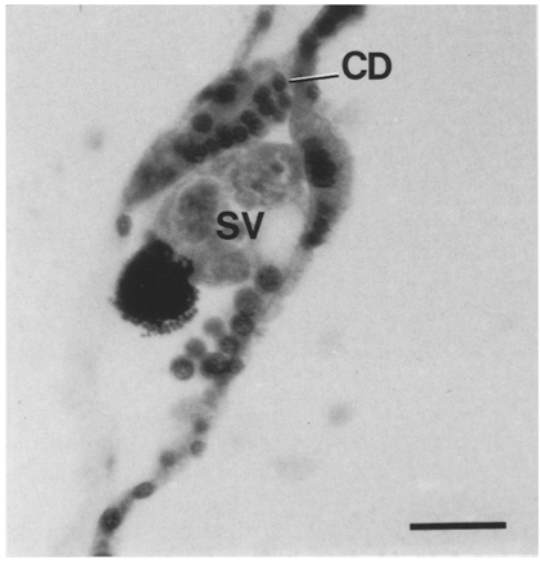

Fig. 5. Section showing the central nervous system at metamorphosis with BrdU-labelled nuclei in most post-metamorphic tissues but not in larval tissues. *SV* Sensory vesicle; *CD* ciliated duct. *Bar:* 15 μm; ×837

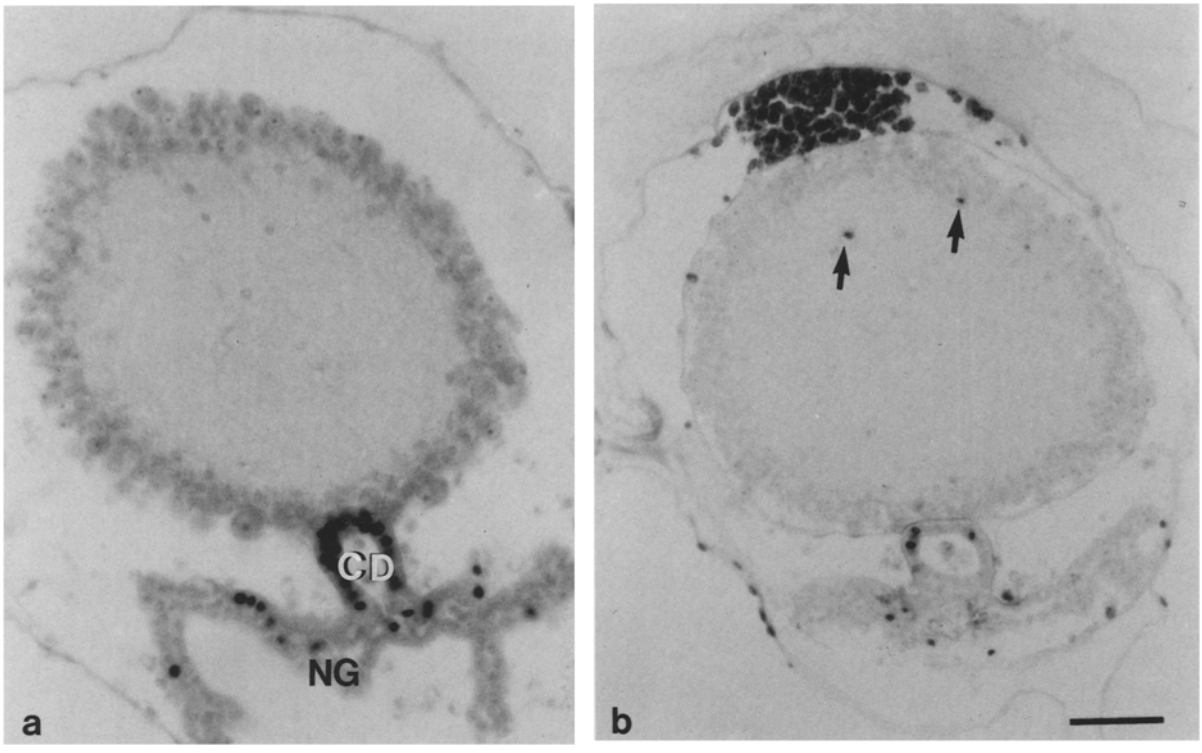

Fig. 6a, b. BrdU incorporation in the neural complex at the 11 to 15-mm stage. *CD* Ciliated duct; *NG* neural gland. **a** Animal sacrificed immediately following a 12-h pulse; labelled nuclei are seen only in the ciliated duct and in the neural gland. **b** Similar section from a specimen sacrificed 5 days after a 12-h pulse showing labelled nuclei in the ganglion (*arrows*), ciliated duct and neural gland. A collection of pigmented blood cells can be seen lying dorsal to the ganglion. *Bar:* 25 μM; × 506

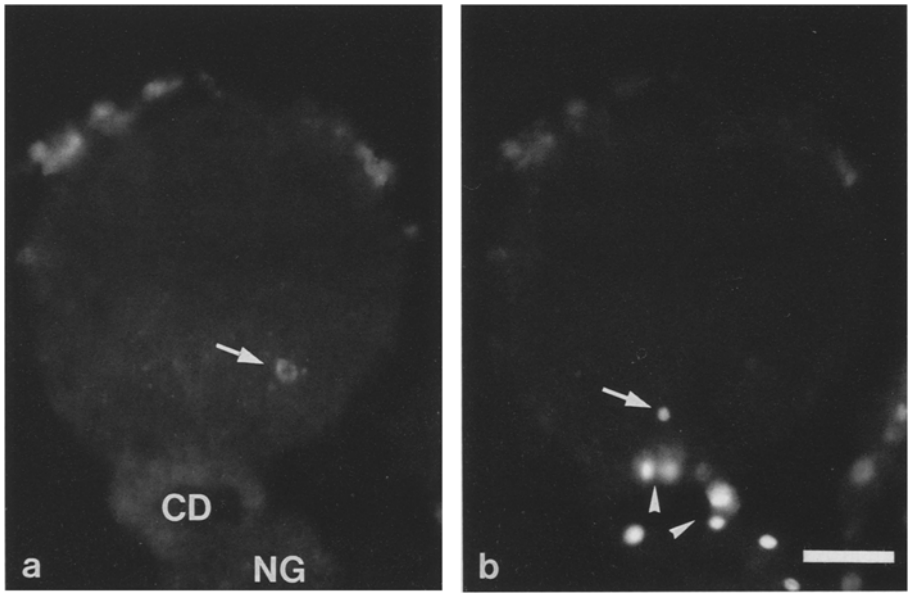

Fig. 7a, b. Section of a ganglion from the 6 to 10-mm stage; double-labelled for detection of SP-li immunoreactivity and BrdU according to method 3. In both pictures, some auto-fluorescence can be seen in the blood cells on the dorsal surface of the ganglion (*top*). *CD* Ciliated duct; *NG* neural gland. **a** *Arrow* shows a SP-li immunoreactive neurone in the inner cortical layer; some transversely cut fibres can also be seen. **b** Labelled nuclei in the ganglion (*arrow*), the ciliated duct and the neural gland (*arrowheads*). *Bar:* 25 μm; × 481

good deal of incorporation was seen in the ciliated funnel, in the region were it tapers off to become the duct, labelling was absent.

No clear distinction could be made between those animals left for 5 to 11 days before fixation. They all showed labelled nuclei in the brain (Fig. 6b), mainly in the mid region but also to some extent in the more distal parts.

Double labelling

Double-labelling immunocytochemistry was performed on specimens of the same three age-groups as above, but not on animals sacrificed immediately following the pulse. In no case was co-localization of BrdU and peptide immunoreactivity observed, although separate BrdU and peptide staining was evident in the brain in

the same sections (Fig. 7a, b). It should be noted here that, in the double-stained preparations prepared according to methods 1 and 2, the density of the immunocytochemical reaction product for the peptide antisera was always weaker compared to preparations that had not undergone processing for BrdU localization. It is possible that these methods reduced peptide immunoreactivity, and thus we were unable to detect co-localization because of weakened peptide staining. The third method using biotin/avidin complexes seemed to produce strong but separate staining of both peptide and BrdU-labelled nuclei, supporting the notion that the peptide-containing cells were born prior to the administration of BrdU in these experiments.

Discussion

This is the first report concerning the appearance of neuropeptide expression during post-metamorphic development in the nervous system of an ascidian. SP-li immunoreactivity is present in animals at the 1-mm stage when the neural complex consists of a cluster of cells with no neuropile. Later in development, cells immunoreactive for SP and CCK can be found in the 3 to 5-mm group, when the ganglion is organized in a more adult-like fashion, with cell bodies surrounding a neuropile of fibres. Because of insufficient material, only the 1-mm stage was investigated for SP-li immunoreactivity and thus the possibility exists that the other peptide also occurs at this stage.

At the 3 to 5-mm stage, both SP-li and CCK-li immunoreactivities are found in cell bodies and in fibres of the neuropile. The clear distributional pattern with CCK-li immunoreactivity predominantly in the anterior part and SP-li immunoreactivity in the cortex indicates that, even at this stage, there are different peptidergic cell populations. As development proceeds, the adult pattern is gradually established whereby CCK-li immunoreactive cells become concentrated at the points of exit of the nerve trunks, whereas SP-li immunoreactive cells are more or less restricted to the inner regions of the cortical zone, with a few cells being scattered in the neuropile.

In a parallel study, we have also considered the distribution of neurons exhibiting GABA-like immunoreactivity (GABA-li) in developing and regenerating ganglia in *Ciona* (Bollner et al. 1993). This classical transmitter, in both normal development and regeneration, is expressed later than the neuropeptides investigated here. This adds creedence to the notion that the neuropeptides presently studied are more important as growth regulators and/or neuromodulators for the early stages of ganglion regeneration and normal development than is GABA. These ideas are in keeping with the possible role of substance P as a growth regulator in other invertebrate systems (Baguñà et al. 1989b).

The BrdU experiments indicate that the cells of the brain arise outside this organ, since no incorporation is seen in the brain when sacrificed immediately after the BrdU pulse. This, together with the finding of labelled nuclei when animals are allowed to survive for a further 5 days, strongly suggests a mechanism whereby cells migrate to the brain from other sites. The origin, or origins, of these cells is not clear but the incorporation of BrdU seen in the ciliated duct epithelium might implicate this as a source of neurons. This is in agreement with the situation in another species, *Ecteinascidia turbinata* (Elwyn 1937). It should be noted that, in the latter species, this process is reported to commence during the embryonic stage, whereas in *Ciona*, our results indicate that it starts during metamorphosis. Certainly, it does not take place in the embryo of this species (Nicol and Meinertzhagen 1991).

At present, we cannot completely rule out other sources for cell recruitment to the developing ganglion and, in this respect, it is of considerable interest that the temporal developmental pattern presently described for SP-li and CCK-li immunoreactive neurons in *Ciona* closely mirrors that seen during the early stages of neural complex regeneration in this species following total ablation central nervous system (Bollner et al. 1992). Here, SP-li immunoreactivity first appears evenly distributed throughout the newly forming ganglion with no discrimination between the cortical and neuropilar zones. Only later in the course of regeneration are the SP-li immunoreactive cells predominantly localized in the ganglion cortex. Similarly, CCK-li immunoreactive neurons in the regenerating ganglion are, from the outset, concentrated at peripheral sites, particularly those associated with the points of anterior nerve trunk emergence (Bollner et al. 1992).

The origin of the neurons in normal development probably lies in a collection of stem cells, as is the case with many other invertebrate developmental systems (Palmberg 1986; Plickert et al. 1988; Baguñà et al. 1989a). In ascidians, these may well be represented by the epithelium described above which on the basis of the BrdU incorporation at least, is a population of actively dividing cells. In regeneration, however, the origin of the new neurones is less certain. In addition to an epithelial stem cell origin, transdifferentiation of other cell types remains a possibility (Bollner et al. 1992, 1993).

Thus, although the present results indicate temporal similarities with respect to the origin and differentiation of neurones during normal development and regeneration, this may be only a superficial resemblance if, in development, the source of the new neurones is exclusively from epithelial stem cells, whereas in regeneration, a combination of stem cells and transdifferentiation contribute to the new tissue. To resolve this problem, it is of importance to recognize neurones, or neuronal precursors, at an earlier stage than is possible by labelling neurotransmitters immunocytochemically. This could be achieved by employing specific monoclonal antibodies to neuronal antigens present prior to neurotransmitter expression, an approach now being undertaken in this laboratory.

The function of both SP and CCK in ascidians is clearly relevant to both normal development and regeneration. Recently, studies in this laboratory have established a fundamental role for these peptides in feeding

and digestion in both *Ciona* and another species *Styela clava*. In the latter, peptides of the gastrin/CCK family are clearly implicated in gut secretory activity (Thorndyke and Bevis 1984). In *Ciona*, substance P has been shown to play a part in the control of contractile activity of the body wall and siphons (Thorndyke and Georges 1988). Thus, in addition to their potential as growth-promoting factors, these peptides are central components in the normal physiology of these animals; one might therefore expect that their early expression would be a priority in the development of a feeding adult from a non-feeding larva.

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