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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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% H₂O₂.

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. 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

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