Distribution of GABA-like immunoreactivity during post-metamorphic development and regeneration of the central nervous system in the ascidian *Ciona intestinalis*

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Abstract. During regeneration of the neural ganglion in *Ciona intestinalis*, the pattern of reappearance of some peptidergic cells is similar to the ontogenetic patterns exhibited by these cell types during normal post-metamorphic development. Using a specific antiserum to gamma-aminobutyric acid (GABA), we describe here the appearance of GABA-ergic cells in *Ciona* during both post-metamorphic development and regeneration of the neural ganglion following total ablation. Post-metamorphic animals were divided into the categories: 1, 3-5, 6-10, 11-15 and 23-27 mm in body length. Regeneration was monitored at 12, 15, 18, 21, 28 and 56 days post ablation. The first appearance of GABA-like immunoreactive cells during normal development were at the 3 to 5-mm stage where they were seen as discrete cells, without processes, evenly distributed in the cortical region throughout the ganglion. Fibres were first seen at the 6 to 10-mm stage. As development proceeded, GABA-like immunoreactive cells became more concentrated near the nerve root exits and along the dorsal rind of the ganglion. In regenerating ganglia, GABA was first detected at 18-21 days post ablation, in cells that lacked any obvious processes and that were distributed in all regions of the ganglion. Fibres were first seen at the 6 to 10-mm stage. As development proceeded, GABA-like immunoreactive cells became more concentrated near the nerve root exits and along the dorsal rind of the ganglion. In regenerating ganglia, GABA was first detected at 18-21 days post ablation, in cells that lacked any obvious processes and that were distributed in all regions of the ganglion. At 28 days post ablation, processes could be detected in the neuropile, and after 56 days GABA cells were found predominantly in the same regions as in the normally developing adult ganglion. Although the overall pattern reflects that in a normal adult, a few differences were detectable. For example, rather more GABAergic cells were concentrated ventrally in the ganglion close to the neural gland.

Key words: GABA — Development, neural ganglion — Neural repair — *Ciona intestinalis* (Urochordata, Tunicata)

Introduction

Ascidians belong to the subphylum Urochordata, and are members of the phylum Chordata. The free-swimming larva with its typical chordate features, e.g., a dorsal hollow nerve tube and a notochord, metamorphoses into a sessile animal with a nervous system of typical invertebrate organization. The adult ascidian nervous system consists of two main parts: a glandular structure (the neural gland) and the ganglion proper with a cortical rind of neurons and central neuropile of fibres (Miller 1953; Berrill 1955).

Although Elwyn (1937) has described the formation of the adult neural complex from the so-called neurohyphophysis of the larva, the developmental changes taking place in the nervous system from metamorphosis to sexually mature adult are poorly understood. For example, little is known about the differentiation of the nervous system and the development of expression of the different putative neurotransmitters known to be present in the adult (for a review, see Thorndyke and Georges 1988). Temporal changes must take place during development, however, since Georges (1985) has demonstrated immunocytochemically the occurrence of serotonin in juvenile *Ciona intestinalis*, although she and others (Welsh and Loveland 1968; Osborne et al. 1979) have failed to find the same substance in the adult brain.

It has long been known that the central nervous system (CNS) of ascidians has considerable powers of regeneration and that the neural complex of *Ciona intestinalis* can regenerate in its entirety following total ablation (Schultze 1899). During regeneration, antisera directed against the peptides substance P (SP) and cholecystokinin (CCK) show staining localized to specific cell populations that re-appear and eventually form a pattern similar to their distribution in the normal adult CNS (Bollner et al. 1992). It is possible that patterns seen during regeneration reflect those of the normal developmental sequence (Purves and Lichtman 1985: Aguayo et al. 1991). In order to study this problem with regard to a classical neurotransmitter, we decided to use...
immunocytochemistry as a tool for identifying neurons during normal growth and in the course of regeneration.

Classical transmitters, such as acetylcholine (ACh), amines and amino acids, have been identified in tunicates by biochemical methods; for example, Florey (1963, 1967), has shown the presence of ACh whereas Osborne et al. (1979), investigating *Ciona intestinalis*, have found several amino acids including gamma-aminobutyric acid (GABA), in homogenates of neural complexes. The latter authors have also demonstrated an effect on cyclic AMP levels in this animal of noradrenaline and dopamine.

The immunocytochemical localization of GABA has also been reported in some invertebrate phyla, e.g., insects (Meyer et al. 1986; Robertson and Wisnioski 1988) and molluscs (Vителaro-Zuccarello and De Biasi 1988). In contrast, despite the detection of GABA in extracts of the brain from *Ciona intestinalis* (Osborne et al. 1979), its precise localization has not been reported in any ascidian species although Bollner et al. (1991) have found GABA immunoreactivity in association with the central nervous system and peripheral neuromuscular terminals, in the nervous system of another urochordate, the appendicularian *Oikopleura dioica*.

Here we have used an antiserum directed against GABA to investigate the pattern of GABA expression in the normal adult neural ganglion of *Ciona* during both post-metamorphic development and regeneration following ablation of the neural complex.

**Materials and methods**

Experiments were performed at the Tjärnö Marine Biological Laboratory in Sweden. For regeneration studies, ablation of the neural complex was carried out as previously described (Bollner et al. 1992) on animals anaesthetised in 0.03% MS222 (Sigma, St. Louis, USA). Animals were kept at approximately 16°C in an open seawater system. Regeneration was monitored by sacrificing animals at 12, 15, 18, 21, 28 and 56 days post ablation (pa). Juvenile animals of different sizes were used to follow the temporal distribution of GABA-like (GABA-li) immunoreactivity during normal development. These were divided into the classes: 1 mm, 2 mm, 3–5 mm, 6–10 mm, 11–15 mm and 23–27 mm in body length. Sexually mature animals of more than 50 mm in body length were considered to represent the final developmental stage. At this length, all examined specimens had gonads producing gametes. All tissues were stored in 3% glutaraldehyde in seawater saturated with picric acid, fixed in 3% glutaraldehyde in seawater saturated with picric acid, or in 4% paraformaldehyde in 0.1M sodium-phosphate buffer at pH 7.4. The specimens were then dehydrated and embedded in paraflin wax. Sections were cut 3–5 mm thick. After antigen recovery, all sections were processed for GABA immunocytochemistry.

Controls included (1) pre-absorption of GABA antiserum with GABA-glutaraldehyde complexes (GABA-G) (Ottersen et al. 1986) at a final GABA concentration of 400 μM, or (2) replacement of the primary antiserum with normal rabbit serum. The immunoreactivity of the antiserum used was also tested by spotting 50 pmol GABA on activated nitrocellulose paper as described in Hodgson et al. (1985).

**Results**

**Normal development**

Neither 1-mm-long nor 2-mm-long specimens revealed any specific immunoreactivity in the central nervous system. The overall background staining in these two groups was higher than later stages even when processed on the same slide, but no difference in staining intensity could be seen between different tissues.

In the 3 to 5-mm group, some specimens revealed specific GABA-li immunoreactive cells in the ganglion. These cells were predominantly located in the periphery rather than the central region (Fig. 1a). All stained cells were of approximately the same size and no obvious axonal processes were seen.

At the 6 to 10-mm stage, GABA immunostaining was found mainly in the areas near the nerve root exits, where they seemed to be the predominant cell type (Fig. 1b). Some of these cells displayed short processes (Fig. 1c). GABA-li immunoreactive cells were also concentrated along the cortical layer on the dorsal side of the ganglion. A few stained cells were observed in the core of the ganglion, the neuropile, which at this stage also displayed a small number of GABA-li immunoreactive fibres. Furthermore, some fibres in the nerve roots leaving the ganglion were also stained with the GABA antiserum (Fig. 1d). In the 11 to 15-mm group, the distribution of GABA-li immunoreactive cells observed in the 6 to 10-mm group was confirmed.

In animals of approximately 25 mm length, the pattern of distribution was identical to that seen in the previous group, the only difference being the occurrence of smaller GABA-li immunoreactive cells together with GABA-li immunoreactive fibres.