Investigation of the contribution from peripheral GnRH-like immunoreactive 'neuroblasts' to the regenerating central nervous system in the protochordate *Ciona intestinalis*

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SUMMARY

The neural ganglion of the ascidian *Ciona intestinalis* regenerates in its entirety within a few weeks of ablation. Here we investigate the role of gonadotropin-releasing hormone-like immunoreactive (GnRH-li) cells in regeneration. Immunocytochemical studies show that in addition to a previously described plexus of GnRH-like neurones located in association with the dorsal strand, the normal adult brain contains GnRH-li neurones. These are predominantly localized to the ventral cortical rind at the posterior of the ganglion. Following ablation, non-process bearing GnRH-li cells appear in the regenerating area within two days. By day 8 post-ablation, process bearing GnRH-li cells are detected close to the regenerating brain. The number of these cells increases at later stages. GnRH-li cells are first detected within the regenerating brain at 14 days post-ablation and their number subsequently increases. These cells are initially concentrated along the ventral surface of the regenerating brain near to the dorsal strand. Double labelling studies with bromodeoxyuridine show that none of the GnRH-li cells are labelled at any stage of regeneration. The data are consistent with a sub-population of the new neurones being derived from GnRH-li neuroblasts born prior to ablation, which migrate from the dorsal strand complex into the regenerating ganglion.

1. INTRODUCTION

In previous studies we have described the regeneration of the central nervous system (CNS) in the protochordate ascidian *Ciona intestinalis*. In this species the neural complex, comprising the ganglion (brain) and neural gland, regenerates within a few weeks following its total ablation. Immunocytochemical studies with antisera directed against selected neuropeptides and γ -aminobutyric acid, as well as monoclonal antibodies to other neuronal components, show that the distributional pattern of a variety of molecules is restored (Bollner *et al.* 1992, 1993*a, b*; Bollner *et al.* 1995).

The cellular origin of the regenerated neural complex is not clear. Studies with bromodeoxyuridine (BrdU) indicate that the regenerating ganglion includes some cells clearly born in response to the ablation. However, a subpopulation of the ganglion cells shows no incorporation of BrdU, indicating that they were born prior to the ablation (Bollner *et al.* 1995). This suggests that at least two different populations of progenitor cells are involved in the regenerative process. The most likely candidates are the dorsal strand cells, which are thought to be of

neural tube origin (Elwyn 1937), and circulating pluripotent stem cells of haemopoietic origin.

The dorsal strand is a hollow structure running posteriorly from the neural gland to the gonads. It is anteriorly extended as the ciliated duct that opens into the lumen of the pharynx as the ciliated funnel (figure 1.)

Associated with the dorsal strand is a nerve plexus comprising neurones situated outside the CNS (Fedele 1938). A recent report (Mackie 1995) demonstrated that these cells show immunoreactivity with an antiserum directed against gonadotropin-releasing hormone (GnRH). Processes from the plexus run along the length of the dorsal strand and some extend into the visceral nerve, one of the posterior nerves leaving the brain. Immunoreactivity was also seen in cells lining the wall of the blood sinuses and in the gill bars of the pharyngeal basket.

The dorsal strand plexus also includes a population of GnRH-li immunoreactive cells lacking neurites, morphologically resembling neuroblasts, situated mainly in the anterior region, near the brain. Close to these were cells displaying short processes suggested to be migratory stages of not fully mature cells (Mackie 1995). It has been proposed that these cells are derived from the dorsal strand through delamination (Fedele 1938), and it is plausible that the dorsal strand plexus

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Figure 1. (a) Diagram of adult *Ciona*, viewed from the left, showing the location of the neural complex (brain and neural gland), dorsal strand with nerve plexus and visceral nerve in relation to the gonads. (b) A ventral view of the neural complex. The ciliated funnel, the ciliated duct and the dorsal strand (shaded) form a continuous epithelium. The neural gland is formed as an evagination from the ciliated duct. The dorsal strand nerve plexus follows the dorsal strand and sends processes into the visceral nerve.

contains a pool of neuroblasts. Such neuroblasts are of interest in the present context as a potential source of new neurones in the regenerating brain.

In this paper we present data on the temporal development of the GnRH-li immunoreactive cells of the dorsal strand plexus during regeneration. We also describe the distribution of GnRH-li immunoreactive cells in the normal adult and regenerating brain of *Ciona intestinalis*.

2. MATERIAL AND METHODS

(a) Animals

Ciona intestinalis were collected by divers at Tjärnö Marine Biological Laboratory, Sweden, and maintained in a running sea water system.

(b) Peptide immunocytochemistry

The animals were anaesthetized in 0.03% MS222 (Sigma, St Louis, USA) prior to fixation in Bouin's fluid made up in sea water. Animals were dissected and the tissue of interest pinned in Sylgard coated dishes before addition of the fixative.

Preparations were dehydrated and embedded in paraffin wax. Serial sections were cut at 8–10 µm, mounted on poly-L-lysine coated glass slides, dewaxed in xylene and rehydrated in a graded ethanol series.

Specimens intended for wholemount preparation were washed in phosphate-buffered saline (PBS) followed by 70% ethanol (to wash out picric acid residues). Specimens were transferred to PBS with 0.5% Triton X-100 for a period of

4 h or overnight, followed by 2 h in blocking solution (PBS with 0.1% Triton X-100 and 2% normal goat serum) and transfer to anti-GnRH antiserum (Bla-5) (dilution 1:800) for 48 h. This was followed by incubation in biotinylated goat anti-rabbit serum (dilution 1:200) (Vector Laboratories, Peterborough, UK) overnight and Texas red conjugated avidin (dilution 1:200) (Vector Laboratories). All dilutions were in blocking solution and antibody incubations were carried out at 4 °C on a rocking table. Each incubation step was separated by 5 × 10 min washes in PBS containing 0.1% Triton X-100 (PBS-TX).

(c) Bromodeoxyuridine labelling of the regenerating 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). Two protocols for incorporation of BrdU were used. In the first, animals were immersed for 24 h in BrdU (250 µM) dissolved in filtered seawater at 2, 8, 14 or 21 days post-ablation, then sacrificed immediately after the pulse. In the second, animals were maintained in BrdU-seawater (7 animals l⁻¹ of seawater) (prepared as above) for seven and 14 days, respectively, and then immediately fixed. Half of the water was replaced every 10–12 hours during the experiment.

(d) BrdU and peptide double labelling immunocytochemistry

Double labelling for GnRH-li and BrdU was carried out as wholemount preparations. Staining for GnRH-li was as described, followed by treatment of preparations with 2 M HCl for 45 min, followed by 1 wash in 0.1 M Borax and 3 washes in PBS-TX. Incubation in monoclonal anti-BrdU was overnight at 4 °C. After 5 × 10 min washes in PBS-TX, FITC-conjugated anti-mouse antiserum was applied overnight at 4 °C followed by 5 × 10 min washes in PBS-TX. The specimens were mounted, ventral side up, in Vectashield (Vector Laboratories) and analysed using a Leica confocal microscope.

3. RESULTS

(a) Normal adult

The results of wholemount studies (data not shown) show that the nerve plexus around the dorsal strand and some fibres in the visceral nerve exhibit prominent GnRH-like immunoreactivity (see also Mackie 1995). However, due to the thickness of the preparation the distribution of GnRH-like immunoreactivity in the brain could not be readily established using this technique. Therefore we used sectioned material to investigate the distribution in the brain.

In the normal adult brain GnRH-like immunoreactivity was found in several morphologically different types of cells. Most of the GnRH-li neurones were located in the cortical rind, at the ventral side, in the posterior part of the brain (figure 2*b–d*). In most of these cells at least one conspicuous process could be seen. Similar cells were found also in the dorsal and anterior cortical layers although in smaller numbers (figure 2*b, c, f*). Smaller-sized immunostained cells were scattered in the neuropile and occasionally also in

Figure 2. Distribution of GnRH-li in the normal adult. (a–e) Series of transverse sections showing a frontal view of GnRH-like immunoreactivity in the normal adult CNS. Note that dark cells laying just under the epidermis (ep) are non-immunoreactive pigmented haemocytetes (bar = 100 μ m). Approximate level of section is indicated in schematic drawing (top left). (a) Section through the area where the two anterior nerves exit the brain. A few fibres can be seen in the nerves and one cell body is located on the dorsal surface of the left anterior nerve (arrowhead) and two smaller immunostained cells can be seen in the right nerve (arrow). (b) Section through the middle part of the brain with a few GnRH-li cells in the cortical layer (arrows) and numerous cross-sectioned fibres in the neuropile (arrowheads). (c) An increasing number of cortical GnRH-li cells (arrows) and fibres (arrowheads) can be seen in the more posterior region. (d) The most posterior part of the brain shows GnRH-li in the ventral cortex and the plexus (arrowheads) can be seen ventral to the brain. (e) As the posterior nerves exit, numerous GnRH-li cell bodies (arrowheads) can be seen ensheathing the right nerve, these are associated with the dorsal strand which makes a loop over this nerve before descending caudally. (f) Detail showing some GnRH-li cells in the cortical layer near the posterior nerve exits (bar = 50 μ m). (g) Transverse section showing GnRH-li cells (arrow) ensheathing the dorsal strand (bar = 50 μ m). (h) High power micrograph of GnRH-li cells lining the dorsal blood sinus (bar = 25 μ m).

the cortical layer. Immunoreactive fibres were prominent in all the nerves (figure 2a, e) and in the neuropile, especially in the posterior region. In transverse sections of the dorsal strand and surrounding tissues GnRH-li cell bodies were clearly seen ensheathing the epithelial

cells of the dorsal strand (figure 2g). The transverse sections through the blood sinuses clearly showed that GnRH-li cells line the interior of the sinus (figure 2h), thus confirming earlier wholemount studies (Mackie 1995).

Figure 3. Confocal images of wholemount preparations two (*a–b*) and eight (*c–d*) days post-ablation, anterior is to the left. (*a*) GnRH-li cells (red) displaying no, or only short, processes can be seen in the regenerating area in the left part of the picture, whereas to the right, the intact plexus with typical plexus GnRH-li cells is evident. (*c*) High magnification of GnRH-li cell in the regenerating area in (*a*) showing the lack of immunostained processes. (*b*) A more posterior part of the plexus and dorsal strand. BrdU-labelled cells (green) can be seen in the dorsal strand (arrows) whereas the plexus neurones lack BrdU labelling. (*d*) BrdU-labelled cells showing the bladder-like structure (arrow) forming at the anterior end of the dorsal strand, the bladder is formed just posterior to the circum-oral blood vessels (arrowheads). (*e*) Detail showing BrdU-labelled (green) cells in the dorsal strand (arrowheads) and other unidentified labelled cells interspersed among GnRH-li (red) plexus cells none of which show the BrdU label.

(b) Regeneration

(i) Two days post-ablation

At the posterior side of the ablation the dorsal strand had grown by proliferation. There was a clear transition between normal and regenerating zones (figure 3*a*). In the undamaged part, GnRH-li cells displayed extensive processes whereas they lacked processes in the regenerating area (figure 3*b, c*). None of these cells showed BrdU incorporation, therefore it is likely that they had migrated into the area.

Extensive mitotic activity could be seen in the anterior-most region of the dorsal strand itself. The mitotic activity declined posteriorly and at approximately 20–25 mm posterior to the site of ablation only a few BrdU-labelled nuclei were detected in the dorsal strand.

(ii) Eight days post-ablation

At this stage a new small neural complex was formed. The epithelium of the dorsal strand had made contact with the pharynx and was expanding laterally. The resulting bladder-like structure occupied the space that was formerly occupied by the neural complex

prior to the ablation (figure 3*d*). These epithelial cells could not be distinguished from the more posterior dorsal strand cells, thus indicating that the gland had not differentiated at this stage. Most of the epithelial cells are BrdU labelled. Some labelled nuclei could also be seen in the regenerating brain.

The dorsal strand plexus now displayed process bearing GnRH-li cells close to the regenerating brain (figure 3*e*). There were also some GnRH-li cells without processes in this region. None of the GnRH-li cells showed BrdU labelling, indicating that they were not born post-ablation.

(iii) Fourteen days post-ablation

By this stage the neural complex had differentiated into a brain and an epithelial-derived neural gland. BrdU-labelled nuclei were prominent in the epithelium of both the gland and the dorsal strand. There was an increase, relative to earlier stages, in the number of GnRH-li cells with processes in the dorsal strand plexus just posterior to the neural complex (data not shown).

In the brain, GnRH-like immunoreactive cells were first seen at 14 d post-ablation. These cells were

Figure 4. Transverse section through the regenerating neural complex at 14 days post-ablation. Several GnRH-like immunostained cell bodies (arrows) can be seen in the brain, some display a strong granular staining in the cytoplasm. Some immunostained beaded fibres can also be seen (arrow heads) (bar = 25 μ m).

predominantly located at the ventral surface, i.e. near the dorsal strand and its associated plexus. Also, immunostained fibres were detected in the neuropile at this stage (figure 4).

Again, none of the GnRH-li cells showed evidence of BrdU incorporation.

(iv) *Twenty-one days post-ablation*

Normal patterns of GnRH-li distribution were almost restored at this stage. The dorsal strand plexus resembled that in the normal adult with an extensive network of GnRH-li cells. There were evidently a larger number of GnRH-like immunoreactive cells in all regions of the brain including the dorsal rind of the cortex. However, as in the normal adult brain, GnRH-like immunoreactive cells were mainly found in the ventral/posterior region. Although no quantitative analysis was made there seemed to be fewer BrdU-labelled cells in the neural complex and dorsal strand compared to the earlier stages indicating that the rate of incorporation of new cells had declined.

4. DISCUSSION

The main purpose of this study was to establish whether the GnRH-like immunoreactive cells of the dorsal strand plexus contribute to the formation of the regenerated CNS. We have used a well characterized antiserum directed against lamprey GnRH (Kelsall *et al.* 1990) to study regeneration of the complete neural complex.

We could find no evidence indicating that any of the GnRH-li cells were born post-ablation. Even when ablated animals were allowed to regenerate their neural complex in the constant presence of BrdU for 15 days, none of the GnRH-li cells exhibited detectable BrdU immunoreactivity. Although, BrdU-positive nuclei are present in the regenerating brain, we do not

Figure 5. Schematic representation of proposed pathway for regenerating GnRH-like immunoreactive neurones. Stem cells in the dorsal strand epithelium delaminate to form GnRH 'neuroblasts' (shaded). The delaminated cells are replaced by mitosis in the dorsal strand epithelial cells. The 'neuroblasts' are then recruited either as GnRH neurones in the brain or in the dorsal strand plexus.

know what kind of cells they are since we have never been able to show neuropeptide-immunoreactivity in any cell that incorporated BrdU. The use of BrdU in double labelling experiments is a well established technique that has provided birth dates for phenotypically identified cells in various systems including hydrozoans and mammals (Kroihner *et al.* 1990; Soriano & Del Rio 1991). Therefore, it seems unlikely that the lack of double labelled cells in this investigation is due to failure of the technique.

To account for the non-BrdU-labelled cells in the brain we have to conclude that there are stem cells present prior to the ablation. However, the origin of the putative stem cells is enigmatic. The GnRH-li 'neuroblasts' described by Mackie (1995) are still plausible candidates. At two days post-ablation we found GnRH-li cells in the regenerating area, most of which were lacking obvious processes, thus resembling the 'neuroblasts' of Mackie (1995). The appearance of these non-dividing cells in the regenerating area clearly indicates their ability to migrate and hence become incorporated into the reforming neural complex. We have no way of tracking these cells as regeneration proceeds but we do find GnRH-li neurones, all born prior to the ablation, in the regenerated brain at 14 days post-ablation and thereafter. The number of GnRH-li cells in the brain increases during the regenerative process and it is possible that these all originate as neuroblasts in the dorsal strand plexus. However, we cannot rule out the possibility that these neuroblasts contribute other types of cells to the regenerated brain.

It has been suggested that the precursor cells, or neuroblasts, are formed through delamination from the dorsal strand epithelium (Fedele 1938) during post-metamorphic development. It is possible that a similar mechanism is involved in the regeneration process. Although none of the GnRH-li plexus cells were labelled for BrdU, the dorsal strand epithelium was shown to be proliferating. Since most of the dorsal strand is left intact by the ablation of the neural complex it is possible that the new plexus cells are derived from this part of the epithelium and that these cells in turn are replaced by proliferation in the epithelium itself (figure 5).

The proposed model would then involve delamination from an epithelial structure followed by migration of cells expressing a neuropeptide before reaching their targets (i.e. the brain or the plexus). Although not typical of developing neurones it is a phenomenon described in other systems. In studies of the development of GnRH neurones in the mammalian brain there is evidence indicating that all GnRH-expressing cells are born in the olfactory pit and subsequently migrate to their final destinations elsewhere in the brain (Schwanzel-Fukuda & Pfaff 1989; Wray *et al.* 1989).

Our data show that the distributional patterns of GnRH-li cells in the normal adult animals are re-established, both in the dorsal strand and in the brain during regeneration. There have been several other studies on the distribution of GnRH-li in the adult ascidian nervous system and the results are not conclusive. There is agreement on the presence of a GnRH-like immunoreactive nerve plexus associated with the dorsal strand in all investigated species. There are, however, recorded differences in the distribution of GnRH-li in the brain. In contrast to the findings in the present report others have not found GnRH-li cell bodies in the brain. Georges & Dubois (1985) found GnRH-li cell bodies only in the juvenile brain of *Ciona*, not in the adult. In *Chelyosoma*, an extensive network of GnRH-li fibres were detected in the brain but no cell bodies (Kelsall *et al.* 1990). Although, the observed difference between *Ciona* and *Chelyosoma* could be explained as a genus difference this cannot account for the lack of immunoreactive cell bodies in the brain of adult *Ciona intestinalis* reported by Georges & Dubois (1985). Another explanation could be differences in sensitivity and specificity of the antisera used. In our investigation we found that the staining of fibres was more intense than that of cell bodies in many preparations. It is therefore possible that the cell bodies contain less of the epitope recognised by the antiserum.

This raises the question as to whether or not ascidians possess more than one form of GnRH (Kelsall *et al.* 1990). If true, the cells in the brain might express a form of GnRH different from that expressed in the cells outside the brain. The recent isolation of two forms of GnRH from the ascidian *Chelyosoma* confirms this possibility (Powell *et al.* 1996). This is in keeping with the situation recognized in most vertebrates where the brain is thought to express more than one form. Here, neurones in the posterior tubercle and those more caudally located express one form (generally referred to as chicken II). The other hypothalamic form of GnRH is thought to be species-specific (Muske *et al.* 1994; Northcutt & Muske 1994).

The differences could also be due to differences in the fixation process. Mackie (1995) found that formaldehyde fixatives containing picric acid produce stronger, i.e. more intense, staining than those without it.

The function of the GnRH-li cells described in *Ciona* and other tunicates is not known. The gonadotropic importance of GnRH is well documented in many higher chordate systems and its association with the dorsal strand, which connects to the gonad, might

indicate a function in reproduction. Spawning in these animals occurs also when the neural complex is removed leaving the dorsal strand and its plexus intact (Hisaw *et al.* 1966).

From the present data we conclude that the GnRH-li 'neuroblasts' are likely to be involved in the regeneration of the neural complex. Whether they contribute to more than the GnRH system in the brain is an open question. The data presented here do not account for all neurones thought to be born prior to the ablation, indicating the complex nature of the regeneration phenomenon.

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