PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Patterns of bromodeoxyuridine incorporation and neuropeptide immunoreactivity during arm regeneration in the starfish Asterias rubens

Claire Moss, A. Jackie Hunter and Michael C. Thorndyke

Phil. Trans. R. Soc. Lond. B 1998 **353**, 421-436
doi: 10.1098/rstb.1998.0220

The Royal Society

Patterns of bromodeoxyuridine incorporation and neuropeptide immunoreactivity during arm regeneration in the starfish *Asterias rubens*

Claire Moss[1], **A. Jackie Hunter**[2] **and Michael C. Thorndyke**[1]*

[1]*School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK*
[2]*SmithKline Beecham Pharmaceuticals Ltd, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, UK*

Regeneration of the arm of the starfish, *Asterias rubens* (L.) (Echinodermata: Asteroidea) was examined using two preparations. The first involved regeneration of the entire arm tip and its associated sensory structures and the second examined regeneration of a small section of radial nerve cord in the mid-arm region. Cell cycle activity was investigated by incorporation of the thymidine analogue, bromodeoxyuridine (BrdU). Details of neuroanatomy were obtained by immunocytochemistry (ICC) using an antiserum to the recently isolated starfish neuropeptide, GFNSALMFamide (Sl). BrdU labelling indicated that initial events occur by morphallaxis, with cell cycle activity first apparent after formation of a wound epidermis. As regeneration proceeded, BrdU immunoreactive (IR) nuclei revealed cell cycle activity in cells at the distal ends of the radial nerve cord epidermis, in the coelomic epithelium, the perihaemal and water vascular canal epithelia, and in the forming tube feet of both preparations. By varying the time between BrdU pulses and tissue fixation, the possible migration or differentiation of labelled cells was investigated. Neuropeptide ICC indicated the extension of Sl-IR nerve fibres into the regenerating area, soon after initial wound healing processes were complete. These fibres were varicose and disorganized in appearance, when compared to the normal pattern of Sl-IR in the radial nerve. Sl-IR was also observed in cell bodies, which reappeared in the reforming optic cushion and radial nerve at later stages of regeneration. Double labelling studies with anti-BrdU and anti-Sl showed no co-localization in these cell bodies, in all the stages examined. It appeared that Sl-IR cells were not undergoing, and had not recently undergone, cell cycle activity. It cannot be confirmed whether Sl-IR neurons were derived from proliferating cells of epithelial origin, or from transdifferentiation of epithelial cells, although the former mechanism is suggested. Differentiation of the regenerating structures to replace cells such as Sl-containing neurons, is thought to involve cell cycle activity and differentiation of epithelial cells in the epidermal tissue, possibly in association with certain types of coelomocytes which move into the regenerating area.

Keywords: regeneration; echinodermata; bromodeoxyuridine; Sl; immunocytochemistry

1. INTRODUCTION

The ability of many animals to regenerate various body parts has been of great scientific and medical interest for some years (Dinsmore 1991). Subsequently it has been discovered that the role of the nervous system during regeneration, and neural regeneration itself, is of fundamental importance for all aspects of regeneration (Singer & Geraudie 1989). Indeed, in invertebrates it has been suggested that the capacity for neural regrowth can be broadly related to their general potential for bodily regeneration (Treherne *et al*. 1988).

Mechanisms of regeneration have been described as comprising one or other of two processes: (i) morphallaxis, where cells differentiate or migrate from existing populations and (ii) epimorphosis, where mitosis occurs and new cells are produced, which either replace those lost directly, or form a 'pool' of precursor cells, which go on to differentiate and replace lost tissues (Wallace 1981). For example, in most cases of vertebrate limb regeneration, the process is epimorphic and dependent on the proliferation of a group of undifferentiated precursor cells, the blastema. However, in invertebrates there is often a more even combination of morphallaxis and epimorphosis (Goss 1969).

Although many studies have centred on vertebrates (Holder 1988), there have also been numerous investigations of invertebrates, including the echinoderms which are able to undergo extensive regeneration (Hyman 1955; Mladenov *et al*. 1989). Many species of echinoderms autotomize appendages, some are capable of evisceration, and some undergo asexual reproduction by fission; these phenomena all require a subsequent regenerative phase. An extreme example of this is the asteroid *Linckia*, which is able to regenerate an entire individual from one arm (Cuenot 1948).

Early studies in echinoderms concentrated on rates of regrowth or on structural changes (Zeleny 1903). Many of these investigations explored the effects of varying levels of

*Author for correspondence (m. thorndyke@rhbnc.ac.uk).

injury on the ability to regenerate after autotomy, fission and experimental injury to spines, test and nerve (Hyman 1955). The importance of the nervous system in arm regeneration has been suggested for various classes, including asteroids (Huet 1975). However, while there are some studies on the origin of regenerating structures in echinoderms, there are few detailed investigations on the origin of proliferating cells and even less which place an emphasis on neural tissue (Candia-Carnevali *et al.* 1995). The nature of neural regeneration in echinoderms, animals which are able to easily regenerate many body parts, is of particular interest.

Neuroanatomical changes in regenerating asteroids have previously been investigated by standard histological procedures, and more recently by ultrastructural studies (Candia-Carnevali *et al.* 1993), but the results are not extensive and do not provide the details of axon profiles and neurons made possible by immunocytochemistry (ICC). The aim of part of this work was to use an antibody raised to a synthetic analogue of GFNSALMFamide (Sl) (Elphick *et al.* 1991) as a neuronal marker for the comparison of normal and regenerative states in the starfish *Asterias rubens*. Sl is known to be present in neurons of the gut, skin (basiepithelial plexus), apical muscle, tube feet and other major nerve tracts of *A. rubens* (Moore & Thorndyke 1993; Newman *et al.* 1995*a*,*b*) as well as in the larval nervous system (Moss *et al.* 1994). Such a ubiquitous distribution of the peptide in asteroid nerves makes it especially useful as a marker of the nervous system, helping to clarify details of neural pathways and connections which have proved difficult to trace by standard techniques or other ICC probes (Cobb 1987). Any change in distribution of this neuropeptide may also be indicative of a role in the regrowth process, as is the case for many other neuropeptides (Strand *et al.* 1991).

There is little information on the origin of cells in the regenerating asteroid arm or the appearance of new neurons in the optic cushion or radial nerve. Questions concerning the mechanisms of regrowth, such as the involvement of stem cells, dedifferentiation of local tissues or transdifferentiation, either in the regenerating arm or nerve, have not been fully investigated. The aim of the second part of this study was to employ the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) to label cell cycle activity during regeneration in *A. rubens*, and to investigate the origin of the regenerated tissue of the arm tip and radial nerve cord. ICC studies using the Sl antiserum have been carried out in combination with BrdU incubation, in order to identify specific cell populations in an attempt to provide a more detailed picture of regeneration. By using arm tip and mid-arm nerve ablation procedures, it was possible to investigate both regeneration of a large area of the arm, including sensory structures, body wall, water and haemal canals, as well as a small area of nerve alone. Mid-arm nerve ablation also allowed some assessment of directionality with regard to the regrowing nerves as it resulted in a proximal and distal nerve stump, whereas the arm tip ablation resulted in only a single distal nerve stump. Such information may be useful when considering the control mechanisms that guide neuronal outgrowth.

The echinoderm nervous system consists of two components, the hyponeural and the ectoneural, which are separated by a basement membrane and thought to be involved in motor and sensory function, respectively (Smith 1966; Cobb 1987). In *A. rubens*, the neuropile of the ectoneural system runs the length of the radial nerve cord and circumoral nerve ring, with neuronal cell bodies scattered throughout the associated nerve epidermis. It is split into superficial and deep fibre layers, based on fibre morphology (figure 1*a*). The hyponeural system appears as clusters of neurons regularly grouped along the radial nerve cord, with fibres extending to effector organs such as the tube feet. In this study it is mainly outgrowth of ectoneural nerves and reappearance of ectoneural cell bodies which is under investigation. The hyponeural system is not present at the very tip of the arm and takes longer to regrow following mid-arm nerve ablation.

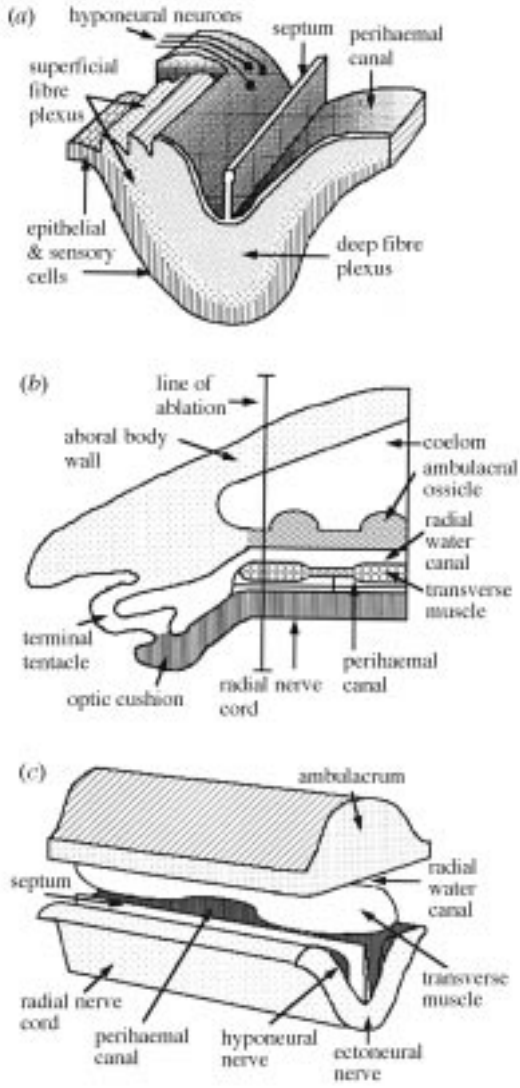

Figure 1. (*a*) Schematic representation of radial nerve cord (RNC) in transverse section showing various regions of neural tissue, after Smith (1966). (*b*) Arm tip in longitudinal section showing nerve, terminal sensory structures and line of ablation. (*c*) Longitudinal representation of RNC showing components of the ambulacral area. All tissue beneath the ambulacrum is removed in mid-arm nerve ablation.

2. METHODS

(a) *Maintenance and sampling of animals*

Specimens of the starfish *Asterias rubens* were collected at Millport Biological Marine Station, Isle of Cumbrae, Scotland, transported to Royal Holloway and maintained in a circulating seawater system at 12 °C. They were fed a diet of mussels once a week and prepared for regeneration experiments approximately 24 h after arrival. Experimental animals were between 10 and 15 cm across, from arm tip to most distant arm tip.

(b) *The arm tip and radial nerve preparation*

To examine the regrowth of the arm of *A.rubens* the arm tip was removed, including the terminal sensory structures of the radial nerve cord (i.e. the optic cushion and terminal tentacle), terminal tube feet and body wall (figure 1*b*). The arm tip was removed by scalpel approximately two-thirds of the way down from the central disc, always using the two arms on either side of the madreporite. After the arm tip had been removed, animals were returned to the aquarium and re-examined at approximately weekly periods up to 50 days after the operation and then monthly up to 90 days. This allowed close observation of the early stages of healing, optic cushion and terminal tentacle reformation, as well as of the less rapid changes associated with the latter stage of regeneration. Controls were carried out by also taking the arm tip from a non-regenerating arm adjacent to the experimental arms, for all stages examined. Further controls were carried out by examining the arm tips of normal animals from the same batch held in the aquarium. At least five animals were observed at each stage, each with two regenerating arm tips.

In order to produce a preparation in which regrowth of the nerve alone could be studied, a system was designed which involved removing a small section of radial nerve from the mid-arm region of *A.rubens*. The animals were anaesthetized in 4% magnesium chloride in seawater for up to 20 min to immobilize the tube feet. The radial nerve, which lies on the oral side of the arm, is separated from the environment by a layer of cells, generally referred to as the epidermis of the nerve (figure 1*a*,*c*). Therefore, no incisions into the main body of the animal were necessary and the nerve and epidermis could be removed in one piece. A section of nerve two-thirds of the way down the arm was ablated in the two arms on either side of the madreporite (figure 3*a*,*b*). The length of nerve removed was always equivalent to the width of two tube feet in each experimental animal, to ensure a comparable injury size between animals. Animals were returned to the aquarium and observed at various stages after nerve ablation. Tissue was taken every 3–4 days, up to 28 days, and then approximately monthly up to 90 days. Controls were carried out by processing the section of nerve which had been removed in the ablation process. At least five animals were used at every stage, each having two regenerating areas. Several preparations were made at earlier key stages of regeneration, for use in whole mount experiments.

(c) *The BrdU/anti-BrdU technique*

The BrdU/anti-BrdU method was used to label DNA synthesis in the regenerating tissue (Gratzner 1982). Animals were incubated in a separate tank of seawater containing BrdU (SIGMA), and methodology and dilutions were based on previous studies on marine animals (Plickert *et al*. 1988; Bollner & Meinertzhagen 1992). To establish the optimal level of BrdU incorporation, animals were incubated in three concentrations of BrdU for four time periods. Optimal conditions could be described as those which gave sufficient levels of incorporation for analysis and for a period which did not have adverse effects on the animal. In adults, the optimal conditions were 36 h incubation at a concentration of 250 µM of BrdU. Incubation was normally two 18 h pulses, with replacement of fresh BrdU/sea water in between. The regenerating tissue was then fixed in Bouin's fluid made up in seawater (Schutte *et al*. 1987) and processed.

Fixation immediately after the BrdU pulse allowed detection of cells active during the previous 36 h. In an attempt to monitor the fate of labelled cells and reveal not only synthetic activity, but also differentiation or migration of cells which had previously divided, we used two other protocols. The first involved a series of staggered (short-term) fixations, where pulsed animals were returned to the aquarium and the regenerating tissue fixed at specified periods after the incubation (tables 1 and 2). The second protocol involved a simultaneous (long-term) fixation of all test animals at a specified period after the BrdU pulse (table 3).

(d) *Immunocytochemistry (ICC)*

Preparations for paraffin embedding and the majority of immunocytochemical tests were fixed in Bouin's fluid. Tissue was left in fixative for 12–18 h, at 4 °C. Following fixation, the calcified structures, such as spines and ambulacra, were removed as far as possible by dissection and tissue was bathed overnight to a maximum of 24 hours in 0.5 M of EDTA in filtered sea water (FSW) at 4 °C, pH 7.8. Most preparations were processed for paraffin wax sections using standard methods. Soft tissue for whole mount ICC was fixed in fresh 4% paraformaldehyde in FSW at 4 °C, for a minimum of 2 h up to a maximum of overnight.

The ABC avidin–biotin visualisation system was used as the standard ICC procedure (Hsu *et al*. 1981). For Sl ICC the antisera used was BLIV (hereafter referred to as anti-Sl) (Elphick *et al*. 1991). Sections were blocked for 1 h in 5% normal goat serum, followed by overnight incubation in the primary antibody. Sections were then rinsed in PBS and incubated in the appropriate biotinylated secondary antibody for 1 h. After washing in PBS, there was a further 1 h incubation in ABC reagent (Vector Labs). Visualization was then by the diaminobenzidine (DAB) (SIGMA)/peroxidase reaction (Sternberger 1979) in Tris-HCl buffer, pH 7.6. For fluorescence labelling, the ABC reagent was replaced with fluorescein or Texas red conjugated avidin (Vector Labs). Primary antisera were generally diluted in PBS with 1% normal goat serum, whereas normal serum, secondary antisera, ABC reagent and fluorescent markers were diluted in PBS alone.

DAB preparations were dehydrated and mounted in DPX. Fluorescent preparations were washed and mounted in 'Vectashield' antifade mountant (Vector Labs). For BrdU ICC, certain modifications were required to the standard ICC technique. Before incubation with normal serum, the sections were immersed in 2N HCl for 25 min to denature the DNA and expose the bases (Moran *et al*. 1985). Immediately following this incubation, the slides were plunged into 0.1 M of borax for 30 s to neutralize the acid (Kono *et al*. 1991), followed by several washes in PBS.

Double labelling studies were also carried out using Sl and BrdU antisera. In all cases these used the ABC avidin–biotin system with some modifications to the standard fluorescence procedure. Processing of the first antisera was as normal, using fluorescein-conjugated avidin to visualize the reaction. The second antibody was diluted in PBS and 0.1% Tween to further block any

Table 1. *BrdU pulses with modified fixation for regenerating arm tips (PA: post-ablation)*

incubation/ days PA	fixation/ days PA	incubation/ days PA	fixation/ days PA
14	18 & 21	27	31 & 34
17	21 & 24	30	34 & 37
20	24 & 27	32	36 & 39
22	26 & 29	35	39 & 42
25	29 & 32		

Table 2. *BrdU pulses with modified fixation for regenerating mid-arm radial nerve (PA: post-ablation)*

incubation/ days PA	fixation/ days PA	incubation/ days PA	fixation/ days PA
4	7 & 9	14	17 & 19
6	9 & 11	16	19 & 21
8	11 & 13	18	21 & 23
10	13 & 15	20	23 & 25
12	15 & 17	22	25 & 27

Table 3. *BrdU pulses for both regenerating arm tip and mid-arm radial nerve with simultaneous fixation (PA: post-ablation)*

incubation/days PA	fixation/ days PA
7	50
14	50
21	50

cross-reactivity between the antisera (Kono *et al.* 1991). Visualization was by Texas red-conjugated avidin D. Anti-BrdU was applied after anti-Sl to avoid any possibility of the acid step affecting the peptide antigenicity. For mid-arm radial nerve whole mount preparations, the standard ICC protocol was modified to allow time for penetration of reagents. After fixation and washing in PBS, tissue was incubated in PBS and 0.5% Triton-X 100 for 2 h and then 5% normal goat serum in PBS and 0.5% Triton X for a further 2 hours at room temperature. Incubation in primary antibody diluted in PBS and 0.1% Triton X was for 36 h at 4 °C. Visualization was as described above, except that incubation periods with secondary antibodies and avidin conjugated to a fluorescent marker were increased to 4 h. Observation was by a Zeiss Axioplan with fluorescence attachments.

ICC controls were by (i) preadsorption with the appropriate antigen, (ii) replacing the primary antisera with non-immune sera of the animal in which the primary antisera were raised, or (iii) by omitting the primary antibody and incubating in PBS and 1% normal goat serum. Preadsorption was performed by mixing two times the working concentration of antisera with an equal volume of 2 × 10 μM of antigen (Polak & Van Norden 1986).

3. RESULTS

(a) *Early events of healing and regeneration: 0–7 days*

(i) *The arm tip*

The normal distribution of Sl in the arm tip is shown in figure 4*a*. Immediately after removal of the arm tip, the wound area contracted, drawing the uppermost body wall towards the undermost severed nerve cord. Thus, the haemal canals were quickly sealed. A thin wound epidermis began to form, apparently from cells of the radial nerve cord epidermis and body wall (figure 2*a*). At 6 days post-ablation (PA), the wound epidermis had thickened to 2–3 cells in width and initial wound healing was complete. There was a disorganized area of cell debris, coelomocytes and possibly connective tissue immediately proximal to the wound (figure 2*a*). The first evidence of cell cycle activity in regenerating tips occurred at this time. Several BrdU labelled nuclei appeared in the wound epidermis, close to the radial nerve cord epidermis, although levels of incorporation were low at this stage (figure 4*c*). At very early stages of regrowth and healing (0–48 h) there was an increased intensity of Sl immunoreactivity (Sl-IR) in the nerve fibres of the nerve stump, when compared to the normal nerve. At 5 days, the staining remained dense at the nerve stumps, although they had contracted slightly, and a layer of cells now covered the exposed nerve profiles (figure 4*e*).

(ii) *The mid-arm radial nerve*

The normal distribution of Sl in the mid-arm radial nerve is shown in figure 4*b*. In mid-arm nerve ablation, healing events are similar, but occur slightly more rapidly than those in the arm tip. By 48 h PA, the epidermis of the radial nerve stumps had extended, flattening in shape and contacting the overlying ambulacrum. Between the nerve stumps there was already a disorganized area of coelomocytes, connective tissue and cellular debris which had remained after ablation, or migrated to the area. Some epidermal cells of the nerve stumps appeared to migrate over this area of disorganized cells. At this time, no nuclei labelled with BrdU could be detected. However, as in the arm tip, the Sl-IR was intense in both the nerve stumps (figure 4*d*).

By 4 days PA, the thin membrane of cells forming the wound epidermis was complete and a few BrdU labelled nuclei could be seen in the wound epidermis. However, by 6–7 days PA, there were numerous stained nuclei in the epidermal tissue of the nerve stumps and some across the wound epidermis (figures 3*c*, 4*g*). The apparent concentration of BrdU labelled nuclei at both nerve stumps indicated activity in both proximal and distal parts of the nerve. Although it was difficult to discern using light microscopy, the BrdU labelled cells all appeared of a uniform size, lying just in from the edge of the radial nerve epidermis, in both arm tip and mid-arm preparations (figure 5*g*). It appeared, therefore, that the labelling was mainly located in the epithelial cells of the epidermis, owing to their size and position, and not in other cells in this layer such as mucous cells, which are larger and more scattered.

By 6 days PA, the tissue across the wound area had broadened and a few Sl-IR nerve fibres could be seen aboral to the wound epidermis, extending from the ectoneural plexus of the nerve stump (figure 3*c*, 4*f*). This outgrowth occurred more rapidly than in the arm tip, with fibres extending to the proximal or distal wound stump after only one week. Outgrowth appeared to occur from both nerve stumps, forming a band of loosely organized peptidergic fibres across the wound site. Whole mount preparations revealed

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

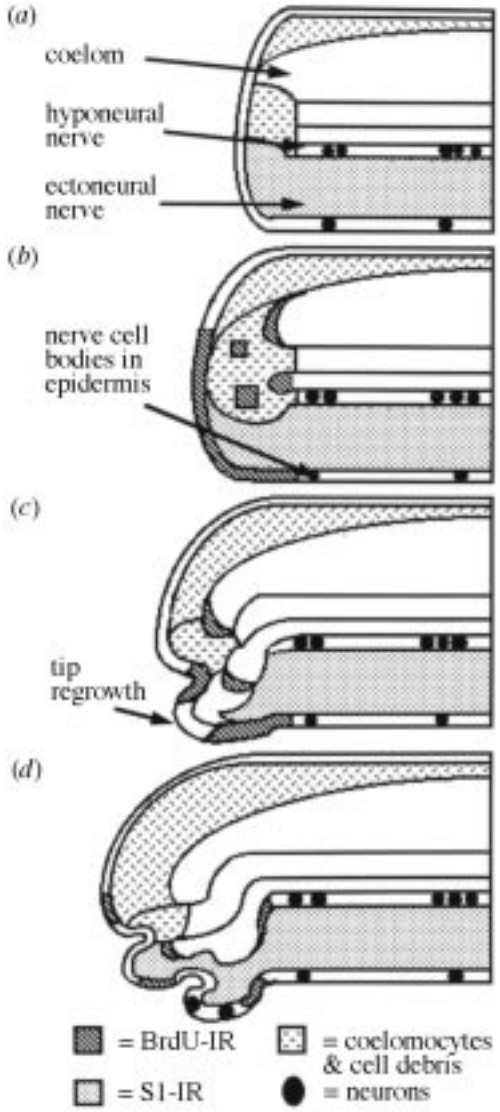

Figure 2. Diagram to show main stages of regeneration in the arm tip. Details of perihaemal canal/radial canal area have been omitted for clarity. (*a*) 0–7 days post-ablation (PA). Blunt ends of severed structures have healed over and coelomocytes and cell debris gathered at the wound site. (*b*) 7–14 days PA. More cells have gathered at the arm tip and cell cycle activity is evident in some tissues. (*c*) 21–28 days PA. Outgrowth of ablated structures is apparent by this stage, with nerve fibres extending into the regenerating tip and cell cycle activity present in epithelial cells of the epidermis, water vascular and perihaemal canals. (*d*) 35–42 days PA. Sensory structures of the arm tip are nearing full reformation and cell bodies have reappeared in the optic cushion.

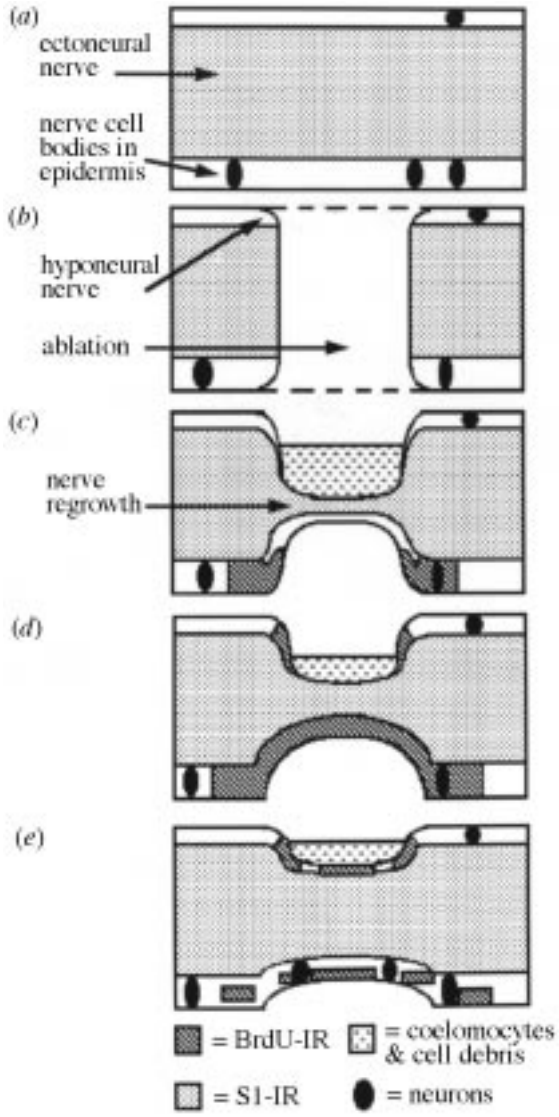

Figure 3. Diagram illustrating main stages of regeneration in mid-arm radial nerve cord (RNC). (*a*) Normal RNC. (*b*) Ablation of rnc forms two nerve stumps. (*c*) During early healing and regeneration the ablated area becomes enclosed by epidermal cells, coelomocytes gather around the wound, and some nerve fibres cross between nerve stumps. (*d*) A phase of active regrowth occurs, with intense cell cycle activity in nerve stump and wound epidermis, accompanied by an increase of neural tissue across the wound. (*e*) At later stages of regrowth, cell bodies reappear in regenerating neural tissue. Some cell cycle activity is still apparent, although the hyponeural nerve is much slower to regenerate.

that most outgrowth was orientated longitudinally, as it would be in the normal nerve, although some fibres did appear to project out into peripheral tissue (figure 4*h*,*i*).

(b) *Intense cell cycle activity: 7–21 days post-ablation*

(i) *The arm tip*

At the arm tip, there was little change in form until approximately 14 days PA, when the tip began to elongate (figure 2*b*). This change was possibly due to the beginning of outgrowth of the ablated structures, indicated by their increased incorporation of BrdU. At 12–14 days PA, numerous cells in the distal ends of the radial water and perihaemal canal epithelia, and in the epidermis of the radial nerve cord at the arm stump were labelled (figure 2*b*, 5*a*). Some labelled cells could also be seen in the heterogeneous cell mass, cell debris and connective tissue behind the wound epidermis (figure 5*b*). In all structures, the distribution of BrdU labelled nuclei was limited to the distal parts of the arm and did not extend further towards the disc, indicating growth at the tip only. At 18 days PA, this distribution of BrdU immunoreactivity (BrdU-IR)

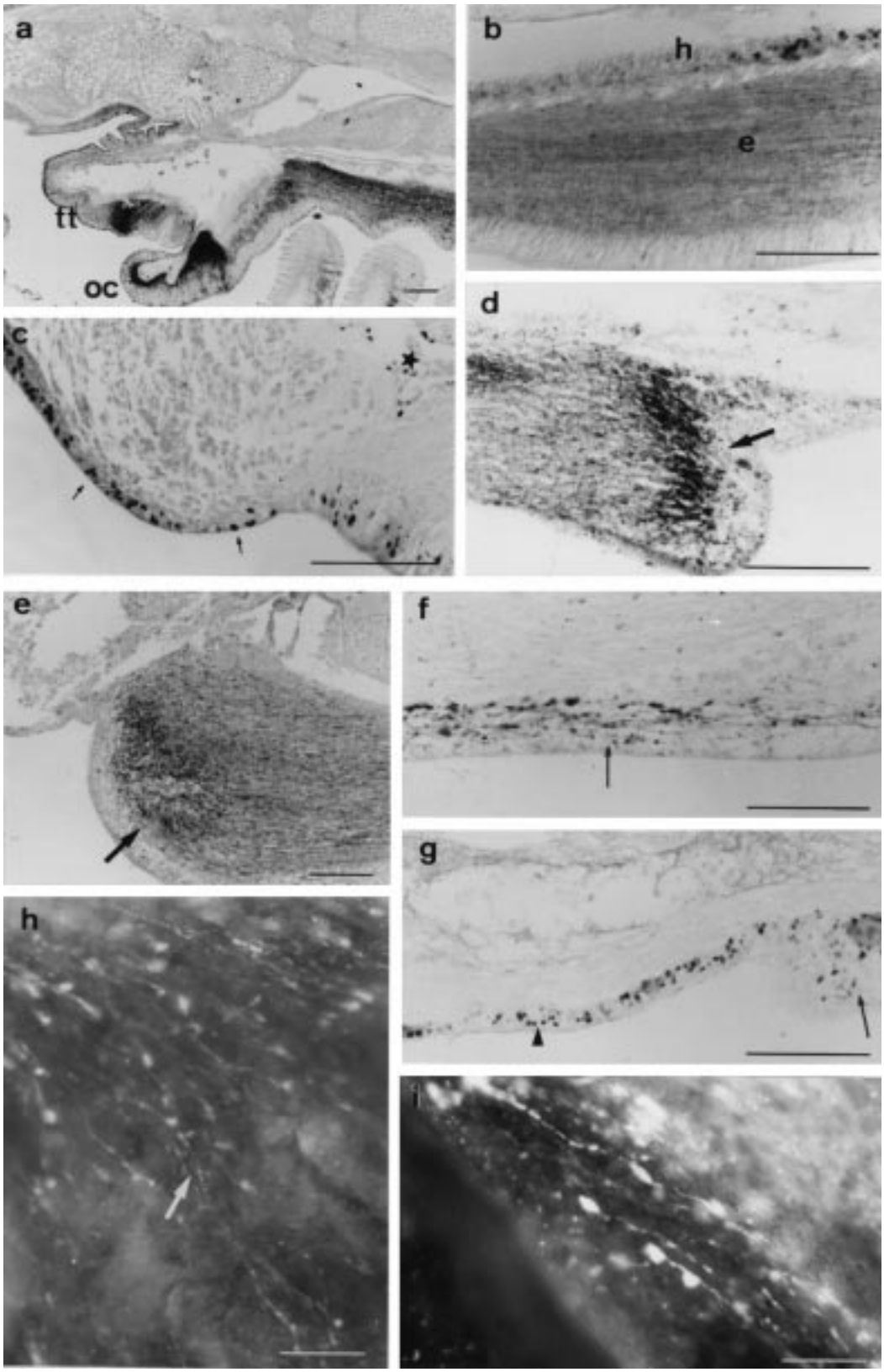

Figure 4. (*a*)–(*g*) Longitudinal sections of arm tip and radial nerve cord (RNC). Bars = 100 µm (*h*)–(*i*) Whole mount preparations of regenerating RNC. Bars = 25 µm. (*a*) Normal arm tip showing S1-IR in RNC, optic cushion (oc) and terminal tentacle (tt) nerve plexi. (*b*) In normal RNC S1-IR is present in ectoneural (e) and hyponeural (h) neural tissue. (*c*) By 6 days post-ablation (PA), BrdU-labelled nuclei can be seen in the wound epidermis (arrows) and at the end of water and haemal canals (star). (*d*) Regenerating mid-arm RNC at 48 hours pa. Epidermal cells have migrated across the nerve stump and S1-IR is intense at the end of severed nerve fibres (arrow). (*e*) After 5 days PA of arm tip showing intense S1-IR in fibres at the nerve stump (arrow). (*f*) After 7 days PA of mid-arm RNC, several S1-IR nerve fibres have crossed the wound site (arrow). (*g*) Mid-arm RNC 7 days PA, with numerous BrdU labelled nuclei within the epidermis of the nerve stump (arrow) and along the wound epidermis (arrowhead). (*h*) S1-IR fibres are projecting out from the main neuropile into surrounding tissue in this whole mount (arrow), 7 days PA. (*i*) As in (*h*) showing intense S1-IR in regenerating nerve fibres.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

remained (figure 5*c*). At this time, the first marked change in shape was observed , in the form of an invagination at the end of the radial nerve (figure 5*c*).

The intense S1-IR at the nerve stump was reduced by 10–14 days PA as the tip healed. S1-IR nerve fibres extended from the nerve stump into the reforming tip at 18–21 days PA, lying above the wound and radial nerve cord epidermis (figure 5*d*). These fibres seemed disorganized, appearing diffuse and loosely woven, with numerous large varicosities not normally observed in the neuropile. The band of ectoneural neuropile was still narrow (25 µm), but had increased in size from earlier observations at 14 days PA (10–15 µm). The endings of these fibres were difficult to identify in paraffin sections, but may have terminated as growth cones within the regenerating neuropile rather than contacting other cells. At this stage there were no peptidergic cell bodies present within the regenerating area, either in the neuropile or in the reformed epidermal layer (figure 2*b*,*c*). This suggests that the fibres originated from cells within the existing nerve or nerve stumps and that they were extending into the reforming area.

(ii) *The mid-arm radial nerve*

In the regenerating mid-arm section of radial nerve, regeneration of the nerve itself had already begun at 7 days PA (figure 3*c*). BrdU incorporation could be seen in the epithelia of the regenerating water vascular and perihaemal canals at 14 days PA, as well as in the epidermis of the radial nerve cord (figure 3*d*). The distribution was no longer concentrated in the nerve stumps, but much more evenly spread across the entire epidermal tissue covering the wound. Numerous cells were labelled with BrdU, suggesting that this was a particularly active period of growth (figure 5*g*). The patterns of BrdU labelling seen at 14 days were still present at 21 days PA. Labelling was seen in the epithelia of the disrupted water and perihaemal canals above the nerve. These epithelia appeared as monolayers of cells extending along the aboral edge of the now reforming neuropile. Some incorporation could also now be seen in the regenerating lower transverse muscle blocks situated oral to the ambulacrum.

At 10 days PA, S1-IR showed the presence of many nerve fibres extending into the regenerating area, suggesting rapid re-establishment of the ectoneural plexus. By 14 days PA, a broader band of S1 fibres was present (figure 3*d* and 5*e*). These formed a disorganized array of diffuse axon profiles with numerous large varicosities (5 µm diameter), quite unlike that seen in the normal nerve (figure 5*f*). Subsequent addition of regenerating fibres appeared to occur at a much lower rate. At this stage there was no indication of reformation of the hyponeural system, although the connective tissue boundary between the ectoneural and hyponeural systems was beginning to reform. As in the arm tip, no S1-IR peptidergic cell bodies were observed at this time.

(c) *Differentiation and complete regeneration: 21–90 days post-ablation*

(i) *The arm tip*

After 21 days of regeneration, the morphology of the regenerating tip indicated that an invagination marked the boundary between the normal nerve cord and the reforming optic cushion. There was further elongation of the tip and, by 28 days PA, some distinction between the terminal tentacle and optic cushion could be seen (figure 2*c*). At 35 days, the tip began to resemble the normal structure, with clear separation of the terminal tentacle and optic cushion (figure 2*d*). This differentiation appeared complete by 42–50 days, with some pigment cells reappearing in the optic cushion.

At 21–28 days, S1-IR was localized to a band of disorganized neuropile stretching into the regenerating sensory structures from the ectoneural tissue of the nerve stump (figure 6*a*). These fibres were diffuse with numerous varicosities, as observed in the regenerating mid-arm radial nerve (figure 6*b*). The first appearance of S1-IR cell bodies associated with the forming optic cushion usually occurred at 28–35 days PA. Initially, only one or two cells could be seen within the forming cushion, with basal projections into the associated band of ectoneural neuropile. At 35 days PA, axons could be seen extending into the modified epithelium of the cushion, coinciding with the onset of reformation of the ocelli cups (figure 6*e*). The S1-IR was now found throughout the ectoneural plexus of both the forming optic cushion and the terminal tentacle (figure 2*d*, 6*c*). There was a gradual increase over time in the number of S1-IR neurons present in the regenerating optic cushion, and long fibres could sometimes be seen extending from these cells into the overlying plexus.

At 42 days PA, the pattern of S1-IR appeared similar to that of the normal arm tip (figure 6*d*) except that ocelli were still forming in the optic cushion. From this time the general distribution of S1 remained the same, with the increasingly orderly appearance of the neuropile and a thickening of the band of ectoneural fibres in the optic cushion. By 80–90 days PA, the S1-IR fibres had regained their more orderly appearance and density, in contrast to the diffuse and varicose arrangement seen in earlier stages. This apparent re-establishment of normal S1 distribution in the regenerating tip suggested that the normal patterns of peptidergic neurons had been regained, even though the tip would continue to grow.

As optic cushion and terminal tentacle reformation advanced (28–35 days PA), BrdU labelled nuclei were present throughout the epithelia of these structures (figure 2*d*). Many cells were still labelled, but there was a reduction in BrdU-IR within the epithelia of the radial water and perihaemal canals and coelom, with a concentration in the epidermal layer of the radial nerve and body wall. By 42 days PA, there was still labelling in the epithelial cells of the optic cushion and terminal tentacle (figure 7*a*). BrdU-IR was also found in the area just proximal to the tip and in the associated canals, but not in the coelomic epithelium or in the tip itself, which showed signs of recalcification. The body wall still contained labelled cells and cell cycle activity could also be seen in the regenerating tube feet just proximal to the tip (figure 7*b*). This level of activity had decreased by 60 days PA, although there were some labelled nuclei within the body wall and scattered in the epidermal tissue. By 90 days PA, few nuclei incorporated BrdU and levels resembled those seen in the control arms, i.e. scattered, infrequent labelled nuclei in the radial nerve epidermis and the tube feet (figure 7*c*).

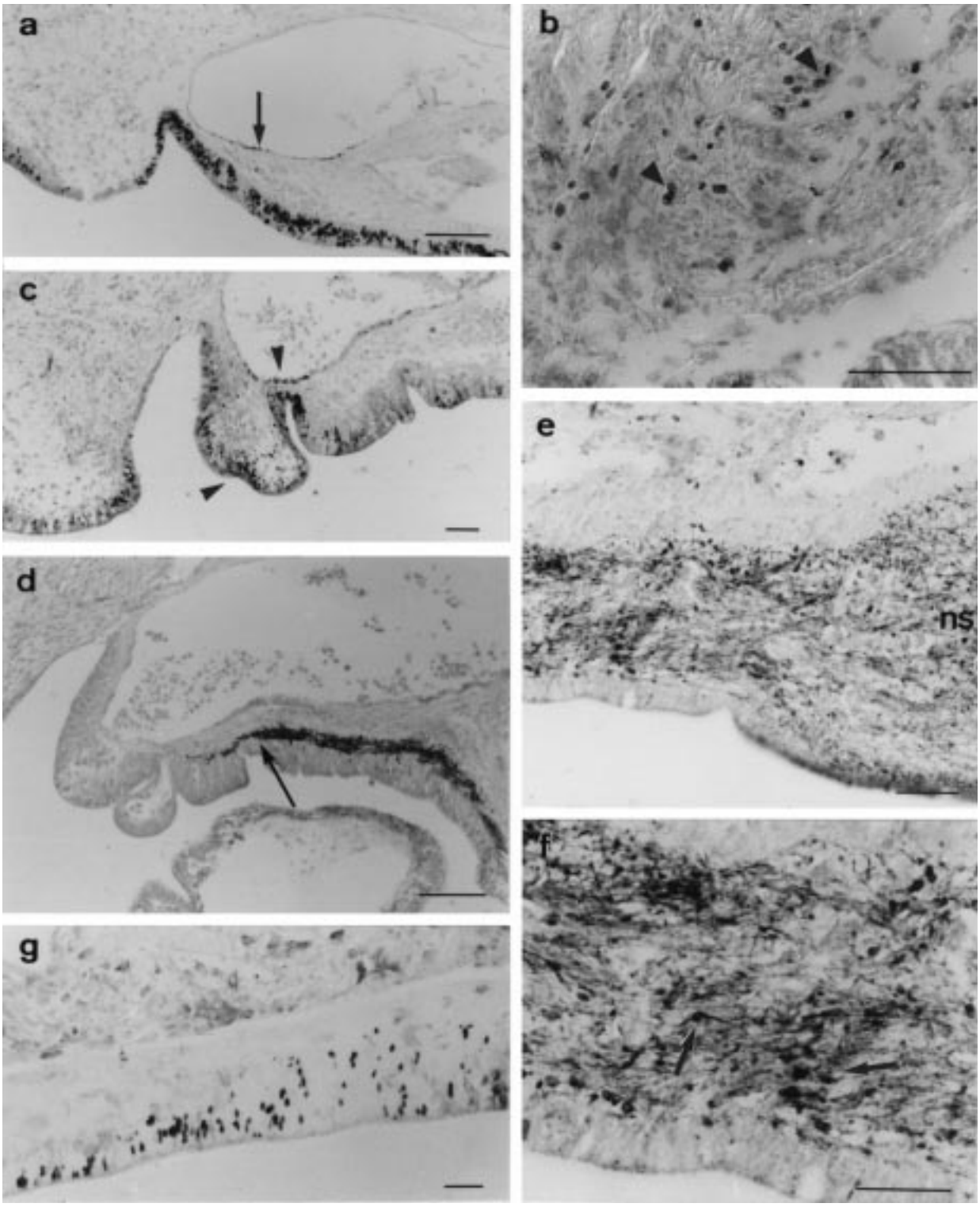

Figure 5. Longitudinal sections of regenerating arm tip and radial nerve cord (RNC). (a) At 14 days PA of the arm tip there are numerous BrdU labelled nuclei in the RNC and wound epidermis and in the radial canal epithelium (arrow). (b) Arm tip at 12 days PA. There are BrdU-labelled nuclei in the area of heterogenous cells at the arm stump (arrowheads). (c) At 21 days PA of arm tip showing BrdU labelling in RNC epidermis and radial canal epithelium (arrowheads). Labelling is also present around the invagination of the rnc. (d) At 21 days PA, S1-IR fibres can be seen extending into the regenerating arm tip (arrow). (e) By 14 days PA of mid-arm rnc numerous S1-IR fibres have extended across the ablated area from the nerve stump (ns). (f) High power of (e), showing the disorganized nature of regenerating fibres, which are diffusely arranged with numerous varicosities (arrows). (g) By 14 days PA, regenerating RNC shows intense BrdU incorporation in epidermal tissue. Scale bars = 50 μm.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

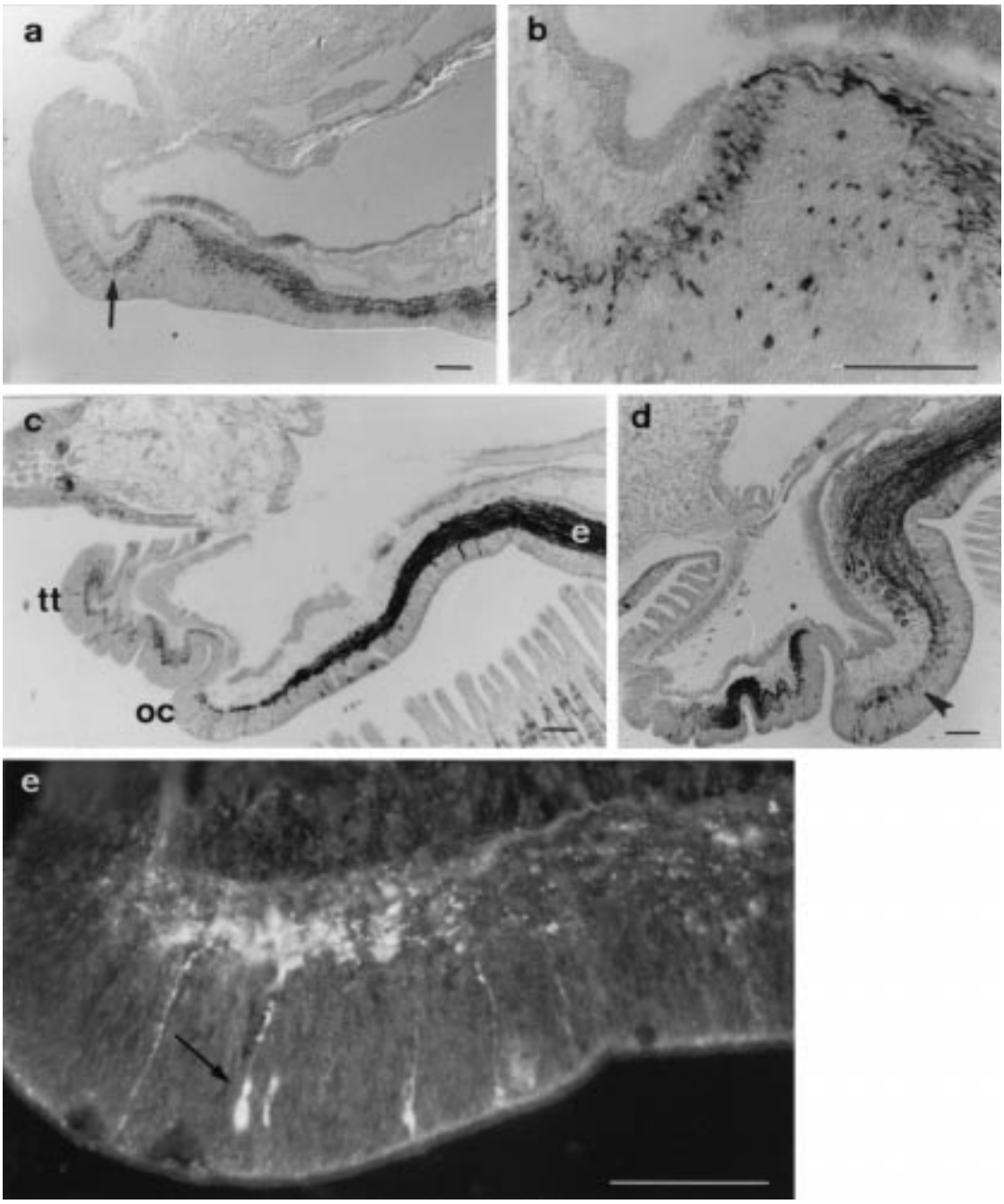

Figure 6. Longitudinal sections of regenerating arm tip stained with S1 antisera. (a) At 28 days PA, S1-IR nerve fibres have extended into regenerating sensory structures of the arm tip (arrow). (b) High power magnification of (a) showing the disorganized and varicose appearance of extending fibres. (c) By 35 days PA, strong S1-IR can be observed in the ectoneural nerve (e), reforming optic cushion (oc) and terminal tentacle (tt). (d) At 42 days PA, S1 distribution is similar to that observed in the normal arm tip, although IR is still reduced in the regenerating optic cushion (arrowhead). (e) At 35 days PA, immunofluorescing cell bodies can be clearly seen in the area of the optic cushion (arrow). Scale bars = 50 μm.

(ii) *The mid-arm radial nerve*

At 21 days, the area above the nerve was still disorganized and some coelomocytes remained between the aboral surface of the nerve and the reforming hyponeural nerve, perihaemal and water canals. Several S1-IR ectoneural cell bodies reappeared in the regenerating area after 28 days PA (figure 3*e*, 8*a*). They were bipolar cells associated with the epidermis of the nerve, and they possessed apical processes which extended into the reforming ectoneural neuropile. Observations at 30 and 35 days PA indicated that the ectoneural fibres were still loosely packed when compared to normal, but they appeared to be becoming more ordered, with increasing numbers of peptidergic cell bodies (figure 8*b*). There was still no S1-IR in the hyponeural tissue which was beginning to reform aboral to the ectoneural nerve and connective tissue layer (figure 8*b*). Preparations made at 60 days PA provided the first indications of hyponeural nerve regeneration, with S1-IR appearing in the hyponeural nerve plexus. Further observations at 80 days PA

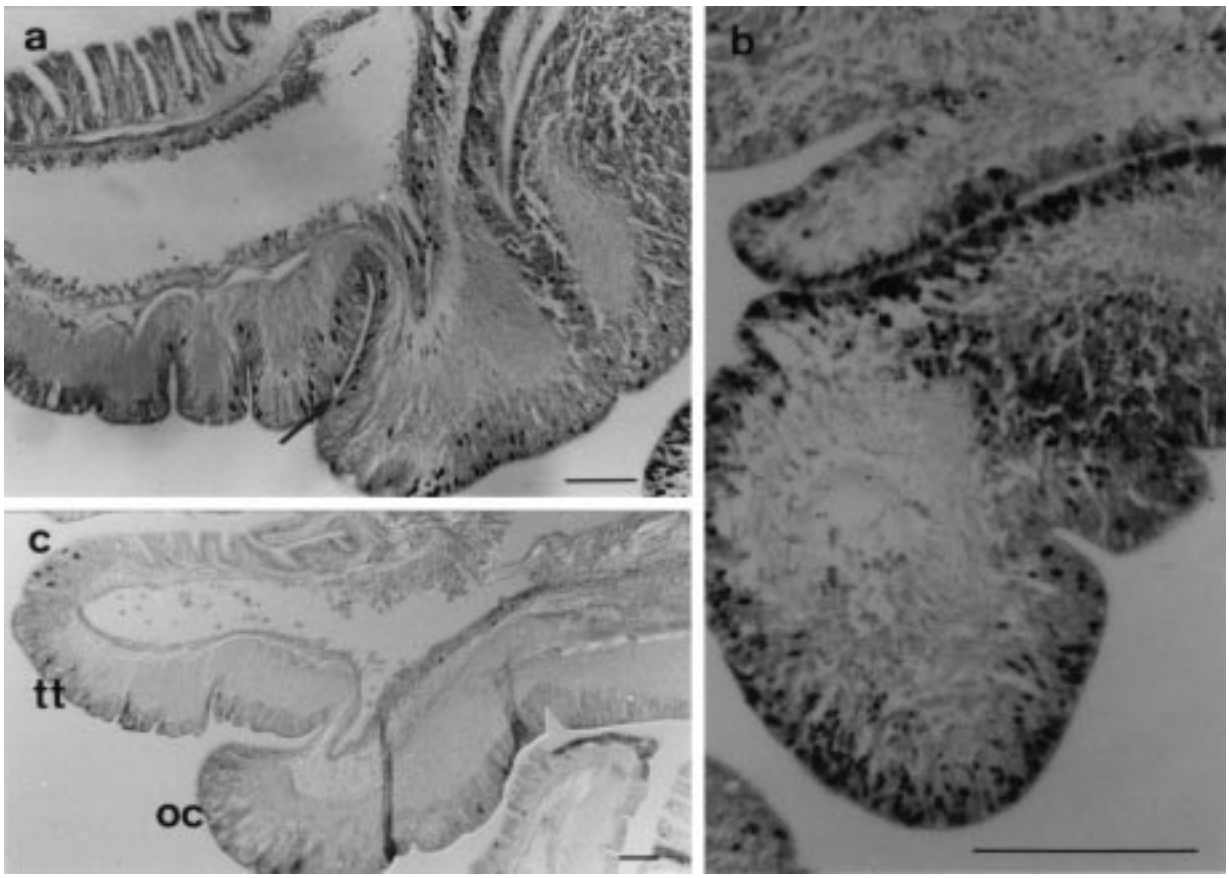

Figure 7. (a) Arm tip at 42 days PA, showing reduced BrdU incorporation in radial nerve cord epidermis and radial canal epithelia, although nuclei are labelled in reforming sensory structures (arrow). (b) New tube foot from an arm tip at 42 days PA, showing BrdU labelling in epidermal tissues. (c) Non-regenerating control arm tip, showing a few BrdU labelled cells in the optic cushion (oc) and terminal tentacle (tt) epidermis. Scale bars = 50 μm.

confirmed hyponeural nerve regeneration, with the appearance of Sl-IR hyponeural cell bodies within the epithelial cell layer (figure 8*d*). The ectoneural neuropile also displayed more organized and closely packed fibres than were seen in earlier stages of regeneration.

At 21–28 days PA there was still evidence of cell cycle activity, with BrdU incorporation in cells of the epidermis of the radial nerve and clear labelling in the reforming perihaemal canal epithelium (figure 8*c*). Later BrdU incubations of the mid-arm regenerating nerve at 35, 42 and 50 days PA indicated a gradual decrease in mitotic activity (figure 3*e*). This incorporation had reduced to control levels by 60 days PA.

Several control experiments were carried out for both normal versus regenerating tissue and ICC techniques. Controls indicated that a few cells in unoperated tissue incorporated BrdU, mainly in the epithelia of the tube feet or radial nerve, a level suggestive of normal mechanisms of growth or repair. There was no difference between control tissue taken from regenerating and non-regenerating animals for either BrdU-IR or Sl-IR, their distributions being clearly distinct from those observed in regenerating tissue. BrdU-IR was always localized in the regenerating area and rarely observed further along the arm. This often pronounced delineation between labelled and unlabelled regions also suggests that the staining patterns observed were genuine and that there was no non-specific reaction between the BrdU antibody and the tissue. The preadsorption controls carried out with anti-BrdU on regenerating arm tips and radial nerve resulted in complete removal of labelled nuclei. Similarly, preadsorption tests with anti-Sl resulted in a reduction of Sl-IR to background levels.

(d) *Double labelling—modified BrdU pulses*

Modified regimes of BrdU pulsing and fixation did not reveal any changes in the general distribution of BrdU-IR nuclei, suggesting little or no movement of labelled cells. However, there appeared to be an increase in the number of labelled nuclei in regimes where tissue was fixed for a short period (e.g. three days) after the pulse. From the incubation regimes, where animals were fixed for between 3 and 28 days after the BrdU pulse, detection of BrdU labelled nuclei indicated that BrdU remained in the cells for around 14 days. BrdU was lost over longer periods, possibly due to numerous divisions which would dilute the BrdU and render it undetectable, or by DNA repair mechanisms which could excise the incorporated molecule (Kirschenbaum & O'Shea 1993).

Double labelling studies were carried out on both regenerating arm tips and mid-arm radial nerve sections to try and identify specific populations of cells which were also

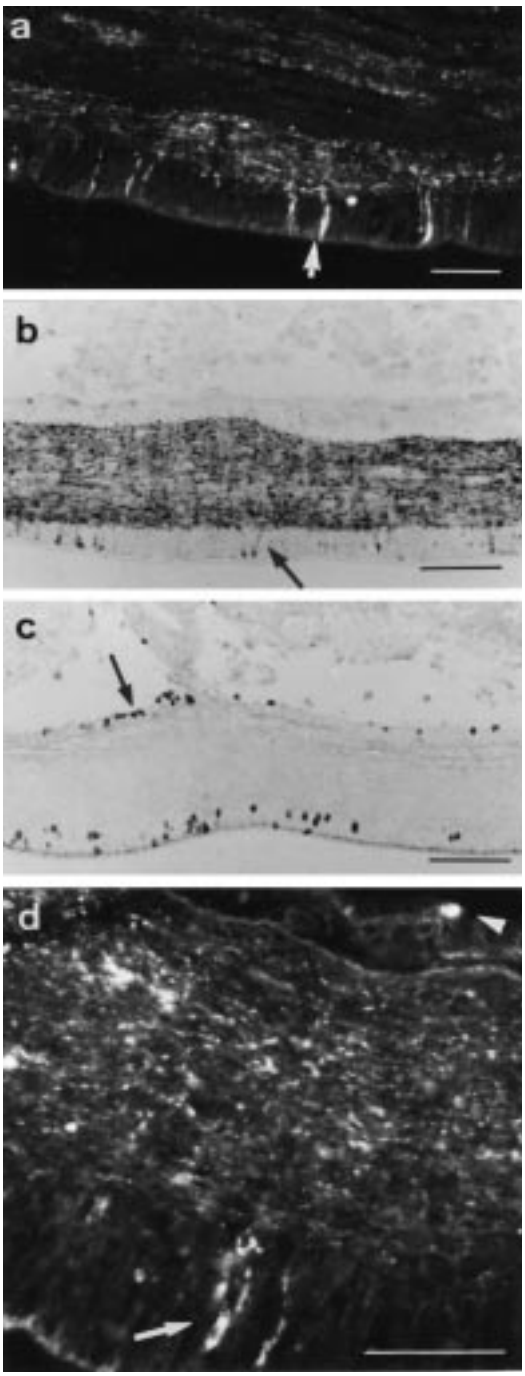

Figure 8. Longitudinal sections of regenerating mid-arm radial nerve cord (RNC). (a) Fluorescent preparation of the nerve at 28 days PA, stained with S1 antisera. Fibres are still disorganized, but cell bodies are once again present in the area (arrow). (b) At 35 days PA, S1-IR nerve fibres show increasing organization and ectoneural cell bodies are apparent in RNC epidermis (arrow). There is no evidence of hyponeural nerve regeneration. (c) At 21 days PA, regenerating mid-arm RNC shows BrdU incorporation in both RNC epidermis and reforming perihaemal canal epithelium (arrow). (d) By 80 days PA, S1-IR cells are present in both ectoneural (arrow) and hyponeural (arrowhead) tissue, indicating further regeneration of the hyponeural nerve. The ectoneural nerve also appears more organized by this stage. Scale bars = 50 µm.

showing cell cycle activity. Fixation immediately after the BrdU pulse would label cells which had been mitotically active during the pulse alone. Therefore, if differentiated S1-containing neurons were active, then it would be likely that co-localization of peptide and BrdU-IR would be seen. However, such co-labelling was never observed in these preparations. In all cases where fixation immediately followed BrdU incubation, neurons that were positive for S1 were not labelled with BrdU (figure 9*a*,*b*).

The short- and long-term BrdU incubation/fixation regimes were tested to allow for the time taken for putative neurons to possibly migrate from elsewhere and/or to differentiate. In protocols where tissue was left for specified periods before fixation, there was no co-localization of BrdU and S1. This was true of all arm tip and mid-arm radial nerve preparations examined. Despite overlaps in exposure to BrdU and observations during periods when S1 neurons were appearing, no cell cycle activity was detected in association with the neurons.

4. DISCUSSION

(a) *Distribution of S1-IR and the putative role of the nerve during regeneration*

The ability to undergo neural regeneration is well known in echinoderms from previous observations, mainly of whole appendages or gut regrowth (Hyman 1955). Mladenov *et al*. (1989) describe a thin layer of nervous tissue extending into the wound area of the regenerating arm tip of *Lepasterias hexactis* as early as 72 h PA. By 11 days there is a disorganized area of nerve distal to the nerve proper, probably corresponding to the diffuse nerve fibres shown by S1-IR in *A. rubens*. Neurosecretory cells are described in this area in *L. hexactis*, and also in association with the regenerating brachial nerve in the crinoid, *Antedon mediterranea* (Candia-Carnevali *et al*. 1993). The changing axonal morphology revealed by the S1 antibody and the intense labelling of all fibre types has allowed more detailed analysis of this phenomenon in *A. rubens*.

The appearance of S1-IR neurons and fibres within the regenerating tissue of *A. rubens* has clearly illustrated the outgrowth of the nerve during regeneration and the reappearance of neurons in the regenerating area. This has also provided a means of monitoring the extent to which the adult pattern is re-established. Newly extending fibres in both arm tip and mid-arm radial nerve regeneration show strong S1-IR at early stages, but this returns to normally observed levels after a broader band of neuropile has formed. This may be due to the breakdown of peptide accumulated in the tip, or to removal of damaged nervous tissue by phagocytosis. The increase in IR seen at early stages in the nerve stump could be indicative of a role in regrowth, although it may also be due to a block in transportation resulting from severed nerves, as was observed in the flatworm *Microstomum lineare* (Palmberg & Reuter 1990). However, it does seem that the regenerating S1-IR fibres, especially at earlier stages of regeneration, have thicker axons, around 2 µm, rather than the 1 µm or less seen in normal fibres (Cobb 1987). This may be due to increased peptide synthesis and transportation. Alternatively, it may be associated with the increased synthesis of cytoskeletal proteins, a feature which has been observed in studies on neural regeneration in mammals (Moskowitz & Oblinger 1995).

There are also intensely stained varicosities within the fibres, a few of which correspond in size to those observed

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

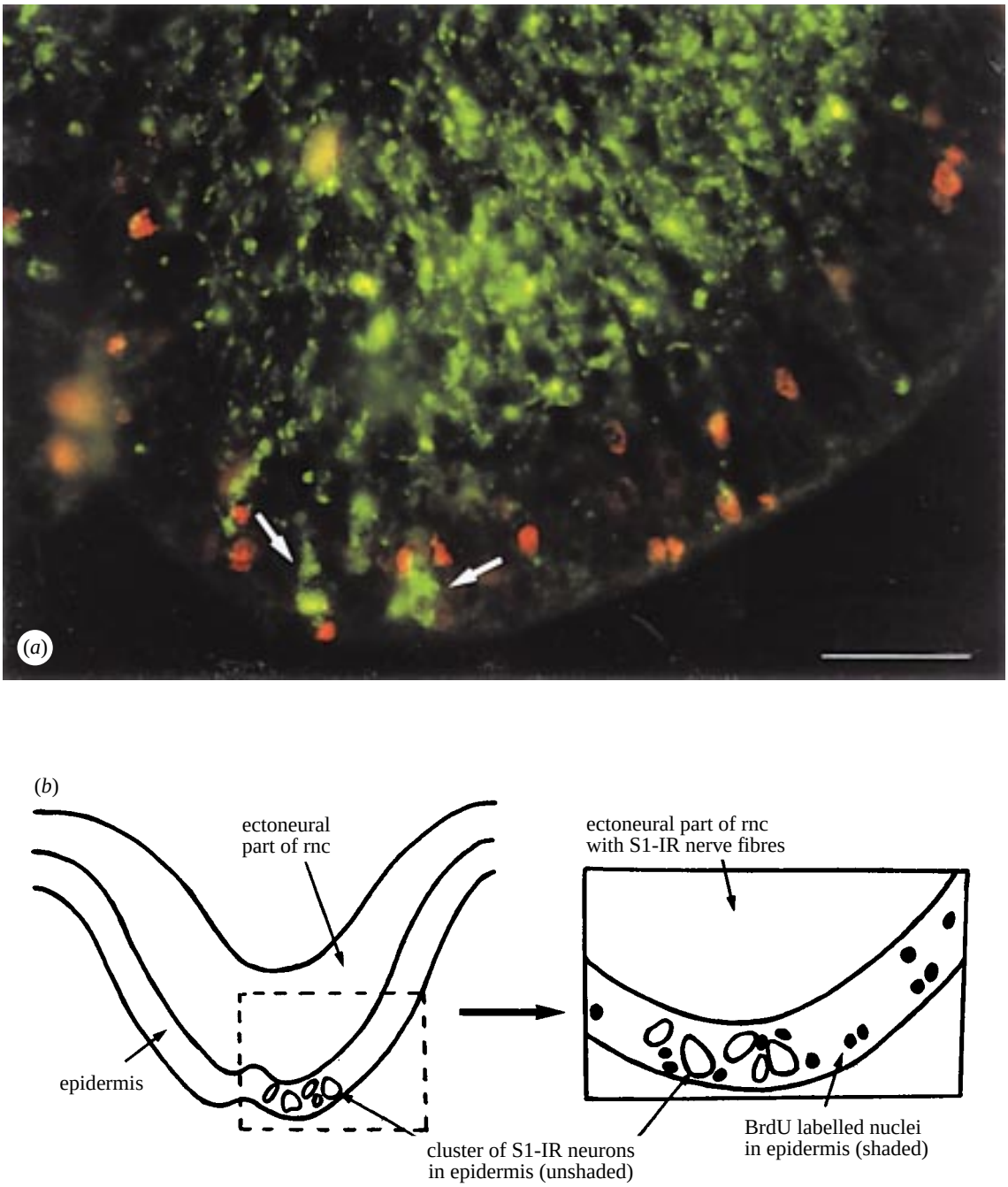

Figure 9. (*a*) Double labelled section of regenerating radial nerve cord at the nerve stump; red = BrdU incorporation, green = S1-IR. Arrows show position of two S1-IR neurons. Scale bar = 50 μm. (*b*) Diagram showing area of the nerve figure 9*a* is taken from, and details of figure 9*a*. BrdU labelled nuclei are restricted to the epidermis underlying the nerve plexus and S1-IR cell bodies within this area are not co-labelled with BrdU.

in the normal nerve (around 3 μm) (Moore 1993), although most are larger (5 μm) and they are more numerous in regenerating tissue. The reason for the large number of these varicosities, which remain in the tissue for a greater part of the regenerative period, is not clear, although varicosities in echinoderm ectoneural nerves have previously been associated with the site of transmitter release (Cobb 1987). It is possible that they play a similar role in the regenerating nerve.

S1-IR fibres are apparent at early stages in the regenerating tissue, whereas cell bodies cannot be visualized in this area until much later. It has been shown that transmitter expression in developing or reforming neurons is normally representative of near or complete differentiation following proliferation and migration (Denburg 1989). It is possible that the expression of S1 in the neurons of regenerating tissue is indicative of a comparatively late stage of regeneration, forming adult structures and patterns of expression. Such information is useful in gauging the temporal changes in the regrowth process at the level of the regenerating neural tissue.

By examining the distribution of the neuropeptide S1 during echinoderm arm regeneration, the intimate association of the nerve with the healing and then reforming

arm tip becomes even more obvious. The studies of Huet (1972, 1975) first suggested that the nerve is needed for normal regeneration. Blocking access of the nerve to the wound site prevented the differentiation of the terminal structures and mitosis was reduced, although the epidermis still formed and cells gathered at the amputation zone. This influence of the nerve, possibly on differentiation, may be mediated by a neurotransmitter, growth factor, a neuropeptide such as S1, or a combination of such molecules (Candia-Carnevali *et al.* 1996). It is now known that the neural tissue plays a role in many regenerative processes and its investigation has become a fundamental underlying feature of regeneration studies (Singer & Geraudie 1989). This role appears to be present in many examples of appendage regeneration, including that seen in annelids, arthropods and fish (Goss 1969). Although its expression does not confirm a physiological role for S1 during regeneration, it is extremely likely that the presence of the nerve itself is of importance.

Most other investigations on invertebrate nerve regeneration are purely morphological. Some annelids, insects and molluscs are capable of nerve outgrowth, and features of this are similar to that observed in *A. rubens* by S1 ICC. For example, in annelids and echinoderms it appears that outgrowth occurs from both the proximal and distal stumps of the ablated nerve; most outgrowth occurs within the first two weeks PA and axon profiles are initially disorganized at the nerve stump (Lyckman *et al.* 1992; this study). The apparent activity of both proximal and distal stumps in mid-arm nerve regeneration provides information which could not be obtained by observing arm tip regrowth. This is not unique to *A. rubens*, although it may be an important aspect in their ability to rapidly regenerate neural tissue. It suggests the absence of a specific chemical gradient to guide regrowth in a proximal to distal direction, as has been shown in other studies (Stocum 1991), and indicates a degree of flexibility in the regeneration mechanism. However, the anatomy of the nervous system also indicates that such outgrowth is not necessarily surprising as somas are scattered along the nerve, rather than located in a specific position, proximal or distal to the wound. Hence, nerve stumps will contain both afferent and efferent fibres. It may be that some proximally located somas send fibres distally to replace ablated nerve fibres and that some distally located ones do the opposite, resulting in bidirectional outgrowth and rapid reconnection of the nerve stumps. Although outgrowth from both nerve stumps suggests the lack of a proximaldistal chemical gradient, the general longitudinal arrangement of the S1-IR axons and their growth towards the opposite stump or along the wound epidermis may indicate that the outgrowth is not random and that physical factors are involved.

Regeneration of the hyponeural nerve (figure 1*a*,*c*), which lies aboral to the ectoneural nerve, is not seen at the tip, although it must progress at a low rate proximal to the tip as the arm begins to regrow. The mid-arm nerve ablation studies indicate that hyponeural fibres and cell bodies do regain their original distribution, although this is a much slower process than that of ectoneural regeneration. This is possibly due to the need for much greater structural recovery before the hyponeural nerve can reform, in terms of replacement of both the underlying neural tissue and also the effector systems, such as the tube feet, which the hyponeural innervates.

(b) *BrdU incorporation and cell cycle activity during regeneration*

The use of BrdU incubations has provided further details concerning the role of epimorphosis in echinoderm regeneration. As well as cell cycle activity, such information can help determine the possible contributions of migration and differentiation during regeneration. For example, in arm tip regeneration there appears to be no true blastema formation, as observed in many other systems, such as amphibian arm regeneration, planarian head reformation or segment addition in regenerating annelids (Goss 1969). Initial healing events occur by morphallaxis, with migration of cells of the radial nerve and body wall epidermis to cover the wound site. Subsequent cell cycle activity is scattered over the area with, apparently, no major accumulation of undifferentiated proliferating cells associated with the regenerating tissue. The appearance of S-phase cells in the areas described confirms the patterns seen in some other echinoderm regeneration studies where cell activity has been considered (e.g. Mladenov *et al.* 1989) and is suggestive of a regrowth process which involves both epimorphosis and morphallaxis.

There are several studies on cell activity during regeneration in echinoderms and, although most of these have focused on holothurians, some work has been carried out on asteroids and crinoids. The autoradiographic studies conducted on arm tip regeneration in the asteroid *L. hexactis* show a similar localization of labelled nuclei in wound and adjacent epidermal regions, parietal peritoneum and water canal epithelia (Mladenov *et al.* 1989). This suggests that previous descriptions of a blastema in asteroid arm regrowth are due to misinterpretation of the area of cell debris and clotted coelomocytes which lie just proximal to the tip (Mladenov *et al.* 1989). The BrdU experiments in the current study confirm the lack of such a centre of proliferation, although the accumulation of cells in the tip area may contribute to the regeneration. In holothurian gut/aquapharyngeal complex regeneration there is no blastema, and mitosis is scattered within the coelomic epithelia and gut lining and appears to play only a partial role in the process (Dolmatov 1992). Notably, the scattered mitotic cells which are present appear to be localized mainly within epithelial tissues, as is the case in both *A. rubens* and *L. hexactis*.

An exception to this is in regeneration of the arm of the crinoid *Antedon mediterranea*, where proliferation studies have suggested the presence of a blastema (Candia-Carnevali *et al.* 1995). A large number of active cells, many of which appear to be coelomocytes, gather beneath the wound epidermis and due to the short duration of the BrdU pulse, it appears that they are dividing in this area. A large number of epithelial cells was also shown to be active in *A. mediterranea* and it does seem that there is still a significant contribution to regeneration by cells of this type, whether directly, or possibly in the production of coelomocytes (Vanden Bosche & Jangoux 1976).

In many cases of echinoderm regeneration it is the proliferation and subsequent differentiation of epithelial cells which is implicated as a primary source for new tissues. This is supported not only by information from

this and other studies (Penn 1982; Anderson, 1962), but also by the failure to detect alternative areas of proliferation in the current investigation. There is no evidence, for example, of a typical undifferentiated 'stem cell' system which may be present in various tissues in the animal, as is the case in regeneration in planarians and coelenterates (Baguna *et al*. 1989; Bosch & David 1991).

The involvement of epithelial cells is also a key feature of asexual budding in sessile ascidians (seasquirts), another large group of invertebrate deuterostomes. This manner of reproduction is a programmed developmental process but, as in regeneration, it involves the formation of new structures from existing adult tissues. The epithelium is capable of proliferation and differentiation, with some of the active epithelial cells dedifferentiating, before differentiating again (Koguchi *et al.* 1993; Kawamura & Nakaguchi 1986). This mechanism appears similar to the processes observed in asteroid regeneration. That is, migration of epithelial cells (morphallaxis), cell cycle activity in epithelial cells (epimorphosis) and possible differentiation into associated cell types, either from undifferentiated cells or through transdifferentiation of existing cells (figure 10). Further studies on juveniles may also indicate whether the patterns of cell cycle activity seen during regeneration are similar during normal growth, albeit at a lower rate.

A potential source of progenitor/stem cells is the numerous coelomocytes which move into the tip and underlying vascular and nervous tissues during healing and regeneration. In both arm tip and mid-arm radial nerve regrowth, coelomocytes are associated with the disrupted structures from the onset of healing until differentiation of sensory structures and reformation of the nerve. They are closely associated with the distal points of the outgrowing tip and lie along the aboral surface of the neuropile in the regenerating nerve. In this study, the coelomocytes within the regenerating area did not appear to be labelled with BrdU, since little labelling was seen in the stump tip, or in the disrupted area above the nerve, both areas where coelomocytes gathered. To have a major role in regeneration in terms of a source of cells for the regenerate, increased production of coelomocytes seems likely and this would probably result in BrdU labelled cells, as has been observed in some studies (Candia-Carnevali *et al.* 1995). An alternative role for coelomocytes could be in the secretion of growth-promoting factors.

It is still possible, however, that some coelomocytes are incorporated in regenerating tissue without passing through the S-phase (figure 10). For example, during budding in some sessile ascidians haemoblasts are thought to differentiate into epithelial cells (Koguchi *et al*. 1993). Also, in crinoid arm regeneration, coelomocytes have been suggested as sources for the regenerating tissue, with amoebocytes, phagocytes, coelomocytes and granule cells all implicated in the process (Candia-Carnevali *et al*. 1993). The apparently large contribution of such cells may be a crucial underlying feature of echinoderm regeneration and, in view of the lack of BrdU labelling of coelomocytes in this study, they would have to be derived from an existing population. It may be possible to test whether coelomocytes are precursor cells by ultrastructural studies, or by raising novel monoclonal antibodies to these cells, which may be detectable during regeneration.

(c) *Putative origin of S1-IR neurons*

At the onset of regeneration in both preparations, no S1-IR neurons were present. First, peptidergic nerve fibres extend from the stumps, followed by more nerve fibres and then finally by the appearance of S1-IR nerve cell bodies. Despite the various regimes of BrdU incubation and fixation, no cells were detected which were double labelled for BrdU and S1. It appears, therefore, that neuronal precursors do not undergo cell cycle activity within the regenerating region and are not born during the BrdU pulse, suggesting that cell migration may be important . Alternatively, if neurons are not born from active cells in the epidermis and neuroepithelial formation is possible without proliferation, transdifferentiation could be the mechanism involved. Such a mechanism is supported by Huet (1972).

An alternative suggestion to the several possible reasons for the lack of double labelling is that the neurons have differentiated from local, mitotically active cells, but that they are not labelled by BrdU. It is perhaps most likely that the incorporation of BrdU into epithelial cells has become undetectable. This may be due to its loss after several divisions, or to DNA repair mechanisms, before nerve cell differentiation has occurred (Kirschenbaum & O'Shea 1993). During the growth of the corpora cardiaca in the locust, the neuroendocrine cells, known to be born in the gland, can be visualized by the BrdU/anti-BrdU technique, and their neuropeptide content by an antiserum to adipokinetic hormone. However, no co-labelling of these cells could be seen unless the animal was left for 14 days after the pulse, and even then numbers of co-labelled cells are very low (Kirschenbaum & O'Shea 1993). Hence, the identification of neuropeptide cells which are derived from BrdU labelled cells has proved difficult in these systems. Double labelling of such cells has been shown to be experimentally viable (Soriano & Del Rio 1991; Kono *et al*. 1991), so it may be a fundamental problem in the time-course of initial BrdU labelling through to differentiation and peptide expression.

The role of neuroepithelial proliferation and differentiation in forming new neurons is also evident in many regrowth processes in vertebrates. For example, reformation of retinal cells from the pigmented epithelium is possible in the amphibian eye (Reh *et al*. 1991), and epithelial supporting cells are capable of dividing and replacing hair cells in the avian ear (Oesterle *et al*. 1993). Regeneration in neuroepithelia is therefore not uncommon. It is also notable that many of these systems occur in tissues which are continually growing and retain the ability to replace nerve cells throughout life. *A. rubens* is a continuously growing animal and it may be that asteroid neuroepithelia retain the ability to add neurons in a similar fashion, i.e. by proliferation and differentiation. This would be in agreement with the results of ultrastructural studies on a tropical asteroid, *Nepanthia belcheri*, which suggest that regeneration of the arm tip is by dedifferentiation, proliferation, and differentiation of epidermal cells into cells of the new optic cushion ocelli (Penn 1982).

There is some evidence to suggest that neurons are derived from locally dividing cells, despite their lack of BrdU labelling. Without further double labelling evidence, it cannot be confirmed whether epithelial cells dedifferentiate, proliferate, and then differentiate into new cell

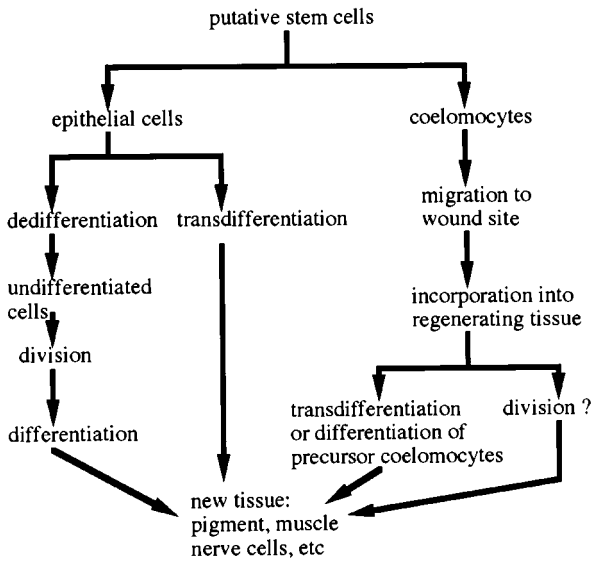

Figure 10. Diagram to show the possible paths of origin of new tissue in both arm tip and radial nerve cord regeneration.

types, or whether they transdifferentiate directly into new cells (figure 10). The present study supports the former mechanism, in view of the intense activity in the neuroepithelium and the apparent continued division of labelled cells in tissue left for various periods after the pulse, as shown by the increasing numbers of BrdU labelled nuclei. This would result in the rapid local production of the cells needed for outgrowth of the ablated structures.

In conclusion, arm regrowth and neural regeneration in asteroids provides an interesting example of non-blastemal, mainly epimorphic regeneration, and further studies on the putative patterning mechanisms involved in this process may reveal that novel growth-related substances are involved. Work has already been undertaken on vertebrate growth factors, in order to explore their effects on echinoderm neural regeneration *in vitro*.

This work was carried out under the tenure of a Science and Engineering Research Council CASE award to C.M. in association with SmithKline Beecham Pharmaceuticals Ltd. Thanks to Zyg Podhorodecki for photographic assistance.

REFERENCES

Anderson, J. M. 1962 Studies on visceral regeneration in seastars. I. Regeneration of the pyloric caeca in *Henricia leviuscula*. *Biol. Bull.* **122**, 321–342.

Baguna, J., Salo, E. & Romero, R. 1989 Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians. *Int. J. Dev. Biol.* **33**, 261–264.

Bollner, T. R. & Meinertzhagen, I. 1992 The patterns of bromodeoxyuridine incorporation in the nervous system of a larval ascidian *Ciona intestinalis*. *Biol. Bull.* **184**, 277–285.

Bosch, T. C. & David, C. N. 1991 Decision making in interstitial stem cells of *Hydra*. *In vivo.* **5**, 515–520.

Candia-Carnevali, M. D., Lucca, E. & Bonasoro, F. 1993 Mechanisms of arm regeneration in the feather star *Antedon mediterranea*: healing of wound and early stages of development. *J. Exp. Zool.* **267**, 299–317.

Candia-Carnevali, M. D., Bonasoro, F., Lucca, E. & Thorndyke, M. C. 1995 Patterns of cell proliferation in the early stages of arm regeneration in the feather star *Antedon mediterranea*. *J. Exp. Zool.* **272**, 464–474.

Candia-Carnevali, M. D., Bonasoro, F., Invernizzi, R., Lucca, E., Welsch, U. & Thorndyke, M. C. 1996 Tissue distribution of monoamine neurotransmitters in normal and regenerating arms of the feather star *Antedon mediterranea*. *Cell. Tiss. Res.* **285**, 341–352.

Cobb, J. L. S. 1987 Neurobiology of Echinodermata. *Nervous systems in invertebrates* (ed. M. A. Ali), pp. 483–527. New York: Plenum Press.

Cuenot, L. 1948 Anatomie, ethologie et systematique des echinodermes. *Traite de zoologie*, (ed. P. P. Grassé). Paris: Masson & Cie.

Denburg, J. L. 1989 The reappearance of a developmental stage specific antigen in adult regenerating neurons of the cockroach. *J. Neurosci.* **9**, 3491–3504.

Dinsmore, C. (ed.) 1991 *A history of regeneration research*. Cambridge University Press.

Dolmatov, I. Y. 1992 Regeneration of the aquapharyngeal complex in the holothurian *Eupentacta fraudatrix* (Holothuria: Dendrochirota). *Keys for regeneration research*. Monographs in Developmental Biology (ed. C. H. Taban & B. Boilly), vol. 23, pp. 40–50. Basel: Karger Press.

Elphick, M. R., Reeve, J. R., Burke, R. D. & Thorndyke, M. C. 1991 Isolation of the neuropeptide SALMFamide-1 from starfish using a new antiserum. *Peptides* **12**, 455–459.

Goss, R. J. 1969 *Principles of regeneration*. New York: Academic Press.

Gratzner, H. G. 1982 Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218**, 474–475.

Holder, N. 1988 Is there a correlation between continuous neurogenesis and directed axon regeneration in the vertebrate nervous system? *TINS* **11**, 94–99.

Hsu, S. M., Raine, L. & Fanger, H. 1981 Use of avidinbiotin peroxidase complex (ABC) in immunocytochemical techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29**, 577–580.

Huet, M. 1972 Etude ultrastructurale et evolution de la cellule neuroepitheliale de l'epiderme du bras de l'etolie de mer, *Asterina gibbosa* (Penn), en regeneration. *Z. Zellforsch. Mikros. Anat.* **126**, 75–89.

Huet, M. 1975 Le role du systeme nerveux au cours de la regeneration du bras chez une etoile de mer: *Asterina gibbosa*. *J. Embryol. Exp. Morph.* **33**, 535–552.

Hyman, L. H. 1955 Echinodermata. *The invertebrates* vol. IV. New York: McGraw-Hill.

Kawamura, K. & Nakaguchi, M. 1986 Mitosis and body patterning during morphallactic development of palleal buds in ascidians. *Devl Biol.* **116**, 39–50.

Kirschenbaum, S. R. & O'Shea, M. 1993 Postembryonic proliferation of neuroendocrine cells expressing adipokinetic hormone peptides in the corpora cardiaca of the locust. *Development* **118**, 1181–1190.

Koguchi, S., Sugino, Y. M. & Kawamura, K. 1993 Dynamics of epithelial stem cell in the process of stolonial budding of the colonial ascidian *Perophora japonica*. *Mem. Faculty Sci, Kochi University*, D, **14**, 7–14.

Kono, T., Takada, M., Wu, J.-Y. & Kitai, S. T. 1991 Double immunohistochemical detection of transmitter phenotype of proliferating cells using bromodeoxyuridine. *Neurosci. Lett.* **132**, 113–116.

Lyckman, A. W., Heidelbaugh, S. M. & Bittner, G. D. 1992 Analysis of neuritic outgrowth from severed giant axons in *Lumbricus terrestris*. *J. Comp. Neurol.* **318**, 426–438.

Mladenov, P. V., Bisgrove, B., Aostra, S. & Burke, R. D. 1989 Mechanisms of arm tip regeneration in the sea star *Lepasterias hexactis*. *Roux's Arch. Devl. Biol.* **198**, 19–28.

Moore, S. M. 1993 An optical and ultrastructural immunocytochemical study on the distribution of SALMFamide neuropeptides in the starfish *Asterias rubens*. Ph.D. thesis, University of London.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Moore, S. M. & Thorndyke, M. C. 1993 Immunocytochemical mapping of the novel echinoderm neuropeptide SALMFamide-1 (S1) in the starfish *Asterias rubens*. *Cell Tiss. Res.* **274**, 605–618.

Moran, R., Darzynkiewicz, Z., Stauano-Coico, L. & Melamed, M. R. 1985 Detection of 5-bromodeoxyuridine (BrdUrd) incorporation by monoclonal antibodies. Role of the denaturation step. *J. Histochem. Cytochem.* **53**, 821–827.

Moskowitz, P. F. & Oblinger, M. M. 1995 Sensory neurons selectively up-regulate synthesis and transport of the beta(III)-tubulin protein during axonal regeneration. *J. Neurosci.* **15**(2), 1545–1555.

Moss, C., Burke, R. D. & Thorndyke, M. C. 1994 Immunocytochemical localisation of the echinoderm neuropeptide GFNSALMFamide (S1) and serotonin in the larvae of the starfish *Pisaster ochraceus* and *Asterias rubens*. *J. Mar. Biol. Ass. UK* **74**, 61–71.

Newman, S. J., Elphick, M. R. & Thorndyke, M. C. 1995*a* Tissue distribution of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. I. Nervous and locomotory systems. *Proc. R. Soc. Lond.* B **261**, 139–145.

Newman, S. J., Elphick, M. R. & Thorndyke, M. C. 1995*b* Tissue distribution of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. II. Digestive system. *Proc. R. Soc. Lond.* B **261**, 187–192.

Oesterle, E. C., Tsue, T. T., Reh, T. A. & Rubel, E. W. 1993 Hair cell regeneration in organ cultures of the postnatal chicken inner ear. *Hear. Res.* **70**, 85–108.

Palmberg, I. E. & Reuter, M. 1990 Neuronal subsets in regenerating *Microstomum lineare*—Immunocytochemistry of FMRF/RF-amide and 5HT. *Acta Acad. Aboensis* Series B **50**, 147–159.

Penn, P. E. 1982 Regeneration of the optic cushion in the tropical intertidal asteroid, *Nepanthia belcheri* (Perrier). In *Echinoderm research* (ed. J. M. Lawrence), pp. 370. Rotterdam: A. A. Balkema.

Plickert, G., Kroiher, M. & Munck, A. 1988 Proliferation kinetics and cell lineages can be studied in whole mounts and macerates by means of BrdU/antiBrdU technique. *Development* **103**, 791–794.

Polak, J. M. & Van Noorden, S. (eds.) 1986. *Immunocytochemisty. Modern methods and applications.* Bristol: John Wright & Sons Ltd.

Reh, T. A., Jones, M. & Pittack, C. 1991 Common mechanisms of retinal regeneration in the larval frog and embryonic chick. In *Regeneration of vertebrate sensory receptor cells.* Ciba Foundation Symposium 160, pp. 171–191. Chichester: Wiley.

Schutte, B., Reynders, M. J., Bosman, F. T. & Blijham, G. H. 1987 Effects of tissue fixation on anti-bromodeoxyuridine immunohistochemistry. *J. Histochem. Cytochem.* **35**, 1343–1345.

Singer, M. & Geraudie, J. 1989 An overview of the historical origin of the nerve influence on regeneration. *Recent trends in regeneration research*, NATO ASI Series 172 (ed. V. Kiortis, S. Koussoulakos & H. Wallace), pp. 1–5. New York: Plenum Press.

Smith, J. E. 1966 The form and function of the nervous system. *Physiology of the Echinodermata* (ed. R. A. Boolootian), pp. 503–512. New York: Interscience.

Soriano, E. & Del Rio, J. A. 1991 Simultaneous immunocytochemical visualisation of bromodeoxyuridine and neural tissue antigens. *J. Histochem. Cytochem.* **39**, 255–263.

Sternberger, L. A. 1979 *Immunocytochemistry.* New York: Wiley.

Stocum, D. L. 1991 Limb regeneration: a call to arms (and legs). *Cell* **67**, 5–8.

Strand, F. L., Rose, K. J., Zuccarelli, L. A., Kume, J., Alves, S. E., Antonawich, F. J. & Garrett, L. Y. 1991 Neuropeptides hormones as neurotrophic factors. *Physiol. Rev.* **71**, 1017–1046.

Treherne, J. E., Smith, P. J. & Howes, E. A. 1988 Neural repair and regeneration in insects. *Advances in insect physiology*, vol. 21, pp. 35–84. New York: Academic Press.

VandenBosche, J. P. & Jangoux, M. 1976 Epithelial origin of starfish coelomocytes. *Nature* **261**, 227–228.

Wallace, H. 1981 *Vertebrate limb regeneration.* New York: Wiley.

Zeleny, C. 1903 A study of the rate of regeneration of the arms in the brittlestar *Ophioglypha lacertosa*. *Biol. Bull.* **6**, 12–17.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES