Journal of Neurocytology 8, 529–548

Axonal–ependymal associations during early regeneration of the transected spinal cord in *Xenopus laevis* tadpoles

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Received 24 October 1978; revised 29 January 1979; accepted 5 February 1979

Summary

The nature and organization of the cellular substrate supporting axonal outgrowth during early regeneration of the spinal cord following transection and/or segment removal were examined in *Xenopus* tadpoles. Longitudinal axonal compartments, formed by radial ependymal processes in unoperated spinal cords, were maintained within the rostral and caudal stumps throughout the early post-operative period. The first neuritic sprouts to appear near the cut ends of the cord were frequently associated with these processes. Between 5 and 7 days after transection either single or multiple cellular aggregates, which consisted primarily of ependyma, began occupying the lesion zone and were encompassed by numerous small fibres. Ependymal processes subsequently extended among the neurites within the lesion gap and re-established longitudinal axonal channels and a surrounding glia limitans.

A concurrent outgrowth of fibres and ependyma from the rostral and caudal stumps was also initiated by 5–7 days following resection of the cord. Axons were seen further within the ablation gap at 10 and 12 days either coursing along the surfaces of the emerging ependyma or organized into small fascicles by the radial processes of these cells. Intermediate stages of axonal fasciculation by the underlying ependyma were also seen. In contrast with previous reports, axonal elongation beyond the cut ends of the cord did not consistently entail the formation of pre-existing ependymal channels. These observations provide additional evidence emphasizing the importance of the ependymal cell surface during axonal outgrowth in the regenerating spinal cord. Guided neuritic elongation, however, does not appear to be dependent upon the prior establishment of a specific type of cytoarchitecture.

Introduction

Several non-mammalian vertebrates possess an impressive capacity for regeneration within the C.N.S. – an ability which contrasts with the tendency toward aborted neuritic outgrowth observed in the mammal (Ramon y Cajal, 1928; Windle, 1956; Clemente, 1964; Guth, 1975; Pettegrew and Windle, 1976; Puchala and Windle,

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1977). In the case of the spinal cord, histological analyses have shown that, after complete transection or segment removal in fish, reptiles and amphibians, anatomical continuity is ultimately restored. Axonal elongation across the lesion site between the cut ends of the cord is also accompanied by a substantial recovery of function (see reviews by Windle, 1956; Clemente, 1964). The cellular mechanisms which underlie successful regeneration in these species remain to be determined.

Observations of spinal cord regeneration following tail amputation in the lizard, *Anolis* (Simpson, 1968; Egar *et al.*, 1970) and urodele, *Triturus* (Egar and Singer, 1972; Nordlander and Singer, 1978) have suggested that ependymal cells, which emerge from the cut end of the cord *prior* to axonal outgrowth, may provide a guiding substrate for neurites. In contrast, much less is known about the degree to which the ependyma plays a role during neuritic outgrowth following transection at higher spinal levels in non-mammalian species. Results of previous studies have not consistently indicated that ependymal cells establish a major terrain for regenerating axons. For example, Butler and Ward (1965, 1967) reported that during reconstitution of ablated spinal segments in larval and adult urodeles, axons bridged the gap between the cut ends of the cord in advance of the emerging ependymal cells. Instead, connective tissue elements appeared to provide the primary cellular substrate for the elongating fibres. Sims (1962) also mentioned that cellular continuity was rarely re-established in the transected spinal cord of *Xenopus* larvae, although small neurites traversed the lesion site. On the other hand, formation of glial–ependymal 'bridges' and fasciculation of axons by processes was described in the regenerating spinal cord of the goldfish following transection (Bernstein and Bernstein, 1969).

In the present ultrastructural study, we have examined the initial period of axonal outgrowth following complete transection and/or segment removal in the spinal cords of *Xenopus laevis* tadpoles. The objective of this investigation was to define more critically the extent to which the ependyma or other cellular elements play a role in regeneration under these experimental conditions. Study of regenerative phenomena in the spinal cord of this species was of particular interest since the observations reported by Sims (1962) suggest that elongating fibres in the transected spinal cord may not utilize an ependymal substrate. Furthermore, various cytological aspects of axonal outgrowth and cellular interactions have been described in the regenerating optic nerves (Reier and Webster, 1974; Reier, 1978, 1979) which, could be applied to this study of a more complex region of the C.N.S.

Preliminary results of this study have been previously summarized (Michel and Reier, 1977).

Materials and methods

Spinal cord transections

Stages 54–56 (Nieuwkoop and Faber, 1956) *Xenopus laevis* tadpoles (NASCO, Fort Atkinson, Wisconsin) were anaesthetized by immersion in a 0.05% solution of tricaine methanesulphonate

(Finquel, Ayerst Laboratories, New York, N.Y.) and a lateral incision was made in the axial musculature. After removing portions of the vertebral column, the exposed spinal cord was completely transected at the level of the lumbar enlargement (that is, between the seventh and ninth body segments) with a pair of fine dissecting scissors. In one series of animals, a 1 mm segment was removed at a comparable level of the cord. The extent of the transection was easily determined by visual inspection through the dissecting microscope while the area was being flushed with Frog Ringer's solution. Following the operation, groups of 8–10 tadpoles were kept in gallon bins of aerated, chlorine-free tap water at 20° C and were fed powdered frog brittle.

Tissue preparation

At post-operative periods of 3, 5, 7, 10, 12 and 14 days, 3–10 tadpoles per interval were perfused through the heart with fixative containing either 0.5% paraformaldehyde and 1.5% glutaraldehyde or 0.5% paraformaldehyde and 4.5% glutaraldehyde. The most uniform preservation was obtained when the former solution contained 2% acrolein. All of the aldehyde solutions were prepared in 0.08 M Sorenson's phosphate buffer at pH 7.6. After perfusion, the dorsal skin was stripped away, and the tadpoles were immersed overnight in fresh fixative kept at room temperature.

A wedge of tissue containing the lesion zone and portions of the adjacent rostral and caudal cord stumps surrounded by associated cartilage and axial musculature, was then obtained and postfixed in 1.0% osmium ferrocyanide (Karnovsky, 1971) at 4° C for 2 h. The tissue was stained *en bloc* with maleate-buffered uranyl acetate, dehydrated in graded concentrations of ethanol and embedded in Epon. The rostral–caudal orientations of each sample was maintained during embedding. Two micrometer sections, obtained at 20–40 μm intervals, were used to determine the general appearance of the cord at various levels within the rostral and caudal regions and intervening lesion gap. Thin sections were taken at levels of specific interest.

For the purposes of the following description, the 'stumps' of the cord refer to 1.5–2.0 mm segments of the intact cord immediately rostral and caudal to the site of transection or segment removal. The 'cut ends' specifically apply to the edge of the stumps immediately adjacent to the lesion. The region between the stumps of the cord represents the intervening lesion gap.

Since all of the animals were sacrificed at early post-operative periods, an evaluation of functional recovery was not possible. In agreement with a previous study (Sims, 1962), the operated tadpoles simply rested at the bottom of the bins during the first two weeks and never showed normal swimming movements. Whenever spontaneous activity did occur, it was usaually limited to gross movement of the trunk.

Observations

DESCRIPTION OF NORMAL SPINAL CORD MORPHOLOGY

The lumbar spinal cord of unoperated stage 54–56 tadpoles could be subdivided into three distinct zones: a pseudostratified, ventricular layer lining the central canal, an intermediate zone of developing grey matter, and an outer marginal region of axons (Fig. 1). Ciliated ependymal cells within the ventricular layer were joined at their apical surfaces by junctional complexes. The basal processes of these cells extended radially toward the periphery of the cord where they expanded into marginal end-feet below a surrounding external basal lamina. Small aggregates of filaments, 8–10 nm in diameter, were characteristically present within the cytoplasm of the ependymal cells. The intermediate layer of the lumbar cord was clearly differentiated into alar and basal plates and consisted of numerous, tightly-packed

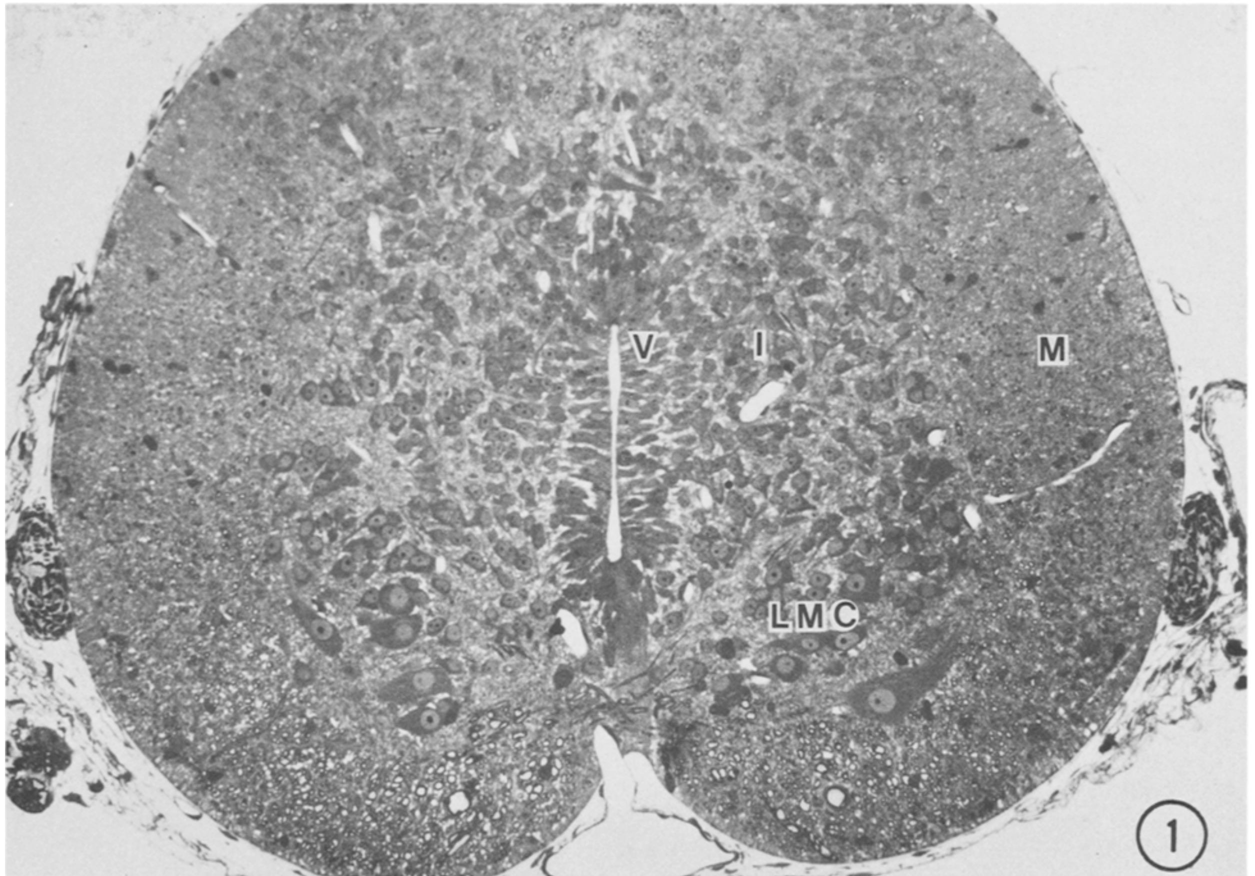
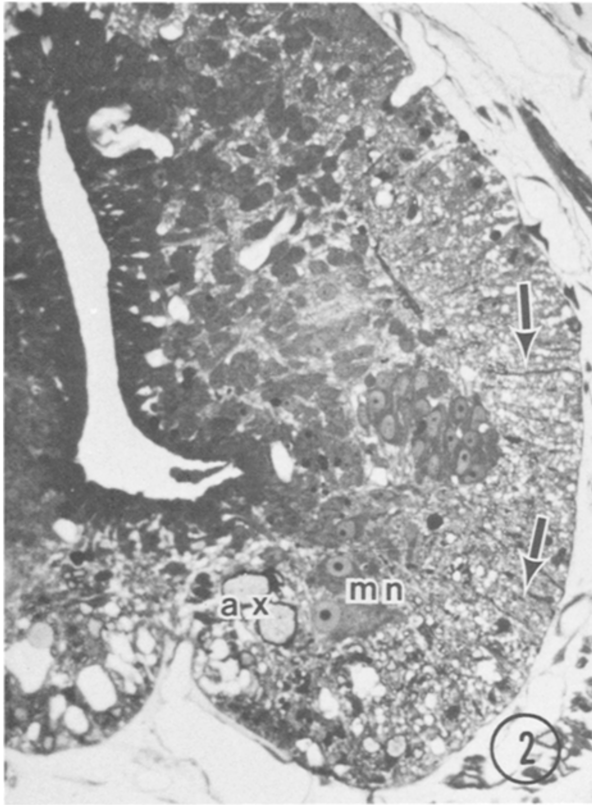


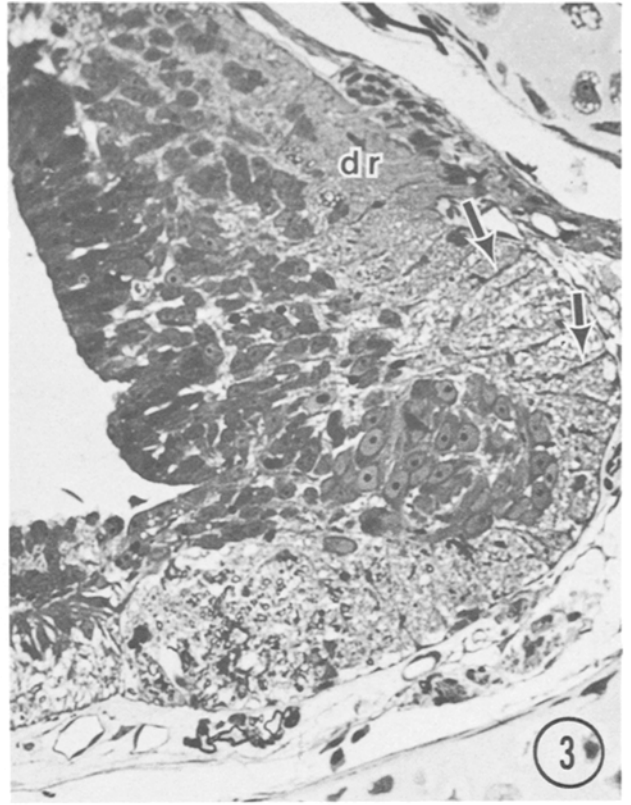
Fig. 1. Illustrated is a transverse section through the lumbar region of a normal, stage 54 *Xenopus* tadpole. Three distinct regions – ventricular (V), intermediate (I) and marginal (M) – can be identified. Several large motor neurons are present within the lateral motor columns (LMC). Numerous myelinated fibres are seen within the ventral marginal region. x 85.

Fig. 2. Three days after simple transection extensive axonal degeneration is evident within the ventral and ventrolateral aspects of the rostral stump. Distortion of the central canal is seen ventrally. Radial glial processes (arrows) and two chromatolytic motor neurons (mn) are also seen. Some large axonal profiles (ax) are still present in the ventral funiculus. x 720.

Fig. 3. Three days after transection, the ventral funiculus caudal to the lesion site contains no large fibre profiles, and only a few small myelinated fibres and cellular debris are visible. Radial glial processes (arrows) are clearly seen extending into the marginal layer. The central canal is distended, and slight evaginations are present along the ependymal wall. A dorsal root entry zone (dr) is also evident in this section. x 80.

Fig. 4. Three days after transection, persisting axonal channels, defined by radial ependymal processes, are seen in the ventrolateral funiculus of the caudal stump. The attenuated ependymal processes (e) occasionally contain debris (arrow). Many of the small, unmyelinated axons appear tightly clustered and these neuritic groups are separated from each other by varying amounts of extracellular space. Most of the fibre aggregates appear to be situated adjacent to cellular processes. x 8200.


ax
mn
2


dr
3

cells. These small cells exhibited large nuclei with prominent nucleoli and scant cytoplasm. The lateral motor columns were comprised of more neurons having axonal and dendritic processes, the usual complement of organelles and a well-defined Nissl substance. Numerous longitudinally-oriented myelinated and unmyelinated axons were seen within the relatively cell-free marginal layer. The ventral and ventrolateral aspects of the marginal layer contained nearly all of the myelinated fibres within the lumbar region (Fig. 1). The radial ependymal processes extending into the marginal zone formed longitudinal 'channels', or fascicles, within which ascending and descending fibres coursed. While many small neurites were present within the marginal zone of the normal cord, profiles resembling growth cones were rarely observed. Oligodendrocytes represented the only identifiable macroglial cell type.

The spinal cord was surrounded by a thin, primitive meningeal sheath which consisted of fibroblasts, collagen and large melanocytes. The cells of the meninges were quite attenuated and long cytoplasmic processes extended from the perinuclear region and overlapped with each other. The cytoplasm of these cells was lucent and contained short cisterns of rough endoplasmic reticulum, ribosomal rosettes and scattered microfibrils. These cells were rarely surrounded by a basal lamina.

DEGENERATIVE CHANGES WITHIN THE TRANSECTED SPINAL CORD

Spinal cords were first examined at three days post-transection (p.t.). In all of the animals studied, complete transection of the cord was confirmed by the separation of rostral and caudal stumps and a partial section of the underlying notochord. An intervening lesion gap (0.25–0.50 mm in length) was usually formed between the cut ends. In some cases, however, separation of the cord was less pronounced, and the tips of the rostral and caudal stumps appeared to overlap. Macrophages, hematogenous elements and large amounts of cellular debris occupied the lesion site.

The general cellular organization of the rostral spinal cord appeared virtually unchanged to a level approximately 0.8–1.0 mm from the lesion site. At this point, several degenerating axons became evident within the dorsal funiculus, and some destruction of fibres and myelin was indicated in the ventral regions of the cord. Approximately 0.5 mm from the lesion, degenerating axons were scattered throughout the marginal layer, but the most extensive axonal destruction occurred in the ventral and ventrolateral areas (Fig. 2 and 3). The incidence of degenerating axons within the rostral and caudal stumps had decreased by 5–7 days p.t. and most necrotic tissue and cellular debris within the lesion zone had been removed.

The grey matter of the rostral and caudal stumps was generally disorganized; however, ventral horn cells frequently remained clustered together, and a few cells near the cut ends showed signs of chromatolysis (Fig. 2). The central canal was distended and often contained small cells and degeneration debris. During the early post-transection periods the radially-oriented ependymal processes were often filled with cellular debris and lipid droplets which were distributed throughout their extent from the perikaryon to the periphery of the cord (Fig. 4).

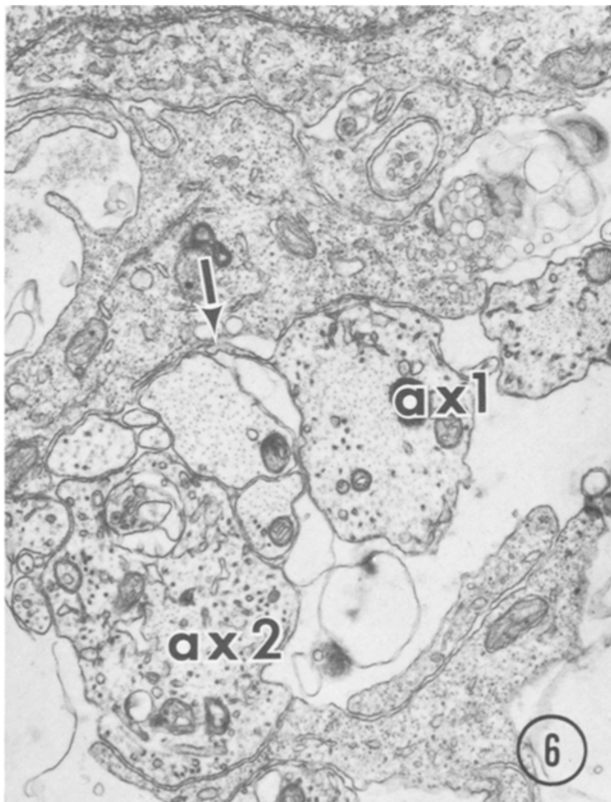


Fig. 5 Within the dorsal region of the caudal stump, five days after transection, several small axonal profiles (e.g., ax1, ax2, ax3) exhibit the characteristic organelle composition (e.g., branched smooth endoplasmic reticulum) of axonal sprouts. Such fibres frequently lie adjacent to ependymal processes (e). A myelinated fibre (MyF), having an axoplasmic appearance similar to the smaller sprouts, is also seen. x 13 500.

Fig. 6. Two small axonal profiles (ax1, ax2) are seen adjacent to ependymal processes within the rostral stump of a transected cord, five days p.t. A tiny filopodium (arrow) extends from one of these profiles (ax1). x 6800.

CELLULAR FRAMEWORKS AND INTERACTIONS DURING AXONAL OUTGROWTH

Rostral and caudal stumps

Longitudinal axonal compartments, defined by radial ependymal processes within the rostral and caudal stumps of the normal spinal cords remained intact throughout the degenerative period. The most dramatic indication of the persistence of axonal compartments was seen in the ventrolateral and lateral regions of the stumps where massive degeneration had occurred (Figs. 2–4). Cellular debris, occasional oligodendrocytes and few fibres were scattered within the extracellular space of these channels. Due to their highly irregular contour, however, considerable expanses of the processes could not always be viewed in single thin sections. Marginal end-feet were frequently attenuated and convoluted and the associated basal lamina was consequently quite tortuous. Numerous marginal end-feet were distributed at the surface of the cord. Thus, a continuous glia limitans was maintained around the cord at the levels of the rostral and caudal stumps (Fig. 4).

At later intervals, the longitudinal compartments were occupied by an increasing population of small fibres which were generally grouped into bundles of varying sizes. The axonal aggregates were separated from each other by large areas of extracellular space, and many were not completely bordered by glial processes. Most bundles, however, were located adjacent to segments of the radial processes or were coursing alongside and between ependymal cell bodies lining the central canal.

Rostral and caudal cut ends

Within the rostral and caudal cut ends the parenchyma of the cord was extremely disorganized and the central canal often contained grey matter which had herniated into the lumen. The majority of axons at the cut ends were organized into tightly-packed clusters which were separated from each other by varying amounts of extracellular space (Figs. 5 and 7). Terminal and preterminal profiles of axonal sprouts were generally observed within this region of the cord by 5–7 days. These processes were characterized by large arrays of branched, smooth endoplasmic reticulum, microtubules, numerous mitochondria and dense bodies distributed within a matrix of neurofilaments (Figs. 5 and 6). In longitudinal sections, some axonal sprouts were observed emerging at nodal regions of myelinated fibres which exhibited similar axoplasmic features at preceding para- and internodal levels (Fig.

Fig. 7. A region of the lateral funiculus in the rostral cut end of the cord at 10 days is shown. The glia limitans (gl) is intact. Groups of tiny neurites (ax) are seen associated with the ependymal extensions (e). x 6900.

Fig. 8. A portion of an ependymal outgrowth located near the cut edge of the cord at six days p.t. is illustrated. The clustered ependymal cells have formed radial processes (e) ending in marginal end-feet at the glia limitans (gl). In this case, longitudinal compartments (lc) are established which contain a few small fibres (arrows). A large axonal profile (ax) containing numerous organelles is also shown. x 9000.

ax
e
e
e
ax
gl
7

ax
lc
e
e
gl
8

5). Frequently, these sprouts were branched and small filopodia extended from their surfaces (Fig. 6). Early stages of myelination were indicated by the presence of thin cuffs of oligodendroglial cytoplasm which surrounded many of these fibres.

Ependymal processes maintained their basic radial distribution at the cut ends and their expanded marginal end-feet established a continuous glia limitans at the periphery of the cord after transection (Fig. 7). Slender ependymal processes extended among the small fibres and partially bordered the bundles of neurites (Fig. 7). Axonal sprouts were frequently aligned in close apposition with the ependymal extensions (Figs. 5 and 6).

Intervening lesion zone

Reconstitution of continuity within the spinal cord following simple transection involved the concurrent outgrowth of axons and emergence of ependymal cells from the cut ends. Furthermore, rapid regeneration of the meninges provided an enveloping sheath which served to confine the majority of fibres to the immediate vicinity of the cut ends of the cord.

By five days p.t., the incidence of mitotic figures within the ventricular region had increased and a substantial population of ependymal cells was present at the cut ends. Often, one or more small aberrant canals branched from the primary canal. The spindle-shaped ependymal cells lining these diverticula were joined apically by junctional complexes, and cilia and microvilli often projected from their luminal surface. Between five and seven days p.t., groups of ependymal cells populated the intervening lesion gap. By later post-operative stages, organized clusters of ependymal cells, which in 2 μm sections appeared to be continuous with cells lining the walls of the primary canal and diverticula within the stumps, appeared in all sections through the lesion zone. In spite of this extensive cellular outgrowth, restoration of a continuous central canal did not occur during the period studied.

Axonal outgrowth into the lesion gap was primarily associated with two distributions of ependymal cells and their processes. The most common pattern involved the formation of a cylindrical reconstituted spinal segment consisting of a central, cellular area surrounded by a marginal layer of small, unmyelinated axons. The cellular core contained spindle-shaped ependymal cells and small neuroblasts resembling those seen in the normal cord. Longitudinal ependymal channels were not as obvious, and the glia limitans was often incomplete.

In some animals the reconstituted segment exhibited a second pattern of cellular organization and distribution of cytoplasmic processes. By six days p.t., a cluster of ependymal cells and a few small neuroblasts was established within the lesion zone. The ependymal cells extended their processes radially and developed a continuous glia limitans (Fig. 8). In this case, the radial processes formed distinct longitudinal channels which contained a sparse population of neurites. Secondary ependymal clusters occasionally arose from this primary cellular aggregate; these cells were separated from each other by varying amounts of extracellular space through which some neurites extended.

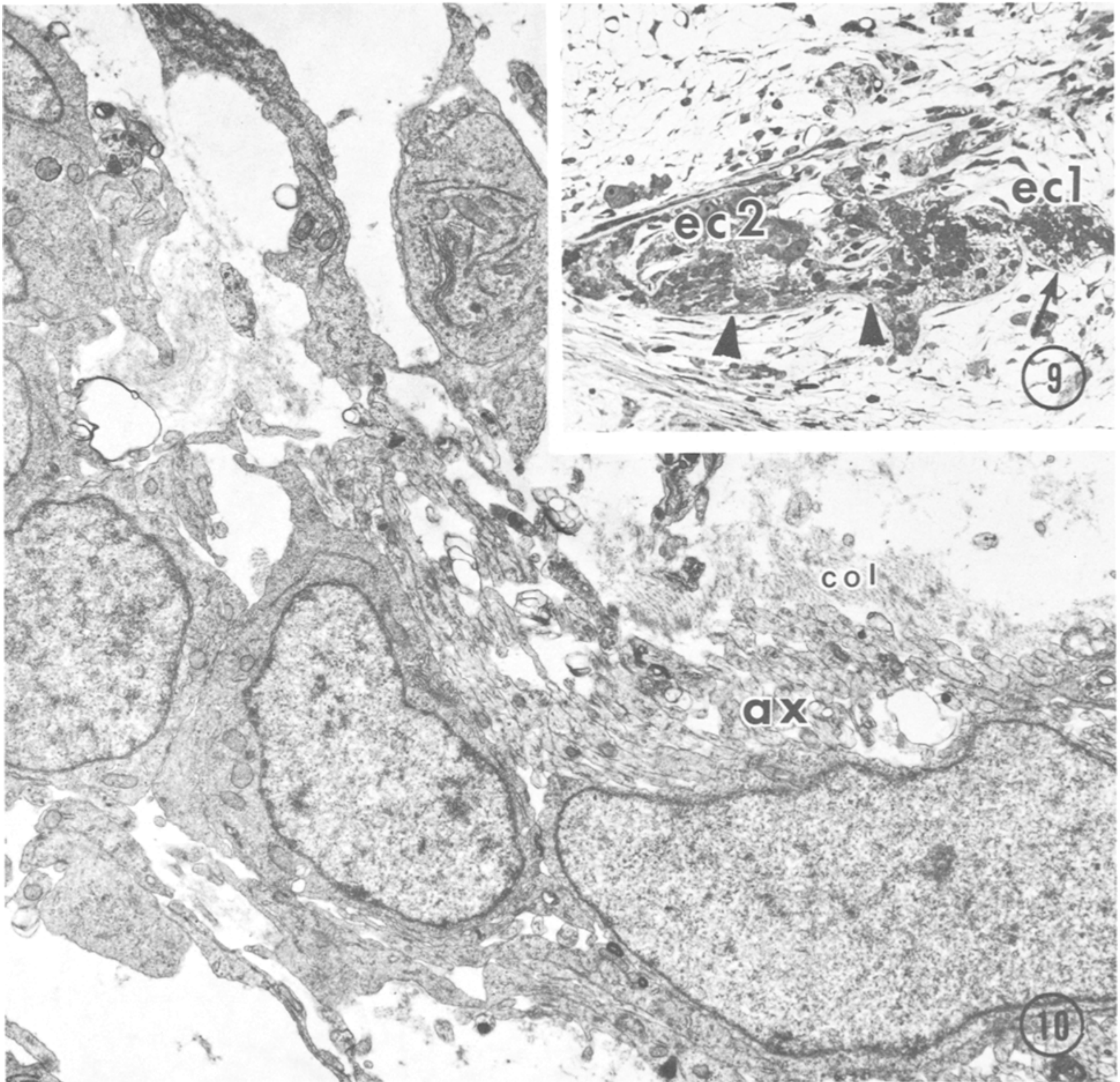


Fig. 9. The general pattern of ependymal cell organization within the lesion gap 12 days following resection of the cord is shown. In one region the ependymal cells have formed a tube (ec1) which appears to be defined by an external membrane (small arrow) at the periphery. The presence of glial channels, similar to those illustrated in Fig. 8, is also suggested within this cellular aggregate at this level of magnification. The remainder of the ependymal outgrowth (ec2) consists of small groups of cells, and an external membrane (arrowheads) partially surrounds this region of the developing ependymal bridge. x 45.

Fig. 10. A row of ependymal cells is shown within the ablation zone 12 days after resection of the cord. Groups of small fibres (ax) course along the surfaces of the cells and accompany the ependymal outgrowth throughout the area. Clumps of collagen (col) are seen near some of the axons. x 13 000.

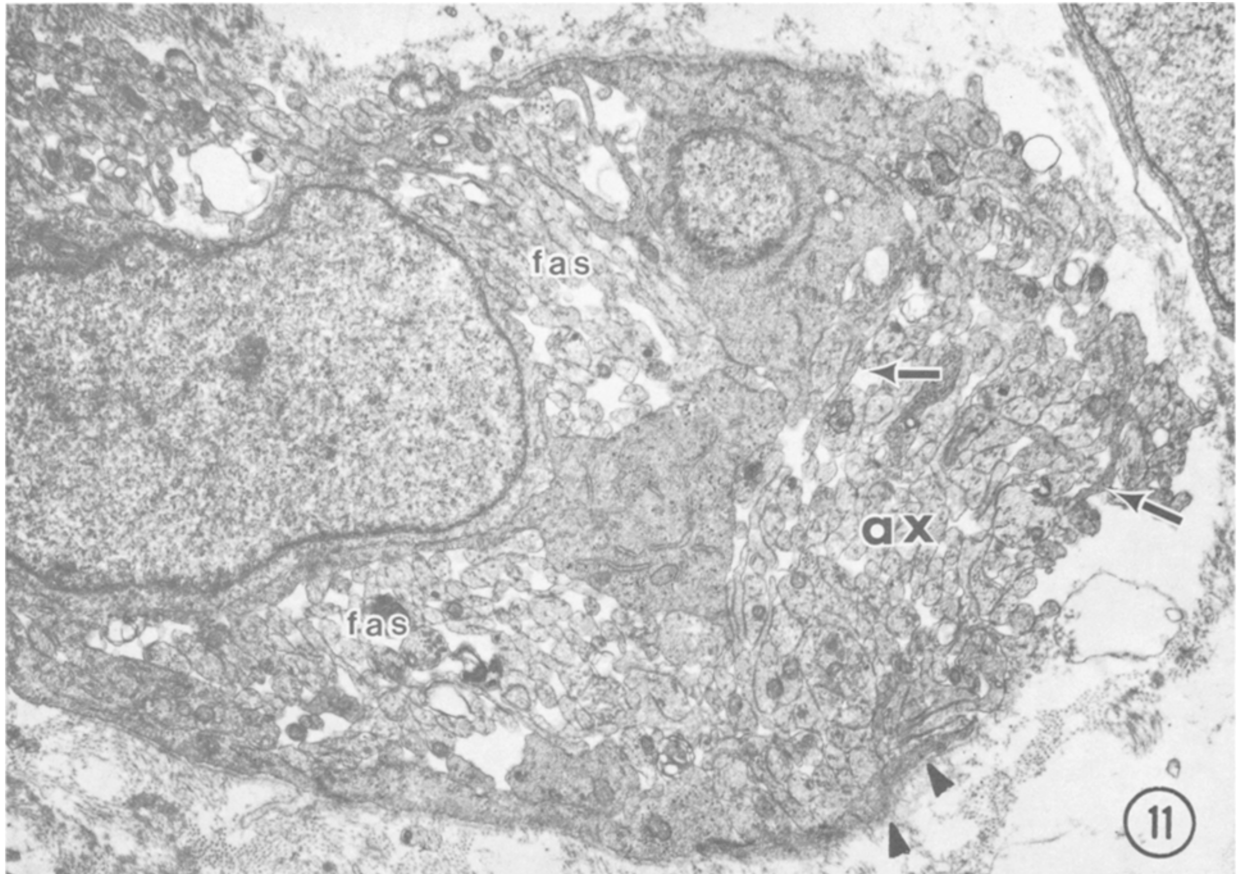


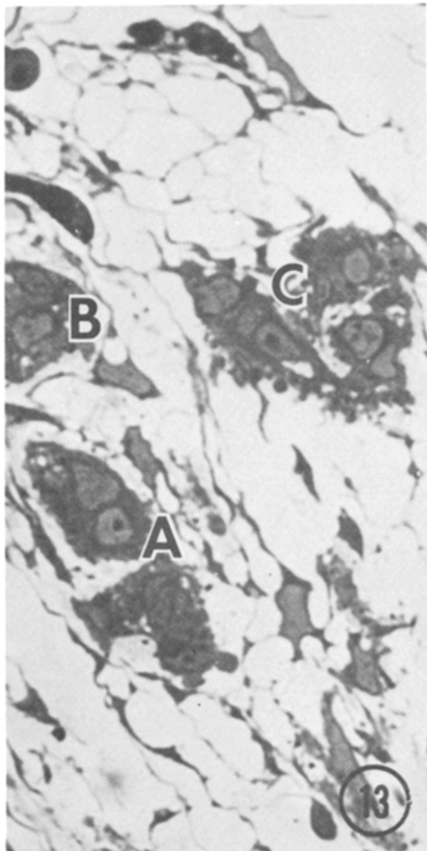
Fig. 11. Another region of the ependymal outgrowth illustrated in Fig. 10 is shown. Many of the neurites are enclosed with fascicles (fas) formed by ependymal processes. One large group of axons (ax) is seen coursing at the surface of the cellular aggregate. Fasciculation of this group is not evident, although some dense ependymal processes (arrows) are distributed among the fibres and a portion of an expanded marginal process (arrowheads) is seen bordering a small region of this fibre bundle. x 12 150.

Fig. 12. A cluster of ependymal cells in the ablation region at 12 days after resection is shown. A few fibres have been fasciculated (fas). Some axons are also seen within intercellular spaces (i.e., channels, arrows). x 13 500.

Fig. 13. At the most advanced level of ependymal outgrowth in the ablation gap of a resected cord, small aggregates of ependymal cells (A, B, C) are present within the connective tissue matrix. x 88.

Fig. 14. An ependymal aggregate (A, illustrated in Fig. 13) is shown. Nearly all of the axons (ax) are distributed along the surface of this cellular cluster. An early stage in the formation of a glia limitans (arrow) is also seen. x 6600.

fas
12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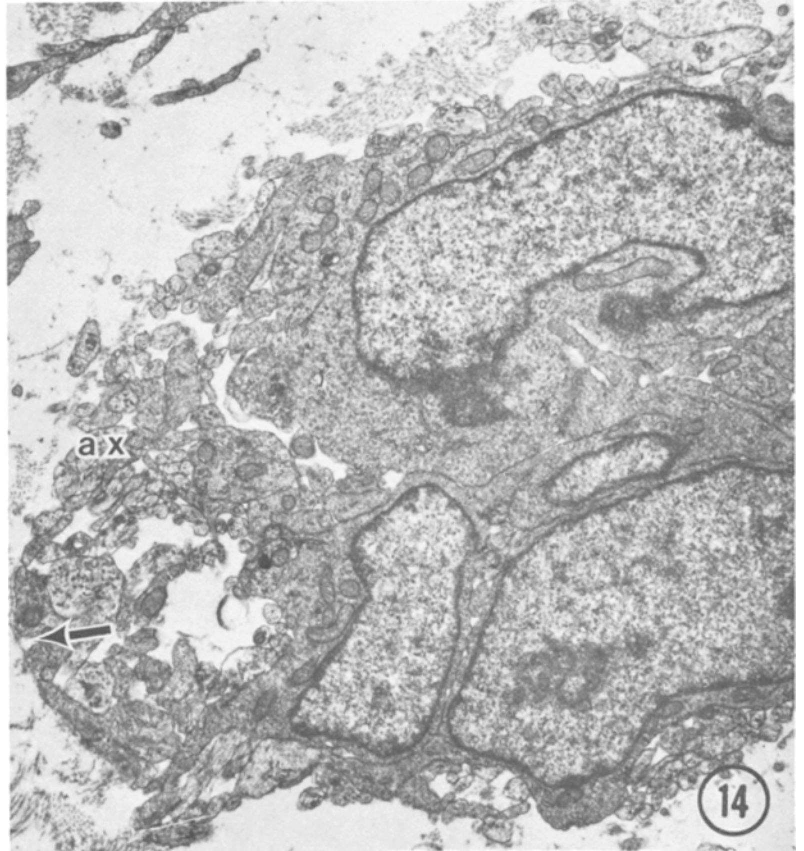
ax
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While our observations of the reconstituted segment suggested that elongating axons were associated with ependymal cells, the close apposition of the cord stumps and frequent displacement of grey matter toward the lesion did not permit an accurate evaluation to be made of the individual role of the ependymal substrate and its structural organization. In order to examine these aspects more directly, a second group of experiments was performed in which the distance between the cut ends of the cord was substantially increased by the resection of a 1 mm segment. A concurrent emergence of ependymal cells and axons from the rostral and caudal stumps of the ablated cords was initiated by 5–7 days. At this time, the majority of neurites were associated with the surfaces of ependymal cells which were rather loosely arranged near the cut edge of the cord. By 10–12 days, outgrowth was evident further within the lesion gap; however, structural continuity between the rostral and caudal stumps was not yet re-established. The pattern of axonal relationships with ependymal cells at these slightly later post-operative intervals were variable, and many resembled those which were seen when the earliest outgrowth of fibres and cells had occurred near the end of the first week.

At 10–12 days, ependymal cells extended from the cut edges of the cord into the mesenchymatous meshwork of the lesion zone where they were organized either in small clusters or rows. In some instances, these cells formed a small ependymal tube which was defined by a surrounding glia limitans (Fig. 9). In other cases, even within the same specimen, cells were more loosely arranged and only a partial glia limitans was re-established (Fig. 9). Axons extending from the stumps of the cord often coursed along the surfaces of the ependymal outgrowths (Fig. 10). In other regions where the cells were more clustered, fibres were gathered into small compartments around the cellular core by the perikarya and short radial processes of the underlying ependyma (Figs. 11 and 12). Intermediate stages of ependymal fasciculation of neurites were also exhibited in these clusters. Some axons distributed at the periphery were only partially enclosed by the marginal end-feet and basal lamina of the underlying ependyma (Fig. 11). Small axonal 'channels', represented by extracellular space between adjacent cells and processes (for example, Nordlander and Singer, 1978), were also associated with the cellular clusters near the cut ends of the cord (Fig. 12). An occasional neurite was observed within these channels. Formation of these discrete intercellular pathways, however, was less common than the more random arrangement described above and seemed only to be associated with cell clusters or tube-like extensions of the ependyma.

At the most distant point of cellular emergence into the lesion gap at 12 days post-resection, axonal–ependymal associations were particularly similar to those observed near the cut ends during initial outgrowth. Small dissociated groups of ependymal cells could be seen within the loose matrix of connective tissue (Fig. 13). Neurites were also present, and the majority of axons was aligned along the surfaces of the ependymal cells (Fig. 14). Fasciculation of axons and the development of ependymal channels were rarely observed at these levels of outgrowth.

During regeneration, connective tissue elements, fibroblasts in particular, formed

a complex trabecular meshwork. Some neurites emerging from the cut ends were aligned adjacent to these cellular processes. In a few instances, Schwann cells, which were probably derived from injured roots and spinal ganglia within the lesion gap, were also associated with these axons. In comparison with the number of axons elongating in relation with ependymal cells, however, these aberrant fibres appeared to represent only a small percentage of the total population of axons within the ablation area.

Discussion

Regeneration of the spinal cord in *Xenopus* tadpoles was characterized by a complex series of cellular events during the initial, two post-operative weeks. The more prominent features associated with the restoration of continuity following either simple transection or segment removal were: a concurrent emergence of axons and ependymal cells from the cut ends of the cord; maintenance of longitudinal axonal channels and a surrounding external limiting membrane at the rostral and caudal stumps; development of a cellular terrain which supported axonal elongation through the lesion zone; and rapid re-establishment of the primitive meningeal sheath.

Ependymal outgrowth into the transection zone

The outgrowth of ependymal cells in the severed spinal cord represented a major aspect of the early restoration of anatomical continuity. Following either simple transection or excision of 1 mm segment of the spinal cord in *Xenopus* tadpoles, proliferation of ependymal cells increased at the rostral and caudal cut ends. By 5–7 days p.t., individual or multiple ependymal clusters occupied the lesion zone. In serial 2 μm sections, these aggregates appeared to be continuous with groups of cells extending from the ependymal layers lining the central canal at the severed ends of the cord. In the case of multiple aggregates, some appeared to arise from the lateral diverticula which may have represented interconnected cavities or branches of the canal (Butler and Ward, 1967).

While an extension of ependymal cells into the lesion zone frequently occurs in non-mammalian species, the pattern of this outgrowth appears to vary. In the regenerating cord of larval and adult urodeles following either ablation of a spinal segment (Butler and Ward, 1965, 1967) or tail amputation (Egar and Singer, 1972; Nordlander and Singer, 1978), the ends of the central canal became extensively dilated. The cut surfaces were healed by ependymal cells, and a terminal vesicle was formed. Re-establishment of the cord's integrity after ablation or elongation of the cord during tail regeneration was achieved by a lengthening of the terminal vesicles. In ablated cords, these vesicles subsequently advanced into the lesion and ultimately fused to form a continuous central canal.

In *Xenopus* tadpoles, formation and movement of enlarged terminal vesicles did not represent a major feature of cellular outgrowth at the stages examined. Rather,

ependymal cells appeared to emerge from the cut ends either individually or in the form of cellular cords. These cells aggregated within the lesion and formed single or multiple bridges between the rostral and caudal stumps. An outgrowth of ependymal cells was also a prominent feature in the regenerating spinal cords of other anuran larvae (Hooker, 1925; reviewed by Piatt, 1955). In striking contrast are the results reported by Sims (1962) which indicated that continuity of the central canal and grey matter rarely occurred during regeneration of the spinal cord in *Xenopus laevis* tadpoles. Our observations in *Xenopus*, however, support the earlier descriptions of spinal cord regeneration in anurans and appear to correspond with recent descriptions of the general mode by which ependymal bridges were established between the rostral and caudal stumps in larval lampreys (Rovainen, 1976; Selzer, 1978) and goldfish (Bernstein and Bernstein, 1969).

Previous analyses of regeneration by light microscopy following ablation of a spinal segment (Butler and Ward, 1965) in larval urodeles have indicated that the outgrowth of axons precedes an emergence of ependymal cells into the lesion gap. Our results after a similar lesion show that during restoration of the spinal cord's continuity the most advanced level of axonal elongation into the lesion is associated with an accompanying outgrowth of the ependyma. These contrasting descriptions of cellular—axonal relationships may be due to basic species variation in such aspects as the composition and organization of the surrounding mesenchymal tissue or other factors which may influence the proliferative and migratory capacities of the ependymal cells and rate of axonal outgrowth. On the other hand, the likelihood exists that ependymal cells in the other investigation, as in our resection material (for example, Fig. 13), were initially few in number and widely scattered within the ablation area; identification of these cells from the surrounding meningeal cells would be consequently more difficult in standard histological preparations.

Axonal associations with the ependymal framework

Following simple transection, ependymal cells within the rostral and caudal stumps maintained their radial processes which in the normal cord formed longitudinal, axonal compartments within the marginal layer. These channels were not destroyed during the early, degenerative period and, consequently, the elongation of axonal sprouts toward the opposite stump was appropriately directed within the persisting compartments. The marginal end-feet of these processes maintained a continuous glia limitans surrounding the parenchyma of the rostral and caudal stumps and thus provided a lateral boundary for the regenerating fibres. The importance of the ependymal framework for growing axons was further suggested by the frequent association of axonal sprouts and bundles with radial processes.

Ependymal—axonal relationships were more evident within the lesion zone following either transection or resection of the cord. Ependymal cells which had extended into this region generally formed a central core around which the accompanying neurites were distributed. Subsequently, radial processes extended among the bundles of axons, and cytoplasmic end-feet developed an enclosing glia

limitans. A comparable pattern of axonal fasciculation has been described during regeneration of the spinal cord in goldfish (Bernstein and Bernstein, 1969).

Compared with reconstitution of the spinal cord during tail regeneration in the newt (Egar and Singer, 1972; Nordlander and Singer, 1978), the pattern of axonal–ependymal associations exhibited in *Xenopus* differed in two major respects. First, a concurrent outgrowth of axons and ependyma was seen. Twelve days after resection of the spinal cord, examination of the most distant level of cellular invasion of the lesion gap showed neurites positioned adjacent to small groups of ependymal cells. In contrast, the caudal extension of ependymal cells into the regenerating tail of the newt occurred prior to the arrival of descending axons. A second difference noted in *Xenopus* was related to the structure of the ependymal outgrowth. In newts, ependymal cells were organized into a single layer which surrounded a central lumen. Cellular processes extending from the centre toward the periphery of this ependymal aggregate were separated by extracellular space. These spaces, which initially lacked axons, subsequently served as longitudinal channels through which regenerating axons elongated. Neither pre-existing ependymal channels nor other forms of specialized cellular organization were as consistently apparent during early regeneration of the spinal cord in the tadpole. Intercellular channels only appeared in regions where the ependymal cells were clustered together and formed a small, tubular outgrowth. Since the development of channels in other species is associated with the elongation of a tube of ependymal cells (for example, Nordlander and Singer, 1978), the variable morphology of the ependymal terrain in *Xenopus* may account for the limited extent to which these intercellular pathways were formed.

While fasciculation of neurites by ependymal processes was seen during initial stages of regeneration, formation of such axonal compartments did not appear to occur prior to the arrival of neurites. For example, in the resected cords, fibres present at the most distant and least mature level of the cellular extension into the lesion gap were rarely fasciculated by ependymal processes. Closer to the cut ends, axonal fasciculation by the ependyma was more frequent; however, many neuritic bundles which were not bound by ependymal cytoplasm were still seen at the periphery of the cellular aggregates. Intermediate stages of fasciculation were indicated by the presence of neuritic groups which were partially bordered by radial ependymal processes and expanded marginal end-feet. These varying degrees of axonal–ependymal association suggest a sequence of events leading to the establishment of longitudinal axonal compartments by ependymal cells either during or subsequent to the arrival of fibres. Based upon this interpretation, the likelihood exists that neurites may stimulate ependymal cells to hypertrophy and extend their radial processes around axonal groups.

These results pertaining to the organization of the cellular substrate in *Xenopus* suggest that an appropriately-directed pattern of axonal elongation can be obtained without requiring prior establishment of a specific type of ependymal cyto-architecture (that is, channels, longitudinal axonal compartments). This consideration, however, does not minimize the significance of an ependymal terrain

since the earliest fibres within the lesion gap utilized the surface of these cells which extended simultaneously from the cut ends of the cord. Eventually, the original population of 'pioneer fibres' which are subsequently contained within ependymal compartments may provide an adequate substrate for fibres which traverse the lesion gap at later times (Young, 1942; Weiss, 1955). Even in the case of the intercellular channels described in other studies, the main consideration may simply be that these spaces between cells permit regenerating fibres to associate with the ependymal surface. Such interactions, as suggested by the presence of numerous membrane specializations (Nordlander and Singer, 1978), may be essential for chemical exchange, among other possibilities, during neuritic outgrowth within the spinal cord.

Restoration of the meninges

Connective tissue cells also contributed in a more general fashion to the development of a cellular framework in *Xenopus* by quickly re-establishing a meningeal sheath which was continuous with the cut ends. Thus, the outgrowth of ependymal cells and axons was relatively confined, and a higher probability for successful restoration of continuity within the spinal cord was obtained. Moreover, the connective tissue elements did not form a dense scar as they do in the transected mammalian spinal cord. The formation of a connective tissue partition in association with glial cell processes between the cut ends of the cord has been frequently regarded as a major barrier limiting the possibility of successful axonal outgrowth in mammals (Brown and McCouch, 1947; Davidoff and Ransohoff, 1948; Windle and Chambers, 1950; Clemente and Windle, 1954). While our findings do not discount the possibility that connective tissue components in this system may also provide a suitable substrate for axonal outgrowth, the greater affinity for the ependymal surface suggests that these cells may play a more specialized role during regeneration.

Concluding considerations

The establishment of cellular substrates for growing axons has consistently characterized regeneration of the spinal cord in various non-mammalian species. Similarly, regeneration within the visual system of these animals (Reier and Webster, 1974; Turner and Singer, 1974; Murray, 1976) frequently entailed formation of longitudinal glial compartments within the degenerated regions of the optic nerve or tract. Whether the absence of such mechanical guides would adversely affect neuritic outgrowth in either the visual system or spinal cord of these animals remains to be determined specifically. There is little doubt, however, that the presence of a cellular substrate facilitates the regenerative process by preventing diffuse spread of growing fibres and by directing the axons to the general region of their appropriate target structures.

Recognition of the potential importance of cellular guides during regeneration has primarily evolved from studies of injured peripheral nerves in which the role of

Schwann cells in directing axonal elongation within the distal stumps has been repeatedly stressed. Ependymal cells in the spinal cord and astrocytes in the optic nerves of non-mammalian species appear to mimic this functional feature. Whether glial and ependymal cells can also subserve a metabolic role in sustaining axonal outgrowth, as suggested by the production of NGF-like proteins by glia within the C.N.S. and P.N.S. (reviewed by Varon and Bunge, 1978), remains to be determined.

Acknowledgements

This work was supported by N.I.H. Grant NS-13836, a grant from the Paralyzed Veterans of America, and a pre-doctoral fellowship awarded to M.E.M. by the Paralyzed Veterans of America. The authors are grateful to Ms Eleanor B. Sourbeer for her excellent technical assistance during the completion of this investigation.

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