Selforganization of ependyma in regenerating teleost spinal cord: evidence from serial section reconstructions

MARILYN J. ANDERSON\textsuperscript{1,2}, CHI Y. CHOY\textsuperscript{2} AND STEPHEN G. WAXMAN\textsuperscript{1,2}

\textsuperscript{1}Department of Neurology, Stanford University School of Medicine, USA
\textsuperscript{2}Veterans Administration Medical Center, 3801 Miranda Avenue, Palo Alto, CA 94304, USA

SUMMARY

Multiple ependymal structures have been observed in regenerating spinal cord of the teleost \textit{Apteronotus albifrons}. Evidence is presented for two modes of formation of the secondary ependymas: budding off from the primary ependyma, and \textit{de novo} origin of a tube-like ependymal structure within a group of undifferentiated cells. Serial sections of regenerated cord provide evidence that undifferentiated cells not in immediate contact with the main ependymal layer can organize and differentiate into an ependymal structure in the regenerating spinal cord. These findings suggest that a significant amount of morphological organization can take place independent of the normal developmental sequence and environment.

INTRODUCTION

The ependyma, or layer of cells surrounding the central canal in the vertebrate central nervous system, is of interest to developmental biologists because of its role in development and in the adult individual. In the embryo this layer, termed the generative neuroepithelium (Jacobson, 1978), is responsible for producing all the cells (both neuronal and non-neuronal) of the growing spinal cord. In the adult vertebrate, the cells lining the central canal (the ependymal cells) have differentiated specialized junctional complexes and cilia at their luminal ends, and may function in regulating the content and circulation of the cerebrospinal fluid.

It has been hypothesized that the ependymal layer in postnatal vertebrates may still harbour undifferentiated ‘stem cells’ which retain a relatively unrestricted potential for mitosis and differentiation under the appropriate circumstances. In fact, production of new cells has been demonstrated in postnatal rat brain (Altman & Das, 1965; Kaplan & Hinds, 1977), in adult rodent olfactory bulb (Graziadei & Graziadei, 1979; Wilson & Raisman, 1980), and in certain brain nuclei in adult birds (Goldman & Nottebohm, 1983). The newly produced cells in these instances include neurones which are recruited into functional circuits (Easter, 1983; Paton & Nottebohm, 1984), glial cells, and ependymal cells which form the wall of the lengthening ependymal tube. The ultimate source of the new cells appears to be

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the ependymal layer, since studies examining \[^{3}H\]thymidine incorporation after a series of intervals (e.g. Altman & Das, 1965; Goldman & Nottebohm, 1983) show labelled cells first in the ependyma or ventricular layer, then later in other brain layers. Thus there is evidence that, under certain circumstances, some of the cells in the ependymal layer of normal adult vertebrates retain the ability to divide and produce new neurones and non-neuronal cells.

The ependyma plays an important role in the regeneration of spinal cord in lower vertebrates (Hughes & New, 1959; Kiortsis & Droin, 1961; Butler & Ward, 1965; Simpson, 1964, 1968, 1983). In the regenerating lizard tail, the presence of ependyma and contact with it have been shown to be necessary in order for spinal cord regeneration to proceed (Kamrin & Singer, 1955). In teleosts and amphibians (Anderson & Waxman, 1981, 1983a; Egar & Singer, 1972; Nordlander & Singer, 1978), ependymal cell mitoses generate the new neurones, glia and ependymal cells of the spinal cord that regenerates after amputation of the tail. In the teleost \textit{Apteronotus albifrons}\(^*\) (Black Ghost Knife fish) (Fig. 1), regeneration of new spinal cord can occur from ependyma at levels up to halfway rostral along the rostrocaudal axis of the fish (Anderson, Waxman & Laufer, 1983). Such a dramatic case of regeneration is possible because in this gymnotiform teleost all of the gut and major organs are located in the rostral third of the fish; the caudal two-thirds of the fish's length contains spinal column, electric organ and muscle. The regeneration of relatively complete spinal cord from rostral nongrowing ependyma in \textit{Apteronotus} indicates that histologically mature, stable ependyma can be stimulated by the amputation (injury) to generate a new ependyma, which in turn generates new neurones, glia and ependymal cells (Anderson \textit{et al.} 1983; Anderson, Waxman & Tadlock, 1984).

In \textit{Apteronotus}, a complete spinal structure, including new nerve cell bodies, neurosecretory cells, axons, and glial components, is regenerated after amputation of the tail (Anderson & Waxman, 1981, 1983a). As in regenerated spinal cord of amphibians and reptiles (Nordlander & Singer, 1978; Egar, Simpson & Singer, 1970), new cells in regenerating \textit{Apteronotus} spinal cord are produced from the ependymal layer in a time-dependent fashion. That is, there is a rostrocaudal gradient of cell production and of differentiation within the regenerating cord (Anderson \textit{et al.} 1983). New cells are generated from ependyma at the caudal extremity of the cord; differentiation proceeds in a rostral-to-caudal direction, so that the rostral portion of regenerated cord contains the 'oldest' and most differentiated tissue. In \textit{Apteronotus}, the processes of differentiation and morphogenesis within the regenerating tissue create a complete spinal cord: \textit{Apteronotus} cord that has regenerated for relatively long time periods (12–29 months) is indistinguishable in morphology from normal cord (Anderson \textit{et al.} 1984; Anderson & Waxman, 1985b).

These studies on regeneration demonstrate the capacity of the intact, adult ependyma of lower vertebrates to generate new spinal neurones and glia \textit{in vivo}

\(^*\) Formerly classified: \textit{Sternarchus albifrons}. 
Selforganization of ependyma

and to direct the organization of a new (regenerating) ependymal and spinal axis. However, several questions arise concerning the developmental potential of these ependymal cells and the relationship between the intact structure of the ependyma and the subsequent morphogenesis of the spinal cord produced during regeneration. One important question concerns whether development of regenerated ependymal structures depends on contact with the pre-existing ependymal tube, which could serve as a template or induction element, or can occur independently as a result of inherent properties of the stem cell population. Recent observations on regenerating spinal cord of *Apteronotus albifrons* have allowed us to address this issue and suggest that the cells generated by the ependyma in this species exhibit an inherent capability for selforganization into differentiated ependymal tubes in the absence of direct contact with a pre-existing ependyma.

**MATERIALS AND METHODS**

Adult *Apteronotus albifrons*, more than 12 cm in length, were used in this study. Spinal cord regeneration was initiated by amputation of the tail. Fish were first anaesthetized in a 1:15 000 solution of tricaine methane sulphonate (MS 222; ‘Finquel’, Ayerst Labs). After amputation of the tail with a razor blade, the fish was returned to its tank and maintained at 26–28°C. After the regeneration period (generally 3–12 months), the fish was reanaesthetized, the regenerated tail was cut off several mm rostral to the previous amputation site, and the spinal cord was dissected out in fixative.

The previous amputation site is clearly visible as a notch in the dorsal aspect of the fish’s tail (Fig. 1), accompanied by a 2–6 mm decrease in size of the tail along the dorsal–ventral axis. In addition, scales covering the regenerated portion of tail are half the size (or less) of the unregenerated scales. Internally at the amputation site there is a tough fibrous scar and the bone of the spinal column forms a very tough, unsegmented mass.

After the amputation the large blood vessels beneath the spinal column were held with a haemostat until bleeding stopped (30–120 s), then the fish was returned to its tank. Triple sulfa

Fig. 1. *Apteronotus albifrons* with 9-month regenerated tail. *Apteronotus* is a weakly electric gymnotiform teleost. The body cavity and organs are located in the first third of the fish, with the anus situated ventrally just behind the mouth. The tail and spinal column of this species readily regenerate in the wild as well as in the laboratory. The previous amputation site in this individual (arrow) is clearly visible as a notch in the top of the tail. Magnification reduced from life size by 30 %.
Fig. 2. Diagram of a 10-month regenerated cord and its subsidiary ependymas. This regenerated cord has subsidiary ependymas both rostral and caudal to a narrow middle section. In the rostral portion, four of the subsidiary ependymas (shown in detail in Figs 3, 4) are located close to the peripheral border of the cord and have no direct connection with the major ependyma. A fifth subsidiary ependyma is present, in continuity with the major ependyma. In the caudal portion of this regenerated cord, several pieces of spinal tissue split off from the major part of the cord. The subsidiary ependymas (1–3) which are observed in these offshoots of spinal tissue are shown in detail in Figs 6, 7.

(Dyna-Pet, Inc.; 100 mg per 5 gallon tank) was added to the tank water as a preventive; in only one case out of over 100 operations has an infection developed after tail amputation. The wound was not sutured because sutures usually pull out within hours, due to the constant undulating motion of the fish (and because it was not necessary). In this fashion, fish can repeatedly regenerate a new tail and spinal cord after repeated amputations. Some of the fish in our laboratory have completed four or five such amputation/regeneration cycles.

The results presented in this paper are based on detailed examination of three fish that had regenerated multiple ependymas. The cord presented in Figs 2, 3, 4, 6 and 7 had regenerated for 10 months and represented the third regeneration cycle for that fish (the tail had been cut and regenerated three times previously). The cord presented in Figs 5 and 8 was from a fish that had regenerated for 20 months and was in its fourth regeneration cycle. The third fish had regenerated for 12 months and was in its fourth regeneration cycle.

Spinal cord was dissected in a solution of 2-5% glutaraldehyde in 0-1 M-sodium phosphate buffer, with 0-08 M-sucrose (pH 7-3; 300 mOs). After dissection, the cord remained in fixative (at room temperature) for 2-3 h. The tissue was then washed in four changes of 0-1 M-phosphate buffer (with 0-08 M-sucrose) over a period of 1 h, and postfixed in 2% OsO4 in phosphate buffer without sucrose (1 h, room temperature). Tissue was washed for 2 h in 0-2 M-acetate-acetic acid buffer (pH 5) and stained en bloc with 2% uranyl acetate. Tissue was embedded in Epon–Araldite and observed with light microscopy and in a JEOL 100 CX electron microscope. In some cases, serial sections were taken, and serial thin sections were examined in the electron microscope.
Selforganization of ependyma
RESULTS

Multiple ependymas

Multiple ependymal tubes are sometimes observed within regenerated spinal cord of *Apteronotus*. These extra ependymal tubes have been studied in three individual fish, each of which was regenerating after three or more amputation/regeneration cycles (described in detail in Materials and Methods). Along the rostrocaudal axis in these regenerated cords, subsidiary ependymas were most frequently observed near the transition zone between regenerated and unregenerated cord (i.e. in rostral regenerated cord) and near the caudal tip. Among the three different cords, 25 separate subsidiary ependymas were observed. These could be subdivided into three groups: those which were clearly formed by budding from the major ependyma (five cases, observed in three different fish); those which appeared to be formed by selforganization (ten cases, observed in two different fish), and those which were of indefinite origin (ten cases, observed in three fish).

The diagram in Fig. 2 schematically depicts one regenerated cord and its subsidiary ependymas. This 10-month regenerated cord, from which Figs 3, 4, 6 and 7 were taken, extended for 4.5 mm. It represents the third cycle of amputation/regeneration for this fish, i.e. the tail had been amputated three times previously. Several subsidiary ependymal tubes were observed in the rostral portion of the regenerated cord, and several others occurred near the caudal tip. Of the extra ependymal tubes in the rostral regenerated cord, all occurred within the major axis of the regenerated cord. This was invariably the case with all the cords we examined: the subsidiary ependymas in rostral cord were located within the major part of the cord. In the caudal region of regenerated cord, however, subsidiary ependymas frequently occurred in pieces of spinal tissue that had separated from the major portion of the cord (as in Fig. 2), as well as within the major portion of the cord. The diagram in Fig. 2 shows the areas of cord from which subsequent figures were taken. It also shows a fifth subsidiary ependyma in rostral regenerated cord (not shown in the subsequent figures) which was formed by budding or direct splitting from the major ependyma.

Fig. 3A shows a transverse section from the rostral portion of the 10-month regenerated cord depicted in Fig. 2. Three subsidiary ependymas can be observed close to the peripheral border of the cord. One additional area (number 2 in Figs 3, 4) shows a subsidiary ependyma at a point approximately 55 μm rostral to this section. These four subsidiary ependymas are tube-shaped cavities which lie parallel to the longitudinal axis of the cord. At each end, these subsidiary ependymal tubes terminate in a group of undifferentiated cells. At the time examined, each of these rostral subsidiary ependymas is totally independent of connection with the main ependyma of the cord.

Examination by electron microscope (Fig. 3B) confirms that the subsidiary structures are true ependymas, having a lumen, typical junctional complexes, and cilia and microvilli extending into the luminal cavity. The ultrastructure of the cilia
Fig. 4A–D. For legend see p. 9
Self organization of ependyma

appears identical to that of normal cilia. The cilia in secondary ependymas emanate from typical basal bodies, and striated fibres can sometimes be observed projecting from the basal body further into the cytoplasm, as in normal ependyma. However, in contrast to normal cord where cilia are restricted to the ependymal cells, cilia in regenerating spinal cord are also observed at distances of up to 65 μm from the ependymal lumen. In regenerating cord, cilia are observed not only on the ependymal cells, but also on cells which contain dense-cored vesicles.

In Fig. 4, serial sections through the rostral region depicted in Figs 2, 3 show that the four peripherally located subsidiary ependymas are the tubular structures, running parallel to the longitudinal axis of the cord. The termination of each ependymal tube in a group of relatively undifferentiated cells with large nuclei can be observed (Fig. 4A–D,H). In a cross-sectional view of the cord, the peripherally located subsidiary ependymas in Fig. 4 are 140–180 μm from the major ependyma.

Self organization of ependyma

Fig. 5 shows the migration and reorganization of ependymal cells in a series of thick sections through a 20-month regenerated cord that represented the fourth regeneration cycle of that fish. In this regenerated cord, the ependymal layer, and presumably cells derived from it, stained more darkly with toluidine blue than the rest of the cells in the cord. In Fig. 5A, darkly stained cells can be observed migrating away from the ependymal layer. In a more caudal section (Fig. 5B), the darkly stained cells are condensed into two discrete groups near the periphery of the cord. Subsequent (more caudal) sections (Fig. 5C) show a central canal and typical ependymal structure within each group of darkly stained cells.

In a second series (Fig. 6), serial thin sections from the caudal region of the regenerated cord illustrated in Fig. 2 document in detail the existence of three subsidiary ependymas within tissue that has separated from the major spinal cord. These subsidiary ependymas have no physical contact with the major ependyma at the time when this cord was fixed. Moreover, serial sections rostral to the series in Fig. 6 (not shown) depict a migration of undifferentiated cells from the major ependyma to the areas of tissue that split off from the major part of the cord (and in which the subsidiary ependymas are located), very similar to the migration of cells depicted in Fig. 5.

In Fig. 6A, two areas of tissue are starting to separate from the major part of the cord. This tissue contains axons, undifferentiated cells and neurosecretory cells and processes. Neurosecretory tissue is normally found in caudal spinal cord of Apterorotus (Anderson & Waxman, 1983b), and other teleosts (Bern & Takasugi, 1983).
Fig. 5. Migration and reorganization of cells from the ependymal layer in a 20-month regenerated cord. This fish was in its fourth regeneration cycle. The series of thick sections (A to C) progresses in a rostral-to-caudal direction and covers a length of 270 μm along the longitudinal axis of the cord. Cells of the ependymal layer of this regenerated cord stained darker (with toluidine blue) than the other cells. In A, darkly staining cells (arrowheads) can be observed apparently migrating away from the ependymal layer. Edge of cord (arrow) is visible at upper left, in the same orientation as in B and C. In B, a section 230 μm caudal to A, where the cord is noticeably smaller in diameter than in A, two groups of darkly staining cells (arrows) are present near the dorsal border. In C, a section 40 μm caudal to B, a lumen (arrow) and ependymal structure are clearly discernible within each of the two groups of dark cells. Magnification ×295. Bar, 50 μm.

1962), as well as in regenerating *Apteronotus* spinal cord (Anderson et al. 1983). The splitting off of spinal tissue, especially neurosecretory tissue, is common in the caudal area of regenerating spinal cord of *Apteronotus*. Following the serial sections in the rostral-to-caudal direction (Fig. 6A–F), subsidiary ependymas are formed in the two pieces of spinal tissue marked 1 and 2 (Fig. 6C). More caudally (Fig. 6E), a third subsidiary ependyma forms in a third area of extraspinal tissue close to areas 1 and 2. These three subsidiary ependymas continue as well-defined ependymal tubes (Fig. 6F), parallel to the major cord, and extend to the end of the tail.

The organization of the subsidiary ependymal structures in Fig. 6 was also followed by electron microscopy (Fig. 7). In rostral sections (at the level of Fig. 6B), the first indication of ependymal structure is the appearance of cilia between the cells. Next (Fig. 7A), a radial array of junctional specializations and cilia is observed. Sections caudal to this (Fig. 7B–D) show that a lumen and the
Fig. 6A–C. For legend see p. 14
Fig. 6D–F. For legend see p. 14
Fig. 7. For legend see p. 14
typical ependymal structure are present. Due to the distance involved between the location of these subsidiary ependymas (Fig. 6, 7) and the major ependyma, their location in spinal tissue that has separated from the major portion of the cord, and the streaming of undifferentiated cells to these areas (more rostrally), we believe that these results strongly suggest that these subsidiary ependymas formed in the absence of contact with the major ependyma.

**Budding from the major ependyma**

The formation of subsidiary ependymas by budding or splitting from the major ependyma has also been observed in regenerating *Apteronotus* spinal cord. In these cases, a subsidiary ependymal tube forms directly from the major ependyma. It may remain in continuity with the major ependyma (as in Fig. 2) or may pinch off creating a separate ependymal tube. Five clear instances of the generation of subsidiary ependymas by budding have been observed in three individual fish. These instances occurred in both caudal and rostral regenerated cord.

**General observations on ependymal structure**

In both the major ependyma and in the secondary ones, differences in staining properties and electron density have been observed between ependymal cells. Some of the ependymal cells stain darker than average, while others stain much more lightly. Correspondingly, at the electron-microscope level, some of the ependymal cells have electron-dense cytoplasm, while others have cytoplasm which is....
Self organization of ependyma

Fig. 8. A section from the 20-month regenerated cord shown in Fig. 5, approximately 500 μm caudal to Fig. 5C. A group of lightly staining cells (arrows) is present in one area of the major ependyma. Other cells appear to be streaming from this to a subsidiary ependyma (sub) located at the peripheral border of this cord. Other undifferentiated cells with large nuclei (arrowheads) are also observed around the abluminal border of the ependyma. One cell with condensed chromatin (bold arrow), most probably in the process of mitosis, has bulged into the central canal. Magnification ×1050. Bar, 10 μm.

very electron lucent. Differences in the density of regenerating ependymal cells have also been noted by Egar & Singer (1972) in the rostral part of regenerating amphibian spinal cord. In several instances in regenerating Apteroneotus spinal cord, we have observed a group of lightly staining ependymal cells in continuity with other lightly staining undifferentiated cells that were streaming away from the major ependyma (Fig. 8). The differential staining properties of these ependymal cells may relate to the cellular processes that underlie the production of new cells, or may relate in some other way to differences in developmental potential or fate of these cells. Fig. 8 also shows a cell in the ependymal layer with condensed chromatin, which is most probably in the process of mitosis. The body of this apparently mitotic cell has bulged into the cavity of the ependymal lumen. This cell is located close to the focus of lucent ependymal cells at an apparent source of migration from the ependymal tube.

DISCUSSION

The results presented here show that multiple ependymas occur in spinal cord of Apteroneotus that is regenerating after a series of previous amputation/
regeneration cycles. Multiple ependymas form both in sections of tissue that have split off from the major spinal cord, and within the major spinal circumference itself. In addition, two modes of production of the secondary ependymas are suggested by the results presented in this paper: (1) budding or direct splitting from the major ependymal structure, and (2) selforganization within a group of undifferentiated cells. If confirmed, this capacity of ependymally derived cells to selforganize into a typical ependymal structure would demonstrate that these cells possess not only a wide range of potentialities with respect to differentiation (e.g. into various spinal cord neurones and glia), but also have extensive potential for morphogenetic organization.

Duplication of the spinal cord can occur spontaneously during embryonic development, and has also been observed in the regenerating tail of the lizard (Simpson, 1964). Duplication of the embryonic spinal cord can also be caused by various chemical or mechanical treatments (Jacobson, 1978). For example, duplication of the notochord and spinal cord in the zebrafish embryo can be induced by exposure to ethanol (Laale, 1971). In all of these cases, the spinal axis splits, producing two whole cords. In this respect, these processes may be qualitatively different from the process in regenerating Apterontus spinal cord, in which numerous subsidiary ependymas can form within a single spinal cord and a whole secondary cord may not result.

The proliferation of ependymal cells and the appearance of ependyma-like rosettes within the spinal cord have been observed in rat spinal cord after transection (Matthews, St. Onge & Faciane, 1979), and in several malignant conditions, including malignancies of the ependyma and subependymal layers (Rubinstein, 1972; Fu, Chen, Kay & Young, 1974; Azzarelli, Rekate & Roessmann, 1977). It should be emphasized that the important difference in the results from Apterontus presented here is that the ependymally derived cells in Apterontus are nonmalignant and that they form into complete ependymal tubes which exist in the correct orientation within the spinal cord.

The multiple ependymas in Apterontus spinal cord have been observed only in cases of repeated amputation and regeneration cycles. The occurrence of multiple ependymas in Apterontus only after multiple injuries implies that the previous amputations have had some effect on the uninjured part of the spinal cord (which is the source of new regeneration). This conclusion is consistent with earlier findings, which suggest that injury 'primes' the Apterontus spinal cord for neurogenesis (Anderson & Waxman, 1985a). So far, the mechanisms underlying the effects of injury are not understood. It is known that in other neuronal systems (McQuarrie & Grafstein, 1981) the process of creating a lesion (injury) can 'prime' the system so that a subsequent lesion will elicit faster regrowth. Thus the process of an initial injury affects not only the regenerating part of the system, but also the adjacent uninjured nerve.

It may also be noteworthy that the multiple ependymas are observed primarily in two locations along the rostrocaudal axis: in rostral regenerated cord near the transition zone (between regenerated and unregenerated cord), and near the
Selforganization of ependyma

caudal almost tip (where new tissue is being generated). It seems reasonable that extra ependymas might be generated at the transition zone, which is the initial site of active proliferation of cells, and which exhibits a morphology characterized by the absence of normal landmarks. Likewise, the caudal tip of regenerating cord remains an active site of cell generation throughout the regeneration process. Hence it, too, is a prime candidate for the generation of secondary ependymas. It is interesting that the newly formed ependymal structures can be located at abnormal sites, i.e. close to the periphery of the cord. The expression of normal ependymal morphogenesis appears to involve processes, e.g. the development of cell polarity, differential cell adhesion, etc., that do not strictly depend on the characteristics of the surrounding milieu.

Our findings emphasize the wide degree of capabilities of the cells produced by the adult teleost ependyma in response to amputation of the cord. They strongly suggest that ependymally derived cells in regenerating Apteronotus spinal cord can selforganize into complete ependymal tubes in the absence of direct contact with the (template of the) major ependyma. These results, we believe, point to the possibility that mammalian ependyma may also contain cells with a wide range of developmental potential if the appropriate modes of stimulation, or conditions to encourage their division and differentiation, can be found.

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