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Transplants of Schwann cell cultures promote axonal regeneration in the adult mammalian brain

(axotomy/central nervous system/extracellular matrix/neurite-promoting factors/peripheral nerve)

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ABSTRACT **Transplantation of embryonic brain tissue or mature peripheral nerves into the adult mammalian central nervous system promotes axonal regrowth from axotomized central nervous system neurons; however, the cellular origin and molecular nature of the factors promoting axonal growth *in vivo* are unknown. To further characterize cellular environments that facilitate regeneration of central nervous system axons, we developed a methodology whereby cultured cell preparations can be transplanted into the brain of mature mammals. For this procedure, lesions are produced in the septal-hippocampal system of adult rats, and selected regions from collagen-supported Schwann cell/neuron cultures (consisting of Schwann cells, extracellular matrix, and degenerating neuronal processes and myelin but devoid of neuronal perikarya and fibroblasts) are positioned within the intracephalic cavity so that they bridge the lesion gap (≈3 mm) separating the septum and hippocampus. At various times up to 3 weeks after transplantation, specimens were prepared for acetylcholinesterase histochemistry and the immunocytochemical localization of laminin (an extracellular matrix protein) and C-4 (a Schwann cell membrane antigen). All specimens (from uninjured controls and from animals with either acellular collagen or mature Schwann cell/extracellular matrix transplants) contained laminin immunoreactivity associated with the meninges, choroid plexus, ependyma, and cerebral blood vessels. All animals with transplants showed prominent laminin staining on astrocytic processes along the intracephalic cavity, but only the Schwann cell/extracellular matrix transplants exhibited dense laminin and C-4 immunoreactivity within the cellular portion of the transplants. Regeneration of acetylcholinesterase-positive septal fibers occurred only in animals containing Schwann cell/extracellular matrix transplants. By 6 days after transplantation, acetylcholinesterase-positive fibers were observed both on laminin-positive cellular tissue strands connecting the septum and the Schwann cell/extracellular matrix transplants and on the initial portions of the transplants. By day 14, acetylcholinesterase-positive fibers traversed the entire lesion cavity in intimate association with the laminin- and C-4-positive cellular layer of the transplants and reinnervated the host hippocampus. However, cholinergic fibers were not associated with all laminin-containing processes along the lesion cavity nor did they grow along acellular collagen transplants. These results indicate the presence of factors in transplants of cultured Schwann cells and their associated extracellular matrix that promote rapid regeneration of central nervous system cholinergic axons *in vivo*.**

It has long been recognized that functional axonal regeneration can occur in the adult mammalian peripheral nervous system (PNS) after peripheral nerve damage (1, 2). In contrast, little spontaneous axonal regeneration occurs following lesions in the mature mammalian central nervous system (CNS) (1, 3, 4). However, recent experimental studies utilizing intracephalic or intraspinal transplants of adult peripheral nerves or embryonic CNS tissue indicate that the cellular environment within the transplanted tissue facilitates axonal growth from a variety of mature CNS neurons within the recipient (5–10). Although classical studies (1, 2) have suggested that Schwann cells (Sc) may be an important component for promoting PNS axonal regeneration, the cellular and/or extracellular factors in PNS and CNS transplants that are responsible for the positive axonal regrowth observed from CNS neurons are still unknown.

Since peripheral nerves possess a relatively well-defined cellular environment composed primarily of axons, Sc, fibroblasts, and extracellular matrix (ECM), this tissue provides an excellent source of material for cell culture preparations that can be used for the isolation and identification of cellular or extracellular factors responsible for promoting axonal growth. Cell culture preparations have been employed to show that neurite growth *in vitro* is enhanced either by culturing neurons in an environment containing heart- or Sc-conditioned-medium factors bound to a culture substratum (11, 12) or by plating CNS neurons onto monolayers of Sc or astrocytes (13, 14). Experiments with nonmammalian vertebrate tissue (15) and predegenerated, frozen–thawed mammalian sciatic nerve (16) indicate that acellular preparations of peripheral nerve ECM also may provide a growth substrate for regenerating peripheral axons *in vivo*. Moreover, one specific ECM component, laminin, has been implicated as a neurite-promoting molecule for both PNS and CNS neurons in culture (17–20).

To successfully promote axonal regeneration in the adult mammalian CNS, it is important to extend the results obtained from *in vitro* preparations to a direct *in vivo* paradigm. Thus, the present study was undertaken to devise a methodology that would combine the use of *in vitro* PNS cell preparations, which can provide selected cellular fractions, with the technique of intracephalic tissue transplantation. By utilizing these combined procedures, it has been possible to demonstrate that transplants of mature cultures of Sc associated with ECM have a profound positive effect on axonal regeneration in the lesioned brain of adult mammals. Moreover, this study establishes a methodology whereby a variety of cellular components can be transplanted in close proximity to lesioned CNS axons in order to evaluate the influence of a specific cellular environment on *in vivo* axonal regeneration from mature CNS neurons.†

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Abbreviations: AcChoEase, acetylcholinesterase; CNS, central nervous system; ECM, extracellular matrix; PNS, peripheral nervous system; Sc, Schwann cell(s).

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†Preliminary reports of this study have been presented at the Fourteenth Annual Meeting of the Society for Neuroscience, Anaheim, CA (21, 22).

MATERIALS AND METHODS

Transplantation Surgery. All surgical procedures were carried out under aseptic conditions while the recipient animals (adult female Sprague–Dawley rats, Charles River Breeding Laboratories; 170–200 g) were maintained under general anesthesia (50 mg of ketamine/kg plus 10 mg of xylazine/kg, given intraperitoneally). During the preparations of the bilateral intracephalic transplantation cavity, the cingulate and parietal cortices overlying the hippocampal fimbria were carefully aspirated, to avoid injury to the superior sagittal sinus, and complete bilateral transections of the dorsal fornix, fimbria, and supracallosal stria were performed. This procedure produced a physical gap of 2–3 mm between the lesioned surfaces of the septum and hippocampus. Gelfoam (Upjohn) soaked in sterile saline solution was used to arrest vascular bleeding within the cavity prior to insertion of the transplants.

During the cavity preparation, the transplants (either an acellular collagen substratum or the non-neuronal portions of a mature Sc/neuron culture grown on a collagen substratum) were cut to an appropriate shape (about 1 × 3 mm) and transferred into the intracephalic cavity with a small spatula. The longitudinal collagen strips were then carefully positioned on the ependymal surface of the dorsal thalamus and oriented with their long axis in contact with the lesioned surfaces of the host septum and hippocampus. Subsequently, the cranial opening was covered with Silastic (Dow–Corning) and the skin was closed with wound clips. To ensure that there was sufficient transplant material to provide a continuous cellular bridge between the septum and hippocampus, some of the animals received a second set of bilateral transplants 5 days after the initial surgery. Thus, these animals possessed double transplants on each side of the brain with two different survival times.

Preparation of the Transplants. Collagen was obtained by acetic acid extraction of rat tail tendons and added to plastic minidishes as described (23). Each dish was coated with a single layer of collagen, which was subjected to ammonia vapor, air-dried, and subsequently covered with a second layer of collagen which was air-dried. This double-layered collagen substratum was rehydrated by the addition of complete medium (medium II, ref. 24) and utilized as control transplants or as a substrate for the cell cultures. The control acellular collagen substratum was incubated for 1 week prior to transplantation, in medium identical to that used for culturing Sc.

Preparations of mature Sc/dorsal root ganglia neuron cultures were obtained as described by Wood (24) and maintained in culture for more than 2 months. Briefly, dorsal root ganglia were dissected from fetal (E15) rats, placed on a double-layered collagen substratum, and incubated in the presence of an antimitotic medium for 1 week. On day 7, the central portion of the explant was extirpated, transferred to a new dish, and incubated in complete medium comprised of a balanced salt solution supplemented with chicken embryo extract and human placental serum (24). Cultures containing only Sc and sensory neurons matured so that the Sc completely differentiated to myelinate large neurites, ensheath smaller axons, and produce a robust ECM (25). Immediately prior to transplantation, the region of the explant containing the neuronal somata was excised and longitudinal strips containing parallel arrays of Sc, ECM, and degenerating neurites and myelin were dissected for transplantation.

Neuroanatomical Procedures. After the appropriate survival periods (1, 6, and 14 days or 14/19 days for double bilateral transplants) the recipient animals were anesthetized and perfused with 250 ml of 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed, equilibrated in 20% (wt/vol) sucrose/phosphate buffer overnight, and prepared for cryostat sectioning. Acetylcholinesterase (AcChoEase) histochemistry (26) was utilized to identify regenerating cholinergic axons within the transplants, since the localization of this enzyme is a good marker for septal cholinergic axons (9, 10). A neurofibrillar silver-staining protocol also was used to identify regenerating axons (27). Immunocytochemical techniques (28, 29) were used to identify the presence and location of two ECM components, laminin (antiserum obtained from Bethesda Research Laboratories) and fibronectin (antiserum supplied by Cappel Laboratories, Cochranville, PA), and to determine the presence of an antigen, C-4 (monoclonal antibody prepared by C.J.C.), specifically present only on Sc associated with ensheathed, unmyelinated axons (30).

RESULTS

Sc/Dorsal Root Ganglion Cultures. Sibling cultures to those used for transplantation were immunohistochemically stained with antibodies directed against fibronectin, laminin, and antigen C-4. The cultures did not stain for fibronectin, demonstrating that they were devoid of fibroblasts (29). Antilaminin immunoreactivity was present in the ECM surrounding both ensheathing and myelinating Sc (29, 30). Antibodies directed against C-4 recognized the surface of ensheathing Sc but not the surface of myelinating Sc (30).

Uninjured Control Specimens. These specimens were analyzed with immunocytochemical procedures to identify intrinsic regions of the rodent CNS that were positive for laminin or C-4. Antilaminin immunoreactivity was associated with several structures: (*i*) the surface meninges of the brain, (*ii*) blood vessels within the subarachnoid space and beneath the pia mater, (*iii*) the vasculature within the brain parenchyma,‡ (*iv*) the choroid plexus, and (*v*) the ependymal lining of the ventricles, which exhibited punctate staining along the basal surfaces of cuboidal ependymal cells and continuous staining along squamous ependymal cells. The monoclonal antibody C-4 did not stain any brain regions, including the meninges, choroid plexus, and ependyma.

Acellular Collagen Transplants. Specimens which received a bilateral fornix/fimbria transection and bilateral transplants of the acellular collagen substratum showed no evidence of regeneration of AcChoEase-positive axons between the septum and the hippocampus at 14 days posttransplantation. Moreover, no axons (identified with both AcChoEase histochemistry and neurofibrillar stains) were associated with the collagen transplants at any survival period examined (up to 3 weeks posttransplantation) nor were they present in the area immediately surrounding the transplants, which contained many cells and cellular processes (Fig. 1*a*). At 14 days posttransplantation there was little, if any, laminin-positive staining in juxtaposition to the collagen transplants (Fig. 1*b*). However, laminin-positive cells and cellular processes were clearly evident along some surfaces of the transplantation cavity (Fig. 1*c*). The laminin reactivity appeared to be associated, at least in part, with the choroid plexus, ependymal cells, and processes of reactive astrocytes that extended into the cavity and along the lesioned surfaces of the brain. Some antilaminin staining also was associated with blood vessels that were near the surface of the cavity, but no laminin immunoreactivity was evident deep within the brain parenchyma of paraformaldehyde-fixed tissue. Adjacent sections reacted for AcChoEase indicated that the few AcChoEase-positive fibers present along the cavity were not necessarily located in regions that exhibited positive laminin

‡Laminin associated with blood vessels deep within the brain parenchyma was visible only in fresh frozen tissues postfixed with acetone (4°C) for 10 min.

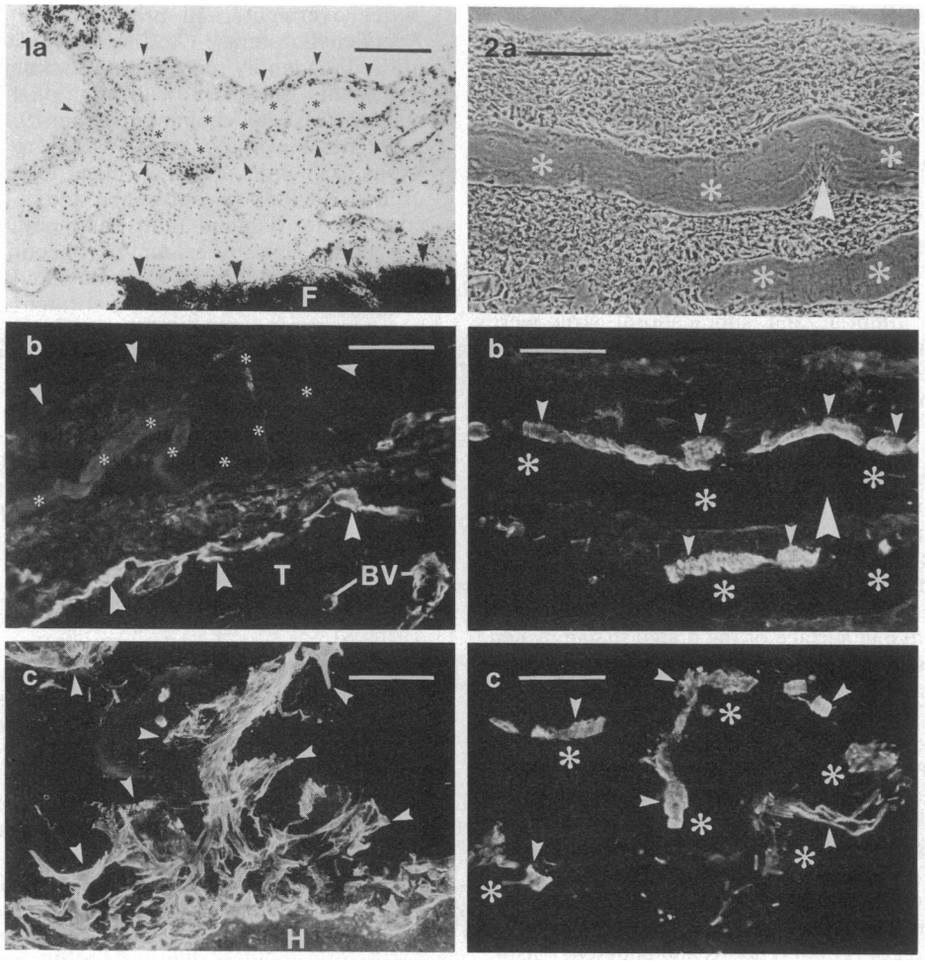

FIG. 1. Photomicrographs of specimens from a rat containing bilateral acellular collagen transplants (14 days after transplantation). (*a*) Histochemical staining for AcChoEase. Although a dense network of AcChoEase-positive fibers (F) is present beneath the lesioned surface (large arrowheads) of the host brain, no AcChoEase staining is present within either the collagen transplant (asterisks) or the cell matrix surrounding the transplant (small arrowheads). (Bar = 200 μm.) (*b*) Section stained for laminin. Immunoreactivity (large arrowheads) is present along the ependymal surface of the host thalamus (T) and along blood vessels (BV). Negligible laminin staining is present along the collagen transplant (asterisks) and within the cells that have migrated along the transplant (small arrowheads). (Bar = 100 μm.) (*c*) Section stained for laminin. This area along the surface of the intracephalic cavity contains an extensive matrix of laminin-positive cells and cellular processes (arrowheads) which emanate from the host brain (H). (Bar = 100 μm.)

FIG. 2. Photomicrographs from rats that received bilateral double Sc/ECM transplants (14/19 days after transplantation). (*a*) The two collagen substrata (asterisks) in this phase-contrast micrograph appear as amorphous strips which are surrounded by a dense matrix of cells and cellular processes. (*b*) Same section area as in *a* viewed with epifluoresence optics to visualize laminin immunoreactivity (small arrowheads) which is localized on only one surface of each collagen substratum (asterisks). Large arrowheads indicate same location in *a* and *b*. (*c*) In this fluorescence micrograph from a different rat, containing a fragmented double Sc/ECM transplant, C-4 immunoreactivity (arrowheads) is localized on cellular zones along the collagen substrata (asterisks). These same cellular zones exhibit laminin and AcChoEase staining in adjacent sections (not shown). (Bar = 100 μm in *a–c*.)

staining. No C-4 immunoreactivity was observed in specimens that received acellular collagen transplants.

Sc/ECM Transplants. Bright-field, phase-contrast optics were used to identify and localize transplants containing Sc within the intracephalic cavities. In these specimens, as in the control transplants, the collagen substratum appeared as an amorphous, acellular strip of material spanning the rostral–caudal extent of the cavity. This collagen substratum bordered a dense layer of Sc and ECM that was always associated with one surface of the collagen substratum (Fig. 2*a*).

Indirect immunohistochemistry was utilized to identify and localize antigens characteristically associated with the PNS. The antilaminin reactivity in the host CNS duplicated that seen in brains containing collagen transplants, but there was more extensive reactivity along the lesioned surfaces of the brain and the associated regions of the ventricles. In addition, an especially dense and conspicuous band of laminin immunoreactivity was intimately associated with the cellular portion of the Sc/ECM transplants at all time points examined (Fig. 2*b*). Moreover, laminin staining was present along cellular tissue strands that were located at the transplant–host transition zones. However, no laminin staining was present within glial or neuronal elements of the host septum or hippocampus. Antibodies directed against C-4 specifically recognized the band of cellular tissue that was associated with only one surface of the collagen substratum (Fig. 2*c*) in the 1- and 14/19-day survival-time specimens. At 6 days posttransplantation, occasional C-4 antibody staining was seen in the anterior end of the cellular transplants. On occasion, when the transplant was fragmented, both the laminin- and the C-4-positive areas were similarly disarrayed but continued to be localized to the identical cellular patches on the surface of the collagen fragments.

As early as 6 days posttransplantation, bundles of AcChoEase-positive fibers were observed in association with

thin cellular strands that traversed the interface between the host septum and transplant (Fig. 3*a*). These cellular strands also stained positively for laminin but lacked C-4 immunoreactivity. In some specimens the AcChoEase-positive fibers extended into the rostral portions of the Sc/ECM transplants. After a survival period of 14/19 days, a pronounced dense AcChoEase staining was associated with the cellular areas of the transplants but not within the collagen substratum (Fig. 3 *a* and *b*). These cellular regions of the transplant also exhibited laminin and C-4 immunoreactivity on adjacent sections. Correlation of the patterns of staining for AcChoEase, laminin, and C-4 on adjacent sections clearly indicated that there was extensive colocalization of these three molecules along the entire rostral–caudal extent of the transplant. In areas where there was disruption of the C-4 (Fig. 2*c*) or laminin pattern, AcChoEase staining was interrupted in an identical fashion. Staining of additional sections with the neurofibrillar method for axons also demonstrated the presence of fine fibers within the cellular layer but not the collagen substratum. At the caudal end of the transplant, AcChoEase-positive axons were observed to extend beyond the transplants for short distances along cellular tissue strands (which were laminin-positive in adjacent sections) to the host dentate gyrus (Fig. 3*c*). At 14 days after transplantation, the AcChoEase-positive fibers had already begun to innervate the dentate molecular layer, hilus, and areas of the host hippocampus bordering the transplant.

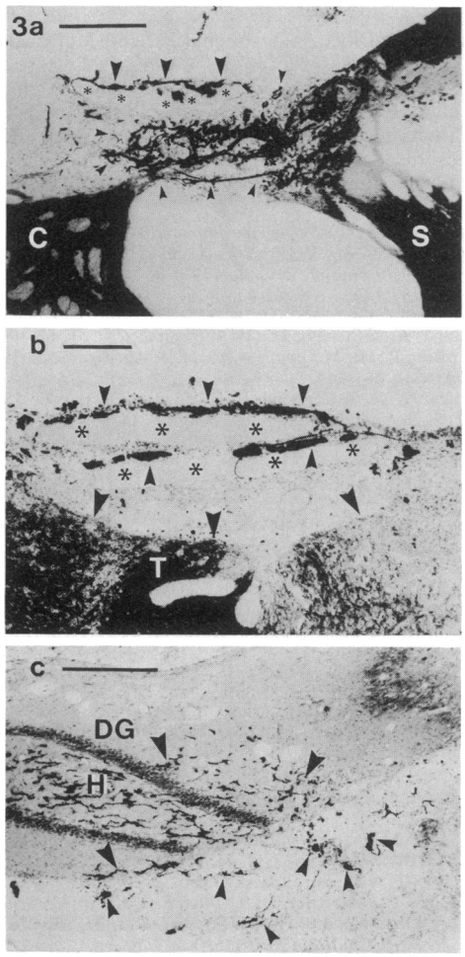

FIG. 3. Photomicrographs of sections from double bilateral Sc/ECM transplants (14/19 days after transplantation), showing staining for AcChoEase. (*a*) Transition zone between the anterior end of a Sc/ECM transplant (asterisks) and the injured surfaces of the host septum (S) and caudate nucleus (C). A dense plexus of AcChoEase-positive fibers (small arrowheads) that originates from the host septum is located within the cellular tissue strands that interconnect the transplant and host CNS. A densely packed band of AcChoEase-positive fibers (large arrowheads) also is present within the laminin- and C-4-positive cell layers along the dorsal surface of the collagen substratum. (Bar = 400 μm.) (*b*) Dense bundles of AcChoEase-positive fibers (small arrowheads) are specifically associated with discrete cellular areas along the dorsal surface of each collagen substratum (asterisks). Those regions that stain for AcChoEase also exhibit laminin and C-4 immunoreactivity in adjacent sections. At this mid-rostrocaudal level, the transplant is positioned above the ventricular surface (large arrowheads) of the host thalamus (T). (Bar = 200 μm.) (*c*) The rostral end of the host dentate gyrus (DG) is connected with the transplant (not present on this section) via cellular tissue strands that contain AcChoEase-positive axons (small arrowheads) that are continuous with those present in the transplant. These fibers enter the dentate neuropil where they form a loose fiber plexus (large arrowheads), especially within the dentate hilus (H). (Bar = 400 μm.)

DISCUSSION

The results show that components of mature Sc/neuron cultures can be successfully implanted and maintained within a lesion cavity in the CNS of an adult mammal. Moreover, these PNS constituents, supported on a collagen substratum, can be properly oriented so that they bridge a lesion-produced gap separating injured surfaces of the host brain, such as the septum and hippocampus. Transplants maintained within the intracephalic cavity for at least 3 weeks can be identified morphologically by the location of the collagen substratum and by immunohistochemical staining of specific markers for Sc (C-4) and ECM (laminin). The cultured Sc survive the transition from their *in vitro* environment to the CNS milieu of the adult recipient, as evidenced by their ability to resynthesize C-4 antigens after contacting regenerating CNS axons. In addition, at least a portion of the Sc-derived ECM is maintained, since selective areas of the transplants exhibit antilaminin reactivity.

A particularly significant finding is the ability of the Sc/ECM transplants to promote the rapid regeneration of CNS cholinergic axons from the host septum into the denervated host hippocampal formation. Within a 14-day period, AcChoEase-positive CNS fibers traverse the lesioned area in direct association with the cellular portion of the cultured transplants and reinnervate the hippocampus. The data indicate that the outgrowth of AcChoEase-positive fibers from the host septum onto the Sc/ECM transplants is initiated between day 1 and day 6 after transplantation. This is consistent with a previous study (9) suggesting that there is a lag period of ≈6 days before the septal cholinergic axons begin to regenerate. Prior to day 6 posttransplantation, C-4 immunoreactivity associated with the Sc disappears, suggesting that there are no viable axons within the transplant at this early time. This conclusion is based on the *in vitro* observation that C-4-positive Sc contacting neurites lose their C-4 immunoreactivity within 1 week after axotomy (unpublished observations). By 14 days posttransplantation, there is an extremely dense AcChoEase-positive fiber plexus within the cellular portions of the Sc/ECM transplants which extends the entire rostral–caudal length of the transplant. Neurofibrillar stained material also demonstrates the presence of axons in the cellular regions of these transplants. There is a concomitant return in the C-4 staining of the transplanted Sc. Immunohistochemical staining of the adjacent tissue sections indicates that there is extensive colocalization of the AcChoEase-positive fibers with those cellular regions of the transplants that also stained for C-4 and laminin. AcChoEase-positive fibers are observed to traverse cellular tissue strands (which are laminin positive but C-4 negative) that interconnect the transplant with the host septum and hippocampus.

All specimens that received bilateral aspiration lesions of the fornix/fimbria and supracallosal stria exhibited a rapid and extensive proliferation of laminin immunoreactivity along the injured surfaces of the brain. Our observations suggest that two cell types within the CNS, ependymal cells and reactive astrocytes, contribute to the laminin staining. In uninjured control specimens, the ependymal cells lining the cerebral ventricles normally are associated with recognizable levels of laminin immunoreactivity. However, after an aspiration lesion, the ependyma near the lesion site exhibit increased antilaminin reactivity. Within 24 hr of the aspiration lesion, reactive astrocytes along the injured brain surfaces also display antilaminin reactivity within their soma and processes. The laminin-positive staining is especially evident along the surface of the lesion and in association with blood vessels near the lesion interface. The ability of astrocytes to produce a laminin-like molecule after a traumatic aspiration lesion of the CNS is consistent with prior observations that reactive astrocytes transiently express laminin immunoreactivity after kainic acid lesions of the neostriatum (31).

To facilitate CNS axonal regeneration, it is essential to determine which cellular and noncellular environments serve as efficient axonal-growth-promoting substrates. Studies by Aguayo and colleagues (6, 8) show that peripheral nerve grafts to the CNS contain a favorable cellular environment for eliciting axonal growth from CNS neurons. At present, several cellular factors are candidates for promoters of axonal growth: (*i*) surface molecules on non-neuronal cells, Sc, and astrocytes (13, 14, 32, 33); (*ii*) soluble factors synthesized by supportive cells (glia) or by non-neuronal target cells (11, 12, 34–36); and (*iii*) ECM molecules including laminin and fibronectin (15–20, 36). Our results indicate that acellular collagen substrates incubated in complete medium do not provide an adequate terrain for rapid axonal regeneration. This is consistent with *in vitro* experiments that indicate that neurites exhibit slow growth on a collagen substratum compared to the growth exhibited when a variety of neurite-growth-promoting factors are present in the culture medium (33, 37). In contrast, long-term (90 days), large collagen bioimplants within the spinal cord are purported to promote regeneration of catecholamine-containing spinal cord axons (38). Since the origin of these fibers was not established, it is unclear whether they were CNS axons or PNS fibers that invaded the implant. Nevertheless, longer survival periods are required to determine whether some CNS axonal growth can occur over collagen transplants in the present paradigm.

Since the cultured cell preparations used in this study contain ECM, degenerating neurites and myelin, and viable Sc, it is not possible to determine which of these components promote rapid axonal growth *in vivo*. However, these transplant preparations are initially devoid of fibroblasts (24), indicating that the molecules associated with these cells are not necessary for CNS axonal regeneration. Thus, the experimental results are consistent with *in vitro* observations that Sc and/or matrix molecules produced by these cells facilitate axonal growth from CNS neurons (11–13, 17–20, 32–36, 39). Our initial observations suggest that laminin alone may not be a sufficient substrate for CNS axonal regeneration *in vivo* because numerous laminin-positive areas along the cavity surface lack associated AcChoEase-positive fiber outgrowth. Although laminin may promote neurite growth from CNS neurons *in vitro* (17–20), a combination of molecular elements may be required to promote CNS axonal regeneration *in vivo*. By analogy, recent evidence indicates that there is a marked enhancement of neurite outgrowth over a laminin substrate when nerve growth factor is also present in the culture medium (40). Cell surface molecules present on the transplanted Sc also are likely candidates as *in vivo* axonal growth promoters, since they appear to facilitate neurite outgrowth *in vitro* (13, 14).

In conclusion, cellular fractions obtained from PNS tissue and maintained in tissue culture produce neurite-promoting factors that facilitate a vigorous axonal regeneration from mature CNS neurons *in vivo*. Furthermore, this experimental paradigm, which utilizes intracephalic transplantation procedures and characterized tissue culture preparations, provides an approach for analyzing which cellular and extracellular components facilitate CNS axonal regeneration *in vivo*.

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