

In Vitro Analysis of Mechanisms Underlying Age-Dependent Failure of Axon Regeneration

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ABSTRACT

Severed axons of the inferior colliculus (IC) commissure can regenerate across a lesion in organotypic cultures from postnatal day (P) 6 gerbils, but this regenerative capacity is lost by P12 (Hafidi et al. [1995] *J Neurosci* 15:1298–1307, [1999] *J Neurobiol* 41:267–280). In the present study, we examined the mechanisms underlying this age-dependent failure of axons to regenerate. In P6–P12 heterochronic cultures, the P12 axons failed to cross the lesion site and project to the contralateral P6 IC lobe. In contrast, axons originating from the P6 lobe could regenerate through the lesion and invade the contralateral P12 IC lobe. To determine whether this age-dependent change in regenerative capacity can develop in organotypic cultures, IC slices with an intact commissure were obtained from P6 animals, grown in vitro for 6 days, and then lesioned at the commissure. In these slices, axon regeneration failure was similar to that observed in normal P12 tissue. Several in vitro treatments enhanced axon regeneration: removal of the entire midline region, inhibition of protein synthesis at the lesion site, and exposure to ABC chondroitinase. Furthermore, when the injured commissural axons were provided with a carpet of C6-R cells (a radial glia-like cell line), significantly more axons projected to the contralateral lobe of the IC. Taken together, these results suggest that the maturation of nonneuronal cells within the lesion site lead to failed axon regeneration in mature animals, and show that ameliorative strategies can be evaluated in vitro. *J. Comp. Neurol.* 470:80–92, 2004. © 2004 Wiley-Liss, Inc.

Indexing terms: repair; glia; injury; C6-R cells; chondroitin sulfate proteoglycan; commissure; cycloheximide

Adult mammalian central neurons display almost no regenerative capacity following injury. The inability of axons to regrow has been attributed, in part, to a cellular response that is elicited by the trauma. Glia have been directly implicated in this posttraumatic reaction (Silver, 1994; Ridet et al., 1998; Stichel and Muller, 1998; Qiu et al., 2000; reviews). Following an injury, astrocytic cells proliferate and become hypertrophic within the lesion site (Miyake et al., 1988; Wang et al., 1994; Kornyei et al., 2000; Liu et al., 2000). Furthermore, reactive astrocytes secrete extracellular matrix molecules such as proteoglycans, laminin, and other diffusible substances (Kliot et al., 1990; Rudge and Silver, 1990; Alonso and Privat, 1993; Irwin and Geisert, 1993; Levine, 1994; Fitch and Silver, 1997; Zuo et al., 1998; McKeon et al., 1999; Asher et al.,

2000). Chondroitin sulfate proteoglycans represent a major component of the brain extracellular matrix that are expressed by astrocytes within the lesion site and can

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inhibit neurite outgrowth in an in vitro assay (Levine, 1994; Smith-Thomas et al., 1995; Zuo et al., 1997, 1998; Lemons et al., 1999; Niederost et al., 1999; Asher et al., 2000; Snow et al., 2001).

Oligodendrocytes and myelin-associated proteins constitute a second class of growth inhibitory cues that may suppress axonal outgrowth (Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Shen et al., 1998; GrandPré et al., 2000; Chen et al., 2000; Fournier et al., 2001). Two principal molecules are implicated in the oligodendrocyte-dependent inhibition of axonal growth, Nogo and MAG (Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPré et al., 2000, 2002).

We described previously the behavior of cut axons in an organotypic culture of the inferior colliculus (IC) from animals of different postnatal ages. IC axons from postnatal (P) day 6 tissue display a robust regeneration following transection (Hafidi et al., 1995). However, the regenerative capacity is significantly diminished when tissue is obtained from P12 animals (Hafidi et al., 1999). Although transected P12 axons are able to grow, they lose the ability to extend across the lesion site (Hafidi et al., 1999). The goals of the present study were to determine whether the growth-inhibitory effect at the lesion site: 1) emerged with tissue age in vitro, 2) was dependent on endogenous properties of the axons, 3) was dependent on protein synthesis or proteoglycan secretion, and 4) could be overcome by the addition of a radial glial cell line (C6-R cells). The present data revealed that age-dependent failure of axon regeneration can emerge in vitro and can be ameliorated by either suppressing protein synthesis, removing proteoglycan activity, or providing a radial glia-like cell line. These results are consistent with the development of nonneuronal characteristics that prevent local outgrowth of severed axons.

MATERIALS AND METHODS

Organotypic cultures

Tissue from gerbils (*Meriones unguiculatus*) at P6 and P12 was used to generate organotypic cultures, as described previously (Stoppini et al., 1991; Hafidi et al., 1995). All protocols were reviewed and approved by the New York University Institutional Animal Care and Use Committee. Animals were anesthetized with hypothermia, alcohol sterilized, decapitated, and the brains were removed and placed in a cooled solution of minimum essential medium (MEM) containing 0.6 g/ml glucose. Vibratome sections of 250 μ m were collected in MEM at 4°C. Well-preserved slices were selected and the pial membrane was removed. Sections were transferred through several drops of the culture medium (50% MEM, 25% Earle's Balanced Salts, 25% horse serum; GIBCO-BRL, Gaithersburg, MD), and placed on 0.2 μ m filter inserts (Millipore, Bedford, MA) within the wells of a 24 chamber plate (Falcon, Lincoln Park, NJ). The culture medium was changed at 1–2-day intervals.

Three types of cultures were used in this study: 1) Complete bilateral slices of P12 IC were obtained and the entire commissure was severed at the midline, resulting in two pieces that were explanted side-by-side (Fig. 1A). These slices were cultured in either N2-defined medium (n = 16) or serum-containing medium (Hafidi et al., 1999;

n = 23). In some experiments (n = 14) a gelatin crystal was soaked in a 1 nM solution of cycloheximide and placed in contact with the lesion site. The cycloheximide-containing gelatin was immediately placed in contact with the cut midline lip of each IC lobe and left in place for 30 seconds. The crystal was then removed, the slices rinsed in fresh media, and the two lobes placed in contact with one another. Pilot studies indicated that longer cycloheximide exposure times resulted in neuron cell death. Therefore, the concentration and exposure period were adjusted to below toxic levels. In another set of experiments (n = 27), chondroitinase ABC (0.1 or 1.0 U/ml) was added to the culture medium. All P12 slice cultures were grown for 7 days in vitro (DIV) prior to fixation. 2) Unilateral slices of P6 and P12 IC were obtained and the lobes cocultured side-by-side, either in serum-containing medium (n = 7) or N2 medium (n = 15). All P6–P12 slice cultures were grown for 7 DIV prior to fixation. 3) Complete bilateral slices of P6 IC with an intact commissural projection were explanted and grown for 6 days. The entire commissure was then severed in vitro and the slices were grown for an additional 6 days in either serum-containing medium (n = 11) or N2 medium (n = 14) before fixation. In experiments that were performed with tissue from a single postnatal age, some cultures were generated from the same animals while other cultures were generated from two different animals. All cultures were grown for 7 DIV prior to fixation.

The survival of P12 cultures was shown previously to require the presence of glutamate receptor antagonists for the first days of explantation (Hafidi et al., 1999). For this reason the growth medium was supplemented with the ionotropic glutamate receptor antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione (100 μ M CNQX, RBI, Natick, MA) and D,L-2-amino-5-phosphonovaleric acid (100 μ M AP5, RBI) during the first 2–3 DIV for P12 cultures or during the 24-hour period following an in vitro commissural lesion.

All axonal projections were visualized using an anti-neurofilament (NF) antibody, as described previously (Hafidi et al., 1999). The NF immunoreactivity revealed axons and a few neuronal cell bodies. At the termination of each experiment, slices were immersion-fixed for 1 hour in 4% paraformaldehyde in 0.12 M phosphate-buffered saline (PBS, pH 7.3 \pm 0.1). Tissue was then rinsed in PBS and incubated in 1% H₂O₂. After several PBS washes, the free-floating sections were incubated in 5% goat serum for 30 minutes and transferred to primary antibody (mouse anti-neurofilament, provided by Dr. D. Dahl, Boston) overnight at 4°C. Slices were washed in several changes of PBS and then incubated in a secondary antibody (goat antimouse, Sigma, St. Louis, MO). Sections were again washed in PBS and incubated in avidin-biotin-horseradish peroxidase complex (ABC-HRP; Vector Labs, Burlingame, CA) for 2 hours. The tissue was washed in 0.1 M Tris-buffered saline (TBS), incubated in diaminobenzidine (DAB; 50 mg/100 ml TBS, pH 7.3 \pm 0.1) for 15 minutes, and then reacted in a DAB solution with 0.03% H₂O₂. Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped with permount.

C6 and C6-R cell line

The generation and characterization of a subclone of the rat C6 glioma cell line (C6-R), with radial glia-like properties has been described previously (Friedlander

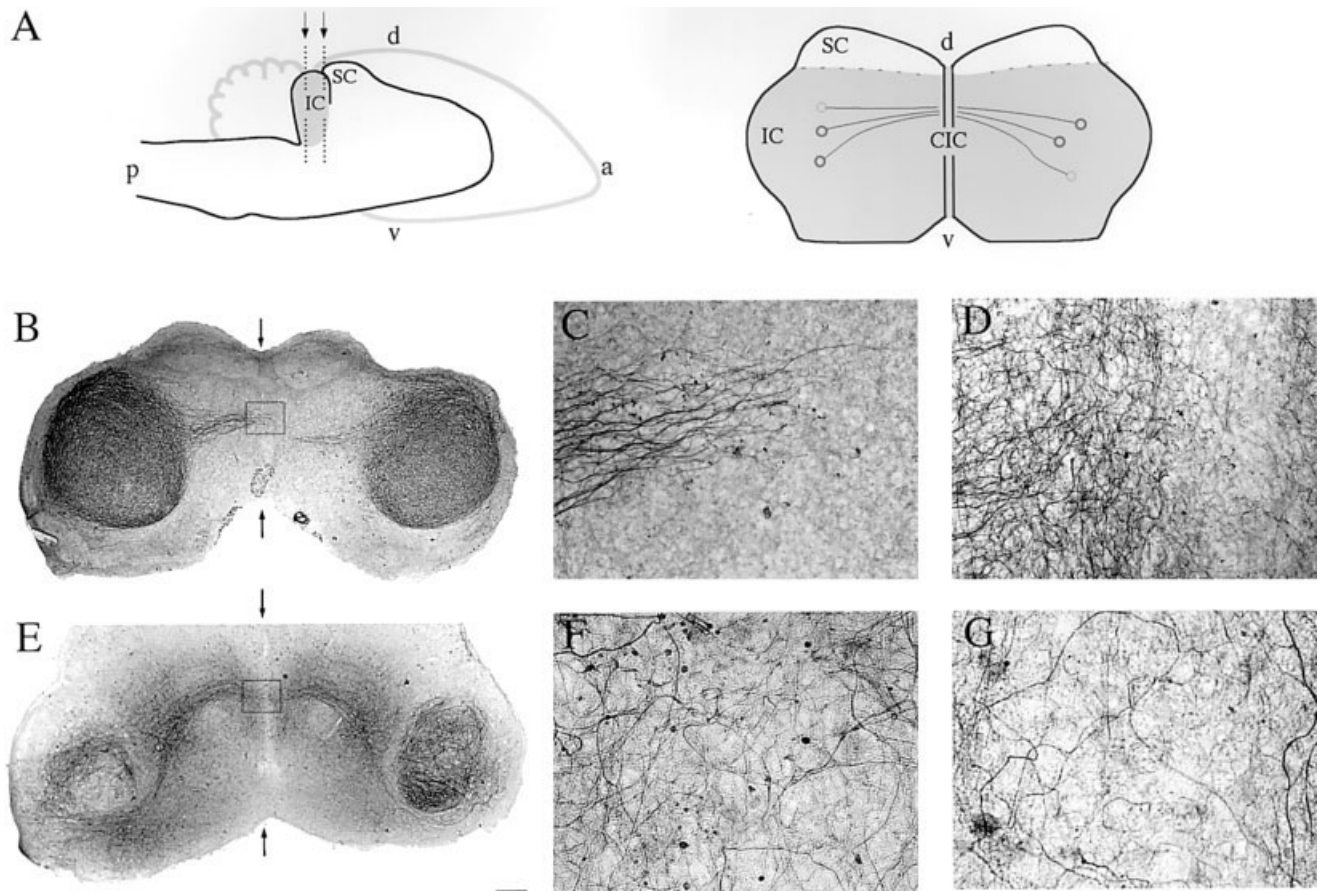


Fig. 1. **A:** Schematic of the experimental preparation. To the left is a sagittal view of the brain showing the position of the inferior colliculus (IC) and the rostrocaudal position of the transverse section that is obtained for organotypic cultures. To the right is a transverse section of the explanted tissue showing the commissural pathway (CIC) between the two lobes of the IC. SC, superior colliculus; a, anterior; p, posterior; d, dorsal; v, ventral. **B–G:** A comparison of P12 IC brain slices with a transected commissure following culture in serum-containing medium (B–D), or N2-defined medium (E–G). Arrows indicated the plane of transection. B: In serum, neurofilament labeling was observed in axonal projections within each lobe of the IC

as well in severed axons at the commissure. At high magnification (C) of the lesion site (square area in B), no axons are seen to cross the lesion site. D: A second example of the lesion site from a transected P12 IC slice grown in serum-containing medium is shown. E: In N2-defined medium, axonal staining was similar to that observed in serum. F: At high magnification of the lesion site (square area in E), several axons were observed to cross the lesion site. G: A second example of transected axons that grew across a lesion site in N2-defined medium. Scale bar = 200 μ m in E (applies to B, E); 20 μ m for C, D, F, G.

et al., 1998). C6 and C6-R cells were plated at 3×10^4 /ml and grown for 5–6 days. They were then exposed to a solution of trypsin/EDTA for 5 minutes to detach the cells from the Petri dish. The reaction was stopped by adding a 10% fetal calf serum-containing DMEM. The cells were then centrifuged and the pellet was resuspended and passaged a second time or used for an IC culture experiment. Three types of experiments were performed: 1) Following transection of the CIC, a pellet of C6-R cells was placed within the lesion site as a bridge between the two lobes of P12 IC. The glioma cell line separated the two edges of the injury site by 100–300 μ m. 2) A pellet of C6-R cells was placed on top of the IC slices at the lesion site. 3) Slices were placed on top of a “carpet” of C6-R cells such that axons at the bottom of the slice were in direct contact with the glioma cell line.

GDNF

We previously showed that neurotrophins did not have any trophic effect on IC neurons in culture (Hafidi et al., 1999). Since GDNF and GDNF-receptors are expressed in the IC at P12 (Hafidi, unpubl. data), we chose to examine the efficacy of GDNF (20 ng/ml) for P12 cultures to determine whether it might enhance regrowth through the lesion site.

Quantitative analyses

NF-stained axons were easily identified in cultures and single axons could be followed over long distances. This permitted very reliable counts of axons within the lesion site. Axons were visualized at the injury site in all cultures at 500–1,250 magnification (40–100 \times objectives, 1.25 \times microscope body magnification factor, and 10 \times ocular objectives). We evaluated the crossing axons along

TABLE 1. Number of Axons Crossing the Lesion Site under Each Experimental Condition

Culture conditions	Slices	Crossing axons ¹	Comparison to P12 in serum	Comparison to P12 in N2
P12 (serum)	23	0.7 ± 0.9	—	$P < 0.0001$ (t = 5.11; df = 37)
P12 (N2)	16	9.6 ± 8.3	$P < 0.0001$ (t = 5.11; df = 37)	—
P6–P12 (serum)				
P6 axons	7	5.6 ± 3.4	$P < 0.0001$ (t = -6.66; df = 28)	$P > 0.05$
P12 axons		0.7 ± 1.0	$P > 0.05$	$P < 0.02$ (t = 2.76; df = 21)
P6–P12 (N2)				
P6 axons	15	7.9 ± 5.3	$P < 0.0001$ (t = 6.45; df = 36)	$P > 0.05$
P12 axons		1.7 ± 1.4	$P > 0.05$	$P < 0.002$ (t = 3.61; df = 29)
P6 + 6DIV (serum)	11	2.3 ± 2.3	$P > 0.05$	$P < 0.01$ (t = 2.81; df = 25)
P6 + 6DIV (N2)	14	5.2 ± 6.9	$P < 0.005$ (t = -3.13; df = 35)	$P > 0.05$
P12 midline removal (serum)	8	8.4 ± 5.8	$P < 0.0001$ (t = 6.36; df = 29)	$P > 0.05$
P12 midline removal (N2)	8	12.8 ± 6.8	$P < 0.0001$ (t = 5.99; df = 29)	$P > 0.05$
P12 Cycloheximide (serum)	14	6.2 ± 5.8	$P < 0.0001$ (t = 4.55; df = 35)	$P > 0.05$
P12 0.01U Chon (serum)	4	6.5 ± 2.6	$P > 0.05$	$P > 0.05$
P12 0.01U Chon (N2)	5	5.2 ± 5.3	$P > 0.05$	$P > 0.05$
P12 1.0U Chon (serum)	8	5.8 ± 4.9	$P < 0.0001$ (t = 4.89; df = 29)	$P > 0.05$
P12 1.0U Chon (N2)	12	6 ± 2.9	$P < 0.0001$ (t = 8.18; df = 33)	$P > 0.05$
P12 C6-R carpet (serum)	26	12.5 ± 9.5	$P < 0.0001$ (t = 5.91; df = 47)	$P > 0.05$
P12 C6-R pellet (serum)	18	4.2 ± 3.8	$P < 0.0001$ (t = 4.37; df = 39)	$P < 0.05$ (t = 2.84; df = 32)
P12 GDNF (serum)	7	9.7 ± 7.5	$P < 0.0001$ (t = 5.88; df = 28)	$P > 0.05$

¹X ± SD.

the entire length of the lesioned midline. Only axons that regrew and crossed into tissue from the second lobe were included in counts of crossing axons. A single observer performed all of the measurements. The number of axons in control P12 tissue was taken from previous work (Hafidi et al., 1999). Montages of cultured slices or individual axons were generated in Adobe PhotoShop from individual JPEG images.

RESULTS

Regeneration improved in serum-free medium

To evaluate whether serum promoted the growth inhibition previously observed in P12 cultures (Hafidi et al., 1999), a set of explants was grown in N2-defined medium. Slices were transected at the midline and placed in vitro with the cut edges in contact (Fig. 1A). Cultures grown in serum-containing medium displayed very few stained axons crossing the lesion site (Fig. 1B–D, republished from figs. 3B,C in Hafidi et al., 1999). The average number of crossing fibers in these cultures was 0.7 axons per slice (n = 23) (Hafidi et al., 1999). As noted previously, some axons terminated as varicosities at the lesion site, while others grew along the lesion site or grew back into the ipsilateral lobe of the IC (Hafidi et al., 1999).

P12 cultures grown in serum-free N2 medium exhibited NF-stained axons within both lobes of the slice. At the commissure lesion site considerably more axons were found to have crossed the midline as compared to cultures grown in serum (Fig. 1E–G, Table 1). However, many commissural axons continued to grow along the lesion without crossing it. The average number of crossing fibers was 9.6 axons per slice (n = 16), although there was a large amount of variability between cultures (from 1–26 axons per slice).

Regeneration in heterochronic explants

To determine whether the failure of axons to regenerate across the lesion depended on the postnatal age of the projecting axons, heterochronic cultures were generated. One lobe of P6 IC was placed in contact with one lobe of P12 tissue and grown for 6 days. The experiment

was performed in both serum-containing and serum-free media. NF staining revealed axons crossing between the two lobes, but the two lobes displayed quite different axonal morphologies. P6 axons were usually thinner than P12 axons and it was possible to distinguish between the regenerating axons from each lobe. As shown in Figure 2, P6 axons were able to grow through the lesion and into P12 IC tissue. However, axonal regeneration through the injury site was not identical to that observed previously in P6 cultures, where many tens of axons were observed coursing along the entire length of the lesion (Hafidi et al., 1995). In heterochronic cultures the P12 axons generally failed to cross the lesion site and behaved in a similar fashion to lesioned axons in P12 slices (Hafidi et al., 1999). The thicker P12 axons either terminated as large varicosities or grew within the ipsilateral IC (Fig. 3).

P6 axons could be followed from the lobe that they emerged from and into the contralateral P12 lobe. As shown in Figures 4 and 5, individual P6 axons from images obtained with a 100× objective were reconstructed over more than 2 mm. The reconstructions begin within the P6 lobe and document the axons as they cross of the severed midline and grow well into the opposite P12 lobe. In Figure 4 the neurofilament stain included the P6 soma. In both Figures 4 and 5 a high-magnification image of the severed P12 commissural axons again document the absence of growth potential. The difference between P6 and P12 axon behavior was observed in the presence or absence of serum. The results indicate that P6 axons are relatively refractory to growth-inhibitory cues as compared to P12 axons (Table 1).

Age-dependent failure to regenerate developed in vitro

To determine whether IC tissue matured in vitro such that the ability of axons to regenerate through the site of trauma was lost, P6 slices with an intact commissure were grown initially for 6 DIV. After this period of in vitro maturation the commissure was severed and the slices were grown for an additional 6 days (until 12 DIV). Since serum was shown to foster growth inhibition at the lesion site (Fig. 1), two culture conditions were employed: In one

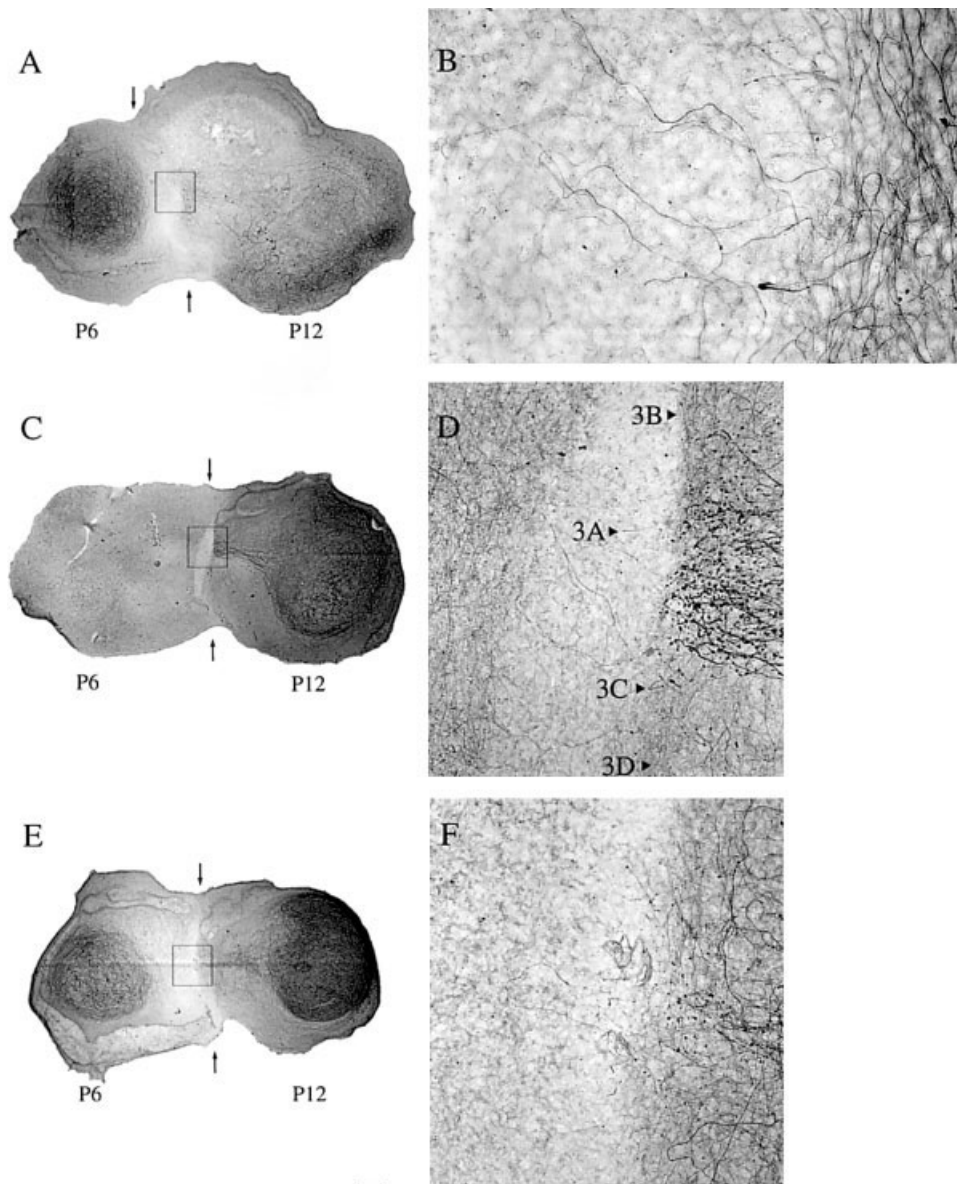


Fig. 2. Three examples of heterochronic P6-P12 cocultures are shown. In each culture the P6 IC lobe is positioned on the left and the P12 IC lobe is situated on the right side of the culture. **A,C,E:** Neurofilament stained fibers were observed in both the P6 and P12 IC lobes. **B,D,F:** High magnification of the lesion sites show stained axons at the site of injury. The major difference between P12 and P6 axons was that the latter were generally thin. Many thin axons originating from the P6 IC lobe were observed to cross the lesion site. In contrast, the thicker P12 axons were observed to stop at the site of injury, or to grow back within the ipsilateral lobe. **D:** Labels and arrowheads indicate the P12 axons that are shown at higher magnification in Figure 3. Scale bar = 200 μ m in E (applies to A, C, E); 20 μ m for B, D, F.

set of experiments, serum was kept in the medium during the entire in vitro period. In the second set, the serum was replaced with N2 when the in vitro commissural lesion was performed at 6 DIV.

For serum-containing medium, there was a complete absence of stained axons within the lesion site when axons were severed in vitro (Fig. 6A-C). As with P12 tissue, axons could be found terminating at the lesion site or sending collaterals along the lesion site without crossing. There were a total of 25 crossing axons in 11 slices, and in the best case 7 axons were observed to cross the lesion of a slice preparation (Table 1).

When serum-free medium (N2) was substituted at the time of lesion (6 DIV), NF-positive axons were subsequently found to cross the lesion site (Fig. 6D-F). Some of these axons could be followed within the contralateral IC central nucleus. There were 99 axons that crossed the injury site in 14 slices. Although defined medium in-

creased the number of crossing axons at the lesion site in these cultures, the difference did not reach significance ($P > 0.05$).

Removal of midline nonneuronal tissue improved regeneration

To assess whether the preexisting cellular environment at the midline was involved in the failure of axon regeneration, the entire midline region was cut away from P12 IC tissue prior to explantation. The two lobes of the IC were placed in culture such that the gray matter was in contact (Fig. 7). These cultures were grown in either serum-containing or N2 defined medium. Under both conditions regeneration was superior to that observed in P12 slices with the midline left intact (Table 1). The number of fibers crossing the injury site was 8.4 per slice in serum-containing medium and 12.8 axons per slice in serum-free medium. There was no significant difference between

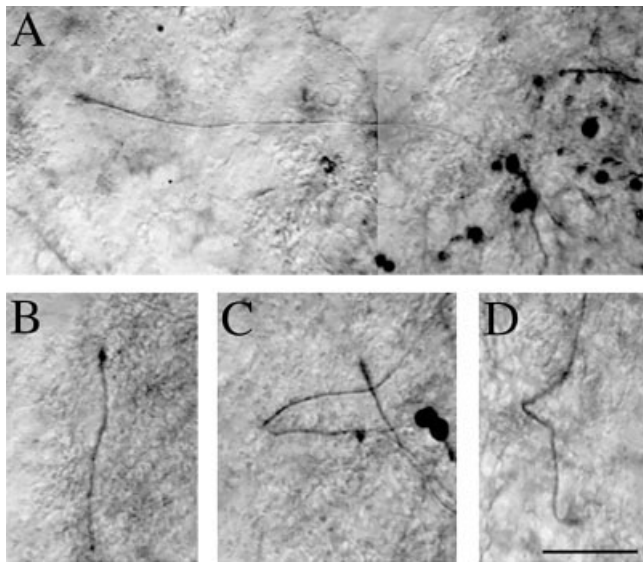


Fig. 3. High-magnification images of severed P12 axons from Figure 2D are shown. **A:** Severed P12 axons generally terminated in large varicosities or terminal bulbs, as shown to the right of this panel. One P12 axon has grown about 40 μm past the population of severed axons and into the lesion site. However, it does not cross the lesion. **B:** A severed P12 axon is observed to grow parallel to the lesion site, which is to the left. **C:** A severed P12 axon is observed to grow in the direction of the lesion, but turn around and grow back into the same side. **D:** A severed P12 axon is observed to grow parallel to the lesion site, which is to the left, and turn back towards the side that it originates from. Scale bar = 10 μm .

these values ($P > 0.05$). In serum, the presumptive regenerating axons were found to have extended around the lesion site, even if they did not cross. In this sense, they displayed greater potential for growth than P12 severed axons that received no further treatment (Fig. 1).

Cycloheximide treatment improved regeneration

Since the removal of midline tissue improved regeneration, we examined whether the synthesis of growth-inhibitory molecules was involved. P12 cultures with a CIC transection were treated with the protein synthesis inhibitor cycloheximide, placed in a gelatin crystal at the lesion site. All cycloheximide-treated cultures were grown in the presence of serum. As shown in Figure 8, most regenerating axons were very thin following cycloheximide exposure. Those axons that were able to regenerate displayed a rather direct trajectory and did not extend axons around the lesion site before crossing. However, there was an increase in the number of crossing fibers (6.2 axons per slice), as compared to untreated tissue (Table 1).

Chondroitinase treatment improved regeneration

To assess whether the failure to regenerate was due to the expression of proteoglycans, P12 cultures with a severed midline were treated with chondroitinase ABC. Cultures were grown in the presence of two different concentrations of chondroitinase (0.01 or 1.0 U), and this treatment was examined in both serum-containing and serum-free medium (Table 1). The axons were generally

thinner when grown in chondroitinase, as compared to untreated cultures (Fig. 9). Furthermore, the midline lesion site (i.e., nonneuronal area between cut axon tips) was much narrower than in untreated cultures. In some cases there was almost no space between the cut terminals from each lobe. In both serum-containing and N2 media the regenerating axons were found to have either extended minimally around the lesion site or crossed the lesion and grew within the contralateral IC lobe. At a quantitative level, chondroitinase ABC treatment was found to improve axon regeneration across the lesion site and the results were similar for both concentrations (Table 1). Outgrowth was also independent of serum- or N2-containing media, suggesting that CSPGs mediate the growth-inhibitory effect of glia. When all chondroitinase-treated cultures were pooled a mean of six axons per slice was found to cross the lesion ($n = 12$).

C6 and C6-R cell line bridges improved regeneration

To assess the growth potential of severed axons, C6 or C6-R cells were placed at the lesioned midline of P12 cultures. In each of these experiments, the glioma cells did not invade the tissue, but rather grew and spread on the top of it. When the C6 or C6-R cell line was placed on top of the slice at the lesion site, or employed as a carpet beneath the slices, many stained axons were found to grow associated with the cell line (Fig. 10), and an average of 12.2 (C6) and 12.5 axons per slice (C6-R) crossed to the contralateral lobe and entered the IC (Table 1). When the C6 or C6-R cell line was placed as a bridge between the two IC lobes, the stained axons displayed three different behaviors. Most axons failed to cross, while some axons crossed the first lip of the lesion, entered the C6 or C6-R bridge, and crossed into the contralateral IC. The number of crossing fibers was 4.1 (C6) or 6.2 axons per slice (C6-R). However, a number of axons crossed the first lip of the lesion, entered the C6 or C6-R bridge, and continued to grow there without crossing to the contralateral side. The number of crossing axons was significantly greater in experiments where a carpet of C6 or C6-R cells was employed as compared to those in which C6 or C6-R cells were placed between the IC lobes (C6-R carpet vs. bridge: $P < 0.002$ ($t = 3.47$; $df = 42$)). This suggests that increased surface area between the IC and C6 or C6-R cells provides a greater opportunity for growth past the lesion site.

GDNF treatment improved regeneration

A member of the transforming growth factor beta, glial cell line derived neurotrophic factor (GDNF) has been shown to have strong trophic and neurite extension actions on a variety of neuronal structures (Airaksinen and Saarma, 2002, for review). Therefore, GDNF was added to the culture medium of P12 slice cultures to determine if it would enhance regrowth of severed axons at the lesion site. As shown in Table 1, an average of 9.7 axons per slice crossed the lesion, and this was significantly greater than control P12-lesioned cultures.

DISCUSSION

This study revealed that the age-dependent failure of axons to regenerate was due, in part, to growth inhibitory factors produced at the midline. When heterochronic P6/

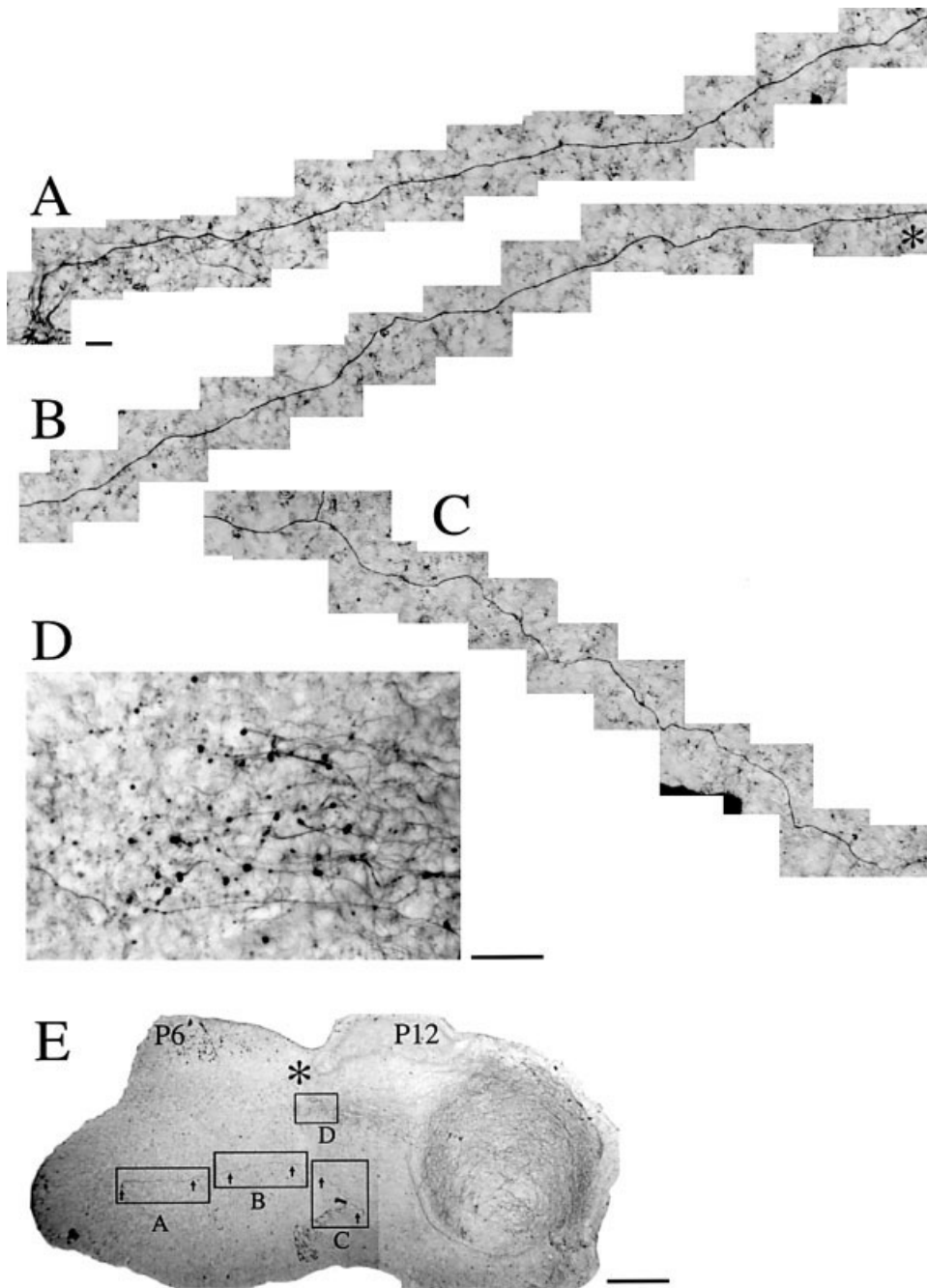


Fig. 4. A heterochronic P6-P12 coculture in which a single P6 axon is reconstructed. **A-C**: A P6 axon was reconstructed from images obtained with a 100 \times objective and is presented in three segments. The soma is present at the left side of the first row. The lesion site is marked by an asterisk (*) to the right side of the second segment. **D**: An image of the severed P12 axons showing that they do not extend into the lesion, but end in varicosities. **E**: The position of each panel is indicated by the boxes in a low-magnification image of the section. Scale bars = 20 μ m in A; 50 μ m in D; 500 μ m in E.

P12 explants were prepared, it was found that severed P6 axons were better able to cross the lesion than P12 axons in the same cultures. This suggests that the younger axons have not yet expressed one or more receptors that mediate growth inhibition. The putative growth inhibitory factors can be partially overcome by removing the midline nonneuronal tissue, treating the tissue with a protein synthesis inhibitor, or treatment with an enzyme that cleaves chondroitin and dermatan sulfates. Finally, the addition of a nonneuronal cell line with radial glia-like properties (C6-R) and a glial cell line (C6) were able to support a greater regenerative response from the severed

axons. These experiments demonstrate that regeneration can be studied experimentally *in vitro* and provide strong evidence for a local growth inhibitory cues at the site of a CNS injury.

Age-dependent failure of axon regeneration

We have shown previously that P6 axons have the capacity to regenerate through a lesion site and make synaptic contacts in a target population (Hafidi et al., 1995). When cultured under identical conditions, P12 tissue fails to regenerate axons through the injury site (Hafidi et al., 1999). In the present study, coculture of P6 and P12 IC

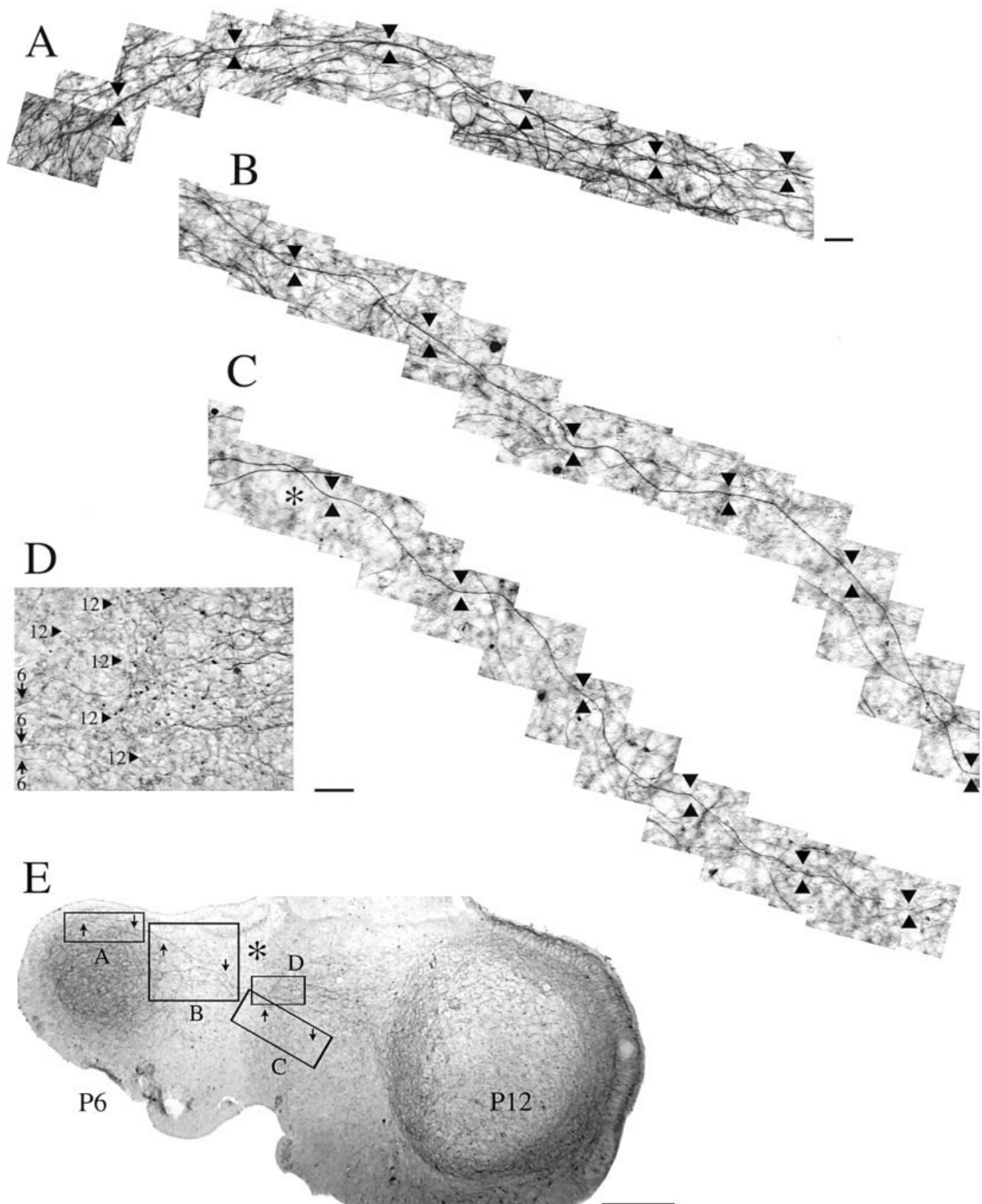


Fig. 5. A heterochronic P6-P12 coculture in which a single P6 axon is reconstructed. **A-C:** A P6 axon was reconstructed from images obtained with a 100× objective and is presented in three segments. The lesion site is marked by an asterisk (*) to the left side of the third segment. **D:** An image of the severed P12 axons (12) showing that they

do not extend into the lesion, but end in varicosities. In contrast, three P6 axons (6) are observed to grow into or beneath the severed P12 commissure. **E:** The position of each panel is indicated by the boxes in a low-magnification image of the section. Scale bars = 20 μm in A; 50 μm in D; 500 μm in E.

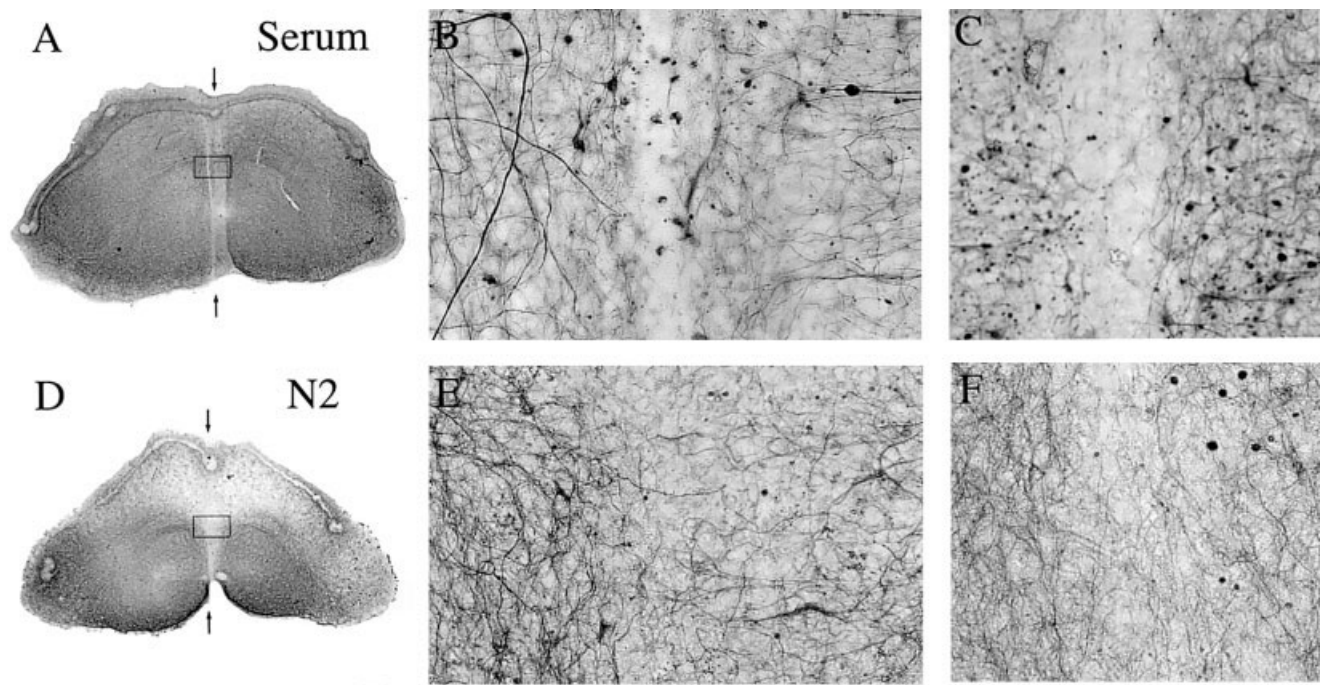


Fig. 6. Tissue from P6 animals was cultured for 6 days in vitro, followed by transection of the CIC fibers at the midline, and then grown for an additional 6 days. **A:** A culture grown in serum-containing medium is shown. **D:** A culture grown in N2 medium is shown. **B,C:** At high magnification, neurofilament-positive fibers were found to stop within the lesion site and form end bulbs in serum. No

axons were observed crossing the injury site in these cultures. **E,F:** Slices grown in N2-defined medium are shown at high magnification and many neurofilament-positive axons were observed to cross the lesion site. Scale bar = 200 μ m in D (applies to A, D); 20 μ m for B, C, E, F.

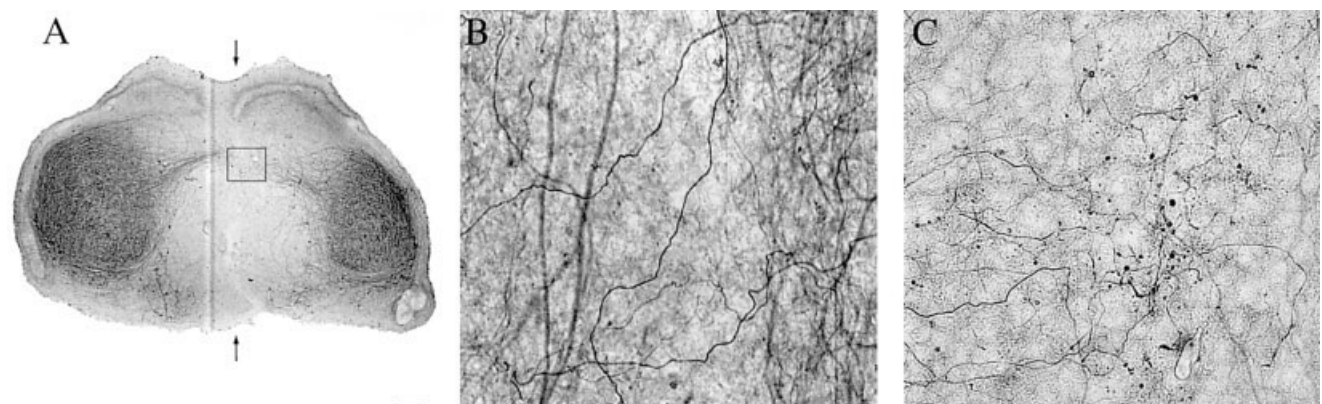


Fig. 7. P12 slices were transected, the midline tissue was removed, and the two IC lobes were placed in apposition to heal. Cultures were grown in serum-containing medium (**A,B**) or N2-defined medium (**C**). Neurofilament-positive axons crossed the lesion site in

both conditions (C and B), although the number of crossing axons was higher in slices grown in the N2-defined medium (see Table 1). Scale bar = 200 μ m in A; 20 μ m for B,C.

lobes revealed that axonal outgrowth by the younger tissue could occur through the injury site and into older tissue. However, there was a near complete failure of the P12 axons to grow across the lesion site and project into the P6 tissue. This result indicates that P6 axons are less sensitive to growth inhibitory signals at the injury site as compared to P12 axons. The P6 IC cultures that received a midline lesion in vitro, 6 days after explantation, also displayed a failure to regenerate. Therefore, we suggest

that there are lesion-induced growth-inhibitory cues at the midline, and that older axons become more sensitive to these cues. The age at which these putative cues are expressed, and the extent to which expression occurs in response to injury, are not yet known.

These findings are in general agreement with previous in vitro studies of regeneration, all of which suggest that growth capacity does not depend on the target tissue (Chen et al., 1995; Li et al., 1995a; Dusart et al., 1997). A

Fig. 8. P12 cultures were transected at the midline and exposed to cycloheximide. **A:** Neurofilament labeled axons were observed within both IC lobes. **B:** At high power (square in A), a few axons were observed to cross the injury site, although others stopped within the vicinity of the lesion. Scale bar = 200 μ m in A; 20 μ m for B.

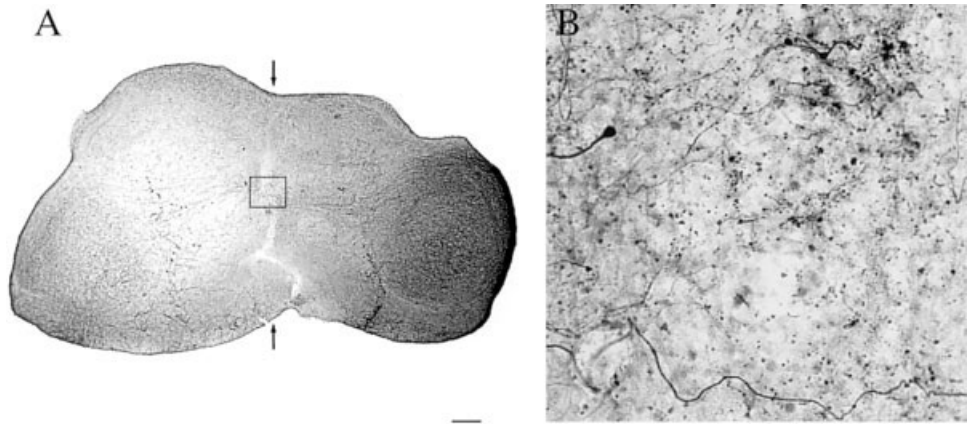
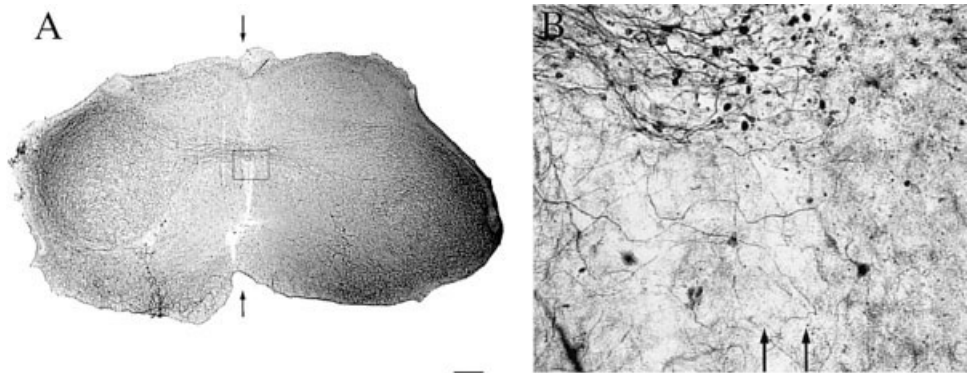


Fig. 9. P12 IC cultures were transected at the midline and grown in the presence of chondroitinase ABC. **A:** Neurofilament staining revealed axonal projections throughout the cultures. **B:** At high magnification (square in A), many axons were seen to cross the lesion site (arrows), although many stopped and formed end bulbs within the injury area. Scale bar = 200 μ m in A; 20 μ m for B.



transient period for axonal regrowth occurs prior to axon myelination (Kalil and Reh, 1982; Xu and Martin, 1989; Shimizu et al., 1990; Jhaveri et al., 1992; Keirstead et al., 1992; Ghooray and Martin, 1993; Hasan et al., 1993; Kapfhammer and Schwab, 1994; Varga et al., 1995; Li et al., 1995a; MacLaren and Taylor, 1995; MacLaren, 1996; Dusart et al., 1997). The main difference between the current findings and the literature is that P6 axons in the heterochronic cultures did not regenerate as robustly as axons from pure P6 cultures (Hafidi et al., 1995). This result suggests indirectly that P6 axons have started to express molecules that interact with growth-inhibitory cues, and that the production of such cues is relatively greater by injured P12 tissue. Thus, the growth-inhibitory potential of axons and of injured nonneuronal tissue can mature in vitro.

Cues within the injury site inhibit axon regeneration

The loss of regenerative capacity is closely associated with inhibitory cues at the injury site in our culture system. Axons in both P12 and P6+6 DIV cultures can grow within one side of the slice, or along the injury site, but not across the lesion. When provided with a favorable growth substrate on the culture dish (Hafidi et al., 1999), or with C6 or C6-R cells (Fig. 10), the P12 explants can exhibit axon outgrowth.

In heterochronic P6–P12 explants the P12 axons probably interacted with local inhibitory cues provided by the

P12 tissue at the lesion site. As a result, P12 axons did not grow across the area of contact to the P6 IC lobe. The P6 axons were not as responsive to these outgrowth inhibitory cues, and more of them extended across the transection and into the P12 IC lobe. However, we did not observe the same robust regeneration obtained in pure P6 cultures (Hafidi et al., 1995). Thus, P6 axons may already express receptors that mediate growth inhibition, but the P6 injury site is not yet able to produce the inhibitory cues. It remains unclear whether the injury site prevents axon outgrowth through membrane contact and/or diffusible factors. Findings from Ichijo and Bonhoeffer (1998) support the presence of diffusible molecules that are secreted at the injured area.

Central axons are known to grow if given a bridge of peripheral nerve or ensheathing glia (Richardson et al., 1980; David and Aguayo, 1981; Berry et al., 1996; Davies et al., 1997; Li et al., 1997; Ramon-Cueto et al., 1998; Xu et al., 1989; Ramon-Cueto et al., 2000). In the present C6 or C6-R cell line experiments, P12 axons regenerated on these cells and often grew into the contralateral IC lobe. When a pellet of C6 or C6-R cells was placed between the transected IC lobes, some axons grew in the cell line, but turned back once they reach the contralateral lip of the injury site. Thus, some regrowing fibers became stuck in the glial cell line bridge. The glial cell line used in this study is known to increase neurite outgrowth (Friedlander et al., 1998) and to promote neuronal migration in vivo

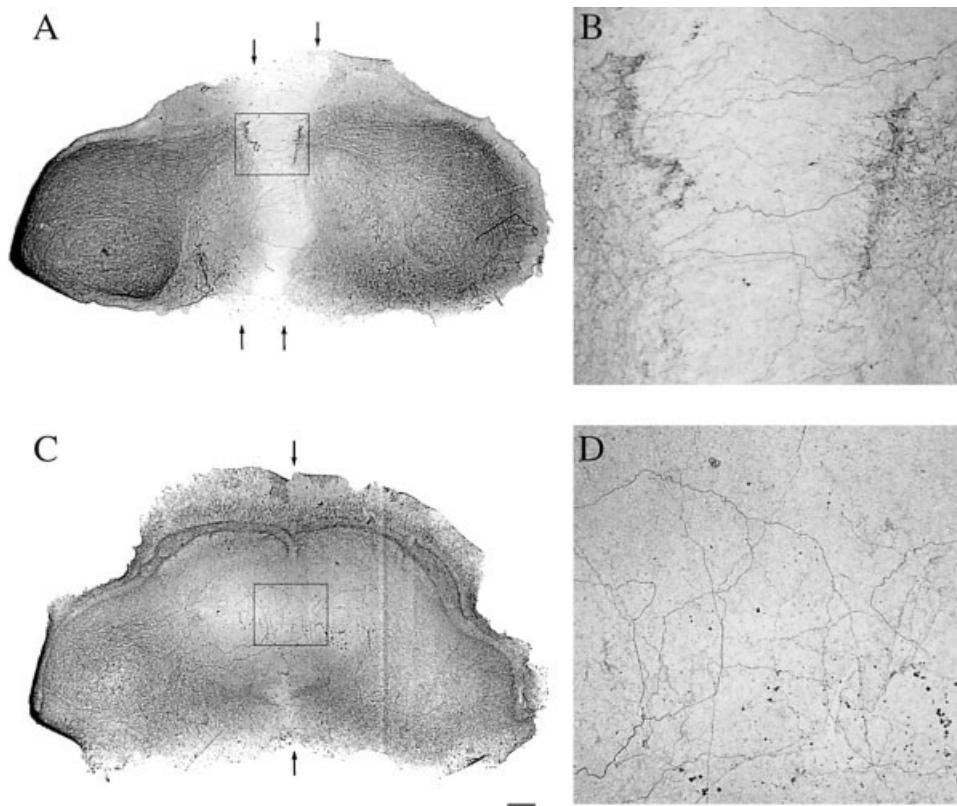


Fig. 10. A graft of C6-R cells were placed between the two lobes of a transected IC (A,B) or on top of the transected commissure (C,D) in P12 cultures. Neurofilament-stained axons in the IC lobes within both side of the graft as well within the C6-R graft. B: At high magnification (square area in A), axons were observed to cross both lips of the IC lesion and course within the C6-R graft. D: A high-magnification image (square area in C) showed that axons grew across the overlaying C6-R graft and grew into the contralateral IC lobe. Scale bar = 200 μ m in C (applies to A, C); 20 μ m for B, D.

(Hormigo et al., 2001). The present results are in general agreement with these previous observations.

The fact that injured axons can grow long distances to the center of the IC lobe suggests that glia at the center of the explant are less inhibitory than those located in the vicinity of the injury. This may be due to neuronal regulation of glial cell proliferation or expression at the center of the IC lobe (Hatten, 1987; Hayashi et al., 1988; Gasser and Hatten, 1990; Mearow et al., 1990; Steward et al., 1991; Chisamore et al., 1996; Pennypacker et al., 1996). This region contains a high density of neurons, whereas the midline is primarily composed of nonneuronal cells. Our study did not focus on the inhibitory effect that oligodendrocytes or myelin-associated molecules might have had on axonal outgrowth (Schwab, 1996). Oligodendrocytes are present in IC tissue at this age and were observed to myelinate some axons in vitro (Hafidi et al., 1999).

Protein synthesis contributed to the inhibition of axon regeneration

The use of cycloheximide permitted a greater number of axons to cross the lesion site and revealed that the inhibitory cues are due, at least in part, to new protein synthesis by the surrounding tissue. One class of molecule that may be involved is the proteoglycans. The use of chondroitinase ABC increased the number of axons that grew across the lesion site (Fig. 9, Table 1). In addition, the chondroitinase ABC data obtained in this study confirmed its general use to help axonal outgrowth in the brain (Smith-Thomas et al., 1995; Zuo et al., 1998; Bradbury et al., 2002).

We do not know the duration of cycloheximide activity, but its action does appear to be prolonged. The axons that do regenerate are thinner than in controls after 6 DIV, and it is likely that this treatment damages both glial and neuronal populations.

The difference between axonal outgrowth through the lesion site in serum-containing medium, as compared to N2-defined medium, may be due to the proliferation of glia at the lesion site. Serum is known to induce the astrocyte proliferation, and these cells are less supportive of neurite outgrowth (Wang et al., 1994; Hou et al., 1995; Vergeli et al., 1995). At P12, it is thought that glia are nonpermissive to axonal outgrowth, perhaps through secretion of molecules that impede growth (McKeon et al., 1991, 1995; Smith-Thomas et al., 1995; Canning et al., 1996; Zuo et al., 1997; Fitch and Silver, 1997).

GDNF helped axons to grow across the lesion site

GDNF has been shown to have neurite extension actions on a variety of neuronal structures (Lin et al., 1993, 1995b; Tomac et al., 1995; Rosenblad et al., 1996; Prang et al., 2001). The addition of GDNF to the IC cultures significantly increased the number of axons that extended across the lesion. GDNF may increase the outgrowth rate of injured axons, hence permitting some of these lesioned axons to cross the lesion site before it becomes highly inhibitory. It remains to be determined whether the enhancement due to GDNF is due to an increase in surviving neurons, a direct effect on cut

axons, or a direct effect on nonneuronal cells at the lesion site.

CONCLUSION

Previous findings from this system show that lesioned P12 axons have the capacity to extend on a permissive substrate when grown in a serum-free medium (Hafidi et al., 1999). The present findings extend these observations by showing that P12 axons are better able to extend across a lesion site in serum-free media and that these effects can be mimicked with treatments that block protein synthesis or enzymatically remove CSPGs. Removal of the midline region also increased regeneration, but did not result in the level of regeneration observed in P6 tissue (Hafidi et al., 1995), suggesting that all P12 IC tissue contains growth-inhibitory cues. Astrocytes at the lesion site are the most probable factor contributing to the failure of axonal regrowth in this model. However, these results in no way contradict an involvement of oligodendrocyte-associated factors in regeneration failure. In fact, the myelin marker MAG is expressed both *in vivo* and *in vitro* at P12 (Hafidi et al., 1995, 1999). Finally, both a glioma and a radial glia-like cell line can restore a significant amount of regrowth. Taken together, these results indicate that multiple strategies are required to help axons extend across a lesion site.

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