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# Changes within Maturing Neurons Limit Axonal Regeneration in the Developing Spinal Cord

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**ABSTRACT:** **Embryonic birds and mammals display a remarkable ability to regenerate axons after spinal injury, but then lose this ability during a discrete developmental transition. To explain this transition, previous research has emphasized the emergence of myelin and other inhibitory factors in the environment of the spinal cord. However, research in other CNS tracts suggests an important role for neuron-intrinsic limitations to axon regeneration. Here we re-examine this issue quantitatively in the hindbrain-spinal projection of the embryonic chick. Using heterochronic cocultures we show that maturation of the spinal cord environment causes a 55% reduction in axon regeneration, while maturation of hindbrain neurons causes a 90% reduction. We further show that young neurons transplanted *in vivo* into older spinal cord can regenerate axons into myelinated white matter, while older axons regenerate poorly and have reduced growth cone motility on a variety of growth-permissive ligands *in vitro*, including laminin, L1, and N-cadherin. Finally, we use video analysis of living growth cones to directly document an age-dependent decline in the motility of brainstem axons. These data show that developmental changes in both the spinal cord environment and in brainstem neurons can reduce regeneration, but that the effect of the environment is only partial, while changes in neurons by themselves cause a nearly complete reduction in regeneration. We conclude that maturational events within neurons are a primary cause for the failure of axon regeneration in the spinal cord.** © 2006 Wiley Periodicals, Inc. J Neurobiol 66: 348–360, 2006

*Keywords:* **spinal cord; axon regeneration; laminin; L1; N-cadherin**

## INTRODUCTION

Axons in the spinal cord of adult birds and mammals fail to regenerate after injury, and correcting this failure is a major goal of neuroscience research. Events in the development of spinal tracts suggest a promising strategy to attack this problem. Unlike adults, embryonic and early postnatal animals show a remarkable ability to heal from spinal injury, displaying long distance axon regeneration and full functional recovery (Bregman et al., 1989; Shimizu et al., 1990; Hasan et al., 1993; Saunders et al., 1998; Fry et al., 2003). The ability to regenerate axons is then lost during a discrete developmental transition. By identifying and reversing the developmental events that restrict axonal regeneration it may be possible to enhance regeneration in the adult.

The majority of spinal injury research has focused on factors in the environment of the spinal cord that inhibit axon growth. The best-studied source of inhibition is myelin. Myelin in the central nervous system (CNS) contains at least three molecules, NogoA, MAG, and OMgp, which bind to the NogoR receptor on injured axons and initiate signaling cascades that reduce axon motility (Fournier et al., 2001, 2003; Domeniconi et al., 2002; Liu et al., 2002; Wang

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et al., 2002). During development, the timing of myelination coincides with the failure of axon regeneration, while treatments that delay myelination in the spinal cord allow some axon regeneration to occur after the time when regeneration is normally restricted (Savio and Schwab, 1990; Keirstead et al., 1992; Vanek et al., 1998). These findings have led to a strong focus on environmental inhibition in general, and myelin in particular, as a main cause for the failure of axon regeneration. In the adult spinal cord, most current work seeks to enhance regeneration by neutralizing inhibitory signals (Bradbury et al., 2002; GrandPre et al., 2002; Fouad et al., 2004) and/or by grafting growth-permissive tissue into the site of injury (Jin et al., 2002; Nikulina et al., 2004; Pearse et al., 2004). Although some axons do regenerate into these improved environments, in the case of descending motor axons, this response is limited to a small number of axons, which elongate relatively slowly. While this limited response may reflect an incomplete reversal of inhibition, it also suggests that axonal regeneration in the spinal cord is prevented by more than inhibition in the environment.

Research on the development of other nervous system tracts suggests that maturing neurons lose a substantial amount of their capacity for successful regeneration. As in the spinal cord, axons in other CNS tracts are capable of regeneration early in development, but then lose this ability during a discrete transition (So et al., 1981; Chen et al., 1995; Dusart et al., 1997; Kawano et al., 2005). Using a strategy of heterochronic cocultures, studies in some of these tracts have shown that age of the neurons has a large effect on the success of axon regeneration (Chen et al., 1995; Li et al., 1995; Dusart et al., 1997; Goldberg et al., 2002). This suggests that besides environmental inhibition, the internal state of neurons can also play a role in limiting axon regeneration, with some neurons showing a reduced capacity for axon regeneration as they age. However, the relative importance of neuronal as compared to environmental maturation appears to vary in different tracts, and, importantly, similar experiments have not been performed in the developing spinal cord. Although in the spinal cord the role of myelin inhibition has received much of the attention (Keirstead et al., 1992; Varga et al., 1995), these findings from elsewhere in the CNS require us to reconsider a fundamental question: is axon regeneration in the developing spinal cord restricted primarily by changes in developing neurons, or by changes in the environment of the spinal cord?

Our aim in this study was to develop quantitative methods to assess axon regeneration in the hindbrain-spinal projection of the embryonic chick, a well-studied model for the developmental failure of axon regeneration. Using age-mismatched slice cultures, *in vivo* transplantation, and video analysis of live growth cones we show quantitatively that changes in both the environment and in neurons restrict axon regeneration, but that changes in maturing neurons by themselves can account for 90% of the reduction in axon regeneration. These data demonstrate that a decline in the neuronal capacity for axon growth plays a critical role in limiting axon regeneration in the spinal cord.

## METHODS

### Retrograde Labeling of Brainstem Projection Neurons

Fertilized White Leghorn chicken eggs were incubated in a humidified incubator at 38°C. As described by Okado and Oppenheim (1985) and Pataky et al. (2000), windows were cut in shells on E5, and small tears were made in the allantoic and amniotic membranes over the cervical spinal cord. For brainstems to be used in axon outgrowth studies, crystals of DiI (Molecular Probes, Eugene, OR) were attached to the tips of insect pins using dried albumin, and then inserted into embryos adjacent to cervical spinal cord. Eggs were sealed with tape and returned to a nonrocking incubator. Survival rates to E15 were around 60%.

### Organotypic Coculture of Brainstem and Spinal Cord

Brainstems containing retrogradely labeled brainstem projection neurons were dissected from E9 and E15 embryos and placed into F12 media (Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (Hyclone, Logan, UT). Brainstems were embedded in 2% low-melting temperature agarose (Sigma, St. Louis, MO), and cut transversely with a set of razor blades separated 300 $\mu$m by silicon spacers (made from microscopy spacers; Molecular Probes). Slices were collected from brainstem tissue rostral to the level of the obex and caudal to the entry of the fifth cranial nerve. Transverse brainstem slices were cut bilaterally on lines from the dorsal midline to the midpoint of the mediolateral ventral surface (See Fig. 1). The dorsal third of the resulting tissue triangle was also removed, and explants were then divided at the midline. These explants contained retrogradely labeled brainstem projection neurons from the raphe nuclei, medullary reticular formation, and pontine reticular formation. To prepare spinal cord explants cervical spinal cord was dissected into F12 media containing 10% calf serum, embedded in 2% low-melting temperature agarose, and then sliced with the same apparatus as brainstem explants to produce longitudinal (sagittally oriented) explants. The two explants nearest the midline were used in experiments.

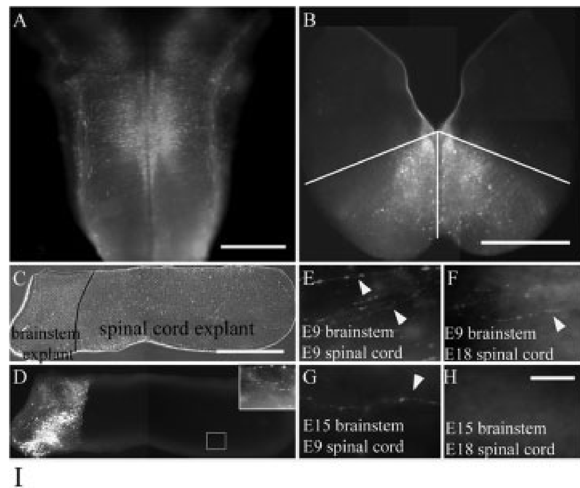


**Figure 1** Explants of older brainstem neurons regenerate fewer axons than young brainstem, regardless of the age of spinal cord target tissue. (A) E9 brainstem, ventral surface, showing spinal projection nuclei retrogradely labeled with DiI. (B) Transverse section of brainstem, dorsal to the top, showing location of spinal projection neurons and cut lines used to prepare explants. (C) Coculture of brainstem and spinal cord. (D) Coculture of E9 brainstem and E9 spinal cord viewed under fluorescence after 3 days in culture, with DiI-labeled regenerating axons in the spinal cord (inset). (E–H) High power views of DiI-labeled brainstem axons regenerating into spinal cord explants (arrowheads). Younger brainstem axons regenerate robustly in young spinal cord, and somewhat less into older spinal cord. Older brainstem neurons regenerate few axons into young spinal cord, and none into older spinal cord. (I) Quantification of axon regeneration, counting the number of DiI-labeled axons that grew more than 1 mm into spinal cord after 3 days in culture. Scale bars are 500 $\mu$m for (A–D), 20 $\mu$m for (E–H). Error bars show SEM. More than 16 cocultures from at least four embryos were measured for each age combination. ***$p < 0.001$, one-way ANOVA.

Explants of brainstem (E9 or E15) and spinal cord (E9 or E18) were rinsed in serum-free Neurobasal media (Invitrogen) and then placed in cell culture inserts (0.4 $\mu$m; Millipore) in 35 mm wells containing 1.5 mL of Neurobasal media with B27 supplements (Invitrogen). In brainstem-spinal cocultures young and old explants were always cultured in the same well to control for the effects of media conditioning. The rostral end of the spinal cord explant was placed in contact with the brainstem explant. After a few hours, brainstem and spinal cord explants of all ages fused. In brainstem-brainstem cocultures, the medial surfaces of E9 and E15 brainstems were apposed. Cultures were maintained for 3 days at 40°C, 5% $CO_2$, with the media refreshed daily, and then fixed in 4% paraformaldehyde. Explants were placed on coverslips and viewed with an Olympus XC-70 inverted microscope at 60X magnification under fluorescence to visualize DiI label in regenerating axons. To quantify axon regeneration of brainstem projection neurons the number of DiI-labeled axons that crossed a virtual line 1000 $\mu$m from the brainstem explant was counted manually.

## Brainstem Explant Culture

Brainstem explants containing retrogradely labeled brainstem projection neurons were prepared from E9 or E15 embryos as described above. Glass coverslips were heated in a 300°C oven for at least 24 h, coated with 100 $\mu$g/mL poly-D-lysine (PDL) (Sigma) overnight, rinsed extensively, and then coated overnight with 100 $\mu$g/mL BSA, laminin (Invitrogen), L1 (R&D Systems, Minneapolis, MN), N-cadherin (R&D), or fibronectin (Sigma). Coverslips were then blocked with 5 mg/mL BSA in PBS (Sigma), and rinsed briefly. In additional experiments with L1, coverslips were coated with nitrocellulose dissolved in 100% amyl acetate and dried after coating with PDL and before coating with L1. Brainstem explants were cultured on coverslips for 3 days in Neurobasal media with B27 supplements, refreshed daily. Explants were fixed in 4% paraformaldehyde, and coverslips were mounted on slides and viewed using an Olympus XC-70 inverted microscope. Digital images of DiI-labeled axons were acquired using a SPOT digital camera and software (Diagnostic Instruments, Sterling Heights, NJ), and the number of axons that regenerated across a virtual line 1 mm from the explant was quantified from these images.

For video analysis glass coverslips were mounted on Petri dishes with holes cut in the bottom, coated overnight with 100 $\mu$g/mL PDL, washed extensively, and coated overnight with 100 $\mu$g/mL laminin. E9 and E15 brainstem explants containing DiI-labeled brainstem projection neurons were cultured on the same coverslips for 1 to 3 days in Neurobasal media with B27 supplements. Coverslips were placed on an inverted microscope stage at 40°C, and fluorescent images were taken to identify regenerating brainstem projection axons. By following the axon back to its explant of origin it was possible to distinguish E9 and E15 axons. Images of identified axons at 20X magnification were taken with visible light each minute for 1 h using a SPOT digital camera and Metavue software (Universal Imaging, West Chester, PA), and the distance moved in 10 min intervals was measured based on the location of the central domain of the growth cone.

## TUNEL Analysis

Explants of brainstem tissue containing DiI-labeled brainstem projection neurons were prepared from E9 or E15 embryos and either fixed immediately in 4% paraformaldehyde or cultured on millicell inserts in Neurobasal media for 1 to 3 days, followed by fixation. To standardize subsequent analysis, tissue from all time points embedded in the same block of OCT and cryostat sections (10 $\mu$m) were collected on the same slide. TUNEL analysis was performed using the Apoptag kit (Serologicals, Atlanta, GA) following the manufacturer's instructions. DAPI (Sigma) was added at 1 $\mu$g/mL in PBS for 10 min, followed by rinsing. The percent of TUNEL-positive brainstem projection neurons was determined, with scoring restricted to DiI-labeled cells with nuclei visible in the plane of the slice.

## *In Ovo* Spinal Cord Transection and Transplantation

For transplantation of E9 brainstem into E15 spinal cord, brainstem projection neurons in E9 brainstem were retrogradely labeled with DiI *in ovo* as described previously, and small explants (<300 $\mu$m diameter) that contained DiI-labeled neurons were selected using a fluorescent microscope. The thoracic spinal cord of E15 embryos was exposed *in ovo* and partly cut using microscissors. E9 brainstem explants were inserted into the resulting hole using a pipette. The injury was then packed with gelfoam (Pharmacia and Upjohn, Kalamazoo, MI), the egg sealed with tape, and returned to the incubator for 3 days. Survival rates were around 50%.

# RESULTS

## Organotypic Coculture of Young and Old Brainstem and Spinal Cord

Descending motor control in the chick comes from reticular, raphe, and vestibular nuclei in the brainstem (ten Donkelaar, 2000). *In vivo*, axon regeneration in descending spinal tracts becomes restricted around embryonic day 13 (E13) (Shimizu et al., 1990; Hasan et al., 1993). To determine whether this transition is caused by changes in the spinal cord environment or changes in brainstem neurons, we used organotypic cocultures in which the age of spinal cord and brainstem could be varied independently. We retrogradely labeled neurons in the brainstem that project axons to the spinal cord by placing DiI crystals into descending axon tracts of ventral spinal cord *in ovo* (first described by Okado and Oppenheim, 1985 and used by Pataky et al., 2000). Embryos injected with DiI then developed normally, and brainstem-spinal projection neurons accumulated DiI [Fig. 1(A)]. On E9 or E15, explants of brainstem containing retrogradely labeled neurons were placed in culture adjacent to spinal cord explants from E9 or E18 embryos that had not received a DiI injection. Axons labeled with DiI grew from the brainstem explants into the spinal cord explants. After 3 days we counted the number of DiI-labeled axons that grew at least 1 mm into spinal cord [Fig. 1(E–H)]. This approach quantified the regenerating brainstem-spinal projection axons, as opposed to newly formed axons or axons from another type of neuron, because only spinally projecting neurons in the brainstem were DiI-labeled, and preparing explants necessarily cut axons that exited the brainstem. DiI label was often dim and difficult to detect against background fluorescence in the spinal explant [Fig. 1(E–H)], and our values likely underestimate the total number of regenerating axons. In control experiments a scalpel blade was passed between the brainstem and spinal cord explant every 12 h. In these experiments DiI-labeled axons were never observed in the spinal cord, showing that DiI was not passively diffusing into spinal cord tissue.

The age of the brainstem explant had a large effect on the success of axon regeneration. As shown in Figure 1, young brainstem explants regenerated an average of 80 axons at least 1 mm into young spinal cord, and when confronted with older spinal cord, young brainstem explants still regenerated an average of 37 axons. In contrast, older brainstem explants regenerated an average of only 10 axons into younger spinal cord, and never regenerated axons into older spinal cord. Thus, between E9 and E15, during the time when regeneration fails completely *in vivo*, maturation of the spinal cord environment caused a 55% reduction in axon growth, and maturation of brainstem neurons caused a 90% reduction in axon growth. Regeneration failed completely only when both brainstem and spinal cord were from mature embryos. Our data suggest that changes in both the environment of the spinal cord and in the internal growth state of neurons are required to cause the complete failure of axon regeneration during development. Importantly, however, our data also suggest that developmental changes in brainstem neurons alone dramatically reduce the success of regeneration, even without any changes in the environment of the spinal cord.

We performed two additional control experiments. First, it is possible that the reduced axon growth from older brainstem explants was caused by a higher rate of cell death in older explants. To test this possibility we performed TUNEL analysis of old and young brainstem projection neurons in culture (Fig. 2) and found similar rates of apoptotic cell death (approximately 30% total over 3 days). A previous study

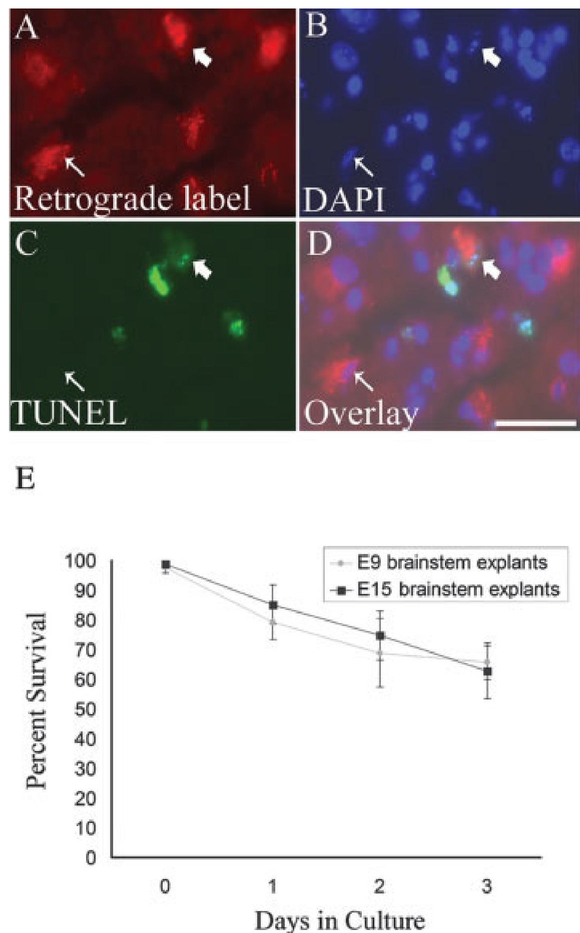


**Figure 2** E9 and E15 spinal projection neurons have similar rates of cell death in culture. (A) Cryostat section of E15 brainstem after 3 days in culture on millicell inserts, with spinal projection neurons identified by retrograde DiI label. (B) DAPI staining of the same section showing condensed (large arrow) and diffuse nuclei (small arrow). (C) TUNEL staining for apoptotic nuclei. Large arrow indicates an apoptotic nucleus; small arrow indicates position of a TUNEL-negative nucleus. (D) Overlay identifying a surviving spinal projection neuron (small arrow) and an apoptotic spinal projection neuron (large arrow). (E) Quantification of cell survival during 3 days in culture, quantifying the percentage of spinal projection neurons with large, diffuse nuclei free of TUNEL stain, and including only cells with nuclei visible in the plane of the slice. In both ages about 10% of the initial population dies each day in culture. Because the rate of cell death is similar in both ages, a difference in cell survival cannot explain the difference in axon regeneration. More than 12 explants from each age were examined for each time point. Error bars show SEM. Error bar is 25 $\mu$m.

using similarly prepared cultures of E9 and E17 chick brainstem assessed cell viability on the basis of nuclear and cell morphology and also concluded that rates of cell survival were similar in the two ages (Borisoff et al., 2000). It remains possible that older neurons could be differentially affected by nonapoptotic cell death pathways. However, our TUNEL analysis, combined with previous findings, suggests that the reduction in axon regeneration from the older brainstem was not secondary to an increase in cell death.

Second, it is possible that reduced axon growth from older brainstem explants is caused not by changes in neurons, but rather by an inhibitory environment within the older brainstem that acts to trap axons within the explant. To determine whether the older brainstem explant was itself inhibitory to axon regeneration we tested the ability of younger brainstem axons to regenerate in the environment of the older brainstem when the two ages of brainstem were combined in our coculture system. As shown in Figure 3, E9 brainstem axons regenerated robustly throughout explants of E15

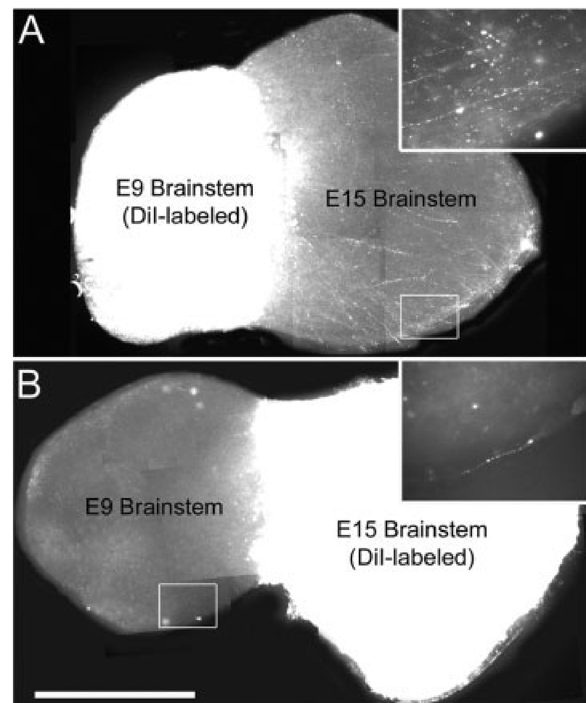


**Figure 3** E9 brainstem-spinal projection axons grow robustly in E15 brainstem. (A) Coculture of E9 brainstem and E15 brainstem after 3 days in culture. E9 brainstem was retrogradely labeled *in ovo* with DiI placed in spinal cord. DiI-labeled axon (inset) grew robustly into E15 brainstem, showing that brainstem-spinal axons were not inhibited by the E15 brainstem environment. (B) Coculture of E9 and E15 brainstem after 3 days in culture. E15 brainstem was retrogradely labeled *in ovo* with DiI placed in the spinal cord to identify spinally projecting neurons. Very few axons grew from E15 brainstem into E9 spinal cord. Scale bar is 1 mm.

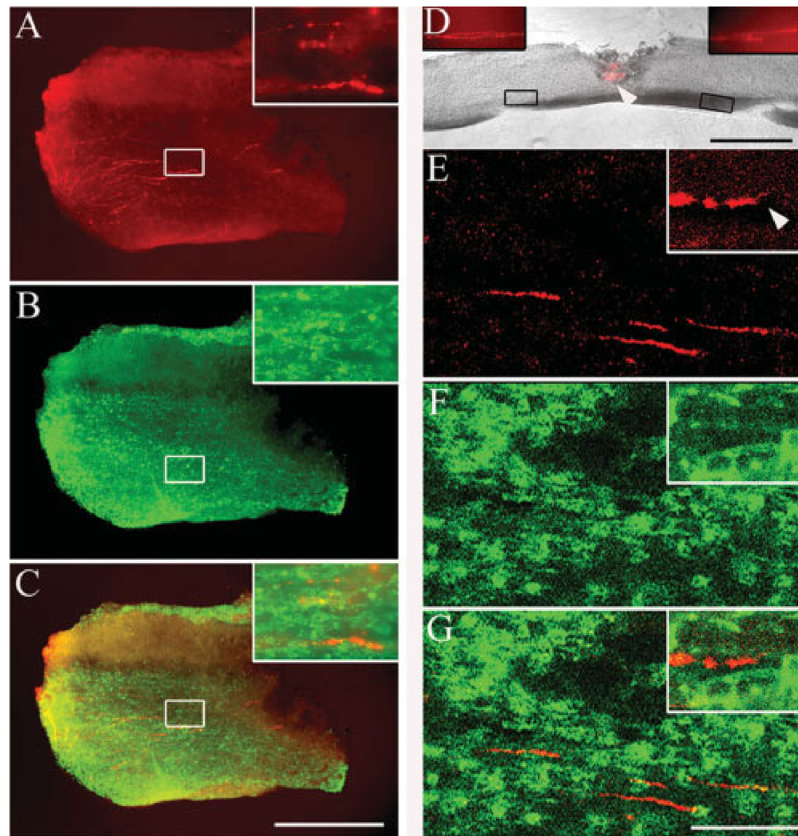


**Figure 4** E9 brainstem-spinal projection neurons regenerate axons into myelinated areas of E18 spinal cord. (A–C) Sagittal section of E18 spinal cord after 3 days in culture with an E9 brainstem explant. Spinal cord is oriented with ventral to the bottom, and the brainstem explant was located to the left of the spinal cord. (A) Spinal projection axons from E9 brainstem neurons, identified by DiI label, regenerating into an explant of E18 spinal cord. (B) Immunohistochemistry for myelin basic protein (MBP) showing location of myelin. The large area of myelination corresponds to ventral-lateral white matter. (C) Overlay showing axons regenerating in myelinated areas of spinal cord explant. (D) Sagittal section of E18 spinal cord 3 days after *in vivo* transplantation of E9 brainstem. Spinal projection neurons in the brainstem explant are labeled with DiI (arrowhead), and DiI-labeled axons are visible in ventral axon tracts up to 2 mm from transplanted explants (insets). (E) Single confocal image showing DiI-labeled axons in E18 spinal cord *in vivo*. Axons are tipped by small growth cones with leading filopodia (inset). (F) MBP immunohistochemistry in the same section showing location of myelin. (G) Overlay showing axons regenerating *in vivo* in close proximity to myelin in E18 spinal cord. Scale bars are 500 $\mu$m in (A–C), 1 mm in (D), 50 $\mu$m in (E–G).

brainstem, while E15 brainstem axons regenerated poorly into E9 brainstem. These data suggest that the low levels of axon regeneration from E15 brainstem explants are not caused by an inhibitory growth environment within the E15 explant itself.

## Young Brainstem Neurons Regenerate Axons into Myelinated Spinal Cord

Our coculture experiments showed that some younger neurons have the ability to regenerate axons in spinal cords from E18 embryos. Myelin is abundant in E18 chick spinal cord (Macklin and Weill, 1985; Keirstead et al., 1992), raising the possibility that younger neurons may be partially resistant to myelin-associated inhibitory cues. However, it is also possible that myelin was degraded in our culture system, or that regenerating axons avoided areas with myelin. We performed immunohistochemistry for myelin basic protein (MBP) to determine the relative position of myelin and regenerating axons in our coculture system. As shown in Figure 4, myelin was abundant in parts of E18 spinal cord explants after 3 days in culture, and axons from E9 brainstem regenerated into areas with myelin. We did not quantify axon growth

in white matter versus grey matter, but qualitatively we observed no indication that regenerating axons avoided areas with myelin.

The ability of younger neurons to regenerate in myelinated spinal cord could also be an artifact of our culture conditions. To test this possibility we transplanted explants of DiI-labeled E9 brainstem into the thoracic spinal cord of living E15 embryos. After 3 days survival we observed DiI-labeled axons that had regenerated into host spinal cord in four of seven embryos, and these axons had grown as far as 2 mm into the cord from the transplant [Fig. 4(D)]. We performed immunohistochemistry for MBP on tissue sections and used confocal microscopy to show that E9 axons regenerated in ventral axon tracts in close proximity to myelin [Fig. 4(E–G)]. Because younger neurons regenerated axons into myelinated environments, we conclude that myelination of the spinal cord is not sufficient to end the permissive period of axon regeneration, and that developmental changes in neurons must also contribute.

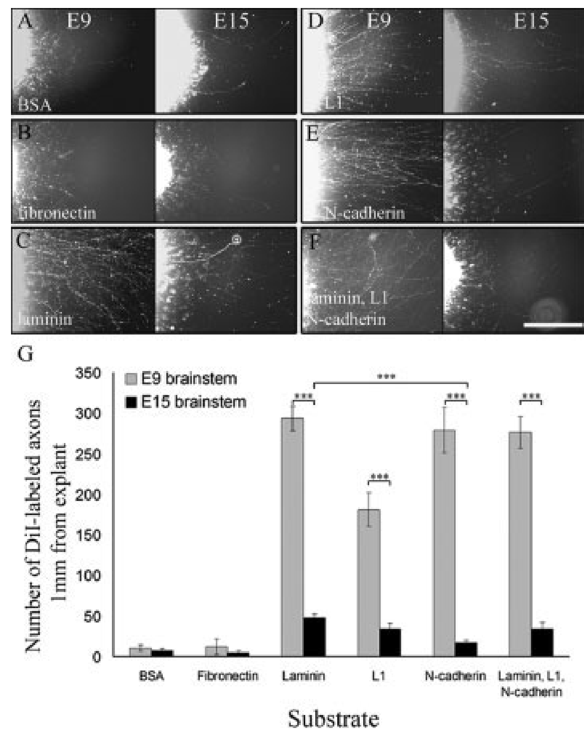


**Figure 5** Laminin, L1, and N-cadherin support axon regeneration from young but not old spinal projection neurons. (A) E9 and E15 brainstem explants on BSA, a nonligand control substrate, showing very little axon regeneration. (B) Brainstem explants on fibronectin, which also supports little regeneration. (C–F) Brainstem explants on laminin (C), L1 (D), N-cadherin (E), or a combination of all three (F), showing robust regeneration of DiI-labeled spinal projection axons from E9 but not E15 brainstem. (G) Quantification of the number of DiI-labeled spinal projection axons from E9 versus E15 brainstem explants that regenerate more than 1 mm. E15 brainstem explants show a substantial reduction in axon regeneration on all substrates. Scale bar is 500 $\mu$m. ***$p < 0.01$, Student's $t$ test with Bonferroni correction for multiple comparisons. More than 12 explants from at least three embryos were analyzed for each age on each substrate.

## Regeneration from Brainstem Neurons on Laminin, L1, N-Cadherin, and Fibronectin

One question raised by the poor axon growth from older explants in our coculture system is whether this reduced growth is due to a general decline in the ability to extend long axons, or whether it reflects a more specific deficiency in the ability to respond to a particular substrate molecule. For instance, retinal ganglion cells of a certain age become unable to regenerate axons on laminin-1 but retain the ability to regenerate on other substrates (Cohen et al., 1986; Calof et al., 1994; Ivins et al., 1998). It has previously been shown that brainstem neurons show an age-dependent decline in axon growth on laminin (Borisoff et al., 2000). To determine whether in brainstem neurons this age-dependent decline in axon growth is general to other ligands, we compared axon regeneration from E9 and E15 brainstem explants on substrates of laminin, L1, N-cadherin, and fibronectin. To quantify axon growth we counted the number of axons that crossed a virtual line 1 mm from the edge of the explant after 3 days in culture.

As shown in Figure 5, E9 brainstem projection axons regenerated axons robustly on substrates of laminin, L1, and N-cadherin. On laminin and N-cadherin, E9 explants grew an average of almost 300 axons a distance of 1 mm from the explant, which is about three times more than the number of axons we observed in explants of younger spinal cord. This difference is likely due to much lower background fluorescence on glass coverslips as compared to spinal explants, allowing detection of more dimly labeled axons. Our methods produced 12 explants that contained reticular and raphe nuclei from each brainstem, so we estimate that the total number of regenerating axons per brainstem is around 3500. Axons were rarely observed to branch, although the high density of axons prevented quantification of branching. We therefore take 3500 as a rough estimate of the number of regenerating neurons in our E9 explants, with the caveat that this estimate could be somewhat inflated if branches from single axons were counted as multiple axons. Previous research has shown that approxi-

mately 7000 neurons in the reticular and raphe nuclei in the chick are retrogradely labeled by spinally injected DiI (Pataky et al., 2000). Because our TUNEL analysis showed that 30% of brainstem projection neurons died in culture, we estimate that 75% of surviving younger neurons regenerated long axons *in vitro* on laminin and N-cadherin.

In initial experiments, substrates of L1 supported somewhat less axon regeneration than did N-cadherin or laminin. However, when L1 was immobilized on coverslips precoated with nitrocellulose instead of PDL, the number of axons regenerating increased from 181 to 236 (±20 SEM, $n = 8$), not significantly different from that seen on laminin or N-cadherin immobilized on nitrocellulose ($p > 0.05$, Student's $t$ test). We conclude that the reduction in axon growth on L1 in our initial experiments is due to inefficient binding of L1 to PDL, or perhaps a binding conformation that obscures critical sites on L1, and does not reflect any substrate preference by young brainstem projection neurons.

To test the possibility that different adhesive ligands recruit growth from different populations of brainstem projection neurons we combined laminin, L1, and N-cadherin on the same coverslip. This treatment resulted in no additive effect on the number of regenerating axons [Fig. 5(G)], suggesting that the same population of neurons responded to all three ligands. Substrates coated with fibronectin supported almost no axon regeneration by brainstem projection neurons [Fig. 5(B,G)], but did support robust growth from explants of dorsal root ganglia, providing a positive control for the performance of fibronectin (data not shown). We conclude that laminin, L1, and N-cadherin, but not fibronectin, are supportive ligands for the regeneration of young brainstem-spinal projection neurons.

Compared to E9 explants, E15 explants regenerated 86 to 93% fewer axons on laminin, L1, and N-cadherin. Axon regeneration from E15 explants on N-cadherin (17.2 ± 3.1 SEM) was significantly lower than on laminin (47.2 ± 4.8) or L1 (34.2 ± 6.7) ($p < 0.05$, Student's $t$ test with Bonferroni correction for multiple comparisons). Based on calculations similar to those described for younger explants (see above) we estimate that no more than 8% of total brainstem-spinal projection neurons regenerated long axons on any substrate tested. Combining laminin, L1, and N-cadherin on the same coverslip did not increase the number of regenerating axons, so as with younger neurons, the same population of neurons was responding to all three adhesive ligands. Growth on fibronectin was no higher than BSA control levels, showing that older neurons are also unresponsive to fibronectin. In sum, these data show that older neurons regenerate poorly on substrates of laminin, L1, and N-cadherin. We conclude that the age-dependent decline in axon regeneration from brainstem-spinal projection neurons is general to a variety of ligands.

### Brainstem Projection Neurons Lose Responsiveness to Laminin over a Six Day Period

Our coculture and *in vitro* data show that older brainstem explants regenerate poorly even when provided with growth-permissive environments free of inhibition, suggesting that the internal state of developing neurons may change in a way that limits their ability to elongate axons. If so, this internal reduction in axon motility would present a major barrier to successful axon regeneration. We therefore set out to characterize more precisely the timing and nature of age-dependent changes in axon motility. To determine the age at which neurons lose the ability to regenerate axons on laminin, we cultured a full time course of brainstem explants between E9 and E15 and quantified the number of axons that regenerated 1 mm from the explant after 3 days. As shown in Figure 6, the number of regenerating axons declined steadily as the age of the brainstem increased. E9 explants regenerated an average of 291 (±21 SEM) axons a distance of 1 mm, while E15 explants regenerated only 30 (±6.7 SEM). These data show that most brainstem projection neurons lose responsiveness to laminin as they mature over a 6 day period.

We performed video analysis of E9 and E15 axons to compare more directly the growth dynamics of young and old axons as they regenerated on laminin. To control for variability of substrates between and within dishes, old and young brainstem explants were cultured in the same dish and, when feasible, videos were taken with axons of different ages in the same field [Fig. 6(B)]. During hour-long analyses, young axons extended an average of twice the distance of older axons. As shown in the histogram of Figure 6(C), 85% of younger axons grew more than 60 $\mu$m, compared to 33% of older axons. When axon movement was quantified over 10 min intervals, we found that younger axons paused or retracted in only 4% of those intervals, compared to 15% for older axons, and that during intervals when axons extended, younger axons grew almost twice the distance of older neurons (16.3 vs. 9.5 $\mu$m, $p < 0.001$ Student's $t$ test). It is important to note that this analysis included only the small number of older neurons whose axons exited the explant and extended on lami-

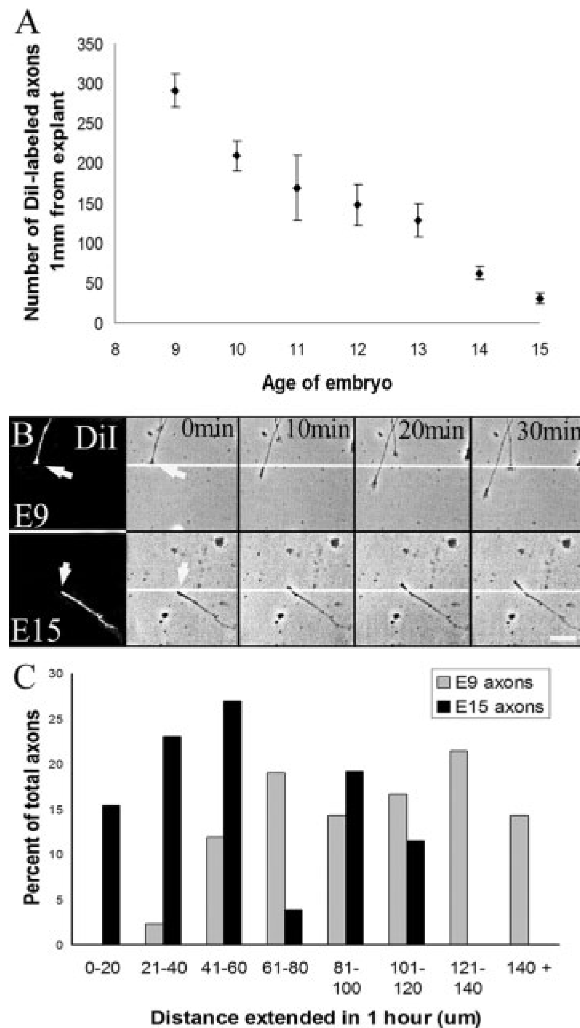


**Figure 6** A time-course and video analysis of the age-dependent reduction of axon motility on laminin substrates. (A) Quantification of the number of spinal projection axons that regenerate 1 mm from brainstem explants over 3 days. The number of regenerating axons declines steadily as the age of the explant increases between E9 and E15. (B) Video analysis of axon motility on laminin. Spinal projection neurons from E9 or E15 brainstem explants were identified with retrograde DiI label (arrows). During 10 min intervals the E9 axon extends rapidly, while the E15 axon extends slowly. (C) Histogram of the total distance moved during 1 h by E9 and E15 axons, showing that younger axons as a population grow farther than older axons. Error bars are SEM. $n \geq 16$ explants from four embryos (A), 49 for E9 axons, 36 for E15 (C). Scale bar is 100 $\mu$m.

nin. Because we could not study the axons of older neurons that do not extend sufficiently to exit the explant, the differences we measured likely underestimate the true differences between the entire population of younger and older neurons. Nevertheless, these observations directly demonstrated an age-dependent reduction in axon motility in a growth-permissive environment free of inhibition, strengthening the notion that developmental changes within neurons reduce axon regeneration.

## DISCUSSION

Our principal conclusion is that between E9 and E15, changes occur within brainstem-spinal projection neurons that substantially limit their ability to regenerate axons, even into permissive environments. E15 explants regenerated about 90% fewer axons than E9 explants, and this reduction was remarkably consistent across diverse substrates including E9 spinal cord, laminin, L1, or N-cadherin. In addition, video analysis showed that axons from older neurons paused more often and advanced more slowly than younger axons on identical laminin substrates. This decline in regeneration on identical substrates must be due to internal differences between old and young neurons.

Interpretation of our results is complicated by our use of explant culture, in which neurons are potentially influenced by signals from other cells. For instance, it is possible that the higher regenerative capacity of younger explants is due to a greater abundance of neurotrophins or other growth-promoting molecules. This would not change our basic conclusion that younger neurons have a higher capacity for axonal regeneration, but because this ability may be regulated by signals external to the neuron we have avoided labeling it as completely intrinsic or cell autonomous (Goldberg, 2004). Conversely, we considered the possibility that the low regenerative capacity of older explants is caused by a growth-inhibitory environment within the explant itself. We consider this unlikely, because axons from younger brainstem do not appear to be inhibited by the environment of older brainstem (Fig. 3). We attempted to address these concerns more directly by performing experiments with dissociated neurons free of cell-extrinsic influences, but older neurons never survived this procedure, despite multiple attempts and methods.

Another complicating factor is that our data do not distinguish different types of brainstem-spinal projection neurons. It is possible that some populations, particularly raphe neurons, maintain a higher growth potential than others (Borisoff et al., 2000). It would be interesting to determine whether the small population of rapidly growing older axons we observed [Fig. 6(C)] was from raphe neurons. However, our data that include all types of projection neurons show large and consistent differences in axon regeneration

between ages. This suggests that the variability between populations is minor compared to the overall effect of neuronal maturation.

Our results are broadly consistent with previous work. Early studies showed that neuronal age can affect both neurite outgrowth and the axonal transport of growth-associated proteins (Skene and Willard, 1981; Collins and Lee, 1982), and subsequent studies in a variety of CNS and PNS neurons have shown a decrease in the neuronal capacity for axon regeneration during the embryonic or early postnatal period (Chen et al., 1995; Li et al., 1995; Dusart et al., 1997; Borisoff et al., 2000; Condic, 2001; Mouveroux et al., 2001). In retinal ganglion cells this reduction is clearly intrinsic to neurons, because it is maintained even in low-density dissociated cultures (Goldberg et al., 2002).

Besides detecting changes in developing brainstem neurons, our approach also allowed us to quantify the effect of maturation in the spinal cord environment. We found that changes in the spinal cord between E9 and E18 caused a partial reduction in axon regeneration, evident in the 55% reduction in axon regeneration from younger neurons as they were confronted by E18 compared to E9 spinal cord explants. However, while changes in the environment of the spinal cord did reduce axon regeneration, almost half of the younger neurons were still able to regenerate long axons into older cord. Given the previous emphasis on myelin inhibition in the developing spinal cord (Keirstead et al., 1992; Varga et al., 1995), it is significant that axons from E9 brainstem were able to regenerate in myelinated white matter, both in slice culture and *in vivo*. This ability of brainstem-spinal projection neurons to regenerate axons into myelinated white matter is consistent with previous findings from other CNS neurons (Li and Raisman, 1993; Davies et al., 1999; Mingorance et al., 2004). We conclude that developmental changes in the spinal cord environment, including myelination, are sufficient to reduce but not to abolish axon regeneration.

Our results differ somewhat from previous research in the embryonic chick spinal cord. Keirstead et al. (1992) found that when myelination is delayed, regeneration succeeds after spinal transections on E15. However, our results would predict that even in the absence of myelin, older neurons would regenerate poorly. One possibility is that the treatments used to delay myelination in that previous study had unexpected effects on neural development, leaving brainstem projection neurons in a more immature state. Myelination itself may be a necessary step in signaling neurons to alter their internal growth potential (Kapfhammer and Schwab, 1994), although this hypothesis is not universally supported (Bouslama-Oueghlani et al., 2003). It is interesting to note that demyelination in posthatch chicks had a markedly smaller effect on axon regeneration, which hints that disruption of myelin delays but does not prevent a neuron-intrinsic switch to poor regeneration (Keirstead et al., 1995).

It has recently been suggested that the primary role of repulsive cues associated with myelin is not to inhibit axon growth, but actually to promote it by channeling axons and preventing excessive adhesion (Raisman, 2004). This model argues that axons are guided between myelin sheaths by a network of astrocytic processes that provide positive growth cues, and that disruption of this geometry is primarily responsible for the failure of axon regeneration following injury. This model would resolve the apparent paradox that robust axon growth is possible in myelinated environments that presumably express inhibitory molecules, as shown here and elsewhere (Li and Raisman, 1993; Davies et al., 1999; Mingorance et al., 2004). It would be interesting in our system to determine whether younger axons regenerating into older spinal cord do so in close apposition to astrocyte processes, as suggested by this model.

Our results have important implications for the treatment of spinal cord injury in the adult. Our data show that during development, maturation of both spinal cord and projection neurons contributes to reducing axon regeneration. Conversely, this suggests that both environmental and neuronal limitations must be removed to fully restore regeneration in the adult spinal cord. This conclusion has been stated previously in qualitative terms, but the strength of our coculture approach is our ability to assign quantitative value to neuronal and environmental maturation. In this regard, our most important finding is that almost 90% of older neurons fail to regenerate axons even when presented with early embryonic spinal cord. These data predict that even with successful treatment of the spinal cord environment, the low growth capacity of mature neurons will limit axon regeneration in the spinal cord to a small fraction of its full potential. Indeed, most spinal injury research supports our conclusions. Despite the wide variety of strategies used to improve the environment of the adult spinal cord, in all cases only a small minority of descending projection neurons respond by regenerating axons (David and Aguayo, 1981; Dyer et al., 1998; Dergham et al., 2002; GrandPre et al., 2002; Jin et al., 2002; Fouad et al., 2004; Kim et al., 2004). Our findings explain this poor response by showing that developmental changes within projection neurons substantially limit axon growth.

The low regenerative capacity of adult CNS is increasingly recognized as a major barrier to axonal regeneration, and a variety of strategies have been attempted to increase the intrinsic regenerative ability of CNS neurons. These include the application of neurotrophins (Kobayashi et al., 1997; Plunet et al., 2002), elevating cAMP levels within injured neurons (Lu et al., 2004; Nikulina et al., 2004), and combinatorial approaches such as exposing neurons to macrophage-derived stimulatory factors while simultaneously decreasing their sensitivity to myelin inhibition (Yin et al., 2003; Fischer et al., 2004a,b). These treatments have succeeded in increasing axon growth, which supports the general notion that a low capacity for growth on the part of CNS neurons does limit axon regeneration. On the other hand, compared to regeneration in the embryonic spinal cord, these approaches have so far succeeded in activating only a fraction of the full regenerative capacity; generally no more than 10% of neurons respond to treatment, and regenerate axons slowly or for short distances. Clearly more work is needed to clarify the mechanisms that regulate the neuronal capacity for axon growth, and one promising strategy is to study neurons during the developmental transition that limits axon growth. Our research provides the first quantitative demonstration of the extent to which changes within neurons limit axon regeneration in the developing spinal cord, and provides a model for further research into this important developmental transition.

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## REFERENCES

Borisoff JF, Pataky DM, McBride CB, Steeves JD. 2000. Raphe-spinal neurons display an age-dependent differential capacity for neurite outgrowth compared to other brainstem-spinal populations. Exp Neurol 166:16–28.

Bouslama-Oueghlani L, Wehrle R, Sotelo C, Dusart I. 2003. The developmental loss of the ability of Purkinje cells to regenerate their axons occurs in the absence of myelin: an in vitro model to prevent myelination. J Neurosci 23:8318–8329.

Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, et al. 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416:636–640.

Bregman BS, Kunkel-Bagden E, McAtee M, O'Neill A. 1989. Extension of the critical period for developmental plasticity of the corticospinal pathway. J Comp Neurol 282:355–370.

Calof AL, Campanero MR, O'Rear JJ, Yurchenco PD, Lander AD. 1994. Domain-specific activation of neuronal migration and neurite outgrowth-promoting activities of laminin. Neuron 13:117–130.

Chen DF, Jhaveri S, Schneider GE. 1995. Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. Proc Natl Acad Sci 92:7287–7291.

Cohen J, Burne JF, Winter J, Bartlett P. 1986. Retinal ganglion cells lose response to laminin with maturation. Nature 322:465–467.

Collins F, Lee MR. 1982. Reversible developmental change in the ability of ciliary ganglion neurons to extend neurites in culture. J Neurosci 2:424–430.

Condic ML. 2001. Adult neuronal regeneration induced by transgenic integrin expression. J Neurosci 21:4782–4788.

David S, Aguayo AJ. 1981. Axonal elongation into peripheral nervous system ''bridges'' after central nervous system injury in adult rats. Science 214:931–933.

Davies SJ, Goucher DR, Doller C, Silver J. 1999. Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. J Neurosci 19:5810–5822.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. 2002. Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22:6570–6577.

Domeniconi M, Cao Z, Spencer T, Sivasankaran R, Wang K, Nikulina E, Kimura N, et al. 2002. Myelin-associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth. Neuron 35:283–290.

Dusart I, Airaksinen MS, Sotelo C. 1997. Purkinje cell survival and axonal regeneration are age dependent: an in vitro study. J Neurosci 17:3710–3726.

Dyer JK, Bourque JA, Steeves JD. 1998. Regeneration of brainstem-spinal axons after lesion and immunological disruption of myelin in adult rat. Exp Neurol 154:12–22.

Fischer D, He Z, Benowitz LI. 2004a. Counteracting the Nogo receptor enhances optic nerve regeneration if retinal ganglion cells are in an active growth state. J Neurosci 24:1646–1651.

Fischer D, Petkova V, Thanos S, Benowitz LI. 2004b. Switching mature retinal ganglion cells to a robust growth state in vivo: gene expression and synergy with RhoA inactivation. J Neurosci 24:8726–8740.

Fouad K, Klusman I, Schwab ME. 2004. Regenerating corticospinal fibers in the Marmoset (Callitrix jacchus) after spinal cord lesion and treatment with the anti-Nogo-A antibody IN-1. Eur J Neurosci 20:2479–2482.

Fournier AE, GrandPre T, Strittmatter SM. 2001. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409:341–346.

Fournier AE, Takizawa BT, Strittmatter SM. 2003. Rho kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23:1416–1423.

Fry EJ, Stolp HB, Lane MA, Dziegielewska KM, Saunders NR. 2003. Regeneration of supraspinal axons after complete transection of the thoracic spinal cord in neonatal opossums (Monodelphis domestica). J Comp Neurol 466:422–444.

Goldberg JL. 2004. Intrinsic neuronal regulation of axon and dendrite growth. Curr Opin Neurobiol 14:551–557.

Goldberg JL, Klassen MP, Hua Y, Barres BA. 2002. Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. Science 296:1860–1864.

GrandPre T, Li S, Strittmatter SM. 2002. Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417:547–551.

Hasan SJ, Keirstead HS, Muir GD, Steeves JD. 1993. Axonal regeneration contributes to repair of injured brainstem-spinal neurons in embryonic chick. J Neurosci 13: 492–507.

Ivins JK, Colognato H, Kreidberg JA, Yurchenco PD, Lander AD. 1998. Neuronal receptors mediating responses to antibodyactivated laminin-1. J Neurosci 18:9703–9715.

Jin Y, Fischer I, Tessler A, Houle JD. 2002. Transplants of fibroblasts genetically modified to express BDNF promote axonal regeneration from supraspinal neurons following chronic spinal cord injury. Exp Neurol 177:265–275.

Kapfhammer JP, Schwab ME. 1994. Inverse patterns of myelination and GAP-43 expression in the adult CNS: neurite growth inhibitors as regulators of neuronal plasticity? J Comp Neurol 340:194–206.

Kawano H, Li HP, Sango K, Kawamura K, Raisman G. 2005. Inhibition of collagen synthesis overrides the age-related failure of regeneration of nigrostriatal dopaminergic axons. J Neurosci Res 80:191–202.

Keirstead HS, Dyer JK, Sholomenko GN, McGraw J, Delaney KR, Steeves JD. 1995. Axonal regeneration and physiological activity following transection and immunological disruption of myelin within the hatchling chick spinal cord. J Neurosci 15:6963–6974.

Keirstead HS, Hasan SJ, Muir GD, Steeves JD. 1992. Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. Proc Natl Acad Sci USA 89:11664–11668.

Kim JE, Liu BP, Park JH, Strittmatter SM. 2004. Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. Neuron 44:439–451.

Kobayashi NR, Fan DP, Giehl KM, Bedard AM, Wiegand SJ, Tetzlaff W. 1997. BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Talpha1-tubulin mRNA expression, and promote axonal regeneration. J Neurosci 17: 9583–9595.

Li D, Field PM, Raisman G. 1995. Failure of Axon Regeneration in Postnatal Rat Entorhinal hippocampal Slice Coculture is Due to Maturation of the Axon, not that of the Pathway or Target. Eur J Neurosci 7: 1164–1171.

Li Y, Raisman G. 1993. Long axon growth from embryonic neurons transplanted into myelinated tracts of the adult rat spinal cord. Brain Res 629:115–127.

Liu BP, Fournier A, GrandPre T, Strittmatter SM. 2002. Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. Science 297:1190–1193.

Lu P, Yang H, Jones LL, Filbin MT, Tuszynski MH. 2004. Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. J Neurosci 24:6402–6409.

Macklin WB, Weill CL. 1985. Appearance of myelin proteins during development in the chick central nervous system. Dev Neurosci 7:170–178.

Mingorance A, Fontana X, Sole M, Burgaya F, Urena JM, Teng FY, Tang BL, et al. 2004. Regulation of Nogo and Nogo receptor during the development of the entorhino-hippocampal pathway and after adult hippocampal lesions. Mol Cell Neurosci 26:34–49.

Mouveroux JM, Lakke EA, Marani E. 2001. Lumbar spinal cord explants from neonatal rat display age-related decrease of outgrowth in culture. Neurosci Lett 311:69–72.

Nikulina E, Tidwell JL, Dai HN, Bregman BS, Filbin MT. 2004. The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. Proc Natl Acad Sci USA 101:8786–8790.

Okado N, Oppenheim RW. 1985. The onset and development of descending pathways to the spinal cord in the chick embryo. J Comp Neurol 232:143–161.

Pataky DM, Borisoff JF, Fernandes KJ, Tetzlaff W, Steeves JD. 2000. Fibroblast growth factor treatment produces differential effects on survival and neurite outgrowth from identified bulbospinal neurons in vitro. Exp Neurol 163:357–372.

Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB. 2004. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 10:610–616.

Plunet W, Kwon BK, Tetzlaff W. 2002. Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. J Neurosci Res 68:1–6.

Raisman G. 2004. Myelin inhibitors: does NO mean GO? Nat Rev Neurosci 5:157–161.

Saunders NR, Kitchener P, Knott GW, Nicholls JG, Potter A, Smith TJ. 1998. Development of walking, swimming and neuronal connections after complete spinal cord transection in the neonatal opossum, Monodelphis domestica. J Neurosci 18:339–355.

Savio T, Schwab ME. 1990. Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. Proc Natl Acad Sci USA 87:4130–4133.

Shimizu I, Oppenheim RW, O'Brien M, Shneiderman A. 1990. Anatomical and functional recovery following spinal cord transection in the chick embryo. J Neurobiol 21:918–937.

Skene JH, Willard M. 1981. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. J Cell Biol 89:96–103.

So KF, Schneider GE, Ayres S. 1981. Lesions of the brachium of the superior colliculus in neonate hamsters: correlation of anatomy with behavior. Exp Neurol 72: 379–400.

ten Donkelaar HJ. 2000. Development and regenerative capacity of descending supraspinal pathways in tetrapods: a comparative approach. Adv Anat Embryol Cell Biol 154:iii–ix, 1–145.

Vanek P, Thallmair M, Schwab ME, Kapfhammer JP. 1998. Increased lesion-induced sprouting of corticospinal fibres in the myelin-free rat spinal cord. Eur J Neurosci 10:45–56.

Varga ZM, Schwab ME, Nicholls JG. 1995. Myelin-associated neurite growth-inhibitory proteins and suppression of regeneration of immature mammalian spinal cord in culture. Proc Natl Acad Sci USA 92: 10959–10963.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z. 2002. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941–944.

Yin Y, Cui Q, Li Y, Irwin N, Fischer D, Harvey AR, Benowitz LI. 2003. Macrophage-derived factors stimulate optic nerve regeneration. J Neurosci 23:2284–2293.