

# Protein Synthesis in Distal Axons is Not Required for Axon Growth in the Embryonic Spinal Cord

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**ABSTRACT:** It is now well established that new proteins are synthesized in the distal segments of elongating axons, where they may play an essential role in some guidance decisions. It remains unclear, however, whether distal protein synthesis also plays an essential role in axon growth *per se*. Previous *in vitro* experiments have shown that blocking protein synthesis in distal axons has no effect on the rate of axonal advance. However, because these experiments were performed *in vitro* and over a relatively short time period, the role of distal protein synthesis over longer periods and in a native tissue environment remained untested. Here, we tested whether protein synthesis in distal axons plays an essential role in the elongation of descending axons in the embryonic spinal cord. We developed an *in situ* model of the brainstem-spinal projec-

tion of the embryonic chick, and developed a split-chamber method in which inhibitors of proteins synthesis could be applied independently to cell bodies in the brainstem or to distal axons in the spinal cord. When protein synthesis was blocked in distal axons, axon growth remained robust for 2 days, which is the length of the experiment. However, when protein synthesis was blocked only in the brainstem, axonal elongation in the spinal cord ceased within 6 h. These data showed that protein synthesis in the distal axon is not essential to continue the advance of axons. Rather, essential proteins are synthesized more proximally and then transported rapidly to the distal axon. © 2007 Wiley

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## INTRODUCTION

During development, axons extend from neuronal perikarya to their synaptic targets, sometimes covering long distances. The spatial separation between the cell body and the distal axon may require that the growth cone, the motile tip of an extending axon, operate with a high degree of autonomy. A recently appreciated facet of this autonomy is the ability of distal axons to locally translate mRNA into protein,

as opposed to relying exclusively on proteins transported from the cell body. It is now clear that a wide variety of vertebrate axons synthesize protein in their distal segments (Davis et al., 1992; Eng et al., 1999; Zheng et al., 2001), and that, at least in culture, the capacity for distal protein synthesis is essential for growth cones to respond to several axonal guidance cues (Campbell and Holt, 2001; Brittis et al., 2002; Wu et al., 2005) or to reform after axotomy (Verma et al., 2005).

These findings raise the question of whether protein synthesis in distal axons plays only a regulatory role in modulating growth cone behavior, or whether it also plays a more fundamental role in supporting the extension of axons. Some have argued, based on the limited half life and slow transport rates of cytoskeletal proteins, that protein synthesis within distal axons must play an essential role in supporting axon extension by replenishing degraded proteins (Twiss and van Minnen, 2006). On the other hand, experiments performed

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*in vitro* have shown repeatedly that when inhibitors of protein synthesis are applied to distal axons, growth cones remain motile and extend at normal rates, although they lose responsiveness to certain guidance cues (Eng et al., 1999; Campbell and Holt, 2001; Verma et al., 2005). These findings suggest that the extension of axons may be independent of distal protein synthesis. However, because most of these experiments lasted for short periods and were performed *in vitro* on simple surfaces, such as laminin coated dishes, it is possible that over longer periods and in native tissue environments, protein synthesis in distal axons might play a more essential role.

Here, we examine the role of distal protein synthesis in growth of CNS axons in the embryonic spinal cord. We developed an *in situ* model of descending axon growth using explants of brainstem and spinal cord from the embryonic chick, and devised a means by which inhibitors of protein synthesis could be applied independently either to the cell bodies of brainstem projection neurons (cell body compartment) or to the spinal cord (axonal compartment). We found that axon growth was reversibly blocked when protein synthesis inhibitors were applied to the cell body compartment, but growth was not inhibited when protein synthesis inhibitors were added only to the axonal compartment. Axon growth in the spinal cord ceased by 6 h after application of inhibitors of protein synthesis to cell bodies alone, and application of inhibitors to both cell bodies and axons did not result in a more rapid cessation of axon growth. These data show that the extension of supraspinal axons in the spinal cord is independent of protein synthesis in distal axons. Rather, proteins that are essential for axonal growth are synthesized in the cell body and then transported rapidly to distal axons. Protein synthesis in growth cones may play important roles in response to local regulatory cues, but not in sustaining axonal elongation.

## METHODS

### Retrograde Labeling and Explant Culture

Fertilized White Leghorn chicken eggs were incubated in a humidified incubator at 38°C. Brainstem-spinal neurons were retrogradely labeled as described (Okado and Oppenheim, 1985; Pataky et al., 2000; Blackmore and Letourneau, 2006). Briefly, on embryonic Day 5 (E5) windows were cut in shells and a crystal of DiI (Molecular Probes, Eugene, OR) was attached to an insect pin using egg albumin and inserted into cervical spinal cord. Embryos were returned to the incubator until E8, when brainstems were dissected and cut into transverse sections of 350  $\mu\text{m}$  thick-

ness, and then microdissected to isolate ventral–medial brainstem, the location of nuclei that provide descending input to the spinal cord. Explants were cultured in Neurobasal media with B27 supplements (Invitrogen, Carlsbad, CA) on glass cover slips that had been coated overnight with poly-D-Lysine (100  $\mu\text{g}/\text{mL}$ ) (Sigma, St. Louis, MO), rinsed extensively, and then coated with laminin (100  $\mu\text{g}/\text{mL}$ ) (Invitrogen).

Explants to be used in immunohistochemistry were cultured for 2 days, with the media refreshed at 24 h and then fixed in 4% paraformaldehyde. In experiments with protein synthesis inhibitors, cycloheximide or anisomycin (Calbiochem, La Jolla, CA) were added to the culture media at a concentration of 10  $\mu\text{M}$ . After 24 and 48 h in culture, explants were viewed under fluorescence using an Olympus XC-70 inverted microscope, and digital images of DiI-labeled axons extending from the explant were acquired using a SPOT digital camera and software (Diagnostic Instruments, Sterling Heights, NJ).

### Immunohistochemistry

After fixation in 4% paraformaldehyde, explants were blocked in 10% normal goat serum (Jackson Immunologicals, West Grove, PA) with 0.03% Triton X100 (Sigma), and then incubated overnight in primary antibodies in blocking solution. Primary antibodies were anti-initiation factor eIF-4E (Santa Cruz Biotechnologies, Santa Cruz, CA, 1:50 dilution), antiphosphorylated eIF4E (Cell Signaling Technologies, Danvers, MA, 1:100 dilution), and antiribosomal protein P0 (Immunovision, Springdale, AZ, 1:500 dilution). Secondary antibodies were Alexafluor 488-conjugated, raised in goat (1:1000, overnight, 4°C; Molecular Probes). Cover slips were mounted in No-Fade media and digital images of DiI-labeled growth cones were acquired using viewed using a SPOT digital camera and software (Diagnostic Instruments, Sterling Heights, NJ) and an Olympus XC-70 inverted microscope.

### Brainstem-Spinal Coculture

To anterogradely label axons that project from brainstem to spinal cord, windows were cut in eggs on E7 and a solution of CM-DiI (10  $\mu\text{g}/\mu\text{L}$  in DMSO) (Molecular Probes) was injected into the brainstem using a pulled glass pipette attached to a picospritzer (General Valve Corporation, Fairchild, NJ). Eggs were then returned to the incubator. The following day, DiI-labeled brainstems and spinal cords were carefully dissected as 1 unit and placed in F12 media (Invitrogen) supplemented with 10% bovine calf serum (Hyclone, Logan, UT). Spinal cords attached to brainstems extended caudally to at least the lumbar enlargement. A second spinal cord from an E8 embryo that had received no DiI injection was also dissected and placed in similar media. The brainstems and spinal cords were cut along the dorsal midline to generate an open-book configuration and placed ventral side down on Millicell culture inserts (30 mm, 0.04  $\mu\text{m}$ , Millipore, Billerica, MA) in 60 mm Falcon

Petri dishes containing 2 mL of Neurobasal media with B27 supplements. The spinal cord attached to the DiI-labeled brainstem was cut with an insect pin at distance of 3 mm from the brainstem. The unlabeled spinal cord was cut in the mid-cervical region, and the rostral end of the unlabeled spinal cord was placed adjacent to the cut end of the labeled spinal cord. This arrangement preserved the geometry found *in vivo*, with a complete transection in the cervical spinal cord. Explants were maintained in culture for the duration of the experiment (2–4 days), with media refreshed daily.

### Split-Chamber System

To create divided culture chambers that would allow drug treatments to be applied only to axons or to cell bodies, Silicone II silicone sealant (GE Sealants and Adhesives, Hunterville, NC) was extruded from a blunt-ended 20 gauge needle to produce a thin line that bisected the sides and bottom of a Falcon 1029 60 mm Petri dish. A thin line of sealant was then applied along the bottom of a 30 mm culture insert, again bisecting the insert into two equal halves. The insert was placed into the center of the Petri dish with the lines of sealant lying atop one another. One milliliter of media was added to each half of the Petri dish. Media contacted the bottom of the culture insert, but remained below the height of the silicon barrier. Explant cultures were placed atop the culture insert. To prevent mixing of media between the two halves of the dish we found that it was essential that the depth of the media not exceed the height of the membrane in the culture insert. If the depth of the media was too great a meniscus of media formed over the explant, allowing diffusion over the silicon barrier.

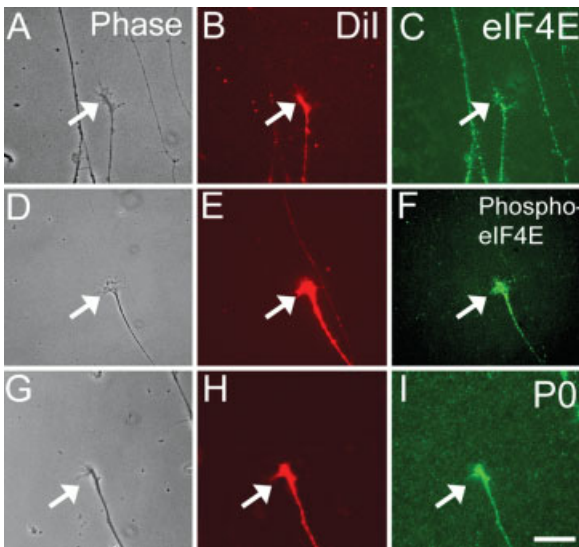
Brainstem-spinal cocultures, prepared as described above, were placed atop the insert with the spinal cord running over the top of the silicon from one half of the dish to the other (see Fig. 4). The compartment that contained the brainstem was designated as the cell body compartment, and the compartment that contained distal spinal cord was designated as the axonal compartment. To verify separation between the two compartments, one of three treatments were applied. First, the fluorescent molecule Alexafluor 546 (Molecular probes) was added at a concentration of 1  $\mu\text{M}$  to the axonal or cell body compartment. Each day for 3 days digital images of each compartment were acquired using with identical acquisition parameters, using an Olympus XC-70 inverted microscope and a SPOT camera and software. Metamorph software (Universal Imaging, West Chester, PA) was used for quantitative image analysis. Average fluorescence intensity was determined for each compartment and normalized to background fluorescence in a separate set of dishes that received no Alexafluor. In a second control, calcein AM (Molecular probes) was added to the axonal compartment at a concentration of 2  $\mu\text{M}$ . Fluorescent images of the spinal cord and brainstem were acquired at 0, 24, and 48 h using an Olympus XC-70 inverted microscope and a SPOT camera and software. Finally, chambers were prepared that contained two brainstem-spinal cord pairs, one with the transection site in the axonal compartment and the other with

the transection site in the cell body compartment. Nocodazole (Sigma), a microtubule depolymerizing drug that blocks axon growth was added to the axonal compartment at a concentration of 1  $\mu\text{M}$  (Gallo and Letourneau, 1999). After 2 days, explants were fixed in 4% paraformaldehyde and DiI-labeled axons viewed under fluorescence with an Olympus XC-70 inverted microscope.

In initial experiments using explants of brainstem on laminin, we found that inhibition of axon growth was maximal and completely reversible at concentrations of 10  $\mu\text{M}$  cycloheximide or 10  $\mu\text{M}$  anisomycin, added at the time of plating and refreshed daily. Ten micromolar of either of the drug was used for all subsequent experiments in the split chamber culture system. Depending on the experiment, inhibitors of protein synthesis were applied to the cell body compartment, the axonal compartment, or both. Compartments that received no inhibitor were treated with an identical volume of DMSO. In experiments that ran multiple days, drugs were refreshed daily. When drugs were washed out, we performed five washes in culture media, with 10 min between each wash.

### RESULTS

In the embryonic chick, motor control is mediated by axons that descended from reticular, vestibular, and raphe nuclei in the brainstem to synapse on targets in the spinal cord (ten Donkelaar, 2000). Descending axon growth can first be detected in the spinal cord on embryonic Day 3 (E3), and axons continue to arrive and extend in the spinal cord until embryonic Day 10 [(Okado and Oppenheim, 1985) and our own unpublished observations]. We first asked whether elements of the protein translation machinery are present in distal axons and growth cones of brainstem-spinal projection neurons. To specifically label neurons in the brainstem that project axons to the spinal cord we used a retrograde prelabeling strategy, described previously (Blackmore and Letourneau, 2006). Briefly, a crystal of DiI was inserted in ovo into the spinal cord on E5. Embryos then developed normally until E8, at which time explants of brainstem tissue were prepared and placed in culture on laminin substrates. Axons grew from the brainstem explants onto the laminin substrate, and some of those axons were labeled with DiI, identifying them as brainstem-spinal projection axons. Examining only DiI-labeled axons, we performed immunohistochemistry for ribosomal protein P0, eIF4E, and phosphorylated eIF4E, elements of the translation machinery that have previously been detected in a variety of peripheral and central axons (Campbell and Holt, 2001; Piper and Holt, 2004; Verma et al., 2005). As shown in Figure 1, brainstem-spinal axons expressed ribosomal protein P0, eIF4E, and phospho-4E-BP1. We



**Figure 1** Brainstem-spinal axons express markers for protein translation. (A,D,G) E9 brainstem axons cultured for 2 days on a laminin substrate. (B,E,H) DiI label in axons, identifying them as brainstem-spinal axons. (C) Immunohistochemistry for eukaryotic initiation factor 4e. (F) Immunohistochemistry for phosphorylated eIF4E, a marker for active protein translation. (G) Immunohistochemistry for ribosomal protein P0. Scale bar is 20  $\mu\text{m}$ . [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

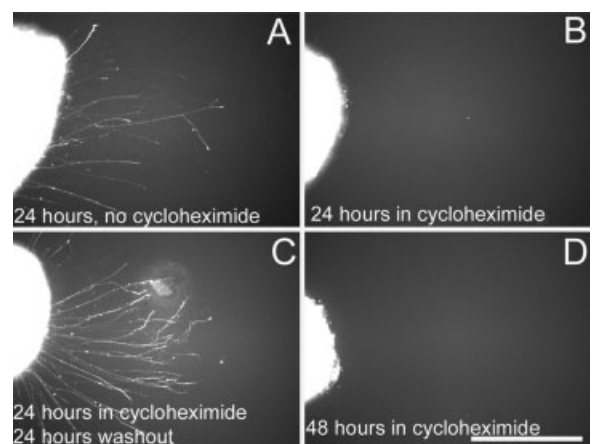
conclude that elements of the translation machinery are present in the distal portions of brainstem-spinal axons as they extend *in vitro*.

To examine the functional role of protein synthesis in axon growth we used two inhibitors of protein synthesis, cycloheximide or anisomycin. We used cycloheximide at a concentration that has previously been shown to inhibit protein synthesis in cultured chick neurons (Luduena, 1973; Oppenheim et al., 1990). In initial experiments we applied inhibitors of protein synthesis to explants of E9 brainstem cultured on laminin substrates. As shown in Figure 2, cycloheximide (10  $\mu\text{M}$ ) completely blocked growth by DiI-labeled brainstem-spinal projection axons. Importantly, axon growth resumed within 24 h after washing out cycloheximide, showed that the inhibition of axonal growth was reversible and not due to cell death. Anisomycin (10  $\mu\text{M}$ ) also completely and reversibly blocked axon growth. These data showed that, as expected, axon growth by brainstem-spinal projection neurons requires new protein synthesis and provide a positive control for the effectiveness of our protein synthesis inhibitors.

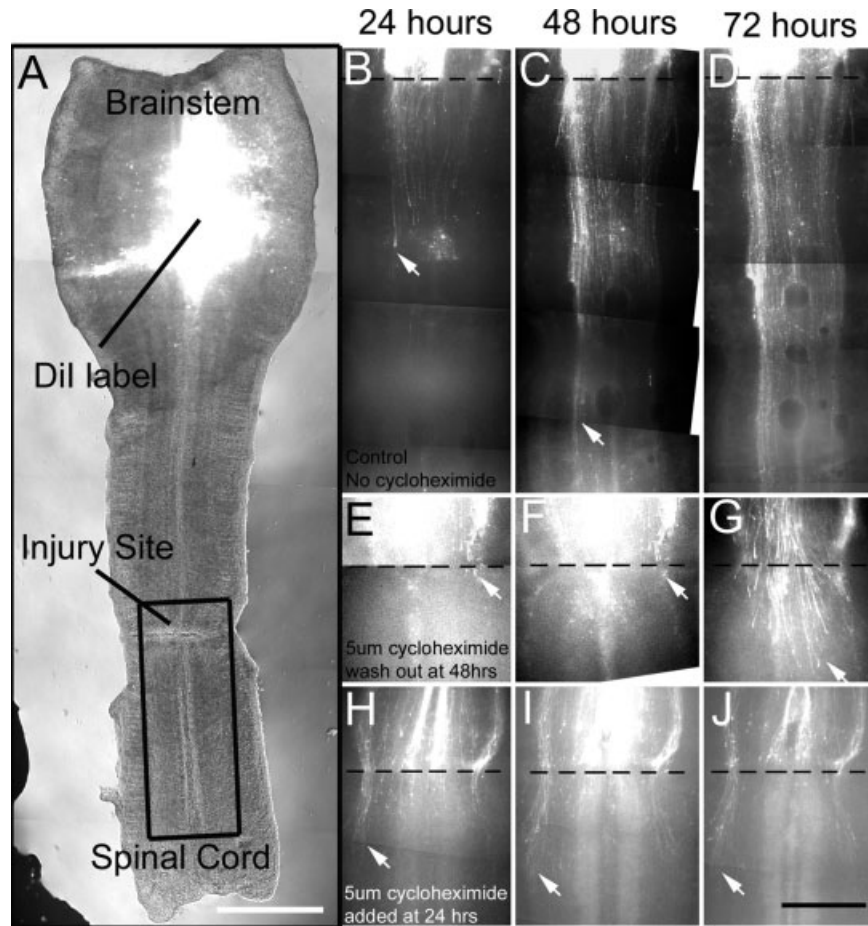
To study the role of protein synthesis in a situation more similar to that found *in vivo*, we developed an *in situ* culture system (Figure 3). On embryonic Day

7, DiI was injected into the brainstem of embryos *in ovo* to label axons that grew from the brainstem down the spinal cord. Twenty-four hours later, brainstems with the spinal cord still attached were dissected as one piece from the embryo and placed on Millicell culture inserts. The spinal cord was cut at a distance of 3 mm from the brainstem, and the cut end of the spinal cord was placed in contact with a spinal explant from another embryo that had received no DiI label. The edges of spinal cord explants began to fuse by 4 h in culture, and DiI-labeled axons had begun crossing the boundary and extending into the unlabeled spinal cord explant after 6 h in culture. At this age, new axons from brainstem neurons are still arriving in cervical spinal cord (Okado and Oppenheim, 1985) (our unpublished observations), and previously grown axons are also capable of regeneration after transection (Shimizu et al., 1990; Hasan et al., 1993; Blackmore and Letourneau, 2006). Therefore, the axons that we observe crossing the boundary between the spinal explants are likely a mix of regenerating axons and newly growing axons that had not yet reached the site of transection.

This *in situ* model offers two important advantages over a simpler *in vitro* model. First, growing axons



**Figure 2** Inhibitors of protein synthesis block the growth of brainstem-spinal axons *in vitro*. E9 brainstem explants containing DiI-labeled spinal projection neurons were cultured on laminin. DiI label is visible in axons, identifying them as brainstem-spinal axons (A) E9 brainstem explant, cultured 24 h in the absence of protein synthesis inhibitors. Axon growth is robust. (B) E9 brainstem explant cultured 24 h in the presence of cycloheximide. No axons regenerate. (C) E9 brainstem explant, cultured 24 h in 10  $\mu\text{M}$  cycloheximide, and then an additional 24 h without cycloheximide. Axon growth recovers when cycloheximide is washed away. (D) E9 brainstem explant cultured 48 h in the presence of cycloheximide. Axon growth is completely blocked. Scale bar is 500  $\mu\text{m}$ .



**Figure 3** Inhibitors of protein synthesis block the growth of brainstem-spinal axons *in situ*. (A) Labeled E8 brainstem with attached spinal cord, cultured 24 h with an unlabeled spinal explant. DiI label was injected into the brainstem. Box shows approximate location of higher power fluorescent images. (B–D) DiI labeled axons at the transection site in the absence of protein synthesis inhibitors after 24 (B), 48 (C), and 72 (D) h. Axon growth is robust. (E,F) DiI labeled axons at the transection site in the presence of cycloheximide after 24 (E) or 48 (F) h. (G) The same transection site as (E) and (F), 24 h after cycloheximide was washed out. Axon growth is now robust. (H) DiI-labeled axons at the transection site in the absence of inhibitors, 24 h after injury. (I,J) Same injury site as (H), 24 (I), or 48 (J) h after cycloheximide was added. Axons extend several hundred microns during the first 24 h in cycloheximide, and then stop elongating during the next 24 h. Scale bars are 1 mm in A, 500  $\mu\text{m}$  in B–J.

encounter a situation close to their naturally complex spinal environment, as opposed to an artificial laminin substrate. Second, at the time of exposure to the drug, the ends of the axons are located many millimeters from the neuronal cell body, instead of only tens of microns typical of a smaller explant.

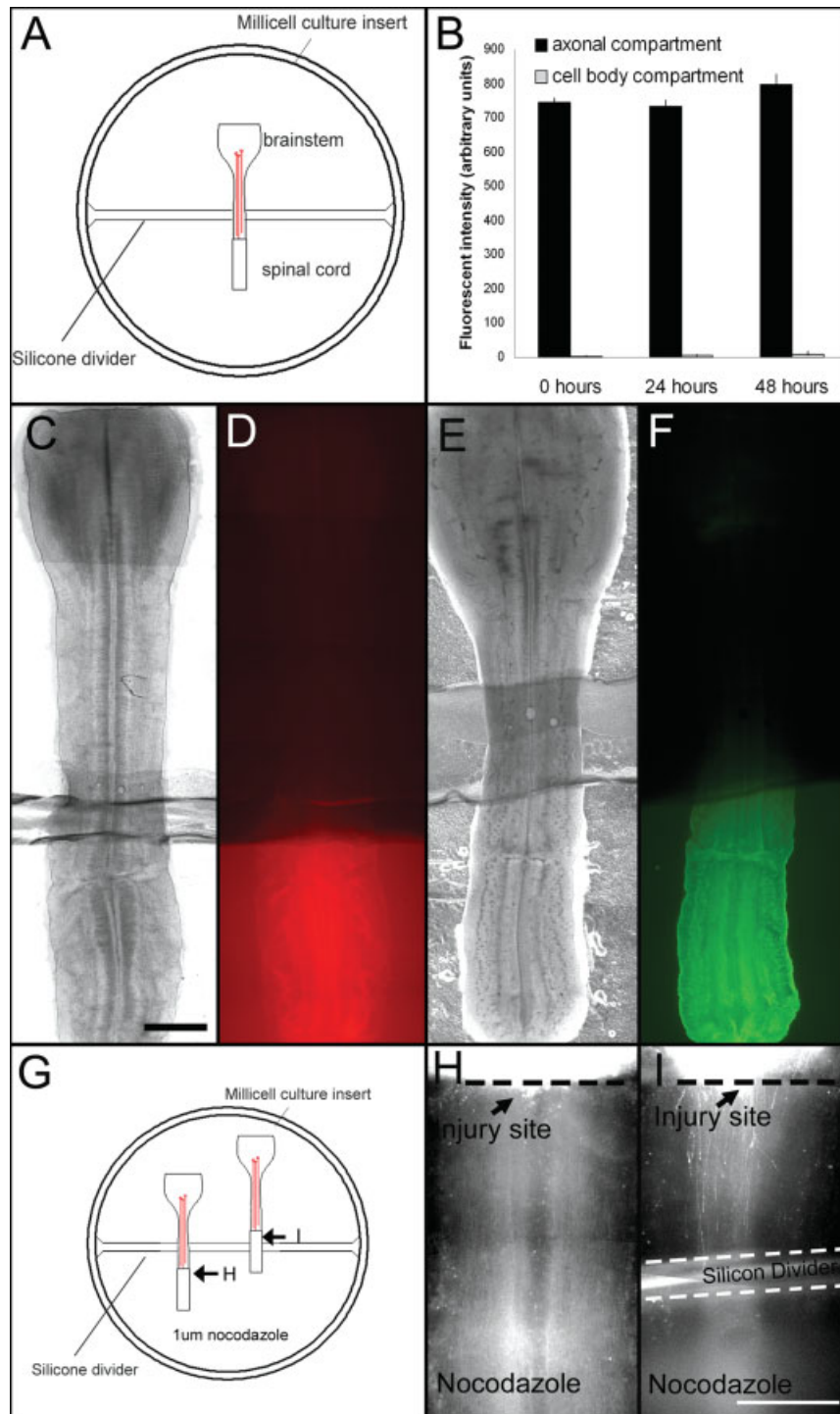
As shown in Figure 3, when protein synthesis inhibitors were applied to our *in situ* model, growth of brainstem axons into the spinal cord explant was completely blocked. In the absence of the inhibitors many labeled axons from brainstem neurons grew more than 1 mm per day into the cocultured spinal explant (10 of 10 explants). Addition of 10  $\mu\text{M}$  cyclo-

heximide or 10  $\mu\text{M}$  anisomycin completely blocked axon growth into the distal spinal explant (4 of 4 cocultures in each drug). When the drugs were washed out after an initial 48 h in culture, axon growth began, showing that the drugs were not acting by killing neurons. Furthermore, if we did not add the inhibitors until 24 h after initiating the cocultures, at a time when many axons had elongated across the boundary, axons grew only a few hundred additional microns, and then ceased elongating. This suggests that protein synthesis is required for the continued extension of axons. We conclude that even when growth cones are located several millimeters from the cell body, new

protein synthesis is required for successful axonal growth in the spinal environment.

The experiments above demonstrate an essential role for newly synthesized proteins in descending axon growth in the spinal cord, but leave unresolved the question of where in the cell these essential pro-

teins are synthesized. To determine whether essential new proteins were synthesized in the cell body or the distal axon, we developed a method of dividing the culture chamber into separate compartments. Our method is similar in principle to the Campenot chamber (Campenot, 1982; Eng et al., 1999), but is used in



**Figure 4** (See legend on following page)

this case for slice culture of CNS neurons. Explants of brainstem with attached spinal cord were placed atop a Millicell insert that was bisected by a strip of silicon sealant. We positioned the explants such that the brainstem lay on one side of the divider (designated the cell body compartment), and the cut ends of the axons with the attached unlabeled spinal cord explant lay on the other side (designated the axonal compartment).

As shown in Figure 4, we performed three control experiments to verify separation between the contents of the two compartments. First, we added the fluorescent marker Alexafluor 568 to the axonal compartment and acquired digital images to compare the fluorescence in the axonal versus cell body compartment. After 2 days, fluorescence in the axonal compartment remained intense, while fluorescence in the cell body compartment remained undetectable. This suggested that media remained separate between the two compartments. We were concerned, however, that drug could leak along the explant itself. We therefore applied calcein AM, a cell-permeant calcium indicator that is taken up by cells and then retained intracellularly, to the axonal compartment. Cells that have access to calcein AM accumulate it and fluoresce. After 2 days in culture, cells in the axonal compartment were brightly labeled, while cell bodies in the brainstem remained unlabeled. Therefore cell bodies in the brainstem did not have access to extracellular calcein AM applied to the axonal compartment, again demonstrating an effective separation between the two compartments.

Finally, as a more direct functional test, we applied nocodazole, a microtubule depolymerizing drug that blocks neurite growth, to one half of our compartmentalized culture dish (Gallo and Letourneau, 1999). In these experiments, we prepared split chambers that contained two brainstem-spinal explants. The spinal

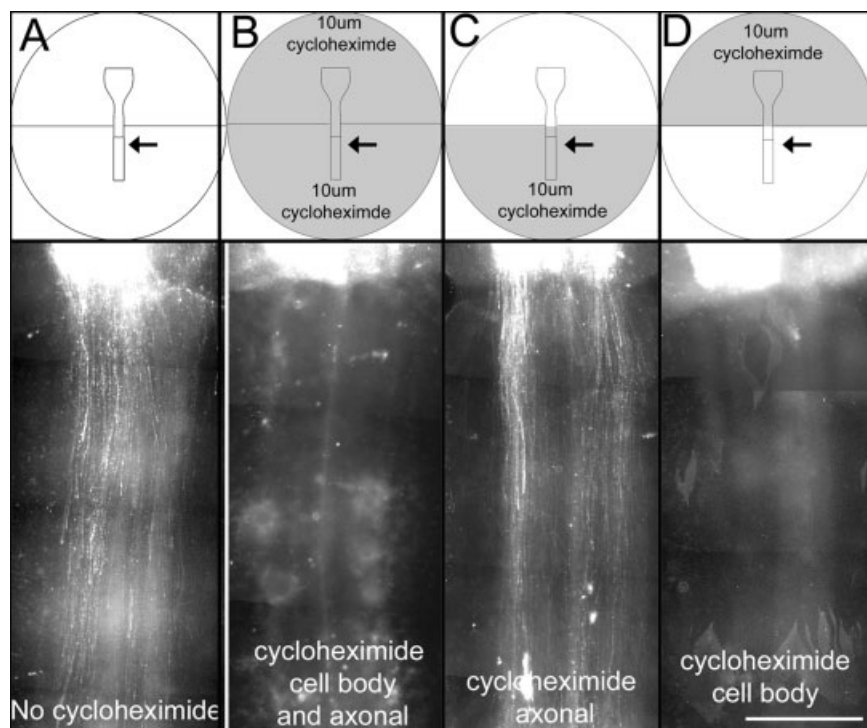
cords of both explants crossed the silicon divider. However, in one case the transection site and apposed spinal cord explant were rostral to the silicone divider, while in the other case the transection site and apposed spinal cord were caudal to the silicone divider, in the other compartment. Nocodazole was added only to the caudal compartment. In this experiment, axon growth was blocked when the transection site was located in the compartment that had received nocodazole, but when the transection site was located in the rostral compartment, there was no inhibition of axonal growth in the rostral compartment, despite the presence of nocodazole in the caudal compartment. This result showed directly that drugs have functional effects only in the chamber compartment to which they are applied.

We next used our divided culture to determine the site of protein synthesis required for axonal growth (Fig. 5). When brainstems with attached unlabeled spinal cords were cut and placed in our divided culture system, axon growth across the transection was robust in the absence of inhibitors, and, as expected, was blocked when inhibitors were added to both the axonal and cell body compartments. When inhibitors of protein synthesis were applied only to the cell body compartment, axon growth was blocked completely (seven of 7-cultures), and axons never crossed into the distal spinal explant. When protein synthesis inhibitors were applied only to the axonal compartment, however, axon growth remained robust (seven of 8-cultures), with many axons extending more than 2 mm into the distal spinal cord over the course of 2 days. These data suggest that protein synthesis in distal axons is not essential for rapid axon growth, while protein synthesis in the cell body or proximal axon is both necessary and sufficient to support axonal growth.

We next examined the temporal dynamics of axon growth and the effects of protein synthesis inhibitors

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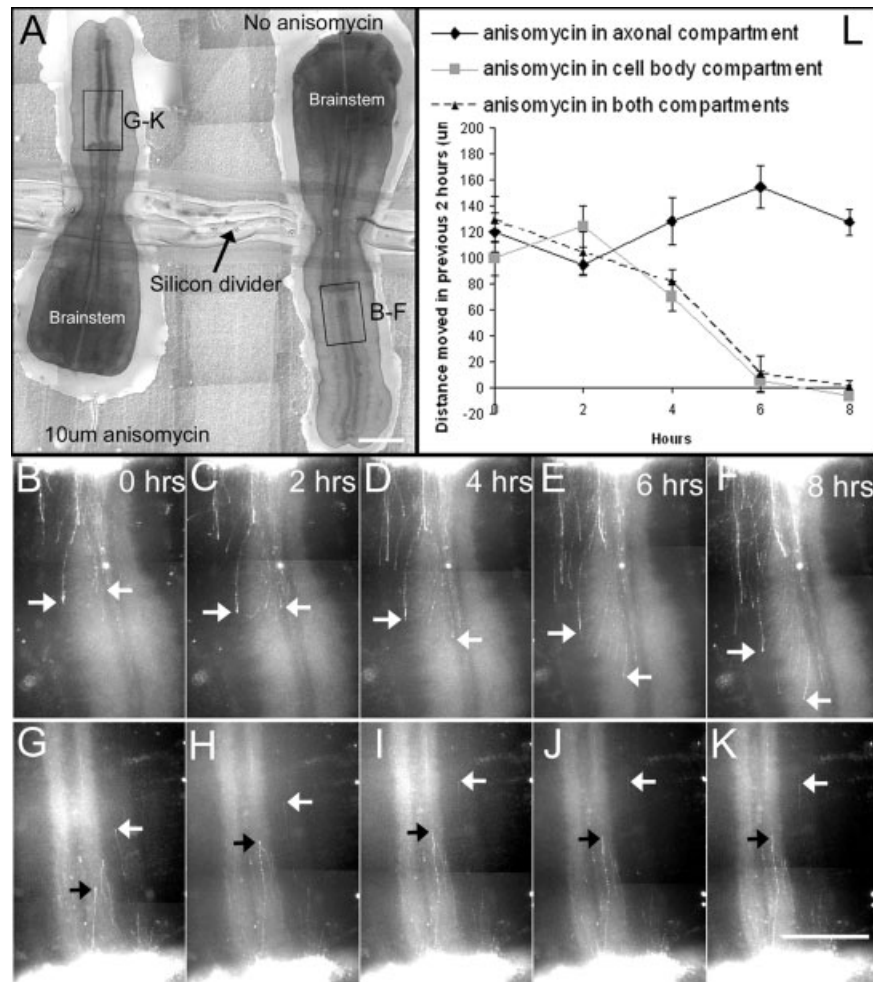
**Figure 4** A split chamber method to isolate brainstem cell bodies and distal axons. (A) Schematic of a chamber split into a cell body and axonal compartment. (B) Quantification of fluorescence after Alexafluor 568 is added to the axonal compartment. Fluorescence remains high in the axonal compartment, and absent from the cell body compartment. (C,D) E8 brainstem with attached spinal cord. (D) Same brainstem as (C), viewed under fluorescence 48 h after alexafluor 568 was added to the axonal compartment. (E) Same brainstem as (C), viewed with fluorescence 48 h after calcein AM was added to axonal compartment. Fluorescence is visible only in the axonal compartment, indicating that cells in the cell body compartment do not have access to molecules added to the axonal compartment. (G) Schematic of split chamber with two brainstem-spinal explants. One spinal cord was cut rostral to the silicone divider, the other was cut caudal to the divider. Nocodazole was added only to the caudal compartment. (H) Transection site in the caudal compartment. Nocodazole completely blocks growth into spinal cord. (I) Transection site in the rostral compartment. Axons have crossed the boundary, showing that nocodazole is confined to the caudal compartment. Scale bar is 1 mm in C–F, 500  $\mu$ m in H–I. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]



**Figure 5** Protein synthesis in cell bodies, and not in axons, is required for axonal growth in the embryonic spinal cord. E8 brainstems with attached spinal cords were cultured in chambers divided into cell body and axonal compartments. All images are of E8 spinal cords in culture, 48 h after injury. DiI label is visible in axons that originate in the brainstem. (A) Axon growth below the transection site is robust in the absence of inhibitors of protein synthesis (B) When cycloheximide was added to both the cell body and axonal compartments, no axons cross the injury site. (C) When cycloheximide was added only to the axonal compartment, axonal growth was robust. (D) When cycloheximide was added only to the cell body compartment, no axons cross the transection site. Scale bar is 500  $\mu\text{m}$ .

(Fig. 6). We prepared divided chambers that contained two brainstem-spinal cultures with opposite orientation. This allowed us to apply protein synthesis inhibitors in the same culture dish to the brainstem of one explant and to the site of spinal transection of another explant. This design provides a strong within-experiment control, and provides additional confidence regarding effective separation of the two chamber compartments. We cultured the explants for 16 h after injury with no protein synthesis inhibitors present, and then added inhibitors to one compartment. We acquired images of living axons for 8 h after the drug was added, and recorded the distance that individual axons extended over 2 h intervals. Prior to the addition of drug, axons extended at an average speed of 62.3 ( $\pm 8.3$  SEM)  $\mu\text{m}$  per hour, which corresponds to a rate of 1.49 mm/day. During the 8 h length of the experiment, protein synthesis inhibitors applied to the axonal compartment did not change this average speed of advance. When protein synthesis inhibitors were applied to the cell body compartment, axon advance was

unchanged for the first 2 h, but was reduced by 47% ( $\pm 6.3\%$  SEM,  $p < 0.01$ ) between 2 h and 4 h. Starting at 4 h, axon growth was dramatically reduced by inhibitors applied only to the cell bodies. Although a few axons advanced between 4 and 6 h, other axons retracted, resulting in an average advance of 0.8  $\mu\text{m}$  ( $\pm 9.3$  SEM)  $\mu\text{m}$ . Between 6 and 8 h no axons moved forward. These data showed that when protein synthesis is blocked in the cell body, the neurons contain sufficient protein stores for only about 4 h of continued axon growth. Importantly, the time at which axonal advance fails is similar when protein synthesis inhibitors are applied only to the cell body compartment or applied to both compartments. Therefore, it appears that if protein synthesis occurs in the distal axon, it cannot act even temporarily to prolong axon growth when the supply of cell body-derived proteins is restricted. These data do not support an essential role for protein synthesis in the distal in supporting axonal elongation. Instead, because axonal elongation at a distance of 3 mm from the cell body fails 4 h after the



**Figure 6** Time course analysis of protein synthesis blockade and axon growth. (A) E8 brainstems with injured spinal cords in a split chamber, oriented such that the brainstem of one explant shares a compartment with the transection site of the other. (B–F) DiI-labeled axons extending in spinal cord when anisomycin is present in the axonal compartment. Axon growth remains constant over 8 h. (G–K) DiI-labeled axons extending in spinal cord when anisomycin is present in the cell body compartment. Axons growth continues for 4 h, after which axons stop extending (white arrow) or retract slightly (black arrow). Quantification of axon extension when brainstem cell bodies, axons, or both are exposed to anisomycin. More than 20 axons from two experiments were analyzed. Scale bars are 1 mm in A, 500  $\mu$ m in B–K.

supply of cell body-derived proteins is interrupted by inhibiting protein synthesis, these data suggest that the cell body continually and rapidly supplies essential proteins to the distal axon.

## DISCUSSION

This study examines the source of new proteins that are essential for the growth of supraspinal axons through the embryonic spinal cord. Our data showed that axon growth in the spinal cord depends on proteins that are synthesized in the cell body (or, conceiv-

ably, the proximal axon) and transported within 4 h to the distal axon. In contrast, protein synthesis within the distal axon is not required for axon growth, and, if it occurs, does not compensate even temporarily for a loss of proteins derived from more proximal sources. This study, which measured growth over 2 days and examined axons in their native CNS tissue environment, is the strongest evidence to date that the cell body, and not the distal axon, is the source of proteins that sustain axon growth in the developing CNS.

This conclusion is largely consistent with other observations. Short-term experiments have demonstrated that axons in culture remain motile and con-

tinue to extend at normal rates in the presence of inhibitors of protein synthesis (Campbell and Holt, 2001; Zheng et al., 2001; Leung et al., 2006; Yao et al., 2006). One strong indication that distal protein synthesis is not required for axonal extension came from an experiment in which inhibitors of protein synthesis were applied to distal axons of sympathetic neurons cultured on collagen surfaces in the split-chamber Campenot system (Eng et al., 1999). Axon growth persisted at normal rates for the 30-h duration of the experiment, demonstrating that similar to our findings, axonal elongation can occur independent of protein synthesis in distal axons. Eng et al. (1999) determined that less than 1% of axonal tubulin and actin could be synthesized in distal axons.

Our data suggest that axonal protein synthesis is not required for axon extension, but, importantly, do not rule out a possible involvement of locally synthesized proteins in other growth cone behaviors. Indeed, previous studies have generally shown that immediate and local protein synthesis is important for some guidance decisions of growth cones (Eng et al., 1999; Campbell and Holt, 2001; Brittis et al., 2002; Piper and Holt, 2004). Our data suggests that any guidance decisions involved in traversing an injury site in the spinal cord and proceeding caudally down the spinal cord do not require distal protein synthesis, but it remains possible that later navigational decisions, such as where to terminate caudal growth and initiate sprouting of side branches into spinal cord gray matter, could be disrupted in the absence of distal protein synthesis. In addition, it is possible that some essential proteins are usually made in distal axons, but when distal protein synthesis is inhibited, a retrograde signal to the perikaryon upregulates synthesis and anterograde transport of these proteins from the cell body.

Our time course analysis provides important information regarding newly synthesized proteins that support axonal growth. These experiments showed that when protein synthesis is blocked only in the cell body compartment, axonal elongation at the tip of axons, 3 mm from the cell body, ceases after about 4 h. Assuming the essential protein is required at the growth cone or axon terminus, in order to become limiting within 4 h, this protein would have to be transported at a rate of at least 3 mm in 4 h, or 12.5  $\mu\text{m}$  per minute. This rate is well within the range of measured rates of transport for a variety of proteins (Lasek et al., 1984; Brown, 2003). The identity of this protein(s) is unclear, but this rate of transport argues that the limiting proteins are not the major cytoskeletal components (i.e., tubulin, actin, or intermediate filaments), whose overall transport rates in axons are much slower (Lasek et al., 1984; Brown, 2003).

Our conclusions must be qualified in light of methodological limitations. First, we did not directly demonstrate that our drug treatments were effective in inhibiting protein synthesis. However, these drugs have been used extensively at these concentrations in neuronal cultures and in the embryonic chick, and their ability to block protein synthesis is well characterized (Luduena, 1973; Oppenheim et al., 1990; Eng et al., 1999; Campbell and Holt, 2001; Piper and Holt, 2004; Verma et al., 2005). A second concern is in our slice culture our method of anterogradely labeling axons does not directly distinguish between regenerative axon growth and the growth of new, uninjured axons. It is possible that newly growing and regenerating axons may differ in their requirement for distal protein synthesis (Verma et al., 2005). We performed time lapse microscopy to observe axons at the boundary between spinal explants in our culture system, and directly observed newly growing, unsevered axons that crossed the injury site, as well as regenerating axons. It remains possible, therefore, that the regeneration of axons was affected differently by the application of protein synthesis inhibitors to the distal axons, than the growth of new axons. Further experiments with older animals in which no new brain stem axons are growing down the spinal cord could more specifically address whether distal protein synthesis is required for axonal regeneration.

As previously demonstrated, we also found that the distal axons of supraspinal neurons contain several components of the machinery for protein synthesis. However, our results clearly demonstrate that long-term axon growth in a native CNS tissue environment does not require proteins synthesized in the distal axons. It is reasonable to assume that the large capacity for protein synthesis in the neuronal perikaryon sustains axonal elongation, while the more limited, and perhaps selective, protein synthesis machinery in growth cones, is dedicated to producing proteins that allow local responses to extrinsic cues or cellular interactions that trigger local growth cone behaviors, such as turning, branching or the transition to a presynaptic terminal.

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