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Protein Synthetic Machinery and mRNA in Regenerating Tips of Spinal Cord Axons in Lamprey

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Abstract

Polyribosomes, mRNA and other elements of translational machinery have been reported in peripheral nerves and in elongating injured axons of sensory neurons *in vitro*, primarily in growth cones. Evidence for involvement of local protein synthesis in regenerating CNS axons is less extensive. We monitored regeneration of back-labeled lamprey spinal axons after spinal cord transection and detected mRNA in axon tips by *in situ* hybridization and micro-aspiration of their axoplasm. Poly(A)+mRNA was present in the axon tips, and was more abundant in actively regenerating tips than in static or retracting ones. Target-specific PCR and *in situ* hybridization revealed plentiful mRNA for the low molecular neurofilament subunit and β -tubulin, but very little for β -actin, consistent with the morphology of their tips, which lack filopodia and lamellipodia. Electron microscopy showed ribosomes/polyribosomes in the distal parts of axon tips and in association with vesicle-like membranes, primarily in the tip. In one instance, there were structures with the appearance of rough endoplasmic reticulum. Immunohistochemistry showed patches of ribosomal protein S6 positivity in a similar distribution. The results suggest that local protein synthesis might be involved in the mechanism of axon regeneration in the lamprey spinal cord.

Graphical abstract

Micro-aspirated tips of regenerating reticulospinal axons in transected lamprey spinal cords contained mRNA for NFL and β -tubulin, but little β -actin mRNA. Static tips contained less

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ROLE OF AUTHORS

All authors had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. Overall design of the project: LQJ, MES. ISH: CRP, KSJ. Molecular cloning: CRP, KSJ, LQJ. Micro-aspiration: LQJ, EM, LQJ. Immunohistochemistry: WR. Global amplification, PCR: CRP, LQJ. Data acquisition and analysis: LQJ, MES. Drafting of the manuscript: LQJ, MES. Final manuscript preparation: LQJ, MES. Obtained funding: LQJ, MES.

CONFLICT OF INTEREST STATEMENT

LQJ, CRP, MES are/were employees of Temple University. KSJ was an undergraduate student. WR is a graduate student. None of the authors have known or potential conflicts of interest, including any financial, personal, or other relationships with other people or organizations within 5 years of beginning the submitted work that could inappropriately influence, or be perceived to influence, this work.

mRNA, while uninjured axons contained almost none. EM of injured axon tips showed polyribosomes and rough ER.

Introduction

Local protein synthesis is reported to contribute to axon regeneration in peripheral nerve (Koenig, 1967; Tobias and Koenig, 1975) and in neuronal cultures (Zheng et al., 2001; Verma et al., 2005; Gumy et al., 2010), whereas in postnatal CNS axons *in vivo*, evidence for local protein synthesis has been sparse. This has suggested the possibility that an absence of local protein synthesis in axons might contribute to failure of axon regeneration in the mammalian CNS (Gumy et al., 2010). The locally synthesized proteins may act as a source of proteins for growth cone re-formation and axon growth (reviewed by (Willis et al., 2011)), or may participate in retrograde signaling of injury to the cell body (Hanz et al., 2003; Perlson et al., 2005). To date, using microarray or high throughput screening in cultured cells whose axons were isolated from their dendrites and perikarya, several thousand mRNAs have been identified in axons of dorsal root ganglion cells (DRG) and retinal ganglion cells (RGC) (Willis et al., 2007; Zivraj et al., 2010; Gumy et al., 2011; Minis et al., 2014) *in vitro*.

In addition to mRNA, there is evidence for the presence of protein synthetic machinery (ribosomes, polyribosomes, ribosomal RNA, ribosomal proteins, and other translation-related molecules) in axons. Early electron microscopy (EM) studies reported ribosomes in peripheral nerves (Zelena, 1970; 1972) and in distal axon segments of cultured DRG (Zheng et al., 2001). Immunohistochemistry (IHC) suggested the presence of several ribosomal proteins, rRNA, and translation factors in peripheral axons (Zheng et al., 2001). Polyribosomes were detected in growth cones of cultured cortical neurons by EM (Bassell et al., 1998), while immunohistochemistry and fluorescence *in situ* hybridization (FISH) suggested the presence of both 5.8S rRNA and 18S (FISH) rRNA in axons of cultured hippocampal neurons (Taylor et al., 2013). Riboprotein S6 was detected by immuno-EM,

and several other components of translational machinery were demonstrated by co-precipitation and other techniques in cultured embryonic spinal cord commissural axons (Tcherkezian et al., 2010).

Difficulty demonstrating local protein synthesis in CNS axons is due in part to the relative difficulty of preparing pure axon fragments and other technical limitations, but it also appears that the translational components are much less abundant than they are in embryonic axons and PNS. Several mRNAs (oxytocin, vasopressin or olfactory marker proteins) were identified using classical techniques such as *in situ* hybridization (ISH) or Northern blotting in normal hypothalamo-hypophyseal tract, and in olfactory axon terminals in the olfactory bulb (Mohr et al., 1991; Ressler et al., 1994; Vassar et al., 1994). However, for the most part, axonal mRNA levels decrease with age, making their identification in adult CNS *in vivo* more difficult. When embryonic cortical and hippocampal neurons were maintained in culture for up to 13 days, axonal mRNA levels for β -actin and synaptophysin decreased dramatically over time (Taylor et al., 2009), or were no longer detected by 10 days *in vitro* (Kleiman et al., 1994). The structural components of the protein synthetic machinery, *e.g.*, ribosomes and polyribosomes, also appear to be less abundant and difficult to demonstrate in mature CNS axons compared with PNS (Gumy et al., 2010). Electron microscopy in monkey and adult rats suggested the presence of ribosomal rosettes only in the initial segment but not in the shaft of CNS axons (Steward and Ribak, 1986), and CNS axons within the optic nerve contained much lower levels of translational components than did sciatic nerve (Verma et al., 2005). More recently, using high resolution ISH techniques capable of detecting single mRNA molecules, Atf4 mRNA has been reported in adult mouse and human brain (Baleriola et al., 2014; Baleriola et al., 2015). In addition, using genetically engineered mice in which the ribosomal protein L10a was tagged with eGFP, immuno-EM for eGFP showed enhanced subaxolemmal staining at nodes of Ranvier in corticospinal tract axons (Walker et al., 2012). The ribosomes themselves were not clearly visible in the axons, neither in wild type animals nor in the transgenic mice, even though in wild type mice, ribosomes were readily seen by conventional EM in cell bodies and dendrites. Thus in mammals, where CNS axon regeneration is poor, local protein synthesis seems to decline after early stages of neuronal development, when axons are undergoing initial outgrowth and pathfinding (Hengst et al., 2006; Hengst and Jaffrey, 2007). While mRNA and other elements of the translational machinery have been reported in CNS axons *in vivo* under some circumstances, their demonstration has required very high resolution or indirect techniques, consistent with the previous conclusions that intra-axonal protein synthesis decreases dramatically after embryonic development.

Recently, association of localized mRNAs with axon regeneration *in vivo* was reported in unidentified (presumably ascending sensory) axons growing through a peripheral nerve graft inserted into a spinal cord injury in rat. GAP43, Neuritin 1 and β -actin mRNAs were detected by quantitative fluorescence *in situ* hybridization (FISH), while ribosomal protein S6 and 5.8S rRNA were detected by IHC in axons within the graft (Kalinski et al., 2015). In order to further explore a possible association between local protein synthesis and the capacity for regeneration of injured, well-developed axons in the CNS, we have looked for mRNA and other elements of protein synthetic machinery in the reticulospinal (RS) axons of large larval lampreys. These animals are 4–5 years old and their large RS axons have long

since (years) extended the entire length of the animal. Many of these axons regenerate after spinal cord transection (TX) (Rovainen, 1976; Selzer, 1978; Yin and Selzer, 1983; Davis and McClellan, 1993), as they do in adult lampreys (Lurie and Selzer, 1991). Several of the RS axons are very large, allowing us to visualize them and micro-aspirate the contents of their regenerating tips in the living animal. We have used these advantages to demonstrate axonally-located mRNAs by PCR amplification and ISH. This advantage allowed us to determine 1) whether the presence of mRNA in an axon tip is indicative of its regeneration status; 2) whether other components of the translational machinery are present in the injured axon tip; and 3) whether the types of cytoskeletal proteins represented by axonal mRNAs mirrors the cytoskeletal protein contents of the growing axon tip.

Materials and Methods

Spinal cord transection and retrograde labeling of axons

A total of 262 larval lampreys (*Petromyzon marinus*) 10–14 cm in length (4–5 years old and in a stable phase of neurological development) were used in these experiments (Table 1). This is the longest phase in the species' life cycle, and is used because of the accessibility of larvae in streambeds. Post-metamorphic adults also show regeneration of RS axons, but live only approximately 2 years (Hardisty and Potter, 1971) and are available in much smaller numbers. They were purchased from Lamprey Services in Michigan and maintained with light cycle in fresh water tanks at 15 °C until use. Lampreys were anesthetized by immersion in saturated aqueous benzocaine for 5 min and pinned to a Sylgard (184 silicone elastomer, Dow Corning) plate filled with ice-cold lamprey Ringer (110 mM NaCl, 2.1 mM KCl, 2.6 mM CaCl₂, 1.8 mM MgCl₂ and 10 mM Tris buffer; Ph 7.4). The spinal cord was exposed *via* a dorsal incision and transected under direct microscopic vision with iridectomy scissors at the level of the 7th gill. Spinal axons and their cell bodies were retrogradely labeled with the fluorescent dye dextran-tetramethylrhodamine (DTMR, 10 kDa, 5% in 0.1 M Tris buffer, pH 7.4) at the time of spinal cord TX by inserting a 0.5 mm dye-soaked Gelfoam pledget into the transection site. The cut axons and their cell bodies became brightly labeled within several hours. The Gelfoam was no longer distinguishable from the overlying scar tissue by 10 days after insertion. Background fluorescence was largely cleared from the area of dye application by 1 week. Although fluorescent label was picked up by glial cells and small neurons in the region adjacent to the application, the resulting low level of fluorescence did not interfere with observation of the large reticulospinal axons and their cut tips. In hemisected cords untransected axons were not labeled by dye applied to the cut edge of the cord. Animals were covered with a lamprey Ringer-moistened paper towel and kept on ice for 3 hours to facilitate clot formation. Animals were returned to fresh water and allowed to recover at 4 °C overnight, then housed at room temperature.

Determination of axon tip growth status

Lampreys were re-anesthetized and their cords re-exposed after 10 to 14 days' recovery. DTMR-labeled axon tips were visualized and their images captured under a fluorescence dissecting microscope and the distances of clearly identified tips from the transection site measured. Fiduciary landmarks such as surface capillaries and local neuronal cell bodies were noted and used for later alignment. Animals were kept anesthetized in lamprey Ringer

at 6–9 °C, and their spinal cords reimaged after 2–4 hr. The growth status of a given axon tip (growing, static, or retracting) during that interval was determined by measuring the change in location of the axon tip relative to fiduciary landmarks. To avoid bias in finding the front edge, images were converted to gray mode and processed using the tool “Find Edges” (Adobe Photoshop CS2). This allowed us to discriminate distances as short as 2–3 μm between observations. A tip was deemed “growing” or “retracting” if it had moved more than 10 μm towards or away from the transection site. The rest were judged “static tips”. We did not encounter ambiguously advancing tips with growth between 3 and 10 μm . Thus the distinction between growing and static was fairly sharp. In 123 animals, visibility of labeled axon tips and of fiduciary landmarks was such that it was possible to determine growth status for 93 axon tips.

Electron microscopy

Spinal cords from 16 animals were surveyed by light microscopy, of which 6 spinal cords were examined by EM to find 33 axon tips. Spinal cords were immersion-fixed with 4.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. After subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 hour at room temperature, and rinsed in dH_2O . After dehydration through serial ethanols, the tissue was infiltrated and embedded in EMBED-812 (Electron Microscopy Sciences). The spinal cords containing labeled axon tips were sectioned longitudinally with a Leica ultramicrotome. Semithin (1 μm) sections were stained with toluidine blue to aid in orientation. Thin sections were mounted on Formvar-coated slot grids, stained with uranyl acetate-lead citrate and examined under the EM (JEOL JEM 1010, Japan) fitted with a Hamamatsu digital camera and AMT Advantage image capture software.

Immunohistochemistry (IHC)

Spinal cords that had been transected and back-labeled with DTMR were checked for growing tips as above and fixed in 4% paraformaldehyde overnight. Then the cords were washed, embedded, and sectioned at 10 μm . The sections were deparaffinized, rehydrated, and washed in PBS. To facilitate antigen retrieval, specimens were immersed in sodium citrate buffer (0.01M, pH 6.0) with 0.05% Tween-20 and heated to boiling temperature by microwave for 20 minutes, and then cooled down at room temperature. After antigen retrieval, the samples were washed in PBS and blocked in solution (0.1% bovine serum albumin in PBS containing 0.2% Triton X-100) for 10 minutes. This was followed by incubation with mouse anti-rpS6 monoclonal antibody (Cell Signaling Technology, #2317) at 1:100 dilution in blocking solution overnight at 4°C. The next morning, sections were washed with PBS and incubated with Alexa Fluor 488 Goat anti-mouse IgG polyclonal antibody (Invitrogen, A11029) at 1:200 in blocking solution for 1 hour at room temperature. Finally, the specimens were washed sequentially with PBS and dH_2O . Slides were mounted with DAPI Fluoromount-G (Southern Biotech, 0100-20) and images were captured using a Nikon Eclipse 80i epifluorescence microscope with a Roper Scientific CoolSNAP digital camera under the control of NIS Elements software. Negative controls employed the same protocol, omitting the primary antibody.

Isolation of the axoplasmic contents from individual axon tips

The lamprey spinal cord is flat and translucent, allowing visualization of cells and fluorescently-labeled axon tips in living preparations (Zhang et al., 2005 ; Jin et al., 2009). The largest of these axons have been traced to the perikarya of large individually identified reticulospinal neurons by serially sectioning through the entire nervous system in a single specimen (Rovainen et al., 1973). With the exception of the Mauthner axon in the lateral tracts, the degree of variability in the arrangement of the large axons in the ventral columns is still not known. Moreover, the narrowing of the axon at the initial segment may also make it difficult to trace even fluorescently labeled axons all the way to their perikaryon in CNS wholemounts. Thus we did not attempt to identify specific individual axons. However, the rate of elongation of a regenerating axon could be discerned in 2–4 hrs (Jin et al., 2009). After axotomy, the sealed proximal tips form spindle-shaped structures, which can be as large as 300 (length) $\times$ 100 (diameter) μ m. This enabled penetration and micro-aspiration through glass micropipettes pulled from 1.5~1.8-mm o.d. microcapillary tubing (KIMAX 51, Kimble, Vineland, NJ), using a Narishige PC-10 puller, and kept in a clean environment before use. Micropipettes were advanced with a Leitz micromanipulator under stereoscopic fluorescence microscopy at 63x magnification, with 8 psi positive pressure applied to prevent entry of contaminants. When a slight blanching of fluorescence indicated dimpling of the axon tip, positive pressure was replaced by negative pressure and the micropipette tip was advanced further. Successfully penetrated axon tips collapsed and the fluorescence was displaced into the micropipette tip, which was quickly withdrawn. The contents were carefully expelled into an autoclaved 0.65 mL microcentrifuge tube with 5 μ L of RNase inhibitor (RNaseOUT, Invitrogen). Samples were frozen immediately in dry ice and kept at -80°C until use. A total of 99 animals were used to harvest axoplasms from 99 axon tips whose growth status had been determined.

Global cDNA amplification and target-specific PCR from single neurons and axon tips

The technique of single-cell cDNA amplification (Brady et al., 1990 ; Kurimoto et al., 2006 ; Kurimoto et al., 2007) was adapted for lamprey axon tips and neuronal perikarya. Positive or negative control tubes in each RT experiment used 1–100 pg of total RNA purified from the lamprey CNS, with and without RT enzyme, respectively. Axoplasm taken from individual axon tips, or from 6 pooled axon tips, was incubated with anchored primer V1(dT)₂₄ [5'-ATATGGATCCGGCGCGCCGTCGAC(T)₂₄-3', IDT] in RT reaction buffer (10 mM DTT, 10 μ M dNTPs, 4 U RNaseOUT). As a control for contamination by RNA from other structures adhering to the micropipette tip, microelectrodes were inserted into the spinal cord but not into axon tips. These pipette tips were dipped into the RNase inhibitor solution and RT-PCR performed as for the axon tip aspirates. Reverse transcriptase (5 U, Invitrogen) was added to the RNA/Oligo(dT) mixture and the tubes incubated at 50°C for 50 min. The reaction then was stopped by heating to 70°C for 10 min and cooled to 4°C . Any DNA in samples and unused V1(dT)₂₄ were digested with (5 U) exonuclease I (5 U, Takara, Japan) at 37°C for 45 min. The enzyme then was inactivated by incubation at 80°C for 25 min. To achieve poly(A) tailing of synthesized cDNA, terminal deoxynucleotidyl transferase (TdT, 5 U, Promega) was added to the reactions containing 1st strand cDNA, 1 $\times$ TdT buffer, 250 μ M dATP, and ribonuclease H (2 U, Invitrogen). The reaction proceeded for 15 min at 37°C , and was stopped by inactivation at 70°C for 10 minutes. An aliquot (5 μ L) of polyadenylated 1st-

strand cDNA was used as template for PCR amplification. PCR reaction consisted of Taq PCR buffer (1×), MgCl₂ (2 mM), dNTPs (0.4 mM), primer V3(dT)₂₄ [0.68 μM, 5'-ATATCTCGAGGGCGCGCCGATCC(T)₂₄-3', IDT], V1(dT)₂₄ (0.68 μM), and GoTaq (0.05 U/μl, Promega) to make a total volume of 25 μl.

The reaction mixture was subjected to the cycling program: 95°C for 3 min, 45°C for 2 min, 72°C 3 min once with V3(dT)₂₄ alone, and then 20 cycles with both primers of 95°C for 30 sec, 60°C for 2 min and 72°C for 3 min, followed by 72°C 7 min and 4°C. At this number of cycles, the relative abundances are well preserved among amplified genes (Iscove et al., 2002).

Global amplification was assessed by target-specific PCR for the lamprey NFL (L-NFL) gene because the axon tips have plentiful NFs (Lurie et al., 1994) and thus L-NFL (Jin et al., 2011) was expected to be present at high levels in lamprey CNS. High sensitivity was reflected by the presence of signal in even low concentrations of positive controls (1 or 10 pg of total RNA isolated from lamprey CNS). Specificity was indicated by the absence of L-NFL in the negative controls (water or non-specifically inserted micropipettes). Validated axonal cDNA preparations were then used for target specific PCRs for other cytoskeletal genes. One μl of globally amplified cDNA (6 pooled samples) was used as a template with sets of primers (Table 2) whose design was based on full-length gene sequences for four lamprey NFs (NF132, DQ398933; NF180, U19361; NF95, DQ398934; and L-NFL, DQ869188) and lamprey vimentin (HQ171987.1), and sequences for lamprey β-actin (ACTB, KM925135), and β-tubulin (TUBB2B, KP728258). Target-specific PCRs for L-NFL, and β-tubulin were further performed on globally amplified products of single axoplasmic samples obtained from 10 growing, 10 static and 10 retracting tips. PCR for other genes were not tested because they were not detected in pooled axoplasmic samples.

Molecular cloning of lamprey β-tubulin, β-actin, and lamprey vimentin full-length genes

A cDNA sequence encoding NF-180 (PMU19361) was cloned previously (Jacobs et al., 1995). Recently, three more lamprey neurofilament (NF) genes have been sequenced and deposited in GenBank [NF-132 (DQ398933), NF-95 (DQ398934), and L-NFL (DQ869188)] (Jin et al., 2011). Genes for three other lamprey cytoskeletal proteins were also cloned.

i. β-tubulin (partial)—The Ensembl *Petromyzon marinus* genome protein database (<http://useast.ensembl.org/index.html>) was searched by BLASTX against human β-III tubulin nucleotide sequence (NM_006086.3, 1794 bp) because this is the subtype most specific to neurons. The search yielded a 1925 bp DNA sequence (ID: ENSPMAT00000000348), although it was more homologous to β-II than to β-III, and thus far, no β-III gene has been found in lamprey. The DNA fragment was used in designing probes to clone the partial lamprey β-tubulin gene (1891 bp) by screening a Uni-ZAP XR lamprey cDNA library, as described previously (Jin et al., 2011). The cloned cDNA consists of 1891 nucleotides and has been deposited in GenBank (KP728258). Although this gene was most homologous to βIIb-tubulin, there was no evidence for multiple β-tubulin subtypes, and we designate it simply as β-tubulin.

ii. β -actin—The most highly conserved regions (229 bp) of β -actin genes were obtained by similarity analysis among 3 species (human, NM_001101, 1852 bp; mouse, NM_007393.3, 1889 bp; and cattle, NM_173979.3, 1948 bp) and used as probe. The lamprey cDNA library was screened to get the full length of the gene. The gene consists of 1961 nucleotide bp and has been deposited in GenBank (KM925135). There was no evidence for multiple β -actin subtypes.

iii. vimentin—A cDNA fragment (717 bp) by-product of the PCR reaction that was performed to clone new NFs (Jin et al., 2011) was highly homologous to the vimentin gene of other species. Several primers were designed to clone the full length of this intermediate filament by PCR, using the lamprey Uni-ZAP XR cDNA library as a template. This vimentin gene consists of 3380 nucleotide bp and has been deposited in GenBank (HQ171987).

***In situ* hybridization with biotin-labeled riboprobes and biotinylated oligo (dT)**

A total of 79 animals were used for ISH on 92 axon tips whose growth status had been determined.

a. Riboprobes—Hybridization of biotin-labeled riboprobes to paraffin sections of lamprey spinal cord was performed as reported previously (Swain et al., 1994). The spinal cord was transected and axons back-labeled with DTMR as above. After 2 weeks' recovery, the spinal cord was reexposed and growth of axon tips was monitored for 2–3 hours under a fluorescence dissecting microscope. Spinal cord segments containing interesting axon tip(s) were removed, pinned flat on Sylgard strips, fixed in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), dehydrated in serial ethanols, cleared in toluene, infiltrated with Paraplast, and embedded in paraffin. Ten μ m coronal paraffin sections were collected, deparaffinized, and rehydrated in xylene and serial ethanols. Biotin-labeled riboprobes for L-NFL were constructed as described previously, but replacing the digoxigenin mixture with a biotin mixture (Roche) (Jin et al., 2011). T7 or SP6 RNA polymerase was used for the sense or antisense probe synthesis. Biotin-labeled Ribo-probes for β -actin, or β -tubulin were prepared by PCR methods (Milligan et al., 1987). One of the PCR primers was tagged with a 30-base nucleotide including the T7 promoter and an adaptor (5'-TAAGCTTTAATACGACTCACTATAGGGAGA-3') at the 5' end. After PCR, cDNA fragments (381 or 411 bp) were synthesized for β -actin and β -tubulin. *In vitro* transcription was performed with an RNA transcription kit (Roche, Nutley, NJ) as recommended by the manufacturer.

Sectioned tissues were pre-hybridized at 55°C in hybridization solution (50% deionized formamide, 5 $\times$ SSC, 100 pg/ml *Torula* yeast RNA, 100 pg/ml wheat germ tRNA, 50 pg/ml heparin, 0.1% Tween-20) followed by hybridization overnight at 55°C in the same solution plus 2 μ g/ml biotin-labeled cRNA. Specimens were washed in hybridization solution, PTw (0.1% Tween-20 in phosphate-buffered saline (PBS)), and PBT (0.1% bovine serum albumin, 0.2% Triton X-100 in PBS). Alkaline phosphatase-conjugated streptavidin (Roche, Nutley, NJ) was diluted (1:500) and applied to tissues for 4 hours at room temperature. Tissue was washed sequentially in PBT and SMT (100 mM NaCl, 50 mM MgCl₂, 100 mM

Tris, pH 9.5, 0.1% Tween-20). The chromogenic reaction was carried out in ice-cold SMT containing 175 pg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 350 pg/ml 4-Nitro blue tetrazolium chloride for 1–2 hrs in the dark at room temperature. Finally, specimens were washed in PBS, dehydrated in serial ethanols, cleared with xylene and mounted under Permount (Fisher scientific). Stained samples were visualized on a Zeiss Axiovert 135 microscope using 25 or 40 × dry objectives. Images were acquired using a Zeiss Hi Resolution color camera and software (Zeiss Axioskop).

b. Biotinylatedoligo(dT)—The experiments were performed as described above, except that sectioned tissue was pre-hybridized at 37°C in hybridization solution, and the hybridized overnight with biotinylated oligo(dT) probe (200 nM, Promega) at 37°C. The fraction of axon tip occupied by ISH label was quantified using the “Polygonal Lasso” and “Histogram” tools (Adobe Photoshop CS2). The “percent filled” was calculated for each tip as the stained area divided by whole tip area.

Western blotting

Ten *Petromyzon marinus* specimens were anesthetized in 0.01% tricaine methanesulfonate and their brains and spinal cords were dissected. Tissues were homogenized by Ultrasonic Processor (FB120, Fisher Scientific) in homogenization buffer consisting of 25 mM HEPES (pH 7.4), 100 mM sucrose, 1 mM EGTA, 0.2% β-mercaptoethanol, 0.04 mM PMSF, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, and 0.01 U/ml soybean trypsin inhibitor. The homogenate was centrifuged at 800 × g for 10 min at 4°C. A first supernatant was collected and centrifuged at 10,000 × g for 15 min. The second supernatant was collected and its protein concentration was quantified by Lowery’s method. Proteins (40 μg) were separated in 12% SDS mini-gel and were electrophoretically transferred onto nitrocellulose membranes (0.45 μm, Bio-Rad) using a Bio-Rad transblot apparatus. The membranes were blocked in Odyssey Blocking Buffer (LI-COR, 927-40000) for 2 hours and incubated with anti-rpS6 monoclonal antibody (Cell Signaling Technology, #2317; 54D2, 1:1000) overnight at 4°C, followed by washing with PBS containing 0.2% Tween 20 (PBST), and incubation with the fluorescently labeled secondary antibody (IRDye800 goat anti-mouse IgG, 1:7,500) for 1 hour at room temperature. The membranes were then rinsed in PBST and scanned by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Antibody characterization

Primary antibodies used in this study are listed in Table 3. Mouse monoclonal anti-S6 (Cell Signaling Technology, Danvers, MA, #2317, RRID: AB_2238583) used at 1:1000 dilution for Western blotting, and 1:100 to label ribosomal protein S6 on IHC. This antibody was raised against a recombinant fusion protein corresponding to full-length human S6 ribosomal protein (manufacturer’s notes). It labels the cytoplasm of neurons and glial cells in lamprey CNS. The antibody recognized a single band of expected size (36 kD) on Western blots of lamprey brain and spinal cord homogenates (see Fig. 5E). LCM3, one of several mouse monoclonal antibodies generated previously in this laboratory against a cytoskeletal fraction of lamprey CNS (Merrick et al., 1995)(RRID: AB_2314735), selectively labels the core of lamprey NFs (Jacobs et al., 1995; Jin et al., 2005). It is phosphorylation-independent and at a dilution of 1:100, labels primarily axons of all sizes in

transverse sections of lamprey spinal cord. Biotinylated goat anti-mouse IgG (H+L) was purchased from Vector Laboratories (BA-9200) and used as a secondary antibody against LCM3 in IHC (RRID: AB_2336171, 1:200). IRDye@800 conjugated goat anti-mouse IgG polyclonal antibody was purchased from Rockland Immunochmicals (Code: 610-132-121, RRID: AB_220347) and used at dilution 1:7,500 as a secondary antibody in Western blotting of ribosomal protein S6. Alexa Fluor@488-labeled goat anti-mouse IgG was produced by Invitrogen (A-11029) and used as secondary antibody in IHC of ribosomal protein S6 (1:200).

Results

Protein synthetic machinery is present in axon tips

i. Electron microscopy (EM)—To evaluate whether structural elements of the protein synthetic machinery are present in the tips of severed CNS axons *in vivo*, we looked for ribosomes, polyribosomes or rough endoplasmic reticulum (rER) in the large reticulospinal axons with EM. Sixteen lamprey spinal cords that were back-labeled with DTMR were studied at 2 weeks post-TX. Six of these spinal cords containing 8 clearly distinguishable back-labeled giant RS axon tips were selected for EM observation. Among them, 4 tips were actively regenerating, 1 was sessile, 1 was retracting, and 2 had undefined growth status because they were partially obscured by overlying soft tissue or blood. Most small axon tips (< 10 μm in diameter) were insufficiently visualized with the dissecting fluorescence microscope to attempt to define their growth status, but we were able to find their tips in the electron micrographs. A total of 33 axon tips were examined by EM. Their diameters varied from 2.5 to 35.5 μm (12.7 ± 1.3 , mean $\pm$ sem). The distal ends of giant axons (shaft > 10 μm) often could be subdivided into three distinct regions: axon shaft, body of axon tip, and a distal-most finger-like protrusion. Small axons were less obviously subdivided. An example of a large axon is shown in Fig. 1. With careful focusing, it was concluded that the distal-most part of the finger-like protrusion had at least 4 lobulated components (silhouettes **a–d**, shown by EM at low-magnification in Fig. 1B, C). These components all appeared to be part of the same tip. At higher magnification, this tip contained a structure that resembles rER (Fig. 2). The growth status of this tip was not determined, but it had features associated with actively growing axons (Jin et al., 2009). This tip arose as a swelling from the axon shaft and had an oval body and a distal finger-like protrusion (*fp*). The tip measured 304 μm in length, from the end of the axon shaft to the distal edge of the *fp*, with a maximum diameter of 105 μm . Of the four components, silhouette **a** was unambiguously part of the axon tip. Silhouettes **b–d** were used for orientation in EM (Fig. 2), but were not examined further. The rER-like structure in this tip (Fig. 2B–F) was located in the proximal compartment (**a**), in obvious continuity with the body of the axon tip. This structure included an interconnected network of flattened, membrane-enclosed sacs (cisternae), high-density particles (ribosomes) studding the cytosolic face of these membranes, and other membranous structures with the appearance of Golgi apparatus located nearby. Neither free nor membrane-associated polyribosomes were seen in the axon shaft proximal to the tip. No other examples of rER were seen.

However, in other 11 axon tips (33%), 6 large RS axons and 5 small axons, we were able to identify ribosomes, polyribosomes (Fig. 3, 4). We did not find ribosomes, polyribosomes or rER in the other 22 tips (67%), although they were not examined in complete serial sections. Of the 4 tips that were known to be growing, we saw polyribosomes in 2. In one sessile and one retracting tip, we found no ribosomes. Thus far, polyribosomes or rER have been seen only in axon tips and in surrounding cell bodies. They have not been seen in axon shafts. The axolemma of the axon tips was often invaginated, forming clefts. Within axon tips, polyribosomes were distributed primarily near the axolemma or near membranous structures close to the distal-most edge, which were often sectioned *en face*, either through the membrane itself or through subaxolemmal cortex (Fig. 3A, B). The absence of membranes separating the compartments on either side of the dense material, and the presence of NFs on both sides, indicated that the compartments are contiguous. Fig. 4 shows ribosomes that were clearly within the axon tip, and far from the axolemma or any invaginated membrane. There was no size difference between axons containing polyribosomes or rER ($11.5 \mu\text{m} \pm 2.1$, $n = 11$) and axons in which we did not see polyribosomes ($13.3 \mu\text{m} \pm 1.7$, $n = 22$, $p = 0.52$). The sizes of ribosomes in the axoplasm (20–27 nm, Fig. 3, 4) were slightly greater than those bound to membranes of the ER-like structure (13–18 nm, Fig. 2), consistent with longstanding findings in mammalian cells that membrane-bound ribosomes are smaller than those in non-membrane fractions (Ramsey and Steele, 1977). Ribosomes in axoplasm were similar in size to those in nearby cells. There were numerous small vesicles (45–65 nm) in many of the axon tips, often in groups and associated with ribosomes (Fig. 3B, 4D). The inner cytoplasmic leaflets in the axon tips often were contacted by clusters of larger vesicles (100–200 nm).

ii. Immunohistochemical evidence for ribosomal protein S6 in axon tips—Two weeks after spinal cord TX, growth status was determined for 13 axon tips in 17 spinal cords. Of these, 6 tips were actively growing at the time of fixation. The fixed spinal cords were embedded in paraffin and the region of injury sectioned horizontally and prepared for IHC, using an antibody that is specific for the ribosomal protein S6. A negative control with primary antibody eliminated (Fig. 5A) showed no staining, even in surrounding cell bodies. In 2 of the growing tips (Fig. 5B, C), there was S6 staining in a distribution that matched that of microtubules as previously described (Jin et al., 2009): i) along the main microtubule bundle that penetrates the center of the enlarged tip from the shaft (Fig. 5B2), ii) along the subaxolemmal space as seen most prominently at the base of the tip (Fig. 5C2), and iii) in the distal part of the tip (Fig. 5B2). The non-growing tips showed little S6 staining, although a faint cytoplasmic stain could not be excluded (Fig. 5D2). In Fig. 5B1, DTMR is largely excluded from a small clear area at the distal-most end of the axon tip (white arrow). This is frequently seen in actively growing tips. It may represent a mechanism of axon growth whereby membrane-bound structures that had excluded the dye eventually fuse with the axolemma at the leading edge (red arrowhead). Specificity of the antibody was demonstrated by Western blotting of lamprey CNS homogenates derived from 10 lampreys. The antibody recognized a single, appropriately-sized band ($\sim 36 \text{ kDa}$; Fig. 5E).

Localization of lamprey mRNAs in axon tips by ISH

To determine whether spinal cord injury induces localization of mRNA in axon tips, we compared Oligo(dT) ISH staining in axons of unlesioned spinal cords and axon tips 2 weeks after spinal cord TX. Oligo(dT) detects the 3'-poly(rA) that is present in most mRNAs. In 4 unlesioned spinal cords, most staining was seen in cells of the gray matter and cells surrounding the central canal, but not in axons in transverse (Fig. 6A) or longitudinal sections (Fig. 6C–E). In spinal cord-TXed animals, the exposed spinal cords were imaged twice 2–3 hours apart to define the axons' growth status (Fig. 7A/B, 7D/E). Some axon tips could no longer be found during the second observation. Among 25 of 41 animals examined for this purpose, 39 axon tips were identified at both times. In the remaining animals, for technical reasons, we were not able to image the same axon twice. The distances of axon tips from fiduciary landmarks revealed that 14 tips were growing forward, 15 were static, and 10 were retracting. Representative growing and static tips are shown in Fig. 7A–C and D–F, respectively. Their staining was quantified and expressed as “percent filled” by two independent observers blind to the experimental design and status of the tip (see Materials and Methods). Actively growing tips were more extensively labeled than static or retracting ones (Fig. 7G). Unlabeled axons passing near axon tips were easily discerned because they were edged with label, due to envelopment by glial processes (Lurie et al., 1994) (Figs. 6C–F, 7F). The very darkly stained structures at the bottom of Fig. 7C are ependymal cells, with their conventional distribution of mRNA in cell bodies and processes, which serve as internal positive controls. In Fig. 7F, the very darkly labeled structures above this tip are cells of the gray matter.

Identification of cytoskeletal mRNAs in pooled axoplasm by global amplification and target-specific PCR

i. Micro aspiration of axoplasm from single axon tips—Brains and spinal cords were back-labeled with DTMR from the site of injury for 2 weeks. Penetration was guided by fluorescence dissecting microscopy. The target region within the axon tips was their caudal-most edge where mRNAs were most likely to be localized. Once the electrode was inside the axon tip, negative pressure was applied to aspirate axoplasm. When the fluorescence disappeared from axon tips (Fig. 8A–C), the electrode was withdrawn instantly. Every aspiration was evaluated by viewing the micropipette under a fluorescence microscope and demonstrating the aspirated fluorescence (Fig. 8D–F). The volume of axoplasm aspirates was 0.095–0.105 μ L, measured by isotope scintillation counting.

ii. Global amplification of mRNA—Analysis of mRNAs with regular PCR requires nanogram amounts of total RNA, but a cell normally contains only 10–30 picograms of total RNA (Nygaard and Hovig, 2006). To overcome this drawback, global amplification of mRNA from small tissue or single cells has been described (Brady et al., 1990; Iscove et al., 2002; Kurimoto et al., 2006; Kurimoto et al., 2007). cDNA product generated by global amplification can be used in analyzing the presence of specific genes (target-specific PCR). In 34 animals, 30 axon tips were penetrated, of which 6 actively growing and 6 static tips were successfully aspirated and separately pooled. Global amplifications were performed on these axoplasms together with positive and negative controls, which contained total RNA purified from lamprey CNS in RT reaction, with or without RT enzyme, respectively. PCR

products were separated in 1% agarose gels after 30 cycles of amplification (Fig. 8G). The wide size distribution of the product (cDNA) indicates multiple mRNAs have been amplified. The heaviest smear, ranging from 300 bp – 1kb, was seen in the positive control lanes (100 pg RNA), followed by individual cytoplasms, and 6 pooled axoplasms. The two clear bands below 200 bp were by-products of unused primers (Kurimoto et al., 2007). Because previous studies on lamprey spinal cord indicated that axon tips are filled with NFs (Lurie et al., 1994; Pijak et al., 1996; Hall et al., 1997) and because NF-L has been suggested to play a central role in the formation of intermediate filaments in neurons (Geisler and Weber, 1981; Liem and Hutchison, 1982), the quality of global amplification product was evaluated by target-specific PCR, using primers for lamprey NF-L (L-NFL, DQ869188). A 389 bp band covering nucleotides 1232–1620 was seen in lamprey CNS homogenates, cytoplasm of single neurons and axon tips, but not in negative controls (Fig. 8H).

iii. Identification of cytoskeletal protein mRNAs in axon tips—Many mRNAs encoding cytoskeletal proteins have been localized to growth cones in cultured neurons, *e.g.*, β -actin, β -tubulin, vimentin, peripherin (Koenig, 1989; Bassell et al., 1998; Zhang et al., 1999; Zheng et al., 2001; Willis et al., 2005). However, we have postulated a mechanism of axon regeneration in CNS that is different from growth cone pulling, and involves NFs (Pijak et al., 1996; Jacobs et al., 1997). In order to determine how the mRNAs in regenerating axons of the lamprey spinal cord correspond to the cytoskeletal contents of axon tips, global amplification PCR products were used as templates for target-specific PCR (Fig. 9). A lamprey cDNA library constructed from polyadenylated RNA isolated from the brain and spinal cord of larval *P. marinus*, PCR products from a previous global amplification of lamprey brain and spinal cord RNA were used as positive controls. We performed target-specific PCR for 7 cytoskeletal protein mRNAs, including lamprey β -actin (KM925135) and β -tubulin (KP728258), 4 previously-cloned NFs – L-NFL (DQ869188), NF95 (DQ398934), NF132 (DQ398933), NF180 (PMU19361), and a class III intermediate filament, vimentin (HQ171987) (Table 2; Fig. 9). L-NFL and β -tubulin mRNAs were present in both growing and static tips, while mRNAs encoding β -actin (faint) and NF95 (faint) were detected only in growing tips. Lamprey NF132 (not shown), NF180 and vimentin mRNAs were not detected in either type of tip.

iv. Sham controls to rule out contamination with mRNA during micropipette passage through the spinal cord—To determine whether micropipettes are contaminated by mRNA from surrounding cells in the course of spinal cord penetration, we performed parallel global amplifications and target-specific PCR for vimentin and L-NFL on samples from real and sham micro-aspirations. Five spinal cords were transected and back-labeled with DTMR (Fig. 10A, C, & E). Two axon tips in two spinal cords were micro-aspirated separately (Fig. 10A/B, C/D). The 3rd spinal cord was used for sham aspirations with 7 electrodes whose tips penetrated the spinal cord but did not enter axons or neurons (Fig. 10F). These micropipette tips were then dipped in RNase inhibitor (RNaseOUT) in the same way as those containing the real samples. No vimentin or L-NFL mRNAs were detected (Fig. 10G, H). Vimentin is widely expressed in lamprey CNS glia (Fig. 11) and thus should have been detected if mRNAs had stuck to the micropipettes during passage.

v. L-NFL and β -tubulin mRNAs were located preferentially in growing axon tips

—We examined axoplasms from 30 axon tips – 10 actively growing, 10 sessile, and 10 retracting. Axoplasms were harvested individually and underwent global amplification for 20 cycles. The resulting cDNAs were used as templates for L-NFL- and β -tubulin-specific PCR. The percentages of actively growing tips containing mRNAs for L-NFL and β -tubulin (90% and 100%, respectively) were significantly greater than in non-growing tips (40%) (Table 4). Signals for β -actin and NF-95 were too low to detect in axoplasm from single axon tips.

Distributions of cytoskeletal protein mRNAs in axon tips by ISH

To localize the mRNAs coding for individual cytoskeletal proteins, we examined axon tips by gene-specific ISH on paraffin sections. Although in general, actively advancing tips contained more ISH product than static or retracting tips, we did not attempt to quantify this for each individual mRNA, as we did with Oligo(dT) ISH.

i. L-NFL mRNA was present in growing tips—Global amplification and target-specific PCR (see above) demonstrated L-NFL mRNA in axon tips. ISH was performed with L-NFL antisense probes on spinal cord sections from 5 animals that contained 5 axon tips at 2 weeks post-TX (Fig. 12). Two growing tips were heavily stained (2/2). L-NFL message was ubiquitous in growing tips, although most heavily concentrated at the leading edge (red arrow in Fig. 12C) and in a central strip arising at the base of the tip (yellow arrow head in Fig. 12C). However, no staining was found in nearby axons (at least 7 axons are seen in Fig. 12D). Heavy staining often reduced the DTMR fluorescence (* in Fig. 12D). No staining was found in 1 static tip (1/1) (Fig. 12E–H) or in 2 retracting tips (0/2; not shown).

ii. β -tubulin mRNA in axon tips—Microtubules are involved in intra-axonal transport and axon regeneration (Gallo, 1998; Rajnicek et al., 2006; Erturk et al., 2007). They are generally synthesized in the cell body, and then transported anterogradely down the axons (Baas, 2002). To determine whether microtubule proteins might potentially be synthesized in the axon, we looked for β -tubulin message by ISH. Seven axon tips from 6 animals were characterized for their growth status (Fig. 13). β -tubulin mRNA was present in 2 growing tips (2/2), 1 static tip (1/3) and 1 retracting tip (1/2). As with L-NFL message, the densest staining was found in the distal-most region of growing tips (Fig. 13C), but also sub-axolemmally in the transition zone between parent axon and the enlarged tip (see below). β -tubulin mRNA was usually less abundant or absent in static tips (Fig. 13A/C, white arrow), but as with Oligo(dT) ISH, this was variable, so that some static tips had more ISH reaction product than some growing tips, (see, *e.g.*, Fig. 13E/G).

iii. Scant presence of β -Actin mRNA in growing tips—F-actin is a prominent component in typical growth cones. However, the growing tips of lamprey RS axons lack lamellipodia and filopodia, and have sparse F-actin, which appears to be associated mainly with vesicle-like structures (Jin et al., 2009). In order to determine whether the paucity of F-actin is mirrored in the mRNA content of the regenerating axon tip, we collected β -actin cDNA amplification products from pooled micro-aspirates. β -actin message was much less abundant than that of L-NFL or β -tubulin, and was not detectable in aspirates of single axon

tips. In ISH studies, 6 axon tips from 5 animals were tested with the β -actin probe. Among them, 1 growing tip (1/2) and 1 static tip (1/3) stained weakly, and a retracting tip (1/1) was not labeled by the β -actin probe (Fig. 14).

iv. mRNAs for L-NFL and β -tubulin are present in some axon shafts—In order to determine whether the mRNA in injured axon tips reflects increased translocation of mRNA from the cell body, or only back-up of a low level of continuously moving message, we looked for ISH evidence of mRNA proximal to the axon tip, discontinuous with the mRNA in the tip. As indicated in Fig. 6A, C, D and E, mRNA levels were very low or undetectable in axons of normal spinal cord. However, 2 weeks after spinal cord TX, axon tips were filled with mRNAs, especially for cytoskeletal proteins. This extended to the region proximal to the axon tip, either continuously or intermittently along the axon, in sections stained with RNA probes for L-NFL (Fig. 15A–D) or β -tubulin (Fig. 15E–H), although only a small fraction of the axons were stained (Fig. 15). We also observed staining in regions between the axons and axon tips in ISH for L-NFL (Fig. 15C) and β -tubulin (Fig. 15G), when the tip was only weakly stained (Fig. 15C), which suggested that upregulated mRNA was being transported anterogradely, but had not yet accumulated in the tip.

Discussion

The present findings show that in the lamprey, an animal that demonstrates axonal regeneration after spinal cord TX, mRNAs are present in the injured axon tips, and are more abundant in the tips of actively growing axons than in static ones. EM observations and immunocytochemistry demonstrated the presence of elements of translational machinery in the axon tips, including ribosomes/polyribosomes, ribosomal protein S6, and in one instance, rER. These results are consistent with the hypothesis that local protein synthesis is involved in the mechanism of CNS axon regeneration.

Localization of ribosomes in axon tips

Indirect evidence for local protein synthesis was first found in peripheral axons of the cat by demonstrating RNA in myelin-free axons (Koenig, 1965). Incorporation of radiolabeled amino acids also was demonstrated in the squid giant axon (Fischer and Litvak, 1967; Giuditta et al., 1968). The incorporation of radio-labeled amino acids into axons was so rapid (2–4 hours) that it suggested pre-existence of protein synthetic machinery in the axon. In anucleated axons of cultured DRG neurons, *de novo* synthesized proteins were identified by mass spectrometry after incubation with ^{35}S -methionine/cysteine (Willis et al., 2005). This was inhibited by local application of cycloheximide, which also caused the growth cones to retract (Zheng et al., 2001). Similarly, local inhibition of protein synthesis also inhibited RGC and DRG growth cone regeneration after axotomy (Verma et al., 2005). In *Xenopus* retinal axons, selective depletion of mRNA coding for lamin B2 caused axon degeneration (Yoon et al., 2012), suggesting that intra-axonal synthesis of lamin-B2 plays a crucial role in axon maintenance. These observations were consistent with conventional EM or electron spectroscopic imaging (ESI) evidence in peripheral neurons (Zelena, 1970; 1972; Koenig et al., 2000; Kun et al., 2007) that ribosomes are present in peripheral nerve *in vivo*, and in RGC (Bassell et al., 1998) and DRG axons (Zheng et al., 2001) *in vitro*. Additional

components involved in intra-axonal protein synthesis, including ribosomal proteins (L17, L29, L4), translation initiation factors (eIF2 α , eIF4e, eIF5), and 28S rRNA, were identified by immunohistochemistry in cultured DRG axons (Zheng et al., 2001).

For CNS neurons, evidence for local translation in axons of mature neurons *in vivo* has been more difficult to find. In a genetically engineered mouse, in which eGFP was fused to the ribosomal protein L10a, dense immunostaining was shown by EM at nodes of Ranvier in corticospinal tract (CST) axons (Walker et al., 2012). The ribosomes themselves were not clearly demonstrated, either by conventional EM or by immuno-EM. Unless electron-dense granules of appropriate size for ribosomes are seen, immuno-EM may not be conclusive, since it cannot be assumed that the target molecules, *e.g.*, ribosomal proteins, all are incorporated into ribosomes. Previous studies had reported failure to find evidence for ribosomes in distal CNS axons *in vivo* (Steward and Ribak, 1986). The difficulty is compounded in regenerating CNS axon tips, which are difficult to identify unequivocally in mammals (Willenberg et al., 2016).

In the present study, most of the ribosomes observed in the axon tips were not bound to membranes. The sizes of the free ribosomes/polyribosomes (20–27 nm) appeared to be slightly greater than the sizes of ER-bound ribosomes (13–18 nm), but similar in size to free ribosomes/polyribosomes seen in nearby cell bodies. The causes of heterogeneity in eukaryotic ribosome structure, composition and function are beyond the scope of this discussion (see (Ramagopal, 1992; Mauro and Edelman, 2002; 2007)), but it was reported early on in liver preparations that membrane-bound ribosomes were smaller than free ribosomes (Ramsey and Steele, 1977). As recently reviewed (Reid and Nicchitta, 2015), free and ER-bound ribosomes tend to translate different proteins, with integral membrane proteins being translated on ER-bound ribosomes, while free ribosomes translate cytosolic proteins, although this distinction is now known not to be absolute. How this may relate to the mechanisms of axon regeneration in our studies is not clear.

Accumulation of mRNA in axon tips

a. Apparent increase in axonal mRNA contents after axotomy—Staining for both poly(A)+RNA and mRNA for individual cytoskeletal proteins was found in axon tips by 10–14 days post-TX. In fact, we have found evidence for accumulation of mRNAs in the distal-most region of cut axon tips within one day post-TX (unpublished). Since there is no DNA in axons, the mRNA is presumably transported from the cell body. Very little mRNA was detected in untransected axons, so this distal accumulation of mRNA is likely due to backup of a very low level of ongoing mRNA transport from the perikaryon into a truncated axon. It also may be that the axon injury causes a signal to be transported retrogradely to the cell body, resulting in increased transcription of some mRNAs, so that the amount of mRNA transported from the perikaryon to the axon is increased. In this case, we would expect mRNA to appear both in the distal tip, and in more proximal portions of axon, with a gap in mRNA content along the axon between. Indeed, some axons stained with probes for mRNAs encoding L-NFL and β -tubulin in more proximal regions, discontinuous from the tip, suggesting that the amount of mRNA transported also may be increased after axotomy. In either case, accumulation of mRNA in severed axon tips might contribute to the conditioning

lesion effect on axon regeneration, whereby prior axotomy potentiates regeneration after a subsequent lesion (McQuarrie et al., 1977; McQuarrie and Grafstein, 1981). This strategy has been used to increase sensitivity of mRNA detection in ascending DRG axons growing into peripheral nerve grafts (Kalinski et al., 2015), and in cultured DRG neurons (Zheng et al., 2001; Verma et al., 2005; Willis et al., 2007; Gummy et al., 2011; Kalinski et al., 2015). The present results suggest that this also may be true in lamprey spinal-projecting axons, but until now a conditioning lesion effect has not been looked for in the lamprey spinal cord.

b. mRNA in axons in vivo—Although mRNAs have been found in axons of some specific brain regions, *e.g.*, oxytocin and vasopressin mRNAs in the hypothalamo-hypothyseal tract (Mohr et al., 1991), and odorant receptor mRNAs in olfactory axon terminals in the olfactory bulb (Ressler et al., 1994), evidence for mRNA in axons of mature CNS neurons *in vivo* has been difficult to obtain, and has required indirect or signal-augmenting procedures. When peripheral nerve segments were grafted into the transected spinal cords of adult rats, invading spinal axons contained 5S rRNA, phosphorylated S6, and multiple translation-related factors (Kalinski et al., 2015). The authors did not look for these translational elements in non-grafted injured spinal cord axons, but the levels of translational machinery were comparable to those of injured sciatic nerve, *i.e.*, much greater than those found by others in the axons of optic nerve (Verma et al., 2005). The same group had previously used transgenic mice in which mRNA for GFP was driven by the 3'-UTR of β -actin in order to track mRNA distribution in spinal cord axons. Ten days after SCI, GFP label had accumulated in the injured axons (Willis et al., 2011). Because the accumulation of mRNA occurred in the absence of a graft, the relationship between translational machinery and axon regeneration in the more recent study is not clear. Evidence that local axonal synthesis of the transcription factor ATF4 is required for retrograde spread of the pathogenic effects of A β ₁₋₄₂ amyloid peptide was obtained by inhibiting local translation with Atf4 siRNA, with subsequent reduction in retrograde neuronal loss (Baleriola et al., 2014). With a high resolution ISH technique, these authors could demonstrate Atf4 mRNA in axons of adult mice and human brain (Baleriola et al., 2015).

Correlation between axon regeneration and mRNA content

The existence of large, identifiable, spinal-projecting neurons in the brainstem of lampreys has enabled us and others to discover heterogeneity in the abilities of individual neurons to regenerate their axons after spinal cord TX (Davis and McClellan, 1994; Jacobs et al., 1997). There is a tendency for axons of small neurons to regenerate better than those of large ones, and most regeneration-incompetent RS neurons (except for the Mauthner axon) are located in the ventromedial axon tracks, while regeneration-competent axons tend to be located in the dorsolateral columns (Rovainen, 1967; Buchanan, 2001). Nevertheless, neither size, position within the brainstem, nor axonal trajectory within the spinal cord can completely account for the differential regenerative abilities of axons. Their regenerative probabilities of these identified neurons have been correlated with their contents of mRNAs for several proteins, and this has generated hypotheses about mechanisms of axon regeneration and neuronal survival after axotomy (Jacobs et al., 1997; Shifman and Selzer, 2000; Shifman et al., 2008; Shifman et al., 2009; Busch and Morgan, 2012; Hu et al., 2013; Zhang et al., 2014). These studies did not assess how mRNA contents were related to whether the axons

of particular neurons had actually regenerated. By contrast, in the present study, we correlated the mRNA contents of some axon tips with their actual growth status at the time of observation, without attempting to trace each axon to its identified perikaryon in the brainstem. We have not yet determined whether a particular molecular characteristic correlates better with the *a priori* regenerative probability of a neuron, or with its actual regeneration. However, because regeneration probabilities are stochastic, if a particular phenotypic feature is essential to the regenerative process, it is logical to expect that the latter correlation would be closer. Indeed, we have recently found that after axotomy, caspase activation and expression of the receptor protein tyrosine phosphatase σ (PTP σ) was seen almost exclusively in neurons whose axons had not regenerated at 7 weeks post-TX. If this finding is confirmed with other molecules, it would be consistent with the view that molecular characteristics are more reliably related to actual regeneration status than to *a priori* probability of regeneration. Nevertheless, our previous observations suggested that axon regeneration in lamprey spinal cord is discontinuous (Jin et al., 2009), *i.e.*, epochs of axon tip advance are interrupted by periods of stasis and even retraction, so it would be surprising if no mRNA were present, even in static and retracting tips.

Implications of the absence of growth cones in regenerating lamprey spinal cord axons

In DRG cultures, mRNA is concentrated most densely in the growth cone. Pharmacological inhibition of protein synthesis after a conditioning axotomy *in vitro* prevented the formation of growth cones (Verma et al., 2005), although local application of protein synthesis inhibitors in compartmented cultures failed to eliminate axon growth (Eng et al., 1999). These observations suggest that in the growth of axons on substrates *in vitro*, growth cone-mediated axon elongation is at least partially dependent on local protein synthesis. Whether local protein synthesis is required for axon regeneration *in vivo* is not known. In the present study, there was more mRNA in those tips that were actively growing than in sessile or retracting tips, even though this growth is very slow, averaging only 5 $\mu\text{m}/\text{hour}$ (Jin et al., 2009). This is consistent with the hypothesis that local protein synthesis contributes to axon regeneration *in vivo*. However, work in the lamprey has suggested that the growing tips of regenerating axons differ markedly from the growth cones of embryonic neurons during development or in tissue culture. Regenerating RS axons lack filopodia and lamellipodia (Lurie et al., 1994; Jin et al., 2009), contain little F-actin (Hall et al., 1997; Jacobs et al., 1997; Jin et al., 2009), and elongate much more slowly than axons *in vitro* (Lurie and Selzer, 1991). Moreover, the growth tips of lamprey spinal axons are densely packed with NFs (Lurie et al., 1994; Pijak et al., 1996; Hall et al., 1997; Jin et al., 2009), which do not invade typical growth cones. These findings have led us to postulate that the mechanism of axon regeneration is different from that of embryonic axon development, in which growth cones rely on an actin-myosin molecular motor to pull the axon in the direction of filopodia anchored to an adhesive substrate. Although it is much more difficult to identify the terminals of axons regenerating in the CNS of mammals, recent observations on CST axons regenerating in the spinal cords of PTEN-deficient mice suggest that their tips have lancinate shapes (Willenberg et al., 2016) similar to those described in regenerating lamprey axons. The abundance of L-NFL, and to a lesser extent β -tubulin, mRNAs by both target-specific PCR and ISH, preferentially in actively elongating axon tips is consistent with a possible role of these molecules in the mechanism of axon regeneration. On the other hand, mRNAs

for β -actin were less abundant in axoplasms of regenerating axon tips. This is somewhat analogous to findings in axons of cortical neurons grown in prolonged cultures in microfluidic chambers. NFL and β -tubulin mRNA levels persisted, while mRNA encoding β -actin decreased significantly from 7 to 13 days in culture. During regeneration following re-axotomy, axonal levels of mRNAs encoding actin and microtubule-associated proteins were reduced, but levels of mRNAs for neuronal intermediate filament proteins were increased (Taylor et al., 2009). It is known that axons of DRG can continue to regenerate even when actin is depolymerized pharmacologically (Marsh and Letourneau, 1984). This was not true in cultures of chick DRG from very young (E7) embryos, but appeared by E14 (Jones et al., 2006). The residual growth was dependent on polymerization of microtubules, but not acutely on their transport function. Similarly, in β -actin knockout mice, motor axon regeneration was unimpaired *in vivo* (Cheever et al., 2011). On the other hand, in cultured rat neurons, increasing axonal synthesis of β -actin and decreasing axonal synthesis of GAP-43 resulted in short, highly branched axons, while decreasing axonal synthesis of β -actin and increasing axonal synthesis of GAP-43 resulted in long axons with few branches (Donnelly et al., 2013). This suggested that β -actin might be more involved in local axon sprouting than long-distance axon growth, and that sprouting may be based on mechanisms more similar to early axon development than axon regeneration. The present findings on mRNA species in regenerating axon tips is consistent with the hypothesis that as axons mature, their ability to grow in response to axotomy is based on mechanisms that involve NFs and microtubules, but not actin-myosin molecular motors, as would be true of conventional growth cones.

Conclusions

The findings in the present study are significant in at least three respects. First, they confirm the existence of mRNAs and elements of protein synthetic machinery in axons of the CNS more directly than has been possible for mammalian CNS. Second, by microaspirating axoplasm from individual axon tips, it has been possible to determine for individual axons which mRNAs are available for translation. Ongoing studies in this laboratory using RNA deep sequencing will allow us to catalog these mRNA species beyond the ones targeted in the present study, as has been done by others for peripheral nerve and axons *in vitro*. Third, we add evidence for a correlation between the presence of protein synthetic machinery and regeneration of axons, thus strengthening the hypothesis that local protein synthesis contributes to the mechanism of axon regeneration *in vivo*, and that the relative inability of axons to target protein synthetic machinery to the injured axon tip may be one reason why axons in the mammalian CNS fail to regenerate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Selection of axon tips for EM analysis

A, live image of the tip (white star) of a large RS axon back-labeled with DTMR at 2 weeks post-TX. **Body** = body of the tip; **fp** = finger-like protrusion; **CC** = central canal. **Blue Inset**: a toluidine blue-stained 1 µm semithin section of the same tip, showing continuity of **fp** with **body**. **B**, EM of the area indicated by the white box in **A**. The green box is the area shown at higher magnification in Fig. 2. Parts of profiles **a**, **c** and **d** are included in Fig. 2. The large (approximately 1 µm), round osmophilic densities (white arrowheads) are intragial lipid droplets (Lurie et al., 1994), and should not be confused with ribosomes, which are much smaller (20–29 nm). **C**, the same EM with outlines around areas that are probably contiguous. The yellow outline represents the boundary of space **a**, and includes the rER-like structure analyzed in Fig. 2. The blue outline encloses spaces **b–d**, which are probably part of the same axon tip (inset), although they appear to be separated from it in this plane of

sectioning. They do not contain any of the structure analyzed in Fig. 2. **Black/white inset:** The original live image of the area covered by EM. In this and subsequent figures, rostral is left. A magenta-green copy is provided in supplementary Fig.S-1.

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Fig. 2. EM evidence for rough endoplasmic reticulum (rER) in an axon tip

A, the boxed region illustrated in Fig. 1B and C shown at higher magnification. Note that it contains three of the related profiles (**a**, **c**, and **d**) of the axon tip. A membrane structure (blue arrowhead) divides compartment **a** into two spaces (**sp1** and **sp2**). The enlargements in subsequent frames show that this structure is part of an assembly that resembles rER. The green, deep blue and light blue boxes are areas imaged at higher magnification in **B**, **C**, and **D** (framed with lines of corresponding colors), respectively. **B**, overall view of an assembly with the appearance of rER. The complete profile consists of membranous structures with Golgi apparatus-like appearance. Regions framed by yellow and red boxes are shown at higher magnification in **E** and **F** respectively (framed with lines of the same colors). In **C&D** and **E&F**, insets are magnifications of the boxed areas, to show the rER-like assembly associated with vacuoles or vesicles. Membranous structures studded with ribosome-like

granules (yellow arrowheads) are shown in the respective insets. The dashed white line in **B** and the white arrow in **F** show a pathway connecting spaces *sp1* and *sp2*. *re* = rER; *nf* = neurofilaments; *mt* = mitochondria; *ga* = Golgi apparatus-like structure; *v* = vesicles; *co* = cisternal organelle. A magenta-green copy is available as supplementary Fig.S-2.

Fig. 3. Polyribosomes near membranous inclusions in axon tips

DTMR-backfilled spinal cords were studied by EM at 2 weeks post-TX. **A**, EM of the junction between the body (*bd*) and a finger-like protrusion (*fp*) of an axon tip, outlined in the inset at the bottom left. The diffuse electron-dense material (black arrow) probably represents *en face* membrane or subaxolemmal cortex, and contains several ribosomes (small white box, enlarged in the inset). Both the *bd* and *fp* are filled with neurofilaments (*nf*; white arrows). **B**, a similar electron-dense plaque contains a crescent-shaped membranous inclusion. Polyribosomes (white arrowheads) are present in or adjacent to this plaque. Cisternal organelles (*co*) and vesicles (*v*) are found in the plaque. Another ribosome-containing plaque was located at the distal edge of the axon tip (black arrowhead in the bottom left inset; not shown by EM). **C**, for comparison, in a nearby unidentified cell, polyribosomes form rosettes (arrowheads), or are associated with membranous structures (arrows).

Fig. 4. Axon tips with ribosomes and polyribosomes

EMs of four transected axon tips, imaged at low magnification in the pale insets. The black-framed boxes in these insets indicate the region of the accompanying EM. **A–C**, the distal axon tips contain ribosomes/polyribosomes (arrowheads). When located in the body (**bd**) of an axon tip, far from membranous invaginations or vesicle-like structures (**v**), most ribosomes are present as single particles or duplets. **nf** = neurofilaments. **D**, clusters of ribosomes (arrowheads) near the axolemma at the tip of a small axon. Polyribosomes were often clustered near vesicle-like membranous structures (white boxed inset). The areas in the small white boxes are further enlarged in the white-framed insets.

Fig. 5. Immunohistochemical demonstration of ribosomal protein S6 in axon tips

Four tips are illustrated **A**, **B**, **C** and **D**. **A1**, **B1**, **C1** and **D1** show DTMR back-labeled axon tips at the time of fixation. **B2**, **C2** and **D2** show staining with an anti-S6 mAb; **A2** is a negative control, with the primary antibody omitted. Note the absence of S6 label, even in the surrounding cells. **A3**, **B3**, **C3** and **D3** show panels **A1/A2**, **B1/B2**, **C1/C2**, and **D1/D2** superimposed, respectively. The growth status of tip **A** was not determined. Tips **B** and **C** were actively growing at the time of fixation. Tip **D** was sessile. S6 label was distributed in areas just subjacent to the axolemma (green arrowheads), or in a linear formation penetrating the central part of the tip with the appearance of a microtubule bundle (yellow arrowhead in **B2**), and in surrounding cell bodies (blue arrowheads in **B2**, **C2**, and **D2**). Note the unstained region at the very caudalmost limit of the axon tip in **B** (white arrow in **B1**), and the absence of staining in the static tip (white stars in **D2**). **E**, the antibody recognized one major band of expected molecular weight on lamprey CNS homogenates. Scale: 50 μ m. A magenta-green copy is available as supplementary Fig. S-3.

Fig. 6. Oligo(dT) staining is absent or very weak in uninjured axons

An oligo(dT) probe was used to label total mRNA by ISH in uninjured lamprey spinal cords.

A, a transverse section of unlesioned spinal cord labeled with an oligo(dT) probe. Only cell bodies were stained. On the left side, the giant Müller RS axons of the ventral columns are indicated by *. **cc**, central canal; **Mth**, Mauthner axon. No staining was found in axons.

Three pale green lines (dorsal to ventral) indicate the approximate levels of the plane of section shown in **C**, **D**, and **E**. **B**, a similar transverse section stained by IHC with LCM3, an anti-NF mAb that primarily labels axons (Jacobs et al., 1995; Merrick et al., 1995). **C**, **D** and **E**, three horizontal sections from a series through the ventral columns in an uninjured spinal cord, showing absence of labeling in the large reticulospinal axons (*), while neurons, glia and ependymal cells were labeled. In **C**, the central canal (**cc**) is bounded by columns of stained ependymal cells (**e**). The thicker columns of labeled cells above and below the central canal are the gray matter (**gm**). Scale: 100 µm. A magenta-green copy is available as supplementary Fig. S-4.

Fig. 7. Oligo(dT) staining is apparent only in injured axons and is stronger in growing than in static axon tips

In two DTMR-backfilled spinal cords at 2 weeks post-TX, a growing tip (**1** in **A–C**) and a static tip (**2** in **D–F**) were monitored and then stained with Oligo(dT). The top panels (**A** and **D**) are live fluorescent images taken at 0 hr. The middle panels (**B** and **E**) are live images taken 2 hours later. The bottom panels (**C** and **F**) are horizontal sections through the axon tips stained with Oligo(dT). Insets in **A**, **B**, **D** and **E** are enlargements of the areas in the white boxes containing the axon tips, used for defining the growth statuses of the tips (blue line), based on fiduciary landmarks (vertical green lines), using the “Find Edges” tool in Photoshop to define the leading edges. The arrow in **C** points to the distal-most edge of the growing axon tip **1**, which contains label throughout most of its area (Percent Filled = 89.1), as compared to the absence of staining in adjacent axon shafts (*). A slender area of gray label to the right (distal) of the arrow makes it appear that the axon has regenerated even further, but careful microscopic focusing showed that it belongs to another axon. The arrow in **F** points to the distal-most edge of static tip **2** (Percent Filled = 3.3). **G**, correlation between growth status and the % of axon tip area occupied by ISH label. Percent Filled (mean ± sem) for growing, static, and retracting axon tips were 57.1 ± 8.1 (n = 14), 18.6 ± 3.7 (n = 15), and 6.8 ± 2.5 (n = 10), respectively. * indicates mean values significantly reduced compared to growing tips (p < 0.05, Kruskal-Wallis one way-ANOVA). **TX**, side where transected; **cc**, central canal. A magenta-green copy is available as supplementary Fig. S-5.

Fig. 8. Aspiration of axoplasm/cytoplasm from axon tips/cell bodies

A–C, a spinal cord viewed under the dissecting fluorescence microscope before and after micro-aspiration. Two axon tips, 1 and 2 in A, were penetrated by separate micropipettes. Each tip disappeared after its axoplasm was aspirated (B and C, respectively). D–F, verification of transfer of cytoplasm to a micro-aspirating electrode. A large reticulospinal neuron, the left B₁ (*), in the brainstem that had been back-labeled with DTMR was micro-aspirated and the transfer of cytoplasm into the electrode tip was observed by light (D) and fluorescence (E) microscopy. F is D and E superimposed, so that red fluorescence is seen within the glass pipet tip. Note: The electrode remained in the B₁ cell after aspiration. G, global amplification of mRNAs. Microaspirated mRNAs in the cytoplasm of a Mauthner cell, and axoplasms of one individual and 6 pooled regenerating axon tips were amplified by RT-PCR with negative (10 pg total CNS RNA, no RT enzyme) and positive controls (10 and 100 pg total CNS RNA, with RT enzyme). H, After global amplification, target-specific PCR showed L-NFL mRNA in the cytoplasms and axoplasms. Cloned L-NFL was a positive

control (**L-NFL**). Scale: 300 μ m. A magenta-green copy is available as supplementary Fig. S-6.

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Fig. 9. Differential expression of cytoskeletal protein mRNAs in growing and static axon tips
 Target-specific primers for lamprey β -actin (**A**), β -tubulin (**B**), vimentin (**C**), L-NFL (**D**), NF95 (**E**), NF180 (**F**) and NF132 (not shown) were used for PCR. Distilled water was the negative control. **G.tips** = globally amplified cDNA from 6 pooled growing axon tips; **S.tips** = globally amplified cDNA from 6 pooled static tips; **Total RNA** = cDNA from total CNS RNA (100 pg); **Library cDNA** = lamprey CNS cDNA library. Both the growing and static tips contained mRNAs encoding parts of L-NFL (389 bp) and β -tubulin (505 bp), but they were sparser in static tips. β -actin (229 bp; loading volume was doubled) and NF95 (417 bp) mRNAs were sparse in growing tips and not detected in static tips. Vimentin (394 bp), NF180 (264 bp) and NF132 (287 bp, not shown) were not detected.

Fig. 10. Vimentin and L-NFL signals were undetectable in electrodes after sham aspiration A/B and C/D, two axon tips (1 and 2) found in 2 spinal cords were micro-aspirated and collected in 2 tubes with RNase inhibitor. Spinal cords were imaged before and after the aspiration (A vs. B; C vs. D). E, a third spinal cord in a region with no axon tips was used as control for sham aspiration. F, penetration of spinal cord without entering axon tips (sham penetration). **Green arrow:** the tip of a sham aspiration electrode viewed by light microscopy. G and H, electrophoresis of target-specific PCR products in 1% agarose gel after amplification of vimentin (F) and L-NFL (G). **Water** = negative control. **Vimentin** = cloned lamprey vimentin gene (5 ng); **L-NFL** = cloned L-NFL gene (5 ng); **1/6 electrodes** = reactions from single or 6 sham-aspiration tips; **axoplasm(1)/(2)** = axoplasm harvested from axon tip 1 (A and B) or axon tip 2 (C and D); * = no band detected in PCR product from

samples of 1 or 6 pooled sham-aspiration electrodes. Scale: 300 μm . A magenta-green copy is available as supplementary Fig.S-7.

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Fig. 11. High vimentin mRNA content in lamprey spinal cord

A, a back-labeled axon tip (*) visualized in the living spinal cord at 2 weeks post-TX. The right side of the spinal cord is partially obscured by muscle. **B**, paraffin section ISH with a vimentin cRNA antisense probe. The axon tip was not labeled by the probe (*). Staining was intense in unidentified adjacent cells (some are neurons – upper blue rectangle in **B**) and cell processes (some are presumed glial processes – both rectangles in **B**). **C**, widespread staining with a vimentin cRNA antisense probe in wholemounted lamprey brainstem. Note the unstained RS neurons, which look like bubbles against the background staining. **Yellow arrowheads** point to mesencephalic Müller cells M2 (upper) and M3 (lower); medial **green arrowhead** is the B1 Müller cell; lateral **green arrowhead** is the Mauthner cell. Scale bar: 300 μ m. A magenta-green copy is available as supplementary Fig.S-8.

Fig. 12. ISH of axon tips with L-NFL probe

A–D, a tip that had grown 21.2 μm in 3.8 hours (**A,B**). The green line connects a stationary fiduciary landmark (small neuron) in the two frames. Insets in **A/B** and **E/F** (bottom left) are enlargements of the boxed areas as in Fig. 7. **C**, the growing tip from **A/B** is labeled intensely by ISH for L-NFL. Note the most intense label is found at the leading edge (red arrow) and in a narrow spine at the base of the tip (yellow arrowhead; presumably a microtubule bundle). More than 7 nearby axons (**D**) were not labeled by the probe (**C**). Binding of the L-NFL probe suppressed **rhodamine** fluorescence (* in **D**). **E–H**, a static axon tip monitored as in **A–D**. No tip displacement was discerned between the images in **E** and **F** (3.6 hrs apart). In **G**, this tip (red arrow) showed little or no labeling for L-NFL mRNA. * In **F** and **H** is a nearby axon tip that is not connected to the one that was

monitored. **Green arrowheads** in **C** and **G** point to small neurons stained by the L-NFL probe. Scale: 200 μm . A magenta-green copy is available as supplementary Fig. S-9.

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Fig. 13. β -tubulin ISH in axon tips

A/B, a growing (1; 31.83 μ m/3.75 hr) and a static (2, white arrow) tip were identified in sequential live images at 2 weeks post-TX, and aligned against fiduciary landmarks (green line) to measure movement of the tip (blue and yellow lines). In **A**, * marks a 3rd axon tip, which was lost at the 2nd imaging. **C**, the spinal cord sectioned and stained with a biotin-labeled β -tubulin RNA probe, which labeled the distal end of the growing tip (red arrow) but not the static tip (white arrow). **D**, blockage of rhodamine fluorescence by presence of the β -tubulin RNA probe. **E–H**, a static axon tip. The β -tubulin probe bound to a broad area of the axon tip (**G**). Sometimes staining was seen even in axons (yellow arrowheads). Insets in **A/B** and **E/F** are enlargements of the boxed areas as in Fig. 7. **H**, DTMR-fluorescence of the section in **G**. Scale: 200 μ m. A magenta-green copy is available as supplementary Fig. S-10.

Fig. 14. ISH of axon tips with a β -actin probe

A/B, two large RS axon tips had their growth status determined over a 4.5 hour interval. The green vertical line locates a fiduciary landmark. The blue and yellow vertical lines mark the distal edges of axon tips 1 and 2 in **A**. Tip 1 was growing (26.52 μ m/4.5 hr). Tip 2 was static. **Insets** in **A** and **B** are magnifications of the areas in the white boxes as in Fig. 7. **Yellow arrowhead** in **B** shows the position of axon tip 2 relative to its position in **A**. **C–F**, in the same spinal cord, the β -actin probe stained the growing tip 1, (**C** and **D**), but not the static tip 2 (**E** and **F**). **Green arrowheads** point to lightly stained local neurons. **D** and **F** are fluorescent images of the sections in **C** and **E**, respectively. The red arrows in **C** and **E** point to the distal-most edges of the axon tips, based on the rhodamine backfilled images in **D** and **F**. Scale: 200 μ m. A magenta-green copy is available as supplementary Fig. S-11.

Fig. 15. Localization of mRNAs for L-NFL and β -tubulin in some injured axons

Two fluorescently back-labeled spinal cords were stained with an L-NFL probe (A and C), and a probe for β -tubulin (E and G). DTMR fluorescent images of the same sections are shown immediately below them in B&D and F&H, respectively. In A/B and E/F, a small fraction of the injured axons were stained with the ISH probes (green stars). White stars identify unlabeled nearby axons. C/D and G/H show mRNA entering axon tips in the sub-axolemmal region (yellow arrowheads). The distal ends of the axon tips are indicated by green arrowheads (C,G). The yellow star in G identifies the positively stained axon just proximal to its stained, growing axon tip, which had been micro-aspirated and is partially collapsed. The green star in G marks a second positively stained axon, whose tip is not in the frame. Scale: 100 μ m. A magenta-green copy is available as supplementary Fig. S-12.

Table 1

Summary of animal use.

Studies	numbers	Body length (cm, mean $\pm$ sem)	Survival time (days) ^a
EM	16	12.1 $\pm$ 0.3	10–14
Immunostaining (SP6)	17	11.9 $\pm$ 0.3	10–14
ISH (Oligo dT)	41	11.9 $\pm$ 0.2	10–14
Aspiration (pooled)	34	12.0 $\pm$ 0.2	10–14
Aspiration (sham)	5	12.7 $\pm$ 0.4	10–14
Aspiration (individual)	60	11.7 $\pm$ 0.1	10–14
ISH (gene specific)	79	12.0 $\pm$ 0.1	10–14
Western blot	10	11.2 $\pm$ 0.3	

^aBetween the day of spinal cord transection and the day of sacrifice after observations or aspiration.

Table 2

Primers used in target-specific PCR

a. L-NFL	
Primer 1:	5'-GACGTTAACACGTTCAACATGCACT-3'
Primer 2:	5'-TCCCGCCTGACCATCAGCTCCTCCT-3'
b. NF95	
Primer 1:	5'-GCAAGGAGCCCAAGAAAGCCGTCAG-3'
Primer 2:	5'-TTTTTTTCTTAACATTCTGTTTTTC-3'
c. NF132	
Primer 1:	5'-AGGCCGGAGCTGAGGAAGAGGACGA-3'
Primer 2:	5'-CTGCTTCTGTGTCGTCTTGTTTCAGC-3'
d. NF180	
Primer 1:	5'-GCAGAGCCGGCGAAGGAAAAACACT-3'
Primer 2:	5'-CGGGGCCGCCACCTTCTCCACTTTC-3'
e. β-Actin	
Primer 1:	5'-CATCATGAAGTGTGACGTGGACATC-3'
Primer 2:	5'-GATCCACATCTGCTGGAAGGTGGAC-3'
f. β-Tubulin	
Primer 1:	5'-GACATCTGCTTCCGCACCCTCAAGC-3'
Primer 2:	5'-GGCAACAGCACGGCCATCCAGGAGC-3'
g. Vimentin	
Primer 1:	5'-ACAGAAGTATACGTGTAGGC-3'
Primer 2:	5'-ACTCCTCTACACCATACAGG-3'

Table 3

Primary and Secondary Antibodies

Name	Host species and antigen	Manufacturer	Dilution	RRID
Anti-S6riboprotein	Mouse monoclonal full-length human recombinant protein	#2317, Cell Signaling Technology	1:1000 (WB) 1:100 (IHC)	AB_2238583
LCM3 (anti-NF180)	Mouse monoclonal lamprey CNS cytoskeletal protein	Author's lab	1:100 (IHC)	AB_2314735
Biotinylated	Goat anti-mouse IgG	BA-9200, Vector Laboratories	1:200 (IHC)	AB_2336171
IRDye@800	Goat anti-mouse IgG	610-132-121, RocklandImmunochemicals	1:7,500 (WB)	AB_220347
Alexa Fluor@488	Goat anti-mouse IgG	A-11029, Invitrogen	1:200 (IHC)	

Table 4
Growing axon tips show increased probability of L-NFL and β -tubulin mRNA presence

Global amplification and gene specific PCR showed that growing tips had a greater likelihood of containing mRNA for L-NFL and β -tubulin than did retracting tips, (^a: $p = 0.02$ for L-NFL by χ^2 test, ^b: $p=0.0003$ for β -tubulin). The increase compared to static tips was not statistically significant ($p > 0.30$), but when static and retracting tips were merged into a single “Non-Growing” group, the difference between growing and non-growing tips was significant for both L-NFL (^c: $p = 0.03$) and β -tubulin (^d: $p = 0.002$).

	Growing (N = 10)		Static (N = 10)		Retracting (N = 10)		All Non-Growing (N = 20)	
	Yes	No	Yes	No	Yes	No	Yes	No
L-NFL	9 ^a	1	6	4	4	6	10	10 ^c
β -tubulin	10 ^b	0	6	4	2	8	8	12 ^d