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The assessment of adeno-associated vectors as potential intrinsic treatments for brainstem axon regeneration

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Abstract

Background—Adeno-associated virus (AAV) vector-mediated transgene expression is a promising therapeutic to change the intrinsic state of neurons and promote repair after central nervous system injury. Given that numerous transgenes have been identified as potential candidates, the present study demonstrates how to determine whether their expression by AAV has a direct intrinsic effect on axon regeneration.

Methods—Serotype 2 AAV-enhanced green fluorescent protein (EGFP) was stereotactically injected into the brainstem of adult rats, followed by a complete transection of the thoracic spinal cord and Schwann cell (SC) bridge implantation.

Results—The expression of EGFP in brainstem neurons labeled numerous axons in the thoracic spinal cord and that regenerated into the SC bridge. The number of EGFP-labeled axons rostral to the bridge directly correlated with the number of EGFP-labeled axons that regenerated into the bridge. Animals with a greater number of EGFP-labeled axons rostral to the bridge exhibited an increased percentage of those axons found near the distal end of the bridge compared to animals with a lesser number. This suggested that EGFP may accumulate distally in the axon with time, enabling easier visualization. By labeling brainstem axons with EGFP before injury, numerous axon remnants undergoing Wallerian degeneration may be identified distal to the complete transection up to 6 weeks after injury.

Conclusions—Serotype 2 AAV-EGFP enabled easy visualization of brainstem axon regeneration. Rigorous models of axonal injury (i.e. complete transection and cell implantation) should be used in combination with AAV-EGFP to directly assess AAV-mediated expression of therapeutic transgenes as intrinsic treatments to improve axonal regeneration.

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Keywords

adeno-associated viral vector; axon regeneration; enhanced green fluorescent protein; Schwann cell; spinal cord injury; Wallerian degeneration

Introduction

Damage to the central nervous system (CNS) results in an abortive regeneration of injured axons and a slow process of Wallerian degeneration. The delayed degeneration of axonal remnants in part contributes to the lack of axon regeneration in the CNS [1]. Given the complex pathophysiology of CNS injury, the successful regeneration of injured axons will ultimately require a combination of treatment strategies. These may include extrinsic strategies such as the modulation of environmental factors [2] and cellular transplantation [3–5], as well as intrinsic strategies that aim to change the phenotypic state of neurons [6–11]. One mechanism to promote the intrinsic growth capacity of neurons is through the expression of transgenes, many of which have been identified as candidates [12–20], including those that modify PTEN/mTOR signaling [21], Krüppel-like factors [22] and mammalian achaete-scute homolog-1 (MASH-1) (R. R. Williams, D. D. Pearce, M. B. Bunge, unpublished data). Before these transgenes can be utilized clinically, however, neuron-specific therapeutic vectors must be developed and tested in animal models of axonal injury. The testing of these vectors will require the co-expression of both a transgene and an anterograde reporter to label the axons. In this way, changes in the regeneration of labeled axons may be directly associated with the neuronal expression of the therapeutic transgene.

Given that most axonal populations do not express cell-type specific antigens for identification with immunohistochemistry, the histological assessment of axon regeneration is often achieved through targeted neuronal tracing techniques. Of these, anterograde tracing allows for the discrete visualization and quantification of axonal processes and their terminations. Although various types of anterograde tracers are available, they are often limited by their use as a single bolus injection and therefore must be applied during an optimal temporal window for detection [23,24]. Furthermore, when utilized to test the efficacy of vectors as intrinsic treatments, axons that are labeled with traditional tracers may not be expressing the transgene of interest. To surmount this issue, therapeutic vectors may be designed to express fluorescent proteins, such as enhanced green fluorescent protein (EGFP) [25]. The continual expression of fluorescent proteins allows them to distribute throughout the neuron so that detailed morphology is easily visualized over the course of weeks to months [26,27]. Thus, fluorescent proteins are useful anterograde tracers to assess the efficacy of therapeutic vectors to promote axon regeneration.

Of the different types of gene therapy strategies, adeno-associated virus (AAV) vectors offer several advantages for the treatment of neurological disorders and, as such, are currently being tested clinically [28–30]. Some recombinant AAV serotypes exhibit neuron-specific tropism in various regions of the CNS [31–40], where they induce long-term gene expression with minimal immune response and toxicity [29,41–44]. AAV-mediated

expression of EGFP functions as an anterograde tracer that fills individual neurons in a Golgi stain-like manner [38,45–48], and transgene expression may be regulated by tetracycline inducible promoters [49,50]. Recombinant AAV vectors do not integrate well into the host genome but, instead, form episomal concatamers through the intermolecular recombination of multiple single-stranded genomes [51,52] and these episomal concatamers are primarily responsible for transgene expression [53]. Thus, upon infection, there is minimal potential for recombinant AAV vectors to alter the phenotype of the neuron, unlike other vectors that integrate into the host cell genome. Another useful feature of concatamers is that each one contains three to six genomes and co-infection of a cell with AAV vectors carrying different transgenes results in the co-expression of both genes [54]. Importantly, this may be exploited to assess AAV vectors as intrinsic treatments to promote axonal regeneration because mixing different AAV vectors may result in the co-expression of a therapeutic transgene and a fluorescent protein. Accordingly, any observed improvement in the regeneration of anterogradely labeled axons directly demonstrates that a given AAV vector is having an intrinsic effect because the somata of the labeled axons also express the therapeutic transgene.

Given the potential for AAV vectors to serve as intrinsic treatments to promote axon regeneration, we developed a novel experimental paradigm for their assessment pre-clinically. This paradigm combines the use of AAV-EGFP as an anterograde tracer with a rigorous method of assessing axon regeneration and degeneration. The injury model we employed was the complete transection of the thoracic spinal cord and implantation of a Schwann cell (SC) bridge [55]. This model was chosen, because, unlike most other CNS injury paradigms, axons identified on the SC bridge have unequivocally regenerated, thus negating issues with sparing and/or sprouting found in other models.

Before the complete transection and implantation of a SC bridge, AAV-EGFP vectors were stereotactically injected into the brainstem. Subsequently, 6 weeks after injury, animals were preserved and EGFP-labeled brainstem axon regeneration into the bridge was assessed. This approach anterogradely labeled numerous brainstem axon populations in the thoracic spinal cord, as well as those that had regenerated into the bridge. This large population of EGFP-labeled axons was readily quantified, and careful analysis revealed that, when continually expressed, EGFP may not be uniformly distributed throughout the axon but may accumulate in distal regions with time. In addition, the use of AAV-EGFP, before injury, resulted in the long-term presence of EGFP-labeled axon remnants in the spinal cord, caudal to the bridge. Because these axon remnants were easily visualized, we propose that this paradigm also offers a new tool to study the process of slow Wallerian degeneration in the CNS. Thus, the preclinical assessment of therapeutic transgenes, expressed by AAV vectors, will require co-expression with a fluorescent protein and the use of rigorous experimental injury models, such as complete transection and cell implantation.

Materials and methods

Experimental design

Twenty young adult (postnatal day 35) female Fischer rats, born on the same day, were obtained from Harlan Laboratories (Frederick, MD, USA). Each rat received two separate

surgeries: first the stereotaxic injection of serotype 2 AAV-EGFP (Figure 1a) and then the complete transection of the spinal cord with the implantation of a SC bridge (Figures 1b and 1c). Each stereotaxic surgery took approximately 90–100 min, and therefore only two rats were injected with AAV-EGFP vectors per day. To achieve maximal transgene expression at the time of injury, all stereotaxic surgeries were performed between 2 and 6 weeks before injury (Figure 1d). Thus, the postnatal age of the rats was in the range of 6–10 weeks on the day that they were injected with AAV-EGFP vectors. The second surgery (i.e. the complete transection of the spinal cord and implantation of a SC bridge) took 20–25 min. To ensure that all the rats were the same age at the time of injury, these surgeries were performed over the course of 2 days (Figure 1d). In this way, the rats were 12 weeks of age and weighed approximately 160–180 g at the time of injury. Six weeks after the complete transection and implantation of a SC bridge, the rats were euthanized for histological analysis (Figure 1d). One animal died during stereotaxic injection and one animal died during transection/SC bridge implantation. In addition, animals were excluded from the study based on the criteria: surgical damage to the spinal cord ($n = 2$), a collapsed polymer channel that surrounded the cellular bridge ($n = 3$), poor rostral stump insertion into the polymer channel ($n = 1$), formation of a large cyst within the polymer channel ($n = 1$) or poor EGFP-labeling of brainstem axons ($n = 1$). In sum, ten animals were used for the quantification of EGFP-labeled axons 6 weeks after the complete transection and implantation of a SC bridge. All procedures were conducted in accordance with animal welfare standards established by the European Communities Council, the USA National Institutes of Health Guide for the Care and Use of Laboratory animals, as well as the Institutional Animal Care and Use Committee at the University of Miami Miller School of Medicine.

Generation of AAV vectors

Serotype 2 AAV vectors were generated by the Miami Project to Cure Paralysis Viral Vector Core, using the AAV Helper-Free System from Stratagene (La Jolla, CA, USA). Briefly, 293 cells were grown to 70–80% confluency, at which point they were transfected with the two helper plasmids and the transgene plasmid for enhanced EGFP (kindly provided by Dr S. Whittemore, University of Louisville, KY, USA) using jetPEI™ (Poly plus Transfection, San Marcos, CA, USA). Transgene plasmids (Figure 1f) were under the transcriptional control of the cytomegalovirus promoter and contained a human growth hormone polyadenylation region (hGH-pA) and the Woodchuck post-transcriptional regulatory element (wPRE) to stabilize the mRNA and for translation efficiency [56,57]. The cells and media were harvested 72 h after transfection and purified for virus using the AAV ViraKit from Virapur (San Diego, CA, USA). There is a dose-dependent effect of AAV on the expression of EGFP [58]. Therefore, all animals were injected with the same titer of AAV-EGFP generated from the same stock. The AAV-EGFP used in the present study was found to have 2.4×10^{10} genomes/ml. In addition, functional titers of AAV-EGFP were determined by infecting HT1080 cells shocked with $0.8 \mu\text{M}$ camptothecin. The use of camptothecin *in vitro* allows for AAV to induce rapid transgene expression in approximately 2 days. Then, EGFP-expressing HT1080 cells were quantified and the transducing units/ml (TU/ml) of AAV-EGFP were found to be 2.6×10^8 TU/ml.

Stereotaxic injection of AAV

The rats were first anesthetized by intraperitoneal injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). Next, the head was secured in a stereotaxic device attached to a micro-manipulator, and a sagittal incision was made to expose the cranial sutures. A micro-drill was used to create a 5-mm wide heart-shaped burr-hole caudal to lambda, exposing the dorsal aspect of the cerebellum as well as the left and right transverse sinus (Figure 1a). Bregma was used as the zero point for bilateral injections into the regions: (i) 2.5 mm lateral, 9.2 mm caudal, with 0.5 μ l injected at 11 mm, 10.5 mm, 10 mm, 7.6 mm and 7.1 mm ventral; (ii) 1.4 mm lateral, 9.6 mm caudal, with 0.5 μ l injected at 10 mm, 9.5 mm, 9.0 mm, 8.5 mm, 8.0 mm, 7.5 mm, 7.1 mm and 6.8 mm ventral; (iii) 0.6 mm lateral, 10.8 caudal, with 0.5 μ l injected at 10.8 mm, 10.4 mm, 10.0 mm, 9.5 mm, 9.0 mm and 8.5 mm. The vector was injected using a sp310i syringe pump (World Precision Instruments, Sarasota, FL, USA) together with a 5- μ l syringe modified with a 5-cm 33-gauge removable needle (Hamilton Company, Reno, NV, USA) at a rate of 0.25 μ l/min. To summarize, the following regions were injected bilaterally with: 2.5 μ l to the ventrolateral pons, 4.0 μ l to the dorsomedial pons and 3.0 μ l to the ventromedial rostral medulla, for a total of 19 μ l in six injection sites. Injections were always performed in a specific order to minimize potential volume lesions (Figure 1a). After surgery, the rats received subcutaneous injections of 3 ml of physiological saline twice a day (b.i.d.) for 3 days to compensate for fluid loss, gentamicin (5 mg/kg) once a day (q.d.) for 7 days to prevent infection, and the analgesic buprenorphine (0.1 mg/kg) three times a day (t.i.d.) for 3 days.

Generation of purified SCs

Purified (95–98%) SC populations were obtained from adult female Fischer rat sciatic nerves as described previously [59,60]. After dissection, nerve explants were placed in D10 [Dulbecco's modified Eagle's medium (DMEM); Invitrogen Corp., Carlsbad, CA, USA; supplemented with 10% fetal bovine serum and gentamicin (50 μ g/ml)]. Contaminating fibroblasts were first removed by transferring the explants to new dishes weekly. Two weeks later, the nerve explants were incubated in 0.25% dispase plus 0.05% collagenase in D10, dissociated by trituration, and plated. After reaching 90% confluency, the SCs were further purified by incubation in conditioned medium from mouse hybridoma cells containing the Thy1.1 antibody and adding rabbit complement to destroy fibroblasts. The SCs were expanded in D10 containing 20 μ g/ml bovine pituitary extract (Invitrogen Corp.), 2.5 nM heregulin (Genentech, San Francisco, CA, USA) and 2 μ M forskolin. SCs were passaged to confluency three times at which point they were harvested by trypsinization and resuspended in DMEM for transplantation.

Spinal cord transection and SC bridge implantation

The rats were anesthetized by intraperitoneal injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). A laminectomy was performed from thoracic vertebrae levels 7 (T7) to T9, with significant lateral exposure. After dural removal from the exposed cord and cutting of the dorsal roots, a single incision was made with angled microscissors to completely transect the spinal cord at T8. Then, the ventral dura was severed and the ventral roots were cut. This procedure led to the retraction of the spinal cord stumps approximately 2–3 mm (Figure 1b).

Completeness of the injury was confirmed by lifting the rostral and caudal stumps. A 5.0 mm long, double-walled polyacrylonitrile/polyvinyl chloride polymer channel, with an inner diameter of 2.7 mm, was positioned in the gap, accommodating rostral and caudal stump insertion approximately 1 mm into each end of the channel. Then, 3.0×10^6 SCs in 15 μ l of DMEM were mixed with 10 μ l of Matrigel (BD Biosciences, San Jose, CA, USA) and immediately injected through the rostral of two holes previously created in the top of the channel (Figure 1c). All SCs were harvested from the same sciatic nerves and passaged at the same time. The SCs were kept on ice for no more than 2 h before implantation. The muscle layers and skin were closed with sutures and Michel clips, respectively. After surgery, the rats received subcutaneous injections of 5 ml of physiological saline b.i.d. for 7 days, gentamicin (5 mg/kg) q.d. for 7 days to prevent urinary tract infection, buprenorphine (0.1 mg/kg) t.i.d. for 3 days to reduce discomfort, and their bladders were expressed manually b.i.d. until they regained micturition.

Tissue processing and immunohistochemistry

The rats were sacrificed by terminal anesthesia (200 mg/kg ketamine and 20 mg/kg xylazine). Their left ventricles were injected with 20 USP units of heparin, and then the rats were perfused transcardially with 200 ml of 4 °C, phosphate-buffered saline (PBS) followed by 400 ml of 4 °C, 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The brains and spinal cords were extirpated and further fixed in PFA overnight and cryoprotected in PBS plus 30% sucrose and 0.025% sodium azide. Tissue blocks were embedded in PBS plus 12% gelatin and 0.025% sodium azide and quick frozen in crushed dry-ice. Brainstems were sectioned into six series at 40 μ m parasagittally using a freezing microtome, and stained as free-floating sections before being mounted on slides. The SC bridges with rostral and caudal spinal cords were serially sectioned into five series at 20 μ m parasagittally using a cryostat and mounted directly on slides.

The antigenic sites were blocked in PBS with 0.5% Triton-X plus 5.0% normal goat serum (NGS) and/or 5.0% normal donkey serum (NDS). Then, a given series of tissue sections was incubated overnight in up to three of the following primary antibodies: GFP (chicken, 3.19 mg/ml, dilution 1:500; Chemicon, Temecula, CA, USA), dopamine β -hydroxylase (D β H; mouse, dilution 1:500; Chemicon), NeuN (mouse, dilution 1:500; Chemicon), glial fibrillary acidic protein (GFAP) (SMI 22, mouse, dilution 1:500; Covance, Denver, PA, USA; rabbit, dilution 1:500; Dako, Carpinteria, CA, USA), S100 (rabbit, dilution 1:500; DAKO) and nonphosphorylated neurofilament (SMI-32, mouse, dilution 1:1000; Covance). Sections were rinsed in PBS and incubated in PBS plus 1% NGS and/or 1% NDS, plus 0.1% Hoechst dye solution (10 mM) and goat anti-mouse, goat anti-rabbit and/or goat anti-chicken secondary antibodies conjugated to 488 or 594 fluorophores (dilution 1:200; Molecular Probes, Eugene, OR, USA) and/or goat anti-mouse or goat anti-rabbit conjugated to Cy-5 (dilution 1:200; Jackson ImmunoResearch, West Grove, PA USA). Finally, the tissue was rinsed in PBS and cover-slipped with Vectashield mounting medium (Vector Labs, Burlingame, CA, USA).

Quantification of axon regeneration

Adhering to guidelines established for the assessment of axon regeneration [61], parasagittal sections of the SC bridge were analyzed via a line-transect method (Figure 1e) using Neurolucida (MBF Bioscience, Williston, VT, USA) together with an Axiophot fluorescence microscope (Zeiss, Thornwood, NY, USA) and MAC 5000 XYZ stage (Ludl, Hawthorne, NY, USA). The extent of host spinal cord tissue inside the polymer channel was defined by the expression of GFAP-positive astrocyte somata, depicted in red at the spinal cord/SC bridge interfaces. The zero points, 0' and 0'', were determined on the tissue sections that contained the tips of rostral and caudal host spinal cord inserted farthest into the polymer channel, respectively. This distance from the ends of the polymer channel was then used to locate 0' and 0'' on the remaining sections. Then, using Hoechst-staining to visualize the tissue, dorsoventral lines were drawn on each section along the rostro-caudal axis at -10.0 mm, 0.25 mm, 0.5 mm, 1.0 mm, 1.5 mm, 2.0 mm and 2.5 mm from 0', as well as 7.0 mm from 0''. The length of these lines at a given location indicated the dorsoventral thickness of bridge tissue. By focusing up and down through the entire tissue section, the number of EGFP-labeled axons (green lines) that crossed a given dorsoventral line was quantified. In this way, a transverse plane of tissue was analyzed and the area was determined by multiplying the tissue section thickness (20 μ m) by the length of the dorsoventral lines at a given location. To normalize for differences in the amount of bridge tissue sampled across animals, this area was used to report counts as EGFP-labeled axons/mm². In addition, axon counts at a given location along the bridge were determined as a percentage of the number of EGFP-labeled axons 10 mm rostral to 0'. As a reference, Table 1 depicts individual animal tissue measurements and quantifications using the line-transect method of analysis. Each of these measurements was used in the statistical analysis.

Statistical analysis

All statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA), using data from the experimental group of ten animals. These included one-tail nonparametric correlations and a one-tail Welch-corrected nonparametric *t*-test with 95% confidence intervals, as well as a two-way analysis of variance (ANOVA). Data are presented as the mean $\pm$ SEM.

Results

AAV-EGFP infects brainstem neurons and anterogradely labels their axons

The stereotaxic injection of serotype 2 AAV-EGFP resulted in the infection of numerous neuronal populations in the pons and medulla (Figure 2). In this way, AAV is capable of creating a transgenic neuronal brainstem. At 2 weeks after infection, there was no evidence of toxicity, inflammatory response, or volume lesion as evident by the absence of both polymorphic neutrophils and ED-1 positive macrophages (data not shown). In the brainstem, AAV-EGFP only infected neurons (Figures 3a to 3d) because EGFP was not found in any cells that did not also express NeuN (compare Figures 3b and 3d). AAV-EGFP infected various neuronal populations, including the D β H-positive noradrenergic neurons in the brainstem (Figures 3e to 3h). Analogous to variability in AAV infectivity of different neuronal sub-types in the retina [62], the intensity of EGFP expression varied across

different neuronal subtypes in the brainstem. In addition, there was variability in expression among brainstem neurons of similar morphology (Figures 3a and 3d; arrowheads) and phenotype, as depicted in the noradrenergic neurons of the locus coeruleus (Figures 3e and 3h; arrowheads). EGFP-labeled neuronal somata were not found outside the pons, medulla or cerebellum. There was no evidence for diffusion of AAV-EGFP through blood vessels or cerebrospinal fluid. Thus, only stereotactically targeted neuronal populations were infected and anterogradely traced with AAV-EGFP.

After injection of AAV-EGFP into the brainstem, numerous EGFP-labeled axon populations were detected throughout both the grey and white matter of the spinal cord rostral to the bridge. These axons exhibited a range of morphologies, ranging from fine and beaded to large. As described previously for GFP-labeled axons [38,46–48,63–65], anti-GFP immunohistochemistry allowed for the discrete and unambiguous visualization of brainstem axons throughout the spinal cord, some of which may not have been detectable by endogenous EGFP expression alone. By 3 weeks after the injection of AAV-EGFP, the number of EGFP-labeled axons/mm² in the thoracic spinal cord of uninjured animals was analogous to animals that received a SC bridge, described below. In animals that received a SC bridge, the expression of EGFP was maintained in brainstem axons for at least 12 weeks (Figures 4a and 4b). In these animals, the line-transect method of analysis determined that the mean number of EGFP-labeled axons 10.0 mm rostral to the SC bridge was 4904 ± 221 axons/mm². Data for individual animals are presented in Table 1. Neither the duration EGFP was expressed after the stereotaxic injection of AAV (8–12 weeks; Figure 4c), nor the postnatal age of the animal (6–10 weeks) at the time of injection (Figure 4d) correlated with the number of EGFP-labeled brainstem axons in the thoracic spinal cord, rostral to the SC bridge. In sum, these data demonstrate that AAV-EGFP functions as a useful anterograde tracer for various brainstem axon populations in the thoracic spinal cord.

The use of AAV-EGFP to quantify brainstem axon regeneration

The injection of AAV-EGFP into the brainstem followed by the complete transection of the thoracic spinal cord and implantation of a SC bridge anterogradely labeled regenerating brainstem axons in the bridge (Figure 5). The combination of these two methods allowed for the unequivocal identification of EGFP-labeled axon regeneration. By sectioning the SC bridge parasagittally and immunostaining for EGFP, EGFP-labeled axons were easily visualized crossing into the bridge. EGFP-labeled brainstem axons were often in close association with SCs (Figures 6a and 6b). Similar to EGFP-labeled somata, the intensity of EGFP often varied in bridge axons that were of similar diameter and morphology (Figure 6b).

Axon regeneration was assessed at progressive distances across the bridge using the line-transect method of quantification. In conjunction with computer-assisted fluorescence microscopy and Neurolucida, dorsoventral lines were drawn at given locations along each tissue section (Figures 1e and 5). For each animal, the lengths of the dorsoventral lines analyzed at a given location and the thickness of the sections (20 μ m) were used to determine the area of tissue sampled across a transverse plane. A mean $\pm$ SEM of 1.5 ± 0.15 mm² of total bridge tissue was sampled for each animal. Although differences in the total

amount of bridge tissue (Table 1) could potentially affect the ability of axons to regenerate, there was no correlation between the absolute number of EGFP-labeled axons that were found to regenerate 2.5 mm into the bridge and the total amount of bridge tissue for that animal (Figure 6c). At 2.5 mm beyond the rostral interface, the number of EGFP-labeled axons was in the range 0–123 per mm² (Table 1) with a mean SEM of 58±16 axons/mm². Analogous to the number of EGFP-labeled axons 10 mm rostral to the SC bridge (Figure 4c), the number of days AAV-EGFP was injected before injury did not correlate with the number of axons/mm² found to regenerate 2.5 mm into the SC bridge (Figure 6d). However, as expected, the total number of EGFP-labeled axons/mm² found to regenerate 2.5 mm into the SC bridge correlated with the total number of EGFP-labeled axons present 10 mm rostral to the bridge (Figure 6e; $p < 0.01$). These data demonstrate that AAV-EGFP may be used to anterogradely trace the regeneration of brainstem axons into SC bridges implanted in the thoracic spinal cord and, not unexpectedly, the number of these EGFP-labeled axons that are found to regenerate is dependent upon the number that are labeled rostral to the site of complete transection.

In an attempt to normalize for potential differences in the number of anterogradely labeled axons across animals, axon regeneration was quantified as a percentage of those that are labeled [61]. Therefore, the quantification of EGFP-labeled axons in the bridge also was determined as a percentage of the EGFP-labeled axons present 10 mm rostral to the spinal cord/SC bridge interface. As expected, this revealed that the percentage of EGFP-labeled axons that regenerated proximally into the bridge (0.25 mm and 0.5 mm) was similar across animals with different numbers of EGFP-labeled axons rostral to the bridge. However, an unexpected result was observed for the percentage of EGFP-labeled axons that regenerated distally into the bridge (1.0 mm, 1.5 mm, 2.0 mm and 2.5 mm). When animals were separated into two groups, those with < 5000 EGFP-labeled axons/mm² ($n = 4$) in the rostral cord exhibited a significantly lower percentage of those axons to regenerate distally into the bridge compared to animals with > 5000 EGFP-labeled axons/mm² ($n = 6$) in the rostral cord (Figure 6f; $p < 0.05$, two-way ANOVA). This was most striking at the farthest measured distance, where there was greater than a five-fold difference in the percentages between groups. At 2.5 mm into the bridge, animals with < 5000 EGFP-labeled axons/mm² in the rostral cord averaged 0.3% of those axons to regenerate compared to an average of 1.6% in animals with 5000 EGFP-labeled axons/mm² (Figure 6f; $p < 0.01$, Welch-corrected nonparametric t -test). As an alternative to grouping animals into those with more or less EGFP-labeled axons rostral to the bridge, the data from individual animals also was analyzed using correlations. Again, at the distal distances, a significant and direct correlation was observed between the number of EGFP-labeled axons rostral to the bridge and the percentage of those axons that were found to regenerate (data not shown). In sum, the percentage of EGFP-labeled axons found to regenerate distally was not equivalent across animals and was associated with the amount of EGFP-labeled axons in the rostral spinal cord.

Pre-labeling with AAV-EGFP enables long-term visualization of axons undergoing Wallerian degeneration

The injection of AAV-EGFP into the brainstem, before complete transection and implantation of a SC bridge, resulted in numerous EGFP-labeled axon remnants in the caudal spinal cord, distal to the site of injury (Figures 7a and 7b). At 6 weeks after injury, animals exhibited up to 10% of their EGFP-labeled axons to have labeled remnants 7 mm distal to the SC bridge (Table 1). They are not likely to be regenerating brainstem axons because they could not be retrogradely traced with Fast Blue injected 7 mm caudal to the SC bridge/host spinal cord interface (data not shown), and they were present 2 days after injury (Figure 7a). The EGFP-labeled axon remnants were present primarily in the white matter of the caudal spinal cord, although some were also found in the grey matter. At 2 days after injury, many of them were long, continuous, and linear (Figure 7a). By 6 weeks, some of the EGFP-labeled axon remnants remained continuous and linear, although others had begun to exhibit a progressive degeneration. Those with progressive degeneration were fragmented, abnormally tortuous, swollen and/or spheroid, and formed linear arrays along the rostro-caudal axis (Figure 7b). In addition, some but not all of the EGFP-labeled axon remnants were immunopositive for SMI-32, an antibody specific for nonphosphorylated epitopes on neurofilament-H [66] and used to identify degenerating axons [67–70]; SMI-32 immunostaining was co-localized but non-uniformly distributed in EGFP-labeled structures (Figures, 7c to 7e), including those with highly tortuous and spheroid morphologies. Together, these data suggest that the EGFP-labeled axon remnants are undergoing the slow process of Wallerian degeneration in the CNS.

Increasing time of EGFP expression before injury strongly correlated with the total number of EGFP-labeled axon remnants that could be identified in the caudal cord ($p < 0.001$; Figure 7f). Thus, animals injected with AAV-EGFP at 42 days before injury contained many more labeled remnants caudal to the SC bridge than animals injected 14 days before injury. This correlation was distinct from that of the EGFP-labeled axons in the rostral spinal cord or those that regenerated into the SC bridge, neither of which correlated with the duration of EGFP expression.

Discussion

The study of therapeutic transgene expression by AAV vectors as potential intrinsic treatments to promote axon regeneration

Numerous therapeutic transgenes have been identified as potential candidates to change the intrinsic state of neurons and thereby promote the regeneration of their axons after injury [12–20], including those that modify PTEN/mTOR signaling [21], Krüppel-like factors [22] and MASH-1 (R. R. Williams, D. D. Pearse, M. B. Bunge, unpublished data). The pre-clinical assessment of these transgenes will require neuron-specific expression to directly demonstrate intrinsic effects on axon regeneration. Although most types of viral vectors exhibit non-neuronal tropism in the CNS [71–73], some recombinant AAV vector serotypes, including serotype 2, are neuron-specific in various regions and have been used to anterogradely label axons with fluorescent proteins [14,38,46–48,62–65,74,75]. In addition, AAV vectors have been demonstrated to induce axonal growth through the expression of

growth factors [63,64] and molecules that modify intracellular signaling [14,74]. These studies, however, did not quantify the regeneration of axons anterogradely labeled with fluorescent proteins. Therefore, the neurons that were observed to regenerate may not necessarily have been induced to express the therapeutic transgene by AAV and a direct intrinsic effect may not be determined. To surmount these issues, the present study used serotype 2 AAV-EGFP to anterogradely label axons in combination with a complete transection injury model, and thereby demonstrated how to determine whether transgene expression by AAV vectors has a direct intrinsic effect on axon regeneration.

The expression of fluorescent proteins in neurons is gaining popularity as a means of anterogradely labeling axons and will likely become a very useful tool for the quantification of axon regeneration. Towards this end, studies with transgenic mice have quantified the regeneration of these labeled axons after the implantation of GFP-labeled DRG neurons [76–79], or transection of the cortico-spinal tract labeled with yellow fluorescent protein (YFP) [80]. A few groups also have begun to use viral vectors to anterogradely trace and subsequently quantify GFP-labeled axon regeneration [65,81–83]. Of these, only one used AAV and found that co-infection of DRG neurons with AAV-GFP and AAV- $\alpha 9$ integrin led to a small population of GFP-labeled sensory axons growing a short distance after crush injury [65]. This is in contrast to the present study, where thousands of axons were labeled with AAV-EGFP and long distance (2.5 mm) regeneration was observed. By labeling such a large number of neurons and performing a complete transection injury, two important variables were revealed concerning the use of fluorescent proteins for the assessment of intrinsic treatments to promote axon regeneration: (i) potential differences in the accumulation of EGFP along an axon and (ii) the long-term presence of EGFP-labeled remnants distal to the site of injury.

EGFP may not be distributed uniformly along an axon and may accumulate distally

The non-uniform distribution of proteins through the axoplasm may result from a continuing rapid anterograde vesicular transport, possibly mediating the distal accumulation of proteins that regulate neurite length [84]. Therefore, when continually expressed, EGFP may build up at the distal ends of axons in an analogous manner. In support of such an accumulation, transgenic mice, expressing YFP in a subset of neurons, exhibit increased distal axon swellings, with homozygotes having many more than heterozygotes [85].

The present study reports additional data suggesting that fluorescent proteins may accumulate at the distal ends of axons. First, the duration of time EGFP was expressed correlated with the number of EGFP-labeled axons/remnants in the spinal cord caudal but not rostral to the SC bridge. Second, depending upon the number of EGFP-labeled axons rostral to the bridge, the percentage of those axons that regenerate into a SC bridge is similar proximally but differs distally. In this way, animals that have increased neuronal expression of EGFP may have an increased accumulation of EGFP at the distal end of their axons, thereby increasing the percentage of those distal ends that are detectable. This has important implications for the comparison of experimental groups that use different AAV vectors to co-express a transgene of interest and a fluorescent protein for the quantification of axon regeneration. These types of studies must ensure that the vectors and/or mixture of vectors in

each experimental group: (i) express an equivalent amount of fluorescent proteins; (ii) have the same functional (TU/ml) and/or vector genomes/ml; and (iii) express the fluorescent proteins for the same amount of time.

Distal EGFP-labeled axon remnants are present for at least 6 weeks after CNS injury

The present study revealed another important variable that must be addressed when using the expression of fluorescent proteins to quantify axon regeneration. This concerns the long-term presence of EGFP in axon remnants distal to the site of injury. Although this is not an issue when the expression of the fluorescent protein begins after injury, many studies that seek to evaluate how a therapeutic vector affects axon regeneration will likely begin by looking for acute effects. Then, if positive results are observed, the temporal expression requirements will be determined.

The presence of fluorescent proteins in distal axon remnants was first reported in transgenic mice [80,86]. Four weeks after injury, the distal YFP-labeled fibers from the corticospinal tract were reported to be dramatically reduced to less than 0.1% [80]. By contrast, the use of AAV-EGFP to label brainstem axons in the present study resulted in the presence of numerous (greater than 10%) labeled distal axon remnants for at least 6 weeks; some of these remnants were continuous and linear and therefore could be mistaken for regenerating axons. Whether or not the observed increase in the sustainability of labeled remnants in the present study is the result of a difference between transgenic mice and AAV vectors or differences in CNS axon populations remains to be determined. Nevertheless, when using fluorescent proteins to label axons before injury, the potential for labeled remnants to be present after injury may be surmounted by using rigorous methods to assess axon regeneration, where axon regeneration is quantified within an implant, such as the SC bridge.

The use of AAV-EGFP to label Wallerian degeneration distal to CNS injury

The use of AAV-EGFP to anterogradely trace axons before CNS injury labels the long-term presence of remnant axons after injury. In the present study, we confirmed that these structures are not regenerating axons because they were visible 2 days after transection and could not be retrogradely traced when Fast Blue was injected 7 mm caudal to the SC bridge/host spinal cord interface. Although the half-life of wild-type GFP in a mammalian cell line has been reported to be approximately 26 h [87], the presence of enhanced EGFP for up to 6 weeks in these distal structures, as well as along the entire length of the brainstem axons, suggests that the half-life of EGFP may be much longer in axoplasm and/or mechanisms that degrade EGFP may not be functioning. An alternative, yet unlikely, explanation is that EGFP mRNA is being translated in the distal axons and remnant structures.

Given that the EGFP-labeled axon-like structures distal to the site of injury are not regenerating or spared axons, they are likely examples of the slow process of Wallerian degeneration in the CNS. This is supported by an increase in the number of remnants with fragmented and spheroid morphologies at 6 weeks compared to 2 days. In addition, some of the EGFP-labeled axons contained nonphosphorylated neurofilament-H, as indicated by SMI-32 immunostaining; SMI-32-positive axons are significantly enriched in regions

undergoing Wallerian degeneration after optic nerve transection [67] and multiple sclerosis [68–70].

The use of transgenic mice that express fluorescent proteins in neurons has been found to aid in the study of axonopathy in models of multiple sclerosis [69], Alzheimer's disease [88] and traumatic injury [89,90]. The presence of YFP-labeled axon remnants, distal to the site of injury, has also been reported in transgenic mice for up to 4 weeks [80,86]. The results in the present study demonstrate that EGFP-label may be present for at least 6 weeks after traumatic injury in the CNS. Given the robust expression at that time, however, it is tempting to speculate that the label may be present until the axon remnants have been completely degraded, possibly years after injury. Thus, the targeted stereotaxic injection of AAV-EGFP before injury offers a new tool to help elucidate the spatial progression and processes of slow Wallerian degeneration in discrete axon populations in the CNS. With this understanding, new therapeutics may be developed to promote the clearance of slowly degenerating remnants, thereby hastening axon regeneration after CNS injury.

Conclusions

The expression of therapeutic transgenes by AAV vectors will likely serve as important treatments that modify the intrinsic state of CNS neurons and subsequently promote regeneration of their injured axons. For example, the regeneration of adult brainstem axons is increased following AAV-mediated expression of a developmental transcription factor, MASH-1, in their somata (R. R. Williams, D. D. Pearce, M. B. Bunge, unpublished data). To assess their potential pre-clinically, AAV vectors carrying therapeutic transgenes should be combined with AAV vectors carrying transgenes for fluorescent proteins, such as EGFP. Then, differences in the amount of EGFP-labeled axon regeneration, across experimental groups, may be directly attributed to the neuronal expression of the therapeutic transgene. When injected before injury, AAV-EGFP is also useful to assess Wallerian degeneration, given that EGFP may be retained in distal axon remnants for at least 6 weeks after injury. In sum, a rigorous injury model, such as complete transection and cell implantation, should be used in combination with AAV-EGFP to directly assess the effectiveness of therapeutic transgenes, expressed by AAV vectors, as intrinsic treatments to promote axonal regeneration.

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Figure 1.

Experimental design. (a) Photograph of the burr-hole created for the stereotaxic injections of AAV-EGFP, illustrating the sites and numerical order of the injections (numbers; scale bar=1 mm). (b) Photograph of the complete spinal cord transection (scale bar=1 mm). (c) Photograph of the SC bridge implantation, showing the dorsal aspect of the rostral and caudal spinal cord stumps inserted into a polymer channel. Two holes (arrows) in the channel were used for the injection of an initially fluid mixture of SCs and Matrigel (scale bar=1 mm). (d) The experiment timeline. Rats were stereotaxically injected between 42 and 14 days before complete transection and implantation of a SC bridge, at time point zero. Animals were maintained for an additional 42 days. (e) The line-transect method was employed for quantification of EGFP-labeled axons (green) in a SC bridge. The polymer channel (thick black lines) and the transverse dorsoventral lines used for quantification (thin

purple lines) are shown. The numbers represent mm from the rostral interface. Here, as well as in all other figures, rostral cord is left, caudal cord is right, dorsal cord is up, and ventral cord is down. (f) Expression cassettes for the AAV vectors, flanked by a left and right internal terminal repeat (ITR). The cassette contained a cytomegalovirus (CMV) promoter, a human β -globin intron (β -Globin Intron), an enhanced GFP (EGFP) sequence, a woodchuck post-translational element (wPRE), and a human growth hormone polyadenylation (hGH-pA) region.

Figure 2.
After the stereotaxic injection of AAV-EGFP into the brainstem, neurons from various regions express EGFP (green). Hoechst-stained nuclei are blue (scale bar=1 mm).

Figure 3.

AAV-EGFP only infects neurons in the brainstem as evident by single Z-plane confocal microscope images. EGFP (a) is found only in NeuN positive neurons (b). Hoechst stained nuclei (c) and the merged confocal image (d) shows cytoplasm and nuclei indicative of co-localization. Note that neighboring neurons of similar morphology exhibit different intensities of EGFP labeling (arrowheads). AAV-EGFP also infects noradrenergic brainstem neurons showing EGFP expression (e), DβH expression (f), Hoechst stained nuclei (g) and a

merged confocal image (h). After the injection of AAV-EGFP, noradrenergic neurons also exhibit different intensities of EGFP labeling (arrowheads; scale bar=5 μ m).

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Figure 4.

The stereotaxic injection of AAV-EGFP into the brainstem functions to anterogradely trace brainstem axons in the thoracic spinal cord. Thousands of EGFP-labeled axons (green) are present 10 mm rostral to the SC bridge in both lateral (a) and medial (b) spinal cord (scale bar=500 μ m). GFAP immunostaining is red and the central canal is indicated by an arrowhead. In animals injected with AAV-EGFP, there is no correlation between the number of EGFP-labeled axons in the rostral spinal cord and the length of time EGFP was expressed (c) or the age of the animal (d).

Figure 5.

The stereotaxic injection of AAV-EGFP into the brainstem functions to anterogradely trace brainstem axons that regenerate into a SC bridge. A confocal image of a SC bridge, showing EGFP-labeled axons (green, arrows) together with immunostaining for GFAP (red) and S100 (blue). The rostral spinal cord stump is delineated by astrocytes that are orange/pink as a result of their coexpression of both GFAP and S100. The polymer tube is indicated by arrowheads and dorsoventral transverse white lines illustrate those used in the line-transect method for axon quantification. The numbers represent distance (mm) from the rostral interface, 0'. Blue SCs immunostained for S100 are seen throughout the bridge as well as through a hole in the polymer channel at the top right. Orange/pink clusters of autofluorescent macrophages are indicated by asterisks (scale bar=0.5 mm).

Figure 6.

EGFP may not be uniformly distributed along a regenerating axon. EGFP-labeled axons (green, arrows) 1.0 mm (a) and 2.5 mm (b) beyond the rostral interface are in close association with S100-positive SCs (blue; scale bar=20 μ m). Note the difference in the intensity of EGFP expression between axons of similar diameter and morphology in (b) (red arrows). The inset indicates where the higher magnification images were obtained (scale bar=1 mm). (c) The number of EGFP-labeled axons that regenerated to the distal end of the SC bridge (2.5 mm) did not correlate with the total area of bridge tissue. (d) The number of

EGFP-labeled axons that regenerated to the distal end of the SC bridge (2.5 mm) did not correlate with the length of time EGFP was expressed. (e) The number of EGFP-labeled axons that regenerated to the distal end of the SC bridge (2.5 mm) correlated with the number of EGFP-labeled axons 10 mm rostral to the bridge ($p < 0.01$; $r^2 = 0.4$). (f) The percentage of EGFP-labeled axons that regenerated to more proximal distances (0.25 mm and 0.5 mm) was similar among animals with greater or less than 5000 EGFP-labeled axons 10 mm rostral to the bridge. However, the percentage of axons found to regenerate to the distal end of the SC bridge (1.0, mm 1.5 mm, 2.0 mm and 2.5 mm) was less in animals with fewer EGFP-labeled axons 10 mm rostral to the bridge (< 5000 axons/mm²) than in animals with more EGFP-labeled axons (> 5000 axons/mm²; $*p < 0.05$; two-way ANOVA). The percentage of axons found to regenerate to the distal end of the bridge (2.5 mm) was five-fold less in animals with fewer EGFP-labeled axons 10 mm rostral to the bridge than in animals with more EGFP-labeled axons ($**p < 0.05$; Welch-corrected nonparametric *t*-test; error bars indicate the SEM).

Figure 7.

The stereotaxic injection of AAV-EGFP into the brainstem, before complete transection of the thoracic spinal cord and implantation of a SC bridge, labels slowly degenerating axon remnants distal to the site of injury. EGFP-labeled axon remnants (green) are visible in the caudal spinal cord 2 days (a) or 6 weeks (b) after the complete transection. GFAP-positive astrocytes are red. Note the presence of linear and continuous remnants at both 2 and 6 weeks (arrows), and the increased number of spheroid and tortuous EGFP-labeled remnants at 6 weeks compared to 2 days (arrowheads; scale bar=20 μ m). At 6 weeks some of the EGFP-labeled remnants (green, c) were immunopositive for nonphosphorylated neurofilaments (SMI-32, red; d) as depicted in the merged image (e; scale bar=1 μ m). (f) The number of EGFP-labeled axon remnants found 7 mm caudal to a SC bridge correlated with the time EGFP was expressed between stereotaxic injection and complete transection/SC bridge surgeries ($p < 0.001$; $r^2=0.5$).

Table 1

Data from ten experimental animals used for histological analysis

Animal	1	2	3	4	5	6	7	8	9	10
Age of animal at time of injection (days)	64	62	43	51	50	50	62	43	56	56
Duration of EGFP expression from time of injection (days)	62	64	83	75	76	76	64	83	70	70
Days between injection and complete transection	20	22	41	33	34	34	22	41	28	28
10.0 mm rostral to 0'										
Tissue area analyzed (mm ²)	0.81	0.83	0.9	0.84	0.78	0.84	0.78	0.74	0.64	0.67
Number of EGFP-labeled axons	5013	4506	4692	4358	4002	4233	3568	3345	2832	2177
Number of EGFP-labeled axons/mm ²	6188.89	5428.92	5213.33	5188.1	5130.77	5039.29	4574.36	4520.27	4425	3249.25
Bridge tissue analyzed (mm ²)										
0.25 mm past 0'	0.3	0.2	0.34	0.43	0.24	0.16	0.4	0.22	0.34	0.21
0.5 mm past 0'	0.3	0.2	0.33	0.4	0.22	0.15	0.39	0.24	0.32	0.15
1.0 mm past 0'	0.31	0.18	0.34	0.34	0.18	0.12	0.35	0.22	0.28	0.14
1.5 mm past 0'	0.31	0.18	0.38	0.27	0.15	0.12	0.32	0.23	0.25	0.14
2.0 mm past 0'	0.34	0.18	0.38	0.3	0.13	0.12	0.27	0.24	0.23	0.14
2.5 mm past 0'	0.34	0.18	0.37	0.3	0.15	0.15	0.24	0.21	0.19	0.14
Total	1.9	1.12	2.14	2.04	1.07	0.82	1.97	1.36	1.61	0.92
Number of EGFP-labeled axons on bridge										
0.25 mm past 0'	76	43	28	90	145	55	62	10	184	33
0.5 mm past 0'	60	41	21	57	103	43	46	7	143	15
1.0 mm past 0'	54	31	13	40	70	26	14	4	96	1
1.5 mm past 0'	57	29	3	32	30	25	10	2	54	1
2.0 mm past 0'	56	26	0	27	20	13	0	0	28	2
2.5 mm past 0'	42	17	1	32	14	16	1	2	8	0
Number of EGFP-labeled axons/mm ² on bridge										
0.25 mm past 0'	253.333	215	82.3529	209.302	604.167	343.75	155	45.4545	541.176	157.143
0.5 mm past 0'	200	205	63.6364	142.5	468.182	286.667	117.949	29.1667	446.875	100
1.0 mm past 0'	174.194	172.222	38.2353	117.647	388.889	216.667	40	18.1818	342.857	7.14286
1.5 mm past 0'	183.871	161.111	7.89474	118.519	200	208.333	31.25	8.69565	216	7.14286
2.0 mm past 0'	164.706	144.444	0	90	153.846	108.333	0	0	121.739	14.2857

Animal	1	2	3	4	5	6	7	8	9	10
2.5 mm past 0'	123.529	94.4444	2.7027	106.667	93.3333	106.667	4.16667	9.52381	42.1053	0
% of EGFP-labeled axons on bridge										
0.25 mm past 0'	4.09336	3.96028	1.57966	4.03428	11.7754	6.8214	3.38845	1.00557	12.23	4.83628
0.5 mm past 0'	3.2316	3.77608	1.22065	2.74667	9.12498	5.68864	2.57848	0.64524	10.0989	3.07763
1.0 mm past 0'	2.81462	3.17231	0.73341	2.26763	7.57954	4.29955	0.87444	0.40223	7.74818	0.21983
1.5 mm past 0'	2.97099	2.96765	0.15143	2.28443	3.89805	4.13418	0.68316	0.19237	4.88136	0.21983
2.0 mm past 0'	2.66132	2.66065	0	1.73474	2.9985	2.14978	0	0	2.75117	0.43966
2.5 mm past 0'	1.99599	1.73966	0.05184	2.05599	1.81909	2.1167	0.09109	0.21069	0.95153	0
Axon remnants 7.0 mm caudal to 0'										
Tissue area analyzed (mm ²)	0.76	0.81	0.64	0.76	0.77	0.68	0.82	0.77	0.83	0.78
Number of EGFP-labeled remnants	82	140	366	224	177	258	57	251	100	48
Number of EGFP-labeled remnants/mm ²	107.895	172.84	571.875	294.737	229.87	379.412	69.5122	325.974	120.482	61.5385
% of EGFP-labeled remnants	1.74336	3.18368	10.9695	5.68102	4.48023	7.52908	1.51961	7.21138	2.72276	1.89393

Age and duration of EGFP expression are presented for each animal, as well as tissue measurements and quantifications using the line-transect method of analysis. The animals were ordered according to their number of EGFP-labeled axons/mm² in the spinal cord rostral to the SC bridge, referred to as 10.0 mm rostral to 0'. Past 0' refers to measurements made in the SC bridge. Measurements made distal to the SC bridge are referred to as 7.0 mm caudal to 0'.