Preferential and Bidirectional Labeling of the Rubrospinal Tract with Adenovirus-GFP for Monitoring Normal and Injured Axons

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Abstract

The rodent rubrospinal tract (RST) has been studied extensively to investigate regeneration and remodeling of central nervous system (CNS) axons. Currently no retrograde tracers can specifically label rubrospinal axons and neurons (RSNs). The RST can be anterogradely labeled by injecting tracers into the red nucleus (RN), but accurately locating the RN is a technical challenge. Here we developed a recombinant adenovirus carrying a green fluorescent protein reporter gene (Adv-GFP) which can preferentially, intensely, and bi-directionally label the RST. When Adv-GFP was injected into the second lumbar spinal cord, the GFP was specifically transported throughout the entire RST, with peak labeling seen at 2 weeks post-injection. When Adv-GFP was injected directly into the RN, GFP was anterogradely transported throughout the RST. Following spinal cord injury (SCI), injection of Adv-GFP resulted in visualization of GFP in transected, spared, or sprouted RST axons bi-directionally. Thus Adv-GFP could be used as a novel tool for monitoring and evaluating strategies designed to maximize RST axonal regeneration and remodeling following SCI.

Key words: adenovirus-green fluorescent protein; labeling; rubrospinal tract; spinal cord injury

Introduction

STUDIES OF REGENERATION AND REORGANIZATION after injury to the mammalian central nervous system (CNS) have relied heavily on analysis of the rodent corticospinal tract (CST) (Bregman et al., 1995; Cheng et al., 1996; Demjen et al., 2004; Li et al., 1997; Liu et al., 2008; Schnell et al., 1994) and rubrospinal tract (RST; Jin et al., 2002; Kobayashi et al., 1997; Kwon et al., 2002; Liu et al., 1999; Ruitenberg et al., 2003; Tobias et al., 2003; Xiao et al., 2005). For these purposes, the CST or RST must be selectively labeled because their axons comprise only a few percent of all spinal axons, and few molecular markers can distinguish them from neighboring axons. The CST is relatively easy to label because it is the only pathway that directly projects from the motor cortex to the spinal cord, and the cortex is on the surface of the brain. However, currently available methods for labeling the RST, which rely on transport or diffusion of injected tracers to the red nucleus (RN) (anterograde), or to RST axonal terminals (retrograde), are limited in several respects. First, the essential surgery is technically difficult to perform. The RN is a prominent but small structure within the rostral midbrain, and it

has a rounded contour with tapering ends and a broad center (Boseila et al., 1975). The adult rat RN is located about 6.2 mm from the surface of the brain; it is therefore difficult to place a needle tip at the very center of the RN. Second, the precise amount of dye to be delivered is difficult to determine; too much dye may label other neighboring neurons/axons, whereas too little dye may cause incomplete labeling of the RST and rubrospinal neurons (RSNs). Slight variations in the location or the amount of dye applied may lead to large variations in the number of RST fibers labeled. Complex normalization and large numbers of animals are therefore needed to obtain statistically valid results. Third, most tracers (e.g., biotinylated dextran amine) currently in use are visualized by immunohistochemistry or enzyme histochemistry methods, which are time consuming (Kerschensteiner et al., 2005). Finally, no specific retrograde tracers are available to label RST axons, although tracers like Fluoro-Gold (FG) can clearly label their cell bodies and primary dendrites.

In recent years, viruses have been developed for tract tracing studies. Compared to conventional tracers, viruses have the ability to traverse multi-synaptic pathways and replicate to amplify signals at each step in the process

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(Kuypers and Ugolini, 1990). Among the many available virus types (e.g., adeno-associated virus, herpes simplex virus, lentivirus, pseudorabies virus, and Sindbis virus), adenoviruses (Adv) have several attractive properties. They can accept large genetic sequences (up to 8 kb), can be concentrated up to 10^{13} pfu/mL, and have a large host range including non-replicative cells (Le Gal La Salle et al., 1993; Quantin et al., 1992; Teschemacher et al., 2005). These important features make adenoviruses an attractive tracer for studying long projecting axonal tracts and their cell bodies in the CNS.

In the present study, we sought to determine whether adenoviruses expressing green fluorescence protein (Adv-GFP) could be used as a retrograde tracer to label multiple propriospinal and supraspinal neurons whose axons project to the lumbar spinal cord. To our surprise, Adv-GFP injections into the second lumbar (L2) spinal cord resulted in specific and exclusive labeling of GFP throughout the entire projection of the RST and RSN within the RN. Importantly, Adv-GFP injection into the L2 spinal cord following graded SCIs at the 10th thoracic (T10) level resulted in the labeling of GFP-positive RST axons at the second cervical (C2) level in a severity-dependent manner, indicating that these axons were spared at, and rostral to, the injury. When Adv-GFP was injected directly into the RN, GFP was anterogradely transported throughout the entire RST that descended in the spinal cord. Finally, following C3 graded hemi-contusive SCIs, anterograde tracing of Adv-GFP resulted in the labeling of RST axons at the injury site (C3), and caudal to it (C4), in a severity-dependent manner. Based on these results, we believe that Adv-GFP holds great promise for assessing experimental therapies for SCI and other white matter pathologies.

Methods

Adenovirus

Replication-defective, temperature-sensitive, recombinant adenoviruses encoding GFP under the control of a CMV promoter were constructed as described previously (He et al., 1998). Recombinant adenovirus was generated using the AdEasy system, in which the vector was modified to include a temperature-sensitive mutation (ts125) within the DNA-binding protein of adenovirus (Romero and Smith, 1998; Tang

et al., 2004). After transfection of 293 cells, virus production was monitored by GFP expression and plaque formation. All plaque-purified adenoviruses were examined for replication-competent adenoviruses by PCR, and were amplified and purified by double cesium chloride gradient ultracentrifugation. The absolute concentration of viral particles was determined by optical absorbency, and the concentration of infectious particles (plaque-forming units) was quantified by viral hexon protein expression in infected HEK293 cells using the Adeno-X Rapid Titer kit (BD Biosciences, Franklin Lakes, NJ). Expression of GFP by virus-infected U373 cells was confirmed 72 h after transfection under fluorescence microscopy. The adenoviruses were re-suspended in OptiMEM (Life Technologies, Paisley, U.K.) before injection.

Animals

A total of 68 adult female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200–220 g were used in this study. For assessment of retrograde labeling of RST/RSNs, Adv-GFP was injected into the L2 spinal cord, followed by FG injection into the L1 cord 4 days later. To examine the time course of retrograde GFP expression, animals received the Adv-GFP injection at L2, followed by perfusion at 3 and 7 days, and 2, 3, 4, and 6 weeks after the Adv-GFP injection. To determine whether Adv-GFP can specifically label the RST in an anterograde manner, Adv-GFP was directly injected into the bilateral RN. Animals that received Adv-GFP injections into the motor cortex served as controls. Finally, to evaluate the potential of applying this technique in SCI research, animals received either lateral hemisection on the right side, graded contusive injuries at T10, or graded hemi-contusion injuries on the right side at C3 (by dropping a 10 g weight from a height of 6.5, 12.5, or 25.0 mm, respectively). In these groups, Adv-GFP was injected into either L2 or the RN after injury for retrograde or anterograde tracing of the RST, respectively (Table 1).

Surgical procedures

All surgical procedures were performed on rats that were under full general anesthesia by an intraperitoneal injection of ketamine (87.7 mg/kg; Ben Venue Laboratories, Bedford, OH), and xylazine (12.3 mg/kg). Cervical/thoracic/lumbar

TABLE 1. EXPERIMENTAL DESIGN AND ANIMAL GROUPS

<i>Animal groups</i>	<i>Injury site</i>	<i>Adv-GFP injection site</i>	<i>FG injection site</i>	<i>Sampling site</i>	<i>Animal number</i>
Retrograde, specificity		L2	L1	Entire spinal cord, brain	6
Retrograde, time course ^a		L2		C2	24
Retrograde, after graded SCI ^b	T10	L2		C2	16
Anterograde, specificity		RN, Ctx		C2, RN, Ctx	10
Anterograde, after graded hemi-contusive SCI ^c	C3	RN		C2, C3, C4	12

^aTime course: 3 and 7 days, and 2, 3, 4, and 6 weeks post-Adv-GFP injection ($n=4$ /group).

^bGraded SCI: a 10-g weight was dropped from a height of 6.5, 12.5, and 25.0 mm onto the dorsal surface of the T10 spinal cord, as well as a lateral hemisection performed on the right side at the same level.

^cGraded hemi-contusive SCI: a 10-g weight dropped from a height of 6.5, 12.5, and 25.0 mm onto the dorsal surface of C3 cord on the right side.

C, cervical spinal cord; T, thoracic spinal cord; L, lumbar spinal cord; FG, Fluoro-Gold; RN, red nucleus; Ctx, motor cortex; Adv-GFP, adenovirus-green fluorescent protein.

spinal cord exposures were conducted as described previously (Howorth et al., 2009). Contusive and hemi-contusive SCI was performed using a New York University (NYU) impactor, as described previously (Gensel et al., 2006; Wang et al., 2006). Lateral hemisection was carried out with a pair of iridectomy scissors as described previously (Arvanian et al., 2009). Spinal or red nuclear Adv-GFP injections were performed immediately after the various injuries. For spinal Adv-GFP/FG injections, a total of 2.5 μ L Adv-GFP (2.46×10^7 pfu/ μ L) or FG (1% in 0.9% saline; Invitrogen, Carlsbad, CA) was slowly injected into the L2 or L1 spinal cord along the midline, as well as 0.8 mm away from midline on each side, using pulled glass capillaries attached to a nanoliter injection device (World Precision Instruments, Sarasota, FL) at a rate of 5 nL/sec. For the midline injection, two deliveries were made at depths of 0.5 and 1.0 mm (0.25 μ L each) from the cord's dorsal surface. For the bilateral injections, five deliveries were made at depths of 0.3, 0.6, 0.9, 1.2, and 1.5 mm (0.2 μ L each). The needle was left in place for an additional 2 min and gradually withdrawn over 1 min after each injection. For red nucleus Adv-GFP injection, a burr hole was made in the skull over the RN with a dental drill at the coordinates described previously (Mori et al., 1997), and 1.2 μ L of Adv-GFP (2.46×10^7 pfu/ μ L) was slowly injected into bilateral red nuclei (0.6 μ L on each side) over 2–3 min at a rate of 5 nL/sec using a nanoliter injection device. For the cortical injection of Adv-GFP, burr holes over the sensorimotor cortex were made prior to the injection of 6.0 μ L of Adv-GFP (2.46×10^7 pfu/ μ L) into the motor cortex bilaterally (0.5 μ L at each site), as described previously (Sivasankaran et al., 2004). After surgery, the rats were placed in a temperature- and humidity-controlled chamber. For rats receiving contusion injuries, manual bladder emptying was performed three times daily until reflex bladder emptying was established. All surgical interventions and postoperative animal care were provided in accordance with the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care and Use Committees of Indiana University.

Tissue preparation

After appropriate survival, the rats were perfused and the spinal cords and brains were removed, cryo-protected, and sectioned as described previously (Sivasankaran et al., 2004; Wang et al., 2006). To ensure that all FG-labeled neurons in the brain were available for visualization, the entire brain was serially sectioned transversely at 20 μ m from the medulla to the motor cortex. Every third section was mounted onto slides in sequence.

Counting of GFP-labeled axons

Sections of GFP-labeled RST axons were photographed from transverse sections of the spinal cord at C2 or C4, or from serial horizontal sections of the spinal cord at C3, using an Olympus BX-60 microscope. For GFP retrograde labeling, GFP-labeled axons after graded-contusion injuries were counted bilaterally from two randomly selected transverse sections at C2 and averaged for each animal. In the spinal hemisection group, only GFP-labeled axons on the ipsilateral side were counted at C2. The data are presented as the number of GFP-labeled axons/unilateral RST/animal. For

GFP anterograde labeling, the GFP-labeled axons were counted from two randomly selected transverse sections and averaged on each side at both C2 and C4, or counted from serial horizontal sections spaced 60 μ m apart and summed on each side at C3. In all quantifications, the investigators who counted the axons were blinded to group assignment. The data were presented as the number of GFP-labeled axons/unilateral RST/cord level/animal. Quantification of GFP-labeled RST axons in this region include (1) the absolute number of GFP-positive axons at ipsilateral C4; (2) the percentage of spared/sprouted GFP-positive axons in the ipsilateral C3 compared with those in the contralateral C3; (3) the percentage of spared/sprouted GFP-positive axons in the ipsilateral C4 (distal to the injury), compared with those in the ipsilateral C2 (proximal to the injury); and (4) the percentage of spared GFP-positive axons in the ipsilateral C4 (distal to the injury), compared with those in the contralateral C4 (undamaged side).

Immunohistochemistry

Before primary antibody incubation, sections were permeabilized and blocked with 0.3% Triton X-100/1% normal donkey serum in 0.01 M PBS for 30 min at room temperature. Different cell-specific and dye-specific antibodies were applied to the sections overnight at 4°C. The cell-specific monoclonal antibodies included rabbit anti-microtubule associated protein 2 antibody (anti-MAP-2, 1:200; Sigma-Aldrich, St. Louis, MO) to identify neurons, mouse anti-glial fibrillary acidic protein antibody (anti-GFAP, 1:200; Sigma-Aldrich) to identify astrocytes, mouse anti-CC1 antibody (1:200; Calbiochem, La Jolla, CA) to identify oligodendrocytes, and mouse anti-ED-1 antibody (1:200; Chemicon International, Temecula, CA) to recognize macrophages and monocytes. The dye-specific antibody included rabbit anti-FG antibody (1:400; Millipore, Billerica, MA) to enhance FG labeling. On the following day, the sections were incubated with rhodamine-conjugated donkey anti-rabbit or rhodamine-conjugated donkey anti-mouse (1:100; Jackson ImmunoResearch, West Grove, PA) antibodies. The sections were washed, mounted, and examined. Primary antiserum omission controls and normal mouse and donkey serum controls were used to further confirm the specificity of the immunofluorescence labeling.

Statistical analysis

Data obtained from the histological assessments were analyzed for significant differences between animal groups using either Student's *t*-test or one-way analysis of variance (ANOVA), with $p < 0.05$ considered statistically significant. All quantitative data were presented as mean $\pm$ standard deviation (SD). The graphs were prepared using Microsoft Excel software.

Results

GFP expression at and around the injection site

Adult rats received injections of 2.5 μ L (2.46×10^7 pfu/ μ L) of recombinant adenovirus containing the GFP gene into the spinal cord at L2. Two weeks later, GFP was highly expressed at the injection site (Fig. 1C), and then tapered down rostrally

FIG. 1. Green fluorescent protein (GFP) expression at and near the injection site 2 weeks after adenovirus-GFP (Adv-GFP) delivery. (A) Schematic diagram of the Adv-GFP injection site (C, the yellow dashed line at L2), as well as sampling sites rostral and caudal to the injection (the black dashed lines B and D). (C) Adv-GFP (2.46×10^7 pfu/ μ L, 2.5μ L) was injected at L2 sites (denoted by yellow dots) through 3 needle tracks (yellow dashed lines). Intense GFP expression was seen at 2 weeks after these injections. (B) At 4 mm rostral to the injection site, strong GFP expression was seen, particularly in the white matter. (D) At 6 mm caudal to the injection site, GFP expression was also seen in the white matter, but it was markedly decreased. Interestingly, ependymal cells surrounding the central canal exhibited strong GFP expression (Q and inset). (E–P) Adv-GFP (arrows) was co-localized in CC1-positive oligodendrocytes (arrows in E–G), GFAP-positive astrocytes (arrows in H–J), MAP-2-positive neurons (arrows in K–M), but not in ED-1-positive macrophages/monocytes (double arrows in O and P; scale bars = 100μ m in B–D, 20μ m in E–P; GFAP, glial fibrillary acidic protein; MAP-2, microtubule associated protein 2). Color image is available online at www.liebertpub.com/neu.

(Fig. 1B) and caudally (Fig. 1D) from it. In sections 4–6 mm away from the injection site (Fig. 1B and D), strong GFP staining was found mainly in the white matter axons, particularly those in the rostral segment, indicating the low resistance to diffusion offered by the axonal tracts, which run longitudinally within the white matter.

To characterize the cell types that expressed GFP at the injection site, we performed immunofluorescence staining with four cell-specific markers. Oligodendrocytes were identified by an antibody against CC1 (Fig. 1F), astrocytes by GFAP (Fig. 1I), neurons by MAP-2 (Fig. 1L), and macrophages/monocytes by ED-1 (Fig. 1O). At the injection site, GFP was co-localized with many CC1⁺ oligodendrocytes (Fig. 1E–G), relatively fewer GFAP⁺ astrocytes (Fig. 1H–J), or MAP-2⁺ neurons (Fig. 1K–M). Notably, GFP was not co-localized with ED-1⁺ macrophages/monocytes (Fig. 1N–P). Interestingly, ependymal cells lining the central canal expressed GFP at and caudal to the injection site (Fig. 1D inset and Q). In the spinal cord, away from the injection site as well as in the brain, no cells expressed GFP except those in the RN.

Retrogradely transported GFP was preferentially expressed in the rubrospinal axons and neurons

In our original design, we sought to determine whether Adv-GFP could be used as a retrograde tracer to label multiple propriospinal and supraspinal neurons whose axons project to the lumbar spinal cord. To our surprise, Adv-GFP injections into the L2 spinal cord resulted in specific and exclusive labeling of GFP in the entire RST (Fig. 2), from the spinal cord to the brainstem (Fig. 2C–H). At the midbrain level, the GFP⁺ RST axons crossed the midline through the ventral tegmental decussation and ended in the red nucleus (Fig. 2C).

To confirm whether the labeling of Adv-GFP in the RST was specific and exclusive, we performed sequential injections of Adv-GFP and FG into the lumbar spinal cord. FG is a widely used retrograde tracer that can non-selectively label neurons whose axons project to the site of injection. In this experiment, Adv-GFP was injected into the L2 spinal cord, and 4 days later, after effective viral infection and transport, FG was injected into the L1 spinal cord. Ten days after the FG

FIG. 2. Retrogradely transported green fluorescent protein (GFP) preferentially expressed in the rubrospinal tract (RST) and rubrospinal neurons (RSNs). (A) Schematic diagram shows the brain and spinal cord with the projection of the RST indicated in blue. Adv-GFP (2.46×10^7 pfu/ μ L, 2.5μ L) was injected into the L2 spinal cord, as indicated in Figure 1, followed by injection of FG (1%, 2.5μ L) into the L1 cord segment at 4 days post-Adv-GFP injection. The animals were sacrificed 10 days later and representative sections at different levels of the spinal cord (G) and brain (B–F) were examined. (B–H, middle column) GFP was exclusively expressed in the RST throughout its entire trajectory. Specifically, the GFP-labeled RST axons were found to originate from the red nucleus, decussate at the ventral tegmental decussation in the midbrain (C), descend in the caudal midbrain (D), pons (E), medulla (F), spinomedullary junction (G), and the spinal cord (H), where it was located in the dorsal portion of the lateral funiculus. (C–G, left column) These are high-magnification images of the circled areas shown in the middle column. In contrast, Fluoro-Gold (FG) labeling was widely distributed in numerous neuronal groups in the brain and spinal cord whose axons project to the L1 level where FG was injected. Double labeling of GFP and FG was found only in RSNs in the red nucleus (RN, middle and right columns), further confirming the specificity of GFP transport in the RST. Except for the sites at or close to the Adv-GFP injections (H; see also Fig. 1B), strongly GFP-labeled RST axons were found at the dorsal portion of the lateral funiculus (G, middle column), the ventrolateral portion of the brainstem (D–F, middle column), the ventral tegmental decussation (C, middle and left columns), and the red nucleus (C, middle and left columns) in the upper midbrain. Co-localization of GFP and FG was only found in neurons within the RN (right column), whereas in other regions such as the motor cortex (Cx, middle and right columns), pedunclopontine tegmental nucleus (PPTg, middle and right columns), lateral vestibular nucleus (LVe, middle and right columns), raphe pallidus nucleus (RPa, middle and right columns), spinal vestibular nucleus (SpVe, middle and right columns), gigantocellular reticular nucleus (Gi, middle and right columns), locus ceruleus (LC, right column), lateral cervical nucleus (LatC, middle and right columns), and medullary reticular nucleus, ventral (MdV, middle and right columns), no GFP and FG co-localization was found, indicating that GFP was not transported by axons of any of these nuclei, although they projected to the site of FG/Adv-GFP injections (scale bars = 1 mm in B–H, 100 μ m in all others).

injection, GFP and FG retrogradely-labeled axons and neurons were examined. We found that GFP was exclusively expressed in the RST throughout its entire trajectory, as described above (Fig. 2C–G). Moreover, double labeling of GFP and FG was found only in RSNs in the red nucleus (Fig. 2C, RN). Except for the sites at or close to the Adv-GFP injection (Fig. 2H; see also Fig. 1B), strongly GFP-labeled RST axons were found at the dorsal portion of the lateral funiculus (Fig. 2G), the lateral portion of the brainstem (Fig. 2D–F), the ventral tegmental decussation (Fig. 2C), and the red nucleus (Fig. 2C) in the midbrain. Co-localization of GFP and FG was only found in neurons within the RN (Fig. 2C, RN). In contrast, in other regions of the CNS, including the motor cortex (Fig. 2B, Cx), pedunculopontine tegmental nucleus (Fig. 2D, PPTg), lateral vestibular nucleus (Fig. 2E, LVe), raphe pallidus nucleus (Fig. 2E, RPa), spinal vestibular nucleus (Fig. 2F, SpVe), locus ceruleus (Fig. 2, LC), gigantocellular reticular nucleus (Fig. 2F, Gi), medullary reticular nucleus, ventral (Fig. 2, MdV), and lateral cervical nucleus (Fig. 2G, LatC), no GFP and FG co-localization was found, indicating that GFP was not transported by axons of any of these nuclei, although they project to the site of FG/Adv-GFP injections.

Time course expression of retrogradely-transported GFP in the RST

To determine the time course of GFP transport in the RST of the spinal cord, we injected Adv-GFP into the normal rat spinal cord at the L2 level and examined GFP expression at the C2 level at 3 and 7 days, and 2, 3, 4, and 6 weeks after the viral injection. No GFP⁺ axons were observed in the RST at 3 days post-injection (Fig. 3A). GFP was weakly expressed at 7 days post-injection (Fig. 3B), and peaked at 2 weeks when intense dot-like GFP profiles were observed within the RST (Fig. 3C). Although GFP expression slightly decreased at 3 and 4 weeks post-injection (Fig. 3D and E), stable GFP expression was observed at 6 weeks post-injection, the latest time point we observed (Fig. 3F). Quantification of the pixel numbers of GFP⁺ RST axons further confirmed that GFP expression peaked at 2 weeks and lasted for at least 6 weeks post-injection (Fig. 3G).

Retrogradely GFP-labeled spared RST axons after SCI were injury severity-dependent

To determine whether the retrograde transport of Adv-GFP could be used as a tool to directly visualize and measure

FIG. 3. Time course expression of retrogradely-transported green fluorescent protein (GFP) in the rubrospinal tract (RST). Adenovirus-GFP (Adv-GFP; 2.46×10^7 pfu/ μ L, 2.5μ L) was injected into the intact rat spinal cord at L2. GFP expression was examined at C2 over a period of 6 weeks after the Adv-GFP injection. (A) At 3 days post-injection, no GFP-positive RST axons were observed in the C2 spinal cord. (B) At 7 days, lightly GFP-labeled axons were found in the RST at C2. (C and D) GFP expression peaked at 2 weeks (C), and remained strongly expressed at 3 weeks post-injection (D). (E and F) Decreased expression of GFP was found at 4 weeks (E), which lasted for at least 6 weeks post-injection (F). (G) Quantification of GFP-positive RST axons in pixel numbers confirmed that the GFP expression peaked at 2 weeks and lasted for 6 weeks post-injection. ($n=4$; mean $\pm$ standard deviation; *** $p < 0.001$ versus the other groups; scale bars = 100μ m in A–F).

spared RST axons after SCI, we performed graded contusive SCI at mild (a 10-g weight dropped from a height of 6.5 mm), moderate (12.5 mm), and severe (25.0 mm) levels using an NYU impact device at T10, and a lateral hemisection at the same level to transect the ipsilateral RST. In these animals, Adv-GFP was injected into the L2 level and retrogradely-transported GFP was examined at the C2 level in cross-sections to determine the spared RST fibers that were GFP-positive. We found that the number of GFP-labeled axons in the RST was in reverse correlation with injury severity (Fig. 4B–E). In fact, with more severe injuries, fewer numbers of GFP-labeled axons were identified (Fig. 4F). When the RST was completely transected by a lateral hemisection, no GFP-labeled axons were found in the RST rostral to the injury (Fig. 4E and F). Thus, retrograde transport of GFP may be

used as a direct measure for therapeutic interventions aimed at protection of RST axons after graded SCI.

Anterograde transport of GFP in the RST with high specificity

To examine whether GFP can be anterogradely transported along the RST, we injected Adv-GFP directly into the red nucleus in the midbrain. Two weeks later, strong GFP expression was found not only in neurons at and around the injection site (Fig. 5A), but also in axons throughout the entire RST that descended in the spinal cord. The cross-section in Figure 5B shows strongly GFP-labeled RST axons at C2. Such axonal profiles exhibited a longitudinal appearance in horizontal sections such as the one shown in the inset (Fig. 5B').

FIG. 4. Graded spinal cord injury resulted in corresponding loss of rubrospinal tract (RST) axons on the ipsilateral side, as visualized by retrograde transport of green fluorescent protein (GFP). (A) This schematic diagram shows the site of spinal cord injury (SCI) at T10, the injection of adenovirus-GFP (Adv-GFP) at L2 immediately after injury, and examination of Adv-GFP labeling at C2 2 weeks after the viral injection. (B–E) Rats were subjected to graded SCI of mild (B, 6.5 mm), moderate (C, 12.5 mm), and severe (D, 25.0 mm) contusions, and complete lateral hemisection (E). Two weeks following SCI and Adv-GFP injection, GFP was expressed and was retrogradely-transported in the spared or sprouted RST axons at C2 (arrows in B–D), with no expression after lateral hemisection (E). (F) Quantitative data show that graded SCIs produced injury severity-dependent decreases in the number of GFP-labeled RST axons at the C2 level (scale bars = 100 μ m in B–E; * p < 0.05; ** p < 0.01).

FIG. 5. Green fluorescent protein (GFP) was anterogradely transported exclusively through the rubrospinal tract. (**A** and **B**) Injection of adenovirus-GFP (Adv-GFP; 2.46×10^7 pfu/ μ L, 1.2 μ L) into bilateral red nuclei (RN) resulted in intensive GFP expression not only in rubrospinal neuronal cell bodies (arrows in **A**), but also in their descending axons within the rubrospinal tract (RST, **B**) at 2 weeks post-viral injection. (**B'**) This inset photomicrograph shows strong GFP expression of the RST at C2 in a representative longitudinal section. (**C** and **D**) In contrast, when Adv-GFP was directly injected into the motor cortex, no GFP expression was observed in layer V cortical neurons (**C**), or their axons descending within the dorsal corticospinal tract (dCST) located within the ventral-most portion of the dorsal funiculus (**D**). A needle tract in **C** clearly shows the residual injected Adv-GFP. Scale bars = 100 μ m in **A–D** and **B'**.

The number and distribution of GFP⁺ axons at the C2 level were similar to those observed after the Adv-GFP retrograde tracing shown in Figure 2G. To determine whether the GFP anterograde transport was specific to the RST, we chose to examine another pathway, the CST, by injecting Adv-GFP directly into the layer V neurons of the motor cortex. Injection of Adv-GFP into the motor cortex did not affect layer V cortical neurons at the injection site (Fig. 5C), or in the dorsal corticospinal tract (dCST) of the spinal cord (Fig. 5D). Thus, injections of Adv-GFP at either innervating terminals or cell bodies of the RST can selectively transport GFP in a retrograde or anterograde manner, respectively. This viral transfection and GFP expression is RST-specific, since injections of the Adv-GFP into terminals or cell bodies of the CST did not elicit such a response.

Graded SCI resulted in severity-dependent loss of anterogradely-labeled GFP⁺ RST axons

Since Adv-GFP can selectively and anterogradely infect the RSN and RST, this approach may be used as a unique tool to characterize SCI or therapy. We therefore performed graded hemi-contusive SCIs at mild (6.5 mm), moderate (12.5 mm), or severe (25.0 mm) injury levels at C3 on the right side using the NYU impactor, followed by injections of Adv-GFP into bilateral RNs (Fig. 6A). Two weeks later, strong GFP expression was found in both the RST contralateral (Fig. 6B–D) and ipsilateral (Fig. 6B'–D') to the injury. At levels rostral to (Fig. 6B and B'), at (Fig. 6C and C'), or caudal to (Fig. 6D and D') the injury, individually labeled axons could be clearly identified

and counted. The graded hemi-contusive SCI at C3 induced a severity-dependent loss of RST axons on the ipsilateral side. Following mild, moderate, and severe injuries at C3, the absolute numbers of spared RST axons at the ipsilateral C4 were 41.75 ± 3.96 , 23.5 ± 3.42 , and 13.0 ± 2.94 , respectively (Fig. 6E; $n=4$ /group); the percentages of spared RST axons at the injury epicenter (C3) were $52.64 \pm 9.59\%$, $29.85 \pm 7.08\%$, and $11.8 \pm 3.56\%$, respectively, compared to those on the uninjured contralateral C3 (Fig. 6F); the percentages of spared axons at the ipsilateral C4 were $13.64 \pm 1.80\%$, $7.68 \pm 1.53\%$, and $4.27 \pm 0.98\%$, respectively, as compared to those at the ipsilateral C2 (Fig. 6G; $n=4$ /group); and the percentages of spared axons at the ipsilateral C4 were $53.16 \pm 9.61\%$, $30.98 \pm 4.76\%$, and $16.48 \pm 3.17\%$, respectively, compared to those at the contralateral C4 (Fig. 6H; $n=4$ /group). The significant differences between the absolute and relative numbers of these three injury groups indicates a strong correlation between injury severity and the number/percent of GFP⁺ RST axons post-SCI. Additionally, this method allows for direct visualization of the spread of RST axons through the site of injury at C3 (Fig. 6C'). Thus, Adv-GFP can be used as a specific anterograde tracer for visualization and quantification of the RST at levels rostral, at, or caudal to a SCI.

Discussion

Adenoviridae is a family of more than 150 non-enveloped double-stranded DNA viruses that infect all vertebrate classes. We constructed a replication-defective, temperature-sensitive recombinant adenovirus encoding GFP under the

FIG. 6. Graded unilateral spinal cord injury (SCI) resulted in a corresponding loss of rubrospinal axons on the ipsilateral side, as visualized by anterograde transport of green fluorescent protein (GFP) at 2 weeks after adenovirus-GFP (Adv-GFP) injection into the red nucleus (RN). (A) Schematic diagram illustrates the sites of hemi-contusive SCI, as well as tissue sampling for GFP visualization. Adult rats were subjected to unilateral graded contusive SCIs (a 10 g weight dropped from a height of 6.5, 12.5, or 25.0 mm) at C3 on the right side, followed by Adv-GFP injections into bilateral red nuclei. (B–D) In the rubrospinal tract (RST) contralateral to the injury (12.5 mm), GFP was strongly expressed at levels rostral to (B; at C2), at C3 (double arrows in C), and caudal to the injury (double arrows in D). (B'–D') In the RST ipsilateral to the injury (12.5 mm), strong RST axonal labeling was seen rostral to the injury (B' at C2). At the injury site, the number of GFP-labeled RST axons was significantly reduced (C' at C3). Interestingly, some of these axons sprouted at the lesion site (arrows in C'). Distal to the injury, the number of GFP-labeled RST axons was also reduced on the ipsilateral side (arrows in D' at C4). (E) Absolute numbers of GFP-positive RST axons in the ipsilateral RST caudal to the injury. (F) Percentage of spared/sprouted GFP-positive axons in the ipsilateral RST at the lesion site (C3), calculated as the number of GFP-positive RST axons on the ipsilateral side divided by those on the contralateral side. Axons were counted in the serial sections and summed. Only those that intersected with the yellow dashed line were counted (C and C'). (G and H) Percentage of GFP-positive axons in the ipsilateral RST caudal to the injury, calculated as the number of GFP-positive RST axons on the ipsilateral side at C4 divided by those either on the ipsilateral side rostral to the injury (G at C2), or the contralateral side at the same level (H at C4). Notably, increasing injury severity resulted in severity-dependent losses of GFP-positive axons in the RST (scale bars = 100 μ m in A–D and B'–D'; * p < 0.05; ** p < 0.01). Color image is available online at www.liebertpub.com/neu.

control of a CMV promoter and found that it can infect neurons, astrocytes, oligodendrocytes, and ependymal cells at and around the injection site. Unexpectedly, we found that Adv-GFP selectively infected and transported GFP throughout the RST in both retrograde and anterograde directions. Importantly, the GFP-labeled axons that traverse through the lesion site can be readily visualized and quantified in a severity-dependent manner. Based on these results, we propose that Adv-GFP can be a useful tool to monitor and evaluate strategies designed to evaluate RST remodeling or regeneration.

Two weeks following the injection of recombinant Adv-GFP into the spinal cord, the bulk of GFP expression was confined within the injection site with a decreasing rostrocaudal extension of 3–4 mm in each direction. This was likely due to the physical diffusion of the virus (Le Gal La Salle et al., 1993). At the injection site, different cell types including neurons, astrocytes, oligodendrocytes, and central canal ependymal cells expressed GFP. However, GFP was not ex-

pressed in macrophages/monocytes. These results are similar to our previous study (Abdellatif et al., 2006), except that in this study a few GFP⁺ oligodendrocytes were observed. This difference might be caused by the different transgenes expressed (GFP versus GFP-D15A), viral volumes (2.5 versus 1.0 μ L), concentrations (2.46×10^7 versus 5×10^5 pfu/ μ L), and sites (L2 versus T8) that were used in the two sets of experiments. For example, in our previous study we found that transgene expression was significantly dependent on the adenoviral titer (Abdellatif et al., 2006).

Our sequential Adv-GFP and FG double retrograde tracing clearly demonstrated that Adv-GFP selectively infected RST, and that the GFP protein was transported retrogradely throughout the entire length of the RST to reach the RSNs. The GFP-labeled pathway matched exactly the anatomical structure of the RST and RSNs. The specificity of the expression was confirmed by the fact that a retrograde tracer FG, when injected near the site of the adenoviral injection, retrogradely labeled numerous propriospinal and supraspinal neurons

whose axons projected to the Adv-GFP/FG injection sites. However, only neurons in the red nucleus were co-localized with the GFP. Given that GFP specifically labels both the RST axons and neurons, this tracer should be superior to conventional retrograde tracers since the latter, such as FG, only retrogradely labels the cell bodies but not axons in a non-specific manner.

Using a recombinant adenovirus carrying a β -galactosidase (β -gal) reporter gene, other investigators found that β -gal expression occurred not only in the red nucleus, but also in regions of the brain including the vestibular nuclei, reticular formation, and locus ceruleus (Liu et al., 1997; Miura et al., 2000; Koda et al., 2004). Additionally, the labeling was found mainly in neurons in the respective nuclei instead of axons (Tsukamoto et al., 2003). One possibility to explain the difference between their observations and ours is that different reporter genes and promoters were used among these studies. Through the use of cell-specific promoters, it is possible to express the gene of interest selectively in a particular phenotype of cells (Howorth et al., 2009; Teschemacher et al., 2005). It should be noted that a staining observed in a particular nucleus may not necessarily represent the retrograde labeling of neurons within that nucleus; instead, labeling of axons passing through that nucleus may occur. For example, we found many GFP⁺ axons in the midst of reticular formation neurons retrogradely labeled by FG. These axons and neurons, however, were not co-localized within the reticular formation, indicating that the GFP⁺ RST axons were passing through instead of originating from reticular formation neurons.

The lack of GFP staining in both the CST axons and neurons in the retrograde experiment provides additional evidence to support that GFP is retrogradely transported by a selected population of neurons, in our case the RSNs, whose axonal terminals are exposed to the Adv-GFP at the injection site. In the anterograde transport study when Adv-GFP was injected into the motor cortex, no GFP⁺ CST neurons were found at the injection site. This implies that the resistance of corticospinal neurons to Adv-GFP infection may possibly be due to the lack of components important for uptake and transport of the virus (Wood et al., 1996).

The reason why Adv-GFP can specifically label the rat RST axons is unclear. It is likely that there is a unique yet unknown uptake or axonal transport mechanism in the RST that renders the observed specificity. We also speculate that a specific receptor may exist on the surface of the rat RST axons or their cell bodies, because binding of virus particles to specific host cell surface receptors is known to be an obligatory step in infection, even though the molecular basis for these interactions is not well characterized. In recent years, coxsackie B and adenovirus type 2 and 5 receptor (CAR) has been identified as the human and mouse cellular receptor of adenovirus (Bergelson et al., 1997; Bewley et al., 1999; Mayr and Freimuth, 1997; Tomko et al., 1997). The binding of adenovirus to CAR may induce downstream signaling (Tamanini et al., 2006), in which CAR's major role is considered primarily as a docking site prior to integrin-mediated internalization. The tissue distribution of CAR is complex and developmentally regulated (Philipson and Pettersson, 2004). Many previous studies characterizing its tissue distribution are based on mRNA expression, and only a few have directly analyzed its protein expression. The tissue distribution of CAR in adult rats,

however, has received little attention. To determine whether CAR was constitutively and specifically expressed in the RST and RSNs of adult rats, we performed anti-CAR immunohistochemistry. Unfortunately, we did not observe definitive co-localization of CAR protein in the rat red nucleus and RST, or in other regions of the brainstem (data not shown). Another possibility is that adenovirus binds directly to the integrin α V/ β 5 receptor (Wickham et al., 1994). Adenovirus has a much lower affinity for this receptor, and typically the fiber of adenovirus binds to the high-affinity CAR, which brings the penton base to the cell surface to induce binding to this integrin receptor (Bergelson, 1999). Binding the integrin α V/ β 5 is required for virus internalization (Bergelson, 1999; Wickham et al., 1994). An extensive examination of integrin expression throughout the CNS revealed moderate expression levels for both the α V and β 5 subunits of integrin (Pinkstaff et al., 1999). This could also explain why relatively high titers of virus are required for labeling neurons and axons from the red nucleus.

Whereas previous studies showed that adenovirus can be preferentially and retrogradely transported within the CNS (Koda et al., 2004; Tsukamoto et al., 2003; Uchida et al., 2008), this study demonstrated for the first time that GFP can not only be retrogradely transported, but it can also be anterogradely transported through the RST. An *in vitro* study also suggested that axonal transport of canine serotype 2 adenovirus vector is bidirectional and vesicular in cultured motor neurons (Salinas et al., 2009).

Adenovirus stimulates a potent inflammatory immune response that may limit transgene expression and makes repeat viral delivery ineffective. Transient immunosuppression has emerged as one technique to prolong adenoviral-mediated transgene expression and enable re-administration of the virus. Cyclosporin A (CsA; Liu et al., 1997), and monoclonal antibodies against T-cell receptors CD4 (W3/25 and OX-38) and CD45 (OX-22; Abdellatif et al., 2006; Romero and Smith, 1998; Ziemba et al., 2008), have been used to suppress any immune response to the adenovirus. In our explorative experiment, CsA was administered subcutaneously at a dose of 10 mg/kg/d 1 day before and then daily after Adv-GFP injection. We found that the GFP expression pattern in distant regions was similar to that of animals without the immunosuppression, although fewer ED-1⁺ cells were seen at and around the injection site of the immunosuppressed rats (data not shown). Focusing on distant GFP expression and expedience urged us not to use CsA in this study. Many other researchers also did not use any immunosuppressants after adenovirus delivery into the CNS (Haase et al., 1997; Koda et al., 2004). Actually, a study showed that CsA had no effect on adenoviral-mediated lac-Z transgene expression (Wilkinson and Euhus, 2001). Although the maximum expression of GFP was found at 2 weeks after injection, stable GFP expression could last up to 6 weeks post-injection, the latest time point that was observed. Due to the immunogenicity of the adenovirus, survival times of greater than 6 weeks may compromise the functional status of the labeled neurons and therefore should be avoided.

The observation that Adv-GFP preferentially, intensely, and bi-directionally labeled the entire length of the RST axons and their cell bodies makes Adv-GFP a powerful tool to study RST axonal remodeling and regeneration. SCI inevitably affects the RST, an important descending motor pathway, either

partially or completely. Therefore, axonal regeneration, plasticity, and sprouting of the RST following SCI have been studied extensively (Harvey et al., 2005; Koda et al., 2004; Kwon et al., 2002; Liu et al., 1999; Ruitenberg et al., 2003; Wu et al., 2009; Xiao et al., 2005; Xu and Martin, 1989, 1991). Using Adv-GFP to study the remodeling and regeneration of the RST is advantageous since it can specifically label RST axons that traverse through the lesion area both anterogradely and retrogradely. In our study, graded spinal cord contusive injury resulted in a severity-dependent axonal loss which can be used as a powerful tool for studying therapeutic interventions following SCI.

The inversely proportional sparing of GFP-labeled RST axons to injury severity, which can also be related to the proportional sparing of white matter to the injury severity in mice (Nishi et al., 2007) and rats (Cao et al., 2005), made these axons an ideal parameter to evaluate SCI and its treatment. When injury was located at the unilateral cervical spinal cord, where RST fibers were apparent and easily counted, the adjacent GFP-positive RST axons such as those above and below the injury site or on the contralateral side can be readily counted and their ratios can be determined as indicators to evaluate survival/damage of the injured RST. More importantly, detailed morphological changes of RST axons as a result of regeneration, plasticity, or sprouting can be directly visualized and quantified at the injury site.

Results of the present study also suggest another possible application, that introducing recombinant adenovirus containing neurotrophin genes into the spinal cord or red nucleus could deliver therapeutic agents to the red nucleus through a similar mechanism. A previous study demonstrated that adenovirus-mediated *in vivo* gene transfer of brain-derived neurotrophic factor can promote RST axonal regeneration and functional recovery after complete transection of the adult rat spinal cord (Koda et al., 2004).

In summary, here we report the development of a recombinant adenovirus carrying a green fluorescent protein reporter gene that can intensely, preferentially, and bidirectionally label the RST. Following SCI, injection of Adv-GFP into the spinal cord or the red nucleus can result in visualization of transected, spared, or sprouted RST axons, retrogradely- or anterogradely-labeled with GFP. Thus Adv-GFP could be used as a novel tool for monitoring and evaluating strategies designed to maximize RST axonal regeneration and remodeling following SCI.

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Author Disclosure Statement

No competing financial interests exist.

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