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C3 transferase gene therapy for continuous conditional RhoA inhibition

Claire-Anne Gutekunst, PhD¹, Jack K. Tung, PhD^{1,3}, Margaret E. McDougal¹, and Robert E. Gross, MD, PhD^{1,2,3}

Claire-Anne Gutekunst: cguteku@emory.edu; Jack K. Tung: jtung@emory.edu; Margaret E. McDougal: mmcdoug@emory.edu; Robert E. Gross: rgross@emory.edu

¹Department of Neurosurgery, Emory University School of Medicine, Atlanta, Georgia

²Department of Neurology, Emory University School of Medicine, Atlanta, Georgia

³Wallace H. Coulter Department of Biomedical Engineering, Emory University School of Medicine and Georgia Institute of Technology College of Engineering, Atlanta, Georgia

Abstract

Regrowth inhibitory molecules prevent axon regeneration in the adult mammalian central nervous system (CNS). RhoA, a small GTPase in the Rho family, is a key intracellular switch that mediates the effects of these extracellular regrowth inhibitors. The bacterial enzyme C3-ADP ribosyltransferase (C3) selectively and irreversibly inhibits the activation of RhoA and stimulates axon outgrowth and regeneration. However, effective intracellular delivery of the C3 protein *in vivo* is limited by poor cell permeability and a short duration of action. To address this, we have developed a gene therapy approach using viral vectors to introduce the C3 gene into neurons or neuronal progenitors. Our vectors deliver C3 in a cell-autonomous (endogenous) or a cell-nonautonomous (secretable/permeable) fashion and promote *in vitro* process outgrowth on inhibitory chondroitin sulfate proteoglycan substrate. Further conditional control of our vectors was achieved via the addition of a Tet-On system, which allows for transcriptional control with doxycycline administration. These vectors will be crucial tools for promoting continued axonal regeneration after CNS injuries or neurodegenerative diseases.

Graphical abstract

Corresponding Authors: Claire-Anne Gutekunst, PhD, Emory University, Department of Neurosurgery, 101 Woodruff Circle, WMB, Suite 6000, Atlanta, GA 30322. cguteku@emory.edu. Robert E. Gross, MD, PhD, Emory University, Department of Neurosurgery, 101 Woodruff Circle, WMB, Suite 6000, Atlanta, GA 30322. rgross@emory.edu.

Author Contributions:

Conception and design: Gutekunst, Gross

Data collection: Gutekunst, Tung, McDougal

Analysis and interpretation: Gutekunst, Tung, McDougal

Manuscript Preparation: Gutekunst, Tung, McDougal, Gross

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Introduction

The Rho family of small GTPases comprises intracellular molecular switches that play critical roles in regulating diverse cellular processes from cell division and migration to axon outgrowth (Luo, 2000, Stankiewicz and Linseman, 2014). Three Rho GTPases – RhoA, Rac1 and Cdc42 – are central to the regulation of the actin and microtubule cytoskeleton involved in axon growth. In simplified terms, Rac1 regulates lamellipodia formation, Cdc42 regulates filipodia, and RhoA regulates axon retraction (stress fiber formation in non-neural cells). As such, RhoA is a pivotal switch in the axonal response to environmental cues that regulate axon extension versus retraction (Gross et al., 2007).

The injured central nervous system (CNS) in the adult contains several types of molecules that inhibit the outgrowth and lead to retraction of axon growth cones, thus contributing to degeneration of fiber pathways and preventing regeneration of CNS pathways after various types of injury. Overcoming inhibitory molecules associated with myelin and the glial scar could greatly improve regeneration in the nervous system (McKerracher and Rosen, 2015). RhoA mediates the effects of diverse extracellular cues present after injury, including the myelin associated inhibitors (e.g. Nogo66), chondroitin sulfate proteoglycans (CSPGs), and some semaphorins that are commonly found in glial scars. Indeed, biochemical blockade of RhoA activity promotes axon growth and increased axon regeneration in the presence of these inhibitory molecules after CNS injury (Niederost et al., 2002, Fu et al., 2007). These promising effects of RhoA blockade are currently being evaluated in human clinical trials for the treatment of spinal cord injury (Fehlings et al., 2011).

C3 transferase (C3) is a bacterial exoenzyme that specifically and irreversibly inhibits activation of RhoA by ADP ribosylation. Direct delivery of C3 to neurons has been shown to promote axon outgrowth (Niederost et al., 2002). However, C3 is not cell-permeable so modifications have been made to improve its entry into cells (Winton et al., 2002, Tan et al., 2007). *In vivo* inhibition of RhoA by direct injection of C3 promotes robust axonal regeneration in the CNS, as demonstrated in models of optic nerve crush (ONC) or spinal cord injuries (SCIs). C3 recombinant protein delivered directly to the injured optic nerve at the crush site allowed processes to extend beyond the lesion site, but was limited by the

short period during which injured axon processes could take up the C3 reagent (Lehmann et al., 1999). A single application of a cell-permeable version of recombinant C3, C3-07, resulted in neuroprotection of RGCs for one week, as well as increased outgrowth of RGC axons across an ONC lesion (Bertrand et al., 2005). Additional injections resulted in improved survival and regeneration over a 2 week period over the single injection (Bertrand et al., 2007). Similarly, groups have documented axon regeneration by RhoA inhibition after SCIs. In rats, permeable C3 was delivered to a T7 dorsal -hemisection SCI model resulting in extensive axonal sprouting into the lesion site and scar. Subsequent SCI studies reconfirmed that a single injection of a cell permeable C3 (Cethrin) was detectable in cells 7 days later and blocked SCI – induced RhoA activation and apoptosis for that period (McKerracher and Higuchi, 2006). Further results following permeable C3 (Cethrin) injections into SCI have yet to be reported, but are the subject of a human clinical trial (Fehlings et al., 2011, McKerracher and Anderson, 2013). Although these modifications have increased the versatility of utilizing C3 for RhoA inhibition, these studies indicate that without a continuous source of cell-permeable C3, its cellular actions are limited to a duration of several days, which is likely insufficient for the regeneration of long axon pathways that are commonly damaged in neurodegenerative diseases.

To address these limitations, we have generated viral vectors expressing C3 transferase to achieve specific, widespread, long term, and conditional RhoA inactivation. These novel vectors express either an endogenous C3 (eC3) or a secretable/permeable C3 (spC3) fused to the green fluorescent protein (GFP). Borrowing from the genetic nomenclature, we have called these C3 variants cell autonomous eC3 for expression within infected cells, and cell nonautonomous spC3, for an effect beyond the infected cells, respectively. We hypothesize that the latter should be able to affect a greater number of neurons than those infected with the cell autonomous approach. To temporally regulate and reduce any potential risks or side effects of C3 expression, we also developed expression vectors that are regulated by doxycycline (Szulc et al., 2006). As proof of principal, we have tested our transgenes *in vitro* and shown that C3 expression from both cell autonomous and cell non-autonomous vectors inhibit RhoA activation and promote neurite growth on inhibitory substrates. C3 expression in the rat striatum could also be conditionally controlled by doxycycline treatment with no significant adverse effects on striatal integrity. This novel toolbox of C3 vectors provides a versatile means of inhibiting RhoA in the nervous system and offers a promising translatable therapy for the regeneration of injured CNS pathways.

EXPERIMENTAL PROCEDURES

Plasmid construction

The myc-tagged C3 gene was polymerase chain reaction (PCR) amplified from the pRK5-mycC3 vector generously provided by Dr. Alan Hall (Aktories et al., 1989) and inserted into the multiple cloning site of the pEGFP-N2 plasmid (Clontech, Mountain View, CA), in frame with the amino terminus of enhanced GFP (EGFP) to generate pEGFP-N2-C3 that expresses an endogenous form of C3GFP (eC3GFP). Next, a vector that expresses a secretable and permeable form of C3GFP was generated. Overlapping primers containing the DNA sequence for the synthetically modified trans-acting activator of transcription

(TATκ) permeability peptide were PCR amplified and inserted at the amino terminus of C3GFP to create pEGFP-N2-TATκC3 (Tunneemann et al., 2006). The cassette containing TATκC3GFP was then PCR amplified and inserted into the multiple cloning site of the pSecTag2 Hygro A plasmid (Invitrogen) to add an immunoglobulin K (IgK) peptide signal sequence to the N-terminus of TATκC3GFP, thus allowing for the secretion of TATκC3GFP (spC3GFP) into the extracellular milieu. A control IgK-TATκGFP (spGFP) cassette was generated in the same fashion. The cassettes containing eGFP, eC3GFP, spGFP and spC3GFP were subsequently cloned into the FUGW lentiviral and the pAAV backbone plasmids for production of 2nd generation lentivirus and adeno-associated serotype 2 virus (AAV2), respectively.

Cell culture

Human embryonic kidney (HEK) 293T cells and NIH3T3 cells were both maintained in a humidified 5% CO₂ atmosphere at 37°C in complete medium consisting of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 international units (IU)/ml penicillin, and 100 µg/ml streptomycin.

Western Blot (WB)

Transient transfections were carried out with the Lipofectamine 2000 reagent (Invitrogen). HEK293 cells were seeded in 6-well plates at 2×10^5 cells per well, grown for 24 hours (hr) and then incubated for 16hr with 1 ml of serum-free medium containing 2.5 µg of plasmid and 5 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After incubation for 16hr, the medium was replaced by fresh medium containing 10% fetal calf serum for 24hr. Cell extracts were prepared in lysis buffer (Promega, Madison, WI) and protein concentration was determined using a bicinchoninic acid protein assay kit (ThermoFisher Scientific, Waltham, MA). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with either rabbit anti-C3 (generously provided by Dr. Lisa McKerracher, Bioaxone, Cambridge, MA (Winton et al., 2002)) or rabbit anti-GFP antibody (Clontech, Mountain View, CA) followed by a donkey anti-rabbit DyLight 800 secondary antibody (Pierce, ThermoFisher Scientific, Waltham, MA). Infrared (IR) detection was performed on an Odyssey IR scanner (Li-cor Biotechnology, Lincoln, NE).

Rhotekin assay

NIH3T3 cells were seeded in 10 cm dishes at 5×10^5 cells per plate and transfected as described above. NIH3T3 cells were incubated with permeable C3 (2 µg/ml for 4hr, Cytoskeleton Inc.), or dimethyl sulfoxide (DMSO). The next day, the effect of C3 expression on RhoA activation was measured using a pull down assay performed according to the manufacturer's recommendations (Cytoskeleton Inc., Denver, CO). Briefly, cells were rinsed with ice-cold phosphate buffer solution (PBS), and then 250 µl of ice-cold 1X lysis/wash buffer was added. Lysates were cleared by centrifugation for 10 minutes. An aliquot of lysate was collected for measuring the total amount of Rho protein. The remaining lysate was added to Rhotekin agarose beads and incubated on ice for 45 minutes and washed three times. Beads and total Rho samples were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with anti-Rho antibody

followed by a DyLight 680 secondary antibody. IR detection was again performed on an Odyssey IR scanner (Li-cor Biotechnology, Lincoln, NE).

Lentiviral vector production

The FUGW and pLVUT-tTR-KRAB (pLVUT) backbones were obtained from Addgene (plasmids 14883 and 11651, respectively). FUGW is a 3rd generation lentiviral plasmid using the human Ubiquitin C (hUbC) promoter to drive enhanced GFP expression (Lois et al., 2002). pLVUT is a Tet-regulated (Tet-on) lentiviral vector for transgene expression under hUbC promoter control and a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Szulc et al., 2006). Lentivirus were produced in-house based on methods described by Dr. Didier Trono (Barde et al., 2010). In brief, HEK293FT cells were grown in multiple 10 cm cell culture dishes to 90% confluency on the day of transfection. Each dish was then transfected with 7.5 µg 8.9 packaging vector, 3 µg VSVG envelope vector, and 10 µg of the transfer vector by calcium phosphate precipitation. Transfection efficiency was confirmed the following day by fluorescence microscopy and lentivirus was subsequently harvested for 2 days following transfection. The harvested lentivirus was purified through a 0.45 µm polyethersulfone (PES) filter and concentrated by ultracentrifugation with a 20% sucrose cushion to an approximate titer of 10⁸–10¹⁰ infectious particles/ml.

AAV viral vector production

The AAV backbone with a chicken β-actin (CBA) promoter and WPRE was generously provided by Dr. Michael G. Kaplitt, Weill Cornell Medical Center, New York, NY. Stock of AAV serotype 2 expressing GFP (AAV-eGFP) was obtained from the University of North Carolina Vector Core (www.med.unc.edu/genetherapy/vectorcore). The laboratory of Dr. Michael G. Kaplitt prepared virus stocks for AAV-eC3GFP, AAV-spGFP and AAV-spC3GFP. Briefly, vector plasmids were packaged into AAV2 particles using a helper-free plasmid transfection system. The vectors were purified by heparin affinity chromatography and dialyzed against PBS. AAV titers were determined by quantitative PCR using primers to a fragment of the AAV backbone and adjusted to 10¹² genomic particles per ml (Sondhi et al., 2012).

Dot blot

Dot blot assay was used to confirm the secretion of spGFP and spC3GFP. HEK293T cells were plated in 10 cm dishes and transfected as described. After several days in culture, cells were incubated overnight in unsupplemented DMEM. The next day, media was collected and concentrated using Amicon Ultra centrifugal filters (EDM Millipore, Billerica, MA). Concentrated samples were then bound to nitrocellulose membrane. The membrane was blocked and incubated with a rabbit polyclonal anti-GFP antibody (632460, Clontech, Mountain View, CA) followed by a donkey anti-rabbit DyLight 800 secondary antibody (Rockland, Limerick, PA). Again, IR detection was performed on an Odyssey IR scanner (Li-cor Biotechnology, Lincoln, NE).

spC3 functional assay

HEK293 cells were plated in a 6 well tray and transfected as described above with spC3GFP or DsRed2 expressing plasmids. Ten hours later, media was changed and DsRed2 expressing cells were passaged and transferred to a well containing either spC3GFP transfected cells or no cells. After 48 hr, the effect of spC3 on process outgrowth of the DsRed2 expressing cells was evaluated by fluorescence microscopy.

CSPG spot assay

Spots were prepared by placing six 2 μ l droplets of Neurobasal media containing mixed CSPGs (50 μ g/ml; CC117, EDM Millipore, Billerica, MA) and laminin (5 μ g/ml; 23017-015, Invitrogen, Carlsbad, CA) onto poly-L-lysine (PLL) (20 μ g/ml; P9155, Sigma-Aldrich, St Louis, MO) pretreated glass coverslips and allowed to dry completely. Rhodamine was added to the droplets at 1 μ g/ml to help visualize the permissive/repulsive interface. Cortical neurons were dissociated from rat embryos at day 18 (E18) as described previously (Gutekunst et al., 2003). Neurons were plated on pre-coated coverslips and transduced with control or C3 expressing viruses. Five days post transduction cells were fixed with 4% paraformaldehyde and coverslipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA) and visualized using a Leica DMREI fluorescent microscope equipped with a QImaging Retiga EXi camera and Simple PCI acquisition software. Expression of the GFP reporter was visible starting 3 days post infection. Twelve and eleven micrographs along the interface between PLL/CSPG were taken at random for eGFP and eC3GF infected cultures respectively, and the number of processes crossing the PLL/CSPG interface counted for each micrograph. For statistical analysis, the mean number of crosses were compared using a Student t-test independent sample, one tail, two sample equal variance with alpha (α)=0.05.

Process outgrowth

Mouse primary cortical neurons were dissociated and plated in 8-well slide chambers (3 chambers/viral vector) precoated with CSPGs (50 μ g/ml; EDM Millipore, Billerica, MA) onto PLL (20 μ g/ml) and infected with control or C3 expressing AAV2 at 10^{10} particles/ml. Three days post infection cells were fixed 4% paraformaldehyde and immunostained using mouse monoclonal anti-acetylated tubulin antibodies (T7451 at 1:500, Sigma-Aldrich, St Louis, MO). Cells were permeabilized in 0.01% Triton-X100 (Sigma-Aldrich, St Louis, MO) in PBS. Length of primary and secondary processes was assessed using free software ImageJ/NeuronJ (NIH) (Schneider et al., 2012). In this study a primary process is defined as directly extending from the cell body, while a secondary process is defined as a branch off the primary. Data are representative of a culture for which each vector was tested in triplicates and primary and secondary processes for 20, 19, and 31 neurons were used for analysis for eGFP, eC3GFP, and spC3GFP, respectively. For statistical analysis, a mixed linear model was used to estimate the means and variances (within- and -between neuron) for ln length (natural log) of the primary processes for each type of expression (eGFP, eC3GFP, and spC3GFP) using the SAS MIXED Procedure. A compound-symmetric variance-covariance form was assumed for the outcome and estimates of the standard errors of parameters were used to perform statistical tests and construct 95% confidence intervals

(Diggle et al., 1994). The model-based means are unbiased with unbalanced and missing data, so long as the missing data are non-informative (missing at random). Since a significant overall difference was detected between the 3 types of expression, t-tests were used to compare the differences between the 3 model-based means for expression. The specific statistical tests were done within the framework of the mixed effects linear model. All statistical tests were 2-sided and unadjusted for multiple comparisons. A value of $P < 0.05$ indicated statistical significance for the overall expression effect. Length are reported as means along with 95% confidence intervals (CI). Similar analyses were performed for secondary processes and for longest length of primary and secondary processes.

Immunocytochemistry

HEK293T cells were prepared in 6-well plates (as described for WB) and infected with control or C3 expressing AAV vectors (final concentration 10^9 particles/ml). Three days post transduction, cells were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min and processed for immunocytochemistry (ICC) using rabbit anti-C3 antibodies. Briefly, cells were permeabilized with 0.01% Triton X-100 in PBS for 5 min, (Sigma-Aldrich, St Louis, MO) in PBS for 5 min, blocked in 4% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove PA) for 30 min and incubated with rabbit anti-C3 antibodies (5 μ g/ml) in 2% NDS in PBS overnight. After several washes, the cells were incubated in Alexa 594 donkey anti-rabbit secondary antibodies (Invitrogen) in PBS for 1hr. Cells were imaged using an inverted Leica DMIRE2 scope equipped with a QImaging Retiga EXi camera and Simple PCI acquisition software. The captured images were stored and processed using Adobe Photoshop software.

In vivo viral vector injection

Adult male Sprague Dawley rats (200–280gm, Charles River Laboratories) were housed in the Emory animal vivarium with a 12hr light/12hr dark cycle and *ad libitum* access to food and water. All procedures were conducted in accordance with approved guidelines from the Emory University Institutional Animal Care and Use Committee. Animals received bilateral injections of either LV-eGFP (n=3), LV-eC3GFP (n=3) or LV-spC3GFP (n=3) at 10^{10} viral particles/ml. Virus were injected (1 μ l and 3 μ l on each side, respectively) using a pulled glass pipette connected to a Nanoject (Drummond Scientific, Bromall, PA) at 280 nl/min at the following stereotactic coordinates: at Bregma; ± 3 mm lateral to midline; 5.2 mm below dura. Starting 5 days post-injection, rats were randomly assigned to drink either sugar water or doxycycline supplemented sugar water (Szulc et al., 2006). For rat experiments, we added 0.2 g/l doxycycline (D3447, Sigma-Aldrich, St Louis, MO) and 5% sucrose into drinking water. The sugar was added to mask the bitterness of the doxycycline.

Immunohistochemistry and double immunofluorescence

After two weeks rats were deeply anesthetized with a lethal dose of Euthasol (130 mg/kg), injected intraperitoneally, and then perfused intracardially with 0.9% NaCl, followed by 4% PFA in 0.1 M PBS at pH 7.2 (PB) for 15 min at a rate of 20 ml per min. Brains were removed and cryoprotected in 30% sucrose at 4°C, sectioned in coronal plane at 50 μ m thickness using a freezing microtome, collected and rinsed in 0.1 M PBS, pH 7.2.

Immunohistochemistry was performed as described previously (Gutekunst et al., 1995, Gutekunst et al., 2010). Free-floating sections were incubated in 0.1% TritonX-100 and 3% hydrogen peroxide to eliminate endogenous peroxidase, rinsed in PBS, and preblocked in 4% normal goat serum (NGS) in PBS for 30 min at room temperature (RT). Sections were incubated in rabbit anti-GFP antibodies (2555, Cell Signaling Technology, Beverly, MA) in PBS containing 2% NGS at 4°C for 48hr, then rinsed and incubated for 1hr at RT in biotinylated anti-rabbit antibody (ABC Elite; Vector Laboratories, Burlingame, CA) in PBS containing 2% NGS. After several rinses in PBS, the sections were incubated in avidin-biotin complex (ABC Elite; Vector) for 90 min at 4°C. Immunoreactivity was visualized by incubation in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.01% hydrogen peroxide in PBS, until a dark brown reaction product was evident (5–10 min). Sections were rinsed and mounted on glass slides, air dried and coverslipped. Sections were visualized using either a Nikon eclipse E400 microscope and images captured using a color digital camera (Nikon Instruments Inc, Melville, NY).

Double immunofluorescence was used to co-localize C3 and KRAB as previously described (Gutekunst et al., 2012). Free-floating striatal sections were rinsed in PBS, blocked in 5% NDS and 0.1% Triton-X for 30 min at RT and rinsed in PBS. After rinses in PBS, sections were incubated overnight at 4°C in mouse anti-GFP and rabbit anti-KRAB (AB72609, Abcam, Cambridge, MA) in PBS containing 1% NDS. Sections were rinsed in PBS and incubated in Alexa Fluor 594 conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West grove, PA) and Alexa Fluor 488 conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories, West grove, PA) secondary antibody in 1% NDS for 1hr at RT. Sections were rinsed with PBS, then mounted on glass slides with hard set Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Sections were visualized using either a Nikon eclipse E400 microscope equipped with 4 fluorescent cubes, a monochrome and color digital camera and Nikon BR software (Nikon Instruments Inc, Melville, NY).

Immunofluorescence was also used to quantify brain response to C3 expression. Free floating sections were stained by immunofluorescence using mouse anti-glial fibrillary acidic protein (GFAP; G3893, Sigma-Aldrich, St. Louis, MO) or mouse anti-rat CD11b/c (OX42, 554859, Pharmingen, SanDiego, CA), followed by an Alexa Fluor 594 conjugated donkey antimouse (Jackson ImmunoResearch Laboratories, West grove, PA) secondary antibody. Sections were mounted on glass slides with hard set Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For each animal, an image was taken at the level of the injection site and GFAP and OX42 immunofluorescence intensity was assessed in 4 random windows per image using free software ImageJ/NeuronJ (NIH) (Schneider et al., 2012). For statistical analysis, mean intensity levels were compared between groups mixed model as described previously.

RESULTS

Development of an endogenous C3GFP (eC3GFP) cassette

To generate a vector co-expressing C3 and GFP as a fusion protein (C3GFP), the C3 cDNA was PCR amplified and inserted into the cloning site of the pEGFP-N2 plasmid encoding an enhanced GFP (EGFP) variant that has been optimized for brighter fluorescence and higher

expression in mammals (Di Polo et al., 1998, Harvey et al., 2009, Hellstrom et al., 2009). Transfected HEK293T cells expressed a band at ~ 60 kDa only in pEGFP-N2-C3 transfected cells, corresponding to the added molecular weights of EGFP and C3, confirming proper expression of the fusion protein (Figure 1A). The effectiveness of the C3GFP cassette in reducing RhoA activation was assessed with a Rhotekin RhoA activation assay. NIH3T3 cells, which are commonly used in Rho activation assays (Zheng et al., 2009), were treated with permeable C3/DMSO, DMSO alone, or transfected with either pEGFP-N2 or pEGFP-N2-C3. Cell lysates were incubated with beads coated with the Rho binding domain (RBD) of the Rho effector protein, Rhotekin, which shows high affinity for GTP-RhoA, and the Rhotekin- RBD/GTP-RhoA complexes bound to the beads were immunoprecipitated with RhoA antibodies; total RhoA (GTP- and GDP- bound) levels were assessed in the absence of immunoprecipitation. As expected, there were equal amounts of total RhoA across samples but decreased levels of active RhoA (RhoA-GTP) in C3 treated and pEGFP-N2-C3 transfected cells as compared to untreated cells and cells transfected with endogenous GFP control (pEGFP-N2) (Figure 1B). Functional cassettes were then moved into an AAV backbone and viral vectors were generated. C3 was only seen in cells transfected with pEGFP-N2-C3 (Figure 1C). Both GFP and C3GFP were present in the cytoplasm, filling the soma and processes consistent with endogenous and cytoplasmic protein expression (Figure 1C).

eC3GFP promotes crossing at PLL/CSPG interface

To confirm the functionality of C3 in the C3GFP fusion protein we used a CSPG spot assay and analyzed the effect of C3 expression on the growth of neuronal processes as they encounter an inhibitory substrate interface. CSPG is a key component of glial scars and a known potent inhibitor of axon regeneration (Fawcett and Asher, 1999, Sandvig et al., 2004, Silver and Miller, 2004, Wang et al., 2008). An interface between PLL and CSPG was created by placing CSPG/laminin-Rhodamine (CSPG-Rhod) or laminin-Rhodamine (Rhod) drops onto laminin coated glass coverslips. Rhodamine was used to visualize the interface and also served as a negative control. Primary dissociated rat E18 cortical neurons were plated on PLL/laminin with a CSPG drop in the center, and after 3 days infected with either eGFP or eC3GFP-expressing AAV. After 5 days most of the processes in cultures infected with eGFP-expressing vector turned at the PLL/CSPG interface, rarely crossing over to the CSPG side. In contrast, processes in cultures transduced with eC3GFP crossed the interface and extended onto the CSPG drop (Figure 2A). The mean number of crosses was 7 times greater in eC3GFP infected cultures compared to eGFP controls (Figure 2B) (Student t-test: $t=3.882$, $df=21$, $p=0.004$).

Development of a secretable/permeable C3GFP (spC3GFP) cassette

The functional eC3GFP cassette was then used to generate a vector that expresses a secreted and permeable form of C3GFP (spC3GFP). The synthetically modified trans-acting activator of transcription (TAT κ) protein transduction domain from human immunodeficiency virus type 1 was used as a permeability tag (Tunnemann et al., 2006). TAT κ is a commonly used transport peptide and has proven effective at transporting cargo to both the cytosol and the nucleus (Fawell et al., 1994, Vives et al., 1997, Schwarze et al., 1999, Flinterman et al., 2009). A secretable IgK signal peptide was then added to the amino terminus of

TATκC3GFP using the pSecTag2 plasmid. C3 antibodies detected appropriately sized (~ 70 kDa) spC3GFP fusion proteins only in HEK293 cells transfected with pSecTag2-TATκ-C3GFP (Figure 3A). The IgK-TATκ-C3GFP cassette was subsequently moved into an AAV backbone; transfection of HEK293T cells with AAV-spC3GFP resulted in expression of spC3GFP, which appeared punctated as expected for secreted proteins (Figure 3B). Proper processing and secretion of spC3GFP was confirmed with a dot blot assay performed on media collected from transfected HEK293 cells (Figure 3C). GFP antibodies detected transgene expression only in the media of spC3GFP-transfected cells with no signal detected in the media of non-transfected cells or cells transfected with endogenous GFP or C3GFP (Figure 3C). The functional property of spC3GFP was also confirmed by culturing HEK293 cells expressing DsRed2 (as a marker) either alone or together with HEK293 cells that had been previously transfected to express spC3GFP. When co-cultured with spC3GFP expressing cells, DsRed-HEK293 cells developed long processes compared to when cultured alone (Figure 3D). The permeable property of the transgenes is again further characterized in a subsequent *in vivo* experiment (described below).

eC3GFP and spC3GFP promote process outgrowth on CSPG substrate

C3 transferase promotes growth of processes on inhibitory substrates. To confirm the functionality of endogenous and secretable/permeable C3GFP cassettes we assessed the growth of processes of cortical neurons growing on CSPG substrate. Primary cortical neurons were plated on CSPG substrate and infected with either eGFP-, eC3GFP- or spC3GFP-expressing AAV vectors. After 3 days, GFP was visible in neurons and cultures were immunostained with tubulin antibodies to visualize and measure primary and secondary processes (Figure 4). The average length of the primary processes was significantly increased in cultures expressing eC3GFP and spC3GFP compared to control cultures only expressing eGFP ($p < 0.05$, t-tests). Whereas the mean length of primary processes in control cultures was $47.8 \mu\text{m}$ 95% CI (36.7, 58.8), the average length was $62.5 \mu\text{m}$ 95% CI (50, 75) and $96.4 \mu\text{m}$ 95% CI (79.8, 113.1) for eC3GFP and spC3GFP infected cultures respectively (Figure 4B). The effect of spC3GFP was significantly stronger than that of eC3GFP ($p < 0.005$, t-test) and the average length of primary processes of spC3GFP cultures was twice as long than in the control cultures ($p < 0.001$, t-test). When considering only the longest primary process for each neuron examined, eGFP and eC3GFP had similar longest primary processes ($159 \mu\text{m}$ and $153 \mu\text{m}$ respectively), whereas the mean longest primary process of neurons in cultures infected with spC3GFP was significantly longer at $242.8 \mu\text{m}$ 95% CI (198.4, 287.1) ($p < 0.05$, t-test) (Figure 4C). Unlike spC3GFP, eC3GFP also had a significant effect in increasing the average length of secondary processes compared to eGFP control (Figure 4B). The average length of secondary processes was $25.6 \mu\text{m}$, 95% CI (21.1, 30) for eGFP, $21.4 \mu\text{m}$, 95% CI (17.4, 25.3) for eC3GFP, compared to $31.5 \mu\text{m}$, 95% CI (27.4, 35.6) for spC3GFP ($p < 0.05$, t-test). The longest secondary processes were also significantly longer in spC3GFP infected cultures compared to control eGFP but not eC3GFP with an average length of $77.0 \mu\text{m}$, 95% CI (59.5, 94.5) ($p < 0.05$, t-test), $46.1 \mu\text{m}$, 95% CI (27.9, 64.3) and $52.5 \mu\text{m}$, 95% CI (28.8, 76.22) respectively (Figure 4C).

In vivo expression of tet-on conditional eC3GFP and spC3GFP

To validate *in vivo* expression, the eGFP, eC3GFP, and spC3GFP cassettes were moved into the tet-on lentiviral plasmid pLVUT for the production of inducible lentiviral vectors (LV). One of the advantages of using pLVUT is its drug-inducible design, which allows control of gene expression in mammalian cells (Szulc et al., 2006). In the absence of doxycycline, the Kruppel associated box (KRAB) transcriptional repressor binds to a tetracycline operator (tetO) and suppresses the expression of the transgene. Upon the addition of doxycycline, the suppression is released (Szulc et al., 2006). The incorporation of such controls in C3 gene therapy would ensure that C3 expression is turned on only during the desired time frame for regeneration. In addition, cells infected with pLVUT express KRAB constitutively (Groner et al., 2012). As such, KRAB can be used as a marker of expression and, for the purposes of this study, can be used to differentiate LV-spC3GFP-infected cells from those surrounding cells that have taken up the secreted and permeable C3GFP in the absence of cell-autonomous expression.

Following injections of either LV-eGFP, LV-eC3GFP or LV-spC3GFP in the striatum, half of the rats received doxycycline-supplemented drinking water for 14 consecutive days. GFP expression was visible only in those animals that had received doxycycline-supplemented water (Figure 5A a–f). GFP immunohistochemistry confirmed GFP expression in cell bodies and processes and uncovered more GFP+ cells and processes than were visible by direct GFP visualization (Figure 5A j–l). More GFP was present in the neuropil of spC3GFP injected striatum (Figure 5A i) compared to eGFP and eC3GFP (Figure 5A g and h). GFP was found in all striatal neuronal cell types including DARPP-32+ medium spiny neurons and large ChAT+ cholinergic interneurons (data not shown). As expected with lentivirus, there was no detectable infection of glial cells.

To further confirm the permeable aspect of secreted spC3GFP, striatal sections from rats injected with spC3GFP were stained for KRAB and GFP (Figure 5B). KRAB immunostaining is visible in the nuclei of many GFP+ cells, but in addition a number of cells positive for GFP but negative for KRAB (Figure 5B) were seen; the latter are most likely cells that are not infected but have taken up the secreted and permeable C3GFP in a cell-nonautonomous manner. The response of glial cells to C3 expression was also examined. Sections with comparable infection with GFP, eC3GFP or spC3GFP expression (confirmed by GFP ICC on adjacent sections) showed no difference in GFAP (reactive astrocytes) or OX42 (microglia) immunoreactivity (Figure 5C), early evidence of absence of inflammation.

DISCUSSION

In the present study we describe the development and validation of several viral vectors for long term and regulated expression of C3 transferase. We designed our first vector to express an endogenous form of full-length C3 transferase fused to GFP. There are only two other studies for which viral vectors have been developed to express C3 transferase. In both studies vectors were engineered to express C3 and GFP separated by an internal ribosomal entry site (IRES) (Fischer et al., 2004, Liu et al., 2005). In addition, for one of the vectors, a sequence encoding the first 10 amino acids of GAP-43 was added to the amino terminus of

C3 to target its localization to the cell membrane (Fischer et al., 2004). Similar to our construct, these vectors express an endogenous form of full-length C3 transferase. However, in our design C3 and GFP are combined as a single fusion protein where GFP can be used as a direct reporter of not only infected cells but also the localization and potential site(s) of action of C3 transferase within the cell compartments. Conversely, with an IRES sequence, GFP can only report which cells have been transduced, and the precise location of C3 requires further manipulation such as immunohistochemistry. Consistent with previous studies, transfection with eC3GFP induced RhoA inhibition, which led to the reorganization of the cytoskeleton and process growth indicating that fusing GFP to C3 did not affect the activity of C3 transferase. Similarly, AAVeC3GFP was able to promote the growth of processes on an inhibitory substrate. This vector can be used as a gene therapy when aiming at inactivating RhoA within a specific cell population. The virus can be injected directly into the CNS or applied to tissue or cells prior to implantation. Although in the present form the construct utilizes the strong ubiquitous chicken β -actin promoter for fast expression with relatively high levels, more specific promoters and/or viral serotypes can be explored to obtain cell type specificity. One limitation of the eC3GFP and the two previously developed vectors is that the transgenes are expressed as cytoplasmic proteins, therefore limiting the effect of C3 to only cells that are transduced.

To enable a more widespread distribution of C3, we designed our second vector to express a secretable/permeable form of full-length C3 transferase fused to GFP. In this approach, a cell non-autonomous spC3GFP cassette was engineered so that cells adjacent to those constitutively expressing C3 can also benefit from its effect. To our knowledge, this is the first development of a secretable/permeable C3 expression system. Similar to eC3GFP, spC3GFP promoted growth of processes on inhibitory substrate but showed an enhanced effect compared to eC3GFP, with 25% and 40% longer growth of primary and secondary processes, respectively. This novel vector can be used as a therapeutic approach when RhoA inhibition is needed in a large population of cells or when the cells needing treatment are too fragile to transduce or perhaps unable to express high levels of transgene. For example, instead of infecting degenerating neurons, a virus could be targeted at the healthy adjacent cells (i.e. different neuronal subtype or glia). Again, specific promoters and/or serotypes would be incorporated in the vector design to achieve specificity.

It has been suggested that C3 may increase the glial inflammatory response post injury (Holtje et al., 2005, Hoffmann et al., 2008), an effect that may intensify with longer-term expression, which distinguishes our experiments from others with short-term direct C3 delivery. In this case, the spC3GFP vector design, which allows for a more widespread distribution of C3, could have deleterious side effects. With this in mind, we developed a third vector designed with a controllable system. Such an approach might maximize effectiveness vs. toxicity and can be used to identify the optimal interval for expression via dose and time–response curves with doxycycline. In addition, KRAB-mediated repression eliminates the leaky, nonspecific expression seen in other conditional systems based on transactivation (Szulc et al., 2006). Of note, this study found that the C3-expressing vectors did not appear to induce any glial response when injected directly into a healthy rat brain. Further studies need to be done to determine their effect in the context of an injury model.

Virally mediated C3 expression can significantly reduce RhoA activation and represents a novel gene therapy approach that could be used alone or in conjunction with other therapies to promote axon regrowth in neurodegenerative disorders or injuries of the CNS. To validate the functionality of the eC3GFP and spC3GFP *in vivo*, the vectors are currently being tested in a rat optic nerve crush (ONC) model. The optic nerve offers several advantages for testing new approaches for CNS regeneration (Vidal-Sanz et al., 1987, Villegas-Perez et al., 1993, Berry et al., 1996, Fournier et al., 2003). Injured optic nerves were treated via intravitreal injection of AAV2-eC3GFP or AAV2-spC3GFP. Both vectors increased RGC survival and axon regeneration compared to controls up to 8 weeks post-injury (submitted). In summary, these new vectors are promising therapeutic approaches to regenerating and protecting neurons after CNS injury or neurodegenerative diseases. Depending on the need, they can be injected directly into the injured tissue to reverse the degenerative process or transduced to stem cells for cell replacement therapies. Further work is underway to test these vectors in other *in vivo* settings including implantation of ex-vivo infected neurons.

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Abbreviations

AAV2	adeno-associated serotype 2 virus
CNS	central nervous system
C3	C3 transferase
CBA	chicken β -actin
CSPGs	chondroitin sulfate proteoglycans
DMEM	Dulbecco's Modified Eagle's medium
eC3	endogenous C3

EGFP	enhanced GFP
HEK	Human embryonic kidney
ICC	immunocytochemistry
ONC	optic nerve crush
SCIs	spinal cord injuries
KRAB	Kruppel-associated box
LV	lentiviral vectors
NGS	normal goat serum
NDS	normal donkey serum
PLL	poly-L-lysine
PCR	polymerase chain reaction
spC3	secretable/permeable C3
TATκ	trans-acting activator of transcription
Tet-on	Tet-regulated
hUbC	Ubiquitin C
WPRE	woodchuck hepatitis virus posttranscriptional regulatory element

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Figure 1. Development of an endogenous C3GFP (eC3GFP) cassette

A) Western blot of cell lysates from HEK293T cells transiently transfected with pEGFP-N2 and pEGFP-N2-C3 and stained with C3 antibodies. A band is visible in the pEGFP-N2-C3 transfected cells only. Molecular weight in kDa. **B)** Western-blot analysis of RhoA activity obtained from lysates of untreated NIH3T3 cells or cells pretreated as indicated. Active RhoA (aRhoA) fraction was isolated from freshly prepared NIH3T3 lysates using GST-Rhotekin-RBD beads following the manufacturer's protocol (upper blot). The total RhoA (tRhoA) levels were determined in all lysates (lower blot). Xfect: transfected with. **C)** Micrographs of HEK293T cells infected with AAV2-eGFP or eC3GFP (green) as indicated and stained with C3 antibodies (red). Nuclei are stained with DAPI (blue). Scale bar: C) 10 μ m.

Figure 2. eC3GFP promotes crossing at PLL/CSPG interface

A) Micrographs of neurons plated on coverslips showing the behavior of neurites at the PLL/CSPG interface. Rhodamine (red) was used to identify the CSPG drop. **B)** Graph of Average number of neurites crossing the PLL/CSPG interface + SEM. Scale bar A) 100 μ m. *** $p < 0.05$, Student t-test.

Figure 3. Development of a secretable/permeable C3GFP (spC3GFP) cassette

A) Western blot of cell lysates from HEK293T cells transiently transfected with pSecTag2 and pSecTag2-TATkC3GFP and stained with C3 antibodies. A band is visible in the pSecTag2-TATkC3GFP transfected cells only. Arrow head shows the molecular weight for pEGFP-N2-C3. Molecular weight in KDa. **B)** Micrographs of HEK293T cells infected with AAV2-spC3GFP (green) and stained with C3 antibodies (red). Nuclei are stained with DAPI (blue). **C)** Dot blots of concentrated media from HEK293T cells transiently transfected with pEGFP-N2 (eGFP), pEGPF-N2-C3 (eC3GFP) or pSecTag2-TATkC3GFP (spC3GFP) as indicated and stained with GFP antibodies. **D)** Micrographs of DsRed2-HEK293 cells growing alone (a–c) or in culture with spC3GFP expressing cells (d–f). DsRed2 fluorescence is shown in grey scale. Extensive processes (arrows) are seen in the cell in co-cultured. Scale bar: B) 10 μ m, D) 100 μ m,

Figure 4. eC3GFP and spC3GFP promote process outgrowth on CSPG substrate

A) Micrographs of neurons infected with AAV-eGFP, AAV-eC3GFP or AAV-spC3GFP plated on CSPG coated coverslips and stained with tubulin (red) for neurite length measurement analysis. **B)** Box and whisker plot showing length of primary and secondary processes with box quartiles (25–75%) and whiskers marking the 5–95% CI **C)** Box and whisker plot showing length of longest primary and secondary process with box quartiles (25–75%) and whiskers marking the 5–95% CI. Scale bar: A) 100 μ m. Statistics: * P<0.05, ** p<0.01, *** p<0.001, t-test.

Figure 5. Conditional expression of eC3GFP and spC3GFP in rat striatum

A) Micrographs of brain sections through the striatum of rats injected with LV-eGFP (a, d, g, j), LV-eC3GFP (b, e, h, k) or LV-spC3GFP (c, f, i, l) and subsequently given ad lib drinking sugar water unsupplemented (a–c) or supplemented (d–i) with doxycycline (Dox). Sections were stained for GFP (a–i) and DAB reaction product is seen in gray (a–f) or brown (g–i). At higher magnification (g–i), DAB reaction product is visible in neuronal cell bodies (g–i), neurite (g–i), and neuropil (i). GFP fluorescence can also be seen in unstained sections (j–l). DAPI (blue) is used as a nuclear marker. **B)** Micrographs of brain section of the striatum of an LV-spC3GFP injected rat immunostained for KRAB (red). GFP is visible in infected KRAB+ neurons (arrows) as well as non-infected KRAB– neurons (arrowheads). Panel a and b show composite images whereas panels c and d show spC3GFP and KRAB ICC respectively. **C)** Micrographs of brain section through the striatum of LV-spC3GFP injected rats receiving Dox or No Dox in their drinking water immunostained for GFAP or OX42 (red). Box and whisker plots showing intensity level of GFAP and OX2 with OX42 in the striatum of rats injected with LV-eGFP, LV-eC3GFP or LV-spC3GFP as indicated with box quartiles (25–75%) and whiskers marking the 5–95% CI. Scale bars: A) a–f: 0.3 mm, g–l: 20 μ m; B) a: 20 μ m, b–d: 10 μ m.