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A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration

PRINCIPAL INVESTIGATOR:

Diane M Snow, Ph.D.

CONTRACTING ORGANIZATION:

University of Kentucky

Lexington, KY 40506

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14. ABSTRACT A major consequence of spinal cord injury (SCI) is the development of a glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory chondroitin sulfate proteoglycans (CSPGs) are elevated in the glial scar and are a major deterrent to successful regeneration. In the present study, we have demonstrated that injured astrocytes produce a wide variety of inhibitory CSPGs. To develop a more efficient method to accomplish CSPG degradation (than the bacterial enzyme chondroitinase), we are addressing a normally occurring catabolic protein for CSPG degradation, the neural <u>aggrecanase, ADAMTS-4</u> . We have begun a two-prong approach, employing studies both <i>in vitro</i> , and <i>in vivo</i> . We have produced recombinant ADAMTS-4 protein for experiments <i>in-vitro</i> , or for injection <i>in vivo</i> , alone and in combination with chondroitinase. Thus far, we have focused mainly on the development of critical reagents and methods, and have tested these in proof of principle assays. In parallel, we have begun to develop a reproducible SCI model and are developing behavioral assessments to validate the success of aggrecanase and chondroitinase treatments. The significance of these stages is that they will lead to an efficient means by which to attenuate axonal inhibition, and thereby promote plasticity and regeneration of adult neurons following SCI.					
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Introduction

Subject. Spinal cord injury (SCI) is a devastating condition affecting as many as 306,000 individuals in the US alone (<http://www.brainandspinalcord.org/spinal-cord-injury/statistics.html>). Beyond US soil, SCI is an all-too-common result of military combat – all with an enormous emotional, social, and financial cost to society. Despite much needed attention over the past few decades and some significant advances, the cellular and molecular mechanisms leading to SCI are not yet clear. **Purpose:** A major consequence of SCI is the development of an astrocytic glial scar. Although the scar has benefits for tissue repair, it also blocks neural regeneration. Inhibitory **chondroitin sulfate proteoglycans (CSPGs)** are elevated in the glial scar and are a major deterrent to successful regeneration. To develop a more efficient method to accomplish CSPG degradation (than the current use of the bacterial enzyme chondroitinase), we are studying a normally occurring catabolic protein for CSPG degradation, the neural aggrecanase, ADAMTS-4. **Scope:** We are using a two prong approach – employing studies both *in vitro*, and *in vivo*. We are isolating, purifying and testing aggrecanase on astrocytes in tissue culture; and we will test aggrecanase lentiviral constructs in an injury model *in vivo*, alone and in combination with chondroitinase, to ameliorate CSPG-induced inhibition. Thus far, we have focused mainly on the experimentation *in vitro* to develop reagents and for proof of principle, but we have also made significant progress regarding our *in vivo* model in parallel with the work *in vitro*.

The following is a Progress Report for CDMRP grant SC090248, entitled "A Better Way to Excise Inhibitory Molecules (CSPGs) from a Spinal Cord Injury Scar to Promote Regeneration". Each point of progress is addressed in relationship to the Statement of Work provided in the original application.

Statement of Work

Overarching goal: *To successfully degrade aggrecan and related CSPGs using naturally occurring ADAMTS-4, alone and in combination with the current "gold standard" (chondroitinase) to attenuate axonal inhibition, and promote plasticity and regeneration of adult neurons of the CNS.*

Task 1. We will determine if aggrecanase and chondroitinase-mediated degradation of CSPGs produced by primary rat cortical astrocytes will foster neurite outgrowth *in vitro*.

1a. We will confirm that primary astrocytes upregulate CSPGs *in vitro* in response to injury (experimentally induced by trauma and/or administration of TGF-beta (Smith and Strunz, 2006). We will also catalogue the specific PGs upregulated and their time course (months 1-4).

Neonatal rat cortical astrocyte cultures were prepared and expanded to 16 T-75 flasks grown to near confluency. The cultures were incubated for 3 days with 2.5 ng/ml TGF-beta in 5 ml of medium. Medium was collected from 16 flasks, and guanidine HCl was added to a final concentration of 4M. The cell layers were washed with PBS, and were lysed in 0.05M sodium acetate, pH 5.8, containing 4M guanidine HCl, 0.5% CHAPS and protease inhibitors. An additional two flasks of cells were lysed, and used for the preparation of total cellular RNA, for quantitative real time PCR analysis of proteoglycan mRNA. Both medium and cell layer samples were chromatographed on Sephadex G-50 equilibrated with 0.05M sodium acetate, pH 6.0 containing 0.5% CHAPS, 0.15 M sodium chloride, and 8M urea. The void fractions were applied to a column of DEAE Sephacel equilibrated in the same buffer. The column was washed, and proteoglycans were eluted using a 0.15 to 1.0 M sodium chloride gradient (**Figure 1A**). Chromatography of medium and cell layers from astrocyte cultures each revealed several peaks that were pooled, buffer-exchanged to remove urea, and concentrated. The multiple peaks are believed to be due to different degrees of sulfation of these proteoglycans. Dot blot analyses of pooled fractions using antibodies specific for different CSPGs were performed (**Figure 1B**). Dot blots were reacted with antibodies to aggrecan, neurocan, phosphacan, NG2, brevican, versican, G3 domain of aggrecan, and aggrecan CS-2 domain aggrecanase-generated neoepitopes GELE and KEEE (mixture of both antibodies). Analysis of the dot blot assays is ongoing, but data indicates that the isolated pools are largely mixtures of genetically distinct proteoglycans. Relevant to this study, we detected aggrecanase generated fragments in the major peak of medium-derived PGs eluting from the DEAE column (reacting with GELE/KEEE antibodies) which was also reactive with an antibody to the aggrecan G3 domain (also consistent with CS-substituted fragments having an intact C-terminus. This pool appeared to be highly inhibitory to neurite outgrowth (**Figure 4C**). All of these proteoglycan pools are being further characterized for sulfation patterns using HPLC sulfated disaccharide analyses (analysis in progress at

Glycotechnology Core Resource, UC Davis). These PG fractions have been used for analysis of neurite outgrowth inhibitory activity using an assay developed in our laboratory.

We have optimized a commercially available assay for high-throughput neurite outgrowth analysis (Cellomics Neurite Outgrowth Kit) to measure the inhibitory effects of substratum bound proteoglycans on neurite outgrowth. In this assay, NeuroscreenTM-1 (NS-1) cells, a derivative of the PC12 cell line, are plated in a 96-well plate, and each well is treated with increasing concentrations of NGF to stimulate neurite outgrowth (**Figure 2**). To test the effect of CSPGs on outgrowth, plates are incubated with serial dilutions of proteoglycan prior to plating. Adsorbed PG is quantified by ELISA assay on duplicate plates. Following two days of outgrowth, cells are fixed and stained to reveal nuclei and neurites, which are detectable at different fluorescent wavelengths. A microscope with a motorized, computer-driven stage is used to obtain multiple images per well of nuclei and neurites. These images have been analyzed using the Neurite Tracer plugin with Image J software. Neurite outgrowth normalized to cell number is plotted vs. NGF concentration for each concentration of proteoglycan adsorbed to the plate surface. In this manner, we have obtained curves to quantify neurite inhibition as a function of proteoglycan concentration. We have carried out experiments during this funding period to examine the inhibitory activity due to chondroitin sulfate, keratan sulfate, and N-linked oligosaccharides (**Figure 3**). We have observed that chondroitinase ABC degradation of aggrecan on the plate surface enhances neurite outgrowth, as expected from the results of this and other laboratories. We have obtained new data suggesting that removal of KS chains further enhances outgrowth. After degrading both chondroitin sulfate and keratan sulfate, we digested the substrate bound aggrecan with peptide-N-glycosidase F, which cleaves N-linked oligosaccharides from the core protein. This treatment resulted in a further enhancement of neurite outgrowth. These observations will be further studied in the coming year.

We have performed neurite outgrowth assays to determine the effect of the different pools of astrocyte proteoglycans shown in Figure 1 upon neurite outgrowth (**Figure 4**). In this experiment we confirmed our previous result showing a dose-response relationship between substrate-bound aggrecan and neurite outgrowth. We have observed that astrocyte cell layer-derived PGs tend to enhance neurite outgrowth, an effect that become more pronounced in the later, more highly negatively charged fractions. We have further observed that medium-derived proteoglycans tend to be inhibitory in the more highly negatively charged fractions. These data will be correlated with results of the ongoing sulfated disaccharide analysis, and the characterization of genetically distinct proteoglycans in each fraction.

1b. Using a lentiviral system, we will induce primary astrocytes to degrade CSPGs via aggrecanase, chondroitinase, or both. Degradation of CSPGs will be confirmed using an anti-C-4-S antibody (2-B-6), which identifies CSPG stubs following chondroitinase cleavage, or by antibodies to neoepitopes that are generated when aggrecanase cleaves the CSPG protein core into specific fragments. (months 5-7).

Cloning of ADAMTS-4 expression constructs. We have generated three ADAMTS-4 expression constructs. The first plasmid (designated 701) contains the ADAMTS-4 coding sequence cloned into the

vector pcDNA3.1/myc-his(-)A. Full sized (90 kDa pro-protein) ADAMTS-4 expressed from this plasmid, as well as the processed 68 kDa secreted form, can be detected with antibodies to ADAMTS-4, myc or His epitopes. The C-terminal His-tag enables purification of the protein on a nickel-chelating resin (i.e. ProBond). The second vector (designated 702) has an ADAMTS-4 insert lacking the N-terminal propeptide, with an N-terminal FLAG sequence adjacent to the first residue of the “processed” 68 kDa ADAMTS-4 sequence. This vector was constructed to determine if an N-terminal FLAG sequence would be more likely to persist in the expressed protein, since autocatalytic C-terminal proteolytic cleavage may remove a C-terminal FLAG sequence. A third construct (designated 703) places an ADAMTS-4 insert having a C-terminal FLAG sequence in the vector pcDNA3.1. The protein product can be affinity purified on a anti-FLAG affinity matrix, and can be detected on a Western blot using an anti-FLAG primary antibody. Each construct can be transiently or stably transfected into mammalian cells.

To produce recombinant ADAMTS-4 protein for experiments *in-vitro*, or for injection into rat spinal cord injury sites (for Task 2a), we have transiently transfected the 703 construct (having a C-terminal FLAG tag) into HEK293T cells for protein expression. This cell line was chosen because it is commonly used for high-level recombinant protein expression. When transfected with the ADAMTS-4 expression plasmid, we found high levels of recombinant protein expression. We found that the C-terminal FLAG tag could not be used for efficient purification of the ADAMTS4 enzyme, due to cleavage occurring near the C-terminus during preparation. We therefore developed a method to purify the recombinant ADAMTS4 using an antibody affinity column (**Figure 5**). This method appears to be successful, and will be used to produce ADAMTS4 for direct infusion into rat spinal cord lesions.

Preparation of pLVX-Tet-On Advanced, and pLVX-Tight-Pur-ADAMTS-4 vectors. We have developed a lentiviral expression system that will enable the inducible expression of ADAMTS4 in primary cultured astrocytes, as well as inducible expression *in vivo*, in a rat model of spinal cord injury. The pLVX-Tet-On Advanced lentiviral vector constitutively expresses the tetracycline-controlled transactivator, rtTA-Advanced. The response lentiviral vector, pLVX-Tight-puro contains a polylinker site for insertion of the gene of interest, the expression of which is controlled by the P_{tight} promoter (a modified Tet-responsive element). Upon induction with doxycycline, rtTA-Advanced expressed from the pLVX-Tet-On lentiviral DNA binds to the P_{Tight} promoter on the response vector, activating transcription of the downstream gene. The ADAMTS-4 insert in the 703-6 (ADAMTS-4 expression) plasmid was excised with EcoRI and XbaI, and was ligated into the pLVX-Tight-puro vector cut with the same enzymes. The ligation product was verified by restriction digestion and sequencing. The pLVX-Tet-On and the pLVX-Tight-puro-ADAMTS-4 plasmids were used to produce lentivirus with the Lenti-X HT packaging system in HEK293T cells. An additional vector was used to prepare a pLVX-tight-puro-luciferase lentivirus for optimizing transactivator and response vector ratios in astrocyte cell cultures. We have used the Lenti-XTM qRT-PCR Titration kit (Clontech) to titer our lentiviral preparations relative to a kit-provided standard having a known copy number. Currently, we have obtained lentivirus titers that are adequate to transduce primary astrocyte cultures. Experiments are in progress to optimize activator-response virus ratios and doxycycline concentrations to activate gene expression. Experiments will be performed to quantify ADAMTS-4 expressed from the lentivirus in primary astrocytes. Proteoglycan degradation will be

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assessed by western blot analysis, as was done for cultures transfected with the ADAMTS-4 plasmid expression construct.

[Note: We had considerable difficulty with this part of the study, and worked continuously with Clontech technical personnel to try to solve issues, only to find just recently that the Packaging Mix from Clontech was faulty. Using their *newly* available Packaging Mix 2 (Clontech, cat # 631260), the protocol was instantly successful and we have generated pLVX-Tet-On viral stocks of sufficient titer to begin transduction experiments.]

1c. Using the above system, we will determine if aggrecanase-mediated degradation of CSPGs in primary rat cortical astrocytes induces growth-inhibiting (CSPG-producing) astrocytes to become growth permissive (CSPG-degraded), and thereby foster regeneration of adult neurons (CST, RST, DC) (months 8-11).

The NS-1 culture system described above provides high through-put results to screen for the effects of various agents. The next step is to examine PG production and enzyme degradation of PGs in a co-culture model using primary neurons and primary injured astrocytes. We have just begun to optimize this co-culture system. We cultured primary rat astrocytes as described previously with and without TGF-beta (activates astrocytes and induces them to upregulate proteoglycans). Primary chicken DRG neurons (E9) were grown on confluent monolayers of these astrocytes. **Figure 6** shows a control culture with healthy DRG neurons (bright green), growing on a monolayer of quiescent astrocytes. Studies being processed now have dual labeling of neurons (red) and astrocytes (green), under both conditions, as well as light microscopy to visualize astrocyte morphology, and are being scored for neurite length. It is possible that neurons grown on TGF-treated astrocytes, i.e. those expressing high levels of aggrecan (and other PGs), will have shorter neurites, and that treatment with aggrecanase and chondroitinase will attenuate this inhibition. Alternatively, we observed that under certain conditions, astrocyte cell layer PGs stimulate neurite outgrowth (Fig. 4B). If this effect is due to the synthesis of heparan sulfate proteoglycans, we may not see an effect of chondroitinase or aggrecanase in this model *in vitro*. We will explore these possibilities. Once optimized, this system can serve to determine the effects of aggrecanase and/or chondroitinase treatments not only on DRG axon outgrowth and regeneration, but also that of other neuronal types (CST, RST, DC; adult).

1d. We will test the responses of other neurons, e.g. 5HT, which have been shown to be robust following SCI in previous studies (months 12-13). Using the NS-1 neurite outgrowth assay, we will determine CSPG production (ELISA), CSPG cleavage (Western blot analyses), and aggrecanase activity (enzyme activity assays and immunostaining for neoepitopes) (Miwa, Gerken et al. 2006; Miwa, Gerken et al. 2006). Further, we will isolate aggrecanase-generated fragments and test their effect(s) on elongating axons in vitro.

Will be addressed in Year 3.

Task 2. We will determine if aggrecanase-mediated degradation of CSPGs produced *in vivo*, in a rat spinal cord injury model system, will permit regeneration (months 13-36), and the effects of combining aggrecanase and chondroitinase treatments.

2a. Lentiviral transfection of ADAMTS-4 in an SCI model system will be performed, using currently approved methods (IACUC Protocol #2010-0702; approved through 8-18-13). We will transduce, using a lentiviral vector, ADAMTS-4 within an injured region of the rat spinal cord (dorsal hemisection), (months 13-18; to adapt the treatment to an established injury model system and develop successful surgical techniques).

Lentiviral preparation is described above in Task 1.

The focus of our SCI work *in vivo* is hand function. Thus, we have relied on our collaborator, Dr. Stephen Onifer, who is optimizing for his own studies, a spinal cord injury at the C-6/7 level in rat. Reproducible, consistent injuries and resulting consistent behavioral deficits are vital for proper determination of the success of our aggrecanase/chondroitinase treatments. For this reason, as stated in the previous annual report, with the guidance of Dr. Onifer, we have been using an injury device fabricated by colleagues at nearby University of Louisville. We moved our animals to the new BioPharm building and created a new surgical suite so that Dr. Onifer and my lab can easily share this piece of equipment. We have now conducted numerous experiments using this device. **Figure 7** (from previous annual report), demonstrates injury depth and width using this device.

The procedure is as follows (IACUC Approval #: Anesthetized adult male Sprague Dawley rats receive an incision in the skin of the back of the shaved and cleansed neck. After muscle dissection, a laminectomy of the dorsal cervical (C6 and C7) vertebrae is performed. A transverse incision is made in the dura with the tip of a sterile 30-G needle between the C6 and C7 dorsal root entry zones. A dorsal-to-ventral laceration of the dorsal columns with a vibrating, custom-made razor blade attached to the LISA-Vibraknife is performed at the level of the dorsal corticospinal tracts. The muscle and skin incisions are closed. Fine forepaw digit use is mildly dysfunctional after the cervical hemisection lesion, as shown in previous studies (Onifer et al., 1997, 2005; Massey et al., 2006; Onifer et al., 2007; Titsworth et al., 2007; Massey et al., 2008). The remainder of forelimb, hindlimb, eating, drinking, respiration, bladder, bowel, and locomotor functions re normal after recovery from anesthesia. Therefore, no additional post-surgery or post-operation veterinary care is required beyond that which is routinely performed for spinal cord injured rats. We are having great success in consistency of injury and of behavioral deficits following the injury (see 2d).

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We will continue using this model through the remainder of the granting period to inject aggrecanase and/or chondroitinase into dorsal hemisected rats, and will closely analyze ADAMTS-4 and chondroitinase expression, as well as aggrecan degradation, using immunohistochemical techniques.

Chondroitinase. A major goal of this study is to use combinational therapy (aggrecanase + chondroitinase (cABC)) to promote regeneration *in vivo*. One issue for this undertaking is that our aggrecanase lentivirus was prepared using a different backbone than the chondroitinase lentiviral vector, supplied by the Smith lab (George Smith, PhD: colleague and previous collaborator). Taking advantage of the lentivirus expertise within SCoBIRC (Charles Mashburn, PhD), our ADAMTS-4 insert has also been cloned into a second construct, pCSC-SP-PW, which is the same construct used to generate the cABC lentivirus. This protocol will result in fewer experimental variables when the reagents are used simultaneously in future experiments.

2b. Aggrecan degradation in the lesion will be monitored with anti-neoepitope antibodies that will recognize aggrecan fragments. Using a variety of microscopy methods and established tract tracing techniques, neurons traversing the glial scar depleted of aggrecan by ADAMTS-4 will be quantified relative to untreated rats. (months 19-24; to gather supportive information to validate results of 3a).

We are just beginning these experiments.

2c. Histological assays to identify all cell types and molecules of interest *in vivo*. (months 25-28)

Some histological analysis has already been performed to identify the lesion volume in dorsal hemisected rats. Post injury histology of Vibraknife treated rat spinal cord at 1, 2 and 3 weeks post injury (**Figure 7** from previous report, and new, **Figure 8**). Longitudinal sections taken at or near midline; n=6.

2d. Behavioral assays. Repeat *in vivo* paradigm and test behavioral recovery using the *Reach, Grasp and Pellet Retrieval* test (motor), the *Grid Walking* test (motor), and the *Sticker Attention* test (sensory) (months 29-36).

Our injury model (see above) resulted in animals that retrieved 0 pellets in the staircase behavior test at week 1 post injury. The animals then showed a slight increase of functionality between weeks 2 to 6 post injury, but had significantly lower performance when compared to baseline training. Two different series of experiments show the efficacy of the Pellet Retrieval behavioral method following dorsal hemisection (**Figures 9 and 10**). For the series of experiments performed in Fig. 9, the Sticker Attention test was performed on sham and dorsal hemisected rats (**Figure 11**), to obtain baseline data, and assess the value of the behavioral test. Data showed that injured rats required approximately 4 times as long to notice and remove the small, plantar surface-adhered stickers than did uninjured rats, which was the

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predicted result. This assay will be used to test sensory deficit and recovery in aggrecanase and/or chondroitinase treated animals.

Injection paradigms. In preparation for introduction of aggrecanase and chondroitinase into the dorsal hemisectioned animals, we used dye injections to simulate enzyme distribution in the lesion and spinal cord tissue. Three days after dye injection, the tissue was sectioned on a cryostat, stained, and examined using epifluorescence microscopy. We observed that the dye dispersed throughout the tissue in a manner consistent with good, but not excellent, tissue delivery (**Figure 12**). Based on the literature, we then tried an osmotic pump method of delivery with an attached intrathecal catheter. We inserted the catheter into the subdural space between the T1 and C7 vertebrae and extending it rostrally into the injury site (**Figure 13**). Tissue sections are being processed now to examine dye distribution using this method. We suspect this method of delivery will give superior results, and if so, will continue to use it throughout the remainder of the studies.

Personnel:

Dr. Thomas Hering was a visiting scientist at UK for a 2 year period. He is now solely associated with Case Western Reserve University, and no longer at UK. However, we will continue to collaborate on the remainder of the studies in this project. He retains a desk here at UK, and will do routine visits to confer.

Dr. Stephen Onifer will be leaving The University of Kentucky for a new position in January 2013. Shared equipment will be remaining at SCoBIRC for our use. We have made all preparations with the help of Dr. Onifer and his lab personnel to assume the responsibility of the studies within our lab. Dr. Onifer will continue to be an off-site collaborator throughout the duration of the grant. Other than this personnel issue, there are no other changes to the proposed studies.

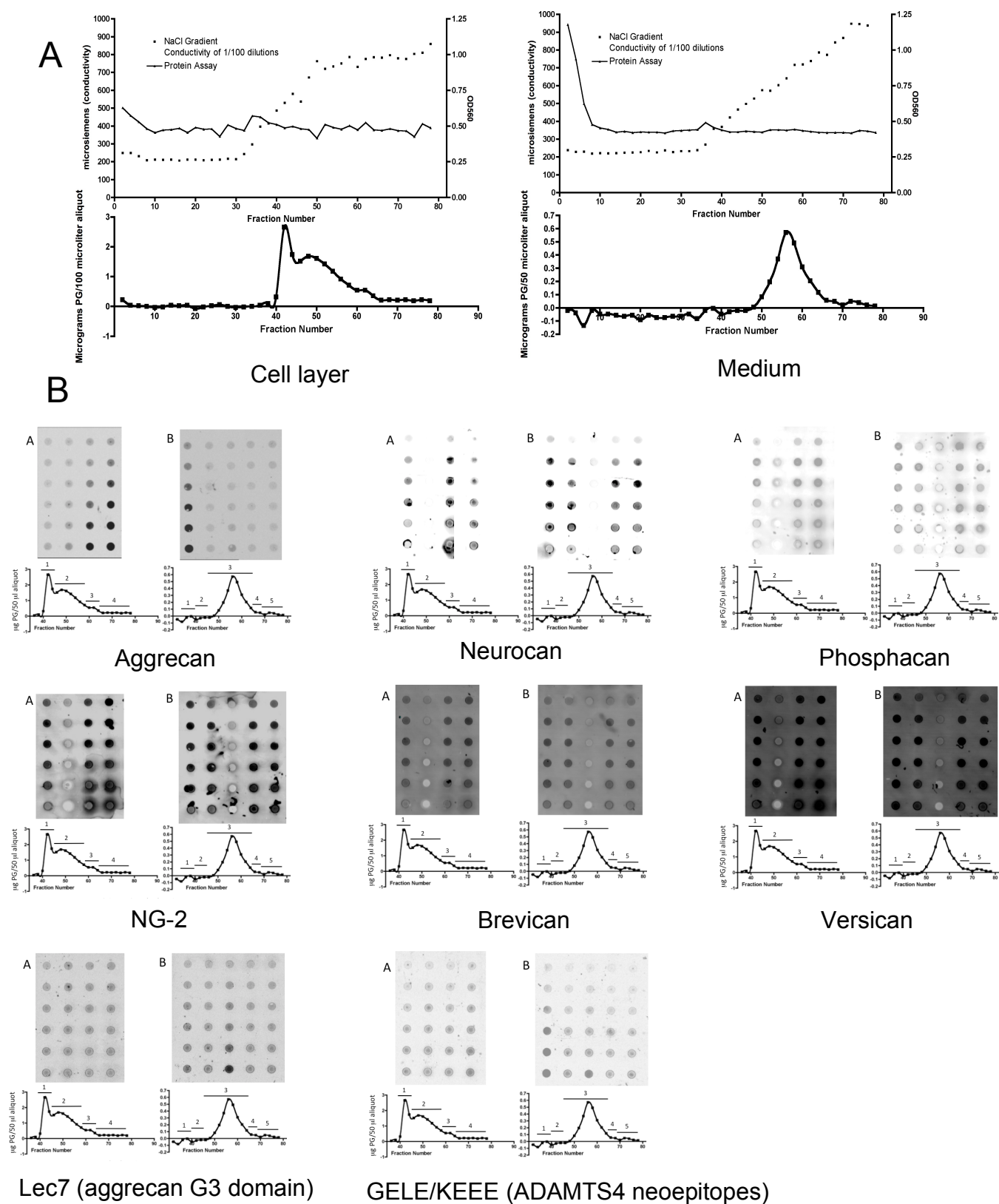


Figure 1. Analysis of proteoglycans produced by activated rat cortical astrocytes. (A) Proteoglycans were purified by size exclusion and ion exchange chromatography. DEAE chromatographic profiles are shown for astrocyte cell layer (A) and medium (B) eluted with a 0.15 to 1.0 M NaCl gradient. (B) Fractions were pooled, concentrated and characterized by dot blot analysis using specific antibodies to different proteoglycans. The dot blot analysis above the chromatogram shows reactivity of serial dilutions of pooled fractions with an antibody to the G1 domain of aggrecan.

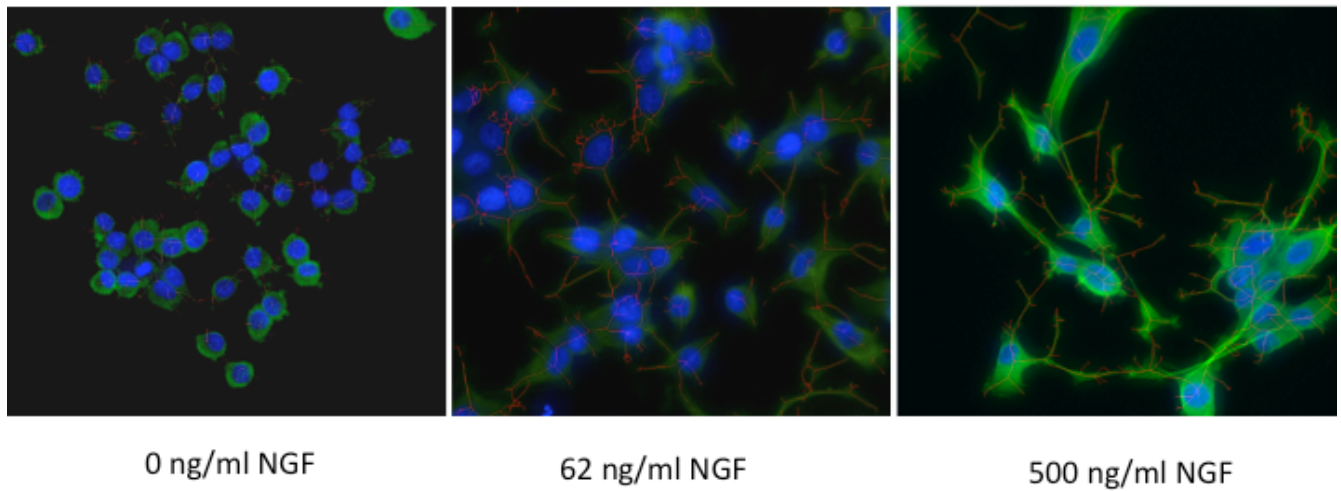


Figure 2. Neurite outgrowth assay: Response of NS-1 cells to NGF treatment. Following culture of NS-1 cells with NGF on proteoglycan-coated substratum, cells were imaged and neurite outgrowth was measured using the Neurite Tracer Image J plugin.

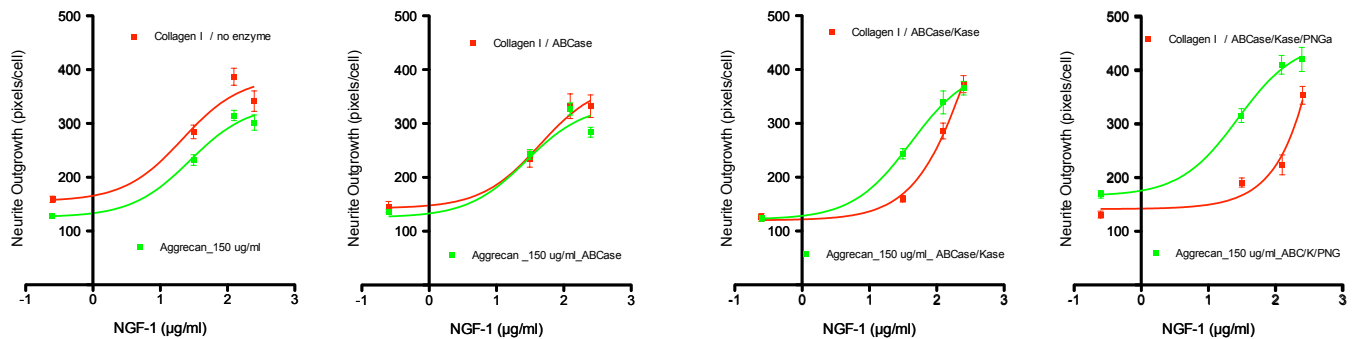


Figure 3. Neurite outgrowth assay: Effect of glycohydrolase treatment of aggreCAN-coated substratum. NS-1 cells were grown on collagen I coated surfaces (red) or on surfaces treated sequentially with either chondroitinase ABC alone (ABCase) chondroitinase ABC and endo-beta-glycosidase to degrade keratan sulfate (K'ase), or ABCase, K'ase, and peptide N-glycosidase F (PNGase).

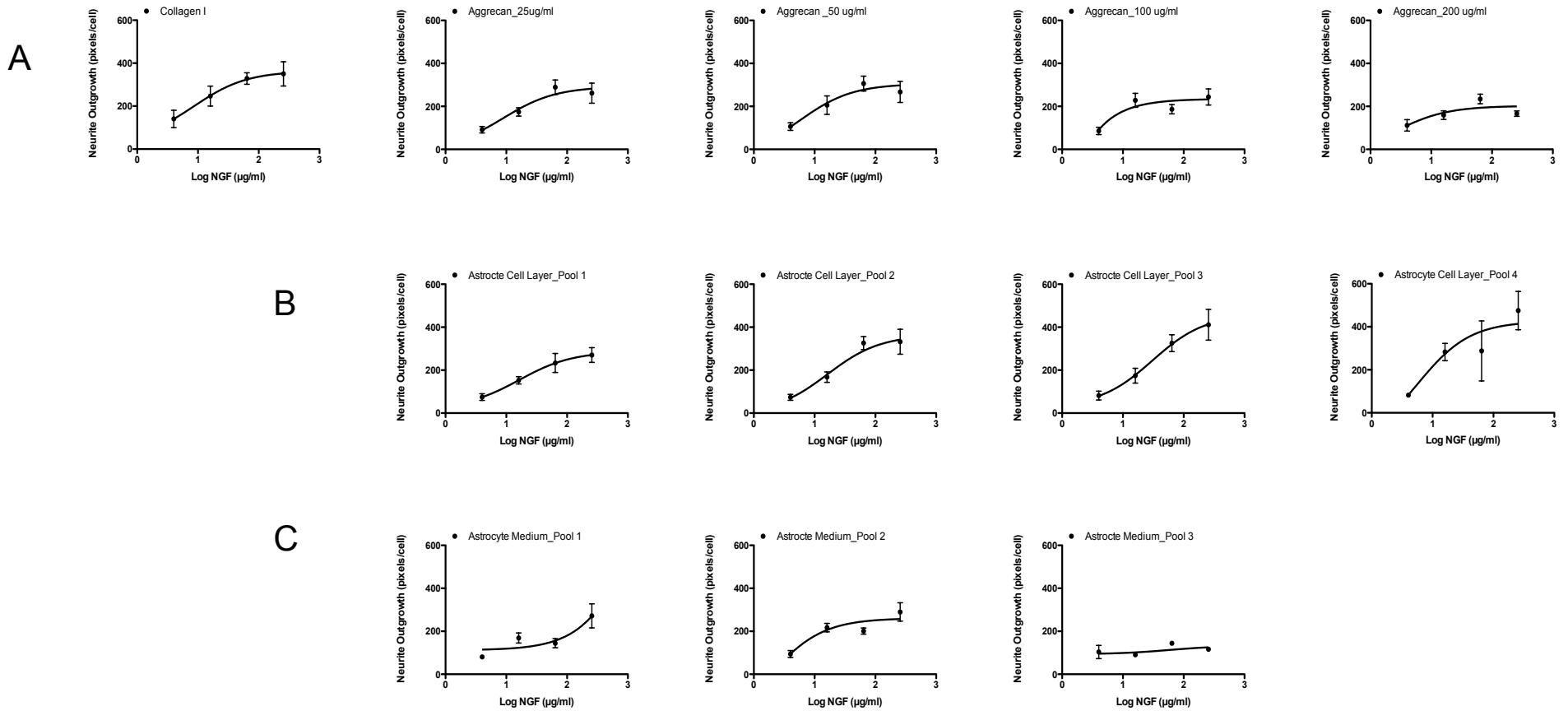


Figure 4. Neurite outgrowth assay: Effect of activated rat cortical astrocyte proteoglycans. (A) bovine cartilage aggrecan control series. Increasing concentrations of aggrecan applied to the culture dish prior to plating and outgrowth of NS-1 cells. (B) Astrocyte cell layer proteoglycan pools (pool #1 to pool #4) from DEAE Sepharose fractionated PGs were applied to the culture dish. Equivalent amounts of GAG from each pool (as determined by uronic acid) were applied. (C) Astrocyte medium proteoglycan pools (pool #1 to pool #3) from DEAE Sepharose fractionated PGs were applied to the culture dish,

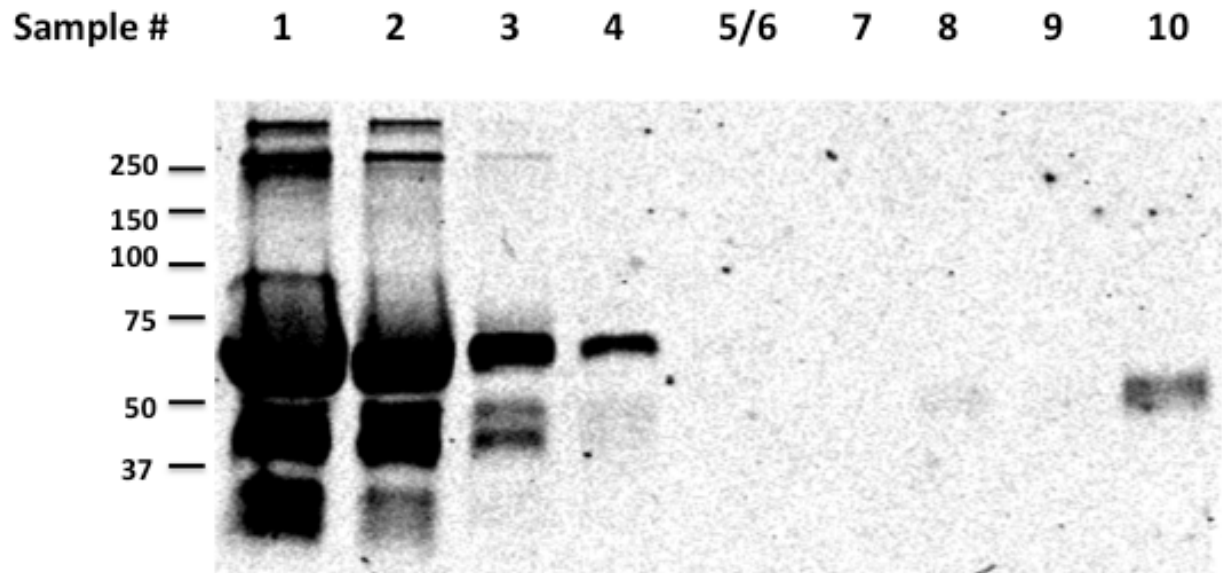
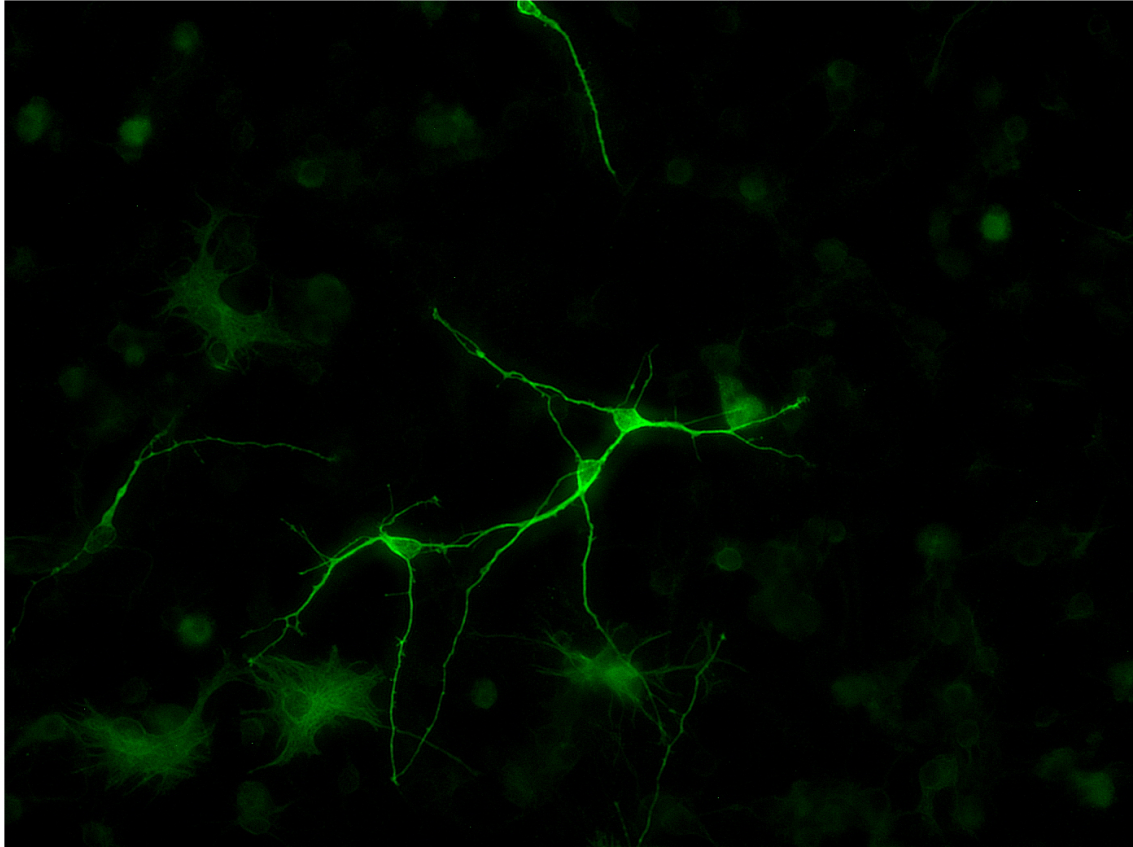


Figure 5 Antibody affinity column chromatography: Purification of recombinant ADAMTS4 expressed in HEK-293T cells. Samples were analyzed by western blot using antibody to epitope on ADAMTS4 (CYNHR) distinct from antibody bound to GlycoLink column resin. Samples are (1) crude unfractionated conditioned medium (2) column flow-through (unbound protein), (3-6) consecutive wash fractions using binding buffer, (7-10) consecutive eluted fractions using elution buffer. Purified ADAMTS4 is seen in lane 10.



Co-culture of chicken DRG neurons (E9) with rat primary astrocytes. Stained using the Neurite Outgrowth kit (Cellomics/Thermo Scientific, Cat # K0700011). Magnification = 20X.

Vibraknife Post-Injury Timecourse

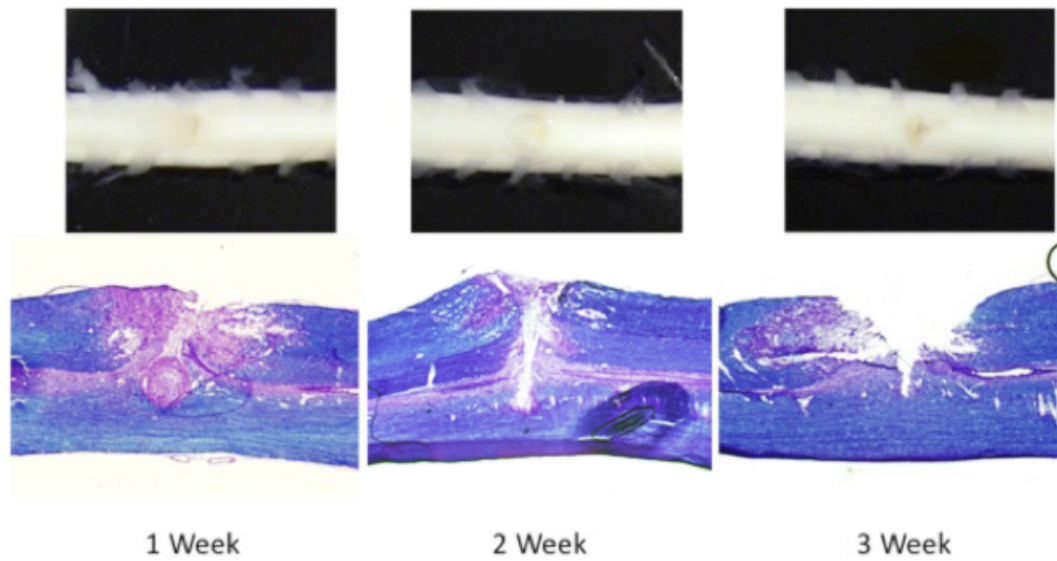


Figure 7. Post injury histology of Vibraknife injured rat spinal cord at 1, 2 and 3 weeks post injury. Longitudinal sections taken at or near midline; n=6.

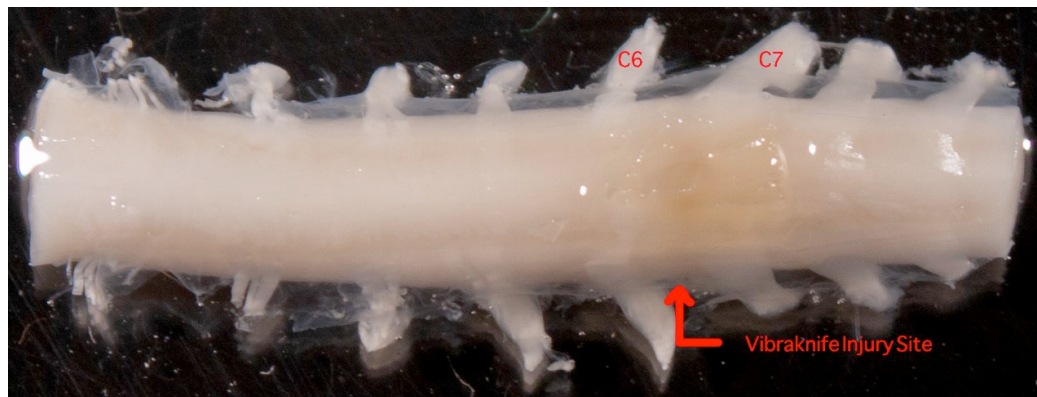


Figure 8. Vibraknife injury site between vertebrae C6 and C7.

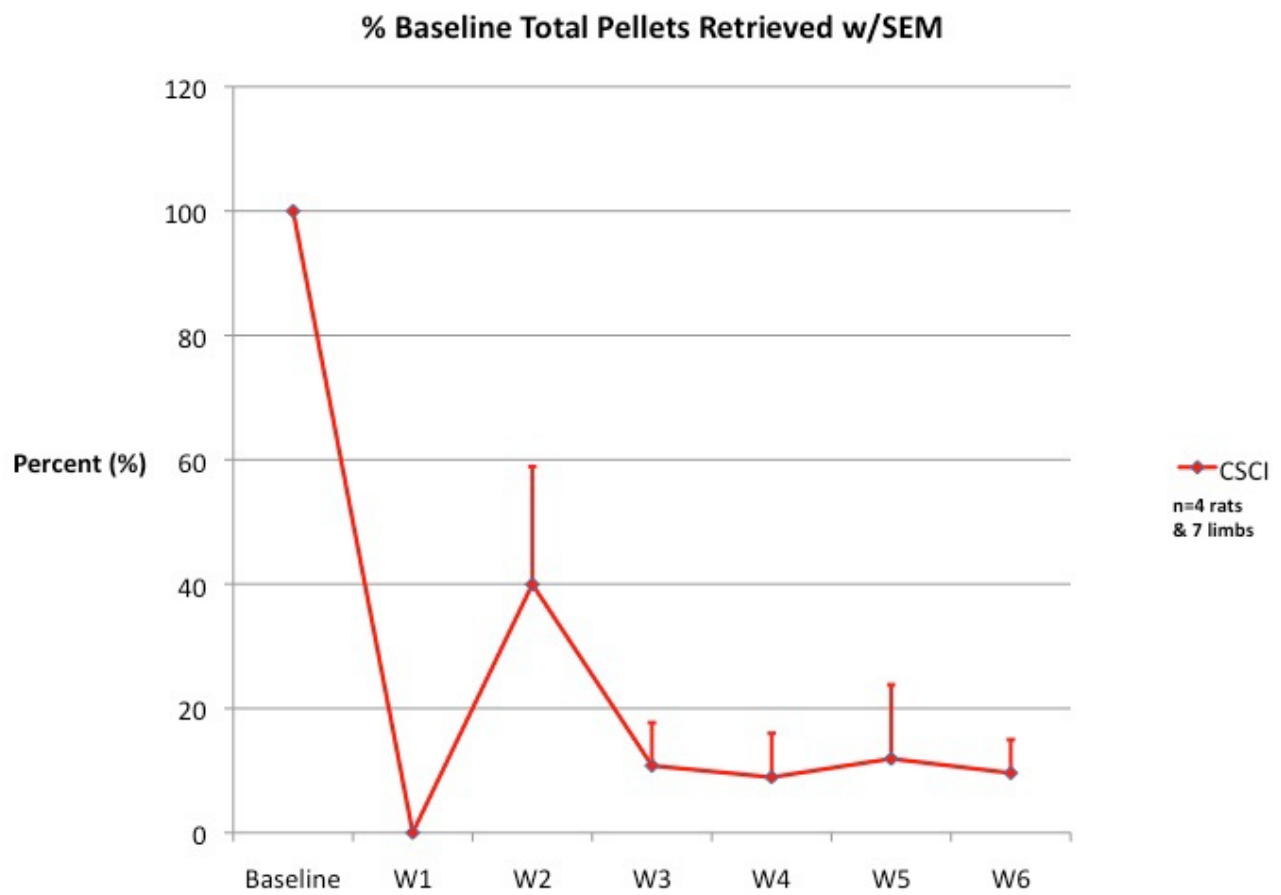


Figure 9

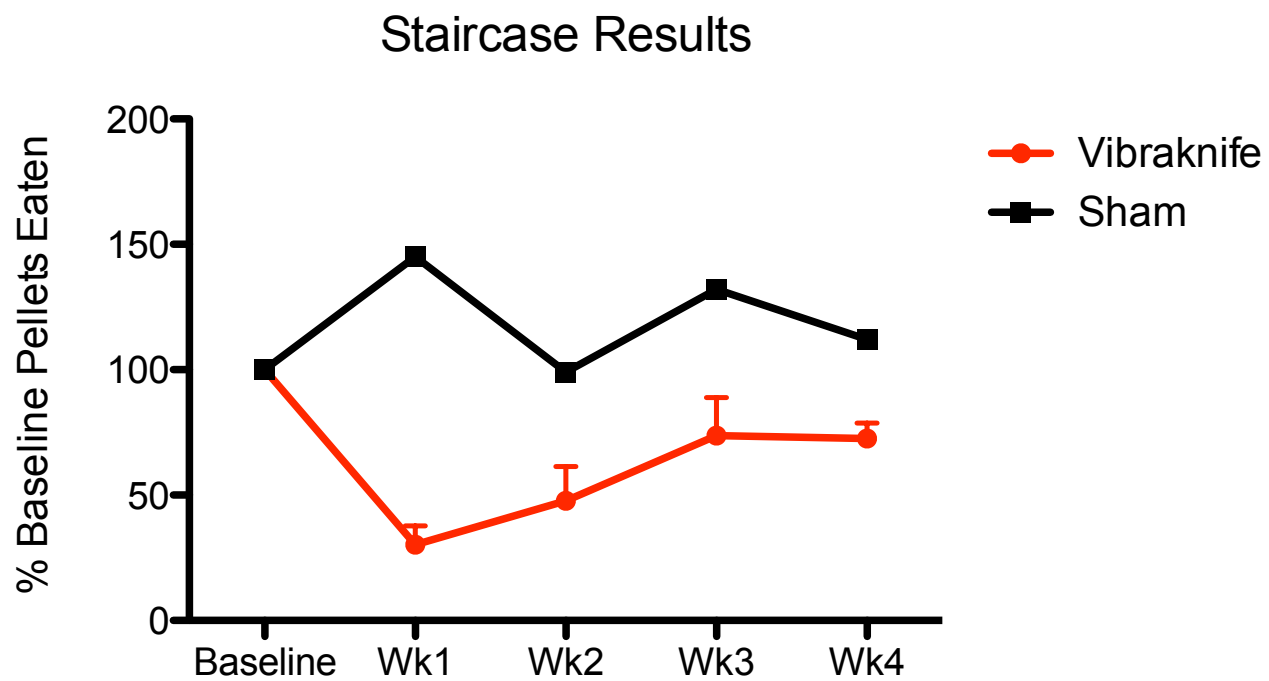


Figure 10

Sticker Test Results

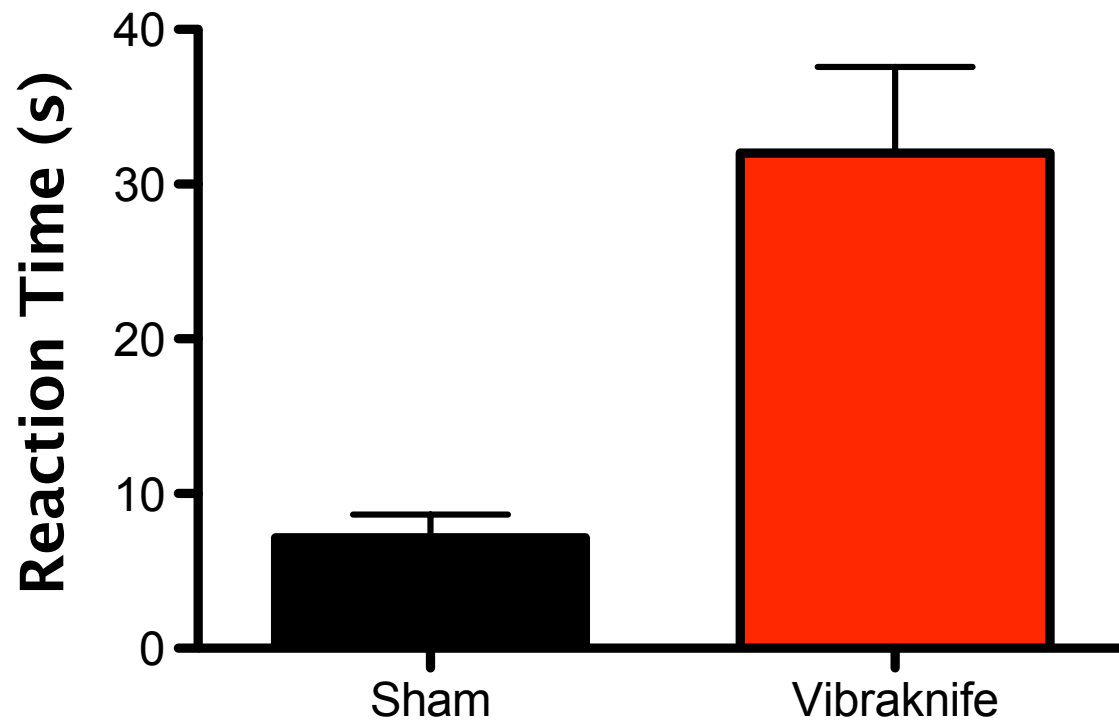


Figure 11

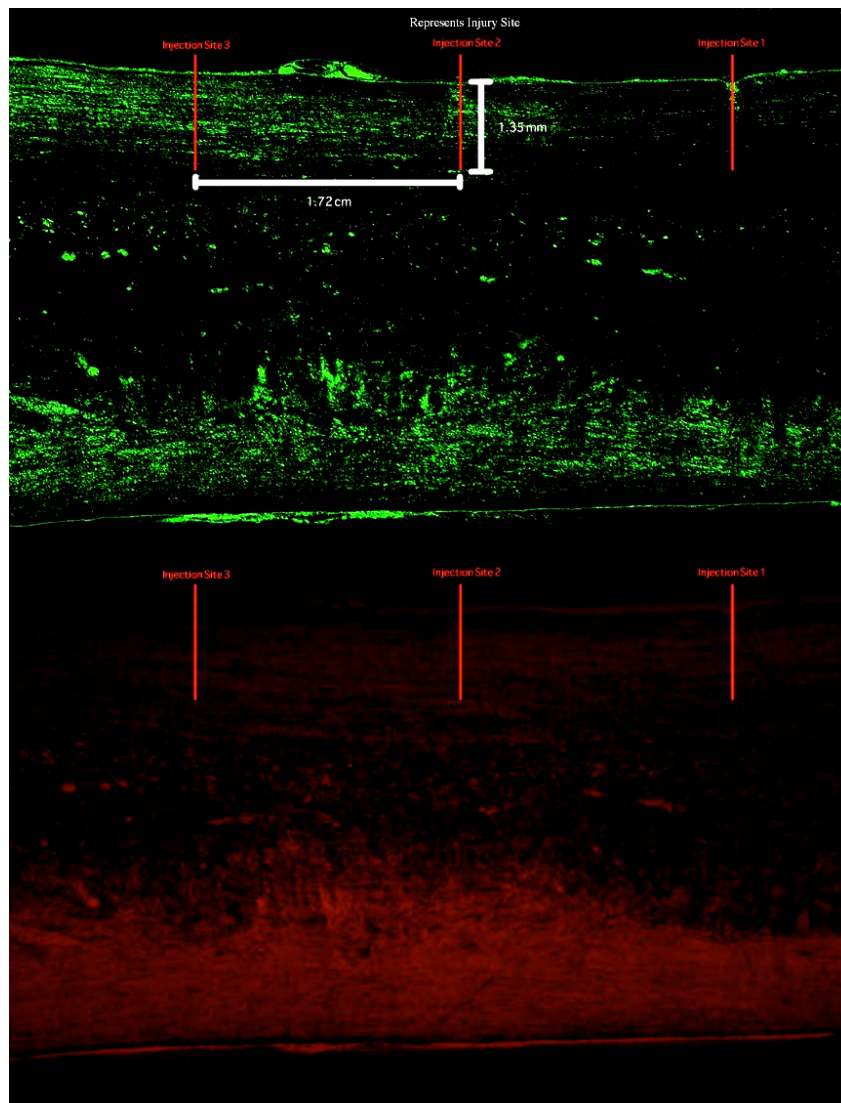


Figure 12

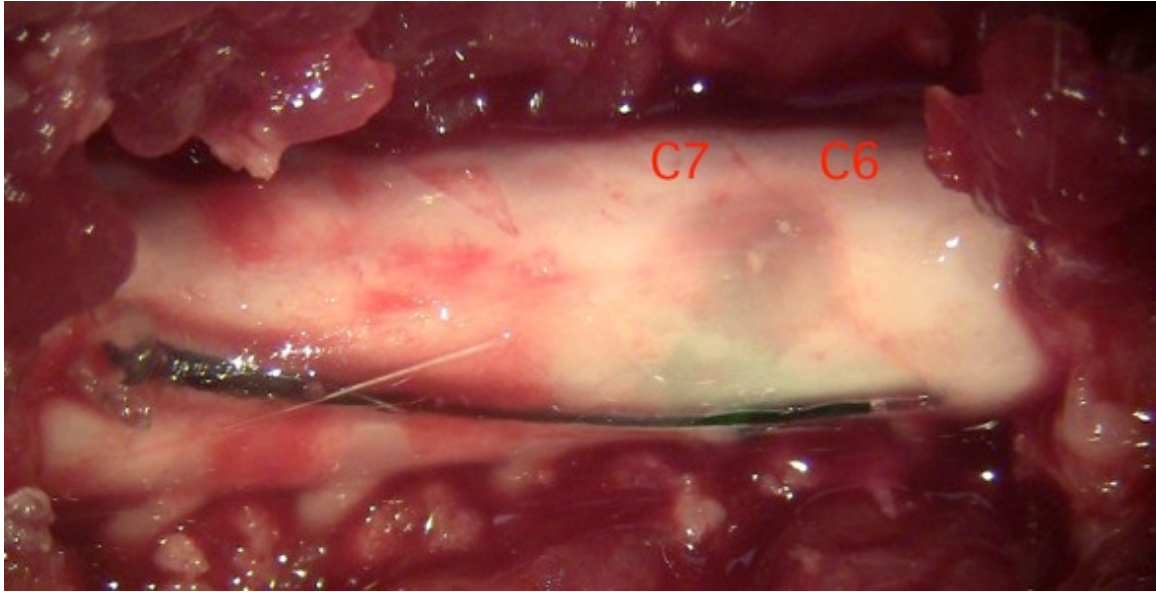


Figure 13. Osmotic mini pump placed at T1 (not shown) and threaded under the dura to administer enzyme at C6/C7. Rates and volumes are currently being optimized.

Key research accomplishments

Provided to us in the progress report review following submission of our year one annual report was the statement: “The PI should note that project milestones, such as completing proposed experiments or recruiting participants, are not acceptable as key research accomplishments. The Key Research Accomplishments section should be a bulleted list of important research findings resulting from the achievement of project milestones”. The majority of our achievements thus far, by this definition, relate to the accomplishment of the statements of work, i.e. we determined the compliment of proteoglycans secreted by injured astrocytes, we created purified enzymes for in vitro and in vivo application, we created lentiviral vectors, we developed a co-culture model and an in vivo injury model and are optimizing them for enzyme application, and so on, and are not true Key Research Accomplishments. However, Key Research Accomplishments may be our discovery that:

- Removal of KS chains further enhanced NS-1 outgrowth, beyond that of chondroitinase treatment alone; and
- Further degradation with peptide-N-glycosidase F, which cleaves N-linked oligosaccharides from the core protein, resulted in a further enhancement of NS-1 neurite outgrowth

These findings are novel and may have an important impact on regeneration research. They will be examined further in the coming year.

Reportable outcomes.

Abstracts related to DOD grant

Transient expression and purification of aggrecanase (adamts-4) from hek293t cells. Jonathan Davies, Diane M. Snow*, and T. M. Hering*. The University of Kentucky, Spinal Cord and Brain Injury Research Center, Lexington, KY 40536.

Beller, JA, Hering, TM, and **Snow, DM**. Biosynthesis of Chondroitin Sulfate Proteoglycans in Human Embryonic Kidney Cells. 29th Natl Neurotrauma Soc Symp, Ft. Lauderdale, FL; June. 10-14, 2011.

Beller, JA, Hering, TM and **Snow, DM**. Biosynthesis of chondroitin sulfate proteoglycans in HEK293T cells. KSCHIRT Symposium, 2011, Louisville, KY.

Hering, TM, Davies, J., **Snow, DM**. Transient expression and purification of aggrecanase (ADAMTS-4) from HEK293T cells. 14th International Symposium for Neural Regeneration, December 7-11, 2011, Monterey, CA.

Beller, JA, Hering, TM, and **Snow, DM**. HEK293T cells produce chondroitin sulfate proteoglycans with varied sulfation patterns, express multiple carbohydrate sulfotransferases, and are a novel system for the production of "Designer PGs". 14th International Symposium for Neural Regeneration, December 7-11, 2011, Monterey, CA.

Conclusions

Importance of implications of completed research: Thus far, our work has generated the specific tools and methodologies necessary to test aggrecanase and chondroitinase *in vitro* and *in vivo*. We have also done preliminary studies to optimize the *in vivo* model and enzyme delivery methods. We are beginning to introduce enzymes into the injury model now, and are processing tissues for observation of cellular and molecular changes, while also assessing short and longer term behavioral results.

Recommended changes: No recommended changes beyond those already implemented (see Body).

“So what?” (*evaluate knowledge gained as a scientific or medical product*): No scientific or medical product is yet evident at this early stage of the project.

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Appendices.

None