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14. ABSTRACT
There are no therapies available that restore motor impairments resulting fromspinal cord injury (SCI). Soldiers with SCI are permanently paralyzed and in needof lifelong care. Promoting axon regeneration after SCI may lead to the formation of axon circuits that may be involved in (or recruited for) motor functions. In the mammalian spinal cord, axon regeneration is frustrated by inhibitors such as chondroitin sulfate proteoglycans (CSPGs) expressed by reactive astrocytes present at the injury site. In adult zebrafish, Danio rerio, some brainstem neuronsare able to grow their axon beyond a spinal cord injury, even though inhibitoryCSPGs are present. Based on these findings we have developed an overall working hypothesis that the ability to grow an axon over CSPGs is intrinsic to the zebrafish brainstem neurons and entails the expression of a distinct set of genes. In Phase 3, we propose to employ an in vitro model system to determine the relationship between L1.1 and the CSPG neurocan, on axon growth from primarybrainstem neurons from adult zebrafish (Specific Aim 1). We also will examine in vivo the role of PTP σ in inhibition of axon regeneration (Specific Aim 2). In addition, we propose to determine the effects of identified transgenic over expressing genes crucial for axon regeneration (Specific Aim 3). The results of the proposed experiments will provide information that may serve as the basis for the development of tailored strategies to promote axon regeneration across injury sites in the spinal cord.

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Contract # W81XWH-11-1-0645

Title: Molecular Determinants Fundamental to Axon Regeneration after SCI

PI: Dr. Jeffrey Alan Plunkett

Final Report of Scientific Progress from Sept 1, 2011 – October 1, 2014

Introduction:

The zebrafish spinal cord model system is unique because of the co-existence of brainstem neurons that do (regenerators) and others that don't (non-regenerators) grow their axon beyond a spinal cord injury. These responses occur in the presence of CS-PGs, which are well-known inhibitors of axon growth in the injured mammalian spinal cord. In this proposal (the first phase of a long-term plan), we will use an *in vitro* and an *in vivo* model system to address the overall hypothesis that *the axon growth response in the injured zebrafish spinal cord is intrinsic to brainstem neurons and entails the expression of a distinct set of genes.* In Specific Aim 1, we will determine the relationship between L1.1 and neurocan and its role in axon regeneration from adult zebrafish brainstem neurons *in vitro*. The experiments in Specific Aim 2 are designed to reveal the involvement of L1.1 in axon growth. In Specific Aim 3, we will identify genes that are fundamental to successful axon regeneration past a CS-PG-rich area in the injured spinal cord.

Keywords:

Axon, Axon regeneration, spinal cord injury, zebrafish, *Danio rerio,* Chondroitin Sulfate proteoglycans, CNS injury

Overall Project Summary:

SOW: Plunkett Laboratory

<u>Specific Aim 1:</u> To determine axon growth from cultured adult zebrafish brainstem neurons with increased L1.1 levels over a neurocan substrate.

L1.1 is the zebrafish homolog of L1, a growth-promoting factor in mammals, and known to be involved in axonal regeneration in adult zebrafish[1]. In our previous studies we are studying if down regulation of L1.1 is implicated in successful axonal regeneration in vitro over neurocan, a well-known axonal growth-inhibitory CSPG. We now propose to study if an increased L1.1 level in brainstem neurons would enhance their ability to regenerate their axon over neurocan in vitro. We will employ our established brainstem neuron culture system and adeno-associated viral vectors to increase L1.1 levels. The degree of axon growth will be determined using quantitative (unbiased) assessment techniques. We predict that higher L1.1 levels further enhance axon growth from brainstem neurons over neurocan, especially from those that otherwise were unsuccessful. The results from our experiments will allow testing our premise that *increasing L1.1 within brainstem neurons elicits axon regeneration over an inhibitory neurocan environment.*

Milestones Specific Aim 1:

During Phase 1 of our grant, we cloned full-length nadl1.1 cDNA (supplied by Thomas Becker, University of Edinburgh) into a pAAV-2A-eGFP dual expression vector. We also transfected pAAV-nadl1.1-2A-eGFP and control pAAV-mCherrry-2A-eGFP constructs into adult zebrafish brainstem cultures using Amaxa-mediated electroporation. Preliminary experiments were performed on laminin and laminin/CSPG substrates ***(Figure 1)****. During this grant period, we have continued to perform experiments to quantify how nadl1.1 overexpression affects cellular responses to CSPGs.*

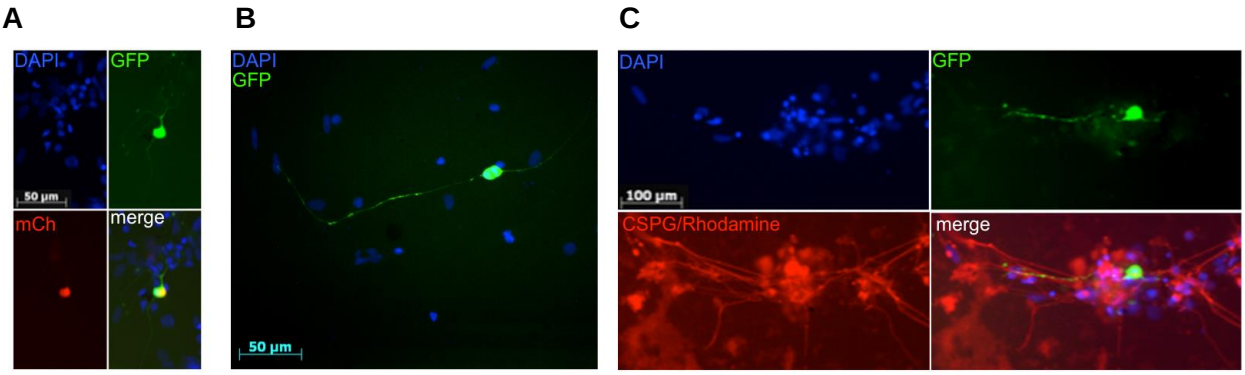


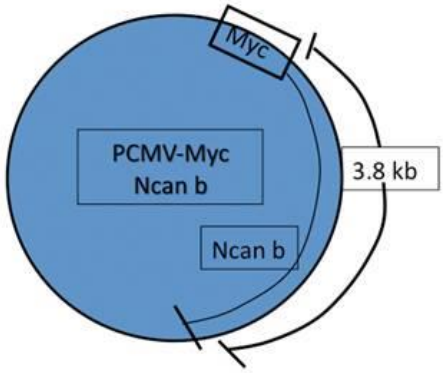
Figure 1. A. pAAV-mCherry-2A-eGFP transfected cell plated on laminin. Individual images from three fluorescent channels and merge. DAPI labels nuclei. GFP and mCherry genes are expressed off the transfected construct. B. pAAV-nadl1.1-2A-eGFP transfected cell plated on laminin. GFP demonstrates construct is expressed. C. pAAV-nadl1.1-2A-eGFP transfected cell interacting with CSPGs, labeled with rhodamine-dextran (red). Individual images from three fluorescent channels and merge.

Zebrafish Neurocan production

During Phase 2 of our grant, we successfully cloned full-length neurocan b (ncanb) cDNA in-frame and downstream of a Myc tag in a pCMV (Clonetech) vector ***(Figure 2)****. In this current phase 3 grant period we continued to characterize our secreted product and analyzing it through Western blot. Using an alkaline-phosphatase conjugated secondary antibody and Western Blue substrate reaction (Promega), anti-c-Myc expression was detected in collected Myc-Ncan transfected 293 media but not in control (untransfected) 293T cell media* ***(Figure 3)****. However, bands did not correspond to the expected ~250 kDa molecular weight band of full-length Myc-NcanB. Three smaller molecular weight bands (arrows), which may arise from Myc-NcanB cleavage, were detected in Myc-NcanB transfected media but not in control media. We hypothesize that we did not make enough full-length Myc-NcanB to detect with the current method. Experiments were repeated using further concentrated media and a more sensitive Western blotting technique, the Odyssey Infrared Fluorescent Imaging system and its accompanying IR-Dye 800 secondary antibodies, which facilitated our quantitative analysis.*

Upon recommendation of two recognized experts in the field of glycobiology, Dr. Herbert Geller of the NIH and Dr. Roman Giger of the University of Michigan, we expressed our product in zebrafish cells and not the mammalian 293T cells that we were using. Reasons for this change would be that they felt confident that expression of the protein core would be correct however, their concern was with the sugar GAG chains that ware added to the neurocan proteoglycan would be of a different composition than that produced in vivo in the fish. In an effort to eliminate this possibility, we transiently transfected primary neuronal and astrocyte cultures from the zebrafish brain. Myc-Ncan B was then be purified from the media using a Myc-column and characterized for molecular weight and the presence of GAG chains. Western blot analysis combined with chondroitinase ABC treatment was utilized. Chondrotinase treatment was expected to produce a downward shift in the molecular weight of intact Myc-NcanB.

A

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Identities = 176/176 (100%), Positives = 176/176 (100%), Gaps = 0/176 (0%)

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Sbjct  121  QVTGVVFHYRAPHNRYALSFSDAKRVCVENSANIATPAQLQATFDDGYDNCDAGWL  176
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Figure 2. A. Plasmid map of pCMV-Myc-ncanb vector showing ncand cloned in-frame and downstream of Myc tag. B. BLAST results confirming 100% identity between the protein sequences of the cloned Ncanb and the amino acid sequence posted for Ncanb (Protein ID: ENSDARP00000020815) on Ensembl Zebrafish Genome Build Zv9.

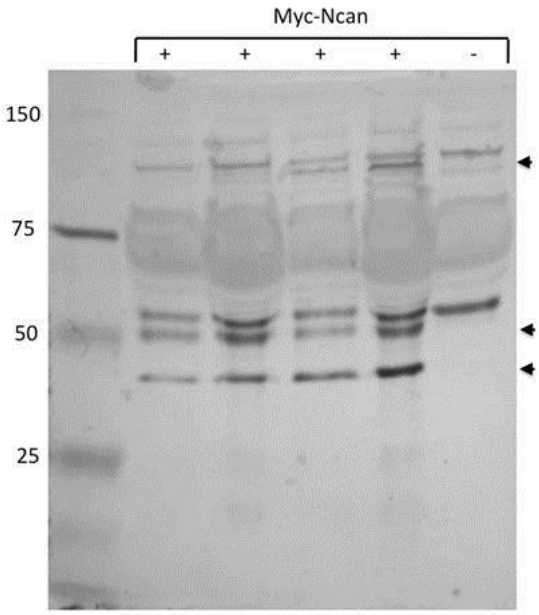

Figure 3. Western blot analyzing Myc-NcanB protein expression in media collected from transfected 293T cells (+) and untransfected media (-). Arrows correspond to differentially expressed bands recognized by anti-c-Myc.

Once Myc-NcanB protein is isolated, it was used as a substrate for zebrafish adult brainstem cultures in vitro. As a preliminary experiment we tested Myc-NcanB produced in 293T cells for inhibitory activity. We exposed zebrafish brainstem neurons to spots of concentrated myc-neurocanB media secreted from transfected 293T cells. Serial dilutions were tested and results demonstrated that high concentrations were inhibitory, as axons failed to cross myc-ncanB borders. Lower concentrations were more permissive and control spots permitted crossing (Figure 4).

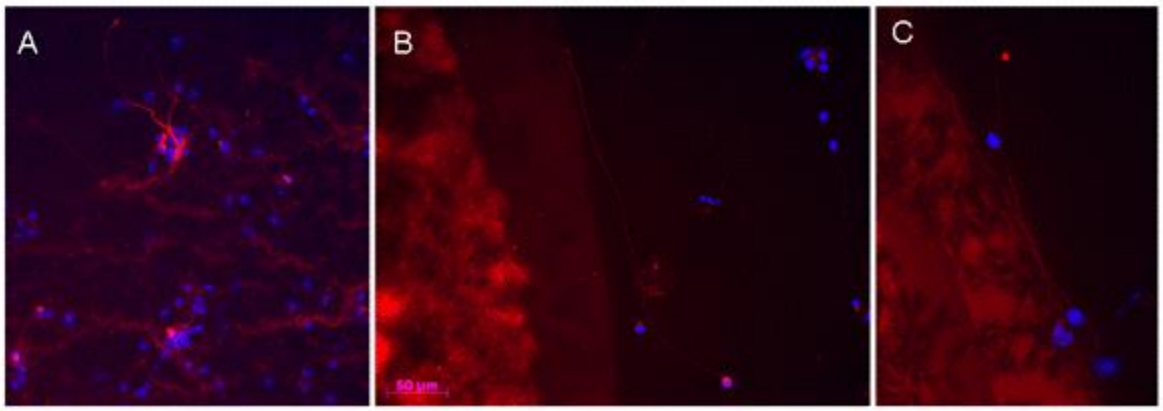

Figure 4. Neuronal cultures with spots of Myc-NcanB conditioned media from 293T cells. A. Control treated cells. B and C. Neurons turning away from concentrated Myc-NcanB spot (labeled red with rhodamine-dextran).

We also tested three antibodies raised against Neurocan to study our secreted product in more detail: MAB5212 (Millipore), 1F6 [Developmental Studies Hybridoma Bank (DSHB)], and 1D1 (DSHB). Neurocan protein expression was examined in adult zebrafish brain tissue lysates collected from control (uninjured) fish as well as from fish 6 days after acute brain injury. As shown in ***Figure 5****, Westerns performed on samples collected from control and injured fish exhibited a ~150kDa band when immunoblotted with 1F6, which recognizes a N-terminal neurocan epitope.*

Preliminary data also suggest that 1F6 recognizes Neurocan protein expression in vivo. Immunoreactivity was observed in adult zebrafish brain tissue harvested three days after acute brain injury ***(Fig 6)****. Taken together, these data suggest we can recognize zebrafish neurocan with an antibody.*

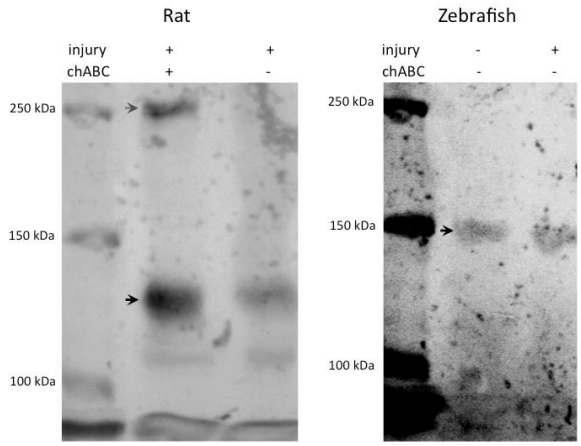

Figure 5. Western blot analysis of Neurocan expression. A 3% SDS-PAGE gel, primary antibody 1F6, and an alkaline phosphatase-conjugated secondary antibody were utilized. Protein extracts were prepared from rat and zebrafish CNS tissues. Spinal cord tissue, harvested from a rat after spinal cord injury (+), served as a positive control for 1F6 immunoreactivity. Samples were either treated (+) with Chondroitinase ABC (chABC) to remove GAG chains or left untreated (-). As expected, 1F6 recognized intact neurocan (gray arrow) and a 130kDa N-terminal processed neurocan fragment (black arrow) in chABC treated samples (Asher et. al., 2000). Zebrafish brain tissue was harvested from control (uninjured) adult fish (-) or from adult fish 6 days after acute brain injury (+). A ~150 kDa band was observed in control and injured samples, but not in a control blot processed without the primary antibody.

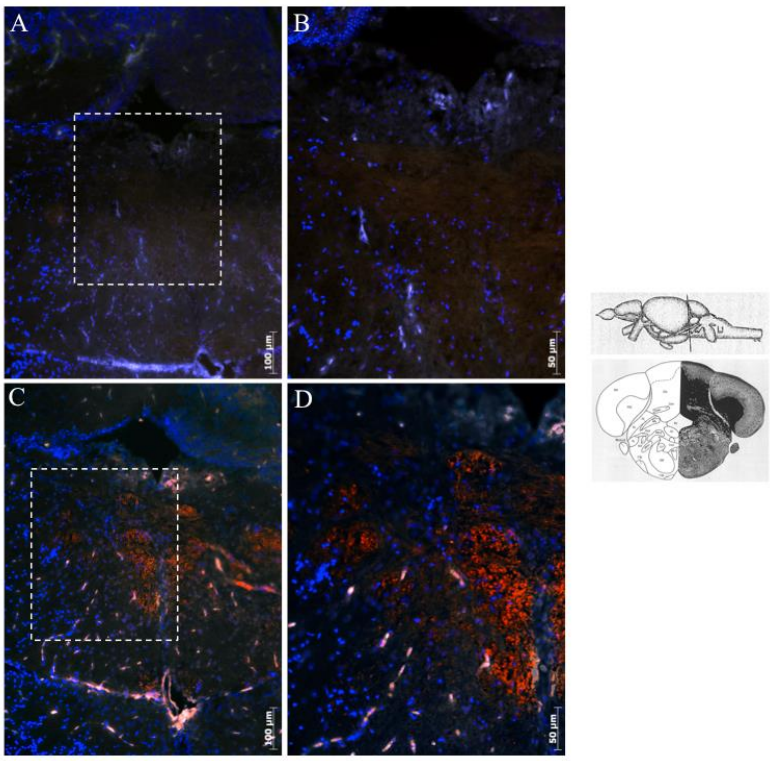

Figure 6. Neurocan expression in the brain of injured adult zebrafish. A and C are adjacent coronal sections harvested 3 days after acute brain injury. Images were taken at 10X. DAPI is shown in blue, and 1F6 in red. Nonspecific staining, which can be seen on both red and green channels (data not shown), is white following background subtraction. B and D are 20x close-ups of the boxed regions in A and C respectively. (A-B) No primary antibody control (only the secondary antibody was added). (C-D) IF6 labeling.

Zebrafish Neurocan Purification

To prepare the zebrafish neurocan, pCMV-Myc-NcanB construct secreted product as a substrate for in vitro culture experiments, we finished work on neurocan protein purification. Myc monoclonal antibody-agarose beads (Clontech 631208) were purchased to purify Myc-NcanB utilizing affinity-chromatography. In an initial experiment shown in ***Figure 7****, potential pCMV-Myc-NcanB construct secreted cleavage products from 293T cells is evidenced by western blot using anti-myc antibodies. We also purified pCMV-Myc-NcanB construct secreted products from 293T cells as well as from whole brain zebrafish cells using an immuno-precipitation strategy. We felt that the combined efforts of the Myc column and immuno-precipitation methodologies using zebrafish brain and 293T cells would yield a neurocan product that is biochemically identical to the native zebrafish neurocan. We would finish characterization and begin to conduct experiments to determine axon growth from cultured adult zebrafish brainstem neurons with increased L1.1 levels over a zebrafish neurocan substrate. .*

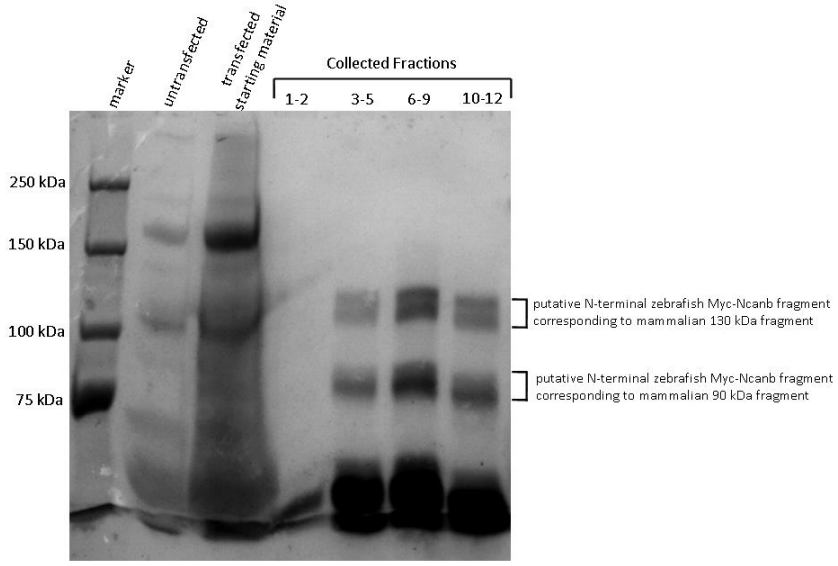

Figure 7. Western blot analyzing Myc-NcanB protein affinity column purification.
Samples were run on a 3% SDS-PAGE gel, transferred to a nitrocellulose membrane, and labeled with anti-c-Myc. Samples include media collected from control (not transfected) 293T cells, pCMV-Myc-NcanB transfected 293T cells, and twelve fractions (pooled as specified) eluted off an anti-c-Myc column. Mammalian neurocan has been described in at least four forms: intact neurocan (observed after chondroitinase ABC treatment as a ~270 kDa band) and three smaller forms resulting from proteolytic cleavage (Asher et. al., 2000). Two N-terminal fragments, 130 kDa and 90 kDa, as well as one C-terminal 170 kDa fragment have been reported. Myc-NcanB encodes a N-terminal Myc-tagged zebrafish NcanB protein. The sequence and position of the described neurocan

cleavage site that produces the 130 and 170 kDa forms is conserved between zebrafish and mammals. Two sets of bands, relatively similar in size to the mammalian 130 and 90 kDa neurocan fragments, were observed in fractions 3-12. These bands were not observed in media collected from pCMV-Myc-NcanB transfected cells prior to concentration (starting material), suggesting Myc-NcanB was too dilute in the sample for labeling.

Zebrafish Neurocan Purification (Part II)

During the next period, we purified Myc-tagged zebrafish Neurocan B (Myc-NcanB) protein from media secreted by 293T cells transfected with the pCMV-Myc-NcanB construct. Purification was accomplished using Myc monoclonal antibody-agarose beads and immunoprecipitation (Fig. 8).

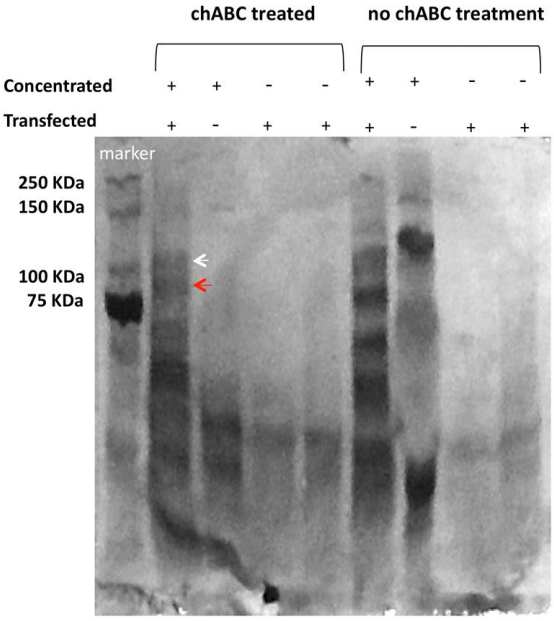

Figure 8. Western blot analyzing Myc-NcanB protein purification.
Samples were run on a 6% SDS-PAGE gel, transferred to nitrocellulose membrane and labeled with anti-c-Myc. Samples include concentrated and not concentrated media collected from control (not transfected) 293T cells and pCMV-Myc-NcanB transfected 293T cells. Media was immunoprecipitated anti-c-Myc agarose beads and eluted off the column. White arrow corresponds to putative N-terminal zebrafish neurocan 130kDa fragment and red arrow corresponds to putative N-terminal zebrafish neurocan 90 kDa fragment observed after chondroitinase ABC (chABC) treatment. Myc-NcanB transfected samples not treated with chABC exhibit bands of a slightly greater molecular weight, as expected from GAG-chain containing N-terminal NcanB fragments. Control samples (not transfected) do not exhibit these same bands. A control blot performed with no primary antibody, exhibited no bands (data not shown).

***In vitro* neuronal response to a purified Myc-NcanB substrate**

Purified Myc-NcanB protein was then tested as an in vitro substrate for adult zebrafish brainstem neurons. Brainstem neurons were exposed to spots of purified Myc-NcanB or control spots. Control spots were prepared from media secreted by control (not transfected) 293T cells, which was immunoprecipitated with anti-c-Myc agarose beads and eluted off the

column. Serial dilutions of both Myc-NcanB and control spots were used as substrate. In addition, a set of cultures grown on Myc-NcanB and control spots were treated with chondrotinase ABC (chABC), an enzyme that degrades glycosaminoglycan (GAG) chains. This allowed us to test whether the response of neurons to Myc-NcanB or control spots was affected by GAG chains. In mammals, GAG chains are partly responsible for CSPG inhibition of axonal growth (reviewed in Kwok et. al., 2008).

Our hypothesis was that Myc-NcanB will be inhibitory to some neuronal populations, but not to others. Our rationale stems from data we have collected about the response of zebrafish brainstem neurons to purified, commercially-available chicken CSPGs, #CC117 Millipore (Table 1). We have found that approximately 50% of cells are repelled by CSPGs, while the remaining 50% can cross into, cross out of, or grow on a CSPG environment. In contrast, control spots, not containing CSPGs, do not show inhibition. Neurons freely cross into, out of, and grown on the control substrate. We believe that neurons that can overcome CSPG inhibition in vitro have an intrinsic regenerative capacity, similar to that reported in vivo, which is not found in neurons repelled by CSPGs.

% repelled	% crossed in	% inside	% crossed out
52	25	15	8

Table 1. Quantification of different neuronal responses to CSPGs.
Five independent experiments were performed to calculate the response of adult zebrafish brainstem neurons to 1ug/mL CSPGs; 1154 cells were analyzed. Numbers refer to the percent of cells in each category.

Consistent with our hypothesis, we observed neurons turning away from Myc-NcanB spots that were not treated with chABC, suggesting that some neurons, as expected, were repelled by the substrate (Fig 9). We also observed populations of neurons crossing into and growing on Myc-NcanB spots, suggesting these neurons were not inhibited by the substrate (Fig 9). Control spots, not containing Myc-Ncan, did not appear to be inhibitory.

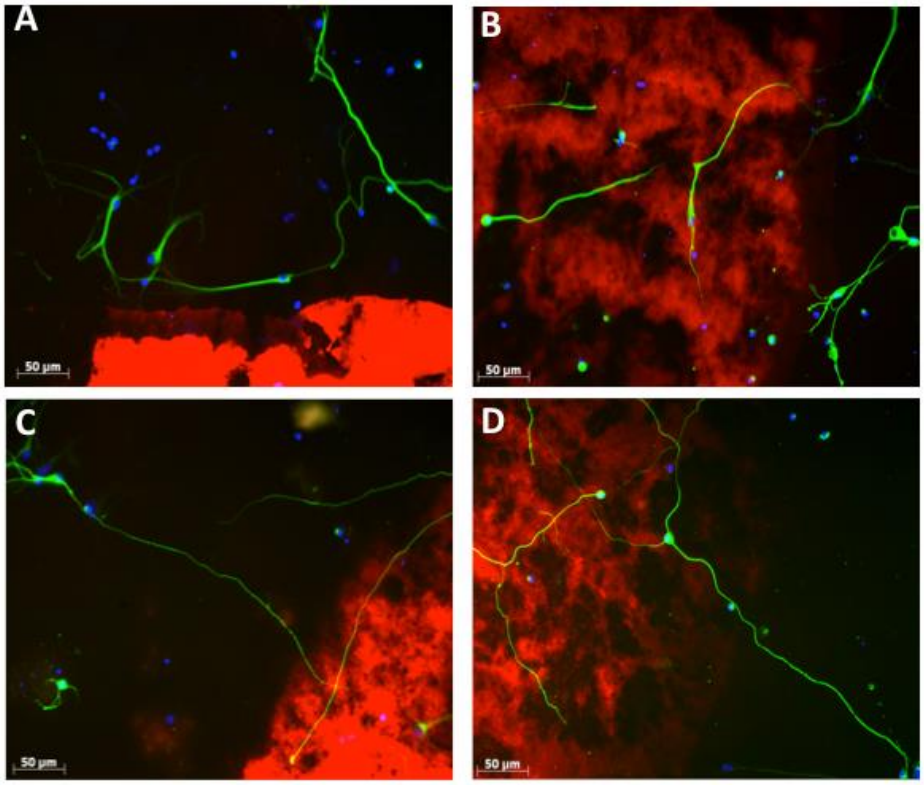

Figure 9. Neuronal response to purified Myc-NcanB.
Adult zebrafish brainstem neurons cultured for 7 days in complete media. Neurons labeled with anti-tubulin (green) and nuclei labeled with DAPI (blue). Rhodamine (red) is used to label Myc-NcanB or control spots (prepared from untransfected cells). (A) Neurons turning away from a Myc-NcanB spot with no chABC treatment. (B) Neurons crossing into and growing on a Myc-NcanB spot treated with chABC. (C) Neuron crossing into a control spot with no chABC treatment. (D) Neurons crossing into and growing on a control spot treated with chABC.

We then continued our development of a zebrafish neurocan-secreting, primary zebrafish CNS cell population, in order to obtain a product with zebrafish-specific glycosylation. As described earlier,transfection of the pAAV-Myc-NcanB-GFP and pCS2-Myc-NcanB expression vectors into primary zebrafish CNS cells was successful. As of the end of the grant period neared we had performed several experiments to examine the overexpression of L1.1 in neurons using zebrafish neurocan as a substrate. These experiments preliminarily demonstrated that overexpression of L1.1 had little or no effect on growth over purified substrates. Future studies beyond the scope of this grant period will explore and seek to understand such phenomena.

Specific Aim 2: To determine the involvement of PTPσ in regeneration from descending adult zebrafish brainstem neurons.

PTPσ is a recently discovered receptor of CSPGs. We showed that PTPσ mRNA is present in adult zebrafish CNS and studied its involvement in axon growth from cultured adult zebrafish brainstem neurons. We now propose to investigate the contribution of PTPσ to axon regeneration in vivo. We will use morpholino-techniques to reduce PTPσ levels in brainstem neurons that project their axon into the spinal cord and assess their regenerative capacity. The extent of axonal regeneration beyond areas of CSPGs within the injured spinal cord will be assessed. We predict that axon regeneration beyond a spinal cord injury is reversely correlated to the availability of PTPσ. Less PTPσ will increase the growth response of axons. The outcomes of these studies will allow testing our premise that *down regulation of PTPσ in adult zebrafish brainstem neurons promotes axonal regeneration in the injured spinal cord*.

Milestones Specific Aim 2:

Establishment of adult zebrafish spinal cord transection technique

Adult zebrafish were deeply anesthetized using 0.033% aminobenzoic acid ethylmethylester (MS222; Sigma). An incision was made approximately 3mm rostral to the dorsal fin at the level of the spinal vertebra. A finely sharpened set of forceps was used to completely transect the spinal cord ***(Figure 10)****. We have established this technique with a success rate of over 70% survival. We then delivered DiI membrane tracers through the use of application of Gelfoam placed within the transection site at the time of injury* ***(Figure 11)****. This technique allowed us to not only establish the surgical technique but also allowed us to trace descending brainstem cells present within the spinal at the time of injury. Future experiments involved a small piece of Gelfoam containing either PTPσ morpholino or control morpholino will be placed in the transection site.*

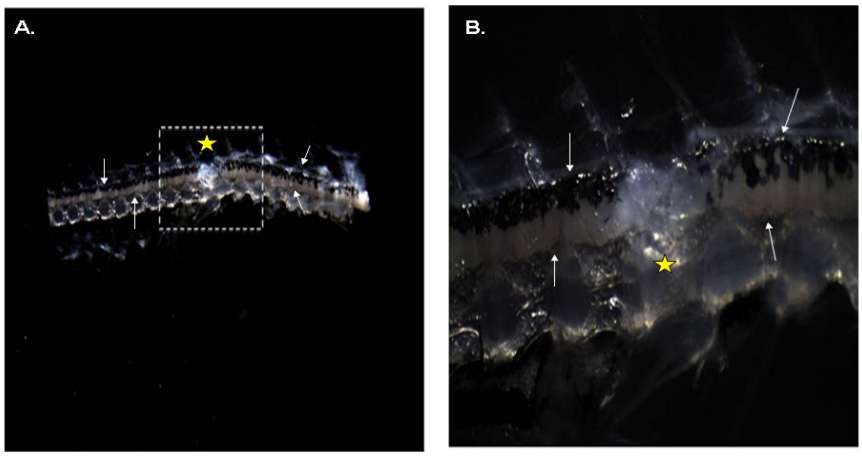

Figure 10. Zebrafish Spinal cord transection. Isolated spinal column of transected zebrafish spinal cord (rostral to left), panel A. Arrows demark CNS spinal tissue. Yellow star indicates area of injury and dashed box of injury area is magnified in panel B. Micrographs were taken two weeks post-injury.

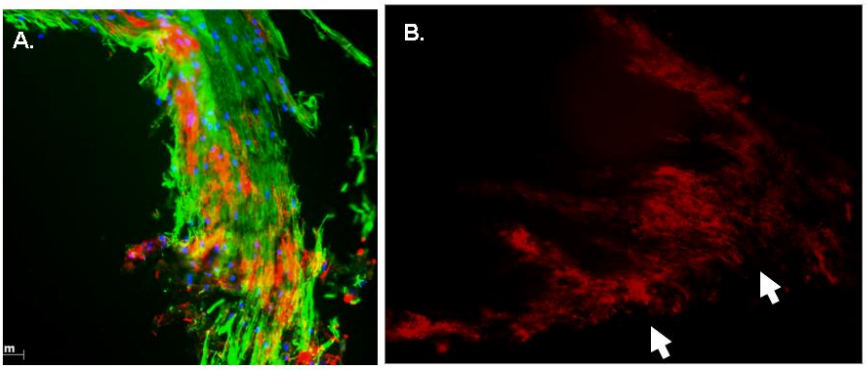

Figure 11. DiI delivery via Gelfoam into zebrafish spinal cord injury site. Fluorescent micrographs of DiI delivered to spinal cord following transection (panels A and B). Panels represent two independent experiments and the caudal border of transected cord is indicated by arrows in panel B.

***Affirmation of Experimental approach**:*

We will knockdown expression of PTPσ within transected descending brainstem neurons using PTPσ antisense morpholino. Zebrafish will receive a complete spinal cord transection and Gelfoam containing PTPσ antisense morpholino. In parallel, control groups will receive control morpholino or Gelfoam alone. Axonal regeneration in the spinal cord and the presence of neurons in a regenerative state will be assessed quantitatively. Together these outcome measures will allow evaluating the effect of PTPσ knock-down on axonal regeneration. Functional (swimming) recovery will be assessed through analysis of a number of parameters related to swimming behavior. Functional recovery will be correlated with axonal regeneration.

Establishment of molecule and PTPσ antisense morpholino delivery to transected axons

Tracing studies were used to determine delivery efficacy of molecules, including targeted morpholinos, to transected axons. We employed DiI strategies with limited success and subsequently employed fluoro-ruby (Oudega Lab reference) to trace brainstem neurons in adult zebrafish with transected spinal cord.

Functional (swimming) Recovery Analysis

In order to determine the efficacy of PTPσ morpholino or control morpholino delivery to an injured spinal cord, functional (swimming) recovery analysis was performed. We established a

low-cost video tracking system that utilizes a video camera and computer monitor to trace the movement of fish over a 5 minute unstimulated period. Fish are recorded while no disturbance or observer stimulus is present. Once recorded, the observer plays back the digital file and simply traces the movements using acetate covering the monitor. As demonstrated in ***Figure 12****, control, uninjured fish move on average 700-800 cm in the 5 minute period (panel A). One day after complete spinal cord transection, fish locomotory behavior is reduced to less than 100cm/ 5 minutes (panel B). Panel C demonstrates that by 28 days following injury, fish recover to an average of 600-700 cm per 5 minute period. An approximate in locomotory behavior of >80% was calculated.*

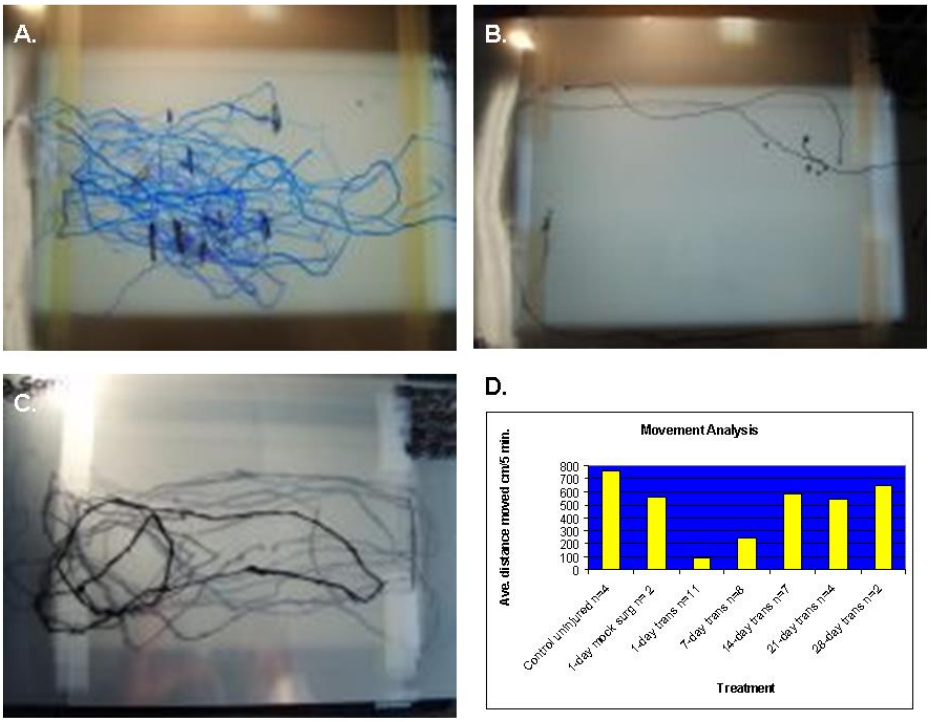

Figure 12. Functional recovery following complete spinal cord transection. Tracing of unstimulated swimming movement over 5 minute period (panels A-C). Uninjured "control" animal (A). One day post-injury (B), and 28 days post-injury (C). Graphic analysis of average movements (D).

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Retrograde labeling of brainstem neurons after spinal cord transection

Fluoro-ruby to trace brainstem neurons, whose axons were transected by spinal cord injury.

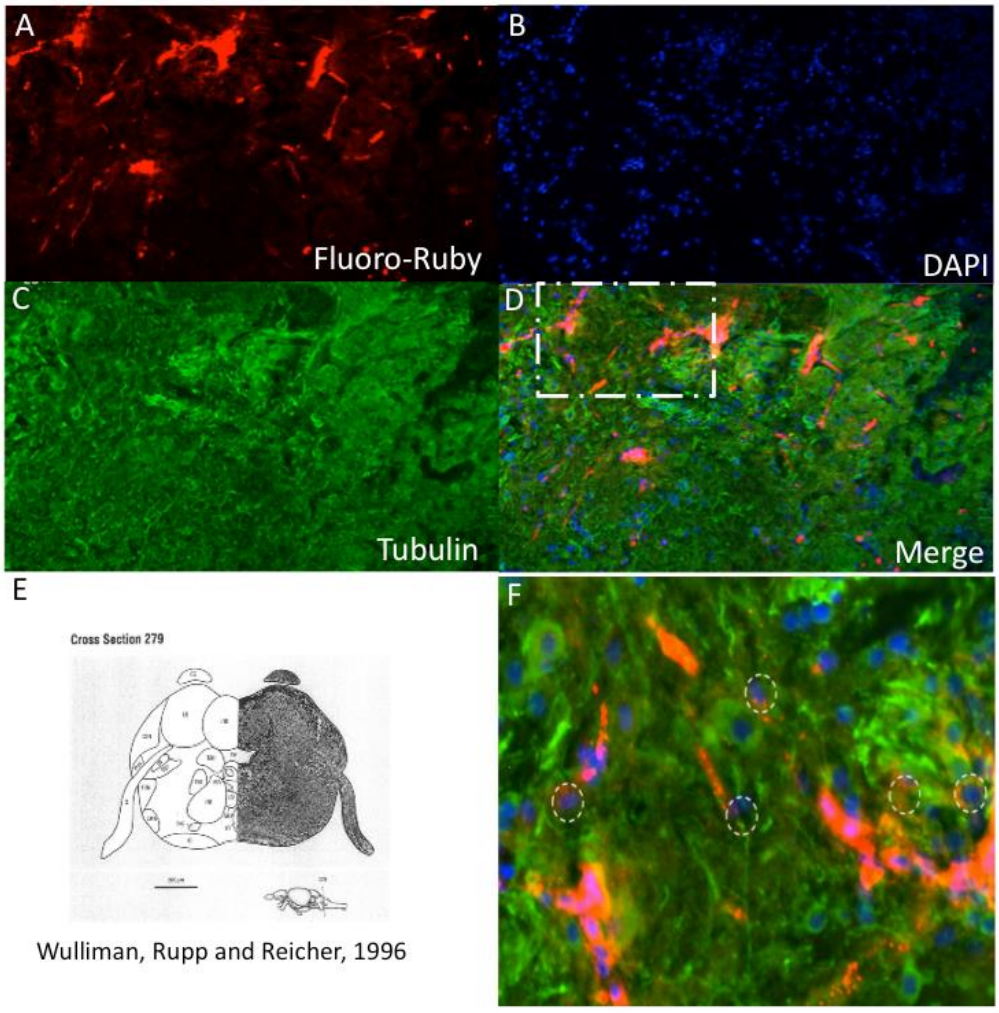

Figure 13. Brainstem neurons labeled with tracer during spinal cord injury.
Brainstem section harvested 1 day after spinal cord injury and fluoro-ruby labeling. (A-D) Each channel is shown individually at 20X and then merged into one image. Fluro-ruby tracer (red), DAPI labeled nuclei (blue), and anti-tubulin labeling cell bodies and processes (green). The area boxed in (D) is shown at 40X magnification in F. The micrographs were taken from a coronal section of the brain corresponding to that shown in E.

PTPσ morpholino characterization *in vitro*

To investigate the contribution of PTPσ to axon regeneration in vivo, we used a morpholino technique to reduce PTPσ levels in brainstem axons transected by spinal cord injury. Prior to using the morpholino in vivo, we characterized its effect on axon growth over CSPGs in vitro.

In Phase 2 of our grant, we quantified the effect of a PTPσ (ptprs) morpholino on the response of adult zebrafish brainstem neurons to 1 ug/mL CSPGs. Our hypothesis was that down regulation of PTPσ will improve axon growth from brainstem neurons that are otherwise unable to grow their axon over CSPGs. However, data revealed there was no significant difference between the response of ptprs and morpholino treated cells to CSPGs (Table 2).

Condition	% Repelled	% Crossed in	% Inside	% Crossed out
control morpholino	51	29	10	10
ptprs morpholino	57	22	11	9

Table 2. Effect of ptprs morpholino on the response of neurons to 1ug/mL CSPGs.
Percent of cells with specific response to CSPGs (1ug/mL). N=3 independent experiments (622 total cells, >300 cells were analyzed for each condition). Chi-square test for independence found no statistically significant difference between the two conditions.

*We quantified the effect of the ptprs morpholino on the response of adult zebrafish brainstem neurons to **0.5 ug/mL** CSPGs. This allowed us to test the morpholino on a less inhibitory substrate than the original 1ug/mL CSPG. The rationale was that the lower CSPG concentration may require less ptprs down regulation (less effective ptprs knock down) to reveal a significant difference between the response of ptprs and control morpholino treated cells to CSPGs. Data revealed no significant difference in the response of ptprs and control morpholino treated cells to CSPGs (Table 3).*

Although the quantitative data demonstrate no significant difference, confirmation of true ptprs knock down through the use of morpholinos is currently being confirmed as described below.

Condition	% Repelled	% Crossed in	% Inside	% Crossed out
control morpholino	*39*	*35*	*12*	*14*
ptprs morpholino	*41*	*33*	*16*	*10*

Table 3. Effect of ptprs morpholino on the response of neurons to 0.5ug/mL CSPGs.
Percent of cells with specific response to CSPGs (0.5ug/mL). N=3 independent experiments (413 total cells, >50 cells were analyzed for each condition in each experiment). Chi-square test for independence found no statistically significant difference between the two conditions.

ptprs morpholino (ptprsMO) knock down confirmation

In order to verify that the ptprs morpholino (ptprsMO) can recognize its target ptprs mRNA sequence and knock down protein translation, we will utilized a reporter construct, which contains the cDNA sequence targeted by the ptprsMO fused to the coding sequence of Green Fluorescent Protein (GFP). Similar constructs to validate morpholino target specificity have been previously described.

We cloned the ptprsMO-GFP construct into a pCS2+ expression vector. Transfected cells were then treated with either the ptprsMO or control morpholino. GFP expression would indicate the morpholino does not bind its target sequence and cannot knockdown protein translation. Preliminary experiments indicated that a population of cells treated with the ptprs morpholino does not express GFP.

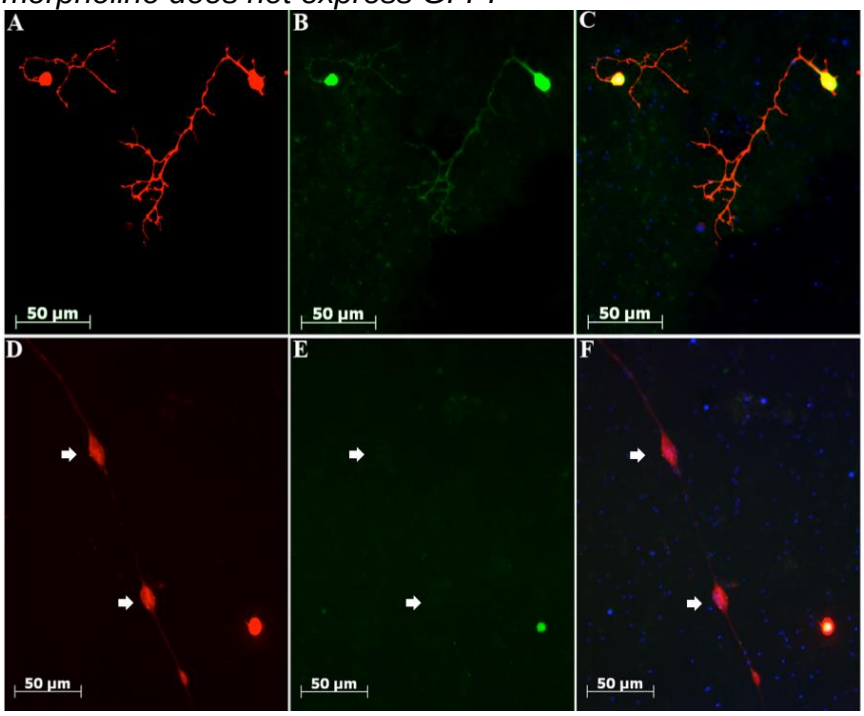

Figure 14. Zebrafish brain cells transfected with the ptprsMO-GFP pCS2 expression vector and incubated with (A-C) control morpholino or (B-D) ptprs morpholino. (A, D) Cells are labeled red. (B, E) GFP expression is shown in green. (C, F) Merge of red and green channels with DAPI (blue) labeled cells. Arrows mark cells with no GFP expression.

We began testing the effect of the PTPσ (ptprs) morpholino on functional swimming recovery after spinal cord injury. We collected data from fish treated with the ptprs morpholino, fish treated with a control morpholino, and fish treated with a morpholino against L1 cell adhesion molecule (nadl1.1), which has been shown to reduce functional swimming recovery. We also continued to collect functional data from injured fish (not treated with morpholinos) at different timepoints. These data are summarized in Figure 15. One-way ANOVA analysis revealed no significant differences among the different 3 or 7-day post-SCI groups, including the 7-day ptprs and nadl1.1 morpholino groups. However, an unpaired t-test did indicate a significant difference ($p<0.01$) between 7-day post-SCI and 7 day PTP morpholino treatment. Published data demonstrates that the nadl1.1 morpholino reduces functional recovery six weeks post-SCI. Hence, it is not surprising that the nadl1.1 morpholino appears to have no effect at the early time points we measured. As described in our previous report, we are also currently testing the efficacy of the ptprs morpholino in vitro. The ability to quantify knockdown efficiency will help us interpret our data. Inadequate knock down of ptprs protein expression could result in a weak or absent phenotype in functional swimming recovery. If this is the case, we will design a new ptprs morpholino or consider reducing ptprs expression levels with an alternative strategy, such as using an RNA interference construct.

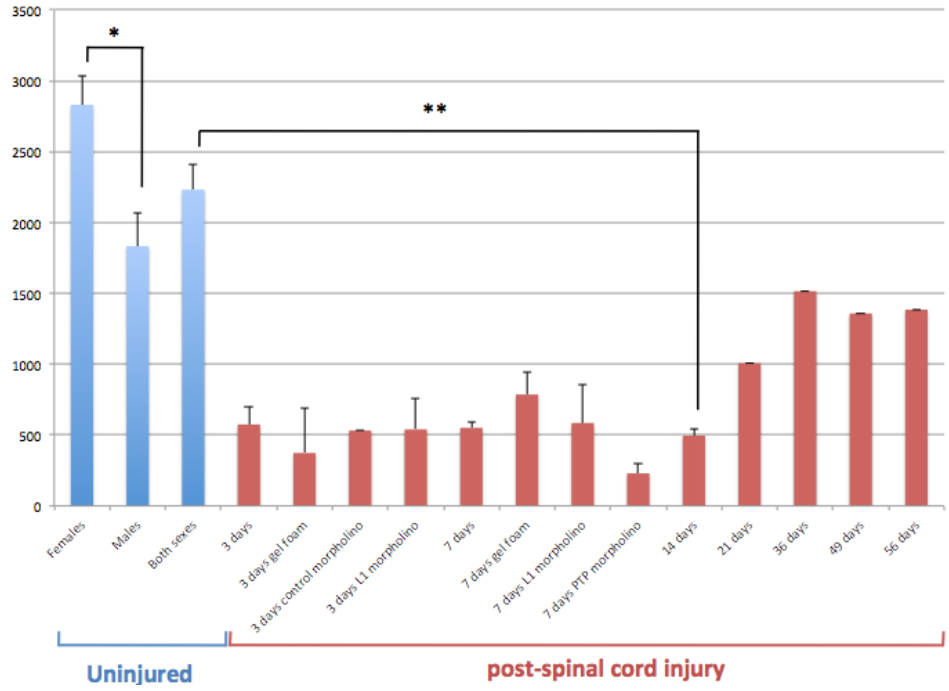

Figure 15. The chart depicts the average distance swam by uninjured and spinal cord injured (SCI) adult zebrafish over a 5 minute interval. The number of days post-SCI are listed. Data was obtained from uninjured females (n=12) and males (n=18). Data was obtained from injured fish at 3 (n=6), 7 (n=4) and 14 days post-SCI

(n=2). Data from only 1 fish was measured for each of the following timepoints: 21, 36, 49, and 56 days post-SCI. Data from gel foam treated fish data were gathered at 3 (n=2) and 7 (n=7) days post-SCI. Data from control morpholino treated fish (n=1) was analyzed at 3 days post-SCI. Data from nadl1.1 (L1) morpholino treated fish were examined at 3 (n=2) and 7 (n=2) days post-SCI. Data from ptprs (PTPσ) morpholino treated fish were examined at 7 days post-SCI (n=4). Error bars refer to the standard error of the mean (SEM). * $p<0.05$; ** $p<0.0001$ based on a One-way ANOVA test, followed by a Turkey-Kramer Multiple Comparison post-test using InStat software.

Up until the end of the project we continued testing the effect of the PTPσ (ptprs) morpholino on functional swimming recovery after spinal cord injury. We collected data from fish treated with the ptprs morpholino, fish treated with a control morpholino, and fish treated with a morpholino against L1 cell adhesion molecule (nadl1.1), which has been shown to reduce functional swimming recovery. We also continued to collect functional data from injured fish (not treated with morpholinos) at different timepoints. We also started to histologically examine spinal cord tissue that has been injured and PTPσ morpholino delivered. As shown in Figure 16 below, we first examind gross morphology and then examined the cord for completeness of recovery and then histologically through serial sectioning for axonal process recovery either positively or negatively. This analysis allowed us to gain a snapshot of axonal growth activity within the cord following SCI and morpholino delivery.

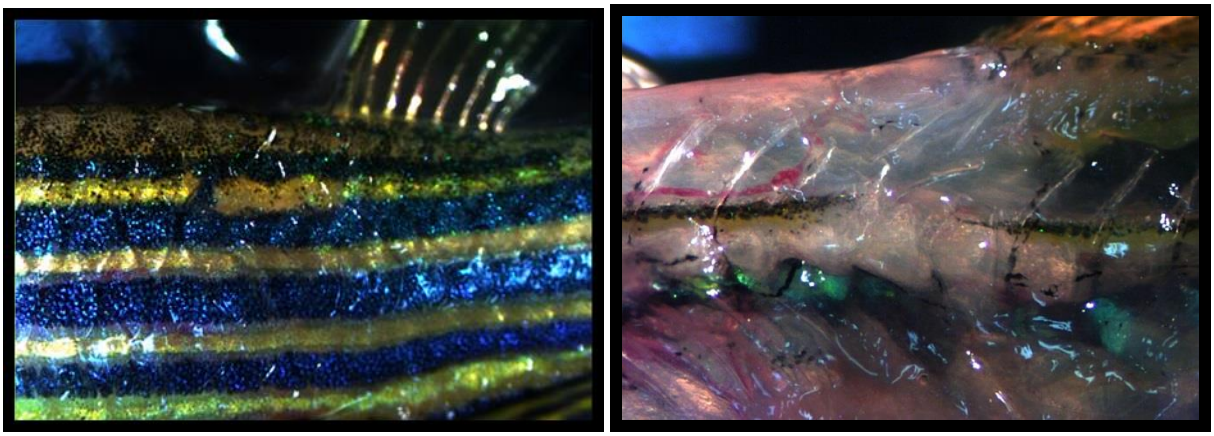

Figure 16. Injury assessment following SCI and morpholino delivery in a 56 day post-SCI animal.

<u>Specific Aim 3:</u> To determine the effects of over expression of genes involved in axonal extension in the transected adult zebrafish spinal cord on axonal regeneration and functional restoration.

Genetic deletion of identified genes involved in axonal regeneration impairs brainstem neurons to extent their axon beyond a CSPG-rich spinal cord transection site. We now propose to over express genes involved in axonal regeneration in adult zebrafish and investigate the effect of this genetic manipulation on axonal regeneration beyond a spinal cord transection. We will determine the degree of axon growth and accompanying functional recovery using tracing, functional testing, and histology. We predict that over expressing the genes will enhance the overall axonal growth process and will be accompanied by improved functional restoration. The results from these studies will allow testing our hypothesis that *axon regeneration from adult zebrafish brainstem neurons depends on the expression of a specific set of genes.*

A large number of tests was necessary to optimize the harvest of brainstem neurons. We tested different retrograde tracers (Fast Blue, Nuclear Yellow, others) at different time points after transection of the spinal cord. Different tracers have different transport characteristics which needed to be determined. This depends on molecular characteristics of the tracer as well as on the organism. Next, the fastest and most efficient tracers were identified and selected. Efficacy (i.e., the number of neurons back-labeled) was the superior criterion (over speed). Once these tracers were known we determine the most optimum time point for administration after spinal cord transection. We have completed this study to provide us with a time line of axon regeneration after complete transection; a manuscript has been published.

Original publication: http://www.ncbi.nlm.nih.gov/pubmed/25157957

In addition we were invited to publish a comprehensive review on axonal regeneration in the zebrafish.

Review article: http://www.ncbi.nlm.nih.gov/pubmed/23893428

All the experiments were fine-tuned by the efforts and results that were presented at the Society for Neuroscience meeting in San Diego (2010). A working collaboration was established with a laboratory at Pitt that owns a LCM device to collect labeled neurons. We could freely use this device. The mRNA of collected neurons was isolated and submitted for quality analysis to the Pittsburgh core proteonomic facility. We isolated RNA from adult zebrafish brains using Qiagen's RNeasy Plus Mini Kit. We also performed reverse-transcriptase PCR reactions to assess quality of our RNA using β-actin primers and the Superscript III First-Strand Synthesis system (Invitrogen). Establishment of these procedures is critical to the development of techniques used in microarray analysis. However, the quality was not up to par. The inaccuracy of the LCM device made us decide to switch to collect the neurons (after appropriate labelling) with FACS.

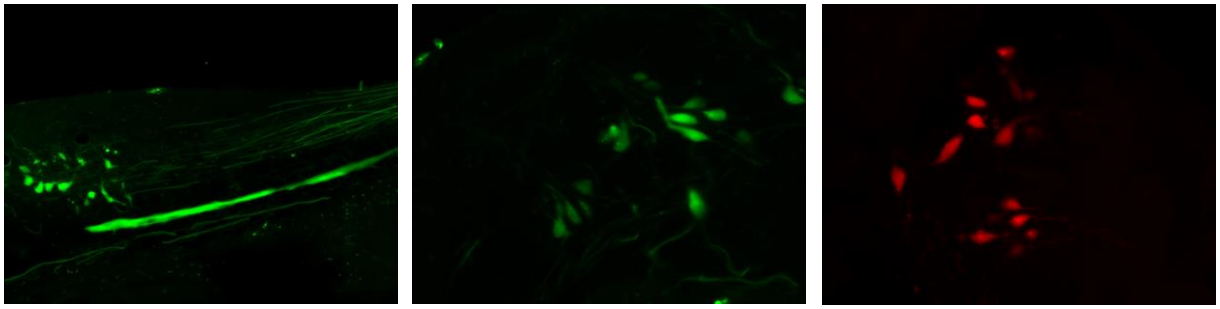

Figure 2. Fluoro-emerald labeled neurons in the adult zebrafish brainstem. The tracer was injected into the spinal cord caudal to the level where a transection would be made. Axons can internalize the tracer and trabnsport it to the cell body. In the left panel, fluoro-emerald-positive axons as well as neurons are depicted. In the middle panel, fluoro-emerald-positive neurons in the brainstem are depicted. In the right panel, a number of fluoro-ruby labeled brainstem neurons are shown.

Using FACS, neurons were collected and RNA quality was tested after zinc-fixation protocols. This is important as time will pass during harvesting of neurons and the already collected RNA needs to be stable. The first results confirmed stable RNA after zinc-fixation, but the brain was less well fixed making the cutting of sections problematic. A number of different zinc-fixation protocols are currently being tested to optimize the end-result, i.e., allowing brainstem cutting as well as proper fixation (stabilization) of brainstem neuron RNA.

Fluoro-emerald and fluoro-ruby were used to trace brainstem neurons in adult zebrafish with transected spinal cord (see figure below). We are currently collecting neurons to be used for RNA isolation and for DNA profiling. We have calculated that a total of 1500 fish will need to be labeled to obtain 50000 brainstem neurons per population. This will yield enough RNA for microarray analysis. We employed fluoro-emerald and fluoro-ruby to trace brainstem neurons in adult zebrafish with transected spinal cord (see figure below). Fluoro-emerald and fluoro-ruby were selected after a comprehensive selection procedure. After recalculation of the number of fish we need to trace to obtain enough RNA from labeled neurons for our gene arrays, we determined that number to be: 1500 fish will need to be labeled to obtain around 50000 brainstem neurons per population. This will yield enough RNA for microarray analysis. This takes time and we have continued to collect labeled brainstem neurons for our microarray analysis.

After enough fish were back-labeled and their neurons collected using FACS and RNA isolated microarray analysis was performed. The results of this final study are being processed. So far, we have identified 25 genes that were crucially up or down regulated in regenerated neurons compared with healthy or non-regenerated neurons. These genes are also expressed in mammals and thus candidate genes to target for enhancement of the axonal growth process after SCI. An article with our results will be published in a peer-reviewed journal.

Key Research Accomplishments:

Specific Aim 1 (Plunkett Lab)

1) Establishment of constructs for secretion of neurcan gene products from both 293 and zebrafish primary cells.

2) Determined that secreted products had an influence on axonal growth *in vitro.*

3) Preliminary study indicated that secreted products had little or no effect on growth of neurons overexpressing L1.1.

Specific Aim 2 (Plunkett Lab)

1) Establishment of spinal cord injury techniques in the Plunkett lab.

2) Establishment of neuronal tracing techniques.

3) Successful delivery of morpholinos to cultured primary cells.

4) Successful delivery of morpholinos to spinal cord tissue.

Specific Aim 3 (Oudega Lab)

1) Establishment of spinal cord injury and neuronal labeling techniques.

2) FACS of back-labeled neurons.

3) Isolation of high-quality RNA for microarrays.

4) Identification of genes crucial for axonal regeneration in the adult zebrafish.

Conclusion:

The different studies within this proposal (in vitro as well as in vivo) some technical/experimental barriers concerning zebrafish neurocan production and purification (specific aim 1) and specific aim 3 were encountered and the problems were addressed. Thus, in conclusion, we accomplished the goals as they were described in our proposal. These data that our line of research generated will lead to many discoveries concerning the interaction of zebrafish neurites with chondroitin sulfates.

Publications, Abstracts and Presentations:

Publications:

1. Katerina Vajn, Jeffery A Plunkett, Alexis Tapanes-Castillo and Martin Oudega.
Axonal regeneration after spinal cord injury in zebrafish and mammals: differences, similarities, translation. Neurosci Bull 2013; 29(4):402-10.

2. Alexis Tapanes-Castillo, Francelethia Shabazz, Mam M'boge, Katarina Vajn, Martin Oudega, and Jeffery A. Plunkett.
Characterization of a novel primary culture system of adult zebrafish brainstem cells. J Neuroscience Methods 2014; 223:11-19.

3. Katarina Vajn, Denis Suler, Alexis Tapanes-Castillo, Jeffery A Plunkett, Martin Oudega.
Temporal profile of endogenous spinal cord repair in the adult zebrafish.
Plos One 2014, Aug 26; 9(8).

4. Alexis Tapanes-Castillo, Francelethia Shabazz, Isaac Chacon, Diana Diaz Martin, Katarina Vajn, Martin Oudega, and Jeffery A. Plunkett. **Axonal growth of primary zebrafish brainstem neurons across inhibitory chondroitin sulfate proteoglycans.**

Manuscript in preparation.

Posters, presentations and Meetings:
2012:

Three undergraduate students from Miami-Dade Honors College conducted research in the Plunkett lab from June-August 2012 through the SRI. They submitted 2 posters and gave one oral presentation at the ***1st Annual Miami Dade College STEM Research Symposium*** *in September 2012. Jonathon Lehrman a Plunkett lab student won Best Oral Presentation.*

Four undergraduate students presented two Plunkett lab posters at the ***2012 University of Miami, Miller School of Medicine Neuroscience Research Day Symposium****.*

-Three posters were presented at the ***Society for Neuroscience Annual Meeting*** *in New Orleans, LA in November 2012. Five undergraduate students from the Plunkett lab were granted authorship due to their significant contributions to the work.*

2013:

- ***January 2013, 46th Annual Winter Conference on Brain Research.*** *A panel session organized by* ***Dr. Jeff Plunkett*** *featured talks from* ***Dr. Martin Oudega*** *(Univ of Pitt),* ***Dr. Herb Geller (NIH), Dr. James Fawcett (Univ. of Cambridge)*** *and* ***Dr. Plunkett*** *on CSPGs. Three posters from the Plunkett/Oudega labs, were also presented. Six undergraduate students from the Plunkett lab were granted authorship due to their significant contributions to the work.*

Winter Brain Panel

CSPGs in CNS injury and repair: a mammalian and non-mammalian perspective.
James W. Fawcett, Herbert M. Geller, Martin Oudega, Jeffery A. Plunkett

Winter Brain Posters

Analysis of putative stem and neural progenitor cell populations following CNS injury in the adult zebrafish
Jeffery Plunkett, Francelethia Shabazz, Katarina Vajn, Alexis Tapanes-Castillo, Martin Oudega

Characterization of a novel primary neuronal culture from adult zebrafish brainstem.
Alexis Tapanes-Castillo, Francelethia Shabazz, Isaac Chacon, Jossias Genao, Arjena Valls, Katarina Vajn, Martin Oudega, Jeffery Plunkett

Keystone Conference "Growing to Extremes: Cell Biology and Pathology of Axons", Tahoe City, CA in March 10-15, 2013.

A. Tapanes-Castillo, F. Shabazz, I. Chacon, J. Genao, A. Valls, K. Vajn, M. Oudega, J.A. Plunkett.
Primary neuronal cultures from the brainstem of adult zebrafish: a novel *in vitro* tool to study axonal growth across inhibitory chondroitin sulfate proteoglycans.

Comment: Dr. Tapanes-Castillo was awarded a $1,200.00 NIH travel fellowship to attend this meeting. Three undergraduate students from the Plunkett lab were granted authorship due to their significant contributions to the work.

Barry University STEM Science Symposium, Miami, FL. March 27th, 2013

Isaac Chacon, Francelethia Shabazz, Jossias Genao, Arjena Valls, Katarina Vajn, Martin Oudega, Alexis Tapanes-Castillo, Jeffery Plunkett.
Characterization of a novel primary neuronal culture from adult zebrafish brainstem.

Comment: Isaac Chacon an undergraduate student in the Plunkett Lab won second place overall for poster presentation.

The following four posters were presented at 8th Annual Southeast Cell Science Undergraduate Research Symposium. Miami, FL April 20, 2013.

Ibis Iser, Haydee Torres, Francelethia Shabazz, Alexis Tapanes-Castillo, Katarina Vajn, Martin Oudega and Jeffery A. Plunkett.
The effect of reducing protein tyrosine phosphatase receptor (ptprs) protein levels on functional recovery after spinal cord injury

Arjena Valls, Alexis Tapanes-Castillo, Francelethia Shabazz, Katarina Vajn, Martin Oudega, Jeffery Plunkett.
The Effects of Zebrafish Neurocan and its Putative Receptor, Protein Tyrosine Phosphatase, on Neurite Outgrowth *in vitro*

Alcides Lorenzo Gonzalez, Aileen Hernandez, Alejandra Cartagena, Lisandra Yut, Francelethia Shabazz, Katarina Vajn, Alexis Tapanes-Castillo, Martin Oudega, Jeffery Plunkett.
The role of central nervous system stem cells in adult zebrafish brainstem neuron axon regeneration
Comment: Won best poster presentation

Isaac Chacon, Francelethia Shabazz, Katarina Vajn, Martin Oudega, Alexis Tapanes-Castillo, Jeffery Plunkett
Behavioral response of adult zebrafish brainstem cells to inhibitory Chondroitin Sulfate Proteoglycans (CSPGs) *in vitro*

Neuroscience 2013
One poster will be presented at the Society for Neuroscience meeting in San Diego CA

Jeffery Plunkett, Francelethia Shabazz, Isaac Chacon, Katarina Vajn, Alexis Tapanes-Castillo, Martin Oudega
The effect of a pre-conditioning spinal cord lesion on the response of adult zebrafish brainstem cells to inhibitory chondroitin sulfate proteoglycans (CSPGs) *in vitro*.

2014:

- March 2014*: Seven undergraduate students from the Plunkett lab presented their work at the* ***Florida Academy of Sciences 78th Annual Meeting at Indian River State College in Fort Pierce, Florida****. One student gave an oral presentation. The rest presented three posters. One student won Second Place for best poster.*

Oral:
Primary neuronal cultures from the brainstem of adult zebrafish: a novel *in vitro* tool to study axonal growth across inhibitory chondroitin sulfate proteoglycans.
Diana Diaz Martin [1], Rolain Pierre [1], Isaac Chacon Rivero[1], Francelethia Shabazz[1], Katarina Vajn[2], Alexis Tapanes-Castillo[1], Martin Oudega[2], Jeffery Plunkett[1]
[1]St. Thomas University, Miami Gardens, FL. [2]University of Pittsburgh School of Medicine, Pittsburgh, PA.

POSTERS:
In vivo and *in vitro* molecular biology techniques to study central nervous system axon growth and regeneration in adult zebrafish.
Alcides Lorenzo Gonzalez[1], Arjena Valls[1], Francelethia Shabazz[1], Katarina Vajn[2], Alexis Tapanes-Castillo[1], Martin Oudega[2], Jeffery Plunkett[1]
[1]St. Thomas University, Miami Gardens, FL. [2]University of Pittsburgh School of Medicine, Pittsburgh, PA.

Alcides Lorenzo Gonzalez was awarded second place in undergraduate Bio section

The role of central nervous system stem cells in adult zebrafish neuron axon regeneration.
Abdiel Badillo[1], Aileen Hernandez*[1], Alcides Lorenzo Gonzalez[1], Alejandra Cartagena[1], Lisandra Yut[1], Francelethia Shabazz[1], Katarina Vajn[2], Alexis Tapanes-Castillo[1], Martin Oudega[2], Jeffery Plunkett[1]
[1]St. Thomas University, Miami Gardens, FL. [2]University of Pittsburgh School of Medicine, Pittsburgh, PA.

Quantification of zebrafish swimming behavior in order to determine the effect of gene knockdown on functional recovery after spinal cord injury.
Haydee Torres[1], Ibis Iser[1], Francelethia Shabazz[1], Katarina Vajn[2], Alexis Tapanes-Castillo[1], Martin Oudega[2], Jeffery Plunkett[1]

[1]St. Thomas University, Miami Gardens, FL. [2]University of Pittsburgh School of Medicine, Pittsburgh, PA.

- April 2014: Keystone Conference, Stem Cells and Reprogramming

Poster
The role of putative stem and neural progenitor cell populations in adult zebrafish axon regeneration. Jeffery Plunkett[1], Aileen Hernandez[1], Alcides Lorenzo Gonzalez[1], Abdiel Badillo[1], Francelethia Shabazz[1], Katarina Vajn[2], Alexis Tapanes-Castillo[1], Martin Oudega[2], [1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL. [2]Departments of Physical Medicine & Rehabilitation, Neurobiology, and Bioengineering, University of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA.

- April 2014: *9th Annual Southeast Cell Science Undergraduate Research Symposium, Miami, Florida*.

Seven undergraduate students from the Plunkett lab presented their work. One student gave an oral presentation and three posters were presented.

Aileen Hernandez won Second Place for best poster.

Inventions, Patents and Licenses: N/A

Reportable Outcomes: Research tool of *in-vitro* cultures of brainstem progenitor neurons that can be used by the research community to study stem/neuronal progenitor cell populations in a relation to alterations in genetic background, substrate and environmental conditions.

Other Achievements:

Undergraduate students from St. Thomas University and the Plunkett Lab have presented their research at the following meetings:

- University of Miami School of Medicine Neuroscience Symposium (Nov 2009-13)
- 2nd Annual STEM Undergraduate Conference at Barry University (March 2010-14)
- Southeast Florida Cell Science Undergraduate Research Symposium (April 2010-14)
- 9th International Meeting on Zebrafish Development and Genetics (June 2010)
- Society for Neuroscience Meetings, (Nov. 2010-13)

Under-represented minority undergraduate students from the Plunkett Lab that have gone to medical, dental or graduate school or careers in science

- Lionel Fonkoua - Medical School at Penn State University
- Frances Brlit - DO program at Nova Southeastern University.
- Anthony Wood- Medical School Morehouse, Atlanta
- Harold Gomez- Medical School University of Michigan
- Emer Balijeous- Dental NYU
- Mam Mboge- Ph.D. program University of Florida
- Arjena Valls – Graduate School Barry University
- Fran Shabazz- Research Technician University of Miami
- Alejandra Cartegena- Dental School University of Detroit Mercy

References: N/A

Appendices: