AWARD NUMBER: W81XWH-11-1-0645

TITLE: Molecular Determinants Fundamental to Axon Regeneration after SCI

PRINCIPAL INVESTIGATOR: Jeffrey Alan Plunkett, Ph.D.

CONTRACTING ORGANIZATION: St. Thomas University, INC.
Opa Locks, FL 33054

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 October 2012	Annual	1 Sep 2011 – 1 Sep 2012

4. TITLE AND SUBTITLE

Molecular Determinants Fundamental to Axon Regeneration after SCI

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0645

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jeffrey Alan Plunkett, Ph.D.

Email: jplunkett@stu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
St. Thomas University, INC.
Opa Locka, FL 33054

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

There are no therapies available that restore motor impairments resulting from spinal cord injury (SCI). Soldiers with SCI are permanently paralyzed and in need of 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 neurons are able to grow their axon beyond a spinal cord injury, even though inhibitory CSPGs 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 primary brainstem 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. The PI is Jeffrey Plunkett, Ph.D.

15. SUBJECT TERMS
Molecular Determinants Fundamental to Axon Regeneration after SCI

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	39	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

Introduction…….. 4

Body…….. 4

Key Research Accomplishments…….. 25

Reportable Outcomes…… 26

Conclusion…… 33

References……. 33

Appendices…… 33

Financials …….. 39

Contract # W81XWH-11-1-0645

Title: Molecular Determinants Fundamental to Axon Regeneration after SCI

PI: Dr. Jeffrey Alan Plunkett

Scientific Progress from Sept. 1, 2011 – Aug.31, 2012 (Months 1-12)

Introduction:
This work represents the third phase in our overall strategy to develop effective treatments for the injured spinal cord. The experiments proposed here continue in the investigations in previous phases and build upon the knowledge that was gathered. We will use an *in vitro* and *in vivo* model system to conduct mechanistically-driven experiments that 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 this stage of our approach, we expect that the results from **Specific Aim 1** will demonstrate that increased levels of L1.1 will improve axonal regeneration over neurocan. We further anticipate that the data obtained in **Specific Aim 2** will demonstrate that regeneration of descending brainstem axons across a CSPG-rich transection site will depend on the availability of PTPσ, a CSPG receptor and that the axonal regeneration response is reversely correlated with the availability of PTPσ. The results from the experiments proposed in **Specific Aim 3** are expected to demonstrate that over expression of genes that are involved in axonal extension in the injured spinal cord will enhance axonal regeneration which will correlate with functional restoration. The overall goals of the proposed experiments are to validate our results from the previous studies, to further establish and employ our *in vitro* and *in vivo* model systems, and to further develop our mechanistic-based models for the successful and failed axonal regeneration response observed in the injured adult zebrafish spinal cord.

Body:

SOW: Plunkett Lab

<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.

Months 1-3: Transfect primary brainstem cultures with control and pAAV-L1cDNA-2A-eGFP vectors.

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)****. In this review period, we have continued to perform experiments to quantify how nadl1.1 overexpression affects cellular responses to CSPGs.*

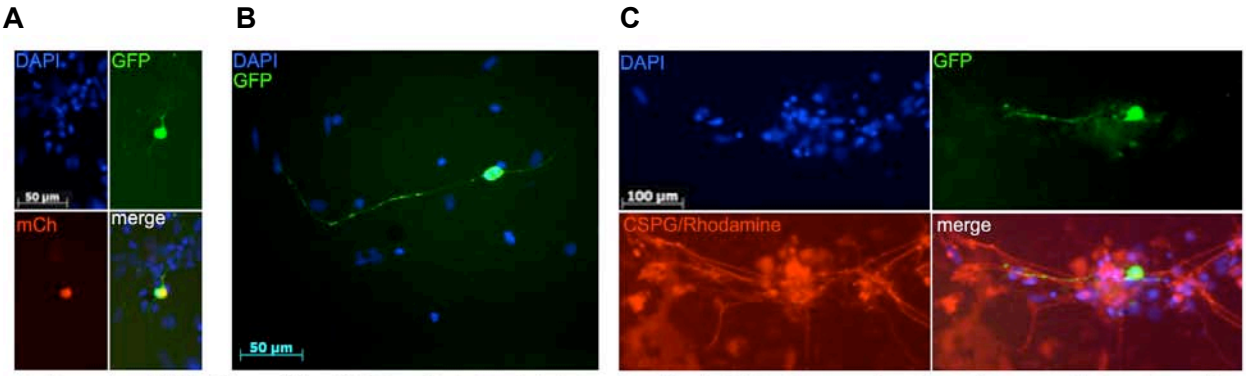


Figure 1. A. pAAV-mCherry-2A-eGFP transfected cell plated on laminin. Individual images from three fluorescent channels and merge. DAPI labels nuclei. GFP and mCherry genes are expressed off the transfected construct. B. pAAV-nadl1.1-2A-eGFP transfected cell plated on laminin. GFP demonstrates construct 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.

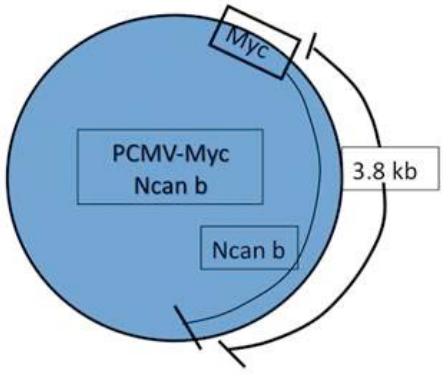
Zebrafish Neurocan production

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 period we are continuing 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 are being 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 facilitates quantitative analysis.

Recently, we presented our data at the Society for Neuroscience Meeting in Washington DC. In speaking with two recognized experts in the field of glycobiology, Dr. Herbert Geller of the NIH and Dr. Roman Giger of the University of Michigan, they both recommended that we express our product in zebrafish cells and not the mammalian 293T cells that we are currently 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 are currently transiently transfecting primary neuronal and astrocyte cultures from the zebrafish brain. Myc-Ncan B will 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 will be utilized. Chondrotinase treatment is expected to produce a downward shift in the molecular weight of intact Myc-NcanB. We should be able to analyze these efforts prior to our next quarterly report. In addition, Dr. Geller has also graciously agreed to not only analyze our cloned/secreted product but also for a possible collaboration on the examination of CSPGs within the zebrafish CNS.

A



B

```
 Score =  369 bits (946),  Expect = 2e-106, Method: Compositional matrix adjust.
 Identities = 176/176 (100%), Positives = 176/176 (100%), Gaps = 0/176 (0%)

Query  1    VNMRKVTHQQVQEPLSGSALLPCVFTLSPSPSHEPPRIKWTKLWGQRGPDGLQKQQSILV  60
            VNMRKVTHQQVQEPLSGSALLPCVFTLSPSPSHEPPRIKWTKLWGQRGPDGLQKQQSILV
Sbjct  1    VNMRKVTHQQVQEPLSGSALLPCVFTLSPSPSHEPPRIKWTKLWGQRGPDGLQKQQSILV  60

Query  61   AKDNIIKVKKAFQGRVTLPGYPGNRYNASLVLTGLRSSDSGMYRCEVVVGINDEQDTVPL  120
            AKDNIIKVKKAFQGRVTLPGYPGNRYNASLVLTGLRSSDSGMYRCEVVVGINDEQDTVPL
Sbjct  61   AKDNIIKVKKAFQGRVTLPGYPGNRYNASLVLTGLRSSDSGMYRCEVVVGINDEQDTVPL  120

Query  121  QVTGVVFHYRAPHNRYALSFSDAKRVCVENSANIATPAQLQATFDDGYDNCDAGWL  176
            QVTGVVFHYRAPHNRYALSFSDAKRVCVENSANIATPAQLQATFDDGYDNCDAGWL
Sbjct  121  QVTGVVFHYRAPHNRYALSFSDAKRVCVENSANIATPAQLQATFDDGYDNCDAGWL  176
```

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.

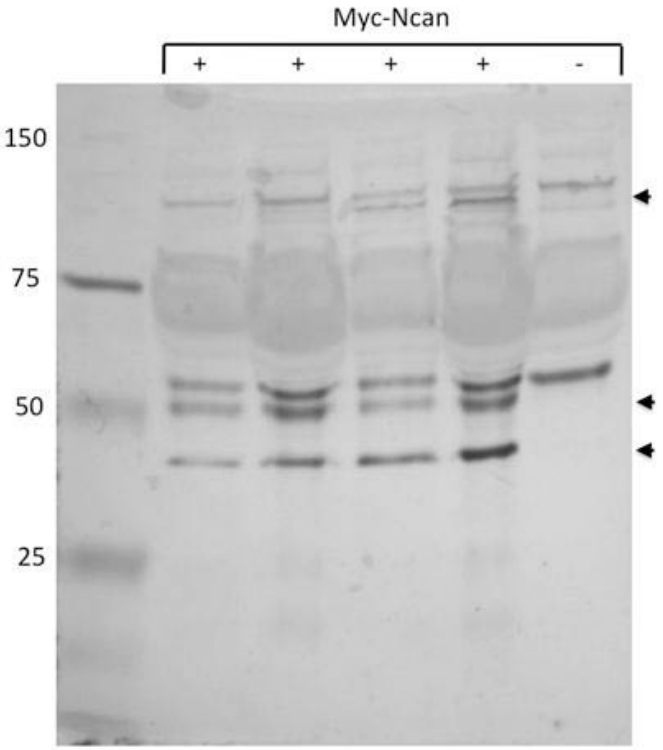


Figure 3. Western blot analyzing Myc-NcanB protein expression in media collected from transfected 293T cells (+) and untransfected media (-). Arrows correspond to differentially expressed bands recognized by anti-c-Myc.

Once Myc-NcanB protein is isolated, it will be used as a substrate for zebrafish adult brainstem cultures in vitro. During this review period, 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).

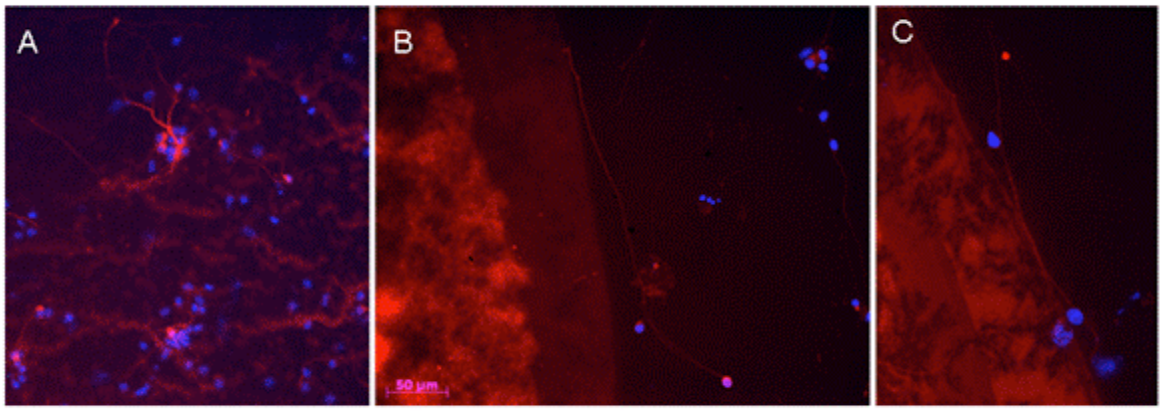


Figure 4. Neuronal cultures with spots of Myc-NcanB conditioned media from 293T cells. A. Control treated cells. B and C. Neurons turning away from concentrated Myc-NcanB spot (labeled red with rhodamine-dextran).

Months 4-6: Analyze L1.1 over expression in cultured brainstem neurons.

During this review period, we continued to transfect our pCMV-Myc-NcanB construct into mammalian 293T cells and collect culture media containing secreted Myc-NcanB. In addition, we followed the suggestions of Dr. Herbert Geller of the NIH and Dr. Roman Giger of the University of Michigan, who both recommended that we express Myc-Ncan in zebrafish cell cultures to maintain the endogenous zebrafish sugar GAG chain composition (personal communications, 2011 Society for Neuroscience meeting). We transfected pCMV-Myc-Ncan into zebrafish brain cultures, and we are currently characterizing the secreted product. We are comparing it to Myc-NcanB collected from 293T cells utilizing Western blot analyses and anti-Myc.

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 show 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.*

Zebrafish Neurocan Purification

To prepare the zebrafish neurocan, pCMV-Myc-NcanB construct secreted product as a substrate for in vitro culture experiments, we began work on neurocan protein purification. Myc monoclonal antibody-agarose beads (Clontech 631208) were purchased to purify Myc-NcanB utilizing affinity-chromatography. Protocols are currently being tested and implemented.

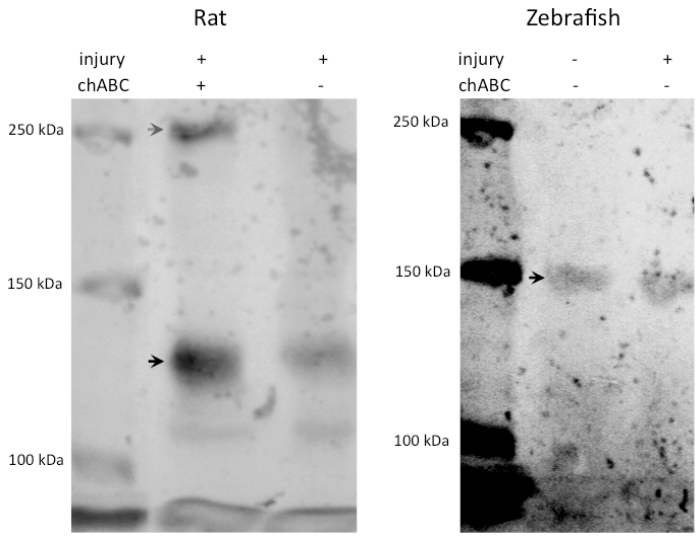


Figure 5. Western blot analysis of Neurocan expression. A 3% SDS-PAGE gel, primary antibody 1F6, and an alkaline phosphatase-conjugated secondary antibody were utilized. Protein extracts were prepared from rat and zebrafish CNS tissues. Spinal cord tissue, harvested from a rat after spinal cord injury (+), 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.

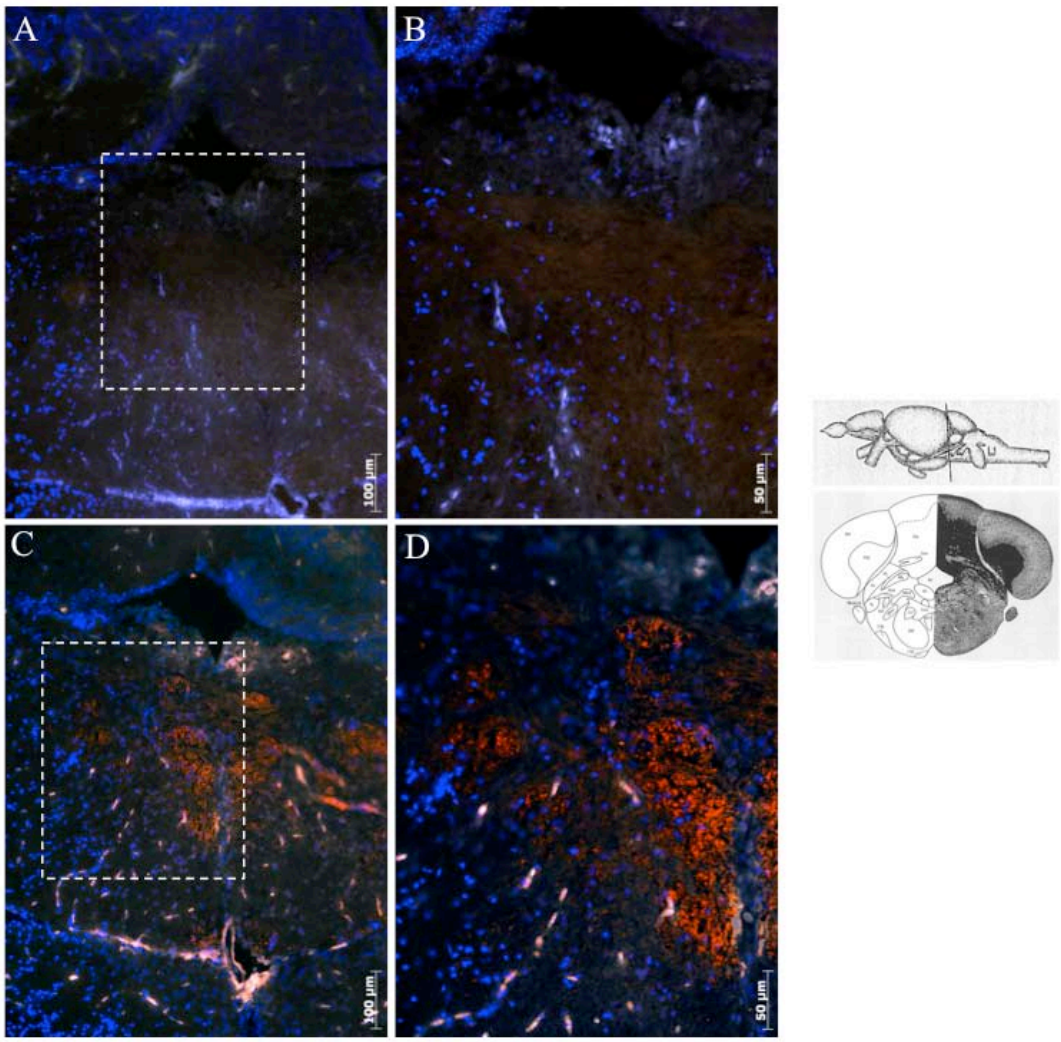


Figure 6. Neurocan expression in the brain of injured adult zebrafish. A and C are adjacent coronal sections harvested 3 days after acute brain injury. Images were taken at 10X. DAPI is shown in blue, and 1F6 in red. Nonspecific staining, which can be seen on both red and green channels (data not shown), 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.

Months 7-9: Establish neurocan and L1 substrate coating procedures.

Zebrafish Neurocan Purification

To prepare the zebrafish neurocan, pCMV-Myc-NcanB construct secreted product as a substrate for in vitro culture experiments, we are finishing 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 are also purifying pCMV-Myc-NcanB construct secreted products from 293T cells as well as from whole brain zebrafish cells*

using an immuno-precipitation strategy. We feel that within the next month (July 2012), the combined efforts of the Myc column and immuno-precipitation methodologies using zebrafish brain and 293T cells will yield a neurocan product that is biochemically identical to the native zebrafish neurocan. We will 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. Finally, although we are a bit behind in this aim, at this time we feel that our efforts during this phase will yield useful data concerning the potential interactions between neurocan and L1 and the role they play in axonal regeneration seen in the zebrafish.

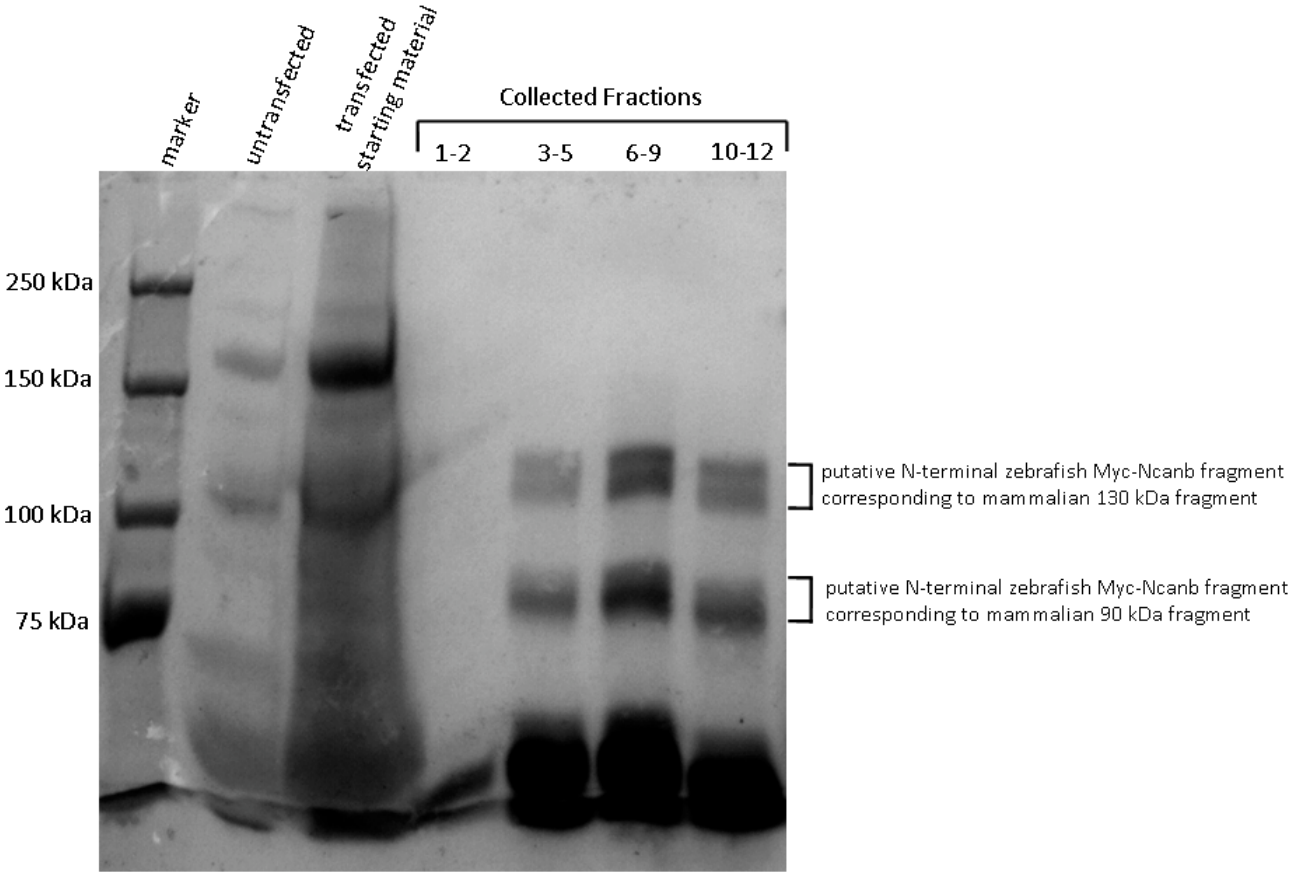


Figure 7. Western blot analyzing Myc-NcanB protein affinity column purification.
Samples were run on a 3% SDS-PAGE gel, transferred to a nitrocellulose membrane, and labeled with anti-c-Myc. Samples include media collected from control (not transfected) 293T cells, pCMV-Myc-NcanB transfected 293T cells, 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.

Months 10-12: Perform control vector transfections and establish transfection conditions.

Zebrafish Neurocan Purification

During this review 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).

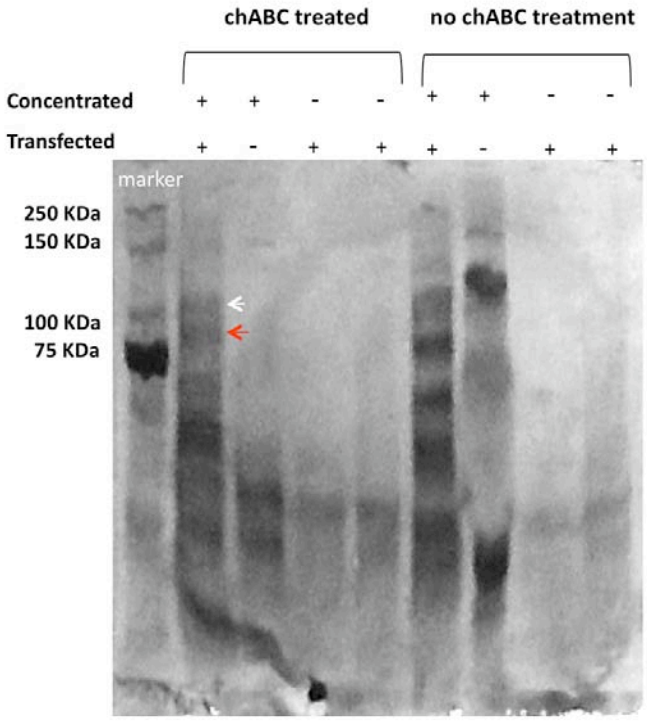


Figure 8. Western blot analyzing Myc-NcanB protein purification.
Samples were run on a 6% SDS-PAGE gel, transferred to nitrocellulose membrane and labeled with anti-c-Myc. Samples include concentrated and not concentrated media collected from control (not transfected) 293T cells and pCMV-Myc-NcanB transfected 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 is 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. We are currently, quantifying our data. This will allow us to calculate the percent of neurons repelled versus not repelled by the Myc-Ncan substrate. It will also us to determine how different concentrations of the Myc-NcanB substrate affect neuronal response. We hypothesize that as the concentration of Myc-NcanB decreases, the substrate will be more permissive for growth and the percent of neurons inhibited by Myc-NcanB will decrease. In addition, quantification will reveal whether chABC treatment, and the resulting loss of GAG chains, affects the response of neurons to Myc-NcanB. We hypothesize that chABC treatment will result in less Myc-NcanB inhibition, and consequently, more cells will cross into and grown on the Myc-NcanB spots.

Once we have finished our quantification studies and determined an optimal concentration of Myc-NcanB for use as a substrate, we will begin to conduct experiments to determine axon growth from cultured adultz zebrafish brainstem neurons with increased L1.1 levels over a zebrafish neurocan substrate.

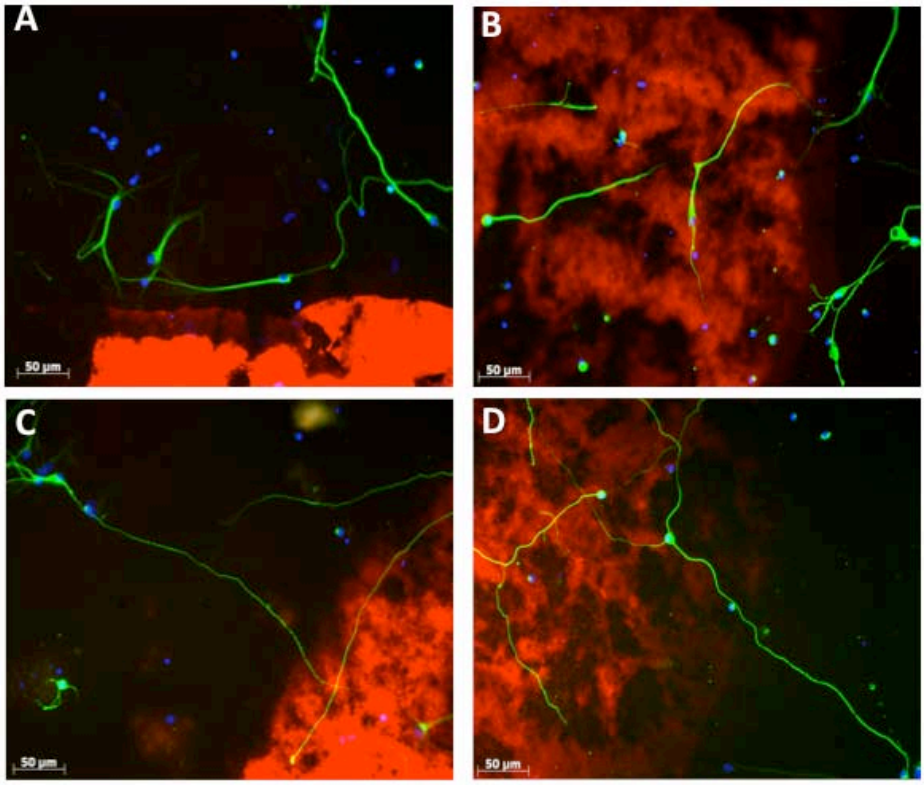


Figure 9. Neuronal response to purified Myc-NcanB.
Adult zebrafish brainstem neurons cultured for 7 days in complete media. Neurons labeled with anti-tubulin (green) and nuclei labeled with DAPI (blue). Rhodamine (red) is used to label Myc-NcanB or control spots (prepared from untransfected cells). (A) 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.

SOW: Plunkett Lab

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:
<u>Specific Aim 2.</u>

Months 1-3: Establish spinal cord transection techniques in Plunkett Lab.
Establishment of adult zebrafish spinal cord transection technique
Adult zebrafish are deeply anesthetized using 0.033% aminobenzoic acid ethylmethylester (MS222; Sigma). An incision is made approximately 3mm rostral to the dorsal fin at the level of the spinal vertebra. A finely sharpened set of forceps is used to completely transect the spinal cord ***(Figure 10)****. During this period of review, we have established this technique with a success rate of over 70% survival. We are currently delivering DiI membrane tracers through the use of application of Gelfoam placed within the transection site at the time of injury* ***(Figure 11)****. This technique will allow us to not only establish the surgical technique but also allow us to trace descending brainstem cells present within the spinal at the time of injury. Future experiments within the next review period will involve a small piece of Gelfoam containing either PTPσ morpholino or control morpholino will be placed in the transection site.*

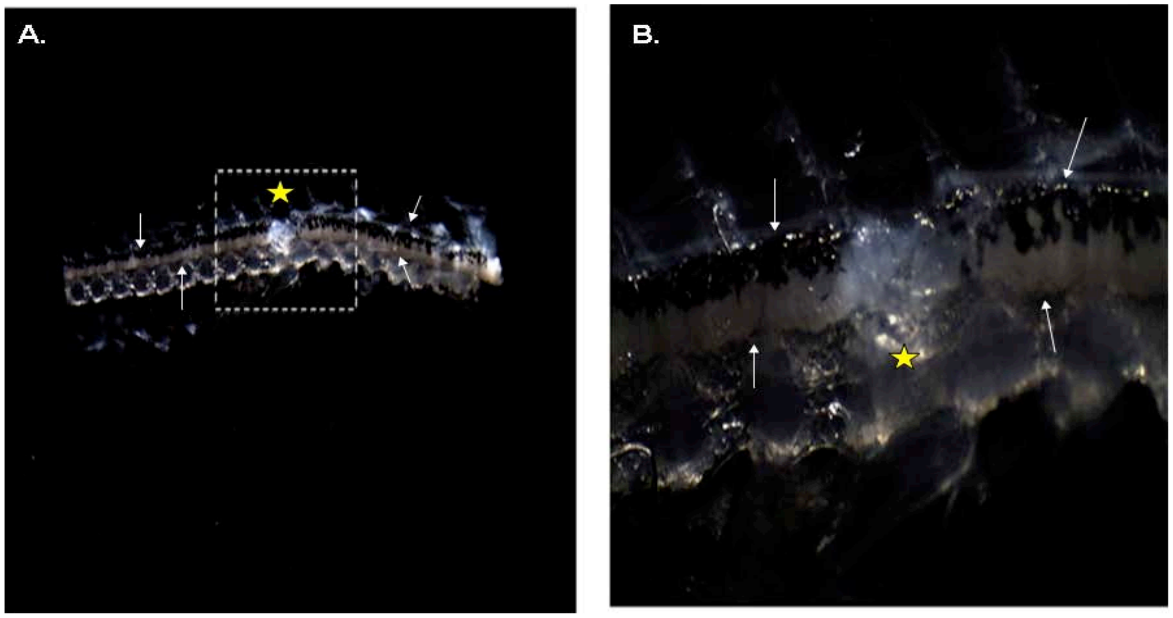


Figure 10. Zebrafish Spinal cord transection. Isolated spinal column of transected zebrafish spinal cord (rostral to left), panel A. Arrows demark CNS spinal tissue. Yellow star indicates area of injury and dashed box of injury area is magnified in panel B. Micrographs were taken two weeks post-injury.

Months 4-6: Establish techniques for delivery of control and PTPσ morpholino to cord.

Establishment of adult zebrafish spinal cord transection technique

We are currently delivering DiI membrane tracers through the use of application of Gelfoam placed within the transection site at the time of injury ***(Figure 5)****. This technique will allow us to not only establish the surgical technique but also allow us to trace descending brainstem cells present within the spinal at the time of injury. Future experiments within the next review period will involve a small piece of Gelfoam containing either PTPσ morpholino or control morpholino will be placed in the transection site.*

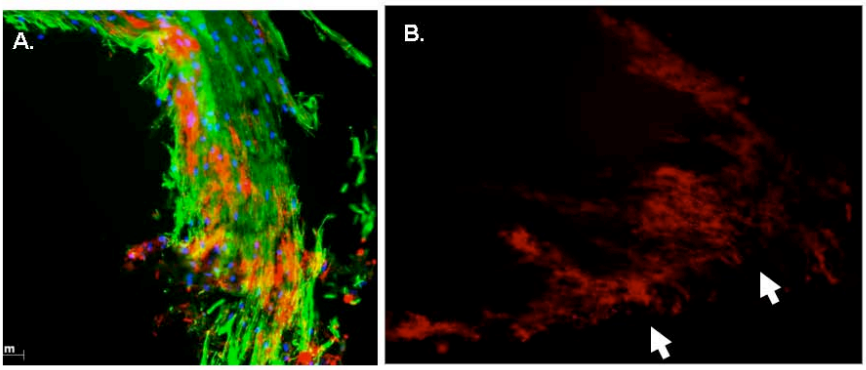


Figure 11. DiI delivery via Gelfoam into zebrafish spinal cord injury site. Fluorescent micrographs of DiI delivered to spinal cord following transection (panels A and B). Panels represent two independent experiments and the caudal border of transected cord is indicated by arrows in panel B.

Months 7-9: Analyze efficacy of morpholino delivery to brainstem neurons.

***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 adult zebrafish spinal cord transection technique done in previous reports

Establishment of molecule and PTPσ antisense morpholino delivery to transected axons

Tracing studies to determine delivery efficacy of molecules, including targeted morpholinos, to transected axons are currently underway. We have employed DiI strategies (previous progress report) with limited success and are currently employing fluoro-ruby (Oudega Lab reference) to trace brainstem neurons in adult zebrafish with transected spinal cord. We are currently analyzing brain sections from fluoro-ruby traced animals. Once establishing delivery

efficacy, experiments within the next review period will involve a small piece of Gelfoam containing either PTPσ morpholino or control morpholino will be placed in the transection site.

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 will be performed. We have 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.*

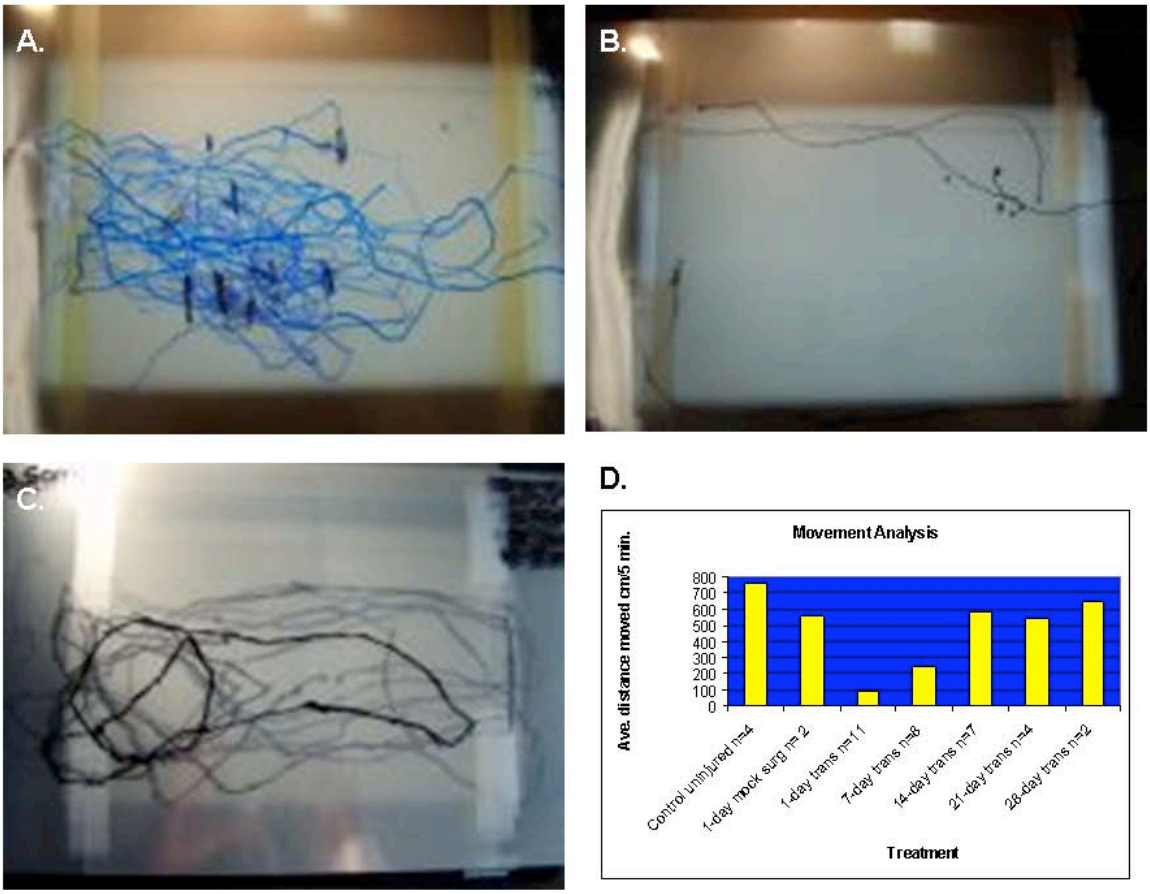


Figure 12. Functional recovery following complete spinal cord transection. Tracing of unstimulated swimming movement over 5 minute period (panels A-C). Uninjured "control" animal (A). One day post-injury (B), and 28 days post-injury (C). Graphic analysis of average movements (D).

Months 10-12: Perform experiments and establish baselines for delivery.

Retrograde labeling of brainstem neurons after spinal cord transection

During this review period, we utilized fluoro-ruby to trace brainstem neurons, whose axons were transected by spinal cord injury.

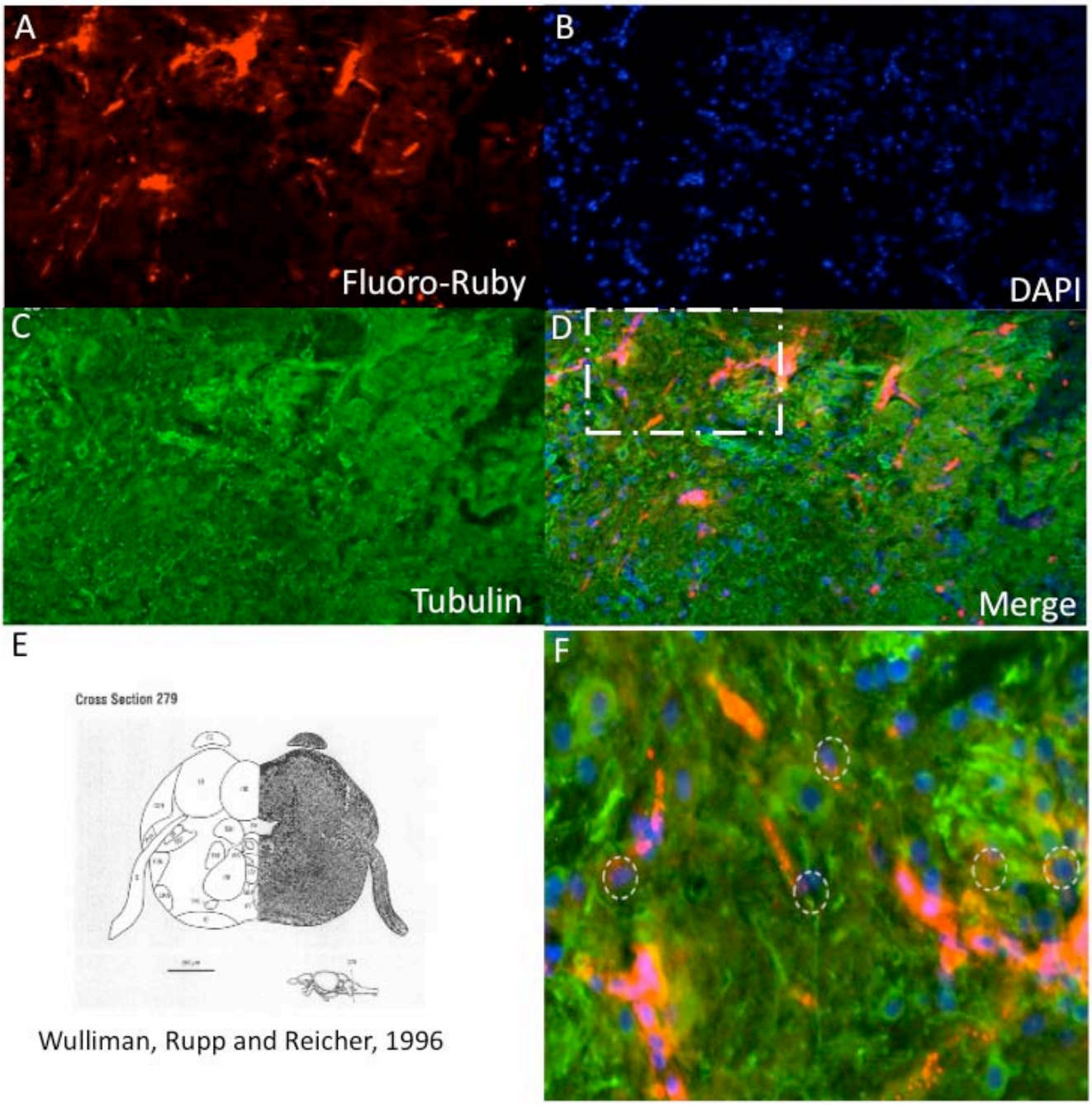


Figure 13. Brainstem neurons labeled with tracer during spinal cord injury.
Brainstem section harvested 1 day after spinal cord injury and fluoro-ruby labeling. (A-D) Each channel is shown individually at 20X and then merged into one image. Fluro-ruby tracer (red), DAPI labeled nuclei (blue), and anti-tubulin 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

To investigate the contribution of PTPσ to axon regeneration in vivo, we are planning to use a morpholino technique to reduce PTPσ levels in brainstem axons transected by spinal cord injury. Prior to using the morpholino in vivo, we have been characterizing 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.

During this review period, we continued to characterize the effects of the ptprs morpholino in vitro. 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. If the efficacy of ptp knock down is insufficient, we will immediately design a new set of morpholinos and conduct the experiment again.

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 utilize 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 (Bresciani et. al., 2010).

We are currently in the process of cloning the ptprsMO-GFP construct into a pCS2+ expression vector. During this review period, we PCR-amplified and purified the ptprsMO binding sequence fused in-frame to GFP cDNA with restriction enzyme sites at both the 5' and 3' end. We also purified the pCS2+ vector backbone. Ligation reactions are expected to produce the ptprsMO-GFP pCS2+ construct, which will then be transfected into zebrafish cells. Transfected cells will then be 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. This result would lead us to design and test a new morpholino to knock down ptprs protein expression. In contrast, GFP knock down would demonstrate the morpholino is capable of recognizing ptprs mRNA and blocking translation. This data would be used to interpret results from our in vitro and in vivo ptprsMO experiments.

SOW: Oudega Lab

<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.*

Milestones:

<u>Specific Aim 3.</u>

Months 1-3: Identify genes essential for brainstem axon growth after SCI.

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.

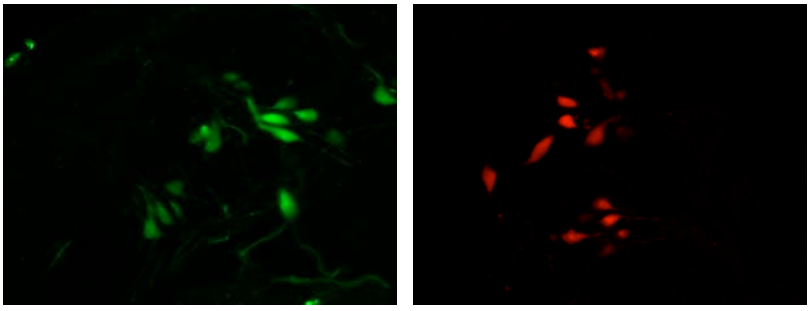


Figure 3. Fluoro-emerald labeled neurons in the adult zebrafish brainstem. The tracer was injected into the spinal cord caudal to the level where a transection would be made. Axons can internalize the tracer and transport it to the cell body. In the left panel, fluoro-emerald-positive axons as well as 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.

A report on the time-course of retrograde labeling of regenerated brainstem neurons is being prepared. Tracer was injected at different times (2-8 weeks) after a complete transection of the spinal cord. Tissue is being processed and labeled neurons quantified to reveal the optimal time for tracer injection. Also, immunostaining was performed for serotonin, tyrosine hydroxylase and glutamate to further characterize the neurons that regenerate and those that

do not. The manuscript is getting close to be finished and submitted but priority goes to labeling and harvesting brainstem neurons for RNA isolation.

Months 4-6: Analyze and finalize microarray and computer 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.

A report on the time-course of retrograde labeling of regenerated brainstem neurons is prepared and currently in pre-review. Tracer was injected at different times (2-8 weeks) after a complete transection of the spinal cord. Tissue is being processed and labeled neurons quantified to reveal the optimal time for tracer injection. Also, immunostaining was performed for serotonin, tyrosine hydroxylase and glutamate to Further characterize the neurons that regenerate and those that do not. 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. 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. The manuscript is getting close to be finished and submitted but priority goes to labeling and harvesting brainstem neurons for RNA isolation.

Months 7-9: Perform knock-in experiments; identify or create transgenic zebrafish.

Fuoro-emerald and fluoro-ruby were used to trace brainstem neurons in adult zebrafish with transected spinal cord. 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.

A report on the time-course of retrograde labeling of regenerated brainstem neurons is prepared and currently in pre-review. Tracer was injected at different times (2-8 weeks) after a complete transection of the spinal cord. Tissue is being processed and labeled neurons quantified to reveal the optimal time for tracer injection. Also, immunostaining was performed for serotonin, tyrosine hydroxylase and glutamate to Further characterize the neurons that regenerate and those that do not. 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. 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. The manuscript is getting close to being finished and submitted but priority goes to labeling and harvesting brainstem neurons for RNA isolation.

Will awaiting the survival of the fish and the collection of the traced neurons, which will take some time due to the required number of fish, we have tested our procedures to isolate high quality RNA. A number of different protocols were tested as these have all different effects on neurons from different species. Mostly rat-protocols were used as there are no standard protocols for zebrafish at present. Some variations of an acquired zebrafish protocol were also tested. We have now identified a protocol that yields high quality RNA from zebrafish neurons.

Months 10-12: Characterize transgenes and functional behavior.

We continue to back-label neurons that do and that do not regenerate across the transection site. The RNA will be purified for which we have now established reliable techniques. We will need 1500 labeled fish to obtain around 50000 brainstem neurons per population. This will yield enough RNA for microarray analysis.

Key Research Accomplishments Specific Aims 1 and 2, Plunkett Lab:

Specific Aim 1:

1. Purified Myc-tagged zebrafish neurocan B (Myc-NcanB) protein.

2. Utilized purified Myc-NcanB protein as a substrate for adult zebrafish brainstem neurons.

3. Determined that adult zebrafish brainstem neurons exhibit different responses to purified Myc-NcanB. Some neuronal populations are inhibited by the protein, while others are able to cross into or grow on Myc-NcanB areas.

Specific Aim 2

1. Spinal cord transection techniques were established in the Plunkett lab.

2. Retrograde neuronal tracing techniques were established in the Plunkett lab to label brainstem neurons transected by spinal cord injury.

3. A PTPσ morpholino was tested *in vitro* to determine how it affects the response of adult zebrafish brainstem neurons to CSPGs.

4. We have begun to prepare a construct, which contains the ptprs morpholino binding sequence upstream of GFP (ptprsMO-GFP pCS2+). Its purpose is to test the efficacy of ptprs knockdown.

Key Research Accomplishments in Specific Aim 3, Oudega Lab :
Specific Aim 3

1. Reliable retrograde tracing techniques have been established.

2. Fish are being traced, survived, and cells are being collected using FACS.

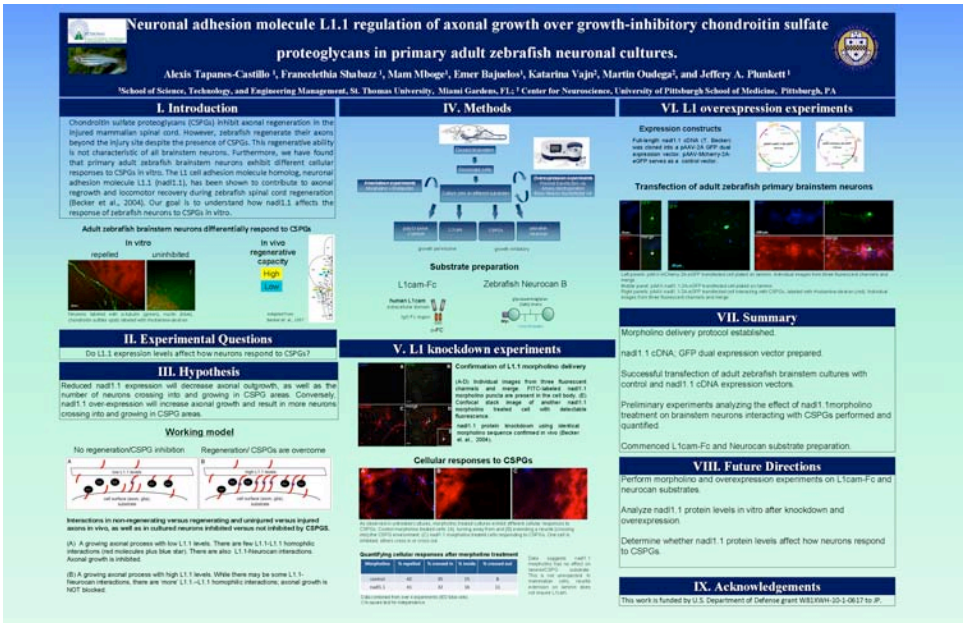
3. Reliable and effective RNA purification techniques have been established.

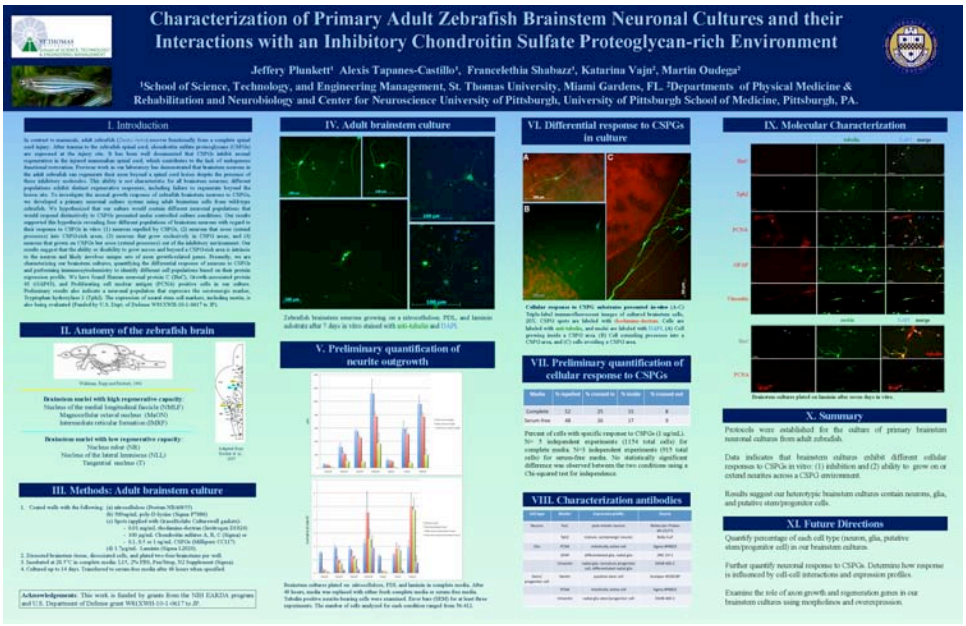
Reportable Outcomes:

Meeting Abstracts:
See Appendix below

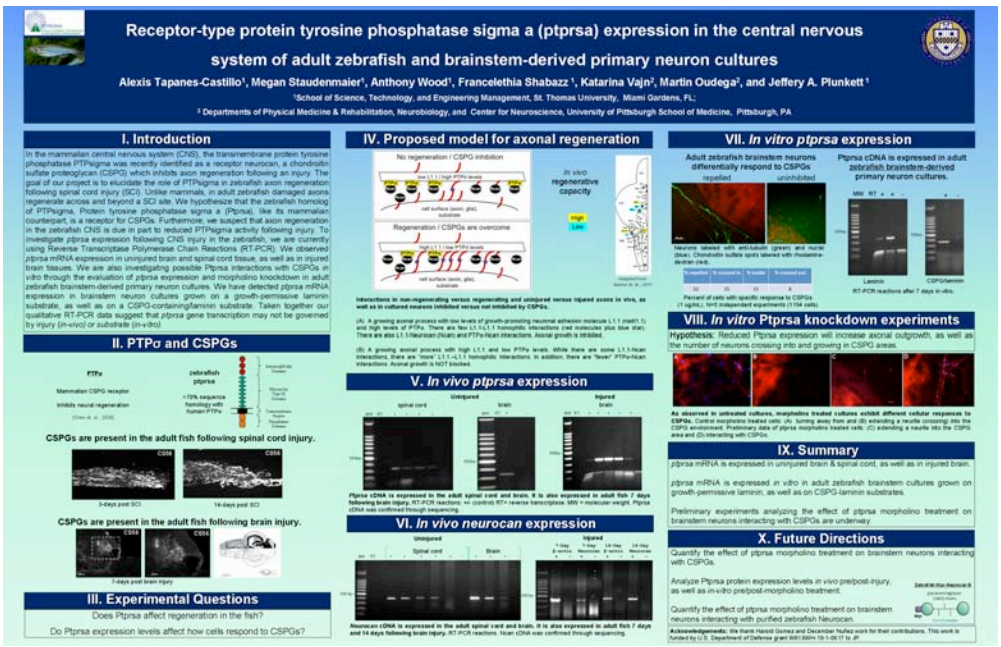
Other Notable Successes:

*- Posters presented at the **Society for Neuroscience Meeting in Washington, D.C. in November 2011**. Three posters were presented from the **Plunkett Lab** and the **Oudega Lab**.*



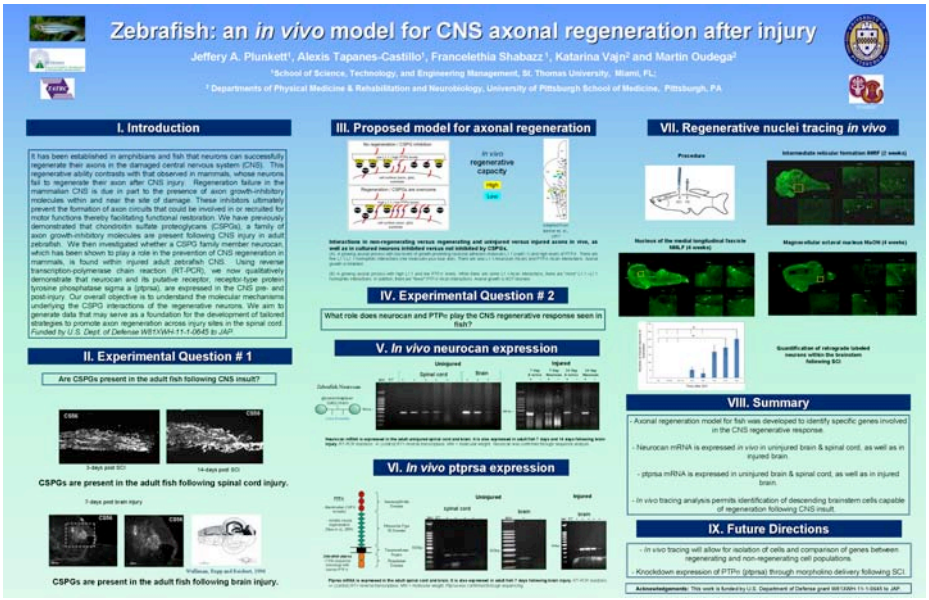


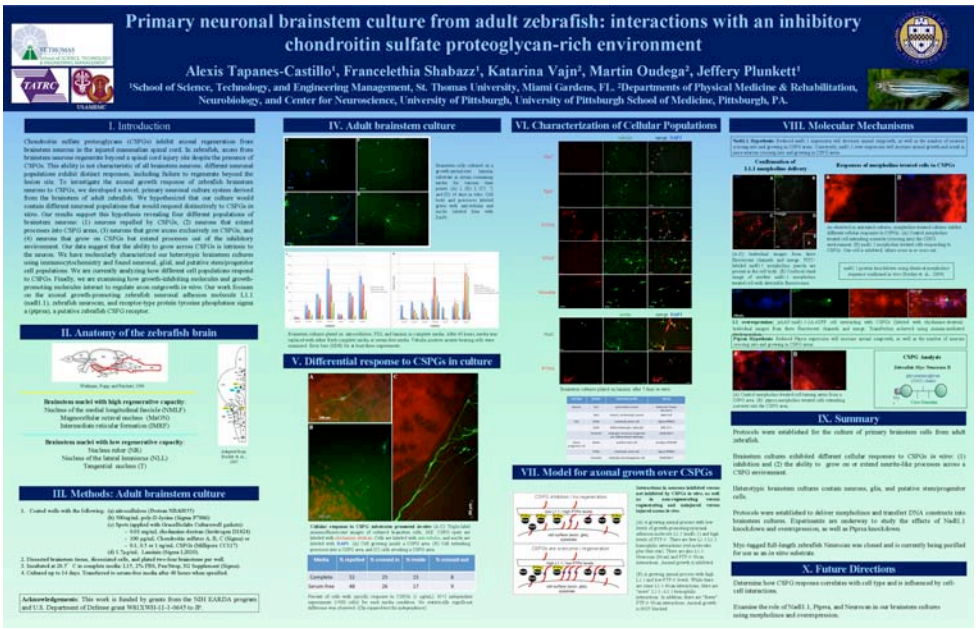
One poster was presented from the Plunkett Lab at the American Society for Cell Biology in Denver, CO (Dec. 2011).



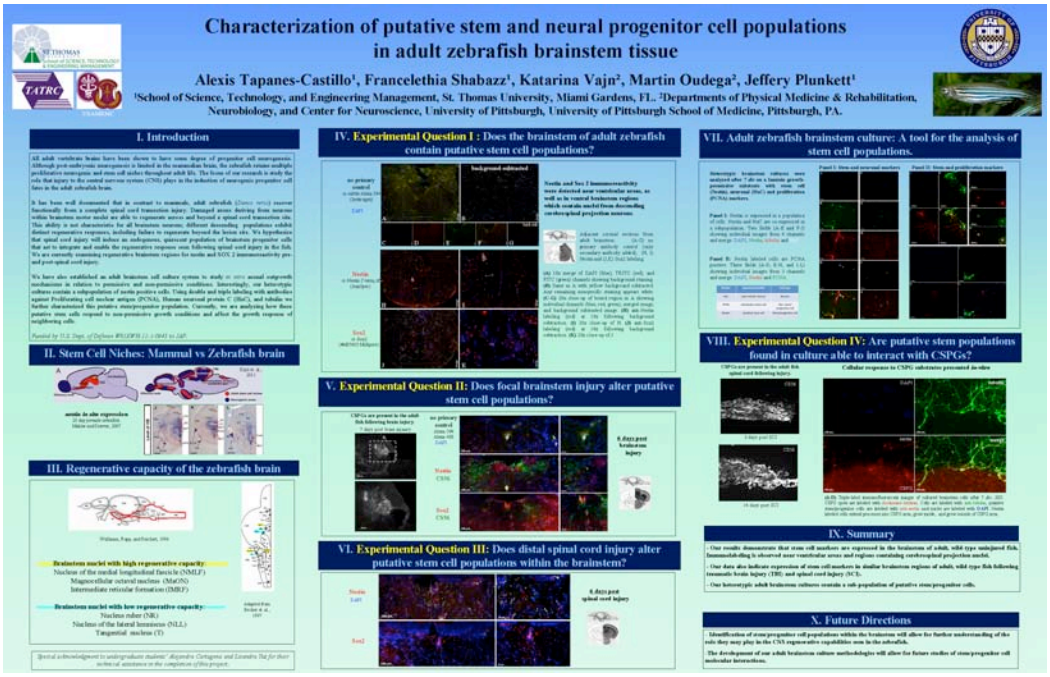
***Dr. Alexis Tapanes-Castillo** from the **Plunkett Lab** was awarded a Minority Affairs Committee (MAC) Travel Fellowship to travel to the American Society for Cell Biology Meeting in Denver, December 2011. She won an award at the MAC poster competition for an outstanding performance.*

Two posters from the Plunkett Lab were presented at the Winter Conference on Brain Research in Salt Lake City, UT (Jan. 2012)





One poster and a poster teaser talk from the Plunkett Lab were presented at the Keystone Symposia The Life of a Stem Cell: From Birth to Death in Olympic Valley, CA (March 2012).



- ***Dr. Tapanes-Castillo*** *from the* ***Plunkett Lab*** *was awarded a Keystone Symposia Travel Fellowship to attend the Keystone Symposia Life of Stem Cell Meeting in Olympic Valley, CA.*

- *Students from the* ***Plunkett Laboratory*** *presented five posters at the* ***4th Annual STEM Symposium*** *held at* ***Barry University in Miami, Florida on April 4th, 2012****. Students from the Plunkett Laboratory won 2 individual awards for their presentations.*

- *Students from the* ***Plunkett Laboratory*** *presented five posters and two oral presentations at the* ***7th Annual Southeast Cell Science Undergraduate Research Symposium*** *held April 16th, 2012 in Miami, Florida.*

Zebrafish: an *in vivo* model for CNS axonal regeneration after injury

Francelethia Shabazz[1], Katarina Vajn[2], Alexis Tapanes-Castillo[1] and Martin Oudega[2], Jeffery A. Plunkett[1]

[1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami, FL;

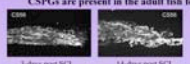
[2] Departments of Physical Medicine & Rehabilitation and Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA

I. Introduction

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury. It has been well documented that chondroitin sulfate proteoglycans (CSPGs), a family of axon growth inhibitory molecules, contribute to the lack of functional restoration in the injured mammalian spinal cord. We recently demonstrated that CSPGs are present following CNS injury in adult zebrafish. Data from reverse transcription-polymerase chain reaction (RT-PCR) experiments show that the CSPG family member neurocan and its putative receptor, receptor-type protein tyrosine phosphatase sigma a (ptprsa), are expressed in the CNS pre- and post-injury. Previous work has also demonstrated that brainstem neurons in the adult zebrafish can regenerate their axon beyond a spinal cord lesion despite the presence of these inhibitory molecules. This ability is not characteristic for all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site. We are currently utilizing our in vivo model to identify specific genes involved in the CNS regeneration response and to determine how axonal regeneration occurs despite the presence of CSPGs. We have established an in vivo tracing protocol that permits identification of descending brainstem cells capable of regeneration following CNS insult. This technique allows isolation of individual cells and comparison of genes between regenerating and non-regenerating cell populations. We aim to understand the molecular mechanisms underlying the CSPG interactions of regenerative neurons. These data may serve as a foundation for the development of tailored strategies to promote axon regeneration across injury sites in the mammalian spinal cord. Funded by U.S. Dept. of Defense W81XWH-11-1-0645 to JAP.

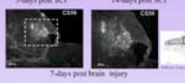
II.Background on CSPGs

CSPGs are present in the adult fish following CNS insult.



3-days post SCI 14-days post SCI

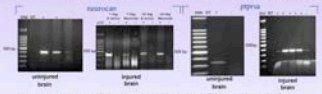
CSPGs are present in the spinal cord of adult fish following spinal cord injury.



7-days post brain injury

CSPGs are present in the brain of adult fish following brain injury.

The CSPG neuron and its putative receptor ptprsa are expressed in vivo



Neurocan and ptprsa mRNA are expressed in the adult fish pre and post-brain injury. RT-PCR reactions: +/- (control) RT+ reverse transcriptase, MW = molecular weight. Neurocan and ptprsa was confirmed through sequencing.

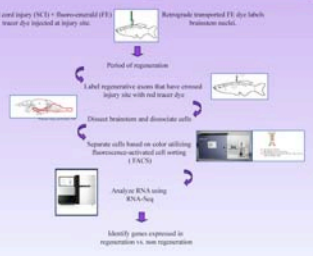
II. Zebrafish brainstem nuclei differ in their regenerative capacity

III.Experimental Question

How do fish cerebrospinal neurons regenerate after injury and overcome CSPG inhibition?

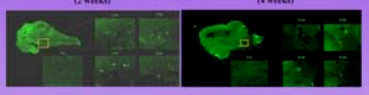
Hypothesis: Regenerating neurons express a specific program of regeneration associated genes.

IV. Experimental approach to identify regeneration associated genes

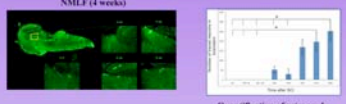


V. Regenerative nuclei tracing *in vivo*

Intermediate reticular formation IMRF (2 weeks) **Magnocellular octaval nucleus MaON (4 weeks)**



Nucleus of the medial longitudinal fascicle NMLF (4 weeks)



Quantification of retrograde labeled neurons within the brainstem following SCI

VI. Summary

- Axonal regeneration model for fish was developed to identify specific genes involved in the CNS regenerative response.

-*In vivo* tracing analysis permits identification of descending brainstem cells capable of regeneration following CNS insult.

-RNA purification techniques optimized.

VII. Future Directions

- *In vivo* tracing will allow for the isolation of labeled brainstem nuclei and the comparison of gene expression between regenerating and non-regenerating cell populations..

Acknowledgements: This work is funded by U.S. Department of Defense grant W81XWH-11-1-0645 to JAP.

Neurocan expression in the CNS of adult zebrafish and its effect on axonal growth of brainstem-derived primary cultures

Harold Gomez[1], Arjena Valls[1], Alexis Tapanes-Castillo[1], Francelethia Shabazz[1], Katarina Vajn[2], Martin Oudega[2], Jeffery Plunkett[1]

[1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL.

[2]Departments of Physical Medicine & Rehabilitation, Neurobiology, and Center for Neuroscience, University of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

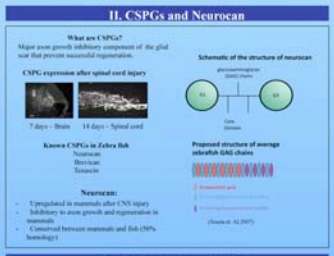
It has been established in amphibians and fish that neurons can successfully regenerate their axons in the damaged central nervous system (CNS). This regenerative ability contrasts with that observed in mammals, whose neurons fail to regenerate their axon after CNS injury. Regeneration failure in the mammalian CNS is due in part to the presence of axon growth-inhibitory molecules within and near the site of damage. These inhibitors ultimately prevent the formation of axon circuits that could be involved in or recruited for motor functions thereby facilitating functional restoration.

We have previously demonstrated that chondroitin sulfate proteoglycans (CSPGs), a family of axon growth-inhibitory molecules are present following CNS injury in adult zebrafish. We then investigated whether a CSPG family member neurocan, which has been shown to play a role in the prevention of CNS regeneration in mammals, is found within injured adult zebrafish CNS. Using reverse transcription-polymerase chain reaction (RT-PCR), we now qualitatively demonstrate that neurocan is expressed in the CNS pre- and post-injury.

We are currently examining neurocan protein expression utilizing Western blot analysis and immunohistochemistry pre- and post-injury. In addition, we have cloned a Myc-tagged full length zebrafish neurocan b construct. We are presently isolating and characterizing the protein for future use in our adult zebrafish brainstem culture system. We hypothesize that zebrafish neurocan b is a growth-inhibitory molecule.

Taken together, the overall objective of this project is to understand the molecular mechanisms underlying the CSPG interactions of the regenerative neurons.

II. CSPGs and Neurocan



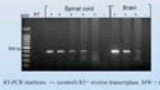
III. Experimental Questions

Is neurocan expressed pre- and post-injury in the adult zebrafish?

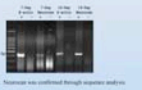
Is zebrafish neurocan inhibitory to axonal growth?

IV. *In vivo* neurocan expression

Neurocan mRNA is expressed in the uninjured spinal cord and brain of adult fish.

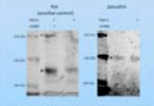


Neurocan is also expressed 7 days and 14 days following brain injury.



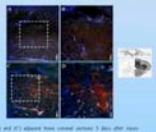
RT-PCR reactions: +/- (control) RT+ reverse transcriptase. MW = molecular weight. Neurocan was confirmed through sequence analysis

Western blot data suggest neurocan protein is expressed in the adult fish brain 3 days after injury.



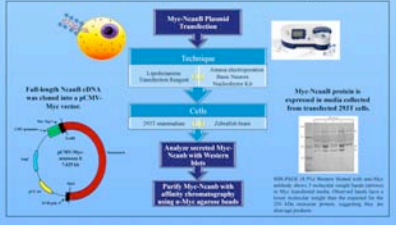
(Left) Rat spinal cord tissue after spinal cord injury (+) served as a positive control for 150 immunoreactivity. 150 recognized intact neurocan (gray arrow) and a 150kDa N-terminal processed neurocan fragment (black arrow) in 293MC treated samples (+). (Right) Brain tissues from adult fish 3 days after brain injury (+) and untreated fish show (-) 150 kDa band that is not observed in a control blot without primary antibody (data not shown).

Immunohistochemistry shows neurocan labeling 3 days post-injury in adult zebrafish brain.



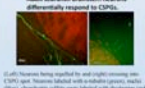
(A) and (C) adjacent brain coronal sections 3 days after injury (150). Neurocan protein is labeled with 150 antibody (red) and the nuclei is labeled with DAPI (blue). Neuropathy staining is white following back-to-back sections. (B) and (D) 20x close-ups of the boxed regions in A and C respectively. cb.Bc No primary antibody control. IC-IM IFG labeling.

V. Neurocan protein purification strategy



VI. Neurocan as an *in vitro* substrate

Adult zebrafish brainstem neurons differentially respond to CSPGs.



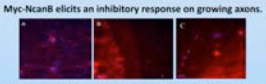
(Left) Neurons being repelled by and (right) crossing into CSPG spot. Neurons labeled with a-tubulin (green), nuclei (blue), chondroitin sulfate spots labeled with rhodamine (red).

Quantification of different responses to CSPGs.

Media	% repelled	% crossed in	% inside	% crossed out
Complete	52	25	15	8
Serum-free	48	26	17	9

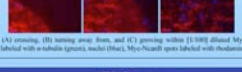
N=3 independent experiments (>900 cells) for each media condition. No statistically significant difference was observed (Chi-squared test for independence).

Myc-NcanB elicits an inhibitory response on growing axons.



(A) Control treated cells . (B) Neurons turning away from concentrated Myc-NcanB spot, panels B and C. Neurons labeled with a-tubulin (red), nuclei (blue), Myc-NcanB spots labeled with rhodamine (red).

Response to Myc-NcanB appears dose-dependent.



Neurons (A) crossing, (B) turning away from, and (C) growing within [1:500] diluted Myc-NcanB spots. Neurons labeled with a-tubulin (green), nuclei (blue), Myc-NcanB spots labeled with rhodamine (red).

IX. Summary

- Neurocan mRNA is expressed in uninjured spinal cord and brain, as well as in injured brain.
- Preliminary data suggest that neurocan protein is expressed 3-days after injury.
- Myc-NcanB was transfected into 293T mammalian cells as well as into fish cells.
- Media collected from cells transfected with Myc-NcanB was tested *in vitro*.

X. Future Directions

- Confirm *in vivo* protein expression pre- and post-injury with Western blot and immunohistochemistry.
- Purify Myc-NcanB with affinity chromatography.
- Analyze how purified Myc-NcanB acts as an *in vitro* substrate.

This work is funded by U.S. Dept. of Defense W81XWH-11-1-0645 to JAP. We would like to thank dr. Yaree Lemmon (UM: Miller School of Medicine – Miami Project to Cure paralysis) for the use of the Amaxa electroporator. Special acknowledgements to Alejandra Carnagena, Lisandra Sal, Anthony Woods, and Megan Stoukidemakos

Primary neuronal brainstem culture from adult zebrafish: interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment

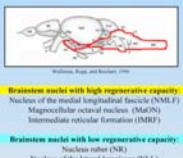
Jossias Genao[1], Isaac Chacon[1], Francelethia Shabazz[1], Alexis Tapanes-Castillo[1], Katarina Vajn[2], Martin Oudega[2], Jeffery Plunkett[1]

[1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL. [2]Departments of Physical Medicine & Rehabilitation, Neurobiology, and Center for Neuroscience, University of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

Chondroitin sulfate proteoglycans (CSPGs) inhibit axonal regeneration from brainstem neurons in the injured mammalian spinal cord. In zebrafish, axons from brainstem neurons regenerate beyond a spinal cord injury site despite the presence of CSPGs. This ability is not characteristic of all brainstem neurons; different neuronal populations exhibit distinct responses, including failure to regenerate beyond the lesion site. To investigate the axonal growth response of zebrafish brainstem neurons to CSPGs, we developed a novel, primary neuronal culture system derived from the brainstem of adult zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs in vitro. Our results support this hypothesis revealing four different populations of brainstem neurons: (1) neurons repelled by CSPGs, (2) neurons that extend processes into CSPG areas, (3) neurons that grow axons exclusively on CSPGs, and (4) neurons that grow on CSPGs but extend processes out of the inhibitory environment. Our data suggest that the ability to grow across CSPGs is intrinsic to the neuron. We have molecularly characterized our heterotypic brainstem cultures using immunocytochemistry and found neuronal, glial, and putative stem/progenitor cell populations. We are currently analyzing how different cell populations respond to CSPGs. Finally, we are examining how growth-inhibiting CSPGs and the axonal growth-promoting zebrafish neuronal adhesion molecule L1.1 (nadl1.1) interact to regulate axon outgrowth in vitro.

II. Anatomy of the zebrafish brain



Brainstem nuclei with high regenerative capacity:
Nucleus of the medial longitudinal fascicle (NMLF)
Magnocellular octaval nucleus (MaON)
Intermediate reticular formation (IMRF)

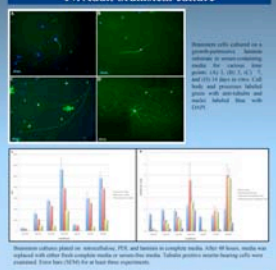
Brainstem nuclei with low regenerative capacity:
Nucleus ruber (NR)
Nucleus of the lateral lemniscus (NLL)
Tangential nucleus (T)

III. Methods: Adult brainstem culture

1. Coated wells with the following: (a) nitrocellulose (Protran NBA0855)
   (b) 500 µg/ml. poly-D-lysine (Sigma P7886)
   (c) Spots (applied with GraceBiolabs Culturewell gaskets):
   - 0.01 mg/ml. rhodamine-dextran (Invitrogen D1824)
   - 100 µg/ml. Chondroitin sulfate A, B, C (Sigma) or
   - 0.1, 0.5 or 1 ug/ml. CSPGs (Millipore CC117)
   (d) 1.7µg/ml. Laminin (Sigma L2020).
2. Dissected brainstem tissue, dissociated cells, and plated two-four brainstems per well.
3. Incubated at 28.5°C in complete media: L15, 2% FBS, Pen/Strep, N2 Supplement (Sigma).
4. Cultured up to 14 days. Transferred to serum-free media after 48 hours when specified.

Acknowledgements: This work is funded by U.S. Department of Defense grant W81XWH-11-1-0645 to JP . We would like to thank Mam Mbogo and Emer Bajaelos for their work on the nadl1.1 project.

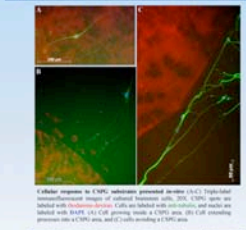
IV. Adult brainstem culture



Brainstem cells cultured on a growth-permissive (…laminin substrate in serum-containing media for various time points: (A) 1, (B) 3, (C) 7, and (D) 14 days in vitro. Cell body and processes labeled green with antitubulin and nuclei labeled blue with DAPI.

Brainstem cultures plated on: mitrocellulose, PDL and laminin in complete media. After 48 hours, media was replaced with either fresh complete media or serum-free media. Tubulin positive neurite-bearing cells were examined. Error bars (SEM) for at least three experiments.

V. Differential response to CSPGs in culture

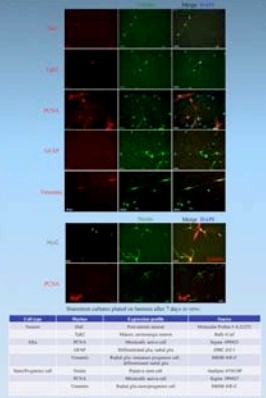


Cellular response to CSPG substrates presented in-vitro (A-C) Triple-label immunofluorescent images of cultured brainstem cells. 20X. CSPG spots are labeled with rhodamine-dextran. Cells are labeled with anti-tubulin, and nuclei are labeled with DAPI. (A) Cell growing inside a CSPG area. (B) Cell extending processes into a CSPG area, and (C) cells avoiding a CSPG area.

Media	% Repelled	% Crossed in	% Inside	% Crossed out
Complete	52	25	15	8
Serum-Free	68	20	12	0

Percent of cells with specific response to CSPGs (1 ug/mL). N=3 independent experiments (>900 cells) for each media condition. No statistically significant difference was observed. (Chi-squared test for independence).

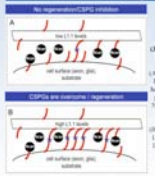
VI. Characterization of Cellular Populations



Brainstem cultures plated on laminin after 7 days in vitro.

Cell type	Marker	Expression profile	Source
Neuron	Hud	Post-mitotic neuron	Monoclonal Molecular Probes # A-21271
	Tuj1	Mature, microtubule neuron	Rabbit #Cat#
Glia	PCNA	Mitotically active cell	Sigma #P8825
	GFAP	Differentiated glia, radial glia	#Ref. #
	Vimentin	Radial glia, immature progenitor cell, differentiated radial glia	SMI-86 #Ab #
Stem/Progenitor cell	Nestin	Putative stem cell	Analysis #1916-M
	PCNA	Mitotically active cell	Sigma #P8825
	Vimentin	Radial glia,stem/progenitor cell	SMI-86 #Ab #

VII. Model for axonal growth over CSPGs

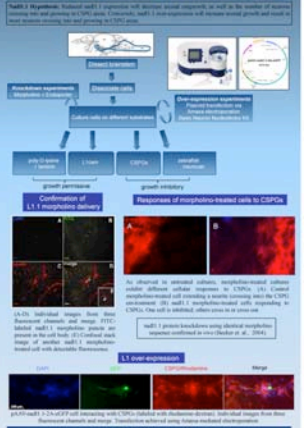


Interactions in (1) non-regenerating versus regenerating axons; (2) uninjured versus injured axons in vivo; and (3) cultured neurons inhibited versus not inhibited by CSPGS.

(A) A growing axonal process with low L1.1 levels. There are few L1.1-L1.1 homophilic interactions (red molecules plus blue star). There are also L1.1-Neurocan (Ncan) and PTPσ-Ncan interactions. Axonal growth is inhibited.

(B) A growing axonal process with high L1.1 levels. While there are some L1.1-Ncan interactions, there are "more" L1.1 –L1.1 homophilic interactions. In addition, there are "fewer" PTPσ-Ncan interactions. Axonal growth is NOT blocked.

VIII. Molecular Mechanisms



IX. Summary

- ✓Protocols were established for the culture of primary brainstem cells from adult zebrafish and for the morpholino delivery.
- ✓Brainstem cultures exhibited different cellular responses to CSPGs *in vitro*: (1) inhibition and (2) the ability to grow on or extend neurite-like processes across a CSPG environment.
- ✓Heterotypic brainstem cultures contain neurons, glia, and putative stem/progenitor cells.

X. Future Directions

- ❑ Determine how CSPG response correlates with cell type and is influenced by cell-cell interactions.
- ❑ Determine whether nadl1.1 protein levels affect how neurons respond to CSPGs.
- ❑ Perform nadl1.1 morpholino and ov-er-expression experiments on L1cam-Fc and Myc-Neurocan substrates.

Receptor-type protein tyrosine phosphatase sigma a (ptprsa) expression in the central nervous system of adult zebrafish and brainstem-derived primary neuron cultures

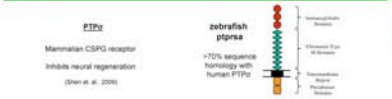
Anthony Wood[1], Megan Staudenmaier[1], December Nuñez[1], Isaac Chacon[1], Francelethia Shabazz[1], Alexis Tapanes-Castillo[1], Katarina Vajn[2], Martin Oudega[2], and Jeffery A. Plunkett[1]

[1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL;
[2] Departments of Physical Medicine & Rehabilitation, Neurobiology, and Center for Neuroscience, University of Pittsburgh School of Medicine, Pittsburgh, PA

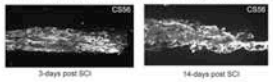
I. Introduction

In the mammalian central nervous system (CNS), the transmembrane protein tyrosine phosphatase PTPsigma was recently identified as a receptor for neurocan, a chondroitin sulfate proteoglycan (CSPG) which inhibits axon regeneration following spinal cord injury (SCI). Unlike mammals, in adult zebrafish damaged axons regenerate across and beyond a SCI site. We hypothesize that the zebrafish homolog of PTPsigma, Protein tyrosine phosphatase sigma a (Ptprsa), like its mammalian counterpart, is a receptor for CSPGs. Furthermore, we suspect that axon regeneration in the zebrafish CNS is due in part to reduced PTPsigma activity following injury. To investigate *ptprsa* expression following CNS injury in the zebrafish, we are currently using Reverse Trranscriptase Polymerase Chain Reaction (RT-PCR). We observed mRNA expression in unjured brain and spinal cord tissue, as well as injured brain tissues. We are also investigating possible Ptprsa interactions with CSPGs *in vitro* through the evaluation of *ptprsa* expression and morpholino knockdown in adult zebrafish brainstem-derived primary neuron cultures. We have detected *ptprsa* mRNA expression in brainstem neuron cultures grown on a growth-permissive laminin substrate, as well as on a CSPG-containing/laminin substrate. Taken together our qualitative RT-PCR data suggest that *ptprsa* gene transcription may not be governed by injury (*in vivo*) or substrate (*in vitro*).

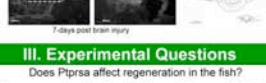
II. PTPσ and CSPGs



CSPGs are present in the adult fish following spinal cord injury.



CSPGs are present in the adult fish following brain injury.

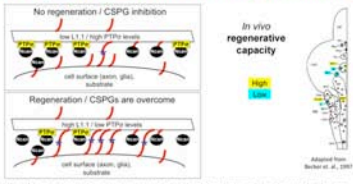


III. Experimental Questions

Does Ptprsa affect regeneration in the fish?

Do Ptprsa expression levels affect how cells respond to CSPGs?

IV. Proposed model for axonal regeneration

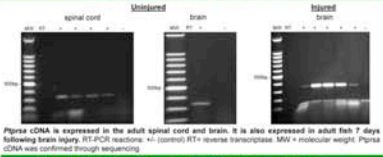


Interactions in non-regenerating versus regenerating and uninjured versus injured axons in vivo, as well as in cultured neurons inhibited versus not inhibited by CSPGs.

(A) A growing axonal process with low levels of growth-promoting neuronal adhesion molecule L1.1 (nadl1.1) and high levels of PTPσ. There are few L1.1-L1.1 homophilic interactions (red molecules plus blue star). There are also L1.1-Neurocan (Ncan) and PTPσ-Ncan interactions. Axonal growth is inhibited.

(B) A growing axonal process with high L1.1 and low PTPσ levels. While there are some L1.1-Ncan interactions, there are "more" L1.1 –L1.1 homophilic interactions. In addition, there are "fewer" PTPσ-Ncan interactions. Axonal growth is NOT blocked.

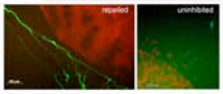
V. *In vivo ptprsa* expression



***Ptprsa* cDNA is expressed in the adult spinal cord and brain. It is also expressed in adult fish 7 days following brain injury.** RT-PCR reactions: +/- (control) RT= reverse transcriptase. MW = molecular weight. Ptprsa cDNA was confirmed through sequencing.

VI. Introduction to Brainstem Culture

Adult zebrafish brainstem neurons differentially respond to CSPGs



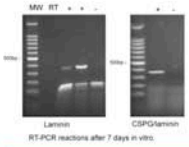
Neurons labeled with anti-tubulin (green) and nuclei (blue). Chondroitin sulfate spots labeled with rhodamine-dextran (red).

% Repelled	% Crossed In	% Inside	% Crossed Out
52	25	15	8

Percent of cells with specific response to CSPGs (1 ug/mL). N=5 independent experiments (1154 cells)

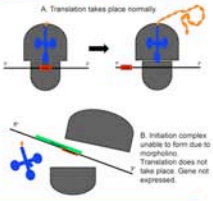
VII. *In vitro ptprsa* expression

Ptprsa cDNA is expressed in adult zebrafish brainstem-derived primary neuron cultures.



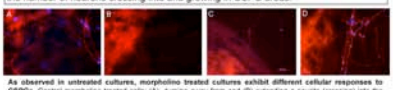
RT-PCR reactions after 7 days in vitro.

VIII. Knockdown mechanism



IX. *In vitro* Ptprsa knockdown experiments

Hypothesis: Reduced Ptprsa expression will increase axonal outgrowth, as well as the number of neurons crossing into and growing in CSPG areas.



As observed in untreated cultures, morpholino treated cultures exhibit different cellular responses to CSPGs. Control morpholino treated cells: (A) turning away from and (B) extending a neurite (crossing) into the CSPG environment. Preliminary data of ptprsa morpholino treated cells: (C) extending a neurite into the CSPG area and (D) interacting with CSPGs.

X. Summary

ptprsa mRNA is expressed in uninjured brain & spinal cord, as well as in injured brain.

ptprsa mRNA is expressed *in vitro* in adult zebrafish brainstem cultures grown on growth-permissive laminin, as well as on CSPG-laminin substrates.

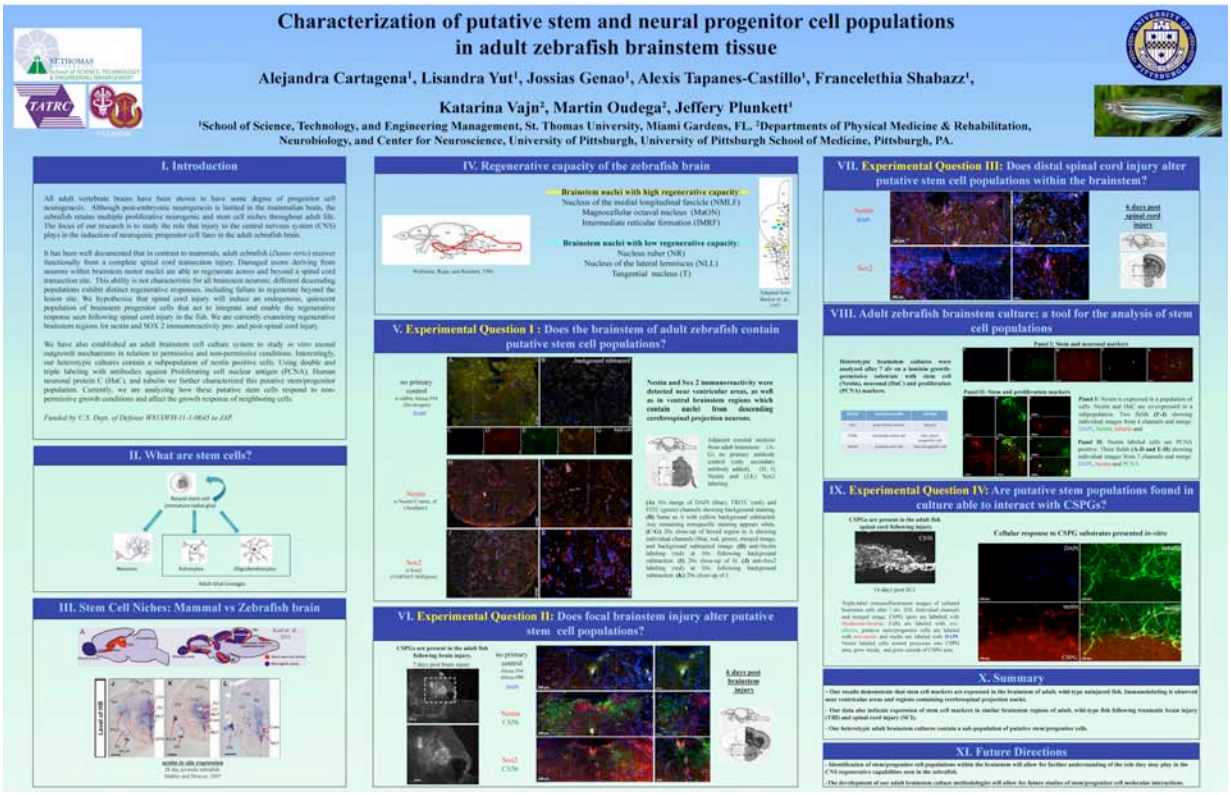
Preliminary experiments analyzing the effect of ptprsa morpholino treatment on brainstem neurons interacting with CSPGs are underway.

XI. Future Directions

Quantify the effect of ptprsa morpholino treatment on brainstem neurons interacting with CSPGs.

Analyze Ptprsa protein expression levels *in vivo* pre/post-injury, as well as *in-vitro* pre/post-morpholino treatment.

Acknowledgements: This work is funded by U.S. Department of Defense grant W81XWH-10-1-0617 to JP.



- ***Student Success: Plunkett Laboratory*** *student Francelethia Shabazz graduated with a BA from St. Thomas University Fall 2011.* ***Plunkett Laboratory students*** *Harold Gomez and Anthony Wood graduated from St. Thomas University in May 2012. Harold graduated with Honors and a BS in Biology. He completed his undergraduate thesis and scored a 32 on the MCAT. He plans to start medical school Fall 2013. Anthony Wood graduated with a BS in Biology and scored a 33 on the MCAT. He also plans to begin medical school Fall 2013.*

- ***SRI, Summer Research Institute at St. Thomas University*** *is a grant-funded program that affords students the opportunity to conduct summer research under the guidance of a St. Thomas professor. The program provides a paid internship for 8 weeks of laboratory research. The* ***Plunkett laboratory*** *hosted 5 St. Thomas and three Miami-Dade College students this past summer. The Plunkett lab also hosted a visiting student to the St. Thomas campus from Stevenson University in Maryland. The Plunkett lab students presented 6 posters and an oral presentation at the SRI Symposia on September 7th, 2012. Students from the Plunkett Laboratory won 3 individual awards for their presentations, including Best Talk and Best Poster.*

- *Three posters from the* ***Plunkett and Oudega Laboratories*** *will be presented the* ***Society for Neuroscience Meeting in New Orleans, LA in October 2012****.*

- A manuscript entitled: **Establishment and characterization of primary brainstem cultures from adult zebrafish brainstem: a novel *in vitro* model.** Tapanes-Castillo A, Shabazz F, M'boge M, Vajn K, Oudega M and Plunkett JA is in preparation. This manuscript is nearly complete and will be submitted October 2012.

Conclusion:

The different studies within this proposal (in vitro as well as in vivo) have been progressing reasonably well according to the described milestones. Some technical/experimental barriers were encountered and these needed to be overcome. This was accomplished for most of them and is still in progress for few. Considering our previous success with surmounting these roadblocks, we are confident that we will be successful. Thus, in conclusion, we are well on our way to accomplish the goals for this first year as they were described in our proposal. This implies that at the end of the 24 month period we will have 1. Analyzed in vitro the role of L1 and zebrafish neurocan in axon regeneration; 2. Examined the role of PTPσ and analyzed the effects of loss of function following SCI; 3. Analyzed gene expression profiles in neurons that do and those that don't regenerate their axon across a spinal cord transection site. These results will set us up to deeper investigations in the third phase of our proposal.

References:

Becker CG, Becker T (2002) Repellent guidance of regeneration optic axons by chondroitin sulfate glycosaminoglycans in zebrafish. J Neurosci 22(3): 842-853

Shen Y, Tenney AP, Busch SA, Horn KP, Cuascut FX, Liu K, He Z, Silver J, Flanagan JG (2009) PTPσ is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. Science **326**, 592.

Kwok JC, Afshari F, Garcia-Alias G, Fawcett JW (2008) Proteoglycans in the central nervous system: plasticity, regeneration and their stimulation with chondroitinase ABC. Restor Neurol Neurosci 26: 131.

Appendix:

Three posters were presented at the Society for Neuroscience Meeting in Washington, D.C. in November 2011.

Neuronal adhesion molecule L1.1 regulation of axonal growth over growth-inhibitory chondroitin sulfate proteoglycans in primary adult zebrafish neuronal cultures.

A. Tapanes-Castillo(1), F. Shabazz(1), M. M'boge(1), E. Bajuelos(1), K. Vajn(2), M. Oudega(2), J.A. Plunkett(1). (1) School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL 33054; (2) Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213.

Axonal regeneration depends on the balance of growth-inhibiting and growth-promoting influences. Chondroitin sulfate proteoglycans (CSPGs) inhibit axonal regeneration in the injured mammalian spinal cord. However, zebrafish regenerate their axons beyond the injury site despite the presence of CSPGs. This ability depends on the expression of the growth-promoting L1 cell adhesion molecule homolog, neuronal adhesion molecule L1.1 (nadl1.1). Our goal is to understand how nadl1.1 affects the response of zebrafish neurons to CSPGs *in vitro*. We hypothesize that reduced nadl1.1 expression will decrease axonal outgrowth, as well as the number of neurons crossing into and growing in CSPG areas. Conversely, nadl1.1 over-expression will increase axonal growth and result in more neurons crossing into and growing in CSPG areas. We are currently using a morpholino-based approach to reduce nadl1.1 expression in primary adult zebrafish brainstem neurons. We also cloned *nadl1.1* full-length cDNA into an adenovirus associated 2A peptide containing expression vector carrying an enhanced *Green fluorescent protein* (*GFP*) reporter gene (*pAAV-nadl1.1-eGFP*). We transfected this construct into adult zebrafish brainstem neurons using Amaxa-mediated electroporation and investigated the responses of both nadl1.1

morpholino-treated cells and nadl1.1 over-expressing (GFP positive) cells to CSPGs presented in culture. *Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JAP.*

Characterization of primary adult zebrafish brainstem neuronal cultures and their interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment

J.A. Plunkett (1), A. Tapanes-Castillo(1), F. Shabazz (1), K. Vajn(2), M. Oudega(2). (1) School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL 33054; (2) Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213.

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury. In zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. CSPGs inhibit axonal regeneration in the injured mammalian spinal cord, which contributes to the lack of endogenous functional restoration. Previous work in our laboratory has demonstrated that brainstem neurons in the adult zebrafish can regenerate their axon beyond a spinal cord lesion despite the presence of these inhibitory molecules. This ability is not characteristic for all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site. To investigate the axonal growth response of zebrafish brainstem neurons to CSPGs, we developed a primary neuronal culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs presented under controlled culture conditions. Our results supported this hypothesis revealing four different populations of brainstem neurons with regard to their response to CSPGs in vitro: (1) neurons repelled by CSPGs, (2) neurons that cross (extend processes) into CSPG-rich areas, (3) neurons that grow exclusively in CSPG areas, and (4) neurons that grown on CSPGs but cross (extend processes) out of the inhibitory environment. Our results suggest that the ability or disability to grow across and beyond a CSPG-rich area is intrinsic to the neuron and likely involves unique sets of axon growth-related genes. Presently, we are characterizing our brainstem cultures, quantifying the differential response of neurons to CSPGs and performing immunocytochemistry to identify different cell populations based on their protein expression profile. We have found Human neuronal protein C (HuC), Growth-associated protein 43 (GAP43), and Proliferating cell nuclear antigen (PCNA) positive cells in our culture. The expression of neural stem cell markers was also examined. Preliminary results also indicate a neuronal population that expresses the serotonergic marker, Tryptophan hydroxylase 2 (Tph2). *Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JAP*.

Temporal characterization of brainstem axon regeneration in the injured spinal cord of adult zebrafish (*Danio rerio*)

K. Vajn[1], D. Suler[1] , A. Betz[1], A. Tapanes-Castillo[3], F. Shabazz[3], JA Plunkett[3], M. Oudega[1,2]
Departments of [1]Physical Medicine & Rehabilitation and [2]Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; [3]Department of Biological Sciences, St. Thomas University, Miami, FL, USA.

Adult zebrafish (*Danio rerio*) recover their swimming ability to near-normal within 6 weeks after spinal cord injury (SCI), which is due at least in part to successful regeneration of brainstem axons across and beyond the injury site. Importantly, not all brainstem neurons involved in swimming regenerate beyond the spinal cord injury site. This remarkable anatomical and functional repair ability of zebrafish after SCI is in sharp contrast with that of mammals with SCI. In the injured mammalian spinal cord axons fail to regenerate and, after SCI, there is very limited functional restoration. We are interested in profiling gene expression in the zebrafish brainstem neuron that do and those that do not regenerate their axon across a spinal cord injury site. A first step toward this is to determine the time period during which regeneration beyond the injury takes place. To this end, we performed a complete spinal cord transection in one-year old zebrafish and applied a retrograde tracer distally at the time of transection, and at 1, 2, 4, 6, and 8 weeks post-injury. The brainstems were histologically processed and the number of retrogradely labeled neurons determined. We used immunohistochemistry to determine the number of axons in the injury site. Our results revealed the time-course of regeneration of axons from brainstem neurons across and beyond the injury site. We have also investigated the relationship between regenerating axons and the injured spinal cord tissue environment.

This work is supported by United States Department of Defense grant W81XWH-10-1-0617 to JAP.

One poster was presented at the American Society for Cell Biology in Denver, CO (Dec. 2011).

Receptor-type protein tyrosine phosphatase sigma a (ptprsa) expression in the central nervous system of adult zebrafish and brainstem derived primary cultures
Alexis Tapanes-Castillo, Megan Staudenmaier, Anthony Wood, Francelethia Shabazz, Katarina Vajn, Martin Oudega, Jeffery A. Plunkett

In mammals, the transmembrane protein tyrosine phosphatase PTPsigma was recently identified as a receptor for the chondroitin sulfate proteoglycan (CSPG), neurocan, which has been shown to inhibit neuronal regeneration. The goal of our project is to elucidate the role of PTPsigma in zebrafish axon regeneration following spinal cord injury (SCI). Unlike mammals, adult zebrafish have the ability to regenerate axons past the SCI site and regain the majority of their motor functions. We hypothesize that the zebrafish homolog of PTPsigma (ptprsa), like its mammalian counterpart, is a receptor for CSPGs. Furthermore, we suspect that axon regeneration in zebrafish is due in part to reduced PTPsigma activity following injury. To qualitatively investigate ptprsa expression following CNS injury in the zebrafish, we are currently using Reverse Transcriptase Polymerase Chain Reactions (RT-PCR). We are also investigating possible PTPsigma (ptprsa) interactions with CSPGs through the evaluation of ptprsa expression in adult zebrafish primary brainstem cultures. We observed ptprsa mRNA expression in uninjured brain and spinal cord tissue as well as injured brain tissues. We have also detected ptprsa mRNA expression in brainstem cultures grown on a growth-permissive laminin substrate, as well as on a CSPG-containing/laminin substrate. Our qualitative RT-PCR data suggest that overall ptprsa gene expression may not be governed by injury (*in-vivo*) or substrate (*in-vitro*). We are currently determining whether ptprsa activity is differentially regulated in the presence versus absence of injury through a post-transcriptional mechanism. Using morpholino knockdown, *in-vitro* experiments are also underway to determine whether ptprsa functionally interacts with zebrafish specific neurocan. *Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JP*.

Two posters were presented at the Winter Brain Research Conference in Snowbird, UT (Jan. 2012)

Zebrafish: an *in vivo* model for CNS axonal regeneration after injury
J.A. Plunkett, A. Tapanes-Castillo, F. Shabazz, K. Vajn, M. Oudega

It has been established in amphibians and fish that neurons can successfully regenerate their axons in the damaged central nervous system (CNS). This regenerative ability contrasts with that observed in mammals, whose neurons fail to regenerate their axon after CNS injury. Regeneration failure in the mammalian CNS is due in part to the presence of axon growth-inhibitory molecules within and near the site of damage. These inhibitors prevent the formation of axon circuits that could be involved in or be recruited for motor functions resulting in functional restoration. We have previously demonstrated that chondroitin sulfate proteoglycans (CSPGs), a family of axon growth-inhibitory molecules are present following CNS injury in adult zebrafish. We then investigated whether a CSPG family member neurocan, which has been shown to play a role in the prevention of CNS regeneration in mammals, is found within injured adult zebrafish CNS. Using reverse transcription-polymerase chain reaction (RT-PCR), we now qualitatively demonstrate that neurocan and its putative receptor, receptor-type protein tyrosine phosphatase sigma a (ptprsa), are expressed in the CNS pre- and post-injury. Our overall objective is to understand the molecular mechanisms through which these regenerative axons interact with CSPGs at the injury site. We aim to generate data that may serve as a foundation for the development of tailored strategies to promote axon regeneration across injury sites in the spinal cord. *Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JAP.*

Primary neuronal brainstem culture from adult zebrafish: interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment

A. Tapanes-Castillo(1), F. Shabazz(1), K. Vajn(2), M. Oudega(2), J.A. Plunkett(1). (1) School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL 33054; (2) Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213.

Chondroitin sulfate proteoglycans (CSPGs) inhibit axonal regeneration from brainstem neurons in the injured mammalian spinal cord. In zebrafish, axons from brainstem neurons regenerate beyond a spinal cord injury site despite the presence of

CSPGs. This ability is not characteristic of all brainstem neurons; different neuronal populations exhibit distinct responses, including failure to regenerate beyond the lesion site. To investigate the axonal growth response of zebrafish brainstem neurons to CSPGs, we developed a novel, primary neuronal culture system derived from the brainstem of adult zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs *in vitro*. Our results support this hypothesis revealing four different populations of brainstem neurons: (1) neurons repelled by CSPGs, (2) neurons that extend processes into CSPG areas, (3) neurons that grow axons exclusively on CSPGs, and (4) neurons that grow on CSPGs but extend processes out of the inhibitory environment. Our data suggest that the ability to grow across CSPGs is intrinsic to the neuron. We have molecularly characterized our heterotypic brainstem cultures using immunocytochemistry and found neuronal, glial, and putative stem/progenitor cell populations. We are currently analyzing how different cell populations respond to CSPGs. Finally, we are examining how growth-inhibiting molecules and growth-promoting molecules interact to regulate axon outgrowth *in vitro*. Our work focuses on the axonal growth-promoting zebrafish neuronal adhesion molecule L1.1 (nadl1.1), zebrafish neurocan, and receptor-type protein tyrosine phosphatase sigma a (ptprsa), a putative zebrafish CSPG receptor. *Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JAP.*

One poster and a poster and an invited talk were presented at the Keystone Symposia The Life of a Stem Cell: From Birth to Death in Olympic Valley, CA (March 2012).

Characterization of putative stem and neural progenitor cell populations from adult zebrafish brainstem tissue
A. Tapanes-Castillo(1), F. Shabazz (1), K. Vajn(2), M. Oudega(2)), J.A. Plunkett(1). (1) School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL 33054; (2) Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213.

All adult vertebrate brains have been shown to have some degree of progenitor cell neurogenesis. Although post-embryonic neurogenesis is limited in the mammalian brain, the zebrafish retains multiple proliferative neurogenic and stem cell niches throughout adult life. The focus of our research is study the role that injury to the central nervous system (CNS) plays in the induction of neurogenic progenitor cell fates in the adult zebrafish brain.

It has been well documented that in contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord transection injury. Damaged axons deriving from neurons within brainstem motor nuclei are able to regenerate across and beyond a spinal cord transection site. This ability is not characteristic for all brainstem neurons; different descending populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site.

We have established an adult brainstem cell culture system to study *in vitro* axonal outgrowth mechanisms in relation to permissive and non-permissive conditions. Surprisingly, in characterizing our heterotypic cultures we found a sub-population of cells that displayed positive immuno-reactivity for an antibody against zebrafish nestin. Using double and triple labeling with antibodies against Proliferating cell nuclear antigen (PCNA), Human neuronal protein C (HuC), and tubulin we are further characterizing this population. We have identified Nestin positive; PCNA positive cells that we hypothesize are stem progenitor cells and/or radial glia. We have also observed Nestin positive; HuC positive cells that likely correspond to early neuroblasts derived from neural progenitor cells.
In the application of our *in vitro* model to CNS injury *in vivo* models, we are currently examining specific regenerative brainstem nuclei for SOX 2 and nestin immuno-reactivity pre- and post-spinal cord injury. We hypothesize that spinal cord injury will induce an endogenous, quiescent population of brainstem progenitor cells that act to integrate and enable the regenerative response seen following spinal cord injury in the fish.

Funded by U.S. Dept. of Defense W81XWH-10-1-0617 to JAP.

Three posters will be presented at the Society for Neuroscience Meeting in New Orleans, LA in October 2012.

Characterization of putative stem and neural progenitor cell populations in adult zebrafish brainstem tissue.

J. A. PLUNKETT[1], A. CARTAGENA[1], L. YUT[1], J. GENAO[1], F. SHABAZZ[1], K. VAJN[2], A. TAPANES-CASTILLO[1], M. OUDEGA[2];
[1]St Thomas Univ., Miami Gardens, FL; [2]Physical Med. and Rehabilitation, Neurobio. and Bioengineering, Univ. of Pittsburgh Sch. of Med., Pittsburgh, PA

Although post-embryonic neurogenesis is limited in the mammalian brain, zebrafish (*Danio rerio*) retain multiple proliferative neurogenic and stem cell niches throughout adult life. The focus of our research is to study how injury to the central nervous system (CNS) affects the induction of neurogenic progenitor cell fates in the adult zebrafish brain. It has been well documented that in contrast to mammals, adult zebrafish recover functionally from a complete spinal cord transection injury. Damaged axons deriving from neurons within brainstem motor nuclei are able to regenerate across and beyond a spinal cord transection site. This ability is not characteristic for all brainstem neurons; different descending populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site. We hypothesize that spinal cord injury will induce an endogenous, quiescent population of brainstem progenitor cells that act to integrate and enable the regenerative response seen following spinal cord injury in the fish. We are currently examining regenerative brainstem regions for stem cell marker expression pre- and post-injury. Prior to injury, Nestin and Sox 2 immunoreactivity were observed near ventricular areas, as well as in ventral brainstem regions, which contain nuclei from descending cerebrospinal projection neurons. These markers were also detected in similar brainstem regions following focal brainstem injury, as well as spinal cord injury. In addition, we have established an adult brainstem cell culture system to study *in vitro* neurite outgrowth mechanisms in relation to permissive and non-permissive conditions. Our heterotypic cultures contain a subpopulation of nestin positive cells. Using double and triple labeling with antibodies against Proliferating cell nuclear antigen (PCNA), Human neuronal protein C (HuC), and tubulin we further characterized this putative stem/progenitor cellular population. Currently, we are analyzing how these putative stem cells respond to non-permissive growth conditions and affect the growth response of neighboring cells. *Funded by U.S. Dept. of Defense W81XWH-11-1-0645 to JAP.*

Neurocan expression in the CNS of adult zebrafish and its effect on neurite outgrowth *in vitro.*

A. TAPANES-CASTILLO[1], H. GOMEZ[1], A. VALLS[1], F. SHABAZZ[1], K. VAJN[2], M. OUDEGA[2], J. A. PLUNKETT[1];
[1]Sch. of Sci., St. Thomas Univ., MIAMI, FL; [2]Physical Medicine, Rehabilitation, Neurobiology, Bioengineering, Univ. of Pittsburgh Sch. of Med., Pittsburgh, PA

It has been established in amphibians and fish that neurons can successfully regenerate their axons in the damaged central nervous system (CNS). This regenerative ability contrasts with that observed in mammals, whose neurons fail to regenerate their axon after CNS injury. Regeneration failure in the mammalian CNS is due in part to the presence of axon growth-inhibitory molecules within and near the site of damage. These inhibitors ultimately prevent the formation of axon circuits that could be involved in or recruited for motor functions thereby facilitating functional restoration. We have previously demonstrated that chondroitin sulfate proteoglycans (CSPGs), a family of axon growth-inhibitory molecules, are present following CNS injury in adult zebrafish. We then investigated whether the CSPG family member neurocan, which has been shown to play a role in the prevention of CNS regeneration in mammals, is found within injured adult zebrafish CNS. Using reverse transcription-polymerase chain reaction (RT-PCR), we now qualitatively demonstrate that neurocan is expressed in the CNS pre- and post-injury. We are currently examining neurocan protein expression utilizing Western blot analysis and immunohistochemistry pre- and post-injury. In addition, we have cloned a Myc-tagged full length zebrafish neurocan b construct. We are presently isolating and characterizing the protein for future use in our adult zebrafish brainstem culture system. We hypothesize that zebrafish neurocan b is a growth-inhibitory molecule. Taken together, the overall objective of this project is to understand the molecular mechanisms underlying the CSPG interactions of the regenerative neurons. Funded by U.S. Dept. of Defense W81XWH-11-1-0645 to JAP.

The role of axon regeneration from brainstem neurons in functional recovery after spinal cord injury in adult zebrafish

K. VAJN[1], D. SULER[1], A. BETZ[1], A. TAPANES-CASTILLO[3], F. SHABAZZ[3], J. PLUNKETT[3], M. OUDEGA[1,2];
[1]Physical Med. and Rehabil., [2]Dept. of Neurobio., Univ. of Pittsburgh, Pittsburgh, PA; [3]Sch. of Science, Technol. and Engin. Mgmt., St. Thomas Univ., Miami, FL

In contrast to mammals, zebrafish (Danio rerio) exhibit remarkable locomotor recovery within six weeks after complete spinal cord injury (SCI). In the first weeks after SCI neurons located in the reticular nucleus (RT), nucleus of medial longitudinal fasciculus (NMLF) and magnocellular octaval nucleus (MaON) regenerate their axons as far as 4000 μm into the caudal spinal cord. Here we investigated if these regenerating axons contribute to the swimming recovery. In adult zebrafish with a complete SCI we investigated the temporal profiles of axonal regeneration from neurons in the RT, NMLF and MaON and of the recovery of swimming. Neurons that regenerated their axon 4000 μm caudal to the transection site were identified using retrograde tracing. We quantified the total number of labeled neurons in the brainstem as well as in individual brainstem nuclei. The total swimming distance was assessed using open field video tracking. Our results demonstrate that the number of neurons in the RT, NMLF and MaON that regenerate their axon increases during the first 8 weeks post-injury. Axons from RT neurons regenerate into the caudal spinal cord before those of NMLF and MaON neurons. The neurotransmitter profile of the regenerating neurons is being investigated. The swimming performance gradually improved as well, with full swimming recovery around 8 weeks post-injury. Our results suggest that the regeneration of different descending tracts contributes to spontaneous swimming recovery after SCI in zebrafish. Current work focuses on revealing the critical molecular determinants underlying the axonal regeneration in the injured spinal cord.

CUMULATIVE EXPENDITURE REPORT
ST. THOMAS UNIVERSITY – Sept. 15, 2012

1. Award No. ____W81XWH-11-1-0645_____2. Report Date _9/30/2012 (annual)

3. Reporting period from __8/1/11_____ to ___8/31//12_____

4. PI ___Dr. Jeffrey Plunkett_____ 5. Telephone No. (305) 628-6572_____

6. Institution_____St. Thomas University_____

7. Project Title____Molecular Determinants Fundamental to Axon Regeneration after SCI

8. Current staff, with percent effort of each on project.

Dr. Jeffrey Plunkett, PI	25% (academic year) + 100% (summer)
Dr. Martin Oudega, Co-PI	40%
Dr. Alexis Tapanes-Castillo (post doc Plunkett lab)	100%
Fran Shabazz (research technician, Plunkett lab)	100%
Katarina Vajn (Post Doc Oudega lab)	100%

9. Award expenditures to date (as applicable):

	This Qtr/Cumulative		**This Qtr/Cumulative**
Personnel	$43,308.46/ $ 43,308.46__	Travel	__$0/ $11,171.71__
Fringe Benefits	$9,677.65 / $9,677.65 ____	Equipment	_$1,379.85 _/ _$8,212.45
Supplies	$6,684.89 _/__$8,179.99__	Other	___$1,507.92__/___$4,482.12_____

	This Qtr/Cumulative
Subtotal	_$62,558.77_____/ _$85,032.38
Indirect Costs	_$25,888.78/ $32,278.84_____
Fee	_____31.25_____/__31.25_____
Total	____$88,478.80_____/__$117,342.47