

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 01-06-2012		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 SEP 2010 - 31 MAY 2012	
4. TITLE AND SUBTITLE Molecular Determinants Fundamental to Axon Regeneration after SCI				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0617	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Jeffrey Plunkett E-Mail: jplunkett@stu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) St. Thomas University Opalocka, 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 We hypothesize that the ability to grow an axon over CSPGs is intrinsic to adult zebrafish brainstem neurons and entails the expression of a distinct set of genes. This premise will be addressed using in vitro adult zebrafish brainstem cell culture systems and in vivo adult zebrafish spinal cord injury model systems. In cultures we have observed three distinct populations of brainstem neurons with regard to their response to chondroitin sulfate proteoglycans (CSPG). Some cells attach, extend processes, and remain exclusively associated with CSPG. Other cells attach outside and extend processes into CSPG-rich areas. A third kind of cell was found to attach outside and extend processes up to but never into CSPG-rich areas. In fact, these processes were clearly repelled by CSPG. We are currently quantifying different aspects of these three adult zebrafish brainstem neuron populations. Thus, these cultures mimic the in vivo behaviors of brainstem populations after SCI. In parallel to these in vitro studies, we have developed minimally invasive spinal cord transection and tracer injection techniques. These are currently employed to investigate the evolution of the scar and the time course of axon regeneration after spinal cord injury. The data from these first in vivo experiments will serve as a basis to optimize our harvest of retrogradely labeled adult brainstem neurons that did or did not regenerate their axon beyond a transection site.					
15. SUBJECT TERMS No subject terms provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
U	U	U	UU	50	

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-32
Key Research Accomplishments.....	33-34
Reportable Outcomes.....	35-50
Conclusion.....	50
References.....	N/A
Appendices.....	N/A

Contract # W81XWH-10-1-0617

Title: Molecular Determinants Fundamental to Axon Regeneration after SCI

PI: Dr. Jeffrey Alan Plunkett

Final Report of Scientific Progress from Sept 1, 2010 – June 1, 2012 (Months 1-21) A three-month extension was granted in Feb. 2012

Introduction:

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

Body:

SOW: Plunkett Laboratory

Specific Aim 1: To determine the relationship between L1.1 and neurocan and its role in axon regeneration from adult zebrafish brainstem neurons *in vitro*.

Increased levels of L1.1 enhance the regenerative capacity of damaged axons in a zebrafish spinal cord repair model¹. L1.1 is the zebrafish homologue of L1 which is present in mammals. In this specific aim, we will employ an adult zebrafish brainstem neuronal culture system to investigate whether L1.1 is involved in the successful growth of an axon over a growth-inhibitory neurocan substrate. Neurocan is a member of the family of growth-inhibitory chondroitin sulfate proteoglycans (CSPGs). We will take advantage of antisense morpholino to down regulate L1.1 levels in cultured neurons and, subsequently, determine axon growth using quantitative (unbiased) assessment techniques. We predict that this intervention will decrease the axonal growth response of brainstem neurons that normally are capable of growing their axon over neurocan. The results from our experiments will allow testing our hypothesis that *cultured adult zebrafish brainstem neurons require L1.1 to grow their axon over neurocan*.

Milestones Specific Aim 1:

- Mo 1-3: Use RT/PCR to show expression of neurocan after SCI.
Mo 4-6: Isolate zebrafish neurocan.
Mo 7-9: Test effects of zebrafish neurocan on axon outgrowth *in vitro*.
Mo 10-12: Test if reducing L1.1 levels decreases outgrowth of axons on neurocan.
Mo 13-15: Analyze results using unbiased quantitative techniques. Start manuscript.
Mo 16-18: Finalize/submit manuscript. Start follow-up experiments.
Extension to Month 21

Months 1-3: Use RT/PCR to show expression of neurocan after SCI.

In adult zebrafish the presence of neurocan after CNS injury was verified using RT-PCR techniques. Zebrafish specific primers were designed using Integrated DNA Technologies SciTools OligoAnalyzer 3.0 (www.idtdna.com).

*Official name: neurocan. Gene ID: 559024. **NcanFor: CCA GAA GCA GCA GTC TAT CC.**
NcanRev: CAGCCGGCATCACAGTTGTC. Expected Product: 373 bp.*

Amplification with primers specific for zebrafish β -actin were used for all control RT-PCR reactions:

*Gene symbol: *bactin1*. Official name: *bactin1*. Gene ID: 57934. **Bactinfor: CGTGCTGTTTTCCCTCC.** **Bactin2rev: CCA TCA CCA GAG TCC ATC AC.** Product: 392 b.*

*As shown in **figure 1**, using zebrafish neurocan specific primers following reverse transcription (RT), we have found neurocan mRNA expression 7 and 14 days post-CNS injury. The data shown represent expression of neurocan mRNA following brain injury. We verified the identity of the zebrafish neurocan product through sequence analysis (**figure 2**). Reasons for using injured brain tissue were due to RNA yield. We initially collected adult zebrafish (n=30) spinal cord tissue samples following spinal cord transection as described in Phase 1, Specific Aim 1. The spinal cord tissues were collected and prepared for standard RT-PCR using commercially available kits and following the guidelines of the manufacturer. Initial experiments indicated that we were unable to isolate enough RNA to perform RT-PCR amplification. We will follow up with analysis of neurocan mRNA expression following SCI through increasing the number of spinal cords harvested.*

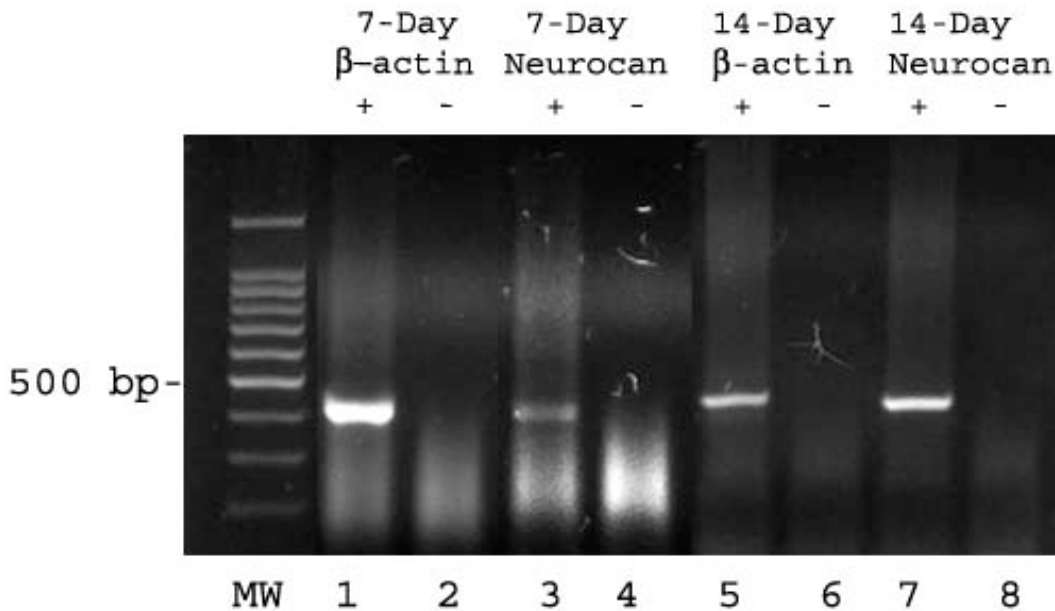


Figure 1. Gel electrophoresis analysis of zebrafish neurocan and β -actin mRNA expression following CNS injury.

Using RT-PCR, neurocan mRNA expression was analyzed 7 and 14 days post-CNS injury (+) indicates the presence of cDNA in the PCR reaction. Using PCR primers specific for zebrafish neurocan, the predicted 373 base pair band was found. β -actin was used for RT controls and (-) cDNA was also used as a control.

Blasted Ncan sequence: 20bp-end against NCBI's complete nucleotide collection (did not specify zebrafish)

Query: Ncan sequence: 20bp-end
Subject: fish ncan

>[ref|XM_682321.3|](#) PREDICTED: Danio rerio neurocan (ncan), mRNA
Length=3162

[GENE ID: 559024 ncan](#) | neurocan [Danio rerio] (10 or fewer PubMed links)

Score = 248 bits (134), Expect = 2e-62
Identities = 137/140 (97%), Gaps = 0/140 (0%)
Strand=Plus/Plus

```

Query 1 TATCGTGCTCCTCACAACCGTTACGCACTTTCTTCTCCGACGCTAAAAGAGTGTGTGTG 60
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 628 TATCGTGCTCCTCACAACCGTTACGCACTTTCTTCTCCGACGCTAAAAGAGTGTGTGTG 687

Query 61 GAAAANTNNGCCAACATCGCCACACCGGCGCAGCTTCAGGCCACGTTTGATGACGGATAC 120
      ||||| | |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 688 GAAAACCTCAGCCAACATCGCCACACCGGCGCAGCTTCAGGCCACGTTTGATGACGGATAC 747

Query 121 GACAACTGTGATGCCGGCTG 140
      |||||||||||||||||||
Sbjct 748 GACAACTGTGATGCCGGCTG 767

```

Figure 2. BLAST results of sequenced zebrafish neurocan product. Sequence results indicate that our amplified product has a 97% identity with zebrafish neurocan.

In conclusion, these data indicate that neurocan is expressed in CNS tissues following injury. Furthermore, initial analysis of un-injured CNS tissues shows no detectable neurocan expression. These data add support to our hypothesis that neurocan is expressed following SCI in the fish and may affect the regenerative capabilities demonstrated in zebrafish brainstem neurons.

Months 4-6: Isolate zebrafish neurocan.

We have previously shown in adult zebrafish the presence of neurocan after CNS injury using RT-PCR techniques.

Ncan Cloning Strategy

Our cloning strategy is to prepare an open reading frame (ORF) clone of zebrafish neurocan based upon the amino acid sequence below. Source Bioscience Imagenes (www.imagenes-bio.de/) will make a synthetic clone of the 1125 aa neurocan protein (sequence above) with an ORF of 3379 bp that corresponds to the amino acid sequence above.

Clone will be optimized for protein expression. The optimization process comprises an adaptation of the codon usage, an optimization of the GC-content and a displacement of such sequences which will decrease the mRNA stability. Furthermore sequence elements which could form secondary structures will be removed. During the optimization process the nucleotide sequence may change from the original ncan exon sequence. However, the aa sequence will be kept at the same. During the production process the synthesized construct will be fully sequenced twice.

Synthetic clone of neurocan ORF has an advantage that the cDNA encoding the predicted ncan protein is 8710bp (no introns included). However, only the first 3379 bp are thought to

encode the protein. The remaining 5.3 kb is supposedly UTR. This would be a large construct for cellular transfection.

Neurocan

Zebrafish Zv9 Genome Build

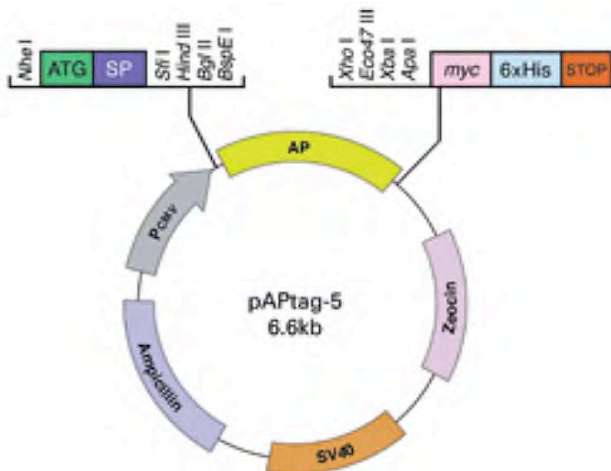
[ncana](#) [[Ensembl gene: ENSDARG00000067820](#)]

Protein: [ENSDARP00000088461](#) 1125aa

Exons Alternating exons Alternating exons Residue overlap splice site

MFHVEAGGIKVLVLLVFSANFGVCSSES AVTVRRVTAPPLRRPLSSSVLLPCVFTLPAA
ASSPPHGPFIHWTHGSGAQQR TVVSARDGVLRVHQAFAGRLRLPGYSKNQLNASLELSRL
RYNDSGTFRCHVALGENYEQD TVHLEVTGVVVFHYS GP AEHYSLSFWEASRACEQNSAVMA
SAEQLWAAFHSSMSNCAAGWISDQSVRYPVQ TPELGCFCGHTEFSRGV RNYGKRDPSELFD
VYCFASDMEGEV FSSSVDPKLT HAEAAAHCESLDARLASVGQLFLAWRSGLEHCGPAWVS
DGTLRVSVGPRGSQCPNEEPGVRRVSPAELSNGT KRFD AFCYRVKKAQKASLPNIMQSL
KPSSEEPSTSPSTSDGQP VVGLDPSVLQVSNWTGRIDLDTESLPAAEPL ELSDEYLTVF
LRAGDSNLDWSQQMDTEDPSI PDQSPGGSAQE EEEEEDEGLSGHSSPTATPEKSKSSI KNI
VDSLWKPWSYLTGGDSEETTTTRTPEMKTSSAPGSGLMSWWSRSWLSVPSSITDKIISPS
TSASSVSPTS VLPTREQSVNQTSPPAQDAPLTTVDWVIVTELPKKT KSTAAVVSTESFSG
ESRVLETEGSSWGDSESLTLETSSNALFINTTDHTLTDSTTNQPKPTDATTSSSSTTSS
TTTSSRTVHGHEARGEIQYRRRN RGKKNQTTTEMSTTPVQD TTTTPFVQSTTDIQSSPEI
HSTTAPSVQTTADIHSTTAPSVQTTADIDTTTSPSVQTTDDIQSTTSPSVQTTDVIQSTT
SPSADTINTETTGM SISTSADQTVSGTTAFIEQHLSTTETIRTKSSTGEAEDDRCSCLHG
GTCLPHGEGFRFCFCPQYSGESCEIDVDDCQSNPCENGGTCIDKEDSFVCLCLPSYSGDR
CERDTEGCEHGWKKFHGH CYRLFRRHTWEDA EKACREHSSHLTSSTSSMEQDFLNLGLH
ENVWIGLNDRTVEEDFQWTDGLDVVYENWRENQPDNFFAGGEDCVVMITREDGKWNDVPC
NYNLPYVCKKGTVMCGTPPAIDNAYLVGRRRSHYDIHAVVRYQCADGFFQRHVPTVRCRP
NGTWERPKI ICTKSRRRAHRYRRQHRSRREHRRHRRHSGHGRD

The synthetic Zebrafish neurocan ORF will be subcloned into an alkaline phosphatase (AP) tagging vector APTag5 (GenHunter, USA: www.genhunter.com) to create a neurocan-AP fusion protein. Using AP-TAG Kit B, the C-terminal end of neurocan will be fused to AP, similarly to the experiment performed with mouse ncan described in Shen et al., 2009 (PTPsigma Science paper). We will then transiently transfect this construct into 293T cells. These transfected cells will be cultured and the secreted fusion protein will be collected from the culture media. The Neurocan-AP fusion protein will be purified using the 6XHis and myc epitope tags. The eluates of the column will be analyzed using SDS-PAGE. The AP-tag will facilitate future cell-free binding assays to test binding interactions between ncan and nad1.1, as well as between ncan and ptpers. The epitope tag will allow us to visualize the ncan fusion protein with Western blots and immunohistochemistry in our cultures.



Months 7-9: Test effects of zebrafish neurocan on axon outgrowth *in vitro*.

Change in Ncan Cloning Strategy

Our cloning strategy since the last quarterly report has changed due to an update in the Ensembl sequence database (Zv9). Since our last report, additional sequence for zebrafish neurocan b was released. This data indicates neurocan b exhibits more homology to mammalian neurocan than neurocan a, the zebrafish paralog we originally planned to clone.

Our new cloning strategy is to prepare an open reading frame (ORF) clone of zebrafish neurocan b based upon the amino acid sequence. We have already ordered a synthetic clone encoding 1266 amino acids from Gene Art Life Technologies (Invitrogen). The clone is currently under construction and should be ready by the end of June 2011.

The synthetic Zebrafish_neurocan b ORF will be subcloned into a pCMV-Myc (Clontech) vector to create a Myc-tagged neurocan b fusion protein. We have already begun preparing the vector backbone for cloning.

Once generated, the pCMV-Myc-neurocan_b construct will be transiently transfected into 293T cells. These transfected cells will be cultured and the secreted fusion protein will be collected from the culture media. Neurocan b protein will be purified using the myc epitope tag. The eluates of the column will be analyzed using SDS-PAGE. The epitope tag will allow us to visualize the ncan_b with Western blots and immunohistochemistry in our cultures.

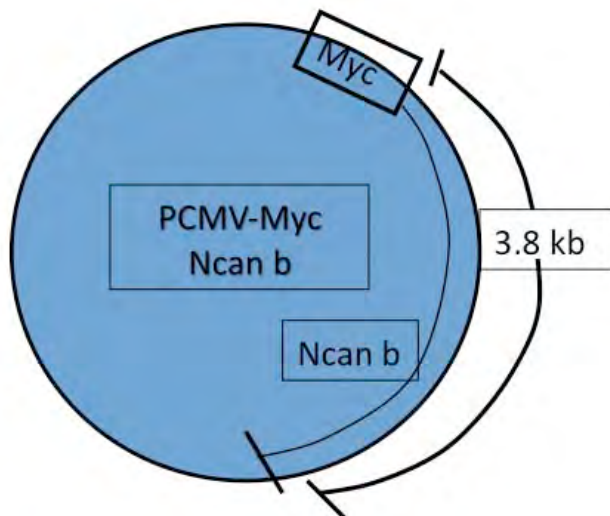
Months 10-12: Test if reducing L1.1 levels decreases outgrowth of axons on neurocan.

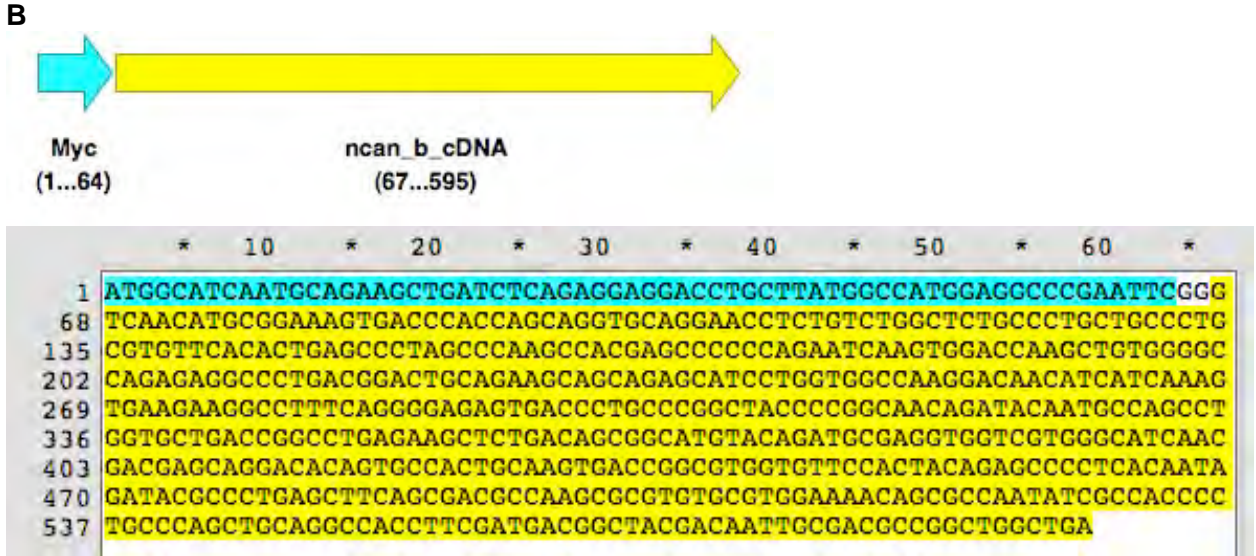
Sequencing results confirmed that we successfully cloned full-length neurocan b cDNA in-frame and downstream of a Myc tag in a pCMV (Clonotech) vector (**Figure 3**).

pCMV-Myc-ncanb was next transiently transfected into 293T cells using Amaxa-mediated electroporation technology: Cell Line Kit V and Program A-23. Media was then collected from cells after 7 days in vitro and concentrated to approximately 30% of the original volume using Centricon Plus-20 centrifugal filter devices (Millipore).

Western blots were run to analyze Myc protein expression using a c-Myc antibody (Clonotech #631206). 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 4**). 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 will be 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. In addition, we plan to perform an experiment in which we treat collected protein with chondroitinase ABC to determine whether GAG chains are being attached to Myc-NcanB. Chondroitinase treatment is expected to produce a downward shift in the molecular weight of intact Myc-NcanB.

A





C

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  VNM RKVTHQQVQEPLSGSALLPCVFTLSPSPSHEPPRIKWTKLWGQRGPDGLQKQQSILV 60
Sbjct 1  VNM RKVTHQQVQEPLSGSALLPCVFTLSPSPSHEPPRIKWTKLWGQRGPDGLQKQQSILV 60

Query 61 AKDNI IKVKKAFQGRVTLPGYPGNRYNASLVLTLGRSSDSGMYRCEVVVGINDEQDTVPL 120
Sbjct 61 AKDNI IKVKKAFQGRVTLPGYPGNRYNASLVLTLGRSSDSGMYRCEVVVGINDEQDTVPL 120

Query 121 QVTGVVPHYRAPHNRYALSFSDAKRVCVENSANIATPAQLQATFDDGYDNC DAGWL 176
Sbjct 121 QVTGVVPHYRAPHNRYALSFSDAKRVCVENSANIATPAQLQATFDDGYDNC DAGWL 176

```

Figure 3. A. Plasmid map of pCMV-Myc-ncanb vector. B. Sequencing results illustrating ncan cDNA (yellow) cloned in-frame and downstream of Myc tag (blue). C. BLAST results confirming 100% identity between the protein sequences of the cloned Ncan b and the amino acid sequence posted for Ncan b (Protein ID: ENSDARP00000020815) on Ensembl Zebrafish Genome Build Zv9.

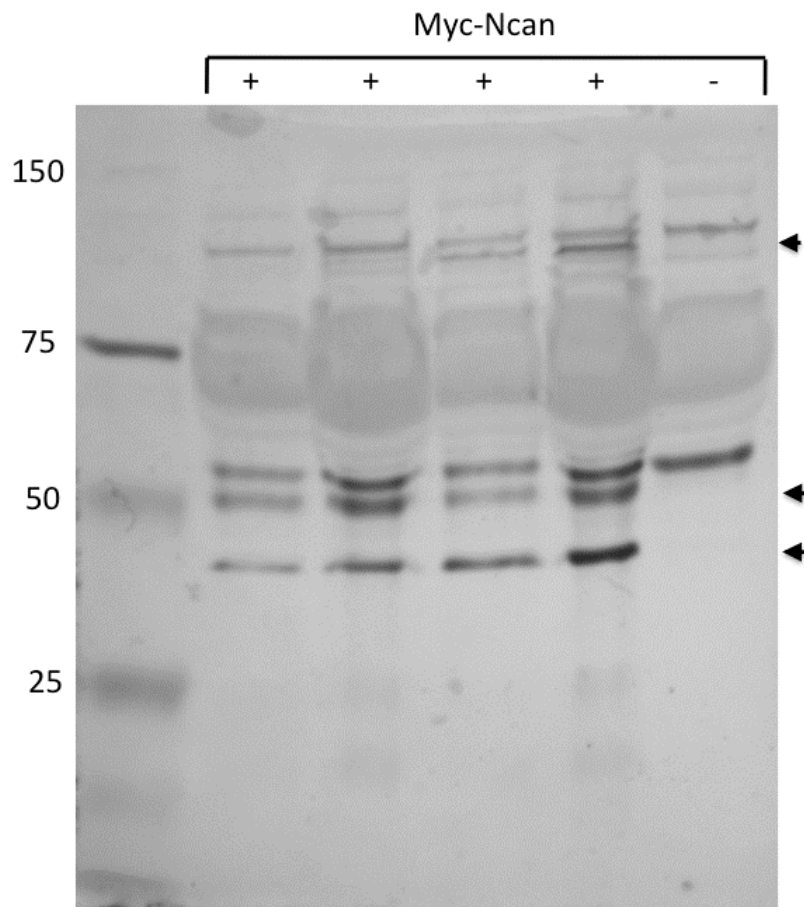


Figure 4. Western blot analyzing Myc-NcanB protein expression in media collected from transfected cells (+) and untransfected media (-). Arrows correspond to differentially expressed bands recognized by anti-c-Myc.

During this and the previous review periods 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 5).

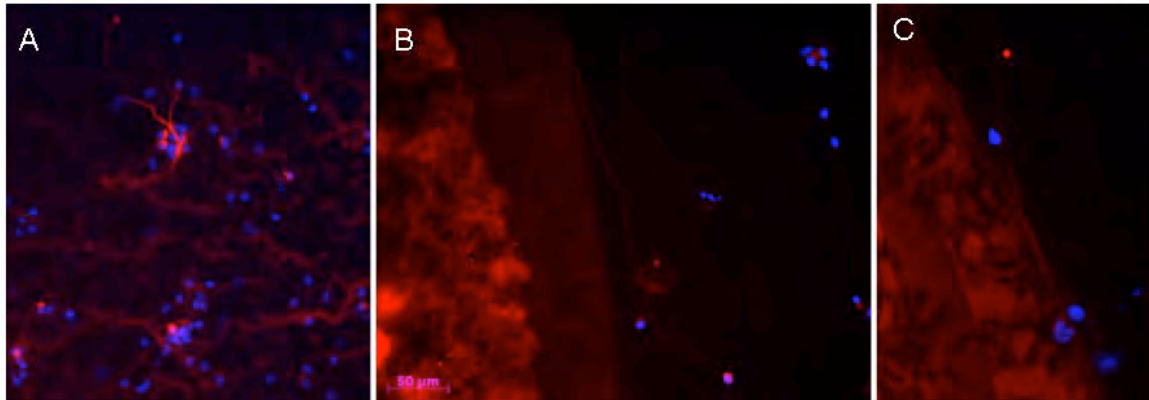


Figure 5. Neuronal cultures with spots of myc-ncanB conditioned media from 293T cells. Control treated cells panel A. Neurons turning away from concentrated myc-ncanB spot, panels B and C.

Once Myc-NcanB protein is isolated, it will be used as a substrate for zebrafish adult brainstem cultures in vitro. In order to have a reference for these future results, we quantified the effect of reducing L1.1 levels on axon outgrowth over commercially available CSPGs (Table 1).

Response	Percent Cells (%)
Repelled	41
Crossing in	16
Crossing out	11
Growing inside	32

Table 1. Response of L1.1 morpholino treated cells to 0.1ug/mL CSPGs after 7 days in vitro. N= 4 independent experiments, 379 total cells.

During Months 10-12 of this review period, we also performed several RT-PCR experiments examining neurocan expression in vivo pre and post-injury. Our qualitative data demonstrates that neurocan mRNA is expressed in un-injured as well as injured brain tissue. Neurocan mRNA is also expressed in un-injured spinal cord (Figure 6).

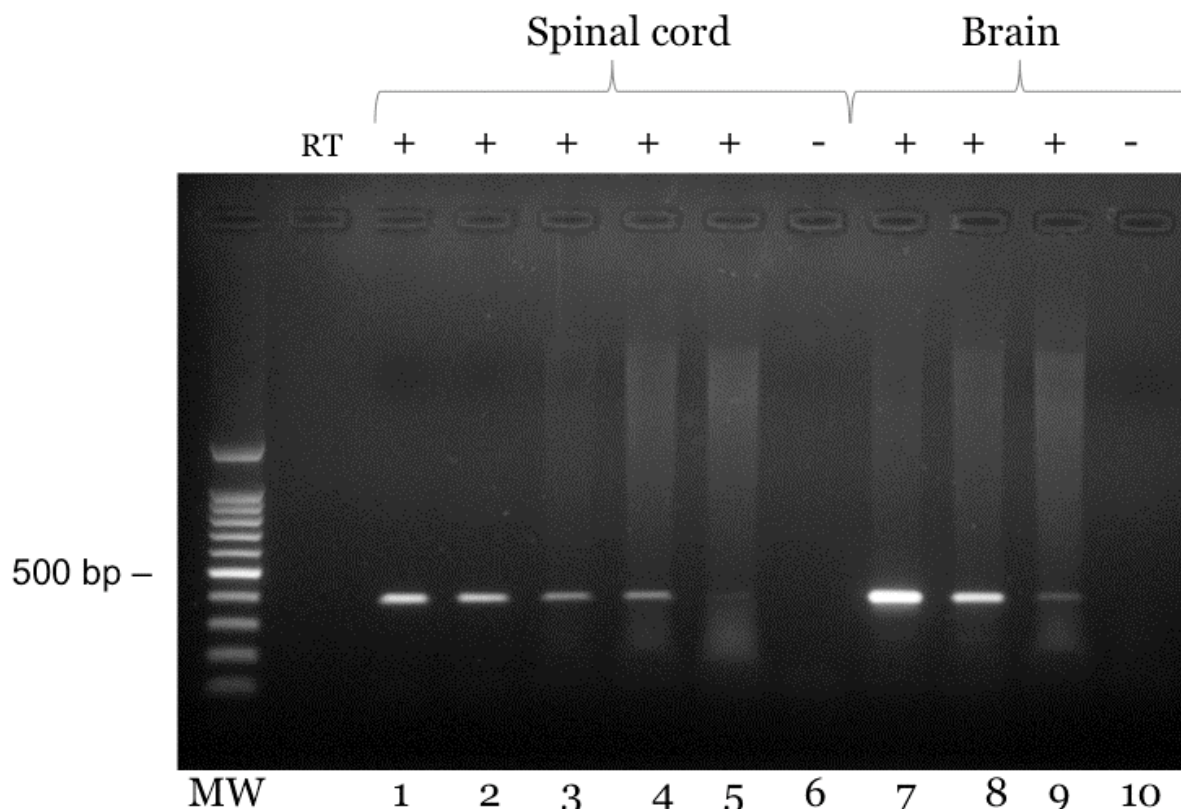


Figure 6. RT-PCR analysis of zebrafish neurocan mRNA in uninjured CNS tissues. Spinal cord (lanes 1-6) and brain (lanes 7-10) samples. Reverse transcriptase (RT) was added to make cDNA (+). Control no cDNA reactions (-) were run without RT.

Months 13-15: Analyze results using unbiased quantitative techniques. Start manuscript.

In the previous period of review, months 9-12 we finished cloning our zebrafish neurocan construct. In this current period we have transfected this construct into mammalian 293T cells and collected culture media from transfected cells. We are also continuing to characterize our secreted product and analyzing it through western blot. We recently 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 Myc-Ncan 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 were added to the neurocan protein core would be of a different composition than that produced in vivo in the fish. In an effort to investigate this possibility, we are currently transiently transfecting primary neuronal and astrocyte cultures from the zebrafish brain. We will be able to analyze these efforts prior to our next quarterly report. 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.

Summary of preliminary data:

Sequencing results confirmed that we successfully cloned full-length neurocan b cDNA in-frame and downstream of a Myc tag in a pCMV (Clonetechn) vector.

pCMV-Myc-ncanb was next transiently transfected into 293T cells using Amaxa-mediated electroporation technology: Cell Line Kit V and Program A-23. Media was then collected from cells after 7 days in vitro and concentrated to approximately 30% of the original volume using Centricon Plus-20 centrifugal filter devices (Millipore).

*Western blots were run to analyze Myc protein expression using a c-Myc antibody (Clonetechn #631206). 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 4**).*

Months 13-15: Analyze results using unbiased quantitative techniques. Start manuscript.

Months 16-18: Finalize/submit manuscript. Start follow-up experiments

In the previous period of review, months 13-15 we finished cloning our zebrafish neurocan construct. In Month 16, we applied for and granted a 3 month extension to complete work specifically in Aims 1 and 2.

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 shown in **Figure 7**, 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 (**Figure 8**). 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.

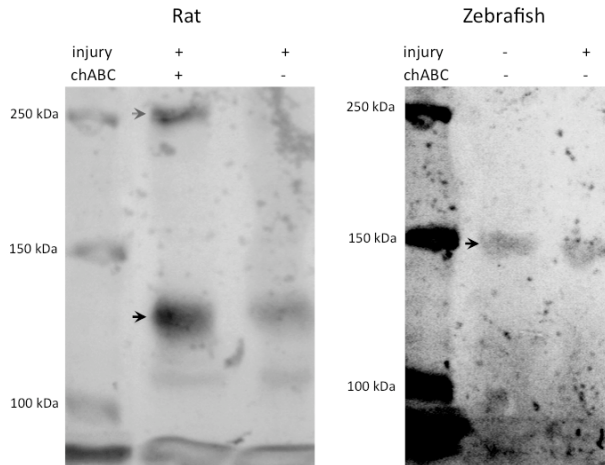


Figure 7. Western blot analysis of Neurocan expression in injured and uninjured CNS tissues.

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.

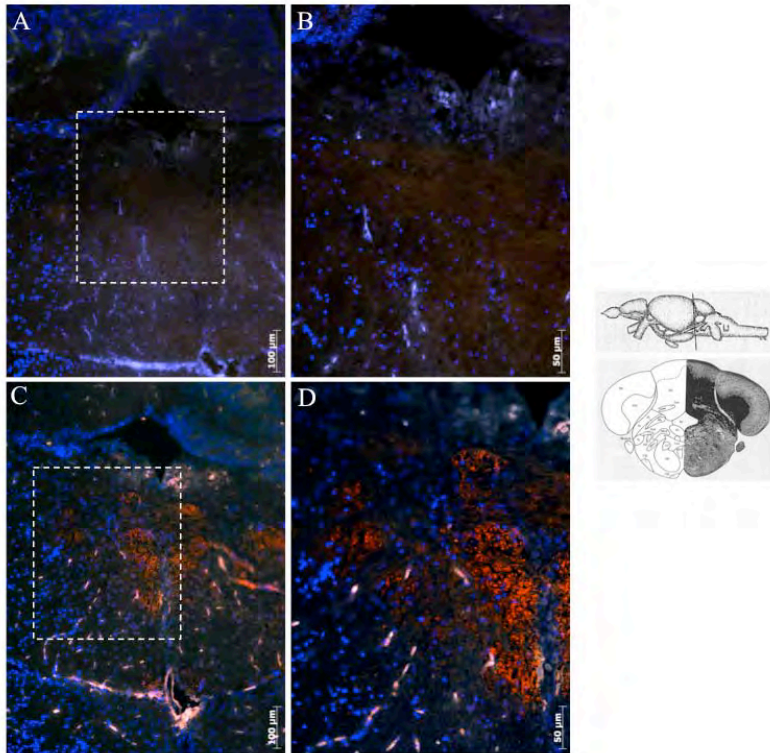


Figure 8. Neurocan expression in the brain of injured adult zebrafish. A and C are adjacent coronal sections harvested 3 days after acute brain injury. Images were taken at 10X. DAPI is shown in blue, and 1F6 in red. Nonspecific staining, which can be seen on both red and green channels (data not shown), 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 19-21: Extension period

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 9**, 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.*

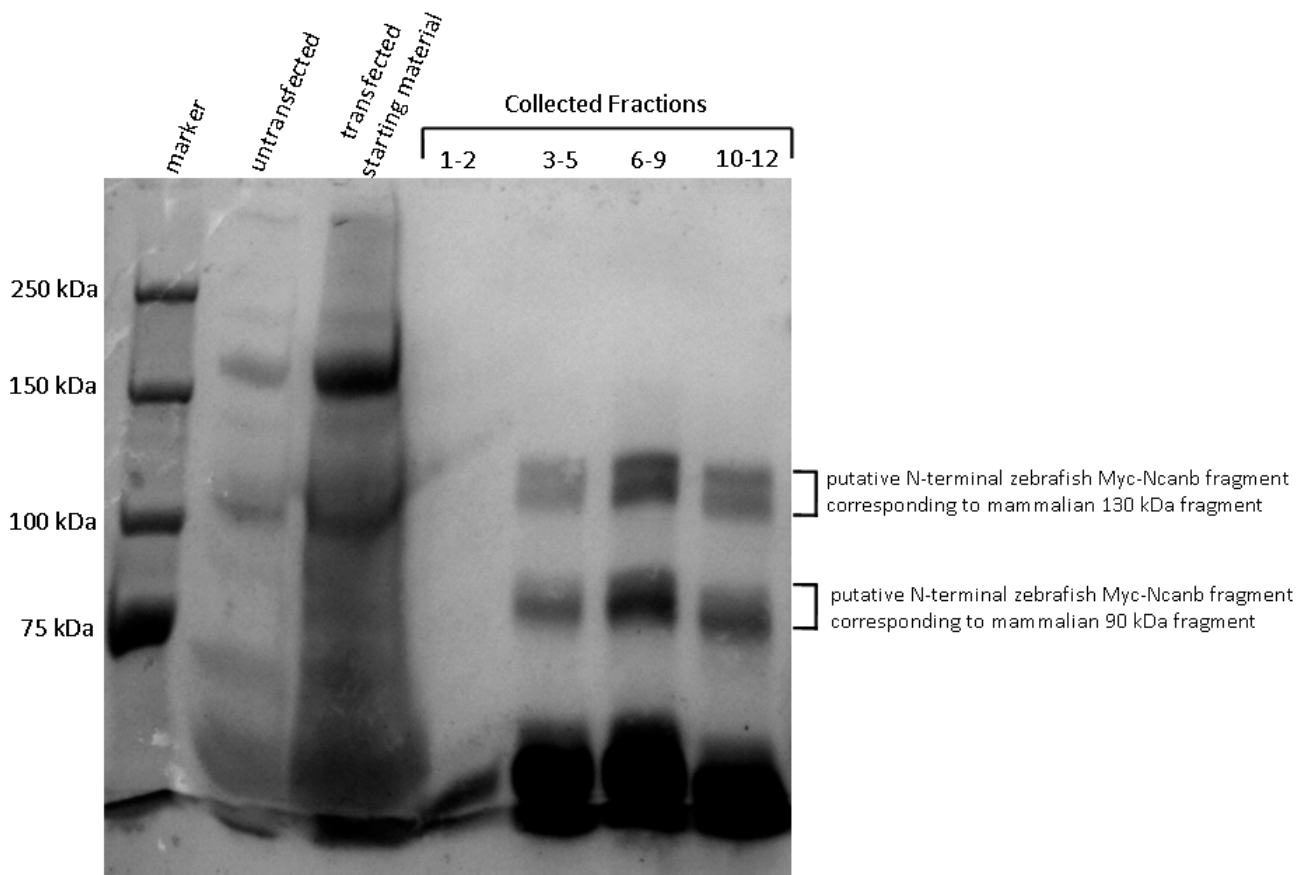


Figure 9. Western blot analyzing Myc-NcanB protein affinity column purification.

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.

SOW: Plunkett Laboratory

Specific Aim 2: To determine the presence and role of PTP σ in CSPG-mediated inhibition of axon regeneration from adult zebrafish brainstem neurons *in vitro*.

The transmembrane protein tyrosine phosphatase, PTP σ has been shown to bind to neural CSPGs². Moreover, genetic deletion of PTP σ elicited axon regeneration in the adult mouse spinal cord^{2,3}. In this specific aim, we will investigate the presence and involvement of PTP σ in failed axon growth from adult brainstem neurons over a CSPG-rich environment. We will use antisense morpholino-based approaches to down regulate PTP σ receptor levels in cultured adult zebrafish brainstem neurons and, subsequently, determine axon growth using quantitative (unbiased) assessment techniques. We expect that down regulation of PTP σ will improve axon growth from brainstem neurons that are otherwise unable to grow their axon over CSPGs. The data from the experiments will allow testing our premise that *PTP σ is involved in CSPG-mediated inhibition of axon growth from cultured adult zebrafish brainstem neurons.*

Milestones Specific Aim 2:

- Mo 1-3: RT/PCR to show PTP σ after SCI. Examine PTP σ in cultured cells.
 - Mo 4-6: Prepare and test PTP σ antisense/control morpholino.
 - Mo 7-9: Test specific conditions for morpholino *in vitro* knockdown.
 - Mo 10-12: Conduct morpholino knockdown using in-situ hybridization.
 - Mo 13-15: Analyze results using unbiased quantitative techniques. Start manuscript.
 - Mo 16-18: Finalize/submit manuscript. Start follow-up experiments.
- Extension to Month 21

Specific Aim 2.

Months 1-3: RT/PCR to show PTP σ after SCI. Examine PTP σ in cultured cells.

*In the adult zebrafish the presence of PTP σ mRNA following SCI was analyzed in un-injured brainstem tissues using RT-PCR. Prior to experimentation, we confirmed the presence of PTP σ within the zebrafish genome using the Zebrafish Zv8 assembly from the Ensembl database at www.ensembl.org (using gene name: *ptprs* and gene ID: 140820).*

Zebrafish specific primers were designed using Integrated DNA Technologies SciTools OligoAnalyzer 3.0 (www.idtdna.com).

*Official gene symbol: *ptprs*. Official name: protein tyrosine phosphatase, receptor type, S. Gene ID: 140820. **PTPRSFor: CCA GCA AGA CTA CGG TGG C (exon 8). PTPRSREV: GTG TGT GTG CTG ATG AAG GTC GC (EXON 9).** 275 bp product expected. We have also designed a forward primer in exon 6 with negative results when amplified with the above reverse primer in exon 9.*

Amplification with primers specific for zebrafish Beta actin were used for all control RT-PCR reactions (as above, specific aim 1)

As shown in **figure 10**, initial experiments indicate the failure to amplify zebrafish $PTP\sigma$ mRNA from un-injured zebrafish tissues. We are currently using this primer set to analyze injured tissue. It should be noted that in using a RT-PCR based strategy, a negative result does not indicate “no expression” as it may be that the particular primer set will not work. In support of this is evidence in mammals that $PTP\sigma$ and other PTPs in the leukocyte antigen-related (LAR) subfamily are extensively alternatively spliced. We designed these primers to amplify a product that spans exon 8 - exon 9 in addition to exon 6 – exon 9. We can hypothesize that unique splice variants may exist in zebrafish between differing tissues. With this knowledge, continued failure to amplify a product using the current primer set will result in the design of new primers from different exons.

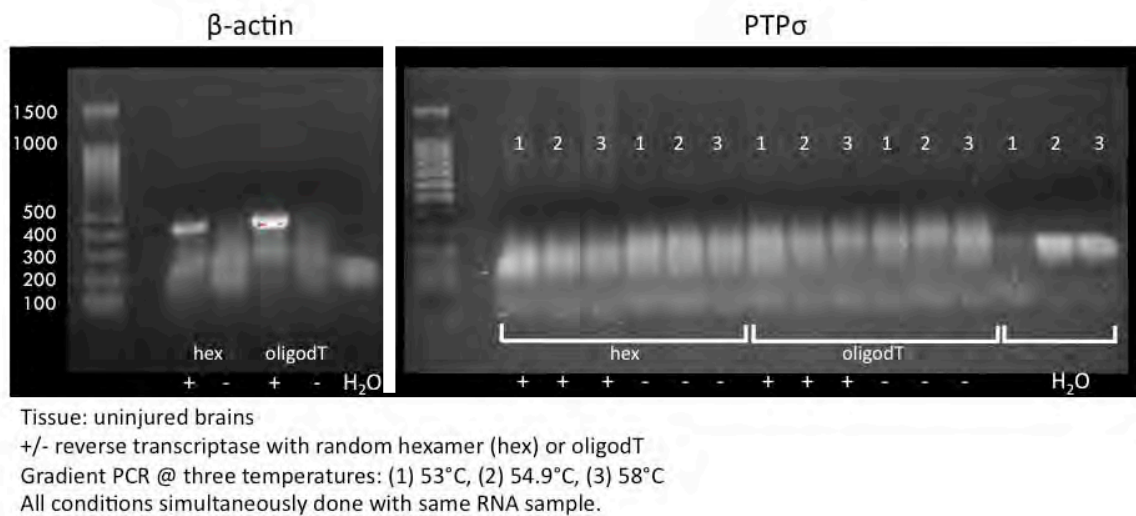


Figure 10. RT-PCR analysis of β -actin and $PTP\sigma$ mRNA expression in uninjured adult brain tissue. β -actin amplification was used for reverse transcription control reactions. Note that $PTP\sigma$ mRNA expression was negative for all conditions tested. Gradient PCR annealing temperatures as well as oligo and random priming were used along with exon 8 – 9 primer set for the above reactions.

Months 4-6: Prepare and test PTP σ antisense/control morpholino.

Since the previous quarterly report we are now able to demonstrate in the adult zebrafish the presence of PTP σ mRNA in un-injured brainstem tissues using RT-PCR. Prior to experimentation, we confirmed the presence of PTP σ within the zebrafish genome using the Zebrafish Zv8 assembly from the Ensembl database at www.ensembl.org (using gene name: *ptprs* and gene ID: 140820). Zebrafish specific primers were designed using Integrated DNA Technologies SciTools OligoAnalyzer 3.0 (www.idtdna.com).

Official gene symbol: *ptprs*. Official name: protein tyrosine phosphatase, receptor type, S. Gene ID: 140820. Forward 5' GTCACCTGCCCATCCTGC-3' Reverse 5'-GCTTGGCTTGGACATTGATTTTC-3' 344 bp product expected. We have designed the forward primer in **exon 1** with the reverse primer in **exon 3**.

Amplification with primers specific for zebrafish Beta actin were used for all control RT-PCR reactions.

As shown in **figure 11**, initial experiments indicate the ability to amplify zebrafish PTP σ mRNA from un-injured zebrafish tissues. We are currently using this new primer set to analyze injured tissue and cultured brainstem neurons. We are also confirming the PTP σ PCR product through sequencing.

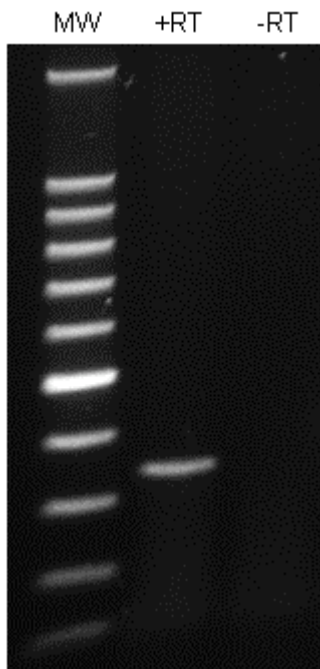


Figure 11. RT-PCR analysis of PTP σ mRNA expression in uninjured adult brain tissue. β -actin amplification was used for reverse transcription control reactions (not shown). PTP σ

mRNA expression was positive when reverse transcription (RT) reaction was included. Gradient PCR annealing temperatures as well as oligo and random priming were used along with exon 1 – 3 primer set for the above reactions.

We have used the RT-PCR data above to design a set of morpholinos to knockdown PTP σ mRNA expression in our cultured cells.

Two morpholinos were designed against PTP σ mRNA (Ensembl transcript ENSDART00000083308) to confirm target specificity. The first morpholino (5'-TGACGCAGATGACCTTTGACCTGGC-3') is a translation-blocking oligo. The second morpholino (5'-TGTTAGCAGTCTCACCTCGATGC-3') is a splice-blocking oligo. It was designed to skip exon 2 during splicing, ligate exon 1 to exon 3 (deleting exon 2), cause a frameshift in the downstream sequence and result in early termination of protein synthesis. Successful splice modification will be verified using RT-PCR.

Both PTP σ morpholinos were designed using GeneTools targeting guidelines to be specific and effective against Ptp σ . We expect that both morpholinos will produce similar phenotypes. This finding would support the hypothesis that the phenotype is due to knockdown of PTP σ , rather than off-target RNA interactions.

Data acquired from cells treated with the PTP σ morpholinos will be compared to that obtained from cells treated with a standard negative control oligo 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

The ability of the PTP σ morpholinos to knockdown protein expression will be tested in vitro. Brainstem neurons will be transfected with a 6XHis/Myc tagged PTP σ -AP protein and treated with PTP σ or control morpholinos. Western blots will be performed with an anti-myc tag antibody to quantify knock down.

We have recently ordered the PTP σ morpholinos and are currently setting parameters for delivery to cultured brainstem neurons.

Months 7-9: Test specific conditions for morpholino *in vitro* knockdown

PTP σ detection in tissue

Prior to experimentation, we confirmed the presence of PTP σ within the zebrafish genome using the Zebrafish Zv8 assembly from the Ensembl database at www.ensembl.org (using gene name: ptp σ and gene ID: 140820). Zebrafish specific primers were designed using Integrated DNA Technologies SciTools OligoAnalyzer 3.0 (www.idtdna.com).

Official gene symbol: ptp σ . Official name: protein tyrosine phosphatase, receptor type, S. Gene ID: 140820.

In a previous quarterly report, we demonstrated in the adult zebrafish the presence of PTP σ mRNA in un-injured brainstem and tissues using RT-PCR.

To date, we have not been able to detect PTP σ in un-injured spinal cord tissue. While control RT-PCR reactions amplified successfully, no PTP σ was detected in un-injured spinal cord. One could hypothesize that the expression of PTP σ in the un-injured brain is due to the prevention of plasticity within the un-injured quiescent tissue.

*Similarly, one might predict that PTP σ expression in uninjured spinal tissue also prevents plasticity. One possibility that exists is that there may be an alternative splice form of the gene within the cord that is distinct from the brain. For this reason, we have performed RT-PCR reactions with additional primer sets, targeting different exons. PTP σ was successfully amplified from uninjured brain using **exon2** forward primer 5'-CTGTAGACATGATCGGCGT-3' and **exon 4** reverse primer 5'-CGGCCGTTACTTGTGTTG-3'.*

We are currently using both primer sets exon1forward/exon3reverse and exon2forward/exon4reverse to analyze un-injured spinal cord tissue, as well as injured brain and spinal cord tissue. We are also currently designing in-situ hybridization experiments to perform further analysis of expression at the cellular level.

PTP σ detection in cultured brainstem cells

Overall, we are using multiple approaches to determine the presence or absence of PTP σ in injured and un-injured brain and spinal cord tissue and in adult brainstem cells grown in culture. We have already successfully performed control RT-PCR reactions from cultured brainstem neurons using β -actin primers. We are currently initiating experiments to analyze PTP σ expression in cultures grown on laminin alone as well as on a CSPG/laminin substrate. Our current approach will allow us to determine if and when PTP σ is expressed in our cultures and how morpholino knock down will affect outgrowth.

PTP σ morpholino approach

We have used the RT-PCR data above to design a set of morpholinos to knockdown PTP σ mRNA expression in our cultured cells.

Two morpholinos were designed against PTP σ mRNA (Ensembl transcript ENSDART00000083308) to confirm target specificity. The first morpholino (5'-TGACGCAGATGACCTTTGACCTGGC-3') is a translation-blocking oligo. The second morpholino (5'-TGTTAGCAGTCTCACCTCGATGC-3') is a splice-blocking oligo. It was designed to skip exon 2 during splicing, ligate exon 1 to exon 3 (deleting exon 2), cause a frameshift in the downstream sequence and result in early termination of protein synthesis. Successful splice modification will be verified using RT-PCR.

Both PTP σ morpholinos were designed using GeneTools targeting guidelines to be specific and effective against Ptp σ . We expect that both morpholinos will produce similar phenotypes.

This finding would support the hypothesis that the phenotype is due to knockdown of PTP σ , rather than off-target RNA interactions.

Data acquired from cells treated with the PTP σ morpholinos will be compared to that obtained from cells treated with a standard negative control oligo 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

The ability of the PTP σ morpholinos to knockdown protein expression will be tested in vitro. Brainstem neurons will be transfected with a construct containing the PTP σ morpholino target sequence upstream of a GFP gene. A reduction in GFP expression would indicate that the morpholino recognizes the PTP σ target.

We have recently ordered the PTP σ morpholinos and are currently setting parameters for delivery to cultured brainstem neurons.

Months 10-12: Conduct morpholino knockdown and using in-situ hybridization.

In months 4-6, we detected the presence of a band corresponding to the expected size of PTP σ mRNA in un-injured brain tissue utilizing RT-PCR, a forward primer in exon 1 and a reverse primer in exon 3. During months 10-12 we cloned this putative PTP σ RT-PCR product into a pCRII vector. Sequencing results revealed that the clone shared 100% identity with ptprsa Transcript ID: ENSDART00000083308 (Ensembl database Zebrafish genome build Zv9). This confirms expression of PTP σ mRNA in un-injured brainstem tissues. Moreover, construction of the pCRII-PTP σ cDNA exons1-3 vector will facilitate future PTP σ in situ studies and PTP σ antibody production.

Alignments

```
>lc1|57657
Length=11592
```

```
Score = 255 bits (138), Expect = 1e-71
Identities = 138/138 (100%), Gaps = 0/138 (0%)
Strand=Plus/Plus
```

```
Query 1 AACGCATCGAGACGATAGAGTTTGTATGAAGGTGCAGGAGCCGTCCTGAGGATCCAGCCTC 60
Sbjct 221 AACGCATCGAGACGATAGAGTTTGTATGAAGGTGCAGGAGCCGTCCTGAGGATCCAGCCTC 280
Query 61 TCCGGGCGCCTCGAGACGAGAACGTCTACGAGTGTCAGGCGGAGAACAGCGAAGGCGAAA 120
Sbjct 281 TCCGGGCGCCTCGAGACGAGAACGTCTACGAGTGTCAGGCGGAGAACAGCGAAGGCGAAA 340
Query 121 TCAATGTCCAAGCCAAGC 138
Sbjct 341 TCAATGTCCAAGCCAAGC 358
```

Figure 12. BLAST nucleotide alignment reveals 100% similarity between sequenced PTP σ cDNA clone and ptprsa Transcript ID: ENSDART00000083308.

During months 10-12, we also demonstrated PTP σ expression in injured adult brain tissue and un-injured adult spinal cord (Figure 9). We also detected PTP σ expression in adult brainstem cells cultured 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).

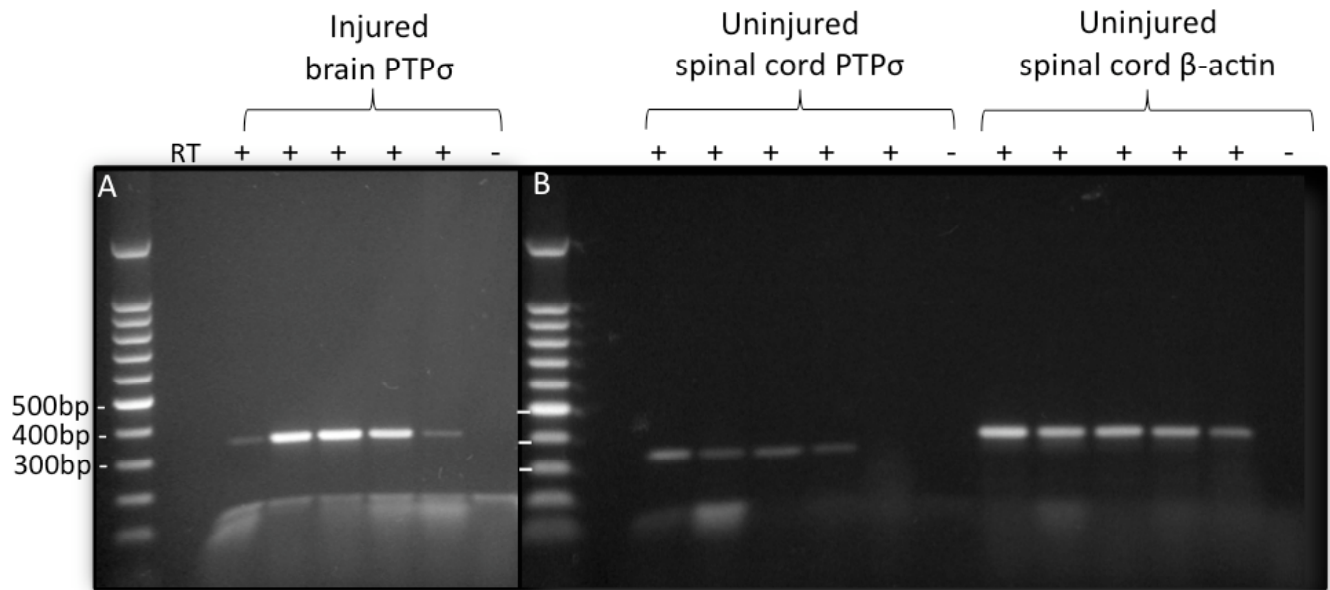


Figure 13. RT-PCR analysis of zebrafish PTP σ mRNA pre- and/or post-CNS injury. (A) Injured brain and (B) spinal cord samples. Reverse transcriptase (RT) was added to make cDNA (+). Control no cDNA reactions (-) were run without RT. Control β -actin RT-PCR reactions were performed on the same samples (shown in same order).

PTP σ morpholino-treated cultures grown on laminin for seven days in vitro are currently being analyzed to determine optimal PTP σ morpholino delivery parameters.

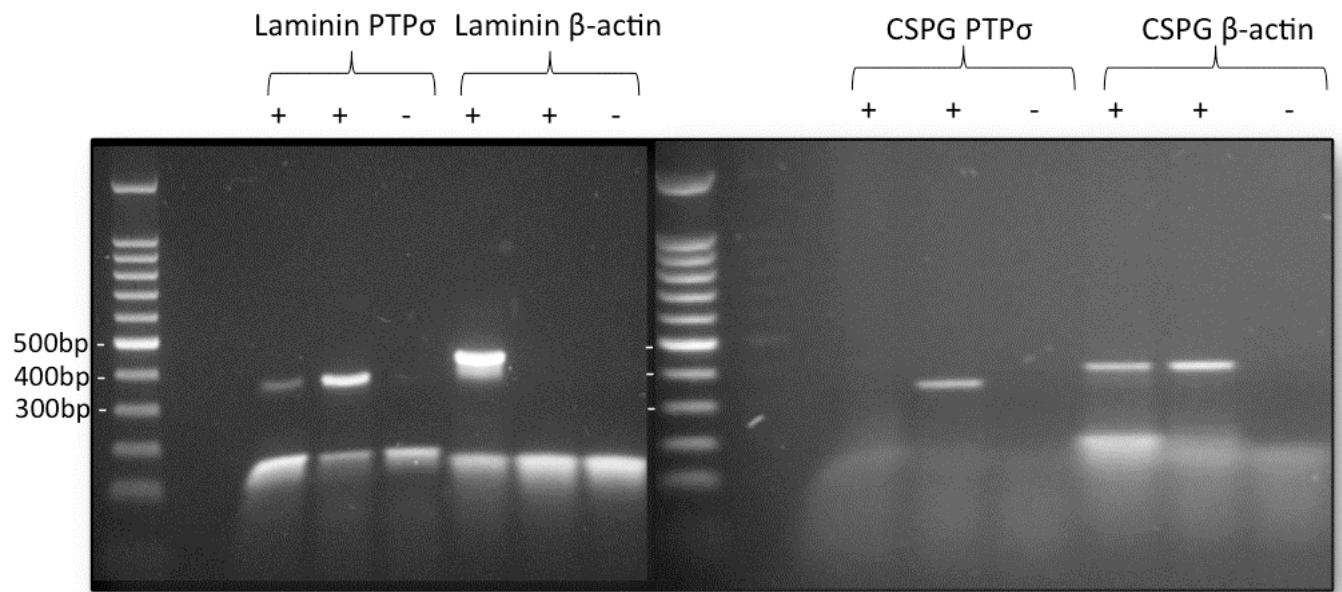


Figure 14. RT-PCR analysis of zebrafish $PTP\sigma$ mRNA in adult brainstem cultures. Cells were grown for 7 days *in vitro* on a growth permissive laminin substrate or on a CSPG/laminin substrate (B). (Reverse transcriptase (RT) was added to make cDNA (+). Control no cDNA reactions (-) were run without RT. Control β -actin RT-PCR reactions were performed on the same samples (shown in same order).

Months 13-15: Analyze results using unbiased quantitative techniques. Start manuscript.

Months 16-18: Finalize/submit manuscript. Start follow-up experiments

Months 19-21: Extension period

*In months 4-6, we detected the presence of a band corresponding to the expected size of $PTP\sigma$ mRNA in un-injured brain tissue utilizing RT-PCR, a forward primer in exon 1 and a reverse primer in exon 3. During months 10-12 we cloned this putative $PTP\sigma$ RT-PCR product into a pCRII vector. Sequencing results revealed that the clone shared 100% identity with *ptprsa* Transcript ID: ENSDART00000083308 (Ensembl database Zebrafish genome build Zv9). This confirms expression of $PTP\sigma$ mRNA in un-injured brainstem tissues. Moreover, construction of the pCRII- $PTP\sigma$ cDNA exons1-3 vector will facilitate future $PTP\sigma$ *in situ* studies and $PTP\sigma$ antibody production.*

During months 10-12, we also demonstrated $PTP\sigma$ mRNA expression in injured adult brain tissue and un-injured adult spinal cord (**Figure 13**). We also detected $PTP\sigma$ expression in adult brainstem cells cultured on a growth-permissive laminin substrate, as well as on a CSPG-containing laminin substrate (**Figure 14**). Our qualitative RT-PCR data suggest that overall *ptprsa* gene expression may not be governed by injury (*in-vivo*) or substrate (*in-vitro*).

PTP σ morpholino-treated brainstem cultures

*PTP σ morpholino-treated cultures grown on CSPG+ laminin for seven days in vitro are currently being analyzed for efficacy of $PTP\sigma$ morpholino delivery (**Figure 15**). To date three experiments have been performed and are currently under analysis.*

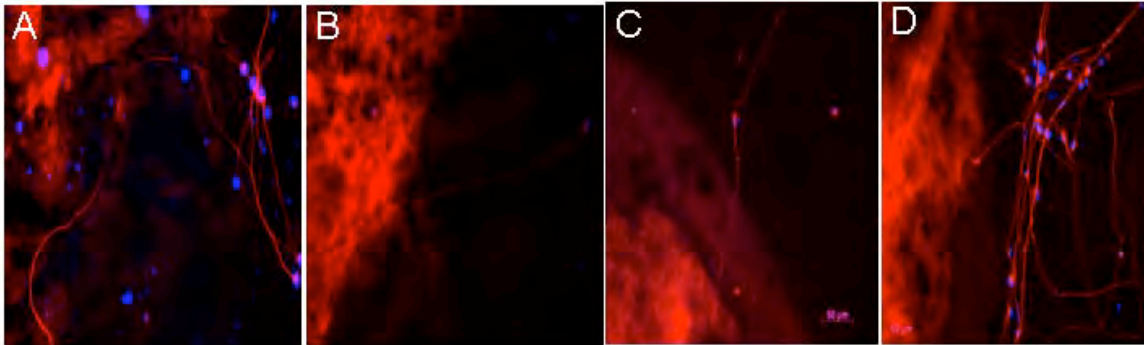


Figure 15. PTP σ morpholino treated brainstem cultures exhibit different cellular responses to CSPGs. Control morpholino treated cells: (A) turning away from and (B) extending a neurite (crossing) into CSPG environment. PTP σ morpholino treated cells: (C) extending a neurite into the CSPG region and (D) interacting with CSPGs.

Quantitative Analysis of Axonal Growth following PTP σ morpholino treatment

Quantitative analysis the *ptprs* morpholino (*ptprsMO*) delivery to cultured brainstem neurons reveals no significant difference in cells ability to cross into the CSPG barrier (**Table 2**). Our original hypothesis was that down regulation of PTP σ will improve axon growth from brainstem neurons that are otherwise unable to grow their axon over CSPGs. The data from the experiments will allow testing our premise that PTP σ is involved in CSPG-mediated inhibition of axon growth from cultured adult zebrafish brainstem neurons. Although the quantitative data demonstrate no significant difference, confirmation of true *ptp* 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.

***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. Upon completion, zebrafish cells will be transfected with *ptprsMO*-GFP and 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* *ptprs*MO experiments.

Table 2

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

Percent of cells with specific response to CSPGs (1 μ g/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.

SOW: Oudega Laboratory

Specific Aim 3: To determine the effects of the knock-out of genes crucial for axon regeneration on spinal cord repair and functional recovery in vivo.

In Phase 1 genes that are crucial for brainstem axon regeneration beyond an injury site in the adult zebrafish spinal cord will be identified. The expression of some of these genes will need to be up regulated whereas that of others will need to be down regulated to obtain optimal axon regeneration. In this specific aim we will focus on the genes whose increased expression accompanies *in vivo* axon regeneration. We will (conditionally) knock-out these genes from the zebrafish genome. These mutant fish will then be subjected to spinal cord transection after which we will determine the extent of axon regeneration (number and length) and the degree of functional (swimming) restoration using quantitative (unbiased) assessment techniques. We expect that the gene deletion will decrease the regenerative response and functional outcome. The results from these studies will allow testing our hypothesis that *axonal regeneration from adult zebrafish brainstem neurons across a spinal cord transection site requires the expression of specific genes.*

Milestones Specific Aim 3:

Dr.Katarina Vajn; Dr. Oudega's laboratory.

- Mo 1-3: Identify genes essential for brainstem axon growth after SCI.
 - Mo 4-9: Perform knock-out experiments; create mutant zebrafish.
 - Mo 7-9: Test the mutant fish for absence genes and functional behavior.
 - Mo 10-12: Perform spinal cord transection surgeries in mutants. Assess function.
 - Mo 13-15: Trace brainstem neurons/regenerating axons. Analyze functional data.
 - Mo 16-18: Analyze axon growth. Write manuscript. Start follow-up experiment.
- Extension to Month 21

Milestones Specific Aim 3:

Dr.Katarina Vajn; Dr. Oudega's laboratory.

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

A large number of experiments necessary to optimize the harvest of brainstem neurons are being performed. We are testing different retrograde tracers (Fast Blue, Nuclear Yellow< others) at different time points after transection of the spinal cord. Different tracers have different transport characteristics which need to be determined. This depends on molecular characteristics of the tracer as well as on the organism. Next, the fastest and most efficient tracer will be selected. Efficacy (i.e., the number of neurons back-labeled) will be a superior criterion over speed. Once this tracer is known we will determine the most optimum time point for administration after spinal cord transection.

Once, the tracer and time point are known we can start back-label brainstem neurons that have grown their axon across an injury as well as brainstem neurons that have failed to do so. For this we use laser capture micro-dissection techniques. The harvested cells (regenerators and non-regenerators) will then be processed for isolation of mRNA and identification of the genes crucial for the growth response (and genes crucial for the failed growth response). All the experiments that are currently ongoing were fine-tuned by the results that were presented

at the Society for Neuroscience meeting in San Diego (10-2010). A working collaboration has been established with a laboratory at Pitt that owns a LCM device. We can freely use this device and have started to do so. The mRNA of collected neurons will be isolated and then submitted for microarray analysis to identify genes that are differentially expressed. In preparation for these experiments, we have begun optimizing our RNA purification techniques. We have isolated RNA from adult zebrafish brains using Qiagen's RNeasy Plus Mini Kit. We have also performed reverse-transcriptase PCR reactions to assess the quality of our RNA using β -actin primers and the Superscript III First-Strand Synthesis system (Invitrogen). Establishment of these procedures is critical to the development of techniques used in microarray analysis.

Months 4-9: Perform knock-out experiments; create mutant zebrafish.

We have tested different tracers during the previous months and have found that fluoro-emerald successfully traces brainstem neurons in the adult zebrafish (Fig. 1). Because we need two tracers to distinguish the two populations of brainstem neurons, fluoro-emerald is currently being tested in combination with fluoro-ruby. If successful, this combination will be used to retrogradely label brainstem neurons in adult zebrafish after spinal cord transection and regeneration. We have calculated that a total of 1000 fish will need to be labeled to obtain 50000 brainstem neurons per population. This will yield enough RNA for microarray analysis.

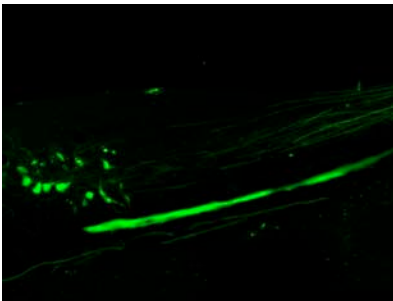


Figure 1. Fluoro-emerald labeled neurons in the adult zebrafish brainstem. The tracer was injected into the spinal cord caudal to the level where a transection would be made. Axons can internalize the tracer and transport it to the cell body.

Dr. Vajn is finishing a report on the time-course of retrograde labeling of regenerated brainstem neurons. 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. An abstract will be presented at the International Zebrafish conference in Glasgow, Scotland.

RNA quality was tested after zinc-fixation protocols. This is important as time will pass during harvesting of neurons and the already collected RNA needs to be stable. The first results have confirmed stable RNA after zinc-fixation, but the brain was less well fixed making the cutting of sections problematic. A number of different zinc-fixation protocols are currently being tested to

optimize the end-result, i.e., allowing brainstem cutting as well as proper fixation (stabilization) of brainstem neuron RNA.

We are finishing up the histology for our time-course study of retrograde labeling of regenerated brainstem neurons including staining for CSPGs and axon markers. This will allow correlation of axons in the spinal cord with cells in the brainstem. ISH on zebrafish is being tested to confirm ICC data.

Mo 7-9: Test the mutant fish for absence genes and functional behavior.

Because we need two tracers to distinguish the two populations of brainstem neurons, fluoro-emerald is currently being tested in combination with fluoro-ruby (Fig. 2). If successful, this combination will be used to retrogradely label brainstem neurons in adult zebrafish after spinal cord transection and regeneration. We have calculated that a total of 1000 fish will need to be labeled to obtain 50000 brainstem neurons per population. This will yield enough RNA for microarray analysis. We continue to collect labeled brainstem neurons for our microarray analysis.

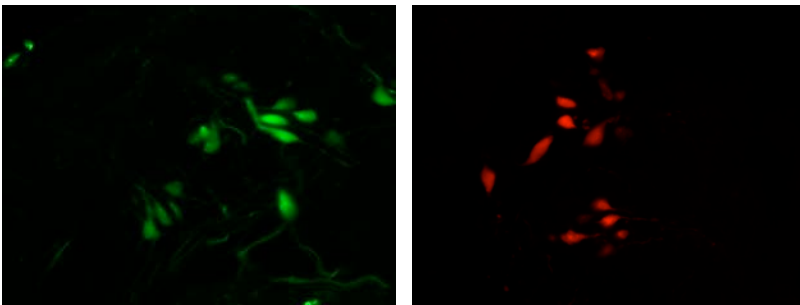


Figure 2. Fluoro-emerald labeled neurons in the adult zebrafish brainstem. In the left panel, fluoro-emerald-positive axons as well as neurons are depicted. In the middle panel, fluoro-emerald-positive neurons in the brainstem are depicted. In the right panel, a number of fluoro-ruby labeled brainstem neurons are shown.

Dr. Vajn is finishing a report on the time-course of retrograde labeling of regenerated brainstem neurons. 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. RNA quality was tested after zinc-fixation protocols. This is important as time will pass during harvesting of neurons and the already collected RNA needs to be stable. The first results have confirmed stable RNA after zinc-fixation, but the brain was less well fixed making the cutting of sections problematic. A number of different zinc-fixation protocols are currently being tested to optimize the end-result, i.e., allowing brainstem cutting as well as proper fixation (stabilization) of brainstem neuron RNA. We are finishing up

the histology for our time-course study of retrograde labeling of regenerated brainstem neurons including staining for CSPGs and axon markers. This will allow correlation of axons in the spinal cord with cells in the brainstem. ISH on zebrafish is being tested to confirm ICC data.

Mo 13-15: Trace brainstem neurons/regenerating axons. Analyze functional data.

Mo 16-18: Analyze axon growth. Write manuscript. Start follow-up experiment.

We employed fluoro-emerald and fluoro-ruby to trace brainstem neurons in adult zebrafish with transected spinal cord (see figure 1 and 2). 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.

Key Research Accomplishments:

Specific Aim 1 (Plunkett Lab)

- (1) Zebrafish neurocan mRNA was found in injured CNS tissues using RT-PCR techniques.
- (2) Myc-tagged full length zebrafish neurocan cDNA cloned into an expression vector to generate pCMV-Myc-NcanB construct.
- (3) Transfection conditions for efficient Myc-NcanB protein production in mammalian 293T cells and zebrafish brain primary cultures were established.
- (4) Myc-NcanB protein purification scheme established using a combination of affinity chromatography and immunoprecipitation.
- (5) Media collected from 293T cells transfected with Myc-NcanB was used as an *in vitro* substrate to examine the behavioral response of zebrafish brainstem neurons to zebrafish neurocan.
- (6) Neurocan mRNA and protein expression was analyzed in adult zebrafish pre- and post-injury.

Specific Aim 2 (Plunkett Lab)

- (1) PTPsigma mRNA expression detected in uninjured brain and spinal cord tissue, as well as in injured brain tissue *in vivo*.
- (2) PTPsigma mRNA expression detected in adult zebrafish brainstem cells *in vitro*.
- (3) Adult zebrafish brainstem cultures were treated with PTPsigma and control morpholinos to determine the effect of morpholino treatment on axon growth over a CSPG-rich environment.
- (4) Data from PTPsigma and control morpholino-treated brainstem cultures was quantified and statistically analyzed.
- (5) Experiments to test the ability of the PTPsigma morpholino to knock down protein expression *in vitro* are underway. A DNA construct to confirm morpholino target specificity is being cloned.

Specific Aim 3 (Oudega Lab)

- (1) Identified retrograde tracers and back-labeled brainstem neurons.
- (2) Isolation of differently labeled neurons.
- (3) High quality yield of RNA from isolated neurons.

(4) Identification proper time point for surgery and tracer injection.

(5). Spinal cord surgeries including tracing for neuron collection for microarrays.

Reportable Outcomes:

- The **2nd Annual Neuroscience Consortium** meeting was held October 1, 2010 at St. Thomas University. This meeting brought in consortium members from University of Pittsburgh to discuss progress of the project. Students and post-docs from both institutes will benefit from the discussions concerning their particular aspect of the project. (Agenda below)



Pictures from the Annual Consortium Meeting

2nd Annual Zebrafish Consortium Meeting

10-01-2010 9:00 AM - 5:00 PM

Rm 111, CCL Science/Technology Bld, St. Thomas University
16400 NW 32nd Ave, Miami, FL 33054. Ph. (305) 628-6572

<p><u>St. Thomas University, Miami, FL:</u> Dr. Jeffery A. Plunkett Dr. Alexis Tapanes-Castillo Students Plunkett laboratory</p>	<p><u>University of Pittsburgh, Pittsburgh, PA:</u> Dr. Martin Oudega Dr. Katarina Vajn</p>
--	---

<u>Time:</u>	<u>Presentation:</u>	<u>Presenter(s):</u>
9:00 - 9:15	Introduction 2 nd ZFM	Dr. Martin Oudega
9:15 – 9:45	Plunkett lab overview	Dr. Alexis Tapanes-Castillo
9:45 – 10:30	Brainstem Culture characterization	Michelle Sierra, Anthony Wood, December Nunez

10:30 – 11:00	L1 Down/Up regulation in cultured brainstem cells	Mam Mboge, Emer Bejuelos
11:00 – 11:15	<i>Coffee break</i>	
11:15 – 11:45	Scar in injured zebrafish spinal cord	Dr. Katarina Vajn
11:45 – 12:30	<u>Discussion:</u> Transition grants, Phase 1 budget	Dr. Jeffrey Plunkett
12:30 – 1:30	<i>Lunch</i>	
1:30 – 2:00	Retrograde tracing in zebrafish spinal cord	Dr. Katarina Vajn
2:00 – 2:30	<u>Discussion:</u> Collecting labeled brainstem cells.	Dr. Katarina Vajn
2:30 – 3:00	<i>Break</i>	
3:00 – 3:30	Neurocan expression following CNS injury	Dr. Jeffrey Plunkett
3:30 – 4:00	Brainstem/CSPG culture	Fran Shabazz
4:00 – 4:45	Conclusions and general discussion	Dr. Jeffrey Plunkett
4:45 - 5:00	Planning/program Third Zebrafish Meeting.	Dr. Martin Oudega

- Members of the Plunkett Lab and Oudega Lab traveled to **The Society for Neuroscience Meeting in San Diego, CA Nov. 13-17, 2010**. Three posters were presented in the *Spinal Cord Injury: Cellular and Molecular Mechanisms: Regeneration Session on Monday Nov. 15th*. The presentations were a complete success and positively reviewed by others in the field.

- Undergraduate students from the Plunkett Lab recently traveled to **the University of Miami Miller School of Medicine, 19th Annual Neuroscience Research Day** and presented 3 posters. This experience was exciting for the students and enabled students to gain presentation experience in front of some of the top neuroscientists from the southeast Florida region.

The role of L1.1 in axonal growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates

Mam M'boqe¹, Emer Bajuelos¹, Francelethia Shabazz¹, Alexis Tapanes-Castillo¹, Katarina Vajn², Martin Oudega², and Jeffery Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL;
²Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

Axon 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. It has been shown that axon growth from zebrafish neurons depends on the presence of the growth-promoting L1 family homing, neuronal adhesion molecule L1.1 (nndl1.1). Our goal is to understand how L1.1 affects the response of zebrafish neurons to CSPGs in vitro. We hypothesize that L1.1 over-expression will result in more neurons crossing into and growing in CSPG areas. Accordingly, reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number of neurons crossing into and growing in CSPG areas.

III. Hypothesis

L1.1 over expression will result in more neurons crossing into and growing in CSPG areas. Accordingly reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number of neurons crossing into and growing in CSPG areas.

V. Results

Delivery of L1.1 morpholino into brainstem neurons

Transfection of brainstem neurons

II. L1.1 Background

Conserved domains of human L1 and zebrafish L1.1 (nndl1.1)

Roles of L1

- Neurite outgrowth, axon fasciculation
- Myelination, cell migration, synaptic function
- Helps promote regenerative growth of mammalian Purkinje cell axons in vivo (Zhang et al., 2003)
- Involved in spinal cord regeneration in adult zebrafish (Becker et al., 2004)

IV. Methods

I. Brainstem culture

1. Coated wells with the following: aminocaproic acid (Protein N18A), 500mg/ml poly-D-lysine (Sigma P7860), 1% Triton X-100 (Sigma L2629)
2. Dissected brainstem tissue, dissociated cells, and plated two brainstems per well.
3. Incubated at 28.5°C in complete media: L15, 2% FBS, Pen/Stap, N2 Supplement.

Gene knockdown experiments

1. Cultured for 48 hours, changed media, and added Endopator (Eton Tools) and morpholinos
2. Lined cultures after 7 days in vitro

L1.1 morpholino: 5'-ATGAAAGAGCCCGACTCCGACAC-3' (Becker et al., 2004)
Control morpholino: 5'-CCCTTACCTGAGTAAATATGAT-3'

Gene overexpression experiments

1. Clone full-length L1.1 cDNA (T. Becker, Univ. of Edinburgh) into Lenti-2A-GFP (V. Lammou, Univ. of Mainz)
2. Transfect primary adult zebrafish brainstem neurons with L1.1 construct or control vector (Lenti and Cherry-2A-GFP) and pMax-GFP using electroporation (Current Transfection protocol: Amata Batis, Nucleofector Kit, Program Cx-1) and 0-05.

II. Immunohistochemistry

Fixed with 4% paraformaldehyde + sucrose. Stained with anti-tubulin (TJ-1+210 (Millipore AB935), DSH Biol. 6G7).

VI. Conclusions

Morpholinos and a GFP expression construct were successfully delivered into primary adult brainstem neurons using Gene Tool's Endopator and Amata electroporation respectively.

VII. Future Directions

- Optimize morpholino delivery and transfection protocols.
- Quantify the effect of up-regulating and down-regulating L1.1 on neuronal morphology.
- Determine how alteration of L1.1 levels affects axon growth over CSPGs.

Neurocan mRNA expression following CNS injury in adult zebrafish (*Danio rerio*)

J.A. Plunkett¹, A. Tapanes-Castillo¹, F. Shabazz¹, K. Vajn² and M. Oudega²

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL;
²Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

It has been established in amphibians and fish that neurons can successfully regenerate their axon in the damaged central nervous system (CNS). This ability often is accompanied by functional recovery. The regenerative ability in amphibian and fish contrasts with that observed in mammals, whose neurons fail to regenerate their axon after 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 thus 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. One of the better known members of this family of growth-inhibitors is neurocan (Ncan). Here, we have expanded our initial observations using molecular biology techniques to investigate the expression profile of Neurocan (Ncan) following CNS injury in adult zebrafish (*Danio rerio*). Using RT-PCR we revealed a specific Ncan mRNA expression pattern in and near damaged CNS tissue. Sequencing of amplified product has confirmed that Ncan is indeed up-regulated in response to CNS insult. Future directions include the development of an alkaline phosphatase-tagged Ncan construct that will be used to investigate specific protein interactions, as well as the response of primary neuronal cultures to purified zebrafish Ncan.

III. Preliminary Data

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

CSPGs are present in the adult fish following brain injury.

V. Experimental Questions?

It is well documented that the CSPG neurocan plays a critical role in the inhibition of regeneration in mammals

Does neurocan affect regeneration in the fish?

Using an RT-PCR strategy, can we amplify the neurocan gene?

II. Introduction to CSPGs

What are CSPGs?
 Major axon growth inhibitory component of the glial scar tissue that prevent successful regeneration.

Known CSPGs in Zebra fish
 Neurocan
 Brevican
 Tenascin

Schematic of the structure of neurocan

Proposed structure of average zebrafish CnG chain

IV. Proposed model for axon regeneration: L1, Neurocan and PTPσ interactions

A. No regeneration

B. Regeneration

Schematic working model of interactions leading to successful axon growth from brainstem neurons in adult zebrafish.

Panel A illustrates a growing axonal process with low L1.1 level and thus few L1.1-L1.1 homophilic interactions (red molecules plus blue stars), growth is blocked by neurocan linked to PTPσ receptor.

Panel B illustrates a growing axonal process with high L1.1 level and thus 'more' L1.1-L1.1 homophilic interactions; growth is NOT blocked the balance between L1.1-mediated growth and neurocan-mediated inhibition has shifted to the L1.1.

VI. Results

Neurocan mRNA is expressed in the adult fish following brain injury.

Zebrafish Neurocan PCR primers
 Forward: CCAGCAATGAGAGTCTTCC
 Reverse: TTTGATGATGCTGCTGCTGCT

Sequencing primers: GAGCAAGATACAGTCCCT

PCR product blasted in NCBI's complete nucleotide collection

VII. Conclusions

- Using an RT-PCR strategy, zebrafish neurocan mRNA was amplified 7 and 14 days following CNS injury.
- DNA sequencing confirmed the identity of our PCR product.

VIII. Future Directions

- Analyze neurocan gene expression following spinal cord injury.
- Examine the effects of zebrafish neurocan on neurite outgrowth
- Determine the role of L1.1, a fish homologue of mammalian L1

IX. Acknowledgements

This work is funded by U.S. Department of Defense grant W81XWH-09-1-0403 to JP.

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

Alexis Tapanes-Castillo¹, Francellethia Shabazz¹, Kararina Vajn², Martin Oudega², Jeffery Plunkett¹
¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL; ²Departments of Physical Medicine & Rehabilitation and Neurobiology and Center for Neuroscience University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

It is well established that axonal growth is inhibited in a complex spinal cord injury (SCI) environment. In the zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are responsible for the inhibitory environment. It has been well documented that CNS axonal growth is inhibited in the injured mammalian spinal cord, thereby contributing to functional motor deficits. Previous work has demonstrated that some mammalian neurons in the adult brainstem regenerate their axons beyond a SCI despite the presence of these inhibitory molecules. The ability to extend axons to all brainstem neurons, although axonal growth is inhibited, suggests a unique ability to regenerate beyond a SCI. To investigate the axon growth response of brainstem neurons to CSPGs, we established a primary neuronal brainstem culture system using GFP-expressing neurons. We investigated the effect of CSPGs on axonal growth in primary neuronal brainstem cultures. We used a GFP-expressing primary neuronal brainstem culture system to investigate the effect of CSPGs on axonal growth in primary neuronal brainstem cultures. We used a GFP-expressing primary neuronal brainstem culture system to investigate the effect of CSPGs on axonal growth in primary neuronal brainstem cultures.

III. Methods: Brainstem culture

1. Zebrafish (Danio rerio) were anesthetized with 0.05% Tricaine and 0.05% Benzocaine.
2. The brainstem was dissected and cultured in DMEM/F12 (1:1) supplemented with 10% FBS.
3. Primary neuronal brainstem cultures were established using GFP-expressing neurons.
4. Cultures were maintained in DMEM/F12 (1:1) supplemented with 10% FBS.
5. Cultures were maintained in DMEM/F12 (1:1) supplemented with 10% FBS.

V. Results: Differential response to CSPGs in culture

Zebrafish brainstem neurons growing on a chondroitin sulfate proteoglycan (CSPG) matrix. The neurons are GFP-expressing (green) and axons are extending through the CSPG matrix (red). The neurons are shown at 7 days in vitro (DIV7) after being cultured with anti-β-tubulin (green) and DAPI (blue).

II. Anatomy of the zebrafish brain

Brainstem nuclei with high regenerative capacity: Nucleus of the medial longitudinal fasciculus (MLF), Magnocellular nucleus (MAG), Intermediate nucleus (IN), Nucleus of the lateral lemniscus (LL), Tegmental nucleus (T).

Brainstem nuclei with low regenerative capacity: Nucleus of the lateral lemniscus (LL), Tegmental nucleus (T).

IV. Results: Preliminary quantification of neuronal outgrowth

Quantification of neuronal outgrowth in primary neuronal brainstem cultures. The graph shows the number of neurons crossing into and growing in CSPG areas under different conditions.

- Undergraduate students from the Plunkett Lab traveled to the **Florida Academy of Sciences Meeting** at FIT on March 11-12, 2011. This was the 75th Anniversary Meeting and the students presented 2 posters. This experience was exciting for the students and enabled students to gain presentation experience in front of some of the top scientists from Florida universities. **Francellethia Shabazz** an undergraduate student/technician from the **Plunkett Lab** won 1st place for “Best Undergraduate Poster”.

The role of L1.1 in axonal growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates

Mam M'boge¹, Emer Bajuelos¹, Francellethia Shabazz¹, Alexis Tapanes-Castillo¹, Kararina Vajn², Martin Oudega², and Jeffery Plunkett¹
¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL; ²Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

Axon 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. It has been shown that axon growth from zebrafish neurons depends on the presence of the growth-promoting L1cam homolog, neuronal adhesion molecule L1.1 (nadh1.1). Our goal is to understand how L1.1 affects the response of zebrafish neurons to CSPGs in vitro. We hypothesize that L1.1 over-expression will result in more neurons crossing into and growing in CSPG areas. Accordingly, reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number of neurons crossing into and growing in CSPG areas. We have successfully delivered morpholino and GFP-expressing constructs into our brainstem neurons using Gene Tools' Endopore and Amara electroporation, respectively. We are currently optimizing procedures to increase the percentage of cells that are morpholino or GFP positive. Once optimized, we will determine how alteration of L1.1 levels within cultured adult brainstem neurons affect axon growth over CSPGs.

III. Hypothesis

L1.1 over expression will result in more neurons crossing into and growing in CSPG areas. Accordingly reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number of neurons crossing into and growing in CSPG areas.

V. Results

Delivery of L1.1 morpholino into brainstem neurons

Transfection of brainstem neurons

II. L1.1 Background

Conserved domains of human L1cam and zebrafish L1.1 (nadh1.1)

Roles of L1

- Neurite outgrowth, axon fasciculation
- Myelination, cell migration, synaptic function
- Helps promote regenerative growth of mammalian Purkinje cell axons in vivo. (Zhang et al., 2005)
- Involved in spinal cord regeneration in adult zebrafish (Becker et al., 2004)

IV. Methods

I. Brainstem culture

1. Control wells with the following: microtubule (Phalloidin, 500mg/ml poly-D-lysine (Sigma P7986), 1.7µg/ml Laminin (Sigma L2020).
2. Dissociated brainstem tissue, dissociated cells, and plated two brainstems per well.
3. Incubated at 28.5°C in complete media: L15, 2% FBS, Pen/Stp, N2 Supplement.

Gene knockdown experiments

1. Cultured for 48 hours, changed media, and added Endopore (Gene Tools) and morpholino.
2. Used 24h culture after 7 days in vitro.

L1.1 morpholino: 5'-ATGAAAACAGCCCGACCTCAGACA-3' (Oikar et al., 2009)

Control morpholino: 5'-ACCTTCTACTGATTC-3'

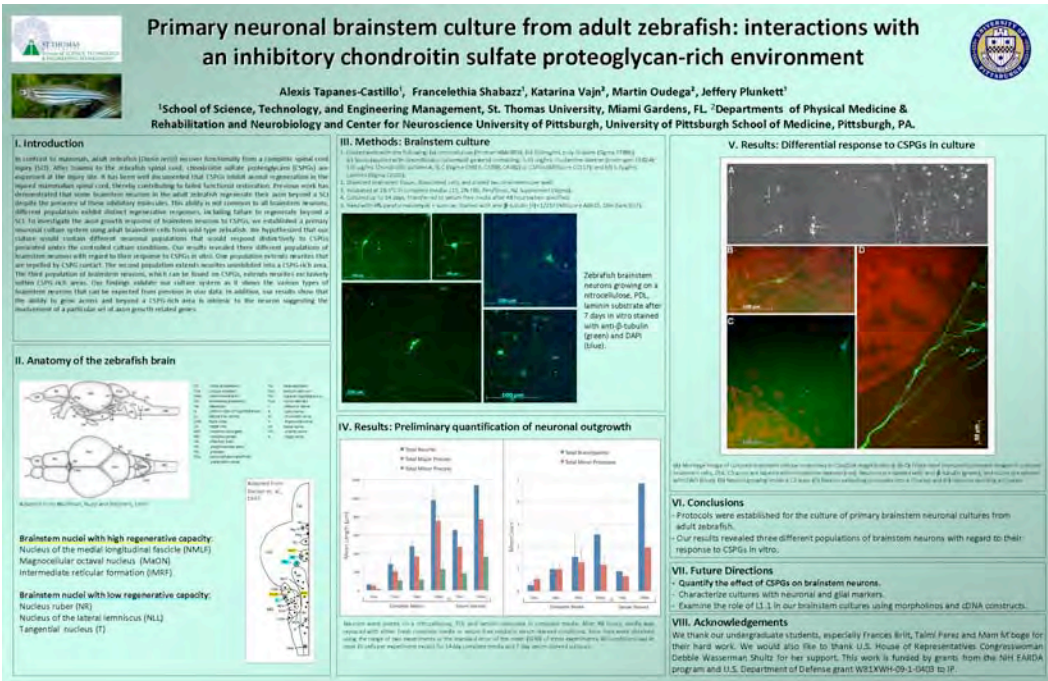
Gene overexpression experiments

1. Clone full-length L1.1 cDNA (T. Becker, Univ. of Edinburgh) into Lenti-2A-EGFP (V. Lantieri, Univ. of Miami).
2. Transfect primary adult zebrafish brainstem neurons with L1.1 construct or control vector (Lentiviral-hygro-2A-EGFP and pMax-GFP) using electroporation.

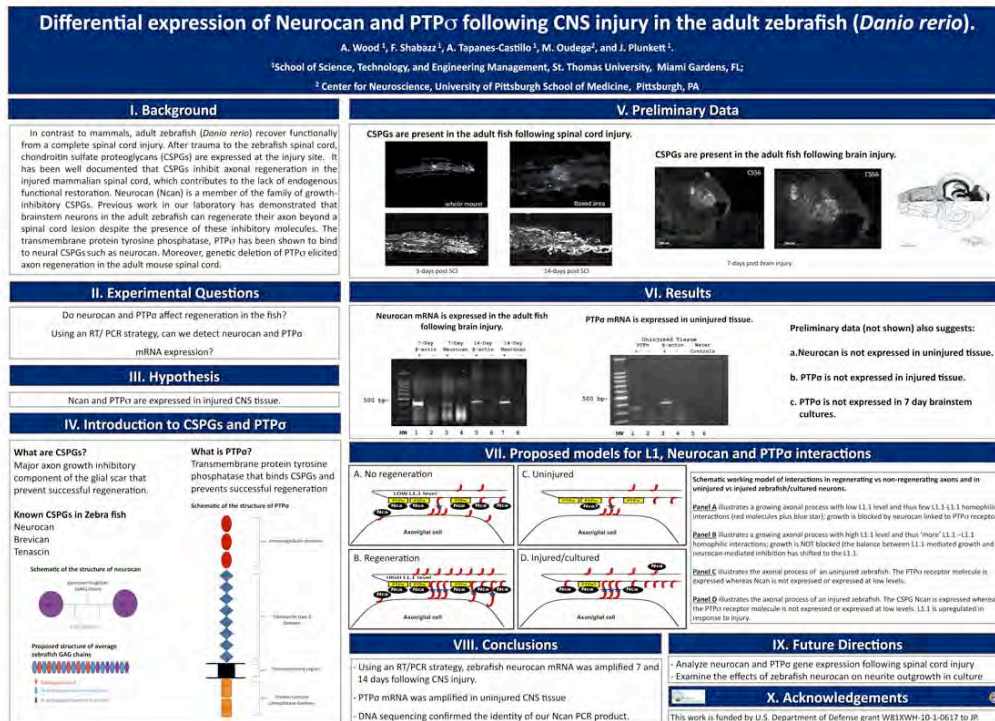
Current translation protocol: Amara Basic Nucleofector Kit, Programs G-13 and G-43.

II. Immunohistochemistry

Fixed with 4% paraformaldehyde + sucrose. Stained with anti-tubulin (TJ-1-250 (Millipore AB913), DAPI (Molecular BioLabs)).



- Students from the Plunkett Laboratory presented five posters and an oral presentation at the 6th Annual Southeast Cell Science Undergraduate Research Symposium held April 16th, 2011 in Miami, Florida.



Molecular characterization of primary neuronal cultures from adult zebrafish

M. SIERRA¹, H. MATA¹, F. SHABAZZ¹, A. TAPANES-CASTILLO¹, M. OUDEGA², and J. PLUNKETT¹. ¹School of Science, Technology, and Engineering Management, St. Thomas University, 16401 NW 37th Avenue, Miami Gardens, FL 33054; ²Center for Neuroscience, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15213.

I. Introduction

Unlike mammals, adult zebrafish (*Danio rerio*) have the remarkable ability to recover function following a complete spinal cord injury (SCI). Our lab has established a primary adult zebrafish brainstem neuronal culture model to study this regenerative response *in vitro*. Our goal is to molecularly characterize these brainstem cultures, as well as to identify and quantify the different cell populations present. We are currently working towards a base-line analysis of cells grown in serum-containing media. We hypothesize that our primary adult zebrafish brainstem cultures contain different types of cells that can be identified with different antibodies. We have tested a panel of antibodies recognizing proteins expressed in differentiating neurons, proliferating cells, and glia. Our data revealed populations of cells that expressed: (1) the neuronal specific marker HuC (Human neuronal protein C), (2) the regeneration-associated protein GAP43 (Growth associated protein 43), and (3) the cellular proliferation marker PCNA (Proliferating cell nuclear antigen) positive cells. Preliminary results also indicate a neuronal population that expresses the serotonergic marker, TPH2 (Tryptophan hydroxylase).

III. Methods

- Coated wells with the following: (a) Nitrocellulose (Protran N80A955), (b) 500 mg/ml poly-D-lysine (Sigma P7886), (c) 1.7 µg/ml Laminin (Sigma L2020)
- Dissected brainstem tissue, dissociated cells, and plated two brainstems per well.
- Incubated at 28.5°C in complete media containing: L15, 2% Fetal Bovine Serum, Pen/Step, N2 Supplement (Sigma)
- Cultured seven days
- Fixed with 4% PFA
- Immunohistochemistry: Anti-β-tubulin (rabbit) [F] = 1/250 (Sigma), Anti-HuC (mouse) [F] = 1/500 (Molecular Probes), Anti-PCNA (mouse) [F] = 1/2000 (Sigma), Anti-TPH2 (rat) [F] = 1/8 (Sigma). Secondary antibodies include: Goat Anti-Mouse Alexa 554, Goat Anti-Rabbit Alexa 488 + DAPI.

IV. Results

GAP 43 Positive: Regeneration Marker

Figure 1. A strong GAP 43 signal, indicator of axonal growth (DAPI - blue, GAP 43 - green)

PCNA Positive: Proliferation Marker

Figure 2. PCNA staining shows proliferating cell nuclei (DAPI - blue, PCNA - green)

HuC Positive: Neuronal Marker

Figure 3. HuC staining in neurons (DAPI - blue, HuC - red, β-tubulin - green)

Positive TPH2: Serotonin Pathway Marker

Figure 4. Three channel image of TPH2 staining neurons (DAPI - blue, TPH2 - red, β-tubulin - green)

Figure 5. No primary antibody (anti-TPH2 control) demonstrates antibody specificity

II. Anatomy of the Zebrafish Brain

Adapted from Beckler et al., 2007

Adapted from Williams, Bupp and Reichert, 1996

Brainstem nuclei with high regenerative capacity:
Nucleus of the medial longitudinal fascicle (NMLF)
Magnocellular octaval nucleus (MaoN)
Intermediate nuclear formation (IMF)

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

V. Conclusions

- Growth-Associated Protein-43 (GAP-43) shows strong signals in the nucleus and neuronal processes.
- Anti-Proliferating Cell Nuclear Antigen (PCNA) indicates proliferating cells in the cultures.
- Human Neuronal Protein (HuC) reveals cells that have differentiated into neurons.
- Tryptophan hydroxylase-2 (TPH2) indicates serotonergic cells.

VI. Future Directions

- To determine which cells belong to the group of high or low regenerative capacity.
- To determine the percent of positive cells in culture.
- Testing additional antibodies for further characterization projects.

VII. Acknowledgments

This work is funded by grants from the NIH/EARDA program and U.S. Dept. of Defense grant W81XWH-10-1-0617 to JP.

The effect of L1.1 down regulation in axonal growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates

Mam M Huggel¹, Francalotta Stralozzi¹, Alexis Tapanes-Castillo¹, Katarina Vajce², Martin Oudega², and Jeffery Plunkett¹
¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL;
²Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

Following spinal cord injury, the mammalian central nervous system is unable to regenerate descending axons past the injury site and regain motor functions. This loss of motor function leads to paralysis. In contrast, the zebra fish used as a model organism has the ability to regenerate after induced spinal cord transection and regain most of its motor functions. The regenerative capacity of neurons is influenced by the balance of both growth promoting and growth inhibiting molecules. In mammals, Chondroitin Sulfates (CSPGs) have been found at the injury site and shown to have an inhibitory effect in axonal regrowth. In contrast to mammals, zebra fish have the ability to re-grow axons past the injury even in the presence of CSPG molecules. This ability is intrinsic to regenerating neurons, and it entails the expression of a definite set of genes. We are currently trying to identify genes involved in this regenerative process. A gene of interest is L1.1, which has been found to be up regulated during axonal regeneration in the zebra fish, and has been hypothesized to contribute to successful re-growth of axons. We are currently using a morpholino based approach to knock down the expression of L1.1 in primary zebrafish brainstem neurons and to study their response to CSPGs *in vitro*. The prediction is that L1.1 gene knockdown will decrease the intrinsic ability of neurons to regenerate an axon over an inhibitory CSPG terrain.

IV. Methods

Brainstem cultures

- Coated wells with the following: nitrocellulose (Protran N80A, 500mg/ml poly-D-lysine (Sigma P7886), 1.7µg/ml Laminin (Sigma L2020). Spots (labeled with GraceBioLabs Culture well gaskets) containing 0.1mg/ml rhodamine-123 (Invitrogen D1824) (1µg/ml CSPGs (Millipore CC117).
- Dissected brainstem tissue, dissociated cells, and plated two brainstems per well.
- Incubated at 28.5°C in complete media: L15, 2% FBS, Pen/Step, N2 Supplement.

Gene knockdown experiments

1. Cultured for 48 hours, changed media, and added Endoporpher (Gene Tools) and morpholino. Ended cultures after 7 days *in vitro*.

L1.1 morpholino: 5'-ATGAAACAGCCCGACTCCAGACA-3' (Becker et al., 2004)

Control morpholino: 5'-CCCTTACCCTCAGTACAAATATA-3'

Immunohistochemistry

Fixed with 4% paraformaldehyde + sucrose. Stained with anti-tubulin [F]=1/250.

VI. Quantification

Figure 1. Significant differences in axonal formation groups at ETag CSPG (n=200 cells, JP + 0.05, n=200 cells, JP + 0.05, n=200 cells, JP + 0.05).

Figure 2. Morpholino significantly affected response groups at ETag CSPG (n=200 cells, JP + 0.05, n=200 cells, JP + 0.05, n=200 cells, JP + 0.05).

Data were collected from four independent experiments (190 cells counted). Chi square non parametric tests were performed using IqTast statistics software.

II. Background

Roles of L1

- Neurite outgrowth, axon fasciculation
- Myelination, cell migration, synaptic function
- Helps promote regenerative growth of mammalian Purkinje cell axons *in vivo* (Zhang et al., 2005)
- Involved in spinal cord regeneration in adult zebrafish (Becker et al., 2004)

Structure of Zebrafish L1.1 (aa111-1caaa)

V. Results

Confirmation of L1.1 morpholino delivery

Differential response to CSPGs in culture

Figure 1. Axonal growth of zebrafish brainstem neurons treated with Endoporpher and L1.1.1 morpholino (A-C) (individual neurons from three independent experiments). (A) Control neurons are similar to wild type (DAPI, anti-HuC, anti-L1.1). (B) Morpholino treated neurons are similar to wild type. (C) Morpholino treated neurons are similar to wild type. (D) Control neurons are similar to wild type. (E) Morpholino treated neurons are similar to wild type. (F) Morpholino treated neurons are similar to wild type. (G) Morpholino treated neurons are similar to wild type. (H) Morpholino treated neurons are similar to wild type. (I) Morpholino treated neurons are similar to wild type. (J) Morpholino treated neurons are similar to wild type. (K) Morpholino treated neurons are similar to wild type. (L) Morpholino treated neurons are similar to wild type. (M) Morpholino treated neurons are similar to wild type. (N) Morpholino treated neurons are similar to wild type. (O) Morpholino treated neurons are similar to wild type. (P) Morpholino treated neurons are similar to wild type. (Q) Morpholino treated neurons are similar to wild type. (R) Morpholino treated neurons are similar to wild type. (S) Morpholino treated neurons are similar to wild type. (T) Morpholino treated neurons are similar to wild type. (U) Morpholino treated neurons are similar to wild type. (V) Morpholino treated neurons are similar to wild type. (W) Morpholino treated neurons are similar to wild type. (X) Morpholino treated neurons are similar to wild type. (Y) Morpholino treated neurons are similar to wild type. (Z) Morpholino treated neurons are similar to wild type.

Figure 2. Morpholino (L1.1) gene knockdown significantly affected response to CSPG. The addition of CSPG to neurons causes a difference in the phenotype behavior of cells treated with L1.1 morpholino and control neurons. Different populations of cells showed different responses to CSPG.

VII. Conclusion


- We are the first to report successful morpholino delivery into primary adult zebrafish brainstem neurons.
- L1.1 morpholino treatment affected how neurons respond to CSPGs *in vitro*.
- Data suggests wild type levels of L1.1 gene expression are necessary for neurite outgrowth *in vitro* over an inhibitory CSPG terrain.

VIII. Future directions

- Perform replication studies to confirm the effect of the L1.1 morpholino on neurite growth over CSPGs.
- Analyze the effect of L1.1 knock down on the morphology of cells.
- Western blot analysis to confirm L1.1 protein knock down.
- Introduce L1.1 as a substrate in the adult zebrafish brain stem culture.

VIII. Acknowledgments

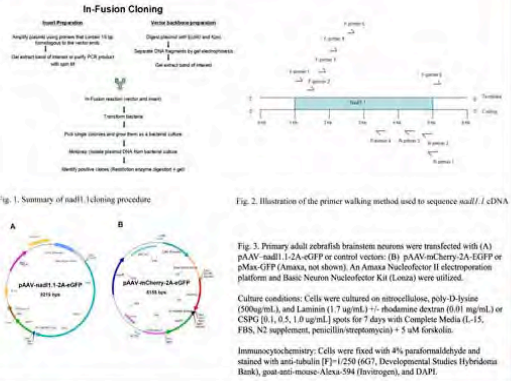
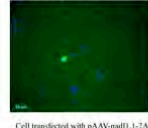
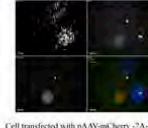

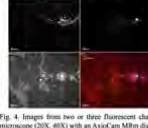
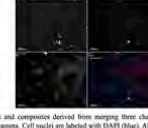
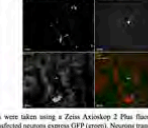
This work is funded by U.S. Department of Defense grant W81XWH-10-1-0617 to J.P. I would like to thank all the Plunkett lab students for their contributions to this project.



The role of L1.1 over-expression in axonal growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates

Emer Bajuelos¹, Francilethia Shabazz¹, Alexis Tapanes-Castillo¹, Martin Oudega², and Jeffery Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL;
²Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA.

<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">I. Introduction</h3> <p style="font-size: 8px;">Axon 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. It has been shown that axon growth from zebrafish neurons depends on the presence of the growth-promoting L1 family homology neuronal adhesion molecule L1.1 (nadh1.1). Our goal is to understand how L1.1 affects the response of zebrafish neurons to CSPGs <i>in vitro</i>.</p>	<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">II. Hypothesis</h3> <p style="font-size: 8px;"><i>nadh1.1</i> over-expression will result in more neurons crossing into and growing in CSPG areas.</p> <h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">III. Specific Aims</h3> <p style="font-size: 8px;">Aim 1: Build dual <i>nadh1.1</i>-eGFP expression vector Aim 2: Transfect vector into adult zebrafish primary brainstem neurons Aim 3: Determine how L1.1 over-expression affects how cells respond to CSPGs</p>	<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">IV. nadh1.1 and L1cam</h3> <p style="font-size: 8px;">• Cell migration • Neurite outgrowth • Axon fasciculation • Promote regenerative growth of mammalian Purkinje cell axons <i>in vivo</i> • Involved in spinal cord regeneration in adult zebrafish</p> <div style="font-size: 8px;"> <p>Human 1257 amino acids</p> <p>Zebrafish 1269 amino acids</p> <p>Immunoglobulin-like superfamily domain</p> <p>Fibronectin domain</p> </div>
<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">V. Methods</h3> <p style="font-size: 8px;">Full-length <i>nadh1.1</i> cDNA was cloned into a dual expression pAAV-eGFP vector. Sanger sequencing was performed (GeneWiz Laboratories) to verify the <i>nadh1.1</i> cDNA sequence.</p> <div style="font-size: 8px;"> <p>In-Fusion Cloning</p> <p>Insert Preparation: Analyze linearizing restriction sites (18 bp long) in the vector. Use a 30 bp overlap of insert and vector PCR product with each end.</p> <p>Vector backbone preparation: Digest (NotI and XbaI) and ligate. Separate DNA fragments to get monomers. Clean up (separate) insert.</p> <p>In-Fusion reaction (vector and insert): Transform bacteria.</p> <p>Pick single colonies and grow them in a bacterial culture. Minimize antibiotic pressure. Extract plasmid. Identify positive clones. Characterize sequence (sequencing).</p> </div> 	<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">VI. Results</h3> <div style="display: flex; justify-content: space-around;"> <div style="text-align: center; font-size: 8px;"> <p>Cell transfected with pAAV-nadh1.1-2A-eGFP plated on Laminin</p>  </div> <div style="text-align: center; font-size: 8px;"> <p>Cell transfected with pAAV-mCherry-2A-eGFP plated on Laminin</p>  </div> <div style="text-align: center; font-size: 8px;"> <p>Cell transfected with pMax-GFP plated on Laminin</p>  </div> </div> <div style="display: flex; justify-content: space-around; font-size: 8px;"> <div style="text-align: center;"> <p>Cell transfected with pAAV-nadh1.1-2A-eGFP approaching CSPGs (1 µg/ml)</p>  </div> <div style="text-align: center;"> <p>Cell transfected with pAAV-mCherry-2A-eGFP repellent CSPGs</p>  </div> <div style="text-align: center; font-size: 8px;"> <p>Cell transfected with pAAV-mCherry-2A-eGFP growing in a rhodamine dextran spot</p>  </div> </div> <p style="font-size: 8px;">Fig. 4. Images from two or three fluorescent channels and composites derived from merging three channels were taken using a Zeiss Axiovert 2 Plus fluorescent microscope (20X, 40X) with an AxioCam M10 digital camera. Cell nuclei are labeled with DAPI (blue). All transfected neurons express GFP (green). Neurons transfected with pAAV-MCherry-2A-eGFP additionally express Mcherry (red). Neurons are labeled with an anti-tubulin antibody and Alexa-594 fluorophore (only). CSPG areas are labeled with rhodamine dextran (red).</p>	
<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">VII. Conclusions</h3> <p style="font-size: 8px;">• <i>nadh1.1</i> cDNA-GFP expressing vector was successfully constructed (pAAV-nadh1.1-2A-eGFP). • We are the first to report successful transfection of adult zebrafish primary brainstem neurons.</p>		
<h3 style="text-align: center; background-color: #e67e22; color: white; padding: 2px;">VIII. Future Directions</h3> <p style="font-size: 8px;">• Determine transfection efficiency. • Quantify the effect of up-regulating <i>nadh1.1</i> on neuronal morphology. • Determine how <i>nadh1.1</i> over-expression affects axon growth over CSPGs.</p>		

- Students from the Plunkett Laboratory presented five posters at the **3rd Annual STEM Symposium** held at Barry University in Miami, Florida on April 20th, 2011. Students from the Plunkett Laboratory won 2 individual awards for their presentations.

- **Mam M'boge** and **Emer Bajuelos** from the **Plunkett Lab** graduated with honors from St. Thomas University in May 2011. Mam graduated with a BS in Biology and is currently employed as a research technician and planning to attend graduate school within the next year. Emer was a double major in Chemistry and Biology and is currently studying for the MCAT and working at the USDA conducting research. Both students also completed undergraduate theses in the Plunkett Laboratory

- **SRI, Summer Research Institute at St. Thomas University 2011** 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** is currently hosting 9 St. Thomas and one Miami-Dade College student for the summer. The Plunkett lab is also hosting a visiting student to the St. Thomas campus from Stevenson University in Maryland.

- **Undergraduate students** from the **Plunkett Lab** traveled to the **Society for Neuroscience Meeting** in Washington D.C. from Nov. 12-16, 2011. Students presented 2 posters. This experience was exciting for the students and enabled students to gain presentation experience in front of some of the top scientists. Francilethia Shabazz, Jill Woodward and Megan Staudenmaier undergraduate students from the Plunkett Lab attended this meeting.

Characterization of Primary Adult Zebrafish Brainstem Neuronal Cultures and their Interactions with an Inhibitory Chondroitin Sulfate Proteoglycan-rich Environment

Jeffery Plunkett¹, Alexis Tapanes-Castillo¹, Françoise Shabazz², Katarina Vajic¹, Martin Oudega¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL; ²Departments of Physical Medicine & Rehabilitation and 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 in the injured mammalian spinal cord. However, zebrafish regenerate their axons beyond the injury site despite the presence of CSPGs. This regenerative ability is not characteristic of all brainstem neurons. Furthermore, we have found that primary adult zebrafish brainstem neurons exhibit different cellular responses to CSPGs in vitro. The L1 cell adhesion molecule-bearing, neuronal adhesion molecule 1.1 (nAd1.1), has been shown to contribute to axonal growth and locomotor recovery during zebrafish spinal cord regeneration (Becker et al., 2004). Our goal is to understand how nAd1.1 affects the response of zebrafish neurons to CSPGs in vitro.

II. Anatomy of the zebrafish brain

Midline longitudinal tract (MLT)
Midline longitudinal tract (MLT)

Lateral longitudinal tract (LLT)
Lateral longitudinal tract (LLT)

III. Methods: Adult brainstem culture

1. Craniotomy and dissection of zebrafish (Danio rerio) brainstem.
2. Dissection of brainstem tissue and preparation of primary brainstem neurons.
3. Culture of primary brainstem neurons on L1cam-Fc and Neurocan substrates.
4. Quantification of neurite outgrowth and cell viability.

IV. Adult brainstem culture

V. Preliminary quantification of neurite outgrowth

VI. Differential response to CSPGs in culture

VII. Preliminary quantification of cellular response to CSPGs

Cell Type	Response to CSPGs
MLT	High
LLT	Low

VIII. Characterization antibodies

Antibody	Target	Application
Anti-nAd1.1	Neuronal adhesion molecule 1.1	Western blot, Immunofluorescence
Anti-L1cam-Fc	L1 cell adhesion molecule	Immunofluorescence
Anti-Neurocan	Neurocan	Immunofluorescence

IX. Molecular Characterization

X. Summary

Primary adult zebrafish brainstem neurons exhibit different cellular responses to CSPGs in vitro: (1) inhibition and (2) ability to grow on an inhibitory substrate across a CSPG environment.

XI. Future Directions

Quantify percentage of each cell type (neuron, glia, positive stem/progenitor cells) in our brainstem cultures.

Further quantify neuronal response to CSPGs. Determine how response is influenced by cell-cell interactions and exposure profiles.

Explore the role of axon growth and regenerative genes in our brainstem cultures using morpholino and overexpression.

IX. Molecular Characterization

X. Summary

Primary adult zebrafish brainstem neurons exhibit different cellular responses to CSPGs in vitro: (1) inhibition and (2) ability to grow on an inhibitory substrate across a CSPG environment.

XI. Future Directions

Quantify percentage of each cell type (neuron, glia, positive stem/progenitor cells) in our brainstem cultures.

Further quantify neuronal response to CSPGs. Determine how response is influenced by cell-cell interactions and exposure profiles.

Explore the role of axon growth and regenerative genes in our brainstem cultures using morpholino and overexpression.

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

Alexis Tapanes-Castillo¹, Françoise Shabazz¹, Mam Mbogo¹, Emer Bajajoch¹, Katarina Vajic¹, Martin Oudega¹, and Jeffery A. Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL; ²Center for Neuroscience, University of Pittsburgh School of Medicine, Pittsburgh, PA

I. Introduction

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 regenerative ability is not characteristic of all brainstem neurons. Furthermore, we have found that primary adult zebrafish brainstem neurons exhibit different cellular responses to CSPGs in vitro. The L1 cell adhesion molecule-bearing, neuronal adhesion molecule 1.1 (nAd1.1), has been shown to contribute to axonal growth and locomotor recovery during zebrafish spinal cord regeneration (Becker et al., 2004). Our goal is to understand how nAd1.1 affects the response of zebrafish neurons to CSPGs in vitro.

II. Experimental Questions

Do L1.1 expression levels affect how neurons respond to CSPGs?

III. Hypothesis

Reduced nAd1.1 expression will decrease axonal outgrowth, as well as the number of neurons crossing into and growing in CSPG areas. Conversely, nAd1.1 over-expression will increase axonal growth and result in more neurons crossing into and growing in CSPG areas.

Working model

IV. Methods

Substrate preparation

L1cam-Fc, Zebrafish Neurocan B

Cellular responses to CSPGs

V. L1 knockdown experiments

Confirmation of L1.1 knockdown delivery

Adult zebrafish brainstem neurons were transfected with control morpholino (MORPH) or nAd1.1 morpholino (MORPH). Cell viability was assessed using propidium iodide (PI) staining. Cell viability was assessed using propidium iodide (PI) staining.

VI. L1 overexpression experiments

Expression constructs

Transfection of adult zebrafish primary brainstem neurons

VII. Summary

Morpholino delivery protocol established.

nAd1.1 cDNA, GFP dual expression vector prepared.

Successful transfection of adult zebrafish brainstem cultures with control and nAd1.1 cDNA expression vectors.

Preliminary experiments analyzing the effect of nAd1.1 morpholino treatment on brainstem neurons interacting with CSPGs performed and quantified.

Completed L1cam-Fc and Neurocan substrate preparation.

VIII. Future Directions

Perform morpholino and overexpression experiments on L1cam-Fc and Neurocan substrates.

Analyze nAd1.1 protein levels in vitro after knockdown and overexpression.

Determine whether nAd1.1 protein levels affect how neurons respond to CSPGs.

IX. Acknowledgements

This work is funded by U.S. Department of Defense grant W81XWH-02-1-0017 to J.P.

IV. Methods

Substrate preparation

L1cam-Fc, Zebrafish Neurocan B

Cellular responses to CSPGs

V. L1 knockdown experiments

Confirmation of L1.1 knockdown delivery

Adult zebrafish brainstem neurons were transfected with control morpholino (MORPH) or nAd1.1 morpholino (MORPH). Cell viability was assessed using propidium iodide (PI) staining. Cell viability was assessed using propidium iodide (PI) staining.

VI. L1 overexpression experiments

Expression constructs

Transfection of adult zebrafish primary brainstem neurons

VII. Summary

Morpholino delivery protocol established.

nAd1.1 cDNA, GFP dual expression vector prepared.

Successful transfection of adult zebrafish brainstem cultures with control and nAd1.1 cDNA expression vectors.

Preliminary experiments analyzing the effect of nAd1.1 morpholino treatment on brainstem neurons interacting with CSPGs performed and quantified.

Completed L1cam-Fc and Neurocan substrate preparation.

VIII. Future Directions

Perform morpholino and overexpression experiments on L1cam-Fc and Neurocan substrates.

Analyze nAd1.1 protein levels in vitro after knockdown and overexpression.

Determine whether nAd1.1 protein levels affect how neurons respond to CSPGs.

IX. Acknowledgements

This work is funded by U.S. Department of Defense grant W81XWH-02-1-0017 to J.P.

-Abstracts that were presented at the Society for Neuroscience Meeting in Washington, D.C. in November 2011 including Oudega group.

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. Vajn¹, D. Suler¹, A. Betz¹, A. Tapanes-Castillo³, F. Shabazz³, J.A. Plunkett³, M. Oudega^{1,2}

Departments of ¹Physical Medicine & Rehabilitation and ²Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; ³Department 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.

- A poster was also presented at the **American Society for Cell Biology in Denver, Colorado December 3-7, 2011**. **Dr. Alexis Tapanes-Castillo** was awarded a \$1,500.00 MAC (Minority Affairs Committee) scholarship to attend this meeting and was also honored with a third place award for Post-doc best poster. Also, two undergraduate students from the **Plunkett lab** were also granted authorship due to their significant contributions to the work.

-Abstract presented at **The American Society for Cell Biology**

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

Alexis Tapanes-Castillo, Megan Staudenmaier, Anthony Wood, Francelethia Shabazz, Katarina Vajn, Martin Oudega, Jeffery A. Plunkett

In the mammalian central nervous system (CNS), the transmembrane protein tyrosine phosphatase PTPsigma was recently identified as a receptor for chondroitin sulfate proteoglycans (CSPGs), such as neurocan, which inhibits axon regeneration following an injury. The goal of our project is to elucidate the role of PTPsigma in zebrafish 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 zebrafish CNS 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 ptprsa interactions with CSPGs through the evaluation of ptprsa expression in adult zebrafish brainstem-derived primary neuron cultures. We observed ptprsa mRNA expression in uninjured brain and spinal cord tissue as well as in injured brain tissues. We have also detected ptprsa mRNA expression in brainstem neuron 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.*

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

Alexis Tapanes-Castillo¹, Megan Staudenmaier¹, Anthony Wood¹, Francellethia Shabazz¹, Katarina Vajn², Martin Oudega², and Jeffery A. Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL
²Departments 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 neuron, a disordered sulfate proteoglycan (CSPG) which inhibits axon regeneration following an injury. The goal of our project is to elucidate the role of PTPsigma in zebrafish 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 Transcriptase Polymerase Chain Reactions (RT-PCR). We observed ptprsa mRNA expression in uninjured brain and spinal cord tissue, as well as in 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).

IV. Proposed model for axonal regeneration

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

(i) A growing axonal process with low levels of growth-promoting neuronal adhesion molecules (L1, ephrins, and Wnt3) and high levels of PTPs. There are few L1/L1, ephrin/ephrin, and Wnt3/Wnt3 interactions. Axonal growth is blocked. There are also L1/L1, ephrin/ephrin, and PTP/Axonal interactions. Axonal growth is blocked.

(ii) A growing axonal process with high L1/L1 and low PTPs levels. While there are some L1/L1 interactions, there are fewer L1/L1, ephrin/ephrin, or Wnt3/Wnt3 interactions. Axonal growth is not blocked.

VII. In vitro ptprsa expression

Adult zebrafish brainstem neurons differentially respond to CSPGs

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

Neurons labeled with anti-laminin (green) and neurofilament (red). Zebrafish culture plate labeled with neurofilament (green) (A).
Neurons labeled with anti-laminin (green) and ptprsa (red) (B).
Percent of cells with specific response to CSPGs (1 right), N1 independent experiments (100 cells).

RT-PCR reactions after 7 days in vitro.

VIII. 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 axons.

As observed in untreated cultures, morpholino treated cultures exhibit different cellular responses to CSPGs. Control morpholino treated cells (A), lacking any form and (B) achieving a neurite crossing into the CSPG environment. Primary outgrowth of ptprsa morpholino treated cells (C) exhibiting a neurite into the CSPG axon and (D) interacting with CSPGs.

IX. 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.

X. 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.

Quantify the effect of ptprsa morpholino treatment on brainstem neurons interacting with purified zebrafish Neurocan.

Acknowledgments: We thank David Gomez and Jacqueline Hufnagel for their contributions. This work is funded by CTS, Department of Defense grant #W81XWH-07-2-0071 to JP.

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?

VI. In vivo neurocan expression

Neurocan 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 of control RT-PCR reactions. 100% neuronal weight. Ptpsa cDNA was confirmed through sequencing.

- Dr. Alexis Tapanes-Castillo and Dr. Plunkett traveled to the the 45th Annual Winter Conference on Brain Research in Salt Lake City, UT. from Jan. 21-26, 2012. Two posters were presented. This meeting allowed Dr. Tapanes-Castillo and Dr. Plunkett the opportunity to network with several top scientist including Dr. Herb Geller from the NIH, Dr. James Fawcett from Cambridge, Dr. Joel Levine from SUNY Stonybrook, Ron Meyer from UC Irvine among others. This was an excellent opportunity to discuss our results with top scientists from across the globe.

45

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

Alexis Tapanes-Castillo¹, Francellethia Shabazz¹, Katarina Vajn², Martin Oudega², Jeffery Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL; ²Departments of Physical Medicine & Rehabilitation, Neurobiology, and Center for Neuroscience, 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 by adsorbing to brainstem neurons and forming a physical barrier to axonal growth. The ability to regenerate beyond a spinal cord injury site depends on the presence of CSPGs. The ability to regenerate beyond a spinal cord injury site depends on the presence of CSPGs. The ability to regenerate beyond a spinal cord injury site depends on the presence of CSPGs.

II. Anatomy of the zebrafish brain

Brainstem nuclei with high regenerative capacity:
Nucleus of the medial longitudinal fasciculus (NMLF)
Nucleus of the reticular formation (NRF)
Nucleus of the reticular formation (NRF)

Brainstem nuclei with low regenerative capacity:
Nucleus of the reticular formation (NRF)
Nucleus of the reticular formation (NRF)

III. Methods: Adult brainstem culture

1. Crude cells with the following:
 - (a) 100,000 cells (100,000 cells)
 - (b) 100,000 cells (100,000 cells)
 - (c) 100,000 cells (100,000 cells)
 - (d) 100,000 cells (100,000 cells)
 - (e) 100,000 cells (100,000 cells)
 - (f) 100,000 cells (100,000 cells)
 - (g) 100,000 cells (100,000 cells)
 - (h) 100,000 cells (100,000 cells)
 - (i) 100,000 cells (100,000 cells)
 - (j) 100,000 cells (100,000 cells)
 - (k) 100,000 cells (100,000 cells)
 - (l) 100,000 cells (100,000 cells)
 - (m) 100,000 cells (100,000 cells)
 - (n) 100,000 cells (100,000 cells)
 - (o) 100,000 cells (100,000 cells)
 - (p) 100,000 cells (100,000 cells)
 - (q) 100,000 cells (100,000 cells)
 - (r) 100,000 cells (100,000 cells)
 - (s) 100,000 cells (100,000 cells)
 - (t) 100,000 cells (100,000 cells)
 - (u) 100,000 cells (100,000 cells)
 - (v) 100,000 cells (100,000 cells)
 - (w) 100,000 cells (100,000 cells)
 - (x) 100,000 cells (100,000 cells)
 - (y) 100,000 cells (100,000 cells)
 - (z) 100,000 cells (100,000 cells)
2. Dissolved brainstem tissue, dissolved cells, and dissolved cells.
3. Standard at 37 °C in complete media (L15, DMEM, F12, Neurobasal, B27, and B27).
4. Culture at 37 °C in complete media (L15, DMEM, F12, Neurobasal, B27, and B27).

Acknowledgments: This work is funded by grants from the NIH (R01NS081919) and U.S. Department of Defense grant W81XWH-11-1-0465 to JP.

IV. Adult brainstem culture

V. Differential response to CSPGs in culture

VI. Characterization of Cellular Populations

VII. Model for axonal growth over CSPGs

VIII. Molecular Mechanisms

IX. Summary

Protocols were established for the culture of primary brainstem cells from adult zebrafish.

Brainstem cultures exhibited different cellular responses to CSPGs in vitro: (1) inhibition and (2) the ability to grow on a coated astrocyte-like processes across a CSPG environment.

X. Future Directions

Determine how CSPG response correlates with cell type and is influenced by cell-cell interactions.

Explore the role of NMLF, NRF, and NRF in our zebrafish cultures using morpholinos and overexpression.

Winter Brain Posters

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

Jeffery A. Plunkett¹, Alexis Tapanes-Castillo¹, Francellethia Shabazz¹, Katarina Vajn² and Martin Oudega²

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami, FL; ²Departments of Physical Medicine & Rehabilitation and Neurobiology, 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 axons 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 and its putative receptor, receptor-type protein tyrosine phosphatase sigma 2 (ptpsr2), are expressed in the CNS pre- and post-injury. Our overall objective is to understand the molecular mechanisms underlying the CSPG interactions of the regenerative neurons. 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-11-1-0465 to JP.

II. Experimental Question # 1

Are CSPGs present in the adult fish following CNS insult?

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

CSPGs are present in the adult fish following brain injury.

III. Proposed model for axonal regeneration

IV. Experimental Question # 2

What role does neurocan and PTPs play the CNS regenerative response seen in fish?

V. *In vivo* neurocan expression

VI. *In vivo* ptpsr2 expression

VII. Regenerative nuclei tracing *in vivo*

VIII. Summary

- Axonal regeneration model for fish was developed to identify specific genes involved in the CNS regenerative response.
- Neurocan mRNA is expressed in *in vivo* uninjured brain & spinal cord, as well as in injured brain.
- ptpsr2 mRNA is expressed in uninjured brain & spinal cord, as well as in injured brain.
- *In vivo* tracing analysis permits identification of descending brainstem cells capable of regeneration following CNS insult.

IX. Future Directions

- *In vivo* tracing will allow for isolation of cells and comparison of genes between regenerating and non-regenerating cell populations.
- Knockdown expression of PTPs (ptpsr2) through morpholino delivery following SCI.

Acknowledgments: This work is funded by U.S. Department of Defense grant W81XWH-11-1-0465 to JP.

- A poster and oral presentation from the **Plunkett Lab** were also presented at the **Keystone Conference "The Life of a Stem Cell"** from March 11-16th in California to present recent findings from the Plunkett lab of putative stem cell populations found in regenerative nuclei of the zebrafish brainstem. Dr. Alexis Tapanes-Castillo was awarded a \$1,200.00 travel fellowship to attend this meeting. Also, two undergraduate students from the Plunkett lab were also granted authorship due to their significant contributions to the work.

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

Alexis Tapanes-Castillo¹, Francellethia Shabazz¹, Katarina Vajn², Martin Oudega², Jeffery Plunkett¹
¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL. ²Departments of Physical Medicine & Rehabilitation, Neurobiology, and Center for Neuroscience, University of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

All adult vertebrate brains have the ability to have some degree of progenitor cell neurogenesis. Although progenitor neurogenesis is limited to the neurogenic zones, the zebrafish brain contains 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 neurogenesis and cell fate 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 and transective injury. Divergent zones during brain neurogenesis within brainstem nuclei are able to regenerate across and beyond a spinal cord transection site. The ability to self-renew and differentiate for all brainstem neurons, different descending projections 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 soon following spinal cord injury in the fish. We are currently examining regenerative brainstem responses for neural and SOX2 immunoreactivity post post-spinal cord injury.

We have also established an adult brainstem cell culture system to study *in vitro* neural outgrowth mechanisms in relation to permissive and non-permissive conditions. Histologically, the brainstem nucleus contains a subpopulation of stem/progenitor cells. Using neural and viral labeling with antibodies against Proliferating Cell Nuclear Antigen (PCNA), Human chorionic gonadotropin (hCG), and tubulin we further characterized this putative neuroprogenitor population. Currently, we are analyzing how brainstem progenitor stem cells respond to non-permissive growth conditions and define the growth response of neighboring cells.

Presented by U.S. Dept. of Defense (W81XWH-02-2-0041) to DARPA.

II. Stem Cell Niches: Mammal vs Zebrafish brain

III. Regenerative capacity of the zebrafish brain

IV. Experimental Question I: Does the brainstem of adult zebrafish contain putative stem cell populations?

V. Experimental Question II: Does focal brainstem injury alter putative stem cell populations?

VI. Experimental Question III: Does distal spinal cord injury alter putative stem cell populations within the brainstem?

VII. Adult zebrafish brainstem culture: A tool for the analysis of stem cell populations.

VIII. Experimental Question IV: Are putative stem populations found in culture able to interact with CSPGs?

IX. Summary

X. Future Directions

-Five Abstracts were submitted to regional meetings by undergraduate students from the **Plunkett Lab** for presentation in April. Students will be attending the **3rd Annual STEM Symposium at Barry University** and **The 7th Annual Southeast Cell Science Undergraduate Symposium in Miami**. This allows undergraduates from the Plunkett Lab the opportunity to present their work from the previous school year.

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

Alejandra Cartagena¹, Lisandra Yut¹, Jossias Genao¹, Alexis Tapanes-Castillo¹, Francellethia Shabazz¹, Katarina Vajn², Martin Oudega², Jeffery Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL. ²Departments of Physical Medicine & Rehabilitation,

Neurobiology, and Center for Neuroscience, University of Pittsburgh, University of Pittsburgh School of Medicine, 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* axonal 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.*

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

Francelethia Shabazz¹, Alexis Tapanes-Castillo¹, Katarina Vajn², Martin Oudega² and Jeffery A. Plunkett¹

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

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 (ptpsa), 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.*

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

Jossias Genao¹, Isaac Chacon¹, Francelethia Shabazz¹, Alexis Tapanes-Castillo¹, Katarina Vajn², Martin Oudega² and Jeffery Plunkett¹

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

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 (nad1.1) interact to regulate axon outgrowth *in vitro*. *Funded by U.S. Dept. of Defense W81XWH-11-1-0645 to JAP.*

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

Anthony Wood¹, Megan Staudenmaier¹, December Nuñez¹, Isaac Chacon¹, Francelthia Shabazz¹, Alexis Tapanes-Castillo¹, Katarina Vajn², Martin Oudega² and Jeffery A. Plunkett¹

¹School 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

In the mammalian central nervous system (CNS), the transmembrane protein tyrosine phosphatase PTPsigma was recently identified as a receptor neurocan, a chondroitin sulfate proteoglycan (CSPG) which inhibits axon regeneration following an injury. The goal of our project is to elucidate the role of PTPsigma in zebrafish 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 *pptrsa* expression following CNS injury in the zebrafish, we are currently using Reverse Transcriptase Polymerase Chain Reactions (RT-PCR). We observed *pptrsa* mRNA expression in uninjured brain and spinal cord tissue, as well as in injured brain tissues. We are also investigating possible Ptprsa interactions with CSPGs *in vitro* through the evaluation of *pptrsa* expression and morpholino knockdown in adult zebrafish brainstem-derived primary neuron cultures. We have detected *pptrsa* 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 *pptrsa* gene transcription may not be governed by injury (*in-vivo*) or substrate (*in-vitro*). 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 axonal growth of brainstem-derived primary cultures

Harold Gomez¹, Arjena Valls¹, Alexis Tapanes-Castillo¹, Francelthia Shabazz¹, Katarina Vajn², Martin Oudega², Jeffery Plunkett¹

¹School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL. ²Departments of Physical Medicine & Rehabilitation, Neurobiology, and Center for Neuroscience, University of Pittsburgh, University of Pittsburgh School of Medicine, 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 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. Funded by U.S. Dept. of Defense W81XWH-11-1-0645 to JAP.

- **December Nunez**, a student in the **Plunkett lab** won **“Best Poster”** at the **7th Annual Southeast Cell Science Undergraduate Symposium in Miami** in April 2012.

- **Francelthia Shabazz** an undergraduate student/technician from the **Plunkett Lab** won second place in the **Aquaneering calendar photo competition** for the submission of a micrograph from our adult zebrafish brainstem cultures. The link to the contest page is below.
http://www.aquaneering.com/2012_calendar_contest_winners.php



- **Anthony Wood, and Harold Gomez** from the **Plunkett Lab** graduated with honors in May 2012. They will both be attending medical school next year. Of note: Anthony scored a 33 on the MCAT which places him in a highly competitive position and Harold just finished his senior thesis which will likely be turned into a manuscript within the next year.

- **Harold Gomez, Jossias Genao and Alejandra Cartejena** from the **Plunkett Lab** were accepted to the University of Miami, Miller School of Medicine Minority Student MCAT preparation program in June 2012. This 8 week program allows the student to not only intensively study for the MCAT but also provides opportunities to shadow Doctors at Jackson Memorial Hospital. The Plunkett Lab has had 4 other students attend this program in past years of which 2 of the students are currently in Medical school.

- A manuscript entitled: **Establishment and characterization of primary brainstem cultures from adult zebrafish; A novel in-vitro model for neurite outgrowth.** Tapanes-Castillo A, Shabazz F, Vajn K, Oudega M and Plunkett JA is finished and will be submitted to the Journal of Neuroscience Research.

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 concerning zebrafish neurocan production and purification (specific aim 1) and specific aim 3 were encountered the problems have been addressed. 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 as they were described in our proposal. With the continuation of this project in Phase 3 through 2014 well underway we feel that our line of research will lead to many discoveries concerning the interaction of zebrafish neurites with chondroitin sulfates.