IDENTIFYING UNIQUE THERAPEUTIC TARGETS TO RESCUE RETINAL GANGLION CELLS FROM DEGENERATION AFTER OPTIC NERVE CRUSH

Tasneem Putliwala Sharma, B.S

APPROVED:

_____

Abbot F. Clark, PhD, Major Professor

_____

Robert Wordinger, PhD, Committee Member

_____

Anuja Ghorpade, PhD, Committee Member

_____

Raghu Krishnamoorthy, PhD, Committee Member

_____

Xiangrong Shi, PhD, University Member

_____

Anuja Ghorpade, PhD, Department Chair of Cell Biology and Immunology

---

Meharvan Singh, PhD, Dean, Graduate School of Biomedical Science

IDENTIFYING UNIQUE THERAPEUTIC TARGETS TO RESCUE RETINAL GANGLION CELLS FROM DEGENERATION AFTER OPTIC NERVE CRUSH

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Tasneem Putliwala Sharma, B.S.

Fort Worth, Texas

April 2014

ACKNOWLEDGEMENTS

I would like to express my innermost gratitude to my mentor Dr. Abbot F. Clark without whom none of this would be possible. His continuous support, patience, enthusiasm and immense insight during my PhD training and research, has provided me with the vision, encouragement and advice necessary for me to proceed through my doctoral program and complete my dissertation. He has been a role model who has been inspirational, supportive, and tolerant, not only in the laboratory but also as a fatherly figure, always striving to be nurturing and caring. I could not be prouder of my academic roots and hope that one day I will be able to fulfill all the scientific dreams that he has instilled in me.

I convey my sincerest appreciation to my co-mentor Dr. Robert J. Wordinger who has been a strong and supportive advisor throughout my graduate school career with special thanks to my advisory committee members Drs. Anuja Ghorpade, Raghu Krishnamoorthy and Xiangrong Shi who have all held me to the highest standards and have given me the confidence and freedom to become an independent thinker and scientist. I would also like to thank Drs. Iok-Hou Pang and Weiming Mao for their professional advice and insight throughout my doctoral education.

My deepest appreciation goes to Dr. Yang Liu whose unflagging encouragement and unconditional assistance in my research has been tremendous. She has been a strong and supportive adviser to me and has always given me great freedom to be a true researcher.

The best and worst moments of my doctoral journey are shared with all my friends and family around the world who always have been sources of laughter, joy, and support. My special thanks goes to Dr. Colleen McDowell for her intuitive scientific encouragement and intense affection, Dr. Jwalitha Shankardas for her perceptive guidance and care, Dr. Ankur Jain for his insightful amity and warmth, Dr. Anupam Sule for his brotherly love, Sean Silverman for his comradeship

and Wanda Medina-Ortiz for her support. It has been a great privilege to be a part of this lab and the UNTHSC family and I am grateful for all the love, patience and support that everyone has provided me. I am happy that our friendships will extend well beyond our shared times here.

Last but not the least, I would like to thank my dearest family: my parents, Mohammed Mansur Arif and Jamila Mansur Putliwala whose care and support has been my greatest inspiration and driving force on this extraordinary journey. I owe them everything for always steering me in the right direction. My love and respect for them knows no bounds when I realize all the sacrifices they have made for me to achieve this goal. My sincerest appreciation to my sister and brother-in-law, Raiqah Mansur Jawadwala and Mustafa Jawadwala, and my brother, Mansur-Ali Mansuri and his family, who have always provided me with unconditional devotion and ever-forgiving love. Their true advice has forever channeled my misgivings towards positive reflections. I would like to specially thank my extended family, Murtaza and Farida Yusufali family including Taher and Insiyah, for their love and guidance throughout my life.

My deepest adoration goes to my husband, Gaurav Sharma, who has been an integral part of my life throughout my PhD career and whose passion and encouragement has allowed me to victoriously complete this journey. Throughout my triumphs and tribulations he has always been my shining star and ceaselessly guided me through every impeding obstacle. I would also like to thank my in-laws, Ashok Sharma and Rama Sharma who have always given me their unconditional support and embraced me as proud parents. My gratitude also goes to my sister and brother-in law, Surbhi Sharma Suthar and Sanjeev Suthar, who have continuously encouraged my ambitions. Finally, completing my PhD degree has probably been the most significant achievement of my life and I would like to dedicate this work to my grandparents who left us too soon. I hope my work makes you proud.

TABLE OF CONTENTS

Page

LIST OF ILLUSTRATIONS

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF TABLES

CHAPTER II

CHAPTER III

CHAPTER I

INTRODUCTION

Central Nervous System Trauma and Neurodegenerative diseases

Prevalence of traumatic brain injury (TBI) has been estimated to be 3.32 to 5.3 million in the United States, while 12,000 to 20,000 spinal cord injury (SCI) cases are reported annually in the United States (Ma, Chan and Carruthers). Costs associated with TBI and SCI exceeds 25 billion dollars causing a significant financial burden on these patients, their families and our society (DeVivo; Rutland-Brown et al.). Brain and spinal cord trauma are predominantly responsible for acute neurological disorders with nerve axonal damage (Knoferle et al.). Axonal pathology is characteristic of even multiple neurodegenerative diseases like Parkinson's, Alzheimer's, amyotrophic lateral sclerosis and ophthalmologic diseases like glaucoma (M. P. Coleman; Williamson and Cleveland; Stokin et al.; Kalesnykas et al.).

Central nervous system (CNS) trauma and neurodegenerative disorders trigger a cascade of cellular events resulting in extensive damage to neurons (Schwartz; Ohlsson, Mattsson and Svensson; Magharious et al.; Wohlfart; Windle). Regenerative failure is a critical endpoint of these destructive triggers culminating in neuronal apoptosis (Monnier et al.; Magharious et al.; Quigley, Nickells, et al.) and inhibition of functional recovery. The non-permissive regenerative environment is due to expression of inhibitory cues (Magharious et al.; Huber and Schwab; Huber et al.; Filbin; S. Tang et al.; Winzeler et al.; Kopp et al.; Sandvig et al.), glial scarring (Silver and Miller; Windle), slow clearance of axonal debris (Lawson et al.), and CNS

inflammation (Lazarov-Spiegler et al.; Jaerve and Muller). Neuronal susceptibility to apoptosis and regenerative failure are crucial endpoints of CNS trauma (Figure 1). We can address these endpoints by: a) understanding global gene expression patterns after trauma, b) delineating pathogenic pathways associated with trauma, and c) discovering a novel therapeutic target for promoting axonal regeneration and neuronal survival.

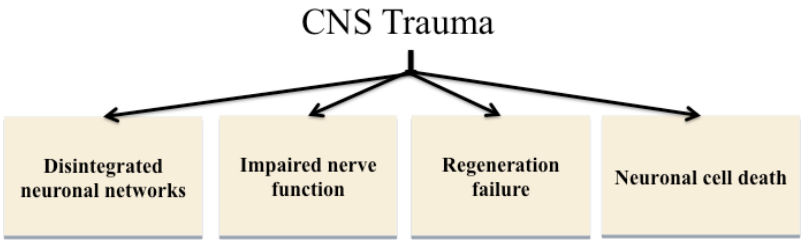


Figure 1: Trauma to the central nervous system causes axonal injury leading to disintegrated neuronal networks, impaired nerve function, regeneration failure and cell death, resulting in long-term neuronal disability

Optic Nerve Crush Model

The well-defined rodent optic nerve crush (ONC) model can be applied to study regeneration failure and CNS trauma (Li, Schlamp and Nickells; Li et al.; Barron et al.; Templeton et al.). The ON and retina are a part of the CNS, and the retinal ganglion cells (RGCs) are the output neurons of the retina. RGC axons comprise the ON, and they transmit signals to the visual centers of the brain (Wohlfart; Bro and Haycock). These visual signals are specifically transmitted from the retina to the lateral geniculate nucleus and superior colliculus (SC) within the brain (Figure 2)

a b

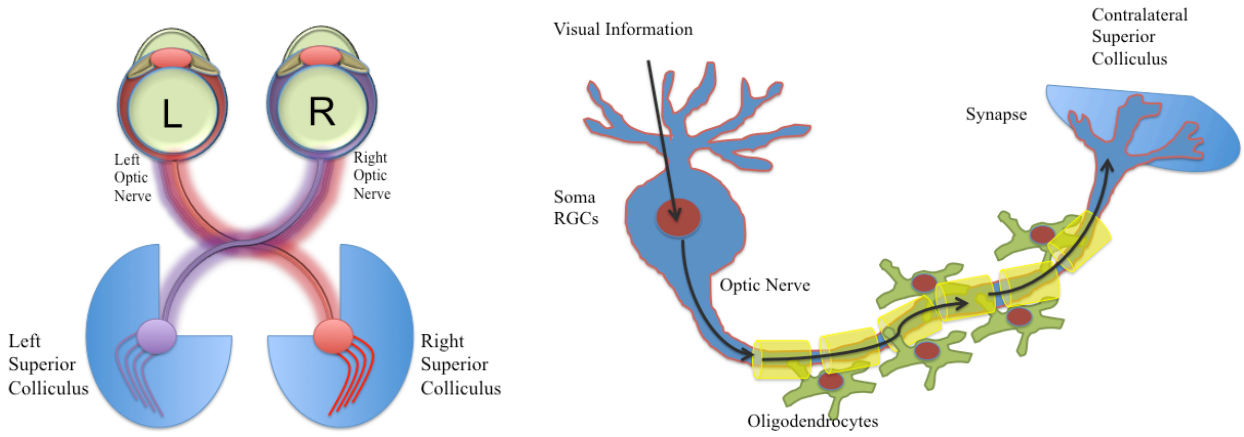


Figure 2. Transmittance of visual signal from the RGCs to the SC in mouse eye a) Visual pathway signal from the retina through the optic nerve to the contralateral SC. b) Visual stimulus from RGC dendrites through the optic nerve, terminating in neurons of the SC

The easy accessibility of the ON and the reproducibility of this model makes it an effective tool to study CNS trauma and will help understand the ensuing traumatic events that activate neuronal apoptosis. Analyzing genes that are regulated due to axonal trauma associated with neuronal loss will provide key therapeutic targets that can rescue neuronal cells like RGCs from death.

Glaucoma and Optic Nerve Crush

Glaucoma represents a group of neurodegenerative diseases identified by structural damage to the ON with slow, progressive death of retinal ganglion cells (Chidlow, Wood and Casson). It is the second leading cause of visual impairment and blindness worldwide and affects around 70 million people globally (Quigley and Broman). Direct costs associated with glaucoma amount to 5.8 billion dollars per year in the United States (Rein). Glaucoma is a multifactorial degenerative

optic neuropathy causing peripheral vision loss that can eventually lead to complete blindness. Clinical features of glaucoma mimic some of the endpoints of axonal injury that include degeneration of retinal ganglion cells (RGC), optic nerve atrophy, and visual field loss (Allingham, Liu and Rhee; Kwon et al.). In addition, the optic nerve head (ONH) has been shown to be the initial site of insult to RGC axons in glaucoma (Quigley and Addicks) (Quigley, Addicks, et al.) (Howell, Libby, et al.) and gene expression changes within the ONH have been studied in ocular hypertension and glaucoma models (Johnson et al.; Howell, Macalinao, et al.; Qu and Jakobs). A major risk factor of glaucoma is elevated intraocular pressure (IOP) (Leske et al.). Most treatments for glaucoma focus on stabilizing IOP. However, even though lowering of IOP slows the progression of the disease, it does not reverse visual field loss and prevent complete loss of RGCs. Therefore, new drug therapies are required for neuroprotection that could possibly save the neurons from degeneration and halt the progression of the disease.

Retinal Ganglion Cell Degeneration

The ONC and subsequent death of RGCs will allow us to understand the molecular mechanisms associated with RGC death. Progressive degeneration of mature retinal ganglion cells (RGCs) has been associated with loss of trophic support (Koeberle and Bahr; Kermer, Klocker and Bahr), stimulation of inflammatory processes/ immune regulation (Koeberle, Gauldie and Ball; Kipnis et al.), and apoptotic effectors (Isenmann et al.; Kermer, Ankerhold, et al.; Kermer, Klocker and Bahr; Kermer, Klocker, et al.; Kikuchi, Tenneti and Lipton). Additionally, axonal injury (Figure 3) causes detrimental effects on the RGCs triggering regenerative failure (Barron et al.; Misantone, Gershenbaum and Murray; Bahr; Klocker et al.). After injury, RGC axons show only a short-lived sprouting response but no long-distance regeneration through the ON (L. Benowitz

and Y. Yin). Factors affecting axonal regeneration after CNS trauma are crucial for recovery, and imbalances within genes involved in axonal plasticity and outgrowth are detrimental to the recovery of the neurons (Aubert, Ridet and Gage; Nicholls and Saunders; Di Giovanni et al.) and need to be identified.

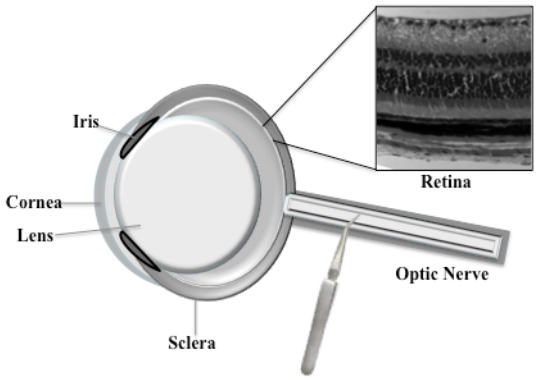


Figure 3: Diagram of the mouse eye with representation of retina layers (inset) and ONC surgical site 0.5mm posterior to the globe.

Optic Nerve Trauma

Optic nerve injuries can be classified into direct and indirect trauma (Knoferle et al.). Direct force injury causes destruction of neurons and vital support elements such as neuroglia at the point of impact. In addition, a successive secondary trauma results in excitotoxicity, free radical production, ischemia, inflammation, and apoptosis (Truettner et al.; Ohlsson et al.; Song et al.). Blunt force trauma, skull fractures or expansive intracranial processes create crush-like trauma instead of transection like injury in the axons making the ONC model applicable for studying axonal injury (Ohlsson et al.). Key features of axonal degeneration are similar despite various

etiologies. Wallerian degeneration (WD) is one critical feature of ON trauma where the injured axon undergoes axonal degeneration at the distal site. Subsequent WD causes rapid degeneration, fragmentation, and blebbing of the remaining axon, microtubule disassembly, and phagocytic clearance at the lesion site (M. Coleman; Kerschensteiner et al.; Knoferle et al.).

Traumatic and spinal cord injury represents classical conditions where nervous system dysfunction is caused by mechanical disruption of axonal structure. Furthermore, axonal degeneration plays a crucial role in the pathogenesis of several neurological disorders as well and normally precedes neuronal cell death resulting in functional disability (Knoferle et al.). The severity of traumatic brain injury ranges from mild (80%) to severe (10%), with most long-term disability caused by moderate to severe injury (Ma, Chan and Carruthers). The incidence of TBI is 1.7 million in the US and prevalence of long-term disability from TBI is around 5 million. The annual incidence of SCI is around 77 million in the US alone (Ma, Chan and Carruthers).

Adeno Associated-Viral Vectors and Current Therapeutic Advances

The ONC presents a unique model system for studying axonal pathology within the CNS. The easy accessibility of the RGC axons and the possibility to manipulate the system via transduction vectors (Harvey, Hellstrom and Rodger; Martin, Klein and Quigley) makes the ONC model distinctive in its applicability. Due to AAV2's lack of pathogenicity and its ability to effectively stably transduce dividing and nondividing cells, these vectors can be used to deliver genes to various tissues. Transduction efficiency and tropism of various intravitreally injected AAV vectors shows highest level of transduction efficiency by AAV2/2 in RGCs (Harvey, Hellstrom and Rodger; Ju et al.). Various groups have previously studied the gene expression changes in the rodent ONC model (Agudo et al.; Templeton et al.; Z. Tang et al.; Lukas et al.). Differential

response of crucial genes after CNS trauma include gap associated protein 43 (*Gap43*) (Blaugrund et al.; Doster et al.; Leon et al.), glial fibrillary acidic protein (*Gfap*) (Ridet et al.; Dibas et al.; Woldemussie, Wijono and Ruiz), and neurofilament deregulation after crush injury (Parrilla-Reverter et al.). Multiple strategies are being developed to promote survival of RGCs and stimulate axonal regeneration after ON trauma using AAV2 vectors.

Current approaches include: a) Peripheral nerve (PN) transplantation and lens injury to regenerate axons of injured ONC adult rat RGCs (Lorber, Berry and Logan), b) promoting survival of RGCs and axonal regrowth in mice after crush and in rats after crush and PN transplantation using ciliary neurotrophic factor viral vectors (AAV-CNTF-GFP) (Leaver, Cui, Plant, et al.), c) injecting bcl2 overexpressing transgenic mice with AAV-CNTF-GFP to increase cell viability and axonal regeneration (Leaver, Cui, Bernard, et al.), d) promoting survival of RGC by brain derived neurotrophic factor (BDNF) using intravitreal injections of AAV-BDNF-GFP (Harvey, Hellstrom and Rodger), e) RGC axonal regrowth after implanting *ex-vivo* PNs grafts transduced with Schwann cell's over-expressing CNTF (Hu et al.), f) experiments with AAV BDNF, CNTF and growth associated protein 43 (GAP43), have shown AAV-CNTF to be the most crucial for promoting both, long-term survival and regeneration (Leaver, Cui, Plant, et al.), g) promoting regeneration in the rat ON using oncomodulin released from microspheres (Yin et al.)and h) increased robust and sustained axon regeneration with simultaneous deletion of both PTEN and SOCS3 and the concurrent activation of mTOR and STAT3 pathways (Sun et al.).

Hypotheses

Although, most studies target neuronal apoptosis by overexpressing intrinsic growth factors and enhancing regeneration in the CNS trauma model, previous studies have established that a multifactorial approach is required for successful and long-lasting therapeutic outcomes (L. I. Benowitz and Y. Yin; Leaver, Cui, Plant, et al.). Current gaps in literature still exist for identification of a key gene that could effectively target neuroprotection, enhance neuron regeneration, and sustain neuronal function. Our study will present an extended outlook on RGC survival and neurodegenerative mechanisms after axonal trauma. To characterize the neurodegenerative mechanisms, the spatial and temporal gene expression changes will need to be explored after CNS trauma. In addition, discovering a therapeutic strategy targeting axonal regeneration after ONC is crucial. This will allow the translation of the proposed research to preventing neurodegeneration caused by CNS trauma and neurodegenerative diseases.

Our global hypothesis is that optic nerve crush leads to distinct temporal retinal and optic nerve gene expression patterns that will represent potential novel therapeutic targets to rescue RGCs from degeneration. Based on this we have two specific hypotheses: **We hypothesize that 1) retinal and optic nerve genes associated with neuronal regeneration and axonogenesis are down regulated after optic nerve crush and that 2) Over-expression of *Nrn1* will protect RGCs from neurodegeneration after ONC trauma.**

Rationale and Specific Aims

To accomplish our study, we addressed the following specific aims:

Hypothesis 1: We hypothesize that retinal and optic nerve genes associated with neuronal regeneration and axonogenesis are down regulated after optic nerve crush

Specific Aim 1: To characterize temporal retinal and optic nerve gene expression profile and "neuronal clusters" in the ONC model.

Rationale: Approximately 50% of the cells in ganglion cell layer (GCL) are RGCs, while the remaining cells consist of displaced amacrine cells, astrocytes, and microglia (Li, Schlamp and Nickells; Haverkamp et al.; Raymond et al.; Masland; Kim et al.). As ONC causes damage to the ON, it eventually leads to the death of RGCs. The cell loss observed by 28 days in this model represents the loss of RGCs (Li, Schlamp and Nickells). To establish temporal differences within genes in retina and ON samples and identify specific gene expression changes taking place after axonal trauma, we used Affymetrix gene chips followed by bioinformatic analysis, including BETR analysis. This mode of evaluation recognizes unique genes in both the retina and ON whose expression leads to neuronal loss, regenerative failure, and degeneration of the axons. Analyzing the retina and ON simultaneously will allow us to investigate temporal responses that initially occur in the optic nerve and how they later translate to the retina and vice versa. In addition, temporal cluster classification is crucial for identifying the neuronal loss mechanisms that are sequentially being regulated after trauma. The pathogenic gene cluster expression changes in our model can be extrapolated to changes occurring after CNS trauma or the neurological loss transpiring in clinical pathological settings.

1.1 To profile the temporal gene expression pattern in retina and ON by microarray analysis

1.2 To identify the specific gene expression changes of ONC model and clusters using bioinformatic tools from the temporal gene expression profile

Specific Aim 2: To identify and validate temporal expression pattern of key retinal and optic nerve genes affected after ONC

Rationale: It is essential to observe temporal changes in our model to identify other gene targets for neuroprotection therapy. By studying "neuronal clusters" associated specific genes after ONC, we will identify the differential response of key genes associated with neurodegeneration and regenerative failure in the BALB/cJ mice. Analysis of pooled microarray samples does not account for the slight variations that exist between samples and negates the individual sample differences. To confirm that individual samples follow the same trend of expression as our microarray data, we will validate individual fold expression of each sample by qRT-PCR. The protein expression changes occurring in the RGCs after crush could be diluted by analysis of the entire retina, so we will analyze specific RGC protein expression changes using immunohistochemistry (IHC) to qualitatively asses the spatial and temporal expression pattern of the key genes (Figure 4).

2.1 To identify the affected key target genes within the neuronal clusters

2.2 To validate the identified genes spatially and temporally after ONC

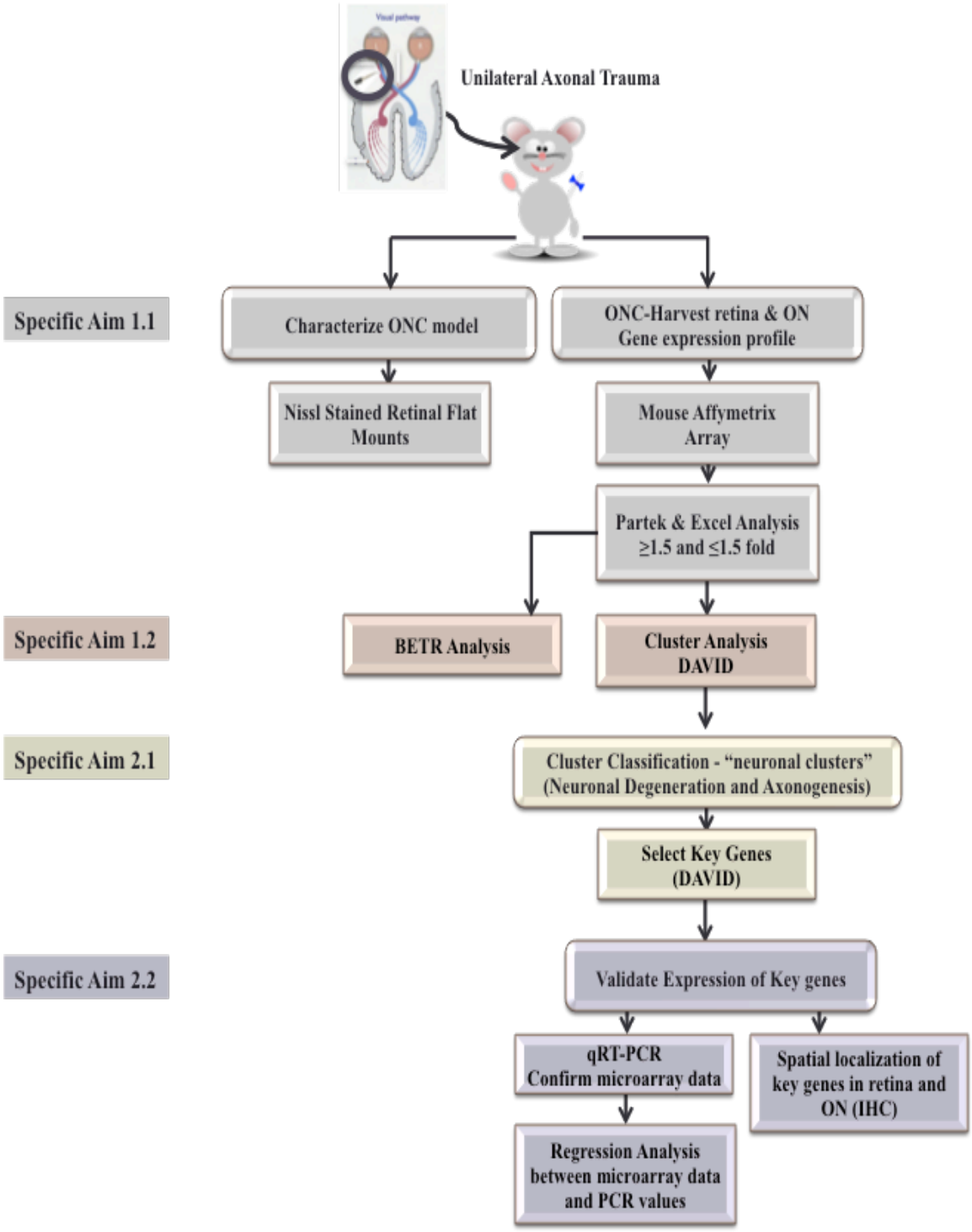


Figure 4: Flowchart presenting Specific Aims of Hypothesis 1

Hypothesis 2: We hypothesize that over-expression of *Nrn1* will protect RGCs from neurodegeneration after ONC trauma.

Specific Aim 1: To determine the neuroprotective role of neuritin 1 (NRN1) on the affected RGCs after ONC.

Rationale: NRN1, also known as candidate plasticity gene 15 (*Cpg15*) is an extracellular, GPI-linked protein, which can be secreted as a soluble form. The gene stimulates axonal plasticity, dendritic arborization, and synapse maturation in the CNS (Cantallops, Haas and Cline). The functional ability of *Nrn1* to promote axonal arborization and synaptic plasticity makes it a unique therapeutic target to address neuronal apoptosis after CNS trauma. In situ hybridization studies with *NRN1* show predominant expression of the gene in the GCL (Fujino et al.). The plasticity associated dynamic regulation of *Nrn1* maybe key to axonal regeneration in the CNS. In addition, *Nrn1* promotes the survival of neural progenitors in early embryonic development (Putz, Harwell and Nedivi). To show the beneficial role of *Nrn1,* we will over-express hNRN1 in RGCs and measure the survival, regeneration, and RGC function in the ONC model (Figure 5).

1.1 To determine expression of *Nrn1* in retina and optic nerve after ONC

1.2 To determine that recombinant human NRN1 increases survival and extends neurite outgrowth in axotomized and growth factor deprived RGCs in culture

1.3 To determine temporal RGC survival, regeneration, and function of RGCs after over expression of *Nrn1* in the ONC model

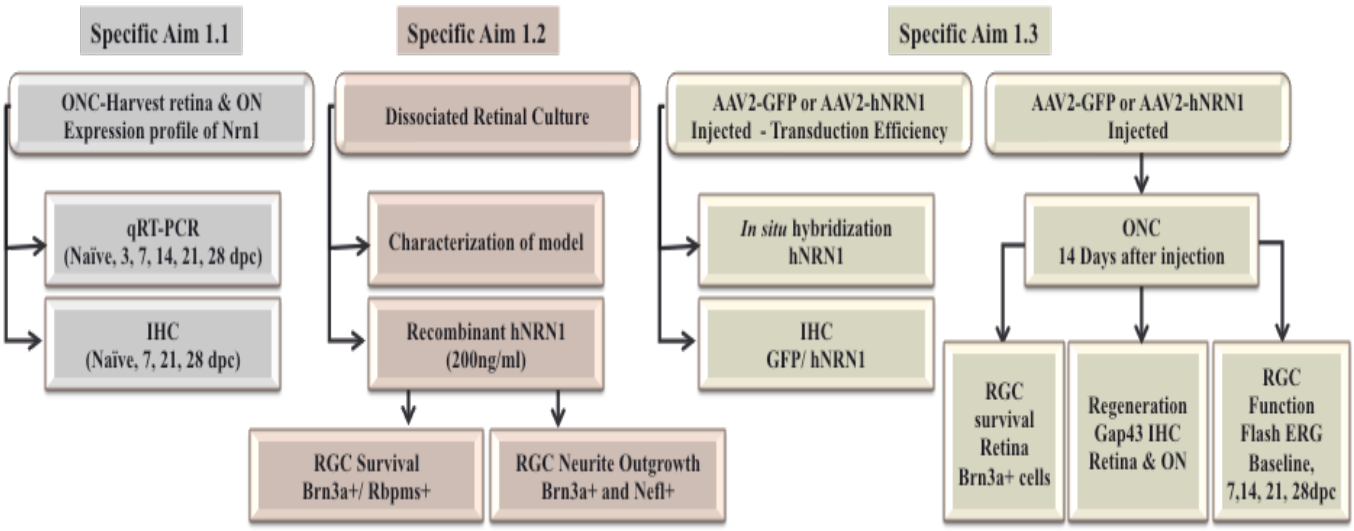


Figure 5: Flowchart presenting Specific Aims of Hypothesis 2

Research Design and Methodology

Detrimental neuronal changes occur after axonal injury, and this study will highlight the distinct and common responses of the retina and ON post crush. To better understand the molecular mechanisms associated with neurodegenerative processes after injury, we created a distinct gene profile using a multi step approach and therapeutically target RGC apoptosis. We first examined the survival of neurons in the ganglion cell layer (GCL) after acute axonal trauma by histological examination of the retinas over an extended 28-day period, which is a well-established time line for RGC death (Li, Schlamp and Nickells). Secondly, we identified significant cluster-based changes occurring sequentially in the retina and ON by meta-analysis of the array data. Thirdly, we identified key clusters associated with neuron degeneration to isolate potential underlying damaging gene expression changes occurring within RGCs. Fourthly, the expression of selective genes was confirmed quantitatively and qualitatively to validate our array data and to examine expression of potential therapeutic targets that are affected by CNS trauma. Lastly, we

therapeutically targeted the RGCs in the mouse ONC model by over-expressing a key axonal regeneration gene (hNrn1) using the AAV2-CAG-hNRN1 viral vector and measured RGC survival, regeneration, and RGC function.

Significance

A detailed molecular gene expression profile of the neuron from the soma to the axons and temporal gene regulation patterns post ONC damage have not been concurrently examined previously. Establishing such an extensive gene expression pattern is crucial for further advancement of the neuroprotection field of research. To the best of our knowledge, this is the first study that will create a transcriptome profile after CNS trauma encompassing neurodegeneration and regenerative failure. Analyzing these mRNA and protein expression patterns allowed the detection and quantification of progressive degenerative changes in the RGCs after ONC. It also enabled the identification of a wide range of therapeutic targets and distinguished *Nrn1* as an effective target to rescue RGCs from degeneration. We present NRN1 as a novel therapy that prevented RGC apoptosis, induced axonal regeneration, and exhibited sustained RGC function after axonal injury.

References

Agudo, M., et al. "Time Course Profiling of the Retinal Transcriptome after Optic Nerve Transection and Optic Nerve Crush." Molecular vision 14 (2008): 1050-63.

Allingham, R. R., Y. Liu, and D. J. Rhee. "The Genetics of Primary Open-Angle Glaucoma: A Review." Exp Eye Res 88.4 (2009): 837-44.

Aubert, I., J. L. Ridet, and F. H. Gage. "Regeneration in the Adult Mammalian Cns: Guided by Development." Current opinion in neurobiology 5.5 (1995): 625-35.

Bahr, M. "Live or Let Die - Retinal Ganglion Cell Death and Survival During Development and in the Lesioned Adult Cns." Trends in neurosciences 23.10 (2000): 483-90.

Barron, K. D., et al. "Qualitative and Quantitative Ultrastructural Observations on Retinal Ganglion Cell Layer of Rat after Intraorbital Optic Nerve Crush." Journal of neurocytology 15.3 (1986): 345-62.

Benowitz, L. I., and Y. Yin. "Combinatorial Treatments for Promoting Axon Regeneration in the Cns: Strategies for Overcoming Inhibitory Signals and Activating Neurons' Intrinsic Growth State." Dev Neurobiol 67.9 (2007): 1148-65.

Benowitz, L., and Y. Yin. "Rewiring the Injured Cns: Lessons from the Optic Nerve." Experimental neurology 209.2 (2008): 389-98.

Blaugrund, E., et al. "Axonal Regeneration Is Associated with Glial Migration: Comparison between the Injured Optic Nerves of Fish and Rats." The Journal of comparative neurology 330.1 (1993): 105-12.

Bro, S., and J. W. Haycock. "Visual Cortex (Translation of S. Ramon Y Cajal) Translated from L'encorce Cerebrale Suivant Les Regions L'ecorce Visuelle, Chapter 25 in "Histologie Du Systeme Nerveux De L'homme Et Des Vertebres," 1911." Behavioral biology 21.4 (1977): 508-28.

Cantallops, I., K. Haas, and H. T. Cline. "Postsynaptic Cpg15 Promotes Synaptic Maturation and Presynaptic Axon Arbor Elaboration in Vivo." Nature neuroscience 3.10 (2000): 1004-11.

Chidlow, G., J. P. Wood, and R. J. Casson. "Pharmacological Neuroprotection for Glaucoma." Drugs 67.5 (2007): 725-59.

Coleman, M. "Axon Degeneration Mechanisms: Commonality Amid Diversity." Nat Rev Neurosci 6.11 (2005): 889-98.

Coleman, M. P. "The Challenges of Axon Survival: Introduction to the Special Issue on Axonal Degeneration." Exp Neurol 246 (2013): 1-5.

DeVivo, M. J. "Causes and Costs of Spinal Cord Injury in the United States." Spinal Cord 35.12 (1997): 809-13.

Di Giovanni, S., et al. "Neuronal Plasticity after Spinal Cord Injury: Identification of a Gene Cluster Driving Neurite Outgrowth." FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19.1 (2005): 153-4.

Dibas, A., et al. "Changes in Ocular Aquaporin Expression Following Optic Nerve Crush." Molecular vision 16 (2010): 330-40.

Doster, S. K., et al. "Expression of the Growth-Associated Protein Gap-43 in Adult Rat Retinal Ganglion Cells Following Axon Injury." Neuron 6.4 (1991): 635-47.

Filbin, M. T. "Myelin-Associated Inhibitors of Axonal Regeneration in the Adult Mammalian Cns." Nature reviews. Neuroscience 4.9 (2003): 703-13.

Fujino, T., et al. "Cpg15 and Cpg15-2 Constitute a Family of Activity-Regulated Ligands Expressed Differentially in the Nervous System to Promote Neurite Growth and Neuronal Survival." The Journal of comparative neurology 507.5 (2008): 1831-45.
Harvey, A. R., M. Hellstrom, and J. Rodger. "Gene Therapy and Transplantation in the Retinofugal Pathway." Progress in brain research 175 (2009): 151-61.
Haverkamp, S., et al. "Expression Analysis of Green Fluorescent Protein in Retinal Neurons of Four Transgenic Mouse Lines." Neuroscience 160.1 (2009): 126-39.
Howell, G. R., et al. "Axons of Retinal Ganglion Cells Are Insulted in the Optic Nerve Early in Dba/2j Glaucoma." The Journal of cell biology 179.7 (2007): 1523-37.
Howell, G. R., et al. "Molecular Clustering Identifies Complement and Endothelin Induction as Early Events in a Mouse Model of Glaucoma." J Clin Invest 121.4 (2011): 1429-44.
Hu, Y., et al. "Lentiviral-Mediated Transfer of Cntf to Schwann Cells within Reconstructed Peripheral Nerve Grafts Enhances Adult Retinal Ganglion Cell Survival and Axonal Regeneration." Molecular therapy : the journal of the American Society of Gene Therapy 11.6 (2005): 906-15.
Huber, A. B., and M. E. Schwab. "Nogo-a, a Potent Inhibitor of Neurite Outgrowth and Regeneration." Biological chemistry 381.5-6 (2000): 407-19.
Huber, A. B., et al. "Patterns of Nogo Mrna and Protein Expression in the Developing and Adult Rat and after Cns Lesions." The Journal of neuroscience : the official journal of the Society for Neuroscience 22.9 (2002): 3553-67.
Isenmann, S., et al. "Up-Regulation of Bax Protein in Degenerating Retinal Ganglion Cells Precedes Apoptotic Cell Death after Optic Nerve Lesion in the Rat." The European journal of neuroscience 9.8 (1997): 1763-72.
Jaerve, A., and H. W. Muller. "Chemokines in Cns Injury and Repair." Cell and tissue research 349.1 (2012): 229-48.
Johnson, E. C., et al. "Global Changes in Optic Nerve Head Gene Expression after Exposure to Elevated Intraocular Pressure in a Rat Glaucoma Model." Invest Ophthalmol Vis Sci 48.7 (2007): 3161-77.
Ju, W. K., et al. "Increased Optic Atrophy Type 1 Expression Protects Retinal Ganglion Cells in a Mouse Model of Glaucoma." Molecular vision 16 (2010): 1331-42.
Kalesnykas, G., et al. "Retinal Ganglion Cell Morphology after Optic Nerve Crush and Experimental Glaucoma." Invest Ophthalmol Vis Sci 53.7 (2012): 3847-57.
Kermer, P., et al. "Caspase-9: Involvement in Secondary Death of Axotomized Rat Retinal Ganglion Cells in Vivo." Brain research. Molecular brain research 85.1-2 (2000): 144-50.
Kermer, P., N. Klocker, and M. Bahr. "Neuronal Death after Brain Injury. Models, Mechanisms, and Therapeutic Strategies in Vivo." Cell and tissue research 298.3 (1999): 383-95.
Kermer, P., et al. "Inhibition of Cpp32-Like Proteases Rescues Axotomized Retinal Ganglion Cells from Secondary Cell Death in Vivo." The Journal of neuroscience : the official journal of the Society for Neuroscience 18.12 (1998): 4656-62.
Kerschensteiner, M., et al. "In Vivo Imaging of Axonal Degeneration and Regeneration in the Injured Spinal Cord." Nat Med 11.5 (2005): 572-7.
Kikuchi, M., L. Tenneti, and S. A. Lipton. "Role of P38 Mitogen-Activated Protein Kinase in Axotomy-Induced Apoptosis of Rat Retinal Ganglion Cells." The Journal of neuroscience : the official journal of the Society for Neuroscience 20.13 (2000): 5037-44.
Kim, C. Y., et al. "Gene Expression Profile of the Adult Human Retinal Ganglion Cell Layer." Molecular vision 12 (2006): 1640-8.

Kipnis, J., et al. "Neuronal Survival after Cns Insult Is Determined by a Genetically Encoded Autoimmune Response." The Journal of neuroscience : the official journal of the Society for Neuroscience 21.13 (2001): 4564-71.
Klocker, N., et al. "Morphological and Functional Analysis of an Incomplete Cns Fiber Tract Lesion: Graded Crush of the Rat Optic Nerve." Journal of neuroscience methods 110.1-2 (2001): 147-53.
Knoferle, J., et al. "Mechanisms of Acute Axonal Degeneration in the Optic Nerve in Vivo." Proc Natl Acad Sci U S A 107.13 (2010): 6064-9.
Koeberle, P. D., and M. Bahr. "Growth and Guidance Cues for Regenerating Axons: Where Have They Gone?" Journal of neurobiology 59.1 (2004): 162-80.
Koeberle, P. D., J. Gauldie, and A. K. Ball. "Effects of Adenoviral-Mediated Gene Transfer of Interleukin-10, Interleukin-4, and Transforming Growth Factor-Beta on the Survival of Axotomized Retinal Ganglion Cells." Neuroscience 125.4 (2004): 903-20.
Kopp, M. A., et al. "Small-Molecule-Induced Rho-Inhibition: Nsaids after Spinal Cord Injury." Cell and tissue research 349.1 (2012): 119-32.
Kwon, Y. H., et al. "Primary Open-Angle Glaucoma." N Engl J Med 360.11 (2009): 1113-24.
Lawson, L. J., et al. "Quantification of the Mononuclear Phagocyte Response to Wallerian Degeneration of the Optic Nerve." Journal of neurocytology 23.12 (1994): 729-44.
Lazarov-Spiegler, O., et al. "Restricted Inflammatory Reaction in the Cns: A Key Impediment to Axonal Regeneration?" Molecular medicine today 4.8 (1998): 337-42.
Leaver, S. G., et al. "Cooperative Effects of Bcl-2 and Aav-Mediated Expression of Cntf on Retinal Ganglion Cell Survival and Axonal Regeneration in Adult Transgenic Mice." The European journal of neuroscience 24.12 (2006): 3323-32.
Leaver, S. G., et al. "Aav-Mediated Expression of Cntf Promotes Long-Term Survival and Regeneration of Adult Rat Retinal Ganglion Cells." Gene therapy 13.18 (2006): 1328-41.
Leon, S., et al. "Lens Injury Stimulates Axon Regeneration in the Mature Rat Optic Nerve." The Journal of neuroscience : the official journal of the Society for Neuroscience 20.12 (2000): 4615-26.
Leske, M. C., et al. "Risk Factors for Open-Angle Glaucoma. The Barbados Eye Study." Arch Ophthalmol 113.7 (1995): 918-24.
Li, Y., C. L. Schlamp, and R. W. Nickells. "Experimental Induction of Retinal Ganglion Cell Death in Adult Mice." Investigative ophthalmology & visual science 40.5 (1999): 1004-8.
Li, Y., et al. "Dominant Inheritance of Retinal Ganglion Cell Resistance to Optic Nerve Crush in Mice." BMC neuroscience 8 (2007): 19.
Lorber, B., M. Berry, and A. Logan. "Different Factors Promote Axonal Regeneration of Adult Rat Retinal Ganglion Cells after Lens Injury and Intravitreal Peripheral Nerve Grafting." Journal of neuroscience research 86.4 (2008): 894-903.
Lukas, T. J., et al. "Early Cellular Signaling Responses to Axonal Injury." Cell communication and signaling : CCS 7 (2009): 5.
Ma, V. Y., L. Chan, and K. J. Carruthers. "The Incidence, Prevalence, Costs and Impact on Disability of Common Conditions Requiring Rehabilitation in the Us: Stroke, Spinal Cord Injury, Traumatic Brain Injury, Multiple Sclerosis, Osteoarthritis, Rheumatoid Arthritis, Limb Loss, and Back Pain." Arch Phys Med Rehabil (2014).
Magharious, M., et al. "Quantitative Itraq Analysis of Retinal Ganglion Cell Degeneration after Optic Nerve Crush." Journal of proteome research 10.8 (2011): 3344-62.

Martin, K. R., R. L. Klein, and H. A. Quigley. "Gene Delivery to the Eye Using Adeno-Associated Viral Vectors." Methods 28.2 (2002): 267-75.
Masland, R. H. "Neuronal Diversity in the Retina." Current opinion in neurobiology 11.4 (2001): 431-6.
Misantone, L. J., M. Gershenbaum, and M. Murray. "Viability of Retinal Ganglion Cells after Optic Nerve Crush in Adult Rats." Journal of neurocytology 13.3 (1984): 449-65.
Monnier, P. P., et al. "Involvement of Caspase-6 and Caspase-8 in Neuronal Apoptosis and the Regenerative Failure of Injured Retinal Ganglion Cells." The Journal of neuroscience : the official journal of the Society for Neuroscience 31.29 (2011): 10494-505.
Nicholls, J., and N. Saunders. "Regeneration of Immature Mammalian Spinal Cord after Injury." Trends in neurosciences 19.6 (1996): 229-34.
Ohlsson, M., et al. "Complement Activation Following Optic Nerve Crush in the Adult Rat." Journal of neurotrauma 20.9 (2003): 895-904.
Ohlsson, M., P. Mattsson, and M. Svensson. "A Temporal Study of Axonal Degeneration and Glial Scar Formation Following a Standardized Crush Injury of the Optic Nerve in the Adult Rat." Restorative neurology and neuroscience 22.1 (2004): 1-10.
Parrilla-Reverter, G., et al. "Time-Course of the Retinal Nerve Fibre Layer Degeneration after Complete Intra-Orbital Optic Nerve Transection or Crush: A Comparative Study." Vision research 49.23 (2009): 2808-25.
Putz, U., C. Harwell, and E. Nedivi. "Soluble Cpg15 Expressed During Early Development Rescues Cortical Progenitors from Apoptosis." Nature neuroscience 8.3 (2005): 322-31.
Qu, J., and T. C. Jakobs. "The Time Course of Gene Expression During Reactive Gliosis in the Optic Nerve." PLoS One 8.6 (2013): e67094.
Quigley, H. A., and E. M. Addicks. "Chronic Experimental Glaucoma in Primates. Ii. Effect of Extended Intraocular Pressure Elevation on Optic Nerve Head and Axonal Transport." Invest Ophthalmol Vis Sci 19.2 (1980): 137-52.
Quigley, H. A., et al. "Optic Nerve Damage in Human Glaucoma. Ii. The Site of Injury and Susceptibility to Damage." Arch Ophthalmol 99.4 (1981): 635-49.
Quigley, H. A., and A. T. Broman. "The Number of People with Glaucoma Worldwide in 2010 and 2020." Br J Ophthalmol 90.3 (2006): 262-7.
Quigley, H. A., et al. "Retinal Ganglion Cell Death in Experimental Glaucoma and after Axotomy Occurs by Apoptosis." Investigative ophthalmology & visual science 36.5 (1995): 774-86.
Raymond, I. D., et al. "Cyan Fluorescent Protein Expression in Ganglion and Amacrine Cells in a Thy1-Cfp Transgenic Mouse Retina." Molecular vision 14 (2008): 1559-74.
Rein, D. B. "Vision Problems Are a Leading Source of Modifiable Health Expenditures." Invest Ophthalmol Vis Sci 54.14 (2013): ORSF18-22.
Ridet, J. L., et al. "Reactive Astrocytes: Cellular and Molecular Cues to Biological Function." Trends in neurosciences 20.12 (1997): 570-7.
Rutland-Brown, W., et al. "Incidence of Traumatic Brain Injury in the United States, 2003." J Head Trauma Rehabil 21.6 (2006): 544-8.
Sandvig, A., et al. "Myelin-, Reactive Glia-, and Scar-Derived Cns Axon Growth Inhibitors: Expression, Receptor Signaling, and Correlation with Axon Regeneration." Glia 46.3 (2004): 225-51.
Schwartz, M. "Optic Nerve Crush: Protection and Regeneration." Brain research bulletin 62.6 (2004): 467-71.

Silver, J., and J. H. Miller. "Regeneration Beyond the Glial Scar." Nature reviews. Neuroscience 5.2 (2004): 146-56.
Song, K. J., et al. "The Relationship between Spinal Stenosis and Neurological Outcome in Traumatic Cervical Spine Injury: An Analysis Using Pavlov's Ratio, Spinal Cord Area, and Spinal Canal Area." Clin Orthop Surg 1.1 (2009): 11-8.
Stokin, G. B., et al. "Axonopathy and Transport Deficits Early in the Pathogenesis of Alzheimer's Disease." Science 307.5713 (2005): 1282-8.
Sun, F., et al. "Sustained Axon Regeneration Induced by Co-Deletion of Pten and Socs3." Nature 480.7377 (2011): 372-5.
Tang, S., et al. "Soluble Myelin-Associated Glycoprotein Released from Damaged White Matter Inhibits Axonal Regeneration." Molecular and cellular neurosciences 18.3 (2001): 259-69.
Tang, Z., et al. "Survival Effect of Pdgf-Cc Rescues Neurons from Apoptosis in Both Brain and Retina by Regulating Gsk3beta Phosphorylation." The Journal of experimental medicine 207.4 (2010): 867-80.
Templeton, J. P., et al. "Differential Response of C57bl/6j Mouse and Dba/2j Mouse to Optic Nerve Crush." BMC neuroscience 10 (2009): 90.
Truettner, J. S., et al. "Subcellular Stress Response after Traumatic Brain Injury." J Neurotrauma 24.4 (2007): 599-612.
Williamson, T. L., and D. W. Cleveland. "Slowing of Axonal Transport Is a Very Early Event in the Toxicity of Als-Linked Sod1 Mutants to Motor Neurons." Nat Neurosci 2.1 (1999): 50-6.
Windle, W. F. "Regeneration of Axons in the Vertebrate Central Nervous System." Physiological reviews 36.4 (1956): 427-40.
Winzeler, A. M., et al. "The Lipid Sulfatide Is a Novel Myelin-Associated Inhibitor of Cns Axon Outgrowth." The Journal of neuroscience : the official journal of the Society for Neuroscience 31.17 (2011): 6481-92.
Wohlfart, G. "Degeneration and Regeneration in the Nervous System. Recent Advances." World neurology 2 (1961): 187-98.
Woldemussie, E., M. Wijono, and G. Ruiz. "Muller Cell Response to Laser-Induced Increase in Intraocular Pressure in Rats." Glia 47.2 (2004): 109-19.
Yin, Y., et al. "Oncomodulin Is a Macrophage-Derived Signal for Axon Regeneration in Retinal Ganglion Cells." Nat Neurosci 9.6 (2006): 843-52.

CHAPTER II

OPTIC NERVE CRUSH INDUCES SPATIAL AND TEMPORAL GENE EXPRESSION PATTERNS IN RETINA AND OPTIC NERVE OF BALB/CJ MICE

Tasneem P. Sharma, Colleen M. McDowell, Yang Liu, Alex H. Wagner, David Thole, Benjamin P. Faga, Robert J. Wordinger, Terry A. Braun and Abbot F. Clark

Abstract

Background

Central nervous system (CNS) trauma and neurodegenerative disorders trigger a cascade of cellular and molecular events resulting in neuronal apoptosis and regenerative failure. The pathogenic mechanisms and gene expression changes associated with these detrimental events can be effectively studied using a rodent optic nerve crush (ONC) model. The purpose of this study was to use a mouse ONC model to: (a) evaluate changes in retina and ON gene expression, (b) identify neurodegenerative pathogenic pathways and (c) discover potential new therapeutic targets.

Results

Only 54% of total neurons survived in the ganglion cell layer (GCL) 28 days post crush. Using Bayesian Estimation of Temporal Regulation (BETR) gene expression analysis, we identified significantly altered expression of 1,723 and 2,110 genes in the retina and ON, respectively. Meta-analysis of altered gene expression (≥ 1.5, ≤ 1.5, $p<0.05$) using Partek and DAVID demonstrated 29 up- and 20 down-regulated retinal gene clusters and 82 up- and 42 down-regulated optic nerve clusters. Regulated gene clusters included regenerative change, synaptic plasticity, axonogenesis, neuron projection, and neuron differentiation. Expression of selected genes (*Vsnl1*, *Syt1*, *Synpr* and *Nrn1*) from retinal and ON neuronal clusters was quantitatively and qualitatively examined for their relation to axonal neurodegeneration by immunohistochemistry and qRT-PCR.

Conclusion

A number of detrimental gene expression changes occur that contribute to trauma-induced neurodegeneration after injury to ON axons. *Nrn1* (synaptic plasticity gene), *Synpr* and *Syt1 (*synaptic vesicle fusion genes), and *Vsnl1* (neuron differentiation associated gene) were a few of the potentially unique genes identified that were down regulated spatially and temporally in our rodent ONC model. Bioinformatic meta-analysis identified significant tissue-specific and time-dependent gene clusters associated with regenerative changes, synaptic plasticity, axonogenesis, neuron projection, and neuron differentiation. These ONC induced neuronal loss and regenerative failure associated clusters can be extrapolated to changes occurring in other forms of CNS trauma or in clinical neurodegenerative pathological settings. In conclusion, this study identified potential therapeutic targets to address two key mechanisms of CNS trauma and neurodegeneration: neuronal loss and regenerative failure.

Keywords: Central nervous system, optic nerve crush, retinal ganglion cell, apoptosis, axotomy, neurodegeneration, regeneration, microarray, gene expression

Background

Central nervous system (CNS) trauma and neurodegenerative disorders trigger a cascade of cellular events resulting in extensive damage to neurons [1-5]. The non-permissive degenerative environment is due to expression of inhibitory cues [3, 6-12], glial scarring [5, 13], slow clearance of axonal debris [14], and CNS inflammation [15, 16]. Regenerative failure is a critical endpoint of these destructive triggers culminating in neuronal apoptosis [3, 17, 18] and inhibition of functional recovery.

The rodent optic nerve crush (ONC) model is an effective model for CNS trauma and regeneration failure [19-25]. The easy accessibility of the ON, an extension of the CNS, and the reproducibility of the ONC model make it an effective tool to study CNS trauma. Changes in gene expression in rodent ONC models have been previously studied [22, 26-30] and include gap associated protein 43 (*Gap43*) [31-33], glial fibrillary acidic protein (*Gfap*) [34-36] and neurofilament deregulation after crush injury [37]. Furthermore, progressive RGC degeneration has been associated with loss of trophic support [38, 39], stimulation of inflammatory processes/ immune regulation [40, 41], and apoptotic effectors [39, 42-45]. In addition, multiple injury models have been utilized to assess the fate of retinal ganglion cells after ocular injuries that include ischemia/reperfusion, ON irradiation, ON transections, and traumatic ON injury in rodent and primate models [22, 30, 46-50]

Although previous studies with CNS trauma models have addressed gene expression changes related to neuronal apoptosis [39], current gaps still exist for identifying long-term neuroprotective and regeneration inducing targets. Additionally, most expression studies for the ONC model have only been performed in the retina or the optic nerve head

[3, 22, 29]. We adopted a distinct strategy from previously published literature by: (a) simultaneously focusing on both the retina and ON, (b) detailing an extended time-course after acute axonal trauma and (c) centering on neurodegeneration and regenerative failure. To pinpoint specific degenerative pathways and identify crucial genes involved with pathological axonal injuries, it is essential to create an extensive molecular gene profile underlying neuronal degeneration and regeneration failure mechanisms. Our study will systematically and temporally identify these degenerative mechanisms that ensue after such an insult. To prevent the progression of the disease, new drug therapies geared towards neuroprotection and effective axonal regeneration are required. The purpose of this study is to detect and quantify progressive temporal degenerative changes by: (a) analyzing gene clusters in the retina and ON using Affymetrix microarrays in the neural, immune, and glial cells following ONC and (b) identifying temporal and spatial expression patterns of key gene targets within the retina and ON after trauma. These data will allow the identification of a wide range of potential therapeutic targets associated with neuronal loss and regenerative failure.

Results

This study highlights common as well as distinct gene expression responses of the retina and ON to ONC injury. To better understand the molecular mechanisms associated with neurodegenerative processes after injury, we first examined the survival of neurons in the ganglion cell layer (GCL) after acute axonal trauma by histological examination of the retinas over an extended 28-day period, which is a well-established time line for RGC death [19]. Second, we identified significant cluster-based changes occurring sequentially in the retina and ON by meta-analysis of the array data. Third, we identified key clusters

associated with neuron degeneration to isolate potential underlying damaging gene expression changes occurring within the retina and ON. Lastly, the expression of selective genes was confirmed quantitatively and qualitatively to validate our array data and examine expression of potential therapeutic targets that are affected by CNS trauma.

Survival of neurons and specificity of gene expression changes following ONC

There was a progressive decrease of neurons in the RGCL following ONC as assessed by Nissl stained retinal flat mounts (Additional Figure 1A). Approximately 50% of the cells in the retinal ganglion cell layer (RGCL) are RGCs, while the remaining cells consist of displaced amacrine cells, astrocytes, and microglia [19, 51-54]. ONC directly damages the ON, eventually leading to the selective death of RGCs. A significant sequential decline of RGCL neurons is seen as early as 14 days post crush (dpc) (81.43% ± 16.9% survival, $p<0.01$) with increased decline by 21 dpc (58.72% ± 5.70% survival, $p<0.001$) and culminating in almost complete loss of RGCs by 28 dpc (54.21% ± 8.27% survival, $p<0.001$) (Additional Figure 1B).

Microarrays were performed following ONC on harvested retina and ON samples from naïve, 3, 7, 14, 21 and 28 dpc mice (n=5). For the analysis, the retina and ON samples were separately pooled for the experimental and control groups at each time point. Time-course microarray data analysis is challenging in pooled data because each sample has slight variations independent of other samples. These errors can be mitigated to an extent by analyzing the significant temporal changes of genes in pooled samples using the BETR analysis [55]. This evaluation allowed us to delineate the differences in percentage of gene changes occurring temporally after ONC within the retinal and ON datasets. BETR probabilities were determined for the total 18,786 genes identified within each

dataset. BETR probabilities ranged from 0 to 1 with 0 being the least significantly changed genes temporally and 1 being the most. Genes were then classified into frequency bins based on the range of BETR probabilities.

In the retinal dataset, only 9.17% (1,723 genes out of 18,786 genes) had the highest BETR probabilities within frequency bin 10 (BETR probability - 0.9 to1.0) indicating only a small specific percentage of total genes were altered temporally after ONC trauma (Additional Figure 2A, Additional Table 1A). Furthermore, within the ON dataset, only 11.23% (2,110 genes out of 18,786 genes) were in the highest BETR probability range (Additional Figure 2B, Additional Table 1B). The small subset of genes identified by BETR analysis correlates with regenerative failure and degeneration that occurs within the retina and ON.

Cluster specific gene classification following ONC

To extract meaningful biological information from the array data, we used the public data-mining tool DAVID to cluster all differentially expressed genes into mechanistic biological categories. Temporal cluster classification is crucial for identifying the neuronal loss mechanisms that are sequentially regulated after trauma. Based on PARTEK fold change levels (≥ 1.5 and ≤ -1.5 compared to the corresponding contralateral control eyes, *q-value* defined by the FDR analogue of the p-value <0.05), we temporally categorized the clusters within three gene ontologies (GO); molecular function (MF), biological process (BP) and cellular component (CC) according to the *Mus musculus* genome within the DAVID database.

A total of 30 up-regulated clusters and 20 down-regulated clusters were significantly identified in the retinal dataset ($p < 0.05$) and 82 up-regulated clusters and 42 down-

regulated clusters were identified within the ON dataset (Table 1). To outline neurodegenerative mechanisms, key clusters were identified relating to neuronal loss and regeneration failure from both the retinal (Figure 1) and ON (Figure 2) clusters previously classified in Table 1. Each of these key clusters contained a group of genes significantly ($p < 0.05$) correlating with that specific cluster. The temporal patterns of the microarray gene ratios were graphed according to their association with these clusters for the retina (Figure 1) and ON (Figure 2).

Retinal clusters associated with neuronal loss and regeneration failure included the clusters neuron projection, regulation of axonogenesis, neuron projection morphogenesis, neuron differentiation and axon clusters (Figure 1). Of particular interest was the gene Neuritin 1 *(Nrn1),* which was identified within the neuron projection morphogenesis and neuron differentiation clusters (Figure 1C, 1D). NRN1 is a secreted GPI-linked protein that stimulates axonal and dendritic arbor growth [56]. Down-regulation of *Nrn1* mRNA expression within the microarray was observed to be biphasic with an initial decline through 7 dpc, a slight increase at 14 dpc and a further decrease by 21 dpc (Figure 1C, 1D). These biphasic patterns may indicate a transient attempt at neuroprotection/neuro-regeneration early in the response to injury.

ON clusters associated with neuronal loss and regeneration identified from the ON cluster tables (Tables 1C and 1D) included positive regulation of axonogenesis, regulation of synaptic plasticity, neuron projection, synaptic transmission and neurofilament cytoskeleton organization (Figure 2).

Neuron projection and synaptic transmission clusters both identified key genes called synaptotagmins *(Syt)* that participate in axonal regeneration, including synaptic projection

and proper axonal targeting. Expression of *Syt* genes was elevated in the ON at 21 dpc (Figures 2C, 2D). Essential proteins associated with synaptic vesicles and synaptic transmission are translated in the soma and get transported to the growing distal ends of extending neurites after crush injury [57, 58]. Eventually, as the RGCs are trying to overcome regenerative failure, they may increase expression of Syt proteins within their axons in attempt to induce synaptic plasticity and transmission at distal ends.

By analyzing the retina and ON simultaneously, we were able to observe the temporal response of gene expression in the retina and ON individually as well as in comparison to each other. Neurofilament (NF) genes were identified in most of the retinal and optic nerve clusters. Decreased expression of neurofilament medium (*Nefm*) and light chain (Nefl) genes in the retina at 3 and 21dpc (Figure 1) preceded neuronal loss after axonal damage (Additional Figure 1B). However, by 28 dpc *Nefm* and *Nefl* expression was elevated in the ON (Figures 2C and 2E). This pattern of NF expression is consistent with previous studies identifying NF dysregulation during neurodegeneration [59-72].

Validation of key target genes having differential expression by qRT-PCR

Analysis of pooled microarray samples does not account for the potential variations that exist between samples and may mask individual sample differences. To confirm individual samples follow the same trend of expression as the microarray data, we used qRT-PCR to determine the expression levels of target genes in each sample. For the retina, we verified two genes (*Nrn1* and *Vsnl1*) that have been previously identified as RGC markers [73, 74]. *Nrn1* had similar expression patterns as *Vsnl1* (Figure 3A and 3B, respectively), and expression of each gene was significantly correlated with the corresponding microarray ratios (*Nrn1* $R^2 = 0.96$, *Vsnl1* $R^2 = 0.73$) ($p < 0.05$) (Additional

Tables 2A and 2B). Both genes displayed a biphasic level of expression with significantly decreased expression from basal naïve levels at 3 and 21 dpc and modestly decreased expression at 14 dpc ($p < 0.05$, n=5) (Figure 3).

In the ON dataset, *Nrn1*, synaptotagmin 1 (*Syt1*) and synaptoporin (*Synpr)* expression levels were validated by qRT-PCR. We observed significantly increased expression of *Nrn1* at 28 dpc versus all time-points ($p < 0.05$, n=5) (Figure 4A). The qRT-PCR results significantly correlate with the microarray data ($R^2 = 0.86$, $p< 0.05$) (Additional Figure 2A and 2B). *Syt1* expression was significantly up regulated at 21 dpc ($p < 0.05$, n=5), in contrast to all the other time points (Figure 4B). These results were similar to *Syt1* microarray ratios, in which *Syt1* expression was elevated only at the 28 dpc period. The shift in the time course of gene up-regulation is most likely due to various individual samples that were masked in the pooled microarray samples. Therefore, the linear regression correlation between both sets of data was less than 0.5 ($R^2 = 0.07$) (Additional Tables 2A and 2B). Increased expression of synaptoporin (*Synpr*) was observed at 28d (Figure 4C) and correlated significantly with gene microarray ratios ($R^2 = 0.71$, $p < 0.05$, n=5) (Additional Table 2B). Expression of *Synpr* has been shown in neurons while *Syt1* has been shown in both neurons and critical in fusion events of astrocytes [75-79]. In addition to synapse formation, *Syt1* has also been shown to regulate the formation of axonal filopodia and branching [78]. The induction of both *Synpr* and *Syt1* expression may be related to synaptic vesicle fusion and release, and the roles of both genes in ONC need to be further explored.

Immunohistochemical analysis of validated gene targets

RGCs comprise only about 0.5% of the whole retina and only approximately 50% of the cells in the retinal ganglion cell layer (RGCL) are RGCs. The remaining cells consist of displaced amacrine cells, astrocytes, and microglia [19, 51-54, 80]. Whole retinas were utilized for microarray analysis, potentially masking the changes specific to the RGCs. To determine temporal protein expression patterns occurring specifically within the RGCs of the GCL, we performed retinal immunostaining. We first tested the expression of Brn3a (brain-specific homeobox/POU domain protein 3A), a well-known marker for RGCs [81-83]. As expected, we observed a progressive decrease in Brn3a expression after ONC within the GCL (Figure 5A and 5D). These results demonstrate a temporal decline in RGCs after axonal injury.

Retinal immunostaining for Vsnl1 and Nrn1 proteins confirmed apparent temporal changes in protein expression after ONC. Within the naïve retinal sections, approximately 50% of the GCL cells were positive for Vsnl1/Brn3a and 47% were positive for Nrn1/Brn3a (Figure 5E and 5F). A biphasic protein expression pattern was observed for Vsnl1 with decreased expression in the nerve fiber layer (NFL) and inner plexiform layer (IPL) at 7 dpc, increased expression at 14 dpc compared to the naive retina, and a complete loss of expression by 28 dpc (Figure 5B). Focusing on the GCL, the staining pattern also changed at 7dpc and became more cytoplasmic, in contrast to the diffuse pattern observed in the naïve retinas (Figure 5E). These data verify the *Vsnl1* mRNA expression data (Figure 3A).

A similar biphasic expression pattern was observed for Nrn1 with peak expression at 14 dpc (Figure 5C) and increased nerve fiber layer staining pattern with the GCL at 7dpc (Figure 5F). Compared to Vsnl1, Nrn1 immunostaining was observed in the ganglion

cells and NFL, but not as extensively within the IPL. In addition, previous studies of retinal *Nrn1 in-situ* hybridization exhibited predominant expression within the ganglion cell layer [84], which agrees with our IHC study.

Temporal Syt1, Synpr and Nrn1 protein expression patterns were determined in longitudinal sections of the ON. Images were examined at each time point for each protein as represented by Figure 6A. At 7dpc, all proteins were individually co-labeled with Nfl to show localization of the axons and the pattern of staining for each protein within the ON (Additional Figure 3G-3I). The expression pattern of Synpr and Nrn1 is not as intense as Syt1 (Additional Figures 3H, 3I) but still colocalized with Nfl staining. In contrast, the staining pattern of Syt1 colocalizes with Nfl and also within the cells surrounding the ON axons (Additional Figure 3G). Increased expression of Syt1 was evident at 14, 21 and 28 dpc (Figure 6B and 6C). Elevated levels of Syt1 were seen within the cytoplasmic region of ON cells through the time course post crush (Figure 6B). Synpr protein expression within the ON was evident at 21 and 28 dpc (Figure 6D and 6E). Similar to Syt1 expression, Synpr cytoplasmic staining was observed within the ON cells (Figure 6D). The temporal protein expression pattern of Syt1 and Synpr followed the mRNA expression patterns (Figure 4B and 4C).

In contrast to Syt1 and Synpr, Nrn1 ON protein expression levels (Figure 6F and 6G) did not compare to the mRNA expression data (Figure 4A). Increased expression of Nrn1 was observed at 14 and 21 dpc (Figure 6F), while increased mRNA expression was observed at 28 dpc (Figure 4A). The offset in the time course for protein expression compared to mRNA expression can be expected due to both mRNA half-life stability and rates of protein synthesis to degradation.

Discussion

Signaling pathways involved in RGC degeneration are quite complex, and identifying correct target molecules that can mitigate neuronal degeneration and failed regeneration are necessary to develop new neuroprotection strategies. We utilized the ONC mouse model to understand the mechanisms involved in RGC death. ONC directly damages the ON, leading to a progressive loss of RGCs. We identified temporal gene expression changes in the retina and ON after ONC. Key genes associated with neuronal loss and regenerative failure were identified in both retina and ON, and the expression changes were validated by qRT-PCR and immuno-staining.

Previous ONC studies have observed changes in gene expression within the retina [22, 85, 86] and glial based responses within the ON [29]. Analyzing the retina and ON simultaneously allowed the identification of individual clusters related to neuronal loss and regenerative failure within each tissue separately as well as allowed us to observe the temporal response of gene expression occurring in both the optic nerve and retina with progressive injury to these two tissues.

Neurofilament genes were identified in both the retina and ON datasets. Atypical accumulations of NFs are associated with several neurodegenerative disorders [59-72], and deregulation of NFs and NF aggregation accompany axonal damage after CNS trauma. NFs have also been associated with CNS diseases and axonal degenerative processes [87]. We show temporal differences in neurofilament expression between the retina and ON suggesting crucial gene changes occur after trauma in the retina and ON. There is progressive decline of retinal *Nfl* expression compared to the elevated expression within the ON out to 28 dpc. These results are consistent with a model in which axonal

damage precedes retinal neuronal degeneration and accumulation of damage associated genes occurs within the ON before soma degeneration. The changes in expression patterns identified in our ONC model correlate with previous studies identifying NF dysregulation during neurodegeneration [59-72].

The RGCL comprises multiple cell types including RGCs, amacrine cells, astrocytes, microglia, and vascular cells that interact with the RGC somas. After ONC, these cells also initiate degenerative pathways causing RGC apoptosis [88, 89]. Thus, the deregulation of genes observed within the retina is not restricted to RGCs and also represent gene expression of the surrounding cells. Glial fibrillary acidic protein (*Gfap)*, a marker of astrogliosis, is up-regulated after CNS trauma and is used as a universal index of retinal injury[34, 89]. *Gfap* is initially up-regulated after ONC [35, 36] and showed a similar expression pattern in our retinal dataset. After injury of CNS axons, glial responses around the affected area are increased, and this may contribute to trauma-induced neurodegeneration [90]. By identifying key clusters associated with degeneration of neurons and axonopathy, we were able to isolate potential target genes (*Vsnl1, Nrn1, Syt1, Synpr)*.

Vsnl1 gene is a member of the neuronal subfamily of EF-hand calcium sensor proteins. These proteins play vital roles in cellular signal transduction and neuroprotection/neurotoxicity and have been implicated in neurodegenerative diseases [91, 92]. Vsnl1 is predominantly expressed in isolated immuno-panned rat RGCs [73] and has also been shown to specifically label the inner retina (amacrine and RGCs) and the inner plexiform layer of rat, chicken, and bovine retinas [93]. In our study, expression of the *Vsnl1* gene is down-regulated after ONC, which may prevent the survival of RGCs.

Although the precise functional roles of *Vsnl1* are still unclear, Vsnl1 proteins may play key roles in membrane trafficking, neuronal signaling, and differentiation [92]. As ON axonal transport is inhibited after ONC, decreased levels of *Vsnl1* may contribute to the deleterious effects on axonal transport mechanisms seen in ONC.

Functionally, Nrn1 acts as a ligand to the insulin receptor [94] and cleavage of the GPI anchor by phospholipase C allows the soluble secreted form to be cell independent [95]. The GPI membrane bound anchor of Nrn1 allows growth promotion as it can stimulate axonal plasticity, dendritic arborization, and synapse maturation in the CNS [56, 95]. Conditional knockout of the *Nrn1* gene delays development, maturation of axons and dendritic arbors, synaptic maturation, and effective learning [96]. Neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) as well as neuronal activity can potentiate the expression of *Nrn1* [97, 98]. NGF induces expression of *Nrn1*, which increases neurite outgrowth in a variety of experimental models [97, 99, 100]. Our studies suggest that after axonal insult, RGCs initially increase *Nrn1* expression for axonal regeneration to overcome obstructed transport mechanisms. These regenerative supportive mechanisms are lost 14 dpc because by then most of the RGCs have been damaged, and the survival of these neurons has progressively decreased. The correlation of retinal protein expression of Nrn1 at 14 dpc mimics the elevated expression of Nrn1 at the same time-point within the ON. Taken together, these data suggest that the dynamic regulation of *Nrn1* may be an effort for axonal regeneration after ONC.

The relative abundance of protein expression may not be proportional to the relative mRNA levels. This lack of correlation in mRNA and protein expression levels could be

due to mRNA stability and/or rates of protein synthesis and/or degradation. The slight increase in retinal mRNA expression at 14 dpc (compared to 3 and 7 dpc) could result in increased the translation of the Nrn1 within the RGCs soma and Nfl, which is then transported downstream to the ON axons.

The optic nerve includes not only the axons of the RGCs but also astrocytes, microglia, and oligodendrocytes that interact with RGC axons as well as each other[29]. Thus, the expression of genes observed within the ON may represent the beneficial or detrimental effects of neighboring cells surrounding the RGC axons. Differentially regulated genes within the ON expression microarray also identified other key genes associated with synaptic transmission (*Syt1* and *Synpr*) and synaptic plasticity gene (*Nrn1)* that participate in axonal regeneration, including synaptic projection, and proper axonal targeting.

A collection of signaling mechanisms link both axonal tips and dendritic terminals to neuronal soma and nucleus by motor-dependent transport machineries. Signaling complexes could be transported either in endosomes, or as non-endosomal complexes associated with importins and dynein [103]. Essential membrane components of synaptic vesicles and synaptic transmission associated proteins are translated in the soma and get transported to the growing distal ends of extending neurites after crush injury [57, 58]. In addition, synaptic vesicles are localized to small vesicles within the neuron, particularly in neuronal axonal processes [104]. Eventually, as axonal transport is inhibited after ONC due to glial scarring [5, 13], there is decreased transport of proteins involved in neuroprotection and synaptic plasticity. This causes deleterious affects, eventually leading to decreased synaptic plasticity and transmission at distal ends.

Syt proteins act as synaptic calcium sensors for vesicle fusion in conjunction with SNAREs that facilitate intracellular membrane fusion events [105-107]. Syts have a conserved mechanism of action and are crucial for neuronal Ca^{2+}-triggered vesicle fusion [108]. Previous studies have shown *Syt1* to participate in axonal regeneration, including synaptic projection and proper axonal targeting [78, 109]. In our study, Syt1 was identified in ON neuron projection and synaptic transmission clusters. It appears that the ON attempts to initiate synaptic projection following ONC trauma as shown by the biphasic mRNA and protein expression of Syt1.

Similarly, *Synprs* are essential membrane components of synaptic vesicles [77]. *Synpr* has restricted distribution within the CNS and is present in the telencephalic structures, hippocampus, olfactory bulb, and retina [75, 110-112]. *Synpr* plays potential roles in the modulation of synaptic transmission and specificity to neuronal circuitry [77]. Increased protein expression of Synpr in the ON was observed at 21 and 28 dpc. The induction of both Synpr and Syt1 expression may be related to synaptic vesicle fusion and release. Elevated expression of Synpr and Nrn1 suggests they are mediating synaptic differentiation as synaptic organizing proteins, but the deregulation of mRNA expression and eventually protein expression may be a futile attempt at ON regeneration in late pathogenesis.

We have explored temporal gene expression changes after ONC axonal injury that can be extrapolated to other CNS traumas. Although there are gene expression differences between the retina and brain, similar differences also occur within discrete regions of the brain as each part of the brain has different motoric, sensory, and cognitive functions. For example, gene expression in the cerebellum differs the most from the other regions of the

brain [113, 114] and has also been reported in inbred strains of mouse brain [115]. In addition, inter-individual differences have also been reported within a species [114]. As is the case while studying any trauma or disease model, only a generic evaluation can be made in terms of relevance to other regions in the CNS. In conclusion, the ONC model has identified two key mechanisms of CNS trauma and neurodegeneration: neuronal loss and regenerative failure. Dysregulation of *Vsnl1*, *Syt1*, *Synpr* and *Nrn1* gene expression may play an important role in neurodegeneration and potentially provide unique targets for intervention.

Conclusions

The current study delineates the gene expression profile associated with neurodegeneration and regenerative failure after ONC-induced CNS trauma. CNS trauma causes degeneration of neurons and axonopathy, which is evident in neurodegenerative diseases such as Parkinson's, Alzheimer's, and glaucoma [1-5]. The susceptibility of the neurons to acute axonal injury allowed the identification of gene expression changes that occur before neuronal loss. Using the reproducible ONC model of CNS trauma, we were able to: (a) examine gene expression changes within the retina and ON, and (b) visualize protein expression patterns of key selected genes associated with neuron loss and regenerative failure within the retina and ON after ONC. BETR analysis of microarray gene expression data was utilized to show that a select small subset of genes was affected at multiple time points following ONC. Bioinformatic meta-analysis identified gene clusters associated with regenerative changes, synaptic plasticity, axonogenesis, neuron projection, and neurodegeneration. A neurite synaptic plasticity gene (*Nrn1*), synaptic vesicle fusion genes (*Synpr* and *Syt1*), and neuron differentiation associated gene (*Vsnl1*)

were a few of the key temporally regulated genes identified in our study. In conclusion, analysis of these gene arrays and protein expression patterns allowed the detection, quantification and visualization of key differentially regulated genes after ONC. This study has identified potential pathogenic genes and possible new therapeutic targets to address two key mechanisms of CNS trauma: neuronal loss and regenerative failure.

Methods

Animals

BALB/cJ mice aged 2-4 months were utilized for all the experiments and were obtained from the Jackson Laboratories (Bar Harbor, ME). The mice were housed and maintained in a 12-hour light/dark cycle and fed ad libitum. All procedures were performed in accordance with Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and the University of North Texas Health Science Center (UNTHSC) Institutional Animal Care and Use Committee regulations (IACUC protocol # 2011/2012-58-A04, approved October 8th 2012).

Mice were divided into three separate groups according to the experiment. The time course of the optic nerve crush (ONC) included six different time-points (naïve (0), 3, 7, 14, 21, 28 days post crush (dpc)). To determine the percentage of neurons surviving after crush, 8-9 mice per time-point were used for retinal Nissl staining. For the microarray studies and qPCR validation, 5 mice were used per time-point. After the retinal and ON tissues were harvested, the samples were divided into two parts. cDNA was made from half of each individual sample for qPCR validation, while the remaining portion of samples were pooled to generate one sample for each experimental time point. Microarray analysis performed on control and ONC retina and ON samples for each time

point. To qualitatively identify protein expression, three mice per time-point were utilized for immunohistochemistry (IHC).

Optic Nerve Crush Model

The ON of the left eye was crushed 0.5mm posterior from the globe for 4 seconds using the Nickell's technique [19]. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and an incision was made along the superior orbital margin. The ON (left) was exposed and crushed using a self-closing jeweler's forceps to ensure reproducibility and constant force. Extreme care was taken not to damage the ocular blood vessels. Indirect ophthalmoscopy was performed to ensure retinal circulation was not blocked. The contralateral eye was used as the uncrushed control.

Characterization of Optic Nerve Crush Model

To quantify cell loss from the retinal ganglion cell layer (RGCL), retinas from fixed eyes were dissected, flat mounted and Nissl stained with cresyl violet stain as previously described [19, 116, 117]. Eyes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; 0.1M phosphate and 100mM NaCl buffer (pH 7.4)) for an hour at room temperature. After fixation, the eyes were rinsed with PBS, and the posterior cup isolated and placed in 0.3% Triton-X 100 PBS for 16 hours at 22°C. The tissues were then placed in 3% H_2O_2 and NaH_2PO_4 overnight. The retinas were dissected, cut into four quadrants, and mounted RGC side up on positively charged glass slides (Fisher Scientific, Chicago, IL). The slides were then air dried and flattened with coverslips using 10g weights. The dried retinas were stained with 1% cresyl violet acetate in 0.25% acetate for 30-45

seconds. After staining, the retinas were dehydrated in 90% and 100% ethanol and cleared with xylene to reduce background staining and mounted with a coverslip.

To determine the density of remaining RGCL neurons within each retina, two digital images from each quadrant (peripheral and mid-peripheral region - four quadrants / retina) were captured at 400 X magnification. A total of 8 images per retina were counted using Adobe Photoshop software. Cell counts were analyzed by comparing the experimental retinas against the contralateral control retinas (cell counts± SD) at each time point. Quantification of percentage neuron survival following ONC from 3 to 28 dpc was performed. Data points (Additional Figure 1B) represent mean ± SD of surviving neurons after crush normalized to contralateral control eyes. Statistics were determined using one-way ANOVA -Tukey, *post hoc* test, ** $p < 0.01$, *** $p < 0.001$, n=8-9 eyes/ time-point.

RNA Processing

Fresh retina and ON samples (from the globe to the chiasm) were cleanly dissected without any contamination from surrounding tissue. In brief, after euthanization each globe was harvested from the mouse eye socket right at the globe and optic nerve head (ONH) juncture. The globe was transferred to a clean petri dish and opened along the limbus. The retina was harvested from the posterior cup and the ONH removed using a trephine. For the ON, the skull was opened and each left and right ON between the globe and chiasm was harvested separately. All samples were collected in 1ml of TRIzol (Invitrogen, Grand Island, NY) and homogenized using 5mm steel beads in the TissueLyser LT (Qiagen, Valencia, CA) for five minutes at 50 oscillations/second. For phase separation 50µl of BAN Phase Separation Reagent (Molecular Research Center,

Cincinnati, Ohio) was added to the homogenized samples, and samples were centrifuged at 14,000rpm for 15 minutes. The upper aqueous phase was transferred to an RNeasy mini column (Qiagen, Valencia, CA) and processed according to the manufacturer's protocol. The total RNA was re-suspended in 20µl of nuclease-free water and quantified using the Thermo Scientific NanoDrop 2000 (NanoDrop products, Wilmington, DE). Integrity of the RNA was measured by calculating the RNA integrity number (RIN) using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), and samples with RIN values greater than 7 were used for microarray analysis.

Affymetrix Gene Chip Arrays

For microarray analysis, 420ng of RNA from each retina sample and 100ng from each ON sample was pooled to a total of 2100ng and 500ng, respectively, and this was performed for each experimental and control group at each time point. Microarray hybridizations were performed at the University of Iowa DNA Core Facility. Total RNA (50ng) was converted to SPIA (Single Primer Isothermal Amplification) amplified cDNA using the WT-Ovation Pico RNA Amplification System, v2 (NuGEN Technologies, San Carlos, CA). The amplified SPIA cDNA product was purified through a QIAGEN QIAquick PCR Purification column (QIAGEN). Five micrograms of this product were fragmented (average fragment size = 85 bases) and biotin labeled using the NuGEN FL-Ovation cDNA Biotin Module (NuGEN Technologies). The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Inc., Santa Clara, CA), placed onto Affymetrix Mouse Gene 1.0 ST arrays and incubated at 45º C for 18 h with 60 rpm rotation in an Affymetrix Model 640 Genechip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin-

phycoerythrin (Molecular Probes, Inc., Eugene, OR), and the signals were amplified with an anti-streptavidin antibody (Vector Laboratories, Inc., Burlingame, CA) using the Affymetrix Model 450 Fluidics Station. Arrays were scanned with the Affymetrix Model 3000 scanner with 7G upgrade, and data were collected using the using the GeneChip operating software (GCOS) v1.4.

Bioinformatic Analysis of Gene Expression Datasets

Microarray data were imported into the Partek Genomics Suite 6.6 software (Partek Inc., Louis, MO) and normalized based on the robust multi-array average (RMA). To further confirm the purity of each extracted tissue, we examined the expression of retina specific genes in the ON tissue and ON genes in the retina tissue. There was greater expression of retina specific markers: *Rho*, *Nr2e3*, *Nrl* and *Crx* in the retinal samples, while these genes were at the lower limits of detection in the ON samples. Conversely, there was greater expression of myelin marker genes *Mag*, *Mobp*, *Mog*, *Mbp* and *Plp1* in the ON samples compared to the retina samples. In addition, we tested expression levels of *Rho* and *Mbp* by qPCR in both tissues (Additional Figure 4).

For the microarray analysis, the ONC samples were compared to the control samples and the microarray ratios and $\log_2$ fold values calculated at each time point. Up and down regulated genes were identified for both datasets (retina and ON) with a selective filter of ≥ 1.5 and ≤ -1.5 fold values. The fold values were based on the *q-value* defined by the FDR analogue of the p <0.05. The genes were further analyzed using the publicly available bioinformatics software Database for Annotation, Visualization and Integrated Discovery (DAVID). Gene ontology (GO) based cluster analysis was performed to identify possible enrichment of genes (GO enrichment score calculated using a χ^2 test)

using differentially regulated genes per time point. The Fishers Exact p value is calculated by DAVID to identify GO enrichment based clusters and any p values <0.05 were considered to be significant based on the Benjamini multiple test correction and were enriched in the annotation category [118, 119]. Neuronal clusters were identified at specific time points and their genes graphed temporally under each GO category.

Identification of specific gene expression changes following ONC by Bayesian Estimation of Temporal Regulation analysis

Analysis of time-course microarray data was performed using Bayesian Estimation of Temporal Regulation (BETR) analysis to account for any variations between individual samples within the pooled samples. The BETR expression probabilities were estimated using Probe Logarithmic Intensity Error with GC-background correction, a routine built into the Affymetrix Power Tools toolkit. Expression estimates for 11 housekeeping genes across all time-points were used to create a linear model between the average expression level and variance of each gene and housekeeping genes. This model was used to simulate additional readings for all estimated transcripts at each time point, which were subsequently used as additional inputs to the BETR [55] R package. From this algorithm output final BETR probabilities were determined for the total 18,786 genes identified within each of the retina and ON gene expression datasets (Additional Table1A, 1B). BETR probabilities ranged from 0 to 1 with 0 being the least significantly changed genes temporally and 1 being the most. Genes were then classified into frequency bins based on the range of BETR probabilities. Each frequency bin identified a range of 0.1 differences in BETR probabilities and bins ranged from the lowest 1 (BETR probability range of 0-0.1) to frequency bin 10 (BETR probability range of 0.9-1) (Additional

Table1A, 1B). We considered low BETR probabilities of frequency bins <5 to reflect no significant changes in gene expression, while high BETR probabilities (0.9-1.0) within frequency bin 10 to represent significant changes in gene expression over time.

Microarray Confirmation Through Real-Time qRT-PCR

Quantitative real-time PCR (qPCR) was used to validate the temporal gene microarray expression ratios for the differentially expressed genes. From the retinal data sets, two genes (*Vsnl1* and *Nrn1*) were selected, while from the optic nerve data set, three genes were chosen (*Syt1, Synpr* and *Nrn1*) (Additional Table 2A). Reverse transcription was performed using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Each sample (500ng of RNA) was reverse transcribed as per manufacturer's protocol. Gene specific primers were designed (MGI database) (Additional Table 3) and PCR products sequenced to confirm the specificity of each primer's transcript (Genewiz Inc, NJ). qPCR was then performed in the BioRad CFX96 real time system (Bio-Rad Laboratories, Hercules, CA) using the SSoAdvanced™ SYBR Green master mix (Bio-Rad Laboratories, Hercules, CA). Cycles for the qRT-PCR were run as described in Additional Table 4. The cycle threshold (C_t) was assigned as $\log_2$ of PCR amplification. Technical duplicates for each sample were averaged, and each ONC and control samples were normalized to their own GAPDH C_t values. The difference between the ONC sample (experimental) and control sample ΔC_t values was used to determine the relative fold change in each sample based on a 2 fold exponential. Control qRT-PCR reactions were performed in the absence of a cDNA template. Gene expression fold changes were graphed temporally for each dataset and compared to temporal microarray ratios from the Partek analysis. Statistical analysis for qPCR was performed using the GraphPad Prism

Software (Mean ± SEM) using one-way ANOVA (Tukey, *post hoc* test) with a p value <0.05 considered statistically significant. Regression analysis was performed between qRT-PCR and microarray ratios, and the R^2 coefficient of determination calculated and p values <0.05 were considered statistically significant (Additional Table 2B).

Immunohistochemistry

IHC was performed to validate protein expression of qRT-PCR confirmed genes and to localize target proteins in the retina and ON. Whole eyes were harvested and fixed in 4% paraformaldehyde for 2 hours at room temperature. After fixation, the tissue was placed in 20% sucrose overnight at 4°C and embedded in optical cutting temperature (OCT) the next day. Sections (10 μm) were cut using a cryostat (Leica Biosystems - Richmond, IL). Cross sections of retina were transferred to Superfrost glass slides (Fisher Scientific - Chicago, IL). Slides were incubated in PBS for 10 minutes and blocked with SuperBlock™ Blocking Buffer (Fisher Scientific, Chicago, IL) at room temperature for one hour. Primary antibodies (Additional Table 5) were diluted in Superblock™. Each slide was probed with the respective primary antibody and incubated overnight at 4°C. Sections were then washed 3 times with PBS for 10 minutes each and incubated with Alexa Fluor secondary antibody (Additional Table 5) for 1 hour at room temperature. Slides were rinsed three times with PBS and mounted with ProLong ® Gold anti-fade reagent with DAPI (Molecular Probes, Grand Island, NY). Sections were observed and captured using a Nikon Eclipse Ti-U Microscope (Nikon, Melville, NY) containing the Nuance Multispectral imaging system and analyzed using Adobe Photoshop CS5 software. Negative control images of retina and ON sections with no primary antibody are presented in Additional Figures 3A-F.

List of Abbreviations

CNS – Central nervous system

TBI –Traumatic brain injury

SCI – Spinal cord injury

ONC – Optic nerve crush

RGC – Retinal ganglion cells

BETR - Bayesian Estimation of Temporal Regulation

DAVID - Database for Annotation, Visualization, and Integrated Discovery

MF – Molecular function

BP – Biological process

CC – Cellular component

NFL – Nerve fiber layer

GCL – Ganglion cell layer

DPC – Days post crush

OCT – Optical cutting temperature

PBS – Phosphate buffer saline solution

IHC – Immunohistochemistry

ANOVA – Analysis of variance

DAPI - 4',6-diamidino-2-phenylindole

qRT-PCR – Quantitative real time polymerase chain reaction

NCBI – National center for biotechnology information

GO – Gene ontology

RIN – RNA integrity number

Competing interests

The authors declare that they have no competing interests

Availability of supporting data

GEO Accession Number: Series GSE44708

Authors Contributions

TPS performed the RNA extraction, tissue sample staining, bioinformatics Partek and DAVID analysis, qRT-PCR and correlation analysis of all optic nerve crush samples; including writing all sections of the manuscript. CM and YL both performed the optic nerve crush and subsequently CM did the Nissl stained retinal flat mount neuron survival analysis while YL prepared the immunohistochemistry sections. BF processed image data. DT performed differential expression analysis and AW analyzed the microarray data using the BETR analysis. AFC, RJW and TAB conceived the study, actively participated in the design and coordination of the study, reviewed all the data, and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by a grant (W81XWH-10-2-0003) from the Department of Defense.

Additional Material

Additional Figure 1A, 1B: Optic nerve crush (ONC) significantly reduces neurons in the retinal ganglion cell layer (RGCL).

Additional Figure 2A, 2B: Frequency distribution of genes altered following optic nerve crush

Additional Figure 3 A-I: Naïve control images and expression of Syt1, Synpr, Nrn1 and Nfl in the ON at 7 days post crush.

Additional Figure 4A, 4B: Expression of tissue specific genes within normal retina and ON samples.

Additional Table 1A, 1B: BETR probabilities based retinal and ON genes distributed within frequency bins

Additional Table 2A, 2B: Microarray ratios and linear regression correlation values of selected target genes

Additional Table 3: Primers for key genes validated from the retina and optic nerve datasets

Additional Table 4: qRT-PCR cycles performed for confirming retina and optic nerve dataset gene expression levels

Additional Table 5: Antibodies against key proteins validated by IHC

Table 1A – Temporal classification of up-regulated retinal gene cluster changes following ONC

GENE ONTOLOGY	CLUSTERS	P VALUE
MOLECULAR FUNCTION	STRUCTURAL EYE PROTEIN	1.90E-06
	EYE DEVELOPMENT	4.50E-03
	EXTRACELLULAR MATRIX BINDING	5.30E-03
	CALCIUM ION BINDING	3.30E-02
	STRUCTURAL EYE LENS PROTEIN	2.20E-11
	STRUCTURAL MOLECULAR ACTIVITY	4.60E-06
BIOLOGICAL PROCESS	RESPONSE TO WOUNDING	1.00E-04
	INFLAMMATORY RESPONSE	3.80E-04
	DEFENSE RESPONSE	5.90E-04
	POSITIVE REGULATION OF IMMUNE SYSTEM RESPONSE	1.20E-02
	RHO PROTEIN SIGNAL TRANSDUCTION	5.90E-03
	REGULATION OF SIGNAL PROLIFERATON	2.70E-02
	DEFENSE RESPONSE	5.40E-04
	INFLAMMATORY RESPONSE	1.60E-02
	RESPONSE TO WOUNDING	4.70E-02
	SENSORY PERCEPTION	8.80E-03
	NEUROLOGICAL SYSTEM PROCESS	2.30E-02
	G-PROTEIN COUPLED RECEPTOR SIGNALING PATHWAY	3.90E-02
	MACROMOLECULAR COMPLEX ASSEMBLY	1.30E-02
	DNA PACKAGING	3.10E-02
	POSITIVE REGULATION OF PROTEIN KINASE ACTIVITY	4.50E-02
CELLULAR COMPONENT	CELL JUNCTION	2.00E-02
	EXTRACELLULAR REGION PART	3.20E-03
	EXTRACELLULAR MATRIX	7.60E-03
	LYSOSOME	3.40E-02
	EXTRACELLULAR REGION PART	3.50E-02
	MICROSOSME	1.70E-02
	INTERMEDIATE FILAMENT	3.70E-02
	RIBOSOME	5.00E-03

Table 1B – Temporal classification of down-regulated retinal gene cluster changes following ONC

GENE ONTOLOGY	CLUSTERS	P VALUE
MOLECULAR FUNCTION	STRUCTURAL EYE LENS PROTEIN	3.80E-15
	STRUCTURAL EYE LENS PROTEIN	2.60E-14
	PATTERN BINDING	3.80E-02
BIOLOGICAL PROCESS	CHROMATIN ASSEMBLY	4.80E-04
	REGULATION OF AXONOGENESIS	7.40E-03
	G-PROTEIN COUPLED RECEPTOR SIGNALING PATHWAY	2.00E-04
	NEUROLOGICAL SYSTEM PROCESS	7.10E-03
	INTERMEDIATE FILAMENT BUNDLE PROCESS	6.40E-05
	MICROTUBULE BASED PROCESS	5.10E-03
	AXONOGENESIS	1.70E-02
	NEURON PROJECTION MORPHOGENESIS	2.00E-02
	NEURON DIFFERENTIATION	4.20E-02
	CELL MORPHOGENESIS INVOLVED IN DIFFERENTIATION	1.20E-02
CELLULAR COMPONENT	NUCLEOSOME	7.50E-05
	NEURON PROJECTION	5.00E-05
	AXON	6.50E-05
	NEUROFILAMENT	1.60E-04
	INTRINSIC TO MEMBRANE	1.30E-02
	NEURON PROJECTION	9.90E-03
	CHROMOSOME	3.10E-02

Table 1C – Temporal classification of up-regulated ON gene cluster changes following ONC

GENE ONTOLOGY	CLUSTERS	P VALUE
MOLECULAR FUNCTION	CHEMOKINE ACTIVITY	4.50E-03
	CHEMOKINE ACTIVITY	6.50E-06
	GROWTH FACTOR BINDING	1.10E-03
	ACTIN BINDING	9.10E-03
	SERINE TYPE ENDOPEPTIDASE INHIBITOR ACTIVITY	7.10E-04
	CHEMOKINE ACTIVITY	6.70E-09
	CYTOKINE ACTIVITY	6.00E-06
	CHEMOKINE ACTIVITY	1.60E-04
	CYTOKINE BINDING	4.30E-04
	ION CHANNEL ACTIVITY	1.20E-09
	CALCIUM ION BINDING	7.70E-07
	GABA RECEPTOR ACTIVITY	4.30E-04
	NEUROTRANSMITTER BINDING	4.00E-03
	CALCIUM CHANNEL ACTIVITY	9.10E-03
	PROTEIN KINASE ACTIVATOR ACTIVITY	8.80E-03
BIOLOGICAL PROCESS	IMMUNE RESPONSE	4.50E-07
	CHEMOTAXIS	3.70E-02
	DEFENSE RESPONSE	6.20E-05
	TRANSLATION	1.20E-03
	CELL CYCLE	3.10E-06
	LEUKOCYTE ACTIVATION	2.10E-04
	ACTIN CYTOSKELETON ORGANIZATION	6.80E-03
	REGULATION OF ADAPTIVE IMMUNE RESPONSE	3.60E-04
	POSITIVE REGULATION OF PROGRAMMED CELL DEATH	1.20E-03
	POSITIVE REGULATION OF AXONOGENESIS	2.10E-02
	SENSORY PERCEPTION	2.90E-02
	IMMUNE RESPONSE	3.40E-08
	CHEMOTAXIS	2.60E-07
	RESPONSE TO WOUNDING	1.10E-06
	CELL ACTIVATION	5.70E-05
	DEFENSE RESPONSE	1.60E-08
	RESPONSE TO WOUNDING	4.00E-06
	CHEMOTAXIS	2.60E-06
	REGULATION OF ADAPTIVE IMMUNE RESPONSE	5.40E-06
	PHAGOCYTOSIS	7.10E-03
	NEUROPEPTIDE SIGNALING PATHWAY	2.50E-02
	ION TRANSPORT	2.70E-06
	TRANSMISSION OF NERVE IMPULSE	6.30E-08
	SYNAPTIC TRANSMISSION	1.20E-06
	SYNAPTIC VESICLE TRANSPORT	4.60E-03
	REGULATION OF SYNAPTIC PLASTICITY	2.40E-03
	REGULATION OF SYNAPTIC TRANSMISSION	6.40E-03
	SYNAPTOGENESIS	1.50E-02
	CELL ADHESION	3.20E-02
CELLULAR COMPONENT	EXTRACELLULAR REGION PART	4.70E-03
	CHROMOSOME	1.50E-09
	EXTRACELLULAR REGION PART	8.80E-08
	COLLAGEN	6.00E-03
	FOCAL ADHESION	7.20E-03
	ANCHORING JUNCTION	2.50E-02
	EXTRACELLULAR REGION	9.80E-04
	EXTRACELLULAR REGION	3.30E-03
	CELL SURFACE	2.80E-04
	EXTRACELLULAR REGION	2.60E-04
	SYNAPSE PART	1.90E-13
	POSTSYNAPTIC MEMBRANE	1.90E-07
	NEURON PROJECTION	3.30E-07
	DENDRITE	2.30E-05
	SYNAPTOSOME	3.10E-04
	POSTSYNATIC DENSITY	5.60E-03
	SYNAPTIC VESICLE	2.60E-03

Table 1D – Temporal classification of down-regulated ON gene cluster changes following ONC

GENE ONTOLOGY	CLUSTERS	P VALUE
MOLECULAR FUNCTION	CALMODULIN BINDING	6.50E-04
	VOLTAGE GATED ION CHANNEL ACTIVITY	6.70E-04
	ION BINDING	3.90E-04
	ENZYME BINDING	5.80E-03
	GABA RECEPTOR ACTIVITY	6.70E-03
	LIGAND GATED ION CHANNEL ACTIVITY	1.80E-02
	CALMODULIN BINDING	1.30E-02
	CALCIUM DEPENDENT PHOSPHOLIPID BINDING	1.40E-04
	NUCLEASE ACTIVITY	2.50E-02
	MICROTUBULE BINDING	2.50E-02
	MOTOR ACTIVITY	1.80E-02
	CYTOKINE ACTIVITY	2.90E-02
BIOLOGICAL PROCESS	REGULATION OF MAPKKK CASCADE	2.00E-02
	POTASSIUM ION TRANSPORT	4.80E-05
	CATION TRANSPORT	5.30E-04
	NEUROTRANSMITTER TRANSPORT	3.80E-03
	SYNAPTIC TRANSMISSION	1.40E-02
	CALCIUM ION TRANSPORT	1.10E-02
	NEUROFILAMENT CYTOSKELETON ORGANIZATION	1.20E-03
	INTERMEDIATE FILAMENT CYTOSKELETON ORGANIZATION	2.00E-03
	NEUROTRANSMITTER TRANSPORT	3.80E-03
	MAPKKK CASCADE	2.40E-02
	MICROTUBULE BASED PROCESS	1.10E-02
	VISUAL PERCEPTION	4.40E-05
	COGNITION	6.50E-03
	NEUROLOGICAL PROCESS	1.40E-02
	MICROTUBULE BASED PROCESS	1.30E-05
	LIPID BIOSYNTHETIC PROCESS	2.20E-02
	VISUAL PERCEPTION	3.20E-10
	SENSORY PERCEPTION	2.90E-02
	EYE DEVELOPMENT	4.80E-05
	IMMUNE RESPONSE	4.70E-02
CELLULAR COMPONENT	SYNAPSE	4.30E-06
	CELL JUNCTION	6.30E-04
	POST SYNAPTIC MEMBRANE	1.10E-03
	PRESYNAPTIC MEMBRANE	5.20E-05
	SYNAPSE PART	2.90E-04
	CELL JUNCTION	3.90E-03
	CELL PROJECTION	3.10E-03
	CLATHRIN COATED VESICLE	1.00E-02
	CYTOSKELETON	2.30E-02
	ANCHORED TO MEMBRANE	4.30E-02

Table 1 Temporal changes in gene clusters after optic nerve crush. Gene expression fold-change values were grouped individually from naïve eyes and ONC eyes out to 28 days post crush (dpc). Genes were highlighted based on fold values for up-regulated (>1.5) datasets and down-regulated (< -1.5) datasets. The selected genes were analyzed by gene ontology (GO) based cluster identification at each time point using DAVID.

Clusters from **(A)** up-regulated retinal, **(B)** down-regulated retinal, **(C)** up-regulated optic nerve and **(D)** down-regulated optic nerve datasets. Significance was determined using the Benjamini multiple test correction, GO enrichment score χ^2 test and Fishers Exact test (p value < 0.05). Time points: 3 dpc (green), 7 dpc (purple), 14 dpc (orange), 21 dpc (grey) and 28 dpc (orange).

Figure 1

Temporal changes of specific retinal gene clusters related to neuronal loss and regeneration failure. Neuron specific and axonal regeneration related neuronal clusters were selected from the retinal GO tables; and the microarray ratios of the genes within each of these clusters were graphed temporally (0 to 28 days post crush (dpc)). Neuronal clusters identified included **(A)** neuron projection, **(B)** regulation of axonogenesis, **(C)** neuron projection morphogenesis, **(D)** neuron differentiation and **(E)** axon. Significance of these clusters was determined using the Benjamini multiple test correction, GO enrichment score χ^2 test and Fishers Exact test (p value < 0.05).

Figure 2

Temporal changes of specific optic nerve gene clusters related to neuronal loss and regeneration failure. Neuron specific and axonal regeneration related neuronal clusters were selected from the optic nerve GO tables and the microarray ratios of the genes within each of these clusters graphed temporally (0 to 28 days post crush (dpc)). Neuronal clusters identified included **(A)** positive regulation of axonogenesis, **(B)** regulation of synaptic plasticity, **(C)** neuron projection, **(D)** synaptic transmission, **(E)** neurofilament cytoskeleton organization and **(F)** visual perception. Significance of these clusters was determined using the Benjamini multiple test correction, GO enrichment score χ^2 test and Fishers Exact test (p value < 0.05).

Figure 3

mRNA expression patterns of selected retinal genes following ONC insult. Pooled microarray mRNA expression changes were validated in individual samples by qRT-PCR.

Relative fold change in each sample was determined based on a 2 fold exponential using mRNA expression values normalized to Gapdh and the contralateral control eye. Fold values of each gene presented as mean ± SEM. **(A)** Visinin like 1 (*Vsnl1)* and **(B)** Neuritin 1 (*Nrn1).* Statistical significance for each time-point determined by one-way ANOVA -Tukey, *post hoc* test, * p value < 0.05, ** p value <0.01, *** p value < 0.001, n=5.

Figure 4

mRNA expression patterns of selected optic nerve genes following ONC insult. Pooled microarray mRNA expression changes were validated in individual samples by qRT-PCR. Relative fold change in each sample was determined based on a 2 fold exponential using mRNA expression values normalized to Gapdh and the contralateral control eye. Fold values of each gene presented as mean ± SEM. **(A)** Synaptotagmin 1(*Syt1),* **(B)** Synaptoporin (*Synpr)*, **(C)** Neuritin 1(*Nrn1)*. Statistical significance for each time-point determined by one-way ANOVA -Tukey, *post hoc* test, * p value < 0.05, ** p value <0.01, *** p value < 0.001, n=5.

Figure 5

Expression of Brn3a, Vsnl1 and Nrn1 in the retina following ONC. Progressive loss of RGCs observed with Brn3a and a biphasic pattern of expression observed for Vsnl1 and Nrn1. Time course of retina sections from naïve, 7, 14, 21 and 28 dpc (days post crush) are indicated on the top of the panel. Fluorescence micrographs of retinal tissue sections were immunolabeled with: **(A)** Brn3a (red), **(B)** Vsnl1 (green) and **(C)** Nrn1

(green). Zoomed 50µm length images of the GCL from each of the respective panels above were cropped to show RGC specific staining for naïve, 7 and 28 dpc: **(D)** Brn3a (red), **(E)** Vsnl1 (green) and **(F)** Nrn1 (green). Blue staining indicates DAPI labeled nuclei and all panels show red immuno-staining for Brn3a. Scale bar = 50µm, n=3. Photomicrographs were captured at 400X original magnification.

Figure 6

Expression of Syt1, Synpr and Nrn1 in the optic nerve following ONC. Differential protein expression of Syt1, Synpr and Nrn1 observed within cytoplasmic region of ON cells following trauma. Optic nerve sections from naïve, 14, 21 and 28 dpc (days post crush) are indicated on the top of the panel. **(A)** Illustrative images captured at each time point for each protein. Fluorescence micrographs of optic nerve tissue sections were were captured at 600x magnification and immunolabeled with: **(B)** Syt1 (green), **(C)** Syt1 and DAPI, **(D)** Synpr (green), **(E)** Synpr and DAPI, **(F)** Nrn1 (green) and **(G)** Nrn1 and DAPI. Blue staining indicates DAPI labeled nuclei. (Scale bar = 100µm (100x), 25µm (600x), n=3). Photomicrographs were captured at 600x original magnification for the selected ON genes.

Figure 1

Temporal changes of specific retinal gene clusters related to neuronal loss and regeneration failure

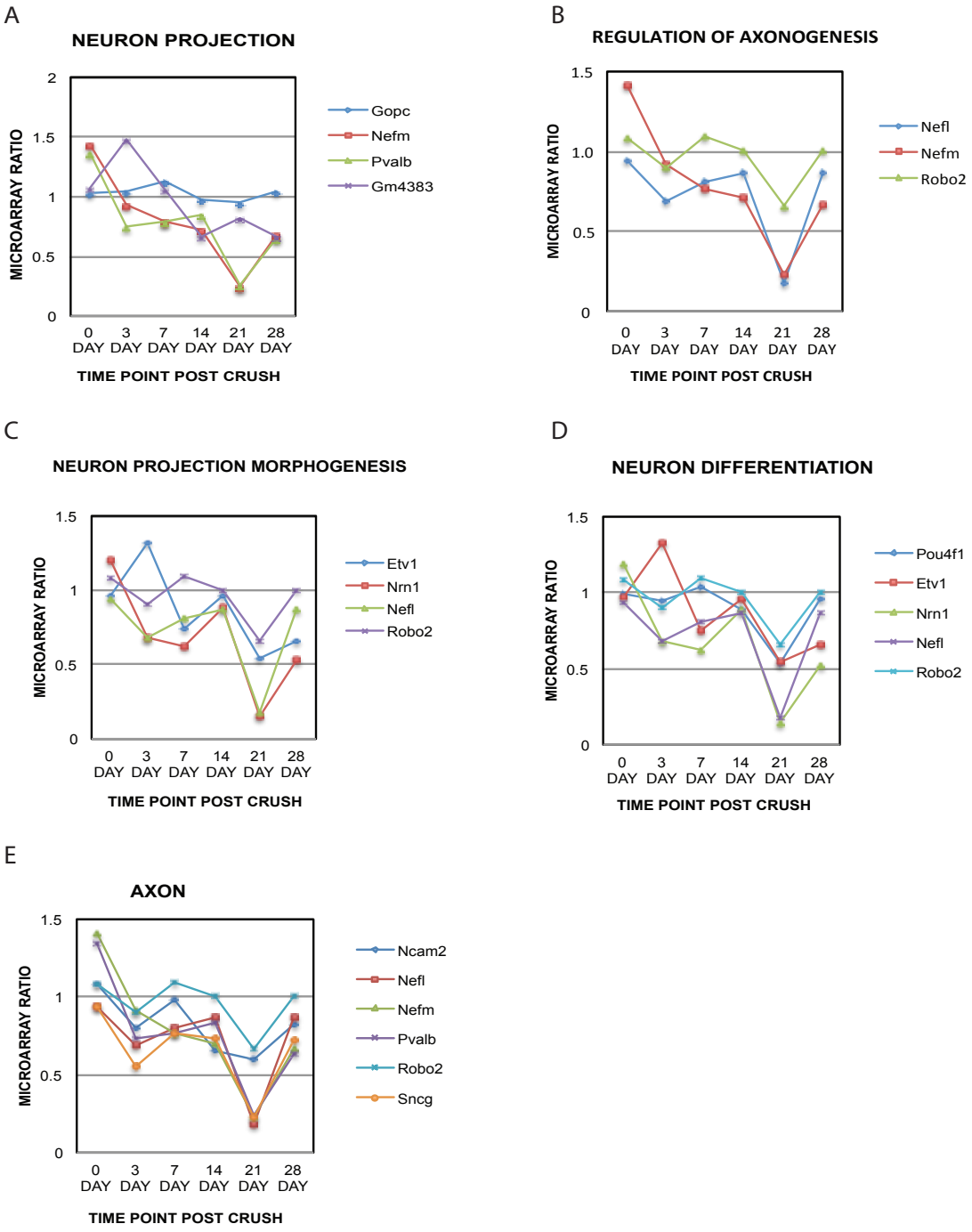


Figure 2

Temporal changes of specific optic nerve gene clusters related to neuronal loss and regeneration failure

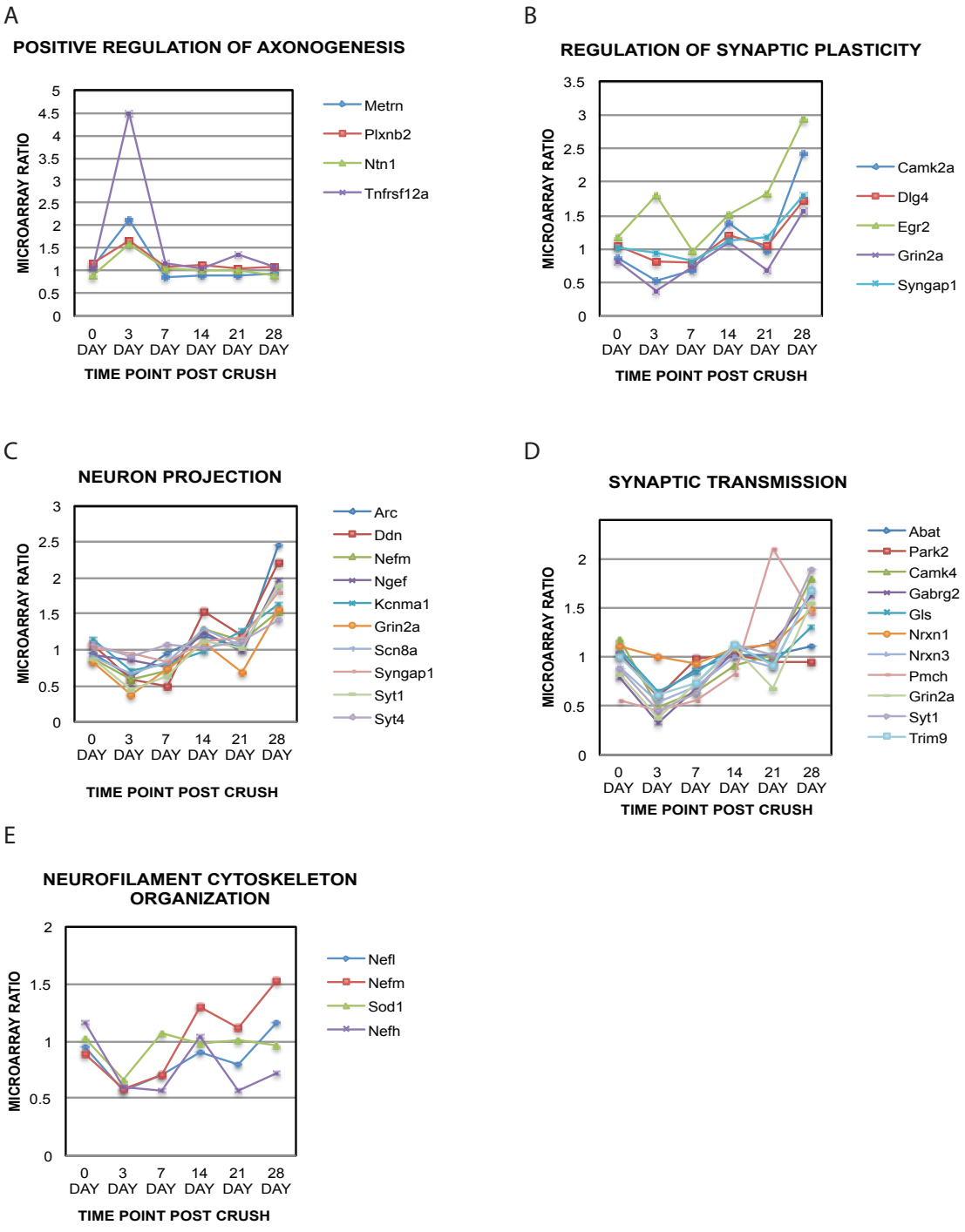


Figure 3

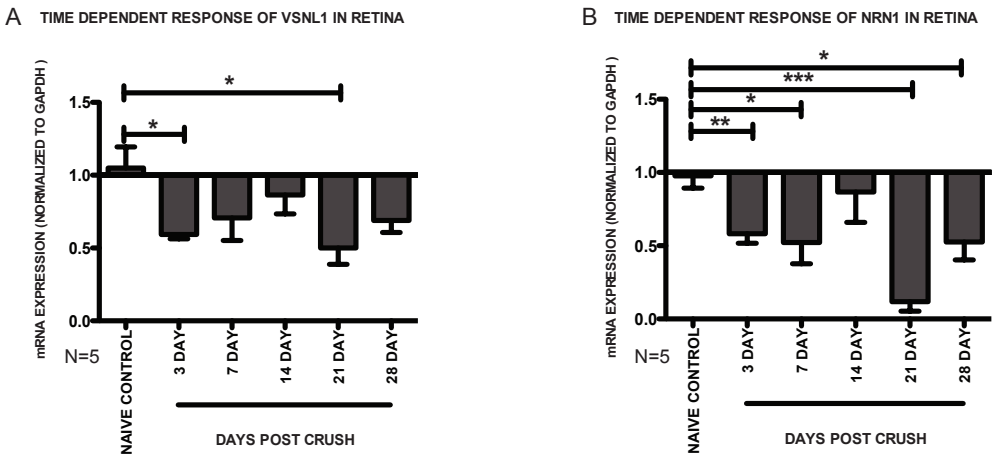

A
TIME DEPENDENT RESPONSE OF VSNL1 IN RETINA
mRNA EXPRESSION (NORMALIZED TO GAPDH)
1.5
1.0
0.5
0.0
*
*
N=5
NAIVE CONTROL
3 DAY
7 DAY
14 DAY
21 DAY
28 DAY
DAYS POST CRUSH
B
TIME DEPENDENT RESPONSE OF NRN1 IN RETINA
mRNA EXPRESSION (NORMALIZED TO GAPDH)
1.5
1.0
0.5
0.0
*
***
*
**
N=5
NAIVE CONTROL
3 DAY
7 DAY
14 DAY
21 DAY
28 DAY
DAYS POST CRUSH

Figure 4

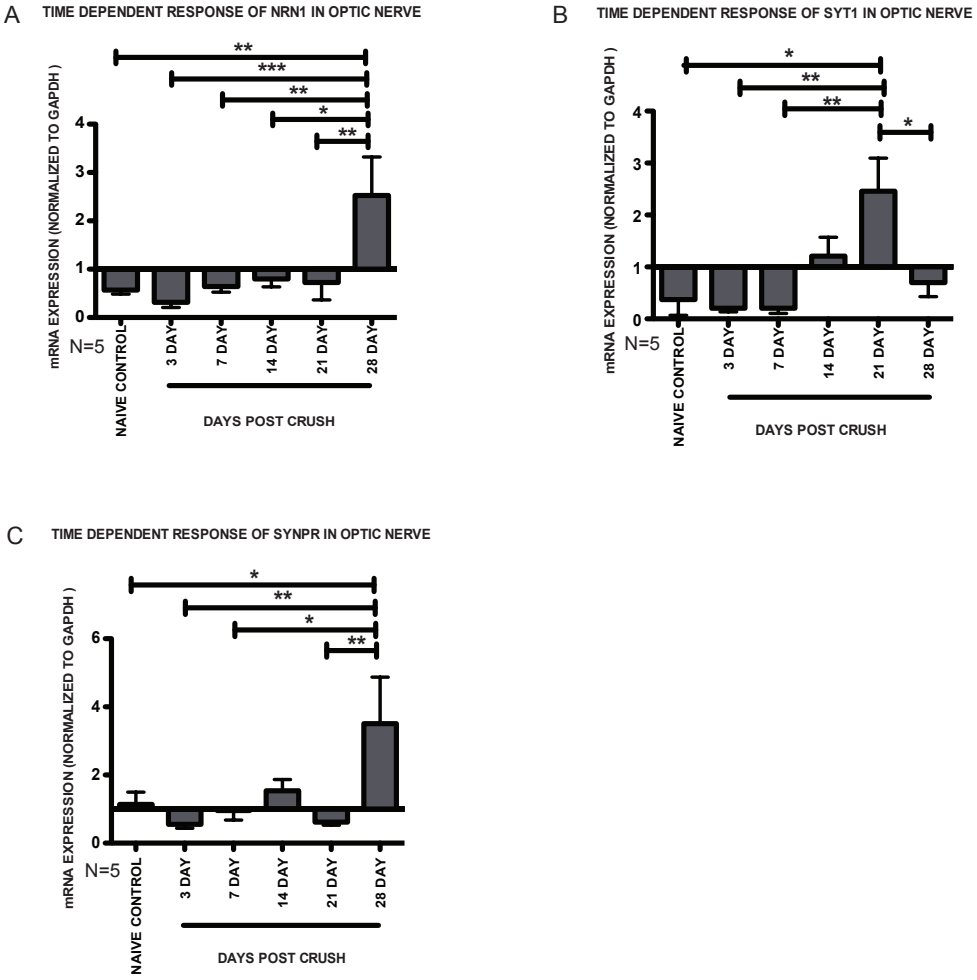

A
TIME DEPENDENT RESPONSE OF NRN1 IN OPTIC NERVE
mRNA EXPRESSION (NORMALIZED TO GAPDH)
N=5
NAIVE CONTROL
3 DAY
7 DAY
14 DAY
21 DAY
28 DAY
DAYS POST CRUSH
B
TIME DEPENDENT RESPONSE OF SYT1 IN OPTIC NERVE
mRNA EXPRESSION (NORMALIZED TO GAPDH)
N=5
NAIVE CONTROL
3 DAY
7 DAY
14 DAY
21 DAY
28 DAY
DAYS POST CRUSH
C
TIME DEPENDENT RESPONSE OF SYNPR IN OPTIC NERVE
mRNA EXPRESSION (NORMALIZED TO GAPDH)
N=5
NAIVE CONTROL
3 DAY
7 DAY
14 DAY
21 DAY
28 DAY
DAYS POST CRUSH

Figure 5

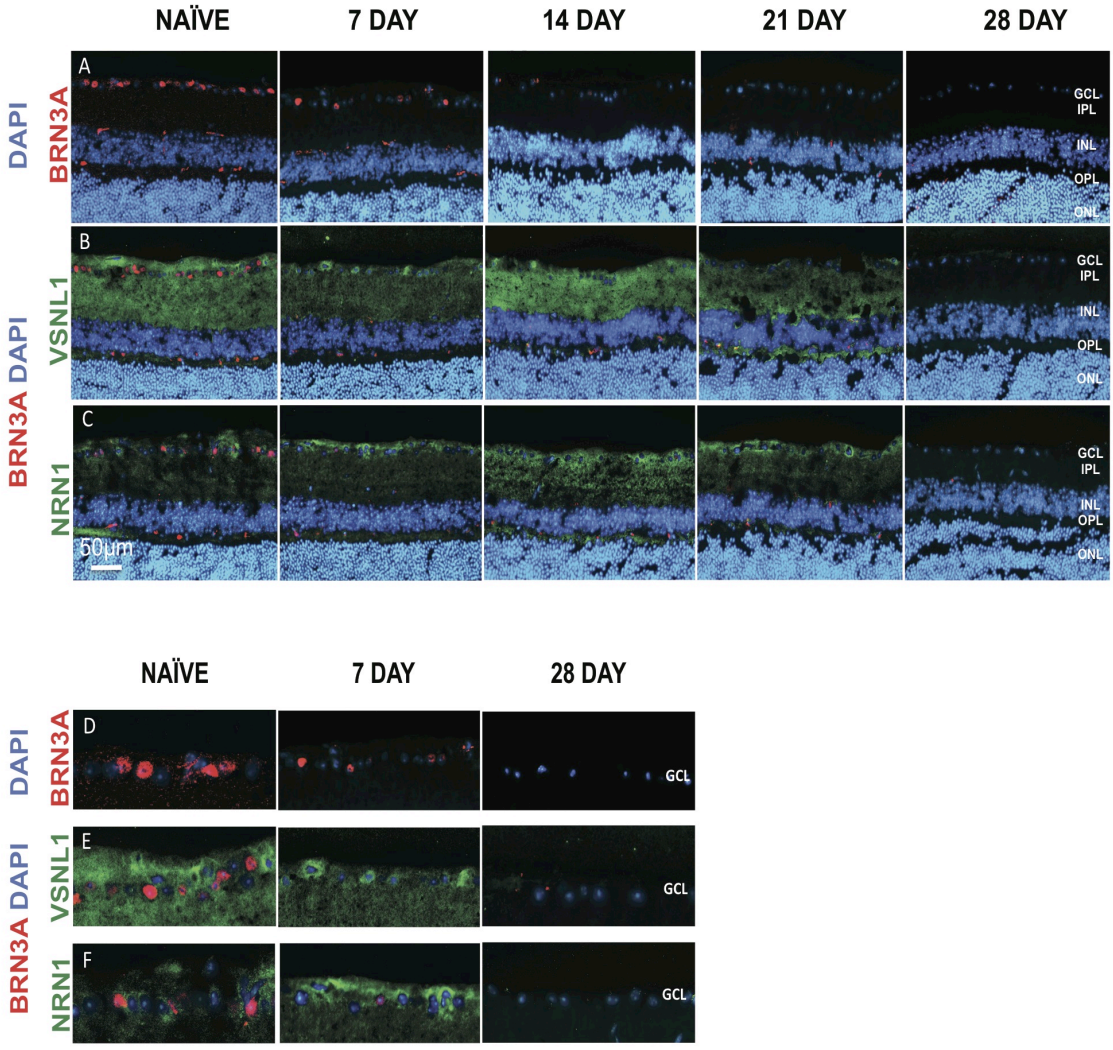
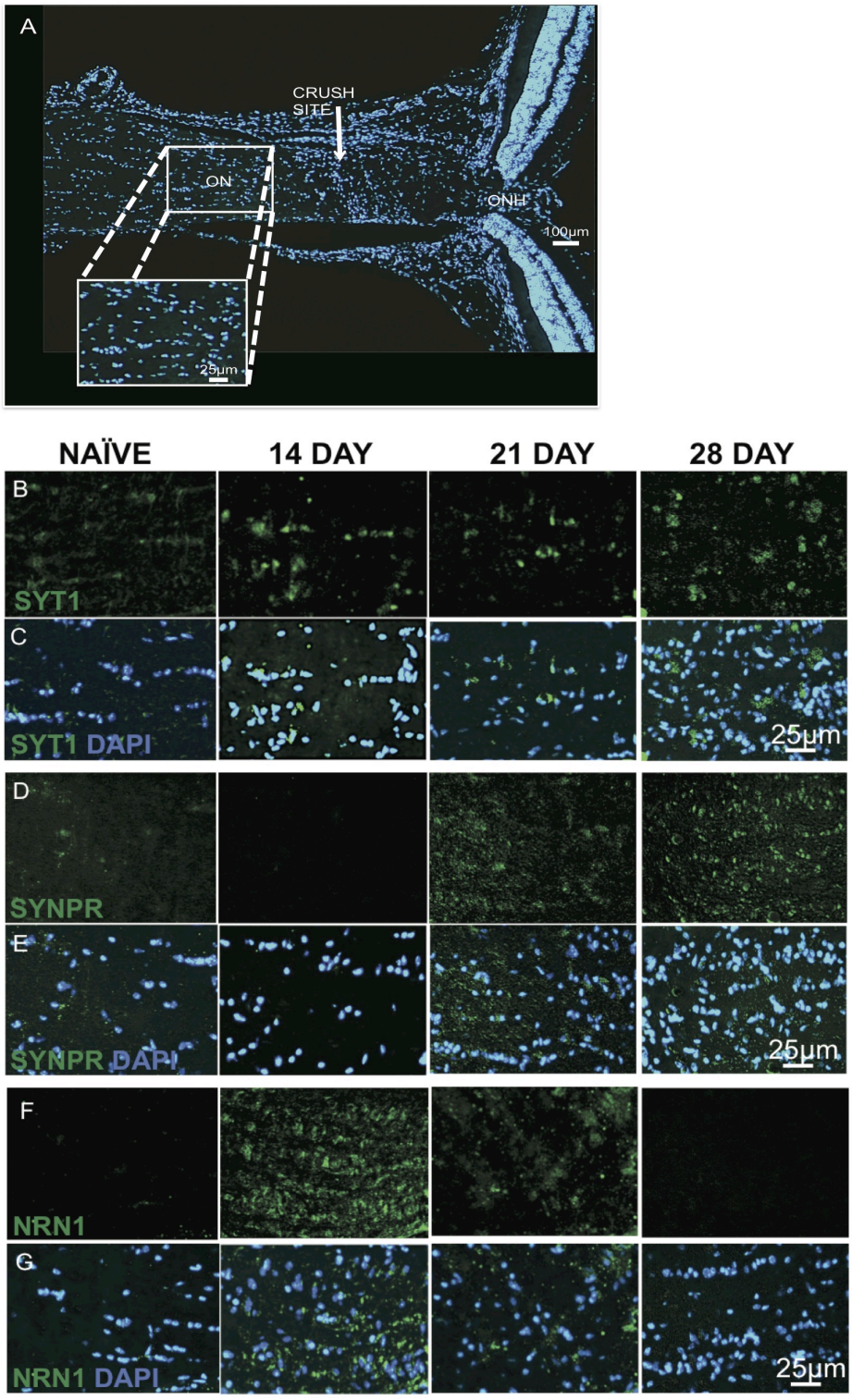
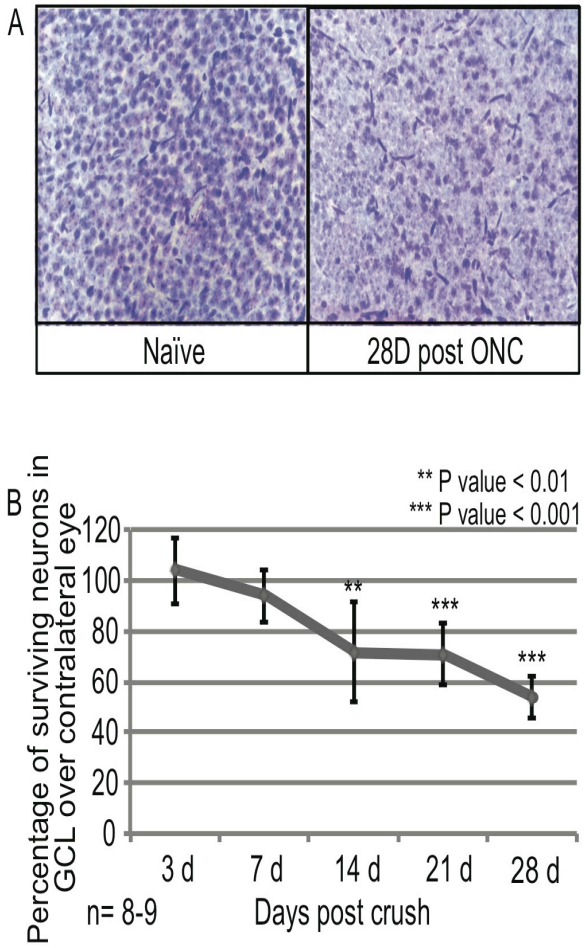

NAÏVE
7 DAY
14 DAY
21 DAY
28 DAY
A
DAPI
BRN3A
B
VSNL1
BRN3A DAPI
C
NRN1
50µm
GCL
IPL
INL
OPL
ONL
NAÏVE
7 DAY
28 DAY
D
E
F
GCL

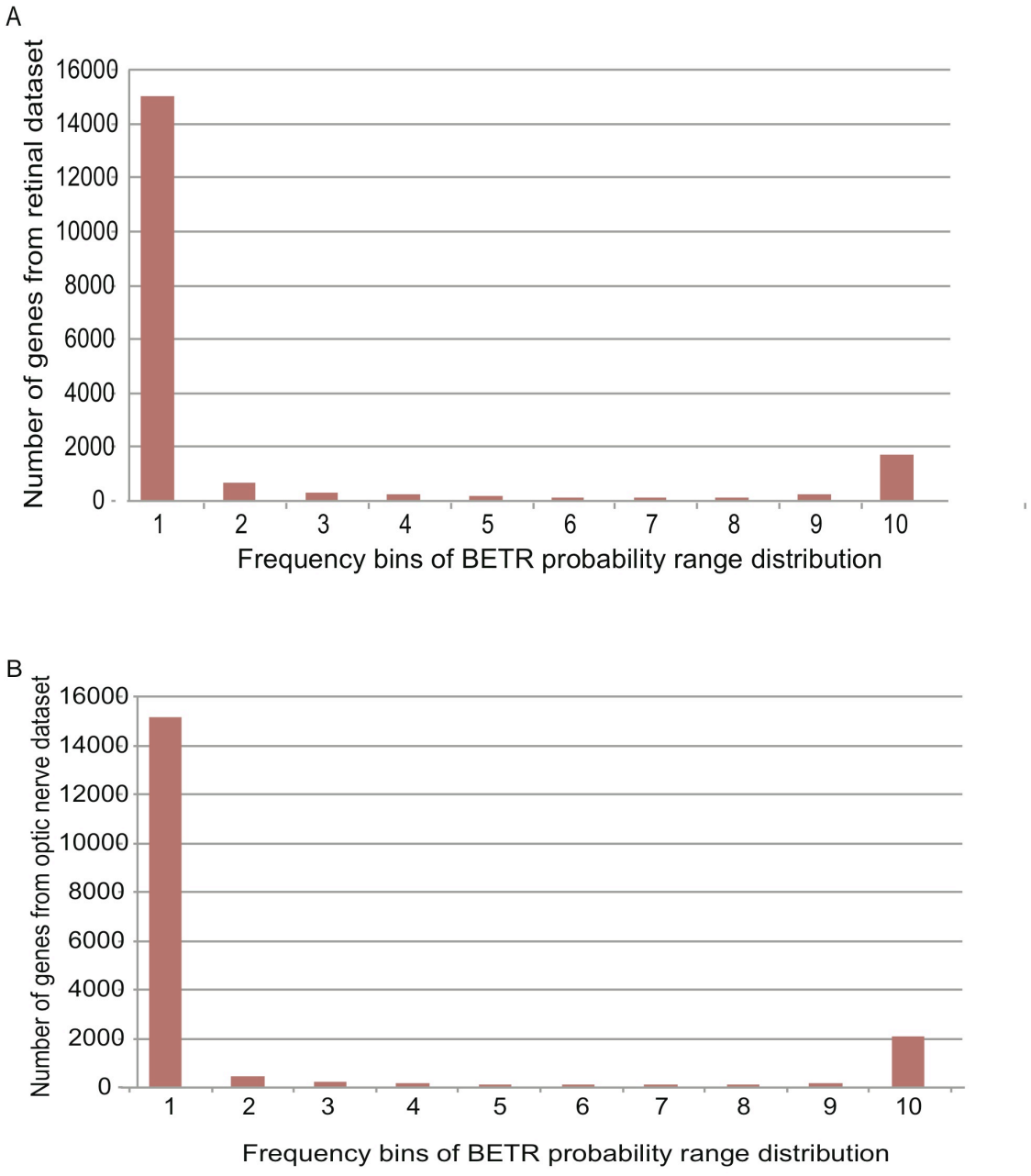
Figure 6


A
CRUSH SITE
ON
ONH
100µm
25µm
NAÏVE
14 DAY
21 DAY
28 DAY
B
SYT1
C
SYT1 DAPI
25µm
D
SYNPR
E
SYNPR DAPI
25µm
F
NRN1
G
NRN1 DAPI
25µm

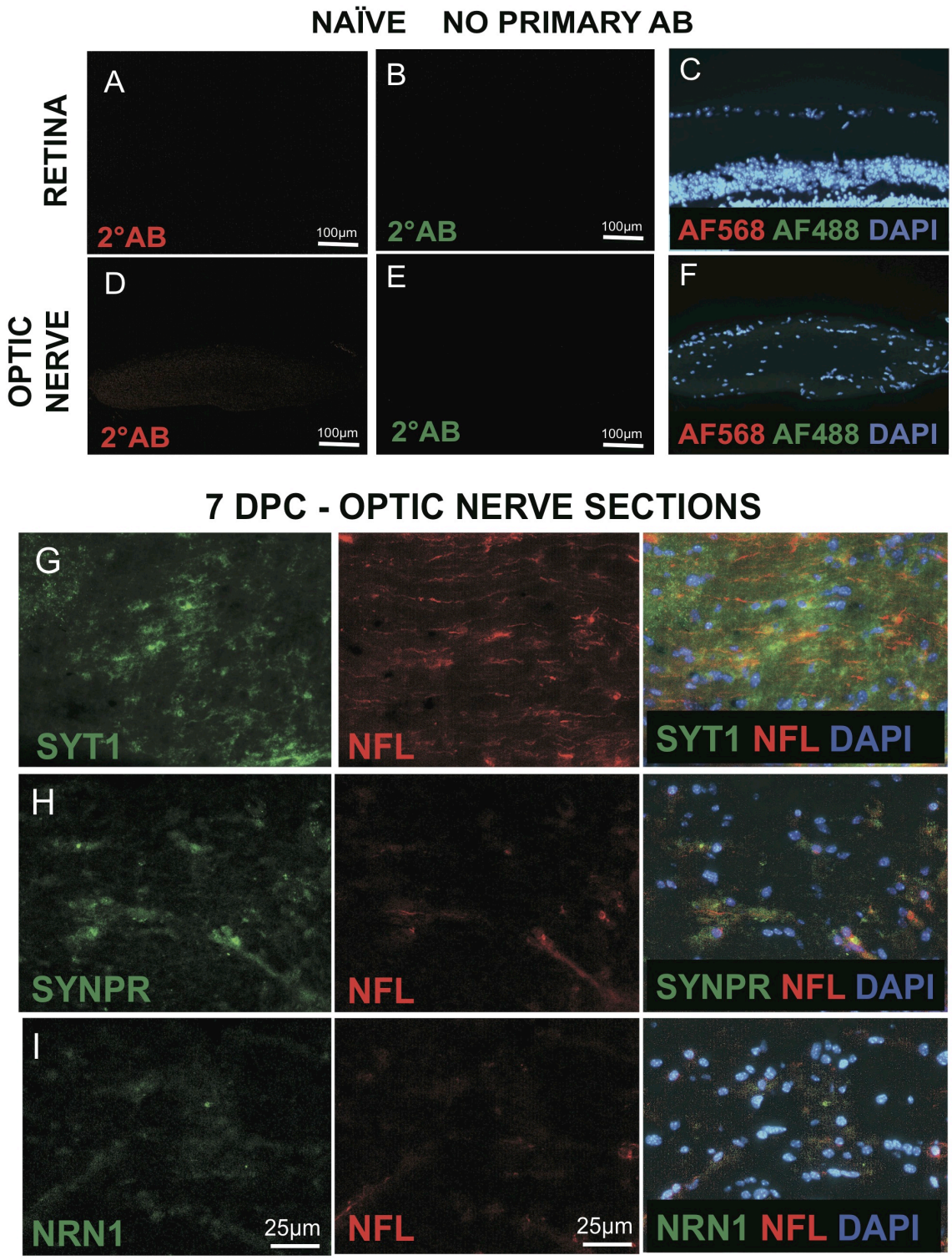
Additional Figure 1



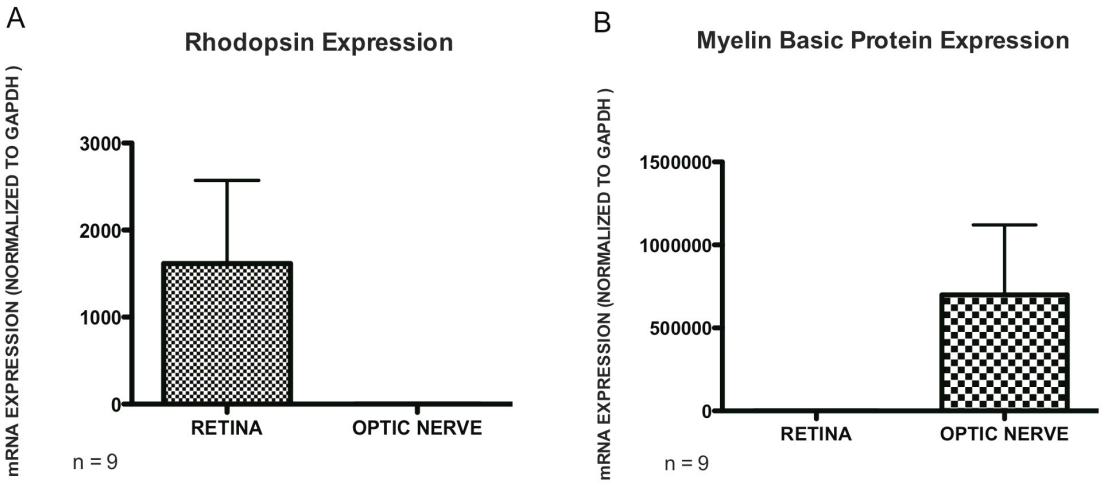
Additional Figure 2



Additional Figure 3



Additional Figure 4



Additional Table 1

Additional Table 1A: BETR probabilities based retinal genes distributed within frequency bins

Frequency Bin	Number of genes	Start BETR Probability	End BETR Probability
1	15012	0	0.1
2	648	0.1	0.2
3	325	0.2	0.3
4	222	0.3	0.4
5	203	0.4	0.5
6	149	0.5	0.6
7	133	0.6	0.7
8	152	0.7	0.8
9	219	0.8	0.9
10	1723	0.9	1.0

Additional Table 1B - BETR probabilities based opticc nerve genes distributed within frequency bins

Frequency Bin	Number of genes	Start BETR Probability	End BETR Probability
1	15187	0	0.1
2	437	0.1	0.2
3	221	0.2	0.3
4	176	0.3	0.4
5	129	0.4	0.5
6	126	0.5	0.6
7	106	0.6	0.7
8	122	0.7	0.8
9	172	0.8	0.9
10	2110	0.9	1.0

Additional Table 2

Additional Table 2A: Microarray ratios of selected target genes

		Microarray Ratios					
	GENES	TIMEPOINTS					
		NAÏVE	3 DAY	7 DAY	14 DAY	21 DAY	28 DAY
Retina	VSNL1	1.66	0.7	0.86	0.68	0.42	0.7
	NRN1	1.19	0.68	0.62	0.88	0.15	0.53
Optic Nerve	SYT1	0.88	0.45	0.64	1.13	1.02	1.9
	SYNPR	1.15	0.63	0.87	0.93	1.08	1.58
	NRN1	0.95	0.46	0.96	0.84	0.72	1.66

Additional Table 2B: Linear regression correlation values of selected target genes

Tissue	Genes	Linear Regression (mRNA values vs Microarray ratios)
		Correlation (R^2)
Retina	VSNL1	0.73
	NRN1	0.96
Optic Nerve	SYT1	0.07
	SYNPR	0.71
	NRN1	0.86

Additional Table 3

Additional Table 3: Primers for key genes validated from the retina and optic nerve datasets

GENE NAMES	ACCESSION NUMBER	GENE	FORWARD PRIMER	REVERSE PRIMER	BP SIZE
Visinin-like 1	NM_012038.4	*Vsnl1*	5' CTGGGCTTGCAGGGCGCAAT 3'	5' GCCCACCATTTTGTAGATAGCCTCG 3'	585
Neuritin 1	NM_153529.2	*Nrn1*	5' GCGGTGCAAATAGCTTACCTG 3'	5' CGGTCTTGATGTTCGTCTTGTC 3'	151
Synaptotagmin 1	NM_009306.3	*Syt1*	5' GGTCCTCGCTCCAGGAAAAG 3'	5' TCGTCATCCTTAAGGGCCTG 3'	563
Synaptoporin	NM_028052.4	*Synpr*	5' CACAGCGCTCTACACTGACT 3'	5' TTGCAGCCTGAATGGGTACG 3'	400
Rhodopsin	NM_145383.1	*Rho*	5' TCAAGCCTGAGGTCAACAAC 3'	5' TCTTGGAAGCGGTGGCAGAG 3'	438
Myelin Basic Protein	NM_010777.3	*Mbp*	5' CAGTCACACTGGAGGGCAAACA 3'	5' TGAGGAGCTGAGAACCCCAGTC 3'	331

Additional Table 4

Additional Table 4: qRT-PCR cycles performed for confirming retina and optic nerve dataset gene expression levels

Cycle		Tissue Specific	Temperature	Time
Step 1	Jump Start		95°C	10 secs
Step 2	Denaturing		95°C	30 secs
Step 3	Annealing	Retina	60°C	30 secs
		Optic Nerve	62°C	30 secs
Step 4	Elongation	Retina	72°C	30 secs
		Optic Nerve	72°C	45 secs
Step 5	39x to Step 1			
Step 6	Melt curve		60-70°C	10 mins

Additional Table 5

Additional Table 5: Antibodies against key proteins validated by IHC

Primary Antibody	Company	Catalog #	Species	Dilution
BRN3A	Chemicon (Millipore)	MAB1585	Mouse	1:50
VSNL1	Novus	NBP1-46282	Rabbit	1:100
NRN1	Abcam	ab64186	Rabbit	1:200
SYT1	Abcam	ab68853	Rabbit	1:300
SYNPR	Synaptic Systems	102 002	Rabbit	1:500

Secondary Antibody	Company	Catalog #	Dilution
Goat anti-mouse AF568	Alexa Fluor	A21043	1:500
Donkey anti rabbit AF568	Alexa Fluor	A10042	1:500
Goat anti-mouse AF488	Alexa Fluor	A11029	1:500
Goat anti-rabbit AF488	Alexa Fluor	A11008	1:500

References

1. Schwartz M: **Optic nerve crush: protection and regeneration**. *Brain research bulletin* 2004, **62**(6):467-471.

2. Ohlsson M, Mattsson P, Svensson M: **A temporal study of axonal degeneration and glial scar formation following a standardized crush injury of the optic nerve in the adult rat**. *Restorative neurology and neuroscience* 2004, **22**(1):1-10.

3. Magharious M, D'Onofrio PM, Hollander A, Zhu P, Chen J, Koeberle PD: **Quantitative iTRAQ analysis of retinal ganglion cell degeneration after optic nerve crush**. *Journal of proteome research* 2011, **10**(8):3344-3362.

4. Wohlfart G: **Degeneration and regeneration in the nervous system. Recent advances**. *World neurology* 1961, **2**:187-198.

5. Windle WF: **Regeneration of axons in the vertebrate central nervous system**. *Physiological reviews* 1956, **36**(4):427-440.

6. Huber AB, Schwab ME: **Nogo-A, a potent inhibitor of neurite outgrowth and regeneration**. *Biological chemistry* 2000, **381**(5-6):407-419.

7. Huber AB, Weinmann O, Brosamle C, Oertle T, Schwab ME: **Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2002, **22**(9):3553-3567.

8. Filbin MT: **Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS**. *Nature reviews Neuroscience* 2003, **4**(9):703-713.

9. Tang S, Qiu J, Nikulina E, Filbin MT: **Soluble myelin-associated glycoprotein released from damaged white matter inhibits axonal regeneration**. *Molecular and cellular neurosciences* 2001, **18**(3):259-269.

10. Winzeler AM, Mandemakers WJ, Sun MZ, Stafford M, Phillips CT, Barres BA: **The lipid sulfatide is a novel myelin-associated inhibitor of CNS axon outgrowth**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011, **31**(17):6481-6492.

11. Kopp MA, Liebscher T, Niedeggen A, Laufer S, Brommer B, Jungehulsing GJ, Strittmatter SM, Dirnagl U, Schwab JM: **Small-molecule-induced Rho-inhibition: NSAIDs after spinal cord injury**. *Cell and tissue research* 2012, **349**(1):119-132.

12. Sandvig A, Berry M, Barrett LB, Butt A, Logan A: **Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration**. *Glia* 2004, **46**(3):225-251.

13. Silver J, Miller JH: **Regeneration beyond the glial scar**. *Nature reviews Neuroscience* 2004, **5**(2):146-156.

14. Lawson LJ, Frost L, Risbridger J, Fearn S, Perry VH: **Quantification of the mononuclear phagocyte response to Wallerian degeneration of the optic nerve**. *Journal of neurocytology* 1994, **23**(12):729-744.

15. Lazarov-Spiegler O, Rapalino O, Agranov G, Schwartz M: **Restricted inflammatory reaction in the CNS: a key impediment to axonal regeneration?** *Molecular medicine today* 1998, **4**(8):337-342.

16. Jaerve A, Muller HW: **Chemokines in CNS injury and repair**. *Cell and tissue research* 2012, **349**(1):229-248.

17. Monnier PP, D'Onofrio PM, Magharious M, Hollander AC, Tassew N, Szydlowska K, Tymianski M, Koeberle PD: **Involvement of caspase-6 and caspase-8 in neuronal apoptosis and the regenerative failure of injured retinal ganglion cells**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011, **31**(29):10494-10505.

18. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ: **Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis**. *Investigative ophthalmology & visual science* 1995, **36**(5):774-786.

19. Li Y, Schlamp CL, Nickells RW: **Experimental induction of retinal ganglion cell death in adult mice**. *Investigative ophthalmology & visual science* 1999, **40**(5):1004-1008.

20. Li Y, Semaan SJ, Schlamp CL, Nickells RW: **Dominant inheritance of retinal ganglion cell resistance to optic nerve crush in mice**. *BMC neuroscience* 2007, **8**:19.

21. Barron KD, Dentinger MP, Krohel G, Easton SK, Mankes R: **Qualitative and quantitative ultrastructural observations on retinal ganglion cell layer of rat after intraorbital optic nerve crush**. *Journal of neurocytology* 1986, **15**(3):345-362.

22. Templeton JP, Nassr M, Vazquez-Chona F, Freeman-Anderson NE, Orr WE, Williams RW, Geisert EE: **Differential response of C57BL/6J mouse and DBA/2J mouse to optic nerve crush**. *BMC neuroscience* 2009, **10**:90.

23. Misantone LJ, Gershenbaum M, Murray M: **Viability of retinal ganglion cells after optic nerve crush in adult rats**. *Journal of neurocytology* 1984, **13**(3):449-465.

24. Bahr M: **Live or let die - retinal ganglion cell death and survival during development and in the lesioned adult CNS**. *Trends in neurosciences* 2000, **23**(10):483-490.

25. Klocker N, Zerfowski M, Gellrich NC, Bahr M: **Morphological and functional analysis of an incomplete CNS fiber tract lesion: graded crush of the rat optic nerve**. *Journal of neuroscience methods* 2001, **110**(1-2):147-153.

26. Agudo M, Perez-Marin MC, Lonngren U, Sobrado P, Conesa A, Canovas I, Salinas-Navarro M, Miralles-Imperial J, Hallbook F, Vidal-Sanz M: **Time course profiling of the retinal transcriptome after optic nerve transection and optic nerve crush**. *Molecular vision* 2008, **14**:1050-1063.

27. Tang Z, Arjunan P, Lee C, Li Y, Kumar A, Hou X, Wang B, Wardega P, Zhang F, Dong L *et al*: **Survival effect of PDGF-CC rescues neurons from apoptosis in both brain and retina by regulating GSK3beta phosphorylation**. *The Journal of experimental medicine* 2010, **207**(4):867-880.

28. Lukas TJ, Wang AL, Yuan M, Neufeld AH: **Early cellular signaling responses to axonal injury**. *Cell communication and signaling : CCS* 2009, **7**:5.

29. Qu J, Jakobs TC: **The Time Course of Gene Expression during Reactive Gliosis in the Optic Nerve**. *PloS one* 2013, **8**(6):e67094.

30. Sharma A, Pollett MA, Plant GW, Harvey AR: **Changes in mRNA expression of class 3 semaphorins and their receptors in the adult rat retino-collicular system**

after unilateral optic nerve injury. *Investigative ophthalmology & visual science* 2012, **53**(13):8367-8377.

31. Blaugrund E, Lavie V, Cohen I, Solomon A, Schreyer DJ, Schwartz M: **Axonal regeneration is associated with glial migration: comparison between the injured optic nerves of fish and rats**. *The Journal of comparative neurology* 1993, **330**(1):105-112.

32. Doster SK, Lozano AM, Aguayo AJ, Willard MB: **Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury**. *Neuron* 1991, **6**(4):635-647.

33. Leon S, Yin Y, Nguyen J, Irwin N, Benowitz LI: **Lens injury stimulates axon regeneration in the mature rat optic nerve**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2000, **20**(12):4615-4626.

34. Ridet JL, Malhotra SK, Privat A, Gage FH: **Reactive astrocytes: cellular and molecular cues to biological function**. *Trends in neurosciences* 1997, **20**(12):570-577.

35. Dibas A, Oku H, Fukuhara M, Kurimoto T, Ikeda T, Patil RV, Sharif NA, Yorio T: **Changes in ocular aquaporin expression following optic nerve crush**. *Molecular vision* 2010, **16**:330-340.

36. Woldemussie E, Wijono M, Ruiz G: **Muller cell response to laser-induced increase in intraocular pressure in rats**. *Glia* 2004, **47**(2):109-119.

37. Parrilla-Reverter G, Agudo M, Nadal-Nicolas F, Alarcon-Martinez L, Jimenez-Lopez M, Salinas-Navarro M, Sobrado-Calvo P, Bernal-Garro JM, Villegas-Perez MP, Vidal-Sanz M: **Time-course of the retinal nerve fibre layer degeneration after**

complete intra-orbital optic nerve transection or crush: a comparative study. *Vision research* 2009, **49**(23):2808-2825.

38. Koeberle PD, Bahr M: **Growth and guidance cues for regenerating axons: where have they gone?** *Journal of neurobiology* 2004, **59**(1):162-180.

39. Kermer P, Klocker N, Bahr M: **Neuronal death after brain injury. Models, mechanisms, and therapeutic strategies in vivo**. *Cell and tissue research* 1999, **298**(3):383-395.

40. Koeberle PD, Gauldie J, Ball AK: **Effects of adenoviral-mediated gene transfer of interleukin-10, interleukin-4, and transforming growth factor-beta on the survival of axotomized retinal ganglion cells**. *Neuroscience* 2004, **125**(4):903-920.

41. Kipnis J, Yoles E, Schori H, Hauben E, Shaked I, Schwartz M: **Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2001, **21**(13):4564-4571.

42. Isenmann S, Wahl C, Krajewski S, Reed JC, Bahr M: **Up-regulation of Bax protein in degenerating retinal ganglion cells precedes apoptotic cell death after optic nerve lesion in the rat**. *The European journal of neuroscience* 1997, **9**(8):1763-1772.

43. Kermer P, Ankerhold R, Klocker N, Krajewski S, Reed JC, Bahr M: **Caspase-9: involvement in secondary death of axotomized rat retinal ganglion cells in vivo**. *Brain research Molecular brain research* 2000, **85**(1-2):144-150.

44. Kermer P, Klocker N, Labes M, Bahr M: **Inhibition of CPP32-like proteases rescues axotomized retinal ganglion cells from secondary cell death in vivo**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1998, **18**(12):4656-4662.

45. Kikuchi M, Tenneti L, Lipton SA: **Role of p38 mitogen-activated protein kinase in axotomy-induced apoptosis of rat retinal ganglion cells**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2000, **20**(13):5037-5044.

46. Galindo-Romero C, Aviles-Trigueros M, Jimenez-Lopez M, Valiente-Soriano FJ, Salinas-Navarro M, Nadal-Nicolas F, Villegas-Perez MP, Vidal-Sanz M, Agudo-Barriuso M: **Axotomy-induced retinal ganglion cell death in adult mice: quantitative and topographic time course analyses**. *Experimental eye research* 2011, **92**(5):377-387.

47. Kim BJ, Braun TA, Wordinger RJ, Clark AF: **Progressive morphological changes and impaired retinal function associated with temporal regulation of gene expression after retinal ischemia/reperfusion injury in mice**. *Molecular neurodegeneration* 2013, **8**:21.

48. Xia Y, Chen J, Xiong L, Liu J, Liu X, Ma L, Zhang Q, You C, Chen J, Liu X *et al*: **Retinal whole genome microarray analysis and early morphological changes in the optic nerves of monkeys after an intraorbital nerve irradiated injury**. *Molecular vision* 2011, **17**:2920-2933.

49. Jehle T, Dimitriu C, Auer S, Knoth R, Vidal-Sanz M, Gozes I, Lagreze WA: **The neuropeptide NAP provides neuroprotection against retinal ganglion cell damage**

after retinal ischemia and optic nerve crush. *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie* 2008, **246**(9):1255-1263.

50. Agudo M, Perez-Marin MC, Sobrado-Calvo P, Lonngren U, Salinas-Navarro M, Canovas I, Nadal-Nicolas FM, Miralles-Imperial J, Hallbook F, Vidal-Sanz M: **Immediate upregulation of proteins belonging to different branches of the apoptotic cascade in the retina after optic nerve transection and optic nerve crush**. *Investigative ophthalmology & visual science* 2009, **50**(1):424-431.

51. Haverkamp S, Inta D, Monyer H, Wassle H: **Expression analysis of green fluorescent protein in retinal neurons of four transgenic mouse lines**. *Neuroscience* 2009, **160**(1):126-139.

52. Raymond ID, Vila A, Huynh UC, Brecha NC: **Cyan fluorescent protein expression in ganglion and amacrine cells in a thy1-CFP transgenic mouse retina**. *Molecular vision* 2008, **14**:1559-1574.

53. Masland RH: **Neuronal diversity in the retina**. *Current opinion in neurobiology* 2001, **11**(4):431-436.

54. Kim CY, Kuehn MH, Clark AF, Kwon YH: **Gene expression profile of the adult human retinal ganglion cell layer**. *Molecular vision* 2006, **12**:1640-1648.

55. Aryee MJ, Gutierrez-Pabello JA, Kramnik I, Maiti T, Quackenbush J: **An improved empirical bayes approach to estimating differential gene expression in microarray time-course data: BETR (Bayesian Estimation of Temporal Regulation)**. *BMC bioinformatics* 2009, **10**:409.

56. Cantallops I, Haas K, Cline HT: **Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration in vivo**. *Nature neuroscience* 2000, **3**(10):1004-1011.

57. Li JY, Dahlstrom A: **Axonal transport of synaptic vesicle proteins in the rat optic nerve**. *Journal of neurobiology* 1997, **32**(2):237-250.

58. Kwon KB, Kim JS, Chang BJ: **Translocational changes of localization of synapsin in axonal sprouts of regenerating rat sciatic nerves after ligation crush injury**. *Journal of veterinary science* 2000, **1**(1):1-9.

59. Carpenter S: **Proximal axonal enlargement in motor neuron disease**. *Neurology* 1968, **18**(9):841-851.

60. Delisle MB, Carpenter S: **Neurofibrillary axonal swellings and amyotrophic lateral sclerosis**. *Journal of the neurological sciences* 1984, **63**(2):241-250.

61. Ishii T, Haga S, Tokutake S: **Presence of neurofilament protein in Alzheimer's neurofibrillary tangles (ANT). An immunofluorescent study**. *Acta neuropathologica* 1979, **48**(2):105-112.

62. Nukina N, Kosik KS, Selkoe DJ: **Recognition of Alzheimer paired helical filaments by monoclonal neurofilament antibodies is due to crossreaction with tau protein**. *Proceedings of the National Academy of Sciences of the United States of America* 1987, **84**(10):3415-3419.

63. Hill WD, Arai M, Cohen JA, Trojanowski JQ: **Neurofilament mRNA is reduced in Parkinson's disease substantia nigra pars compacta neurons**. *The Journal of comparative neurology* 1993, **329**(3):328-336.

64. Fabrizi GM, Cavallaro T, Angiari C, Bertolasi L, Cabrini I, Ferrarini M, Rizzuto N: **Giant axon and neurofilament accumulation in Charcot-Marie-Tooth disease type 2E**. *Neurology* 2004, **62**(8):1429-1431.

65. Bigio EH, Lipton AM, White CL, 3rd, Dickson DW, Hirano A: **Frontotemporal and motor neurone degeneration with neurofilament inclusion bodies: additional evidence for overlap between FTD and ALS**. *Neuropathology and applied neurobiology* 2003, **29**(3):239-253.

66. Cairns NJ, Perry RH, Jaros E, Burn D, McKeith IG, Lowe JS, Holton J, Rossor MN, Skullerud K, Duyckaerts C *et al*: **Patients with a novel neurofilamentopathy: dementia with neurofilament inclusions**. *Neuroscience letters* 2003, **341**(3):177-180.

67. Josephs KA, Holton JL, Rossor MN, Braendgaard H, Ozawa T, Fox NC, Petersen RC, Pearl GS, Ganguly M, Rosa P *et al*: **Neurofilament inclusion body disease: a new proteinopathy?** *Brain : a journal of neurology* 2003, **126**(Pt 10):2291-2303.

68. Asbury AK, Gale MK, Cox SC, Baringer JR, Berg BO: **Giant axonal neuropathy--a unique case with segmental neurofilamentous masses**. *Acta neuropathologica* 1972, **20**(3):237-247.

69. Medori R, Autilio-Gambetti L, Monaco S, Gambetti P: **Experimental diabetic neuropathy: impairment of slow transport with changes in axon cross-sectional area**. *Proceedings of the National Academy of Sciences of the United States of America* 1985, **82**(22):7716-7720.

70. Medori R, Jenich H, Autilio-Gambetti L, Gambetti P: **Experimental diabetic neuropathy: similar changes of slow axonal transport and axonal size in different**

animal models. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1988, **8**(5):1814-1821.

71. Perrot R, Berges R, Bocquet A, Eyer J: **Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration**. *Molecular neurobiology* 2008, **38**(1):27-65.

72. Huang X, Kong W, Zhou Y, Gregori G: **Distortion of axonal cytoskeleton: an early sign of glaucomatous damage**. *Investigative ophthalmology & visual science* 2011, **52**(6):2879-2888.

73. Ivanov D, Dvoriantchikova G, Nathanson L, McKinnon SJ, Shestopalov VI: **Microarray analysis of gene expression in adult retinal ganglion cells**. *FEBS letters* 2006, **580**(1):331-335.

74. Farkas RH, Qian J, Goldberg JL, Quigley HA, Zack DJ: **Gene expression profiling of purified rat retinal ganglion cells**. *Investigative ophthalmology & visual science* 2004, **45**(8):2503-2513.

75. Grabs D, Bergmann M, Schuster T, Fox PA, Brich M, Gratz M: **Differential expression of synaptophysin and synaptoporin during pre- and postnatal development of the rat hippocampal network**. *The European journal of neuroscience* 1994, **6**(11):1765-1771.

76. Singec I, Knoth R, Ditter M, Hagemeyer CE, Rosenbrock H, Frotscher M, Volk B: **Synaptic vesicle protein synaptoporin is differently expressed by subpopulations of mouse hippocampal neurons**. *The Journal of comparative neurology* 2002, **452**(2):139-153.

77. Sun T, Xiao HS, Zhou PB, Lu YJ, Bao L, Zhang X: **Differential expression of synaptoporin and synaptophysin in primary sensory neurons and up-regulation of synaptoporin after peripheral nerve injury**. *Neuroscience* 2006, **141**(3):1233-1245.

78. Greif KF, Asabere N, Lutz GJ, Gallo G: **Synaptotagmin-1 promotes the formation of axonal filopodia and branches along the developing axons of forebrain neurons**. *Developmental neurobiology* 2013, **73**(1):27-44.

79. Malarkey EB, Parpura V: **Temporal characteristics of vesicular fusion in astrocytes: examination of synaptobrevin 2-laden vesicles at single vesicle resolution**. *The Journal of physiology* 2011, **589**(Pt 17):4271-4300.

80. Jeon CJ, Strettoi E, Masland RH: **The major cell populations of the mouse retina**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1998, **18**(21):8936-8946.

81. Quina LA, Pak W, Lanier J, Banwait P, Gratwick K, Liu Y, Velasquez T, O'Leary DD, Goulding M, Turner EE: **Brn3a-expressing retinal ganglion cells project specifically to thalamocortical and collicular visual pathways**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2005, **25**(50):11595-11604.

82. Johnson TV, Martin KR: **Development and characterization of an adult retinal explant organotypic tissue culture system as an in vitro intraocular stem cell transplantation model**. *Investigative ophthalmology & visual science* 2008, **49**(8):3503-3512.

83. Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, Nieto-Lopez L, Canovas-Martinez I, Salinas-Navarro M, Vidal-Sanz M, Agudo M: **Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve-injured retinas**. *Investigative ophthalmology & visual science* 2009, **50**(8):3860-3868.

84. Fujino T, Wu Z, Lin WC, Phillips MA, Nedivi E: **cpg15 and cpg15-2 constitute a family of activity-regulated ligands expressed differentially in the nervous system to promote neurite growth and neuronal survival**. *The Journal of comparative neurology* 2008, **507**(5):1831-1845.

85. McCurley AT, Callard GV: **Time Course Analysis of Gene Expression Patterns in Zebrafish Eye During Optic Nerve Regeneration**. *Journal of experimental neuroscience* 2010, **2010**(4):17-33.

86. Agudo-Barriuso M, Lahoz A, Nadal-Nicolas FM, Sobrado-Calvo P, Piquer-Gil M, Diaz-Llopis M, Vidal-Sanz M, Mullor JL: **Metabolomic changes in the rat retina after optic nerve crush**. *Investigative ophthalmology & visual science* 2013, **54**(6):4249-4259.

87. Yuan A, Rao MV, Sasaki T, Chen Y, Kumar A, Veeranna, Liem RK, Eyer J, Peterson AC, Julien JP *et al*: **Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2006, **26**(39):10006-10019.

88. Kielczewski JL, Pease ME, Quigley HA: **The effect of experimental glaucoma and optic nerve transection on amacrine cells in the rat retina**. *Investigative ophthalmology & visual science* 2005, **46**(9):3188-3196.

89. Kirsch M, Trautmann N, Ernst M, Hofmann HD: **Involvement of gp130-associated cytokine signaling in Muller cell activation following optic nerve lesion**. *Glia* 2010, **58**(7):768-779.

90. Aldskogius H, Kozlova EN: **Central neuron-glial and glial-glial interactions following axon injury**. *Progress in neurobiology* 1998, **55**(1):1-26.

91. Bernstein HG, Baumann B, Danos P, Diekmann S, Bogerts B, Gundelfinger ED, Braunewell KH: **Regional and cellular distribution of neural visinin-like protein immunoreactivities (VILIP-1 and VILIP-3) in human brain**. *Journal of neurocytology* 1999, **28**(8):655-662.

92. Braunewell KH, Klein-Szanto AJ: **Visinin-like proteins (VSNLs): interaction partners and emerging functions in signal transduction of a subfamily of neuronal Ca2+ -sensor proteins**. *Cell and tissue research* 2009, **335**(2):301-316.

93. De Raad S, Comte M, Nef P, Lenz SE, Gundelfinger ED, Cox JA: **Distribution pattern of three neural calcium-binding proteins (NCS-1, VILIP and recoverin) in chicken, bovine and rat retina**. *The Histochemical journal* 1995, **27**(7):524-535.

94. Yao JJ, Gao XF, Chow CW, Zhan XQ, Hu CL, Mei YA: **Neuritin activates insulin receptor pathway to up-regulate Kv4.2-mediated transient outward K+ current in rat cerebellar granule neurons**. *The Journal of biological chemistry* 2012, **287**(49):41534-41545.

95. Nedivi E, Wu GY, Cline HT: **Promotion of dendritic growth by CPG15, an activity-induced signaling molecule**. *Science* 1998, **281**(5384):1863-1866.

96. Fujino T, Leslie JH, Eavri R, Chen JL, Lin WC, Flanders GH, Borok E, Horvath TL, Nedivi E: **CPG15 regulates synapse stability in the developing and adult brain**. *Genes & development* 2011, **25**(24):2674-2685.

97. Naeve GS, Ramakrishnan M, Kramer R, Hevroni D, Citri Y, Theill LE: **Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(6):2648-2653.

98. Nedivi E, Fieldust S, Theill LE, Hevron D: **A set of genes expressed in response to light in the adult cerebral cortex and regulated during development**. *Proceedings of the National Academy of Sciences of the United States of America* 1996, **93**(5):2048-2053.

99. Javaherian A, Cline HT: **Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo**. *Neuron* 2005, **45**(4):505-512.

100. Cappelletti G, Galbiati M, Ronchi C, Maggioni MG, Onesto E, Poletti A: **Neuritin (cpg15) enhances the differentiating effect of NGF on neuronal PC12 cells**. *Journal of neuroscience research* 2007, **85**(12):2702-2713.

101. Vogel C, Marcotte EM: **Insights into the regulation of protein abundance from proteomic and transcriptomic analyses**. *Nature reviews Genetics* 2012, **13**(4):227-232.

102. Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, Chen X, Li L, Wu S, Chen Y *et al*: **How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes**. *Acta biochimica et biophysica Sinica* 2008, **40**(5):426-436.

103. Fainzilber M, Budnik V, Segal RA, Kreutz MR: **From synapse to nucleus and back again--communication over distance within neurons**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011, **31**(45):16045-16048.

104. Morfini G, Szebenyi G, Elluru R, Ratner N, Brady ST: **Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility**. *The EMBO journal* 2002, **21**(3):281-293.

105. Kochubey O, Lou X, Schneggenburger R: **Regulation of transmitter release by Ca(2+) and synaptotagmin: insights from a large CNS synapse**. *Trends in neurosciences* 2011, **34**(5):237-246.

106. Chen YA, Scheller RH: **SNARE-mediated membrane fusion**. *Nature reviews Molecular cell biology* 2001, **2**(2):98-106.

107. Martens S, Kozlov MM, McMahon HT: **How synaptotagmin promotes membrane fusion**. *Science* 2007, **316**(5828):1205-1208.

108. Vrljic M, Strop P, Ernst JA, Sutton RB, Chu S, Brunger AT: **Molecular mechanism of the synaptotagmin-SNARE interaction in Ca2+-triggered vesicle fusion**. *Nature structural & molecular biology* 2010, **17**(3):325-331.

109. Vennekate W, Schroder S, Lin CC, van den Bogaart G, Grunwald M, Jahn R, Walla PJ: **Cis- and trans-membrane interactions of synaptotagmin-1**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(27):11037-11042.

110. Marqueze-Pouey B, Wisden W, Malosio ML, Betz H: **Differential expression of synaptophysin and synaptoporin mRNAs in the postnatal rat central nervous system**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1991, **11**(11):3388-3397.

111. Bergmann M, Schuster T, Grabs D, Marqueze-Pouey B, Betz H, Traurig H, Mayerhofer A, Gratzl M: **Synaptophysin and synaptoporin expression in the developing rat olfactory system**. *Brain research Developmental brain research* 1993, **74**(2):235-244.

112. Brandstatter JH, Lohrke S, Morgans CW, Wassle H: **Distributions of two homologous synaptic vesicle proteins, synaptoporin and synaptophysin, in the mammalian retina**. *The Journal of comparative neurology* 1996, **370**(1):1-10.

113. Khaitovich P, Muetzel B, She X, Lachmann M, Hellmann I, Dietzsch J, Steigele S, Do HH, Weiss G, Enard W *et al*: **Regional patterns of gene expression in human and chimpanzee brains**. *Genome research* 2004, **14**(8):1462-1473.

114. Khaitovich P, Weiss G, Lachmann M, Hellmann I, Enard W, Muetzel B, Wirkner U, Ansorge W, Paabo S: **A neutral model of transcriptome evolution**. *PLoS biology* 2004, **2**(5):E132.

115. Sandberg R, Yasuda R, Pankratz DG, Carter TA, Del Rio JA, Wodicka L, Mayford M, Lockhart DJ, Barlow C: **Regional and strain-specific gene expression mapping in the adult mouse brain**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(20):11038-11043.

116. Dietz JA, Li Y, Chung LM, Yandell BS, Schlamp CL, Nickells RW: **Rgcs1, a dominant QTL that affects retinal ganglion cell death after optic nerve crush in mice**. *BMC neuroscience* 2008, **9**:74.

117. Libby RT, Li Y, Savinova OV, Barter J, Smith RS, Nickells RW, John SW: **Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage**. *PLoS genetics* 2005, **1**(1):17-26.

118. Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA: **DAVID: Database for Annotation, Visualization, and Integrated Discovery**. *Genome biology* 2003, **4**(5):P3.

119. Ryan JC, Morey JS, Bottein MY, Ramsdell JS, Van Dolah FM: **Gene expression profiling in brain of mice exposed to the marine neurotoxin ciguatoxin reveals an acute anti-inflammatory, neuroprotective response**. *BMC neuroscience* 2010, **11**:107.

CHAPTER III

NEURITIN1 PROMOTES RETINAL GANGLION CELL SURVIVAL AND AXONAL REGENERATION FOLLOWING OPTIC NERVE CRUSH

Tasneem P. Sharma, Yang Liu, Robert J. Wordinger, Iok-Hou Pang and Abbot F. Clark

Abstract

Neuritin1 (Nrn1) is an extracellular GPI-linked protein that stimulates axonal plasticity, dendritic arborization, and synapse maturation in the central nervous system (CNS). The purpose of this study was to evaluate the neuroprotective and axogenic properties of Nrn1 on axotomized retinal ganglion cells (RGCs) *in vitro* and on the *in vivo* optic nerve crush (ONC) mouse model. Axotomized cultured RGCs treated with recombinant hNRN1 significantly increased survival of RGCs by 29% ($n=8$, $p<0.01$) and neurite outgrowth by 261% compared to controls ($n=5,7$, $p<0.05$). RGC transduction with AAV2-CAG-hNRN1 prior to ONC promoted RGC survival (42%, $n=5$-8, $p<0.05$) and significantly preserved RGC ERG function by 41% until 28 days post crush (dpc) ($n=6$, $p<0.05$) compared to the control AAV2-CAG-GFP transduction group. Significantly elevated levels of RGC marker, Rbpms (42%, $n=5$-8, $p<0.001$) and growth cone marker, Gap43 (27%, $n=3$, $p<0.01$) were observed 28 dpc in the retinas of the treatment group compared to the control group. Significant increase in Gap43 (100%, $n=5$-6, $p<0.05$) expression was observed within the optic nerves of the AAV2-hNRN1 group compared to the controls. In conclusion, Nrn1 exhibited neuroprotective, regenerative effects and preserved RGC function on axotomized RGCs *in vitro* and after axonal injury *in vivo*. Nrn1 is a potential therapeutic target for CNS neurodegenerative diseases.

Keywords

Central nervous system, Neuritin1, optic nerve crush, retinal ganglion cell, neuroprotection and regeneration

Abbreviations

AAV, Adeno Associated-Viral Vector; bFGF, Basic Fibroblast Growth Factor; BDNF, Brain-Derived Neurotrophic Factor; BRN3A, Brain-Specific Homeobox/POU Domain Protein 3A; CNTF, Ciliary Neurotrophic Factor; CNS, Central Nervous System; dpc, Days Post Crush; fERG, Flash Electroretinogram; GAP43, Growth Associated Protein 43; GCL, Ganglion Cell Layer; GFP, Green Fluorescent Protein; GPI, Glycophosphatidylinositol; IHC, Immuno-histochemistry; IPL, Inner Plexiform Layer; IVT, Intravitreal; NFL, Nerve Fiber Layer; NGF, Nerve Growth Factor; NRN1, Neuritin 1; NT-3, Neurotrophin-3; NT-4, Neurotrophin-4; ON, Optic Nerve; ONC, Optic Nerve Crush; PFA, Paraformaldehyde; PN, Peripheral Nerves; pSTR, Positive Scotopic Threshold Response; RBPMS, RNA Binding Protein with Multiple Splicing; RGC, Retinal Ganglion Cell; SC, Superior Colliculus.

Introduction

Central nervous system (CNS) trauma and neurodegenerative disorders trigger a cascade of intrinsic and extrinsic cellular events resulting in regenerative failure and subsequent damage to neurons [1-5]. The intrinsic factors include deregulation in growth-promoting factors, apoptotic factors, intracellular signaling molecules and trophic factors [6]. Conversely, the extrinsic factors correlate to growth inhibition due to inhibitory cues [3, 8-14] that include myelin and myelin associated inhibitors, glial scarring [5, 7], slow clearance of axonal debris [8], incorrect development of neuronal projections [6] , and CNS inflammation [16, 17]. Progressive degeneration of mature retinal ganglion cells (RGCs) has been associated with loss of trophic support [9, 10], stimulation of inflammatory processes/ immune regulation [11, 12], and apoptotic effectors [10, 13-16].

After injury, mammalian RGC axons show only a short-lived sprouting response but no long-distance regeneration through the optic nerve (ON) [17]. Glial responses around the affected area are initiated by injured CNS axons [18]. Axons undergoing Wallerian degeneration are surrounded by astrocytes that up-regulate *Gfap* expression contributing to trauma-induced neurodegeneration [19]. Glial scarring inhibits axonal transport after optic nerve crush (ONC) [5, 7] decreasing transport of proteins involved in neuroprotection and synaptic plasticity. Regenerative failure is a critical endpoint of these destructive triggers culminating in neuronal apoptosis [3, 20, 21] and inhibition of functional recovery. Intrinsic factors affecting axonal regeneration after CNS are crucial for recovery and dysregulation of genes involved in axonal plasticity and outgrowth can prove detrimental to the neuronal recovery [22-24].

Current neuroprotection approaches include promoting survival of RGCs by intraocular injections of recombinant factors like ciliary neurotrophic factor (CNTF) and peripheral nerve (PNs) transplantations *in vitro* [25] and *in vivo* after injury [29] _ENREF_30. Intraocular administration of neurotrophin-4 (NT-4) and brain derived neurotrophic factor (BDNF) after ON transection also exerted neuroprotective effects on axotomized RGCs. In addition, PNs transplanted adjacent to ONs, *ex vivo* PNs grafts with lenti-viral transduced Schwann cells, and lens injury have the ability to regenerate axons of injured RGCs [26] [27]. Furthermore, using adeno-associated viral vector (AAV) therapy, AAV injections of CNTF in bcl2 overexpressing transgenic mice increases cell viability and axonal regeneration [28], whereas BDNF promotes survival of RGCs [29]. Likewise, experiments with AAV-BDNF, -CNTF and -growth associated protein 43 (GAP43) have shown that AAV-CNTF was the most crucial for promoting both long-term survival and regeneration [30]

Although, most studies target neuronal apoptosis by overexpressing intrinsic growth factors and enhancing regeneration in CNS trauma models, previous studies have established that a multifactorial approach is required for successful and long-lasting therapeutic outcomes [6, 30]. Currently gaps still exist for a key gene that could effectively target neuroprotection, enhance neurogenesis, and sustain neuronal function.

One key gene implicated in neuronal plasticity is Neuritin 1 (*Nrn1*), also known as candidate plasticity gene 15. It has multiple functions and was first identified and characterized when screening for candidate plasticity genes in the rat hippocampal dentate gyrus activated by kainate [31-33]. *Nrn1* is highly conserved across species[34] and translates to an extracellular, GPI-linked protein, which can be secreted as a soluble form.

Nrn1 (protein) stimulates axonal plasticity, dendritic arborization and synapse maturation in the CNS [35]. During early embryonic development, Nrn1 promotes the survival of neural progenitors and differentiated neurons [36], while later in development it promotes axonal and dendritic growth and stabilization, allowing maturation and formation of synapses [32, 35, 37]. In the adult brain, Nrn1 has been correlated with activity-dependent functional plasticity [34, 38] and is expressed in post mitotic neurons.

Nrn1 may be a crucial gene for neuroprotection and regeneration because growth factors such as nerve growth factor (NGF), BDNF and neurotrophin-3 (NT-3) as well as neuronal activity can potentiate the expression of *Nrn1* [33, 39]. In addition, we reported *Nrn1* mRNA expression appears to be biphasic after ON axonal trauma, indicating a transient attempt by RGCs at neuroprotection/neuroregeneration in response to ONC injury (unpublished data). The dynamic regulation of *Nrn1* coupled with neurotrophic effects may promote axonal regeneration in the CNS. To overcome CNS trauma, a new therapy geared towards neuroprotection and effective axonal regeneration is required. The purpose of this study is to evaluate the therapeutic effects of Nrn1 in mouse RGC cultures as well as in the mouse ONC model. We have identified a unique neuroprotective and regenerative strategy that prevents neurodegeneration after ON injury. AAV2-hNRN1 expression vectors partially rescued RGCs from apoptosis, maintained RGC ERG responses, and initiated regeneration of injured axons.

Results

Recombinant human neuritin increases RGC neuroprotection and neurite outgrowth *in vitro*

Our mixed retinal cell culture was characterized by using markers (Supplemental Table S1) of different retinal cell types (Supplemental Figures S1a-h). We used two different markers to identify RGCs within these cultures. We observed a higher percentage of RNA binding protein with multiple splicing (Rbpms) positive cells (35.75 ± 1.39%, n=8) compared to Brain-specific homeobox/POU domain protein 3A (Brn3a) (17.24 ± 1.04%, n=8) (Supplemental Figure S1i). In addition, the majority of cells were RGCs, although we observed other retinal cell types (amacrine cells, astrocytes, Müller cells, microglia, bipolar cells) (Supplemental Figures S1a-h) within the mixed culture.

To test the neuroprotective and regenerative effects of Nrn1 *in vitro,* we exposed the cells to medium without growth factors and treated the axotomized RGCs in the mixed culture with recombinant hNRN1 (200ng/ml). After treatment, phase contrast images of cultures showed robust cells with increased neurite outgrowth (Figures 1a and b). NRN1 treatment significantly increased the number of Brn3a positive RGCs by 29% (24.27 ± 1.72%, mean ± SEM, RGCs in NRN1 treated cultures versus 17.25 ± 1.05% RGCs in control cultures, n=8; $p<0.01$) (Figures 1c-e). In addition, NRN1 also significantly increased the number of Rbpms positive RGCs by 32% (46.45 ± 1.69% RGCs in NRN1 treatment, n=8 versus the 31.26 ± 5.94% RGCs in control cultures, n=6; $p<0.05$) (Supplemental Figures S2a-c).

Compared to the control, recombinant NRN1 increased RGC neurite outgrowth (Figures 2a and b). Neurite and nuclear tracings (Figures 2c and d) also showed a higher neurite tracing pattern after treatment. No significant difference in total DAPI cell counts was observed between control (251.8 ± 11.2 cells, n=6) and treatment (335.0 ± 34.3 cells, n=7) (Figure 2e). Quantification of total neurite length per 39100μm^2 area showed

significantly increased neurite outgrowth by 261% ($p<0.05$) with NRN1 treatment (24290 ± 60 μm, n=6) compared to control cells (9383 ± 15 μm n=5) (Figure 2f).

AAV2 mediated over-expression of hNrn1

We performed *in situ* hybridization to test mRNA expression of human *NRN1* in AAV2-hNRN1 injected mouse eyes. No expression was observed in the naïve retinas (Figure 3a). Two weeks after injection, we observed robust selective staining predominantly in the ganglion cell layer (GCL) suggesting that RGCs and/or displaced amacrine cells are the major retinal cell types expressing NRN1 (Figure 3b). The expression was maintained through six weeks after injection (Figures 3c-e).

Over-expression of NRN1 was also confirmed by immunohistochemical (IHC) staining. Increased hNRN1 was specifically detected as early as 2 weeks in the nerve fiber layer (NFL) and the cells of the GCL (Figure 4a). Since NRN1 is a secreted protein [32], slight expression was also observed within the inner and outer plexiform layers (Figure 4a). Similarly, within the ON there was increased expression 2 weeks after intravitreal injection (IVT) compared to the naïve non-injected control eyes, and immuno-reactivity was maintained at 3 weeks (Figure 4b). Similar to NRN1 expression, our control vector (AAV2-GFP) also presented robust expression at 3 weeks post IVT in the retina and ON (Supplemental Figures S3a and b).

To determine whether the transgene proteins were anterogradely transported through the visual pathway, we tested expression of GFP and hNRN1 in the superior colliculus (SC). Visual signals are transmitted from the retina to the lateral geniculate nucleus and SC through the RGC axons. In rodents, most retinal inputs project to the contralateral SC with minor innervation to the ipsilateral SC [40]. We observed increased NRN1 staining in

the contralateral SC and faint staining within the ipsilateral SC, which was still maintained at 3 weeks (Figures 4c and d). The AAV2-GFP virus also showed marked GFP expression at 3 weeks in the contralateral SC compared to the naïve animals (Supplemental Figures S3c and d).

AAV2-hNRN1 mediated neuroprotection and axonal regeneration after axonal trauma

To analyze RGC survival, retinal sections were examined for Brn3a immuno-reactivity from ora serrata to ora serrata through the central region of the mouse eye (Figure 5a). Quantitative analysis of Brn3a revealed more surviving RGCs in the AAV2-hNRN1 injected eyes compared to the control AAV2-GFP eyes. There was a significant increase in RGCs (33%, $p<0.05$) at 7 days post crush (dpc) in the AAV2-hNRN1 group (21.25 ± 1.96, n=6) compared to the AAV2-GFP controls (14.27 ± 1.62, n=5) and a 42% increase ($p<0.05$) at 28 dpc within the AAV2-hNRN1 eyes (12.76 ± 1.69, n=7) versus the controls (7.36 ± 0.84, n=4). Although, there was a trend of increased survival in the AAV2-hNRN1 group compared to the control AAV2-GFP group at 14 dpc (30%) and 21 dpc (20%), these increases were not statistically significant (Figure 5b).

Gap43 is a classical marker of axonal regeneration [41-43]. Cytoskeletal proteins transport Gap proteins to the injured end of the axon, where they are incorporated into the membranes of growth cones [44, 45]. An active RGC regenerative state was induced by over-expression of hNRN1 in our ONC model as seen by increased immunoreactivity of Gap43 within the retina and ONs after crush. Gap43 immunoreactivity in our control group showed expression patterns similar to those observed previously in other axonal injury studies [42, 46]. In the ONC control mice, we observed elevated Gap43 expression in

the NFL and inner plexiform layer (IPL) at 7 dpc. This expression was diminished from 14 to 28 dpc (Figure 6a), signifying decreased regeneration due to the extensive RGC loss. In contrast, the NRN1 group showed sustained elevation of Gap43 expression from 7 dpc through 28 dpc (Figure 6b).

Similar expression patterns were observed for the ONs in the ONC control group, where there was increased Gap43 expression at 7 dpc but not at subsequent time-points (Figure 6c). However, Gap43 expression was markedly increased within the ONs of NRN1 treated mice. At 7 dpc, elevated Gap43 immunoreactivity was observed within the ON that was sustained through 28 dpc. Moreover by 21 dpc, Gap43 expression was observed posterior to the lesion site (Figure 6d).

To further confirm the beneficial effects of hNRN1 after ONC, retinas and ONs were examined 28 dpc for Rbpms and Gap43 expression by western immunoblotting. Retinal protein lysates revealed increased expression of Rbpms and Gap43 in retinas after AAV2-hNRN1 injection compared to the ONC control AAV2-GFP retinas (Figure 7a). Quantification of Western blots revealed a significant 42% increase ($p<0.001$) in Rbpms expression in AAV2-hNRN1 treated retinas (0.26 ± 0.01, $n=8$) compared to the AAV2-GFP controls (0.15 ± 0.02, $n=5$) (Figure 7b). The regenerative marker Gap43 also showed a significant 27% increase ($p<0.01$) in the experimental AAV2-hNRN1 group (2.15 ± 0.09, $n=3$) compared to the AAV2-GFP group (1.57 ± 0.08, $n=3$) (Figure 7c).

Rbpms is predominantly expressed in the RGC soma (retinal lysates) but not in RGC axons (ON lysates) (Figure 7d). ON Gap43 expression significantly increased 100% ($p<0.05$) in the AAV2-hNRN1 group (0.32 ± 0.04, $n=6$) versus the control AAV2-GFP

group (0.16 ± 0.02, n=5) (Figure 7e). The increase demonstrated the promotion of regeneration within the axons of the RGCs.

AAV2-hNRN1 sustains RGC function after axonal trauma

The baseline positive scotopic threshold response (pSTR) amplitudes were recorded 10 days after IVT. The baseline pSTR values were similar in the AAV2-GFP (21.05 ± 2.20μV, n=6) and AAV2-hNRN1 (22.33 ± 2.05μV, n=6) injected mice prior to ONC. Compared to the control GFP group, all the hNRN1 injected mice maintained pSTR amplitudes similar to the initial baseline throughout the 28-day time course after ONC (Figure 8). At 7 dpc, a significant decrease (38%, $p<0.05$) in amplitude was observed in the control group (13.51 ± 2.62μV, n=6) compared to the hNRN1 treated group (21.71 ± 2.46μV, n=6). At 14 dpc, the average amplitude in the hNRN1 group (18.56 ± 1.37μV, n=6) was still significantly greater (34%, $p<0.05$) than the control group (12.31 ± 1.71μV, n=6). At 21 dpc, pSTR amplitudes were significantly decreased (42%, $p<0.01$) in the control group (11.80 ± 1.09μV, n=6) versus the hNRN1 treated group (20.26 ± 2.14μV, n=6) group. This difference was still maintained at 28 dpc where a highly significant decrease (41%, $p<0.001$) was observed in the control group (12.73 ± 0.99μV, n=6) in contrast to the hNRN1 treated group (21.63 ± 0.45μV, n=6). Representative amplitudes and graphs of each time-point are presented in Supplemental Figure S4. Although there was only partial protection of RGC counts with NRN1 treatment, we still observed sustained RGC function as assessed by flash electroretinograms (fERGs). This suggests that the remaining RGCs elicit an effective functional response that maintains amplitudes similar to baseline values.

Discussion

During early developmental embryonic stages, *Nrn1* promotes survival of neural progenitors and differentiated neurons [36], while in later stages it promotes axonal and dendritic growth and stabilization, promoting maturation and formation of synapses [32, 35, 37]. We demonstrated that recombinant hNRN1 stimulated the survival of axotomized RGCs and increased neurite outgrowth *in vitro*. Further, over-expression of hNRN1 *in vivo* promoted survival of the RGCs and revived the regenerative ability of the injured axons. Finally, the preserved neurons maintained functional light response after ON injury as indicated by fERGs.

Recombinant human neuritin increases neuroprotection and neurite outgrowth in axotomized RGCs

Functionally, Nrn1 acts as a ligand to the insulin receptor [47], and cleavage of the GPI anchor by phospholipase C allows the soluble form to be secreted and activate downstream pathways [32]. The neurotrophic growth factor, NGF can induce the transcription and translation of *Nrn1*, which increases neurite outgrowth in cultured rat embryonic hippocampal and cortical neurons [33], motor neurons of *Xenopus* [37] and PC 12 cells [48]. This increased neurite outgrowth occurs via mitogen-activated protein kinase or phosphatidylinositol-3 kinase activation [49].

To analyze both the neuroprotective and regenerative effects of NRN1 *in vitro*, we treated RGCs cultured without growth factors with recombinant hNRN1. We investigated RGC survival using the RGC markers Brn3a and Rbpms. Brn3a is expressed in a smaller subpopulation of RGCs [50], while Rbpms is expressed in almost all RGCs [51]. Regardless of the marker used, our data showed increased RGC survival after NRN1 treatment. In

addition, we observed 61% increase in RGC neuritogenesis after application of hNRN1 for 10 days, indicating that prolonged exposure of NRN1 promotes RGC regeneration.

Neuroprotection and axonal regeneration mediated by AAV2-hNRN1 after axonal trauma

Previously, *in-situ* hybridization has shown predominant *Nrn1* expression in the GCL [34], and immunoreactivity with NRN1 has also been specifically used to identify RGCs [52]. In addition, the regenerative role of NRN1 has been reported in previous studies, where it is a downstream effector of neurite outgrowth [32, 35,38]. NRN1 enhances the development of motor neuron axon arbors by promoting neuromuscular synaptogenesis and new axon branches [37]. *Nrn1* mRNA expression shifts predominantly from cell body to axon after nerve crush injury, suggesting the encoding of a growth-associated protein [53]. NRN1 is also up regulated after spinal cord injury [24] and stimulates regeneration of the peripheral neurons [54]. Silencing of neuritin using siRNA abolished NGF-mediated neurite outgrowth in an experimental diabetic neuropathy model, demonstrating the crucial effect of neuritin in regeneration [49]. Furthermore, conditional knockout of the *Nrn1* gene in mice delays development, maturation of axons and dendritic arbors, synaptic maturation and effective learning [55].

Prior studies performed by our group showed down-regulation of *Nrn1* within the retina and ON after ON crush injury. In addition, we showed that *Nrn1* exhibits a biphasic pattern of expression after axonal insult. Axotomized RGCs initially increase *Nrn1* expression in attempt to induce axonal regeneration and overcome obstructed transport mechanisms. These regenerative supportive mechanisms are lost 14 dpc because by then

most of the RGCs have been damaged, and the survival of these neurons has progressively decreased (unpublished data).

To test the neuroprotective and regenerative effects of hNRN1, we used an AAV2 delivery vector because of its transduction efficiency and tropism for RGCs [29, 56]. We found that hNRN1 promotes RGC survival by 42% at 28 dpc compared to the control GFP vector after axonal trauma. In addition, Gap43 is normally expressed by RGCs during development, and expression is increased transiently after axotomy in mature RGCs [57]. The expression is further elevated when injured RGCs are stimulated experimentally to regrow their axons [42]. In our study, there was increased Gap43 expression in growth cones, even past the lesion site at 21 dpc. In addition, previously it has been shown that the GPI membrane bound anchor of NRN1 allows it to confer spatial specificity and direct growth promotion [32]. These data indicate that neuritin may play important roles in neuronal differentiation and survival as well as in neurite outgrowth and axonal regeneration.

RGC functional activity sustained by AAV2-hNRN1 after axonal trauma

The functional ability of Nrn1 to promote axonal arborization and synaptic plasticity make it a unique therapeutic target to rescue RGCs from apoptosis, prevent regenerative failure and also overcome the detrimental loss in RGC function due to ONC. Previous studies have shown that axonal injury following ONC leads to severe visual deficits [58] so we analyzed the effects of hNRN1 on preserving RGC function following optic nerve injury. There was significant increase of 41% in the pSTR amplitude of the NRN1 group compared to the control GFP group at 28 dpc. The maintenance of RGC function by NRN1 treatment was comparable to the pre-ONC baseline further emphasizing the

beneficial effects of NRN1 to protect RGCs, enhance regeneration and maintain proper RGC neuronal targeting. Other studies have addressed RGC survivability and axonal regeneration with CNTF and other growth factors [29, 30], but to the best of our knowledge, this is the first study that shows a single gene exhibiting different aspects of overcoming ONC injury, which include RGC survival, axonal regeneration and sustained RGC function.

After the initial insult of axotomy, degenerative pathways ensue and during this phase of degeneration, it is crucial for cells to overcome cell death mechanisms and potentiate neurite extensions for survivability and accurate neuronal targeting. *Nrn1* gene therapy was aimed to increase survival and regeneration of axotomized RGCs both *in vitro* and *in vivo.* Although our experiments were crucial in identifying a gene target that could address the different facets of neurodegeneration and regenerative failure, further experiments need to be performed to test correct innervation of traumatized axons to target neurons. In addition, these studies have been only performed in the ONC model and could be further extrapolated to other CNS trauma models as well as neurodegenerative models.

In conclusion, our study discovered a unique neuroprotective and regenerative strategy to prevent RGC degeneration. Over-expressing of hNRN1 allowed the rescue of RGCs from apoptosis, regenerated injured axons, and maintained RGC function in an ON injury model.

Materials and Methods

Animals

BALB/cJ mice aged 2-4 months were utilized for all the experiments and were obtained from the Jackson Laboratories (Bar Harbor, ME). The mice were housed and maintained in a 12-hour light/dark cycle and fed ad libitum. All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and the University of North Texas Health Science Center (UNTHSC) Institutional Animal Care and Use Committee regulations.

Retinal Cell Culture

Isolation and culture of retinal cells was modified from a previously reported protocol [59]. Briefly, retinas from postnatal day p5-p7 BALB/cJ mice were dissociated with papain solution (2 mg papain/mL (Sigma, St. Louis, MO), 0.4 mg/mL DL-cysteine (Sigma), and 0.4 mg/mL bovine serum albumin (Sigma) in neurobasal medium (Gibco/Invitrogen, Carlsbad, CA)), for 5 min at 37°C. Dissociated retinas were further dispersed using a 1000µl pipettor and RGC culture medium [59] ((Neurobasal/B27 medium with 100 units/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 1 mM pyruvate (Gibco/Invitrogen), 2 mM glutamine (Gibco/Invitrogen), 5 µg/mL insulin (Sigma), 100 µg/mL transferrin (Sigma), 100 µg/mL bovine serum albumin (Sigma), 60 ng/mL progesterone (Sigma), 16 µg/mL putrescine (Sigma), 40 ng/mL sodium selenite (Sigma), 40 ng/mL thyroxine (Sigma), 40 ng/mL tri-iodothyronine (Sigma), 50 ng/mL BDNF (Biosource, Camarillo, CA), 10 ng/mL CNTF (Biosource), 10 ng/mL bFGF (Biosource), 5 µM forskolin (Sigma), and 1% fetal calf serum (Atlas Biologicals, Fort Collins, CO)) was added to neutralize the papain. The supernatant was discarded after centrifugation (3000 r.p.m.) to remove any extra papain. RGC medium was added to the pellet and cells

were dispersed into a single cell suspension by pipetting. Cells were cultured on poly-D-lysine- and laminin-coated 8-well chambered culture slides (BD Biosciences, San Jose, CA) and incubated in 5% CO_2 /95% air at 37°C in a humidified incubator. Cells were maintained in two different mediums: Medium+GFs (RGC medium with BDNF, bFGF, CNTF and forskolin) or Medium+NoGFs (RGC medium with no growth factors) for various treatments

Characterization of Retinal Cell Cultures

Dissociated retinal cells were cultured on the chamber slides for six days in Medium+GFs and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. After fixation, the cells were blocked with SuperBlock™ Blocking Buffer (Fisher Scientific, Chicago, IL) at room temperature for 1 h. Primary antibodies (Supplemental Table S1) were diluted in SuperBlock™ and incubated overnight at 4°C. Wells were washed and incubated with Alexa Fluor/TRITC secondary antibody (Supplemental Table S1) for 1 h at room temperature. Slides were then rinsed again and mounted with ProLong ® Gold anti-fade reagent with DAPI (Molecular Probes, Grand Island, NY). Images were captured at 400x magnification using a Nikon Eclipse Ti-U Microscope (Nikon, Melville, NY) with the Nuance Multispectral imaging system and analyzed using Adobe Photoshop CS5 software. Images were acquired from random fields of view for every well, and the procedure was repeated in independent culture experiments (8 images/well, n=8). Mean number of Brn3a and Rbpms positive RGCs were counted and quantified over total number of DAPI cells per image and data are presented as Mean ± SEM.

Recombinant Neuritin1 Treatment – *in vitro* RGC Survival and Neurite Outgrowth

Dissociated retinal cells were cultured on the chamber slides for six days in culture medium (Medium+GFs). They were then switched to Medium+NoGFs with or without treatment with hNRN1 (200ng/ml). Medium was replaced every 48 h for the next 10 days. After 10 days, the cells were fixed with 4% PFA for 30 min. Immunocytochemistry staining was performed for Brn3a and Rbpms (for RGC survival, n=6) or Brn3a and Nefl (for neurite outgrowth, n=5-8) and slides mounted with anti-fade reagent containing DAPI. The RGC survival and neurite outgrowth experiments were performed independently.

For each experiment (survival and neurite outgrowth), images were captured and analyzed at 400x magnification by fluorescent microscopy for control and treatment conditions (8 images/well). Each experiment was independently repeated at least three times. Masked image capture and analysis were performed to remove any bias as to the treatment condition. Cell counts were analyzed using Adobe Photoshop software. Data are presented as Mean ± SEM of replicate wells. RGC survival was evaluated by counting Brn3a/Rbpms positive cells over total number of DAPI positive cells. For regeneration studies, RGCs were photographed and neurite length determined using ImageJ software with the NeuriteTracer plugin. Processing of nuclei and neuron images was compiled using the plugin with normalization and standardization procedures in place. Total neurite length per 39,100 μm^2 area was calculated for the control and NRN1 treated cells.

AAV2 Production and Intravitreal Administration

For AAV2 production, we used a pCMV6-XL5 plasmid (Origene, Rockville, MD) carrying the hNRN1 (NM_016588) cDNA clone. Vector Biolabs (Philadelphia, PA)

performed the AAV cis cloning, cis-plasmid preparation, viral packaging, viral purification and GC titration of AAV2-CAG-hNRN1-WPRE. AAV2-GFP with the CAG/CBA promoter was also ordered from Vector Biolabs.

For AAV2 intravitreal injections, mice were anesthetized by intraperitoneal (ip) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and 2µl of AAV2-CAG-GFP or AAV2-CAG-hNRN1 (10^{10} GC) was injected into the left eye using a Hamilton syringe. The contralateral eye was left untreated. To study transduction efficiency, single intravitreal AAV2 injections were administrated and mice harvested at 2, 3, 4 and 6 weeks post injection (n=4). For studying RGC survival (n=7-8), axonal regeneration (n=7-8) and RGC function (n=6), single AAV2 injections were administered 2 weeks prior to ONC surgery and mice harvested or tested at 7, 14, 21 and 28 dpc.

Optic Nerve Crush Model

The ON of the left eye was crushed 0.5mm posterior from the globe for 4 s using the Nickell's technique [60]. Briefly, mice were anesthetized by ip injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The ON (left) was exposed and crushed intraorbitally using self-closing forceps to ensure reproducibility and constant force. Extreme care was taken not to damage the ocular blood vessels. Indirect ophthalmoscopy was performed to ensure retinal circulation was not compromised.

Immunohistochemistry

IHC was performed on retina and ON cryo-sections and SC paraffin sections, to validate protein expression of Brn3a (RGC survival, n=7/8), NRN1 and GFP (AAV2 induced over-expression, n=4), and Gap43 (regeneration after IVT and ONC, n=7/8). Whole eyes

with ONs were harvested and fixed in 4% PFA for 2 h at room temperature. After fixation, the tissue was placed in 20% sucrose overnight at 4°C and embedded in optimum cutting temperature (OCT) the next day. Sections (10 μm) were cut using a cryostat (Leica Biosystems - Richmond, IL). Cross sections of retina were transferred to Superfrost glass slides (Fisher Scientific - Chicago, IL). Slides were incubated in PBS for 10 min and blocked with SuperBlock™ Blocking Buffer at room temperature for 1 h. Primary antibodies (Supplemental Table S1) were diluted in SuperBlock™. Each slide was incubated with the respective primary antibody and incubated overnight at 4°C. Sections were then washed and incubated with Alexa Fluor secondary antibody (Supplemental Table S1) for 1 h at room temperature. After rinsing, slides were mounted with ProLong ® Gold anti-fade reagent with DAPI. Sections were observed and captured using a Nikon Eclipse Ti-U Microscope containing the Nuance Multispectral imaging system and analyzed using Adobe Photoshop CS5 software.

Survival counts of the retinal ganglion cells *in vivo*

RGC survival counts were determined by counting Brn3a positive cells in a masked manner from six retinal sections from ora serrata to ora serrata through the optic nerve head. Cell counts from all six sections were averaged per retina and the mean of all retina counts determined for both experimental conditions: control (AAV2-GFP, n=7) and treatment (AAV2-hNRN1, n=8) for each time-point post crush (7,14, 21 and 28 dpc). The data (Mean ± SEM) were analyzed by comparing both the groups: treatment (AAV2-hNRN1) versus control (AAV2-GFP).

RNA *In Situ* Hybridization

RNA *in-situ* hybridization was performed to verify effective transduction of AAV2-GFP and AAV2-hNRN1 (n=4). Cryo-fixed retina cross-sections were subjected to protein digestion using proteinase K stock (Panomics, Santa Clara, CA) diluted 1:100 for 20 min at room temperature. *In situ* hybridization was performed using Type 1 probes for hNRN1 (VAI-15422) designed by Panomics following manufacturer's protocols. Briefly, probes were diluted in 1:50 in hybridization buffer and sections incubated at 40°C for 3 h and then overnight at room temperature. The sections were then washed and hybridized in succession after each of the following treatment: PreAmp1 QF (1:100) at 40°C (25 min), Amp1 QF (1:100) at 40°C (15 min), Label Probe AP (1:1000) at 40°C (15 min). Sections were finally incubated with AP Enhancer solution for 5 min at room temperature and Fast Red substrate (chromogenic dye) at 40°C (30 min). Slides were washed and mounted with ProLong ® Gold anti-fade reagent with DAPI (Molecular Probes, Grand Island, NY). Sections (three sections/mouse, n=4) were observed and captured at 400X magnification using a Nikon Eclipse Ti-U Microscope (Nikon, Melville, NY) containing the Nuance Multispectral imaging system and analyzed using Adobe Photoshop CS5 software.

Western Blot Assay

For retinal and ON lysate preparation, mice were euthanized, eyes enucleated and retinas and ON were carefully dissected. Each isolated tissue sample was homogenized in MPER lysis buffer (Fisher Scientific, Pittsburgh PA) with 1% protease inhibitor cocktail (Pierce Technology, Rockford, IL) and the lysate supernatant collected after centrifugation at 5000 r.p.m. (10 min at 4°C). Protein concentration was determined by the BioRad Dc Protein Assay Kit (Bio-Rad Laboratories). Proteins were separated on 15%

polyacrylamide gels with 35µg protein applied per lane, and transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK). Membranes were blocked (5% milk in TBST buffer) and incubated overnight at 4^{o}C with primary antibody (Supplemental Table S1). The membranes were washed with TBST and probed with horseradish peroxidase-conjugated secondary antibody (Supplemental Table S1) in 5% non-fat milk in TBST for 1 h at room temperature. Proteins were detected using the ECL reagent SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL) and a Fluor ChemTM 8900 imager (Alpha Innotech, Santa Clara, California). Immunoreactivity was quantified using the FluorChem spot densitometry software.

Full field flash ERG recording

Mice were dark-adapted overnight prior to ERG recording. The next day all the procedures were performed in a dark room with a dim red safe light as needed. The mice were anesthetized during the recording procedure by Isoflurane (Bulter Schein Animal Health, Dublin, OH) inhalation. Their body temperature was maintained at 37°C by a heating pad and monitored using a rectal probe. A Handheld Multi-species-ElectroRetinoGraph (HMsERG) unit (Ocuscience, Kansas City, MO), with thread electrodes was utilized to perform the full field flash ERG. Pupils were dilated with 2.5% phenylephrine ophthalmic eye drops. The thread electrodes were carefully placed on top of each cornea. The cornea was moistened with 2.5% hypromellose ophthalmic solution (Arkon, Lake Forest, IL) and covered with a 2.5 mm clear contact lens. The corresponding stainless steel reference electrode was placed under the skin in the area below each eye. Another grounding needle electrode was inserted subcutaneously above the tail. Simultaneous bilateral ERG recordings were performed on the mouse eyes.

The light stimuli impulse was generated in a Ganzfeld doom with light emitting diodes (LED). The scotopic ERG responses were recorded by stimulating the retina with a flash intensity of 3×10^{-5} cd·s·m^{-2}. Responses from 30 flashes with 2 s intervals were averaged. The amplitudes of pSTRs were measured from the baseline to the positive peak of each waveform and latency measured by time-to peak major positive deflection. The data (Mean ± SEM) were analyzed by comparing both the groups: treatment (AAV2-hNRN1) versus control (AAV2-GFP).

Statistical analysis

One-way ANOVA followed by Tukey *post hoc* test was utilized to analyze intragroup differences across time-points and unpaired Student's t-test was used to analyze data between two groups. Data are presented as mean ± SEM and a p value <0.05 was considered statistically significant.

Titles and Legends to Figures:

Figure 1

Increased survival of axotomized RGCs by recombinant human Nrn1 *in vitro*. Dissociated retinal cultures were treated with vehicle (control) or recombinant hNRN1 (200ng/ml) (treatment) for 10 days in culture. Phase contrast images of (a) control and (b) treatment. Fluorescence micrographs of dissociated retinal ganglion cells immunolabeled with Brn3a and DAPI (c) control and (d) treatment. Blue staining indicates DAPI labeled nuclei with red immuno-staining for Brn3a expression. Scale bar = 50μm. Photomicrographs were captured at 400X original magnification. e) Quantification of Brn3a stained RGCs per well. Values represent the mean of 8 wells from three independent experiments per group. Data presented as mean ± SEM. Statistical significance between treatment conditions determined by unpaired Student's t-test, ** p value <0.01, n=8.

Figure 2

Recombinant human NRN1 increases neurite outgrowth in axotomized RGCs *in vitro*. Dissociated retinal cultures were treated with vehicle (control) or recombinant hNRN1 (200ng/ml) for 10 days in culture. Fluorescence micrographs of neurite outgrowths in dissociated retinal ganglion cells immunolabeled with Brn3a, Nefl and DAPI in (a) control and (b) NRN1 treated cells. Blue staining indicates DAPI labeled nuclei, red immunostaining for Brn3a and green for Nefl. Processed nuclear and neuronal tracings in (c) control and (d) NRN1 treated cells. Scale bar = 50μm. Photomicrographs were captured at 400X original magnification. Quantification of total number of (e) DAPI positive cells and (f) total neurite length in retinal cultures as described in a-d. Values

represent the mean of total cells and total neurite length (μm) for eight random images captured in 39100μm^2 area for 5-7 wells from three independent experiments per group. Data presented as mean ± SEM. Statistical significance between treatment conditions determined by unpaired Student's t-test, * p value <0.05, n.s.= not significant, n=5-7.

Figure 3

Increased retinal neuritin mRNA expression after AAV2 mediated NRN1 overexpression. BALB/cJ mice received intravitreal injections of AAV2-hNRN1. *In situ* hybridization was performed on retinal sections probed for *NRN1*. *NRN1* signals are present in the ganglion cell layer. Retinal sections from (a) naïve, (b) 2 weeks, (c) 3 weeks, (d) 4 weeks and (e) 6 weeks after intravitreal injection. Blue staining indicates DAPI labeled nuclei, all panels show red immuno-staining for hNRN1 in the RGC layer. Scale bar = 50μm, n=4. Photomicrographs were captured at 400X original magnification.

Figure 4

Increased expression of hNRN1 *in vivo* after intravitreal injection of AAV2-CAG-hNRN1. BALB/cJ mice received intravitreal injections of AAV2-hNRN1 and were harvested at naïve, 2 and 3 weeks after injection. Fluorescence micrographs show over-expression of hNRN1 in longitudinal (a) retinal, (b) retina and ON and (c-d) superior colliculus coronal sections. Green=NRN1; red=Brn3a; blue= DAPI. Time point post injection indicated on top of the panel. RSC (right contralateral superior colliculus) and LSC (left ipsilateral superior colliculus). Scale bar = 50μm (a,b), 100μm (c,d), n=4. Photomicrographs were captured at 400X and 100x original magnification.

Figure 5

AAV2 mediated delivery of NRN1 *in vivo* promotes and sustained survival of axotomized retinal ganglion cells. Animals were intravitreally injected with either AAV2-GFP or AAV2-hNRN1. Two weeks later, animals were subjected to optic nerve crush and whole eyes harvested at 7, 14, 21 and 28 days post crush. (a) Representation of retinal tissue sections counted from ora serrata to ora serrata in the central region of each mouse retina (six sections/eye). Fluorescent micrographs of Brn3a and Brn3a with DAPI images at 7 days post crush show representative image captured at each point throughout the whole retina to count the Brn3a positive cells (400x magnification). (b) Quantification of Brn3a positive cells. Naïve retinas presented a mean of 103.73 ± 8.65 cells (n=3). Data presented as mean ± SEM. Statistical significance between treatment conditions determined by unpaired Student's t-test, * p value < 0.05, n=7-8.

Figure 6

Retinal ganglion cell axonal growth is stimulated by AAV2-CAG-hNRN1 in ONC model. Animals were intravitreally injected with either AAV2-GFP or AAV2-hNRN1. Two weeks later, animals were subjected to optic nerve crush and whole eyes harvested at 7, 14, 21 and 28 days post crush. Fluorescence micrographs of longitudinal retinal sections immunolabeled with Gap43 and DAPI (a) AAV2-GFP, (b) AAV2-hNRN1 and optic nerve sections (c) AAV2-GFP and (d) AAV2-hNRN1. Blue staining indicates DAPI labeled nuclei and red immunostaining for Gap43. Time point post optic nerve crush indicated on top of the panel. Dotted line represents crush site within each ON image. Scale bar = 50μm (a,b), 100μm (c,d), n=7-8. Photomicrographs were captured at 400X and 100x original magnification.

Figure 7

AAV2-CAG-hNRN1 mediated survival and regeneration of RGCs in the *in vivo* optic nerve crush model. Animals were intravitreally injected with either AAV2-GFP or AAV2-hNRN1. Two weeks later, animals were subjected to optic nerve crush and retinas and ONs harvested 28 days post crush. (a) Western blot analysis of retinal and ON lysates from AAV2-GFP and AAV2-hNRN1 animals using antibodies against Rbpms and Gap43. β-actin served as loading control. Quantification by densitometry of retinal lysates between groups for (b) Rbpms, n=5-8, (c) Gap43, n=3, and of ON lysates for (d) Rbpms, n=3 and (e) Gap43, n=5-6 normalized to β-actin. Data presented as mean ± SEM. Statistical significance between treatment conditions determined by unpaired Student's t-test, * p value < 0.05, ***P*<0.01; ****P*<0.001.

Figure 8

AAV2-CAG-hNRN1 induced sustained RGC function after crush. Animals were intravitreally injected with either AAV2-GFP or AAV2-hNRN1. Two weeks later, animals were subjected to optic nerve crush and fERGs analyzed at baseline (before crush) and at 7, 14, 21 and 28 days post crush. AAV2-hNRN1 sustains RGC function after optic nerve crush. Data presented as mean pSTR amplitudes ± SEM. Statistical significance between treatment conditions determined by unpaired Student's t-test and across time-points determined by one-way ANOVA -Tukey, *post hoc* test, * p value < 0.05, ** p value <0.01, *** p value < 0.001, n=6.

Figure 1

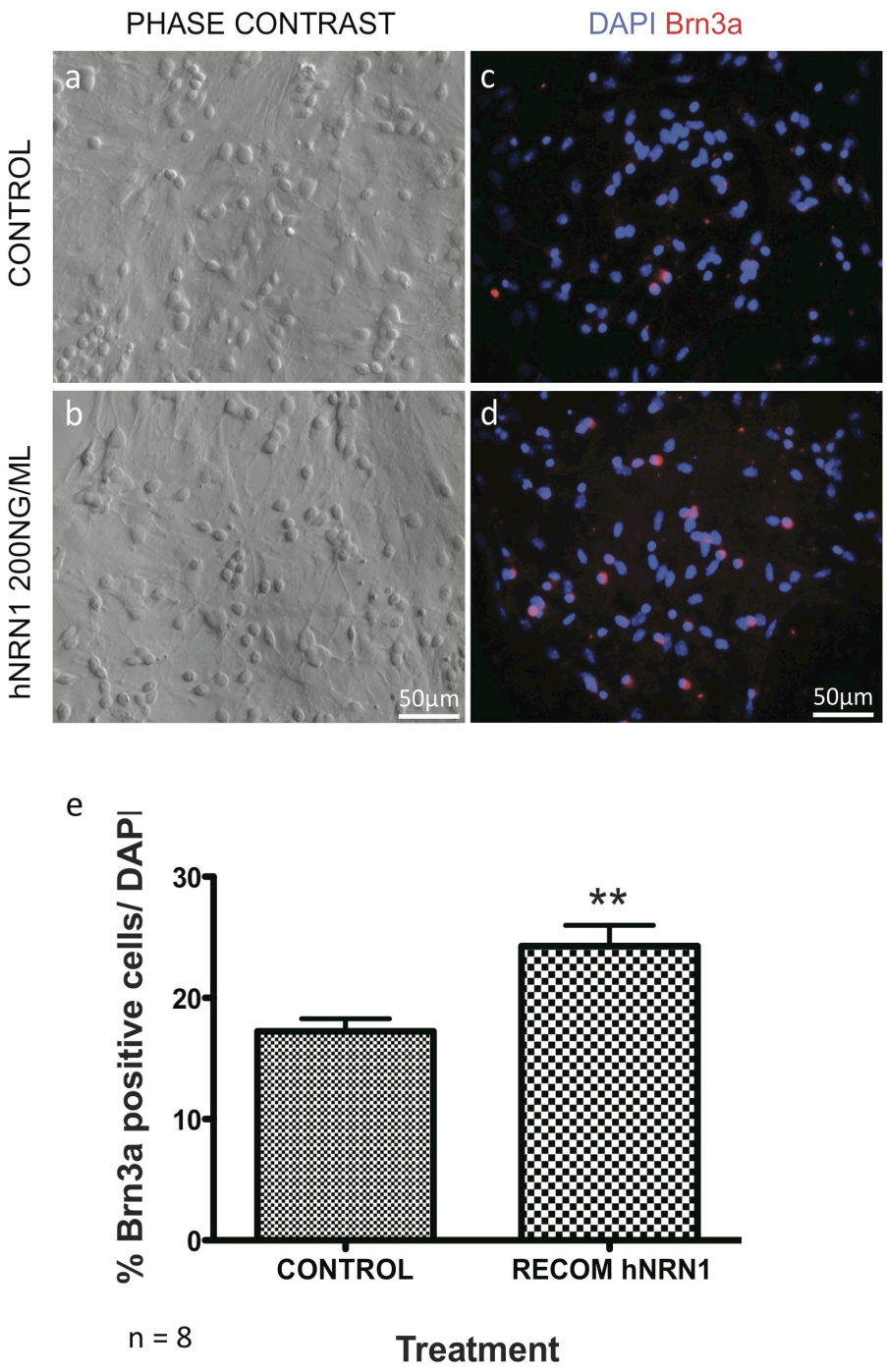

PHASE CONTRAST
DAPI Brn3a
CONTROL
hNRN1 200NG/ML
a
b
c
d
50µm
50µm
e
% Brn3a positive cells/ DAPI
30
20
10
0
**
CONTROL
RECOM hNRN1
n = 8
Treatment

Figure 2

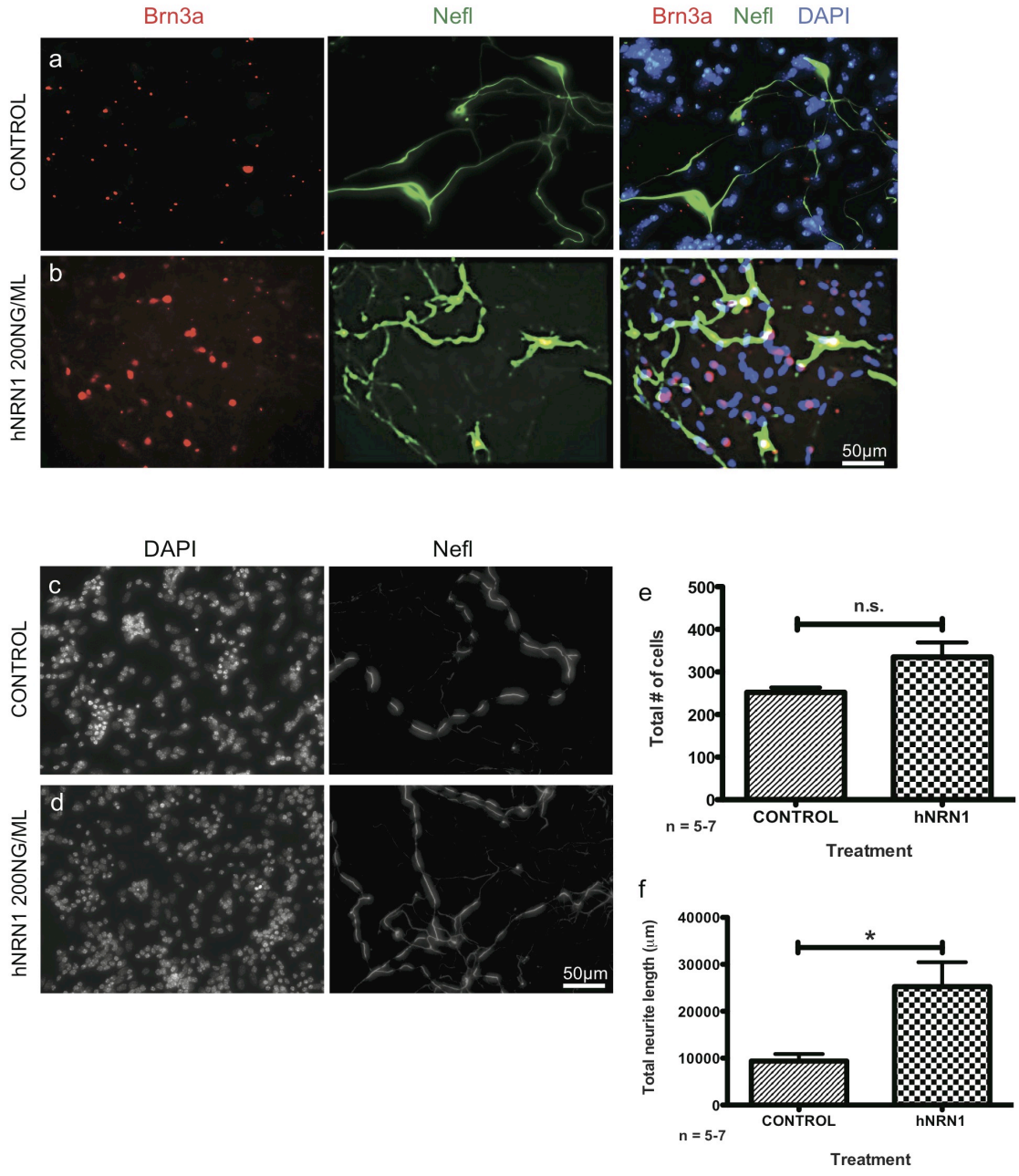

Brn3a
Nefl
Brn3a Nefl DAPI
a
b
CONTROL
hNRN1 200NG/ML
50µm
DAPI
Nefl
c
d
CONTROL
hNRN1 200NG/ML
50µm
e
Total # of cells
500
400
300
200
100
0
n.s.
n = 5-7
CONTROL
hNRN1
Treatment
f
Total neurite length (µm)
40000
30000
20000
10000
0
*
n = 5-7
CONTROL
hNRN1
Treatment

Figure 3

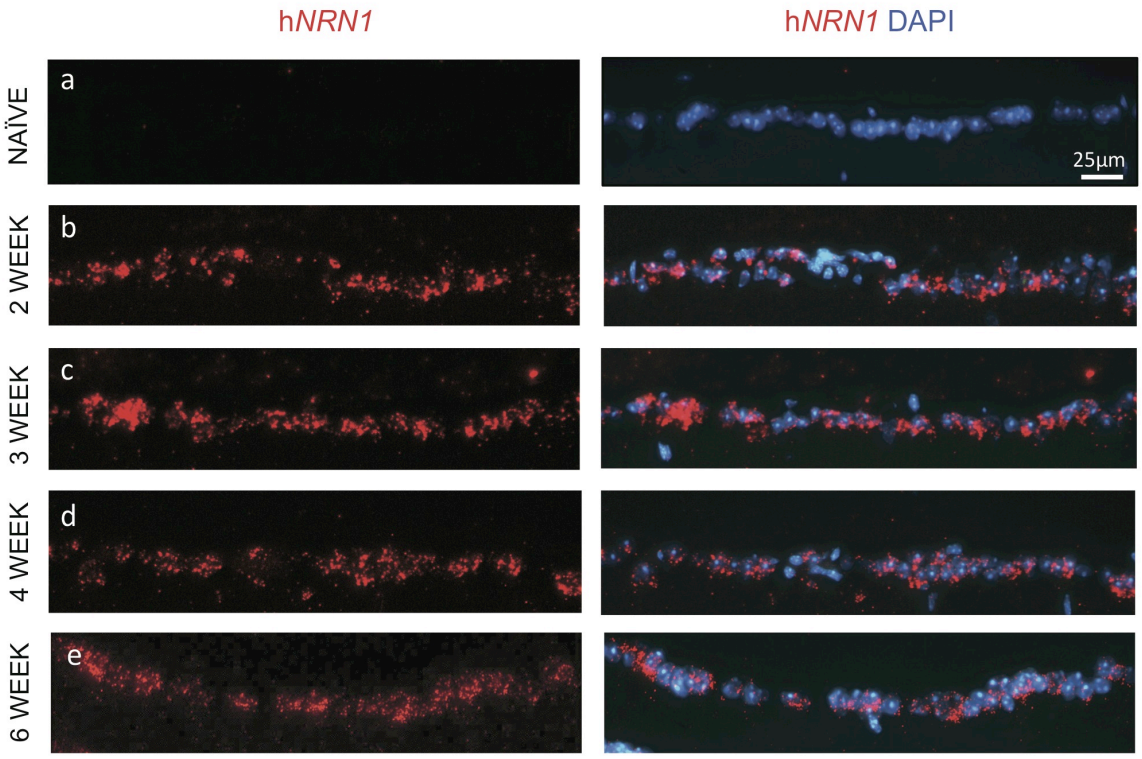


Figure 4

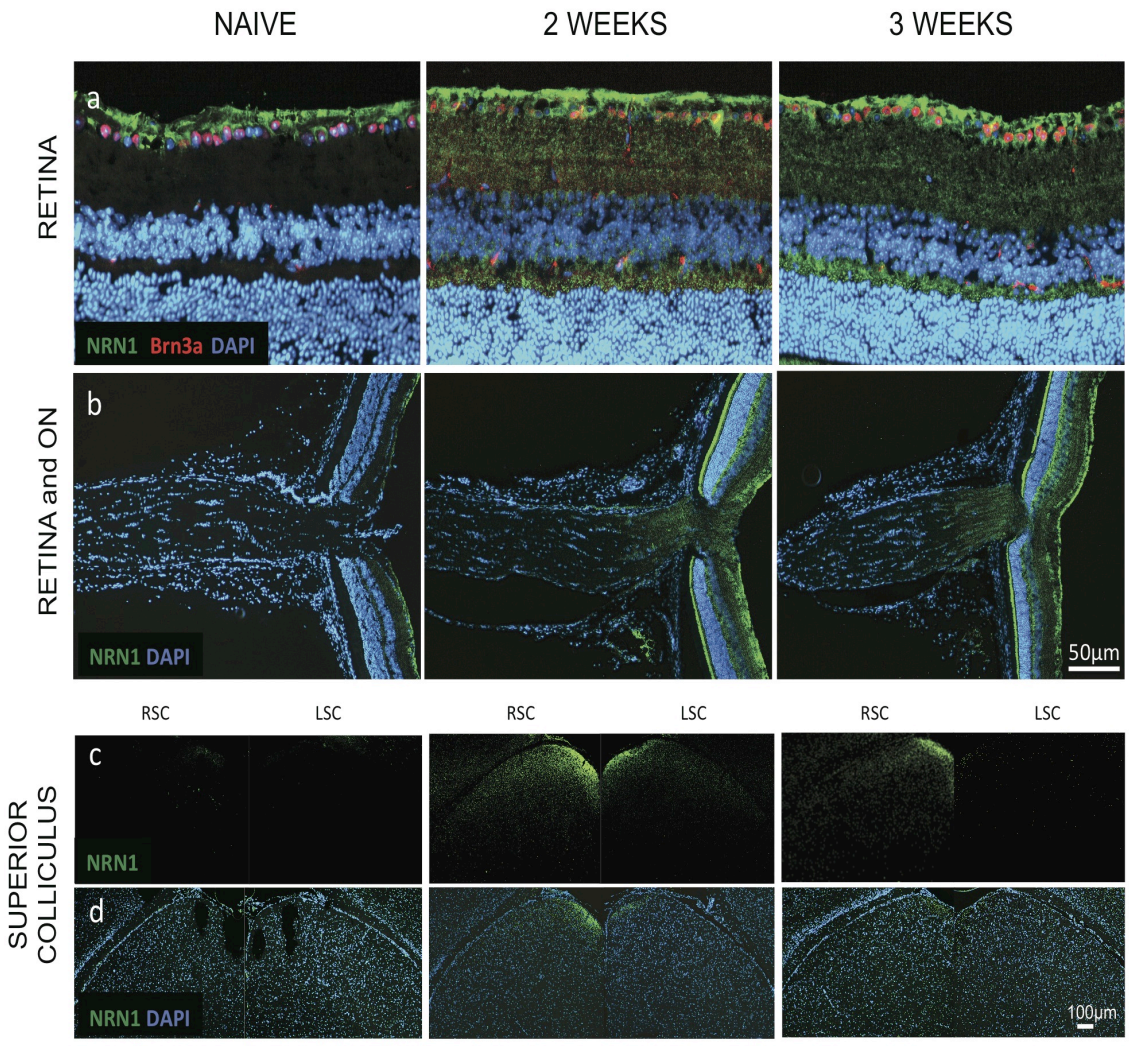

NAIVE
2 WEEKS
3 WEEKS
RETINA
a
NRN1 Brn3a DAPI
RETINA and ON
b
NRN1 DAPI
50µm
RSC
LSC
RSC
LSC
RSC
LSC
SUPERIOR COLLICULUS
c
NRN1
d
NRN1 DAPI
100µm

Figure 5

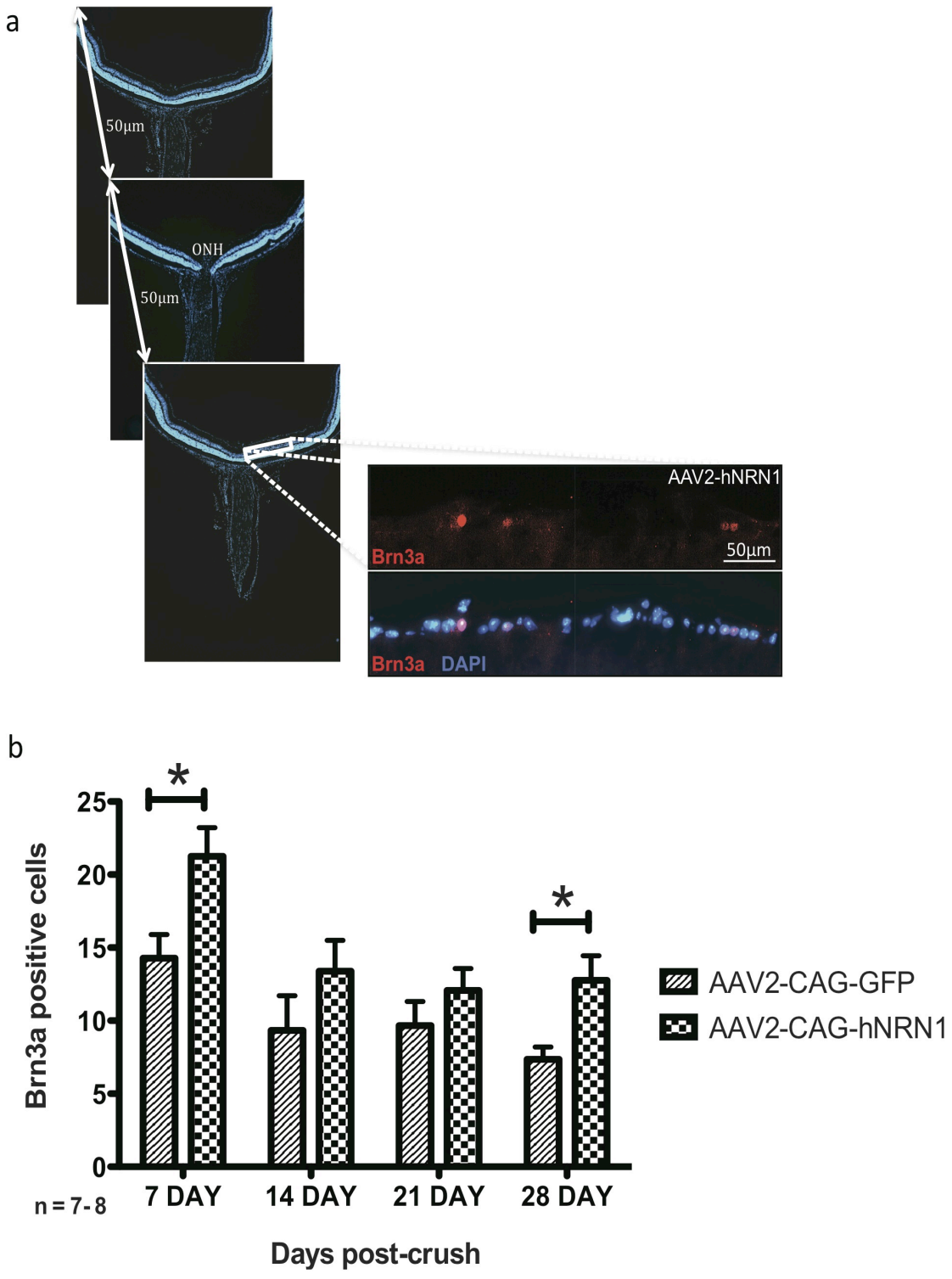

a
50µm
ONH
50µm
AAV2-hNRN1
Brn3a
50µm
Brn3a DAPI
b
Brn3a positive cells
25
20
15
10
5
0
*
*
n = 7-8
7 DAY
14 DAY
21 DAY
28 DAY
Days post-crush
AAV2-CAG-GFP
AAV2-CAG-hNRN1

Figure 6

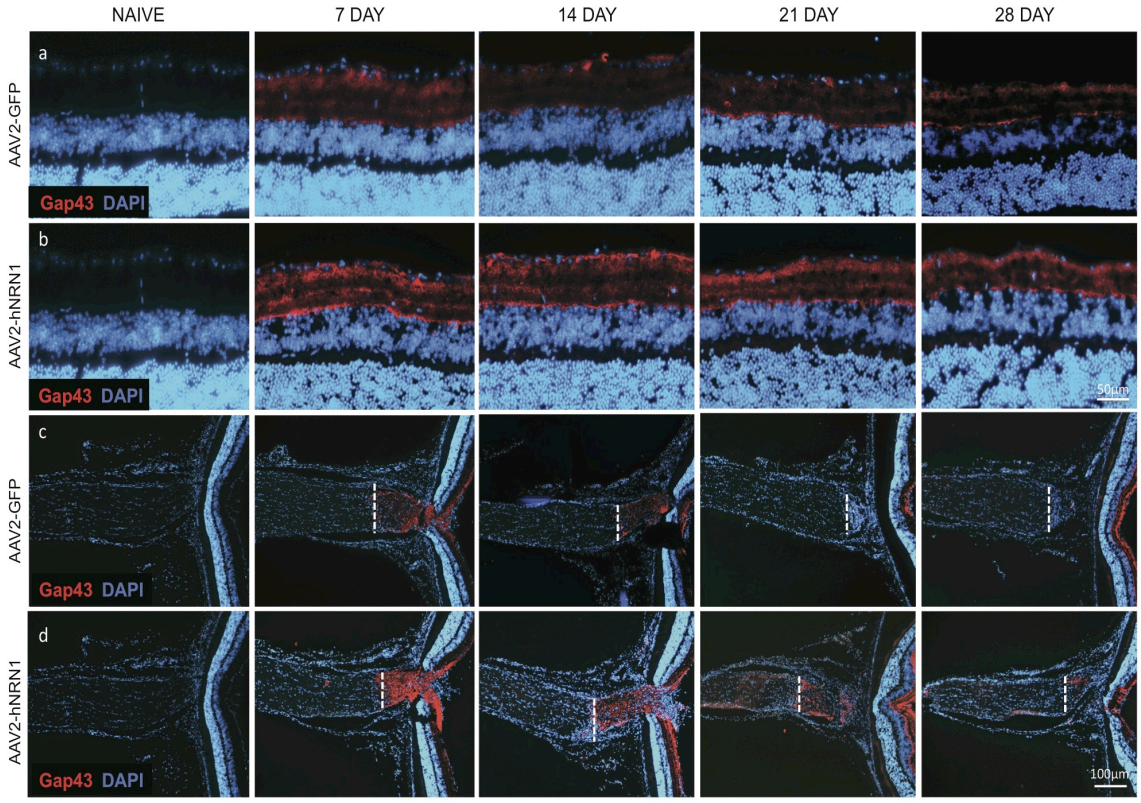

NAIVE
7 DAY
14 DAY
21 DAY
28 DAY
a
AAV2-GFP
Gap43 DAPI
b
AAV2-hNRN1
Gap43 DAPI
50μm
c
AAV2-GFP
Gap43 DAPI
d
AAV2-hNRN1
Gap43 DAPI
100μm

Figure 7

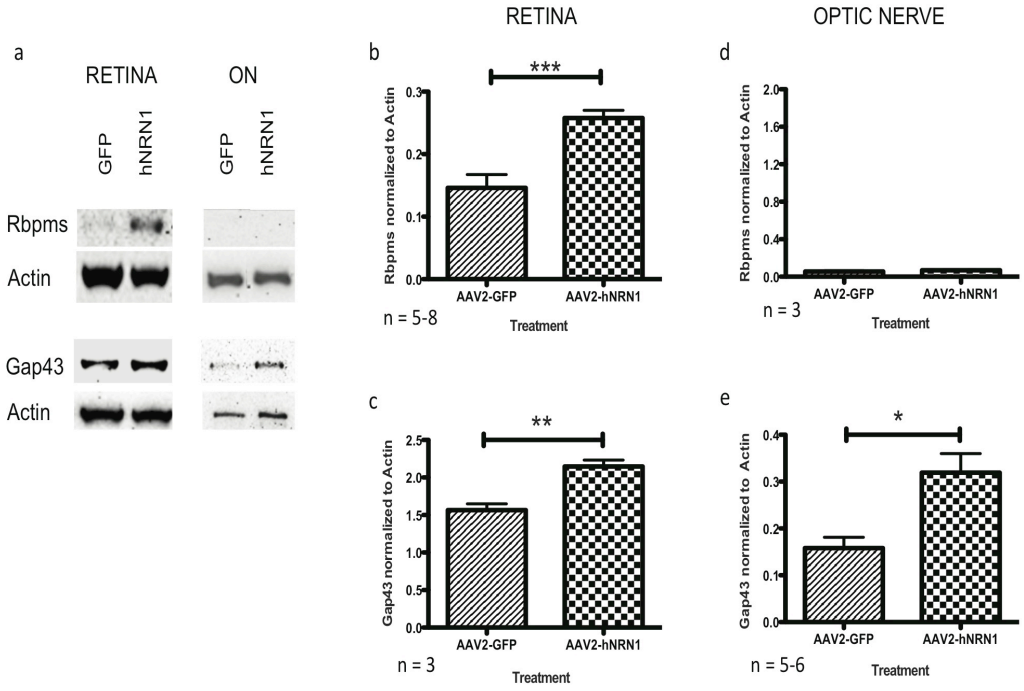
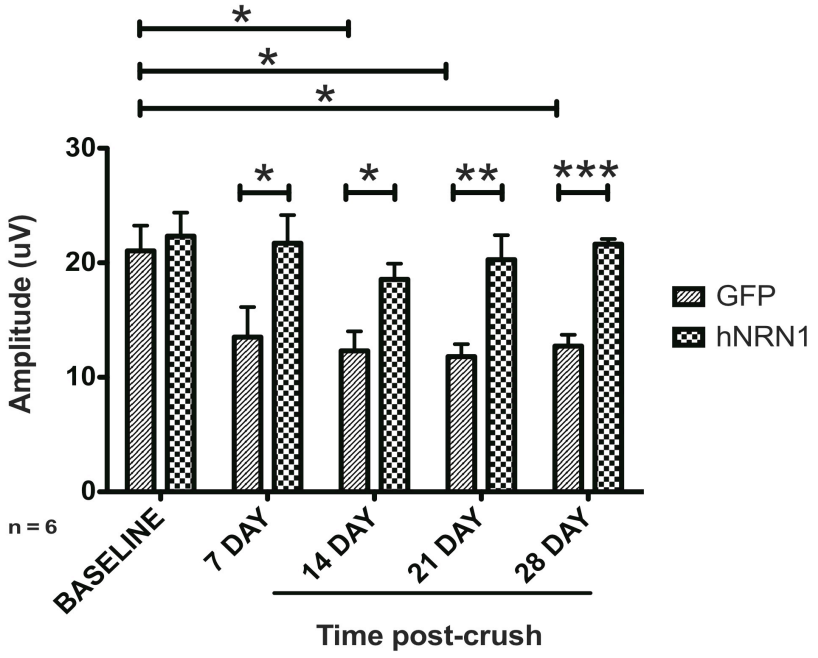


Figure 8



Supplemental Figure S1.jpg - Characterization of cells and quantification of RGCs within the *in vitro* mixed retinal cell culture. Photomicrographs were captured at 40X original magnification. Values represent the mean of 8 wells from three independent experiments per group. Data presented as mean ± SEM, n=8.

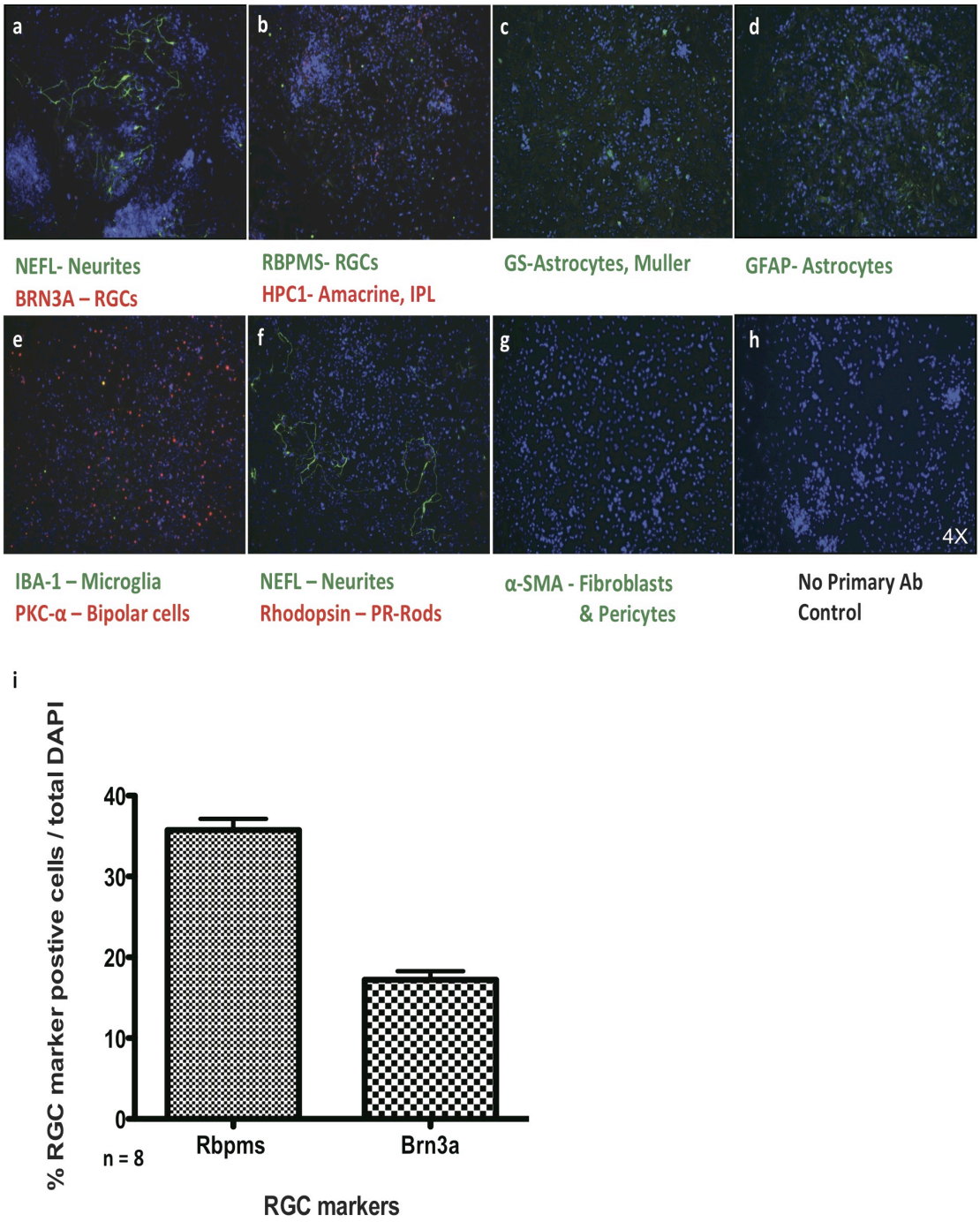
Supplemental Figure S2.jpg - Increased survival of RGCs by recombinant exogenous hNrn1 *in vitro*. Dissociated retinal cultures were treated for 10 days with vehicle or recombinant hNRN1 (200ng/ml). Fluorescence micrographs immunolabeled with Rbpms and DAPI and quantification of RGC survival. Mean ± SEM. Unpaired Student's t-test, * p value <0.05, n=6-8.

Supplemental Figure S3.jpg - *In vivo* expression of GFP using AAV2 viral vectors. BALB/cJ mice received intravitreal injections of AAV2-GFP. Retinas, ONs and SC harvested at naïve and 3 weeks after injection. Fluorescence micrographs showed over-expression of GFP (3 tissues). Red= Brn3a, blue = DAPI. Scale bar = 50μm, 100μm, n=4.

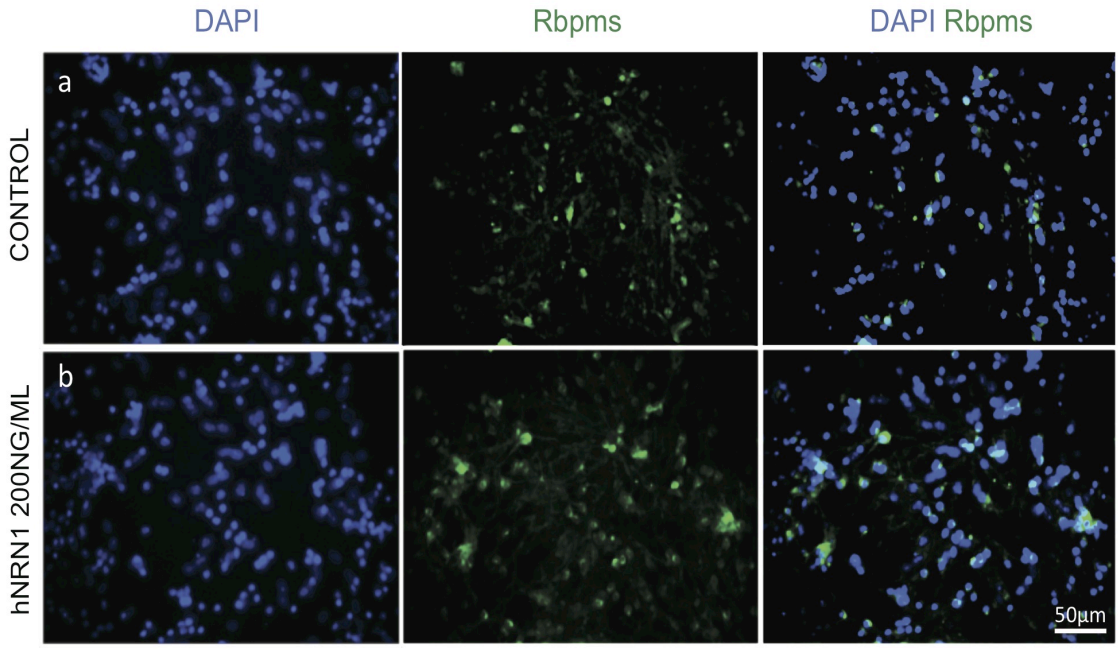
Supplemental Figure S4.jpg - Amplitudes and graphs of pSTRs after AAV2 mediated GFP or hNRN1 injections *in vivo*. Animals were intravitreally injected with either AAV2-GFP or AAV2-hNRN1. Two weeks later, ONC performed and pSTRs analyzed at baseline, 7, 14, 21 and 28 dpc. Mean pSTR amplitude ± SEM graphically presented, * p value<0.05, n=6.

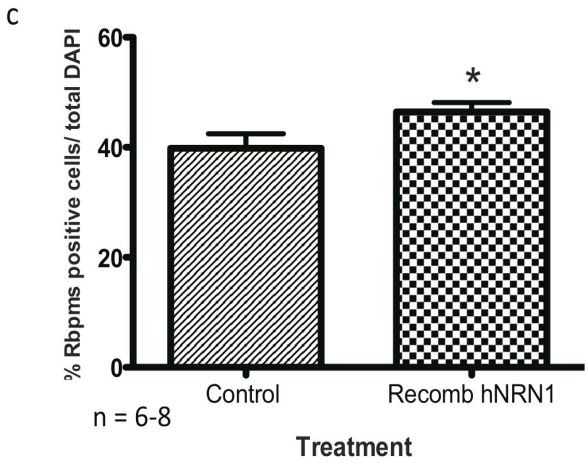
Supplemental Table S1.jpg - Antibodies and recombinant protein table. Information and dilutions of primary antibodies, secondary antibodies and recombinant protein presented in tabular format.

Supplemental Figure S1

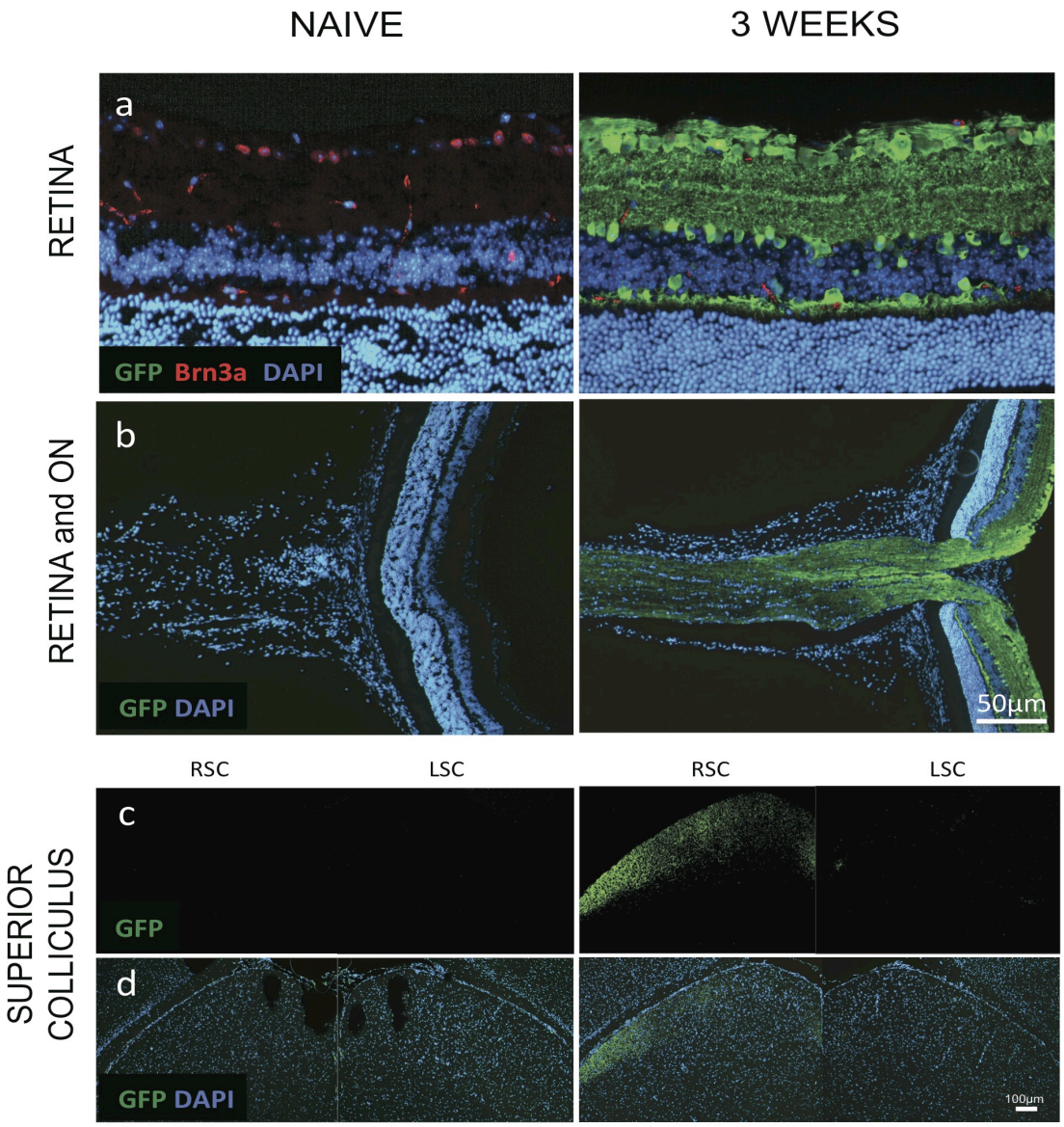


Supplemental Figure S2

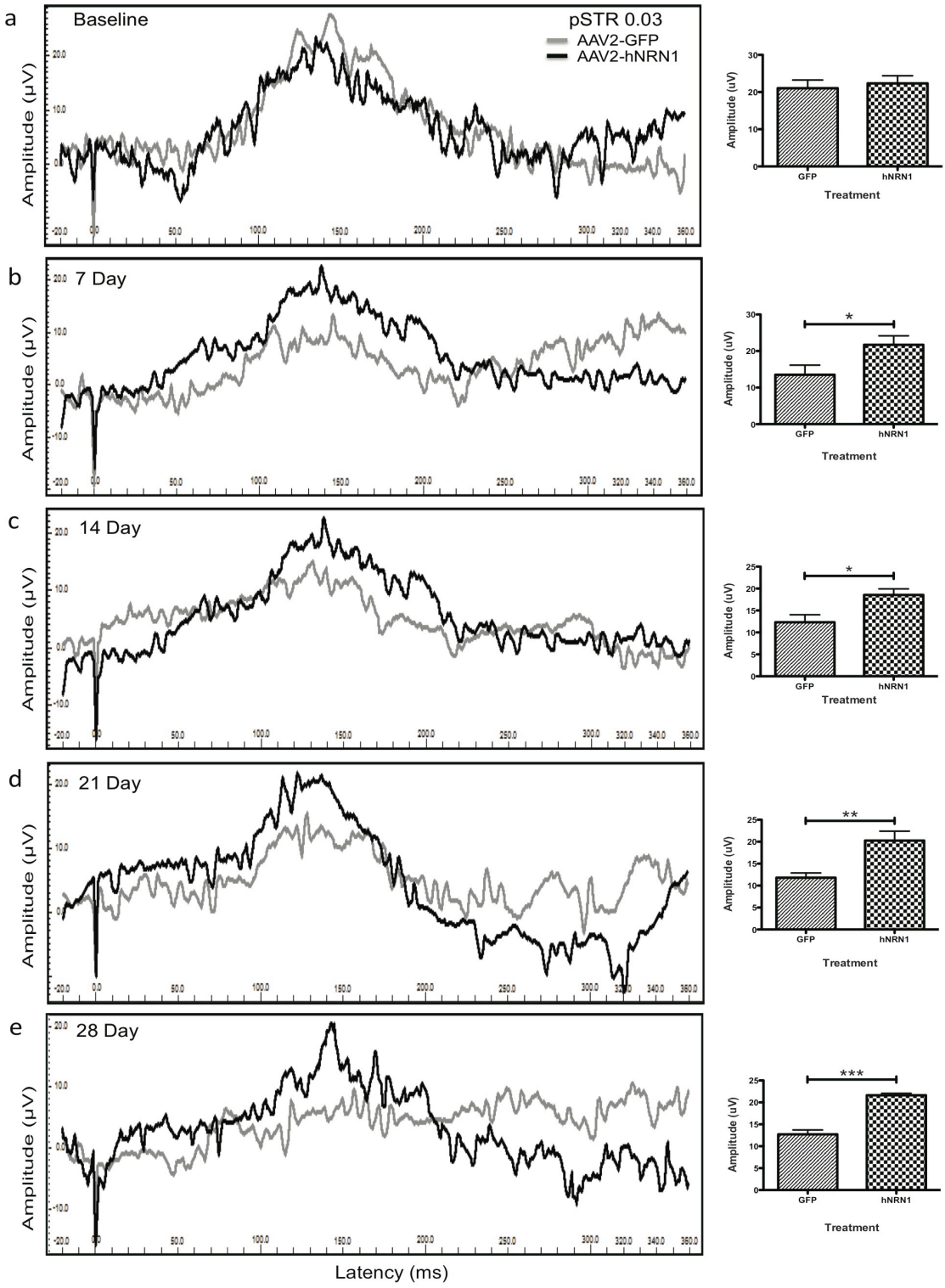




Supplemental Figure S3



Supplemental Figure S4



Supplemental Table S1

Supplemental Table S1 – Antibodies and recombinant protein table

Primary Antibody	Company	Catalog #	Species	Dilution	Marker
BRN3A	Chemicon (Millipore)	MAB1585	Mouse	1:50	RGCs
NEFL	Cell Signaling	C28E10	Rabbit	1:100	Neurites
RBPMS	GeneTex	GTX 118619	Rabbit	1:200	RGCs
HPC1	Sigma	m4858	Mouse	1:100	Amacrine, IPL
GS	Abcam	ab73593	Rabbit	1:1000	Astrocytes, Muller
GFAP	Novus	NB300-141	Rabbit	1:500	Astrocytes
IBA-1	Wako	019-19741	Rabbit	1:150	Microglia
PKCα	Abcam	ab11723	Mouse	1:100	Bipolar Cells
RHO	Inhouse	1D-4	Mouse	1:1000	Photoreceptors - Rods
α-SMA	Sigma	1A4	Mouse	1:50	Fibroblasts
NRN1	Abcam	ab64186	Rabbit	1:200	Neuritogenesis
GAP43	Cell Signaling	D9C8	Rabbit	1:200	Growth-associated protein

Secondary Antibody	Company	Catalog #	Dilution
Goat anti-mouse AF568	Alexa Fluor	A21043	1:500
Donkey anti rabbit AF568	Alexa Fluor	A10042	1:500
Goat anti-mouse AF488	Alexa Fluor	A11029	1:500
Goat anti-rabbit AF488	Alexa Fluor	A11008	1:500
Goat anti-mouse TRITC	Life Technologies	T2762	1:500

Recombinant	Company	Catalog #	Final Concentration
hNRN1	Sino Biologicals	12722-H08B	200ng/ml

Conflict of Interest

The authors declare that they have no competing interests

Supplemental Information

Supplemental information is available at Cell Death and Disease's website'

Acknowledgements

The authors would like to thank Dr. Robert Nickells (University of Wisconsin) for showing us his ONC technique. This work was supported by a Department of Defense grant (W81XWH-10-2-0003).

References

1. Schwartz M. Optic nerve crush: protection and regeneration. *Brain research bulletin* 2004 Feb 15; **62**(6)**:** 467-471.

2. Ohlsson M, Mattsson P, Svensson M. A temporal study of axonal degeneration and glial scar formation following a standardized crush injury of the optic nerve in the adult rat. *Restorative neurology and neuroscience* 2004; **22**(1)**:** 1-10.

3. Magharious M, D'Onofrio PM, Hollander A, Zhu P, Chen J, Koeberle PD. Quantitative iTRAQ analysis of retinal ganglion cell degeneration after optic nerve crush. *Journal of proteome research* 2011 Aug 5; **10**(8)**:** 3344-3362.

4. Wohlfart G. Degeneration and regeneration in the nervous system. Recent advances. *World neurology* 1961 Mar; **2:** 187-198.

5. Windle WF. Regeneration of axons in the vertebrate central nervous system. *Physiological reviews* 1956 Oct; **36**(4)**:** 427-440.

6. Benowitz LI, Yin Y. Combinatorial treatments for promoting axon regeneration in the CNS: strategies for overcoming inhibitory signals and activating neurons' intrinsic growth state. *Developmental neurobiology* 2007 Aug; **67**(9)**:** 1148-1165.

7. Silver J, Miller JH. Regeneration beyond the glial scar. *Nature reviews Neuroscience* 2004 Feb; **5**(2)**:** 146-156.

8. Lawson LJ, Frost L, Risbridger J, Fearn S, Perry VH. Quantification of the mononuclear phagocyte response to Wallerian degeneration of the optic nerve. *Journal of neurocytology* 1994 Dec; **23**(12)**:** 729-744.

9. Koeberle PD, Bahr M. Growth and guidance cues for regenerating axons: where have they gone? *Journal of neurobiology* 2004 Apr; **59**(1)**:** 162-180.

10. Kermer P, Klocker N, Bahr M. Neuronal death after brain injury. Models, mechanisms, and therapeutic strategies in vivo. *Cell and tissue research* 1999 Dec; **298**(3)**:** 383-395.

11. Koeberle PD, Gauldie J, Ball AK. Effects of adenoviral-mediated gene transfer of interleukin-10, interleukin-4, and transforming growth factor-beta on the survival of axotomized retinal ganglion cells. *Neuroscience* 2004; **125**(4)**:** 903-920.

12. Kipnis J, Yoles E, Schori H, Hauben E, Shaked I, Schwartz M. Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2001 Jul 1; **21**(13)**:** 4564-4571.

13. Isenmann S, Wahl C, Krajewski S, Reed JC, Bahr M. Up-regulation of Bax protein in degenerating retinal ganglion cells precedes apoptotic cell death after optic nerve lesion in the rat. *The European journal of neuroscience* 1997 Aug; **9**(8)**:** 1763-1772.

14. Kermer P, Ankerhold R, Klocker N, Krajewski S, Reed JC, Bahr M. Caspase-9: involvement in secondary death of axotomized rat retinal ganglion cells in vivo. *Brain research Molecular brain research* 2000 Dec 28; **85**(1-2)**:** 144-150.

15. Kermer P, Klocker N, Labes M, Bahr M. Inhibition of CPP32-like proteases rescues axotomized retinal ganglion cells from secondary cell death in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1998 Jun 15; **18**(12)**:** 4656-4662.

16. Kikuchi M, Tenneti L, Lipton SA. Role of p38 mitogen-activated protein kinase in axotomy-induced apoptosis of rat retinal ganglion cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2000 Jul 1; **20**(13)**:** 5037-5044.

17. Benowitz L, Yin Y. Rewiring the injured CNS: lessons from the optic nerve. *Experimental neurology* 2008 Feb; **209**(2)**:** 389-398.

18. Aldskogius H, Kozlova EN. Central neuron-glial and glial-glial interactions following axon injury. *Progress in neurobiology* 1998 May; **55**(1)**:** 1-26.

19. Engelmann R, Dieterich DC, Bien A, Kreutz MR. A different retinal glia response to optic nerve injury/lipopolysaccharide administration in hooded and albino rats. *Brain research* 2001 Jan 19; **889**(1-2)**:** 251-255.

20. Monnier PP, D'Onofrio PM, Magharious M, Hollander AC, Tassew N, Szydlowska K*, et al.* Involvement of caspase-6 and caspase-8 in neuronal apoptosis and the regenerative failure of injured retinal ganglion cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011 Jul 20; **31**(29)**:** 10494-10505.

21. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Investigative ophthalmology & visual science* 1995 Apr; **36**(5)**:** 774-786.

22. Aubert I, Ridet JL, Gage FH. Regeneration in the adult mammalian CNS: guided by development. *Current opinion in neurobiology* 1995 Oct; **5**(5)**:** 625-635.

23. Nicholls J, Saunders N. Regeneration of immature mammalian spinal cord after injury. *Trends in neurosciences* 1996 Jun; **19**(6)**:** 229-234.

24. Di Giovanni S, Faden AI, Yakovlev A, Duke-Cohan JS, Finn T, Thouin M*, et al.* Neuronal plasticity after spinal cord injury: identification of a gene cluster driving neurite outgrowth. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2005 Jan; **19**(1)**:** 153-154.

25. Jo SA, Wang E, Benowitz LI. Ciliary neurotrophic factor is an axogenesis factor for retinal ganglion cells. *Neuroscience* 1999 Mar; **89**(2)**:** 579-591.

26. Lorber B, Berry M, Logan A. Different factors promote axonal regeneration of adult rat retinal ganglion cells after lens injury and intravitreal peripheral nerve grafting. *Journal of neuroscience research* 2008 Mar; **86**(4)**:** 894-903.

27. Hu Y, Leaver SG, Plant GW, Hendriks WT, Niclou SP, Verhaagen J*, et al.* Lentiviral-mediated transfer of CNTF to schwann cells within reconstructed peripheral nerve grafts enhances adult retinal ganglion cell survival and axonal regeneration. *Molecular therapy : the journal of the American Society of Gene Therapy* 2005 Jun; **11**(6)**:** 906-915.

28. Leaver SG, Cui Q, Bernard O, Harvey AR. Cooperative effects of bcl-2 and AAV-mediated expression of CNTF on retinal ganglion cell survival and axonal regeneration in adult transgenic mice. *The European journal of neuroscience* 2006 Dec; **24**(12)**:** 3323-3332.

29. Harvey AR, Hellstrom M, Rodger J. Gene therapy and transplantation in the retinofugal pathway. *Progress in brain research* 2009; **175:** 151-161.

30. Leaver SG, Cui Q, Plant GW, Arulpragasam A, Hisheh S, Verhaagen J*, et al.* AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. *Gene therapy* 2006 Sep; **13**(18)**:** 1328-1341.

31. Nedivi E, Hevroni D, Naot D, Israeli D, Citri Y. Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature* 1993 Jun 24; **363**(6431)**:** 718-722.

32. Nedivi E, Wu GY, Cline HT. Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. *Science* 1998 Sep 18; **281**(5384)**:** 1863-1866.

33. Naeve GS, Ramakrishnan M, Kramer R, Hevroni D, Citri Y, Theill LE. Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 1997 Mar 18; **94**(6)**:** 2648-2653.

34. Fujino T, Wu Z, Lin WC, Phillips MA, Nedivi E. cpg15 and cpg15-2 constitute a family of activity-regulated ligands expressed differentially in the nervous system to promote neurite growth and neuronal survival. *The Journal of comparative neurology* 2008 Apr 10; **507**(5)**:** 1831-1845.

35. Cantallops I, Haas K, Cline HT. Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration in vivo. *Nature neuroscience* 2000 Oct; **3**(10)**:** 1004-1011.

36. Putz U, Harwell C, Nedivi E. Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis. *Nature neuroscience* 2005 Mar; **8**(3)**:** 322-331.

37. Javaherian A, Cline HT. Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron* 2005 Feb 17; **45**(4)**:** 505-512.

38. Lee WC, Nedivi E. Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of cpg15-like genes. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2002 Mar 1; **22**(5)**:** 1807-1815.

39. Nedivi E, Fieldust S, Theill LE, Hevron D. A set of genes expressed in response to light in the adult cerebral cortex and regulated during development. *Proceedings of the National Academy of Sciences of the United States of America* 1996 Mar 5; **93**(5)**:** 2048-2053.

40. Haustead DJ, Lukehurst SS, Clutton GT, Bartlett CA, Dunlop SA, Arrese CA*, et al.* Functional topography and integration of the contralateral and ipsilateral retinocollicular projections of ephrin-A-/- mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2008 Jul 16; **28**(29)**:** 7376-7386.

41. Blaugrund E, Lavie V, Cohen I, Solomon A, Schreyer DJ, Schwartz M. Axonal regeneration is associated with glial migration: comparison between the injured optic nerves of fish and rats. *The Journal of comparative neurology* 1993 Apr 1; **330**(1)**:** 105-112.

42. Doster SK, Lozano AM, Aguayo AJ, Willard MB. Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. *Neuron* 1991 Apr; **6**(4)**:** 635-647.

43. Leon S, Yin Y, Nguyen J, Irwin N, Benowitz LI. Lens injury stimulates axon regeneration in the mature rat optic nerve. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2000 Jun 15; **20**(12)**:** 4615-4626.

44. Udvadia AJ, Koster RW, Skene JH. GAP-43 promoter elements in transgenic zebrafish reveal a difference in signals for axon growth during CNS development and regeneration. *Development* 2001 Apr; **128**(7)**:** 1175-1182.

45. McCurley AT, Callard GV. Time Course Analysis of Gene Expression Patterns in Zebrafish Eye During Optic Nerve Regeneration. *Journal of experimental neuroscience* 2010 Jul 13; **2010**(4)**:** 17-33.

46. Chidlow G, Ebneter A, Wood JP, Casson RJ. The optic nerve head is the site of axonal transport disruption, axonal cytoskeleton damage and putative axonal regeneration failure in a rat model of glaucoma. *Acta neuropathologica* 2011 Jun; **121**(6)**:** 737-751.

47. Yao JJ, Gao XF, Chow CW, Zhan XQ, Hu CL, Mei YA. Neuritin activates insulin receptor pathway to up-regulate Kv4.2-mediated transient outward K+ current in rat cerebellar granule neurons. *The Journal of biological chemistry* 2012 Nov 30; **287**(49)**:** 41534-41545.

48. Cappelletti G, Galbiati M, Ronchi C, Maggioni MG, Onesto E, Poletti A. Neuritin (cpg15) enhances the differentiating effect of NGF on neuronal PC12 cells. *Journal of neuroscience research* 2007 Sep; **85**(12)**:** 2702-2713.

49. Karamoysoyli E, Burnand RC, Tomlinson DR, Gardiner NJ. Neuritin mediates nerve growth factor-induced axonal regeneration and is deficient in experimental diabetic neuropathy. *Diabetes* 2008 Jan; **57**(1)**:** 181-189.

50. Badea TC, Nathans J. Morphologies of mouse retinal ganglion cells expressing transcription factors Brn3a, Brn3b, and Brn3c: analysis of wild type and mutant cells using genetically-directed sparse labeling. *Vision research* 2011 Jan 28; **51**(2)**:** 269-279.

51. Kwong JM, Caprioli J, Piri N. RNA binding protein with multiple splicing: a new marker for retinal ganglion cells. *Investigative ophthalmology & visual science* 2010 Feb; **51**(2)**:** 1052-1058.

52. Ivanov D, Dvoriantchikova G, Nathanson L, McKinnon SJ, Shestopalov VI. Microarray analysis of gene expression in adult retinal ganglion cells. *FEBS letters* 2006 Jan 9; **580**(1)**:** 331-335.

53. Merianda TT, Gomes C, Yoo S, Vuppalanchi D, Twiss JL. Axonal localization of neuritin/CPG15 mRNA in neuronal populations through distinct 5' and 3' UTR elements. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2013 Aug 21; **33**(34)**:** 13735-13742.

54. Xi S, Liu W, Zhang H, Gao R, Huang J. [Effect of Neuritin protein on axonal regeneration after acute spinal cord injury in rats]. *Zhongguo xiu fu chong jian wai ke za*

zhi = Zhongguo xiufu chongjian waike zazhi = Chinese journal of reparative and reconstructive surgery 2009 Oct; **23**(10)**:** 1219-1223.

55. Fujino T, Leslie JH, Eavri R, Chen JL, Lin WC, Flanders GH*, et al.* CPG15 regulates synapse stability in the developing and adult brain. *Genes & development* 2011 Dec 15; **25**(24)**:** 2674-2685.

56. Ju WK, Kim KY, Duong-Polk KX, Lindsey JD, Ellisman MH, Weinreb RN. Increased optic atrophy type 1 expression protects retinal ganglion cells in a mouse model of glaucoma. *Molecular vision* 2010; **16:** 1331-1342.

57. Reh TA, Tetzlaff W, Ertlmaier A, Zwiers H. Developmental study of the expression of B50/GAP-43 in rat retina. *Journal of neurobiology* 1993 Jul; **24**(7)**:** 949-958.

58. Rousseau V, Sabel BA. Restoration of vision IV: role of compensatory soma swelling of surviving retinal ganglion cells in recovery of vision after optic nerve crush. *Restorative neurology and neuroscience* 2001; **18**(4)**:** 177-189.

59. Pang IH, Zeng H, Fleenor DL, Clark AF. Pigment epithelium-derived factor protects retinal ganglion cells. *BMC neuroscience* 2007; **8:** 11.

60. Li Y, Schlamp CL, Nickells RW. Experimental induction of retinal ganglion cell death in adult mice. *Investigative ophthalmology & visual science* 1999 Apr; **40**(5)**:** 1004-1008.

CHAPTER IV

CONCLUSION

CNS trauma causes axonopathy and degeneration of neurons, which is evident in neurodegenerative diseases such as Parkinson's, Alzheimer's, and glaucoma. Victims of CNS injury or certain neurodegenerative diseases are unable to fully overcome sensory, motor, cognitive, and/or autonomic functions (Benowitz and Yin). These destructive insults are triggered by a cascade of intrinsic and extrinsic events resulting in damage to neurons and subsequent regenerative failure (Schwartz; Ohlsson, Mattsson and Svensson; Magharious et al.; Wohlfart; Windle). The intrinsic factors include deregulation in growth-promoting factors, apoptotic factors, intracellular signaling molecules and trophic factors, while the extrinsic factors correlate to growth inhibition by myelin, myelin associated inhibitors, glial scarring and development of neuronal projections (Benowitz and Yin).

Our study focused on both events simultaneously as we observed gene expression changes within the whole retina and ON. The detrimental retinal gene expression changes that we observed were not restricted to the RGCs, but also included other retinal cells such as astrocytes, microglia, and vascular cells that interact with the RGC somas. After ONC, these cells also initiate degenerative pathways and exude extrinsic molecular factors causing RGC apoptosis (Kielczewski, Pease and Quigley; Kirsch et al.). The initial trauma of axotomy causes degenerative pathways to ensue and during this phase of

degeneration, it is crucial for cells to overcome cell death mechanisms and potentiate neurite extensions for survivability and accurate neuronal targeting. Further, the ON includes RGC axons, astrocytes, microglia, and oligodendrocytes that interact with each other (Qu and Jakobs). The expression of genes observed within the ON may represent the beneficial or detrimental effects of neighboring cells surrounding the RGC axons. Glial responses around the affected area are initiated by injured CNS axons (Aldskogius and Kozlova). After injury, microglia proliferation occurs. Axons undergoing Wallerian degeneration are surrounded by astrocytes that up-regulate *Gfap* expression contributing to trauma-induced neurodegeneration (Engelmann et al.). Glial scarring inhibits axonal transport after ONC (Silver and Miller; Windle) decreasing transport of proteins involved in neuroprotection and synaptic plasticity. This causes deleterious affects, eventually leading to decreased synaptic plasticity and transmission at distal ends (Figure 1).

Using the reproducible ONC model of CNS trauma, we were able to effectively examine the crucial intrinsic factors that get down regulated after axonal trauma and how this then distresses the soma of the RGCs. In addition, other pathways were also highlighted that could be regulating the extrinsic factors that lead to RGC apoptosis. In brief, we were able to: (a) simultaneously examine gene expression changes within the retina and ON, and (b) visualize protein expression patterns of key selected genes associated with neuron loss and regenerative failure within the retina and ON after ONC. Furthermore, we were able to test the over-expression effects of one target gene in RGCs for neuroprotection and regenerative efficacy after axonal trauma.

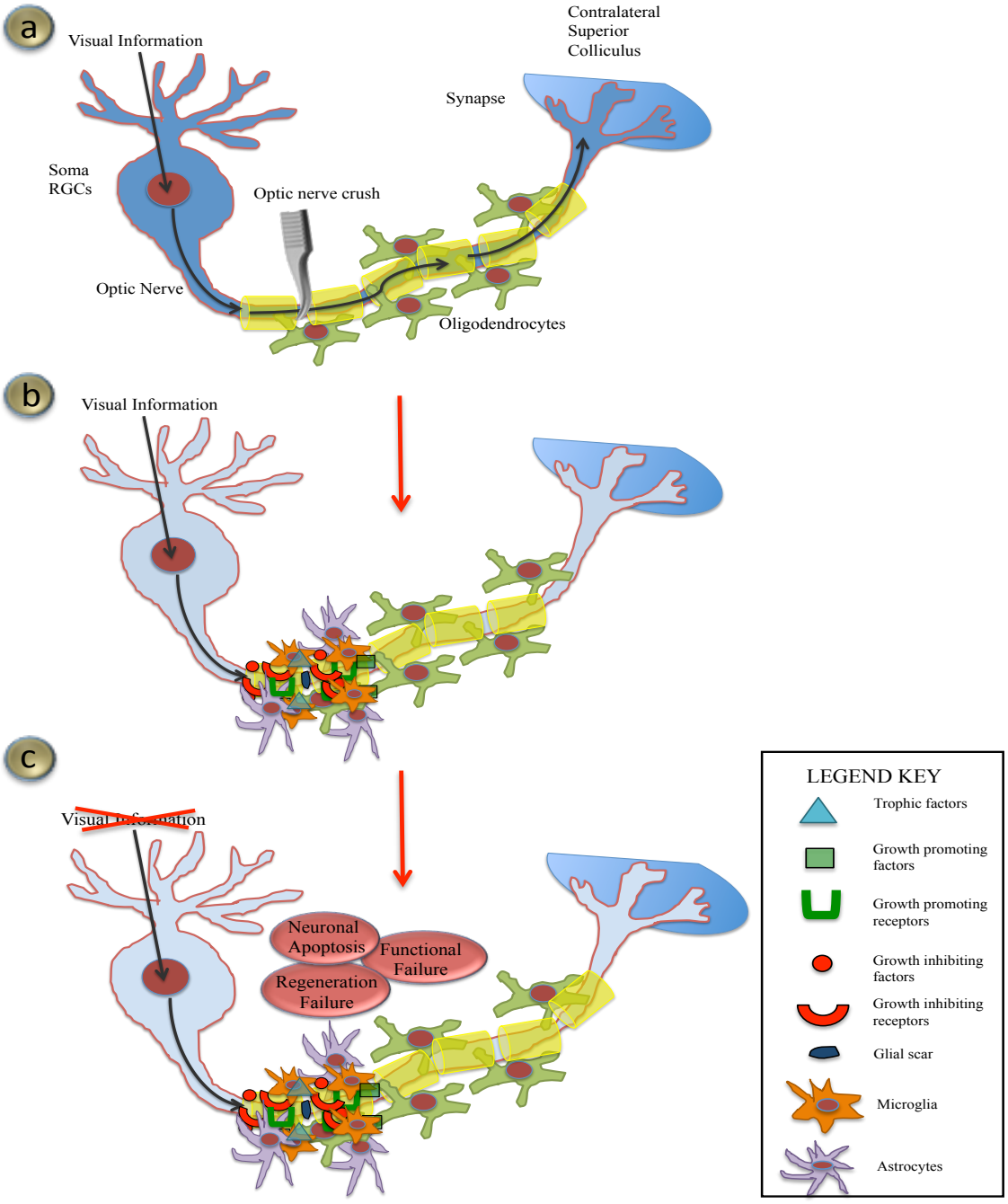


Figure 1: Optic nerve crush causes axonopathy and degeneration of neurons. Representation of (a) axonal injury to RGC, (b) cellular events after axonal injury and (c) culmination of events that result in neurodegeneration and regenerative failure.

Bioinformatic meta-analysis of our data identified significant tissue-specific and time-dependent gene clusters associated with regenerative changes, synaptic plasticity, axonogenesis, neuron projection, and neurodegeneration. We identified a number of detrimental gene expression changes contributing to trauma-induced neurodegeneration. *Nrn1* (synaptic plasticity gene), *Synpr* and *Syt1 (*synaptic vesicle fusion genes), and *Vsnl1* (neuron differentiation associated gene) were a few of the potentially unique genes identified that were down regulated spatially and temporally in our rodent ONC model.

Vsnl1, *Syt1* and *Synpr* all have calcium-sensing components and are associated with synaptic vesicles. Synaptic vesicles are localized to small vesicles within the neuron, particularly in neuronal axonal processes (Morfini et al.). A collection of signaling mechanisms link both axonal tips and dendritic terminals to neuronal soma by motor-dependent cytoskeletal transport machineries. Essential membrane components of synaptic vesicles and synaptic transmission are translated in the soma and transported to the growing distal ends of extending neurites after crush injury (Li and Dahlstrom; Kwon, Kim and Chang). All three genes either have an important role in cellular signal transduction for neuroprotection/neurotoxicity (Bernstein et al.; Braunewell and Klein-Szanto) or participate in axonal regeneration, including synaptic projection and proper axonal targeting (Vennekate et al.; Greif et al.). Their critical role in synaptic transduction and down regulation of these genes after trauma, make them candidates for potential gene therapies that can be over-expressed to provide plausible beneficial treatment options in the future.

Nrn1 is a vital player in neuritogenesis and neuroplasticity. The gene has shown a biphasic recovery pattern in the rodent spinal cord injury model (Di Giovanni et al.)

paralleling the recovery pattern observed in our axonal trauma model. Within our model, the expression of *Nrn1* was similar to other neuronal associated axonogenesis and axon architecture related genes: *Nefl*, *Nefm* and *Sncg*. Expression of all these genes mimicked the progressive loss of RGCs we observed after ONC. During the time course post crush, we observed a biphasic pattern of gene expression correlating very closely to the primary and secondary phase of RGC death. RGC degeneration has a two-phase longitudinal profile after ONC (Levkovitch-Verbin et al.). It has been proposed that a second phase of RGC death occurs after the primary insult to the ON (Yoles and Schwartz; Leung et al.). Once primary axonal injury occurs, a successive secondary trauma causes excitotoxicity, free radical production, ischemia, inflammation, and apoptosis (Truettner et al.; Ohlsson et al.; Song et al.). The biphasic pattern of expression after axonal trauma indicates a transient attempt at neuroprotection and regeneration by RGCs during the primary and secondary trauma response.

Furthermore, *Nrn1* coupled with neurotrophic effects is essential in CNS trauma. Various neurotrophic factors such as BDNF, GDNF, NT-4/5, CNTF and NGF exert short-lived neuroprotective effects both *in vivo* and *in vitro*. These molecules bind to two receptor types: tyrosine kinase receptors (Trk-A, -B and -C); and common neurotrophin receptors ($p75^{NTR}$) (Lykissas et al.). NGF, BDNF and NT-3 selectively bind to Trk receptors inducing a multitude of downstream signaling cascades by the activation of MAPK, PI3K and PKC (Hackam) (Kalb). These signaling pathways activate ERK1/2/MAPK and CREB, which enhance neuroprotection, facilitate branch formation, and induce neurite outgrowth after injury (Kalb; Markus, Patel and Snider; Prokosch et al.; Edstrom and

Ekstrom). NGF, BDNF and NT-3 can all potentiate the expression of *Nrn1* (Naeve et al.; Nedivi et al.) (Figure 2).

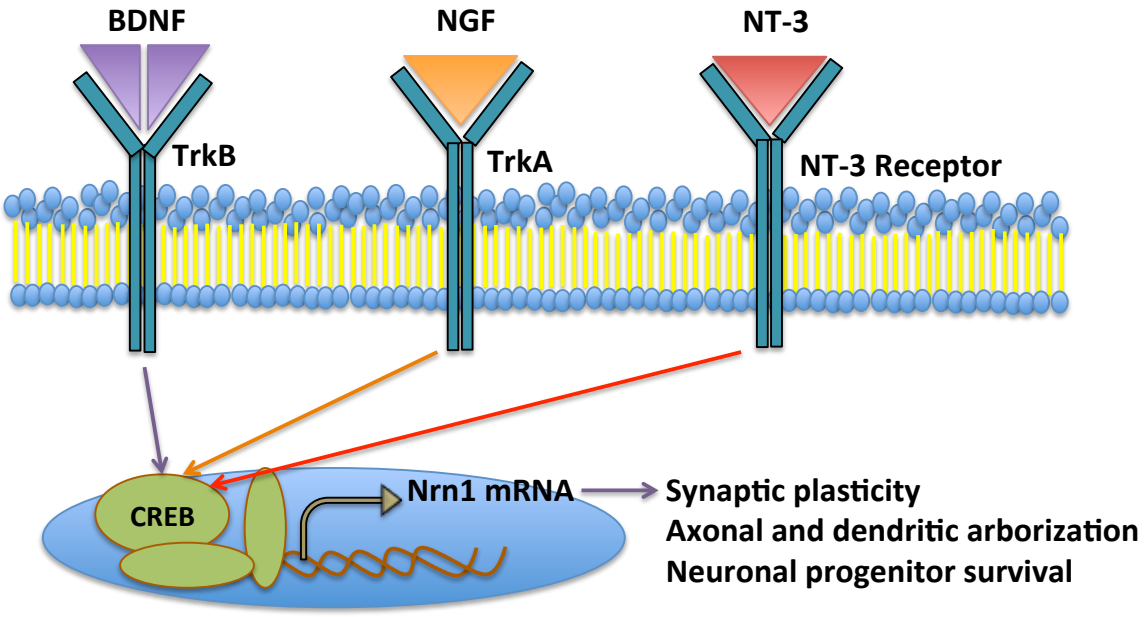


Figure 2: Schematic for neuritin induction. NGF, BDNF and NT-3 can all potentiate the expression of *Nrn1*

The neuroprotective and regenerative effects of these neurotrophic factors may be mediated by *Nrn1* and proved to be a crucial target gene in CNS axonal regeneration. Functionally, Nrn1 acts as a ligand to the insulin receptor (Yao et al.), and cleavage of the GPI anchor by phospholipase C allows the soluble form to be secreted and activate downstream pathways (Nedivi, Wu and Cline). The neurotrophin, NGF can induce the transcription and translation of *Nrn1* which increases neurite outgrowth in cultured rat embryonic hippocampal and cortical neurons (Naeve et al.), motor neurons of *Xenopus* (Javaherian and Cline), and PC 12 cells (Cappelletti et al.). This increased neurite outgrowth occurs via mitogen-activated protein kinase or phosphatidylinositol-3 kinase activation (Karamoysoyli et al.) (Figure 3).

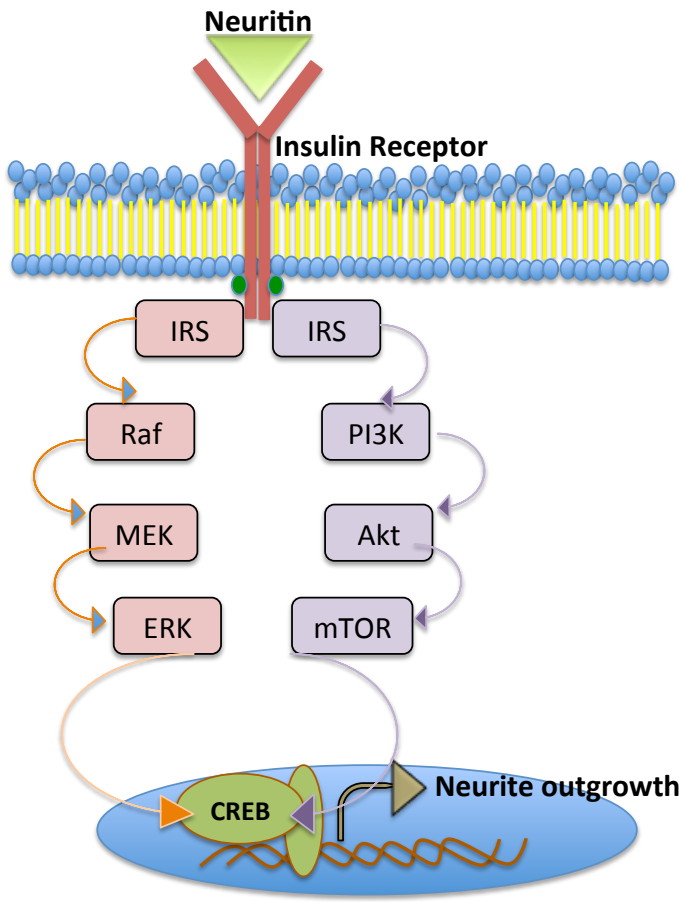


Figure 3: Nrn1 signaling pathway. Nrn1 binds to the insulin receptor and activates both ERK and PI3K signaling.

The ability of Nrn1 to promote axonal arborization and synaptic plasticity makes it a unique therapeutic target to address neuronal apoptosis and regenerative failure after CNS trauma. We designed an AAV2 based hNRN1 therapy to target RGCs and observed the effects of NRN1 over-expression in the axotomized RGCs. Our studies indicated that NRN1 exhibited neuroprotective, regenerative effects and sustained RGC function on axotomized RGCs *in vitro* and after axonal injury *in vivo* (Figure 4). These data imply that neuritin may play important roles in neuronal differentiation and survival as well as in neurite outgrowth and axonal regeneration

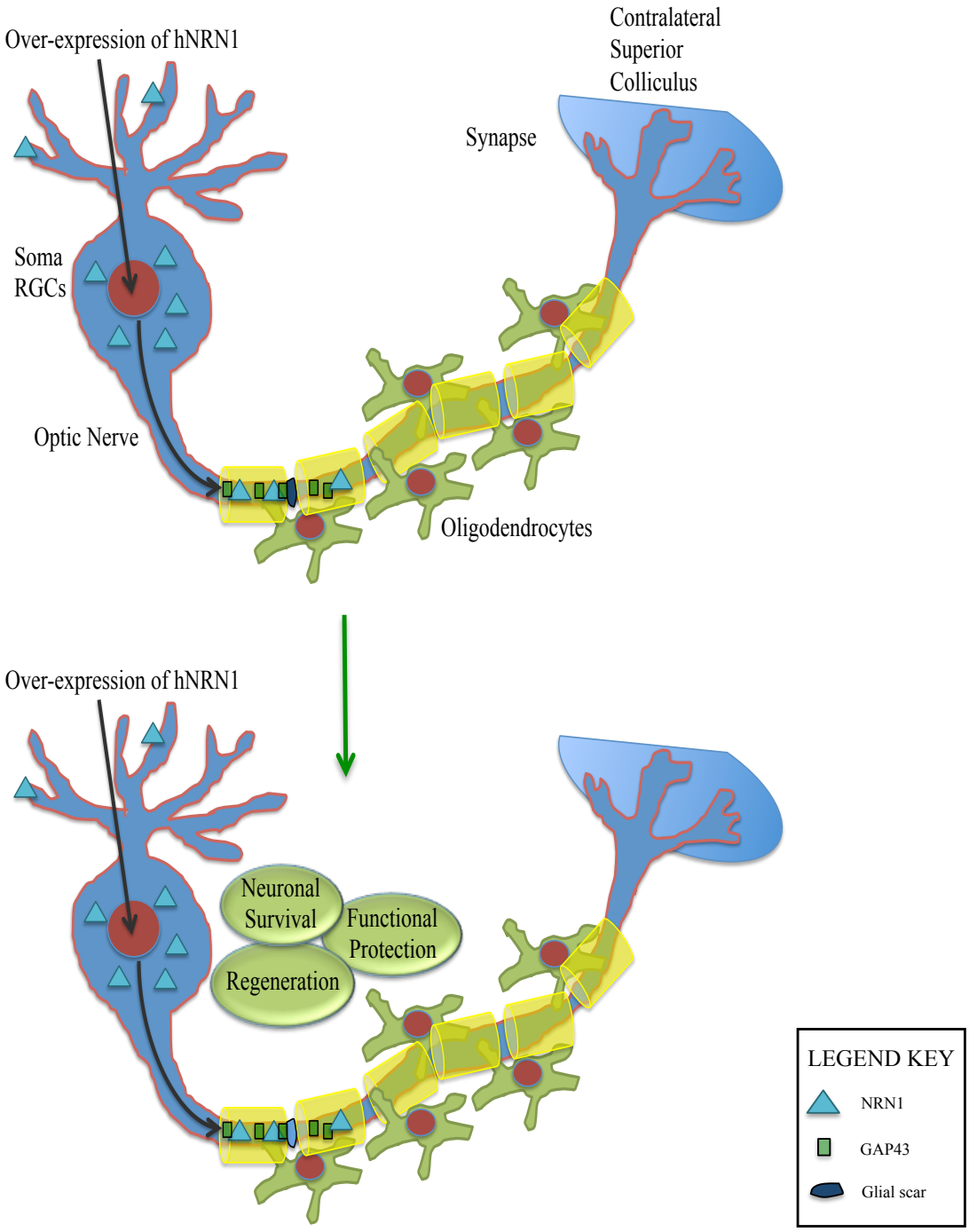


Figure 4: AAV2-hNRN1 delivery to RGCs exhibited beneficial effects, including neuroprotection, regeneration and sustained functional response of RGCs. Gap43 mediates active RGC regenerative state past lesion site

In conclusion, we identified potential pathogenic genes and possible new therapeutic targets to address two key mechanisms of CNS trauma: neuronal loss and regenerative failure. Our study presented potential target genes and allowed the development of a unique neuroprotective and regenerative gene therapy strategy to prevent RGC degeneration. The differences in gene expression between different injury models and between injury to the brain parenchyma and the optic nerve are noteworthy. Although there are gene expression differences between the retina and brain, similar differences also occur within discrete regions of the brain as each part of the brain has different motoric, sensory, and cognitive functions. For example, gene expression in the cerebellum differs the most from the other regions of the brain (Khaitovich, Muetzel, et al.; Khaitovich, Weiss, et al.) (Sandberg et al.). In addition, inter-individual differences have also been reported within a species (Khaitovich, Weiss, et al.). As is the case while studying any trauma or disease model, only a generic evaluation can be made in terms of relevance to other regions in the CNS. However, our study reports significant comparison to CNS associated degeneration because the retina and ON are in essence a segment of the CNS. The novel findings of our study show the critical gene expression changes occurring after axonal injury and present conceivable therapies that can be extrapolated to other forms of CNS trauma and neurodegenerative diseases.

References

Aldskogius, H., and E. N. Kozlova. "Central Neuron-Glial and Glial-Glial Interactions Following Axon Injury." Progress in neurobiology 55.1 (1998): 1-26.

Benowitz, L. I., and Y. Yin. "Combinatorial Treatments for Promoting Axon Regeneration in the Cns: Strategies for Overcoming Inhibitory Signals and Activating Neurons' Intrinsic Growth State." Dev Neurobiol 67.9 (2007): 1148-65.

Bernstein, H. G., et al. "Regional and Cellular Distribution of Neural Visinin-Like Protein Immunoreactivities (Vilip-1 and Vilip-3) in Human Brain." Journal of neurocytology 28.8 (1999): 655-62.

Braunewell, K. H., and A. J. Klein-Szanto. "Visinin-Like Proteins (Vsnls): Interaction Partners and Emerging Functions in Signal Transduction of a Subfamily of Neuronal Ca2+ -Sensor Proteins." Cell and tissue research 335.2 (2009): 301-16.

Cappelletti, G., et al. "Neuritin (Cpg15) Enhances the Differentiating Effect of Ngf on Neuronal Pc12 Cells." Journal of neuroscience research 85.12 (2007): 2702-13.

Di Giovanni, S., et al. "Neuronal Plasticity after Spinal Cord Injury: Identification of a Gene Cluster Driving Neurite Outgrowth." FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19.1 (2005): 153-4.

Edstrom, A., and P. A. Ekstrom. "Role of Phosphatidylinositol 3-Kinase in Neuronal Survival and Axonal Outgrowth of Adult Mouse Dorsal Root Ganglia Explants." J Neurosci Res 74.5 (2003): 726-35.

Engelmann, R., et al. "A Different Retinal Glia Response to Optic Nerve Injury/Lipopolysaccharide Administration in Hooded and Albino Rats." Brain research 889.1-2 (2001): 251-5.

Greif, K. F., et al. "Synaptotagmin-1 Promotes the Formation of Axonal Filopodia and Branches Along the Developing Axons of Forebrain Neurons." Dev Neurobiol 73.1 (2013): 27-44.

Hackam, A. S. "Regulation of Neurotrophin Expression and Activity in the Retina." Adv Exp Med Biol 613 (2008): 343-9.

Javaherian, A., and H. T. Cline. "Coordinated Motor Neuron Axon Growth and Neuromuscular Synaptogenesis Are Promoted by Cpg15 in Vivo." Neuron 45.4 (2005): 505-12.

Kalb, R. "The Protean Actions of Neurotrophins and Their Receptors on the Life and Death of Neurons." Trends Neurosci 28.1 (2005): 5-11.

Karamoysoyli, E., et al. "Neuritin Mediates Nerve Growth Factor-Induced Axonal Regeneration and Is Deficient in Experimental Diabetic Neuropathy." Diabetes 57.1 (2008): 181-9.

Khaitovich, P., et al. "Regional Patterns of Gene Expression in Human and Chimpanzee Brains." Genome Res 14.8 (2004): 1462-73.

Khaitovich, P., et al. "A Neutral Model of Transcriptome Evolution." PLoS Biol 2.5 (2004): E132.

Kielczewski, J. L., M. E. Pease, and H. A. Quigley. "The Effect of Experimental Glaucoma and Optic Nerve Transection on Amacrine Cells in the Rat Retina." Invest Ophthalmol Vis Sci 46.9 (2005): 3188-96.

Kirsch, M., et al. "Involvement of Gp130-Associated Cytokine Signaling in Muller Cell Activation Following Optic Nerve Lesion." Glia 58.7 (2010): 768-79.

Kwon, K. B., J. S. Kim, and B. J. Chang. "Translocational Changes of Localization of Synapsin in Axonal Sprouts of Regenerating Rat Sciatic Nerves after Ligation Crush Injury." J Vet Sci 1.1 (2000): 1-9.
Leung, C. K., et al. "Longitudinal Profile of Retinal Ganglion Cell Damage after Optic Nerve Crush with Blue-Light Confocal Scanning Laser Ophthalmoscopy." Investigative ophthalmology & visual science 49.11 (2008): 4898-902.
Levkovitch-Verbin, H., et al. "Optic Nerve Transection in Monkeys May Result in Secondary Degeneration of Retinal Ganglion Cells." Invest Ophthalmol Vis Sci 42.5 (2001): 975-82.
Li, J. Y., and A. Dahlstrom. "Axonal Transport of Synaptic Vesicle Proteins in the Rat Optic Nerve." J Neurobiol 32.2 (1997): 237-50.
Lykissas, M. G., et al. "The Role of Neurotrophins in Axonal Growth, Guidance, and Regeneration." Curr Neurovasc Res 4.2 (2007): 143-51.
Magharious, M., et al. "Quantitative Itraq Analysis of Retinal Ganglion Cell Degeneration after Optic Nerve Crush." Journal of proteome research 10.8 (2011): 3344-62.
Markus, A., T. D. Patel, and W. D. Snider. "Neurotrophic Factors and Axonal Growth." Curr Opin Neurobiol 12.5 (2002): 523-31.
Morfini, G., et al. "Glycogen Synthase Kinase 3 Phosphorylates Kinesin Light Chains and Negatively Regulates Kinesin-Based Motility." EMBO J 21.3 (2002): 281-93.
Naeve, G. S., et al. "Neuritin: A Gene Induced by Neural Activity and Neurotrophins That Promotes Neuritogenesis." Proceedings of the National Academy of Sciences of the United States of America 94.6 (1997): 2648-53.
Nedivi, E., et al. "A Set of Genes Expressed in Response to Light in the Adult Cerebral Cortex and Regulated During Development." Proceedings of the National Academy of Sciences of the United States of America 93.5 (1996): 2048-53.
Nedivi, E., G. Y. Wu, and H. T. Cline. "Promotion of Dendritic Growth by Cpg15, an Activity-Induced Signaling Molecule." Science 281.5384 (1998): 1863-6.
Ohlsson, M., et al. "Complement Activation Following Optic Nerve Crush in the Adult Rat." Journal of neurotrauma 20.9 (2003): 895-904.
Ohlsson, M., P. Mattsson, and M. Svensson. "A Temporal Study of Axonal Degeneration and Glial Scar Formation Following a Standardized Crush Injury of the Optic Nerve in the Adult Rat." Restorative neurology and neuroscience 22.1 (2004): 1-10.
Prokosch, V., et al. "Deciphering Proteins and Their Functions in the Regenerating Retina." Expert Rev Proteomics 7.5 (2010): 775-95.
Qu, J., and T. C. Jakobs. "The Time Course of Gene Expression During Reactive Gliosis in the Optic Nerve." PLoS One 8.6 (2013): e67094.
Sandberg, R., et al. "Regional and Strain-Specific Gene Expression Mapping in the Adult Mouse Brain." Proc Natl Acad Sci U S A 97.20 (2000): 11038-43.
Schwartz, M. "Optic Nerve Crush: Protection and Regeneration." Brain research bulletin 62.6 (2004): 467-71.
Silver, J., and J. H. Miller. "Regeneration Beyond the Glial Scar." Nature reviews. Neuroscience 5.2 (2004): 146-56.

Song, K. J., et al. "The Relationship between Spinal Stenosis and Neurological Outcome in Traumatic Cervical Spine Injury: An Analysis Using Pavlov's Ratio, Spinal Cord Area, and Spinal Canal Area." Clin Orthop Surg 1.1 (2009): 11-8.
Truettner, J. S., et al. "Subcellular Stress Response after Traumatic Brain Injury." J Neurotrauma 24.4 (2007): 599-612.
Vennekate, W., et al. "Cis- and Trans-Membrane Interactions of Synaptotagmin-1." Proc Natl Acad Sci U S A 109.27 (2012): 11037-42.
Windle, W. F. "Regeneration of Axons in the Vertebrate Central Nervous System." Physiological reviews 36.4 (1956): 427-40.
Wohlfart, G. "Degeneration and Regeneration in the Nervous System. Recent Advances." World neurology 2 (1961): 187-98.
Yao, J. J., et al. "Neuritin Activates Insulin Receptor Pathway to up-Regulate Kv4.2-Mediated Transient Outward K+ Current in Rat Cerebellar Granule Neurons." J Biol Chem 287.49 (2012): 41534-45.
Yoles, E., and M. Schwartz. "Degeneration of Spared Axons Following Partial White Matter Lesion: Implications for Optic Nerve Neuropathies." Exp Neurol 153.1 (1998): 1-7.

CHAPTER V

FUTURE DIRECTIONS

Neuronal cell death associated pathogenic gene expression clusters identified in the ONC model can be extrapolated to other forms of CNS trauma and neurodegenerative diseases. Our detailed gene expression profile identified crucial targets that could have therapeutic potential. Some of these targets have already been discussed and include: *Nrn1* (synaptic plasticity gene), *Synpr* and *Syt1 (*synaptic vesicle fusion genes), and *Vsnl1* (neuron differentiation associated gene).

We selected one of the gene therapy targets to explore as a plausible future therapy. With hNRN1, we demonstrated increased survival and regeneration of axotomized RGCs both *in vitro* and *in vivo*. Although our experiments were crucial in identifying a gene target that could address the different facets of neurodegeneration and regenerative failure, further experiments need to be performed to test the full functionality of the gene both *in vitro* and *in vivo*.

1. Experiments could be performed *in vitro* for highlighting the signaling pathways used by neuritin and the effect on RGCs. NRN1 acts as a ligand to the insulin receptor (IR) (Yao et al.) and cleavage of the GPI anchor by phospholipase C allows the soluble form to be secreted and activate downstream pathways (Nedivi, Wu and Cline). This increased neurite promotion occurs via mitogen-activated

protein kinase (MAPK) or phosphatidylinositol-3 kinase (PI-3K) activation (Karamoysoyli et al.).

a. We could investigate if RGCs treated with recombinant neuritin activate and induce phosphorylation of ERK (pERK), Akt (pAkt), and mammalian target of rapamycin (pmTOR).

b. Previous studies have shown a crucial role of ERK cascades after synaptic activation (Ji et al.; Sweatt). The mTOR pathway through the AKT kinase has also recently been shown to induce synaptic plasticity (Hay and Sonenberg). In addition, NRN1 induces increased mRNA and protein expression of Kv4.2 channels by up-regulating these pathways in rat cerebellar granule neurons (Yao et al.). We could first examine the expression levels of Kv4.2 in RGCs treated with recombinant NRN1 and then silence the activation of each pathway with the following: MEK inhibitor (U0126-ERK), rapamycin (mTOR) or LY294002 (AKT) as well as evaluate expression of Kv4.2.

c. We could transfect RGCs in culture with siRNA against the insulin receptor and then add recombinant neuritin to observe the downstream effects of neuritin on MAPK and PI-3K pathways. Expression levels of multiple effectors like Ras, Raf, extracellular signal-regulated kinase (ERK), and AKT could be measured. This would delineate if truly neuritin is acting through IR and if it is activating these particular pathways in the RGCs.

2. In the ONC model, RGCs over-expressing AAV2-CAG-hNRN1 could be tested for effective transport mechanisms and correct innervation to target neurons in the SC. Currently we have just tested the intrinsic effects of NRN1 but further studies need to be performed to ensure proper axonal targeting and also show if any extrinsic factors are influencing axonal projection.

    a. Anterograde transport of fluorescent cholera toxin B (CTB) (injected intravitreally) through the ON can be quantified by measuring average fluorescence intensity along the ON, through the optic chiasm, and into the superficial layers of the SC.

    b. An extended time course needs to be observed after AAV2 mediated over-expression to analyze if NRN1 induces regeneration at later time points and quantify expression of Gap43 past the lesion site.

    c. Ephrin receptors are arranged in a complementary fashion in RGCs and these are crucial for topographic organization of the retinal projections to the SC and LGN (Braisted et al.). Specific ephrin ligands (secreted from midline glia at the chiasm) that target RGC ephrin receptors determine axonal projection (Nakagawa et al.). Extensive studies can be performed on Ephrin ligand and receptor expression within the ON and SC, after over-expression of hNRN1.

3. For our current experimental model, NRN1 was over-expressed before axonal injury. Our ONC gene expression studies suggested that after axonal insult, RGCs initially increased *Nrn1* expression for axonal regeneration to overcome obstructed transport mechanisms. This supportive mechanism of regenerative

cues gets impeded, as the deterioration of the neuron progressively worsens. The ONC model is an acute trauma model and the insult is quite extensive with significant death of RGCs observed as early as 14dpc. To ensure that the beneficial effects of NRN1 could induce RGC survival and prevent apoptosis, the RGCs were transduced with AAV2-hNRN1 2 weeks before crush. This experimental design enabled effective transduction of RGCs for promoting survival. However, to mimic clinical conditions of CNS trauma, it would be crucial to explore the effect of over-expression on survival, regeneration and RGC function after the insult.

4. *Cpg15*$^{-/-}$ mice lack the exons 2-3 of *Nrn1* gene, eliminating the gene's expression in this strain. These mice exhibit a delay in axonal and dendritic arborization and maturation of excitatory synapses. RGCs within these mice would not express *Nrn1* and by measuring electrical function (ERG) of these RGCs, we could further analyze if the gene is critical for synapse formation and maturation of the excitatory synapses in the retinal neurons.

5. In addition, our current studies have been only performed in the ONC model and could be further extrapolated to other CNS trauma models as well as neurodegenerative diseases.

    a. Glaucoma is a degenerative optic neuropathy causing peripheral vision loss that can eventually lead to complete blindness. Most treatments for glaucoma focus on stabilizing IOP and even though lowering of IOP slows the progression of the disease it does not reverse visual field loss and prevent complete loss of RGCs. Therefore new drug therapies are required

for neuroprotection that could possibly save the neurons from degeneration and halt the progression of the disease. The neuroprotective and regenerative effects of over-expressing AAV2-CAG-hNRN1 could be studied in a few of these glaucoma models which include:

i. Transgenic mutant myocilin mice

ii. Laser photocoagulation induced OHT

iii. DBA/2J strain of naturally occurring glaucoma

iv. Microbead-induced OHT

b. Other CNS trauma models like traumatic brain injury and spinal cord injury models could also be additionally tested:

i. Fluid percussion injury model

ii. Controlled cortical impact model

iii. Weight drop impact acceleration models

iv. Blast injury models

v. Contusion models of spinal cord injury

6. Plausible clinical trial studies could be further pursued with *Nrn1* which include:

a. Encapsulated human cells genetically modified to secrete NRN1 could be implanted in regions of CNS trauma or neurodegeneration. The outer membrane of the semipermeable encapsulated cell implant would allow NRN1 to reach the target area.

b. Local application of exogenous NRN1 has shown to promote axonal regeneration and recovery in locomotor function in rats after acute spinal cord injury (Xi et al.). Similarly we could apply recombinant NRN1 directly to the site of CNS injury.

c. Gene therapy with viral vectors over-expressing NRN1. Certain viral vectors have selective tropism for specific cell types, so this approach could deliver targeted therapy.

References

Braisted, J. E., et al. "Graded and Lamina-Specific Distributions of Ligands of Ephb Receptor Tyrosine Kinases in the Developing Retinotectal System." Dev Biol 191.1 (1997): 14-28.

Hay, N., and N. Sonenberg. "Upstream and Downstream of Mtor." Genes Dev 18.16 (2004): 1926-45.

Ji, R. R., et al. "Erk Map Kinase Activation in Superficial Spinal Cord Neurons Induces Prodynorphin and Nk-1 Upregulation and Contributes to Persistent Inflammatory Pain Hypersensitivity." J Neurosci 22.2 (2002): 478-85.

Karamoysoyli, E., et al. "Neuritin Mediates Nerve Growth Factor-Induced Axonal Regeneration and Is Deficient in Experimental Diabetic Neuropathy." Diabetes 57.1 (2008): 181-9.

Nakagawa, S., et al. "Ephrin-B Regulates the Ipsilateral Routing of Retinal Axons at the Optic Chiasm." Neuron 25.3 (2000): 599-610.

Nedivi, E., G. Y. Wu, and H. T. Cline. "Promotion of Dendritic Growth by Cpg15, an Activity-Induced Signaling Molecule." Science 281.5384 (1998): 1863-6.

Sweatt, J. D. "The Neuronal Map Kinase Cascade: A Biochemical Signal Integration System Subserving Synaptic Plasticity and Memory." J Neurochem 76.1 (2001): 1-10.

Xi, S., et al. "[Effect of Neuritin Protein on Axonal Regeneration after Acute Spinal Cord Injury in Rats]." Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 23.10 (2009): 1219-23.

Yao, J. J., et al. "Neuritin Activates Insulin Receptor Pathway to up-Regulate Kv4.2-Mediated Transient Outward K+ Current in Rat Cerebellar Granule Neurons." J Biol Chem 287.49 (2012): 41534-45.

LIST OF PUBLICATIONS

Bollinger KE, Crabb JS, Yuan X, **Putliwala T**, Clark AF, Crabb JW. Quantitative Proteomics: TGFβ_2 Signaling In Trabecular Meshwork Cells. Invest Ophthalmol Vis Sci. 2011 Oct 21;52(11): 8287-94. doi: 10.1167/iovs.11-8218

McDowell CM, Luan T, Zhang Z, **Putliwala T**, Wordinger RJ, Millar JC, John SW, Pang IH, Clark AF. Mutant Human Myocilin Induces Strain Specific Differences In Ocular Hypertension And Optic Nerve Damage In Mice. Exp Eye Res. 2012 Jul;100:65-72. doi: 10.1016/j.exer.2012.04.016.

Bollinger KE, Crabb JS, Yuan X, **Putliwala T**, Clark AF, Crabb JW. Proteomic Similarities In Steroid Responsiveness In Normal And Glaucomatous Trabecular Meshwork Cells. Mol Vis. 2012;18:2001-11. Jul 20, 2012.

Wordinger RJ, **Sharma TP**, Clark AF The Role of TGF-β2 and Bone Morphogenetic Proteins (BMPs) in the Trabecular Meshwork and Glaucoma. J Ocul Pharmacol Ther. 2014 Feb 11

Sharma TP, McDowell CM, Liu Y, Wagner AH, Thole D, Faga BP, Wordinger RJ, Braun TA, Clark AF Optic nerve crush induces spatial and temporal gene expression patterns in retina and optic nerve of BALB/cJ mice. (Molecular Neurodegeneration 2013)

Sharma TP, Liu Y, Wordinger RJ, Pang IH, Clark AF Neuroprotective and regenerative role of neuritin1 following optic nerve crush (Cell Death and Disease 2014)

Liu Y, **Sharma TP,** Mao W**,** Wordinger RJ, Gorbatyuk MS, Clark AF Gene delivery of grp78/bip promotes retinal ganglion cell survival following optic nerve crush (Journal of Neuroscience 2014)