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14. ABSTRACT
The overall goal of the VISION project is to discover neuroprotective strategies in three separate mouse models of injury to the visual axis, in order to identify potential candidates for the treatment of combat eye injuries and preserve vision in our injured warfighters. We have established three different mouse models of ocular injury with different injury- initiating mechanisms (i.e. optic nerve crush, retinal ischemia/reperfusion, and chronic ocular hypertension). We have developed techniques to quantify damage to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus) that are damaged in these three models. We are testing neuroprotective agents and strategies, including neuroprotective estrogens, sigma-1 agonists, Brn3b, inhibitors of Jun N-terminal kinase (JNK), and inhibitors of protein stress to determine their efficacy in protecting the retina, optic nerve and superior colliculus from the damage induced by each of the 3 models.

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Introduction

In combat situations, traumatic eye injuries are frequent, leading to irreversible damage to the visual axis (retina, optic nerve, and visual centers in the brain). Although many of these injuries are treated surgically, there is a definite need for new therapeutic agents that protect and restore normal functions to ocular and brain neural tissues following traumatic injury to the eye. In addition, our models of ocular trauma mimic some features of common degenerative ocular diseases, so our discovery of pathogenic pathways and new therapeutic agents may also identify new therapeutic interventions for these diseases, which are prevalent in our veterans.

The overall goal of the VISION project was to discover neuroprotective strategies in three separate rodent models of injury to the visual axis, in order to identify potential candidates for the treatment of combat eye injuries and preserve vision in our injured warfighters. We established and used three different in vivo rodent models of ocular injury with different injury-initiating mechanisms (i.e. optic nerve crush, retinal ischemia/reperfusion, and chronic ocular hypertension). We developed techniques to quantify damage to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus) damaged in these three models.

Identification of pathogenic pathways that are involved in traumatic injury to the retina, optic nerve, and visual centers in the brain will lead to the discovery and development of new neuroprotective and regenerative strategies for treating combat related ocular injuries and degenerative ocular diseases. This initiative focused on addressing "Inadequate mitigation and treatment of traumatic injuries, war-related injuries, and diseases to ocular structures and the visual system". We proposed two aims that use rodent models of ocular injury (optic nerve crush and ischemia reperfusion) to: (1) test 6 neuroprotective strategies that will either delay or prevent further damage to the retina, optic nerve and visual centers of the brain, and (2) use genomic (transcriptomic) techniques to identify and characterize early and late pathogenic pathways involved in damage to the retina, optic nerve and visual centers of the brain. We used AAV2 delivery of agents that led to neuroprotection and neuroregeneration and also validated new therapeutic targets by genetic depletion of candidate pathogenic genes. Looking at initial and progressive damage to all these tissues helped us better understand the pathophysiology of neuronal damage and led to the design and testing of novel neuroprotective strategies.

We initially evaluated six specific neuroprotective strategies (neuroprotective estrogens, inhibitors of Jun N-terminal kinase (JNK), the sigma-1 receptor, Brn3b, C1q, and methylene blue) to determine their efficacy in protecting the retina, optic nerve and superior colliculus from neurodegenerative damage induced our 3 models. In addition, we evaluated time dependent, injury-induced changes in gene expression in the effected tissues and identified major pathogenic pathways involved in neurodegeneration in order to develop new therapeutic approaches for neuroprotection and neuroregeneration. This approach has allowed us to identify four new molecular pathogenic pathways, including neuritin-1, CHOP, caspase 7, and BiP. Overall, we evaluated ten different neuroprotective/neuroregenerative strategies, all of which provided some degree of neuroprotection and several of these strategies provided profound neuroprotection to the visual axis. We readily shared our discoveries through presentations at scientific meetings, published meeting abstracts, and peer-reviewed scientific publications.

This is the final report for the VISION (Vision Integrating Strategies in Ophthalmology and Neurochemistry) project at UNTHSC. Although our original proposal was based on 5 years of funding, we only received funding for the first two years, and we have received no cost extensions for years 3-5. We made a number of important discoveries despite the reduced funding for this overall project.

Key Words

- Neuroprotection
- Retina
- Optic nerve
- Visual centers of the brain
- Axonopathy
- Ischemia/reperfusion injury
- cJun N-terminal kinase inhibition
- Sigma-1 receptor
- Brn3b
- Neuritin-1
- CHOP
- BiP
- Caspase 7
- Complement factor C1q
- Transcriptomics

Overall Project Summary

We feel that overall this project was very successful for the following reasons: (1) Ten different neuroprotective agents were tested in different models of injury to visual axis neurons (retina, optic nerve, and neurons in the visual centers of the brain). Several of these treatments provided profound neuroprotection from severe neuronal insults. (2) We have shared our discoveries with the scientific community via 39 presentations and abstracts at national/international scientific meetings as well as 23 peer-reviewed scientific publications. We sincerely hope that one or more of these discoveries will be tested and developed clinically for treating individuals, including warfighters, with traumatic neuronal injury. ***The following is a brief synopsis of our discoveries based on our 23 peer-reviewed publications. Please note that the cited figures (in bold) refer to the figure numbers in the referenced publications.***

Establishment and characterization of rodent models of neuronal injury to the visual axis: We established and characterized three mouse models of damage to visual axis (retina, optic nerve and visual centers in the brain). Optic nerve crush (ONC) is an acute model of axonal injury leading to subsequent degeneration of retinal ganglion cell (RGC) somas (**Figures 1 & 3**), loss of RGC function (**Figures 5-6**), as well as target neurons in the superior colliculus of the brain (**Figure 2**)(**Liu et al. IOVS 2014**). Retinal ischemia/reperfusion (I/R) injury is an acute model of ischemic damage to the inner retina, followed by loss of inner retinal function (**Figures 6-7**) and degeneration of inner retinal neurons (**Figures 2-5**) (**Kim et al. Mol Neurodegen 2013**). I/R injury also damages contralateral superior colliculus neurons (**Figure 10**) (**Kim et al. 2016**). We also developed quantitative methods to access neuronal damage to the visual axis in all three models, including non-invasive techniques to measure neuronal morphological changes in retinal layer thickness using spectral domain-optical coherence tomography (**Figure 3 in Kim et al. Mol Neurodegen 2013**) (**Figure 3 in Liu et al IOVS 2014**) and functional changes to the retina by electroretinography (ERG)) (**Figures 6-7 in Kim et al. Mol Neurodegen 2013**) (Figures 5-6 in Liu et al. IOVS 2014).

Neuroprotection by JNK inhibition: The cJun N-terminal kinase (JNK) is a major signaling hub that can regulate cell proliferation, differentiation, or apoptosis, depending on the circumstances and environment. We have shown early activation (phosphorylation) of JNK and its downstream substrate JUN in retinas within 1 hour of retinal I/R injury (**Figures 2-5 in Kim et al Mol Neurodegen 2016**). This activation is most prominent in retinal ganglion cells (RGCs) and precedes the progressive loss of RGCs (**Figure 8**), thinning of inner retinal layers (Figures 6-7), decreased retinal function (ERG b-waves) (**Figure 9**), and neuronal loss in the superior colliculus (vision center in the brain innervated by RGC axons) (**Figures 10-11**). Daily systemic administration of the JNK inhibitor SP600125 totally protected the retina and RGCs from I/R induced retinal injury (**Figures 6-11 in Kim et al. Mol Neurodegen 2016**). It truly is remarkable that this therapy provided complete structural and functional protection of retinal and brain neurons from this severe insult.

Neuroprotection and neuroregeneration by gene therapy with retinal transcription factor Brn3b: Brn3b is a transcription factor responsible for the development of the majority of RGCs. This transcription factor protects and enhances neurite outgrowth in cultured neurons (**Figures 1-2, 4 & 6**) and neurons exposed to hypoxia (**Figures 4-7 in Phatak et al. Cell Mol Neurobiology 2015**). A viral Brn3b expression vector (AAV2.Brn3b) was constructed to transduce RGCs in vivo (**Figure 1 in Stankowska et al. IOVS 2015**). Over-expression of Brn3b in the retina protected RGCs from chronically elevated IOP in a rat model of glaucoma (**Figures 7-8**). Brn3b also appeared to enhance RGC axonal regeneration through the optic nerve head (**Figures 3 & 6 in Stankowska et al. IOVS 2015**), which is the initial site of glaucomatous damage. Bcl-2, Bcl-xL, and p-AKT appear to be involved in the neuroprotective effects of Brn3b in this glaucoma models (**Figures 2-3 & 5-6 in Phatak et al. Mol Vis 2016**).

Neuroprotection by activation of the sigma-1 receptor: The sigma-1 receptor is an endoplasmic reticulum chaperone protein that modulates intracellular calcium signaling and inhibits voltage gated K^+ channels. This receptor is linked to a number of additional signaling transduction pathways. The sigma-1 receptor is expressed on RGCs and attenuates the calcium response in cultured RGCs via interaction with voltage gated calcium channels and attenuates the NMDA induced calcium influx in RGCs (**Figures 2-7 in Mueller et al. Exp Eye Res. 2013**). Sigma- receptor stimulation protects RGCs via activation of the Erk1/2 signaling pathway. Sigma-1 receptors also enhance RGC synapse formation by mediating mitochondrial fusion (**Yorio et al. ARVO 2011; Mueller et al, ARVO 2012**). Stimulation of the sigma-1 receptor protects cultured RGCs from ischemic damage by activating ERK1/2 (**Figures 2-8 in Mueller et al Exp Eye Res 2014**). Sigma-1 activation protects cultured RGCs from oxygen glucose deprivation by stabilizing the mitochondrial membrane potential (**Figures 3-6 & 8 in Ellis et al. IOVS 2017**). Additional in vivo studies are being conducted in the ONC and retinal I/R models using a sigma-1 receptor agonist (pentazocine) as well as in Sigma-1 receptor deficient mice.

Neuroprotection by targeting complement factor C1q: The complement cascade plays a major role in both innate and acquired immune responses. Relatively recently, complement components C1q and C3 have been shown to play a role in developmental dendritic pruning of RGCs and their target neurons in the brain. We have shown increased expression of C1q in the retinal and superior colliculus neurons after retinal I/R injury (**Figures 1-2**) that correspond to glial activation in the retina and superior colliculus (**Figures 3-4**). I/R retinal injury is significantly reduced morphologically (protection of retinal thickness and RGCs) (**Figures 5-6**) and functionally (ERG) (**Figure 7**) in mice deficient in C1q (*C1q$^{+/-}$* and *C1q$^{-/-}$* mice) (**Silverman et al., Mol Neurodegen 2016**). C1q deficiency also suppressed microglial activation due to I/R injury (**Figure 8**). This strongly suggests that C1q plays an important pathogenic role in neurodegeneration of the visual axis induced by ischemia.

Neuroprotection with non-feminizing estrogens: Estrogens are well known as female sex hormones, but recent evidence also supports their neuroprotective activities, which often can be separated from their feminizing activities. Non-feminizing estrogens can protect cultured retinal neurons from excitotoxic injury (**Figures 4-7 in Nixon & Simpkins IOVS 2012**). However, a single systemic dose of estradiol prior to ONC injury failed to protect RGCs from progressive degeneration. Therefore, no further in vivo studies were conducted.

Neuroprotection with methylene blue: Methylene blue has been reported to protect a variety of cells from ischemic damage, including brain neurons, via targeting mitochondrial respiration. Treatment of primary rat RGC cultures with methylene blue protects these cells from rotenone, staurosporine and hypoxia cytotoxicity (**Figures 3-8 in Daudt et al. IOVS 2012**). Methylene blue also delayed senescence of neural progenitor cells (**Figures 1-6 in Xie et al. Frontiers in Cell Neurosci 2014**). Further evaluation of this compound for activity in our in vivo models was not possible given the decreased funding support for the VISION project.

Approach to discover new therapeutic targets: In addition to testing the 6 neurprotective therapeutic targets listed above, we also used a variety of methods to discover new potential therapeutic targets in our models of neurodegeneration of the visual axis. We used transcriptomics to identify temporal changes in gene expression in the retina, optic nerve, and superior colliculus in our two acute models of injury to the visual axis (i.e. ONC and retinal I/R induced injury). We reported temporal changes in retinal gene expression after ONC (**Figures 1-6 and Tables 1-4 Sharma et al. Mol Neurodegen 2014**) that led to the discovery of neuritin-1 as a new therapeutic target (**Sharma et al. Cell Death Disease 2015**) (see below). We also used transcriptomics to discover changes in retinal gene expression after retinal I/R injury (**Figures 8-10 and Tables 1-2 Kim et al. Mol Neurodegen 2013**).

In addition, we found that the unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD) pathways were activated after acute retinal damage induced by I/R or ONC. This discovery led us to evaluate key targets associated with these pathways (i.e. CHOP, Caspase 7, and BiP) for potential neuroprotective activities (see below).

Neuroprotection and neuroregeneration by Neuritin-1 (NRN1) gene therapy: Examination of changes in retinal gene expression after ONC lead to the discovery that *Nrn1* expression was significantly down-regulated shortly after ONC (**Figures 1 & 3-6 Sharma et al. Mol Neurodegen 2014**). Neuritin-1 promotes neuritogenesis of neurons during development, appears to play a role in neuronal plasticity in adults, and is expressed in RGCs. Given the functions of neuritin-1, we hypothesized that increasing NRN1 expression in RGCs would protect these neurons from axonopathy induced neurodegeneration. We constructed a viral vector (AAV2.NRN1) to transduce mouse eyes and over-express NRN1 in RGCs (**Figures 3-4 in Sharma et al. Cell Death Disease 2015**). NRN1 partially protected RGCs from ONC-induced neurodegeneration (**Figures 1 & 5**), induced RGC axonal regeneration (**Figures 2 & 6-7**) and more importantly functionally protected the RGC from ERG b-wave deficits (**Figure 8**) (**Sharma et al. Cell Death Dis. 2015**).

Neuroprotection by targeting CHOP: Endoplasmic reticulum (ER) stress occurs during a wide variety of neuronal insults. One downstream effector of ER stress is CHOP (DDIT3), which is a C/EBP transcription factor that can activate neuronal death via apoptosis. The expression of retinal CHOP is significantly increased within 3 days of retinal I/R injury, with expression mostly localized to RGCs (**Figures 1 & 3**). Deletion of CHOP expression in $Chop^{-/-}$ mice partially protected RGCs morphologically (**Figure 4**) and functionally (**Figure 5**) from I/R induced damage (**Nashine et al. IOVS 2014**). This clearly demonstrates that ER stress plays a major role in retinal I/R induced neurodegeneration and suggests other potential ER stress associated therapeutic targets.

Neuroprotection by targeting Caspase 7: Caspase 7 is an effector caspase induced by ER stress that is involved in apoptotic cell death. Little was known about its potential involvement in neuronal cell death, especially in the retina. ONC injury in mice increases expression and activation of caspase 7 in retinal ganglion cells (**Figures 1-2**) as well as increased caspase 7 substrate cleavage (**Figure 4**). In addition, deletion of caspase 7 expression in $Casp7^{-/-}$ mice partially protected the retina from ONC induced retinal thinning (**Figure 6**), loss of RGCs (**Figure 5**) and functional deficits (ERG pSTR) (Figure 7) (**Choudhury et al. Mol Neurodegen 2015**). This demonstrates that RGCs can be protected by targeting ER stress induced apoptosis.

Neuroprotection by BiP (GRP78) gene therapy: BiP is an ER molecular chaperone that plays a protective role during ER stress by translocating misfolded proteins out of the ER for proteolytic degradation. Expression of BiP in RGCs is increased shortly after ONC injury in an attempt to provide endogenous protection. We generated a viral vector to transduce and over-express BiP in RGCs (AAV2.BiP). Over-expression of BiP significantly reduced RGC loss post-ONC and partially protected ERG pSTR amplitudes, providing both morphological and functional protection (**Liu et al. ARVO 2014**). This demonstrates that ONC associated ER stress causes pathogenic neurodegeneration, which can be prevented by gene therapy with the molecular chaperone BiP. We did not further pursue this neuroprotective target due to the funding cut for this project.

Key Research Accomplishments

(1) The VISION research project was a multi-PI effort to discover new neuroprotective strategies to protect the visual axis (retina, optic nerve, and visual centers in the brain) from traumatic injury. We proposed to evaluate specific neuroprotective strategies using small molecules, gene therapy, and genetic deletion of target genes. In addition, we evaluated molecular changes in the visual axis over the course of traumatic injury in order to discover and test new therapeutic strategies. The VISION project was an ambitious 5-year project initially involving 6 faculty members, 5 postdoctoral fellows, 7 graduate students, and 4 research associates. However, we only receiving funding for the first 2 years of this 5-year project and had to make major adjustments to our budget, personnel, and research plan. Over the 5 years of this project, we evaluated 10 different therapeutic approaches and made a number of significant discoveries, despite this major budget reduction.

(2) We established and characterized three mouse models of damage to visual axis (retina, optic nerve and visual centers in the brain). Optic nerve crush (ONC) is an acute model of axonal injury leading to subsequent degeneration of retinal ganglion cell somas as well as target neurons in the superior colliculus of the brain. Retinal ischemia/reperfusion (I/R) injury is an acute model of ischemic damage to the inner retina, followed by degeneration of retinal and superior colliculus neurons. We also developed an inducible model of elevated intraocular pressure that causes optic neuropathy and retinopathy that progressively damages retinal ganglion cells. In addition, we developed quantitative methods to access structural and functional neuronal damage to the visual axis in all three models.

(3) We initially evaluated six different therapeutic neuroprotective strategies, including JNK inhibition, Brn3b, sigma-1 receptor, C1q, neuroprotective estrogens, and methylene blue. All six strategies gave varying degrees of neuroprotection. JNK inhibition totally protected the visual axis (retina and superior colliculus) structurally and morphologically from retinal ischemia/reperfusion injury. Genetic depletion of complement component C1q also protected the retina from ischemia/reperfusion injury. Gene therapy with Brn3b significantly protected retinal ganglion cells from ocular hypertension induced damage and appeared to stimulate axonal regeneration. Sigma-1 receptor agonists and methylene blue are neuroprotective in cultured neurons. We plan on testing the potential neuroprotective role of the sigma-1 receptor in our in vivo models.

(4) We used transcriptomics to identify additional neuroprotective targets, including NRN1, CHOP, caspase 7, and BiP. Gene therapy with NRN1 or with BiP partially protected the retina from ONC-induced retinal ganglion cell loss and partially protected RGC function. NRN1 also induced optic nerve axonal regeneration, so this gene is both neuroprotective and neuroregenerative. Genetic knockout of CHOP structurally and functionally protected the retina from ischemia/reperfusion injury, while genetic depletion of caspase 7 protected the retina from ONC injury.

(5) We have presented our research findings at national and international visual sciences meetings (39 presentations and published abstracts). We also have published 18 and submitted an additional 2 peer-reviewed scientific manuscripts related to this work.

(6) Over the course of this 5 year project, we have trained and at least partially supported 5 postdoctoral fellows and 7 graduate students.

Conclusion

Through the generous support of TATRC and the Department of Defense, the VISION project discovered and evaluated 10 different therapeutic neuroprotective approaches to treat traumatic injury to neurons in the visual axis (brain, optic nerve, and visual centers in the brain). Two of our experimental in vivo models caused acute and severe traumatic damage to the visual axis. We showed total structural and functional protection with one therapy (JNK inhibition), and to our knowledge no previous therapeutic approach has provided this level of protection. Several of our gene therapy approaches not only provided neuroprotection, but also showed neuroregeneration of optic nerve axons showing that we can regrow injured nerve axons. Since the retina and optic nerve are extensions of central nervous system (CNS) and we also evaluated neurons in the visual centers of the brain, our discoveries would likely also apply to other CNS neurons in the brain and spinal cord. We have readily shared all our discoveries with the scientific community through 39 presentations and published abstracts as well as 20 peer-reviewed publications.

Personnel Supported by VISION Project

<u>Faculty (Principal Investigators)</u>
- Abbot Clark, PhD (Program Director) (2010-2015)
- Marina Gorbatyuk, PhD (2010-2012)
- Raghu Krishnamoorthy, PhD (2010-2013)
- James Simpkins, PhD (2010-2011)
- Robert Wordinger, PhD (2010-2013)
- Thomas Yorio, PhD (2010-2015)

<u>Postdoctoral Fellows</u>
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- Shreyasi Choudhury (2011-2014)
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- Sonali Nashine (2011-2014)
- Yong Park (2011-2015)
- Nitasha Phatak (2010-2013)
- Tasneem Sharma (2010-2014)
- Sean Silverman (2011-2015)

<u>Technical Support</u>
- Terri Beckwith (2011-2013)
- Sherri Harris (2014-2015)
- Sandra Neubauer (2010-2015)
- Holly Tebow (2010-2014)

Publications, Abstracts, and Presentations

Abstracts and Presentations (39)

Phatak NR, Minton AZ, Mireles CE, Krishnamoorthy R. Overexpression of POU domain transcription factor, Brn3b, cuased neurite outgrowth and axon elongation in cultured transformed 661W cells. 2011 ARVO Annual Meeting, Abstract 2659

Mueller B, Krishnamoorthy R, Daudt D, Ma H-Y, Yorio T. Interaction of sigma-1 receptors with voltage gated calcium channels attenuates calcium response in primary retinal ganglion cells. 2011 ARVO Annual Meeting, Abstract 2657

Yorio T, Daudt D, Mueller B. Effects of sigma-1 receptors on mitochondrial fusion in retinal ganglion cells. 2011 ARVO Annual Meeting, Abstract 4619

Nixon ES, Simpkins JW. Neuroprotective effects of non-feminizing estrogen analogues in retinal neurons. 2011 Society for Neuroscience Abstract 895.01.

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Choudhury S et al. Role of ER stress-induced caspase7 in retinal degeneration of T17M rhodopsin transgenic mice. 2012 ARVO Annual Meeting Abstract 6446

Kunte M et al. ER stress is involved in retinal degeneration induced by human T17M mutant rhodopsin. 2012 ARVO Annual Meeting Abstract 6453

Nashine S et al. Deficiency in the pro-apoptotic CHOP protein, an UPR downstream marker, does not prevent vision loss in T17M Rho retina. 2012 ARVO Annual Meeting Abstract 6457

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Ellis, Dorette Z; Li, Linya; Park Yong H.; Mueller, Brett; Ma, Hai-Ying; Yorio, Thomas, "Sigma-1 Receptor-Induced Neuroprotection: Correlation between Mitochondrial Membrane Potential and Caspase Activity in Glucose Oxygen Deprived Retinal Ganglion Cells" (2014), Association for Ocular Pharmacology and Therapeutics, Charleston, SC

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Park, Yong H.; Mueller, Brett H.; Yorio, Thomas, "NMDA and AMPA Receptor Stimulation in Retinal Ganglion Cells Induces Prolonged Phosphorylation of CREB and Increases Resistance to Apoptosis" (2014), International Society for Eye Research, San Francisco, CA

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