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14. ABSTRACT

Background: The neurons in the eye called retinal ganglion cells (RGCs) send visual information through nerve fibers that travel into the optic nerve to reach the brain. Damage to the optic nerve resulting from traumatic brain injury (TBI) and traumatic optic neuropathy (TON) can result in the death of these neurons, and subsequent visual impairment. There is no treatment available to restore vision once the damage is done. We have previously discovered specific genes that induce robust optic nerve regeneration in mice. Additionally, we have demonstrated that genetic modification of cell death-related genes render RGCs highly resistant to injury.

Objective/Hypothesis: The objective of this proposal is to determine the ability of combinatorial strategies to rescue RGCs and improve optic nerve regeneration in clinically-relevant models of optic nerve injury. The hypotheses of our study are: i) using a combinatorial treatment strategy comprised of hypothermia exposure and neuroprotective gene therapy will further improve RGC survival after TBI, and ii) regenerative gene therapy will promote optic nerve regeneration and restoration of lost vision after clinically-relevant optic nerve injury generated close to the brain.

15. SUBJECT TERMS
TBI, axon regeneration, hypothermia, optic neuropathy, PTEN, apoptosis, axon injury, retinal ganglion cells

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1. Introduction

Vision, the ability to see, is perhaps one of the most important senses in our lives, as it is critical for navigation and survival. Military service members are more likely than non-military civilians to encounter traumatic events that can result in brain injury. Traumatic brain injury (TBI) is a debilitating, multifaceted trauma that frequently occurs in the military patient population. One of the facets of TBI is damage to the optic nerve, which can result in significant visual impairment. About 75% of active military personnel subjected to trauma suffer from progressive glaucoma or optic nerve injury (also known as optic neuropathy). The optic nerve works like a "highway", connecting the eye to the brain. When it is damaged, such as what often occurs in TBI, the eye no longer can send visual information to the brain, resulting in irreversible blindness. Currently, there is no treatment available to patients that can regenerate the damaged optic nerve needed to reverse blindness. There has been some progress in animal research aimed at finding a cure for repairing the damaged optic nerve. One promising and relatively safe method is hypothermia exposure (cooling of the body) with beneficial effects observed in preclinical animal models to reduce the rate of nerve damage. Another therapeutic approach is the use of gene therapy to provide nerve protection and permit regeneration. However, these approaches, when given individually, have limited therapeutic effects. An optimized approach would be to combine the individual treatments together, ultimately leading to additive and synergistic effects. Such combinatorial approaches have never been tested in animal models of TBI-induced optic nerve injury. Our proposed study will explore this exciting possibility. The main objective of this study is to determine whether our unique combinatorial strategies rescue cells and promote optic nerve regeneration with a greater efficacy in clinically-relevant models of optic nerve injury. To this end, we will use cutting-edge tissue imaging techniques, genetically-modified mice, and innovative gene therapy approaches.

2. Keywords

Keywords TBI, axon regeneration, hypothermia, optic neuropathy, PTEN, apoptosis, axon injury, retinal ganglion cells (RGCs).

3. Accomplishments

Below is the list of important activities and timeline approved SOW.

Specific Aim 1: Systematically characterize the site, type and time course of damage in the visual pathway, and long term subtype-specific RGC loss following TBI.	**Proposed Timeline**	**Progress**
Major Task 1: Systematically document axonal damage in Thy1-YFP mice with CTB injection.	Months	
Local IBC/IACUC Approval	1-2 months	Completed.
Milestone Achieved: Local IBC/IACUC/ACURO Approval	1-4 months	Completed.
Subtask 1: Breed Thy1-YFP mice (up to 4 breeding pairs 4 males and 4 females= 8 mice total)	2-6 months	Completed.
Subtask 2: Intravitreal injection of CTB. TBI, image whole tissues and document site, type and time course of axonal damage (5 mice x 3 time points 2, 14 and 56 days = 15 mice total)	2-6 months	Analysis underway. Using immunohistochemistry, we assessed RGC survival and cell stress markers in the retina, and signs of inflammation in the retina as well as in the optic nerves. Labeling of axons with CTB was performed, and the tissue sections were analyzed for signs of axonal transport disruption.
Subtask 3: No CTB injection animals. TBI, image whole tissues and document site, type, and time course of axonal damage (5 mice x 3 time points 2, 14 and 56 days = 15 mice total)	2-12 months	Analysis underway.

Subtask 4: TBI, assessment of RGC types' differences in survival (10 mice x 4 groups = 40 mice total)	2-12 months	Completed. Different RGC markers were used to assess survival different RGC types after injury.
Milestone(s): Have the 3D imaging Have the analysis on axonal damage profiles	12 months	Completed, and analysis underway.
Milestone(s): Have the RGC types' survival rates determined	12 months	Completed, and analysis underway.

Specific Aim 2: Assess the individual and combined effects of hypothermia and gene therapy in preventing retinal ganglion cell (RGC) death after TBI.		
Major Task 2: Determine RGC survival after combinatorial treatment		
Subtask 1: Generate AAV2-shRNA against CHOP and AAV2-XBP1	12-16 months	Completed. We have AAVs including AAVs targeting XBP1, CHOP as well as AAV-shRNAs against numerous genes for further examination.
Subtask 2: Generate animals subjected to TBI, optic nerve injury and intravitreal injection of AAV with or without hypothermia (8 mice x 7 groups x 2 survival time points = 112 mice total)	13-18 months	Some completed, and analysis is underway.
Subtask 3: Immunohistochemistry to examine RGC survival in various animal groups	13-20 months	Some completed, and analysis is underway.
Milestone(s) Achieved: Have the RGC quantification completed	20 months	Some completed, and analysis is underway.
Milestone(s) Achieved: Submit manuscript for publication on the results from 3D imaging in Specific Aim 1	20-28 months	One manuscript has been accepted for publication.
Specific Aim 3. Assess RGC axon regeneration and functional recovery after pre-chiasmic nerve crush.		
Major Task 3: Assess the extent of RGC axon regeneration		
Subtask 1: Generate AAV2-shRNA against PTEN, AAV2-STATca and AAV2-MEKca	20-23 months	Completed
Subtask 2: Generate animals subjected to prechiasmic crush and intravitreal injection of AAV (8 mice x 2 groups. 12 mice x 2 groups = 40 mice total)	21-24 months	Completed
Subtask 3: Tissue sections to examine RGC axon regeneration and reconnection in the chiasm and into the brain	22-30 months	Analysis underway.
Milestone(s) Achieved: Document regeneration profiles, target innervation and potential synapse formation by the regenerated axons	30 months	Analysis underway.

Major Task 4: Assess restoration of visual functions in mice		
Subtask 1: Behavioral assays including circadian rhythm, pupil reflex, visual cliff test (12 mice x 2 groups = 24 mice total)	22-32 months	Not yet commenced.
Subtask 2: Prepare and submit publications on RGC survival and axon regeneration	36 months	Not yet commenced.
Milestone(s) Achieved: Complete functional behavioral assays Milestone(s) Achieved: Submission of at least one research paper Milestone(s) Achieved: Review of potential IP, patent, and potential transition plan for next step in translation into clinics	36 months	Not yet commenced.

In the years 2020 and 2021, there has been a significant slowdown in initiating and completing some of the proposed animal experiment due to the restrictions resulting from the COVID19. Nonetheless, we have managed to carry out substantial experiments during the last two years of funding period. We have initiated and completed the listed subtasks above. Specifically, the main goals of this reporting period were to; i) test the TBI models in our hands and assess the degree of visual system damage using tissue sections, and in whole tissues (i.e., subtasks 1, 2 and 3). We have generated several animal groups that were subjected to either sham surgery or TBI. Several days and weeks after TBI, mice were humanely euthanized. Eyes, optic nerves, and brains were removed from these animals for analysis. Retinas were processed for immunostaining and stained with antibodies against RBPMS (RGC marker), reactive astrocytes (i.e., GFAP) and immune cells (IBA1). RGC numbers were counted, and signs of inflammation were assessed. Some animals received intravitreal injection of cholera toxin beta subunit (CTB) prior to TBI to label RGC axons. CTB labeling was used to assess the effects of TBI on axonal transport and examine for signs of axonal damage. Some animals with CTB injection were perfused, then processed for tissue clearing and whole tissue imaging. Using immunohistochemistry, we assessed RGC survival (i.e., using RBPMS antibody) and cell stress markers (ATF3 and c-Jun expression) in the retina, and signs of inflammation in the retina as well as in the optic nerves. Labeling of axons with CTB was performed, and the tissue sections were analyzed for signs of axonal transport disruption. We see some degree of visual system damage in these mice.

In addition to the genes that we proposed to examine (e.g., XBP1 and CHOP), we sought to examine other genes that might have even stronger neuroprotective effects on mouse RGCs. To this end, we have searched in our RNAseq data protein coding genes as well as non-coding RNAs (e.g., microRNAs and long non-coding RNAs (lncRNAs)) that are differentially expressed in the injury-resilient RGC types (i.e., ipRGCs). Accordingly, we find dozens of transcription factors and lncRNAs that might be good candidates for further testing in vivo. These genes include Taf7l, Phf10 and Mef2b, and several lncRNAs whose functions are completely unknown. We have started to generate shRNAs and Crispr/Cas9 to silence these genes, generate AAVs and injected in adult mice to assess whether they promote RGC survival after optic nerve injury. Excitingly, our preliminary data show that one lncRNA (we call it “lncRNA 2021” or Onil1, optic nerve injury induced lncRNA1) has strong neuroprotective effects of RGCs in vivo (Figure 1). A part of this initial finding was submitted to the journal *BMC Genomics* and was recently accepted for publication. To our knowledge, this is the first demonstration that silencing a single lncRNA protects adult RGCs after optic nerve damage.

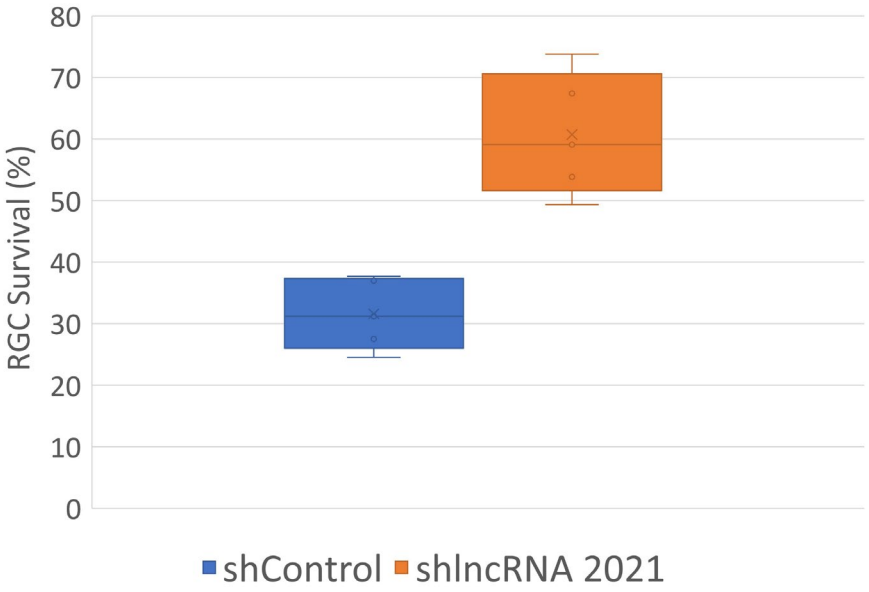

Figure 1. Novel neuroprotective lncRNA against optic nerve damage in adult mice. Animals were given intravitreal injection of AAV expressing shRNA against lncRNA 2021. Control animals received injection of AAV expressing scramble shRNA. Animals were given intraorbital optic nerve lesion, and perfused 2 weeks after injury. shlncRNA animals show about 2-fold increase in RGC survival compared to the control group. (n=5 mice/group).

We find that shRNA against one lncRNA (Onil1) exhibit remarkable neuroprotection of RGCs; approximately 70-90% of RGCs survived compared to 30% survival seen in the control animals receiving control AAV injection. Our results demonstrate for the first time, that silencing a single lncRNA prevents RGC death after axonal injury. We find that this neuroprotective effect is far greater than that seen from targeting coding genes including CHOP and XBP1.

During this reporting period, we generated additional animals to examine whether the lncRNA gene therapy can protect RGCs for a long time. To this end, animals received optic nerve injury and were left to survive for another 2 months. We will be analyzing these animals in the next few weeks.

Additionally, as described in our last report, we have tested whether knocking down different transcription factors (TF) that we found to be highly induced after optic nerve injury will promote RGC survival after TBI and after optic nerve crush. We generated shRNAs against these genes and inserted them into AAVs like we described above. Interestingly, we find that knocking down one TF (for the time being we are calling this gene Survival Regulating Transcription Factor 1 or SRTF1) in RGCs promote strong RGC protection. Our data show that AAV-shRNA against SRTF1 results in about 60% RGC survive compared to 20-30% in control animals after optic nerve injury. Thus, we have discovered **at least two novel genes** (i.e. Onil1 and this SRTF1) that regulate RGC survival after injury. As far as we know, there is nothing known about these genes for their roles in RGC survival. During this reported period, we have generated additional animals to confirm these preliminary results.

In sum, we continue to optimize TBI models to best characterize neuroprotective strategies. We continue to assess whether gene treatments protect RGCs. During this process, we have generated long-term survival animals and additional animals to confirm our results that silencing an lncRNA and a TF provides remarkable RGC protection after optic nerve damage.

4. Impact

There is currently no treatment that can prevent retinal ganglion cell (RGC) death and restore vision after traumatic brain injury (TBI and traumatic optic neuropathy (TON). Hypothermia and gene therapy are viable therapeutic options and have already been tested in other pathological conditions in the central nervous system. The combined effects of these strategies have not been tested before. The potential RGC protection and regeneration conferred by these strategies will be significantly impactful in the field of neuroscience and for the treatment of TON patients. We are currently processing and analyzing the tissues and samples from animals generated for subtasks above. The results from these sets of animals will create strong foundation for our future experiment proposed in this study.

5. Changes/Problems

COVID 19 during 2020 and 2021 has significantly limited our ability to generate animals, and perform analyses on the processed tissues. Fortunately, most restrictions were lifted off in the later part of 2021, allowing staff to return to work, and generate animals, perform experiments. We had continued to work with the guidelines provided by the School, and sought to remain as strategic and productive as possible to complete the proposed studies.

6. Products

We had one manuscript accepted for publication, partially supported by this grant.

Manuscript title: Identification of Long Noncoding RNAs in Injury-Resilient and Injury-Susceptible Mouse Retinal Ganglion Cells. ***BMC Genomics***, 2021, PMID: 34649511

7. Participants & Other Collaborating Organizations

Name:	**Kevin Park**
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Contribution to Project:	**Dr. Park has designed the experiment, and trained students and lab technicians.**

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Nearest person month worked:	**1**
Contribution to Project:	**Dr. Blaya has performed TBI, processed tissues and trained students.**

Name:	**Ana Ayupe**
Project Role:	**Investigator (Postdoctoral fellow)**
Researcher Identifier (ORCID ID):	
Nearest person month worked:	**4**
Contribution to Project:	**Dr. Ayupe processed tissues, analyzed the samples and trained students.**

8. Special Reporting Requirements N/a

9. Appendices N/A