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TITLE: Novel Combinatorial Approaches to Repair Visual System after Optic Nerve Damage

PRINCIPAL INVESTIGATOR: Kevin K. Park

CONTRACTING ORGANIZATION: University of Miami Miller School of Medicine
Miami Project to Cure Paralysis
1095 NW 14th Ter. LPLC 4-20
Miami, FL 33136

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Kevin Park
E-Mail: kpark@miami.edu

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University of Miami Miller School of Medicine
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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. The specific aims are:
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. Currently, there are gaps in the knowledge of the type and extent of axonal damage that occurs in the optic pathway after TBI. Therefore, more comprehensive animal studies are needed to better understand the pathophysiology of optic nerve damage and visual impairment after TBI.
Aim 2. Assess the individual and combined effects of hypothermia and gene therapy in preventing RGC death after TBI. We reason that hypothermia will reduce the rate of cell death in the acute stages after injury, while gene therapy will provide long-term neuroprotection of RGCs, and we expect a synergistic effect with combinatorial treatment versus single treatments alone.
Aim 3. Assess RGC axon regeneration and functional recovery after optic nerve crush adjacent to the brain. Nearly all of the previous animal studies aimed at examining optic nerve regeneration have used a model in which the injury is generated in optic nerve regions close to the eye. This is simply because these regions are easy to access during animal surgery. However, optic nerve damage, particularly that resulting from TBI, occurs most frequently in optic nerve regions near the brain. Thus, we will test the exciting possibility that gene therapy will be effective in restoring vision in a clinically-relevant crush model in which optic nerve injury is performed near the brain.
Study Design:
Aim 1: To characterize visual system damage following TBI, we will subject adult mice to TBI and perform cutting-edge whole tissue imaging. We will systematically analyze signs of axon severance over several time points after TBI. Since there are different types of RGCs present, we will use immunostaining to examine which types of RGC die or survive after TBI.
Aim 2: We will assess for the first time the combinatorial effects of hypothermia and gene therapy in protecting RGCs after TBI. Animals will undergo hypothermia for several hours and gene therapy that targets cell death-associated genes will be administered into the eye. Using tissue staining, we will determine whether these treatment paradigms administered within hours or days after TBI improves RGC survival.
Aim 3: Animals will receive optic nerve crush immediately before the chiasm. Gene therapy that targets several different regeneration-associated genes will be given to the injured animals. Optic nerve regeneration will be assessed in tissue sections. Restoration of visual functions in animals will be assessed by monitoring various visually-guided behaviors.
Impact: There is currently no treatment that can prevent RGC death and restore vision after TBI and TON. Hypothermia and gene therapy are viable therapeutic options and have already been tested in other pathological conditions in the CNS. 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.
Military Relevance: Military service members are at a greater risk of incurring optic nerve damage. This proposal will investigate combinatorial therapeutic interventions to rescue dying neurons, promote regeneration, and restore vision, with the added potential to be administered in the clinic and on the battlefield

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.	**Timeline**	**Investigator**
Major Task 1: Systematically document axonal damage in Thy1-YFP mice with CTB injection.	Months	
Local IBC/IACUC Approval	1-2 months	Dr. Park
Milestone Achieved: Local IBC/IACUC/ACURO Approval	1-4 months	Dr. Park
Subtask 1: Breed Thy1-YFP mice (up to 4 breeding pairs 4 males and 4 females= 8 mice total)	2-6 months	Dr. Park
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	Dr. Park
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	Dr. Park/Dr. Bramlett/ Dr. Tsoulfas
Subtask 4: TBI, assessment of RGC types' differences in survival (10 mice x 4 groups = 40 mice total)	2-12 months	Dr. Park/Dr. Bramlett/
Milestone(s) Achieved: Have the 3D imaging completed. Have the analysis on axonal damage profiles completed	12 months	Dr. Park/Dr. Bramlett/ Dr. Tsoulfas
Milestone(s) Achieved: Have the RGC types' survival rates determined	12 months	Dr. Park/Dr. Bramlett

Local IBC/IACUC Approval	**Completed**
Milestone Achieved: Local IBC/IACUC/ACURO Approval	**Completed**
Subtask 1: Breed Thy1-YFP mice (up to 4 breeding pairs 4 males and 4 females= 8 mice total)	**Breeding started and ongoing.**
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)	**Animals received TBI and some of these animals have been processed for tissue staining and histology for the early survival time points. 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)	**Animals received TBI and some of these animals have been processed for tissue staining and histology for the early survival time points. Using immunohistochemistry, we are assessing RGC survival and cell stress markers in the retina, and signs of inflammation in the retina as well as in the optic nerves.**

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 several experiments during the first year 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 but more mice and longer survival time points are needed to conclude. Increase in N value and the longer survival study are underway as planned. We are currently in the process of counting cells, and imaging the tissue sections, and analyzing the stained cells,

4. Impact

There is currently no treatment that can prevent retinal ganglion cell (RGC) death and restore vision after traumatic brain injury (TBI0 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 2 and 3 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 situation in the last several months has significantly limited our ability to generate animals, and perform analyses on the processed tissues. Fortunately, restrictions were lifted off to some extent in the last few months, allowing staff to return to work, and generate animals, perform experiments.
Having said that, our research activities are still suboptimal. This is partly because the school has placed limits on the number of research personnel allowed in given spaces. COVID 19 situation may not improve, and there is possibility that restrictions impacting the lab work will continue to be in place, which will slow the progress of our studies. We will continue to work with the guidelines provided by the School, and seek to remain as strategic and productive as possible to complete the proposed studies.

6. Products

We recently published our work, partly supported by this grant, in the journal *eNeuro* (see below).
Márcio Ribeiro, Konstanin Levay, Benito Yon, Ana Ayupe, Yadira Salgueiro, Kevin K. Park. Neural Cadherin Plays Distinct Roles for Neuronal Survival and Axon Growth under Different Regenerative Conditions. *eNeuro* (in press). ENEURO.0325-20.2020

7. Participants & Other Collaborating Organizations

Name:	**Kevin Park**
Project Role:	**PD/PI**
Researcher Identifier (ORCID ID):	0000-0003-4796-3894
Nearest person month worked:	**1**
Contribution to Project:	**Dr. Park has designed the experiment, and trained students and lab technicians.**

Name:	**Meghan Blaya**
Project Role:	**Investigator (Assistant Scientist)**
Researcher Identifier (ORCID ID):	0000-0002-1722-7872
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