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TITLE: Targeted treatment of traumatic optic neuropathy inspired by neuroprotective adaptations of hibernation

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The primary objective of this research is to translate adaptive strategies used by hibernators to prevent vision loss associated with head injuries resulting in damage to the optic nerve. In Aim 1 (CY 19), we sought to optimize the selected drugs to maximize the viability of ganglion cells following mechanical compression of the optic nerve. Comparisons were made to determine whether irreversible or reversible protease inhibition would be

15. SUBJECT TERMS

Traumatic Optic Neuropathy (TON), Hibernation, Retinal Ganglion Cells (RGCs), Neuroprotection

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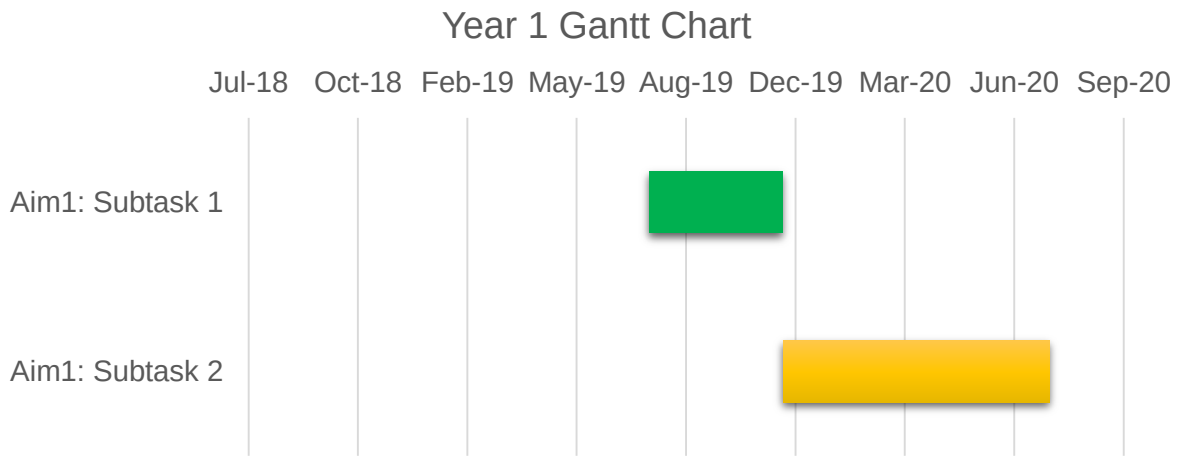
TABLE OF CONTENTS

INTRODUCTION: The innate ability of hibernators to respond uniquely to optic nerve injury and prevent permanent loss of vision due to retinal ganglion cell (RGC) loss has prompted the ambitious goal of translating the cellular strategies involved in hibernation to preserve vision in soldiers that have experience blunt or blast induced trauma to the optic nerve. Recently we have made substantial progress in understanding the mechanisms that contribute to oxidative stress and cell death and have identified two pharmaceutical interventions that mimic the protective effects of hibernation. Our goal in the proposed work is to advance the development of these drugs and their delivery for human use in order to promote RGC neuroprotection. These efforts will culminate in the evaluation of their efficacy in an experimental blast model of ocular injury.

KEYWORDS: Traumatic Optic Neuropathy (TON), Hibernation, Retinal Ganglion Cells (RGCs), Neuroprotection

ACCOMPLISHMENTS:

- *In this reporting period (Year 1) we have successfully tested single dose administration of BAM15 and PI cocktail, BAM15 and reversible protease inhibitors, and DMM post optic nerve crush (ONC) injury.*
- *We explored the use of other pharmaceutical agents that target the same identified pathways including methylene blue, acriflavine, and resveratrol.*
- *We have also evaluated repeated intraocular injections with DMM at either 0.44M and 0.88M.*
- *To date the BAM15 PI cocktail has shown the most promise.*
- *Once COVID-19 restrictions on travel have been lifted we anticipate our post doc will be able to participate in professional development workshops and relevant scientific conferences such as the Association for Research in Vision and Opthalmology (ARVO).*
- *The project was presented internally at our weekly lab meeting for discussion and to the Ophthalmic Genetics and Visual Function Branch meeting in order to gain valuable feedback from both scientists and clinicians.*
- *As the project develops, we will be in contact with the National Eye Institute Office of Communications regarding a press release to publicize the findings to the public.*
- *Future experiments will focus on testing whether multiple injections of BAM15/PI can provide advanced ganglion cell protection marking the completion of Specific AIM 1. We will then begin AIM2 (Year 2) focusing on using BAM15+PI in conjunction with the cell penetrating peptides to evaluate the success of eye drop delivery. We will also begin the process of obtaining IACUC approval for the blast injury model in preparation for AIM3 (Year 3). Descriptions of AIMs 1-3 are provided below along with the new findings from fourth quarter (Year 1).*



Milestone(s) Achieved: determination whether reversible protease inhibitors delivered in conjunction with BAM15 further improve ganglion cell viability. Identification of optimal dose/timing of delivery for DMM and BAM15/PI following optic nerve crush injury.

Major Goals of the Project

Specific Aim 1: To translate adaptive strategies employed by hibernators and demonstrate the feasibility of using hibernation-mimicking drugs to promote retinal ganglion cell survival.

Major Task 1: Optimize selection, dose, and timing of pharmaceutical agents using optic nerve crush injury model

Subtask 1: Evaluate (histological analysis/pERGs) whether the addition of reversible protease inhibitors to BAM15 (1μM) delivered by intraocular injection is more effective at preserving ganglion cell viability at 21d following optic nerve crush injury. Results to be compared to preliminary data that used a commercially available protease inhibitor cocktail that contained irreversible protease inhibitors.

Projected Milestone (1-6 months)

From 1st quarterly report: The experiments were conducted using other funds with an existing, approved animal use protocol (mouse). 9 mice were divided into 3 groups (Untreated, BAM15+PI reversible, BAM15+PI cocktail). Each mouse was subjected to unilateral crush injury of the optic nerve (left eye). The contralateral, uninjured optic nerve (right eye) served as a control. At the time of injury the mice were intraocularly injected with either 2μL of sham: HBSS, 1μM BAM15+Luepeptin (20μM)+Pepstatin A (10μm), or 1μM BAM15+ Halt Protease Inhibitor cocktail (1x). Pattern ERGs were recorded 24 hrs post injury and weekly up to 4 weeks after the injury to monitor changes in ganglion cell function. At day 28 the animals were euthanized and the eyes enucleated and fixed in preparation for staining. Surviving ganglion cells were selectively stained with RBPMS (RNA binding protein with multiple splicing) and imaged on a confocal microscope. Figure 1, pattern ERG responses in the a) affected eyes of C57bl6 mice treated with BAM15+PI cocktail shows a ~3-fold improvement ($p<0.05$) in amplitude 2 weeks post optic nerve crush (ONC) injury compared to Sham treated or BAM15+PI reversible. Additional doses may be beneficial and will be explored in the next report. b) Unaffected eyes share similar response properties across all groups. c) Contour map plot of ganglion cells stained positive for RPBMS 4 weeks post ONC injury. Highest densities of surviving ganglion cells are shown in Yellow-Red. d) Plot of average number of RPBMS+ ganglion cells per treatment group. Image analysis performed in imageJ revealed ~1000 more ganglion cells in the BAM15+PI cocktail group compared to untreated controls ($p<0.05$).

The use of a protease inhibitor cocktail (which includes reversible as well as irreversible protease inhibitors) in conjunction with BAM15 shows improved neuroprotection following ONC compared to BAM15 with the addition of reversible protease inhibitors.

Subtask 2: Identify appropriate dose/timing of delivery of pharmaceutical agents. Effective doses of DMM (2mM) and BAM15 (1μM) have been determined from preliminary data.

Timing of delivery will be varied over the first 5 days post injury during which time there is typically a significant loss in ganglion cell number.

Projected Milestone (7-12 months)

From 2nd quarterly report: The experiments were conducted using other funds with an existing, approved animal use protocol (mouse). 8 mice were evenly divided into 2 treatment groups (DMM, Acriflavine + Methylene Blue + Resveratrol). Preliminary data provided in the proposal showed that 0.88M DMM was effective at improving ganglion cell viability following optic nerve crush in awake 13-lined ground squirrels. In those experiments DMM was given prior to optic nerve injury and subsequently every other day following the injury for up to 5 days.

The following experiment was performed to determine whether a single injection of 0.44M DMM after optic nerve injury provided protection against microglial-induced ganglion cell death. Additionally, as stated in the proposal we "concurrently explored the use of other pharmaceutical agents that target the same identified pathways in the event that another drug has improved bioactivity (works at lower concentrations) or has improved delivery." In this regard we investigated whether a cocktail that included Acriflavine+Metheylene Blue+Resveratrol offered improved ganglion cell protection. Whereas DMM targets succinate oxidation which leads to ROS production, stabilization of hif-1alpha, and microglia activation, Acriflavine directly prevents hif-1 alpha dimerization preventing microglial-induced ganglion cell death. Acriflavine has also been shown to be deliverable through eye drops in the suppression of choroidal neovascularization and thus may have improved delivery. Methylene Blue targets tau accumulation in neurodegenerative disorders such as Alzheimer's disease. Tau recently has been shown to be involved in the death of retinal ganglion cells following optic nerve crush in rats (Oku et al., 2019) and thus Methylene Blue may provide additional cellular protection. Lastly, resveratrol was shown to delay RGC loss following traumatic optic injury in mice (Zuo et al., 2013) and we have shown that it can dramatically improve cell viability in vitro in response to other stressors such as cold exposure (4 degrees) mimicking the ability of 13-lined ground squirrel derived cells. Each mouse was subjected to unilateral crush injury of the optic nerve (left eye). The contralateral, uninjured optic nerve (right eye) served as a control. At the time of injury the mice were intraocularly injected with 2uL of either 0.44M DMM or 400uM Acriflavine + 20uM Metheylene Blue + 60uM Resveratrol. Pattern ERGs were recorded prior to injury and 24 hrs post injury and weekly up to 2 weeks after the injury to monitor changes in ganglion cell function. At day 14 the animals were euthanized and the eyes enucleated and fixed in preparation for staining.

Surviving ganglion cells were selectively stained with RBPMS (RNA binding protein with multiple splicing) and imaged on a confocal microscope. Figure 1a, pattern ERG responses in the a) affected eyes of C57bl6 mice treated with a single injection of DMM shows reduced pERG response 13 days post optic nerve crush (ONC) injury. b) pERG responses in uninjured/untreated eyes. Unaffected eyes share similar response properties across all groups. c) Acriflavine + Methylene Blue + Resveratrol were injected at Day 0 and again 4 days later. The pERG response amplitude is preserved post injury at 13 days. d) pERG amplitudes plotted as a function of time post injury showing that Acriflavine + MB + Resv provide 2.5-fold greater pERG response amplitudes compared to DMM or Sham injected. e) Contour map plot of ganglion cells stained positive for RPBMS 2 weeks post ONC injury. Highest densities of surviving ganglion cells are shown in Yellow-Red. f) Plot of average number of RPBMS+ ganglion cells per treatment group. Image analysis performed in imageJ revealed ~1000 more ganglion cells in the DMM group compared to Acriflavine+MB+Resv. Its possible that the DMM administered not only metabolically inhibited the activation of microglial cells but also inhibited retinal ganglion cell responses.

From 3rd quarterly report:

The experiments were conducted using other funds with an existing, approved animal use protocol (mouse). 8 mice were divided into 2 groups (Sham, 0.44M DMM). Each mouse was subjected to unilateral crush injury of the optic nerve (right eye). The contralateral, uninjured optic nerve (left eye) served as a control. One injection of either sham PBS or 0.44M DMM (2µL) is provided the day prior to ONC injury. Pattern ergs were recorded 24hr later prior to injury to assess effect of DMM on ganglion cell function. No detriment to the PERG amplitude was detected with 0.44M DMM. Follow up injections
were performed using a nanoinjector 2 days post injury and 5 days post injury. Retinas were again evaluated on day 7 with pattern ERG prior to being euthanized. The eyes were enucleated and fixed in preparation for staining. Surviving ganglion cells were selectively stained with RBPMS (RNA binding protein with multiple splicing) and imaged on a confocal microscope. Figure 1, pattern ERG responses in the ONC eyes of C57bl6 mice treated with a) PBS or b) DMM 1-week after injury. c) PERG response amplitude data summarized in the bar chart shows no significant improvement (p=0.54) in the amplitude of DMM treated animals 1-week post optic nerve crush (ONC) injury compared to Sham treated. Figure 2, Contour map plot of ganglion cells stained positive for RPBMS 1-week post ONC injury in a) Sham and b) DMM injected mice. Highest densities of surviving ganglion cells are shown in Yellow-Red. c) Plot of average number of RPBMS+ ganglion cells per

treatment group. Image analysis performed in imageJ revealed similar number of surviving ganglion cells in the DMM group compared to untreated controls (p=0.63) in agreement with the functional recordings (PERG).

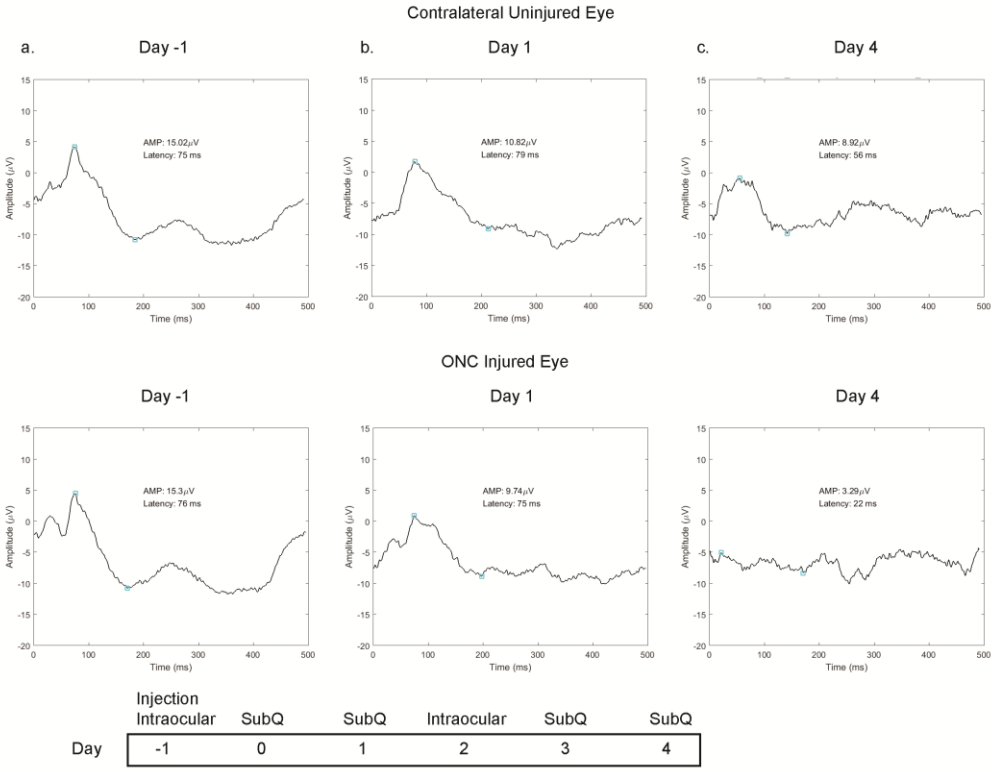
Recent Advances (4th quarter):

A higher dose, 0.88M DMM, was tested to determine if higher doses of DMM could recreate the protection observed in awake 13 lined ground squirrels. 4 mice were subjected to unilateral crush injury of the optic nerve (right eye). The contralateral, uninjured optic nerve (left eye) served as a control. One injection of .88M DMM (2µL) is provided the day prior to ONC injury. An additional intraocular injection was provided 2 days post ONC. On days when no intraocular injection was provided, a subcutaneous injection of 580mg/kg (100uL) was provided beneath the lateral skin in front of the hind leg.

Pattern ergs were one day prior to initial DMM intraocular injection and 24hr later prior to injury to assess effect of DMM on ganglion cell function. No detriment to the PERG amplitude was detected within 24hr following injection with 0.88M DMM. Follow up injections were performed using a nanoinjector 2 days post injury. Retinas were evaluated on day 4 post injury with pattern ERG prior to being euthanized. The eyes were enucleated and fixed in preparation for staining. Surviving ganglion cells were selectively stained with RBPMS (RNA binding protein with multiple splicing) and imaged on a confocal microscope.

Figure 1A and 1B, pattern ERG responses in the ONC eyes of representative C57bl6 mice (Fig. 1A, mouse #1444; Fig. 1B, #1448): a) baseline response prior to injury, b) 24h following initial DMM intraocular injection, c) 4 days after injury. The injection scheme is shown at the bottom. Figure 2, PERG response amplitudes summarized in the graph shows significant loss in the amplitude of DMM treated animals 4 days post optic nerve crush (ONC) injury compared to uninjured contralateral eyes. Figure 3, immunofluorescent images of retinal ganglion cells stained with RBPMS (red) 4 days post ONC. Figure 4, contour map plot of retinal ganglion cells shown in Figure 3 in a) uninjured contralateral eyes and b) ONC-injured DMM injected eyes. Highest densities of surviving ganglion cells are shown in Yellow-Red where 40 on the color scale corresponds to 5,800 cells/mm^2. c) Plot of average number of $RPBMS^+$ ganglion cells per treatment group. Image analysis performed in imageJ revealed 15,482 +/- 1499 surviving ganglion cells in the DMM treated group compared to 33,722 +/- 2129 RGCs in the uninjured control eyes (p=0.0004). Figure 5, immunofluorescent images of $IBA1^+$ microglial cells (green) shows microglia accumulation in the ganglion cell layer is inhibited by DMM administration. However, this suggests that although the cell bodies are preserved by DMM, long term treatment with DMM may silence ganglion cell activity.

Figure 1A. Representative recording of Pattern ERGs following ONC injury (Mouse #1444)



	Injection					
	Intraocular	SubQ	SubQ	Intraocular	SubQ	SubQ
Day	-1	0	1	2	3	4

Figure 1B. Representative recording of Pattern ERGs following ONC injury (Mouse #1448)

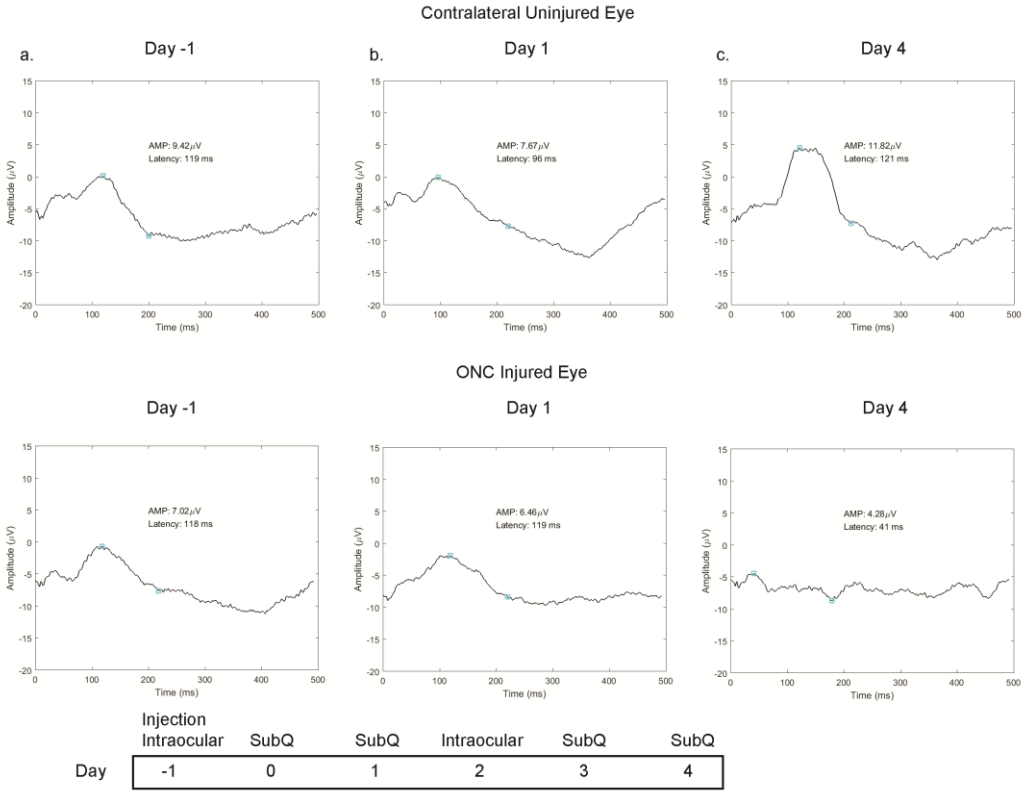


Figure 2. Pattern ERG Response Amplitudes

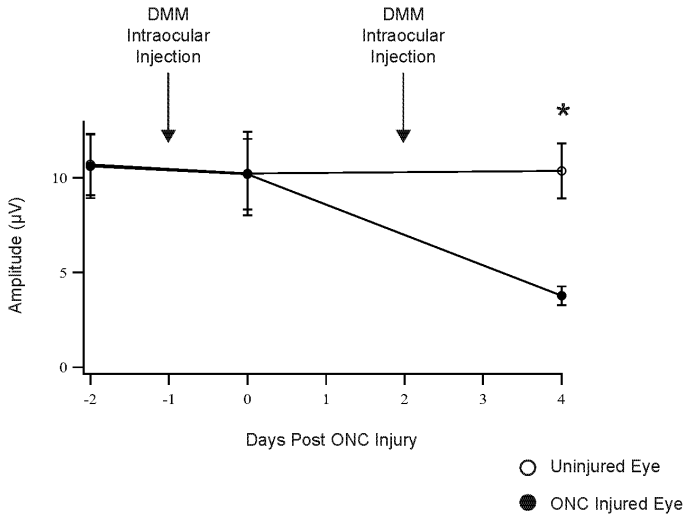


Figure 3. Immunofluorescent images of RGCs stained with RBPMS (red) 4 days after ONC.

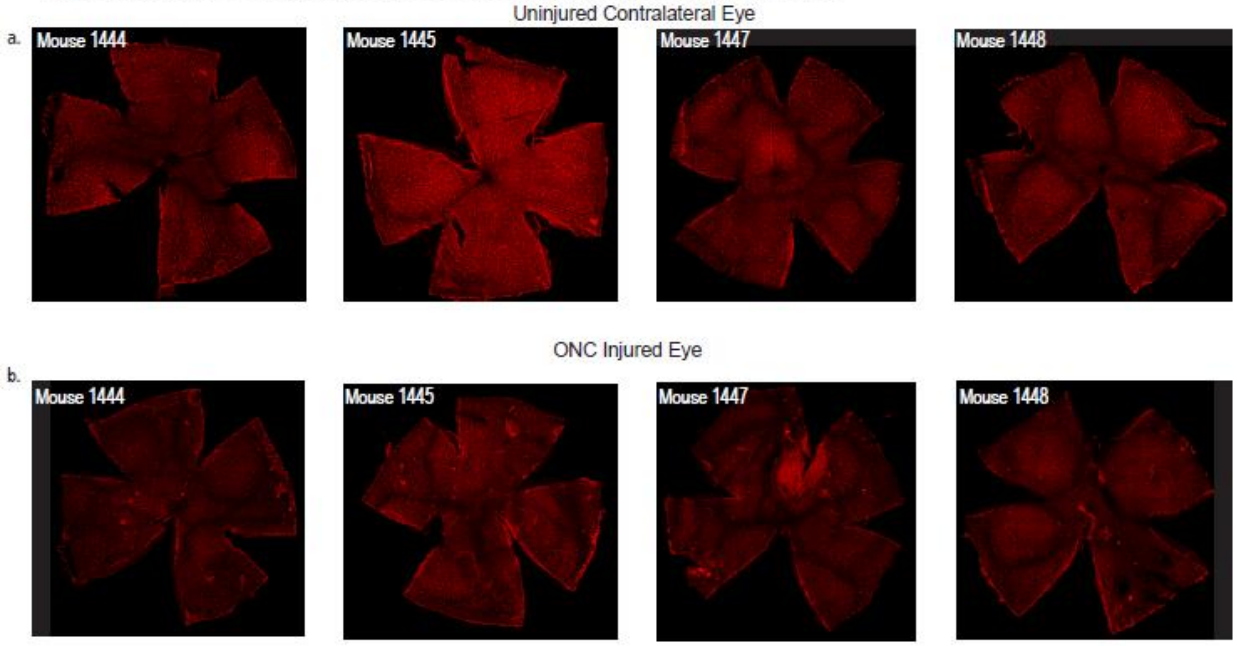


Figure 4. Contour Plots of RGC density 4 days after ONC.

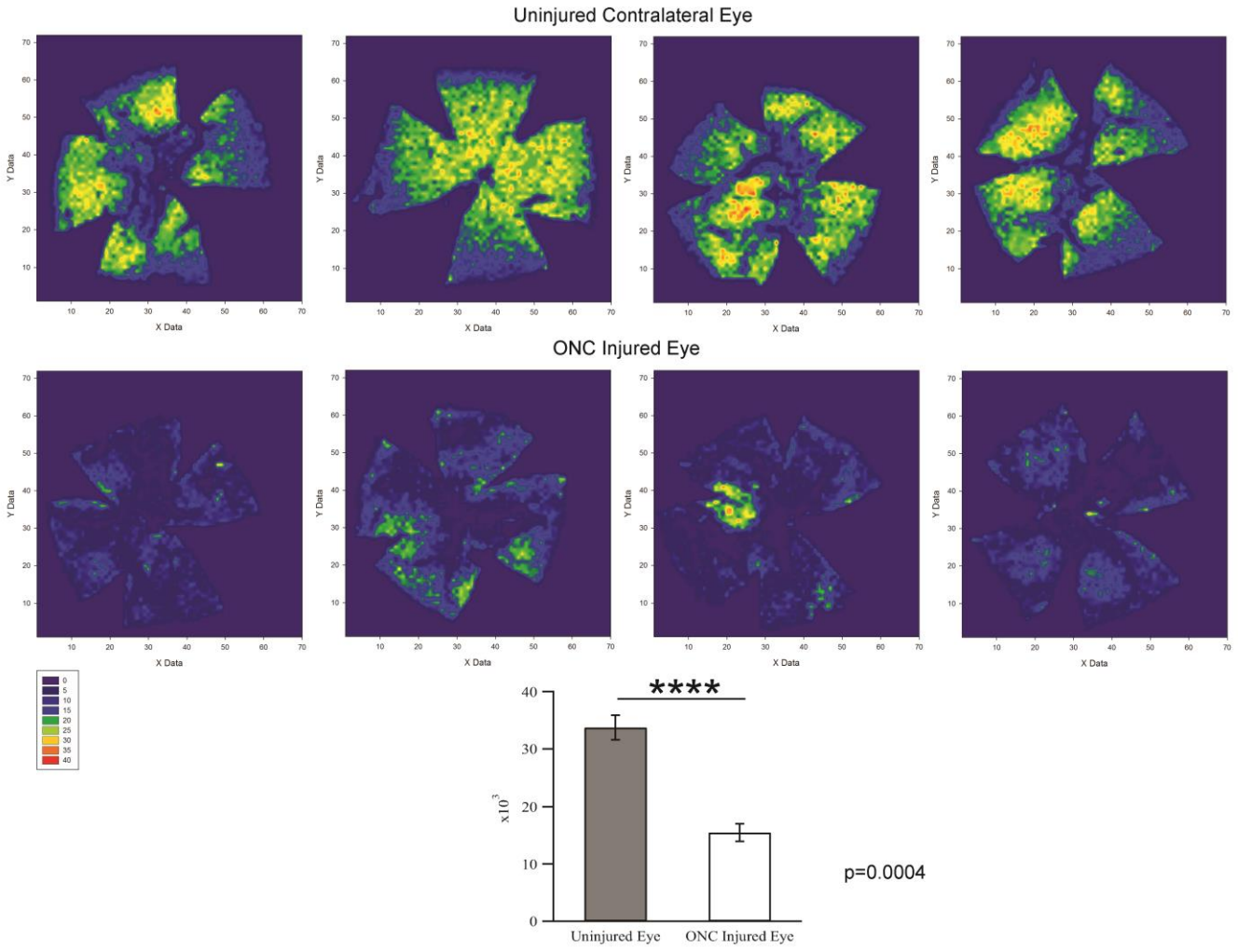
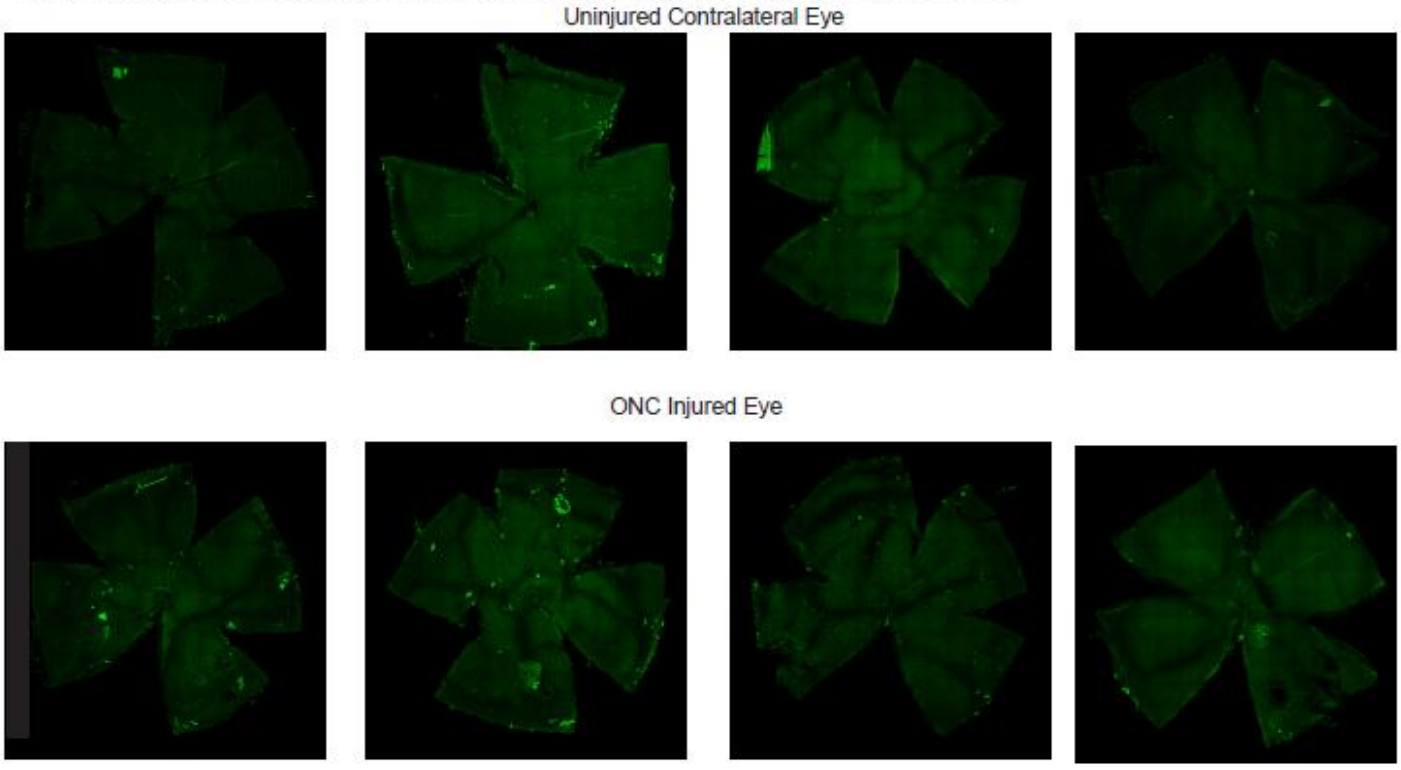


Figure 5. Immunofluorescent images of microglia cells stained with IBA1 (green) 4 days after ONC.



Work to be performed in CY 2020 and 2021:

Specific Aim 2: Development and optimization of hibernation-mimicking drugs delivery system

Major Task 2: Evaluate use of cell penetrating peptides in ocular delivery.

Subtask 1: Determine whether cell penetrating peptides can reach the neural retina in the ground squirrel model. FITC conjugated cell penetrating peptides (R-8; poly-arginine) 2.5ug/mL will be delivered to the animals 40uL drop/each eye for 15 min while under anesthesia. Animals will be evaluated 24 or 48 hours post eye drop delivery by fundus imaging or visualized post mortem with retinal flatmounts.

Subtask 2: Determine frequency of instilling drops containing cell penetrating peptides required to deliver DMM or BAM15/PI to the ganglion cells to adequately improve ganglion cell viability following optic nerve crush injury.

Milestone(s): Determination of feasibility of using cell penetrating peptides to deliver DMM or BAM15/PI to the ganglion cell layer at effective concentrations following optic nerve injury.

Specific Aim 3: Demonstrate broad applicability of treatment by evaluating the pathological changes underlying RGC death and treatment efficacy in a blast injury model.

Major Task 3: Evaluate topical eye drop delivery of hibernation inspired drugs to treat blast induce traumatic optic neuropathy

Subtask 1: Submit documents for IACUC approval at USU for animal use in the advanced blast simulator (ABS).

Milestone(s): Obtain IACUC approval

Subtask 2: Determine optimal parameters to produce blast induced detectable deficits in optic nerve function. Subject animals to ABS exposures.

Subtask 3: Assess deficits in optic nerve function by pERG and ultra high-resolution OCT (Bioptigen).

Subtask 4: Using blast parameters established in subtask 2, evaluate the effectiveness of the hibernation inspired treatments using the eye drop delivery system established in Aim2 to facilitate delivery of DMM or BAM15/PI. Assessed by pERG and ultra high-resolution OCT (Bioptigen). Post mortem retinal whole mounts (7 days/14 days/21days) will be stained for ganglion cells in order to quantify and compare to prior results using the optic nerve crush model.

Milestone(s): Demonstration of successful delivery of hibernation mimicking drugs and preservation of retinal function following blast injury to optic nerve; publication of 1-2 peer reviewed papers

IMPACT:

The short-term impact of this study on the field of vision research will be the development of novel hibernation-inspired neuroprotective drugs for the preservation of retinal ganglion cells following traumatic optic neuropathy. Currently there is no consensus treatment for vision loss attributed to direct or indirect injury to the optic nerve and thus this study meets an unmet clinical need. The long-term impact of such study would lead to the prevention of debilitating vision loss and significantly increase vision-related quality of life following traumatic optic neuropathy.

Patients that have sustained head injuries with acute trauma to the optic nerve will benefit from having an experimentally proven treatment plan that will lead to improved prognosis. The likelihood that a successful outcome of the proposed research project will lead to a practical application to preserve eyesight in events of trauma are high given the sufficient evidence and preliminary data that demonstrates that the use of pharmaceutical intervention to mimic hibernation dramatically improves ganglion cell viability. This research into developing and testing a novel eye drop delivery system will be beneficial to the vision research community as it is also broadly applicable for delivering targeted therapies to affected ocular tissues in inherited or age-related retinal degenerations that affect millions world-wide.

The ideas central to this project: 1) harnessing the neuroprotective effects of hibernation and 2) the topical eye drop delivery of retinal drugs hold the promise to change the standard of care and further our understanding of cellular adaptive strategies that enable hibernation and lead to profound, transformative discoveries in medicine and stimulate economic growth.

There has been nothing to report regarding the impact on technology transfer as the project is just entering Year 2. There has also been nothing to report regarding the impact on society beyond science and technology although we expect upon successful completion of the project a publication to of the results of the study will be a valuable contribution to the vision research community.

CHANGES/PROBLEMS: Nothing to report

PRODUCTS: Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS: No Change

Dr. Wei Li
Project Role: PI
Researcher Identifier (ORCID ID): 0000-0002-2897-649X
Contribution to Project: Provided project direction.

Dr. Steven Stasheff
Project Role: Clinician
Researcher Identifier (ORCID ID):
Contribution to Project: Supplied literature review and suggestions for improving pattern ERGs on mice.

Dr. Francisco Nadal Nicolas
Project Role: Post-Doctoral Fellow
Researcher Identifier (ORCID ID): 0000-0003-4121-514X
Contribution to Project: Performed optic nerve crush injuries and imaged immunostaining (Rbpms)

Dr. Kiyoharu J. Miyagishima
Project Role: Co-PI/Staff Scientist
Researcher Identifier (ORCID ID): 0000-0002-9744-3152
Nearest Person month worked: 2
Contribution to Project: Recorded pattern ERGs and performed data analysis, immunostaining, and imaging. Provided project reports.

Dr. John Ball
Project Role: Staff Scientist
Researcher Identifier (ORCID ID):
Contribution to Project: Will investigate alternative methods to assess blast injury parameters with the goal of reducing the number of animals.

SPECIAL REPORTING REQUIREMENTS: Quad Chart (see attached)

Targeted Treatment of Traumatic Optic Neuropathy Inspired by Neuroprotective Adaptations of Hibernation
Log Number: VR180285
Award Number: CDMRPL-18-0-VR180205

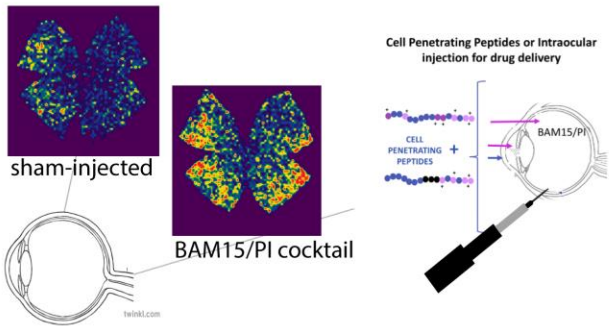
PI: Dr. Wei Li **Org:** National Institutes of Health **Award Amount:** $500,000 total cost dollars

Study/Product Aim(s)

* Evaluation of hibernation-mimicking drugs to promote RGC survival.
* Development and optimization of hibernation-mimicking drugs delivery system.
* Demonstration of our treatment's broad applicability by showing that pathological changes similar to those in the ONC model also underlie RGC death in a blast injury model.

Approach

We propose to use the identified mechanisms underlying neuroprotection in hibernating ground squirrels to halt mitochondrial metabolic changes prior to the onset of inflammation resulting from optic nerve injury. Preliminary work identified several compounds that successfully target these differentially regulated pathways including dimethyl malonate (DMM) and BAM15 + protease inhibitors. Cell penetrating peptides particularly those developed from the human immunodeficiency virus (Tat – trans-activator of transcription, poly arginine) have previously been shown to efficiently enter the ocular posterior segment when applied topically and will be used to formulate eye drops containing our hibernation-mimicking compounds.



Accomplishment: To date BAM15/PI cocktail has shown the most promise in preserving retinal ganglion cells following ONC injury and will be further evaluated to determine whether multiple doses supplied with cell penetrating peptides can improve viabilibty.

Timeline and Cost

Activities CY	19	20	21
Evaluation of hibernation-mimicking drugs to treat optic nerve crush			
Development of topical ocular drug delivery system			
Evaluation of hibernation-mimicking drugs to treat blast-induced ocular trauma			
Estimated Budget ($K)	**$433000**	**$33500**	**$33500**

Updated: (08/11/20)

Goals/Milestones

CY19 Goal Optimize selection, dose, and timing of pharmaceutical agents using optic nerve crush injury model

- ☒ Evaluate whether reversible protease inhibitors improves neuroprotection.
- ☒ Identify appropriate dose/timing of delivery of pharmaceutical agents.

CY20 Goals Development and optimization of hibernation-mimicking drugs delivery system

- ☐ Determine whether cell penetrating peptides (CPPs) can reach the neural retina
- ☐ Determine frequency of instilling drops with CPPs to adequately deliver drugs

CY21 Goal Evaluate topical eye drop delivery of hibernation inspired drugs to treat blast induce traumatic optic neuropathy

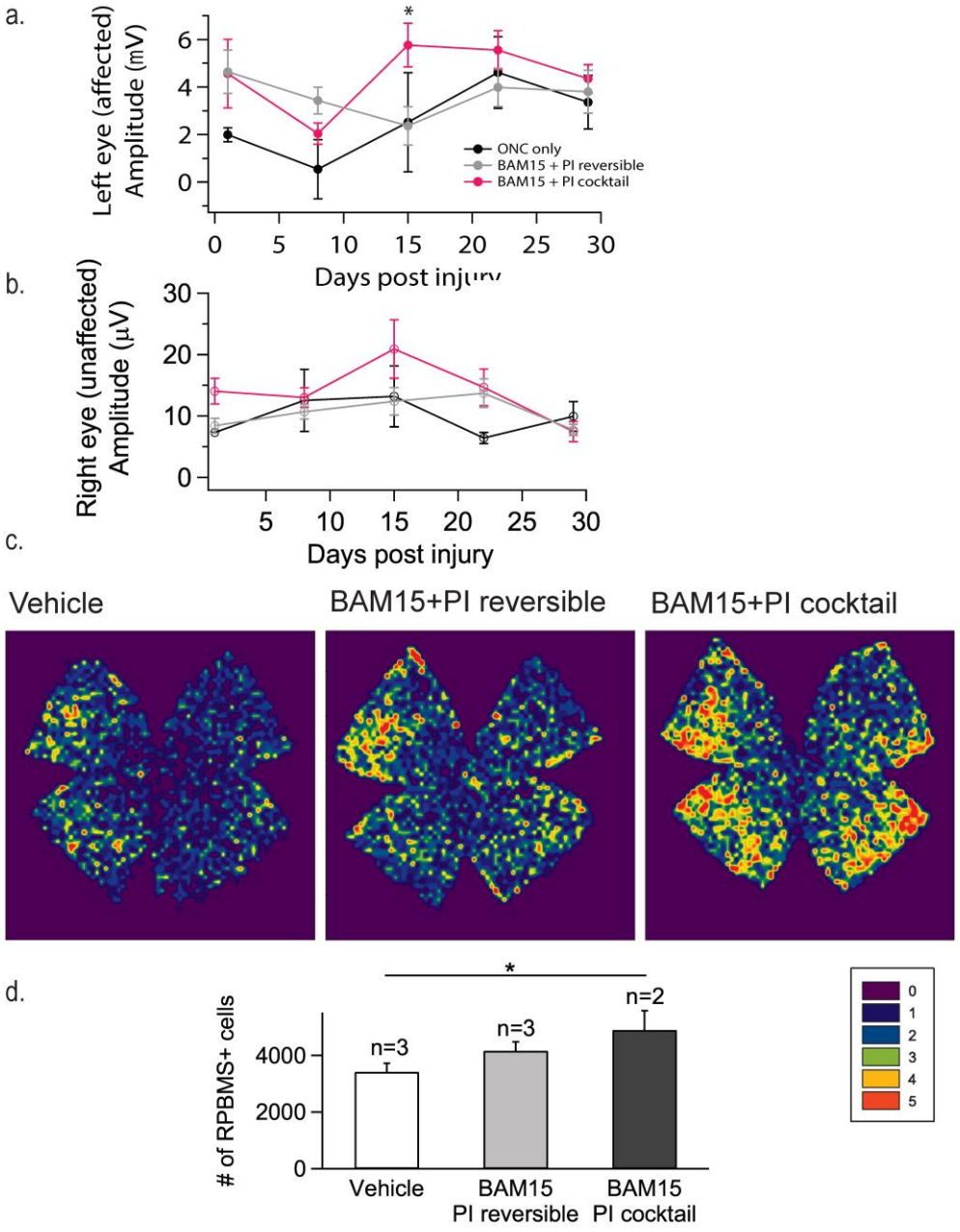
- ☐ Submit documents for IACUC approval at USUHS for advanced blast simulator
- ☐ Determine parameters to produce blast induced deficits in optic nerve function
- ☐ Evaluate drugs effectiveness to treat blast-induced ocular injury to optic nerve

Budget Expenditure to Date
Projected Expenditure: $500,000 total cost (3 years)

APPENDICES: Accompanying figures for previous Quarterly Reports

From 1st quarterly report:

Figure 1



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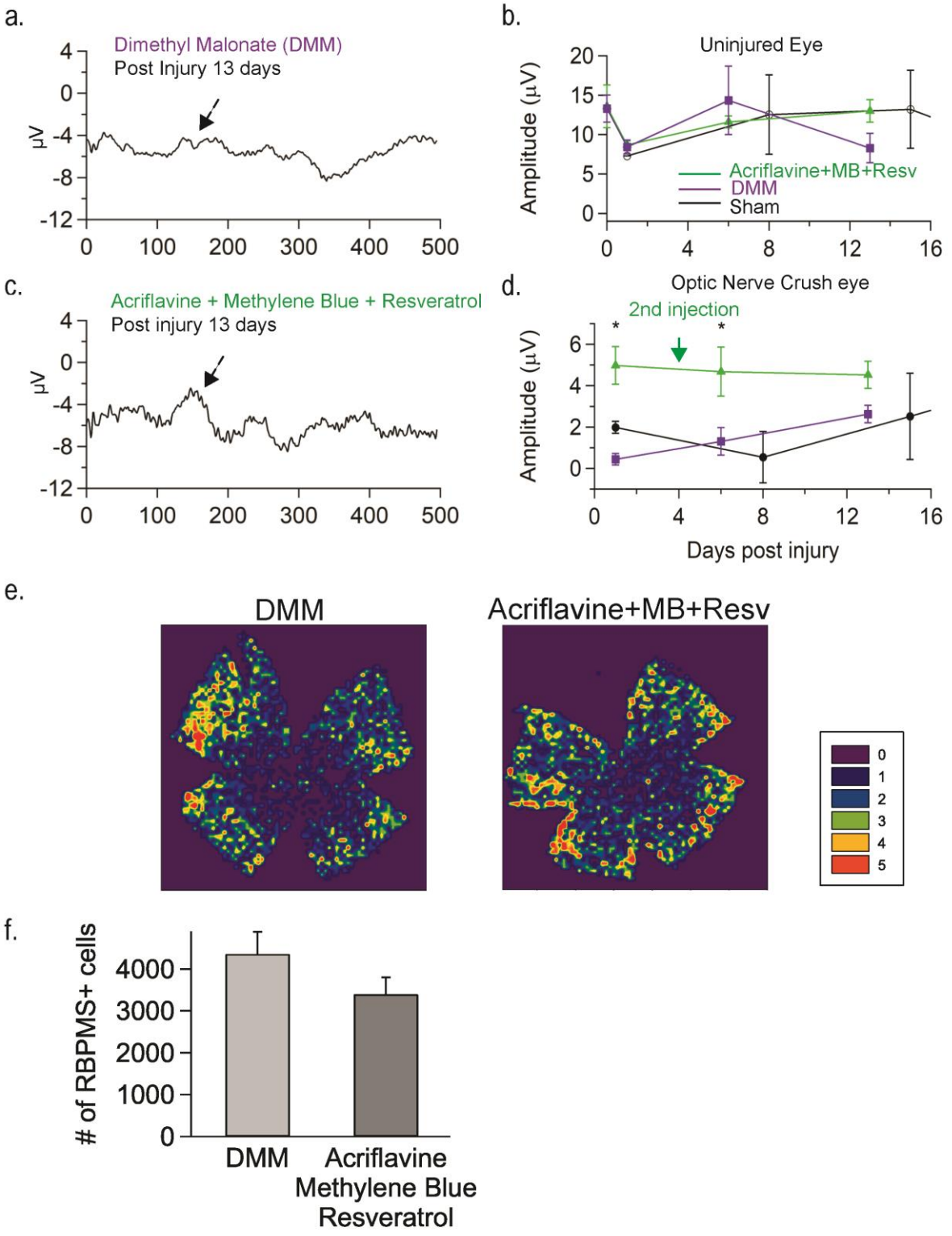
From 2nd quarterly report:

Figure 1



From 3rd quarterly report:

Figure 1.

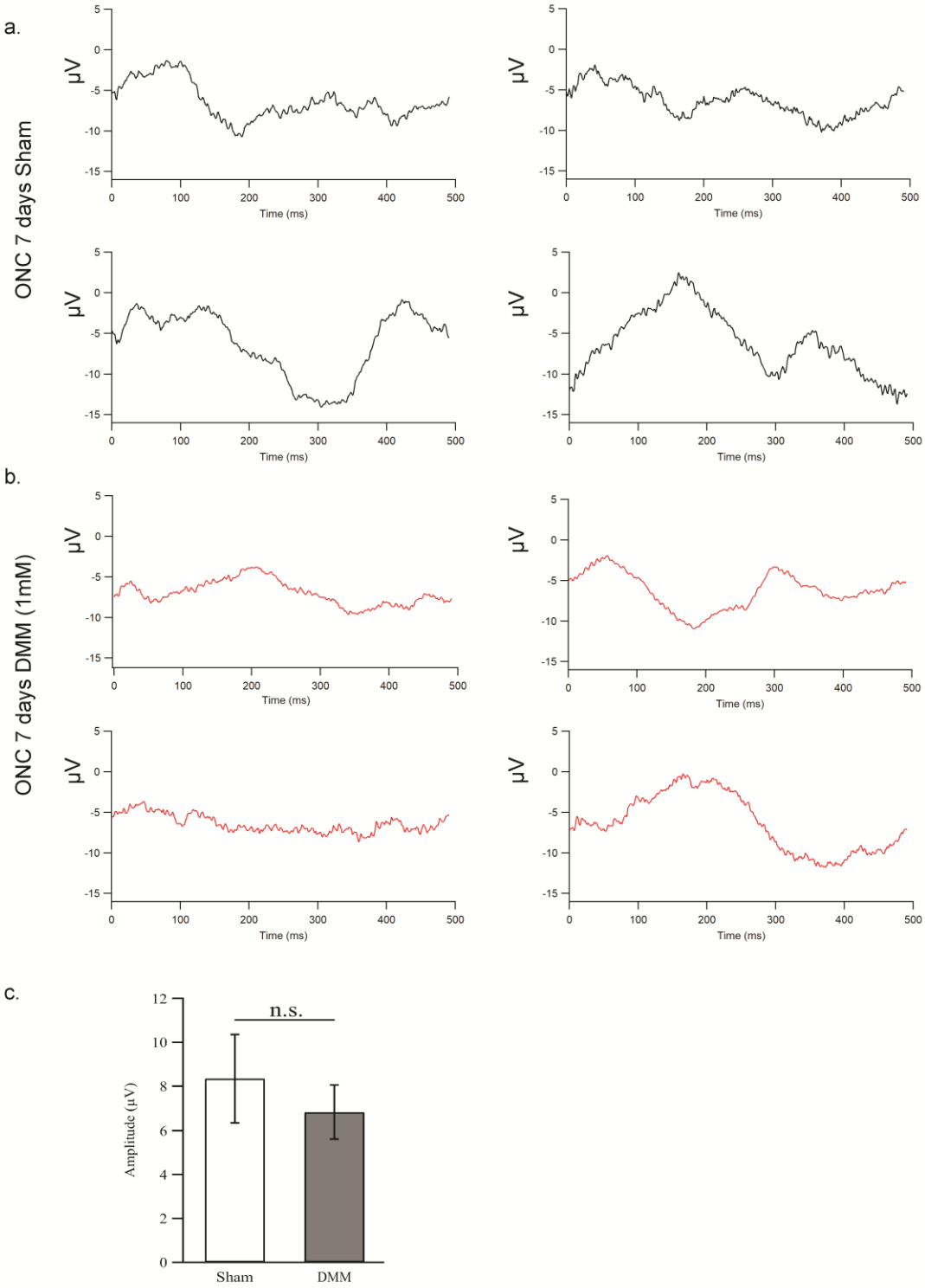
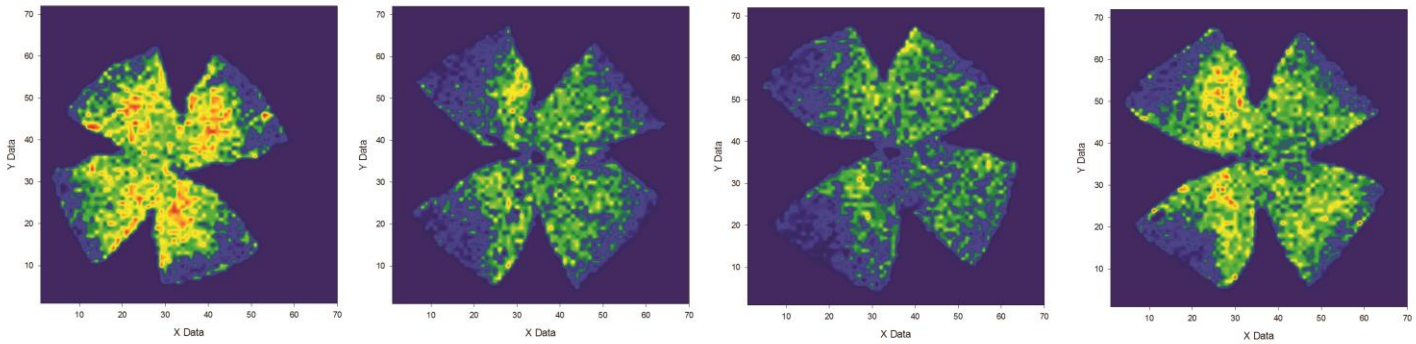
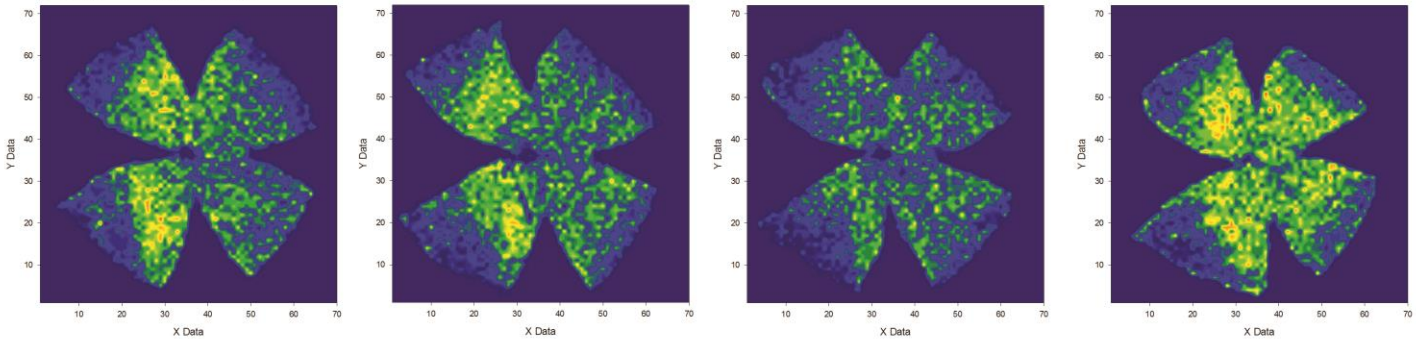


Figure 2.

a. ONC + Sham



b. ONC + DMM (1mM)



c.