MILITARY INTERDEPARTMENT PURCHASE REQUEST:

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

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CONTRACTING ORGANIZATION: Department of the Army, US Army Medical Research and Development Command (USAMRDC)

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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. Presently there is no consensus on the appropriate treatment for traumatic optic neuropathy. Damage to the optic nerve is irreversible as the nerve fibers don't have the capacity to regenerate on their own, thus preservation of the ganglion cells and their axons under these adverse conditions by mimicking hibernation would represent a novel way to prevent vision loss.

15. SUBJECT TERMS
Traumatic Optic Neuropathy (TON), Hibernation, Retinal Ganglion Cells (RGCs), Neuroprotection

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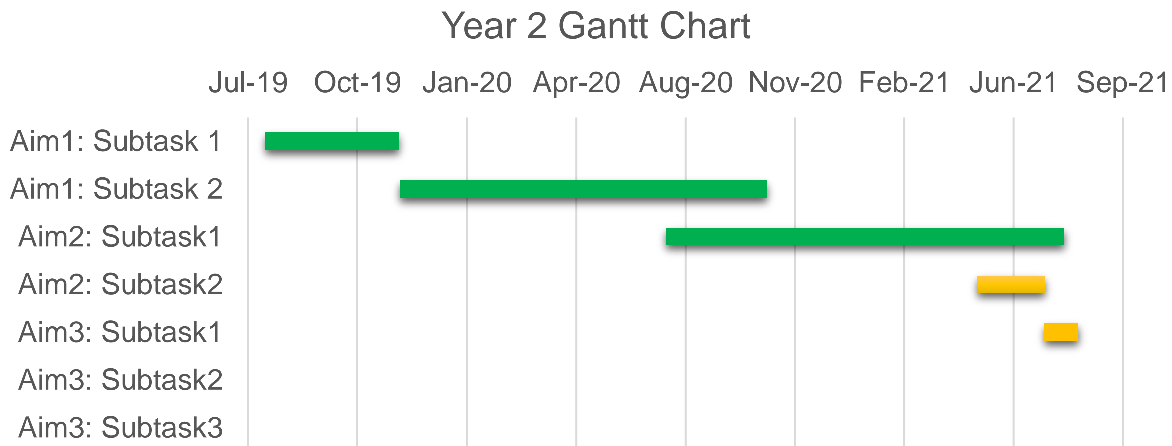
INTRODUCTION: The innate ability of hibernators to respond uniquely to optic nerve injury and prevent permanent loss of vision due to retinal ganglion cell (RGC) loss has prompted the ambitious goal of translating the cellular strategies involved in hibernation to preserve vision in soldiers that have 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 2) we have successfully optimized the dose and timing of administration of DMM post optic nerve crush (ONC) injury to protect RGCs.*
- *We explored the use of other pharmaceutical agents including icilin, acriflavine, and mitoQ which targets the same identified pathways by inhibiting microglia activation.*
- *We have explored DARC (detection of apoptosing retinal ganglion cells) as a method to noninvasively monitor RGC viability in vivo as a tool for screening drug efficiency.*
- *We published a manuscript entitled, "Establishing the ground squirrel as a superb model for retinal ganglion cell disorders and optic neuropathies" that provides a detailed anatomical comparison of the RGCs in ground squirrels with other rodent models. The number and distribution of RGCs in squirrels are more closely matched to primates and share a similar interlocking pattern between RGC axons and astrocytes in the retina nerve fiber layer – an anatomical relationship not found in other rodent models. We also fully characterized the optic nerve crush injury model and exploited the unique optic nerve in ground squirrels to devise a partial injury model where the extent of the injury to the RGCs could be precisely controlled to spare a portion of the retina in the same eye as an ideal control.*
- *We are preparing another manuscript for submission detailing the use of DMM to inhibit microglial cell activity to protect RGCs after optic nerve injury.*
- *COVID-19 restrictions on travel have prevented our fellow from participating in in-person professional development workshops and attending scientific conferences. However, he presented our recent work at the International Hibernation Symposium (2021).*
- *The project was presented internally at our weekly lab meeting for discussion 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.*
- *We have completed Specific Aim 1 (Optimize selection, dose, and timing of pharmaceutical agents using optic nerve crush injury model) and made significant progress in Specific Aim 2 (Development and optimization of hibernation-mimicking drug delivery system). We have reached out to companies that specialize in protein synthesis and the conjugation of CPPs to DMM does not seem possible since there is no active group on the compound. The dimethyl malonate is a diester derivative of malonic acid. It is only possible to conjugate the malonic acid monomer but not the dimer.*
- *Further we evaluated the use of CPPs to deliver L-AP4 (which blocks ON-bipolar responses) to the retina as a proof of concept with a reliable readout (reduction of the b-wave of the ERG) and have shown that in the ground squirrel that CPP delivery of L-AP4 at bioactive concentrations was not feasible.*
- *Future experiments will focus on establishing the blast model of traumatic optic nerve injury in ground squirrels and determining whether the DMM treatment can protect RGCs*

- *We will begin AIM3 (Year 3) focusing on using DMM (intravitreal injection using alternate-day administration) which we have shown works well in the optic nerve injury model as well as a hydrogel approach (single intravitreal injection) to evaluate the effectiveness of a single injection. We have begun the process of obtaining IACUC approval for the ground squirrel blast injury model in preparation for AIM3. We are scheduled to receive rat or mouse cadaver eyes from Dr. McCabe at USUHS in order to provide evidence to the NIH Veterinary Research and Resource Section that traumatic optic neuropathy can be achieved in this blast model without extensive collateral bodily injury.*
- *Descriptions of AIMs 1-3 are provided below along with the new findings from fourth quarter (Year).*



Milestone(s) Achieved: (Year 1) 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. (Year 2) Determination of feasibility of using cell penetrating peptides to deliver therapeutics to the ganglion cell layer at effective concentrations following optic nerve 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 2: Identify appropriate dose/timing of delivery of pharmaceutical agents. Timing of delivery will be varied over initial days post injury during which time there is typically a significant loss in ganglion cell number.

Projected Milestone (7-12 months)

<u>From 1st quarterly report</u>: The experiments were conducted using other funds with an existing, approved animal use protocol (mouse). 4 mice were selected for testing the following treatment Acriflavine (400µM)+MitoQ (0.15µM). 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. All injections (2µL) were provided post injury beginning approximately 1-2 hours post injury and follow up injections were performed using a nanoinjector on post injury days 1, 3, and 5. Animals were euthanized on day 7. One animal had an inflamed eye and could not be injected on days 3 and 5 and was removed from the study. Transcardial perfusion was performed and 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 activated microglia were stained with CD68 and imaged on a confocal microscope. Figure 1A and 1B, representative immunofluorescent images of RBPMS labeled RGCs quantified using the custom matlab software described in Guymer et al 2020. RGC isodensity maps

are provided with the highest densities of surviving ganglion cells shown in yellow. Despite treatment with acriflavine (hif-1alpha inhibitor) and mitoQ (mitochondrial-specific antioxidant) the microglial inflammatory response (CD68+ cells shown in green) was not abated. Figure 1C, quantification of the number of surviving RGCs is provided in these mice in comparison to the sham control mice (ONC 7days, PBS injected) described in the previous reporting period. Note: Multiple injections of acriflavine/mitoQ appears to make the lens opaque perhaps due hypoxia's suggested role in lens fiber differentiation and maintenance of lens transparency. This discovery can be explored further in a separate study on cataractogenesis.

In a separate study 3 mice were selected for testing whether multiple injections of the BAM15 (1µM) /PI (1x) treatment described in Report #1 could provide additional benefit. All injections (2µL) were provided post injury beginning approximately 1-2 hours post injury and follow up injections were performed using a nanoinjector on post injury day 1, 3, and 5. Animals were euthanized on day 7. Unfortunately, only one mouse survived due to cage flooding. Figure 2, the RBPMS stained RGCs, the automated cell count mask, and the isodensity plots are provided along with the CD68+ microglia staining.

Figure 3, FITC conjugated cell penetrating peptides (R-8; poly-arginine) 5.0ug/uL were delivered to 3 animals, 5uL drop/each eye, for 15 min while under anesthesia. In Figure 3A cell penetrating peptides (CPPs) can be seen in the anterior chamber of an undilated mouse between the cornea and the iris (yellow arrow). Images were taken using a Heidelberg Spectralis OCT with anterior segment module. In Figure 3B, in mice subjected to dilation the cell penetrating peptides can be observed both above and below the iris suggesting dilation may facilitate the uptake and penetration of CPPs. In Figure 3C, the fluorescence of the eye is notably brighter following uptake of FITClabeled CPPs.

This study explored the use of other therapeutics similar to DMM aimed at minimizing ROS production and microglia activation but caused unintended side effects and resulted in minimal neuroprotection.

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

Subtask 2: Determine minimal frequency of instilling drops containing cell penetrating peptides

required to deliver compounds to the retinal ganglion cells to adequately improve ganglion cell

viability following optic nerve crush injury.

<u>From 2nd quarterly report:</u> The experiments were conducted using other funds with an existing, approved animal use protocol (mouse). In preliminary experiments, 5 mice were selected for testing. N=3 mice were subjected to optic nerve crush injury alone (untreated) and N=2 mice were provided Icilin (300µM) that was delivered in suspension using 5µL FITC conjugated cell penetrating peptides (R-8; poly-arginine; 2.5ug/ml). Animals were euthanized on day 7. Transcardial perfusion was performed and 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 activated microglia were stained with CD68 (inset) and imaged on a confocal microscope. Figure 1A, representative immunofluorescent images of RBPMS labeled (red) RGCs. Figure 1B, RGCs were quantified using the custom matlab software described in Guymer et al 2020. Figure 1C, RGC isodensity maps are provided with the highest densities of surviving ganglion cells shown in yellow. Note: microglial inflammatory response in treated animals (inset, CD68+ cells shown in magenta) was not abated. Although the average cell count did not rise to the level of significance between ONC untreated (15089.3 +/- 1775.7) and icilin/CPPs treated (19942.5 +/- 1897.5) likely due to the limited sample size, the intensity of RBPMS staining was noticeably brighter in icilin treated mice indicating higher expression and potentially improved cell viability.

To explore the potential for this drug further, 8 additional mice subjected of ONC injury were selected for testing of Icilin delivery with (n=6) or without CPPs (n=2). 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. Icilin (5µL) was applied to the corneas of the injured eyes post injury beginning approximately 10-15 min post injury and a second drop was applied ~30 min later.

This study explored the use of icilin, a TRPM8 activator, that stimulates a "cold-response" that appears to increase the number of surviving RGCs by ~5,000 (a larger sample size is required to reach statistical significance) prompting further investigation into the utility of this drug.

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.

From 3rd quarterly report:

Anterior FA/OCT imaging of the ground squirrel eye was performed to determine whether FITC conjugated cell penetrating peptides (R-8, poly arginine) can be imaged noninvasively to monitor drug delivery. Figure 1A. shows a ground squirrel positioned in front of a Spectralis anterior segment module. Figure 1B. shows fluorescence images before and after application of the FITClabeled CPPs. The brightness of the FITC-CPPs is notable around the eyelids and toward the nasal part of the eye due to the animal's position. Figure 1C. We attempted OCT to detect FITC-labeled CPPs in the anterior chamber, and similar to de Cogan et al., 2017, we identified structures resembling particles (white arrow). Figure 1D. We performed additional tests using a Kimwipe paper with and without a drop of FITC-CPPs. The FA image shows an increase in fluorescence in the paper with FITC-CPPs however no visible change is associated with the OCT image. Figure 1E. We also performed time lapse imaging of the application of FITC-CPPs to the ground squirrel eye. The FITC-CPPs are extremely small and quickly envelop and disperse across the eye. We anticipate that the resolution required to image CPPs directly may surpass the limits of OCT.

Thus, we tested the feasibility of delivering a retinal marker (e.g. Annexin V) via intraocular injection to mice with optic nerve injury (day 8) to label apoptosing RGCs. This approach uses what is known as DARC (detection of apoptosing retinal ganglion cells) imaging and labels the dying cells with AnnexinV-conjugated to Alexa488. Figure 2A. FA images of the contralateral uninjured eye and the ONC (8d) 2h after injection with AnnexinV-488 demonstrating that dying RGCs can be labeled and imaged noninvasively. Figure 2B shows FA (left) and OCT (right) images indicating that the RGC labeling with AnnexinV precedes changes in retinal thickness as measured by OCT.

We plan to attempt AnnexinV delivery to optic nerve injured ground squirrel to determine if CPP delivery can facilitate RGC labeling and compare it to intraocular injection of Annexin V. This technique can also be used as an in vivo readout of RGC viability to assess our pharmacological treatments. We are in the process of updating our NIH protocol and will submit an amendment to ACURO before we begin.

Recent Advances (4th quarter):

The experiments were conducted using a combination of DOD funded (squirrel) and other funds with an existing, approved animal use protocol (mouse). In preliminary experiments, 10 mice were selected for testing. N=4 mice were subjected to optic nerve crush injury alone (untreated) and N=6 mice were injected with DMM (0.875M, 1uL) delivered on days 0, 2, 4 post ONC. Animals were euthanized on day 5. Transcardial perfusion was performed, and the eyes were enucleated and fixed in preparation for staining. Surviving ganglion cells were selectively stained with Brn3a and imaged on a confocal microscope. Figure 1A-B, representative RGC isodensity maps are provided with the highest densities of surviving ganglion cells shown in yellow (max: 5800 RGCs/mm^2). Figure 1C, the average cell count was significantly higher in DMM treated (32213 +/- 1640.6) compared to untreated (27785.2 +/- 1089.2)($p<0.01$).

In follow-up experiments, 12 mice were selected for testing. N=6 mice were subjected to optic nerve crush injury alone (untreated) and N=6 mice were injected with DMM (0.875M, 1uL) delivered on days 0, 3, 7 post ONC. Animals were euthanized on day 11. Transcardial perfusion was performed, and the eyes were enucleated and fixed in preparation for staining. Surviving ganglion cells were selectively stained with Brn3a and imaged on a confocal microscope. Figure 2A-B, representative RGC isodensity maps are provided with

the highest densities of surviving ganglion cells shown in red (max: 1700 RGCs/mm^2). Figure 2C, the average cell count was significantly higher in DMM treated (10187.5 +/- 1990.3) compared to untreated (4940.3 +/- 1160.3)($p<0.001$).

We also repeated this same experiment increasing the number of injections to every other day. Here N=5 mice were subjected to optic nerve crush injury and injected with DMM (0.875M, 1uL) delivered on days 0, 2, 4, 6, 8, 10 post ONC. Animals were euthanized on day 11. Transcardial perfusion was performed, and the eyes were enucleated and fixed in preparation for staining. Surviving ganglion cells were selectively stained with Brn3a and imaged on a confocal microscope. Figure 3A-B, representative RGC isodensity maps are provided with the highest densities of surviving ganglion cells shown in red (max: 1700 RGCs/mm^2). Figure 3C, the average cell count for DMM delivery every other day (eod) (9572.6. +/- 959.7) was similar to the DMM treatment described above for delivery on days 0,3,7. Thus additional injections of DMM did not enhance neuroprotection beyond the 0,3,7 day delivery strategy.

In Figure 4, we further explored the use of detection of apoptosing retinal ganglion cells (DARC) imaging as an in vivo assessment of our drugs on retinal ganglion cell viability. Figure 4A shows the fluorescence angiography image of annexin-488 conjugate labeled celled cells and the accompanying OCT which shows that the density of the bright scattering particles (Annexin positive cells) are above the retinal nerve fiber layer.Figure 4B layers the fluorescence angiography image over the OCT image to help visualize the position of the annexin positive cells in both images. Figure 4C, following *in vivo* imaging, we euthanized the animal and performed transcardial perfusion fixation. The eyes were enucleated and a retinal flat mount was in imaged on a confocal to identify the annexin positive cells. Since the vitreous above the retinal ganglion cell layer is removed for imaging RGCs in whole mounts we found that many of the annexin positive cells were removed in the process. Those that remained were clearly above the layer of retinal ganglion cells and their shape and morphology suggested they were likely microglial cells.

In Figure 5, we performed a proof-of-concept study to assess the ability of cell penetrating peptides to deliver drugs to the neural retina at bioactive concentrations. Figure 5A-B, here we used L-AP4 which is an inhibitor of ON-bipolar responses which can be easily measured by the b-wave of an ERG. Figure 5C-D, we measured the baseline ERG responses prior to drug delivery (t=0). We then delivered 2mM L-AP4 with CPPs or through intravitreal injection in light adapted ground squirrels to determine if L-AP4 would reduce the b-wave response. Figure 5E, at 30min post drug delivery we measured a decrease in the b-wave of the intravitreal injection but no change in the amplitude of the topically applied CPPs with L-AP4. The inhibition of the b-wave persisted even 60 min post intravitreal injection.

In Figure 6, we explored whether icilin, an activator of TRPM8, could be used to inhibit microglial activation *in vitro* and *in vivo*. We researched TRPM8 expression in retinal cells (right of plot, Figure 6A) and found that it is more highly expressed in these cell types compared to other tissues (eyeIntegration v1.05). In Figure 6B, we used BV2 microglial cells challenged with LPS to determine whether varying concentrations of icilin had an anti-inflammatory effect. We found that working concentrations between 100-300uM were optimal at inhibiting BV2 microglial inflammatory cytokine secretion (IL1B, IL6, TNFalpha). Figure 6C, we performed preliminary studies injecting icilin (300uM, 2ul intravitreally on days 0,2,4,6) into 2 mice following ONC (7d) to determine how well repeated intravitreal injection of icilin is tolerated. We plan to dismount and restain these slides to assess microglial activity using CD68 as a marker. Figure 6D, we have also begun writing our own retinal ganglion cell quantification software using Nikon's NIS Elements General Analysis (GA3) module which uses multiple conventional segmentation and AI tools that can be combined to create customized data measurement routines.

In the next phase of this project, we will attempt to deliver DMM in a smart hydrogel that is thermosensitive reducing the number of injections required to a single post injury injection. We plan on obtaining preliminary data from cadaver mouse/rat eyes to demonstrate that the blast injury model can be safely adapted to induce traumatic optic neuropathy in our ground squirrel animal model. Once we have IACUC and ACURO approval we will begin testing the effectiveness of DMM treatment in the blast injury model. Icilin will continue to be explored as a potential neuroprotectant.

Work to be performed in CY 2022:

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 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 optimized delivery system established in Aim2 to facilitate delivery of DMM. 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 3. Although the studies evaluating the utility of DARC (detection of apoptosing retinal ganglion cells) technology, revealed that the past decade of research in this field has been somewhat misleading. We found the annexin labeling which was presumed to label apoptosing retinal ganglion cells to be situated above the nerve fiber layer and most likely labeled inflammatory myeloid cells or microglia participating in phagocytosis of dying RGCs. This calls into question the application and interpretation of this technology in the clinical diagnosis of glaucoma currently in clinical trials.

There has also been nothing to report regarding the impact on society beyond science and technology although we recently published a paper describing the ground squirrel as an ideal model system to study traumatic optic neuropathies and we plan on submitting another manuscript on the neuroprotective effects of DMM for optic nerve injuries – the results of these studies will be valuable contribution to the vision research community.

CHANGES/PROBLEMS: As mentioned in our research proposal. If cell penetrating peptides could not confidently deliver the appropriate bioactive therapeutics to the neural retina we would explore sustained release options including hydrogels for delivery. We will make the appropriate changes to our animal protocol to include this change when we submit the addition of the blast injury model.

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). Also performed and trained Dr. Miyagishima on intravitreal injections for drug delivery. Assisted with testing DARC technology on mice and GS.

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: Performed optic nerve crush surgeries, recorded pERGs, performed data analysis, immunostaining, and imaging. Optimized analysis procedures using ImageJ, Matlab, and Nikon NIS Elements Analysis software. 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 have previously been shown to efficiently enter the posterior ocular segment when applied topically and will be used to formulate eye drops containing our hibernation-mimicking compounds. If CPP delivery of our drugs is unsuccessful, we will explore the use of controlled release materials following a single intraocular injection.

sham-injected
DMM-injected
(days 0, 3, 7)
Drug and polymer
in solution
20°C
37°C
Drug loaded in
thermoresponsive
hydrogel
polymer
drug

Accomplishment: DMM has been optimized to preserve retinal ganglion cells following ONC injury and will be evaluated in blast injury model. Bioactive concentrations have not been obtainabled with CPPs. A smart hydrogel will be explored for controlled release.

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: (08/19/21)

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

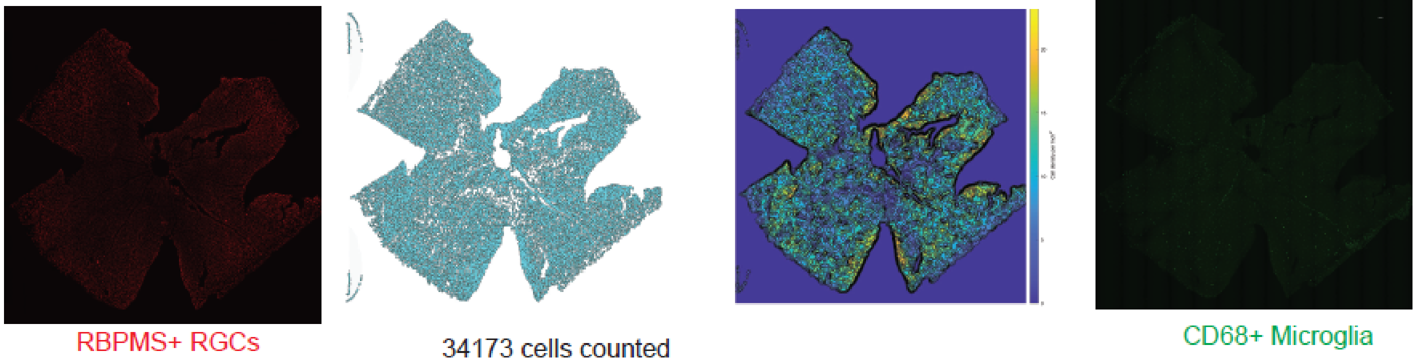
Budget Expenditure to Date
Projected Expenditure: $500,000 total cost (3 years)

APPENDICES: Accompanying figures for previous Quarterly Reports

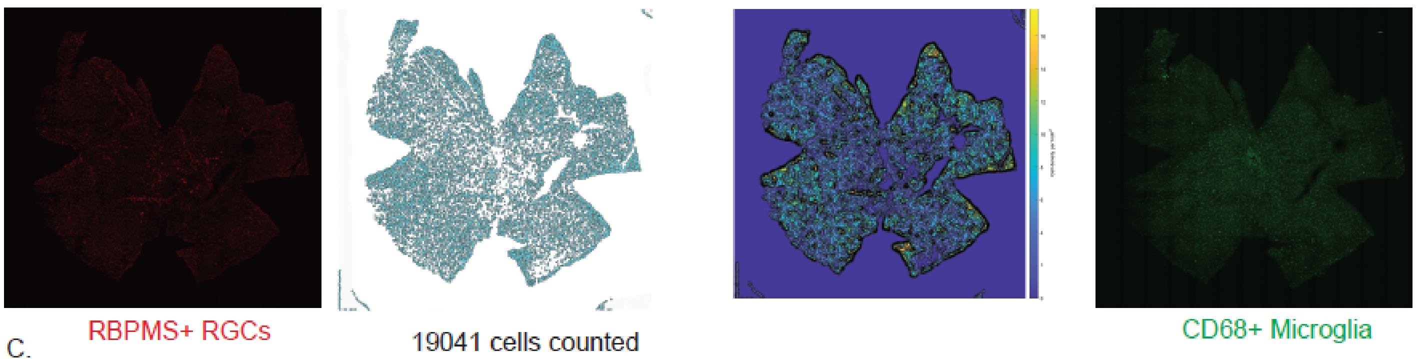
From 1st quarterly report:

Figure 1

A. Uninjured contralateral eye



B. ONC 7d + Acriflavine & Mito Q intravitral injection Day 0, 1, 3, 5



C.

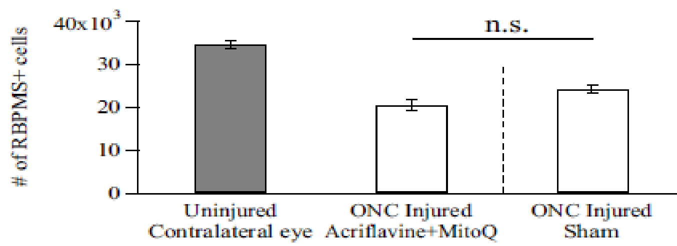
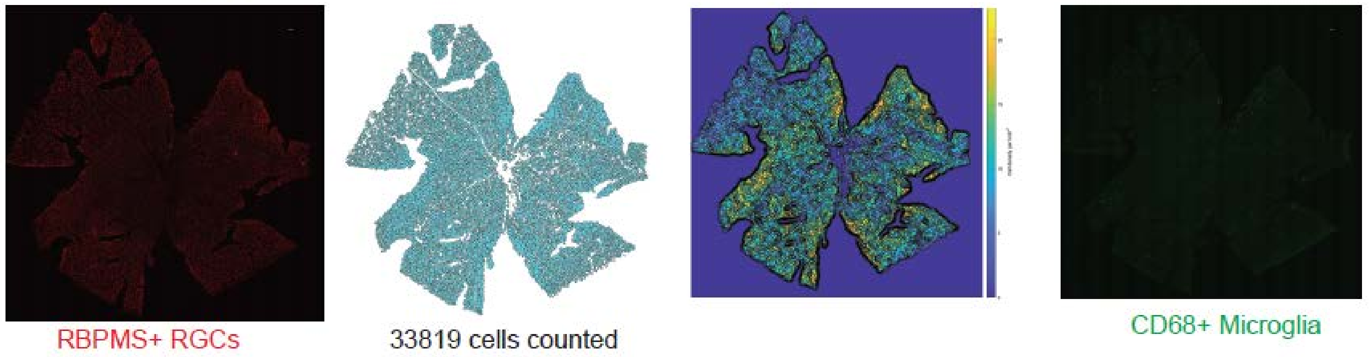


Figure 2

A. Contralateral Uninjured Eye



B. ONC 7d + BAM15/PI intravitral injection Day 0, 1, 3, 5

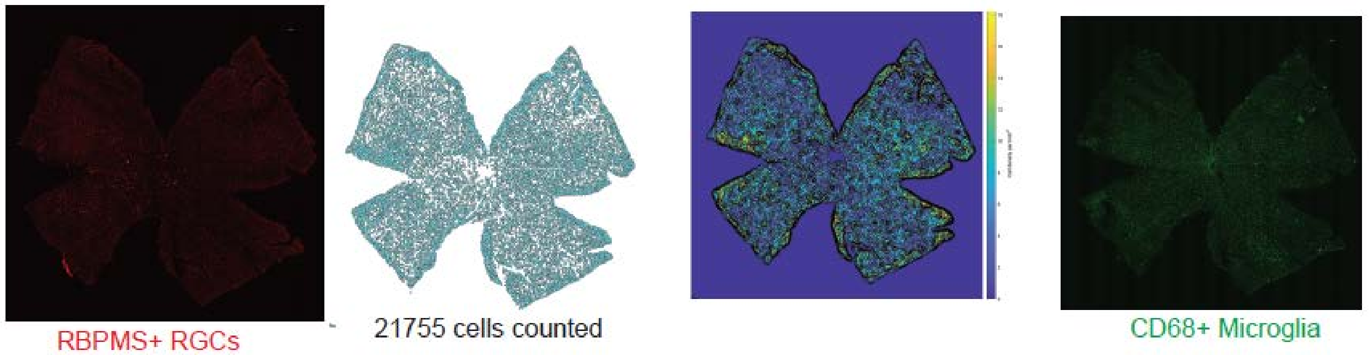
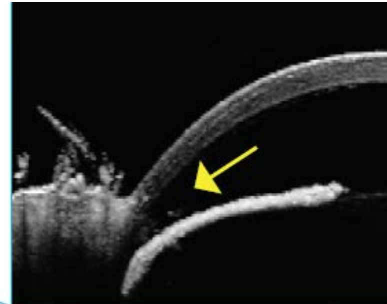
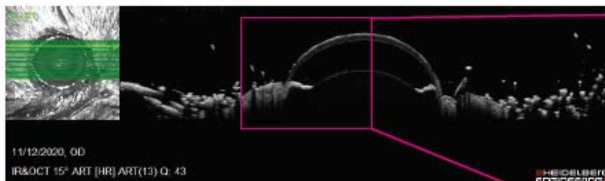


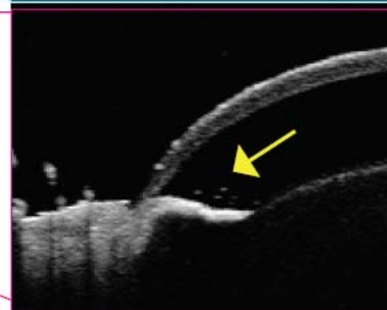
Figure 3

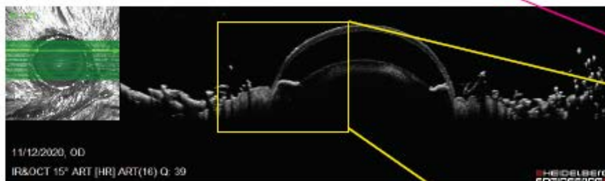
A. No dilation, FITC R8 CPPs added

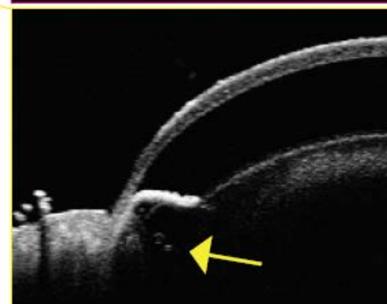


B. Dilation with phenylephrine (2.5%) and tropicamide (1%), FITC R8 CPPs added

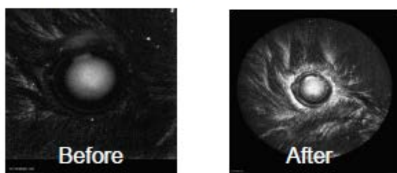








C. Fluorescence of Eye (Before and after FITC R8)

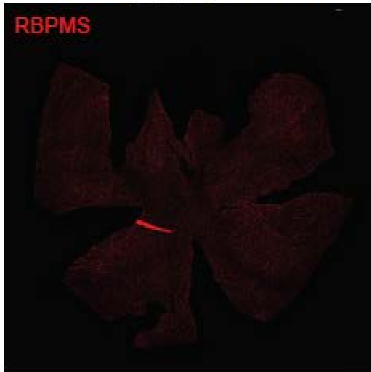


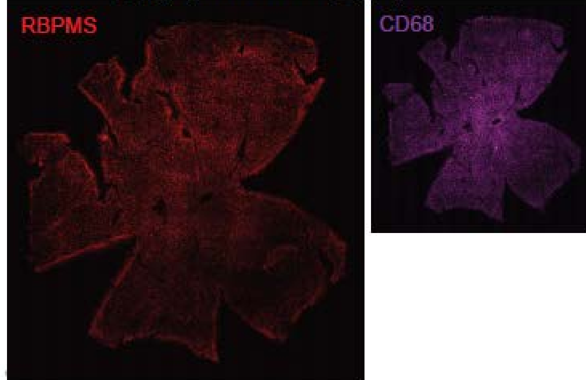
From 2nd quarterly report:

Figure 1.

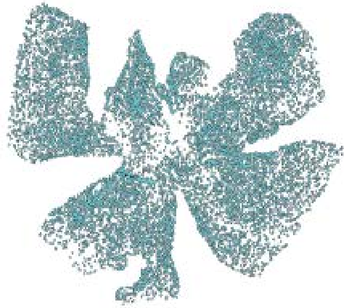
A. ONC injury (8d), Untreated

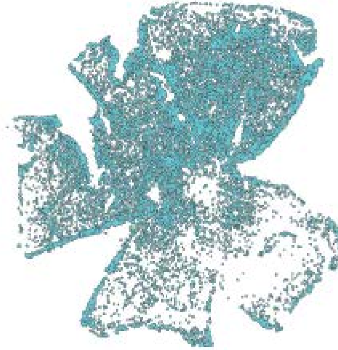
ONC injury (8d), Icilin treated (CPPs)



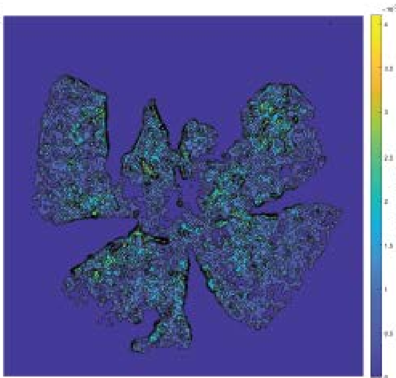


B.





C.



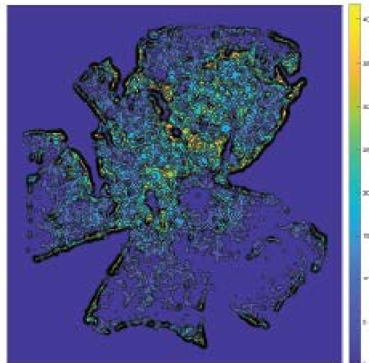
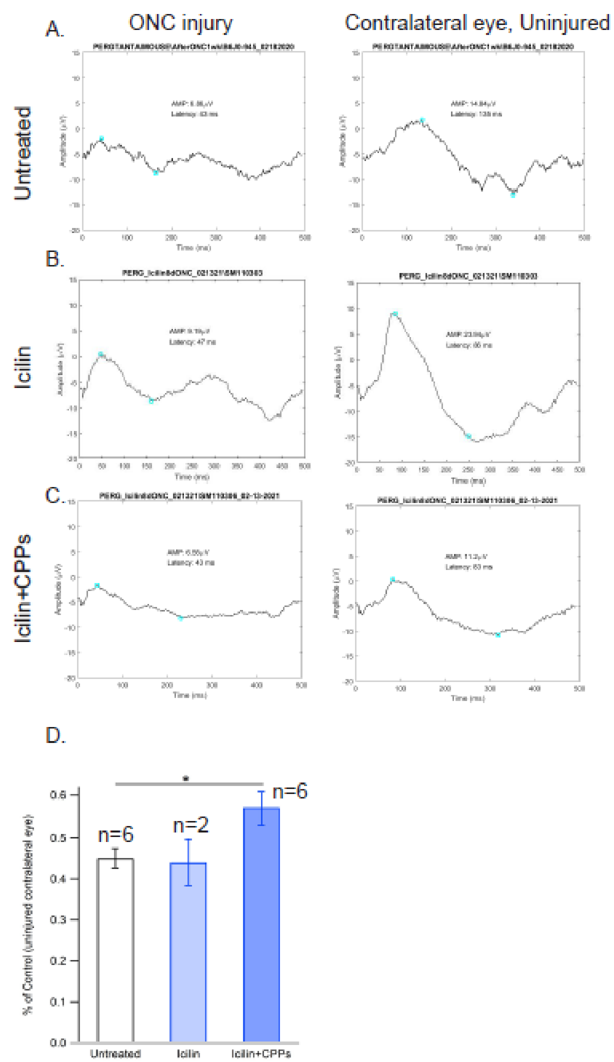


Figure 2



From 3rd quarterly report:

Figure 1.

A.

B.

C.

Refraction correction failed.

5/12/2021, OD

IR&OCT 20° ART [HR] ART(60) Q: 45

HEIDELBERG

D.

E.

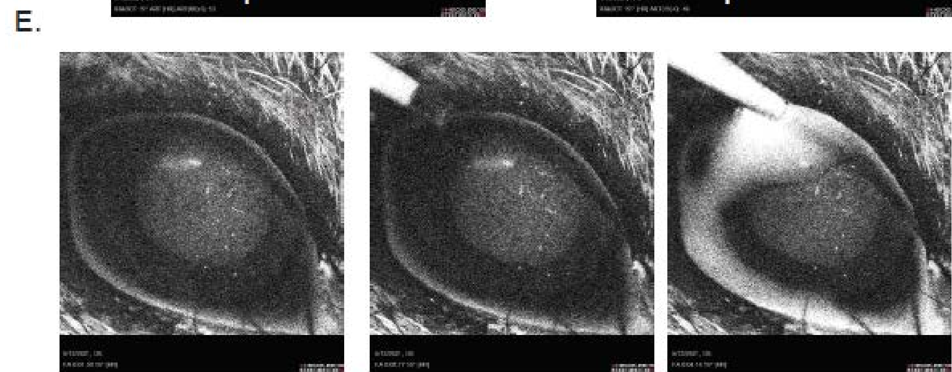


Figure 2.

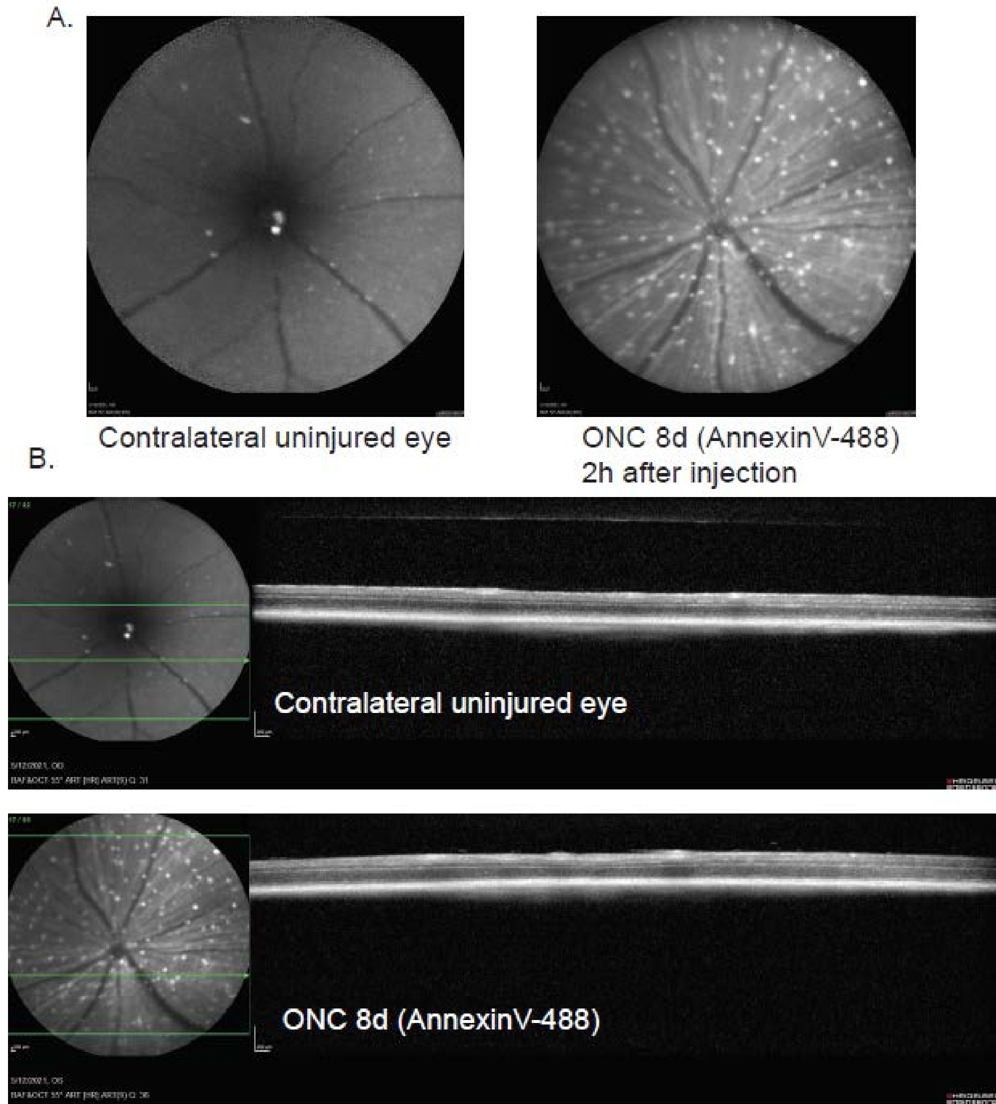
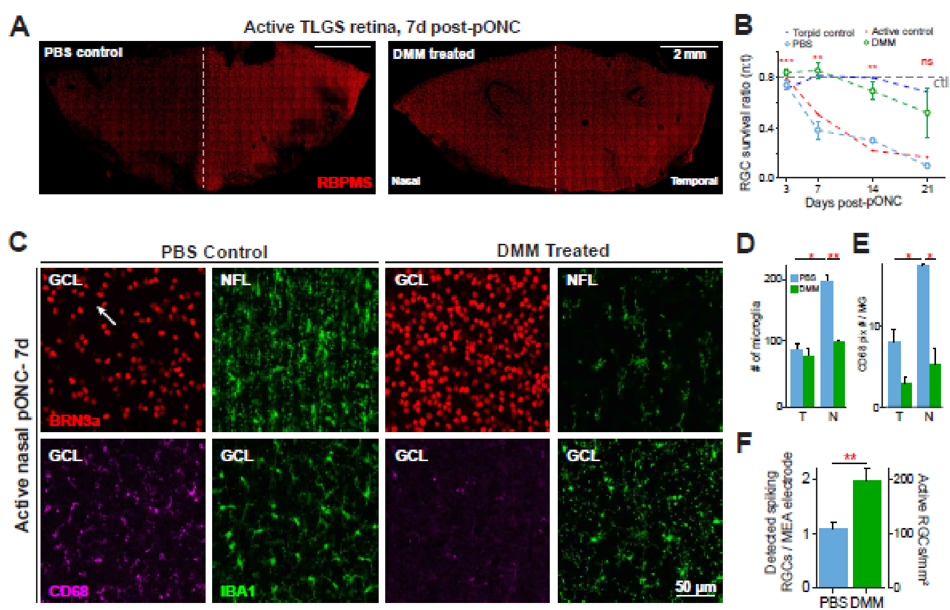


Figure 3.



Recent advances 4th quarter:

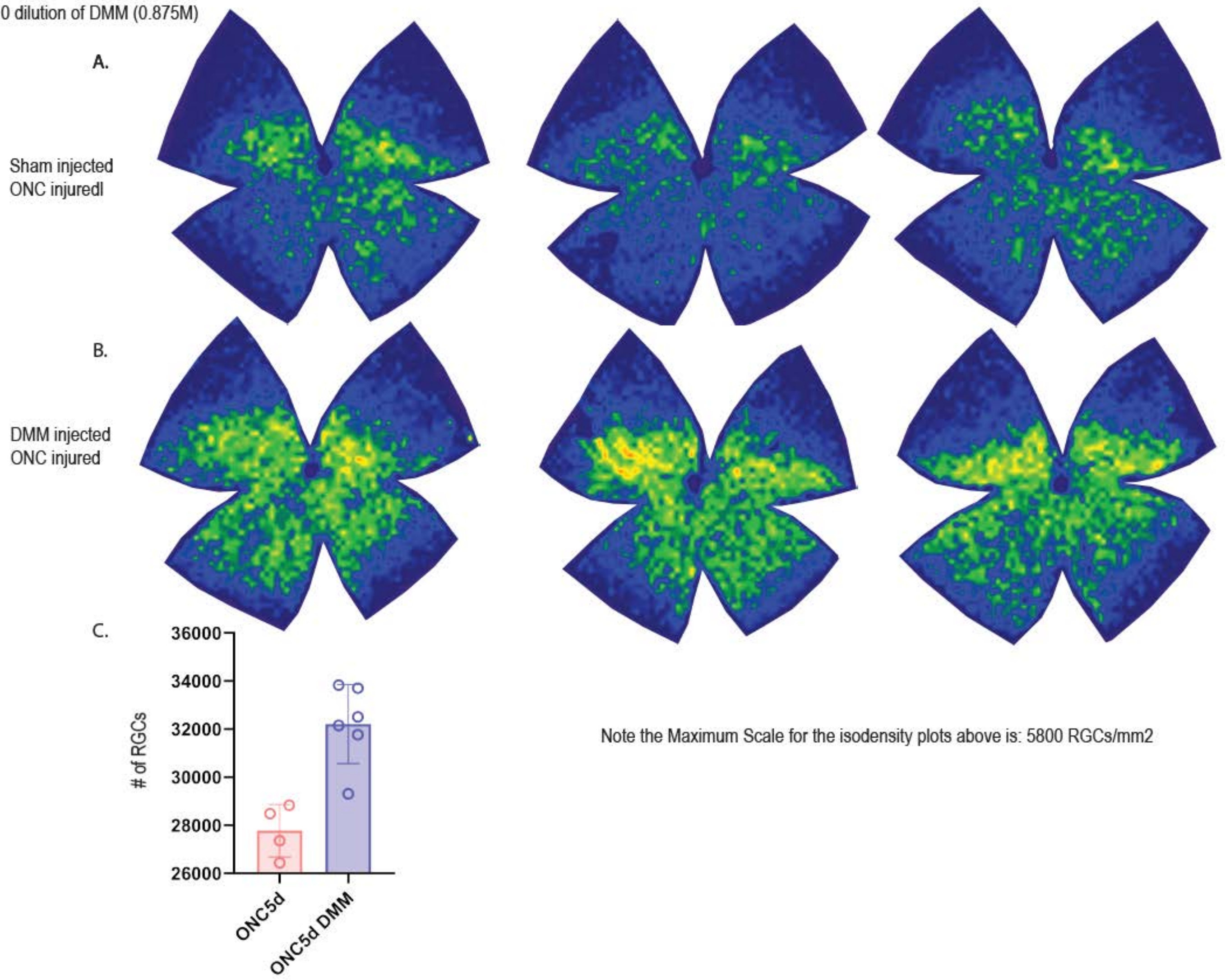


Figure 2. ONC 11d (DMM injected on days 0, 3, 7)
1ul of 1:10 dilution of DMM (0.875M)

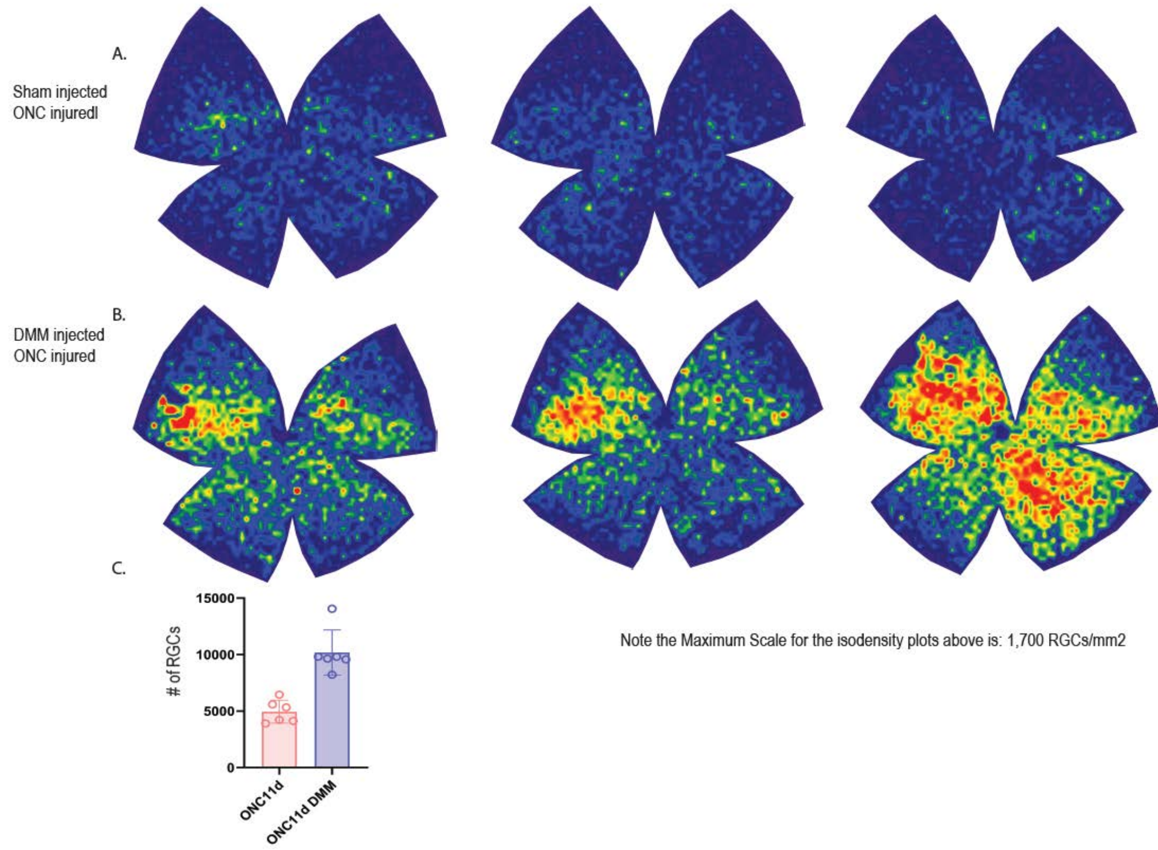


Figure 3. ONC 11d (DMM injected on every other day (eod: 0, 2, 4, 6, 8, 10)
1ul of 1:10 dilution of DMM (0.875M)

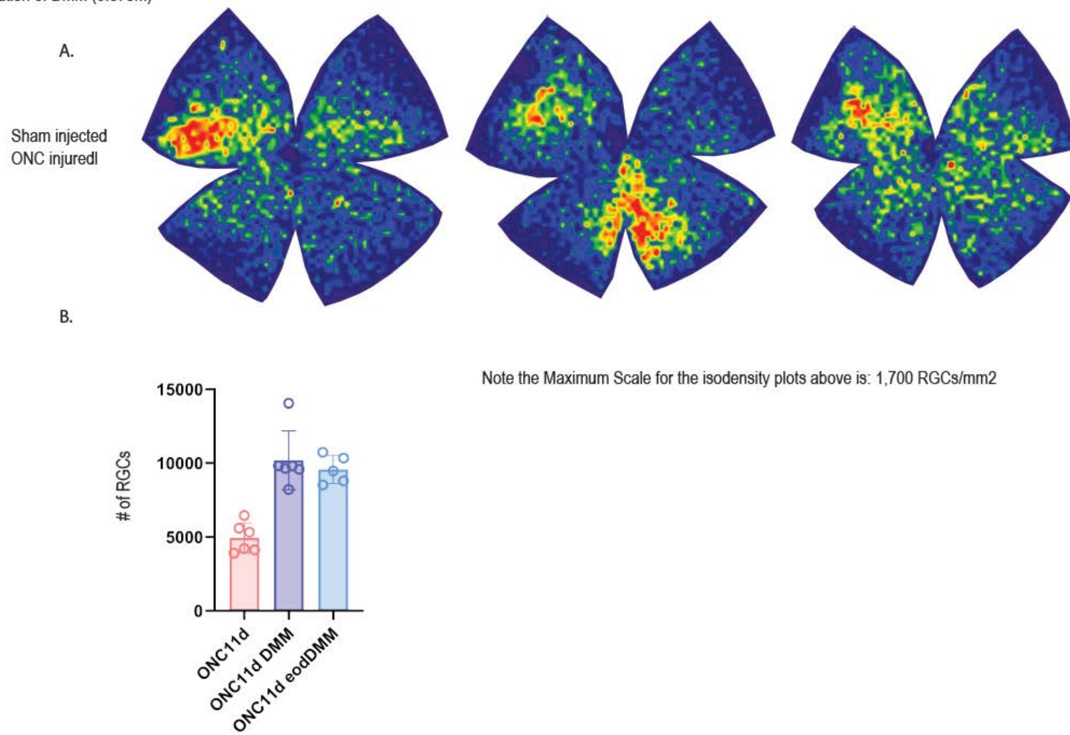
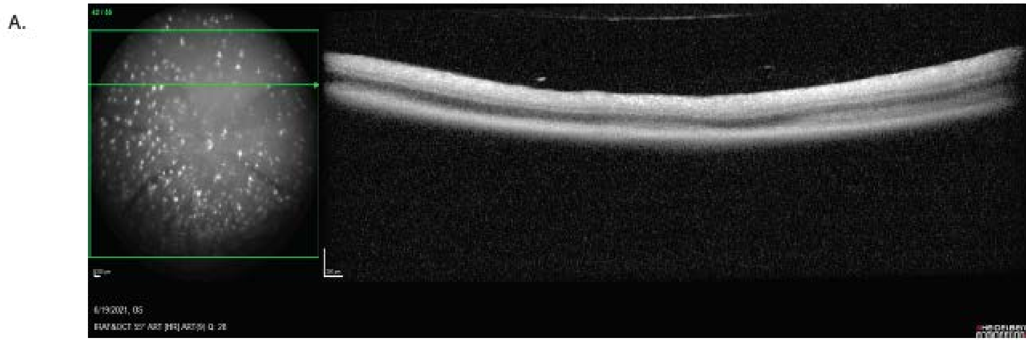
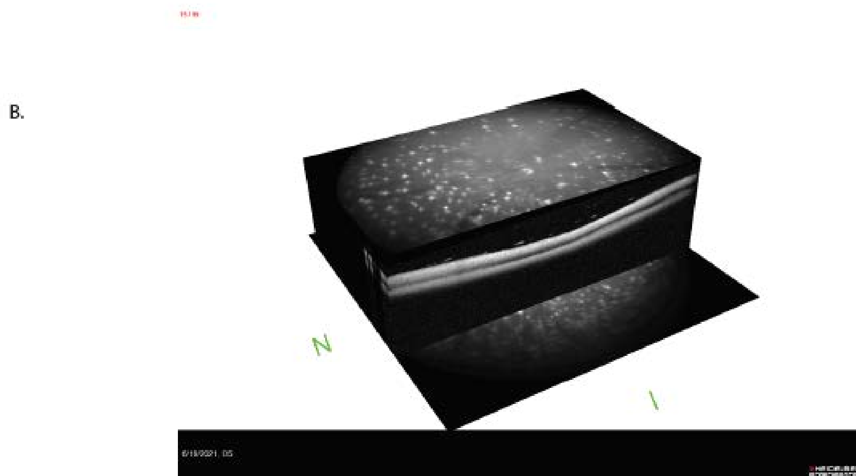


Figure 4. ONC 4d Intravitreal injection of Annexin 488 conjugate
Imaged using Spectrali angiography (fluorescein setting)

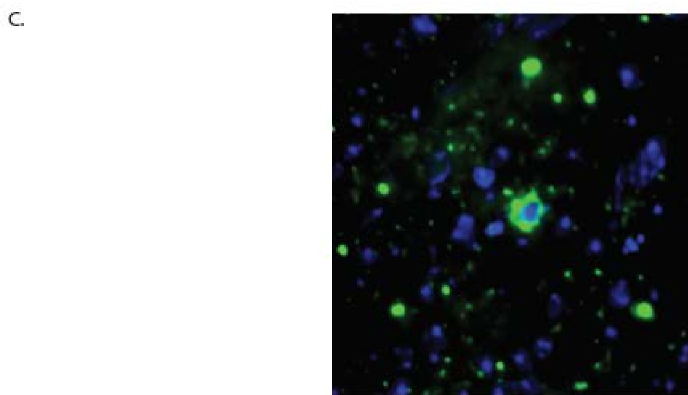
A.



B.



C.



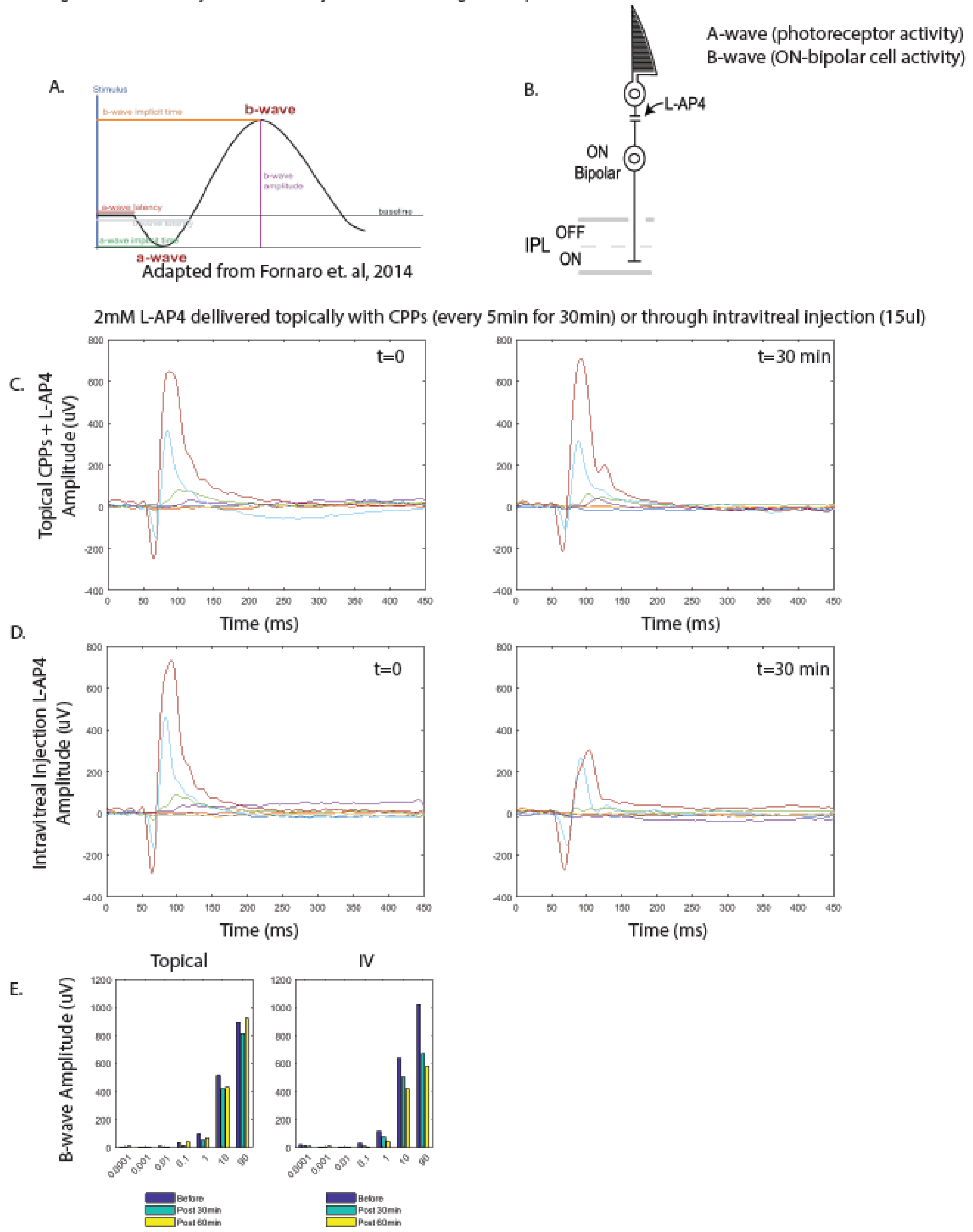


Figure 5. CPP delivery or Intravitreal Injection of L-AP4 to ground squirrel retina

Figure 6. Neuroprotective effect of icilin in vitro and in vivo