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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.

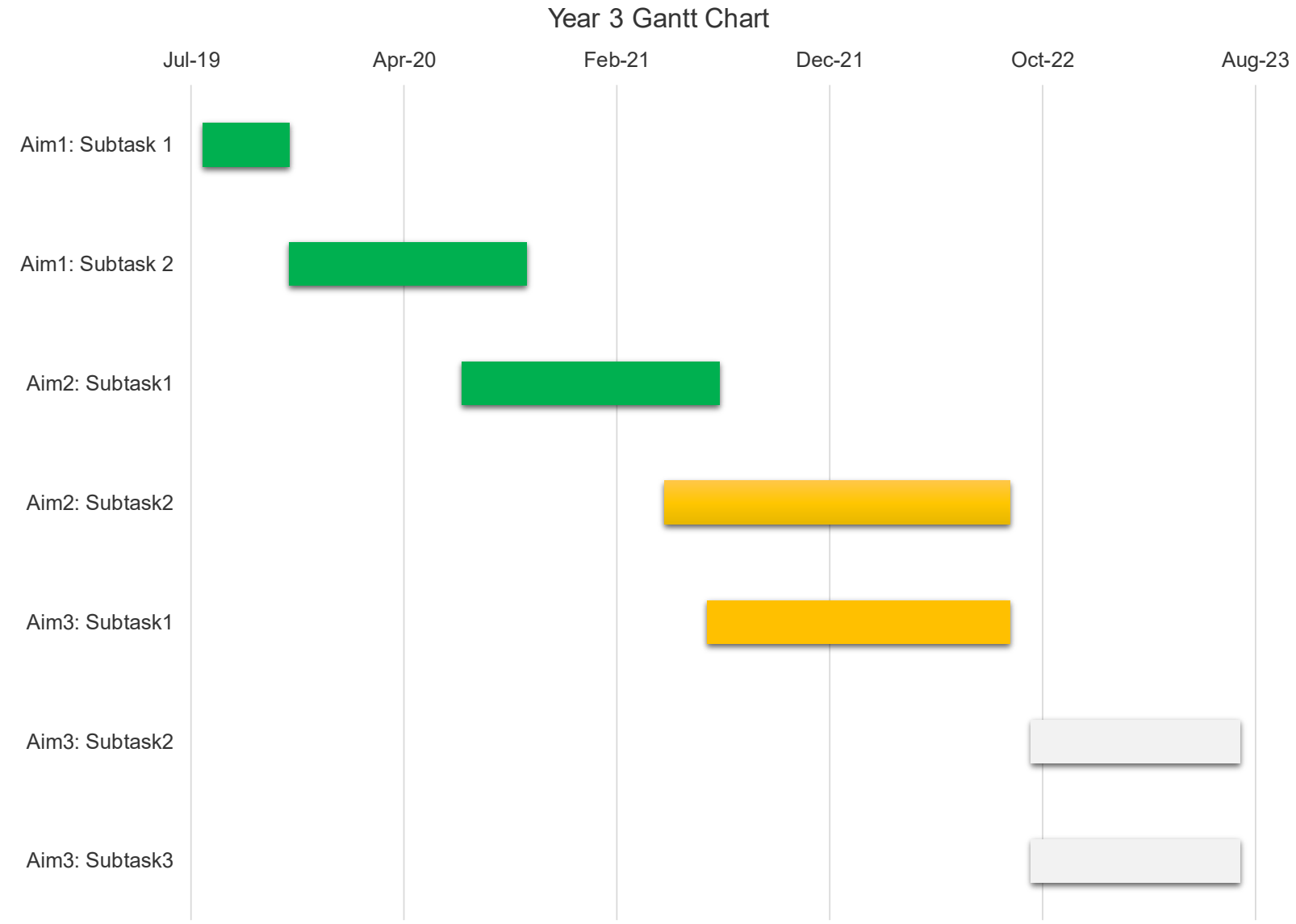
KEYWORDS: Traumatic Optic Neuropathy (TON), Hibernation, Retinal Ganglion Cells (RGCs), Neuroprotection

ACCOMPLISHMENTS:

- *In this reporting period we received rat cadaver eyes from Dr. McCabe at USUHS in order to provide evidence to the NIH Veterinary Research and Resource Section that the blast model can be performed without extensive collateral bodily injury. Having met this requirement, we were able to draft an animal protocol to cover the work to be performed in AIM 3. The animal protocol was reviewed by our IACUC committee and amendments were made. An MOU was drafted between NIH and USUHS outlining the responsibilities of each party with respect to the animals and work to be performed. The Animal protocol will be finalized once all signatures are obtained.*
- *We explored the use of other pharmaceutical agents including MAM, which targets succinate dehydrogenase (similar to DMM) to inhibit microglia activation.*
- *We have further 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've identified APO-15 as a potential non calcium dependent method to label locally activated immune cells and apoptosing RGCs. Efforts were made to optimize APO-15 for use in the TLGS model.*
- *We performed additional experiments toward finalizing a manuscript for submission detailing the use of DMM to inhibit microglial cell activity to protect RGCs after optic nerve injury. We showed that addition of cell permeable succinate (succinate-nv) to hibernating ground squirrels eliminated the intrinsic neuroprotection of hibernation following optic nerve injury.*
- *Continued COVID-19 restrictions on travel have prevented our fellow from participating in in-person professional development workshops and attending scientific conferences.*
- *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 made significant progress toward completing Specific Aim 2 (Development and optimization of hibernation-mimicking drug delivery system). We have optimized the protocol for production of a thermosensitive hydrogel that is capable of delivering our hibernation mimicking drugs through a 27 gauge needle. In several animals we observed reduced ERG responses following intravitreal injection of the hydrogel suggesting retroorbital delivery may be preferable. Further studies will determine the efficacy of retroorbital delivery.*
- *We also collected retinas from uninjured and optic nerve crushed squirrels (awake, hibernating, awake + DMM treatment) at various time points (24hrs, 3d, 7d) after injury to perform an untargeted metabolomics study. Samples are being prepared to be shipped to our collaborator for analysis.*
- *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 Year 4 focusing on using DMM in a hydrogel approach (single retroorbital injection) to evaluate the effectiveness of a single targeted injection. We will finalize IACUC approval for the ground squirrel blast injury model and complete AIM3.*

- *The Milestones Achieved, Gantt Chart, and SOW (with % completion, dates) are provided below along with the new findings from fourth quarter (Year 3).*

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. (Year 3) Significant progress was made toward developing the thermosensitive hydrogel delivery system and in obtaining IACUC approval.



Research-Specific Tasks:			% Complete	Date Complete
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 in *awake* 13-lined ground squirrels (GS). Results to be compared to preliminary data that used a commercially available protease inhibitor cocktail that contained irreversible protease inhibitors. Animals used: [4 GS per group x 2 groups = 8 GS total]	1-6	Dr. Wei Li	100%	November-19
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. Animals used (DMM): [3 GS per group x 9 groups = 27 GS total] Animals used (BAM15): [3 GS per group x 9 groups = 27 GS total]	7-12	Dr. Wei Li	100%	October-20
Milestone(s) Achieved: determination whether reversible protease inhibitors delivered in conjunction with BAM15 further improve ganglion cell viabillity. Identification of optimal dose/timing of delivery for DMM and BAM15/PI following optic nerve crush injury.	12		100%	October-20
Specific Aim 2: Development and optimization of hibernation- mimicking drugs delivery system				
Major Task 2: Evaluate use of cell penetrating peptides in ocular delivery in the ground squirrel.				
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. Animals used: [4 GS per group x 2 groups = 8 GS total]	13-16	Dr. Wei Li	100%	July-19
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. Animals used: [4 GS per group x 6 groups = 24 GS total]	16-24	Dr. Wei Li	80%	In progress
Milestone(s) Achieved: 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.	24		80%	In progress
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).	16-24	Dr. Wei Li	100%	August-22
Milestone(s) Achieved: Obtain IACUC approval	24		90%	In progress
Subtask 2: Determine optimal parameters to produce blast induced detectable deficits in optic nerve function. Subject animals to ABS exposures. Animals used: [3 GS per group x 4 groups = 12 GS total]	24-28	Dr. Joseph McCabe	0%	In progress
Subtask 3: Assess deficits in optic nerve function by pERG and ultra high-resolution OCT (Bioptigen). Animals used: [3 GS per group x 4 groups = 12 GS total]	24-28	Dr. Wei Li	0%	In progress
Subtask 4: Using blast parameters established in subtask2, 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. Animals used: [6 GS per group x 3 groups = 18 GS total]	28-36	Dr. Wei Li	0%	In progress
Milestone(s) Achieved: 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	36		0%	In progress

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Major Goals of the Project

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

Major Task 2: Development and optimization of hibernation-mimicking drugs delivery system. Explore use of controlled release materials: single intraocular injection to the targeted cells at the back of the eye to improve ganglion cell viability.

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 hibernation inspired drugs to treat blast induced traumatic optic neuropathy
Subtask 1: Submit documents for IACUC approval at USUHS to use the advanced blast simulator

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 the initial days post injury during which time there is typically a significant loss in ganglion cell number.

Projected Milestone (24 months)
From 1st quarterly report: Major Task 3: Unlike birds, rats, and mice (which are commonly used in research), the 13-lined ground squirrels are not excluded from the US Animal Welfare Act. As such it is necessary to ensure that the blast injury will produce the desired optic nerve injury while minimizing collateral damage. Before granting us permission to submit an amendment to our animal protocol, our Animal Program Director, Dr. James Raber has requested that an initial study be performed in rats, which are comparable in size to the 13-lined ground squirrels. This study was performed under the protocol of our collaborator Dr. McCabe (at USUHS). The rats (all female) were euthanized by Dr. McCabe's team and provided to us for whole animal fixation via transcardial perfusion. There were two experimental groups: 24h following blast injury and 7d following blast injury. Each group consisted of two age matched animals one subjected to a blast and one sham. The animals in the 7d group were 3 months old at the time of euthanasia whereas the animals in the 24h group were approximately 7.5 months old. The animals were positioned "nose first" in a velcro mesh holder suspended within the blast chamber to ameliorate peripheral organ effects. The blast wave was approximately 20 psi. Following fixation, the eyes were removed for immunostaining of RGCs. The animal carcasses were submitted to DVR for general diagnostic necropsy to check the rest of the body for any other blast related damage. Results are pending.

Additionally, to assess the potential risk of unwanted pathogens introduced into our established squirrel colony from the potential movement of animals to and from USUHS we were asked to perform swab tests of surfaces the animals may come into contact with to test for microbial contamination and PCR swabs to test for possible murine pathogens. These samples were submitted to DVR for analysis and the results are pending.

Figure 1. The fixed eyes were dissected and the cornea and lens were photographed (ezImageX3, Aven mighty scope) noting A. the absence of corneal trauma commonly associated with pneumatic pressure induced models of blast injury. B. The lens capsules showed no signs of rupture.

Major Task 2: We have begun formulating a hydrogel to deliver DMM through a single intraocular injection. The hydrogel tested consisted of 2.5% chitosan (C), 1% gelatin (G) dissolved in acetic acid 0.1M. The C/G solution was further diluted 1:1 with water, as it was too viscous to pass through a 30g syringe necessary for intraocular injection. 1mL of glycerol 2-phosphate disodium salt hydrate (GP) (44.4% w/v) was added to 5mL of C/G dilute solution to bring the pH between 7-7.5. As a test for thermosensitivity samples placed in a 37C incubator overnight solidified into a gel whereas samples placed in a 4C fridge remained liquid. We will improve the homogeneity of the solution next time by dissolving the chitosan completely before adding the gelatin. This will help prevent the syringe needle from becoming clogged. We have also begun testing the use of MAM, which is more potent than DMM because of its faster esterase hydrolysis. Both inhibit succinate dehydrogenase

which prevents oxidation of succinate and ROS byproducts that trigger microglial activation. The caveat is that MAM is highly insoluble in many solvents. However, we have identified and tested a solvent Transcutol HP that is biocompatible, transparent, and maintains the bioactivity of MAM.

Figure 1. Images showing BV2 cells under the following conditions: (C. control, D. LPS (6h), E. LPS + 100uM MAM (6h), F. LPS + 20uM MAM (6h). High concentrations of MAM were toxic and triggered cell death (e.g. 100uM). We found that at 20uM MAM was well tolerated by BV2 microglial cells and inhibited inflammatory activation (G. IL1b, $p<0.001$; H. TNFalpha, $p<0.001$; I. IL6, $p<0.01$) in response to LPS. Our institute earlier this week approved our amendment to the animal protocol to test the hydrogel-based ocular delivery system and the MAM (diacetoxymethyl malonate diester and we will perform in vivo tests in our next report.

This study gathered preliminary data on rats subjected to blast injury to satisfy the request by the NIH Veterinary Research and Resource Section in order to obtain authorization to submit an animal protocol for review. Also as an alternative strategy for drug delivery a hydrogel was formulated and tested for thermosensitive properties. MAM also was tested in vitro on BV2 microglial cells as an additional treatment option to inhibit microglial activation.

Major Task 3: Evaluate hibernation inspired drugs to treat blast induced traumatic optic neuropathy
Subtask 1: Submit documents for IACUC approval at USUHS to use the advanced blast simulator

Major Task1: Optimize selection, dose, and timing of pharmaceutical agents using ONC injury model.
Subtask 2: Identify appropriate dose/timing of delivery of pharmaceutical agents.

From 2nd quarterly report: This study was performed under the protocol of our collaborator Dr. McCabe (at USUHS). The rats (all female) were euthanized by Dr. McCabe's team and provided to us for whole animal fixation via transcardial perfusion. The blast wave was approximately 20 psi.
The animal carcasses submitted to DVR for general diagnostic necropsy found no gross abnormalities resulting from blast related damage. Swab tests are in the process of being reanalyzed by PCR, but initial tests appeared to be free from murine pathogens.

Figure 1. The fixed eyes were immunostained for RBPMS to enable the quantification of RGCs using the Nikon Elements General Analysis 3 software. No changes in the RGC number were found at 24h or 7d post ABS injury suggesting the animal's orientation may need to be positioned lateral to the blast. Additionally, the injury may take longer to develop.

In lieu of further preliminary studies, the NIH Veterinary staff has agreed to allow us to move forward with the animal protocol amendment in order to position the animals lateral to the blast inducing optic neuropathy as described in Evans et al. 2021. A draft has been completed and is being revised by Veterinary staff. Under the recommendation of Dr. McCabe we are also including a CHIMERA (close head impact model of engineered rotational acceleration) injury model for the 13-lined ground squirrel in our amendment. Upon completion these amendments will be submitted to ACUC and to ACURO for approval.

During this period, we continued research on alternative hibernation mimicking drugs that could be used for neuroprotection. Previously our in vitro data identified MAM, a succinate dehydrogenase inhibitor that works similar to DMM, as a possible drug that could be used to inhibit the activation of microglia that lead to RGC death. Here 6 mice were selected for testing the following treatment: MAM (20uM). 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. All injections (1µL) were provided 0.5h post injury. Animals were euthanized on day 11.

Surviving ganglion cells were selectively stained with RBPMS (RNA binding protein with multiple splicing) and imaged on a confocal microscope. Figure 2, representative immunofluorescent images of RBPMS labeled RGCs quantified using the Nikon Elements. Isodensity maps generated in Matlab. The highest densities of surviving ganglion cells shown in red/yellow.

Bottom bar graph shows the quantification of the number of surviving RGCs in these mice in comparison to uninured contralateral eyes, untreated control mice (ONC 11 days), and DMM treated mice (ONC 11days)

described in a previous reporting period. MAM (p=0.0002) was similarly effective as DMM (p=0.0005) at protecting RGCs after optic nerve crush injury.

To confirm our previous data regarding the importance of inhibiting succinate dehydrogenase in preventing ROS accumulation and microglial activation following traumatic optic neuropathy we performed the following experiments. In hibernating ground squirrels, which have very low levels of succinate compared to awake animals, we injected cell permeable succinate at days: -1, 0, 4, and 9 following partial ONC. Animals were collected on day 14 and stained with RBPMS. The nasal crush side showed a marked increase in RGC death when succinate was delivered compared to hibernating animals that were intrinsically protected. The white arrow indicates the crush site. The dorsal crush vs uncrushed side had 35.5 +/- 19.0 % (n=3) as many RGCs as the uncrushed side.

This study quantified RGC loss following frontal blast injury and was used to justify the need for a new animal protocol allowing for animals to be positioned lateral to the blast. MAM was evaluated in vivo following optic nerve injury and showed similar neuroprotection to DMM. The role of succinate in driving adverse microglial activation following optic nerve injury by injecting cell permeable succinate into hibernating TLGS causing them to lose their intrinsic neuroprotection.

Major Task 3: Evaluate hibernation inspired drugs to treat blast induced traumatic optic neuropathy
Subtask 1: Submit documents for IACUC approval at NIH for animal use in the advanced blast simulator (ABS).

From 3rd quarterly report:

Blast experiments have been on hold until ACUC approves our animal protocol.
The following experiments were conducted using other funds with an existing, approved animal use protocol (squirrel). In order to demonstrate that inhibiting succinate dehydrogenase with our Dimethyl Malonate treatment can prevent metabolic reprogramming of microglia toward proinflammatory responses to axonal injury we have begun optimizing a protocol to collect retinas from awake and hibernating 13-lined ground squirrels under the following conditions:

Baseline Controls (no injury)
Awake n=4
Hibernating n=4

Total Optic Nerve Crush (6h post injury)
Hibernating n=4
Awake n=4
Awake + DMM treatment n=4

Total Optic Nerve Crush (3d post injury)
Hibernating n=4
Awake n=4
Awake + DMM treatment n=4

Total Optic Nerve Crush (7d post injury)
Hibernating n=4
Awake n=4
Awake + DMM treatment n=4

We would like to include several time points to capture acute changes immediately after the injury (6h post) and (3d/7d post) to observe metabolomic changes once the microglia are activated from the ROS production.

We are collaborating with the NIH Metabolomics Core facility (NIEHS) to perform an untargeted metabolomics screen as it is broader in scope and may reveal unknown metabolites in the hibernating condition that may be interesting to pursue identification in follow-up studies.

The data shown is a preliminary experiment to determine if a single retina is enough material to acquire data and if the method will yield technically sound data with high reproducibility.

Three samples were provided for analysis.
Sample 1 – left retina from animal A
Sample 2 – left retina from animal B
Sample 3 (not analyzed) – two retinas together from animal A and B
*Sample 3 was not analyzed as it was too much material for the homogenization tube.

The base peak chromatograms for the positive (Figure 1A) and negative (Figure 1B) ionization mode are shown. Different compounds are detected in the positive and negative mode – following typical acid-base chemistry – cations in positive mode and anions in negative mode. Note, the plots doesn't detail the full extent of chemicals detected as the actual compounds are detected across ~4 orders of magnitude which cannot be accurately plotted along the y-axis. There are likely 1000s of chemicals detected in the data.
In assessing the technical reproducibility of our method, a pooled sample (generated by taking an aliquot from each sample) was analyzed in triplicate (Figure 2A-B). The individual base peak chromatograms are overlaid onto of one another. The data were highly reproducible.

In addition to the above metabolic study, we continued to optimize real-time imaging of retinal ganglion cell (RGC) apoptosis in the thirteen lined ground squirrel (TLGS) model. Our previous studies showed that annexin fails to label apoptosing RGCs in the TLGS. Here we tested a calcium independent probe (apotracker) in mice and in TLGS (Figure 3).

This study optimized a protocol to collect retinas for untargeted metabolomic analysis. Preliminary experiments show high reproducibility. We also continued optimizing real-time imaging of apotosing retinal ganglion cells in TLGS using a calcium independent probe that labeled phosphatidyl serine (cell death marker) similar to annexin.

Major Task 3: Evaluate hibernation inspired drugs to treat blast induced traumatic optic neuropathy
Subtask 1: Submit documents for IACUC approval at USUHS to use the advanced blast simulator

Major Task1: Optimize selection, dose, and timing of pharmaceutical agents using ONC injury model.
Subtask 2: Identify appropriate dose/timing of delivery of pharmaceutical agents.

Recent Advances (4th quarter):

Our new animal protocol which includes the work to be performed by Dr. McCabe's lab at USUHS (ABS blast injury model and CHIMERA (closed head impact model of engineered rotational acceleration was reviewed by our IACUC. All recommendations by the IACUC committee were made. Approval is pending the inclusion of an MOA between USUHS and NIH. This MOA is in the process of being signed from officials on both sides.

During this period, we also continued research on improving the thermosensitive hydrogel.
Efforts were made to dissolve the chitosan (C) fully in acetic acid first leaving it with a stir bar for 24-48h followed by the addition of gelatin (G). The C/G suspension was thoroughly dissolved on a stir plate before autoclaving. β-Glycerophosphate disodium salt hydrate is added to adjust the pH to 7.4
The final hydrogel is then filtered through a 100um cell strainer.

The DMM was then added (1:10 dilution). The hydrogel at RT successfully passed through a 30g Hamilton syringe and retained its thermosensitive properties. The sol-gel transition at 37°C took approximately one minute when tested in a bench top incubator assessed by inverting the tube. Initial injections (1uL) of the hydrogel were successful in entering and solidifying into a gel in WT mice (naïve – no injury). However, in the 5 eyes of mice intravitreally injected with the hydrogel we observed opacity of the cornea possibly from damage to the lens during the injection. ERG recordings showed that this blocked light from reaching the retina (Figure 1 A). No a-waves or b-waves were present in the hydrogel injected eyes 6 days post injection. In 3 mice subjected to optic nerve crush (ONC) injury one mouse displayed ERG responses (Figure 1B) suggesting that it might be possible to inject the gel without injuring the lens and causing opacity.

In the mouse that retained ERG responsiveness we waited until ~day 11 to collect the optic nerve crushed eye to qualitatively compare the ganglion cell number (29,551 RGCs) and microglia morphology to data previously reported (Figure 1 C). RGC appears to be denser compared to untreated ONC11d although an accurate cell count is obscured by damage to some areas during dissection and several image artifacts.

To test whether the hydrogel can be more successfully delivered in a larger animal model where the vitreous volume is greater, we injected a 13-lined ground squirrel with the hydrogel (10ul) and recorded light adapted ERG responses. The a-wave and b-wave amplitudes of the hydrogel injected eye were similar in amplitude to the naive contralateral eye suggesting that the hydrogel does not block light entry to the eye in this model. We have previously experimentally measured the volume of the TLGS vitreous to be ~280ul. In comparison the vitreous volume of the mouse is ~ 4ul of which the lens makes up a large majority of the volume.

We will continue testing the hydrogel loaded with DMM in TLGS subjected to ONC. Additionally, we have finished collecting all the samples for the comparative metabolomics study of optic nerve injury (hibernating vs awake vs DMM treatment) and are confirming the total optic nerve crush by imaging the damage site and staining for several cellular markers (Figure 1 E-G). We will be preparing to ship these samples for analysis in the coming weeks as we await our animal protocol to be approved.

Work to be performed in CY 2022:

Specific Aim 2: Development and optimization of hibernation-mimicking drugs delivery system
Milestone(s): Determination of feasibility of using hydrogel 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 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: We requested a no cost extension because our institute had delayed the process of allowing us to submit an animal protocol for IACUC review.

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
Contribution to Project: Provided clinical insight and references.

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). Assisted with testing DARC technology on GS. Helped collect samples for metabolomics study

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 on mice, performed data analysis, immunostaining, and imaging. Optimized analysis procedures using Nikon NIS Elements Analysis software. Provided project reports.

Dr. Joseph McCabe
Project Role: Collaborating PI
Contribution to Project: Provided blast injured rat cadavers as part of the preliminary study required by NIH prior to submitting an animal protocol for TLGS.

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

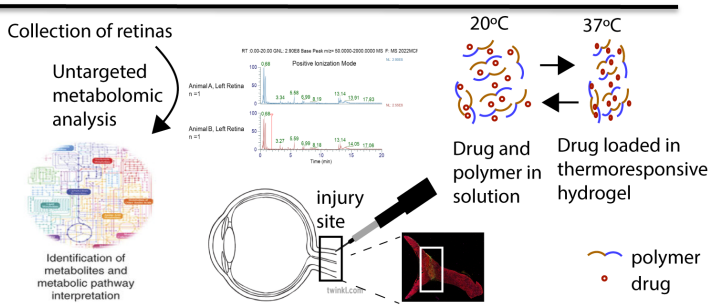
PI: Dr. Wei Li **Org:** National Institutes of Health **Award Amount:** $500,000 total cost dollars

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.



Accomplishment: Established MOA with USUHS. TLGS animal protocol (ABS & CHIMERA) submitted for approval. Preliminary blast exposures performed on rats. Collected retinas for untargeted metabolmic profiling of optic nerve injury in summer active, hibernating, and DMM treated TLGS. DMM was successfully incorporated into a thermosensitive hydrogel. Retrobulbar delivery will be explored.

Timeline and Cost

Activities CY	19	20	21	22
Evaluation of hibernation-mimicking drugs to treat optic nerve crush				
Development of ocular drug delivery system				
Evaluation of hibernation-mimicking drugs to treat blast-induced ocular trauma				
Estimated Budget ($K)	**$433000**	**$33500**	**$33500**	**$0**

Updated: (08/19/22)

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 Goal- 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
  - ↳ Alternatively: Develop thermosensitive hydrogel for drug delivery

CY21 Goal- Evaluate drug delivery of hibernation inspired drugs to treat blast induced traumatic optic neuropathy

- ☒ Submit documents for IACUC approval at USUHS for advanced blast simulator

CY22 Goal

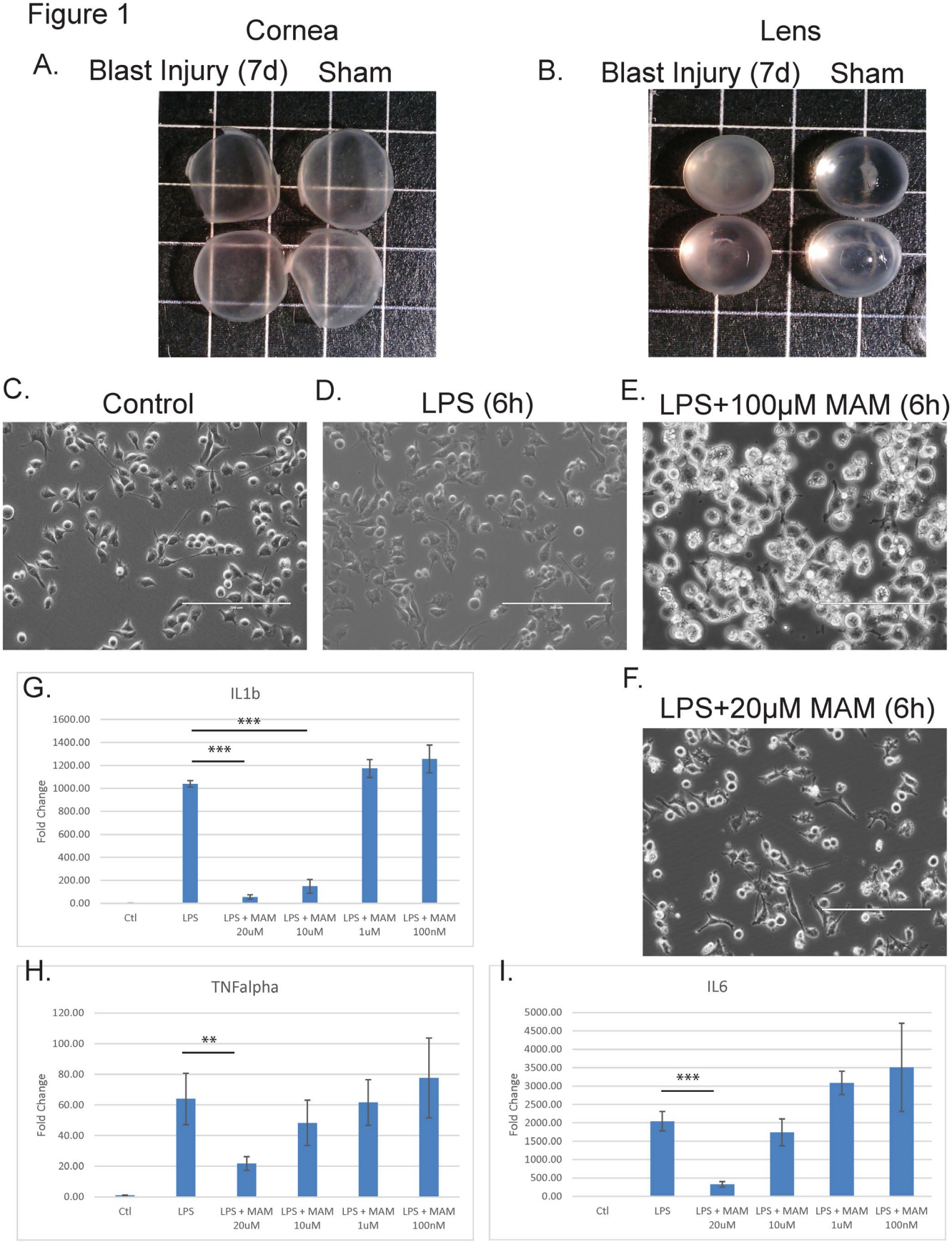
- ☐ Determine parameters to produce blast induced deficits in optic nerve function
- ☐ Evaluate drugs effectiveness to treat blast induced ocular injury to optic nerve

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. Female Rats exposed to ~ 20PSI blast overpressure (ABS)

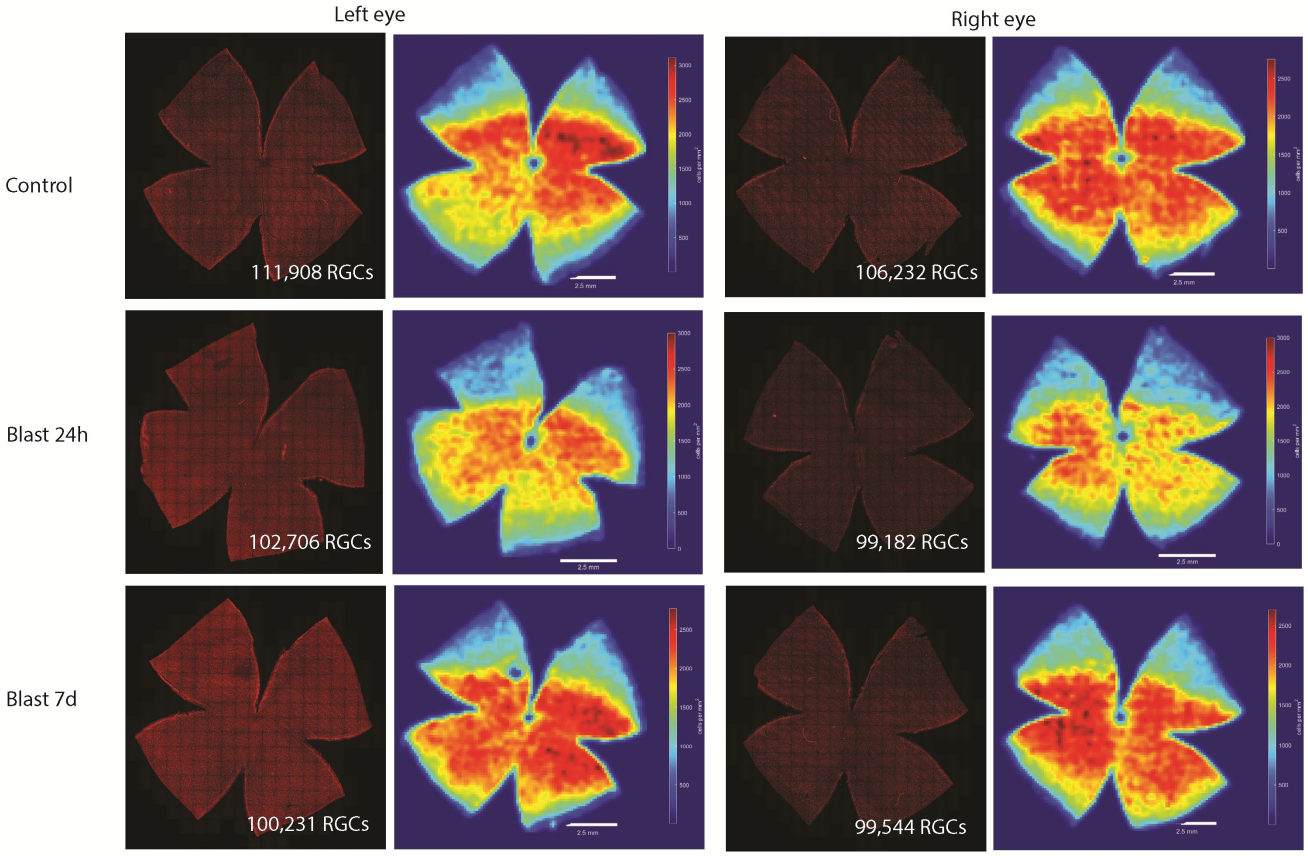
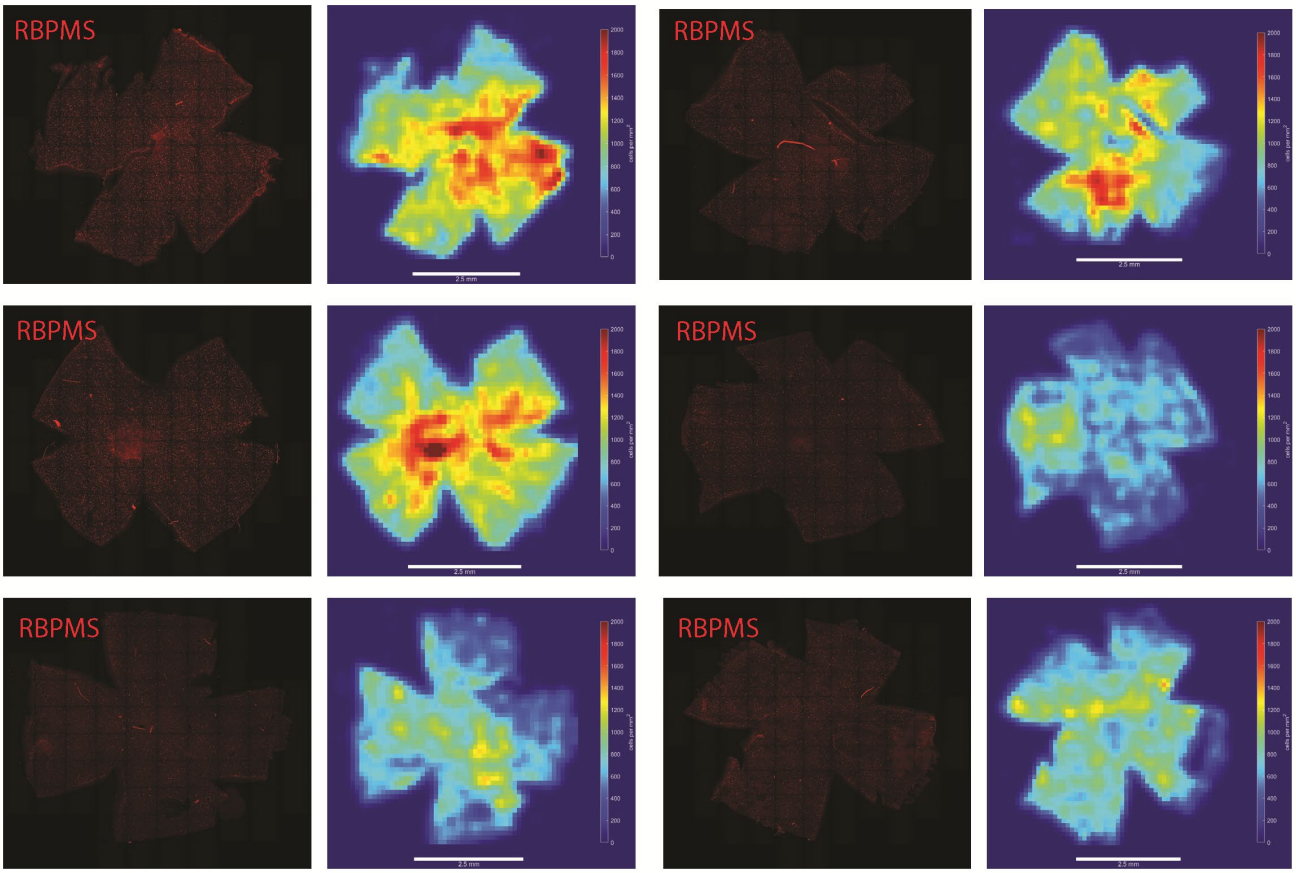


Figure 2. MAM (20uM) injected intravitreally into mice post ONC injury at day 0. Retinas collected at 11 days post ONC.



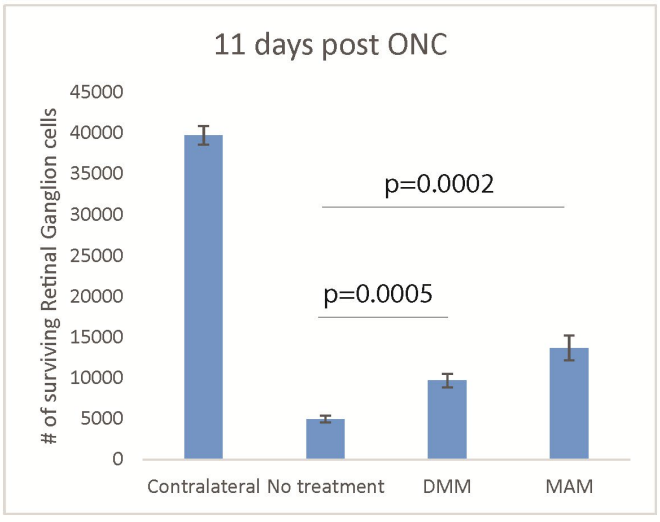
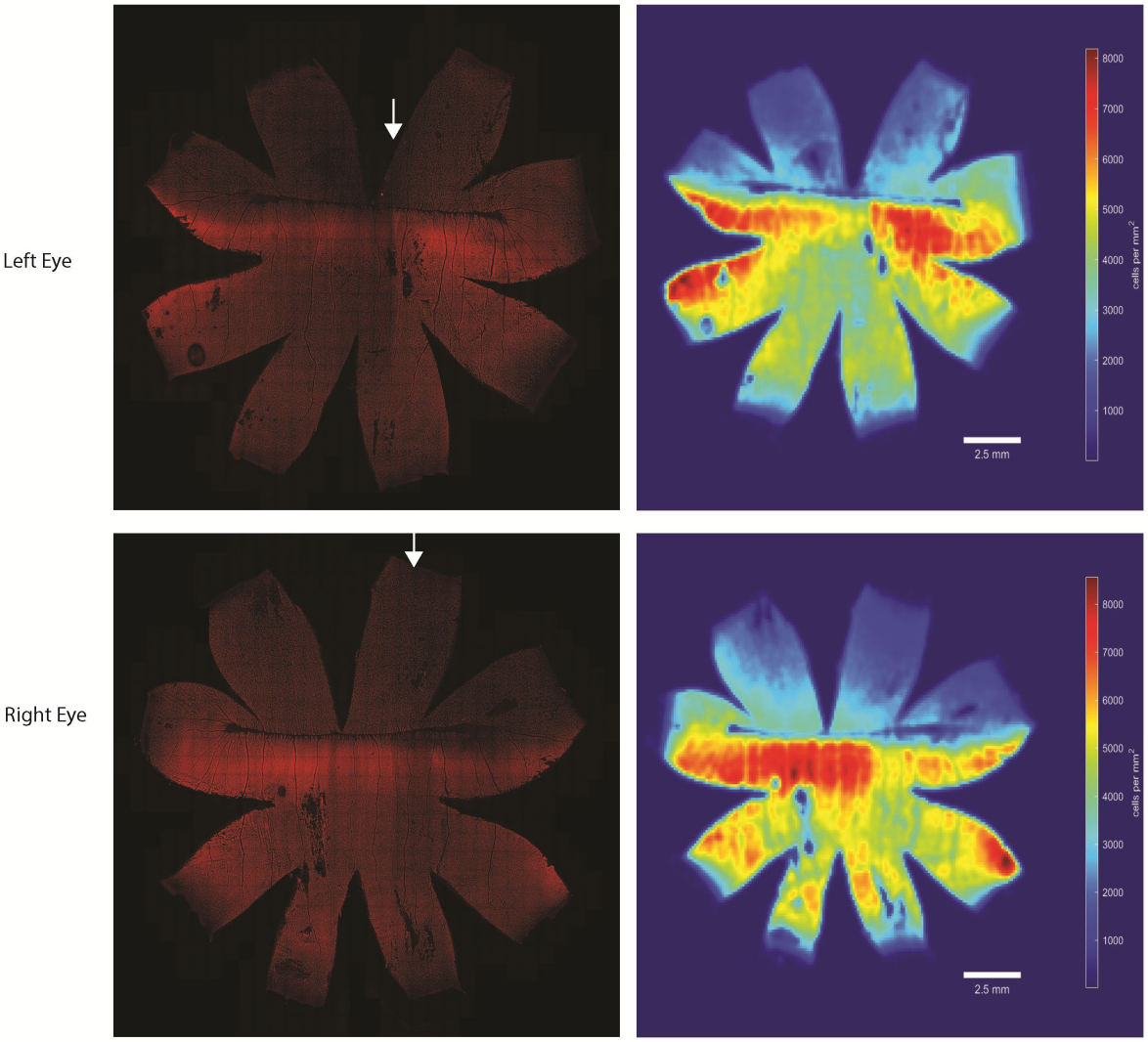


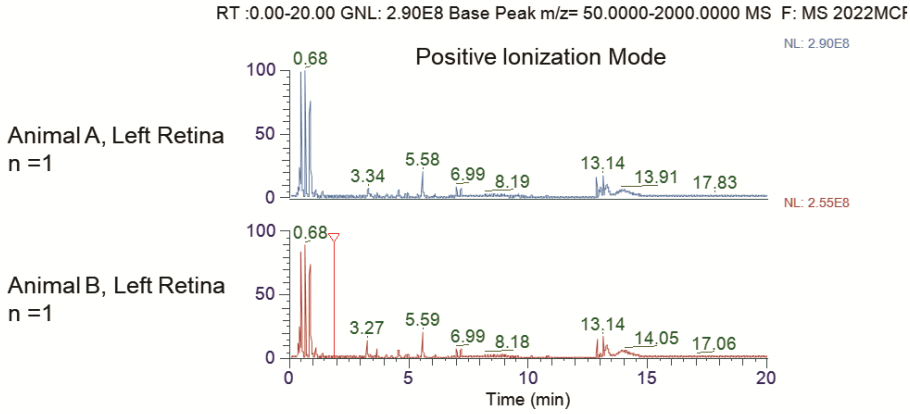
Figure 3. Hibernating TLGS partial ONC 14d injected with succinate NV (-1d, 0d, 4d, 9d)



From 3rd quarterly report:

Figure 1.

A.



B.

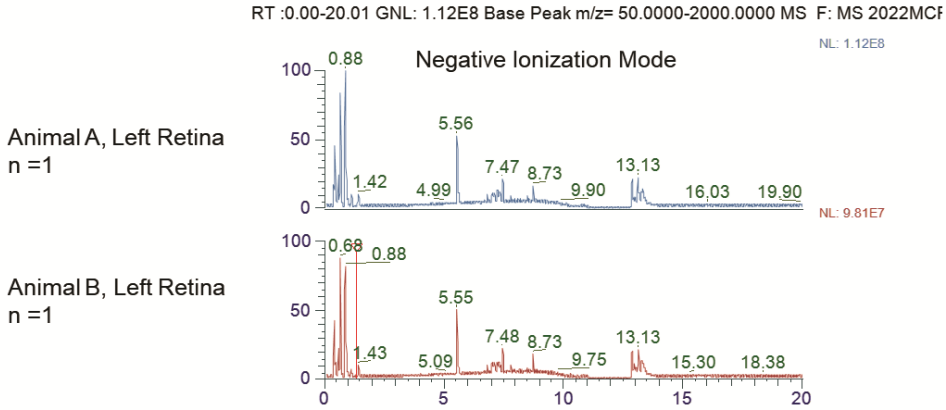


Figure 2.

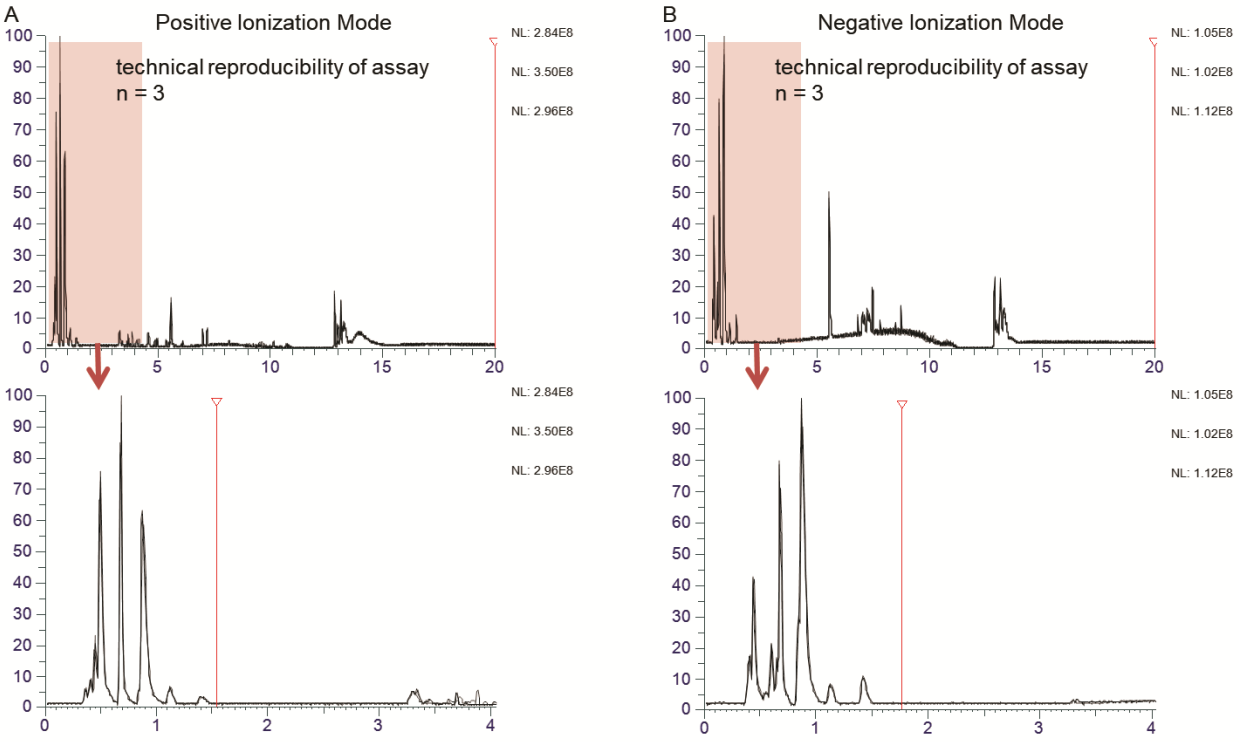
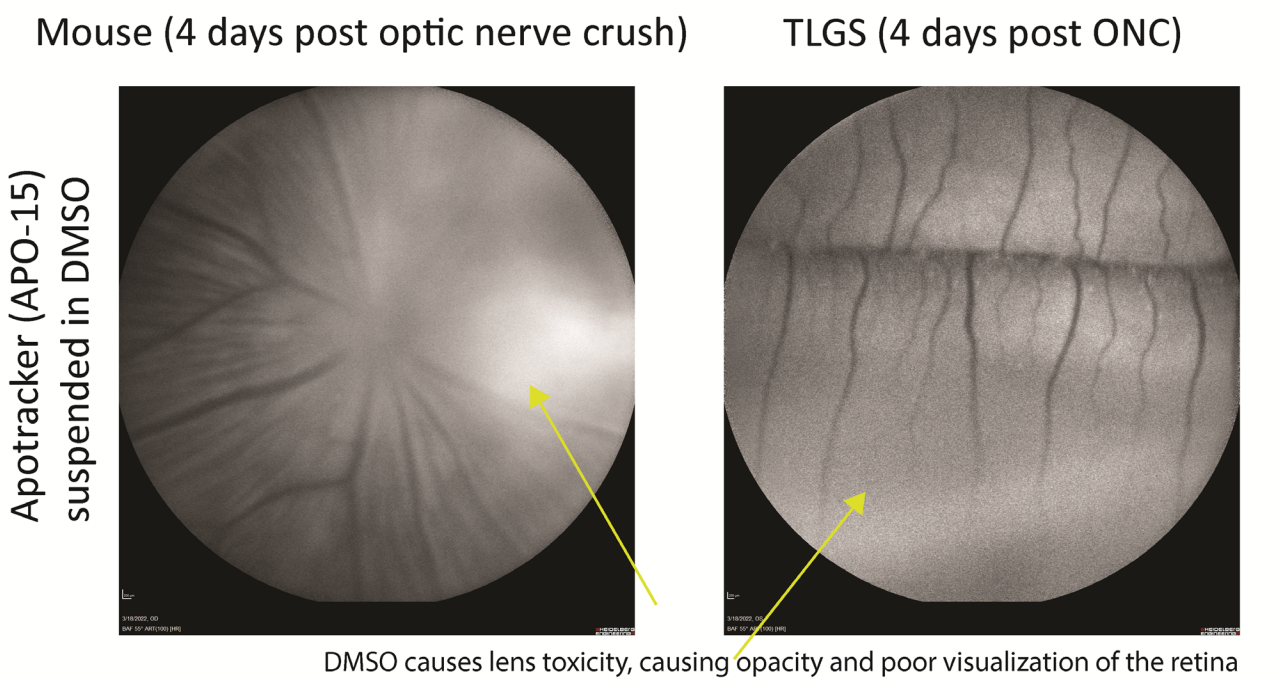
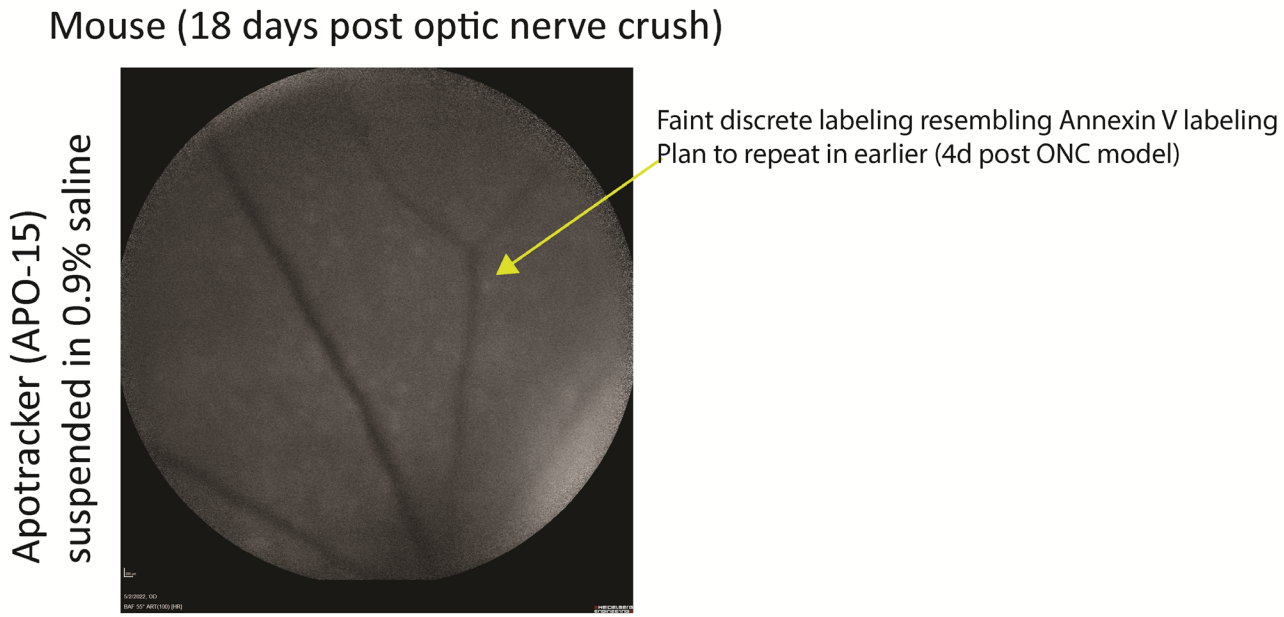

A
Positive Ionization Mode
technical reproducibility of assay
n = 3
NL: 2.84E8
NL: 3.50E8
NL: 2.96E8
B
Negative Ionization Mode
technical reproducibility of assay
n = 3
NL: 1.05E8
NL: 1.02E8
NL: 1.12E8

Figure 3.

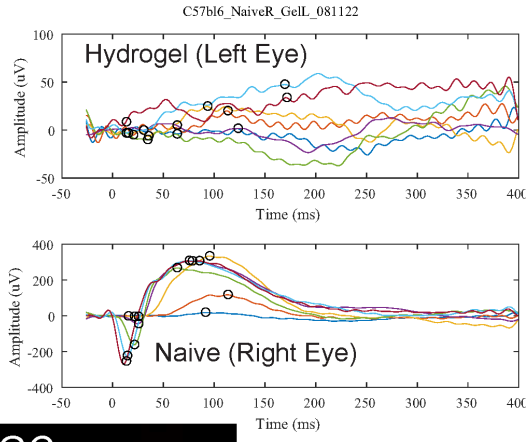
- Apo-15 shares similar excitation/emission as fluorescein (500nm/520nm)
- Detects apoptotic cells similar to annexin (PS to the cell surface)
- Calcium-independent probe
- Mouse intravitreally injected with 1uL of Apotracker (vitreous volume 4ul),
- Thirteen lined ground squirrel (TLGS) intravitreally injected with 15ul of Apotracker (vitreous volume 280ul)



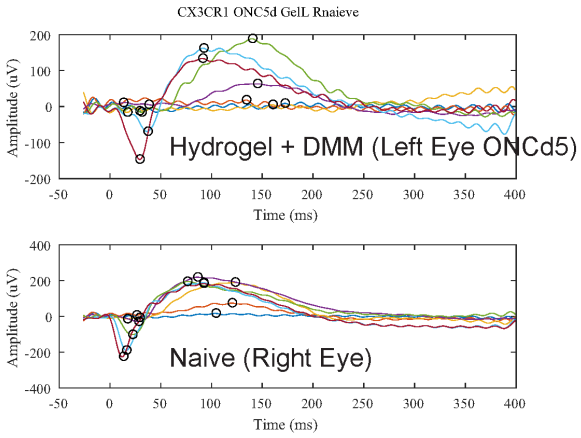


Recent advances 4th quarter:

Figure 1 A.

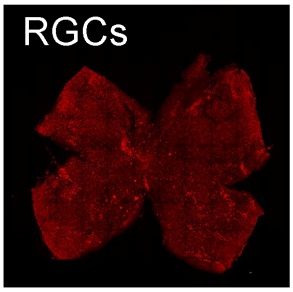


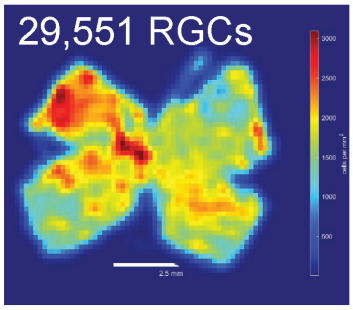
B.



C.

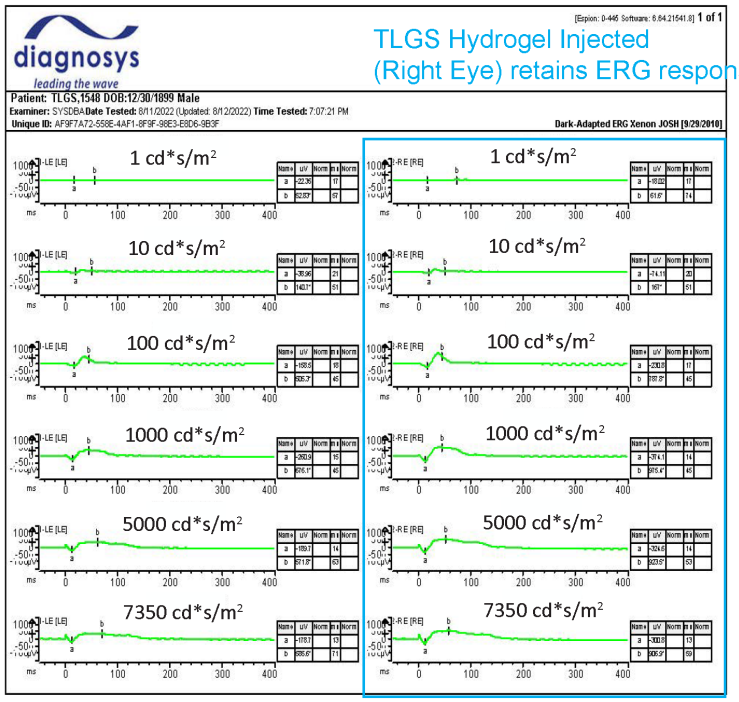
RBPMS





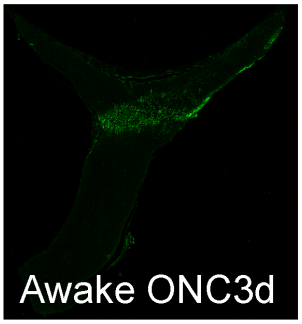
CX3CR1-GFP

D.



E.

CD68



F.

GFAP

G.

IBA1