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TITLE: Role of Endoplasmic Reticulum Stress and Systemic Inflammation in Blunt TBI-Induced Optic Nerve Injury

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CONTRACTING ORGANIZATION: Children's Hospital, Cincinnati

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14. ABSTRACT

Traumatic brain injury (TBI) is a major cause of injury in American military personnel. TBI caused by blunt head trauma is often associated with vision loss, due to injury of the optic nerve. Such vision loss can be severe. Up to approximately 75% of military personnel with TBI report visual symptoms, and such symptoms lead to significantly reduced quality of life measures. In spite of the magnitude of this problem, there are currently no treatments for optic nerve trauma that are backed by scientific evidence. This work is focused on better understanding pathological mechanisms active in a murine model of traumatic optic neuropathy from head trauma. Specifically, we aim to better understand the roles of endoplasmic reticulum stress and peripheral cytokine-mediated inflammation on retinal injury resulting from blunt head trauma. We are approaching the goals of this application via the following specific aims: Aim 1: To determine which element(s) of the endoplasmic reticulum stress response are most relevant to degenerative injury after optic nerve trauma. Aim 2: To determine the degree to which the peripheral inflammatory response contributes to outcomes after optic nerve trauma. This research will help clarify what should be targeted after blunt head injury to improve visual outcomes after TBI.

15. SUBJECT TERMS
Traumatic brain injury; traumatic optic neuropathy; head trauma; endoplasmic reticulum stress; inflammation; cytokines; mice

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1. INTRODUCTION:

This work is focused on better understanding pathological mechanisms active in a murine model of traumatic optic neuropathy from head trauma. Specifically, we aimed to better understand the roles of endoplasmic reticulum stress and peripheral cytokine-mediated inflammation on retinal injury resulting from blunt head trauma. We approached the goals of this application via the following specific aims: Aim 1: To determine which element(s) of the ER stress response are most relevant to degenerative injury after optic nerve trauma. Aim 2: To determine the degree to which the peripheral inflammatory response contributes to outcomes after optic nerve trauma.

2. KEYWORDS:

Traumatic brain injury; traumatic optic neuropathy; head trauma; endoplasmic reticulum stress; inflammation; cytokines; mice

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Test activators and inhibitors of arms of the ER stress response for effect on visual outcomes after blunt TBI. Months 1-16
Completed

Major Task 2: Determine blunt head TBI visual outcomes after treatment with immune modulating drugs. Months 12-24
Completed

What was accomplished under these goals?

Specific Aim 1: Test activators and inhibitors of arms of the ER stress response for effect on visual outcomes after blunt TBI.

These results are shown in the draft manuscript contained in Appendix 2. This manuscript will soon be submitted for publication.

Specific Aim 2: Determine blunt head TBI visual outcomes after treatment with immune modulating drugs.

First, we subjected mice to head trauma leading to traumatic optic neuropathy (TON), then obtained serum samples 6 hours after injury to assess systemic inflammation caused by head trauma. We found that there is significant upregulation of multiple cytokines at 6 hours after head trauma/TON. Specifically, IL-1beta, IL-6, IL-8, and VEGF levels were increased, while TNF-alpha levels were reduced, and IL-10 levels were not altered (Fig. 1).

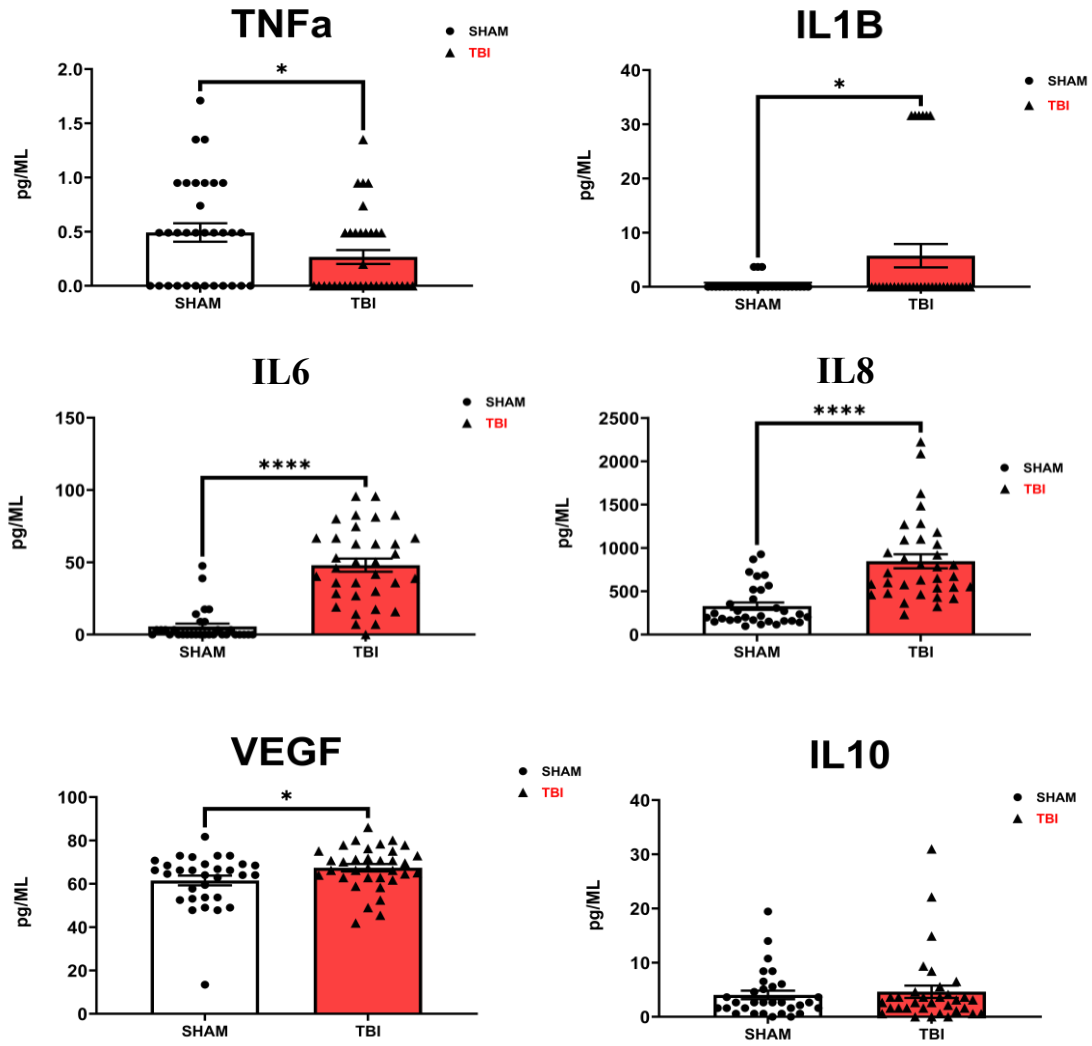


Fig. 1. Serum cytokine levels, 6 hours after TON. * $p < 0.05$; **** $p < 0.0001$ vs sham controls.

After this, we used several cohorts of mice subjected to TON or sham, then treated groups with non-specific IgG (control) or monoclonal antibodies directed against IL-6, TNF-alpha, or IL-1beta by intraperitoneal injection 24 hours after injury, and tested for effect of this treatment on TON outcomes at 7 days post-injury. We found that there was relative preservation of retinal ganglion cells (assessed by Western blot against RBPMS protein) in animals treated with any of the three anti-cytokine antibodies, but not in those treated with nonspecific IgG (Fig. 2). We also assessed axonal degeneration in optic tracts in these mice, using the neurodegeneration stain Fluoro-jade B. There was not a significant reduction in neurodegeneration with any of these treatment, although the increase in Fluoro-jade B staining in anti-IL-6 treated mice was not statistically significant from sham animals in this group, suggesting a possible partial response (Fig. 3). There was not a significant effect of any of these treatments on retinal ER stress markers at this time point (Fig. 4).

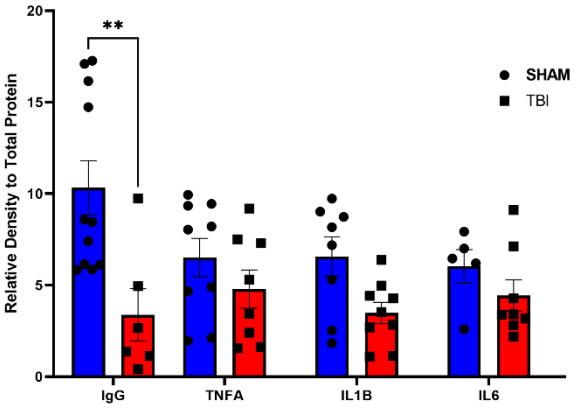


Fig. 2. Retinal RBPMS expression is reduced by TON, but this reduction is eliminated by neutralizing antibodies against TNF-alpha, IL-1beta, or IL-6, compared to nonspecific immunoglobulin (IgG) treatment.

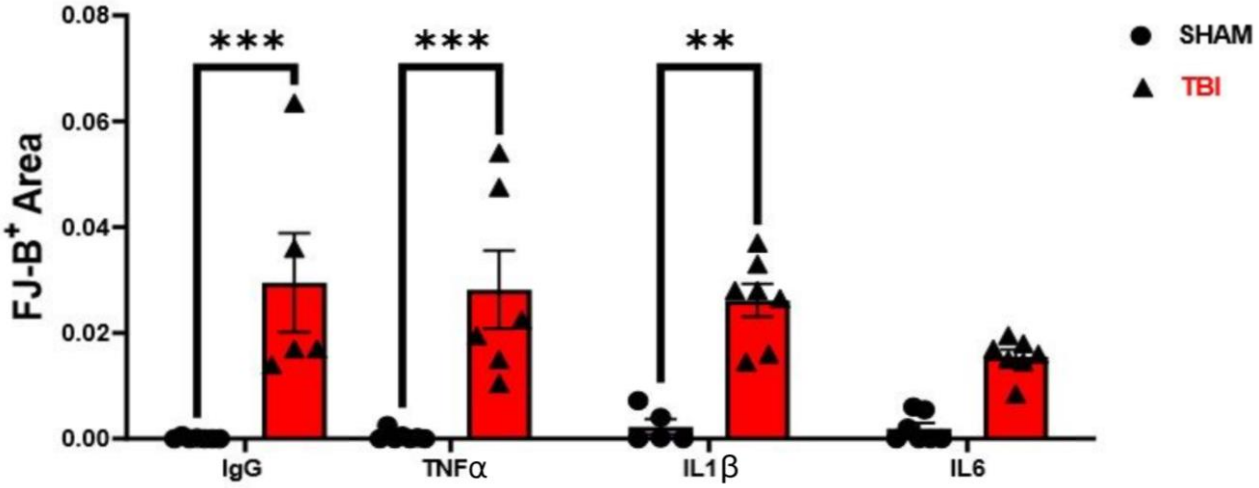


Fig. 3. Neurodegeneration in optic tracts, assessed via Fluoro-jade B staining. In TON mice, there was significant elevation of staining in mice treated with non-specific IgG, anti-TNF-alpha, or anti-IL-1beta. In mice treated with anti-IL-6, the increase was not statistically significant. ** $p < 0.01$. *** $p < 0.001$.

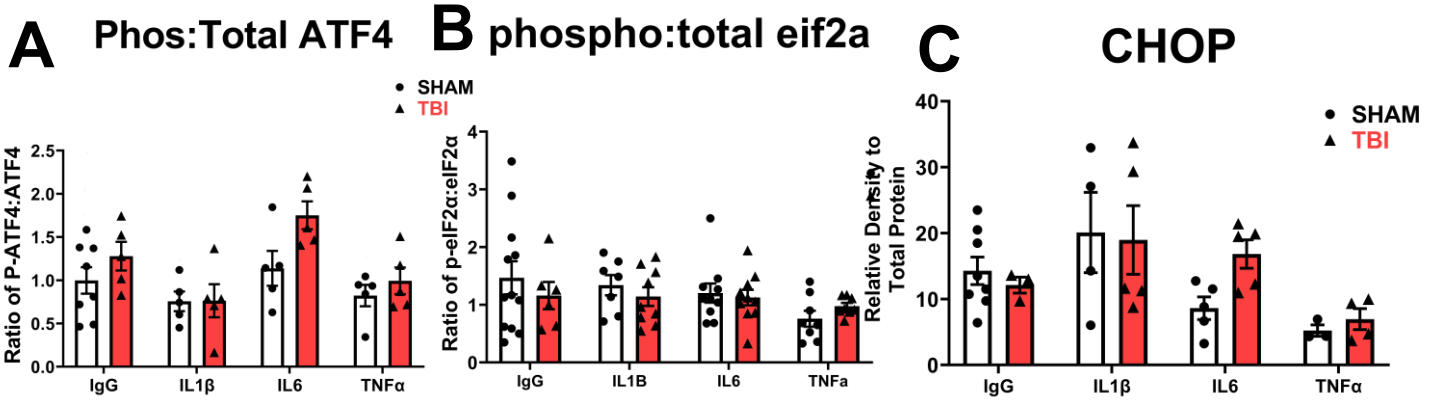


Fig. 4. Western blotting for retinal ER stress relevant proteins. There was not a significant effect of any anti-cytokine treatment on levels of phosphorylated. ATF4, phosphorylated eIF-2alpha, or CHOP

We also examined optomotor function in animals under these treatment conditions, at 7 and 30 days after TON. Compared to non-specific antibody treatment (IgG), these treatments improved optomotor behavior in some conditions. Anti-TNF-alpha improved function at 7 days post injury (DPI) in the 0.26 cycles per degree grating. At 30 DPI, anti-IL-1beta and anti-TNF-alpha improved function in the 0.26 cycles per degree grating. However, in some of these groups, mice appeared to do worse than control treated mice, which we interpret as representing some complexity in the effects of the systemic immune responses. Fig. 5 shows the results of optomotor testing.

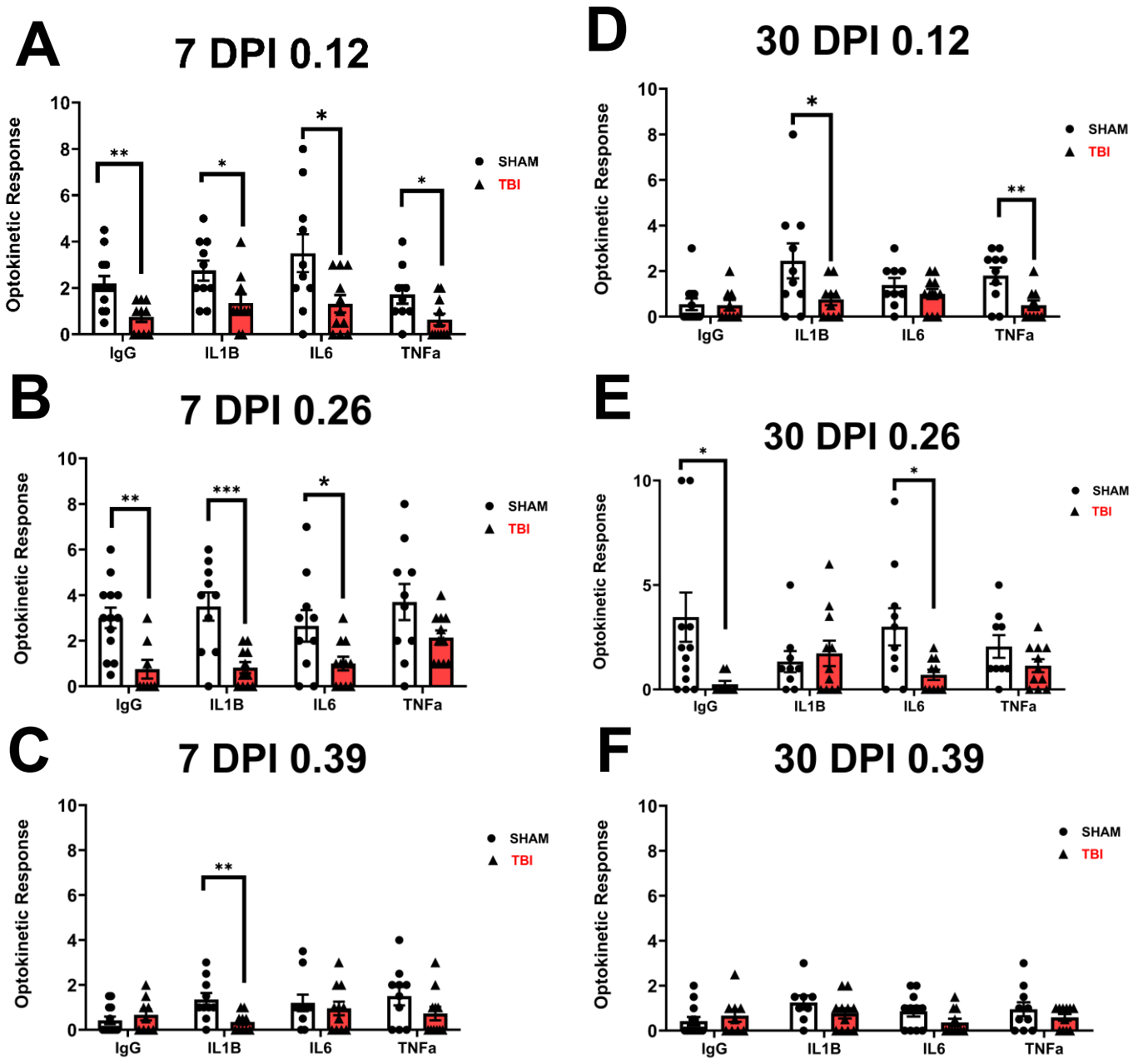


Fig. 5. Optomotor testing of mice at 7 and 30 days after TON (7 DPI and 30 DPI), after treatment with non-specific immunoglobulin (IgG) or monoclonal antibodies against IL-1beta, IL-6, or TNF-alpha. 0.12, 0.26, and 0.39 denote the optical grating thickness, and are measures of cycles per degree. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to sham animals in the same treatment group.

What opportunities for training and professional development has the project provided?

Nothing To Report

How were the results disseminated to communities of interest?

Results were presented at several international meetings including MHSRS (see below for details).

In addition, two manuscripts are currently being prepared for publication, including the data shown above and in Appendix 2, and are expected to be published within the next year.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The studies in Aim 1 showed that although there is a role of ER stress in optic nerve injury associated with head trauma, modulating ER stress did not appear to significantly improve outcomes after TON, at least within the parameters that we tested. However, we also observed an apparent compartmentalization of ER stress responses, that is different in retinal ganglion cell bodies than it is in the proximal or distal segments of the optic nerve. This difference in compartmentalization appears to suggest a role for ER stress responses in the distal axon in mediating degeneration of the distal axon after injury. There appear to be roles for glial cells including microglia and astroglia in this process. Better understanding of this compartmentalization may lead to effective interventions using this pathway that do not appear to work when given to the whole animal. In any case, these results are expected to improve our understanding of the pathophysiology of axon injury in TON.

Studies in Aim 2 show that there is a role of the systemic immune response on progression of TON after head trauma. In particular, results are promising in that blocking systemic cytokine systems appears to improve the pathology in this injury. The results also suggest that the role of systemic cytokines is complex, and that successful treatment of TON by modulating cytokine responses will likely require targeting multiple cytokines, and may require precision in timing of the treatments. These results overall support peripheral inflammation as a viable treatment target for TON associated with head trauma.

What was the impact on other disciplines?

The findings in Aim 1 of this project suggest that ER stress mechanisms may play a role in vision in the uninjured state. This finding may contribute to the field of eye physiology, by improving our understanding of ER stress in normal eye function.

The findings in Aim 2 of this project suggest that systemic immune responses to head trauma likely influence the outcome of eye injury. They also suggest that systemic inflammation driven by cytokine levels is not uniformly good or bad in the case of neurologic injury. This observation may improve the intersection of immunology with neurotrauma in the context of optic nerve injury.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects
Nothing to report.

Significant changes in use or care of vertebrate animals
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report - although one manuscript is nearly ready for submission (see Appendix 2 for the current draft).

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

1. **Evanson, N.K.**, Hetzer, S.M., and Torrens, J. Peripheral inflammatory cytokines in a murine model of TBI-associated traumatic optic neuropathy [poster presentation] presented at Military Health Service Research Symposium (Orlando, FL), 8/15/2023.
2. **Evanson, N.K.** Insights into traumatic optic neuropathy from animal research [invited symposium speaker] presented at American Academy of Optometry (New Orleans, LA), 10/12/2023.
3. **Evanson, N.K.** Insights into traumatic optic neuropathy from animal research [invited symposium speaker] presented at American Academy of Ophthalmology (San Francisco, CA), 11/5/2023.

- **Website(s) or other Internet site(s)**

  Nothing to report.

- **Technologies or techniques**

  Nothing to report.

- **Inventions, patent applications, and/or licenses**

  Nothing to report.

- **Other Products**

  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Nathan K. Evanson MD, PhD
No change

Name: Shelby Hetzer
No change

Name: Jordyn Torrens
No change

Name: Macy Urig
Project Role: Research Associate
Researcher Identifier: none
Nearest person month worked: 4
Contribution to Project: Ms. Urig assisted Jordyn Torrens in performing the experiments on peripheral inflammation and ER stress.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

See Appendix 1.

9. APPENDICES:

Appendix 1: Updated quad chart
Appendix 2: Near-final Manuscript for Aim 1 results, to be submitted for publication soon.

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ☑ or **No** ☐
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

1. ER stress per se does not appear to be a viable treatment target for TON, at least with the timing and dosage we used.
2. ER stress responses do appear to be compartmentalized, with different responses in the cell bodies as compared to axons in traumatic optic neuropathy. This finding may lead to better understanding of how to intervene in traumatic optic neuropathy.
3. Systemic inflammation does appear to be a viable treatment target for traumatic optic neuropathy.
4. Successful treatment of traumatic optic neuropathy via systemic inflammation will likely require targeting multiple cytokines and/or pathways simultaneously to obtain the best results.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

Yes - applied to CDMRP Vision Research Program. Total budget requested is $750,000.
Title: Targeting Peripheral Inflammation in Blunt Head Injury-induced Optic Neuropathy

4. What will be **the next step(s)** for this project?

The first step in the ongoing project will be to finish submitting the results for publication. Next, pending funding of the grant application submitted for this, will be to better understand the time course of systemic cytokine production after head trauma, then determine whether blocking multiple cytokine systems will lead to better improvement in visual system pathology and visual function than what was seen with blocking single cytokine systems. This project will also include more functional measures of vision including electroretinograms, optical coherence tomography, and pupillometry as endpoints. Including these techniques will increase the sensitivity for improvement with treatment.

5. How would you classify your **lead candidate product**? f

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify: Preclinical knowledge to identify and characterize potential treatment target(s)

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

Successful targeting of systemic inflammation after traumatic optic neuropathy has the potential to improve visual function after blunt head trauma. Given that vision loss after traumatic brain injury is a significant problem in warfighters, veterans, and civilian populations, the ultimate results of this research is expected to improve visual outcomes in all three of these populations.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

The next steps to move this product forward are to finish characterizing the target (systemic inflammation/cytokines) for how they can best be targeted by a therapeutic. Once this is completed, and specific targets are understood, we will need to identify candidate therapeutics (most likely biologics) that can target these. As treatments are currently available to target multiple cytokine systems, it is relatively likely that treatments will already exist that can target the identified cytokines, and that only the specific combination needs to be identified. From that point, translational and clinical trials will need to be performed.

At this stage in research, as we do not yet have a therapeutic identified, intellectual property issues are not yet at play. At future stages in this research, however, this will need to be explored. This will be done in cooperation with the Cincinnati Children's Hospital Medical Center Innovation Ventures team when a potential approach has been identified.

Role of endoplasmic reticulum stress and systemic inflammation in blunt TBI-induced optic nerve injury

W81XWH2110907-VR200166

PI: Nathan K. Evanson **Org:** Cincinnati Children's Hospital Medical Center **Award Amount:** $260,000

Study/Product Aim(s)

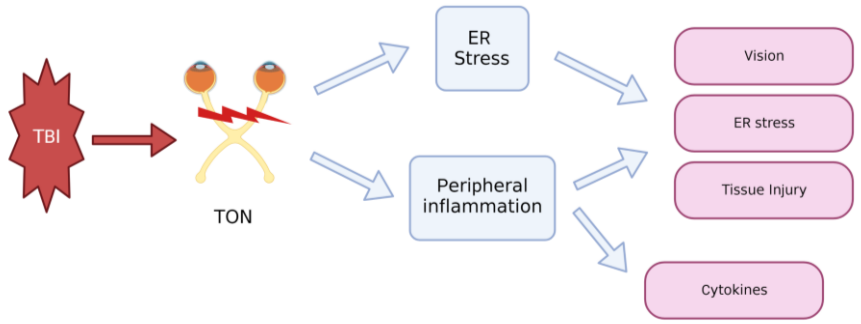
- Aim 1: To determine which element(s) of the ER stress response are most relevant to degenerative injury after optic nerve trauma.
- Aim 2: To determine the degree to which the peripheral inflammatory response contributes to outcomes after optic nerve trauma.

Approach

We are using a murine closed head weight drop model of head trauma, which results in bilateral optic nerve injury.

- Optokinetic nystagmus for visual function
- Histologic measures of tissue injury (brain and retina)
- Western Blotting for ER stress measures
- Serum cytokine measures for Aim 2
- Antibody-mediated neutralization of peripheral cytokines

Endoplasmic reticulum stress and peripheral inflammation in traumatic optic neuropathy.



TNF-alpha appears to be most important peripheral cytokine for driving visual deficits early after injury

Timeline and Cost

Activities	CY 21	22	23	
Specific Aim 1				
Specific Aim 2				
Data analysis and reporting				
Estimated Budget ($K)	**$32K**	**$130K**	**$98K**	

Updated: 12 Jan 2024

Goals/Milestones (Example)

CY21 Goal – Initiation of project
☑ Obtain regulatory approval, hire staff, get first cohorts
CY22 Goals –
☑ Initiate experimental procedures Aim 1
☑ Initiate experimental procedures Aim 2
CY23 Goal –
☑ Complete experiments and data analysis
☐ Submit results for publication (in process)

Budget Expenditure to Date

Projected Expenditure: $260K
Actual Expenditure: $260K

Targeting eIF2α in TBI-induced traumatic optic neuropathy: Effects of Salubrinal and the Integrated Stress Response Inhibitor (ISRIB).

Shelby M. Hetzer[1], Rohan Bellary[2], and Nathan K. Evanson[3, 4]

[1] University of Cincinnati, Neuroscience Graduate Program

[2] University of Louisville, College of Medicine

[3] Cincinnati Children's Hospital and Medical Center, Division of Pediatric Physical Medicine & Rehabilitation

[4] University of Cincinnati, Department of Pediatrics

Abstract

Traumatic brain injury (TBI) induces acute primary injuries and long-term injury associated with secondary cellular responses. One common result of primary injury is traumatic axonal injury (TAI) which can occur in the optic nerve. Optic nerve injuries, termed Traumatic optic neuropathies (TON), occur in up to 5% of TBI cases and lead to irreversible visual deficits. TBI-induced TON triggers the Endoplasmic Reticulum (ER) stress response including activation of both the PERK and IRE1a arms. TON-induced phosphorylation of eIF2α, a downstream ER stress activator in the PERK pathway, is a double-edged sword. eIF2a phosphorylation can lead to apoptosis or activate the cell's adaptive unfolded protein response. So, it follows that interventions exist to both increase or decrease phosphorylation of this factor, and animal models of TBI report similar benefits after the use of both interventional types, but no one has compared the two interventions against each other in the same model. We hypothesized that dephosphorylation, rather than phosphorylation, of eIF2α would lead to reduced apoptosis and improved visual performance and retinal cell survival. To test this, adult male mice were injected with Salubrinal (increases p-eIF2α), ISRIB (decreases p-eIF2α), or vehicle (6.25% DMSO in sterile saline) 60 minutes post-injury then tested visual performance, ER stress responses, and cell death. While both drugs improved visual acuity, neither rescued a loss in OKR and both impaired OKRs in control mice. Western blot analyses confirmed appropriate effects of both drugs in the retina but did not increase RGC survival. In addition to probing the retina for ER stress, we also collected optic nerves and were surprised to discover differences in eIF2a phosphorylation, antioxidant response, and protein folding chaperones between the regions and dependent on intervention. These results reveal important compartmentalized ER stress responses to axon injury and suggest that interventions in the PERK pathway may alter necessary homeostatic regulation of the UPR in the retina.

Introduction

Traumatic brain injuries (TBIs) affect approximately 2.9 million people yearly.(Taylor CA, 2017) TBI first induces a primary injury that manifests directly then latent secondary effects arise that tend to cause long-term complications due to molecular signaling cascades, leading to chronic neurological complications.(Ladak et al., 2019) Moreover, the results of TBI are widespread and not limited to any one brain-controlled system. This variability or lack of regional specificity results from the inherent diversity of TBIs where no one is identical to

another. Importantly, many of the widespread, divergent symptomologies are likely driven by diffuse axonal injury which has an estimated incidence of 73% of all TBI cases.

One way that axonal injury impacts TBI patients can be seen through the array of visual defects that arise in 50-60% of patients, (Ventura et al., 2014) with 2-3% attributed to injury of the optic nerve.(Chen et al., 2022) This optic nerve axonal trauma, or traumatic optic neuropathy (TON), is one pathophysiological outcome associated with TBI.(Steinsapir and Goldberg, 2011) As a result of injury to the optic nerve, patients experience a range of symptoms that include progressive retinal thinning, visual acuity decreases, or outright vision loss. Some longitudinal case studies report progressive visual changes as far after injury at 30 years.(Chen et al., 2017; Chan et al., 2019) Due to the concern surrounding this condition and the lack of knowledge regarding the mechanisms, treatment, or chronic consequences, there is a clear need to discover potential interventions.(Bastakis et al., 2019; Karimi et al., 2021; Wladis et al., 2021)

Nevertheless, TON remains understudied in this context despite emerging evidence that it is likely a more prevalent co-morbidity of TBI than previously assumed.(Evans et al., 2021; Hetzer et al., 2023) Our weight-drop TBI model provides an opportune system for exploring the effects of traumatic axonal injury to the optic nerve as we have shown that our closed-head injury produces replicable damage to the optic nerve with subsequent death of retinal ganglion cells (RGCs). We also previously found that TBI-induced TON leads to elevation of endoplasmic reticulum (ER) stress markers in the retina post-injury.(Hetzer et al., 2021; Torrens et al., 2023) Of the three ER stress receptors activated under duress (IRE1α, PERK, and/or ATF6), both the inositol-requiring enzyme 1 alpha (IRE1α) and PERK pathways were elevated up to 30 days post-injury. However, the PERK pathway was more sensitive to chronic upregulation and a brief-oxygen intervention in our model.(Torrens et al., 2023) so we decided to intervene in this pathway to assess effects on visual outcomes. There are two readily available pharmaceuticals previously tested in the context of TBI – Salubrinal,(Rubovitch et al., 2015; Logsdon et al., 2016; Tan et al., 2018; Wang et al., 2019) and the Integrated Stress Response Inhibitor (ISRIB).(Chou et al., 2017; Wenzhu Zhou et al., 2023) Both drugs target PERK's downstream eukaryotic translation initiation factor, eIF2α.

Once phosphorylated, following the heterodimerization of PERK, the alpha subunit of the tertiary eIF2 complex can act to improve ER stress by halting protein translation and/or promoting transcription of downstream effector mRNAs like antioxidants and apoptotic factors like CHOP (C/EBP Homologous Protein). Phosphorylated

eIF2α (p-eIF2α) inhibits binding of its guanine nucleotide exchange factor, eIF2B.(Konieczny and Safer, 1983) Without eIF2B, eIF2α cannot be returned to its active GTP-bound state, thus halting transcription due to a lack of eIF2's complete ternary complex. (Bogorad et al., 2018) Interestingly, depending on the severity of the stress in the ER, eIF2α can lead to translation of open reading frames for proteins that can assist with both cell survival (e.g., antioxidants, metabolic support, nutrient uptake, mitochondrial function) and cell death (e.g., CHOP, Caspase 12, autophagy genes(Bond et al., 2020)). eIF2α, however, is not solely associated with ER stress, but is also the crux of the Integrated Stress Response, which can respond to oxidative stress, heat shock, DNA damage, hypoxia, amino acid deprivation, viral infection, and ultraviolet light.(Wek, 2018; Bond et al., 2020) Upregulation of PERK-phosphorylation with eIF2α, along with IRE1α pathway activation, indicates ER stress, which is what we have shown in our model.

Given that the PERK pathway is upregulated in TON-induced visual deficits and sustained retinal cellular loss post-TBI,(Hetzer et al., 2021; Torrens et al., 2023) the PERK-eIF2α pathway poses a potential control point in preventing the secondary cellular outcomes of TON in retinal cells. From this duality of eIF2α-based stress control, we chose to compare effects of contrasting drugs, Salubrinal and ISRIB. Salubrinal promotes the continued phosphorylation of eIF2α to maintain decreased protein translation and reduce the burden on the ER during injured or diseased states. In contrast, ISRIB is a small-molecule inhibitor that prevents the formation of stress granules impairing eIF2α's ability to translate downstream factors like the pro-apoptotic Activating transcription factor 4 (ATF4)-CHOP cascade.(Sidrauski et al., 2015) ISRIB appears to only induce this downregulation of mRNA translation in stressed cells, leaving unstressed cells unaltered.(Sidrauski et al., 2015) As noted above, both of these drugs show promise in treating experimental TBI but neither has been tested in the context of TON or other models of optic nerve injury.

We hypothesized that manipulating the PERK pathway via ISRIB-mediated dephosphorylation of eIF2α would lead to increased retinal ganglion cell survival and improved visual function in a TBI-induced TON mouse model. Conversely, we predict opposite effects with Salubrinal, which we hypothesize will lead to increased apoptotic factors to induce ER-mediated cell death of RGCs (Figure 1).

Methods

Animals and Interventions

Eight-week old, male mice (C57Bl/6J) underwent a closed-head weight drop TBI as previously described.(Evanson et al., 2018) TBI occurred on day 0 of experimental procedures (Figure 2c). SHAM mice were anesthetized but did not undergo TBI. Mice were then injected intraperitoneally one-hour post-injury with a single dose of Salubrinal (1.5mg/kg), ISRIB (2.5 mg/kg), or Vehicle (6.25% DMSO in sterile saline). Doses were chosen based on previously published research to be sufficient to produce beneficial effects(Chou et al., 2017; Wang et al., 2019) and one-hour post injury dosing was chosen because preliminary data suggested that ER stress begins to elevate one hour post injury and remains elevated until at least 3 hours post injury (Figure 2d). Mice were thus split into six cohorts – Salubrinal (n=12), ISRIB (n= 12), or Vehicle (n= 12) intervention plus or minus TBI (TBI n = 36, SHAM n=36). Supplementary Figure 1 shows a detailed timeline of experimental procedures.

Optokinetic Response (OKR) Testing

As previously described,(Hetzer et al., 2021) mice underwent four days of behavioral testing for optokinetic nystagmus function using a custom built optokinetic behavioral assay (Figure 2b). Briefly, optokinetic testing utilizes the manipulation of sine-wave gratings ranging from wider to thinner alternating black and white bars to assess involuntary visual responses in mice (0.12, 0.26, 0.32, and 0.39 cycles per degree [cpd]). Mice are placed on an immobile platform as the gratings rotate around them in both clockwise and counterclockwise direction at two rpm for two minutes in each direction. A visual response in this task is the involuntary visual tracking of the moving stimulus, termed an optokinetic response (OKR). Thus, this device can be used to assess the basic functionality of the optokinetic response (I.e., is there one or is it blunted) in addition to visual acuity. Visual acuity is the ability of a subject to detect distinct visual stimuli (i.e., distinguishing the white and black bars as separate). This was assessed by the varying the cycles/degree to determine thresholds of the OKR. These gratings are shown on the x-axis of Figure 3. Higher cpds were indicative of thinner bars that are more “difficult” for the subject to discern, thereby providing us with information on the threshold of visual acuity, the frequency at which OKRs stop. (Thaung et al., 2002)

Western Blotting

Mice were euthanized by rapid decapitation seven days post injury, and retinas and optic nerves were extracted into lysis buffer (RIPA Buffer, Sigma Aldrich, CAT: R0278) plus Halt protease and phosphatase inhibitor (Thermo Fisher, CAT: 78440) and frozen on dry ice. Retinas and optic nerves were then lysed, and a BCA assay was used to determine protein concentrations and subsequent loading amounts (20-30µg). Controls of a sham brain (intermembrane) and water (negative) were used on each blot. Twenty µg retinal samples and 15ug nerve samples were loaded into SDS-PAGE gels and transferred onto Amersham Hybond-P 0.45 µm PVDF membranes (GE Life Sciences, Pittsburgh, PA; CAT: GE 10600029). Membranes were incubated in Fisher No-Stain Total Protein Stain (Thermo Fisher, CAT: A44449) as per manufacturer instructions, blocked in either 5% non-fat milk or 5% BSA (manufacturer dependent) for 1 hour at room temperature, followed by overnight incubation in primary antibodies at 4ºC (see antibody table 1). Membranes were rinsed in TBST then incubated in anti-rabbit HRP for two hours at room temperature. Blots were imaged using an iBright™ Imaging System (Thermo Fisher) and analyzed using Image J. Some blots were stripped following imaging in stripping buffer (β-mercaptoethanol, 20% Sodium Dodecyl Sulfate, and 1M Tris-HCl pH 6.8) for 30 minutes at 37ºC, washed, re-blocked, and exposed to the same immunoblotting steps as above.

Statistical Analysis

Optokinetic behavioral data were analyzed utilizing a 2-way ANOVA within each grating for each drug separately (injury x Sal/Veh or injury x ISRIB/Veh). This is done because gratins are expected to differ from each other. Results are presented with each grating in one graph for ease of interpretation. Western blot data were also analyzed by 2-way ANOVA for each protein of interest and graphed with both drugs on the same plot for ease. A critical significance level, α, was set at $p \leq 0.05$ for all statistical analyses, with a $\beta = 0.08$. Fisher's LSD was used for post-hoc analyses when relevant.

Results

Visual Function after TBI is differentially improved with Salubrinal and ISRIB.

We first began our assessment of ER stress inhibition by observing the effects of each drug intervention on overall visual response as indicated by the optokinetic behavioral assay. This assay was designed not only

to assess overall performance/retention of optokinetic nystagmus but also to ascertain any injury-induced drop offs in visual acuity.

Starting with ISRIB intervention (Figure 3a), overall results show no improvement in OKR. However, there is an increase in visual response in TBI+ISRIB mice at higher spatial frequencies (i.e., more difficult visual gratings). A main effect of TBI was observed across all gratings (0.12, 0.26, 0.32 and 0.39) with injury significantly reducing the overall number of OKRs recorded. The main effect of drug was found only at 0.26 and 0.32 cpd ("optimal/highest" range of mouse acuity as verified in our previous publications) with a significant interaction at 0.26 and 0.39. The interaction at 0.26 cpd was driven largely by a significant reduction in the total number of OKRs recorded by SHAM mice given ISRIB compared to SHAM+Vehicle ($p=0.02$) while both injured groups performed significantly worse than both SHAMs. In contrast, the interaction at 0.39 cpd was driven by TBI+ISRIB mice, who were not significantly different from SHAM+ISRIB mice ($p=0.6$), and performed better than TBI+Vehicle mice ($p=0.03$), though not significantly. Importantly, SHAM+Vehicle responses were not significantly different from SHAM+ISRIB at all gratings except 0.26 cpd. Table 2 provides a full accounting of all statistics for these data.

Given Salubrinal, similarly non-significant results were found (Figure 3b). A main effect of injury was observed across all gratings of 0.12, 0.26, 0.32 and 0.39. A main effect of drug was only observed at higher spatial resolution gratings (0.32 cpd $p=0.02$ and 0.39 cpd $p=0.04$). Like ISRIB, Salubrinal-injected SHAM mice performed worse than vehicle controls at one of the four gratings (0.39) and generally show fewer responses than controls. Despite overall drug effects, there were no improvements between injured vehicle and Salubrinal mice, but there were several instances where injured mice given salubrinal were no longer impaired compared to SHAM + Salubrinal mice (0.26 and 0.32). Finally, a significant interaction only occurred at 0.39 cpd where Salubrinal induced significant decrease in OKRs between sham groups. Table 3 provides a full accounting of all statistics for these data.

Salubrinal and ISRIB have long lasting predicted effects on eIF2α phosphorylation in the retina but not in the optic nerve.

Injury elevated the ratio of p-eIF2α to total eIF2α in both retinas and optic nerves (Figure 4). There were no significant increases given ISRIB in the retina ($p =0.3$) nor in the nerve ($p=0.07$), in line with its mechanism of action. Conversely, Salubrinal maintained elevated levels of p-eIF2α following TBI, as shown by a main effect of

injury (p=0.04) in the retina. However, in the nerve, TBI + Salubrinal mice showed no increase in p- eIF2α ratio despite an increase in TBI + Vehicle mice. Further, there were no outright differences in total eIF2α in either tissue, but in the retina there was a significant increase in p-eIF2α given TBI + Salubrinal (main effect of drug 0.04) (supplementary figure 2a-d). Detailed statistics for all western analyses are presented in tables two and three.

Neither Salubrinal nor ISRIB prevent retinal cell loss or affect apoptosis.

RBPMS western blot analysis (figure 5a,b) displayed no retinal ganglion cell preservation given either drug. A main effect of injury was observed as expected except between SHAM and TBI. Although TBI+ISRIB clearly showed reduced RBPMS expression compared to SHAM+ISRIB, this was technically insignificant (p=0.06). Additionally, there was no upregulation of apoptotic markers. While our lab has shown upregulation of Caspase-3 at this time point in adolescent mice, this has not been the case with our adult animals (figure 5c,d). However, we do repeatedly find that peak cell death has usually occurred by 7 days post injury with most active cell-death likely complete before this timepoint, so this result is unsurprising. We also measured a well-described pro-apoptotic factor downstream of eIF2α and ATF4, CHOP (C/EBP Homologous Protein; aka GADD153).(Marciniak et al., 2004) We previously reported elevated CHOP expression in this model given injury,(Hetzer et al., 2021) and our results support that this is likely still true; for, although not significant in this experiment (p=0.06), TBI+Vehicle CHOP is elevated compared to SHAM (figure e,f). Because this was insignificant, it is difficult to draw conclusions from the lack of significance found between controls and our drug conditions. Finally, Caspase-12 has been argued to be an ER-specific caspase,(Rao et al., 2002) so we looked for elevation in this apoptotic factor. Although there was a significant interaction when assessing ISRIB intervention, post hoc tests revealed no significant t-tests (figure 5g,h). Additionally, a main effect of drug was found for Salubrinal, but post hoc tests revealed no significance. Because these markers are predominantly associated with somatic responses, they were not analyzed in nerve samples.(Rao et al., 2002; Sanges et al., 2006)

Only some downstream effectors of the PERK, and not the ER-stress specific IRE1α, pathway are affected by drug, and this is region dependent.

Because of the overlapping nature of ER stress with the Integrated Stress response, we wanted to confirm activation of a second ER stress pathway – IRE1α. To do this we examined the downstream X-box

Binding protein 1 (XBP1), which is spliced upon IRE1α oligomerization allowing it to upregulate genes involved in both maintenance of proteins and their degradation.(Hetz et al., 2020) Additionally, we chose XBP1 as an indicator of ER stress as there is crosstalk and parallel activation of XBP1 by the third branch of the UPR, ATF6.(Walter and Ron, 2011) Unspliced XBP1 (XBP1u) and spliced XBP1 (XBP1s) were not different between any groups in the retina or the nerve (Supplementary Figure 1e-h). We found no difference in the ratio of the two (figure 6a-d).

As previously described, the PERK pathway hinges on eIF2α phosphorylation, after which markers like ATF4 and nrf2 (Nuclear factor erythroid 2-related factor 2) can be upregulated to respond to the stress at hand. ATF4, a transcription factor, can be assessed in total and phosphorylated forms. There were no changes in either form in the retina and this did not change with intervention (Supplementary Figure 1i-j) or as a ratio (Figure 6e-f). In the nerve, we show no change in total ATF4, but a significant main effect of injury ($p=0.02$) for phosphorylated ATF4 (Supplementary Figure k-l). Moreover, there was a main effect of Salubrinal ($p=0.04$) and injury ($p=0.003$) when considering the ratio between the two within-subjects (Figure 6g-h). Post-hoc analyses revealed lower rates of ATF4 phosphorylation given Salubrinal, but only in SHAM mice who had a significantly lower ratio compared to their injured counterparts ($p= 0.004$). There were no effects of ISRIB on ATF4 expression in the nerve (figure 6g-h).

Further downstream, beneficial factors like nrf2 can be translated to reduce oxidative burden. In the retina, there was a significant interaction for both drugs driven largely by the significant increase in nrf2 expression given both injury and either ISRIB or Salubrinal, though Salubrinal increases nrf2 more drastically (figure 6i-j).. A complete inverse was true of the optic nerve, whereby there were main effects of injury revealing a significant reduction in overall nrf2 expression regardless of intervention (figure 6k-l). Because we observed an increase in nrf2 in the retina, we checked for a commonly upregulated oxidative stress marker in TBI – NADPH oxidase 2 (NOX2) (Chuang et al., 2013). There was a main effect of ISRIB ($p=0.02$), and a main effect of injury ($p=0.05$). Post-hoc tests revealed a significant increase in NOX2 in SHAM+ISRIB mice compared to TBI + Vehicle ($p=0.009$) and SHAM + Vehicle ($p=0.03$), which was then reduced in TBI + ISRIB mice ($p=0.04$; figure 6m-n). There were no significant differences when using Salubrinal (figure 6m-n). Lack of sufficient protein prevented us from looking at NOX2 in the optic nerve.

ER protein folding chaperone, ERO1La, is differentially affected by injury, drug, and location.

Two key protein folding chaperones reside in the ER to produce the necessary disulfide bonds for proper protein folding. We only had sufficient protein to assess one of these chaperones, so we chose ERO1α. If done under non-reducing conditions, three bands can be distinguished via western blot – a low twice-oxidized (Ox2) band at 45 kDa, a middle once-oxidized (Ox1) band around 50 kDa, and a reduced (R) band around 60 kDa.(Benham et al., 2013a) In the retina, only the oxidized forms of ERO1α were detected. We show no differences in the Ox1 band for either drug. For ISRIB, we found main effects of both drug ($p=0.02$) and injury ($p=0.002$) for the Ox2 band (Figure 7a). Injury significantly reduced overall ERO1α Ox2, but ISRIB was able to significantly increase this between TBI+Veh and TBI+ISRIB ($p=0.04$). For Salubrinal, there was a significant interaction ($p<0.0001$). Injury reduced overall ERO1α Ox2 ($p=0.0005$), and salubrinal alone decreased ERO1 Ox2 between SHAM vehicle and Salubrinal mice ($p=0.007$). However, given injury, Salubrinal had the opposite effect whereby it significantly increased ERO1α-Ox2 between TBI + Vehicle and TBI+Salubrinal groups ($p=0.0003$; Figure 7c)

We also found significant changes in ERO1α in the optic nerve. Here, there was a main effect of injury ($p=0.01$) on ERO1α-Ox2 (Figure 7b). Both injured vehicle ($p=0.03$) and injured ISRIB ($p=0.04$) mice had less ERO1α-Ox2 than SHAMs. This reduction was not seen in injured Salubrinal mice ($p=0.9$). As in the retina there were no changes in the Ox1 band (Figure 7d). However, there was a detectable reduced ERO1α band in optic nerve blots. Analyses revealed no effect of TBI unless given ISRIB, in which case TBI+ISRIB mice had elevated levels of reduced ERO1α compared to TBI+Veh and SHAM+ISRIB ($p<0.05$) and SHAM + Veh ($p=0.05$; Figure 7e).

Discussion

We show that interventions targeting eIF2α in a model of traumatic optic neuropathy do not improve visual function nor retinal ganglion cell survival but do reveal intricacies in compartmentalized ER stress responses and effects of ISRIB alone that TBI further impacts. Despite appropriate regulation of eIF2α phosphorylation based on the mechanisms of Salubrinal and ISRIB, there were few significant differences in ER stress response and in downstream integrated stress response factors. Instead, the protein folding machinery of the ER, particularly the primary oxidase ERO1α, might be more sensitive to/revealing about these manipulations.

We further show that ER and oxidative stress associated protein expression is unique based on which compartment of the neuron we studied with retinal extracts revealing predicted downstream proteins like nrf2 with upregulation of p-eIF2α while the opposite was true of the optic nerve.

Although some literature suggests that PERK pathway stimulation via interventions like Salubrinal can be neuroprotective via prolonged increases in p-eIF2α (Athanasiou et al., 2017; Starr and Gorbatyuk, 2019) or nrf2,(Comitato et al., 2020) we do not show similar protection in this model. Nor do we show protection when decreasing activation of this pathway, as others have shown with both ISRIB (Chou et al., 2017; Wenzhu Zhou et al., 2023) and other interventions against downstream proteins like ATF4 or CHOP in models of TBI.(Song et al., 2008; Pitale et al., 2017; Hu et al., 2019; Neill and Masson, 2023) Optomotor response data showed that ISRIB and Salubrinal led to modest improvements in visual acuity but did not pose a direct benefit on OKRs. These impacts on visual acuity suggest that either drug may play a role in the restoration/preservation of photoreceptors (cones/rods) following injury rather than RGCs. Indeed, ISRIB may preferentially preserve cone function despite loss of rods in a model of inherited retinal degeneration.(Gorbatyuk et al., 2020)

Our western blot findings that neither intervention prevented loss of RGCs could also support this line of thinking. Inhibition of the apoptotic factor, CHOP, occurred in both drug conditions despite an increase in TBI+vehicle mice. This indicates that even though interventions lower ER-mediated apoptosis at 7 days post injury, this was not sufficient to prevent equal loss of RBPMS expression. This becomes a more likely theory when considering our unpublished electroretinogram findings. In a small cohort of control versus injured mice, we showed significant delays in both A wave (a rod-driven response) and B wave (a bipolar cell/cone driven response) latency at 7 DPI. This deficit was not seen at 24 hours, 3 days, 14 days, or 30 days post injury, suggesting that 7 days post injury is a critical time point for photoreceptors (Supplementary Figure x). Unfortunately, limited tissue prevented us from determining the effects of our interventions on photoreceptors, which also respond to ER stress and ISRIB treatment.(Gorbatyuk et al., 2020)

It is also important to note that there were inhibitory effects of both ISRIB and Salubrinal on SHAM mouse OKR, potentially through undescribed ER stress mechanisms. The combination of injury and ISRIB, in particular, revealed novel interactions. ISRIB alone increased expression of the ER-mediated pro-apoptotic caspase 12 and oxidative stress marker, NADPH oxidase 2 (NOX2). Due to the complex nature of the Integrated Stress response, which, similarly to ER stress, hinges on eIF2α phosphorylation, but has many more channels for

activation, ISRIB could be dampening the regulatory need of the system for p-eIF2α. This makes sense, for in the eye the oxidative environment is inherently higher than in other tissues, so it is capable of managing higher levels of oxidative stress. Perhaps inhibiting a pathway for dealing with oxidative stress, protein folding, and metabolic regulation could prove more detrimental in the eye than in the optic nerve. For example, it was found that targeting p-eIF2α, both by systemic GADD34 knockout and photoreceptor specific PERK knock out, in a model of inherited retinal degenerative disease was not protective. Instead, retinal cells might be required to activate alternative pathways for protein synthesis control.(Starr and Gorbatyuk, 2019)

Additional studies using models of retinitis pigmentosa in age-related macular degeneration report similar negative findings when p-eIF2α is increased. This is in contrast to work performed in hippocampal cultures which showed that Salubrinal-induced upregulation of p-eIF2α and ATF4 led to subsequent antioxidant defense increase and resistance to a number of cellular stressors.(Lewerenz and Maher, 2009) Based on our acute findings and these negative effects, it might be that ISRIB/Salubrinal administration may be more harmful to photoreceptors than beneficial for RGCs. Further support for this idea comes from research suggesting that photoreceptors and the retinal pigment epithelium responds poorly to these drugs(McLaughlin et al., 2022) and that basal/homeostatic upregulation of ATF4 can be beneficial for photoreceptor regeneration.(Bhootada et al., 2016)This contrasts with the brain or tumor cells where we were unable to find reports of similar effects for either drug. In fact, ISRIB is described as being able to avoid affecting unstressed cells.(Sidrauski et al., 2015)

This lack of ER stress upregulation, functional improvement, and RGC preservation in the retina nearly led us to move on from this study. But recent research has taken notice of proximal axon signaling, and it inspired us to probe our collected optic nerve samples. This seemed more important to do once we showed that our model provides a unique opportunity to dissect out proximal axon responses over distal, degenerative mechanisms because axon breakage occurs more distal to the eye than in most other optic nerve injury models. While optic nerve crush, transection, and ocular blast injures optic nerve axons in or behind the eye, we injure the optic nerve in a replicable 1-1.5 mm section just proximal to the optic chiasm.(Hetzer et al., 2023) Thus, most of what we know about the proximal axon is from *in vitro* or *ex vivo* studies.(Mok et al., 2009; Baleriola et al., 2014; Hao et al., 2016; Pathak et al., 2016; Almasieh et al., 2017; Hao et al., 2019) This distinction is made more important because differences between signaling in distal and proximal axon segments have been unearthed including retrograde phosphatidyl serene externalization,(Almasieh et al., 2017) upregulation of the DLK-cJUN

pathway,(Larhammar et al., 2017; Asghari Adib et al., 2018; Kievit, 2019; Ugbode et al., 2019) local calcium signaling,(Calixto et al., 2012; Frati et al., 2017) and more. It has also been proposed that the autonomous ER may be a critical mechanism for injury signaling in the peripheral nervous system where models of axon injury are made easier to study than in the central nervous system.(Ying et al., 2014; Farley and Watkins, 2018; Ohtake et al., 2018) This distinction is important because one process can be regenerative in the PNS, while the same process is degenerative in the CNS.(Farley and Watkins, 2018)

Therefore, our ability to study the proximal portion of the optic nerve over the degenerating distal end in the brain/past the optic chiasm could reveal unique ER-stress related injury responses. We might expect some differences between smooth and rough ER as rough ER, predominantly associated with ER stress and protein synthesis,(Kuijpers et al.; Pryme, 1986; Almanza et al., 2019) is located only in the soma. In contrast, the axon contains only smooth ER with free floating ribosomes.(Kuijpers et al.; Voeltz et al., 2002) Ozturk, et al. have shown that axonal ER might be less involved in the canonical ER stress response and might, instead, respond to perturbations via Ca^{2+} signaling, lipid synthesis, or communication with mitochondria at mitochondrial-associated membranes.(Leitman et al., 2013; Villegas et al., 2014; Sun et al., 2017; Carreras-Sureda et al., 2019; Fan and Simmen, 2019; Öztürk et al., 2020) Furthermore, an argument can be made for the importance of this interconnected meshwork of ER in axonal degeneration as axonal ER can participate in anterograde and retrograde transport and has processes necessary to repair or communicate breakage along the whole of the neuron.(Öztürk et al., 2020) It is, therefore, likely that differences exist in how that axon might respond to injury and interventions against ER-stress signaling compared to the soma.

We first assessed eIF2α phosphorylation in the nerve and found increased phosphorylated eIF2α given injury, while ISRIB seemed to reduce this phosphorylation as predicted. However, Salubrinal either had no effect or also reduced p-eIF2α as TBI and sham mice were not different from each other. As in the retina, there was no increase in XBP1s, so we began to look at PERK's downstream cascade. One of the first factors that eIF2α upregulates upon phosphorylation is ATF4, which targets genes involved in apoptotic, autophagy, antioxidant, and other stress response functions. We previously showed that increased phosphorylation of ATF4, likely indicative of ATF degradation,(Lassot et al., 2001) was associated with less axonal degeneration in the optic tract given a brief-oxygen intervention.(Torrens et al., 2023) Here we show a similar increase in ATF4 phosphorylation rather than total ATF4 in the optic nerve after salubrinal treatment. This is interesting because

prolonged eIF2α phosphorylation typically leads to prolonged upregulation of total ATF4 translation. However, a recent meta-analysis of ATF4 post translational modifications shows that ATF4 phosphorylation at serine 215, rather than S219, increases ATF4 activation/activity.(Neill and Masson, 2023) Conversely, we found an insignificant increase in ATF4 phosphorylation in the retina after ISRIB + Injury which makes sense if one follows the previously proposed line of thinking (i.e., reduced p-eIF2α would lead to reduced ATF4 translation or to signals for its degradation upon cessation of ER burden). It could be argued that this difference may have been significant given a higher n or a more sensitive assay. There are luciferase assays for ATF4 that more clearly reveal phosphorylation states and other modifications to better answer the question of what these increases mean that should be considered for future experiments.

This is critical to accurately assess the function of ATF4 in these studies as ATF4's function is dependent on its binding partners, post-translational modifications, or histone modifications. Within phosphorylation state alone, p-ATF4 could be destined for ubiquitination by SCF E3 ligase,(Lassot et al., 2001) decreased transcription of its downstream targets, or overt downregulation.(Bagheri-Yarmand et al., 2015; Park et al., 2019) Additionally, we did not collect fixed tissues for imaging in this study, which would be necessary to assess axonal degeneration or changes in the brain. This will be critical in future studies to understand both ER stress localization and whether Salubrinal or ISRIB is more beneficial on the distal side of injury rather than the proximal/somal side that we have analyzed here.

ATF4 can instigate the expression of the antioxidant nrf2, as can the inherent activation of the PERK receptor, tightly inking ER stress to oxidative stress responses. But little is currently known about this ER stress-oxidative stress relationship, particularly under pathological conditions. Studied alone, oxidative stress is a major mechanism of injury pathology associated with TBI,(Kontos and Povlishock, 1986; Khatri et al., 2018; Ismail et al., 2020; Chandran et al., 2021) ocular diseases,(Masuda et al., 2017; Rohowetz et al., 2018; Chan et al., 2020) and TON.(Bricker-Anthony et al., 2014; O'Hare Doig et al., 2014; Tao et al., 2017; Bernardo-Colón et al., 2018; DeJulius et al., 2021) As predicted, Salubrinal most significantly increased nrf2 in injured mice. Perhaps more intriguing is that this increase was not seen in the optic nerve. Although nrf2 requires nuclear translation and this could explain a lack of nrf2 in the axon, it seems odd that it would not be shuttled down the axon to the primary site of injury or expressed in glial cells in the nerve. However, our samples are from seven days post injury, which could be too late to see a need for antioxidants in the nerve. Support for this possibility comes from a variable

time course of ROS elevation, which can range from one hour to 28 days,(Lieven et al., 2006; Kanamori et al., 2010; Bricker-Anthony et al., 2014; O'Hare Doig et al., 2014) but this research is limited to understanding the retinal response not the axonal response. Future studies will need to probe the axon for ROS markers and perhaps visualize where and when exactly oxidative stress occurs in each compartment of the injured neuron (i.e., somal versus proximal versus distal).

We were able to assess one ROS producing enzyme, Nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase 2 (NOX2). NOXs have been associated with TBI and reviewed by many.(Khatri et al., 2018; Yao et al., 2021) Compared to other NOXs after TBI, NOX2 appears most consistently upregulated in the central nervous system, (Chuang et al., 2013) where it is localized in the plasma membrane of neurons and glial cells.(Ibi and Yabe-Nishimura, 2020) Moreover, ER stress-mediated NOX2 upregulation is more likely to lead to cell death than other NOXs like NOX4, which can be both pro-survival and proapoptotic.(Laurindo et al., 2014) As mentioned above, ISRIB treatment significantly increased NOX2 expression in control mice while TBI nearly significantly decreased overall NOX2 expression with or without ISRIB treatment. Perhaps using ISRIB when there is no increase in ER/oxidative stress prevents the homeostatic functions of this combined system. When ER stress would otherwise have led to oxidative stress, ISRIB might be able to prevent NOX2 expression. Support for linkage of these two pathways comes from a study by Li et al., showing that genetic deletion of NOX2 blocked ER-mediated apoptosis, (Li et al., 2010) similar to our finding of blocked CHOP increase after ISRIB treatment. Here we must note that retinal and optic nerve tissue samples were limited, so by the time these questions became relevant, our sample numbers were significantly reduced.

Like all biological pathways, overlap is bound to arise, and many roads point to a critical relationship between ER stress and oxidative stress. Not only do NOXs generate hydrogen peroxide but so does the increase in protein folding that occurs with ER stress. The ER folding chaperones ERO1α and protein disulfide isomerase (PDI) create H_2O_2 upon disulfide bridge formation during folding, and PDI is also sensitive to NOX signaling.(Laurindo et al., 2014; Zito, 2015) Thus, it is surprising that we found no upregulation in NOX2 despite changes in ERO1α. ERO1α is critical for the maintenance of the ER's oxidizing environment (Pollard et al., 1998) where a too-reducing environment can disrupt bond formation and a too-oxidizing environment can induce improper/excess folding.(Sevier and Kaiser, 2008) ERO1α exists in three recognized states: an inactive oxidized (Ox2) form, an active partially oxidized (Ox1) form, and a reduced (R) form. When assessed by western blot

under non-reducing conditions, each of these isoforms can be analyzed.(Benham et al., 2013b) In response to a changing redox environment, ERO1α must fluctuate between active and inactive states, which it can do within minutes, positioning it as a crucial indicator of the ER's redox environment.(Benham et al., 2013b)

In the retina, where the rough ER resides and the brunt of protein folding takes place, we show that there is no change in the active Ox1 form of ERO1α, nor is there any detectible reduced ERO1α. Instead, injury significantly reduced the inactive form of ERO1α and both ISRIB and Salubrinal increased inactive ERO1α -Ox2. It is odd that there is not an accompanying increase in active/reduced ERO1α with this decrease in the inactive isoform given injury. Still, an increase in Ox2 with ISRIB and Salubrinal suggests that these interventions influence the return of ER to equilibrium as higher levels of inactive Ox2 indicate an increasing oxidizing environment. Increased ERO1α -Ox2 might also imply that there is reduced oxidative stress as decreased ability of ERO1α to interact with PDI would lead to reduced production of H_2O_2 and increased reduced glutathione (GSH). It has also been suggested that when the ER becomes over oxidized by excess reactive oxygen species, ERO1-Ox2 dissociation from PDI allows ERO1a to travel to the Golgi complex and further reduce the hyper-oxidation of the ER under duress.(Kakihana et al., 2013) Future experiments will need to clarify the binding state and localization of ERO1a to better understand its role in the ER stress response.

In the axon, it is theorized that ERO1 may be less crucial to protein folding maintenance and more important for Ca^{2+} maintenance (Li et al., 2009; Anelli et al., 2011) since smooth ER is enriched with mitochondrial ER-associated membranes (MAMs). In these regions, ERO1 and PERK have been shown to interact with each other to regulate ER-mitochondrial calcium dynamics and metabolically adapt mitochondria via increased MAM contact sites under ER stress in an attempt to restore/moderate ROS levels.(Bassot et al., 2023) It is, therefore, interesting that we see no changes in axonal ERO1 after injury unless ISRIB is administered. ISRIB decreased phosphorylation of eIF2α, which would prevent eIF2α's ability to inhibit protein translation, allowing the ER to continue producing and folding proteins under ER stress and reducing PERK activation. We did show that ISRIB prevents p-eIF2α increase in the nerve, so it is possible that the increase in ERO1-Ox2 and reduced ERO1, could indicate that the ER is unable to control the redox environment under these conditions. Future studies will need to better assess PERK changes, MAMs, calcium flux, and the redox state of the ER.

Limitations

Overall, these data helped us to confirm the acute (i.e., 7 day) effects of Salubrinal and ISRIB on eIF2α and on visual system functioning. Moving forward with this study, the first thing that needs to be reexamined is the dose-response. This study utilized a single dose that occurred one-hour post-TBI. Salubrinal has a half-life of 1.2 hours in vivo (Zhang et al., 2012) and ISRIB has a half-life of about 8 hours.(Sidrauski et al., 2013) Our tissues were extracted 7 days after injury/injection occurred. Thus, multiple dose administration needs to be explored as well as a dose response to assess whether more pronounced effects could be seen including improved RGC and photoreceptor survival/function.

Next, it should be noted that while cell death markers such as Caspase 3 and Caspase 12 were examined using western blotting during this report, these caspases should be analyzed using a more accurate enzyme assay. Caspases are proteolytic enzymes, and while western blot analysis is appropriate if completed in conjunction with cleaved to total caspase ratios, an enzyme assay would provide the best analysis of Caspase activation rather than simply the quantity of protein present. Similarly, only RBPMS expression was utilized to measure retinal cells within this study. We were interested in RGCs as they are the cells that undergo axonal damage following TBI, but no other cell types were studied, and our functional analysis cannot necessarily differentiate among cell types. In future studies, an electroretinogram should be implemented to gain a better idea of retinal cell function.

Further visualization of ER damage with electron microscopy and assessment of MAMs and PERK or IP3 receptor alterations could provide more insight into rough versus smooth ER response to injury since this has not yet been considered in other models of TON. Finally, it should be noted that this study was performed only in adult male mice. Further studies should examine the effects of TBI-induced TON within a more diverse demographic of animals, including females and adolescents. Both of these populations could prove informative as we have shown significant differences between adult and adolescent populations in our model. Adolescents appear to produce a more consistent ER stress response after optic nerve injury, while adults, as were used in this study, have less robust upregulation of the IRE1α pathway. This could be a result of differences in protein translation that come with age, whereby adolescents have a higher rate of protein synthesis and turnover than adults which could be more sensitive to axon injury(Kim et al., 2023). Moreover, we have yet to characterize effects of TON in females, but others have found that oxidative stress responses after TBI, particularly involved

with mitochondrial dysfunction,(Gupte et al., 2019) are affected by sex through inherent antioxidant properties of female hormones, which could influence ER stress and the redox state of the ER.(Morrow et al., 1992; Bayir et al., 2004)

Conclusions

Overall, our data suggested that both drugs control PERK pathway regulation long after they have presumably been fully metabolized. Although this did not result in pronounced functional changes or altered RGC survival, we do see novel alterations in redox-sensitive factors that suggest that the primary mechanism of axon signaling in TON is more likely the integrated stress response or a combination of both ER and oxidative stress responses. These data also show that the eye is highly sensitive to changes in the PERK pathway since interventions in control mice both worsened performance and increased oxidative and apoptotic markers in the absence of injury. We further confirm that axon and soma do not respond identically to axon injury. Of import to past and future treatment strategies, this difference may be critical to understanding why a therapy might work in one scenario but not another. Thus, compartmentalization of neuronal signaling mechanisms will be an important piece of the puzzle as we continue searching for treatments for TON and traumatic axonal injury.

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Tables

Table 1 Antibodies

Table 1							
Antibody	**Concentration**	**Host Species**	**Molecular Weight (kDa)**	**Supplier**	**CAT**	**RRID**	**Immunogen**
ATF4	1:2000	Rbt Poly	48, 60	Fisher	10835-1-AP	AB_2058600	UniProt AG1279
Caspase 3 (Cas 3)	1:2500	Rbt Poly	30	CST	9662	AB_331439	synthetic peptide corresponding to residues surrounding the cleavage site of human caspase-3
Caspase 12 (Cas 12)	1:1000	Rbt Poly	42, 55	CST	35965	NA	synthetic peptide corresponding to residues surrounding Lys163 of mouse caspase-12 protein
CHOP/ GADD153	1:1000	Rbt Poly	23	Novus Bio	NBP2-13172		UniProt P35638
eIF2α	1:2500	Rbt Poly	38	CST	5324	AB_10692650	UniProt P05198
P-eIF2α	1:500	Rbt Poly	38	CST	3398	AB_2096481	UniProt P05198
ERO1L α	1:1000	Rbt Mono	50	Fisher	702709	AB_2716886	Protein corresponding to human ERO1L (aa22-aa468)
nrf2	1:1000	Rbt Poly	55	Novus Bio	NBP1-32822	AB_10003994	Recombinant protein encompassing a sequence within the center region of human NRF2.
NOX2	1:500	Rbt Poly	58	Novus Bio	NBP2-41291	NA	Antibody was raised against a 15 amino acid peptide near the amino terminus of human NOX2. The immunogen is located within amino acids 140 - 190 of NOX2. Amino Acid Squence: NFARKRIKNPEGGLY
PERK	1:1000	Rbt Poly	140	CST	5683	AB_10841299	UniProt Q9NZJ5
RBPMS	1:1000	Rbt Poly	28	Fisher	PA5-31231	AB_2548705	Recombinant protein fragment corresponding to a region within amino acids 28 and 196 of Human RBPMS
XBP1U	1:500	Rbt Poly	38	Fisher	25977-1-AP	AB_2880326	XBP1 Fusion Protein Ag21714 (167-261 aa encoded by BC000938)
XBP1s	1:500	Rbt Poly	45/55	ProteinTech	24868-1-AP	AB_2879766	24868-1-AP is XBP1S-specific Fusion Protein expressed in E. coli
anti-Rbt HRP secondary	1:500-1:3000	goat	N/A	CST	7074	AB_2099233	

Table 2								
SALUBRINAL								
Grating 0.12								
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 13.63	**0.006**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM Sal**	**SHAM Veh vs TBI Sal**	**TBI Veh vs SHAM Sal**	**TBI Veh vs TBI Sal**	**SHAM Salvs TBI Sal**
Drug	F(1,44)=1.05	0.31	**0.007**	0.66	0.06	**0.002**	0.32	**0.02**
Interaction	F(1,44)=0.16	0.69						
Grating 0.26								
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 3.96	**<0.0001**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM Sal**	**SHAM Veh vs TBI Sal**	**TBI Veh vs SHAM Sal**	**TBI Veh vs TBI Sal**	**SHAM Salvs TBI Sal**
Drug	F(1,44)=0.04	0.83	**<0.0001**	0.12	**0.0001**	**0.0006**	0.21	**0.01**
Interaction	F(1,44)=0.16	0.05						
Grating 0.32								
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 24.05	**<0.0001**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM Sal**	**SHAM Veh vs TBI Sal**	**TBI Veh vs SHAM Sal**	**TBI Veh vs TBI Sal**	**SHAM Salvs TBI Sal**
Drug	F(1,44)=5.4	**0.02**	**0.0004**	0.06	**<0.0001**	0.29	0.91	0.07
Interaction	F(1,44)=0.19	0.17						
	Grating 0.39							
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 6.81	**<0.0001**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM Sal**	**SHAM Veh vs TBI Sal**	**TBI Veh vs SHAM Sal**	**TBI Veh vs TBI Sal**	**SHAM Salvs TBI Sal**
Drug	F(1,44)=0.04	**0.04**	**<0.0001**	**0.008**	**<0.0001**	**0.02**	0.99	**0.03**
Interaction	F(1,44)=6.81	**0.01**						

Table 3

ISRIB								
Grating 0.12								
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 36.72	**<0.0001**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM ISRIB**	**SHAM Veh vs TBI ISRIB**	**TBI Veh vs SHAM ISRIB**	**TBI Veh vs TBI ISRIB**	**SHAM ISRIBvs TBI ISRIB**
Drug	F(1,44)=3.42	0.07	**0.02**	0.07	**0.02**	**<0.0001**	0.99	**<0.0001**
Interaction	F(1,44)=2.66	0.11						
Grating 0.26								
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 35.80	**<0.0001**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM ISRIB**	**SHAM Veh vs TBI ISRIB**	**TBI Veh vs SHAM ISRIB**	**TBI Veh vs TBI ISRIB**	**SHAM ISRIBvs TBI ISRIB**
Drug	F(1,44)=4.83	**0.03**	**<0.0001**	**0.02**	**<0.0001**	**0.04**	0.99	**0.03**
Interaction	F(1,44)=4.2	**0.04**						
Grating 0.32								
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 30.22	**<0.0001**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM ISRIB**	**SHAM Veh vs TBI ISRIB**	**TBI Veh vs SHAM ISRIB**	**TBI Veh vs TBI ISRIB**	**SHAM ISRIBvs TBI ISRIB**
Drug	F(1,44)=5.77	**0.02**	**0.0007**	0.15	**<0.0001**	0.14	0.58	**0.005**
Interaction	F(1,44)=0.32	0.57						
	Grating 0.39							
	Test Statistic	***p* value**	**Post Hoc Comparrisons p values**					
Injury	F (1,44) = 15.9	**0.0002**	**SHAM Veh v TBI Veh**	**SHAM Veh vs SHAM ISRIB**	**SHAM Veh vs TBI ISRIB**	**TBI Veh vs SHAM ISRIB**	**TBI Veh vs TBI ISRIB**	**SHAM ISRIBvs TBI ISRIB**
Drug	F(1,44)=0.002	0.96	**0.0005**	0.37	**0.03**	**0.03**	0.44	0.58
Interaction	F(1,44)=0.03	**0.03**						

Table 4	Western Test Results Retina				
Protein	**Comparrison**	**Test Statistic**	***p* value**	**Test Statistic**	***p* value**
		ISRIB		**Salubrinal**	
ER Stress/ISR					
XBP1U	*Interaction*	F (1, 11) = 0.0005629	0.9815	F (1, 13) = 0.1140	0.7411
	Drug	F (1, 11) = 2.354	0.1532	F (1, 13) = 0.5985	0.453
	Injury	F (1, 11) = 0.06145	0.8088	F (1, 13) = 0.0001685	0.9898
XBP1s	*Interaction*	F (1, 11) = 0.04805	0.8305	F (1, 13) = 0.01969	0.8905
	Drug	F (1, 11) = 3.399	0.0923	F (1, 13) = 0.6754	0.426
	Injury	F (1, 11) = 0.007783	0.9313	F (1, 13) = 0.0002662	0.9872
XBP1s:U	*Interaction*	F (1, 11) = 0.3482	0.567	F (1, 13) = 0.3845	0.5459
	Drug	F (1, 11) = 0.3070	0.5906	F (1, 13) = 0.4081	0.534
	Injury	F (1, 11) = 0.1777	0.6814	F (1, 13) = 0.08235	0.7787
PERK	*Interaction*	F (1, 29) = 4.908	**0.0347**	F (1, 29) = 1.093	0.3046
	Drug	F (1, 29) = 0.1837	0.6713	F (1, 29) = 0.0006656	0.9796
	Injury	F (1, 29) = 0.4797	0.4941	F (1, 29) = 3.864	0.06
eIF2α	*Interaction*	F (1, 29) = 3.743	0.0628	F (1, 29) = 0.06321	0.8033
	Drug	F (1, 29) = 0.6074	0.4421	F (1, 29) = 0.06780	0.7964
	Injury	F (1, 29) = 3.006e-007	0.9996	F (1, 29) = 3.755	0.0625
p-eIF2α	*Interaction*	F (1, 24) = 0.8700	0.3603	F (1, 22) = 0.1425	0.7095
	Drug	F (1, 24) = 0.1640	0.689	F (1, 22) = 4.482	**0.0458**
	Injury	F (1, 24) = 2.961	0.0982	F (1, 22) = 1.464	0.2392
eIF2α phos:total	*Interaction*	F (1, 14) = 1.807	0.2003	F (1, 16) = 0.1957	0.6641
	Drug	F (1, 14) = 0.2628	0.6162	F (1, 16) = 0.6975	0.4159
	Injury	F (1, 14) = 1.148	0.3022	F (1, 16) = 4.557	**0.0486**
ATF4 phosphorylated	*Interaction*	F (1, 11) = 0.04494	0.836	F (1, 13) = 0.05385	0.8201
	Drug	F (1, 11) = 0.0005626	0.9815	F (1, 13) = 0.9477	0.3481
	Injury	F (1, 11) = 0.01846	0.8944	F (1, 13) = 0.1987	0.6631
ATF4 total	*Interaction*	F (1, 11) = 0.8291	0.382	F (1, 13) = 1.498	0.2428
	Drug	F (1, 11) = 0.8514	0.376	F (1, 13) = 0.5722	0.4629
	Injury	F (1, 11) = 2.271	0.16	F (1, 13) = 0.2146	0.6508
ATF4 P:T	*Interaction*	F (1, 11) = 2.031	0.1819	F (1, 13) = 0.8200	0.3817
	Drug	F (1, 11) = 2.226	0.1638	F (1, 13) = 0.1917	0.6687
	Injury	F (1, 11) = 3.115	0.1053	F (1, 13) = 0.2116	0.6531
ERO1Lα - Reduced (55/60 kDa)	*Interaction*	*no band*			
	Drug				
	Injury				
ERO1Lα - Ox1 Active (50 kDa)	*Interaction*	F (1, 11) = 0.1286	0.7267	F (1, 13) = 1.614	0.2262
	Drug	F (1, 11) = 0.05687	0.8159	F (1, 13) = 0.8653	0.3692
	Injury	F (1, 11) = 2.631	0.1331	F (1, 13) = 0.01241	0.913
ERO1Lα - Ox2 Inactive (45 Kda)	*Interaction*	F (1, 10) = 0.3950	0.5438	F (1, 12) = 34.49	**<0.0001**
	Drug	F (1, 10) = 7.428	**0.0214**	F (1, 12) = 1.772	0.2079
	Injury	F (1, 10) = 17.30	**0.002**	F (1, 12) = 0.7721	0.3968
Oxidative Stress					
nrf2	*Interaction*	F (1, 13) = 39.25	**<0.0001**	F (1, 13) = 7.011	**0.0201**
	Drug	F (1, 13) = 4.368	0.05	F (1, 13) = 0.6250	0.4434
	Injury	F (1, 13) = 0.001097	0.9741	F (1, 13) = 1.113e-005	0.9974
NOX2	*Interaction*	F (1, 12) = 0.2033	0.6601	F (1, 13) = 3.706	0.0764
	Drug	F (1, 12) = 6.112	**0.0294**	F (1, 13) = 0.001735	0.9674
	Injury	F (1, 12) = 4.712	0.05	F (1, 13) = 1.187	0.2957
Cell Death/Apoptosis					
RBPMS	*Interaction*	F (1, 32) = 0.1296	0.7212	F (1, 30) = 0.02084	0.8862
N=8	*Drug*	F (1, 32) = 3.246	0.081	F (1, 30) = 0.06585	0.7992
	Injury	F (1, 32) = 14.68	**0.0006**	F (1, 30) = 14.94	**0.0006**
CHOP/GADD34	*Interaction*	F (1, 25) = 1.244	0.2753	F (1, 22) = 1.575	0.2226
N=7	*Drug*	F (1, 25) = 0.3298	0.5709	F (1, 22) = 0.03103	0.8618
	Injury	F (1, 25) = 3.630	0.0683	F (1, 22) = 2.648	0.1179
Cas12 n=9	*Interaction*	F (1, 27) = 5.861	**0.0225**	F (1, 27) = 1.083	0.3074
	Drug	F (1, 27) = 0.7367	0.3983	F (1, 27) = 5.021	**0.0335**
	Injury	F (1, 27) = 0.1597	0.6926	F (1, 27) = 0.6172	0.4389
Cas3 n=8	*Interaction*	F (1, 38) = 1.387	0.2462	F (1, 37) = 0.1879	0.6672
	Drug	F (1, 38) = 0.5047	0.4818	F (1, 37) = 0.5142	0.4778
	Injury	F (1, 38) = 1.179	0.2843	F (1, 37) = 0.2591	0.6137

Table 5	Western Test Results Optic Nerve				
Protein	**Comparrison**	**Test Statistic**	***p* value**	**Test Statistic**	***p* value**
		ISRIB		**Salubrinal**	
ER Stress/ISR					
XBP1U	*Interaction*	F (1, 18) = 0.1063	0.7482	F (1, 17) = 2.966	0.1032
	Drug	F (1, 18) = 1.815	0.1946	F (1, 17) = 1.638	0.2178
	Injury	F (1, 18) = 0.5380	0.4727	F (1, 17) = 0.4823	0.4967
XBP1s	*Interaction*	F (1, 19) = 0.5050	0.4859	F (1, 19) = 0.06137	0.807
	Drug	F (1, 19) = 0.1004	0.7548	F (1, 19) = 0.1986	0.6609
	Injury	F (1, 19) = 0.07764	0.7835	F (1, 19) = 0.03683	0.8498
XBP1s:U	*Interaction*	F (1, 19) = 0.0002031	0.9888	F (1, 18) = 2.345	0.143
	Drug	F (1, 19) = 1.260	0.2756	F (1, 18) = 0.5275	0.477
	Injury	F (1, 19) = 0.7670	0.3921	F (1, 18) = 0.5025	0.4875
eIF2α	*Interaction*	F (1, 19) = 0.09178	0.7652	F (1, 19) = 0.001620	0.9683
	Drug	F (1, 19) = 0.2259	0.64	F (1, 19) = 1.566	0.226
	Injury	F (1, 19) = 0.7450	0.3988	F (1, 19) = 0.8346	0.3724
p-eIF2α	*Interaction*	F (1, 17) = 2.900	0.1068	F (1, 17) = 3.652	0.073
	Drug	F (1, 17) = 0.4879	0.4943	F (1, 17) = 0.1830	0.6742
	Injury	F (1, 17) = 12.41	**0.0026**	F (1, 17) = 7.567	**0.0136**
eIF2α phos:total	*Interaction*	F (1, 17) = 2.900	0.1068	F (1, 17) = 3.652	0.073
	Drug	F (1, 17) = 0.4879	0.4943	F (1, 17) = 0.1830	0.6742
	Injury	F (1, 17) = 12.41	**0.0026**	F (1, 17) = 7.567	**0.0136**
ATF4 phosphorylated	*Interaction*	F (1, 15) = 1.667	0.2163	F (1, 16) = 0.05202	0.8225
	Drug	F (1, 15) = 1.378	0.2587	F (1, 16) = 0.006613	0.9362
	Injury	F (1, 15) = 1.190	0.2926	F (1, 16) = 7.693	**0.0136**
ATF4 total	*Interaction*	F (1, 15) = 1.858	0.1929	F (1, 16) = 2.031	0.1733
	Drug	F (1, 15) = 0.6380	0.4369	F (1, 16) = 2.210	0.1566
	Injury	F (1, 15) = 0.07886	0.7827	F (1, 16) = 0.01475	0.9049
ATF4 phos:total	*Interaction*	F (1, 15) = 0.02129	0.8859	F (1, 16) = 1.970	0.1796
	Drug	F (1, 15) = 0.06102	0.8082	F (1, 16) = 5.203	**0.0366**
	Injury	F (1, 15) = 3.480	0.0818	F (1, 16) = 12.27	**0.0029**
ERO1Lα - Reduced (60 kDa)	*Interaction*	F (1, 15) = 4.433	0.05	F (1, 16) = 2.753	0.1166
	Drug	F (1, 15) = 1.108	0.3091	F (1, 16) = 0.6658	0.4265
	Injury	F (1, 15) = 3.201	0.0938	F (1, 16) = 1.205	0.2886
ERO1Lα - Ox1 Active (50 kDa)	*Interaction*	F (1, 15) = 1.530	0.2351	F (1, 16) = 0.9116	0.3539
	Drug	F (1, 15) = 0.3492	0.5634	F (1, 16) = 0.4368	0.5181
	Injury	F (1, 15) = 1.123	0.306	F (1, 16) = 0.5388	0.4735
ERO1Lα - Ox2 Inactive (45 Kda)	*Interaction*	F (1, 13) = 9.772	**0.008**	F (1, 14) = 0.9692	0.3416
	Drug	F (1, 13) = 11.09	**0.0054**	F (1, 14) = 0.7395	0.4043
	Injury	F (1, 13) = 11.55	**0.0047**	F (1, 14) = 2.178	0.1621
Oxidative Stress					
nrf2	*Interaction*	F (1, 8) = 0.07074	0.797	F (1, 8) = 1.039	0.338
	Drug	F (1, 8) = 0.1226	0.7353	F (1, 8) = 0.5821	0.4674
	Injury	F (1, 8) = 13.27	**0.0066**	F (1, 8) = 27.79	**0.0008**
NOX2	*Interaction*	*Insufficient protein for analysis*			
	Drug				
	Injury				

Supplementary Table 1. Sex Differences

Optic Nerve TUJ1 + Grp78 + ATF4			Optic Nerve S100β + ATF4			Retina TUJ1 + Grp78 + ATF4		
ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value
Axonal ATF4 Sex Effects			***ATF4 Inside S100β Sex Effects***			***Grp78 IPL Central Sex Effects***		
Row factor	F (3, 81) = 1.467	0.230	Location	F (1.448, 31.86) = 5.114	**0.020**	Interaction	F (1, 24) = 0.2131	0.649
Injury	F (1, 81) = 0.4716	0.494	Injury	F (1, 26) = 13.34	**0.001**	Sex	F (1, 24) = 7.392e-006	0.998
Sex	F (1, 81) = 0.2921	0.590	Sex	F (1, 26) = 1.661	0.209	Injury	F (1, 24) = 0.7879	0.384
Row factor x Injury	F (3, 81) = 2.650	0.054	Location x Injury	F (3, 66) = 2.928	**0.040**	Residual		
Row factor x Sex	F (3, 81) = 1.989	0.122	Location x Sex	F (3, 66) = 0.3538	0.787			
Injury x Sex	F (1, 81) = 0.7292	0.396	Injury x Sex	F (1, 26) = 0.3492	0.560	***Grp78 IPL MidPeripheral Sex Effects***		
Row factor x Injury x Sex	F (3, 81) = 0.9921	0.401	Location x Injury x Sex	F (3, 66) = 0.4769	0.699	Interaction	F (1, 27) = 2.567	0.121
Total ATF4 Vol Sex Effects			***Total ATF Sex Effects***			Sex	F (1, 27) = 0.2144	0.647
Location	F (2.697, 47.65) =	0.249	Location	F (1.459, 28.70) = 5.263	**0.019**	Injury	F (1, 27) = 2.363	0.136
Injury	F (1, 25) = 7.580	0.011	Injury	F (1, 26) = 10.06	**0.004**	Residual		
Sex	F (1, 25) = 2.209	0.150	Sex	F (1, 26) = 0.4367	0.515			
Location x Injury	F (3, 53) = 2.215	0.097	Location x Injury	F (3, 59) = 2.969	**0.039**	***Grp78 IPL Peripheral Sex Effects***		
Location x Sex	F (3, 53) = 1.883	0.144	Location x Sex	F (3, 59) = 0.004251	1.000	Interaction	F (1, 25) = 0.1923	0.665
Injury x Sex	F (1, 25) = 3.113	0.090	Injury x Sex	F (1, 26) = 0.3034	0.586	Sex	F (1, 25) = 0.7883	0.383
Location x Injury x Sex	F (3, 53) = 0.8640	0.466	Location x Injury x Sex	F (3, 59) = 0.1976	0.898	Injury	F (1, 25) = 0.2039	0.656
Axonal Grp78 Volume Sex Effects						Residual		
Location	F (2.387, 63.66) =	0.495	**Optic Nerve IBA1 + ATF4**					
Injury	F (1, 80) = 4.962	**0.029**	***ATF4 Inside IBA1 Sex Effects***			***Grp78 GCL Central Sex Effects***		
Sex	F (1, 80) = 2.960	0.089	Location	F (1.493, 37.31) = 1.475		Interaction	F (1, 24) = 0.004956	0.945
Location x Injury	F (3, 80) = 0.7114	0.548	Injury	F (1, 31) = 9.478	0.241	Sex	F (1, 24) = 0.3401	0.565
Location x Sex	F (3, 80) = 0.7712	0.514	Sex	F (1, 31) = 0.2786	**0.004**	Injury	F (1, 24) = 1.032	0.320
Injury x Sex	F (1, 80) = 0.2844	0.595	Location x Injury	F (3, 75) = 1.299	0.601	Residual		
Location x Injury x Sex	F (3, 80) = 0.06213	0.980	Location x Sex	F (3, 75) = 0.1517	0.281			
Relevant post-hoc tests			Injury x Sex	F (1, 31) = 0.3179	0.928			
Injury:TBI Male vs. Injury:TBI Female		**0.028**	Location x Injury x Sex	F (3, 75) = 0.1728	0.577			

Supplementary Table 1. Sex Differences Continued…								
ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value	ANOVA table	F (DFn, DFd)	P value
Axonal Grp78 MFI Sex Effects			***Total ATF Sex Effects***			***Grp78 GCL MidPeripheral Sex Effects***		
Location	F (3, 56) = 0.5088	0.678	Location	F (2.818, 68.56) = 2.702		Interaction	F (1, 27) = 2.265	0.144
Injury	F (1, 24) = 0.00797	0.930	Injury	F (1, 31) = 24.45	0.056	Sex	F (1, 27) = 9.898e-006	0.998
Sex	F (1, 24) = 8.214	**0.009**	Sex	F (1, 31) = 0.2288	**<0.0001**	Injury	F (1, 27) = 0.007638	0.931
Location x Injury	F (3, 56) = 1.201	0.318	Location x Injury	F (3, 73) = 2.104	0.636	Residual		
Location x Sex	F (3, 56) = 1.685	0.181	Location x Sex	F (3, 73) = 0.3418	0.107			
Injury x Sex	F (1, 24) = 3.510	0.073	Injury x Sex	F (1, 31) = 0.2930	0.795	***Grp78 GCL Peripheral Sex Effects***		
Location x Injury x Sex	F (3, 56) = 0.8201	0.488	Location x Injury x Sex	F (3, 73) = 0.4398	0.592	Interaction	F (1, 28) = 3.392	0.076
Relevant post-hoc tests					0.725	Sex	F (1, 28) = 2.798	0.106
Chiasm:SHAM Male vs. Chiasm:SHAM Female		**0.024**				Injury	F (1, 28) = 6.146	0.020
Eye:SHAM Male vs. Eye:SHAM Female		**0.004**				Residual		
Perinuclear Grp78 Volume Sex Effects								
Location	F (2.281, 44.11) =	0.778				***ATF4 GCL Central Sex Effects***		
Injury	F (1, 24) = 0.9307	0.344				Interaction	F (1, 25) = 1.021	0.322
Sex	F (1, 24) = 4.017	0.057				Sex	F (1, 25) = 0.9154	0.348
Location x Injury	F (3, 58) = 1.069	0.369				Injury	F (1, 25) = 1.059	0.313
Location x Sex	F (3, 58) = 2.581	0.062				Residual		
Injury x Sex	F (1, 24) = 3.950	0.058						
Location x Injury x Sex	F (3, 58) = 1.247	0.301				***ATF4 GCL MidPeripheral Sex Effects***		
Perinuclear Grp78 MFI Sex Effects						Interaction	F (1, 28) = 1.248	0.274
Location	F (2.574, 48.06) =	0.516				Sex	F (1, 28) = 2.437	0.130
Injury	F (1, 24) = 0.2328	0.634				Injury	F (1, 28) = 0.4390	0.513
Sex	F (1, 24) = 2.913	0.101				Residual		
Location x Injury	F (3, 56) = 4.545	**0.006**						
Location x Sex	F (3, 56) = 0.8994	0.447				***ATF4 GCL Peripheral Sex Effects***		
Injury x Sex	F (1, 24) = 0.5348	0.472				Interaction	F (1, 26) = 1.284	0.268
Location x Injury x Sex	F (3, 56) = 1.959	0.131				Sex	F (1, 26) = 0.5598	0.461
Relevant post-hoc tests						Injury	F (1, 26) = 3.821	0.062
Proximal:SHAM Male vs. Proximal:SHAM Female		**0.010**				Residual		
Eye:SHAM Male vs. Eye:SHAM Female		**0.029**						

Figures

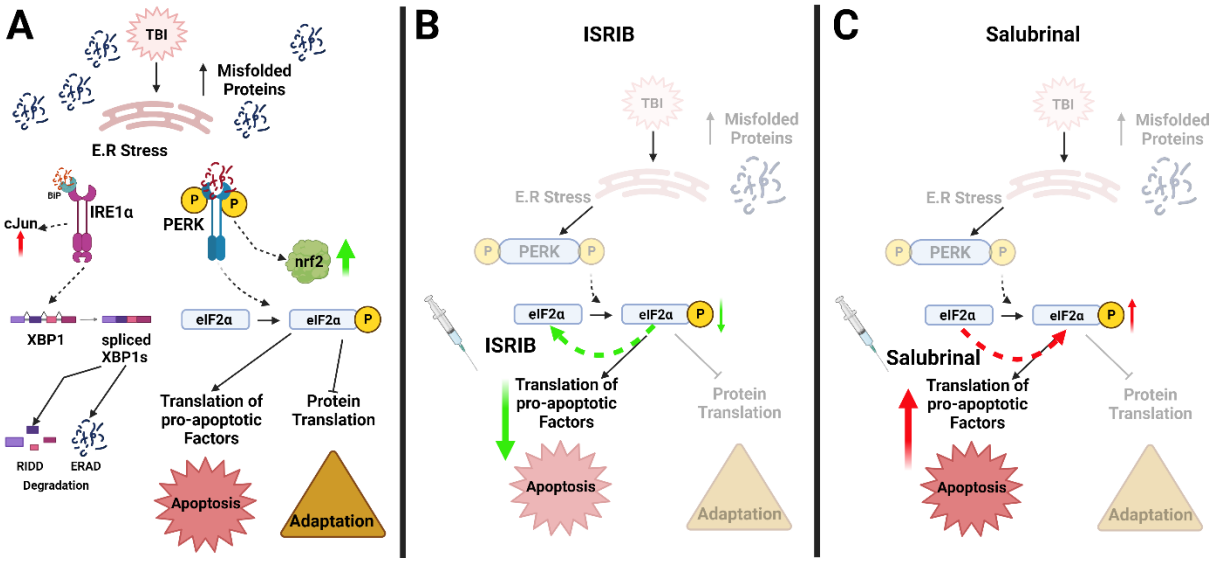


Figure 1. Predicted effects of Salubrinal and ISRIB. (A) Depicts the basic pathways for ER-Stress mediated activation of IRE1α and PERK pathways. Upon binding of misfolded proteins to IRE1α or PERK (or their pre-bound Binding Immunoglobulin Protein, BiP), these receptors can initiate cascades for both adaptation to injury by reduction of ER burden via mRNA degradation and protein translation inhibition or apoptosis. IRE1α will splice XBP1 allowing it to initiate mRNA degradation (regulated IRE1α-dependent mRNA decay [RIDD]) or tag proteins for exportation and degradation by the proteosome (ER associate degreddation [ERAD]). Upon too great or prolonged ER stress, IRE1α can also activate the apoptotic cJun pathway. PERK phosphorylated eIF2α, which is able to halt protein translation (adaptive to an extent) while also facilitation translation of a select few factors like ATF4, which can then go on to the nucleus to initiate translation of apoptotic genes (e.g., CHOP) autophagy genes, biosynthetic genes, and antioxidant genes (e.g., nrf2). **(B)** We hypothesize that ISRIB-mediated dephosphorylation of eIF2α wil be most beneficial by preventing the upregulation of proteins like ATF4 and CHOP as well as potential feedback for inactivation of the IRE1α pathway. **(C)** In contrast, we hypothesize that increasing eIF2α phosphorylation with Salubrinal will lead to worse outcomes through increased apoptotic protein translation.

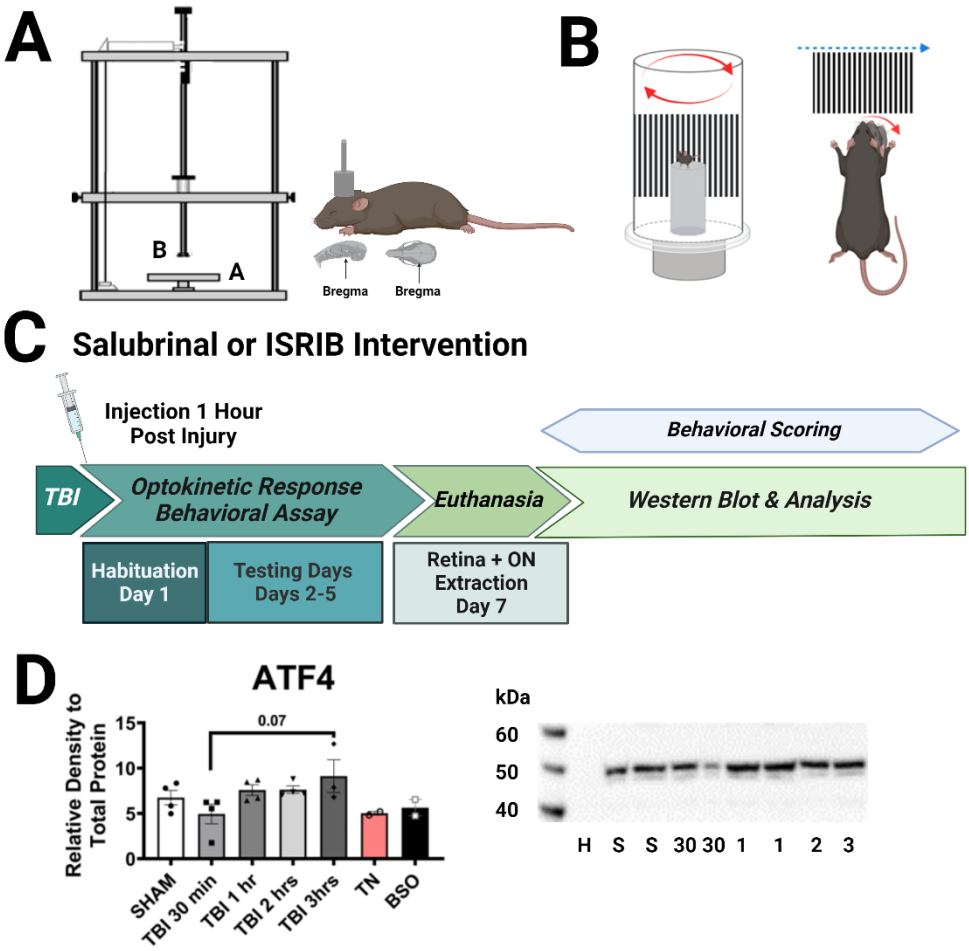


Figure 2. Experimental Timeline and Procedures. In **(A)** we show a depictions of our weight drop device along with the rough positioning of the weight atop the head. Within **A** the smaller "A" represents the platform and "B" the weight. We also show a depiction of our optokinetic device **(B)** and our timeline of procedures **(C)**. In order to decide on a time point of injurection, we examined a small cohort of mice at 30 mins, 1hr, 2hrs, and 3hrs post TBI and then looked at the downstream, traditionally nuclear UPR marker ATF4. **(D)** ATF4 was increased by 3 hours post injury. H = empty well H2O, S = SHAM, 30= 30 min injury, 1= 1hr injury, 2 = 2hr injury, 3= 3 hour injury, TN=tunicamycin positive control, BSO = buthionine sulfoxamine positive control.

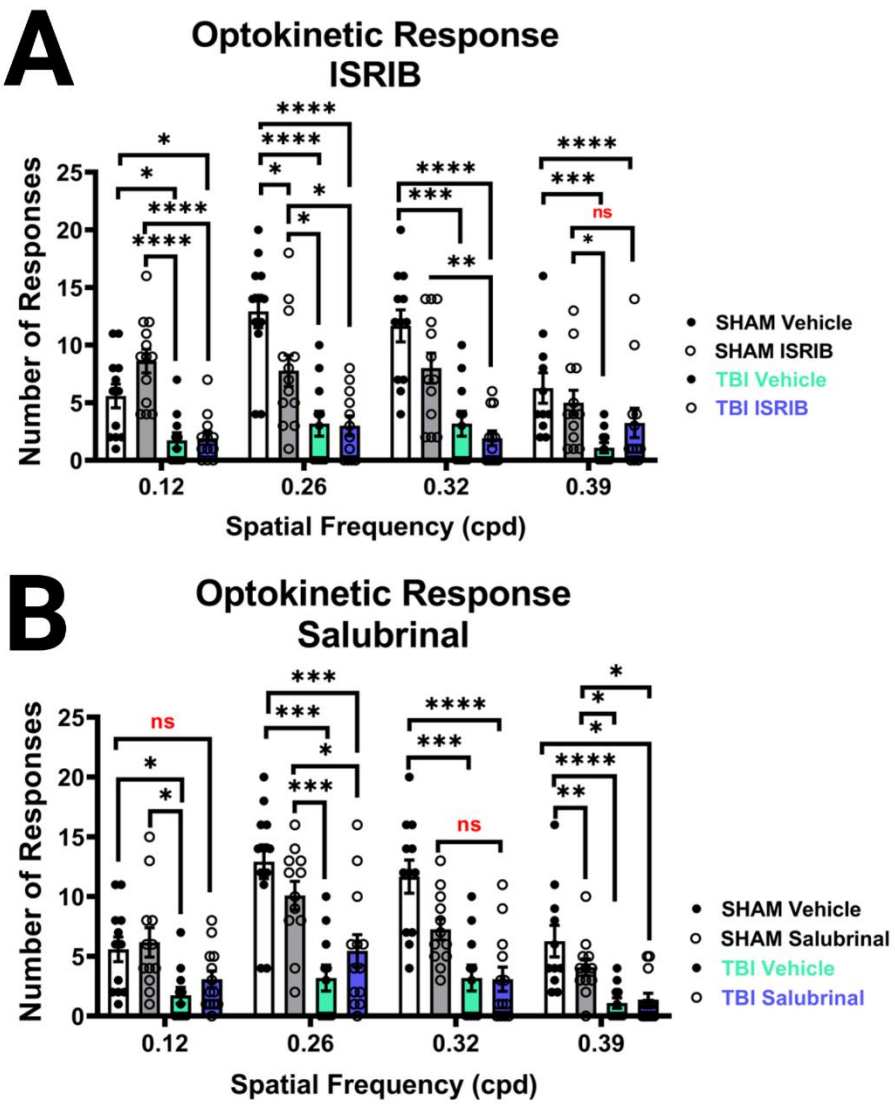


Figure 3. Optokinetic Function. (A) ISRIB did not improve optokinetic performance within the first 7 days post injury. Instead, we show that ISRIB may have been more detrimental to retinal cell function as SHAM ISRIB mice performed worse than vehicle controls. Still, ISRIB may better preserve visual acuity as TBI ISRIB mice performed similarly (red ns) to controls at 0.39cpd. **(B)** Salubrinal results are largely the same, suggesting that there are inherent/homeostatic needs in the retina for PERK pathway function. * p<0.05, ** p<0.01, *** p<0.001, **** P<0.0001

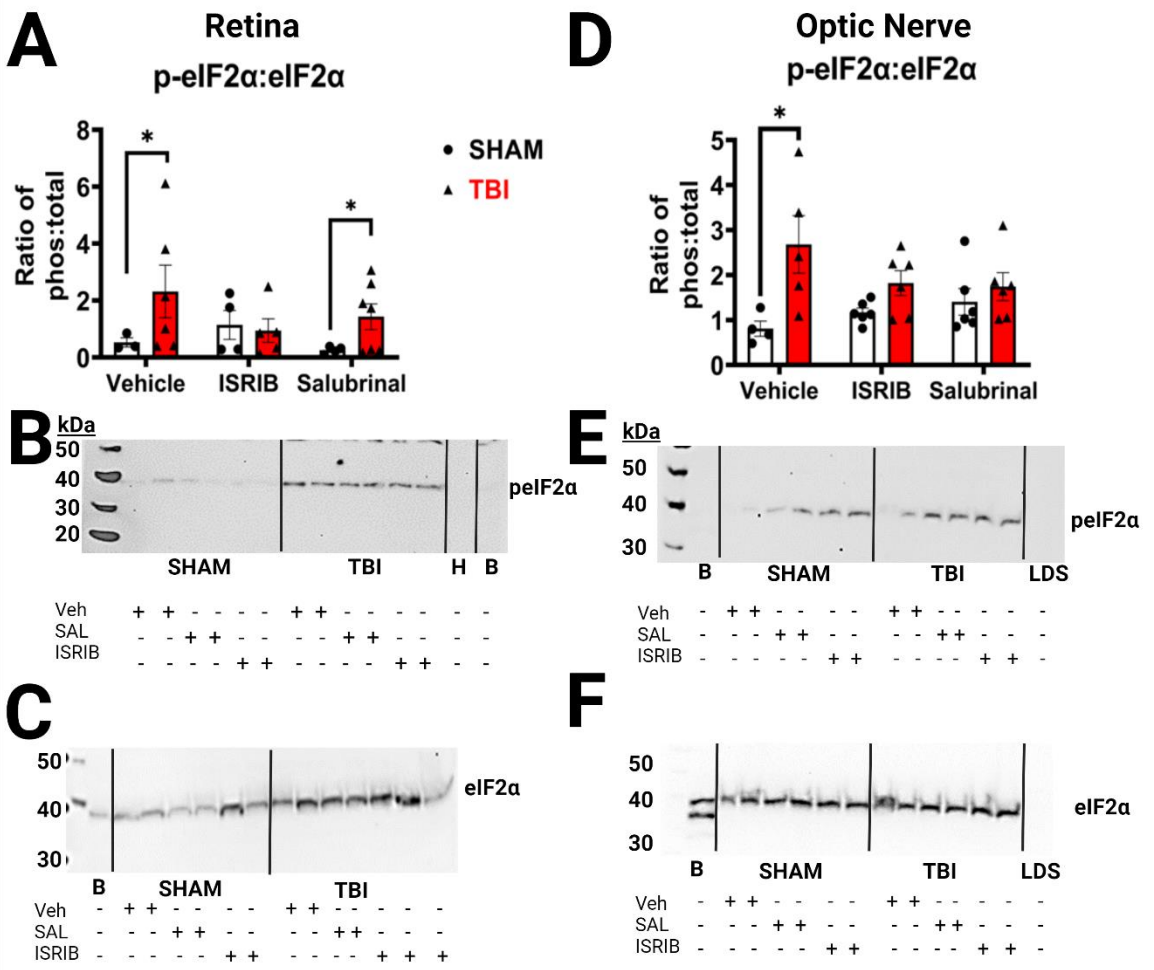


Figure 4. eIF2α phosphorylation in the retina and optic nerve. (A) Retinal lysate revealed that both ISRIB and Salubrinal performed as anticipated with long-lasting suppression of eIF2α phosphorylation with ISRIB in injured mice and maintained p-eIF2α with Salubrinal. **(B & C)** Show representative retinal western blots separated by condition with molecular weight (kDa) identification provided using a Magic Mark ladder labeled on the left of the blots. **(D)** Although p-eIF2α was increased with injury, we did not see the same increase in the retina given Salubrinal. **(E & F)** Show representative optic nerve western blots. B=intermembrane control sham brain homogenate, H=H_2O empty lane, L= ladder, LDS=LDS empty lane, SAL= Salubrinal, Veh = Vehicle. * $p<0.05$

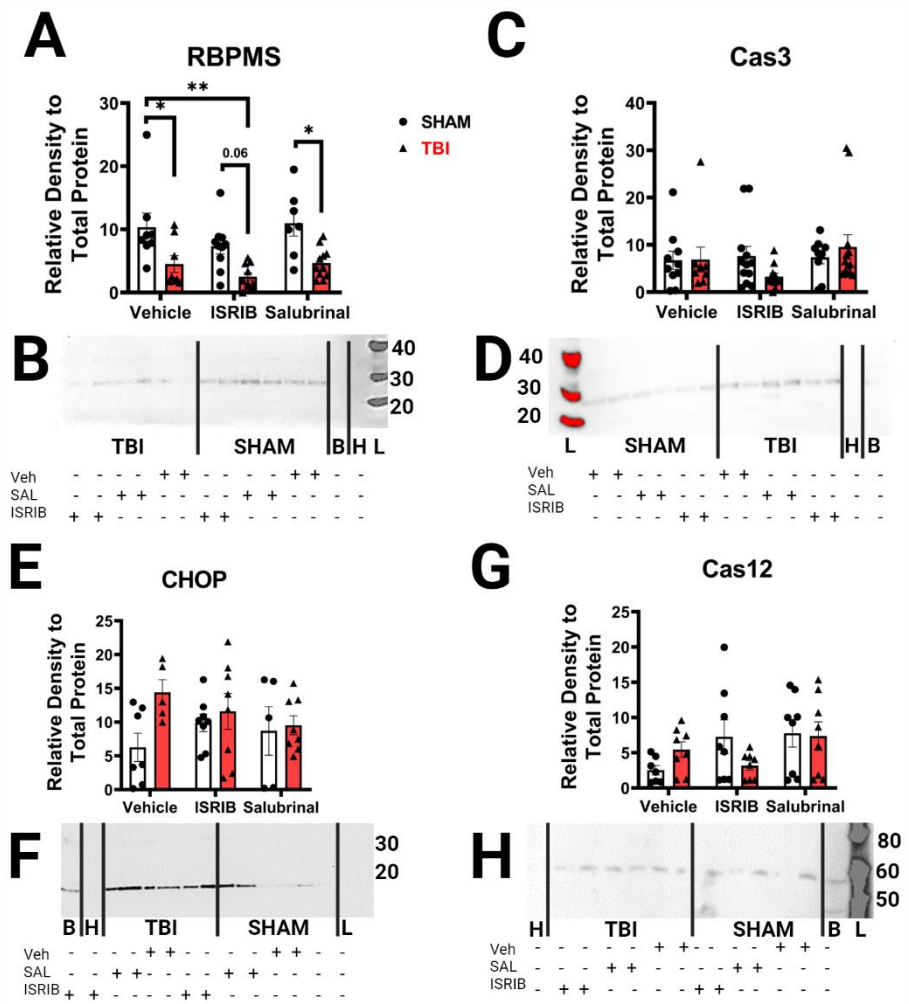


Figure 5. Retinal Apoptosis and RGC protein expression. (A) RGC-specific protein RBPMS was decreased in all conditions. Although CHOP expression **(E)** increased with injury, there were no significant differences in any of the apoptosis-related markers examined including **(C)** Caspase 3 **(E)** CHOP or **(G)** Caspase 12. Representative blots for each marker are shown below their respective graphs in **(B,D,F, and H)**. B=intermembrane control sham brain homogenate, H=H_2O empty lane, L= ladder, LDS=LDS empty lane, SAL= Salubrinal, Veh = Vehicle. * $p<0.05$, ** $p<0.01$

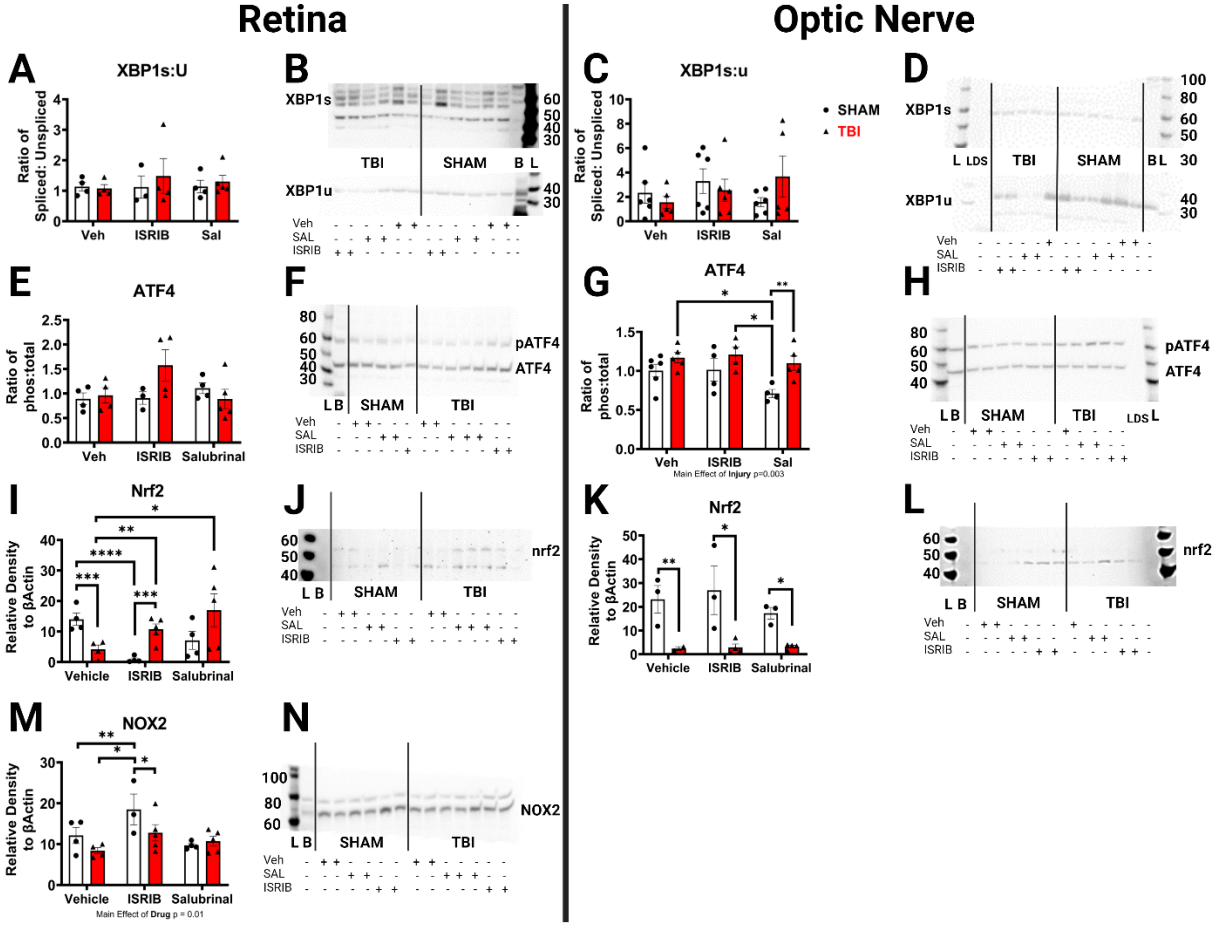


Figure 6. UPR and Oxidative Stress Markers. The ratio of spliced XBP1 to total was no different between groups in retinal samples **(A,B)** or optic nerve samples **(C,D).** The ratio of total to phosphorylated ATF4 was also not changed in the retina **(E,F)** but it was elevated given Salubrinal in the optic nerve **(G,H).** The antioxidant nrf2 was decreased after injury and both ISRIB and Salubrinal increased expression in the retina **(I,J)** but not in the nerve where it remain depressed given injury **(K,L).** ISRIB alone increased the oxidative stress marker NOX2 in control mice and TBI decreased it **(M,N).** There was insufficient optic nerve lysate remaining to assess NOX2 in the nerve. B=intermembrane control sham brain homogenate, H=H_2O empty lane, L= ladder, LDS=LDS empty lane, SAL= Salubrinal, Veh = Vehicle. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001.

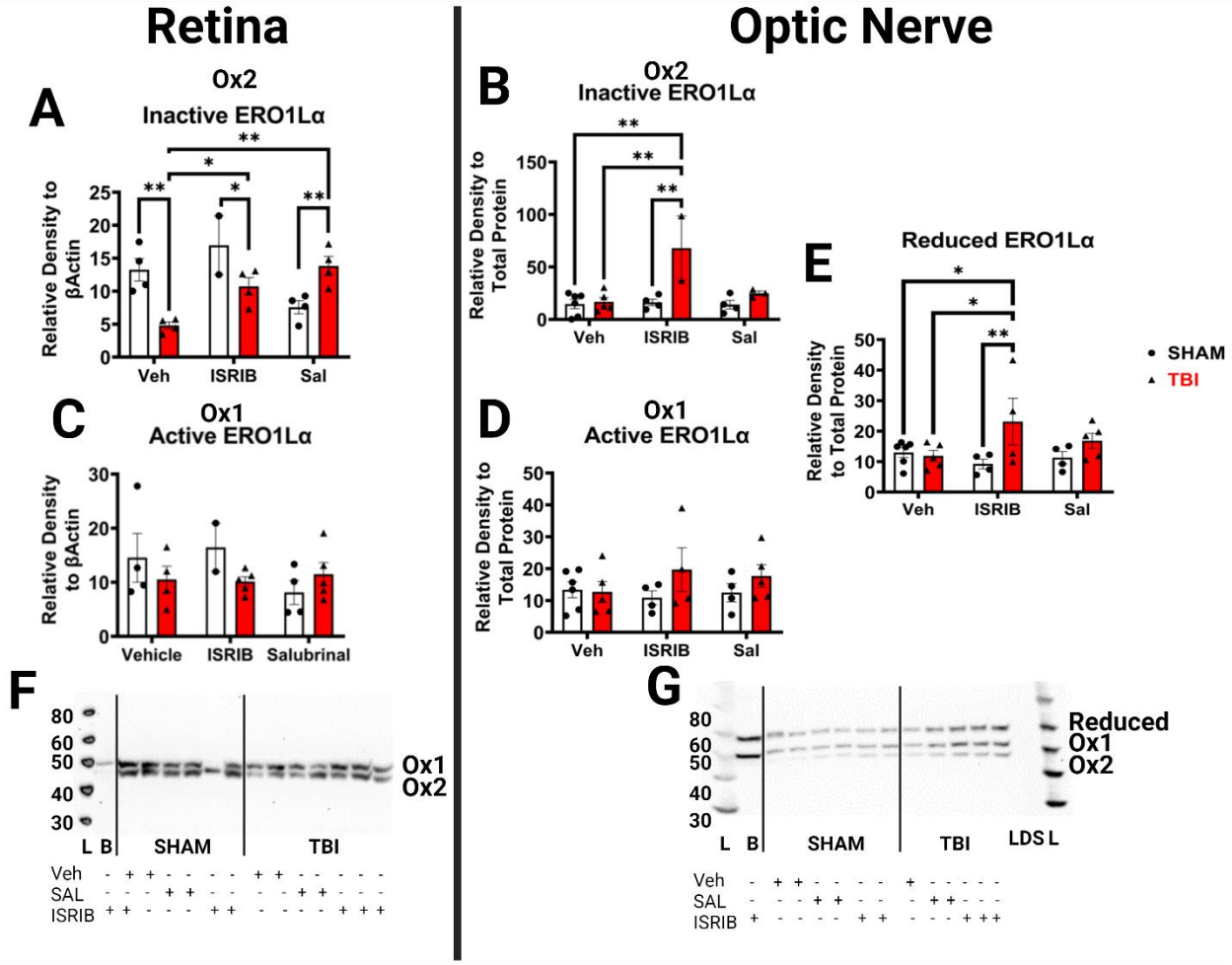
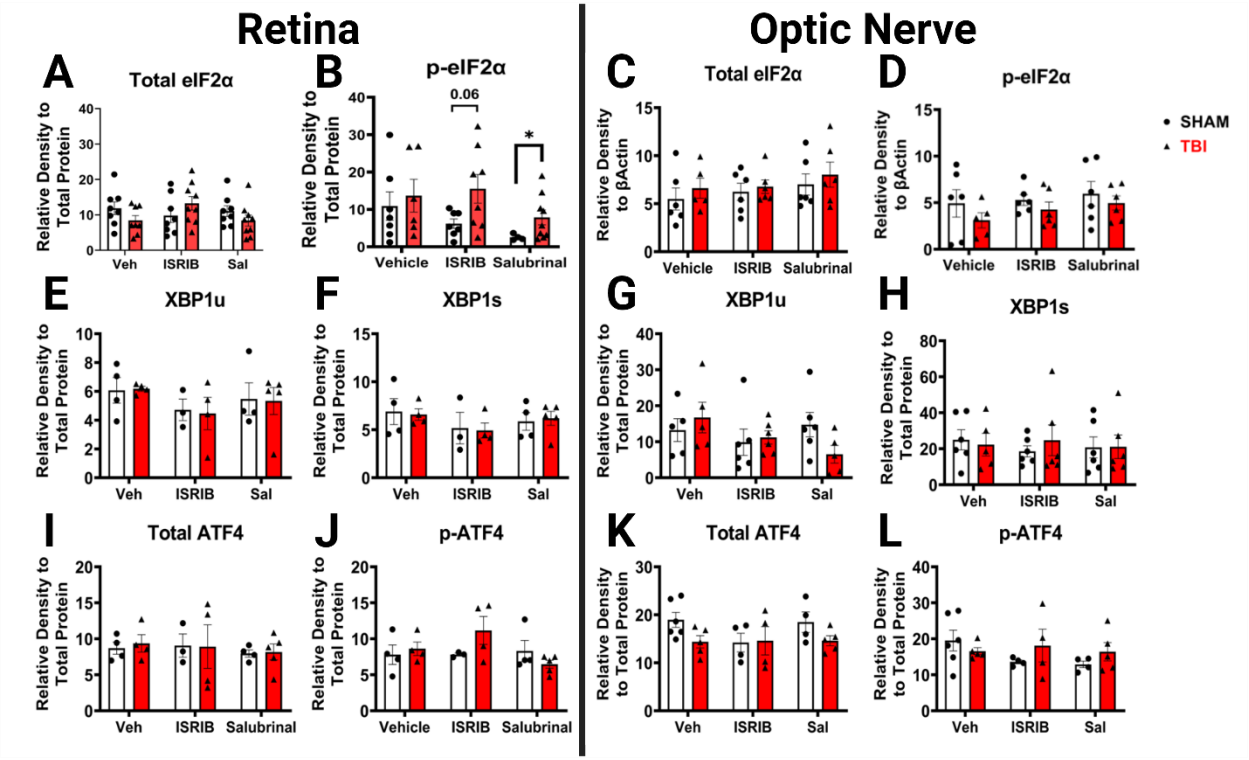


Figure 7. ERO1α expression. In the retina ERO1α-Ox was reduced after injury **(A)** despite no compensatory increase in the active Ox1 variant **(C).** ISRIB increased ERO1α-Ox2 and TBI then decreased it, while Salubrinal increased ERO1α-Ox2 in injured mice **(A)**. In the nerve, only ISRIB+TBI increased this Ox2 variant **(B)** and the reduced form or ERO1α **(E),** but there were still no changes to active ERO1α-O1 **(D).** Representative blots are provided in for retinal ERO1α **(F)** and nerve **(G).** B=intermembrane control sham brain homogenate, L= ladder, LDS=LDS empty lane, SAL= Salubrinal, Veh = Vehicle. * $p<0.05$, ** $p<0.01$.



Supplementary Figure 1. Additional Western Blot Data. There were no differences found when measuring total eIF2α in the retina **(A)**, but Salubrinal did significantly increase with Injury given Salubrinal **(B).** No differences were found for total **(C)** or p-eIF2α **(D)** in the optic nerve. There were also no differences in unspliced XBP1 **(E)** or sliced XBP1 **(F)** in the retina or nerve **(G & H** respectively). Finally, there were no differences in total ATF4 **(I)** or p-ATF4 **(J)** in the retina or nerve **(K & L** respectively). Veh = Vehicle, Sal = Salubrinal. *p<0.05

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