Drug manipulation of the PERK-eIF2a pathway post-TBI modestly improves visual deficits without preserving retinal cells.

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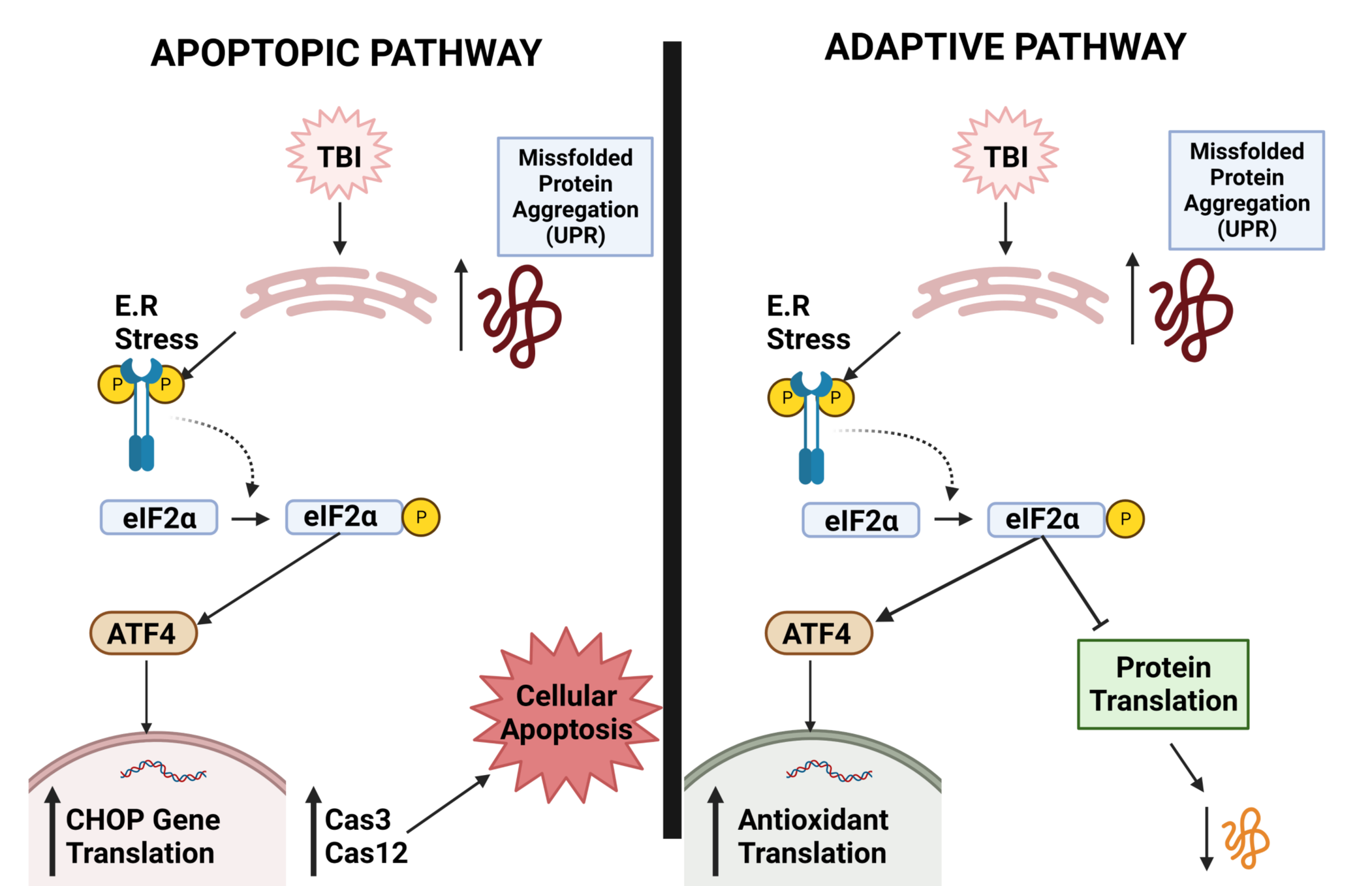
Introduction

- Traumatic Brain Injury (TBI) effects approximately 2.9 million people yearly (12,13)
- TBI results in primary (acute) injuries as well as secondary injuries (i.e., molecular signaling cascades) (12)
- 50-60% of TBI patients report visual deficits
  - "Traumatic Optic Neuropathy" (TON) describes injury specific to the optic nerve and the loss of visual function that follows (10)
- Our lab has shown that TBI induced TON leads to activation of the endoplasmic reticulum (ER) stress pathway and the unfolded protein response (UPR) with specific activation of the PERK pathway. (10,11)
- PERK phosphorylates elongation initiation factor two alpha (eIF2α), which can shift cellular responses towards adaptive or apoptotic fates (Fig. 1)(9)
- It's not yet clear whether disease type would make increased phosphorylation or reduced phosphorylation more likely to ship the cell towards adaptive responses and survival." (2,10,11)
- Currently there are no drug therapeutics used to treat the secondary effects of TBI/TON.

Figure 1

Figure 1. eIf2α acts as a switch to direct cells toward or away from apoptosis. A graphic depiction of the PERK branch of ER stress is depicted. The left diagram depicts downstream factors translated after eIf2α is phosphorylated that are associated with apoptosis. The right panel depicts those factors and mechanisms associated with adaptation. *Created using Biorender*



Hypothesis

Manipulation of the PERK pathway, via ISRIB eIF2a dephosphorylation, will increase Retinal Ganglion Cell (RGC) survival and improve visual outcomes by reducing endoplasmic reticulum stress. (see Figure 3)

Methods

Animals
- Adult male C57Bl/6J mice

Traumatic Brain Injury (Figure 1a,b)
- Closed head weight drop model of TON
- A 400g metal rod positioned 1.5 cm above bregma

•SHAM mice are anesthetized but not injured.

Drug Interventions
- Drugs: Salubrinal (1.5 mg/kg), ISRIB (2.5 mg/kg)
- Vehicle (6.25% DMSO in saline)
- Injected 60 minutes post injury

Optokinetic Response Assay (OKR) [Figure 4a.]
- Involuntary optokinetic nystagmus
- (similar to smooth pursuit) assessed days 2-5 post injury
- 4 contrast gratings (0.12, 0.26, 0.32, & 0.39 cycles per degree [cpd])

Western Blot Analysis
- Retinal protein samples (20µg determined by BCA Assay)
- Proteins analyzed to confirm markers for:
  - ER Stress/UPR: PERK, eIF2a, P-elf2a
  - Apoptosis: Caspase-3, Caspase-12, CHOP
  - Retinal Ganglion Cell Loss: RBPMS

Data Analysis
- Optokinetic behavioral data analyzed via 3-way repeated measures ANOVA
- Western blot data analyzed via (ImageJ), and stats used 2-Way ANOVA

Figure 2

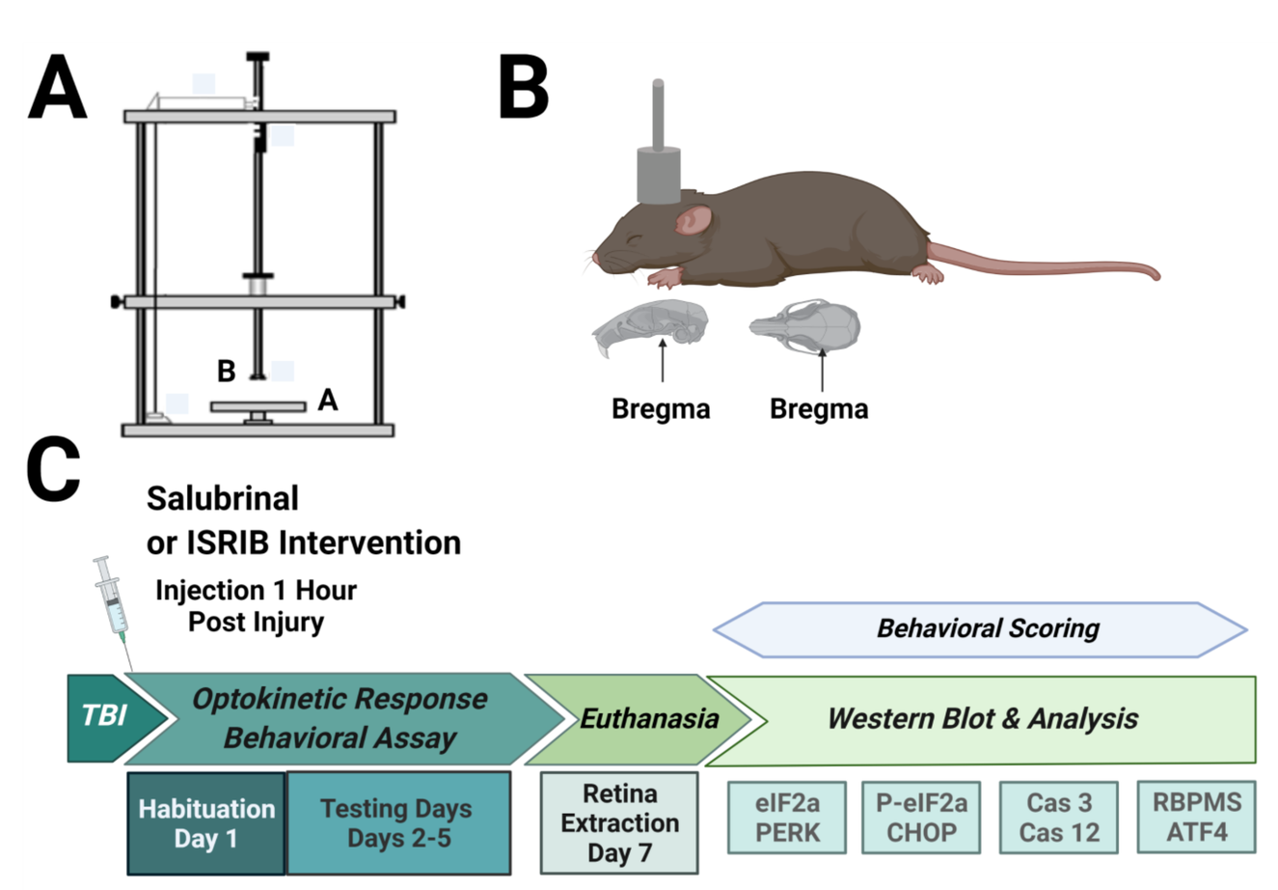


Figure 2. Methods and Timeline. (A) Depiction of our weight-drop TBI device (a) is the corkboard platform, and (b) is the weight. **(B)**Depiction of rough injury location. **(C)** Experimental Timeline. Created using *Biorender*

Results

Figure 3

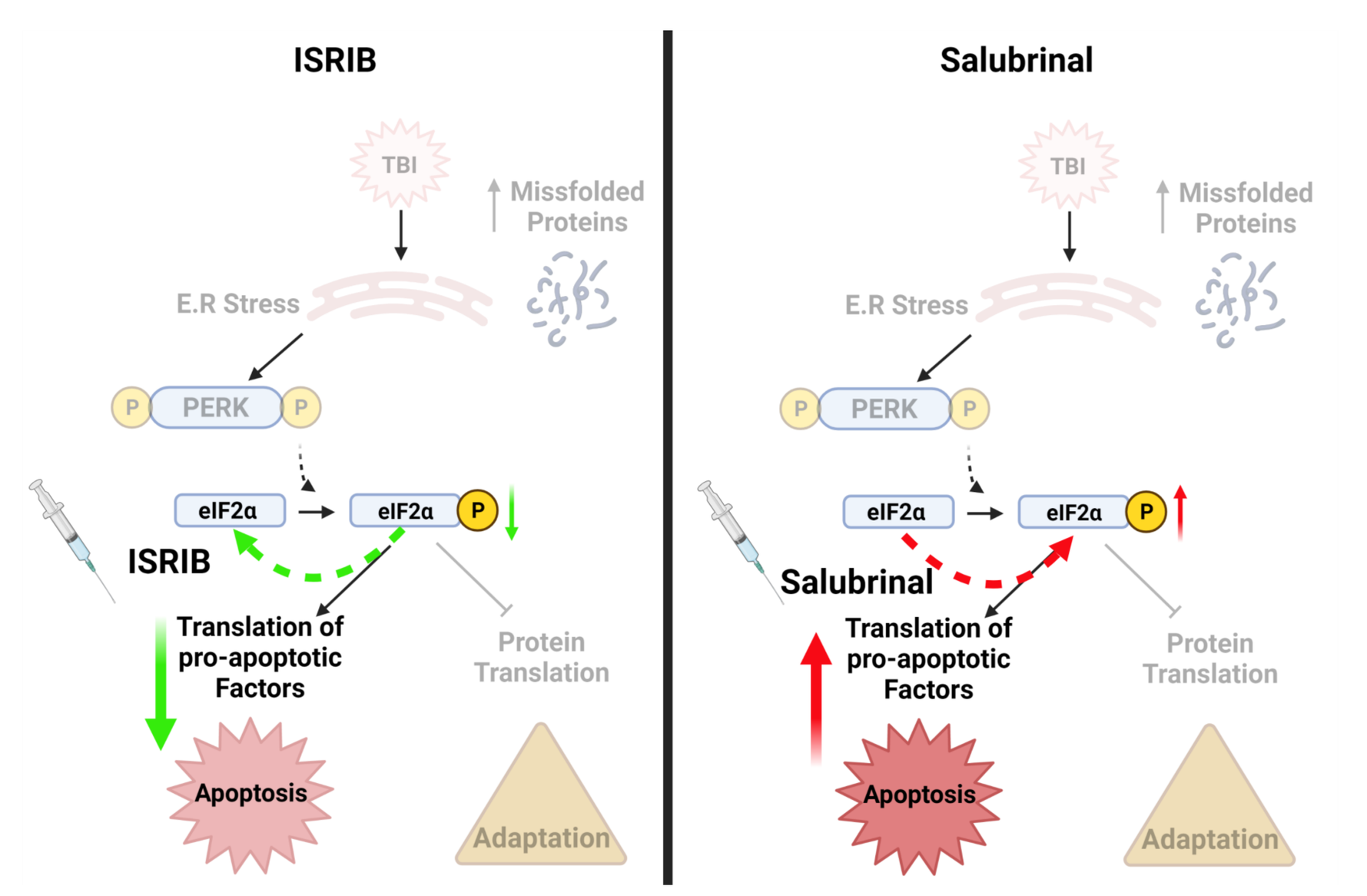


Figure 3. PERK Pathway diagram with hypothesized effects of drug intervention. Diagram displays the effects of drug therapeutics within the context of PERK manipulation post-TON and hypothesized effects on cellular outcomes. ISRIB intervention is shown on the left. Intervention is hypothesized to lead to a reduction in phosphorylated eIF2a leading to a reduction of downstream apoptotic factor translation. Salubrinal intervention is shown on the right. Through increased phosphorylation of eIF2a, there will be increased downstream activation of apoptotic products. *Created using Biorender*

Figure 4. ISRIB modestly improves Visual acuity, Salubrinal moderately helps to improve overall optokinetic response rate. (A) Image of optokinetic testing device. Mice are placed in center while the cylinder spins directionally to test for OKR response. **(B)** Depiction of interior contrast grating bars. Higher levels of contrast indicate reduced width of black bars and less discrepancy between white and black bar visual stimuli. **(C)** Optokinetic response rate following ISRIB intervention (N = 12). TBI displayed a main effect at all levels of CPD. TBI+ISRIB shown to have a maintained visual response rate at higher levels of contrast (0.39 CPD)(ns differences compared to SHAM +ISRIB conditions, p >0.05). ISRIB showed no effects at 0.12 and 0.32 CPD. Data at 0.26 CPD show decrease in response rate between SHAM + Vehicle and SHAM + ISRIB conditions (p<0.05), indicating less effect at lower levels of contrast. **(D)** Optokinetic response rate following Salubrinal intervention (N=12). TBI showed a main effect. TBI + Salubrinal is shown to have no significant difference from SHAM + Vehicle conditions at 0.12 and 0.26 CPD (p >0.05), indicating increase in visual response. Data at 0.32 CPD shows no difference between SHAM + Salubrinal and TBI + Vehicle conditions as well as no difference between SHAM + Salubrinal and TBI + Salubrinal conditions. Data at 0.39 CPD display significant reduction in response rate between SHAM + Vehicle and SHAM + Salubrinal conditions with no differences between TBI +/- Salubrinal conditions.

Figure 4

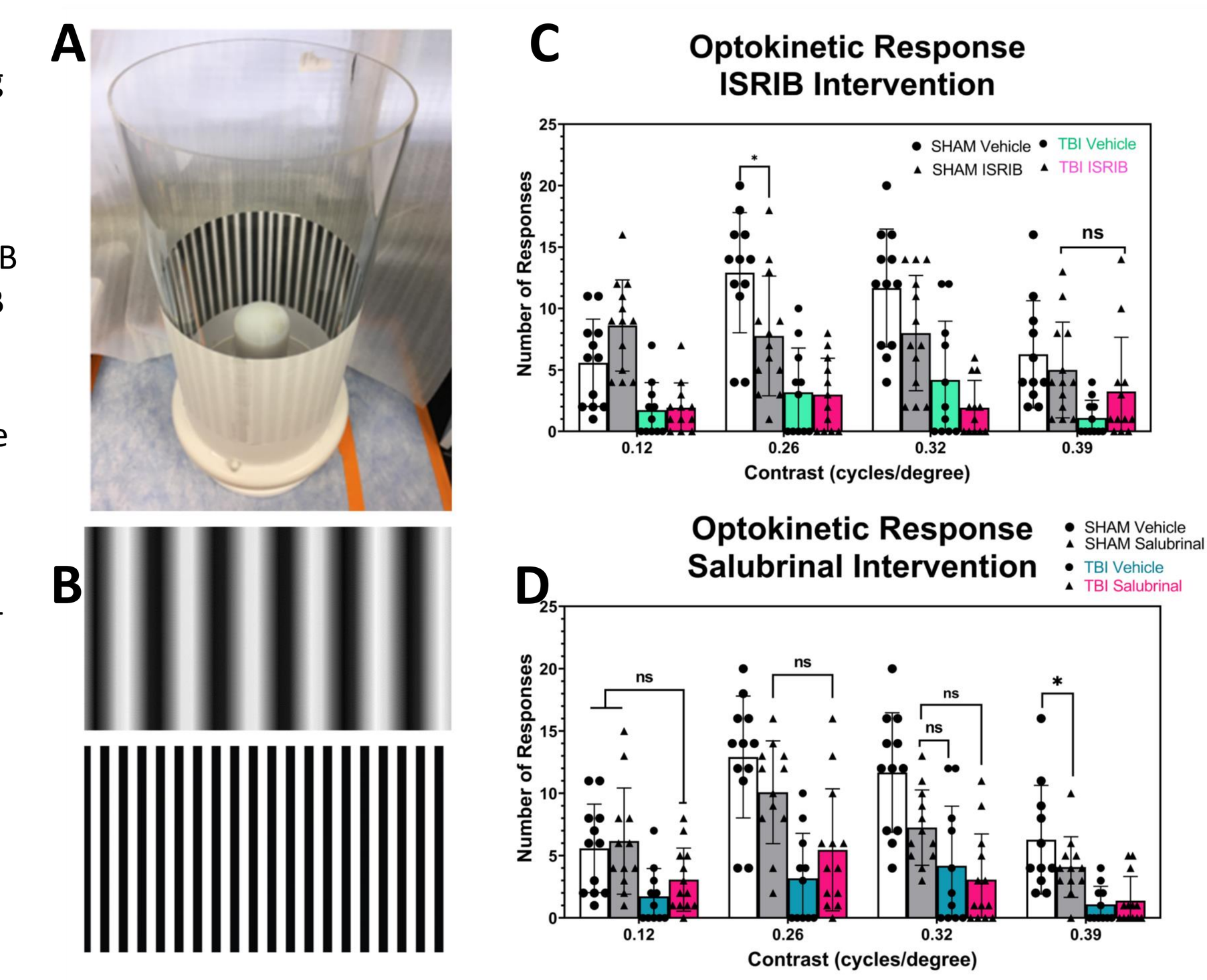
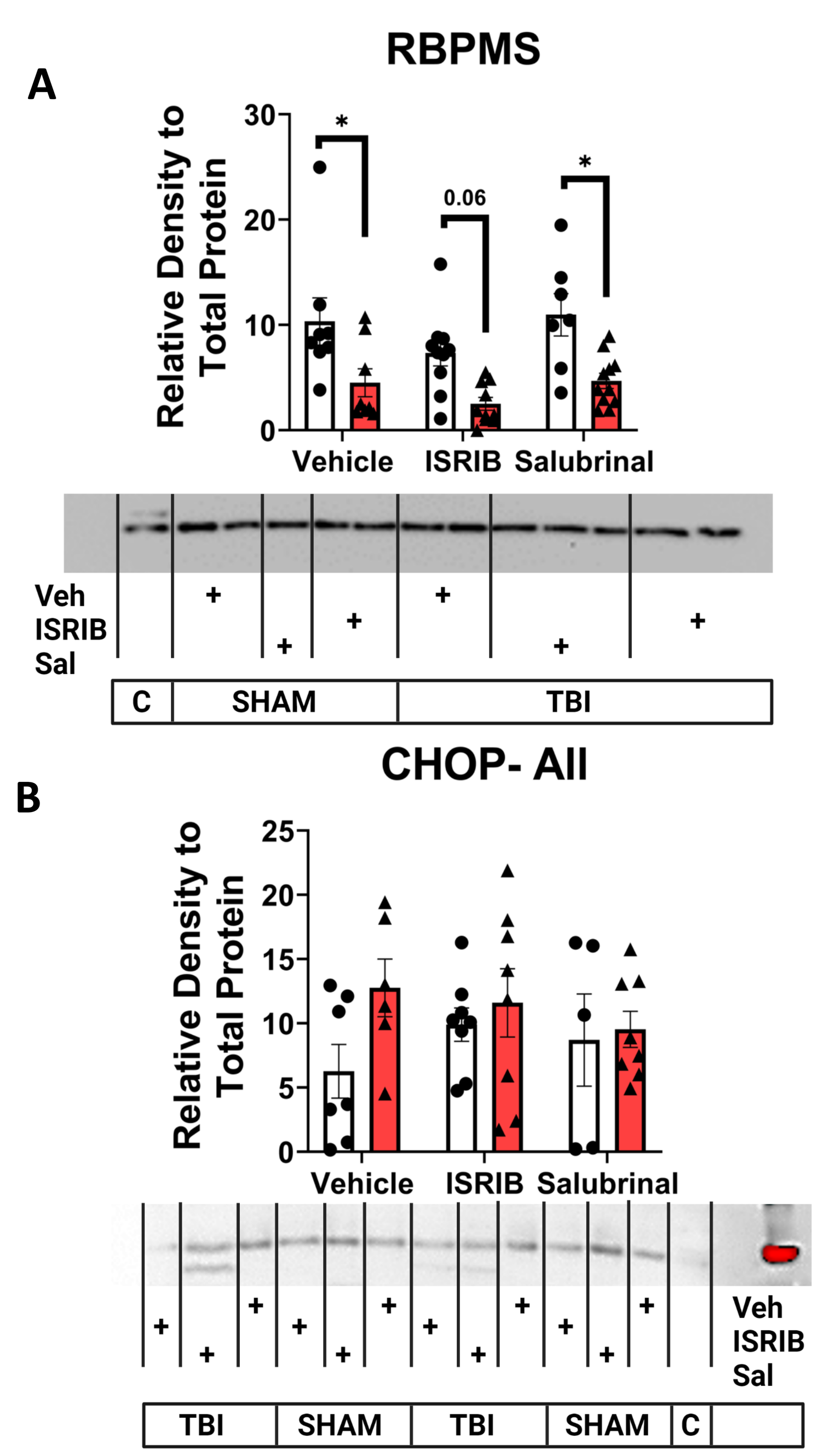


Figure 5



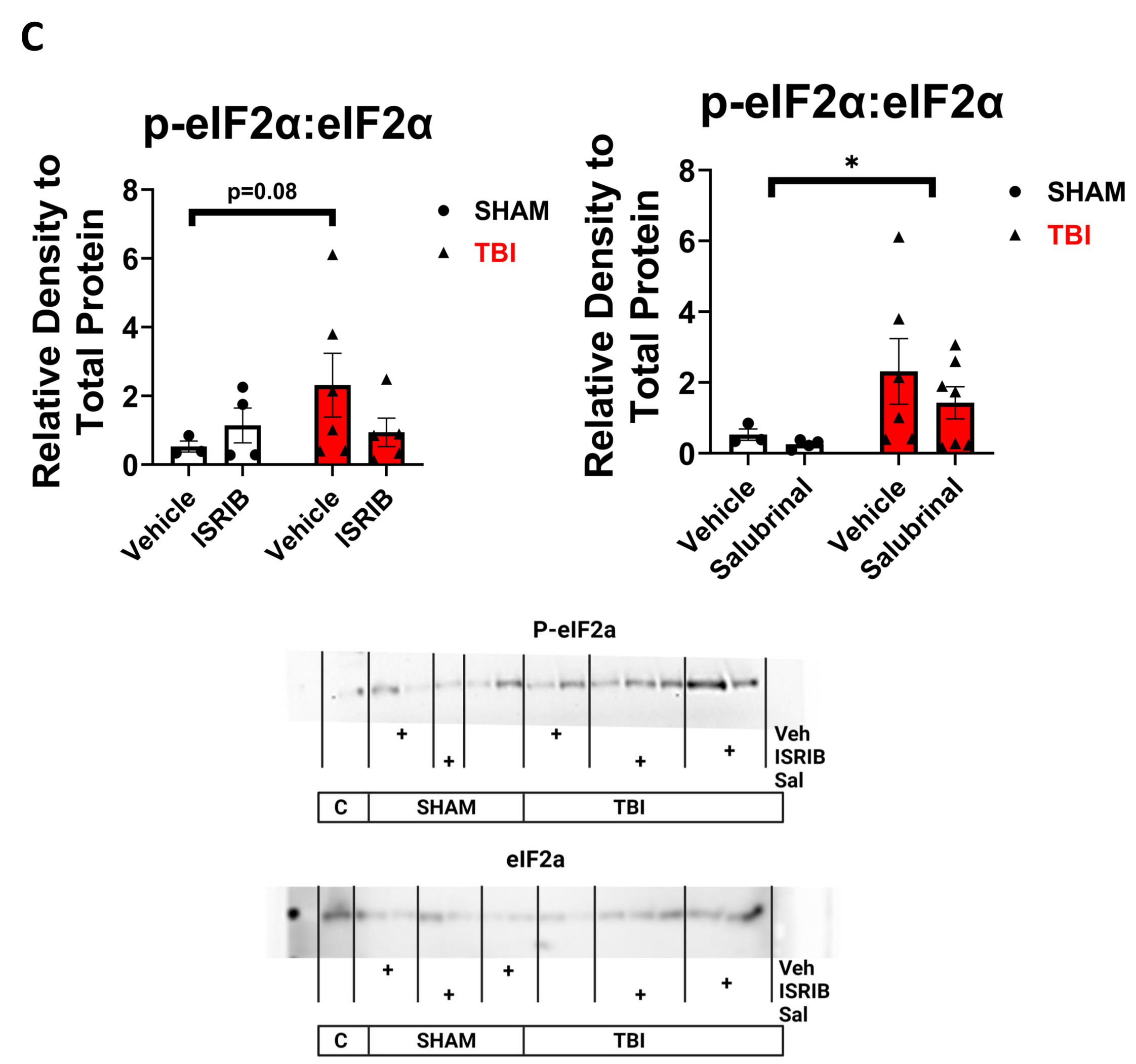


Figure 5. Western blot analysis of RBPMS, CHOP, P-eIF2a, e-IF2a. * = p < 0.05. Data is Mean +/- SEM for all sets. N Values listed for each condition. Western blot data shown below graph with conditions labeled for each cohort.

Figure 5a. Neither Salubrinal or ISRIB offers RGC saving functions 7 DPI. N = 8 for all conditions. Data shows main effect due to TBI in TBI + Vehicle as well as TBI + Salubrinal conditions as expected. TBI + ISRIB revealed a trend in reduction of RBPMS (p = 0.06) but not to a level of significance. No significant recovery was found in the Salubrinal or ISRIB cohort when compared to SHAM controls. Results indicate lack of RGC saving function at experimental dose 7 DPI.

Figure 5c. ISRIB has no effect on phosphorylation ratios of eIF2a, while Salubrinal maintained levels of TBI phosphorylation as expected. N = 3 for SHAM + Vehicle, N = 6 for TBI + Vehicle, N = 4 SHAM + ISRIB and TBI + ISRIB and SHAM + Salubrinal, N = 7 for TBI + Salubrinal. ISRIB intervention (left) showed no effects of injury or drug intervention (p > 0.05) between SHAM + Vehicle or TBI + Vehicle; No differences between SHAM + ISRIB and TBI + ISRIB cohorts. Reduced level of ratio is observed in TBI + ISRIB condition but due to low N, no significance was observed. Salubrinal Intervention (right) shows main effect of injury on ratio (p>0.05) as expected. TBI + Salubrinal showed elevated ratio compared to Vehicle + TBI condition, but not to a level of significance, higher N values might reveal higher significance if further testing occurs.

Figure 5b. Neither Salubrinal or ISRIB elevates or demotes CHOP expression, 7 DPI. N= 7 for all conditions. CHOP, a translated downstream apoptotic marker, was expected to be increased due to TBI effects as well as through Salubrinal intervention. No significant main effect of injury was observed between TBI and SHAM conditions (p > 0.05). No significant effect of drug was observed on CHOP elevation or demotion compared to SHAM conditions across both Salubrinal and ISRIB cohorts. (p>0.05).

Figure 6

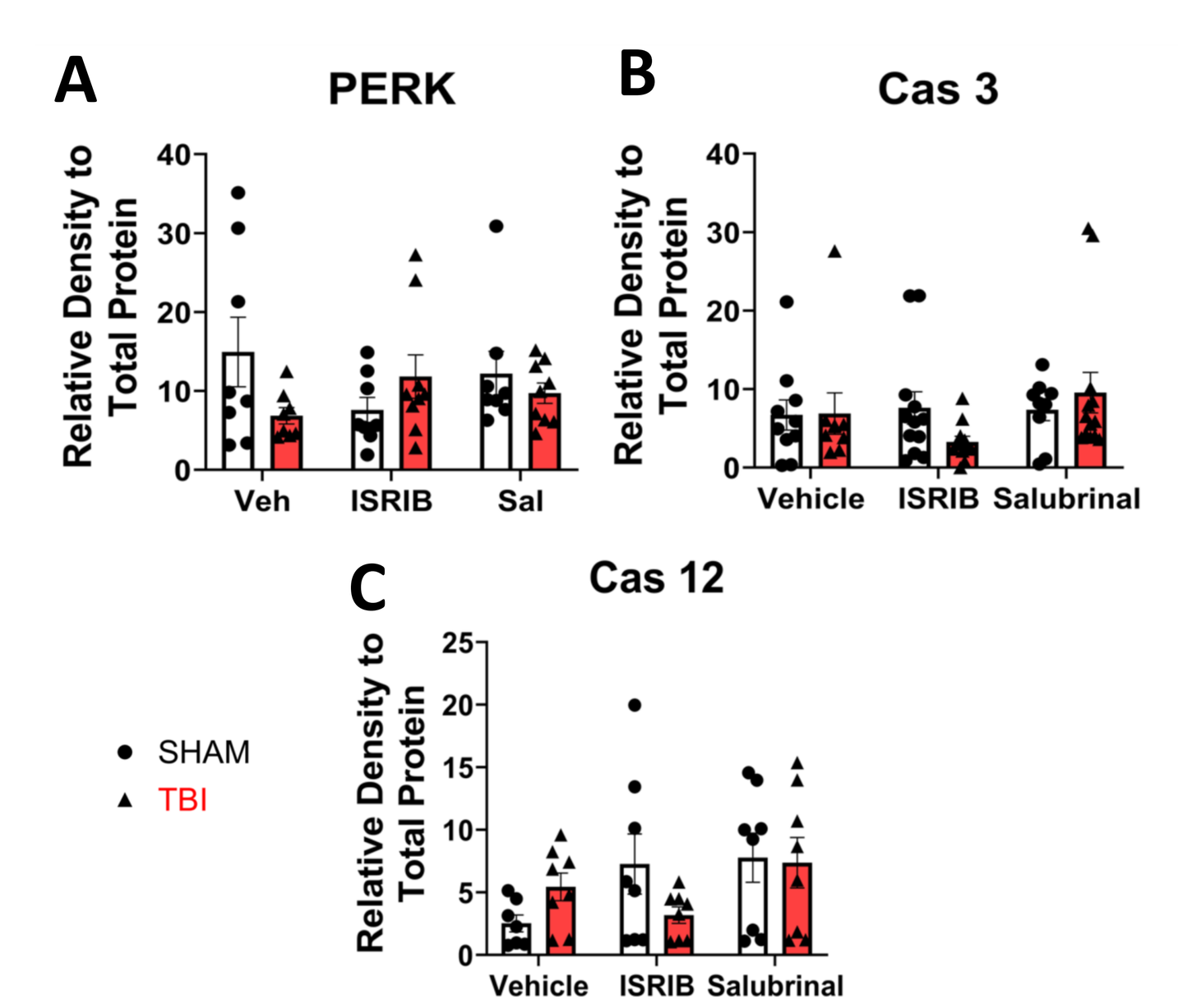


Figure 6. Neither Salubrinal or ISRIB altered PERK pathway modulation or cell death levels 7 DPI. Western Blot analysis of PERK (A), Caspase 3 (B), Caspase 12 (C). N values = 7, 9, 8 for PERK, Cas3, and Cas 12 respectfully. PERK (6A) represents a downstream protein kinase of the ER stress pathway. Caspase 3 (6B) is a downstream marker of cell death hypothesized to be cleaved during TBI induced apoptosis. Caspase 12 (6C) like caspase 3, is another downstream marker of cell death. Results yielded no effect of main effect, TBI vs. SHAM conditions (p > 0.05) for any of the tested factors. Similarly, no effect of Salubrinal or ISRIB +/- TBI was observed to be significantly different when compared to their Vehicle + SHAM counterpart (p>0.05). Data suggests lack of drug efficacy 7 DPI.

Discussion

- Modest Behavioral Effects:
  - ISRIB improved Visual Acuity at high CPD – could suggest effects on different visual system elements (cones vs. Rods)
  - Salubrinal improved OKR performance at low CPD (maybe RGC targeted)
- Retinal Cell Loss
  - Neither ISRIB or Salubrinal was able to restore levels of RGC's to pre TBI conditions
  - Suggests proactive effects on other retinal elements rather than retinal cells as a whole
- ER Stress/UPR Activation
  - ISRIB kept elf2α phosphorylation down (i.e., not different from sham levels). Salubrinal +TBI had significantly increased ratio of phosphorylated eIF2α compared to sham groups.
  - Supported the mechanisms of action for these drugs within the PERK-eIF2α system.
  - There were no changes in overt PERK levels, but we were unable to measure phospho-PERK – poses questions around drug efficacy 7 DPI.
- Apoptosis:
  - CHOP was elevated after TBI, but no groups showed different levels of pro-apoptotic markers 7 DPI.
  - Analysis of total caspases 3 and 12 were also examined but showed no differences between SHAM and TBI.
  - Suggests effects of drugs were no longer effective 7 DPI.

Limitations/Future Directions

- Age and Sex of mice:
  - Only adult male mice utilized/Explore females and adolescent models
- Analysis of other ER stress pathways: IRE1 & ATF6
- Need for cleaved Caspase data
- Examination of other retinal cells (i.e., rods and cones)
- Dosing strategy
- Effects outside on brain visual pathophysiology
- Examination of retinal cell function (e.g., electroretinogram)

Acknowledgments

Shelby Hetzer (BS, PhD) Research and Lab Mentor, Jordyn Torrens (BS) Peer Mentor, Dr. Nathan Evanson (MD,PhD) Faculty Mentor, Macy Urig (Peer), Nicholas Goatley (BS) Peer, Vivian Lallo (Peer)

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