

AWARD NUMBER: W81XWH-16-1-0076

TITLE: CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma

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REPORT DATE: January 2021

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE January 2021		2. REPORT TYPE Final Report		3. DATES COVERED 15Mar2016-14Sep2020	
4. TITLE AND SUBTITLE CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma				5a. CONTRACT NUMBER W81XWH-16-1-0076	
				5b. GRANT NUMBER MR141242	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Anton Reiner E-Mail: areiner@uthsc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Tennessee Health Science Center Memphis, TN 38163				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Visual deficits after traumatic brain injury (TBI) or after non-rupturing ocular trauma are highly common in the military, but interventions that limit the post-trauma visual impairments have not been identified. We have found that treatment with a CB2 cannabinoid receptor inverse agonist for 2 weeks after closed-head blast TBI greatly attenuates the visual deficits and retinal pathology produced by mild traumatic brain injury in mice, apparently by modulating the otherwise deleterious role of microglia in the injury process after trauma. The drug we have used (SMM189), however, has not yet been approved for human use. Raloxifene is an FDA-approved estrogen receptor drug that is used to treat osteoporosis, but also shows noteworthy CB2 inverse agonism. In our studies, we tested the benefit of raloxifene for reducing visual deficits and visual system damage from TBI or closed-globe ocular injury in mice, when delivered daily after TBI produced using left side focal cranial blast or dorsal cranial impact injury or after single or repeat ocular blast injury (OBI). Visual system injury and its abatement with raloxifene was assessed by functional testing (visual acuity, contrast sensitivity, the scotopic electroretinogram, pupil light response, and light aversion) and morphological analysis of retina, optic nerve, optic tract, and central visual structures. We found that raloxifene delivered by this regimen reduced the functional deficits and mitigated the underlying visual system pathology caused by focal cranial blast TBI, dorsal cranial impact TBI, single OBI or repeat OBI. The benefit could be obtained even with treatment delayed for 48 hours. Neurochemical analysis showed that the benefit stemmed from modulating microglia to a more beneficial M2 state, and thereby arresting the secondary injury caused by pro-inflammatory microglial M1 activation. Our studies support testing of raloxifene in phase 2 human clinical trials to determine its efficacy in treating visual system injury after brain and/or eye trauma. Such testing could lead to repurposing as a treatment for military trauma victims within the near future. In that context, it could be adopted as a routine treatment administered by medical personnel shortly after trauma, and thereby prevent or reduce the harmful consequences of the trauma for vision.					
15. SUBJECT TERMS traumatic brain injury, ocular blast injury, raloxifene, CB2 receptors, CB2 inverse agonist, visual system, retina, optic nerve, microglia, neuroinflammation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 48	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. Introduction

Visual deficits after traumatic brain injury (TBI) or after non-rupturing ocular trauma (by itself or as a TBI comorbidity) are highly common in the military, often leading to inability to return to service and/or life-long impairments. Interventions that limit the post-trauma visual impairments, however, have not been identified. We have found that treatment with a cannabinoid type-2 receptor (CB2) inverse agonist for 2 weeks after closed-head blast TBI greatly attenuates the visual deficits and retinal pathology produced by mild traumatic brain injury in mice, apparently by modulating the otherwise deleterious role of microglia in the injury process after trauma. The drug we have used (SMM189), however, has not yet been approved for human use. Raloxifene is an FDA-approved estrogen receptor drug that is used to treat osteoporosis, but has been found to also show noteworthy CB2 inverse agonism. In our studies, we tested the benefit of raloxifene for reducing visual deficits and visual system damage caused by TBI or closed-globe ocular injury in mice. In the case of TBI, we used two different standardized injury models - our left side focal cranial blast model of TBI, and a dorsal cranial impact model of mild TBI. Our goal was to determine if raloxifene reduced visual deficits and pathology, when delivered daily after the TBI produced in either case. In the case of ocular blast injury (OBI), our goal likewise was to determine if raloxifene reduces visual deficits and pathology, when delivered daily after the trauma. In the case of OBI, we examined raloxifene benefit after both single (1x) and repeat (5x) blast. Visual system injury and its abatement with raloxifene was assessed by functional testing (acuity, contrast sensitivity, the scotopic electroretinogram, the pupil light response, and light aversion) and morphological analysis of retina, optic nerve, optic tract, and central visual structures. In addition, morphological and biochemical analysis was used to show the role of microglial biasing from the harmful M1 phenotype toward the protective M2 phenotype in raloxifene benefit. Effective raloxifene dose and duration of treatment was determined as well. In brief, we found that raloxifene greatly attenuated visual deficits and visual system injury after blast TBI, impact TBI, 1x OBI and 5x OBI. Moreover, we found that the benefit arose from a modulation of microglia away the harmful M1 phenotype toward the protective M2 phenotype, and the benefit could be achieved even if treatment was not initiated until 48 hours after injury. We also found that raloxifene reduced depression and fearfulness stemming from TBI. Our studies showing benefit of raloxifene in preventing deficits and reducing neural tissue damage injury after TBI or OBI suggest it should be tested in phase 2 clinical trials to determine its efficacy in treating human visual injury after brain and/or eye trauma. This could lead to its approval for the use in military trauma victims. In that context, it could be adopted as a routine treatment administered by medical personnel shortly after trauma, and thereby prevent or reduce the harmful consequences of the trauma for vision, as well as perhaps for cognition and mood.

2. Keywords

Traumatic Brain Injury
Ocular Blast Injury
Raloxifene
CB2 receptors
CB2 receptor inverse agonist
Visual system
Retina
Optic nerve
Visual acuity
Electroretinogram (ERG)
Pupil light response
Light aversion
Microglia
Neuroinflammation

3. Accomplishments

The three tasks (Aims) making up the **Major Goals** of our project and each of their subgoals are listed below.

Major Goals for Task 1:

Task 1: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a left side focal cranial mouse blast model of TBI (Aim 1). This Task was divided into three subtasks as described below.

Subtask 1-1: Determine if raloxifene reduces the visual deficits and pathology in our focal blast model of TBI, when delivered daily after the TBI event. We have in fact shown that raloxifene reduces visual deficits and pathology in our focal blast model of mild TBI, as further detailed below. This subtask is 100% complete.

Subtask 1-2: Determine if raloxifene benefit in our focal blast model of TBI stems from modulation of microglia to the beneficial M2 phenotype. We have in fact shown that this is the case, as further detailed below. This subtask is 100% complete.

Subtask 1-3: Determine the treatment window for the raloxifene benefit in our focal blast model of TBI. We found treatment remained effective even when treatment initiation was delayed 48 hours. This subtask is 100% complete, as further detailed below.

Major Goals for Task 2:

Task 2: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a mouse dorsal cranial impact model of TBI (Aim 2). This Task was divided into three subtasks as described below.

Subtask 2-1: Determine if raloxifene reduces the visual deficits and pathology in a dorsal cranial impact model of TBI, when delivered daily after the TBI event. Our studies show that raloxifene reduces visual deficits after impact TBI, as further detailed below. This subtask is 100% complete.

Subtask 2-2: Determine if raloxifene benefit in a dorsal cranial impact model of TBI stems from modulation of microglia to the beneficial M2 phenotype. We have in fact shown that this is the case, as further detailed below. This subtask is 100% complete.

Subtask 2-3: Determine treatment window for the raloxifene benefit in a dorsal cranial impact model of TBI. The findings in Subtask 1-3 sufficed to address this subtask.

Major Goals for Task 3:

Task 3: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a mouse model of ocular blast injury (OBI) (Aim 3). This Task was divided into three subtasks as described below.

Subtask 2-1: Determine if raloxifene reduces the visual deficits and pathology in a model of OBI, when delivered daily after the TBI event. We found that raloxifene reduces visual deficits and pathology after 1x and 5x OBI, as detailed below. This subtask is 100% complete.

Subtask 2-2: Determine if raloxifene benefit in a model of OBI stems from modulation of microglia to the beneficial M2 phenotype. We have in fact shown that this is the case, as further detailed below. This subtask is 100% complete.

Subtask 2-3: Determine treatment window for the raloxifene benefit in a model of OBI. The findings in Subtask 1-3 sufficed to address this subtask.

Accomplishments for each Goal

Task 1 – Raloxifene Benefit for Blast TBI

1) Major Activities. We conducted behavioral, electrophysiological, morphological and biochemical studies in mice to evaluate the benefit of raloxifene in alleviating visual deficits and visual system injury after TBI produced using our blast system (Subtask 1-1). We evaluated raloxifene benefit for the following visual deficits or abnormalities after focal cranial blast TBI in mice: 1) a reduction in visual acuity; 2) a reduction in contrast sensitivity; 3) a reduction in the A-wave of the scotopic ERG; 4) a reduction in the B-wave of the scotopic ERG; 5) an increase in light aversion; and 6) abnormality in the pupil light reflex. Morphological studies assessed the rescue by raloxifene of structural damage to the visual system by blast TBI (Subtask 1-1). Neurochemical and biochemical studies assessed the modulatory influence of raloxifene on microglia – in particular the ability of raloxifene to bias microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 1-2). Functional and morphological studies assessed the effective treatment window for raloxifene (Subtask 1-3). We published a manuscript in *Experimental Neurology* on these findings (Honig et al. *Exp Neurol* 2019, PMID: 31518568), available as an epub in September 2019 and published in print in December 2019.

Task 2 – Raloxifene Benefit for Impact TBI

1) Major Activities. We conducted behavioral, electrophysiological, and morphological studies in mice to evaluate the benefit of raloxifene in alleviating visual deficits and visual system injury after TBI produced using an impact system (Subtask 2-1). We evaluated raloxifene benefit for the following visual endpoints after impact mild TBI: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex. Morphological studies assessed the rescue by raloxifene of structural damage to the visual system by impact TBI (Subtask 2-1). Neurochemical and biochemical studies assessed the modulatory influence of raloxifene on microglia – in particular the ability of raloxifene to bias microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). Studies to assess the effective treatment window for raloxifene (Subtask 2-3) were addressed by Subtask 1-3.

Task 3 – Raloxifene Benefit for OBI

1) Major Activities. We conducted behavioral, electrophysiological, and morphological studies in mice to evaluate the benefit of raloxifene in alleviating visual deficits and visual system injury after single (1x) blast and 5x blast OBI (Subtask 3-1). We evaluated raloxifene benefit for the following visual endpoints after 1x and 5x OBI: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex. Morphological studies assessed the rescue by raloxifene of structural damage to the visual system by 1x and 5x OBI (Subtask 3-1). Neurochemical and biochemical studies assessed the modulatory influence of raloxifene on microglia – in particular the ability of raloxifene to bias microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 3-2). Studies to assess the effective treatment window for raloxifene (Subtask 3-3) were addressed by Subtask 1-3.

Specific Objectives for each Goal

Task 1 – Raloxifene Benefit for Blast TBI

2) Specific Objectives. Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after mild TBI created using our focal blast model (Subtask 1-1). In the case of behavioral and physiological assessments, we sought to show that raloxifene alleviates: 1) a reduction in visual acuity following blast mild TBI; 2) a reduction in contrast sensitivity following blast mild TBI; 3) a reduction in the A-wave of the scotopic ERG following blast mild TBI; 4) a reduction in the B-wave of the scotopic ERG following blast mild TBI; 5) an increase in light aversion following blast mild TBI; and 6) an abnormality in the pupil light reflex following blast mild TBI. In the case of our morphological studies, we sought to show that raloxifene rescues structural damage to the visual system caused by blast mild TBI (Subtask 1-1).

In the case of the modulatory influence of raloxifene on microglia, we sought to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 1-2). Functional and morphological studies sought to determine the effective treatment window for raloxifene (Subtask 1-3). All of these objectives have been achieved, and the findings published (Honig et al., 2019, Experimental Neurology).

Task 2 – Raloxifene Benefit for Impact TBI

2) Specific Objectives. Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after TBI caused by an impact device (Subtask 2-1). In the case of behavioral and physiological assessments, we sought to determine if raloxifene shows a benefit for reducing impact TBI-induced abnormalities in the following endpoints: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex. In the case of our morphological studies, our goal was to determine if raloxifene rescues structural damage to the visual system caused by impact mild TBI (Subtask 2-1). As detailed below, these objectives have been achieved, and a manuscript describing them is in preparation. In the case of the modulatory influence of raloxifene on microglia, our goal was to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). These objectives too have been achieved, and the aforementioned manuscript in preparation will include these results. Functional studies to determine the effective treatment window for raloxifene after impact TBI (Subtask 2-3) were addressed by Subtask 1-3.

Task 3 – Raloxifene Benefit for OBI

2) Specific Objectives. Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after OBI (Subtask 3-1). In the case of behavioral and physiological assessments, we sought to determine if raloxifene shows a benefit for reducing 1x or 5x OBI-induced abnormalities in the following endpoints: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex. As detailed below, these objectives are achieved. In the case of our morphological studies, our goal was to determine if raloxifene rescues structural damage to the visual system caused by OBI (Subtask 3-1). This objective too was achieved. In the case of the modulatory influence of raloxifene on microglia, our goal was to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 3-2). A manuscript describing the functional studies, morphological studies, and biochemical studies is in preparation. Functional studies to determine the effective treatment window for raloxifene following OBI (Subtask 3-3) were addressed by Subtask 1-3.

Significant Results or Key Outcomes for each Goal

Significant Results or Key Outcomes for Task 1 – Raloxifene Benefit for Blast TBI Subtasks 1-1 (Benefit), 1-2 (Microglial Modulation) and 1-3 (Treatment Window)

1A. Completion of Subtasks 1-1, 1-2, and 1-3. Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after TBI created using our focal blast model. In the case of behavioral and physiological assessments, we have shown that raloxifene alleviates: 1) a reduction in visual acuity following blast mild TBI; 2) a reduction in contrast sensitivity following blast mild TBI; 3) a reduction in the A-wave of the scotopic ERG following blast mild TBI; 4) a reduction in the B-wave of the scotopic ERG following blast mild TBI; 5) an increase in light aversion following blast mild TBI; and 6) an abnormality in the pupil light reflex following blast mild TBI. In the case of our morphological studies, we have shown that raloxifene rescues structural damage to the visual system caused by blast mild TBI, in particular optic nerve axon loss and oculomotor nucleus neuron loss. In the case of the modulatory influence of raloxifene on microglia, we have provided biochemical and immunohistochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the

helpful M2 phenotype. Finally, functional and morphological studies have determined the effective treatment window for raloxifene (treatment needs to begin within 48 hours of injury for optimal benefit). Our findings for Task 1 (Aim 1) were presented at the 2019 ARVO Annual Meeting in May, 2019. Additionally, we published a manuscript in *Experimental Neurology* based on these findings, and presented these findings at an In-Progress Review at Fort Detrick, MD for the Clinical and Rehabilitative Medicine Research Program of the DOD on 10/29/2019. The presentation was entitled: CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma. This presentation also reported on raloxifene benefit following impact TBI and following ocular blast injury (OBI). We were also invited to submit a summary of our progress on blast TBI for the FY19 and FY20 Blast Injury Research Coordinating Office Blast Report by the US Army. In summary, all studies for Task 1 (Aim 1) have been completed. The citation information for the ARVO meeting presentation, published paper, and invited summaries follow. Additionally, we have also shown in yet unpublished studies that raloxifene reduces depression and fearfulness after blast TBI, using the same simple tests for which we had shown that the novel synthetic CB2 inverse agonist SMM189 also reduces depression and fearfulness after blast TBI (Reiner et al, *Int J Mol Sci* 2015, PMID: 25561230). These additional findings on raloxifene mitigation of post-TBI depression and fearfulness are detailed below in section 1B, and a manuscript describing them is in preparation.

1. Reiner A, Del Mar N, Henderson D, Perry A, Ragsdale T, Doty J, Driver J, Guley N, Mitchell W, Li C, Moore BM, Honig M. 2019. Raloxifene through its cannabinoid type-2 receptor inverse agonism mitigates visual deficits and pathology after mild TBI. *ARVO Abst* # 4405.
2. Honig MG, Del Mar NA, Henderson DL, Ragsdale TD, Doty JB, Driver JH, Li C, Fortugno AP, Mitchell WM, Perry AM, Moore BM, Reiner A. 2019. Amelioration of visual deficits and visual system pathology after mild TBI via the cannabinoid type-2 receptor inverse agonism of raloxifene. *Exp. Neurol.*, 322: 113063, 2019, PMID: 31518568.
3. Reiner, A. 2020. CB2 receptor therapy using the FDA-approved drug raloxifene to mitigate visual deficits after mild TBI and/or ocular trauma. In: FY19 Blast Injury Research Coordinating Office Blast Report. US Army.
4. Reiner, A. 2021. CB2 receptor therapy using the FDA-approved drug raloxifene to mitigate visual deficits after mild TBI and/or ocular trauma. In: FY20 Blast Injury Research Coordinating Office Blast Report. US Army.

1B. Additional Raloxifene Benefit After Blast TBI. To evaluate whether raloxifene benefit extended to the emotional disturbances seen in mice after blast TBI, some of the mice used in the visual system analysis also underwent testing for depression and fear. For these evaluations, we used the same simple and brief tests for which we had shown that the novel synthetic inverse agonist SMM189 reduces depression and fearfulness after blast TBI (Reiner et al, *Int J Mol Sci* 2015, PMID: 25561230). For evaluating depression, we used the tail suspension test, in which depressed mice progressively struggle less than control mice to free themselves over the course of the 5-minute test. As in our prior study, blast TBI caused a significant increase in depression as measured by the tail suspension test (i.e. showed increased immobility), but both 5 mg/kg and 10 mg/kg raloxifene delivered daily for the two weeks after blast fully rescued depression.

We have also previously shown that three to eight weeks after 50–60 psi left cranial blast, mice show increased retention of learned fear in a tone-shock fear conditioning paradigm (Heldt et al, *Frontiers Neurol* 2014, PMID: 24478749). We subsequently showed that SMM189 reduces this fearfulness (Reiner et al, *Int J Mol Sci* 2015, PMID: 25561230). In our studies of raloxifene benefit for fearfulness after blast TBI, we confirmed that blast mice showed significantly greater fear responding during the tone conditioned stimulus (CS) presentation than did sham mice. The lower dose of raloxifene did not prevent this, as their responses were significantly greater than sham and similar to blast, but the higher dose did, as their fear responses were not different from sham, but significantly less than in blast mice. Moreover, the two raloxifene groups differed significantly from each other in their fear responding. Thus, raloxifene at the higher dose alleviated the depression and fearfulness stemming from blast TBI, and the lower dose prevented the depression. Given the psychological disturbances that ensue from TBI in humans, it is of note that raloxifene may also be able to remedy them. Whether this benefit is limited to treatment just after TBI or whether a benefit could also be achieved with treatment days or weeks after TBI would of interest to determine, as raloxifene then might be useful in mitigating the

persistent PTSD-like consequences of TBI.

Significant Results or Key Outcomes for Task 2 – Raloxifene Benefit for Impact TBI

Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after TBI in mice caused by an impact device (Subtask 2-1). In the case of behavioral and physiological assessments, we sought to determine if raloxifene shows a benefit for reducing TBI-induced abnormalities in the following endpoints: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex. In the case of our morphological studies, our goal was determine if raloxifene rescues structural damage to the visual system caused by impact TBI (Subtask 2-1). As detailed below, these objectives have been achieved. In the case of the modulatory influence of raloxifene on microglia, our goal was to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). The findings for Task 2 (Aim 2) were presented among the abstracts for the 2020 ARVO Annual Meeting, scheduled for May, 2020. Note that the meeting itself was canceled due to the Covid-19 pandemic but the abstracts were nonetheless published. We are also preparing a manuscript for publication of these findings. The citation information for the ARVO abstract on the impact TBI work follows. We also presented these findings at the In-Progress Review at Fort Detrick Meeting on 10/29/2019.

1. Reiner A, Del Mar N, Henderson D, Perry A, Doty J, O'Neal D, Li C, Moore BM, Honig M. 2020. Raloxifene through its cannabinoid type-2 receptor inverse agonism mitigates visual deficits and pathology after impact TBI. *ARVO Abst # 689. Invest. Ophthalmol. Vis. Sci.*, 61(7):689.

2. Honig MG, Del Mar NA, Henderson DL, Doty JB, O'Neal D, Cox R, Li C, Perry AM, Moore BM, Reiner A. 2021. Amelioration of visual deficits and visual system pathology after impact TBI via the cannabinoid type-2 receptor inverse agonism of raloxifene. In prep.

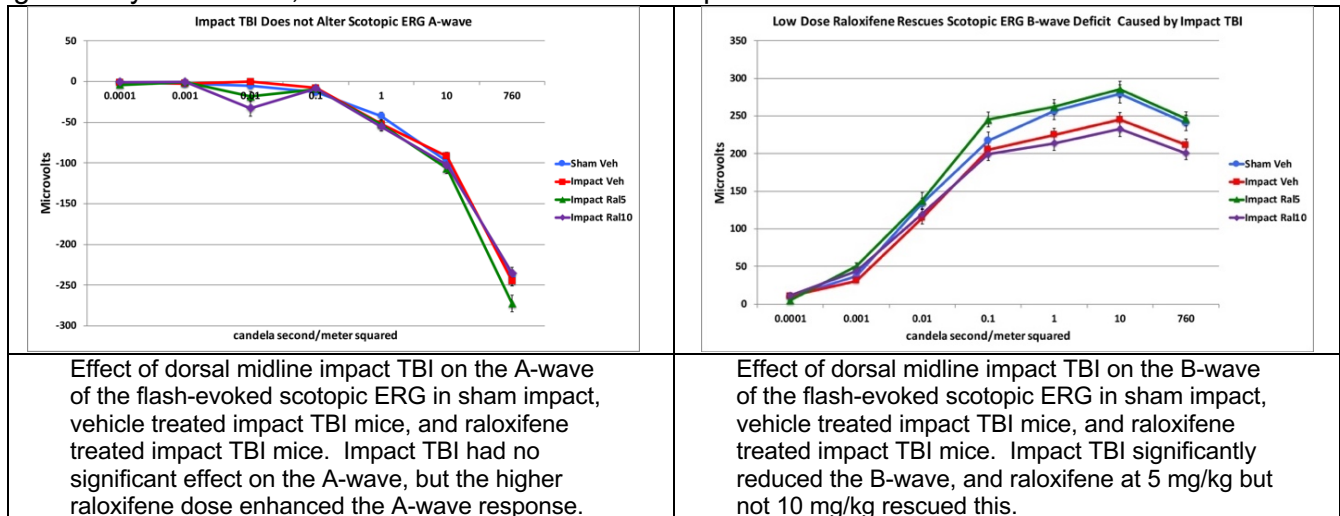
Subtask 2-1: Functional and Morphological Studies of Impact TBI

2-1A. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after Impact TBI. We assessed visual acuity and contrast sensitivity deficits after impact TBI and their amelioration by raloxifene in 21 sham impact mice treated with vehicle, 26 impact TBI mice treated with vehicle, 22 impact mice treated with 5 mg/kg raloxifene, and 14 impact mice treated with 10 mg/kg raloxifene. Treatment was begun two hours after impact and then daily for two weeks thereafter. TBI was produced in anesthetized mice using an Impact One Stereotaxic Impactor (Leica Biosystems, Buffalo Grove, IL) at a strike velocity of 5 m per second, strike depth of 1.0 mm, and dwell time of 200 milliseconds, to the shaved mouse head at 1.5 mm caudal to Bregma and centered on the midline. Note that as the injury is at the midline, the eyes, optic nerves, and two sides of the brain are equally affected. Thus, impact TBI data are presented for the two eyes pooled within group. Impact TBI produced a significant deficit in contrast sensitivity with the impact-vehicle mice needing greater contrast to detect the moving stripes than the sham mice. The 5 mg/kg raloxifene impact mice and the 10 mg/kg raloxifene impact mice exhibited better contrast sensitivity than the impact-vehicle mice, and were not significantly different than the sham mice. Thus, raloxifene rescued the deficit in contrast sensitivity produced by TBI. Thus, as with blast TBI, raloxifene rescued a post-TBI contrast sensitivity deficit when treatment was begun two hours after impact and then daily for two weeks thereafter. In the case of visual acuity, a reduction was observed in the impact-vehicle mice compared to the sham-vehicle mice that trended toward but did not achieve significance. Visual acuity for the eyes from the impact mice treated with both doses of raloxifene was slightly better than for the impact-vehicle mice and not significantly different than for the sham mice, suggestive of improvement.

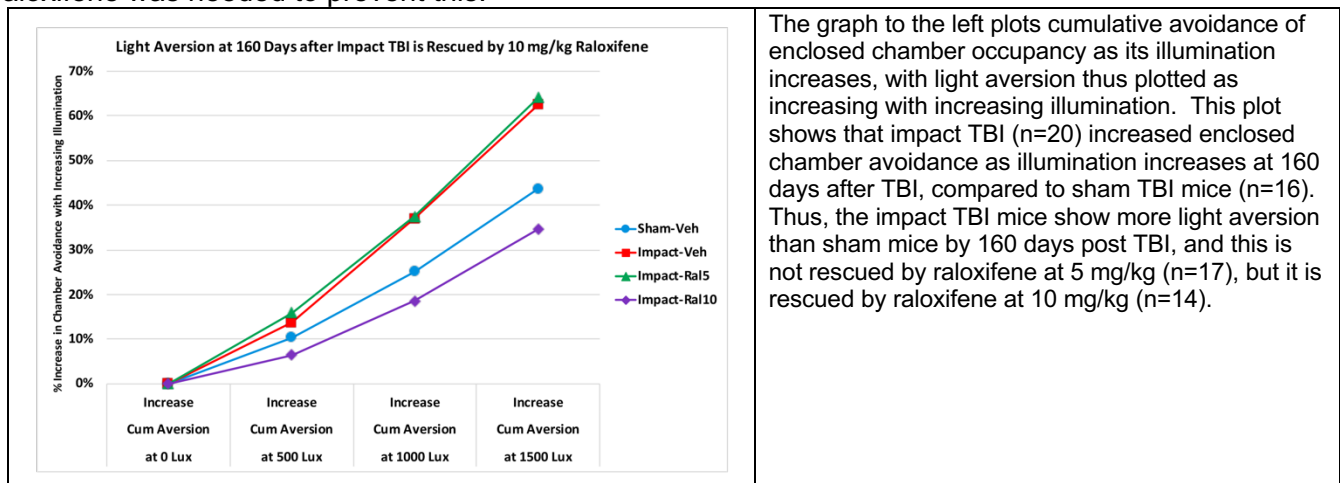
2-1B. Raloxifene Benefit – Scotopic Light-adapted ERG A-wave and B-wave after Impact TBI.

The average peak amplitudes of the A-wave were similar in impact-vehicle mice and sham mice. This was unchanged by 10 mg/kg raloxifene, but 5 mg/kg raloxifene significantly enhanced the A-wave response over the three brightest light intensities. By contrast, impact TBI did produce a B-wave deficit, with the average peak amplitudes of the B-wave over the brightest 6 intensities significantly reduced in impact-vehicle mice compared to sham mice. Raloxifene at 5 mg/kg yielded prominent rescue, and in fact, the B-wave responses across the brightest 6 intensities were significantly greater in the 5 mg /kg raloxifene-treated impact mice than in the vehicle-treated impact TBI mice and even greater than in

sham mice. Surprisingly, raloxifene at 10 mg/kg did not rescue the B-wave deficit, as it differed significantly from sham, but not from vehicle-treated impact TBI mice.



2-1C. Raloxifene Benefit – Light Aversion in Impact TBI Mice at 160 Days after TBI. We evaluated Light aversion at ~5 months after impact by allowing each mouse to move freely between two chambers, an open chamber kept dark and an enclosed chamber with variable illumination. As shown below, increased illumination in the enclosed chamber resulted in greater avoidance for all groups of mice. The impact-vehicle mice, however, spent significantly less time than sham mice in the increasingly brighter enclosed chamber, as did the impact mice treated with 5 mg/kg raloxifene, and impact-vehicle and impact-raloxifene5 mice did not differ significantly from one another. Treatment with 10 mg/kg raloxifene rescued this heightened light aversion, as blast-raloxifene10 mice occupied the illuminated enclosed chamber significantly less than the impact-vehicle mice and trended as even less than in the sham mice. Thus, impact TBI caused increased light aversion, and the higher dose of raloxifene was needed to prevent this.



2-1D. Raloxifene Benefit – Pupil Light Reflex after Impact TBI. To provide insight into the increased light aversion mice exhibit as a consequence of TBI, we assessed the pupil light reflex (PLR) ~7 months after impact. We found that pupil diameter was significantly 15-30% greater in impact TBI-vehicle mice before red light, during red light, after red light, and during and after blue light compared to sham mice, but the percent change in constriction from baseline was not significantly different than in sham. Thus, the pupil was excessively dilated at rest and during light-evoked constriction in mice that had been subjected to impact TBI, but the relative magnitude of the dynamic constriction to red light (driven by photoreceptors) and to blue light (driven by intrinsically photosensitive retinal ganglion cells) were similar to that in sham mice. Treatment with 5 mg/kg raloxifene did not rescue the enlarged resting pupil size compared to sham or the excessive dilation during blue light compared to sham,

although there was some rescue (i.e. restored to being similar to sham) of the excessive dilation during red light that was seen in impact-vehicle mice compared to sham, and the percent constriction from baseline to both red light and blue light did not differ from sham). For impact mice treated with 10 mg/kg raloxifene, resting pupil size was again significantly greater than for sham mice, but pupil size during red light was not significantly different than in sham and during blue light was even significantly less than in sham. Thus, 10 mg/kg raloxifene did not rescue the increase in resting pupil size caused by impact TBI, but it did normalize the absolute magnitude of constriction caused by red light, and yielded hyperconstriction to blue light. As a result of this differential pattern of rescue, the percent pupil constriction from baseline in response to blue light was significantly greater in impact mice treated with 10 mg/kg raloxifene than in sham mice, as was the percent constriction to red light. Thus, raloxifene at 10 mg/kg, and to a much smaller extent at 5 mg/kg, rescued the pupillary defect (i.e. excessive dilation) caused by impact TBI, and caused an exaggerated percent constriction from baseline.

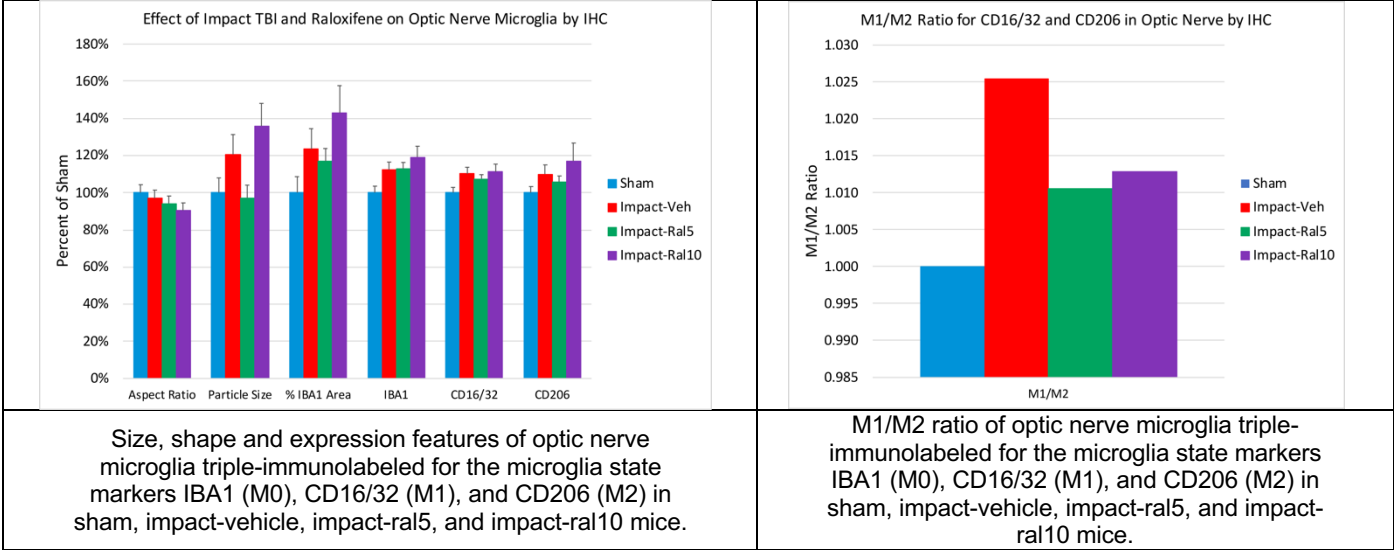
2-1E. Raloxifene Benefit in Mice with Impact TBI – Optic Nerve. For 80 impact TBI mice (21 sham with vehicle, 25 TBI with vehicle, 21 TBI with 5 mg/kg raloxifene, and 13 with 10 mg/kg raloxifene) for which functional assessment of raloxifene benefit was performed, we harvested optic nerves and performed blinded computer-assisted axon counts. We found a significant 21.8% loss of optic nerve axons in the impact-vehicle mice compared to sham mice. By contrast, axon abundance in the impact mice treated with 5 mg/kg raloxifene was 7.4% less, but not significantly different than in sham mice, and greater than for the impact-vehicle mice. Raloxifene at 10 mg/kg was yet more effective for axon rescue, showing no significant loss and an axon abundance indistinguishable from that in sham mice and significantly greater than in the impact-vehicle mice. Optic nerve axon abundance did not correlate with either contrast sensitivity threshold or visual acuity across all animals.

2-1F. Raloxifene Benefit in Mice with Impact TBI – Oculomotor Nucleus. To help understand the basis of eye movement abnormalities in TBI victims and assess their potential alleviation by raloxifene, we examined the oculomotor nucleus in our TBI mice using immunolabeling for choline acetyltransferase (ChAT) to detect oculomotor neurons. We focused on the oculomotor nucleus (cranial nerve nucleus III), because it innervates 4 extraocular muscles, and performed blinded counts of cholinergic neurons in the cell group and measurement of the area of the cell group at a matched standard level across cases. Analysis was performed at about 30 weeks post impact trauma to assess loss and rescue for 20 sham mice, 25 TBI mice without drug, 19 mice with 5 mg/kg raloxifene, and 14 with 10 mg/kg raloxifene. Unlike in blast TBI, however, no significant loss of oculomotor neurons was seen with impact TBI in the impact-vehicle group (97.5% of sham). We thus could not evaluate raloxifene rescue of oculomotor neurons after impact TBI.

Subtask 2-2: Neurochemical Studies of Microglial Modulation by Raloxifene in Impact TBI

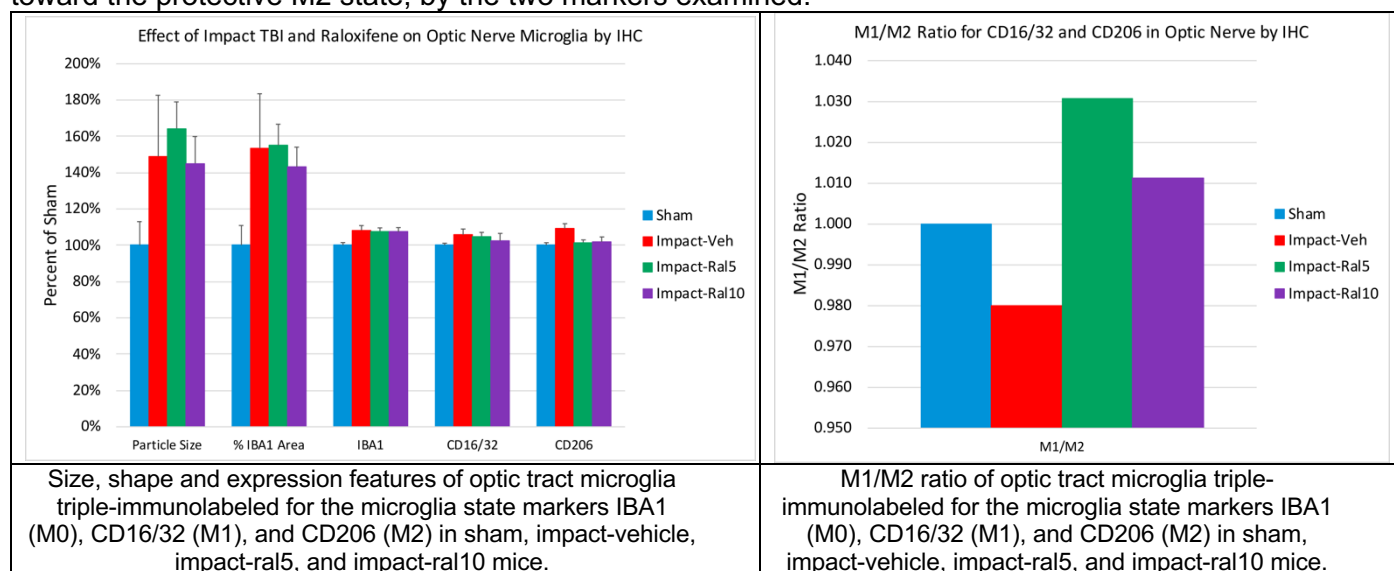
2-2A. Raloxifene Benefit in Mice with Impact TBI – Immunohistochemical Studies of Optic Nerve. Our examination of cryostat sections from eyes with optic nerve attached revealed occasional axon bulbs scattered along the intraorbital part of the optic nerve at 3 days after impact. Axon bulbs were largely absent at the optic nerve head following impact TBI, in contrast to the many we saw previously at the optic nerve head with focal cranial blast TBI (Guley et al, J Neurotrauma 2016, PMID: 26414413). Most strikingly, and reminiscent of what we also saw with focal cranial blast TBI (Honig et al. Exp Neurol 2019, PMID: 31518568), axon bulbs after dorsal cranial impact TBI tended to be concentrated in a ~500 µm length of nerve corresponding to a level somewhat beyond the extraocular muscle cone and thus within, or slightly past, the bony optic canal. Immunofluorescent staining for IBA1 showed that the prominent axon bulbs at this level were frequently associated with microglia possessing large cell bodies and shortened thick processes, which are typically thought to be reactive. In some cases, microglia engulfed the bulbs. To assess the modulation of optic nerve microglia by impact TBI and raloxifene, we examined IBA1-immunolabeled microglia for colocalization of the M1 marker CD16/32 versus the M2 marker CD206 in mice sacrificed 3 days after impact, focusing on the part of the optic nerve proximal to the extraocular muscle cone (i.e. closer to brain). We found that after TBI alone, expression by optic nerve microglia of IBA1, CD16/32, and CD206 was increased, with the increase

greater for CD16/32 than CD206, resulting in an increased M1/M2 ratio compared to sham (see Figure below left). Impact TBI also increased the total optic nerve area occupied by microglia and the roundedness of their cell bodies. The lower dose of raloxifene greatly reduced IBA1 and CD16/32 immunolabeling relative to impact-vehicle mice, but also decreased CD206 closer to sham levels. Because the relative reduction in CD16/32 was greater than the CD206 reduction, the M1/M2 ratio was slightly less than in impact-vehicle mice. The area occupied by microglia and their roundedness was similar to that in impact-vehicle mice. The higher raloxifene dose exerted a very prominent activating effect on microglia that appears to have been highly protective, based on the efficacy of this dose in rescuing optic nerve axons. Not only was CD206 greatly elevated above both sham and impact-vehicle levels, but CD16/32 was also elevated comparably to that in impact-vehicle optic nerves, resulting in an M1/M2 ratio that was only slightly less than in the impact-vehicle mice. Moreover, the area occupied by microglia and their roundedness was yet greater than in impact-vehicle mice. Activated microglia in impact-raloxifene10 mice nerves were common in fields with SMI-32+ axon bulbs. In some nerves in impact-raloxifene10 mice, rounded activated microglia were found in clusters in which axon bulbs were relatively scarce, suggesting that the microglia at these sites had effected rescue or had cleared axonal debris. These results show that raloxifene did modulate microglia in optic nerves damaged by impact TBI, and the higher dose fostered engagement of these microglia with damaged axons in a way that axon counts suggest was therapeutic.



2-2B. Raloxifene Benefit in Mice with Impact TBI – Immunohistochemical Studies of Optic Tract. To further evaluate how raloxifene affects microglia within a field of axons damaged by impact TBI, we also examined the optic tract, where reactive microglia are known to be abundant during the first week after impact TBI. We used the same mice as in our above study of optic nerve, and again used multiple immunofluorescent labeling for IBA1 to visualize all microglia, as well as the M1 marker CD16/32 and the M2 marker CD206. We found that activated microglia were far less common in optic tract than after focal cranial blast injury, suggesting that impact TBI may primarily cause its damage to optic axons at the level of the optic nerve, and that blast may cause part of its injury to axons of retinal origin at the level of the optic tract. Nonetheless, optic tract microglia were affected by dorsal midline cranial impact TBI, as we found that it increased the size of IBA1+ microglia and their occupancy of optic tract by about 50% and 60%, respectively, with the effect being bilateral as expected in keeping with dorsal midline nature of the impact injury. Moreover, as shown below, the intensity of IBA1, CD16/32, and CD206 immunolabeling was greater by 10% in the impact-vehicle mice than in sham-vehicle mice. Since CD16/32 and CD206 immunolabeling were similarly increased in the impact-vehicle mice, the M1/M2 ratio for microglia in the impact-vehicle mice was similar to that in sham mice, despite the clear evidence of microglial activation. Microglia traits for the lower raloxifene dose were similar to those in impact-vehicle mice, but the higher dose slightly reduced microglial size, abundance and the intensity of IBA1, CD16/32, and CD206 immunolabeling, more so CD206 than CD16/32. As a result, the M1/M2

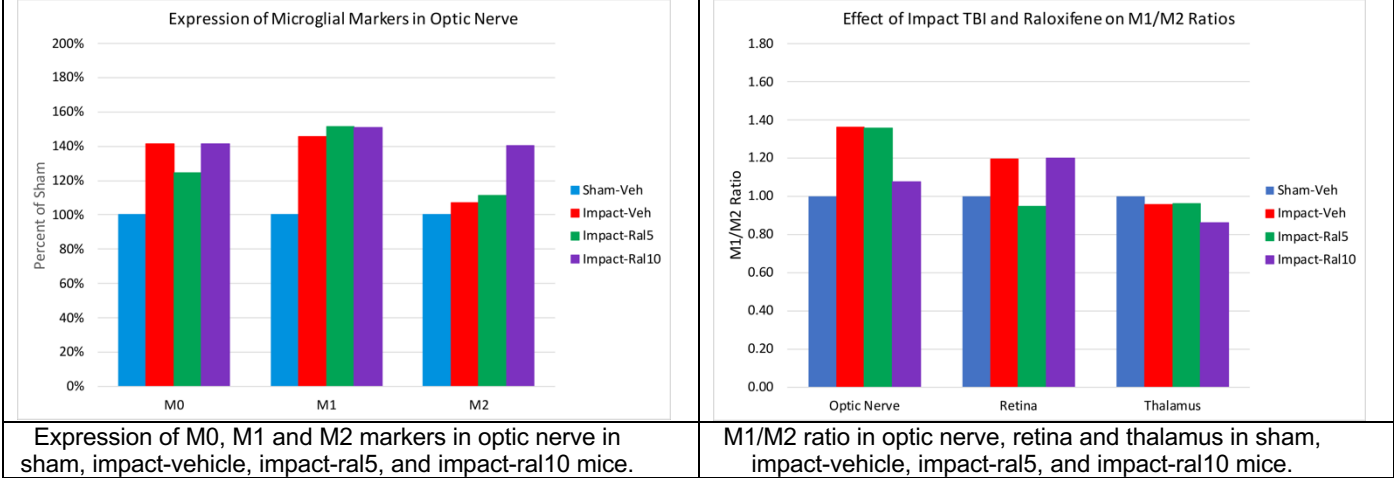
ratio was slightly greater than that for sham and impact-vehicle mice. Thus, raloxifene treatment appeared to partially reverse microglial activation in the optic tract at the higher dose, but at 3 days post impact TBI the impact did not bias microglia away from harmful M1 state, nor did raloxifene bias them toward the protective M2 state, by the two markers examined.



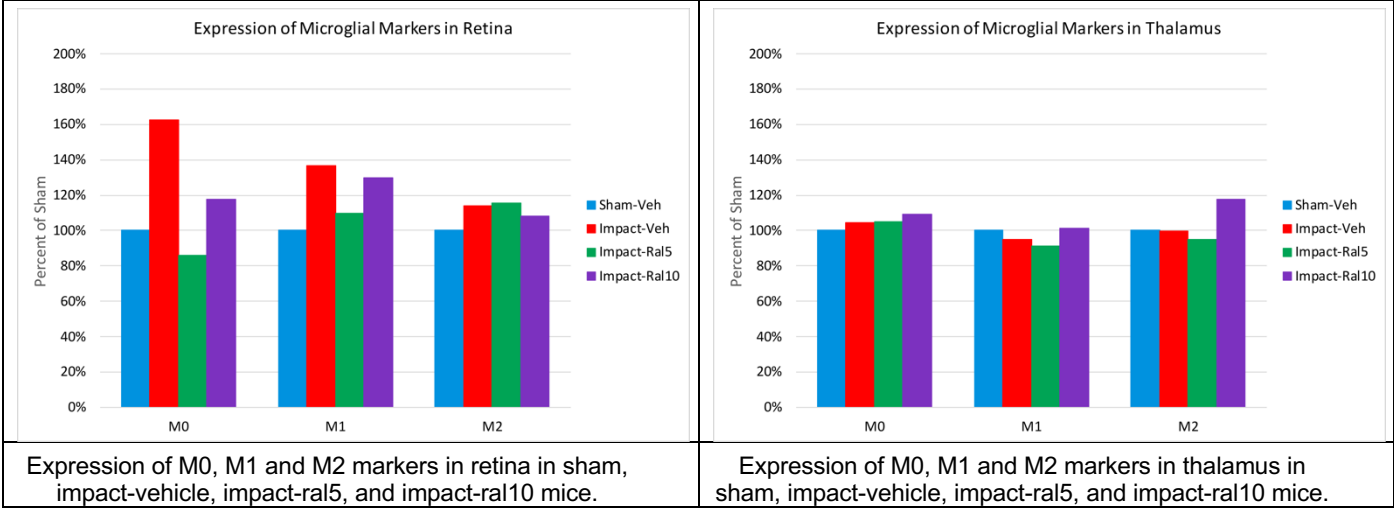
2-2C. Raloxifene Benefit in Mice with Impact TBI – Biochemical Studies. We also performed biochemical studies on microglial modulation in the optic nerves, retina, and thalamus by impact TBI and by raloxifene at 5 mg/kg and 10 mg/kg, measuring expression of markers informative for microglial M0, M1, and M2 activation state. For these studies, mRNA was harvested three days after impact TBI, with each mouse receiving 4 daily treatments with vehicle or one of the two raloxifene doses. The first dose was delivered 2 hours post impact, and the other three daily until sacrifice on the third day after impact. We used two approaches for assessing microglial gene expression. As one approach, we used a technology called the NanoString nCounter system, a novel platform that can be used for direct quantification of mRNA levels. This technology works by directly capturing, imaging, and counting fluorescent barcodes tagged to individual mRNA molecules, without the need for amplification or reverse transcription. As the other approach, we used TaqMan qPCR, as we had in our studies of blast TBI (Honig et al, Exp Neurol 2019, PMID: 31518568). Our studies in blast TBI using qPCR showed that microglia are shifted by blast TBI from the M0 quiescent state to the M1 pro-inflammatory state, and that raloxifene biases M1 microglia toward the pro-healing M2 state, more so the higher dose. For impact TBI, because the injury is dorsal midline and thus affects the sides of the brain and the two eyes equally, in our analysis of retina, optic nerve and thalamus, the two sides were pooled. For qPCR, we assessed expression of the following microglia markers: 1) M0 – ionized calcium-binding adapter molecule 1 (IBA1), transmembrane protein 119 (Tmem119), purinergic receptor P2Y12 (P2ry12), and transforming growth factor-beta (TGFβ); 2) M1 – Fc gamma receptor 2 (CD32), interferon-gamma (IFNγ), interleukin 1-beta (IL1β), tumor necrosis factor-alpha (TNFα), and inducible nitric oxide synthase (iNOS); and 3) M2 - Arginase-1 (Arg1), chitinase-like protein 3 (Ym1), interleukin-10 (IL10), and triggering receptor expressed on myeloid cells 2 (TREM2). In the case of NanoString analysis, we assessed the following microglial markers to determine the effect of impact TBI and of raloxifene on their expression, some of which were also examined by qPCR and immunolabeling of the optic nerve and tract: 1) for M0: MERTK, P2ry12, SPARC, and Tmem119; 2) for M1: CD16, CD32, IFN-gamma; IL1-beta, IL6, iNOS, CD86, IL12p40, and Tlr2; and 3) for M2: Arg-1, CD206, IL10, Trem2, Ym1, Fizz1, CD36, IL13, IL4r-alpha. In the below description, we have combined the qPCR and NanoString expression data, averaging the results in the case of those markers that were examined by both approaches. The combined results for the markers assessed in common by qPCR and NanoString were largely similar to those obtained for the specific M0, M1 and M2 markers IBA1, CD16/32 and CD206 by immunolabeling and by the two types of expression analysis.

The results for optic nerve in the impact-vehicle mice showed upregulation of M0 markers, and upregulation of M1 markers relative to M2 markers, yielding an increased M1/M2 ratio for the impact-

vehicle mice (see figures below). For optic nerve, the lower raloxifene dose had little effect on M1 or M2 markers, yielding an M1/M2 ratio similar to that in impact-vehicle mice. At the higher dose, as it had in our immunolabeling studies of optic nerve, raloxifene increased M1 markers and even more so increased M2 markers relative to impact-vehicle mice, yielding an M1/M2 ratio considerably lower than in impact-vehicle mice, consistent with the greater benefit of the higher dose for optic nerve rescue.



The results for retina in the impact-vehicle mice showed upregulation of M0 markers, and upregulation of M1 markers relative to M2 markers, yielding an increased M1/M2 ratio for the impact-vehicle mice (see figures below). The lower dose of raloxifene reduced M1 but not M2 markers relative to impact-vehicle mice. As a result, the lower raloxifene dose yielded a lower M1/M2 ratio for retina than seen in the impact-vehicle mice. The higher raloxifene dose only modestly reduced M1 expression relative to impact-vehicle mice, but also reduced M2 markers, yielding an M1/M2 ratio similar to that in impact-vehicle mice. Thus, the lower dose was more effective at beneficial microglial modulation in retina, perhaps explaining why the lower but not higher dose rescued the B-wave deficit.



In the case of thalamus (which contains the optic tracts), the results for impact-vehicle mice indicated that for thalamus as a whole, dorsal cranial impact TBI did not cause noteworthy microglial activation, although our immunolabeling studies showed clear evidence of microglial activation at least in the optic tracts. In particular, only a small increase in thalamic M0 markers was seen in impact-vehicle mice, and M1 and M2 markers were reduced, M1 more so, yielding an M1/M2 ratio slightly less than in sham mice. The lower raloxifene dose yielded largely similar results, while the higher raloxifene dose increased M0 and M1 marker expression above that in impact-vehicle mice. An increase in M2 markers was especially prominent with the higher raloxifene dose, yielding an M1/M2 ratio that was less than that in impact-vehicle mice. The overall results of our biochemical studies indicate that the higher raloxifene beneficially modulated microglia toward the M2 state in optic nerve and thalamus,

while the lower dose did so for retina. The immunolabeling studies for IBA1, CD16/32 and CD206 confirmed this outcome for microglia of optic nerve.

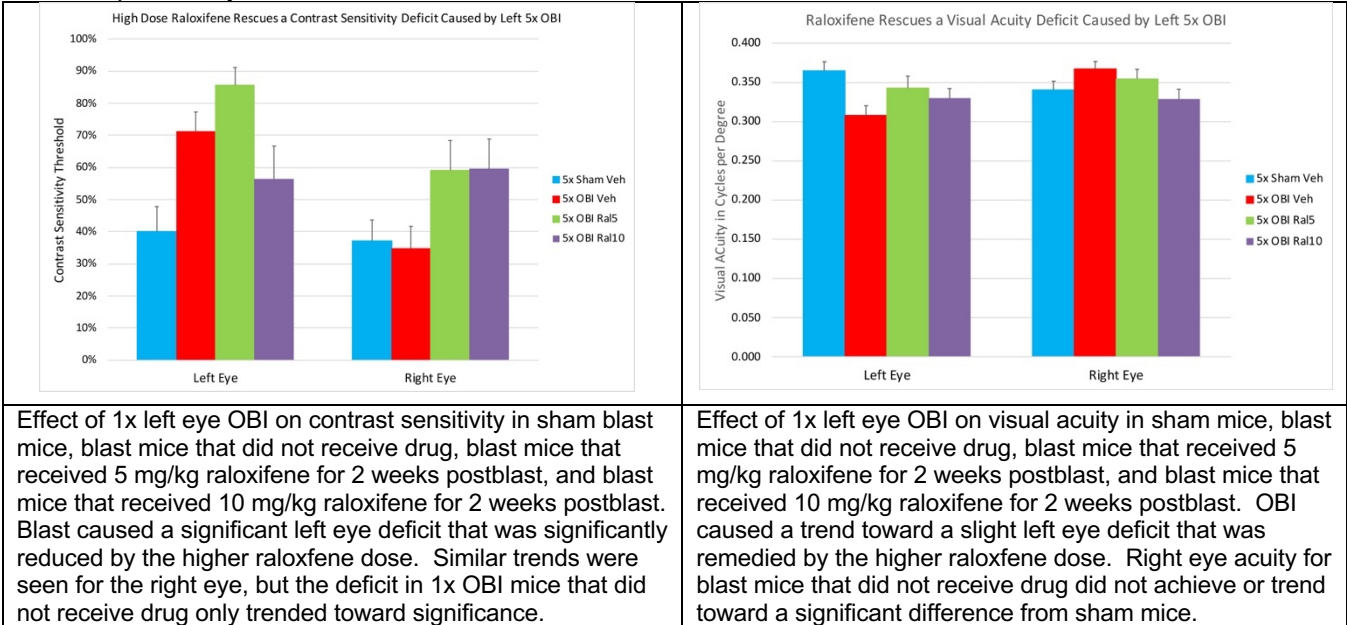
Significant Results or Key Outcomes for Task 3 – Raloxifene Benefit for OBI

Our objective was to show that raloxifene alleviates visual deficits and visual system injury after single 25-psi ocular blast injury (OBI) in mice. In the case of behavioral and physiological assessments, we sought to determine if raloxifene shows a benefit by reducing OBI-induced abnormalities in the following endpoints: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex (PLR). These objectives have been achieved for single blast OBI. We found a small deficit in contrast sensitivity after single 25-psi OBI that was rescued by both raloxifene doses, but no deficit for acuity. We also found no ERG deficit after single 25-psi OBI. At 180 days after single 25-psi OBI, light aversion was evident in the 1x OBI-vehicle mice, and 5 mg/kg raloxifene as well as 10 mg/kg prevented this abnormality. Our results on PLR show slight bilateral hyperconstriction in response to red light that was rectified best by the higher raloxifene dose. Because the functional deficits tended to be small after single blast 25-psi OBI, we also studied mice with 5x 25-psi OBI (one minute between blasts). As discussed below, these mice show more substantial visual deficits than the 1x OBI mice, and we assessed their functional outcomes with and without raloxifene, showing a raloxifene benefit on the same parameters as for 1x OBI. A manuscript currently in preparation on our 1x and 5x OBI findings.

1. Honig MG, Del Mar NA, Henderson DL, O’Neal D, Cox R, Li C, Perry AM, Moore BM, Reiner A. 2021. Rescue of visual deficits and visual system pathology after single and repeat ocular blast injury via the cannabinoid type-2 receptor inverse agonism of raloxifene. In prep.

Subtask 3-1A: Functional and Morphological Studies of 1x OBI

3-1A-1. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after 1x OBI. In an initial cohort of 5 male BL6 mice, we assessed deficits in contrast sensitivity threshold and visual acuity after left eye 1x OBI at 18-psi using OptoMotry about one month after OBI, and compared the outcome to the results we had previously obtained for sham blast TBI. We found that left eye OBI at 18-psi caused a significant left eye contrast sensitivity deficit, but not in the non-blasted right eye. The left eye deficit was lesser than we had seen with blast TBI (Guley et al. Exp Eye Res 2019, PMID: 30922891; Honig et al. Exp Neurol 2019, PMID: 31518568). We then assessed raloxifene benefit in an additional 14 1x OBI sham mice, 13 left eye 1x OBI mice treated with vehicle, 12 left eye 1x OBI mice treated with 5 mg/kg raloxifene, and 15 left eye 1x OBI mice treated with 10 mg/kg raloxifene. For these mice, we used 25-psi left eye blast, since we wanted to further enhance the occurrence of blast-related deficits.



We found similar results in the 1x OBI-vehicle group as with 18-psi left eye 1x OBI, and thus combined the 18-psi mice with the 25-psi vehicle mice for the blast 1x OBI control group (14 mice total) (see

above graphs). We observed a significant contrast sensitivity deficit for the left eye in the 1x OBI group with no raloxifene compared to the sham OBI mice. Raloxifene at 10 mg/kg rescued the 1x OBI deficit, as the left eye contrast sensitivity did not differ between sham and OBI mice with raloxifene at 10 mg/kg, but did differ significantly between 1x OBI mice with vehicle and those with raloxifene at 10 mg/kg. The lower dose of raloxifene also exhibited some rescue, as the left eye contrast sensitivity deficit also did not differ significantly from sham. Thus, raloxifene does rescue the left eye 1x OBI contrast sensitivity deficit, at 10 mg/kg. Unlike with left side 50-psi focal cranial blast TBI, we did not see a significant right eye deficit for contrast sensitivity with left eye 1x OBI, although there was perhaps a slight trend in that direction. This is in keeping with the apparent tendency of inflammation to spread between eyes (Honig et al. Exp Neurol 2019, PMID: 31518568). Both raloxifene doses ameliorated this right eye tendency toward worsening.

In the same left 1x OBI mice, we did not find a significant worsening of left eye acuity (see above right graph), comparing the blast left eyes in mice that did not receive drug to sham eyes, although there was a weak trend in that direction. A similar trend toward worsening of acuity was seen for blast left eyes in mice treated with 5 mg/kg raloxifene, but not 10 mg/kg raloxifene. Thus, there was a possible slight trend toward a left eye acuity deficit in left 1x OBI mice, and a trend toward its rescue with the higher raloxifene dose. For the right eyes, no significant difference or trend toward such was seen between sham eyes and blast no-drug eyes in visual acuity. Similarly, visual acuity in right eyes from blast mice treated with raloxifene also showed no significant difference sham, or any difference from blast no-drug. Thus, 1x left eye OBI did not yield a right eye acuity deficit.

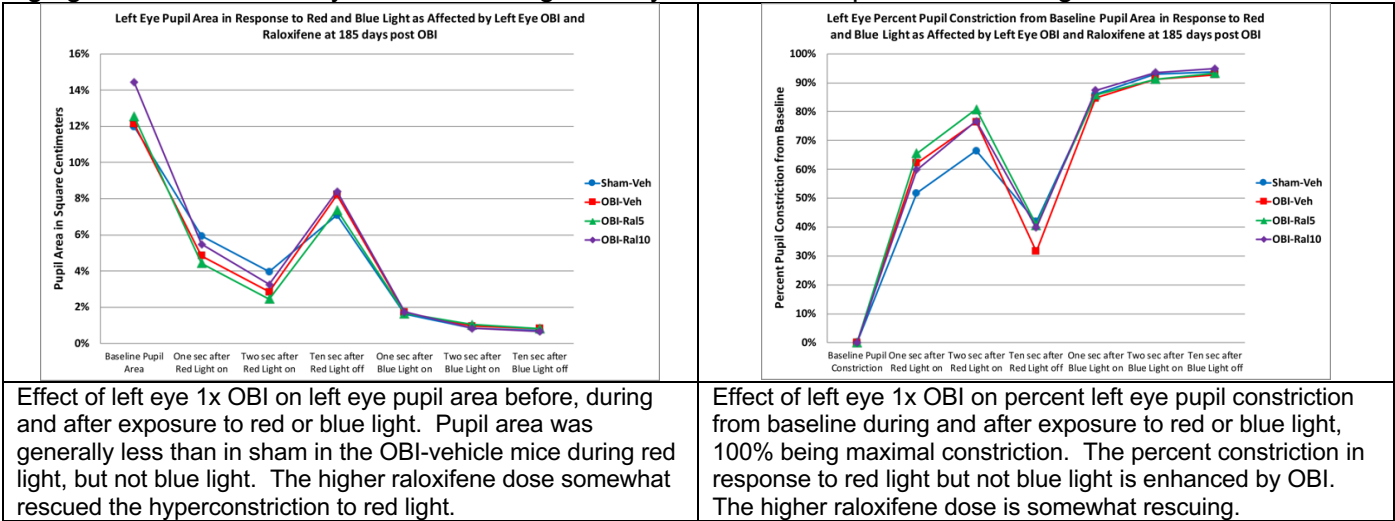
3-1A-2. Raloxifene Benefit – Scotopic Light-adapted ERG A-wave and B-wave after 1x OBI. As part of evaluating raloxifene efficacy in mitigating visual system injury after left eye 1x 25-psi OBI, we measured the A-wave and B-wave amplitudes for the flash-evoked scotopic ERG at about 5 weeks after OBI. We analyzed 9 sham mice, 8 1x OBI mice that received vehicle, 5 1x OBI mice that received 5 mg/kg raloxifene, and 3 1x OBI mice that received 10 mg/kg raloxifene. To assess OBI-induced deficits and rescue with raloxifene, we compared left and right eyes for each animal, and compared the left-eye right eye difference across groups. Our expectation was that if left eye OBI produced an ERG defect, it should be apparent as a right eye – left eye difference. We chose this strategy for analysis because Optomotry for contrast sensitivity and acuity had shown that any functional deficits were clear in the left eye but were absent in the right eye. There were no noteworthy or significant differences between the 3 groups of mice with OBI (i.e. 25psi-Veh, 25psi-Raloxifene 5mg/kg, and 25psi-Raloxifene 10mg/kg) for the A-wave, nor were any of the groups (including the OBI-vehicle mice) significantly different from sham for the A-wave. Similarly, for the B-wave, there were no noteworthy or significant differences between the 3 groups of mice with 1x OBI, nor were any of the groups (including the OBI-vehicle mice) different from sham. In all cases, the difference from sham was insignificant - less than 20 microvolts. Thus, 1x left eye OBI did not produce an ERG deficit.

3-1A-3. Raloxifene Benefit – Light Sensitivity after 1x OBI. We assessed light aversion at several different times after blast in sham mice receiving vehicle, 1x OBI mice receiving vehicle, and 1x OBI mice receiving either of the two doses of raloxifene. At 95 days post-OBI, the sham-vehicle mice spent significantly more time in the enclosed box at 0 lux than the 1x OBI mice that received 5 mg/kg raloxifene or 10 mg/kg raloxifene, but the sham-vehicle and OBI-vehicle mice did not differ. Moreover, the 1x OBI-vehicle mice spent significantly more time in the enclosed box at 0 lux than the 1x OBI mice that received 5 mg/kg raloxifene or 10 mg/kg raloxifene. Since enclosed box occupancy at 0 lux reflects anxiety, these results suggest that although 1x OBI itself did not increase anxiety, raloxifene reduced it. We next evaluated the effects of increasing illumination in the enclosed box to assess light aversion at 95 days post 1x OBI. The results show that OBI-vehicle mice showed significantly greater light aversion than sham-vehicle mice. This was rescued by both raloxifene doses, which decreased light avoidance to even less than in sham-vehicle mice. The difference between 1x OBI-raloxifene5 mice and sham-vehicle mice was highly significant, as was the difference between 1x OBI-raloxifene5 mice and 1x OBI-vehicle mice. The 1x OBI-raloxifene10 mice also showed significantly less light aversion than the sham-vehicle and also showed significantly less light aversion than OBI-vehicle mice. These results suggest that raloxifene might help alleviate light aversion in the first months after 1x OBI.

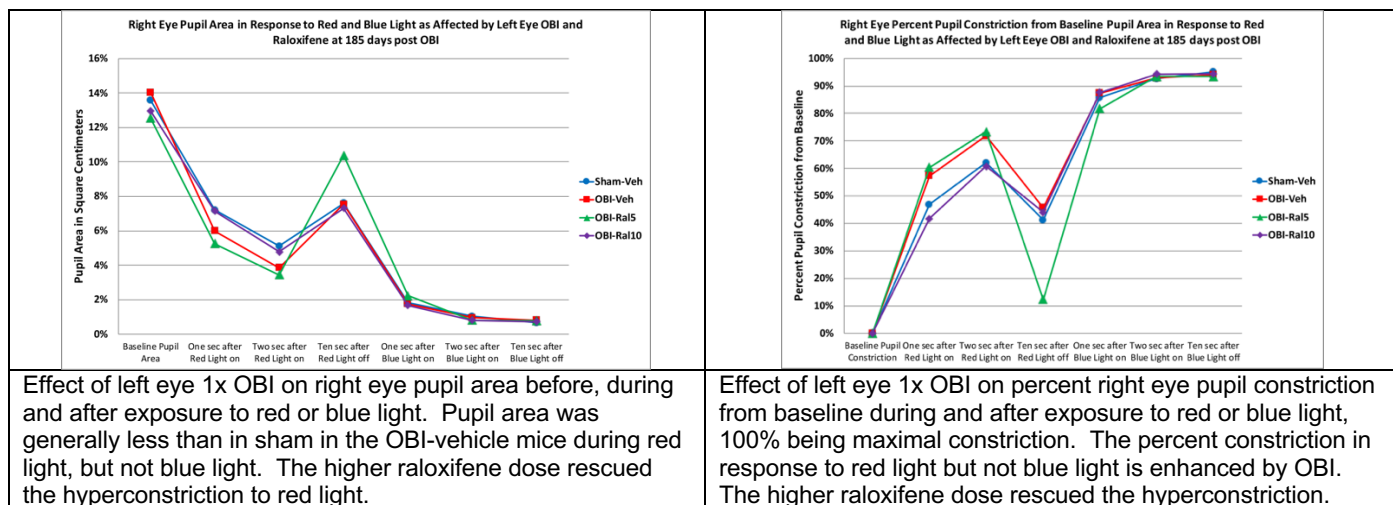
We also evaluated anxiety and light aversion at 180 days post blast in the same mice as at the 95-day time point post 1x OBI. By 180 days post 1x OBI, the anxiolytic effect of raloxifene was not evident, since the groups were no longer distinguishable in the percent of the time they spent in the enclosed box at 0 lux – all were around 55% occupancy. The findings for light aversion also differed from those at 95 days. By 180 days, light aversion remained evident in the 1x OBI-vehicle mice, as they avoided the lighted box as its illumination increased significantly more than did sham-vehicle mice. Raloxifene prevented the light aversion caused by 1x OBI, as light avoidance for the 5 mg/kg dose was not significantly different from sham. Similarly, light avoidance for the 10 mg/kg dose was not significantly different from sham. Moreover, light avoidance was significantly less than in the 1x OBI-vehicle mice for both the 1x OBI mice treated with 5 mg/kg raloxifene, as well as those treated with 10 mg/kg. Thus, 1x OBI caused a long-term effect that yielded light aversion long after the injury, and raloxifene treatment for the two weeks after injury prevented this.

3-1A-4. Raloxifene Benefit – Pupil Light Reflex after 1x OBI. These studies were carried out at ~26 weeks after 1x OBI, and in total involved analysis of both eyes in 14 sham-vehicle mice, 14 1x OBI-vehicle mice, 15 1x OBI-raloxifene5 mice, and 15 1x OBI-raloxifene10 mice. Surprisingly, left eye 1x OBI affected pupil responses in both eyes in a similar way, with no effect on resting pupil diameter, but somewhat enhancing percent pupil constriction to red but not blue light. The enhanced response to red light was more prominent for the right eye and was rescued with the raloxifene.

The figure below left shows pupil area for the left eye. We found that the resting pupil area of the left eye before the red light stimulus was not significantly different from sham for the 1x OBI-vehicle, the 1x OBI-raloxifene5, or the 1x OBI-raloxifene10 mice, although the pupil trended toward being slightly more dilated in 1x OBI-raloxifene10 mice than sham mice. During red light exposure, the constricted pupil size was largely similar for all groups, but there was a trend toward increased constriction from baseline compared to sham in the 1x OBI-vehicle mice. After red light off but before blue light on, neither absolute pupil size nor percent constriction from pre red-light baseline (Below right) differed between the groups for the left eye. In the case of the blue light response, both during and after blue light off, 1x OBI-vehicle mice did not differ significantly in absolute pupil size or percent constriction compared to shams, nor did the 1x OBI mice treated with the higher dose of raloxifene. The mice receiving the lower dose of raloxifene, however, had a significantly reduced left eye constriction to blue light, both in terms of absolute pupil area and in terms of percent constriction from pre red-light baseline. As the diminished constriction to blue light caused by 5 mg/kg raloxifene for the left eye is a small effect, and less than the rescue of the slight 1x OBI-induced left eye hyperconstriction to red light, the action of 5 mg/kg raloxifene on left eye PLR after single left eye OBI is more positive than negative.



The figure below left shows the resting pupil area of the right eye. The results show that before the red light stimulus, resting pupil area of the right eye was not significantly different from sham for the 1x OBI-vehicle, 1x OBI-raloxifene5, or 1x OBI-raloxifene10 mice. Thus, the resting pupil size of the right eye was not significantly affected by left eye 1x OBI in any group. During red light exposure, the right eye



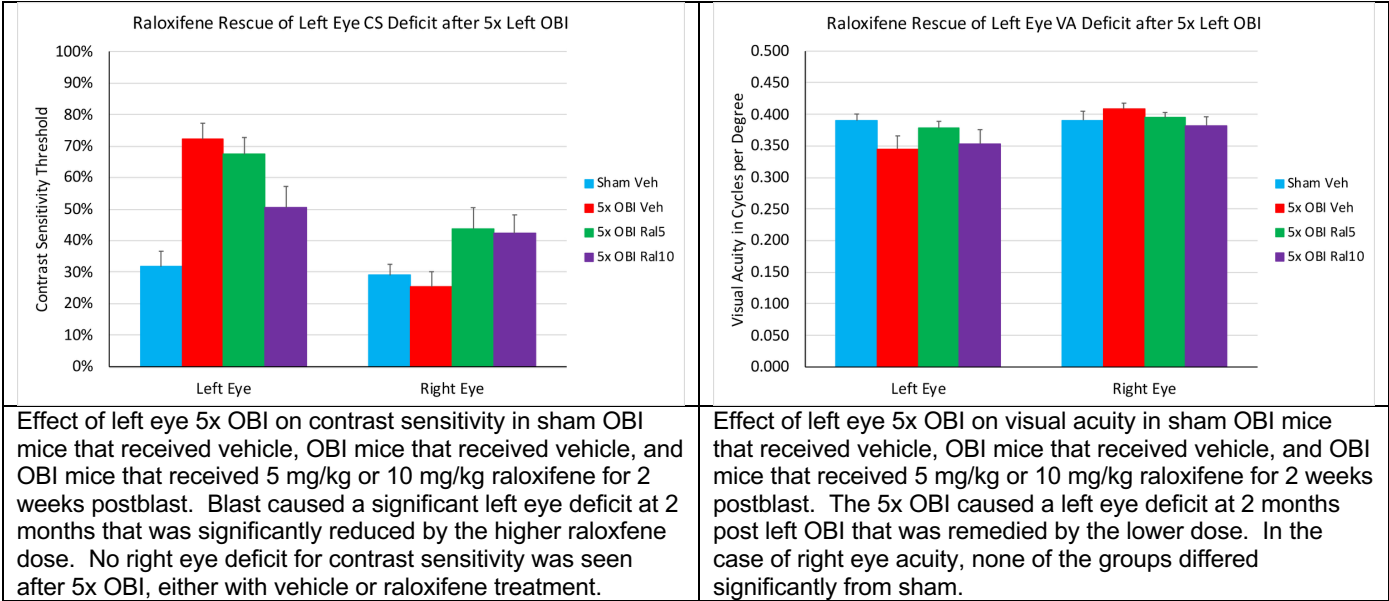
pupil in the 1x OBI-vehicle mice had a significantly higher percent constriction from baseline than did right pupil in sham mice. Percent constriction from baseline, however, did not differ from sham for either the OBI-raloxifene5, or OBI-raloxifene10 mice during red light. Thus, there was a right eye hyperconstriction to red light caused by left eye 1x OBI, and this was rescued by both raloxifene doses. In the case of the right eye response to blue light, percent constriction from baseline was not significantly different from sham for the 1x OBI-vehicle, 1x OBI-raloxifene5, or 1x OBI-raloxifene10 mice, as shown in the above graph to the right. Thus, left eye 1x OBI augments constriction of the right pupil to red light but not blue light, and both doses of raloxifene rescue the red light hyperconstriction.

3-1A-5. Raloxifene Benefit in Mice after 1x OBI – Optic Nerve. We observed significant 17% loss of left optic nerve axons by 7 months after 1x left OBI and vehicle treatment, compared to sham mice with vehicle treatment. Raloxifene at the lower dose did not rescue the loss, but did at the higher dose. The left optic nerve axon abundance across groups was slightly inversely correlated with the contrast sensitivity threshold ($r=-0.327$). We examined right optic nerve in several mice with 1x left OBI and vehicle treatment, and found axon abundance to be indistinguishable from the left eye in sham mice in the present study, and from left and right eye in sham mice in our prior studies on blast TBI. Thus, 1x left OBI does yield discernible axon loss in left optic nerve, and the higher raloxifene dose rescued it.

Subtask 3-1B: Functional and Morphological Studies of 5x OBI

3-1B-1A. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after 5x OBI. Because the functional deficits tended to be small after 1x OBI, we also studied mice with 5x 25-psi OBI (1 minute interblast interval), which in the case of OptoMotry assessment included 14 sham 5x OBI mice treated with vehicle, 22 5x OBI mice treated with vehicle, 15 5x OBI mice treated with 5 mg/kg raloxifene, and 17 5x OBI mice treated with 10 mg/kg raloxifene. We found at 2 months after blast that 5x 25-psi OBI caused a substantial and significant left eye contrast sensitivity deficit (see below left graph) and a significant left eye visual acuity deficit (see below right graph), but no deficit for either contrast sensitivity or acuity was seen in the right eye of 5x 25-psi OBI mice. Raloxifene at 5 mg/kg did not rescue the left eye contrast sensitivity deficit, since it remained significantly worse than in sham, and not significantly different from 5x OBI-vehicle. By contrast, raloxifene at 10 mg/kg did yield rescue, as the left eye contrast sensitivity threshold was less significantly different from sham and significantly better than in 5x OBI-vehicle mice. Both doses of raloxifene rescued the left eye acuity deficit from 5x OBI. Thus, 5x OBI does produce significant deficits in contrast sensitivity and visual acuity in the left eye at 2 months after blast, but not the right eye, and these left eye deficits were rescued by raloxifene. We re-examined the deficits and raloxifene rescue at 3 months post blast in a subset of the mice, to assess the endurance of the OBI deficits and raloxifene rescue. As at 2 months post 5x OBI, a significant left eye deficit in contrast sensitivity was seen compared to sham mice. Although left eye visual acuity at 3 months only trended toward being less than sham, a significant left eye deficit in visual acuity was evident by a paired t-test with the right eye in 5x OBI-vehicle mice. As at 2 months, no right eye deficit was seen in 5x OBI-vehicle mice at 3 months. Also as at 2 months, the 10 mg/kg

dose but not the 5 mg/kg dose rescued the contrast sensitivity deficit in the left eye, since for the lower dose contrast sensitivity remained significantly worse than in sham and not significantly different from 5x OBI-vehicle, and for the higher dose it was not significantly different from sham and significantly better than in 5x OBI-vehicle mice. Both doses rescued the left eye acuity deficit. Thus, the deficits in the untreated mice persisted and the rescue effects persisted at 3 months as well, with the higher dose of raloxifene needed to rescue both deficits.

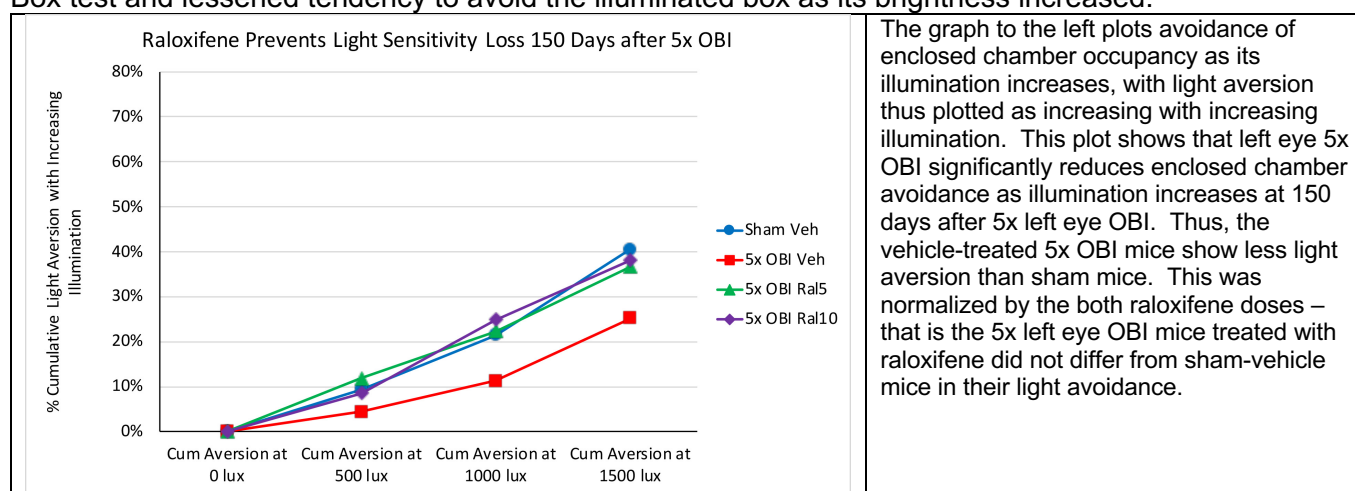


3-1B-1B. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after 5x OBI - Effect of Gender and Age. Although the majority of the mice used in the 5x OBI study were males and were blasted around 3 months of age, one set of contemporaneously blasted mice included some females and some mice of both genders in which blast was performed at 6 months of age. Although only a few of the total mice were females and only a few were 6 months old at time of blast, this nonetheless allowed us to assess the impact of gender and age on the outcome from 5x OBI and raloxifene treatment. For the males and females created and tested contemporaneously, we found that the magnitude of the contrast sensitivity deficit after 5x OBI-vehicle compared to sham was similar in males and females, as was the rescue with raloxifene. For these mice, however, the visual acuity deficit was greater in female than male 5x OBI-vehicle mice. Moreover, the rescue with the lower raloxifene dose was greater in females for both contrast sensitivity and visual acuity than it was in males. Thus, while there may be some gender differences, 5x OBI is injurious to the visual system in males and females, and raloxifene rescues in both. No deficits were seen for either gender in the right eye for contrast sensitivity or visual acuity. We also evaluated the effect of blast age on the outcome in the same set of mice. The left eye contrast sensitivity deficit in the 5x OBI-vehicle mice compared to sham mice was similarly large in the 3-month blast age group and 6-month blast age group, as was the rescue with higher raloxifene dose. The lower dose was, however, much more effective at the older blast age. At neither age did left eye 5x OBI yield a contrast sensitivity deficit in the right eye. In the case of visual acuity, the left eye deficit after left eye 5x OBI-vehicle was greater at the younger blast age.

3-1B-2. Raloxifene Benefit – Scotopic Light-adapted ERG A-wave and B-wave after 5x OBI. Dark-adapted full-field ERGs were recorded for the 5x OBI mice about 5 months after blast. We found that left eye 5x OBI yielded significant reductions in both the A-wave and B-wave peak amplitudes for the left eye compared to sham left eye. The A-wave was significantly reduced at the brighter light intensities for the left eye of 5x left OBI mice, while the B-wave was significantly reduced across all light intensities for the left eye of 5x left OBI mice. Both doses of raloxifene provided significant but incomplete rescue of the A-wave deficit, with amplitudes for the lower dose and the higher dose significantly better than in sham, although a significant deficit compared to sham, nonetheless, remained for the lower and higher dose. Neither dose of raloxifene rescued the left eye B-wave deficit,

as the B-wave remained significantly lower than in sham left eye across all light intensities at both raloxifene doses, and for neither dose was it significantly better than in the 5x left OBI-vehicle mice.

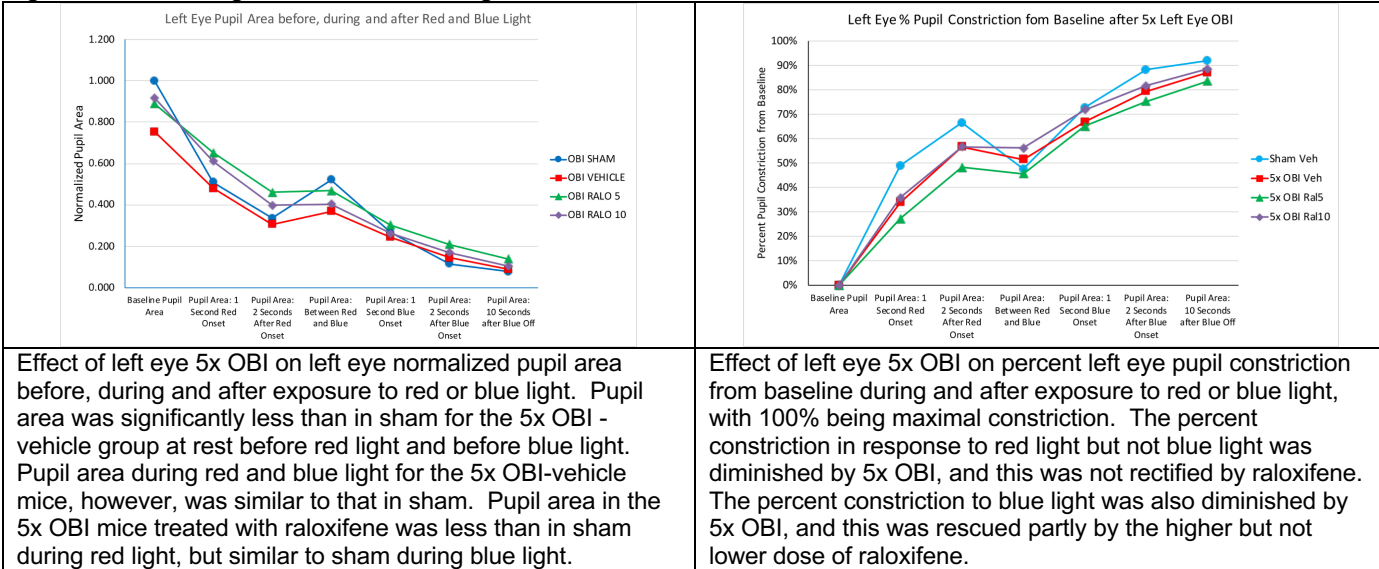
3-1B-3. Raloxifene Benefit – Light Sensitivity after 5x OBI. We evaluated light aversion for the 5x 25-psi OBI mice at 150 days after left eye 5x OBI. As shown below, we found that light sensitivity was significantly less in the 5x OBI mice treated with vehicle than in sham mice – the 5x OBI mice treated with vehicle spent more time in the light box as its illumination increased. The 5x OBI mice treated with either raloxifene dose did not differ significantly from sham 5x OBI mice in light aversion but did differ from 5x OBI-vehicle mice, thus showing rescue of the light insensitivity caused by 5x OBI. For example, 5x left OBI mice with the lower raloxifene dose did not differ from sham, but differed significantly from 5x OBI-vehicle mice. Similarly, 5x left OBI mice with the higher raloxifene dose also did not differ from sham, but differed significantly from 5x OBI-vehicle mice. As the 5x OBI-vehicle mice had shown a substantial left eye contrast sensitivity deficit and an ERG A-wave deficit over the same time frame, it seems likely that the reduced light aversion seen in our Light/Dark Box test reflects the visual disability caused by the left 5x OBI, manifesting in this case as an insensitivity in the Light/Dark Box test and lessened tendency to avoid the illuminated box as its brightness increased.



3-1B-4. Raloxifene Benefit – Pupil Light Reflex after 5x OBI. We analyzed the pupil light reflex at 6 months post blast in the 5x OBI mice for which we also had previously analyzed visual acuity, contrast sensitivity, the ERG, and light aversion (9 sham, 12 5x OBI-vehicle, 10 5x OBI-ralo5, and 9 5x OBI-ralo10). We found that left eye 5x OBI in vehicle-treated mice had a significant impact on the resting pupil size prior to red light exposure and after red light exposure in the left but not right eye (see below left graph), with resting pupil size in left eye of 5x left OBI mice prior to red light at 75.4% of sham-vehicle left eye and after red light at 70.7% of sham left eye. By contrast, the resting pupil size in left eye of 5x OBI-raloxifene5 mice and 5x OBI-raloxifene10 mice was not significantly different than in sham-vehicle left eye, although there was a trend toward a reduction for the higher dose. In the case of the right eye 6 months after 5x left OBI, 5x OBI-vehicle mice did not differ from sham either prior to red light (or after red light, although a trend toward reduction was seen for the latter). The right eye for 5x OBI-ralo5 mice also did not differ from sham prior to red light or after red light. The right eye pupil area for 5x OBI-ralo10 mice was, however, significantly less than in sham prior to red light but not after. Thus, 5x OBI caused the blasted left eye but not the non-blasted right eye to be significantly more constricted in its resting state than sham.

The constricted state of the left pupil in 5x OBI – vehicle mice diminished the magnitude of the percent constriction to red and blue light, although the absolute pupil size achieved in 5x OBI – vehicle mice was the same as in sham – vehicle mice. This can be noted in the graph below left, in which pupil area in the left eye of 5x OBI – vehicle mice was less than in sham at rest (i.e. before red light) and after red light (but before blue light), but no different than in sham during red light and during blue light and the

10 minutes afterward. Thus, the left pupils of the 5x OBI – vehicle mice could reach a state of constriction that was no different than in sham during light stimulation. They could not, however, reach a normal state of dilation in the dark, and as a result their percent constriction from baseline was clearly and significantly less than in sham during red light and blue light (see graph below right). Note that although the left eyes in the raloxifene-treated 5x OBI mice were comparable to sham in the dark (i.e. at rest), their left eyes did not constrict to the same extent as in sham, and thus their percent constriction from baseline was less than in sham during both red light and blue light. In the case of the higher raloxifene dose, the left eyes were comparable to sham in the dark (i.e. at rest), but their left eyes did not constrict to the same extent as in sham during red light but did during blue light, and thus their percent constriction from baseline was significantly less than in sham during red light but not blue light. In summary, 5x left OBI caused a slightly constricted left pupil at rest but the magnitude of the constriction achieved during red and blue light was similar to that in sham. Raloxifene rectified the resting pupil defect, but the lower dose yielded deficient constriction during red and blue light, and the higher dose during red but not blue light.



The right eye pupil response after left eye 5x OBI was also altered from sham. As noted above, for the right eye 6 months after left eye 5x OBI, neither 5x OBI-vehicle nor 5x OBI-raloxifene5 differed significantly from sham-vehicle mice in resting pupil area prior to any light stimulation. This was also largely true for the period between red and blue light presentations, during which the right pupil in the 5x OBI-vehicle mice trended toward being more constricted (smaller area) than in sham mice. This was a general pattern for the right eye in 5x OBI-vehicle mice, which tended to be more constricted than in sham even during red and blue light presentation, as was also true of the 5x OBI mice treated with the higher raloxifene dose. It thus appears to be the case that 5x left eye OBI has an effect on the pupil response of the right eye. This was also evident in the percent constriction for the right eye in the 5x OBI – vehicle mice during red and blue light, which was slightly but not significantly greater than in sham during both red and blue light. The percent constriction for the right eye in the 5x OBI was no different than in sham during red and blue light with both raloxifene doses.

3-1B-5. Raloxifene Benefit in Mice with 5x OBI – Optic Nerve. We observed a significant 15% loss of left optic nerve axons by 11 months after 5x left OBI and vehicle treatment, compared to sham mice. We examined right optic nerve in two mice with 5x left OBI and vehicle treatment, and found the axon abundance to be indistinguishable from the left eye in sham mice in the present study, and from left and right eye in sham mice in our prior studies (Honig et al. Exp Neurol 2019, PMID: 31518568). Both doses of raloxifene partially rescued the left optic nerve axon loss, the higher dose somewhat more so. For example, in 5x OBI-ral5 mice, left nerve axon abundance was 90.6% of sham, which did not differ significantly from sham. Similarly, in 5x OBI-ral10 mice, left nerve axon abundance was 92.7% of sham, which did not differ significantly from sham. Axon abundance in left optic nerves across the four groups was significantly inversely correlated with contrast sensitivity threshold ($r=-0.4293$), meaning the fewer the axons the higher the threshold and the worse contrast sensitivity.

Subtask 3-2: Neurochemical Studies of Microglial Modulation by Raloxifene in Impact TBI

3-2A. Raloxifene Benefit in Mice with 5x OBI – Biochemical Studies. To assess if blast shifts microglia toward expression of M1 markers, and raloxifene shifts such blast-affected microglia toward M2 marker expression, qPCR was used to determine the expression of M0, M1, and M2 markers in left optic nerve, left retina, and right thalamus, using the same approach as for our published study on raloxifene benefit for blast TBI (Honig et al. Exp Neurol 2019, PMID: 31518568). In the left optic nerve, all five of the M1 markers increased with 5x OBI-vehicle when compared to sham. With raloxifene treatment (10 mg/kg), all five of these M1 markers decreased to below 5x OBI-vehicle levels but still above sham levels. The mean for the M2 markers in the left optic nerve showed an unexpected increase in 5x OBI-vehicle mice due to greatly elevated expression of Arg-1 and YM1. Raloxifene treatment (10 mg/kg) decreased the expression of Arg-1 and YM1 substantially below the levels seen in the 5x OBI-vehicle mice, but increased the expression of the other two M2 markers, Trem2 and IL10, above that seen in the 5x OBI-vehicle mice. In the left retina, 5x OBI-vehicle increased the expression of all five M1 markers compared to sham while raloxifene treatment reduced iNOS and CD32 to below 5x OBI-vehicle but not sham levels, and IFN γ and TNF α were even reduced to below sham levels. Mean M2 expression in the left retina was also increased by 5x OBI-vehicle and further increased with raloxifene treatment after 5x OBI, but this raloxifene effect was driven by a large increase in IL10 expression. Raloxifene actually decreased expression of Arg-1, YM1, and Trem2 below the levels in 5x OBI-vehicle mice. In the right thalamus, three of the four markers were increased by 5x OBI (not P2ry12), and all four were reduced below sham levels by raloxifene. M1 expression in right thalamus was increased by 5x OBI-vehicle for four out of five markers (all except IFN γ). With raloxifene treatment, the M1 expression was decreased below 5x OBI-vehicle for all markers except IFN γ . The M2 marker mean for the right thalamus was slightly decreased with 5x OBI-vehicle but increased with 10 mg/kg raloxifene treatment, an effect driven by YM1 and IL10 increases. When comparing the marker expression as an M1:M2 ratio, the left retina and right thalamus behave similarly. In both, 5x OBI-vehicle caused a ratio favoring the M1 state, and that ratio was then reduced to a ratio favoring the M2 state with 10 mg/kg raloxifene treatment. The left optic nerve favors the M2 state in both the OBI-vehicle group and the OBI-raloxifene treated group due to the unexpected increase in M2 expression in 5x OBI-vehicle mice driven by the large increases in Arg-1 and YM1. Excluding these from the ratio for all groups for optic nerve yields an M1/M2 ratio that shows relative M1 elevation with 5x OBI-vehicle and relative M2 favoring with 5x OBI-raloxifene.

Task 1: What opportunities for training and professional development has the project provided?

The project was not intended to provide training and professional development opportunities, and so there is Nothing to Report.

Task 2: What opportunities for training and professional development has the project provided?

The project was not intended to provide training and professional development opportunities, and so there is Nothing to Report.

Task 3: What opportunities for training and professional development has the project provided?

The project was not intended to provide training and professional development opportunities, and so there is Nothing to Report.

Task 1: How were the results disseminated to communities of interest? We published a manuscript in Experimental Neurology based on our findings on raloxifene benefit in blast TBI, and presented these findings at an In-Progress Review at Fort Detrick, MD for the Clinical and Rehabilitative Medicine Research Program of the DOD on 10/29/2019. The presentation was entitled: CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma. This presentation also reported on raloxifene benefit following impact TBI and following ocular blast injury (OBI). We were also invited to submit a summary of our progress on blast TBI for the FY19 and FY20 Blast Injury Research Coordinating Office Blast Report by the US Army. We also presented our findings on this topic at the 2019 Annual Meeting of the Association for Research in Vision and Ophthalmology (Abstract # 4405).

Task 2: How were the results disseminated to communities of interest? We presented our findings on raloxifene benefit in impact TBI as well at the In-Progress Review at Fort Detrick, MD for the Clinical and Rehabilitative Medicine Research Program of the DOD on 10/29/2019. We are preparing our findings on impact TBI for publication, and presented them at the 2020 Annual Meeting of the Association for Research in Vision and Ophthalmology (Abstract # 689).

Task 3: How were the results disseminated to communities of interest? We are preparing our findings on OBI for publication.

Task 1: What do you plan to do during the next reporting period to accomplish the goals?

This is the final report, so there is nothing to report in this regard.

Task 2: What do you plan to do during the next reporting period to accomplish the goals?

This is the final report, so there is nothing to report in this regard.

Task 3: What do you plan to do during the next reporting period to accomplish the goals?

This is the final report, so there is nothing to report in this regard.

4. Impact

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

Due to our evidence for a robust benefit of raloxifene in mitigating visual deficits and anxiety after blast TBI, and mitigating visual deficits after impact TBI, we have initiated planning a randomized phase 2 clinical trial in collaboration with Dr. Martin Croce, trauma surgeon at UTHSC and Medical Director of the Elvis Presley Memorial Trauma Center at Regional One Health and Dr. Marcia Honig, a co-investigator on the current award. A large community of researchers and clinicians at UTHSC is dedicated to the study and treatment of head trauma, and the Elvis Presley Memorial Trauma Center is one of the busiest trauma centers in the country, with about 5,000 annual trauma admissions. Approximately 15% of these blunt injured patients suffer a traumatic brain injury. The PIs of the Phase 2 Clinical Trial (Drs. Reiner, Honig, and Croce) will work with several of Dr. Croce's clinician colleagues in developing the protocols and recruitment strategy for a phase 2 trial of raloxifene efficacy for visual and cognitive deficits after head trauma. We have sought funding to support this effort, which has largely been put on hold due to the Covid19 pandemic.

What was the impact on other disciplines?

Nothing to Report.

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.

There have been no changes in approach, objectives or scope during the reporting period. Thus, Nothing to Report.

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

During the first three months of the project (period 1), delays were encountered for several reasons. First, we could not purchase animals or raloxifene until the grant had started. As a result, the start of animal testing was delayed a few weeks until the receipt of mice (April 4) and raloxifene (March 31). Two members of our research staff (Del Mar and Li) were moved into two of the staff positions on this project, and upon the start of the project the process of hiring to fill the third staff position was initiated

and completed, with Desmond Henderson joining our team on June 7. Additionally, because the first set of mice did not show substantial deficits in contrast sensitivity with 50-psi blast, we needed to devote time to recalibrate our blast system. Although the initial delays slowed the project, the first task is nearly complete. The impact studies of the second year were delayed in their start until June 2017, which was the soonest that our consultant Dr. Rad Tzekov could visit to instruct us on use of the impact TBI system. Progress was also unavoidably slowed because one of the staff conducting these studies, Dr. Nobel Del Mar, suffered a severe left shoulder injury after he slipped and fell on ice in January 2018. This impaired his work performance, and he ultimately went on disability leave and had rotator cuff surgery in early March, and returned in late May 2018. We made up for the resulting delays during the fourth no-cost extension year of the project. Due to the Coronavirus pandemic, we were unable to conduct laboratory research from mid-March to mid-May, 2020. Nonetheless, the project has been completed, and it is now clear that raloxifene treatment begun within 48 hours of brain or eye trauma and continued for 2 weeks greatly mitigates functional deficits and nervous system damage.

Changes that had a significant impact on expenditures.

There have been no changes that have had a significant impact on expenditures. Nothing to Report.

Significant changes in use or care of human subjects.

No human subjects are used. 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.

No biohazards or select agents are used. Nothing to Report.

6. Products

Publications, conference papers, and presentations

Our findings for **Task 1** (Aim 1) were presented at the 2019 ARVO Annual Meeting in May, 2019. Additionally, we published a manuscript in *Experimental Neurology* based on these findings, and presented these findings at an In-Progress Review at Fort Detrick, MD for the Clinical and Rehabilitative Medicine Research Program of the DOD on 10/29/2019. The presentation was entitled: CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma. This presentation also reported on raloxifene benefit following impact TBI and following ocular blast injury (OBI). We were also invited to submit a summary of our progress on blast TBI for the FY19 and FY20 Blast Injury Research Coordinating Office Blast Report by the US Army. The citation information for the ARVO meeting presentation, published paper, and invited summary follow. Additionally, we have also shown in yet unpublished studies that raloxifene reduces depression and fearfulness after blast TBI, using the same simple tests for which we had shown that the novel synthetic CB2 inverse agonist SMM189 also reduces depression and fearfulness after blast TBI (Reiner et al, *Int J Mol Sci* 2015, PMID: 25561230). A manuscript on this is in preparation.

1. Reiner A, Del Mar N, Henderson D, Perry A, Ragsdale T, Doty J, Driver J, Guley N, Mitchell W, Li C, Moore BM, Honig M. 2019. Raloxifene through its cannabinoid type-2 receptor inverse agonism mitigates visual deficits and pathology after mild TBI. *ARVO Abst* # 4405.
2. Honig MG, Del Mar NA, Henderson DL, Ragsdale TD, Doty JB, Driver JH, Li C, Fortugno AP, Mitchell WM, Perry AM, Moore BM, Reiner A. 2019. Amelioration of visual deficits and visual system pathology after mild TBI via the cannabinoid type-2 receptor inverse agonism of raloxifene. *Exp. Neurol.*, 322: 113063, 2019, PMID: 31518568.
3. Reiner, A. 2020. CB2 receptor therapy using the FDA-approved drug raloxifene to mitigate visual deficits after mild TBI and/or ocular trauma. In: FY19 Blast Injury Research Coordinating Office Blast Report. US Army, in press.

4. Reiner, A. 2021. CB2 receptor therapy using the FDA-approved drug raloxifene to mitigate visual deficits after mild TBI and/or ocular trauma. In: FY19 Blast Injury Research Coordinating Office Blast Report. US Army, in press.
5. Honig MG, Del Mar NA, Henderson DL, Moore BM, Reiner A. 2021. Rescue of emotional deficits after focal blast traumatic brain injury via the cannabinoid type-2 receptor inverse agonism of raloxifene. In prep.

The findings for **Task 2** (Aim 2) were presented among the abstracts for the 2020 ARVO Annual Meeting, scheduled for May, 2020. Note that the meeting was canceled due to the Covi-d19 pandemic but the abstracts were nonetheless published. We have also begun drafting a manuscript on these findings. The citation information for the ARVO abstract on the impact TBI work follows. New findings during year 4 are summarized below.

1. Reiner A, Del Mar N, Henderson D, Perry A, Doty J, O'Neal D, Li C, Moore BM, Honig M. 2020. Raloxifene through its cannabinoid type-2 receptor inverse agonism mitigates visual deficits and pathology after impact TBI. ARVO Abst # 689. Invest. Ophthalmol. Vis. Sci., 61(7):689.
2. Honig MG, Del Mar NA, Henderson DL, Doty JB, O'Neal D, Cox R, Li C, Perry AM, Moore BM, Reiner A. 2021. Amelioration of visual deficits and visual system pathology after impact TBI via the cannabinoid type-2 receptor inverse agonism of raloxifene. In prep.

The findings for **Task 3** (Aim 3) will be published in a manuscript currently in preparation.

1. Honig MG, Del Mar NA, Henderson DL, O'Neal D, Cox R, Li C, Perry AM, Moore BM, Reiner A. 2021. Rescue of visual deficits and visual system pathology after single and repeat ocular blast injury via the cannabinoid type-2 receptor inverse agonism of raloxifene. In prep.

Website, Technologies/Techniques, Inventions, Patent Applications, Licenses, and Other Products

Nothing to Report.

7. Participants and other Collaborating Organizations

What individuals have worked on the project?

Name:	Anton Reiner
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	1.2
Contribution to Project:	Dr. Reiner has overseen the research and interpreted results.

Name:	Marcia Honig
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	1.2
Contribution to Project:	Dr. Honig has aided in overseeing the research and interpreting results.

Name:	Bob Moore
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	0.6
Contribution to Project:	Dr. Moore formulated the raloxifene for in vivo testing in mice.

Name:	Nobel Del Mar
Project Role:	Technical Director
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	5.4
Contribution to Project:	Dr. Del Mar performed the TBI/OBI and animal testing.

Name:	Chunyan Li
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	5.4
Contribution to Project:	Dr. Li animal testing and morphology.

Name:	Desmond Henderson
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	6.0
Contribution to Project:	Mr. Henderson performed TBI and animal testing.

Name:	Rachel Cox
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	2.0
Contribution to Project:	Rachel performed TBI and animal testing.

PR Name:	Hongbing Wang
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2
Contribution to Project:	Dr. Wang analyzed microglia biochemistry.

Name:	Aaron Perry
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month per year worked:	2.2
Contribution to Project:	Aaron analyzed microglia biochemistry.

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: The current award is not a collaborative award, so there is Nothing to Report.

QUAD CHARTS: An updated Quad Chart is attached to this submission.

9. Appendices

1. Honig et al. Exp Neurol 2019, PMID 31518568 is attached.

CB2 Receptor Therapy Using the FDA-approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma

Funding Opportunity Number: W81XWH-14-CRMRP-NSRRA



PI: Anton Reiner

Org: University of Tennessee Health Science Center

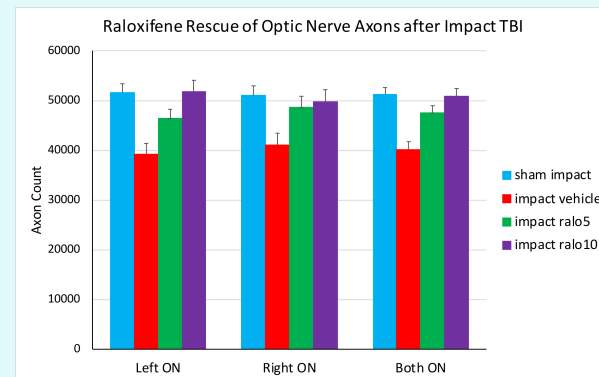
Requested Amount: \$1,346,882

Study Aim

- Visual disabilities after brain and/or closed-globe ocular trauma from blast injury are highly common in military personnel, but effective treatments are lacking. We have found that early post-injury treatment with CB2 cannabinoid receptor inverse agonists mitigates visual impairments caused by mild TBI in a preclinical model.
- We hypothesize that the recently discovered CB2 receptor inverse agonism of raloxifene, which is FDA approved for treatment of osteoporosis, will make it effective for mitigating visual disabilities after brain and/or closed-globe ocular trauma. We will test its effectiveness in doing so in preclinical models.

Approach

We test the efficacy of raloxifene for reducing retinal and optic nerve damage and visual deficits stemming from mild TBI in two different standardized mouse models and in closed-globe ocular injury in mice.



- Example of raloxifene benefit in impact TBI benefit: Rescue of ~20% optic nerve axon loss after dorsal midline cranial impact TBI.

Accomplishment: We found that raloxifene administered daily after left side cranial blast TBI, dorsal cranial midline impact TBI, or left eye ocular blast injury in mice reduces visual deficits and visual system pathology.

Timeline and Cost

Activities	CY16	CY17	CY18	CY19
Confirm visual benefit of raloxifene in blast mild TBI				
Confirm visual benefit of raloxifene in impact mild TBI				
Confirm visual benefit of raloxifene in ocular blast injury				
Estimated Total Budget (\$K)	Dir: \$285K ID: \$146K	Dir: \$305K ID: \$153K	Dir: \$301K ID: \$157K	NCE

Goals

- **Study 1 (CY16-17):** Confirm effectiveness of the CB2 receptor inverse agonism of raloxifene in mitigating visual deficits in blast model of mild TBI in mouse. Refine dose and timing of treatment, and perform mechanistic studies to demonstrate role of microglial modulation in benefit. This Aim is complete.
- **Study 2 (CY17-19):** Confirm effectiveness of raloxifene in mitigating visual deficits in dorsal cranial impact model of mild TBI in mice, and role of microglial modulation in benefit. This Aim is 100% complete.
- **Study 3 (CY18-20):** Confirm effectiveness of raloxifene in mitigating visual deficits in models of 1x or 5x ocular blast injury in mice, and role of microglial modulation in benefit. This study is 100% complete.

Comments/Challenges/Issues/Concerns

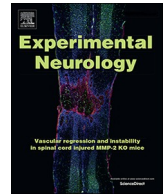
- None.

Budget Expenditure to Date

Projected Expenditure: \$888,877 Direct Cost

Actual Expenditure: \$888,877 Direct Cost

Updated: (12/29/2020)



Research paper

Amelioration of visual deficits and visual system pathology after mild TBI via the cannabinoid Type-2 receptor inverse agonism of raloxifene

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ARTICLE INFO

Keywords:

TBI
Visual deficits
Microglia
CB2 receptors
Therapy

ABSTRACT

Visual deficits after traumatic brain injury (TBI) are common, but interventions that limit the post-trauma impairments have not been identified. We have found that treatment with the cannabinoid type-2 receptor (CB2) inverse agonist SMM-189 for 2 weeks after closed-head blast TBI greatly attenuates the visual deficits and retinal pathology this otherwise produces in mice, by modulating the deleterious role of microglia in the injury process after trauma. SMM-189, however, has not yet been approved for human use. Raloxifene is an FDA-approved estrogen receptor drug that is used to treat osteoporosis, but it was recently found to also show noteworthy CB2 inverse agonism. In the current studies, we found that a high pressure air blast in the absence of raloxifene treatment yields deficits in visual acuity and contrast sensitivity, reductions in the A-wave and B-wave of the scotopic electroretinogram (ERG), light aversion, and increased pupil constriction to light. Raloxifene delivered daily for two weeks after blast at 5–10 mg/kg mitigates or eliminates these abnormalities (with the higher dose generally more effective). This functional rescue with raloxifene is accompanied by a biasing of microglia from the harmful M1 to the helpful M2 state, and reductions in retinal, optic nerve, and oculomotor nucleus pathology. We also found that raloxifene treatment is still effective even when delayed until 48 h after TBI, and that raloxifene benefit appears attributable to its CB2 inverse agonism rather than its estrogenic actions. Our studies show raloxifene is effective in treating visual injury after brain and/or eye trauma, and they provide basis for phase-2 efficacy testing in human clinical trials.

1. Introduction

Traumatic brain injury (TBI) is a frequent consequence of motor vehicle accidents, falls, and sports activities for the general population, as is exposure to explosive blasts in the case of members of the military. Even mild TBI, with a very brief or no loss of consciousness (i.e. concussion), can produce adverse sensory, motor, cognitive and emotional outcomes. Although resting is often sufficient treatment for symptoms to resolve within a few weeks, some individuals are burdened by persistent problems (Hiplaylee et al., 2017). Among these are visual deficits, including impairments in acuity, contrast sensitivity, convergence, accommodation, eye movements, and pupillary constriction, which often result in difficulty reading, double vision, and light aversion (Du et al., 2005; Goodrich et al., 2013; Jacobs and Van Stavern, 2013; Capó-

Aponte et al., 2017; Armstrong, 2018). For individuals who experience persisting visual deficits, the use of eyeglasses with specially prescribed tints and prism combinations currently provides the best solution (Armstrong, 2018), as effective treatments for TBI per se are lacking.

As part of a concussive event, whether from sudden impact, acceleration-deceleration, or blast pressure waves, the resulting forces are transmitted across the skull and cause brain tissue compression, stretching, and dynamic shear. Axons are especially vulnerable to deformation, and so damage to the optic nerves, optic radiation and/or cranial oculomotor nerves is frequently observed in people with mild TBI and contributes significantly to their visual deficits (Lachapelle et al., 2004; Bruce et al., 2006; Caeyenberghs et al., 2010; Goodrich et al., 2013; Jacobs and Van Stavern, 2013).

Axonal injury sets off a wave of secondary injury cascades, one of

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<https://doi.org/10.1016/j.expneurol.2019.113063>

Received 3 June 2019; Received in revised form 9 August 2019; Accepted 7 September 2019

Available online 10 September 2019

0014-4886/ © 2019 Published by Elsevier Inc.

the more prominent being microglial activation, which in turn causes further damage and worsens the outcome of the initial trauma (Kumar and Loane, 2012; Kumar et al., 2016; Donat et al., 2017). Microglial activation is thus an attractive therapeutic target and drugs acting on activated microglia may provide an effective approach for reducing the injury. Cannabinoid type-2 receptors (CB2) are normally expressed at low levels in the brain, but at higher levels on microglia than on neurons (Ashton and Glass, 2007; Stella, 2010). Activated microglia rapidly increase their expression of CB2 receptors (Ashton and Glass, 2007; Stella, 2010; Schomberg and Olson, 2012; Donat et al., 2014), and so drugs acting on CB2 receptors selectively target microglia (Stella, 2010). CB2 inverse agonists show particular promise (Lunn et al., 2006, 2008). CB2 inverse agonists stabilize CB2 receptors, which are otherwise constitutively active, in an inactive state and reduce adenylyl cyclase inhibition, thereby increasing cyclic adenosine monophosphate (cAMP) production (Atwood et al., 2012). This in turn leads to downstream activation of protein kinase A, which phosphorylates the cAMP response element binding protein (CREB). The transcriptional activity of phosphorylated-CREB (pCREB) has the overall effect of biasing activated microglia from the pro-inflammatory M1 state toward the protective M2 state, thereby underlying the beneficial effects of CB2 inverse agonists (Lunn et al., 2006, 2008; Presley et al., 2015).

We have previously shown that the selective CB2 inverse agonist SMM-189 reduces the M1 features and increases the M2 features of human and murine microglia in vitro (Presley et al., 2015; Reiner et al., 2015). Moreover, daily treatment of mice with SMM-189 for 2 weeks following focal cranial blast TBI greatly attenuates the visual, motor and emotional deficits, neuron loss, and electrophysiological abnormalities that are typically present at 1–2 months (Heldt et al., 2014; Reiner et al., 2015; Bu et al., 2016; Liu et al., 2017; Guley et al., 2019). As part of our SMM-189 studies, we showed that SMM-189 treatment increases levels of nuclear pCREB and biases microglia toward an M2 state in vivo (Bu et al., 2016; Guley et al., 2019), and that the 2-week SMM-189 treatment regimen rescues contrast sensitivity deficits, optic nerve axon loss, and retinal pathologies the mice would otherwise exhibit a month after TBI (Guley et al., 2019). SMM-189, however, is an experimental drug that has not been approved for human use. By contrast, raloxifene, which was recently found to have noteworthy CB2 receptor inverse agonism (Kumar and Song, 2013), is already approved by the Food and Drug Administration (FDA), though due to its action as a selective estrogen receptor modulator. Given that raloxifene is proven safe for human use, if shown to be effective in animal models of TBI, it could be fast-tracked for repurposing as a TBI therapy. Here we sought to evaluate the benefit of raloxifene for mitigating several visual deficits and the associated visual system pathology after mild TBI using our focal blast mouse model and to examine how raloxifene treatment affects microglia in the retina, optic nerve, and brain.

2. Methods

2.1. Animals, TBI and drugs

2.1.1. Animals

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) received either 50-psi or sham blast at about 3 months of age, and they were then injected over the next two weeks with either raloxifene or vehicle. A series of functional tests were administered to the mice beginning at 30 days after blast. Mice were typically perfused at ~ 8 months post-blast when functional testing was complete, and the eyes, optic nerves and brains dissected and prepared for histological evaluation. Optic nerves from additional mice, some at ~ 2 months and others at ~ 16 months after injury were also used for axon counts, and included in the analysis as we found that axon abundance was not correlated with post-blast survival time or with the age of the animal at sacrifice for any of the experimental groups. A cohort of 15 mice was sacrificed 5 days after blast for histological analysis. Eyes, optic nerves

and brains were separately collected from additional small cohorts of mice at 1, 3, and 5 days after blast for qPCR analysis. All experiments were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research, with National Institutes of Health (NIH), Department of Defense (DOD), and The University of Tennessee Health Science Center institutional guidelines, and with institutional and DOD approval.

2.1.2. Blast TBI device and blast administration

Our blast model of mild TBI has been previously described in considerable detail (Guley et al., 2016). In brief, the blast device is a modified, horizontally mounted paintball gun that emits a brief high-pressure air blast calibrated to the desired pressure, in this case 50 pounds per square inch (psi) above atmospheric pressure, to consistently produce a moderate level of injury with nearly 99% survival. Mice were anesthetized with Avertin, placed on a cushioned sling, and then inserted into protective tubing that shielded the mouse, except for a 7.5 mm diameter area halfway between the ear and the eye on the left side of the head. Mice that received a sham (0-psi) blast were handled in the identical way, but with a metal plate inserted between the barrel of the paintball gun and the mouse holder. After blast, mice were kept warm and recovered from the anesthesia in 15–30 min. Tylenol was provided in the drinking water at 35 mg/ml for 24 h before and after blast.

2.1.3. Raloxifene and vehicle administration

Raloxifene was prepared in a vehicle containing ethanol:Cremophor:0.9% saline (5:5:90) and administered at a dose of 5 or 10 mg/kg of body weight. Mice were injected intraperitoneally (ip) with either raloxifene or vehicle, beginning at 2 h after blast, and again at approximately the same time every day (± 1 h) for the next 14 days (15 doses in total), unless they were sacrificed at an earlier time point. For brevity, mice that received blast and vehicle will henceforth be referred to as blast-vehicle, sham mice that received vehicle as sham-vehicle or simply sham, mice that received blast and drug as blast-raloxifene, or more specifically blast-raloxifene5 for the lower dose and blast-raloxifene10 for the higher dose.

2.1.4. Additional drugs

To determine if raloxifene benefit stems from its agonist effects on β -type estrogen receptors (ER- β), or its antagonism at α -type estrogen receptors (ER- α) (Habib and Beyer, 2015; Frick et al., 2015), we performed experiments using several drugs that are selective for these two types of estrogen receptors. These were the ER- β antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) (1 mg/kg; Sigma-Aldrich), the selective ER- β agonist 2,3-Bis(4-hydroxyphenyl)propionitrile (DPN) (1 mg/kg; Sigma-Aldrich), and the selective ER- α antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride hydrate (MPP) (1 mg/kg; Sigma-Aldrich) (Li et al., 2017). The MPP and PHTPP doses were 3-fold greater than those shown to be effective in prior studies (Li et al., 2017), and the DPN dose was the same as or as much as 10-fold greater than the effective dose used in studies by others (Waters et al., 2009; Pisani et al., 2016). In an additional study, to verify that CB2 inverse agonism accounts for the benefit of raloxifene, we administered the selective CB2 inverse agonist SR144528 at 6 mg/kg (Cayman Chemical). In all cases, these drugs were prepared in the same vehicle as raloxifene, the injections were ip, and administration began 2 h after blast and continued for an additional 14 days.

2.2. Functional testing

2.2.1. Contrast sensitivity and visual acuity

The optokinetic reflex was used to determine the contrast sensitivity threshold and visual acuity of mice before and about one month post-

blast, using a Cerebral Mechanics OptoMotry system (CerebralMechanics Inc.: www.cerebralmechanics.com). The test was administered in a double-blinded manner, with the experimenter unaware of both the stimulus presented to the mouse and the eye being tested at any given time, as well as the treatment group. Contrast sensitivity threshold was determined at a 0.042 c/d spatial frequency and visual acuity was assessed at 100% contrast, as in our prior studies (Reiner et al., 2015; Guley et al., 2016, 2019). As there were no significant pre-blast differences between experimental groups for either eye, the post-blast data for the groups were normalized relative to sham pre-blast performance to better evaluate the effect of TBI.

2.2.2. Electretinogram (ERG)

For full-field ERG recordings, mice were dark adapted for 12 h, anesthetized with ketamine-xylazine, and the pupils dilated with 1% cyclopentolate hydrochloride. A drop of methylcellulose solution (Celluvisc; Allergan, Irvine, CA) was applied to each eye to protect it and help maintain a good electrical connection. Animals were kept on a warm (38 °C) pad during the procedure. Full-field ERGs were recorded from both eyes using the Diagnosys LLC system. Gold wires placed on each eye served as the corneal electrodes, and a steel subdermal needle served as the reference electrode. For grounding, a steel needle was placed in the tail. Animals received the following series of dim flashes to assess the scotopic (rod) A-wave and B-wave responses: 1) 0.00001 cd.sec/m² (15 trials with a 5 s interstimulus interval, ISI); 2) 0.0001 cd.sec/m² (10 trials with a 5 s ISI); 3) 0.001 cd.sec/m² (3 trials with a 10.1 s ISI); 4) 0.01 cd.sec/m² (3 trials with a 15.1 s ISI); 5) 0.1 cd.sec/m² (2 trials with a 20 s ISI); 6) 1 cd.sec/m² (2 trials with a 20 s ISI); and 7) 2.88 cd.sec/m² (2 trials with a 20 s ISI). Note that there were fewer trials and longer ISIs at the higher light intensities to limit rod photopigment bleaching, which would then affect subsequent trials. We measured the A-wave and B-wave amplitudes before blast and about 6–8 weeks after blast, and normalized data from the different groups relative to sham pre-blast performance to better assess TBI effects. No significant pre-blast differences were found between groups for either eye.

2.2.3. Light/dark testing to assess light aversion

The light-dark test arena consisted of a clear-walled test arena containing two equally sized compartments – an open area surrounded by the clear Plexiglas walls of the overall test arena and an enclosed chamber with black Plexiglas walls. An opening in the latter allowed the mice free passage from one chamber to the other. Infrared laser beams throughout the arena detected mouse movement and location, and a program automatically measured how much time the mouse spent in each compartment. The enclosed chamber contained a light bulb that could provide illumination at 500 lux, 1000 lux or 1500 lux, but was otherwise at 0 lux. The entire test arena was covered by a black drape during the testing, to prevent the mouse from being distracted by room cues or room lighting. The illumination of the open chamber was slightly > 0 lux when draped and ranged from 2 to 10 lux as illumination of the enclosed chamber was increased. Mice, which are nocturnal, tend to spend more time in the enclosed chamber under normal circumstances, as it provides a place the mouse can hide. Mice were habituated to the arena at ~100 days after blast to reduce the anxiety they would have otherwise demonstrated when exposed to a novel environment (Matynia et al., 2012), and tested about a month later. For the test, the mouse was placed in the open side and the arena covered with the drape. Each test began with 5 min of no light in the enclosed chamber (0 lux), followed by 5 min each of 500, 1000 and 1500 lux. As mice are naturally averse to bright light, they spend less time in the enclosed chamber as its illumination increases. Any increased avoidance of the enclosed chamber was interpreted as increased light aversion.

2.2.4. Pupillometry

Pupillometry was carried out under scotopic conditions on awake mice that were held using minimal manual restraint, having been previously habituated by extensive handling. We used a Melan-100 instrument (BioMed Vision Technologies, Ames, IA), equipped with two diode-based light sources: 630 nm for red light (200 kilocandela per square meter, kcd/m²) and 480 nm for blue light (200 kcd/m²). Red light elicits pupillary constriction mediated by rods and/or cones, whereas blue light elicits a response attributable to rods/cones and to intrinsically photosensitive retinal ganglion cells (ipRGCs) during light exposure, with the ipRGC response persisting after light offset. Pupil responses were recorded using a digital infrared video camera (Sony Handycam; Sony Corporation). An image of baseline pupil diameter was first taken in the dark. One eye was illuminated at a time, at a distance of 4 cm for 2 s, beginning with the red light, and then after a 10-s recovery period, with the blue light. Five minutes was allowed to elapse before testing the other eye. Frames from the captured movies were analyzed with Image J to measure pupil diameter and area before, during and after the light stimulus.

2.3. Morphological and immunohistochemical studies

2.3.1. Animal sacrifice

Mice were deeply anesthetized (Avertin; 0.2 mL/g body weight), the chest opened, and 0.1 mL of heparinized saline (800 U.S.P. units/mL) injected into the heart. Mice were then perfused transcardially with 30 mL of 0.9% NaCl in 0.1 M sodium phosphate buffer at pH 7.4 (PB), followed by 60 mL of 4% paraformaldehyde, 0.1 M lysine-0.1 M sodium periodate in 0.1 M PB at pH 7.4 (PLP). Brains were removed, and a pin inserted longitudinally into the right side of each brain, so that the left and right sides of the brain could be distinguished after sectioning. The brain was placed in PLP overnight 4 °C to post-fix, and the following day transferred to a 20% sucrose/10% glycerol solution and stored at 4 °C until sectioned. Each eye was removed from its socket, infused with PLP and post-fixed for 2 h at 4 °C. The lens and cornea were then removed. The eye cup was transferred to 0.1 M PB/0.01% sodium azide at 4 °C for storage, while the optic nerve was post-fixed in 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M PB, for later embedding in plastic.

2.3.2. Tissue sectioning and immunolabeling

Fixed brains were frozen with dry ice and sectioned on a sliding microtome in the transverse plane at 35 µm. Eyes were immunostained as eye cups and prepared as retinal flat-mounts, as described in more detail below. Plastic-embedded optic nerves were sectioned transversely at 1 µm and stained with 1% *p*-phenylenediamine in 50% methanol. Immunohistochemistry on free-floating brain sections was performed by peroxidase-antiperoxidase labeling or by multiple immunofluorescence, as described in our prior studies (Reiner et al., 2015; Guley et al., 2019). Image analysis was performed by individuals blind to treatment group.

2.3.3. Optic nerve axon counts

We viewed optic nerve sections using an Olympus BH2 light microscope with S Plan Apochromat objectives and an achromatic condenser (Olympus Corporation, Tokyo, Japan) and captured images with a SPOT Idea™ camera (Diagnostic instruments, Inc., Sterling Heights, MI), as described previously (Guley et al., 2019). A low-power image of each optic nerve was first captured using a 20x objective to measure its cross-sectional area and to divide the nerve into quadrants. An image of a subfield within each quadrant was then captured near its mid-point, using a 100x oil immersion objective. The image of each subfield was overlain with a 4 × 6 grid of twenty-four 100 µm² counting boxes. A random number generator was used to select 2 boxes per row per grid in which to count axons. Axon density was calculated for each quadrant, the densities for the 4 quadrants were averaged, and then the total

number of axons was estimated by multiplying the optic nerve cross sectional area by the axon density.

2.3.4. Analysis of ipRGCs

To help in understanding the basis for changes in light aversion and pupil constriction, we assessed melanopsin expression by intrinsically photosensitive retinal ganglion cells (ipRGCs) using the intensity of immunolabeling as a readout of relative expression levels. To do this, we immunostained eye cups for melanopsin, in combination with s-opsin, and prepared retinal flat-mounts. For s-opsin, we used a rabbit polyclonal antibody (Abcam #81017), raised against a synthetic peptide from the internal region of the human protein. S-opsin immunostaining specifically labels the outer segments of short wavelength cone photoreceptors, which are more abundant in inferior retina than superior retina (Ouk et al., 2016), thereby allowing us to determine the orientation of each retina. For melanopsin, we used a rabbit polyclonal antibody (Abcam #19306), raised against a synthetic peptide corresponding to amino acids 1–19 of the rat protein (Boudard et al., 2009; Benedetto et al., 2017). As this immunogen is the same as that used to generate a rabbit polyclonal antibody sold by Thermo Fisher (#PA1-780; Lin and Peng, 2013) and nearly identical to the immunogen for #UF006 (Advanced Targeting Systems; Hughes et al., 2013; Ouk et al., 2016), albeit with an additional 4 amino acids, we expect that our melanopsin immunolabeling detected M1 and M2 type ipRGCs (Baver et al., 2008; Berson et al., 2010; Lin and Peng, 2013). Note that although both the melanopsin and the s-opsin antibodies were rabbit polyclonals and visualized with the same secondary antibody, melanopsin+ ipRGCs and s-opsin+ outer segments were nonetheless readily distinguishable by their locations in the inner and outer retina, respectively, and by their distinct morphologies.

The immunostained eye cups were mounted onto slides, flattened, and coverslipped and viewed with a Zeiss 710 confocal microscope (LSM 800, Carl Zeiss AG, Oberkochen, Germany). Images were captured with a 10x, 0.45 numerical aperture objective at 0.8x zoom, and a pinhole setting of 2 Airy units, using the tile capture function of the Zen software (Zen Black Version 2.1, Carl Zeiss AG, Oberkochen, Germany) to allow efficient visualization of the entire retinal surface. Laser power and gain were adjusted to optimize image quality and were standardized across all images for each marker. A set of 3–4 z-stacks was acquired at 6 μm intervals through the inner retina, and another set of 5–6 z-stacks through the outer retina. The z-stack inner retina images and the 3 z-stack outer retina images containing the majority of melanopsin+ cells were used to generate separate maximum intensity projection images with the Zen software, and exported as tiff files for analysis with Adobe Photoshop and FIJI. Each image of inner retina was rotated in Adobe Photoshop so that the region of densest s-opsin immunostaining was positioned toward the bottom, and the least dense toward the top (Fig. 1A). The melanopsin image was then rotated the same number of degrees.

As melanopsin expression is known to vary along the superior-inferior axis of the retina (Hughes et al., 2013; Ouk et al., 2016), we took the following approach in our analysis. First, we divided the retinas into counting sectors that were consistent from mouse to mouse, by placing a dot in the center of optic nerve head, and drawing a 2400 μm $\times$ 2400 μm square centered on that dot in a separate layer of the Photoshop file. We drew horizontal lines at 600 μm above and below the center, so that the counting area was divided into 4 regions: 600–1200 μm superior retina, 0–600 μm superior retina, 0–600 μm inferior retina, 600–1200 μm inferior retina (henceforth called mid-superior, lower superior, upper inferior, mid-inferior, respectively). We circled all the melanopsin+ ipRGCs as shown in Fig. 1B, using the freehand pencil tool in a different Photoshop layer to facilitate later identification. Each cell was then drawn with a fine pencil in another layer in Photoshop to create a mask, with the opacity of the drawing layer set at 20–30% so that the image could still be seen (Fig. 1C, D). The mask and image layers were opened in FIJI, and the masks selected

by thresholding. We next directed the Analyze Particles function to the original melanopsin image to obtain cell body area and optical density (OD) for each melanopsin+ cell. To correct for background OD, which varied across the counting areas and between retinas, we made 3 copies of the mask layer, displacing each in a different direction $\sim$ 2–5 cell diameters away from the measured ipRGC cells, and measured the OD under each displaced mask for each cell. The average of the 3 background measurements was subtracted from the measured cell OD. In the occasional cases in which a displaced mask was positioned over another melanopsin+ cell or one of the relaxing cuts in the retina, so that its OD was not representative of the background adjacent to the cell of interest, that measurement was excluded.

2.3.5. Oculomotor nuclei

To understand the basis of eye movement abnormalities, we immunolabeled brain sections for choline acetyltransferase (ChAT), using a goat polyclonal antibody (Chemicon #AB144) as in prior studies (Deng and Reiner, 2016). Images were captured at a standardized level through the midbrain, the area of the oculomotor nucleus determined for that section using Image J, and ChAT+ perikarya counted.

2.3.6. Microglia in the optic tract

To begin to assess the effect of raloxifene on microglia, we sacrificed a cohort of 15 mice at 5 days after blast for immunofluorescence analysis. We immunostained sections through the brains of these mice for IBA1, to visualize all microglia, in combination with the M1 marker CD16/32, and the M2 marker CD206, using rabbit anti-IBA1 (Wako Chemicals #019-19,741), rat anti-CD16/32 (Abcam #ab25235), goat anti-CD206 (R&D Systems #AF2535), respectively, as in our previous studies (Guley et al., 2019). We directed our attention to the right optic tract, where reactive microglia are abundant during the first week after 50-psi blast (Guley et al., 2016, 2019). Images were captured with the Zeiss 710 confocal microscope, using a 20x, 0.8 numerical aperture objective, a pinhole setting of 2 Airy units, and the tile capture function of the Zen software. Laser power and gain were adjusted to optimize image quality and were standardized across all images for each marker. A set of 3 z-stacks was acquired at 2 μm intervals, used to generate a maximum intensity projection image with the Zen software, and exported as a tiff file for analysis with Adobe Photoshop and FIJI.

We took the following approach in our analysis. First, we outlined the right optic tract using the freehand pencil tool in Photoshop to determine its area and restrict subsequent analysis to this region. Second, we measured the background labeling intensity in 3 small areas without microglia within the optic tract for each channel for each image, and used the channel average to adjust each channel to a background OD level of 10 (1 = black, 255 = white), using the Math function in FIJI. Third, we then measured the overall mean OD within the right optic tract for each channel for each image. Fourth, for each image we thresholded the IBA1 channel to a standard level, and then used the Analyze Particles function in FIJI with a minimum particle size of 100 pixels (17.4 μm^2) to create a mask of the IBA-1 labeling above threshold. The IBA1 mask was then imported back into Photoshop, where the freehand pencil tool was used to separate any small, closely spaced sets of microglial cells that had been incorrectly joined in FIJI. The corrected mask was then reopened in FIJI and that software used to measure the area above threshold and count the number of particles.

2.4. Pharmacological and qPCR studies

2.4.1. ACTOne cell-based cAMP assay

The functional activity of raloxifene and SMM-189 at CB2 receptors was evaluated using the ACTOne cell-based cAMP assay in HEK-CNG + CB2 cells, as described in Presley et al. (2015). In brief, HEK-CNG + CB2 cells (HEK – human embryonic kidney; CNG – cyclic nucleotide-gated) were plated into clear poly-D-lysine coated 96-well plates at 50,000 cells per 100 μL culture medium with 10% serum the

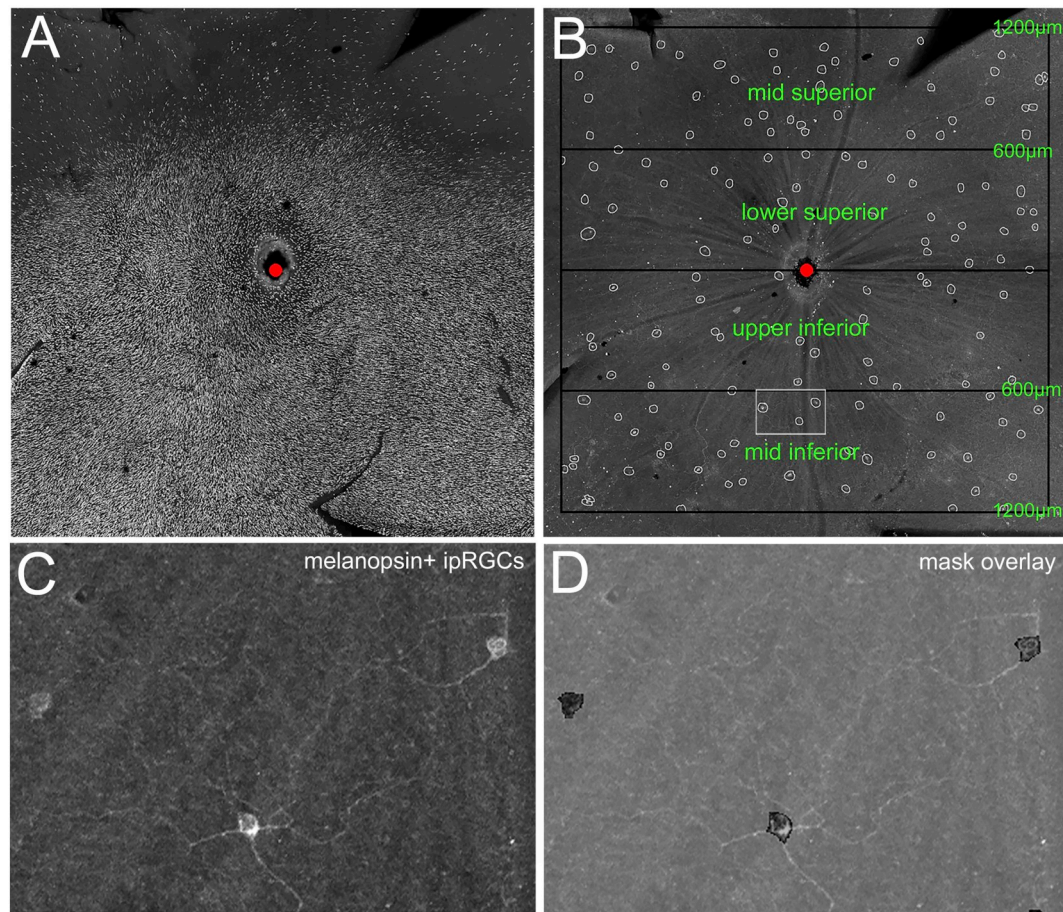


Fig. 1. Approach for analysis of melanopsin + ipRGCs. **A.** Confocal image of a retinal flat mount showing immunolabeling for S-opsin. The image was rotated to the correct orientation as determined by the high abundance of S-opsin + outer segments in ventral retina. The location of the optic nerve head is marked by a red dot. **B.** Melanopsin immunolabeling of the same retina shown in A. A 2400 $\mu\text{m} \times 2400 \mu\text{m}$ box, centered on the optic nerve head (red dot), was drawn in a separate layer in Photoshop, divided into 4 sectors, and ipRGCs circled for identification. Peripheral retina is beyond the field of view and was not analyzed. **C.** High magnification view of the area marked by the small rectangle in B, showing the labeled cell bodies and processes of three melanopsin + ipRGCs. **D.** Same field of view as C. Labeled cell bodies were drawn to create a mask to allow measurement of their optical densities. The mask layer is shown overlaid on the image layer at 30% opacity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

day before experiments were performed. The day of the experiment, 100 μL of warmed ACTOne formulation Membrane Potential Dye was added to each well, and incubated for 1 h in the dark at room temperature. Drug was then added in Dulbecco's phosphate buffered saline (DPBS) with 2.5% (v/v) dimethyl sulfoxide (DMSO), with 25 $\mu\text{mol/L}$ Ro 20-1724 (selective inhibitor of cAMP-specific phosphodiesterase), and 800 nmol/L forskolin. Raloxifene and SMM-189 were tested at final concentrations of 10^{-10} to 10^{-5} to span the range from roughly one log unit above and below the previously determined SMM-189 EC50. Plates were read 50 min after raloxifene or SMM-189 addition using a BioTek (Winooski, VT) plate reader (Ex 540 nm, Em 590 nm). At least six biological replicates were used for subsequent data analysis.

2.4.2. qPCR studies

We used quantitative PCR (qPCR) to assess how microglia are modulated by blast TBI and raloxifene treatment. For these studies, the left and right optic nerves, retina, and thalamus were each separately dissected and the tissue homogenized in TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's recommendations. Chloroform was added to the homogenate, centrifuged, and the upper colorless phase transferred to a clean tube. Precipitation was with 100% isopropanol and linear acrylamide. Precipitate was washed with 75% ethanol and the final mRNA pellet was suspended in DEPC (diethylpyrocarbonate) treated water. RNA concentration was assessed by analysis on Qubit Spectrophotometer and RNA purity by a NanoDrop

Spectrophotometer. Reverse transcription (RT) was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany), combining 100 ng of RNA with reaction buffer and enzyme mix, following manufacturer's directions. As it was necessary to linearly amplify the cDNA to produce an adequate amount of cDNA for qPCR from the optic nerve, with its limited volume of tissue and low mRNA harvest, this was done for all tissues using TaqMan® PreAmp Master Mix (Thermo Fisher Scientific, Waltham, MA). To assess the various states of microglia, we used primers for the following transcripts: 1) M0 – ionized calcium-binding adapter molecule 1 (IBA1), transmembrane protein 119 (Tmem119), purinergic receptor P2Y12 (P2ry12), and transforming growth factor-beta (TGF β); 2) M1 – Fc gamma receptor 2 (CD32), interferon-gamma (IFN γ), interleukin 1-beta (IL1 β), tumor necrosis factor-alpha (TNF α), and inducible nitric oxide synthase (iNOS); and 3) M2 – Arginase-1 (Arg1), chitinase-like protein 3 (YM1), interleukin-10 (IL10), and triggering receptor expressed on myeloid cells 2 (TREM2). Plates were run using Roche® LightCycler 480 and data were analyzed using the Comparative C_T ($\Delta\Delta C_T$) Method. Primers and probes are shown in Table 1 in the supplementary material. As seven of the thirteen transcripts examined are unique to microglia in these tissues (IBA1, Tmem119, P2ry12, CD16/32, IL1 β , Ym1, and TREM2), our data provide evidence on the effects of blast and raloxifene on microglia. While the remaining 6 transcripts are typically considered to be microglial markers, they can also be expressed by astrocytes, oligodendrocytes, and/or neurons. Although the relative

levels of expression of these 6 transcripts for the different cell types in the various tissue sources is not known, it is important to note that there are no neurons in the optic nerve and no oligodendrocytes in the retina.

2.5. Statistical analysis

One-way ANOVA followed by planned comparisons using post-hoc Fisher PLSD (Protected Least Significant Difference) tests was used to analyze behavioral and histological data, unless otherwise stated. Results are presented as group mean $\pm$ standard error of the mean (SEM).

3. Results

In the work reported here, we examined the consequences of blast TBI and the benefit provided by raloxifene treatment in terms of visual function and associated morphological changes. Mice were tested for contrast sensitivity and visual acuity at one month after blast, followed by ERGs at $\sim$ 2 months, light aversion at $\sim$ 4 months, and pupillometry at $\sim$ 7 months, before being sacrificed for histological analyses. A few additional subsets of mice were subjected to blast with and without treatment and sacrificed within a few days for immunohistochemical or qPCR analysis to evaluate microglial state.

3.1. Functional benefit of raloxifene

3.1.1. Contrast sensitivity and visual acuity

We have previously reported that a focal blast delivered to the left side of the cranium produces deficits in contrast sensitivity in both eyes (Guley et al., 2016, 2019). In the current series of experiments, as expected, we again found significant increases in contrast sensitivity thresholds for both eyes following blast (Fig. 2A). Contrast sensitivity thresholds for the blast-raloxifene5 mice, however, were significantly lower than in the blast-vehicle mice and similar to the sham mice, both comparisons indicating that contrast sensitivity was improved. The blast-raloxifene10 mice showed a similar pattern of results, with contrast sensitivity thresholds for both eyes restored to a similar level as in sham mice. For this smaller set of animals (10 mice for 10 mg/kg as compared to 22 mice for 5 mg/kg), the improvement in contrast sensitivity compared to blast-vehicle mice was significant for the right eye and trended toward, but did not reach, statistical significance for the left eye. Thus, blast TBI produced bilateral deficits in contrast sensitivity and raloxifene treatment alleviated those deficits.

Optomotor testing of visual acuity for the same animals did not

reveal any deficit for the left eye as a consequence of blast. By contrast, visual acuity was significantly reduced for the right eye of blast-vehicle mice compared to sham mice. Visual acuity was improved by both doses of raloxifene, as it was not significantly different than for sham-vehicle mice for both doses, with the blast-raloxifene5 mice also significantly different than the blast-vehicle mice (Fig. 2B).

3.1.2. Scotopic dark-adapted ERG A-wave and B-wave

Scotopic ERGs showed reductions in the peak amplitudes of the A-wave for both eyes and the B-wave for the left eye as a consequence of blast and rescue of these deficits with raloxifene treatment (10 mice per experimental group; Fig. 3). Specifically, the A-wave peak for the 4 brightest light intensities for both eyes was significantly reduced for blast-vehicle mice compared to sham, while blast-raloxifene mice at both doses did not differ from sham. The left eye B-wave peak for the 6 brightest light intensities was also reduced for blast-vehicle mice compared to sham and restored to sham levels in the drug-treated mice. By contrast, the B-wave peak for the right eye did not show significant changes with blast or with drug treatment.

3.1.3. Light aversion

Light aversion was assessed by allowing each mouse to move freely between two chambers, an open chamber kept dark and an enclosed chamber with variable illumination. As shown in Fig. 4, increased illumination in the enclosed chamber resulted in greater avoidance for all groups of mice. The blast-vehicle mice, however, spent significantly less time than sham mice in the increasingly brighter enclosed chamber. Treatment with 10 mg/kg raloxifene rescued this heightened light aversion, as blast-raloxifene10 mice and sham mice occupied the enclosed chamber to similar extents. The blast-raloxifene5 mice, however, still differed significantly from sham. Thus, blast TBI caused light aversion, and the higher dose of raloxifene was needed to prevent this.

3.1.4. Pupil light reflex

To provide insight into the increased light aversion the mice exhibited following blast TBI, we conducted pupillometry. Surprisingly, we found abnormalities in the pupil light reflex (PLR) of the right eye following exposure to red light and to blue light, but not for the left eye (Fig. 5). Pupil constriction for the right eye was significantly enhanced in blast-vehicle mice compared to sham mice, for the 2 s during exposure to red light and during and for up to 10 s after exposure to blue light (Fig. 5B). Treatment with 5 mg/kg raloxifene partly normalized the red light but not the blue light response, whereas 10 mg/kg raloxifene normalized the increased pupil constriction for both red and blue

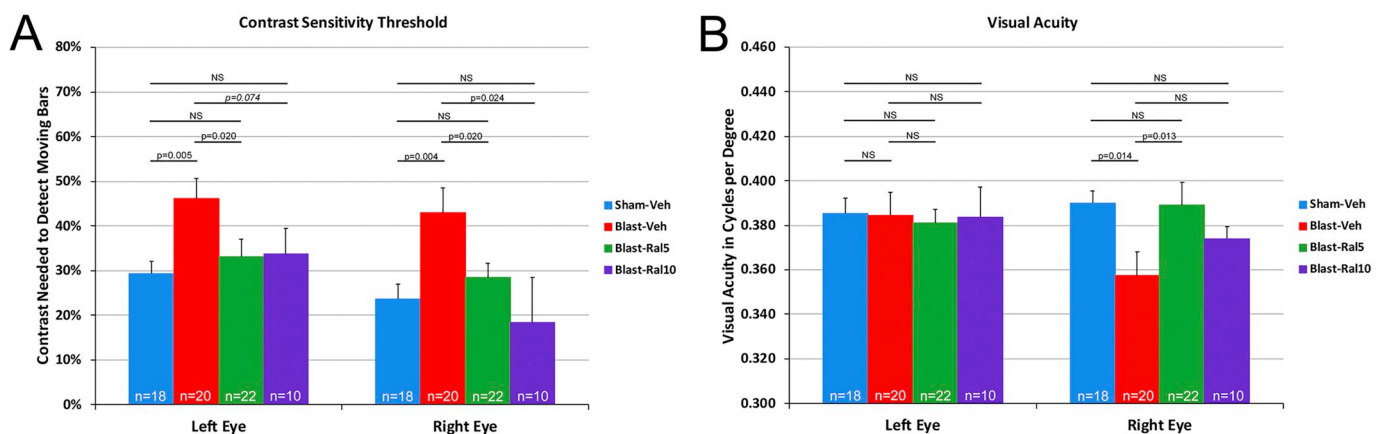


Fig. 2. Contrast sensitivity and visual acuity as measured using Optomotry a month after blast. **A.** The contrast sensitivity thresholds were significantly more in blast-vehicle mice than in sham-vehicle mice for both eyes. Mice treated with raloxifene were similar to sham and improved over blast-vehicle mice, either significantly or in the case of the left eye of the blast-ral10 mice approaching significance. **B.** The right eye of blast-vehicle mice showed a significant loss of acuity compared to sham mice, which was rescued by raloxifene treatment. Acuity for the left eye was similar for the blast-vehicle, blast-raloxifene, and sham mice. N = the number of eyes. Errors bars are SEMs. *p* values that are close to statistical significance are shown italicized.

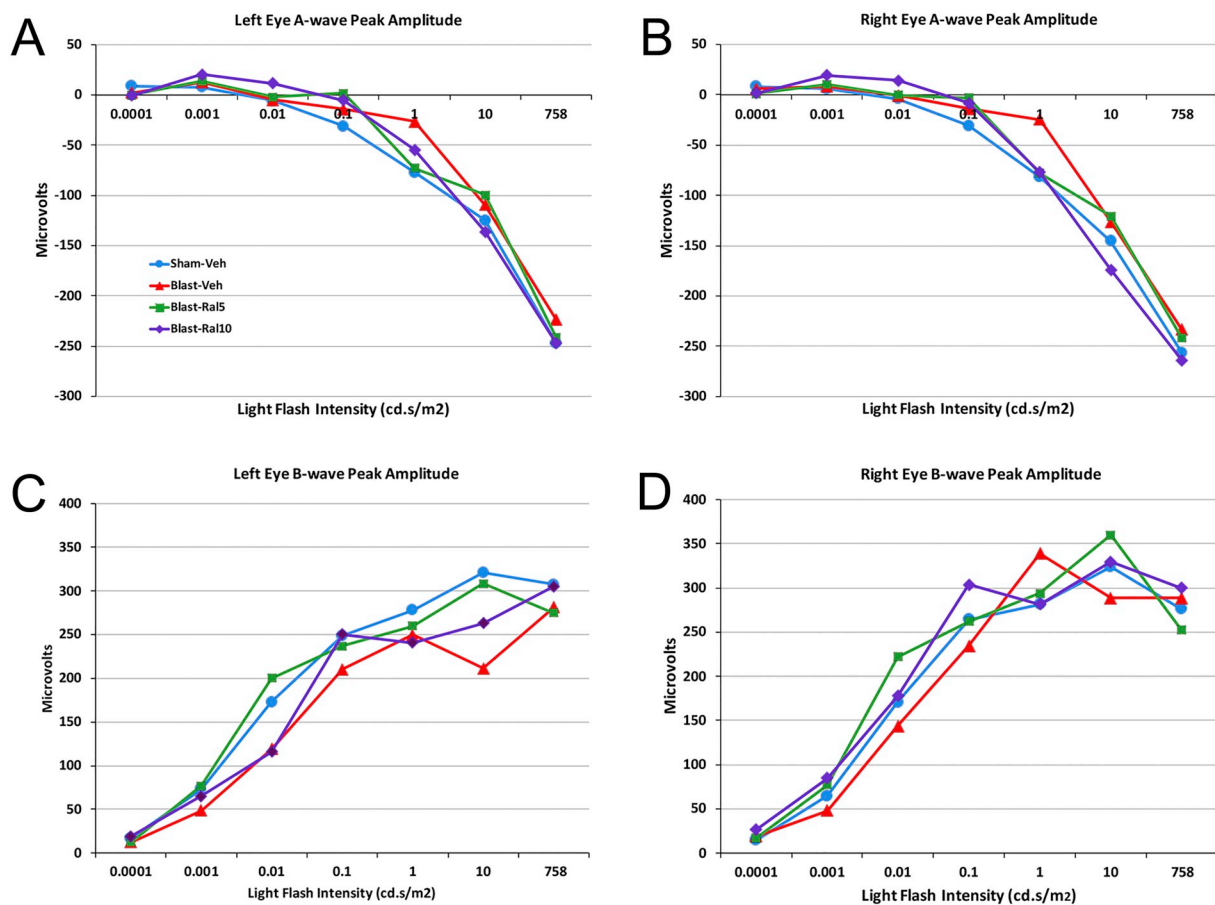


Fig. 3. Average flash-evoked scotopic ERG peak A-wave and B-wave amplitudes at 7 light intensities at ~2 months after blast. Values for each mouse were normalized to the sham pre-blast baseline. **A, B.** The A-wave peak for the 4 brightest light intensities was reduced for blast-vehicle mice compared to sham mice. The reduction for the left eye was significant ($p = .0343$), while blast-raloxifene mice were statistically indistinguishable from sham ($p = .1787$ for blast-raloxifene5 compared to sham, $p = .4664$ for blast-raloxifene10 compared to sham). The right eye showed a similar reduction in the A-wave peak with blast compared to sham ($p = .0251$), and a restoration to sham values in raloxifene-treated mice ($p = .2254$ for blast-raloxifene5 compared to sham; $p = .8602$ for blast-raloxifene10 compared to sham). **C, D.** For the left eye, the B-wave peak, measured from baseline, for the 6 brightest light intensities was reduced for blast-vehicle mice compared to sham mice ($p = .0038$), and blast-raloxifene mice were statistically indistinguishable from sham ($p = .6806$ for blast-raloxifene5 compared to sham; $p = .0867$ for blast-raloxifene10 compared to sham). The B-wave peak for the right eye did not show a significant deficit with blast ($p = .7053$), and blast-raloxifene mice were not significantly different than sham ($p = .3366$ for blast-raloxifene5; $p = .2553$ for blast-raloxifene10).

light (Fig. 5D).

3.2. Morphological benefit of raloxifene

3.2.1. Optic nerve

The left optic nerve of blast-vehicle mice showed a significant 10% loss of axons (Fig. 6). Similar loss was found in the blast mice treated with 5 mg/kg raloxifene, whereas treatment with 10 mg/kg raloxifene yielded full rescue. Notably, left optic nerve axon abundance across all 4 experimental groups was significantly correlated with that eye's contrast sensitivity threshold ($r = 0.248$), suggesting that optic nerve axon loss resulting from the blast injury contributes to the contrast sensitivity deficit and that the rescue of axons with raloxifene treatment contributes to the functional rescue. By contrast, axon abundance was not decreased in right optic nerve of blast-vehicle mice, or in that of raloxifene-treated blast mice, compared to sham. Accordingly, the contrast sensitivity deficit for the right eye, as well as the right eye acuity deficit, must be attributable to something other than optic nerve axon loss.

3.2.2. Melanopsin-expressing ipRGCs

To help elucidate the basis of the changes in light aversion and pupil constriction after blast and with raloxifene treatment, we examined

melanopsin-immunolabeled ipRGCs. Given that ipRGC size and melanopsin expression vary with location in the retina, we divided the central two-thirds of the retina into 4 sectors from superior to inferior, as shown in Fig. 1B, and compared only those ipRGCs located in the same sector. As the changes in ipRGC size and melanopsin immunolabeling after blast and with raloxifene treatment were similar across sectors, the data in Fig. 7 are shown relative to the sham values for that sector for each eye. Because there were no consistent differences between the raloxifene doses, we combined the data for the two. For the left eye, ipRGC soma size was significantly decreased and melanopsin immunolabeling was significantly increased for blast-vehicle mice as compared to sham mice. Raloxifene treatment reversed both changes: ipRGC soma size was significantly greater and melanopsin immunolabeling was significantly less for blast-raloxifene mice than for blast-vehicle mice for all sectors. In addition, ipRGC soma size was not significantly less for blast-raloxifene mice than for sham mice for three of the four sectors. The right eye showed similar changes, i.e. a decrease in ipRGC soma size and an increase in melanopsin immunolabeling, which were smaller in extent than for the left eye but still significant for all sectors, and raloxifene rescue of melanopsin expression that reached statistical significance for three of the sectors.

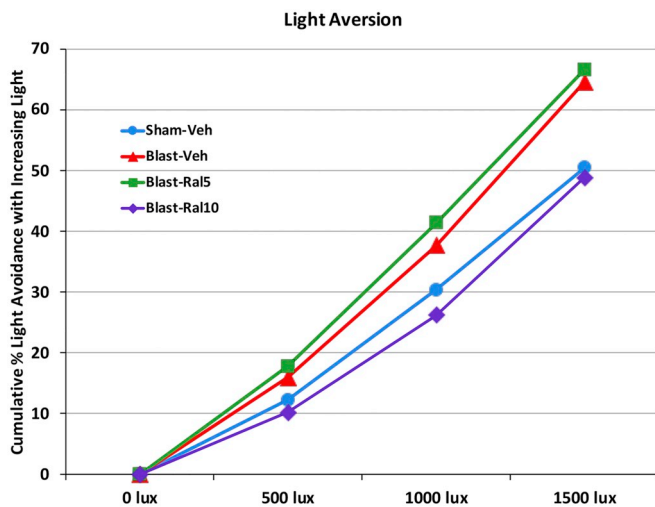


Fig. 4. Light aversion, tested at ~4 months after blast, plotted as cumulative avoidance of an enclosed chamber with increasing brightness, relative to an adjacent dark chamber. Blast-vehicle mice showed greater avoidance of the enclosed chamber as light intensity increased than did sham-vehicle mice (chi-square analysis; $p = .0326$). The amount of enclosed chamber occupancy with increased light intensity for blast-raloxifene10 mice was similar to sham mice ($p = .6242$), but significantly different between blast-raloxifene5 mice and sham ($p = .0029$). Thus, raloxifene at 10 mg/kg, but not at 5 mg/kg, rescued the increase in light aversion.

3.2.3. Oculomotor nucleus

To understand the basis of eye movement abnormalities, we examined the oculomotor nucleus, which innervates 4 of the 6 extraocular muscles, using ChAT immunolabeling. The area occupied by the oculomotor nucleus area was reduced bilaterally by 40.2% and neuron abundance by 32.6% in the blast-vehicle mice compared to sham (Fig. 8). Treatment with 5 mg/kg raloxifene restored OM area and neuron count to levels similar to sham mice and significantly greater than blast-vehicle mice (Fig. 8). Raloxifene at 10 mg/kg also produced significant improvement, but was seemingly somewhat less effective as the lower dose for area. More specifically, OM neuron count for the blast-raloxifene10 mice did not differ significantly from sham mice and was also significantly greater than for blast-vehicle mice, whereas OM area was significantly larger than for blast-vehicle mice but still smaller than for sham mice (Fig. 8).

3.3. Microglial modulation by raloxifene

3.3.1. Effect of raloxifene on optic tract microglia

We examined the right optic tract in tissue sections immunolabeled for IBA1, to visualize all microglia, the M1 marker CD16/32, and the M2 marker CD206, in 5 blast-vehicle mice, 5 blast mice treated with 5 mg/kg raloxifene, and 5 sham mice sacrificed 5 days after blast TBI. We found that blast substantially increased the size of IBA1 + microglia, in accord with our previous findings that microglia in the right optic tract are activated following blast TBI, presumably by molecules released from adjacent damaged axons, and contribute to further axonal

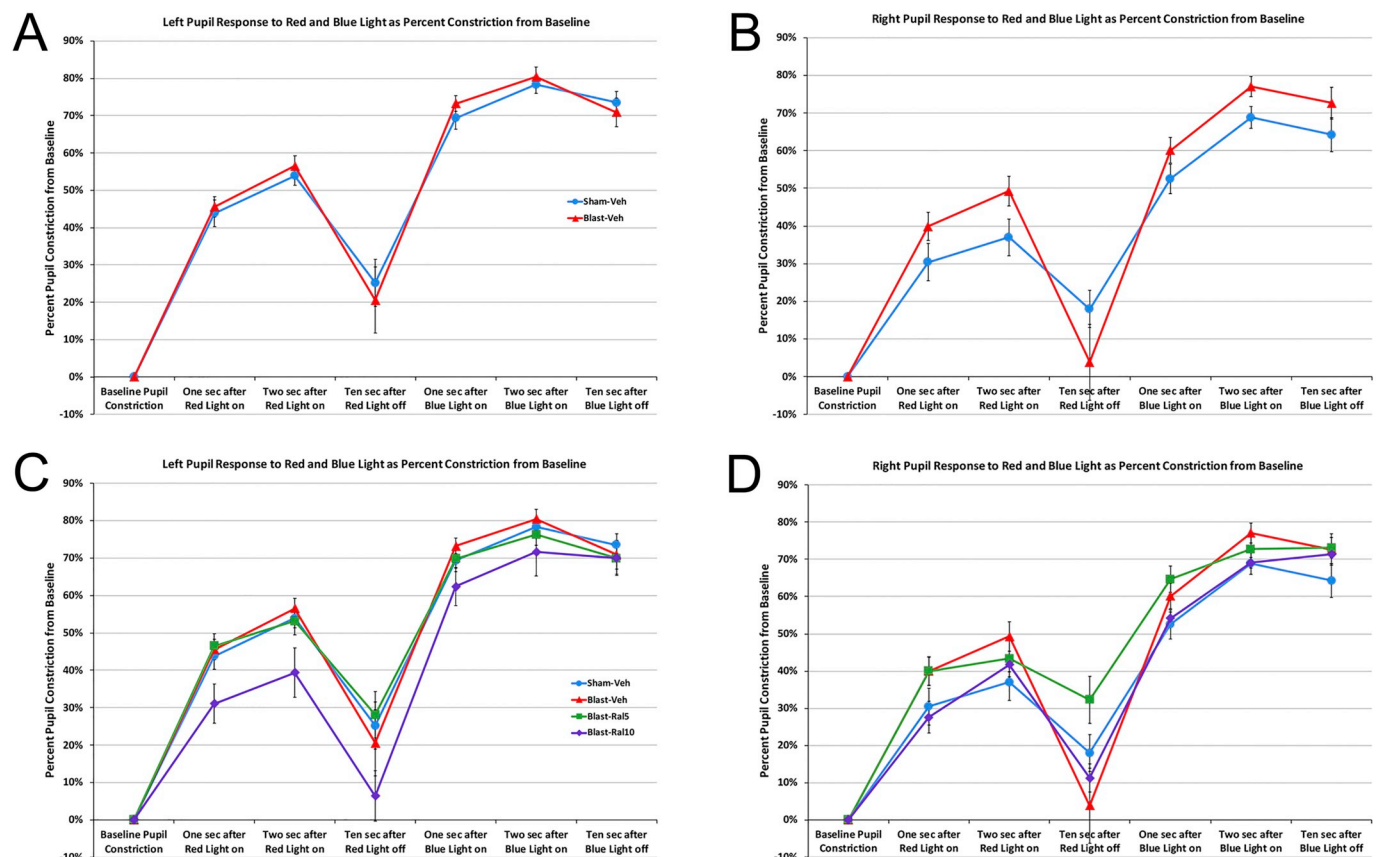


Fig. 5. Pupil responses, plotted as % constriction (i.e. pupil area reduction) from pre-illumination baseline, to red and blue light. **A, C.** Left eye pupil constriction was no different in blast-vehicle mice than in sham mice (**A**), but blast mice treated with 10 mg/kg raloxifene showed a significantly diminished response to red light ($p = .0012$; **C**). **B, D.** For the right eye, pupil constriction was significantly enhanced in blast-vehicle mice compared to sham mice during the 2 s of exposure to red light ($p = .0105$), and during and for up to 10 s after exposure to blue light ($p = .005$; **B**). The pupil responses of mice treated with 10 mg/kg raloxifene were similar to sham for both red light ($p = .836$) and blue light ($p = .363$), whereas pupil constriction for blast-raloxifene5 mice was similar to sham for red light ($p = .0575$), but still enhanced compared to sham for blue light ($p = .0026$; **D**). Errors bars are SEMs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

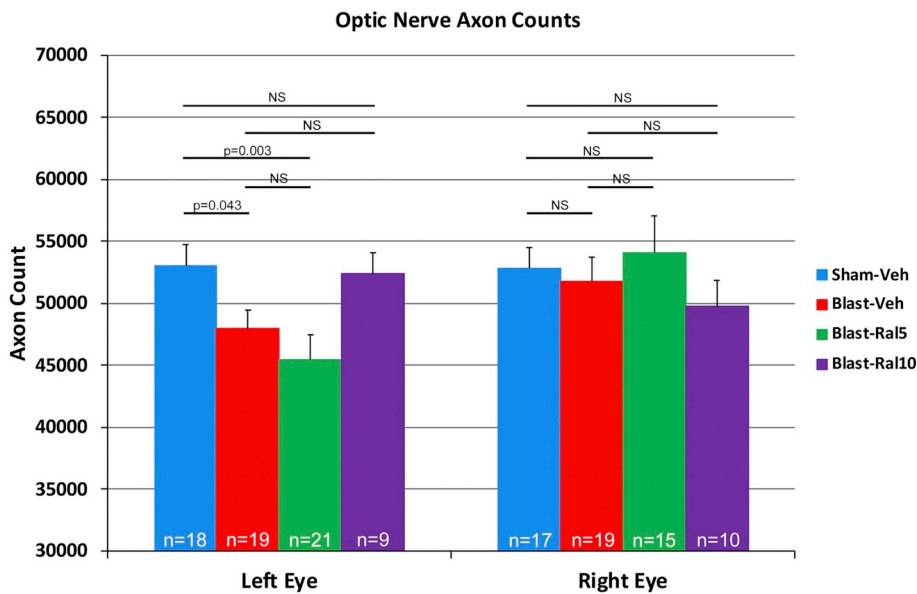


Fig. 6. Optic nerve axon counts. The left optic nerve of blast-vehicle and blast-ral5 mice showed a significant loss of axons. Treatment with 10 mg/kg raloxifene yielded full rescue. The right optic nerve showed no axon loss following blast and none of the differences between experimental groups were significant. N = the number of optic nerves. Errors bars are SEMs.

injury (Guley et al., 2019). Moreover, as shown in Fig. 9, the intensity of IBA1 and CD16/32 immunolabeling was greater in the blast-vehicle mice than in sham-vehicle mice. The intensity of CD206 immunolabeling was also increased, although to a slightly smaller extent than CD16/32 immunolabeling, resulting in a higher M1/M2 ratio for microglia in the blast-vehicle mice than in sham mice (Fig. 9). Quantification of the IBA1 immunolabeling by thresholding and particle analysis also revealed post blast increases in the area occupied by microglia and the density of IBA1+ profiles > 100 pixels in size ($\sim 17.4 \mu\text{m}^2$). Chi-square analysis showed that microglia in blast-vehicle mice differed highly significantly ($p < 2.0 \times 10^{-107}$) from microglia in sham mice for this combination of six traits. By contrast, the intensity of IBA1 immunolabeling, the areal coverage by microglia and the density of IBA1+ profiles in the right optic tract of blast mice treated with 5 mg/kg raloxifene were all decreased in comparison to blast-vehicle mice (Fig. 9). Further, CD16/32 expression was decreased relative to blast-vehicle mice, while CD206 expression was increased, reducing the M1/M2 ratio to a level similar to that for the sham mice. Chi-square analysis for the six traits showed a significant difference ($p = .0051$) between microglia in the blast-raloxifene mice and the blast-vehicle mice. Thus, raloxifene treatment appears to partially reverse microglial activation, and bias microglia toward the protective M2 state.

3.3.2. qPCR studies of microglia modulation over time after blast TBI

To expand our understanding of microglial modulation after blast beyond our current and previous findings using immunofluorescent labeling (Bu et al., 2016; Guley et al., 2016, 2019), we used qPCR to quantify the expression of 4 M0 markers, 5 M1 markers, and 4 M2 markers in left and right optic nerve, retina, and thalamus at 1, 3, and 5 days after blast (Fig. 10A-F). The M1 markers tended to show the greatest changes over this 5-day time period, with multiple M1 markers being elevated for each tissue at least at one time point. In particular, each of the 5 M1 markers was increased > 50% in left optic nerve (LON), left retina, and both left and right thalamus at 3 days, and 3 of the 5 markers (TNF α , IFN γ and IL1 β) were increased > 50% in right optic nerve (RON) and right retina at 5 days (Tables 2–4 in the supplementary material). The expression of the 5 M1 markers averaged together exhibited a larger peak increase during the 5-day period after blast than did the expression of the M2 (and M0) markers for both sides of the retina and the thalamus. By contrast, the M2 markers showed a larger peak increase than the M1 (and M0) markers for left and right optic nerves. In fact, the M0 markers changed fairly little after blast (< 50%), the only exception being the left retina.

The increase in M1 marker expression was transient over the 5-day time period we examined. M1 transcript levels in LON and left and right thalamus increased from day 1 to day 3, and declined by day 5. The left retina also showed an M1 decline at day 5, although its M1 expression was already high at day 1, and remained high at day 3. In contrast, M1 expression in right retina was low at day 1, slightly elevated at day 3, and considerably increased by day 5, and RON showed only small changes in M1 expression but followed a similar temporal pattern, with M1 levels higher at day 5 than at day 3. Changes in M2 expression also appeared to be transient in the case of RON and right thalamus, for which the expression of the 4 M2 markers averaged together increased from day 1 to day 3 but declined by day 5. In contrast, for LON, left and right retina, and left thalamus, M2 expression was low at day 3 but increased by day 5. Whether this increase in M2 expression persisted beyond day 5, or whether M2 levels showed a rapid decline similar to M2 expression for RON and right thalamus between days 3 and 5 is uncertain.

To help visualize the temporal changes in relative M1 versus M2 expression levels, we calculated M1/M2 ratios for each time point for each tissue. Left optic nerve, retina and thalamus exhibited an M1/M2 peak at 3 days followed by decline at 5 days, whereas M1/M2 ratios were higher at 5 days than at 3 days for these same tissues on the right side (Fig. 10G). Thus, the natural progression in microglial behavior appears to entail M1 activation during the first 3 days after injury, with a gradual overtaking of M1 markers by M2 markers by 5 days for the directly targeted left side and a delayed increase in M1 expression relative to M2 expression on the contrecoup right side, indicating different pathogenic processes on the right than on the left.

3.3.3. qPCR studies of raloxifene effects on microglial modulation after blast TBI

To examine how raloxifene affects microglia, mice were treated 2 h after blast, again on days 1–3, sacrificed, and qPCR performed as described above. As shown in Fig. 11A-F, raloxifene treatment generally decreased the expression of M1 markers and increased the expression of M2 markers over their respective levels in blast-vehicle mice, with the higher dose generally being more effective. More specifically, 10 mg/kg raloxifene decreased M1 expression and increased M2 expression for the retina and the thalamus on both sides, whereas 5 mg/kg raloxifene increased M2 markers but did not decrease M1 markers in the retina, and decreased M1 markers but did not notably increase M2 markers in the thalamus. In the case of the left optic nerve, the higher dose of raloxifene decreased M1 expression but did not increase M2 expression,

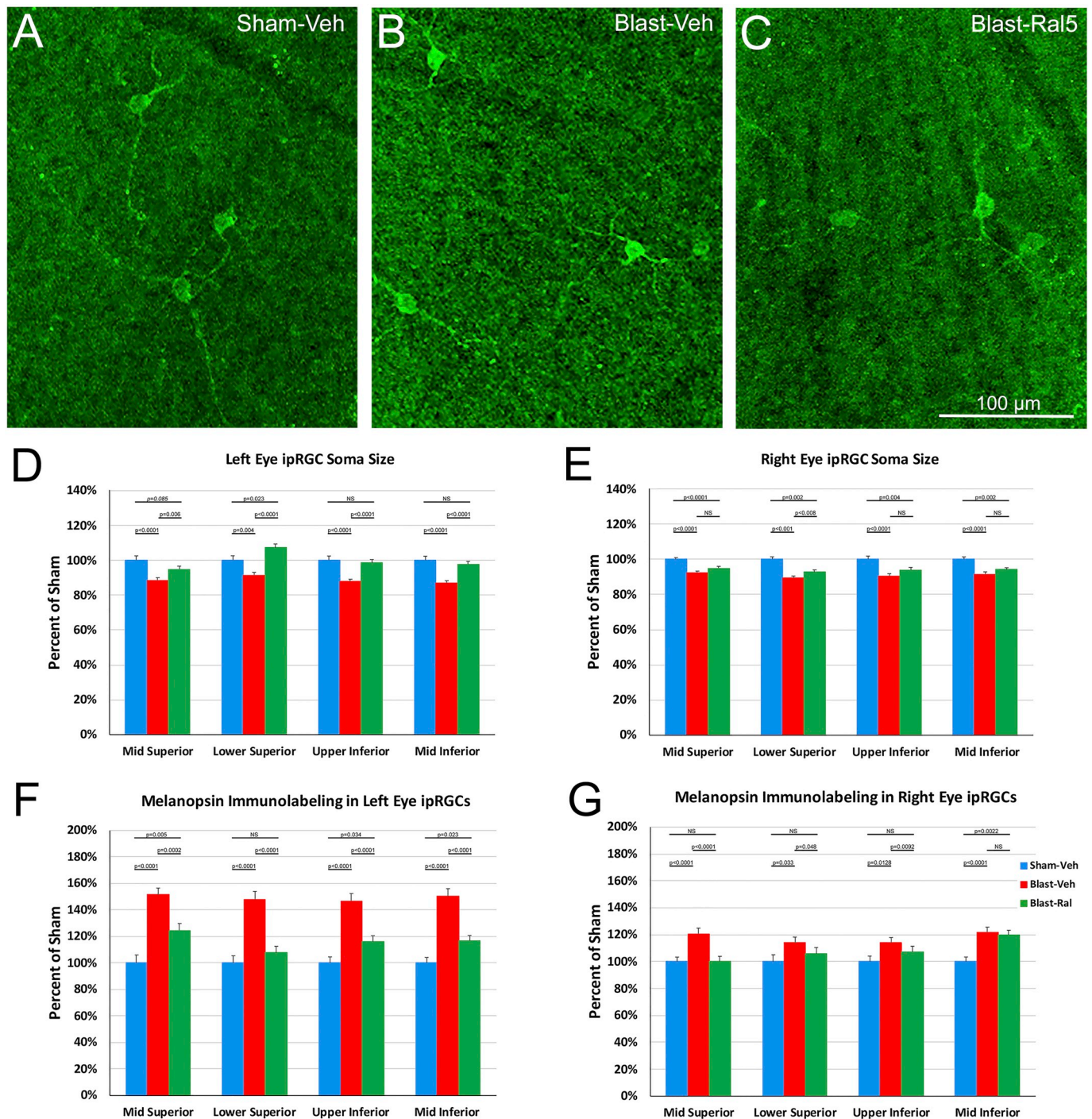


Fig. 7. Melanopsin+ ipRGC analysis. A-C. Confocal images of melanopsin immunolabeling, all taken from the mid-inferior sector of the left eye. ipRGC cell bodies in the blast-vehicle retina were more intensely immunolabeled for melanopsin and smaller than in the sham. These changes were largely reversed in raloxifene-treated mice. Scale bar in C applies to A-C. D-G. Quantification of soma size and melanopsin immunolabeling, shown relative to the sham value for each sector for each eye. Blast significantly reduced ipRGC size and increased melanopsin immunolabeling for both eyes. Raloxifene treatment consistently lessened the effect of blast. Errors bars are SEMs. *p* values that are close to statistical significance are shown italicized.

while the lower dose increased M2 expression but did not decrease M1 expression. For the right optic nerve, both doses increased M2 expression, the higher dose to a greater extent, but both had little effect on M1 expression (Fig. 11G). M1/M2 ratios for the blast-vehicle mice were > 1 for all tissues, with the exception of RON, consistent with the early modulation of microglia toward the M1 state following injury. M1/M2 ratios for the raloxifene-treated mice were lower than for the blast-vehicle mice for all 6 tissues for both doses, consistent with raloxifene modulating microglia toward the M2 state. Moreover, the M1/

M2 ratios were lower for the 10 mg/kg dose than the 5 mg/kg dose for all but LON, suggesting the higher dose is generally more effective than the lower dose in modulating microglia. With respect to specific transcripts, the two doses of raloxifene exhibited some similarities and some differences (Tables 5–7 in the supplementary material). For example, both doses increased all M2 markers in left retina (note IL10 levels were too low to detect in the raloxifene10 retinal samples), and all but TREM2 in RON. However, while the lower dose increased all M2 markers in LON and left thalamus, the higher dose increased only 3 of

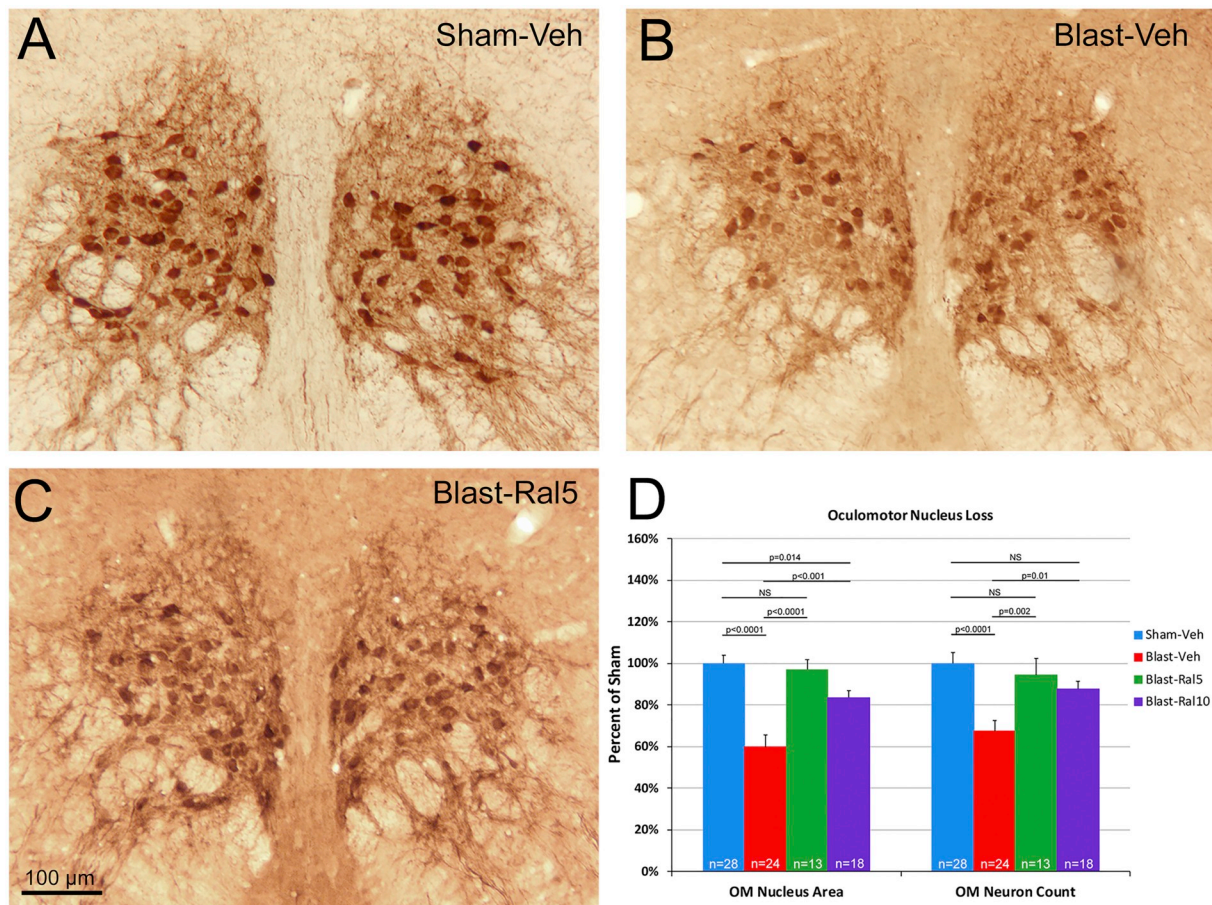


Fig. 8. Oculomotor nuclei. **A-C.** Sections of midbrain immunostained for choline acetyltransferase to reveal motoneurons. The area occupied by the oculomotor (OM) nucleus and its neuron abundance were less on both sides of the blast-vehicle mouse than in the sham, whereas mice treated with 5 mg/kg raloxifene were similar to sham. Scale bar in C applies to A-C. **D.** Quantification of OM nucleus area and number of neurons. The left and right sides for each mouse were pooled for statistical analysis. Raloxifene treatment yielded rescue, with 5 mg/kg being somewhat more effective than 10 mg/kg for both parameters. N = number of OM nuclei analyzed. Errors bars are SEMs.

the 4 M2 markers in these same tissues (all but YM1 in LON and all but TREM2 in left thalamus).

3.4. CB2 inverse agonist specificity of raloxifene benefit

3.4.1. CB2 receptor pharmacology

We previously demonstrated the inverse agonism of SMM-189 at CB2 receptors by assessing its ability to increase cAMP in cells expressing CB2 receptors (Presley et al., 2015). To confirm raloxifene action as an inverse agonist at CB2 receptors (Kumar and Song, 2013), we used the ACTOne cell-based cAMP assay in HEK-CNG + CB2 cells. Raloxifene caused a dose-dependent increase in cAMP-driven fluorescence with 1 μ M of drug producing a level of fluorescence over baseline 5-fold greater than that for SMM-189 (Fig. 12). Thus, raloxifene appears to provide greater efficacy as a CB2 inverse agonist than SMM-189.

3.4.2. Raloxifene benefit is mimicked by the CB2 inverse agonist SR144528

We next evaluated if the selective CB2 inverse agonist SR144528, which we have previously shown increases the levels of M2 markers on the surfaces of cultured microglia (Presley et al., 2015), similarly yields rescue for the contrast sensitivity deficit after blast. As shown in Fig. 13A, SR144528 rescued the TBI-induced contrast sensitivity deficit for both eyes.

3.4.3. Raloxifene benefit does not depend on its estrogenic effects

In principle, raloxifene benefit could stem, not from its action on

CB2 receptors, but rather via its agonist effects on β -type estrogen receptors, or its antagonism at α -type estrogen receptors (Frick et al., 2015; Habib and Beyer, 2015). To test the first possibility, we treated some blast mice with 5 mg/kg raloxifene immediately followed by the ER- β antagonist PHTPP. To test both possibilities, we injected other mice successively with a selective ER- β agonist (DPN) and a selective ER- α antagonist (MPP). An additional set of mice received 5 mg/kg raloxifene and then a saline injection. Drug administration followed our typical two-week schedule. Subsequent contrast sensitivity testing showed that, as expected, raloxifene followed by saline rescued the deficit for both eyes (Fig. 13B). The ER- β antagonist PHTPP did not attenuate the rescuing effect of raloxifene for either eye, and the combination of DPN and MPP did not replicate raloxifene rescue. Our findings that blocking the ER- β agonism of raloxifene does not prevent benefit, and mimicking the ER- β agonism and selective ER- α antagonism of raloxifene does not replicate raloxifene benefit, indicate that the estrogenic actions of raloxifene are not the basis of its ability to mitigate visual deficits after blast TBI.

3.5. Raloxifene treatment window for functional benefit after blast TBI

To determine if delaying administration of raloxifene still yields benefit, we used 5 mg/kg as the standard dose and varied the treatment regimen as follows. One group of mice first received raloxifene one day after blast (thus missing treatment on day 0, the day of blast), and was then treated daily for 14 days (blast-ral day 1–14). A second group of

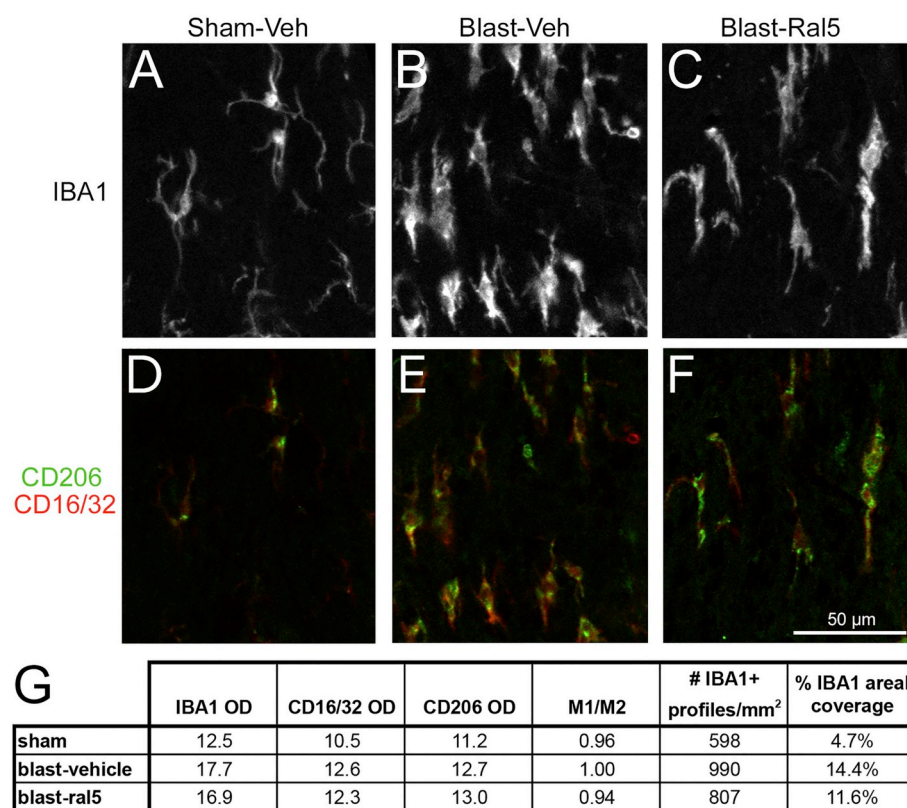


Fig. 9. Microglia in right optic tract. A-E. Confocal images showing the right optic tract of a Sham-Veh mouse (A, C), Blast-Veh (B, E), and a Blast-Ral5 mouse (D, F), simultaneously immunolabeled for Iba-1 to visualize microglia, for CD16/32 to detect the M1 phenotype, and for CD206 to detect the M2 phenotype. The upper panels show the IBA1 immunolabeling pseudo-colored white, while the lower panels are of the same field of view as the panel immediately above and show the merge for the M1 and M2 markers. The red CD16/32 M1 labeling predominates more in the Blast-Veh image in E than in the corresponding Blast-SMM image in F, where the green CD206 M2 labeling is more readily seen. The scale bar in F applies to A-F. G. Quantification of the IBA1, CD16/32 and CD206 immunolabeling. The intensity of IBA1, CD16/32 and CD206 immunolabeling, the M1/M2 ratio, microglial abundance and areal coverage are all increased by blast, as also shown in B and E compared to A and D, respectively. Raloxifene treatment decreased microglial abundance and areal coverage, and the intensity of IBA1 and CD16/32 immunolabeling compared to Blast-Veh, but increased CD206 immunolabeling intensity and thus decreased the M1/M2 ratio, as seen in images C and F compared to B and E, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mice was not treated until the second day after blast (blast-ral day 2–14) and a third group not until the third day (blast-ral day 3–14). For comparison, a fourth group of mice received only 4 daily treatments (blast-ral day 0–3). As shown in Fig. 14, the left eye contrast sensitivity threshold for mice first treated the day after blast, or two days after blast did not differ significantly from sham mice. However, the left eye contrast sensitivity threshold for mice first treated on the third day of blast differed significantly from sham mice, as did that for mice treated for only the first 3 days. Thus, a delay in raloxifene treatment until the second day after blast still yielded rescue of the blast-induced contrast sensitivity deficit for the left eye, whereas a longer delay or a shorter treatment failed to provide benefit. By contrast, the contrast sensitivity and visual acuity deficits for the right eye were both rescued by all 4 treatment regimens, indicating a wider treatment window pertains for some TBI outcomes.

4. Discussion

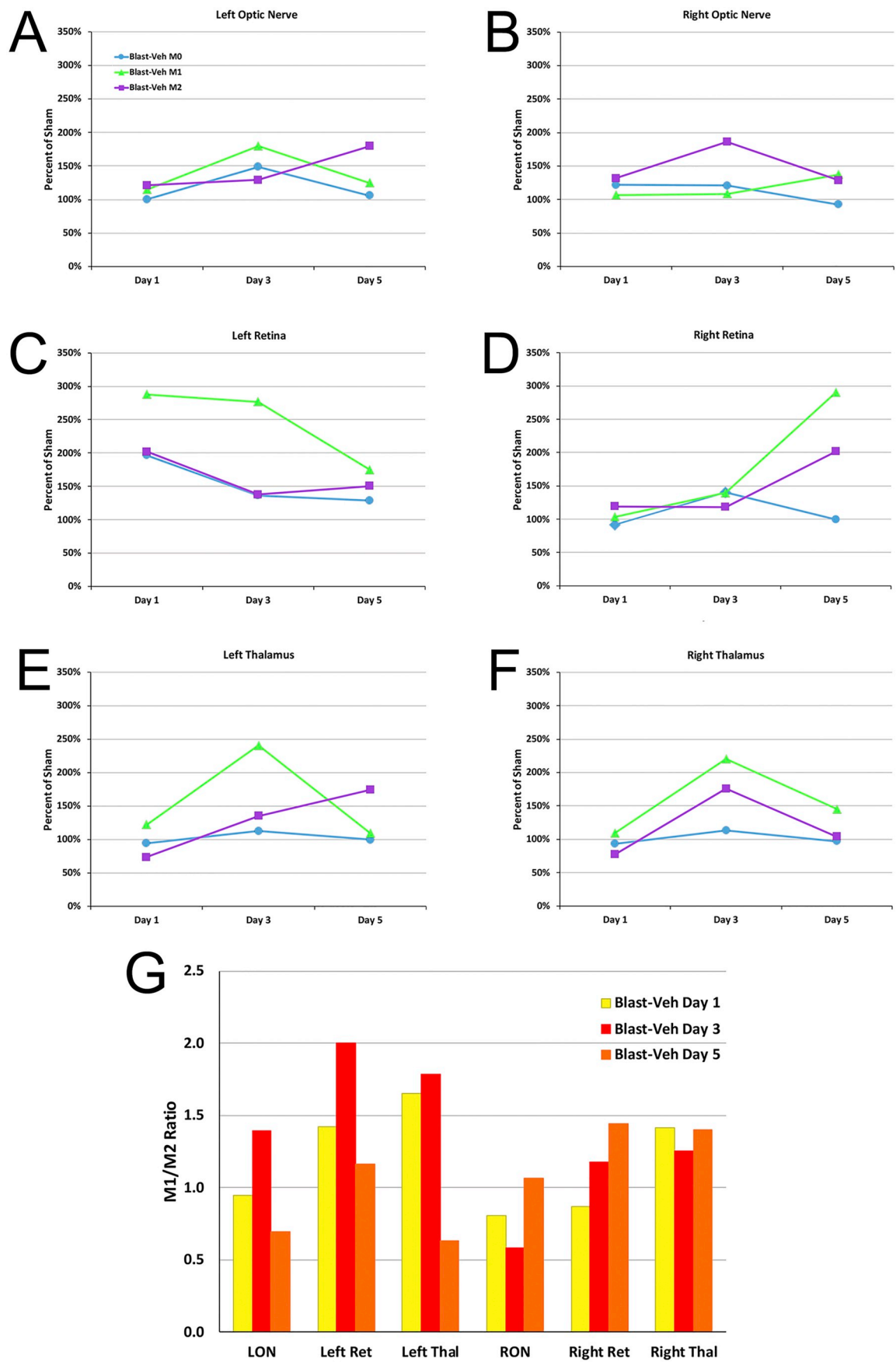
In the work described here, we report that raloxifene rescues a variety of visual deficits and associated pathologies that would otherwise result from TBI. Below we describe these deficits and pathologies, the relationships between them, and their rescue by raloxifene. We then move on to discuss the mechanism of raloxifene action and its potential as a therapy for patients suffering from TBI.

4.1. Visual deficits and pathology associated with blast

As previously described for our left-side focal cranial blast TBI model (Guley et al., 2016), as the blast pressure wave crosses the skull and passes from left to right, the brain is likely to move rightwards in the cranium and then recoil. The associated stretch, stress, and shear forces injure axons, as is evidenced by the presence of swollen axonal bulbs a few days after the injury. The damaged axons are conspicuously associated with microglia that have been activated by molecules released from the axons and their ensheathing myelin. These microglia

are primarily biased toward the pro-inflammatory M1 state, exacerbating the injury (Loane and Kumar, 2016; Donat et al., 2017) and serving as the target of our raloxifene treatment strategy. We have found damaged RGC axons, surrounded by activated microglia, primarily at three sites in our focal cranial blast model: as they exit the left eye to enter the optic nerve, as they traverse the narrow bony optic nerve canal, and as they project along the right optic tract (Guley et al., 2019). Some of the initial axonal injury reflects damage severe enough to progress to degeneration, as ~10% of axons in the left optic nerve are lost by 30 days after blast (here and Guley et al., 2019). The RGC axon loss appears to contribute to the contrast sensitivity deficit for the left eye, based on the significant correlation between these two parameters (here and Guley et al., 2019). In addition, raloxifene treatment rescued both the optic nerve loss and the contrast sensitivity deficit, suggesting that the functional rescue, at least in part, stems from the rescue of optic nerve axons. A similar relationship does not pertain to the right eye, which shows a contrast sensitivity deficit, but little, if any, loss of optic nerve axons (here and Guley et al., 2019). The right eye contrast sensitivity deficit may instead be caused by retinal injury (discussed below), optic nerve dysfunction not manifesting as loss, and/or injury to central visual areas on the left (blast) side of the brain to which the right eye projects. Raloxifene rescue would then result from the amelioration of the relevant perturbation.

The biomechanical forces resulting from the blast pressure wave could potentially cause injury to, not just the optic nerve, but other cranial nerves as well. Of importance for visual function, these include the oculomotor, trochlear, and abducens nerves, which together innervate the six extraocular muscles. Consistent with this possibility, we found a > 30% loss of motoneurons in the oculomotor nucleus (which innervates 4 of the 6 extraocular muscles). That the extent of this neuron loss was the same for the two sides of the brain and considerably greater than the 10% loss of left optic nerve axons raises the additional possibility that oculomotor cell bodies, despite their location some distance from the blast, were directly damaged. Indeed we have previously found bilateral neuron loss in the cortex and striatum (20% and



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Fig. 10. The mean expression of M0, M1, and M2 markers with time after blast. **A–F.** Transcript levels were determined by qPCR and are expressed relative to transcript levels for the same tissue from sham-vehicle mice. The blast-induced changes in transcript levels were the largest for M1 markers, and the smallest for M0 markers. Additionally, M1 marker increases were greater on the left side than on the right side for corresponding tissues at 3 days, but greater on the right at 5 days. **G.** Transcript levels are expressed as M1/M2 ratios to help visualize temporal changes in expression. For the left side, the M1/M2 ratio peaks at 3 days. By contrast, for right side the M1/M2 ratio is higher at 5 days than at 1 or 3 days (optic nerve and retina), or fairly similar at all time points (thalamus).

30%, respectively; Bu et al., 2016), even among cortical neurons with axons projecting locally rather than along a white matter tract. Regardless of the mechanism(s) of injury, the loss of oculomotor innervation would lead to impaired control of the extraocular muscles and thereby probably contribute to the deficits in fixation, pursuit, saccades, and vergence frequently exhibited by human victims of TBI (Goodrich et al., 2013; Capó-Aponte et al., 2017). Importantly, we found that raloxifene treatment prevented the loss of oculomotor neurons, as we have previously shown for SMM-189 in the case of cortical and striatal neurons (Bu et al., 2016), suggesting that microglial activation somehow contributes to neuron loss. Treatment with raloxifene might thereby reduce eye movement dysfunction and the various visual deficits mentioned above in human TBI victims.

Despite the eyes being shielded from the blast, we also observed retinal injury in the form of reductions in peak amplitude of the photoreceptor-driven A-wave for both eyes and in the bipolar-cell driven B-wave for the left eye. The reduction of both ERG components for the left retina is consistent with our previous observations of increased microglial abundance at 3 and 30 days (Guley et al., 2019), upregulation of glial fibrillary acidic protein (GFAP) at 30 days (Guley et al., 2019), and thinning, of inner retina in particular, by 11 weeks in the left eye following left side focal cranial blast (Reiner et al., 2015). The damage to the left eye may originate from the torsion/stretch of soft tissues accompanying the transmission of blast wave through the orbit, and may in turn factor into the impairment of contrast sensitivity. For the right retina, we previously reported two signs of injury: an increase in microglial abundance at 30 days (Guley et al., 2019) and thickening of the inner retina at 11 weeks (Reiner et al., 2015). Pathology for the right eye appears to be delayed and/or progress more slowly than for the left eye (Reiner et al., 2015; Guley et al., 2019) and the underlying mechanism(s) is/are uncertain. One possibility is that the right eye is subjected to compressive forces as the head is pushed toward the right during left side blast (Bricker-Anthony and Rex, 2015). We have not detected retinal detachment or obvious damage to the cornea, as reported after a direct ocular blast at 26-psi (Hines-Beard et al., 2012; Bricker-Anthony et al., 2014, 2017) but not at 15-psi blast (Vest et al., 2019), perhaps because the right side of the mouse head is cushioned by soft foam in our experimental setup. Nonetheless, the thickening of inner retina we have observed, possibly reflecting an underlying edema, may contribute to the right eye contrast sensitivity and visual acuity deficits. Importantly, raloxifene rescued the ERG deficits for both eyes, despite the differing retinal pathologies, suggesting that microglial responses contribute to pathogenesis in the retina and poor visual function in both eyes following TBI.

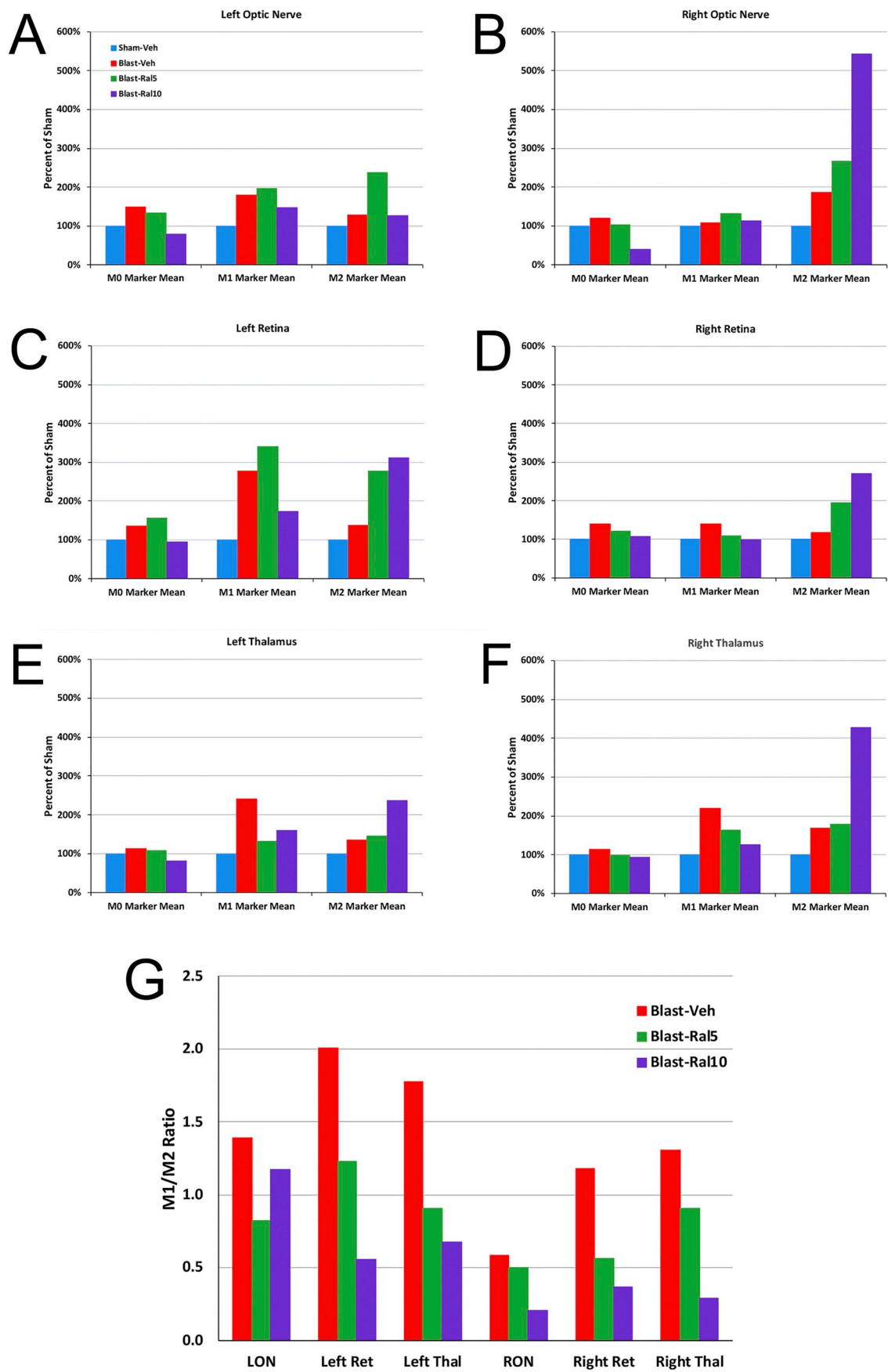
The mice subjected to blast also exhibited increased light aversion and enhanced pupil constriction of the right eye. To help understand the basis of these functional impairments, we focused our retinal analysis on melanopsin-expressing ipRGCs, which constitute 2–3% of the total population of RGCs (Hughes et al., 2013). IpRGCs have been implicated in light aversion (Collison et al., 2015; Matynia et al., 2012) and, through their projections to the olivary pretectal nucleus (Baver et al., 2008), mediate pupillary responses to blue light (Schmidt et al., 2011). Moreover, the downregulation of melanopsin levels by ipRGCs is associated with diminished pupil light responses (Ouk et al., 2016). Here we found ipRGCs in both eyes increased their levels of melanopsin and became smaller in size after blast, with changes for left retina greater than for right retina. Raloxifene rescued the increased light aversion and the enhanced right eye pupil constriction, as well as the upregulation of melanopsin levels and the decrease in ipRGC cell size

for both eyes. One possible scenario is that the increased melanopsin expression contributes to the greater light aversion and to the enhanced pupil constriction for the right eye after blast, while the potential for hyper-pupil responsiveness is counteracted by optic nerve axon loss for the left eye. Alternatively, or in addition, the increased light aversion after blast may stem from a putative similar upregulation of melanopsin expression by the recently identified subpopulation of nociceptive trigeminal neurons localized primarily to the ophthalmic branch of the trigeminal nerve (Matynia et al., 2016), which innervate cornea. Additional trigeminal afferent fibers supplying the conjunctiva, cornea, sclera, and/or uvea may also transmit pain signals that contribute to photophobia (Matynia et al., 2015).

4.2. Microglial modulation by TBI and raloxifene

As described above, microglia in the retina, optic nerve and brain become activated after blast TBI, and confirmed here for the right optic tract. While some investigators have reported benefit using CB2 agonists (Magid et al., 2019), CB2 inverse agonists have the advantage of stabilizing CB2 receptors in an inactive state, which leads to transcriptional changes whereby microglia are biased away from the pro-inflammatory M1 state and toward the protective M2 state (Lunn et al., 2006, 2008; Lawrence and Natoli, 2011; Franco and Fernández-Suárez, 2015). We previously showed that the CB2 inverse agonist SMM-189 has this effect on microglia in vitro using biochemical approaches (Presley et al., 2015; Reiner et al., 2015) and in vivo using immunostaining (Bu et al., 2016; Guley et al., 2019). Importantly, SMM-189 treatment rescued various sensory, motor and emotional deficits that mice otherwise exhibit after TBI and the accompanying axon and neuron loss (Reiner et al., 2015; Bu et al., 2016; Guley et al., 2019). SMM-189, however, has not undergone any human testing, and so the possibility that it could be used as a human TBI therapy is at least a decade off. Similarly, the commercially available CB2 inverse agonist SR144528, although effective in vitro (Presley et al., 2015) and in rescuing deficits in contrast sensitivity (Fig. 13 in the current studies), is not approved for human use. Such impediments do not apply to raloxifene, which is already FDA-approved as a selective estrogen receptor modulator, but also possesses CB2 inverse agonism, as first shown by Kumar and Song (2013) and confirmed here using the AC-TOne assay.

Although we describe microglia here as having two distinct polarization states, we well realize that this is an oversimplification. Rather, microglia appear to be remarkably diverse, comprising a multi-dimensional spectrum of phenotypes when numerous characteristics of individual cells are profiled (Hanisch, 2013; Franco and Fernández-Suárez, 2015; Jha et al., 2016; Morganti et al., 2016; Fernández-Arjona et al., 2017). In the current work, we used qPCR to characterize microglia, so that we could simultaneously assess expression of a greater number of markers than in our previous and current studies using immunostaining (Bu et al., 2016; Guley et al., 2019). While our approach has the disadvantages that we could not examine microglia for this number of markers on an individual basis and that changes in transcript levels (of TGFβ, for example) might in some cases reflect not just microglia, but other cell types as well (Doyle et al., 2010; Dobolyi et al., 2012), it provides a more comprehensive view of the overall inflammatory/protective milieu. Note, however, that seven of the 13 transcripts we examined are unique to microglia (IBA1, Tmem119, P2ry12, CD16/32, IL1β, Ym1, and TREM2). Further, the immunolabeling results we present here indicate that blast and raloxifene



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Fig. 11. The mean expression of M0, M1, and M2 markers at 3 days after blast. **A-F.** Transcript levels were determined by qPCR and are expressed relative to transcript levels for the same tissue from sham-vehicle mice. Expression of M1 markers relative to M2 markers was increased following blast for all tissues except RON. M1 expression levels were decreased by raloxifene at the higher dose, and in some cases at the lower dose. Raloxifene also increased M2 expression in 4 of the 6 tissues, with the higher dose being more effective than the lower dose, except for LON. **G.** Raloxifene reduced the M1/M2 ratio below blast-vehicle for optic nerve, retina and thalamus on both sides, with the higher dose being more effective than the lower dose, with left optic nerve being the only exception.

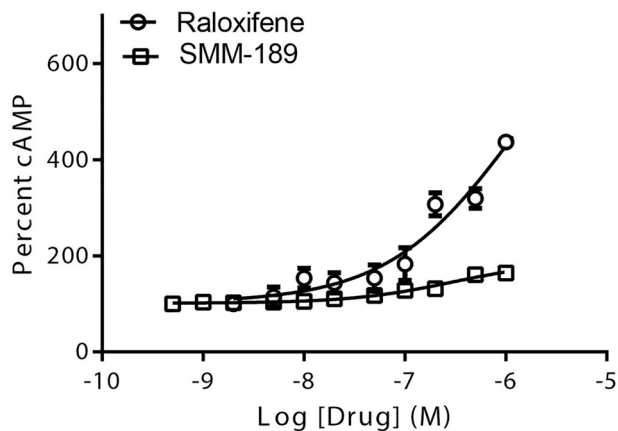


Fig. 12. Comparison of raloxifene and SMM-189 activity in HEK-CNG + CB2 cells using the ACTOne assay. Raloxifene caused a dose-dependent increase in cAMP-driven fluorescence over baseline that was about 5-fold greater than that with SMM-189 at a concentration of 1 μ M.

exert effects on microglia in the right optic tract and are in concordance with the qPCR results for the markers examined in common (IBA1 and CD16/32). In our qPCR studies, we found considerable variation between the different tissues and the sides of the animal (coup-contrecoup) in the time course of changes in microglial markers, yet several patterns were evident. M0 markers changed little after blast, rarely by > 50%, while M1 markers often increased 2–3-fold. M2 marker expression tended to be low initially after blast, especially compared to M1 markers, and later increased. This pattern was most evident for the left optic nerve, retina, and thalamus, for which the M1/M2 ratio peaked at 3 days and declined below the 1-day level by 5 days (Fig. 10G). Thus, the natural progression in microglial behavior for the directly targeted left side appeared to entail M1 activation during the first 3 days after injury, with a gradual decline of M1 markers and an increase in M2 markers by 5 days. Interestingly, a similar pattern of initial M1 activation followed by M2 activation has been reported

following controlled cortical or closed head impact TBI (Morganti et al., 2016; Kumar et al., 2016; Madathil et al., 2018), which produce greater injury than our air blast model. By contrast, M1 expression and the M1/M2 ratio for RON and right retina did not increase until or continued to increase at day 5, indicating a different and slower pathogenic mechanism for the contrecoup side, either because the level of pressure reaching the right side is less or because the tissue distortion caused by left side blast differs for the two sides. Temporal changes in microglial expression for the right thalamus differed from that of the other 5 tissues in that its M1/M2 ratio was high for all 5 days after blast, perhaps because the right thalamus contains the brain continuation of the directly damaged left optic nerve (i.e. the right optic tract) and its central targets, and thus exhibits both coup and contrecoup characteristics.

Our qPCR results further showed that the expression of microglial markers at 3 days after blast was modulated by raloxifene treatment. Both doses of raloxifene reduced the M1/M2 ratio for all tissues below that for blast-vehicle mice and one or both doses reduced the M1 expression below blast-vehicle levels in 5 of the 6 tissues, the higher dose generally being more effective. For example, 10 mg/kg raloxifene decreased M1 expression and increased M2 expression for both sides of retina and thalamus, whereas 5 mg/kg raloxifene effects were limited to increasing M2 markers in the retina, and to decreasing M1 markers in the thalamus. The results for raloxifene effects on the left optic nerve were different than for all the other tissues in that not even the higher dose both decreased M1 expression and increased M2 expression, but it nonetheless rescued the loss of left optic nerve axons. It is possible that treatment with the higher dose for more than the 4 days used in the case of the qPCR studies would be effective in decreasing M1 markers and increasing M2 markers, as treatment continued for 15 days in the case of all the histological assessments and functional testing. Indeed, only the higher dose was able to rescue the increase in light aversion, and microglial activation clearly persists for longer than a few days after blast injury. It should, however, also be noted that for some outcomes, for example right eye visual acuity, the lower dose possibly provided better rescue than the higher dose. The reasons for the variation in the amount of rescue for the two doses we tested are not clear, although they do not appear to reflect any drug toxicity, as the raloxifene-treated

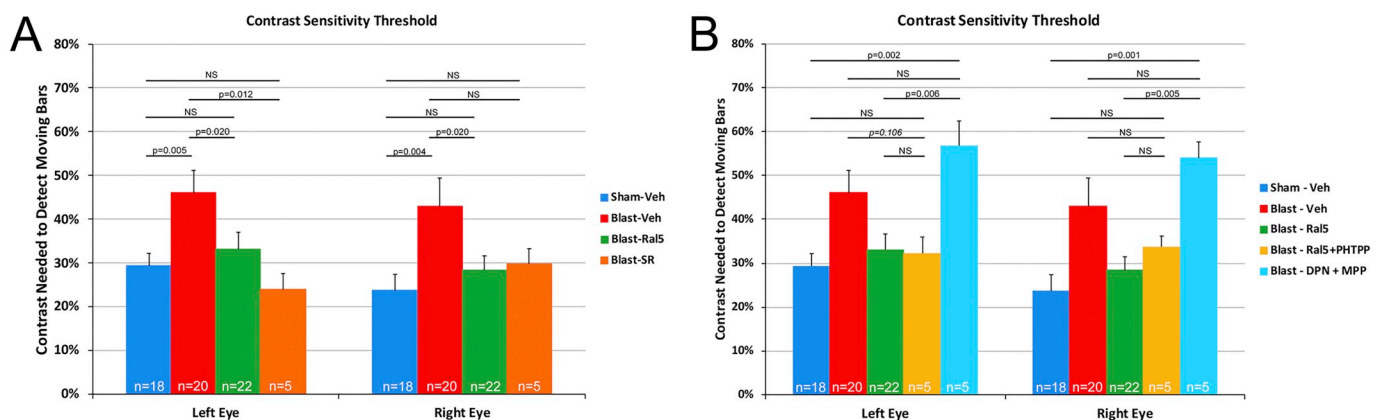


Fig. 13. Comparison of raloxifene with other drugs on the rescue of contrast sensitivity deficits produced by blast TBI. **A.** Comparison of raloxifene and the CB2 inverse agonist SR144528. The SR144528 benefit was similar to that observed with raloxifene. **B.** Comparison of raloxifene with drugs acting at estrogen receptors. Injection of the ER- β antagonist PHTPP immediately following injection of 5 mg/kg raloxifene did not lessen the benefit of raloxifene. Injection of the ER- β agonist DPN followed by the selective ER- α antagonist PHTPP yielded no benefit, rather than duplicating raloxifene action. Statistical comparisons between sham-vehicle, blast-vehicle, and blast-ral5 are the same as in panel A, but for simplicity, are not shown in panel B. N = the number of eyes. Errors bars are SEMs. p values that are close to statistical significance are shown italicized.

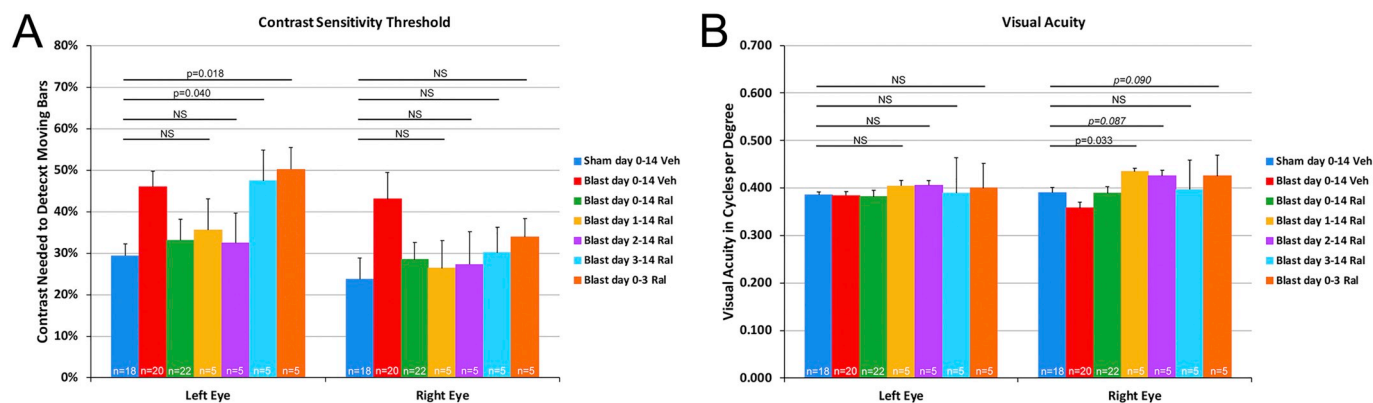


Fig. 14. Effective treatment windows for raloxifene rescue of deficits in contrast sensitivity and acuity. Raloxifene treatment was delayed for 1, 2 or 3 days in separate groups of mice or discontinued starting four days after blast. **A.** The contrast sensitivity deficit for the left eye was rescued even when raloxifene treatment was delayed for 2 days after blast, but not when treatment was either delayed for 3 days or did not continue past the third day. By contrast, the contrast sensitivity deficit for the right eye was rescued regardless of treatment regimen. **B.** The visual acuity deficit for the right eye was rescued by all 4 of the treatment schedules. There was no visual acuity deficit for the left eye. N = the number of eyes. Errors bars are SEMs. p values that are close to statistical significance are shown italicized.

mice (at either dose) did not weigh less than sham or blast-vehicle mice at the time of sacrifice. Moreover, mice have been shown to survive doses as high as 5000 mg/kg without obvious untoward effects (Raloxifene FDA Drug Approval Package, 1999). Nonetheless, optimization of raloxifene dosage will require further attention and would certainly be a component of any future human trials, as discussed in the following section.

4.3. Considerations for human use

In principle, raloxifene benefit could stem, not from its action on CB2 receptors, but rather from its estrogenic effects. This does not seem to be the case, since we found that the ER- β antagonist PHTPP did not block, and the ER- β agonist DPN in combination with the selective ER- α antagonist did not duplicate the raloxifene rescue of the contrast sensitivity deficit after blast TBI. Thus, the benefit we observed with raloxifene in mitigating visual system dysfunction and injury appears to derive from its CB2 actions rather than its estrogenic effects. Moreover, another CB2 inverse agonist, SR144528, duplicated the benefit of SMM-189 and raloxifene for the contrast sensitivity deficit after TBI in our blast model.

Raloxifene was first approved by the FDA to treat postmenopausal osteoporosis nearly twenty years ago and has since been approved to reduce the risk of invasive breast cancer in postmenopausal women. Importantly, raloxifene has no evident adverse hormonal side effects and is safe for use in men, having been tested in clinical trials to treat bone fracture (Uebelhart et al., 2009), prostate cancer (Smith et al., 2004), and acromegaly (Dimaraki et al., 2004). The present findings show that raloxifene reduces visual deficits and visual system injury after focal cranial blast TBI in mice. Although the doses we used here are 5–10-fold higher than that used to treat osteoporosis and cancer in humans, the higher doses are also known to be safe in humans (Draper et al., 1996). Moreover, according to the FDA filing for raloxifene (Evista), no mortality was seen after a single oral dose in rats or mice at 5000 mg/kg or in monkeys at 1000 mg/kg. It is additionally important to note that we have shown that SMM-189 is effective in reducing increased fearfulness and depression stemming from brain trauma in our mouse model of focal cranial blast TBI (Reiner et al., 2015; Bu et al., 2016), and our preliminary results suggest raloxifene has similar benefit in this model. Even mild TBI in humans commonly leads to persistent and psychologically debilitating symptoms that can include anxiety, fearfulness, and depression (Bombardier et al., 2010; Bazarrian et al., 2013). In this regard then, a regimen of raloxifene delivered in the aftermath of head trauma may also help reduce the adverse psychological outcomes from TBI in humans.

An important consideration for treating TBI in humans is when treatment must be begun and how long it must be continued. Consistent with the peak pro-inflammatory behavior of microglia in left retina and optic nerve during the first 3 days after injury, we found that treatment must be initiated no later than day 3 for maximum benefit. Microglial activation in the right eye and optic nerve appears to develop more slowly, and at least some right eye deficits could be rescued even with treatment begun at 3 days. We do not know if a full two weeks of treatment is needed for full benefit and, while the higher dose generally appears more effective, further work is required to optimize dosage. Taken together our findings that raloxifene rescues functional deficits and pathological changes following TBI in our mouse model support further consideration of raloxifene as a TBI therapy. With its FDA approval as safe for humans, a phase 2 clinical trial could start at any time, and if found effective, raloxifene could be repurposed for human use relatively quickly.

5. Conclusions

We found that 5–10 mg/kg raloxifene delivered daily for two weeks after blast mitigates or eliminates visual deficits that would otherwise be exhibited and decreases retinal, optic nerve, and oculomotor nucleus pathology, with the higher dose generally more effective. This rescue with raloxifene appears attributable to its CB2 inverse agonism and is accompanied by a biasing of microglia from the harmful M1 to the helpful M2 state. Raloxifene treatment is still effective even when delayed until 48 h after TBI. Our studies provide a strong basis for phase-2 testing of raloxifene efficacy in reducing TBI deficits and pathology.

Declaration of Competing Interest

None.

Acknowledgements

Special thanks to Marion Joni, Conor Dorian, and Dr. Natalie Guley for technical assistance. The research was supported by The Methodist Hospitals Endowed Professorship in Neuroscience (AR), UTHSC Neuroscience Institute (CL), NIH Medical Student Research Fellowship Program at UTHSC (JBD & JHD), and DoD grant W81XWH-16-1-0076 (AR, MGH).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

doi.org/10.1016/j.expneurol.2019.113063.

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