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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 was recently found to show noteworthy CB2 inverse agonism. In the current studies, we are testing the benefit of raloxifene for reducing visual deficits and retinal and optic nerve damage from mild TBI and closed-globe ocular injury in mice. In the fourth year of the project, we have been determining if raloxifene reduces visual deficits and pathology, when delivered daily after TBI produced using dorsal cranial impact injury or 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, central visual structures and oculomotor nerves. We found that raloxifene for impact TBI and OBI reduces functional deficits, and mitigates the underlying visual system pathology and inflammatory response, seemingly by modulating microglia to a more beneficial state. Our proposed animal studies continue to show benefit of raloxifene in preventing visual deficits and injury after brain and/or ocular trauma, and they support its testing in phase 2 human clinical trials to determine its efficacy in treating visual injury after brain and/or eye trauma, leading to its approval for 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

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Table of Contents

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 was recently found to also show noteworthy CB2 inverse agonism. In the current studies, we are testing the benefit of raloxifene for reducing visual deficits and retinal and optic nerve damage from mild TBI and closed-globe ocular injury in mice. In the case of TBI, our plan is to use two different standardized models - our focal blast model of mild TBI, and an impact model of mild TBI that also yields optic nerve injury. Our goal is to determine if raloxifene reduces visual deficits and pathology, when delivered daily after TBI produced in both cases. In the case of ocular blast injury, our goal is likewise to determine if raloxifene reduces visual deficits and pathology, when delivered daily after the trauma. Visual system injury and its abatement with raloxifene is being 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, central visual structures and oculomotor nerves. In addition, morphological and biochemical analysis is being used to assess 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 is being determined as well. If our proposed animal studies show benefit of raloxifene in preventing visual deficits and injury after brain and/or ocular trauma, it will next be tested in phase 2 human clinical trials to determine its efficacy in treating visual injury after brain and/or eye trauma, speeding its eventual 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.

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

Accomplishments

Our efforts during the fourth year of the project focused on publishing the studies of Task 1 (Aim 1 – Blast TBI studies), and on pursuing studies of Task 2 (Aim 2 – Impact TBI studies) and Task 3 (Aim 3 – ocular blast injury studies). The studies of Task 1 have been published, and those of Tasks 2 and 3 are nearly complete. The completed studies from both Tasks 1 and 2 show the benefit of raloxifene in reducing visual deficits after TBI, and those from Task 3 show its benefit in ocular blast injury. We separately discuss progress below on these three Tasks.

Major Goals for the First Year of the Project as listed in SOW:

Overall Major Task 1: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a mouse blast model of mild 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 mild TBI, when delivered daily after the TBI event. This was to be carried out in the first 6 months of the project. We have in fact shown that raloxifene reduces visual deficits and pathology in our focal blast model of mild TBI. Most of this functional analysis was completed during year 2 of the project, and the remainder was completed in year 3. The pathology studies were also largely completed during year 2, but some were completed in year 3. This subtask is 100% complete.

Subtask 1-2: Determine if raloxifene benefit in our focal blast model of mild TBI stems from modulation of microglia to the beneficial M2 phenotype. These studies were initiated during the second six months of the project, and they were completed during year 3.

Subtask 1-3: Determine treatment window for the raloxifene benefit in our focal blast model of mild TBI. This was begun in year 1 and completed during year 2 of the project.

Major Goals for the Second Year of the Project as listed in SOW:

Overall Major Task 2: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a mouse impact model of mild 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 focal impact model of mild TBI, when delivered daily after the TBI event. The functional and pathological studies have been completed. Our studies show that raloxifene reduces visual deficits after impact TBI, as detailed below. This subtask is 100% complete.

Subtask 2-2: Determine if raloxifene benefit in a focal impact model of mild TBI stems from modulation of microglia to the beneficial M2 phenotype. This task was to be completed during the second 6 months of the second year of the project. These studies are nearly completed.

Subtask 2-3: Determine treatment window for the raloxifene benefit in a focal impact model of mild TBI. These studies have not been completed but the issue they address has largely been answered by the findings in Subtask 1-3.

Major Goals for the Third Year of the Project as listed in SOW:

Overall Major 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 have found in these yet ongoing studies that raloxifene reduces visual deficits and pathology after OBI, as detailed below. The functional studies have been completed, and the pathology studies are in progress. We estimate this subtask is 95% complete.

Subtask 2-2: Determine if raloxifene benefit in a model of OBI stems from modulation of microglia to the beneficial M2 phenotype. These studies are ongoing, are 90% complete, and will be completed this summer.
Subtask 2-3: Determine treatment window for the raloxifene benefit in a model of OBI. These studies are ongoing, but the issue they address has largely been answered by the findings in Subtask 1-3.

What was accomplished under these goals?
We discuss accomplishments under Tasks 1, 2 and 3 below, beginning with Task 1.

Task 1 – Raloxifene Benefit for Blast TBI
1) Major Activities. We have 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 have evaluated raloxifene benefit for the following visual deficits or abnormalities after blast mild TBI: 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. These studies were initiated in the first year and have been completed during the third year. Morphological studies assessed the rescue by raloxifene of structural damage to the visual system by blast mild TBI (Subtask 1-1). Neurochemical and biochemical studies have 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 have assessed the effective treatment window for raloxifene (Subtask 1-3). We published a manuscript in Experimental Neurology based on these findings, 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 have 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 have conducted studies to evaluate 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 have assessed the rescue by raloxifene of structural damage to the visual system by blast mild TBI (Subtask 2-1). Neurochemical and biochemical studies have 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) are addressed by Subtask 1-3.
Task 3 – Raloxifene Benefit for OBI
1) Major Activities. We have conducted behavioral, electrophysiological, and morphological studies in mice to evaluate the benefit of raloxifene in alleviating visual deficits and visual system injury after single blast and 5x blast OBI (Subtask 3-1). We have conducted studies that to evaluate 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 are ongoing to assess the rescue by raloxifene of structural damage to the visual system by 1x and 5x OBI (Subtask 3-1). Neurochemical and biochemical studies are ongoing to assess 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). Functional studies will assess the effective treatment window for raloxifene (Subtask 3-3).

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 have 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 have 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 have 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 have 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 mild TBI caused by an impact device (Subtask 2-1). In the case of behavioral and physiological assessments, we have 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 has been 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 largely been achieved. In the case of the modulatory influence of raloxifene on microglia, our goal is to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). Functional studies were planned to determine the effective treatment window for raloxifene (Subtask 2-3), but are 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 have sought to determine if raloxifene shows a benefit for 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. As detailed below, these objectives are largely achieved. In the case of our morphological studies, our goal is to determine if raloxifene rescues structural damage to the visual system caused by OBI (Subtask 3-1). In the case of the modulatory influence of raloxifene on microglia, our goal is to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 3-2). Functional studies are intended to determine the effective treatment window for raloxifene (Subtask 3-3), but are addressed by Subtask 1-3.

3) Significant Results or Key Outcomes. In the description below, we briefly summarize our findings for blast TBI, as presented in our published paper, Honig et al., 2019. Additionally, our findings from the fourth year for impact TBI and OBI are summarized below in detail.

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 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 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). These additional findings are detailed below in section 1B.

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.

1B. Additional Raloxifene Benefit After Blast TBI. Our studies in mice showed a considerable benefit of raloxifene treatment after blast TBI for visual system dysfunction and injury. 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 (p=0.000002) 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 (as shown in Figure 1 below).

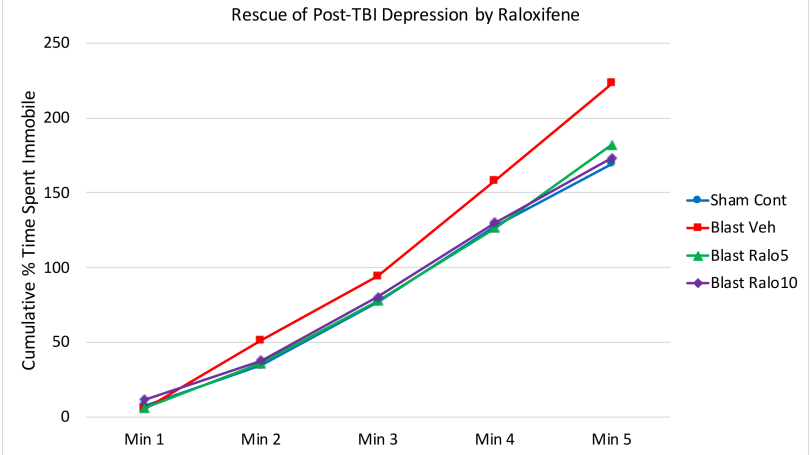


Figure 1. Effects of 50-psi blast compared to 0-psi sham blast on the tail suspension test of depression six to eight weeks after blast. Data are presented as cumulative immobility per consecutive one-minute block. Note that immobility, reflecting depression, is increased in blast mice compared to sham blast mice. By contrast, depression is normalized in blast mice treated with either 5 mg/kg and 10 mg/kg raloxifene.

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 (Figure 2 below), we confirmed that blast mice showed significantly greater fear responding during the tone conditioned stimulus (CS) presentation than did sham mice (p=0.002705). The lower dose of raloxifene did not prevent this, as their responses were significantly greater than sham (p=0.001778) and similar to blast (p=0.954492), but the higher dose did, as their fear responses were not different from sham (p=0.457874), but significantly less than in blast mice (p=0.02290). Moreover, the two raloxifene groups differed significantly from each other in their fear responding (p=0.018681). Thus, raloxifene at the higher dose alleviated the depression and fearfulness increases 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 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.

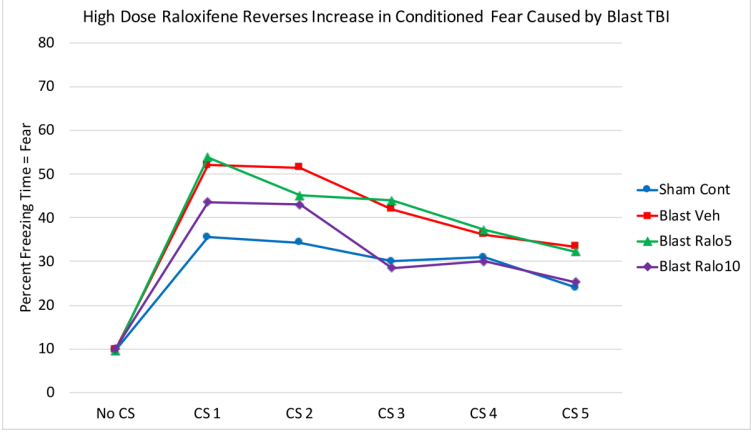


Figure 2. Effects of 50-psi blast compared to 0-psi sham blast in mice compared to 50-psi mice treated with raloxifene on fear conditioning, as revealed by fear responses to the conditioned stimulus (CS) alone, the day after acquisition of conditioned fear to an auditory CS signaling impending shock, 6-8 weeks after blast. Data are presented for the one-minute block prior to CS presentation, and for the mean of successive blocks of 3 CS presentations. Blast significantly increases fear responses above sham, and 10 mg/kg raloxifene reverses this. Fear responses are aligned on the last 3 pre-CS blocks.

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 mild TBI caused by an impact device (Subtask 2-1). In the case of behavioral and physiological assessments, we have 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 has been 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 largely been achieved. In the case of the modulatory influence of raloxifene on microglia, our goal is to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). Functional studies were planned to determine the effective treatment window for raloxifene (Subtask 2-3). 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 have also begun drafting a manuscript on 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. 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.

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

2-1A. Raloxifene Benefit – Scotopic Light-adapted ERG A-wave and B-wave after Impact TBI. During period 13, we completed analysis of scotopic flash-evoked ERG data on the effect of impact TBI and raloxifene at 10-12 weeks after the impact TBI or sham impact, bringing the total number of animals to 15 sham impact mice treated with vehicle, 17 impact TBI mice treated with vehicle, 17 impact mice treated with 5 mg/kg raloxifene, and 14 impact mice treated with 10 mg/kg raloxifene. Left eye data did not differ from right eye data, as expected given the dorsal midline nature of the impact TBI, and we thus pooled left and right eyes per group. We observed that the mean post-blast A-wave peaks in the impact TBI mice with vehicle at all light intensities tested were similar to those in the sham impact mice, and no significant difference was seen between these two groups by ANOVA over the last three light intensities, at which the A-wave becomes manifest (Fig. 3A). This was unchanged by 10 mg/kg raloxifene, but 5 mg/kg raloxifene slightly but significantly enhanced the A-wave response over the three brightest light intensities ($p=0.00059$). Although no impact TBI-related deficit was seen for the A-wave, the ERG results for the B-wave show that the average post-blast B-wave peak amplitudes were reduced in vehicle-treated impact TBI mice compared to the vehicle-treated sham mice (Fig. 3B), and differed significantly by ANOVA for the brightest 6 intensities ($p=0.000017$). Raloxifene at 5 mg/kg yielded prominent rescue of this B-wave deficit, and in fact, the responses across the brightest 6 intensities were significantly greater in the raloxifene-treated mice than even in the sham mice ($p=0.0437$). They were also significantly much greater than in the vehicle-treated impact TBI mice ($p=0.0001 \times 10^{-6}$). Surprisingly, raloxifene at 10 mg/kg did not rescue the B-wave deficit, as the responses differed significantly from sham ($p=0.000002$), but not from the vehicle-treated impact TBI mice ($p=0.4652$).

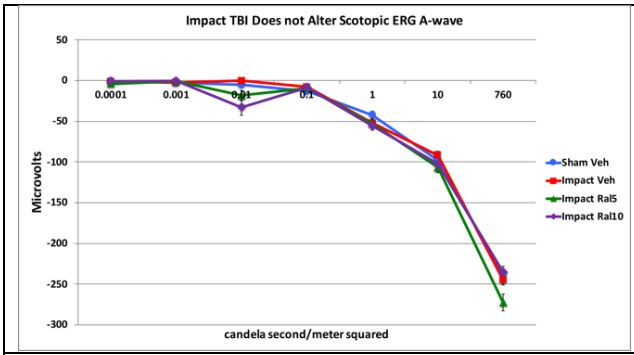


Figure 3A. Effect of dorsal midline impact TBI on the A-wave of the flash-evoked scotopic ERG in sham impact, vehicle treated impact TBI mice, and raloxifene treated impact TBI mice. Impact TBI had no significant effect on the A-wave, but the higher raloxifene dose enhanced the A-wave response.

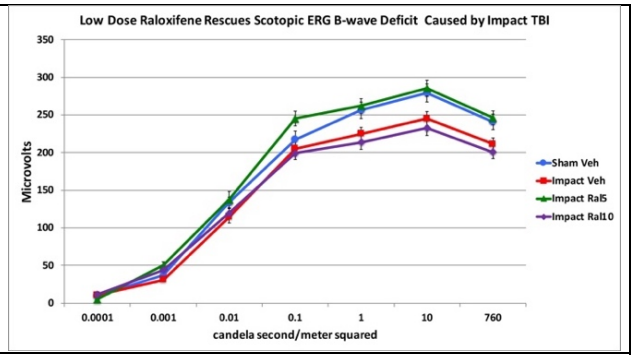


Figure 3B. Effect of dorsal midline impact TBI on the B-wave of the flash-evoked scotopic ERG in sham impact, vehicle treated impact TBI mice, and raloxifene treated impact TBI mice. Impact TBI significantly reduced the B-wave, and raloxifene at 5 mg/kg but not 10 mg/kg rescued this.

2-1B. Raloxifene Benefit – Light Aversion in Impact TBI Mice at 160 Days after TBI. We evaluated Light-Dark Box behavior for impact TBI mice at 160 days post-TBI. Increased light aversion compared to sham was manifest for the impact-vehicle mice, which was not rescued by raloxifene at the 5 mg/kg. Impact TBI mice that received the higher raloxifene dose showed that (like for blast TBI) the higher dose prevents the significant increase in light aversion caused as a long term consequence after impact TBI. In fact, light aversion in the impact TBI mice receiving 10 mg/kg raloxifene showed even less light aversion than the sham mice did. The results are illustrated in Figure 4 below.

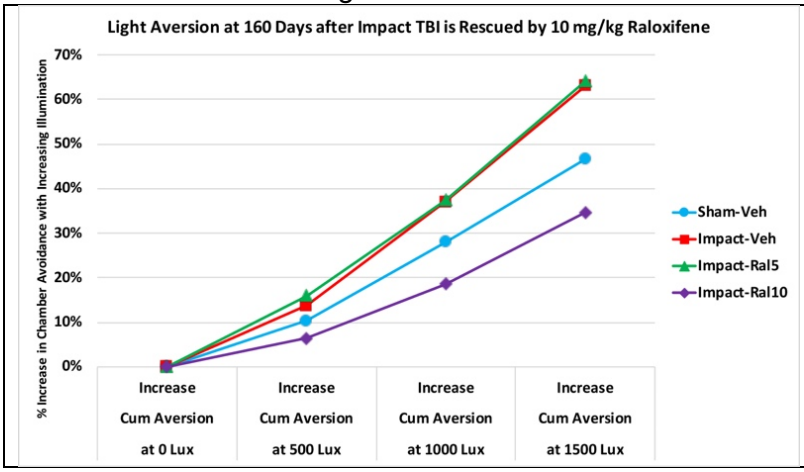


Figure 4. The graph to the left plots cumulative avoidance of enclosed chamber occupancy as its illumination increases, with light aversion thus plotted as increasing with increasing illumination. This plot shows that impact TBI (n=20) increases enclosed chamber avoidance as illumination increases at 160 days after TBI, compared to sham TBI mice (n=16). Thus, the impact TBI mice show more light aversion than sham mice by 160 days post TBI, and this is not rescued by raloxifene at 5 mg/kg (n=17), but it is rescued by raloxifene at 10 mg/kg (n=14).

2-1C. Raloxifene Benefit – Pupil Light Reflex after Impact TBI. Because the TBI impact was dorsal midline and equally affects both eyes, the eyes were pooled within group for the analysis of effects of impact TBI on the pupil light reflex (PLR). These studies were carried out at about 28 weeks (200 days) after impact TBI, and they were completed by analysis of a final set of mice during period 13 of the project. The final tally involved analysis of both eyes in 21 sham-vehicle mice, 25 TBI-vehicle mice, 22 TBI-5mg/kg raloxifene mice, and 14 TBI-10mg/kg raloxifene mice. In brief, we found that pupil diameter was significantly 20-30% greater in impact TBI-vehicle mice before, during and after both red or blue light compared to sham mice, but percent constriction from baseline was not significantly different than in sham. Thus, the pupil was excessively dilated after impact TBI at rest, but neither the relative magnitude of the dynamic photoreceptor-driven constriction to red light nor the dynamic intrinsically photosensitive retinal ganglion cell-driven constriction to blue light was lessened. The 5 mg/kg raloxifene dose did not rescue enlarged resting pupil size after impact TBI, nor did it rescue the excessive dilation during blue light. There was, however, some rescue of the excessive dilation

during red light. Nonetheless, percent constriction from baseline was normal in impact TBI mice treated with 5 mg/kg raloxifene. For impact TBI mice treated with 10 mg/kg raloxifene, resting pupil size was again significantly greater than in sham, but pupil size during and after red light, and during and after blue light was 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 extent of constriction caused by red light, and yielded hyperconstriction to blue light. As a result of this differential pattern of rescue with 10 mg/kg raloxifene, the percent pupil constriction from baseline in response to red or blue light was significantly greater in TBI mice treated with 10 mg/kg raloxifene than in sham mice. Thus, as with blast TBI, raloxifene at 10 mg/kg, but much less so at the lower dose, rescued a pupillary defect caused by TBI. These findings are illustrated in Figures 5A and 5B below.

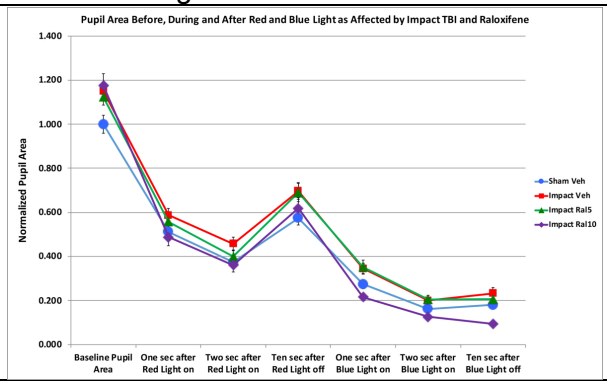


Figure 5A. Effect of impact TBI on pupil area before, during and after exposure to red or blue light. Resting pupil area is greater than in sham in the impact TBI mice, regardless of treatment. Although the pupil constricts well in response to red and blue light in impact TBI mice, the absolute magnitude of the constriction is reduced. Raloxifene at both doses rescues the deficit in absolute constriction for red light, but only the higher dose rescues the deficit in absolute constriction to blue light caused by impact TBI.

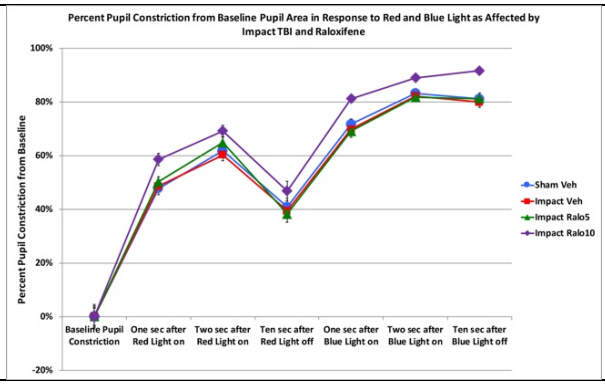


Figure 5B. Effect of impact TBI on percent pupil constriction from baseline during and after exposure to red or blue light, 100% being complete pupil closure and 0% being baseline pupil area. The percent constriction in response to red light and blue light does not differ significantly from sham for either the TBI-vehicle mice or the TBI-5mg/kg raloxifene mice. Thus, although pupil size is greater after impact TBI, the per cent constriction is no different than in sham. Percent constriction was, however, enhanced in 10mg/kg mice.

2-1D. 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 complete, we harvested optic nerves and performed blinded computer-assisted axon counts. Both the left and right optic nerves showed substantial axon loss in the TBI-vehicle group, and we thus pooled left and right optic nerves within group in our further analysis (note that impact TBI is dorsal midline cranial and the effects are expected to be bilaterally symmetrical). We found a significant and substantial loss (21.8%) of optic nerve axons in the TBI-vehicle optic nerves compared to the sham-vehicle optic nerves (p=0.0000009) (Figure 6). By contrast, no significant loss was seen in the TBI – 5 mg/kg raloxifene nerves (p=0.083598), although axon abundance remained somewhat less (7.4%) than in sham. Raloxifene at 10 mg/kg was more effective for axon rescue after TBI, showing no significant loss and an axon abundance indistinguishable from that in sham impact optic nerves (p=0.84165). Moreover, optic nerve axon counts for both the 5 mg/kg impact TBI nerves (p=0.000751) and the 10 mg/kg impact TBI nerves (p=0.000025) were significantly more than in the TBI-vehicle optic nerves.

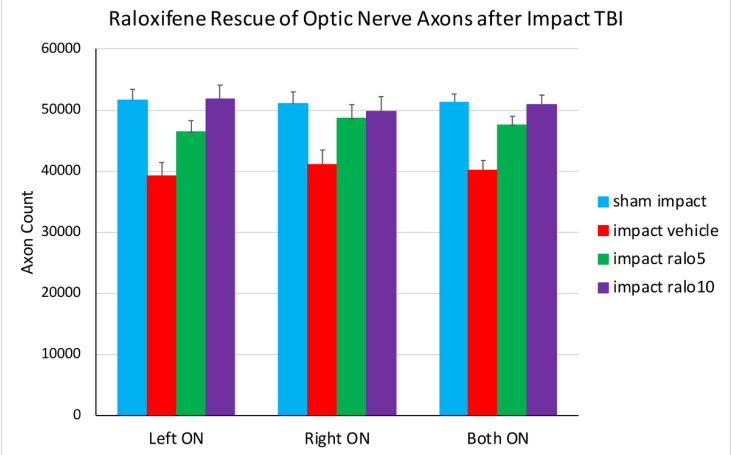


Figure 6. The graph to the left shows the results for blinded computer-assisted axon counts for left and right optic nerves in impact TBI mice, separately and combined. Because the impact was dorsal midline and equally affects light and right optic nerves, the axon loss was very similar for left and right. The results indicate significant loss of axons in mice receiving vehicle after impact TBI, but no significant loss in mice receiving either 5mg/kg or 10 mg/kg raloxifene after impact TBI. Thus, raloxifene for 2 weeks post TBI greatly lessens optic nerve axon loss, with the higher dose eliminating detectible loss.

2-1E. 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 have examined the oculomotor nucleus in our TBI mice using immunolabeling for choline acetyltransferase (ChAT) to detect oculomotor neurons. We have 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. As in our prior studies of impact TBI as well as in our prior blast studies of oculomotor nucleus, data for the oculomotor nucleus of the two sides of the brain were pooled, as they did not differ. No significant reduction in oculomotor nucleus area was seen after impact TBI in either the vehicle or 5 mg/kg raloxifene treated mice (Figure 4 below), although area was significantly 11% reduced for oculomotor nucleus in mice treated with 10 mg/kg raloxifene (p=0.00448). Oculomotor neuron abundance also did not differ among sham impact, untreated impact TBI mice, and impact mice treated with 5 mg/kg raloxifene. Oculomotor neurons were slightly more abundant in the mice treated with 10 mg/kg raloxifene than in sham mice (p=0.02263). Thus, impact TBI did not cause oculomotor neuron loss or oculomotor area reduction. As a result, raloxifene rescue could not be assessed, although raloxifene clearly did not make the effect of impact TBI on the oculomotor nucleus worse.

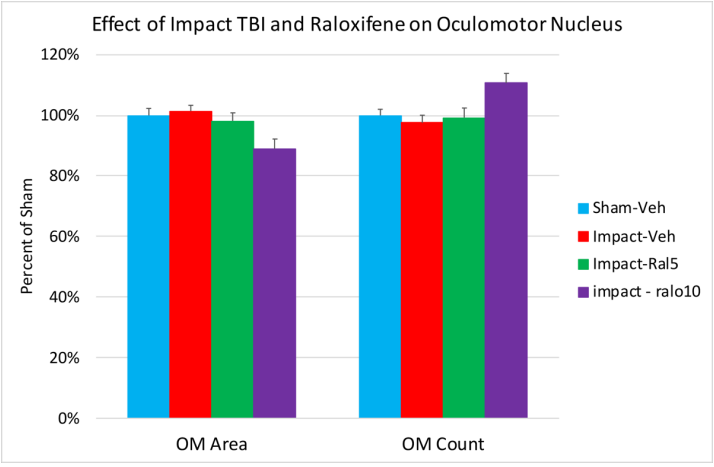


Figure 7. The graph to the left shows the results for blinded computer-assisted analysis and neuron counts for left and right oculomotor nucleus in impact TBI mice. Because the impact was dorsal midline and equally affects the oculomotor nuclei of both sides of the brain, their results were combined for the analysis shown in the graph to the left. The results indicate no reduction in oculomotor nucleus (OM) area after impact TBI. They also show no significant loss of OM neurons in mice receiving vehicle after impact TBI, as well as in mice receiving raloxifene after impact TBI at either dose. Thus, raloxifene for 2 weeks post impact TBI did not obviously worsen long term OM neuron injury, but in the absence of oculomotor nucleus injury rescue with raloxifene could not be assessed.

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

2-2A. Raloxifene Benefit in Mice with Impact TBI – Biochemical Studies. We performed studies during Year 4 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. This system is available at the J.S. and Bobbi Allen Gene Expression Analysis Laboratory in the Cancer Research Building of UTHSC. As the other approach, we used TaqMan qPCR, as we had in our studies of blast TBI (Honig et al., 2019). 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 (Honig et al, Exp. Neurol. 2019). For impact TBI, because the injury is dorsal midline and thus expected to affect the sides of the brain and the two eyes equally, in our analysis of retina, optic nerve and thalamus, the two sides were pooled.

We assessed the following microglial markers by NanoString: 1) for M0: IBA1, Mertk, P2ry12, SPARC, and Tmem119; for M1: CD16, CD32, IFN-gamma; IL1β, IL6, iNOS, CD86, IL12p40, and Tlr2; and for M2: Arg-1, CD206, IL10, Trem2, YM1, Fizz1, CD36, IL13, IL4rα. For optic nerve we found that, averaging the full set of M0 markers, that impact TBI increased M0 marker expression 22.0% above sham. Both doses of raloxifene reversed this increase. In the case of the M1 markers in optic nerve, impact TBI increased their expression by 49.5%. The four daily raloxifene treatments normalized M1 expression for the lower dose but not the higher dose. For the M2 markers, we did not observe increased average expression with either raloxifene dose. Examination of the raw data revealed, however, that many of the M2 markers that were informative in our blast TBI study, as detected by qPCR and reported in Honig et al (2019), appeared to have expression below a reliable detection limit for NanoString. Excluding the low expressing genes did yield an M2 marker increase with the higher raloxifene dose. In the case of retina, NanoString revealed that impact TBI increased M0 marker expression 68.2% above sham, with both doses of raloxifene reversing this increase. In the case of the M1 markers in retina, impact TBI increased their expression by 31.6%, and again both raloxifene treatments normalized the M1 expression, but the lower dose more so. Neither dose increased M2 marker expression above that in sham and impact mice. Again, this may stem from expression below a reliable NanoString detection limit for the more informative M2 genes. Finally, impact TBI did not increase M0 or M1 markers in thalamus, and raloxifene did not increase M2 markers in thalamus, by NanoString. These NanoString studies show, nonetheless, that for optic nerve and retina, raloxifene normalizes elevated M0 and M1 marker expression caused by impact TBI, especially the lower dose.

Our qPCR analysis for retina showed that impact TBI increased expression of the M0 markers (P2ry12, Tmem119, TGFβ and IBA1) and M1 markers (CD32, IL1β, IFNγ, iNOS and TNFα) examined, as also true for the NanoString analysis. Impact TBI decreased M2 marker expression (Arg-1, YM1, Trem2, and IL10), resulting in a 3.3 M1/M2 ratio for impact TBI. The lower raloxifene dose greatly reduced M0 and M1 marker expression below even sham levels but did not increase M2 marker expression, resulting nonetheless in a much lower M1/M2 ratio (1.2) than for impact TBI – vehicle mice. The higher raloxifene dose normalized M0 but not M1 marker expression, but did nearly double M2 expression compared to the impact – vehicle mice, resulting in a lower M1/M2 ratio than in the impact TBI mice (1.7). Note that for the markers

examined with NanoString, impact increased the M1/M2 ratio above sham, and both raloxifene doses reduced it below impact alone levels. In the case of optic nerve, qPCR analysis showed that impact TBI increased M0 and M1 marker expression but not M2 marker expression in impact TBI – vehicle mice, but the higher raloxifene dose substantially increased M2 marker expression. In the case of thalamus (which contains optic tract), the higher raloxifene dose substantially increased M2 marker expression, but impact TBI alone did not. The overall qPCR and NanoString results for the effects of impact TBI and raloxifene treatment 3 days after TBI are consistent with a modulatory effect on microglia that mitigates their M1 features and increases their M2 features. The effects are, however, not as great as at the same time point for blast TBI. This may reflect a slightly different time course of injury after dorsal cranial impact than after left side focal cranial blast. It may be that studies at a later time point after impact TBI, after more days of raloxifene treatment, would reveal a stronger M2 biasing effect of the drug. In any case, our functional and morphological data show a strong rescuing effect with the higher dose of raloxifene when delivered for two weeks post injury.

2-2B. Raloxifene Benefit after Impact TBI – Effects on Retina, Optic Nerve and Optic Tract Microglia. We have also created an additional set of impact TBI mice, consisting of six sham mice, 8 TBI-vehicle mice, 8 TBI-ralo5 mice, and 8 TB-ralo10 mice, that were sacrificed at 3 days after TBI and are being used for immunohistochemical analysis of axon injury and microglial activation state in retina, optic nerve and optic tract. As part of assessing microglial activation, we are determining if blast shifts microglia toward expression of M1 markers, and raloxifene shifts such blast-affected microglia toward M2 marker expression.

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). As detailed in our Progress Report for year 3, 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 during year 4 show slight bilateral hyperconstriction in response to red light that is rectified best by the higher raloxifene dose. Because the functional deficits tended to be small after single blast 25-psi OBI, we subsequently 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 have further assessed their functional outcomes with and without raloxifene during year 4.

Subtask 3-1: Functional and Morphological Studies of Ocular Blast Injury (OBI)

3-1A. Raloxifene Benefit – Pupil Light Reflex after 1x OBI. These studies were carried out at about 26 weeks (180 days) after single 25-psi OBI, and were completed during period 13 of the project, and in total involved analysis of both eyes in 14 sham-vehicle mice, 14 1x OBI-vehicle mice, 15 1x OBI-5mg/kg raloxifene mice, and 15 1x OBI-10mg/kg raloxifene mice. Surprisingly, left eye 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 rescued with the raloxifene, as detailed below.

Figure 8A below shows pupil area data 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-5mg/kg raloxifene, or the 1x OBI-10mg/kg raloxifene mice, although the pupil tended toward being slightly more dilated in 10 mg/kg 1x OBI mice ($p=0.0886$). During

red light exposure, the constricted pupil size was largely similar for all groups, but there was a trend toward increased constriction from baseline in the 1x OBI-Vehicle mice (p=0.0902). After red light off but before blue light on, neither absolute pupil size nor percent constriction from pre-red light baseline (Fig. 8B) differed between the groups for the left eye. Similarly, during blue light and after, 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 (p=0.0060) and in terms of percent constriction from pre-red light baseline (p=0.0149). 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 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.

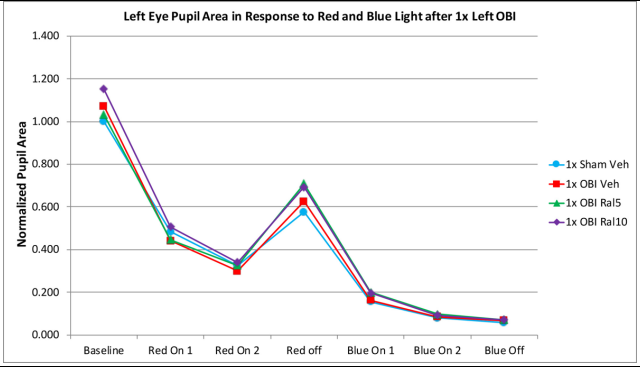


Figure 8A. Effect of left eye single blast OBI on left eye pupil area before, during and after exposure to red or blue light. Pupil area was slightly less than in sham in the OBI-vehicle mice during red light exposure, but not blue light exposure. Raloxifene at both doses rescued the slight hyperconstriction to red light in the OBI-vehicle mice.

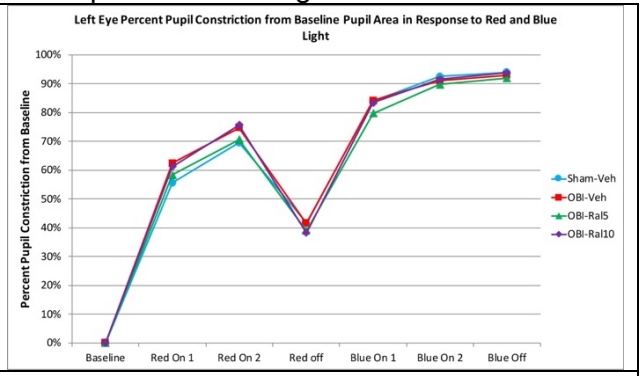


Figure 8B. Effect of left eye single blast OBI on percent left eye pupil constriction from baseline during and after exposure to red or blue light, with 100% being maximal constriction. The percent constriction in response to red light but not blue light was slightly enhanced by OBI. The lower raloxifene dose rescued the hyperconstriction to red light, but yielded a slightly reduced constriction to blue light.

Figure 9A below 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 (p=0.17508), 1x OBI-5mg/kg raloxifene (p=0.82823), or 1x OBI-10mg/kg raloxifene mice (p=0.28674). Thus, the resting pupil size of the right eye was not significantly affected by left 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 for (p=0.03408). Percent constriction from baseline, however, did not differ from sham for either the OBI-5mg/kg raloxifene (p=0.11561), or OBI-10mg/kg raloxifene mice (p=0.58283) 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 (p=0.65847), 1x OBI-5mg/kg raloxifene (p=0.27060), or 1x OBI-10mg/kg raloxifene mice (p=0.70323), as shown in Figure 9B. 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.

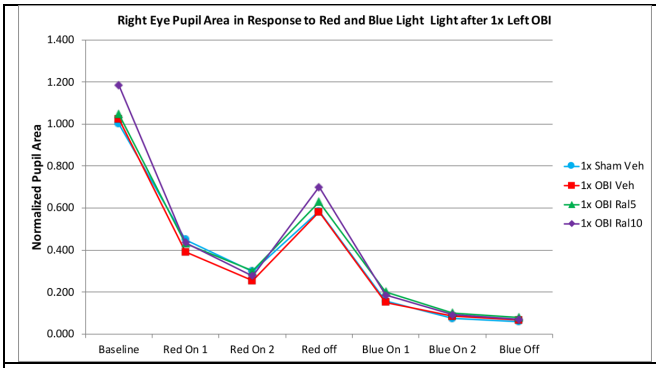


Figure 9A. Effect of left eye single blast OBI on right eye pupil area before, during and after exposure to red or blue light. Pupil area was not significantly different than in sham for any group before or during red light, or during and after blue light. Note, however, that pupil area tended to be slightly greater prior to red light for the OBI-vehicle mice, but constrict equally to sham.

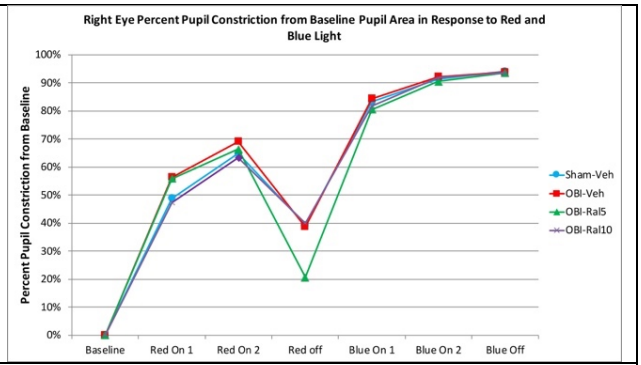


Figure 9B. Effect of left eye single blast OBI on percent right eye pupil constriction from baseline during and after exposure to red or blue light, 100% being maximal constriction. The percent constriction in response to red light but not blue light was enhanced by OBI. Both raloxifene doses rescued the hyperconstriction during red light exposure, the higher dose more so.

3-1B. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after 5x OBI. Because the functional deficits tended to be small after single 25-psi OBI, we additionally studied mice with 5x 25-psi OBI (1 minute interblast interval), which includes 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 that 5x 25-psi OBI caused a significant left eye contrast sensitivity deficit ($p=0.0000055$) (Fig. 10A) and a significant left eye visual acuity deficit ($p=0.017931$) (Fig. 10B), but no deficit for either contrast sensitivity or acuity was seen in the right eye of 5x 25-psi OBI, at 2 months after blast. Although raloxifene at 5 mg/kg did not rescue the left eye contrast sensitivity deficit, since it was significantly worse than in sham ($p=0.000042$), and not significantly different from 5x OBI-vehicle ($p=0.533059$). By contrast, raloxifene at 10 mg/kg did yield rescue, as the left eye contrast sensitivity was less significantly different from sham ($p=0.037529$) and significantly better than in 5x OBI-vehicle mice ($p=0.012953$). 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, and these were rescued by raloxifene. We re-examined the deficits and raloxifene rescue at 3 months post blast in a major 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 and a significant left eye deficit in visual acuity were present in 5x OBI-vehicle mice. 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, and 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. We tested again at 7 months post blast in the same subset, using an automated Optomotry system called the OptoDrum (Striatech, Germany). The results showed that the left eye contrast sensitivity deficit persisted at 7 months after the 5x OBI, and the raloxifene rescue persisted as well, in this case with both doses effective at reducing the deficit. The visual acuity deficit, however, was no longer evident at 7 months after the 5x OBI, at least not with this automated technology.

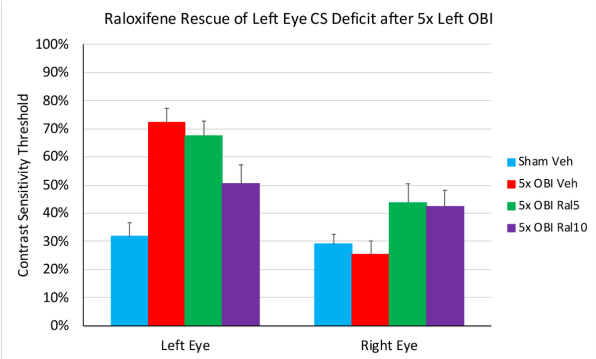


Figure 10A. Effect of left eye 5x OBI on contrast sensitivity in sham OBI mice that received vehicle, OBI mice that received vehicle, and OBI mice that received 5 mg/kg or 10 mg/kg raloxifene for 2 weeks postblast. Blast caused a significant left eye deficit that was significantly reduced by the higher raloxfene dose. No right eye deficit for contrast sensitivity was seen after 5x OBI, either with vehicle or raloxifene treatment.

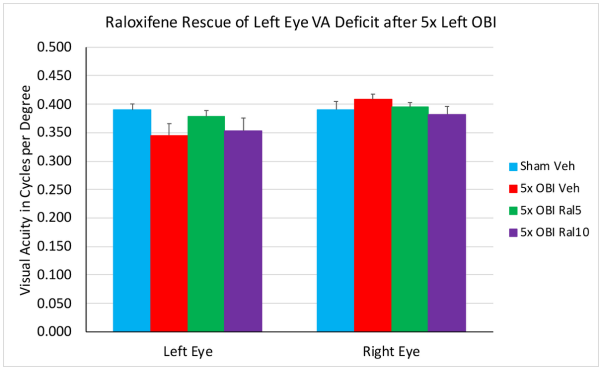


Figure 10B. Effect of left eye 5x OBI on visual acuity in sham OBI mice that received vehicle, OBI mice that received vehicle, and OBI mice that received 5 mg/kg or 10 mg/kg raloxifene for 2 weeks postblast. The 5x OBI caused a left eye deficit that was remedied by the lower dose. In the case of right eye acuity, none of the groups differed significantly from sham.

3-1C. Raloxifene Benefit 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, also included among the 5x OBI mice were 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 at blast on the outcome from 5x OBI and raloxifene treatment. For the cohort of males and females created and tested contemporaneously, we found that the magnitude of the contrast sensitivity deficit after 5x OBI-vehicle was similar in males and females, as was the rescue with raloxifene (Figure 11A). For this set of mice, 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. Because our sample of animals blasted at 3 months in this cohort was small, we examined blast age effects in our overall set of 5x OBI mice (Figure 11B). The left eye contrast sensitivity deficit in the 5x OBI-vehicle mice was similarly large in the 3-month blast age and 6 month blast age groups, 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.

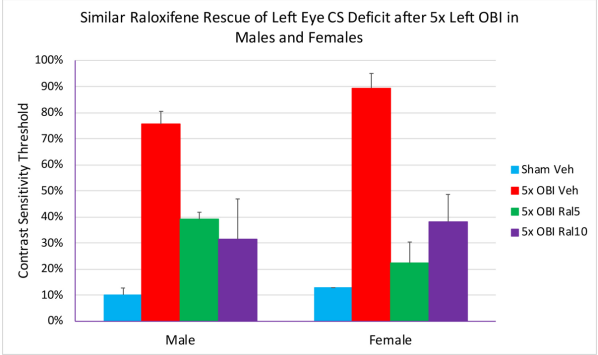


Figure 11A. Effect of left eye 5x OBI on contrast sensitivity in male versus female sham OBI mice that received vehicle, male versus female OBI mice that received vehicle, and male versus female OBI mice that received 5 mg/kg or 10 mg/kg raloxifene for 2 weeks postblast. Blast caused a significant left eye deficit that was significantly reduced by both raloxifene doses to a relatively equal extent in males and females. No right eye deficit for contrast sensitivity was seen after 5x OBI in either gender, either with vehicle or raloxifene.

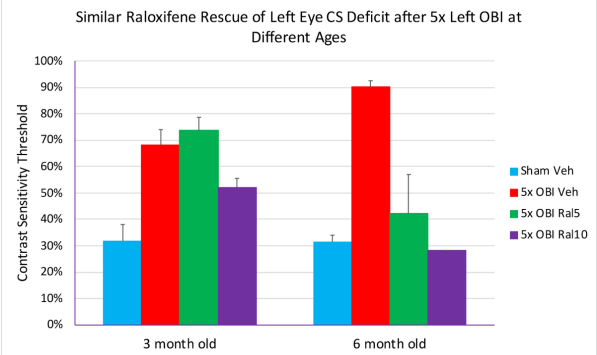


Figure 11B. Effect of left eye 5x OBI on contrast sensitivity in sham OBI mice that received vehicle, OBI mice that received vehicle, and OBI mice that received 5 mg/kg or 10 mg/kg raloxifene for 2 weeks postblast, comparing mice that were blasted at 3 months of age to others that were blasted at 6 months of age. The 5x OBI caused a left eye contrast sensitivity deficit at both blast ages, which was remedied by the higher raloxfene doses at both ages. The lower dose was also effective in older blast mice, perhaps because females predominated in that group.

3-1D. Raloxifene Benefit – Scotopic ERG after 5x OBI. Dark-adapted full-field ERGs were recorded for the 5x OBI mice about 5 months after blast, using a Celeris rodent ERG system. We found that left eye 5x OBI yielded significant reductions in both the A-wave and B-wave peak amplitudes. The A-wave was significantly reduced at the brighter light intensities, while the B-wave was significantly reduced across all light intensities. Both doses of raloxifene provided significant but incomplete rescue of the A-wave deficit, but they did not rescue the B-wave deficit, as shown below (Figure 12A, B).

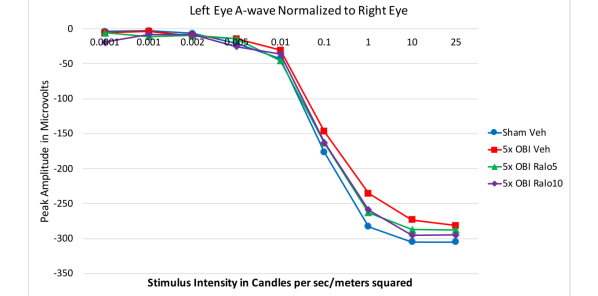


Figure 12A. Effect of 5x left eye OBI on the left eye A-wave of the flash-evoked scotopic ERG in sham OBI, vehicle-treated OBI mice, and raloxifene (5 mg/kg or 10 mg/kg) treated OBI TBI mice, 5 months after OBI. Left eye 5x OBI significantly reduced the left eye A-wave, and raloxifene at both doses provided partial rescue.

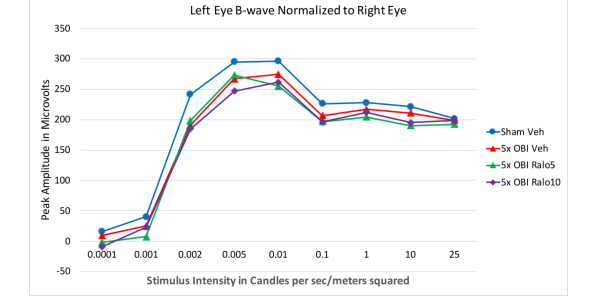


Figure 12B. Effect of 5x left eye OBI on the left eye B-wave of the flash-evoked scotopic ERG in sham OBI, vehicle treated OBI mice, and raloxifene (5 mg/kg or 10 mg/kg) treated OBI TBI mice, 5 months after OBI. Left eye 5x OBI significantly reduced the B-wave, but raloxifene at neither dose provided rescue.

3-1E. Raloxifene Benefit – Light Aversion after 5x OBI. We evaluated light aversion for the 5x 25-psi OBI mice at 150 days after left eye 5x OBI to assess how it evolved over time. As shown in the graph below Figure 13), 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 less time in the light box as its illumination increased. The 5x OBI mice treated with either raloxifene dose did not differ significantly from sham OBI in light aversion, showing rescue of the light insensitivity caused by 5x OBI. 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 5x OBI, manifesting in this case as an insensitivity on the Light/Dark Box test.

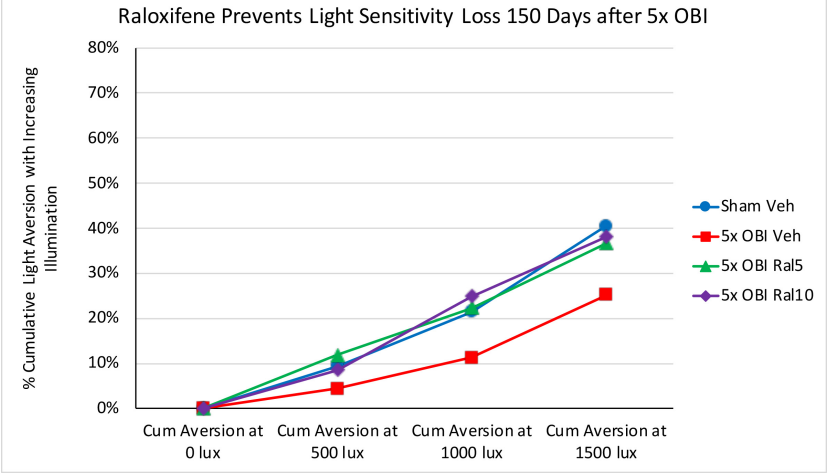


Figure 13. The graph to the left plots avoidance of enclosed chamber occupancy as its illumination increases, with light aversion thus plotted as increasing with increasing illumination. This plot shows that left eye 5x OBI significantly reduces enclosed chamber avoidance as illumination increases at 150 days after 5x left eye OBI. Thus, the vehicle-treated 5x OBI mice show less light aversion than sham mice. This was normalized by the both raloxifene doses – that is the 5x left eye OBI mice treated with raloxifene did not differ from sham-vehicle mice in their light avoidance.

3-1F. Raloxifene Benefit – Pupil Light Reflex after 5x OBI. We analyzed the pupil light reflex in 5x OBI mice for which we also analyzed acuity, contrast sensitivity, the ERG, and light aversion (9 sham, 12 5x OBI-vehicle, 10 5x OBI-ralo5, and 9 5x OBI-ralo10) at 6 months post blast. The pupil responses to illumination were recorded digitally, and the images of the responses were analyzed to determine the pupil responses to red versus blue light. We found that 5x left eye OBI in vehicle-treated mice had a significant impact on the resting pupil size in the left but not right eye (Fig. 14) – resting pupil size in left eye of 5x left OBI mice was 75.4% of sham-vehicle left eye (p=0.01021). By contrast, the resting pupil size in the left eye of 5x OBI-ralo5 mice and 5x OBI-ralo10 mice was not significantly different sham-vehicle left eye (Fig. 14). In the case of right eye 6 months after left eye 5x OBI, neither 5x OBI-vehicle, 5x OBI-ralo5, nor 5x OBI-ralo10 differed significantly from sham-vehicle mice. Thus, 5x OBI caused the blasted eye but not the opposite eye to be constricted in its resting state. The basis of this is uncertain, but it could reflect damage to the sympathetic input to dilator muscles of the pupil.

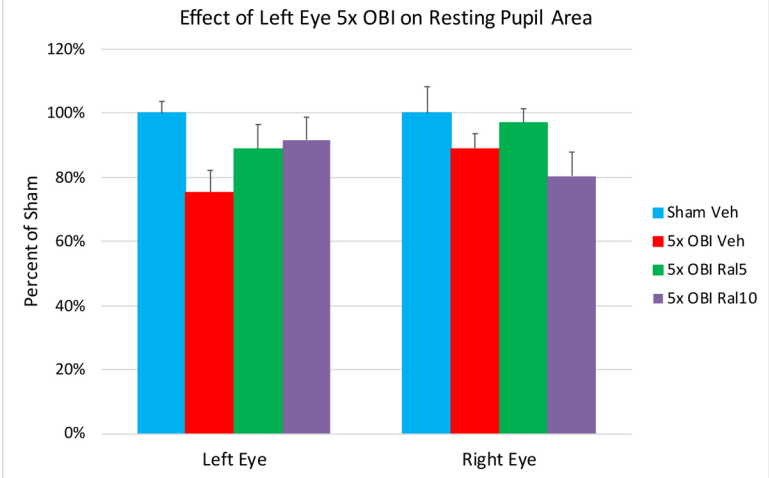


Figure 14. Effect of left eye 5x OBI on left eye pupil area before exposure to red or blue light (i.e. resting pupil size) 6 months after blast. Pupil area was significantly 25.6% less than in sham – vehicle mice, but not in 5x OBI mice treated with 5 mg/kg raloxifene or 10 mg/kg raloxifene. By contrast, in the case of right eye 6 months after left eye 5x OBI, neither 5x OBI-vehicle, 5x OBI-ralo5, nor 5x OBI-ralo10 differed significantly from sham-vehicle mice.

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 Figure 15A below, 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), 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 notably less than in sham (Fig. 15B). 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.

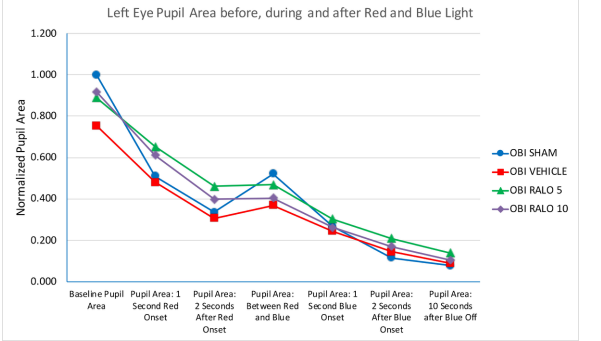


Figure 15A. Effect of left eye 5x OBI on left eye normalized pupil area before, during and after exposure to red or blue light. Pupil area was significantly less than in sham for the 5x OBI - vehicle group at rest before red light and before blue light. Pupil area during red and blue light for the 5x OBI-vehicle mice, however, was similar to that in sham. Pupil area in the 5x OBI mice treated with raloxifene was less than in sham during red light, but similar to sham during blue light.

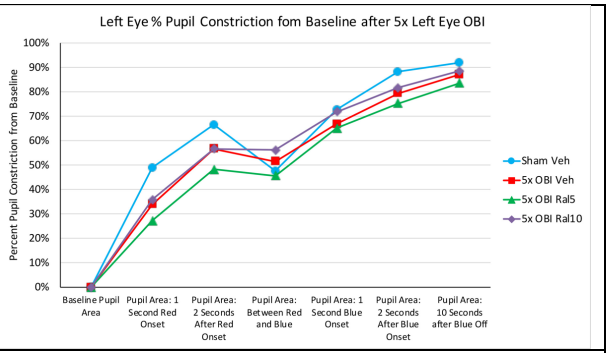


Figure 15B. Effect of left eye 5x OBI on percent left eye pupil constriction from baseline during and after exposure to red or blue light, with 100% being maximal constriction. The percent constriction in response to red light but not blue light was diminished by 5x OBI, and this was not rectified by raloxifene. The percent constriction to blue light was also diminished by 5x OBI, and this was rescued partly by the higher but not lower dose of raloxifene.

The right eye pupil response after left eye 5x OBI was also altered from sham. As noted above, the right eye 6 months after left eye 5x OBI, neither 5x OBI-vehicle, 5x OBI-ralo5, nor 5x OBI-ralo10 differed significantly from sham-vehicle mice in resting pupil area prior to any light stimulation. Although this was also true for the period between red and blue light presentations, during this period the right pupil in the 5x OBI-vehicle mice trended toward being more constricted (smaller area) than in sham mice (Fig. 15A). 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 (Fig. 16B). It may thus be the case that 5x left eye OBI has an effect on right eye, perhaps via inflammation that travels from the left to the right eye. Note, however, the percent constriction for the right eye in the 5x OBI – vehicle mice during red and blue light was largely similar to sham, as were the responses in the raloxifene mice.

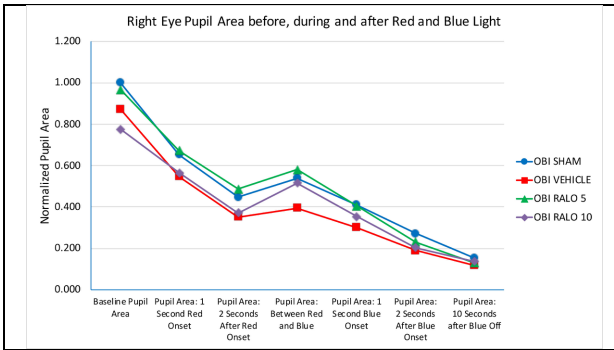


Figure 16A. Effect of left eye 5x OBI on right eye normalized pupil area before, during and after red or blue light. Pupil area was not significantly different than in sham for any 5x OBI - group before red light, but it trended toward less than sham for the vehicle-treated 5x OBI mice before blue light. Pupil area during red and blue light for the 5x OBI-vehicle mice and 5x OBI – ralo10 mice, however, was less than in sham.

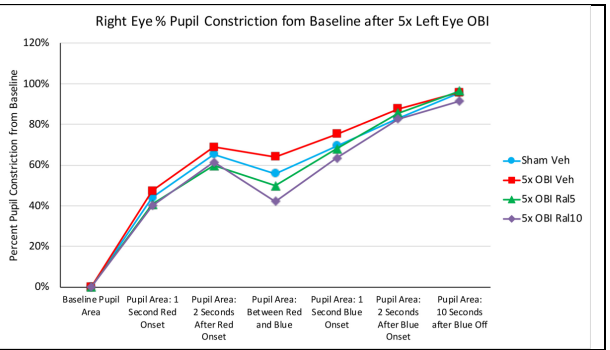


Figure 16B. Effect of left eye 5x OBI on percent right eye pupil constriction from baseline during and after exposure to red or blue light, with 100% being maximal constriction. The percent constriction in response to red and blue light in 5x OBI – vehicle mice was similar to that in sham, but similar to sham in the raloxifene-treated 5x OBI groups. Prior to blue light, per cent constriction was less than sham for the raloxifene mice.

3-1G. Raloxifene Benefit – Optic Nerve Axon Loss after repeat OBI. We compared left and right optic nerves after left eye 3x 25-psi OBI in 5 mice at 2 weeks post OBI and after left eye 5x 25-psi OBI in 2 mice at two months after OBI, using blinded computer-assisted axon counts. We found that axon abundance for both left and right optic nerves was normal at 2 weeks post 3x OBI, and few actively degenerating axons were seen in either left or right optic nerves. By contrast, at 2 months after left eye 5x 25-psi OBI, we observed a 27.8% reduction in axon abundance in the left optic nerve compared to the right optic nerve, which had normal abundance. Thus, 3x 25-psi left eye OBI did not cause axon loss at 2 weeks post OBI, but 3x 25-psi left eye OBI did cause substantial loss by 2 months. As discussed below, this is consistent with the functional visual deficits we observed with left eye 5x OBI. Moreover, the axon loss was highly correlated with contrast sensitivity at 2 months after OBI, ascertained shortly before sacrifice for histology.

3-1H. Raloxifene Benefit after 5x OBI – Effects on Microglia. We have created an additional set of 5x OBI mice, consisting of seven sham mice, six 5x OBI-vehicle mice, seven 5x OBI-ralo5 mice, and six 5x OBI-ralo10 mice, that were sacrificed at 3 days after OBI. This set includes both males and females, and was blasted at about 8 months of age, to assess age and gender effects on axonal injury and microglial activation in the retina, optic nerve, and optic tract after 5x OBI, using immunolabeling. As part of assessing microglial activation, we are determining if blast shifts microglia toward expression of M1 markers, and raloxifene shifts such blast-affected microglia toward M2 marker expression.

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 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, 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 still collecting data on OBI and but plan to submit our findings for publication during the current project period.

Task 1: What do you plan to do during the next reporting period to accomplish the goals?
The goals have been accomplished.
Task 2: What do you plan to do during the next reporting period to accomplish the goals?
We will complete analysis of the effect of raloxifene on microglia following impact TBI, and submit our findings for publication.
Task 3: What do you plan to do during the next reporting period to accomplish the goals?
We will complete analysis of the morphological benefit of raloxifene following OBI, complete analysis of the effect of raloxifene on microglia following OBI, and submit our findings for publication.

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.

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.

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 should 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.

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.

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 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 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). These additional findings are detailed below in section 1B.

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.

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.

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

Nothing to Report.

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 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 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 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 worked:	5.0
Contribution to Project:	Dr. Del Mar performed the TBI and animal testing.

Name:	Chunyan Li
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.0
Contribution to Project:	Dr. Li performed TBI and animal testing.

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

Name:	Aaron Perry
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.0
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.

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.

Appendices

1. A PDF of our Honig et al. 2019 and our 2020 ARVO abstract are 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

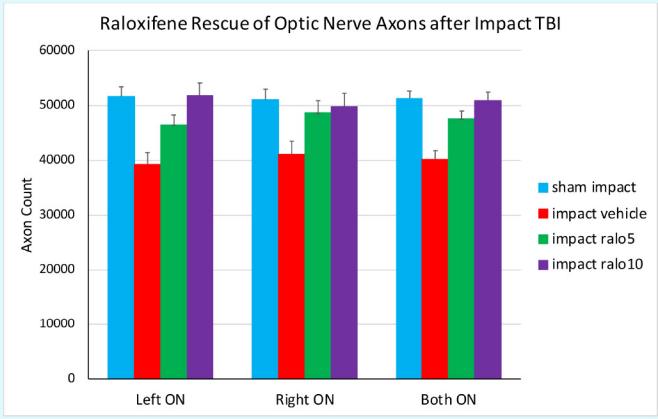
Study Aim

•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 will 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

Updated: (04/14/2020)

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. Additionally, refine dose and timing of treatment, and perform mechanistic studies to demonstrate the role of microglial modulation in the benefit. This Aim is complete.
- **Study 2 (CY17-19):** Confirm effectiveness of raloxifene in mitigating visual deficits in a standardized dorsal cranial impact model of mild TBI in mice. This Aim is 95% complete.
- **Study 3 (CY18-20): C**onfirm effectiveness of raloxifene in mitigating visual deficits in standardized models of single or repeat ocular blast injury in mice. This study is 90% complete.

Comments/Challenges/Issues/Concerns

- None.

Budget Expenditure to Date

Projected Expenditure: $888,877 Direct Cost

Actual Expenditure: ~$863,133 Direct Cost

ARVO 2020

View Abstract

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Study Group: Anton Reiner

ABSTRACT

TITLE: Raloxifene through its Cannabinoid Type-2 Receptor Inverse Agonism Mitigates Visual Deficits and Pathology after Impact TBI

ABSTRACT BODY:

Purpose: We have previously shown that treatment for 2 weeks with the cannabinoid type-2 receptor (CB2) inverse agonist SMM189 attenuates the visual deficits and retinal pathology produced by closed-head focal cranial blast in mice, by modulating the otherwise deleterious effect of microglia in secondary pathogenic processes. SMM189, however, has not yet been approved for human use. Raloxifene is FDA approved (as an estrogen receptor drug to treat osteoporosis) but also acts as a CB2 inverse agonist, and similarly attenuates the visual deficits and retinal pathology produced by closed-head focal cranial blast in mice. Here we assessed if raloxifene also reduces the visual deficits and retinal pathology that result from impact traumatic brain injury (TBI).

Methods: Single impact TBI was produced in anesthetized male C57BL/6 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. Mice were injected with vehicle or raloxifene at 5 or 10 mg/kg daily for 2 weeks. Sham male mice were anesthetized, secured in the Impact One device but not impacted, and subsequently injected with vehicle. Functional tests at >1 month assessed visual acuity and contrast sensitivity, the scotopic ERG, light aversion, and the pupil light reflex. Morphological analysis assessed optic nerve injury.

Results: We found that central midline impact TBI yielded bilateral deficits in visual acuity and contrast sensitivity, a reduction in the B-wave amplitude of the scotopic ERG, light aversion, decreased pupil constriction to light, and a 20% loss of optic nerve axons. Daily treatment with both doses of raloxifene for 2 weeks post blast reduced or eliminated the deficits in acuity, contrast sensitivity, light aversion, pupil constriction, and optic nerve axon loss (with the higher dose generally more effective). The B-wave deficit was rescued by the lower dose, but surprisingly not by the higher dose.

Conclusions: Our studies show that raloxifene ameliorates visual system deficits and injury after impact TBI.

Our findings provide support for phase-2 efficacy testing in clinical trials, which if effective could lead to the rapid repurposing of raloxifene for treating mild TBI in people.

(No Image Selected)

Layman Abstract (optional): Provide a 50-200 word description of your work that non-scientists can understand. Describe the big picture and the implications of your findings, not the study itself and the associated details.: We have shown that treatment for 2 weeks with the cannabinoid type-2 receptor (CB2) inverse agonist SMM189 greatly reduces the visual deficits and retinal pathology resulting from traumatic brain injury (TBI) in a mouse model of cranial blast. CB2 inverse agonists have this action because they convert microglia from being helpful rather than harmful in the immediate aftermath of the trauma. SMM189, however, is a novel drug that has not yet been approved for human use. Because raloxifene is FDA-approved as an estrogen receptor drug to treat osteoporosis but also acts as a CB2 inverse agonist, we assessed its utility in treating TBI in mice. Our previous work has shown raloxifene benefit in our blast model. The current studies show that raloxifene ameliorates the visual system deficits and injury that result from an impact to the head. Our findings provide basis for phase-2 efficacy testing of raloxifene benefit after TBI in human clinical trials. If raloxifene proves effective in reducing TBI deficits in such trials, it could lead to its rapid repurposing for use in the immediate aftermath of TBI for mitigating deficits in humans.

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TRAVEL GRANTS and AWARDS APPLICATIONS

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AFFIRMATIONS

Affirmations: Affirmation of compliance with ARVO's Statement for Use of Animals.
Affirmations: Affirmation to reveal essential structure, novel compound elements, or identify new gene compounds.
Affirmations: Affirmation of compliance with ARVO's Statement for Use of Human Subjects and/or Declaration of Helsinki.
Affirmations: Affirmation that submission of this abstract has been approved by the Principal Investigator.
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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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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 (Hiploylee 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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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. Electroretinogram (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*/m^2 (15 trials with a 5 s interstimulus interval, ISI); 2) 0.0001 cd.sec/m^2 (10 trials with a 5 s ISI); 3) 0.001 cd.sec/m^2 (3 trials with a 10.1 s ISI); 4) 0.01 cd.sec/m^2 (3 trials with a 15.1 s ISI); 5) 0.1 cd.sec/m^2 (2 trials with a 20 s ISI); 6) 1 cd.sec/m^2 (2 trials with a 20 s ISI); and 7) 2.88 cd.sec/m^2 (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/m^2) and 480 nm for blue light (200 kcd/m^2). 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 μm^2 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 × 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 ~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

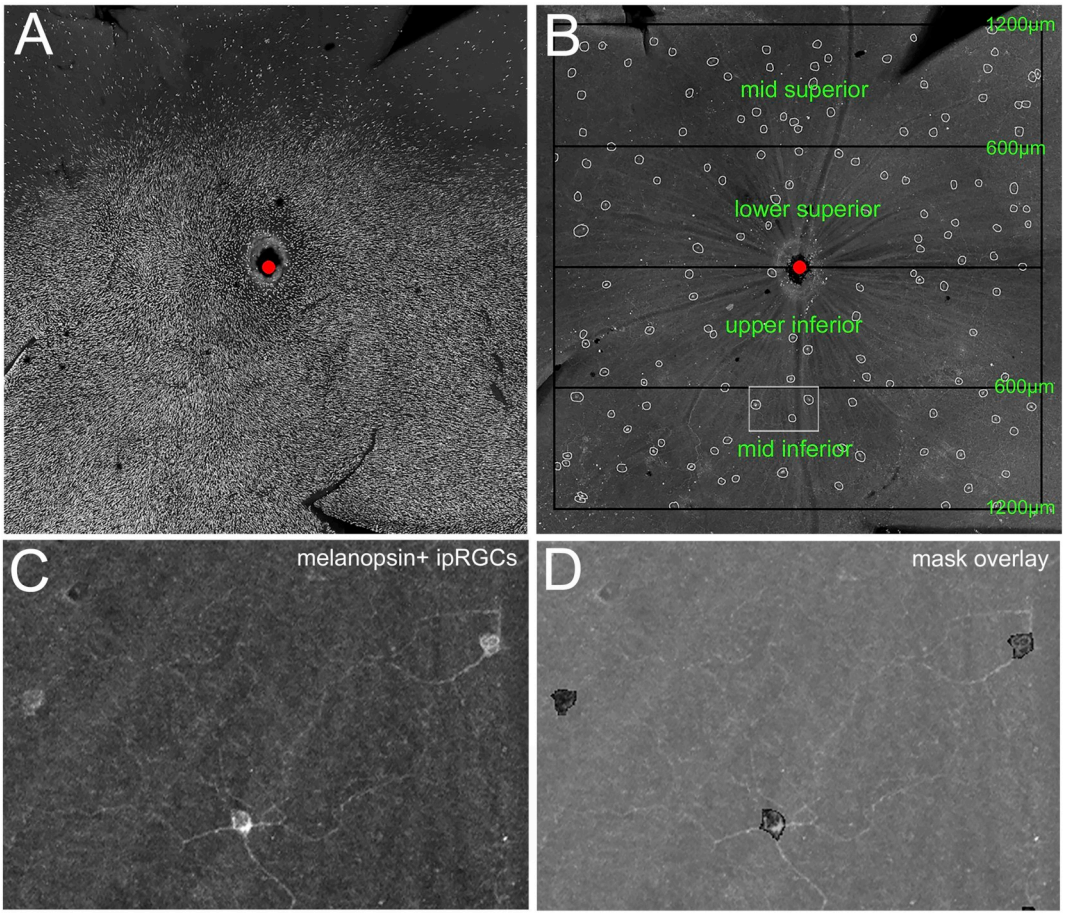


Fig. 1. Approach for analysis of melanopsin+ ipRGCs. **A.** Confocal image of a retinal flat mount showing immunolabeling for S-opsin. The image was rotated to the correct orientation as determined by the high abundance of S-opsin+ outer segments in ventral retina. The location of the optic nerve head is marked by a red dot. **B.** Melanopsin immunolabeling of the same retina shown in A. A 2400 µm × 2400 µm 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 µmol/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 (TGFβ); 2) M1 – Fc gamma receptor 2 (CD32), interferon-gamma (IFNγ), interleukin 1-beta (IL1β), tumor necrosis factor-alpha (TNFα), and inducible intric 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, IL1β, 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 ± 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 ~2 months, light aversion at ~4 months, and pupillometry at ~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

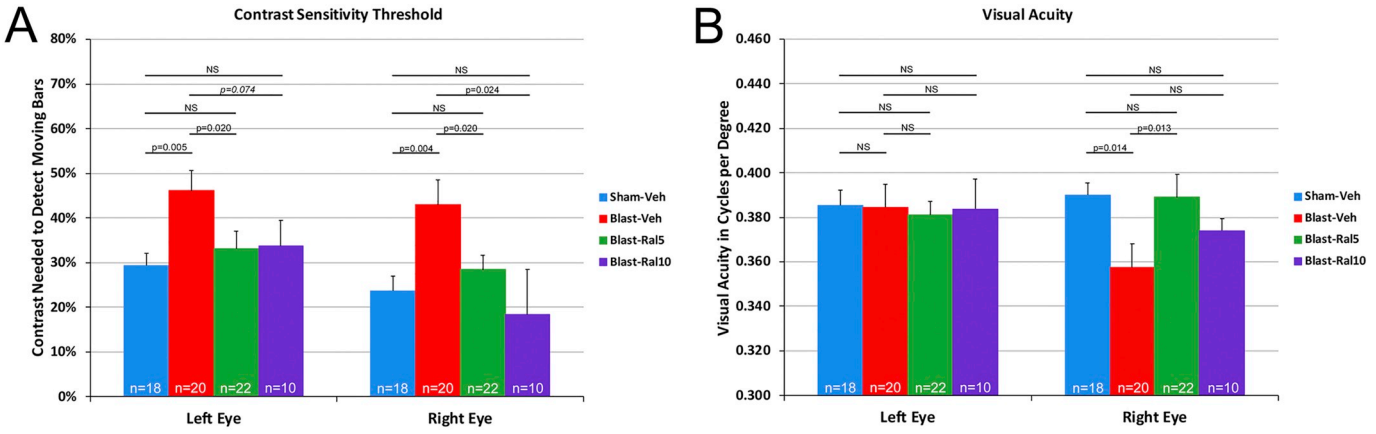


Fig. 2. Contrast sensitivity and visual acuity as measured using Optomotry a month after blast. **A.** The contrast sensitivity thresholds were significantly more in blast-vehicle mice than in sham-vehicle mice for both eyes. Mice treated with raloxifene were similar to sham and improved over blast-vehicle 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.

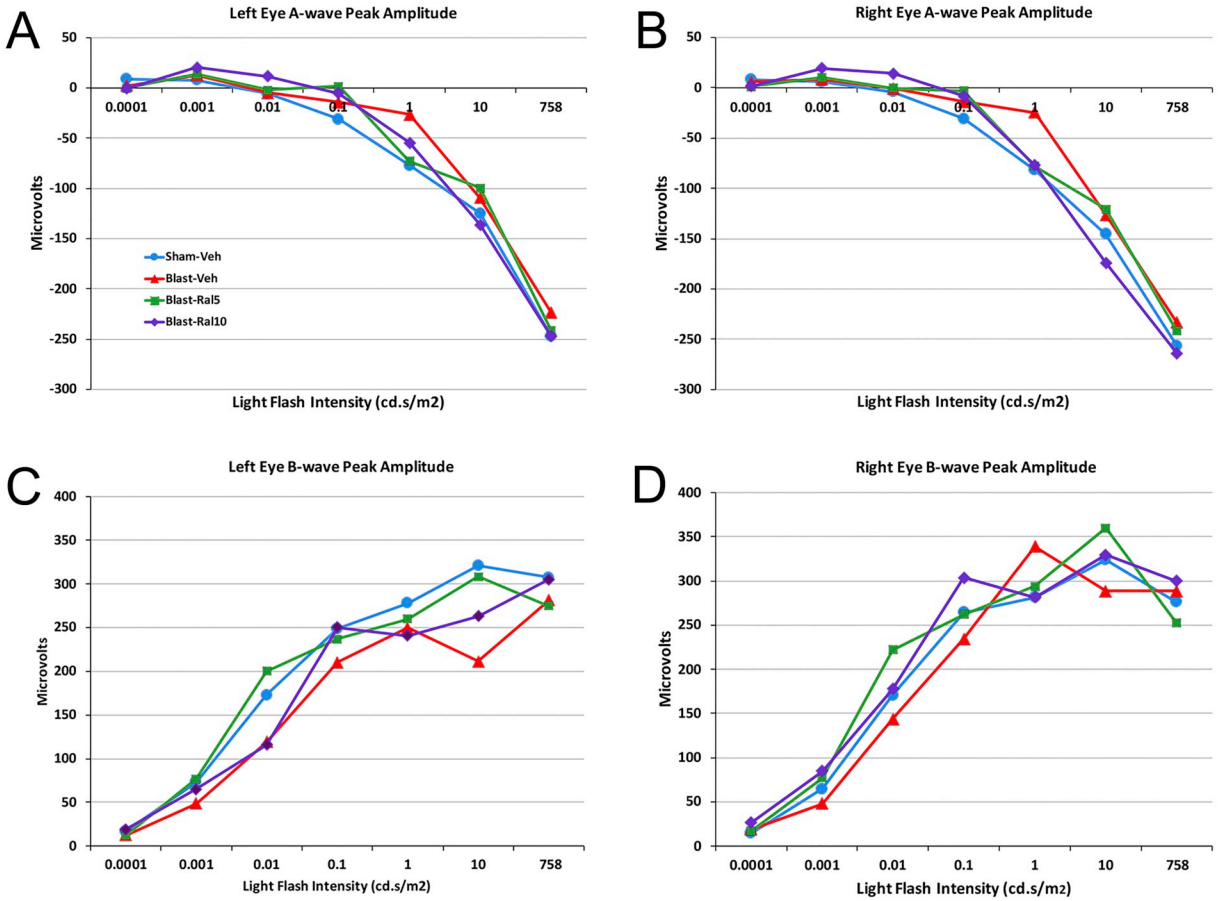


Fig. 3. Average flash-evoked scotopic ERG peak A-wave and B-wave amplitudes at 7 light intensities at ~2 months after blast. Values for each mouse were normalized to the sham pre-blast baseline. **A, B.** The A-wave peak for the 4 brightest light intensities was reduced for blast-vehicle mice compared 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.

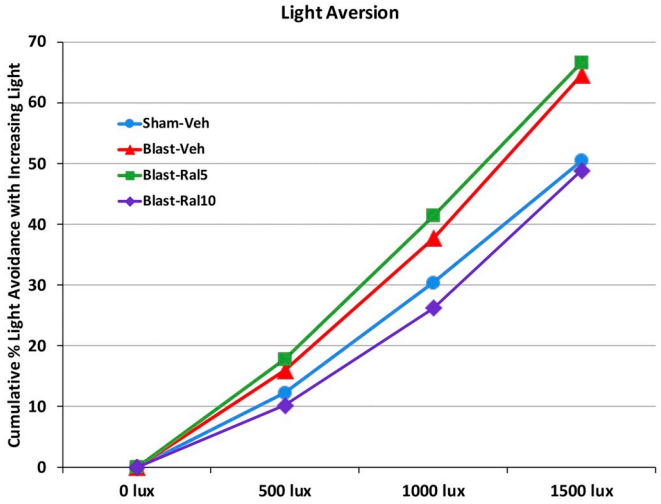


Fig. 4. Light aversion, tested at ~4 months after blast, plotted as cumulative avoidance of an enclosed chamber with increasing brightness, relative to an adjacent dark chamber. Blast-vehicle mice showed greater avoidance of the enclosed chamber as light intensity increased than did sham-vehicle mice (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

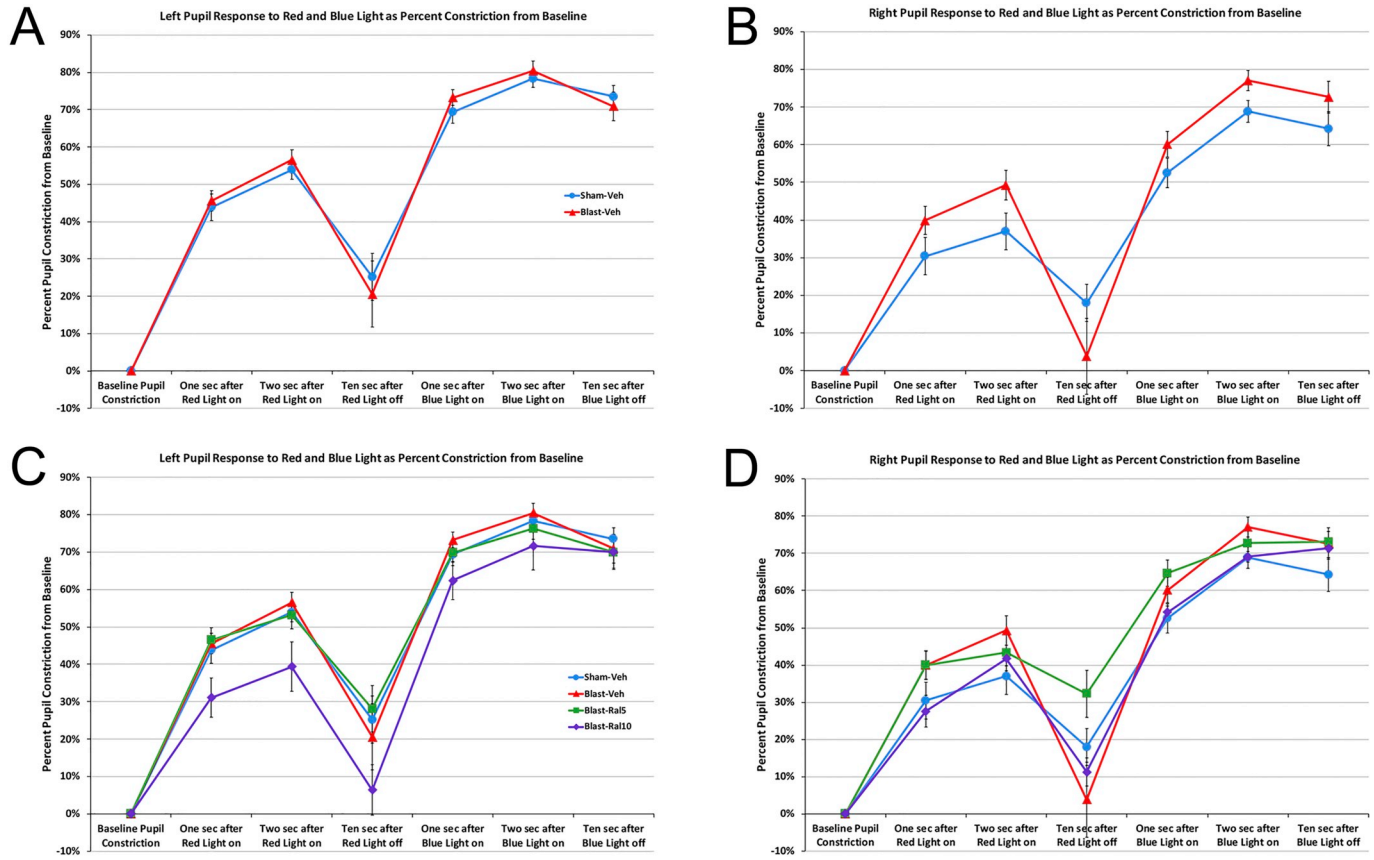


Fig. 5. Pupil responses, plotted as % constriction (i.e. pupil area reduction) from pre-illumination baseline, to red and blue light. **A, C.** Left eye pupil constriction was no different in blast-vehicle mice than in sham mice (A), but blast mice treated with 10 mg/kg raloxifene showed a significantly 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.)

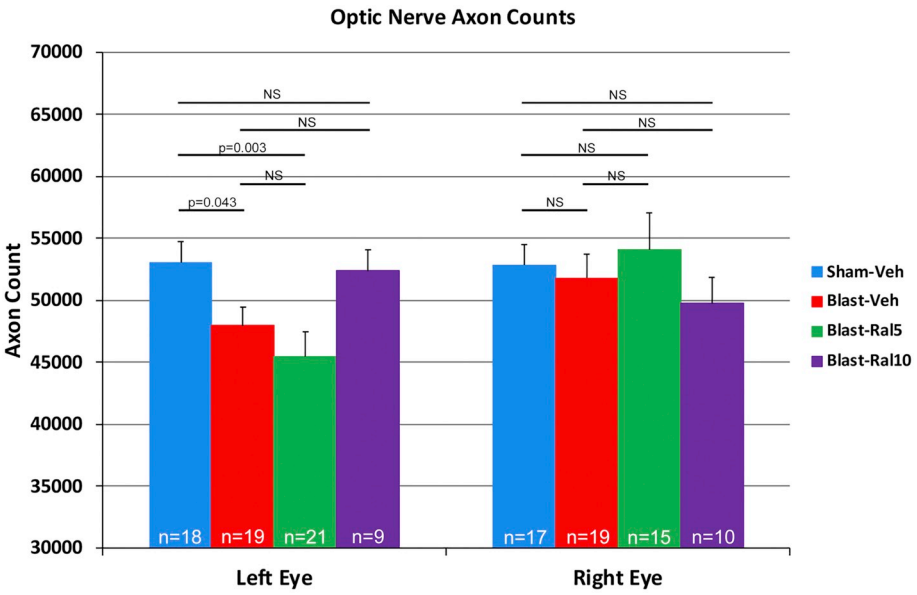


Fig. 6. Optic nerve axon counts. The left optic nerve of blast-vehicle and blast-ral5 mice showed a significant loss of axons. Treatment with 10 mg/kg raloxifene yielded full rescue. The right optic nerve showed no axon loss following blast and none of the differences between experimental groups were 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 (~17.4 μm^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 (TNFα, IFNγ and IL1β) 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,

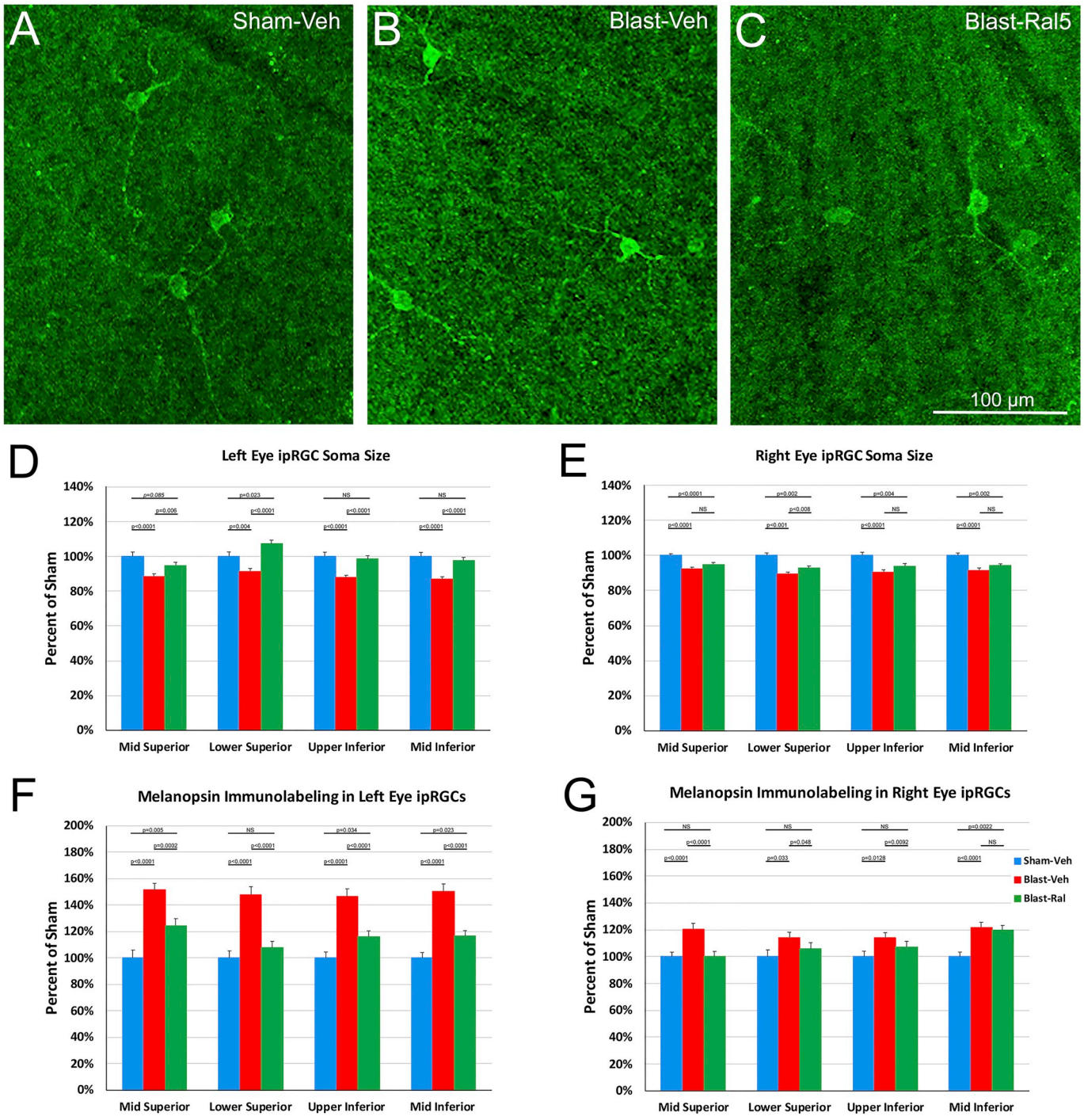


Fig. 7. Melanopsin + ipRGC analysis. **A-C.** Confocal images of melanopsin immunolabeling, all taken from the mid-inferior sector of the left eye. ipRGC cell bodies in the blast-vehicle retina were more intensely immunolabeled for melanopsin and smaller than in the sham. These changes were largely reversed 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

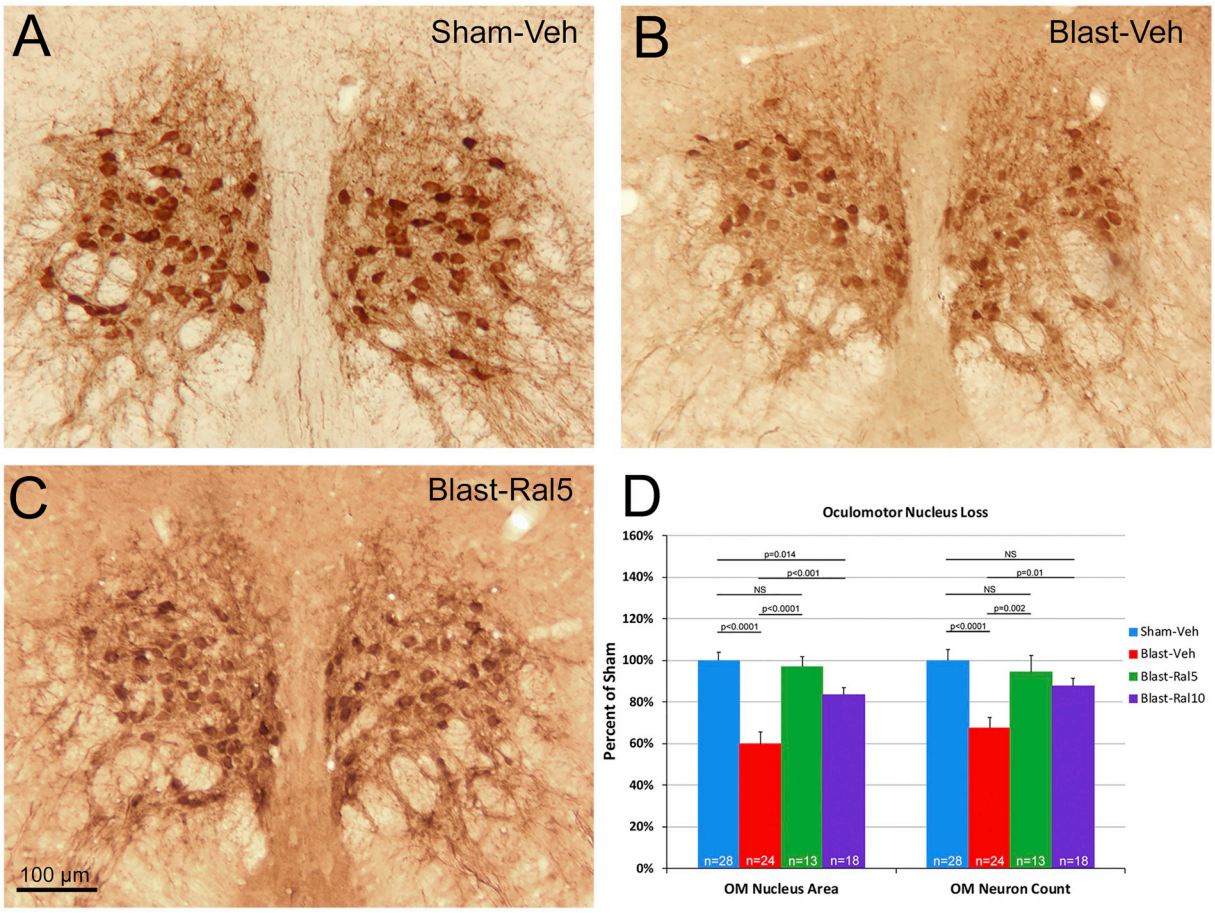


Fig. 8. Oculomotor nuclei. **A-C.** Sections of midbrain immunostained for choline acetyltransferase to reveal motoneurons. The area occupied by the oculomotor (OM) nucleus and its neuron abundance were less on both sides of the blast-vehicle mouse than in the sham, whereas mice treated with 5 mg/kg 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 μM 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

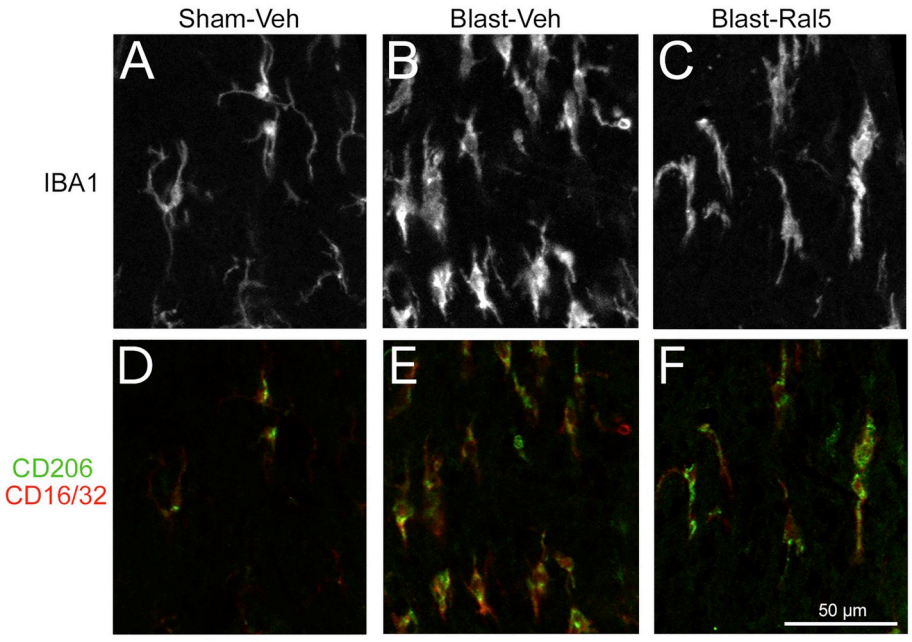
We next evaluated if the selective CB2 inverse agonist SR144528, which we have previously shown increases the levels of M2 markers on the surfaces of cultured microglia (Presley et al., 2015), similarly yields rescue for the contrast sensitivity deficit after blast. As shown in Fig. 13A, SR144528 rescued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



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	IBA1 OD	CD16/32 OD	CD206 OD	M1/M2	# IBA1+ profiles/mm^2	% IBA1 areal coverage
sham	12.5	10.5	11.2	0.96	598	4.7%
blast-vehicle	17.7	12.6	12.7	1.00	990	14.4%
blast-ral5	16.9	12.3	13.0	0.94	807	11.6%

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 immmunolabeling. 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

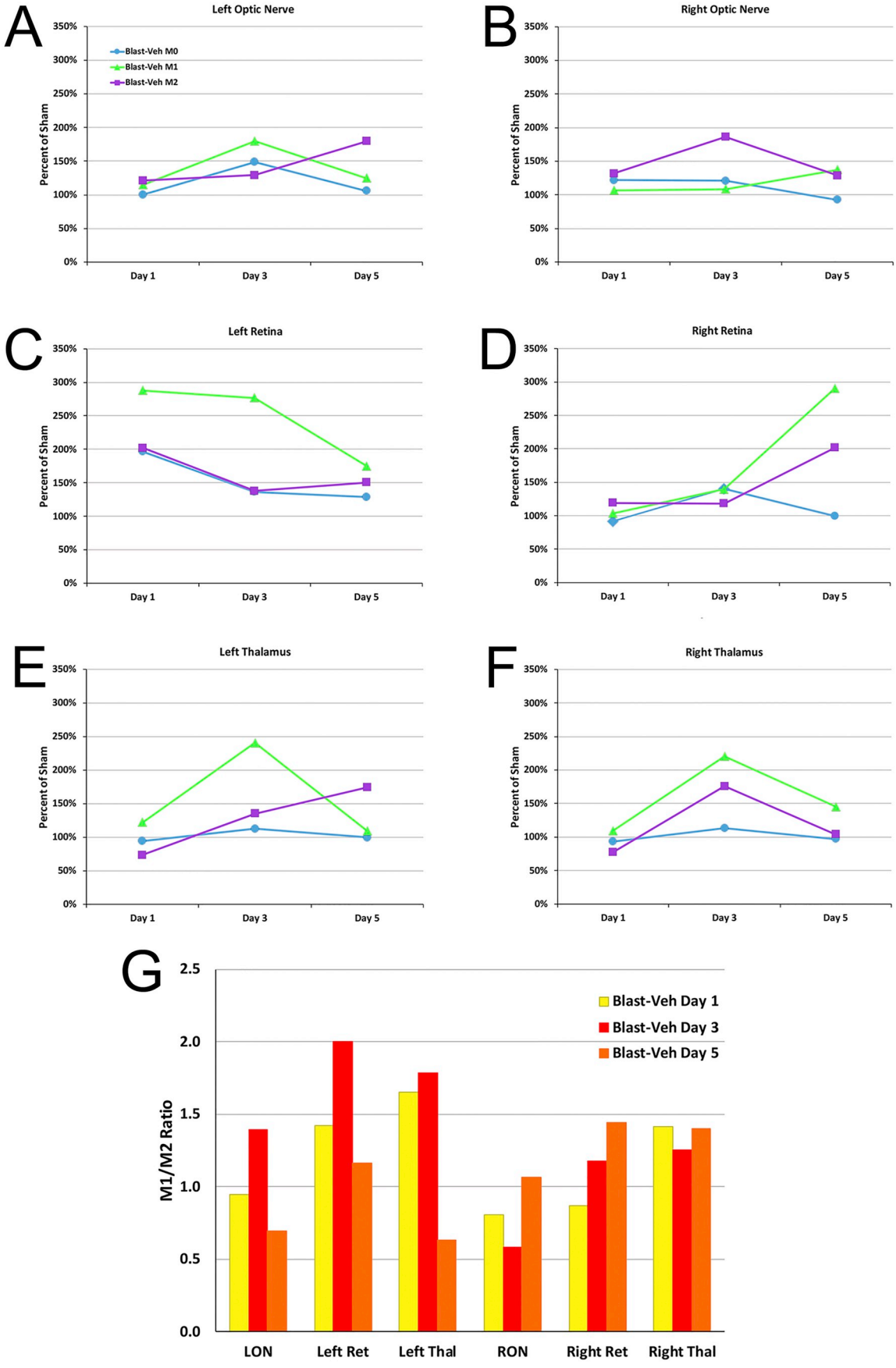

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Fig. 10. The mean expression of M0, M1, and M2 markers with time after blast. **A-F.** Transcript levels were determined by qPCR and are expressed relative to transcript levels for the same tissue from sham-vehicle mice. The blast-induced changes in 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 ACTOne 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 multidimensional 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

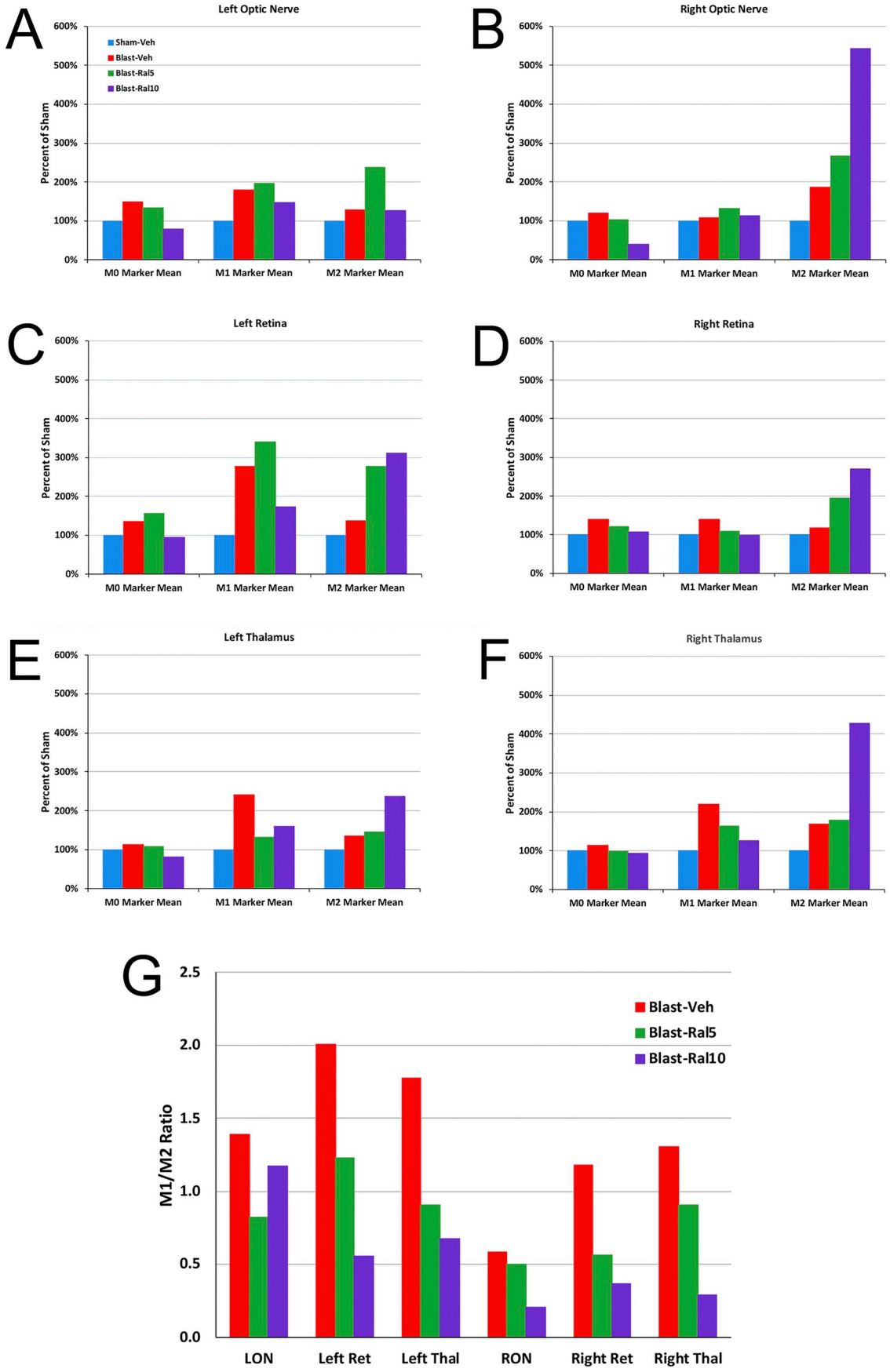

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Fig. 11. The mean expression of M0, M1, and M2 markers at 3 days after blast. **A-F.** Transcript levels were determined by qPCR and are expressed relative to transcript levels for the same tissue from sham-vehicle mice. Expression of M1 markers relative 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.

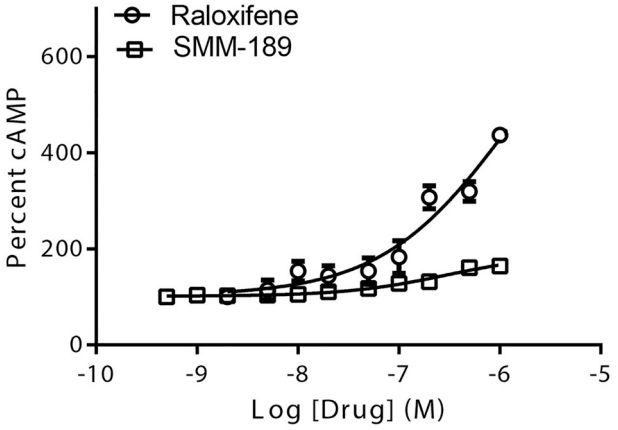

Fig. 12. Comparison of raloxifene and SMM-189 activity in HEK-CNG + CB2 cells using the ACTOne assay. Raloxifene caused a dose-dependent increase in cAMP-driven fluorescence over baseline that was about 5-fold greater than that with SMM-189 at a concentration of 1 μM.

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

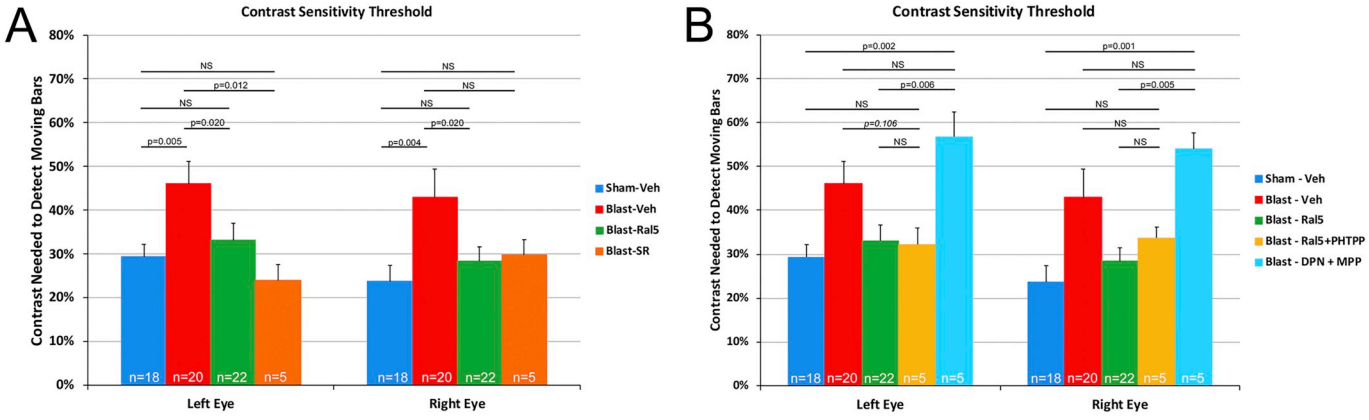

Fig. 13. Comparison of raloxifene with other drugs on the rescue of contrast sensitivity deficits produced by blast TBI. **A.** Comparison of raloxifene and the CB2 inverse agonist SR144528. The SR144528 benefit was similar to that observed with raloxifene. **B.** Comparison of raloxifene with drugs 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.

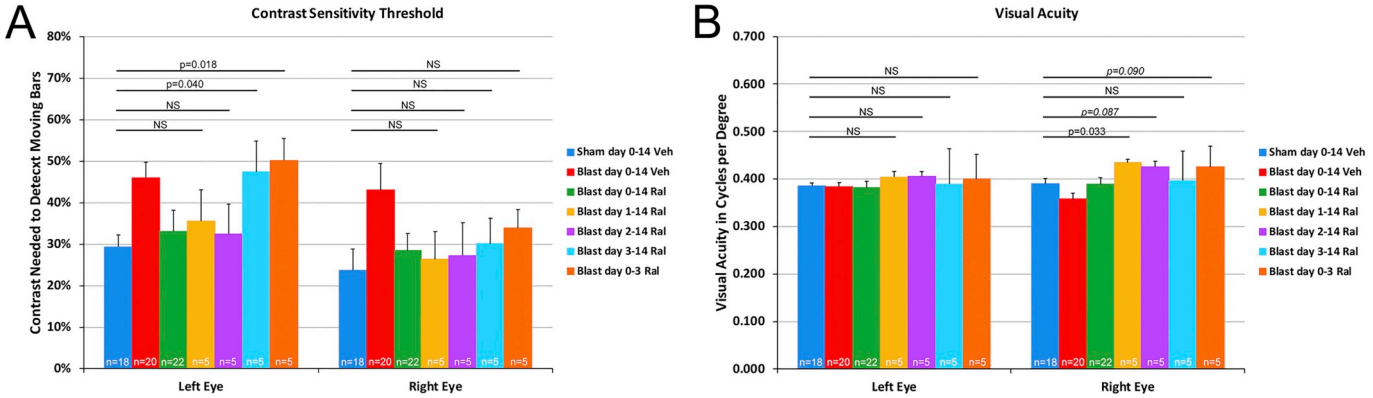

Fig. 14. Effective treatment windows for raloxifene rescue of deficits in contrast sensitivity and acuity. Raloxifene treatment was delayed for 1, 2 or 3 days in separate groups of mice or discontinued starting four days after blast. **A.** The contrast sensitivity deficit for the left eye was rescued 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; Bazarian 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.

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Appendix A. Supplementary data

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

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