ANIMAL MODELS

Lasting Retinal Injury in a Mouse Model of Blast-Induced Trauma

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Traumatic brain injury due to blast exposure is currently the most prevalent of war injuries. Although secondary ocular blast injuries due to flying debris are more common, primary ocular blast exposure resulting from blast wave pressure has been reported among survivors of explosions, but with limited understanding of the resulting retinal pathologies. Using a compressed air-driven shock tube system, adult male and female C57BL/6 mice were exposed to blast wave pressure of 300 kPa (43.5 psi) per day for 3 successive days, and euthanized 30 days after injury. We assessed retinal tissues using immunofluorescence for glial fibrillary acidic protein, microglia-specific proteins Iba1 and CD68, and phosphorylated tau (AT-270 pThr181 and AT-180 pThr231). Primary blast wave pressure resulted in activation of Müller glia, loss of photoreceptor cells, and an increase in phosphorylated tau in retinal neurons and glia. We found that 300-kPa blasts yielded no detectable cognitive or motor deficits, and no neurochemical or biochemical evidence of injury in the striatum or prefrontal cortex, respectively. These changes were detected 30 days after blast exposure, suggesting the possibility of long-lasting retinal injury and neuronal inflammation after primary blast exposure. (*Am J Pathol* 2017, 187: 1459–1472; <http://dx.doi.org/10.1016/j.ajpath.2017.03.005>)

The nature of 21st century military conflict has led to a dramatic increase in exposure of military personnel and civilians to blast wave pressure, leading to traumatic brain injury (TBI).¹ The retina is part of the central nervous system; as such, it is vulnerable to injuries similar to those that affect the brain.² Today, >80% of military personnel experiencing TBI also exhibit symptoms of visual dysfunction.³ Primary ocular blast exposure resulting from blast wave pressure has been reported among survivors of explosions, but with limited understanding of the resulting retinal pathologies; therefore, therapeutic interventions are currently out of reach.⁴ Previous consolidation of data from the US Department of Defense shows a high percentage of visual field deficits, photophobia, oculomotor dysfunction, and decrease in contrast sensitivity in service members, 45 to 60 days after blast-induced TBI, supporting the need for a chronic blast wave pressure injury model.⁵ Existing rodent models of blast injury show the susceptibility of the retina to the effects of low-level (120 ± 7 kPa) and high-level (≥ 180 kPa) blast wave pressure, including glial cell activation

in the ganglion cell layer, inner nuclear layer, and outer nuclear layer, with an overall increase in biomarkers of inflammation and apoptosis.^{2–4} Existing models differ significantly in intensity and duration; however, long-lasting effects of blast wave pressure have not been reported.

We conducted a study in which a compressed air-driven shock tube system was calibrated to deliver blast wave pressure of 300 kPa (43.5 psi) each day for 3 successive days to mice. Herein, we show that 30 days after exposure to successive blast wave pressure, the retinas of exposed mice present with glial cell activation, microglial activation, photoreceptor cell loss, and an increase in phosphorylated

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tau in the outer plexiform layer. Proximity of the eye to blast wave pressure had a substantial effect on the severity of these retinal responses. There was a notable difference in the response of the retina on the side of the mouse ipsilateral to blast exposure in comparison to the retina on the contralateral side. Behavioral parameters, including the Morris water maze, rotarod, and open-field activity, showed no deficits in cognitive or motor function. Neurochemical assessment of the striatum, as well as composition of astrocytes, microglia, and phosphorylated tau within the prefrontal cortex showed no indication of damage induced by blast wave pressure. To the best of our knowledge, this report is the first to compare neurological and retinal effects of blast injury. This study is also the first to show prolonged effects of blast wave pressure on specific retinal cell types. Specifically, we report Müller glia hypertrophy, microglial activation, photoreceptor cell loss, and an increase in phosphorylated tau in both retinal neurons and glial cells. This blast wave pressure model may provide insight into the underlying pathological mechanisms and help to identify markers of long-lasting retinal injury due to blast exposure.

Materials and Methods

Ethics Statement

These experiments were performed in accordance with NIH's *Guide for the Care and Use of Laboratory Animals*⁶ and were approved by the Iowa State University Animal Care and Use Committee (protocol 4-11-7123-M).

Exposure to Blast Wave Pressure

Exposure to blast wave pressure was conducted using an open-ended shock tube, as described by Shah et al.⁷ In this model, a 0.3-m driver section is rapidly pressurized with compressed helium until it ruptures four 0.35-mm-thick mylar membranes (burst pressure, approximately 9.9 MPa), propelling a shock wave down a 3-m, open-ended driven section (ID, 3.6 cm). Briefly, 20 adult male or female C57BL/6 mice were randomly divided into blast wave pressure or sham-exposed groups. Ten mice were exposed to blast wave pressure of 300 kPa (peak pressure means $\pm$ SD, 297 ± 15 kPa; positive duration, 146 ± 6 μ seconds) each day for 3 successive days, and euthanized at 30 days after injury (Supplemental Table S1). The remaining 10 mice served as control. Mice were anesthetized with 4% isoflurane in 2 L/minute oxygen for 90 seconds in the induction chamber, then supplemented with 2% isoflurane in 2 L/minute oxygen via nose cone in blast animal holder until blast delivery (approximately 20 seconds). Gas anesthesia was withdrawn immediately before triggering blast. Blast winds accompanying intense blast overpressures can lead to substantial head acceleration, generating severe or lethal injuries; therefore, the animals were placed 45 degrees lateral to the shock tube axis to avoid blast wind exposure.

The body was constrained securely inside a padded metal holding tube to limit blast wave pressure exposure primarily to the head. The head was constrained to limit any movement, and prevent injury due to head rotation. Control mice received anesthesia and were constrained the same way, except for exposure to blast wave pressure. Mice were placed at a distance of 15 cm from the open end of the shock tube, with the right side of the head (ipsilateral eye) facing the blast wave (Figure 1). The angle and distance from the shock tube's open end determined the peak overpressure and positive duration experienced by the mouse,⁷ both of which were measured by a pressure transducer located 1.75 cm below the mouse's head. On average, recovery from the blast exposure and anesthesia, indicated by upright posture and ambulation, occurred after approximately 30 seconds.

Histopathology and Immunohistochemistry

Blast-exposed and sham mice were anesthetized i.p. with 200 mg/kg ketamine and 20 mg/kg xylazine, followed by supplementation with isoflurane, and perfused transcardially with 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline. Ipsilateral globes were post-fixed in 4% paraformaldehyde. After 24 hours, each lens was removed and globes were subjected to a sucrose gradient (10%, 20%, and 30% all in 0.1 MPO₄ buffer), embedded in OCT, and frozen using dry ice. Sagittal sections (9 μ m thick) of the ipsilateral retina were collected onto superfrost plus glass slides. Globes contralateral to the blast were post-fixed in Bouin's fixative for 24 hours, embedded in paraffin, and divided into sections sagittally (4 μ m thick) onto superfrost plus glass slides. Paraffin-embedded sections of the retina were rehydrated using xylene, followed by a decreasing ethanol concentration gradient (100%, 90%, and 70%), and a final wash with diH₂O. Heat-mediated

Figure 1 Schematic representation of blast exposure. Exposure to blast wave pressure was conducted as described by Shah et al.⁷ Animals were placed 38 degrees lateral to the shock tube axis, 15 cm from the Mylar membrane, with the right (R) side of the head (ipsilateral eye) facing the blast wave. Control mice were restrained the same way, but were not exposed to blast wave. L, left.

antigen retrieval was performed using EDTA buffer (10 mmol/L Trizma base, 1 mmol/L EDTA solution, and 0.05% Tween 20, pH 9.0) in an autoclave for 30 minutes. OCT-embedded sections of the retina were hydrated for 15 minutes using tris-buffered saline with 0.05% Tween 20. Paraffin and OCT-embedded tissues were incubated with Background Buster (Innovex Biosciences Inc., Richmond, CA) for 1.5 hours. Primary antibodies against glial fibrillary acidic protein (GFAP; 1:500; Dako, Carpinteria, CA), Iba1 (1:500; Wako Chemicals USA, Inc., Richmond, VA), CD68 (1:100; Wako Chemicals USA, Inc.), AT-270 (tau pThr181; 1:100; Thermo Fisher Scientific, Inc., Rockford, IL), AT-180 (tau pThr231; 1:100; Thermo Fisher Scientific, Inc.), tau clone 39E10 (1:250; BioLegend, San Diego, CA), and calbindin (1:1000; Dako) were diluted in blocking solution containing 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 0.04% Triton X-100 (Thermo Fisher Scientific, Inc.), and 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in phosphate-buffered saline and incubated for 48 hours at room temperature, then 24 hours at 4°C. Tissues were washed with tris-buffered saline with 0.05% Tween 20 (6 × 5 minutes), and incubated with a secondary antibody cocktail including Cy3 and/or Alexa Fluor 488—conjugated AffiniPure secondary antibodies (1:300; Jackson ImmunoResearch), and DAPI (1 µg/mL; Sigma-Aldrich) for 1.5 hours. After another wash, slides were mounted with Vectashield HardSet antifade mounting medium (Vector Laboratories Inc., Burlingame, CA). Negative controls were processed in parallel by omission of the primary and/or secondary antibody.

Behavioral Studies

Social Discrimination Test

The social discrimination test assesses the ability of a mouse to discriminate between its own bedding and the bedding of a mouse of the opposite sex. The test is performed inside a narrow rectangular (44.5 × 10.8 cm) chamber made of clear Plexiglas. A small container filled with bedding is fixed at either end of the chamber. Each container is sealed except for a 1-cm diameter hole on top to allow sniffing without disturbing the bedding. A 3-minute test session began by placing a mouse in the middle of the chamber facing his own bedding container. Movements inside the test chamber are video-tracked from above via webcam connected to ANY-maze software version 4.99 (ANY-maze Behavioral Tracking Software, Wood Dale, IL). The sniffing zone was defined by a circle 1 cm wider than the outside circumference of the bedding container, and time spent with head inside the sniffing zone was used to test for group differences.

Morris Water Maze

We assessed the impact of successive blast wave pressure exposure on learning and memory by subjecting mice to the Morris water maze, according to the rapid 2-day protocol described by Gulinello et al.⁸ with some modification. The apparatus comprised a round, 1.15-m diameter galvanized

stock tank filled with water mixed with white tempera paint, allowing the movements of mice to be tracked by a webcam connected to ANY-maze software. Several highly visible cues (references) were posted just outside the tank above each of four quadrants. Water temperature was maintained at 22°C to 24°C. In this 2-day rapid Morris water maze protocol, mice were subjected to 1 day of five 60-second training trials preblast wave exposure and 1 day of five 60-second training trials 30 days after exposure. During all five trials for both sessions, the 11.2-cm diameter platform (1% of pool area) was submerged 1 cm below the surface and remained in one fixed location. For trial 1 only, the platform was made visible by a bright green vertical center post marking its location. Furthermore, each mouse was placed on the platform for 10 seconds before initiating each trial. Every trial began with the mouse facing the tank wall, and trials ended with the mouse resting on the platform for 20 seconds, having either reached the platform on its own or being placed there by the experimenter after the 60-second trial ended. After each trial, mice were returned to their cages, which were placed on heating pads where they remained for 10 to 15 minutes, allowing enough time for their fur to dry before the next trial began. We assessed maze performance by measuring the time taken to first reach the platform.

Rotarod

Locomotor coordination was measured on the accelerating rotarod (pc Rota Rod IV; Accuscan Instruments, Columbus, OH), which could test four mice per run. At 30 days after blast wave pressure exposure, mice were placed on the 3.2-cm diameter rotarod as it rotated at $0.000286 \times g$. Then, after a 90-second acclimation, the rotarod began accelerating to $0.007155 \times g$ during a 1 minute (16 rpm/minute) period before ramping up to $0.007155 \times g/\text{minute}$ for an additional 2 minutes, thus reaching a maximum speed of $0.064397 \times g$ after 3 minutes. By accelerating to high rpm in a relatively short time, we intended this to be a demanding motor coordination task to better discriminate subtle treatment effects while simultaneously minimizing the fatigue seen in prolonged constant speed trials. Latencies to fall are reported as the average of three trials, which were executed consecutively with a 20-second gap (at $0.000286 \times g$) between acceleration trials. A trial ended when infrared (IR) sensors below the rod registered a fall or when the experimenter triggered the sensors manually to terminate passive rotations. In the latter case, even though the trial was terminated, mice were left on the rotarod until the trial terminated for all mice.

Open-Field Test

The spontaneous locomotor activity and thigmotaxis (wall-hugging or open-field anxiety) of mice exploring a novel open field was measured in a 40 × 40-cm (1600-cm²) clear Plexiglas chamber that fit inside a VersaMax activity monitor (model VMM; Accuscan Instruments). The monitor is equipped with two 40 × 40-cm square arrays of IR beams. The IR light-emitting diodes within each array are 2.5 cm apart laterally,

with the lowermost array monitoring the animal's horizontal x-y position at 1.5 cm high, whereas the uppermost array at 10.5 cm high registered vertical movements. Horizontal and vertical activity scores represent the number of IR beam breaks in the lower and upper rows of beams, respectively. The IR beam break data were acquired via the VersaMax Analyzer (model VMAUSB; Accuscan Instruments). We used VersaMap to define time spent in the center to be determined by beam breaks >10 cm (>4 IR beams) from walls. All VersaMax activity monitoring for pretreatment and post-treatment sessions lasted 12 minutes, with only the last 10 minutes used for analysis. The first 2 minutes, representing within-session acclimation, were truncated.

HPLC Analysis of Striatal Neurotransmitter Levels

Tissue concentrations of i) dopamine and its two main metabolites, 3,4-dihydroxyphenyl-acetic acid and homovanillic acid, ii) serotonin and its main metabolite, 5-hydroxyindoleacetic acid, and iii) norepinephrine were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. After 30 days post blast wave exposure, samples from the striatum were prepared and quantified as described previously.⁹ Briefly, four female and four male mice randomly selected from each group were sacrificed via carbon dioxide inhalation, followed by exsanguination by cardiac puncture. Target tissues dissected from the extracted brains were weighed and then immediately frozen on dry ice after suspending in 0.1 mol/L perchloric acid solution containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅ before transferring them to -80°C. Tissue homogenates were centrifuged through 0.22-μm filters before diluting 1:9 in the phosphate-buffered acetonitrile mobile phase MD-TM (ESA Inc., Chelmsford, MA). The primary analytes dopamine, serotonin, norepinephrine, and the metabolites 3,4-dihydroxyphenyl-acetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid were separated isocratically by injecting 20 μL through a C-18 reversed-phase column (Microsorb-MV 100-3; 100 × 4.6 mm; 3-μm particles) using a flow rate of 0.6 mL/minute on an HPLC system (UltiMate 3000; Dionex, Madison, WI) coupled to an analytical thermostatted autosampler (WPS-3000TSL; Dionex). The electrochemical detection system consisted of a coulometric array detector (CoulArray 5600A; Dionex) with a guard cell (model 5020; Thermo Scientific, Chelmsford, MA) and an analytical cell (model 5014B; ESA Inc.). The data acquisition and analysis were performed using CoulArray Data Station Software version 3 (ESA Inc.).

Western Blot Analysis of Brain Tissues

Tissue homogenates were prepared using modified radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA), as previously described.¹⁰ Briefly, prefrontal cortex, ipsilateral to the blast was dissected and homogenized in

modified radioimmunoprecipitation assay. Protein concentrations were determined with the Bradford protein assay. Homogenates containing equal amounts of protein were separated on a 10% to 15% SDS-polyacrylamide gel. After separation, proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with LI-COR blocking buffer for 1 hour. The membranes were then incubated with primary antibodies directed against tau PHF-1 (pSer396,404; rabbit monoclonal; 1:2000 dilution; a gift from the laboratory of Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY), PHF-tau clone 39E10 (mouse monoclonal; 1:2000 dilution; BioLegend, San Diego, CA), Iba1 (goat monoclonal; 1:1200 dilution; Abcam, Cambridge, MA), or GFAP (mouse monoclonal; 1:1200 dilution; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. The primary antibody treatments were followed by treatment with IR800-conjugated anti-rabbit or Alexa Fluor 680-conjugated anti-mouse secondary antibody (1:5000; Abcam) for 1 hour at room temperature. To confirm equal protein loading, blots were reprobed with β-actin antibody (1:15,000 dilution; Abcam). Western blot images were captured with the Odyssey IR Imaging system (LI-COR Biosciences), and data were analyzed using Odyssey 2.0 software (LI-COR Biosciences, Lincoln, NE).

Quantitative and Statistical Analysis

Fluorescence images were taken at ×20 using a commercial upright microscope system (Zeiss AxioPlan 2 Microscope Imaging System; Oberkochen, Germany). Confocal images we captured using a Nikon A1R+ Resonant Scanning Confocal System with a Ti-E inverted microscope and laser lines 405, 488, 561, and 640 nm (Nikon Instruments Inc., Melville, NY). Three different sites of the retina were analyzed to gauge for differences in immunoreactivity: central retina at the thickest section of the retina adjacent to the optic nerve head, and two peripheral retinal locations on opposite sides of the retina within one 20× field of view from the peripheral margin. Micrographs were captured using a commercial photo-editing system [Adobe Photoshop and Adobe Illustrator (CC); Adobe Systems, San Jose, CA]. For quantification of GFAP, Iba1, AT-270, AT-180, and CD68 immunoreactivity, the percentage of the total image area thresholded (outer limiting membrane to inner limiting membrane of the retina) was analyzed using ImageJ version 1.47v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Sex versus treatment interaction effect via two-way analysis of variance was used to determine whether sexes should be pooled. For all behavioral as well as histological studies, the ratio of males/females was equal ($n = 5$ males and 5 females). Preliminary analyses for behavioral and histological studies ($P \leq 0.05$) showed the two sexes did not respond differently to the same treatment (Supplemental Table S2); therefore, sexes were pooled. Pooled rotarod data were analyzed using an unpaired *t*-test. All other pooled behavioral data were analyzed using two-way analysis of variance, and Tukey's multiple comparisons test

(post-hock). HPLC data were analyzed using an unpaired *t*-test. Quantified histological data were analyzed using two-way analysis of variance, and Tukey's multiple comparisons test (post-hock). Thickness of the outer nuclear layer was measured by counts of cell bodies spanning the layer. For each animal ($n = 20$), the central retina within a cross section was used. Retinal thickness was analyzed using an unpaired *t*-test. Prism 6 for Windows (Graph Pad Software, La Jolla, CA) was used for statistical analysis.

Results

Blast Wave Pressure Induces Activation of Retinal Glial Cells

Müller glia are the principal glial cells of the retina, with processes projecting radially through the thickness of the retina, and provide limits at the outer and inner limiting membrane. In the healthy retina, GFAP in Müller glia is localized to the end feet at the retina's inner limiting membrane.¹¹ In response to retinal injury or stress, thick GFAP immunoreactive processes span the retina from the inner limiting membrane to the external limiting membrane, concomitant with hypertrophied processes of Müller glia.¹² We assessed the distribution of GFAP immunoreactivity to detect activation of Müller glia after blast exposure. GFAP immunoreactivity in sham animals with retinas ipsilateral (Figure 2A) and contralateral (Supplemental Figure S1A) to blast exposure was localized to the Müller glia end feet, and astrocytes in the optic fiber layer. When quantified, the distribution of GFAP immunoreactivity in retinas of sham animals accounted for approximately $2.60\% \pm 0.39\%$ (means $\pm$ SEM) of total area (Figure 2C and Supplemental Figure S1C). Thirty days after blast exposure, the area of GFAP immunoreactivity in retinas ipsilateral to blast exposure was increased and consistently spanned the retina from the Müller glia end feet in the inner limiting membrane to the outer plexiform layer, with some apical processes visible in the outer nuclear layer (Figure 2B). There were no significant differences in the area of GFAP immunoreactivity between peripheral and central retinas of blast-exposed mice ($22.79\% \pm 3.19\%$ and $29.41\% \pm 2.35\%$ of total area, respectively) (Figure 2C). In contrast, GFAP immunoreactivity in retinas contralateral to blast exposure remained localized to the Müller glia end feet, with occasional processes extending past the optic fiber layer (Supplemental Figure S1B). When quantified, the area of GFAP immunoreactivity in retinas contralateral to blast exposure was $4.52\% \pm 0.77\%$ and $4.32\% \pm 0.74\%$ of total area in peripheral and central retinas, respectively (Supplemental Figure S1C).

Successive Blast Exposure Induces an Inflammatory Response in the Retina

The prevalence and morphology of retinal microglia in response to blast injury was assessed using Iba1

Figure 2 Distribution of GFAP in retinas ipsilateral to blast exposure, and quantification of the area of GFAP immunoreactivity. **A:** GFAP localizes to the optic fiber layer (OFL) of retinas of sham mice. **B:** Thirty days after blast exposure, the distribution of GFAP immunoreactivity increases, spanning the retina from the OFL to the outer nuclear layer (ONL). **C:** Bar graphs show a significant increase in the percentage area of GFAP immunoreactivity in peripheral and central retina after blast exposure. Data are expressed as means $\pm$ SEM (**C**). $n = 10$ mice per group. $**P < 0.01$ versus peripheral sham; $++++P < 0.0001$ versus central sham. Scale bars = $20\ \mu\text{m}$ (**A** and **B**). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer.

immunoreactivity. In sham animals, Iba1 immunoreactivity showed ramified microglia with thin irregular processes and small somata (occasionally lacking processes), within the inner and outer plexiform layers (Figure 3A). Iba1 immunoreactivity occupied $0.31\% \pm 0.06\%$ of total area in peripheral retina, and $0.37\% \pm 0.11\%$ of total area in central retina (Figure 3C). Iba1 immunoreactivity in the retinas ipsilateral to blast exposure showed an amoeboid-like morphology with thicker, and longer processes, and swollen cell bodies, all characteristics of activated microglia.¹³ Iba1 immunoreactivity was localized to the inner and outer plexiform layers (Figure 3B). When quantified, Iba1 immunoreactivity accounted for $0.93\% \pm 0.19\%$ and $0.93\% \pm 0.18\%$ of total area in peripheral and central retinas, respectively (Figure 3C). Iba1 immunoreactivity in retinas contralateral to blast exposure was not significantly

Figure 3 Distribution of Iba1 and CD68 in retinas ipsilateral to blast exposure, and quantification of microglial activation. There are distinct morphological differences between microglia of control and blast-exposed retinas. **A:** Microglia of sham retinas are ramified with small cell bodies, and thin processes (arrows, main image and inset). **B:** Microglia of blast-exposed retinas are amoeboid with swollen soma, and long thick processes (arrows, main image and inset). Microglia of both control and blast-exposed retinas are localized to the outer plexiform layer (OPL) and inner plexiform layer (IPL). **Insets:** High magnification images of microglial morphology. **D** and **E:** CD68 immunoreactivity shows activated microglia localized to the OPL and IPL (**E**), compared to the lack of activated microglia in sham retinas (**D**). **C** and **F:** Bar graphs show a significant increase in area of Iba1 and CD68 immunofluorescence in peripheral and central retina after blast exposure. Data are expressed as means $\pm$ SEM (**C** and **F**). $n = 10$ mice per group. * $P < 0.05$ versus peripheral sham, **** $P < 0.0001$ versus peripheral sham; † $P < 0.05$ versus central sham, †††† $P < 0.0001$ versus central sham. Scale bars = 20 μ m (**A**, **B**, **D**, and **E**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

different from retinas of sham animals; however, morphological differences of microglia in retinas from blasted animals were noted (**Supplemental Figure S2**, A–C). The cell surface antigen CD68 is a common marker expressed on macrophages¹⁴; therefore, immunoreactivity for CD68 was used to examine blast-exposed retinas for proinflammatory microglial activation. No CD68 immunoreactivity was detected in the retinas of sham-exposed mice (**Figure 3D** and **Supplemental Figure S2D**). The retinas of exposed mice from the side ipsilateral to the blast were positive for CD68 immunoreactivity in the inner and outer plexiform layers (**Figure 3E**). When quantified, CD68 immunoreactivity was $1.22\% \pm 0.12\%$ and $1.16\% \pm 0.15\%$ of total area in peripheral and central retinas, respectively (**Figure 3F**). In contrast, values for CD68-positive microglia in the retinas contralateral to blast exposure were significantly lower than those of retinas ipsilateral to blast exposure, making up $0.32\% \pm 0.03\%$ and $0.30\% \pm 0.06\%$ of total area in peripheral and central retinas, respectively (**Supplemental Figure S2**, D–F).

Successive Blast Exposure Results in an Increase in Phosphorylated Tau Species Detected in Retinal Neurons and Glia

Hyperphosphorylated tau is a major component of neurofibrillary tangles involved in the pathology of Alzheimer disease and other neurodegenerative conditions.¹⁵ Antibodies against AT-270 and AT-180 (detects paired helical filament with a phosphorylated threonine in position 181 and 231, respectively) were used to evaluate the presence of phosphorylated tau species in the retinas of animals exposed to blast wave pressure. There was little AT-270 and AT-180 immunoreactivity in retinas of sham animals. AT-270 immunoreactivity comprised $0.04\% \pm 0.04\%$ of total area in peripheral retinas, and $0.09\% \pm 0.07\%$ of total area in central retinas, whereas AT-180 immunoreactivity made up $0.02\% \pm 0.01\%$ of total area in peripheral retinas, and $0.03\% \pm 0.02\%$ of total area in central retinas (**Figure 4**, A, C, D, and F). However, 30 days after successive blast exposure, retinas ipsilateral to blast exposure showed robust AT-270 and AT-180 immunoreactivity localized to the optic fiber layer, and outer plexiform layer (**Figure 4**, B and E). When quantified, AT-270 immunoreactivity was approximately $6.34\% \pm 0.90\%$ of total area in peripheral retinas, and $5.72\% \pm 0.84\%$ in central retinas (**Figure 4C**). In contrast, values for AT-270 in the retinas contralateral to blast exposure were significantly lower than those of retinas ipsilateral to blast exposure (**Supplemental Figure S3**). AT-180 immunoreactivity was approximately $3.67\% \pm 0.90\%$ of total area in peripheral retinas, and $3.65\% \pm 0.53\%$ in central retinas (**Figure 4F**). An antibody against total tau demonstrated there was no appreciable difference in total tau immunoreactivity when comparing retinas from blast animals to sham animals (**Supplemental Figure S4**). The outer plexiform layer contains numerous neuronal

Figure 4 Expression and distribution of AT-270 and AT-180 in retinas ipsilateral to blast exposure and quantification of immunoreactivity. **A, B, D,** and **E:** Thirty days after successive blast exposure, retinas ipsilateral to the blast show that intense AT-270 and AT-180 immunoreactivity for phosphorylated tau epitopes is localized to the outer plexiform layer (OPL) and optic fiber layer (OFL; **B** and **E**), whereas no AT-270 or AT-180 immunoreactivity is detectable in sham retinas (**A** and **D**). **B** and **E:** Phosphorylated tau in blast-exposed retinas is also occasionally localized to the IPL, with sparse immunoreactivity. **C** and **F:** Bar graphs show an increase in total fluorescence area for both phosphorylated tau epitopes in both peripheral and central retina after blast exposure. Data are expressed as means $\pm$ SEM (**C** and **F**). $n = 10$ mice per group. **** $P < 0.0001$ versus peripheral sham; ††† $P < 0.001$, †††† $P < 0.0001$ versus central sham. Scale bars = 20 μ m (**A, B, D,** and **E**). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.

processes, including those of horizontal cells. Calbindin immunoreactivity in the outer plexiform layer is specific for horizontal cell processes.¹⁶ Double immunofluorescence and confocal microscopy were used to determine whether phosphorylated tau was present in horizontal cell processes. Retinas ipsilateral to blast exposure showed colocalization of immunofluorescence for the two phosphorylated tau epitopes (AT-270 and AT-180), and calbindin, confirming that these phosphorylated tau species were present in horizontal cells (Figure 5). Because a similar pattern of GFAP immunoreactivity was also detected in activated Müller glia, double labeling with GFAP and AT-270, assessed with confocal microscopy, also revealed colocalization of the two proteins in the outer plexiform layer (Figure 6, A–F) and optic fiber layer (Figure 6, A–C and G–I), suggesting Müller glia also contain phosphorylated tau in retinas of blast-exposed mice. Double labeling with GFAP and AT-180 revealed colocalization in the outer plexiform layer (Figure 6, J–L).

Successive Blast Exposure Causes a Loss of Photoreceptors

To determine whether blast exposure resulted in changes in retinal morphology, we assessed the thickness of the outer nuclear layer, as measured by number of photoreceptor cell nuclei. Thirty days after blast exposure, the mean thickness of the outer nuclear layer of retinas ipsilateral to blast exposure was significantly decreased from approximately 14 cell nuclei in sham retinas to 10 cell nuclei in retinas of blast-exposed mice (Figure 7). Outer nuclear layer thickness in retinas contralateral to blast exposure trended to decrease from 13 cell nuclei in sham animals to 11 cell nuclei; however, the decrease did not reach significance (Supplemental Figure S5).

Blast Wave Pressure Does Not Significantly Affect Cognitive or Motor Function

To provide context for retinal changes we report, the following are results from assessment of cognitive and motor function as well as striatal neurotransmitter and GFAP and Iba-1 levels in the frontal cortex.

To assess the effect of blast wave pressure on cognitive function, olfactory recognition memory, and spatial learning, we used the social discrimination test (Figure 8A) and Morris water maze (Figure 8B), respectively. Cognitive function was assessed 30 days after exposure to blast wave pressure. Olfactory recognition memory refers to the ability to distinguish between familiar and unfamiliar stimuli, in this case the ability of adult male and female mice to discriminate between their own bedding and the bedding of a mouse of the opposite sex. Olfactory recognition was measured according to the time a mouse spent within the sniffing zone of its bedding. Preliminary results showed the two sexes did not respond differently to the same treatment;

Figure 5 Colocalization of phosphorylated tau and calbindin in retinas ipsilateral to blast exposure. **A–C:** Double labeling with AT-270 and calbindin shows colocalization of the two proteins in the outer plexiform layer (OPL). **D–F:** Confocal imaging confirms colocalization in the OPL, suggesting that phosphorylated tau (pThr 181) is present in horizontal cells. **G–I:** Double labeling with AT-180 and calbindin also shows colocalization. Scale bars: 20 μ m (**A–C** and **G–I**); 40 μ m (**D–F**). Original magnification, $\times 40$ (**D–F**). GCL, ganglion cell; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.

therefore, sexes were pooled (data not shown). We show no significant difference in time spent in the sniff zone, between sham, and mice exposed to blast (Figure 8A). The Morris water maze was used to assess the effect of blast wave pressure on memory, by measuring latency to platform, or the time it took for a mouse to find and reach the platform after training sessions. Our data showed no significant difference in performance between sham and blast-exposed groups (Figure 8B). A series of tests were performed to characterize the impact of blast wave pressure on locomotion. Thirty days after blast exposure, mice were tested using the Versamax locomotor activity monitor and the rotarod. No deficit in motor function was detected in

blast-exposed mice when compared to sham, as seen with behavioral parameters, including the rotarod test (Figure 8C), open-field activity (Figure 8D), horizontal activity (Figure 8E), or vertical activity (Figure 8F). Together, our results suggest that blast wave pressure does not cause deficits in cognitive or motor function.

Blast Wave Pressure Does Not Cause Damage to Striatal or Cortical Neurons

To further probe the effect of blast wave pressure on the brain, we measured striatal dopamine and its metabolites, 3,4-dihydroxyphenyl-acetic acid and homovanillic acid;

Figure 6 Colocalization of phosphorylated tau and GFAP in retinas ipsilateral to blast exposure. **A–I:** Double labeling with AT-270 and GFAP shows colocalization of the two proteins in the outer plexiform layer (OPL) and optic fiber layer (OFL). **D–I:** Confocal imaging confirms colocalization in the OPL (**D–F**) and OFL (**G–I**), suggesting that phosphorylated tau (pThr 181) is present in Müller glia. **J–L:** Double labeling with AT-180 (pThr 231) and GFAP shows colocalization, primarily in the OPL. Scale bars: 20 μ m (**A–C** and **G–I**); 40 μ m (**D–F**). Original magnification, $\times 40$ (**D–I**). GCL, ganglion cell; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.

Figure 7 Measurement of retinal thickness. Successive blast exposure causes a decrease in retinal thickness of retinas ipsilateral to blast exposure. Thickness of outer nuclear layer (ONL) is expressed as number of cell bodies spanning the thickness of the layer. Mean retinal thickness of sham retinas is 13.83 ± 0.31 nuclei. In retinas ipsilateral to blast exposure, mean retinal thickness is 10.33 ± 0.84 nuclei. Data are expressed as means $\pm$ SEM. $n = 10$ mice per group. ** $P < 0.01$ versus sham.

serotonin and its metabolite, 5-hydroxyindoleacetic acid; and norepinephrine by HPLC (Figure 9). Our results show no significant changes in striatal neurotransmitter levels between sham and blast-exposed groups. To assess for blast-induced pathology in the prefrontal cortex, we measured protein levels of GFAP, Iba1, p-tau, and total tau. Western blot analysis revealed no significant differences in levels of Iba1 or phosphorylated tau in the prefrontal cortex 30 days after exposure to blast wave pressure (Figure 10, A, C, and D), although GFAP expression in the prefrontal cortex was decreased 30 days after exposure to blast wave pressure (Figure 10, A and B).

Discussion

This study demonstrates that three daily successive exposures to 300 kPa (43.5 psi) blast wave pressure induces long-lasting glial cell activation, inflammation, neuronal loss, and an increase in phosphorylated tau in the mouse retina. Despite these retinal effects, no significant changes in cognitive or motor function were detected. This suggests that robust retinal pathology was not due to extreme severity of blast wave pressure. We show no significant changes in striatal neurotransmitters, and we did not detect significant differences in levels of Iba1, or phosphorylated tau in the prefrontal cortex. There was an observed decrease in GFAP in the prefrontal cortex of these animals, which may be because of remodeling of the astrocyte network due to stress.¹⁷ Thus, in this model, the retina may serve as a central nervous system compartment that is more vulnerable, and therefore may be an effective and more sensitive indicator of low-level injury due to blast wave pressure.

This work used a compressed air–driven shock tube system to expose mice to blast wave pressure of 300 kPa per day for 3 successive days. A compressed shock tube system provides an instantaneous increase in pressure, immediately followed by a decrease below the surrounding ambient pressure that occurs within 2 milliseconds, a range of time that closely reflects a real explosive blast.¹⁸ In addition, the mice were not exposed to the blast wind to prevent injury due to head rotation, thus ensuring that injury is due to neural tissue deformation resulting from the blast wave pressure. Our model provides for controlled investigation of the chronic effects of high-level blast wave pressure without the confounding effects of secondary blast injury due to flying debris, or injury due to head acceleration/deceleration.

The current study reports a change in distribution of GFAP immunoreactivity in Müller glia, indicating activation in the retinas of blast-exposed mice. Activation of Müller glia was greater in retinas ipsilateral rather than contralateral with respect to the direction of the blast wave. Hypertrophy of Müller cells, indicated by an increase in the distribution of GFAP immunoreactivity, is a widely reported pathology after exposure to blast wave pressure; however, most are reports of acute trauma. A number of studies report an increased distribution of GFAP immunoreactivity in Müller cell processes 7 days after exposure.^{2,4,19} Increased distribution of GFAP throughout Müller glia is a ubiquitous response to stress observed in various retinal degenerative diseases.²⁰ Müller glia serve to maintain neuronal homeostasis, including degradation of foreign material; therefore, a stress response evident by swollen Müller end feet and hypertrophied processes 30 days after blast exposure suggests a fundamental role in response to retinal trauma.²⁰ Although glial cell activation immediately after retinal injury is important for the repair and protection of retinal neurons, studies show that diabetic retinopathy and other retinal degenerative diseases may be exacerbated by reactive gliosis causing dysplasia and overall progression of neuronal degeneration.^{21–23} Therefore, insight into the regulation of this process may be necessary to determine the role of Müller glia in long-lasting retinal injury due to successive blast wave pressure.

Our results using Iba1 immunoreactivity indicate activation of microglia with an amoeboid-like morphology and thick processes localized to the outer plexiform and inner plexiform layers of retinas exposed to blast. Thirty days after blast exposure, Iba1 protein levels in the prefrontal cortex were comparable to sham. Others have shown prevalence of microglia 7 days after blast exposure, specifically in the pyramidal tract of the pons and cerebellar white matter as well as the optic tract. This reactivity, however, was no longer evident after 2 weeks.²⁴ The presence of reactive microglia in the retina with an amoeboid-like appearance, beginning at 3 days after blast exposure, has also been reported.¹⁹ Anti-CD68 was used to evaluate a proinflammatory response because of local proliferation,

Figure 8 Successive blast exposure does not affect cognitive or motor function. **A** and **B**: Performance parameters using the VersaMax infrared computerized activity monitoring system and Morris water maze (MWM) were used as a measure of cognitive function. The social discrimination test assesses olfactory recognition memory (**A**), whereas the MWM assesses for overall memory retention (**B**). **C–F**: Motor function was measured using the rotarod and VersaMax infrared computerized activity monitoring system. VersaMax data showing latency to fall on rotarod (**C**), center time in VersaMax open-field (**D**), horizontal activity (**E**), and vertical activity (**F**). **D**: Center time in VersaMax open field is also a correlate of open-field anxiety. Analysis of spontaneous locomotor activity shows no significant difference between sham and mice exposed to blast wave pressure. Data are expressed as means $\pm$ SEM (**A–F**). $n = 10$ mice per group.

and enhanced phagocytosis.¹⁴ CD68 immunoreactivity showed a prominent macrophagic response of microglia in the inner and outer plexiform layers of blast-exposed retinas, compared to the absence of CD68-positive microglia in sham animals. Microglia comprise the majority of tissue macrophage population within the central nervous system;

therefore, activation represents a common pathomechanism in a variety of retinal degenerative diseases, often parallel to chronic inflammation and the onset of retinal cell death.¹³ Without a strict regulatory mechanism to limit this immunological cascade, microglial activation may significantly contribute to furthering retinal tissue damage.^{14,25} This may be the reason for the presence of activated microglia 30 days after exposure to blast wave pressure. Further studies to assess the functional phenotype of microglial activation, whether classic or alternative, are necessary to evaluate the specific role of microglia in long-lasting retinal injury due to blast wave pressure.

We report increased tau phosphorylation (Thr231 and Thr181) in retinas of blast-exposed mice. Phosphorylation of Thr231 is shown to reduce levels of acetylated tubulin, inhibiting microtubule assembly and stabilization.²⁶ Accumulation of neurofibrillary tangles is a hallmark feature of neurodegenerative disorders, all of which present with neuronal tau-positive inclusions as a predominant feature.^{15,27} In the healthy brain, tau localizes to axons to promote neuronal integrity and axonal transport; however, a stepwise process, including phosphorylation of specific sites, and later aggregation into neurofibrillary tangles, or oligomers, can lead to neurodegeneration.^{28,29} Most tauopathies are characterized by the abnormal accumulation of tau in both neurons and glial cells in contrast to the healthy brain, where tau expression is minimal in glial cells. Glial tau phosphorylation occurs most often in oligodendrocytes, astrocytes, and to a lesser extent in microglia.^{27,30,31} Studies in blast models demonstrate elevated levels of

Figure 9 Blast wave pressure does not deplete striatal neurotransmitter levels. Thirty days after blast exposure, levels of striatal dopamine, norepinephrine, serotonin, and its metabolite levels were measured using high-performance liquid chromatography. Compared to the striata of control mice, exposure to blast does not significantly alter levels of striatal dopamine (DA), 3,4-dihydroxyphenyl-acetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), 5-hydroxytryptamine (5-HT, serotonin), or 5-hydroxyindoleacetic acid (5-HIAA), as measured by HPLC. Data are expressed as means $\pm$ SEM. $n = 4$ mice per group.

Figure 10 Western blot analysis of GFAP, Iba1, and p-tau protein expression levels in the prefrontal cortex. **A:** GFAP (50 kDa), Iba1 (15 kDa), p-tau (50 kDa), tau (50 kDa), and actin (37 kDa) immunoreactive bands. **B–D:** Representative bar graphs showing quantitative densitometric analysis of GFAP/actin (**B**), Iba1 (**C**), and p-tau/tau ratio (**D**) in prefrontal cortex of six mice per group. Data are given $\pm$ SEM (**B–D**). * $P < 0.05$ versus sham.

phosphorylated tau expressed by pyramidal neurons of the hippocampus and Purkinje and basket cells of the cerebellum 2 weeks after blast exposure, but the presence of phosphorylated tau species in the retina has not been investigated.³² Huber et al³² report elevated levels of cleaved tau in the brain 30 days after a single blast exposure event, therefore setting the stage for chronic neurofibrillary tangle formation. Similarly, as part of the central nervous system, the retina is also susceptible to such damage. Müller glia undergo activation in response to retinal damage, which exacerbates pathology¹²; therefore, an increase in tau phosphorylation in Müller glia may also be a response to stress. Furthermore, although glial activation has proved protective in some contexts, glial cells are often implicated as secondary effectors of toxicity through the release of proinflammatory cytokines.^{33,34} Perhaps the increase of phosphorylated tau in Müller glia contributes to the pathological response to stress. Retinal horizontal cells are a class of interneurons that regulate input and output between photoreceptor cells and bipolar cells via feedback and feed-forward inhibition.³⁵ As horizontal cells modulate signal transmission from photoreceptors to bipolar cells, continued degeneration of horizontal cells due to tau accumulation may cause disruption of this signal, and contribute to retinal dysfunction.³⁶ Gupta et al³⁷ show a significant increase in tau protein (isoform AT8, phosphorylated at serine 202 and threonine 205) colocalized with parvalbumin in horizontal cells, within the posterior retina of surgical glaucoma specimens. The significance of phosphorylated tau in Müller glia and horizontal cells in response to retinal damage caused by blast wave pressure requires further investigation.

We report a decrease in retinal thickness, specifically of the outer nuclear layer of the retinas of mice ipsilateral to blast exposure. By 30 days after blast exposure, the thickness of the outer nuclear layer decreased by approximately 28.5%. Because the outer nuclear layer contains cell bodies of photoreceptor cells, rods, and cones, a decrease in thickness indicates photoreceptor cell death. Previous studies show decreased axon density and minimal degeneration 10 months after blast injury; however, data on the loss of specific cell types and the thickness of retinal layers were not reported.¹⁸

We also report that blast wave pressure of 300 kPa (43.5 psi) per day for 3 successive days does not induce deficits in cognitive function, including olfactory recognition or spatial memory, evidenced by normal performance in the social discrimination test and the Morris water maze. Consistent with this, the mice exhibited normal function on rotarod, as well as in motor parameters in open field. Prior work has reported that mild TBI, caused by repeated overpressure of 50 to 60 psi, results in open-field anxiety, as well as motor deficits at 1 to 2 weeks after exposure.^{24,38} Mild TBI caused by 50 to 60 psi blasts in mice induced sustained cognitive and motor deficits in mice, coupled with mild neuronal loss and diffuse axonal injury, similar to pathology observed in human mild TBI.³⁸ In addition, Gullotti et al³⁹ concluded that 415 ± 41 kPa (60 ± 6 psi) is necessary to generate a significant behavioral deficit in terms of TBI severity and survivability. This study demonstrates no detectable changes in striatal neurotransmitters that regulate general cognitive and motor ability. Together, these data suggest that even with three daily successive exposures, the overpressure used in this

study (43.5 psi) was not enough to generate prolonged cognitive or motor deficits.

Because of considerable concern in regard to the high frequency of TBI causing visual dysfunction in humans, there are various existing rodent models of blast injury to the retina, but reports demonstrating long-lasting retinal injury are sparse. In this approach, blast wave pressure was targeted to the right side of the head, with the mice positioned to avoid blast wind-induced head rotational injuries that are likely severe or lethal at the high blast overpressures tested. One research group has characterized a model in which a total of 5 kg of 2,4,6-trinitrotoluene with a penta-erythritol tetra-nitrate booster was detonated to deliver high blast wave pressure of 180 KPa, and 480 KPa, whereas another group used an air paintball gun to directly target the eyes of mice with a puff of carbon dioxide (160 to 200 kPa), leading to corneal edema, and deficit in visual acuity.^{4,40} Repeated exposure to blast wave pressure originating from 2,4,6-trinitrotoluene (approximately 120 ± 7 kPa) has been shown to induce glial cell activation in the retina, specifically in the ganglion cell layer and inner nuclear layer, inflammation, and compromised vascular permeability detected 4 to 72 hours after exposure; however, prolonged or long-term effects using either model were not demonstrated.⁴

We demonstrate long-lasting effects of blast wave pressure on specific retinal cell types. Up-regulation of GFAP by Müller glia is a robust indicator of retinal stress that may contribute to and exacerbate prolonged retinal pathology due to blast injury. We show elevated species of phosphorylated tau in horizontal cells and in regions of activated Müller cells. Horizontal cells contribute to the ganglion cell receptive field surround, which is essential for contrast sensitivity. Our results suggest that phosphorylated tau accumulation, as well as corresponding glial cell activation, may set the stage for decreased contrast sensitivity commonly reported by service members. In addition, we show detectable photoreceptor cell loss 30 days after blast exposure, indicating a need for early intervention to counter primary blast effects on the retina. This mouse model of blast-induced retinal trauma provides insight into chronic retinal pathologies caused by blast wave pressure, without injury to the brain. Our work will aid in efforts not only to understand and potentially prevent retinal damage due to blast exposure, but also to assess the magnitude of exposure and identify individuals who may need or benefit from proactive treatments as they become available.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.03.005>.

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