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# Molecular mechanisms of increased cerebral vulnerability after repeated mild blast-induced traumatic brain injury☆

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ABSTRACT

The consequences of a mild traumatic brain injury can be especially severe if it is repeated within the period of increased cerebral vulnerability (ICV) that follows the initial insult. To better understand the molecular mechanisms that contribute to ICV, we exposed rats to different levels of mild blast overpressure (5 exposures; total pressure range: 15.54–19.41 psi or 107.14–133.83 kPa) at a rate of 1 per 30 min, monitored select physiological parameters, and assessed behavior. Two days post-injury or sham, we determined changes in protein biomarkers related to various pathologies in behaviorally relevant brain regions and in plasma. We found that oxygen saturation and heart rate were transiently depressed following mild blast exposure and that injured rats exhibited significantly increased anxiety- and depression-related behaviors. Proteomic analyses of the selected brain regions showed evidence of substantial oxidative stress and vascular changes, altered cell adhesion, and inflammation predominantly in the prefrontal cortex. Importantly, these pathological changes as well as indications of neuronal and glial cell loss/damage were also detected in the plasma of injured rats. Our findings illustrate some of the complex molecular changes that contribute to the period of ICV in repeated mild blast-induced traumatic brain injury. Further studies are needed to determine the functional and temporal

*Abbreviations:* AD, amygdala; BBB, blood brain barrier; bTBI, blast-induced TBI; CNS, central nervous system; DHC, dorsal hippocampus; ICV, increased cerebral vulnerability; mTBI, mild traumatic brain injury; OF, open field; PFC, prefrontal cortex; PTSD, post-traumatic stress disorder; rmTBI, repeated mild traumatic brain injury; TBI, traumatic brain injury; USU, Uniformed Services University; VHC, ventral hippocampus.



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relationship between the various pathomechanisms. The validation of these and other markers can help to diagnose individuals with ICV using a minimally invasive procedure and to develop evidence-based treatments for chronic neuropsychiatric conditions.



## 1. Introduction

Mild traumatic brain injuries (mTBIs) constitute approximately 80% of all traumatic brain injuries (TBIs) [1]. Among civilians, mTBIs (better known as concussions) affect ~1.3 million individuals in the US annually, mostly during contact sports such as boxing, hockey, and football [1,2]. In the military, ~266,000 service members suffered mTBIs between the years 2000 and 2012 with the overwhelming majority of injuries being blast-induced (http://www.dvbic.org/dod-worldwide-numbers-tbi). Because mTBIs typically result in transient and very mild symptoms, many injured individuals return to the same activity—sports and/or military duty—and significant numbers are re-injured, some multiple times causing repeated mild TBI (rmTBI).

A single mTBI can result in neurobehavioral problems such as increased anxiety and cognitive impairment, but the probability of short- as well as long-term impairment is significantly higher following rmTBI [3]. The exposure to a single mTBI triggers a complex cascade of pathological (as well as restorative) responses during what is known as the secondary injury process. Depending on the severity of the insult, some or most of these changes dissipate over time especially after mTBIs. However, re-injury during the ongoing secondary injury process can result in a disproportionally severe—even fatal—outcome and significantly increase the probability of long-term damage. While severe TBIs have unique symptoms (early malignant cerebral edema and delayed severe vasoconstriction), mTBI, symptoms and likely the components of its secondary injury process are shared with other forms of closed head TBIs. However, one important unknown is whether the onset and extent of the individual pathologies are shared among the different forms of mTBIs.

Clinical and experimental data have demonstrated that the interval between insults is a key factor in determining outcome severity after rmTBIs [4,5]. The interval after the initial insult during which re-injury causes disproportionately adverse effects is referred to as the period of increased cerebral vulnerability (ICV). While the exact pathomechanism of ICV is currently unknown, two key studies using different models of rmTBI have shown that decreased cerebral glucose metabolism and increased axonal and vascular vulnerability are major contributors to ICV [6–8]. The authors have concluded that these otherwise transient changes predispose the brain for additional damage if subsequent insults take place within the period of ICV. Whether or not other components of the secondary injury process contribute to ICV as well as the exact "window period" of vulnerability have yet to be determined [5,9].

Previous studies, including our own, have identified several of the pathobiologies involved in blast-induced TBI (bTBI), which may contribute to ICV. These include oxidative stress, metabolic and vascular changes, altered cellular adhesion, inflammation, as well as axonal, neuronal and glial cell damage [10,11]. Accordingly, we have selected protein biomarkers that are representative of the various functional clusters listed above and assayed them using reverse phase protein microarray (RPPM). While we have successfully utilized RPPM for the identification of blast-induced changes in brain tissue, cerebrospinal fluid, plasma and/or serum [10,12–16], the method does not provide absolute protein values like other antibody-based assays (e.g., ELISA) and its use is limited to the availability of specific antibodies. However, RPPM offers superior sensitivity, specificity, a dynamic range, and high throughput ideal for assaying large numbers of samples.

Our previous works using the rodent model of bTBI identified some of the functional deficits sustained following single and repeated mild blast overpressure exposure as well as several of the molecular and cellular changes associated with each type of injury [11]. We found that when rats were exposed to mild blast overpressure (~138 kPa) once per day for five consecutive days, the resultant damage (as indicated by specific protein biomarker levels in the plasma and in brain tissue) was only moderately greater in multiple blast-injured rats compared to single-injured rats at 2 h post-injury, 22 days, or even 42 days after the injury. Consistent with the observations from the abovementioned studies, our data indicated that the period of ICV after blast-induced mTBI is considerably shorter than 24 h in rats.

To better understand the biology of ICV, in this study we exposed rats to a total of five mild blasts at the rate of 1 per 30 min. As described herein, this increased frequency/shortened interval of insults resulted in significant alterations in physiological parameters, neurobehavioral deficits, and extensive molecular changes that can be detected in functionally relevant brain regions as well as in circulating blood. The detected changes in protein biomarker levels implicate oxidative stress, vascular pathologies, and inflammation in the cerebral vulnerability that follows blast-induced mTBI. These markers can be tested in humans who have suffered an mTBI and if validated they will aid in identifying individuals with ICV and in determining a "safe return to duty/play".

## 2. Materials and methods

### 2.1. Animals

A total of 22 male Sprague Dawley rats (weight at arrival: 280–300 g; approximate age in days: 63–67) (Charles River Laboratories, Wilmington, MA) were used in our study. Upon arrival at the Uniformed Services University (USU; Bethesda, MD), animals were housed in pairs in standard rat cages in a reverse 12 h-light 12 h-dark cycle with food and water ad lib. During the 5-day acclimation period, animals were handled for 5 min each day prior to undergoing baseline physiological monitoring and behavioral testing. Baseline horizontal activity results

were used to create the experimental groups: naïve ($n = 4$), sham ($n = 6$), and injured ($n = 12$) with no statistical significance among them. Animals were handled according to protocol, approved by the Institutional Animal Care and Use Committee at USU.

### 2.2. Experimental manipulations and injury

For the duration of the study, naïve animals were kept in the animal facility at USU without any manipulation except on physiological monitoring and behavioral testing days as described later. On the day of the injury, animals in the sham and injured groups were transported from USU to Walter Reed Army Institute of Research (Silver Spring, MD). Sham rats were anesthetized for 6 min in an induction chamber with 4% isoflurane (Forane; Baxter Healthcare Corporation, Deerfield, IL) and placed into a shock tube holder in a transverse prone position without being exposed to blast overpressure. Sham rats were kept in the procedure room adjacent to the shock tube for the length of the injured animals' exposures [11].

Injured animals underwent the same procedures as their respective sham group in addition to being exposed to 5 mild blasts of varying pressures at a rate of 1 per 30 min (*blast no. 1*: Mylar membrane gauge thickness = 750 [average peak total pressure: 15.54 psi or ~107.14 kPa, total overpressure duration: 9.01 ms]; *blast no. 2*: Mylar membrane gauge thickness = 1400 [average peak total pressure: 19.41 psi or ~133.83 kPa, total overpressure duration: 10.60 ms]; *blast no. 3*: Mylar membrane gauge thickness = 1000 [average peak total pressure: 17.78 psi or ~122.59 kPa, total overpressure duration: 9.22 ms]; *blast no. 4*: Mylar membrane gauge thickness = 750; *blast no. 5*: Mylar membrane gauge thickness = 1000). Blast injury was administered to rats (weight at injury: 320–360 g) while wearing chest protection using a compressed air-driven shock tube as described earlier in detail [11,17]. Mortality was approximately 8% (i.e., 1 rat). All animals were transported back to the USU animal facility at the conclusion of the exposures.

### 2.3. Physiological parameters

Physiological parameters were measured non-invasively under isoflurane anesthesia at baseline, immediately following injury (×5 for injured rats), and 24 h after blast (or sham) exposure using the MouseOx® Pulse Oximeter adopted for rats (Starr Life Sciences Corp., Oakmont, PA, USA) [10]. The selected parameters included: (a) arterial oxygen saturation, a ratio of oxyhemoglobin to total hemoglobin concentration in arterial blood (normal $O_2$ levels: 95–100%; low $O_2$ levels: <95%); (b) heart rate, the number of heartbeats per min (reference range for Sprague Dawley rats: 250–450 beats per min); (c) pulse distention: a measure of local blood flow; (d) breath rate, the number of breaths taken per min (reference range for Sprague Dawley rats: 70–115 breaths per min).

### 2.4. Behavioral tests: open field

All animals were tested at baseline and 24 h post-injury (or sham) using the open field (OF) system, which measures naturally occurring behaviors that are exhibited when an animal explores and interacts with its surroundings [18]. Variables extracted from the animal's movement within the chambers (i.e., horizontal movement, vertical movement, and time spent in the center of the chamber) provide information about gross motor performance, depression-related behavior, and anxiety-related behavior.

OF activity was measured using the Accuscan Superflex Sensor Version 2.2 infrared photocell system in the Accuscan Instruments Standard Animal Cage (measuring 40 × 40 × 30 cm; Accuscan Instruments Incorporated, Columbus, OH) located in a dedicated room designed to minimize acoustic interruptions. The animal's locomotion is captured by three, paired 16-photocell Superflex Sensors that transmit the location data to the Accuscan Superflex Node located on the upper-rear of the chamber. The Superflex Node transmits the OF data from each of the 16 chambers to a computer located within the test room where the data is processed and aggregated by the Accuscan Fusion Software (Version 3.4). The OF activity of each rat was measured for 1 h during its active period (dark cycle).

### 2.5. Blood and tissue collection

Animals were terminated 1 day following the completion of the behavioral testing (i.e., 48 h post-injury or sham). For protein measures, rats ($N = 14$; naïve = 4, sham = 4, injured = 6) were deeply anesthetized with isoflurane inhalant until a tail or toe pinch produced no reflex movement. Blood was obtained by cardiac puncture, and samples were promptly centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants (plasma) were aliquoted, flash-frozen, and stored at −80 °C until processing. Animals were then decapitated using a guillotine (Harvard Apparatus Co.; Dover, MA) and the brains were immediately removed. The prefrontal cortex (PFC), amygdala (AD), ventral hippocampus (VHC), and dorsal hippocampus (DHC) were dissected over wet ice and the dissected brain regions were then flash-frozen and stored at −80 °C until processing [14,11].

### 2.6. Protein measures

Relative concentrations of the selected protein biomarkers (Supplementary Table) were determined using RPPM. Sample preparation, printing, scanning, and data analysis were performed as follows and as described earlier [14,15,19,20]. Dissected brain regions were pulverized in liquid nitrogen using a mortar and pestle. Two hundred milligrams of the powder were transferred into 1 mL of Tissue-Protein Extraction Reagent (T-PER) lysis buffer (78510; Thermo Scientific, Waltham, MA) with EDTA-free Halt Protease and Phosphatase Inhibitor Cocktail (78441; Thermo Scientific) in 1.5 mL tubes on ice. The suspensions were kept on ice and sonicated in a Misonix S-4000 automated sonicator (Misonix, Farmingdale, NY) at an amplitude of 20 using 12 repeats of 10 s bursts with 50 s cooling breaks between bursts. Samples were then centrifuged at 4 °C at 15,000 × *g* for 15 min and the supernatants were aliquoted and stored at −80 °C until use. Tissue protein concentrations were measured using a bicinchoninic acid (BCA) assay (PI-23250, Thermo Scientific).

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.trprot.2013.11.001.

Tissue samples were thawed on ice and diluted in print buffer to a final protein concentration of 1 mg/mL; 240 μg of protein (by corresponding volume) were mixed with 80 μL of 3× print buffer (30% glycerol, 0.15% SDS, and 150 mM DTT in 3× TBS) and the final volume was adjusted to 240 μL with the appropriate amount of water (270733; Sigma Aldrich, St. Louis, MO). Plasma samples were diluted 1:10 by volume; 24 μL of plasma, 80 μL of 3× print buffer, and 136 μL of water for a final volume of 240 μL. Individual samples were then subjected to an 11-point serial 1:2 dilution in 96-well plates using a JANUS Varispan Integrator and Expanded Platform Workstation (PerkinElmer, Waltham, MA) and transferred into 384-well microarray plates (X7022; Molecular Devices, Sunnyvale, CA). Plates were centrifuged at 4 °C at 1500 × *g* for 5 min and transferred into an Aushon 2470 Arrayer (Aushon Biosystems, Billerica, MA) for printing.

Samples were printed on Grace Bio-Labs ONCYTE® NOVA (plasma; 505177) or AVID (tissue; 305177) single-pad nitrocellulose coated glass slides. The Aushon Arrayer was programmed to use 16 pins and set for a single deposition per dot for plasma and double for brain tissue. Each sample was printed in 12 dilutions (12 rows) and in triplicates (3 columns) resulting in a block of 3 × 12 dots. The spot diameter was set to 250 nm with a spacing of 500 nm between dots on the *x*-axis and 375 nm on the *y*-axis. Wash time was set at 1.2 s for tissue and 2 s for plasma without delays.

Primary antibodies were diluted in antibody incubation buffer (0.1% BSA, EDTA-free Halt Protease and Phosphatase Inhibitor Cocktail, 1× TBS, 0.5% Tween-20) and used in the dilutions listed (Supplementary Table). All antibodies were pretested for specificity by Western Blot analysis using tissue extracts (or plasma) along with a specific positive control [16]. The slides were blocked with 5% dried milk in 1× TBS with 0.1% Tween-20 (TBST). Two hundred μL of the primary antibody solution was then distributed over the entire nitrocellulose surface of the slide, covered with a cover slip (Nunc* mSeries LifterSlips, Fisher Scientific, Pittsburgh, PA), and incubated in a humidity chamber at 4 °C overnight while being gently rotated. The next day, slides were washed 3 times in 0.1% TBST for 10 min each, and then incubated with the secondary antibodies Alexa Fluor® 633 donkey anti-sheep (A-21100), Alexa Fluor® 635 goat anti-mouse (A-31574), Alexa Fluor® 647 goat anti-rabbit (A-21245) or rabbit anti-goat IgG (A-21446) from Invitrogen at a 1:6000 dilution in antibody incubation buffer for 1 h at room temperature. Slides were washed 3 times in 0.1% TBST 10 min each, and 1 time in 1× TBS. Slides were then allowed to fully air dry in the dark.

The fluorescent signals were measured by scanning the slides in a Scan Array Express microarray scanner (Perkin Elmer, Waltham, MA). Data were imported into a Microsoft Excel-based bioinformatics program developed in house for analysis [14–17]. The linear regression of the log–log data was calculated after the removal of flagged data, which include signal to noise ratios of <2, spot intensities in the saturation or noise range, or high variability between duplicate spots (>10–15%). The total amount of antigen is determined by the Y-axis intercept or Y-cept [16].

### 2.7. Data comparison and statistical analyses

All animals ($N = 21$; naïve = 4, sham = 6, injured = 11) were used for the physiological and the behavioral analyses. Paired *t*-tests were used to assess changes in arterial oxygen saturation, heart rate, pulse distention, and breath rate within each group (baseline vs. 24 h); all post-injury values were compared to baseline values in injured rats. To determine differences (if any) in the measured physiological parameters among the three groups, a one-way ANOVA followed by Tukey's HSD test was conducted at baseline and at 24 h. ANOVA (covarying for baseline) followed by Tukey's HSD test were conducted for each of the behavioral variables. OF activity scores were separated into three subscales: horizontal activity, vertical activity, and center time. Comparisons were performed for *Condition* and *Time*, and statistically significant differences were reported where applicable.

Fourteen animals (naïve = 4, sham = 4, injured = 6) were used for the protein analyses. Differences in the mean protein biomarker levels measured in plasma and in brain tissue were analyzed with ANOVA followed by Tukey's HSD test. Statistical significance was reported for naïve vs. injured and sham vs. injured. For protein levels in the plasma, statistically significant differences are indicated in boldface. For protein levels in the brain, a two sided $p$ value of <0.05 is depicted by one special character, $p < 0.01$ by two, and $p < 0.001$ by three. All statistical analyses were performed using IBM SPSS Statistics 20 software. Tests were two tailed using $\alpha = 0.05$; data are presented as the mean ± S.E.M.

## 3. Results

### 3.1. Oxygen saturation and heart rate are transiently depressed following repeated mild blast sustained at short intervals

A pulse oximeter was used to measure arterial $O_2$ saturation, heart rate, pulse distension, and breath rate at baseline, after each blast exposure for injured rats, and 24 h post-injury (or sham). Of the four physiological parameters, only arterial $O_2$ saturation levels and heart rate changed considerably in response to the injuries (Fig. 1A–D). Both arterial $O_2$ saturation and heart rate significantly decreased in the injured rats compared to baseline values. However, this depression was transient as it was followed by a full recovery at 24 h (Fig. 1A and B). No significant injury-induced changes were measured in pulse distension after any of the exposures while changes in breath rate were minimal and similarly transient (Fig. 1C and D).

### 3.2. Repeated mild blast overpressure exposure adversely affects motor activity and functional outcome

Using an open field system we determined the effect of repeated mild blast exposure on gross motor activity as well as anxiety- and depression-related behavior. Fig. 2A summarizes the amount of horizontal activity, an index of general health and movement, at baseline and 24 h following injury (or sham). Twenty-four hours post-injury, sham animals

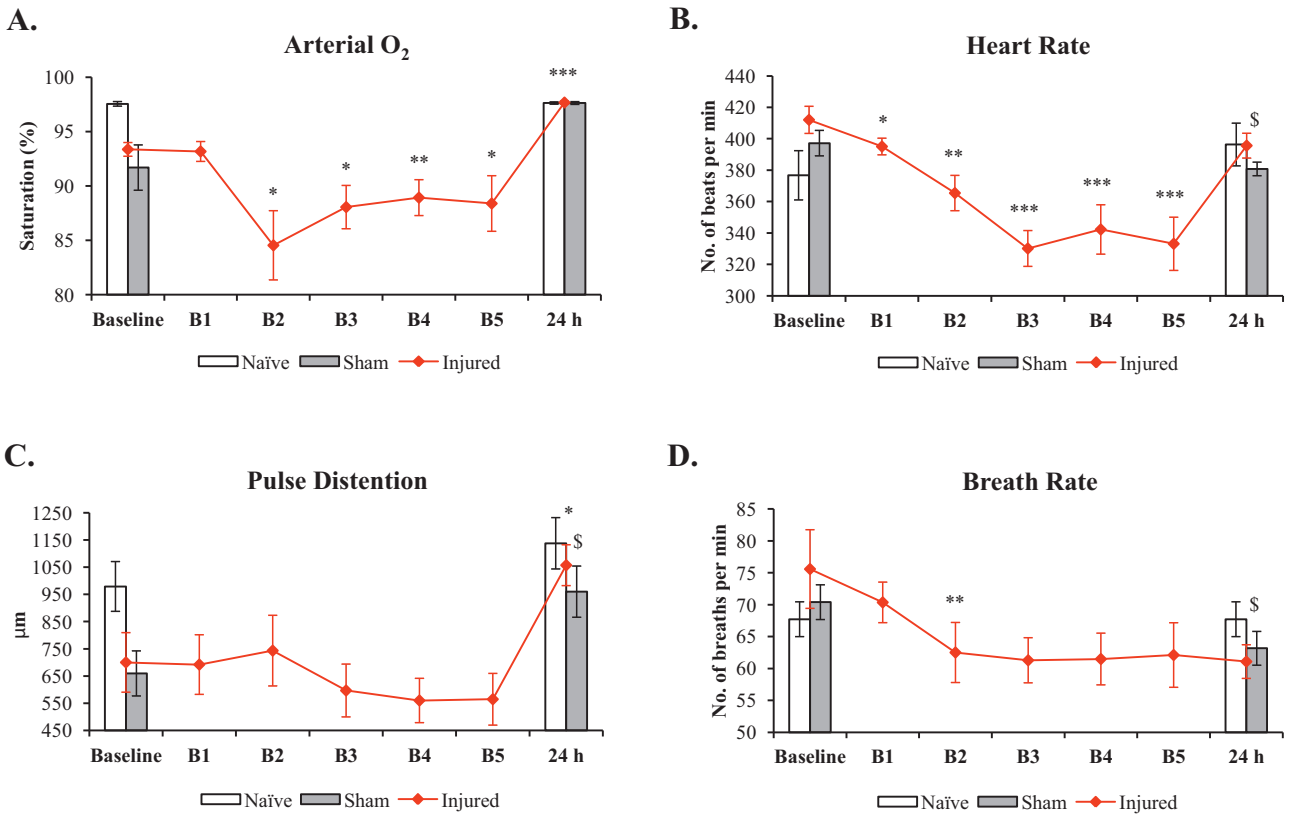


**Fig. 1 – Significant albeit transient reductions in arterial oxygen saturation and heart rate were measured in injured rats. (A) Arterial oxygen saturation levels (%), (B) heart rate (number of beats per min), (C) pulse distension (μm), and (D) breath rate (number of breaths per min) were obtained under isoflurane anesthesia at baseline, immediately following injury (×5 for injured rats), and at 24 h post-injury. Data are presented as the mean ± S.E.M. $^{\$}p < 0.05$ for sham pre- vs. post-injury values; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.01$ for injured pre- vs. post-injury values.**

(8504.16 ± 901.74) were not significantly different from naïve animals (10,661.92 ± 1103.70), but there was a significant effect of injury such that injured animals (6777.67 ± 665.50) had significantly less horizontal activity than naïve animals.

Fig. 2B shows the amount of vertical activity, an index of depression-related behaviors, where less vertical activity indicates more depression-related behaviors. At 24 h post-injury, sham animals (1288.55 ± 209.48) were not significantly different from naïve animals (1862.90 ± 256.25). However, injured animals (887.01 ± 154.38) had significantly less vertical activity than naïve animals. Injured animals (70.86 ± 22.44) also spent significantly less time in the center of the chamber, an index of anxiety-related behaviors (more time in the center indicates less anxiety-related behaviors), than naïve animals (236.73 ± 36.37) (Fig. 2C). Naïve animals were not significantly different from sham animals (140.63 ± 30.12).

### 3.3. *The exposure to mild blasts at short intervals induces complex changes in protein biomarker levels in functionally-relevant brain regions*

Two days following injury (or sham), we dissected the PFC, AD, DHC, and VHC and determined the effect of repeated mild blast exposure on tissue levels of select protein biomarkers related to oxidative stress and vascular functions, cellular adhesion and the extracellular matrix, inflammation, and glial cell response.

Of the two biomarkers related to oxidative stress, 4-hydroxy-2-noneal (HNE) and hypoxia-inducible factor 1α (HIF1α), HNE was significantly increased in all four of the tested brain regions whereas changes in HIF1α tissue levels were limited to the AD and the DHC of injured rats (Fig. 3A and B). Similar to HNE, vascular endothelial growth factor (VEGF) and aquaporin 4 (AQP4) levels were significantly increased in all brain regions of the injured rats. On the other hand, tissue levels of von Willebrand factor (vWF) were most significantly changed in the PFC only (Fig. 3C–E).

We also detected significant injury-induced changes in the tissue levels of cellular adhesion and extracellular matrix molecules. While integrin α6 tissue levels were mainly elevated in the PFC and the AD of injured rats, TIMP metallopeptidase inhibitor 1 (TIMP1) concentrations were significantly elevated in all brain regions but the DHC (Fig. 4A and B). Of the two extracellular matrix proteins, only TIMP metallopeptidase inhibitor 4 (TIMP4) was significantly increased in all of the tested brain regions compared to naïve and/or sham animals (Fig. 4C).

Among the tested inflammatory biomarkers, only galectin-1 (Gal-1) and macrophage inflammatory protein 1α (MIP1α) were significantly higher in all four brain regions of exposed

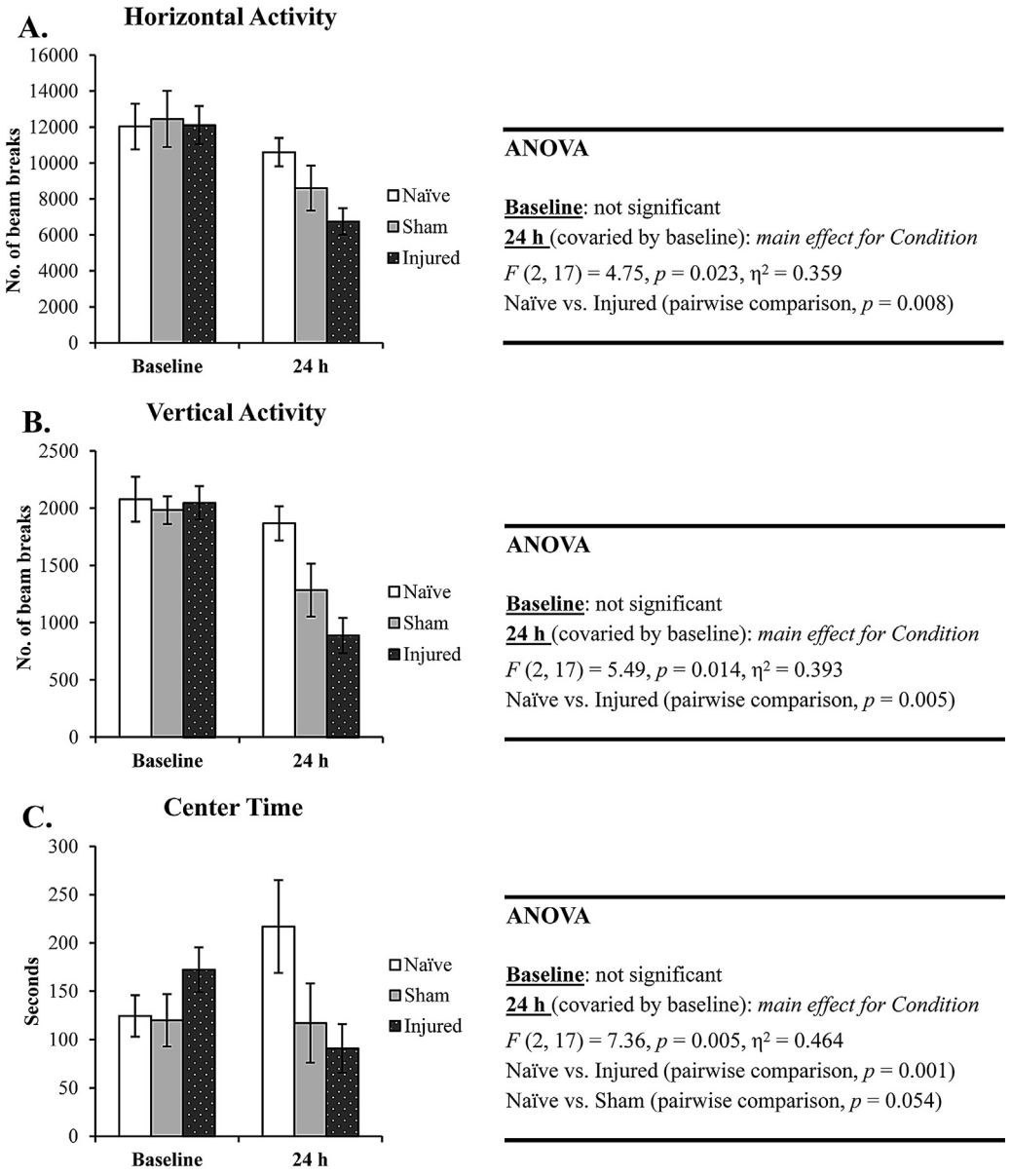


**Fig. 2 – Injured rats exhibit a decline in gross movement and an elevation in depression- and anxiety-related behaviors. An open field system was used to measure (A) horizontal activity (number of beam breaks), (B) vertical activity (number of beam breaks), and (C) center time (s) at baseline and 24 h following blast (or sham) exposure. Data are presented as the mean ± S.E.M.**

rats (Figs. 4E and 5C, respectively). Matrix metalloproteinase 8 (MMP8) and chemokine (C–C motif) receptor 5 (CCR5) levels were significantly elevated in all brain regions but the AD (Figs. 4D and 5D, respectively) while toll-like receptor 9 (TLR9), p38 mitogen-activated protein kinase (p38 MAPK), and osteopontin (OPN) tissue levels were elevated in all brain regions but the DHC (Figs. 4F and 5A and B, respectively).

Both microglia- and astroglia-specific markers (CD53 (OX44) and glial fibrillary acidic protein (GFAP), respectively) showed significant injury-induced increases in injured rats compared to naïve and/or sham animals. OX44 levels were increased modestly in all brain regions but the AD (Fig. 5E). By contrast, GFAP tissue levels increased in all of the analyzed brain regions with the change being among the most robust of the tested biomarkers (Fig. 5F).

### 3.4. *Some of the molecular changes induced by repeated, high frequency mild blast exposure are reflected in altered plasma levels of protein biomarkers*

Plasma levels of the three tested oxidative stress-related markers, HNE, HIF1α, and ceruloplasmin, and several vascular protein biomarkers—VEGF, vWF, aquaporin 1 (AQP1), AQP4, fetal liver kinase 1 (FLK1/VEGF receptor 2), and claudin 5—were significantly increased in injured rats compared to naïve, sham, or both control groups (Table 1). Plasma concentrations of cell adhesion and extracellular matrix proteins,

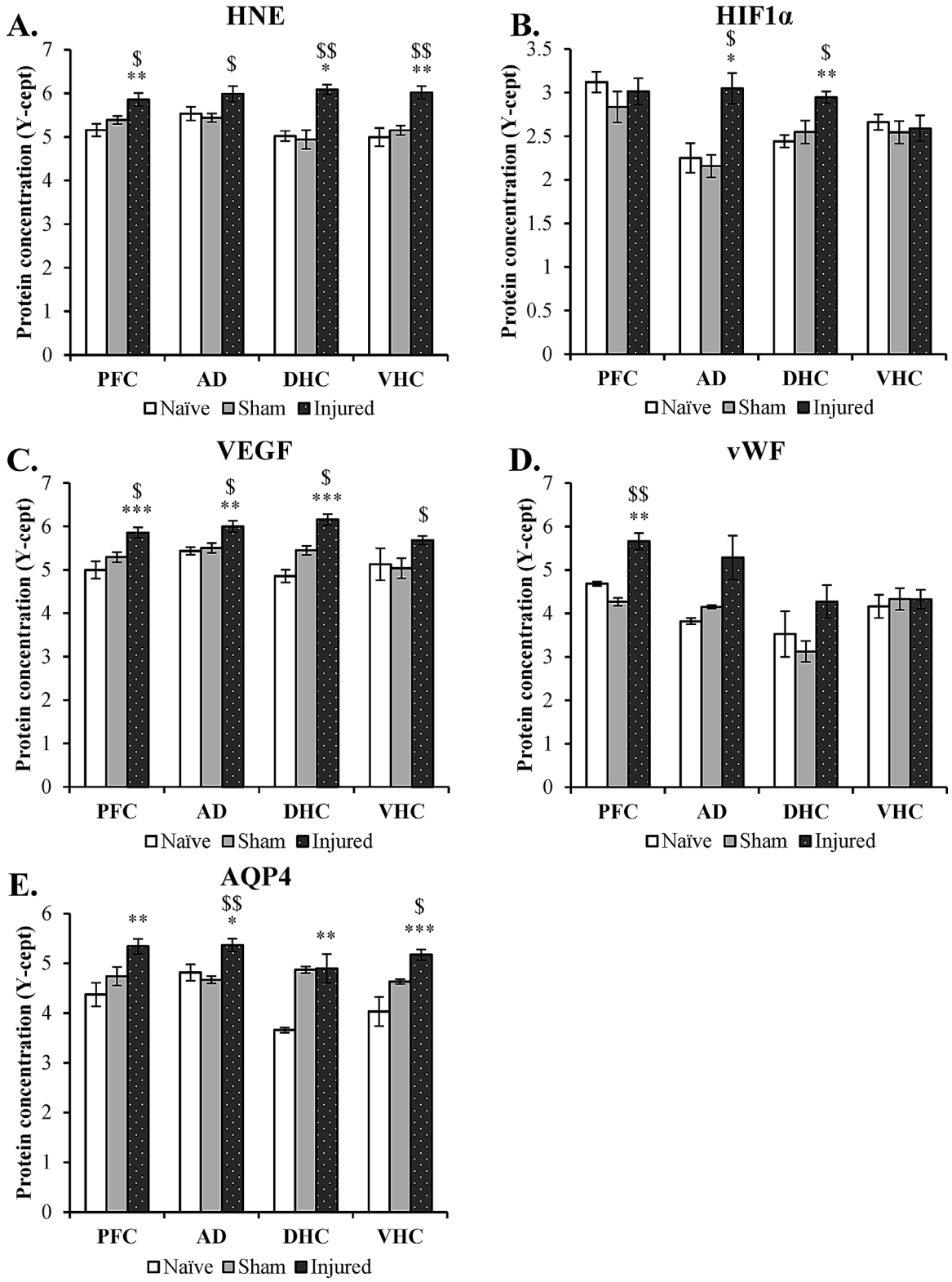


**Fig. 3 – Brain tissue levels of oxidative stress and vascular protein biomarkers in the selected brain regions. Tissue extracts were prepared from the prefrontal cortex (PFC), amygdala (AD), dorsal and ventral hippocampus (DHC and VHC, respectively) of naïve, sham, and injured rats. The reported Y-cept values (log 10) indicate relative protein concentrations; data are presented as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ for naïve vs. injured; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ for sham vs. injured.**

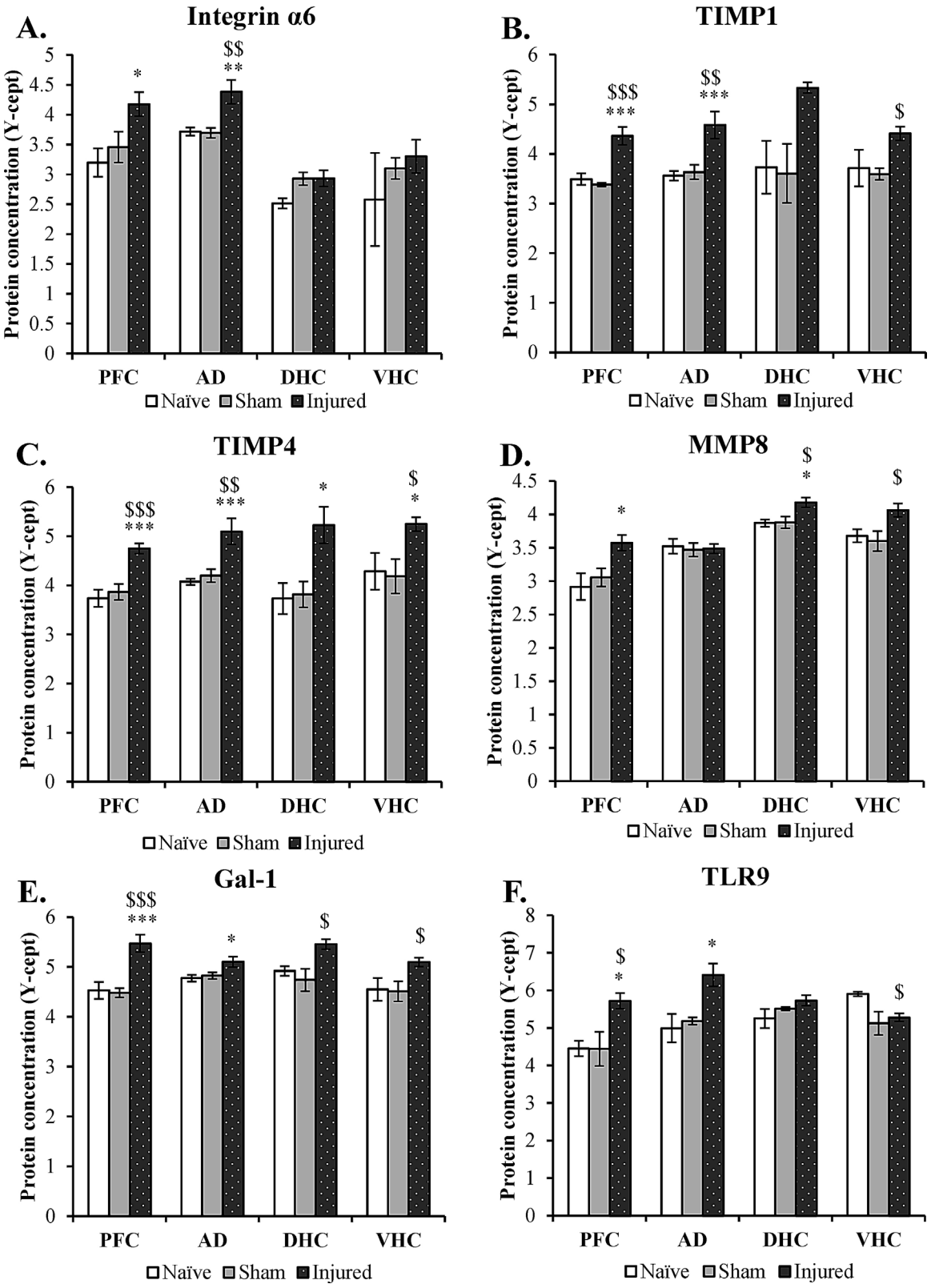


**Fig. 4 – Brain tissue levels of proteins related to cellular adhesion, extracellular matrix, and inflammation in the selected brain regions. Tissue extracts were prepared from the prefrontal cortex (PFC), amygdala (AD), dorsal and ventral hippocampus (DHC and VHC, respectively) of naïve, sham, and injured rats. The reported Y-cept values (log 10) indicate relative protein concentrations; data are presented as the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for naïve vs. injured; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ for sham vs. injured.**

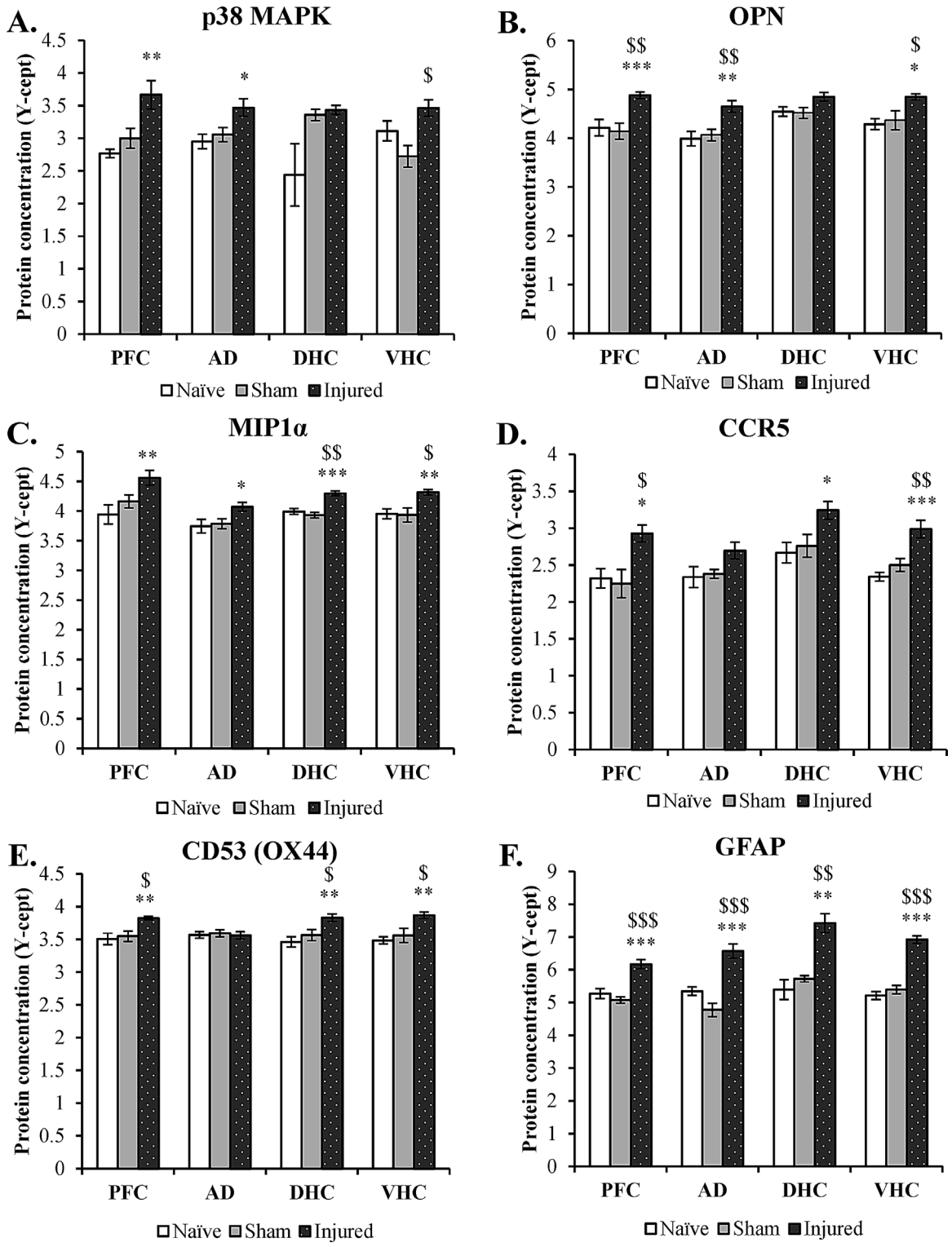


**Fig. 5 – Brain tissue levels of protein biomarkers related to inflammation and glial cell response in the selected brain regions. Tissue extracts were prepared from the prefrontal cortex (PFC), amygdala (AD), dorsal and ventral hippocampus (DHC and VHC, respectively) of naïve, sham, and injured rats. The reported Y-cept values (log 10) indicate relative protein concentrations; data are presented as the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for naïve vs. injured; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, and $^{\$\$\$}p < 0.001$ for sham vs. injured.**

**Table 1 – Oxidative stress and vascular protein biomarker levels in the plasma.**

| | | | | | Comparison of means | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ANOVA | | Naïve vs. injured | | Sham vs. injured | |
| | Group | Mean ± S.E.M. | *F* value | *p* Value | Mean diff. | *p* Value | Mean diff. | *p* Value |
| HNE | Naïve | 6.355 ± 0.12 | 12.769 | 0.0002 | −0.508 | **0.0003** | −0.399 | **0.0040** |
| | Sham | 6.464 ± 0.08 | | | | | | |
| | Injured | 6.863 ± 0.06 | | | | | | |
| HIF1α | Naïve | 4.289 ± 0.30 | 6.303 | 0.0061 | −0.986 | **0.0118** | −0.878 | **0.0261** |
| | Sham | 4.397 ± 0.21 | | | | | | |
| | Injured | 5.275 ± 0.19 | | | | | | |
| Ceruloplasmin | Naïve | 6.469 ± 0.04 | 19.622 | 0.0001 | −0.485 | **0.0001** | −0.375 | **0.0004** |
| | Sham | 6.579 ± 0.04 | | | | | | |
| | Injured | 6.955 ± 0.07 | | | | | | |
| VEGF | Naïve | 5.793 ± 0.08 | 9.664 | 0.0008 | −0.918 | **0.0042** | −0.998 | **0.0029** |
| | Sham | 5.713 ± 0.03 | | | | | | |
| | Injured | 6.711 ± 0.23 | | | | | | |
| vWF | Naïve | 4.095 ± 0.14 | 6.014 | 0.0086 | −0.684 | **0.0096** | −0.539 | 0.0673 |
| | Sham | 4.239 ± 0.12 | | | | | | |
| | Injured | 4.779 ± 0.17 | | | | | | |
| AQP1 | Naïve | 5.119 ± 0.26 | 6.050 | 0.0075 | −0.902 | **0.0160** | −0.851 | **0.0234** |
| | Sham | 5.169 ± 0.25 | | | | | | |
| | Injured | 6.021 ± 0.15 | | | | | | |
| AQP4 | Naïve | 6.037 ± 0.05 | 12.450 | 0.0002 | −0.413 | **0.0003** | −0.335 | **0.0042** |
| | Sham | 6.114 ± 0.06 | | | | | | |
| | Injured | 6.450 ± 0.07 | | | | | | |
| FLK1 | Naïve | 4.868 ± 0.17 | 8.117 | 0.0022 | −0.745 | **0.0033** | −0.576 | **0.0233** |
| | Sham | 5.036 ± 0.08 | | | | | | |
| | Injured | 5.612 ± 0.14 | | | | | | |
| Claudin 5 | Naïve | 5.221 ± 0.12 | 11.453 | 0.0003 | −0.797 | **0.0003** | −0.482 | **0.0312** |
| | Sham | 5.537 ± 0.07 | | | | | | |
| | Injured | 6.018 ± 0.13 | | | | | | |

Mean protein values (log 10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface. For *p* values < 0.0001, the minimum value is listed as *p* = 0.0001.

**Table 2 – Cell adhesion and extracellular matrix protein biomarker levels in the plasma.**

| | | | | | Comparison of means | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ANOVA | | Naïve vs. injured | | Sham vs. injured | |
| | Group | Mean ± S.E.M. | *F* value | *p* Value | Mean diff. | *p* Value | Mean diff. | *p* Value |
| Integrin α6 | Naïve | 4.209 ± 0.12 | 18.567 | 0.0001 | −1.379 | **0.0003** | −1.363 | **0.0001** |
| | Sham | 4.224 ± 0.20 | | | | | | |
| | Injured | 5.587 ± 0.19 | | | | | | |
| TIMP1 | Naïve | 4.178 ± 0.18 | 9.777 | 0.0009 | −1.439 | **0.0009** | −1.043 | **0.0192** |
| | Sham | 4.574 ± 0.25 | | | | | | |
| | Injured | 5.617 ± 0.27 | | | | | | |
| TIMP4 | Naïve | 4.650 ± 0.08 | 14.195 | 0.0001 | −0.564 | **0.0002** | −0.460 | **0.0022** |
| | Sham | 4.753 ± 0.07 | | | | | | |
| | Injured | 5.213 ± 0.09 | | | | | | |

Mean protein values (log 10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface. For *p* values < 0.0001, the minimum value is listed as *p* = 0.0001.

integrin α6, TIMP1, and TIMP4, were also significantly elevated in injured animals (Table 2).

Inflammatory markers Gal-1, p38 MAPK, MIP1α, CCR5, monocyte chemotactic protein 1 (MCP1), cytokine-induced neutrophil chemoattractant 1α (CINC1α), fibrinogen, C-reactive protein (CRP), and N-formyl peptide receptor (FPR) were significantly increased in the plasma of injured rats compared to naïve and/or sham animals (Table 3). Consistent with the abovementioned biomarkers, plasma concentrations of glial—GFAP, OX44, and S100 calcium binding

**Table 3 – Inflammatory protein biomarker levels in the plasma.**

| | | | ANOVA | | Comparison of means Naïve vs. injured | | Sham vs. injured | |
|---|---|---|---|---|---|---|---|---|
| | Group | Mean ± S.E.M. | *F* value | *p* Value | Mean diff. | *p* Value | Mean diff. | *p* Value |
| Galectin | Naïve | 5.079 ± 0.05 | 6.134 | 0.0068 | −0.286 | **0.0404** | −0.354 | **0.0101** |
| | Sham | 5.011 ± 0.11 | | | | | | |
| | Injured | 5.365 ± 0.07 | | | | | | |
| p38 MAPK | Naïve | 3.732 ± 0.39 | 8.502 | 0.0018 | −1.396 | **0.0051** | −1.317 | **0.0081** |
| | Sham | 3.811 ± 0.29 | | | | | | |
| | Injured | 5.128 ± 0.20 | | | | | | |
| MIP1α | Naïve | 4.690 ± 0.16 | 4.513 | 0.0241 | −0.371 | **0.0416** | −0.353 | 0.0672 |
| | Sham | 4.707 ± 0.09 | | | | | | |
| | Injured | 5.060 ± 0.06 | | | | | | |
| CCR5 | Naïve | 4.026 ± 0.20 | 5.170 | 0.0176 | −0.896 | **0.0165** | −0.586 | 0.1626 |
| | Sham | 4.337 ± 0.13 | | | | | | |
| | Injured | 4.922 ± 0.22 | | | | | | |
| MCP1 | Naïve | 4.053 ± 0.09 | 6.449 | 0.0057 | −0.552 | **0.0060** | −0.387 | 0.0605 |
| | Sham | 4.218 ± 0.11 | | | | | | |
| | Injured | 4.605 ± 0.13 | | | | | | |
| CINC1α | Naïve | 3.860 ± 0.06 | 37.765 | 0.0001 | −1.224 | **0.0001** | −1.337 | **0.0001** |
| | Sham | 3.746 ± 0.11 | | | | | | |
| | Injured | 5.083 ± 0.14 | | | | | | |
| Fibrinogen | Naïve | 4.840 ± 0.22 | 6.932 | 0.0046 | −0.722 | **0.0073** | −0.595 | **0.0377** |
| | Sham | 4.968 ± 0.09 | | | | | | |
| | Injured | 5.563 ± 0.13 | | | | | | |
| CRP | Naïve | 5.154 ± 0.17 | 3.864 | 0.0357 | −0.474 | **0.0395** | −0.362 | 0.1559 |
| | Sham | 5.266 ± 0.08 | | | | | | |
| | Injured | 5.628 ± 0.12 | | | | | | |
| FPR | Naïve | 4.831 ± 0.31 | 4.166 | 0.0381 | −0.498 | 0.4472 | −0.917 | **0.0310** |
| | Sham | 4.412 ± 0.36 | | | | | | |
| | Injured | 5.329 ± 0.08 | | | | | | |

Mean protein values (log 10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface. For *p* values < 0.0001, the minimum value is listed as *p* = 0.0001.

protein β (S100β)—and neuronal protein markers—neuron-specific enolase (NSE), neurofilament-heavy chain (NF-H), creatine kinase-brain type (CK-BB), and tau—were all significantly elevated in the injured animals two days after blast overpressure exposure (Table 4).

## 4. Discussion

In this work we have shown that mild blast overpressure repeated at the rate of 1 per 30 min causes significant physiological, neurobehavioral, and molecular changes using our well-established rat model of bTBI. Our findings suggest that oxidative stress, vascular pathologies, and inflammation are critical components of the molecular pathology underlying ICV in blast-induced rmTBI. The biomarkers used in this study are candidates for clinical testing and if verified, they can be developed into blood-based diagnostics to aid in the identification of individuals with ICV.

We selected to monitor changes in physiological parameters using non-invasive methods similar to those utilized in clinical settings. Injury-induced changes in heart and respiratory rates, $O_2$ arterial saturation, and pulse distention are used as indicators of potential intracerebral pathologies such as disturbed cerebral blood flow, elevated intracranial pressure, and ischemia especially in severe and moderate TBI. These transient post-injury changes, particularly post-traumatic hypoxia, can contribute to ICV. We found that while heart rate remained within the normal range for rats, it was significantly decreased after mild blast overpressure exposure compared to baseline. Similarly, arterial $O_2$ saturation was significantly lower after exposures compared to pre-injury values. The mild and transient hypoxia we measured is consistent with previous observations identifying metabolic depression as one of the underlying causes of ICV. Thus, it may be useful to monitor vital signs as they can aid in the detection of altered physiology and the degree of ICV.

Behavioral assessments have shown that mood changes and increased anxiety are two of the major symptoms following bTBI [21,22]. We previously observed such changes when we exposed rats to a single or multiple mild blasts 24 h apart and found that anxiety- and depression-related changes manifest shortly after injury and are transient in nature [11]. However, we failed to detect a cumulative effect of repeated blast (and consequently more severe behavioral symptoms) in multiple-injured rats relative to single-injured rats at that "low" blast frequency. In our current study, we also found significantly increased anxiety- and depression-related

**Table 4 – Glial and neuronal protein biomarker levels in the plasma.**

| | | | ANOVA | | Comparison of means: Naïve vs. injured | | Comparison of means: Sham vs. injured | |
|---|---|---|---|---|---|---|---|---|
| | Group | Mean ± S.E.M. | *F* value | *p* Value | Mean diff. | *p* Value | Mean diff. | *p* Value |
| GFAP | Naïve<br>Sham<br>Injured | 4.725 ± 0.11<br>4.608 ± 0.13<br>5.281 ± 0.13 | 8.785 | 0.0014 | −0.556 | **0.0102** | −0.673 | **0.0029** |
| CD53 (OX44) | Naïve<br>Sham<br>Injured | 4.612 ± 0.21<br>4.436 ± 0.16<br>5.024 ± 0.12 | 3.787 | 0.0366 | −0.411 | 0.1801 | −0.587 | **0.0383** |
| S100β | Naïve<br>Sham<br>Injured | 4.163 ± 0.11<br>3.967 ± 0.08<br>5.428 ± 0.10 | 68.988 | 0.0001 | −1.264 | **0.0001** | −1.460 | **0.0001** |
| NSE | Naïve<br>Sham<br>Injured | 3.541 ± 0.09<br>3.594 ± 0.06<br>4.670 ± 0.15 | 26.101 | 0.0001 | −1.129 | **0.0001** | −1.076 | **0.0001** |
| NF-H | Naïve<br>Sham<br>Injured | 5.732 ± 0.05<br>5.687 ± 0.05<br>5.928 ± 0.05 | 6.310 | 0.0063 | −0.196 | **0.0329** | −0.241 | **0.0110** |
| CK-BB | Naïve<br>Sham<br>Injured | 4.342 ± 0.14<br>4.426 ± 0.11<br>4.910 ± 0.11 | 6.930 | 0.0040 | −0.568 | **0.0072** | −0.484 | **0.0229** |
| Tau | Naïve<br>Sham<br>Injured | 4.454 ± 0.14<br>4.540 ± 0.27<br>6.089 ± 0.15 | 30.884 | 0.0001 | −1.635 | **0.0001** | −1.549 | **0.0004** |

Mean protein values (log 10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface. For *p* values < 0.0001, the minimum value is listed as *p* = 0.0001.

behaviors in injured animals but no conclusions can be made about the role of ICV in the cumulative effect of repeated mild blast exposure due to the absence of a 24 h interval blast group. The only other study assessing the effects of "tightly coupled" mild blast exposures using the rotarod test showed a moderate, albeit transient cumulative effect of multiple exposures where animals gradually recovered starting at day 5 post-injury [23]. Unfortunately, no behavioral assessments were performed in the two key studies that directly address the involvement of metabolic depression and axonal and vascular changes in ICV [6,8], although a previous work by the same group has shown increased cognitive deficits after repeated TBI [7].

Clinical observations have indicated that the behavioral symptoms of mTBIs partly overlap with those of post-traumatic stress disorder (PTSD) [21]. However, available experimental data indicate that the molecular pathologies of PTSD and bTBI may be distinct [14,24,25]. The observed increase in anxiety indicates that the prefrontal cortex and/or its functionally related structures (the amygdala and the hippocampus) are adversely affected by repeated injury. Experimental research and clinical imaging studies have shown that the PFC is involved in mediating symptoms associated with PTSD as well as TBI, and that the PFC is especially susceptible to noxious insults including trauma [26–29]. Accordingly, our proteomics analyses of the PFC, AD, DHC and VHC showed that of the 17 protein biomarkers measured in brain tissue, the expression of 16 changed significantly in the PFC of injured rats. The second most affected brain region was the VHC in which 14 of the 17 markers changed significantly in response to repeated injury followed by the AD (13 markers) and the DHC (11 markers).

The detected changes in tissue levels of the measured markers suggest complex pathological processes as a result of repeated blast overpressure exposure. This includes oxidative stress, which can be related to and/or triggered by the relative hypoxia as discussed above. HNE, a byproduct of lipid peroxidation [30,31], was significantly elevated in all four brain regions of injured rats. Interestingly, HIF1α was the only marker with unchanged tissue levels in the PFC in response to repeated injury although its levels were significantly elevated in the AD and in the DHC. HIF1α, a transcription factor, is part of the adaptive and restorative cellular mechanisms that follow various noxious neuronal insults involving hypoxia (e.g., TBI and stroke) [32]. A potential explanation for why HIF1α levels remained unchanged in the PFC is the relatively early activation of HIF1α in response to hypoxia, which is within hours and thus outside of our current termination time point (2 days).

Tissue levels of the three markers associated with various aspects of vascular function were significantly elevated in the PFC of injured animals. These findings are consistent with previous observations implicating altered vascular function in the period of ICV. Using impact acceleration injury, Fujita et al. (2012) demonstrated that the initial mild insult can result in ICV via vascular hypersensitivity [6]. When the injury was repeated within 3 h of the initial insult, the second mild injury triggered vascular dysfunction as measured by

the hypersensitivity of brain vasculature to acetylcholine stimulation. No such hypersensitivity was detected when re-injury took place 10 h after the initial injury.

In our study, we found that the expression of VEGF, a mediator of multiple endothelial functions including vascular permeability and endothelial proliferation [33,34], increased significantly in all tested brain regions. Similar to VEGF, AQP4 was elevated in all four regions. Increased tissue levels of AQP4, a main water channel of the central nervous system (CNS) [35,36], indicate altered blood brain barrier (BBB) function. Brain edema has been observed after various brain insults including subarachnoid hemorrhage and TBI. The significance of altered BBB permeability following blast-induced rmTBI is currently not known, but increased BBB permeability has been demonstrated after repeated mild athletic TBI. Of the three vascular markers, vWF tissue levels were only significantly elevated in the PFC. In addition to its major role in regulating blood coagulation, vWF has additional functions in the inflammatory response to vascular damage [37].

Neuroinflammation, a hallmark of TBI, can be triggered by multiple upstream pathological changes such as vascular damage and altered cellular adhesion [38,39]. Abnormal cell surface interactions and the activation of various extracellular matrix proteins have been found after CNS injury [40]. The involvement of the extracellular matrix in the pathobiology of blast-induced rmTBI is indicated by the brain region specific increase in integrin α6. As a member of the transmembrane receptor family, integrin α6 plays a vital role in cell adhesion and cell–cell interactions as well as signal transduction from the extracellular milieu into the cell [41]. The role of the extracellular matrix in ICV is further demonstrated by the substantial increase in brain tissue levels of TIMP1 and TIMP4, inhibitors of matrix metalloproteinases [42,43]. The expression of TIMP1 and TIMP4 in the PFC as well as the AD (and to a lesser extent in the hippocampal sub-regions) is induced at the transcriptional level by several cytokines linking cell surface remodeling to inflammation.

The selected markers perform various functions in the inflammatory process. Consistent with altered TIMP1 and TIMP4 expression, we found elevated tissue levels of MMP8, a matrix metalloproteinase involved in tissue remodeling after injury and chemokine/cytokine activation and inactivation [44]. Gal-1, a β-galactoside-binding protein with intra- and extracellular functions, is heavily involved in the initiation, propagation, and overall mediation of inflammatory processes [45]. Gal-1 overexpression in the PFC of injured animals is indicative of pathological progression in this vulnerable region. Another molecule with a role in the initiation of the neuroinflammatory process is TLR9, a member of the toll-like receptor family [46,47]. Cytokine signaling by the TLRs and other proteins activates p38 MAPK in the injured tissues. Both TLR9 and p38 MAPK were similarly elevated in the PFC, the AD, and the VHC.

OPN plays a number of roles in immune function that include cytokine production, cell attachment, and cell activation [48]. Another molecule involved in the synthesis and the delivery of pro-inflammatory cytokines is MIP1α, a member of the Macrophage Inflammatory Protein family of pro-inflammatory chemokines [38,39,49–52]. MIP1α mediates immune response by activating inflammatory cells that release additional pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α [53,54]. Chronically elevated MIP1α levels are found in immune disorders of the CNS such as multiple sclerosis. In the injured CNS, MIP1α and OPN are likely produced by microglia, the resident immune cell of the brain [55,56]. CCR5 is a receptor for various chemokines including MIP1α, MIP1β, and RANTES [57]. Changes in brain tissue levels of CCR5 in response to repeated blast injury were similar to those of MMP8 as it was significantly elevated in the PFC and the two hippocampal sub-compartments but not in the AD. CCR5, a G-protein coupled receptor, is also expressed by macrophages and by other cells with signaling functions between the nervous and immune systems [53,58].

CD53 is a marker of microglia and macrophages [52]. While it is possible that there was a macrophage response at this level of repeated injury, we cannot confirm this in the absence of a detailed immunohistochemical analysis. Nonetheless, we detected significantly increased CD53 (OX44) levels in all brain regions but the AD. Another cell type involved in the neuroinflammatory process is the astrocyte marked by GFAP expression [59]. Tissue levels of GFAP were significantly elevated in all brain regions of injured animals. However, the change in GFAP levels was the most robust suggesting that astroglial response (i.e., gliosis) is a more global response in the injured brain than some of the markers we as well as others have analyzed. This global glial response to injury is consistent with the numerous and somewhat diverse functions astroglia play in the normal and injured CNS [60–64].

Of the markers related to metabolic changes/oxidative stress and vascular functions, only vWF levels were not significantly increased in injured rats relative to their sham counterparts. This finding is in contrast with the significant increases in vWF levels in the PFC of injured rats compared to both naïve and sham animals. However, this supports our previous findings that experimental manipulations alone (i.e., sham conditions) can trigger pathological changes due to the role of stress as a cofactor in bTBI [14,17]. These findings also suggest that some of the changes can be spatially/anatomically restricted and might not be reflected in the systemic circulation. This notion is supported by the finding that plasma levels of several inflammatory markers (MIP1α, CCR5, MCP1 and CRP) were unchanged while there were significant changes in their tissue levels, again mostly in the PFC. As discussed above, these markers are involved in mediating the injury process at various levels.

Elevated plasma levels of the measured cell surface markers (integrin α6, TIMP1 and TIMP4) are likely indicative of the increases measured in brain tissue [43,65–67]; these markers can also be of extracellular origin. All brain-specific proteins were significantly elevated in the plasma of injured animals relative to sham animals. The increases in neuronal and glial markers suggest complex neuropathologies that include glial loss/damage as indicated by elevated GFAP and S100β levels. The correlation between elevated serum levels of GFAP and S100β and injury severity and outcome have been extensively studied in experimental and in clinical TBI [68–71]. Elevated levels of S100β are frequently found during the acute phase of TBI [71]. However, this early increase can be (and typically is) of extracranial origin because damage to other organs results in increased levels of circulating S100β. Consistent with most

clinical studies, we found no correlation between injury severity and S100β plasma levels in our earlier experimental work [15]. Nevertheless, a recent article showed that the temporal pattern of circulating S100β correlates very well with injury outcome if patients are monitored for an extended period of time [72]. While increased serum levels of GFAP have a limited prognostic value alone, they can provide a significant predictive value in combination with other markers such as microtubule-associated protein 2 (MAP2) [73], ubiquitin C-terminal hydrolase L1 (UCH-L1), and αII-spectrin breakdown product (SBDP145) [74].

Similar to GFAP and S100β, NSE, NF-H and CK-BB have been extensively tested in different TBI studies [75–77]. These markers have also been tested in clinical studies with varying extents of correlation between measured sera levels and injury severity and/or outcome. Increased plasma levels of these proteins reflect neuronal damage and/or loss. However, elevated serum NSE levels have been previously detected after extracranial insults such as cardiac arrest [78,79]. Our earlier work using the porcine model of bTBI has showed that the temporal profile of serum NF-H levels correlates with injury severity [15]. One of the most substantive increases in plasma biomarker levels was of tau protein, indicating axonal damage. This finding may be especially significant since tau pathologies have been suspected to play a key role in the development of chronic neurodegenerative conditions (e.g., chronic traumatic encephalopathy) following rmTBI [80–85].

In summary, we found that: (1) ICV in the rodent model of blast-induced rmTBI is measured in hours, which roughly translates to days in humans. (2) A number of complex molecular pathologies contribute to ICV in blast-induced rmTBI; these include metabolic changes/oxidative stress, vascular dysfunction, altered cellular adhesion and interaction, inflammation, as well as neuronal and glial damage and/or loss. (3) Virtually all of the molecular pathologies affected the PFC, which may potentially account for the increased anxiety- and depression-related behaviors exhibited by injured rats; this observation maybe related to the PFC's anatomical localization and vulnerability to physical injuries, especially if they are repetitive in nature. (4) These pathologies are also reflected in altered plasma levels of biomarkers that can be validated in humans and developed into blood-based diagnostics for the purpose of identifying individuals with ICV. A limitation of our current study is that injury-induced changes in marker plasma levels were determined at a single, terminal time point. Accordingly, serial blood sampling would provide invaluable information about the temporal pattern of molecular changes that may be critical for the identification of the period of ICV in rmTBIs.

## Conflict of interest statement

The views, opinions, and/or findings contained herein are those of the authors and should not be construed as an official position, policy, or decision of the Uniformed Services University of the Health Sciences, Department of the Army or the Department of Defense. The authors have no financial disclosures.

Animal handling and treatments were conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations related to animals and experiments involving animals, and adhered to principles stated in the Guide to the Care and Use of Laboratory Animals, National Research Council. The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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