NEUROSENSORY ALTERATIONS FROM BLAST EXPOSURE AND BLUNT IMPACT

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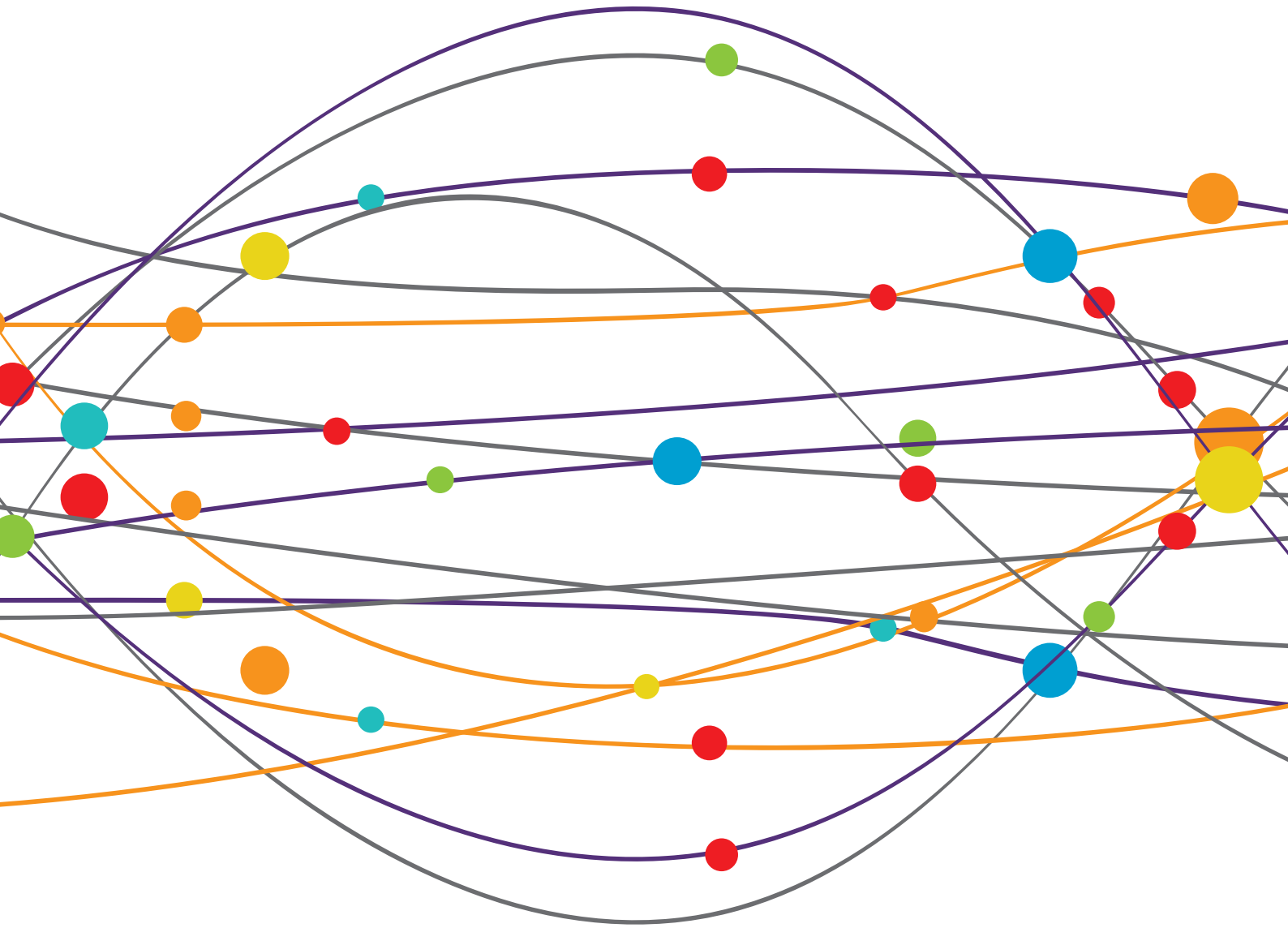

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NEUROSENSORY ALTERATIONS FROM BLAST EXPOSURE AND BLUNT IMPACT

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EDITORIAL
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Editorial: Neurosensory Alterations From Blast Exposure and Blunt Impact

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Editorial on the Research Topic

Neurosensory Alterations From Blast Exposure and Blunt Impact

Neurosensory perturbations are the most common symptomatology observed following blast and blunt trauma to brain (1–5). Glasgow coma scale (GCS) for civilian injuries and threshold of injury in military personnel heavily rely on the perturbations associated with neurosensory systems to diagnose mild to severe traumatic brain injury. In addition, a growing body of evidence is revealing gender differences in the experiences of men and women following a brain injury (6, 7). The 11 papers presented on the Research Topic of Neurosensory Alterations from Blast Exposure and Blunt Impact highlight the challenges in understanding the disruptive effects on neurosensory systems following traumatic brain injuries experienced by both civilians and military personnel.

CLINICAL STUDIES

Lumba-Brown et al. examined head injury following a blunt trauma in college athletes and documented the prevalence of oculomotor, vestibular, and auditory impairments among concussed athletes, and the effect of sex and sport on symptom prevalence. They found females tended to have a higher incidence of abnormal outcomes on particular concussion assessment tasks as compared- to males. Sergio et al., using fMRI, showed different sex-related patterns of brain activity during rule-based skill performance and suggested that injury to the brain could result in different visuomotor related impairments in males and females. Both these studies indicate that more research is needed to help better understand the relationship between sex, gender and brain injury to improve diagnosis and treatment for both sexes. Modica et al. found that career military breachers tended to report a greater sensitivity to noise or light as well as irritability when compared to non-breachers. In addition, they found a higher level of self-reporting of tinnitus within this group and discussed the challenges encountered when conducting these studies in order to fully characterize long term exposure to blast. Kuchinsky et al. examined service members and veterans with and without a history of TBI and with and without a history of blast exposure to determine the contribution of auditory, cognitive, and posttraumatic stress disorder symptoms—to difficulties understanding speech in complex environments. They determined that TBI status and cognitive function predicted objective speech recognition performance, exposure to blast—subjective hearing complaints, and PTSD symptoms- hearing complaints. Tinnitus was associated with TBI severity and cognitive performance. The authors discuss the limitations of standard audiometric assessments of service members and veterans with a history of blast exposure and/or

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TBI and why it is important to use more than just pure-tone audiograms when evaluating chronic effects of TBI.

Vartanian et al. used a cross-sectional study design to compare neuropsychological and neurocognitive profiles of breaching instructors and range staff to non-breaching military controls. While there were differences between the groups on several of the tests, the authors concluded that these results should be carefully considered as these disturbances may not be unique to the breaching environment but rather to the broader life experience of the individual. Carr et al. used a retrospect chart review study design to compare a group of soldiers who, by virtue of their occupation, were exposed to multiple blast events over time but were not associated with a diagnosis from acute blast exposure to a group of soldiers who were likely to be deployed, but unlikely to be exposed to any blast. The two groups of soldiers were compared on a wide range of factors such as military occupational specialty (MOS) assignment, diseases, hospitalizations, ambulance encounters, and disability status. Self-reporting of tinnitus appeared to be the one factor that was clearly different between the two groups. These two studies highlight just some of the complexities of studying the impact of occupational blast exposure on health and performance in military personnel. Complementary the these studies is Wang et al., using blood samples from breachers with repeated blast exposures throughout their military careers, identified DNA methylation changes in genes that were linked to symptoms commonly reported by the study participants such as sleep disturbances, tinnitus and headaches. Their results provide some insights on the neurobiological mechanism(s) underlying mild TBI induced by chronic exposure to blast. Thangavelu et al. studied whether TBI-associated blood based biomarkers (such as GFAP, UCHL-1, Nf-L, tau, and amyloid beta peptides), that are commonly linked with exposure to blast overpressure, could also be measured in sniper trainees who were exposed to low levels of overpressure caused by their weapons, specially 0.50 caliber rifles. Data from neurocognitive testing and self-reporting of symptoms were collected to compare with the blood biomarker findings. Serum levels of amyloid beta peptides were found to be elevated in the sniper trainees following low levels of overpressure exposure from rifle fire even with infrequent TBI symptomology.

PRECLINICAL STUDIES

Using a rodent model of blast injury, Dickerson et al. examined neuropathological changes within the thalamus and amygdala following repetitive blast events. Blast exposed animals were shown to have a decrease in performance on a test routinely used for measuring motor function and balance and a significant increase in glial activation in the thalamus, but not in the amygdala. The authors discuss the possibility that the thalamic glial changes could be contributing to the vestibulomotor impairment. Seno et al., using a laser-induced shock wave model, have demonstrated that selective serotonin reuptake inhibitors decreased depression like behavior through hippocampal neurogenesis mediated by brain-derived neurotrophic factor and serotonin.

COMPUTATIONAL STUDIES

Traumatic optic neuropathy refers to optic nerve injury resulting from direct and indirect head and facial trauma. A direct and an indirect traumatic event can affect the optic nerve, causing impairment of vision (8, 9). Li et al. developed a computational head model with a biofidelic orbit which included the entire length of the optic nerve. This model was used to perform simulations to better understand the mode and location of injury to the optic nerve following an impact to the head from different directions using either a rigid or compliant impactor. The simulated data suggested that impacts to the forehead (vs. the side or back of the head) are more likely to cause optic nerve injury. It was proposed that the mechanisms of injury could be due to "the uneven deformation of the optic canal which induces deformation of the optic nerve and the tugging between the brain and optic nerve." The development of models (animal models or computational models) of indirect traumatic optic neuropathy (ITON) could prove to be beneficial in understanding the mechanisms of damage as well as potential therapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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The Biomechanics of Indirect Traumatic Optic Neuropathy Using a Computational Head Model With a Biofidelic Orbit

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Indirect traumatic optic neuropathy (ITON) is an injury to the optic nerve due to head trauma and usually results in partial or complete loss of vision. In order to advance a mechanistic understanding of the injury to the optic nerve, we developed a head model with a biofidelic orbit. Head impacts were simulated under controlled conditions of impactor velocity. The locations of impact were varied to include frontal, lateral, and posterior parts of the head. Impact studies were conducted using two types of impactors that differed in their rigidity relative to the skull. The simulated results from both the impactors suggest that forehead impacts are those to which the optic nerve is most vulnerable. The mode and location of optic nerve injury is significantly different between the impacting conditions. Simulated results using a relatively rigid impactor (metal cylinder) suggest optic nerve injury initiates at the location of the intracranial end of the optic canal and spreads to the regions of the optic nerve in the vicinity of the optic canal. In this case, the deformation of the skull at the optic canal, resulting in deformation of the optic nerve, was the primary mode of injury. On the other hand, simulated results using a relatively compliant impactor (soccer ball) suggest that primary mode of injury comes from the brain tugging upon the optic nerve (from where it is affixed to the intracranial end of the optic canal) during *coup countercoup* motion of the brain. This study represents the first published effort to employ a biofidelic simulation of the full length of the optic nerve in which the orbit is integrated within the whole head.

Keywords: finite element methods, brain injury, vision loss, optic nerve, head trauma, concussion, biomechanics, optic neuropathy

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INTRODUCTION

Indirect traumatic optic neuropathy (ITON) is a mechanical impact-induced injury to the optic nerve that often results in partial or complete loss of visual function to one or both the eyes (1, 2). It is distinct from direct traumatic optic neuropathy, a condition in which a fragment from a fracture of the skull or a penetrating foreign body directly damages the optic nerve. One estimate reports that ITON occurs in 0.5 to 5% cases of closed head trauma (3). ITON is an important medical problem associated with non-penetrating injury to the head because of the potential for devastating

effects on vision. In this study, we perform mechanics-based simulations to quantify the likelihood and predict the location of ITON from impact forces to the head.

The incidence of ITON has not been directly reported in a large civilian population. However, surveillance studies of traumatic optic neuropathy of any type have been reported for pediatric and adult populations in England. For both adults and children, the overall incidence of either direct or indirect traumatic optic neuropathy is ∼1/million (4, 5).

The mechanism of injury associated with ITON is poorly understood. Neither the location along the length of the optic nerve where the injury occurs nor the magnitude or type of forces that cause the injury are known. It has been suggested that there might be direct damage to the optic nerve axons and/or to the vascular supply of the optic nerve (6–8) and it is clear that there is ultimately retrograde damage to- and loss of retinal ganglion cell axons (9–11). Diffusion tensor imaging studies (DTI) have demonstrated reduced fractional anisotropy along the nerve (12) and ocular coherence tomography suggests that the retinal nerve fiber layer may acutely thicken, as well (13). Currently, there are no confirmed protocols for prevention, mitigation or treatment of ITON (2).

Two aspects of ITON that seem to be widely accepted are that it seems more likely to occur after frontal injury and that it can occur from a relatively minor impact insufficient to cause facial fractures (14). However, these observational findings have neither been fully confirmed nor explained. Because the vision loss associated with ITON can be devastating, understanding the biomechanics of this condition in humans is imperative to guiding preventive, mitigating, and therapeutic intervention.

We hypothesize two modes of optic nerve injury leading to the development of ITON: (a) Focused impingement of *stress* waves from the skull at the optic canal; and (b) Stretching deformation of the optic nerve between brain and the optic-canal due to *coup-countercoup* motion of the brain. We further hypothesize that impacts to the forehead are more susceptible to optic nerve injury compared to the lateral and dorsal impacts.

BACKGROUND

There are a number of suggested mechanisms by which blunt trauma leads to ITON (3), including (1) shear stress damage to the optic nerve sheath (along with its trabeculations and capillaries) and axons (14), (2) tugging of optic nerve between brain and the optic canal (15), and (3) nerve sheath hematoma and/or edema within the optic canal and chiasm (1). Clinical or cadaveric data that might validate one of more of these mechanisms is lacking. It has been proposed that the fusion of bone periosteum and optic nerve dura at the entrance, exit and within the optic canal make the optic nerve more susceptible to deformation of the optic canal and the relatively lower elastic tissues adjacent to it (10). Adding to this complexity is that ITON may be accompanied by damage to the orbit making it difficult to distinguish and enumerate the effects of direct and indirect energy to the nerve. Furthermore, the concussion associated with ITON may be accompanied by transient amnesia, making it difficult for patients to recount the event and provide information that might be valuable in understanding the mechanism of ITON. While CT and MRI are conventionally employed for diagnosing optic nerve and canal injuries (1), these modalities cannot detect ITON in the acute setting.

Finite element (FE) simulation with high-fidelity computational models based upon MRI data have been commonly regarded as a powerful mathematical method to evaluate the effects of traumatic injury. Uchio et al. (16) firstly developed an elaborate eyeball model and conducted the quasi-static uniaxial strip tests to measure the material properties and failure strains of the cornea and sclera. Stitzel et al. (17) considered the material of the eyeball computational model as non-linear elasticity and set up a validation protocol to predict globe rupture injury after comparing between their simulations and experiments. In addition, Cirovic et al. (18) included the globe, the orbital fat, the extra-ocular muscles and the optic nerve in their computational model to study the protective mechanism of eye stability during head trauma. The incompressibility of the orbital fat and the rigidity of the orbital walls can effectively restrict the excessive distortion of the eye and the optic nerve. Subsequently, several finite element models were developed with more accurate anatomy structures to fit different purposes such as the effects of impact, blast, and shaking, as well as eye mechanics and accommodation, respectively (19–22).

With the development of a traumatic brain injury (TBI) simulation, i.e., the reconstruction of an entire head model assembled with an orbital model, studies of indirect trauma on the globe have become possible (23–26). Huempfner-Hierl et al. (27) developed a simplified skull model to simulate the anterior impact on the forehead and showed that stressors propagated toward the optic foramen and the chiasm through the orbital ceiling. Notably, the majority of these computational models were employed to study globe rather than nerve injury. The etiology of ITON is still unclear due to the absence of a complete optic nerve FE model.

In this study, we developed a complete FE ITON computational model that includes the entirety of both optic nerves, globes and the supporting components, such as the orbital fat, extra-ocular muscles (EOM), as well as the brain, meninges and cerebrospinal fluid (CSF). We then used this model to explore the mechanics of ITON comparing a variety of injury sites and impact angles for a rigid and compliant impactor.

METHODS

The Computational Head Model With High-Fidelity Orbit

The head model was built from MRI images of a 50th percentile male based upon the Visible Human Project supported by NIH. The Visible Human Project provides complete, anatomically detailed 3D human body images to the public. The MRI data used in our study consisted of axial slices of a male head and neck at 4 mm intervals. The MRI images were 256 by 256 pixel resolution with each pixel made up of 12 bits of gray tone (28). Materialize Mimics, a 3D medical image processor, was used to segment the

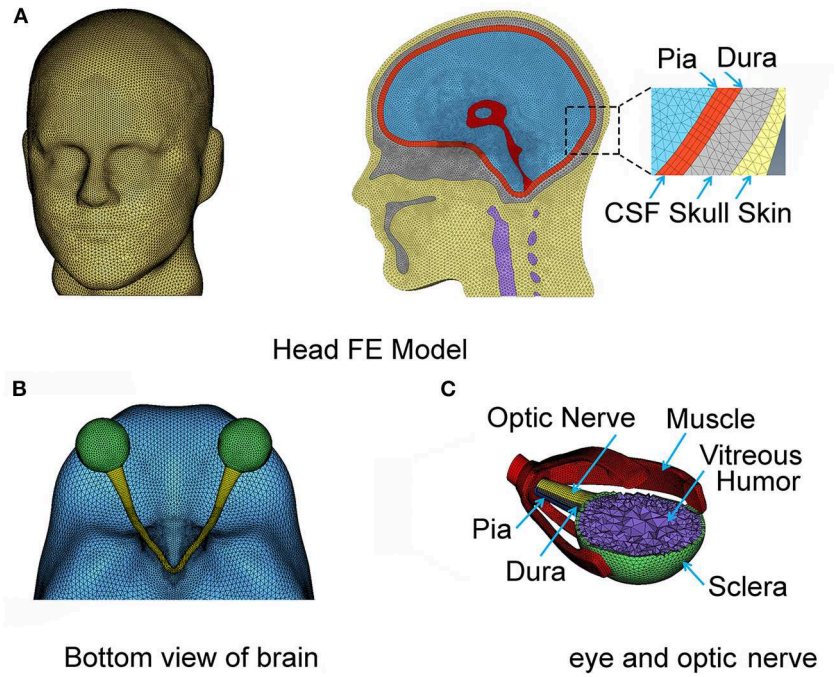

FIGURE 1 | The complete FE head model with the meninges and CSF **(A)**. The eyeball and optic nerve are also included in the head model **(B,C)**, respectively.

head images to different components and convert the selected components to a surface model, which were then converted to volumes. These volumetric FE models were meshed in Altair Hypermesh software based upon the surface model. MRI (T1-weighted) images were segmented into different substructures, which included brain, ventricles, cerebrospinal fluid (CSF), subarachnoid structure (SAS), neck, and skull. Selected orbital substructures that were reproduced from MRI include fat, optic nerve, globe, bone, and extraocular muscles (EOMs).

Thinner structures such as the meninges were not resolved in the MRI images. Therefore, we manually created elements for the dura and pia by making a copy of the topology of surfaces in their neighborhood. Dura was created by shrinking the inner surface of the skull, and pia was created by dilating the outer surface of the brain (and the optic nerve). The skin and the cornea-sclera were developed from MRI images. Neighboring substructures that were in contact share common finite element node forming a perfect / no-slip interface, e.g., between fat and the orbit.

As shown in **Figure 1A**, the skin model is meshed through to the skull and the cervical vertebrae. The CSF and the ventricles are modeled as soft solids, using a very low value of shear modulus that yields a corresponding Young's modulus of 1 kPa. This approximation allows us to consider the compliance of the fluid, circumventing the challenges associated with having to model the solid-fluid interface. The dura and the pia are assigned as membrane elements with thicknesses of 0.3 (29) and 0.13 mm (30), respectively. The arachnoid mater of the brain is not explicitly modeled; instead we homogenized the properties of sub-arachnoid space and CSF. Since CSF flows in the sub-arachnoid space, we assumed that the net compliance of the homogenized space can be approximated as a relatively soft solid (31). The CSF is commonly regarded as a soft solid in our model, with a bulk modulus of 2.1 GPa (32–34). The mechanical properties of these layers are poorly known, and thus most previous studies have made similar approximations when investigating the shear motions of the brain during mild accelerations.

The right and left hemispheres of the brain were separated by the falx cerebri as the mid-sagitally-oriented extension of the dura. The tentorium cerebelli is an axially-oriented extension of the dura that distinguishes the cerebrum and the cerebellum. The FE nodes of the falx and tentorium are numerically "tied" (i.e., no slip contact) to the finite elements of the substructure in the immediate vicinity. The biofidelic model of the brain sub-structures utilized in this work, including the meninges, was rigorously validated against *in situ* measurements of 3D brain deformations under mild angular accelerations of the head under sagittal and axial rotations (33, 35). It is recognized that the orientation of axonal fiber bundles within the white matter of the optic nerve is important for estimating the axial strains along the fiber.

The geometry of the eye and the optic nerve with its surrounding orbital tissues was also digitally reconstructed using Hypermesh software (by Altair Hyperworks), guided by the MRI images, as shown in **Figure 1B**. Since this study focuses on the optic nerve, the globe is simplified in our model with only a sclera and cornea layer enveloping vitreous humor. The EOMs are attached to the sclera anteriorly and extend posteriorly to the annulus of Zinn (a ring of fibrous tissue surrounding the optic nerve at the anterior opening of the optic canal). The extracranial

TABLE 1 | Material properties of the computational head model.

	Element type and number		Density(kg/m^3)	Material properties	References
Skull	Solid, C3D10M	1,056,814	1,300	E = 14.5 GPa, $\nu = 0.35$;	(36)
Brain	Solid, C3D10M	3,262,394	1,040	$E_0 = 3.1$ kPa, $\nu \sim 0.5$; $g_1 = 0.450$, $\tau_1 = 0.5$; $g_2 = 0.365$, $\tau_2 = 50$	(37)
Skin	Solid, C3D10M	311,390	1,200	E = 1 MPa, $\nu = 0.45$;	(38)
Dura	Membrane, M3D4R; thick 0.3 mm	52,303	1,130	E = 31.5 MPa, $\nu \sim 0.5$;	(29)
Pia	Membrane, M3D4R; thick 0.13 mm	77,107	1,130	E = 10.8 MPa, $\nu \sim 0.5$;	(30)
Ventricles, SAS and CSF	Fluidic Solid, C3D8R	1,019,914	1,000	K = 2.1GPa, $G_0 = 500$Pa, $G_\infty = 0$ Pa	(39)
Optic nerve	Solid, C3D10M	33,782	1,040	$E_\infty = 30$ kPa, $\nu \sim 0.5$; $g_1 = 0.450$, $\tau_1 = 0.5$; $g_2 = 0.365$, $\tau_2 = 50$	(40)
Cornea-Sclera layer	Solid, C3D8R	26,856	1,400	K = 3.571 GPa; $\nu \sim 0.5$	(41, 42)
Vitreous humor	Solid, C3D10M	152,104	1,006	K = 2.272 GPa; $\nu \sim 0.5$	(24)
Rectus muscles	Solid, C3D10M	51,604	1,200	E = 11 MPa, $\nu = 0.4$;	(43)
Oblique muscles	Shell, S3R; thick 5 mm	1,044	1,200	E = 11 MPa, $\nu = 0.4$;	(43)
Orbital fat	Solid, C3D10M	846,714	1,000	$E_\infty = 1.5$ kPa, $\nu \sim 0.5$; $g_1 = 0.9$, $\tau_1 = 0.5$; $g_2 = 0.5$, $\tau_2 = 50$	(44)
Steel Impact	Solid, C3D10M	85,040	8350	E = 100 GPa, $\nu = 0.37$	(45, 46)
Soccer Ball	Shell, S3R; thick 5 mm	3,516	553	E = 100 MPa, $\nu = 0.45$, m = 0.45 kg, $P_{inner} = 0.11$ MPa	(47)

optic nerve is connected to the posterior sclera of the globe and extends to the intraorbital opening of the optic canal where the nerve sheath attaches and remains attached until the nerve exits at the intracranial opening of the canal. The nerve then continues until it forms with optic chiasm by connecting with the optic nerve from the fellow eye as shown in **Figure 1C**. Notably, the length of the nerve in the orbit is longer than the distance from the eye to the globe, allowing the nerve to be slack within the orbit; the intracranial portion of the nerve is not slack. The nodes on the surface of the optic chiasm are numerically "tied" (no-slip contact) to the finite elements of the brain.

Normally, the dura and pia mater extend into the optic canal and envelop the optic nerve. We assumed that the SAS between the dura and the pia had a relatively lesser role in load transfer of the optic nerve, because of its smaller thickness compared to the SAS of the brain. For that reason, the SAS between the dura and pia of the optic nerve were excluded in the model.

All six EOMs muscles were included within the model for the orbit. These muscles are important for the motion of the globe within the orbit during normal functions. In addition, these muscles offer stiffness to the connection between the globe and the skull. The four rectus muscles (superior, lateral, medial, and inferior) are modeled using a solid finite element. Thicknesses of these muscles were explicitly modeled by referencing MRI images. The rectus muscles connect the globe to the posterior orbit. The two oblique muscles (superior and inferior) were relatively difficult to identify from the MRI images because of the constrained space in which these are located within the orbit. We employed a simplified structural form of the oblique muscles using a shell form of finite element with a finite thickness (5 mm, from MRI) to implicitly model the stiffness of their connection between the globe and the skull. The superior oblique muscle runs from the globe through a pulley-like trochlear notch in the superonasal orbit to the posterior orbit. The inferior oblique runs under the globe from its lateral aspect to an attachment in the inferonasal orbit. There are 12 main substructures to our computational head model as listed in **Table 1**, and each of these substructures is numerically tied with the its neighboring components by sharing the common nodes at the interface.

Supplementary Figure 1 shows biofidelic construction of cervical vertebral structure for the neck, developed from the MRI. Vertebrae, from C1 to a partial structure of C5, are modeled as a fused bone structure in our computational model. Since we did not include the entire spine in our model, the soft tissue surrounds the vertebral structure.

The entire computational head was meshed using 6,840,422 finite elements, and a finite element convergence test was performed on the mesh size for the optic nerve to numerically obtain mesh-independent results on impact loading. The finite element mesh was imported in a non-linear finite element analysis package (Abaqus software by Simulia). Because we assumed that the whole head is symmetric about the sagittal plane, we developed a half model on one side of the sagittal plane and then mirrored the other side. The coordinate system is referenced as Anterior-Posterior (AP axis), Right-Left (RL axis), and Superior-Inferior (SI axis).

Material Property

A complete list of material properties is listed in **Table 1**. All substructures were modeled using isotropic and homogenous material properties. An elastic material requires two independent constants: the Poisson's ratio (ϑ) and the Young's modulus (E) values; these were assigned based upon measurements reported in the literature. Soft substructures, such as brain, were modeled using a value of Poisson's ratio that is approximately 0.5 (e.g., 0.49999975 in Abaqus FE software). This is a relatively high value,

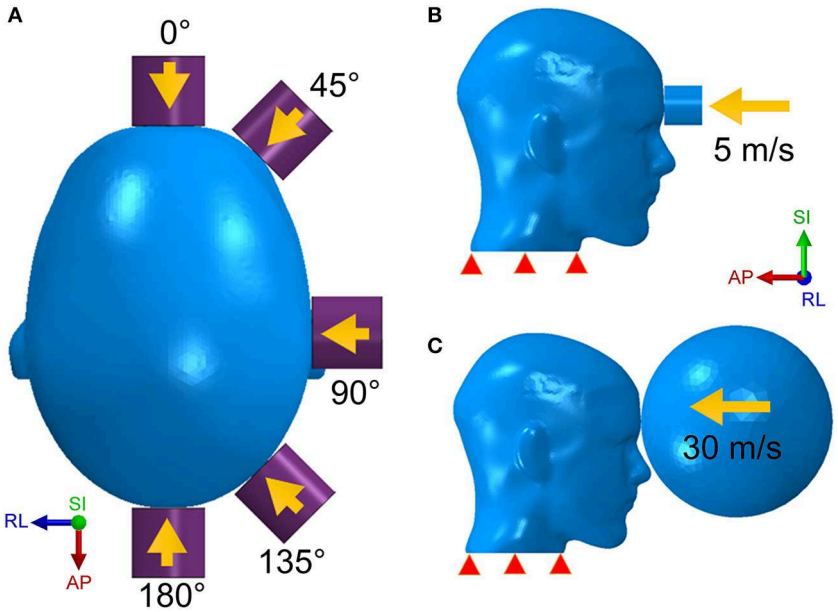

FIGURE 2 | The head model is respectively struck by the cylinder impactor from five directions **(A)** with an initial speed of 5 m/s **(B)** and by the soccer ball with a speed of 30 m/s **(C)**.

signifying incompressibility of brain, fat, vitreous humor, and the ventricles, as opposed to more compressible structures such as skin and the muscles. The resulting value of the bulk modulus for individual substructures is calculated using $E = 3K(1 - 2\vartheta)$ and is presented in **Table 1**. The bulk modulus is an average value for the brain and is consistent with values adopted in previous studies (33).

Viscoelastic material properties were assigned to relevant substructures (brain, fat, and optic nerve), since measurements were available in the literature from studies employing nano-indentation and other experimental techniques; these were commonly used in published simulation studies (36, 37, 44, 48, 49). We adopted a linear viscoelastic model to describe the biomechanical behavior of all the tissue components in the head model. For a linear viscoelastic model, the time-dependent shear modulus (relaxation modulus) is independent of the strain magnitude. Although non-linear viscoelastic model would be more appropriate for parts of the orbit that are anticipated to experience magnitudes of strain >20% (of the original length), material properties for a non-linear model are unavailable in the literature.

In linear viscoelasticity, the relaxation shear modulus $G_R(t)$ is determined by the dimensionless function, $g_R(t)$, expressed as a Prony series of N viscoelastic constants $\bar{g}_i^P$ and τ_i^G. The dimensionless function is given by

$$g_R(t) = 1 - \sum_{i=1}^{N} \bar{g}_i^P (1 - e^{-t/\tau_i^G}) \quad (1)$$

where $g_R(t) = G_R(t)/G_0$, and G_0 is the instantaneous shear modulus. The long-term shear modulus $G_\infty = 1 - \sum_{i=1}^{N} \bar{g}_i^P$ can be obtained when time tends to a long-term value, $t \to \infty$.

Loading Setup for Realistic Impact Analysis

The impact analysis was performed using a dynamic module in Abaqus FE software, employing an explicit numerical time-stepping procedure. We referred to earlier investigations for a variety of impactors employed during studies of head trauma (20, 45, 46). It is commonly accepted that the mass and momentum (product of mass and velocity) of impactors play an important role in defining the energies of the impactor. However, studies employing the effect of different relative stiffnesses of the impactors have not been reported. Therefore, we modeled impactors with two different stiffness values, i.e., one with greater and the other lesser stiffness than the skull.

We modeled the stiffer impactor as a cylinder with a density of 8,350 kg/m^3, a Young's modulus of 100 GPa, a Poisson ratio of 0.37 and a finite element type as solid. Five impact regions as well as five impact angles were studied in the simulation (**Figure 2A**). The initial velocity of the impactor was set along the impact direction at 5 m/s (**Figure 2B**), which correlates with a general minor-impact type of blow such as from a fisticuff (50).

The less stiff impactor is made of a deformable elastomer sphere and was meant to simulate a soccer ball strike on the human head. The speed of a soccer ball can be as high as 25–35 m/s for case of high-powered shots taken by professional players (51). However, unlike the cylinder impact that strikes on a rather narrow area, the impact form of the soccer ball covers a wider area of the head due to the deformable nature of the soccer ball. In addition, the size of the soccer ball is comparable to the human head. For this case of loading, we built a hollow soccer ball FE model using a shell type finite element mesh with a diameter of 0.215 m, a mass of 0.45 kg and a fixed internal pressure of 110 kPa

(51). As shown in **Figure 2C**, the soccer ball is placed in front of the forehead with an initial speed of 30 m/s.

RESULTS

Cylinder Impactor

Figure 3 shows snapshots of maximum principal strain distribution along the entire optic nerve under an impact of the cylinder at several different impactor orientations relative to the head. Bain and Meaney performed the tension experiments on the optic nerve of a guinea pig *in-situ* at strain rates of 30–60 s^{-1} to measure the functional and morphological tissue-level threshold strains for axonal injury due to stretch (52). In general, the functional threshold strains (corresponding to electrophysiological impairment) were less than morphological threshold strains (determined by immunohistochemical staining). Through regression analysis, liberal, conservative, and optimal strain thresholds of 28, 13, and 18%, respectively, were

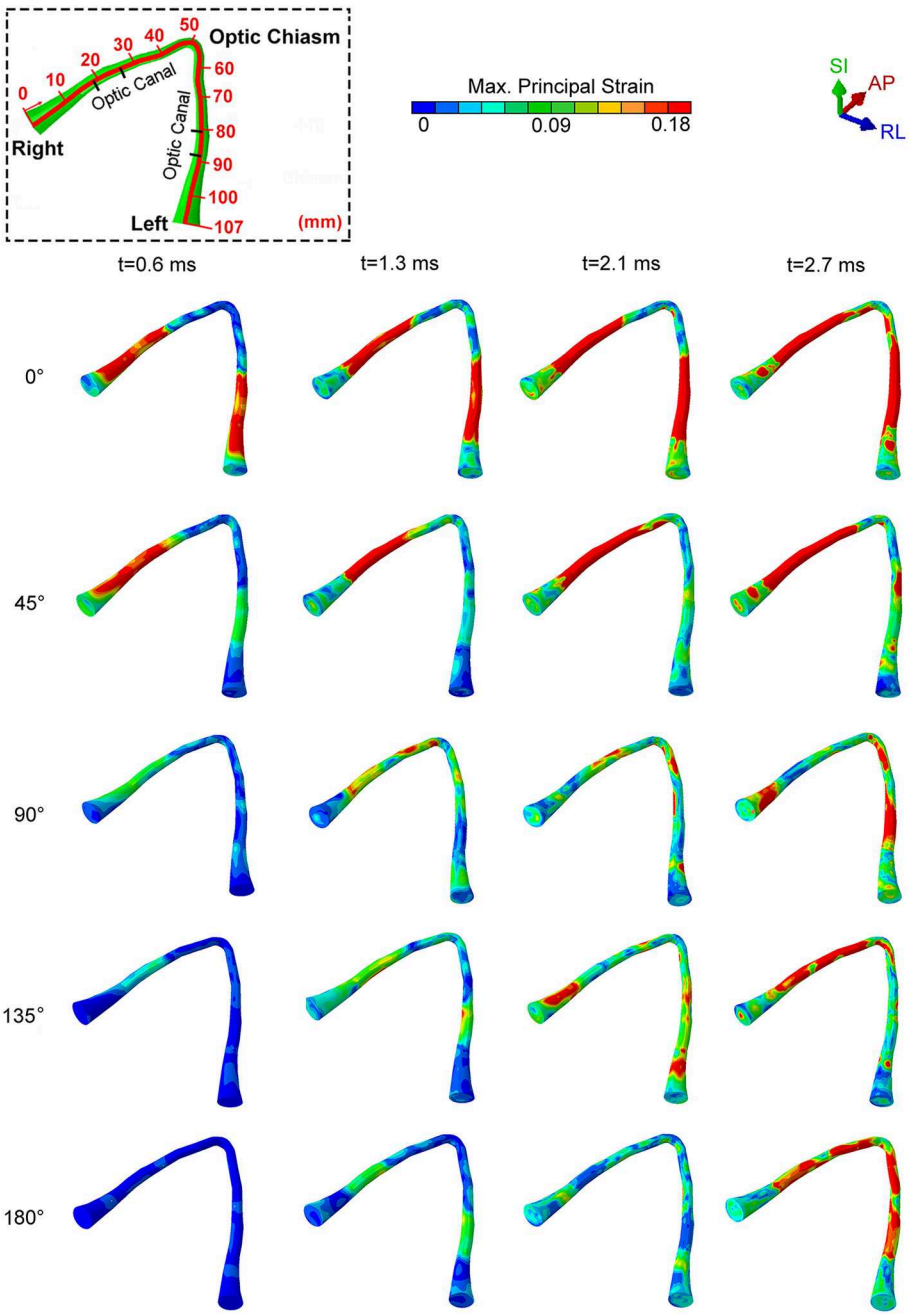

FIGURE 3 | The locations of maximum principal strain of the optic nerve during cylinder impact for different impact directions.

obtained for the onset of electrophysiological impairment. Based upon the previously successful application of the optimal strain threshold for injury analysis of the brain white matter (15, 34), we choose the same optimal strain threshold of 18% as a criterion for functional injury of the optic nerve. We assume a strong likelihood of functional injury if the strain at anytime during the dynamic analysis overshoots this strain threshold. We also assume that the likelihood of injury increases with an increasing extent of the optic nerve that is subjected to strain above this threshold. The dashed box on the top left part of the figure shows the position of the optic canal and optic chiasm relative to the optic nerve to aid in distinguishing those various regions of the optic nerve (i.e., intra-orbital, -canalicular, and -cranial).

For an impact direction (angle) of 0°, the optic nerve shows injury by 0.6 ms, and the injury sites initially occur within the optic canal, depicted by red colored regions in **Figure 3**. Subsequently, the likelihood of injury spreads rapidly to the remaining portions of the nerve, except the optic nerve head and the optic chiasm (**Figure 3**, t = 2.7 ms). The impact from 0° leads to a bilaterally symmetric injury of the optic nerves.

A 45° impact during which the impactor is brought to the right side of the forehead above the right eye resulted in a markedly asymmetric distribution of injury. In this case, there was injury only to the right optic nerve.

For the three other impact directions (i.e., 90°, 135° and 180° to the forehead), the optic nerve is subjected to strain below the injury threshold until 1.3 ms, but eventually shows some injured areas by 2.7 ms. Differences in the injury sites vary substantially for these three cases. At 90° and 135°, the impacts cause an asymmetrical strain distribution at 2.7 ms. The 90° impact leads to an injury near the optic nerve head for the optic nerve ipsilateral to the impact, while the injured regions are more distal to the optic nerve head for the optic nerve contralateral to impact. The 135° impact injures the ipsilateral nerve near or within the optic canal. In contrast, an impact at 180° causes symmetric injury to the optic nerves. However, unlike the 0° impact, the injured regions are more likely to lie in the posterior part of the optic canal, closer to the optic chiasm. In addition, the injury for the 180° impact develops much later in time (by almost 2 ms) compared to the 0° impact.

Figure 4 shows graphical representations of the average quantitative strain histories starting from the right optic nerve (secondary to a right-sided impacts angled at 45, 90° and 135°) and ending on the left optic nerve; this spans a length of approximately 100 mm, as shown in **Figure 4A**. The average is taken over four paths around the circumference (top, bottom, inside, outside) to demonstrate the distribution of strain over the length of the optic nerve, shown in **Supplementary Figure 2**. The strain value in the optic nerve increases with time. Impacts at both 0° and 45° cause higher strain value than the three other directions of impact. Paths corresponding to the 0° and 45° impacts shows elevated levels of strain in the vicinity of the optic canal, while the optic chiasm (at ∼50 mm from the origin of the right optic nerve) is subjected to lower magnitudes of strain. **Figure 4** also shows that there is symmetric pattern for strain associated with the 0° impact and an asymmetric pattern for strain associated with the 45° impact. In addition, the patters persist for the greatest duration compared to the other angles of impact. The greatest strain is seen for the 45° impact at 2.7 ms on the intracranial optic nerve ipsilateral to the impact, suggesting this is the most deleterious situation in terms of causing ITON, while the 0° impact is the next most severe case.

Further understanding on the role of stress waves in deforming the optic nerve between the 45° and 0° impact was obtained by analyzing the stress distribution in the skull from the moment of impact. **Figure 5** shows the evolution of stress distribution over time on the skull for the 0° and 45 impact. Colors indicate magnitude of von Mises stress, a scalar measure obtained from the three-dimensional nature of the stress tensor (9 components in a 3 × 3 matrix). For both cases, the figure shows that the stress travels along the orbital ceiling and reaches the optic canal. For the 0° impact, a similar magnitude of stress reaches both orbits. On the other hand, for the 45° impact more stress reaches to orbit ipsilateral to the side of impact.

Figure 6A depicts the potential locations of the bone deformations that might play a role in compression or shearing deformations of the optic nerve or optic canal. Displacements were measured at four locations: the intra-orbital (i.e., anterior) rim (point A for upper rim, point B for lower rim) and the intra-cranial (i.e., posterior) rim (point C for upper rim, point D for lower rim) of the canal ipsilateral to the impact. **Figure 6B** graphically portrays the differences in the displacements (i.e., relative motion) between respective upper and lower points, indicating the degree of deformation of the orbit. It should be noted that the relative displacement between points A and B along the SI axis will indicate a tendency to induce radially oriented compression/decompression deformations of the optic nerve, while displacement along the AP axis and RL axis will indicate a tendency to induce shearing deformations. When there is no relative displacement, then the displacement graph lines for the respective upper and the lower points will overlap. Further, differences between displacements of points A and C or between points B and D will indicate a tendency to induce either compression/decompression or bending of the optic nerve along AP-axis and RL-/SI-axes, respectively.

During the 0° impact, relative displacement of points A and B (also between C and D) along the AP-axis is higher than that along the SI- and RL-axis, indicating a predominant shearing force applied to the optic nerve along its length (within plane AP-SI). The difference in displacements can be noticed between 0.25 to 1 ms and again between 2 and 3 ms. Within these time windows, minor differences in displacements between points A and C along the SI-axis are also apparent, indicating a tendency for the optic canal to undergo bending in plane AP-SI. The deformations and displacements of the points along the RL-axis are relatively insignificant. Similar trends are seen with the 180° impact, although, the deformations of points A and B as well as points C and D along the AP-axis are smaller compared to the 0° impact. In contrast, the deformations for the 45° impact along the RL-axis are larger than those from the 0° impact. Displacement is apparent between points A and C and also points B and D,

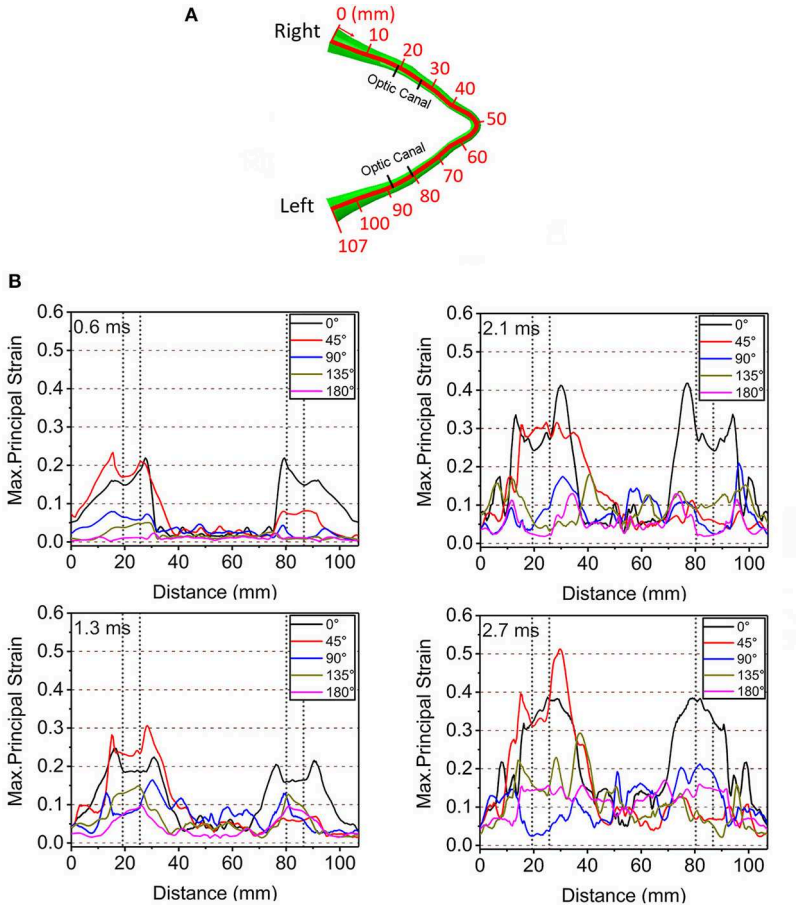

FIGURE 4 | (A) Length scale along the optic nerve. **(B)** The maximum principal strain along the optic nerve at corresponding times of 0.6, 1.3, 2.1, and 2.7ms.

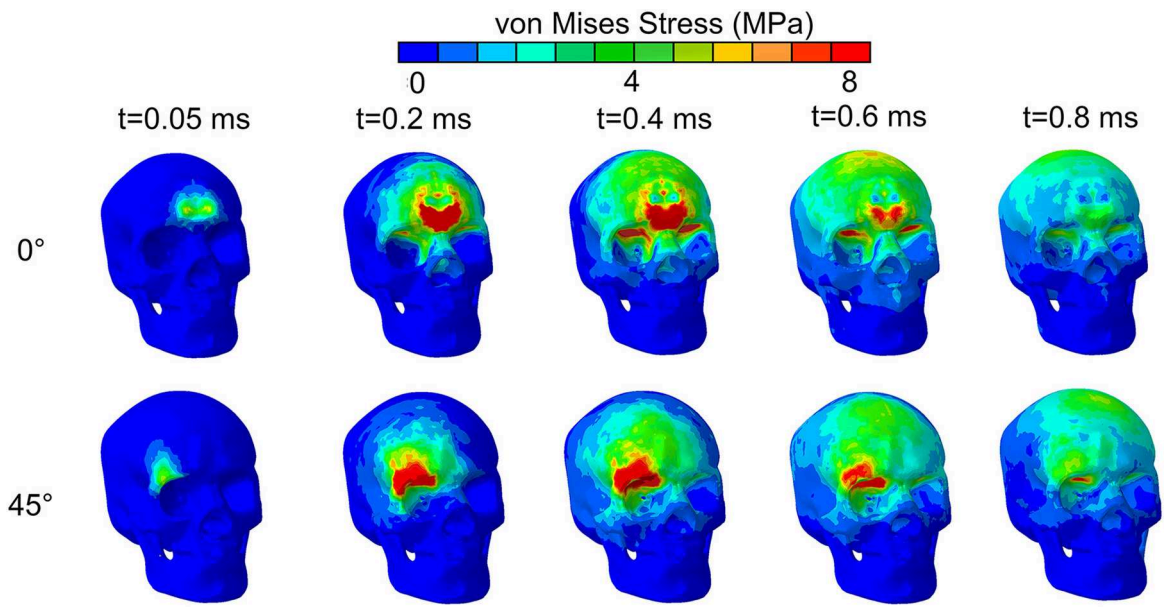

FIGURE 5 | von Mises stress distribution on the skull for impact directions of 0° and 45° by an impact from a relatively stiff cylinder.

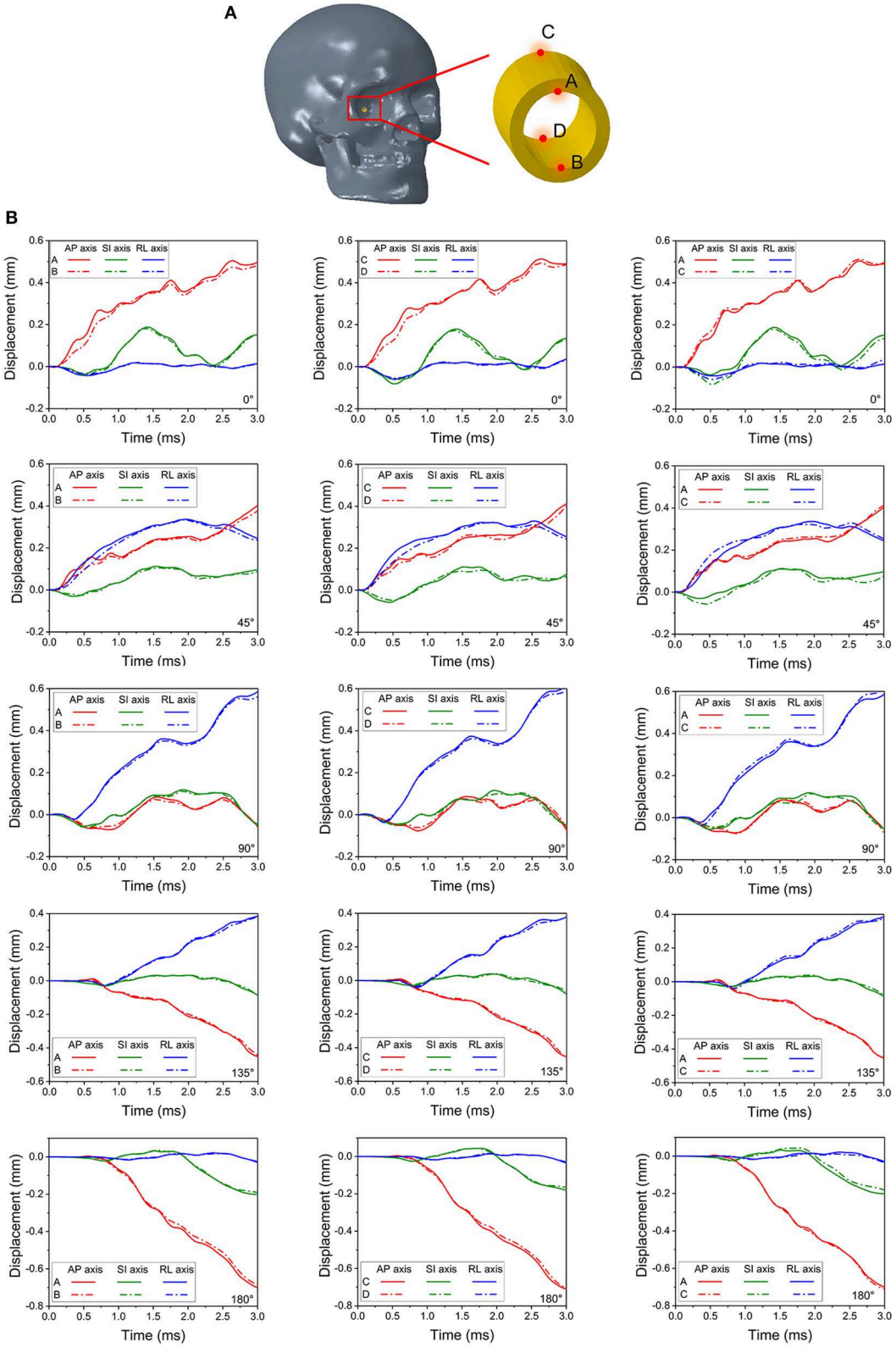

FIGURE 6 | Four monitor points shown in **(A)** are set on the rim of the right (struck-side for 45°, 90° and 135°) optic canal indicate deformations of the canal in **(B)**.

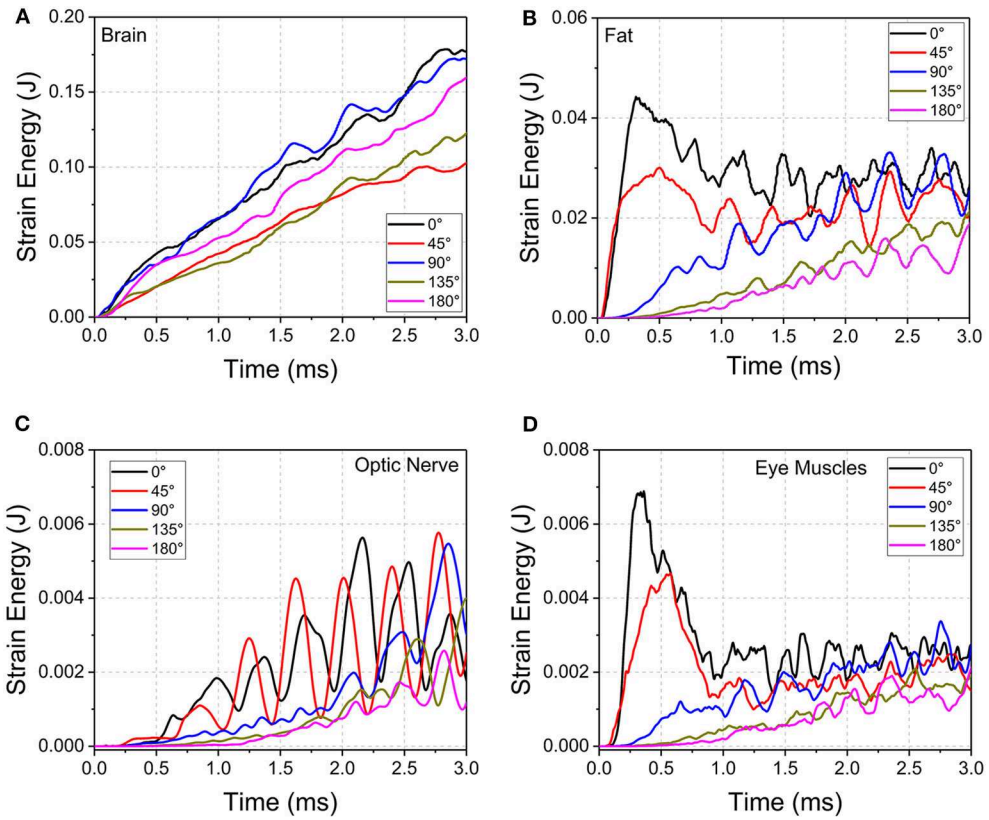

FIGURE 7 | Strain energy development of **(A)** brain, **(B)** orbital fat, **(C)** optic nerve, and **(D)** eye muscles during cylinder impact for the different impact directions.

along the RL- and SI-axes, as well as between A and B and also points C and D along the SI- and AP-axis. This indicates that all deformations modes of the optic canal are active, i.e., shearing, compression/decompression, and bending. This further suggests that the 45° impact is indeed more deleterious compared to the 0° impact. Deformations for the impact at 90°, 135° and 180° progressively lessen and are smaller than those of the 45° and the 0° impact cases.

The evolution of strain energy (stored energy due to deformation) of the brain, orbital fat, optic nerve, and eye muscles were tracked for the entire duration of impact (**Figure 7**). The strain energy of the brain continuously increases for all the cases during the simulations. Notably for the 45° impact, the strain energy absorbed by the brain is the lowest compared to all other impact orientations, indicating an increased role of other substructures (besides the brain) in absorbing the impact strain energy. The 0° and 90° impacts led to the highest strain energies for the brain although the pattern of strain development is similar for all impact directions in that strain energy steadily rises over time.

For the orbital fat, optic nerve and eye muscles, the strain energies decrease as the angle of impact moves from 0° to 180° between 0 and 2 ms. However, the evolution of strain development differs among tissues and angle of impact. For fat and muscle, the strain energy peaks within 0.5 ms for 0° and 45° impact and then plateaus with oscillations while the strain energy for the other three directions steadily rises. For the optic nerve, the strain energy steadily grows from 0 to 3 ms for all angles of impact, although for 0° and 45° the strain energy demonstrates wide oscillations during this increase. The 45° impact led to the highest strain energy for the optic nerve, followed by the 0° impact.

Oscillations in strain energy development indicate the presence of stress waves traveling over the length of the optic nerve, which would induce oscillating deformations. The period of oscillations in the optic nerve are larger compared to the period of oscillations in the EOMs. This effect may be explained based on the characteristic material wave speed, defined as the square root of the ratio of material elastic modulus divided by material density. Deformation waves traveling back and forth over the length of the optic nerve result in the oscillations of deformation, and hence the oscillations in strain energy. Since the elastic modulus of the muscles is higher than that for the optic nerve by two orders of magnitude (**Table 1**), wave speed in the muscle tissue is higher compared to the wave speed in the optic nerve. Upon impact, the EOMs tend to absorb more strain energy, but this more quickly dissipates to substructures of the orbit because of the higher wave speed of the muscle. This would suggest that EOMs play a role in protecting the optic nerve during trauma.

Soccer Ball Impact

We performed simulations using the soccer ball for the same five impact angles explored with the cylinder. **Figure 8** shows the maximum principal strain on the optic nerve during the soccer ball impact from 0° impact (**Figure 8A**) and 45° impact (**Figure 8B**). Comparison of these plots shows that the 0° impact demonstrates higher strains, indicating higher likelihood of injury compared to the 45° impact. Time histories of strain for all five angles of soccer ball impact are shown in **Figure 8C**. The 0° impact shows an oscillation of the strain forces; the magnitude of strain elevation is higher intraorbitally at 2 and 6 ms while it is higher intracanalicularly at 4 and 8 ms. The 180° shows a similar pattern of elevation at 2, 4, and 6 ms, but at 8 ms, the strain in the intraorbitally continues to rise. For both impact angles, there does not appear to be substantial differences between right and left nerves. The 90° impact shows higher strains later in time, indicating likelihood of unilateral injury after 6 ms. The 45° and the 135° impacts show the least magnitudes of strains among all the impact angles—this is in contrast with the case of cylinder impact.

Figure 9 shows the length scale along the optic nerve (**Figure 9A**) and the maximum principal strain for four different paths along the length of the optic nerve optic nerve at times of 2 ms, 4 ms, 6 ms and 8 ms obtained from a 0° impact of a soccer ball (**Figure 9B**). **Supplementary Figure 2a** shows chosen paths along four regions around the circumference (top, bottom, inside, outside) for distribution of strain along the length of the optic nerve. As with the cylinder impactor, it is presumed that the threshold for strain injury from soccer ball impact is 18% (34). These results show that strain at 2 ms is maximal at 30 mm and 90 mm, which are anatomically located within the intracranial region of the optic nerve. The result also shows that as time progresses the strain seems to increase near the chiasm so that by 8 ms it is greatest therein.

Figure 10 shows maximal strain along the course of the optic nerve (**Figure 10A**), the von Mises Stress over the skull (**Figure 10B**) and the displacement for the points A-B and C-D (locations shown in **Figure 6A**) from the 0° impact angle with a deformable soccer ball (**Figure 10C**). Injurious levels of strain are first demonstrated by 4 ms from the time of impact. Injurious levels of strain are maximal at 6 ms and these energies are concentrated at the point of the optic where it exits the optic canal intracranially, then spreading posteriorly to include the optic chiasm.

Figure 10B shows that the stress wave propagates along the orbital ceiling, but the stress value is lower than that of the corresponding cylinder impact (**Figure 5**). Concerning potential deformation of the optic canal, no obvious displacement gaps can be found along SI- and RL- axes between the upper and lower rim (**Figure 10C**). There are some noticeable displacement differences between point A and B (also between points C and D) along the AP-axis, indicating a shearing mode of the optic nerve. These values indicate that the part of the optic nerve within the optic canal is indeed subjected to a finite strain, although the strain remains below the injury threshold for the most part of the impact duration, except at 6 ms (**Figure 9B**).

Figure 11 compares the displacement of the head and brain along different axes for both the cylinder and soccer ball impactor at 0° impact. To clarify the role of brain motion in inducing injury to the optic nerve, the displacement along the AP axis for six (anterior, posterior, superior, inferior, right, and left) monitor locations are summarized and compared between the two types of impactors. Each location consists of a pair of points, one on the skull and another on the surface of the brain, as shown in **Figure 11A**. The difference between these corresponding points is essentially a measure of deformation of the sub-arachnoid space, i.e., the motion of the brain with respect to the skull. **Figure 11B** graphically depicts the motion of the brain-skull points for the two impactors between 0 and 10 ms. The absence of any gaps between the skull and brain graphs for the anterior, posterior and superior locations indicate negligible deformation. In the inferior region, the brain oscillates so that it is displaced more or less than skull, depending on the time. In the left and right locations, the displacement of the brain is initially greater than the skull until approximately 4 ms and then it is less than that of the skull. For the left, right and inferior locations, the differential displacement of the skull with respect to the brain could be expected to create forces that are transmitted to the intracranial optic nerve since the optic nerve is fixed at both the optic chiasm (to the brain) and intracranial optic canal (to the skull).

Concerning the overall movement of the head, in the cylinder case, the AP axis displacement indicates that the head initially moves posteriorly until $\sim$ 3 ms and then rebounds, albeit with oscillations, particularly for anterior and inferior locations. On the other hand, the head motion in the soccer ball impact case completes only half a cycle movement, moving posteriorly until 8 ms. The maximal head movement is greater for the cylinder than for the soccer ball only until $\sim$3 ms. Thereafter, the movement of the head continues to increase posteriorly for the soccer ball impact to a maximum of $\sim$2 mm. Finally, it should be noted that the effect of relatively low shear modulus of the brain-skull interface compared to either brain or skull (**Table 1**) can be seen in **Figure 11B**. In particular, this figure shows the displacement difference at the brain-skull interface is highest at the inferior monitoring point, indicating that the inferior region of the brain-skull interface may be particularly prone to shear strain; it is in this location that the optic chiasm is located.

DISCUSSION

The simulations suggest that more than one possible injury mechanism might result in ITON. Notably, these results are in agreement with both static loading studies (6) and the blunt trauma simulations (27) that conclude that the forehead impact would lead to a stress wave that propagates through the orbital roof and concentrates at the orbital apex. However, the presence of an explicit model for an optic nerve in our case enabled us to perform advanced analyses of optic nerve injury site, pattern, and mechanisms of injury. In our simulations for the cylinder impact, the 0° and 45° cases demonstrated that the

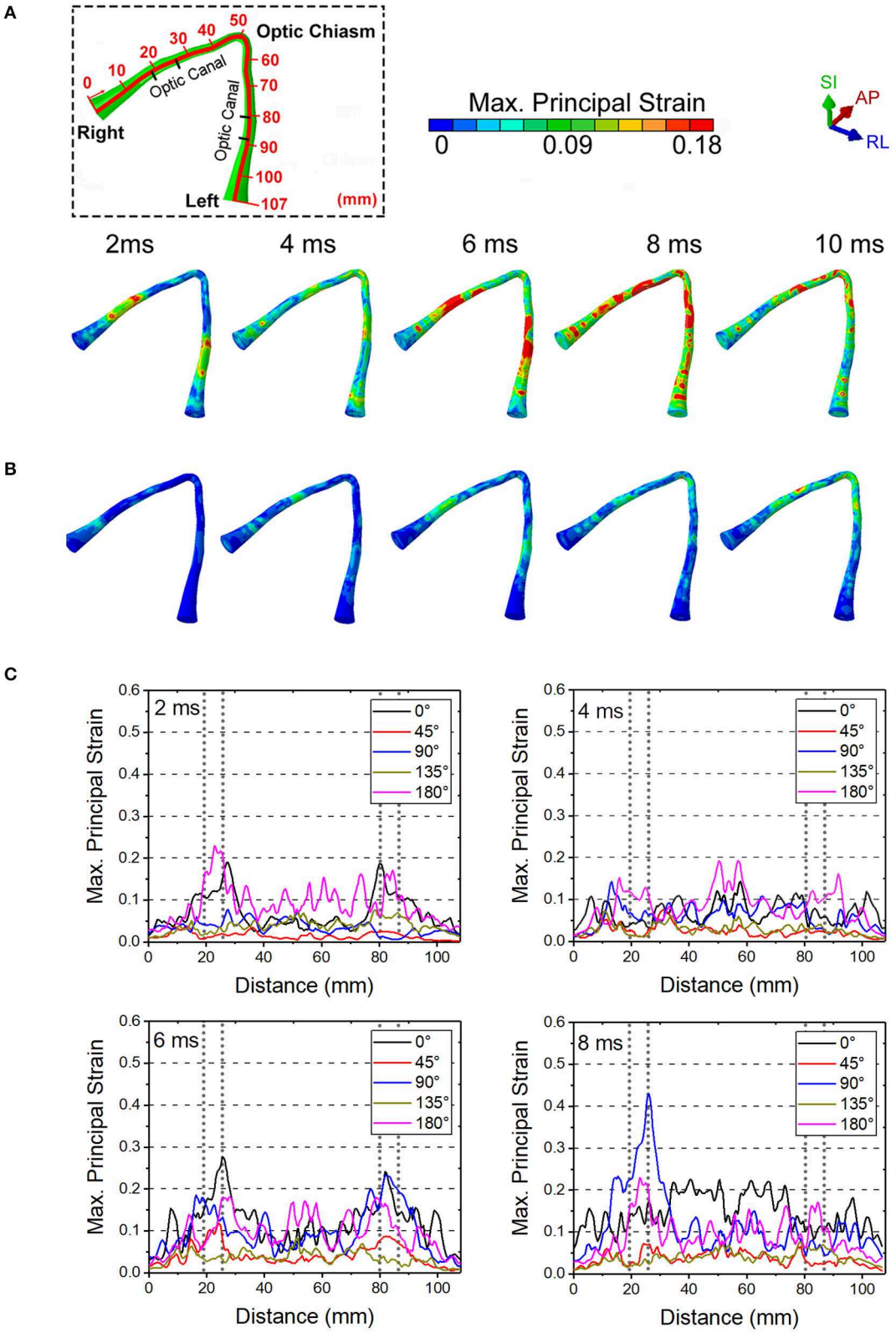

FIGURE 8 | The maximum principal strain on optic nerve during the soccer impact from **(A)** 0 deg and **(B)** 45 deg. **(C)** Time history of strains for all five angles of soccer impact.

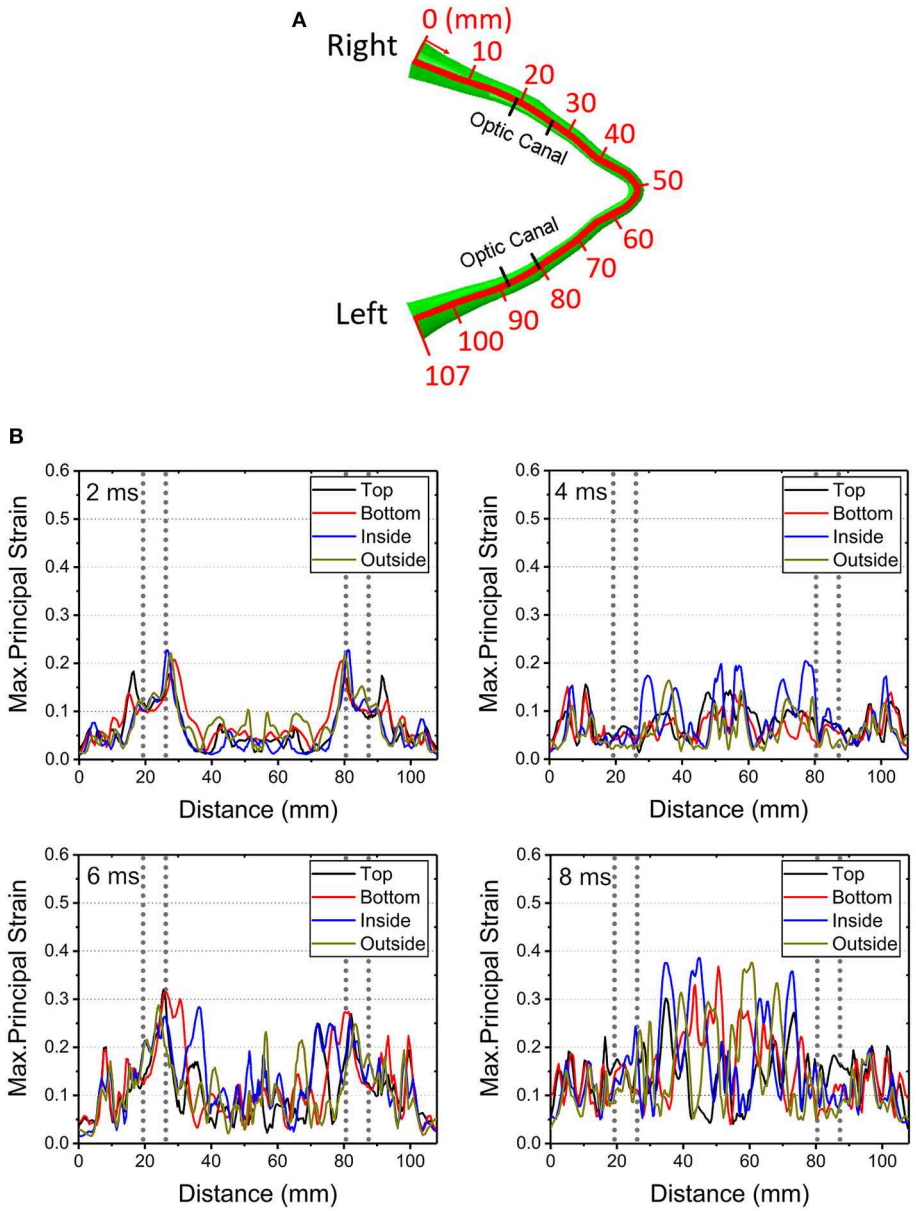

FIGURE 9 | (A) Length scale along the optic nerve. **(B)** The maximum principal strain for four different paths along the length of the optic nerve optic nerve at times of 2, 4, 6, and 8 ms obtained from a 0° impact of a soccer ball.

stress wave propagates from the superior orbital rim to the optic canal along the orbital roof (**Figure 5**). This stress wave resulted in an uneven deformation of the upper and lower rim of the optic canal that would cause a predominantly shearing type injury in the optic nerve. Notably, Santos-Bueso et al. (53) have reported a clinical case of ITON in which a patient suffered a right frontal impact, which is very similar with the 45° case in our simulation.

It also appears that the likelihood of injury to the intracanalicular optic nerve is higher than that for the intracranial and intraorbital portions of the nerve with the cylinder impact. The impact energy was propagated from the orbital ceiling to the upper rim of the optic canal, which leads to optic canal diameter reduction and damage to the optic nerve. Furthermore, the relatively higher strain energy of the orbital fat for the 0° and 45° impact cases indicates that the blunt impact around the orbital rim is more likely to cause injury at these angles than at 90°, 135° and 180°. Indeed, the corresponding deformations of the optic canal from cylinder impacts at 90°, 135° and 180° appear to be insufficient to cause injury within

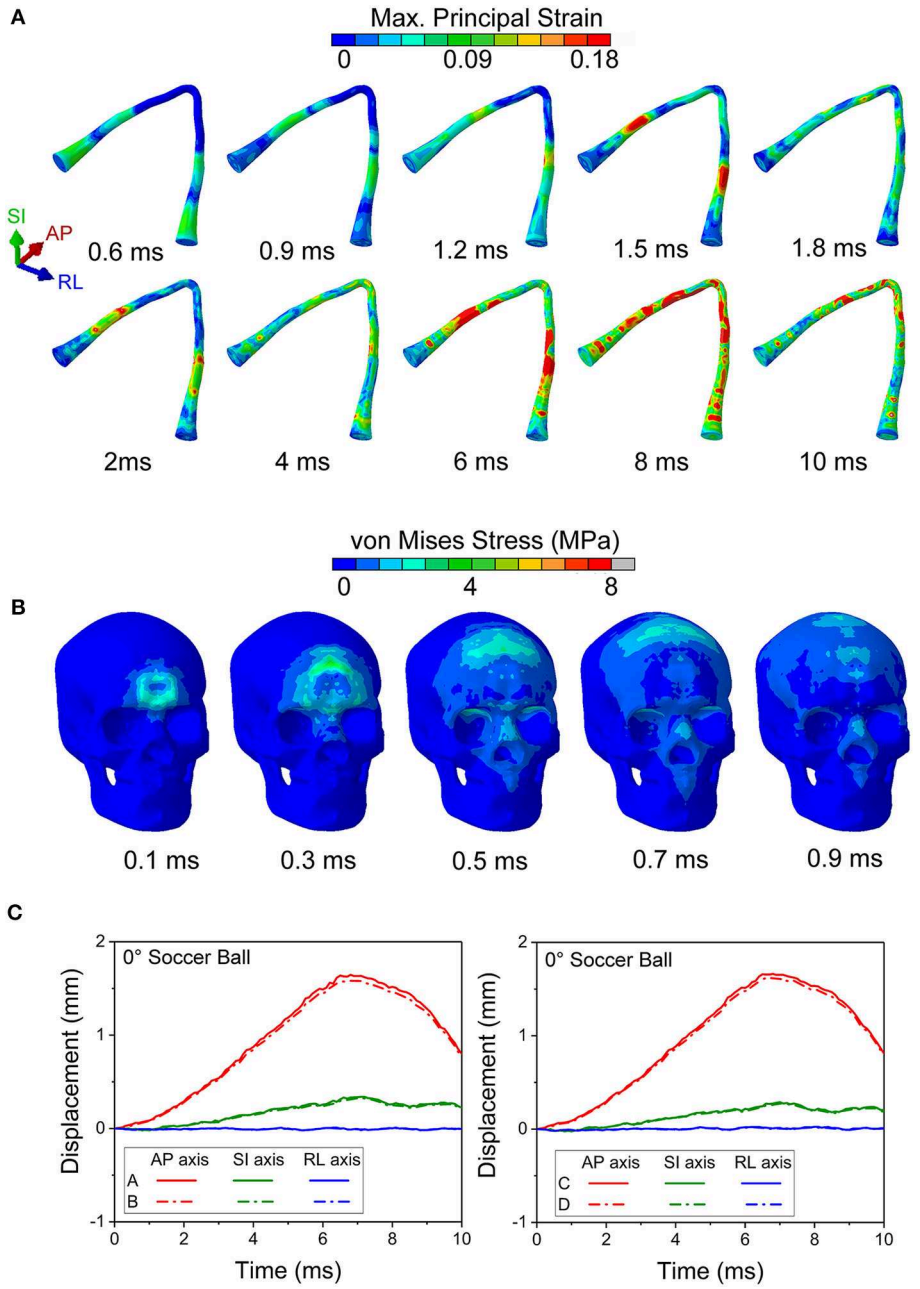

FIGURE 10 | (A) Maximum principal strain of the optic nerve is presented during the 0° soccer ball impact. **(B)** Mises stress distribution of the skull during the 0° soccer ball impact. **(C)** Displacement of the optic canal during the 0° soccer ball impact.

the optic canal (**Figure 6**). Moreover, the strain energy of the orbital fat is also extremely low at the beginning of the impact, supporting the idea that the deformation of the optic canal is not likely to cause any optic nerve injury in the 90°, 135° and 180° cases.

Concerning the less stiff impactor, we chose a soccer ball because TBI is unfortunately common in ball sports. The impact of the ball with the head can induce deformations of brain substructures beyond the functional injury thresholds (47). Indeed, TBI has been ranked as the most important cause of injuries in soccer (54, 55). Because of the deformable nature of the soccer ball, the skull stress is much lower compared to the 0° impact with the rigid cylinder. Although the initial impact area of the soccer ball is small (at 0.1 ms), this impact area keeps increasing since the ball deforms. The stress wave also propagates along the orbital roof, but the stress tends to dissipate before it arrives at the orbital apex. The symmetric displacement of the optic canal rim also suggests that there is no obvious

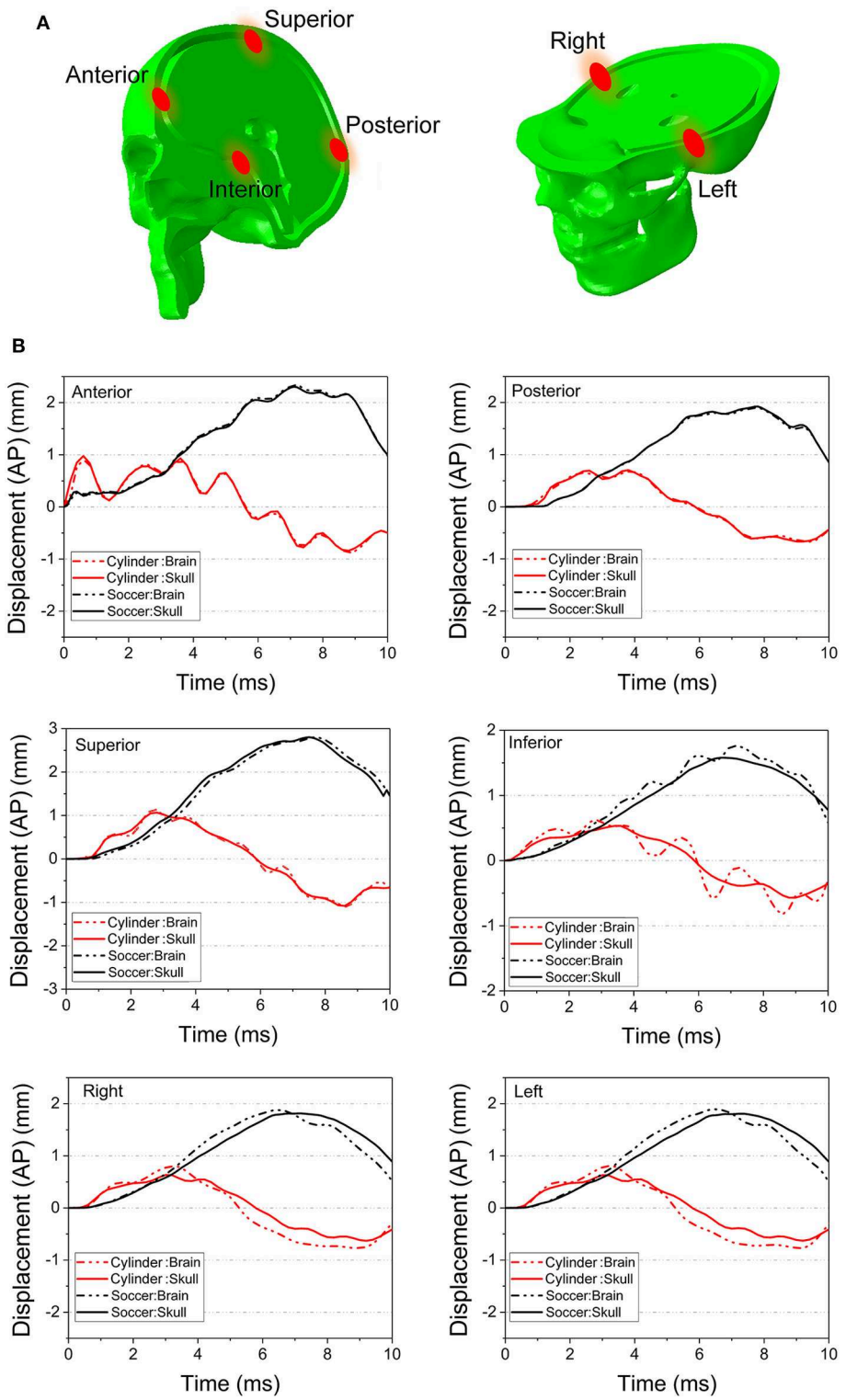

FIGURE 11 | (A) Several pairs of monitor points are set nearby on the different area of the skull and the brain. **(B)** The displacement along AP axis of the monitor points are compared between the 0° cylinder impact and soccer ball impact.

deformation of the optic canal in case of the soccer ball impact. Rather, the inconsistent AP axis displacement of the skull and brain and the fact that levels of energy sufficient to damage the nerve injury are reached at approximately 7 ms suggest that ITON in this case is most likely caused by motion of the brain subjecting the intracranial optic nerve to tension. This idea is

supported by the finding that large and often times oscillatory (i.e., *coup contrecoup)* motion of the brain develops after soccer ball impact (**Figure 11B**); such motion would cause the brain to pull on the intracranial optic nerve. This tugging damage could also be exacerbated by the continuous deformation of the brain resulting from the increasing strain energy of the brain with soccer ball impact.

We understand that there are limitations to this study. Even the best simulations require assumptions, particularly with employing those literature-derived metrics used to build the whole-head model. Furthermore, as mentioned previously, creating a nonlinear viscoelastic model for substructures of the orbit that experience magnitudes of strain $>$ 20% (of the original length) could improve the accuracy of the model. It is also germane to mention that our anatomic assumptions made about the dura and pia reflect data reported for the brain but that these may be less accurate for the optic nerve itself.

It has been reported that ∼60% of the extra-fascicular matrix of the human optic nerve is made of varying types of connective tissue (56). Therefore, the assumption that the human optic nerve has a Young's modulus of 30 kPa may be low. Published data of the cow optic nerve reports a Young's modulus of 5.2 MPa (57). This measurement by Shin et al. (57) on the intraorbital part of the cow optic nerve corresponds to the composite property of the optic nerve, including the pia. Results from our computational model using the higher end of the modulus, 5.2 MPa (57) are shown in **Supplementary Figure 3**. This simulated result indicates that the optic nerve does not lead to any injury to the intraorbital part of the optic nerve for the case of 45° impact at 50 m/s impact velocity, which is 10 times the injurious velocity (5 m/s) for the case with lower modulus (30 kPa). At such a high velocity, our simulations suggested the likelihood of skull fracture (judging from maximum principal stress exceeding fracture stress of the skull), while the injury of the optic nerve was precluded. Thus, a constant modulus of 5.2 MPa over the length of the optic nerve overestimates the stiffness of the optic nerve in our simulations.

On the other hand, *in vivo* shear wave elastography studies on normal humans indicated that the mean Young's modulus of the distal nerve was 17.3 kPa (58), which is comparable to our estimate of long-term viscoelastic modulus of 30 kPa. In our study, the composite modulus of the optic nerve, including the pia, is 0.67 MPa. Moreover, the modulus of the optic nerve is likely to vary over it's length, since the fraction of collagen in the optic nerve is known to decrease from intraorbital part to intracranial part (56). In addition, the amount of collagen also varies between animals and humans (56). With the lack of consistent and detailed measurements on the viscoelastic properties of the optic nerve, we use a constant value for the viscoelastic long-term modulus, 30 kPa, over the length of the optic nerve. Regardless of the actual value of the Young's modulus, the *relative* distribution of forces along the varying segments of the optic nerve measured in this simulation is likely to remain unchanged, as would be the conclusion that impacts at certain angles are more likely to cause ITON. The authors recognize that the current ITON head model still requires validation through animal (59) and cadaveric-based (60) experiments; this research is ongoing.

Future efforts with this model could be applied to other medical concerns that arise in patients with mild TBI. For example, our finding that the skull base substructures (such as pituitary gland) may be prone to injury could help clarify the biomechanics leading to neuroendocrine disorders reported after mild TBI (61–63).

In summary, we have created the first human whole head finite element model to include the orbits and the entire lengths of the optic nerves. This model has helped create data to suggest that impacts at the superior orbital rim (forehead) appear be those most likely to cause ITON. In addition, it indicates that there are two major injury mechanisms: (1) the uneven deformation of the optic canal, which further induces deformation of the optic nerve, and (2) the tugging between the brain and the optic nerve.

CONCLUSION

For this study, a high-fidelity orbit model within a whole head model including the brain was developed and used to study the biomechanics of ITON. The cylinder impact on the ITON model from different directions have been simulated. The simulation results provide analysis of the maximum principal strain, von Mises stress, the strain energy, and the displacements within the optic canal and the head-brain interface, supporting the most likely cause for injury mechanism of the optic nerve.

The shear effect at the optic canal is the most likely cause of injury from forehead impacts (0° and 45°) received from stiffer objects, compared with other impact directions (e.g., back or side of the head). The impact stress wave propagates along the orbital ceiling to the optic apex. The onset of injury occurs at intracanalicular region of the optic nerve. More specifically, relatively higher strains are seen at the optic nerve where the nerve exits on either side of the canal, since the nerve is tethered by the canal. In the case of an impact to the forehead by a relatively compliant object (relative to the skull), the tugging (tension) of the optic nerve is the most likely mechanism of optic nerve injury. The motion of the brain within the skull is the cause of tensile mode of deformation of the optic nerve. This injury is most likely to occur in the intracranial region of the optic nerve. The *coup countercoup* mechanism of injury is dominant in this case of soccer ball impact and so one would expect the impact to the back of the head to have the same likelihood of causing injury as the forehead impact.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00346/full#supplementary-material

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frontiers in Neurology

BRIEF RESEARCH REPORT
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Objective and Subjective Auditory Effects of Traumatic Brain Injury and Blast Exposure in Service Members and Veterans

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Service members and veterans (SMVs) with a history of traumatic brain injury (TBI) or blast-related injury often report difficulties understanding speech in complex environments that are not captured by clinical tests of auditory function. Little is currently known about the relative contribution of other auditory, cognitive, and symptomological factors to these communication challenges. This study evaluated the influence of these factors on subjective and objective measures of hearing difficulties in SMVs with and without a history of TBI or blast exposure. Analyses included 212 U.S. SMVs who completed auditory and cognitive batteries and surveys of hearing and other symptoms as part of a larger longitudinal study of TBI. Objective speech recognition performance was predicted by TBI status, while subjective hearing complaints were predicted by blast exposure. Bothersome tinnitus was associated with a history of more severe TBI. Speech recognition performance deficits and tinnitus complaints were also associated with poorer cognitive function. Hearing complaints were predicted by high frequency hearing loss and reports of more severe PTSD symptoms. These results suggest that SMVs with a history of blast exposure and/or TBI experience communication deficits that go beyond what would be expected based on standard audiometric assessments of their injuries.

Keywords: speech perception, hearing, tinnitus, traumatic brain injury, blast exposure, service members and veterans

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INTRODUCTION

Traumatic brain injury (TBI) is a common injury among military service members and veterans (SMVs) (1). There has been increasing awareness that physical, sensory, cognitive, and/or affective symptoms are often reported many months or years following injury [e.g., (2)] or develop following subconcussive blast exposure (3). Critical for improving patient care is our ability to comprehensively assess the range of problems that individuals with a TBI or blast exposure experience. This goal is complicated by variability in the causes and symptoms associated with these deficits.

Of particular challenge has been assessing TBI- and blast-associated deficits in communication. Hearing loss and tinnitus are among the most prevalent service-connected disabilities for veterans (4) and the incidence of hearing difficulties in service members, particularly those deployed (5), exceeds that of the U.S. working population (6). The monumental increase of blast exposure in deployments has led to an increased incidence of TBI diagnoses and associated auditory impairment (7). Permanent sensorineural hearing loss is reportedly the most prevalent type of auditory impairment in blast trauma, accounting for 35–54% of auditory injury (8). Chandler (9) estimated that 64% of blast-injured service members being treated at a large, U.S. military treatment facility had ongoing hearing loss. Bothersome tinnitus is also often experienced in people with trauma-related injuries, exposure to high levels of occupational noise, and hearing loss; all of which are common in the military population. Approximately 20% of people with chronic tinnitus have bothersome tinnitus that can promote cognitive difficulties, mental health disorders, insomnia, and decreased psychosocial functioning (10–12).

SMVs with a history of TBI and/or blast exposure often report even greater difficulties understanding speech in noisy environments than would be predicted from clinical audiometric assessments, such as pure tone thresholds (13). Clinical tests are often not sensitive enough to quantify speech recognition difficulties (14). In general, individuals with a TBI may appear normal in clinical exams, but suffer in more complex, real-world environments (15).

These findings suggest multiple sources of impairment may occur with TBI or blast exposure that exacerbate speech understanding difficulties in challenging conditions. Though the mechanisms are still under investigation, damage along the peripheral to central auditory pathway may place greater demands on top-down, cognitive systems to compensate, especially in adverse conditions. Individuals with damage to these domain-general systems, as can occur with TBI or blast exposure, may thus be particularly unable to compensate. Indeed, a tight link between auditory and cognitive impairments has been noted in the epidemiological literature (16). Additionally, small-scale studies of civilians have observed associations between auditory and cognitive function in assessing auditory processing abilities (13, 17, 18), though these assessments did not consistently distinguish individuals with and without a history of TBI.

Accurate assessment of communication challenges is critical for mitigating the potential negative social and cognitive consequences of auditory dysfunction including poorer quality of life (19) and job performance and promotion (20). The presence of bothersome tinnitus can have a detrimental impact on a person's emotional, social, mental, and professional life. Tinnitus secondary to blast injury may even be more detrimental due to its sudden emergence instead of gradual onset with progressive sensorineural hearing loss (21).

Complicating the assessment of the impact of TBI and blast exposure on communication is the variability in the causes and symptoms associated with these injuries. For example, mild TBI (mTBI) resulting from blast exposure has been associated with more self-reported hearing difficulty than mTBI resulting from a non-blast mechanism (22). Comorbidities may also cloud our understanding of symptoms of TBI reported years following a TBI. Over 40% of soldiers with mTBI have a comorbid post-traumatic stress disorder (PTSD), and a number of health problems reported by service members with mTBI are strongly influenced by PTSD or depression (23, 24).

Due to increased concern of mental health disorders in the military population independent of auditory status (25, 26), it is imperative that those with bothersome tinnitus and auditory dysfunction are given timely and appropriate treatment options. However, tests of speech recognition in noise and other complex environments as well as tinnitus evaluations are often not part of a standard audiological evaluation.

Given the diversity of factors contributing to challenging speech understanding and hearing and tinnitus problems, we present an initial analysis of a large-scale study of SMVs. This study aims to highlight domains that may be important for comprehensive assessments of the subjective and objective hearing and tinnitus problems of SMVs with or without a history of TBI and/or blast exposure.

METHOD

Participants

SMVs underwent auditory and neuropsychological testing at Walter Reed National Military Medical Center (WRNMMC) as part of the Congressionally mandated 15-Year Longitudinal TBI Study (Sec721 NDAA FY2007) by the Defense and Veterans Brain Injury Center (DVBIC). Details on inclusion criteria, group definition, and recruiting procedures are found in Lange et al. (2). In the current analyses, SMV's first session of complete data was included, yielding 278 participants. Eighteen participants were excluded due to having an equivocal or unknown TBI history. Individuals were also excluded because of invalid cognitive test scores (e.g., performance validity testing) or exaggerated symptom reporting (2, 27) ($n = 46$) or invalid auditory test scores ($n = 2$).

Of the remaining 212 participants, 40% had a history of an uncomplicated mild TBI (mTBI), 29% had greater than an uncomplicated mTBI (i.e., $n = 16$ complicated mTBI, $n = 14$ moderate TBI, $n = 15$ severe TBI, $n = 16$ penetrating TBI), and 31% of had no history of TBI (details in Measures section). 40% of all participants responded on a screening question as having been close enough to an explosive blast to self-report symptoms of a "possible" alteration of consciousness (Blast Exposure question described below). These SMV participants were 93% male and ranged in age from 19.57 to 61.97 years ($M = 37.69$, $SD = 10.25$). Individuals with a history of TBI were tested at least 2.5 months after their date of injury ($M = 7.33$ years, $SD = 8.15$).

Measures

Audiological Screening

Otoscopy was performed to confirm no abnormalities of the tympanic membrane, ear canal, or presence of occluding cerumen. Clear visualization of the tympanic membrane was noted during otoscopy for all participants. Tympanometry measured middle ear function to assess tympanic membrane mobility and compliance and ensured there were no active

tympanometric abnormalities. Pure tone air conduction thresholds were measured at octave/interoctave frequencies of 0.25, 0.5, 1, 2, 3, 4, 6, and 8 kHz to determine the degree of hearing loss in each ear. Standard Pure Tone Averages were computed for low frequencies (PTA LF: 0.5, 1, 2 kHz) and high frequencies (PTA HF: 3, 4, 6, 8 kHz) in the better and in the worse ear.

Speech Recognition Composite Score

Previous studies have shown that there can be substantial variability in the performance of individuals on different standardized speech tests (28). To obtain a comprehensive estimate of speech-in-noise performance, each participant was tested with five speech-in-noise measures and a composite score was obtained by transforming test scores to have the same polarity (lower scores = better performance), z-transforming, and then averaging. The five measures were: (1) Modified Rhyme Test, (2) Standard and (3) Time-Compressed/Reverberant Quick Speech-in-Noise Test, (4) Listening in Spatialized Noise, and (5) High/Low Context Sentences.

Modified rhyme test (MRT)

The MRT (29) is a consonant perception test that requires listeners to identify a monosyllabic word from six alternatives that differ only by the first or last consonant. Each listener completed 40 MRT trials in each ear. Stimuli were masked by speech-shaped noise. Half the trials were presented at a +4 dB SNR and half at a −4 dB SNR. Median response time (MRT RT) was also recorded for each participant.

Standard and time compressed/reverberant quick speech-in-noise (QSIN) tests

Each participant completed an adaptive tracking task using IEEE sentences from the Modified QSIN test (30). Separate tracks were used to estimate the 50% speech reception threshold (SRT) for the standard test (diotic speech in four-talker babble) and the speeded-reverb test (time-compressed speech with 4-talker babble at +90 degrees, 4-talker babble at −90°, and a target talker at 0 degrees).

Listening in spatialized noise (LISN-S)

In the LISN-S (31), participants repeat target sentences in the presence of two competing talkers who are speaking sentences that could easily be confused with the target speech. Only the high-cue condition of the test was administered, where that target talker was a different sex than the masking talkers and the masking talkers were separated 90 degrees to the left and right of the target. The test estimates the SRT where listeners identify 50% of the words in the target sentences.

High/low context sentences (HLCS)

HLCS utilizes the Revised Speech Perception in Noise Test (R-SPIN) sentences (32) to assess comprehension of high and low context sentences in multitalker background noise at a standard or time compressed rate. Participants repeat the entire sentence, which is scored for key words correct to generate a percent correct for each context condition.

Subjective Auditory Complaints

Tinnitus and hearing survey (THS)

The THS (33) differentiates problems caused by hearing loss from tinnitus or hyperacusis. Participants rate how problematic their hearing or tinnitus has been in myriad situations within the last week (0–4). Hearing and Tinnitus subscores each comprise four questions, with a higher score indicating greater problems.

Neurological Symptoms

TBI history and severity

TBI severity was non-normally distributed, with greater representation of uncomplicated mTBI. Thus, TBI history (present vs. absent) and TBI severity (no more than uncomplicated mTBI vs. complicated mTBI or more severe) were treated as binary factors. Details of TBI severity categorization are in Lange et al. (2). In sum, TBI severity was classified as: *uncomplicated mTBI* (i) Glasgow Coma Scale (GCS) = 13–15, Post-Traumatic Amnesia (PTA) <24 h, Loss of Consciousness (LOC) <30 min, and/or Alteration of Consciousness (AOC) present, and (ii) no trauma-related intracranial abnormality on CT or MRT; *complicated mTBI* (i) GCS = 13–15, PTA <24 h, LOC <30 min, and/or AOC present, and (ii) trauma-related intracranial abnormality on CT or MRI; *moderate TBI:* LOC 1–24 h, PTA 1–7 days, and ICA present or absent; *severe TBI*: LOC >24 h, PTA >7 days, and ICA present or absent; *penetrating TBI*: breach of the cranial vault and/or dura mater by external object (e.g., bullet, shrapnel) and/or skull fragment (i.e., skull fracture). Individuals with no history of TBI included 41 injured controls (orthopedic/soft tissue injury with no evidence of AOC, LOC, or PTA as result of injury) and 25 non-injured controls.

PTSD checklist-civilian version (PCL-C)

The PCL-C is a self-administered questionnaire (34) with 17 items designed to evaluate self-reported PTSD, patterned after the DSM-IV-TR (35) symptom criteria for PTSD. The PCL-C is not limited to military experiences, but open to any traumatic event experienced in their lifetime. Participants rate each item (1–5), with a higher total score indicating greater severity of symptoms.

TBI quality of life depression scale (TBIQOL-DEP)

The TBI Quality of Life measurement system (36) assesses self-reported quality of life problems in individuals with a history of TBI. Higher t-scores on the depression scale (TBIQOL-DEP) indicate more severe depressive symptoms.

Blast exposure

Participants responded to a question based on the Ohio State University Traumatic Brain Injury Identification Method (37): "*Have you ever been nearby when an explosion or blast occurred, that resulted in you feeling confused, disoriented, or having a loss of memory for a few seconds or minutes (or longer)? Think about any combat-related incidents.*" Participants indicating "yes" were categorized as having been exposed to a blast. While this question screens for self-reported blast exposure, individuals may not have met diagnostic criteria for AOC as revealed through in-depth interviews with the study team.

Cognitive Domains

Cognitive function was assessed through neuropsychological testing including components of the Connor's Continuous Performance Test-2 (CPT-II) (38), Wechsler Adult Intelligence Scale-IV (WAIS-IV) (39), Delis-Kaplan Executive Function System (D-KEFS) (40), Trail Making Test (41), and Neuropsychological Assessment Battery [NAB; (42)]. Cognitive domains that have been shown to contribute to speech understanding in adverse conditions [e.g., (43, 44)] were included in analyses. Domain composite scores (27) were calculated by averaging the scaled scores ($M = 10$, $SD = 3$) associated with the following subtests, with higher scores indicating better performance. Tests that do not produce scaled scores were converted prior to calculating the composite domains.

Attention and working memory domain

CPT-II Omissions and Hit Reaction Time Standard Error and WAIS-IV Digit Span and Letter-Number Sequencing.

Processing speed domain

WAIS-IV Coding and Symbol Search, D-KEFS Color-Word Condition 2, and Trail Making Test Trial A.

Executive functioning domain

D-KEFS Verbal Fluency Category Switching and Color Word Interference Test Inhibition, NAB Categories Test, and WAIS-IV Similarities.

Verbal fluency domain

D-KEFS Verbal Fluency Letter Fluency and Category Fluency.

Analyses

One challenge for studies with many predictors is that traditional regression models may be underpowered to test the role of each variable. Building predictive models using stepwise procedures may be subject to over-fitting and sensitivity to multicollinearity, thus limiting generalizability to the population. Here, we employ a penalized regression model (least absolute shrinkage and selection operator, LASSO) that allows for testing large numbers of predictors while minimizing model error and potential for over-fitting (45). LASSO has been used to examine questions about the role of cognitive, sensory, and demographic factors in predicting clinical outcomes for individuals with schizophrenia (46). It has also been used to assess the relative roles of objective and self-reported auditory and cognitive functions in hearing aid outcomes (47).

LASSO beta coefficients for variables that contribute less to the model are forced to be exactly zero via a shrinkage penalty (lambda), allowing for concurrent variable selection and parameter estimation. Only the most contributive variables remain in the final model. The Bayesian version of the LASSO (48) has the added advantage of providing standard errors and a more flexible way of estimating tuning parameters and predictors.

Bayesian LASSO regressions were run in R [version 3.6.0; (49)], using mostly default settings of the blasso function in the package movomvn (version 1.9-13) (50). Models were run with Gibbs sampling, uninformative gaussian priors, and hyperparameters recommended by Park and Casella (48). 10,000 Markov Chain Monte Carlo (MCMC) samples of the model were drawn to achieve stable estimates of predictors and tuning parameters. Beta values represent the median values of the posteriors for each predictor after 1,000 discarded burn-in samples. Resulting estimates of lambda and variance are reported.

Predictors and dependent measures were scaled and centered prior to entering in the model. The predictors for the speech recognition composite model included: age (years), TBI history (0/1), TBI severity (0/1), Blast Exposure (0/1), MRT RT (ms), pure tone average (dB HL) in the better and worse ears for low frequencies (i.e., PTA LF BE, PTA LF WE) and high frequencies (i.e., PTA HF BE, PTA HF WE), PCL-C score, TBIQOL-DEP score, and domain scores for Attention/WM, Processing Speed, Executive Function, and Verbal Fluency. Models for THS Hearing and Tinnitus scores also included the speech composite score as a predictor. Beta estimates indicate the contribution of each variable to the measure of interest, with an associated probability that its contribution is not 0 (contributing factors >0.50).

Variance inflation factors (VIFs) were initially calculated to assess potential multicollinearity across regression predictors before entering them into the models. A VIF > 10 is often used as an indicator of collinearity that could impact model stability. All predictors had a VIF <5.82.

RESULTS

Objective Speech Recognition Performance

The results of the Bayesian LASSO predicting speech recognition composite scores are in **Table 1** and **Figure 1**. Factors associated with worse speech recognition performance (higher score) were decreasing age, a history of TBI, slower MRT RT, poorer hearing thresholds for the better and worse ears in the lower frequencies and for the better ear in the high frequencies, and worse executive function. The median variance and lambda penalty parameters were 0.50 and 0.13, respectively. Deviations of the fitted values from the raw values are approximated by an R-square of 0.50.

Subjective Hearing Difficulties

The results of the Bayesian LASSO predicting self-reported difficulties with hearing are in **Table 1** and **Figure 2**. Factors associated with more hearing problems (higher THS score) were a history of self-reported blast exposure, poorer hearing thresholds for the better and worse ears in the higher frequencies, and greater reports of symptoms of PTSD. Median variance and lambda penalty parameters were 0.55 and 0.18, respectively. Deviations of the fitted values from the raw values were approximated by an R-square of 0.46.

Subjective Tinnitus Difficulties

The results of the Bayesian LASSO predicting self-reported difficulties with tinnitus are in **Table 1** and **Figure 3**. Factors associated with more tinnitus problems (higher THS score) were the presence of a more severe TBI, greater reports of symptoms of PTSD, and poorer processing speed. TBI severity

was not independent from TBI history due to controls having a value of 0 for both. In this case, LASSO shrinks the less contributive factor to 0. Median variance and lambda penalty parameters were 0.89 and 0.57, respectively. Deviations of the fitted values from the raw values were approximated by an R-square of 0.12.

TABLE 1 | Results of Bayesian LASSO regressions.

Parameter	Beta (probability ≠ 0)		
	Speech recog composite	**THS Hearing**	**THS Tinnitus**
Age	**−0.0069 (0.71)**	0 (0.32)	0 (0.41)
TBI	**0.14 (0.70)**	0 (0.32)	0 (0.50)
TBI severity	0 (0.32)	0 (0.38)	**0.41 (0.96)**
Blast	0 (0.35)	**0.074 (0.62)**	0 (0.41)
MRT response time	**0.020 (0.98)**	0 (0.43)	0 (0.38)
PTA LF BE	**0.0060 (0.62)**	0 (0.36)	0 (0.38)
PTA LF WE	**0.020 (0.97)**	0 (0.49)	0 (0.41)
PTA HF BE	**0.030 (>0.99)**	**0.016 (0.93)**	0 (0.50)
PTA HF WE	0 (0.41)	**0.0078 (0.82)**	0 (0.47)
PCL-C	0 (0.29)	**0.035 (1.00)**	0 (0.37)
TBIQOL-DEP	0 (0.35)	0 (0.51)	0 (0.38)
Attention WM domain	0 (0.34)	0 (0.50)	**−0.0058 (0.60)**
Processing speed domain	0 (0.33)	0 (0.32)	**−0.048 (0.81)**
Executive function domain	**−0.098 (0.98)**	0 (0.40)	0 (0.49)
Verbal fluency domain	0 (0.30)	0 (0.39)	0 (0.38)
Speech recog composite	N/A	0 (0.50)	0 (0.41)

Beta values represent the median values of the posteriors for each predictor (contributive factors in bold).

DISCUSSION

SMVs with a history of TBI or blast exposure often report difficulties understanding speech in adverse conditions that are difficult to capture with standard audiometric tests. This suggests multiple factors contribute to their speech recognition problems. This study's results are consistent with this: individuals with a self-reported history of blast exposure also reported more severe auditory symptoms than those without, but only those with a history of TBI exhibited significantly worse performance on an objective measure of speech recognition. Furthermore, having a history of a more severe TBI was associated with greater tinnitus complaints (though this model explained little variance overall).

As expected, impairments in pure tone audiometric thresholds contributed to objective and subjective hearing performance. Three of the four thresholds contributed to predicted objective performance. At high frequencies, speech-in-noise performance was dominated by the thresholds in the "better" ear, which reflects that listeners can extract speech information from one ear for binaurally-presented speech. At low frequencies, speech-in-noise performance was primarily determined by thresholds in the "worse" ear. This reflects the important role that low-frequency binaural processing plays in the perception of speech stimuli in complex auditory environments. The performance benefit that listeners get when a noisy signal is spatially separated from a target (i.e., binaural release from masking) depends on the auditory system's ability to compare the amplitude and phases of low-frequency sounds arriving at the two ears. Thus, one would expect performance to be limited by the fidelity of the neural representation of the sound in the worse ear. Previous studies have shown that binaural tasks like auditory localization tend to degrade when the hearing threshold at 500 Hz exceeds 40 dB in the worse ear (51).

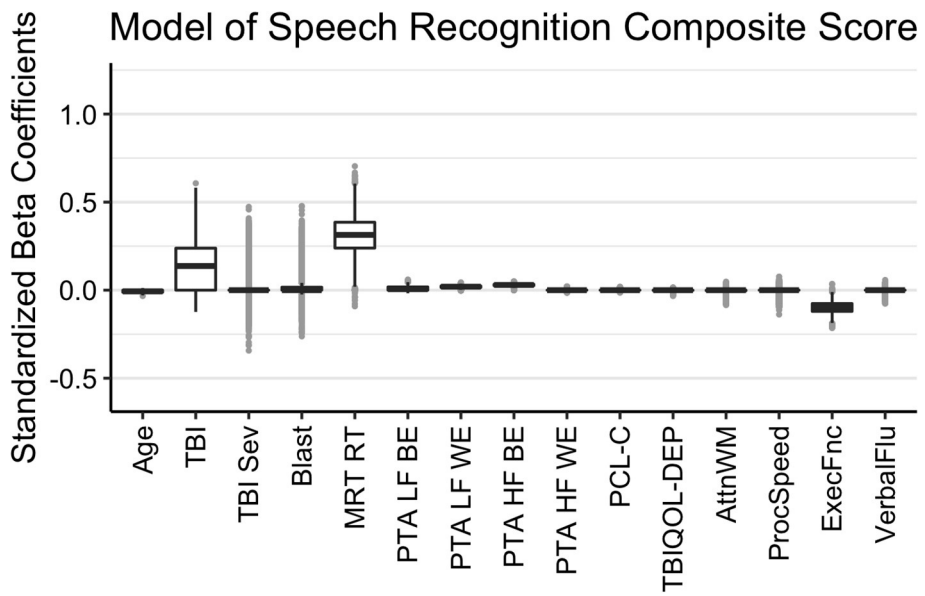

FIGURE 1 | Regression coefficients for the Speech Recognition Composite model. Median intercept (mu) = −0.10 (Q1 = −0.47, Q3 = 0.27).

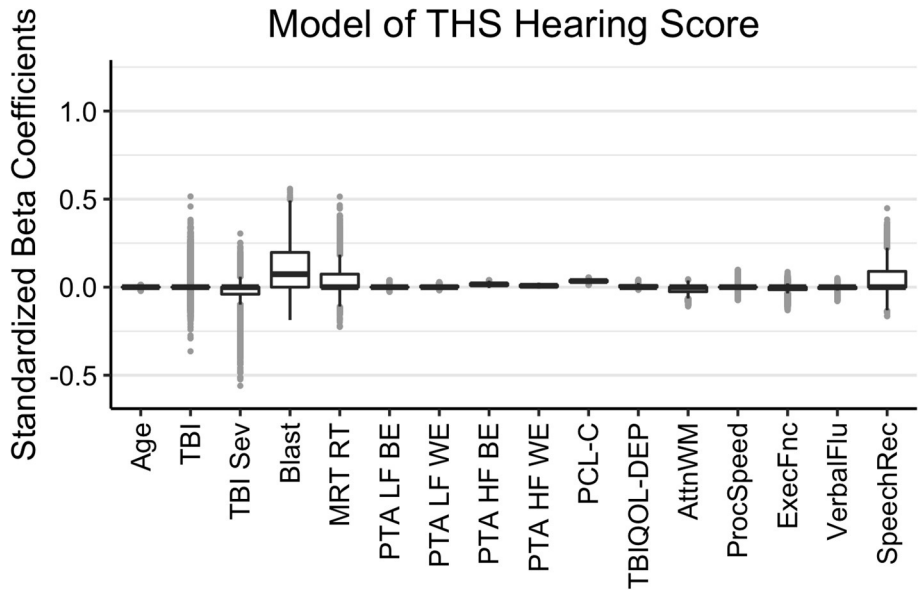

FIGURE 2 | Regression coefficients for self-reported difficulties with hearing (THS Hearing). Median intercept (mu) = −1.53 (Q1 = −1.86, Q3 = −1.21).

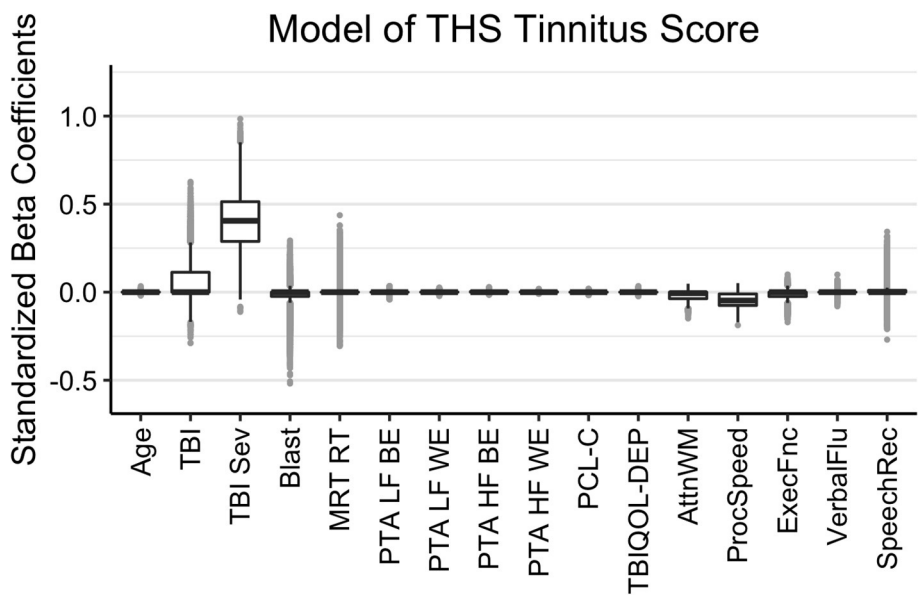

FIGURE 3 | Regression coefficients for self-reported difficulties with tinnitus (THS Tinnitus). Median intercept (mu) = 0.50 (Q1 = 0.14, Q3 = 0.84).

MRT RT and executive function also predicted objective speech-in-noise recognition. These results align with previous findings that cognitive factors influence performance on speech-in-noise tests [e.g., (43, 44)]. They also extend the results of small-scale studies of civilians with and without TBI (each total $n < 35$) that observed associations between auditory and cognitive function in assessing speech-in-noise abilities (13, 18).

Surprisingly, an increase, rather than a decrease, in speech-in-noise performance was predicted with aging. This was one of the weaker effects in the model and likely reflects an offset related to another predictor that naturally increases with age, like hearing thresholds. This interpretation is supported by a weak, positive correlation between age and speech composite score [$r_{(210)} = 0.14$, $p = 0.04$] in the overall data when other factors are not partialed out.

In contrast to objective recognition, subjective hearing difficulties were predominantly predicted by thresholds in the better ear at high and low frequencies. Notably, the actual

performance level on the objective speech-in-noise tests was not a significant predictor. Responses to THS Hearing questions may have been more likely to be influenced by the audibility of soft sounds than by speech-in-noise understanding. Objective speech-in-noise tests in this study were generally presented at a high enough level to ensure audibility, which could explain why low frequency thresholds in the worse ear contributed more to the objective than the subjective speech measure. Additionally, symptoms of PTSD were related to subjective hearing problems. However, even when including these factors, self-reported blast exposure was still related to hearing complaints. This follows from the finding that mTBIs caused by blast exposure are associated with more self-reported hearing difficulty than those not caused by blast (22). Because blast exposure often accompanies acoustic noise exposure, more detailed blast and noise exposure history information will be needed to better understand this link.

In addition to greater TBI severity, subjective tinnitus complaints were also associated with measures of processing speed and attention/working memory. This finding aligns with research suggesting that bothersome tinnitus coincides with deficits in attention and executive functions (52). However, few factors predicted tinnitus problems in this sample, and the model explained a small amount of variance.

The results of this study highlight that standard audiometric measures may be insufficient to characterize the hearing-related problems of SMVs with a history of TBI or blast exposure. Although the mechanisms are unclear, these data suggest that SMVs with blast exposure or TBI suffer from hearing deficits that go beyond what would be expected from increased hearing thresholds, elevated PTSD and depressive symptoms, and degraded cognitive function that might result from their injuries. Indeed, there was an observable relationship between hearing difficulties and TBI or blast history even when these other factors were included in the models.

One takeaway from this data is that objective speech-in-noise performance, subjective hearing and tinnitus were associated with different injury mechanisms. Objective speech-in-noise performance was best predicted by the presence of any TBI. Subjective hearing was best predicted by a self-reported history of blast exposure and tinnitus was best predicted by the presence of severe TBI. Military audiologists have anecdotally noted that blast-exposed patients tend to report hearing problems that are difficult to validate with clinical tests of speech-in-noise recognition. These data are consistent with those anecdotal observations. However, it is not clear whether increased patient complaints reflect a true performance deficit that was not detected by our speech-in-noise tests or whether it reflects a tendency for blast-exposed listeners to experience greater listening effort even when they achieve the same level of objective performance.

This preliminary, cross-sectional analysis has focused on identifying broad factors that might differentiate the objective hearing performance and subjective hearing complaints of SMVs with TBI from those of SMVs who have not suffered head injuries. As data collection progresses, we hope to be able to identify more specific tests or combinations of tests that might be sensitive to the unique hearing pathologies that exist in this population. The longitudinal nature of the study will also make it possible to track how these hearing problems progress over time and the extent to which they might contribute to the overall quality of life experienced by SMVs with TBI. Perhaps most importantly, we hope to be able to conduct more nuanced analyses to help identify the specific exposures or injury mechanisms that might be responsible for the excess hearing difficulties attributed to blast exposure or TBI in this sample. In the short term, however, these results may serve to highlight the importance of including audiological measures beyond the pure-tone audiogram in studies evaluating chronic effects of TBI. Including cognitive and symptomological assessments may help to better characterize and ultimately better remediate these deficits. However, more work is needed to fully account for the challenges that SMVs with a history of TBI or blast exposure face.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because Department of Defense policy prohibits sharing of sensitive data for this DoD-funded research. Requests to access the datasets should be directed to Stefanie E. Kuchinsky, stefanie.e.kuchinsky.civ@mail.mil.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at Walter Reed National Military Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RL, LF, and TB designed and directed the larger longitudinal project. RL, LF, TB, SL, DB, and ME contributed to the overarching design of this study. DB, SK, and ME conceptualized the specific research question. SK conceptualized and implemented the analysis plan. SK, DB, and ME interpreted the results and wrote the initial draft of the paper. All authors contributed to the article and approved the submitted version.

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Glial Activation in the Thalamus Contributes to Vestibulomotor Deficits Following Blast-Induced Neurotrauma

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Vestibular impairment has become a frequent consequence following blast-related traumatic brain injury (bTBI) in military personnel and Veterans. Behavioral outcomes such as depression, fear and anxiety are also common comorbidities of bTBI. To accelerate pre-clinical research and therapy developments, there is a need to study the link between behavioral patterns and neuropathology. The transmission of neurosensory information often involves a pathway from the cerebral cortex to the thalamus, and the thalamus serves crucial integrative functions within vestibular processing. Pathways from the thalamus also connect with the amygdala, suggesting thalamic and amygdalar contributions to anxiolytic behavior. Here we used behavioral assays and immunohistochemistry to determine the sub-acute and early chronic effects of repeated blast exposure on the thalamic and amygdala nuclei. Behavioral results indicated vestibulomotor deficits at 1 and 3 weeks following repeated blast events. Anxiety-like behavior assessments depicted trending increases in the blast group. Astrogliosis and microglia activation were observed upon post-mortem pathological examination in the thalamic region, along with a limited glia response in the amygdala at 4 weeks. These findings are consistent with a diffuse glia response associated with bTBI and support the premise that dysfunction within the thalamic nuclei following repeated blast exposures contribute to vestibulomotor impairment.

Keywords: thalamus, amygdala, blast, vestibulomotor, microglia, astrocytes, traumatic brain injury

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INTRODUCTION

Neurosensory deficits such as vestibular impairment are a frequent outcome following traumatic brain injury (TBI) and if not treated can lead to long-term disability (1). In military populations, more than 25% of the Veterans are suffering from closed head injuries due to blast exposures during combat (2, 3). Transference of blast wave energy into the brain results in neurological deficits leading to the diagnosis of blast-related traumatic brain injury (bTBI). There is a growing concern that there may be detrimental effects within the brain following multiple low- to medium-level blast exposures during military training and combat. The neurosensory sequelae following blast exposure can include auditory, sleep, vestibular and visual impairments (4–7). These neurosensory conditions have become increasingly prevalent in military personnel exposed to blast events

and are a common comorbidity of bTBI. Acute vestibulomotor deficits have been noted in 98% of patients diagnosed with bTBI and 72% of these patients report long-term vestibular impairment (8, 9). Clinical manifestations of vestibular damage includes motor impairments leading to imbalance, motion intolerance, postural instability and dizziness (1, 10). In most cases, there are also significant behavioral concerns that overlap with blast-related impairments such as anxiety, attention, fear, memory, and problem-solving deficits (11–13). Due to their unique combat experiences, Veterans face many long-term health challenges that result from the blast trauma including neurosensory deficits.

The transmission of neurosensory information often involves a pathway from the cerebral cortex to the thalamus (14, 15). This indicates that the thalamus serves crucial integrative functions within vestibular processing. Peripheral vestibular stimulation has been shown to cause strong activation within the thalamus, with the ventrolateral nuclei (VL), laterodorsal nuclei (LD), and central medial nuclei (CM) of the thalamus receiving inputs from the bilateral superior vestibular nucleus (SuVN) and the contralateral medial vestibular nucleus (MVN). The VL, LD, and CM project to the primary motor and premotor cortices suggesting that the circuitry between these nuclei represents a major vestibulomotor pathway. A study in rats showed that lesions in the LD impair spatial learning and memory, suggesting that the LD is part of novel processing involved in spatial orientation and learning to sensory cues (16). Since the CM receives inputs from various vestibular nuclei, lesions in the CM have been linked to impairments in working memory and motor control (17).

The influence of vestibular stimulation on behavior can be mediated through the projections of nuclei from the vestibular system, through the thalamus, into amygdala cells (18). The amygdala is known to integrate and process information pertinent to reward and emotions such as fear and anxiety (19). Specifically, the basolateral amygdaloid complex (BLA) integrates information regarding fear and anxiety-inducing stimuli, regulating emotional and behavioral responses (20). Furthermore, the BLA receives sensory information, such as vestibular outputs, through axons networking through the superior and lateral vestibular nuclei, which then project through the thalamic nuclei to the BLA (21). Evidence linking pathological dysfunction of the amygdala and thalamus to sensory impairments is important to further understanding the mechanisms associated with bTBI.

Studies have demonstrated lesions within the vestibular nuclei, the thalamic nuclei, and the BLA correlate to vestibulomotor and stress induced deficits (22–24). However, an understanding of how the glial cells (astrocytes and microglia) contribute to the morbidities in these specific brain regions is lacking. Astrocytes are the most numerous cells in the human central nervous system (CNS) and carry out many homeostatic functions crucial for normal brain function. Astrocytes associated with injured tissue often termed reactive or gliotic astrocytes, are characterized by profound changes in protein and gene expression leading to hypertrophy, increased expression of intermediate filaments [glial-fibrillary acidic protein (GFAP), nestin and vimentin] and increased proliferation (25, 26). These changes ultimately result in homeostatic deficits, including dysregulation of critical ions and neurotransmitter uptake capacities, contributing to neuropathology. Reactive astrocytes are also characterized by a combination of structural and functional changes, which include thickening and retraction of primary, secondary and tertiary processes (27, 28). It is these fine processes in a healthy brain that are intimately associated with over 90% of functional synapses in the CNS (29). Accumulating evidence from preclinical and clinical studies suggest reactive astrocytes contribute to the TBI sequelae (30, 31). Microglia compose of approximately 10% of the total glia of the brain and function as the innate immune system in the CNS (32). They are the first line of defense, playing a critical role in neuroinflammation following injury. Microglia become activated adapting both pro- and anti-inflammatory phenotypes which produce high levels of cytokines and oxidative metabolites that are important in phagocytic activity that eliminate extracellular debris, apoptotic cells, and increases tissue remodeling (33). Additionally, it has been hypothesized that a larger number of pro and anti-inflammatory microglia would be located around traumatic lesions, and take on morphological changes in order to respond to these lesions (34, 35). Microglia are known to convert from a "healthy" ramified shape to a reactive hypertrophic, "bushy" morphology, or become "rod-like," with thin somas and polarized processes aligning adjacent to neuronal processes (36, 37). Activated microglia have also been associated with an amoeboid morphology that further aids in phagocytic properties, which either lead to cumulative neuronal loss, or promote neuroplasticity, and axonal regeneration (38).

There is significant clinical and preclinical support for the premise that blast exposure leads to neuroinflammation. Gill et al. reported finding elevated serum levels of IL-6 and TNF-alpha acutely in a population of military personnel exposed to a blast insult (39). A report by Rusiecki et al. measured serum levels of pro- and anti-inflammatory cytokines pre- and post-deployment of those who had been diagnosed with mild and moderate bTBI (40). They found chronic changes in several inflammatory markers (MMP3, IL-1α, IL-4, IL-6, and IL-8) indicating a long-term response to blast exposure. Preclinical studies not only show elevated levels of cytokines but extend to histological measures of neuroinflammation and reactive gliosis within animals exposed to blast events (41–46). Collectively, these studies depict a significant contribution of neuroinflammation to the enduring complications of bTBI. Identifying the mechanisms that contribute to the pathological changes in the brain that link to these symptoms remains complicated. Limited attention has been given to vestibular injuries associated with bTBI. Blast waves are known to cause inner ear damage but recent debates question whether the injury is more widespread, and whether various brain regions are being affected by bTBI contributing to neurosensation deficits (47, 48). Arun et al. found significant neuromotor impairments occurring up to 6 months following repeated blast exposures in rats (49). As neurobehavioral deficits are being presented following blast exposure, identifying and studying the pathological changes that contribute to these shortfalls is

imperative. We aimed to characterize neuropathological changes within the thalamus and amygdala following repeated blast exposures, thus providing more data to assist the mechanistic understanding of the vestibular impairment that presents clinically following a blast injury.

MATERIALS/METHODS

Animals and Blast Exposure

The study described herein was carried out in accordance with experimental protocols approved by the University Institutional Animal Care and Use Committee at Virginia Tech. Prior to any experimentation, male Sprague Dawley rats (Envigo, Dublin, VA, USA) weighing approximately 250–300 g were acclimated for several days (12 h light/dark cycle) with food and water provided *ad libitum*.

The blast wave was generated using a custom Advanced Blast Simulator (ABS) (200 cm × 30.48 cm × 30.48 cm) located at the Center for Injury Biomechanics at Virginia Tech University. The ABS consisted of three distinct sections to create, develop, and dissipate the blast wave (**Figure 1**). The blast wave developed following a helium-driven rupture of calibrated acetate membranes. The passive end-wave eliminator was located downstream of the test location to facilitate the dissipation of the blast wave through a series of baffles. As a result, the test location was exposed to a single peak overpressure representing a free-field blast exposure. Pressure measurements were collected at 250 kHz using a Dash 8HF data acquisition system (Astro-Med, Inc., West Warwick, RI, USA). Analysis of pressure profiles was conducted using a custom MATLAB script to calculate impulse and duration of the positive and negative phases and rise time. Peak overpressure was determined using the Rankine—Hugoniot relations and observed wave speed at the animal test location within the ABS.

Prior to blast exposure, animals were anesthetized with 3% isoflurane and placed in the ABS. Each animal was supported in the prone position inside the ABS facing the oncoming shock front using a mesh sling. The sling was designed to minimize flow hindrance and isolate primary blast injury by eliminating acceleration/deceleration injuries. Animals were exposed to three blasts (16.62 psi ± 2.27 psi) separated by 1 h each (3 × 1 h) ($n = 10$). There was also a sham group ($n = 10$) that received all the same procedures with the exception of blast exposure. Following the sham or blast procedures, animals were observed through the recovery stages of injury and anesthesia.

BEHAVIORAL ASSESSMENTS

Accelerating Rotor Rod Task (RR)

Sensorimotor coordination and motor learning post-blasts were assessed using the RR (San Diego Instruments, San Diego, CA). Pre-training sessions were completed before blast exposure to ensure that animals were able to adequately perform the task and that all motor deficits would solely be due to bTBI and subsequent injury progression. In the pre-training sessions, the animals were taught to stand on the stationary rod. Once this was achieved, the rod was turned on so the animals would learn to walk at constant speeds between 3 and 21 revolutions per minute (RPM). The animals were placed back on the rod upon falling. The animals were also introduced to the accelerating protocol and a baseline reading was obtained. During testing, the animals were placed on the RR which accelerated 3 RPM every 12 s, starting at 3 RPM and finishing at 30 RPM. The maximum amount of time allotted for each trial was 120 s. Latency to fall, total distance traveled, and maximum RPM was recorded for each trial using the manufacturer's software. The RR task was performed 1 and 3 weeks following blast exposure, with the animal performing the task for a total of three trials at each time point.

Open Field Test (OFT)

Blast induced anxiety-like behavior was measured using the OFT. The animal was placed in an arena (80 cm^2) in a low-light room. The animal was allowed to explore the arena for 5 min. The investigator was not present inside the room at any point throughout the trial. Three-point tracking was performed using EthoVision XT and included tracking of the tip of the nose, center of the body, and base of the tail (Noldus Information Technology, Leesburg, VA, USA). Each trial was recorded at 30 frames per second and proper tracking was confirmed by an investigator blind to treatments. Anxiety is measured as thigmotaxic behavior within the OF environment (50). Therefore, the fraction of time spent along the walls of the arena was calculated and used to represent anxiolytic behavior. Locomotor function was also measured in the open field arena by measuring the total distance traveled. The OF test was administered prior to blast exposure then biweekly following blast exposure for the 1 month study.

IMMUNOHISTOCHEMISTRY (IHC)

Four weeks following blast exposure, animals were euthanized by transcardial perfusion of saline and 4% paraformaldehyde. Following perfusion, brains were collected and stored in 4% paraformaldehyde fixative solution. After 24 h in the fixative, whole brains were cryoprotected in a 30% sucrose solution for tissue sectioning preparation. Once whole brains were completely submerged in the sucrose solution (∼48 h), tissues were then embedded in Tissue-Tek optimal cutting temperature (OCT) embedding medium (Sakura Finetek USA, Inc., Torrance CA) and frozen at −80° for cryostat processing. Brains were sectioned at 40 μm in the coronal plane and sections including the CM, LD, VL, and BLA were chosen (∼-3.00 mm posterior from Bregma), with two random sections placed per slide for staining. Samples were rinsed three times with phosphate-buffered saline (PBS) and were permeabilized in PBS with 0.3% Triton (PBX) for 30 min at room temperature. The samples were then incubated in 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Sections were incubated for 16–18 h at 4°C with primary antibodies glial fibrillary acidic protein (GFAP. 1:500; Invitrogen, Carlsbad, California), and ionized calcium-binding adaptor molecule 1 (IBA-1, 1:300; Biocare Medical, Concord, California). The following day, sections were washed three times for 5 min in PBX and incubated for 1.5 h at room temperature with secondary antibodies (Alexa Fluor 555 anti-rabbit IgG antibody and Alexa Fluor 488 anti-mouse IgG

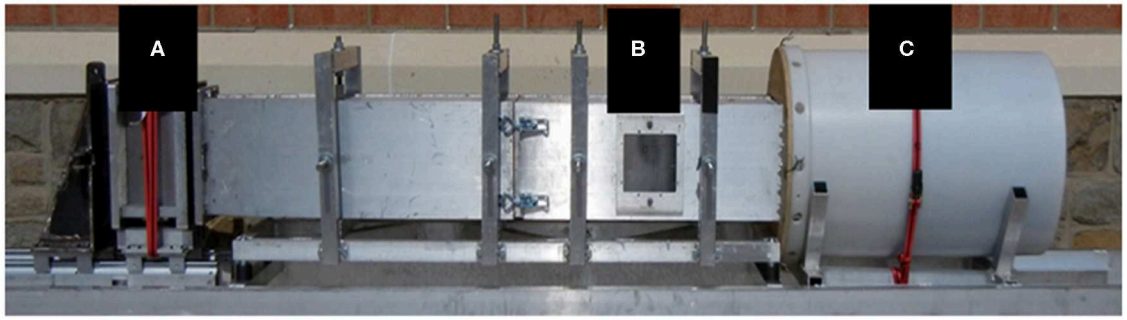

FIGURE 1 | The ABS was used to re-create free field blast exposures. Acetate membranes are passively ruptured following pressurization using helium gas in the driver section **(A)**. The blast wave reaches the animal located in the test section **(B)**, and is dissipated in the end-wave eliminator **(C)**.

antibody; Invitrogen, Carlsbad, California). After three more 5 min PBX washes, samples were mounted and cover slipped with Slow Fade Reagent with DAPI (Invitrogen, Carlsbad, CA). Sections were then imaged under a Zeiss fluorescence microscope at 20X magnification.

To provide a comprehensive analysis of the glial pathology, we quantified four specific parameters using ImageJ software; area fraction, count per area, integrated density of fluorescence and mean area per cell. Area fraction quantifies the percentage of positive signal within the region of interest. Count per area represents the total number of positive cells divided by the area. Integrated density of fluorescence measures the level of fluorescence intensity in the positive signal using gray pixel intensity. Mean area per cell provides detail to the average cell soma size normalized to the area, giving the average area of the cell. Count per area and mean area per cell were completed by using the "analyze particles" function with a pixel area size threshold of 0.004 to exclude small pixel noise and extract objects of interest. Mean brain region values were derived from a minimum of four images for each animal per stain.

STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA). Statistical differences between groups were assessed by the student's *t*-test. Statistical differences between groups and multiple time points were assessed by two-way ANOVA with repeated measures applying *post-hoc* tests where appropriate. Further analysis of significance and variability was done by calculating the effect size between treatment groups (ω^2). The Shapiro-Wilk test and Levene's test were used to verify assumptions of normality and equality of variances, respectively. Data that did not pass normality or equal variance assumptions were assessed using either Welch's correction *t*-test or Mann-Whitney's non-parametric test. Data were considered statistically significant with $p < 0.05$ and trending at $p < 0.1$. All histology data was normalized to respective shams. All data is represented as the mean ± standard error of the mean, or SEM.

TABLE 1 | Summary of blast wave characteristics.

Treatment	Peak pressure (psi)	Duration (ms)	Impulse (psi*ms)	Rise time (ms)
3 × 1 h blast	16.62 ± 2.27	2.25 ± 0.10	12.06 ± 2.64	0.049 ± 0.036

Sprague Dawley rats (male, 250–300 g) were anesthetized and exposed to three blast insults separated by 1 h each. Sham animals underwent all procedures with the exception of the blast insult. The average peak pressure resulted in a blast wave magnitude of ~17 psi which induces a mild TBI in rodents. Results are represented as Mean ± SEM.

RESULTS

Blast Event and Animal Recovery

Blast Animals ($n = 10$) were exposed to three blast events 1 h apart. Blast wave parameters are described in **Table 1**. Sham animals ($n = 10$) were exposed to all procedures with the exception of the blast exposures. Following exposures, no obvious external signs of injury were discernible. Over the 4 week period, there was no significant difference in the weights observed in the blast group when compared to the sham group. The average weight of the repeated bTBI animals was 361.8 g ± 10.0, while the sham group average was 349.9 g ± 15.7. The percentage of weight gain for the blast group was 1.24% when normalized to shams.

Blast-Induced Vestibulomotor Deficits

The accelerating RR task is an established test that is an effective measure of motor function and balance impairments in rodents (51–53). Animals exhibiting motor deficits show a decrease in time on the RR beam (latency to fall), decreased distance traveled, and decrease maximum RPM on the beam compared to their sham counterparts. Results of the repeated blast exposures on RR performance are shown in **Figure 2**. From the repeated measures ANOVA, the blast injury had a significant effect on RR performance at both 1 and 3 weeks. Repeated bTBI animals showed a significant decrease in latency to fall ($p < 0.05$) compared to the shams, with an effect size of 0.11 (**Figure 2A**). Additionally, the repeated bTBI group showed a significant decrease in distance traveled and maximum RPM at both time points (**Figures 2B,C**), with the effect size for both parameters also found to be 0.11. There was no interaction between time and

blast for latency to fall, distance traveled, and maximum RPM on the RR. Time itself also did not have a significant effect on either parameter measured for RR.

Anxiety-Like Behaviors

The effect of multiple blast exposures was assessed using OFT at 1 and 3 weeks (**Figure 3**). Statistical analysis of the effects of repeated blast exposures on OFT performance depicted that time alone had a significant effect on the time spent exploring the center for blast and sham animals. Statistical analysis of the effects of repeated blast exposures on OFT performance depicted that time alone had a significant effect on the time spent exploring the center for blast and sham animals. No significance was observed in the interaction between time and blast (**Figure 3A**). A significant increase in the maximum velocity of blast animals was found at 3 weeks compared to shams. Even though this was observed, the overall treatment effect (blast) was not found to be significant. Time was also not found to be significant for maximum velocity (**Figure 3B**). No significance in interaction between time and blast was observed for total distance traveled, and time and blast alone was not found to be significant (**Figure 3C**). A summary of all behavioral analyses is found in **Table 2**.

Immunohistochemistry (IHC)

Elevated Levels of Microglia Found Within the Thalamus

To identify areas of potential molecular mechanisms responsible for the observed vestibulomotor deficits, we examined the level of IBA-1 in three regions of the thalamus; CM, LD, and VL. IBA-1 is a common marker for microglia as it is involved in phagocytosis and actin reorganization in microglia. It is constitutively expressed in microglia and is elevated when microglia are activated in injuries such as blast (54–56). We therefore performed IHC and compared levels of IBA-1 in both blast and sham brains (**Figure 4A**). There was a significant increase ($p < 0.05$) in the bTBI compared to sham groups. Specifically, the integrated density of fluorescence was significantly increased in the CM region of the thalamus in blast animals in comparison to shams (**Figure 4B**). IBA-1 expression was also measured through the percentage of the positive signal within a given area (area fraction). There was a significant increase in area fraction of IBA-1 within the VL region of the thalamus for blast animals, with a trending increase in the LD ($p = 0.0908$) for blast animals compared to shams (**Figure 4C**). Quantification of averaged cell somas (mean area per cell) showed a trending increase in the CM in blast animals compared to the sham, with no significant differences found in the LD and the VL regions. There were no significant differences in the CM or VL regions of the thalamus between blast and shams in the number of cells per area of interest (count per area), with a trending increase in count per area in the LD region in blast animals compared to shams. The significant increase in IBA-1 expression between treatment groups suggests a compromised thalamus following repeated blast exposure.

Since the amygdala is associated with the sensorimotor complex, levels of IBA-1 in the BLA were measured. We found trending increases of IBA-1 in the integrated density of fluorescence in blast animals compared to sham ($p = 0.0568$). Increasing trends of IBA-1 were also observed in the area fraction

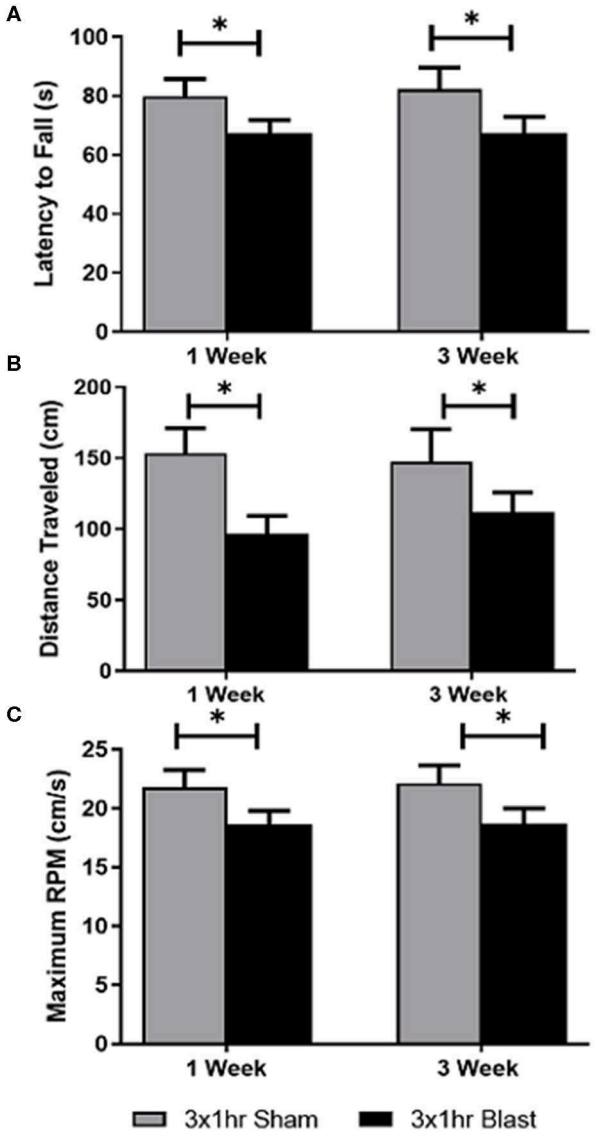

FIGURE 2 | Vestibulomotor function was impaired following blast exposure. Using the accelerating rotor rod task; latency to fall, total distance traveled, and maximum RPM were quantified at 1 and 3 weeks following repeated blast exposure. A two-way ANOVA with repeated measures indicated significant differences in the three parameters. **(A)** A significant decrease in latency to fall in blast animals compared to shams was observed. No significance was found in the interaction between time and blast, or in time itself. The blast animals spent less time on the beam before falling, suggesting vestibulomotor impairment following blast injury. **(B)** A significant decrease in the distance traveled in blast animals compared to shams was also observed. No significance in the interaction between time and treatment, or in time itself was found. These decreases suggests motor impairment due to postural instability, dizziness, or balance issues. **(C)** Blast animals operated on the accelerating rotor rod at a slower RPM than sham animals. These findings were found to be significant. No significance in the interaction between time and blast or in time itself was found. *$p < 0.05$. Mean ± SEM.

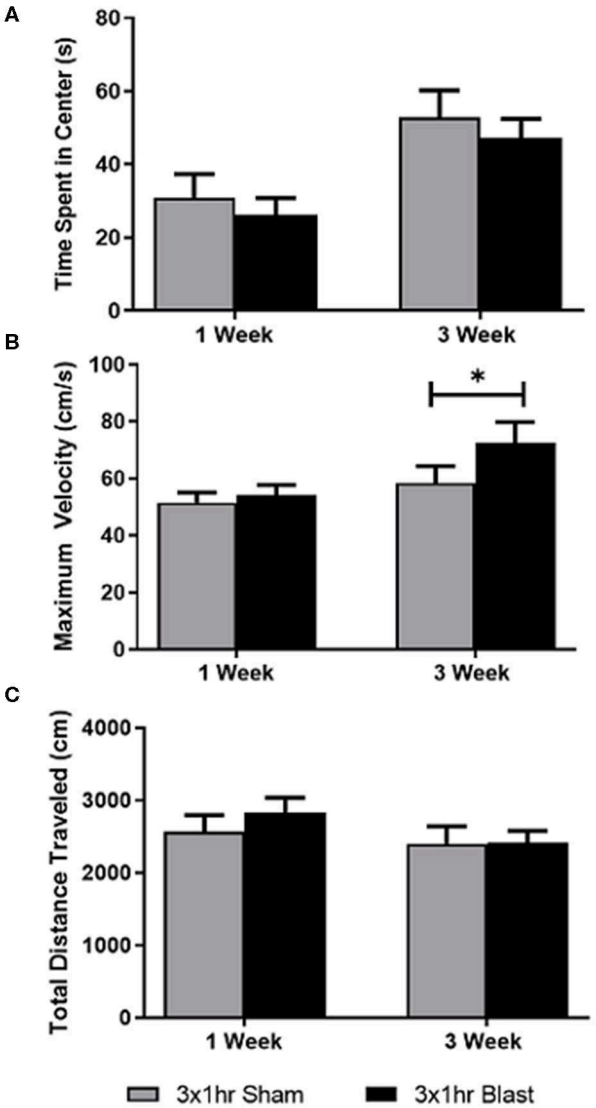

FIGURE 3 | Testing anxiety-like behaviors following repeated bTBI. Using OFT, time spent in the center, maximum velocity, and total distance traveled were quantified at 1 and 3 weeks following injury. A two-way ANOVA with repeated measures was used to assess differences in the three parameters. **(A)** Time itself had a significant effect on the time spent in the center of the arena. No significance was found in the interaction between time and blast, or in treatment itself. **(B)** Analysis of maximum velocity indicated a significant difference between blast and sham groups at 3 weeks, but overall, blast and time alone did not reach significance. No significant interaction between time and blast was observed. **(C)** Significance in the interaction between time and blast was also not observed for total distance traveled. Significance in time and blast itself was also not found. $^*p < 0.05$. Mean ± SEM.

($p = 0.0891$) and mean area per cell ($p = 0.0739$) in blast animals. A summary of all IBA-1 analyses is found in **Table 3**.

Astrocyte Reactivity Was Elevated Within the Thalamus

To determine if the observed behavioral deficits are associated with astrocyte reactivity, we performed IHC to quantify the levels of GFAP in three regions of the thalamus; CM, LD, and VL (**Figure 5**). GFAP is an extensively studied biomarker of brain injury and has been commonly observed to be elevated in the brain following blast exposure. In rats exposed to repeated blast injury, there was prominent astrogliosis within the thalamus (**Figure 6A**). Astrocyte reactivity (area per cell) within the CM region was significantly increased ($p < 0.05$) due to repeated bTBI, with a trending increase in the LD region ($p = 0.079$). However, there were no significant differences observed in the VL region (**Figure 6B**). The increased soma of the astrocytes suggests changes in size (hypertrophy) taking place due to reactivity in response to the injured region. There were trending increases in the amount of GFAP signal (area fraction) and the count per area in the CM region of blast animals, however, there were no significant trends in integrated density in either regions of the thalamus. When examining the level of GFAP within the BLA, we found no significant differences in either of the parameters measured for GFAP, indicating limited astrocyte reactivity within the BLA at 1 month following injury. A summary of all GFAP analyses is found in **Table 3**.

DISCUSSION

In this study, we found vestibulomotor deficits in an early chronic phase of blast TBI. We showed that animals subjected to three repetitive blast events with an inter-blast interval of 1 h displayed decreased ability to complete the rotor rod tasks. These impairments were associated with glial pathology within the thalamus, with trends toward microglia activation taking place in the amygdala. Results from the study indicate that the blast exposures lead to long-term consequences that resemble those reported in Veterans with bTBI.

Few preclinical studies have reported on vestibular disorders following bTBI. Lien and Dickman investigated vestibular injury following low intensity blast exposure using the rotor rod behavioral task (10). They found a significant reduction in the ability of the animals to perform the balance task on the rotating rod for several weeks following blast exposure. In our current study, at 1 and 3 weeks following injury, blast animals showed significant decreases in RR tasks in comparison to sham animals, suggesting vestibular injury. Subsequently, there were pathological changes in the thalamus that may be linked to the vestibulomotor deficits seen in this behavior task. Elevated levels of IBA-1 in the thalamic nuclei suggests that there is an increase in the inflammatory response that aids in eliminating cell debris and the tissue repair process. Astrogliosis was also elevated in the thalamic nuclei. More specifically, the mean area per cell was increased in blast compared to sham animals, a sign of hypertrophy. As hypertrophy of astrocyte cell bodies and processes have been associated with reactive astrogliosis following blast exposure (57), this may indicate that these sequelae are taking place in the thalamus of blast-injured animals.

Limited studies on glial pathology within the thalamus following blast injury have been reported. A study by Studlack et al. focused on linking thalamic sensitization to headache and pain following blast injury (8). Their investigation included

TABLE 2 | Vestibulomotor deficits persist in repeated blast animals, while anxiety-like behavior is not detectable 1 and 3 weeks following injury.

Accelerating rotor rod

	Latency to fall (s)		Distance traveled (cm)		Maximum RPM	
	One week	Three weeks	One week	Three weeks	One week	Three weeks
Sham	79.93 ± 5.87	82.40 ± 7.12	151. 7 ± 18.63	147.4 ± 23.09	21. 80 ± 1.47	22.11 ± 1.57
Blast	67.26 ± 4.63*	67.40 ± 5.54*	111.2 ± 12.59*	111.9 ± 14.03*	18.64 ± 1.17*	18.73 ± 1.29*

Open field thigomotaxis

	Time spent in center (s)		Distance traveled (cm)		Maximum velocity (cm/s)	
Sham	26.64 ± 5.23	56.88 ± 6.93	2570 ± 225	2399 ± 243	51.58 ± 3.51	53.63 ± 3.90
Blast	26.16 ± 4.69	47.30 ± 5.13	2829 ± 210	2410 ± 167	54.11 ± 3.67	64.48 ± 6.19*

Accelerating rotor rod

Latency to fall (s)		Distance traveled (cm)		Maximum RPM	
Source	***p*-value**	**Source**	***p*-value**	**Source**	***p*-value**
Time × Blast	0.8408	Time x Blast	0.8408	Time x Blast	0.9369
Time	0.8738	Time	0.7674	Time	0.884
Blast	0.0242	Blast	0.0223	Blast	0.0219

Open field thigomotaxis

Time spent in center (s)		Distance traveled (cm)		Maximum velocity	
Time × Blast	0.3651	Time x Blast	0.5595	Time x Blast	0.3464
Time	<0.0001	Time	0.1716	Time	0.163
Blast	0.4125	Blast	0.527	Blast	0.134

*Vestibulomotor impairment and anxiety-like behaviors were measured using RR and OFT at 1 and 3 weeks following repeated blast exposure. The results for the RR task were expressed as latency to fall, distance traveled, and maximum RPM. OFT data were expressed as time spent in the center, distance traveled, and maximum velocity. A two-way ANOVA with repeated measures for rotor rod indicated significance in treatment at 1 and 3 weeks, with no significance in the interaction between time and treatment or in time itself. OFT showed that time had a significant effect on time spent in the center at 1 and 3 weeks, with no significance in the interaction between time and treatment, or in treatment individually. *$p < 0.05$, mean ± SEM.*

characterizing the astrocytic response within the VPM and the posterior thalamus (PO) which are associated with pain transmission. Brains examined 9 weeks following blast injury did not show elevated levels of astrocyte expression or reactive microglia in the PO or VPM. Further, no significant changes in gliosis within the PO was reported by a subsequent study (58). For both of these studies, the blast device/methods, behavioral assessments and timing of histological observation differed from our study, thus the results are difficult to compare.

Perez-Polo et al. characterized a rodent blast model using behavioral and neuropathological assays that included the thalamus and amygdala (59). They found that motor coordination (via beam-balance and foot-fault assays) was impaired following blast exposure. They reported elevated IBA-1 labeling in the thalamus as early as 6 h and lasting through 30 days following injury. Similarly, our group previously demonstrated increased astrocyte and microglia labeling in other brain regions such as the hippocampus up to 3 months following blast neurotrauma (43, 60). The results from this study suggest that glial activation in the rat thalamus may be similar to the gliosis occurring throughout the brain and likely influences the functional outcomes from this diffuse injury. Sajja et al. investigated amygdalar vulnerability following a single blast exposure (43). They found significant anxiety-like behaviors 1 week following a single bTBI that was associated with elevated levels of GFAP and IBA-1 within the amygdala. While the present study did not show significant anxiety-like behaviors 1 or 3 weeks following repeated blast injury, animals did present with trending increased levels of IBA-1 in the amygdala.

Although there is information identifying regional changes occurring within the brain, there is still a lack of understanding of how the affected areas work together to cause vestibular disorders. Injuries to the vestibular system can be related to pathology from the vestibular labyrinth of the inner ear to the transmission of the nerve impulses being carried by the vestibular nerve to many brain regions including the brain stem, thalamus, and cerebellum. Some fibers ascend into the vestibular area of the cerebral cortex after relaying information in the ventrolateral nuclei, laterodorsal nuclei, and central medial nuclei of the thalamus. The vestibular system itself contains many structures that are vulnerable to blast injuries (61). Damage to neuronal projections connecting the vestibular system with the thalamic and amygdala nuclei have been identified as contributing to vestibulomotor deficits seen as common

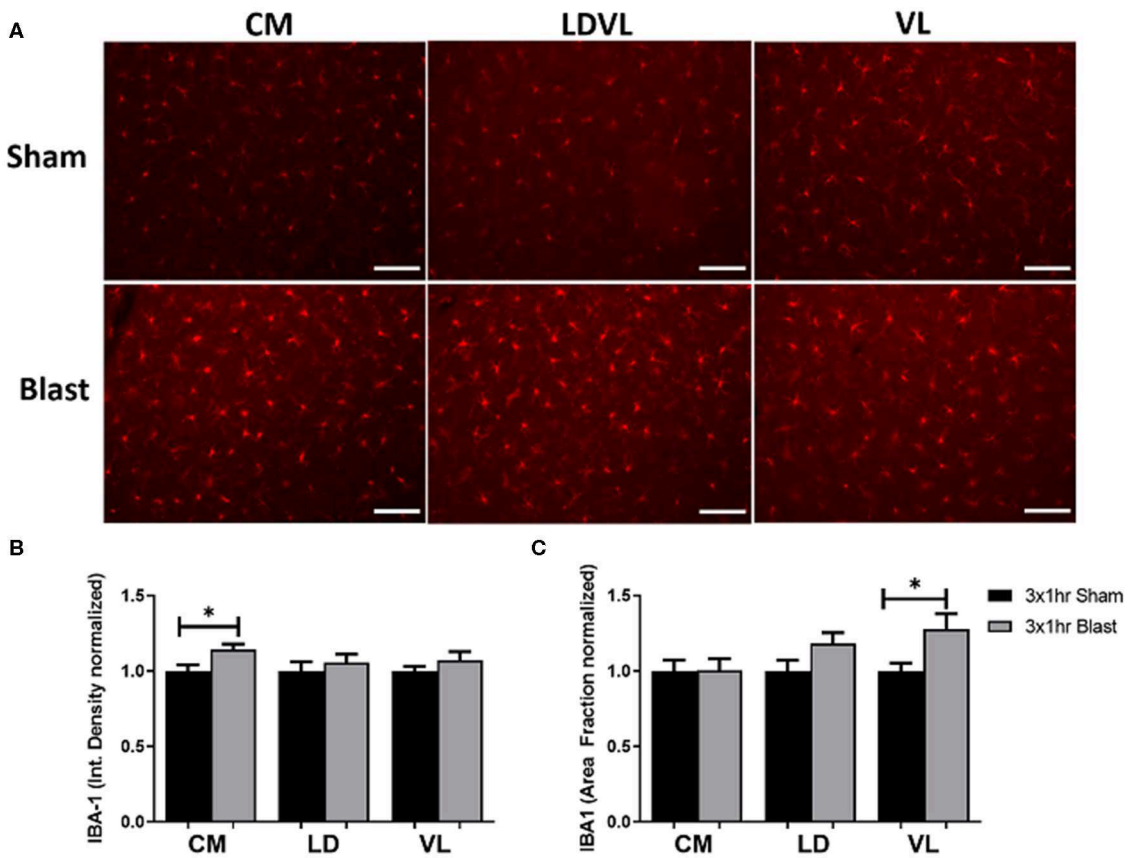

FIGURE 4 | Increased IBA-1 expression and elevated levels of microglia following repeated blast exposure. **(A)** Elevated staining intensities of IBA-1 were observed in the blast animals as compared to the respective sham. Magnification is at 20x and scale bar = 100 μm. **(B)** The level of IBA-1 expression was quantified via the integrated density of fluorescence. A significant increase of IBA-1 was found in the CM region of the thalamus of blast animals compared to shams. Higher intensity of fluorescence may indicate activation of microglia following injury as upregulation of IBA-1 is found in activated cells. **(C)** The area fraction that the IBA-1 signal occupied signifies the percent of positive signal within a given region. A significant increase in the area fraction of IBA-1 was found in the VL region of the thalamus in the blast animals. A trending increase was noted in the LD region of the blast animals (0.0908). Increased area fraction may indicate proliferation of microglia to the injured regions. $n = 10$ per group. Mean ± SEM, $^*p < 0.05$ and data was normalized to shams.

sequelae of TBI (8, 62–64). While neuronal damage has been implicated, the mechanism of how glial cells contribute to the long-term recovery of the vestibular system is unknown. Moreover, astrocytes and microglia play important roles in the recovery of injured tissues. Reactive astrocytes work to return the brain environment back to its healthy state by balancing homeostatic deficits and mitigating oxidative stress. Activation of microglia and subsequent inflammatory response takes place as microglia accumulate and work to remove the extracellular debris or apoptotic cells following injury. At all stages of repair, glia help regulate the inflammatory response through the release of pro- and anti-inflammatory cytokines following injury.

Glial responses have been identified at the acute phase of injury following bTBI, however there is still limited knowledge on the sub-acute and early chronic outcomes, and how they contribute to vestibulomotor and behavioral deficits. Previous studies report on acute changes (2–72 h) of glial dysfunction (elevated levels of reactive astrocytes and activated microglia) in brain regions such as the hippocampus, amygdala, and the pre-frontal cortex (57, 60, 65). Sub-acute (3–14 days) results have also provided insight regarding the dynamic glial response occurring in the brain following blast injury (45, 66). Due to the gap in data from the early chronic stage of repair (longer than 14 days), this study was conducted to provide more evidence regarding how neurosensory changes, primarily glial dysfunction in the thalamus and amygdala, are contributing to vestibulomotor and behavioral deficits.

Clinical studies have indicated that thalamic damage transpires following both blast and impact-related injuries and have an impact on motor and cognition impairments (67). Neurosensory outcomes such as vestibulomotor impairment have become increasingly common in military personnel, Veterans and civilians diagnosed with TBI (1, 61). However, those exposed to blast events appear to have a unique set of outcomes compared to those involved in impact-related injuries. Hoffer et al. conducted a clinical study to investigate this premise (68). They examined the vestibular-ocular and vestibular-spinal reflexes in two separate cohorts of mild TBI (mTBI) patients;

TABLE 3 | Increased levels of IBA-1 and GFAP expression can be found across thalamic regions.

	Mean area per cell		Count per area		Integrated density		Area fraction	
	Mean ± SEM	***p*-value**	**Mean ± SEM**	***p*-value**	**Mean ± SEM**	***p*-value**	**Mean ± SEM**	***p*-value**
GFAP								
CM Sham	7.941 ± 1.48	0.0239*	0.005 ± 0.001	0.1049	790147 ± 83864	0.3587	7.436 ± 1.36	0.0513
CM Blast	14.62 ± 2.265		0.011 ± 0.002		995887 ± 1998610		12.63 ± 1.91	
LD Sham	7.610 ± 0.57	0.079	0.059 ± 0.010	0.1708	560345 ± 44038	0.2614	11.83 ± 2.23	0.2734
LD Blast	9.649 ± 0.94		0.033 ± 0.010		648680 ± 60688		8.946 ± 1.25	
VL Sham	7.927 ± 1.09	0.2401	0.073 ± 0.020	0.6508	568916 ± 52308	0.627	11.58 ± 2.57	0.6727
VL Blast	9.96 ± 1.28		0.045 ± 0.014		611167 ± 67609		10.32 ± 1.45	
BLA Sham	58.73 ± 1.64	0.8305	0.0015 ± 0.001	0.5588	14557027 ± 986680	0.4879	8.879 ± 0.34	0.2491
BLA Blast	57.99 ± 3.40		0.0016 ± 0.001		13663037 ± 930213		8.608 ± 0.20	
IBA-1								
CM Sham	7.70 ± 0.33	0.0864	0.006 ± 0.002	0.3723	814061 ± 34270	0.0206*	4.41 ± 0.32	0.9794
CM Blast	8.74 ± 0.32		0.005 ± 0.002		930026 ± 30207		4.42 ± 0.35	
LD Sham	9.21 ± 1.13	0.8263	0.006 ± 0.001	0.0696	779684 ± 48406	0.5105	3.48 ± 0.25	0.0908
LD Blast	9.07 ± 0.48		0.005 ± 0.001		823351 ± 43806		4.116 ± 0.25	
VL Sham	8.81 ± 0.37	0.4537	0.005 ± 0.001	0.1444	951827 ± 30880	0.2977	3.96 ± 0.21	0.0283*
VL Blast	9.27 ± 0.47		0.006 ± 0.002		1020798 ± 54538		5.07 ± 0.39	
BLA Sham	129.4 ± 9.87	0.0739	0.004 ± 0.000	0.7859	937989216 ± 6193055	0.0568	13.67 ± 1.10	0.0891
BLA Blast	159.4 ± 12.84		0.004 ± 0.000		115647537 ± 85823062		18.27 ± 2.27	

*Several parameters were measured for IHC in order to quantify both astrogliosis and microglia activation. Regions of the brain that are listed include the CM, LD, and VL regions of the thalamus, and the BLA region of the amygdala. Astrogliosis can be detected in all regions of the thalamus, with no significant differences or trends found in the BLA region of the amygdala. Microglia activation was also indicated in all three regions of the thalamus, with trending increases in IBA-1 expression found in the BLA region of the amygdala. All p-values represented were measured using the student t-test between the comparing of the sham and blast animal (n = 10 per group). Significance was observed at *p < 0.05 and trends were observed at p < 0.1 Data is also represented as Mean ± SEM.*

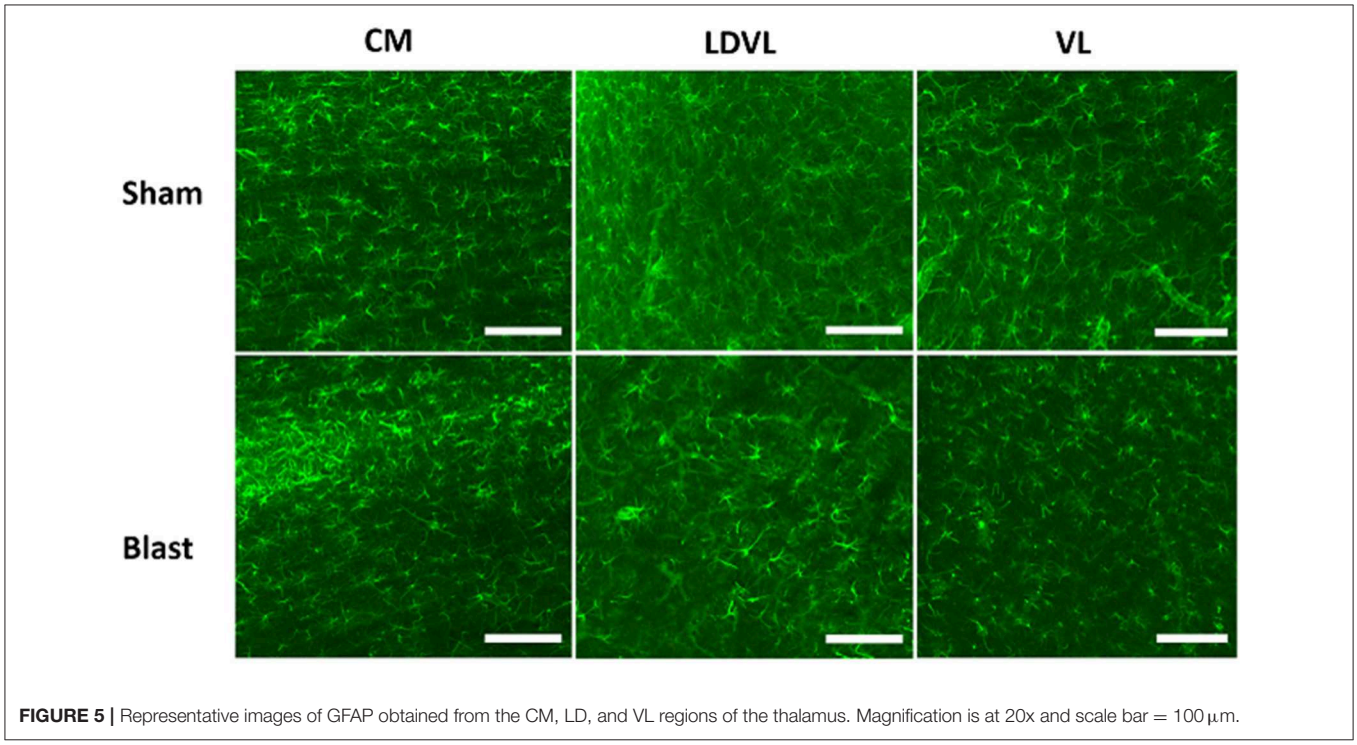

FIGURE 5 | Representative images of GFAP obtained from the CM, LD, and VL regions of the thalamus. Magnification is at 20x and scale bar = 100 μm.

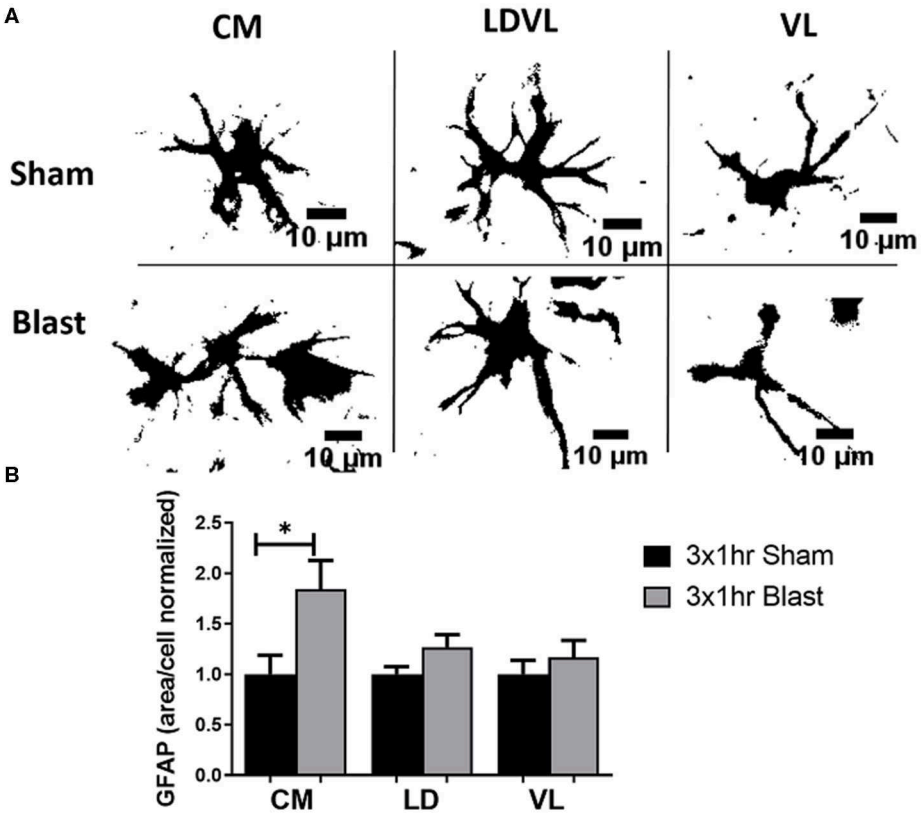

FIGURE 6 | Significant astrogliosis after blast injury. **(A)** A view of individual astrocytes (GFAP) in both sham and repeated bTBI animals showed various sizes of cell bodies. Blast animals demonstrated larger areas of cells than shams. Scale bar = 10 µm. **(B)** A significant increase in GFAP mean area per cell was noted in the CM region of the thalamus of blast animals compared to shams. A trending increase in the mean area per cell was noted in the LD region ($p = 0.079$). This signifies that astrocytes are becoming reactive, changing size in response to injury. *$p < 0.05$, Mean ± SEM, data was normalized to shams.

blunt and blast head trauma. They found that a higher percentage of blast-exposed patients exhibited a trend toward low-frequency phase lag on evaluation. A subsequent study completed by the same group was completed that studied active military personnel exposed to blast (69). They performed vestibular function and auditory tests, subsequently compared results to those with impact-related head injuries. They found that vestibular function significantly worsened in blast-exposed patients as a function of time between injury and presentation. They also noted that the blast group presented with a unique set of vestibular disorders and associated symptoms as compared to the impact-related group. These studies identify a distinct difference between impact and blast injured mTBI patients and provide evidence that treatment strategies should be individualized on the basis of each mechanism of injury. This suggests that the mechanism of vestibular injury differs between blast and impact-related TBI. Understanding the differences between these two distinct types of injury would lead to a more focused approach by clinicians to develop better treatment strategies for those exposed to blast injury.

The debate on the biomechanical transmission of blast wave energy to the brain is also ongoing, but many accept that the injury mechanism differs from impact-related head injuries. Explanations point to a dynamic skull deformation theory that produces high-speed compression leading to shear stress between fluid and tissue interfaces (70). Brain tissue at interfaces with fluid, such as that bordering the cerebrospinal fluid-filled ventricles or blood-filled sinuses, are thought to be particularly susceptible to primary blast injury due to the reflection of blast waves at borders of materials with differing densities. This stress likely contributes to the cellular response triggered by blast exposure. While areas of the thalamus may be protected from the fluid-tissue interface stress as its location is in the center of the brain, transmission of compressive forces may also explain the inner brain region damage seen in some models. Animal models of both single and repeated blast exposure also indicated neuronal injury and glial dysfunction due to the biomechanical stress transmission.

As we advance the generation of clinically relevant data to decipher blast injury mechanisms, we will be able to assist in further understanding the differing outcomes and neuropathy observed between blast and impact injury modes, bTBI is characterized as a diffuse injury that presents with persistence gliosis (71). A limitation of the current study is the use of IBA-1

to detect microglia in the brain. IBA-1 has an affinity to both microglia and monocyte-derived macrophage surface markers (72–74), thus the use of IBA-1 cannot fully distinguish between the local and systemic inflammatory response. The timing of when the systemic macrophage resolve from the brain injury is debated. Studies have emerged reporting that CCR2-dependent macrophages are recruited from the periphery then dissipate in the early chronic stages of injury (75, 76). These reports support the hypothesis that the key players of the inflammatory response 4 weeks after injury is the resident microglia and astrocytes, making glial contributions the focus of our studies. Future work should test this hypothesis by using microglia-specific markers for a confirmation of glial characterization following bTBI. Transmembrane 119 (TMEM 119) is an example of a recently identified microglia marker that has been used by multiple sclerosis researchers and could be applied to TBI studies (77). Collectively, the current study has proven that glial activation within the thalamus is contributing to the ongoing vestibulomotor deficits following blast induced injury, especially at the chronic stages. We hope that the advancement of these studies will lead to further strategies that will aid in long-term healthcare for bTBI patients, ultimately improving their quality of life.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Virginia Tech Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MD was responsible for analysis of results, interpretation of results, and preparation of manuscript. ZB was responsible for data collection, analysis of data, and interpretation of results. SM and MU was responsible for data collection and analysis of data. PV was responsible for study design, securing funding, interpretation of results, and preparation of manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Overpressure Exposure From .50-Caliber Rifle Training Is Associated With Increased Amyloid Beta Peptides in Serum

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Background: Overpressure (OP) is an increase in air pressure above normal atmospheric levels. Military personnel are repeatedly exposed to low levels of OP caused by various weapon systems. Repeated OP may increase risk of neurological disease or psychological disorder diagnoses. A means to detect early phase effects that may be relevant to brain trauma remain elusive. Therefore, development of quantitative and objective OP-mediated effects during acute timeframes would vastly augment point-of-care or field-based decisions. This pilot study evaluated the amplitude of traumatic brain injury (TBI)–associated biomarkers in serum as a consequence of repeated OP exposure from .50-caliber rifle use over training multiple days.

Objective: To determine the acute temporal profile of TBI-associated serum biomarkers and their relationship with neurocognitive decrements or self-reported symptoms among participants exposed to low-level, repeated OP from weapons used in a training environment.

Methods: Study participants were enrolled in .50-caliber sniper rifle training and exposed to mild OP (peak pressure 3.8–4.5 psi, impulse 19.27–42.22 psi-ms per day) for three consecutive days (D1–D3). Defense automated neurobehavioral assessment (DANA) neurocognitive testing, symptom reporting, and blood collection were conducted 2–3 h before (pre-) and again 0.45–3 h after (post-) OP exposure. The TBI-associated serum biomarkers, glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1 (UCH-L1), neurofilament light (Nf-L), tau, and amyloid beta peptides (Aβ-40 and Aβ-42) were measured using digital ELISAs.

Results: Serum GFAP decreased on D1 and D3 but not D2 after OP exposure. Nf-L was suppressed on D3 alone. Aβ-40 was elevated on D2 alone while Aβ-42 was elevated each day after OP exposure. Suppression of GFAP and elevation of Aβ-42 correlated to OP-mediated impulse levels measured on D3.

Conclusions: Acute measurement of Aβ-peptides may have utility as biomarkers of subconcussive OP caused by rifle fire. Fluctuation of GFAP, Nf-L, and particularly Aβ peptide levels may have utility as acute, systemic responders of subconcussive OP exposure caused by rifle fire even in the absence of extreme operational deficits or clinically defined concussion.

Keywords: .50 caliber rifle training, overpressure, low-level blast, occupational exposure, traumatic brain injury, serum biomarkers, amyloid beta peptides

INTRODUCTION

Overpressure (OP) is an increase in air pressure above atmospheric levels (1, 2). Military and law enforcement personnel are routinely exposed to OP within training environments and in-theater. OP-mediated effects are most widely understood in the context of moderate-to-high levels of blast caused by improvised explosive devices (IEDs) (3–5). However, operational training involving .50-caliber rifle systems are also capable of generating repeated OP exposure (6). OP levels consistently exceed 4 psi, an "above safe" level, which is dependent upon rifle configuration (e.g., muzzle devices and ammunition type) as well as environment. For e.g., prone firing positions and shots taken from atop hard surfaces (e.g., vehicles or concrete) are associated with even greater OP levels (7).

OP exposure can cause brain trauma and may start underlying pathology that leads to neurodegenerative disorders (8); however, the exposure thresholds at which injuries occur are not yet defined. Blast-related mild traumatic brain injury (mTBI) is often identified by symptoms, but there is concern that blast exposures that do not result in symptom reporting or mTBI, referred to as subconcussive exposures, may also be associated with neurotrauma (9). Effects of repeated OP exposure have been measured and include neurocognitive decrements, blood-based biomarker level changes, and manifestation of symptoms (10), which are similar to those observed among mild traumatic brain (mTBI) or concussed patients. These effects are observed in absence of a diagnosable injury, and personnel remain fit for duty. Symptom reporting among operators with repetitive blast exposures are characterized, in part, by headaches, dizziness, taking longer to think, and tinnitus (11–13). Detection, prevention, or mitigation of these subconcussive, subacute, and chronic outcomes may be met by assessment of OP-mediated health effects that are measured acutely. Symptomology is often variable, subjective, and may be underreported (14, 15). Therefore, objective measurements that identify OP-mediated health effects present an unmet operational need and have become a health care priority (https://www.congress.gov/bill/115th-congress/senate-bill/2883/all-info) such that routine monitoring of health-related effects from operational exposure has become a topic of interest.

The use of blood-based biomarkers may augment the ability to objectively measure the effects of OP inclusive of neurocognitive decrement and concussion-like symptoms. Several proteins of the central nervous system (CNS) are used to identify TBI and neurological disease. Glial fibrillary acidic protein (GFAP) is an abundant astroglial protein within the cytoskeleton (16). Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a deubiquitinating enzyme enriched in neuronal cell bodies (17). GFAP and UCH-L1 are the most widely used biomarkers for acute, moderate-to-severe TBI (18–21). Tau, an important microtubule-associated structural element of the neuronal cytoskeleton (22). Neurofilament light chain (Nf-L), another central nervous system–enriched protein, is also a component of the axonal cytoskeleton that is primarily expressed in large-caliber, myelinated axons. Nf-L has been identified in peripheral blood collected weeks or months after TBI (23). Tau and Nf-L are currently the most widely used biomarkers applied to acute–subacute mTBI or concussion with variable results (24–27). Amyloid precursor protein (APP), an integral membrane protein predominantly expressed in the synapses of neurons, is the precursor of amyloid beta (Aβ) peptides, which have been shown to be elevated in blood of both patients and animal models after TBI (28). Overall, evaluation of serum-based biomarkers are capable of providing objective measures.

We sought to determine if acute assessment of these TBI-associated proteins were applicable to low levels of OP caused by weapons use. This study measured serum biomarker levels (GFAP, tau, Nf-L, UCH-L1, and Aβ peptides), reaction time (a metric of neurocognitive performance), and concussion-like symptomology among military personnel exposed to mild, repeated OP caused by .50-caliber rifle discharge. Early and objective quantitation of OP-mediated peripheral biomarkers may aid in rapid decision making and identify systemic effects even in the absence of a definitive mTBI or concussion diagnosis.

METHODS

Study Participants

Male, active-duty, law-enforcement personnel ($n = 15$) engaged in a 3-day (D)-long training course within a single site. The daily interval of training was ∼24 h, during which .50-caliber rifle firing, neurocognitive testing, blood sampling, and symptomology assessment were conducted before and after each daily training session. Informed consent was obtained prior to all procedures and testing. The protocol was approved by the Walter Reed Army Institute of Research Institutional Review Board (WRAIR protocol #2304). All participants were assigned unique identification numbers such that data was deidentified prior to collecting field-based metrics, symptom surveys, and biological samples used in this study. All outcome metrics,

including details regarding rifle systems, are indicated for each study participant (**Supplementary Table 1**). Rifle systems, configurations, and ammunition types varied slightly between participants based on departmental resources, but all systems ranged from 20- to 29-inch barrels, generally fired unsuppressed, using a mixture of 690–750 grain (gr) ammunition. The most common ammunition was Hornady .50 caliber AMAX 750 gr, and the most common rifle systems were the Barrett M82A1 20- and 29-inch configuration or the M107A1. Most systems were semiautomatic and can have a different pressure signature at the ear due to the cycling of the action than other systems with some bolt-action platforms, and no uniform trends were noted, thus prohibiting grouped analyses based on barrel length. At pre- and post-OP exposure time-points, participants completed DANA tasks, blood collection, and symptom survey assessment. Participants did not report adverse health conditions, e.g., dehydration, that would be cause for removal from the study. Weather conditions were moderate and did not affect the study or the participants.

Overpressure Measurements

OP measurements were conducted as previously described (29). All participants were exposed to 3-days of OP events, firing 4–50 rounds per day from a variety of positions (prone, seated, kneeling, standing, and supported standing from barricade). All training days were consecutive. OP was measured as pounds per square inch (psi) using the B3 Blast Gauge sensor (generation 6, BlackBox Biometrics, Rochester, NY) mounted on the left shoulder of each participant. The sensor from the left shoulder best approximates incident orientation to the blast wave (30). The B3 is a small, lightweight, accurate, disposable, and off-the-shelf exposure measurement device that can assess OP and impulse exposures for a blast event; the sensors are positioned on the subject such that they best approximate incident orientation. It has been developed to be worn by participants in complex environments and assess OP exposure. It records and collects data from the blast wave: overpressure, acceleration (rate at which speed changes), and impulse (time exposed to certain levels of OP). Participants are static during training; the B3 sensor are not designed to measure motion or movement of the user (e.g., study participant). Peak pressure is the maximum overpressure peak recorded by the B3 during a blast event per individual per day. Cumulative impulse (psi-ms) is derived from the summation of psi-ms signals per participant per day. Overpressure (psi) and impulse (psi multiplied by milliseconds [psi-ms]) are displayed for each incident and used to calculate the peak overpressure and cumulative impulse values for the training session (**Supplementary Table 1**).

Assessment of Neurocognitive Performance

The defense automatized neurocognitive assessment (DANA) tool was administered prior to (pre-OP: −3 to −2 h) and after (post-OP: +0.45 to +3 h) OP exposure to mirror blood-draw time. The DANA consists of three subtasks conducted with a hand-held device and monitor screen. (1) Simple reaction time (SRT) measures pure reaction time when the participant was required to tap on the location of the yellow asterisk symbol as quickly as possible each time it appeared. (2) Procedural reaction time (PRT) is a choice reaction time that measures accuracy, reaction time, and impulsivity. The screen displays one of four numbers for 3 s. The participant is required to press on a left button ("2" or "3") or right button ("4" or "5") depending on the number pressed. This choice reaction time task targets simple executive functioning with easy decision-making capabilities. (3) Go-no-go (GNG) is a forced choice reaction-time task. A picture of a house is presented on the screen. Either a "friend" (green) or "foe" (white) appear in a window. The respondent must push a "fire" button only when a "foe" appears. The choice reaction time measures sustained attention and impulsivity. The test quantifies speed and accuracy of target omissions and commissions.

Symptom Reporting

Participants completed a 32-item, paper-and-pencil health symptom survey before (pre-) and after (post-) OP exposure within the same timeframe as DANA assessment and blood collection for a total of two surveys per day over 3 consecutive days. The symptoms on the survey are similar to that of the Rivermead instrument (31, 32) but with additional survey questions and responses relevant to operational blast OP training in addition to those of concussion (33). Participants were instructed to use a 5-point Likert scale (0 = "not experienced at all," 1 = "no more of a problem than before training," 2 = "mild problem—present but don't really notice and doesn't concern me," 3 = "moderate problem—I can continue what I am doing, but I notice the problem," 4 = "severe problem—constantly present, feels like it could affect my performance").

Serum Collection and Preparation

Venous blood was collected directly into BD Vacutainer SST™ Serum Separation Tubes (Fisher Scientific, Waltham, MA) prior to (pre-OP: −3 to −2 h) and after (post-OP: +0.45 to +3 h) OP exposure to mirror DANA assessment. Serum was processed within 30 min according to the manufacturer's instructions. Samples were centrifuged at 1,000 × g for 10 min at room temperature. Samples were stored in 1-mL aliquots, supplemented with HALT protease/phosphatase inhibitors (Fisher Scientific, Waltham, MA) and stored at −80°C until use.

Quantitative Biomarker Measurements

Glial fibrillary acidic protein (GFAP), ubiquitin carboxyl-terminal esterase L1 (UCH-L1), neurofilament light polypeptide (Nf-L), tau, and amyloid beta (Aβ)-40 and−42 were measured by multiplex digital immunoassay using a single molecule array technology with the SiMoA HD-1 instrument (Quanterix Corporation, Billerica, MA). All assays were performed based on manufacturer's recommendations as previously reported (34). Briefly, serum was thawed on ice, then centrifuged at 10,200 × g for 10 min at 4°C. Thereafter, 120 μL of serum supernatant was directly loaded onto a 96-well plate and diluted 1/4 during the assay. Each serum sample, standard, or internal control was tested in duplicate. All reported biomarker values were within the limits of detection reported, and

TABLE 1 | Demographic data of military personnel exposed to overpressure from munitions.

Number of subjects (*n*)		**15**	
Age (years)			
Mean (SD)		42.3 (5.7)	
Range [Min–Max]		33–52	
Duration of Service (years)			
Mean (SD)		14.7 (6.7)	
Range [Min–Max]		8–26	
Sample Collection Time (h)			
Event	**Pre-exposure**	**Post-exposure**	
Mean (SD)	−2.48 (0.59)	1.40 (1.3)	
Range [Min–Max]	−3.16 to −2.1	0.46 to 2.90	
Peak pressure (psi)	**Day-1**	**Day-2**	**Day-3**
Mean (SD)	3.86 (1.04)	3.82 (0.42)	4.52 (1.59)
Cumulative Impulse (psi × time) (milliseconds)	**Day-1**	**Day-2**	**Day-3**
Mean (SD)	42.2 (18.7)	19.3 (7.37)	34.1 (13.4)

Demographic Characteristics of Study Participants and Biosample Collection Timelines. Participants' age, duration of service, as well as pre-OP and post-OP exposure time-point for DANA, blood collection, and symptom surveys are indicated. Levels of OP exposure derived from left shoulder B3 sensors from all participants are displayed as the peak pressure (psi) and cumulative impulse (psi × time) levels are displayed for each day (mean + SD each day, based on all participants).

internal quality controls were consistent (CV < 1%) for each biomarker tested (**Supplementary Table 2A**). Inter- or intra-participant variation is provided for the biomarker analysis (**Supplementary Table 2B**). Curve fitting analysis was conducted using preset programs designed by the manufacturer.

Data Management and Statistical Analysis

Data analysis was conducted with Prism version 8.2.1 (GraphPad, La Jolla, CA) under the assumptions that all participants underwent similar OP exposure conditions although barrel length and bullet weights are known to affect the exposure conditions for weapon systems. DANA metrics (SRT, PRT, and GNG) and biomarker levels are displayed as the median + interquartile range (IQR). Quantitative measurements were tested for normality and found to fit a non-normal distribution per the D'Agostino & Pearson normality test. Data was evaluated using non-parametric RM-ANOVA (Friedman's Test) with Dunn's *post-hoc* test for multiple comparison across days and within-subjects per day (levels: pre-D1, post-D1, pre-D2, post-D2, pre-D3, post-D3) (biomarker concentration [pg/mL], $^{*}p \leq 0.05$). For comparisons between OP levels (peak psi or cumulative impulse) or symptoms, biomarker values were transformed into a delta (post- minus pre-OP exposure) prior to determining the two-tailed Spearman rank correlation coefficient ($^{*}p \leq 0.05$).

RESULTS

Demographic information of participants, the time of serum sample collection and survey assessment as well as the peak pressure or impulse values derived from left shoulder B3 sensors are shown (**Table 1**). Participants ($n = 15$) were males aged 33–52 (mean +/– SD: 42.3 +/– 5.7 years) with variable duration of service (mean +/– SD: 14.7 +/– 6.7 years; range: 8–26 years). DANA, symptom reporting, and serum sampling occurred before (pre-OP: mean: −2.48 h, range: −3.16–−2.10) and after (post-OP: mean: 1.40 h, range: 0.46–2.90 h) .50-caliber rifle firing. Peak pressures for D1 (mean +/– SD: 3.86 +/– 1.04 psi) and D2 (mean +/– SD: 3.82 +/– 0.42 psi) were similar but slightly elevated on D3 (mean +/– SD: 4.52 +/– 1.59 psi). Cumulative impulse values were highest on D1 (mean +/– SD: 42.2 +/– 18.7 psi-ms), compared to D2 (mean +/– SD: 19.3 +/– 7.37 psi-ms) and D3 (mean +/– SD: 34.1 +/– 13.4 psi-ms).

Neurocognitive assessment was performed using the DANA. SRT, PRT, and GNG assessments were generally not negatively (or adversely) affected by OP exposure (**Supplementary Table 3**). Symptom reporting was highly variable over time (**Figure 1**). Headaches (7/15, 47%) and feeling frustrated or impatient (6/15, 40%) were the two most frequently reported after OP events on D1. On D2 and D3, the above symptoms resolved. Few subjects reported other symptoms as a consequence of OP events during training. Therefore, symptoms were not further evaluated as a consequence of peak pressure, impulse levels, or for relationships with changes in biomarker levels.

Next, TBI-related biomarkers were measured in serum collected before and after daily OP (**Figure 2**). The Dunn's multiple comparison test for daily changes in biomarkers are shown (**Supplementary Table 4**). GFAP levels fell significantly on D1 (pre-OP—median: 84.7, IQR: 64.8–139 pg/mL; post-OP—median: 58.3, IQR: 41.8–93.4 pg/mL, $p \leq 0.05$). Values were not affected on D2 (pre-OP—median: 91.8, IQR: 55.9–117 pg/mL; post-OP—median: 89.2, IQR: 57.6–120 pg/mL, p = NS) but were suppressed again on D3 (pre-OP—median: 84.8, IQR: 55.2–114 pg/mL; post-OP—median: 62.4, IQR: 51.9–74.3 pg/mL, $p \leq 0.05$) (**Figure 2A**). Nf-L levels were not affected (D1: pre-OP—median: 8.69, IQR: 7.03–9.43 pg/mL; post-OP—median: 7.69, IQR: 7.07–11.2 pg/mL, NS; D2: pre-OP—median: 7.82, IQR: 6.4–9.84 pg/mL; post-OP—median: 8.8, IQR: 6.21–9.02 pg/mL, NS) until D3 of OP exposure (pre-OP—median: 7.29, IQR: 6.63–9.60 pg/mL; post-OP—median: 6.94, IQR: 5.95–8.3 pg/mL, $p \leq 0.05$) (**Figure 2B**).

OP-mediated elevation of Aβ peptide levels was more robust. Aβ-40 showed a somewhat progressive, upward trend each day (D1: pre-OP—median: 146, IQR: 134–163 pg/mL; post-OP—median: 167, IQR: 141–219 pg/mL, NS; D2: pre-OP—median: 138, IQR: 117–155 pg/mL; post-OP—median: 169, IQR: 145–196 pg/mL, $p \leq 0.05$; D3: pre-OP—median: 154, IQR: 140–169 pg/mL; post-OP—median: 177, IQR: 143–198 pg/mL, NS) (**Figure 2C**). Similarly, Aβ-42 levels were also higher as a consequence of daily OP exposure (D1: pre-OP—median: 3.41, IQR: 2.67–4.7 pg/mL; post-OP—median: 6.52, IQR: 5.19–7.89 pg/mL, $p \leq 0.05$; D2: pre-OP—median: 2.31, IQR: 1.34–5.32 pg/mL; post-OP—median: 6.17, IQR: 5.68–7.71 pg/mL, $p \leq 0.05$; D3: pre-OP—median: 4.81, IQR: 3.35–5.93 pg/mL; post-OP—median: 6.66, IQR: 5.34–7.36 pg/mL, $p \leq 0.05$) (**Figure 2D**). Further, Aβ-42/Aβ-40 ratios showed a significant upward trend after the each consecutive OP exposure event (D1: pre-OP—median: 0.021, IQR: 0.016–0.029 pg/mL; post-OP—median:

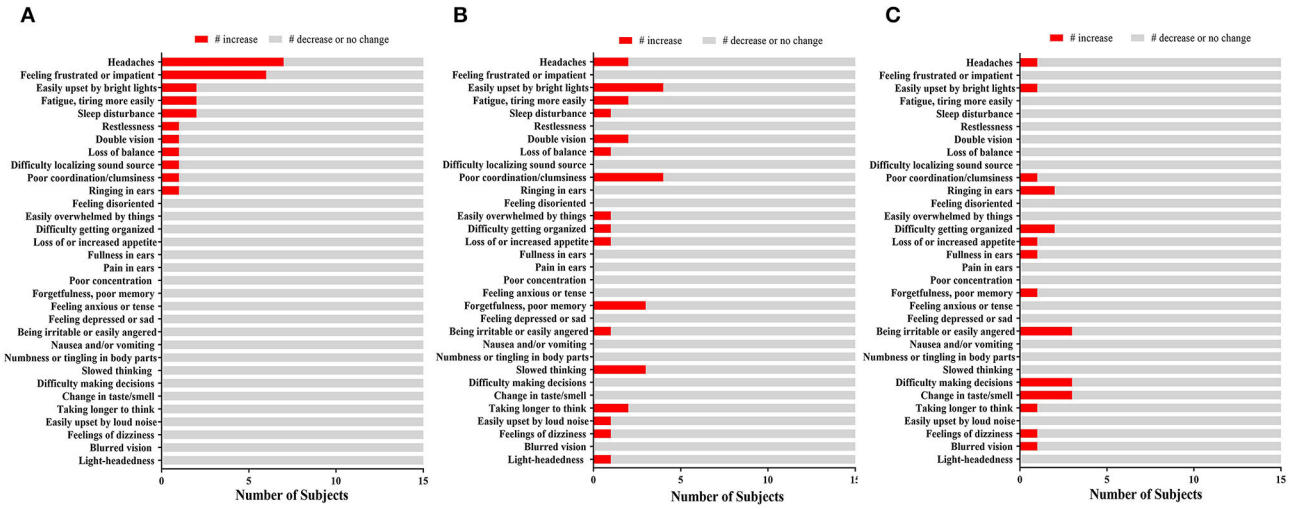

FIGURE 1 | The Effect of OP Exposure on Symptom Reporting. Bar chart indicating the number of participants (*x*-axis) and self-reported symptoms (*y*-axis) on **(A)** Day 1, **(B)** Day 2, **(C)**, and Day 3. Those who reported an increase (red) compared those who did not (gray) are shown.

0.037, IQR: 0.031–0.043 pg/mL, $p \leq 0.05$; D2: pre-OP—median: 0.018, IQR: 0.012–0.030 pg/mL; post-OP—median: 0.038, IQR: 0.033–0.045 pg/mL, $p \leq 0.05$; D3: pre-OP—median: 0.030, IQR: 0.023–0.037 pg/mL; post-OP—median: 0.037, IQR: 0.031–0.042 pg/mL, $p \leq 0.05$) (**Supplementary Figure 1A**). There were no appreciable changes in the levels of UCH-L1 (range: 0–30.9 pg/mL) (**Supplementary Figure 1B**) or tau (range: 0–0.502 pg/mL) (**Supplementary Figure 1C**) on either day.

The changes in biomarker levels were compared to peak OP and cumulative impulse levels on each training day. Spearman rank correlations are indicated for all comparisons (**Table 2**). On D1, peak pressure (psi) levels were directly proportional to increased dNf-L ($r = +0.55$, $p = 0.03$) although the median response in levels detected pre-OP and post-OP were not significant as previously shown. Biomarker changes were not associated with peak-OP or cumulative impulse values evaluated on D2. In contrast, the negative change in dGFAP was aligned with peak OP ($r = -0.54$, $p = 0.04$) and dAβ-42 ($r = +0.65$, $p = 0.01$) correlated to cumulative impulse levels on D3. The highest cumulative impulse levels from rifle fire on D1 were associated with increased serum Nf-L (positive dNf-L). On D2, the lowest cumulative impulse levels were recorded and did not correlate to changes in biomarker levels. In contrast, the second highest cumulative impulse levels recorded on D3 were in accordance with a drop in GFAP (e.g., negative dGFAP) and elevation of Aβ-42 (e.g., positive dAβ-42). Next, the cumulative effect of exposures (Sum-OP or Sum-impulse) was examined by comparing changes in biomarker levels on D3 post-OP to those measured in blood collected on D1 pre-OP to OP or impulse levels on D1–D3. Spearman rank correlations are indicated for all comparisons (**Table 3**). Biomarker changes were not associated with the Sum-OP. The changes in dGFAP and dNf-L did not correlate with the Sum-impulse levels. However, dAβ-40 showed moderate correlation ($r = +0.53$, $p = 0.042$) and dAβ-42 showed strong correlation ($r = +1.00$, $p = <0.0001$) with Sum-impulse. The changes in the biomarker levels were not significantly correlated with number of shots fired on either day (**Supplementary Table 5**). Finally, the changes in biomarker levels were not significantly correlated with SRT, PRT, or GNG DANA metrics or to symptoms reported by the study participants (data not shown).

DISCUSSION

Repeated OP exposure is associated with nervous system–related health effects. Yet objective measurements that may be used to define a trauma effect, particularly in the acute timeframe that is applicable to monitoring, remain difficult to define. Therefore, this study evaluated acute serum biomarker profiles, neurocognitive metrics, and reported symptomology among participants exposed to daily OP from .50-caliber rifle fire during a 3-day training period.

Quantitation of peak pressure and/or impulse caused by gunfire (not the number of shots fired) is the definitive metric that represents exposures derived from rapid-fire weapons per DoD requirements associated with health assessment as provided by Health and Human Services, Centers of Disease Control, and the National Institute for Occupational Health and Safety (https://www.cdc.gov/niosh/hhe/reports/pdfs/2013-0124-3208.pdf). Our current study shows that low levels of OP from .50-caliber rifle fire training did not have a substantive negative effect upon neurocognitive subtasks from the DANA. The null change in SRT is not surprising. SRT is similar among concussed vs. non-concussed athletes (35), and the participants in this study were exposed to subconcussive overpressure conditions. The decrease in PRT and GNG subtasks is indicative of a slightly faster response that has been previously reported to occur among nearly 67% of study participants (29) and may be

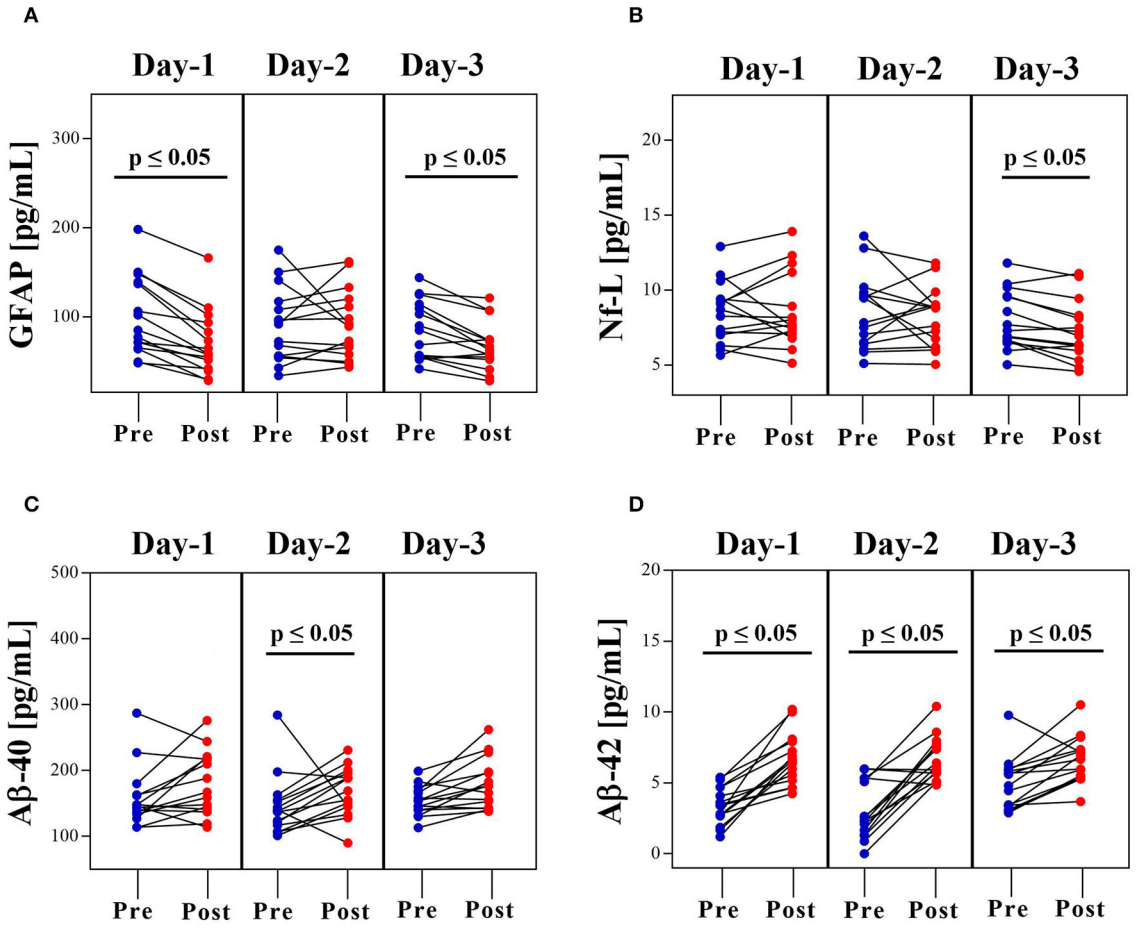

FIGURE 2 | Acute Dynamics of Serum Protein Biomarkers before and after OP Exposure. Concentration of **(A)** GFAP, **(B)** Nf-L, **(C)** Aβ-40, and **(D)** Aβ-42 in serum collected before (pre-OP range: −3.16 to −2.10) and after (post-OP range: 0.45–3 h) OP exposure. Data is shown as concentration [pg/mL] (RM-ANOVA with Dunn's *post-hoc* test).

reflective of a "practice effect" among persons without a clinically defined concussion (36). Essentially, the participants are likely paying more attention to the task itself, which is common. Few subjects reported headaches, the top-ranked symptom. The levels recorded for median peak OP among all participants was <4 psi, which is often cited as a "safe level" in regards to disruption of the ear drum among subject matter experts (37). Repeated exposure to moderate-to-high OP (≥5 psi) leads to persistent changes in symptomology and neurocognitive performance (38, 39), which we found not to occur in these participants. Thus, the observations shown herein are aligned with recent studies indicating that exposure to low levels of OP from .50-caliber rifles is common and does not largely affect DANA or symptoms although precautions to avoid higher levels of exposure are suggested due to peak pressure (psi) exposure guidelines.

The association between repeated OP and TBI is a well-accepted hypothesis (2, 40). Therefore, biomarkers are generally expected to increase as a consequence of OP exposure. UCH-L1 is well-known to be elevated within 24 h after severe, not subconcussive, TBI (41–43). Nf-L and tau are typically elevated during much later timeframes and associated with CT abnormalities (25, 44, 45). Serum tau may also be elevated acutely after exposure to moderate psi levels in rodent models (46). This study shows that serum UCH-L1 and tau were generally unaffected by repeated OP exposure after .50-caliber rifle fire. The lack of a UCH-L1 or tau response after low-level OP in this context is fitting for a subconcussive exposure that lacks to the need to employ the concussion protocol or imaging as well an exposure paradigm that does not lead to a moderate-to-severe head wound.

A small (albeit potentially non-significant) increase in GFAP or Nf-L was also expected after daily OP exposure. Serum GFAP typically increases after acutely after moderate-to-severe TBI or CT-positive closed head injury (47). Among boxers, mild head impact is associated with higher levels of serum Nf-L in serum collected >7-days (48). In contrast, Nf-L and (to a greater extent) GFAP were found to be decreased in subjects exposed to subconcussive levels of OP in this study. The biological

TABLE 2 | The relationship between peak OP levels (psi) and cumulative Impulse (psi-ms) and biomarker changes is indicated.

Biomarker Change	Day	Peak OP (psi)		Cumulative Impulse (psi-ms)	
		Spearman *r*	*p*-value	Spearman *r*	*p*-value
dGFAP	D1	−0.39	NS	−0.08	NS
	D2	0.11	NS	−0.44	NS
	D3	**−0.54**	**0.04**	−0.09	NS
dNfL	D1	**0.55**	**0.03**	−0.46	NS
	D2	0.10	NS	−0.25	NS
	D3	0.28	NS	0.01	NS
dAβ-40	D1	0.20	NS	−0.10	NS
	D2	−0.30	NS	−0.45	NS
	D3	0.32	NS	.50	NS
dAβ-42	D1	0.24	NS	−0.28	NS
	D2	−0.31	NS	−0.47	NS
	D3	0.20	NS	**0.65**	**0.01**
dAβ-42/40 Ratio	D1	−0.33	NS	0.11	NS
	D2	−0.21	NS	0.45	NS
	D3	−0.01	NS	0.10	NS

The correlation coefficient is shown in bold (2-tailed, Spearman r).

TABLE 3 | The relationship between the summations of either OP levels (psi) or impulse (psi-ms) determined during training (D1, D2, and D3) and biomarker changes defined by the last day of training (D3 Post-OP) to the first day before training (Day1 Pre-OP) is indicated.

Biomarker Change (D3 Post OP–D1 Pre OP)	Summation OP (psi)		Summation Impulse (psi-ms)	
	Spearman *r*	*p*-value	Spearman *r*	*p*-value
dGFAP	−0.32	NS	−0.27	NS
dNfL	−0.01	NS	0.18	NS
dAβ-40	0.37	NS	**0.53**	**0.042**
dAβ-42	0.14	NS	**1.0**	**<0.0001**

The correlation coefficient is shown in bold (2-tailed, Spearman r).

relevance of serum GFAP (D1 and D3) and Nf-L (D3 only) suppression as well as the correlation between decreased GFAP and peak OP levels is not yet known and potentially confounding, when viewed in the context of longer timeframes or clinically diagnosed, moderate-to-severe TBI as a predetermined endpoint. However, it is interesting to note that Nf-L levels decreased 1–12 h within serum collected from hockey players post–game play (25). This time-dependent observation is often overlooked and may indicate that acute (e.g., hourly) temporal dynamics of Nf-L may be useful in monitoring early phase OP-mediated effects when compared to a participant's basal biomarker levels, whereas leakage of a fairly large protein from the CNS is useful in the context of a known variable (e.g., clinical TBI diagnosis) during longer timeframes (e.g., weeks–months). Our group has previously reported that serum GFAP levels also decreased in accordance with persistent concussion-like symptomology after mild-to-moderate OP exposure caused by blast (34). Replication of both observations with larger cohorts and variation of peak OP levels and assessment of medical relevance compared to basal levels is ongoing.

Elevation of Aβ peptides was most robust after OP overpressure exposure due to .50-caliber rifle fire. These trends were generally associated with higher peak OP psi or impulse levels on the last day of training. In addition, Aβ-40 elevation correlated positively with the sum of impulse levels measured throughout the 3-day course, indicating that there may be a cumulative effect. This work is the first to explicitly report elevation of both Aβ-40 and−42 peptides within h of low-level OP exposure caused by rifle fire. Aβ-40 and−42 are well-understood as a key pathological component in chronic, neurodegenerative disease progression (49). Aβ levels are increased acutely among brain trauma patients, even those with diffuse injury compared to controls (50). Furthermore, Aβ-42 is higher in plasma exosomes collected from military personnel with mTBI compared to non-TBI controls (51). Transient leakage of small peptides, such as Aβ, from the CNS is feasible in the context of acute, subconcussive OP. Gap junctions and the blood–brain barrier are transiently affected by OP caused by blast in rodent models (52, 53). Therefore, acute elevation of amyloid beta peptides in serum may be sensitive responders of low-level, subconcussive OP. A potential caveat is that Aβ peptides and their precursor, APP, are also expressed outside of the CNS, including the epidermis, adipose tissue, and muscle (54, 55) and may be affected by circadian rhythms (56), leading to changes throughout the day (57). Tissue injuries were not reported and sleep disturbance were not significant among participants. Thus, circadian rhythms are not a likely confound. Overall, the changes in serum Aβ levels may have utility as biomarkers of low-level OP caused by .50-caliber rifle fire.

This preliminary work is not without a few limitations that are common to sampling within the context of real-world scenarios. First, the overall sample size ($N = 15$) is small. The participants of this study consist of an extraordinarily unique group due to the nature and context of specialized operational training. Second, the biomarker values are variable among non-injured controls or presumably healthy individuals (58) due to expected biological variance, how or which samples are defined as controls (59) and which quantitative assays (e.g., colorimetric vs. digital) are used (60, 61). Third, Aβ-40 and−42 peptides are produced by a wide variety of tissues inclusive of the CNS and periphery (62). In addition, Aβ peptide fluctuation may occur due to diet, medication, and stress. The current study cannot distinguish between the central and peripheral sources of the biomarker changes. Additional studies are in progress to address these potential confounds. To that end, we found that biomarker levels detected in commercially available controls were lower compared to levels among participants of this study and a previous report (38). Incorporation of control groups consisting of participants who conduct the same activities without firing .50-caliber weapons would be highly valuable. Due to the requirement that each participant actively engages in training, these groups do not yet exist. Instead, this study is structured such that each participant is compared to himself before OP exposure. A means to establish control groups that

consist of personnel similar to those within this study that meets study guidelines, training requirements, and availability of participants is in progress. Lastly, future studies will seek to evaluate of biomarker changes in the context of operationally safe (<4 psi) or above safe (≥4 psi) peak pressure (63) as well as low (<25 psi-ms) vs. high (≥25 psi-ms) impulse levels, which has been suggested by a working group, but impulse thresholds are experimental (39).

CONCLUSIONS

Subconcussive, low-level OP exposure caused by .50-caliber rifle fire is associated with daily fluctuation of serum biomarkers although symptomology is infrequent. This preliminary work indicates that acute elevation of Aβ peptides (and the Aβ-42/40 ratio) in serum may have utility as biomarkers of subconcussive OP relevant to impulse levels and that measurement of basal or pre-OP exposure biomarker levels serve as a reference for acute, post-OP exposure effects. The rifle systems and ammunition used among study participants offer authentic, real-world, scenarios akin to those that are likely to occur in combat. Therefore, assessment of dynamic biomarker changes, particularly when evaluated before and after OP exposure in the absence of a clinically defined TBI or concussion, are poised for additional evaluation and may be adaptable to operationally relevant health monitoring.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The protocol was approved by the Walter Reed Army Institute of Research Institutional Review Board (WRAIR protocol #2304). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB, CL, ME, and GK designed the study. CL, ME, and GK conducted DANA, survey, biological sample collection, and data collection. BT conducted biological sample processing and SiMoA biomarker assays. BT, AB, CL, and JN conducted data management and statistical analysis. BT, AB, CL, ME, and GK prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00620/full#supplementary-material

Supplementary Figure 1 | Acute Dynamics of Additional Serum Protein Biomarkers before and after OP Exposure. Graphs are shown for the **(A)** the Aβ 42/40 ratio as well as concentrations of **(B)** UCH-L1 and **(C)** Tau in serum collected before (pre-OP range: −3.16 to −2.10) and after (post-OP range: 0.45–3 h) OP exposure. UCH-L1 and tau data is shown as the concentration [pg/mL] (*$p \leq 0.05$, RM-ANOVA with Dunn's *post-hoc* test).

Supplementary Table 1 | Detailed Characteristics of Study Participants and Study Outcome Metrics.Each participants' age, duration of service, peak overpressure (psi), and cumulative impulse from left shoulder B3 sensors (time and delta), pre-OP and post-OP exposure time-points (time and delta), DANA metrics (milliseconds and delta), individual serum biomarker levels (pg/mL and delta), and transformed symptom survey responses (0 = decrease or no change; +1 = increase) are shown.

Supplementary Table 2 | Variation in Biomarker Analysis. **(A)** Assay limits are shown as provided by the manufacturer. **(B)** The coefficient of variation (CV %) in the biomarker assays is shown for internal quality controls (QC1 and QC2), the expected concentration is provided by Quanterix per assay lot. **(C)** Daily coefficient of variation of biomarker concentrations among study participants.

Supplementary Table 3 | The Effect of OP Exposure on DANA Metrics. Values (milliseconds, ms) of (A) SRT (B) PRT (C) GNG were determined before (pre-) and after (post-) OP exposure for each participant and each day is displayed as the median + interquartile range (IQR) (*$p \leq 0.05$, RM-ANOVA with Dunn's *post-hoc* test).

Supplementary Table 4 | Dunn's multiple comparisons for biomarkers across three days (*$p \leq 0.05$, RM-ANOVA with Dunn's *post-hoc* test).

Supplementary Table 5 | The relationship between number of shots fired each day and biomarker changes is indicated. (*$p \leq 0.05$, 2-tailed, Spearman *r*).

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Association of MOS-Based Blast Exposure With Medical Outcomes

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The study of effects associated with human exposure to repeated low-level blast during training or operations of select military occupational specialties (MOS) challenges medical science because acute negative effects that might follow such exposures cannot be expected to be clear or prevalent. Any gross effects from such occupational blast exposure on health or performance should be expected to have been already identified and addressed by affected military units through changes to their standard training protocols. Instead, effects, if any, should be expected to be incremental in nature and to vary among individuals of different susceptibilities and exposure histories. Despite the challenge, occupational blast-associated effects in humans are emerging in ongoing research. The purpose of the present study was to examine medical records for evidence of blast-associated effects that may have clinical significance in current standard of care. We hypothesized that populations exposed to blast by virtue of their military occupation would have poorer global medical outcomes than cohorts less likely to have been occupationally exposed. Records from a population of 50,254 service members in MOSs with a high likelihood of occupational blast exposure were compared to records from a matched cohort of 50,254 service members in MOSs with a lower likelihood of occupational blast exposure. These two groups were compared in hospitalizations, outpatient visits, pharmacy, and disability ratings. The clearest finding was higher risk among blast-exposed MOSs for ambulatory encounters for tinnitus, with adjusted risk ratios of 1.19 (CI 1.03–1.37), 1.21 (CI 1.16–1.26), and 1.31 (CI 1.18–1.45) across career time points. Other hypothesized effects (i.e., neurological outcomes) were smaller and were associated with acute exposure. This study documents that service members in occupations that likely include repeated exposure to blast are at some increased risk for neurosensory conditions that present in medical evaluations. Other hypothesized risks from occupational exposure may manifest as symptomology not visible in the medical system or current standard of care. Separate studies, observational and epidemiological, are underway to evaluate further the potential for occupational risk, but the evidence presented here may indicate near-term opportunities to guide efforts to reduce neurosensory risk among exposed service members.

Keywords: blast, military, epidemiology, occupation, healthcare

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INTRODUCTION

The term "occupational blast exposure" is intended to denote repeated exposures to low-level explosive blast events that occur as part of training and operational activities experienced by personnel in designated roles in the military and law enforcement. Such roles include indirect fires (artillery, mortar), explosive breaching, and antiarmor weapon operation. These occupational roles will differ in blast exposure magnitude, frequency, periodicity, or concomitant factors such as acoustic insult, aerobic exertion, and psychological stress. The common factor across occupational roles, *repeated blast exposure*, has been of increasing concern as a cause of negative neurological effects, especially in context of similar increasing concern for brain injury from repeated head impacts in contact sports like American football. Exposure to occupational blast (i.e., low-level blast) is not known to result in acute injury; neurological effects, if any, would be cumulative in nature and not recognizable as diagnosis of traumatic brain injury (TBI) in standard of care medicine. Anecdotal reports suggest that service members with particularly high levels of occupational blast exposure, chronic exposure, experience negative neurological effects, but relevant research has not shown conclusive evidence of such effects (1, 2). The work presented here is an examination of medical records for clinical evidence for hypothesized occupational blast exposure effects.

After years of repeated occupational exposure to explosive events used in close proximity (high explosive or propellant combustion in heavy weapons), some individuals report symptoms consistent with concussion (e.g., memory deficits, headache, dizziness, difficulty concentrating). Those symptoms are reported as experienced to a greater degree during periods of repeated exposure to blast in training. The anecdotally reported occupational blast-related symptomology has been supported by a symptom survey among a blast-exposed professional community (3), by pilot study evidence that included cognitive performance and blood-based neurotrauma biomarkers collected during training programs involving explosives (4), and by symptom inventory in other field studies of operational training (5–9). In addition to symptom reporting, research observations of low-level blast-associated effects have included deficits in cognitive function (10), cellular changes in peripheral blood (11–15), and neuroimaging evidence that blast exposure may negatively affect neurophysiological functioning in simple tasks requiring memory of visual stimuli (6). It is important to note that none of these cited studies included blast exposure association with diagnosed injury—the focus was on blast exposures considered low level in magnitude. This growing body of evidence is suggestive of an association between occupations that have a likelihood of repeated exposure to explosive blast and negative effects on health, but the entirety of this evidence has been subclinical, unassociated with medical diagnosis of injury.

In contrast, populations that do receive clinical diagnosis of TBI following acute exposure to significant blast events in a combat setting show clear evidence of blast-related neurotrauma, directly supporting the diagnosis. The clinical relevance of repeated low-level blast, such as experienced in routine training in some occupational specialties, is unknown. Effects from such low-level exposures may present differently than effects from acute exposure to significant blast events and thus, are not identified as diagnosable TBI but may be diagnosed as other conditions. A corollary condition may be chronic traumatic encephalopathy, recently observed among athletes in contact sports who sustain many hundreds or thousands of subconcussive blunt impacts to the head (16). Chronic traumatic encephalopathy, or other neuropathology from repeated subconcussive events, can present as symptomology during life but is not diagnosed until after death, upon postmortem exam. Research is needed to understand the clinical presentation of such conditions in standard of care medicine. Longitudinal or cross-sectional comparisons among cohorts of interest would be an important addition to current findings. Here, we addressed that gap for the risk associated with repeated exposure to low-level blast, which is not known to be currently associated with a clinical diagnosis. Professional communities exposed to blast in their occupational roles may have exposures during tactical operations, but they will all have exposures during routine training, for acquiring needed skills as well as maintaining those skills over time. Occupation-based estimates of risk from exposure history have been revealed for military occupational specialties (MOSs) in previous studies (17–19) and could serve to prevent injury as has been recommended for contact sports (20).

This study was a subproject to the Accession Medical Standards Analysis and Research Activity (AMSARA) CORE protocol (21) and utilized data already collected for other purposes from AMSARA and the Tri-Service Disability Evaluation Systems Database Analysis and Research (DES), which is part of the same contract as AMSARA.

MATERIALS AND METHODS

Study Design

This matched cohort study compared healthcare utilization, prescription drug utilization, and disability discharge between Soldiers with specific combat arms occupations, with MOS serving as a proxy for occupational exposure to explosive blast, vs. Soldiers with occupations that are likely to deploy to a combat zone but less likely to be occupationally exposed to blast. The inclusion of several categories of medical outcomes in this design was to increase sensitivity of finding an occupation-based chronic exposure effect not associated with acute diagnosis, within datasets that are coded according to diagnoses. To assess the short-, medium-, and long-term effects of occupational exposure to blast, three time periods of military service were used for ascertaining the study outcomes: first 12 months, from 1 to 7 years, and from 8 to 14 years of service. These time periods will reflect conditions at baseline and initial training, conditions from a full tour of duty, and conditions beyond one tour of duty.

This study was performed under a minimal risk human use WRAIR protocol (#2023.05) reviewed and approved by the Walter Reed Army Institute of Research Institutional Review Board.

TABLE 1 | Department of the Army Pamphlet 611–21 (Smartbook) (2017) "Military Occupational Classification and Structure" descriptions of basic level major duties for example MOS in the Exposed and the Unexposed groups.

Exposed group example MOS **[emphasis added]**:
10–13B. MOS 13B—Cannon Crewmember, CMF 13
a. Major duties. The cannon crewmember supervises or serves as **a member of field artillery cannon section** or ammunition section.
(1) MOSC 13B1O. Integral member of a crew that operates high technology cannon artillery weapon systems. Load and **fire howitzers**. Sets fuse and charge on a variety of munitions, including high explosive artillery rounds, laser guided projectiles, scatterable mines, and rocket assisted projectiles. Uses computer generated fire direction data to set elevation of cannon tube for loading and firing. **Employ rifles, machine guns, and grenade and rocket launchers in offensive and defensive operations**. Drives and operates heavy and light wheeled trucks and tracked vehicles. Transports and manages artillery ammunition. Participate in reconnaissance operations to include security operations and position preparation. Operate in reduced visibility environments with infrared and starlight enhancing night vision devices and other equipment. Coordinate movement into position. Camouflages position area. Communicate using voice and digital wire and radio equipment. Use critical combat survival skills to operate in a hostile environment. Maintain operational readiness of vehicles and equipment.
Unexposed group example MOS:
10–92A. MOS 92A—Automated Logistical Specialist (Auto Log Spec) CMF 92
a. Major duties. The automated logistical specialist supervises and performs management or stock record/warehouse functions pertaining to receipt, storage, distribution, and issue and maintains equipment records and parts. Duties for MOS 92A at each level of skill are:
(2) MOSC 92A1O. Establishes and maintains stock records and other documents such as inventory, materiel control, accounting and supply reports. Establishes and maintains automated and manual accounting records, posts receipts, and turn-ins and performs dues-ins and dues-outs accounting. Correct error and exception documents. Reviews and verifies quantities received against bills of lading, contracts, purchase requests and shipping documents. Unloads, unpacks, visually inspects, counts, segregates, palletizes, and stores incoming supplies and equipment. Maintains stock locator system and administers document control procedures. Repairs and constructs fiberboard or wooden containers. Packs, crate, stencil, weigh and band equipment and supplies. Construct bins, shelving and other storage aids. Processes request, and turn-in documents at direct support level through warehousing section. Processes inventories, surveys and warehousing documents. Performs prescribed load list (PLL) and shop stock list (SSL) duties in manual and automated supply applications. Prepares, annotates and distributes shipping documents. Breaks down and distributes field rations. Operate material handling equipment (MHE). Perform accounting and sales functions in self-service supply. Perform Standard Army Maintenance System Enhanced (SAMS-E) duties in automated applications. Simplifies and standardizes the collection and use of maintenance data. Improves readiness management and visibility by providing equipment status and asset data. Raise the quality and accuracy of performance, cost, backlog, man-hour, and parts data through improved maintenance management.

Study Population

All active duty enlisted US Army men who initially entered service from fiscal year (FY) 2000 to 2013 (October 1, 1999 to September 30, 2013) were eligible for inclusion in this study. Records prior to 1999 were not included because there was less consistent digitization of records at those earlier dates. Records later than 2013 were not included because this study was initially designed in 2015, and AMSARA uses a 2-year time lag in epidemiological studies to accommodate time for medical records to be completed, digitized, and centralized.

Eligible Soldiers for the Exposed group were excluded if they received a preaccession disqualification or medical waiver for tinnitus, headache, or sleep disturbance, or if their records were missing any variables that were of interest in this study. Those hospitalized for severe or penetrating TBI or traumatic amputation were excluded because these injuries indicated a single exposure to a high-energy blast. The primary purpose of this study was to examine the health effects of Soldiers occupationally exposed to blast over time and without clearly associated diagnosis from acute blast exposure—to include Soldiers with major medical conditions or diagnosed injuries directly associated with high-energy blast events would bias the results and would not be consistent with the primary purpose of the study. TBI that was not severe was not a criterion for exclusion. Excluding Soldiers with mild TBI from the study would have made the population less representative of the MOSs. The population of Soldiers eligible for the Exposed group had military occupations that were likely to be occupationally exposed to blast by virtue of MOS descriptions and training required for major duties and included Cannon Crewmembers, Explosive Ordinance Disposal Specialists, Indirect Fire Infantrymen, Combat Engineers, and Special Forces. Descriptions for major duties of these MOSs are available in the Department of the Army Pamphlet 611–21 (Smartbook) (22) and are reflected in a number of other sources, including public domain websites (e.g., army-portal.com). Each of the five MOSs listed here has descriptions that stipulate explosives or heavy weapons in the basic level of MOS major duties (see **Table 1** for example).

Soldiers eligible for the Unexposed group had military occupations that were likely to deploy to a combat zone but less likely to be occupationally exposed to blast, especially during training. These occupations included Quartermaster, Military Intelligence, Signal, Field Mechanical Maintenance, Engineers other than combat, Psychological Operations, or those who are Motor Transport Operators, Radar Operators, Military Police, or Chemical/Biological/Radiological/Nuclear Specialists. Eligible Soldiers for the Unexposed group were individually matched to the Exposed group on fiscal year of and age at military entry and history of deployment (yes/no). Those matched Soldiers were then randomly sampled to yield an equivalent number for the Unexposed group.

In the three stratified time periods (first 12 months, years 1–7, years 8–14) for both groups, the first time period included the full study population, while the second time period included only Soldiers who did not attrit within the 1st year of service. The third time period (years 8–14) includes those who entered military service prior to FY 2008 and have at least 8 years of military service.

Data Sources

The Defense Manpower Data Center (DMDC), Seaside, CA, provided entry dates, loss dates, deployment dates, and locations, military occupation, age, sex, race, education, and marital status. The US Military Entrance Processing Command (USMEPCOM) provided data from study subjects' medical examination prior to military entry, specifically examination dates, medical qualification status (fully qualified, medical disqualification, administrative qualification), and where relevant, medical diagnoses based on International Classification of Diseases, Ninth Revision (ICD-9) codes. The US Army Recruiting Command, Fort Knox, Kentucky provided data on recruits who had a medical disqualification at the pre-enlistment medical examination and sought a medical waiver. These data included medical waiver action (approved, denied) and disease/disorder in the form of ICD-9 diagnosis codes.

Data on medical encounters occurring at military treatment facilities (MTFs) during the study period were provided by the Defense Health Agency, and prescriptions filled at MTFs since 2002 were provided by the Pharmacy Data Transaction Service via the Military Health System (MHS) Data Repository. These data included encounter dates, count of bed days, and ICD-9 disease/disorder codes for each medical encounter or fill dates, drug type, drug category, and days supply for pharmacy data.

Data on disability discharge considerations were provided by the US Army Physical Disability Agency (PDA) and included demographic characteristics at the time of disability evaluation as well as information pertaining to the disability evaluation including dates, disposition, percent rating, and the diseases or disorders for which the Soldier was deemed unfit. Diseases and disorders present at disability evaluations are coded based on the Veterans Affairs Schedule for Rating Disabilities (VASRD) in lieu of ICD-9 codes.

Measures

The three time periods of military service (first 12 months, from 1 to 7 years, and from 8 to 14 years of service) were calculated using the date of the Soldier's first military entry and date of the study outcome, which included the date of the healthcare encounter, the date of the prescription fill, or the date of disability discharge. Time in years to first deployment was calculated as the duration between the date the Soldier's initial military entry and date of his first deployment. Time in months on deployment was calculated as duration of every unique deployment, using deployment begin and end dates. Length of service was the duration between a Soldier's earliest date of military entry and the most recent date of military exit. A Soldier was categorized as having been deployed if deployed in support of Overseas Contingency Operations/Global War on Terrorism at any time during the study period. Deployment count was determined by the number of unique deployments based on deployment date.

Overall healthcare utilizations, including the total number with an encounter and the average number of encounters per person, were determined by counting all unique hospitalizations or ambulatory encounters for Soldiers with at least one encounter for each time period. Ambulatory encounters were identified as unique using both date and appointment identification number, allowing for multiple ambulatory encounters on the same day. Inpatient encounters were counted as unique by date of admission; however, an admission date within a week of a prior hospitalization was counted as one hospitalization. Previous examination of data from military hospitals found hospital readmissions within 7 days of a prior admission's discharge date were most likely to be a transfer to another military hospital.

Specific disorders, disease or disorder subcategories by system, and drug classes were chosen as outcomes of interest based on the etiopathophysiological pathways consistent with or possibly related to the consequences of blast exposure. The proportion of Soldiers with a healthcare encounter and the average number of encounters per specific disorder or subcategory were calculated using all unique hospitalizations or ambulatory encounters for Soldiers with at least one encounter for that specific disorder or subcategory in any diagnostic position stratified by time period. All bed days for all hospitalizations per Soldiers were totaled to examine the average number of bed days overall and by specific disorder or subcategory for each time period. Due to the large number of prescription drug classes, drug classes were combined into general therapeutic classes (e.g., cardiac drugs). The general therapeutic classes examined in this study were skeletal muscle relaxants, cardiac drugs, hypotensive agents, vasodilating agents, central nervous system agents, analgesics/antipyretics, anticonvulsants, psychotherapeutic agents, and anxiolytics/sedatives/hypnotics. The total number of fills and the total number of days supply for prescription drugs, both overall and by therapeutic class, were calculated by counting all unique drug fills, based on the date of the drug fill and the name of the drug, for each time period.

A Soldier may be considered unfit for military service and disability discharged due to either a single disease/disorder or their combined effect. The medical conditions are based on the VASRD codes, which were designed for the purpose of disability rating and compensation rather than medical diagnoses, and do not necessarily correlate directly to ICD-9 diagnostic codes. In this study, multiple VASRDs were combined to create the disability subcategories of interest, which were based on diseases or disorders possibly related to the consequences of blast exposure. A total disability rating, calculated by the PDA, is based on disability ratings for each individual medical condition and is expressed as a percentage. The total disability rating is then used to assign a disability disposition. Service members receiving a rating of 20% or less are usually separated with a one-time severance payment, while those receiving a rating of 30% or greater are eligible for disability retirement benefits, which include lifetime monthly retirement pay and access to MHS medical care. The disability evaluation board may deem a Soldier's medical condition(s) as not unfitting; these Soldiers are designated as fit and may continue military service. Soldiers may be evaluated for disability more than once, particularly if the severity of their disease or disorder could change over time. In these cases, the final disposition, disability rating, and medical conditions were collected from the most recent disability record.

Statistical Analysis

Univariate analysis was used to characterize and compare the distribution of variables of interest between the exposed and unexposed populations. Frequencies and proportions were utilized to examine the categorical variables, including demographic (e.g., race), military (e.g., MOS), deployment (e.g., number of deployments), and disability discharge (e.g., disability rating) characteristics. Means and standard deviations were calculated for continuous variables, including total length in service, total deployment duration, total number of conditions at disability evaluation, and length in service to first deployment or to disability evaluation. Cox proportional hazards regression models calculated crude and adjusted relative risks and associated 95% confidence intervals and were utilized to determine which factors are more likely to occur in the Exposed group than the Unexposed group (23). The adjusted regression models controlled for potential confounding from race category or educational level at military entry.

Univariate analysis was also used to compare healthcare utilization patterns, including the average number of healthcare encounters and the average number of bed days, as well as prescription drug utilization, including the average number of fills and the average days' supply, which were characterized overall and by group of pathology (e.g., respiratory) or specific nosology (e.g., headaches). To assess whether certain disorders, disease or disorder subcategories by system, or prescription medication use are significantly more common among those occupationally exposed to blast from a clinical standpoint, relative risks, and associated 95% confidence intervals were calculated using Cox proportional hazards regression. Adjusted relative risks and associated 95% confidence intervals, controlling for race and education, were also calculated.

All statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). Given that results are reported as risk ratios and confidence intervals, *p*-values are not reported separately.

RESULTS

Table 2 describes and compares the demographic, deployment, and military characteristics of the population occupationally exposed to blast (Exposed group; $n = 50{,}254$) and the population less likely to be occupationally exposed to blast (Unexposed group; $n = 50{,}254$). The occupation with the largest proportion within the Exposed group was Cannon Crewmember (43%), followed by Combat Engineer and Special Forces, which each comprised 20% of the Exposed group. The most common occupations in the Unexposed group were Quartermaster (29%), Field Mechanical Maintenance (21%), Signal (14%), Motor Transport Operators (11%), and Military Intelligence (11%). The group occupationally exposed to blast was slightly more likely to be white and have a higher education level at military entry than the Unexposed group. Of those who were deployed, both groups were deployed a similar number of times, yet the Exposed group was deployed, on average, slightly earlier (Exposed = 1.75 years of service; Unexposed = 1.90 years of service) and for a slightly longer duration (Exposed = 15.2 months; Unexposed = 14.8 months) than the Unexposed group. Soldiers occupationally exposed to blast had a slightly shorter time in military service than those unlikely to be exposed to blast (Exposed = 4.5 years; Unexposed = 4.9 years). In regard to attrition for each group across the three time periods, attrition rates for Exposed and

TABLE 2 | Demographic and military characteristics of the study population.

	Exposed ($n = 50{,}254$)	Unexposed ($n = 50{,}254$)	Adjusted RR*	95% CI
	%	%		
Race at entry				
White (ref)	83.54	66.95	1.00	–
Black	10.73	26.19	0.52	0.50–0.53
Other	5.73	6.86	0.81	0.78–0.85
Education at entry				
<HS	1.02	0.90	1.06	0.97–1.17
HS Diploma/GED (ref)	81.98	84.58	1.00	–
Some college	10.21	9.20	1.09	1.06–1.12
Bachelor's or higher	5.97	4.28	1.21	1.16–1.26
Missing	0.82	1.05	–	–
Occupation at entry				
Cannon crewmember	43.48	–	–	–
Combat engineer	20.38	–	–	–
Special forces	20.01	–	–	–
EOD specialist	12.89	–	–	–
Indirect fire infantry	3.24	–	–	–
Quartermaster	–	29.13	–	–
Field mechanical maintenance	–	21.40	–	–
Signal	–	13.95	–	–
Motor transport operator	–	11.05	–	–
Military intelligence	–	10.99	–	–
Military police	–	7.32	–	–
CBRN specialist	–	2.24	–	–
Engineers other than combat	–	1.97	–	–
FA radar operator/surveyor	–	1.01	–	–
Dog handler	–	0.56	–	–
Psychological operations	–	0.40	–	–
Deployment count				
0	41.91	41.91	–	–
1	33.02	35.46	–	–
2	15.75	14.59	–	–
3+	9.32	8.04	–	–
Time in years to first deployment (mean ± SD)	1.75 ± 1.21	1.90 ± 1.42	0.95	0.94–0.95
Total months deployed (mean ± SD)	15.23 ± 10.04	14.77 ± 9.56	1.01	1.00–1.01
Years in service (mean ± SD)	4.54 ± 3.12	4.87 ± 3.19	0.98	0.97–0.98

HS, high school; EOD, explosive ordinance disposal; FA, field artillery; CBRN, chemical, biological, radiological, nuclear.

**Adjusted models control for race and education at military entry.*

Unexposed groups at years 1–7 were 11 and 9%, respectively, and at years 8–14 were 86 and 85%, respectively.

There was no difference between the two groups for the average numbers of hospitalizations and the average number of bed days for each disease or disorder in all time periods. There was no difference in the risk of hospitalization of both groups overall and for disorder subcategory across all time periods, with the exception of a hospitalization for injury or poisoning (**Table 3**). The Exposed group was 16–25% more likely to be hospitalized for an injury or poisoning than the Unexposed group. When adjusted for demographic characteristics, the Exposed group continued to be more likely to be hospitalized for an injury or poisoning up to the first 7 years in service. The most common reasons for an injury/poisoning-related hospitalization in both groups were ankle fractures and heat stroke, which are not typical presentations of occupational exposure to blast. TBI diagnosis is associated with a specific traumatic event rather than chronic exposure, and in these data, TBI was not among the five most common reasons for an injury/poisoning-related hospitalization, but we included it in **Table 3** as a specific diagnosis. The ICD-9 codes associated with TBI were assigned more frequently to the Exposed group than to the Unexposed group, and the adjusted risk ratio increased with time. **Table 4** shows the three most common ICD-9 codes associated with TBI for each group and each time period.

Exposed Soldiers were not more likely than Unexposed Soldiers to have a larger average number of ambulatory encounters for each disorder or subcategory in all time periods. Soldiers occupationally exposed to blast had either the same or lower risk of having an ambulatory encounter both overall and for all disease or disorder subcategories than Soldiers unlikely to be exposed to blast, with the exception of diseases of the circulatory system (**Table 5**). The risk of an encounter for a circulatory system disease was higher in only the first 12 months of service (aRR = 1.21, 95% CI = 1.18–1.25). With regard to specific disorders likely to be associated with chronic exposure to blast, the Exposed group was more likely to have an ambulatory encounter for tinnitus in the first 12 months of service (aRR = 1.19, 95% CI = 1.03–1.37), between the first and 7th years of service (aRR = 1.21, 95% CI = 1.16–1.26) and after the 8th year of service (aRR = 1.31, 95% CI = 1.18–1.45) (**Figure 1**). As with the hospitalization data, TBI was not among the five most common reasons for an injury-/poisoning-related ambulatory encounter, but we included it in **Table 5** as a specific diagnosis. As with the hospitalization data, the ICD-9 codes associated with TBI were assigned more frequently to the Exposed group than to the Unexposed group, and the adjusted risk ratio increased with time. **Table 6** shows the three most common ICD-9 codes associated with TBI for each group and each time period.

The Exposed group was not more likely to have more prescription fills or be prescribed for a longer duration of time (days' supply) for any of the drug classes of interest than the Unexposed group at any time period in both the crude and adjusted models.

Approximately 9.5% of the Exposed group and 8.7% of the Unexposed group were evaluated for disability discharge, and the Exposed group was slightly more likely to be evaluated

TABLE 3 | Risk of hospitalization overall and by disease/disorder.

	First 12 months				Years 1–7				Years 8–14			
	Exposed (*n* = 50,254)	Unexposed (*n* = 50,254)	aRR*	95% CI	Exposed (*n* = 44,520)	Unexposed (*n* = 45,937)	aRR*	95% CI	Exposed (*n* = 6,791)	Unexposed (*n* = 7,725)	aRR*	95% CI
	%	%			%	%			%	%		
Any disease/disorder	3.73	3.63	1.01	0.96–1.05	9.11	9.00	1.01	0.97–1.04	4.46	4.58	1.02	0.91–1.15
Specific disorder												
Headache	0.05	0.09	0.77	0.53–1.13	0.23	0.25	0.96	0.80–1.17	0.34	0.35	1.00	0.66–1.51
Sleep disturbance	0.02	0.04	0.65	0.33–1.24	0.23	0.26	0.92	0.76–1.11	0.19	0.23	0.89	0.52–1.54
Tinnitus	<0.01	0	1.83	0.36–13.02	0.02	0.01	1.25	0.65–2.40	0.04	0.01	1.42	0.46–4.39
TBI	0.08	0.05	1.23	0.90–1.66	0.32	0.17	1.32	1.12–1.55	0.16	0.03	1.85	1.02–3.34
Disease/disorder subcategory												
Respiratory	0.78	0.95	0.89	0.81–0.99	1.13	1.20	0.99	0.90–1.08	0.68	0.76	1.02	0.76–1.36
Nervous system/sense organs	0.23	0.22	0.99	0.83–1.19	1.32	1.13	1.07	0.99–1.16	1.24	1.20	1.03	0.83–1.28
Psychiatric	1.27	1.22	1.01	0.93–1.09	4.13	4.30	0.98	0.94–1.03	2.46	2.61	0.99	0.85–1.15
Endocrine, nutritional, immunity	0.22	0.21	1.02	0.85–1.23	0.77	0.72	1.03	0.92–1.14	0.59	0.61	1.06	0.77–1.45
Circulatory	0.25	0.21	1.10	0.93–1.32	0.95	1.04	0.99	0.90–1.09	0.88	0.91	1.00	0.76–1.31
Digestive	0.61	0.69	0.93	0.83–1.04	1.95	2.13	0.95	0.89–1.02	1.28	1.26	1.03	0.83–1.27
Symptoms/Ill-defined conditions	0.55	0.62	0.94	0.84–1.06	1.66	1.80	0.97	0.90–1.04	1.19	1.09	1.10	0.89–1.38
Injury/poisonings	1.18	0.78	1.19	1.10–1.29	3.76	2.76	1.15	1.09–1.20	1.27	0.85	1.23	0.99–1.52

**Adjusted models control for race and education at military entry.*

TABLE 4 | Most common traumatic brain injury (TBI) international classification of diseases, ninth revision (ICD-9) codes at hospitalizations.

	Exposed	Unexposed	RR	95% CI
First 12 months	***n* = 41**	***n* = 26**		
Concussion	43.90%	38.46%	1.14	0.63–2.07
Head injury, unspecified	21.95%	23.08%	0.95	0.38–2.36
Fracture of base of skull	9.76%	11.54%	0.84	0.21–3.48
Years 2–7	***n* = 143**	***n* = 76**		
Concussion	59.44%	50.00%	1.19	0.91–1.55
Intracranial injury nos	17.48%	10.53%	1.66	0.79–3.50
Fracture of base of skull	10.49%	15.79%	0.66	0.33–1.35
Years 8–14	***n* = 13**	***n* = 3**		
Post-concussion syndrome	38.46%	0.00%	–	–
Concussion	23.08%	100.00%	0.23	0.09–0.62
Intracranial injury nos	23.08%	0.00%	–	–

Categories are not mutually exclusive.

for disability than the Unexposed group (aRR = 1.05, 95% CI = 1.02–1.08) (**Table 7**). The majority of those evaluated for disability discharge in both exposure groups was separated with a one-time severance payment (48%) or medically retired (45–47%). Overall, the Exposed and Unexposed groups had a similar distribution for dispositions and ratings, and both groups had, on average, 1.7 conditions that were evaluated for disability. The group occupationally exposed to blast had a slightly shorter term of service until evaluation for disability (aRR = 0.98, 95% CI = 0.97–0.99) and had a higher risk of being evaluated for a nervous system- or sense organ-related disability between their 1st and 7th year of military service (aRR = 1.15, 95% CI = 1.07–1.25) than the Unexposed group (**Table 8**). No differences in the risk of disability evaluation for any of the other disease or disorder subcategories of interest in any time period met statistical criterion.

DISCUSSION

Considering the wide range and large number of endpoints considered, there were surprisingly few differences between the exposure groups that met statistical criterion. There were no substantial differences, even when statistically significant, in number of deployments, time to first deployment, duration of deployment, and time in service, and most other factors considered.

Frequency of hospitalization was rare for both groups and did not differ between exposure groups except for the injury or poisoning subcategory. Because the Exposed group included only combat arms occupations while the Unexposed group included only combat support and combat service support

TABLE 5 | Risk of an ambulatory encounter overall and by disease/disorder.

	First 12 months				Years 1–7				Years 8–14			
	Exposed (*n* = 50,254)	Unexposed (*n* = 50,254)	aRR*	95% CI	Exposed (*n* = 44,520)	Unexposed (*n* = 45,937)	aRR*	95% CI	Exposed (*n* = 6,791)	Unexposed (*n* = 7,725)	aRR*	95% CI
	%	%			%	%			%	%		
Any disease/disorder	98.95	99.10	0.92	0.84–1.00	95.37	96.36	0.87	0.83–0.91	66.04	69.73	0.89	0.89–1.00
Specific disorder												
Headache	5.23	6.66	0.90	0.86–0.93	11.20	14.69	0.87	0.85–0.90	10.19	11.57	0.98	0.90–1.06
Sleep disturbance	1.23	1.53	0.88	0.81–0.95	14.42	16.84	0.91	0.89–0.94	14.86	17.02	0.95	0.89–1.02
Tinnitus	0.39	0.24	1.19	1.03–1.37	5.38	3.33	1.21	1.16–1.26	5.71	3.20	1.31	1.18–1.45
TBI	1.11	0.91	1.09	1.00–1.18	7.63	5.65	1.15	1.11–1.19	7.17	4.92	1.23	1.12–1.35
Disease/disorder subcategory												
Respiratory	50.34	52.40	0.96	0.94–0.97	40.04	49.51	0.82	0.81–0.84	25.36	33.79	0.82	0.77–0.87
Nervous system/sense organs	47.62	51.72	0.92	0.90–0.93	57.25	61.92	0.90	0.88–0.92	38.43	43.63	0.93	0.88–0.98
Psychiatric	18.65	20.80	0.92	0.90–0.94	49.66	52.69	0.94	0.93–0.96	35.05	37.80	0.97	0.92–1.02
Endocrine, nutritional, immunity	6.25	7.04	0.93	0.90–0.97	19.33	25.53	0.82	0.80–0.84	16.35	22.20	0.83	0.78–0.89
Circulatory	9.99	6.57	1.21	1.18–1.25	18.45	23.06	0.88	0.85–0.90	16.05	20.28	0.90	0.84–0.96
Digestive	17.23	20.93	0.89	0.87–0.91	32.00	42.55	0.79	0.78–0.81	19.76	25.22	0.87	0.82–0.93
Symptoms/Ill-defined conditions	30.65	36.20	0.88	0.88–0.91	55.81	63.56	0.86	0.85–0.88	39.36	44.88	0.92	0.88–0.97
Injury/poisonings	40.49	40.58	1.00	0.98–1.02	55.86	61.71	0.89	0.87–0.91	31.06	35.02	0.94	0.89–0.99

**Adjusted models control for race and education at military entry.*

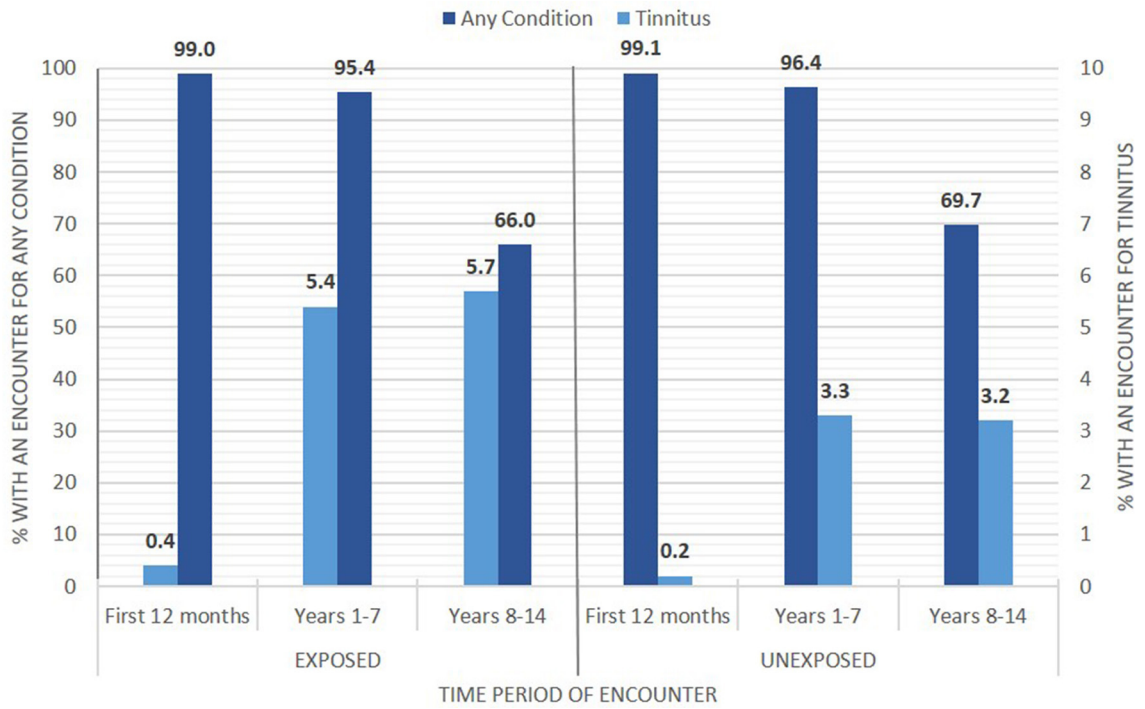

FIGURE 1 | Ambulatory encounters across three time ranges of military service showing rates for tinnitus diagnosis and for any other diagnosis for each group: Exposed (left) and Unexposed (right).

occupations, this difference is not unexpected. The primary reasons for injury or poisoning were ankle injury and heat stroke, which could reflect different training and operational environments. An infrequent reason for hospitalization was TBI. Although infrequent, there was a difference between groups in hospitalization for TBI in that the Exposed group showed the higher frequency at the later two time periods examined, suggesting a relationship to chronic exposure within those MOSs. TBI, in this case concussion most frequently, is associated with a specific exposure rather than chronic exposure, so the relevance of this association to the primary hypothesis is not obvious. It has been suggested elsewhere (24, 25) that chronic exposure to blast, such as is characteristic of some MOSs, may increase vulnerability to future TBI. The association in the hospitalization data we report here may be further evidence for that hypothesis.

In the ambulatory encounter data, the Exposed group had a higher frequency of circulatory system diseases; however, the risk of encounters was only higher for the first 12 months of service. For all other periods, it was lower among the Exposed group. The findings regarding circulatory diseases may reflect random statistical variation across many potential endpoints.

The findings regarding tinnitus are more interesting, as there is clear biological plausibility for a causal relationship between exposure and endpoint. The risk was higher among exposed Soldiers at every period of follow-up. Further investigation of tinnitus (see **Supplemental Tables and Figure**) was conducted to assess the overall risk of tinnitus diagnosis, regardless of the diagnosis order (hospitalization or ambulatory encounter). This supplemental analysis similarly found that exposed Soldiers are at an increased risk of being diagnosed with tinnitus during service [relative risk (RR), 1.75; 95% CI, 1.65–1.85], and an analysis of exposure time found the highest period of risk of diagnosis at 3–4 years of service (RR, 1.89; 95% CI, 1.62–2.18). Among those Soldiers diagnosed with tinnitus, the Exposed group was more likely to be disability discharged (RR, 1.59; 95% CI, 1.45–1.76) or attrit from service (RR, 2.42; 95% CI, 2.17–2.70) than the Unexposed group.

The findings regarding TBI are an echo of the hospitalization findings, in that there is an elevation of risk that occurs with more years of service and, assumedly, more years of exposure in the MOSs selected for occupational blast. There is an interesting difference in the ambulatory encounter data on TBI. Concussion is the most frequently appearing code, but the code for post-concussion syndrome also appears in the top three occurring codes for the majority of Soldiers with TBI. This seems reasonable because post-concussion syndrome is unlikely to result in hospitalization, but this may also be the evidence of a medical outcome associated with chronic exposure to blast. Post-concussion syndrome is associated with a specific traumatic event rather than chronic exposure, but post-concussion syndrome is divorced in time from the traumatic event, with symptoms that can be present weeks or months after injury. Furthermore, those symptoms are consistent with symptoms reported by Soldiers exposed to occupational blast (e.g., headache, dizziness, sleep difficulty, concentration difficulty). Greater frequency of post-concussive syndrome was

TABLE 6 | Most common traumatic brain injury (TBI) international classification of diseases, ninth revision (ICD-9) codes at outpatient encounters.

	Exposed	Unexposed	RR	95% CI
First 12 months	***n* = 559**	***n* = 455**		
Concussion	53.67%	45.71%	1.17	1.03–1.33
Head injury, unspecified	37.21%	46.37%	0.80	0.69–0.93
Post-concussion syndrome	14.67%	12.75%	1.15	0.84–1.57
Years 2–7	***n* = 3,398**	***n* = 2,596**		
Concussion	57.83%	55.32%	1.05	0.99–1.09
Post-concussion syndrome	29.27%	26.25%	1.11	1.02–1.21
Late effect of intracranial injury w/out mention of skull fracture	19.75%	14.75%	1.34	1.19–1.50
Years 8–14	***n* = 487**	***n* = 380**		
Concussion	46.41%	48.16%	0.96	0.84–1.11
Intracranial injury nos	33.88%	27.89%	1.21	0.99–1.49
Late effect of intracranial injury without mention of skull fracture	33.06%	35.26%	0.94	0.78–1.13

Categories are not mutually exclusive.

TABLE 7 | Risk of disability and disability characteristics of the study population.

Risk of disability in full population	**Exposed (*n* = 50,254)**	**Unexposed (*n* = 50,254)**	**aRR***	**95% CI**
	%	**%**		
Never evaluated for disability (ref)	90.49	91.34	1.00	–
Evaluated for disability	9.51	8.66	1.05	1.02–1.08
Disability characteristic	**Exposed (*n* = 4,781)**	**Unexposed (*n* = 4,350)**	**aRR***	**95% CI**
	%	**%**		
Disability disposition				
Retired	47.08	45.49	1.01	0.95–1.08
Separated with severance (ref)	48.04	48.64	1.00	–
Separated w/out benefits	3.14	3.59	1.02	0.86–1.21
Fit	0.71	1.20	0.76	0.54–1.07
Other	1.02	1.06	0.99	0.75–1.32
Combined rating				
0	7.84	6.78	1.09	0.97–1.23
10 (ref)	26.23	26.69	1.00	–
20	13.68	15.03	0.95	0.87–1.05
30	9.12	10.32	0.95	0.85–1.06
40	7.82	6.67	1.07	0.95–1.20
50	9.16	8.67	1.00	0.90–1.12
60	6.59	6.21	1.03	0.91–1.17
70	7.74	6.87	1.04	0.92–1.17
80	2.95	3.08	0.97	0.81–1.16
90	1.23	1.01	1.06	0.81–1.38
100	2.26	2.60	0.95	0.78–1.16
Unrated	4.48	5.24	0.98	0.84–1.13
Missing	0.90	0.83	–	–
Rating by disability eligibility				
<30% (ref)	53.23	54.71	1.00	–
≥30% (disability retirement)	46.77	45.29	1.01	0.95–1.08
# of conditions evaluated (mean ± SD)	1.77 ± 1.21	1.71 ± 1.12	1.02	0.99–1.04
Time in service to disability evaluation in years (mean ± SD)	4.65 ± 3.09	4.86 ± 3.08	0.98	0.97–0.99

**Adjusted models control for race and education at military entry.*

also observed in the hospitalization data in the Exposed group for the longest time period of service, but the low number of persons in those data did not warrant standalone inference.

Exposed group members were slightly more likely to be evaluated for medical disability, but not more likely to receive any particular disability disposition or to have a higher-rated disability. They were more likely to be evaluated for a nervous system/sense organs system condition between 1 and 7 years of service, which is consistent with the observations for tinnitus in the ambulatory encounter data.

The non-specific and generally negative findings of these analyses do not support broad detrimental effects of occupational exposure to blast on health care or disability outcomes of Soldiers. The finding of tinnitus, however, does reflect specific detrimental effects that may be associated with blast exposure, particularly in the observation that odds of tinnitus diagnosis increase with apparent duration of exposure. This finding in clinical records is consistent with previous research showing self-reported tinnitus symptomology association with chronic exposure to blast (3).

The findings of TBI and post-concussion syndrome as associated with chronic blast exposure echo the pattern of tinnitus, but TBI and post-concussion syndrome are rarer conditions in these data. These findings were overlooked in initial analyses, partially due to the comparatively larger associations with musculoskeletal injury and partially due to the nature of these diagnoses' association with exposure to specific traumatic events rather than chronic exposure. In follow-up analyses, the association between exposed MOSs and TBI and post-concussion syndrome emerged. Interpreting these conditions as risks from chronic exposure to occupational blast must be considered alongside the potential that these conditions are confounding factors, potentially serving as additional causes of other conditions associated with blast exposure. That consideration seems more relevant for TBI than post-concussion syndrome, which seems more likely to be greater in frequency for blast-exposed MOS as a result of their chronic exposure. Taken together, these findings suggest particular attention to tinnitus, TBI, and post-concussion syndrome by medical personnel in evaluations of Soldiers with some routine exposure to explosives and heavy weapons, in both combat and training environments. This suggestion may not seem surprising, but this study was conducted because it was unknown if or how effects from

TABLE 8 | Risk of evaluation for disability by disease/disorder subcategory.

Disability disease/disorder subcategory	First 12 months				Years 1–7				Years 8–14			
	Exposed	Unexposed	aRR*	95% CI	Exposed	Unexposed	aRR*	95% CI	Exposed	Unexposed	aRR*	95% CI
	(*n* = 263)	(*n* = 229)			(*n* = 3,779)	(*n* = 3,401)			(*n* = 715)	(*n* = 705)		
	%	%			%	%			%	%		
Psychiatric	6.46	3.49	1.58	0.89–2.81	26.07	25.05	1.01	0.93–1.09	55.66	48.65	1.10	0.94–1.27
Nervous system/sense Organs	7.60	7.42	1.04	0.64–1.68	20.40	15.32	1.15	1.07–1.25	29.37	23.40	1.15	0.97–1.35
Digestive	1.14	0.44	1.14	0.36–3.67	1.93	1.88	0.99	0.78–1.25	2.80	1.84	1.21	0.76–1.93
Respiratory	3.04	3.06	1.07	0.52–2.23	4.42	5.23	0.97	0.83–1.14	2.24	3.69	0.87	0.52–1.43
Circulatory	1.90	1.75	1.15	0.46–2.88	1.69	2.09	0.94	0.73–1.21	1.40	2.70	0.69	0.37–1.30
Endocrine/immunity	2.28	2.18	1.02	0.41–2.50	1.19	1.59	0.92	0.68–1.23	1.26	1.84	0.90	0.46–1.75

**Adjusted models control for race and education at military entry.*

repeated exposure to low-level blast may be captured in standard of care medical records.

Another consideration from these results, based on analyses of data drawn from standard of care medicine, is that blast-related effects from lower-level exposures may be below threshold in standard of care. The anecdotal reports and research evidence suggest that effects from low-level blast, if any, are small and accrue over long duration of repeated exposures and, thus, may require longitudinal evidence during clinical encounters for detection, especially with populations who are high functioning at baseline (26). There are current initiatives for such longitudinal assessment programs, and research evidence provides rationale for further exploration. According to Public Law 116-92 Section 717, the Department of Defense is to develop and employ a systematic means of objectively recording blast exposures among all service members or among those in select communities, yielding an exposure-based record system that would provide significant power in the evaluation of risk from exposure to blast, for both acute and chronic effects. There are limitations in epidemiological studies, and further research is warranted. A key limitation in this design was the use of MOS as a proxy for exposure to blast at an occupational level. MOS lacks precision in this approach, but there is not a separate measure of blast exposure currently available. The support for this use of MOS is in the associated training requirements and in the sample size for this study. Our use of multiple MOSs instead of a single MOS did likely introduce further limitations in precision for blast exposure history. The detonation of high explosive in training with hand grenades or in explosive breaching yields a different blast overpressure wave than does combustion of propellant in artillery or heavy weapons. However, for the purposes of the present study, we adopted the position that the differences in exposures between types of blast events were small relative to differences between our categories of MOSs, Exposed vs. Unexposed. From the examples in **Table 1**, following basic training, the experiences and further training for Cannon Crewmembers and Quartermasters are quite different. The size achieved for the study group increased statistical power in the analysis, needed to compare these groups for which there was no known clinical difference. The detection of a difference in tinnitus shown in these results suggests that this method for discovery was effective. In addition, this finding was consistent with a previous epidemiological study that showed association between combat arms MOSs (infantry) and risk for auditory injury (17), although that study approach used injury codes to search for MOS association rather than the approach presented here, using MOS categories to evaluate differences in injury rates. To further assess the potential for risk from occupational exposure to blast, following studies could explore annual hearing assessment results in conjunction with MOS as an additional proxy for longitudinal exposure to blast. Assays of periodically collected biological samples in the Department of Defense Serum Repository could play a similar role. Although physiological markers of low-level blast exposure have been elusive (7), there is emerging evidence that blood-based assays among personnel with a history of exposure to blast shows epigenetic differences from comparable personnel who do not have history of blast

exposure (27). At minimum, the exploratory research design employed here with Soldiers could be replicated with comparable US Marine Corps populations.

In addition, the role(s) of TBI diagnoses should be followed up with studies designed for that examination. In the data presented here, TBI diagnosis occurred at rates of 7% and less across the exposure groups and time periods compared, and those comparison conditions were not balanced in size. Drawing inferences regarding TBI as an exposure effect, or cause of other outcomes, or both would be better served by a study designed for that purpose. Such future studies could also explore the role of exposure to high-energy blast exposure events in greater detail, including as design parameters length of deployment as well as time period and location of deployment. These considerations were outside the scope of the study presented here.

One further challenge encountered in the research presented here was the rate of attrition observed across the 14-year span of the studied records. The neurological insults hypothesized to result from exposure to occupational blast have been compared to chronic traumatic encephalopathy (CTE) diagnosis, and CTE among athletes has been identified as having an average latency of 15 years between exposure and symptomology, or 8 years after retirement from activity (28). In the data presented here, the average service duration for over 100,000 Soldiers was <5 years, which is consistent with other estimates of average length of service for enlisted personnel at 7 years (29). A longitudinal exposure monitoring program for active duty US military populations such as that described in Public Law 116–92 would face a challenge parallel to a challenge in the data presented in this study, the limited ability to longitudinally track health-related phenomena that have relatively long incubation periods. Veterans Affairs records may offer some advantages to protect study designs against the attrition rate observed here, but those records are not complete capture of population data in the same way as active duty military medical records. The present study was an exploration for evidence of occupational blast-related changes that reached clinical significance for active duty personnel.

In further studies of outcomes related to occupational blast exposure, low-level blast exposure, finding no clinically relevant occupational blast exposure-related effects would be welcome. Effects from blast exposure that are limited to transient phenomena (e.g., effect on immediate performance) and that are entirely reversible would be able to be managed differently. However, studies such as the one presented here are necessary to learn if injury is associated, especially as definitions of injury can change with time and advances in medical science. In the near term, this study points to opportunities for providers to monitor closely hearing conservation programs and for developers to enhance hearing protection and mitigate the elevated risk for tinnitus in these occupational blast-exposed populations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available because they are drawn from medical records systems and the Privacy Act applies. Requests to access the datasets should be directed to each of the repositories identified in the article as a data source.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Walter Reed Army Institute of Research Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

WC conceived of the presented idea. WC, AK, and NW developed the theory. AK, CT, and NW and performed the computations and verified the analytical methods. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00619/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sex-Related Differences in Neurosensory Alterations Following Blunt Head Injury

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Background: There is heterogeneity in neurosensory alterations following mild traumatic brain injury. Commonly assessed neurosensory symptoms following head injury include symptom reports and measures of oculomotor impairment, auditory changes, and vestibular impairment.

Hypothesis/Purpose: Neurosensory alterations are prevalent acutely following mild traumatic brain injury secondary to blunt head trauma during collegiate varsity sports and may vary by sex and sport.

Study Design: Retrospective study of a large collegiate athletic database.

Methods: Analyses were performed using an established single University dataset of 177 male and female collegiate varsity athletes who were diagnosed with concussion/mild traumatic brain injury between September 2013 and October 2019. Descriptive and comparative analyses were performed on individual and grouped acute concussion assessments pertaining to neurosensory alterations obtained within 72 h of injury using components of the Sports Concussion Assessment Tool Version 5 and Vestibular/Ocular-Motor Screening.

Results: Females had significantly more abnormal smooth pursuit (p-value: 0.045), convergence (p-value: 0.031), and visual motion sensitivity tests results (p-value: 0.023) than males. There were no differences in neurosensory alterations when grouped by *overall* auditory, vestibular, or oculomotor impairments. The majority of sports-related concussions occurred during football (50, 28.25%), wrestling (21, 11.86%), water polo (15, 8.47%), and basketball (14, 7.91%). Abnormal vestibular assessments were high in these top four sports categories, but statistically significant differences in overall auditory, vestibular, or oculomotor impairments were not reached by individual sport. However, water polo players had higher abnormal individual assessments related to balance reports on the sideline (60.00%, p-value: 0.045) and in the clinic setting (57.14%, p-value: 0.038) as compared to all other sports.

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Conclusion: While neurosensory alterations are prevalent in both male and female athletes acutely post-concussion, females have a higher incidence of abnormalities in smooth pursuit, convergence, and visual motion sensitivity and may benefit from early rehabilitation.

Keywords: concussion, mild traumatic brain injuries, vestibular, oculomotor, subtypes, neurosensory alterations, sports related concussion

INTRODUCTION

Neurosensory alterations following blunt head trauma can be debilitating because sensory input informs how we interact with the world. Heterogeneity in neurosensory alterations following mild traumatic brain injury (TBI), including concussion, may be related to premorbid conditions, medications, injury-related factors, and other modifying factors such as co-existing injuries or intoxication (1, 2). Some neurosensory-related symptoms are assessed in common post-concussion symptom scales and measures of vestibulo-oculomotor impairment following mild TBI in children and adults (3–5).

Neurosensory alterations are examined to various degrees in a variety of concussion assessments employed acutely, throughout recovery, and in the long-term setting. For example, the assessment of symptom burden and symptom provocation following vestibulo-oculomotor assessment has been reported in pediatric athletes in the acute setting (5, 6). Visual processing impairments are identifiable in adults following childhood concussion, upon long-term assessment (7). Though outcomes of tests used for concussion assessment have been reported at varying time-points following injury, there is limited data reporting thematically-grouped neurosensory alterations using subjective and objective measures following acute blunt head injury resulting in sports-related concussion in men and women.

Understanding the comparative prevalence of neurosensory alterations in physically fit individuals in the acute setting is particularly important to inform the range of acute impairments that may be seen in soldiers injured in austere environments, where traditional concussion assessment may not be feasible. We hypothesized that neurosensory alterations are acutely prevalent in physically fit, varsity collegiate athletes following on-field, blunt head injury resulting in sports-related concussion and that these symptoms and impairments would be higher in sports with more frequent person-to-person blunt impacts such as football and wrestling. This study presents the immediate, on-scene reports of neurosensory symptoms in varsity collegiate athletes, as well as reports from the initial concussion clinic visit within 72-h of injury.

METHODS

This study was a retrospective review of an existing sports concussion database of collegiate varsity athletes sustaining concussion at a single university. All athletes are evaluated by a concussion specialist (a neurosurgeon or sports-medicine physician) and diagnosed with concussion in accordance with the 5th Consensus Statement on Concussion in Sport recommendations upon their first clinic visit within 72-h of injury (8). Athletes completed post-concussion symptom assessments on the sideline, immediately following injury, as well as at the first concussion clinic visit. Clinic assessments included the complete Sports Concussion Assessment Tool Version 5 (SCAT5), Vestibular/Ocular-Motor Screening (VOMS), and eye-tracking utilizing virtual reality goggles. Athletes sustaining concussion from 2013 to 2019 were included in this analysis.

Measures

The SCAT5 is a concussion assessment tool that includes a post-concussive symptom evaluation, memory and cognitive screen, Glasgow Coma Scale score, and balance assessment (9). The symptom evaluation employs a rating scale of 22 symptoms with severity ratings from 0 (no symptom) to 6 (maximum symptom). Balance is assessed at the clinic visit using the Balance Error Scoring System (BESS) and on the sideline using the modified BESS.

The Vestibular Ocular Motor Screening (VOMS) measures symptom provocation while testing the ability of a patient to complete a set of balance, vision, and movement-integrated tasks (10). In our cohort, this assessment was completed by physical therapists in the initial concussion clinic visit. Patients were prompted to rate their symptom levels on a scale from 0 to 10 before testing began and following each task. The 4 patient-reported symptoms queried are "headache," "dizziness," "nausea," and "fogginess." Our cohort performed 7 tests related to the VOMS: smooth pursuit, horizontal and vertical saccades, convergence, horizontal and vertical VOR, and visual motion sensitivity.

Our institution's internal review board approved the population of this research database (IRB-47124) and de-identified data analysis was adjudicated as exempt.

Dichotomization and Statistical Analysis

Continuous scores from the SCAT5 and individual tests comprising the VOMS were dichotomized into two groups: normal and abnormal. Modified BESS and sideline symptoms were taken from the SCAT5, whereas clinic BESS, clinic symptoms, and VOMS were taken from the physical therapy screening performed within 72 h of injury at the initial concussion clinic visit with the diagnosing neurosurgeon or sports medicine physician. Sideline modified BESS and clinic BESS scores of $\geq$5 and $\geq$15, respectively, were designated as abnormal (11). The presence of a symptom, of any severity, at sideline or clinic was labeled as abnormal. For each VOMS

assessment, any single symptom with a severity ≥2 or a near point convergence of ≥5 cm was marked as abnormal (10).

A novel categorization system was used to dichotomize the BESS, VOMS, and reported symptoms as normal or abnormal based on auditory, vestibular, or oculomotor impairments. Abnormal vestibular assessments included any abnormal scoring on components of the VOMS, BESS, or symptoms of "dizziness" and/or "balance problems." Abnormal oculomotor assessments included abnormal scoring on components of the VOMS or symptoms including "vision changes," "light sensitivity," or "blurred vision." Abnormal auditory reports were designated as the presence of "noise sensitivity" or "ringing in ears."

Descriptive statistics were calculated for the subjects' demographics and scores on concussion assessments. Dichotomized variables were analyzed using chi squared or Fisher's exact test, as appropriate. Logistic regression was used to assess effect size, and Levene's test was used to asses equalities in variance between males and females with regards to outcomes. Analyses were run using Stata 14/SE for Windows (StataCorp, LP College Station, TX).

RESULTS

We identified 177 varsity collegiate athletes diagnosed with sports-related concussion/mild TBI following blunt head injury from 2013 to 2019. Within this population, 103 (58.19%) were male, and 74 (41.81%) were female, as reported in **Table 1**. The subjects participated in a variety of sports, with the majority of sports-related concussion occurring from football (50, 28.25%), wrestling (21, 11.86%), water polo (15, 8.47%), and basketball (14, 7.91%).

TABLE 1 | Demographics and subject characteristics.

Characteristic	*N* (177)	Percentage
Sex	**177**	
Male	103	58.19%
Female	74	41.81%
Sports	**177**	
Baseball	4	2.26%
Basketball	14	7.91%
Beach volleyball	6	3.39%
Fencing	4	2.26%
Field hockey	4	2.26%
Football	50	28.25%
Golf	1	0.56%
Gymnastics	6	3.39%
Lacrosse	7	3.95%
Rowing	8	4.52%
Sailing	7	3.95%
Soccer	8	4.52%
Softball	5	2.82%
Swimming and diving	7	3.95%
Synchronized swimming	1	0.56%
Track and field	1	0.56%
Volleyball	8	4.52%
Water polo	15	8.47%
Wrestling	21	11.86%

The bolded numbers represent total number of subjects related to that column.

Sex-based differences in specific portions of the VOMS were identified. Females had consistently more abnormal VOMS measurements with statistical significance being reached for smooth pursuit (*p*-value: 0.045), convergence (*p*-value: 0.031), and visual motion sensitivity tests (*p*-value: 0.023), as depicted in **Table 2**. Though males consistently had higher rates of light sensitivity and females had higher rates of noise sensitivity, these differences were not statistically significant, as reported in **Tables 2**, **3**. No significant differences were found in *grouped* vestibular, oculomotor, or auditory assessments between males and females, described in **Table 4**.

Logistic regression was used to assess neurosensory alteration differences by sex. Male athletes had 0.43 times the odds of vestibular impairment (95%CI: 0.13–1.40, *p*-value: 0.163) and 0.61 times the odds of oculomotor impairment (95%CI: 0.26–1.44, *p*-value: 0.261) relative to females. However, males had 1.10 times the odds of having hearing issues following a blunt head injury relative to females (95%CI: 0.59–2.06, *p*-value: 0.769).

Levene's test was used to assess for variance equality between groups and their outcomes. We found no significant differences in variances between sex and vestibular (*p*-value: 0.155), oculomotor (*p*-value: 0.261), and hearing (*p*-value: 0.770) impairments.

When comparing overall and grouped assessments of vestibular, oculomotor, or auditory impairments, football players had higher rates of abnormal assessments, however, statistical significance was not reached, as reported in **Table 5**. Among the sports with the highest internal concussion rates (football, wrestling, basketball, and water polo), water polo players had significantly higher rates of abnormal BESS assessments (57.14%, *p*-value: 0.038) and reports of balance problems (60.00%, *p*-value: 0.045), in the clinic and sideline settings, respectively, as reported in **Table 6**. Abnormal VOMS and other oculomotor assessments were common in the top four sports, but statistically significant differences were not reached. Overall, abnormal vestibular and oculomotor assessments were more common than reports of abnormal auditory symptoms.

DISCUSSION

This study demonstrated that females have higher rates of abnormal outcomes on specific measures of the VOMS (smooth pursuit, convergence, and visual motion sensitivity tests) as compared to males, which has been reported previously (12). The pathophysiology of this finding has yet to be determined. Females appear to be more susceptible to certain neurosensory alterations related to oculomotor control and motion perception, representing a potential injury vulnerability. Clinical assessment in the acute post-injury period is warranted to allow for the potential benefit of early rehabilitation. There is no difference in the *grouped* neurosensory alterations by sex, underscoring the importance of considering all outcomes derived from larger concussion assessment batteries.

The vast majority of concussions included in this dataset were sustained by subjects while playing football, wrestling,

TABLE 2 | Vestibular and oculomotor impairment report by sex.

Impairment report	Male		Female		*p*-value
	Abnormal		Abnormal		
Vision changes					
Sideline "blurred vision"	33	39.47%	18	32.93%	0.447
Clinic "vision change"	1	0.96%	4	5.34%	0.162
Light sensitivity					
Sideline	36	43.08%	31	56.96%	0.108
Clinic	10	9.62%	10	13.36%	0.430
VOMS (clinic)					
Smooth pursuit	58	67.98%	49	82.81%	0.045
Horizontal saccades	59	69.16%	50	83.10%	0.056
Vertical saccades	58	67.98%	47	80.77%	0.089
Convergence	64	75.08%	52	89.50%	0.031
Horizontal VOR	63	73.03%	49	84.26%	0.112
Vertical VOR	57	67.60%	46	81.88%	0.060
Visual motion sensitivity	55	70.25%	48	87.07%	0.023
BESS					
Sideline	34	40.67%	22	41.06%	0.950
Clinic	22	23.46%	26	35.79%	0.081
Dizziness					
Sideline	48	57.54%	32	58.82%	0.868
Clinic	35	33.76%	29	38.87%	0.477
Balance impairment					
Sideline	27	32.66%	24	43.99%	0.175
Clinic	2	1.92%	0	0.00%	0.511

TABLE 3 | Auditory impairment report by sex.

Hearing report	Male		Female		*p*-value
	Abnormal		Abnormal		
"Noise sensitivity"					
Sideline	33	39.47%	23	42.14%	0.742
Clinic	7	6.74%	8	10.68%	0.344
Clinic "ringing in ears"	1	0.96%	0	0.00%	1.000

TABLE 4 | Neurosensory reports by sex.

Neurosensory measurement	Male	Percentage	Female	Percentage	*p*-value
Vestibular	**103**		**74**		0.190
Normal	12	11.65%	4	5.41%	
Abnormal	91	88.35%	70	94.59%	
Oculomotor	**103**		**74**		0.258
Normal	19	18.45%	9	12.16%	
Abnormal	84	81.55%	65	87.84%	
Auditory	**103**		**74**		0.769
Normal	66	64.08%	49	66.22%	
Abnormal	37	35.92%	25	33.78%	

The bolded numbers represent total number of subjects related to that column.

water polo, and basketball, which is consistent with previous reports of concussion incidence in sport. These sports also had the highest incidence of repeat concussions, though statistical significance when grouped by neurosensory alterations was not reached. This study reported greater neurosensory alterations in football, wrestling, water polo, and basketball compared to other sports present in our dataset including baseball, beach volleyball, fencing, field hockey, golf, gymnastics, lacrosse, sailing, softball, swimming and diving, synchronized swimming, track and field, rowing, soccer, and volleyball. Statistically significant differences in grouped auditory, vestibular, or oculomotor impairments were not reached by individual sport suggesting that mechanisms of injury in relation to a specific sport may not affect neurosensory alteration patterns. When examining individual outcomes by sport, water polo players had relatively more abnormal balance reports than other sports. Differences in balance assessments and training have been queried in previous literature, including comparing land to water-sports, but the significance is unclear (13, 14).

The strengths of this research are that 100% of subjects had complete reports of pre-defined neurosensory alterations pertaining to groupings of oculomotor, vestibular, and auditory symptoms and impairments at sideline and initial concussion clinic assessment. Much data has been reported on neurosensory alterations in sub-acute and chronic settings, especially pertaining to children (3–5, 7); however, this work represents the first study to report and compare grouped neurosensory outcomes in the immediate-acute time frame in highly physically fit collegiate athletes, by sex and sport. It also supports recent work identifying sex-based differences in vestibular-oculomotor assessments.

This study was limited by a small number of subjects populating individual "other sports" which included: baseball, beach volleyball, fencing, field hockey, golf, gymnastics, lacrosse, sailing, softball, swimming and diving, synchronized swimming, track and field, rowing, soccer, and volleyball. Identifying specific differences in neurosensory alterations in association with these sports requires a larger sample size pertaining to these sports. This study was also limited by the examination of inherently subjective and semi-objective measures, such as balance assessments and symptom scale reports, which may not sufficiently reflect the scope of symptomatology or impairment (14–16). Objective evaluation of neurosensory alterations, such as pin-prick testing, hearing examinations, and Snellen's chart for vision testing, were not obtained in this sample, limiting further interpretation of more objective impairments.

Concussion causes immediate neurosensory alterations, most commonly represented by vestibular and oculomotor symptoms and impairments, followed by auditory symptoms. While concussion recovery is traditionally defined by the resolution of post-concussive symptoms, bridging the gap between symptomatology and objective measures is critical to understanding the pathophysiology of injury and its recovery (16). Future research should aim to elucidate the pathophysiology of sex-based differences in order to address more personalized care including injury prevention strategies.

TABLE 5 | Neurosensory alterations by sport.

	Abnormal vestibular		*p*-value	Abnormal oculomotor		*p*-value	Abnormal auditory		*p*-value
Sport categorized	**161**		0.152	**149**		0.196	**62**		0.801
Football	42	26.09%		39	26.17%		19	30.65%	
Wrestling	18	11.18%		16	10.74%		7	11.29%	
Water polo	15	9.32%		15	10.07%		3	4.84%	
Basketball	13	8.07%		12	8.05%		5	8.06%	
Top 4 sports combined	**88**		0.424	**99**		0.186	**39**		0.665
Other sports	**73**		0.527	**17**		0.948	**5**		0.267

The bolded numbers represent total number of subjects related to that column.

TABLE 6 | Vestibular impairment reports by sport.

Sport	Football		Wrestling		Water Polo		Basketball		Other Sport		*p*-value 1	*p*-value 2
	Abnormal		Abnormal		Abnormal		Abnormal		Abnormal			
VOMS												
Smooth pursuit	28	66.67%	9	75.00%	12	85.71%	9	64.29%	49	79.03%	0.448	0.524
Horizontal saccades	28	66.67%	8	66.67%	12	85.71%	9	64.29%	52	82.54%	0.207	0.548
Vertical saccades	28	66.67%	8	66.67%	12	85.71%	8	61.54%	49	79.03%	0.351	0.524
Convergence	30	71.43%	10	83.33%	13	92.86%	11	84.62%	52	83.87%	0.439	0.374
Horizontal VOR	29	69.05%	10	76.92%	11	84.62%	10	71.43%	52	83.87%	0.404	0.786
Vertical VOR	27	64.29%	9	75.00%	10	83.33%	9	69.23%	48	78.69%	0.514	0.692
Visual motion sensitivity	27	69.23%	8	72.73%	10	83.33%	8	72.73%	50	83.33%	0.490	0.850
BESS												
Sideline	18	43.90%	7	38.89%	2	22.22%	3	33.33%	26	44.07%	0.797	0.708
Clinic	10	22.73%	2	11.76%	8	57.14%	3	21.43%	25	32.89%	0.058	0.038
"Dizziness"												
Sideline	21	52.50%	13	68.42%	6	60.00%	4	44.44%	36	61.02%	0.691	0.584
Clinic	14	28.00%	10	47.62%	6	40.00%	6	42.86%	28	36.36%	0.557	0.390
"Balance problem"												
Sideline	11	28.21%	10	52.63%	6	60.00%	1	11.11%	23	38.98%	0.089	0.045
Clinic	2	4.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0.461	1.000

As our study demonstrated, important impairments may be determined from specific tests within larger, commonly used concussion assessments. In order to advance the field and allow for continued scientific hypotheses, the outcomes of specific tests within common concussion assessments should be analyzed for secondary outcomes, such as sex-based differences. Additionally, further research is needed to understand how targeted treatments may address these complaints early on, improving symptoms and quality of life in the acute setting, as well as shortening recovery trajectories in both females and males. Finally, research identifying neurosensory alterations contributing to common concussion subtypes, considering the presence or absence of cervical strain as an associated condition, will further inform overlap between physiologic systems and direct targeted treatments (1, 2).

DATA AVAILABILITY STATEMENT

The data analyzed in this study was subject to the following licenses/restrictions: the dataset has not been approved by Stanford University for dissemination. Requests to access these datasets should be directed to falene@stanford.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stanford University Internal Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AL-B, JC, and JG designed and conducted the study. AL-B, CM, and KN authored the manuscript. All authors contributed to major edits and revisions, and agree to be accountable for the content of the work.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Effects of Mild Traumatic Brain Injury on Cognitive-Motor Integration for Skilled Performance

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Adults exposed to blast and blunt impact often experience mild traumatic brain injury, affecting neural functions related to sensory, cognitive, and motor function. In this perspective article, we will review the effects of impact and blast exposure on functional performance that requires the integration of these sensory, cognitive, and motor control systems. We describe cognitive-motor integration and how it relates to successfully navigating skilled activities crucial for work, duty, sport, and even daily life. We review our research on the behavioral effects of traumatic impact and blast exposure on cognitive-motor integration in both younger and older adults, and the neural networks that are involved in these types of skills. Overall, we have observed impairments in rule-based skilled performance as a function of both physical impact and blast exposure. The extent of these impairments depended on the age at injury and the sex of the individual. It appears, however, that cognitive-motor integration deficits can be mitigated by the level of skill expertise of the affected individual, suggesting that such experience imparts resiliency in the brain networks that underly the control of complex visuomotor performance. Finally, we discuss the next steps needed to comprehensively understand the impact of trauma and blast exposure on functional movement control.

Keywords: movement, cognition, eye-hand coordination, sensorimotor integration, concussion, blast exposure, neuroimaging, motor psychophysics

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A COMPLEX SKILL REQUIRES A COMPLEX BRAIN: AN INTRODUCTION TO THE NEURAL CONTROL OF RULE-BASED SKILLED PERFORMANCE

Imagine

At work, you operate a drone aircraft. You have one joystick that sends the drone up or down and turns it in a circle, and another that flies it forward, back, or side to side, and all the while you look at a computer monitor showing images from the drone's camera to allow you to direct its movement. On break you enjoy a cup of tea, reaching your arm forward to pick up your teacup.

Operating the drone and reaching for the cup are examples of visually-guided arm movement that vary widely in their complexity. How are you able to perform these tasks proficiently and relatively effortlessly? Reaching for and interacting with objects in the environment is a common daily task, but one that can vary widely depending on task conditions. Looking at and then reaching for a teacup is an example of a direct interaction. The visual stimulus guiding the action is itself

the target of the action. In contrast, remotely operating a drone is an example of an indirect interaction: there is an intermediary between the action and the target. Direct interactions are guided by standard sensorimotor mappings within the brain. Indirect interactions are guided by non-standard sensorimotor mapping, and rely on different neural computations that must incorporate the spatial dissociation of gaze, attention, and overt motor output (1).

"Transformational" non-standard mappings use specific spatial algorithms to relate the position of the visual cue to the direction of action, such as relating horizontal mouse movement to vertical cursor movement. The different levels of visuomotor compatibility in transformational mappings are achieved by two sensorimotor strategies: sensorimotor recalibration and visuomotor adaptation. Sensorimotor recalibration allows for adaptation to spatial orientation differences by remapping between two sensory modalities (2–4). Because this is an implicit recalibration, there are after-effects if the source of recalibration is removed. Think of the lightness you feel for the first few steps after taking a long trek with a heavy backpack. In comparison, visuomotor adaptation, or "strategic control" (5) relies on a mental rotation to align the required limb movement to the spatial location of a target. It is more explicit, does not produce after-effects, and can require rule integration. Note that these terms are not used consistently across the motor adaptation literature [for comprehensive reviews see (6, 7)]. Lastly, in order to execute skilled movements smoothly and accurately, the brain must often account for various rule-based situations. Combining thought and action is a process known as cognitive-motor integration (CMI). CMI is essential for the completion of skilled activities that involve non-standard mappings and other complex skills, making it crucial for work, duty, sport, and daily life. While healthy adults perform CMI with little conscious awareness, these skills are not innate and can be affected by brain injury. In this paper, we review three related lines of research: sport-related head impact and motor behavior, blast-related impact and motor behavior, and functional brain neurophysiology and motor behavior. The linking theme is the neural control of rule-based sensory-guided movement in health and following mild brain injury.

A FAILURE TO COMMUNICATE: THE EFFECT OF MILD BRAIN INJURY ON COGNITIVE-MOTOR INTEGRATION BEHAVIOR

While some acquired brain injury can impose distinct focal damage to specific brain regions, it is a more diffuse injury that typically arises from blast and concussive impact (8, 9). The acceleration and deceleration forces of concussive trauma initiate a "neurometabolic" cascade. This cascade invokes a state of energy crisis from both an increased glucose requirement—needed to restore ion homeostasis—and a decreased supply of glucose through reduced cerebral blood flow (10, 11). Further, axonal injury occurs due to the mechanical shearing and tensile strain from acceleration, deceleration, and rotational forces (12). Such forces lead to altered axonal membrane permeability and ionic disruption, as well as cytoskeletal breakdown (10, 11), all of which can impair neurotransmission. The time frame, however, for the recovery from these effects and their exact relationship to concussion symptoms is not fully understood.

We propose that widespread injury can reduce rule-based skilled performance through a failure to communicate between brain networks. Cognitive brain network alterations have been observed following concussion (13–17). To assess the communication between brain areas for movement control, our group developed a touch screen based functional assessment tool using a visuomotor task that incorporates two forms of non-standard mapping: explicit rule integration (strategic control), and implicit spatial vision-to-hand-motion realignment (sensorimotor recalibration). Portable software applications to assess brain function following injury are increasingly being developed, and offer a useful, pragmatic approach to providing information to the user and their caregivers (18–22). In our task, a basic standard mapping condition has the participant place their finger over a central start target on a vertical touch screen, immediately slide their finger on that same screen directly toward a target that appears, and hold it there. There is universal agreement among the hundreds of youth who have done this task that "it is the most boring video game ever." It does get more interesting for them, however. In the remaining three cognitive-motor integration (CMI) conditions, one or both forms of non-standard mapping are introduced. In the first CMI condition, the target and cursor are viewed on the vertical touch screen, but the motion of the cursor is reversed from the motion of the finger on that screen. We often see participants talking themselves through this condition ("target is down…slide up slide up!"), suggesting that we are indeed tapping into explicit rule integration associated with this mental rotation. In the second CMI condition, the touch screen recording finger movement is now laid flat in front of them, and the targets are still presented in the same vertical location as before (on a tablet or monitor). This condition requires a more implicit sensorimotor recalibration since the guiding visual information and required motor goal are in different spatial locations. Those who have grown up with computers and video games barely register this condition as a challenge. To this blasé tech-savvy group, however, we throw our third CMI condition, a combination of the two other CMI conditions: one must now think their way through moving opposite to an extrafoveal target (**Figure 1A**). There is, without fail, a reaction of surprise to this condition. Try it yourself now if you are at a computer with a mouse! Physically turn your mouse around, grab it with the bottom end pointing out, and move your cursor to an icon on your screen. Congratulations, you have just performed cognitive-motor integration using two forms of non-standard mapping. Continue reading to find out what your brain just accomplished.

How Does Concussion History Influence CMI Performance? Are They Really "Recovered"?

There is evidence that individuals with concussion—a form of mild traumatic brain injury (mTBI)—have reduced capacity

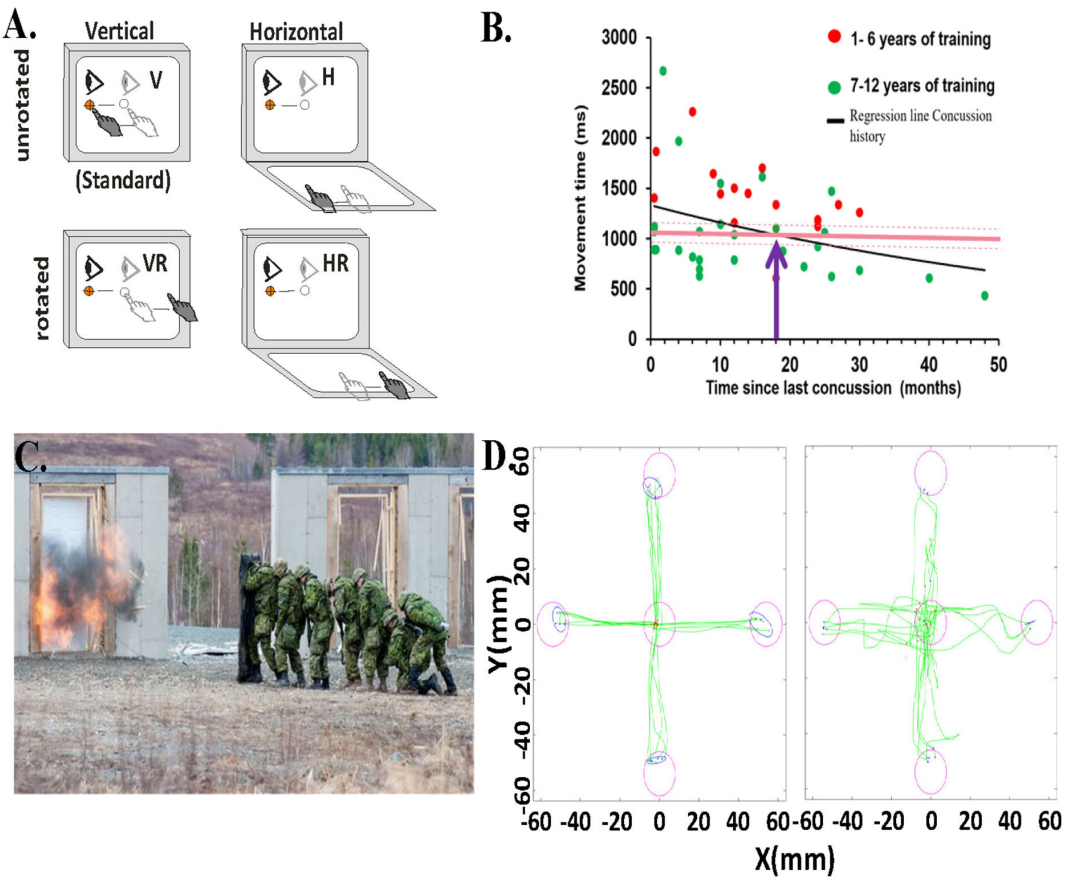

FIGURE 1 | Behavioral task and data. **(A)** Schematic of experimental conditions. Visual stimuli were presented on a vertical touchscreen. Motion was recorded on either that touch screen or a second one placed horizontally in front of the participant. Light gray eye and hand symbols indicate the start position. The dark gray eye and hand symbols depict the movement from start position toward the target. The target was presented in one of four locations (right, left, up, or down). H, horizontally placed touchscreen; V, vertically placed touch screen; R, rotated (180º) visual feedback. **(B)** Relationship between the total movement time in the CMI condition and time since last concussion for each child with concussion history and asymptomatic (dots), represented by the regression line (black line). Red dots are those children with 1–6 years of training, green dots are those with 7 or more years of sport experience. Also included is the mean total movement time of the CMI condition of the healthy children (8–16 years old) with no concussion-history (dark pink horizontal line). The light pink dotted lines indicate the upper and lower confidence intervals of the control group's total movement time. The purple arrow denotes where the two lines of the concussion history and no-history controls cross. **(C)** Canadian Forces School of Military Engineering personnel participating in breaching exercises. Photo courtesy of Haley Voutour, 5th Canadian Division Support Group. **(D)** Examples of hand movement trajectories (green) in the Standard (direct interaction) and in the Plane Change+Feedback Reversal condition ("horizontal rotated") from one control participant (left panel) and from one breacher participant (right panel).

for executive function, slower cognitive processing speeds, and attention deficits (23). These data suggest that task switching, required when performing two tasks within the *same* domain ("dual-tasking"), will be more difficult for those with concussion. Concurrent task paradigms using both task switching and integration tasks (combining behaviors from two *different* domains) have been proposed as a means to assess concussion recovery. For example, there are subtle changes in oculomotor function, gait, and balance after concussion, which become more pronounced during both dual-task and integration paradigms (23, 24). Determining if an individual has recovered from concussion is a challenge from a risk management perspective. Clinicians understand the risk of returning athletes too soon to sport and yet the tools used to determine recovery are inadequate, testing one domain at a time (e.g., motor, cognition) or relying on self-report (25, 26). Thus, many individuals are returned to work and sport with impairments that may leave them vulnerable to further injury (27, 28).

We have used our task in several sport concussion studies (29–32), and always observe significant differences in performance outcomes related to both timing and accuracy in concussed athletes vs. controls. We compared youth athletes (8–16 years old) with a history of concussion who were asymptomatic and deemed recovered (33) to age-matched controls with no history of concussion (30). There were no differences between groups on the simple, standard task. In contrast, in the non-standard task, youth who reported a previous concussion were significantly slower in moving from the start to their first

stopping point ("movement time," or MT), which was often before they got to the target. They were also slower in moving from the start to the end targets, a movement which comprised the initial ballistic MT plus the post-pause corrective movement to get to the final target (30). This finding indicates that concussion history is associated with slower non-standard skilled performance in these children, compared to their non-concussed peers. This finding was replicated in a similar cohort of youth athletes who also showed decreased accuracy and increased path variability (31). The significant differences we found in the group with a previous concussion did not subside and return to the level of the matched control group until on average 18 months post-concussion (**Figure 1B**); that's nearly two seasons later! This finding is concerning given that most youth will have returned to activity within this time frame and thus are likely more vulnerable to re-injury, concussive, or otherwise.

When we examined concussion history effects on CMI performance at the elite level, we found more subtle differences (32). In a study of 102 National Hockey League (NHL) draft prospects (mean age = 17 years, all asymptomatic) we observed that previously concussed participants had significantly decreased reaction times (RT) on the non-standard task compared to the control group, indicating that they were slower to respond to the peripheral stimulus when it appeared (32). Given the dramatic increase in speed of the game between recreational-level and NHL-caliber athletes, we suggest that even minor changes in RT to a given stimulus may represent both a hazard to player safety and an influence on player performance. Noteworthy though are the attenuated effects of concussion on CMI in these elite vs. the non-elite young athletes in our other studies. Experience in complex tasks may be linked to a neurological "reserve" that appears to compensate for behavioral performance (32). The concept of "reserve" is one that is more commonly used with respect to cognition (34, 35), posited to provide a protective effect against cognitive decline associated with aging or disease. One can think of enhanced skilled performance through years of training as offering this same level of protection against performance decline following brain injury, via more efficient and resilient brain networks needed in the control of complex skills.

What About Low-Level Blast Exposure and Cognitive-Motor Integration Performance?

In a recent study in collaboration with Development and Research Defense Canada, we examined the impact, literally, of repeated blast-wave exposure over a period of years. We did this by looking at the CMI performance of 19 breaching instructors (those who teach others how to forcefully open closed/locked entryways) and range staff having many years of service, which came with many years of exposure to low level blast explosions (**Figure 1C**). We compared their performance to that of 19 age- and sex-matched Canadian Armed Forces non-breacher control participants. Similar to what we observed in experienced athletes, our preliminary results show a significantly greater variability in reaction time ($p < 0.05$, equality of variance test) in the most challenging CMI condition in the breacher group (36). We also observed slower reaction times and worse accuracy in the standard (Cohen's D: 1.11, $p < 0.01$) and horizontal (Cohen's D: 0.94, $p < 0.01$) visuomotor conditions in the breacher group compared to controls (**Figure 1D**). The large variation in reaction time within the breacher group could be due to the differences in years of exposure to low levels of blast explosions during the breacher courses (37–40). Note that the source of performance decline may have come from subtle brain network alterations due to blast exposure (41), or could have arisen due to reduced function at the sensory input or neuromuscular output levels (or a combination of all these things). A comprehensive sensory, cognitive, and motor examination of this group (ongoing) will provide these important answers. However, it is also noteworthy that there were overall few CMI deficits in this group of breachers, similar to what we observed in the previously concussed elite-level athletes. Hence, these data suggest the presence of a motor skill reserve reflecting resilient neural movement control networks in these highly trained service members.

Overall, these studies highlight the importance of testing and assessing those affected by blast exposure and mild brain trauma with outcomes that require coordinated brain activity. Assessing motor or cognitive tasks alone do not demonstrate the ability to pick up on the interconnectivity impairments that appear to exist following concussive injuries. Put simply, we believe that multi-domain tasks reveal a failure to communicate, communication here referring to the interaction between different brain areas responsible for rule-based skilled performance.

WHAT'S GOING ON IN THERE? THE NEURAL CORRELATES OF COGNITIVE-MOTOR INTEGRATION

We know from behavioral studies that damage to the brain often results in deficits in CMI performance while leaving standard reaching intact. This observation suggests that the neural correlates of CMI differ from those used to produce standard reaching movements. To understand the additional neural control mechanisms involved in movement control using these different levels of gaze/reach dissociation, neuroimaging work in our laboratory has directly compared brain activity in tasks requiring CMI with standard visuomotor mapping. These data provide fundamental information about the neural control of complex movement, and provide insight into what is driving behavioral impairment following diffuse injury from mild head impact.

Many studies suggest that the brain couples eye and arm movements so that they are made to the same spatial location by default (42–44). Within the brain, neural signals associated with the control of the eyes and the arm converge in several regions. For example, posterior parietal cortex regions known to be essential for producing reaching movements encode reaches

in an eye-centered frame of reference (45–47). Similarly, the production of arm moments influences activity within brain areas thought to be specific for eye position control (48, 49). The existence of neural circuitry that couples reach to gaze during standard tasks predicts the existence of regions that can inhibit this pairing (50). In a recent functional MRI study, we compared brain activity during standard reaching to activity associated with a CMI task in which saccades were made toward the cued location, but arm movements were made 180° away from that location (51). This task required both decoupling reach from gaze, and incorporating an explicit rule to move the arm away from the cued target location. Patterns of activity within the cuneus region of the occipital cortex, an important sensorimotor hub (52–54), strongly distinguished between the two conditions, predicting whether the eyes and hand were going to move to the same or opposite target locations. Others have observed that damage to the cuneus is a common precursor to the development of optic ataxia (55–58), whereby patients show extremely poor accuracy when reaching for non-foveal targets (59–62). Together, these data suggest that the cuneus is likely crucial for decoupling reach from gaze during CMI tasks. We also observed that like the cuneus, spatial patterns of activity in the medial premotor cortex strongly distinguished between the CMI and standard tasks. Others have reported that when non-human primates learn two different CMI tasks, "context-dependent" cells in this region only fire for one of the two tasks (63). Further, when activity in this region of the brain is suppressed, deficits in switching between rules for different visuomotor associations occur (64). Taken together, these findings suggest that while the cuneus plays a role in allowing us to inhibit the default coupling of the eyes and arm, the medial premotor cortex is involved in incorporating explicit rules into motor plans that determine specifically how these effectors should be dissociated to satisfy the context of a given CMI task.

CMI tasks also require implicit sensorimotor recalibration. The relationships between visual information, patterns of muscle activity, and proprioceptive feedback are altered. We examined the effect of implicit recalibration in another fMRI study comparing standard hand movements to visual targets on a touch screen in the vertical plane to non-standard ones where the hand moved on a horizontally-placed touch screen (65). Both conditions generated almost completely overlapping activity in the typical motor, somatosensory, premotor, parietal, occipital, and cerebellar regions associated with visually guided reaching, and no differences in the amplitude of task-related activity. However, multi-voxel pattern analyses revealed that within these regions, patterns of activity strongly discriminated between the two tasks. As trials progressed from presentation of a cued target, to a delay period, to motor execution, task discrimination occurred in an increasing number of brain regions, eventually encompassing the majority of regions active in the two tasks (**Figures 2A–C**). In other words, although both conditions activated similar regions of the brain, the nature of this activity differed in ways that were strongly predictive of which visuomotor mapping was being performed. Taken as a whole, our imaging results in healthy adults demonstrate that each of the additional components required for CMI (i.e., inhibiting the default coupling of reach and gaze, incorporation of task-specific rules, and sensorimotor recalibration) exerts a distinct influence on how movements are accomplished by the brain.

Sex- and Injury-Related Effects on the Control of Cognitive-Motor Integration

We have observed that both CMI and standard visuomotor tasks evoke a notably more bilateral pattern of activity in premotor and parietal regions in women when compared to men. This phenomenon has been observed using both fMRI (66) and EEG (67). Such bilateral activity in reach-related regions in women might provide protective functional redundancy capable of compensating for decreased activity in either hemisphere, as demonstrated by a study in which one such region (dorsal premotor cortex) was inhibited via transcranial magnetic stimulation (68). Importantly, these sex-related differences are observed even when men and women demonstrate equal skill levels in the tasks (**Figures 2D,E**). Further, the nature of sex differences can depend upon the specific type of CMI task performed. For example, men had greater lateral sulcus activity than women for CMI tasks in which eye and hand movements were made in opposite directions but not when they moved in the same direction. Others have also observed a pattern of more bilateral brain activity in women relative to men when processing language (69, 70), emotion (71–73), and music (74). Therefore, the phenomenon of relatively bilateral patterns of activity in women is not limited to processes associated with motor control. Different sex-related patterns of brain activity imply that damage to the brain could result in different visuomotor-related deficits in men and women. These observations have important clinical implications and strongly indicate that neurological assessments and rehabilitation must ultimately be customized to best serve the needs of both male and female patients.

Preliminary imaging work has also begun to reveal how a history of concussion affects the neural correlates of CMI. Anatomical images were compared between women who were diagnosed with post-concussion syndrome (PCS) and women with no history of brain trauma (75). When comparing PCS participants to controls, we observed volume decreases in cerebellar regions that serve a variety of diverse roles including cognitive, sensorimotor, and vestibular functions (76). This wide-range of functions could be a factor in the observation that symptoms associated with PCS are quite variable (77). Diffusion-weighted images were also collected, and showed decreased fractional anisotropy (FA) that was associated with poorer CMI performance in several white matter tracts across all study participants (78). Taken with our previous observations that CMI relies upon numerous cortical regions throughout the brain, this finding emphasizes that healthy white matter connections within this network are also crucial for successful cognitive-motor integration.

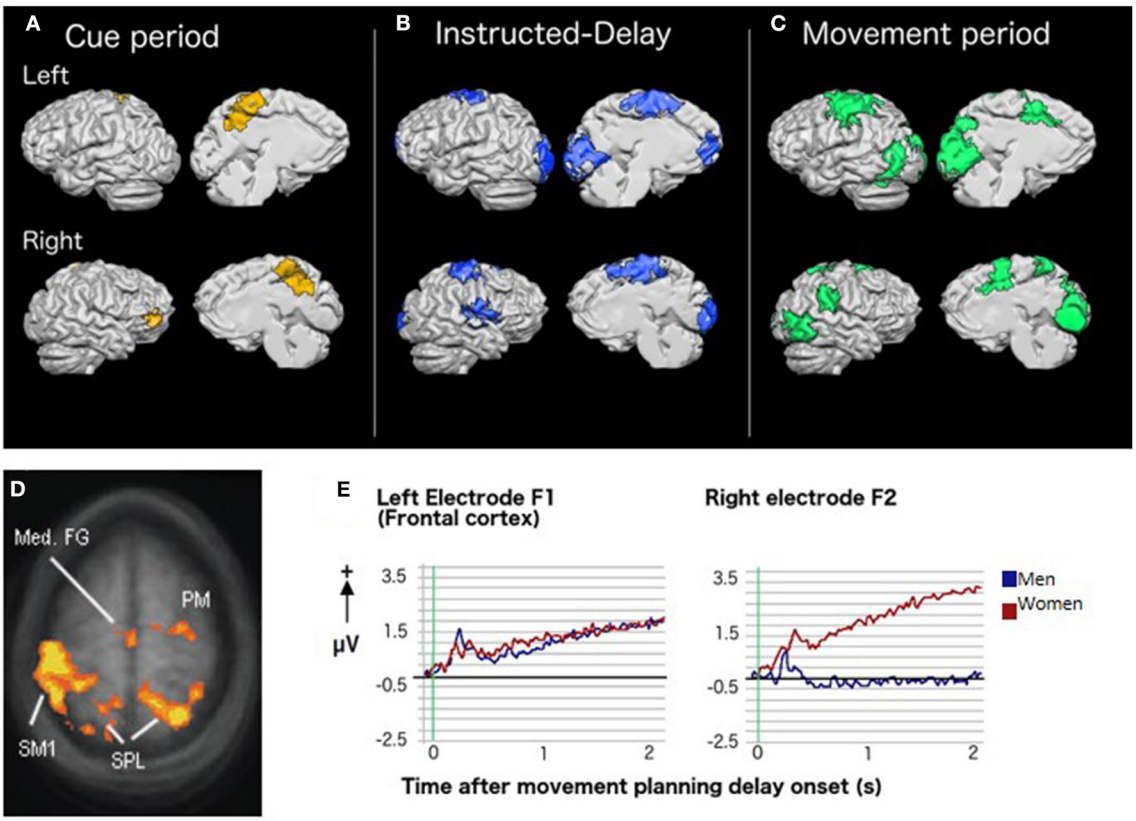

FIGURE 2 | Brain activity and CMI: Results of group whole-brain recursive feature elimination multi-voxel pattern analysis. Colored regions represent voxels in which significant decoding of the standard vs. plane-change visuomotor mapping conditions occurred during **(A)** the cue period (yellow), **(B)** the instructed-delay period (blue), and **(C)** the movement period (green). Regions are shown overlaid on a Talairach-normalized brain from one participant. Within each panel, the left hemisphere is shown at the top and the right hemisphere is shown on the bottom. Lateral surfaces are shown on the left of each panel and medial surfaces on the right [adapted with permission from (65)]. **(D)** Sex-related differences in brain activity during rule-based skill performance. Frontal and parietal cortex regions with significantly higher fMRI BOLD signal in women relative to men during non-standard movement planning [adapted with permission from (66)]. **(E)** EEG slow cortical potentials over left and right frontal cortex during movement planning showing a more bilateral pattern of activity in women than men [adapted with permission from (67)].

MOVING FORWARD, IN A THOUGHTFUL WAY

As always with science, our research leaves us with more questions than answers. Moving forward, we believe it is important to quantify the effects of various factors that impact one's movement control response to blast exposure and blunt head trauma. Our findings around sex-related differences in the neural control of skill highlight the importance of examining factors such as hormonal influences on injury response, and sex-related vs. gender-related differences in brain network organization. The basic age-related differences we see in our younger vs. working-aged individuals in the studies reviewed here highlights the need to study the interactions between natural aging, skill reserve, and the long-term effects of mild brain injury through longitudinal research. What we are most enthusiastically pursuing at the moment, however, is research into counteracting the effects of compromised brain health through cognitive-motor interventions designed to strengthen the very neural control networks that appear to be affected by concussive injury and neuropathology (79, 80). Such a targeted approach holds promise as an effective means of stabilizing and improving one's functional abilities in the face of injury, allowing for a more enriched daily life.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by York University Human Participants Research Committee and the Human Research Ethics Committee of Defence Research and Development Canada. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LS conceptualized the review. LS, DG, MA, and DD conceptualized, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DNA Methylation Patterns of Chronic Explosive Breaching in U.S. Military Warfighters

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Background: Injuries from exposure to explosions rose dramatically during the Iraq and Afghanistan wars, which motivated investigation of blast-related neurotrauma. We have undertaken human studies involving military "breachers" —exposed to controlled, low-level blast during a 3-days explosive breaching course.

Methods: We screened epigenetic profiles in peripheral blood samples from 59 subjects (in two separate U.S. Military training sessions) using Infinium MethylationEPIC BeadChips. Participants had varying numbers of exposures to blast over their military careers (empirically defined as high ≥ 40, and conversely, low < 39 breaching exposures). Daily self-reported physiological symptoms were recorded. Tinnitus, memory problems, headaches, and sleep disturbances are most frequently reported.

Results: We identified 14 significantly differentially methylated regions (DMRs) within genes associated with cumulative blast exposure in participants with high relative to low cumulative blast exposure. Notably, *NTSR1* and *SPON1* were significantly differentially methylated in high relative to low blast exposed groups, suggesting that sleep dysregulation may be altered in response to chronic cumulative blast exposure. In comparing lifetime blast exposure at baseline (prior to exposure in current training), and top associated symptoms, we identified significant DMRs associated with tinnitus, sleep difficulties, and headache. Notably, we identified *KCNN3, SOD3, MUC4, GALR1*, and *WDR45B*, which are implicated in auditory function, as differentially methylated associated with self-reported tinnitus. These findings suggest neurobiological mechanisms behind auditory injuries in our military warfighters and are particularly relevant given tinnitus is not only a primary disability among veterans, but has also been demonstrated in active duty medical records for populations exposed to blast in training. Additionally, we found that differentially methylated regions associated with the genes *CCDC68* and *COMT* track with sleep difficulties, and those within *FMOD* and *TNXB* track with pain and headache.

Conclusion: Sleep disturbances, as well as tinnitus and chronic pain, are widely reported in U.S. military service members and veterans. As we have previously demonstrated, DNA methylation encapsulates lifetime exposure to blast. The current

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data support previous findings and recapitulate transcriptional regulatory alterations in genes involved in sleep, auditory function, and pain. These data uncovered novel epigenetic and transcriptional regulatory mechanism underlying the etiological basis of these symptoms.

Keywords: blast exposure, breacher, epigenetics, DNA methylation, tinnitus, sleep, pain

INTRODUCTION

Injuries from exposure to explosive blasts rose dramatically during Operation Iraqi Freedom and Operation Enduring Freedom (OIF, OEF) due to the increased use of improvised explosive devices (IEDs) in military settings and in civilian populations through acts of terrorism (1, 2), which have motivated investigations of blast-related neurotrauma. Despite this increase in occurrences, our understanding of the effects of blast and the mechanisms behind subsequent brain injury remains limited (3, 4). Toward this effort, the John S. McCain National Defense Authorization Act (NDAA) for Fiscal Year 2019 (5) passed by Congress emphasized the importance of preventing blast-related traumatic brain injury (TBI) in both combat and training sessions. The bill called for a review of the cognitive effects of blast exposure including both the effects of successive blast events, and the feasibility of understanding the cumulative (lifetime or annual) limits of blast exposure (2). Further, in recognition of the latent residual effects of exposures to blast, the 2020 NDAA passed by U.S. Congress also ordered the history of blast exposure and blast duration from both combat exposure and trainings to be included in medical histories of Service Members in order to inform future risk mitigation and determination of injury and related sequelae (6).

In an effort to understand the acute and chronic physiological and cognitive effects of blast exposure in military personnel, we have focused on military Breachers, a unique population who are, by occupational definition, typically in close proximity to controlled, low-level blast during explosive breaching operations and training, and repeatedly exposed to primary blast overpressure waves. Breachers may apply explosives as a means of gaining access to barricaded or hardened structures, where they can be exposed to as many as a dozen 0.3–10 lb charges per day during training exercises and larger numbers and magnitudes per day during military operations.

Exposure to blast often leads to polytrauma (multiple traumatic injuries) and a multisystem response [for an in-depth review see (7)]. Shock waves from explosive blasts can damage both gas- and fluid-filled structures of the body, including the lungs, intestines, brain, eyes, nose, and middle ear (7–11). Specifically, damage to the auditory system can be the consequence of either direct exposure of the auditory canal to blast shock waves or TBI and neurological impairment affecting central auditory processing involving different brain regions after blast exposure (12). It can be difficult to determine which neurologic symptoms are linked to blast-related TBI separate from those that may be related to other kinds of injury from the blast that affects the nervous system secondarily. Depending on blast injury severity, neuropathological and clinical symptoms can include neuronal swelling, subdural hematomas, myelin deformation, inflammation, loss of consciousness, temporary disorientation, sleep disturbances, memory deficits, and tinnitus (ringing in the ear) (12–20).

Based on concerns for potential injury by the cadre of breachers and instructors, the Department of Defense (DOD) has been conducting studies on the bio-effects from repeated exposure, and findings from our own group (21) and others (22, 23) have begun to show that blast exposure during training is capable of inducing changes in DNA methylation and gene expression in military breachers (22) that track with the physiological symptoms of blast injury (24). In the present study of military breachers, we set out to replicate previous findings, investigating effects of acute and chronic blast exposure on DNA methylation and reported symptoms at baseline across two military training sites–representing the largest DNA methylation study of breachers to date. In previous work, we found no significant DNA methylation changes associated with acute blast exposure (3-days post blast) (21). In the present study, we investigated whether blast induced DNA methylation changes could be detected at a more proximal time point, within 2-h post-blast exposure. Additionally, in line with our ongoing work in understanding the chronic biosignatures of accumulative blast exposure, we investigated whether DNA methylation from peripheral blood captures chronic cumulative exposures to blast and associated symptoms in this independent cohort of breachers. To study the chronic effects of blast exposure and associated biological mechanisms, we examined baseline (i.e., before training) transcriptional regulatory profiles in operational blast training, comparing participants with low vs. high cumulative lifetime blast exposures. We further examined the high vs. low cumulative exposed groups' reported physiological and psychological symptoms and aim to identify DNA methylation changes that associate with frequently reported symptoms by breacher training participants, which included sleep disturbance, tinnitus, and headache.

METHODS

Samples Demographics and Symptoms

All subjects consented to participate in the study and the human use protocol for interaction with the subjects was approved by Institutional Review Board (IRB) of the Walter Reed Army Institute of Research (Silver Spring, MD) and chains of command prior to data collection. The procedures were followed in accordance with the ethical standards of the IRB, Army Regulation 70-25, and the Helsinki Declaration.

Data were collected over 3-days at two training sites, situated at Fort Leonard Wood, MO, from 59 male subjects. Demographic information including sex, age, lifetime operational exposure to blast, and self-reported lifetime TBI history were recorded at the start of the training (see **Supplementary S1**). Also, self-report symptom assessments were completed at each time point that blood was collected. The self-report symptom assessment included a range of symptoms related to blast injury, including tinnitus, sleep difficulties, and headache, as well as additional symptoms based on the Rivermead Post-Concussion Symptom Questionnaire (RPSQ) (25) and surveys derived from aggregations of concussion symptomology present in current clinical and research findings, along with relevant Veterans Affairs and CDC Annual Report materials (24, 26, 27). The symptom assessment was developed to be administered on a single page and for minimal disruption of, or interference with, the operational duty of the personnel whose responsibility first and foremost is training. This is due to the fact that it is not feasible to incorporate administration of detailed and time-consuming clinician administered assessments that would interfere with operational duties and responsibilities of the participants. Despite its brevity, the assessment is an inclusive list of blast symptomology, with the benefit of being neutrally worded to ameliorate potential underreporting by participating military service members (24). The neutrality of this survey is crucial given the possibility of underreporting military operation-associated symptomology by service members, particularly for symptoms associated with mental health status, given the potential stigma around possible fitness for operational duties (24, 28).

DNA Methylation Sample Processing and Quality Control (QC)

Whole blood was collected using PAXgene blood DNA tubes (PreAnalytix), and stored at −80°C. Genomic DNA was isolated using PAXgene Blood DNA kit (PreAnalytix). Genomic DNA was bisulfite converted (Zymo Research) and CpG methylation determined using Illumina Infinium HumanMethylation EPIC BeadChip microarrays, as described previously (29). Data and QC analyses was performed using R Language 3.4.2 (30), an environment for statistical computing, and Bioconductor 2.13 (31), and all raw data files (.idat) processed by the minfi package (32). All samples were subjected to quality control procedures for sample tracking and sex prediction analyses as follows. All samples but one displayed >99% of probes that passed detection call < 0.00005 (**Figure S1**), and the one sample did not pass was dropped and was not included in downstream analyses. Sex QC analysis also confirmed methylation-based sex prediction with those reported (all male, **Figure S2**). It should be noted that a single reference female sample was included for the sex QC analysis (**Figure S2**). For QC sample tracking of pre- vs. post-blast exposure breacher training, we used the 59 single nucleotide polymorphism (SNP) probes included in the Human MethylationEPIC BeadChip, confirming that the subjects from FLWB training site for which multiple samples at multiple time points were assayed grouped together (**Figure S3**).

DNA Methylation Data Analysis

For all DNA methylation analyses, we used the matrix of M-values (logit transformation of beta-values) which correspond to methylation levels. Surrogate variable analysis (SVA) was performed to add surrogate variables and rule out potential batch effects. A linear model was used for the binary variable of interest, while including age and history of TBI as covariates in the model. Performing the comparative analysis in limma (33) implemented in *R*, we obtained *t*-statistics and associated *p*-values for each CpG site. The point-wise *p*-values, were then used for the identification of differentially methylated regions (DMRs) using the combined-*p*-value tool (34), and all DMRs identified by the combined-*p* value tool are all significant. For baseline low vs. high cumulative blast symptom DNA methylation analyses, we used the same matrix of *M*-values as above, where for each symptom, a vector of symptom scores at day 1 is added to the design matrix as a new variable to perform linear regression in limma, with point-wise *p*-value and significant CpG sites done in the same manner. Moreover, for the pre-post blast symptom analysis, we used the matrix of change in *M*-values as in pre-post methylation analyses and dichotomized the symptom variable where for each selected/filtered symptom we compared symptom score pre-post; if the score increased, the variable was set to equal one, otherwise it was set to zero.

Furthermore, due to the potential contribution of cellular heterogeneity of blood sample specimens and its effect on DNA methylation patterns, we examined whether the variability in cell proportions may be a potential confound between methylation and our factors of interest. Therefore, we used Horvath's DNA methylation age calculator (35), a tool to estimate DNA methylation age and cell proportions for samples on the Illumina Infinium platform (36) in order to calculate the cell proportion estimates of six cell types (CD4 T-cell, CD8 T-cell, natural killer, B-cell, monocytes, and granulocytes), and compared each cell's proportions between groups in both studies (low vs. high cumulative blast exposure and acute pre- vs. post-blast exposure). None of the cell types showed different cell proportions within the low-high study, while CD4T, natural killer and B-cell showed significant differences between the pre- and post-exposure samples; and thus their proportion differences (post- minus pre-) were added as covariates in subsequent DNA methylation analyses. Specifically, for the pre- and post-blast exposure analysis, a matrix of difference *M*-values (post-minus pre-blast) was used in the linear model, including the additional covariates from the cell proportion estimates. Lastly, for replication of findings across independent data sets, DMRs were compared such that if the directionality of gain or loss of DNA methylation within the differentially methylated region(s) and associated CpG sites were consistent across both datasets, it was considered a replication.

RESULTS

In the present study we examined genome-scale DNA methylation patterns using the Illumina Infinium HumanMethylation EPIC BeadChip at baseline (pre-breacher

training) amongst two cohorts of military trainees in 3-days period during an explosive breaching course at Fort Leonard Wood (FLW). This consisted of 28 participants from one training session (FLWA) and 31 participants from second, separate session (FLWB), totaling 59 sample subjects (for demographic breakdown of each subject per site and respective breaching history, see **Table S1**). In an attempt to replicate our previous findings, for one of these sites (FLWB), we also investigated DNA methylation changes following exposure to operational blast exercises, which corresponded to 29 participants with available biospecimens that passed QC [see Methods DNA methylation sample processing and Quality Control (QC)].

DNA Methylation Changes Are Not Significantly Associated With Acute Blast Exposure

We previously reported that DNA methylation changes do not track with acute exposure to blast (21), and our present findings further support this in that DNA methylation appears to not be altered acutely pre- vs. post-blast exposure. As noted, for this experiment we used biospecimens from one site, FLWB, and examined DNA methylation changes in day-2 of training, pre vs. post blast exposure (approximately within 2 h post blast exposure, see **Figure 1A** for training protocol). DNA methylation assays were conducted from whole blood samples. When accounting for intra-individual differences in cell proportions (as described in Methods DNA methylation data analysis), we were unable to detect significant changes in DNA methylation before and after exposure to blast (see **Table S2**).

Lifetime Cumulative Blast Exposure Alters DNA Methylation in High Relative to Low Blast-Exposed Groups

We have previously shown that lifetime cumulative blast exposure is capable of altering DNA methylation in our investigations of military breachers with high relative to low lifetime blast exposures at baseline among military service members participating in a 10-days explosive breaching training course (21). Here, we also investigate at baseline, alterations in DNA methylation in 59 military trainees from two independent cohorts over a 3-days period during the explosive breaching course at Fort Leonard Wood (FLWA and FLWB sites, **Figure 1A**). The history of number of self-reported career breaches–that is, number of breaching exposures throughout a career in military service–were recorded at baseline for the two separate 3-days training cohorts reported here (59 participants total), as well as self-reported history of TBI (shown together in **Figure 1B** and **Table S1**). However, we first determined whether participants from these two separate training sessions were comparable to be combined for subsequent analyses in this study. In particular, we found no significant difference between the two sites based on the participant's age (t-test, $p = 0.3666$), lifetime history of mild TBI (χ^2, $p = 0.1424$), or lifetime career breaching history (χ^2, $p = 0.8214$). As such, all data including DNA methylation data for the two sites were combined for subsequent downstream analyses. For the comparative analyses, we considered a total of 33 participants with reported low number of lifetime career breaches (≤40), and 26 trainees with reported high number of career breaches, ranging from 40 to

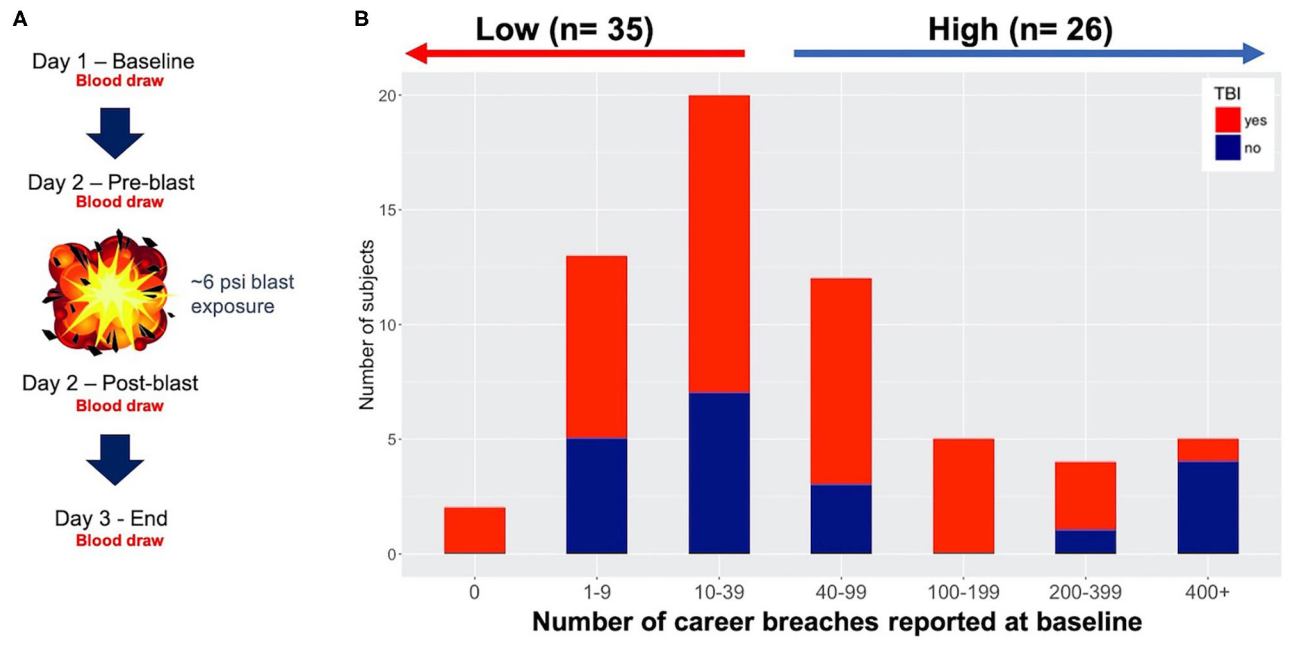

FIGURE 1 | Study design and participants. **(A)** 3-day protocol showing blast exposure and blood draws throughout the study. In this study we focus on baseline data only using specimens from the pre-blast timepoint. **(B)** Number of career breaches reported at baseline (61 participants) with and without history of traumatic brain injury (TBI) (red and blue, respectively), with ≤39 blasts empirically defined as low blast, and 40≤ career breaches considered high exposure to blast.

more than 400 breaches (**Figure 1B**). The criterion for low vs. high cumulative career breaching experience used here was empirically defined, and was based on our previous work (21). It should be noted that estimation of cell proportions in whole-blood specimens, showed no significant differences by cell types within the low vs. high groups (data not shown), and thus were not adjusted for in the analytical models going forward. Also, there was no correlation between history of TBI and the total number of lifetime career breaches ($\chi^2 = 9.15$, $p = 0.1655$).

Comparing DNA methylation changes in high relative to low cumulative blast exposure groups at baseline, we identified six significantly differentially methylated regions (DMRs) that passed rigorous multiple testing corrections (**Table S3**), with four DMRs overlapping with regulatory and protein coding regions, (i.e., promoter and genic features, shown in **Figure 2**). Notably, DMRs overlapping with loci associated with autoimmune disorders (37–39), *ZKSCAN4* (zinc finger with *KRAB* and *SCAN* domains 4) and *PSORS1C3* (psoriasis susceptibility 1 candidate 3), localized to the major histocompatibility complex region on chromosome 6 show a loss of methylation in the *ZKSCAN4* DMR and a gain of methylation in the *PSORS1C3* DMR in the group with high cumulative exposure to blast (**Figure 2**). Additionally, DMRs overlapping with the loci *SPON1* (spondin-1), with loss of methylation, and NTSR1 (neurotensin receptor 1), with gain of methylation, were also identified in subjects with high cumulative exposure to blast (**Figure 2**). Both *NTSR1* and *SPON1* are implicated in circadian rhythm cycles and dysregulated sleep.

Symptoms Associated With Cumulative Blast Exposure and Associated Differentially Methylated Regions in High Relative to Low Blast-Exposed Groups

Self-reported neurological and physiological symptoms endorsed by participants in the breacher cohorts were ascertained at the start of the training (prior to blast exposure) and we utilized this information to track DNA methylation changes that co-occured with symptoms associated with cumulative exposure to blast.

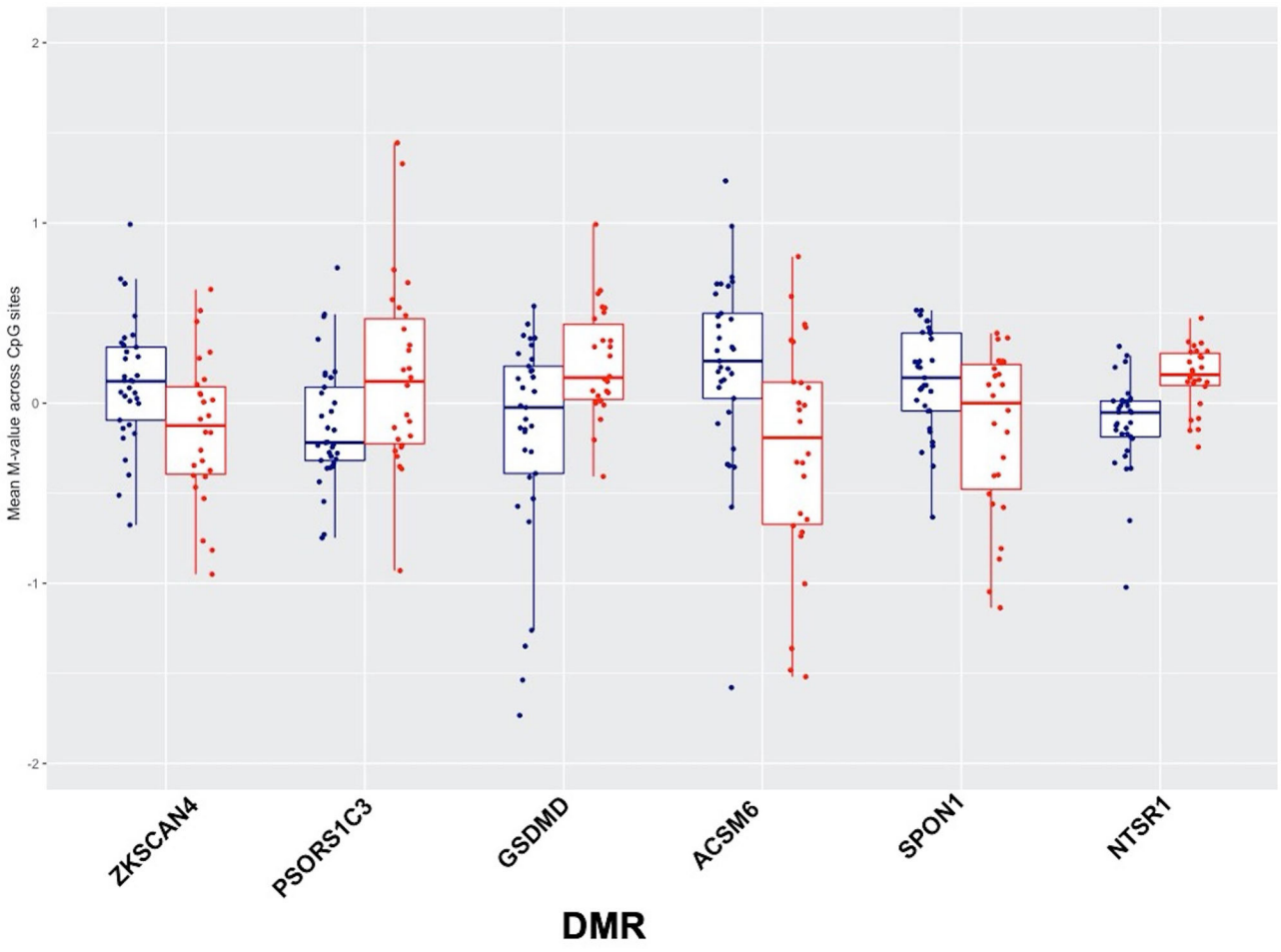

FIGURE 2 | Six significantly differentially methylated regions (DMRs) identified using the combined *p*-value tool in high relative to low-blast exposed groups, showing low blast exposed groups in blue and high blast exposed groups in red, with mean methylation (represented by *M*-values) across CpG sites for each subject on the y-axis.

We previously demonstrated that symptoms in high relative to low blast exposed groups tracked with DNA methylation patterns (21), where tinnitus (ringing in the ear) was significantly associated with changes in 18 differentially methylated regions. In the present study, tinnitus was the top reported symptom at baseline, with 49% of participants reporting tinnitus at "baseline" (assessed pre-blast in day-2 of training depicted in **Figure 1A** protocol, with symptom frequencies shown in **Figure 3A**). The subsequent top symptoms (as also shown in **Figure 3A**) were forgetfulness (34% reported), headache (35% reported), and sleep disturbances (25% reported). No significant DMRs were identified for the reported symptom of forgetfulness that tracked with cumulative blast.

DNA methylation analyses at baseline, in high relative to low cumulative blast exposure groups with the reported symptom of tinnitus identified 14 DMRs (**Table S4**), with 11 of these DMRs overlapping with promoter and genic features of protein coding genes (or experimentally validated loci **Figure 3B**). Remarkably, we found that five out of 11 (45%) of these DMRs were associated with genes involved in auditory functioning or hearing loss through this unbiased genome wide approach. These include the genes KCNN3 (potassium calcium-activated channel subfamily *N* member 3), MUC4 (mucin 4, cell surface associated), SOD3 (superoxide dismutase 3), WDR45B (WD repeat domain 45B), which show a loss in methylation in the high cumulative exposed group, and GALR1 (galanin receptor 1), which show a gain in methylation. Additionally, we observed 10 DMRs that tracked with the reported symptom of sleep difficulty in high relative to low lifetime blast exposed participants (**Table S4**), with 8-DMRs overlapping with genic and promoter regulatory regions (**Figure 3C**). Notably, two loci COMT (catechol-O-methyltransferase) and CCDC68 (coiled-coil domain containing 68) have previously been associated with sleep disturbance and insomnia. Both show a gain of methylation that track

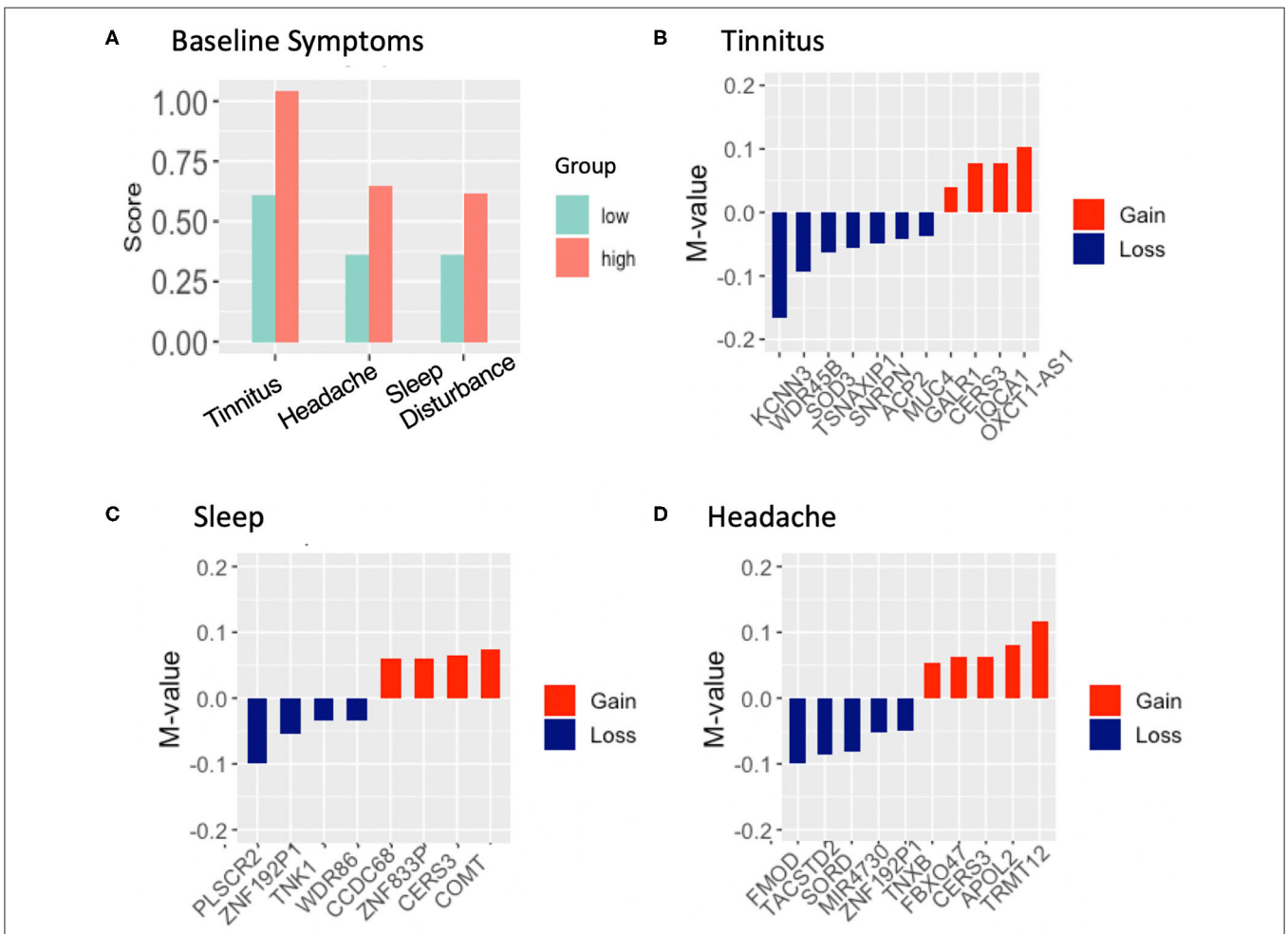

FIGURE 3 | (A), Frequency of endorsed symptoms at baseline in 61 subjects across high and low blast exposed groups. Tinnitus (ringing in the ear) was the highest reported symptom, endorsed by 29 participants; **(B–D)**, differentially methylated regions and associated genes that track with the reported symptom of tinnitus, headache, sleep difficulties in high relative to low lifetime blast exposed groups, showing gain of methylation in red and loss of methylation in blue, with mean methylation (represented as *M*-values) across CpG sites (between the high group relative to the low blast exposed group) shown on the y-axis.

with sleep disturbance in those with high accumulative blast exposure. Finally, headache, the third symptom that passed filtering and tracked with DNA methylation patterns in high vs. low blast exposed groups, yielded 13 DMRs (**Table S4**), and of these, 10-DMRs overlapped with genes and promoter regulatory regions (**Figure 3D**). Although no previous studies have linked these loci to headache symptoms, we did identify two loci FMOD (fibromodulin), which shows a loss in methylation, and conversely TNXB (tenascin XB), which shows a gain in methylation in high blast exposed groups that have been previously investigated in studies involving temporomandibular joint dysfunction (TMJ) and chronic pain (40).

DISCUSSION

We identified changes in DNA methylation and gene expression in a 10-days explosive breaching training course (21), emphasizing the effects of cumulative blast on sustained DNA methylation alterations, specifically related to chronic symptoms of sleep disturbances and tinnitus in our previous work. Here, we examined DNA methylation data from 59 military trainees with varying lifetime histories of exposure to blast during a 3-days training session, in conjunction with symptom and demographic information, in order to determine novel DNA methylation signatures in association with blast exposure and blast-related symptoms, and importantly attempt to replicate our previous findings in studies of breachers (21). In line with our previous findings, we showed that DNA methylation changes do not encapsulate physiological changes acutely, pre-post blast exposure, suggesting other molecular mechanisms for symptoms experienced acutely following exposure. However, also as shown previously (21), we found that DNA methylation signatures appear to encapsulate long-term chronic exposure to blast.

DNA Methylation Alterations Associated With Cumulative Exposure to Blast

Similar to our previous work, in comparing DNA methylation changes in high relative to low cumulative blast exposure groups at baseline (21), we identified six significant DMRs that passed multiple testing corrections, wherein *SPON1* and *NTSR1* were identified as significantly differentially methylated in the low vs. high blast exposure analysis (**Figure 2**). Both *NTSR1* and *SPON1* are implicated in circadian rhythm cycles (41, 42), further confirming that chronic exposure to blast may dysregulate sleep. A study of intrinsically photosensitive retinal ganglion cells in the suprachiasmatic nucleus identified *F-spondin* as significantly enriched in the suprachiasmatic nucleus (SCN). *F-spondin* deficient mutants ($spon^{-/-}$) demonstrated severely disrupted "free running" rhythmicity, suggesting a novel role for F-spondin in maintaining intrinsic circadian rhythm cycles (42). Our low vs. high career breaching DNA methylation analyses also identified *NTSR1* as significantly differentially methylated. In animal studies of NTSR1, NTSR1 knockout mice had a lower percentage of time spent in REM sleep relative to wild-type (41). Furthermore, following sleep deprivation, *NTSR1* knockout mice (C57BL/6N wild-type mice with a targeted *Ntsr1* mutation) exhibited more wake and less NREM rebound sleep, and also showed increased anxiety and despair behaviors (41).

To confirm previous findings, we examined whether data from our prior and current studies replicate. Comparing DNA methylation data between our previous findings (21) and the present study using an independent cohort, we investigated DNA methylation patterns in *PAX8-AS1*, an antisense transcript of *PAX8*, a transcription factor associated with thyroid function. Genome-wide association studies have implicated variants associated with *PAX8* and sleep duration (43–45). We observed similar trends in terms of gain of methylation associated with high levels of lifetime blast exposure in this study for the *PAX8-AS1* gene, such that mean methylation levels in *PAX8-AS1* were higher in breachers with high lifetime cumulative blast exposure in both previous and present findings (**Figure 4A**). However, the antisense RNA, *PAX8-AS1*, was not differentially methylated in low vs. high cumulative blast groups following correction for multiple testing.

We also investigated DNA methylation patterns of *NTSR1* and *SPON1* genes across the two breaching protocols in the current FLW and previously published study. We found that the methylation pattern of the *NTSR1* DMR replicated across both studies such that breachers with high lifetime exposures to blast had higher mean-methylation of *NTSR1* (**Figure 4B**). Yet, we did not observe the same directionality in gain of methylation in the *SPON1* DMR in the present study relative to our previously published data (**Figure 4B**). However, it is possible that the methylation change is not high enough to be detected across all CpG sites. Our findings on sleep related genes in the breacher populations are highly translational, given the high prevalence of insomnia in service members and veterans (46, 47). Amongst a cohort of Veterans who had recently incurred combat-related mild TBIs (both blast and blunt trauma-induced), nearly all endorsed sleep issues, but those with specifically blast-induced mild TBI developed higher rates of anxiety and insomnia than those with blunt injuries (48). Taken together, these data suggest that the mechanism of blast injury may differently influence underlying molecular interactions, wherein recognition of the mode of injury may be crucially important for development of targeted clinical treatment and rehabilitation.

DNA Methylation Tracks With Chronic Exposure to Blast and Related Symptoms

We identified changes in DNA methylation that tracked with top self-reported symptoms of tinnitus, sleep difficulties, and headache (**Figure 3A**).

Tinnitus

Notably, tinnitus symptom analyses identified regions with differential methylation in genes *KCNN3*, *MUC4*, *GALR1*, *SOD3*, and *WDR45B* (in the low vs. high cumulative blast exposed groups (**Figure 3B**). Again, to confirm our previous findings, we examined whether the tinnitus findings from our prior and current studies replicate. We first investigated DNA methylation patterns in *KCNN3*, *MUC4*, *GALR1*, *SOD3*, and *WDR45B* genes that are implicated in auditory functioning and hearing loss across the two breaching protocols in the current FLW and

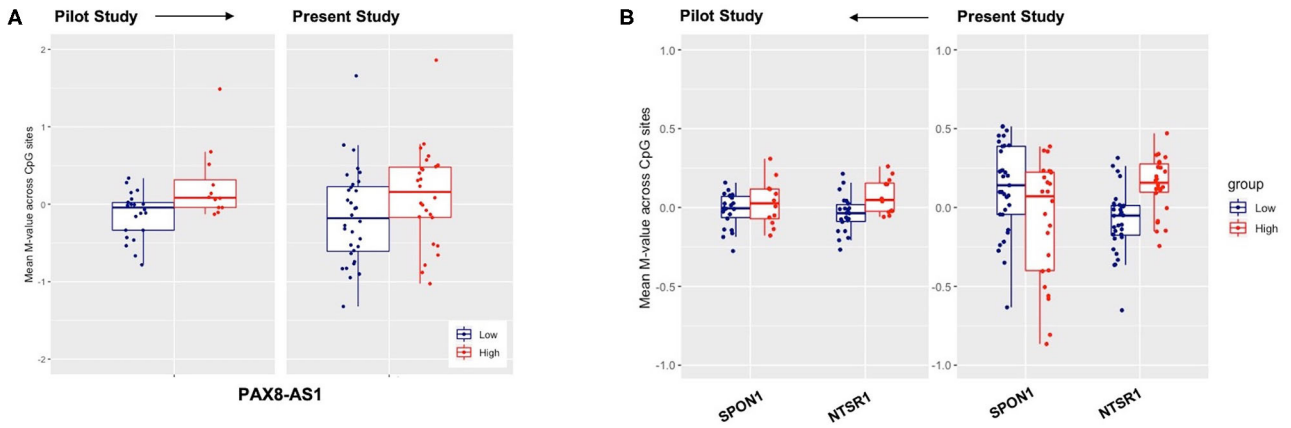

FIGURE 4 | Replication of previously reported genes for low-high blast exposure analyses of genes **(A)** *PAX-AS1* and **(B)** *SPON1* and *NTSR1*, with mean methylation *M*-values across CpG sites on the y-axis and the respective mean *M*-value across CpG sites for both low-(blue) and high (red) lifetime blast exposed individuals.

previously published studies, and found that the methylation patterns of the DMRs in these loci did not replicate. However, comparing DNA methylation data from our previous findings (21) with the current FLW dataset, we observed that the genes Cytochrome P450 Family 2 Subfamily *E* Member 1 (*CYP2E1*) showed the same pattern in gain of methylation, and the gene dual specificity phosphatase 22 (*DUSP22*) showed the same pattern in loss of methylation, amongst breachers with high cumulative exposure to blast. Relevant to the symptoms of tinnitus, deletions of the *DUSP22* gene in humans results in severe intellectual disability and deafness (49, 50), while *CYP2E1* is integral to the metabolism of acrylonitrile, which has been shown in rodents to potentiate damage to hair cells in the inner ear.

Importantly, we have identified novel genes differentially methylated that are associated with tinnitus symptoms in breachers with cumulative exposure to blast in the present study (**Figure 3A**). Specifically, potassium channel genes, along with *KCNNN3* and other genes in this family, play a critical role in defining the electrophysiological properties involved in the response patterns of auditory neurons (51, 52). The electrophysiological properties of auditory neurons are governed by the neuronal circuitry, cellular morphology, and patterns of potassium channel subunit expression. The cochlear nucleus is the only central nervous system region to receive direct innervation from the auditory nerve, and a recent systematic gene expression profiling study in rodents identified transcripts for 51 potassium channels (including *KCNN3*) within the cochlear nuclei subdivisions (i.e., the anterior ventral, posterior ventral, and dorsal), showing highest KCNN3 gene expression in the anterior ventral cochlear nuclei (53). Data from the present study provides the first association of *KCNN3* gene with the symptom of tinnitus in human samples.

To our knowledge, this study also provides the first human data supporting the association of *SOD3* with tinnitus symptoms in breachers with repeated blast exposure. In a rodent study, mice with repeated exposure to blasts showed injury to the auditory cortex and coordinated gene expression changes in genes known to be involved in age- or noise-induced hearing impairment across multiple brain regions, where increase *SOD3* expression in the hippocampus was observed following blast exposure (12). This increase in *SOD3* expression is potentially a protective response to blast injury, since superoxide dismutase are critical antioxidants that work against oxidative stress in the body (54). Indeed, the functional role of reactive oxygen species and the protective efficacy of antioxidants in noise-induced hearing loss are well-established (55–57). Repeated blast injury can induce production of reactive oxygen species (58) leading to oxidative stress, suggested as a possible mechanism for tinnitus, given that oxidative stress can impact hair cells, cochlear degeneration, and neural-auditory pathways (59–61).

Furthermore, *GALR1* and *WDR45* loci identified in the present study have been linked to congenital developmental auditory dysfunction. A large mutation screening of 307 deafness genes in patients with microtia identified *GALR1* as a strong candidate gene (amongst others) with novel mutations associated with microtia (62). Microtia is a malformation of the external ear that ranges in severity from mild differences in auricular shape and size to complete absence of the external ear with atresia or stenosis of the auditory canal that may be caused by genetic and/or environmental factors (63, 64). Microtia patients can suffer from range of symptoms including conductive hearing loss and sensorineural hearing loss (65). Interestingly, a case report of a patient with congenital aural atresia (typically resulting in unilateral or bilateral ear malformation) caused by chromosome 18q deletion, also contained the *GALR1* gene (66). Additionally, mutations in the *WDR45* loci have resulted in beta-propeller protein-associated neurodegeneration (BPAN), a rare form of neurodegeneration resulting from an accumulation of iron in the brain (67). Patients with WDR45 mutation also exhibit lateral sensory neural hearing loss and auditory agnosia (68) Similarly, in rodents *WDR45* knockout mice exhibit either an increased

or absent auditory brainstem response when assessed using an 18 kHz-evoked Auditory Brain Stem Response Threshold test, compared to wildtype animals (69).

Lastly, the *MUC4* gene also was found to track with symptom of tinnitus in breachers, and has been previously implicated in mucoid otitis media (MOM) (70) a common otological disease that can result in hearing loss. MOM is a chronic condition that can persist long-term leading to conductive hearing loss (71). Pathologically, MOM is characterized by accumulation of mucous effusion in the inner ear that reflects high concentrations of mucins, including *MUC4*. Mucin hyperproduction involving *MUC4* overexpression have been observed under inflammatory conditions (72–74), which is in line with the inflammatory response also observed following acoustic injury (75), suggesting that immune responses may underlie biological and molecular processes associated with acoustic trauma. These findings are particularly relevant because blast exposure is reported to cause auditory impairment in a large population of service members (76, 77). Auditory/vestibular injuries from blast traumatic brain injury (TBI) can cause increased incidence of tinnitus and hearing loss, which when left untreated can worsen over time (76–79). In fact, tinnitus is the most prevalent service-connected disability of all Veterans Benefit Administration compensation recipients (80), in addition to being the most commonly reported symptom by breachers in former and this present study.

Sleep Disturbance

In addition to DMRs that tracked with the symptom of tinnitus, we also identified DMRs that tracked with the symptom of sleep difficulties (**Figure 3C**). Interestingly, the gene CCDC68, coiled-coil domain containing 68, has been shown to be significantly associated with the sleep disturbance phenotype in genome wide association studies (81–83). Moreover, *PLSCR2*, phospholipid scramblase 2, and TNK1, tyrosine kinase non-receptor 1, are involved in phospholipid metabolism, which is significant because lipid signaling and is associated with both sleep and synaptic function (84). *ACSF3*, an acyl co-A synthetase, was also identified as differentially methylated and plays a role in fatty acid synthesis (85), which is a critical process that sustains brain energy metabolism during sleep (86). Interestingly, we also observed *COMT* as differentially methylated and significantly associated with the reported symptom of sleep disturbance, and COMT has been implicated in and circadian physiology (87–89). The catechol-*O*-methyltransferase enzyme modulates dopamine levels within the prefrontal cortex (PFC), where it metabolizes dopamine and renders it inactive (87, 88). A single nucleotide polymorphism within the *COMT* gene With a valine (Val) to methionine (Met) substitution at codon 158 (Val158Met) (90) differentially affect COMT's enzymatic activity and thus dopamine levels within the PFC. Specifically, the Met allele results in a ∼4-fold reduction of the enzymatic activity compared to the Val allele, leading to increased dopamine within the PFC (87, 90, 91). In a study involving subjects with sleep deprivation, those with the Val allele showed greater impairment in adaptive decision making as compared to those with the Met allele (92). Using actigraphy data another study showed that Val/Val and Met/Met homozygotes habitually prolonged sleep on rest days compared to workdays, whereas Val/Met heterozygotes did not significantly increase their sleep duration (93) suggesting that the Val/Met polymorphism may be associated with inter-individual differences in distinct aspects of sleep-wake regulation and physiology. The association between chronic lifetime cumulative blast exposure and changes in DNA methylation in these loci previously implicated in sleep and circadian function is highly relevant, because sleep disturbances are commonly reported in military service members within both active duty and post-deployment settings (94–96).

Headache and Chronic Pain

A number of genes were also identified that tracked with the reported symptom of headache (**Figure 3D**). In particular, the gene *FMOD* (fibromodulin) a collagen-binding molecule expressed in connective tissues is particularly interesting given its role in pathobiology in osteoarthritis of the temporomandibular joint (40, 97). A number of rodent models involving *FMOD* gene knockouts have shown accelerated osteoarthritis in the temporomandibular joint (40), as compared to wildtype animals. Temporomandibular joint disorder has been reported by service members and veterans with exposure to IEDs and blast injury (98) and posttraumatic stress disorder (PTSD) (99). Of relevance to the data presented, headaches are the most commonly reported condition associated with temporomandibular joint disorders (100). Veterans with blast mTBI report high prevalence of chronic daily headaches and migraines, as compared to the general population with TBI and concussion (101–103). Another gene *TNXB* (tenascin XB) was also found in the present study to track with headache in breachers with high cumulative blast exposure. Mutations in the *TNXB* locus are associated with joint hypermobility syndrome, which is a connective tissue disorder characterized by chronic musculoskeletal pain due to joint hyperextensibility (104). This is considered a milder form of Ehlers-Danlos syndrome, in which *TNXB* locus is also implicated (104, 105). These disorders have highly variable clinical presentations, which include chronic pain and headaches (104). Headaches can be the primary complaint or refractory due to tension, temporomandibular joint dysfunction, or unilateral myofascial pain etc. (104, 106). The two key loci *FMOD* and *TNXB* identified here that track with headache and chronic pain may provide insight to the etiological basis of chronic pain reported by our service members and veterans with blast related mild TBI (107, 108).

Study Limitations

This study has a number of limitations. First, this study lacks representation of both sexes, for all our participants were male; therefore, our findings may not be generalizable to the small (but growing) population of female service members and veterans. The growing increase in female population in the United States Armed Forces and recent inclusion of women in combat roles investigation of sex differences can be the focus of future studies. Additionally, as noted in Methods samples demographics and symptoms, the self-report symptom assessment used was based on the RPSQ and surveys derived from aggregations of symptoms associated with clinical concussion symptomology, supported by

clinical and research findings (24, 26, 27) and these measures likely include a number of separable factors [e.g., (109)]. Therefore, a larger sample size may be necessary to fully develop associations within distinct domains. The sample sizes used in the present study are small given the potential heterogeneity that exists within the epigenetic and symptom data across the participants, therefore, these findings warrant replication and confirmation in larger cohorts. Further, for feasibility of collecting data in settings for military training, there was only limited opportunity available for capturing lifetime history information. More detailed information on not only frequency of blast exposures, but also magnitude of exposures, as well as other factors such as lifetime trauma and military related post-traumatic stress, was not practical to obtain and thus reduces the precision in this work. Finally, although the differentially methylated loci identified link to relevant clinical features related to chronic symptoms of tinnitus, sleep difficulties, and headache reported by the breachers, it is important to demonstrate in future studies whether these transcriptional regulatory changes confer coordinated changes in gene expression *via* whole genome RNA sequencing within the same samples and subjects, thus allowing for establishment of functional association.

CONCLUSIONS

The findings from this study have broad implications, which have led to identification of molecular signatures in military service members that track with chronic symptoms related to cumulative exposure to blast. These molecular signatures are measured through changes in DNA methylation, which is a highly stable epigenetic mark that can encapsulate a lifetime of environmental exposures. DNA methylation alterations in genes identified in this study, which tracked with chronic symptoms of tinnitus, sleep disturbance, and pain prevalent in service members and veterans, provides mechanistic insight on the impact of repeated blast injury and related sequelae, thus, allowing for consideration of prevention measures during training and deployment of our Warfighters, as well as informing future development of biomarkers in clinical care for our veterans.

DATA AVAILABILITY STATEMENT

The datasets have been uploaded to the GEO (accession #: GSE155426).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of the Walter Reed Army Institute of Research (Silver Spring, MD). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB, CL, GK, and WC contributed to conceptualization of study protocol, recruitment, to phenotypic, and biospecimen collection. FH, YG, CW, and ZW contributed to study-design and conducted experiments. JN inventoried and shipped the samples. CW conducted sample processing and quality control. ZW performed DNA methylation and symptom data analyses with statistical/analytical guidance from YG. CW, ZW, and FH prepared the manuscript. All authors reviewed and approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.01010/full#supplementary-material

Figure S1 | Quality control, detection *p*-value. Bar graph showing the proportion of probes with poor quality ($p > 0.0005$) probes for each biological sample. All samples had high quality data, with at least 99% of probes passing criteria.

Figure S2 | Quality control, gender prediction. Plot demonstrates consistency for predicted and reported sex, using Chromosome X and Y median intensity.

Figure S3 | Quality control, genotype consistency check with paired samples pre- and post-training exposure. The dendrogram shows clustering of individuals by genotypes derived from the 65 SNP probes for biological samples collected pre/post training. The y-axis shows Euclidean distance and samples that show no clustering were those for which no post-training biological samples were available.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Blast in Context: The Neuropsychological and Neurocognitive Effects of Long-Term Occupational Exposure to Repeated Low-Level Explosives on Canadian Armed Forces' Breaching Instructors and Range Staff

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Currently, there is strong interest within the military to better understand the effects of long-term occupational exposure to repeated low-level blast on health and performance. To gain traction on the chronic sequelae of blast, we focused on *breaching*—a tactical technique for gaining entry into closed/blocked spaces by placing explosives and maintaining a calculated safe distance from the detonation. Using a cross-sectional design, we compared the neuropsychological and neurocognitive profiles of breaching instructors and range staff to sex- and age-matched Canadian Armed Forces (CAF) controls. Univariate tests demonstrated that breaching was associated with greater post-concussive symptoms (*Rivermead Post Concussion Symptoms Questionnaire*) and lower levels of energy (*RAND SF-36*). In addition, breaching instructors and range staff were slower on a test that requires moving and thinking simultaneously (i.e., cognitive-motor integration). Next, using a multivariate approach, we explored the impact of other possible sources of injury, including concussion and prior war-zone deployment on the same outcomes. Concussion history was associated with higher post-concussive scores and musculoskeletal problems, whereas deployment was associated with higher post-concussive scores, but lower energy and greater PTSD symptomatology (using PCL-5). Our results indicate that although breaching, concussion, and deployment were similarly correlated with greater post-concussive symptoms, concussion history appears to be uniquely associated with altered musculoskeletal function, whereas deployment history appears to be uniquely associated with lower energy and risk of PTSD. We argue that the broader injury context must, therefore, be considered when studying the impact of repetitive low-level explosives on health and performance in military members.

Keywords: TBI, blast, concussion, military personnel, cognitive motor integration

INTRODUCTION

Recent military engagements in Iraq and Afghanistan have been associated with significant rates of blast-induced traumatic brain injury (TBI). For example, according to statistics from the Department of Defense (DoD), 14% of TBI cases encountered in Operation Enduring Freedom (OEF) and/or Operation Iraqi Freedom (OIF) were due to blast exposure (1). Indeed, based on data compiled by the Department of Veterans Affairs, nearly three-quarters of all combat-related injuries over the period 2005–2009 were due to explosions (2). Importantly, 10–15% of TBI cases from those theaters of war continue to report persistent post-concussive symptoms following the resolution of the initial symptoms (3), indicating that TBI represents an enduring public health concern for our service members and Veterans.

Accordingly, neurological impairments, including mild TBI, are increasingly recognized as an occupational health and performance concern within the Canadian Armed Forces (CAF) and the Canadian Special Operations Forces Command (CANSOFCOM) (4, 5). However, isolating the effects of low-level blast in theater has proven difficult because of the tremendous heterogeneity that exists in the nature of explosions and their effects on individuals in combat settings (6). For example, it is recognized that blast-induced TBI can result from multiple factors, such as direct exposure to the explosive wave, projectiles that penetrate the skin, structural collapse or displacement of the body, and/or indirect effects such as thermal exposure—referred to as primary, secondary, tertiary and quaternary effects of blast exposure, respectively (7). In this sense, it is difficult to tease apart and measure the effects of primary blast exposure from other accompanying factors in combat settings.

Because the conditions that characterize blast exposure in operational settings complicate one's ability to study the effects of primary blast *in situ*, researchers have explored surrogate settings wherein the effects of exposure to blast can be assessed in an operationally realistic, yet scientifically more controlled manner. One such context involves explosive breaching, which is a tactical technique used to gain entry into a closed or blocked space using explosives [see (8)]. The procedure involves the placement of explosives and the maintenance of a calculated distance away from the source during detonation (**Figure 1**). Exposure levels during breacher training can vary depending on the charge weight, reflective surfaces in the environment, the geometry of the structures involved, and location of the exposed individual relative to the explosion. Nevertheless, breacher training is regulated by guidelines in order to limit hazardous exposure to blast overpressures in trainees, breaching instructors and range staff. For example, according to the Canadian Army's tactical breaching manual, breachers should not be exposed to blast overpressures that exceed a threshold of 3 pounds per square inch (psi) (9). However, a recent preliminary examination using blast gauges mounted on Canadian Forces School of Military Engineering (CFSME) instructors and range staff during breacher training revealed that ∼12% of blast events exceeded 3 psi (10). This naturalistic observation suggested that despite adherence to guidelines that govern breacher training, it is nevertheless possible for individuals to be exposed to potentially hazardous levels of blast, with possible downstream effects on health and performance.

FIGURE 1 | Canadian Forces School of Military Engineering personnel participating in breaching exercises. Photo courtesy of Haley Voutour (5th Canadian Division Support Group).

Although the precise nature of the relationship between long-term exposure to repetitive low-level blast and human health remains unknown (11–13), data suggest that long-term exposure to blast events can have adverse effects on the nervous system (14, 15), and can be associated with alterations in cerebral metabolism, diffuse white matter disruption, chronic neuroinflammation (16, 17), or perturbations to circulating levels of neurological injury biomarkers (8, 18–21). Indeed, a major theme in the literature revolves around whether blast injuries represent a different mechanism of injury than acceleration–deceleration injuries, by virtue of their physical dynamics. This idea is plausible, given the effect that explosives can have on both air-filled organs and/or organs surrounded by fluid-filled cavities within the body (22, 23), and remains an important area of study [see Belding et al. (24)].

Critically, despite lack of clarity regarding the underlying mechanism of injury, self-reports of breachers reveal concussion-like symptoms including headaches, sleep disturbances, and memory impairments that can interfere with daily activity (19, 25). In addition, and particularly relevant to the present purposes, there is reason to believe that the impairments do not arise in relation to acute exposure, but rather accumulate as a function of repetitive, cumulative exposure to low-level blast over the course of one's career. For example, the number and severity of symptoms reported by breachers increases with their history of chronic blast exposure (19). In addition, it has been shown that it is breaching instructors who oversee training regularly, rather than students who partake in as few as a single training exercise, that exhibit impairments in various memory tasks and alterations in brain function. Specifically, there was greater activation in the prefrontal cortex when performing a working memory task in instructors but not students following participation in a 2-week breacher course, compared to baseline (25). Because the impairment and associated neural alterations were specific to instructors, it appears that they emerge in response to repeated occupational exposure to low-level blast in the course of one's career, rather than acutely following exposure to isolated blast events [see also (15)].

Present Study

The objective of the present study was to investigate the impact of long-term occupational exposure to repeated low-level blast on health and performance in CAF members. Toward that end, we administered a battery of neuropsychological and neurocognitive measures to breaching instructors and range staff from CFSME, and compared their scores and performance to a group of sex- and age-matched CAF controls with no occupational experience with breaching. The selection of measures was largely informed by the literature on blast-induced TBI and concussion in sports [see (26)]. Regarding the latter, we administered a measure derived from the sports concussion literature that has not been used to study the impact of blast on performance in the past. Specifically, the *Brain Dysfunction Indicator* (BrDI) is a device that measures performance on a task that requires moving and thinking at the same time—known as 'cognitive-motor integration'. BrDI has been shown to be sensitive to movement control impairments in individuals with a history of concussion. These impairments are apparent in several aspects of the task such as movement reaction time, completion time, accuracy, and precision, and are detected despite the individuals showing no impairments in other tests that are currently available for assessing concussion recovery. Its ability to sensitively detect performance impairment in cognitive-motor integration has been shown for elite and competitive athletes—both adult (27, 28) and youth (29, 30). In addition, there is recent evidence to suggest an association between cognitive-motor performance and white matter integrity (31)—a structural neural index that might be affected by repetitive exposure to low-level blast. BrDI was, therefore, included in our task battery because of its sensitivity to detect concussion in athletes, and also to complement the remainder of our neurocognitive tasks, all of which measured various aspects of cognition and perception exclusively, rather than integrated.

Aside from our focal interest on the impact of repetitive exposure to low-level blast on health and performance, we were also cognizant of the fact that the same outcome measures could be affected by other sources of (head) injury—in particular concussion and deployment to a war zone. There were two reasons for this conjecture; first, as described above, some of our metrics for measuring the impact of blast in military personnel were informed by the sports literature on concussion. As such, one might expect that a history of concussion will influence those outcomes. Second, because war-zone deployment can be associated with a variety of health hazards, it is possible that any measure that reflects impairments in health might also be affected by one's deployment history. More broadly, we understand that blast effects on health and performance occur within a larger professional and personal context, and that it is important to probe those pathways as well, in order to obtain a more holistic picture of the impact of blast on military personnel exposed to explosives.

We hypothesized that compared to sex- and age-matched CAF controls, breaching instructors and range staff would exhibit impairments measured by tests of neuropsychological and neurocognitive function. As part of our battery we also included a measure of clinical posttraumatic stress disorder (PTSD) symptomatology [PCL-5, (32)]. This measure was included because previous studies with service members and veterans have shown that there might be comorbidity between blast-induced TBI and PTSD and/or depression, among other clinical symptoms [e.g., (33–36), see also (37)]. In the present study we did not expect to see any differences between the two groups on PCL-5, since we had no a priori reason to believe that exposure to breacher training *per se* is an emotionally traumatic experience. However, regardless of breaching, we did suspect that deployment to a war zone would be associated with elevated scores on the PCL-5.

METHOD

Participants

The study protocol was approved by the Human Research Ethics Committee of Defence Research and Development Canada. Potential participants were recruited via an electronic recruitment poster that was circulated among CFSME staff (for breaching instructors and range staff) and at Denison Armory (for controls). If interested in participating in the study, members were asked to email the PI. The participants were breaching instructors and range staff ($n = 19$) from CFSME, and sex- and age-matched CAF controls with no occupational experience as breachers ($n = 19$). Their demographics and service history appear in **Table 1**.

There has been no prior quantification of the amount of blast that instructors and range staff are exposed to in the course of their careers at CFSME, although some parameters can be used to contextualize the problem space. CFSME administers between 8 and 20 breaching courses per year. In turn, each course includes 1–2 days of breaching on the range (see **Figure 1**). Typically, instructors (also sometimes referred to as Assistant Range Safety Officers [ARSOs]) and range staff form a "cell" that administers the courses together for a period of 1–3 years. In that period, and unless there is a scheduling conflict, members of each cell will be at the range together. Nevertheless, the specific amount of blast overpressure that members within the same cell are exposed to can vary, depending on various factors such as one's geographic position, functional role, and the geometry of the space (which can impact wave reflection and re-convergence, etc.), among other factors. The instructors are integrated into the breaching stack for both wall and door breaches, although their position within the stack can vary depending on the condition. According to current breaching guidelines, the maximum number of exposures an instructor can be exposed to is limited to six blast events per day. After this limit has been reached, the instructors are rotated out of the stack and serve other functions on the range further away from the source of the blast in order to limit additional exposure. In turn, range staff who are not instructors but fulfill other roles on the range (e.g., Officer in Charge of the Range, Range Safety Officer, Ammunition NCO, etc.) are typically further away from the source of the detonations, and therefore receive relatively less exposure than instructors. However, they may be exposed to more than six blasts events per day. In summary, the magnitude and number of blast events that breaching instructors and range

TABLE 1 | Demographics and service history.

Variables	Breachers/range staff ($n = 19$)	CAF controls ($n = 19$)	Mean difference (95% CI)	Bootstrap ratio	*P*
Age (years)	33 (27-38)	32 (27.5–35.5)	0.8 (−4.4–5.7)	3.2	0.742
Sex–(*n*, % male)	17 (89.5)	17 (89.5)	0 (−21.1–21.1)	0	0.790
Military service (years)	11.3 (9–14.5)	5 (1.5–10.2)	6.4 (3.2–10.3)	3.7	**<0.001**
Exposure to explosives (years)	10 (7.5–12)	0 (0–0)	10.4 (8–13.1)	8.0	**<0.001**
Breaching (years)	7 (4.5–10)	0 (0–0)	7.1 (5.2–9.3)	7.2	**<0.001**
Combat deployment	11 (64.7)	0 (0)	68.2 (47.4–89.5)	6.3	**<0.001**
Status					
Regular Force	9 (47.4)	8 (42.1)	5.8 (−21.1–31.6)	0.4	0.546
Reservist	10 (52.6)	11 (57.9)	5.8 (−21.1–31.6)	0.4	0.546
Rank					
Junior NCM	5 (26.3)	13 (68.4)	−42.4 (−68.4 to −10.5)	2.8	**0.004**
Senior NCM	12 (63.2)	0 (0)	63 (42–84.5)	5.4	**<0.001**
Junior Officer	2 (10.5)	6 (31.6)	−20.6 (−47.4–10.5)	1.4	0.098
Education					
High School	6 (31.6)	4 (21.1)	10.6 (−21.1–42.1)	0.7	0.378
College	6 (31.6)	4 (21.1)	10.5 (−15.8–36.8)	0.8	0.310
Undergraduate	5 (26.3)	10 (52.6)	−26 (−52.6–5.3)	1.7	0.056
Graduate	1 (0.5)	1 (0.5)	0.4 (−15.8–15.8)	0	0.658
None	1 (0.5)	0	5.2 (0–15.8)	1	0.312

*Continuous/integer data presented as the median and interquartile range–med (iqr); categorical data presented as the frequency and percent = n (%). CAF, Canadian Armed Forces; NCM, Non-commissioned member. Significance corrected at a false discovery rate of p = 0.05 (**bold p-values**), derived from bootstrapped mean difference testing. For categorical variables, the mean difference was evaluated on the percent of individuals categorized to each outcome (see section Methods).*

staff are exposed to can vary, the quantification of which will be an important step for improving our understanding of the etiology of blast-induced TBI.

Materials and Procedure

All data were collected in a single session for each participant. CFSME breachers and range staff were tested at Canadian Forces Base Gagetown (CFB Gagetown). Sex- and age-matched CAF controls were tested at DRDC (Toronto Research Center). The measures included the neuropsychological and neurocognitive tasks discussed here, as well as a suite of physiological indices (i.e., blood biomarkers, hearing, vestibular function, and postural tremor). Findings in relation to physiological measures will be discussed in a separate manuscript.

The *Background Health Questionnaire* included six questions to assess history of prior head injury (**Table 2**). The participants completed a battery of neuropsychological measures. The *RAND SF-36 Health Survey* (38) has 36 items aggregated into 8 health-related scales, where a score of 100 indicates optimal functioning in that health category. The *Short Musculoskeletal Function Questionnaire* [SMFQ; (39)] generates scores on two indices: The *Dysfunction Index* (DI) assesses the participant's perceptions of his or her functional musculoskeletal performance, whereas the *Bother Index* (BI) assesses how much the participant is bothered by musculoskeletal problems. The participants completed a modified version of the *Rivermead Post Concussion Symptoms Questionnaire* [RPQ; (40)]. Specifically, rather than asking participants to compare themselves to a time prior to the accident, for each symptom they were asked to indicate whether they had experienced it as a function of injury to the head. Next, symptomatic criteria for PTSD were assessed using the 20-item *Post-Traumatic Checklist* (PCL-5), according to the Diagnostic and Statistical Manual of Mental Disorders [DSM-5, (32)].

Neurocognitive function was assessed using the Cognitive Test Software (41). This involved the computerized administration of four measures in sequence: (1) *Delayed matching-to-sample (dMTS)*: This test assessed short-term visual (iconic) memory and pattern recognition (42). (2) *Four-choice reaction time task (4-choice RT task)*: This test assessed the ability to respond rapidly and accurately to simple visual stimuli presented on a computer screen (43). (3) *n-back*: This is a test of working memory performance, and requires the maintenance and updating of dynamic rehearsal sets (44). In the present study, *n* had a range of 1-3. (4) *Stroop*: This is a test of executive functions, specifically inhibition (45). Finally, the participants completed BrDI (**Figure 2**). For that task participants were seated at a desk with a touch-sensitive computer tablet connected to an external monitor. While wearing a touch-screen glove on their dominant hand, participants were instructed to place their finger on a central spot on the horizontally placed computer tablet and move the cursor as accurately and quickly as possible across the screen into the target. In two of the four conditions, participants viewed the targets directly on the tablet while sliding their finger in the same or opposite direction to move the cursor

TABLE 2 | History of prior head injury.

Variables	Breachers/range staff ($n = 19$)	CAF controls ($n = 19$)	Mean difference (95% CI)	Bootstrap ratio	*P*
Concussion	8 (44.4)	5 (26.3)	21 (−5.3–47.4)	1.5	0.088
Physical impact to head	9 (47.4)	11 (57.9)	−10.4 (−36.8–15.8)	0.7	0.402
MVA	14 (73.7)	9 (47.4)	2.6 (−5.3–52.6)	1.7	0.066
Fallen as child	8 (42.1)	6 (31.6)	10.4 (−10.5–31.6)	1	0.206
Physical fight	13 (68.4)	15 (78.9)	−10.6 (−31.6–15.8)	0.9	0.258
Blast exposure	19 (100)	2 (10.5)	89.2 (73.7–100)	12.5	**<0.001**

CAF, Canadian Armed Forces; MVA, motor vehicle accident. Data presented as the frequency and (%). Significance corrected at a false discovery rate of p = 0.05 (bold p-values), derived from bootstrapped mean difference testing evaluated on the percent of individuals categorized to each outcome (see section Method).

toward the target. In the other two conditions, participants viewed the targets and cursor on an external monitor in the vertical upright position while moving their finger in the same or opposite direction. Each participant performed 5 trials in each of 4 randomly presented conditions. Finally, all participants completed *Cognistat* (46–48) which is a measure used to assess cognitive function in five distinct ability areas, including their subcomponents (language, spatial-constructional skills, memory, calculations, and reasoning and judgment). The test requires 15–20 min for completion. The test format was paper-and-pencil, administered individually by one of two experimenters who were trained to criterion in advance.

Statistical Analysis

Prior to statistical analysis, all variables were checked for deviations from normality through testing the skewness and kurtosis for each variable against a random gaussian noise model (1,000 iterations). Skewness in the breacher/range staff group ranged from 0.2 ($p = 0.630$) to −2.6 ($p < 0.001$), whereas kurtosis ranged from 2.4 ($p = 0.997$) to 15.5 ($p < 0.001$). In CAF controls, skewness ranged from 0 ($p = 0.834$) to 3 ($p < 0.001$), and kurtosis ranged from 3 ($p = 0.871$) to 23 ($p < 0.001$). Hence, before statistical testing, variables exhibiting moderate normality deviations were transformed by winsorization (10%), whereas variables that severely deviated from normality were rank transformed.

Univariate, between-group comparisons for continuous/interval variables (demographic, psychological and cognitive measures) were conducted using a bootstrapped mean difference test (1,000 resamples), run in a repeated-measures framework to account for CAF subject matching. Briefly, a distribution of mean difference scores for each variable was created to identify the average and 95% confidence interval of the difference between groups; percentile *p*-values were obtained by computing the fraction of bootstrapped coefficient values not enclosing zero effect in a two-tailed framework, which were then corrected at a false discovery rate (FDR) of 0.05. Standardized effect sizes were defined in terms of bootstrap ratios (BSR) which were calculated by dividing the bootstrapped mean of the differences by the standard error of the mean for each comparison. For categorical variables, the mean difference test was evaluated on the percent of individuals categorized to each outcome. For example, given a binary variable with two possible outcomes (0 or 1), the difference in the percent of individuals with outcome 1 was calculated between groups.

To compare psychological and cognitive test profiles (1) between breachers/range staff and CAF controls, (2) between personnel with vs. without a history of concussion, and (3) between personnel who were deployed vs. never deployed to a war zone, a partial least squares discriminant analysis (PLSDA) test was employed. PLSDA is a classification algorithm that seeks to maximize covariance between a set of predictor variables (cognitive and psychological test scores) and a single binary response variable (breacher/non-breacher; concussion/no concussion; war zone/no war zone). By creating latent variables comprised of individual variable weights, the PSLDA is optimized to handle collinearity, and is well-posed for a low ratio of subjects-to-variables. The PLSDA was run in a bootstrapped framework (1,000 iterations), followed by the generation of effect sizes as bootstrap ratios (BSR: mean/standard error) and percentile *p*-values, corrected at an FDR of 0.05. Model performance was evaluated via predication accuracy (Accur) and posterior probability (PProb) estimates, procured in a leave-two-out resampling framework. Accur was evaluated by assigning each subject to the outcome group with the most similar PLS score, and then quantitating the percent of correctly classified subjects. PProb was derived via the calculated likelihood of the PLS model in identifying the correct outcome conditioned on observed subject scores under a Gaussian noise model. Prior to PLSDA analyses, the potential confounding influence of age was adjusted for by partial regression on all affected variables, and any variables with near-zero variance were removed prior to analysis. All data were analyzed and graphed using R (RStudio, version 1.2.1335, Boston, United States).

RESULTS

Demographics and Service History

Demographic and service history variables in breachers/range staff and CAF controls can be seen in **Table 1**. Both groups were predominantly male (89.5%), with breachers/range staff reporting significantly more years of military service (BSR = 3.7, $p < 0.001$), years of breaching (BSR = 7.2, $p < 0.001$) and a history of combat deployment (BSR = 6.39, $p < 0.001$) than CAF controls. Breachers/range staff were comprised of a

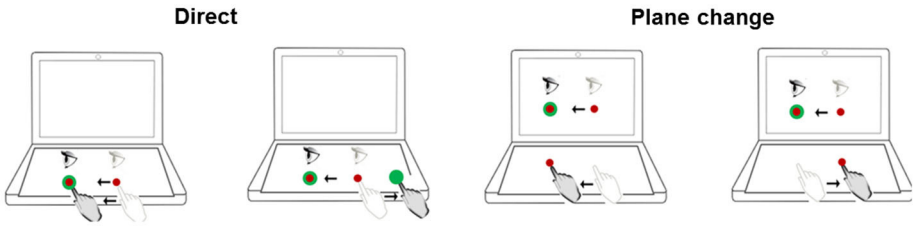

FIGURE 2 | Schematic of experimental conditions for BrDI. BrDI, Brain Dysfunction Indicator. Visual stimuli were presented either directly on the computer tablet (same plane; Direct) or on the attached monitor (plane change). Light gray eye and hand indicate the start position. The dark gray eye and hand symbols depict the movement from start position toward the target. The target was presented in one of four locations (right, left, up or down).

higher proportion of Senior NCM ranked personnel compared to CAF controls (BSR = 5.4, $p < 0.001$), whereas CAF controls were comprised of a higher proportion of Junior NCM ranked individuals (BSR = 2.8, $p = 0.004$).

History of Prior Head Trauma

Head trauma history in breachers/range staff and CAF controls are displayed in **Table 2**. There were no differences in prior head trauma history between the two groups with the exception of blast exposure which, as expected, was prevalent in 100% of breachers/range staff but only in 10.2% of CAF controls (BSR = 12.5, $p < 0.001$).

Neuropsychological and Neurocognitive Measures

Breachers/range staff and CAF controls' neuropsychological and neurocognitive scores can be seen in **Table 3**. Breachers/range staff scored significantly lower on the Energy subscale of SF-36 (BSR = 2.2, $p = 0.022$) compared to CAF controls. Rivermead scores were analyzed using two methods. First, responses to the initial three items of the questionnaire (headache, feelings of dizziness, nausea/vomiting) generated RPQ-3 that captures *early* post-concussive symptoms (i.e., symptoms that tend to present themselves closer to the time of injury), whereas responses to the next thirteen items (e.g., sleep disturbance) generated RPQ-13 that captures *late* post-concussive symptoms (i.e., symptoms that tend to present themselves later following the injury) (49). Second, we sorted the items into cognitive, emotional, and somatic symptoms. Items in each category were summed, omitting scores of "1" (40). Following Verfaellie et al. (50), we divided the total score for each category by its number of items. Breachers/range staff scored significantly higher on both the RPQ3 (BSR = 3.8, $p < 0.001$) and RPQ13 (BSR = 4.0, $p < 0.001$) compared to CAF controls; scores were also higher for Rivermead's somatic (BSR = 3.6, $p = 0.004$), cognitive (BSR = 2.9, $p = 0.004$) and emotional (BSR = 3.4, $p < 0.001$) test components in breachers/range staff compared to CAF controls.

Examples of hand movement trajectories associated with performance on BrDI are illustrated in **Figure 3**. The dependent variable for this task consisted of RT associated with the four conditions. BrDI direct plane veridical (BSR = 2.7, $p = 0.004$) and differential plane veridical (BSR = 3.0, $p = 0.002$) times were significantly higher in breachers/range staff compared to CAF controls (**Figure 4**).

Tasks on the neurocognitive task battery were scored as follows: For dMTS, the dependent variable was accuracy (i.e., percentage correct out of 25 trials). For the 4-choice RT task, the dependent variable was the RT associated with correct responses. For Stroop, the dependent variable was the difference in RT for correctly identifying the color of incongruent word trials (e.g., the word RED appearing in blue) vs. RT for correctly identifying the color of congruent word trials (e.g., the word RED appearing in red). For the n-back, the dependent variable was *d'* (i.e., sensitivity) (51). Higher *d'* values reflect greater sensitivity, whereas a *d'* nearing zero reflects chance performance. None of these measures appeared sensitive to the effects of blast. However, on Cognistat, CAF controls displayed significantly higher failure rates on tests of memory and comprehension compared to breachers/range staff (BSR = 1.9, $p < 0.001$ for both tests).

PLSDA plots displaying psychological and cognitive test profiles in three separate classification analyses are shown in **Figure 5**. All analyses were adjusted for the effects of age. RPQ3 (BSR = 4.5, $p < 0.001$) and RPQ13 (BSR = 5.1, $p < 0.001$) scores contributed significantly to class separation between breachers/range staff ($n = 19$) and CAF controls ($n = 19$), with higher scores found in the former; the PProb of the model was 0.73, and Accur was 0.78 (**Figure 5A**). When all participants were taken into consideration (i.e., breachers/range staff and CAF controls combined), those with a history of concussion ($n = 13$) displayed higher scores on RPQ3 (BSR = 3.6, $p = 0.006$) and RPQ13 (BSR = 4.5, $p = 0.002$), as well as higher scores on SMFA's Bother Index (BSR = 3.8, $p < 0.001$) and Dysfunction Index (BSR = 3.2, $p < 0.001$), compared to those with no history of concussion ($n = 24$); model PProb was 0.65, and Accur was 0.68 (**Figure 5B**). Finally, compared to those who had never deployed to a war zone ($n = 25$), deployment to a war zone ($n = 11$) was associated with lower scores in the SF-36 Energy subscale (BSR = 3.3, $p = 0.006$), as well as higher scores in the PCL-5 (BSR = 4.8, $p < 0.001$), the RPQ3 (BSR = 4.7, $p < 0.001$) and RPQ13 (6.2, $p < 0.001$); model PProb was 0.72 and Accur was 0.75 (**Figure 5C**).

DISCUSSION

Our study was conducted to test the hypothesis that compared to sex- and age-matched CAF controls, breachers and range

TABLE 3 | Neurocognitive and neuropsychological measures.

Variables	Breachers/range staff ($n = 19$)	CAF controls ($n = 19$)	Bootstrap ratio	Fractional *P*
Neuropsychological Measures				
RAND SF-36				
General health	75 (67.5–80)	80 (65–92.5)	0.7	0.478
Physical functioning	95 (92.5–100)	100 (95–100)	1.6	0.120
Emotional well-being	80 (66–88)	84 (68–88)	0.4	0.676
Social functioning	100 (87.5–100)	100 (7–100)	0.3	0.786
Pain	90 (80–90)	90 (85–100)	1.5	0.126
Energy	50 (37.5–67.5)	65 (60–80)	2.2	0.022
Role limitations (physical health)	100 (100–100)	100 (100–100)	0	0.952
Role limitations (emotional problems)	100 (100–100)	100 (66.7–100)	1.2	0.224
SMFA				
Function index	40 (37–45)	34 (34–41)	2.4	0.016
Bother index	15 (13–16.5)	12 (12–16)	1.8	0.072
Rivermead				
RPQ3	2 (1–5.5)	0 (0–2)	3.8	**<0.001**
RPQ13	7.0 (1.5–15.5)	0 (0–2.5)	4.0	**<0.001**
Somatic	0.6 (0.1–1.1)	0 (0–0.1)	3.6	**0.004**
Cognitive	0 (0–1.3)	0 (0–0)	2.9	**0.004**
Emotional	0 (0–0.9)	0 (0–0)	3.4	**<0.001**
PCL-5	7 (0.5–10.5)	0 (0–6)	1.9	0.068
Cognistat–frequency (%)				
Consciousness	19 (100)	19 (100)	–	–
Attention	16 (84.2)	16 (84.2)	0.1	0.742
Memory	19 (100)	16 (84.2)	1.9	**<0.001**
Comprehension	19 (100)	16 (84.2)	1.9	**<0.001**
Repetition	16 (84.2)	16 (84.2)	0	0.810
Naming	13 (68.4)	11 (57.9)	0.8	0.306
Constructional ability	17 (89.5)	16 (84.2)	0.6	0.362
Calculations	14 (73.7)	16 (84.2)	0.8	0.346
Similarities	18 (94.7)	15 (78.9)	1.4	0.126
Judgement	13 (68.4)	16 (84.2)	1.2	0.150
Neurocognitive measures				
4-choice RT task (ms)	451 (406–519.5)	450 (425.5–517)	0.7	0.538
n-back (d')				
1-back	4.7 (3.8–4.7)	4 (3.4–4.7)	1.2	0.218
2-back	2.5 (2.1–3.1)	2.8 (2.2–3.5)	0.7	0.490
3-back	1.2 (0.9–1.6)	1.3 (1.2–1.7)	1.1	0.254
dMTS_(% correct)	68 (56–78)	72 (66–82)	1.6	0.094
Stroop (ms)	48 (16.2–80.2)	49 (38–62.5)	0.1	0.918
BrDI (msec)				
Same plane veridical	365.8 (335.6–409.3)	343.2 (282.6–358.9)	2.7	**0.004**
Same plane reversed	511.9 (461.0–560.6)	479.6 (397.6–528.3)	1.3	0.182
Differential plane veridical	386.3 (355.0–452.5)	340.2 (304.7–369.4)	3.0	**0.002**
Different plane reversed	528.9 (460.8–645.1)	477.3 (409.6–514.4)	2.2	0.034

Interval and continuous data presented as the median and interquartile range–med (iqr); categorical data presented as the frequency and percent–n (%). BrDI, Brain Dysfunction Indicator; RT, reaction time; ms, milliseconds; dMTS, delayed matching-to-sample task. For n-back and dMTS the numbers indicate accuracys (percentage); for Stroop the numbers indicate reaction time (seconds). Significance corrected at a false discovery rate (FDR) of p = 0.05 (bold p-values), derived from bootstrapped mean difference testing. For categorical variables, the mean difference was evaluated on the percent of individuals categorized to each outcome (see section Methods).

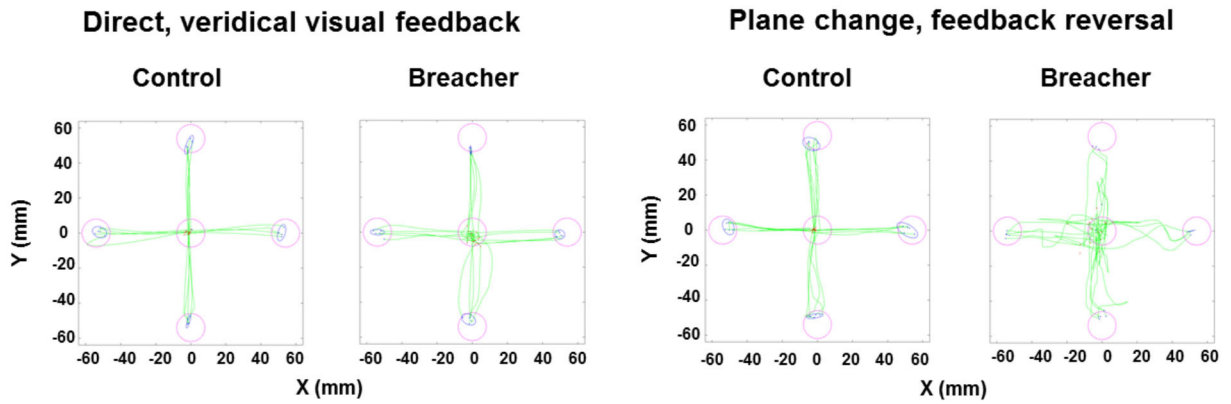

FIGURE 3 | Examples of hand movement trajectories for BrDI. BrDI, Brain Dysfunction Indicator. Examples of hand movement trajectories (green) in the Direct condition (i.e., movements in target direction and in the same plane) and in the Plane change feedback reversal condition (i.e., movements in the opposite direction of the presented target and in a different plane) from one control participant (left side of each condition panel) and from one breaching instructor or range staff (right side of each condition panel). Magenta circles represent the location of targets. Red dots indicate the starting position of the finger for each trial. Blue ellipses represent the 95% confidence intervals for the final endpoint positions of movements (blue dots).

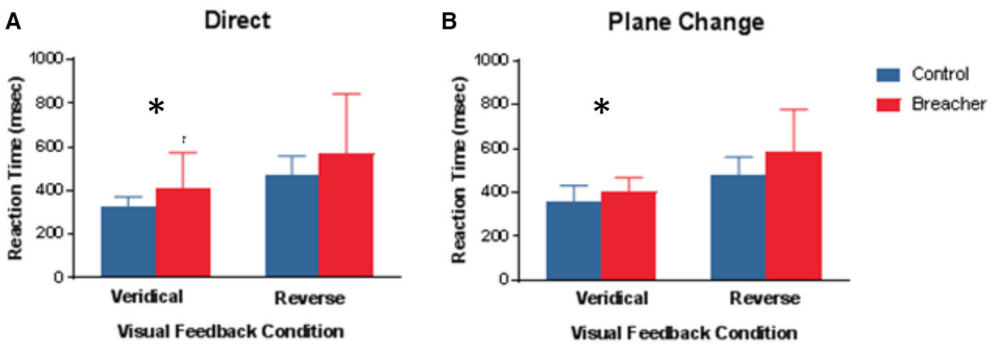

FIGURE 4 | Mean Reaction time under 4 different conditions for BrDI. BrDI, Brain Dysfunction Indicator. **(A)** Same plane with veridical (left panel) or reversed (right panel) visual feedback to move the cursor into the target. **(B)** Different plane with veridical (left panel) or reversed (right panel). Error bars represent ± SD. Significant difference between breaching instructors and range staff vs. controls (see text) $^{*}p < 0.01$.

staff would exhibit functional impairments measured on standardized neuropsychological and neurocognitive tests. Indeed, univariate analyses demonstrated that compared to CAF controls, breachers and range staff reported significantly greater post-concussive symptoms (*Rivermead Post Concussion Symptoms Questionnaire*), as well as lower levels of energy (*RAND SF-36 Health Survey*). These results suggest that repetitive exposure to low-level blast is associated with impairments in health and function as measured by self-report neuropsychological measures.

None of our standard tests of neurocognitive function that measure short-term visual memory, choice reaction time, or executive functions proved sensitive to the effects of repetitive exposure to low-level blast. In contrast, breachers and range staff exhibited longer RT in two conditions on BrDI task. This novel finding suggests that cognitive-motor integration might actually be an ability that is affected adversely by blast exposure, and that BrDI represents a useful method for detecting the impact of blast exposure over and above what can be obtained using standard neurocognitive tasks alone. Accordingly, there has been considerable fundamental research on the underlying brain activity during cognitive-motor integration, and how this is distinct from brain activity associated with thinking alone or moving alone. These studies used tasks that are laboratory versions of what is tested with BrDI, and support the scientific concept underlying the unique nature of this approach (52–57). Although no previous human studies in military blast have specifically examined this functional measure, a considerable body of work suggests that there is a unique additive value to including tasks that measure cognitive-motor integration in studies of the effects of blast exposure (12, 37).

Importantly, despite impairment in cognitive-motor integration, breaching instructors and range staff are nevertheless able to perform demanding jobs. Hurtubise et al. (28) have noticed a similar pattern in concussed elite athletes. Specifically,

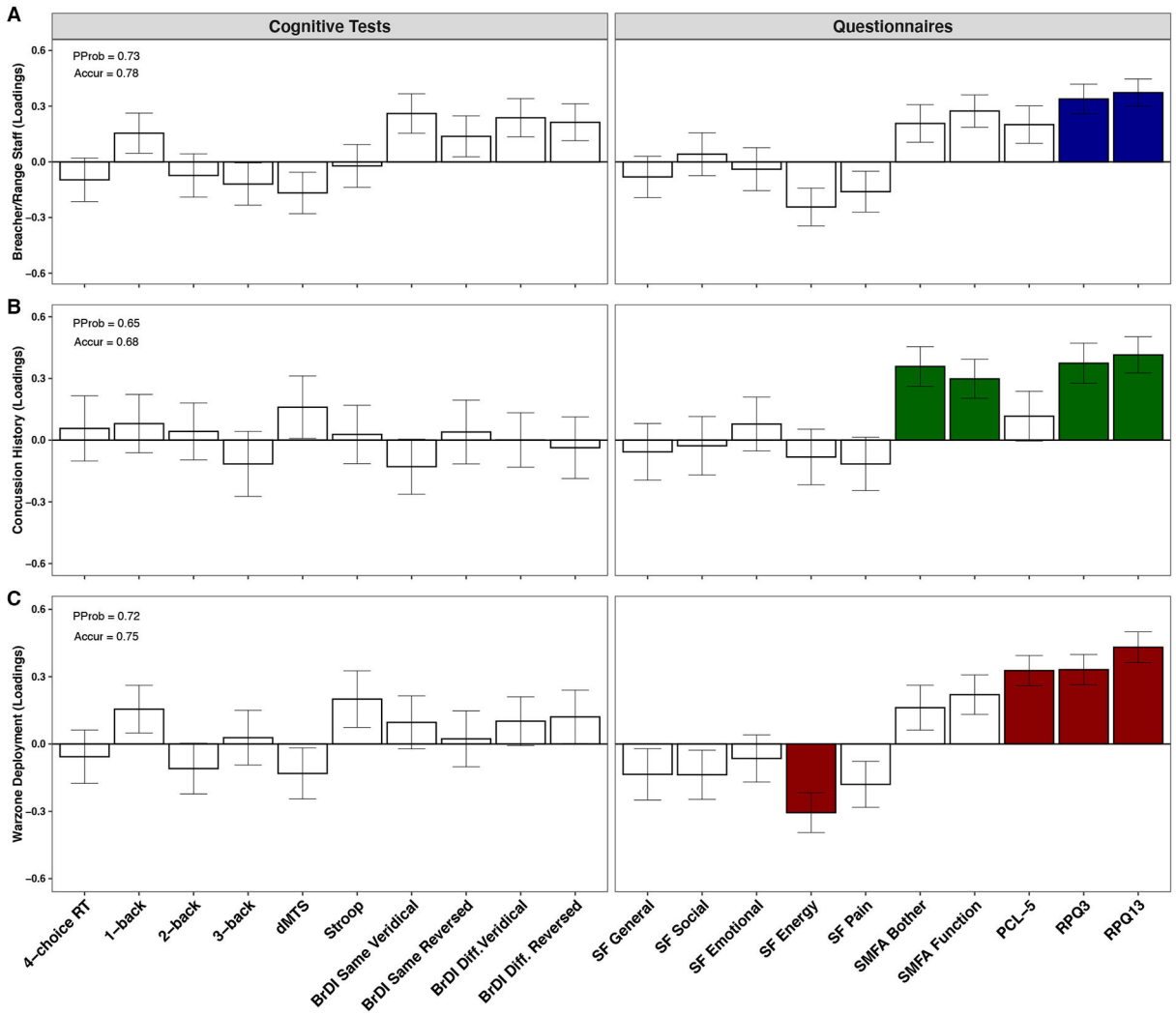

FIGURE 5 | Psychological and cognitive test profiles of military personnel. PProb, posterior probability; Accur, accuracy; RT, reaction time; dMTS, delayed matching-to-sample task; BrDI, brain dysfunction indicator; SMFA, selective functional movement assessment; PCL-5, Posttraumatic stress disorder (PTSD) checklist for diagnostic and statistician's manual (DSM-5); RPQ, Rivermead post-concussion symptoms questionnaire. Plots show the contributions of psychological and cognitive measures toward class separation in **(A)** breachers/range staff ($n = 19$) vs. CAF controls ($n = 19$), **(B)** personnel with ($n = 13$) vs. without ($n = 24$) a history of concussion, and **(C)** personnel deployed to a war zone ($n = 11$) vs. never deployed ($n = 25$), by partial least squares discriminant analysis (PLSDA). Bars represent biomarker loadings and the standard error derived from bootstrapped resampling (1,000 samples). Colored bars = significant at a false discovery rate (FDR) < 0.05.

they reported noticeable behavioral deficits in elite vs. non-elite concussed athletes, despite elite athletic performance in the former group. It was proposed that high-level athletes possess a superior fronto-parietal network connectivity due to their higher level of training, and are, therefore, able to compensate for the mild brain injury. Similarly, it is possible that CAF breachers and range staff have also built-up superior fronto-parietal networks following years of military training, and can thereby perform at high levels, occupationally. This interpretation is consistent with their scores on Cognistat, where they performed better on the subcomponents of Memory and Comprehension than CAF controls. These findings suggest that there might indeed be components of cognitive function that could be enhanced by the occupational demands of breaching, the mechanisms for which require further study.

An important aspect of our approach in this study was to examine the impact of blast within the larger occupational context of military service and injury. Our multivariate approach demonstrated that the disturbances found in breachers and range staff do not appear to be unique to breaching, as they were also observed, to a similar extent, in military personnel with a history of concussion as well as those who have been deployed to a war zone. Specifically, it appears that post-concussive symptoms are associated with all three conditions: breaching, concussions, and war-zone deployment (**Figure 5**). However,

within a non-matched, age-adjusted model, reported concussion history appears to uniquely alter SMFA scores, whereas war-zone deployment appears to uniquely alter perceived energy and risk of PTSD. In the case of PCL-5 measures, individuals with multiple deployments showed consistently higher scores (median value of 7), which corresponds with greater PTSD symptomatology (58). Although well-below the threshold for clinical diagnosis (i.e., PCL-5 score $\geq$ 33), this observation is consistent with a large body of research suggesting a link between multiple deployments, mTBI and increasing vulnerability to developing PTSD and other mental health problems (59, 60). These findings should prove useful as researchers work toward developing improved diagnostic tools for distinguishing between the effects of these three conditions that frequently overlap in this population exposed routinely to low-level blast (61, 62).

It is important to exercise caution in interpreting our findings. First, although our two groups were comparable in terms of demographic and past brain injury indicators, they differed on a number of factors that might have affected our findings, including greater number of years of service in the military, as well as a greater frequency of deployment to war. Second, because we employed a quasi-experimental cross-sectional design, it is not possible to draw any causal inference from our findings. However, we do hope that our findings will motivate longitudinal studies that are better suited for isolating the effects of long-term occupational exposure to repeated low-level blast in operators [see Kamimori et al. (21)]. Third, because the precise mechanism(s) underlying blast-related neurological injury remains unknown, additional work on that fundamental problem is necessary for gaining a better understanding of the injury pathway (12, 37). Fourth, our sample reflects an armed forces population that is mostly male, and as such the findings may not be entirely representative of females (63–66). Despite these limitations, our results suggest that long-term occupational exposure to repeated low-level blast is a phenomenon that requires further systematic study, and that outcomes associated with it might not be necessarily unique to the breaching environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data for this study were collected from members of the Canadian Armed Forces (CAF). Permission from the CAF's chain of command will be required to make the generated dataset available for public access. Requests to access the datasets should be directed to Oshin Vartanian, oshin.vartanian@drdc-rddc.gc.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Defence Research and Development Canada Human Research Ethics Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OV, CT, SR, AN, LS, DF, AC, and RJ designed the study. OV, CT, SR, AN, KK, QL, and DS collected the data. OV, AD, DG, and KK analyzed the data. All authors contributed to writing and editing the manuscript.

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BRIEF RESEARCH REPORT
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Hearing Loss and Irritability Reporting Without Vestibular Differences in Explosive Breaching Professionals

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Background: Blast exposure is a potential hazard in modern military operations and training, especially for some military occupations. Helmets, peripheral armor, hearing protection, and eye protection worn by military personnel provide some acute protection from blast effects but may not fully protect personnel against cumulative effects of repeated blast overpressure waves experienced over a career. The current study aimed to characterize the long-term outcomes of repeated exposure to primary blast overpressure in experienced career operators with an emphasis on the assessment of hearing and vestibular outcomes.

Methods: Participants included experienced "breachers" (military and law enforcement explosives professionals who gain entry into structures through controlled detonation of charges) and similarly aged and experienced "non-breachers" (non-breaching military and law enforcement personnel). Responses to a clinical interview and performance on audiological and vestibular testing were compared.

Results: Hearing loss, ringing in the ears, irritability, and sensitivity to light or noise were more common among breachers than non-breachers. Breachers reported more combat exposure than non-breachers, and subsequently, memory loss and difficulty concentrating were associated with both breaching and combat exposure. Vestibular and ocular motor outcomes were not different between breachers and non-breachers.

Conclusion: Hearing-related, irritability, and sensitivity outcomes are associated with a career in breaching. Future studies examining long-term effects of blast exposure should take measures to control for combat exposure.

Keywords: hearing loss, blast overpressure, symptom reporting, career breaching, vestibular

INTRODUCTION

In operations and training, U.S. military personnel are exposed to blast overpressure and associated sound pressure insults. These exposures arise from multiple sources, to include improvised explosive devices, ordnance (breach explosives, hand grenades), and weaponry (heavy shoulder-fired weapons, high-powered rifles). It can be difficult to control circumstances surrounding operational assaults, but training environments, on the other hand, typically employ measures to prevent exposures from ordnance or weaponry that may result in an injury. However, there is a growing concern regarding the long-term effects of cumulative subconcussive blast events. Recently, the U.S. Congress directed the Department of Defense to study the effects of blast on military personnel (1, 2). There is particular concern over outcomes from repetitive subconcussive low-intensity blast exposure where symptoms may manifest over time (3).

"Breachers" are military and law enforcement personnel who use controlled explosive breaching charges to quickly ingress into a fortified structure. Some breachers report deleterious effects which they believe are associated with their exposure and coined the term "breacher's brain" to describe a symptom array that includes fatigue, thinking difficulty, and headache (4). Despite this awareness, the effects of blast on military personnel have been difficult to characterize. Assessments of breachers have been inconsistent in detecting changes in neuroimaging, symptom-reporting, neurocognitive performance, or biomarkers of brain injury (5–7).

More recently, LaValle et al. found that exposure to high blast overpressure in breaching training (above 34.47 kPa) led to a transient, but measurable, effect on neurocognitive performance (8). In another recent study examining relatively lower blast overpressure readings at a grenade range training course (0.97–2.89 kPa), Sajja et al. found that post-training neurosensory symptoms were associated with low-level sound overpressure exposure (9). Specifically, headache and thinking difficulty, followed by lightheadedness, ringing of the ears (i.e., tinnitus), restlessness, frustration, and irritability. The authors noted that sound pressure was likely influential in generating the symptoms, as acoustic sensors in the field recorded sound pressure ranging from 153.72 to 163.22 dB peak (9). These readings were in excess of the Department of Defense 2015 Regulation (No. 385-1-89) impulse noise safety limit of 140 dB peak (10). This suggests that sound pressure may be contributing to observed long-term outcomes associated with blast exposure.

The vestibular system may be affected when the auditory system is transducing high-decibel sound waves, as dizziness or imbalance are symptoms commonly reported after blast. In a review of studies examining vestibular metrics, up to half of blast-exposed individuals exhibit vestibular, balance, or ocular motor dysfunction (11). Our goal was to investigate whether career breachers demonstrated auditory or vestibular system dysfunction, not from acute blast exposure or a recent concussion, but from cumulative controlled low-level blast exposure during the course of their career. The data presented here are a component of a large multi-institutional effort to assess the effects of repetitive blast exposure in experienced breachers.

METHODS

Participants

This study was approved by Institutional Review Boards (IRB) at the Naval Medical Research Center (NMRC.2011.0002), the Walter Reed Army Institute of Research (WRAIR #1796), and the Central Nervous System IRB of the National Institutes of Health. Participants traveled to Bethesda, Maryland for several days of data collection at the National Institutes of Health Clinical Center. Methods on neuroimaging, blood component, and neuropsychological data collected from this study, as well as neuroimaging analysis, have been described in a publication authored by Stone et al. (12). Future publications or reports will focus on analysis of blood, neuropsychological, and posture/balance data collected in this study. Participants were recruited from military and law enforcement communities (active duty and prior service). Breachers (n = 20), were categorized as individuals with careers utilizing explosives to gain entry into structures. Specifically, breachers met the inclusion criteria of at least 4 years of experience with breaching operations occurring at least annually or had exposure to at least 400 breaching blasts over their careers. Non-breachers ($n = 14$) were recruited to match a similar age, education, and years in career to the breachers. Thus, inclusion criteria for non-breachers consisted of at least 4 years of military or law enforcement experience, and exposed to no more than 40 breaching blasts over their careers. All participants were male due to breaching careers only recently opening up to female service members. Exclusion criteria included history of diagnosis of moderate to severe brain injury, central nervous system disorder, medical conditions affecting cerebral metabolism, recent concussion, or injury including loss of consciousness >5 min.

Clinical Interview

Participants underwent a demographic and historical clinical interview and a medical history and neurological examination. Clinical interview questions and summary responses are detailed in the Appendix (**Supplementary Material**) and **Supplementary Table 1**. Responses were recorded and whenever possible, questions were coded into "yes/no" responses. In

addition to demographics, participants were asked about their past weapons use and combat experiences. Participants were asked to recount each large weapon or explosive they had interacted with and the number of times they were exposed to its detonation. Responses regarding small arms were not consistently detailed or discussed during interviews, so any responses which were recorded were excluded from analysis. Weapons were grouped into categories of heavy weapons, artillery, small explosives, and large explosives (excluding breaching charges). If a participant reported more than 10 instances of any of the categorized weapons, they were scored as having exposure to that category.

For combat experiences, participants were administered a 34-item Combat Exposure Checklist, a modification of the Walter Reed Army Institute of Research Combat Experiences Scale [described by Guyker et al. (13)] (grammatical tense was adjusted to account for all deployments, three questions related to crime were removed, four questions associated with recent conflicts were added, and answer choices were adjusted). The list contained experiences common to modern battlefields. Participants responded on an ordered scale: experience had never occurred (1), occurred once (2), occurred between two and four times (3), or occurred ten or more times (4). These responses were scored as: 0 = zero experiences; 1 = one experience; 3 = two through four experiences; 10 = 10 or more experiences. Scores for each participant were summed and treated as a scalar variable in the range of 0–340.

Audiometric, Vestibular, and Balance Data Collection

Audiology and vestibular data were collected and processed in the Audiology Unit at the National Institute on Deafness and Other Communication Disorders. All audiologic evaluations were conducted using GSI-61 (Grason-Stadler, Eden Prairie, MN) clinical audiometers with the patient in double-walled sound treated rooms, both of which met American National Standards Institute criteria (14, 15). Audiological measurements included speech reception thresholds and pure-tone air conduction thresholds measured in octave band frequencies from 250 to 8,000 Hz and including interoctave assessment at 3,000 and 6,000 Hz; bone conduction pure-tone thresholds from 250 to 4,000 Hz were evaluated when air-conduction thresholds exceeded 25 dB HL. Tympanograms were acquired using a Grason-Stadler Tympstar immittance bridge in response to a standard 226-Hz probe tone.

Vestibular testing included measurement of the vestibulo-ocular reflex elicited by stimulation of the horizontal semicircular canal during bi-thermal caloric irrigations and sinusoidal harmonic acceleration using a rotary chair. Sinusoidal harmonic acceleration stimuli were presented using a calibrated Neuro Kinetics (Neuro Kinetics, Inc.; Pittsburgh, PA) Neuro-Otologic Test Center via VEST™ software at octave frequencies from 0.01 to 0.64 Hz, and bithermal air caloric irrigations were delivered via an ICS Medical Chartr NCA-200 irrigator. Ocular motor stimuli were presented in the NOTC light-proof enclosure (Neuro Kinetics, Inc.; Pittsburgh, PA). Eye tracking for all assessments were measured with Neuro Kinetics (Neuro Kinetics, Inc.; Pittsburgh, PA) binocular infrared digital 250-Hz video-goggles via I-Portal-VOG® software.

Cervical vestibular evoked myogenic potentials (cVEMPs) were elicited via an air-conducted 500 Hz tone burst (Blackman gating, 2 ms rise/fall time, 0 ms plateau) presented monaurally via insert earphones at 100–107 dB nHL and a rate of 5.1/s (Intelligent Hearing Systems; Miami, FL). Myogenic activity was recorded from surface electrodes placed on the ipsilateral sternocleidomastoid muscle (reference), the sternum (active), and the forehead (ground). Cervical VEMP responses were accepted only when sternocleidomastoid myogenic activity was between 50 and 100 μV. The cVEMP was interpreted based on presence or absence of the bi-phasic P1-N1 peak response and interaural symmetry ratio of the P1-N1 amplitude.

Statistics

To compare demographic information and responses to clinical interview questions between breachers and non-breachers, categorical variables were compared by Chi square test, ranked variables were compared by Mann Whitney *U*-test, and scalar variables were compared by one-way ANOVA. Answers to clinical interview questions varying between groups were probed for associations with combat exposure: combat exposure scores were compared by ANOVA between participants reporting "yes" vs. those reporting "no." After data collection, one breacher participant was discovered to have a vestibular schwannoma; none of his audiological or vestibular data were analyzed as a result. His self-reported clinical interview responses were included, as exclusion of it did not change results. Audiometric and vestibular data were compared by one-way or two-way ANOVA between groups with Bonferroni post-test, depending on whether the test had a single measurement or multiple measurements taken, respectively. Pure tone assessment was analyzed by repeated measures two-way ANOVA as each frequency is dependent on the others; standardized residuals for each ear were compared by one-way ANOVA to compare individual frequencies.

RESULTS

Race, ethnicity, marital status, handedness [$p > 0.05$: $\chi^2_{(4,N=34)} = 2.893$; $\chi^2_{(1,N=34)} = 0.068$; $\chi^2(3, N = 34) = 0.971$; $\chi^2_{(1,N=34)} = 0.146$], age, years of education, and years of service [$p > 0.05$: $F_{(1,32)} = 0.078$; $F_{(1,32)} = 0.058$; $F_{(1,32)} = 1.463$] were not different between breachers and non-breachers. It should be noted that most participants in either group were right-handed (18 out of 20 breachers and 12 out of 14 non-breachers). Breachers were a mean of 39.7 ± 8.3 years of age (ranging age 26–54 years) and served for 16.8 ± 6.7 years while non-breachers were 38.9 ± 7.8 years of age (ranging 27–53 years) and served for 13.9 ± 7.0 years. Unexpectedly, breachers reported having more head injuries in comparison to non-breachers [1.1 ± 1.0 to 0.3 ± 0.5, $F_{(1,32)} = 7.712$, $p = 0.009$]. In terms of breaching experience, total years exposed to breaches averaged 14.4 ± 7.6 in breachers in comparison to 0.04 ± 0.13 years in non-breachers [$F_{(1,32)}$

$= 50.323$, $p < 0.001$]. Approximate number of total breaches experienced ranged from 100 to 34,800 among breachers, but only 0-15 among non-breachers ($U = 0$, $p < 0.001$).

In terms of other service experiences, breachers were more likely than non-breachers to have exposure to large explosives (besides breaches) [$\chi^2_{(1,N=34)} = 4.568$, $p = 0.033$], but no differences were detected among exposure to small explosives, artillery, or heavy weapons. In addition, breachers reported significantly higher combat exposure scores than non-breachers [158.6 ± 65.1 to 60.4 ± 50.0, $F_{(1,32)} = 22.526$, $p < 0.001$]. To address the potential confounding of combat exposure in our comparisons, we attempted to compare only those breachers with similar combat exposure scores to non-breachers by limiting the combat exposure score to 160, the highest non-breacher score. However, even when comparing this sub-group of 11 breachers with all 14 non-breachers, the average combat exposure score among breachers was still higher when compared to non-breachers [109.0 ± 25.5 to 60.4 ± 50.0, $F_{(1,23)} = 8.597$, $p = 0.007$].

From the clinical interview, self-report measures that did not associate with combat exposure were compared between groups. More breachers reported experiencing tinnitus [$\chi^2_{(1,N=34)} = 4.371$, $p = 0.037$] and irritability [$\chi^2_{(1,N=34)} = 5.781$, $p = 0.016$] than non-breachers (**Figures 1A,B**). When comparing only the breachers with a matching range of combat exposure scores to non-breachers, low combat breachers reported more sensitivity to light and noise [$\chi^2(1, N = 25) = 4.957$, $p = 0.026$, **Figure 1C**]. Memory problems [$\chi^2_{(1,N=34)} = 4.371$, $p = 0.037$] and difficulty concentrating [$\chi^2_{(1,N=34)} = 5.781$, $p = 0.016$] were reported more among all breachers, but these responses were also associated with higher combat exposure scores. The combat exposure score was significantly higher among breachers reporting memory problems [189.3 ± 59.2 to 101.6 ± 23.6, $F_{(1,18)} = 13.883$, $p = 0.002$], and among breachers reporting difficulty concentrating [187.9 ± 64.8 to 122.8 ± 46.7, $F_{(1,18)} = 6.359$, $p = 0.021$]. In contrast, these associations were not seen in breachers reporting ringing in the ears [173.5 ± 67.8 to 131.0 ± 53.3, $F_{(1,18)} = 2.044$, $p = 0.170$], irritability [170.5 ± 64.0 to 144.1 ± 67.1, $F_{(1,18)} = 0.803$, $p = 0.382$], or sensitivity to light and noise [115.0 ± 29.5 to 104.0 ± 23.3, $F_{(1,9)} = 0.480$, $p = 0.506$]. Additionally, breachers reported exercising more hours per week than non-breachers ($U = 77.500$, $p = 0.028$), but this effect was driven by three breachers reporting 14–30 h per week (all other participants reported 12 or less), so activities which qualify as exercise may have been interpreted differently among participants. No other responses from the clinical interview were statistically significant between groups ($p > 0.05$, χ^2 or U; responses summarized in **Supplementary Table 1**).

Breachers exhibited poorer hearing thresholds in the right ear as analyzed from effect of group contribution to a two-way repeated measures ANOVA [$F_{(1,31)} = 4.884$, $p = 0.035$, **Figure 2A**]. This effect was not seen in identical analysis performed on threshold data from the left ear [$F_{(1,31)} = 3.079$, $p = 0.089$, **Figure 2B**]. *Post-hoc* analysis of the right ear residuals by ANOVA revealed the right ear group effect was most driven by hearing thresholds at 2,000 and 3,000 Hz [2,000 Hz: breacher residual = 0.450 ± 1.01, non-breacher residual = −0.610 ± 0.58, $F_{(1,31)} = 12.222$, $p = 0.001$; 3,000 Hz: breacher residual = 0.343 ± 1.11, non-breacher residual = −0.466 ± 0.59, $F_{(1,31)} = 6.118$, $p = 0.019$, **Figure 2A**]. The pattern of how hearing thresholds varied across the entire frequency spectrum was not different between breachers and non-breachers in either ear, as evidenced by a null interaction effect of frequency by group when analyzing the split-plot interaction of all variables in the aforementioned two-way repeated measures ANOVA (right ear $F = 1.498$, $p = 0.213$; left ear $F = 1.716$, $p = 0.151$).

No significant differences were observed for any vestibular or ocular motor outcome measures between breachers and non-breachers. Non-significant vestibular outcomes measures included VOR gain, phase and symmetry from rotational testing, caloric testing, and cervical VEMP P1-N1 amplitude and amplitude ratio. Overall mean smooth-pursuit eye movement parameters (velocity saccade percent, velocity gain asymmetry, and phase) and saccade eye movement parameters (accuracy grand mean, latency grand mean, and final accuracy grand mean) were not different between groups.

DISCUSSION

Recently, similar but more subtle and still deleterious symptoms of reduced hearing as well as cognitive deficits and increased audiologic complaints have been reported from individuals exposed to low-level overpressure environments (9). As such, it has become important to further elucidate, and even quantify, such adverse effects observed during low-level blast exposure, particularly as it relates to a growing concern for what constitutes safe occupational and militaristic operations when obligated to perform under controlled overpressure environments. For example, tinnitus is the most pervasive service-connected disability (16). In 2018, tinnitus was ranked first as the most prevalent disability for new Veterans Administration (VA) recipients (16). Similarly within our cohort, participants were more likely to report experiencing ringing in the ears than 11.2% of adults aged >18 in the general population (17).

Similar differences in subjective symptom reporting between breachers and non-breachers were observed in other circumstances as well. For example, the increased prevalence of self-reported photo- and phono-sensitivity among low-combat breachers vs. non-breachers was significantly higher. While it remains uncertain with respect to the degree that hearing differences cause breachers to report a greater sensitivity to noise, irritability might also have a concomitant impact on sensitivity to uncomfortable stimuli. However, it is important to consider that irritability might be associated with head injuries or military stress; a neuropsychological complement to this study will be discussed in future work.

The lack of difference between patterns of pure tone thresholds suggests that hearing loss occurs in a similar way among both groups, with some frequencies being more vulnerable than others. The significant group contribution to the model, at least in certain higher frequencies in the right ear, however, shows that breachers exhibit poorer hearing than non-breachers. This could be a result of a mostly right-handed sample:

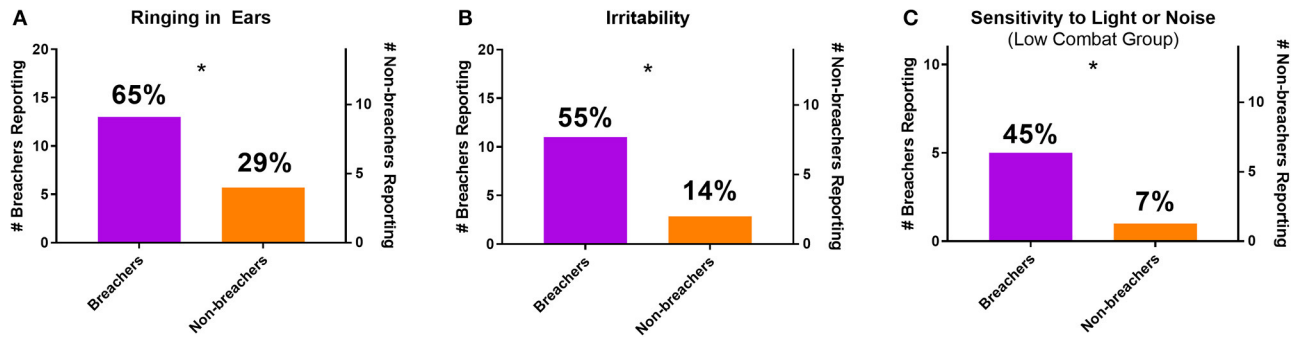

FIGURE 1 | Number and percentage of breachers and non-breachers reporting **(A)** ringing in the ears, **(B)** irritability, and **(C)** sensitivity to light or noise (including only participants with combat exposure scores of 160 or less). *$p < 0.05$, χ^2.

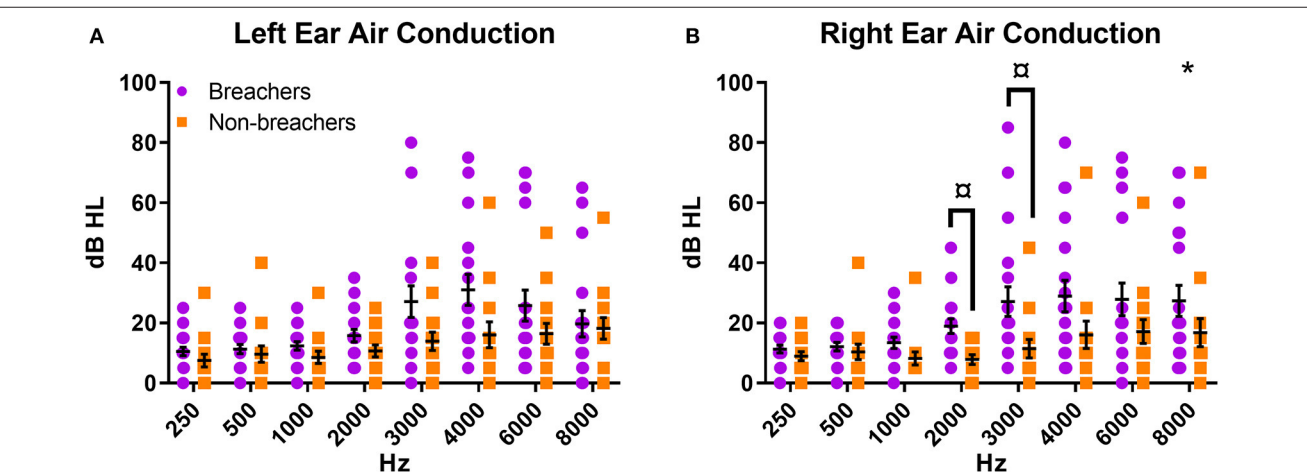

FIGURE 2 | Air conduction pure tone thresholds for **(A)** left ear and **(B)** right ear. *$p < 0.05$ for group factor in repeated measures two-way ANOVA; ¤$p < 0.05$ for individual frequency comparison between groups in one-way ANOVA on the residuals.

some weapons fired on the right side of the body of a right-handed individual may contribute, or hearing protection may more commonly be removed from the right ear of a right-handed individual when straining to hear or understand something. In breachers specifically, the increased hearing loss in the right ear might be an effect of training to face left prior to detonation.

These data support previous findings that identify subtle cognitive, otologic, and audiologic differences within a cohort of individuals subjected to controlled low-level sound overpressure. Such studies are often difficult to design and succeed in effective recruiting given the heterogenous nature of occupational experience, frequency of blast exposure, types of blast exposure, and any co-morbid historical or medical diagnoses such as childhood concussion. As such, the sample size for this study may limit the power of these results and should be interpreted with some caution. However, these findings are consistent with other recent work (18, 19), though it should be noted that breachers did not report headaches at a higher rate. In addition, self-reported responses are subjective and can be influenced by motivation to be perceived by others as ill or injured, or actually being perceived as such. Future blast and hearing research should take measures to include validated tinnitus scales and clinical emotion testing.

Finally, the lack of control for combat exposure was a shortcoming in characterizing long term exposure to blast. The recruitment of career breachers presents a robustly reliable blast exposure signal in the sample. However, the inherent nature of breaching involves entering structures and being within meters of combative individuals and combat environments. The Combat Exposure Checklist queries about sights and sounds experienced firsthand by the participant. By not controlling for combat exposure among the non-breachers in the sample, it is likely we recruited individuals who perform operations dozens or thousands of meters away from combat that can been seen and heard. Due to the presumed psychological impact of combat exposure on behavioral outcomes, it becomes challenging to attribute self-reported differences between the [otherwise well-matched] groups on breaching (and, by extension, blast exposure). Therefore, while worth mentioning the differences found in self-reported memory loss and difficulty concentrating, it may be just as likely these outcomes are associated with combat exposure as they are long term blast exposure. Future studies should include the Combat Exposure Checklist, or another scale like it, in order to better control for combat exposure or even to screen for participants. In a similar regard, until more is known

about cumulative effects of exposure, studies should characterize and quantify all types of blast exposure so that even small arms exposure can be controlled for.

CONCLUSIONS

When compared to non-breaching service and law enforcement members, breachers had more instances of deleterious hearing-related outcomes, irritability, and photo/phono-sensitivity while having no differences in vestibular or ocular motor responses. Breachers exhibited higher rates of pure tone hearing loss in one ear for frequencies commonly associated with noise exposure, as well as self-reported ringing in the ears, irritability, and sensitivity to light or noise. Vestibular and ocular motor outcome measures did not vary between groups. Breachers more often reported memory and concentration problems, but these outcomes were also associated with high combat exposure. Since the breachers sampled were characterized by higher combat exposure than the non-breachers, these outcomes require more research.

DATA AVAILABILITY STATEMENT

The dataset presented in this article is not yet available at the time of publication due to an ongoing study in progress. At the completion of the study, data will be uploaded to FITBIR. Requests to access the dataset can be directed to FITBIR-ops@mail.nih.gov at that time. In the meantime, requests can be sent to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Naval Medical Research Center; Walter Reed Army Institute of Research and the Central Nervous System IRB of the National Institutes of Health. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CM and BJ prepared the initial draft. CM and BJ did the analyses. The authors that contributed in the writing and editing are: CM, BJ, CZ, KK, CB, JK, AY, ML, PW, KD, EP, AQ, BA, EW, JS, SA, and WC. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.588377/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Effects of Selective Serotonin Reuptake Inhibitors on Depression-Like Behavior in a Laser-Induced Shock Wave Model

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Primary blast injury can result in depression-like behavior in the long-term. However, the effects of the selective serotonin reuptake inhibitor (SSRI) on the depression induced by mild blast traumatic brain injury (bTBI) in the long-term remain unclear. We generated a mouse model of mild bTBI using laser-induced shock wave (LISW) and administered an SSRI to mice by oral gavage for 14 days after LISW exposure. This study aimed to investigate the mechanisms of SSRI-mediated alleviation of depression-like behavior induced by mild bTBI. Animals were divided into three groups: sham, LISW-Vehicle, and LISW-SSRI. LISW was applied to the head of anesthetized mice at 0.5 J/cm^2. Twenty-eight days after the LISW, mice in the LISW-SSRI group exhibited reduced depression-like behavior, a significant increase in the number of cells co-stained for 5-bromo-2'-deoxyuridine (Brd-U) and doublecortin (DCX) in the dentate gyrus (DG) as well as increased brain-derived neurotrophic factor (BDNF) and serotonin levels in the hippocampus compared to the sham and LISW-Vehicle groups. Additionally, levels of phosphorylated cAMP response element binding protein (pCREB) in the DG were significantly decreased in the LISW-Vehicle group compared to that in the sham group. Importantly, pCREB levels were not significantly different between LISW-SSRI and sham groups suggesting that SSRI treatment may limit the downregulation of pCREB induced by mild bTBI. In conclusion, recovery from depression-like behavior after mild bTBI may be mediated by hippocampal neurogenesis induced by increased BDNF and serotonin levels as well as the inhibition of pCREB downregulation in the hippocampus.

Keywords: mild blast traumatic brain injury, selective serotonin reuptake inhibitor, depression, neurogenesis, phosphorylated cAMP response element binding protein, brain-derived neurotrophic factor, laser-induced shock wave

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INTRODUCTION

In recent years, the number of patients injured by bombs has increased globally (1). Many soldiers suffering from mild blast traumatic brain injury (mild bTBI) from the wars in Iraq and Afghanistan experience chronic mental disorders such as depression and cognitive impairments after returning to the United States (2–4). However, the mechanisms that lead to depression after mild bTBI remain unclear (2–5).

It is well established that neurogenesis occurs in the dentate gyrus (DG) and subventricular zone (SVZ) after traumatic brain injury (TBI). Neurogenesis persists for at least 1 month in the hippocampus and 2 weeks in the SVZ, although it can last for up to 1 year in humans (6). As previously reported, there is a negative relationship between depression and neurogenesis (7–12). In fact, novel theories regarding the mechanism of action of antidepressant drugs highlight activation of neurogenesis in the hippocampus. For example, stress can attenuate neurogenesis while the administration of antidepressant drugs activates neurogenesis in the hippocampal DG (7–11). Several previously published molecular studies may shed light on the relationship between depression and the hippocampal DG. Neurogenesis is also closely related with brain-derived neurotrophic factor (BDNF) (9, 11, 12). Regulation of BDNF expression involves phosphorylated cyclic adenosine monophosphate response element binding protein (pCREB) which acts as a gene transcription factor in cells. Chronic administration of antidepressant drugs increases pCREB, but the underlying mechanisms remain unclear (13, 14). Additionally, pCREB is thought to play an important role in hippocampal neurogenesis after TBI, but its precise mechanisms have not been fully elucidated (15–19).

Sato et al. developed a rat model of bTBI using laser-induced shock wave (LISW) (20). LISW is compact in size, easy to use and control, and has superior safety and versatility. Moreover, LISW enables the selection of specific energies or regions of application in animal models. Tomura et al. proposed a mild bTBI mouse model which recapitulates depression-like symptoms in the chronic phase after LISW exposure (21). However, the efficacy of early treatment with a selective serotonin reuptake inhibitor (SSRI) on depression-like behavior induced by mild bTBI in the chronic phase remain unclear. We hypothesized that any antidepressant-like effects would be closely related to neurogenesis, focusing especially on changes in BDNF, serotonin, and pCREB. Therefore, we administered an SSRI, which has been approved as the first-line drug for posttraumatic stress disorder (PTSD) and depression in the United States, in this mild bTBI mouse model to investigate the mechanisms of recovery from depression-like behaviors induced by mild bTBI (22).

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (aged 8 weeks, weighing 22–25 g) were obtained from SLC Japan (Shizuoka, Japan). Mice were housed at 22–24°C with food and water available *ad libitum*. Animals were divided into three groups as follows; sham, LISW-Vehicle, and LISW-SSRI. Sertraline (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was selected as the SSRI and administered at 15 mg/kg to the SSRI group by oral gavage for 14 days after LISW exposure (23–26). The dose of sertraline was decided following a review of the literature (23, 24). Mice in the Vehicle and sham groups were administered distilled water instead of sertraline by oral gavage for 14 days. Sertraline or distilled water was administered to mice 24 h after LISW exposure on day 1. The sham-operated group underwent anesthesia and shaving of the head, but no LISW was applied (**Figure 1**). In this study, the mortality rate of the mice was zero. The study design was approved by the Ethics Committee of National Defense Medical College at the time of study initiation (approval number: 16010).

LISW

The second harmonics of a 532-nm Q-switched Nd: YAG laser (Brilliant b, Quantel, Les Ulis Cedex, France; pulse width, 6 ns) was used as described previously (20, 21, 27). A laser target comprising a light-absorbing material (0.5-mm-thick natural black rubber disk) on which an optically transparent material (1.0-mm-thick polyethylene terephthalate) was adhered with adhesive and placed on the tissue. The laser pulse was absorbed by the black rubber to induce plasma, and its expansion generated a shock wave (LISW) (27). The laser pulse was focused with a plano-convex lens to a 6-mm diameter spot on the target. The targets were carefully maintained using forceps when LISW was applied to the mice. Tomura et al. reported that LISW levels of 0.6 J/cm^2 (peak pressure: 58.0 Mpa) and higher induced obvious skull bone fractures and brain surface hemorrhage (21). LISW levels of 0.5 J/cm^2 (peak pressure: 48.6 Mpa) did not induce head fracture or hemorrhage. We therefore selected 0.5 J/cm^2 as the threshold for inducing TBI to avoid traumatic injury to the head.

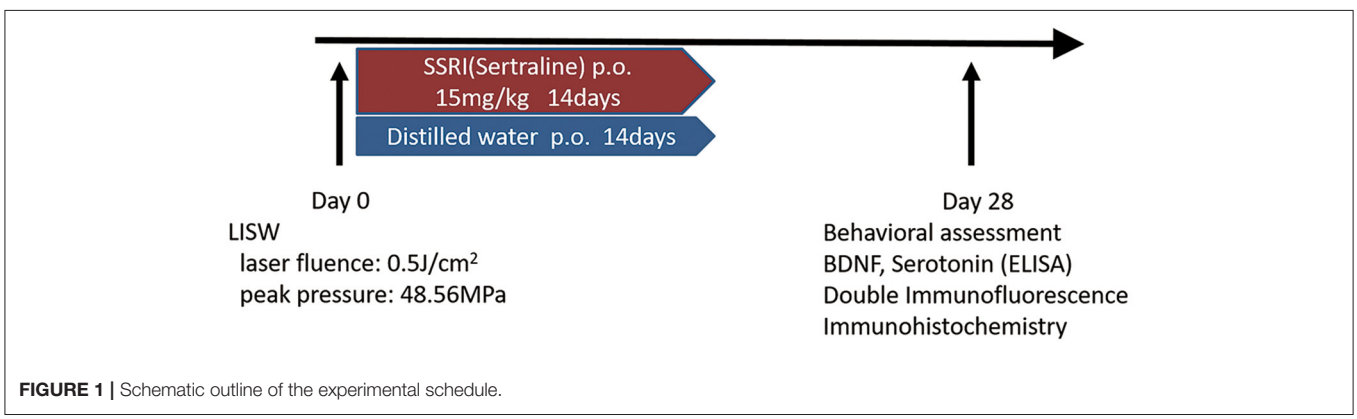

FIGURE 1 | Schematic outline of the experimental schedule.

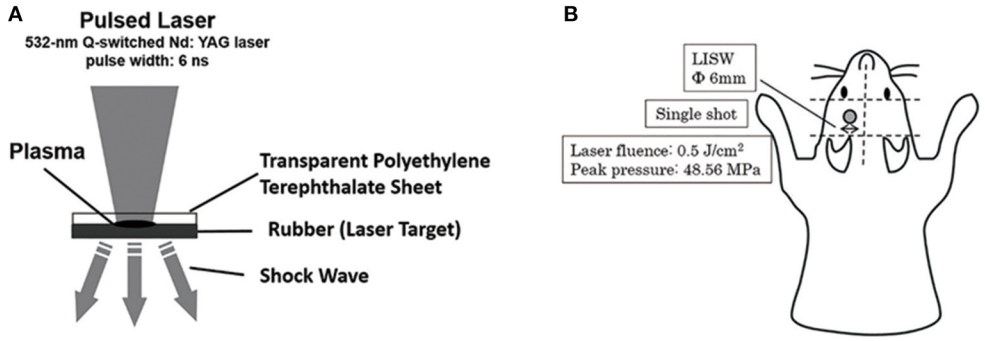

FIGURE 2 | (A) Schema for the generation of laser-induced shock wave (LISW). A laser target comprising a light-absorbing material (0.5-mm-thick natural black rubber disk) on which an optically transparent material (1.0-mm-thick polyethylene terephthalate) was adhered with adhesive, was placed on the tissue. The laser pulse was absorbed by the black rubber to induce plasma and its expansion generated a shock wave (LISW). **(B)** This schema shows the region of LISW exposure. Single pulse by LISW was applied on the skin surface of the left parietal region. The laser target was 6-mm in diameter. Laser influence was 0.5 J/cm^2, and peak pressure was 48.56 Mpa.

In this study, LISW levels of 0.5 J/cm^2 were used (21). A single LISW pulse was applied to the skin surface of the left parietal region (**Figure 2**).

Behavioral Assessment

The tail suspension test and forced swimming test were performed as behavioral assessments at 28 days after LISW exposure. Both the tail suspension test and forced swimming test are well-established methods for assessing depression-like behavior (28–30). In the tail suspension test, mice were suspended by their tails using an elastic band attached to the tail by adhesive tape (approximately 1 cm from the tip of the tail), and the elastic band was hooked on a horizontal rod. Immobility time within a 6-min period was scored for each mouse after mice were suspended by a blinded observer (28, 29). In the forced swimming test, mice were placed in plastic cylinders (height, 30 cm; diameter, 20 cm) containing water (25 ± 1°C; depth, 15 cm). Immobility time within a 6-min period was scored for each mouse after they were placed in the water by a blinded observer (29).

The Y-maze test was also performed. The detailed protocols and results are presented in the **Supplementary Material**. Different animals were used in each behavioral experiment.

ELISA Analysis

At 28 days after LISW exposure, mice were deeply anesthetized with a mix of ketamine and xylazine (ketamine: 100 mg/kg, xylazine: 10 mg/kg, intramuscular injection). Euthanasia was performed by cervical dislocation. The left total hippocampal area was rapidly dissected out and homogenized in RIPA buffer (Pierce® RIPA Lysis buffer; Pierce Biotechnology, Rockford, IL) containing protease inhibitors (The cOmplete®; Roche, Penzberg, Germany). Samples were centrifuged at 4°C and 15,000 rpm for 10 min. The supernatants were collected for enzyme-linked immunosorbent assay (ELISA) analysis and stored at −80°C. The total protein concentration was determined using a BCA protein assay kit (Pierce™ BCA Protein Assay kit; Pierce Biotechnology).

BDNF protein levels in hippocampal homogenates were measured using the Mature BDNF Rapid™ ELISA kit (biosensis, Thebarton, South Australia) according to the manufacturer's instructions. Hippocampal homogenates (100 μL) were diluted with sample diluent and added to duplicate wells of plates pre-coated with an antibody against mature BDNF. After washing, an antibody against mature BDNF was added to the wells, and the plates were incubated for 30 min. After washing, a streptavidin-HRP conjugate was added to the wells, and the plates were incubated for 30 min. After washing, tetramethylbenzidine substrate was added to the wells for 8 min in the dark, then stop solution was added, and the plates were subsequently read on a plate reader at 450 nm. The calibration curve was plotted with the mean absorbance for the calibrator on the y-axis and concentration (0–500 pg/mL) of BDNF on the x-axis.

Serotonin levels in hippocampal homogenates were measured using the Abnova® Serotonin ELISA kit (Abnova, Taipei City, Taiwan) according to the manufacturer's manual. Hippocampal homogenates (100 μL) were diluted with sample diluent and added to duplicate wells of plates pre-coated with acylation reagent. Acylation buffer was added to the wells, and the plates were incubated for 30 min. Acylated samples were then added to duplicate wells in plates pre-coated with the serotonin antigen. A rabbit anti-serotonin antibody was added to the wells and plates ware incubated overnight at 4°C. After washing, goat anti-rabbit immunoglobulin conjugated with peroxidase was added to the wells, and the plates were incubated for 30 min. After washing, tetramethylbenzidine substrate was added to the wells for 30 min, stop solution was added, and the plates were read on a plate reader at 450 nm. The calibration curve was plotted using the mean absorbance for the calibrator on the y-axis against concentration (0–2.5 ng/mL) of serotonin on the x-axis. Each absorbance was read using SPECTRA max PLUS 384® (Molecular Devices Japan, Tokyo, Japan).

Immunofluorescence Staining

At 28 days after LISW exposure, a separate group of mice were deeply anesthetized with a mixture of ketamine and xylazine

(ketamine: 100 mg/kg, xylazine: 10 mg/kg, intramuscular injection) and transcardially perfused with normal saline followed by 4% paraformaldehyde. 5-Bromo-2'-deoxyuridine (Brd-U) staining was performed on paraffin sections using a Brd-U Labeling & Detection Kit II (Roche). Brd-U was injected intravenously (10 mL/kg) 24 h prior to euthanasia. Fifty micrometer-thick coronal sections were prepared from mouse brains, and slides were deparaffinized. For antigen retrieval, sections were autoclaved using a Decliaking Chamber™ (Biocare Medical, Pacheco, CA) with 10 mM citrate buffer (pH 6.0, Emergo Europe, Haag, Netherlands) for 15 min at 121°C. The sections were washed with tris-buffered saline (TBS) and incubated with blocking buffer (3% skim milk) for 30 min at 37°C. The sections were incubated overnight at 4°C with primary antibodies against DCX (1:800, Abcam, Cambridge, UK). The sections were washed with tris-buffered saline with tween (TBS-T) and TBS, and incubated for 60 min at 37°C with secondary antibodies (donkey anti-rabbit IgG antibody; 1:500, Abcam). The sections were washed with TBS-T and TBS, and incubated with blocking buffer (3% skim milk) for 30 min at 37°C. The sections were incubated for 30 min at 37°C with primary antibodies against Brd-U (Brd-U Labeling & Detection Kit II; Roche). The sections were washed with TBS-T and TBS and incubated for 30 min at 37°C with secondary antibodies (goat anti-mouse IgG antibody; 1:1,000, Abcam). The number of cells stained with both Brd-U and DCX in the left hippocampal DG was counted in a blinded manner using BZ-X710® (Keyence, Osaka, Japan). Six DG regions were selected from −2.06 to +2.54 mm anteroposterior to bregma in each mouse (21, 31).

Immunohistochemical Staining

Coronal sections (50 μm-thick) were prepared for immunohistochemical analysis. Slides were deparaffinized and hydrated. For antigen retrieval, sections were autoclaved using a Decliaking Chamber™ (Biocare Medical) with 10 mM citrate buffer (pH 6.0, Emergo Europe) for 10 min at 110°C. Endogenous peroxidases were blocked with 3% hydrogen peroxide in methanol for 5 min. Further incubation with 10% goat serum (Nichirei Biosciences, Tokyo, Japan) was performed for 30 min at 37°C. The sections were incubated with primary antibodies against pCREB (1:5,000, Abcam) overnight at 4°C. The form of pCREB which we used was phosphorylated at Ser 133. The primary antibodies were diluted in 1% bovine serum albumin (Sigma-Aldrich Japan, Tokyo, Japan). The sections were washed with PBS and incubated with N-Histofine® Simple Stain Mouse MAX-PO (Nichirei Biosciences) for 30 min at room temperature. Peroxidase activity correlating to pCREB expression was visualized with diaminobenzidine (DAB; Histofine®, Nichirei Biosciences). One section per one animal was used for the analysis. For an evaluation of morphological changes, adjacent sections were counterstained with hematoxylin. The number of cells stained with DAB in the left hippocampal DG was counted in a blinded manner using BZ-X710® (Keyence). The cells stained with hematoxylin in the left hippocampal DG were counted for the total cells using BZ-X710®.

Statistical Analysis

To estimate sample size, we referred to published data examining a mild bTBI model, wherein the immobility time in the forced swim test increased from 132.0 s in the sham group to 223.4 s in the LISW group (21). With an effect size of 1.36 and a standard deviation (SD) of 67.1, a sample size of 10 was estimated to provide 90% power in a two-sided test. All outcome measures were analyzed using one-way repeated measures analysis of variance, followed by *post hoc* analysis using Tukey's test. All data are expressed as mean ± standard deviation (± SD). Differences with $p < 0.05$ were considered statistically significant. The mice which could not swim were excluded in the forced swimming test. Other exclusion criteria did not exist. All statistical analyses were performed using SPSS ver. 24.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Behavioral Assessment

In both the tail suspension and forced swimming tests, longer immobility time reflects greater depressive-like behavior (28–30). In the tail suspension test, immobility time was 168.0 ± 23.9, 201.2 ± 36.0, and 152.7 ± 28.6 s for sham, LISW-Vehicle, and LISW-SSRI groups, respectively, at 28 days after LISW exposure ($n = 10$). Immobility time was significantly longer in the LISW-Vehicle group than in the sham group ($F_{2,27} = 6.876$, $p < 0.05$). No significant differences between sham and LISW-SSRI groups were observed (**Figure 3A**).

In the forced swimming test, the immobility time was 167.0 ± 58.9, 232.5 ± 56.1, and 156.2 ± 61.5 s for sham, LISW-Vehicle, and LISW-SSRI, respectively, at 28 days after LISW exposure ($n = 10$–11). Immobility time was significantly longer in the LISW-Vehicle group than in the sham group ($F_{2,29} = 5.373$, $p < 0.05$). No significant differences between sham and LISW-SSRI groups were observed (**Figure 3B**).

BDNF and Serotonin in the Left Hippocampal DG

At 28 days after LISW exposure, BDNF protein levels in the left hippocampal DG was 497.6 ± 145.4, 484.2 ± 87.2, and 675.8 ± 198.1 pg/mg protein in sham, LISW-Vehicle, and LISW-SSRI groups, respectively ($n = 9$–10). BDNF protein levels in the LISW-SSRI group were significantly higher than those in the sham and LISW-Vehicle groups ($F_{2,25} = 4.620$, $p < 0.05$). No significant differences between sham and LISW-Vehicle groups were noted (**Figure 4A**).

At 28 days after LISW exposure, serotonin levels in the left hippocampal DG were 3,364.8 ± 1,050.2, 3,253.2 ± 1,047.6, and 4,692.2 ± 1,311.7 pg/mg protein in sham, LISW-Vehicle, and LISW-SSRI groups, respectively ($n = 9$–10). Serotonin levels in the LISW-SSRI group were significantly higher than those in the sham and LISW-Vehicle groups ($F_{2,26} = 4.768$, $p < 0.05$). No significant differences between sham and LISW-Vehicle groups were noted (**Figure 4B**).

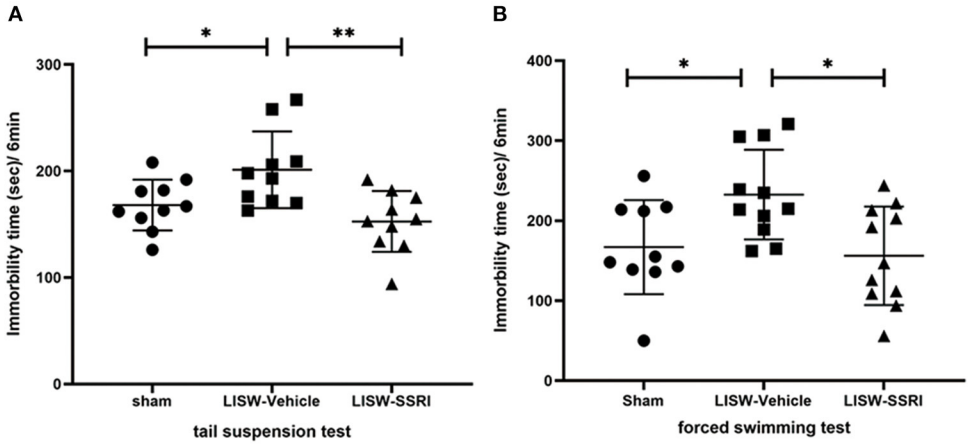

FIGURE 3 | Results of behavioral assessments. In both the tail suspension test **(A)** and forced swimming test **(B)**, the immobility time in LISW-Vehicle group was significantly higher than that in the sham group. There was no significant difference between sham and LISW-SSRI groups; $n = 10$ **(A)**, $n = 10$–11 **(B)**, $^{**}p < 0.001$, $^{*}p < 0.05$.

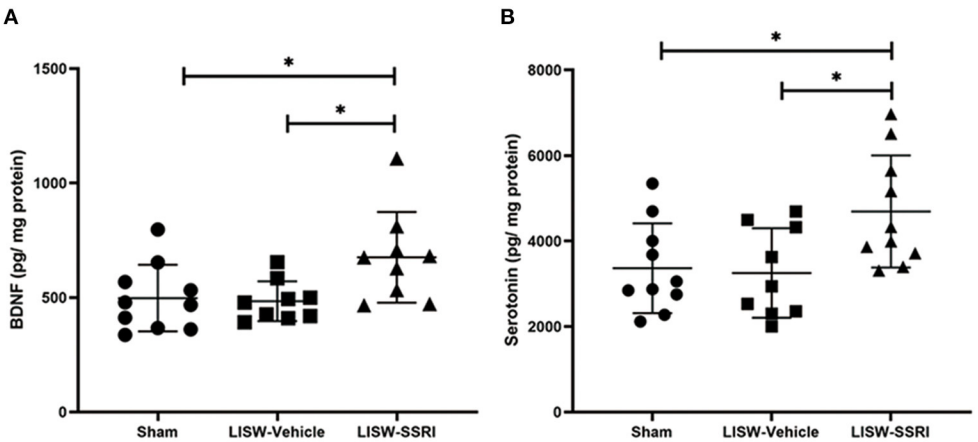

FIGURE 4 | Graphical presentation of BDNF and serotonin levels in the left hippocampal DG. **(A)** BDNF protein levels in the left hippocampus in the LISW-SSRI group were significantly higher than those in the sham and LISW-Vehicle groups. There was no significant difference between sham and LISW-Vehicle groups. **(B)** Serotonin levels in the left hippocampus of the LISW-SSRI group were significantly higher than those of the sham and LISW-Vehicle groups. No significant differences between sham and LISW-Vehicle groups were noted; $n = 9$–10 for each test, $^{*}p < 0.05$.

Co-expression of Brd-U and DCX in the Left Hippocampal DG

Brd-U is incorporated into newly synthesized DNA during the S period. DNA containing Brd-U can be detected using an anti-Brd-U antibody, which enables the detection of proliferating cells that replicate DNA (32). Doublecortin (DCX) is expressed by differentiated neurons. As such, co-staining with both Brd-U and DCX enables the analysis of proliferated neurons, indicating neurogenesis (15, 33).

At 28 days after LISW exposure, we compared cells stained with both Brd-U and DCX in the left hippocampal DG. For immunofluorescence staining, cells stained for Brd-U are indicated in green and those stained for DCX are indicated in red. Double-labeled cells appear yellow (**Figure 5A**).

The number of cells stained with both Brd-U and DCX was 34.7 ± 6.6, 36.0 ± 6.3, and 45.1 ± 5.2 for sham, LISW-Vehicle, and LISW-SSRI, groups, respectively ($n = 7$). The number of cells stained with both Brd-U and DCX in the LISW-SSRI group was significantly higher than that in sham and LISW-Vehicle groups ($F_{2,18} = 6.208$, $p < 0.05$) (**Figure 5B**).

Ratio of pCREB-Positive Cell Counts in the Left Hippocampal DG

At 28 days after LISW exposure, we compared the number of cells positive for pCREB based on DAB staining in the left hippocampal DG. We calculated the ratio (number of cells with positive staining using the pCREB antibody/total number of cells) in a blinded manner using BZ-X710® (Keyence). We

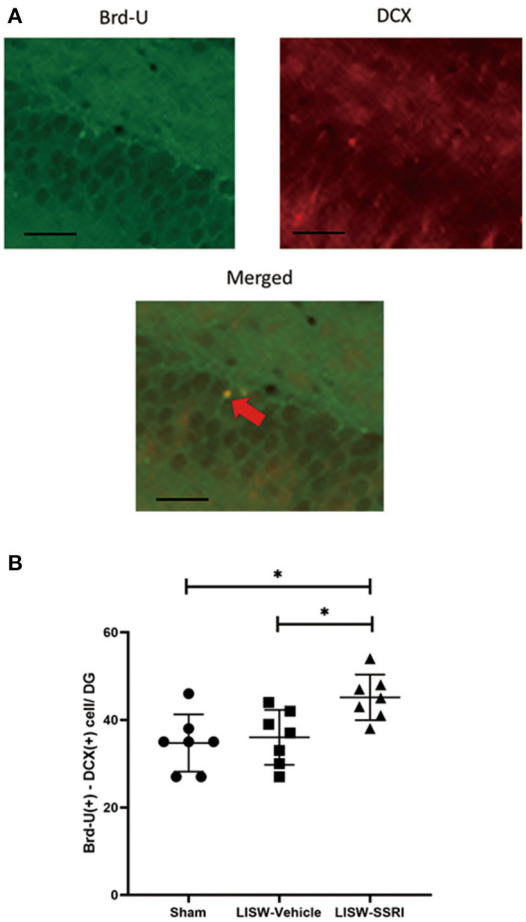

FIGURE 5 | Co-expression of Brd-U and DCX in the left hippocampal DG. **(A)** For immunofluorescence staining, cells stained with Brd-U are colored in green and those stained with DCX are colored in red, Double-labeled cells stained with both Brd-U and DCX appear yellow. **(B)** In the left hippocampal DG, the number of cells stained with both Brd-U and DCX in the LISW-SSRI group was significantly higher than that in the sham and LISW-Vehicle groups; scale bars = 25 μm, $n = 7$, $^{*}p < 0.05$.

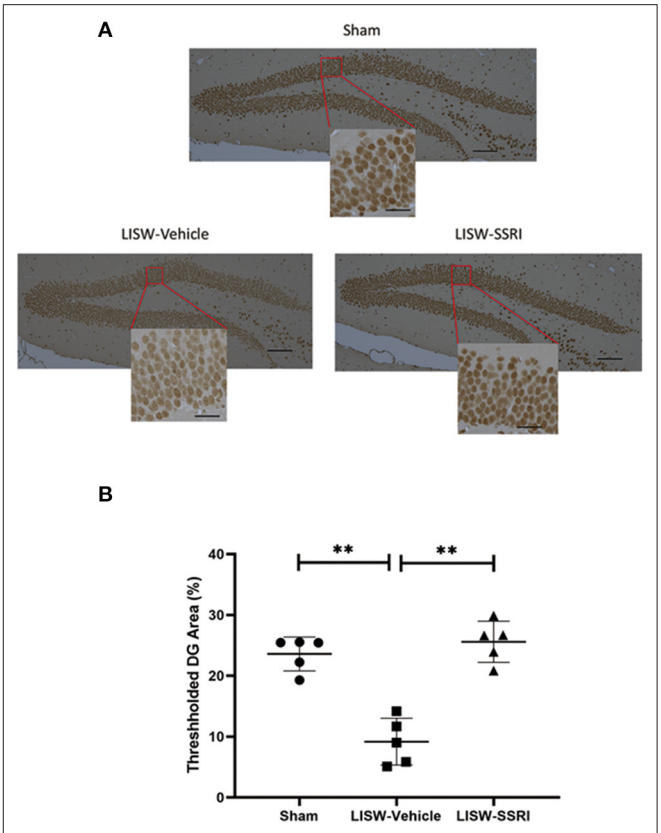

FIGURE 6 | Ratio of pCREB-positive cell counts in the left hippocampal DG. **(A)** The ratio of pCREB-positive cells (number of positive cells stained with pCREB antibody / total number of cells) in the left hippocampal DG was calculated in a blinded manner using BZ-X710® (Keyence). **(B)** The ratio of positive cells in the LISW-Vehicle group was significantly lower than that in the sham and LISW-SSRI groups. There was no significant difference between sham and LISW-SSRI groups; scale bars = 100 and 25 μm, $n = 5$, $^{**}p < 0.001$.

compared the ratio of positive cells in the left hippocampal DG (**Figure 6A**), which was 23.6 ± 2.8%, 9.2 ± 3.8%, and 25.6 ± 3.4% for sham, LISW-Vehicle, and LISW-SSRI, groups, respectively ($n = 5$). The ratio of positive cells was significantly lower in the LISW-Vehicle group than in the sham and LISW-SSRI groups ($F_{2,12} = 35.457$, $p < 0.001$). No significant differences between the sham and LISW-SSRI groups were observed (**Figure 6B**).

DISCUSSION

In this study, we examined the effects of SSRI administration for 2 weeks following TBI on behavior at 1 month post injury in a mouse model of mild bTBI using LISW. Our data showed that administration of an antidepressant drug alleviated depression-like behavior at 28 days after LISW exposure. When given prior to the development of symptoms, SSRIs may be an effective therapeutic strategy for prevention of depression during the chronic phase of a mild TBI. SSRIs increase the extracellular levels of the neurotransmitter serotonin by limiting reuptake into presynaptic cells, thereby increasing the level of serotonin in the synaptic cleft. The half-life of sertraline is 22–24 h. The theory of the monoamine hypothesis is that the depletion of serotonin and other monoamines is the pathophysiology underling depression (34). However, the monoamine hypothesis has several contradictions. For example, the elevation of monoamines in the synaptic cleft occurs within a short period of time after the administration of an antidepressant drug. Despite this rapid elevation in monoamines from the first administration, typical antidepressants take weeks to months of chronic use to exert their therapeutic effects (34, 35). In this study, the serotonin levels in the LISW-Vehicle group were the same as those of the sham group at 28 days after LISW exposure. Our results contradict the monoamine hypothesis. Some literature has reported the utility of sertraline for prophylaxis following TBI (36, 37). In this study, sertraline was administered to the mice for

14 days, not for 28 days. Van Dyke et al. reported that the effects of elevated serotonin accumulate slowly *in vivo* and may account for the delay in relief of depression (38). Our sertraline treatment might partially prevent the development of depression and may have mechanisms other than that of the monoamine hypothesis.

In recent years, increasing focus has been placed on the role of neurogenesis in anti-depressant mechanisms. Therefore, we focused on neurogenesis as the potential mechanism of recovery from depression-like behavior induced by mild bTBI. Neurogenesis is the physiological phenomenon by which new neurons differentiate from neural stem cells or precursor cells. It occurs in embryonic and fetal periods. Neurogenesis plays an important role in the formation and development of the brain. New neurons decrease as an individual develops, but neurogenesis persists in the hippocampus and SVZ even after maturation (11). We observed an increase in the number of cells stained with both Brd-U and DCX in the hippocampal DG of the LISW-SSRI group 28 days after LISW exposure. This suggests that neurogenesis in the DG was activated by early treatment with an SSRI. This neurogenesis at 28 days might be strongly related to the SSRI treatment, not due to mild bTBI. As described in the Introduction, exposure to stress reduces neurogenesis, but the administration of antidepressant drugs activates neurogenesis in the DG (7–10). For example, Malberg et al. reported that chronic administration of an SSRI activated neurogenesis in the hippocampal DG (8). Therefore, the antidepressant-like effects in the SSRI-LISW group observed in this study may have been influenced by neurogenesis induced by SSRI administration.

The detailed mechanisms underscoring neurogenesis are not fully known, but our understanding of the relationship between neurogenesis and signaling molecules is growing. In this study, we focused on BDNF, serotonin, and pCREB, which are factors related to neurogenesis in the hippocampus. BDNF is a neurotrophic factor belonging to the neurotrophic family, which also includes nerve growth factor. It acts to maintain neural cells, promote neurite outgrowth, and regulate neurotransmitter synthesis. BDNF and serotonin levels were increased in the hippocampus of the SSRI-LISW group. This is likely due to the fact that SSRI administration limits the reuptake of serotonin in the synaptic cleft. SSRIs also contribute to elevation of BDNF in the synaptic cleft, and BDNF can interact with serotonin in the synaptic cleft (11, 39). In short, BDNF and serotonin interact each elevating the level of the other in the synaptic cleft (39). In this study, serotonin may have interacted with BDNF in the hippocampus after SSRI administration, which could have induced an activation of neurogenesis in the hippocampal DG.

The pCREB is associated with BDNF gene expression in the cytoplasm via various pathways. The pCREB can be activated after phosphorylation by several signaling molecules via the BDNF – pCREB pathway. Under TBI conditions, the basal levels of pCREB decrease over several months post-trauma (40). Wu et al. reported that pCREB was downregulated in the hippocampus after TBI (15). Similarly, we found that pCREB was significantly lower in the hippocampal DG of the LISW-Vehicle group compared to the sham group. This result suggests that LISW exposure could influence the downregulation of pCREB. Conversely, there was no significant difference between sham and LISW-SSRI groups. Therefore, SSRI administration may have limited the downregulation of pCREB induced by mild bTBI. SSRI administration promotes the elevation of BDNF and serotonin in the synaptic cleft. The elevation of BDNF and serotonin could also play an important role in the downregulation of pCREB. However, the relationship between BDNF or serotonin and downregulation of pCREB is unknown thus requiring further examination in the future.

In this study, we used LISW to investigate the effects of early SSRI treatment on bTBI-induced depression-like symptomology 28 days later. The shock tube or open field blast models are often used in studies on blast injuries (41, 42). However, compared to conventional instruments, LISW has higher controllability of various parameters such as pressure, impulse, frequency, and regions (20, 27). While we applied a single pulse by LISW on the skin surface of the left parietal region in mice, it is important to note that alterations in various conditions such as species, side, frequency, or region may provide even more insight into the mechanisms of bTBI. For instance, rats could be used instead of mice; the LISW could have performed on the right side, cerebellum, or frontal lobe; or a more severe bTBI model could have been used instead of a mild bTBI model. Therefore, future experiments using different parameters or bTBI models should be compared to those from this model in an attempt to better understand the mechanisms involved. Moreover, future studies should use another control group where shams are treated with the SSRI, or use female mice, or different strains instead of male C57BL/6 mice.

We chose to study the effects of an SSRI in this study given that they are often used as the first-line pharmacological treatment for PTSD and depression in the United States (22). However, it will be important to investigate other pharmacological treatments with different mechanisms of action other than SSRI's using our model in the future. This could help shed further light on the mechanisms involved in the successful recovery from bTBI-induced depression. It is also important to note that other factors such as tropomyosin receptor kinase B (TrkB) in the BDNF - pCREB pathway have been previously associated with depression (43). Therefore, novel mechanisms may also be identified by investigating the relationship between these other factors and neurogenesis. Further, SSRIs have been shown to play an important role in transporting other monoamines such as dopamine or noradrenaline, making it important to examine other monoamine systems in the future (44).

This study has several limitations. First, we only analyzed a single time point 28 days after LISW exposure. This time point was selected because many soldiers who suffered from mild bTBI experienced depression during the chronic phase, not the acute phase (2–4). Nevertheless, studies geared at examining changes in neurogenesis during acute phase are still warranted. Hence, we should check other time points, such as 7 or 14 days. It will be interesting to compare the findings

at 28 days and other time points. Another limitation is that we only examined the ipsilateral side of the hippocampus after LISW. We selected the hippocampus because it is one of the most well established sites of neurogenesis in TBI (6). Sahay et al. revealed that the heterogeneity of depression indicates that its origin may lie in dysfunction of multiple brain regions (45). SSRI treatment also impacts the entire brain. This model is limited in that although bTBI affects all brain regions, the exposure disproportionately affects the hippocampus. Hence the effect of exposure to other brain regions is not included in this study. It is possible that exposure to all regions may have an effect, but we expect that to be limited since anxiety and depression predominantly occur due to hippocampal dysfunction. Moreover, we only examined depression, and other deficits such as anxiety should also be examined. The study of various deficits in mice may clarify the mechanism of SSRIs.

In conclusion, early administration of an SSRI produced an elevation in BDNF and serotonin and attenuated pCREB downregulation in the hippocampus after LISW exposure. These changes may underlie the increase in neurogenesis observed after SSRI treatment in the hippocampal DG and ultimately lead to the amelioration of depression-like behavior induced by mild bTBI.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of National Defense Medical College (approval number: 16010).

AUTHOR CONTRIBUTIONS

SSe was the primary investigator of this study and was thus responsible for all the study processes. ST contributed to study design, data interpretation, and revision of the manuscript. HM contributed to data collection and data interpretation. SSa contributed to resources and study design. DS contributed to study design, statistical analysis, data interpretation, revision of the manuscript, and provided final approval to submit the manuscript for publication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.602038/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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