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Mikrostruktur des Gehirns und neuropsychologische Funktionsfähigkeit bei Veteranen

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List of abbreviations

AD	*Axial diffusivity*
ALLO	*Allopregnanolone*
ANDRO	*Androsterone*
CBT	*Cognitive behavioral therapy*
CT	*Computer tomography*
DAI	*Diffuse axonal injury*
dMRI	*Diffusion-weighted magnet resonance imaging*
DSM	*Diagnostic and Statistical Manual of Diseases*
DTI	*Diffusion tensor imaging*
FA	*Fractional ansisotropy*
FAt	*Fractional ansisotropy of the tissue*
FDA	*Food and Drug Administration*
FW	*Free-water*
GABA	*Gamma-aminobutyric acid*
GCS	*Glasgow coma scale*
HPA	*Hypothalamic-pituitary-adrenal axis*
INTRuST	*Injury and Traumatic Stress Clinical Consortium*
MD	*Mean diffusivity*
MRI	*Magnet resonance imaging*
mTBI	*Mild traumatic brain injury*
OEF	*Operation Enduring Freedom*
OIF	*Operation Iraqi Freedom*
ONF	*Operation New Dawn*
PCS	*Postconcussive symptoms*
PREGNA	*Pregnanolone*
PREGNE	*Pregnenolone*
PTSD	*Posttraumatic Stress Disorder*
RD	*Radial diffusivity*
ROI	*Region of interest*
SSRI	*Selective serotonin reuptake inhibitors*
TRACTS	*Translational Research Center for TBI and Stress Disorders*
VA/DoD	*Veterans Administration/Department of Defense*
Voxel	*Volume of a pixel*

Publication record of the presented work

The presented work is based on the following two papers:

1. **Umminger LF**, Rojczyk P, Seitz-Holland J, Sollmann N, Kaufmann E, Kinzel P, Zhang F, Kochsiek J, Langhein M, Kim CL, Wiegand TLT, Kilts JD, Naylor JC, Grant GA, Rathi Y, Coleman MJ, Bouix S, Tripodis Y, Pasternak O, George MS, McAllister TW, Zafonte R, Stein MB, O'Donnell LJ, Marx CE, Shenton ME, Koerte IK. **White Matter Microstructure Is Associated with Serum Neuroactive Steroids and Psychological Functioning.** J Neurotrauma. 2023 Apr;40(7-8):649-664. doi: 10.1089/neu.2022.0111. Epub 2023 Jan 6. PMID: 36324218
    - Web of Science Core Collection Impact factor 2022: 4,869

2. Kaufmann E, Rojczyk P, Sydnor VJ, Guenette JP, Tripodis Y, Kaufmann D, **Umminger L**, Seitz-Holland J, Sollmann N, Rathi Y, Bouix S, Fortier CB, Salat D, Pasternak O, Hinds SR, Milberg WP, McGlinchey RE, Shenton ME, Koerte IK. **Association of War Zone-Related Stress With Alterations in Limbic Gray Matter Microstructure**. JAMA Netw Open. 2022 Sep 1;5(9):e2231891. doi: 10.1001/jamanetworkopen.2022.31891. PMID: 36112375.
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Summary

The cumulative dissertation is based on two original papers published in 2022 in the scientific journals *Journal of Neurotrauma* (first authorship) and *JAMA Network Open* (co-authorship). The overall research goal of the two studies is to assess microstructural brain alterations in veterans. The assessment and visualization are performed using a particularly sensitive technique based on magnetic resonance imaging (MRI), known as diffusion tensor imaging (DTI). Both studies use a specific advancement of DTI, free-water diffusion tensor imaging, which corrects the DTI metrics for the effects of extracellular free water and thus provides additional information compared to conventional diffusion measurements. The studies both address the question, whether microstructural brain alterations in veterans are related to neuropsychological function. In both studies, the influence of a common diagnosis in veterans, mild traumatic brain injury (mTBI), is also considered. In addition, the two studies each address specific aspects, that have not been adequately addressed in previous studies.

Original article 1 (First authorship)

This first project aims to address the question, whether neuroprotective and centrally acting hormones, so-called neuroactive steroids, play a role in the pathophysiology of a variety of neuropsychiatric symptoms in veterans. The research question is based on studies on the genesis of neuropsychiatric diseases, which discuss not only the influence of microstructural brain alterations, but also the significance of neuroendocrine dysregulations in stress-associated diseases. Previous research on health outcomes in veterans has focused primarily on the most common neuropsychiatric diagnoses, including mTBI and post-traumatic stress disorder (PTDS). However, given the large overlap of multiple neuropsychiatric symptoms in veterans, our study pursues a novel approach by examining the overall psychological functioning across veterans. Specifically, this study aims to determine whether associations between serum levels of neuroactive steroids, whole brain white matter microstructure, and psychological functioning can be established. In addition, we determine whether these associations are influenced by common and often comorbid diagnoses, mTBI and/or PTSD. 163 subjects from the INTRuST consortium project were included in the study. Subjects underwent neuropsychological assessment using clinical questionnaires, as well as MRI imaging and blood sampling to determine levels of the neuroactive steroids allopregnanolone (ALLO) and pregnenolone (PREGNE). Based on selected psychological questionnaires, factor analysis was performed. The analysis confirmed that the neuropsychological symptoms measured by the questionnaires can be attributed to a common factor that captures psychological functioning. Regression analysis demonstrated that serum levels of ALLO are

positively associated with whole brain white matter microstructure. A moderation analysis revealed that this association was more pronounced in individuals with a comorbid mTBI and PTSD diagnosis. In addition, a positive association was established between white matter microstructure and psychological functioning. Thus, this study shows that neuroactive steroids have a protective effect on white matter microstructure. Moreover, the results suggest that a dysregulation of the endocrine stress response plays a crucial role in the progression of widespread neuropsychiatric impairments in veterans.

Original article 2 (Co-authorship)

To date, research investigating brain function and structure in veterans has been conducted almost exclusively in the context of war zone-related neuropsychiatric diagnoses such as PTSD and/or mTBI. Considerably fewer studies have examined what likely constitutes a common, underlying contributor to neuropsychiatric impairment in veterans: war zone-related traumatizing experiences. In light of this research gap, this project addresses the question whether war zone-related stress is associated with brain structural alterations and to what extent this association is influenced by a diagnosis of mTBI. Since studies on macrostructural gray matter alterations after combat exposure have shown a decrease in limbic and paralimbic volumes, this study focuses on 8 limbic and paralimbic regions of the gray matter in each brain hemisphere. Expanding on previous studies, gray matter was analyzed using DTI, which enables the analysis of gray matter at a microstructural level. In addition, we examined whether a link can be established between microstructural gray matter alterations and neuropsychological function. Our sample consists of 168 male veterans from the INTRuST study. The veterans underwent a neuropsychological examination based on clinical questionnaires as well as MRI imaging. The findings indicated on the one hand, that greater war zone-related stress is associated with gray matter microstructural abnormalities in the bilateral cingulate, bilateral orbitofrontal, and right parahippocampal gyrus. Altered gray matter microstructure in the cingulate/orbitofrontal gyri was in turn associated with poorer response inhibition. On the other hand, greater war zone-related stress was related to altered gray matter microstructure in the amygdala-hippocampal complex. The altered microstructure in the amygdala-hippocampal complex was linked to better short-term memory and higher processing speed. In addition, a history of mTBI did not affect the relationship between war zone-related stress and gray matter microstructure. The study is highly relevant because it reveals pathophysiological mechanisms behind the adverse health effects of war zone-related stress. Thus, microstructural alterations of the limbic gray matter microstructure were identified as a major factor mediating the relationship between war zone-related stress and neuropsychological consequences.

Zusammenfassung

Die kumulative Dissertation basiert auf zwei Originalarbeiten, welche 2022 in den wissenschaftlichen Fachzeitschriften Journal of Neurotrauma (Erstautorenschaft) und JAMA Network Open (Mitautorenschaft) veröffentlicht wurden. Das gemeinsame Forschungsziel der beiden Studien besteht darin, mikrostrukturelle Gehirnveränderungen bei Kriegsveteranen darzustellen. Die Quantifizierung und Darstellung erfolgt mittels einer besonders sensitiven, auf der Magnetresonanztomographie (engl. Magnet resonance imaging, MRI) basierenden Technik, der sog. Diffusions-Tensor-Magnetresonanztomographie (engl. Diffusion tensor imaging, DTI). Beide Studien verwenden dabei eine spezielle Weiterentwicklung der DTI, die free-water Bildgebung, welche die DTI-Messwerte um die Effekte des extrazellulären freien Wassers korrigiert und somit zusätzliche Informationen im Vergleich zu konventionellen Diffusionsmessungen liefert. Die Studien gehen zudem beide der Fragestellung nach, inwieweit die mikrostrukturellen Gehirnveränderungen bei Kriegsveteranen mit neuropsychologischen Funktionen zusammenhängen. In beiden Studien wird außerdem der Einfluss einer häufigen kriegsassoziierten Diagnose, das leichte Schädel-Hirn-Trauma (engl. Mild traumatic brain injury, mTBI), berücksichtigt. Zusätzlich behandeln die beiden Arbeiten jeweils spezielle Teilaspekte, welche in bisherigen Studien nicht hinreichend adressiert wurden.

1. Arbeit

Das erste Projekt möchte insbesondere die Fragestellung testen, inwieweit neuroprotektive und zentral wirksame Hormone, sogenannte Neurosteroide, bei der Pathophysiologie vielfältiger neuropsychiatrischer Symptome bei Veteranen eine Rolle spielen. Die Fragestellung knüpft an Studien zur Genese neuropsychiatrischer Erkrankungen an, welche neben dem Einfluss mikrostruktureller Gehirnveränderungen auch die Bedeutsamkeit neuroendokriner Dysregulationen bei stressassoziierten Erkrankungen diskutieren. Bisherige Forschung zu den Gesundheitsfolgen bei Veteranen fokussierte sich hauptsächlich auf die häufigsten neuropsychiatrischen Diagnosen, wozu insbesondere das mTBI und die Posttraumatische Belastungsstörung (engl. Posttraumatic stress disorder, PTSD) zählen. Da die vielfältigen neuropsychiatrischen Symptome bei Kriegsveteranen jedoch eine große Schnittmenge aufweisen, verfolgt unsere Studie den neuen Ansatz, die gesamte psychologische Funktionsfähigkeit der Veteranen zu untersuchen. Speziell soll untersucht werden, ob Zusammenhänge zwischen Serumspiegel von Neurosteroiden, der Mikrostruktur der gesamten weißen Substanz (engl. White matter) und der psychologischen Funktionsfähigkeit nachgewiesen werden können. Zudem soll beantwortet werden,

ob diese Zusammenhänge durch die zentralen kriegsassoziierten Diagnosen, mTBI und/oder PTSD, beeinflusst werden. 163 Probanden wurden in die Studie eingeschlossen. Bei den Probanden erfolgte eine neuropsychologische Untersuchung mittels klinischer Fragebögen, eine MRT-Bildgebung sowie eine Blutabnahme zur Bestimmung der Neurosteroide Allopregnanolon (engl. Allopregnanolone, ALLO) und Pregnenolon (engl. Pregnenolone, PREGNE). Basierend auf ausgewählten psychologischen Fragebögen führten wir eine Faktorenanalyse durch. Diese bestätigte, dass die mithilfe der Fragebögen gemessenen neuropsychologischen Symptome auf einen gemeinsamen Faktor zurückgeführt werden können, welcher die psychologische Funktionsfähigkeit erfasst. Mithilfe von Regressionsanalysen konnte nachgewiesen werden, dass die Serumspiegel von ALLO mit der Mikrostruktur der gesamten weißen Substanz assoziiert sind. Eine Moderationsanalyse konnte zeigen, dass dieser Zusammenhang bei Personen mit einer komorbiden mTBI und PTSD Diagnose verstärkt ist. Zudem konnte eine positive Assoziation zwischen der Mikrostruktur der weißen Substanz und der psychologischen Funktionsfähigkeit hergestellt werden.
Diese Studie zeigt, dass Neurosteroide einen protektiven Effekt auf die Mikrostruktur der weißen Substanz haben. Zudem deuten die Ergebnisse darauf hin, dass die Dysregulation der endokrinen Stress-Antwort eine entscheidende Rolle bei der Entwicklung weitreichender neuropsychiatrischer Beeinträchtigungen bei Kriegsveteranen spielt.

2. Arbeit

Die Erforschung veränderter Gehirnfunktionen und Strukturen bei Kriegsveteranen erfolgte bisher fast ausschließlich im Zusammenhang mit kriegsassoziierten neuropsychiatrischen Diagnosen wie PTSD und/oder mTBI. Weitaus weniger Studien untersuchten dabei die gemeinsame, zugrundeliegende Ursache der neuropsychiatrischen Beeinträchtigung bei Veteranen: den erlebten, kriegsbedingten Stress. Vor dem Hintergrund dieser Forschungslücke behandelt dieses Projekt die Teilfrage, inwieweit kriegsbedingter Stress mit veränderten Gehirnstrukturen einhergeht und inwieweit diese Assoziation durch die Diagnose eines mTBI beeinflusst wird. Da Studien zu makrostrukturellen Veränderungen der grauen Substanz nach Kriegsexposition eine Reduktion der Volumina limbischer und paralimbischer Strukturen nachweisen konnten, konzentriert sich diese Studie auf 8 limbische und paralimbische Regionen der grauen Substanz in jeder Gehirnhälfte. Als Erweiterung zu bisherigen Studien soll die graue Substanz mithilfe der DTI untersucht werden, wodurch die Analyse der grauen Substanz auf mikrostruktureller Ebene ermöglicht wird.

Darüber hinaus soll geprüft werden, ob eine Verbindung zwischen mikrostrukturellen Veränderungen der grauen Substanz und neuropsychologischen Funktionen hergestellt werden kann.

Unsere Stichprobe besteht aus 168 männlichen Kriegsveteranen. Bei den Probanden wurde sowohl eine neuropsychologische Untersuchung mittels klinischer Fragebögen als auch eine MRT-Bildgebung durchgeführt. Die Ergebnisse zeigen zum einen, dass größerer kriegsbedingter Stress mit Anomalien der Mikrostruktur der grauen Substanz im bilateralen cingulären, bilateralen orbitofrontalen und rechten parahippocampalen Gyrus verbunden ist. Die veränderte Mikrostruktur der grauen Substanz im cingulären/orbitofrontalen Gyrus war wiederum mit einer schlechteren Reaktionshemmung verbunden. Zum anderen konnte gezeigt werden, dass stärkerer kriegsbedingter Stress mit einer veränderten Mikrostruktur der grauen Substanz im Amygdala-Hippocampus-Komplex zusammenhängt. Die veränderte Mikrostruktur im Amygdala-Hippocampus-Komplex war mit einem besseren Kurzzeitgedächtnis und einer höheren Verarbeitungsgeschwindigkeit verknüpft.

Zudem stellte sich heraus, dass ein mTBI in der Vorgeschichte keinen Einfluss auf den Zusammenhang zwischen kriegsbedingtem Stress und der Mikrostruktur der grauen Substanz hatte.

Die Studie hat insofern eine große Relevanz, als sie pathophysiologische Mechanismen hinter den gesundheitsschädlichen Auswirkungen von kriegsbedingtem Stress entschlüsselt. So konnten mikrostrukturelle Veränderungen der grauen Substanz des limbischen Systems als ein wesentlicher Faktor ausgemacht werden, welche den Zusammenhang zwischen kriegsbedingtem Stress und neuropsychologischen Folgen vermitteln.

I. Introduction

I.1. Background on the veteran population and military-related health outcomes

Mental health in the veteran population has been a neglected area of interest for a long time [1].
However, after the Vietnam War and with the inclusion of post-traumatic stress disorder (PTSD) in the Diagnostic and Statistical Manual of Diseases (DSM) in 1980, the mental health of the veteran population has received increasing attention in research [2]. The subsequent wars in Afghanistan and Iraq led to a further variety of studies examining the mental and behavioral health problems of veterans and their contributing factors [2].
The wars in Afghanistan and Iraq differ from previous wars in many ways. They represent one of the longest enduring U.S. military operations ever, and the United States has deployed more than 2.7 million men and women in support of combat operations in Iraq and Afghanistan [2,3]. Combat operations included “Operation Enduring Freedom” (OEF), which is the official name for the war in Afghanistan from October 2001 to December 2014 [3]. Subsequently, troops continued to be deployed in Afghanistan until 2021, mainly helping to train and support Afghan security forces [4]. The war in Iraq is called "Operation Iraqi Freedom" (OIF) and began in March 2003. Since September 2010, ongoing operations in Iraq have been given the new name "Operation New Dawn" (OND) due to reductions in U.S. forces. OND ended with the termination of the war in Iraq in December 2011 [3]. In contrast to previous wars, more women, parents of young children, and Reserve and National Guard soldiers have been deployed. In some cases, deployed personnel were exposed to longer deployments and shorter periods at home between deployments than in previous wars [5].
By now, increasing evidence on OEF/OIF/OND veterans points to the long-term effects of deployment to combat on psychological health, interpersonal and economic functioning [6–8]. The consequences for society are devastating, as veterans are often unable to integrate into social and working life after deployment [7,8]. Of note, veterans represent a clinically complex group with multiple comorbidities [9]. Among the most prevalent and serious health sequelae affecting OEF/OIF/OND veterans are mTBI and PTSD [10,11]. The high incidence of these two diseases can be attributed to the use of improvised explosive devices and the constant threat during these operations [12]. Apart from mTBI and PTSD, a variety of other health conditions have also been associated with combat exposure [1,13,14]. These include various psychiatric conditions such as depressive disorders, substance use disorders, suicidal attempts, and anxiety disorders [14]. Additionally, veterans are at increased risk for behavioral disorders such as chronic pain [15] and sleep disorders [16].

Symptoms of these above mentioned psychiatric and behavioral conditions often overlap, making it difficult to diagnose and treat diseases in veterans [11]. Given the far-reaching consequences and the associated challenges of the veteran population, consortia have been established, particularly in the United States, to develop new treatments or preventive measures that reduce the impact of military-related psychological health problems.

I.2 Signature injuries of war: mTBI and PTSD

1.2.1 Prevalence and Diagnosis

Mild traumatic brain injury

Mild TBI is considered to be one of the leading causes of disability in veterans, with rates of around 15% among American service members [17].

Although there is a wide range of definitions regarding mTBI, consensus has been reached on the diagnostic criteria as proposed by the Mild Traumatic Brain Injury Committee of the American Congress of Rehabilitation Medicine [18] (see table). There are three criteria that are consistently used to diagnose mTBI, including the Glasgow Coma Scale (GCS) score, duration of loss of consciousness and post-traumatic amnesia [18]. Usually, symptoms that occur in the context of mTBI dissolve within days to weeks [19]. However, about one third of the affected individuals experience persistent post-concussive symptoms (PPCS) that last beyond 5 months [20]. PPCS include somatic symptoms (e.g. nausea, dizziness, headache, and light or sound sensitivity), cognitive impairment (e.g. concentration, attention and memory deficits), and emotional alterations (e.g. irritability, frustration and depression) [21] (see **Table 1**).

Mild traumatic brain injury (American Congress of Rehabilitation Medicine)	
	traumatically induced physiological disruption of brain function (one of the following) 1. period of loss of consciousness 2. loss of memory for events immediately before or after the accident 3. alteration in mental state at the time of the accident (e.g. feeling dazed, disoriented, or confused) 4. focal neurological deficit(s) that may or may not be transient
Mild severity	- loss of consciousness < 30 minutes - after 30 minutes, initial GCS: 13–15 - posttraumatic amnesia (PTA) < 24 hours

Inclusion Criteria	1. the head being struck 2. the head striking an object 3. brain undergoing an acceleration/deceleration movement (i.e. whip-lash) without direct external trauma to the head
Exclusion Criteria	- stroke anoxia, tumor, encephalitis
Post-concussive symptoms	1. physical symptoms of brain injury - nausea, vomiting, dizziness, headache, blurred vision, sleep disturbance, quickness to fatigue, lethargy, or other sensory loss 2. cognitive deficits - e.g. involving attention, concentration, perception, memory, speech/ language, or executive functions 3. behavioral changes /alterations in emotional responsivity - e.g. irritability, quickness to anger, disinhibition, or emotional lability

Table 1: Diagnostic criteria of mTBI according to the American Congress of Rehabilitation Medicine

Posttraumatic Stress Disorder

PTSD is defined by the development of characteristic symptoms following exposure to one or more traumatic events [22]. The current PTSD prevalence averages 1.1% [23]. However, the lifetime prevalence is substantially higher and varies between 13.0-20.4% for women and 6.2-8.2% for men [24].

In the population of OIF and OEF veterans, every 11-20 out of 100 veterans receives a diagnosis of PTSD in a given year [25,26].

Particularly characteristic of PTSD is the recurrent, involuntary, intrusive and distressing re-experiencing of aspects of the traumatic event, including flashbacks and nightmares [22,27]. A further core symptom is the avoidance of situations, people or circumstances associated with the trauma (C-Criterion).

Indicative for PTSD is also persistent hyperarousal, which manifests itself in irritable behavior, hypervigilance, concentration problems, and sleep disorders. The 5th edition of the DSM also includes a symptom cluster of negative cognition (e.g. amnesia regarding important parts of the event, exaggerated negative beliefs) and emotional disruptions (e.g. negative emotions, loss of interest, sense of detachment from others) (see **Table 2**).

	DSM-V	ICD-10 research diagnostic criteria
Duration	Symptoms present for at least 1 month	Symptoms should usually arise within 6 months of the traumatic event
Stressor Criterion	A. Exposure to actual or threatened death, serious injury, or sexual violence	A. Event or situation of exceptionally threatening or catastrophic nature likely to cause pervasive distress in almost anyone
Symptoms	B. Intrusion symptoms - One of five C. Avoidance - One of two D. Negative alterations in cognitions and mood - Two of seven E. Persistent hyperarousal - Two of six	B. Persistent re-experiencing of the stressor C. Avoidance D. Either 1.) Inability to recall important aspects of the stressor 2.) Persistent hyperarousal - Two of five E. Clinically significant functional impairment
Further criteria	- Distress/ functional impairment (e.g. social, occupational) - No substance abuse/ general medical condition	- Clinically significant functional impairment

Table 2 : Diagnostic criteria of PTSD according to ICD-10 and DSM-5

Mild traumatic brain injury and Posttraumatic Stress Disorder

A large proportion of OEF/OIF/OND veterans with positive mTBI diagnosis also suffer from PTSD, with prevalence rates between 26 and 44 % [17,28]. Further, the high comorbidity of mTBI and PTSD is plausible given the bilateral risk of the two diseases. On the one hand, TBI is often associated with potentially traumatic conditions [29] and may increase the risk of developing PTSD by causing neural disruption [30]. On the other hand, PTSD is a predictive factor for the development of persistent symptoms following TBI, especially when the TBI is mild [11,31]. In addition to the high comorbidity, PTSD and mTBI also manifest in similar symptoms, making clinical distinctions and effective interventions difficult [10–12]. Whereas the somatic symptoms of brain injury are relatively distinctive of persistent post-concussive symptoms [12], the non-specific symptoms associated with milder TBI show a large overlap with the PTSD symptomatology [10,11,32] (**Figure 1**).

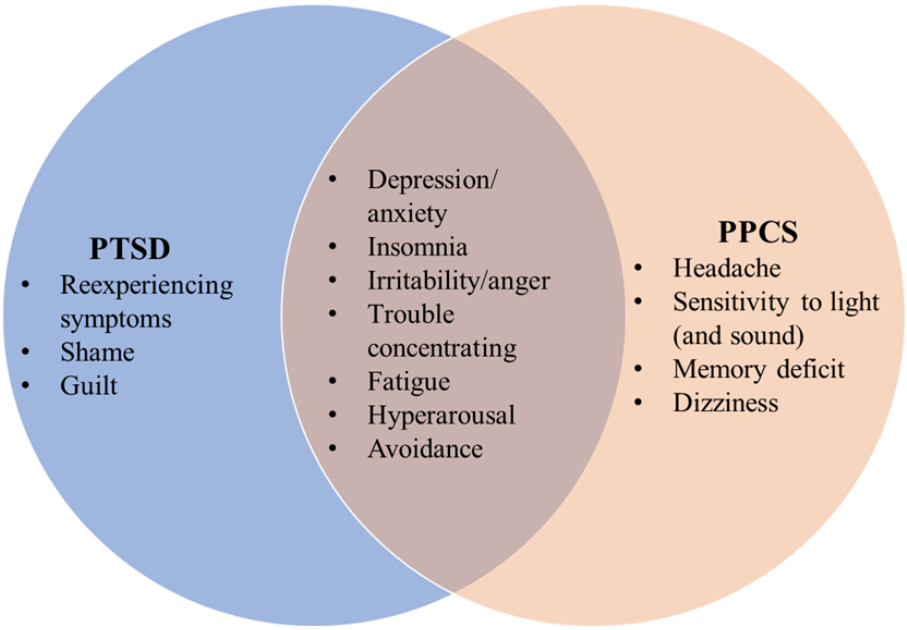

Figure 1: Symptoms of PTSD and PPCS and their overlap [10]
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1.2.2 Pathogenesis

Due to the development of in vivo functional neuroimaging and endocrinological studies, the understanding of the common diagnoses in veterans has evolved considerably in recent years. In the following section, I will review the pathogenesis of the two most prevalent disorders in veterans: mTBI and PTSD. The neuroendocrine and brain structural pathologies of military-related neuropsychiatric disorders will be addressed in the following sections I.3 and I.5.

Mild traumatic brain injury

Mild TBI is caused by shearing forces due to sudden acceleration-deceleration effects on the brain [33]. These shearing forces lead to diffuse injuries of axons within the white matter tracts of the brain [34]. Axonal injury causes localized transport failure and might have the effect of local swelling [33,34]. In addition, the release of glutamate and other excitatory neurotransmitters from the synapse of damaged axons causes a rapid ion shift across the cell membrane [33,35].

Alterations in metabolic reactions affect glucose metabolism and oxygen levels and lead to increased energy demands and a period of metabolic crisis. Furthermore, mTBI is associated with an initial decrease in cerebral blood flow followed by vasodilatation, which further exacerbates the mismatch between energy supply and demand [33,35–37].

This energy deficit may, in turn, result in structural and functional alterations in mTBI.

In addition to the acute pathomechanisms following mTBI, there are also chronic consequences that emerge over the course of weeks and months. Inflammatory processes are viewed as important secondary mechanisms after mTBI [38]. The initial mechanical damage caused by the neurotrauma initiates an immune response. Immune cells release pro-inflammatory cytokines and chemokines, leading to cerebral edema and elevated intracranial pressure. As a result of mTBI, the blood-brain barrier may also be damaged, facilitating the invasion of circulating immune regulators that further amplify the inflammatory response [39,40]. Such a prolonged immune response may also favor neurodegenerative processes [39]. Neurodegenerative effects after brain trauma include the accumulation of beta-amyloid precursors and tau proteins [41,42]. The pathophysiological alterations also have clinical implications, as mTBI is associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease [43]. The more progressive consequences of brain injury also include axonal demyelination and white matter atrophy as a result of primary axonal damage or the death of myelinating cells [39].

Posttraumatic Stress Disorder

The pathogenesis of PTSD is a complex process that has been increasingly unraveled, primarily through the findings of genetics, neuroendocrinology (section I.3) and neuroimaging (section I.5). Genetics research has shown that both environmental influences and hereditary factors play a role in the development of PTSD [44–46].

As far as environmental influences are concerned, numerous studies have shown that the type, timing, intensity and duration of the trauma are crucial factors in predicting the proneness to the disease [47]. Regarding genetic influences, twin and family studies revealed that the overall heritability of PTSD is around 30% [48]. The genetic vulnerability remains even after accounting for genetic factors (e.g., personality traits) that influence exposure to potentially traumatic events such as combat [49].

I.3 Military associated neuropsychiatric disorders: Neuroendocrinological processes

The vulnerability of veterans to multiple neuropsychiatric conditions can be attributed to the extreme stress of the war experience and the corresponding dysregulations of various neuroendocrine processes [50]. It is known that stress-related conditions lead to neuroendocrinological dysregulations, which include upregulations of the hypothalamic-pituitary-adrenal (HPA) axis [46,51,52] and associated glucocorticoid elevations [53]. In addition, alterations in neuroactive steroid levels in response to stress have also been demonstrated [54].

Neuroactive steroids, in particular ALLO and its precursor PREGNE [55–57], are positive Gamma-aminobutyric acid (GABA) receptor modulators and reveal many neuroprotective effects [56,58] such as anti-inflammatory [59–61], anti-apoptotic [62], anti-depressant [63–65], anxiolytic [58,66–69] and positive cognitive effects [70–77]. In addition, the neuroactive steroid ALLO yields pronounced neuroprotective actions in TBI. Animal models have shown that ALLO increases in several brain regions in response to a brain injury [78], leading to enhanced neurogenesis [79], reduced neuroinflammation [59,61] and ischemic infarct volume [80]. Importantly, both ALLO and PREGNE play a significant role in neuroendocrine stress regulation by normalizing the hyper-activated HPA axis (**Figure 2**) [81]. Thus, the neuroactive steroids constitute an endogenous autoregulatory mechanism to restore homeostasis and produce neuroprotection in response to acute stress [66,81–86] . However, in contrast to acute stress, it is proven that chronic stress reduces neuroactive steroid levels [68,87–93] and leads to impaired negative feedback regulation of the HPA axis [91,92,94].

Indeed, decreased levels of ALLO have been found in service members with a chronic stress-related disorder such as PTSD, which were inversely correlated with re-experiencing and depressive symptoms of PTSD [95,96]. Correspondingly, reduced levels of neuroactive steroids such as pregnanolone (PREGNA) and androsterone (ANDRO) have been documented in veterans with a history of blast-related TBI compared to veterans without TBI [97].

However, the association between neuroactive steroids and structural brain alterations has been poorly investigated so far. To date, only one recent study has shown the neuroprotective effect of neuroactive steroids on gray matter in comorbid mTBI and PTSD [98]. Additionally, there is evidence from one research group presented as an abstract demonstrating that neuroactive steroids may also unfold neuroprotective effects on white matter [96].

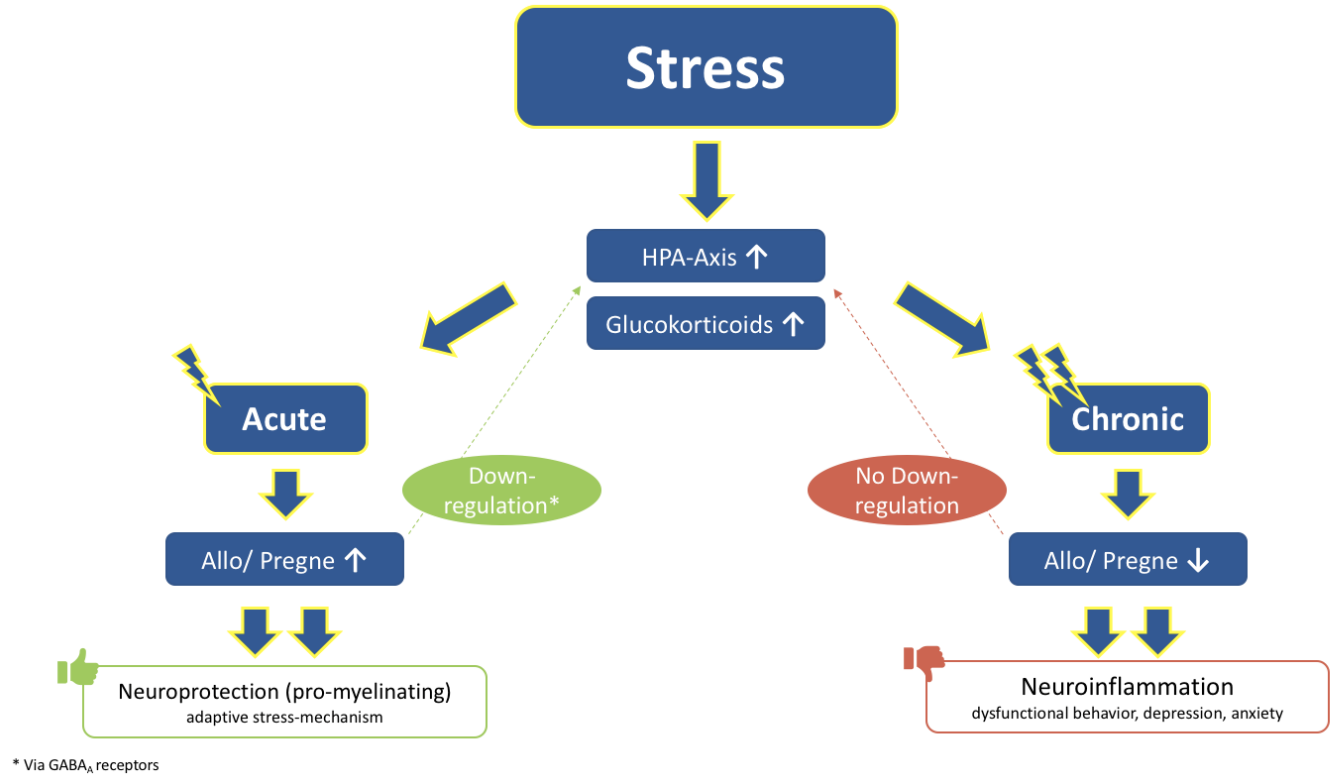

Figure 2: Summarized illustration of central neuroendocrinological processes in response to traumatizing stress or brain trauma and the role of neuroactive steroids (ALLO and PREGNE)

I.4 Treatment needs of veterans

Regarding the treatment of the most common neuropsychiatric diseases in veterans, remarkable efficacy has been demonstrated for psychotherapeutic interventions in combination with psychotropic medication [99–102]. Regarding the treatment of PTSD, there is particularly strong evidence for behavioral therapies that involve cognitive restructuring and exposure to the trauma memory [11]. On the pharmacological side, selective serotonin reuptake inhibitors (SSRIs), a class of antidepressants, are the only drugs approved by the Food and Drug Administration (FDA) for the treatment of PTSD. However, complete remission rates in patients only range between 20% and 30% [103], and particularly low response rates to SSRIs are reported in veterans [104,105]. Furthermore, it must be taken into account that PTSD is often comorbid and occurs especially with mTBI, depression, and substance abuse [99]. Although the psychological treatments were not initially directed at patients with an additional mTBI diagnosis [11], similarly high response rates to cognitive behavioral therapies are shown for patients with PTSD and comorbid mTBI, compared to those with PTSD only [106,107]. According to the Veterans Administration/Department of Defense (VA/DoD) Clinical Practice Guidelines, comorbid conditions should be treated simultaneously [99]. The VA/DoD Clinical Practice Guidelines for Major Depressive

Disorder recommend cognitive behavioral therapy (CBT), interpersonal psychotherapy, problem-solving therapy, and client-centered counseling as psychotherapeutic interventions for moderate and major depression [100]. Electroconvulsive shock therapy can be used for very severe, psychotic, and treatment-resistant major depression. Moreover, the use of psychotropic medication (antidepressants) is recommended for moderate and severe major depression [100].

I.5 Neuroimaging

I.5.1 Methods

Diffusion tensor imaging

MRI was developed in the mid-1980s [108] and has become a fundamental tool for neurological diagnosis [109]. MRI is more sensitive than computer tomography (CT) in detecting subtle abnormalities [110,111] as it has a better contrast resolution, especially for soft tissue. It is able to detect subacute hemorrhages and macroscopic areas of gray and white matter damage [108].

A special type of MRI sequence that was introduced in 1994 [112] is DTI. Whereas CT and MRI only reveal macroscopic brain alterations, DTI offers the most powerful tool for studying white matter and microstructural alterations in vivo [108,109,112,113]. DTI provides information about tissue structure based on the diffuse motion of water molecules (Brownian motion). It measures not only the rate but also the directionality of diffusion [108,114]. For each volume of a pixel (voxel) of the brain, a diffusion tensor (three-dimensional vector) is calculated describing the diffusion characteristics. Eigenvectors ($\lambda 1$, $\lambda 2$, $\lambda 3$) are the axes of the three-dimensional vector and describe the orientation of diffusion, while eigenvalues represent the length of their measure and thus describe the magnitude of diffusion. When the diffusivity is the same in all directions (e.g., in cerebrospinal fluid), diffusion is called isotropic and can be visualized as sphere [114]. The diffusion of water molecules in structured tissue such as white matter is restricted, and water molecules diffuse most likely along the main direction of fibers and rarely perpendicular to them [112,115] which is called anisotropic diffusion [115]. Anisotropic diffusion can be visualized as an ellipsoid (**Figure 3**). Diffusion parameters can be calculated from the diffusion tensor based on the eigenvalues.

Relevant diffusion parameters are fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD). FA is an index of anisotropic diffusion or directionality, ranging from 0 (isotropic diffusion) to 1 (anisotropic diffusion) [109,116]. It is the most commonly used diffusion value, as it is assumed to be a summary measure of white matter organization [117].

MD represents the sum of all three eigenvalues, divided by three. It expresses the average diffusivity of water molecules [118]. Typically, FA and MD are inversely related [108].

AD is equal to the largest eigenvalue (λ1), which is oriented parallel to the axonal structures [114]. It is considered that AD is a measure of axon integrity [119]. The average of the diffusivities in the two minor axes (λ2, λ3) is called RD and describes the diffusion perpendicular to the main diffusion direction [109]. RD is an indicator of myelin integrity [119].

However, even if DTI parameters provide important information about white matter microstructure, it must be taken into account that they only represent an indirect and non-specific measure of white matter microstructural properties [108,120]. A reduced FA can be caused by different pathologies, including demyelination [121,122], edema [123] or gliosis [124]. In 2009, Pasternak et al. presented a promising advancement of the DTI method, which is called free-water imaging [125]. This two-compartment model of water diffusion separately determines the amount of free water in the extracellular space and in the vicinity of cellular tissue [125]. The two most relevant parameters that are obtained when using free-water imaging are free water (FW) and fractional anisotropy of the tissue (FAt). The FW parameter measures the fractional volume of unrestricted free water in the extracellular space. FAt represents the diffusion tensor, which is corrected for the contribution of free water. Thus, free-water imaging improves the specificity of DTI indices and provides additional information about white matter microstructure [125,126].

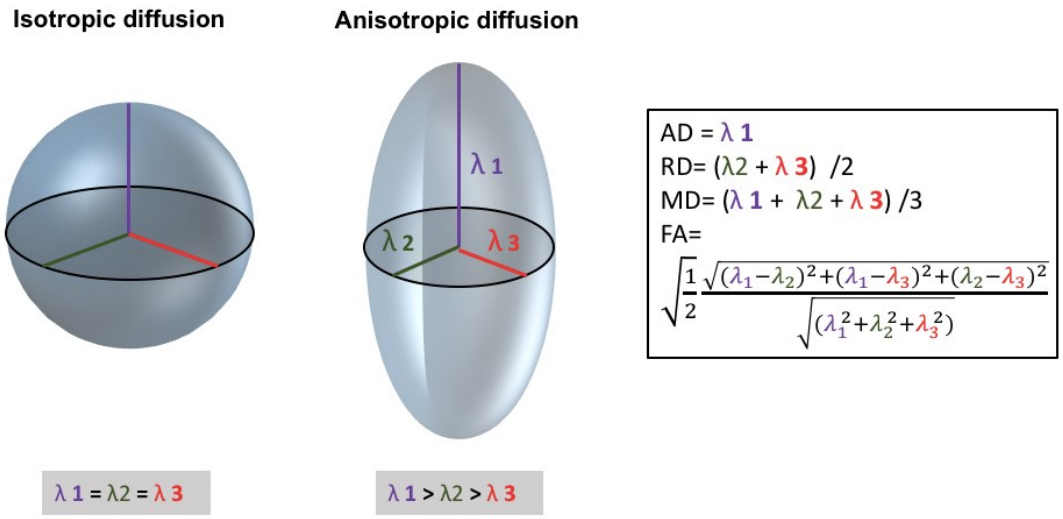

Figure 3: Isotropic and anisotropic diffusion [Figure modified after 114]

Region of interest (ROI) analysis

The ROI analysis focuses on specific regions based on a priori formulated hypotheses [114]. The predefined regions of interest can be obtained either manually or by (semi-)automated segmentation [114,127]. For each subject, the diffusion values are extracted and averaged for the selected regions. Of note, the method can be utilized for both white and gray matter studies [128]. Due to its high sensitivity, ROI analysis is best applied when there is a clear hypothesis about the expected differences in white or gray matter in a well-defined brain region [127].

Tractography

Fiber tractography represents another post-processing method that enables the 3D visualization and quantification of an entire white matter tract [108,114,129]. Thus, it provides important information about the connectivity of different brain regions. Connectivity between the voxels can be determined based on the anisotropic diffusion of water [130]. Fiber tracking uses the diffusion tensor of each voxel to follow the main diffusion direction from voxel to voxel through the brain [129]. Similar to ROI-approaches, a region of interest is defined as a starting point. In addition, inclusion and exclusion regions can be assigned to describe the trajectory of the fibers more precisely [114]. However, in contrast to ROI approaches, tractography can only be used for white matter analysis.

Fiber Clustering

Common methods for extracting fiber bundles based on tractography require the manual determination of multiple regions of interest. Instead of this manual method, fiber clustering approaches have been proposed [131]. Fiber clustering is a fully automatic and unguided method that groups white matter fibers according to their shape and spatial position, thus avoiding the application of an inflexible geometric scheme [131–133]. The fiber clustering approach uses an algorithm that applies a pre-generated whole-brain white matter atlas of the entire brain to processed cerebral MRI images in order to identify subject-specific white matter tracts [134,135]. The atlas was trained using 100 MRI images of healthy subjects from the Human Connectome Project and provides a whole brain white matter parcellation into 800 fiber clusters [134]. This approach does not require an a priori hypothesis about the location of the pathology, as it is entirely data-driven [136]. Recent findings have shown that clustering approaches extract the most consistent white matter tracts across all subjects compared to multiple ROI- and atlas-based approaches [137].

I.5.2 Previous findings

Neuroimaging findings in mTBI

Advanced neuroimaging has been widely utilized to study common neuropsychiatric disorders in veterans, including PTSD and mTBI [138–147.] In mTBI, several gray matter regions such as the thalamus [148–150], hippocampus [148,149], putamen [149] and insula [150] have shown to be affected. In addition to gray matter findings in mTBI, white matter alterations were observed in various regions, including the corpus callosum [151–156], centrum semiovale [152,153], forceps major [157], internal capsule [152,153], fornix [154] and cingulum bundle [156]. These white matter changes are predominantly characterized by reduced FA and increased MD and AD values, although some studies indicate deviating alterations in diffusion values [108,158]. Among the large number of affected white matter regions in mTBI, the corpus callosum seems to be the most commonly impaired structure [109]. Interestingly, studies have shown that white matter microstructure of the corpus callosum is also associated with cognitive outcome after mTBI [159].

Neuroimaging findings in PTSD

In PTSD, reduced gray matter volume has been found particularly in frontal and limbic regions [160,161] such as the anterior cingulate cortex [162,163], medial prefrontal cortex [164,165], hippocampus [163,166] or amygdala [167]. Furthermore, studies have shown that posttraumatic stress symptoms in veterans are inversely correlated with limbic gray matter volumes [168].

Considering white matter changes in PTSD, FA decreases and MD increases are present in several regions, such as the cingulum bundle [139,169], superior longitudinal fascicle [139,170], corpus callosum [171] and uncinate fascicle [172]. Further, diffusion measures of the uncinate fascicle were associated with anxiety symptoms and amygdala activity [172]. However, results are even more inconsistent than mTBI findings, with some studies showing higher FA in the anterior cingulate cortex [173], temporal cortex [174] and higher generalized FA in the right frontotemporal pathways [138] in patients compared to controls.

Neuroimaging findings in comorbid mTBI and PTSD

The comorbid condition of mTBI and PTSD is associated with a particularly high risk of white matter abnormalities [144,145]. One MRI study of OEF/OIF/OND veterans with comorbid PTSD and mTBI found a reduction in bilateral anterior amygdala volume in the comorbid group compared to veterans with neither condition. Interestingly, the reduced amygdala volume was linked to poorer inhibitory behavioral control [175]. Further studies have found altered white matter microstructure in the uncinate fascicle [176] and CB [146] in veterans with mTBI and PTSD relative to those with mTBI only. Additionally, diffusion measures in the bilateral uncinate fascicle were associated with PTSD symptoms [176], indicating poorer recovery in patients with a comorbid mTBI and PTSD diagnosis [146].

Moreover, to date, only one study has used the more sensitive method of free-water imaging to examine gray matter alterations in the context of comorbid mTBI and PTSD [177]. However, the free-water imaging method has not been used to assess alterations in white matter microstructure in patients with comorbid mTBI and PTSD so far.

I.6 Motivation for this work

The overall aim of this work is to investigate neuropsychological, endocrine, and brain structural alterations in a population of OEF/OIF/OND veterans. Our study is intended to provide a better understanding of the complex endocrine and brain structural pathomechanisms underlying the various neuropsychiatric symptoms in veterans and ultimately pave the way for targeted biology-based treatments. Veterans are a highly vulnerable cohort and present with multiple comorbidities above and beyond the main diagnoses mTBI and PTSD [2,14,178]. The influence of the various conditions and their interaction has not yet been sufficiently disentangled, therefore focusing solely on diagnoses does not capture the complexity of the cohort.

Against this background, the aim of our first study was to apply a transdiagnostic approach to assess the overall psychological health of veterans and link it to neuroendocrine and brain structural correlates.

As research increasingly points to the importance of neuroendocrine processes in connection with stress-related conditions [58,81,179,180], this new and important aspect was also part of our research interest. Based on the previous literature, we examined the hypotheses that:

1. Serum neuroactive steroids are positively associated with psychological functioning.
2. Serum neuroactive steroids are associated with whole brain white matter microstructure.
3. White matter microstructure is associated with psychological functioning.
4. The associations between serum neuroactive steroids, whole brain white matter microstructure, and psychological functioning are moderated by an mTBI and/or PTSD diagnosis.

To address these hypotheses, state-of-the-art methods of fiber clustering and free-water imaging were chosen to obtain an accurate understanding of the white matter alterations in veterans. Given the inconsistencies of previous imaging studies in veterans [108,181], we examined the whole brain white matter to get a comprehensive picture.

Similar to our first study, our second study also follows a transdiagnostic approach by focusing on the common risk factor and associated microstructural pathomechanisms underlying veterans' increased vulnerability for neuropsychiatric disorders. Specifically, in our second study, we hypothesized that:

1. War zone-related stress is associated with microstructural alterations in the limbic gray matter.
2. Alterations in limbic gray matter microstructure are linked to neuropsychological functioning.
3. Associations between war zone-related stress and limbic gray matter microstructure are modulated by a history of mTBI.

To address these hypotheses, we applied free-water imaging, as we did in our first study. In contrast to our first study, we examined gray matter microstructure, looking specifically at the classic limbic and paralimbic brain regions. Importantly, studies examining the microstructure of gray matter are quite rare and enable the detection of previously unnoticed gray matter alterations in veterans.

II Paper 1

II.1 Background

OEF/OIF/OND veterans are at increased risk for far-reaching neuropsychological health impairments after deployment [6,182], impeding their social and work reintegration [7,8]. The predominant diagnoses in veterans are PTSD and mTBI [183,184], but a variety of other neuropsychiatric diagnoses are also associated with war experiences [2,13]. These different comorbidities all interact with each other and share a large number of common symptoms [185]. However, research has mainly focused on the most common diagnoses in this cohort, thereby failing to address the complexity of neuropsychological symptom burden. Thus, there is an urgent need to examine the overall psychological functioning of veterans at risk for extensive mental health issues.

Regarding the potential factors influencing poor psychological functioning in veterans, there is evidence that neuroendocrine dysregulations play an important role [97,186]. As major contributors to neuroendocrinological stress regulation, the neuroactive steroids ALLO and its precursor PREGNE [55–57] exert many neuroprotective effects and play a critical role in regulating the stress-response [58,68,81,82,86,187]. Another way to further investigate the pathophysiological basis of adverse health outcomes in veterans is to examine brain structure and function. For this purpose, MRI is used, and specifically DTI is sensitive enough to detect subtle microstructural changes in white matter [108,188].
Taken together, there is evidence of psychological, neuroendocrine, and structural brain alterations in veterans at risk for mental health issues and comorbidities. However, previous studies have not linked the different research fields together to examine the underlying pathomechanisms in a more comprehensive way.
In order to fill this gap, we have chosen a comprehensive approach to investigate potential associations between neuroactive steroid levels, whole-brain white matter microstructure, and psychological functioning by using a highly sensitive method. Additionally, effects of mTBI and PTSD on these associations will be assessed.

II.2 Methods

We examined 163 subjects in our study, based on data collection by the Injury and Traumatic Stress (INTRuST) Clinical Consortium at six sites in the United States.
Psychological questionnaires were used to assess the diagnoses of PTSD, mTBI, and alcohol or drug addiction and to assess psychological functioning of the participants.

The questionnaires were collected as part of a comprehensive neuropsychiatric test battery as part of the INTRuST data collection. For our study, we included all subjects with both high-quality MRI data and neuroactive steroid measurements available. Quantification of the neuroactive steroids ALLO and PREGNE was performed in serum.

The subjects' dMRI sequences were acquired using 3 Tesla scanners (GE 750, General Electric, Chicago, USA; Achieva, Philips Healthcare, Best, Netherlands; Tim Trio, Siemens Healthineers, Erlangen, Germany). The dMRI data obtained by the different scanners were harmonized using a validated algorithm to adjust for scanner-specific differences. We performed the fiber clustering method and extracted subject-specific average whole-brain FAt values (**Figure 4**). Statistical analyses were performed using SPSS, and a Bonferroni-corrected value of <.05 was considered statistically significant. We performed factor analysis using the Anderson-Rubin method to derive an underlying psychological functioning factor based on the psychological questionnaires. Regression models were used to examine associations between serum neuroactive steroid levels, psychological functioning, and whole brain white matter microstructure. Moderation models tested the influence of mTBI and comorbid post-traumatic stress disorder (PTSD) and mTBI on these associations.

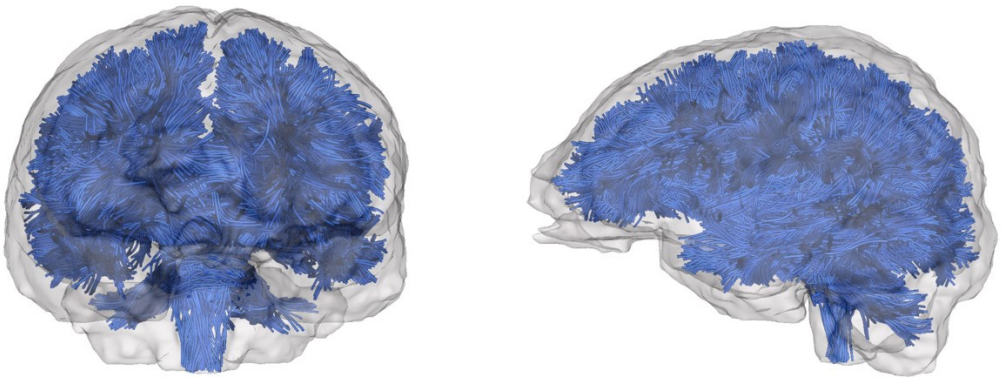

Figure 4: Whole-Brain White Matter of one participant as modeled by the fiber clustering method [adapted from paper 1].

II.3 Results

Psychological functioning

Factor analysis revealed one underlying psychological functioning factor based on the selected tests of psychological symptoms, functional impairment, and health-related quality of life.

Association Between Serum Neuroactive Steroids and Psychological Functioning

We did not detect any association between ALLO or PREGNE and the psychological functioning factor**.**

Association Between Serum Neuroactive Steroids and Whole-Brain FAt

Serum ALLO was associated with whole brain FAt. This association was significantly modulated by a comorbid diagnosis of PTSD and mTBI, whereas an mTBI diagnosis alone had no significant effect on this association (**Figure 5**). However, serum PREGNE was not significantly associated with whole brain FAt.

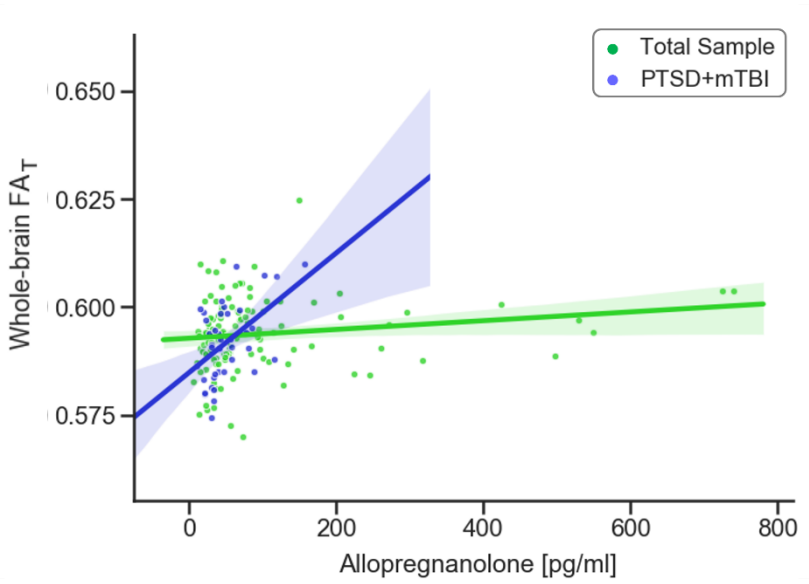

Figure 5: Scatter plot illustrating the association between ALLO and whole-brain FAt in the total sample and the moderating effect of PTSD+mTBI comorbidity on this association [adapted from paper 1].

Association Between Whole-Brain FAt and Psychological Functioning

A lower whole-brain FAt score was linked to poorer psychological functioning. In this case, an mTBI or a comorbid PTSD and mTBI diagnosis did not affect the association.

II.4 Discussion

The aim of the study was to examine the relationship between serum levels of neuroactive steroids, psychological functioning, and whole-brain white matter microstructure and the additional influence of mTBI and PTSD on these associations. The three main results are presented below and summarized in **Figure 6**.

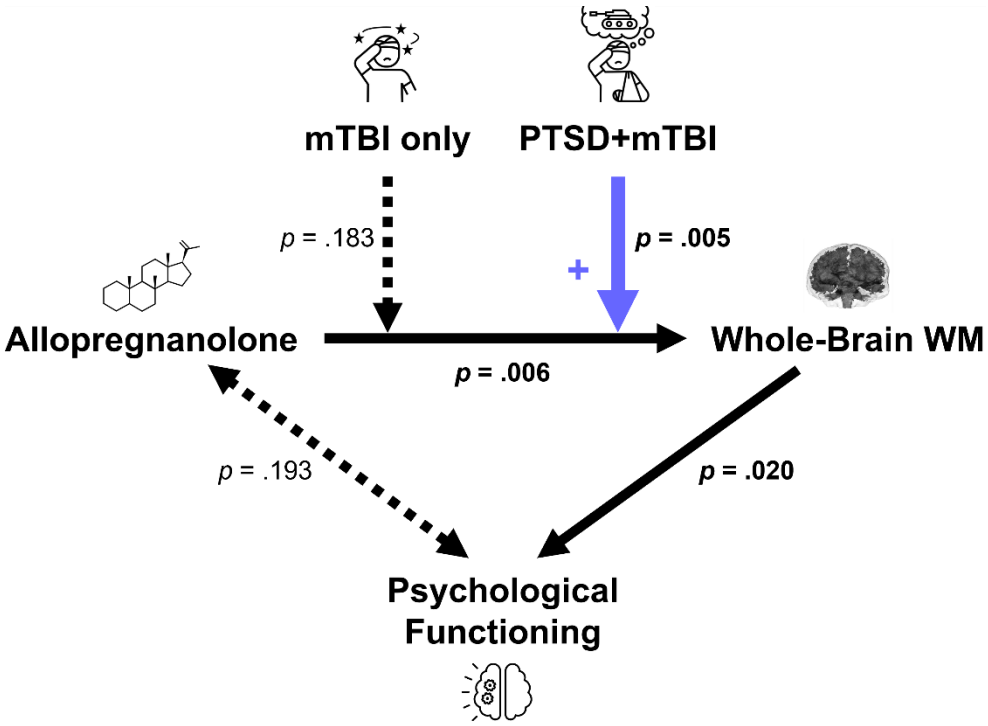

Figure 6: Summary of Findings [adapted from paper 1].

Association Between Serum Neuroactive Steroids and Psychological Functioning

As hypothesized, factor analysis revealed that the various psychological dimensions assessed in our study can be explained by one common factor. This finding confirmed that symptoms of common psychiatric diagnoses in veterans do not reflect distinct constructs [185], but rather share a common origin.

However, contrary to our hypothesis, we did not detect direct associations between serum neuroactive steroids and psychological functioning. This result contradicts the majority of previous research, which has been able to establish a link between neuroactive steroids and various psychological outcomes [91,92,94,95,189–194]. However, these studies are not directly comparable to ours due to differences in study design and a focus on specific facets of psychological functioning.

Association Between Serum Neuroactive Steroids and Whole-Brain White Matter

We confirmed an association between white matter microstructure and psychological functioning. The beneficial effects of ALLO on white matter may likely be explained by neuroprotective processes [179], increasing the integrity of the fiber protecting myelin sheath and axonal density.

In addition to the first result, we also showed that the association between ALLO and white matter microstructure was strengthened by a comorbid PTSD and mTBI diagnosis.

This result demonstrates that decreased ALLO levels may have a more pronounced detrimental impact on white matter in individuals with comorbid PTSD and mTBI, compared to individuals with mTBI only or healthy individuals, which is also supported by various studies [145–147].
In contrast to individuals with mTBI only, individuals with an additional PTSD diagnosis tend to have particularly pronounced stress and trauma related endocrine dysregulations, which in turn lead to stronger impairments of white matter microstructure.

Association Between Whole-Brain White Matter Microstructure and Psychological Functioning
In our study, we found an association between decreased white matter microstructure and poorer psychological functioning. This association may be explained by a disruption of myelinated pathways in the brain leading to altered white matter connectivity and thus to a functional disruption of networks involved in emotion regulation [195,196]. Our result is consistent with the literature, indicating that different neuropsychiatric disorders are linked to white matter impairments in veterans [145,176,197,198].

Conclusion

This study is a first step towards understanding the structural and functional pathomechanisms in veterans at risk for various psychiatric conditions and suggests that by protecting white matter microstructure, neuroactive steroids may have a therapeutic role in the treatment of psychological symptoms in veterans. Future research should focus on studying the causal and dynamic relationships between neuroactive steroids, white matter, and psychological functioning.

II.5 Own contribution

My contribution to work 1 is composed of the following parts:
Literature research and formulation of working hypotheses, data curation, assessment, and statistical analysis of the data, writing the manuscript of the publication, critical revision of the manuscript.
I participated in work 1 as a shared first author with Ms. Philine Rojczyk.
Based on an extensive literature review and the identification of research gaps, I developed the hypotheses of the study. During data preparation, I validated the data sets and checked for completeness, selected subjects with complete data sets, and integrated multiple data sets.

Processing of the dMRI data to generate diffusion parameters as well as statistical analysis were accomplished with consultation and validation from Yorghos Tripodis, Professor of Biostatistics at Boston University. Processing of the dMRI data was also assisted by the Psychiatry Neuroimaging Laboratory (PNL) team at Harvard Medical School in Boston.

The critical discussion and interpretation of the data were done in close exchange with Philine Rojzyk. I wrote the manuscript together with Philine Rojzyk and critically revised it with the help of several authors, mainly of the cBRAIN team at Ludwig-Maximilians-University in Munich. I wrote the first draft of the manuscript and then focused on finalizing the introduction and discussion parts, while Philine Rojzyk was involved in the method and result parts. In addition, I prepared the paper for submission and answered the queries in the review process. The entire process from hypothesis generation to the completion of the manuscript, as well as the review process, was supervised by Prof. Dr. med. Inga K. Koerte.

III Paper 2

III.1 Background

There is substantial evidence linking combat exposure to poor mental health in veterans returning from war [2,101,199]. In particular, psychiatric disorders in veterans [14] and their far-reaching consequences for social and personal life [6–8] have been well studied. However, the pathomechanism behind the high prevalence of psychiatric disorders in veterans has not been sufficiently explored so far. An established risk factor for the development of psychiatric illness is the intense psychological stress in relation to combat exposure [200]. In addition, veterans who sustained an mTBI also have an increased risk of suffering from psychiatric illness and neurocognitive impairment for several reasons [11,29]. Nevertheless, very few studies have focused on the influence of war zone-related stress on brain structure and function, and in particular, no validated questionnaires have been applied. Moreover, the impact of an mTBI diagnosis on the relationship between war zone-related stress and brain structure and neuropsychological functioning remains to be elucidated.

In particular, dMRI, a specific MRI sequence, is able to detect microstructural alterations and has been mainly used to study white matter [108,188]. Although microstructural white matter alterations have been demonstrated in the common neuropsychiatric disorders affecting veterans [138–147], studies on the impact of war zone-related stress on white matter microstructure are scarce. Notably, the microstructure of gray matter has not been studied in this context at all.

Therefore, the aim of this study was to investigate whether war zone-related stress is associated with microstructural alterations in gray matter and whether this association is modulated by an mTBI diagnosis. Additionally, we aimed to examine whether gray matter changes are associated with neuropsychological functioning.

III.2 Methods

Our study used data from 168 male veterans with available high-quality MRI's and clinical data collected as part of the TRACTS (Translational Research Center for TBI and Stress Disorders) study. The clinical assessment of this study comprised the assessment of psychiatric disorders (Mood Disorders, Substance Use Disorders, Anxiety Disorders, Eating Disorders, Adjustment Disorders), mTBI, war zone-related stress, as well as functional and neurocognitive outcome using validated questionnaires.

Study participants were scanned on a 3-Tesla Siemens TIM Trio MRI scanner (Siemens Healthcare, Erlangen, Germany) and Diffusion MRI were acquired using a single-shot echo-planar sequence with a twice refocused spin-echo pulse.

The average of the free water corrected FA measure (FAt) was derived for eight preselected limbic and paralimbic gray matter regions in each hemisphere (16 regions of interest in total). Statistical analyses were conducted utilizing SPSS, and a p-value of <0.05 was considered statistically significant. A false discovery rate (FDR[201]) of 5% was determined to adjust for multiple comparisons. Generalized linear models (GLM) were applied to assess the link between war zone-related stress and diffusion measures. We added the number of lifetime mTBIs both as a fixed effect and as a modifier of the main effect to investigate whether mTBI has an influence on the relationship between war zone-related stress and gray matter diffusion. The diffusion measures that were significantly associated with war zone-related stress were analyzed post-hoc to examine the association between these diffusion measures and functioning.

III.3 Results

Effects of War zone-related Stress on Limbic Gray Matter Diffusion

We established a negative association between greater war zone-related stress and FA_T in the bilateral cingulate gyri, bilateral orbitofrontal gyri and right parahippocampal gyrus. As opposed to our first finding, war zone-related stress was positively associated with FA_T in the right amygdala-hippocampus complex.

Impact of mTBI on the Association of war zone-Related Stress and Limbic Gray Matter Diffusion

The diagnosis of mTBI did not impact the relationship between war zone-related stress and limbic gray matter FA_T.

Association of Limbic Gray Matter Diffusion and Functional Outcome

Decreased FAt in the cingulate and orbitofrontal gyri was related to reduced response inhibition but to enhanced fronto-temporal functions, i.e., verbal short-term memory performance and processing speed. At the opposite, decreased FA_T in the amygdala-hippocampal region was associated with better response inhibition and impaired performance of verbal short-term memory and processing speed (**Figure 7**). A link between limbic gray matter FA_T and neurobehavioral symptoms or clinical functioning could not be confirmed.

III.4 Discussion

Our study demonstrates that greater war zone-related stress is related to decreased FA_T in the bilateral cingulate, bilateral orbitofrontal , and right parahippocampal gyrus. Decreased FA_T in the cingulate/orbitofrontal gyrus was in turn linked to impaired response inhibition.

In contrast, greater war zone-related stress was associated with higher FA_T in the amygdala-hippocampus complex, which in turn was related to better short-term memory and processing speed. Of note, mTBI did not significantly alter the relationship between war zone-related stress and limbic gray matter structure.

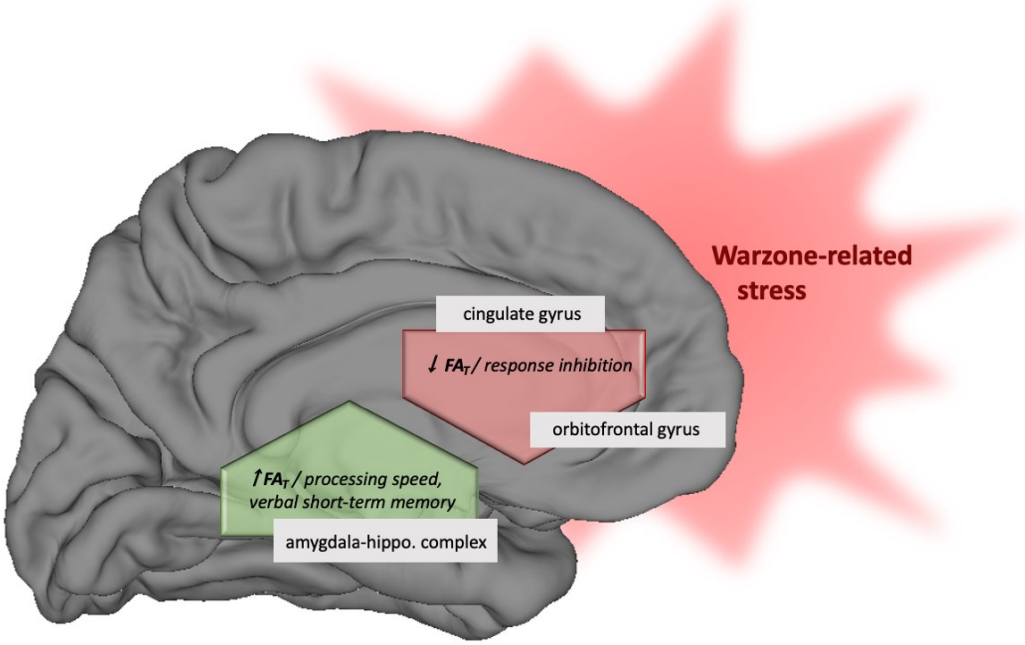

Figure 7: Effects of war zone-related stress on limbic and paralimbic brain areas and associated cognitive functions adapted from paper 2

War zone-related Stress and Limbic Gray Matter Diffusion

Our study is one of the rare ones investigating the microstructure of gray matter, and accordingly, there has been little research that elucidates the pathophysiological basis of gray matter microstructural alterations. However, a reasonable explanation for the association between war zone-related stress and decreased FA_T in the cingulate, orbitofrontal, and right parahippocampal gyrus might be a decrease in astrocytes [202] and/or neurons [203], which are the major components of gray matter.

In contrast, the positive association between higher war zone-related stress and FA_T in the amygdala-hippocampus complex might be grounded in neuroplastic remodeling processes, as indicated by previous research [203].

Association between Limbic Gray Matter Diffusion and Functional Outcome

Increased FA_T in the amygdala-hippocampus complex was not only associated with greater war zone-related stress but also with better fronto-temporal brain functions. This may be due to chronic excessive activation of fronto-temporal brain functions such as short-term memory and processing speed [204–208]. This overactivation leads to improved function of fronto-temporal regions by triggering neuroplastic processes [209]. A potential clinical manifestation could be, for example, a hypervigilant state and readiness to respond, which is an advantageous adaptation mechanism in combat situations, but a disadvantage if persisting in civilian life.

Furthermore, an association between impaired prefrontal/cingulate functions (response inhibition) and reduced FA_T in prefrontal regions was found, which may be due to the phenomenon of interference. In this case, the predominant use of certain brain functions, such as fronto-temporal functions, results in worse functioning of other cognitive tasks, such as response inhibition [210–212].

Conclusion

The findings indicate that the adverse health consequences of experienced war zone-related stress may be attributed to alterations in limbic gray matter microstructure. The importance of early preventive treatment for veterans is demonstrated.

III.5 Own contribution

Concerning this second paper, I contributed to the analysis, interpretation, and editing of the data.

Based on my extensive literature review of brain alterations in veterans, I provided suggestions for the analysis and interpretation of the data.

Further, I was involved in editing the manuscript. I read through the manuscript several times, and provided critical revisions by making suggestions for content, the figures, as well as formal improvements throughout the text. I was particularly involved in the revision of the introduction.

IV Original articles

White Matter Microstructure is Associated with Serum Neuroactive Steroids and Psychological Functioning

Journal of Neurotrauma
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Journal of Neurotrauma

ORIGINAL ARTICLE IMAGING

White Matter Microstructure Is Associated with Serum Neuroactive Steroids and Psychological Functioning

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Abstract

Military service members are at increased risk for mental health issues, and comorbidity with mild traumatic brain injury (mTBI) is common. Largely overlapping symptoms between conditions suggest a shared pathophysiology. The present work investigates the associations among white matter microstructure, psychological functioning, and serum neuroactive steroids that are part of the stress-response system. Diffusion-weighted brain imaging was acquired from 163 participants (with and without military affiliation) and free-water-corrected fractional anisotropy (FA_T) was extracted. Associations between serum neurosteroid levels of allopregnanolone (ALLO) and pregnenolone (PREGNE), psychological functioning, and whole-brain white matter microstructure were assessed using regression models. Moderation models tested the effect of mTBI and comorbid post-traumatic stress disorder (PTSD) and mTBI on these associations. ALLO is associated with whole-brain white matter FA_T ($\beta = 0.24$, $t = 3.05$, $p = 0.006$). This association is significantly modulated by PTSD+mTBI comorbidity ($\beta = 0.00$, $t = 2.50$, $p = 0.027$), although an mTBI diagnosis alone

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did not significantly impact this association ($p = 0.088$). There was no significant association between PREGNE and FA_T ($p = 0.380$). Importantly, lower FA_T is associated with poor psychological functioning ($\beta = -0.19$, $t = -2.35$, $p = 0.020$). This study provides novel insight into a potential common pathophysiological mechanism of neurosteroid dysregulation underlying the high risk for mental health issues in military service members. Further, comorbidity of PTSD and mTBI may bring the compensatory effects of the brain's stress response to their limit. Future research is needed to investigate whether neurosteroid regulation may be a promising tool for restoring brain health and improving psychological functioning.

Keywords: diffusion tensor imaging; mild traumatic brain injury; military service members; neuroactive steroids; post-traumatic stress disorder; psychological functioning

Introduction

Military personnel returning from Operation Enduring Freedom (OEF), Operation Iraqi Freedom (OIF), or Operation New Dawn (OND) show high rates of health problems in general[1,2] and mental health problems in particular.[3] Post-traumatic stress disorder (PTSD) and mild traumatic brain injury (mTBI) are the most common diagnoses and have therefore been described as ''signature wounds'' of military service members.[4,5] Further, comorbidity with other mental disorders such as depression, substance abuse, or anxiety disorder is common.[6–9] In fact, up to 30% of veterans[8,9] are diagnosed with a mental disorder compared with 4–12% among the general population.[10]

Importantly, even those service members who do not meet the diagnostic criteria for a mental disorder are at risk for experiencing low quality of life and psychological distress, which may lead to problems in their social and work life.[11,12] The largely overlapping symptoms of various psychiatric conditions in veterans, including general distress, exaggerated startle, sleep disturbances, depressed mood, anxiety, and impaired social functioning,[8] suggest a common underlying pathophysiology.[13,14] However, to date, most research has been focused on specific psychiatric diagnoses according to categorical diagnostic criteria, neglecting the complexity of comorbidity as well as the overlap in clinical features across diagnoses.[15]

Given the high overlap of symptoms among various conditions, a second approach that has become more popular is to investigate psychosocial functioning on a continuum.[15] In fact, there is evidence that impairments in psychological functioning – the interplay of an individual's overall mental health, behavior, and social skills[16] – underlies almost all mental disorders,[15] suggesting a shared common pathomechanism.[15] Shared underlying symptom clusters can be identified using factor analysis, which has been widely used to facilitate the interpretation of multiple related variables of interest by reducing multicollinearity.[15,17–19] Summarizing the clinical characteristics of military service members into one common factor that explains the majority of symptoms could relate them to objective measures of brain structure and function, thereby elucidating potentially common pathophysiological processes.

A major common feature underlying many psychiatric conditions is the dysregulation of the stress response system[20,21] which may explain similar symptom clusters among various mental disorders. Military service members in particular commonly face extended periods of stress associated with deployment, which activates their stress response system.[22] It is of note here that the neurosteroid allopregnanolone (ALLO) and its precursor pregnenolone (PREGNE) are involved in the neuroendocrinological stress regulation[23–25] to normalize the hyperactivation in the hypothalamic–pituitary–adrenal (HPA) axis.[26–28] In response to traumatizing stress or brain trauma, ALLO and PREGNE exert neuroprotective effects by promoting anti-inflammatory,[25,29–32] anti-apoptotic,[33,34] and pro-myelinating processes.[35] Moreover, ALLO binds to γ-aminobutyric acid type A ($GABA_A$) receptors, which leads to a positive receptor modulation and induces anxiolytic and analgesic effects.[28,36]

Although acute stress stimulates an upregulation of neuroactive steroids, chronic stress has been shown to result in downregulation of ALLO levels.[37–44] This may be explained by the fact that GABA receptor sensitivity decreases following repeated stress exposure as a result of excessive neurosteroid binding.[36] Therefore, it is possible that neurosteroid levels are compensatorily downregulated during chronic stress, to restore GABA receptor sensitivity.[36] Indeed, service members with TBI,[22] PTSD,[45] or comorbid mTBI and PTSD[46] exhibit decreased neurosteroid levels compared with healthy controls. In turn, the chronic stress-induced decrease in neurosteroid concentrations leads to hypersensitivity to new stressors,[42,43,47] dysfunctional behavior,[48] memory deficits,[49] sleep problems,[50–52] and depressive symptoms,[53] thereby perpetuating the endocrine dysregulations.

Although neurosteroid dysregulations have been shown to lead to poor psychological outcome and impaired behavior,[42,43,47,48,50–53] their underlying pathomechanisms remain largely unknown. There is initial evidence to suggest that brain structure likely plays a crucial mediatory role between neurosteroid dysregulation and psychological functioning. In fact, we recently revealed an association between neurosteroid levels and cortical thickness in veterans.[46] Additionally, animal studies of neurodegenerative diseases have

reported an association between increased ALLO levels with markers of myelin and white matter regeneration.[54] The brain's white matter may in this way be responsible for some of the deficits in psychological functioning observed in service members.[55] Further, dense myelination is associated with faster signal transmission,[56] whereas impairments in myelination have been linked to impaired stimulus conduction. The latter translates into impairments in psychological functioning, as seen in various neuropsychiatric disorders.[55]

Diffusion-weighted magnetic resonance imaging MRI (dMRI) has been used to study white matter microstructure in the most common diagnoses in military service members, including mTBI and PTSD.[57–66] Most dMRI studies report widespread abnormalities of fractional anisotropy (FA),[67,68] suggesting demyelination or axonal degeneration.[69] However, the relationship between serum neurosteroid levels and white matter microstructure and its association with psychological functioning remains to be elucidated.

The aims of this study are to investigate (1) whether serum levels of ALLO and PREGNE are associated with psychological functioning, (2) whether serum levels of ALLO and PREGNE are associated with white matter microstructure, and (3) whether changes in white matter microstructure are associated with psychological functioning. Moreover, we will assess whether these associations are moderated by the most common diagnoses in military service members, mTBI and/or PTSD.

Methods

Ethics approval

This study was approved by the institutional review boards of all involved sites and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants before enrollment.

Study design and participants

Participants were recruited as part of the Injury and Traumatic Stress (INTRuST) Clinical Consortium (W81XWH-08-2-0159, intrust.sdsc.edu), which was funded by the Department of Defense and consists of 10 sites across the United States. The overarching aim of the consortium was to improve both understanding and treatment of PTSD and mTBI.[23,45,46,64,70–75]

To be included in the INTRuST study, participants had to be between 18 and 70 years old and have English as their primary language. Exclusion criteria were English as a second language acquired after the age of 5 years; history of a learning disability; uncontrolled hypertension; taking more than one antihypertensive medication; diagnosis of bipolar I disorder, psychotic, delirium, or dementing disorders; uncontrolled chronic disease; history of moderate to severe TBI; oral or intramuscular steroid use within the last 4 months; or currently taking medication affecting brain function (other than psychotropic medications). Exclusion criteria specific to participants who underwent MRI included general MRI contraindications, disorders of the central nervous system affecting the brain, and pregnancy/lactation.

Study enrollment was open from 2008 to 2013, and a total of 771 participants (both with and without military affiliation) were included. Four hundred and twenty-six participants underwent cranial MRI at 6 of the 10 study sites. In the present study, we included participants with both available MRI and serum neurosteroid data, resulting in a sample of 205 participants. MRI data was excluded from another 42 participants because of insufficient MRI data quality, yielding a final sample of 163 participants. Participants were to complete self-report questionnaires and imaging as well as blood draw within 30 days. The final sample did not differ significantly from the excluded participants in demographics (age, sex, race, education, income, employment status), PTSD and mTBI diagnosis, and psychological functioning (PTSD and depressive symptoms, insomnia, alcohol and drug use, functional disability, and health-related quality of life).

Clinical assessments and questionnaires

Assessment of PTSD. PTSD diagnosis was based on the Mini-International Neuropsychiatric Interview (MINI)[76] in 22 subjects, the PTSD Checklist (PCL)[77] in 21 subjects, the Clinician-Administered PTSD Scale for DSM-5 (CAPS)[78] in 9 subjects, and the Structured Clinical Interview for DSM-4 (SCID)[79] in 1 subject.

Assessment of mTBI. History of mTBI was assessed with a self-report mTBI screening instrument consisting of three items assessing (1) past brain injury, (2) immediate loss or alteration of consciousness or unawareness of the event, and (3) amnesia before or after the event. The INTRuST mTBI Screening Instrument was developed following the diagnostic criteria by the American Congress of Rehabilitation Medicine[80] and has been used in previous publications of the INTRuST Clinical Consortium.[46,64,71,74,75]

Assessment of addiction. Alcohol or drug addiction was assessed according to the Drug Abuse Screening Test (DAST-20)[81] and the Alcohol Use Identification Test (AUDIT-10).[82]

Assessment of psychological functioning. From the comprehensive INTRuST Clinical Consortium psychological test battery, questionnaires assessing psychiatric symptoms, functional impairment, and health-related quality of life were chosen in the present study (Table 1).

Neurosteroid quantification

Serum neurosteroid quantifications were performed by gas chromatography/mass spectrometry (GC/MS) preceded by high-performance liquid chromatography (HPLC) as

Table 1. Psychological Functioning

PCL-C	Assessing the presence and severity of post-traumatic stress disorder (PTSD) symptoms within the last month
PHQ-9	Assessing depressive symptoms
BSI-18	Assessing psychological distress
ISI	Assessing nature, severity, and impact of insomnia
SDS	Assessing functional impairment in work, social and family life
SF-12	Assessing health-related quality of life

PCL-C, PTSD Checklist Civilian;[83] PHQ-9, 9-Item Patient Health Questionnaire;[84] BSI-18, Brief Symptom Inventory;[85] ISI, Insomnia Severity Index;[86] SDS, Sheehan Disability Scale;[87] SF-12, Short Form Health Survey.[88]

reported elsewhere.[46] Neurosteroids were run in one batch and quantified blind to condition. Serum samples had been frozen between 6 and 42 months prior to neurosteroid quantification. One milliliter of serum was extracted three times in ethyl acetate prior to HPLC purification using tetrahydrofuran, ethanol, and hexane in the mobile phase. Heptafluorobutyric acid anhydride (HFBA) was used to derivatize the samples. The samples were injected onto an Agilent 5973 MS coupled to an Agilent 6890N GC equipped with an Agilent HP-5MS $30\,m \times 0.250\,mm \times 0.25\,\mu m$ capillary column, and analyzed in the positive ion-electron impact ionization mode with helium as the carrier gas. The definitive structural identification of each neurosteroid was provided by both its GC/MS retention time and unique mass fragmentation pattern. MS single ion monitoring was used to focus on the most abundant ion fragment for each HFBA derivative (ALLO 496.2, PREGNE 298.2). Twenty percent of serum samples were run in duplicate. Intra-assay coefficients of variation were 4.4% for ALLO and 2.0% for PREGNE. The inter-assay coefficient of variation (CV) for batch-to-batch runs was 14.0% for ALLO and 13.9% for PREGNE. Deuterated internal standards were utilized: D4-allopregnanolone for ALLO and D4-pregnenolonenolone for PREGNE.

A constant amount of deuterated internal standard was combined with varying known quantities of steroids (Steraloids) to prepare the standard curve for the steroid of interest. Identical to the experimental samples, each standard curve sample was extracted three times in ethyl acetate prior to HPLC purification and GC/MS injection; standard curve $r^2 = 0.99$ for each neurosteroid. The area under the peak of a known quantity of each steroid was divided by the area under the peak of the internal standard. The resulting ratio was plotted on the y-axis against known quantities of each steroid, generating a standard curve. Only integrated peaks with a signal-to-noise ratio ≥5:1 were integrated. The limit of neurosteroid quantification with this methodology is 1 pg for ALLO and PREGNE (femtomolar sensitivity).

MRI

Image acquisition. The current study used dMRI sequences acquired on three different types of 3-Tesla scanners (Tim Trio, Siemens Healthineers, Erlangen, Germany; GE 750, GE Healthcare, Chicago, IL, USA; or Achieva, Philips Healthcare, Best, The Netherlands) at 6 out of the 10 INTRuST acquisition sites (for imaging sequence details for each MRI system see Table 2).

Table 2. Acquisition Parameters for Diffusion-Weighted Magnetic Resonance Imaging (dMRI)

	GE	*Siemens*	*Philips*
Orientation	Axial	Axial	Axial
Phase encoding direction	Left/right	Anterior/posterior	Posterior/anterior
Field of view (in mm)	256	256	256
TE	83ms	87ms	73 ms
TR	10s	10s	10 sec
Number of directions	64	64	64
b-value	900	900	900
Number of b0	7	7	7
Resolution matrix	128x128	128x128	128x128
Voxel size (in mm^3)	2x2x2	2x2x2	2x2x2
Number of slices	73	73	73
Acquisition time (in min)	14:40	14:08	14:21

Multi-site study; dMRI data acquisition on Tim Trio, Siemens Healthineers, Erlangen, Germany; GE 750, GE Healthcare, Chicago, IL, USA; Achieva, Philips Healthcare, Best, The Netherlands.
TE, echo time; TR, repetition time.

Image processing. *Image harmonization and preprocessing.* Data harmonization is indispensable when attempting to accurately analyze a large data sample acquired through different types of MRI scanners. Therefore, dMRI data were harmonized across the six data acquisition sites using a validated harmonization algorithm.[72] The harmonization approach accounts for scanner-specific differences such as spatial variability of the diffusion signal in different brain areas, while at the same time, the inter-subject variability is maintained at each site and scanner.[72] Pre-processing of the harmonized dMRI data was performed using scripts of our in-house image processing pipeline (https://github.com/pnlbwh/pnlutil/blob/master/pipeline/README.md). First, the images were axis-aligned, centered, and motion-corrected. Next, eddy current correction was applied with an affine registration of each gradient-weighted image to the baseline using FMRIB Software Library, version 5.1 (FSL; The Oxford Centre for Functional MRI of the Brain, Oxford, UK; http://fsl.fmrib.ox.ac.uk). Images were visually inspected for artifacts, such as motion artifacts, ringing, or ghosting of the skull or eyeballs, using 3D Slicer (version 4.5, http://www.slicer.org),[89] leading to the exclusion of 42 participants. Diffusion masks covering the entire brain were created from the dMRI data and manually corrected in 3D Slicer where necessary (e.g., in case of incomplete coverage of the brain) by a trained PhD level researcher.

White matter fiber clustering. White matter fiber clustering was conducted according to an open-source

pipeline of the whitematteranalysis software (https://github.com/SlicerDMRI/whitematteranalysis). The white matter fiber clustering groups fibers according to their anatomical shape and spatial position and extracts a large number of fiber tracts from the entire brain. This is a major advantage over previous automated fiber tracking approaches, which are limited to the extraction of only the major fiber tracts, thereby failing to cover the entire brain's white matter (including the cerebellum, brainstem, and superficial tracts).[90]

The white matter fiber clustering bases the white matter parcellation of each subject on a pre-provided fiber clustering atlas: a neuroanatomist-curated set of tracts covering the white matter (http://dmri.slicer.org/atlases/).[90] First, unscented Kalman filter (UKF) tractography (https://github.com/pnlbwh/ukftractography)[91] was performed in all subjects using established fiber tracking parameters as follows. Fiber tracking was seeded in all voxels within the brain mask where FA was >0.18 (default). Tracking stopped in voxels where the FA value fell <0.15 (default) or the sum of the normalized signal across all gradient directions fell <0.1 (default) (a parameter to distinguish between white/gray matter and cerebrospinal fluid [CSF] regions). In addition to these major parameters, the UKF method uses other parameters to fine tune the fiber tracking result, including: Qm to control process noise for angles/direction, Ql to control process noise for eigenvalues, and Rs to control for expected noise in the diffusion signal. These three parameters were well adjusted according to the dMRI data properties under study and set to 70, 0.001, and 0.015, respectively. Visual and quantitative quality control of the generated tractography data for all subjects under study was performed using a semi-automated quality control tool in the whitematteranalysis software (https://github.com/SlicerDMRI/whitematteranalysis).

Next, each subject's tractography was registered with the pre-provided atlas tractography. By following up with this step we were able to largely reduce the known tractography issue of false-positive tracking.[92] False-positive fiber tracking is a contributing factor affecting white matter parcellation reproducibility.[92] With our approach, false positive fibers in the atlas have been annotated and rejected via expert judgment.[90] Usage of the atlas therefore can ameliorate potential subject-specific false-positive fibers that are inconsistent with respect to known neuroanatomical knowledge. In this study, subject-specific fibers that had improbable fiber geometric trajectories were automatically removed.

For each subject, we performed atlas-based white matter parcellation[90,93,94] using a robust machine learning approach that has been shown to consistently identify white matter tracts across the full human lifespan, across health conditions including brain tumors, and across different image acquisitions.[90] This approach produces consistent tracts across subjects,[95] is reproducible in test-retest data sets[96] and is robust to anatomical variability.[94] The approach has also been employed for quantitative tractography analyses[97] in many recent studies.[98–105]

Subject-specific anatomical fiber tract identification was conducted for both hemispheres by linking the registered tractography to the annotated atlas clusters for each tract. Fiber tracts of the entire brain (Fig. 1) were combined into one whole-brain white matter variable, given that we aimed to investigate global white matter effects in association with neuroactive steroids and psychological functioning and did not specify hypothesis for individual tracts. A quantitative quality assessment of the number of streamlines (NoS) and FA was performed to make sure that there were not individual subjects with outlier values. A visual quality assessment of each subject's whole-brain white matter tracts was also performed to ensure anatomical correctness. These quality check processes follow best practices in recent studies.[98,102,106–109] All data passed quality checks.

Diffusion parameter extraction. Diffusivity of the extracted whole-brain white matter tracts was calculated and corrected for the relative contribution of extracellular free water in each voxel using free-water modeling.[110] The resulting diffusivity represents the tissue compartment in each voxel from which the tissue's free-water-corrected FA (FA_{Tissue}, i.e., FA_T) was calculated. Compared with FA, FA_T serves as a more accurate marker for cellular white matter microstructure that is less susceptible to partial volume effects with CSF, and is thus more sensitive to the degree of myelinization of fiber tracts, axonal density, and fiber orientation.[111] Subject-specific average whole-brain FA_T values were extracted from the whole brain's white matter fiber clustering output.

Statistical analysis

SPSS software (version 25.0; IBM Statistics for Mac, Armonk, NY, USA) was used for all statistical analyses. A Bonferroni-corrected p value of <0.05 was considered statistically significant.

Psychological functioning

Given the variety of psychological symptoms among veterans, we investigated whether a common psychological functioning construct was underlying the different psychological dimensions present in the current sample (assessed with the questionnaires presented in Table 1). A factor analysis using the Anderson–Rubin method to extract one or more underlying psychological functioning factors was performed. Varimax rotation was applied to ensure the orthogonality of the estimated factors. Only factors with an eigenvalue >1 were extracted. The assumptions for the conduction of a factor analysis were investigated using Bartlett's tests of sphericity.

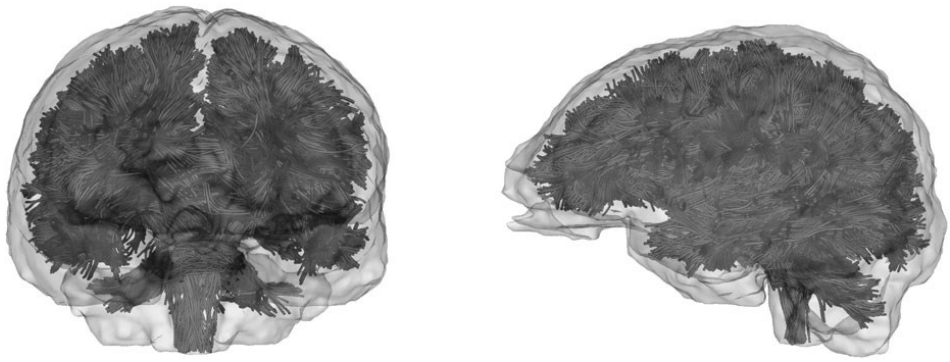

FIG. 1. Whole-brain white matter tractography. This figure shows the whole-brain white matter of one participant derived from the fiber clustering output (in the sagittal and coronal plane).

Association between serum neuroactive steroids and psychological functioning

The association between serum neurosteroid levels and psychological functioning was assessed using two multiple regression models with independent variable serum ALLO/PREGNE and dependent variable psychological functioning ($p < 0.025$, Bonferroni-corrected for two tests). Age, sex, and alcohol and drug use were included as covariates. In case of a statistically significant association, we additionally assessed the effect of mTBI and PTSD+mTBI comorbidity on the association between serum neurosteroid levels and psychological functioning using Hayes PROCESS[112] (double moderation model – Model 2). Bonferroni correction was applied for two tests ($p < 0.025$). PTSD diagnosis without mTBI comorbidity was not investigated as an individual effect because of the small number of participants with PTSD only ($n = 10$).

Association between serum neuroactive steroids and whole-brain FA_T

The association between serum neurosteroid levels and whole-brain FA_T was analyzed by conducting two multiple regression models with independent variable serum ALLO/PREGNE and dependent variable whole-brain FA_T ($p < 0.025$, Bonferroni-corrected for two tests). Age, sex, and alcohol and drug use were included as covariates. In case of a statistically significant association, we additionally assessed the effect of mTBI and PTSD+mTBI comorbidity on the association between serum neurosteroid levels and whole-brain white matter FA_T using Hayes PROCESS[112] (double moderation model – Model 2). Bonferroni correction was applied for two tests ($p < 0.025$).

Association between whole-brain FA_T and psychological functioning

The association between psychological functioning and whole-brain FA_T was investigated using a multiple regression model with whole-brain FA_T as independent variable and psychological functioning as dependent variable. Age, sex, and alcohol and drug use were included as covariates. In case of a statistically significant association, we additionally assessed the effect of mTBI and PTSD+mTBI comorbidity on the association between whole-brain FA_T and psychological functioning using Hayes PROCESS[112] (double moderation model – Model 2).

Results

Sample characteristics

A sample of 163 participants from the INTRuST study were included. The demographic characteristics of the participants are displayed in Table 3. Neuropsychiatric comorbidities among the sample are visualized in Figure 2.

Psychological functioning

Bartlett's test of sphericity was used to test the overall significance of all correlations within the matrix, which was statistically significant ($\chi^2[15] = 1010.27$, $p < 0.001$). As significant correlations between all variables were shown, conducting a factor analysis to identify the underlying factor behind the correlating variables is statistically justified. The factor analysis revealed one underlying psychological functioning factor based on PTSD symptoms (PCL-C); depression (Patient Health Questionnaire [PHQ]-9); psychological distress (Brief Symptom Inventory [BSI]); insomnia (Insomnia Severity Index [ISI]); functional impairment of work, social, and family life (SDS), and health-related quality of life (Short Form 12

Table 3. Sample Characteristics

	Total		*PTSD*		*mTBI*		*PTSD+mTBI*		*No PTSD or mTBI*		*mTBI vs. PTSD+mTBI vs. no PTSD or mTBI*	*Post-hoc PTSD+mTBI vs. mTBI*	*Post-hoc mTBI vs. no PTSD or mTBI*	*Post-hoc PTSD+mTBI vs. no PTSD or mTBI*
											AN(C)OVA			
	n	*ME±IQR*	n	*ME±IQR*	n	*ME±IQR*	n	*ME±IQR*	n	ME±IQR	F(df), p	p	p	p
Age	161	32.00±21.00	10	33.00±17.00	33	34.00±23.00	43	35.00±16.00	75	28.00±19.00	6.42(2, 148),0.002	0.654	0.014	0.001
Education (years)	159	14.00±3.00	9	14.00±2.00	33	15.00±3.00	42	13.50±3.00	75	15.00±3.00	4.23(2, 147), 0.016	0.038	0.788	0.005
ALLO (pg/ml)	161	47.30±53.65	10	44.55±96.95	34	37.00±38.60	41	40.60±34.65	76	62.00±64.15	1.38(2,144), 0.255	0.975	0.168	0.169
PREGNE (pg/ml)	163	425.50±415.00	10	266.25±547.88	34	370.20±353.38	43	382.60±414.00	76	460.65±427.18	0.29(2,146), 0.748	0.897	0.490	0.571
Whole-brain FA_T	163	0.59±.01	10	0.59±.01	34	0.59±.01	43	0.59±.01	76	0.59±.01	2.32(2,146), 0.102	0.077	0.991	0.050
PTSD (PCL_C)	152	21.00±35.00	6	64.00±30	33	30.00±26.00	37	61.00±18.00	76	17.00±1.00	221.63(2, 139), <0.001	< 0.001	< 0.001	< 0.001
Depression (PHQ-9)	161	2.00±11.00	10	14.00±11.00	34	4.50±8.00	41	14.00±7.00	76	0.00±1.00	171.52(2, 144), <0.001	< 0.001	< 0.001	< 0.001
General distress (BSI)	160	21.00±18.00	10	42.00±24.00	34	27.00±13.00	40	47.00±20.00	76	18.00±1.00	116,16(2, 143), <0.001	< 0.001	< 0.001	< 0.001
Insomnia (ISI)	156	5.00±15.00	8	18.00±16.00	34	9.00±10.00	39	22.00±6.00	75	1.00±3.00	137.56(2, 141), <0.001	< 0.001	< 0.001	< 0.001
Disability in family, social, and work life (SDS)	163	0.00±16.00	10	18.50±17.00	34	9.00±17.00	43	19.00±9.00	76	0.00±.00	93.62(2, 146), <0.001	< 0.001	< 0.001	< 0.001
General health and well-being (SF-12)	160	55.52±17.25	10	44.74±17.79	34	44.74±10.78	41	44.75±15.09	75	55.52±6.47	44.21(2, 143), <0.001	< 0.001	< 0.001	< 0.001
Psychological functioning (Factor)	148	-0.58±1.56	5	1.56±1.34	33	0.01±1.24	35	1.46±.95	75	-0.78±.13	217.75(2, 136), <0.001	< 0.001	< 0.001	< 0.001
Alcohol use (AUDIT-10)	155	3.00±6.00	7	6.00±28.00	33	3.00±6.00	40	3.00±8.00	75	2.00±4.00	4.32(2, 141), 0.015	0.875	0.022	0.011
Drug use (DAST-20)	163	0.00±1.00	10	0.50±5.00	34	0.00±1.00	43	1.00±2.00	76	0.00±0.00	2.02(2, 146), .136	0.172	0.666	0.050
											Fisher's exact test			
	n	%	n	%	n	%	n	%	n	%	χ^2, p	p	p	p
Military (military/civilian/ unknown)	66/ 76/21	40.49/ 46.63/12.88	6/1/3	60.00/ 10.00/30.00	14/ 17/3	41.18/ 50.00/8.82	30/ 4/9	69.77/ 9.30/20.93	16/ 54/6	21.05/ 71.05/7.89	46.14, < 0.001	< 0.001	0.033	< 0.001
Sex (male/female)	102/61	62.58/37.42	10	60.00/40.00	34	67.65/32.35	43	86.05/13.95	76	47.37/52.63	18.73, < 0.001	0.049	0.038	< 0.001
Race											10.11, 0.156	0.598	0.075	0.196
African American	26	16.00	3	30.00	4	11.76	7	16.67	12	16.90				
Asian	6	3.68	-	-	-	-	1	2.38	5	7.04				
Native	2	1.23	-	-	-	-	2	4.76	-	-				
Unknown	3	1.84	-	-	2	5.88	1	2.38	5	7.04				
White	120	73.62	7	70.00	28	82.35	31	73.81	54	76.06				
Handedness											4.30, 0.636	0.520	0.318	0.976
Right	115	70.55	6	60.00	23	67.65	32	74.42	54	71.05				
Left	19	11.66	-	-	7	20.59	4	9.30	8	10.53				
Both	3	1.84	-	-	1	2.94	1	2.33	1	1.32				
Unknown	26	15.95	4	40.00	3	8.82	6	13.95	13	17.11				

SD, Standard deviation; PTSD, Posttraumatic stress disorder; mTBI, Mild traumatic brain injury; ME; IQR, interquartile range; ALLO, allopregnanolone; PREGNE, pregnenolone; AUDIT-10, Alcohol Use Identification Test; DAST-20, Drug Abuse Screening Test. For other test acronyms, see Table 1. Analyses of covariance (ANCOVAs) were corrected for age and sex (except for age and education).

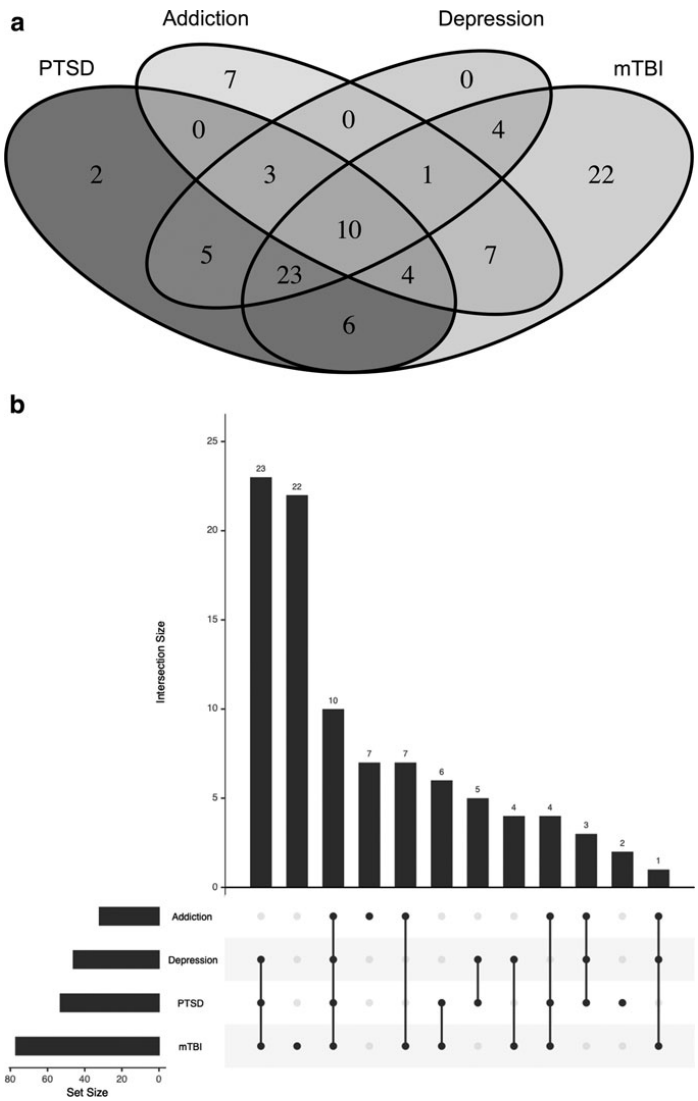

FIG. 2. Neuropsychiatric comorbidities. **(a)** (Venn diagram) and **(b)** (UpSet plot) display the comorbid neuropsychiatric disorders in the present sample ($n = 163$). Particularly, participants with both PTSD and mTBI have high numbers of comorbidity with alcohol/drug addiction and depression. mTBI, Mild traumatic brain injury; PTSD, Post-traumatic stress disorder..

Health Survey, General Health [SF12-GH]), with factor loadings between -0.75 and 0.96, and an eigenvalue of 4.97, accounting for 82.85% of the variance in the data (Table 4). The next factors had an eigenvalue of 0.41 and 0.24 and explained 6.8% and 4% of the total variance.

Association between serum neuroactive steroids and psychological functioning

There was no significant association between serum ALLO ($p = 0.193$) or PREGNE ($p = 0.703$) and the psychological functioning measure derived by the factor analysis.

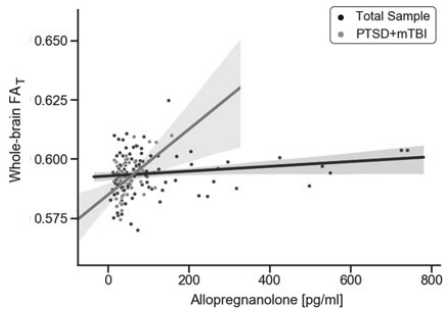

FIG. 3. Association between allopregnanolone (ALLO) and whole-brain FA_T. This figure illustrates the significant moderating effect of PTSD+mTBI comorbidity on the association between ALLO (pg/mL) and whole-brain FA_T. mTBI, Mild traumatic brain injury; PTSD, Post-traumatic stress disorder; FA_T, Fractional anisotropy $_{\text{Tissue}}$

Association between serum neuroactive steroids and whole-brain FA_T

The multiple regression analyses revealed a significant positive association between serum ALLO and whole-brain FA_T ($\beta = 0.24$, $t = 3.05$, $p = 0.006$). There was no statistically significant association between serum PREGNE and whole-brain FA_T ($p = 0.380$).

The moderation analysis showed that the association between serum ALLO and whole-brain FA_T was significantly moderated by PTSD+mTBI comorbidity ($b = 0.00$, $t = 2.50$, $p = 0.027$, Figs. 3 and 4), whereas an mTBI diagnosis alone did not impact this relationship ($p = 0.088$).

Association between whole-brain FA_T and psychological functioning

There was a significant negative association between whole-brain FA_T and psychological functioning ($\beta = -0.19$, $t = -2.35$, $p = 0.020$, Fig. 4). Lower scores on the psychological functioning scale represent better functioning. Mild TBI ($p = 1.000$) or PTSD+mTBI comorbidity ($p = 1.000$) did not significantly alter this relation, suggesting that psychological functioning is associated with white matter structure independently of these diagnoses.

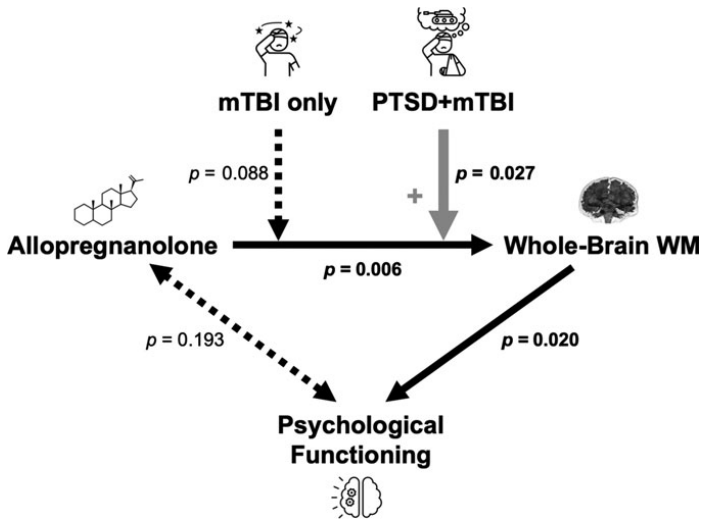

FIG. 4. Summary of findings. This figure illustrates the significant associations between allopregnanolone (ALLO) and whole-brain white matter and between whole-brain white matter and psychological functioning. Comorbidity of PTSD and mTBI significantly alters the strength of the observed relation between ALLO and whole-brain white matter. PTSD, Post-traumatic stress disorder; mTBI, Mild traumatic brain injury.

Discussion

This study investigated the association among serum levels of the neuroactive steroids ALLO and PREGNE, psychological functioning, and whole-brain white matter microstructure. Moreover, effects of mTBI and comorbidity of PTSD and mTBI on these associations were assessed.

Higher serum levels of ALLO were associated with increased white matter FA_T. This suggests that higher levels of ALLO may have neuroprotective effects on white matter microstructure. Further, our results demonstrate that the association between ALLO and white matter FA_T is stronger in individuals with PTSD and mTBI comorbidity (Figs. 3 and 4), indicating that a decrease in ALLO leads to a stronger decrease in FA_T in these individuals. We suspect that these clinically highly burdened individuals are more sensitive to alterations in neuroactive steroids. Importantly, lower FA_T is associated with poor overall psychological functioning in the entire sample and independent of mTBI or PTSD diagnosis (Fig. 5).

Association between serum neuroactive steroids and psychological functioning

We demonstrate that a common factor is underlying the different symptom domains of psychological functioning.

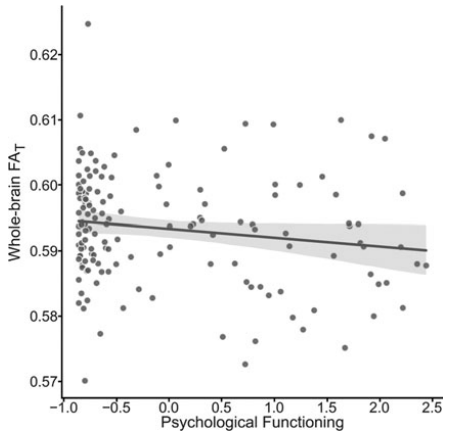

FIG. 5. Association between psychological functioning and whole-brain FA_T. This figure shows the association between the psychological functioning and whole brain FA_T ($\beta = -0.20$, $t = -2.38$, $p = 0.019$). Lower scores on the psychological functioning scale represent better functioning. Scores are standardized z-scores with a mean of 0 and a standard deviation of 1.

This result suggests that the different psychological questionnaires measure a common overall construct. This result is also in line with previous research showing that psychological symptoms in military service members assessed with different diagnostic tools are strongly correlated,[15] emphasizing further the shared nature of symptoms.

Contrary to previous findings,[42,43,47,48,50–53,113,114] however, we did not detect a statistically significant association between serum neuroactive steroids and psychological functioning. Although most research studies to date have shown a link between neuroactive steroids and several dimensions of psychological functioning, one other study also did not find a direct influence of neuroactive steroids on psychological symptoms.[115] It should be noted that many of the previously published studies were conducted in animal models and that most studies examined group comparisons rather than correlations.[42,43,47] Moreover, previous research has focused only on specific facets of psychological functioning (such as in PTSD,[113,114] depression,[116] or sleep[50–52]), whereas studies on overall psychological functioning in relation to neuroactive steroids are currently missing. Further, the few studies with human subjects examined either men[114] or women[113] and included much smaller sample sizes than those in our study.[53,113,114]

It is not yet clear to what extent sex might have affected the association between neuroactive steroids and psychological functioning. Therefore, future research should specifically investigate whether the measured values are affected by sex, using larger sample sizes.

Association between serum neuroactive steroids and whole-brain white matter microstructure

We demonstrate an association between serum levels of the neurosteroid ALLO and white matter microstructure. Previous research has attributed the positive effects of ALLO on white matter microstructure to anti-inflammatory,[25,29–31] anti-apoptotic,[33,34] and promyelinating[35] effects previously reported for ALLO. In fact, ALLO increases markers of myelination[117] and reduces inflammatory cytokines in the brains of mice.[29] Moreover, ALLO promotes proliferation of neural progenitor cells and regulates cell-cycle gene and protein expression,[118] thereby further benefitting white matter microstructure.

Notably, the association between ALLO and white matter microstructure was significantly stronger in participants with the comorbidity of PTSD and mTBI, indicating that a decrease in ALLO levels may potentially lead to a stronger negative effect on white matter microstructure in these clinically highly burdened individuals. This is in line with previous research demonstrating that white matter abnormalities are more severe in those with PTSD

and mTBI than in those with either or neither condition.[63–66] Additionally, a study in this same cohort found an association between neuroactive steroids and cortical thickness only among individuals with PTSD and mTBI comorbidity, but not in those with mTBI only or in healthy controls.[46]

In contrast to comorbid PTSD and mTBI, mTBI alone did not show a statistically significant effect on the association between neuroactive steroids and white matter alterations. It is of note that although most of the participants with mTBI alone in our study still had prolonged post-concussive symptoms, those who additionally had PTSD were more severely impacted. Service members with an additional current diagnosis of PTSD face ongoing stress-related endocrine dysregulations, which adds to their brain trauma sustained a decade ago.

Our findings thus support the hypothesis that neuroactive steroids reveal their neuroprotective effects particularly in stress-related conditions. Stress and trauma cause a variety of acute responses, such as upregulation of the HPA axis[119,120] or neurodegenerative processes.[121,122] In an attempt to regulate the stress response, neuroactive steroids counteract these dysregulations by promoting neuroregeneration and release neuroprotective effects on white matter microstructure.[27,28,123–127] Thus, the association between neuroactive steroids and white matter microstructure may be most apparent in individuals with PTSD and mTBI comorbidity, as endocrine dysregulation[46] and consequently brain alterations[63–66] are most pronounced in these individuals.

Moreover, there could potentially be a threshold effect of neuroactive steroids, meaning that they only exert adverse brain effects when concentrations are at a critical level.[128] Individuals with a PTSD diagnosis in addition to mTBI may be more likely to reach a critically low level of neuroactive steroids than individuals with a distant history of mTBI or healthy individuals,[46] so that neuroprotective mechanisms are no longer effective enough. Additionally, individuals with a comorbid mTBI and PTSD diagnosis also have a high prevalence of other comorbidities, such as depression[5,129–131] and alcoholism,[132,133] which may further increase the overall stress burden and consecutive stress response and possible endocrine dysregulation.[113,134]

Association between whole-brain white matter microstructure and psychological functioning

In the present study, we found an association between greater white matter microstructure alteration (decrease in FA_T) and worse psychological functioning. Our finding is in line with previous studies, showing that various psychopathological conditions such as depression[64] or PTSD[135,136] are related to alterations in white matter tracts in military service members.[64,66,135,136] Moreover, our comprehensive approach adds to the existing literature by showing that whole-brain white matter microstructure is associated with overall psychological functioning, independent of mTBI or PTSD diagnosis. This association highlights the essential role of white matter microstructure for psychological functioning in general and, more importantly, suggests a common pathophysiology of psychological symptoms in military service members.

There is a growing research interest in redefining neuropsychiatric diseases as symptomatic expressions of cellular and molecular dysfunctions of brain circuits.[137] The association between white matter changes and psychological functioning can be attributed to the fact that white matter fiber tracts connect various nodes of brain networks involved in psychological functioning.[138,139] Therapeutics that enhance white matter microstructural integrity may thus also facilitate intra- and intercommunication of brain networks and consequently benefit psychological functioning.

Initial evidence of neuroactive steroids as a favorable treatment option for service members comes from a pilot randomized controlled trial with an 8-week course of exogeneous PREGNE administration after mTBI that reported enhanced psychological functioning compared with administration of a placebo.[45] The findings suggest that exogenous supplementation with neuroactive steroids after trauma may benefit brain health and ultimately also benefit psychological outcome. Future research is needed to further investigate the therapeutic potential of neuroactive steroids in the context of brain trauma by also relating the effects of therapeutically administered neuroactive steroids to neuroimaging findings.

Limitations

We acknowledge several limitations of this study. We were not able to separately examine the effect of PTSD because of the very small number of participants with PTSD only ($n = 10$). Therefore, we cannot entirely rule out that PTSD and not the comorbidity of PTSD and mTBI accounted for some of our findings. Moreover, we were not able to assess differences between participants with PTSD+mTBI and those with PTSD+mTBI+ depression given the limited sample sizes. Future research should consider depressive disorders in the relationship between neurosteroids and brain structure.

Similarly, we were not able to assess the effect of current or past military service (e.g., active duty or veteran, branch, deployment, and combat exposure) on our outcome measures because of missing information. Future studies are, therefore, needed to compare neuroactive steroid levels, brain structure, and psychological functioning between military and civilian PTSD and mTBI samples.

Moreover, although the INTruST mTBI screening tool is a validated instrument, the newest gold standard for retrospective TBI assessment post-combat is the Boston

Assessment of Traumatic Brain Injury-Lifetime (BAT-L)[140] and the Ohio State University Identification TBI Method (OSU-TBI-ID)[141] for both civilian and military TBI.

Further, we note that diffusion weighted imaging provides only an approximation of neural pathology. However, the correction for extracellular free-water adds to the specificity of diffusion tensor imaging (DTI) metrics. FA_T is a more accurate marker of cellular processes than the conventional FA and, therefore, an improved index of white matter health in the living.

It should also be noted that we measured serum levels of ALLO and PREGNE and not CSF levels. Although studies in mice suggest that peripheral markers of neuroactive steroids are adequate proxies for central processes,[142–144] additional research is needed. Further, ALLO and PREGNE levels might be influenced by factors that we are not accounting for, such as the time point of blood draw, the menstrual cycle phase,[145,146] or oral contraception use in women.[115,147] It is of note that there is some evidence that basal neurosteroid concentrations are higher in women than in men and that neurosteroid concentrations in women are more impacted by stress.[148] The limited female sample size in our study, however, prevented us from examining the sexes independently and should be targeted in future studies. Psychiatric medication use was not consistently assessed. We were therefore not able to control for this potential confounder.

Finally, although we report a relationship between neurosteroid levels and white matter microstructure, the interpretation of causal relationships is limited, given the cross-sectional study design. However, it should be noted that the present study represents one of the most extensive studies investigating the association between neuroactive steroids and brain structural alterations and is consistent with previous research regarding additive effects of mTBI and PTSD on brain structure.[57,63,149] Longitudinal research is needed to further explore the relationship among neuroactive steroids, white matter microstructure, and psychological functioning.

Conclusion

We report that higher neurosteroid levels are associated with increased FA_T of the whole brain's white matter. This result underscores previous reports on the neuroprotective effect of neuroactive steroids. Importantly, white matter alterations are associated with worse psychological functioning.

Further, results from this study suggest that comorbidity of PTSD and mTBI may bring the compensatory effects of the brain's stress response to their limit, where lower levels of neuroactive steroids are associated with an even steeper increase in alterations of white matter microstructure. Thus, this study provides insight into what could potentially be a common pathophysiological mechanism underlying the high risk for various psychiatric symptoms and diagnoses in those with PTSD and mTBI: a dysregulated stress response system. Future research is needed to investigate whether neurosteroid regulation may be a promising tool for preserving or restoring brain health and for improving psychological functioning in veterans.

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Author's Contributions

L.F.U. was responsible for conceptualization and design; data curation; formal analysis and interpretation; writing – original draft, review and editing; and visualization. P.R. was responsible for conceptualization and design; data curation; formal analysis and interpretation; writing – original draft, review and editing; and visualization. J.S.H. was responsible for conceptualization and design; formal analysis and interpretation; and supervision, writing – original draft, review and editing. N.S. was responsible for conceptualization and design; writing – original draft, review and editing; and supervision. E.K. was responsible for conceptualization; formal analysis and interpretation; and writing – review and editing. P.K. was responsible for conceptualization; data curation; and writing - review and editing. F.Z. was responsible for methodology; software; and writing – review and editing. J.K. was responsible for conceptualization; formal analysis; and writing – review and editing. M.L. was responsible for formal analysis; and writing – review and editing. C.L.K. was responsible for visualizaition; and writing – review and editing. T.L.T.W. was responsible for visualization; and writing – review and editing. J.D.K. was responsible for acquisition and analysis; and writing – review and editing. J.C.N. was responsible for acquisition and analysis; and writing – review and editing. G.A.G. was responsible for conceptualization and design; acquisition and interpretation; and writing – review and editing. Y.R. was responsible for conceptualization and design; acquisition and analysis; and writing – review and editing. M.J.C. was responsible for conceptualization and design; acquisition and analysis; and writing – review and editing. S.B. was responsible for conceptualization and design; data curation; acquisition and analysis; and writing – review and editing. Y.T. was responsible for formal analysis; software; and writing – review and editing. O.P. was responsible for conceptualization and design; software; acquisition and analysis; and writing – review and editing. M.S.G. was responsible for conceptualization and design; acquisition; and writing – review and editing. T.W.M. was responsible for conceptualization and design; acquisition and interpretation;

and writing – review and editing. R.Z. was responsible for concetualization and design; acquisition and interpretation; and writing – review and editing. M.B.S. was responsible for conceptualization and design; acquisition; and writing – review and editing. L.J.O. was responsible for conceptualization and design; methodology; software; and writing – review and editing. C.E.M. was responsible for conceptualization and design; acquisition; formal analysis and interpretation; writing – review and editing; and supervision. M.E.S. was responsible for conceptualization and design; acquisition; analysis and interpretation; writing – review and editing; and supervision. I.K.K. was responsible for conceptualization and design; analysis and interpretation; writing – original draft; review and editing; and supervision. This manuscript is part of the dissertation of L.F.U.

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Author Disclosure Statement

Murray B. Stein has in the past three years received consulting income from Acadia Pharmaceuticals, Actelion, Aptinyx, atai Life Sciences, Boehringer Ingelheim, Bionomics, BioXcel Therapeutics, Clexio, Eisai, Empower-Pharm, Engrail Therapeutics, GW Pharmaceuticals, Janssen, Jazz Pharmaceuticals, and Roche/Genentech. Dr. Stein has stock options in Oxeia Biopharmaceuticals and EpiVario. He is paid for his editorial work on *Depression and Anxiety* (Editor-in-Chief), *Biological Psychiatry* (Deputy Editor), and *UpToDate* (Co-Editor-in-Chief for Psychiatry). Christine E. Marx is a co-applicant on pending patent applications focusing on neurosteroids and derivatives in central nervous system (CNS) disorders. No patents have been issued. No licensing is in place. VA 208 waiver is in place. The other authors report no biomedical financial interests or potential conflicts of interest.

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Association of War Zone–Related Stress With Alterations in Limbic Gray Matter Microstructure

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Abstract

IMPORTANCE Military service members returning from theaters of war are at increased risk for mental illness, but despite high prevalence and substantial individual and societal burden, the underlying pathomechanisms remain largely unknown. Exposure to high levels of emotional stress in theaters of war and mild traumatic brain injury (mTBI) are presumed factors associated with risk for the development of mental disorders.

OBJECTIVE To investigate (1) whether war zone–related stress is associated with microstructural alterations in limbic gray matter (GM) independent of mental disorders common in this population, (2) whether associations between war zone–related stress and limbic GM microstructure are modulated by a history of mTBI, and (3) whether alterations in limbic GM microstructure are associated with neuropsychological functioning.

DESIGN, SETTING, AND PARTICIPANTS This cohort study was part of the TRACTS (Translational Research Center for TBI and Stress Disorders) study, which took place in 2010 to 2014 at the Veterans Affair Rehabilitation Research and Development TBI National Network Research Center. Participants included male veterans (aged 18-65 years) with available diffusion tensor imaging data enrolled in the TRACTS study. Data analysis was performed between December 2017 to September 2021.

EXPOSURES The Deployment Risk and Resilience Inventory (DRRI) was used to measure exposure to war zone–related stress. The Boston Assessment of TBI-Lifetime was used to assess history of mTBI. Stroop Inhibition (Stroop-IN) and Inhibition/Switching (Stroop-IS) Total Error Scaled Scores were used to assess executive or attentional control functions.

MAIN OUTCOMES AND MEASURES Diffusion characteristics (fractional anisotropy of tissue [FA_T]) of 16 limbic and paralimbic GM regions and measures of functional outcome.

RESULTS Among 384 male veterans recruited, 168 (mean [SD] age, 31.4 [7.4] years) were analyzed. Greater war zone–related stress was associated with lower FA_T in the cingulate (DRRI-combat left: $P = .002$, partial $r = -0.289$; DRRI-combat right: $P = .02$, partial $r = -0.216$; DRRI-aftermath left: $P = .004$, partial $r = -0.281$; DRRI-aftermath right: $P = .02$, partial $r = -0.219$), orbitofrontal (DRRI-combat left medial orbitofrontal cortex: $P = .02$, partial $r = -0.222$; DRRI-combat right medial orbitofrontal cortex: $P = .005$, partial $r = -0.256$; DRRI-aftermath left medial orbitofrontal cortex: $P = .02$, partial $r = -0.214$; DRRI-aftermath right medial orbitofrontal cortex: $P = .005$, partial $r = -0.260$; DRRI-aftermath right lateral orbitofrontal cortex: $P = .03$, partial $r = -0.196$), and parahippocampal (DRRI-aftermath right: $P = .03$, partial $r = -0.191$) gyrus, as well as with higher FA_T in the amygdala-hippocampus complex (DRRI-combat: $P = .005$, partial $r = 0.254$; DRRI-aftermath: $P = .02$, partial $r = 0.223$). Lower FA_T in the cingulate-orbitofrontal gyri was associated with impaired

(continued)

Key Points

Question Is war zone–related stress associated with limbic gray matter (GM) microstructure?

Findings In this cohort study of US veterans, exposure to war zone–related stress was associated with alterations in limbic GM microstructure, independent of the diagnosis of mental disorder or mild traumatic brain injury. Furthermore, GM microstructure was associated with cognitive functioning.

Meaning These findings suggest that war zone–related stress may lead to limbic GM microstructure alterations, which may underlie the deleterious outcomes of war zone–related stress on brain health and that military service members may benefit from early therapeutic interventions following deployment.

+ Supplemental content

Author affiliations and article information are listed at the end of this article.

Abstract (continued)

response inhibition (Stroop-IS left cingulate: $P < .001$, partial $r = -0.440$; Stroop-IS right cingulate: $P < .001$, partial $r = -0.372$; Stroop-IS left medial orbitofrontal cortex: $P < .001$, partial $r = -0.304$; Stroop-IS right medial orbitofrontal cortex: $P < .001$, partial $r = -0.340$; Stroop-IN left cingulate: $P < .001$, partial $r = -0.421$; Stroop-IN right cingulate: $P < .001$, partial $r = -0.300$; Stroop-IN left medial orbitofrontal cortex: $P = .01$, partial $r = -0.223$; Stroop-IN right medial orbitofrontal cortex: $P < .001$, partial $r = -0.343$), whereas higher FA_T in the mesial temporal regions was associated with improved short-term memory and processing speed (left amygdala-hippocampus complex: $P < .001$, partial $r = -0.574$; right amygdala-hippocampus complex: $P < .001$, partial $r = 0.645$; short-term memory left amygdala-hippocampus complex: $P < .001$, partial $r = 0.570$; short-term memory right amygdala-hippocampus complex: $P < .001$, partial $r = 0.633$). A history of mTBI did not modulate the association between war zone–related stress and GM diffusion.

CONCLUSIONS AND RELEVANCE This study revealed an association between war zone–related stress and alteration of limbic GM microstructure, which was associated with cognitive functioning. These results suggest that altered limbic GM microstructure may underlie the deleterious outcomes of war zone–related stress on brain health. Military service members may benefit from early therapeutic interventions after deployment to a war zone.

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Introduction

Military personnel serving in theaters of war are at increased risk for physical and mental health problems following deployment.[1-3] Mental health–related disorders are pervasive; up to 30% of service members returning from Operation Enduring Freedom (OEF), Operation Iraqi Freedom (OIF), or Operation New Dawn (OND) receive a diagnosis of a mental illness, such as posttraumatic stress disorder (PTSD), anxiety, or depression.[4-6] Known factors associated with postdeployment mental disorders include combat exposure and associated psychosocial stressors.[7-9] Importantly, service members exhibit symptoms related to war zone stress and experience low quality of life even if they do not meet the diagnostic criteria for a mental disorder.[10] Furthermore, despite the prevalence and adversity of war zone–related stress, the majority of previous studies have not specifically investigated the impact of war zone–related stress, and even fewer have used quantitative questionnaires such as the Deployment Risk and Resilience Inventory (DRRI) to quantify perceived war zone–related stress.[11-14] Although mental health problems are highly prevalent in postdeployed military service members[15] and war zone–related stress has been discussed as a risk factor, the underlying pathomechanisms remain poorly understood.

Furthermore, approximately 12% to 35% of OEF, OIF, and OND veterans have sustained a mild traumatic brain injury (mTBI).[16-19] Evidence suggests that mTBI is not only a highly prevalent comorbidity but is also considered a potential risk factor for the development of mental disorders. In fact, service members who have sustained mTBI have a significantly increased risk for developing PTSD[1,16,20-22] and depression.[1,23,24] Moreover, they exhibit poorer neurocognitive functioning, worse long-term recovery,[25] and more severe neurological impairment[26,27] compared with those who have not sustained mTBI. However, it is unknown whether comorbidity with mTBI modulates a possible association between war zone–related stress and alterations of brain structure and neuropsychological functioning. A better understanding of the outcomes of war zone–related stress on brain microstructure and function is critical for improving long-term health and quality of life of military service members returning from theaters of war.

Magnetic resonance imaging (MRI) provides a noninvasive way to study brain alterations as it allows for the in vivo, 3-dimensional investigation of brain macrostructure and microstructure.[28] Neuroimaging studies have linked neuropsychiatric disorders, including PTSD and mTBI, to

macrostructural brain alterations.[29] However, although an association between diagnoses and abnormal brain structure has been established, research on the outcomes of war zone–related stress on brain structure is sparse. Combat exposure has been found to be associated with lower volume of limbic or limbic-associated gray matter (GM) regions, such as the amygdala,[30] hippocampus,[31,32] orbitofrontal gyrus,[33] posterior insula,[34] ventromedial prefrontal cortex, and dorsal anterior cingulate cortex.[35] Of note, although lower limbic GM volumes have been associated with PTSD symptom severity and extent of alcohol use, other disorders commonly seen in this population have previously not been considered.

Diffusion-weighted MRI (dMRI) has been shown to be sensitive to subtle microstructural brain alterations associated with neuropsychiatric disorders, such as PTSD and mTBI.[29] Complementary to volumetric measures, dMRI has the potential to reveal alterations in tissue composition (eg, glial changes[36-38] and atrophy[39]) and tissue morphologic changes (eg, alterations in dendritic arborization[40-42]), thereby providing insight into underlying pathomechanisms. Although most research to date has focused on the microstructure of connecting white matter (WM) fiber tracts,[43-46] studies on the limbic GM microstructure are sparse. Importantly, to our knowledge, no study to date has investigated the association between combat exposure and limbic GM diffusion, although limbic GM constitutes an essential neuroanatomical correlate of mental and neuropsychological functioning as suggested previously by volumetric studies[31,32,47] of limbic system structures in postdeployed veterans. The aim of this study is to investigate (1) whether war zone–related stress is associated with microstructural alterations in limbic GM independent of mental disorders, (2) whether associations between war zone–related stress and limbic GM microstructure are modulated by a history of mTBI, and (3) whether alterations in limbic system GM microstructure are associated with neuropsychological functioning.

Methods

This cohort study was approved by the institutional review board of human studies research at the Veterans Affair Boston Healthcare System and all participants provided written informed consent. The study follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for observational studies.

Participants

The Translational Research Center for TBI and Stress Disorders (TRACTS) study is a longitudinal prospective cohort study that aims to assess and track the potential outcomes of psychologically and physically traumatic experiences related to military deployment over time. Inclusion criteria for enrollment into the TRACTS study were (1) age 18 to 65 years, (2) male sex, and (3) service in OEF, OIF, or OND, or scheduled deployment.[48] Exclusion criteria were (1) history of neurological illness other than TBI; (2) current diagnosis of schizophrenia spectrum or other psychotic disorders; (3) current diagnosis of bipolar or related disorders; (4) active suicidal and/or homicidal ideation, intent, or plan requiring crisis intervention; and (5) cognitive disorder due to general medical condition other than TBI. Parameters with potential impact on cerebral microstructure and resilience such as eduction, socioeconomic status, race and ethnicity were collected via interview.

Of the first 384 consecutively recruited veterans, 273 consented to share their data with investigators outside of TRACTS. Of these 273 veterans, several had to be excluded from the present study for the following reasons: predeployment status (ie, military service members who had not yet been deployed to combat zones) (15 participants), postenrollment report of neurological disorders (ie, history of meningitis, or brain surgery; 4 participants), history of moderate or severe TBI (15 participants), and exposure to neurotoxic chemicals or anoxia (30 participants). Another 26 cases did not pass the rigorous quality control of the MRI data, and 15 cases had missing clinical variables required for this study. The selection process is summarized in **Figure 1**.

Diagnostic and Clinical Assessment

Assessment of Psychiatric Disorders

The nonpatient edition of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I/NP)[49] was used to detect the presence of psychopathological disorders. The following modules were administered: module D, mood disorders; module E, substance use disorders; module F, anxiety disorders (except PTSD); module H, eating disorders; and module I, adjustment disorders. Presence and history of PTSD were determined according to the Clinician-Administered PTSD Scale (CAPS)[50] using the *Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition)* (*DSM-IV*) standard scoring rule.[51]

Assessment of mTBI

The Boston Assessment of TBI-Lifetime (BAT-L)[17] was conducted to diagnose lifetime history of TBI. Specifically, mTBI was defined by the following criteria: loss of consciousness for 30 minutes or less, posttraumatic amnesia for 24 hours or less, or altered mental status for 24 hours or less.[17]

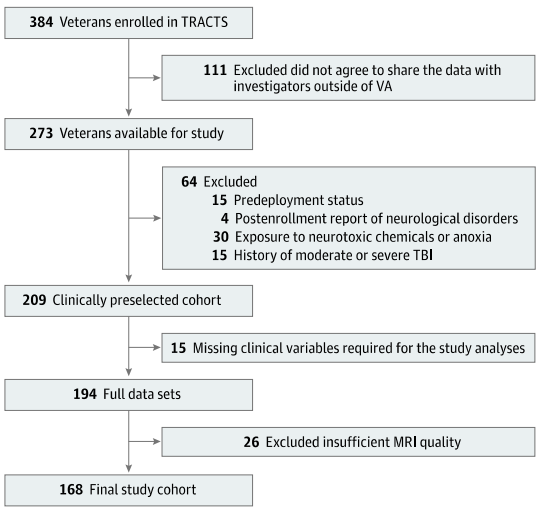
Assessment of War Zone–Related Stress

Stressors associated with deployment to war zones were assessed via selected scales from the DRRI.[52] The combat experiences and aftermath of battle scales were used to assess perceived war zone–related stress. Both DRRI subscales (called hereafter DRRI-combat and DRRI-aftermath) consist of 16 questions concerning combat or war zone–related events. The DRRI-combat uses a 5-point Likert frequency scale (0 = never; 4 = daily or almost daily), yielding a maximum possible score of 64 points. The DRRI-aftermath scale uses a binary response (0 = no and 1 = yes), resulting in a maximum score of 16 points. Higher scores on both the DRRI-combat and DRRI-aftermath scale reflect greater exposure to deployment-related stressors.

Assessment of Functional Outcome

The World Health Organization Disability Assessment Schedule II (WHODAS II)[53] is a 36-item self-report questionnaire that was designed to measure disability associated with all physical and mental disorders including cognition, mobility, self-care, getting along, life activities, and participation. Functional impairments within the last 30 days are rated on a 5-point scale (0 = no disability;

Figure 1. Flowchart of the Cohort Selection Process



MRI indicates magnetic resonance imaging; TBI traumatic brain injury; TRACTS, Translational Research Center for TBI and Stress Disorders; VA, Veterans Affairs.

4 = extreme disability/cannot do). A total disability score is calculated by summing the scores across all subscales. Higher scores reflect greater disability.

The Neurobehavioral Symptom Inventory (NSI) is a 22-item self-report questionnaire used to assess postconcussion symptoms following TBI.[54] Tested symptoms include sensory, affective, vestibular, and cognitive symptoms, rated on a 5-point Likert scale (0 = none; 4 = very severe). Higher scores reflect more severe neurobehavioral symptoms.

According to identified limbic regions with GM diffusion alterations, the Digit Span Total Score (DSTot) and the Coding Raw Scores[55] were chosen from the comprehensive neuropsychological test battery,[48] as they reflect functions of the frontal and temporal lobe (ie, verbal short-term memory performance and processing speed). In addition, Stroop Inhibition (Stroop-IN) and Inhibition/Switching (Stroop-IS) Total Error Scaled Scores[56] were selected to assess more specifically executive or attentional control functions associated with the prefrontal and cingulate cortex,[57-62] whereby higher Total Error Scaled Scores reflect impaired response inhibition and vice versa.

Assessment of Hypervigilance

The CAPS criterion D was used to assess the frequency and intensity of symptoms of hypervigilance at postdeployment, including difficulty sleeping, irritability, difficulty concentrating, hypervigilance, and exaggerated startle response. Answers were rated on a 5-point Likert scales ranging from 0 to 4 and summarized in a total score, resulting in a maximum score of 40 points.

Effort Testing

Performance validity was assessed via the Verbal Multiple Symptom Validity Test (MSVT).[63] The MSVT evaluates verbal learning, memory, and response consistency. It is composed of the subtests immediate recall, delayed recognition, consistency of responding across immediate recall, and delayed recognition, as well as paired associates and free recall. Study participants who failed the MSVT (8 participants) were excluded from the post hoc analyses as they were suspected of potential reduced effort or malingering.

MRI Acquisition and Data Processing

MRI of the brain was performed using a 3-Tesla TIM Trio scanner (Siemens Healthineers) located at the VA Medical Center in Boston, Massachusetts. T1-weighted (T1w) gradient-echo sequence parameters were field of view, 256 mm; 256 sections; inversion time, 1.000 ms; repetition time, 2.530 ms; echo time, 3.32 ms; flip angle, 7°; and isotropic resolution, 1 × 1 × 1 mm^3. dMRI was acquired using a single-shot, echo-planar sequence with a twice-refocused spin-echo pulse and the following parameters: field of view, 256 mm; 64 axial sections with no intersection gap; 60 gradient directions with a b-value of 700 seconds/mm^2; 10 b = 0 volumes; repetition time, 10 000 ms; echo time, 103 ms; and isotropic resolution, 2 × 2 × 2 mm^3.

dMRI data were corrected for motion and eddy current distortions via affine registration to the first b = 0 volume using FMRIB Software Library, version 5.1 (The Oxford Centre for Functional MRI of the Brain).[64] Brain masks were created and manually edited in 3D Slicer, version 4.5 (Surgical Planning Laboratory, Brigham and Women´s Hospital).[65] Automated segmentation of brain regions from the T1w data was performed using FreeSurfer[66] (version 5.1.0).[67]

Free water (FW)–corrected diffusion tensor measures were derived from dMRI using in-house software.[68] FW imaging separates the dMRI signal into 2 compartments: a FW and a tissue compartment. FW in the brain is expected where water molecules are free to diffuse, such as in cerebrospinal fluid, and large extracellular spaces. We calculated a fractional anisotropy of tissue (FA_T) map from the FW-corrected diffusion tensor, which serves as a more accurate marker of anisotropy in brain tissue than the conventional FA measure. To obtain diffusion metrics for selected regions, FreeSurfer parcellation label maps were nonlinearly registered from the individual T1w space to the respective dMRI space to obtain diffusion metrics for selected regions. Eight limbic and

paralimbic GM regions in each hemisphere were evaluated—that is, cingulate gyrus, amygdala-hippocampus complex, parahippocampal gyrus, entorhinal cortex, lateral and medial orbitofrontal cortex, insula, and temporal pole. Amygdala and hippocampus were combined into 1 region of interest to ensure higher parcellation accuracy.[69] For each of these 8 bihemispheric regions of interest (16 in total), the mean of the diffusion measure (FA_T) was calculated.

Statistical Analysis

Statistical analyses were performed using SAS statistical software version 9.4 (SAS Institute). Means and SDs are displayed for continuous parameters, while absolute and relative frequencies are provided for noncontinuous variables. Generalized linear models for repeated measures using the restricted maximum likelihood approach and an unstructured covariance matrix across brain regions were used to evaluate the association of war zone–related stress with regional diffusion measures. The following parameters were selected a priori as covariates: age, diagnosis of current PTSD, mood, anxiety, substance use disorder, and weight-corrected lifetime drinking history (LDH). To test the outcomes of mTBI on the association between war zone–related stress and limbic GM diffusion, the number of lifetime mTBIs was added as fixed effect as well as modifier to the main effect.

Post hoc analyses were conducted to test for associations between diffusion measures that were significantly associated with war zone stress and neurobehavioral symptoms (NSI), cognitive (DSTot, Coding Raw Score, and Stroop IN/IS Total error scaled score), and disability (WHODAS). Participants who failed error testing (MSVT) were excluded from the post hoc analyses. Age, diagnosis of current PTSD, mood, anxiety, and substance use disorder, and LDH were included as covariates.

A false discovery rate[70] was set at 5% to correct for multiple comparisons, using the Benjamini-Hochberg method. A corrected 2-tailed $P < .05$ was considered significant. Data were analyzed December 2017 to September 2021.

Results

The final study cohort encompassed 168 male veterans with a mean (SD) age of 31.4 (7.4) years. Sample demographic characteristics are summarized in **Table 1**. The vast majority of participants were White (130 participants [77%]), followed by 24 Hispanic participants (14%) and 11 Black participants (6%) (Table 1). Although the level of education was balanced across the cohort (mean [SD] 13.9 [1.9] school years), potentially relevant differences were observed for the family status as only 38% (64 participants) were married or cohabiting.

Associations of War Zone–Related Stress With Limbic GM Diffusion

In the cohort of 168 veterans, greater war zone–related stress as assessed by DRRI-combat and DRRI-aftermath was negatively associated with FA_T in the bilateral cingulate gyri (DRRI-combat left: $P = .002$, partial $r = -0.289$, $df = 167$; DRRI-combat right: $P = .02$, partial $r = -0.216$, $df = 167$; DRRI-aftermath left: $P = .004$, partial $r = -0.281$, $df = 167$; DRRI-aftermath right: $P = .02$, partial $r = -0.219$, $df = 167$) and bilateral medial orbitofrontal gyri (DRRI-combat left medial orbitofrontal cortex: $P = .02$, partial $r = -0.222$, $df = 167$; DRRI-combat right medial orbitofrontal cortex: $P = .005$, partial $r = -0.256$, $df = 167$; DRRI-aftermath left medial orbitofrontal cortex: $P = .02$, partial $r = -0.214$, $df = 167$; DRRI-aftermath right medial orbitofrontal cortex: $P = .005$, partial $r = -0.260$, $df = 167$; DRRI-aftermath right lateral orbitofrontal cortex: $P = .03$, partial $r = -0.196$, $df = 167$). Notably, these associations were observed while controlling for age, PTSD diagnosis, mood disorder, anxiety disorder, and substance use disorder as well as LDH.

Moreover, a negative association was observed between DRRI-aftermath and the right lateral orbitofrontal gyrus FA_T and right parahippocampal gyrus FA_T ($P = .03$, partial $r = -0.191$, $df = 167$). In contrast, a positive association was found for both measures of war zone–related stress and FA_T in

the right amygdala-hippocampus complex (DRRI-combat: $P = .005$, partial $r = 0.254$, $df = 167$; DRRI-aftermath: $P = .02$, partial $r = 0.223$, $df = 167$). Results are summarized in **Table 2**.

Outcomes of mTBI on the Association of War Zone–Related Stress and Limbic GM Diffusion

The majority of veterans (109 of 168 [64.9%]) sustained at least 1 mTBI before or during deployment. They reported having experienced a mean (SD) of 1.38 (2.23) mTBIs throughout life with a maximum number of 18 mTBIs. Number of lifetime mTBIs was not associated with limbic GM diffusion and did not mediate the association between war zone–related stress and limbic GM FA_T.

Table 1. Demographics, Deployment-Related Factors, and Postdeployment Characteristics of Study Cohort

Variable	Participants, No. (%) (N = 168)
Demographics	
Age, mean (SD), y	31.36 (7.43)
Race and ethnicity	
Asian	2 (1.19)
Black	11 (6.55)
Hispanic	24 (14.29)
Unknown	1 (0.60)
White	130 (77.38)
Education mean (SD), school years	13.86 (1.93)
Married or cohabitating	64 (38.10)
Deployment factors	
OEF, OIF, or OND deployments, mean (SD), No.	1.4 (0.7)
Other stressful deployments, mean (SD), No.	0.41 (0.79)
Duration of OEF, OIF, or OND deployments, mean (SD), mo	13.82 (8.45)
Service in army branch	101 (60.12)
DRRI total score, mean (SD)	
Combat experience (DRRI-combat)	17.31 (12.02)
Aftermath exposure (DRRI-aftermath)	7.65 (4.7)
Military mTBIs, mean (SD), No.	0.63 (1.53)
Wounded or injured in combat	35 (20.83)
Postdeployment characteristics	
Time since last deployment, mean (SD), mo	40.07 (29.98)
Disorder	
Mood	35 (20.83)
Anxiety	28 (16.67)
PTSD diagnosis	112 (66.67)
Clinician-Administered PTSD Scale, mean (SD)[a]	78.35 (22.9)
Substance use disorder	25 (14.88)
Lifetime drinking history, weight corrected, mean (SD)	1790.6 (2092.7)
Lifetime TBIs, mean (SD)	1.38 (2.23)

Abbreviations: DRRI, Deployment Risk and Resilience Inventory; mTBI, mild traumatic brain injury; OEF, Operation Enduring Freedom; OIF, Operation Iraqi Freedom; OND, Operation New Dawn; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a] Clinician-Administered PTSD Scale score was evaluated for 112 veterans who met diagnostic criteria for postdeployment PTSD.

Association of Limbic GM Diffusion and Functional Outcome

Results of the post hoc analysis of diffusion and associated functioning are shown in **Table 3**. Decreased FA_T in the cingulate gyri and the medial orbitofrontal cortex was associated with impaired response inhibition (Stroop-IS left cingulate: $P < .001$, partial $r = -0.440$, $df = 151$; Stroop-IS right cingulate: $P < .001$, partial $r = -0.372$, $df = 151$; Stroop-IS left medial orbitofrontal cortex: $P < .001$, partial $r = -0.304$, $df = 151$; Stroop-IS right medial orbitofrontal cortex: $P < .001$, partial $r = -0.340$, $df = 151$; Stroop-IN left cingulate: $P < .001$, partial $r = -0.421$, $df = 151$; Stroop-IN right cingulate: $P < .001$, partial $r = -0.300$, $df = 151$; Stroop-IN left medial orbitofrontal cortex: $P = .01$, partial $r = -0.223$; $df = 151$; Stroop-IN right medial orbitofrontal cortex: $P < .001$, partial $r = -0.343$, $df = 151$), but with better frontotemporal functions (DSTot left amygdala-hippocampus complex: $P < .001$, partial $r = -0.574$, $df = 159$; DSTot right amygdala-hippocampus complex: $P < .001$, partial $r = 0.645$, $df = 159$; short-term memory left amygdala-hippocampus complex: $P < .001$, partial $r = 0.570$, $df = 156$; short-term memory right amygdala-hippocampus complex: $P < .001$, partial $r = 0.633$, $df = 156$). In contrast, impaired response inhibition and improved verbal short-term memory

Table 2. Association of War Zone–Related Stress and Limbic Gray Matter Diffusion Using Fractional Anisotropy of Tissue

	Combat exposure (DRRI-combat)				Aftermath exposure (DRRI-aftermath)			
	Left hemisphere		Right hemisphere		Left hemisphere		Right hemisphere	
Region	Partial r[a]	FDR corrected P value	Partial r[a]	FDR corrected P value	Partial r[a]	FDR corrected P value	Partial r[a]	FDR corrected P value
Amygdala-hippocampus complex	0.158	.09	0.254	.005[b]	0.136	.14	0.224	.02[b]
Cingulate gyrus	−0.289	.002[b]	−0.216	.02[b]	−0.281	.004[b]	−0.219	.02[b]
Entorhinal cortex	0.020	.80	0.121	.21	−0.023	.88	0.049	.65
Insular cortex	−0.058	.52	−0.057	.52	−0.138	.14	−0.061	.57
Lateral orbitofrontal cortex	−0.081	.43	−0.151	.10	−0.083	.41	−0.196	.03[b]
Medial orbitofrontal cortex	−0.222	.02[b]	−0.256	.005[b]	−0.214	.02[b]	−0.260	.005[b]
Parahippocampal gyrus	−0.059	.52	−0.166	.08	−0.009	.97	−0.191	.03[b]
Temporal pole	−0.089	.40	0.053	.52	−0.224	.39	−0.003	.97

Abbreviations: DRRI, Deployment Risk and Resilience Inventory; FDR, false discovery rate.

[a] The higher the partial r, the stronger the linear association between 2 variables. Positive values represent positive correlations, and negative values represent negative or inverse correlations.

[b] Denotes significant results.

Table 3. Association of Limbic Gray Matter Diffusion Using Fractional Anisotropy of Tissue and Cognitive Functioning

					Stroop inhibition			
	Digit Span Total Score		Coding Raw Score		Total error scaled score		Switching total error scaled score	
Region	Partial r[a]	FDR corrected P value	Partial r[a]	FDR corrected P value	Partial r[a]	FDR corrected P value	Partial r[a]	FDR corrected P value
Left amygdala-hippocampus comp	0.574	<.001[b]	0.570	<.001[b]	0.443	<.001[b]	0.483	<.001[b]
Left cingulate gyrus	−0.393	<.001[b]	−0.330	<.001[b]	−0.421	<.001[b]	−0.440	<.001[b]
Left lateral orbitofrontal cortex	−0.058	.74	−0.006	.94	−0.036	.79	−0.044	.78
Left medial orbitofrontal cortex	−0.202	.02[b]	−0.193	.03[b]	−0.223	.01[b]	−0.304	<.001[b]
Left parahippocampal gyrus	0.042	.80	0.007	.94	0.059	.79	0.013	.95
Right amygdala-hippocampus comp	0.645	<.001[b]	0.633	<.001[b]	0.500	<.001[b]	0.518	<.001[b]
Right cingulate gyrus	−0.290	<.001[b]	−0.237	.007[b]	−0.300	<.001[b]	−0.372	<.001[b]
Right lateral orbitofrontal cortex	0.041	.80	0.024	.76	−0.038	.79	0.005	.95
Right medial orbitofrontal cortex	−0.263	.002[b]	−0.262	.003[b]	−0.343	<.001[b]	−0.340	<.001[b]
Right parahippocampal gyrus	−0.001	.99	−0.021	.79	−0.032	.79	−0.103	.35

Abbreviation: FDR false discovery rate.

[a] The higher the partial r, the stronger the linear association between 2 variables. Positive values represent positive, and negative values represent negative or inverse correlations.

[b] Denotes significant results.

performance and processing speed were associated with increased FA_T in the amygdala-hippocampal region (**Figure 2**). No significant associations were revealed for limbic GM diffusion and (postconcussion) neurobehavioral symptoms or disability (eTable 1 in the Supplement).

Association of Limbic GM Diffusion and Hypervigilance State

Hypervigilance at postdeployment was positively associated with FA_T in the amygdala-hippocampal region (left: $P < .001$, partial $r = 0.325$, $df = 165$; right: $P < .001$, partial $r = 0.309$; $df = 165$) and negatively associated with FA_T in the cingulate gyri (left: $P < .01$, partial $r = -0.253$ $df = 165$; right: $P < .01$; partial $r = -0.261$ $df = 165$). The results are summarized in eTable 2 in the Supplement.

Discussion

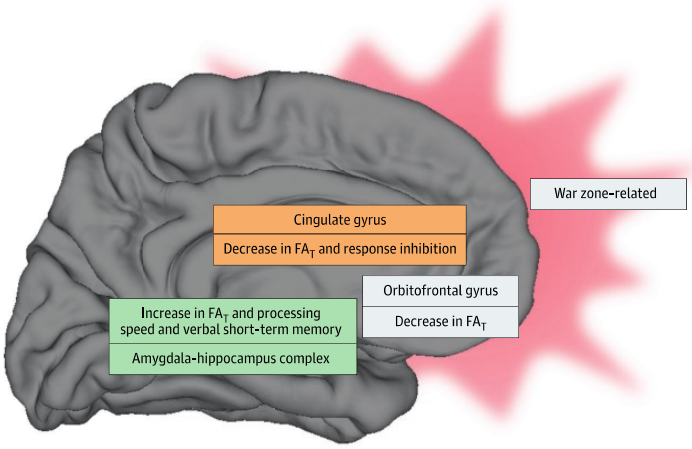
This cohort study found an association between war zone–related stress and microstructure of limbic GM in veterans. Importantly, these findings were observed while accounting for common comorbidities, including PTSD, mood, anxiety, and substance use disorder. Furthermore, mTBI had no significant effect on the association between war zone–related stress and limbic GM microstructure. Finally, characteristics of limbic GM microstructure were associated with cognitive performance including verbal short-term memory, processing speed, and response inhibition, while no associations with overall disability and neurobehavioral symptoms were found.

War Zone–Related Stress and Limbic GM Diffusion

This study revealed a co-occurring decrease and increase in limbic GM FA_T. More specifically, the greater the experienced war zone–related stress, the lower FA_T was in the cingulate gyri, the medial orbitofrontal gyri, the right lateral orbitofrontal gyrus, and the right parahippocampal gyrus. Moreover, the greater the experienced war zone–related stress, the higher the FA_T in the amygdala-hippocampus complex. Importantly, associations described previously were independent of diagnosis of mental disorders as well as mTBI.

The interpretation of diffusion measures in GM is challenging as data linking diffusion to histologic profile is sparse.[37,71-74] FA in GM likely reflects diffusion properties of the main GM components (ie, astroglia, neurons, and axons). For example, a study in mice reveals an association

Figure 2. Model of Structural Brain Alterations and Associated Cognitive Function in the Context of War Zone–Related Stress



War zone–related stress was associated with decreased fractional anisotropy of tissue (FA_T) in cingulate and orbitofrontal cortex, as well as increased FA_T in the amygdala-hippocampus complex. Limbic gray matter FA_T measures were further associated with cognitive function (ie, impaired response inhibition as well as improved verbal short-term memory and processing speed). Taken together with the current literature on functional imaging in posttraumatic stress disorder, we propose that the observed diffusion alterations may result from a functional shift from frontolimbic toward mesial temporal structures (shift of functional demand from cingulate and orbitofrontal regions toward mesial temporal regions).

between decreased FA and decreased astrocyte density in the hippocampus.[71] Astrocytes play a crucial role in complex brain functions, such as neurotransmitter homeostasis and blood-brain barrier maintenance.[75] Moreover, a decrease in astrocytes predisposes the brain to inflammatory states.[75,76] Another dMRI study[77] in a murine model of Parkinson disease found an association between decreased FA_T in the substantia nigra and neuronal loss. Taken together, the association between war zone–related stress and decreased FA_T in the cingulate, orbitofrontal gyri, and right parahippocampal gyrus may potentially be due to a decrease in astrocytes and/or neurons.

Interestingly, a positive association was found for greater war zone–related stress and higher FA_T in the amygdala-hippocampus complex. Increased FA_T in GM and WM has been associated with neuroplastic remodeling.[72,73] In rodents, long-term learning and memory tasks induced an FA increase, particularly in limbic system structures such as the amygdala, the parahippocampal gyrus, and the cingulate cortex, which correlated with an increase in a myelin marker (myelin basic protein) in the histological analysis.[72,73] The authors[72,73] hypothesized that oligodendrocytes, which form the myelin sheaths in the central nervous system, produced more myelin basic protein postlearning to allow for the required flow of information. Taken together, findings of our study suggest regional differences in the association between war zone–related stress and alterations in GM microstructure that may be due to neurodegenerative and neuroplastic processes.

Association Between Limbic GM Diffusion and Functional Outcome

We observed improved frontotemporal brain functions (ie, short-term memory and processing speed) in association with increased FA_T in the amygdala-hippocampal complex (Figure 2), which is in line with previous studies that report a link between processing speed and hippocampal FA.[78-80] Our study results further suggest an association between improved frontotemporal brain functions with war zone–related stress.[81] It has been hypothesized that hypervigilance and readiness to respond to combat-related challenges may be advantageous adaptations to the highly stressful environment. However, it may be challenging to transition back to normal states of alertness when returning from deployment. The chronic activated state may consequently lead to a functional overuse of frontotemporal brain functions. This overuse may induce neuroplastic changes as suggested by the increased FA_T in the amygdala-hippocampal complex[73] found in this study. This hypothesis is supported by our finding of a significant association between hypervigilance state at postdeployment and increased FA_T in the amygdala-hippocampal complex.

At the same time, we observed impaired prefrontal-cingulate functions (response inhibition) in association with lower FA_T in prefrontal regions. This is thought to result from functional (emotional or stress) overuse of mesial temporal structures, as described previously, which may, in turn, lead to poorer performance in other cognitive tasks, a phenomenon called interference.[82-84] Interference or shift of emotion and cognition has previously been described in patients with PTSD[85] as well as in veterans. More specifically, impaired memory consolidation and reduced learning speed were observed in veterans returning from OEF, OIF, or OND.[86,87] Of note, those functions are typically associated with the prefrontal-cingulate cortex,[86-89] regions that have been found to have lower FA_T in association with war zone stress in the current study.

Taken together, we hypothesize that the outcomes of war zone–related stress outlast deployment, leading to attentional interference with increased functional use of mesial temporal structures and decreased use or impaired retrieval of prefrontal-cingulate functions. This hypothesis is further supported by functional MRI studies,[90] which have reported a hypoconnectivity of mesial temporal and prefrontal brain regions under conditions of stress. The functional interference may, in turn, lead to microstructural adaptations, reflected by increased FA_T in the amygdala-hippocampus complex and decreased FA_T in the cingulate and orbitofrontal gyri (Figure 2). This biological adaptive response may potentially, in addition to preexisting biological predisposition for deployment, mean that service members with outstanding processing speed and verbal short-term memory might be more likely to join the military and to be deployed.

No significant associations were found between limbic GM diffusion and more general measures of functional outcome following mTBI (ie, the WHODAS and NSI). We thus speculate that abnormalities in the limbic system may need to be more severe to cause impairments in everyday functioning. Furthermore, the observed limbic alterations may represent a minor contributor to everyday functioning as assessed using WHODAS and NSI, whereas the individual comorbidities may be the main drivers of the functional impairment.

Limitations

Our study has limitations. We investigated a representative subsample of OEF, OIF, or OND veterans[48] and we accounted for common comorbidities in the statistical analysis. However, we used dichotomous variables based on the *DSM-IV* classifications to account for the presence of psychopathologic disorders. Future studies should consider using dimensional assessments of psychopathologic disorders, to further investigate the spectrum of psychopathologic disorders. Furthermore, we did not account for service branch, race, or socioeconomic status,[91-98] which might be of importance for resilience, stress exposure, management, and rehabilitation and should be considered in future analyses. The vast majority of participants were White, followed by Hispanic and Black participants (Table 1). Although the level of education was balanced across the cohort, potentially relevant differences were observed for the family status as only 38% were married or cohabiting. A further limitation is that this study was limited to male participants only. The cross-sectional design of this study further limits the interpretation of our findings as well as the identification of additional factors associated with risk and causal relationship between war zone–related stress and alterations in limbic GM may not be drawn. Moreover, we did not differentiate between the amygdala and hippocampus as we aimed for the highest possible accuracy in the segmentation. Previous research of imaging data has demonstrated that the use of the combined amygdala-hippocampus complex represents a methodologically more rigorous and accurate approach of segmentation using FreeSurfer.[69] Against the background of our study findings, future studies should strive to retest our hypothesis on manually segmented limbic GM. Additionally, although all interviews were conducted by doctoral level psychologists, their administration at long-term follow-up might have been inevitably biased by participant subjective memory and reporting. Of further note, multishell dMRI data would have improved the FW model fit but was not available in the study. In addition, the analysis of GM is highly sensitive to misalignment of the diffusion space and T1 space, which may have caused inflation in the FW measure. Despite the FW-correction, the FA_T measures remain unspecific and can only serve as a gross estimation of the underlying microstructure.

Conclusions

In this study, war zone–related stress was associated with alterations in limbic GM microstructure, which, in turn, were associated with cognitive function independent of the diagnosis of mental disorders and mTBI commonly observed in this population. Taken together, findings from this study suggest that alterations in limbic GM microstructure may underlie the deleterious outcomes of exposure to war zone–related stress. Thus, military service members exposed to war zone–related stress may benefit from early therapeutic intervention even in the absence of a diagnosed mental disorder.

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SUPPLEMENT.

eTable 1. Association of Limbic Gray Matter Diffusion (FAT) and Disability/Neurobehavioral Symptoms

eTable 2. Association of Limbic Gray Matter Diffusion (FAT) and Hypervigilance State

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