A multi-modal structural neuroimaging study of the amygdala in PTSD

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ABSTRACT

Posttraumatic stress disorder (PTSD) is a devastating condition that is often associated with dysfunction in the amygdala's structure and function. In this manuscript, we explored both the structure and metabolic activity of the amygdala in the form of volumetric subnuclei analyses, single-proton magnetic resonance spectroscopy of the amygdalohippocampal region, and diffusion weighted imaging of the uncinate fasciculus, a major axon tract that connects the prefrontal cortex (by way of the subgenual anterior cingulate cortex) to the amygdala, in a cohort of combat veterans with and without PTSD. Each of these veterans has a monozygotic twin who has never been exposed to combat. This allowed us to look for potential vulnerability effects in the amygdala that may have predisposed our sample of combat veterans to develop PTSD, as well as what amygdalar abnormalities are acquired as a result of trauma or having PTSD. We found preliminary evidence to suggest that there is a vulnerability effect of the cortical amygdala volume such that smaller cortical amygdala volumes may be a sign of potential vulnerability to developing PTSD following a major traumatic event. Our diffusion tractography study in the uncinate fasciculus indicated that decreases in fractional anisotropy, mean diffusivity, and radial diffusivity may be a neural signature related to combat exposure, with a trend towards fractional anisotropy being a vulnerability factor to the development of PTSD. These diffusion results correlated with mood disturbances that are often seen in PTSD. No significant results emerged from our spectroscopy findings. Potential implications for individuals like our participants who underwent combat trauma, as well as implications for any individual with posttraumatic stress disorder, are discussed.

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GENERAL INTRODUCTION

Posttraumatic Stress Disorder

Posttraumatic stress disorder (PTSD) is a psychiatric disorder affecting people who are exposed to actual or threatened death, serious injury or sexual violence. Symptoms of PTSD include the experience of recurrent, involuntary and distressing recollections, nightmares, and flashbacks of the traumatic event. Patients also avoid trauma-related cues and experience negative alterations in thinking and feeling. Patients with PTSD also have signs and symptoms of hyperarousal and have increased startle responses, suggesting an abnormality in the hypothalamic-pituitary adrenal axis (HPA axis; American Psychiatric Association, 2013). The debilitating effects of PTSD are a social, economic, and public health concern: for example, individuals with PTSD are at increased risk of suicide, which is as high as 13% in one study of 431 veterans (Jakupcak et al., 2010; Sareen et al., 2007). Further, one study of homeless individuals found that 12.3% of those who were homeless were combat veterans, whereas combat veterans themselves make up less than 1% of the population (Henry, Cortes, and Morris, 2013). This implies that the experience of combat and the PTSD that could develop place a differential burden on those who have experienced it. Furthermore, the prevalence of PTSD among veterans ranges from 11 to 30% based on the area of service. While a cornerstone symptom of posttraumatic stress disorder (PTSD) is the recurrent, involuntary, and distressing recollections, nightmares, and flashbacks, people with PTSD share many symptoms with mood and anxiety disorders such as emotion regulation issues and persistent negative thoughts about themselves, the world, and other people (American Psychiatric Association, 2013). In addition, many people with a history of psychological trauma report difficulties concentrating and paying

attention, so much so that the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) includes difficulty concentrating as a symptom of the illness (American Psychiatric Association, 2013). Those with PTSD may struggle to establish and maintain healthy social connections due to their heightened emotional reactivity and irritability (American Psychiatric Association, 2013). In fact, research by Sippel et al. (Sippel et al., 2015) demonstrated that individuals with PTSD exhibited increased levels of interpersonal conflict and decreased social support compared to individuals without PTSD. Such difficulties in relationships can further contribute to feelings of isolation and exacerbate the overall distress experienced by individuals with PTSD.

The Neuroanatomy of PTSD: A Brief Introduction

The hypervigilance, exaggerated startle responses, anxiety, and overall heightened reactivity that nearly all individuals with PTSD experience in some form has led neuroscientists to investigate the autonomic nervous system in individuals with PTSD, and later the limbic system in the brain, which are closely tied together. The sympathetic nervous system (SNS) plays a significant role in the physiological response to stress and the regulation of the "fight-or-flight" response, as well as the parasympathetic nervous system (PNS) that acts as the SNS's counterpart. In PTSD, there is evidence of dysregulation in the SNS, leading to heightened arousal and reactivity to traumatic reminders (American Psychiatric Association, 2013). Individuals with PTSD often exhibit increased sympathetic activity reflected by heightened heart rate, blood pressure, and cortisol levels, even in the absence of immediate threat or danger (Schnurr & Jankowski, 1999). Often activated in tandem with the SNS is the hypothalamic-pituitary-adrenal axis (HPA axis), which ultimately releases hormones such as norepinephrine (otherwise known as adrenaline) and cortisol. Researchers have found abnormalities in noradrenergic and glucocorticoid responses in individuals with PTSD, though the actual

directionality of those abnormalities often varies for reasons that are not yet known but are likely multifaceted (Southwick et al., 1999). Moreover, studies utilizing measures such as skin conductance response (SCR) and heart rate variability (HRV) that largely measure PNS activity, have demonstrated abnormalities in autonomic functioning (Pole et al., 2009; Rabe et al., 2008).

The limbic system, in turn, is a complex network of brain structures involved in emotional processing, memory formation, and motivation. It works with the SNS and PNS to regulate heart rate, respiration rate, blood pressure, and gastric motility, among other things. In addition, because of its role in "fight or flight" behaviors, it becomes heavily involved in emotions, connecting sensory information with emotional responses, and integrating cognitive and emotional processes. The key structures within the limbic system include the amygdala, hippocampus, hypothalamus, and parts of the thalamus and prefrontal cortex such as the anterior cingulate cortex and the ventromedial prefrontal cortex (American Psychiatric Association, 2013).

The amygdala is a subcortical region of the brain involved in the processing and regulation of emotions, particularly fear and threat responses. It plays a critical role in the formation and consolidation of emotional memories, including those associated with traumatic events (LeDoux, 2000). The amygdala also interacts with other regions of the brain, such as the prefrontal cortex, to modulate emotional responses and facilitate the expression of appropriate behaviors in response to emotional stimuli (Phelps & LeDoux, 2005). It is often considered the "hub" for limbic functioning, since almost all key regions in the central nervous system have a direct or second-order connection to the amygdala (Hariri, 2015). It interacts extremely closely with the hippocampus: situated in the medial temporal lobe immediately next to the amygdala, the amygdala serves as the hippocampus's salience detector, allowing it to play a key role in the

encoding, consolidation, and retrieval of episodic and declarative memories (Eichenbaum, 2000). Additionally, the hippocampus is involved in contextual processing and the differentiation of familiar and novel environments, which contributes to the regulation of emotional responses and the ability to adapt to changing circumstances (Fanselow & Dong, 2010). For this reason, this study focuses in-depth on the amygdala.

Background: The Amygdala in Traumatic Stress Disorders and other Psychiatric Illness

The amygdala is a brain region commonly known for its role in gating fear, arousal, and anxiety responses (LeDoux 1996; Sander et al. 2003), making it a key brain region to study in the presence of a psychiatric illness. The amygdala is involved in the assessment of potential threat and/or biologically relevant ambiguity (Nutt & Malizia, 2004; Pitman et al., 2001; Rauch & Foa, 2006). Hyper-responsivity of the amygdala to threat-related stimuli, with deficient top-down regulation over the amygdala by the medial prefrontal cortex, is thought to trigger fight-or-flight states, hypervigilance, and an exaggerated startle response (Nutt & Malizia, 2004; Pitman et al., 2001; Rauch & Foa, 2006). The first human neuroimaging studies of the amygdala focused on fear and anxiety because a large corpus of anatomical, behavioral, and electrophysiological literature in animals and humans described a central role for the amygdala in fear and anxiety states. Over time, the conception has broadened such that neuroimaging studies began to demonstrate greater amygdala responses to not just fearful stimuli, but any stimuli that possess an emotional valence or are novel to the participant (Goldstein, 1992; LeDoux, 1996). For example, studies followed demonstrating that amygdala activity also increased to positively valenced faces (Breiter et al., 1996; Canli et al., 2002) and scenes (Hamann et al., 2002), and that novelty detection was an independent function of the amygdala, distinct from its established role in processing emotional

stimuli. Swanson and Petrovich (1998) defined the amygdala as neither a structural nor a functional unit and highlighted converging evidence to suggest that the amygdala is an evolutionarily conserved structure. Earlier, research on structural organization of the amygdala in different amniotic vertebrates revealed a common pattern of organization, along with shared functional roles, and recent studies have shown a homology between the amygdalae of higher order animals (such as mammals, birds, or reptiles), as well as in amphibious species (see Pabba, 2013, for a comprehensive review). For example, an automated meta-analysis generated using Neurosynth (Yarkoni et al., 2011) reveals that studies tagged with the keywords 'fear' and/or 'anxiety' consistently reveal activation near the amygdala in animal models. In rats who underwent trauma in infancy, social play activated corticotropin-releasing hormone (CRH)-expressing neurons in the amygdala. Further, this study showed aberrant functional connectivity of pleasure/reward and fear circuits: using diffusion tensor imaging tractography, researchers found increased structural connectivity of the amygdala to the medial prefrontal cortex in these rats (Bolton et al., 2018).

Classic histology studies in post-mortem human tissue and behavioral studies in animals have shown that the amygdala is composed of discrete nuclei with distinctive characteristics (Blair & Fanselow, 2014). The amygdala contains two primary anatomical subdivisions: the basolateral (BLA) and centromedial (CMA) amygdala. The BLA receives and contains primarily excitatory (glutamatergic) projection neurons that resemble cortical pyramidal cells as well as dopaminergic neurons, whereas CMA contains primarily inhibitory (GABAergic) projection neurons that resemble medium spiny cells of the striatum, as well as a smaller amount of dopaminergic neurons (Swanson & Petrovich, 1998; Xiang et al., 2008). The BLA is partitioned into lateral and basal subnuclei, both of which play important roles in emotion and motivation. Principal neurons of the BLA receive inputs from and project to multiple brain regions that relay

sensory, motivational, and mnemonic information into the amygdala (Aggleton & Young, 2000). The BLA contains bidirectional excitatory inputs to the ventromedial prefrontal cortex (vmPFC), an area that is key to top-down regulation over limbic system activation, the thalamus, and the hippocampus which is critical for integrating memories. The BLA also has excitatory projections to the entorhinal cortex, which aids in memory integration. It also receives neuromodulatory afferent signaling from the locus coeruleus (LC). The BLA then projects to the CeA, which projects back to the LC for further neuromodulation. Given that LC is a major region for norepinephrine and dopamine production, it underscores the amygdala's involvement in sympathetic and parasympathetic activity (Aggleton & Young, 2000). Inputs to the BLA arrive at modifiable glutamatergic synapses on the dendrites of BLA neurons, which can undergo associative plasticity, and these projections continue to more medial or central nuclei of the amygdala. The passage of information from the basal nuclei to the central nuclei is regulated by small clusters of GABA-ergic inhibitory cells, called the intercalated cell masses. The intercalated cell masses attenuate the glutamatergic signal from the BLA directly and indirectly, decreasing the excitatory output which triggers autonomic components of fear and novelty response (Aghajani et al., 2016). If this inhibition is reduced, as it may be in patients with smaller amygdala, the fear response and novelty response signal could be amplified, causing pathological dysregulation of autonomic cues in response to neutral sensory input. The CMA is partitioned into medial (MeA) and central (CeA) subregions, where the CeA is especially involved in regulating fear-motivated defensive behaviors. The CeA is traditionally considered the final output station of the amygdala and, importantly, its GABA-ergic neurons provide feed-forward inhibition within the amygdala (Lee et al., 2013; Partridge et al., 2016). The central nuclei then carry the signal downstream to evoke the hallmark autonomic responses to fear,

arousal, and novelty by projecting to the hypothalamus, pons, periaqueductal gray, and medulla (Zhang et al., 2021). Dopaminergic neurons are also present in smaller amounts throughout the amygdala to aid in neuromodulatory signaling. Epigenetic PCR studies have shown that dopamine receptor 2 mRNA is the most abundant in the basal nucleus, meaning that D2 is produced more in the basal nucleus than any other nucleus in the amygdala. Levels of D4 mRNA were highest in the basal and central nuclei (Xiang et al., 2008).

In addition, increased activation in the dorsal amygdala (including the central and medial amygdala) is associated with elevated signs and symptoms of arousal in response to acute threat (e.g., Pavlovian threat cues; Cheng et al., 2006, 2007; Knight et al., 2005; Kragel & LaBar, 2016; LaBar et al., 1998; van Well et al., 2012; Wood et al., 2014). It has also been shown that delivery of orexin (typically delivered from the lateral hypothalamus to multiple areas of the central nervous system) enhances fear expression, while applying orexinergic antagonists to the central nucleus of the amygdala reduces fear expression (Salehabadi et al., 2020). In human research into mood and anxiety disorders, one study found that the expression of mRNA for dopaminergic transmission is greater for individuals with mood disorders in the basal part of the amygdala (Xiang et al., 2008).

Figure 1. Major amygdala subnuclei and their afferent and efferent connections in mood, anxiety, and traumatic stress disorders.

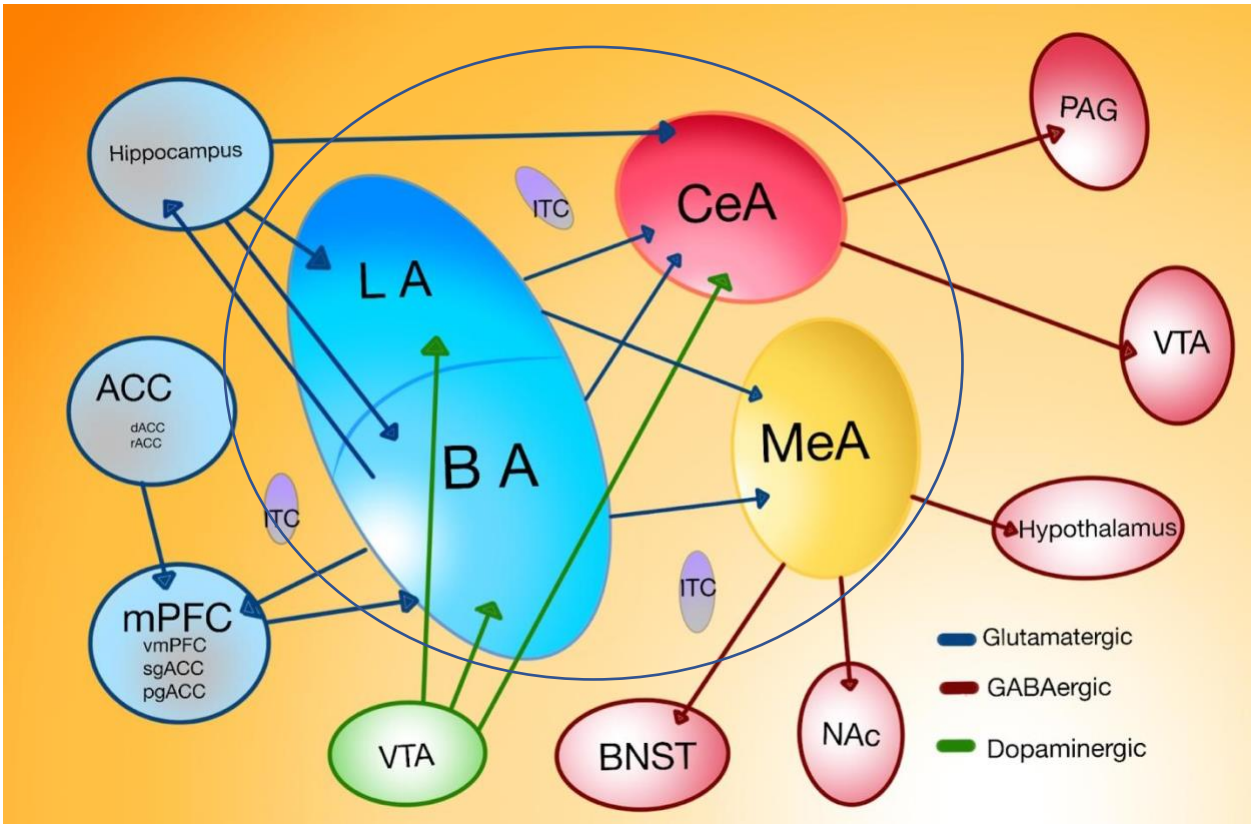


Figure 1. A diagram showing the afferent and efferent flow through the major nuclei of the amygdala, depicting both the centromedial complex (central amygdala: CeA and medial amygdala: MeA, red and yellow) and the basolateral complex (basal nucleus: BA and lateral nucleus: LA, blue) as well as some major functions of efferent connections. Abbreviations: BF, basal forebrain; BNST, bed nucleus of the stria terminalis; GABAergic, gamma-aminobutyric acidergic; LC, locus coeruleus; MeA, medial amygdala; mPFC, medial prefrontal cortex; NAC, nucleus accumbens; PAG, periaqueductal gray; VTA, ventral tegmental area.

In animal models of mood, anxiety, and traumatic stress disorders, distinct amygdala subnuclei and their projections have shown differences that could eventually aid in isolating mechanisms of action for the disease states in humans. In human research into mood and anxiety disorders, one study found that the expression of mRNA for dopaminergic transmission is greater for individuals with mood disorders in the basal part of the amygdala (Xiang et al., 2008), which could be tested in humans using a volumetric study.

From a connectivity standpoint, several key afferent and efferent connections emerge between the amygdala and the cortex that are frequently shown to display abnormalities in mood, anxiety and traumatic stress psychopathology, particularly between the amygdala, the cingulate cortex, and the medial prefrontal cortex (mPFC) which is often further divided into regions such as the ventromedial prefrontal cortex (vmPFC, whose homologue in animals is typically the infralimbic cortex), dorsomedial prefrontal cortex (dmPFC), among others. Connections between the BLA and mPFC, as well as the BLA and hippocampus, support the inhibition of fear (Orsini & Maren, 2012; Senn et al., 2014). Specifically, infralimbic - BLA projection neurons have been shown to play a role in a critical circuit for fear extinction (Bukalo et al., 2015; Cho et al., 2013; Senn et al., 2014; Strobel et al., 2015; Vogel et al., 2016) making it integral to the understanding of disorders of anxiety and traumatic stress. For example, Maymon et al. (Maymon et al., 2020) exposed rats to the "shock and reminders" model of PTSD and tested for hyperarousal and depression-like behaviors 3 weeks later. They showed that by increasing levels of neuropeptide Y and endocannabinoids within the BLA (both of which can have anxiolytic effects in increased amounts; Abush & Akirav, 2013; Bortolato & Piomelli, 2008; Ganon-Elazar & Akirav, 2009, 2012, 2013) they were able to prevent both the hyperarousal and anhedonic behaviors in the exposed rats often seen in experiments that provoke fear behavior. In a different mouse anxiety model induced by chronic stress, Liu et al. (2020) found that projection neurons from the dorsomedial prefrontal cortex (dmPFC) to the BLA show dysfunction (rather than those efferently connected with dmPFC) such that there is increased presynaptic glutamate release. This is thought to be the prefrontal cortex's attempt to exert top-down regulation over an overexcited amygdala response to stress. To underscore this point, this same group found that excitatory signaling is correlated with the increased anxiety-like behavior in stressed mice.

Regarding the CeA's connectivity, work in monkeys using FDG-PET shows that activity in the CeA's efferent connections (such as the bed nucleus of the stria terminalis) may be associated with heritable individual differences in fear and anxiety (Fox et al., 2015). This same area appears to be involved in elevated fear and anxiety behaviors following threat exposure (Ressler, 2010; Shackman et al., 2017). The CeA is directly involved in autonomic functions such as heart rate and respiration via projections to areas such as the nucleus of the solitary tract (Ressler, 2010). The periaqueductal gray (PAG)-projecting cells in the CMA neurons are also known to trigger freezing behaviors in animal models (Viviani et al., 2011; Zhang et al., 2021). For mood disorders, reducing the expression of CRH, a hormone secreted primarily in the paraventricular nucleus of the hypothalamus and is implicated in the activation of the hypothalamic pituitary adrenal (HPA) axis, which regulates fight or flight responses, inside the CeA reversed trauma-induced anhedonia in rats without influencing other emotional measures in mouse models (Bolton et al., 2018). In addition, areas such as the bed nucleus of the stria terminalis and locus coeruleus work to control autonomic functions like heart rate and respiration, key in fight or flight responses (Zhang et al., 2021).

In addition to connectivity differences, models of anxiety and mood symptomatology show some consistent abnormalities in the metabolic activity of the subnuclei of the amygdala. Heightened metabolic activity in the CeA in rodents is associated with elevated defensive responses during sustained exposure to an unfamiliar testing cage (i.e., in the absence of intruder threat; Fox et al., 2008; Kalin et al., 2005).

The Current Study: A Roadmap

In posttraumatic stress disorder, the DSM-5 says that a traumatic or stressful event must have occurred, and psychological distress following exposure to a traumatic or stressful event is required, albeit quite variable depending on the diagnosis itself. In some cases, symptoms can be well understood within an anxiety- or fear-based context. However, many individuals who have been exposed to a traumatic or stressful event exhibit a phenotype in which, rather than anxiety- or fear-based symptoms, the most prominent clinical characteristics are anhedonia and dysphoric symptoms, externalizing, angry, and aggressive symptoms, or dissociative symptoms (Feeny et al. 2000; Malta et al. 2009; Harnett et al. 2021). It is not uncommon for an individual to present with some combination of the above symptoms (with or without anxiety- or fear-based symptoms). Due to PTSD's symptomatology, the amygdala has been studied in vivo in humans with PTSD as soon as the technology was available, and amygdala may be critical to our understanding of traumatic stress disorders, especially when studied in-vivo using neuroimaging. Neuroimaging has been an instrumental tool in understanding the structure, function and metabolic activity in both the amygdala and its surrounding connections to other regions of the brain. Therefore, the modes of analysis used in this work are structural magnetic resonance imaging (MRI) to understand the size of the amygdala, especially regarding its individual subnuclei and how its structure might be perturbed in PTSD patients compared to control patients. We also used diffusion weighted imaging (DWI) to understand how the amygdala's connections are disrupted in PTSD compared to controls, as well as single-proton magnetic resonance spectroscopy (MRS) to look at differences in metabolic activity in PTSD compared to controls. To better understand the origins of the abnormalities in the amygdala that researchers see in PTSD participants compared to controls, we implemented a twin design, discussed in detail in the chapter "The Origin of Abnormalities in PTSD."

AMYGDALA SUBNUCLEI FINDINGS IN PTSD

As expected from animal literature showing deviations of the connections between the BLA, CMA and the remainder of the cortex (specifically the cingulate and dmPFC), functional MRI (fMRI) has shown that resting-state fMRI brain voxel-wise functional connectivity of BLA and CMA complexes differed between PTSD and controls. In one such study, the PTSD group had stronger resting-state functional connectivity of the left BLA with the pregenual ACC (pgACC) and dorsomedial PFC (dmPFC) than the trauma-exposed control group (Brown et al., 2014). In the same study, the PTSD group had stronger resting-state functional connectivity between the right BLA and the dorsal ACC compared to the control group who does not have PTSD. It further showed that the trauma-exposed control group had greater resting-state functional connectivity between the right BLA and the left inferior frontal gyrus compared to the PTSD group.

In contrast to Brown et al., another study examined functional connectivity in adolescents with a history of PTSD and found that, compared to age and sex-matched controls with no history of significant trauma, adolescent PTSD patients had decreased right BLA connectivity with the ACC: the ACC cluster included the dorsal and ventral portions of the ACC and MPFC (Aghajani et al., 2016). This is the opposite pattern that was seen in adult trauma. Two studies on early life trauma have also shown that patients had increased left CMA connectivity with a cluster that includes the orbitofrontal and subcallosal cortices compared to controls (Aghajani et al., 2016; Grant et al., 2015). Another study found that stronger CMA connectivity with the orbitofrontal/subcallosal region was correlated with more severe PTSD symptoms (Aghajani et al., 2016) which implies that the OFC in these participants may be exerting increased top-down

control over the amygdala. In a dynamic causal functional connectivity study, individuals *without* early life stress (ELS) had fewer significant connections emerge from defined BLA-driven intra-amygdaloid paths. Individuals without ELS also had fewer significant connections between the orbitofrontal cortex and the CeA than the ELS group. The ELS group had a higher number of robust CeA-facilitated intra-and extra-amygdaloid paths compared to the non-ELS group. Negative causal paths (meaning an increase in activation in one region causes a decrease in activation in the other) from OFC/BA32 to BLA predicted modulation of threat among non-ELS, while a unique within-amygdala path predicted modulation of threat among ELS (Grant et al., 2015).

Regarding volumetric analyses, individuals with PTSD show smaller laminar and paralaminar nuclei compared to trauma-exposed controls, but larger left and right central, medial, and cortical nuclei ($p < .05$, false discovery rate corrected; Morey et al., 2020). This same study broke the participants' PTSD symptoms into their subcategories based on the DSM-5 and showed that reexperiencing, avoidance, and hyperarousal symptoms showed significant (FDR-corrected) positive associations with left central, left medial, and left cortical nuclei and right whole-amygdala volumes such that an individual with greater reexperiencing symptoms is more likely to have greater volumes in these nuclei.

Interestingly, relative to controls, adolescents with PTSD had smaller gray matter volumes in the right BLA and bilaterally in the CMA (Aghamohammadi-Sereshki et al., 2021). Further, in opposition to what has been seen in adult trauma compared to controls, one study in adolescents saw a reduction in the CMA (Oshri et al., 2019). One study indicated that a history of greater childhood maltreatment (total CTQ-25 score) was negatively associated with the right BLA, basal, and accessory basal amygdala, meaning that greater trauma corresponded to smaller

subnuclei volumes (Aghamohammadi-Sereshki et al., 2021). Two studies similarly found that higher adverse childhood experiences (ACE) scores are significantly related to reduced volume of the right BLA (Oshri et al., 2019; Veer et al., 2015). Further, the reduction in volume of the right basolateral amygdala was associated with increased anxiety, depressive symptoms, and alcohol use (Oshri et al., 2019).

Studying amygdala nuclei in vivo in humans has been a challenge because it requires both high field MRIs and hand parcellation of the subnuclei, which is labor-intensive, susceptible to rater error, and not easily applied to large samples of clinical interest. In addition, while animal research is vital for developing testable hypotheses in humans, animal models of disease can only capture a discrete subset of symptomatology that is seen in human psychiatric disease and cannot capture the entirety of an illness of this kind. However, a relatively new software (Saygin et al., 2017) allows for in-vivo human imaging of subnuclei: it visualizes and labels nine amygdala nuclei and twelve hippocampal subfields. By scanning postmortem brains at both high resolution (100-150μm) and using high MR field strength (7T; n = 10), an atlas was created from these labels using a recently developed algorithm based on Bayesian inference. This atlas can be used to automatically segment nine amygdala nuclei from a 3T resolution structural MR image. The new ex vivo atlas significantly outperformed estimations of the whole amygdala derived from the traditional segmentation. This new atlas creates the ability to explore the volume, function, and connectivity of the human amygdala and hippocampus with unprecedented detail. This adds to work already completed by FSL's Juliech pipeline (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Atlases/Juelich) which allowed segmentation into both BLA and CMA subnuclei only, based on a parcellation completed by Amunts et al. (2005).

The advent of automated subnuclei parcellation software that is adaptable to lower field MRI has allowed for the in-vivo testing of animal models of psychiatric disorders in large cohorts of human clinical research participants. Like what was seen in animal models of fear, stress, anhedonia, and trauma, we would expect to see the major amygdala complexes (basolateral and centromedial) differences in both structure and function, in addition to abnormalities in their afferent and efferent projections to and from the cortex. One would expect to find differences in connectivity between the mPFC and the amygdala, specifically in afferent projections to the BLA from the prefrontal cortex, as well as the efferent connections from the CeA to areas regulating HPA axis activation and autonomic functioning. As for the structure of the amygdala itself, since the amygdala is hyperactive in anxiety and mood disorders, one might expect either increases in volume and/or structural connections due to increased glutamatergic and/or dopaminergic metabolism (for example in the BLA), though it is also possible to see decreases in structure volume due to decreases in GABA-ergic neurons that could also increase functional activation of the amygdala (for example, in the CMA).

In short, the literature on amygdala subnuclei is a burgeoning field, especially when it comes to clinical research. Thus far, work in PTSD shows that there are altered functional pathways, as well as larger central, medial, and cortical nuclei in PTSD participants compared to controls. The goal of the present analysis is to examine how the subnuclei might vary in older adults with combat PTSD, and also utilize the twin study to establish abnormalities in PTSD participants that may have already been present before their trauma, as opposed to acquired abnormalities that followed the traumatic event.

SPECTROSCOPY FINDINGS IN PTSD

Magnetic resonance spectroscopy has been instrumental in understanding some of the metabolic activity in the amygdala as a whole volume, as well as the amygdalohippocampal region (depending on the spatial resolution of the scan) in posttraumatic stress disorder. A systematic review of 24 MRS studies that were performed between 1998 and 2017 showed that participants with PTSD have lower N-acetylaspartate (NAA) levels in the amygdalohippocampal region and anterior cingulate cortex, with and without atrophic change. While the exact function of NAA varies depending on the exact process being studied, it is often understood to be a marker of axonal density and viability, while decreases in NAA can be considered a marker of glial cell density and inflammatory activity, especially with increases in creatine and choline in the same region (Ousdal et al., 2018). These results have been shown to be related to symptomatology: one study for example found were decreased NAA levels in bilateral hippocampus in the PTSD group relative to the healthy control group and those NAA levels were negatively correlated with re-experiencing symptoms in PTSD (Ham et al., 2007). However, these results are not devoid of conflict: for example 71 non-PTSD age- and sex-matched control participants, all of whom had suffered the same earthquake about one year had increased creatine (Cr) concentration in the left amygdala, and increased myoinositol (mI) concentration in the right amygdala, compared to non-PTSD controls. The PTSD group showed significantly decreased volumes of bilateral amygdala compared to non-PTSD controls, but amygdala volumes were not correlated with metabolite concentrations. (Su et al., 2018)

Largely conflicting results, this 2018 review states, have been reported in choline-containing metabolites as well. With that said, some of these findings may be due to differences in analysis metrics. Another study echoed these results in patients with post-traumatic stress disorder and 50 gender-and age-matched controls, and they found that the values of NAA/Cr

ratios in hippocampus gyrus were significant lower in patients with post-traumatic stress disorder compared to controls, but there was no significant difference in the values of Cho/Cr in hippocampus between PTSD and controls (Guo et al., 2008). Research that does not use ratios with NAA or Cr is more conflicting (Quadrelli et al., 2018). This same review goes on to show that recent studies, using more advanced techniques and modern hardware, have shown evidence of glutamatergic dysfunction and differences in gamma-aminobutyric acid (GABA) levels in the brain of patients with PTSD. Myoinositol and creatine are largely unchanged in the majority of studies (Quadrelli et al., 2018).

In pediatric patients, the picture is more complicated: for example, 28 pediatric PTSD patients (11 boys, 17 girls) and 24 matched trauma-exposed control participants (9 boys, 15 girls) underwent magnetic resonance brain imaging and 1H-MRS of the bilateral amygdalae. The concentrations of N-acetyl aspartate (NAA), myoinositol (mI), total creatine (tCr) and total choline (tCho) in the right amygdala were significantly increased in PTSD patients compared with trauma-exposed control participants. There were significant group-by-age interactions in the left amygdala NAA and right amygdala mI concentrations: older pediatric patients with PTSD had higher left amygdala NAA concentration and younger patients had higher right amygdala mI concentration than trauma-exposed control participants. There was also a significant correlation between right mI concentration and time since trauma in PTSD patients. Finally, there was significant group-by-age interaction in the left amygdala volume; intragroup analysis revealed that the right amygdala volume was significantly lower than the left in the PTSD group, but not in the control group (Wang et al., 2019). This illustrates the multifaceted nature of NAA in the amygdalohippocampal region that requires future study to understand its role throughout the lifespan.

While the literature on spectroscopy in PTSD is quite mixed, in general findings have shown abnormalities in NAA, creatine and choline that could represent abnormal glial cell and inflammatory activity in the amygdala. It is less understood why results are largely mixed, and it is certainly not well understood how these differences in metabolic activity emerged in individuals with PTSD compared to controls.

DIFFUSION FINDINGS IN PTSD

Posttraumatic stress disorder (PTSD) is associated with decreased white matter integrity in the brain of tracts that connect the prefrontal cortex with limbic regions, referred to as the uncinate fasciculus (UF), connecting the subgenual regions of the cingulate cortex to the basolateral portion of the amygdala (Harnett et al., 2020).

Using diffusion weighted imaging (DWI), researchers have shown that abnormalities in the uncinate fasciculus are present in multiple age groups of individuals with PTSD and across multiple trauma types. For example, typhoon survivors with PTSD (n = 27), trauma-exposed controls (TEC) (n = 33), and healthy controls (HCs) (n = 30) underwent DWI and researchers found that decreases in integrity of the right UF differentiated the PTSD group and TEC group from the HC group. Further decreases in integrity of the left UF differentiated the PTSD group from the other two groups (Zhang et al., 2022). Another study backed up this claim, where trauma exposed participants with and without PTSD were identified to have significant disruption in white matter (WM) integrity as indexed by decreased fractional anisotropy (FA) in the uncinate fasciculus (UF) compared to the healthy controls. Significant negative correlations were found between total Clinician Administered PTSD scale (CAPS) lifetime clinical sub scores and FA values of PTSD participants in the right UF and an analysis between UF gray matter in the cingulate values found a negative correlation between the two in PTSD participants

(O'Doherty et al., 2018). In a group of police officers, officers with PTSD showed significantly less white matter integrity of the right uncinate fasciculus compared to officers without PTSD (Koch et al., 2017). In a study of Iraq and Afghanistan service members who had recently returned from deployment and did not meet criteria for clinical PTSD, correlations were found between left uncinate fasciculus' (UF) white matter tract integrity and total PTSD symptoms; the left UF and hyperarousal symptoms; right UF integrity and total PTSD symptoms; right UF integrity and hyperarousal symptoms; and left UF and startle during early extinction. The results indicate that compromise of UF tract frontal-limbic connections are associated with greater PTSD symptom severity and lower startle response during extinction (Costanzo et al., 2016).

Compared to a trauma exposed non-PTSD group, treatment-naïve PTSD showed lower fractional anisotropy accompanied by higher radial diffusivity and mean diffusivity in the left uncinate fasciculus and decreased white matter integrity was associated with increased PTSD symptom severity across all subscales of the CAPS (Suo et al., 2022).

To summarize, individuals with PTSD compared to control participants show decreased white matter integrity in the uncinate fasciculus compared to control participants, and we would expect to see this in our combat veterans as well. What is less well understood is both the origins of these abnormalities, and how these findings may change with different demographics of individuals with PTSD.

THE ORIGIN OF ABNORMALITIES IN PTSD

While neuroimaging can be instrumental in proposing potential mechanisms underlying some of the behavioral and neurological changes that can be seen in PTSD, MRI cannot determine what caused these abnormalities in brain activation: are they inherited and present as familial vulnerability factors before a person undergoes significant trauma, or do these

abnormalities develop after trauma has already taken place and PTSD develops? The most direct way to test for familial vulnerability factors is to study monozygotic twins in which one twin underwent a clinically significant trauma and one did not (Shin et al., 2011). If the structural or functional abnormality is also seen in the co-twins of the individuals with PTSD, that would suggest a familial vulnerability factor that predisposed the twins with PTSD to develop the condition. We will use a cohort of Vietnam combat veterans with and without PTSD, as well as their combat-unexposed identical co-twins without PTSD in the hopes of discovering the origin of these abnormalities in brain activation.

We have reason to believe that symptoms of PTSD may reflect familial vulnerabilities. For example, the heritability of PTSD is thought to be between 30 and 40% (Segman & Shalev, 2003; Yehuda et al., 2001). While scarce, some studies have suggested that the abnormalities that we see in neuroimaging in PTSD may be present in the individual before the traumatic event occurs, making them more vulnerable to developing PTSD in the future: for example, multiple studies have found that increased fMRI activation in the dorsal anterior cingulate cortex was a vulnerability factor for the development of PTSD in the future (Shin et al., 2009, 2011) and one study has shown that the amygdala and hippocampus's fMRI activations may be related to an individual's vulnerability to stress (Admon et al., 2009). Some evidence suggests that the white matter integrity of certain regions is heritable; results of one meta-analysis indicate nearly 70% heritability (Jahanshad et al., 2013) and there is a link between this metric and FKBP5 (Carballedo et al., 2013; Fani et al., 2014; van Zuiden et al., 2012). A second study examined DTI within 48 hours of a motor vehicle accident and found that decreased UF radial diffusivity predicted PTSD development and correlated with CAPS scores at follow-up post PTSD diagnosis, implying that the structural difference was already present (Hu et al., 2016). Integrity

of the UF shortly after a trauma occurred also predicted re-experiencing PTSD symptoms at six months post-trauma. These results were supported in an independent replication analysis by the same author (Fani et al., 2019). A longitudinal study found that white matter microstructure (assessed < 1-month post-trauma) of the uncinate fasciculus varied with acute posttraumatic stress severity, such that decreased integrity was related to greater posttraumatic stress symptoms (assessed < 1-month post-trauma). However, the test to determine whether UF predicted posttraumatic stress symptoms at 3 months post trauma was not significant. The results suggest white matter architecture of the prefrontal cortex amygdala network could play an important role in the development of trauma and stress-related disorders after the trauma has already occurred, but that effect may have already been present (Harnett et al., 2020).

On the other hand, some of the abnormalities in PTSD may be acquired: one study found that the prefrontal cortex fMRI activation during a script-driven imagery task was an acquired characteristic of PTSD (Dahlgren et al., 2017). Another longitudinal study imaged trauma exposed participants at one-month post-trauma and conducted clinical assessments at one- and six-months post-trauma. Probabilistic tractography was used to examine connectivity of select pathways. Logistic regression results indicated that, after accounting for acute stress symptoms and other clinical risk factors, the integrity of the uncinate fasciculus (UF) uniquely predicted the presence of posttraumatic anhedonia at six months post-trauma, but the test was not significant at one month, implying there may have been an acquired effect of PTSD on the participants. Together, these factors contributed to 76% of the variance in posttraumatic anhedonia. One study looked at gray matter in the pregenual anterior cingulate cortex using voxel-based morphometry in monozygotic twin pairs, such that one twin was a Vietnam War veteran (either with or without PTSD) and one twin was not. They found that decreases in gray matter in the PTSD group

compared to the control group were an acquired characteristic of PTSD (Kasai et al., 2008). One study that assessed participants before and after trauma: in a group of adolescent earthquake survivors who were imaged before the quake occurred, researchers found that the state anxiety level after the earthquake was positively associated with increased FA changes from before to after the earthquake in the left uncinate fasciculus, implying that changes in the UF occurred after the earthquake occurred in these survivors, and that change was positively correlated with increased state anxiety in those adolescents (Sekiguchi et al., 2014). This study provides more conclusive evidence for an acquired result, but it is only one study with a narrow demographic. UF abnormalities were also seen in a pediatric PTSD sample, such that children with PTSD showed a lack of development in the UF compared to healthy age-matched controls after the trauma has already occurred (Russell et al., 2021).

HYPOTHESES

Based on previous literature examining the amygdala and its connections in PTSD, and the existing literature on potential vulnerability vs. acquired characteristics of PTSD, we expected the following results from our analyses:

1. For our subnuclei analyses, we expected larger central, medial and cortical subnuclei volumes in combat veterans with PTSD compared to combat veterans without PTSD, and we expected increases in those subnuclei to be positively correlated with the participants' behavioral metrics for PTSD symptoms. Because the literature does not point us in any one direction on whether these effects would be vulnerability or acquired characteristics of PTSD, we approached this analysis with an exploratory mindset.

2. For our spectroscopy analysis, we expected to see decreases in NAA, as well as increases in creatine and choline in the amygdalohippocampal region of combat veterans with PTSD compared to combat veterans without PTSD. Because decreases in UF integrity may be directly related to metabolic activity within the amygdalohippocampal region of the brain, we also expect these results to be vulnerability factors that predispose an individual to developing PTSD in the future. We expected decreases in NAA to correspond with increases in PTSD symptoms and increases in creatine and choline to correlate positively with increases in PTSD symptoms.
3. For our diffusion analyses, we expected to see decreases in WM integrity marked by fractional anisotropy, medial, mean, axial, and/or radial diffusivity. Because the majority of the literature we found on diffusion analyses in the UF implied that UF differences were vulnerability characteristics that were present before the trauma occurred, we expected this result to be a vulnerability factor that predisposes an individual to developing PTSD in the future. We expected FA values to be inversely correlated with PTSD behavioral scores such that increases in PTSD symptoms corresponds to decreases in FA. We also would expect that increases in mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity (AD) would correspond to greater PTSD symptoms.

METHODS

MRI Acquisition in the Twins

Each participant underwent a multi-echo MPRAGE (MEMPRAGE) structural scan on a 3T Siemens Trio Tim scanner (128 sagittal slices; 1 mm3 isotropic voxels; TR=2530 ms; TE 1–4= 1.64/3.5/5.36/7.22 ms; flip angle 7°, bandwidth 651 Hz/Px), a T2-weighted structural scan. (TR=3200ms, TE=870ms, flip angle = 90°, bandwidth = 651 Hz/Px), a diffusion-weighted imaging scan with 60 diffusion directions and field-mapping (TR=8020ms, TE=83ms, FOV=256mm, voxel=2mm^3 isotropic, b value=0 for 8 frames, then 700 s/mm^2 for 62 frames), and two chemical-shift-imaging scans, with and without water suppression sagittal 15-mm thick slice across the amygdala-hippocampal area using a point-resolved spectroscopy (PRESS) sequence with an echo time of 30 ms (TR 1700 ms, 6 acquisitions (weighted), FOV 160 × 160 mm2, voxel size 6.7 × 6.7 × 15 mm^3) on a 3T Siemens Trio Tim scanner at the Martinos Center for Biomedical Imaging in Charlestown, MA.

Statistical Analysis

For all neuroimaging analyses, participants' data were submitted to a statistical analysis of variance (ANOVA) using linear mixed effects modeling. Each analysis was run separately. Exposed versus unexposed co-twins were treated as a repeated measure (i.e., main effect of combat exposure). In addition, we treated the twin pairs in which the combat exposed twin had PTSD as a separate group from the twin pairs in which the exposed twin never had PTSD.

A significant difference between these two groups of twin pairs would be consistent with a familial risk factor (as long as there was also no interaction between PTSD Diagnosis and Exposure). This finding would indicate that the combat-exposed twins with PTSD have the same

functional abnormality as their unexposed co-twins without PTSD. A significant PTSD Diagnosis x Exposure interaction reflecting an abnormality in only the exposed twins with PTSD would indicate an acquired sign of PTSD. Lastly, a significant main effect of combat exposure, meaning a significant difference between all combat-exposed twins as compared to all combat unexposed twins, in the absence of an interaction would suggest that the functional abnormality is associated with exposure to combat and unrelated PTSD.

Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) Correlations

For all significant findings, we will correlate the significant metric with the Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) from the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (American Psychiatric Association, 2000). The CAPS contains 3 subscales that assign a numerical value to the severity of symptoms surrounding the traumatic event itself (subscale B), negative mood and cognitions outside of the traumatic event (subscale C), as well as symptoms of impulsivity (subscale D). These three scores can be added together to get a total CAPS score, meant to represent the severity of posttraumatic stress symptoms for that person.

CAPS correlations will be run for the entire participant pool, for just the combat exposed participants, and to further investigate the potential for vulnerability or protective factors, we will run a final set of correlations with the combat unexposed participants using the CAPS scores from their combat exposed cotwin.

Amygdala Subnuclei Methods

Preprocessing

Both T1 and T2 weighted structural images were used to generate estimations of the amygdala subnuclei volumes. Automated segmentation and labeling of subcortical volumes and estimation of total intracranial volume from T1 images were performed using the FreeSurfer version 7.0 image analysis suite (http://surfer.nmr.mgh.harvard.edu) and its library tool recon-all. Amygdala subregion segmentation was performed using the function segmentHA_T1.sh (Saygin et al., 2017). Amygdala volumes from the left and right hemispheres were generated in each participant for the basal, lateral, accessory basal, central, medial, cortical, and paralaminar nuclei as well as the corticoamygdaloid transition area, anterior amygdaloid area, and the whole amygdala. Left and right substructures were analyzed separately. Protocols for quality control and image analysis were adapted from the subcortical and hippocampal subfields developed by the ENIGMA (Enhancing Neuroimaging Genetics Through Meta-analysis) Consortium (http://enigma.ini.usc.edu/protocols/imaging-protocols/). A priori subnuclei volumes were extracted for linear mixed-effects analyses using lme4 in R (R Core Team, 2021) with age, handedness, and total intracranial volume as covariates of no interest, per convention for analysis of volumetric data between patients and controls (see O'Brien et al., 2011, for an in-depth review).

Myriad researchers have, in the past, opted to use the whole amygdala volume as a covariate of no interest as well when doing subnuclei analysis (see Morey et al., 2020) while some have not. We opted to omit the whole amygdala volume. However, we recognize that subnuclei analyses are a burgeoning field and that standardized recommendations have not yet been made.

For our CAPS correlations, we know that raw amygdala volumes are collinear with regressors of no interest such as age, total intracranial volume, and handedness in our sample (O'Brien et al., 2011). Therefore, we residualized the mixed effects models by these covariates of no interest to make sure there was no undue influence of these variables.

MR Spectroscopy Methods

Preprocessing

The MRS raw data were exported and analyzed by LCModel (Version 6.3–1H) using a PRESS basis set yielded by the same sequence and the same parameters (Provencher, 1993). Eddy-current correction and water reference scaling were performed to improve spectral quality. Metabolites selected for further analysis were NAA, glutamate, creatine and choline. In accordance with current recommendations, only values with a fitting error < 15% will be accepted for further statistical analysis as described above (Provencher, 1993).

Diffusion Imaging Methods

Preprocessing

Motion and eddy current effects were reduced via registration to a b0 image as implemented using dt_recon in Freesurfer version 7.0 (http://surfer.nmr.mgh.harvard.edu) and metrics of fractional anisotropy, radial diffusivity, mean diffusivity and axial diffusivity were taken at each voxel throughout the whole brain. DWI images were processed in the TRACULA stream of Freesurfer to analyze the uncinate fasciculus' shape and size: briefly, the TRACULA pipeline used for this analysis began with FSL's eddy current correction (version 6.0.7), then the b0 images (including the T2 scan) were registered to the cross-sectional T1 image using

FMRIB's Linear Image Registration Tool (FLIRT), and tensor fitting using DTFIT was completed (fits a diffusion tensor model at each voxel). Finally, the uncinate fasciculus was reconstructed for each individual using FSL's bedpost-X pipeline. Monte Carlo Markov chains were used to estimate posterior probability distributions for the fractional anisotropy, radial diffusivity, mean diffusivity and axial diffusivity measurements. The weighted average for the left and right uncinate fasciculus' statistics were extracted and submitted to a linear mixed effects model as described above.

RESULTS

Demographics

26 male monozygotic twin pairs (n=52) underwent MRI scanning as described above:

Table 1.

Group means and standard deviations of combat-exposed Vietnam veterans with and without PTSD and their combat-unexposed, identical co-twins

	PTSD Pairs (N=13)				Non-PTSD Pairs (N=13)				Mixed Model ANOVA					
	Exposed		Unexposed		Exposed		Unexposed		Diagnosis		Exposure		Interaction	
Measure	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F	p	F	p	F	p
Age	61.23	6.34	61.23	6.34	62.73	3.86	62.73	3.86	0.58	0.45	--	--	--	--
Education	13.85	2.73	13.77	3.68	15.57	3.64	14.8	2.73	1.42	0.244	1.17	0.29	0.82	0.371
CAPS														
Re-experiencing	16.85	10.3	2.77	4.53	1.2	2.27	0.53	2.07	**34.38**	**<.001**	**19**	**<.001**	**15.3**	**<.001**
Avoidance	21.77	8.42	1.92	4.84	1.27	2.46	0.67	2.58	**70.34**	**<.001**	**37**	**<.001**	**32.2**	**<.001**
Hyperarousal	21	8.79	1.77	6.38	1.47	2.8	0.47	1.25	**52.18**	**<.001**	**33.4**	**<.001**	**26.2**	**<.001**
Total	59.62	23.5	6.46	15.2	3.93	6.72	1.67	3.89	**66.07**	**<.001**	**36.9**	**<.001**	**30.3**	**<.001**
MAST	5.5	5.9	3.54	5.49	2	2.62	2.6	3.74	1.79	0.191	<.01	n.s.	<.01	n.s
Medication use	0.83	0.39	0.15	0.38	0.13	0.35	0.14	0.36	**8.1**	**0.008**	<.01	n.s.	<.01	n.s.
CTQ	42.42	11.8	41.24	13.6	37.79	8.07	39.12	11.1	0.69	0.413	0.02	0.92	0.35	0.561
BDI	11.54	10	4	7.75	4.4	4.14	3.47	3.34	3.64	0.067	**6.55**	**0.02**	3.68	0.066

Table 1. Demographics table for n=52 participants in this cohort (13 PTSD pairs, 13 Control pairs). CAPS = Clinician Administered PTSD Scale. CTQ: Childhood Trauma Questionnaire. MAST: Michigan Alcohol Screening Test. BDI: Beck Depression Inventory.

Amygdala Subnuclei Results

Demographics

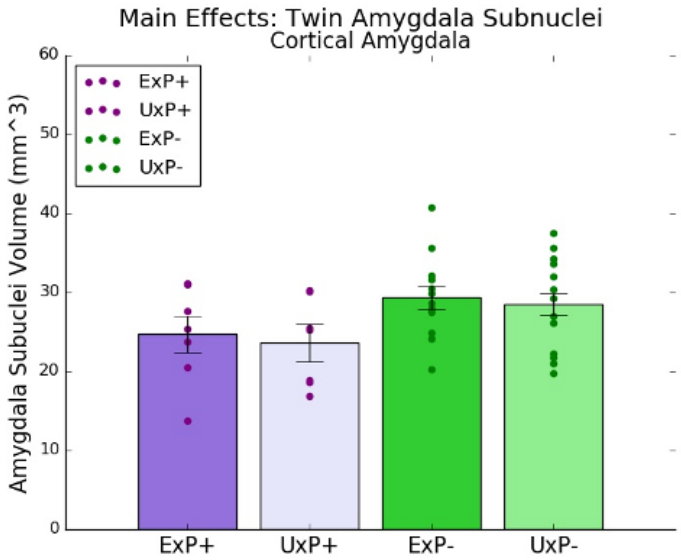
40 participants' data (13 Control pairs, 7 PTSD pairs) were able to be successfully reconstructed in Freesurfer for the subnuclei analysis. This means that (1) they had T1 and T2 scan data that passed a manual quality control assessment and were able to be successfully coregistered to each other in the surface and volume-based reconstructions. In addition, amygdalohippocampal reconstructions were able to be conducted for the amygdala subnuclei and

hippocampal subfields. 12 participants' (5 from the PTSD group with 3 combat exposed, 7 from the control group with 3 combat exposed) data had to be omitted due to either missing a T2 scan or poor quality/coregistration statistics between the T1 and T2 data.

Cortical Subnuclei

Our mixed effects ANOVA revealed a main effect of Diagnosis in the left cortical nuclei of the amygdala such that participants with PTSD and their co-twins have smaller left cortical nuclei than the participants without PTSD and their co-twins ($F(1, 39) = 5.04$, $p = .035$; Figure 1). The main effect of Exposure ($F(1,39) = .05$, $p = .825$) and the Diagnosis x Exposure interaction ($F(1,39) = .09$, $p = .773$) were not significant.

Figure 2. Main Effects: Cortical Amygdala



Note. This main effect of Diagnosis shows that individuals with PTSD and their co-twins have smaller left cortical amygdalae than individuals who did not get PTSD and their co-twins, implying smaller cortical nuclei may be a vulnerability factor to the development of PTSD in the future. ExP+ = twin exposed to combat and developed PTSD; UxP+ = the trauma unexposed cotwin of the person who developed PTSD; ExP- = twin exposed to combat who did not develop PTSD; UxP- = the trauma unexposed cotwin of the person who did not develop PTSD.

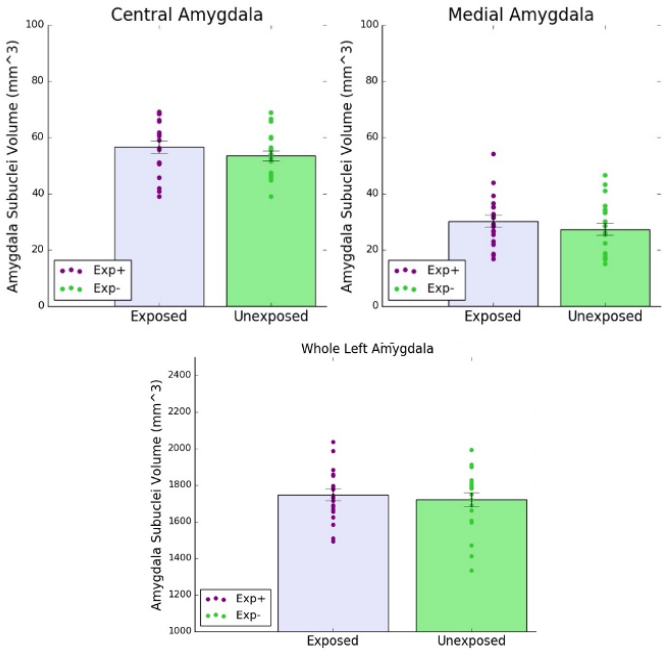
Medial Amygdala

Analyses revealed a main effect of Exposure in the left medial amygdala ($F(1, 39) = 34.93$, $p = .0294$) such that participants who were exposed to combat have larger left medial amygdalae than participants who were not exposed to combat. The main effect of Diagnosis ($F(1, 39) = 1.32$, $p = .2634$) and the Diagnosis x Exposure interaction ($F(1,39) = 2.80$, $p = .1148$) were not significant.

Central Amygdala

There was a main effect of Exposure in the left central amygdala ($F(1,39) = 10.96$, $p = .004$, Figure 2) such that participants who were exposed to combat have larger left central amygdalae than participants who were not exposed to combat. However, that effect was superseded by a Diagnosis x Exposure interaction effect in the left central amygdala ($F(1,39) = 5.05$, $p = 0.04$, Figure 3) such that control participants had significantly larger central amygdalae than their cotwins or the PTSD participants and their cotwins. The main effect of Diagnosis was not significant ($F(1,39) = 1.85$, $p = .1863$).

Figure 3**. Main Effects of Exposure: Twin Amygdala Subnuclei.**



From Figure 3 (below), it is apparent that there is a significant outlier in the participants who went to combat and developed PTSD (indicated by "ExP+" in Figure 3). When that outlier is removed, however, there are no new main effects or interactions that emerge in the central nucleus. All other effects remain significant after the outlier was removed from the central amygdala analysis (main effect of Exposure post removal: $F(1,39) = 17.96$, $p = .0005$; Diagnosis x Exposure interaction post removal: $F(1,39) = 14.16$, $p = .0015$).

Figure 4. Diagnosis by Exposure interaction effect in the left central amygdala subnuclei.

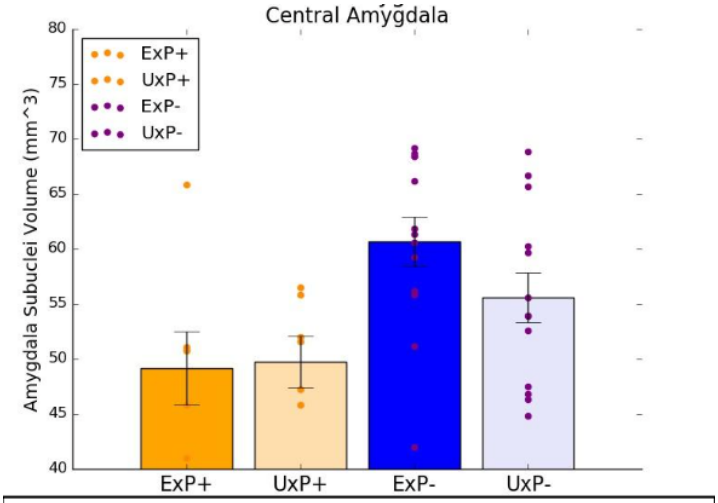


Figure 4. The Diagnosis x Exposure interaction effect in the central amygdala shows significant variability but shows that combat exposed veterans without PTSD have larger central nuclei than their cotwins and the PTSD twins.

Left Whole Amygdala

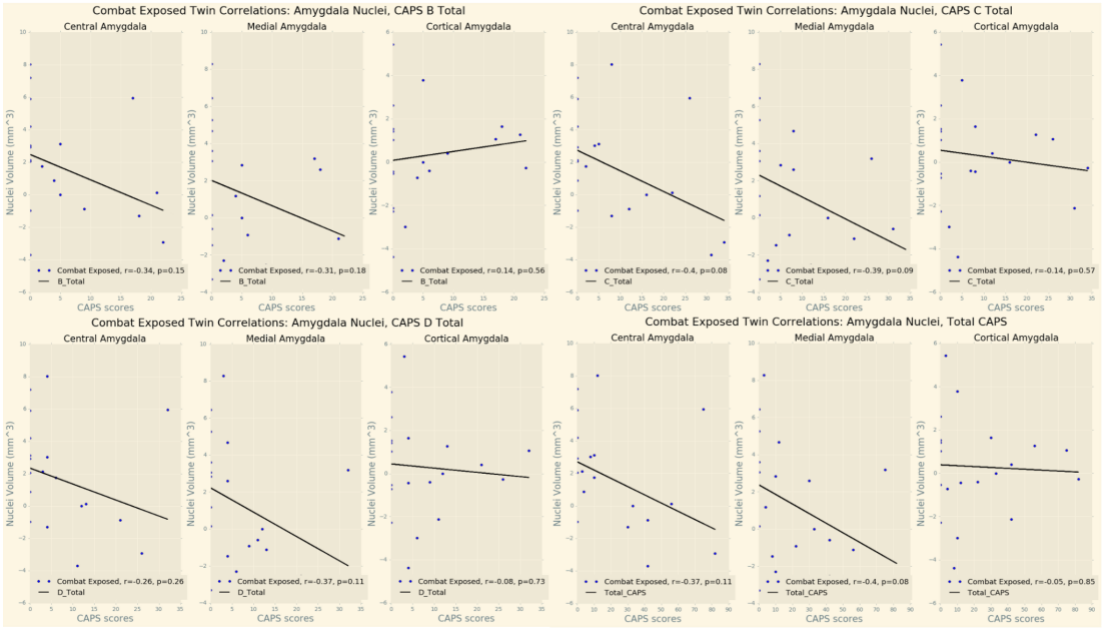
There is also a main effect of Exposure for the left whole amygdala, such that individuals who were exposed to combat had larger amygdala volumes in total than individuals who were not exposed to combat ($F(1,39) = 5.05$, $p = .04$). The main effect of Diagnosis ($F(1,39) = 1.07$, $p = .31$) and the Diagnosis x Exposure interaction ($F(1,39) = 3.10$, $p = .098$) were not significant.

Correlations between Subnuclei Volumes and Participants CAPS scores

No correlations between residualized subnuclei volumes and CAPS scores (subscores or total scores) were found to be significant when the whole group was included ($abs(r(39)) < .29$, $p > 0.22$), nor were there significant correlations for the group of participants who were exposed to combat ($abs(r(19)) < .4$, $p > 0.08$), nor when we use the CAPS scores for the combat exposed participants and correlate those with their combat unexposed cotwin's subnuclei volumes in their

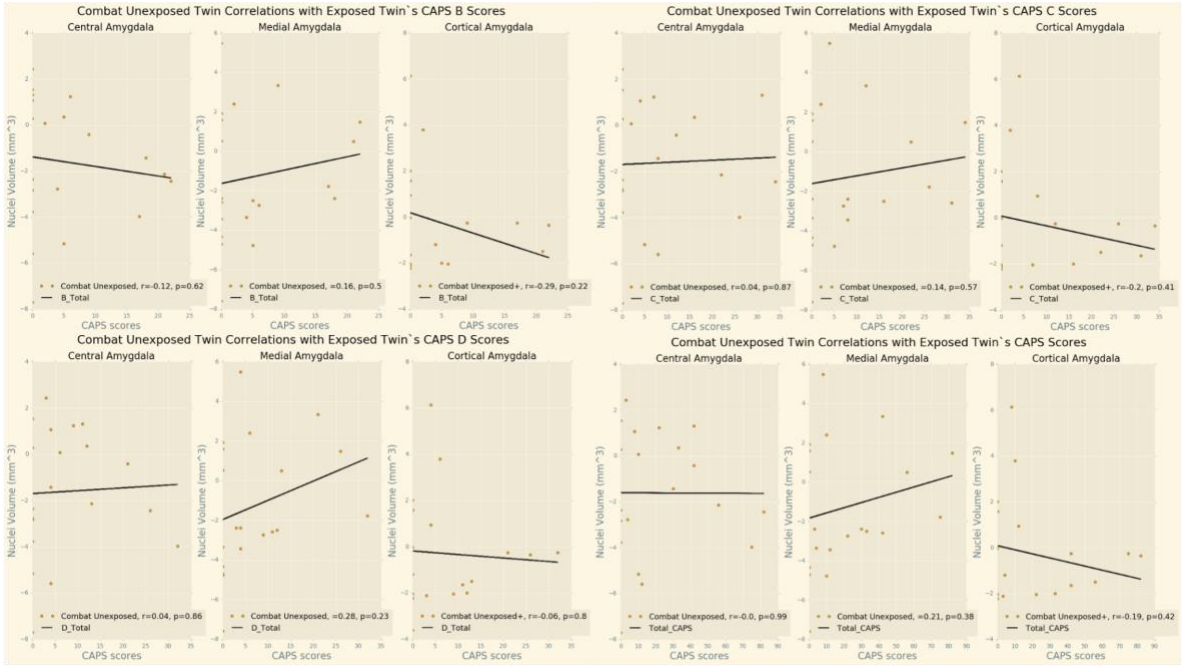
Ux co-twins (abs($r(19)$) < .4, p > 0.08, Figure 7). A full breakdown of correlations can be found in Figures 5-7. While many of the correlations trend in the opposite direction of what we would have expected, these trends appear to be driven by outliers in the data (Figures 5-7).

Figure 5. Amygdala subnuclei correlations with Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) for all combat exposed participants.



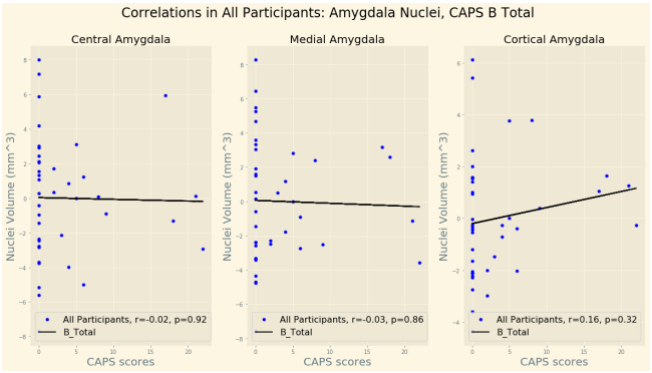
Note. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

Figure 6. Amygdala subnuclei of combat unexposed participants correlations with Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) from their combat-unexposed cotwin.



Note. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

Figure 7. Amygdala subnuclei correlations with the Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) for all participants with PTSD.

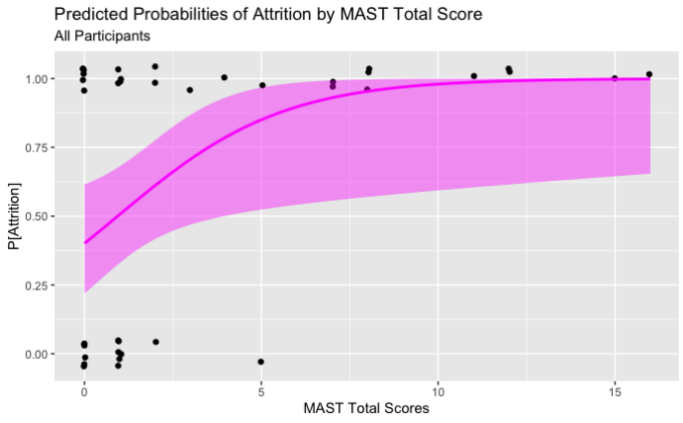


Note. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

Spectroscopy Results

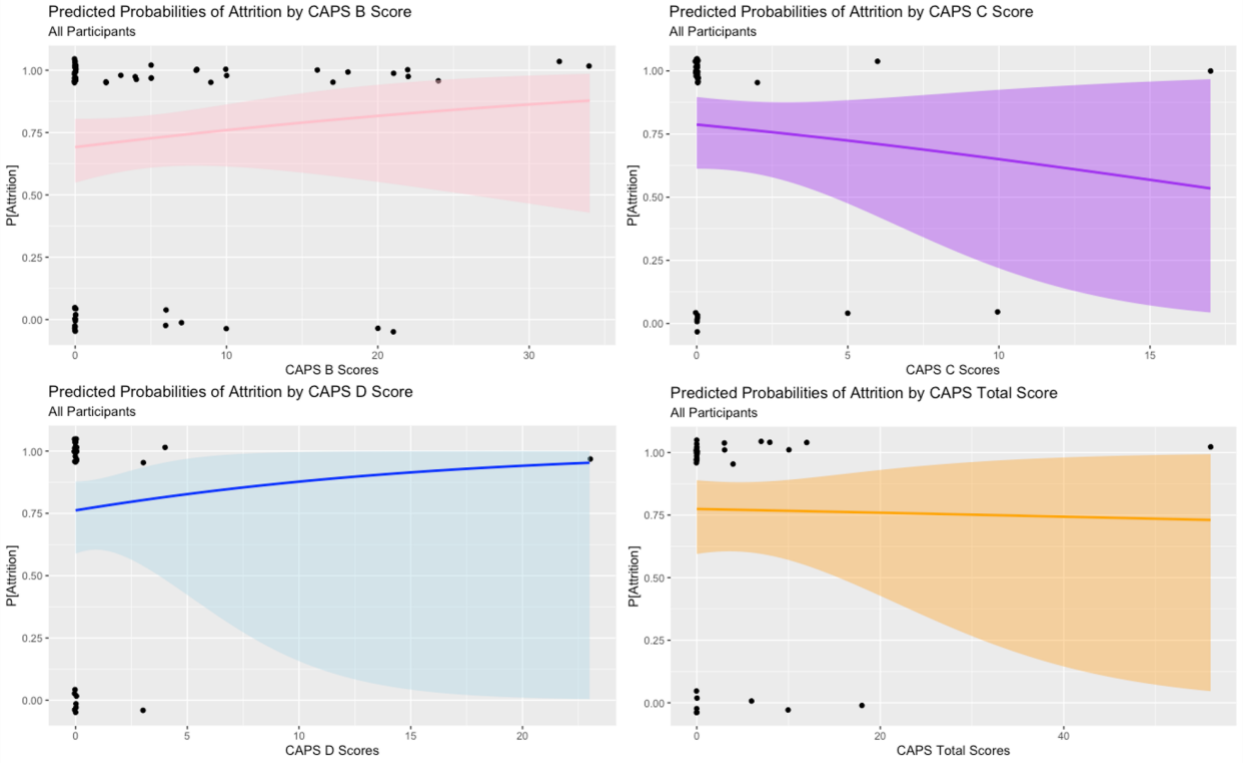
Because our MRS scan was the last scan of our imaging study, of the 52 participants who were imaged for this study, only 19 participants had usable spectroscopy data for analysis. This dropout did not substantially alter any demographics data listed above, and a test for selective attrition bias using logistic regression showed that no one group of individuals (ExP+, ExP-, etc.) drove that attrition (all *p*s > .3). In addition, for all participants, when we look only at participants exposed to combat, and when we look at combat unexposed participants' dropout rates using the combat exposed cotwin's CAPS scores, the relationship between CAPS scores did not predict a person's likelihood to drop out of the study before the MRS scan could be completed, nor did the Beck Depression Inventory (BDI; all *p*s > .25, Figures 9-11). However, the Michigan Alcohol Screening Test (MAST) did predict participant attrition for all participants (z=2.151, p = .0314; Figure 8).

Figure 8. Predicted Probabilities of Study Attrition based on the Michigan Alcohol Screening Test using logistic regression.



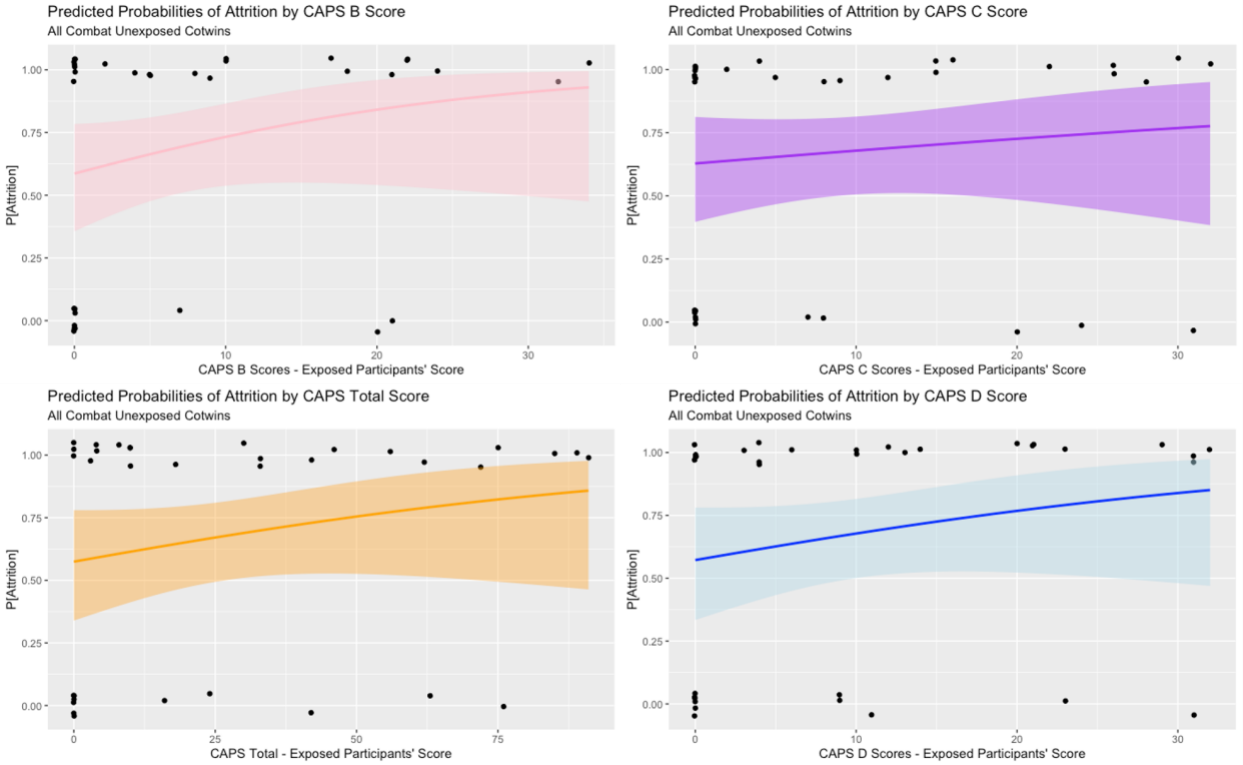
Note. MAST: Michigan Alcohol Screening Test.

***Figure 8*. Predicted Probabilities of Study Attrition based on the Clinician Administered Posttraumatic Stress Disorder scale for all participants using logistic regression.**



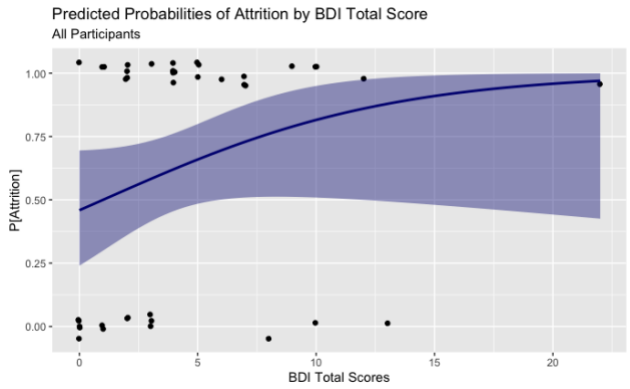
Note. All p values are not significant. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

Figure 9. Predicted Probabilities of Study Attrition based on the Clinician Administered Posttraumatic Stress Disorder scale (CAPS) for all combat unexposed participants using the combat exposed cotwin's CAPS scores using logistic regression.



Note. All *p* values are not significant. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

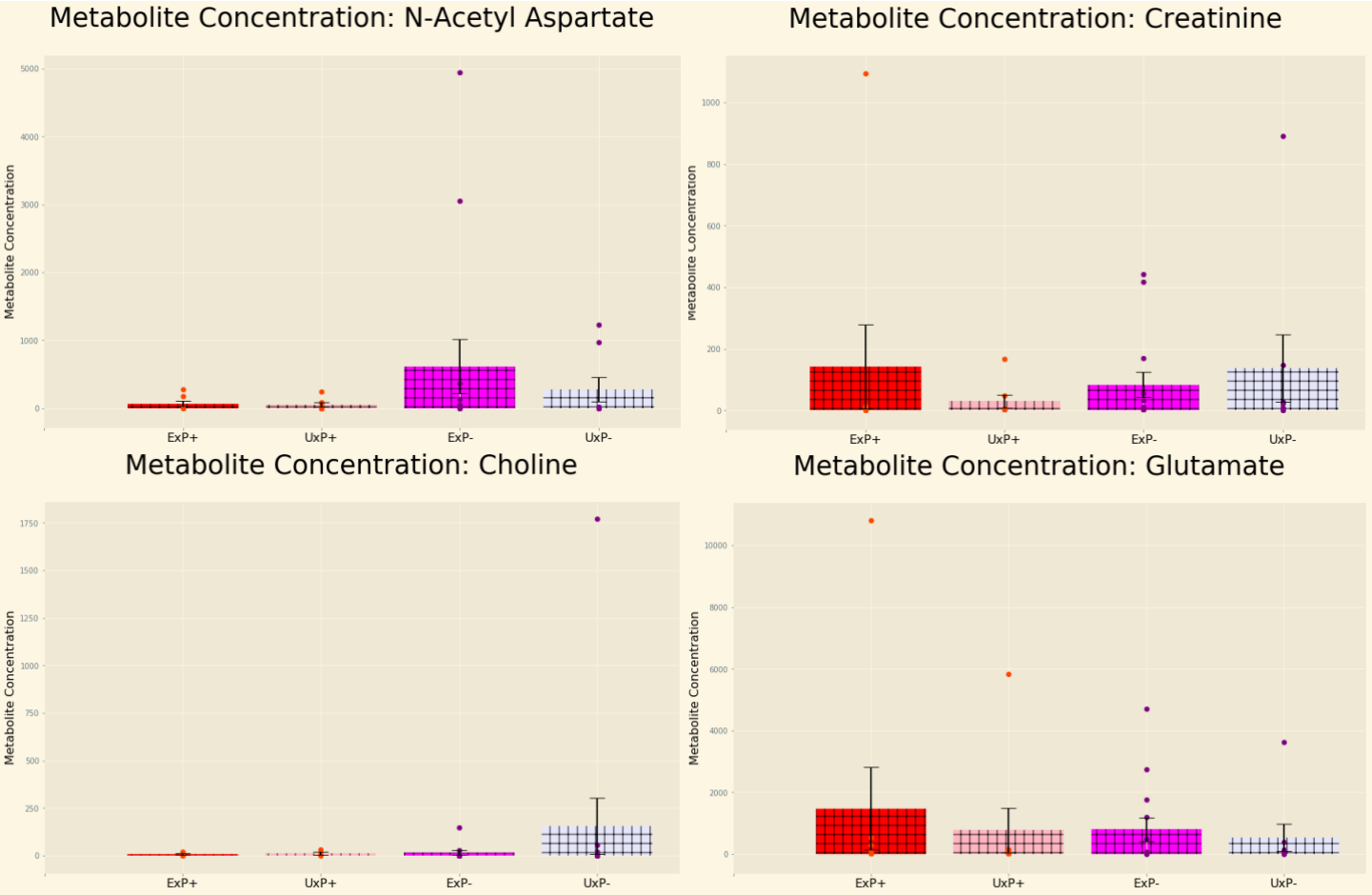
Figure 10. Predicted Probabilities of Study Attrition based on the Beck Depression Inventory using logistic regression.



Note. All *p* values are not significant. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

From our mixed-effects analysis, no significant differences were detected in metabolite concentrations for our a priori hypotheses for N-acetyl aspartate (Diagnosis: $F(1,18) = .235$, $p = .631$; Exposure: $F(1,18) = .65$, $p = .425$; Diagnosis x Exposure: $F(1,18) = .197$, $p = .660$), creatine (Diagnosis: $F(1,18) = .85$, $p = .363$; Exposure: $F(1,18) = .28$, $p = .602$; Diagnosis x Exposure: $F(1,18) = 1.14$, $p = .2917$), choline (Diagnosis: $F(1,18) = .60$, $p = .449$; Exposure: $F(1,18) = 1.18$, $p = .289$; Diagnosis x Exposure: $F(1,18) = .28$, $p = .601$), or glutamate (Diagnosis: $F(1,18) = .04$, $p = .842$; Exposure: $F(1,18) = .11$, $p = .7445$; Diagnosis x Exposure: $F(1,18) = .09$, $p = .7643$), however the model showed significant outliers. Instead of trimming outliers in an already small sample, we performed a robust linear mixed effects model using the same model design as listed previously, with p values estimated via bootstrapping, however there remained no significant differences in metabolite concentrations for our a priori hypotheses (all *t* values < 1).

***Figure 11*. Metabolite concentrations by group.**

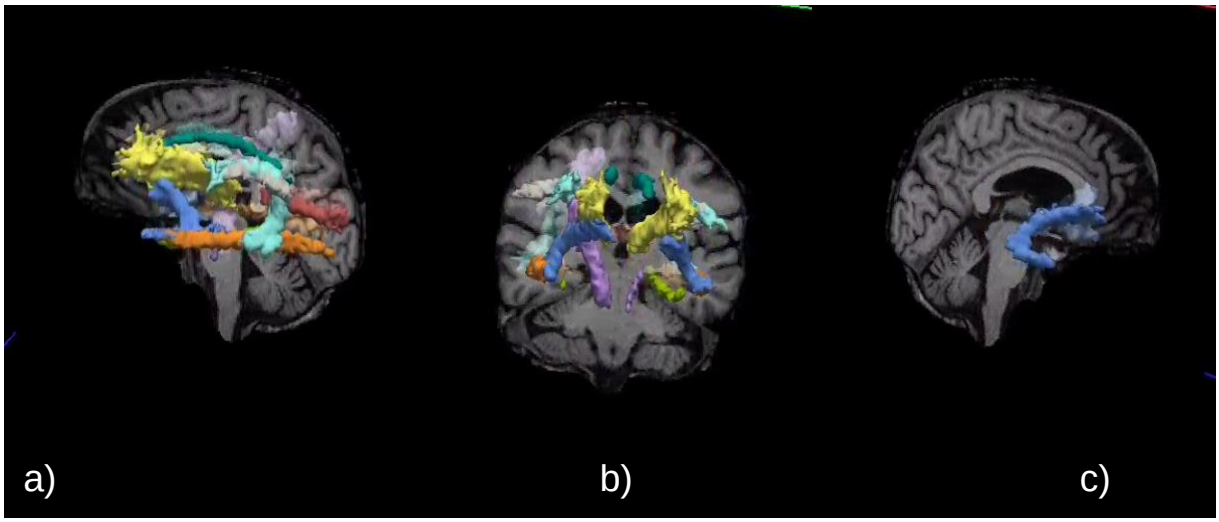


Note. ExP+: Combat exposed participant who developed PTSD; ExP-: combat exposed participant who did not develop PTSD; UxP+: monozygotic cotwins of individuals who went to combat and developed PTSD; UxP-: monozygotic cotwins of individuals who went to combat and did not develop PTSD.

Diffusion Results

A total of 38 participants (9 PTSD twin pairs, 8 Control pairs, 4 singletons whose twin could not be analyzed) had usable T1, T2 and diffusion weighted images that were successfully processed in TRACULA.

Figure 13. White matter reconstruction pathways in TRACULA



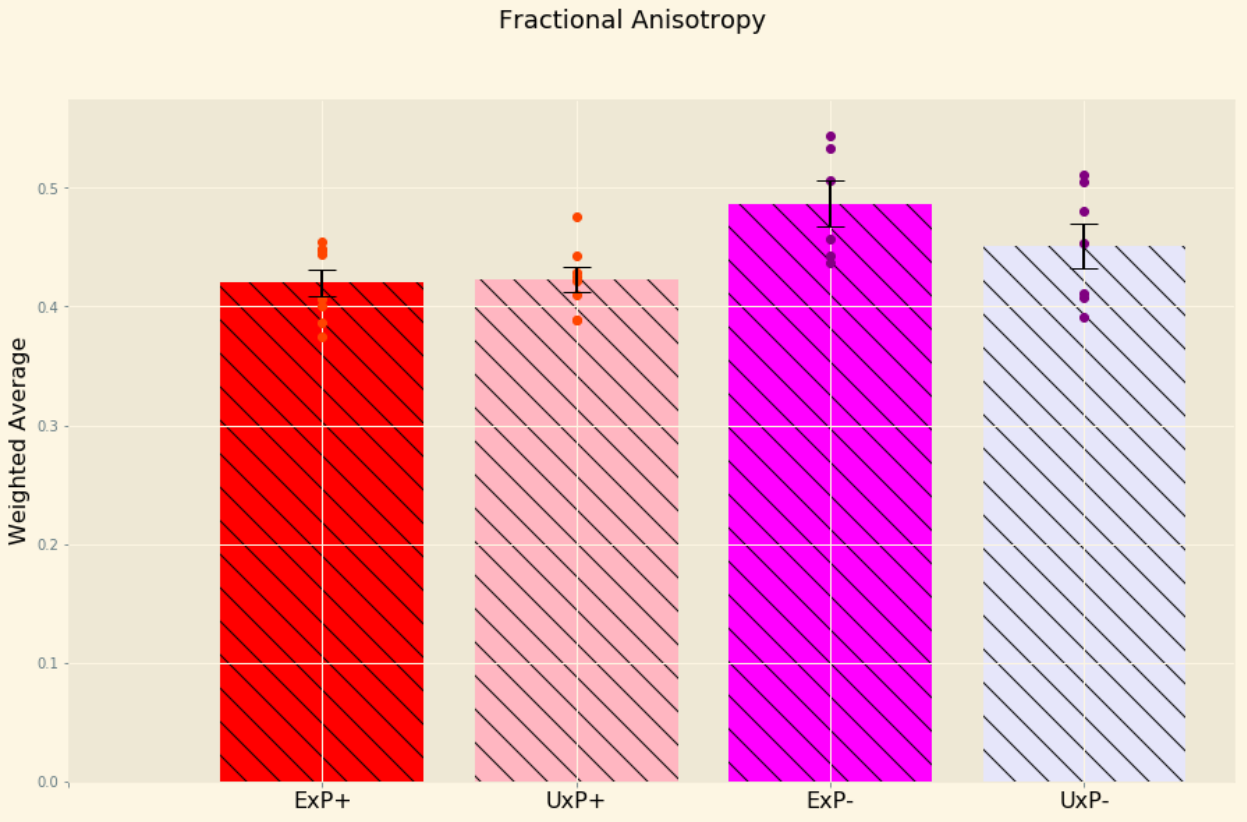
Note. An example participant showing a) 18 reconstructed white matter tracts superimposed over that participant's T1 image in both the sagittal and b) axial plane, as well as a highlight on c) the uncinate fasciculus.

Fractional Anisotropy (FA)

In the left uncinate fasciculus, we found a significant main effect of Exposure such that individuals with combat exposure showed increased FA compared to individuals who were not exposed to combat ($F(1,37) = 10.43$, $p = .00823$; Figure 11). There was also a trending main effect of Diagnosis for FA, such that individuals who were exposed to combat and developed PTSD as well as their cotwins had decreased FA values compared to individuals who did not ($F(1,37) = 2.58$, $p = .12581$). However, these effects are superseded by a significant Diagnosis by

Exposure interaction (F(1,37)=4.97, *p* = .0469; Figure 11) such that individuals who were exposed to combat and did not develop PTSD showed the highest FA values compared to all other participants.

Figure 14. Fractional Anisotropy by group.



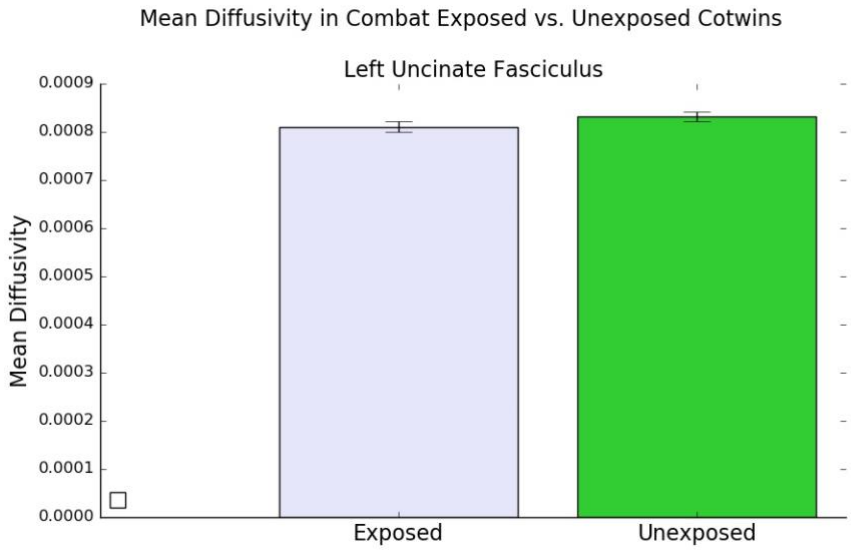
Note. ExP+: Combat exposed participant who developed PTSD; ExP-: combat exposed participant who did not develop PTSD; UxP+: monozygotic cotwins of individuals who went to combat and developed PTSD. UxP-: monozygotic cotwins of individuals who went to combat and did not develop PTSD.

Mean Diffusivity (MD)

In the left uncinate fasciculus (UF), we found a main effect of Exposure, such that the individuals who were exposed to combat showed decreased MD compared to the individuals who were not exposed to combat ($F(1,37) = 5.803$, $p = 0.0353$). This implies that decreased MD

in this area may have come about as a result of undergoing significant combat trauma. The main effect of Diagnosis ($F(1,37) = 1.37$, $p = .2562$) as well as the Diagnosis x Exposure interaction ($F(1,37) = 1.49$, $p = .2469$) were not significant.

Figure 15. Mean Diffusivity by group.



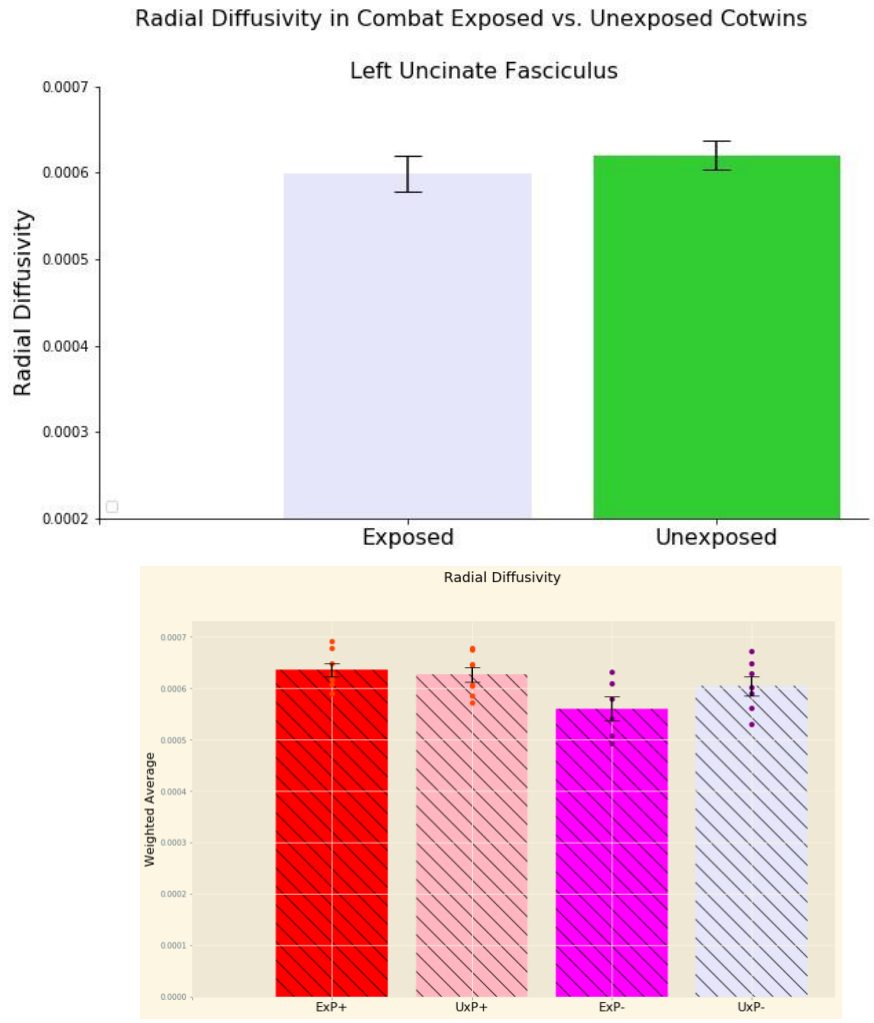
Note. ExP+: Combat exposed participant who developed PTSD; ExP-: combat exposed participant who did not develop PTSD; UxP+: monozygotic cotwins of individuals who went to combat and developed PTSD. UxP-: monozygotic cotwins of individuals who went to combat and did not develop PTSD.

Radial Diffusivity (RD)

There was a significant main effect of Exposure such that the individuals who were exposed to combat showed decreased radial diffusivity (RD) compared to the individuals who were not exposed to combat ($F(1,37) = 12.40$, $p = 0.0049$) in the left uncinate fasciculus. This implies that decreased RD in this area may have come about as a result of undergoing significant combat trauma. The main effect of Diagnosis ($F(1,37) = 2.18$, $p = .1568$) and the Diagnosis x Exposure interaction ($F(1,37) = 4.78$, $p = .0508$), although trending in an expected direction such

that the combat exposed twins with PTSD and their combat unexposed cotwins showed increased RD compared to controls and their cotwins, with the combat exposed twins without PTSD showing the least RD, were not significant.

Figure 16. Radial diffusivity by group.



Note. ExP+: Combat exposed participant who developed PTSD; ExP-: combat exposed participant who did not develop PTSD; UxP+: monozygotic cotwins of individuals who went to combat and developed PTSD. UxP-: monozygotic cotwins of individuals who went to combat and did not develop PTSD.

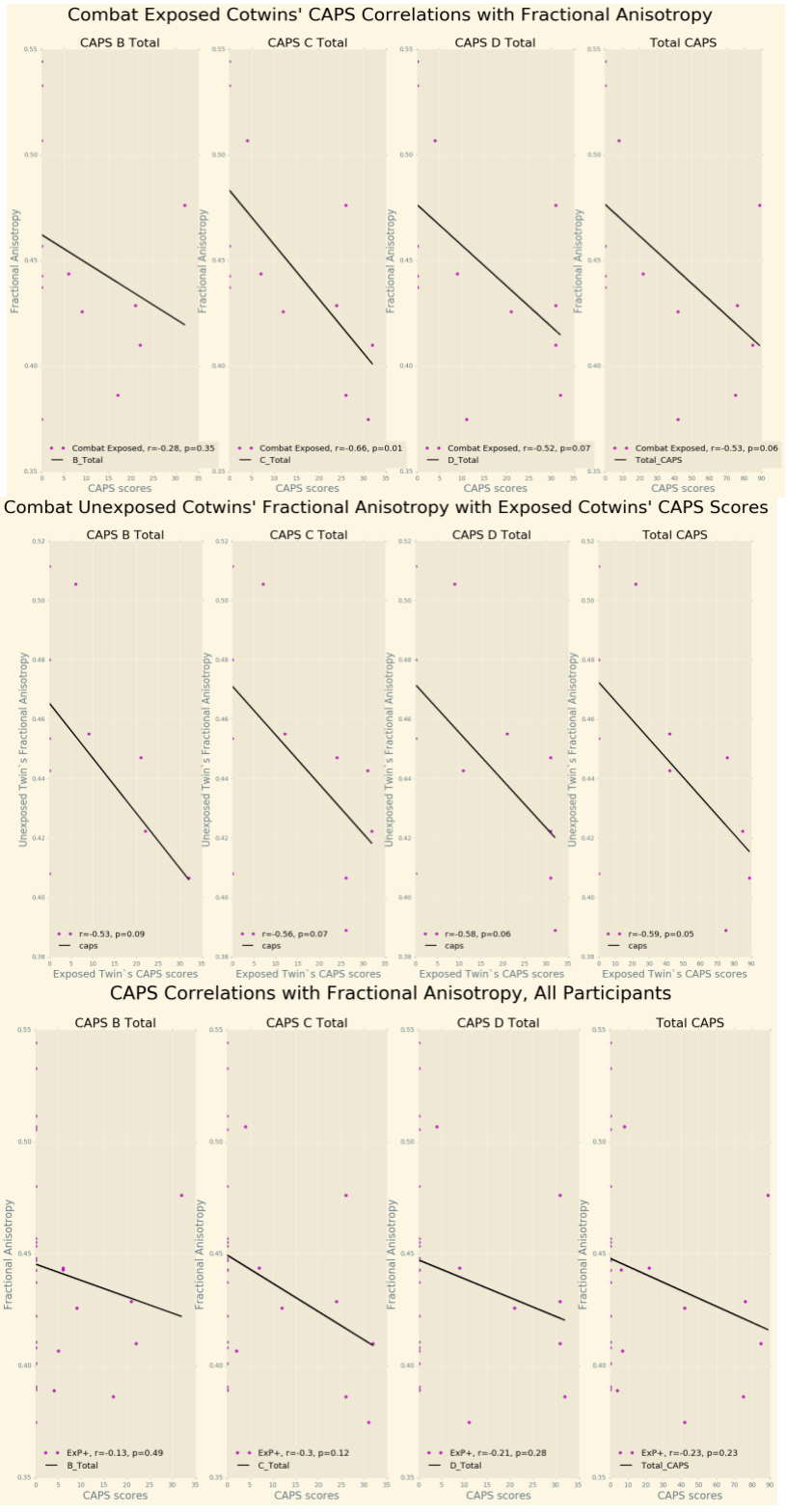
Axial Diffusivity (AD)

There are no significant AD findings to report. The main effect of Diagnosis for AD ($F(1,37) = .032$, $p = .859$)), the main effect of Exposure ($F(1,37)) = .001$. $p = .972$) and the interaction of Diagnosis by Exposure effects ($F(1,37) = .304$, $p = .592$) were not significant.

CAPS Correlations

All CAPS correlations trended in the directions we would expect for our significant findings, such that decreased FA and increased MD and RD were correlated with increased symptomatology (Figures 17-19). Several correlations emerged significant: for FA values in the left uncinate, there were significant correlations for CAPS C scores when we looked at only our combat exposed participants. These measures quantify an individual's degree of negative cognitions about the self, world and others, and they predicted a decrease in diffusion across the principal diffusion direction in those participants (CAPS C: r(16) = .66, p = .01, Figure 17). This same trend was seen for the CAPS D and total CAPS for combat exposed participants (CAPS D: r(16) =-.52 , p = .07; Total CAPS: r(16) = -.53, p = .06, Figure 17). The same trend was seen for RD and MD such that increases in these values corresponded to an increase in PTSD symptoms, however the correlation was only significant for the CAPS C score in RD (r(16) = .63, p = .02, Figure 19). A trend for CAPS C was seen with MD values for our combat exposed participants (r(16) = .53, p = .06, Figure 18).

Figure 17. Clinician Administered Posttraumatic Stress Disorder Scale Correlations with tractography metrics for Fractional Anisotropy.



Note. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

Figure 18. Clinician Administered Posttraumatic Stress Disorder Scale Correlations with tractography metrics for Mean Diffusivity.

Note. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

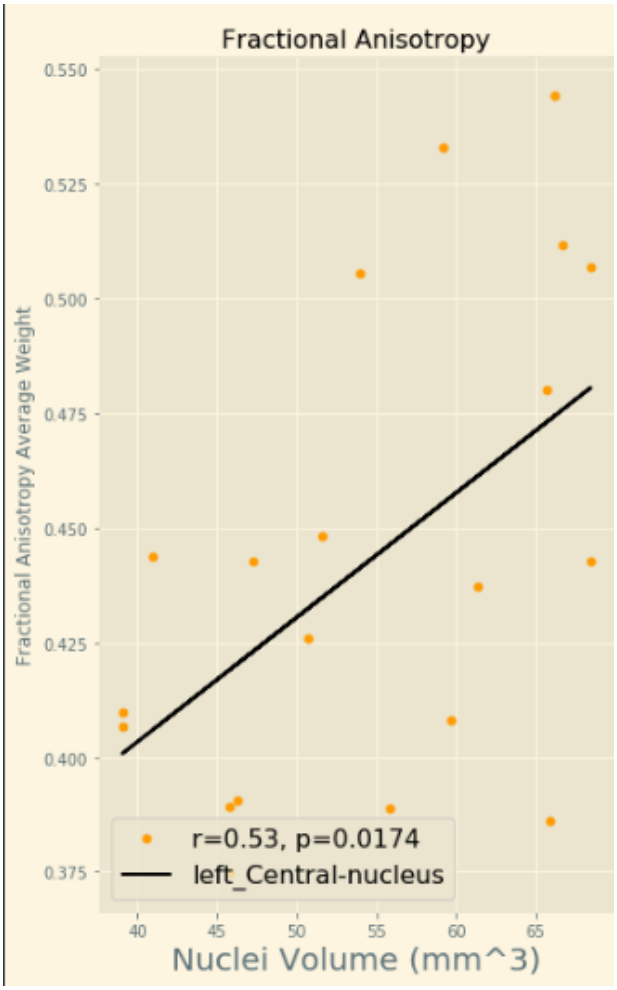
Figure 19. Clinician Administered Posttraumatic Stress Disorder Scale Correlations with Radial Diffusivity.

Note. CAPS B: intrusive thoughts and experiences surrounding the traumatic event; CAPS C: negative cognitions about oneself, the world or other people; CAPS D: impulsivity and exaggerated startle responses; Total CAPS: sum of CAPS B-D.

Cross- Dependent Variable Correlations

For a cross dependent variable analysis, we correlated our fractional anisotropy findings with our findings from the central amygdala. 20 participants had both amygdala subnuclei and diffusion findings to correlate. Results are as we would have expected given our results, that increases in fractional anisotropy correspond to increases in the central amygdala's volume ($r(19) = .53$, $p = .0174$, Figure 17).

Figure 20. Correlation between central amygdala volumes and fractional anisotropy in all participants.



DISCUSSION

Amygdala Subnuclei Discussion

From this volumetric analysis, the cortical, medial and central amygdala subnuclei emerged from our analysis as potential regions-of-interest for future studies and could be of clinical benefit for either primary prevention of PTSD (preventing the onset of the disease) or as secondary prevention (reducing the impact of the disease through early intervention). The central and medial amygdala subnuclei findings further reinforce what has already been seen in the literature when reviewing MRI scans of participants with PTSD and comparing them to either trauma-exposed non-PTSD controls or controls who have never been exposed to a serious traumatic event: the medial and central amygdala subnuclei were found to be larger on average in our twin participants who were exposed to combat, compared to the participants who did not go to combat, meaning that exposure to a traumatic event itself may cause changes in these amygdala subnuclei that were not previously there, since these differences were not seen in twins who had not gone to combat. With that said, we found an interaction effect in the central amygdala such that the individuals who went to combat and did not get PTSD showed greater central amygdala volumes compared to all the other groups. This was not expected; however, it is possible that this finding in the central amygdala is part of an underlying resilience factor present in this group. Further study will be needed to evaluate if this result replicates and if it tends to correspond to better clinical outcomes in combat exposed veterans.

Our cortical amygdala finding contributes a novel result that warrants future study: we found that the cortical amygdala was smaller both in the participants who were exposed to combat and developed PTSD as well as their identical co-twins who were never exposed to

combat. This would imply that the cortical amygdala's volume (or lack thereof) might be a pre-existing vulnerability factor for the development of PTSD such that, upon a significant trauma, that person would be more likely to develop the disease. The cortical amygdala finding was incidental, and it would not survive a multiple comparison correction for false positives. In addition, there was no significant correlation between CAPS scores in the exposed twins and the residualized amygdala volumes in the unexposed twins, thus undermining the impact of the finding as a potential vulnerability factor Due to our low N, our niche population, and the nascence of studying amygdala subnuclei in vivo in humans, it is our opinion that the finding would still merit further investigation and future study as a vulnerability factor.

The cortical amygdala is a subnucleus commonly studied in combination with the central and medial amygdala as part of the centromedial complex of the amygdala (Noto et al. 2021). While both the cortical and medial subnuclei are three layered structures that are part of the primary olfactory cortex in humans, and the medial amygdala is often implicated in studies of PTSD, little attention has been given to the cortical amygdala and its potential to play a role in PTSD. The cortical amygdala has been shown to be involved in not just olfaction but learned behavior and threat avoidance in rodent models. The two subnuclei seem to work together during memory consolidation; most notably during long-term potentiation (Noto et al. 2021; Hakim et al. 2022).

In humans, few studies report cortical amygdala findings in imaging, however a nontrivial portion of those findings involve a correlation with social and emotional processing, although these findings were outside the scope of PTSD. In temporal lobe epilepsy involving hippocampal sclerosis, a reduction in cortical amygdala volume was also seen compared to non-epilepsy controls, and those individuals did show deficiencies in verbal memory (Ballerini et al.

2023). A different study looked at amygdala subnuclei not for the formation of memories, but emotional processing and alexithymic traits, and measured it both through the lifespan and as a point prevalence (Malykhin et al. 2023). Other studies have noted cortical amygdala reductions in volume were related to poorer performance on emotional recognition tasks (Hrybouski et al. 2016; Aghamohammadi-Sereshki et al. 2019). Therefore, it may be of use to explore the emotional processing in adults with combat PTSD, especially if this finding in the cortical amygdala is replicated. Further, other disorders that see disturbances in mood, anxiety and social/emotional performance have seen abnormalities in the cortical amygdala compared to healthy controls. For example, individuals with frontotemporal dementia, a neurodegenerative disease marked by emotional lability and social disturbances in addition to more common dementia symptoms, there is amygdalar atrophy specifically within the cortical amygdala. Individuals with autism spectrum disorder show a different pattern: while their symptom severity does correlate with cortical amygdalar volumes, larger cortical amygdala volumes are seen in individuals who have greater autism symptoms (Seguin et al. 2021). Finally, neonates who are very pre-term show cortical amygdala atrophy that correlates with overall social and emotional deficits commonly seen with premature birth (Mueller et al. 2022). While the study of the cortical amygdala in PTSD is relatively new, its involvement with the hippocampus and its implications in social and emotional performance make it a promising candidate for future study.

Spectroscopy Discussion

We set out to analyze the neurometabolites N-acetyl aspartate (NAA), creatine, choline, and glutamate, with the a priori hypothesis that, based on similar studies with combat veterans, decreases in NAA, as well as increases in creatine, choline and glutamate in the amygdalohippocampal region would occur in combat veterans with PTSD compared to combat

veterans without PTSD. We expected these results to be vulnerability factors that predispose an individual to developing PTSD in the future. Those results were not significant in the sample we studied. It is possible that a contributing factor to our lack of findings is the low N. Tests for selective attrition bias were not significant, meaning that the participants who dropped out before the MRS scan could be conducted were not significantly different from the participants who finished the full scanning protocol, based on our demographic and clinical measures. With that said, it is still possible that the lack of participants prevented us from detecting smaller effects that exist in this population, as no known MRS research in PTSD has explored the unique effect that age might have on neurometabolites in the hippocampus.

It is possible that the functional and structural effects seen in PTSD become less pronounced as a person ages. In general, older adults present lower rates of PTSD than the general population (Lapp, Agbokou, & Ferreri, 2011). However, many will argue that this number is misleading due to under-reporting and differing presentations of PTSD across generations and the lifespan: the elderly may attribute or experience their PTSD symptoms as somatic rather than psychological (van Zelst et al., 2003).There is evidence for symptom stability (Dirkzwager, Bramsen, & van der Ploeg, 2001), exacerbation (Joffe, Brodaty, Luscombe, & Ehrlich, 2003; Solomon & Mikulincer, 2006) reduction (Tennant & Hughes, 1997), and fluctuation (Dirkzwager et al., 2001) of previously existing PTSD, leading to a complicated picture of what we should expect PTSD, specifically within the framework of our dataset, to look like over time (Lapp et al., 2011).

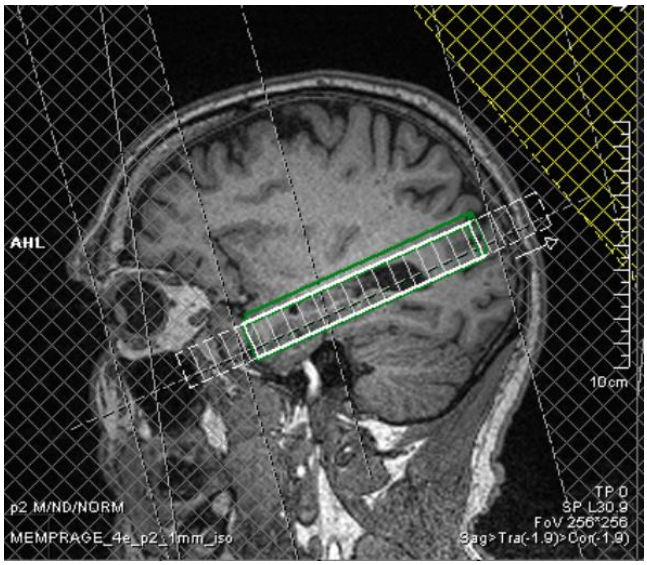
However, there is evidence to suggest that the elderly may be at more risk, or at least may present additional complexity, regarding the development of psychopathology if they have a history of a traumatic event. Compared with the general population, the elderly more often suffer

from chronic physical and mental disorders; social networks tend to narrow with time; and financial means are often reduced (Averill & Beck, 2000). This is especially true regarding a recurrence of symptoms and/or new onset of symptoms related to a past traumatic event. As far as the HPA axis, many studies suggest that diurnal cortisol may increase with age, leading to a decrease in glucocorticoid receptor sensitivity over time, however, the results are inconsistent (Golier, Caramanica, & Yehuda, 2012; Gupta & Morley, 2014). One study has also shown that individuals with PTSD have advanced DNA methylation age over time, which could provide evidence for increased HPA axis activation (Rachel Yehuda, Halligan, Grossman, Golier, & Wong, 2002) along with other studies that have found other markers of accelerated aging in PTSD (Yaffe et al., 2010). It is also unclear precisely when and if PTSD leads to an increase in glucocorticoid sensitivity or a decrease in this population, or if that effect changes as participants age. While many studies have shown increased glucocorticoid receptor sensitivity, a few have shown decreases as well (Golier et al., 2012; R. Yehuda, 2009). There are also myriad other biological mechanisms that could either create or maintain increases in HPA axis activity (see Yehuda, 2009 for a review) and this may also depend on the person's age, gender, and other demographic predictors at the time of trauma. Furthermore, the type of trauma a person experiences may have differential effects on their bodies, specifically in the hippocampus (Hinojosa 2022).

It is also possible that the hippocampal atrophy itself muddled our findings: while most of our participants had hippocampi that could be imaged using 6 -10 6.7mm long voxels, some hippocampi were only 3–4 voxels long, with significant infiltration from the surrounding cerebral spinal fluid due to a lack of hippocampal tissue. Should there be neurometabolite differences in aging adults with combat PTSD compared to controls, it is possible that advances

in MRS in the future will be better equipped to deal with profound heterogeneity in hippocampal atrophy across the study and detect effects smaller than those we were capable of detecting with the technology we had at the time of scanning.

Figure 7. Hippocampal atrophy in our monozygotic twin sample



Note. Example participant with suspected age-related hippocampal atrophy.

These aging factors may have continued to muddle the spectroscopy portion of the study. There is significant overlap between the neurometabolites implicated in PTSD and the neurometabolites implicated in both normal and pathological aging, and with the growing number of combat veterans in the US it will be important to disentangle how PTSD might affect this population throughout their lifespan and the most appropriate methods of intervention. Aging effects seem to be prominent within the amygdalohippocampal region: not only are decreases in hippocampal volume commonly noted in both normal and pathological aging (Lazarov et al. 2024), it has also been seen in PTSD (see Hinojosa 2022 for a comprehensive review). Many studies have found that both aging symptoms and familial incidence of dementia

scores were related to higher myoinositol, choline, total creatine, glutamate-glutamine, and GABA, many of these effects driven by older age and a higher body mass index even above genetic predictors such as apolipoprotein ε4 genotype (Dounavi et al. 2024, Schreiner et al. 2024). There are also known metabolic issues that can be highlighted in the whole brain of older adults: preliminary findings indicate that there are significant age-related differences in several key indicators of neural membrane turnover and mitochondrial function. While we did not get a whole-brain spectroscopy scan in our study, future studies may want to consider cellular metabolism and how it might differentially affect PTSD. It is possible we did not see results because age related changes supersede any changes that might occur due to PTSD.

Diffusion Discussion

Our tractography analysis revealed that individuals who experienced combat related trauma showed decreased mean and radial diffusivity of the left uncinate fasciculus compared to individuals who did not undergo combat trauma. Due to the nature of our twin study, we can elucidate that these abnormalities may have come about as the result of combat trauma itself as opposed to a pre-existing vulnerability. However, regarding fractional anisotropy in the left uncinate fasciculus, we found a significant Diagnosis by Exposure interaction; inspection of the means revealed that this effect was due to the combat exposed Control group having relatively greater FA values than the other groups. These diffusion results correlated with mood disturbances that are often seen in PTSD, as was quantified by the CAPS. Fractional anisotropy (FA) is a measure of the magnitude of the preferred direction of water's diffusion at a particular voxel in the brain. It is a value that ranges from 0 to 1, with 0 being completely isotropic diffusion, with water moving equally in all possible directions, and 1 being the most direction-driven diffusion possible (Lenglet 2015). Mean diffusivity (MD) is defined as the overall average

magnitude of diffusion: it is quantified as the arithmetic mean of the three principal eigenvectors of the diffusion tensor (Lenglet 2015). It is often thought that MD would increase as myelin starts to break down, as diffusion would then become more isotropic, implying an often inverse relationship between MD and fractional anisotropy (Lenglet 2015). Therefore, the fact that we see decreased MD in the group of participants who we would expect to have poorer neuronal health outcomes is puzzling. A similar story can be said of radial diffusivity (RD), which measures the second and third eigenvalues of the diffusion tensor and can be used to quantify crossing white matter fibers that run perpendicular to the primary direction of diffusion. As RD increases, water is more isotropically diffuse in that particular voxel in the brain, implying some degree of damage to the myelin along the principal fiber tract (Lenglet 2015). We again saw a decrease in RD in the combat exposed participants compared to controls, which we would not have expected.

It is possible that the white matter has underwent an accelerated aging process for our combat exposed participants compared to the unexposed cotwins. White matter undergoes a variety of changes with aging: overall, the white matter volume decreases, microstructural properties are lost, and lesions of many kinds may begin to accumulate (Cox et al., 2016; Davis, 2009; de Leeuw, 2001; Westlye et al., 2010; Ylikoski et al., 1995). However, these changes do not occur uniformly, being greater in anterior than posterior brain regions (Kochunov et al., 2007; Pfefferbaum & Sullivan, 2005; Sullivan & Pfefferbaum, 2006), the thalamic radiations and association fasciculi (Cox et al., 2016; Slater et al., 2019). This white matter aging pattern follows what has been referred to as a last-in-first-out hypothesis (Raz & Daugherty, 2018), since the white matter tracts that mature the most slowly are also the first to experience the effects of aging. For example, the thalamic radiations and association fibers (including the UF) show

protracted maturation in early life. These patterns of matter loss with aging are associated with worsening cognitive performance affecting processing speed, primarily impairing executive functions (Kennedy & Raz, 2009; Tubi et al., 2020). Episodic memory function in the cognitively healthy elderly is also negatively associated with white matter microstructural properties of the uncinate, as well as the inferior and superior longitudinal fasciculus, thalamic radiations, and dorsal cingulum bundle (Lockhart et al., 2012; Sasson, Doniger, Pasternak, Tarrasch, & Assaf, 2013; Ziegler et al., 2010). Therefore, it is entirely possible that the UF has begun some demyelination in these participants due to age: the outstanding question is whether we might expect a differential aging process between the PTSD participants and healthy controls. In one attempted meta-analysis, researchers looked for neuroimaging studies that relate to PTSD and aging: because they only found seven studies and the regions implicated were quite heterogeneous, the meta-analysis could not be conducted (Alves de Araujo Junior et al. 2023). Researchers instead implicate various frontal, temporal and parietal regions for future study and, like us, call for future study to examine the nature of PTSD in older adults, especially as veterans from several major US wars are entering this stage of life. However, typically one would also see an increase in MD and RD values that would accompany this change. It is reassuring that our MD and RD measures in our participants correlated with their behavioral measures of PTSD in the direction we would expect, meaning that increased in MD and RD corresponded to worsening PTSD symptoms, however it only serves to further highlight that something other than an accelerated aging process is occurring in the combat exposed participants, or that age and combat exposure are interacting to create . One possible theory is that instead of a differential aging process, there are simply a fewer number of neurons present in the areas of the uncinate that would primarily drive the diffusion process. It is also possible the uncinate is simply smaller

in structure in the combat exposed participants compared to controls. Future research is needed to 1) see if this result replicates in older adults with PTSD and 2) to determine what exactly the nature of this abnormality is.

There is no shortage of analyzes that we can run to follow up on our findings and further quantify how the uncinate fasciculus and other tracts might be behaving in this group of participants. In addition to running a whole-brain analysis in which we look at whole brain values for the FA, MD, RD and AD at each voxel individually, we can quantify changes in the uncinate's shape, size and diffusion directions at individual points throughout the tract in an analysis called Pointwise Assessment of Streamline Tractography Attributes (PASTA; Jones et al. 2005). PASTA, while a more computationally intense analysis than the tractography analysis we completed, could give a better idea of exactly where the differences in the uncinate lie and allow for a better understanding of the disease process. Using a weighted average value for diffusion metrics is standard practice in tractography as we are assuming some degree of uniformity in the form and function of an individual tract, with exceptions, it is possible that the disease process of PTSD affects some portions of the UF more than others, most notably at the beginning and end of the fiber tracts (Yendiki et al. 2011). This might also help explain our more unexpected findings in our tractography analysis.

Lastly, we ran this analysis using TRACULA, which offers users minimal guidance as to how best to assess data for correctable quality assurance issues within its pipeline, such as poor registration or poorly reconstructed white matter tracts. As TRACULA is a data-driven, probabilistic pipeline constrained by prior knowledge of neuroanatomy, the most common recommendation when encountering tract reconstruction issues is to rerun previous analysis steps and, if the tracts still do not process correctly and/or the issue cannot be diagnosed, it is often

recommended to omit this participant from future diffusion analyses. One 2021 paper (He et al. 2021), however, provides a series of recommendations for troubleshooting and quality assurance. Our tractography analysis lost two participants due to tract reconstruction issues that were not diagnosable by the research team: future work will attempt these recommendations in the hopes of preserving more participant data and increasing power for this study.

CONCLUSIONS AND FINAL CONSIDERATIONS

In summary, we performed a three-part magnetic resonance imaging study in a cohort of monozygotic identical twin pairs. One twin in the twin pair was exposed to combat-related trauma whereas the other twin was not. In doing so, we were afforded the opportunity to determine whether any common neurobiological markers of PTSD, such as those in the amygdalohippocampal region as well as the uncinate fasciculus, are potential familial vulnerability factors that are present before a trauma occurs and make a person more likely to develop PTSD if and when they encounter a significant trauma in their lifetime. We found preliminary evidence to suggest that decreased cortical amygdala volumes might serve as a vulnerability factor because not only do the individuals who developed combat PTSD have smaller amygdala volumes compared to those who did not develop combat PTSD, but this neural signature was seen in the identical co-twin of the PTSD participants who never went to combat, meaning that neither combat trauma nor the disease process of PTSD could have created this signature. Alternatively, we also could determine what neuroimaging markers were effects of exposure to combat, meaning that the combat-exposed individuals show a certain neuroimaging effect compared to the combat unexposed individuals. This type of exposure effect was seen in medial amygdala volumes. We can also determine what neuroimaging markers are only seen in

individuals with PTSD, and this could show us what types of imaging markers are present as part of the disease process of PTSD. No results in this manuscript fit this latter signature. Finally, in our tractography study, we isolated the uncinate fasciculus to determine whether any familial vulnerability patterns might emerge, and we found that the left uncinate fasciculus showed decreased MD and RD in combat exposed participants compared to combat unexposed cotwins. These results did significantly correlated with participants' CAPS-C scores, specifically the subscale that measures a participant's mood and anxiety symptoms. In addition, we found an effect in the participants FA values in the left uncinate that found that the participants who went to combat and did not get PTSD had the highest FA values compared to the other groups, with combat exposed individuals showing significantly lower FA than the combat unexposed individuals. This is a potential sign that the individuals who went to combat and did not get PTSD possess some kind of protective factor that may have both preserved their FA values and precluded the development of PTSD.

At multiple points throughout the manuscript, we mention the participants' age as a potential significant variable in this particular group of individuals. This is for a few reasons: to begin, it is not typical for an imaging study that is not studying age related effects to image individuals exclusively in their early 60s. Secondly interestingly, it could also mean that the effects of age are blunting the neural effects of PTSD in these individuals despite nearly completely stable PTSD status in the individuals who were measured more than once. In our sample, two twin pairs had a twin who entered partial remission for their PTSD symptoms, and only one twin pair moved from the control group to the PTSD group between the previous imaging study and the present study.

In addition, as with any psychiatric study that has both multi-day components and

neuroimaging components that require participants to lie completely still in a small space for multiple hours, sometimes over multiple days, it is also possible that our neuroimaging results are not representative of combat veterans in general because these studies require participants to experience a level of physical and psychological discomfort that may not be possible in the more severe cases of PTSD. For example, in our particular sample, measures of problematic alcohol consumption predicted participant attrition from the study, and future studies should investigate how and if that relationship could be something that could be used for participant benefit. In addition, major depressive disorder is another common comorbidity of PTSD, as one of the major subcategories of PTSD involves distorted negative thinking about one's own self, the world around them and other people (American Psychiatric Association, 2013), so much so that it is sometimes argued that the mood disturbances that emerge as a result of PTSD, even if they meet criteria for major depressive disorder, are part of the same construct and that separating them may be arbitrary. We did see some correlations with negative alterations in mood related to PTSD with regards to our diffusion analysis. With the advent of stronger and faster MRI technology, as well as the advent of open MRI machines to allow claustrophobic or otherwise anxiety-prone individuals to undergo MRI scanning in a more comfortable setting, future studies may end up recruiting more severe patients to see how our results might compare to their studies.

In addition, it is important to consider the effects that we may have either missed or overstated due to PTSD being a largely heterogeneous illness that affects different genders and races differently. Our sample might differ from other individuals with different races, genders, types of trauma and ages of onset of the illness and those differences may have different psychological and neurological outcomes. From a gender-based perspective, several factors that might influence the severity and course of PTSD are outlined in detail in Christiansen and Berke

(2020), among other comprehensive reviews (Irish et al. 2011; Kimerling et al. 2018) and the implications of their findings on our work are briefly discussed here:

It is thought that sex and gender ultimately work together to create differences not only in human behavior, but psychopathology on the whole. These differences that will have downstream effects on the underlying biology and anatomical architecture (Christiansen 2015). For example, the prevalence of PTSD in women is approximately twice that found in men (Kessler et al. 2005; Kilpatrick et al. 2013), even when one considers differences in trauma type, differences in diagnostic criteria, culture, and publication biases (Tolin and Foa 2006; Kilpatrick et al. 2013). In addition to being more prevalent, PTSD in women also tends to be more severe, the course of the illness tends to be longer, and women have higher comorbidity rates compared to men (Kessler et al. 2005; Kilpatrick et al. 2013). All that said, women generally respond better to treatment than men (Ogrodniczuk 2006; Wade et al. 2016), creating a muddled picture for how our sample of men and their biology might compare to other individuals.

From a neuroscience perspective, there are several neuroendocrine markers to consider that might play a role in qualifying some of the results we see in our sample of male Vietnam War veterans. For example, testosterone, while known for an increase in arousal and impulsivity, has also been found to have anxiolytic effects, possibly by reducing HPA reactivity to stress and trauma (Christiansen and Hansen 2015; McHenry et al. 2014; Reijnen et al. 2015). Because the amygdala contains various androgen receptors, it is known as a sexually dimorphic region such that the male amygdala is larger on average than the female amygdala due to the increase in free-floating androgens that were not otherwise converted to estradiol. In addition, variations in estradiol and progesterone levels related to the female menstrual cycle have previously been found to affect multiple neurotransmitter systems and the HPA-axis' response to stress (Maddox

et al. 2018; Nillni et al. 2015). Furthermore, estrogen and progesterone have been implicated in cognitive-emotional processes underlying PTSD, such as conditioning and extinction, formation and retrieval of memories, and processing and interpretation of danger cues (Maddox et al. 2018; Pineles et al. 2016). Finally, prior studies have found that progesterone predicts participants' subjective ratings of visual imagery related to their trauma (Wassell et al. 2015) and that high levels of estradiol attenuate brain response and negative affect in response to psychosocial stress (Albert et al. 2015). Therefore, any results seen in the amygdala in our sample should be verified with other genders to see if different results emerge, as sexually dimorphic hormones may have direct effects on the shape, size and function of the amygdala and all downstream regions and processes. In addition to specific gonadal hormones, allopregnanolone and pregnanolone (ALLO) are metabolites of progesterone and are sometimes correlated with symptoms of distress (Pineles et al. 2018). The proposed mechanism by which this occurs is that ALLO serves as a modulator for the anxiolytic effects of GABA, and that ALLO levels, like progesterone, fluctuate throughout the menstrual cycle, creating a varying profile of risk for distress related to PTSD in individuals who menstruate (Kelley et al. 2011).

Finally, it is important to examine the generalizability of our study to other races, cultures and socio-economic backgrounds. Promisingly, illnesses akin to PTSD have been described outside western culture and western medicine, and while the use of magnetic resonance imaging to quantify the nuances of limbic system perturbations is nearly exclusively reserved for higher socio-economic classes due to the cost barrier alone, measuring limbic activity and autonomic dysregulation can be as simple and cost-effective as recording electrical measurements of skin conductance, heart rate, heart rate variability, and orthostatic hypotension, among others. Regarding heart rate variability, while some of the literature is divided (Shah and Vaccarino

2015), many studies have found that individuals with PTSD show decreased heart rate variability and increased skin conductance responses compared to controls (Dennis et al. 2016; Liddell et al. 2016; Critchley et al. 2003; Mather and Thayer 2018; Chang et al. 2013) and this result generalizes to multiple non-Western societies (Hinton et al. 2009; Hinton et al. 2007; Hinton et al. 2002). One study was even able to use heart rate variability as a biofeedback mechanism to help reduce symptoms of PTSD (Tan et al. 2011), and a final study showed that heart rate variability was a vulnerability factor to developing PTSD after combat exposure (Minassian et al. 2015). Furthermore, one study found that not only were skin conductance responses increased in PTSD participants compared to controls, their respiratory sinus arrhythmia was decreased, implying dysfunction of the autonomic nervous system in individuals with PTSD (Seligowski et al., 2019). Therefore, we know there are commonalities among the neurobiology of PTSD across cultures and socio-economic classes, however to what extent the brain changes with these differences is not yet known.

One way that we know that PTSD looks different on different groups of individuals is from the study of the interaction between trauma and race-related stressors. Race-related stress is an oft unrecognized source of physical and emotional distress that could predispose a person to develop PTSD and/or present additional levels of complexity when treating the condition and understanding its etiology. Individuals who underwent increased race-related stress show greater PTSD symptoms both in general and related to racial trauma (Elbasheir et al. 2024). In addition, the course of PTSD for minority persons looks different: minority individuals with PTSD are more likely to experience suicidal ideation as a result of the illness (Jeon et al. 2024) and are more likely to endure a more chronic course of PTSD compared to white counterparts (Birk et al. 2024; Torres et al. 2024). In addition, Black women who underwent increased race-related

stressors show increased epigenetic aging signatures (Elbasheir et al. 2024). This effect may have an intergenerational component as well, even outside the scope of race-related trauma: both Black and Hispanic/Latinx veterans were significantly more likely to have an infant born at lower gestational age and lower infant birth weight as compared with White veterans in covariate-adjusted models. These factors put infants at increased risk for negative health outcomes in the future (Nillni et al. 2024). From a neuroimaging perspective, Black children showed lower amygdala, hippocampus, and prefrontal cortex gray matter volumes compared with White children, and the volumes of the prefrontal cortex and amygdala varied with metrics of childhood adversity, with income being the most common predictor of brain volume differences (Dumornay et al. 2023). Importantly, controlling statistical analyses for differences in childhood adversity qualified some of the race-related differences in gray matter volume. Another study showed that increases in racial disparities are correlated with decreased FA in the uncinate fasciculus among Black women ages 18-62 (Fani et al. 2022). Given that we have evidence to suggest that racial disparities pose the potential to affect neuroanatomical structure and subsequent function, it is reasonable and imperative to approach this topic with nuance and not generalize the results of a predominantly White cohort of combat veterans in their early 60s to other populations, as we will do a disservice to the overwhelming majority of individuals seeking mental health treatment.

To summarize, we conducted a structural cross-modal neuroimaging study of post-combat veterans roughly 40 years after their exposure to combat trauma, each of whom has a monozygotic twin pair to allow us to assess if the differences in brain structure we see are directly the result of trauma exposure, the result of the disease process of PTSD, or if there is a pre-existing familial vulnerability that could, in the future, allow us to assess an individual's risk

for developing PTSD before their exposure to a trauma as significant as combat. We found that the volume of the cortical amygdala served as a pre-existing vulnerability factor and that the volume of the medial amygdala, as well as the mean and radial diffusivity of the uncinate fasciculus showed differences in individuals who were exposed to combat compared to individuals who were not exposed to combat. We saw an interaction effect in the uncinate fasciculus such that individuals who went to combat and did not develop PTSD had the highest FA values, indicating a potential protective factor against the development of PTSD in these individuals. The effects we found may have been affected by the older age of our participants, but this opens up an interesting question of how PTSD might affect a person throughout their lifespan. Caution should be observed when interpreting these results outside the scope of the demographics of our sample, replication attempts are needed, and further follow-up regarding quality assessment and assurance will be conducted with the information we have at present, and we will continue to do so as the field of neuroimaging rapidly (and seemingly exponentially) grows and evolves.

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