CHARACTERIZATION OF DNA METHYLATION AND GENE EXPRESSION PROFILES IN POST-TRAUMATIC STRESS DISORDER

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

Post-traumatic stress disorder (PTSD) is a debilitating mental disorder precipitated by trauma exposure and characterized by involuntary re-experiencing of the traumatic event, avoidance of trauma-associated stimuli, negative alterations in mood and cognition, and alterations in arousal/reactivity. While most individuals are exposed to a potentially traumatic event at some point in their lives, only a small subset develop PTSD. This suggests that trauma exposure is necessary, but not sufficient, for the pathogenesis of PTSD, and that many other pre-existing factors, including genetic background, play an influential role in determining individual response to trauma.

PTSD is a complex syndrome with a great degree of heterogeneity in presentation within and across populations. Similarly, biological correlates associated with PTSD, specifically DNA methylation (DNAm) and gene expression profiles, exhibit tissue specificity and are comprised of two dynamic components: 1) profiles reflecting cellular composition and 2) alterations in DNAm levels at CpG sites and transcriptional activity. In this work, I examine DNA methylation and gene expression profiles that reflect the pathophysiology of PTSD while considering fundamental population contexts and sources of heterogeneity that have not been adequately addressed in the existing PTSD literature – sex, ancestry, and cellular composition.

After an overview of relevant literature in Chapter 1, the subsequent two chapters examine sex-specific profiles associated with PTSD. Chapter 2 disentangles the contribution of cellular composition from DNAm alterations in methylomic profiles to discern sex-specific leukocyte composition associated with PTSD using a combined dataset from two independent cohorts. Chapter 3 assesses the relationship between blood and brain transcriptomic profiles in a

rat model of PTSD (i.e., individual response to recent trauma exposure) using a publicly available expression dataset. Chapter 4 implements an integrative systems-level approach to investigate the genomic context underlying leukocyte methylomic and transcriptomic profiles in an understudied, at-risk population of African ancestry, and to identify regulatory epigenetic marks that putatively regulate leukocyte gene expression in PTSD. Chapter 5 provides a discussion of the implications of these findings and their contributions to the existing PTSD literature.

This work uses genomic approaches to generate mechanistic hypotheses regarding dysregulation observed in PTSD. It contributes novel insights to the current PTSD literature by considering sex-, ancestry-, and cell/tissue-specific contexts, which have historically been overlooked, and by doing so provides input for understanding findings that are often inconsistent across published DNA methylation and gene expression studies of PTSD.

ACKNOWLEDGMENTS

To my knowledge, the vast majority of PhDs do not consider their PhD experience to have been a cake walk. There seems to be a set of typical and less typical, but not necessarily surprising, challenges PhD students encounter, find personally trying, and handle/deal with in their own way. Despite recognizing that many other graduates had similar struggles and also seriously considered dropping out at multiple points during their training, I still truly believe *my* thesis completion/PhD was a miracle that could not have been possible without a number of people who demonstrated an enormous amount of patience, compassion, and understanding over my years of graduate training.

First and foremost, I thank my advisor, Monica Uddin, for her support/guidance and for sticking with me for the last four and a half years. She deserves much credit for the completion of this dissertation. When I was at my wits' end and had completely depleted all mental/emotional reserves, she managed to talk me back into rolling forward again. She figured me out and convinced me that I was *really* close and that the rest of the road was paved/flat. In reality, there was a lot left to do in the last ABD year, but I didn't need to know that at the time; she knew my tendencies and coached me to the finish line according to my specific needs.

I would also like to express my sincere gratitude to the rest of my doctoral committee: Profs. Rodney Johnson, Janice Juraska, and Lisa Stubbs, for their insights and guidance in the form of reading lists, suggested courses/conferences, and discussions. They also served on my committee since I started the neuroscience program, and their suggestions and comments throughout my training provided the direction that enabled me to consider my research from various perspectives. When I started the program, my goal was to become some sort of

interdisciplinary unicorn by the time I graduated. I soon realized this was ridiculously unrealistic and that the PhD training was supposed to be a launchpad for this lifelong mission; my doctoral committee directed that launch.

I'm blessed to have been in supportive programs run by seasoned disaster management experts and counselors. In the NSP office, Sam Beshers advised me on many crucial decisions, including who I should reach out to for my doctoral committee. Actually I sought him out years before transferring to the NSP and am forever grateful for his counsel and apologetic for my episodes of incessant jabbering. Stephanie Pregent handled all NSP-related things, big and small, with care and thought; just hanging around and chatting with her had a restorative effect on me. While most students don't require intervention from their program director, there's one pivotal moment when I did. Aside from her capable leadership, Martha Gillette somehow catalyzed a turning of tides in my training, without which I probably would not have finished. The MSP office remained steadfast for the last eight years, including through my 20s, and cared for my development as an individual, not as a statistic. The level-headed Jims (i.e., Jim Slauch & Jim Hall) remained cool, calm, collected through problem-solving what felt like a soap opera of a graduate journey and supported/respected my decisions and needs. Nora Few provided the guidance, backing, and sanity support I needed; she centered me when I couldn't tell left from right. Heather Wright came into the picture later exuding a ton of passion, energy, positivity and brilliance. Every visit and chat with her brightened my day, and she's simply been a "one of a kind" positive force in my graduate experience.

I'd like to thank all my lab members and give special thanks to some key labmates. Don Armstrong was a know-all wizard who dedicated many hours of his limited time teaching me coding basics/tips that would have taken me much longer to figure out on my own. He was

someone I could bounce ideas off of and provided many helpful discussions. Angie Bustamante was key for getting me started in the lab and was the supportive "lab elder" that I could turn to for lab help and could enjoy hours of chatting with outside the lab. John Pfeiffer has been a solid bro – less hours of chit-chat, but a pillar of support telling me I'd be awesome and reminding me to be confident. He's been an awesome lab mate with his straight talk, clear-mindedness, intelligence and honesty, and I really couldn't have asked for more.

In addition to labmates, I had theme-mates/cubicle-mates whose company I've enjoyed, including Priya, Will, Jess, and Yunshu. They've advised me through dry runs and were also there for milestones, IGB-related events, and hangouts/chats. Throughout my years at Illinois, I've enjoyed many other fond memories hanging out with wonderful classmates, colleagues, and roommates, including many who graduated ahead of me over the years and some who I met later in my training and wish I had spent more time with. I expect I'll run into quite a few later down the road, but I'll name two constant sources of support and encouragement who were with me since the beginning: Olivia Cangellaris and Libby Joachim. Eight years equals most of our twenties and some. A lot of life happened with chapters where we saw each other every day, some where we saw each other less often but celebrated huge life milestones and others where I may have seen them once or twice in a full year. Even during those periods of self-imposed isolation, it was comforting to know they understood me and that I could text them to touch base, meet up, and/or borrow one of their dogs for a walk.

I'm blessed to have similar constant VIPs outside of Illinois, including gmont, bchevv, TB, caps, Lynn, Laura, who kept tabs on me to make sure I was still alive, encouraged me through burnouts, made sure I had a good time during my occasional escapes, and stuck with me despite my difficult periods of ranting, followed by ghosting… I was not a great friend and was

especially frustrating to deal with for some of my grad school years, but somehow there are good people standing by me to continue friendships spanning 14+ years and that I can rely on. There are longtime friends, a tight-knit extended family with awesome cousins, and then there's the nuclear family – love/hate relationships since birth that are almost impossible to get rid of. Eight years ago, I didn't realize or appreciate that they were the greatest source of unconditional love, support, and encouragement. Nor did I know that those relationships – with my parents, Irene and Thomas, and my sister, Theresa, would evolve for the better. This thesis could not have been finished without the direct involvement of my parents during my last ABD year. They made sure I ate well (essentially spoon-fed me). My mom made sure I was working, and my dad made sure there was a constant supply of coffee and Häagen Dazs in the freezer. Theresa dumped/loaned her adorable cats to go on a yearlong cross-country road trip and turned my work sanctuary into a zoo. In retrospect, despite the madness, having two dogs and two cats in the house may have done more good than harm; at least four shots of oxytocin a day while under self/thesis-imposed house arrest. I'm fully aware that I've been blessed and seriously spoiled.

I wish to acknowledge the financial support and opportunities provided by the CompGen Fellowship, which helped me get started. The fellowship provided an intellectual resource and facilitated the valuable discussions I've had with co-advisors, Victor Jongeneel and Saurabh Sinha, through the fellowship. I'd like to thank collaborators and co-authors, including Fei Xue for helpful discussions. Finally, I'd like to express my appreciation for the time and effort of study participants, staff, volunteers, and investigators of the Detroit Neighborhood Health Study, supported by the National Institutes of Health [R01DA022720, R01DA022720-S1, RC1MH088283, R01 MD011728, 3R01MD011728-02S1], and the Grady Trauma Project (Aim 1), supported by the National Institute of Mental Health [MH096764 and MH071537].

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CHAPTER 1: INTRODUCTION

1.1 Overview

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that is precipitated by a traumatic event involving direct or indirect exposure to actual or threatened death, serious injury, or sexual violence[1]. It is characterized by intrusive and persistent re-experiencing of the traumatic event, avoidance of distressing, trauma-associated stimuli, negative alterations in cognition and mood, and alterations in arousal/reactivity that persist for longer than a month[1]. While most people experience a traumatic event at some point in their lives (~50-90% of US population[2-4], ~70% world-wide[5,6]), only a small subset develop PTSD over a lifetime (~7% and ~4% lifetime prevalence of PTSD in the US[7,8] and across countries[5,9], respectively). This suggests that exposure to trauma is necessary, but not sufficient, for the development of PTSD, and that pre-existing factors, such as genetic background and early life experiences, interact with trauma exposure to precipitate a distinct inability to reinstate homeostasis after trauma[10].

Epigenetic mechanisms, such as DNA methylation, may serve as a key link between pre-existing factors and individual response to trauma exposure. In addition to their role in the onset of PTSD, they may continue to play a role in the pathophysiology of the disorder and may even continue to influence physiology after the disorder remits, in large part through their influence on gene expression profiles, which culminate the effects of functional genetic variants and regulatory epigenetic marks and serve as a useful biological readout for understanding the pathophysiology of PTSD.

However, there are major challenges to understanding PTSD that stem from its heterogeneity and evolving definition. PTSD is a complex syndrome with a great degree of heterogeneity in presentation within and across populations, and trauma-exposed individuals with vastly distinct symptom presentations can qualify for the same diagnosis of PTSD based on the Diagnostic and Statistical Manual, which is revised with each edition[11]. This heterogeneity in presentation may reflect subtypes of PTSD that vary by extent of dysregulation in implicated biological pathways, which in turn may be based on many functional genetic variants of modest effects that interact with each other and with an individual's environmental exposures, particularly the causal traumatic event[12]. Type, duration, frequency, and intensity of the trauma exposure(s), comorbid conditions, as well as interindividual variability in other exposures (e.g., chronic stressors, social support) also contribute to the heterogeneity of PTSD[12]. Furthermore, differences in prevalence and presentation of PTSD have been noted between sexes/genders[2-4,8,13-16] and across racial/ethnic groups[17-20], but the biological processes underlying differences have not been adequately investigated.

This dissertation work seeks to address heterogeneity in PTSD that stems from multiple sources: sex, tissue, and ancestral background. Our studies in humans examine the association between lifetime PTSD and the immune system, which may reflect longstanding physiological changes that occur as a consequence of developing PTSD or a pre-existing physiological difference/setpoint associated with greater vulnerability for developing PTSD. In particular, we investigate sex differences in leukocyte composition using methylomic profiles, which may reflect sex-specific immune processes – putatively involved in the well-known two-fold higher risk of PTSD in females compared to males[2-4,8,13-15,21-23]. While profiling from leukocytes in peripheral blood may inform our understanding of the systemic nature of PTSD and allow us to

investigate the key role of the peripheral immune system in modulating behaviors underlying PTSD, the brain is most directly associated with psychiatric symptoms and defining manifestations of PTSD. Since the brain is inaccessible in living humans, we use a rodent model of PTSD to investigate the relationship between brain and blood transcriptomic profiles associated with individual behavioral differences in response to stress exposure. The final empirical chapter addresses potential genomic sources for the known health disparity in PTSD, namely the greater prevalence in African-Americans that exists even when controlling levels of trauma exposure[18], from a genomic perspective. We identify PTSD-relevant genetic variants in an African-American cohort that may putatively regulate DNA methylation and gene expression and investigate the association between differentially methylated CpGs and gene expression in lifetime PTSD, thereby elucidating biological sources of increased vulnerability—and resilience—to this disorder within an understudied population.

1.2 Societal significance of understanding PTSD

While PTSD is estimated to rank among the top 20 leading causes of disease burden in the general US population[24], some argue that the level of disease burden can be considered comparable to depression, the second leading cause of disability burden in the world[25], when work impairment, symptom chronicity, decrement in health status/increased risk of chronic disease, drain on healthcare resources, and treatment resistance, are taken into full consideration[26-28]. Globally, the aftereffects of recent wars, such as the Syrian refugee crisis, have highlighted the necessity for considering mental health and post-traumatic psychopathology in public policymaking[29]. This is particularly pertinent to host countries grappling with the challenges of accommodating and quickly integrating a large influx of refugees and migrants,

most of whom have experienced traumatic incidents and are at increased risk for developing psychiatric disorders, including PTSD[30]. While this post-conflict and displacement context illustrates an especially substantial disparity between available and needed mental health service capacity, mental health services are generally limited in both developing and developed countries and require development and implementation of triage systems to prioritize care to those who need it most. Investigation of environmental influences, root causes, and risk and protective factors of disorders will enable informed decision making and allow us to work towards prevention and early intervention. This is in fact one of the key research thrusts of the Grand Challenges in Global Mental Health initiative, which identified PTSD as a basic and clinical research priority for making an impact on population health in both developing and developed countries[31].

1.3 Sex/gender differences in PTSD: animal and human work

Epidemiological studies in the general population have consistently shown lifetime prevalence of PTSD to be about twice as high in women than men, even when risk of exposure and types of trauma are taken into consideration[2-4,8,13-15,21-23]. This gender difference in disease prevalence is also observed in depression[32] and other commonly occurring mood and anxiety disorders[33]. While women have a significantly higher risk for developing mood and anxiety disorders, men have significantly higher risk for impulse-control and substance-use disorders[7], which suggests the involvement of sexually dimorphic stress response mechanisms and sex differences in stress-related dysregulation of involved biological processes and neural circuits.

In addition to prevalence/conditional risk for developing PTSD, sex/gender differences have been reported in other aspects of PTSD, such as 1) chronicity, where women report

considerably longer duration of PTSD than men (independent of the type of precipitating trauma)[3,14]; 2) effect of childhood trauma exposure, where females have much higher risk of developing PTSD in childhood (≤ 15 years) than males[13]; 3) nature of co-morbidity with other stress-related conditions and disorders, such as depression, where women exhibit a bidirectional relationship between PTSD and depressive symptoms (i.e., PTSD symptoms predict later depression symptoms, and vice versa), while men only show a significant path from PTSD to depressive symptoms[34,35]; 4) emotional regulation, where women exhibit greater sensitivity to and intolerance of negative emotions, while men report greater impulsivity in response to emotions[16]; 5) cognitive symptoms, where women show stronger association with post-trauma, dysfunctional beliefs, such as negative appraisals about the self/world and self-blame[36], and stronger perceptions of threat and loss of control[37]; and 6) heritability estimates, where women have higher heritability estimates for PTSD than men, as supported by twin studies[38] and a genome-wide association (GWA) study[39], which reported molecular-genetics-based heritability estimates (h^2_{SNP}) to be 29% in females and indistinguishable from 0% in males of European-American descent .

Although it is difficult to disentangle gender (i.e., an individual's self-representation based on biology and sociocultural influences) from sex (i.e., an individual's biological status determined most prominently by their DNA) in humans, supporting evidence from animal models suggest that there are robust sex differences that contribute to the greater vulnerability of women to PTSD and stress-related psychopathology, including sexually dimorphic organization of brain circuitry and stress response mechanisms[23,40,41]. These sex differences are believed to be driven by the organizational and activational effects of gonadal steroid hormones and genes (especially those on sex chromosomes) during developmentally sensitive and environmentally

responsive periods (i.e., perinatal period and puberty), which shape sexually dimorphic processes throughout the lifespan[41].

However, some caveats need to be noted. While animal models of PTSD[42-44], predominantly in rodents, have been and will continue to be key for understanding the mechanisms underlying biochemical and physiological alterations most directly relevant to endophenotypes of PTSD, it is important to consider the translatability and validity of models carefully, especially with regards to sex differences. The majority of animal research on stress and PTSD have, unfortunately, been conducted on male animals using stress models developed for male animals, which has impeded our progress in understanding the mechanisms underlying sex differences[45]. Furthermore, while robust sex differences have been observed in the limited studies that investigated both sexes, findings have often been inconsistent and correspondence with human studies have been ambiguous[45,46]. Thus, careful consideration for possible sex specificity in animal models and in interpretation of human studies is needed. Here, I will touch on a few findings with supporting evidence in both the animal and human literature that highlight the importance of gonadal steroids, genetics, and epigenetics in PTSD.

Sex-specific mechanisms have been observed in endophenotypes of PTSD – broadly divided into dysregulation of fear memory (i.e., fear conditioning and fear extinction)[47] and stress response[41]. Fear extinction, a form of learning involved in the suppression of learned fear responses, is the basis of exposure-based cognitive behavioral therapy (CBT) and has been found to be modulated by circulating gonadal hormones, especially estrogen in females[48,49]. Fear extinction learning during high ovarian hormone levels (i.e., proestrus phase or hormone injection prior to learning in metestrus phase) was determined to be more fully consolidated (i.e., low freezing during recall), while fear extinction during low ovarian hormone levels (i.e.,

metestrus phase or estrogen/progesterone receptor blockade during proestrus) impaired extinction consolidation (i.e., significantly higher freezing during recall)[50]. Another study further revealed that estrogen plays a more prominent role than progesterone in fear extinction, specifically through estrogen receptor beta[51]. Follow-up studies in humans were consistent with these findings and showed that higher estradiol during extinction learning enhanced extinction recall, while low levels impaired this process[52]. Furthermore, low estradiol levels have been linked with fear extinction deficits in women with PTSD, but not in women without PTSD[48,53], suggesting estrogen may be a promising therapeutic adjuvant for use during exposure-based CBT in women with PTSD.

More recently, a study explored the genetic and epigenetic context behind estrogenic mediation of fear regulation in women with PTSD, with supporting evidence in a rodent model[54]. They found significant overlap between CpG sites associated with PTSD and those that associated with serum estradiol levels at nominal significance, supporting an epigenetic link between estradiol-mediated signaling and PTSD in women. The most robust finding revealed higher methylation of CpG sites in the histone deacetylase 4 (*HDAC4*) gene in blood samples of PTSD cases than controls, with significance remaining in cg22937172 after Bonferroni correction. Higher methylation at this site was also associated with lower serum estradiol levels. Additionally, this CpG region is found near binding sites for glucocorticoid and estrogen receptors and a common single nucleotide polymorphism (SNP), rs7570903. The C/C genotype at this SNP was found to be associated with higher cg22937172 methylation and lower *HDAC4* expression levels. Since *HDAC4* is involved in long-term memory processes, further follow-up determined the relationship between genetic variation at this locus and fear-related traits in both human and animal models. Women with the C/C genotype at rs7570903 showed enhanced fear

expression and this genotype interacted with PTSD diagnosis to predict increased fear conditioning and higher fear-potentiated startle during early extinction. Neuroimaging assessment of highly traumatized women revealed that this risk genotype is associated with increased resting-state functional connectivity between the amygdala and cingulate cortex, which are brain regions implicated in fear memory. Additionally, auditory fear conditioning increased *Hdac4* expression in amygdala of ovariectomized and low estrogen (metestrous) cycle, but not high estrogen (proestrous) cycle female mice, relative to homecage controls. In all, this study supported a mediating role for estrogen in the regulation of *HDAC4* expression, which is associated with fear learning processes and may contribute to PTSD in women. It illustrated the genetic and epigenetic context by drawing associations between a genetic risk variant and DNA methylation mark that regulate *HDAC4* expression in blood with fear regulation and estrogen levels.

In addition to its significant mediating role in fear learning, gonadal hormones have also been implicated in the regulation of sex-specific stress response mechanisms in PTSD, which are largely thought to be mediated by the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes[55-57]. Of note, a functional SNP variant (rs2267735) in a putative oestrogen response element within the *ADCYAP1R1* gene was associated with PTSD diagnosis, PTSD symptoms, and fear discrimination (i.e., fear potentiated startle), and showed a strong association between PTSD symptom severity and genotype by trauma exposure interaction only in women[58,59]. The *ADCYAP1R1* gene encodes the pituitary adenylate cyclase-activating polypeptide (PACAP/PAC1) receptor, which interacts with PACAP to regulate neuroendocrine stress circuits and cellular stress response[60,61]. Sex-specific effects of this regulatory system were reported in both brain and blood[59]. In the brain, a sex-by-genotype interaction was found to

regulate *ADCYAP1R1* expression (i.e., mRNA levels), such that females with the C/C genotype expressed significantly less *ADCYAP1R1* than males or females with the C/G genotype. In blood, PACAP levels were associated with fear physiology, PTSD diagnosis, and symptoms, in heavily traumatized women. Additionally, in rodent models, fear conditioning and oestrogen replacement were found to induce *ADCYAP1R1* mRNA expression in amygdala and bed nuclease of stria terminalis (BNST), respectively, providing further evidence for the role of estrogen in stress response via regulation of the *ADCYAP1R1* gene[59]. Finally, while not as fully explored, a SNP (rs523349) in the *SRD5A2* gene, which encodes steroid 5-α-reductase type 2, was associated with PTS symptom severity and risk for PTSD only in men[62], and is suspected to differentially regulate stress and sex steroid metabolism (for testosterone, cortisol, and progesterone) between sexes. So far, sexually dimorphic stress-response mechanisms are largely attributed to the crosstalk between glucocorticoid receptors and estrogen receptors[63].

However, immune signaling is also a key domain in the study of sex differences in PTSD. Like sex differences in brain circuitry and stress response mechanisms, prominent immunological sex differences are also mediated by genes, hormones, and environmental exposures[64], and may play a key role in sex-specific brain development implicated in sexually dimorphic stress response and vulnerability for psychiatric disorders[65]. So far, work at the intersection of stress and psychoneuroimmunology has focused on male animals and sex differences in stress-related neuroinflammation have not been explored. Similarly, investigation of sex differences in the PTSD transcriptomic literature, which is largely based on blood tissue (e.g., leukocytes, PBMCs), is sparse. Two studies that isolated $CD14^+$ monocytes from peripheral blood noted sex differences in transcriptional regulation and gene expression associated with chronic PTSD in monocytes[66,67]. In contrast, a recent mega-analysis of the

PTSD blood transcriptome that considered sex and type of traumatic event in co-expression network analyses reported some distinct gene expression perturbation modules, but generally a high degree of sharing of transcriptional dysregulation across sex and modes of trauma in PTSD, converging on common signaling cascades, including cytokine, innate immune, and type I interferon pathways[68]. Further investigation of sex differences in immune dysregulation is warranted.

Investigating sex-specific trajectories of the brain and immune system across the lifespan, particularly in the context of stress and trauma, may help inform how the crosstalk between the brain and immune system influence sex differences in behavior and vulnerability/resilience for PTSD and other stress-related disorders. It will allow discovery of robust predictive biomarkers that can be used for implementing early interventions, and development of improved, and possibly sex-appropriate preventative or therapeutic strategies. This, in turn, has immense implications for reducing stress-related disease burden.

1.4 Racial/ethnic disparities in PTSD

Lifetime prevalence of PTSD differs across racial/ethnic groups and is higher among at-risk minority groups, including African-Americans (AA) and Latinos, compared to Caucasians[14,18,69-71]. This health disparity may reflect biological as well as interrelated sociocultural differences, which include differences in trauma exposure, socioeconomic status (e.g., education, income, employment status), access to health care/treatment seeking behavior, discrimination, and cultural factors (e.g., reporting style, cultural interpretations of symptoms and distress, coping styles)[17]. Indeed, at-risk ethnic minorities, most notably economically disadvantaged AAs living in urban settings, have higher levels of trauma exposure, particularly

assaultive violence, compared to Caucasians[14,19,72,73]. However, the higher lifetime prevalence and conditional risk of PTSD in AAs compared to Caucasians, is not entirely accounted for by differences in trauma exposure, suggesting biological and environmental risk factors, such as low socioeconomic status and racial discrimination, may account for this disparity[18].

Racial/ethnic differences have also been reported in the expression and severity of PTSD symptoms, the course of disease, and the magnitude of associations between PTSD and chronic medical conditions, such as diabetes[17,20,74-76]. While these differences have largely been attributed to sociocultural risk factors in epidemiological studies, the biological mechanisms by which these sociocultural determinants translate into health disparities, are largely unknown. The concept of allostatic load models sociocultural risk factors as chronic exposure to social and environmental stressors that lead to accumulation of "wear and tear" in the body, which manifests as physiological changes that increase vulnerability to disease and stress-related psychopathology among socially disadvantaged groups, including at-risk racial/ethnic minorities[77-79]. Thus, racial/ethnic disparities in PTSD may be attributable to a larger allostatic load driven by higher exposure to social adversity in these at-risk groups.

These racial/ethnic differences in allostatic load may contribute to racial/ethnic differences in gene-by-environment (GxE) interactions and epigenetic processes. Higher allostatic load due to social adversity in at-risk groups is likely to be reflected in epigenetic processes that biochemically encode these exposures into the genomic architecture of each cell to modulate gene expression, and, by extension, cell functioning and phenotype. Similarly, higher allostatic load may also interact with functional genetic variants to moderate risk for, or resilience to, development of PTSD. While biological mechanisms underlying the regulatory effect of genetic variants are likely to be shared, the allele frequency of genetic variants may

differ greatly across different racial/ethnic groups[80]. This means PTSD-associated functional genetic variants with different allele frequencies across populations may be more relevant for some racial/ethnic groups than others. Further studies that explore the role of GxE and epigenetic processes in the development and pathophysiology of PTSD should consider the implications of these various racial/ethnic differences.

1.5 Summary

This work uses genomic approaches to generate mechanistic hypotheses regarding dysregulation observed in PTSD. It contributes novel insights to the current PTSD literature by considering sex-, ancestry-, and tissue-specific contexts, which have largely been overlooked, and by doing so provides input for understanding findings that have been inconsistent across published studies.

CHAPTER 2: METHYLOMIC PROFILES REVEAL SEX-SPECIFIC LEUKOCYTE COMPOSITION ASSOCIATED WITH PTSD[1]

2.1 Abstract

Post-traumatic stress disorder (PTSD) is a debilitating mental disorder precipitated by trauma exposure. However, only some persons exposed to trauma develop PTSD. There are sex differences in risk; twice as many women as men develop a lifetime diagnosis of PTSD. Methylomic profiles derived from peripheral blood are well-suited for investigating PTSD because DNA methylation (DNAm) encodes individual response to trauma and may play a key role in the immune dysregulation characteristic of PTSD pathophysiology. In the current study, we leveraged recent methodological advances to investigate sex-specific differences in DNAm-based leukocyte composition that are associated with lifetime PTSD. We estimated leukocyte composition on a combined methylation array dataset (483 participants, ~450k CpG sites) consisting of two civilian cohorts, the Detroit Neighborhood Health Study and Grady Trauma Project. Sex-stratified Mann-Whitney U test and two-way ANCOVA revealed that lifetime PTSD was associated with a small but significant elevation in monocyte proportions in males, but not in females (Holm-adjusted p-val < 0.05). No difference in monocyte proportions was observed between current and remitted PTSD cases in males, suggesting that this sex-specific difference reflects a long-standing trait of lifetime history of PTSD, rather than current state of PTSD. Associations with lifetime PTSD or PTSD status were not observed in any other leukocyte subtype and our finding in monocytes was confirmed using cell estimates based on a

[1] This chapter is available on bioRxiv preprint (10.1101/594127) and is under review at Brain, Behavior, and Immunity.

different deconvolution algorithm, suggesting that our sex-specific findings are robust across cell estimation approaches. Overall, our main finding of elevated monocyte proportions in males, but not in females with lifetime history of PTSD provides evidence for a sex-specific difference in peripheral blood leukocyte composition that is detectable in methylomic profiles and that reflects long-standing changes associated with PTSD diagnosis.

2.2 Introduction

Post-traumatic stress disorder (PTSD) is a debilitating mental disorder that is precipitated by a traumatic experience involving direct or indirect exposure to actual or threatened death, serious injury, or sexual violence[1]. PTSD presents with intrusive and persistent re-experiencing of the traumatic event, avoidance of distressing, trauma-associated stimuli, negative alterations in cognition and mood, and alterations in arousal/reactivity that persist for longer than a month[1]. While most individuals are exposed to a potentially traumatic event at some point in their lives, only some develop PTSD[2-8], suggesting that the disorder reflects a distinct inability to reinstate homeostasis after psychological trauma in vulnerable individuals[10].

Epidemiological studies have identified sex to be a significant vulnerability factor for developing PTSD, with women twice as likely to have lifetime PTSD than men, even when risk of exposure and types of trauma are taken into consideration[3,4,8,13-15]. This sex bias in disease prevalence is also observed in other stress-related mood and anxiety disorders[33], including depression[32]. Preclinical and clinical investigations have identified sexual dimorphism in stress response systems that may be involved in the increased prevalence of stress-related psychopathologies in women[23,40]. Furthermore, in addition to sexual dimorphism in the neurobiological underpinnings of stress/trauma response, recent animal studies suggest that

behavioral response to traumatic stress is fundamentally different between males and females and should be considered in interpretation of results[81]. In humans, response to stress/trauma exposure involves both biological and social contributors corresponding to sex and gender-related variables. While the effects of sex and gender are difficult to disentangle, investigations stratified by biological sex, understood to interact with gender-related variables, are warranted to improve our currently limited understanding of the sex-specific biological processes dysregulated in PTSD pathophysiology.

Mounting evidence suggests a key role for stress-induced inflammation and immune alteration in the development and maintenance of PTSD and other stress-related psychiatric disorders. Although findings from human literature have been mixed, PTSD has generally been associated with increased pro-inflammatory tone, basally and in response to immune challenge, via both cytokine signaling and changes in immune cell distribution/function[82-98]. Investigations in animal models, primarily in male rodent studies, have provided mechanistic insights into how peripheral immune cell response/signaling and distribution is linked with microglial activation and neuroinflammatory dynamics to trigger stress-induced anxiety behavior and memory impairment[99-105].

Epigenetic mechanisms have emerged as important regulators of PTSD-associated immune dysregulation and inflammation[106-111], and are particularly significant for the study of PTSD because they capture the interactions among pre-disposing genetic/environmental risk factors and the precipitating trauma exposure. Individual response to trauma exposure can be encoded as short-lived or persistent epigenetic changes that reflect and may contribute to posttraumatic physiological changes, some of which remain after remission of PTSD symptoms. DNA methylation (DNAm) has been the most widely studied epigenetic mechanism and

evidence from both animal and human models point to its key role in stress regulation[10,112-114], fear memory[115-119], and immune function[108-111,120-123], in both brain and blood. Exploring PTSD-associated DNAm profiles in blood may inform our understanding of mechanisms underlying immune dysregulation, particularly those that coordinate peripheral immune-neuroimmune crosstalk[124,125], and given the well-known sex difference in PTSD prevalence and in immune response[64,126], sex-stratified investigation is warranted.

Peripheral blood-based methylomic profiles are comprised of two dynamic components: 1) profiles reflecting proportions of immune cell subtypes (i.e., leukocyte composition) and 2) alterations in DNAm levels at CpG sites genome-wide (i.e., differential methylation). Epigenome-wide association studies (EWAS) often seek to identify dynamic differential methylation marks and treat cellular heterogeneity as a major confound that must be addressed to improve signal detection. However, differences in leukocyte subtypes provide key insights into immunological changes and warrant examination themselves. Recent developments in deconvolution algorithms and cell-type discriminating reference databases have improved estimates[127-130] and enabled utility of DNAm-based leukocyte subtype estimates as proxies for white blood cell differential-based metrics[131,132]. Leveraging these recently developed methods, here we use leukocyte-derived methylomic profiles combined from two civilian cohorts to investigate our hypothesis that PTSD is associated with sex-specific differences in leukocyte composition, detectable by DNAm-based estimates. To our knowledge, this study is the first to investigate leukocyte composition profiles in PTSD using these new DNAm-based approaches for immune profiling.

2.3 Methods

Study Participants

Samples from trauma-exposed, adult participants with available Illumina HumanMethylation450 (450K) BeadChip array data were selected from two predominantly African-American, community-based cohorts examining biological and environmental correlates of PTSD, namely the Detroit Neighborhood Health Study (DNHS; n=192) and Grady Trauma Project (GTP; n=422). The DNHS, based in Detroit, MI, was approved by the institutional review boards of the University of Michigan and University of North Carolina at Chapel Hill. The GTP, based in Atlanta, GA, was approved by the institutional review boards of Emory University School of Medicine and Grady Memorial Hospital. All participants provided written informed consent prior to data collection. Details regarding the DNHS[73,108,133] and GTP[72,134,135] were published previously. While neither study excluded participants based on illness, women known to be pregnant (in the GTP) were excluded from estimation and analyses, due to well-known/known significant differences in leukocyte composition during pregnancy[136]. Collected demographic data included self-reported gender, race, age, and current smoking, which was defined as any cigarette smoking in the past 30 days.

Assessment of PTSD

Study participants were assessed for PTSD, as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)[137]. In the DNHS, study participants were assessed for PTSD using the well-validated self-report PTSD Checklist, Civilian Version (PCL-C)[138-141] and additional questions about duration, timing, and impairment due to symptoms, via structured telephone interviews[14,108,142]. Participants who met all six DSM-IV criteria in

reference to their worst traumatic event or to a randomly selected traumatic event (if the participant experienced more than one trauma), were considered affected by lifetime PTSD. Those that met criteria based on symptoms reported within the past month were considered affected by current PTSD. Analysis of data from the clinical interviews showed that the PTSD instrument used during structured telephone interviews had excellent internal consistency and high concordance[108,143]. The PCL-C yielded a Cronbach coefficient alpha (α) of 0.93. Using cluster scoring based on DSM-IV criteria (i.e. to be a case, the participant's symptoms had to meet all six criteria), the instrument had a specificity (SP) of 0.97, sensitivity (SE) of 0.24, positive predictive value (PPV) of 0.80, negative predictive value (NPV) 0.72, and an area under the ROC curve (AUC) of 0.76, as previously reported[108,143]. In the GTP, [144]study participants were assessed for lifetime and current PTSD using the Clinician-Administered Post-traumatic Stress Disorder Scale (CAPS, DSM-IV), a structured interview administered by a clinician that has previously been shown to have excellent reliability (i.e., consistent scores across items, raters, testing occasions) and excellent convergent and discriminant validity in large scale psychometric studies[145,146]. For lifetime analyses, participants meeting criteria for PTSD at any point (including current and past PTSD) were considered cases and compared to trauma-exposed controls who lacked a history of PTSD at any point in their lifetime. For comparisons of current PTSD with remitted PTSD and trauma exposed controls, participants with lifetime PTSD were further separated into two groups: those with current PTSD and those with lifetime (but not current) PTSD.

Sample Processing

In the DNHS, samples were obtained via an in-home blood draw performed by a clinician, processed in the lab within two hours, and stored at -20°C until testing. Detailed methods regarding biospecimen processing in the DNHS are available in a separate publication[147]. In the GTP, DNA was extracted from whole blood, aliquoted and frozen at -80°C within two hours of collection. Genomic DNA was extracted from peripheral blood using the DNA Mini Kit (Qiagen, Germantown, MD) in the DNHS and the Gentra Puregene Kit (Qiagen, Germantown, MD) in the GTP. Both studies bisulfite-converted the DNA samples using the Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA) and used 500 ng of DNA per sample for whole-genome amplification, fragmentation, and hybridization to the Illumina Human Methylation 450K BeadChip array (Illumina, San Diego, CA), according to the manufacturers' recommended protocols. Sample processing procedures have been published previously for both the DNHS and GTP[123,135,148-150].

Quality control and pre-processing of 450K Data

The raw .idat files were imported into R (version 3.5.1)[151], using the *minfi*[152] Bioconductor (version 3.7)[153,154] package, for all subsequent data processing and analyses. After quality control (QC)[152,155,156], data pre-processing[157-162] was conducted on all QC'ed samples (DNHS: n = 187; GTP: n = 416). This included duplicates (n=12) in the DNHS and participants with known pregnancy (n = 26) or missing PTSD phenotype data (n = 82) in the GTP. Analyses were conducted on unique participants that passed QC, as described below, and had PTSD data available (Table 2.1).

For data quality assessment, samples were checked for 1) low total signal (mean signal intensity less than half of the overall median, after setting probes with detection p-value > 0.001 or < 2,000 arbitrary units to missing); 2) > 1% of failed probes (detection p-value > 0.001); 3) outlying beta value distribution (i.e., smaller than three times interquartile range (IQR) from the lower quartile or larger than 3 times IQR from the upper quartile); 4) greater than three standard deviations of the mean bisulfite conversion control probe signal intensity[130]. Additionally, samples were checked for gender discordance based on median total intensity of X and Y-chromosome mapped probes (as implemented in *minfi*[152]) and removed if predicted sex differed from self-reported gender. Five samples were removed among DNHS samples for gender discordance, and six samples were removed among GTP samples (two for data quality and four for gender discordance). After within-array background correction and dye-bias equalization using out-of-band control probes (ssNoob[158,163]; *minfi*), probes with detection p-value > 0.001 in more than 10% of samples[155] and cross-reactive probes[159] (i.e., cross-hybridized between autosomes and sex chromosomes) were removed. Beta-mixture quantile (BMIQ) normalization (*ChAMP*[161,162]) was used to correct for type II probe bias[160].

To control for technical artifacts (e.g., sample processing and imaging batch effects), principal components (PCs) based on non-negative control probe signal intensity[156] were removed from BMIQ-normalized M-values (i.e., logit-transformed beta-values) separately for each study. PC correlation heatmaps were used to check for successful removal/reduction of batch effects, especially chip and row effects, while maintaining signal from biological variables. The DNHS and GTP datasets were then combined and an empirical Bayes method (i.e., ComBat[164] in the *sva* package[165]) was used on the combined M-values to account for study effects while controlling for sex and age. Data quality assessment, QC probe filtering, and first

step of batch removal were study-specific, while pre-processing steps (ssNoob+BMIQ) implemented within-array approaches unaffected by study. Only probes that passed QC in both studies (n = 455,072 probes) were included in the combined dataset.

Leukocyte Composition Estimation

Leukocyte composition was estimated on ComBat-adjusted beta-values using the *EpiDISH*[128] reference database, which is informed by cell-type specific DNase hypersensitive sites (DHS; based on the NIH Epigenomics Roadmap database[166]) and is optimized for discriminating granulocytes, $CD14^+$ monocytes, $CD8^+$ T cells, $CD4^+$ T cells, $CD19^+$ B cells, and $CD56^+$ natural killer cells. Robust partial correlation (RPC; robust multivariate linear regression, non-constrained projection) was used as the primary deconvolution algorithm and *EpiDISH's* implementation of linear, constrained projection (CP), originally introduced by Houseman et al. (2012)[167], was used to calculate a second set of estimates for comparison.

Ancestry Estimation

DNAm-based ancestry PCs were derived on cleaned beta-values after regressing out sex and age from batch-adjusted M-values. Ancestry PCs were calculated on a subset of 2,317 ancestry informative CpG probes included in two published ancestry informative CpG lists that accounted for confounders[168] and that included probes within 10 base pairs (bp) of single nucleotide polymorphisms (SNPs)[169]. The first 2 PCs based on this subset of probes were used as ancestry PCs (ancPCs) after checking for strong association with self-reported race and effective separation of self-reported races.

Statistical Analysis

The Shapiro-Wilk test was used to assess normality and Levene's test was used to compare equality of variance among groups. Since cell estimates had dissimilar, non-normal distributions, non-parametric tests were used for all initial group comparisons of leukocyte subtypes. The two-sample Kolmogorov-Smirnov (KS) test was used to compare distribution of cell estimates when variances were unequal between groups and Mann-Whitney U test was used to compare mean ranks of cell estimates otherwise. Spearman's rank correlation was used to assess agreement between estimates based on RPC and CP deconvolution approaches. A threshold of 0.05 was used for p-values and p-values were adjusted for multiple comparisons using Holm's method[170], unless otherwise specified.

To test our main hypothesis—that PTSD is associated with sex-specific differences in leukocyte composition—initial sex-stratified analyses were conducted on all leukocyte subtypes using the non-parametric Mann-Whitney U test. For leukocyte subtypes determined to be significantly associated with lifetime PTSD in either sex based on initial Mann-Whitney U tests, a two-way analysis of covariance (ANCOVA; Type III) controlling for age, ancestry (based on DNAm ancestry PCs), and current smoking, was performed with post-hoc comparison of estimated marginal means to examine the effects of sex and lifetime PTSD on transformed cell estimates. Transformation for cell estimate was conducted to meet modeling assumptions for ANCOVA and was informed by Tukey's Ladder of Powers. Power parameter (λ) was computed to maximize normality based on the Shapiro-Wilks W statistic. Sex-stratified Kruskal-Wallis and post-hoc Dunn tests were conducted as additional follow-up to investigate possible

differences in cell proportions by PTSD status (i.e., trauma-exposed controls, remitted PTSD, and current PTSD).

2.4 Results

Demographic characteristics of sampled study participants from the DNHS and GTP

The demographic characteristics of study participants included in primary analyses investigating sex-specific associations between DNAm-based cell estimates and lifetime PTSD are presented in Table 2.1. Of the 483 participants from the combined DNHS and GTP sample, 57.3% had a lifetime diagnosis of PTSD, 68.3% were female, and 38.7% were current smokers. The study population was predominantly African-American (89.2%), based on self-reported race, and the median age was 48 years (IQR: 17.5; 37.5-55 years).

Comparison of leukocyte subtype estimates by deconvolution approach

Good overall agreement was observed between RPC and CP estimates, as measured by Spearman's correlation (i.e., RPC-CP correlation), but CD8T cells showed much poorer agreement, $\rho_s(481) = 0.83$, relative to the other leukocyte subtypes, $\rho_s(481) > 0.94$ (Figure 2.1). Since the main objective of this study was to investigate sex-specific differences in leukocyte composition, comparison of RPC and CP estimates was stratified by sex. Sex-stratified RPC-CP correlation revealed that the largest difference in RPC-CP correlation between sexes was also found in CD8T cells, $|\Delta\rho_s| = 0.07$, such that females showed poorer correlation, $\rho_s(328) = 0.80$, than males, $\rho_s(151) = 0.87$. For the other leukocyte subtypes, the difference in RPC-CP correlation between sexes ($|\Delta\rho_s|$), ranged from 0.01 to 0.03, with $CD56^+$ natural killer (NK) cells having the second largest difference in correlation between sexes (female: $\rho_s(328) = 0.93$; male: $\rho_s(151) = 0.96$). In all leukocyte subtypes, except $CD19^+$ B cells, females had lower correlation

coefficients than males. Detailed results for RPC-based cell estimates are reported below and corresponding results based on CP-based estimates are reported in *Appendix A - Supplementary Materials*, due to strong agreement between findings from both sets of estimates.

Comparison of leukocyte subtype estimates by sex and lifetime PTSD

Cell estimates were compared by sex, lifetime PTSD, and study in each leukocyte subtype to establish overall differences. Significant overall sex differences were observed in the distributions of $CD56^+$ natural killer (NK) cell (KS: $D = 0.19$, *adj*. $p = 0.007$) and $CD8^+$ T cell (CD8T) proportions (KS: $D = 0.16$; *adj*. $p = 0.04$) in RPC estimates. Males showed greater variability than females for both NK and CD8T cells (male vs. female - IQR_{NK}: 6.2% vs. 4.15%; IQR_{CD8T}: 9.5% vs. 6.2%), as well as higher median NK (5.5% vs. 4.4%) and lower median CD8T (9.0% vs. 9.7%) cell proportions (Figure 2.2). No significant overall differences (i.e., in both sexes combined) were observed between lifetime PTSD cases and trauma-exposed controls in any leukocyte subtype (Mann-Whitney). Additional analyses comparing leukocyte subtype proportions between participating cohorts and assessing age effects in each cell type are reported in Appendix A.

Elevated monocyte proportions were associated with lifetime PTSD in males, but not females

Sex-stratified Mann-Whitney U tests revealed a significant difference in monocyte proportions between PTSD cases and controls in males (Figures 2.3 and 2.4). Males with lifetime PTSD had higher median monocyte proportions than trauma-exposed controls, $U = 2100$, $Z = -2.9$, $p = 0.004$, *adj*. $p = 0.026$, $r = 0.23$. In contrast, no difference in monocyte estimates was found between groups in females, $U = 13000$, $Z = -0.58$, $p = 0.6$, *adj*. $p = 1$, $r =$

0.03. Lifetime PTSD-associated differences were not observed in any other leukocyte subtypes in either sex.

A two-way ANCOVA was conducted to investigate whether sex moderated the effects of lifetime PTSD on transformed monocyte estimates, while accounting for age, ancestry, and current smoking (Table 2.2). Monocyte estimates were square root transformed for the ANCOVA to meet model assumptions (i.e., normality) and were informed by Tukey's Ladder of Powers (RPC: $\lambda = 0.43$; CP $\lambda = 0.5$). A significant interaction was found between sex and lifetime PTSD, $F(1, 461) = 4.89$, $p = 0.027$, $\eta_p^2 = 0.011$. Post-hoc comparison of estimated marginal means (EMMs) for lifetime PTSD by sex (Figure 2.5; Table 2.3) showed a significant mean difference between lifetime PTSD cases and controls in males, $\Delta EMM = 0.26$, $SE = 0.08$, $t(461) = 3.32$, $p = 0.001$, where mean monocyte estimates were higher in lifetime PTSD cases than controls. No significant mean difference was observed between PTSD cases and controls in females, $\Delta EMM = 0.05$, $SE = 0.05$, $t(461) = 0.89$, $p = 0.37$, confirming findings from initial sex-stratified analyses. Together, our results suggest that male PTSD cases have significantly elevated monocyte proportions compared to trauma-exposed controls and that this lifetime PTSD-associated difference is not observed in females.

Association between monocyte proportions and lifetime PTSD in males is independent of current PTSD status

To investigate whether participants with current PTSD exhibited a different monocyte profile from those with remitted PTSD, a sex-stratified Kruskal-Wallis test was conducted for PTSD status (i.e., trauma-exposed controls, remitted PTSD, and current PTSD; Figure 2.6). A significant difference in monocyte estimates was observed in males, $H(2) = 8.2$, $p = 0.017$, but

not in females, $H(2) = 1.1$, $p = 0.59$, confirming findings from analyses for lifetime PTSD. The post-hoc Dunn test revealed significant differences between PTSD case groups and trauma-exposed controls (current PTSD vs. controls: $Z = 2.31$, $p = 0.021$, *adj.* $p = 0.042$, $r = 0.23$; remitted PTSD vs controls: $Z = 2.40$, $p = 0.016$, *adj.* $p = 0.049$, $r = 0.22$), but no significant difference between current and remitted PTSD groups, $Z = 0.18$, $p = 0.86$, *adj.* $p = 0.86$, $r = 0.02$. These findings suggest that the association between monocyte proportions and lifetime PTSD in males is independent of current PTSD state and may reflect long-standing changes associated with lifetime history of PTSD diagnosis.

Comparative analyses based on CP monocyte estimates showed similar results to RPC-based results (see *Appendix A - Supplementary Materials*); however, CP-based results consistently presented smaller effect sizes than RPC-based results across all analyses and follow-up comparisons between PTSD case groups and trauma-exposed controls did not reach significance after p-value adjustment in post-hoc Dunn test using CP monocyte estimates in males.

2.5 Discussion

Methylomic profiles derived from peripheral blood offer a wealth of information and can be harnessed to detect two dynamic measures of immune state: 1) differences in leukocyte composition (i.e., proportions of peripheral immune cell subtypes); and 2) true alterations in methylation involved in epigenetic regulation of immune processes. They are particularly well-suited for investigating PTSD because DNAm encodes individual response to trauma and may play a key role in PTSD-associated immune dysregulation. Given the prominent sex differences in both PTSD prevalence[3,4,8,13-15] and immune response[64,126], the primary goal of the present

study was to investigate whether PTSD is associated with sex-specific differences in leukocyte composition, detectable by DNAm-based estimates. We found that lifetime PTSD was associated with a small but significant elevation in monocyte proportions in males, but not females. No difference in monocyte proportions was observed between current and remitted PTSD cases in males, suggesting that this sex-specific difference reflects a long-standing trait of lifetime history of PTSD diagnosis, rather than current state of PTSD. These findings were observed in both the primary RPC and comparison CP-based sets of cell estimates, which were derived using non-constrained vs. constrained projection deconvolution algorithms, respectively. Overall, our main finding of elevated monocyte proportions in males, but not females with lifetime history of PTSD provides evidence for a sex-specific difference in peripheral blood leukocyte composition that likely reflects long-standing changes associated with PTSD diagnosis and is detectable in methylomic profiles, consistently across different deconvolution algorithms.

In our study, we leveraged recent advances in reference-based deconvolution methods[128-130] – specifically the *EpiDISH* algorithm[128], which (i) employs DNase hypersensitive site (DHS) data of leukocyte subtypes to inform probe selection for their reference database and (ii) introduces RPC, a non-constrained projection approach for reference-based deconvolution. A comparative validation study on in-silico mixtures of purified cell DNAm profiles previously showed this newer RPC approach to consistently perform better than the widely used CP approach[167], based on root mean square error (RMSE) and R2, at low noise levels[128] typically encountered in real data[128,130]. Relevant to our results, the study showed the difference in RMSE and R2 to be the most prominent in CD8T cells[128]. This is consistent with our comparison between RPC and CP estimates, which showed CD8T cells to exhibit poorer correlation between RPC and CP estimates relative to other leukocyte subtypes and the largest difference in RPC-CP

correlation between sexes. Similarly, the validation study reported better performance of RPC, compared to CP, in monocytes, with higher RMSE and lower R2 in CP compared to RPC, suggesting RPC-based estimates were more robustly associated with true weights. In light of the validation study, this suggests that our RPC-based estimates were better able to resolve male-specific association of monocyte proportions with lifetime PTSD. In all, our results were consistent with the previously published validation study[128] and favored use of RPC estimates for modeling leukocyte composition. However, as these methods have been developed recently, further validation and comparative studies are warranted.

Comparison of leukocyte subtype estimates by sex revealed significant baseline sex differences in the distributions of NK cell and CD8T cell proportions, with males showing greater median NK and lower median CD8T cell proportions, using both RPC and CP based estimates. This finding is consistent with a previous study that reported sex differences in both leukocyte subtypes using estimates based on *minfi's* implementation of the Houseman approach[171] and with immunology literature that reported higher NK cell counts and proportions in males compared to females[172]. A recent study that modeled cell-specific methylation profiles also reported robust sex differences in $CD56^{+}$ NK methylation patterns[173], suggesting that this leukocyte subtype may be regulated by DNAm in a sex-specific manner. Additionally, DNAm dynamics have been found to drive effector functions in CD8T cells after stimulation[174,175]. Development of reference databases that resolve the six main leukocyte subtypes to consider proportions of subsets with shared lineage but different functionality/phenotype (e.g., naïve vs memory vs regulatory subtypes) may allow us to explore this hypothesis and would greatly enrich our understanding of immune activity.

Our main finding of elevated monocyte proportions in male lifetime PTSD cases is consistent with a previous study of Gulf War Illness (GWI) in a predominantly male veteran cohort, which reported an association between GWI and increase in monocyte count[176]. However, comparable studies reporting monocyte counts from differential leukocyte count are generally lacking; the majority of immune studies of PTSD have focused on inflammatory markers (e.g., cytokine levels) and cellular activity, including spontaneous and stimulated cytokine production, and studies of cell counts/proportions have focused on lymphocytes, particularly T-cell subsets[82,84,177-180]. Two PTSD studies that reported monocyte counts based on white blood cell differential count found no significant difference in monocyte proportions[181,182], which is consistent with our results in both sexes, but did not conduct sex-stratified analyses. Additionally, a small study in adult females that matched PTSD participants and controls for phase of menstrual cycle agreed with our female-specific results and reported no difference between PTSD subjects and controls in percentage of any lymphocyte subsets or total numbers of leukocytes, neutrophils, lymphocytes, or monocytes[183]. PTSD studies investigating peripheral lymphocyte numbers have reported mixed findings[85,107,178,179,181,184-188], but more recent studies that further resolved T-cell subpopulations support PTSD-associated differences in T-cell composition indicative of pro-inflammatory skew[94,107,188] and immunological aging[91]. Many of these studies were conducted predominantly in one sex (often in male veteran cohorts[107,184-188] and those based on both sexes did not conduct sex-stratified analyses[91,94,181]. To our knowledge, no authoritative study of sex differences in complete blood counts in PTSD has yet been published, and studies of sex differences in PTSD have generally been lacking, with a number of large-scale studies conducted in predominantly male military cohorts (or on female-only cohorts, e.g. Nurses' Health Study II[189,190]).

While not for PTSD, a study of depression that examined white blood cell differential count noted a significant increase in monocyte count and proportions among males with major depressive disorder (MDD), but not females, and a significant sex by diagnosis interaction[191]. Likewise, a separate longitudinal study following MDD inpatients also reported elevated monocyte counts in patients compared to controls, and this was driven by men[192]. Additionally, a decrease in depression severity was associated with a decrease in monocyte counts[192], suggesting that monocytes may be related to clinical improvement. Similarly, the presence and severity of atherosclerosis, another condition linked to PTSD via systemic low-grade inflammatory state[193], are also associated with an increase in monocyte count in males, but not females[194].

Further prospective investigation of PTSD is needed to determine whether the higher monocyte proportion observed in males reflects an increased susceptibility for developing PTSD or if it reflects an immunological shift in response to the precipitating trauma associated with PTSD psychopathology. However, studies in a male rodent model provide strong evidence for the latter and have been important for establishing the relationship between peripheral immune cells and the brain in the context of psychosocial stress and associated behavior. Repeated social defeat (RSD) was found to induce myelopoiesis and release inflammatory ($Ly6C^{hi}$) monocytes into circulation via sympathetic signaling, and this increased level of circulating peripheral monocytes was associated with recruitment of pro-inflammatory monocytes/macrophages to the brain and neuroinflammation[99,195,196]. Increased proportion of these peripheral monocytes and macrophage recruitment to the brain were also demonstrated to correspond with development, maintenance, and re-establishment of RSD-induced anxiety-like behavior; blockade of this recruitment (via splenectomy or β-adrenergic receptor blockage) before RSD was found to

prevent development of anxiety-like behavior[101,102]. Additionally, a recent paper discerned that stress-induced anxiety-like behavior and social avoidance are dependent on an increase in IL-6 after stress exposure, which induces a primed transcriptional profile in monocytes recruited to the brain and propagates IL-1β mediated inflammation associated with anxiety-like behavior[197]. These studies implicate peripheral monocytes in directly affecting relevant PTSD-like behavior after stress exposure in males[198,199]. Very recently, the first study using a modified version of the RSD paradigm was conducted in female mice and reported a similar onset of anxiety-like and social avoidance behavior, increase in myelopoiesis, increase in peripheral monocyte proportions, and recruitment of peripheral myeloid cells to the brain, 14 hours after the last RSD cycle[105]. Continued work based on this paradigm at multiple time points may be fruitful for investigating if there are sex differences in the kinetics of leukocyte trafficking and tissue distribution, especially since recent investigations in other PTSD-relevant rodent models suggest fundamental sex differences in neurobiological response to trauma exposure[81] and in regulation of stress/trauma-induced neuroinflammatory priming/neuroimmune alterations[200,201]. Furthermore, a social stress paradigm in pregnant rats reported lower numbers of monocytes in stressed females than control females rats[202], illustrating the importance of considering different paradigms and breeds/species.

Although no studies of PTSD have investigated sex differences in monocyte counts or methylomic profiles, chronic PTSD-associated sex differences were noted in transcriptional regulation[67] and gene expression[66] of $CD14^+$ monocytes isolated from peripheral blood. Given the inherent sex differences in innate immune response[64,203], as understood in the context of infection, injury, and treatment of inflammatory disorders, sexually dimorphic dynamics and effects may also exist in the context of neuroimmune response to stress/trauma exposure[201]. One

relevant sex difference in monocytes involves the expression of IL-6, which was suggested to be important for stress-induced anxiety-like behavior and social avoidance in the RSD model[197]. Independent of reproductive hormones (i.e., estradiol, dehydroepiandrosterone [DHEA], progesterone), women were shown to have greater monocyte expression of IL-6 across a circadian period than men, and sympathovagal balance was negatively associated with monocyte IL-6 expression only in women[204]. On the other hand, a study examining sex differences in regulation of inflammatory cell recruitment and cytokine synthesis found that ovarian hormones regulate phenotype, function, and numbers of macrophages, but not T lymphocytes, in females[205]. This fundamental sex difference may underlie more efficient recognition and elimination of infectious stimuli without recruitment of circulating neutrophils or excessive cytokine production in females, compared to males[205], and may also have implications in the context of psychosocial stress exposure. Relatedly, statin, which has anti-inflammatory activity, modulates monocyte migration in a sex-specific manner, such that both spontaneous and LPS-induced migration of isolated monocytes were found to be inhibited by statin in women, but not men[206].

Our observation of male-specific increase in monocyte proportions associated with lifetime PTSD may reflect fundamental sex differences in leukocyte trafficking, tissue distribution, and thus composition in blood, with implications for stress/trauma-induced neuroimmune alterations and behavior. Of note, while the effect size detected in males translates to an absolute difference of only 1.3% in monocyte proportions between participants with vs. without PTSD (~8.1% vs. 6.8%; Table 2.3), it also corresponds to an increase of approximately 19% in overall monocytes among men with lifetime PTSD. Furthermore, the lack of difference between remitted and current PTSD (in male-specific elevation of monocyte proportions) may

have a number of implications for PTSD pathophysiology, including adverse health consequences associated with PTSD across the life course in men, which may be distinct from PTSD-associated health trajectories in women[22,207,208].

Although the current dataset combined two cohorts and known pregnancies were excluded from our study, sample size and unavailable phenotype data on pregnancy, timing of the menstrual cycle, hormonal birth control use, well-harmonized measures of depression, and health status, as well as gender-related variables, such as coping mechanisms, are all limitations of this study. Future studies that account for hormone levels and other fundamental physiological sex differences may help identify female-specific associations between PTSD and leukocyte composition and clarify if hormone-dependent processes influence leukocyte composition dynamics. Additionally, both cohorts included in this study are civilian, urban, and predominantly African-American, so generalizability of our findings may be limited to this population.

Overall, our study implements current state-of-the-art methods to illustrate feasibility of using DNAm-based leukocyte composition estimates to probe immune profiles. Our literature-supported finding of male-specific elevation in DNAm-based monocyte proportions may be an informative metric to include as part of a diagnostic biomarker panel for PTSD in males, and future study in females, with consideration for hormonal status, may elucidate a female-specific panel as well. Differential methylation markers discovered in sex-stratified EWAS, which account for these cell estimates as covariates, are other prime candidates to be included in such sex-specific biomarker panels. Furthermore, in addition to being able to infer leukocyte composition when complete blood count data is not available, these DNAm-based estimates of leukocyte composition can be used to determine cell-specific differential methylation profiles.

In fact, methods and validation for cell-specific differential methylation analyses have been published very recently[209,210] and may enable the next significant advance in extracting insights from methylomic profiles by contextualizing how differential methylation in specific leukocyte subtypes alter regulatory dynamics in the immune system. Ultimately, this work may help to shape future studies designed to determine whether sex-specific methylomic metrics of peripheral immune status can inform us about sex differences in neuroinflammation and corresponding behavior in response to trauma exposure.

2.6 Conclusion

By combining DNA methylation datasets from two civilian cohorts, the current study found a small but significant elevation in monocyte proportion associated with lifetime PTSD in males, but not females. This sex-specific difference in peripheral blood leukocyte composition reflects a long-standing trait of PTSD diagnosis, rather than current state of PTSD. While this finding was confirmed using two different cell estimation approaches (i.e., deconvolution algorithms), the recently developed non-constrained projection approach (RPC) appears better suited for modeling leukocyte composition. Methylome-based characterization of immune profiles holds special promise for the study of PTSD and continued development of reference databases and validation of methods will build on these recent improvements to enrich our understanding of sex-specific immune dysregulation associated with PTSD.

2.7 Figures and Tables

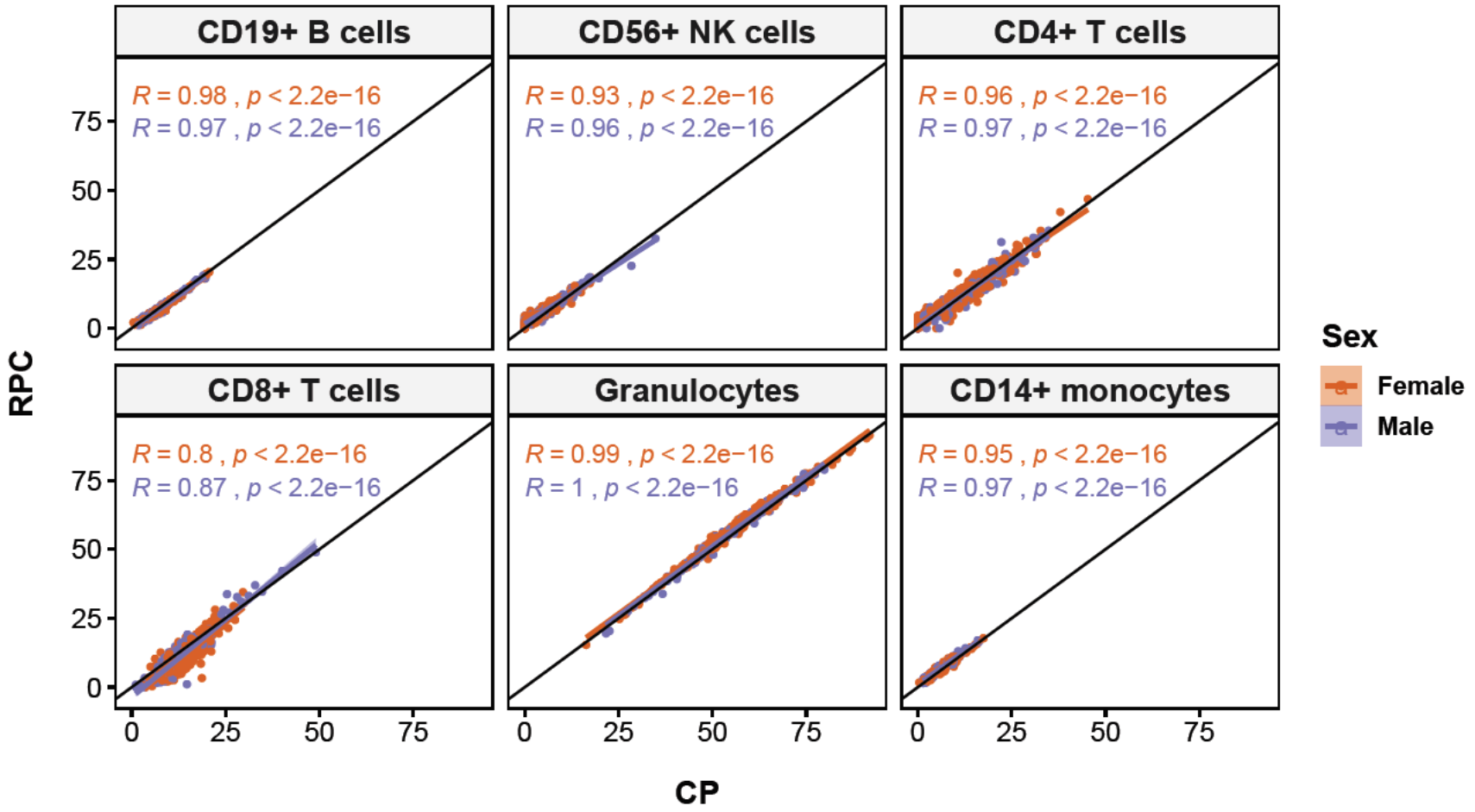


Figure 2.1: Correlation (Spearman's rho) between cell estimates derived using robust partial correlation (RPC) and constrained projection (CP) deconvolution algorithms is high in all leukocyte subtypes, with $CD8^+$ T cells (CD8T) showing the worst agreement at $R = 0.8$ in females and $R = 0.87$ in males. CD8T cells also showed the largest discrepancy in sex-specific correlation of estimates. RPC-CP correlation was > 0.9 and difference in RPC-CP correlation between sexes was between 0.01 and 0.03 for all other leukocyte subtypes.

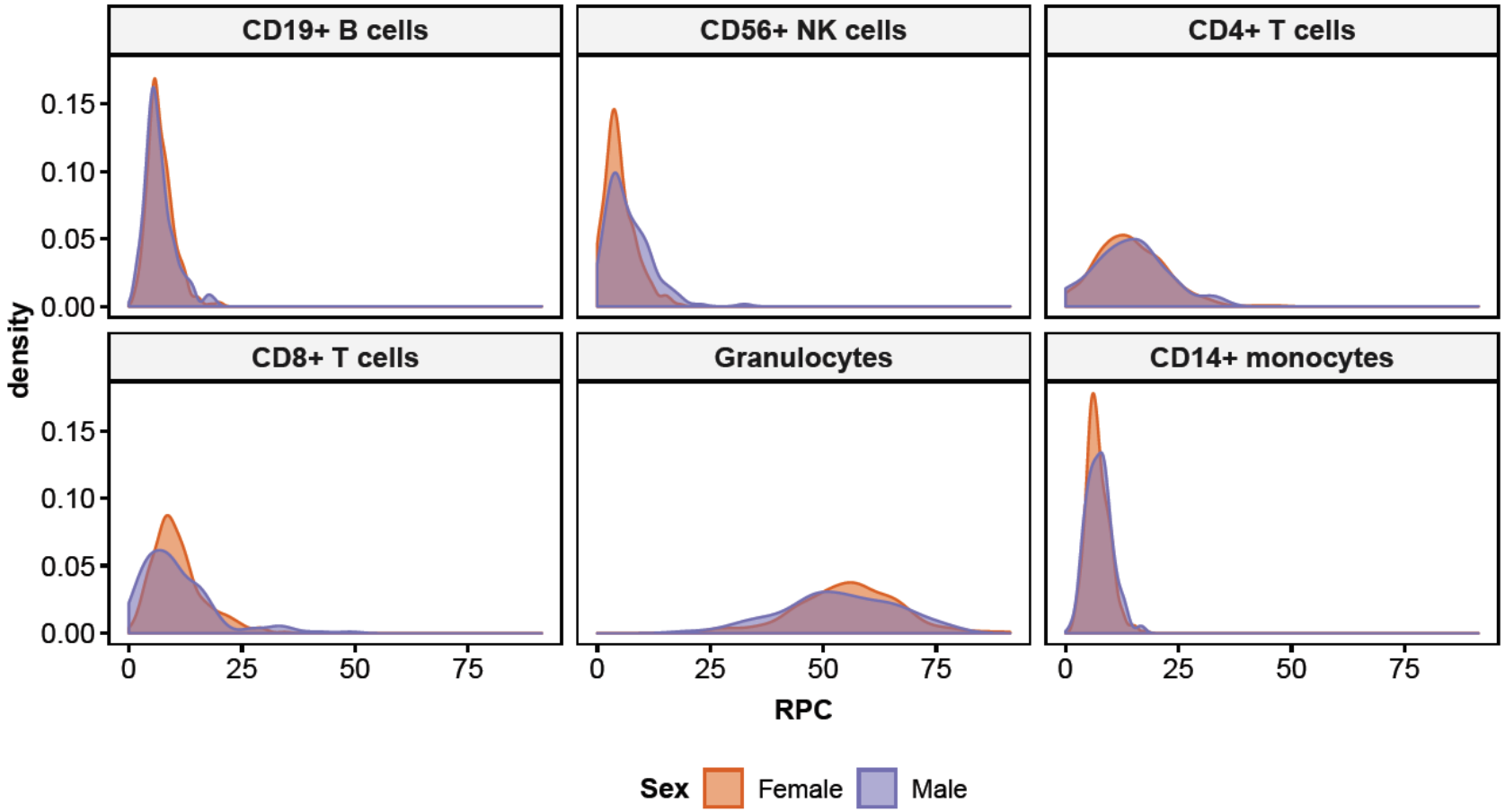


Figure 2.2. Distribution of leukocyte subtypes based on robust partial correlation (RPC) estimates, by sex. Sex differences in $CD8^+$ T and $CD56^+$ NK cell distributions were found to be prominent.

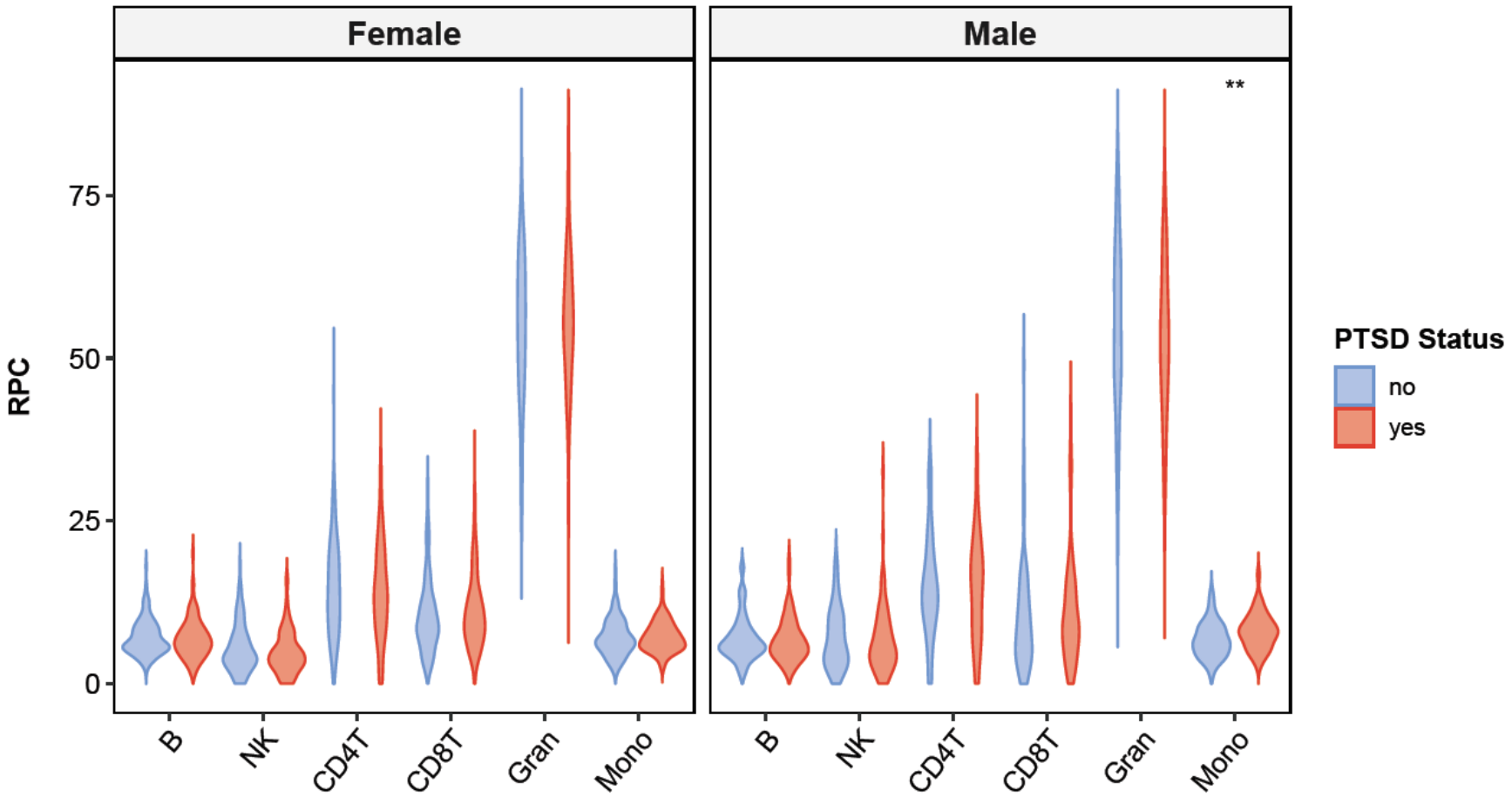


Figure 2.3: Violin plots of RPC estimates for lifetime PTSD cases and controls, stratified by sex. Only monocyte proportions in males showed statistically significant difference between lifetime PTSD cases and controls, based on Mann-Whitney U test (p-value = 0.004; Holm-adjusted p-value = 0.026). For figure labels on x-axis: B = $CD19^+$ B cells; NK = $CD56^+$ NK cells; CD4T = $CD4^+$ T cells; CD8T = $CD8^+$ T cells; Gran = Granulocytes; Mono = $CD14^+$ monocytes.

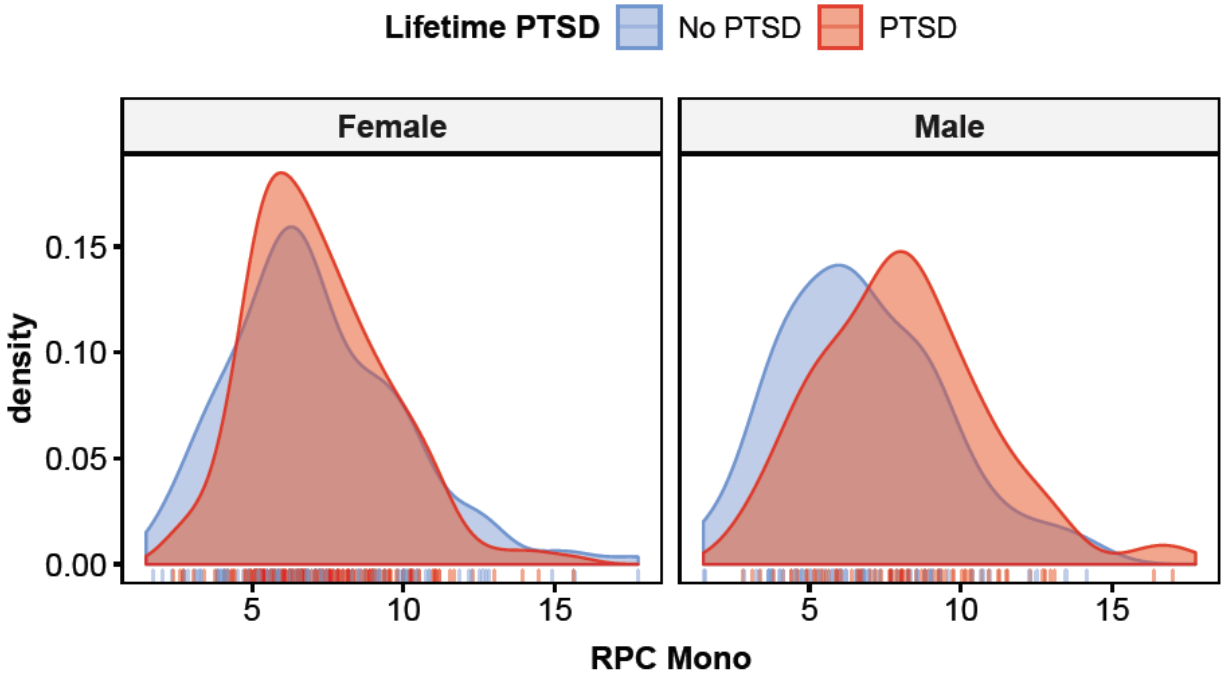


Figure 2.4: Density plots for RPC monocyte estimates in lifetime PTSD cases and controls, stratified by sex, show distinctly higher monocyte levels in males with lifetime PTSD compared to trauma-exposed cases. This difference in monocyte levels between lifetime PTSD case and controls is not observed in females.

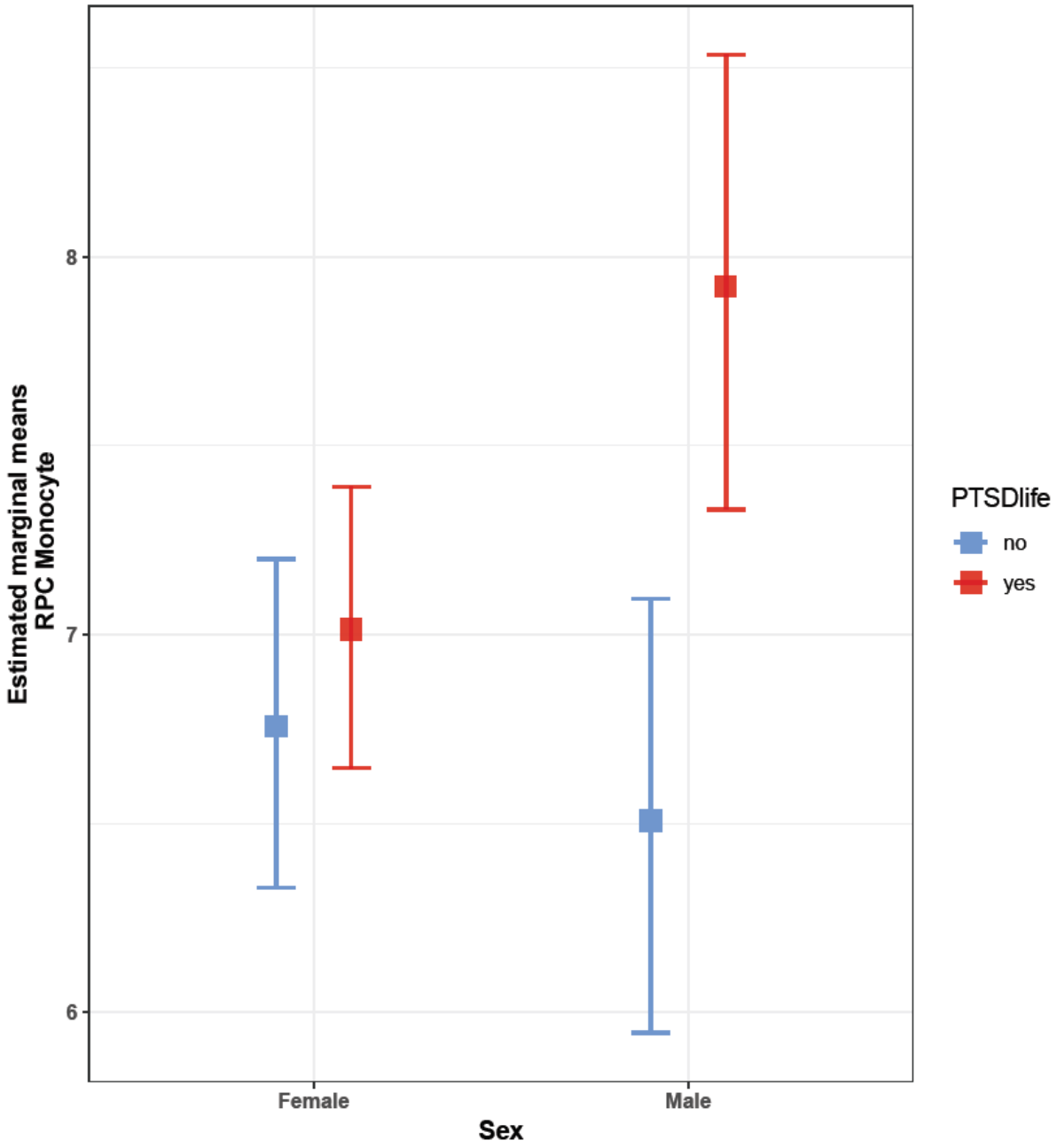


Figure 2.5: Lifetime PTSD by sex interaction plot for estimated marginal means (EMMs) of RPC monocyte estimates. Interaction plot shows a significant EMM difference between lifetime PTSD cases (red) and controls (blue) in males, where mean monocyte estimates are higher in cases than controls. No significant EMM difference was observed between PTSD cases and controls in females.

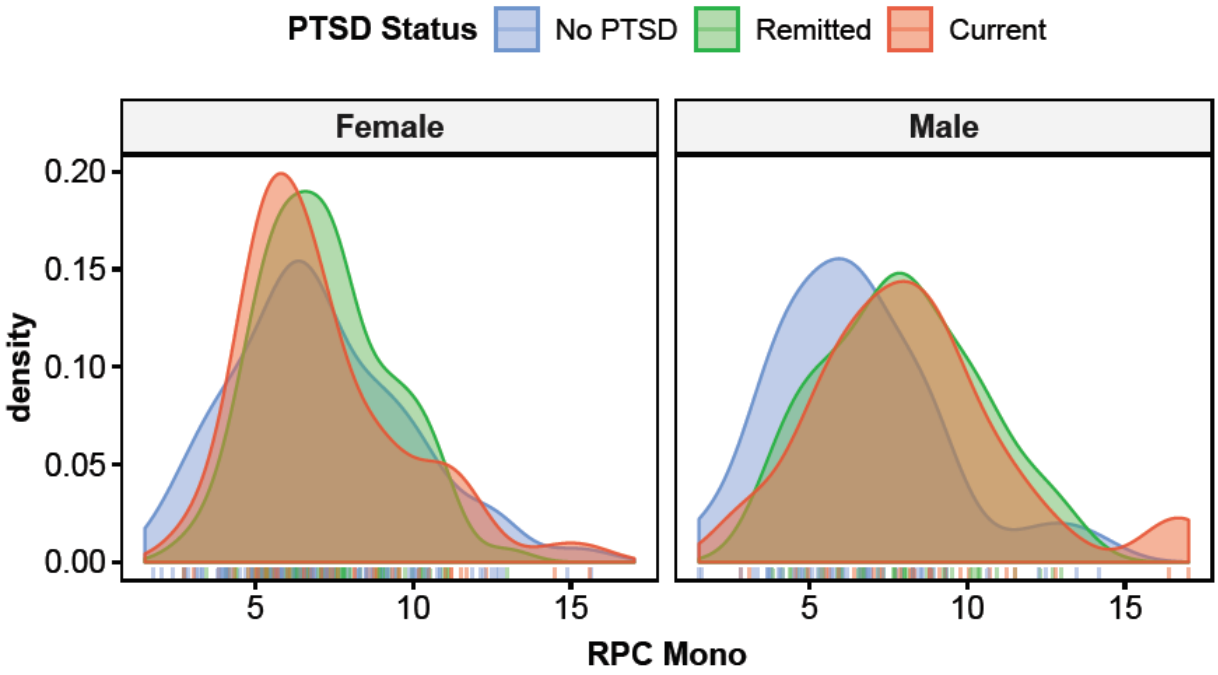


Figure 2.6: Density plots for RPC monocyte estimates comparing those with current PTSD, remitted PTSD, and trauma-exposed controls, stratified by sex. Distinguishing between current and remitted PTSD cases suggests that the significant peak difference in male PTSD cases is associated with long-standing PTSD trait, rather than current PTSD state. Corresponding post-hoc Dunn test revealed no significant difference between current and remitted PTSD cases and significant differences between PTSD case groups and trauma-exposed controls. Again, no significant differences were observed in females.

Table 2.1: Key demographic characteristics, by Sex

	Female (n = 330)	Male (n = 153)	Total (n = 483)
Study			
DNHS	111 (33.6%)	64 (41.8%)	175 (36.2%)
GTP	219 (66.4%)	89 (58.2%)	308 (63.8%)
Race			
AA	291 (88.2%)	140 (91.5%)	431 (89.2%)
CA	33 (10.0%)	11 (7.2%)	44 (9.1%)
Other	6 (1.8%)	2 (1.3%)	8 (1.7%)
Median Age			
	49.0 (38.0 - 55.0)	48.0 (36.0 - 56.0)	48.0 (37.5 - 55.0)
Current Smoking			
no	210 (63.6%)	72 (47.1%)	282 (58.4%)
yes	109 (33.0%)	78 (51.0%)	187 (38.7%)
Missing	11 (3.3%)	3 (2.0%)	14 (2.9%)
Lifetime PTSD			
no	135 (40.9%)	71 (46.4%)	206 (42.7%)
yes	195 (59.1%)	82 (53.6%)	277 (57.3%)

This table describes the subset of participants included in primary analyses investigating sex-specific associations between DNAm-based cell estimates and lifetime PTSD, by sex. For the 2-way ANCOVA, 14 participants were excluded due to missing current smoking data.

DNHS: Detroit Neighborhood Health Study; GTP: Grady Trauma Project

Table 2.2: Two-way ANCOVA Table for RPC monocyte estimates (n = 469)

Terms	Type III Sum of Squares	*df*	Mean Square	*F*	*p*	$_{\text{partial}}\eta^2$
Sex	0.336	1	0.336	1.469	0.226	0.003
PTSDlife	2.379	1	2.379	10.407	0.001***	0.022
Age	2.309	1	2.309	10.101	0.002***	0.021
ancPC1	0.000	1	0.000	0.001	0.977	0.000
ancPC2	0.219	1	0.219	0.957	0.329	0.002
Smoking	0.060	1	0.060	0.261	0.610	0.001
Sex:PTSDlife	1.146	1	1.146	5.011	0.026*	0.011
Residuals	105.399	461	0.229			

$^{**}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

Table 2.3: Summary for RPC monocyte estimates by group

Sex	PTSD	n	mean	SE	EMM	SE_{EMM}	lower.CL	upper.CL
Female	no	135	7.113	0.2443	6.758	0.2214	6.330	7.200
Female	yes	184	7.182	0.1691	7.014	0.1893	6.647	7.391
Male	no	70	6.803	0.3228	6.507	0.2926	5.945	7.095
Male	yes	80	8.103	0.3147	7.921	0.3063	7.331	8.535

This table describes untransformed RPC monocyte estimates by group (i.e., sex and lifetime PTSD); n = count per group; EMM = estimated marginal means (i.e., least squares means); SE = standard errors for regular means; SE_{EMM} = standard errors for EMM.

Lower and upper confidence limits are for 95% level. EMM and intervals were back-transformed from the square root scale to the original scale of cell subtype proportions (%). Significance level of alpha = 0.05 was used for EMM comparisons. Results for pairwise comparison were averaged over levels for current smoking. Degree of freedom was 461 and male lifetime PTSD cases were significantly different from other groups.

CHAPTER 3: SEX-SPECIFIC AND SHARED EXPRESSION PROFILES OF VULNERABILITY AND RESILIENCE TO TRAUMA IN BRAIN AND BLOOD

3.1 Abstract

While post-traumatic stress disorder (PTSD) is defined by behavioral/cognitive symptoms most directly relevant to brain function, it can be considered a systemic disorder characterized by a distinct inability to reinstate homeostasis after trauma. In this study, we investigated expression profiles of key PTSD-relevant tissues, namely blood, amygdala, and hippocampus, from a rat model of PTSD, to identify sex-specific and shared processes associated with individual differences in response to recent trauma exposure. Our findings suggest both shared and sex-specific mechanisms underlying individual differences associated with vulnerability and resilience to trauma in hippocampus, amygdala, and blood. By disentangling cell composition from transcriptional changes, we found higher proportions of hippocampal oligodendrocytes in the PTSD-like, extreme behavioral response (EBR) group for both sexes and also identified modules for transcriptional activity associated with group differences (ie., response to trauma) in the hippocampus that appeared to be sex-specific. By contrast, we found prominent sex differences, but no group differences, in amygdalar cell composition, and both shared and sex-specific modules representing PTSD-relevant transcriptional activity in the amygdala. Across amygdala and hippocampus, both sex-specific and shared processes were relevant to an overarching framework for EBR implicating disrupted TNFα/NFκB signaling and excitatory/inhibitory imbalance in dysregulated synaptic/structural plasticity with important implications for fear learning and memory. Our main finding in peripheral blood was consistent with the human literature and identified wound healing processes and hemostasis to be

upregulated in the resilient, minimal behavioral response (MBR) group across sexes, but disrupted in a sexually dimorphic manner in the EBR group. Unlike the varied characterization of the EBR group, characterization of MBR across blood, amygdala, and hippocampus suggests a common theme of upregulated wound healing and extracellular matrix (ECM) remodeling shared between sexes. In all, we identified differential oligodendrocyte proportions in hippocampus between PTSD-like EBR and resilient MBR, and identified processes and pathways that characterize the EBR and MBR-associated transcriptional changes across hippocampus, amygdala, and blood. The sex-specific mechanisms involved in EBR may contribute to the pronounced disparity in risk for PTSD, with women much more likely to develop PTSD.

3.2 Introduction

Identifying robust peripheral biomarkers that can be used for implementing early intervention and developing improved preventative or therapeutic strategies would be valuable for managing post-traumatic stress disorder (PTSD), especially since accessing human brain tissue is often not a viable option. This requires understanding the relationship between brain and peripheral tissue, such as blood, in the context of stress/trauma exposure. Evidence from the past decade has demonstrated a key role for immune dysregulation in PTSD[211,212]. In fact, while PTSD is defined by behavioral/cognitive symptoms most directly associated with brain function, it can be considered a systemic disorder involving physiological changes throughout the body across all stages of PTSD[213]. PTSD symptoms reflect a distinct inability to reinstate homeostasis after trauma, and they involve bidirectional crosstalk between the brain and the rest of the body,

prominently via peripheral blood, which serves as a conduit for neuroendocrine and immune signaling.

Notably, systemic inflammation may underlie the pathophysiology of PTSD, as well as the consistent link between PTSD and chronic medical conditions associated with aging, such as cardiovascular, metabolic, autoimmune, and neurodegenerative diseases[190,207,214-216], and other markers of accelerated aging[91,123,215-220]. This dysregulated inflammatory state is itself partially coordinated by maladaptive alterations of hypothalamic-pituitary adrenal (HPA) axis activity and sympathetic nervous system (SNS) sensitivity/responsivity[221,222], which affect both peripheral immune cells in blood and neuroimmune dynamics in brain. Since these systemic regulatory processes coordinate both brain and blood, ultimately, we would like to determine if transcriptomic signatures of peripheral immune status can inform us about neuroinflammation in the brain and corresponding behavior in response to stress. Furthermore, while not extensively studied in the context of stress and psychoneuroimmunology, these regulatory systems, involved in stress and immune response, are known to have prominent sex differences[64,65,203]. Thus, considering sex-specific mechanisms in cross tissue investigations is warranted for characterizing the alterations in peripheral and central nervous system immune states (e.g., neuroinflammation), in response to disrupted HPA, SNS, and immune signaling.

Gene expression (GE) profiles serve as a useful biological readout; taking GE "snapshots" across peripheral blood and key brain regions can help identify processes and pathways disrupted in PTSD that are shared across different tissues, particularly between easily accessible peripheral blood and brain. Animal models of PTSD are key resources that provide access to both blood and brain tissues and allow control of stress/trauma exposure. In this study, we draw from a publicly available dataset[223] that models PTSD using a cut-off behavioral

criteria, to assign outbred rats as "vulnerable" or "resilient" based on their behavioral response after stress/trauma exposure (i.e., predator scent stress)[44,224]. This model is able to differentiate individual behavioral response to stress from general stress response by grouping two extremes of behavior using "diagnostic" inclusion-exclusion criteria, to capture PTSD-like (avoidance/hyperarousal) and resilient/resistant[225] phenotypes as groups distinct from the general population (i.e., middle 50%, not included)[226]. By also including unexposed controls, we are able to distinguish: 1) differential response to stress exposure (vulnerable vs. resilient), which is analogous to human studies of PTSD cases and trauma-exposed controls; 2) individual response to stress exposure (vulnerable exposed vs. unexposed or resilient exposed vs. unexposed); and 3) general response to trauma exposure (exposed [vulnerable and resilient] vs. unexposed).

Here, we examine expression profiles of blood, amygdala, and hippocampus from this rat model of PTSD[223,226], to identify sex-specific and shared pathways associated with group differences in response to recent trauma exposure, in each of these key PTSD-relevant tissues. In addition to capitalizing on the strengths of this animal model, we disentangle transcriptional activity from cell composition (and/or other covariates) in the bulk expression profile and assess the contributions of cell composition and transcriptional activity to group differences in each tissue. In order to gain a systems-level understanding of transcriptional activity occurring in each tissue, we implement an unsupervised co-expression network approach – to identify groups of genes within a network (i.e., gene modules) and explore their association with sex-stratified group. Finally, we examine the identified modules across tissues to look for common themes.

3.3 Methods

Dataset

Non-normalized microarray datasets, deposited by the Daskalakis et al. (2014)[223] study, were obtained from the NCBI GEO database[227]. They consisted of 47 blood (GSE60280; 8 samples per group per sex, with one sample missing), 30 amygdalar (GSE60302; 5 samples per group per sex), and 30 hippocampal (GSE60303; 5 samples per group per sex) samples derived from a rat model of PTSD (detailed below) and acquired using the Illumina RatRef-12 v1.0 Expression BeadChip Array platform. Details regarding the dataset and sample processing protocols are published in the original paper[223]. All data import, processing and analyses were conducted in R (version 3.5.1)[151].

Animal Model of PTSD

The animal model of the PTSD used in Daskalakis et al. (2014)[223] was developed by Cohen and Zohar[226] and involved brief exposure (10 min) of adult outbred Sprague-Dawley rats to predator-scent stress (PSS), an ecologically valid stressor that mimics a life-threatening situation for rodents. Seven days after PSS exposure, the animals were tested for behavioral and physiological response to provocation, and those at either end of the response distribution were categorized as being either vulnerable (EBR; extreme behavioral response) or resilient (MBR; minimal behavioral response), based on statistically validated cut-off behavioral criteria (CBC)[224]. The elevated plus-maze (EPM) and acoustic-startle response (ASR) behavioral tests were used to assess anxiety and arousal, respectively. Comparisons of expression profiles were also made against PSS-unexposed controls (CON).

Quality Control and Data Processing

Minimal probe detection and inter-array correlation (IAC; Pearson's) were assessed to identify sample outliers. One blood and one hippocampus sample were excluded due to low signal (less than 4000 significantly detected probes at detection p-val < 0.01). Then, one sample outlier was identified and removed for each tissue dataset, based on low mean IAC calculated for all pairs of samples within each tissue dataset (standard deviations from mean IAC < -2.5). After sample removal, 45 out of 47 blood samples, 28 out 30 hippocampal samples and 29 out of 30 amygdalar samples were retained. Table 3.1 presents the breakdown of samples used in analyses by tissue, group and sex.

Expression datasets for each tissue were *normexp* background-corrected, quantile normalized, and $\log_2$ transformed, separately using *neqc* in the *limma* package[228,229]. For background correction, observed intensities were fit to a normal+exponential convolution model, estimating model parameters using detection p-value to infer mean and variance of negative control probes. Probes were filtered for low expression in each tissue dataset and annotation quality (out of 22,523 total probes). Retained probes had a detection p-value < 0.01 in at least 10% of subjects, consistent chromosome mapping between annotation sources, and mapping to human orthologs. For 1:1 gene:probe mapping, genes mapped to multiple probes were represented by the probe with highest mean expression levels across samples in each tissue dataset. After data processing, each tissue dataset retained 4,901 expressed genes (represented by 5,065 probes) in blood, 6,653 genes (represented by 6,869 probes) in hippocampus, and 6,811 genes (represented by 7,029 probes) in amygdala.

Annotation

Probe annotations were based on platform, organism, and transcriptome level annotations packaged in Bioconductor (version 3.7)[153,154] databases. Re-annotated gene symbols (based on re-mapping of Illumina Rat v1 probe sequences with ReMOAT[230]; *illuminaRatv1.db*) were used to retrieve updated NCBI Entrez Gene IDs (April 2018; *org.Rn.eg.db*). Entrez Gene IDs were used to map genes to UCSC rn5 genome coordinates (*TxDb.Rnorvegicus.UCSC.rn5.refGene*; April 2018) and predicted human orthologs in the Molecular Signatures Database (MSigDB v6.2.1, released July 2018; *msigdbr*). For multi-mapping genes, human ortholog is chosen within MSigDB based on support by the largest number of databases (eggNOG, Ensembl Compara, HGNC, HomoloGene, Inparanoid, NCBI Gene Orthology, OMA, OrthoDB, OrthoMCL, Panther, PhylomeDB, TreeFam and ZFIN).

Brain Cell Proportion and Surrogate Variable Estimation

Since no validated reference datasets were available for peripheral blood and brain cell types in rats, cell estimation was attempted using marker sets constructed in other species. Brain cell subtypes were estimated using the *BRETIGEA* package, which includes well-conserved consensus brain cell marker sets identified across mouse and human datasets[231] and implements a singular value decomposition approach (SVD), based on CellCODE[232]. This marker set from combined human and mouse measurements contains 1000 markers each for six brain cell types: astrocytes (ast), endothelial cells (end), microglia (mic), neurons (neu), oligodendrocytes (oli), and oligodendrocyte progenitor cells (opc). Of these, only markers expressed in each brain dataset were used for cell estimation (Table 3.2).

Leukocyte cell estimation was attempted using a recently published mouse reference signature matrix, consisting of 511 distinguishing genes for 25 immune cell types[233], modeled after the human LM22 signature matrix constructed for use with the CIBERSORT[234] deconvolution approach. However, this reference dataset was inadequate for deriving reliable leukocyte cell estimates: only 142 out of the 511 signature genes were expressed in our blood dataset. Comparison between mouse and rat genomes have previously revealed significant genomic differences in immune system-related genes between the two rodent models[235]. Notably, rats have higher evolutionary rates for these genes and thus possess immune-related genes not found in mouse[235]. Thus, cell estimates were only calculated for brain tissues (i.e., hippocampus and amygdala).

In addition to estimation of brain cell proportions, we conducted surrogate variable analysis[236] on processed expression datasets to derive surrogate variables (SVs) for each tissue, while accounting for sex-stratified group, defined as each individual response category by sex (six levels, e.g.., female EBR, male MBR, female CON). There were 4 SVs identified in hippocampus, 5 SVs identified in amygdala, and 9 SVs identified in blood. Computed SVs were used to account for expression heterogeneity associated with technical and biological artefacts, and are assumed to proxy contributions of cell composition to expression heterogeneity (among other latent sources of confounding)[237].

Cell Composition Analysis in Brain Tissue

Shapiro-Wilk test was used to assess normality and Levene's test (using median as center; modified Brown-Forsythe) was used to compare equality of variance among groups. Since not all cell subtype estimates had normal distributions (by population), Mann-Whitney U

test was used to compare mean ranks of cell estimates for initial comparisons by trauma-exposed group (EBR vs MBR) and sex, across cell subtypes, when equality of variance was met. Two-sample Kolmogorov-Smirov (KS) test was used to compare distribution of non-normal cell estimates when variances were unequal between groups. For cell subtypes of interest that met modeling assumptions, one-way analysis of covariance (ANCOVA) was used to test main effect of each variable (EBR vs MBR). Kruskal-Wallis and post-hoc Dunn tests were used for group comparisons including trauma un-exposed controls. A threshold of 0.05 was used for p-values and p-values were adjusted for multiple comparisons using Holm's method[170] in cell composition analyses.

Differential Expression and Gene Set Enrichment Analyses in Blood

Since we were not able to estimate cell proportions in blood and had more samples in the blood dataset than in datasets from brain tissues, we conducted differential expression analyses in blood, to cross-check with network analyses. Association between group and gene expression due to transcriptional changes was assessed by fitting a linear regression model of gene expression as a function of sex-stratified group while accounting for SVs as covariates in the model. Sex-stratified contrasts were applied to the model fit to investigate EBR vs. MBR, EBR vs. CON, and MBR vs. CON differences. The linear model and sex-stratified contrasts were fitted using an empirical Bayes approach in *limma* to compute moderated t-statistics and $\log_2$ fold change (logFC)[228,238] for each probe in each comparison, to determine differential expression (DE) for each sex-stratified comparison (FDR-adjusted p-value < 0.05).

Pre-ranked gene set enrichment analysis (GSEA)[239] was conducted for each sex-stratified comparison using a sorted vector of logFC as input. The *fgsea* algorithm for fast gene set

enrichment analysis[240] was implemented via *clusterProfiler*[241] for gene sets in the hallmark gene set collection from the Molecular Signatures Database (MSigDB v6.2, released July 2018)[239,242] that had a minimum of 10 and maximum of 500 genes. The p-value of the enrichment score (representing the degree to which a gene set is over-represented at the top or bottom of the ranked logFC list) was calculated by permutation test (1000 permutations) and adjusted for multiple hypothesis testing using the Benjamini-Hochberg (BH) method[243] (*adj p* < 0.05).

Gene Co-expression Network Analysis

Signed co-expression gene network construction and gene module discovery were conducted using the *CEMiTool* package[244], which implements a novel soft threshold (β) selection algorithm, distinct from the original weighted gene co-expression network analysis (WGCNA) approach[245]. The β power parameter selection was conducted after variance-based filtering of expression data, using default settings. For construction of signed co-expression networks, the weighted network adjacency matrix was calculated by raising the co-expression similarity measure to the selected β, and biweight midcorrelation (bicor) was used as the co-expression measure, unless otherwise noted. For module detection, genes were assigned to modules using the dynamic tree cutting method[246] on branches of the hierarchical clustering dendrogram of the signed topological overlap dissimilarity matrix (1-TOM). Modules with eigengene correlations > 0.8 were merged and minimum module size was set to 30 genes. Group differences for activity of identified gene modules (i.e., upregulation or downregulation; induced/repressed) were determined via *CEMiTool* using the *fgsea* algorithm[240], treating genes in co-expressed modules as gene sets and the z-score normalized expression of samples within each group as rankings on the analysis. Unsupervised network analyses in brain tissues were conducted on

both SV and cell proportion-adjusted expression data, while analyses in blood were conducted on SV-adjusted data only. Network analyses were also conducted on unadjusted expression data for all tissues for comparison, to infer whether modules represented general processes independent of cell proportions or if they may be linked to cell proportions and other covariates.

Gene set analyses were conducted for MSigDB v6.2 gene set collections (not including cancer-specific collections)[239,242] using hypergeometric testing (over-representation analysis) as implemented in *clusterProfiler*[241], for modules of interest identified in gene co-expression network analyses (*adj p* < 0.05; BH p-val adjustment; gene set with 10-500 genes; qval cutoff 0.2). To identify potential hubs, module graphs were constructed for protein-protein interactions (PPI) from the human PPI dataset on BioGRID[247,248]. Appendix B contains all gene co-expression network analysis and gene set analysis results, including CEMiTool reports for all analyses with modules detected and tabulated results for gene set analyses of all MSigDB collections with significant results.

3.4 Results

Higher proportions of hippocampal oligodendrocytes observed in EBR compared to MBR and CON groups, in both sexes

Non-parametric Mann-Whitney U test revealed higher median proportions of hippocampal oligodendrocytes in the extreme behavioral response (EBR; PTSD-like) group compared to the minimal behavioral response (MBR; resilient) group in both sexes, $Z = -2.78$, *Holm-adj* $p = 0.024$, $r = 0.66$ (Figure 3.1). ANCOVA testing the effects of group and sex confirmed significant group differences (EBR vs MBR), $F(1,15) = 11.4$, $p = 0.004$, and no significant sex differences, $F(1,15) = 0.05$, $p = 0.82$, in oligodendrocyte proportions (Table 3.3).

A small, yet significant, difference in hippocampal oligodendrocyte proportions was also observed between EBR and trauma-unexposed control (CON) groups by Kruskal-Wallis, $H(2) = 9.3$, $p = 0.01$, and post-hoc Dunn test (Table 3.4). No significant difference was observed between MBR and CON groups.

Additionally, unsupervised network analysis of hippocampal expression levels before cell subtype adjustment identified a gene module significantly associated with upregulation in EBR and downregulation in MBR groups of both sexes, as well as downregulation in the female CON group (M2: 104 genes; Figure 3.2A). While the module was not significantly enriched for GO terms, in the curated chemical and genetic perturbation gene set collection (C2 CGP) from the MSigDB database, the top hit enriched in this EBR – MBR module was for oligodendrocyte markers (*adj p* = 0.009; 7 out of 45 genes in *LEIN_OLIGODENDROCYTE_MARKERS*). Comparison of modules from network analyses before and after cell subtype adjustment confirmed the module enriched for oligodendrocyte markers was lost after adjustment, suggesting the enrichment was related to hippocampal cell proportions. Together, results from cell composition and unsupervised network analyses suggest that an increase in hippocampal oligodendrocyte proportions occur in the PTSD-like EBR group, but not the resilient MBR group, in response to trauma exposure. Group differences were not detected in any other hippocampal cell subtype.

PTSD-relevant hippocampal gene expression modules are sex-specific

Network analyses conducted on the full (both sexes), cell-adjusted hippocampal expression data identified a module associated with upregulation in the female EBR group, weaker upregulation in the male MBR group and downregulation in the unexposed CON group

from both sexes (M1: 165 genes; Figure 3.2B). Top GO terms enriched in this module were cellular response to endogenous stimulus, response to organic cyclic compound, and response to lipid. Relatedly, top hits from other gene set collections identified enrichment for genes up-regulated after stimulation with NRG1 and EGF in the C2 CGP collection and TNFα signaling via NFκB pathway in the MSigDB Hallmark collection, implicating activation of ligand-receptor signaling as one possible mechanism for significant processes identified among GO terms. While there were no other modules based on adjusted hippocampal data to confirm the MBR – CON comparison in males, the EBR – CON comparison in females was confirmed in modules identified from SV-adjusted hippocampal data (female subset and full set). The EBR – CON module identified from the female subset of SV-adjusted hippocampal data (M2: 144 genes; Figure 3.3A) was significantly enriched for all the top gene sets identified with the M1 module from cell-adjusted hippocampal data (Table 3.5) and the female EBR – CON module based on the full SV-adjusted dataset (M1: 81 genes; Figure 3.2C) was significantly enriched for the top hallmark and C2 CGP terms, but not GO terms. Of note, the gene set for genes upregulated after NRG1 stimulation was identified as the top hit in the C2 CGP collection in all three EBR – CON modules and module gene members (i.e., genes in the module that overlap with genes in gene set) in the EGF signaling gene set were a subset of module gene members in the NRG1 signaling gene set. Thus, while both gene sets were top hits, upregulation by NRG1 signaling is endorsed over EGF signaling in our results. In summary, analyses of both cell and SV-adjusted hippocampal data provided consistent evidence implicating upregulation of genes after NRG1 stimulation, and TNFα signaling via NFKB pathway in the female EBR group, but not in the male EBR group; in fact, the M1 module based on full, cell-adjusted data suggests that enriched processes are associated with the MBR group in males.

Sex-stratified network analyses on SV-adjusted data detected a second module in the female subset, which was associated with upregulation in MBR and downregulation in the EBR group (M1: 170 genes; Figure 3.3B). This female-specific MBR – EBR module was significantly enriched for neurological system process, proteinaceous extracellular matrix (ECM), extracellular structure organization, ensheathment of neurons, and long-term synaptic potentiation among GO terms (Figure 3.4). ECM relevant terms were corroborated by significant enrichment for ECM receptor interaction among KEGG pathway gene sets (*adj p* = 0.009, 6/31genes). Interestingly, the top hit in the C2 CGP collection was for genes with high-CpG density promoters (HCP) bearing histone H3K4me3 and H3K27me3 in brain (adj p = 0.0012, 28/387 genes; *MEISSNER_BRAIN_HCP_WITH_H3K4ME3_AND_H3K27ME3*). In embryonic stem cells, genes containing HCPs enriched with both H3K4me3 and H3K27me3 histone marks (associated with transcription initiation and repression, respectively) were found to be highly regulated developmental genes[249], and this histone state is itself dynamic and correlated with DNA methylation levels[250]. Thus, enrichment for this gene set suggests the module is dynamically regulated by this epigenetic mechanism.

Network analyses on the male subset of SV-adjusted hippocampal expression data most notably identified one module associated with upregulation in the EBR group (M2: 101 genes; NES 1.75; Figure 3.3C). This EBR-related module was significantly enriched for regulation of GABAergic synaptic transmission, the TCR pathway, G alpha I signaling events, G-protein coupled receptor (GPCR) signaling, and transcription factor targets (TFTs) for GR, CEBP, and LFA1 (Table 3.6).

Prominent sex differences in amygdalar cell composition is accompanied by both shared and sex-specific PTSD-relevant expression modules

While no significant group differences were detected for any cell subtype in the amygdala (*Holm-adj* $p < 0.05$), prominent sex differences were observed in the distribution of cell subtype proportions, based on Levene's and Kolmogorov-Smirnov tests (Table 3.7). Females, regardless of group, showed significantly greater variance in distribution of relative cell proportions for astrocytes (ast), neurons (neu), oligodendrocytes (oli), and oligodendrocyte progenitor cells (opc), compared to males (Figure 3.5).

Network analysis of amygdalar expression levels after cell subtype adjustment identified three modules putatively associated with group, either shared between sexes or sex-specific (Figure 3.6). Module M2 (59 genes) was associated with upregulation of MBR compared to CON in both sexes, and was enriched for GO terms related to extracellular matrix (ECM), including collagen fibril organization (Figure 3.7A), which were corroborated by significant enrichment to matrisome-related terms in the C2 CP collection and ECM organization among REACTOME pathways. Module M3 (55 genes) was weakly associated with downregulation in EBR groups of both sexes and upregulation in the female MBR group. The top GO terms enriched in this module were for GPCR signaling pathway, synaptic signaling and somatodendritic compartment (Figure 3.7B). Enrichment results in the REACTOME pathway collection corroborated significant GO hits and suggested enrichment for regulation of insulin secretion by glucagon-like peptide1 and GABAβ receptor activation (Figure 3.8A). In all, cell-adjusted network analyses suggest significant upregulation of processes involved in ECM organization in the MBR relative to unexposed CON, and downregulation of GPCR and synaptic signaling in the EBR group in the amygdala, which is shared by both sexes. However, we note

downregulation of these pathways/processes in EBR is differentiated from the MBR group only in females.

Sex-stratified network analyses on cell-adjusted amygdalar expression levels did not identify any notable PTSD-relevant module in the male subset but identified one module in the female subset associated with upregulation in the MBR group and downregulation in the EBR group (M2: 40 genes). Top GO terms enriched in this module were GPCR signaling pathway, neurological system process, and behavior (Figure 3.8B); these top GO hits were corroborated by significantly enriched terms among REACTOME gene sets. Additionally, the module was enriched for *BRAIN_HCP_WITH_H3K4ME3_AND_H3K27ME3* (C2 CGP; *adj p* = 0.0003; 13/422 genes).

Network analyses based on the full SV-adjusted dataset did not identify any notable PTSD-relevant modules, but sex-stratified network analyses on SV-adjusted data identified one large module, using Spearman correlation as the co-expression measure, in the male amygdalar subset and three modules in the female subset (Figure 3.9). In females, module M3 (108 genes), associated with downregulation in EBR and upregulation in MBR, was significantly enriched for GO terms related to TF activity (RNAPII core promoter, proximal region sequence-specific binding), particularly for transcriptional activator activity, and muscle tissue/structure development. Analyses in other gene set collections identified top enrichment for TNFα signaling via NFκB (*adj p* = 1.5e-08, Hallmark), four gene sets associated with the SRF transcription factor (C3 TFT), and genes downregulated in neurons after NPAS4 knockdown by RNAi (*adj p* = 1.34e-06).

In males, the identified PTSD-relevant module was associated with downregulation in the EBR and relative upregulation in the CON group (M1: 453 genes). The top GO term enriched in

this module was GPCR signaling pathway, which was corroborated by significant terms from the REACTOME gene set collection and agreed with the top GO term associated with module M3 from cell-adjusted amygdalar data, suggesting this pathway may be generally downregulated in the EBR group from both sexes. Other GO terms significantly enriched in this male M1 module were response to organic cyclic compound/lipid/(steroid) hormone/nitrogen compound. The module was also significantly enriched for neuroactive ligand receptor interaction (KEGG, *adj p* = 0.016), TNFα signaling via NFκB (Hallmark, *adj p* = 0.002), hypoxia (Hallmark, *adj p* = 0.03), and *MEISSNER_BRAIN_HCP_WITH_H3K4ME3_AND_H3K27ME3* (C2 CGP, top hit, *adj p* = 5.06e-09).

Wound-healing processes in blood are upregulated in MBR across sexes, but sexually dimorphic in EBR

While no modules were identified on the full SV-adjusted data, sex-stratified network analyses of SV-adjusted blood expression levels (Figure 3.10A,B) identified a module upregulated in the MBR group compared to the unexposed CON group in both males (M1: 134 genes) and females (M1: 186 genes). In both sexes, the module was significantly enriched for GO terms relevant to wound healing, such as platelet activation, response to wound healing, and hemostasis, as well as regulation of body fluid levels (Figure 3.11), and these terms were corroborated in other gene set collections for platelet-specific genes (C2 CGP, top hit in both), hemostasis (REACTOME). Interaction networks constructed from sex-stratified SV-adjusted blood expression levels identified *FOS*, *ALOX12*, *PCMT1*, *YWHAH*, and *SSX2IP* as common hub genes in both male and female subsets (Figure 3.12).

Sex-specific enrichment was also observed in this MBR – CON module. In males, the module was significantly enriched for GPCR signaling pathway and this was corroborated by significant hits from the REACTOME gene set collection. Additionally, the top hit among KEGG pathways was vascular smooth muscle contraction, which had 7 out of 39 genes in common with the male MBR – CON module. Of these, *PPP1CC* was identified as a significant interaction hub gene in the module's interaction network (Figure 3.12A) and was notably found to be differentially expressed between MBR and CON groups in males, but not in females. Furthermore, this gene was not included in the female MBR – CON module, suggesting *PPP1CC* may be a key gene overexpressed in the male MBR group and relevant to male-specific differences in individual response to trauma.

In females, the MBR – CON module was enriched for biological adhesion and extracellular space and significant hits among REACTOME pathways corroborated GO terms, involving cell junction organization, cell-cell communication, and hemostasis. This module was also enriched for the complement pathway in the hallmark collection and for TFTs of MEF2, HNF1, and SRF, implicating these transcription factors in putatively coordinating MBR-associated upregulation in females.

No modules were detected before SV adjustment in males, but the MBR – CON module was identified in unadjusted blood expression levels for both the female subset (M1: 157 genes; Figure 3.10C) and full set (M2: 89 genes; Figure 3.10D). In fact, the modules based on unadjusted data were more significantly enriched for shared GO terms and other top hits. This indicates that MBR upregulation of wound healing processes, particularly involving hemostasis/platelets, and regulation of body fluid levels are independent of MBR associations with other covariates, which were modeled as 9 SVs in blood. We also observed this module

was more strongly enriched in females (MBR.F: NES = 3.7; CON.F: NES = -3.44) than in males (MBR.M: NES = 1.66; CON.M: NES = -2.2).

Interestingly, while upregulation in the MBR group is shared between sexes, the M2 module based on the full unadjusted blood expression data shows sex differences in the EBR group, such that female EBR is upregulated while male EBR is downregulated for this wound process-related module (Figure 3.10D). In fact, the downregulation in the male EBR group has a greater effect than the male control group (EBR.M: NES = -2.64; CON.M: NES = -2.2). This suggests there may be a sex-specific EBR response to stress/trauma for the processes represented in this module. As this sexually dimorphic association is observed only in this module on the full, unadjusted data and not detected in sex-stratified SV-adjusted modules, enrichment terms unique to this module may be most relevant for these sexually dimorphic EBR effects. The reactive oxygen species (ROS) pathway was identified as a top hit only in the full, unadjusted M2 module, implicating response to ROS/oxidative stress as a high priority pathway for sex differences in the EBR group.

In addition to these MBR-related findings, a module associated with downregulation in EBR and relative upregulation in unexposed CON group was identified in females (M2: 112 genes; Figure 3.10B). This female-specific CON – EBR module was significantly enriched for GO terms related to immune and defense response, as well as coagulation, IL6-JAK-STAT3 signaling, and interferon gamma response in the hallmark gene set collection. These hallmark processes were also significantly associated with female EBR vs. MBR and EBR vs. CON contrasts in gene set enrichment analyses of differential expression results (Appendix B). Notably, interferon gamma (IFNγ) response was the top hit in gene set enrichment analyses of

both the female EBR vs. MBR and EBR vs. CON comparisons, suggesting IFNγ may be a key player involved in PTSD-like response to trauma in females.

3.5 Discussion

In this study, we investigated expression profiles of key PTSD-relevant tissues, namely blood, amygdala, and hippocampus, from a rat model of PTSD, to identify sex-specific and shared processes associated with individual differences in response to recent trauma exposure. By estimating cell proportions from brain expression profiles, we found higher proportions of hippocampal oligodendrocytes in the PTSD-like EBR group compared to the resilient MBR and unexposed CON group in both sexes, and this was supported by enrichment for oligodendrocyte markers in network analyses on unadjusted hippocampal data. While no group-related differences in cell proportions were detected in the amygdala, prominent sex differences were noted, with females showing significantly greater variance in distribution of relative cell proportions for astrocytes, neurons, oligodendrocytes, and oligodendrocyte progenitor cells. Cell proportion estimates and surrogate variables (SVs) were also used for data adjustment in network analyses to identify gene modules reflecting transcriptional activity, rather than coordinated expression reflecting a mix of cellular composition and transcriptional activity. By accounting for cell proportions and surrogate variables (SVs), we were able to identify a number of shared and sex-specific gene expression modules reflecting group differences in transcriptional activity, in hippocampus, amygdala, and blood.

Hippocampus

Our finding of higher hippocampal oligodendrocyte proportions in the EBR group is consistent with reports from a recent study that used a metric based on magnetic resonance imaging (MRI) to estimate hippocampal myelination in male veterans with and without PTSD (due to combat trauma)[251]; veterans with PTSD had significantly more hippocampal myelin than trauma-exposed controls, and there was a positive correlation between hippocampal myelination estimates and PTSD symptom severity[251]. Relatedly, in rats, immobilization stress and corticosterone have been demonstrated to induce oligodendrogenesis in the dentate gyrus (DG) of adult hippocampus[252], suggesting stress induces hippocampal myelin formation. In our study, post-hoc Dunn test for hippocampal oligodendrocytes showed a stronger effect size in the EBR vs. MBR comparison than the EBR vs. CON comparison and no significant difference between the MBR and CON groups, suggesting that differences in hippocampal oligodendrocyte proportions reflect PTSD-relevant differences in response to trauma (consistent with the study in humans) rather than differences due to trauma exposure (consistent with the study in rats). Diffusion tensor imaging studies have endorsed significant alterations in white matter (WM) integrity, as measured by fractional anisotropy (FA), which are associated with trauma exposure, current PTSD diagnosis, current and lifetime PTSD symptom scores, PTSD status (e.g., remitted vs. persistent), and treatment outcome[253-256]. Both decrease[253,256] and increase[254,255] in FA were reported in different brain regions/tracts and contexts, with WM changes suggested to change over the course of PTSD. Moreover, investigation in oligodendrocyte precursor cell (OPC) culture demonstrated an interplay between pro-inflammatory cytokines and corticosteroids, such that IFNγ and TNFα impair survival and maturation of OPCs and co-administration of corticosteroids, most prominently dexamethasone, counters these deleterious effects[257]. Taken together, these studies support a model where dynamic, brain region-specific changes in white

matter associated with stress/trauma exposure and PTSD pathophysiology reflect stress and neuroinflammation-dependent remodeling at the cellular/molecular level via dynamics in myelination and OPC survival, proliferation, and differentiation/maturation.

Findings from our hippocampal network analyses endorse group differences in transcriptional activity that fit into the framework of this model and the broader PTSD literature on hippocampus that have consistently endorsed diminished neuronal and functional integrity [258]. Furthermore, our results suggest that there may be sex differences. Among the modules identified across hippocampal network analyses (i.e., full dataset vs. sex-stratified, SV-adjusted vs. cell estimate-adjusted), we highlight a module associated with upregulation in the EBR group in each sex and one notable module significantly associated with upregulation in MBR and downregulation in EBR that was detected only in females.

First, the female-specific MBR – EBR module was significantly enriched for extracellular structure organization, ECM receptor interaction, long-term synaptic potentiation, ensheathment of neurons, neurological system processes, and gliogenesis. When taken together, these enrichment results endorse group differences in the ECM and associated signaling, which may be related to glial regulation of hippocampal synaptic plasticity, specifically long-term synaptic potentiation which has implications for learning and memory, via ensheathment of neurons. As oligodendrocyte proportions were found to be higher in the EBR than the MBR group, enrichment for gliogenesis and ensheathment of neurons may potentially reflect involvement of other glial cells, such as astrocytes that may provide non-myelinating ensheathment of neuronal synapses[259]. Since our cell proportion estimates only capture the major brain cell types, it is unable to inform changes in subpopulations of the major brain cell types. Given the diversity of astrocytes as a cell population, our results could indicate

gliogenesis of relevant astrocyte subtypes (e.g., protoplasmic) or other glial cells that may not be captured by estimation of major brain cell types and imply participation of multiple glial cell types. Interestingly, this hippocampal female MBR – EBR module was also significantly enriched for genes with high-CpG density promoters (HCPs) bearing histone H3K4me3 and H3K27me3 marks in brain, suggesting dynamic regulation of trauma-exposed group differences in female hippocampus via this epigenetic mechanism. In summary, findings from this module suggest differences in individual response to trauma may involve glial processes supporting synaptic connectivity in the hippocampus, putatively via astrocyte-synapse interactions, specifically in females. These processes, which are upregulated in the resilient MBR group and downregulated in the PTSD-like EBR group, may safeguard fear extinction learning in the MBR group and be implicated in impaired fear extinction learning in the EBR group.

Next, consistent evidence across three network analyses, in both the SV- and cell-adjusted full dataset and female subset of SV-adjusted data, endorsed increased expression of genes regulated by nuclear factor-κB (NFκB) in response to tumor necrosis factor-α (TNFα) and upregulation of genes after neuregulin-1 (NRG1) stimulation in the EBR group relative to the unexposed CON group in females, but not males. In fact, the network analysis on full, cell-adjusted data suggested this module may be associated with upregulation in the male MBR group; however, we note this was not endorsed by any other module identified in sex-stratified or full network analyses. Stress has been shown to induce increased levels of proinflammatory cytokines, including TNFα, which activates NFκB, a key inflammatory transcription factor[260]. The TNFα signaling via NFκB pathway has various roles in the brain and is involved in regulating many signaling events, including growth/process development (e.g., dendritic arborization, axonal growth), and modulation of neuronal excitability/vulnerability of neurons to

excitotoxicity[260,261]. In the hippocampus, the TNFα signaling via NFκB pathway has been implicated in regulating synaptic plasticity and memory[261], and stress has been shown to activate NFκB signaling and decrease proliferation of neural stem-cell like cells in adult hippocampus[262]. Administration of an NFκB inhibitor has been demonstrated to block stress-induced inhibition of adult hippocampal neurogenesis, suggesting NFκB signaling may be a critical mediator of cellular (e.g., antineurogenic) consequences of stress that are linked to depressive-like behavior[262].

Relatedly, this module associated with female EBR – CON was also consistently enriched for genes up-regulated after stimulation with NRG1. While this gene set was based on experiments conducted on a breast cancer cell line (MCF-7)[263] and caution should be exercised in interpreting implications in brain, NRG1 is generally involved in activation of proliferation, survival, and differentiation. Thus, upregulation of identified gene module members may reflect a general response to NRG1 that is not cell-line specific. NRG1 is a member of the EGF family of receptor tyrosine kinase protein ligands with multiple isoforms and is known to be an important neurotrophic factor in the brain with key roles in neural development, synaptic plasticity, brain activity homeostasis, and neuroinflammation[264,265]. Disruption of normal NRG/ERBB signaling has been implicated in impaired brain functioning and a number of psychiatric disorders[264], with the *NRG1* gene most notably implicated in schizophrenia[266,267]. In hippocampus, NRG1 is suggested to regulate synapse development, particularly formation and maturation of inhibitory synapses, and has been shown to reduce expression of γ-aminobutyric acid (GABA) receptor α subunits in hippocampal slices[268].

In males, the module upregulated in the EBR group was associated with genes involved in the regulation of GABAergic synaptic transmission as well as those involved in the T-cell

receptor (TCR) signaling pathway. In addition to its inhibitory role in synaptic transmission, GABAergic signaling plays a parallel role in the immune system[269] and has been shown to inhibit adaptive inflammatory response to myelin proteins in an animal model of multiple sclerosis (i.e., experimental autoimmune encephalomyelitis [EAE])[270]. Similarly, relevant to this male EBR-associated module, GABAergic signaling negatively regulates T cell-mediated immunity by its modulatory effect on T-cell proliferation and cytokine production[271], and has been shown to inhibit TCR-mediated T-cell cycle progression *in vitro*[272]. Thus, GABAergic signaling may putatively play a dual role in the regulation of both synaptic transmission and T-cell programming in this male EBR module.

The male EBR module was also significantly enriched for GPCR signaling and for genes with 3'UTR containing motif matching annotations for GR (glucocorticoid receptor, *NR3C1*), CEBPα (CCAAT/enhancer binding protein), and LFA-1 (lymphocyte function-associated antigen-1). While GPCR signaling is ubiquitous, in the context of tissue and other significant enrichment hits for this module, fits a narrative of G-protein mediated signaling promoting initial adhesion of inflammatory T-cells on blood brain barrier endothelium (BBB) for transendothelial migration to hippocampus, with a putative role for LFA-1 in this process, under neuroinflammatory conditions[273]. In parallel, stimulation of LFA-1 also modulates transcriptional programming for a number of key pathways, including activation of T lymphocyte differentiation into effector subsets Th1, Th17, and induced regulatory T cells, for tuning of T-cell functioning[274]; this modulation of transcriptional programming is the function directly implicated by our enrichment results. In a similar vein, CEBPα is involved in inflammatory response and regulate expression of many pro-inflammatory genes shown to have increased expression after injury to aged brain[275]. Finally, GR is an important mediator of stress

response that is implicated in stress-related psychiatric disorders. Glucocorticoids in the hippocampus, distinct from their effect in hypothalamus, have been shown to play an important role in the development of hypothalamus-pituitary adrenal (HPA) axis hyperactivity[276]. Of note, acute stress has been shown to increase interaction of GRs with their genomic targets in the hippocampus, in a gene-dependent manner[277] and chronic stress has been implicated in dysfunction of GABAergic interneurons in the hippocampus[278].

In summary, our results in hippocampus have detected distinct, putatively sex-specific, transcriptional activity characteristic of the PTSD-like EBR group and differential response to trauma in females involving glial processes supporting hippocampal synaptic connectivity that may be implicated in fear extinction learning. EBR in females was characterized by stress-induced upregulation of the TNFα signaling via NFκB pathway and disruption of NRG1/ERBB signaling, which may be involved in dysregulated synaptic plasticity and development in the hippocampus. Notably, these mechanisms may be implicated in the formation and maturation of inhibitory GABAergic synapses, with consequences for modulation of neuronal excitotoxicity. EBR in males was explicitly associated with increased regulation of GABAergic synaptic transmission and was also characterized by upregulated T-cell receptor signaling. Additionally, three transcription factors implicated in stress, inflammatory response, and T-cell functioning, namely GR, CEBPα, and LFA-1, were identified as candidates that may coordinate these processes.

Amygdala

While no group differences were observed in brain cell subtype proportions, cell composition analyses revealed prominent sex differences in the distribution of amygdalar cell

subtype proportions, such that females, regardless of group, showed significantly greater variance in distribution of relative cell proportions for astrocytes (ast), neurons (neu), oligodendrocytes (oli), and oligodendrocyte progenitor cells (opc), compared to males. The original Daskalakis et al. (2014)[223] study that deposited this dataset did not mention controlling for or tracking cycle or conducting ovariectomies, indicating the female rats in this dataset were free-cycling. Thus, the broader distribution of these cell proportions may potentially reflect sensitivity of these amygdalar cell types to gonadal steroid hormones.

Extensive sex differences are found at the cellular and physiological level in the amygdala, most notably in the posterodorsal medial amygdala (MePD), and are associated with organizational and activational effects of gonadal steroid hormones[279-285]; they are observed prior to puberty[279,286,287], become more extensive during puberty[288], and demonstrate steroid-dependent plasticity during adulthood[280-285]. In rat MePD, which is important for sex-specific behavior, studies have reported more neurons and astrocytes in males than in females, and additional sex differences in size and morphology of cell bodies and processes, which also differ by laterality[281,286,287,289]. While not specific to amygdala, sexual dimorphism of oligodendrocyte response to sex hormones has been reported in cell culture with females consistently showing greater sensitivity and responsiveness to hormones than males[290]. To our knowledge, brain region-specific differences have not yet been investigated.

Network analyses in amygdala detected both shared and sex-specific gene modules. The resilient MBR group in both sexes was characterized by increased expression of ECM-relevant genes (e.g., cytoskeletal proteins) and upregulation of ECM organization/remodeling, with collagen fibril organization specifically implicated. Additionally, enrichment for epithelial mesenchymal transition implicated enhanced migratory capacity and stem cell-like phenotype

with processes in the likeness of wound healing and tissue repair in response to stress-induced neuroinflammation[291]. The second shared module was significantly associated with downregulation in EBR for both sexes and relative upregulation in females; thus, our results endorse this module to reflect group differences in response to trauma in females, but only supports downregulation of this module to be characteristic of the EBR group in males.

With respect to the PTSD-like EBR group, this shared module was associated with downregulation of GPCR signaling, synaptic signaling/transmission, GABAβ receptor activation, and exploration behavior, as well as decreased regulation of insulin secretion by glucagon-like peptide 1, protein kinase A (PKA) signaling, and cAMP metabolic process. GPCR signaling, PKA signaling, and cAMP metabolic processes are ubiquitous and involved in signaling transduction; in the cell, cAMP targets PKA, which serves as the principal effector mechanism for GPCRs linked to adenylate cyclase[292]. The cAMP/PKA signaling pathway is involved in the regulation of glucose homeostasis[293] and relevant to the decreased regulation of insulin secretion by glucagon-like peptide 1. Additionally, the cAMP/PKA signaling pathway plays an essential, evolutionarily conserved role in the mediation of neural processing of threat-related stimuli (i.e., fear learning), consolidation of fear memory, and fear-related behavioral response in the amygdala[292]. Targeted activation of cAMP/PKA signaling in the lateral amygdala has been shown to increase neuronal excitability and lead to generalized fear[294], while targeted inhibition of cAMP/PKA activity in the lateral amygdala immediately after fear conditioning impaired fear memory retention[295]. Additionally, stress modulates fear memories; β-adrenoceptor-mediated activation of the cAMP/PKA pathway has been shown to enhance fear memory consolidation and glucocorticoids (GCs) interact with the noradrenergic signaling pathway to modulate this activation in the basolateral amygdala (BLA)[292]. Targeted inhibition of

either β-adrenoceptor or cAMP/PKA in the BLA before or immediately post inhibitory avoidance training has been shown to block memory consolidation and administering a GR antagonist before this training was able to block the noradrenergic-dependent enhancement of memory retention[296]. Fear learning and memory is also linked to other anxiety-related behavioral responses, including exploration behavior, which was also significantly downregulated in this module. Thus, dysregulation of this pathway in the amygdala is directly relevant for pathogenesis of PTSD.

Furthermore, the module was associated with the downregulation of synaptic signaling, specifically implicating disrupted GABAβ receptor activation and opioid signaling in the EBR group. GABAβ receptor activation in the BLA has been shown to inhibit glutamatergic excitatory transmission[297] and is essential for maintaining balance between neuronal excitation and inhibition[298]. Stress has been shown to disrupt GABAergic inhibitory signaling in the amygdala in a projection-specific manner, leading to hyperexcitability in the BLA, and this effect has in turn has been implicated in increased anxiety/depression, emotional dysregulation, and the development of psychiatric diseases[299]. Relevant to PTSD, functional neuroimaging studies have identified increased activation of the amygdala as a neural correlate for PTSD symptoms[258,300,301]. Opioid signaling in the amygdala, particularly by kappa-type opioid receptors (κ-OR), is also involved in modulation of synaptic signaling and circuitry underlying fear learning and anxiety-like behaviors[302,303]. Notably, a key gene downregulated was prodynorphin (*PDYN*), which encodes a preproprotein that is processed into endogenous opioid peptides that preferentially bind to κ-OR[302]. In line with our results, reduced *PDYN* mRNA expression in discrete nuclei of the amygdaloid complex was found in postmortem samples from subjects with major depression and bipolar disorder[304]. Additionally, *Pdyn* gene knockout was

found to alter $GABA_A$ receptor expression in the amygdala, increase anxiety-like behaviors, [305], enhance cue-dependent fear conditioning, and delay contextual fear extinction[306]. Blockade of κ-ORs before extinction trials also produced similar effects and in humans, subjects with a functionally relevant *PDYN* polymorphism showed reduced fear extinction and significantly blunted functional connectivity between the amygdala and ventromedial prefrontal cortex[306]. Thus, dynorphin/κ-OR signaling play a role in fear extinction and disrupted κ-OR signaling impairs extinction learning. In all, disruption of GABAβ receptor activation and κ-OR signaling lead to dysregulation of synaptic signaling in the amygdala, which is implicated in PTSD-relevant symptoms, including impaired fear extinction.

In males, one module was significantly associated with downregulation in EBR and relative upregulation in CON, suggesting that this module highlights EBR response to trauma exposure. Accordingly, a number of the terms significantly enriched with the module were related to response a molecular factor, notably to lipid, hormone, and steroid hormone, implicating enrichment for genes downregulated in response to glucocorticoids (GCs). This male-specific EBR module was also significantly associated with downregulation of neuropeptide hormone activity, which included decreased expression of cholecystokinin (*Ckk*), neuropeptide Y (*Npy*), and vasoactive intestinal peptide (*Vip*). These neuropeptides define classes of BLA interneurons that provide feedforward and feedback inhibition to projection neurons and other interneurons locally and are likely to be involved in higher-order contextualized behavior such as fear extinction[307,308]. Thus, downregulation of these neuropeptides may be indicative of a decrease in these inhibitory amygdalar interneurons and disruption of excitatory/inhibitory balance.

Of these neuropeptides, NPY has been studied the most in relation to stress-related psychiatric disorders, as its release is induced by stress and has anxiolytic properties[309]. Notably, a study of NPY using the same predator-scent stress (PSS) paradigm found lower NPY levels in the amygdala of the male EBR group compared to MBR or CON and showed administration of NPY one hour post-exposure to significantly reduce prevalence rates of EBR and trauma-cue freezing responses compared to controls[310]. This suggests 1) downregulation of NPY is a robust characteristic of EBR directly linked to the behavioral response, and 2) NPY may serve not only as a diagnostic marker but also as a treatment candidate that can prevent development of PTSD-like symptoms and promote resilience. Interestingly, low NPY expression may be a predisposing risk factor. A human genetic imaging study identified an *NPY* haplotype predictive of *NPY* mRNA expression in postmortem brain and lymphoblast and plasma NPY levels; using functional (fMRI and PET) imaging, they found low NPY haplotype individuals exhibited greater amygdalar reactivity in response to threat-related facial expression, had lower activation of pain/stress-induced μ-opioid system activation, and reported more negative emotional experiences during a painful stressor compared to high NPY haplotype individuals[311].

The male EBR module was also characterized by decreased expression of genes regulated NFκB in response to TNFα, which is the opposite direction of effect seen in hippocampus for the female EBR group. Both the hippocampus and amygdala are stress-sensitive brain regions where NFκB plays a critical role in memory and is necessary for fear memory consolidation and reconsolidation[312-314]. However, the exact region- and sex- specific differences in downstream effects of TNFα/NFκB signaling are unknown and require further investigation. In sum, blunted GC response, disrupted inhibitory modulation of amygdalar circuitry, diminished NPY levels,

and dysregulation of genes downstream of TNFα/NFκB signaling are implicated in the male EBR group.

More PTSD-relevant modules were identified in females; two modules (based on cell and SV-adjusted female subset) were associated with upregulation in MBR and downregulation in EBR, with the cell-adjusted module largely overlapping with the shared module identified in the full, cell-adjusted dataset. This module was significantly enriched for the previously identified gene sets and was also enriched for additional terms. Notably, significant enrichment for inhibition of insulin secretion by adrenaline/noradrenaline and behavior/associative learning elaborated a potential role for nor/adrenergic signaling in glucose homeostasis and strengthened the implications in fear learning and anxiety-related behavioral response. Additionally, downregulation of dopamine receptor signaling, specifically for dopamine D_2 receptor (*Drd2*), was significantly associated with this module. The *DRD2* gene is a popular gene candidate for PTSD and a meta-analysis of dopaminergic system genes identified a significant association for *DRD2* rs1800497[315]. Other genetic variants of *DRD2* have also been implicated as a risk factor for PTSD, such as the A1 allele of the TaqA1/A2 polymorphism[316,317] and C957T SNP (rs6277)[318,319]. Dopaminergic signaling plays an important modulatory role in fear learning/memory and anxiety. D2 receptors in the amygdala are endorsed to have context-specific effects and an important role in regulating fear/anxiety responses[320,321], with putatively differential functions in the central versus the lateral amygdala[322], which receives key dopaminergic innervations from the ventral tegmental area[323] and mediates dopaminergic gating of LTP induction necessary for fear conditioning[324]. Notably, dopaminergic innervation of the amygdala is suggested to be more responsive to stress exposure than other limbic brain regions[325], implying dopamine signaling in the amygdala may be important in stress response.

Thus, differential dopaminergic and GABAergic signaling in the amygdala may underlie important group differences in the modulation of fear learning and memory between resilient and PTSD-like females.

The second female-specific module strongly endorses *Srf* as a key driver for trauma-exposed group differences in the female amygdala. In the adult brain, the *Srf* transcription factor activates immediate early genes (IEGs) and mediates cytoskeletal dynamics at neuronal processes[326]. Enrichment results support differential regulation of both these effects, which suggests fundamental group differences in experience-dependent modulation of synaptic plasticity involving structural changes[327]; in amygdala, this has implications for long-term fear learning and memory. Notably, the top hit in the C2 CGP collection indicates enrichment for genes downregulated in neurons after *Npas4* knockdown, suggesting downstream effects of *Srf* disruption in the female EBR group may involve disruption of *Npas4* activation. In the amygdala, *Npas4* expression was found to be selectively induced under conditions of associative learning and is suggested to be important for fear memory consolidation/reconsolidation[328]. It has been demonstrated to regulate activity-dependent development of GABAergic inhibitory synapses via transcriptional regulation of the activity-dependent genes directly involved[329], which fits the theme of disrupted excitatory-inhibitory balance in the amygdala. Interestingly, cross-disorder investigation of transcriptome-wide dysregulation in postmortem brain of autism spectrum disorder (ASD), schizophrenia (SCZ), and bipolar disorder (BP) also identified *NPAS4* and other classic early response genes in a neuronal module differentiating BD and SCZ from ASD[330], suggesting disruption of fundamental experience-activated plasticity mechanisms in stress-related neuropsychiatric disorders. Continuing with the theme of group differences in excitatory-inhibitory balance, the next top enrichment hit in the C2 CGP collection was for genes

upregulated after NRG1 stimulation, suggesting NRG1 stimulation in the female MBR group. NRG1/ErbB4 signaling was found to be critical in the maintenance of GABAergic activity in the BLA, and this was associated with modulation of anxiety-like behaviors[331]. Moreover, administration of NRG1 into the BLA had anxiolytic effects and enhanced GABAergic neurotransmission in high-anxiety mice[331], suggesting a protective role of NRG1 stimulation in the MBR group. Since the C2 CGP gene set collection does not include gene sets for perturbations in the opposite direction we cannot confirm what implications *Npas4* upregulation and NRG1 inhibition may carry.

Additionally, the module was indicated for differential expression of genes regulated by TNFα signaling via NFκB. This is in line with our results for amygdala in the male EBR group, which was also characterized by downregulation of this gene set. However, opposing direction of effect was observed for this gene set (of genes regulated by TNFα signaling via NFκB) in the female EBR group across amygdala and hippocampus. Surprisingly, inspection of overlapping gene module members revealed there were more genes overlapping between the hippocampal and amygdalar modules with opposite directions of effect in females than between the male and female amygdalar modules with same direction of effect, suggesting putative sex and brain region-specific regulation in response to TNFα signaling via NFκB. The different direction of effect between amygdala and hippocampus in females parallels previous studies demonstrating stress to increase synaptic plasticity, BDNF levels, and induce dendritic hypertrophy in the amygdala, while having the opposite effect in hippocampus and medial prefrontal cortex[332-335]. This is also endorsed by the one amygdalar module, detected in females, associated with upregulation in EBR relative to the unexposed CON group; this female EBR module was characterized by upregulation of genes involved in the ECM, stem cell/NPC proliferation,

positive regulation of neuron differentiation, and, positive regulation of dendritic spine development. Thus, TNFα signaling via NFκB may be involved in the regulation of stress-mediated synaptic/structural plasticity in both the hippocampus and amygdala, but have region-specific effects. Additionally, there may be more nuanced sex differences in genes/processes altered by TNFα signaling via NFκB.

In summary, our results in amygdala support fundamental sex differences in cell composition that may be significantly influenced by sex hormones. While a number of sex-specific modules are endorsed, some group-relevant modules were shared across sexes. The resilient MBR group was characterized by upregulation of ECM organization/remodeling, illustrating processes similar to wound healing and tissue repair in response to stress-induced neuroinflammation in the amygdala. The PTSD-like EBR group was characterized by disruption of cAMP/PKA signaling, GABAβ receptor activation, and κ-OR signaling in the amygdala, which lead to dysregulation of synaptic signaling, impaired fear learning/memory, and anxiety-related behavioral response. Additionally, TNFα signaling via NFκB was indicated to play a major role in mediating stress-induced synaptic/structural plasticity in both sexes, but implicated for sex-specific downstream effects. In males, EBR was also characterized by decreased expression of neuropeptide hormones, including those use to define classes of BLA interneurons, suggesting an additional male-specific mechanism involved in the disruption of excitatory/inhibitory balance. Notably, downregulation of NPY may be directly linked to behavioral response and was only detected in male, but not female, amygdala. On the other hand, in females, disruption of dopamine D_2 receptor signaling and the Srf transcription factor were suggested to be key drivers for trauma-exposed group differences in the amygdala, where they are purported to shape differences in excitatory/inhibitory balance, differentially activate

IEGs and differentially mediate cytoskeletal dynamics in neuronal processes. Together they may drive fundamental group differences in experience-dependent modulation of structural plasticity, and this has implications for long term fear learning and memory. Additionally, we noted these PTSD-relevant modules in amygdala were significantly enriched for genes with HCPs bearing H3K4/27me3 in brain and were stronger in sex-stratified modules, except in the female module characterized by transcriptional regulation via Srf. Thus, epigenetic regulation of transcriptional activity is endorsed in the amygdala, in both sexes.

Blood

A module characterizing MBR in blood was identified for each sex. In both sexes, this module was associated with general upregulation of wound healing processes, particularly platelet activation/hemostasis, and increased regulation of body fluid levels. These processes were also identified using unadjusted expression levels, suggesting they are independent of MBR associations with other covariates modeled by SVs. Interestingly, the unadjusted module also revealed that while upregulation of this module is shared across sexes in the MBR group, stress response in represented processes are sexually dimorphic in the EBR group, such that the module is upregulated in the female EBR and downregulated in male EBR group.

The findings from this module for males directly mirror gene network analyses conducted in humans (using blood)[68,336]. A prospective cohort study in US Marines pre- and post-deployment identified a module after combat trauma exposure (post-deployment) associated with PTSD resiliency signatures and an upregulation of genes involved in hemostasis and wound responsiveness[336]. A more recent mega-analysis combined five independent blood transcriptome datasets and investigated three PTSD case-trauma-exposed control groups, stratified by type of

trauma exposure and sex, specifically men with combat trauma, men with interpersonal (IP) trauma, and women with IP trauma. They identified a wound-healing and coagulation module downregulated in men with PTSD and combat trauma (relative to trauma-exposed control males with combat trauma). This module was not relevant to women, which is in line with our results that indicated upregulation in both the female MBR and EBR groups. Interestingly, the module also was not identified in men with IP trauma either, suggesting associated processes may be trauma-exposure specific. A parallel validation study using a prospective female military cohort would enable us to similarly substantiate our female results in humans and help advance our understanding of the effect of trauma type on gene expression.

Furthermore, our results suggest response to ROS/oxidative stress may be a candidate pathway to target in the study of sex differences in PTSD. Sex differences in oxidative stress have previously been implicated in the study of cardiovascular diseases, which also have a sex/gender bias in disease prevalence[337] and are often co-morbid with PTSD[215,216]. Sex differences in response to oxidative stress have also been reported in the brain, notably in the hippocampus, in response to ethanol withdrawal[338] and prenatal stress[339]. Moreover, these sex differences appear to be evolutionarily conserved with reports of sex-specific adaptation to oxidative stress even reported in fruit flies[340].

The sex-stratified MBR – CON modules also endorsed some processes to be sex-specific. Vascular smooth muscle contraction is supported to be a process more relevant in males with *PPP1CC* identified as a candidate hub gene upregulated in the male MBR group. Upregulation of the complement pathway and is supported to be more relevant in females with HNF1 and SRF found to be candidate transcription factors involved in coordinating this MBR module in females. Additionally, a module identified only in females suggested female EBR may be

characterized by downregulation of immune and defense responses, which may involve impairment of coagulation, IL6-JAK-STAT3 signaling, and interferon gamma (IFNγ) response, with IFNγ particularly implicated as a key player involved in PTSD-like response to stress in females. This is in line with a study that reported several sex-specific effects of IFNγ treatment on monoaminergic activity in key limbic regions and also demonstrated an IFNγ by stress (acute restraint stress) interaction to increase corticosterone levels in plasma, with larger effects in males[341].

In summary, we found wound healing processes and hemostasis to be upregulated in the resilient MBR group across sexes, but disrupted in a sexually dimorphic manner in the EBR group. These findings, particularly in males, are consistent with the PTSD literature. Response to oxidative stress and IFNγ are endorsed as candidate processes to target in the study of sex differences in PTSD.

Cross-Tissue and Integrated Remarks

Systemic stress hormones, particularly glucocorticoids (GCs), act in both blood and brain. The hippocampus and amygdala are highly plastic and stress-sensitive limbic regions central for learning and memory associated with strong emotions – prominently fear memories, which have direct implications for PTSD symptoms. Stress-induced TNFα/NFκB signaling, which plays a key role in the regulation of synaptic plasticity and fear learning/memory, is suggested to be a key pathway differentially disrupted in the EBR group in both brain regions. Although the specific downstream disruption is region-specific, a common theme of excitatory/inhibitory imbalance, involving disruption of GABAergic signaling, is advocated. In

females, differential NRG1/ERBB signaling is implicated with region-specific effects in hippocampus and amygdala.

Another notable commonality across hippocampus and amygdala was upregulation of ECM-regulated genes and ECM organization/remodeling in the resilient MBR group, in response to stress-induced neuroinflammation. This is akin to a wound healing/tissue repair response in the brain and parallels significant upregulation of wound healing processes characteristic of MBR in blood. Notably upregulation of ECM/wound healing is shared across sexes in both amygdala and blood, of both MBR groups. While upregulation of ECM was identified in a female-specific hippocampal module, no MBR upregulated module was detected for males among adjusted, sex-stratified network analyses and there was generally more difficulty identifying PTSD-relevant hippocampal modules in males than females. This challenge may stem from the loss of two male hippocampal samples during QC, which resulted in four male hippocampal samples in the EBR and MBR groups (8 samples total). Thus, sex-specificity needs to be revisited with a larger sample size, especially in hippocampus. In general, upregulated ECM remodeling and wound healing processes appears to be a common MBR response to stress/trauma that is observed across tissues and shared between sexes.

As mentioned, a major limitation of this study was sample size, with some sex-stratified groups in brain having only four samples, namely male EBR and male MBR groups in hippocampus and female MBR group in amygdala. By identifying co-expressed gene modules using an unsupervised network analysis approach and converging evidence across analyses based on sex-stratified and full datasets adjusted for SV and cell estimates (in brain) we were able to identify the gene modules that were most consistently supported across analyses. Additionally,

further validation and investigation is required for cell estimation approaches, particularly in rats, due to lack of reference datasets.

Our findings suggest both shared and sex-specific mechanisms underlying individual differences associated with vulnerability and resilience to trauma in blood and two key limbic areas, namely hippocampus and amygdala. By disentangling cell composition from transcriptional changes, we found higher proportions of hippocampal oligodendrocytes in the EBR group for both sexes and also identified modules for transcriptional activity associated with group differences (ie., response to trauma) in the hippocampus that appeared to be sex-specific. By contrast, we found prominent sex differences, but no group differences, in amygdalar cell composition, and both shared and sex-specific modules representing PTSD-relevant transcriptional activity in the amygdala. Across amygdala and hippocampus, both sex-specific and shared processes were relevant to an overarching framework for EBR implicating disrupted TNFα/NFκB signaling and excitatory/inhibitory imbalance in dysregulated synaptic/structural plasticity with important implications for fear learning and memory. Our main finding in peripheral blood was consistent with the human literature and identified wound healing processes and hemostasis to be upregulated in the MBR group across sexes, but disrupted in a sexually dimorphic manner in the EBR group. Unlike the varied characterization of the EBR group, characterization of MBR across blood, amygdala, and hippocampus suggests a common theme of upregulated wound healing and ECM remodeling shared between sexes. In all, we identified differential oligodendrocyte proportions in hippocampus between PTSD-like EBR and resilient MBR, and identified processes and pathways that characterize the EBR and MBR-associated transcriptional changes across hippocampus, amygdala, and blood. The sex-specific mechanisms

involved in EBR may contribute to the pronounced disparity in risk for PTSD, with women much more likely to develop PTSD.

3.6 Figures and Tables

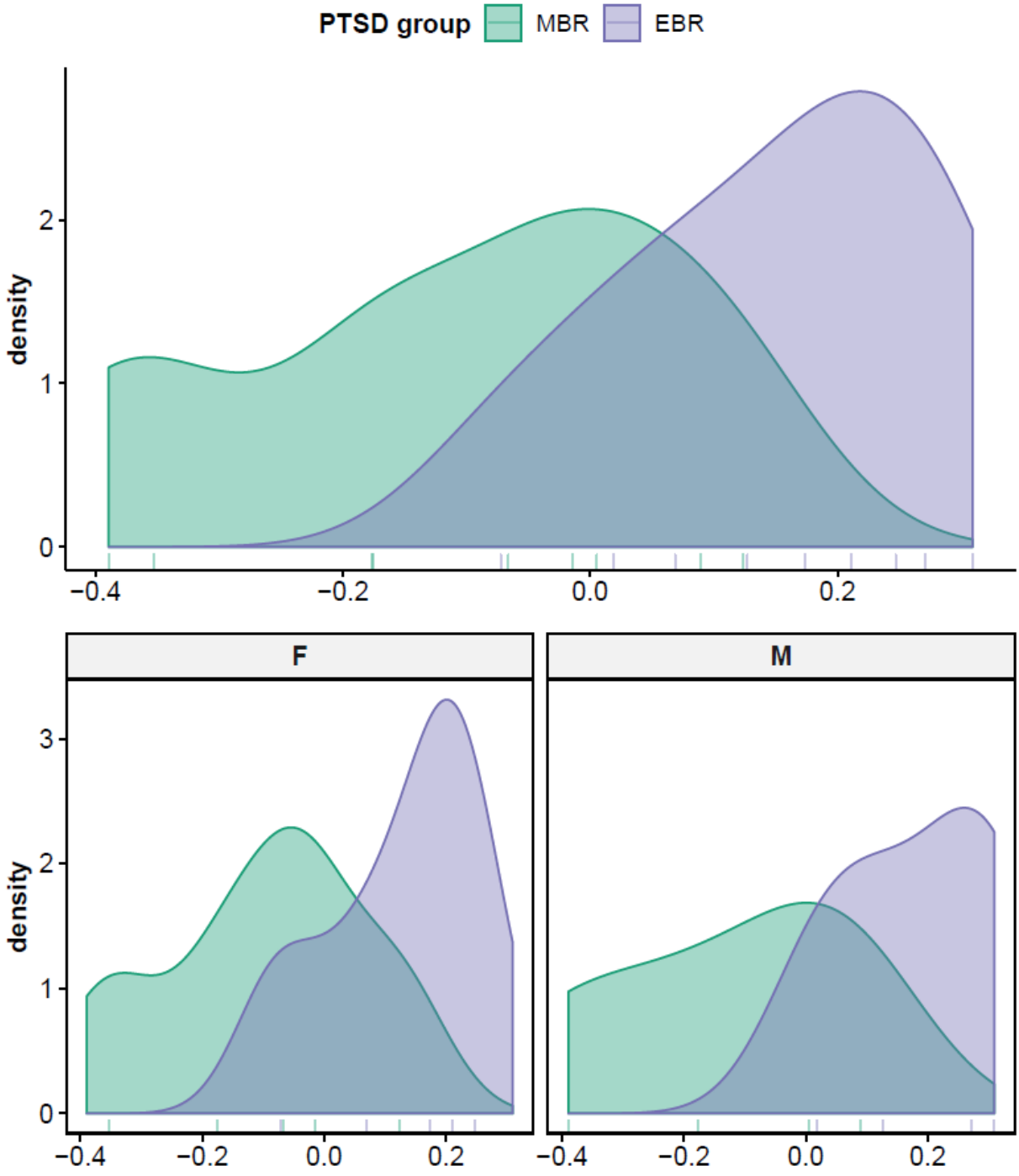


Figure 3.1. Density plots of hippocampal oligodendrocyte estimates in EBR (purple) and MBR (green) groups, show significantly higher proportions in EBR compared to MBR group in both sexes (top). Sex-stratified density plots (bottom) confirm this group difference is not sex-specific.
Note: EBR: extreme behavioral response (PTSD-like); MBR: minimal behavioral response (resilient).

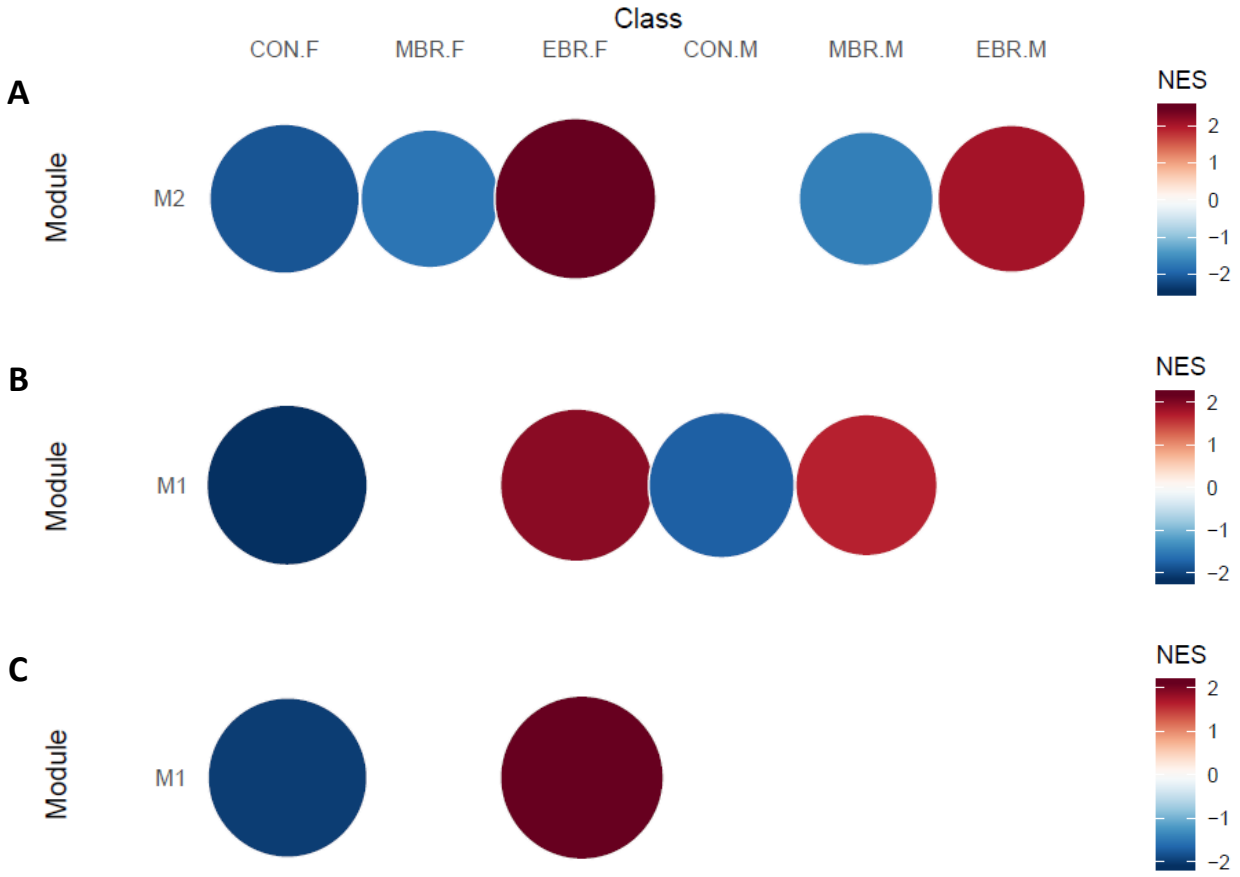


Figure 3.2. Module enrichment plot for PTSD-relevant modules identified in network analyses of hippocampus. Analyses on full expression dataset (both sexes) that has been (A) unadjusted for cell proportion estimates or surrogate variable (SV) estimates; (B) adjusted for cell proportion estimates; (C) adjusted for SV estimates.
(A) unadjusted: Module M2 consists of 104 genes; upregulation in EBR and downregulation in MBR groups in both sexes, and downregulation in female CON group.
(B) cell-adjusted: Module M1 consists of 165 genes; upregulation in female EBR and male MBR groups with relative downregulation in CON group in both sexes.
(C) SV-adjusted: Module M1 consists of 81 genes; upregulation in female EBR and downregulation in female CON group.
The size and intensity of the circles correspond to the normalized enrichment score (NES) for the module in each sex-stratified group (normalized by the number of genes in the module).

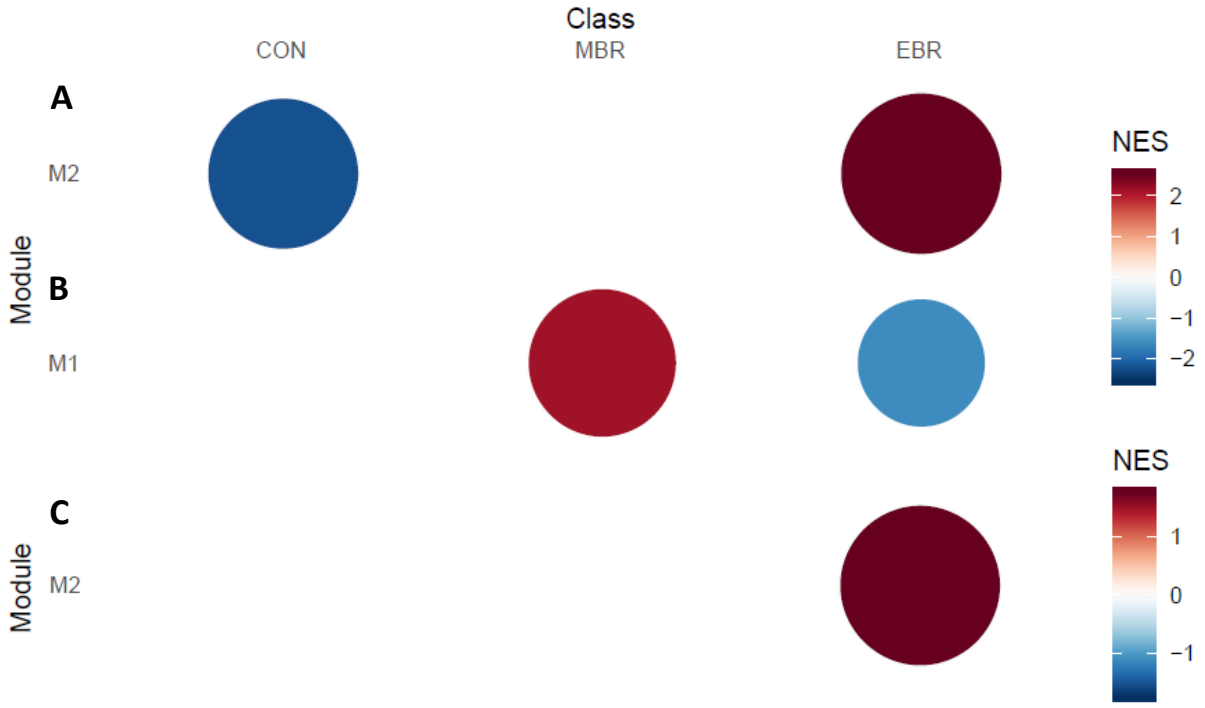


Figure 3.3. Module enrichment plot for PTSD-relevant modules identified in sex-stratified network analyses of hippocampus.
In the SV-adjusted female subset: (A) Module M2 consists of 144 genes and is associated with upregulation in EBR and relative downregulation in CON; (B) Module M1 consists of 170 genes and is associated with upregulation in MBR and downregulation in EBR.
In the SV-adjusted male subset: (C) Module M2 consists of 101 genes and is associated with upregulation in EBR
The size and intensity of the circles correspond to the normalized enrichment score (NES) for the module in each sex-stratified group (normalized by the number of genes in the module.

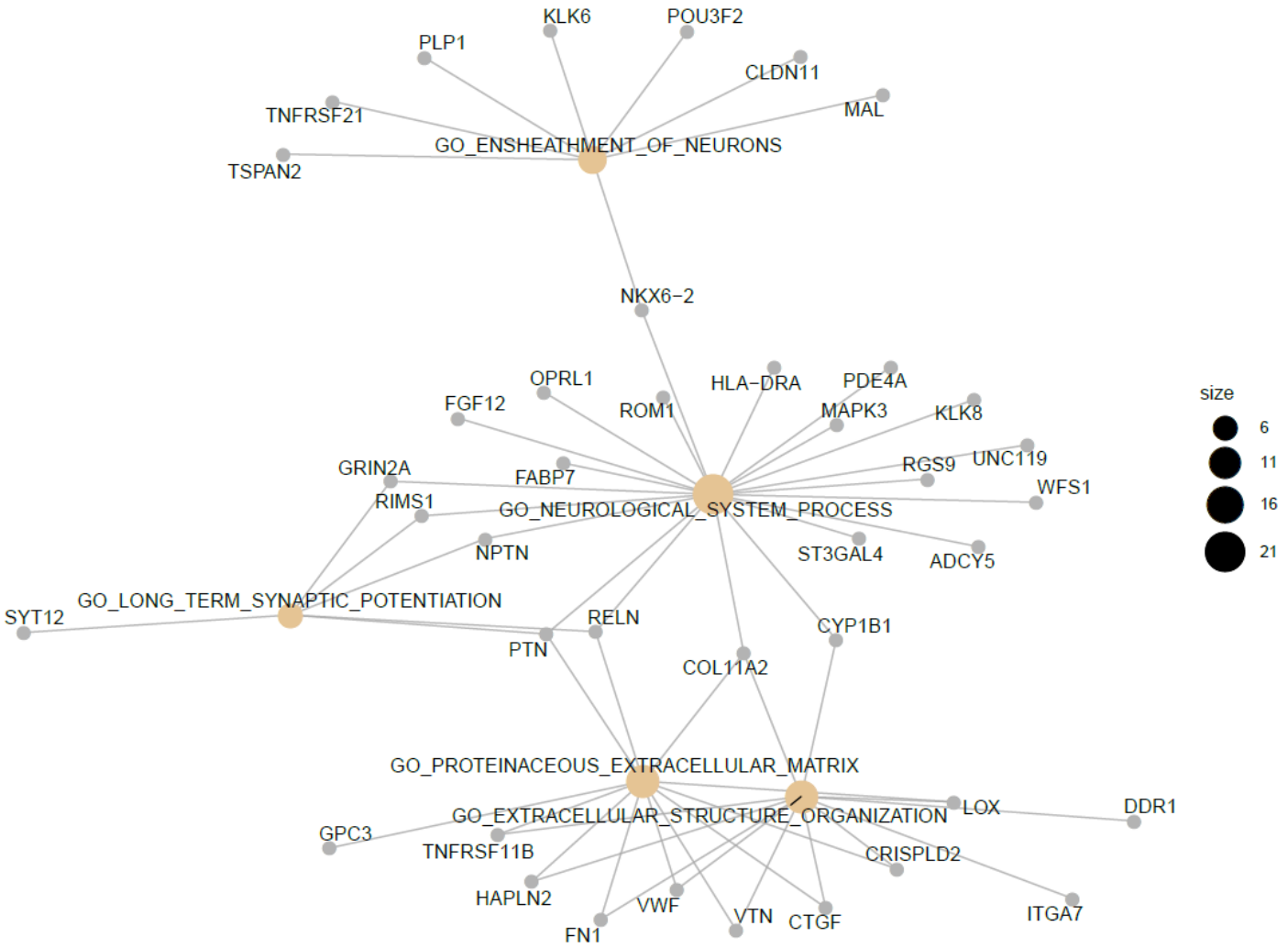


Figure 3.4. Gene-concept network plot for top GO terms enriched in female-specific MBR – EBR module derived from female subset of SV-adjusted hippocampal expression data (M1: 170 genes). Edges show associations between GO terms based on overlapping module genes. Size of circles, representing GO terms, reflect gene count.

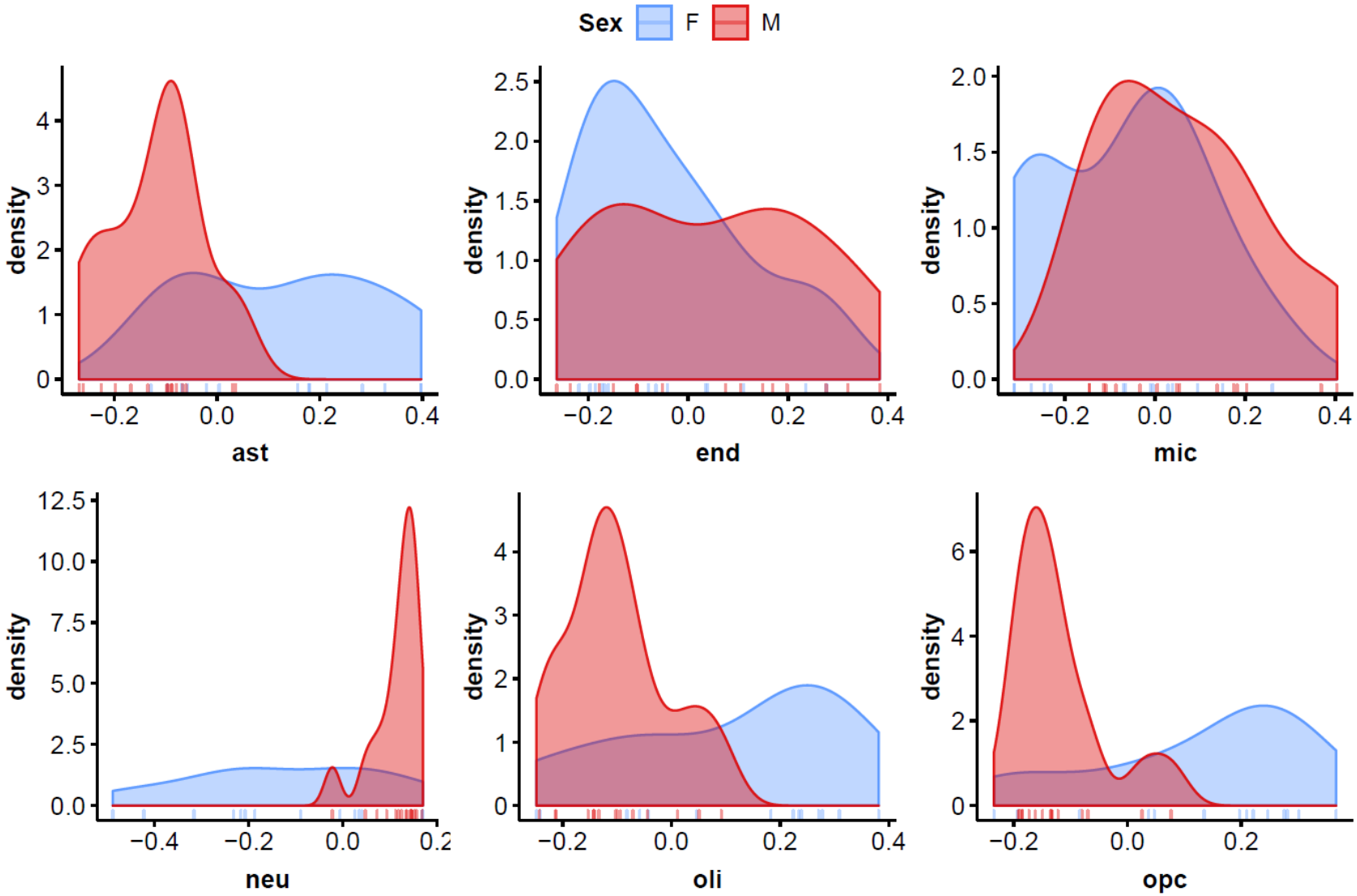


Figure 3.5. Density plots of amygdalar cell subtypes by sex, show prominent sex differences amygdalar cell composition, significant in astrocytes (ast), neurons (neu), oligodendrocytes (oli), and oligodendrocyte progenitor cells (opc), with females (blue) showing broad distributions (greater variance) and males (red) displaying sharp peaks. Males had relatively higher neu proportion and lower ast, oli, opc proportions.

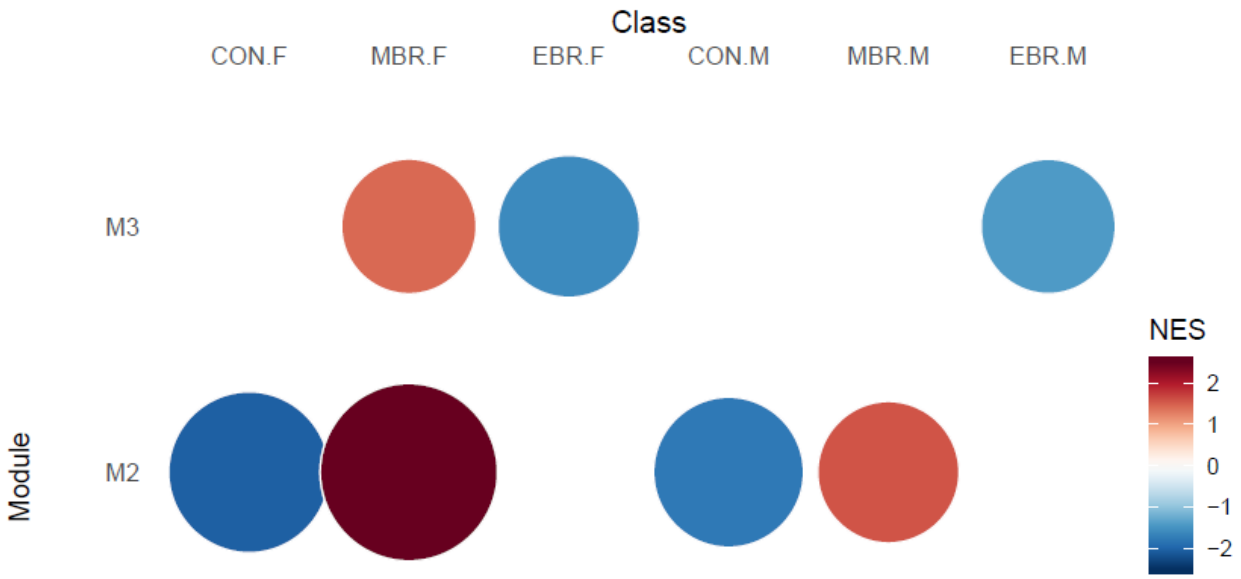


Figure 3.6. Module enrichment plot for PTSD-relevant modules identified in network analysis of cell-adjusted amygdalar expression levels (both sexes).
Module M2 (middle row) consists of 59 genes and is associated with upregulation in MBR and relative downregulation in CON, in both sexes.
Module M3 (top row) consists of 55 genes and is weakly associated with downregulation in EBR groups of both sexes and upregulation in the female MBR group.
The size and intensity of the circles correspond to the normalized enrichment score (NES) for the module in each class (normalized by the number of genes in the module.

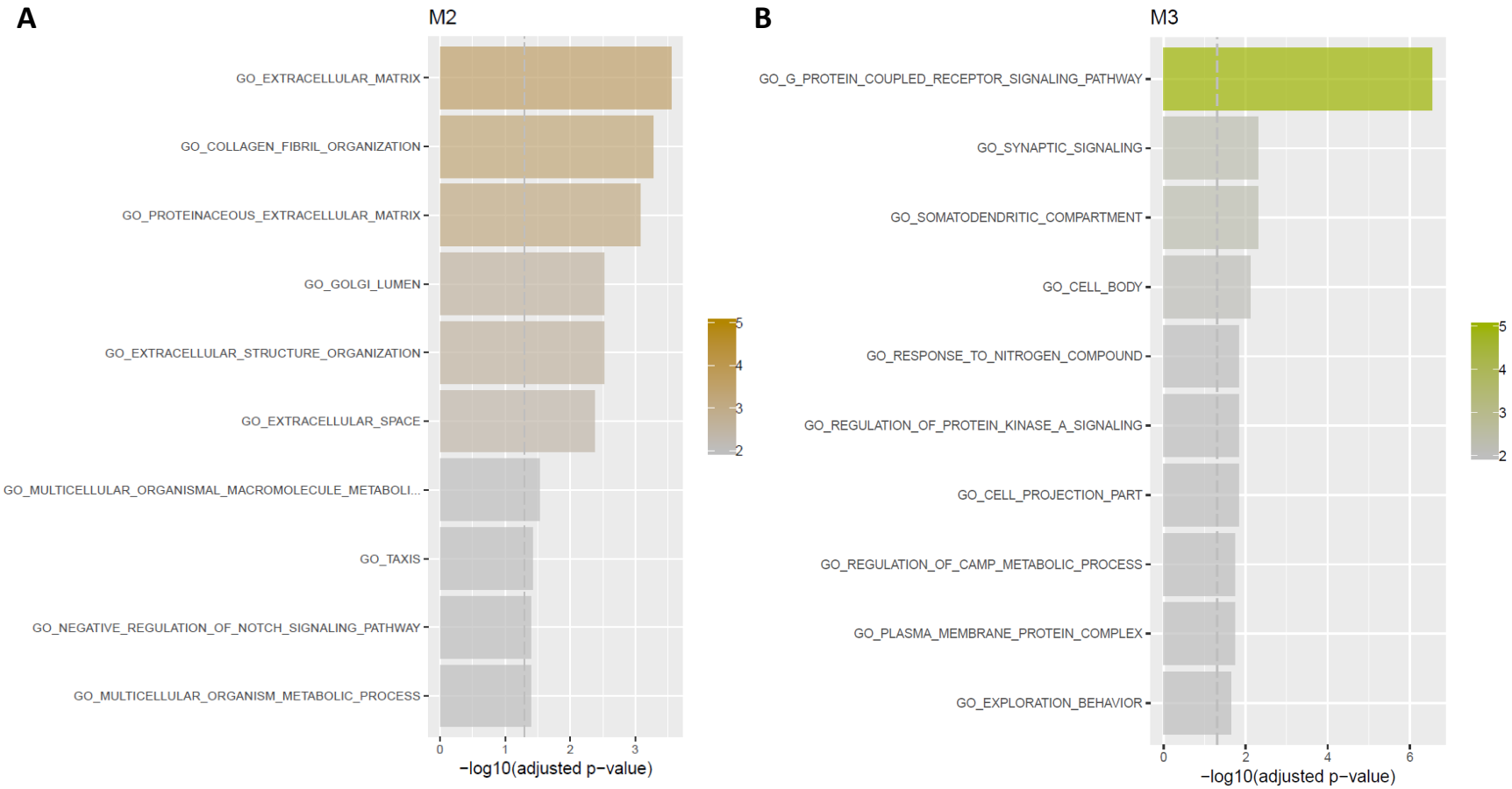


Figure 3.7. Barplot for top GO terms enriched in (A) M2 and (B) M3 modules identified in network analysis of cell-adjusted amygdalar expression levels.
x-axis and color transparency display -$\log_{10}$ of the Benjamini-Hochberg (BH)-adjusted p-value. Dashed vertical line indicates BH-adjusted p-value threshold of 0.05.

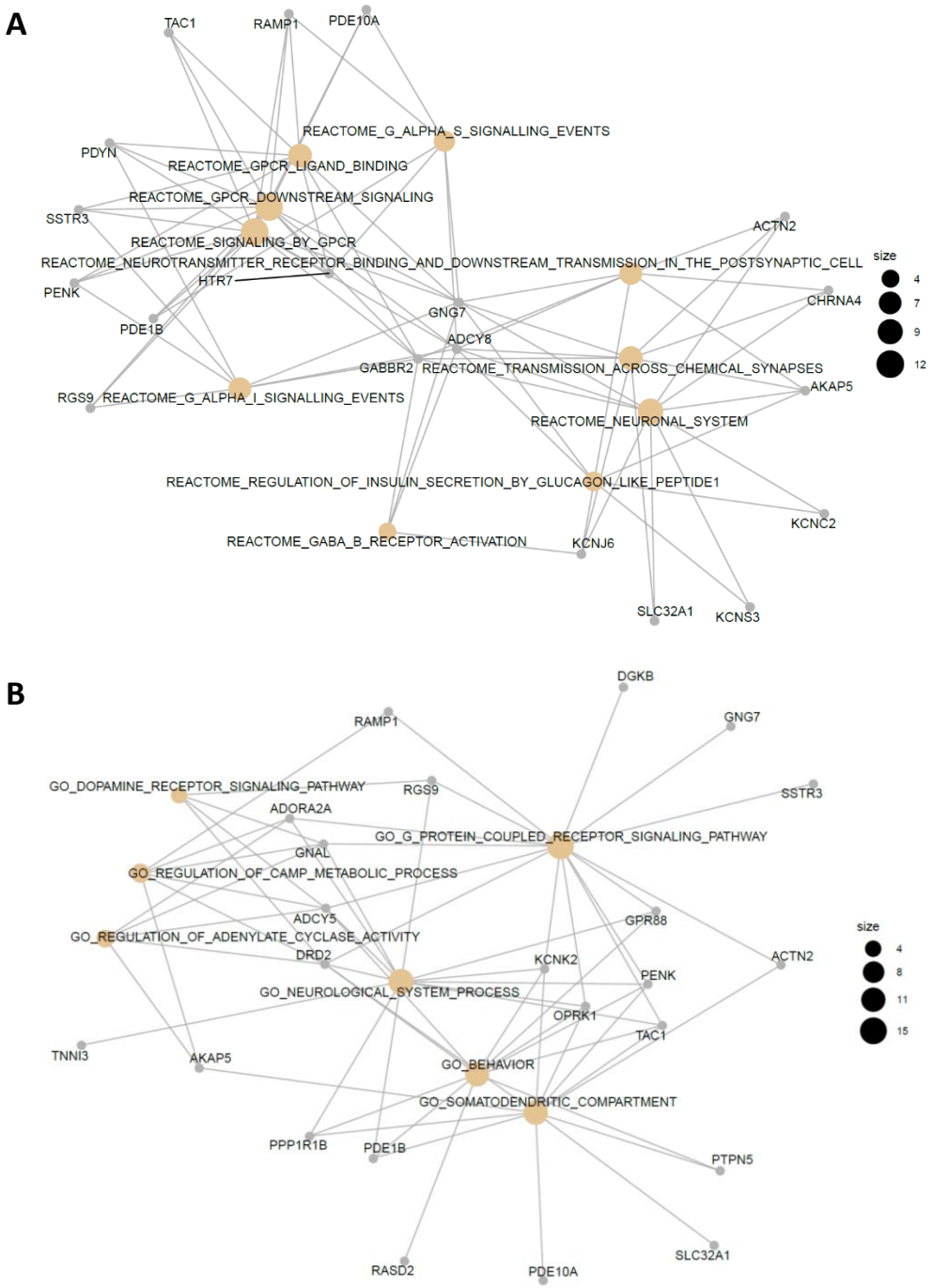


Figure 3.8. Gene-concept network plot for (A) top REACTOME terms enriched in M3 module of cell-adjusted amygdalar expression levels (55 genes), associated with downregulation in the EBR group for both sexes, with relative upregulation in the female MBR group; (B) top GO terms enriched in M2 module derived from female subset of cell-adjusted amygdala (40 genes). M2 is upregulated in MBR and downregulated in EBR, in females. Size of circles representing terms refers to gene count.

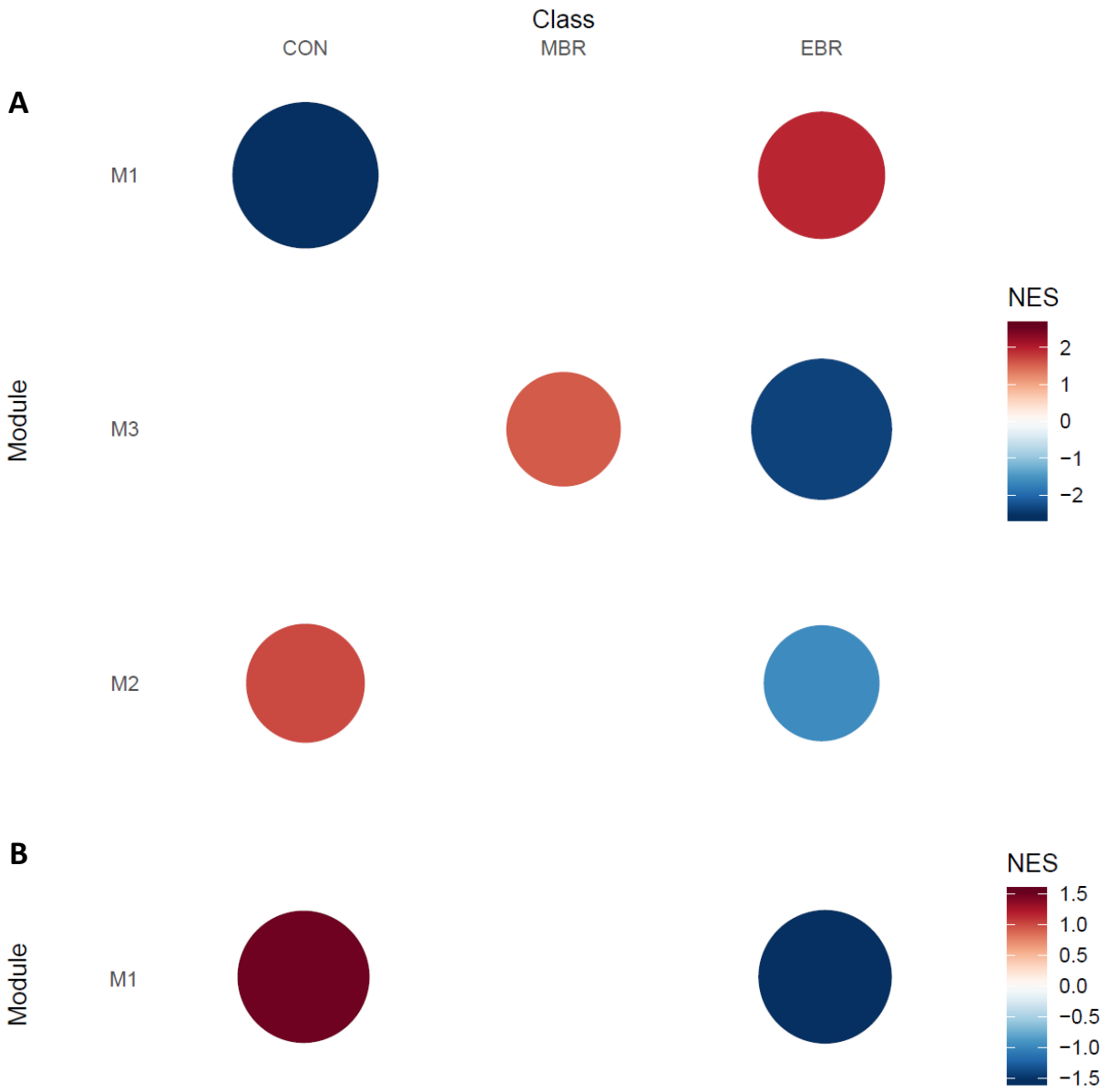


Figure 3.9. Module enrichment plot for modules identified in sex-stratified network analyses of SV-adjusted amygdalar expression levels in (A) females and (B) males.
The size and intensity of the circles correspond to the normalized enrichment score (NES) for the module in each class (normalized by the number of genes in the module.

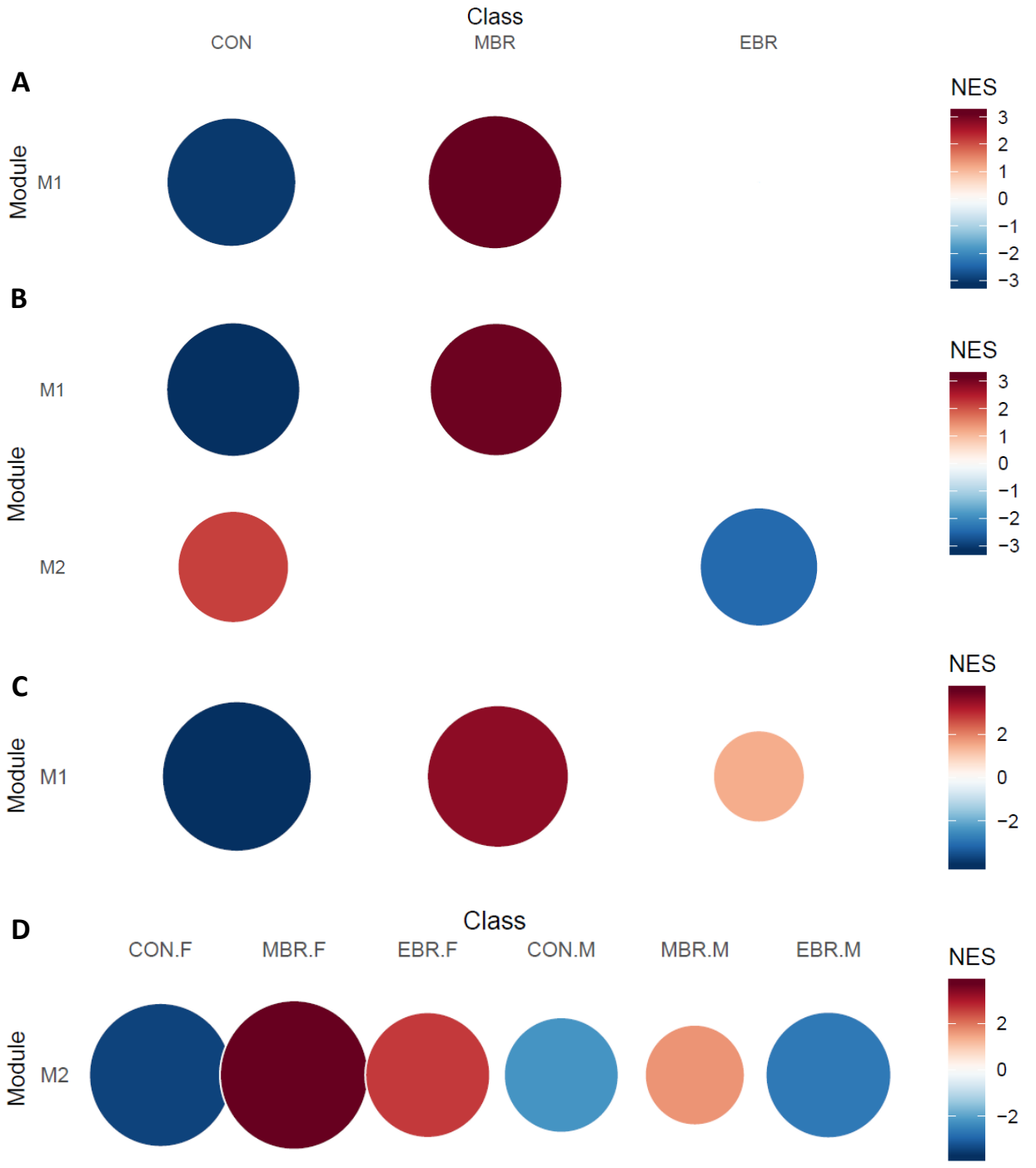


Figure 3.10. Module enrichment plot for modules identified in blood-based network. Sex-stratified analyses of SV-adjusted data in (A) males and (B) females identified a shared module upregulated in MBR relative to CON. This module was also identified in un-adjusted data in the (C) female subset and (D) full set (both sexes). Notably, this module also revealed sex-specific group differences in the EBR group and a second module was identified in the SV-adjusted female subset relevant to downregulation in female EBR, relative to CON.
The size and intensity of the circles correspond to the normalized enrichment score (NES) for the module in each class (normalized by the number of genes in the module.

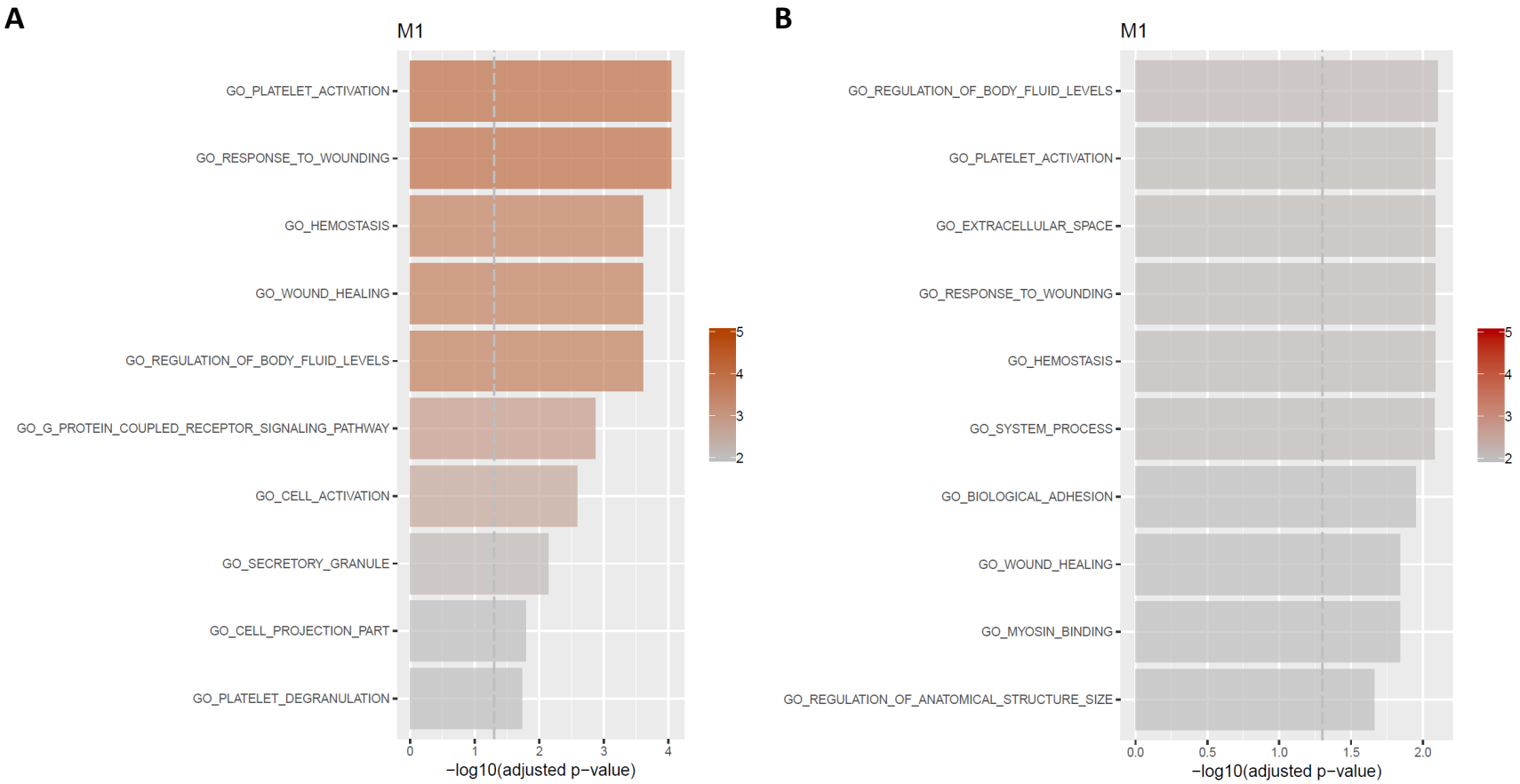


Figure 3.11. Barplot for top GO terms enriched in M1 modules identified in sex-stratified network analyses of SV-adjusted blood expression levels in (A) males and (B) females. Both modules were upregulated in the MBR group compared to the unexposed CON group. x-axis and color transparency display -$\log_{10}$ of the Benjamini-Hochberg (BH)-adjusted p-value. Dashed vertical line indicates BH-adjusted p-value threshold of 0.05.

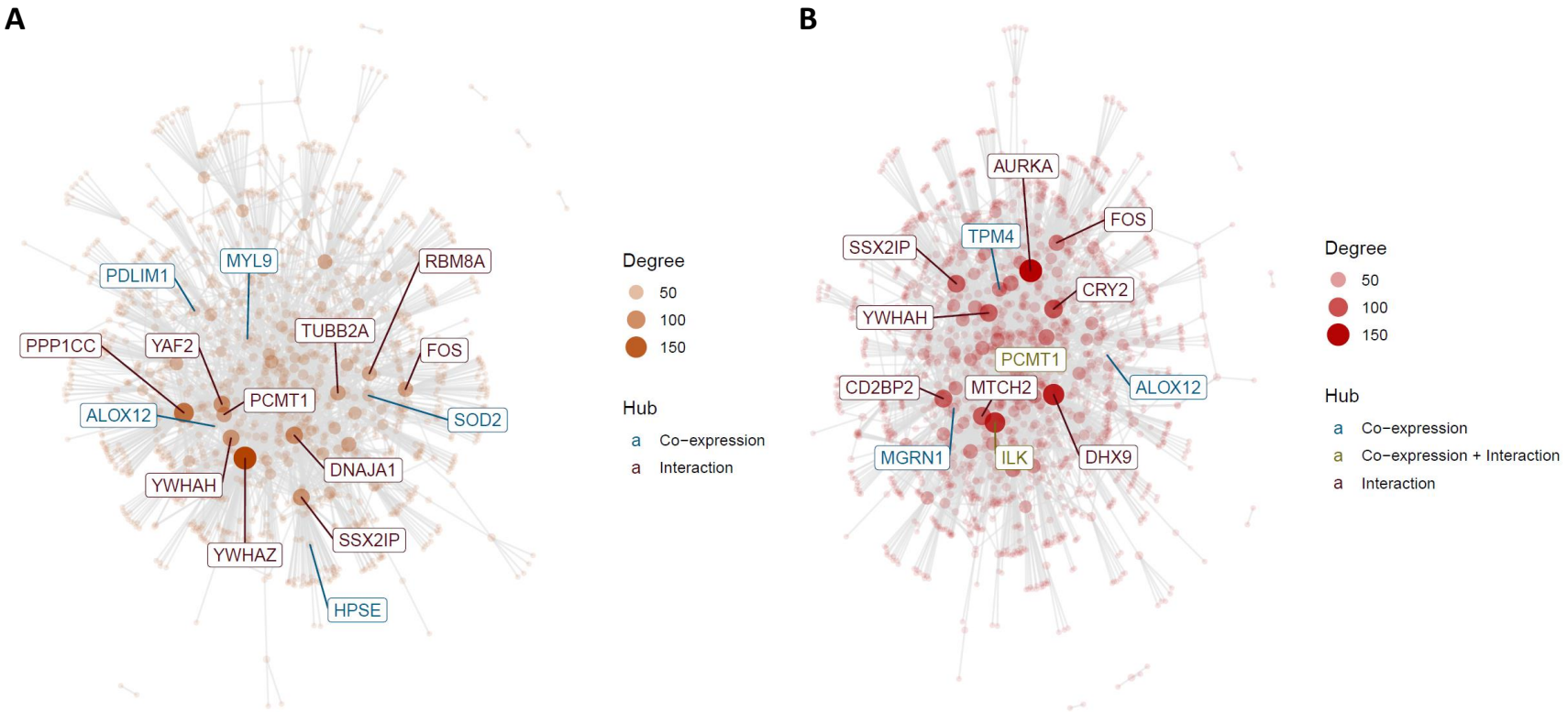


Figure 3.12. Interaction networks for M1 modules identified in sex-stratified network analyses of SV-adjusted blood expression levels in (A) males and (B) females. Both modules were upregulated in the MBR group compared to the unexposed CON group.
Circles identify hub genes in the module and color of their labels indicate nature of associations.

Table 3.1: Sample Characteristics by Tissue, Sex, and group, after processing

Tissue	Group	Count
Hippocampus	EBR	9 (M: 4; F: 5)
	MBR	9 (M: 4; F: 5)
	CON	10 (M: 5; F: 5)
Amygdala	EBR	10 (M: 5; F: 5)
	MBR	9 (M: 5; F: 4)
	CON	10 (M: 5; F: 5)
Blood	EBR	15 (M: 7; F: 8)
	MBR	16 (M: 8; F: 8)
	CON	14 (M: 8; F: 6)

Count contains number of samples included in analyses, after quality control and data processing, by tissue and group. The breakdown by sex is included in parentheses (M = male; F = female).

EBR: extreme behavioral response
MBR: minimal behavioral response
CON: trauma unexposed controls

Table 3.2: Brain cell subtype markers used for cell proportion estimates

Tissue	ast	end	mic	neu	oli	opc
Hippocampus	386	352	338	476	460	334
Amygdala	392	372	353	497	468	349

Table reports the number of markers/genes expressed out of 1,000 markers per cell type, curated in *BRETIGEA*. These markers were used for cell proportion estimates.
ast: astrocytes; end: endothelial cells; mic: microglia; neu: neurons; oli: oligodendrocytes; opc: oligodendrocyte progenitor cells.

Table 3.3: ANCOVA Table - Hippocampal Oligodendrocytes EBR vs. MBR (n = 18)

Terms	Sum of Squares	*df*	Mean Square	*F*	*p*	$_{\text{partial}}\eta^2$
PTSD group	0.297	1	0.297	11.410	0.004***	0.432
Sex	0.001	1	0.001	0.053	0.822	0.004
Residuals	0.390	15	0.026			

***$p < 0.005$

Table 3.4: Dunn test for Kruskal-Wallis multiple comparison - Hippocampal Oligodendrocytes

Comparison	*Z*	*p (unadj)*	*p (adj)*	r^2
CON - EBR	-2.31	0.021	0.042	0.28
CON - MBR	0.66	0.508	0.508	0.02
EBR - MBR	2.89	0.004	0.011	0.46

Note: p-values adjusted with the Holm method across group comparisons.
Direction of effect for EBR vs CON comparison is the inverse of CON vs EBR comparison.
Estimate of percentage variance explained r^2, is calculated from the *Z*-score: , where N is the total number of observations.

Table 3.5: Enrichment for M2 module associated with EBR vs CON in female subset of SV-adjusted hippocampal expression data

	Gene Ratio	Bg Ratio	adj p	geneID
C2: CGP (top hit)				
NAGASHIMA_NRG1_SIGNALING_UP	18/144	99/6477	5.3e-09	SGK1/EGR1/JUNB/TOB2/PER1/ARC/DUSP8/NFIL3/SIK1/NR4A2/EGR4/IER2/FOS/IER5/KDM6B/GADD45B/KLF2/ZFP36
NAGASHIMA_EGF_SIGNALING_UP	10/144	36/6477	2.7e-06	EGR1/JUNB/ARC/SIK1/NR4A2/EGR4/IER2/FOS/KDM6B/ZFP36
H				
TNFA_SIGNALING_VIA_NFKB	17/52	94/2230	2.5e-10	SGK1/EGR1/NFKBIA/JUNB/PER1/NFIL3/SIK1/CFLAR/NR4A2/KLF4/IER2/FOS/IER5/KDM6B/GADD45B/KLF2/ZFP36
C5: GO BP (top hits)				
CELLULAR_RESPONSE_TO_ORGANIC_CYCLIC_COMPOUND	18/132	216/5807	0.0025	APLP1/SGK1/EGR1/PDE2A/NTS/NFKBIA/RAE1/IRF3/SIRT2/ARG1/CDKN1B/NR4A2/KLF4/HCN2/BMP7/KLF2/KCNJ11/RAMP3
CELLULAR_RESPONSE_TO_ENDOGENOUS_STIMULUS	26/132	466/5807	0.0055	APLP1/SGK1/EGR1/PDE2A/NTS/WNT4/JUNB/MYO5A/ACVR2B/SIRT2/CDH13/ARG1/SIK1/PIK3R2/CACNA1H/NR4A2/KLF4/HCN2/RRAGD/FOS/BMP7/ENPP1/KLF2/KCNJ11/ASNS/RAMP3

Note: p-values adjusted with Benjamini-Hochberg method.
Consider gene sets with a minimum of 10 and maximum of 500 genes. Universe of background genes is based on overlap of genes expressed in hippocampus (n = 6,489) with gene set collection.
First number in Gene Ratio refers to number of genes overlapping between term/gene set and module (i.e., gene count), while second number refers to number of genes overlapping between gene set collection and the module.
First number in Background Ratio (Bg Ratio) refers to universe of expressed genes in the gene set, while second number refers to universe of expressed genes in the gene set collection.
geneID lists genes in gene module that overlap with genes in gene set/term.
All gene set collections (blue rows) were from the Molecular Signatures Database (MSigDB v6.2, released July 2018).
C2: CGP = chemical and genetic perturbations sub-collection in curated gene set collection; 3,433 gene sets.
H = hallmark gene set collection; 50 gene sets.
C5: GO BP = biological process sub-collection in GO gene set collection; 4,436 gene sets.

Table 3.6: Enrichment for M2 module associated with EBR in male subset of SV-adjusted hippocampal expression data

	Gene Ratio	Bg Ratio	adj p	geneID
C2: CP REACTOME				
G_ALPHA_I_SIGNALLING_EVENTS	7/42	69/2433	0.021	CXCL12/RGS4/SST/PENK/PDYN/CNR1/RGS8
GPCR_DOWNSTREAM_SIGNALING	10/42	178/2433	0.04	CXCL12/PRKCE/NGEF/RGS4/SST/PENK/VIP/PDYN/CNR1/RGS8
SIGNALING_BY_GPCR	11/42	216/2433	0.04	CXCL12/PRKCE/NGEF/RGS4/SST/WNT4/PENK/VIP/PDYN/CNR1/RGS8
C3 TFT				
GR_Q6	8/82	106/5191	0.041	TYRO3/WNT4/KLF5/DLX1/NNAT/CNTN4/NCAM1/LRRTM3
CEBP_Q2_01	8/82	107/5191	0.041	STMN1/TNNC2/KLF5/DLX1/CALML4/PTEN/EFEMP1/LRRTM3
LFA1_Q6	8/82	107/5191	0.041	WNT4/ANXA4/LCAT/DLX1/PDYN/ACVR1C/GSS/TMEM132E
C5: GO				
REGULATION_OF_SYNAPTIC_TRANSMISSION_GABAERGIC	5/96	20/6137	0.02	PRKCE/HAP1/PTEN/PLCL2/CNR1

Note: p-values adjusted with Benjamini-Hochberg method.
Consider gene sets with a minimum of 10 and maximum of 500 genes. Universe of background genes is based on overlap of genes expressed in hippocampus (n = 6,489) with gene set collection. First number in Gene Ratio refers to number of genes overlapping between term/gene set and module (i.e., gene count), while second number refers to number of genes overlapping between gene set collection and the module. First number in Background Ratio (Bg Ratio) refers to universe of expressed genes in the gene set, while second number refers to universe of expressed genes in the gene set collection. geneID lists genes in gene module that overlap with genes in gene set/term.
All gene set collections (blue rows) were from the Molecular Signatures Database (MSigDB v6.2, released July 2018).
C2: CGP = chemical and genetic perturbations sub-collection in curated gene set collection; 3,433 gene sets.
C2: CP REACTOME = REACTOME gene sets in canonical pathways sub-collection in curated gene set collection; 674 gene sets.
C3 TFT = transcription factor targets sub-collection in motif gene set collection; 615 gene sets. Significant gene sets suggest module enrichment for genes having at least one occurrence of transcription factor binding sites for VGR_Q6, VCEBP_Q2_01, and V$LFA1_Q6 (v7.4 TRANSFAC), in the regions spanning up to 4 kb around their transcription start sites.
C5: GO = GO gene set collection; 5,917 gene sets total.

Table 3.7: Sex differences in distribution of amygdalar cell subtype proportions

	Levene's Test for Equality of Variances		Two-sample Kolmogorov-Smirnov Test	
Cell subtype	***F***	***p***	***D***	***adj p***
ast	7.92	0.009	0.652	0.01
end	1.92	0.18	0.319	0.52
mic	0.011	0.92	0.357	0.52
neu	23.28	4.87e-05	0.790	4.82e-04
oli	5.47	0.027	0.571	0.037
opc	5.18	0.005	0.719	0.003

Note: In two-sample Kolmogorov-Smirnov test, p-values were adjusted using Holm's method across cell subtypes. Nominal p-value is shown for Levene's test (df: 1,27).
ast: astrocytes; end: endothelial cells; mic: microglia; neu: neurons; oli: oligodendrocytes; opc: oligodendrocyte progenitor cells.

CHAPTER 4: GENE REGULATORY MECHANISMS UNDERLYING PTSD IN AFRICAN AMERICANS

4.1 Abstract

Although post-traumatic stress disorder (PTSD) is moderately heritable, robust replicable genetic variants have eluded detection due to challenges managing heterogeneity and gene-environment interactions. Implementing an integrative systems level approach can help infer regulatory contexts for genetic variants and epigenetic marks. In this study we conducted a preliminary investigation of the gene regulatory mechanisms underlying PTSD, specifically in African-Americans (AA) – an understudied, at-risk population. We identified a putatively causal methylation quantitative trait locus (meQTL) located in the *CAST* gene; specifically, we found that rs151835 may be mediated by DNA methylation levels at cg13012653. Further investigation revealed that *CAST* is located within topologically associated domains (TADs), suggestive of shared regulatory mechanisms across tissue, and a separate resource mapping the correlation of methylation levels (CpG) between brain and blood showed a significant correlation in CpG levels between brain and blood. SNPs that do not endorse a causal effect on PTSD themselves may still have a functional effect regulating CpGs and gene expression levels (GE) involved in the pathophysiology of PTSD. By including eQTL and meQTL SNPs as covariates in differential analyses, we identified differentially methylated (DM) CpGs and expressed (DE) genes that previously could not be detected. On a related but separate note, we also identified one eQTM association independent of any SNP between a cell-specific DM (csDM) CpG, suggested to be on $CD8^+$ T cells, and a DE gene. Lastly, we examined overlap of these PTSD-relevant associations found in blood with previous reports in relevant brain regions (ie.,

frontal/temporal cortex, hippocampus) to identify a set of PTSD-relevant eQTL and meQTL associations in both blood and brain. Continued studies examining the implications of this overlap may help identify PTSD-relevant eQTL and meQTL associations in blood that can make inferences in brain.

4.2 Introduction

While most people experience a traumatic event at some point in their lives, only a small subset develop PTSD over a lifetime, suggesting that trauma exposure is necessary but not sufficient for the development of PTSD and that pre-existing factors, such as genetics, contribute significantly. Although PTSD is moderately heritable[342-345], contributing genetic variants have been difficult to detect and replicate due to a number of challenges. In addition to heterogeneity derived from the constantly evolving diagnostic criteria that encompass a diverse array of symptom presentations, PTSD has a highly complex, polygenic genomic architecture that needs to be considered in the context of trauma exposure[346] – a prerequisite PTSD diagnostic criterion. In other words, there are a large number of genes with small effects that contribute in different and interactive ways (e.g., gene-by-gene (GxG) interaction) towards disease susceptibility, which is compounded by the importance of considering gene-by-environment (GxE) interactions to model how the environment (e.g., trauma exposure) influences PTSD risk by interacting with contributing genetic factors[347]. Thus, identifying and assigning causality to specific genetic variants is challenging for polygenic, complex trait conditions in general[348], and particularly complicated in the case of PTSD due to the importance of context (e.g., trauma type) and cumulative combination of both genetic and non-genetic factors.

One important consideration applied to genetic studies of PTSD is comparison of PTSD cases against similarly trauma-exposed controls. However, single data-type approaches such as large-scale genome-wide association studies (GWAS) and candidate gene studies have still had limited success, to date, in identifying replicable risk variants[39], due to the challenges of considering these interactions. An integrative systems level approach that incorporates genomic, epigenomic, and transcriptomic data can help achieve a more thorough and informative interrogation of genotype-phenotype associations and put putative regulatory roles of genetic variants and epigenetic marks (e.g., methylated CpG sites) in context.

Another challenge of genomic studies is confounding due to population stratification (i.e., population differences in SNP variation) in samples of diverse genetic ancestry. To address this, populations based on ancestry are often analyzed separately and statistical techniques controlling for population stratification are used to increase likelihood of finding genetic variation relevant to the phenotype rather than spurious associations to ancestry markers. However, much of the genomic literature has been grounded in studies of European-ancestry individuals and our understanding of genomic contexts derived from these studies cannot be generalized to other ancestry groups[349]. Lack of population diversity in GWAS is a major concern in genomic medicine with significant implications for health disparities, and particularly recognized for those of African ancestry[350-352]; this lack of diversity has explicitly been recognized in genomic PTSD research as well[353]. African ancestry populations have the greatest genomic diversity compared to all other world populations and a number of examples have illustrated the functional consequences and African-specific health implications, notably for cardiometabolic and immune traits relevant to diabetes, cardiovascular disease, obesity/inflammation, and autoimmune diseases[354-357].

Ancestry-specific differences in genomic context may be due to population differences in allele frequency at functional genomic variants or other mechanisms involving other layers of genetic regulation. A prominent example illustrating a fundamental immune system consequence of functional genetic variation is the Duffy Null polymorphism, which is > 90% difference in frequency between West Africans and European Americans and predictive of white blood cell and neutrophil count[358,359]. Notably for epigenetic regulation, DNA methylation has been shown to contribute to population differences, with the majority of population-specific CpG sites associated with genetic variation[360-363]. Such genomic variants, or methylation quantitative trait loci (meQTLs), underlie allele-specific methylation patterns at a given locus and are thought to mediate inter-individual methylation differences that may be invariant across tissues[364]. In other words, since genetic variants/SNPs do not vary within an individual, DNA methylation loci regulated by these SNPs may share patterns across tissues. Thus, identifying functionally relevant genetic variants (SNPs) that share regulatory contexts across tissues of interest (e.g., blood and brain) may provide insights into disease-relevant changes in less accessible tissues of interest (e.g., brain).

In this study, we investigate the genomic context underlying DNA methylation and gene expression patterns associated with post-traumatic stress disorder (PTSD), specifically in African Americans, who have been shown to have greater conditional risk for developing PTSD following trauma exposure[18]. Specifically, we implement an integrative -omics framework to 1) identify genetic variants (SNPs) that putatively regulate DNA methylation and gene expression in PTSD, 2) investigate methylated CpG marks associated with leukocyte gene expression relevant to PTSD, and 3) identify SNPs that putatively regulate DNA methylation and gene expression associated with PTSD in peripheral blood and determine if the same regulatory

context has been identified in relevant brain tissues in previous studies[364,365], to infer PTSD-associated changes that may be shared in both blood and brain.

4.3 Methods

Study Participants and Datasets

Adult participants of the Detroit Neighborhood Health Study (DNHS; described previously) with available genotype, DNA methylation, and/or gene expression (GE) microarray data were included in this study. Datasets were derived from whole blood samples that were processed to isolate DNA, for genotyping and DNA methylation assays, and RNA for gene expression measurements at the Applied Genomics Technology Facility (Wayne State University, Detroit). Genotype, DNA methylation (DNAm), and GE were assayed using the Illumina OmniExpress, HM 450K, and HT-12 BeadChip arrays, respectively, in consenting DNHS participants. For expression quantitative trait loci (eQTL) analyses, there were 81 African-American (AA) participants with both genotype and GE data, balanced for sex and lifetime PTSD status. For methylation quantitative trait loci (meQTL) analyses, there were 124 AA participants with both DNAm and genotype data. For expression quantitative trait methylation loci (eQTM; i.e., sites showing correlated expression and methylation) analyses, there were 99 participants (AA: n = 82) with both DNAm and GE data. All controls were trauma exposed. Trauma exposure and PTSD histories were collected via structured telephone interviews.

Lifetime PTSD diagnosis was assessed by the well-validated self-report PTSD Checklist, Civilian Version (PCL-C)[138-140] based on symptoms defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)[14,108,142]. Participants who met all six

DSM-IV criteria in reference to their worst traumatic event or to a randomly selected traumatic event (if the participant experienced more than one trauma), were considered affected by lifetime PTSD. The survey-based diagnoses were validated in a random subsample of participants via in-person clinical interview and showed high internal consistency and concordance[108,143]. Smoking status was defined as smoking at least one day within 30 days of survey data collection, as reported by the participant.

Sample Processing

Genomic DNA was extracted from peripheral blood using the DNA Mini Kit (Qiagen, Germantown, MD). DNA samples were bisulfite-converted using the Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA) and whole-genome amplified, fragmented, and hybridized to the Illumina Human Methylation 450K BeadChip array (Illumina, San Diego, CA), according to the manufacturers' recommended protocols, as published previously[148,149]. RNA was extracted from leukocytes using Leukolock kits (Ambion, Austin, TX) following the manufacturer's alternative protocol, to preserve total RNA. RNA samples that met quality control criteria for RNA quality and purity: RNA integrity number (RIN) ≥5 and 28s/18s ratio ≥1.0, as determined by Agilent 2100 Bioanalyzer (Agilent, Wilmington, DE), and 260/280 ratios ≥1.7, as determined by NanoDrop 1000 (ThermoScientific, Wilmington, DE), were used for gene expression profiling. For microarray processing, 220ng of RNA from each participant was supplied to the Wayne State University Core facility, Applied Genomics Technology Center (AGTC), and each chip of up to 12 samples was matched for PTSD cases and trauma-exposed controls.

Quality Control, Processing, and Analysis of -Omic Data

An overview of the computational workflow, described in more detail below and referenced in each subsection, is summarized as a flow chart (Figure 4.1). Unless otherwise indicated for genotype quality control (QC) and processing, all QC, processing, and analyses were conducted in R, version 3.5.1[151], Bioconductor version 3.7[153,154].

Quality Control and Processing of Genotype Data

Quality-control (QC), imputation, and calculation of ancestry PCs from genotype data are described in Duncan et al. (2017)[39]. Briefly, QC procedures were conducted in PLINK[366]; samples and probes were removed based on the following criteria, sequentially: 1) Monomorphic SNPs and SNPs with missingness > 0.05 (i.e., call rate < 95%); 2) Individuals with missingness > 0.02 (i.e., call rate < 98%); 3) Individuals with heterozygosity $|F_{het}| > 0.2$ (autosomal heterozygosity deviation); 4) Individuals failing sex check; 5) SNPs with missingness > 0.02 (call rate < 98%); 6) SNPs with differential missingness between cases and controls > 0.02; 7) SNPs failing Hardy-Weinberg equilibrium (controls: $p < 1x10^{-6}$; cases: $p < 1x10^{-10}$). Pre-phasing and genotype imputation to the 1000 Genomes[367] phase 1 reference panel was performed according to the PGC pipeline[368] using SHAPEIT for phasing[369] and IMPUTE2[370,371] for imputation. Imputation was performed with a chunk size of 3 Mb and default parameters on the full set of 2,186 phased haplotypes (release v3.macGT1, August 2012).

Additional QC was conducted using the *SNPRelate* package[372] to perform identity-by-descent (IBD) relatedness analysis on 631 samples in the dataset to check for cryptic relatedness and duplicates, using probes retained after linkage disequilibrium (LD) -based SNP pruning (LD threshold = 0.2; kinship threshold ≥ 0.1). IBD coefficients were computed using PLINK's

method of moments procedure. After removing 4 samples based on IBD analysis, 627 samples were retained, of which 596 participants self-reported as African-American and non-Hispanic. SNPs were limited to those with a minor allele frequency (MAF) > 0.1 for genetic association tests and eQTL/meQTL analyses.

Quality Control and Processing of DNA Methylation Data

QC and data processing for the DNHS 450K dataset followed the workflow described in Chapter 2. Briefly, five samples were removed for gender discordance in the QC step, before ssNoob[158,163] background correction, probe filtering based on detection (p-value > 0.001 in more than 10% of samples[155]) and cross-reactive probes[159] (i.e., cross-hybridized between autosomes and sex chromosomes), and beta-mixture quantile (BMIQ) normalization (*ChAMP*[161,162]). To control for technical artifacts (e.g., sample processing and imaging batch effects), four PCs based on non-negative control probe signal intensity[156] were removed from BMIQ-normalized M-values (i.e., logit-transformed beta-values), while controlling for lifetime PTSD and age. PC correlation heatmaps were used to check for successful removal/reduction of batch effects, especially chip and row effects, while maintaining signal from biological variables.

Leukocyte Composition and Ancestry Estimation Based on 450K Data

Leukocyte composition was estimated on batch-adjusted beta-values using robust partial correlation (RPC; robust multivariate linear regression, non-constrained projection) as the deconvolution algorithm and the *EpiDISH*[128] reference database, which is informed by cell-type specific DNase hypersensitive sites (DHS; based on the NIH Epigenomics Roadmap database[166])

and is optimized for discriminating granulocytes, $CD14^+$ monocytes, $CD8^+$ T cells, $CD4^+$ T cells, $CD19^+$ B cells, and $CD56^+$ natural killer cells.

DNAm-based ancestry principal components (PCs) were derived on cleaned beta-values after regressing out sex and age from batch-adjusted M-values. Ancestry PCs were calculated on a subset of 2,317 ancestry informative CpG probes included in two published ancestry informative CpG lists that accounted for confounders[168] and that included probes within 10 base pairs (bp) of single nucleotide polymorphisms (SNPs)[169]. The first 2 PCs based on this subset of probes were used as ancestry PCs after checking for strong association with self-reported race. Scatter plots confirmed effective separation of self-reported race.

Quality Control and Processing of Gene Expression Data

Raw control and sample probe profiles were imported into R. Samples were removed based on low sample quality and QC metrics (i.e., RIN ≤ 5; inter-array correlation (IAC), std dev > -2.5; sex check fail). Out of 129 samples, 123 were retained after sample QC. (One removed for not having RIN data, 4 removed as outliers based on inter-array correlation (IAC), 1 removed for failing sex check.) Pre-processing involved *normexp* background correction using negative control probes, quantile normalization, and $\log_2$ transformation using the *neqc* function in the *limma* package[228,229]. Probes were filtered based on expression (detection p-value < 0.01 in at least 10% of subjects) and processed expression data was adjusted for plate effects, while accounting for lifetime PTSD, sex, and age, using ComBat[165] in the *sva* package.

Probes were annotated based on three database sources available on Bioconductor: 1) ReMOAT re-annotated Illumina HT12-v4 probe annotations in *illuminaHumanv4.db*[230]; 2) UCSC Known Gene table on the hg19 genome assembly, mapped by Entrez Gene ID

(*TxDb.Hsapiens.UCSC.hg19.knownGene*); and 3) Illumina HT12-v4 annotations from Ensembl via BioMart (GRCh37.p13/hg19. Ensembl 95).

Leukocyte Composition Estimation Based on HT-12 Data

Relative leukocyte subtype proportions were estimated using the CellCODE approach to compute surrogate proportion variables (SPVs)[232], which reflect relative differences in cell-type composition between groups. SPV estimation was based on ComBat-corrected expression data and guided using tag markers for B cells, $CD8^+$ T cells, $CD4^+$ T cells, natural killer (NK) cells, monocytes, and granulocytes, based on HaemAtlas[373], a publicly available, pure cell reference dataset built on the Illumina HumanWG-6 v2 BeadChip array (ArrayExpress E-TABM-633). Before use in tag marker selection, the HaemAtlas dataset was corrected for array effects while accounting for cell-type using ComBat and probes were filtered to exclude probes that mapped to sex chromosomes and that had poor annotation quality (i.e., inconsistent chromosome mapping across annotation databases, UCSC knownGene table, hg19 or Ensembl 95). Expression of retained probes were averaged within each cell type to build the reference dataset used for tag marker selection.

Up to 10 tag markers were selected for each cell type based on ranking by highest expression value that exceeded the other five cell types by a cutoff of 1 in $\log_2$ space. The tag markers estimated to be the most transcriptionally stable (80% of markers) for each cell type were used to guide singular value decomposition (SVD) of the sex-stratified dataset structure and the first right singular vector (i.e., eigengene) was used as the SPV corresponding to relative proportion for that cell type. The SPV estimates were incorporated into the linear model for our differential expression analyses to adjust for cell-type proportions.

Data Preparation for Cross-Omic Analyses

Additional data preparation steps were conducted before cross-omic analyses. HT12 v4 expression probes were further filtered based on four criteria. Probes were removed if they 1) had poor quality annotation, based on no mapping to Entrez Gene ID and inconsistent chromosome mapping between the Illumina annotation and either UCSC knownGene table and/or Ensembl annotation sources; 2) mapped to non-autosomal chromosomes (i.e., sex chromosomes); and 3) overlapped with SNPs that had MAF ≥ 0.01 in the African superpopulation, based on 1000 Genomes Project, Phase 3 data[374]. Additionally, for genes that mapped to multiple probes, the probe with the highest average intensity/expression level was selected to represent the gene (Entrez Gene ID) for 1:1 probe:gene mapping. HM450K methylation probes were also filtered based on mapping to non-autosomal chromosomes and overlap with SNPs that had MAF ≥ 0.01 in the African superpopulation. In total, 1,102,581 SNPs, 9,055 genes, and 328,492 CpGs were retained for analyses.

Inverse-rank normalized residual expression, adjusted for lifetime PTSD, sex, age, ancestry PCs, CellCODE SPV estimates ($CD8^+$T cells, $CD4^+$T cells, B cells, NK cells, monocytes, and granulocytes), current smoking, and RNA integrity number (RIN), was used for eQTL and eQTM analyses. Similarly, inverse-rank normalized residual M-values (logit-transformed beta values), adjusted for lifetime PTSD, sex, age, DNAm-based ancestry PCs, RPC cell estimates ($CD8^+$T, $CD4^+$T, NK cells, monocytes, B-cells), and current smoking, was used for meQTL and eQTM analyses. Genotype-derived ancestry PCs were used for covariate adjustment in eQTL and meQTL analyses and DNAm-based ancestry PCs were used for eQTM analyses.

xQTL Association Analyses: PTSD-Agnostic eQTL. meQTL, and eQTM mapping

Cross-omic quantitative trait loci (xQTL) associations were identified using the BootstrapQTL[375] package, built on the MatrixEQTL[376] framework, separately for SNP-GE (cis-eQTL), SNP-CpG (cis-meQTL), and CpG-GE (cis-eQTM) associations. Briefly, the BootstrapQTL method implements a hierarchical procedure to control for multiple testing of variants at each gene (local correction) before controlling for multiple testing across all genes (global correction), and also corrects for effect size overestimation using a bootstrap method[375]. The cis-eQTL and cis-eQTM analyses were conducted on the full set of genes and SNPs within 250kb of the Illumina Human HT12-v4 probe ends, mapped to expressed probes on the HT12 BeadChip array. To alleviate the burdensome number of associations between 1,178,530 SNPs and 328,492 CpGs, meQTL association analyses were conducted in two sets: set 1 was filtered by SNPs identified as eQTLs in eQTL association analyses and set 2 was conducted on an LD-pruned set of SNPs (i.e., those in linkage equilibrium), which was identified by iteratively removing adjacent SNPs that exceed an LD threshold of 0.8 in a sliding window. The first meQTL set was used towards cross-omic integrative analyses (i.e., identification of SNP-CpG-GE associations) and the second, LD-filtered meQTL set was used for meQTL-specific differential methylation analyses. Both meQTL sets used a distance threshold of 100kb between SNPs and CpGs.

Since CpGs and genes with eQTL/meQTL associations are associated with multiple SNPs, some of which may be in linkage disequilibrium, we identified a set of effective eQTL and meQTL associations for leading SNPs based on best (minimum) p-value per CpG or gene. For CpGs and genes that had associations in both directions (i.e., both positive and negative

directions of effect), we identified secondary SNPs with minimum p-value among SNPs with opposite direction of effect from the primary leading SNP per CpG/gene. We used these effective sets of associations in subsequent differential analyses (Figure 4.1). Additive modeling was used for genotype.

Genetic Association Tests for lifetime PTSD

Simple genetic association tests based on Cochran-Armitage test (1 df) and conventional Pearsonian test (2 df; 3x2 contingency table) were run on the larger DNHS genotype dataset of African-American participants (n = 596) for SNPs with MAF > 0.1, using *snpStats* to identify SNPs suggestively associated with lifetime PTSD ($p < 0.01$ for either association test). Genomic inflation factor (λ) for both tests was 1.03.

Differential Methylation and Expression Analyses for lifetime PTSD (without accounting for SNP)

Genes independent of eQTLs (in eQTMs) were identified as PTSD-relevant if they were significantly associated with lifetime PTSD based on standard differential expression analysis (FDR < 0.05) conducted on the subset of eQTM participants (n = 99). Gene expression was modeled as a function of lifetime PTSD, accounting for sex, age, DNAm-based ancestry PCs, current smoking status, RIN and CellCODE SPVs. The linear model was fit genewise (on the full set of genes, n= 9,055) using an empirical Bayes approach in *limma* to compute moderated t-statistics and $\log_2$ fold change[228,238]. CpGs independent of meQTLs (in eQTMs) were identified as PTSD relevant if they were significantly associated with lifetime PTSD based on cell-type specific differential methylation (csDM) analysis (FDR < 0.05) conducted on the full DNHS

HM450K dataset (n = 174). This recently published approach, described in Zheng et al. (2018)[209], models beta-values as the interaction between each leukocyte subtype proportion (RPC-based cell estimate) and lifetime PTSD, accounting for sex, age, current smoking, and DNAm-based ancestry PCs.

Causal Inference Test and Mediation Analyses for lifetime PTSD

Causal inference test (CIT)[377] and mediation analyses[378] were conducted for identified eQTL and meQTL associations separately at SNPs suggested to associate with lifetime PTSD in our larger genotype dataset of African-American participants (n = 596). CIT implements formal statistical hypothesis testing to quantify uncertainty in causal inference using an intersection-union test that combines a series of four statistical tests for a "chain" of mathematical conditions to form an omnibus test that determines if a potential mediator is causal for the trait[377]. Causal mediation is supported by a significant omnibus p-value ($p\_cit < 0.05$), which represents the maximum of the p-values for the four component tests.

Initial CITs were run without permutation using the *cit* package, accounting for covariates in the model. For CITs on eQTL associations, covariates included were sex, age, CellCODE SPVs, current smoking and RIN. For CITs on meQTL associations, covariates included were sex, age, RPC cell estimates and current smoking. For significant causal hits identified in initial CIT, follow-up CIT was run with 10,000 permutations, based on the original code published in Millstein et al. (2009)[377].

Additionally, causal mediation analyses were run with non-parametric bootstrapping (10,000 Monte Carlo draws) for significance testing and estimation of the average causal mediation effects (ACME), using the *mediation* package[378]. In the outcome model for these

analyses, lifetime PTSD is regressed on both the SNP and mediator (GE or CpG), using a binomial model with probit link function, to confirm that the mediator is a significant predictor of lifetime PTSD and that the significant association between lifetime PTSD and SNP is lost (full mediation) or greatly reduced (partial mediation) after accounting for the mediator. Causal mediation analysis was conducted for treatment comparisons between control genotype with no minor alleles to one minor allele and between genotype with no minor alleles to two minor alleles. In addition to estimating ACME, mediation analysis reports the average direct effects (ADE), total effect (tau; combined indirect and direct effects), and the ratio of these estimates (i.e., proportion mediated).

Adjusted expression levels and M-values were used for both CIT with permutation and causal mediation analyses. Expression levels were adjusted for sex, age, ancestry PCs, CellCODE SPVs, current smoking and RIN, while accounting for lifetime PTSD, and inverse-rank normalized. M-values were adjusted for sex, age, ancestry PCs, RPC cell estimates, and current smoking, while accounting for lifetime PTSD, and inverse-rank normalized.

Identification of xQTL associations relevant to lifetime PTSD

To investigate relevance of xQTL associations to PTSD, we identified CpGs differentially methylated (DM) and genes differentially expressed (DE) by lifetime PTSD. Since SNPs significantly influence associated DNA methylation levels (CpGs) and gene expression levels (GE), differential methylation and differential expression was considered for eQTMs independent of SNPs (i.e., without associated meQTL or eQTL) separately from CpG and GE significantly associated with meQTLs and eQTLs. As described above, CpG and GE

independent of meQTLs and eQTLs, respectively, were identified using cell-specific DM and standard linear model DE analyses that did not account for SNP as a covariate.

CpGs and gene expression probes associated with meQTLs/eQTLs were identified as PTSD-relevant if they were significantly associated with lifetime PTSD while accounting for meQTL/eQTL, based on two tests: 1) the relevant component test in the CIT framework (association conditioned on SNP; without permutation); and 2) likelihood ratio test comparing models with and without lifetime PTSD as a predictor ($p < 0.01$ for both tests). Overlap between PTSD-relevant xQTL associations identified genomic variants that acted as both eQTLs and meQTLs (putatively regulating both DNAm and gene expression) and significant SNP-CpG-gene associations. Finally, PTSD-relevant associations were queried against previously published xQTL associations in brain tissues (i.e., hippocampus and frontal/temporal cortex)[364,365] to determine if any putatively share regulatory contexts in blood and brain.

4.4 Results

Identification of xQTL associations in the DNHS

In cis-eQTL analyses, 3,476 significant cis-eQTLs were identified for 3,421 unique SNPs (MAF > 0.1) among associations between 9,055 genes and 1,102,581 SNPs. Of the 3,421 SNPs associated with gene expression (GE), 4,075 significant SNP-CpG (cis-meQTL) associations were identified for 1,649 SNPs and 343 CpGs. These SNPs were associated with 117 unique genes through their cis-eQTL association and considered cis-e/meQTLs associated with both CpG and GE. In our second meQTL analysis on LD pruned subset of SNPs (n = 285,812), 35,997 significant cis-meQTLs were detected. Across both sets of meQTL analyses, 39,668 distinct meQTLs were detected and 1,649 SNPs were identified as cis-e/meQTLs with both

DNAm and GE associations. There were 4,104 e/meQTL associations for these SNPs to 343 CpGs and 117 genes. After selecting strongest associations based on minimum p-val per CpG/gene (in each direction of effect), there were 265 eQTL and 18,415 meQTL associations in effective xQTL sets used in subsequent analyses combining xQTL and differential analyses (i.e., Step 2 in Fig. 4.1).

Additionally, eQTM analyses detected 356 significant CpG-GE associations. Of these, the CpGs in 167 eQTM associations also had meQTL associations and GE in 97 eQTM associations also had eQTL associations. Investigating the overlap across eQTL, meQTL, and eQTM associations identified 1,512 significant SNP-CpG-GE associations. These cross-omic associations consisted of 985 unique eQTL associations, 1,500 meQTL associations, and 80 eQTM associations, across 973 SNPs, 79 CpGs, and 42 unique genes.

Identification of individual PTSD-relevant features

Genetic association tests (1 and 2 df) were used to identify candidate SNPs suggestively associated with lifetime PTSD. In the full SNP set (typed and imputed), 19,327 out of 1,182,646 SNPs with MAF > 0.1 were identified to have a suggestive association with lifetime PTSD ($p<0.01$). Genetic association tests on the LD pruned subset of SNPs (n = 285,812) detected 4,717 SNPs that were suggestively associated with lifetime PTSD ($p < 0.01$).

Standard linear model differential expression analyses identified 1,104 DE genes associated with lifetime PTSD (FDR < 0.05). Due to challenges identifying differentially methylated (DM) genes using standard linear model analyses, we implemented a recently published cell-specific DM approach reported to achieve high sensitivity and specificity in

scenarios where DM was not identified[209]. Using this approach, we identified 8,356 csDM CpGs associated with lifetime PTSD in at least one of the leukocyte subtypes (FDR < 0.05).

rs151835 identified as a putative causal variant for PTSD mediated by DNAm levels at cg13012653

There were 26 cis-eQTL SNPs suggested to be associated with lifetime PTSD based on genetic association tests conducted in the larger genotype dataset. However, none of the genes among candidate cis-eQTL associations were identified as differentially expressed by lifetime PTSD and no causal variants were identified in initial CITs for cis-eQTL associations. In contrast, among cis-meQTL associations, there were 268 SNP-CpG associations for 212 SNPs suggested to be associated with lifetime PTSD.

One cis-meQTL, rs151835, was identified as a putative causal variant for PTSD mediated by DNAm levels at cg13012653 in initial CIT (p_cit = 0.026; Table 4.1). Participants in the lifetime PTSD group were more likely to have the G/G genotype at rs151835 (Cochran-Armitage Test: $\chi^2(1, N = 596) = 6.87$, p = 0.009; Pearsonian Test: $\chi^2(2, N = 596) = 7.34$, p = 0.025), and this genotype was associated with lower methylation levels at cg13012653 (meQTL: corrected β = -0.8, SNP p < 2.7e-7, CpG p < 1.4e-6), contributing to lower methylation levels in the PTSD group (LRT: p = 0.29; CIT: p = 0.026; Figure 4.2). This cis-meQTL, rs151835, is an intron variant located 7,852 bp upstream of cg13012653.

Follow-up CIT with 10,000 permutations using the original test published in Millstein et al. (2009)[377] continued to support a causal pathway (p = 0.048). Causal mediation analyses for both comparisons (mod1: comparing 0 vs 1 minor alleles; mod2: 0 vs 2 minor alleles) showed a significant effect of cg13012653 on the relationship between rs151835 and lifetime PTSD

(mod1: ACME = -0.1, p = 0.024; mod2: ACME = -0.2, p = 0.026) with no direct effect of SNP (mod1: ADE = -0.05, p = 0.50; mod2: ADE = -0.12, p = 0.53) and a significant total effect (tau: mod1: p = 0.026; mod2: p = 0.023). Thus, a total mediation effect of cg13012653 on rs151835 was supported for both comparisons (Figure 4.3) and all CIT and mediation analyses consistently supported rs151835 to have a causal effect on PTSD mediated via cg13012653.

Integrated xQTL and differential analyses identify putative PTSD-relevant associations

SNPs that do not endorse a causal effect on PTSD themselves may still have a functional effect regulating CpGs and genes involved in the pathophysiology of PTSD. By integrating xQTL and differential analyses, we identified xQTL associations putatively relevant to lifetime PTSD. All tables summarizing results for each set of PTSD-relevant associations are in Appendix C.

Among the effective set of SNP-GE associations, we identified 28 eQTL associations differentially expressed by lifetime PTSD. Of these, 18 were newly detected after accounting for SNP and 10 were detected in standard DE analysis even without accounting for SNP. Two of the differentially expressed genes, *FAM198B* and *AHSA2*, were associated with a secondary SNP with the opposite direction of effect from lead SNP (i.e., associated with two SNPs of opposing effects on GE), supporting more complex regulatory mechanism for these genes. Additionally, none of the SNPs were suggestively associated with lifetime PTSD based on genetic association tests.

Among effective set of SNP-CpG associations, we identified 804 meQTL associations that putatively regulate CpGs associated with lifetime PTSD. Of these, 20 were also detected in cell-type specific DM analysis without accounting for SNP while the majority were newly

detected after accounting for SNP. Additionally, 46 CpGs were associated with two SNPs of opposing effects on CpG methylation levels and 15 SNPs suggestively associated with lifetime PTSD based on genetic association tests.

Among the set of 356 CpG-GE associations, there were 3 PTSD-relevant CpG-Gene associations (eQTM; Table 4.2). One eQTM independent of SNPs (i.e., not associated with eQTLs or meQTLs, n = 249) showed a positive association (corrected β = 0.28, eCpG p = 8.1e-4, eGene p = 0.039, r = 0.41) between cg12324708 and *LYRM7*, a significant DE gene. Cell-type specific DM analyses supported cg12324708 to be hypermethylated in $CD8^+$ T cells by lifetime PTSD status. Additionally, based on HM450K annotation, cg12324708 is located in a cell-specific differentially methylated region (cDMR).

A negative eQTM association (corrected β = -0.32, eCpG p = 7.6e-4, eGene p = 0.038, r = -0.44) between cg22801149 and *VRK3* was identified and both CpG and GE were significantly associated with lifetime PTSD after accounting for SNP. Expression levels for *VRK3* was also significantly associated with lifetime PTSD in standard DE analysis without accounting for SNP. Additionally, cg22801149 - *VRK3* was associated with 49 SNPs (SNP-CpG-Gene associations) and had different lead SNPs for eQTL (rs113293707) and meQTL (rs4009626).

A positive eQTM association (corrected β = 0.46, eCpG p = 1.0e-5, eGene p = 0.001, r = 0.50) between cg09251680 and *SACM1L* was identified as PTSD-relevant: cg09251680 was associated with lifetime PTSD after accounting for its meQTL and *SACM1L* was not associated with any SNP but found to be significant in standard DE analysis.

Finally, while not identified as a significant eQTM association, two e/meQTLs (i.e., SNPs associated with both CpG and GE), rs12310416 and rs6488618, were associated with both

cg23793961 and *CLEC4C*. Both cg23793961 and *CLEC4C* were associated with PTSD after accounting for SNP.

Cross-tissue overlap of PTSD-relevant eQTL/meQTL associations between blood and brain

PTSD-relevant associations were checked against previously published associations in relevant brain regions (i.e., hippocampus and frontal/temporal cortex)[364,365]. While there was no cross-tissue overlap for eQTM associations, there were a number of PTSD-relevant eQTL and meQTL associations shared between blood and brain regions or in linkage disequilibrium with reported associations in brain.

Notably, there were 5 significant eQTL associations (Table 4.3) in both the frontal and temporal cortex that overlapped with the PTSD-relevant SNP-CpG-Gene associations identified in blood for *VRK3*. An eQTL for *CEP192*, identified to be PTSD-relevant in blood, was also detected in temporal cortex. Among meQTLs identified to be PTSD-relevant in blood, one overlapped with temporal cortex (rs10496439 - cg08023751) and 17 meQTLs overlapped in hippocampus (Table 4.4).

4.5 Discussion

In this study we conducted a preliminary investigation of the gene regulatory mechanisms underlying PTSD, specifically in African-Americans (AA) – an understudied, at-risk population. We identified a putatively causal SNP variant, rs151835, mediated by DNA methylation levels at cg13012653. Both the SNP and CpG site are located in the *CAST* gene on chromosome 5 and, based on SCREEN ENCODE[379], in candidate cis-regulatory elements (ccRE; EH37E1250073, EH37E1250077). The *CAST* gene, which encodes calpastatin, was located within the

topologically associated domain (TAD) for both ccREs. As TADs are largely invariant across different cell types[380], this is suggestive of shared regulatory mechanisms. Using a recently available resource[381], we found the CpG in this meQTL, cg13012653, to have significantly correlated methylation levels between blood and brain (*rho* = 0.6, $p = 0.044$), suggesting its utility for inferring brain CpG levels using blood CpG levels. Notably, calpastatin inhibits calpain, which has been implicated in a number of neurodegenerative disorders and is believed to play a role in neuronal death[382]. Of note, rs151835 specifically has been associated with Parkinson's disease in two independent datasets[382].

In addition to this suggestively causal meQTL, we sought to identify other PTSD-relevant associations based on differential methylation or expression of associated CpG or Genes. As mentioned, SNPs that do not endorse a causal effect on PTSD themselves may still be relevant for regulating CpGs and gene expression levels (GE) involved in the pathophysiology of PTSD. Although not detectable in our model, it is possible that the association of SNP with PTSD was not detected/hidden because our model did not account for expected interaction effects with relevant experience-related variables, such as childhood maltreatment or trauma burden. Here, we focused on identifying differential methylated CpGs and differential expressed genes by informing DM and DE models of the SNP as a covariate. We identified a number of DM CpGs and DE genes that were not identified without accounting for the meQTL/eQTL SNPs respectively. Notably, one specific set of 49 e/meQTLs was also identified as an eQTM, cg22801149-*VRK3*, where both CpG and GE were differentially methylated and expressed. Five of the participating eQTL associations were also identified in both the temporal and frontal cortical brain regions. The *VRK3* gene encodes a member of the vaccinia-related kinase (VRK) family of serine/protein kinases, is widely expressed in tissues, and has previously been

implicated in autism spectrum disorders; mice deficient in *VRK3* were shown to exhibit autism-like behavior, with decreased dendritic spine number and arborization in the hippocampus[383].

In addition to the eQTLs for *VRK3* that overlapped between blood and brain cortical tissue (frontal/temporal), we identified another PTSD-relevant eQTL and 18 meQTLs in blood that also had parallel eQTL and meQTLs identified in hippocampus and temporal cortex. Recently, a report produced a map of systemic interindividual variation of methylation levels at CpGs and showed that methylation levels in one tissue can predict expression for associated genes in other tissues[363]. Further investigation of the genomic context and region for this list of associations, including TADs, CpG methylation level correlation between blood and brain, will help identify what inferences can be made in relevant brain regions. Since genomic variants are constant across tissue, PTSD-relevant eQTLs/meQTLs that overlap between brain and blood may provide a shared genetic regulatory mechanism by which CpGs/genes in leukocytes may be correlated with those in brain. It is also important to keep in mind that the PTSD-relevant associations that we have identified and characterized provide only a glimpse of the gene regulatory context involved in PTSD. The complexities of regulatory mechanisms pose modeling challenges and cross-omic associations are not expected to be linear in many contexts. For example, the relationship between CpGs and gene expression are dependent on genomic context and have been found to be non-linear outside of classical promoter regions (eg., TSS, first exon, 3' end)[384].

Since sample size and, relatedly, use of one cohort, were major limitations of our study, we utilized integrative approaches and relied on convergence of evidence across multiple sets of analyses to support our findings. In all, our preliminary investigation of the gene regulatory mechanisms underlying PTSD in African-Americans (AA) provides proof of concept for the

implementation of an integrative genomic approach to identify candidates that may be used for blood-brain inference. Most notably, we found a causal variant in the *CAST* gene putatively mediated via DNA methylation that may have significant correlation between blood and brain. While this variant was not found to have different allele frequencies in African ancestry populations, implementing a population-specific approach is necessary for identifying functionally relevant population differences in gene regulatory mechanisms. Given the profound impact and implications of these population differences, our African ancestry-specific study seeks to lessen the gap in knowledge implicated in health disparities. Further examination of allele frequencies, putative population differences, and mechanistic inferences are warranted for the candidate PTSD-relevant eQTL and meQTLs shared between key brain regions and blood. Continued work investigating these preliminary results require replication in other data sets and validation using other approaches (e.g., functional assays).

4.6 Figures and Tables

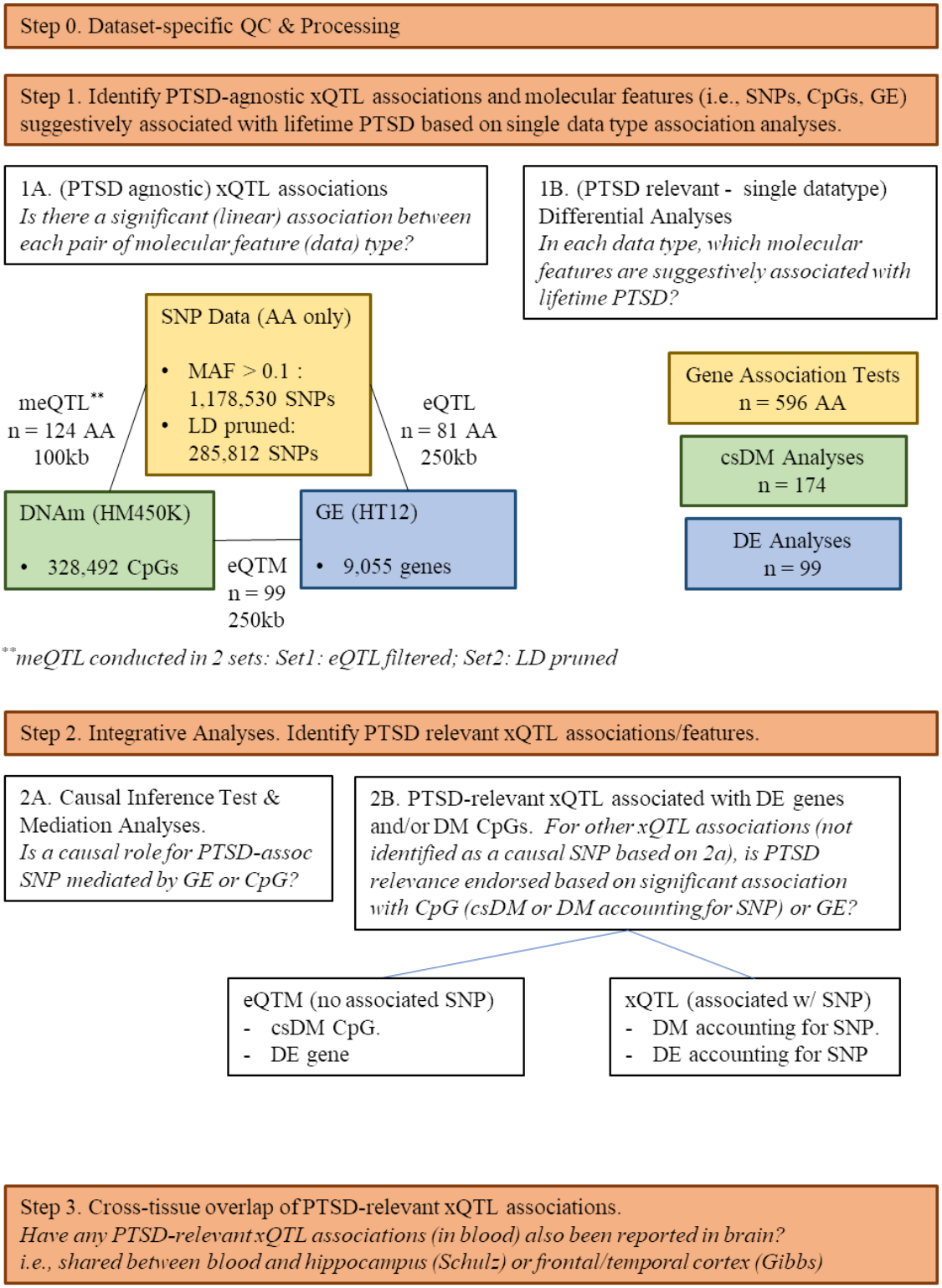
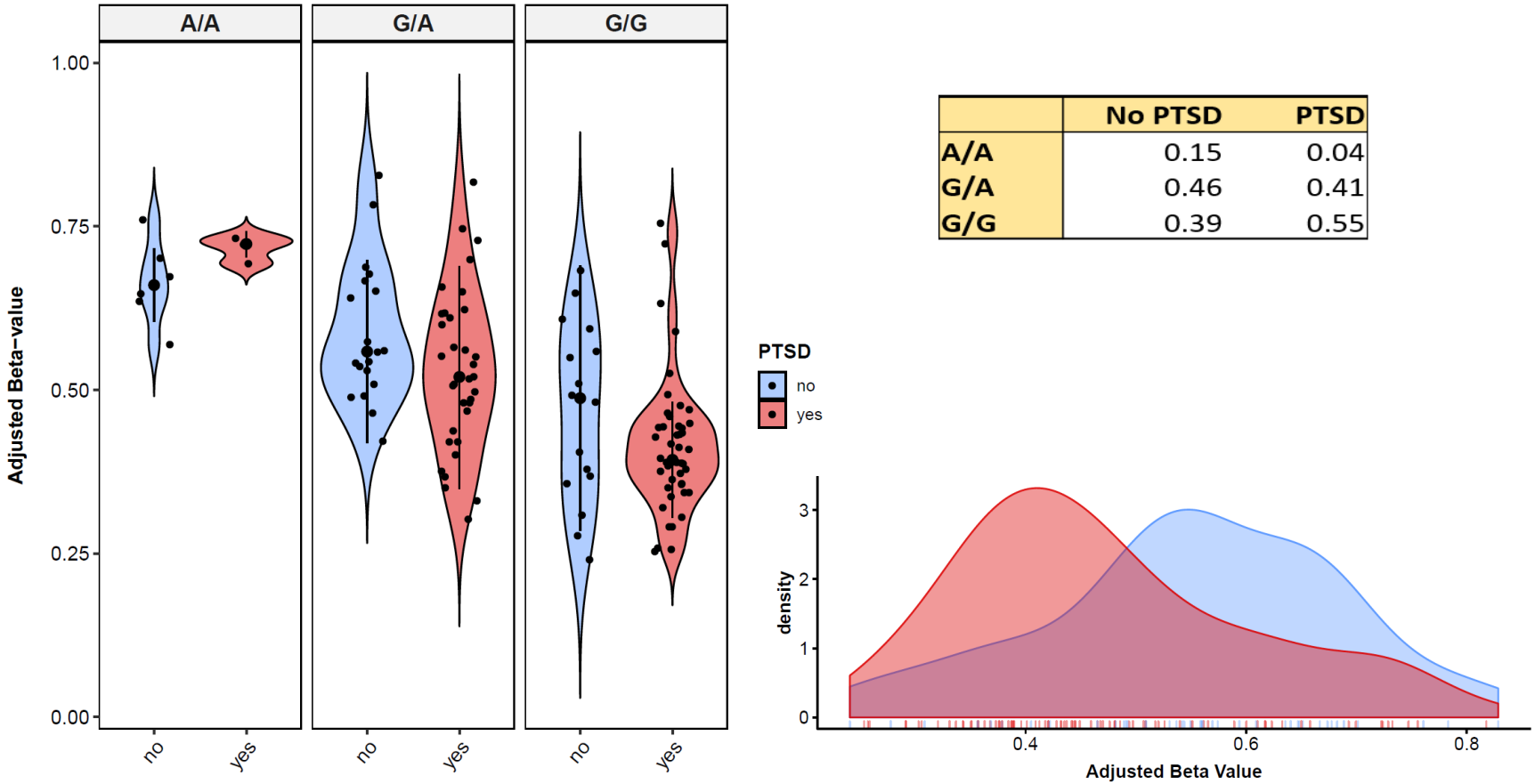


Figure 4.1. Study Design Schematic.



	No PTSD	PTSD
A/A	0.15	0.04
G/A	0.46	0.41
G/G	0.39	0.55

Figure 4.2. Candidate causal meQTL (rs151835) mediated by cg13012653.
Violin plot (left) for adjusted beta-values at cg13012653 in trauma-exposed controls (blue) and lifetime PTSD cases (red), by rs151835 genotype. Table (top right) shows proportion of genotypes by PTSD status (i.e., trauma-exposed controls and lifetime PTSD cases). Density plot (bottom right) shows distribution of adjusted beta-values at cg13012653 for each PTSD group. PTSD cases are more likely to have the G/G genotype at rs151835 and lower methylation levels at cg13012653. Beta-values are adjusted for sex, age, current smoking, and leukocyte proportions (based on RPC estimates).

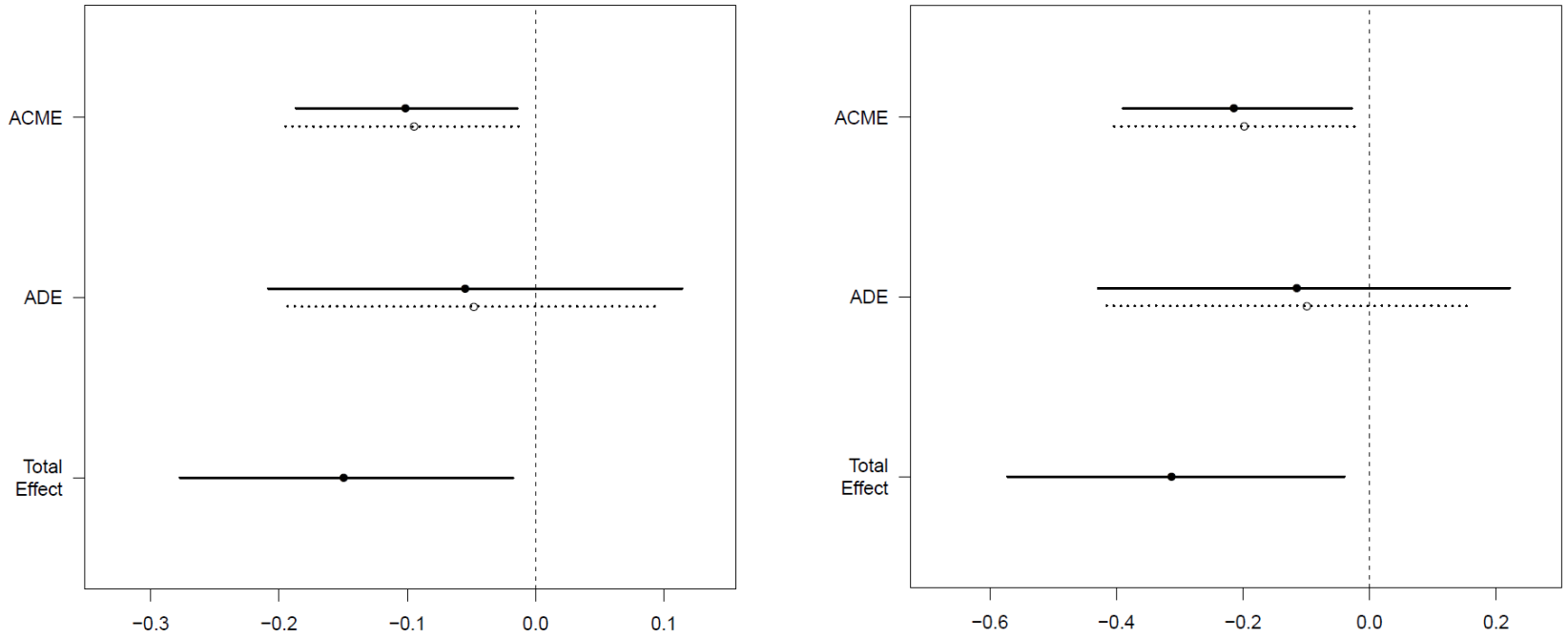


Figure 4.3. Effect Plot for Causal Mediation Analysis: rs151835 - cg13012653.
Effect estimates with 95% confidence intervals (non-parametric bootstrap) are shown for average causal mediation effect (ACME), average direct effect (ADE) and total effect of model 1, comparing control genotype (0 minor alleles) to 1 minor allele (left), and model 2, comparing 0 vs 2 minor alleles. ACME and ADE estimates for treatment group (i.e., lifetime PTSD) are shown by the solid line and estimates for control group (trauma-exposed controls) are shown by the dotted line. Total effect estimates are the same for both groups.

Table 4.1: Initial causal inference test results for significant CIT meQTL (rs151835 - cg13012653)

model	p_cit	p_TassocL	p_TassocGgvnL	p_GassocLgvnT	p_LindTgvnG	SNP	CpG
1	0.026	0.021	0.026	2.54E-05	0.001	rs151835	cg13012653
2	0.045	0.005	0.045	2.70E-07	0.002	rs151835	cg13012653

Note: Model 1 does not include ancestry PCs as a covariate, while Model 2 does. In meQTL-based CIT models, lifetime PTSD is the trait/outcome of interest (T), tested SNP is the locus/instrumental variable (L), and test CpG is the potential causal mediator (G).
p_cit: CIT (omnibus) p-value. Significance suggests mediation of causal effect of SNP on lifetime PTSD via CpG.
p_TassocL: component p-value for test of association between PTSD (T) and SNP (L)
p_TassocGgvnL: component p-value for test of association between PTSD (T) and CpG levels (G), given SNP (L)
p_GassocLgvnT: component p-value for test of association between CpG levels (G) and SNP (L), given PTSD (T)
p_LindTgvnG: component p-value for equivalence test of SNP (L) independent of PTSD (T) given CpG (G)

Table 4.2: PTSD-relevant eQTM associations

Gene	CpG	statistic	Nominal β	Corrected β	nominal pval	CpG pval	Gene pval	SE (β)	r
LYRM7	cg12324708	4.48	0.41	0.28	2.07E-05	8.07E-04	0.039	0.063	0.41
VRK3	cg22801149	-4.89	-0.44	-0.32	4.01E-06	7.61E-04	0.038	0.066	-0.44
SACM1L	cg09251680	5.77	0.51	0.46	9.36E-08	1.01E-05	0.001	0.080	0.50

Table 4.3: PTSD-relevant eQTL associations shared between blood and brain

SNP	Gene	Chr	Brain Region	Ref
rs12978378	*VRK3*	19	fctx; tctx	Gibbs
rs12984158	*VRK3*	19	fctx; tctx	Gibbs
rs12609054	*VRK3*	19	fctx; tctx	Gibbs
rs12611411	*VRK3*	19	fctx; tctx	Gibbs
rs12978357	*VRK3*	19	fctx; tctx	Gibbs
rs11663049	*CEP192*	18	tctx	Gibbs

fctx: frontal cortex
tctx: temporal cortex

Brain Reference: Gibbs et al. (2010)[364]

Table 4.4: PTSD-relevant meQTL associations shared between blood and brain

SNP	CpG	Associated Gene	Chr	Brain Region	Ref
rs10496439	cg08023751	*MERTK*	2	tctx	Gibbs
rs17674346	cg25716820	*CELF5*	19	hippo	Schulz
rs4640536	cg10954944	*TRNA_Asp*	3	hippo	Schulz
rs2526748	cg05623727	*RBM5*	3	hippo	Schulz
rs2856234	cg05623727	*RBM5*	3	hippo	Schulz
rs10897276	cg00685014	*SCGB1A1*	11	hippo	Schulz
rs9970286	cg15591803	*DENND2D*	1	hippo	Schulz
rs6573939	cg26146732	*ADAM21P1*	14	hippo	Schulz
rs2287783	cg00339695	*SLC5A11*	16	hippo	Schulz
rs11607954	cg17139436	*KRTAP5-6*	11	hippo	Schulz
rs10897276	cg16509239	*SCGB1A1*	11	hippo	Schulz
rs6907558	cg26242687	*DQ141194*	6	hippo	Schulz
rs2247114	cg21159260	*BC013821*	5	hippo	Schulz
rs7765522	cg06139720	*IPCEF1*	6	hippo	Schulz
rs7213791	cg21287519	*SGSM2*	17	hippo	Schulz
rs1205259	cg25302603	*PCNXL3*	11	hippo	Schulz
rs2883956	cg21191810	*CEP85L*	6	hippo	Schulz
rs10223063	cg18373318	*SNX18*	5	hippo	Schulz

tctx: temporal cortex
hippo: hippocampus

Brain References: Gibbs et al. (2010)[364]; Schulz et al. (2017)[365]

CHAPTER 5: CONCLUSION

5.1 General Discussion

This body of work used genomic approaches to generate mechanistic hypotheses regarding dysregulation observed in post-traumatic stress disorder (PTSD), with a focus on characterizing sources of heterogeneity that are known to underlie the pathophysiology associated with the disorder. It characterized both methylomic and transcriptomic profiles that reflect the pathophysiology of PTSD, in both brain and blood, and explored the genomic and epigenomic players underlying leukocyte transcriptomic profiles in PTSD to gain systems-level insights into sex-, ancestry-, and tissue-specific mechanisms involved in this disorder.

A major overarching theme is the significance of cellular heterogeneity in methylomic and transcriptomic profiles. While epigenomic and transcriptomic studies have focused on the identification of dynamic differential methylation marks and transcriptional activity, respectively, understanding changes in cellular composition provide key insights and warrant examination themselves. In our first study, we leveraged a recently developed methods in cell deconvolution approach for DNA methylation-based immune profiling of lifetime PTSD and found higher monocyte proportions in males, but not females with a lifetime history of PTSD. In our second study, we leveraged recently developed brain cell reference dataset to uncover higher levels of hippocampal oligodendrocytes in the PTSD-like group compared to the resilient or unexposed group in a rat model of PTSD. While this finding of increased hippocampal oligodendrocytes is shared between sexes, profound sex differences in cell composition was revealed in amygdala. Accounting for cell composition we further identified both sex-specific and shared networks of transcriptional activity associated with individual differences in stress

response. Together, this discovery of sex-specific differences in leukocyte composition and sex-specific gene networks of transcriptional activity in brain highlight the importance of approaching sex as more than a covariate in the study of PTSD.

The second theme considers the relationship between blood and brain. While PTSD is defined by behavioral/cognitive symptoms most directly relevant to brain function, it can be considered a systemic disorder characterized by a distinct inability to reinstate homeostasis after trauma. In our second study investigating expression profiles of blood, amygdala, and hippocampus, we found the common subject shared across blood and brain actually characterized the resilient, rather than the PTSD-like, response to stress: the resilient group was characterized with increased wound healing response in blood and increased ECM reorganization in both brain regions. An overarching set of pathways disrupted in PTSD was identified across amygdala and hippocampus as well. Across these key limbic regions, we found PTSD-like response to stress to involve disrupted TNFα/NFκB signaling and excitatory/inhibitory imbalance in dysregulated synaptic/structural plasticity with important implications for fear learning and memory. Our third study approached the blood-brain topic from a gene regulatory context. DNA methylation levels (CpG) and gene expression levels (GE) are often cell-specific, but genetic variants, such as single nucleotide polymorphisms, (SNPs) are not. Thus, CpG and GE regulated by SNPs have an "anchor" to base inferences of CpG and GE across blood and brain. Most notably, we identified a putatively causal SNP, rs151835, in the *CAST* gene mediated by DNA methylation levels at cg13012653, which is suggested to be correlated between blood and brain. Additionally, we identified a subset of PTSD-relevant SNP-CpG and SNP-GE associations shared between blood and key brain regions that require further investigation.

On the other side of the coin, although PTSD is moderately heritable, robust replicable genetic variants have eluded detection due to challenges managing heterogeneity and gene-environment interactions. Ancestry-specific investigation implementing an integrative systems level approach can help characterize regulatory contexts for genetic variants and epigenetic marks. Our last study conducted a preliminary investigation of the gene regulatory mechanisms underlying PTSD, specifically in African-Americans (AA) – an understudied, at-risk population. While our primary causal SNP candidate in the *CAST* gene was not found to have different allele frequencies in African ancestry populations, implementing a population-specific approach is necessary for identifying functionally relevant population differences in gene regulatory mechanisms. Given the profound impact and implications of these population differences, our African ancestry-specific study sought to lessen the gap in knowledge implicated in health disparities.

While this work is informative for understanding how trauma exposure precipitates a distinct physiological state in vulnerable individuals, future work that associates genomic profiles with symptom clusters rather than PTSD diagnosis may provide better resolution insights into pathways specifically associated with individual PTSD symptom clusters. Expanding on this, a transdiagnostic symptom cluster approach may further help disentangle the substantial heterogeneity and co-morbidity observed among stress-related disorders [385] by providing intermediate phenotypes more closely associated with biological processes than diagnostic categories. In fact, the National Institute for Mental Health (NIMH) recognized the need for a paradigm shift in psychiatric nosology from being based on descriptive, clinical observations to pathophysiology informed largely by clinical neuroscience and genomics research[386-388]. To support the eventual development of such a diagnostic system, the NIMH

launched the Research Domain Criteria (RDoC) initiative, which provides a research classification framework whereby multi-level, multidimensional data can be leveraged to understand the biological basis of behavioral/cognitive domains dysregulated in mental disorders[386-389].

Applying the RDoC framework to longitudinal studies that investigate symptoms and physiological states, in the context of genetics, life experiences/exposures, and sociocultural context, over a life course[390], will enable us to understand the role of each determinant in influencing mental health trajectories and to discern biological correlates that reflect pre-existing risk factors from those that occur as a response to trauma at different clinical stages of PTSD[213,391]. Ultimately, the hope is that our improved mechanistic understanding will lead to the discovery of robust predictive biomarkers that can be used for implementing early intervention and development of improved preventive and therapeutic strategies that cater to individual differences, including sex/gender- and ancestry/ethnic- specific considerations. This, in turn, has immense implications for reducing stress-related disease burden globally.

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APPENDIX A: SUPPLEMENTARY MATERIALS – CHAPTER 2

I. Results for main analyses, based on CP estimates

II. Supplementary Figures and Tables for CP-based results of main analyses

III. Comparison of leukocyte subtype proportions between participating cohorts

IV. Assessment of age effects, including supplementary figures

I. Results for main analyses, based on CP estimates

A second set of cell estimates, derived using the popular constrained projection (CP) deconvolution approach, were compared against robust partial correlation (RPC) based cell estimates. Results from analyses conducted on both sets of cell estimates were checked for agreement. The main text details findings based on RPC estimates and this supplementary materials section describes corresponding findings based on CP estimates.

Comparison of leukocyte subtype estimates by sex, lifetime PTSD, and study using CP estimates

As observed in RPC estimates, there were significant sex differences in CP-based distributions of both NK (KS: $D = 0.21$, *adj* $p = 0.001$) and CD8T (KS: $D = 0.25$, *adj* $p = 4.21\times10^{-5}$) cell estimates, with males again showing greater variability than females for both NK and CD8T cells (male vs. female - IQR_{NK}: 7.3% vs. 5.2%; IQR_{CD8T}: 6.4% vs. 5.1%). Additionally, there were higher median NK (5.0% vs. 2.9%) and lower median CD8T (11.6% vs. 13.5%) cell proportions in males compared to females (Figure A.1). Again, no significant overall differences (i.e., in both sexes combined) were observed between lifetime PTSD cases and trauma-exposed controls in any leukocyte subtype (Mann-Whitney).

CP estimates confirm male-specific association between lifetime PTSD and DNAm-based monocyte proportions

As observed previously, males with lifetime PTSD had higher monocyte proportions than trauma-exposed controls, $U = 2200$, $Z = -2.5$, $p = 0.01$, $r = 0.20$, while no difference was found between PTSD cases and controls in females, $U = 13000$, $Z = -0.15$, $p = 0.88$, $r = 0.008$ (Figure A.2). A two-way ANCOVA on square root transformed CP-based estimates, accounting for age, ancestry, and current smoking (Table A.1), showed a significant interaction between sex and lifetime PTSD, $F(1,461) = 3.99$, $p = 0.046$, $\eta_p^2 = 0.009$. Post-hoc comparison of EMMs by sex (Figure A.3; Table A.2) confirmed a significant mean difference between lifetime PTSD cases and controls in males, with higher mean monocyte estimates in PTSD cases, $\Delta EMM = 0.24$, $SE = 0.09$, $t(461) = 2.85$, $p = 0.005$. Again, no significant mean difference between PTSD cases and controls was found in females, $\Delta EMM = 0.04$, $SE = 0.06$, $t(461) = 0.63$, $p = 0.53$. Smaller difference in EMM (ΔEMM) and larger EMM standard error were observed in male CP estimates, compared to RPC estimates.

Finally, sex-stratified Kruskal-Wallis tests for PTSD status using CP-based monocyte estimates (Figure A.4) also suggested significant differences in males, $H(2) = 6.5$, $p = 0.038$, but not females, $H(2) = 0.74$, $p = 0.69$. While no difference was observed between current and remitted PTSD groups, $Z = 0.24$, $p = 0.81$, *adj.* $p = 0.81$, $r = 0.03$, in the post-hoc Dunn test, differences between PTSD cases and trauma-exposed controls showed smaller effect sizes and only nominal significance (current PTSD vs controls: $Z = 2.11$, $p = 0.035$, *adj.* $p = 0.10$, $r = 0.21$; remitted PTSD vs controls: $Z = 2.10$, $p = 0.036$, *adj.* $p = 0.071$, $r = 0.19$).

II. Supplementary Figures and Tables for CP-based results of main analyses

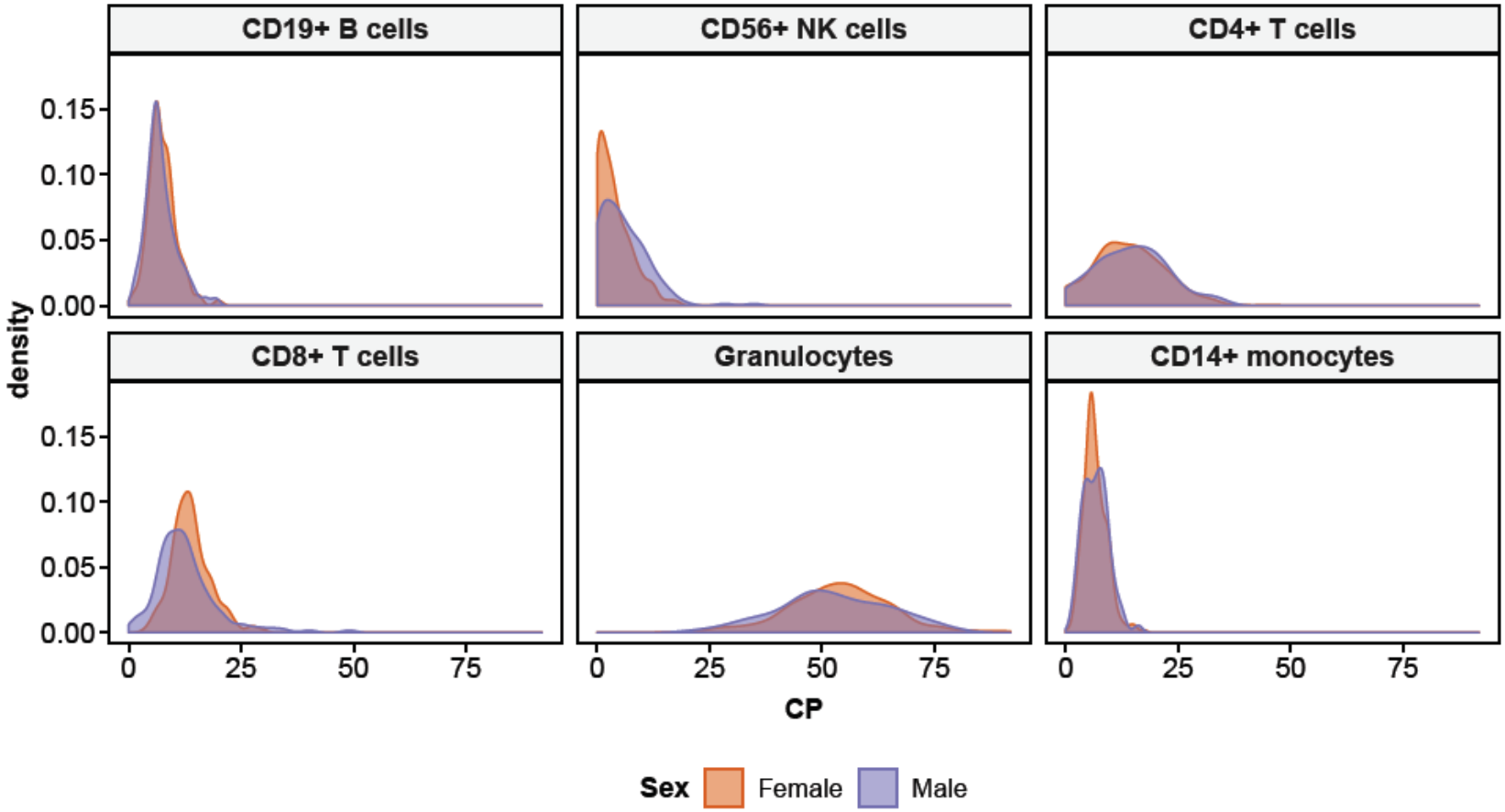

Figure A.1: Distribution of leukocyte subtypes based on constrained projection (CP) estimates, by sex. As noted in RPC estimates, sex differences in CD8+ T and CD56+ NK cell distributions were found to be prominent.

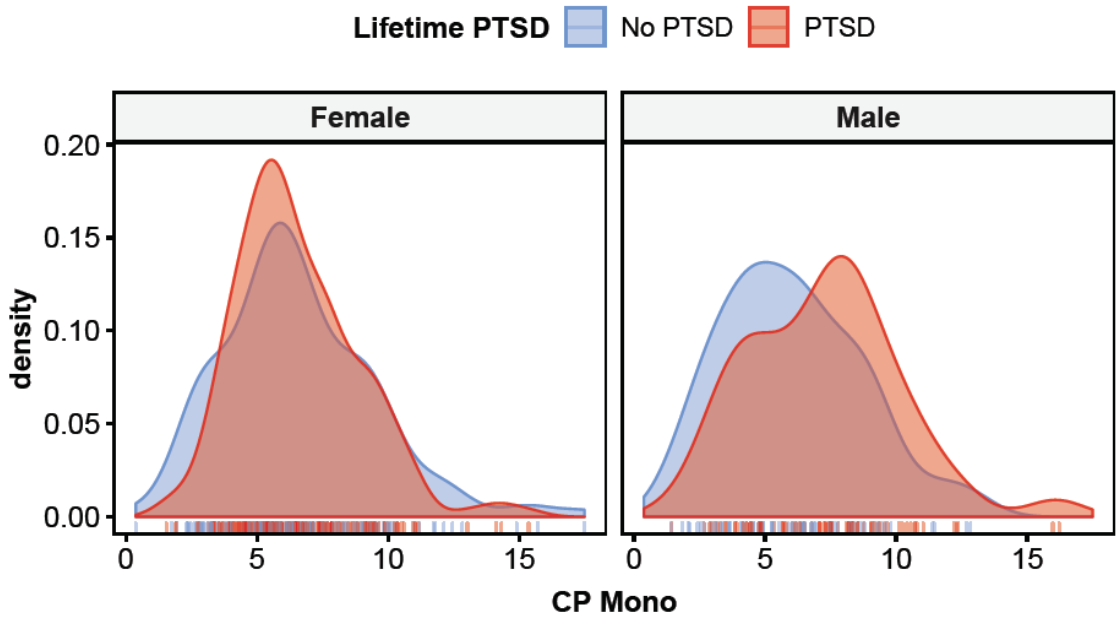

Figure A.2: Density plots for CP monocyte estimates in lifetime PTSD cases and controls, stratified by sex, show distinctly higher monocyte levels in males with lifetime PTSD compared to trauma-exposed cases. This difference in monocyte levels between lifetime PTSD case and controls is not observed in females, mirroring findings based on RPC estimates.

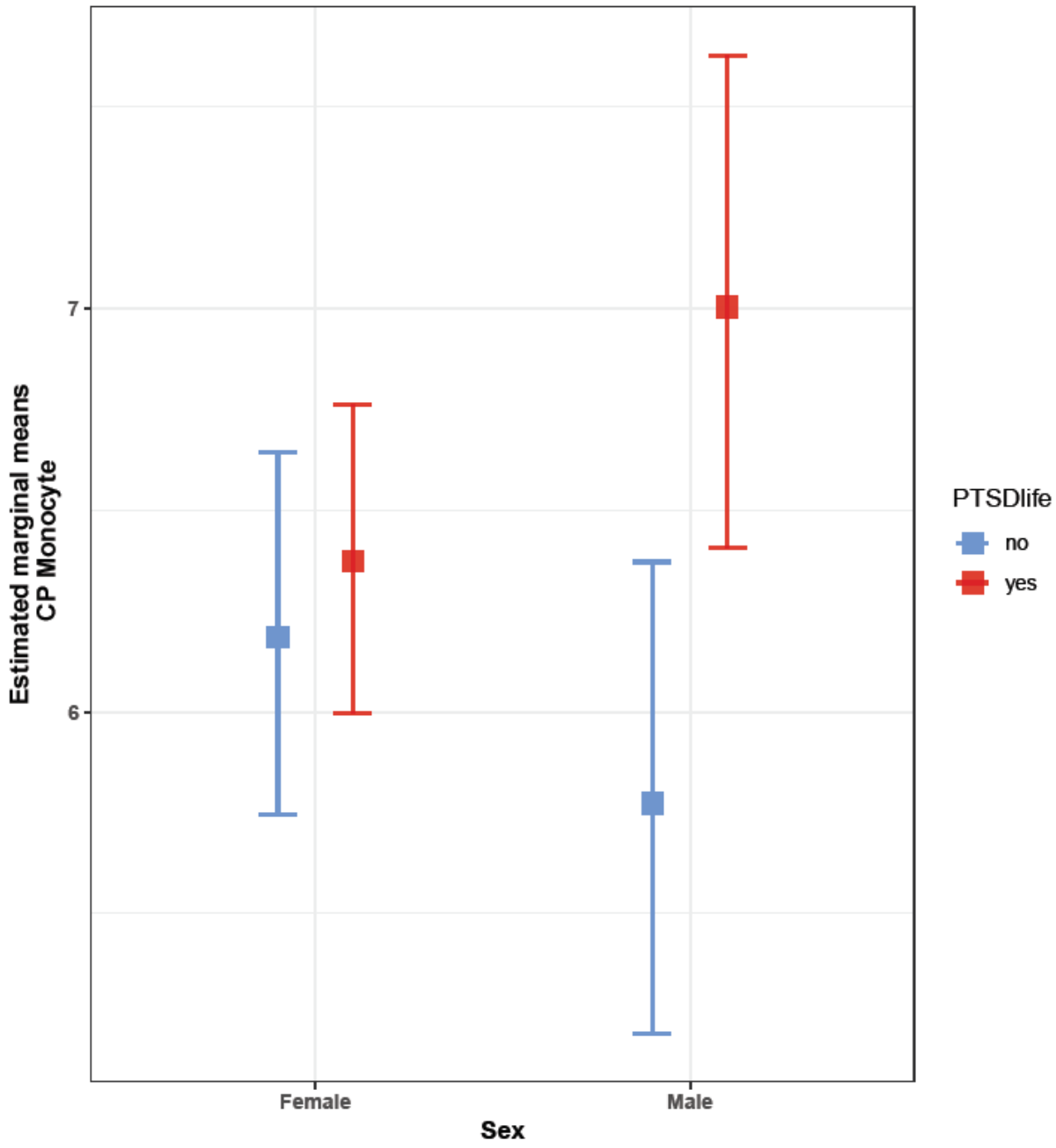

Figure A.3: Lifetime PTSD by sex interaction plot for estimated marginal means (EMMs) of CP monocyte estimates. Interaction plot shows a significant EMM difference between lifetime PTSD cases (red) and controls (blue) in males, where mean monocyte estimates are higher in cases than controls. No significant EMM difference was observed between PTSD cases and controls in females.

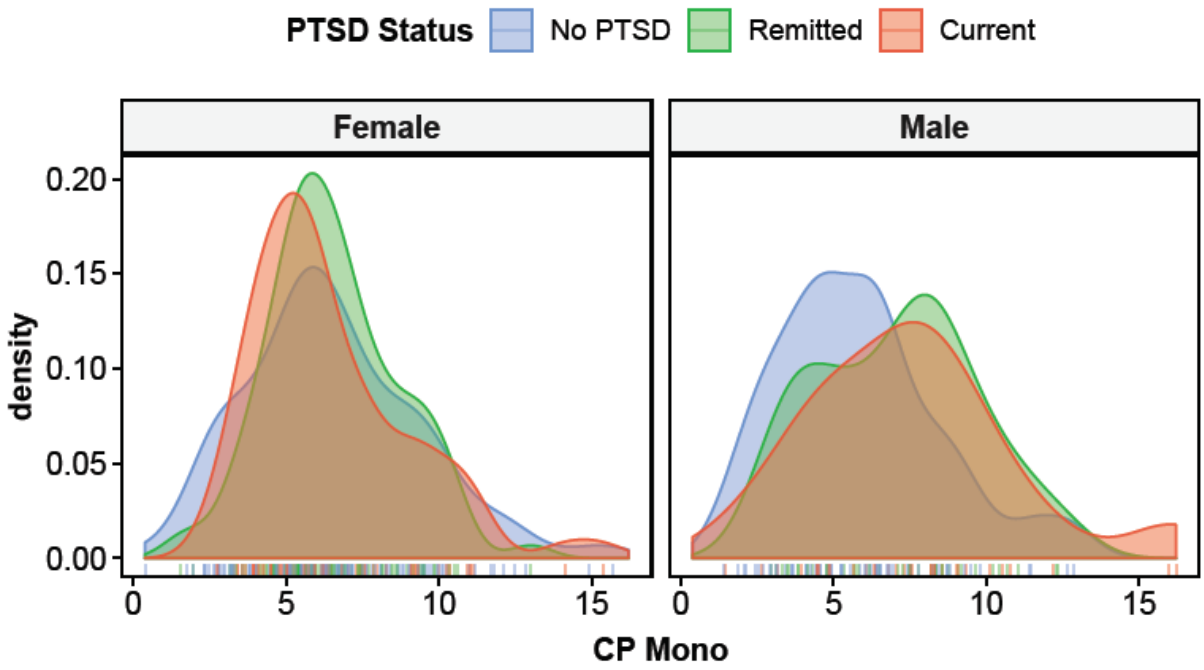

Figure A.4: Density plots for CP monocyte estimates comparing those with current PTSD, remitted PTSD, and trauma-exposed controls, stratified by sex. No significant difference is observed between current and remitted PTSD cases, which suggests that the significant difference in male PTSD cases is associated with long-standing PTSD trait, rather than current PTSD state. However, unlike RPC-based estimates, difference between PTSD case groups and trauma-exposed controls was only nominally significant in corresponding post-hoc Dunn test. No significant differences were observed in females.

Table A.1: Two-way ANCOVA Table for CP monocyte estimates (n = 469)

Terms	Type III Sum of Squares	*df*	Mean Square	*F*	*p*	$_{\text{partial}}\eta^2$
Sex	0.034	1	0.034	0.130	0.719	0.000
PTSDlife	1.929	1	1.929	7.256	0.007**	0.015
Age	2.964	1	2.964	11.150	0.001***	0.024
ancPC1	0.036	1	0.036	0.137	0.712	0.000
ancPC2	0.063	1	0.063	0.236	0.627	0.001
Smoking	0.000	1	0.000	0.000	0.987	0.000
Sex:PTSDlife	1.060	1	1.060	3.987	0.046*	0.009
Residuals	122.555	461	0.266			

**$p < 0.05$, **$p < 0.01$, ***$p < 0.005$

Table A.2: Summary for CP monocyte estimates by group

Sex	PTSD	n	mean	SE	EMM	SE_{EMM}	lower.CL	upper.CL
Female	no	135	6.564	0.2499	6.186	0.2285	5.745	6.643
Female	yes	184	6.551	0.1735	6.373	0.1946	5.996	6.761
Male	no	70	6.072	0.3165	5.772	0.2972	5.203	6.371
Male	yes	80	7.270	0.3248	7.004	0.3106	6.407	7.628

This table describes untransformed CP monocyte estimates by group (i.e., sex and lifetime PTSD); n = count per group; EMM = estimated marginal means (i.e., least squares means); SE = standard errors for regular means; SE_{EMM} = standard errors for EMM.

Lower and upper confidence limits are for 95% level. EMM and intervals were back-transformed from the square root scale to the original scale of cell subtype proportions (%). Significance level of alpha = 0.05 was used for EMM comparisons. Results for pairwise comparison were averaged over levels for current smoking. Degree of freedom was 461 and male lifetime PTSD cases were significantly different from other groups.

III. Comparison of leukocyte subtype proportions between participating cohorts

We assessed study differences in leukocyte subsets using Mann-Whitney U test (Holm-adjusted $p < 0.05$). For RPC estimates, comparison between DNHS and GTP cohorts revealed a significant difference in median CD8T-cell proportions, $U = 22{,}634$, $Z = -2.9$, $p = 0.003$, *adj.* $p = 0.021$, $r = -0.13$, in the overall dataset (both sexes), but not in sex-stratified subsets. Similarly, comparison between DNHS and GTP cohorts using CP-based estimates revealed a significant difference in median CD8T-cell proportions, $U = 22{,}739$, $Z = -2.9$, $p = 0.004$, *adj.* $p = 0.026$, $r = -0.13$, in the overall dataset (both sexes), as observed using RPC estimates. However, unlike RPC estimates, a significant study difference in CD8T-cell proportions was also observed in the male subset, $U = 1{,}972$, $Z = -3.24$, $p = 0.001$, *adj.* $p = 0.007$, $r = -0.26$. Study differences were not observed for any other leukocyte subtype, in the overall or sex-stratified sets, for each set of cell estimates.

These differences may reflect demographic differences between the DNHS and GTP cohorts, in our dataset. Chi-squared test (simulated p-value, based on 2000 replicates) indicates that self-reported race has a trending association with cohort ($\chi = 5.42$, $p = 0.07$), with GTP contributing more AA participants, and lifetime PTSD had a significant association with cohort ($\chi = 8.96$, $p = 0.003$), with DNHS contributing fewer trauma-exposed controls. Thus, to benefit from increase in sample size while placing less relevance on possible cohort differences, datasets were combined at the individual level before deriving estimates and conducting analyses on the "mega" dataset.

IV. Assessment of age effects

Spearman's rank correlation was used to explore age effects in each leukocyte subtype, with proportion estimates based on RPC (Figure A.5) and CP (Figure A.6). The direction of effect for each leukocyte subtype was consistent between RPC and CP estimates. At Holm-adjusted $p < 0.05$, we observed a small but significant decline in CD8T, $\rho_s(481) = -0.19$, *adj p* = 0.0002, and CD4T , $\rho_s(481) = -0.13$, *adj p* = 0.015, cell proportions and a small but significant increase in monocyte , $\rho_s(481) = 0.13$, *adj p* = 0.015, proportions with age. Correlation tests for age based on CP estimates were consistent with results based on RPC estimates. For CP estimates, we also observed a small but significant decline in CD8T, $\rho_s(481) = -0.16$, *adj p* = 0.002, and CD4T , $\rho_s(481) = -0.12$, *adj p* = 0.046, cell proportions and a small but significant increase in monocyte , $\rho_s(481) = 0.14$, *adj p* = 0.013, proportions with age. Comparison of age effects from our DNAm-based estimates with a published study[2], based on Coulter counter (complete blood cell count, CBC) and flow cytometry data, revealed age-related changes in CD8T and monocyte proportions to be consistent for the age group corresponding to our study (i.e., between adult and elderly group). However, no significant change between adult and elderly groups for CD4T proportions was found in the published study. Effect of age was consistent by sex and sex by age interaction was not endorsed for any cell subtype. However, we observed the decrease of CD8T cell proportions by age was slightly greater in males (RPC: $\rho_s(151) = -0.24$; CP: $\rho_s(151) = -0.23$) than females (RPC: $\rho_s(328) = -0.17$; CP: $\rho_s(328) = -0.15$), using both RPC (Figure A.7) and CP estimates (Figure A.8).

[2] Valiathan, R., Ashman, M. & Asthana, D. Effects of Ageing on the Immune System: Infants to Elderly. *Scand J Immunol* **83**, 255-266, doi:10.1111/sji.12413 (2016).

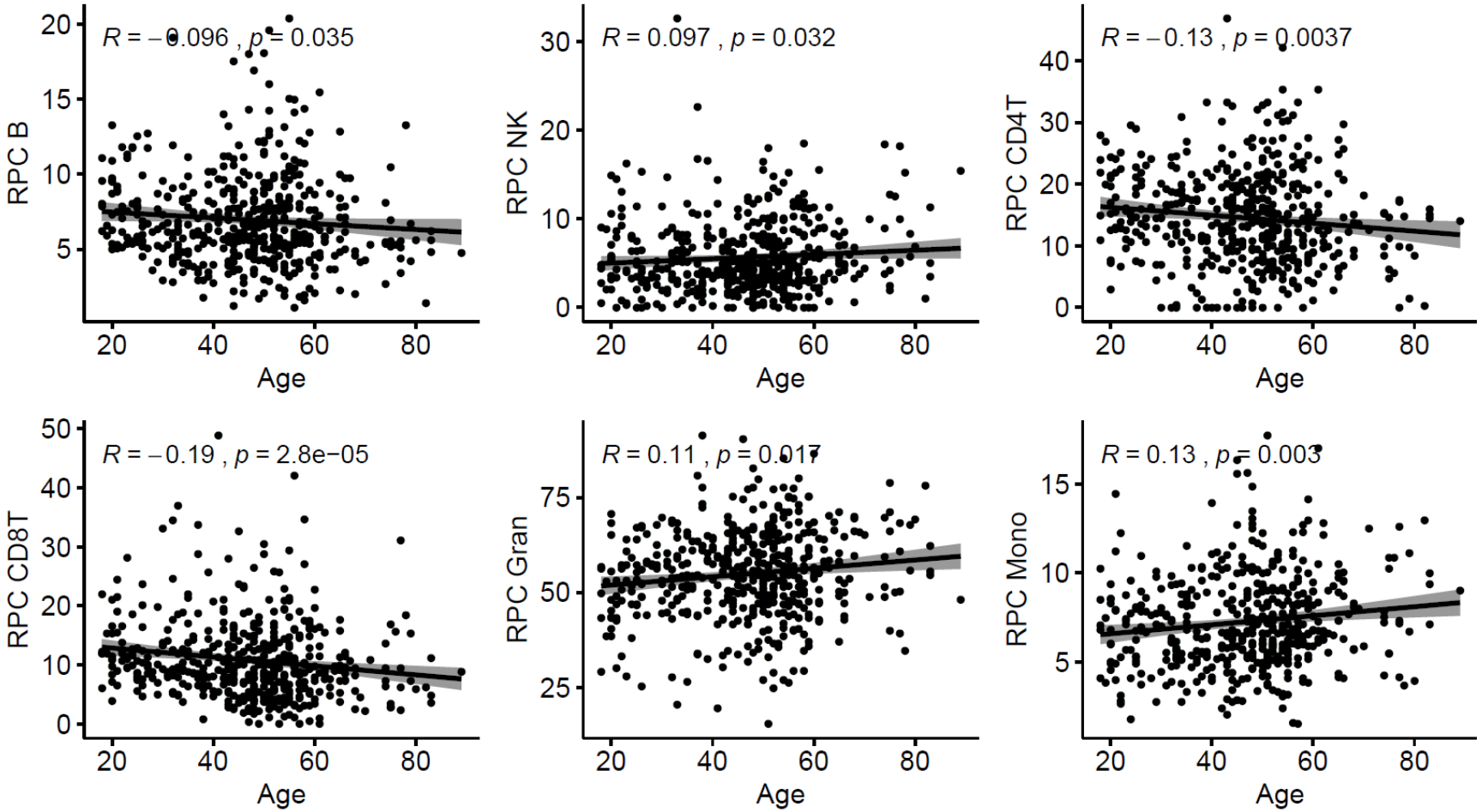

Figure A.5: Effect of age on proportion of leukocyte subtypes, based on RPC. Spearman's correlation and nominal p-value are reported towards top of each scatter plot. Significant decrease in CD8T and CD4T cells and significant increase in monocytes is detected with increasing age, at Holm-adjusted p-value < 0.05.

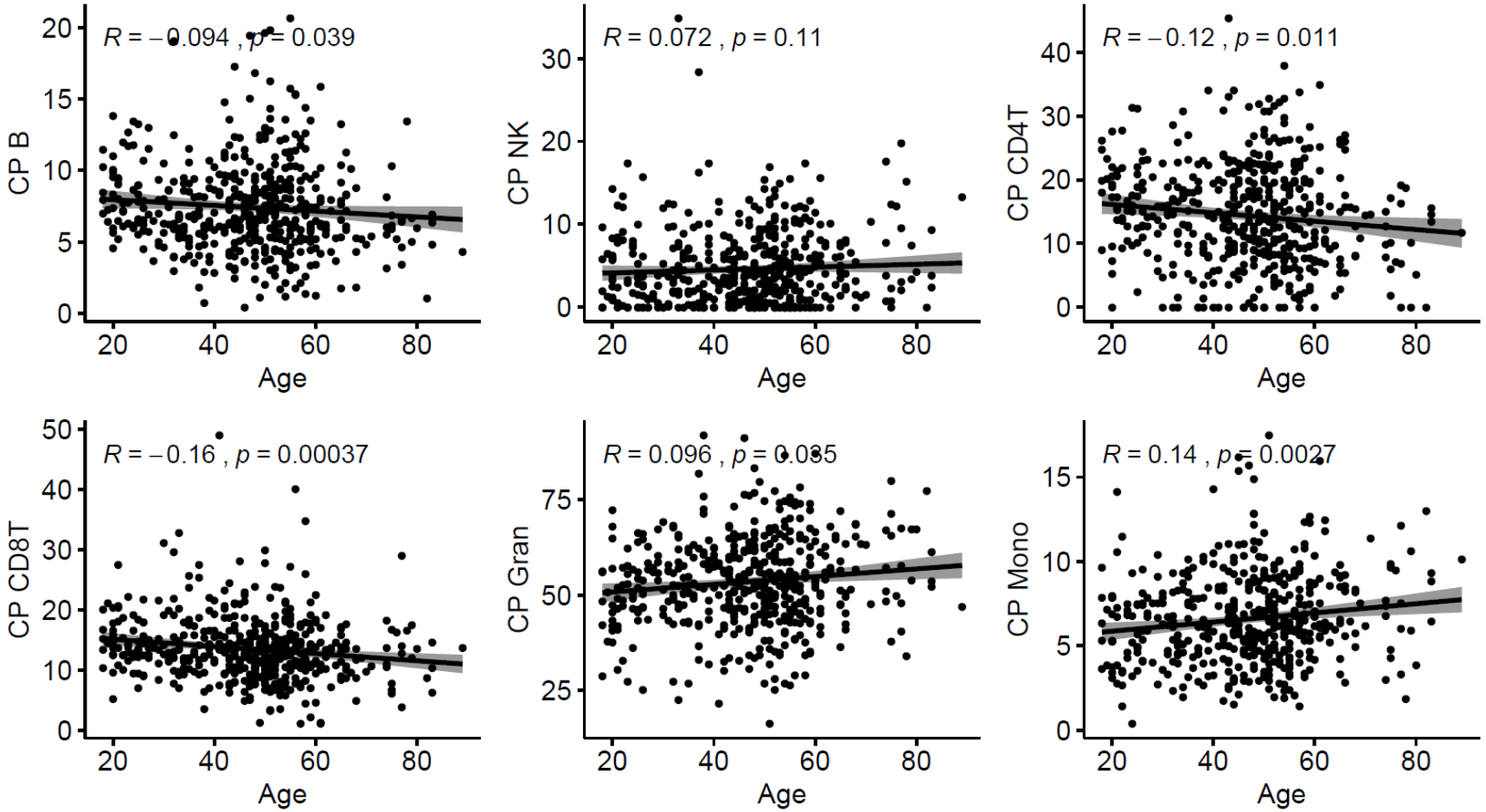

Figure A.6: Effect of age on proportion of leukocyte subtypes, based on CP. Spearman's correlation and nominal p-value are reported towards top of each scatter plot. Similar to RPC, significant decrease in CD8T and CD4T cells and significant increase in monocytes is detected with increasing age, at Holm-adjusted p-value < 0.05.

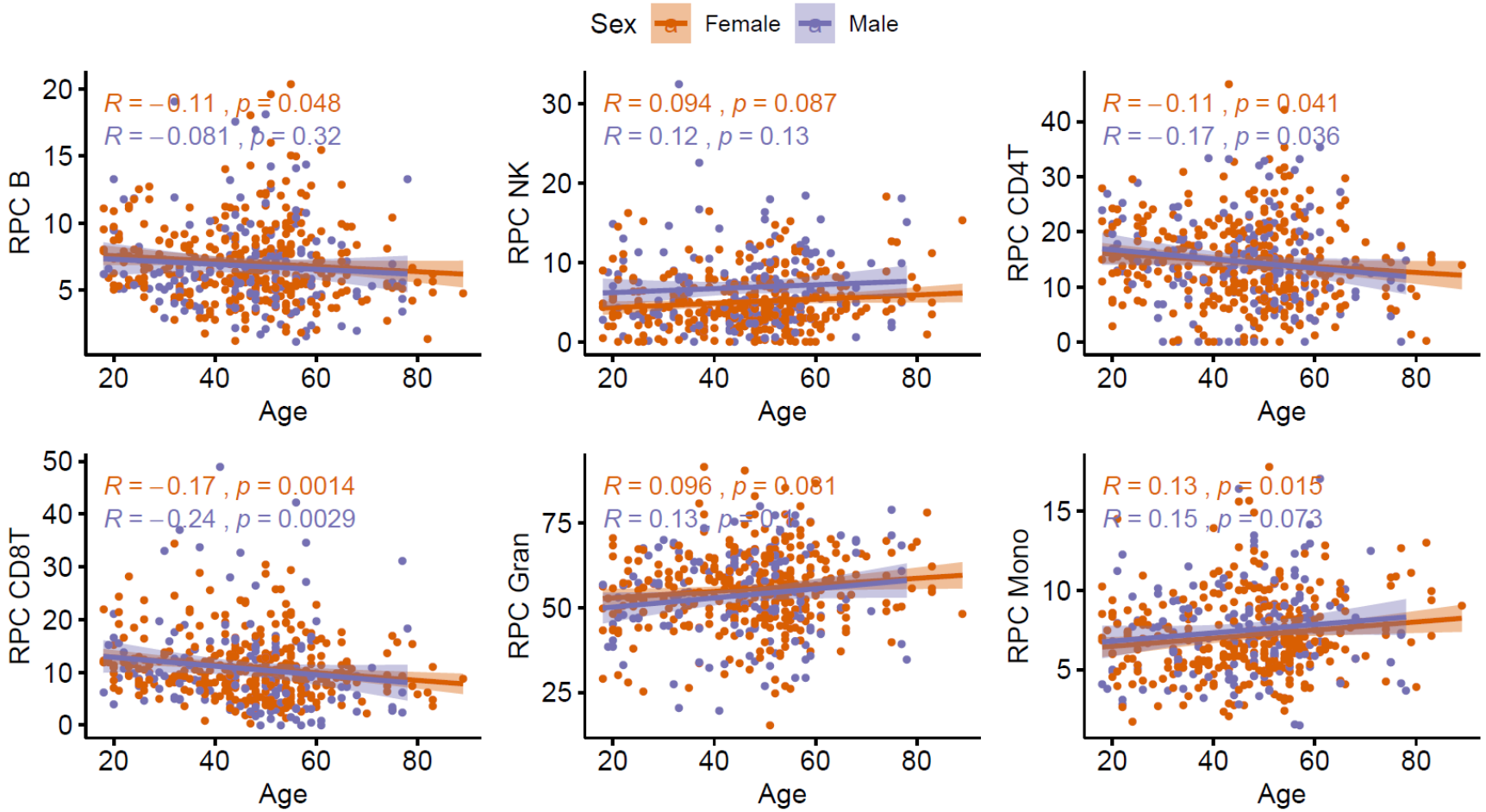

Figure A.7: Sex-stratified effect of age on proportion of leukocyte subtypes, based on RPC. Spearman's correlation and nominal p-value are reported towards top of each scatter plot, for each sex (female: orange; male: purple).

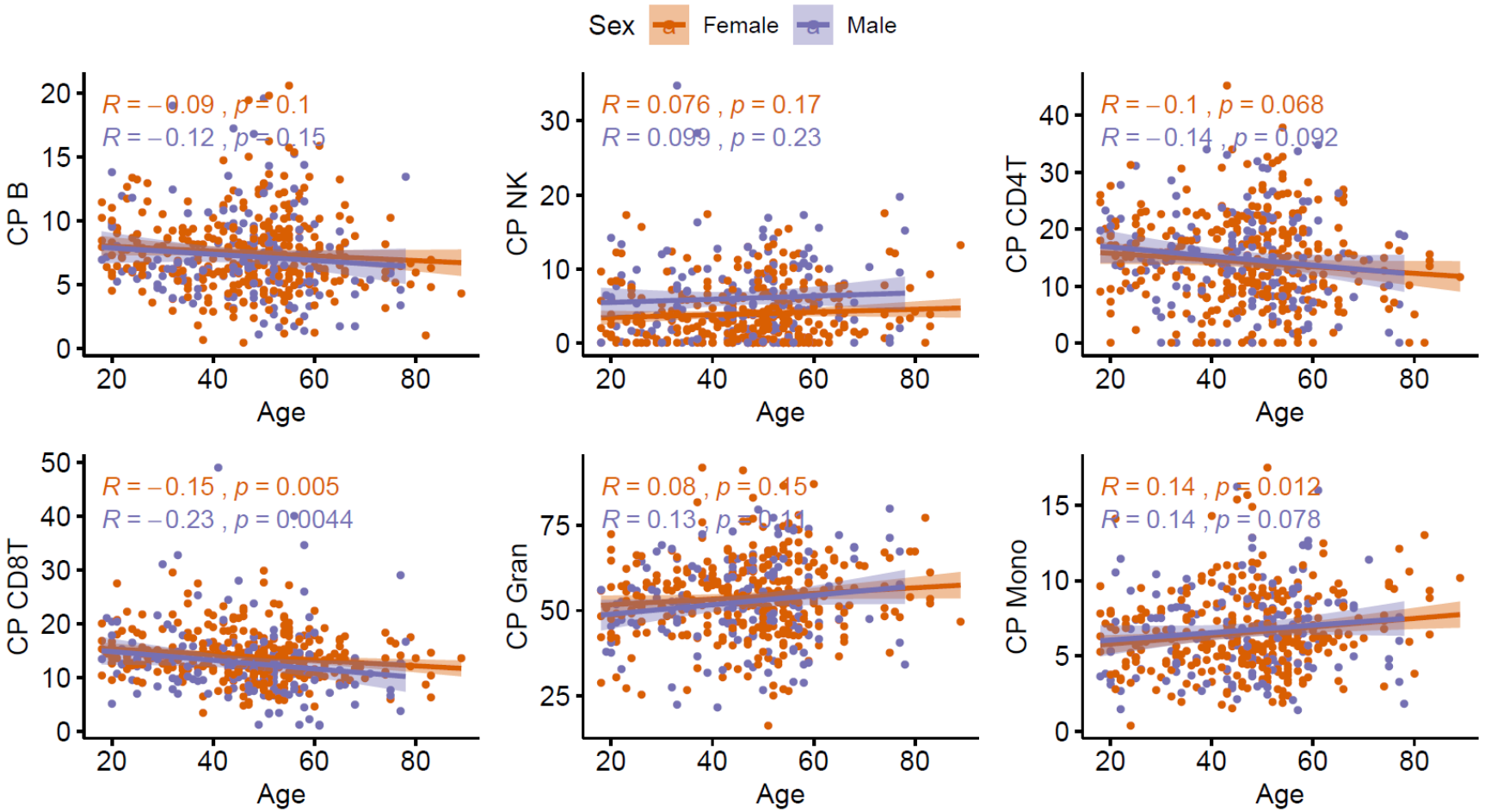

Figure A.8: Sex-stratified effect of age on proportion of leukocyte subtypes, based on CP. Spearman's correlation and nominal p-value are reported towards top of each scatter plot, for each sex (female: orange; male: purple).

APPENDIX B: CEMITOOL REPORTS & GENE SET RESULTS – CHAPTER 3

The supplementary zip file ("AppendixB_Chap3Results.zip") includes results from network and gene set analyses for all tissues. The main directory is organized by tissue. CEMiTool reports are located in the "CEMiTool Reports/" subdirectory for each tissue. This html report generated using *CEMiTool* includes network analysis parameters, group associations for modules, barplots showing top gene set results in the GO collection, and interaction networks. Gene set enrichment results for all MSigDB collections associated with each analysis (e.g., full vs. sex-stratified, unadjusted vs. SV-adjusted vs. cell estimate adjusted) are included in their own subdirectories, with separate csv files for each module and gene set collection. Additional notes are included in a plain text README file located in the top directory.

APPENDIX C: SUPPLEMENTARY MATERIALS – CHAPTER 4

The supplementary zip file ("AppendixC_Chap4Results.zip") includes the tabulated set of results for PTSD-relevant xQTL associations, which are in separate .csv files. A plain text README file located in the top directory contains the description for each .csv file.