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The epigenetic consequences of trauma

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DEDICATION

I would like to dedicate this work to my family, mentors, and friends for their continued and unwavering support. I wouldn't be here without them.

THE EPIGENETIC CONSEQUENCES OF TRAUMA

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ABSTRACT

Epigenetics is a rapidly growing field that has provided insight into the etiology of many physiological mechanisms. Research around post-traumatic stress disorder (PTSD) has evolved immensely since expanding to include an epigenetic lens. Researchers have studied which gene loci are associated with PTSD to understand how genes can become either over or under expressed when exposed to trauma. The three main epigenetic factors that assist with regulating the genome are: DNA methylation, histone modification (including methylation and acetylation), and noncoding RNA. Each factor utilizes a different mechanism to help with either the upregulation or downregulation of a specific gene.

Within PTSD research, the impacts of these genome modifications have been studied to understand how they regulate the common physiological symptoms associated with PTSD diagnoses. These symptomologies include decreased basal cortisol levels, decreased cardiovascular health, decreased immune function, and increased mortality. Many epigenetic studies have explored how changes in specific gene loci contribute to these physiological dysregulations. Some genes of interest include nuclear receptor subfamily 3 group C member 1 (NR3C1), FK506 binding protein 5 (FKBP5), and spindle and kinetochore-associated protein 2 (SKA2). Many studies have been conducted examining the DNA methylation activity of each gene in those with PTSD diagnoses and those without. However, research continues to produce mixed results. While some studies

show an increase of DNA methylation for a specific gene in subjects with PTSD, other studies evidence a decrease of DNA methylation for the same gene.

Examining the reasons for conflicting evidence is valuable to further understand the epigenetic mechanisms that occur. After conducting a literature review, four confounding factors have been identified as contributors to such mixed results. The first factor is the difference in each study's definition of trauma, as well as the diagnostic tools they use to identify subjects with PTSD. The second factor is the samples used to detect epigenetic changes. Most samples collected in epigenetic studies of PTSD include whole blood samples, salivary samples, and only rarely, brain tissue samples. These different sample types, when cross-compared, can contribute to discrepancies in DNA methylation data. Furthermore, whole blood samples are not only vulnerable to intrinsic factor variabilities, but external factor variabilities. The third factor is a difference in subject population across the literature. Many studies are focused on either combat-veterans (with all male subjects) or child cohorts. These differences in demographics make it difficult to compare groups, as research indicates several epigenetic factors such as DNA methylation activity are sex, ethnicity, and age dependent. Finally, the fourth confounding factor is age at onset of trauma. Many studies show that trauma exposure in childhood leads to more severe symptoms compared to trauma exposure in adulthood.

It is important to consider these factors and account for confounding variables when conducting future research. In doing so, more robust and accurate research can be produced. A more refined understanding of the epigenetic etiology of PTSD, as well as

its epigenetic biomarkers, will likely yield greater insight into PTSD diagnoses, as well as best treatment practices.

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LIST OF ABBREVIATIONS

ADCY8 ..... Adenylate cyclase 8

ADCYAP1R1 ..... Adenylate cyclase activating polypeptide 1 receptor 1

ACTH ..... Adrenocorticotropic hormone

BDNF ..... Brain derived neurotropic factor

CTQ ..... Childhood Trauma Questionnaire

CAPS ..... Clinician Administered PTSD Scale

CRH ..... Corticotropin releasing hormone

DNAm ..... DNA methylation

DNMTs ..... DNA methyltransferases

DSM-5 ..... Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

DHT ..... Dihydrotestosterone

EWAS ..... Epigenome-wide association study

EB ..... Estradiol benzoate

FKBP5 ..... FK506 binding protein 5

G×E ..... Gene by Environment

GR ..... Glucocorticoid receptor

H3K9 ..... Histone 3-Lysine 9

H3K4 ..... Histone 3-Lysine 4

H3K27 ..... Histone 3-Lysine 27

H3K79 ..... Histone 3-Lysine 79

H4K20 ..... Histone 4-Lysine 20

5-HTT ..... Human serotonin transporter

SLC6A4 ..... Human serotonin transporter gene

HPA ..... Hypothalamic-pituitary-adrenal

IKK ..... IKB Kinase

IKBα ..... Inhibitor of nuclear κB

IL ..... Interleukin

ICD ..... International Classification of Diseases and Related Health Problems

lncRNA ..... Long noncoding RNA

MDD ..... Major Depressive Disorder

MeCP2 ..... Methyl CpG-binding protein 2

miRNA ..... Micro RNA

NIK ..... NF-kappa-B inducing kinase

ncRNA ..... Noncoding RNA

NR3C1 ..... Nuclear receptor subfamily 3 group C member 1

snoRNA ..... Nucleolar RNA

PBMCs ..... Peripheral blood mononuclear cells

piRNA ..... Piwi-interacting RNA

PTSD ..... Post-traumatic stress disorder

siRNA ..... Small interfering RNA

sncRNA ..... Small noncoding RNA

SES ..... Socioeconomic status

SKA2 ..... Spindle and kinetochore-associated protein 2

TRAILS ..... Tracking Adolescents' Individual Lives

TWAS ..... Transcriptome-wide association study

HTQ ..... Traumatic Life Events Questionnaire

TNF ..... Tumor necrosis factor

WHO ..... World Health Organization

INTRODUCTION

This thesis will begin with an overview of the frameworks with which I conceptualize trauma –biologically and psychologically. The study of epigenetic relationships to trauma, namely its physiological and psychological impacts, is a complex and controversial one. While many studies have investigated the potential bidirectional relationship between epigenetics and trauma, these studies have returned mixed and inconclusive results.

Many theories on how information is passed from one organism to another have been put forth. Jean-Baptiste Lamarck and Charles Darwin presented independent theories of inheritance in the early and mid-19th century, respectively. Both postulated that acquired traits, developed in response to environmental factors, can be inherited by offspring (Gowri & Monteiro, 2021). August Weismann presented his own opposing theory in 1885, notably known as the "Weismann Barrier." By cutting off the tails in five consecutive generations of mice, Weismann observed that no mouse was born with a tail abnormality, despite the previous generations having had their tails artificially stubbed (Tollefsbol, 2017). The Weismann Barrier proposed only germ cells (and not somatic cells) can hold hereditary information, and that any environmental changes made to somatic cells would not impact an organism's germ cells nor its offspring (Gowri & Monteiro, 2021).

For many years, the Weismann Barrier was a well accepted theory. Then, in 1942, Conrad Waddington coined the term "epigenetics" to explain the process and mechanisms through which genotypes influence phenotype changes (Waddington, 1942).

Waddington used epigenetics to explain how phenotypic changes could occur during an organisms development, without incurring any changes to its genotype (Allis & Jenuwein, 2016; Waddington, 1942). In the many years since epigenetics was initially conceptualized, it has developed more modern definitions. Today, epigenetics is defined as mechanisms that occur to alter gene expression and activity without changing the DNA sequence itself (Alkuraya, 2014; Jaenisch & Bird, 2003). Furthermore, the study of epigenetics focuses on the ways in which environmental factors influence heritable molecular mechanisms (Alkuraya, 2014).

Molecular Mechanisms of Epigenetics

Epigenetic mechanisms influence gene expression, thereby impacting protein levels and phenotype (Pishva et al., 2017). Changes in phenotypes can occur due to changes in epigenetic variations which are either randomly determined or influenced by an organism's environment. In order to investigate how epigenetic mechanisms and environmental factors can influence one another bi-directionally, it is important to understand the epigenetic pathways by which DNA activity is regulated. These pathways include: DNA methylation, histone modifications, chromatin remodeling, and more recently emphasized in literature, noncoding RNA (Schaefer & Thompson, 2017).

DNA Methylation

The most well studied epigenetic marker is DNA methylation, a process that occurrs in animals, plants, and fungi (Tollefsbol, 2017). In this thesis, DNA methylation will be discussed within the context of CpG islands, CpG island shores, gene bodies, and

repetitive sequences. The most frequent form of DNA methylation is the reversible covalent addition of a methyl group (CH_3) to the fifth carbon of a cytosine base. (Nabais et al., 2023; Tollefsbol, 2017). This cytosine methylation is almost exclusively limited to cytosines of CpG dinucleotides, which typically exist in "CpG islands," clusters of CpG dinucleotides typically located in the 5'-UTR (Arechederra et al., 2018). More specifically, CpG islands denote regions of approximately 200 bases where GC base pairing makes up at least 50% of the ratio (Capell & Berger, 2022; Portela & Esteller, 2010). Separate from CpG islands, CpG island *shores* (in line with their name) are regions that sit approximately 200 bp from CpG islands and consist of 1/10 the CpG density of the neighboring island (Irizarry et al., 2009) While methylation of both CpG islands and CpG island shores prevents gene expression, methylation of CpG island shores is uniquely tissue-specific (Irizarry et al., 2009; Portela & Esteller, 2010). Methylation at CpG island and CpG island shores reduces gene expression by blocking transcription factor binding to corresponding gene promotor regions (Tyrka et al., 2016).

While DNA methylation is typically associated with transcriptional inhibition, as are the cases with CpG islands and CpG island shores at 5'-UTRs, DNA methylation of gene bodies is associated with transcriptional activation (Jones, 2012). Most gene bodies have very low CpG density. When these CpG sites are methylated, they have been shown to promote transcriptional activity. Theories behind this mechanism include gene body methylation relating to elongation as well as encouraging transcription to occur at the correct site (Portela & Esteller, 2010).

Lastly, methylation at repeat regions like centromeres encourages chromosomal stability while discouraging transposable element activity (Jones, 2012).

While discussing the various patterns of DNA methylation, it is important to understand the family of enzymes responsible -DNA methyltransferases (DNMTs). DNMTs have been observed to behave in either a *de novo* fashion or maintenance fashion (Portela & Esteller, 2010). The *de novo* DNMTs are DNMT3a and DNMT3b and are present in embryonic stem cells. The maintenance DNMT is DNMT1 and is the most common form. Importantly, during mitosis, DNMT1 ensures that DNA daughter strands maintain the methylation patterns of their parent strands (Tammen et al., 2013).

While DNA methylation plays an important role in epigenetic modifications, it is not the only pathway to influencing gene expression.

Histone Modification

Histone modifications also play a key role in regulating the genome. Histones form nucleosomes which the DNA wrap around. The nucleosome is made up of one four different histones: H2A, H2B, H3 and H4 (Portela & Esteller, 2010). The nucleosome is made up of two H2A-H2B dimers and one H3-H4 tetramer. While DNA wraps 1.65 turns around this histone octamer, a fifth histone, H1(known as the linker histone), separates the DNA wrapped around two adjacent histone complexes (Portela & Esteller, 2010; Tollefsbol, 2017).

Chromatin exists in two functional forms, euchromatin and heterochromatin (Tollefsbol, 2017). Heterochromatin is a highly condensed form in which DNA regulatory activity is minimal if at all. Euchromatin is a lesser condensed form of

chromatin, where DNA regulatory processes (i.e. transcription) can occur. The different functional forms of chromatin enable the ability to control gene expression. Through modifications to histones, chromatin can switch between heterochromatin and euchromatin forms. Heterochromatin formation takes hold when there are high levels of methylation to H3-Lysine 9 (H3K4), H3K27, and H4K20. In addition to high levels of methylation, low levels of acetylation on H3K4, H3K6, and H3K79 promote heterochromatin formation (Portela & Esteller, 2010). Euchromatin formation occurs when the opposite is true. Specifically, high levels of acetylation and low levels of methylation to the corresponding histone proteins stimulate euchromatin and therefore, DNA transcription. These histone modifications are an important part of the regulation of gene expression. DNA methylation, histone methylation, and acetylation are the most studied mechanisms in genome modification. However, recent studies have shown that noncoding RNA also plays an important role.

Noncoding RNA

Regions of DNA that do not code for proteins used to be identified as junk DNA. However, scientists have since discovered important roles that these noncoding regions play in genome expression. It is now widely understood that there are many types of RNA (aside from mRNA) that are transcribed from DNA. (Tollefsbol, 2017). However, unlike mRNA, these RNA strands are not translated into proteins. Rather, they assist with genome regulation. This group of RNA is referred to as noncoding RNA (ncRNA). ncRNAs smaller than 200 nucleotides are small noncoding RNAs and ncRNAs greater than 200 nucleotides are long noncoding RNAs (lncRNA). Small ncRNAS can further be

broken down into microRNAs (miRNAs) small interfering RNA (siRNA), piwi-interacting RNA (piRNA), and small nucleolar RNAs (snoRNA) (Portela & Esteller, 2010; Tollefsbol, 2017). More information on the various noncoding RNAs and their function can be found in Table 1.

Table 1. Function of noncoding RNAs

(A) Type	Length (in nucleotides)	Function	Number in humans
miRNA (microRNA)	19–24	mRNA degradation or repression of translation	~2,500
siRNA (short-interfering RNA)	20–25	mRNA degradation	Not known
piRNA (piwi interacting RNA)	26–31	Transposon silencing, germline development	~23,000
snoRNA (small nucleolar RNA)	60–150#	Modification of rRNA	~400
lncRNA (long noncoding RNA)	> 200	Chromatin reprogramming, Precursors of small RNAs	~120,000

Adapted by (Tollefsbol, 2017)

Gene by Environment

Finally, when studying the bidirectional relationship between epigenetics and trauma, it is important to understand gene by environment interactions (G×E). G×E is defined as "a different effect of an environmental exposure on disease risk in persons with different genotypes," (Ottman, 1996, p. 1). Interestingly, because genetic and environmental factors are not independent of one another, the genotype of an individual will influence the magnitude of the environmental impact on the phenotype of that individual (Penner-Goeke & Binder, 2019). While the impacts of epigenetic mechanisms like DNA methylation or histone modification can be studied within the context of either genetics or environment, the interplay of the two provides a more nuanced understanding. This is demonstrated by Czamara et. al, who studied prenatal environmental factors and genotypes of over 2,000 women to determine the varying influences each would have on

DNA methylation of neonatal blood (Czamara et al., 2019). DNA methylation was examined at CpG-sites demonstrating high methylation variability. The authors examined several environmental factors including but not limited to maternal age, maternal smoking, gestational diabetes, maternal anxiety and depression scores, and infant sex. 10,452 variable CpGs were recognized and sorted into 2,683 variably methylated regions. Four models were used to identify the explanation for the variability of DNA methylation at the CpG sites: genotype factors (G), environmental factors (E), additive impacts (G+E), and interactive impacts (G×E) (Czamara et al., 2019). DNA methylation patterns were best explained using either (G+E) and (G×E), emphasizing the value of utilizing gene by environment models in future research when evaluating risk for disease.

G×E interactions are a valuable model to utilize when assessing risk for disease, particularly in psychiatric research. Caspi (2003) conducted a critical study which utilized G×E interactions to assess behavior. In a prospective longitudinal study, participants were organized into groups based on their varied functional polymorphisms in the promotor region of the human serotonin transporter gene. (Caspi et al., 2003). The human serotonin transporter gene (SLC6A4) houses the noncoding polymorphic region (5-HTTLPR), of the human serotonin transporter (5-HTT). 5-HTTLPR is made up of two variants, the “short” allele and “long” allele, where the “short” allele is observed less frequently than the “long allele” (Wendland et al., 2006). Data was later collected on the likeliness of participants to obtain a depression diagnosis or depressive symptoms (Caspi et al., 2003). Individuals who had short/short or short/long genotypes were more likely

than individuals with long/long genotypes to receive a depression diagnosis or present with depressive symptoms.

In a later study conducted with rhesus macaques, macaques were separated into two groups: those raised with their mothers, and those raised with their peers (Barr et al., 2004). Within both groups, macaques were identified as having a 5-HTTLPR genotype of either short/long or long/long. After exposure to a stress induced environment later in life (i.e. separation from mother or peer), adrenocorticotropic hormone (ACTH) levels were measured. In peer-reared groups, those with long/long genotypes had significantly lower ACTH levels than those with short/short genotypes. Interestingly, in mother-reared groups, there was no significant difference in ACTH levels regardless of genotype (Barr et al., 2004). Study results are depicted in Figure 1.

Figure 1. Genotype impacts on behavior in Rhesus Macaques

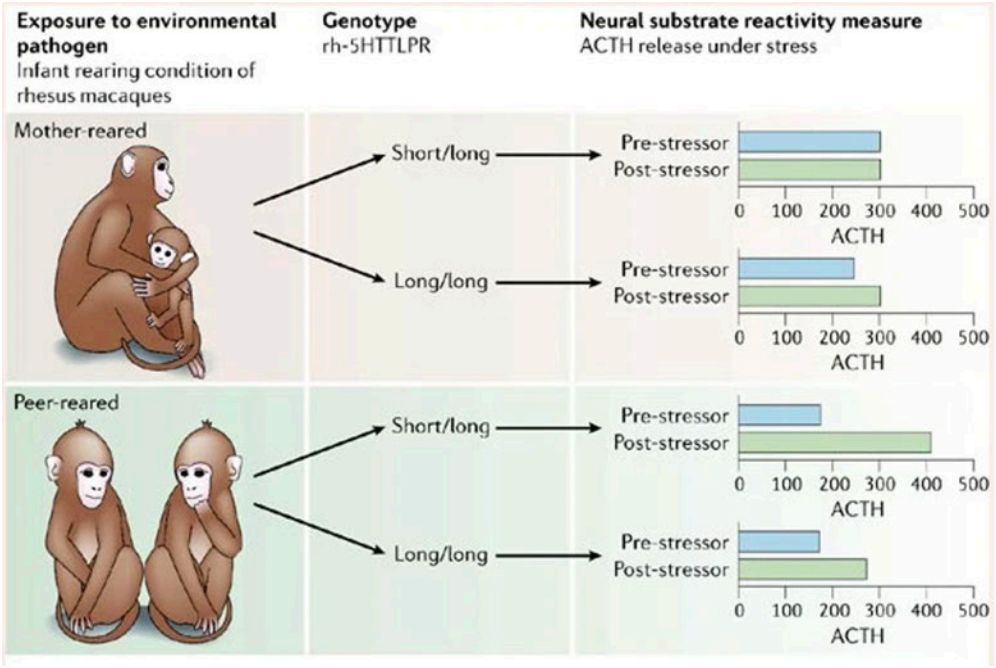

Figure adapted by (Caspi et al., 2003)

These examples document the importance of investigating G×E interactions. In understanding that environment and genes influence one another, researchers can investigate the potential bidirectional relationship between epigenetics ("gene") and trauma ("environment").

Defining Trauma and PTSD

To further investigate the relationship between epigenetics and trauma, it is important to understand the many ways in which trauma has been defined in academia, clinical settings, and psychiatric research.

In 1871 Dr. Jacob Mendez Da Costa studied 300 soldiers returning from the American Civil War. These soldiers presented with both cardiac and respiratory symptoms (Wolf & Morrison, 2017). In 1916, Sir James Mackenzie elaborated on similar symptoms he had observed, particularly echoing Da Costa's description of the "soldier's heart," or "irritable heart of soldiers,"(Mackenzie, 1916, p.1). All of the 300 soldiers Mackenzie's studied received medical treatment for their cardiac symptoms. However, when continuing with treatment like that of a patient suffering from heart failure, around 90% of the treated cases showed little effect. Mackenzie further advised different treatment methods for these soldiers. Interestingly, Mackenzie noticed a unique trend with these soldiers; their onset of symptoms were almost always delayed and suddenly brought on (Mackenzie, 1916).

There are two leading clinical definitions to help diagnose PTSD – The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, (DSM-5) and the World Health Organization's International Classification of Diseases and Related Health

Problems (ICD-11). While the DSM-5 typically uses a more extensive and nuanced approach to obtaining a PTSD diagnosis, the ICD typically utilizes a simpler process to ease the burdens of those attempting to diagnose patients in disadvantaged populations (Bryant, 2019). According to the DSM-5, in order to receive a diagnosis of PTSD, a person must first experience or witness a traumatic event and experience a variety of symptoms including but not limited to: intrusive thoughts or dreams, avoidance/numbing of internal thoughts or conversation regarding the trauma, negative changes in cognition/mood, and irritable behavior or hyperarousal (Al Jowf et al., 2021; American Psychiatric Association, 2013; Bryant, 2019). Most notably, these symptoms must persist for a minimum of one month following the traumatic event in order to be within the DSM-5 standard of a PTSD diagnosis. The ICD-11 diagnosis centers around individuals who, after experiencing a traumatic event, continue to re-experience memories of the event, exhibit avoidance, experience traumatic thoughts/memories, and maintain a heightened sense of arousal (Reed et al., 2019). An additional diagnosis denoted as "complex PTSD" was added in the ICD-11. Complex PTSD applies to individuals who have experienced continued stressors, beyond the initial, traumatizing event. Those with a complex PTSD diagnosis have been shown to have greater and more persistent symptoms when compared to those with a traditional PTSD diagnosis (Brewin et al., 2017).

Historically, there has been much debate on whether exposure to an isolated incident of extreme stress, or chronic, low-grade stress yields different psychiatric symptoms. Today, trauma —and its prospective effects —are recognized as a distinct psychological phenomenon (Ebert et al., 2018). Furthermore, not all acute high stress

events fall under the category of trauma. The DSM-5 defines trauma as involving "actual or threatened death, serious injury, or sexual violence," (American Psychiatric Association, 2013, p. 271). Non-acute high stress events, even those that are life-threatening, are excluded from the DSM-5 definition of trauma, as it pertains to a PTSD diagnosis. For example, suffering from cancer would not comprise trauma in this definition. In their definition of complex PTSD, the ICD-11 defines traumatic stressors as including, but not limited to: genocide, slavery, prolonged domestic violence, and child abuse.

Epidemiologic studies from the World Mental Health Survey Consortium have shown that over 70% of the general population will encounter a traumatic event at some point (Benjet et al., 2016). Interestingly, while so many will encounter a traumatic event, most will not develop any long-term adverse symptoms from the experience. The lifetime prevalence of post-traumatic stress disorder (PTSD) as studied by the National Comorbidity Survey is just 7.8% (Kessler et al., 1995). In more recent studies conducted by the World Health Organization (WHO), this number has decreased to 3.9% of the population (Koenen et al., 2017).

Why is there such a drastic discrepancy between the number of individuals who encounter a traumatic event, and the number of individuals who experience post-traumatic stress? Why do some individuals who experience trauma develop PTSD, while many others do not? How can individuals who undergo very similar stressors experience vastly different outcomes. In order to better treat and prevent PTSD, it is important to understand both its environmental and epidemiologic risk factors at play.

CLINICAL IMPACTS OF PTSD

To best examine the interplay between epigenetics and post-traumatic stress disorder, it is important to outline the physiological manifestations of PTSD. Several clinical studies have demonstrated that PTSD incurs detrimental impacts to an individual's stress levels, cardiac health, immunity, and mortality.

Cortisol Levels

In times of acute stress, serotonin (5-HT) is released in the amygdala and activates corticotropin-releasing hormone (CRH) in the hypothalamus (Heisler et al., 2007). CRH subsequently acts upon adrenocorticotropic hormone (ACTH) in the anterior pituitary (King, 2022). CRH stimulates ACTH to be at its highest levels right before waking and slowly declines throughout the day. ACTH will then activate the release of cortisol from the adrenal cortex, working as negative feedback for ACTH secretion (King, 2022; Tyrka et al., 2016). Yehuda et. al (1995) sought to observe the relationship between Holocaust survivors with PTSD and changes to this hypothalamic-pituitary-adrenal (HPA) axis. Subjects were organized into three groups: Holocaust survivors with a PTSD diagnosis, Holocaust survivors without a PTSD diagnosis, and individuals who were similar in race, age, and religion, but who had not experienced the Holocaust (Yehuda et al., 1995). PTSD diagnoses were obtained utilizing the Civilian Mississippi PTSD Scale and urine samples were collected from each subject. Surprisingly, the research demonstrated a negative correlation between cortisol levels and PTSD diagnoses: Holocaust survivors with PTSD had lower urinary cortisol levels when compared to the other two groups. The authors noted the importance of these results as it replicated their previous study measuring cortisol levels of combat veterans with PTSD whose traumas were of a

different nature (Yehuda et al., 1995). Because the study was conducted several decades after the Holocaust (the traumatic event of note for the subjects) the authors suggested that participants' low cortisol levels resulted from chronic PTSD (Yehuda et al., 1995).

A later study was conducted to measure the difference in cortisol levels in individuals during various times of the day. Participants were organized into three groups: trauma-exposed with a PTSD diagnosis, no trauma-exposure with PTSD diagnosis, and non-trauma exposed (Wessa et al., 2006). Subjects measured their salivary cortisol levels upon awakening through 8pm. Results showed a significant negative correlation between PTSD diagnosis and overall cortisol level, particularly 30-60 minutes after awakening. Overall, baseline cortisol levels were not significantly different between the three groups. However, specifically after 30 minutes to one hour after awakening, a drastic variability was identified. The authors suggest that lower levels of cortisol in PTSD subjects could possibly be a response to higher glucocorticoid receptor sensitivity.

Immunity

O'Toole & Catts (2008) designed a cohort study to measure the physiological impacts of Australian Vietnam Veterans with PTSD. In this study, exposure to trauma, frequency of health visits, and recent health history were established through participant self-reporting (O'Toole & Catts, 2008). A strong relationship between PTSD and subsequent asthma diagnoses was discovered. One hypothesis for this correlation posited by the authors, is that PTSD influences inflammatory responses causing immunological impairment. Several other studies have linked PTSD to immunological decline. Another epidemiologic study linked PTSD in Vietnam veterans to a higher prevalence of autoimmune disease (Boscarino, 2004). These included thyroid disease, rheumatoid

arthritis, and psoriasis. Importantly, veterans with PTSD showed higher T-cell counts and immunoglobulin-M levels than did their veteran counterparts without PTSD.

A later study demonstrated similar results in a cohort of male and female refugees with and without PTSD. Subjects were recruited by the Psychotrauma Research and Outpatient Clinic for Refugees in Germany (Gola et al., 2013). PTSD diagnoses were determined by the Clinician Administered PTSD Scale (CAPS). Those with a positive PTSD diagnosis also met the DSM-IV criteria for having a current major depressive episode. Basal plasma cytokine measurements were then taken from all subjects. Results showed generally low basal plasma cytokine levels in PTSD subjects and non-PSTD controls. While blood leukocyte distribution was the same in both groups, isolated peripheral blood mononuclear cells (PBMCs) showed higher levels of spontaneously produced interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α even when adjusted for multiple covariates like sex and smoking status. The authors suggest that these findings are evidence of those with PTSD suffering from low-grade inflammation (Gola et al., 2013).

Cardiac Health

One of the major negative downstream consequences of PTSD is cardiac health decline. Many studies have examined the cardiovascular impacts of those with PTSD and without PTSD. By extension, a direct correlation in mind for PTSD and developing hypertension (McFarlane, 2010). One study focusing on refugees in the US with PTSD diagnosis, noted the prevalence of hypertension and diabetes to be 42% and 15.5% respectively (Kinzie et al., 2008). The prevalence was noted to be statistically significant

from the general US population. When further separated into groups of "high-trauma" and "low-trauma," high-trauma groups consistently showed higher rates of hypertension.

In a prospective cohort study conducted by the US Department of Veterans Affairs, incidence of coronary heart disease was measured among veterans with PTSD (Kubzansky et al., 2007). Participants included US male veterans in the Greater Boston area who did not have any preexisting coronary heart disease. PTSD diagnoses were established using either the Mississippi Scale for Combat-Related PTSD or the Keane PTSD scale. The results established an association between PTSD and coronary heart disease. While this study used only male participants, a later study measured the correlation of PTSD and coronary heart disease in both men and women. The meta-analysis assessed 47 previous prospective studies measuring PTSD and coronary heart disease to analyze if PTSD diagnoses independently showed positive correlations with subsequent coronary heart disease (Akosile et al., 2018). The authors identified a positive correlation, and proposed the reason was due to a very common symptom of PTSD being increased hyperarousal. As such, basal activation of the sympathetic nervous system would be higher than it typically would otherwise, causing an increase in blood pressure and resting heart rate, as well as a decrease in heart rate variability (Akosile et al., 2018).

Two important studies that help strongly demonstrate the relationship with PTSD and cardiovascular disease are twin and sibling studies in two different populations. One of these studies was a sibling-controlled cohort study conducted in Sweden. More than 100,000 participants diagnosed with stress-related disorders including PTSD and acute stress reaction were assessed alongside their corresponding full sibling who did not have

a stress-related diagnosis (Fang et al., 2019). Patient information was taken from the Swedish National Patient Registrar from the years 1987 to 2013. The authors determined that PTSD is strongly associated with developing cardiovascular disease later in life, independent of family background. The second prospective twin study looked at middle-aged male twins utilizing the Vietnam Era Twin Registry. Twins who did not already have a self-reported preexisting condition of cardiovascular diseases were assessed. Results also demonstrated a strong comparison between PTSD and subsequently developing cardiovascular disease and/or having a myocardial infarction.

These studies truly emphasize the importance to not only further investigate the correlation with PTSD and cardiovascular disease, but also to determine the mechanism by which this occurs.

Mortality

Unfortunately, studies have documented the consistent association between PTSD and increased mortality. In a study used to understand this dynamic, a random sample of US Vietnam veterans was examined measuring for PTSD and post-service mortality. Initially, each participant filled out a self-reported survey between 1985-1986 regarding PTSD symptoms, health history (including use of cigarettes), demographic information, and military history (Boscarino, 2006a). Each participant was followed and assessed for cause of death. When reporting on the study in 2006, the authors noted that PTSD-positive veterans were more likely to have died since the start of the study. The reasons for each death were organized into all-cause, cardiovascular, cancer, and external cause-of-death. Vietnam Theater veterans with PTSD showed higher incidences of mortality in

each category. Boscarino (2006b) conducted a similar follow up study to further understand the relationship between PTSD and mortality due to external factors. A sample of over 15,000 US Army veterans were assessed 30 years after completing their military service (Boscarino, 2006b) . Even when controlling for several variables like age, race, anti-social and thrill-seeking personality types, there remained a statically significance between PTSD and external causes of mortality. The author acknowledged that the reason for the significance remains unclear, but that a potential reason could be due to behavioral risk factors related to trauma exposure like alcohol and drug abuse. While the reasons for external related deaths are multifactorial, it is important to understand the epigenetic factors that contribute as they can determine if such epigenetic factors contribute to behavioral risk factors after trauma exposure.

RELATIONSHIP BETWEEN EPIGENETICS AND PTSD

While breakthroughs have been made in discovering various associations between PTSD and physiological aberrations, no one has tried to understand the mechanism by which this occurs until recently. In utilizing epigenetics, much more can be understood not only how certain genomic variations predispose certain individuals to PTSD, but also how PTSD can influence the genome.

Because the study of epigenetics is a young and rapidly growing field, many studies have been put forth in order to piece together the potential bidirectional PTSD-epigenetic relationship. However, studies have consistently shown conflicting results and contradictory evidence. After previously examining the ways in which PTSD have been linked to changes in cortisol levels, decreased cardiac health, decreased immunity, and

increased mortality, this thesis will now examine PTSD with these same four factors through the lens of the recent (and often contradictory) epigenetic studies that have been put forth.

Cortisol Levels

The hypothalamic-pituitary-adrenal (HPA) axis has been studied to be the reason for changes in cortisol due to PTSD. To examine the validity of this theory, genetic and epigenetic mechanisms regulating the HPA axis have been tested to determine the relationship between PTSD and the HPA axis. Substantial evidence indicates that PTSD can cause DNA methylation changes and impact the HPA axis (Kuan, Waszczuk, Kotov, Marsit, et al., 2017). Two important genes of relevance are NR3C1 and FKBP5.

NR3C1

The NR3C1 gene is located on chromosome 5q31-32 (Tyrka et al., 2016). NR3C1 codes for the glucocorticoid receptor (GR) that binds to cortisol (Persaud & Cates, 2022). In a previously mentioned study by Yehuda et. al (1995), it was hypothesized that PTSD-positive individuals had lower levels of cortisol when compared to their PTSD-negative counterparts due to having “stronger negative feedback of cortisol” as well as an “increased number and sensitivity” of glucocorticoid receptors (Yehuda et al., 1996 p.8). By investigating the impacts of NR3C1 on those suffering with PTSD, researchers have been able to explore if this hypothesis is valid. In particular, studies have been conducted to determine the methylation status of this gene and its overall expression (Persaud & Cates, 2022).

As previously discussed, DNA methylation at CpG islands and CpG island shores are associated with gene silencing (Portela & Esteller, 2010). Therefore, to confirm the postulation that PTSD is correlated with low cortisol levels due to an increased number of glucocorticoid receptors, studies must show less methylation activity observed at the NR3C1 gene at CpG islands and CpG island shores.

One study does, in fact, validate this hypothesis. The methylation activity of NR3C1 was measured in combat veterans with PTSD in comparison to combat veterans without PTSD in order to determine the epigenetic relationship between trauma and the HPA axis (Tyrka et al., 2016; Yehuda et al., 2015). Peripheral blood mononuclear cells (PBMCs) were acquired from each subject. Results revealed that methylation to the NR3C1-1F promotor was inversely correlated to PTSD associated symptoms. While several studies continue to demonstrate that PTSD is associated with hypomethylation of NR3C1, conflicting research has shown the opposite to be the case (Palma-Gudiel et al., 2015; Persaud & Cates, 2022).

An example of such contradictory results, the TRAILS (Tracking Adolescents ' Individual Lives) Survey, examined the methylation of the NR3C1 gene subjects who had experienced traumatic events at differing points in their lives (van der Knaap et al., 2014). Data was taken from the TRAILS study, a prospective study of the Dutch population from pre-adolescence through adulthood. DNA was extracted from whole-blood samples. Three genomic regions within CpG island of the NR3C1 promoter region were examined. Subjects who reported stressful life events or traumatic youth

experiences showed increased hypermethylation at exon 1F (van der Knaap et al., 2014). This finding is consistent with several other studies.

While there is conflicting evidence around the impact that PTSD has on the methylation status of the NR3C1 gene, further research is continuing to be done. To fully understand the connection between PTSD and the HPA axis, researchers not only continue to study the NR3C1 gene, but the FKBP5 gene as well.

FKBP5

The FKBP5 gene is located on chromosome 6p21.31 (Mendonça et al., 2021). Increased expression of the FKBP5 gene is correlated with several psychiatric diseases (Blair et al., 2019). The FKBP5 gene encodes FK506 binding protein 5 (FKBP5) (Persaud & Cates, 2022). FKBP5 is a co-chaperone that assists in modulation the NR3C1 glucocorticoid receptor (Mendonça et al., 2021). A co-chaperone can be defined as "proteins that participate in the function of chaperone activity" (Caplan, 2003). Chaperones assist the folding and trafficking of a protein, typically a transcription factor or protein kinase. Because FKBP5 assists with the folding of the NR3C1 glucocorticoid receptor, the intracellular concentration of FKBP5 will influence the affinity of the glucocorticoid receptor to glucocorticoids (Mendonça et al., 2021). Several studies have explored how PTSD impacts the methylation activity of FKBP5.

Zannas et. al 2019 compared two groups, those with prolonged early childhood stressors (i.e. separation from parents) with those who did not experience prolonged early childhood stressors, and compared each groups methylation activity at FKBP5 (Zannas et al., 2019). Whole blood DNA was sampled from the participants. The results of this study

revealed reduced methylation at FKBP5 (thereby an increase in expression of FKBP5) in the group exposed to prolonged early childhood stressors.

In an attempt to minimize confounding factors, a study was conducted of subjects who were exposed to a single trauma: the World Trade Center attacks on 9/11 (Yehuda et al., 2009). Psychologists administered the Clinician Administered PTSD Scale (CAPS) as well as the Structured Clinical Interview for the DSM-IV to organize subjects into PTSD-positive or PTSD-negative cohorts. Subjects also completed the Trauma History Questionnaire and Childhood Trauma Questionnaire. Fasted whole blood samples were obtained from each participant and a genome wide expression analysis was performed. Significant decreases in FKBP5 expression were observed in participants with PTSD. The authors proposed that such reduced expressions in FKPB5 enhance glucocorticoid receptor response — thereby leading to lower cortisol levels in those with PTSD (Yehuda et al., 2009). However, it is important to note that associations between cortisol level and PTSD continue to produce mixed results.

A later study was conducted of subjects who were also exposed to the World Trade Center attacks, although this time with a focus on first responders. Responders were placed into two groups, those that developed PTSD and those that did not (Kuan, Waszczuk, Kotov, Marsit, et al., 2017). A diagnosis was determined by Master's level clinical evaluators trained to administer the Major Depressive Disorder (MDD) and PTSD assessments. The methylation activity of each subject was profiled using whole blood samples and the Epigenome Wide Association Study (EWAS) design. The EWAS enabled the authors to examine the epigenetic patterns of individual subjects without

prior knowledge of disease. It allowed the authors to take “an agnostic approach to identifying genetic variants for disease” (Koenen et al., 2013 p. 1). The authors reported null findings (Kuan, Waszczuk, Kotov, Marsit, et al., 2017). Results brought back no significant relationship between epigenetic alternation and PTSD.

The authors conducted a follow-up study to gain a more comprehensive understanding of their previous findings. World Trade Center responders were once again recruited and divided into those with PTSD and those without. Transcriptome-wide expression study using RNA-Seq was later done as a follow-up study (Kuan, Waszczuk, Kotov, Clouston, et al., 2017). Rather than using a genome-wide or epigenome-wide approach, a transcriptome-wide association study (TWAS) adds another layer to examine gene expression (Li & Ritchie, 2021). Master’s level clinical evaluators trained to administer PTSD assessments were utilized to provide PTSD diagnoses to the participants. Whole blood samples of World Trade Center responders with without PTSD were taken (Kuan, Waszczuk, Kotov, Clouston, et al., 2017). Blood cell type proportions were also taken into account during analysis (i.e. proportions of CD8T, CD4T, natural killer, and granulocytes) When comparing transcriptome gene expressions, FKBP5 was among the top overexpressed gene in those with PTSD.

These are examples of many studies that have been conducted to understand the association between PTSD and the HPA axis. While results are contradictory, it is important to understand the limitations and confounding factors of each study, leading to mixed results. This will be discussed in EXAMINING DISCREPANCIES IN EPIGENETIC-PTSD RESEARCH. While many researchers studied the connection

between PTSD and the HPA axis, others focused on other negative physiological associations with PTSD.

Immunity

The epigenome itself is impacted by inflammation, and yet, epigenetic mechanisms can be important agents in activating chronic inflammation (Alam et al., 2017). Studies have demonstrated a negative correlation with PTSD and overall immunity (Boscarino, 2004; O'Toole & Catts, 2008). More recent work has also demonstrated the epigenetic influence on this association.

In search of understanding the PTSD-epigenetic relationship, DNA methylation activity of specific immune related gene promotors were observed in military service members with and without PTSD (Rusiecki et al., 2013) A cohort of male and female military personal were followed pre- and post-deployment and monitored for development of PTSD. The International Classification of Diseases Ninth Edition (ICD-9) was utilized to ensure no subject had pre-existing symptoms of PTSD or depression prior to the study. PTSD diagnoses based on ICD-9 codes were also utilized to determine development of PTSD post deployment. Serum samples were simultaneously collected pre- and post-deployment to measure changes in DNA methylation at specific gene promoter sites. These sites included: insulin-like growth factor 2 (IFG2), long non-coding RNA transcript H19, interleukin-8 (IL8), interleukin-16 (IL16), and interleukin-18 (IL18). Results showed that, post-deployment, the control group (those who did not develop PTSD) had significantly decreased methylation activity of H19, but the PTSD positive group did not. Furthermore, those that developed PTSD post-deployment showed

increased DNA methylation activity of H19 and IL18. This work demonstrated the epigenetic impact of PTSD on the immune response.

In a later study, Zannas et al (2019) utilized a large-scale genome-wide analysis rather than select for specific gene promotor regions, in order to determine the epigenetic relationship of PTSD and the immune response. Zannas et al (2019) recognized a relationship between the regulation of FKBP5 (a highly studied gene in relation to trauma and PTSD) and the NF-κB-driven inflammatory response (Zannas et al., 2019). NF-κB is a DNA binding transcription factor. NF-κB signaling and activity impacts inflammation, apoptosis, development, and overall immunity (Kang, 2019) NF-κB is regulated by the cytoplasmic inhibitor "inhibitor of nuclear facto κB" (IKBα). IKBα regulates NF-κB by binding to it and keeping NF-κB inactive in the cytoplasm (Barrett et al., 2017). In order to activate NF-κB, IKBα must be phosphorylated by IKB kinase (IKK). IKK is comprised of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ) (Kang, 2019). NF-kappa-B-inducing kinase (NIK) helps to regulate the NF-κB pathway by phosphorylating IKKα and therefore activating IKKα activity, leading to activation of the NF-κB pathway (Zannas et al., 2019).

The study conducted by Zannas et. al (2019) was a large-scale genome wide analysis and mechanistic investigation of human cohorts. The cell culture experiments that were run included healthy donors with wildtype FFKBP5 and FKBP5 knockout Jurkat cells. Upregulation of FKB5 was tested by stimulating cell types with DEX, which has been studied to induce FKBP5 expression (Menke et al., 2012). The authors analysis demonstrated higher FKBP5 expression was correlated with increased inflammation and

increased activity of the NF-κB pathway (Zannas et al., 2019). The mechanism behind this utilizes the NIK-IKKα interaction. Upregulation of the FKBP5 gene strengthens NIK-IKKα binding, therefore increasing the levels of phosphorylated IKKα (pIKKα). This, in turn, stimulates NF-κB pathway signaling (Zannas et al., 2019).

The relationship between FKBP5 and NF-κB is an important, as it open a window into understanding the relationship with PTSD and inflammation. As previously stated, studies have shown that PTSD correlates with methylation changes in FKBP5 (Kuan, Waszczuk, Kotov, Clouston, et al., 2017, 2017; Yehuda et al., 2009; Zannas et al., 2019). While the data is unclear as to when PTSD causes a dampening or stimulation of FKBP5, it is evident that this change in expression can have downstream impacts on the inflammatory response.

Cardiovascular Health

There is an increasingly growing amount of research which links PTSD symptoms to cardiometabolic traits, including metabolic syndrome, cardiovascular disease, and type 2 diabetes (Sumner et al., 2017). Pollard et. al (2016) conducted a review of 106 studies investigating candidate risk genes for PTSD (Pollard et al., 2016). In his review he reported thirteen genes to have been shown to have an association by more than one investigator. These genes include, but are not limited to, adenylate cyclase 8 (ADCY8), adenylate cyclase activating polypeptide 1 receptor 1 (ADCYAP1R1), brain derived neurotropic factor (BDNF), NR3C1, and FKBP5.

Zannas et al (2019) described the connection between FKPB5 and cardiovascular disease. Aging and stress related upregulation to the FKNBP5-NF-κB signaling pathway

not only stimulates inflammation but also contributes to cardiovascular disease (Zannas et al., 2019). This occurs by proinflammatory states having an increased levels of interleukin (IL)-8 as well as an increased granulocyte-to-lymphocyte ratio. These biomarkers are highly correlated with cardiovascular risk (Zannas et al., 2019). While evidence shows that an upregulation in FKBP5 corresponds to heightened risk of cardiovascular disease, it is still uncertain how FKBP5 is correlated to PTSD. As stated previously, many studies have produced mixed results on the relationship of FKBP and PTSD. While some studies show that PTSD corresponds to a downregulation of FKBP, others have shown that PTSD corresponds to an upregulation of FKBP5 (Kuan, Waszczuk, Kotov, Clouston, et al., 2017; Yehuda et al., 2009; Zannas et al., 2019).

Mortality – Genetic Aging

A major topic of research is the epigenetic impact of aging and mortality. Several studies have been conducted uncovering the effects of post-traumatic stress on accelerated aging in the epigenome (Lohr et al., 2015). A genetic metric that has historically been used to measure cellular aging is the length of telomeres. Telomeres are specialized DNA structures made up of DNA repeat sequences that sit at the ends of chromosome (King, 2022; Wolf & Morrison, 2017). Telomeres ensure chromosomal integrity by maintaining preventing necessary coding regions of the genome from shortening during cellular replication. Studies have been conducted showing the association between psychiatric disorders (including depressive disorder, anxiety disorder, and PTSD) and telomere length (Darrow et al., 2016).

However, there has been some critique as to whether or not telomeres are an accurate measure of accelerated aging (Wolf & Morrison, 2017). One of these reasons is that telomeres show a weak association with chronological age. Another critique of this metric is that after conducting meta-analyses on the correlation between stress and PTSD and telomere length, negative correlations resulted, but the correlations were weak and often not significant (Wolf & Morrison, 2017).

A newer metric that has been used within the research community has been the use of DNA methylation as a marker for cellular aging. Unlike telomeres, DNA methylation activity has shown strong associations with chronological age (Christensen et al., 2009). Before examining the various studies that seek to understand the correlation between PTSD and extracellular aging, it is important to examine the algorithms that are used in these studies. Four algorithms have been used as the standard metrics for estimating DNA-methylation age data. These algorithms are Horvath, Hannum, GrimAge, and PhenoAge. Each model has been used in recent years as an "epigenetic clock" to help determine the correlation between environmental factors and genetic aging (Wolf & Morrison, 2017 p. 5).

DNA Methylation – Epigenetic Clocks

In 2013, Steve Horvath and Gregory Hannum independently developed two DNA methylation (DNAm) age estimation algorithms known as Horvath and Hannum, receptively. Horvath identified 353 CpG sites within, or proximal to, 353 different genes. These 353 CpG sites were combined into a weighted score to predict chronological age with a correlation of $r = 0.96$ (Horvath, 2013; Wolf & Morrison, 2017). Horvath used multiple tissue types as the basis for his pan-tissue model. Hannum, on the other hand,

developed his model for predicting DNAm age utilizing CpG methylation sites that are within or near genes known specifically for age-related functions (Hannum et al., 2013; Wolf & Morrison, 2017). Hannum only used whole blood samples as the basis for his model. Hannum argued that using whole blood as the basis for his model (rather than multiple tissue types) was advantageous for those collecting and testing samples to be used in future studies (Hannum et al., 2013). Hannum focused on identifying common changes in DNAm, rather than tissue-specific changes (Bell et al., 2019). Hannum's model, like Horvath's, predicts chronological age with a correlation of $r = 0.96$ (Hannum et al., 2013; McCrory et al., 2021). Horvath and Hannum are recognized as the first-generation clocks (McCrory et al., 2021). While still used to today to measure DNA methylation age, The Horvath and Hannum models "exhibited only weak associations with clinical measures of physiological dysregulation" (McCrory et al., 2021 p.2). The more recent models, GrimAge and PhenoAge, have gained more traction in epigenetic studies.

The second-generation clocks include GrimAge and PhenoAge. GrimAge and PhenoAge account for the physiological dysregulation shortcomings by incorporating correlations of morbidity and mortality into their algorithms.

The GrimAge model was developed using a two-step approach. First, surrogate biomarkers of physiological risk and stress factors were identified. The biomarkers consisted of seven plasma proteins known to be associated with morbidity and mortality (Lu et al., 2019). Because smoking is a significant risk factor of morbidity and mortality, a DNAm-based estimation of smoking pack-years was determined. The models for

estimating DNAm of physiological risk factors and smoking pack-years were then combined into a single model (Lu et al., 2019).

The PhenoAge model was also developed also using a two-step approach. First, a weighted combination of 10 clinical characteristics (including chronological age, lymphocyte percentage, and mean cell volume) were used to develop a phenotypic age estimator. Second, this phenotypic age estimator was regressed onto DNAm levels identified in 153 CpG sites (Lu et al., 2019; McCrory et al., 2021).

Epigenetic Clocks and PTSD

Wolf et al. (2019) conducted a study utilizing Horvath and Hannum models to determine the relationship between PTSD and accelerated aging (Wolf et al., 2019). This two-year longitudinal study recruited United Sates war veterans of Iraq and Afghanistan. Subjects were categorized into those with PTSD and those without PTSD. Subjects were interviewed utilizing the Traumatic Life Events Questionnaire (TLEQ), a self-assessment, to determine trauma exposure. PTSD was determined via a Clinician Administered PTSD Scale for DSM-IV. DNA was then extracted from peripheral blood samples where Horvath and Hannum DNAm age estimations were made. The authors found that alcohol use and avoidance and numbing symptoms associated with PTSD correlated with increased cellular aging for the Horvath model, but not for the Hannum model (Wolf et al., 2019).

The first study to measure DNAm Grim against PTSD was done by Yang et. al (2020). The authors were interested in the hypothesis that PTSD is a condition of "accelerated biological aging" (Yang et al., 2021). Participants were combat trauma-exposed male veterans with and without PTSD. Subjects were placed in the PSTD group

based on an assessment utilizing the Clinician Administered PTSD Scale (CAPS) diagnostic. All subjects were of similar chronological age. Utilizing the DNAm Grim model, those in the PTSD group showed a significantly accelerated epigenetic age compared to those in the control group.

Accelerated cellular aging was also measured utilizing the PhenoAge model. In a follow-up to his earlier study on the impacts of PTSD on World Trade Center responders, Kuan et al. (2021) explored the correlation between responders with PTSD and accelerated aging (Kuan et al., 2021). World Trade Center responders were organized into those with PTSD and those without PTSD. Diagnoses were provided via Master's level psychologists who administered the Structured Clinical Interview for DSM-I (SCID). Whole blood samples were then obtained from subjects in each group. DNAm activity was compared utilizing the Horvath, Hannum, GrimAge, and PhenoAge models. The authors concluded that the GrimAge model showed a high correlation between PTSD and accelerated cellular aging. However, Horvath, Hannum, and PhenoAge did not show accelerated cellular aging as corresponding with a PTSD diagnosis.

While the epigenetic clocks are valuable models to help determine how environmental factors influence the genome, more work needs to be done to understand the reasons behind the mixed results that are demonstrated across several studies.

INTERGENERATIONAL AND TRANSGENERATIONAL INHERITANCE

While evidence shows that gene by environment interactions (G×E) can have an impact on the cellular level of individuals, there is growing debate around changes to epigenetic profiles in humans impacting offspring and future generations. This is

understood as intergenerational and transgenerational epigenetics. This phenomenon has been most studied in plants, however, there have been an increasing amount of animal model and human studies (Tuscher & Day, 2019). In order to examine the research dedicated to this phenomenon, it is important to understand the nuanced distinction between intergenerational and transgenerational inheritance.

Intergenerational inheritance occurs when environmentally induced epigenetic alterations in parental generations are inherited by a generation of offspring that could have potential direct exposure to the environmental factor in utero or through germ line cells (Al Jowf et al., 2021). The environmental change can be made up of many factors including drug and alcohol abuse, diet, stress, and PTSD (Tuscher & Day, 2019). In F0 males and F0 non-pregnant females, intergenerational inheritance includes epigenetic changes to F1 offspring (Skinner, 2008). In F0 pregnant females, however, intergenerational inheritance includes epigenetic changes to both the F1 and F2 offspring (Al Jowf et al., 2021; Tuscher & Day, 2019).

Transgenerational inheritance relies strictly on the germline inheritance of epigenetic changes due to there being no direct environmental exposure by the offspring generation (Tuscher & Day, 2019). Simply put, transgenerational inheritance refers to F0 males and F0 non-pregnant females passing down epigenetic alterations to their F2 generation (Skinner, 2008). In F0 pregnant females, transgenerational inheritance occurs when the epigenetic alterations are inherited by their F3 generation. If an epigenetic alteration is passed down to generations that had no potential direct environmental

exposure, it is considered transgenerational inheritance. Figure 2 demonstrates the differences between intergenerational and transgenerational inheritance.

Figure 2. Differences between Intergenerational and Transgenerational Inheritance

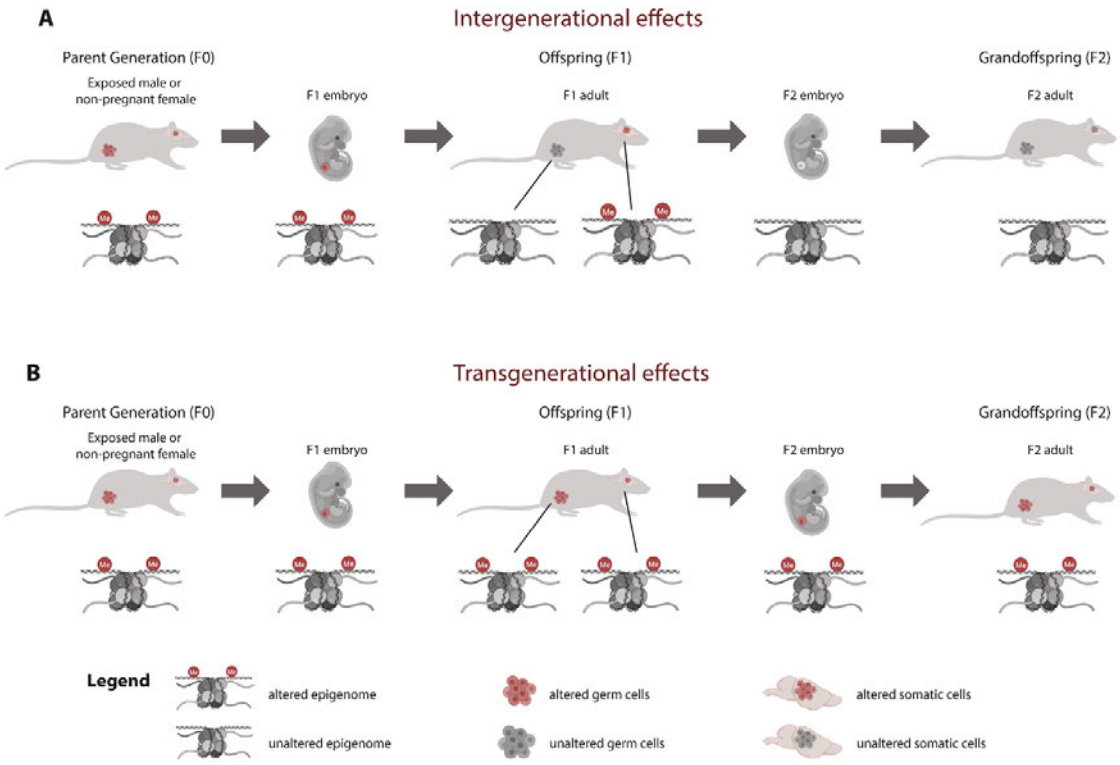

Figure adapted by (Tuscher & Day, 2019)

Intergenerational Inheritance and PTSD

Interested in studying the ways PTSD impacts epigenetic intergenerational inheritance, Yehuda et al (Yehuda et al., 2016) studied Holocaust survivors and their offspring. The authors had previously studied how exposure to trauma by the parental generation enhances the risk for development of PTSD and mood disorders to occur in

their offspring (Yehuda et al., 1998). Now, the authors are looking to understand the epigenetic ramifications of these findings. Holocaust survivors, their offspring, and a comparable control group where the parental generation was not exposed to the Holocaust were recruited (Yehuda et al., 2016). PTSD was confirmed in Holocaust survivors via a psychiatric diagnosis using the Structured Clinical Interview for DSM-IV. The Childhood Trauma Questionnaire (CTQ) was completed by the F1 generation to avoid any confounding factors of PTSD in offspring due to direct environmental exposure. Whole blood and salivary cortisol samples were collected from all groups. The results demonstrated unique epigenetic changes between Holocaust survivors and their offspring. Both Holocaust survivors and their offspring showed methylation differences at the functional intron 7 region of the FKBP5 gene in comparison to their respective control groups. However, the methylation activities were not identical. Rather, in Holocaust survivors, methylation at the intron 7 region was higher than the F0 control group. But in Holocaust survivor offspring, the methylation at intron 7 was lower than the F1 control group. The authors suggest that a reason for the difference in methylation activity could be due to an "intergenerational epigenetic priming of the physiological response" (Yehuda et al., 2016 p.8). This form of intergenerational epigenetic inheritance is in line with the theory of F1 offspring receiving potential direct trauma exposure (or any environmental factor) via in utero or germline cells (Skinner, 2008).

Transgenerational Inheritance and PTSD

Several studies on chronic stress, PTSD, and its transgenerational impacts have been conducted with animal models. In example, a study with Drosophila explored whether learned behaviors in response to stress could be passed down to offspring. The

F0 generation of Drosophila were separated into three groups: exposure to prolong appetitive-olfactory conditioning, exposure to aversive-olfactory conditioning, and a control group with no exposure (Williams, 2016). The appetitive-olfactory conditioned group were exposed to a specific odor for the ten minutes of every hour where they had access to their food. In the aversive-olfactory conditioned group, Drosophila were placed on electric copper grid and an odor was released with the deliverance of a mild electric shock every five minutes. The F1 and F2 generations of Drosophila were then separated from the F0 generation to ensure no behaviors were influenced by environmental factors. The results of the experiment demonstrated sensorimotor behavioral changes in both F1 and F2 offspring of the two conditioned groups, indicative of transgenerational inheritance.

In search of understanding the effects of PTSD on epigenetic transgenerational inheritance, a study was conducted with mice exposed to chronic stress. The goal of this study was to assess if acquired DNA methylation changes were inherited in future offspring (Franklin et al., 2010). An F0 generation of mice were exposed to chronic and unpredictable maternal separation from postnatal day 1 to day 14. The F0 generation was then mated with a control F0 group. Both behavioral tests and DNA methylation assays were conducted in F1, F2, and F3 generations, of which none experienced the maternal separation. Behavioral assessments included forced swim tests and open field observation in comparison to control groups of the corresponding generation. The authors noted that although the F1, F2, and F3 generations did not experience unpredictable maternal separation, they still demonstrated depressive-like behaviors akin to those of F0 (Franklin

et al., 2010). Additionally, DNA methylation assays of sperm samples were conducted, demonstrating an upregulation of methylated CpGs at the gene coding for methyl CpG-binding protein 2 (MeCP2) in F1 sperm. MeCP2 is linked to regulation of stress with a decrease in MeCP2 (i.e. via DNA methylation) correlating with increased stress (Urdinguio et al., 2009).

Interestingly a more recent studies have utilized mouse models to find a correlation between small noncoding (sncRNA) and long noncoding RNA (lnRNA) with transgenerational inheritance (Gapp et al., 2020). In multiple studies, Katharina Gapp has investigated how ncRNA in sperm is a carrier of heritable information. Furthermore, this ncRNA is susceptible to changes from environmental factors (like high stress environments) in mice. Gapp randomly selected a litter of mice who were subjected to unpredictable separation from their mother via cage changes. ncRNA fractions from sperm from exposed male mice were then collected and injected into naïve fertilized oocytes. F0 noncoding RNA was then observed in F1 and F2 offspring, signifying transgenerational inheritance (Gapp et al., 2020). While this research is fairly new, and no specific studies to my knowledge have been conducted on transgenerational inheritance in humans, it is a potential key into understanding more about the environmental impacts on transgenerational inheritance in humans.

Although studies demonstrate evidence of environmental factors impacting epigenetic inheritance, it is important to consider potential confounding factors in these studies. In previous psycho-analytic research, intergenerational trauma was demonstrated to be passed down in humans through attachment relationships (Colangeli, 2020). The

challenge to consider when reflecting on results of these studies is to ensure that when determining the transmission of trauma in humans, studies are able to disconnect the trauma experienced by the parents from the traumatic behaviors either learned or endured by the offspring at an early age (Colangeli, 2020).

EXAMINING DISCREPANCIES IN EPIGENETIC-PTSD RESEARCH

There has recently been rapid growth in the field of epigenetics and trauma. With this outpouring of new literature, however, has come conflicting results on the mechanisms by which PTSD and the epigenome influence one another. Specifically, there has been conflicting evidence on the changes to cortisol levels and methylation activity of the FKBP5 gene in those with PTSD compared to those without PTSD. In examining the literature, I identified seven factors that authors consistently list as limitations to their work. The following four factors will be examined as potential reasons for the mixed results: defining/measuring trauma, measuring epigenetic changes, differences subject population, and timing of trauma.

Defining / Measuring Trauma

When conducting studies investigating the relationship between PTSD and the epigenome, a confounding variable across the literature is the different ways in which PTSD and trauma exposure are diagnosed. In this review, the different protocols that have been used to identify and diagnose PTSD include the Structured Clinical Interview for DSM-IV, Clinician Administered PTSD Scale (CAPS), PCL-C Checklist, Trauma History Questionnaire, Traumatic Life Events Questionnaire (TLEQ), Childhood Trauma Questionnaire, Mississippi Scale for Combat-Related PTSD, Civilian Mississippi PTSD

Scale, and the Keane PTSD scale. These diagnostic tools range from self-reporting to clinician administered. Several other screening tools continue to be used including but not limited to the Harvard Trauma Questionnaire (HTQ) and the Impact of Event Scale (Magwood et al., 2023). While method has pros and cons, the differences in diagnosing PSTD could have the potential of leading to different results across studies. For example, the DSM-V requires a minimum of one month of demonstrating PTSD symptoms before a diagnosis can be given, whereas the ICD-9 does not require this timeline. These variations in standards could potentially lead to different subjects being included or excluded in a study depending on the diagnostic tool used.

Many systematic reviews have been conducted to validate the effectiveness of each of these screening tools. Many demographics, like refugees and military personnel, have been found to have accurate diagnostic results when using diagnostic tools tailored to their population (Mughal et al., 2020). While this ensures accuracy when comparing results of subjects within the same demographic, accuracy across demographics is diminished. This includes comparing those who have experienced mass trauma, like genocide or terrorism, to those who have experienced personal trauma like physical abuse or domestic violence. While this does not imply that no comparative analysis can be conducted, it is important to understand the potential confounding variables when conducting direct comparisons.

Measuring Epigenetic Changes

Another variant in the literature is the use of multiple sample types to determine DNA methylation activity. Across the literature, the most widely used samples consist of

whole blood, saliva, and brain tissue. One study looked at the epigenetic variation of the spindle and kinetochore-associated protein 2 (SKA2) gene in those with PTSD vs those without PTSD (Kaminsky et al., 2015). SKA2 was investigated as a biomarker for predicting suicidal behavior in individuals with PTSD. SKA2 has been shown to be an important regulator of the HPA axis by stimulating glucocorticoid receptors' nuclear transactivation (Rice et al., 2008). Furthermore, SKA2 expression reduces HPA axis ability to suppress cortisol via negative feedback after stress (Kaminsky et al., 2015). Utilizing this data, the authors wanted to determine what samples could be used to predict suicidal behavior. Subjects were recruited from the Grady Trauma Project, a cohort of predominantly black, low socioeconomic status (SES) individuals. Saliva, whole blood, and brain samples were collected. Results demonstrated a similar predictive efficacy between whole blood and saliva samples. The authors noted that this was due to around 74% overlap of cell type between both samples (Kaminsky et al., 2015). However, it was demonstrated that saliva samples were better surrogates for brain tissue compared to whole blood samples, with this theory supported by previous studies of buccal tissue deriving from the same primary germ layer as brain tissue. The authors also note that the tissue sample type are of most importance when utilizing biomarkers impacted by tissue specific responses to the epigenome (Kaminsky et al., 2015).

Another study was done demonstrating the variation in DNA methylation activity between brain and whole blood samples. The goal of the study was to measure the variation across blood, prefrontal cortex, entorhinal cortex, superior temporal gyrus, and the cerebellum (Hannon et al., 2015). Brain samples were obtained by the MRC London

Neurodegenerative Disease Brain Bank. Brain tissue samples were dissected by trained specialists and matched whole blood samples were collected prior to death. The results revealed that for the majority of the genome, DNA methylation variations between individuals in "whole blood is not a strong predictor interindividual variation in the brain" (Hannon et al., 2015 p.1). This is especially true in disorders where the brain is the tissue of most interest. In fact, interindividual variation of DNA methylation sites was only correlated at a small number of loci across both tissues. The authors conclude by stating the continuing use whole blood samples for neuropsychiatric disorders will provide limited data in helping understand the pathological mechanism.

An example of a well-studied gene that is potentially influenced by tissue specific responses is FKBP5. Animal model studies have been used to demonstrate the relationship between chronic stress and FKBP5 to further understand the mechanisms by which stress and trauma impact the HPA axis. Stress induced mice showed an increased expression of FKBP5 in the paraventricular nucleus and the central amygdala. However, this increase in expression was not observed in in the hippocampus (Mendonça et al., 2021). One reason why the increase in expression was not observed in the hippocampus might be due to the fact that FKBP5 has an already high basal level expression (Scharf et al., 2011). Importantly, it can be understood that FKBP5 expression can potentially be tissue specific.

Difference in Subject Population

In examining the bidirectional relationship of PTSD and epigenetics, several studies have produced conflicting or inconsistent results. One of the potential

confounding factors leading to such discrepancies are the wide range of subject demographics that have not been accounted for.

DNA Methylation Changes by Demographics

The most common sample used throughout the literature are whole blood samples. Nabais et al (2023) points out the importance of considering intrinsic and extrinsic factors upon examining DNA methylation of bulk tissue like blood (Nabais et al., 2023). Intrinsic factors are changes to DNA methylation that are directly associated with the phenotype being investigated. Extrinsic factors refer to the differences in cell-type proportions, an important measure since many DNA methylation activities are cell-type dependent. Barfield et al. (2014) explains that a nuanced but important difference between classic genome wide association studies (GWAS) and genome wide studies of DNA methylation is that that methylation data does not solely reveal genetic inherited genetic information, but is also influenced by other factors including cell type heterogeneity (Barfield et al., 2014).

Horvath et al. (2016), demonstrated that when utilizing the Horvath and Hannum epigenetic clocks, differences in methylation activity were shown when accounting for sex and race/ethnicity (Hannum et al., 2013; Horvath, 2013; Horvath et al., 2016). In this study, whole blood, saliva, and brain samples were obtained from seven different racial/ethnic groups. In blood samples, Hispanic and Tsimane Amerindians subjects had lower intrinsic but higher extrinsic epigenetic aging rates than their counterparts (Horvath et al., 2016). Black subjects had lower extrinsic epigenetic aging rates in blood samples. Male subjects had higher epigenetic aging rates than women in all whole blood, saliva, and brain tissue samples. This study demonstrated not only the importance of considering

the collected tissue sample when administering further studies, but to also look at how subject demographics can influence study results. When comparing results, it is important to adjust for these differences before determining the data as inconclusive.

Risk for PTSD Potentially Sex Dependent

Studies have increasingly showed that there are sex differences in the epigenetic programing of stress pathways (Bale, 2011). Further research has been conducted on the correlation between sex and PTSD. While epidemiologic studies have detected a higher prevalence of PTSD in women when compared to men, only recently has there been insight into why this might be the case (Breslau et al., 1998). As previously stated, adenylate cyclase activating polypeptide 1 receptor 1 (ADCYAP1R1) is one of the main genes associated with PTSD (Pollard et al., 2016). ADCYAP1R1 regulated by an estrogen response element and has been linked to PTSD only in women (Daskalakis et al., 2018; Ressler et al., 2011). ADCYAP1R1 is the receptor to pituitary adenylate cyclase-activating polypeptide (PACAP)1. While PACAP1 is known for having a role in regulating the cellular stress response, it was unclear if it played a role in the human psychological stress response like PTSD. Ressler et al. (2011) found that ADCYAP1R1 gene expression is dynamically regulated by estrogen24. (Ressler et al., 2011). In this civilian, cross-sectional study, research interviews and salivary DNA and blood samples were collected.

A later review was conducted to determine further sex differences in DNA methylation that could contribute to varying outcomes when exposed to trauma (Uddin et al., 2013). The authors proposed that by examining the sex differences in DNA

methylation of genes that regulate brain development, more can be understood about how sex influences neuropsychiatric disorders. As previously discussed, DNA methyltransferase 3A (DNMT 3A) and DNA methyltransferase 1 (DNMT1) encode for enzymes that conduct *de novo* methylation of CpG sites during development (Tammen et al., 2013). Because DNMTs are involved in the mechanism of DNA methylation, sex dependent DNMT regulation is an important factor to consider when studying disease (Uddin et al., 2013).

One study conducted looked at variations in DNMT3a expression in rat amygdala during development (Kolodkin & Auger, 2011). In this study, female and male rats were analyzed to look at sex-dependent mechanisms in development. Male and female rats were collected through rapid decapitation. DNMT mRNA and protein expression were measured and compared between males and females. Females expressed significantly higher levels of DNMT3a in the amygdala compared to males. Expression of DNMT3a and DNMT1 were then examined in female rats who were administered subcutaneous injections of either estradiol benzoate (EB), dihydrotestosterone (DHT), or oil (O). Injections were administered 24 hours after birth and collections were taken 48 hours after birth. Oil treated females expressed significantly higher rates of DNMT3a in the amygdala in comparison to EB or DHT treated rats. DNMT1 expression did not show any significant differences across the three injection groups. Overall, the evidence showed that female rats had significantly higher rates of DNMT3a in females compared to males within the first 24 hours after birth, but that this difference did was not

significant in older rats. The authors link this to hormonal changes having an impact on the expression levels of DNMT3a (Kolodkin & Auger, 2011).

Because DNMT3a has shown to have varied expression levels dependent upon sex, it is important to understand how this might impact future studies and reviews. Firstly, when comparing various studies on PTSD and the epigenome, many of the studies have all male subjects. This is in part since several studies involve combat veterans or military personnel, which are a majority male demographic. Therefore, when drawing comparison between studies, it is possible for methylation activity to vary for causes other than symptoms of depression or PTSD.

Timing of Trauma

When comparing studies on the epigenetic consequences of PTSD, it is important to examine not only the demographic of subjects, but the timing of when the traumatic events at hand have occurred within the subjects 'lives. Epidemiologic studies have shown that early childhood is a time of increased risk for negative life experiences to lead to development of psychiatric disorders like anxiety, major depression, and PTSD in adulthood (Heim & Nemeroff, 2001). For example, a study of around 2000 adult women reviewed history of trauma and neuropsychiatric diagnoses. Subjects who experienced a personal trauma like childhood sexual or physical abuse were much more likely to develop signs of depression and anxiety when compared to women who experienced the same traumatic event in adulthood (McCauley et al., 1997). To investigate how these early childhood traumatic exposures can cause neurobiological impacts, another study was done assessing the salivary cortisol concentrations of institutionalized infants in

Romania who experienced different types of early life stress at different developmental stages (Carlson & Earls, 1997). Subjects were between the ages of 2-9 months old and categorized into two groups, the intervention group and the control group. The intervention group had access to a caretaker whereas the control group were left in their standard living conditions. Studies revealed a correlation between age and salivary cortisol levels in the infants(Carlson & Earls, 1997). Younger children who experienced traumatic events had decreased levels of cortisol, indicating dysregulation in the HPA axis (Heim & Nemeroff, 2001). A similar study, previously discussed, on rhesus macaques studied this dysregulation of the APA axis (Barr et al., 2004). Those who were separated from their mothers and raised alongside their peers had lower basal levels of ACTH (leading to lower levels of cortisol) compared to those raised by their mothers.

The dysregulation in the HPA axis activity can be explained as the HPA axis develops from infancy to early adulthood (Agorastos et al., 2019). Along with the HPA axis, the amygdala and hippocampus develop during the same period. This implies that greater HPA axis plasticity during this period of life may lead to heightened vulnerability and therefore more dramatic symptoms in adulthood.

To examine the epigenetic etiology of the dysregulation of the HPA axis, a study was done examining the differences that timing of childhood maltreatment had on DNA methylation activity (Dunn et al., 2019). This study examined if trauma had time-dependent effects on DNA methylation. The authors discovered that the variability examined across studies on DNA methylation and trauma are strongly correlated with the

developmental stage of life when the trauma occurred as rather than accumulation of trauma encounters or recency to the exposure (Dunn et al., 2019).

Most recently, Zhange et al (2022) further studied this idea by investigating the reason why multiple studies have produced mixed results when studying trauma and NR3C1 DNA methylation. Several candidate genes associated with trauma were assessed, included NR3C1, encoding the glucocorticoid receptor. The authors concluded that while there were inconsistencies in DNA methylation across all candidate genes, NR3C1 DNA methylation levels were the exception. More specifically, NR3C1 DNA methylation levels at exon F1 were only consistent when comparing subjects of childhood trauma (Y. Zhang & Liu, 2022). This gives insight into the potential reason for the discrepancies previously discussed regarding DNA methylation of NR3C1 (Gladish et al., 2022). Yehuda et al. (2015) and van der Knaap (2014) both studied the effects of trauma on NR3C1 DNA methylation activity. However, one cohort consisted of combat soldiers who experienced trauma as adults, while the other cohort utilized the TRAILS (Tracking Adolescents 'Individual Lives) Survey whose subjects experienced trauma as children (van der Knaap et al., 2014; Yehuda et al., 2015). These changes to DNA methylation activity in NR3C1 could be correlated to the plasticity of the HPA axis during development.

It is important when conducting a meta-analysis of future studies, to understand that these differences might not imply inconclusive results, but rather point to different underlying mechanisms that occur based on point in development that the trauma occurred.

CONCLUSION

The study of the bi-directional relationship between epigentics and PTSD continues to rapidly develop. Though PTSD used to be viewed from a strictly physiological and psychological standpoint, utilizing genetic and epigenetic research will not only enable a greater understanding of the genetic etiology of PTSD, but also the potential for more optimal treatments for those suffering from the neuropsychiatric disorder. Testing for epigentic changes in a patient's genome can eventually be used as a supplement to current treatments and therapies (like Cognitive Behavioral Therapy) by helping assess a patient's progress via biological markers.

Lerhner & Yehuda (2014) write that once we have a more thorough understanding of the epigenetic consequences of PTSD, clinicians will be able to utilize genome testing as a tool for future diagnosis (Lehrner & Yehuda, 2014). Many patients with PTSD have a difficult time describing their symptoms and this could be a tool to help those who are struggling.

PTSD has been linked to having epigenetic impacts on genome loci that regulate cortisol, immunity, cardiovascular health, and overall acceleration of cellular aging. Though there have been some discrepancies in the literature, understanding the confounding factors that lead to mixed results will allow for more robust research methods in future work. This will enable not only more accurate testing, but replicable studies to ensure validity.

To better understand the relationship between specific gene loci (like NC3R1 and FKBP5) and PTSD, further research should be conducted on the tissue-specific

regulations of these regions. Furthermore, studies examining brain tissue in comparison to whole blood samples will provide a better understanding on which epigenetic aspects are ubiquitous and which are not. Studies should also continue to adjust for various population demographics, including by sex, race, and age at onset of traumatic event, as these factors have shown to cause variations in results.

Lastly, although research on transgenerational inheritance is fairly new in animal models, further research should be conducted on the relationship between ncRNAs and heritability in humans. This includes performing longitudinal studies on the impact of ncRNAs in those exposed to trauma who develop PTSD, those exposed to trauma who do not develop PTSD, and those not exposed to trauma. The more that is understood of the epigenetic mechanisms that change by environmental exposure, the more we can understand the true consequences of PTSD.

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