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DNA Methylation Correlates of PTSD: Recent Findings and Technical Challenges

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Abstract

There is increasing evidence that epigenetic factors play a critical role in posttraumatic stress disorder (PTSD), by mediating the impact of environmental exposures to trauma on the regulation of gene expression. DNA methylation is one epigenetic mark that has been highly studied in PTSD. This review will begin by providing an overview of DNA methylation (DNAm) methods, and will then highlight two major biological systems that have been identified in the epigenetic regulation in PTSD: (a) the immune system and (b) the stress response system. In addition to candidate gene approaches, we will review novel strategies to study epigenome-wide PTSD-related effects, including epigenome-wide algorithms that distill information from many loci into a single summary score (e.g., measures of “epigenetic age” which have been associated with PTSD). This review will also cover recent epigenome wide association studies (EWAS) of PTSD, and biological pathway models used to identify gene sets enriched in PTSD. Finally, we address technical and methodological advances and challenges to the field, and highlight exciting directions for future research.

1. Introduction

Posttraumatic stress disorder (PTSD) is a highly debilitating condition that is defined by reexperiencing of a traumatic event (e.g. nightmares and flashbacks), avoidance of cues and reminders linked to the trauma, negative alterations in cognition and mood (e.g. negative beliefs, anhedonia), and arousal and reactivity (e.g. aggression, irritability, startle, hypervigilance, and sleep disturbances) (American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 2013). Compared to other psychiatric disorders, PTSD is unique in that its development is by definition, a consequence of exposure to a

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traumatic event. Although experiencing a traumatic event is common, with estimates suggesting that 90% of individuals will experience a traumatic event within their lifetime (1), the prevalence of PTSD is much lower: approximately 7% of the general population (2), 23% of combat veterans of the recent conflicts in Iraq and Afghanistan (3), and up to 46% of individuals living in neighborhoods with high levels of violence (4). The discrepancy in the prevalence of trauma exposure compared to that of PTSD suggests that individual vulnerability and resilience to trauma are key factors in the development of the disorder. In line with this observation, studies of monozygotic and dizygotic twins suggest that 30–40% of the variance in PTSD is attributable to genetic factors (5–10), thus highlighting the genetic contribution to increasing risk for PTSD, and also indicating that environmental factors play a key role in its development and course. In fact, the largest genome wide association study (GWAS) to date of PTSD (N=20070) reported a molecular genetics-based heritability estimate of 29% for PTSD for European American females, and also reported overlapping genetic risk of PTSD with both schizophrenia and bipolar disorder (11).

There is mounting evidence that epigenetic processes play an important role in linking environmental exposures to basic biology, and given this, may be relevant to the etiology of psychiatric disorders (12–14), including PTSD. While a number of prior reviews have summarized epigenetic changes in PTSD (15–17), the goal of this review was to extend prior summaries by integrating findings into a broader perspective. Specifically, after briefly reviewing DNA methylation (DNAm) methods, we highlight two major biological processes that have been identified in epigenetic regulation in PTSD: (a) the immune system and (b) the stress response. We describe novel approaches to studying PTSD-related epigenetic effects that span the epigenome, ranging from biological pathway models to the use of epigenome-wide algorithms that distill information from many loci into a single summary score (e.g., measures of “epigenetic age,” which have been associated with PTSD). Finally, we address technical and methodological advances and challenges to the field, and highlight exciting directions for future research. Although this review will focus specifically on the role of DNAm in PTSD, there is evidence that other epigenetic mechanisms (e.g. histone modifications and non-coding RNAs) also play a role in PTSD (18) and we direct the reader to a number of excellent reviews and primary literature sources for more information (19–31). We focus on DNAm because it is the epigenetic mechanism that has been most studied in PTSD, likely due to its overall stability as an epigenetic mark and due to advances in the technology that facilitate its study and replication across groups (e.g. microarrays, discussed below).

1.1 DNA methylation Overview

DNA methylation refers to the process by which a methyl group is added to the carbon-5 position of cytosine residues of the dinucleotide CpG in DNA to form 5-methylcytosine (5mC). DNA methylation occurs most abundantly at cytosine-phosphate-guanine (CpG) sites. CpG islands were originally described by Gardiner-Garden and Frommer as being sequences in the genome that have G+C content greater than 50% for 200 base pairs or more, and observed CpG to expected CpG ratio of greater than or equal to 0.6 (32). This definition however, did not exclude most Alu-repeat-associated regions (some of which have high GC content). To account for these regions, the definition for CpG islands was further

modified to include the following requirements: 1) regions of DNA greater than 500 base pairs, 2) G+C content greater than or equal to 55%, and 3) ratio of observed CpG to expected CpG of 0.65 (33). More recently, Bock et al. created an epigenome prediction pipeline that incorporated epigenetic and functional states to generate quantitative scores of “CpG island strength” (34). Mapping CpG islands via epigenome prediction avoids the use of arbitrary threshold parameters and accounts for heterogeneity among CpG islands. CpG islands are most frequently unmethylated, and are typically present in the promoter regions of downstream genes. DNA methylation of a promoter region has been inversely correlated with the level of gene transcription, thus DNA methylation has been most typically associated with silencing gene expression (35, 36), however, this is not always the case (36, 37). In addition to CpG methylation, hydroxymethylation (hmC) is another modification that can occur at the carbon-5 position of cytosine residues (38), and studies have found this mark to be particularly abundant in brain tissues relative to other tissues (39, 40). More recent studies have suggested the 5mC and 5hmC are dynamic and reversible modifications (41, 42). An important, but as yet unanswered question for the field is whether different types of DNA modifications such as 5mC and 5hmC lead to differing transcriptional outcomes. Preliminary research has suggested that 5hmC is associated with increased gene transcription (43–45) and we direct the reader to prior reviews for additional information (46). Technical approaches employing assays based on bisulfite conversion (described below), which are most commonly used by the field, do not differentiate between these two modifications. Additionally, future studies are needed to determine the potential role of methylation at sites other than CpG dinucleotides. Other biological processes that regulate methylation and have been investigated in the context of trauma and stress include DNA methyltransferases (DNMTs) (47) (48), specifically DNMT1 (traditionally considered the primary maintenance methyltransferase) and DNMT3A and 3B (traditionally considered responsible for de novo methylation (49), and Tet methylcytosine dioxygenases (TETs), which sequentially oxidize methyl modifications and return the cytosine to an unmodified state (46).

1.2 Technical methods to assess DNAm

There are several approaches for studying epigenetic variation in samples. Affinity-based methods quantify and assess modified DNA from unmethylated DNA by immunoprecipitation (antibody based methods that recognize specific methylation marks such as 5mc or 5hmc); these approaches include chromatin immunoprecipitation sequencing (ChIP-Seq), methylated DNA immunoprecipitation (meDIP-Seq), methyl-binding domain sequencing (MBD-Seq), and variation on those techniques. Limitations to these approaches are that only methylation of discrete regions can be determined rather than individual CpG sites.

Bisulfite conversion has been a steadfast and widely-used method to assess base-specific methylation levels and works by preferential deamination of unmodified (unmethylated) cytosines to uracils; these uracils are then copied as thymines during subsequent amplifications (50, 51). During this process, methylated and hydroxymethylated cytosines are protected from deamination (and are not converted to uracil) and are thus identifiable during sequencing. The level of methylation is determined from the number of sequencing

reads (e.g. counting the number of cytosines and thymines) at a specific site. Due to the time intensiveness of these methods, these approaches only allow coverage of a small genomic region, and are thus primarily used to validate and gain a more in depth spatial resolution of methylation at a specific target identified using micro-array based methods. Whole-genome bisulfite sequencing (WGBS) has been introduced, however, these studies are also large undertakings and concerns remain over insufficient sequencing depth to adequately assess statistically significant differences between groups (at least 1 billion of 100 base pair reads are needed for ~30X coverage for WGBS) (52–54).

High-density microarray chips have been the primary technical approach used to assess DNAm. This approach relies on bisulfite conversion of sample DNA followed by hybridization to oligonucleotide probes on a microarray slide. Detection probes are complementary to bisulfite-converted fragments, which originally contained either unmethylated cytosines, or methylated/hydroxymethylated cytosines. Recent advances in BeadChip technology have led to the introduction of DNA methylation BeadChips which can inexpensively measure methylation at hundreds of thousands of genomic sites per sample, yielding an estimate of the proportion of DNA that is methylated across cells (referred to as the β) at each site. These BeadChips have become the tool of choice for large-scale studies of methylation in psychiatric disorder case-control samples, as in the studies reported in the sections below. Another method to quantitatively assess methylation of CG cytosines is bisulfite pyrosequencing, which is a “sequencing by synthesis” approach (55, 56). This technology relies on bisulfite conversion and PCR amplification of the region of interest, followed by the stepwise incorporation of deoxynucleotide triphosphates (dNTPs), and light detection (due to a chain reaction wherein pyrophosphate is released).

2. Advantages and disadvantages of candidate vs. epigenome-wide analytic approaches

Candidate gene studies refer to the use of prior knowledge and literature to identify genes of interest and investigate the contribution of their variation to a given disorder. These targeted studies have been criticized for increasing the risk of false positive results as a genome-wide level of statistical significance is generally not used to identify associated variants. However, they have contributed substantial knowledge concerning mechanistic links between the genome and psychiatric conditions (57–59). In contrast, genome-wide association (GWAS), or in the context of this review, epigenome-wide association studies (EWAS), take an unbiased approach towards identifying epigenetic variation in specific genes and their contribution to specific phenotypes or diagnoses; they traditionally set a much more stringent bar for significance (typically $p < 5 \times 10^{-8}$). Genome-wide studies, too, have been criticized for frequently producing results that are of a small magnitude of effect and that do not replicate across cohorts (60). Here we will review recent findings supporting a role for genes involved in: (a) the immune system; and (b) the hypothalamic pituitary adrenal (HPA) axis in the regulation of PTSD.

2.1 Candidate gene studies supporting immune system dysregulation in PTSD

A growing body of research suggests that trauma exposure and PTSD may be associated with epigenetic changes in genes related to inflammatory responses and the immune system (16, 61, 62) (Table 1). One of the most widely-studied markers of peripheral inflammation, *C-reactive protein* (CRP), has also been linked to PTSD in recent studies (63–66). In a candidate gene study focused on associations between peripheral CRP and PTSD in a sample of N=286 veterans with a high prevalence of PTSD, Miller et al. (67) found that PTSD severity was positively associated with CRP levels ($P=0.004$), and negatively associated with DNAm at *cg10636246* in the *Absent in Melanoma 2* (*AIM2*) gene ($P=0.009$). *AIM2* is an inflammasome receptor for double stranded DNA activating cascades that has been previously implicated in host defense mechanisms against pathogens in the innate immune response, and has been implicated in other diseases (68). This locus has also previously been linked to CRP levels through results from a large-scale epigenome-wide association study (69). Miller et al. further showed that the relationship between current PTSD severity and serum CRP was statistically mediated by the methylation levels at that locus ($P=0.017$), suggesting that PTSD may be related to increased inflammatory markers, specifically CRP, by way of altered DNAm at *AIM2*, although the causal direction of this association has not been tested.

Several additional candidate gene studies have supported associations between PTSD diagnosis and methylation changes in immune-related genes (70–74). In a longitudinal study of N=150 U.S. military service members pre- and post-deployment, serum methylation levels (%5-mC) of the gene encoding interleukin 18 (IL18) and of the maternally imprinted long non-coding RNA transcript H19 were increased post-deployment in individuals who developed PTSD (for IL18, cases: 1.39%, controls: -3.83%, $p=0.01$; for H19, cases: +0.57%, controls: -1.97%, $p=0.04$) (28). Differences in methylation for H19 were found to be driven primarily by significant decreases in %5-mC for control cases, while the patterns for IL18 were driven by both the decrease in %5-mC for controls, as well as the increase in %5-mC for cases. Together, these studies point toward an important role of the immune system and its regulation by epigenetic processes in PTSD. Stress and trauma induced activation of the immune system results in the peripheral release of cytokines and other immune-related molecules (66, 71), which have the potential to enter the central nervous system (CNS) and influence circuits regulating mood and behavior (75). Furthermore, the chronic inflammatory state accompanying PTSD may influence epigenetic modifications of genes within this system.

2.2 Candidate gene studies investigating epigenetic regulation of the HPA axis in PTSD

Dysregulated HPA axis signaling has been widely implicated in PTSD and other trauma and stress-related disorders (76–78). Although not without controversy, the literature suggests that PTSD is associated with suppressed cortisol responses in dexamethasone suppression tests, reflecting increased negative feedback of the HPA axis (79–81). These effects are thought to be regulated by the glucocorticoid receptor (GR; encoded by the gene nuclear receptor subfamily 3 group C member 1, *NR3C1*), which mediates negative feedback of the HPA axis, and in chronic PTSD, may act to suppress stress-related release of cortisol. A number of studies have shown decreased methylation levels of *NR3C1* promoter regions in

individuals with PTSD compared to those without PTSD (82–84). Furthermore, methylation of the *NR3C1* exon 1F promoter regions has been reported to be inversely correlated with PTSD symptom severity and dexamethasone suppression of cortisol. Increased methylation of this region has also been associated with improvements in PTSD symptoms in response to prolonged exposure therapy (85). However, there have also been contradictory findings reporting increased *NR3C1* exon 1F methylation in PTSD (86), highlighting the complexity of this system and the need for further study. For example, a recent longitudinal study investigated methylation of *NR3C1* exon 1F prior to and following a 4-month military deployment in N=92 male subjects and demonstrated an association of post-deployment mental health problems with increases in methylation at loci associated with *NR3C1* exon 1F expression (87).

Another gene that has been reported to play a key role in stress-related disorders is the gene encoding the steroid receptor chaperone FK506 binding protein 51 (FKBP51), *FKBP5* (88–92). FKBP51 is a co-chaperone of the glucocorticoid receptor that regulates GR sensitivity by creating an intracellular ultra-short negative feedback loop (93, 94). Prior work has shown that polymorphisms in *FKBP5* may interact with early childhood trauma to predict PTSD, suicide attempts, and major depression in adulthood (57, 95–99). Additionally, Klengel et al. (58) showed that allele-specific, childhood trauma-dependent regulation of *FKBP5* was associated with increased risk for adulthood stress-related psychiatric disorders. CpG sites located near glucocorticoid response elements (GREs) were reported to be selectively demethylated in risk allele carriers with childhood but not adult trauma. In *in vitro* studies using human progenitor hippocampal neuronal cells, DNA methylation changes were also observed after glucocorticoid exposure.

Recent studies have also reported epigenetic regulation of the stress-responsive gene encoding pituitary adenylate cyclase-activating polypeptide (PACAP, encoded by the gene *ADCYAP1*) in PTSD (59, 100–104). Ressler et al. (59) reported that PACAP levels in peripheral blood were associated with PTSD diagnosis and symptoms, but only in women with high levels of trauma. Methylation of the gene encoding the receptor for PACAP (*ADCYAP1R1*) was associated with PTSD symptoms in both men and women (N=107, P<0.0005). Follow up experiments in rodent models reported increases in *ADCYAP1R1* transcription following fear conditioning.

Finally, PTSD symptom severity has also been positively associated with blood methylation levels of the spindle and kinetochore associated protein 2 (*SKA2*) gene, which plays a role in glucocorticoid receptor transactivation (105) and was previously identified as a biomarker for suicide (106). Increased *SKA2* methylation has been associated with PTSD symptoms, and with decreased cortisol stress reactivity (107). Furthermore, DNA methylation at the *SKA2* locus cg13989295 was associated with bilateral reductions in cortical thickness of frontal regions, and PTSD symptom severity was negatively correlated with cortical thickness in identified regions, potentially via positive associations with *SKA2* DNA methylation (108).

3. Epigenome-wide studies of PTSD

3.1 Epigenome-wide association studies (EWAS) studies of PTSD

Genome-wide DNAm studies have identified novel targets for future investigation. To date, there have been 3 EWAS studies of PTSD using the early HumanMethylation27 BeadChip (27K) (109–111) and 6 EWAS studies of PTSD using either the EPIC (850K) or 450K BeadChip methylation microarrays (112–117). Uddin et al., (109, 111) and Smith et al., (110) were among the first genome-wide DNAm studies of PTSD; both these studies used the HumanMethylation27 BeadChip methylation microarray to assay ~27,000 CpG sites across the genome (~14,000 genes). Specifically, using data from a sample of N=100 trauma-exposed participants (n=23 PTSD-affected and n=77 PTSD-unaffected) from an urban community sample, Uddin et al. (109) examined a limited set of genomic loci and classified methylation probes as unmethylated if they had beta values <0.2, or methylated if they had beta values >0.8. There was no significant difference in the number of uniquely methylated genes overall between PTSD-affected and –unaffected individuals. With respect to the number of uniquely methylated genes, there was a significant difference between PTSD-affected and –unaffected individuals ($P<0.0001$). Using functional annotation clustering to query the biological relevance of these uniquely methylated and unmethylated genes, the authors found that immune system related genes figured prominently in the functional annotation clusters from uniquely unmethylated genes in PTSD-affected individuals. The investigators also reported a positive association between PTSD and elevated antibody levels to cytomegalovirus (CMV), suggesting compromised immune reactivity. In a follow up study, Uddin et al., (111) investigated whether socioeconomic position (SEP) moderates the relationship between gene methylation profiles and PTSD (both lifetime diagnosis and symptom severity) in a sample of N=100. When assessing either PTSD lifetime diagnosis or symptom severity, the authors reported significant interactions of methylation x SEP (uncorrected $P<0.01$) for 119 and 55 CpG sites, respectively, associated with genes related to nervous system functioning. In another study, Smith et al. (110) found CpG sites in five genes (translocated promoter region, *TPR*, $P=1.9 \times 10^{-6}$; annexin A2, *ANXA2* $P=9.3 \times 10^{-6}$; C-type lectin domain family 9, member A, *CLEC9A* $P=4.3 \times 10^{-6}$; acid phosphatase 5, tartrate resistant *ACP5*, $P=8.0 \times 10^{-6}$; toll-like receptor 8, *TLR8* $P=1.1 \times 10^{-5}$) that were differentially methylated in PTSD cases versus controls after correcting for experiment-wide multiple-testing in a sample of N=110. Methylation of one CpG site located near neuropeptide FF receptor 2 (*NPFFR2*) was inversely associated with total life stress (TLS) scores ($P=6.6 \times 10^{-7}$). *TLR8*, *ACP5*, and *NPFFR2*, have all been linked in independent studies to inflammatory processes (118–125). In a subset of the above cohort (n=51) along with an additional n=126 independent subjects (total N=177), the authors also investigated blood-based inflammatory biomarkers and found decreased levels of plasma IL4 in individuals with PTSD (adjusting for history of suicide attempts), and increased levels of plasma TNFa associated with history of child abuse and increased TLS scores (adjusting for history of substance abuse).

In a sample of N=169, Mehta et al. (117) used the 450K BeadChip to investigate gene expression and DNAm profiles in individuals with PTSD (with and without histories of childhood abuse) compared to trauma exposed controls. Gene expression profiles were

almost completely nonoverlapping (overlap 2%) between PTSD patients with and without histories of childhood abuse. Furthermore, changes in gene expression were more associated with changes in DNAm in the same gene in the PTSD group with histories of child abuse (69% of cases) compared to the PTSD group without histories of child abuse (34% of cases). In a sample of Australian Vietnam War veterans (N=96), Mehta et al. (115) used the Illumina EPIC chip and identified 5 CpG sites spanning the genes BR serine/threonine kinase 1 (*BRSK1*), nerve growth factor (*NGF*), lipocalin 8 (*LCN8*), and dedicator of cytokinesis 2 (*DOCK2*), whose methylation was significantly associated with PTSD symptom severity; only the *DOCK2* finding was replicated in an independent cohort (N=115 males from a community cohort that was part of the Grady Trauma Project, using the 450K BeadChip) (115). *DOCK2* has been previously implicated in studies of Alzheimer's disease pathology and neuroinflammation (126), and it is expressed in the brain, cells of the immune system (127) and some cancer cells (128). In another recent EWAS study, Rutten et al. (116) investigated genome-wide blood DNAm in a cohort of N=93 subjects 1 month prior to- and 6 months post-deployment using the 450K BeadChip. The authors reported that the emergence of PTSD symptoms over time was significantly associated with DNAm alterations at 17 genomic positions (termed "differentially methylated positions", or "DMPs") and 12 genomic regions (termed "differentially methylated regions", "DMRs"). Significant associations were further investigated in a replication cohort of N=98 male US Marines, and findings of decreased DNAm at *ZFP57*, *RNF39*, and *HIST1H2APS2* were replicated. *ZFP57* encodes zinc-finger protein 57 and plays a role in genomic imprinting in stem cells during development and adulthood (129, 130), and *RNF39* encodes ring finger protein 39, which plays a role in synaptic plasticity and long-term potentiation (131) and has been previously implicated in GWASs of schizophrenia (132), anger (133), and risk for major psychiatric disorders (134). Other EWASs studies of PTSD have found no significant epigenome-wide associations (112, 114), or have used pathway approaches to data analysis, as described below. As well, Chen et al., (112) investigated DNAm (using the 450K BeadChip) in n=12 participants with PTSD and n=12 trauma exposed control participants, and found no corrected significant effects. Kuan et al., (114) investigated DNAm (using the 450K BeadChip) associated with PTSD and MDD in a sample of N=473 individuals who were exposed to the September 11th, 2011 World Trade Center disaster, and found no corrected significant epigenome-wide associations for PTSD or major depressive disorder (MDD).

3.2 Gene network approaches in the study of PTSD

Recent studies have taken "knowledge-driven data-mining" approaches such as gene set enrichment analyses (GSEAs) and functional network analyses to further probe available data sets that may be limited by small sample sizes, or that have not identified epigenome-wide significant associations. GSEA and functional network analyses use computational methods to determine whether sets of genes show statistically significant differences between phenotypic groups. For example, in additional analyses to the above-described Mehta et al. study (115), the authors investigated PTSD-associated biological pathways by examining broader patterns of DNAm enrichment among their top results. Using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database via the Webgestalt interface (135), the authors reported that their significantly associated genes were enriched for pathways

related to regulation of actin cytoskeleton and focal adhesion pathway in individuals with PTSD (enrichment evaluation analyses used a hyper geometric test and Bonferroni-adjusted P -value of 0.01). Furthermore, although the Kuan et al., (96) study did not report any significant epigenome-wide associations for PTSD, using pathway and gene ontology based approaches (using the *gometh* function in the Bioconductor package *missMethyl* (136) and reporting gene sets significant at FDR 0.05), significant enrichment was observed for a number of KEGG pathways (top KEGG pathways included oxytocin and MAPK signaling, insulin resistance, cholinergic synapse and inflammatory bowel disease pathways) (114). Similarly, the above-described Rutten et al. study (98) also reported enrichment of several gene pathways relevant to PTSD (including, but not limited to, pathways involved in circadian rhythm, IL17 signaling, dopaminergic and serotonergic signaling, Wnt signaling, sleep regulation, and complement activation). How these identified pathways play a role in PTSD remains an open question for future investigation.

In another genome-wide DNAm study in whole blood, Hammamieh et al. (113) investigated epigenetic functional networks of PTSD in a training set of trauma-exposed combat veterans with and without PTSD (48 PTSD+/51 PTSD-) and identified approximately 2800 differentially methylated genes (DMGs), of which 84.5% were hypermethylated in cases with PTSD (using the Agilent whole genome array containing ~27K CpGs). Forty-two of these DMGs were selected from the initial training set (based on methylation status and implication in PTSD), and methylation was verified using targeted bisulfite sequencing. The authors validated the identified networks in a follow-up test set (31 PTSD+/28 PTSD-), as well as with a combined training and testing dataset run on a distinct methylation platform, the Illumina 450K BeadChip. For DMG analyses, $p < 0.05$ used to screen for differentially methylated genes, and Bonferroni-adjusted genome-wide significance was set at $p < 1.16e - 07$. For functional network analyses, ClueGo v2.1.2 and Ingenuity pathway analysis were used for network construction, with significant pathways meeting a cutoff of $p < 0.05$. Consistent with the altered patterns of immune activation highlighted in the studies above, innate-immunity associated genes accounted for 60% of the differentially methylated genes in individuals with PTSD. Additional networks identified included those relevant to aging, neurogenic functional pathways, and HPA axis (i.e., stress responding) functions and the synthesis of its key regulators. Approaches such as GSEA and those described above offer unique methods to further probe existing data sets that may be otherwise limited by sample size; they offer the potential to identify novel gene candidate sets and pathways for further research, potentially combining the strengths of epigenome-wide investigations with subsequent candidate gene studies. However, caution must be taken to avoid over-interpreting these findings without mechanistic follow-up, validation, and replication, especially as these studies can be subject to biases (137).

In total, there is broad evidence for the role of inflammation and HPA axis signaling in PTSD. Nonetheless, key challenges to both gene-targeted and epigenome-wide studies have been the lack of replication of studies, and findings that have not consistently implicated a primary set of PTSD risk loci. Ongoing efforts to increase sample sizes and work with consortia to combine data sets and increase sample size and variability are steps that will likely yield new insights.

3.3 Epigenome-wide DNAm based indices of cellular aging: the epigenetic clock in PTSD

3.3.1 Overview of DNAm indices of cellular age—In addition to evaluation of broad epigenetic pathways and differentially methylated regions in PTSD, another approach for evaluating epigenome-wide data in association with PTSD is the use of DNAm scores that combine methylation levels at select loci across the epigenome into a single, weighted index. The most extensively studied example of this is the use of DNAm data to examine associations between cellular aging and PTSD. In particular, DNAm levels at select loci from across the epigenome can be combined to form a weighted index based on the strength of their associations with chronological age (138, 139) and then used to accurately predict chronological age in independent datasets. One of these indices, the Horvath algorithm, includes 353 DNAm loci that together are correlated with chronological age at $r = .96$ across multiple human tissue types. A second index, the Hannum algorithm, was developed in whole blood and includes 71 loci that, when combined, are also associated with chronological age at $r = .96$. Despite these strong associations with chronological age, some individuals have over-predicted age (i.e., DNAm age exceeds chronological age) while others have under-predicted age (i.e., DNAm age is less than chronological age) and this discrepancy can be used to index accelerated versus decelerated cellular age, respectively. These indices of advanced cellular age are meaningful because they have been associated with shortened time until death (140), and a variety of pathological conditions including cancer (141, 142), and Alzheimer's cognitive impairment (143).

3.3.2 DNAm age studies of PTSD—A number of studies have suggested that traumatic stress is associated with advanced cellular age in the epigenome relative to chronological age and this may link trauma-related psychopathology with premature onset of age-related medical conditions (144). Zannas et al. (145) and Jovanovic et al. (146) reported that life stress and childhood exposure to violence, respectively, were associated with advanced Horvath DNAm age among adults, and children, respectively. In contrast, Boks et al. (147) suggested that increasing PTSD symptoms from pre-to-post combat deployment was associated with decreasing Horvath DNAm age (and, similarly, with lengthened telomeres over the same time period). Wolf et al., (148, 149) did not observe associations between trauma exposure or PTSD and advanced Horvath DNAm age, but did report associations between PTSD symptoms (and specifically, hyperarousal symptoms) and advanced Hannum DNAm age among military veterans. In an effort to address this variability in results across studies, Wolf et al. (150) conducted a meta-analysis across nine cohorts contributing to the Psychiatric Genomics Consortium PTSD Workgroup and found that childhood trauma and lifetime PTSD symptom severity were associated with advanced Hannum (but not Horvath) DNAm age. The studies reviewed thus far were all cross-sectional in design, thus the direction of the association between traumatic stress and advanced DNAm age was not clear. In a more recent study, Wolf et al. (151) reported that post-trauma alcohol-use disorders and PTSD avoidance and numbing symptoms predicted an increasing pace of the Horvath epigenetic clock over the course of two years, raising the possibility of a causal link between psychiatric symptoms and accelerated epigenetic aging.

3.3.3 Future directions for DNAm age studies—One criticism leveled at DNAm age studies is that the strong association between chronological age and DNAm age yields

little remaining variance for use in evaluating accelerated or decelerated aging, and this may account for small effect sizes with psychiatric symptoms and inconsistent effects across studies. To address this concern, Levine et al. (152) recently developed a new DNAm algorithm to predict advanced cellular age (as opposed to predicting chronological age). The authors referred to this metric as “DNAm PhenoAge.” To develop this index, they first developed a measure of “phenotypic age” by identifying biological variables that predicted mortality over a specified period of time (23 years in the discovery sample, 12 years in the validation sample). The strongest predictors were chronological age, and blood-based markers of albumin, creatinine, glucose, C-reactive protein, lymphocytes, mean cell volume, red cell distribution width, alkaline phosphatase, and white blood cells. Then, these variables that predicted mortality were combined into a weighted score for use as a new phenotype and epigenome-wide DNAm values were examined as predictors of this new variable. Only DNAm loci included on all three Illumina BeadChips (e.g., the EPIC, 450k, and 27k) were considered so that the algorithm could be applicable to multiple BeadChip platforms. This analysis identified 513 DNAm loci that predicted phenotypic age and these were combined into a new weighted epigenetic score and tested in independent datasets as predictors of a variety of other age-related health conditions (e.g., immune markers, disease prevalence, mortality), controlling for chronological age. Levine et al. found that the new epigenetic DNAm PhenoAge calculator out-performed both the Hannum and Horvath advanced DNAm age metrics in association with 10- and 20-year mortality risk. The Levine DNAm PhenoAge index also showed increased DNAm age in post-mortem brain tissue in individuals diagnosed with Alzheimer’s disease compared to controls. Socioeconomic status was negatively related to DNAm PhenoAge, adjusting for chronological age (152), raising the possibility that education and income-related disparities in health outcomes may also be reflected in the epigenome. Levine et al. also conducted a variety of analyses to examine the impact of advanced DNAm PhenoAge on gene expression. Results suggested that advanced DNAm PhenoAge was correlated with changes in gene expression in blood and that the pattern of expression changes was generally consistent with expression patterns known to be associated with age. However, DNAm PhenoAge-related expression changes were more extreme (e.g., more upregulated or downregulated) than what would be expected based on age alone. This suggests that advanced DNAm PhenoAge may represent an acceleration of the pace of biological aging in the epigenome, rather than a distinct pathological process. Further, analysis of expression data suggested enrichment of pro-inflammatory pathways in the genes that were over-expressed in association with advanced DNAm PhenoAge.

This new epigenetic biomarker has yet to be evaluated in independent research groups, but it holds substantial promise for understanding the biology of advanced cellular age and its link to morbidity and mortality, for identifying the environmental and psychological factors that accelerate cellular aging, for tracking changes in cellular age over time, and for testing interventions that might normalize cellular aging and thereby contribute to extended years of healthy, disease-free living. These studies also highlight a unique approach to managing epigenome-wide DNAm data via the use of a weighted index score that moves beyond the limitations of candidate gene studies but also reduces epigenome-wide data down to a single scale, preserving information from across the genome without raising the risk of multiple-testing-related false positives. We may someday develop other DNAm algorithms to serve as

epigenome markers of other important variables (e.g., obesity,) relevant to PTSD, or to develop an epigenetic score for PTSD itself.

4. Technical limitations of DNAm studies

Many of the EWAS and epigenetic clock studies reviewed above have been performed using the Illumina Infinium HumanMethylation450 BeadChip (450K), which assesses DNA methylation at nearly 500,000 sites. More recently, this has been supplanted by the Illumina MethylationEPIC BeadChip (EPIC), which assesses methylation at nearly 850,000 sites. The EPIC chip includes 90% of sites assessed by the 450K chip, and excludes sites from the 450K chip that were deemed poorly performing. Early assessments of the performance of the EPIC BeadChips focused on the correlation across sites for a single sample assessed twice, once on the 450K chip and once on the EPIC chip (153–155). These studies found that this correlation was uniformly high ($r > 0.99$ for most samples examined). However, a recent study went further and examined the correlation for specific sites that had been measured on both chips in 145 whole-blood samples (156). The authors found that the per-site correlation varied substantially, but was low for the majority of sites assessed ($r < 0.20$ for 55.6% of sites). The authors attributed the low correlation to the low variability of a large proportion of the sites measured using the EPIC chips in blood samples. That is, most low correlation sites were nearly entirely methylated or entirely demethylated in the blood samples (95.8% of sites with $r < 0.2$ had median $\beta < 0.05$ or median $\beta > 0.95$). This finding should not be considered a shortcoming of EPIC chip design, based on the idea that sites will differ by tissue, and studies of other tissue types are performed with these chips. Instead, this finding should be considered an opportunity, as it raises the possibility of a similarly performing, but lower cost BeadChip which focuses its assessment on sites that are variable in a particular tissue, providing lower per-sample cost without compromising the amount of quality information obtained. Overall, these chip-based approaches are advantageous due to their relatively low cost per sample, straightforward workflow, and amenability to high throughput analyses of large numbers of samples. At the same time, the primary limitations of these approaches should be highlighted: arrays only assess CG sites, and fewer sites are covered compared to whole genome bisulfite sequencing approaches.

5. Future directions

5.1 Increasing sample sizes and decreasing variability across studies: Consortia-lead efforts

The literature reviewed to date makes it clear that large consortium studies and a common analysis pipeline are necessary to produce reliable genetic and epigenetic associations for psychiatric disorders in general (157) and PTSD in particular (158). Consortia provide an opportunity for this but also face challenges due to issues such as population stratification (i.e., ancestry), as well as variability in DNAm methods (for example, chip variability, the position on the chip to which each sample is assigned (159), and the heterogeneity in the mixture of cell types that make up a sample (160, 161). Recently, Ratanatharathorn et al. (162) presented the results of the development and validation of a quality control (QC) and analysis pipeline that can be used to reliably remove batch effects and aggregate information

across cohorts to perform large-scale methylation studies of PTSD. Using 450K BeadChip data from seven cohorts (total n of over 1,100 samples), this study compared alternate data cleaning, normalization, and analysis procedures with particular attention to reliably removing effects of ancestry within cohorts, preserving a suitable genome-wide distribution of p -values under the null (genome wide inflation), and eliminating technical artifacts. As a positive control, they assessed the degree to which methylation effects due to age were reliably observed across cohorts. The final Psychiatric Genetics Consortium (PGC)-PTSD EWAS pipeline included a normalization step using the Beta Mixture Quantile (BMIQ) method (163) and removal of batch effects based on the ComBat procedure (159). The pipeline analysis was performed using the limma package (164). Possible population stratification was addressed by including principal components for ancestry as covariates, either computed from genome-wide SNP data when available, or calculated from the methylation data at sites that have nearby or overlapping SNPs (165). As blood is a heterogeneous tissue, the analysis also incorporated covariates for the proportion of different types of white blood cells (CD8, CD4, NK, etc.) which were estimated from the methylation data itself (166). This pipeline was shown to control for genome-wide deflation and inflation of p -values better than a Functional Normalization (Funnorm) QC pipeline (167) and additionally was shown to consistently (across cohorts) detect many age-associated methylation sites across the genome. The utilization of a consistent QC and analysis pipeline across participating studies will increase the likelihood that robust and replicable associations are generated in EWAS studies of PTSD.

5.2 Postmortem human brain approaches

The majority of the studies reviewed above were performed in mixed tissues such as peripheral blood and buccal cells, based on their accessibility for molecular investigation. While system level approaches using postmortem tissue have shown promising results for other psychiatric disorders (168), few studies have investigated epigenetic variation in postmortem human brain tissue in PTSD due to the relative lack of availability of brain tissue from individuals with histories of PTSD. The need for postmortem studies of PTSD is evident (169), and a number of recent studies have investigated gene expression changes in postmortem tissue samples from individuals with PTSD and reported differential regulation of HPA axis and immune-related genes (170–173, 196). To our knowledge, no studies thus far have investigated epigenetic variation in postmortem brain samples of individuals with PTSD relative to control samples. The VA National PTSD Brain Bank was recently established (174) in response to this identified gap in PTSD research. The psychiatric disorders and conditions described here are fundamentally brain disorders, and thus future investigations using postmortem brain samples are critical. Additionally, future studies are needed to understand how changes in the periphery (e.g. from blood and saliva) relate to and reflect changes in the brain; such an understanding will be important for understanding the pathophysiology of PTSD, and for identifying accessible biomarkers. Recent studies suggest that some signaling pathways are common across tissues (175), such as GR activation. Stress activation of the HPA axis results in an increase in cortisol, and there are both tissue-specific and tissue-wide GR binding sites that have been described (176). Prior studies have also suggested changes in DNA methylation in NGF1-A binding sites in both postmortem hippocampus and peripheral blood (77, 177). Future work is needed to understand the extent

to which changes in peripheral tissues reflect changes in the brain. In addition to postmortem studies, other approaches that may bridge this gap include generating neuronal cells using induced pluripotent stem cells (iPSCs) or brain organoids (178), both of which are exciting avenues for future research that allow for alternate access to neuronal tissue.

5.3 Cell-type specific approaches

Another technical challenge relevant for peripheral and postmortem brain studies is that cell tissues are a heterogeneous mixture of differing cell types and DNAm levels may differ by specific cell type and a PTSD-associated signal may be cell-type specific. One new method to address this heterogeneity is to profile individual neurons using single-cell RNA sequencing approaches (179, 180). These methods offer the potential to better characterize the contributions of cell-type specific populations to specific phenotypes and disorders. Studies have demonstrated feasibility of assessing methylation patterns from single cells (161–168). Moving forward, single cell based approaches will likely provide tremendous insight and advances to the field.

5.4 Longitudinal studies

Methylation changes are dynamic and thus longitudinal studies are needed to study how epigenetic factors may change across the lifespan, following trauma, and following treatment interventions. The need for longitudinal studies also extends to the need for examination of distinct developmental critical periods and how trauma exposure during specific sensitive periods of development is associated with epigenetic changes (117). Foundational studies of maternal stress in rodent models (117, 181) and studies of stress during human pregnancy in humans (182) suggest that in utero developmental periods are sensitive to epigenetic programming (183). Furthermore, a number of intriguing recent studies have also suggested that stress and trauma exposure in a parental generation, prior to conception, may be transmitted to subsequent generations via epigenetic mechanisms in the germline (184–188). These studies require additional investigation and replication, but nonetheless are important considerations for understanding and extending human studies of PTSD.

5.5 Potential methylation-based treatment approaches

The dynamic and reversible nature of epigenetic modifications due to environmental factors raises the exciting possibility of directly or indirectly manipulating these marks to attenuate psychiatric symptoms. Antidepressants and drugs such as valproic acid are known to possess epigenetic effects (189), and endogenous enzymes such as DNMTs, histone acetyl transferases (HATs), and histone deacetylases (HDACs) are intriguing agents based on their ability to manipulate epigenetic marks. However, the targeting of specific epigenetic modifications within the genome remains a challenging undertaking due to lack of clarity regarding how particular cell types and brain regions contribute to psychopathology, and the difficulty in predicting directionality in functional outcomes and gene transcription. Furthermore, many drugs targeting epigenetic processes have substantial side effects resulting from non-selective modes of action. Other technological advances that could allow for more targeted manipulation of epigenetic marks in a temporally and spatially controlled manner include the RNA-guided clustered regularly interspaced short palindromic repeats

(CRISPR/Cas9) systems, and protein-guided transcriptional activator like effectors (TALEs) (190, 191). In the case of CRISPR/Cas9, recent advances have been made in fusing epigenome-modifying proteins to the Cas9 protein, thus allowing for manipulation of epigenetic marks at specific locations in the genome (192). Of note, a high level of caution must be exerted with respect to implementing these approaches in a clinical setting; many drugs targeting epigenetic processes have shown undesirable side effects resulting from non-selective modes of action, as well as off-target effects.

Advances in precision medicine approaches and methods used to study pharmacogenetics (193) could similarly be applied to the study of epigenetic factors. Epigenetic modifications could be used to predict and better monitor therapeutic interventions (85, 194, 195). One example of such an approach is in work by Powell et al., in which DNA methylation of the cytokine IL11 was associated with antidepressant treatment response (195). Epigenetic and genetic profiling prior to treatment could be used to identify how an individual would respond to a particular intervention, and thus reduce the chances of treatment failure and give treatment providers a more informed method to prescribe a treatment course.

6. Conclusion

Increasing evidence suggests that epigenetic mechanisms play an important role in the pathophysiology of PTSD and other stress-related disorders. A better understanding of these epigenetic mechanisms will provide insight into these disorders, and will pave the way for biomarker development and the identification of novel treatment interventions. Candidate gene as well as epigenome wide studies have highlighted the contribution of genes involved in the HPA axis and immune system in PTSD. We have also highlighted the use of DNAm age scores; weighted indices derived from methylation levels at specific loci across the epigenome that have been used to investigate cellular age in trauma-related disorders. Consortia-lead efforts such as the PGC's PTSD workgroup, are currently working to bring together multiple investigators and data sets. Such efforts will not only increase sample size, but will also increase consistency in data collection and analysis processing pipelines. A crucial next step for novel gene targets that meet genome wide statistical significance will be to translate findings to animal models for further manipulation and mechanistic investigation. Finally, follow-up studies should include parallel approaches using neuroimaging genetics in humans, and postmortem human brain bank analyses using recent brain bank initiatives.

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Abbreviations:

5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine

ACP5	Acid phosphatase 5, tartrate resistant
AIM2	Absent in Melanoma 2
ANXA2	Annexin A2
BRSK1	BR serine/threonine kinase 1
ChIP-Seq	Chromatin immunoprecipitation sequencing
CLEC9A	C-type lectin domain family 9, member A
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CRISPR	clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
DMG	Differentially methylated gene
DMP	Differentially methylated positions
DMR	Differentially methylated regions
DNA_m	DNA methylation
DNMT	DNA methyltransferases
dNTP	Deoxynucleotide triphosphates
DOCK2	Dedicator of cytokinesis 2
EWAS	Epigenome wide association study
FKBP5	FK506 binding protein 51
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
GSEA	Gene set enrichment analyses
GWAS	Genome wide association study
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HPA	Hypothalamic pituitary adrenal axis
HPA	Hypothalamic pituitary adrenal
IL	Interleukin
iPSCs	Induced pluripotent stem cells

KEGG	Kyoto Encyclopedia of Genes and Genomes
LCN8	Lipocalin 8
MBD-Seq	Methyl-binding domain sequencing
MDD	Major depressive disorder
meDIP-Seq	Methylated DNA immunoprecipitation
NGF	Nerve growth factor
NPFFR2	Neuropeptide FF receptor 2
NR3C1	Nuclear receptor subfamily 3 group C member 1
PACAP	Pituitary adenylate cyclase-activating polypeptide
PGC	Psychiatric Genetics Consortium
PTSD	Posttraumatic stress disorder
QC	Quality control
RNF39	Ring finger protein 39
SKA2	Spindle and kinetochore associated protein 2
SNP	Single nucleotide polymorphism
TALE	Transcriptional activator like effectors
TET	Tet methylcytosine dioxygenases
TLR8	Toll-like receptor 8
TLS	Total life stress
TPR	Translocated promoter region
WGBS	Whole-genome bisulfite sequencing
ZFP57	Zinc-finger protein 57

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Highlights:

- There is mounting evidence that epigenetic processes play an important role in PTSD.
- We will highlight the role immune system and the stress response in the epigenetic regulation in PTSD.
- Approaches for studying PTSD-related epigenetic effects that span the epigenome are reviewed.
- Technical and methodological advances and challenges to the field, and directions for future research are addressed.

Table 1.

Selected Human Studies Focused on Epigenetics and Immune Dysregulation in PTSD

Reference	Sample size (N)	Approach	Method of DNA Methylation Investigation	Identified genes	P-value (Cases vs. Controls)	Summary of Findings
Uddin et al., 2010	100	Epigenome-wide	Bisulfite, Infinium HumanMethylation 27K BeadChip (Illumina, San Diego, CA)	Genes related to inflammatory and immune response	Methylation probes classified as unmethylated if they had beta values <0.2; classified as methylated if they had beta values >0.8	Significant difference between PTSD-affected and-unaffected individuals ($P<0.0001$) for the number of uniquely methylated genes immune system; genes figured prominently in the functional annotation clusters from uniquely unmethylated genes in PTSD-affected individuals.
Smith et al., 2011	110	Epigenome-wide	Bisulfite, Infinium HumanMethylation 27K BeadChip	TPR (cg24577137) CLEC9A (cg20098659) APC5 (cg07967308) ANXA2 (cg08081036) TLR8 (cg07759587)	1.9E-06 4.3E-06 8.0E-06 9.3E-06 1.1E-05	Subjects with PTSD had increased global methylation and differential methylation of genes related to Inflammation. (Analysis cut-off: $FDR<0.05$)
Rusiecki et al., 2013	150	Candidate gene	Bisulfite and pyrosequencing	H19 IL18	4.0E-02 1.0E-02	Post-deployment, IL18 methylation was increased in individuals who developed PTSD, and H19 and IL18 methylation was decreased in those who did not develop PTSD
Kuan et al., 2017	473	Epigenome-wide	Human Methylation 450K BeadChip (Illumina, San Diego, CA)	ZDHHC11 (cg05693864) CSMD2 (cg06182923) COL9A3 (cg08696494) Intergenic (cg25664402) PDCCD6IP (cg05569176) TBC1D24 (cg09370982) FAM164A (cg07654569)	1.73E-06 4.73E-05 5.39E-05 5.80E-05 7.82E-05 8.97E-05 9.91E-05	No corrected significant epigenome-wide associations for PTSD. Significant enrichment was observed for a number of KEGG pathways (including oxytocin and MAPK signaling, insulin resistance, cholinergic synapse and inflammatory bowel disease pathways) in PTSD. Nominal significance P-value (0.0001) that did not reach significant FDR cutoff (0.055).

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Reference	Sample size (N)	Approach	Method of DNA Methylation Investigation	Identified genes	P-value (Cases vs. Controls)	Summary of Findings
Miller et al., 2018	286	Candidate gene	HumanOmni2.5–8 microarrays (Illumina, San Diego, CA)	AIM2 (cg10636246)	9.0E-03	PTSD severity was positively associated with CRP levels, and negatively associated with DNAm of AIM2, an inflammation related gene. PTSD severity and CRP levels were mediated by methylation levels at the AIM2 locus.