Dialogues in clinical neuroscience

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Editorial

Epigenetics: the missing link between genes and psychiatric disorders?

Florence Thibaut, MD, PhD - Editor in chief

Most studies describing epigenetic modifications have focused on DNA methylation, but fewer studies have focused on histone modifications and noncoding RNAs. Chromatin architecture and CCCTC-binding factor represent important noncoding regulatory elements that warrant further investigation in order to improve our understanding of the genomic basis of complex diseases such as psychiatric disorders.

Keywords: epigenetics; histones; DNA methylation; noncoding RNA; CTCF

Psychiatric disorders are polygenic and multifactorial disorders resulting from a complex interplay between genetic and nongenetic environmental factors. The highest heritability estimates have been observed in schizophrenia, bipolar disorders, or attention-deficit-hyperactivity disorders (60% to 80%) as compared with depression (~40%) or addictive disorders (~50%).1 (Penner-Goeke and Binder, in this issue, p 397). The lack of underpinning identified biological markers make the genetic studies more difficult as compared with other polygenic and multifactorial diseases such as diabetes. In fact, there is a large heterogeneity between clinical phenotypes. Genome-wide association studies conducted in large case–control populations have led to the identification of numerous single-nucleotide polymorphisms. Most of these common at-risk variants do not alter protein structure but rather have diverse regulatory functions. Moreover, some of these variants are common to different psychiatric diseases. In schizophrenia, common variants explain only 30% to 50% of the variance. Rare de novo copy number variants may also significantly increase the risk of schizophrenia as well as of bipolar disorder or autism when they are present. Finally, “nongenetic” processes such as gene-environment interactions contribute significantly. Epigenetics, through DNA methylation, histone post-translational modifications, or noncoding RNAs, may induce changes in gene expression without any variation in the DNA sequence. Epigenetic processes are mainly influenced by environment. They are stable and can be transmitted through cell division but might also be reversible. Most of these epigenetic changes are tissue-specific, and postmortem studies are important in order to identify them. In addition to neuronal cells, glial cells can also be involved in epigenetic changes.

The epigenetics of stressful early life adversity has been extensively studied in anxiety and depressive disorders. In this regard, the brain-derived neurotrophic factor (BDNF), hypothalamic–pituitary–adrenal (HPA) axis, and FKBP5 (a critical regulator of the HPA cortisol response) genes, as well as the gene encoding the serotonin transporter, have been extensively studied as candidate genes. Interestingly, variants in genes encoding for epigenetic modifiers have also been reported on. Epigenome-wide associated studies (EWAS) were also recently conducted in large populations. Several papers in this issue will review these topics. Most studies describing epigenetic modifications have focused on DNA methylation, but fewer studies have focused on histone modifications and noncoding RNAs. Most studies have found that...
have been conducted using peripheral tissues which do not necessarily reflect brain epigenetic changes. In addition, controlling environmental factors that may contribute to epigenetic changes is not easy in large cohorts.

In addition to these latter epigenetic changes, there is a higher-order chromatin organization within the nucleus; the human genome folds in three dimensions to form thousands of chromatin loops. Superimposed upon nucleosomes are topologically associating domains (TADs). Sequences within TADs are more likely to come into contact with each other than with loci from outside domains. Moreover, TAD boundaries and chromatin loop formations are often delimited by CTCF (DNA binding proteins). CCCTC-binding factor (CTCF) is an important epigenetic regulator, widely expressed in the tissues of vertebrates, which modifies the transcription of genes by altering their location within the nucleus. In fact, chromatin loops allow distal regulatory elements to come into contact with gene promoters in order to regulate gene expression. CTCF is also required for inter-chromosomal interactions such as pairing of the X chromosomes. Furthermore, point mutation and loss of heterozygosity of CTCF is associated with human cancer. In addition, robust and intact CTCF looping is required for the induction of a rapid and accurate myocardial stress response in animal models, which may play a role in heart failure. This may open new pathways to pharmacological treatments in cardiac diseases. In summary, chromatin architecture and CTCF binding are important noncoding regulatory elements that are already investigated in oncology and cardiology and that warrant further investigation to help understanding of the genomic basis of complex diseases such as psychiatric disorders.

Some pharmacologic treatments such as DNA methyltransferase inhibitors or histone acetylase inhibitors (valproic acid) are already used in psychiatry and oncology. The clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9)—CRISPR-dCas9—an epimodifier complex, has been shown to demethylate the BDNF gene specifically, activating its expression (see also Day, in this issue p 359).

Epigenetics opens up new pathways in the understanding of complex diseases and in the search for biological markers and, possibly, new treatments.

References

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Epigenetics

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Unraveling the epigenetic landscape of depression: focus on early life stress

Angélica Torres-Berrío, PhD; Orna Issler, PhD; Eric M. Parise, PhD; Eric J. Nestler, MD, PhD

Depression is a devastating psychiatric disorder caused by a combination of genetic predisposition and life events, mainly exposure to stress. Early life stress (ELS) in particular is known to “scar” the brain, leading to an increased susceptibility to developing depression later in life via epigenetic mechanisms. Epigenetic processes lead to changes in gene expression that are not due to changes in DNA sequence, but achieved via modulation of chromatin modifications, DNA methylation, and noncoding RNAs. Here we review common epigenetic mechanisms including the enzymes that take part in reading, writing, and erasing specific epigenetic marks. We then describe recent developments in understanding how ELS leads to changes in the epigenome that are manifested in increased susceptibility to depression-like abnormalities in animal models. We conclude with highlighting the need for future studies that will potentially enable the utilisation of the understanding of epigenetic changes linked to ELS for the development of much-needed novel therapeutic strategies and biomarker discovery.

Keywords: chromatin remodeling; histone modification; DNA methylation; noncoding RNA; early life stress

Introduction

Epidemiological and preclinical studies have identified stress as an important risk factor for the onset of an episode of major depressive disorder (MDD), a highly debilitating psychiatric condition characterized by persistent alterations in mood, motivation, or cognitive function. Depression occurs across the lifespan, from childhood through late life, but the peak of incidence is observed during peripubescence.

Early life is a critical period of plasticity for brain development and is highly sensitive to adverse experiences such as stress exposure. Indeed, the consequences of stress on mental health are more severe when experienced early in life. Early life stress (ELS), including child maltreatment, parent neglect, undernutrition, or sexual abuse, increases the risk for depression and other stress-related disorders later in life by two- to fourfold. Furthermore, in people subjected to ELS, the early presence of depressive events is a predictor of recurrent episodes and severity of depression.

Susceptibility to MDD is partly mediated by genetic factors with an estimated 35% heritability, mediated by hundreds of genomic variations, each of very small effect. This genetic predisposition, in combination with exposure to stressful life events, can lead to the development of MDD via epigenetic mechanisms, or changes in gene expression that are not due to DNA sequence variation. These epigenetic changes are mediated by several mechanisms, including histone modifications, DNA methylation, noncoding RNAs, and changes in the 3D structure of chromatin, that consequently lead to changes in brain function. Notably, epigenetic changes caused by exposure to ELS in genetically prone
individuals can leave a “molecular scar” that leads to the development of MDD, particularly following exposure to additional profound stress throughout life.9

Functional and molecular alterations in the prefrontal cortex (PFC), ventral tegmental area (VTA), nucleus accumbens (NAc), hippocampus (HPC), and amygdala (AMY), collectively known as the mesocorticolimbic system, have been consistently associated with MDD.10 These brain regions are known to be involved in the regulation of cognitive function, emotion, motivation, and mood and are highly sensitive to the effects of stress.11 Indeed, the protracted development of the mesocorticolimbic system renders it highly sensitive to environmental insults during early life.12,13

This review discusses recent advances in the study of epigenetics and depression, with a special focus on ELS. In the first section, we introduce the basic mechanisms of epigenetic regulation, including histone modifications, chromatin remodeling, DNA methylation, and noncoding RNAs. In the second section, we present evidence from rodent and human studies showing alterations in these epigenetic mechanisms that are associated with ELS-induced vulnerability to MDD. We highlight studies exploring the effects of stress from birth to periadolescence and how the consequences of such stress can be observed in adulthood and even subsequent generations. Finally, we suggest future directions in which further work is needed to better understand the deleterious effects of ELS on MDD risk.

Epigenetic mechanisms

Histone modifications

The human genome contains over 3 billion base pairs of DNA reaching ~2 meters in length, that is condensed into the cell nucleus, with a diameter of ~6 μm. This requires intricate organization that is obtained by compacting DNA onto histone proteins, thus creating chromatin. Nucleosomes are the basic repeating unit of chromatin fibers, consisting of ~147 base pairs of DNA wrapped around a single histone octamer comprised of two copies of each of four core histones H3, H4, H2A and H2B (Figure 1). Assembly of nucleosomes is achieved through linker histone H1 and a short linker DNA segment, allowing for interaction between neighboring nucleosomes. This nucleosomal organization of DNA is responsible for the higher-order chromatin structure, thereby facilitating or preventing access of gene regulatory machinery to fine-tune gene expression through post-translational modifications (PTMs; eg, acetylation, methylation, phosphorylation, and ubiquitination) on histone N-terminal tails which face outward from the nucleosome core.14,15

Histone acetylation at lysine residues is generally associated with transcriptional activation by loosening DNA-histone binding and, therefore, increasing spacing between nucleosomes. This state facilitates the binding of transcription factors and other regulatory proteins.15 Histone methylation results from the addition of one, two, or three methyl groups to lysine or arginine residues of histone tails. This PTM can either promote transcriptional repression or activation depending on the modified amino acid.14 Histone acetylation is controlled by two enzymes: histone acetyltransferases and histone deacetylases (HDACs), whereas histone methylation is controlled by lysine or arginine histone methyltransferases (HMTs) and histone demethylases.16 Enzymes that add PTMs are considered “writers,” those that remove PTMs “erasers,” and proteins that recognize these PTMs to direct transcriptional outputs are referred to as “readers.”

Several histone variants have been identified, including H2A.Z, H2A.X, and H3.1–3. These variant histone proteins structurally differ from the canonical histones, ranging from a few amino acids up to entire protein domains. Histone variants are also subjected to PTMs and thus influence chromatin dynamics.17

Chromatin remodeling and 3D chromatin structure

Another mechanism by which chromatin structure can be modified is through the action of proteins known as chromatin remodelers. These protein complexes, including SWI/SNF, CHD, ISWI, and INO80 families, mobilize or reposition histone octamers along the linear DNA and thereby regulate the spacing of nucleosomes and DNA accessi-
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Early life stress: epigenetic fingerprint in depression - Torres-Berrío et al

There is evidence that functional alternations in chromatin remodeling complexes in the brain are associated with psychiatric disorders, including MDD, schizophrenia, and autism. Likewise, the 3D structure of chromatin is subject to dynamic regulation. This organization allows distant regions of the genome to come into close approximation and contribute to the regulation of gene expression.

DNA methylation

DNA methylation—5-methylcytosine (5mC)—is a well-described epigenetic mechanism classically associated with suppression of gene transcription. This process occurs predominantly within cytosine-guanine (CpG) dinucleotide sequences and is catalyzed by DNA methyltransferases (DNMTs). More recent research has demonstrated 5mC at non-CpG sites as well as methylation of other nucleotides. Moreover, variant forms of 5mC, such as 5-hydroxymethylcytosine, have been shown to play important roles in CNS function.

DNMTs are dynamically regulated throughout development and play an important role in guiding DNA methylation patterns in the CNS. DNMT1 is considered a maintenance DNMT as it perpetuates methylation in a newly synthet-

Figure 1. Chromatin structure and epigenetic regulation of gene expression. Gene expression is regulated by DNA methylation, histone post-translational modifications (PTMs), and the actions of non-coding RNAs, among other mechanisms. To fit within the nucleus, DNA is wrapped around histone proteins creating higher order chromatin structure, which can facilitate or prevent access of gene regulatory machinery through steric mechanisms. DNA methylation is canonically associated with suppression of gene transcription. Deposition of methyl groups is catalyzed by DNA methyltransferases (DNMTs). DNMT1 regulates maintenance, while DNMT3a/b deposit de novo methyl groups. Removal of these marks, or demethylation, is facilitated by ten eleven translocation (Tet1-3) proteins, cytidine deaminase (AID), and growth arrest and DNA damage-45 (GADD45). PTMs control chromatin states through modifications, including methylation and acetylation, of histone residues. Histone acetylation is most commonly associated with chromatin relaxation enabling transcriptional activation through greater accessibility of transcriptional machinery. Histone methylation is associated with either transcriptional repression (by promoting increased chromatin compaction) or transcriptional activation, depending on the site undergoing modification. Histone acetylation is mediated by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). Histone methylation is controlled by lysine or arginine histone methyltransferases (HMTs), while histone demethylation is mediated by histone demethylases (HDMs). Lastly, miRNAs are short RNAs capable of regulating gene expression post-transcriptionally. miRNAs target mRNAs to promote their degradation or inhibit their translation. miRNAs also interact with IncRNAs which are regulatory transcripts longer than 200 b that can act as molecular decoys, scaffolds, or guides – adding another layer of epigenetic regulation.
sized complementary DNA strand that is generated during DNA replication. In contrast, DNMT3a and 3b are de novo DNMTs, as they methylate previously unmethylated regions of the DNA. The molecular mechanisms of DNA demethylation have recently been established. They involve enzymes implicated previously in DNA repair, such as ten eleven translocation (TET1-3) proteins, growth arrest and DNA damage-45 (GADD45), and activation-induced cytidine deaminase (AID).

Noncoding RNAs

In the last few decades the central dogma of biology has changed, acknowledging that RNA molecules are key effectors in cells and not simply intermediate messengers in protein generation. Such noncoding RNAs (ncRNAs) are involved in many molecular processes in development and adulthood, in health and disease. ncRNAs are divided into classes by their length, with 200 bases set as an arbitrary cutoff between short (sncRNAs) and long noncoding RNAs (lncRNAs). sncRNAs include microRNAs (miRNAs), short interfering RNAs, and piwi-interacting RNAs. Of these, miRNAs are the most studied biotype in depression and therefore considered in this review. Notably, multiple classes of ncRNAs can be detected in circulation and body fluids. Indeed, there is evidence that circulating ncRNAs transfer signal between organs, and that their levels in circulation can be used as biomarkers of disease states.

miRNA biogenesis and function

miRNA biogenesis is a multistage process extensively reviewed in ref 29. Briefly, miRNAs arise from RNA hairpin structures and are cleaved into a mature miRNA that forms silencing complex with associated proteins. This complex targets the 3'-untranslated region (3'UTR) of mRNAs to promote their degradation or inhibit their translation. Since miRNAs target mRNAs of complementary sequence, it is possible to use bioinformatics to predict miRNA targets. A single mRNA can be targeted by multiple miRNAs and, conversely, a miRNA can target multiple mRNAs or interact with lncRNAs, which act as sponges of miRNAs.

lncRNA evolution and function

We now know that the majority of the human genome is transcribed and gives rise to more lncRNAs than protein-coding genes. Comparative genomic studies indicate that evolutionarily higher organisms have larger portions of noncoding transcripts in their genome. Specifically, a third of lncRNAs have arisen within the primate lineage and ~40% of lncRNAs are expressed only in brain, suggesting a key role for lncRNAs in the evolution of higher brain function. lncRNAs have similar structural properties to those of protein-coding genes, both at the chromatin level, with similar histone modification patterns, and at the transcript level, as being formed from multiple exons that are subjected to alternative splicing. Functionally, lncRNAs play regulatory roles as decoys, scaffolds, or guides at the transcriptional, post-transcriptional or post-translational levels interacting with DNA, RNAs or proteins. Such interactions can lead to either repression or activation of the interaction patterns. To date, there is no systematic method to predict lncRNA targets or molecular function other than empirically.

Epigenetic alterations and depression in the context of ELS

The use of animal models is a powerful tool for understanding the link between ELS-induced vulnerability to depression and epigenetic molecular adaptations. In rodents, ELS can be modeled by inducing variations to the early caregiving environment, including maternal separation (MS), variations in the levels of maternal care (MC), or periadolescent exposure to several stressors including social isolation (SI) or chronic social defeat stress (CSDS). These manipulations reprogram numerous brain regions such as the hypothalamus–pituitary–adrenal (HPA) axis, leading to enhanced vulnerability to adult stress, which can be reversed by environmental enrichment or pharmacological manipulations. A comparative study conducted by our group showed that different protocols of ELS can induce an array of phenotypic outputs. In this section, we describe recent implicating epigenetic processes within the mesocorticolumbic reward system underlying the lifelong effects of ELS (Table I).

Histone modifications

Histone acetylation

Mounting evidence suggests that ELS alters acetylation of histones and the expression of HDAC enzymes leading to transcriptional, structural, and physiological changes in several key brain regions. For example, subjecting mice to combined MS and SI induces a rapid increase in histones H3 and H4 acetylation in HPC, concomitant with
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Early life stress: epigenetic fingerprint in depression - *Torres-Berrio et al*

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<th>EPIGENETIC MARK</th>
<th>SPECIES</th>
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<td>24 h MS</td>
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<td>AKAP79/150 and Bdnf</td>
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<td>PND5 to PND10</td>
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<td>Bdnf</td>
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*Table I (continued overleaf). Alterations in histone post-translational modifications and associated enzymes in rodent models of early life stress.*
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Table I (continued). Alterations in histone post-transcriptional modifications and associated enzymes in rodent models of early life stress.

high corticosterone levels. This effect correlates with elevated expression of the immediate early genes activity-regulated cytoskeleton-associated protein (Arc) and early growth response 1 (Egr1), increased H4 acetylation at the Arc and Egr1 gene promoters and higher dendritic complexity of hippocampal CA3 neurons. In the central AMY and bed nucleus of the stria terminalis, MS elevates oxytocin receptor mRNA and H3 acetylation at lysine (K) 14 (H3K14ac) specifically within the promoter region of the oxytocin receptor gene, coinciding with increased ultrasonic vocalizations in early life as an indicator of enhanced stress-reactivity. In contrast, decreased acetylation of exon IV of the brain-derived neurotrophic factor (Bdnf) gene in rat medial PFC (mPFC) results from early maltreatment. Intriguingly, a brief protocol of MS decreased H3 acetylation at the promoters of genes encoding the dopamine receptor 1 (DRD1) and dopamine- and cAMP-regulated phosphoprotein of 32 kD (DARPP-32) in HPC, effects associated with decreased depression-like behaviors in adult mice, suggesting that the severity of the ELS protocol contributes to the lasting behavioral outputs associated with histone acetylation.

In rodents, the quality of MC and subsequent performance of offspring in stress-related tasks is mediated by sustained epigenetic changes in dorsal HPC (dHPC) and mPFC. Adult offspring from mothers displaying high MC (eg, high licking and grooming of the pups) show better learning performance, lower stress sensitivity and reduced fearfulness compared with offspring of low MC mothers. These behavioral changes are associated with H3 acetylation in dHPC in stress- and plasticity-associated genes, including those encoding the glucocorticoid receptor (GR; Nr3c1 gene), protocadherins-α, -β, and -γ and metabotropic glutamate receptor 1. Synaptic plasticity in the VTA dopamine (DA) system is sensitive to changes in histone acetylation induced by ELS. For example, a single episode (24 hr) of MS leads to reduced levels of H3 acetylation at lysine 9 (H3K9ac).
and increased expression of HDAC2 in the VTA. These epigenetic changes correlate with high expression of the postsynaptic scaffolding A-kinase anchoring protein 150 (AKAP150), which is known to control GABAA receptor trafficking in VTA DA neurons. Indeed, MS induces long-term depression (LTD) at GABAergic synapses onto VTA DA neurons in an AKAP150-dependent manner. Remarkably, the selective class I HDAC inhibitor, CI-994, reverses MS-induced GABAergic abnormalities both in vitro and in vivo, increasing the levels of H3K9ac and subsequently normalizing expression levels of AKAP150 in VTA. These findings illustrate functional alterations induced by ELS and highlight the therapeutic potential of HDAC inhibitors.

ELS alters acetylation in non-neuronal cells as well. One study showed that MS decreases HDAC1/2 expression and affects myelination of mPFC across the lifespan. The morphological alterations were associated with impaired mPFC-dependent cognitive function in adult rats. Furthermore, MS reduced the number of mature oligodendrocytes via HDAC1/2-induced inhibition of WNT signaling. Postmortem studies in humans with a history of child abuse provide further support for the involvement of epigenetic regulation in oligodendrocytes, showing DNA hypermethylation of genes related to myelin and oligodendrocytes in the anterior cingulate cortex, as well as increased numbers of mature myelinating oligodendrocytes and decreased numbers of oligodendrocyte-lineage cells in ventromedial PFC. Combined, this suggests that epigenetic reprogramming of oligodendrocytes induced by ELS may produce persistent dysregulation to cortical myelination and alter the connectivity within the mesocorticolimbic reward system.

As mentioned, the effects of ELS can vary greatly depending on genetic sensitivity, and there is ample evidence to suggest the involvement of histone acetylation processes when comparing mouse strains. For example, BALB/C mice are behaviorally more sensitive to the effects of ELS and adult stress in comparison to C57BL/6 mice. Adult stress decreases H3 acetylation at the promoter of the glial cell-derived neurotrophic factor gene (Gdnf) in NAc of BALB/C mice, while increases H3 acetylation in C57BL/6 mice at the same locus. Furthermore, BALB/C mice exposed to MS display increased cortical expression of acetylated histone H4 proteins, specifically, H4K5ac, H4K8ac, H4K12ac, and H4K16ac, along with decreased expression of different HDACs.

Collectively, these findings support the idea that the early environment controls activation of synaptic plasticity genes, which in turn disrupts the proper development of stress-sensitive brain structures via mechanisms sustained at least partly via epigenetic mechanisms like histone acetylation. Importantly, pharmacological manipulations using HDAC inhibitors (HDACis) alone or in combination with fluoxetine, regulate both acetylation patterns and stress-related behaviors in rodents exposed to MS, further supporting the potential therapeutic effects of HDACis to prevent or treat the deleterious effects of ELS in vulnerable individuals.

Histone methylation

Patterns of histone methylation are influenced by ELS and, similar to acetylation, correlate with genetic-based stress sensitivity. High-stress sensitive rats, which are prone to depression- and anxiety-like behaviors, display a global increase in levels of trimethylation of H3 at lysine 9 (H3K9me3), and in particular a reduction of this mark in proximity to stress-related genes, including those encoding the GR and fibroblast growth factor 2, in HPC, AMY, and NAc. Similarly, stress sensitive BALB/C mice exhibit reduced H3K4me3 expression in NAc following chronic stress. Indeed, alteration in methylation of H3K9 or H3K4 seem to be key players in regulating vulnerability to stress across the lifespan. For example, early MS induces opposite patterns of histone methylation in HPC in early adolescence versus adulthood. Adolescent rats previously exposed to MS display decreased H3K9me2, but increased BDNF, expression and enhanced HPC neurogenesis. By contrast, adult rats exposed to MS exhibit impaired spatial memory, decreased neurogenesis and increased performance in H3K9me2 expression.

MS has also been shown to produce persistent down-regulation of numerous HMTs within mPFC, including SMYD3 and SUV420H1, which lasts into adulthood and may underlie some of the altered structural and functional plasticity induced by ELS. Moreover, adult male mice with a history of MS exhibited increased H3K4me3 in the promoter region of DNA damage induced apoptosis suppressor (Ddias) and phosphatidylinositol-5-phosphate 4-kinase type 2 alpha (Pip4k2a) in mPFC. Yet another study using a rat model of ELS found that impaired social...
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Not all consequences of ELS are deleterious, as potential epigenetic mechanisms underlying the beneficial impact early life adversity have been reported. For instance, male mice exposed to MS along with unpredictable maternal stress (MSUS) show increased performance in anxiety-

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Limitations</th>
<th>Example data</th>
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<tbody>
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<td>WGBS</td>
<td>Detects DNA methylation genome wide at single-base resolution</td>
<td>• Requires very deep sequencing&lt;br&gt;• Analysis is challenging</td>
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<td>ChIP-Seq</td>
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<td>• Analysis is challenging&lt;br&gt;• No locus-specific information</td>
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<td>GAM</td>
<td>Cryosectioning based mapping of genome-wide chromosomal interactions</td>
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Figure 2. Common high-throughput techniques for probing the epigenome. Owing to methodological advances, the toolbox for studying the dynamics of epigenetic modifications is rich and constantly evolving. Several approaches exist to assess changes in DNA methylation including whole genome bisulfite sequencing (WGBS), methylated DNA immunoprecipitation (MeDIP) and methylation-sensitive restriction enzyme sequencing (MRE-Seq). At the level of RNA: next generation RNA sequencing (RNA-Seq) is widely used to probe changes in gene expression. Recently m6A/m sequencing has been utilized to test changes in RNA methylation. PTM of histones can be detected using chromatin immunoprecipitation coupled with DNA sequencing (ChIP-Seq) or mass spectrometry (MS) to detect multiple histone PTMs simultaneously. Chromosome conformation capture (Hi-C) can be used to uncover contacts between distant genomic loci that affect gene expression and/or chromatin structure. Assay for transposase-accessible chromatin sequencing (ATAC-Seq) can be used to determine genome-wide chromatin accessibility and genome architecture mapping (GAM) allows for the mapping of genome-wide chromosomal interactions. Together, as each tool has its pros and cons, combining multiple assays can enable profiling the changes in the epigenome, allowing a more thorough understanding of potential mechanisms contributing to MDD.

behavior in adulthood was correlated with elevated levels of H3K4me2/me3 and H3K9me2/me3 in AMY. These data complement work mentioned earlier showing that offspring of high MC mothers also display increased levels of H3K4me3 in dHPC.41
related tasks and goal directed-behavioral assays, as well as increased behavioral flexibility.56 This increased overall performance is associated with decreases in H3K4me2 and H3K36me3 at an intragenic region of the gene encoding the mineralocorticoid receptor within HPC. While these data support the possibility that some forms of ELS can promote resilience through epigenetic-based mechanisms, future studies are needed to provide causal evidence, including how, when, and which forms of ELS promote susceptibility/resilience and the specific underlying epigenetic mechanisms involved.

**Histone variants**

The substitution of histone variants for canonical plays a critical role in chromatin function. While these variants are highly conserved across species little is known about their role in the sustained changes in gene expression induced by ELS. In adults, the histone variant H3.3 is increased in NAc of depressed humans and in adult, susceptible mice exposed to CSDS.58 This study analyzed the developmental dynamics of H3.3, and the canonical histones H3.1 and H3.2, in the mouse NAc in response to ELS. ELS induced a sustained elevation of H3.3 and a loss of H3.2 levels in adult mice, indicating that reduced histone turnover may contribute to increased stress susceptibility in adulthood.

In summary, histone modifications are robustly regulated by ELS across brain regions; however, the significance of these changes are region-specific. Existing data suggest that, overall, increased histone acetylation, which would favor an open chromatin conformation and increased transcription, is antidepressant. More work is needed to understand how histone acetylation and methylation patterns regulate expression of specific gene targets implicated in depression and whether this pathway can be targeted for treatment. This may be achieved through future studies geared toward combining genome-wide histone code profiling with RNA-seq (Figure 2) to determine causal relationships between epigenetic modulation and gene expression.

**Chromatin remodeling**

Factors that regulate chromatin remodeling might be associated with depression. For example, the chromatin-remodeling factor, SMARCA3, mediates neurogenesis and the antidepressant effects induced by fluoxetine.59 We recently demonstrated that ATP-utilizing chromatin assembly and remodeling factor (ACF) is necessary for stress-induced depressive-like behaviors.60 This study shows that upregulation of ACF1, a subunit of the ACF complex, in NAc is linked to stress vulnerability in adult mice and depressed humans. Additionally, altered levels of several ATP-dependent chromatin remodeling factors, including SNF2H in AMY and CHD3 and CHD5 in ventral HPC, are seen in highly stress-sensitive mice.61 However, to date there is no information regarding the role of chromatin remodeling complexes in ELS. Similarly, further work is needed to examine effects of ELS and adult stress on 3D chromatin structure genome-wide.

**DNA methylation**

Several lines of research demonstrate that early life adversity leads to global alterations in DNA methylation and levels of DNMTs that can be observed across the lifespan, and even into subsequent generations (Table II). Indeed, patterns of DNA methylation induced by early adversity alter HPA axis programming and brain structures that regulate stress-induced negative feedback.62 For example, early MS induces long-lasting hyperactivity of the HPA axis by decreasing DNA methylation at the enhancer region of the arginine vasopressin gene and increases its expression in the paraventricular nucleus of the hypothalamus, an effect that persists 1 year after MS.63 Similarly, neuropeptide receptor-1 (NTSR1) levels are reduced in AMY of adult rats exposed to MS. This effect was linked to increased DNA methylation in the promoter region of Ntsr1, enhanced fear conditioning and HPA axis reactivity.64 In addition, FK506 binding protein 5 (FKBP5; also known as FKBP51), a chaperone protein that modulates translocation of GR to the nucleus upon ligand binding, is altered by ELS in humans and rodents.65-67 Indeed, individuals with a single nucleotide polymorphism in the intronic region of the FKBP5 gene that were exposed to early trauma exhibit increased risk for post-traumatic stress disorder (PTSD). This polymorphism reduces DNA methylation in the promoter and intronic regions of the FKBP5 gene and increases mRNA expression of FKBP5 in vitro and in blood cells from PTSD subjects. Importantly, the reduced methylation levels correlated with the severity of PTSD.67

Variations of MC or MS alter DNA methylation patterns in the adult HPC and mPFC that result in impaired activation of the stress-induced negative feedback.68 Offspring experiencing low MC display high levels of hippocampal DNA methylation of the GR gene, concomitant with reduced expression of GR mRNA.69,70 This is in line with classic...
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Table II. Alterations in DNA methylation in rodent models of early life stress.
findings that associate such changes in GR with heightened HPA activity. Social isolation (SI) during peri-adolescence produces glucocorticoid-induced DNA methylation changes in VTA DA neurons. In one study, disrupted-in-schizophrenia 1 (Disc1) mutant mice were subjected to stress by SI in adolescence. Disc1 mice exhibited high-stress sensitivity and developed stress-induced depression-like behaviors. Interestingly, Disc1 mice exhibited decreased extracellular levels of DA in cortex and hypermethylation of the tyrosine hydroxylase (TH) gene promoter in VTA. These epigenetic patters were observed even 12 weeks post-SI and were specific to DA neurons projecting to PFC. Interestingly, Disc1 mice also show increased methylation in the intronic glucocorticoid response element (GRE) of the Bdnf gene, but decreased methylation in the intronic GRE of the Fkbp5 gene, suggesting involvement of GR as a transcription factor. Indeed, in studies administering the GR antagonist, RU38486, during early adolescence reported reversal of DA alterations induced by SI in Disc1 mice.

ELS alters methylation of genes that play important roles in neurodevelopment, neurogenesis or synaptic plasticity. For example, variations in MC increased binding of nerve growth factor-inducible protein A (NGFI-A) to the GR gene, which determines its methylation and acetylation patterns, whereas the promoter of the Glial cell line-derived neurotrophic factor (Gdnf) gene, which regulates VTA development, is hypermethylated in MS rats and plasma levels of DA and GDNF inversely correlated with depression-like behaviors. Moreover, several studies report alterations in BDNF signaling and Bdnf methylation in HPC, PFC, AMY, and VTA after ELS. For example, ELS causes long-lasting decreases in Bdnf mRNA levels, along with increased DNA methylation of the Bdnf gene in PFC. These alterations in methylation were observed even one generation after ELS, suggesting persistent and transgenerational changes in DNA methylation.

Disruption of the caregiving environment by low nesting material or early maltreatment affect methylation patterns of exon IV of Bdnf in PFC, AMY, and HPC. It was noted that this is a sex-specific effect that varies across development. Similarly, mice exposed to MS display increased methylation of exon IX of Bdnf in HPC, suggesting that methylation of Bdnf occurs at several regions of the gene. Together, these findings support human studies demonstrating that individuals with Val66Met polymorphism in BDNF are more sensitive to childhood adversity and depression in adulthood.

Early adversity induces age-dependent and sex-specific effects on several regulators of DNA methylation, including DNMTs, and DNMT inhibitors have been used to prevent the deleterious effects of ELS and adult stress in rodents. One study found that MS induces a short-term increase in expression of Dnmt1, Dnmt3a, and Dnmt3b in rat mPFC, an effect that depends on GR binding. Similarly, adolescent males previously exposed to early maltreatment display decreased mRNA levels of MECP2 (a methylated DNA binding protein) in mPFC. In adulthood, levels of Dnmt1, Dnmt3a, Mecp2, Gadd45b, and Hdac1 mRNA were decreased in male rats with early maltreatment, whereas adult females only exhibit a significant decrease in Gadd45b. Gadd45b is highly regulated by maternal interactions and adult stress. Indeed, rewarding maternal contact increases Gadd45b expression, induces β1 adrenergic receptor gene hypomethylation, and enhances noradrenergic signaling in mPFC of adult rats. In HPC, MS increased DNMT1 expression, which is associated with increased methylation at the promoter of the retinoic acid receptor-α (RARα) gene, along with decreased RARα expression. This effect leads to reduced neurogenesis by attenuating neural differentiation of adult neural precursor cells. Adolescent stress (AS) induces global reductions in HPC DNA methylation in females compared with controls, an effect linked to differential regulation of the estrogen receptor 1 (Esr1) gene. Variations in MC also alter DNA methylation of the Esr1 gene and affect maternal behavior in females. Importantly, Esr1 has been causally linked to sex differences in stress responses, suggesting that it may be a promising target for the lasting effects of ELS.

There is also promising evidence that changes in DNA methylation in brain caused by exposure to ELS are associated with alternations in DNA methylation in blood. One study showed that prenatal exposure to bisphenol A (BPA), an agent that disrupts neurodevelopment, induced lasting sex-specific changes in DNA methylation of Bdnf both in mouse HPC and blood. These changes are consistent with methylation alterations at BDNF in cord blood of humans exposed to high maternal BPA levels in utero. These results
Box 1. OTX2 programs lasting stress susceptibility. Adverse early life experiences increase the risk for MDD and other stress-related disorders by inducing long-lasting changes in the function and connectivity of key brain structures. These alterations are mediated in part by master regulators of transcription during this critical period of development.\(^\text{18}\) One of these regulators is the transcription factor orthodenticle homeobox 2 (OTX2) that has a key role in development of the ventral tegmental area (VTA) by controlling the differentiation of neuronal progenitors into dopaminergic neurons.\(^\text{16}-\text{18}\) In VTA, \(Otx2\) mRNA—which is highly enriched in dopamine neurons in this brain region—is high during early postnatal age, but decreases in adolescence and remains low through adulthood.\(^\text{48}\) Our group recently demonstrated that OTX2 in VTA programs long-lasting effects of early life stress (ELS) in mice (panel A). Specifically, Peña et al established a “two-hit” stress model, in which juvenile mice were exposed to ELS first-hit by an adapted protocol of maternal separation. In adulthood, these mice were further exposed to a second-hit of stress, namely, chronic social defeat stress (CSDS). We found that ELS increases susceptibility to adult stress by inducing a transient reduction of OTX2 levels in the juvenile VTA. Viral-mediated manipulations demonstrated that juvenile overexpression or downregulation of \(Otx2\) in VTA prevents or promotes, respectively, susceptibility to adult stress. Reduced OTX2 expression induced by ELS is associated with reduced binding to regulatory regions of critical target genes involved in VTA development, including \(Sema3c\) and \(Wnt1\) and thereby leads to a long-lasting transcriptional programming in VTA that renders individuals more susceptible to a second-hit of stress even though OTX2 levels themselves recover. Current research is focused on understanding the nature of the “chromatin scar” that leaves these OTX2 target genes impaired for a lifetime. In a follow up translational study,\(^\text{58}\) Kaufman et al. examined whether peripheral markers of OTX2 methylation and OTX2-regulated genes as well as resting-state functional connectivity were associated with vulnerability or resilience to depression in children that experienced maltreatment (panel B). The authors reported that the degree of DNA methylation at the OTX2 gene in blood positively correlated with history of maltreatment and predicted depression in children. Moreover, increased OTX2 methylation (which would be expected to decrease OTX2 expression as seen in mice) was associated with increased functional connectivity between key brain structures implicated in depression, including the right ventromedial PFC and bilateral regions of medial frontal cortex and cingulate gyrus. Future experiments are needed to understand whether and how alterations of OTX2 protein signaling in the juvenile VTA leads to global changes in brain circuitry and connectivity. Collectively, preclinical and clinical evidence support the role of OTX2 as a master developmental regulator of enduring alterations in transcription induced by ELS and a promising predictive biomarker and therapeutic target.

suggest *BDNF* DNA methylation in blood as a biomarker for the early detection of psychopathology. Another prospective study profiled CpG methylation genome wide in a large cohort of humans using microarrays on cord blood and on leukocytes later in childhood. The authors identified 38 sites with changed methylation at childhood that are associated with exposure to early life adversity, particularly adversity before the age of 3. Peripheral changes in methylation are of high translational relevance due to their potential use as biomarkers for detecting exposure to stress. Indeed, understanding how alterations in DNA methylation occurring in brain are functionally associated with vulnerability to depression offers novel therapeutic strategies. An example of this approach, focusing on the ELS-regulated gene, orthodenticle homeobox 2 (*OTX2*), is given in Box 1.

### Noncoding RNAs

#### miRNAs

In the last decade there has been an increased interest in understanding the role of miRNAs in regulating depression and other stress-related disorders. Advances have been possible since large subsets of miRNAs are evolutionarily conserved between rodents and humans. Several studies tested the effects MS on specific miRNA-target interactions in male rat brain. Uchida et al found that MS leads to increased levels of several miRNAs in mPFC when measured in early life, namely miR-132, miR-124, miR-212, miR-9 and miR-29. Upregulation of the first 3 miRNAs was sustained into adulthood. The authors suggest that these miRNAs are downstream of repressor element-1 silencing transcription factor splice variant 4 (REST4). Indeed, when REST4 is overexpressed in rat mPFC, levels of a subset of these miRNAs is upregulated as well, along with increases in depression-like behaviors. Another study explored the effects of MS and several antidepressant treatments on HPC miRNA expression using microarrays. The authors highlighted downregulation of miR-451 by MS, an effect that was reversed by fluoxetine, and upregulation of miR-598 that was common to fluoxetine and electroconvulsive seizures.

Others tested the effects of MS combined with CUS in adulthood on regulation of miRNAs in brain. Bai et al found that MS, but not CUS, upregulated miR-16 in male rats HPC, along with downregulation of BDNF. The authors suggest that this may be caused by miR-16-*Bdnf* interactions miR-16 has been implicated in depression by regulating the serotonergic system. Zhang et al found that MS and CUS increased DA receptor 2 (DRD2) levels, and inversely decreased miR-9 expression in striatum. The combination of MS and CUS had an augmented effect on this miRNA and target regulation. In vitro assays demonstrated that miR-9 and DRD2 directly interact. In addition, the study showed that miR-326 is downregulated in striatum by MS and that treatment with escitalopram rescues its dysregulation.

Stress in adolescence can also alter miRNA expression in brain. Exposure of male rats to AS downregulates miR-135a levels in mPFC, which coincides with increased serotonin receptor 1A levels. This dysregulation was normalized by paroxetine or by a corticotropin-releasing hormone antagonist. This miRNA-target interaction was originally identified in other stress models in raphe nucleus. In another study, Xu et al tested the effects of AS on male rats’ basolateral AMY miRNA expression patterns. They focused on the GR-FKBP5 pathway and miR-18a and miR-124a, which are known regulators of GR. The authors found that AS increased miR-124a expression levels in the basolateral AMY in adolescence, and the change was sustained to adulthood, along with an increase in miR-18a expression during adolescent only. Levels of GR and FKBP5 were inversely downregulated, as expected. miR-15a was identified in another study as part of the AMY response to chronic stress. The authors reported that miR-15a is elevated in peripheral human blood of patients in response to acute stress and following childhood trauma. This study set an example for the potential use of miRNAs as biomarkers for the long-lasting effects of early life adverse events.

In summary, to date there is evidence for the involvement of central miRNAs in epigenetically mediating the effects of ELS and facilitating the development of stress-related psychopathologies. Yet, there is a need for high-throughput screens profiling the developmental changes in miRNA expression levels caused by exposure to ELS both in brain and blood followed by causal mechanistic studies.

Transgenerational regulation of sperm miRNAs by ELS

Converging evidence suggests a highly controversial concept, that a stress induced phenotype is partly transfer-
rable to offspring via epigenetic mechanisms.\(^{100-112}\) These studies are focusing on paternal transmission and several of them highlighted sperm miRNAs as the molecular mediators of this effect. Gapp et al showed that exposure of male mice to ELS transgenerationally reduced anxiety-like behavior, increased depression-like behaviors, reduced insulin levels, and blunted glucose tolerance in the offspring. The authors showed that these phenotypic changes are associated with altered miRNA expression in sperm and hippocampus of the stressed mice and their progeny. Injection of several regulated miRNAs into oocytes was sufficient to mimic the stress phenotype, suggesting that sperm miRNAs mediate transgenerational effects of stress.\(^{112}\) These findings are in line with earlier work showing changes in offspring sperm and central miRNAs caused by parental stress exposure, with some overlap in the highlighted miRNAs, for example upregulation of miR-375.\(^{111}\) The same group transgenerationally passed aspects of paternal stress to offspring by manipulating several sperm miRNAs.\(^{113}\)

Another study tested whether the effects of ELS on the sperm epigenome is relevant to humans: they assessed miRNA expression in sperm of subjects exposed to adverse childhood experience. They tested several miRNAs based on the literature and noted that members of the miR-449 and miR-34 family were downregulated in men exposed to early life trauma. In the same study, these two miRNAs were downregulated in sperm of ELS-exposed mice and in offspring embryos.\(^{114}\) These findings highlight the translational value of studying regulation of miRNAs by ELS.

**LncRNAs**

By comparison to miRNAs, research on the function of LncRNAs in stress and depression is in its infancy. This is due to the novelty of this class of ncRNA as well as the challenges in studying them at the conceptual, bioinformatics, and experimental levels. Since many LncRNAs arose in primates, and the majority are not conserved between humans and rodents, it is challenging to perform and interpret causal experiments using animal models. Still, a few studies have looked at stress-induced regulation of LncRNAs in rodent brain\(^ {115,116}\) and antidepressant models.\(^ {117}\) Few human studies have explored the regulation of LncRNAs in human depression in brain\(^ {118}\) and the circulation,\(^ {119}\) and notably several of the recent genome-wide association study (GWAS) hits are in LncRNAs genes.\(^ {120,121}\) Only one study profiled the effects of MD on the PFC transcriptome including LncRNAs and noted differential expression caused by ELS\(^ {122}\); hence this avenue is open for exploration.

**Future directions and concluding remarks**

As our understanding of epigenetic regulation has expanded, it has become exceedingly clear that this regulation plays a crucial role in the pathophysiology of neuropsychiatric disorders, including MDD. Notably, alterations to the epigenetic landscape early in life may produce lasting abnormalities that prime the individual for increased susceptibility to stress and depression later in life. While this provides us with a framework for future investigation, there remain many hurdles. For example, nearly all studies to date have assessed epigenetic modifications in heterogeneous populations of cells, but with recent advancement in technology a high priority becomes investigation of specific cell populations. Already the epigenetic regulation of non-neuronal cell populations has been implicated in stress responses and depression, including increased microglial-specific histone modifications\(^ {123}\) or increased DNA methylation and histone modifications in astrocytes,\(^ {124,125}\) as well as unique global astrocytic DNA methylation patterns in the brains of MDD patients postmortem.\(^ {126}\) Even less is known about the contributions of cell-type specific epigenetic modulations after ELS, although a couple of recent studies have implicated epigenome changes in oligodendrocytes as being important.\(^ {44,45}\)

Women are twice as likely to suffer from depression than men.\(^ {127,128}\) There is a dire need to better understand the inherent sex differences associated with epigenetic regulation, during ELS as well as MDD in general, since most research has focused exclusively on males.\(^ {128}\) Recent studies conducted in rodents exposed to MS or AS have begun to reveal global epigenetic patterns that are sex-specific.\(^ {58,90,94}\) Estrogen receptors emerge as potential therapeutic targets.\(^ {90,95}\) In addition, functional studies using more advanced molecular techniques, including viral-mediated CRISPR-Cas9 systems,\(^ {129}\) will enable the manipulation of specific epigenetic modifications associated with target genes and assess where and how ELS-induced changes in the epigenetic landscape orchestrate the spatiotemporal organization of neural connectivity throughout life and in a sex-specific manner.

Taken together, improving our understanding of epigenetic mechanisms involved in ELS using genome-wide studies
with special emphasis on sex- and cell- type specificity across the lifespan can pave the way for the development of therapeutic and diagnostic interventions. Given the complexity of epigenetic regulation resulting from ELS, more advanced analytic models, including machine learning and bioinformatics, are urgently needed.

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Early life stress: epigenetic fingerprint in depression - Torres-Berrio et al

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Genetic and epigenetic editing in nervous system

Jeremy J. Day, PhD

Numerous neuronal functions depend on the precise spatiotemporal regulation of gene expression, and the cellular machinery that contributes to this regulation is frequently disrupted in neurodevelopmental, neuropsychiatric, and neurological disease states. Recent advances in gene editing technology have enabled increasingly rapid understanding of gene sequence variation and gene regulatory function in the central nervous system. Moreover, these tools have provided new insights into the locus-specific functions of epigenetic modifications and enabled epigenetic editing at specific gene loci in disease contexts. Continued development of clustered regularly interspaced short palindromic repeats (CRISPR)-based tools has provided not only cell-specific modulation, but also rapid induction profiles that permit sophisticated interrogation of the temporal dynamics that contribute to brain health and disease. This review summarizes recent advances in genetic editing, transcriptional modulation, and epigenetic reorganization, with a focus on applications to neuronal systems and potential uses in brain disorders characterized by genetic sequence variation or transcriptional dysregulation.

Keywords: neuroepigenetics; CRISPR/Cas9; epigenetic editing; gene regulation; transcription

Introduction

Gene regulatory mechanisms play an essential role in the nervous system. In the developing brain, intricate and temporally coordinated gene expression programs produce the immense cellular diversity of the nervous system, creating hundreds of distinct cell types. This coordinated regulation is required for the structural, physiological, and functional properties of all neuronal circuits, and dysregulation of these processes is a common feature of neurodevelopmental disorders and intellectual disability. In the adult brain, the same mechanisms remain dynamically responsive to environmental stimuli, and are critical for neuronal plasticity, memory formation and maintenance, and experience-dependent behavioral changes. Moreover, altered epigenetic patterns and transcriptional dysregulation are hallmarks of numerous neuropsychiatric and neurological disorders, suggesting that treatments targeting aberrant gene expression are relevant to mental health and neurodegenerative conditions.

One critical aspect of gene regulation and chromatin reorganization in the nervous system is the temporally dynamic nature of this process. For example, rapid experience-dependent changes in gene expression shape network formation in the developing brain, and even “stable” epigenetic modifications like DNA methylation can be rapidly reprogrammed at thousands of sites across the genome within hours of a significant behavioral experience. These changes occur at specific genes and with a high degree of cellular precision. However, until recently, approaches used to alter transcriptional and epigenetic states in the nervous system suffered from lack of temporal precision, lack of genetic precision, or both (Figure 1). For example, even
substrate-specific pharmacological approaches alter experience-dependent gene expression and epigenetic profiles in complex ways at hundreds or even thousands of genes, potentially due to the numerous off-target or compensatory effects that arise with such approaches. Likewise, knockdown or overexpression of chromatin regulators likely affects thousands of loci in the genome, and the temporal resolution of these manipulations is poor. These limitations, together with the central importance of epigenetic regulation in the nervous system highlighted throughout this issue of *Dialogues in Clinical Neuroscience*, highlights the need for site-specific gene modulation and epigenetic editing approaches. This review will summarize recent breakthrough advances in genetic and epigenetic editing approaches, with a specific focus on clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) systems that have enabled both the genetic and temporal precision required for novel breakthrough discoveries in this field.

**CRISPR/Cas9 systems for gene editing and gene regulation**

The first approaches to enable customizable DNA sequence editing or localized epigenetic editing were based on zinc-finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs). While ZFNs contain individual zinc finger domains that recognize specific DNA trinucleotide sequences, TALENs contain DNA binding domains that are selective for individual DNA bases. In both cases, these DNA binding domains can be customized into an array to recognize specific (and genetically unique) DNA sequences of interest. Fusion of restriction enzymes or other effector proteins to these sequence-specific arrays has enabled customizable DNA sequence editing, gene activation, gene repression, epigenetic editing, and even splice variant regulation. However, these systems also harbor several weaknesses, including a laborious synthesis process. To date, these challenges have limited integration of these tools into the neuroscience community.

**Cas9-based gene editing**

CRISPR/Cas gene editing systems were initially discovered as an RNA-guided endonuclease mechanism used by many species of bacteria and archaea against foreign DNA material from invading viruses and plasmids. Type II CRISPR/Cas innate immunity systems encode sequences of invading DNA as arrays of CRISPR RNAs (crRNAs) in the host genome. Together with a trans-activating RNA (tracrRNA) and Cas9 nuclease also expressed from the host genome, these crRNAs form a stable complex with Cas9 that seeks out the complementary target sequence in foreign DNA and initiates cleavage of both strands of DNA. One requirement for this double-strand break (DSB) is a protospacer adjacent motif (PAM), a 2-5 nucleotide sequence in DNA that must appear adjacent to the crRNA targeting locus. This motif helps to prevent unintended cleavage of the host DNA, but also imposes some limits in terms of genomic targeting in other species. However, in the most commonly used type II system, from *Streptococcus pyogenes*, the required motif of N-G-G is a relatively common sequence.

Since their discovery in bacteria, CRISPR/Cas9 systems have rapidly been adapted to perform similar roles in eukaryotes. In the engineered systems currently used for DNA targeting in mammalian genomes, the tracrRNA and crRNA sequences have been combined into a single guide RNA (sgRNA), which performs both DNA targeting and Cas9 complex formation functions. This arrangement has simplified both the design and generation of CRISPR targeting strategies, as it requires only a 20-nt RNA sequence for targeting instead of long amino acid chains used by ZFN and TALEN approaches. This ease in...
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Engineering of CRISPR sgRNAs to target selected DNA sequences of interest has become routine and commonplace across nearly all subfields of biology, and CRISPR/Cas9 systems have now outpaced TALEN and ZFN approaches for gene editing applications.

The Cas9 nuclease contains two distinct nuclease domains – the HNH nuclease targets the complementary (sgRNA-targeting) DNA strand, whereas the RuvC nuclease cleaves the non-template strand (Figure 2a). The resulting DSB can be repaired by one of two cellular mechanisms – the nonho-

Figure 2. Emerging toolbox for genetic and temporal precision of CRISPR-Cas9 based approaches for studying gene regulation. a, Top, use of CRISPR-Cas9 protein to induce double-strand breaks (DSB) at specific sites based on complementarity of the sgRNA target and location of a protospacer-adjacent motif (PAM). Bottom, catalytically inactive dCas9 protein creates a modular genomic anchor for fused effector proteins. b, dCas9-effector toolbox enables robust bidirectional transcriptional regulation, epigenetic editing of chromatin or DNA, fusion of modular scaffolds or fluorescent proteins, and recruitment of long non-coding RNAs. c, Strategies for genetic, chemical, and light-activated control of dCas9-effector expression and/or localization. See text for details and abbreviations.
mologous end joining (NHEJ) pathway or the homology directed repair (HDR) pathway. The NHEJ pathway results in relatively random mutations, including base substitutions, small insertions, and deletions. This pathway is most active in nondividing cells and is frequently used for gene disruption experiments in neuronal systems by creating large deletions, frameshift mutations, or early stop codons. In contrast, the HDR pathway uses a donor DNA fragment as a template for DNA repair, resulting in a precise insertion/correction event. However, this pathway is largely only active in dividing cells, and thus is not commonly employed in terminally differentiated neurons.

**dCas9-based transcriptional modulation**

In addition to its common use in DNA cleavage, the Cas9 nuclease can be inactivated via two simple amino acid substitutions (D10A in the RuvC nuclease and H840A in the HNH nuclease of the *Streptococcus pyogenes* Cas9). These mutations render the Cas9 catalytically inactive, and thus this version is called “dead” Cas9, or dCas9. However, this does not alter formation of complexes between the sgRNA and dCas9, and does not alter genome-targeting efficiency. Therefore, whereas Cas9 can be used for genome editing, dCas9 can be used as a global genomic anchoring system for stable and site-specific DNA targeting12-14 (Figure 2a). This anchor can be used as a modular scaffold for tethered effector proteins with many different functions. Most commonly, these effectors are fused directly to the dCas9, enabling direct genomic targeting as a modular scaffold that can accept nearly any fusion protein (Figure 2b). However, other systems utilize RNA extensions from the sgRNA that contain RNA binding motifs (such as MS2 loops) to direct effectors fused to MS2 proteins directly to the locus of interest.15

An initial realization after generation of dCas9 variants was that even without a fused protein, targeting of dCas9 to the non-template strand of DNA just downstream of transcription start sites was sufficient to disrupt gene transcription and/or elongation, causing robust decreases in expression of target genes.16,17 Subsequently, transcriptional inactivation domains (such as the KRAB domain closely associated with histone 3 lysine 9 trimethylation) were tethered directly to the dCas9, and these dCas-KRAB fusion variants yielded even more robust decreases in target gene expression than dCas9 alone.18,19 This CRISPR “interference” (CRISPRi) strategy has now been employed with large sgRNA libraries to identify key regulatory elements and screen for individual genes involved in critical cellular processes.18,20 More relevant to this review, this approach has also successfully been adapted for neuronal systems,21,22 revealing nearly complete gene silencing and extremely high on-target specificity that is sensitive to even a single nucleotide substitution in the sgRNA sequence. Moreover, CRISPRI is more effective at gene knockdown than previous gold-standard technology (RNAi), enabling complex dissociation of single gene contributions to neurotransmission as well as multiplexed gene regulation in the adult brain.22

While previous strategies for gene repression relied on expensive knockout technology or RNAi approaches that are subject to off-target effects, available tools for gene overexpression have been even more limited. Typical approaches rely on driving exogenous expression of cDNA from a viral vector, which makes titration of gene expression profiles or exploration of specific transcript variants difficult. In addition, these approaches are limited by viral vector capacity, and thus longer neuronal genes have not been amenable to this overexpression approach. Fortunately, CRISPR/dCas9 strategies can also be employed for this goal via fusion of transcriptional activators such as the viral protein VP64 to achieve gene induction from the endogenous gene locus.19,23,24 Like CRISPRi, this CRISPR “activation” (CRISPRa) approach is highly selective at induction of the target gene, and has increasingly been used for cellular reprogramming, genome-scale CRISPR screens, and dissection of functional elements in the genome25-28 (Carullo et al, unpublished). While initial gene regulation levels with CRISPRa fusion approaches were somewhat modest, recent development of multi-part activators (such as VP64-p65-Rta [VPR], a fusion of three distinct transcriptional activation domains) has dramatically increased the efficiency of this strategy.28 CRISPRa has now been adapted for effective use in primary neuronal cultures and the adult brain,21,29 demonstrating the ability to target many different genes regardless of length or cellular function, as well as upregulation of specific transcript variants from complex genes such as brain-derived neurotrophic factor (*Bdnf*). Likewise, targeted transcriptional activation of the circadian and activity-responsive *Per1* gene with dCas9-VP64 rescued age-related loss of this gene in the hippocampus and restored normal long-term memory.30 Similarly, CRISPRa targeting to an enhancer near an obesity-linked gene in a haploinsufficient mouse model was shown to rescue deficient *Sim1* gene expression and reverse obesity phenotypes.31
CRISPR epigenetic editing

As highlighted in the previous section, epigenetic modifications are highly dynamic in the central nervous system, where they respond to specific activity states and experiences, are altered during aging, and are dysregulated in diseases that affect brain function. Moreover, instead of affecting epigenetic landscapes globally, these processes produce gene-specific changes in epigenetic states. Thus, another goal of developing dCas9-based tools has been centered upon establishing site-specific epigenetic modifications via fusion of chromatin modifiers to dCas9. Typically, these efforts have been focused on either writers or erasers of specific epigenetic modifications, which are the most likely to produce sustained results at the associated gene locus.

Among the first epigenetic CRISPR effectors to be engineered was a dCas9 fusion to the histone acetyltransferase p300, which catalyzes acetylation of H3K27. This mark is commonly found at active gene promoters, but is also enriched at enhancer elements in the genome and has been used as a readout of enhancer activity state. Notably, Gersbach and colleagues found that dCas9-p300 fusions were capable of upregulating H3K27ac at enhancers and promoters, and increasing expression from linked genes with genome-wide specificity. Interestingly, enhancer-based gene regulation effects could not be reproduced with previous generations of CRISPRa tools (dCas9-VP64 fusions) and was dependent on the catalytic activity of the p300 histone acetyltransferase domain, suggesting that chromatin remodeling may be a critical event in activation of distal regulatory loci. More recently, this approach was used at activity-regulated enhancers near the *Fos* gene locus in neurons, revealing for the first time that experience-dependent gene regulation in the nervous system is finely tuned by H3K27ac dynamics at genomic enhancers.

Whereas dCas9-p300 fusions were found to broadly increase gene expression at both promoters and enhancers, site-specific removal of histone acetylation using dCas9-HDAC fusions has led to more mixed results. The initial report demonstrating a dCas9-HDAC3 fusion revealed that effects of local histone deacetylation were highly dependent on the specific target sequence, with some sgRNAs producing robust effects and others failing to alter gene expression at all. Likewise, targeting dCas9-HDAC8 to enhancers at the *Fos* gene locus in neurons revealed modest decreases in baseline H3K27ac, but this only altered expression of *Fos* mRNA under stimulation-dependent conditions (not at baseline). Active enhancers are also marked by H3K4 methylation, and other reports have obtained similar results via targeted recruitment of dCas9-LSD1 (a lysine demethylase) to enhancer loci in the genome, but observed no effects when dCas9 alone or dCas9-KRAB fusions were targeted to the same sites. Together, these results suggest that the effect of epigenetic editing is largely dependent on local chromatin landscape and cellular state, and indicates that targeting endogenous modifications at specific regulatory sites may be required for desired readouts.

CRISPR-based tools provide a versatile, multiplexable, and inexpensive technology for the systematic interrogation of genetic and epigenetic contributions to neuronal function

Perhaps most of the effort in development of dCas9-based epigenetic effectors has focused on cytosine methylation, which is catalyzed by DNA methyltransferases (DNMTs) and removed as part of an oxidation pathway by ten-eleven translocation enzymes (TETs). Fusion of the active methyltransferase domain from DNMT proteins to dCas9 has been reported to result in robust increases in methylation within a defined ~100bp window surrounding the location of the sgRNA. Critically, these changes were not observed with a catalytically inactive dCas-DNMT mutant, and increased cytosine methylation was also associated with decreased expression of target genes. Conversely, directing dCas9-TET fusions to specific gene promoters results in decreased methylation and increased expression of target genes in postmitotic neurons, demonstrating bidirectional regulation with this approach. While dCas9-DNMT fusions have subsequently been shown to have potential off-target effects in methylation depleted cells, these tools have already been employed for early stage proof-of-principle experiments in disease models.

A powerful example of the potential for CRISPR-based epigenetic editing is Fragile X Syndrome (FXS), an
X-linked neurodevelopmental disorder characterized by intellectual disability. FXS is caused by methylation-mediated silencing of the FMR1 gene, which occurs due to a CGG trinucleotide repeat expansion in the FMR1 promoter. FXS has no cure and there are currently no treatments. Remarkably, Rudolf Jaenisch and colleagues demonstrated that recruitment of dCas9-TET fusions to CGG repeats caused robust loss of methylation at the FMR1 promoter in human cells, which was associated with almost complete restoration of FMR1 protein expression and normalization of FXS related physiological and cellular deficits. Notably, rescued expression of FMR1 persisted even after removal of active dCas9 binding to the FMR1 locus, suggestive of enduring maintenance of altered DNA methylation status. Finally, these authors also used RNA sequencing and whole genome bisulfite sequencing (to measure DNA methylation changes), demonstrating that transcriptional and epigenetic effects were highly specific to the targeted FMR1 locus. Overall, these results provide a powerful example of the potential for this type of technology in disease states linked to epigenetic dysregulation, setting the stage for investigations into more complex polygenic disorders. Additionally, this general strategy can be used for recruitment of additional effectors, such as long noncoding RNAs that are increasingly being viewed as key regulators of chromatin states and neuronal function (Carullo et al, unpublished).

**Inducible CRISPR/Cas9 gene regulation systems**

Constitutively expressed CRISPR/Cas9 gene editing and gene regulation systems have already proven to be useful for understanding the function of specific genes, disease-linked mutations, and regulatory elements. However, these tools come with a central limitation, in that their constitutive expression means that temporally specific gene expression patterns cannot be recapitulated or blocked. This element is crucial in the nervous system, given the overwhelming evidence for rapid and coordinated changes in gene expression or chromatin regulatory dynamics. Additionally, these tools are often driven via robust promoters that are active in all cell types, making it difficult to ascertain the cell-specific functions of gene expression programs. To overcome these challenges, the core CRISPR/Cas9 tools highlighted above have undergone considerable reengineering in the past few years, resulting in unprecedented temporal and cellular precision of genetic, transcriptional, or epigenetic editing.

**Cellular specificity**

Initial Cas9 and dCas9 tools were driven by robust artificial or nonselective promoters, enabling strong expression in diverse cell lines and mammalian cell types. However, continued refinement of this approach has brought about improved cell type resolution, for example by using cell-type specific promoters. In a recent publication, the human Synapsin (hSyn) promoter was used to drive expression of a dCas9-VPR transgene, revealing selective induction of targeted genes across multiple primary neuron types and neuron-specific expression in the adult brain via lentiviral delivery. Similarly, unique promoters for other distinct cell types (eg, GFAP for glia, MBP for oligodendrocytes) could be useful for driving dCas9 fusion proteins in a more selective fashion. One advantage of this strategy is that it does not require a specific transgenic animal, and thus is compatible with almost any animal model system, cell line, or induced stem cell.

A more common approach involves combination of CRISPR technology with cell-specific expression of Cre recombinase, either via transgenic Cre driver lines or virally mediated expression of Cre recombinase under the control of a cell-specific promoter. Cre recombinase performs cleavage and ligation reactions at specific DNA elements called “lox” sites, which are most commonly inserted flanking a specific transgene of interest. Lox sequences are directional, and the action of Cre recombinase therefore varies based on the orientation of lox pairs relative to each other. For example, insertion of lox sites facing the same direction will result in excision of DNA between these sites. Likewise, insertion of lox sites facing each other will result in inversion and ligation of DNA between these sites, effectively flipping the transgene. The existence of heterotypic lox sites (eg, loxP and lox2272) increases combinatorial power of this system, as recombination events are specific within heterotypic pairs. Insertion of lox sites flanking CRISPR transgenes can therefore be used to gain Cre-dependent control over expression of CRISPR machinery. This approach has taken two basic strategies. In one strategy, a lox-stop-lox cassette is inserted ahead of the dCas9 fusion transgene (or functional Cas9), which prevents Cas9/dCas9 expression until the lox-stop-lox cassette is excised by Cre recombinase. Similarly, an inverted Cas9/dCas9 cassette can be flanked by heterotypic lox sites, which requires two recombination events for normal Cas9 expression. A lox-stop-lox approach has been used for generation of mouse and rat...
lines for Cas9-mediated genome editing in specific cell populations,48,49 and more recently for development of a mouse line for CRISPRa in targeted cell populations.30 A second, alternate approach is to drive the targeting sgRNA in a Cre-dependent fashion by placing a lox-stop-lox cassette upstream of the sgRNA and enabling ubiquitous expression of the Cas9 or dCas-fusion protein.48 Critically, either of these strategies requires access to a cell-specific Cre driver transgenic animal or co-delivery of Cre recombinase vectors driven by cell-specific promoters. However, in all cases these manipulations produce irreversible recombination events, meaning that CRISPR transgenes cannot be controlled in a temporally specific fashion.

Temporal specificity and reversible activation
A classic way to limit transgene expression to a defined temporal window is to use a tetracycline-inducible promoter. Tetracycline expression systems use a bacterial tetracycline response element, which contains repeats of a tetracycline operator sequence. Recent reports have used this strategy for temporally specific control of sgRNA expression by using a constitutively expressed Tet repressor (TetR). In the presence of tetracycline derivatives like doxycycline, TetR cannot bind to tetracycline operator sequences, relieving repression of sgRNA expression.50 This inducible sgRNA vector is capable of initiating gene editing both in vitro and in vivo, with effects occurring in the adult brain as soon as 1 day after addition of doxycycline to the diet. Although a similar approach has been used to regulate expression of Cas9 protein in other somatic tissues and cell lines,51,52 this similar approach has been used to regulate expression of Cas9 in neuronal systems.53,54 Although a split Cas9 system that employed fusion of N-terminal and C-terminal components of Cas9 or dCas9 to distinct rapamycin binding domains of the mammalian target of rapamycin (mTOR) enabled rapamycin-dependent gene editing and transcriptional activation.54 Like ERT2-based tools, this system also presented substantially lower efficiency than constitutive expression of wild-type Cas9 and was hampered by leaky gene regulation in the absence of rapamycin. Additionally, the reversibility of splitCas9 tools has not been systematically assessed.

Perhaps the most widely used approach for rapid control of CRISPR tools is light delivery, which is not only faster than chemical or genetic manipulations, but also rapidly reversible. These efforts drew on a rich history of using light to control protein interactions and gene regulation in simple cellular systems, but also on rapidly developing advances in optogenetic tools for delivery of light to specific organs, including the mammalian brain.55,56 The first demonstration of customizable light-induced gene targeting used TALE systems in which Cryptochrome 2 (Cry2), a blue light-sensitive protein from Arabidopsis Thaliana, was fused to a site-specific TALE array, enabling light-independent recruitment of Cry2 to a DNA locus. Instead of fusing an effector directly to the DNA binding TALE, the transcriptional activator VP64 was instead fused to CIB1, a binding partner that interacts with Cry2 only in the presence of blue wavefront light (due to a conformational change in Cry2 protein). Remarkably, this system enabled light-induced transcriptional modulation via recruitment of VP64 in the presence of blue light, and similar results were obtained when VP64 was fused to Cry2 and CIB1 was fused to the TALE array.57 Moreover, this approach supported light-activated epigenetic editing with fused histone methyltransferases and histone deacetylases, revealing the possibility for light-activated “optoepigenetic” approaches for precise and specific epigenetic editing with temporal and spatial precision.

Following this landmark demonstration, similar results were reported for light-based transcriptional activation using custom zinc finger proteins,58 and then for dCas9-based approaches.59 This toolbox has rapidly grown in the past 5 years,60-62 giving rise to additional blue-light sensitive effectors for genetic and transcriptional regulation.
These include a photoswitchable Cas9 (psCas9) that uses two photo-dissociable fluorescent proteins to obscure the DNA binding domain of Cas9 under normal conditions, preventing gene editing or Cas9 targeting. These domains dissociate in the presence of blue wavelength (500 nm) light, enabling Cas9 targeting or dCas9-effector recruitment for robust regulation of gene expression. A similar strategy has been employed in the recently described CASANOVA system, which ingeniously uses anti-CRISPR (Acr) proteins discovered in bacteriophages. These naturally occurring proteins bind to Cas9 with subnanomolar affinity in the region that normally interacts with the PAM motif, providing a mechanism for bacteriophages to escape gene editing functions of CRISPR/Cas9. However, these proteins are also active and provide robust inhibition of Cas9 and dCas9 in mammalian cells. In the CASANOVA (which stands for “CRISPR–Cas9 activity switching via a novel optogenetic variant of AcrIIA4”) system, a light-sensitive LOV2 domain from Avena Sativa was fused to the anti-CRISPR protein AcrIIA4. The N and C termini of the LOV2 protein are in close proximity in darkness but unravel upon exposure to blue light, disrupting AcrIIA4 interactions with Cas9. This switch allows for nearly complete inhibition of gene editing or gene regulation functions of Cas9 and dCas9 in baseline dark states, but activation in the presence of blue light.

One limitation to blue light sensitive Cas9 or dCas9 tools is that this wavelength of light does not penetrate deeply into tissues, and in some cases can damage tissues (or neurons) with increased light dosage. In contrast, near infra-red (NIR) light penetrates deeply into mammalian tissues and does not present robust phototoxicity complications. Recently described strategies have adapted light-inducible CRISPR systems for NIR light using either BphS protein (a NIR-sensitive c-di-GMP synthase) to signal to a c-di-GMP responsive transcription factor to initiate transcription of CRISPR effectors, or by NIR-sensitive nanocarriers of sgRNA/Cas9 complexes that release CRISPR cargo upon NIR light stimulation. However, these systems are in the early design phases and have not undergone systematic testing in different tissues and with different dCas9 effectors. Nevertheless, these approaches may eventually provide a path towards noninvasive gene and transcriptional editing in the brain, given the capacity for these wavelengths to penetrate transcranially into deep brain structures.

Conclusions and future directions

The research tools outlined above have enabled rapid transformation of a burgeoning subfield that previously lacked necessary genetic and temporal specificity. These emerging tools set the stage for causal, mechanistic dissection of key regulatory events linked to experience-dependent changes in neuronal function and dysregulation of gene expression in brain disease states. Although these approaches are likely many years away from translation to clinical applications, it is noteworthy that many CRISPR-based approaches are already in early clinical or preclinical trials to treat numerous genetic diseases, including inherited childhood blindness, sickle-cell disease, and Duchenne muscular dystrophy. While numerous hurdles exist for delivery of CRISPR machinery to the CNS, these examples offer hope that CRISPR-based genetic editing, transcriptional modulation, or epigenetic editing could be used in human diseases that affect brain function. However, regardless of potential clinical applications, these tools provide a versatile, multiplexable, and inexpensive technology for the systematic interrogation of genetic and epigenetic contributions to neuronal function.

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Genetic and epigenetic editing in nervous system - Day


The epigenetics of perinatal stress

Moshe Szyf, PhD

Early life adversity is associated with long-term effects on physical and mental health later in life, but the mechanisms are yet unclear. Epigenetic mechanisms program cell-type-specific gene expression during development, enabling one genome to be programmed in many ways, resulting in diverse stable profiles of gene expression in different cells and organs in the body. DNA methylation, an enzymatic covalent modification of DNA, has been one of the principal epigenetic mechanisms investigated. Emerging evidence is consistent with the idea that epigenetic processes are involved in embedding the impact of early-life experience in the genome and mediating between social environments and later behavioral phenotypes. Whereas there is evidence supporting this hypothesis in animal studies, human studies have been less conclusive. A major problem is the fact that the brain is inaccessible to epigenetic studies in humans and the relevance of DNA methylation in peripheral tissues to behavioral phenotypes has been questioned. In addition, human studies are usually confounded with genetic and environmental heterogeneity and it is very difficult to derive causality. The idea that epigenetic mechanisms mediate the life-long effects of perinatal adversity has attractive potential implications for early detection, prevention, and intervention in mental health disorders will be discussed.

Keywords: early life stress; perinatal stress; DNA methylation; epigenetics; early life adversity; NR3C1; glucocorticoid

Introduction

Early life stress and social adversity have been associated with behavioral disorders later in life. What are the mechanisms that mediate between experiences early in life and changes in stable phenotype later in life? Since these phenotypes are apparent a long time after the early experience, the changes in gene expression programming must be stable. It is postulated here that epigenetic mechanisms which evolved to confer cell-type-specific gene expression during embryonal and postnatal development are also involved in conferring experiential-specific gene expression profiles, mediating the phenotypic consequences of early life stress.

Epigenetic mechanisms

Epigenetic mechanisms explain how identical genes are differentially expressed in space and time in different cell types in the same individual. Epigenetic mechanisms establish cell-type-specific long-term states of gene expression during embryonal and postnatal development. The focus in this review is on DNA methylation, which is a chemical covalent modification of the genetic “hardware” itself and has been the most studied epigenetic mechanism in relation to early-life stress.

DNA methylation and hydroxymethylation

Both adenine and cytosine bases in DNA could be enzymatically modified by DNA methylation. The methyl donor for the reaction is S-adenosyl methionine (SAMe). The role of cytosine methylation in gene regulation has been extensively studied in mammalian DNA while the presence of N(6)-methyladenine in vertebrates and human DNA was discovered recently and its role in gene regulation and cellular differentiation is yet unclear. In vertebrates, DNA methylation is prevalent in the dinucleotide...
DNA methylation plays a causal role in regulation of gene expression

The inverse correlation between DNA methylation of promoters and transcription suggested that DNA methylation in promoters silences gene expression. The main evolutionary lesson derived from studying bacterial restriction-modification systems was that DNA methylation interfered with the interaction of proteins and their recognition elements in DNA; bacterial restriction enzymes did not cleave their recognition sequence when it was methylated. Similarly, it was shown that DNA methylation interferes with binding of transcription factors to their methylated recognition sequence, resulting in inhibition of transcription initiation. DNA methylation also attracts a specific class of proteins to methylated DNA; methylated DNA binding proteins. These proteins such as MeCP2 recruit chromatin inactivation complexes to promoter resulting in a silencing chromatin configuration. Three lines of evidence support a causal role for DNA methylation in gene expression. First, in vitro methylation of promoters silences reporter gene expression when transfected into cells. Second, treating cells with DNA methylation inhibitor 5-azacytidine activates gene expression. Third, knockdown of DNMT1 in cells or genetic knockout in mice results in changes in gene expression. There is still no evidence that demethylation of a specific set of sites can induce gene expression.

However, recent genome-wide correlations between promoter methylation and gene expression reveal a modest inverse correlation between gene expression and DNA methylation of promoters. It should be noted that the simplistic idea that DNA methylation in promoters is just a mere inverse image of steady-state mRNA levels that dominates the literature is inconsistent with the data and the complexity of epigenetic regulation. First, DNA methylation controls transcription turn-on while most gene expression studies measure steady-state mRNA. Second, expression and DNA methylation are heterogeneous in almost any population of cells. High expression might be occurring in a small fraction of cells that are hypomethylated, while the majority of cells are methylated and silenced. In such a

Epigenetic mechanisms mediate life-long effects of perinatal adversity, and this has attractive potential implications in mental health disorders

CG, which is a palindromic sequence (5’CG3’/5’GC3’). This enables semiconservative replication of DNA methylation from the template DNA (5’CG3’) to the nascent DNA (5’GC3’) by the DNA methyltransferase I (DNMT1) enzyme, a maintenance DNMT1. The maintenance of DNA methylation in dividing cells implies that a new methylation introduced into a CG sequence in DNA will be inherited through many cell divisions. A different set of enzymes, de novo methyltransferases DNMT3a and DNMT3b, introduce methyl groups to DNA irrespective of the DNA methylation state of the template, which will be maintained in following cell divisions by the maintenance DNMT1. Thus, a change in DNA methylation in response to a transient signal introduced by early life stress can be “memorized” in the genome and serve as a genomic memory of the functional state of the gene. Cell-specific DNA methylation patterns are formed during development, are involved in programming cell-type-specific gene expression profiles, and are essential for vertebrate development. Examination of methylation patterns of genes in different tissues revealed an inverse correlation between DNA methylation of promoter regions and gene activity. A recent survey of the state of methylation of all promoters in the brain that were physically engaged in active transcription revealed that all active promoters are unmethylated without exception. In contrast, methylation in gene bodies containing the coding regions of the gene, the exons, and the introns seems to positively correlate with gene expression. Interestingly, recently discovered N(6)-Methyladenine is poised at exons of transcribed genes and positively correlates with gene expression.

The methyl moiety on cytosine is further modified by oxidation to 5-hydroxymethyl-cytosine, 5-formylcytosine and 5-carboxylcytosine by ten-eleven translocation (TET 1-3) monoxygenases. The role that these modifications play in controlling gene expression is unclear; however, it is plausible that these oxidized modifications of the methyl moiety provide further fine tuning of epigenetic regulation of genes and are implicated in gene activation during development.
case overlaying methylation and expression, as is commonly done, provides a misleading picture of a highly methylated gene that is highly expressed.\textsuperscript{13} RNApolII that is phosphorylated at serine 5 (PS5) is the form of RNApolII present on promoters that is turning on transcription.\textsuperscript{23} Bisulfite mapping of PS5 bound DNA and input DNA revealed that promoters engaged in transcription were invariably unmethylated. Even when the overall methylation of a promoter at a brain sample was high, PS5 bound DNA was invariably unmethylated (Figure 1).\textsuperscript{13} Thus, the methylation level of a promoter provides a digital count of the fraction of cells in a population where this promoter is silenced. Third, although loss of methylation in a promoter is necessary for expression, it is not sufficient. Demethylation removes a barrier for expression, but expression might be realized at the right time or context when the needed factors or signals are present.\textsuperscript{24} For example, prenatal demethylation of an enhancer of the tyrosine aminotransferase gene in the liver programs the gene to respond to glucocorticoids stimulus postnatally.\textsuperscript{25} This is important for our discussion of how early life stress triggers phenotypes in adulthood.

Gene expression is responsive to time- and context-dependent signals such as neurotransmitters or hormones. DNA methylation anticipates future transcriptional response to triggers; comparing steady-state expression with DNA methylation does not capture the full meaning and scope of the regulatory roles of differential methylation (Figure 2).\textsuperscript{13}

Although this review focuses on DNA methylation and no other epigenetic modifications, it should be noted that DNA methylation and chromatin modifications are interrelated. For example, histone acetylation at H3K9 is often correlated with regions of DNA hypomethylation and both are associated with gene expression (for a review see ref 26). Histone methylation at H3K4, which is a marker of active promoters, inhibits binding of DNA methylating enzymes DNMT3A and DNMT3B, thus keeping these active regions hypomethylated. On the other hand, histone methylation at H3K36 which occurs at actively transcribed gene bodies serves to recruit DNA methylating enzymes DNMT3A and 3B to methylated gene bodies of actively transcribed genes (for a review see ref 27).

**Figure 1.** The same gene could be methylated and silenced in most neurons and unmethylated and highly transcriptionally active in few neurons. Measuring DNA methylation and mRNA expression from such a sample will show high methylation and high expression. However, truly all the expression that we measure comes from a few cells, while in most cells in the sample the gene promoter is methylated and expression is silenced. RNApolII, RNA polII transcription initiation complex; horizontal arrow, transcription; red balloons, methylated sites; horizontal curved lines, mRNA molecules.
Evidence for epigenetic programming by early life social environment; the role of the mother

The first evidence that DNA methylation might be mediating long-term programming of DNA function in response to time-limited social exposure came from studies of the effects of differences in maternal care on life-long stress responsivity in rats. Animals that were reared by a high licking and grooming mother exhibited a more measured stress responsiveness than animals reared by a low licking and grooming mother. The differences in stress behavior between offspring of high and low maternal care were not genetically determined, since this phenotype was transferred by a foster mother as well as the biological (genetic) mother. Differences in maternal care are associated with differences in DNA methylation, histone acetylation, transcription factor binding at the glucocorticoid hormone receptor (NR3C1) gene promoter, and expression in the hippocampus of the adult offspring. Animals that received high maternal care exhibited increased H3K9 acetylation, reduced DNA methylation, and increased expression of the glucocorticoid receptor gene in the hippocampus in comparison with the low-care animals. H3K9 acetylation is a hallmark of active genes and there is a close bilateral relation between increased histone acetylation and reduced DNA methylation.

Epigenetic marks are laid down and maintained by enzymes that either add or remove epigenetic modifications and are therefore potentially reversible in contrast to genetic changes. There is therefore a potential therapeutic angle to the discovery of a causal epigenetic driver of behavioral phenotypes. Indeed, treatment of adult rats with methionine, an upstream source of methyl moieties for methylation reac-

Figure 2. Experience triggered, systemic epigenetic alterations anticipates response to future signals. A model for epigenetic programming by early life stress. Perinatal stress perceived by the brain triggers release of glucocorticoids (GC) from the adrenal in the mother prenatally or the newborn postnatally. GC activate nuclear glucocorticoid receptors across the body, which epigenetically program (demethylate) genes that are targets of GR in brain and white blood cells (WBC). The demethylation events are insufficient for activation of these genes. A-brain specific factor (TF) is required for expression and will activate low expression of the gene in the brain but not in blood. During adulthood a stressful event transiently triggers a very high level of expression of the GR regulated gene specifically in the brain. Horizontal arrow, transcription; circles, CpG sites; CH3 in circles, methylated sites; empty circles, unmethylated CpG sites; horizontal curved lines, mRNA.
tions including DNA methylation, reversed the behavior of high maternal care offspring to a behavior characteristic of low maternal care offspring, while trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), which elevated histone acetylation, triggered loss of DNA demethylation and activation of the glucocorticoid receptor reversed the behavior of offspring of low maternal care, which became similar to offspring of high maternal care mothers. These studies support a causal role for epigenetic programming by maternal behavior and point to potential epigenetic therapeutics for behavioral/psychiatric disorders.

These early studies also provided a plausible mechanism and a working paradigm for further exploration of the molecular links between experience and epigenetic reprogramming. Stimulation of a serotoninergic cAMP-dependent signaling pathway in the hippocampus of offspring by maternal care leads to activation of the transcription factor nerve growth induced factor A (NGFIA), which in turn recruits epigenetic factors such as the histone acetyl transferase (CREB binding protein) CBP and the methylated DNA binding domain protein MBD2 to the Nr3c1 gene resulting in epigenetic programming of the gene.

**Early life adversity and DNA methylation in animal models**

Animal studies confirmed DNA methylation programming by early life experience. For example, methylation of brain-derived nerve growth factor (BDNF) is altered in response to an abusive caretaker in the prefrontal cortex, and arginine vasopressin (Avp) is demethylated in the paraventricular nucleus in response to early life stress. Maternal separation in two strains of mice triggered sex and strain specific anxiety and heightened stress responsivity later in life and DNA methylation differences in Nr3c1, Avp, and Nrda4 promoter regions. These are involved in regulation of the HPA axis: the observed changes in methylation potentially affect regulation of expression of these genes and as a consequence the HPA axis. Prenatal stress in rats was shown to affect DNA methylation and expression in the hippocampus and prefrontal cortex of the Glycoprotein M6A (Gpm6a) gene, which is involved in filopodium motility and possibly synaptic extension. Animal models were also used to link prenatal stress and schizophrenia-like phenotypes later in life in mice such as deficits in social interaction, prepulse inhibition, and fear conditioning. In these animals DNMT1 and 3α and hydroxy-methylation and methylation in the reelin and Gad67 promoters are elevated in GABAergic neurons in the frontal cortex. The effects of reelin and GAD 67 would be consistent with impaired GABA-ergic inhibition, which is seen in models of schizophrenia.

**Early life stress impacts a broad transcriptome and DNA methylation landscape in brain and other tissues; alterations in DNA methylation follow a developmental trajectory and are sex-specific**

Maternal care affects a wide transcriptomic landscape in the hippocampus and broad genomic regions including the entire protocadherin α, β, and γ gene families. Several of the protocadherin genes are associated with neurodevelopment and neuropsychiatric disorders consistent with functional implications for these epigenetic alterations. The response to early life stress and maternal behavior is also not limited to the brain and involves at least the immune system as well. Studies of rhesus macaques revealed differentially methylated regions in both T cells and prefrontal cortex in monkeys who were separated from their mothers after birth. There was a small overlap between differentially methylated regions in brain and immune cells; however, there were notably many tissue specific differentially methylated sites. The immune system has been long known to be impacted by early life stress in both animal models and in humans. The response to behavioral experiences, stress, and adversity early in life is system-wide and is not limited to the brain, while most probably driven by sensing mechanisms in the brain. Several lines of study suggest a bilateral dialogue between immune and neuroendocrine functions in humans and animals and a crosstalk which affects behavior immunity and inflammation.

The placenta is also impacted by maternal social experience and early life stress. Maternal social rank differences in nonhuman primates were associated with broad changes in DNA methylation in placentae. The changes in DNA methylation that were associated with maternal rank found in placentae overlapped with gene expression alterations associated with social rank in adult rhesus monkeys discovered in a different, independent study. These cross-sectional analyses are consistent with the hypothesis that epigenetic markers of early social experience in placentae are predic-
Evidence for epigenetic programming by early life stress in humans

In comparison with animal studies, it is more difficult to obtain evidence in humans for associations of DNA methylation with early life stress. Heterogeneity of human populations results in confounding genetic and environmental factors. Moreover, since the brain is inaccessible for DNA methylation analysis in living humans, most studies are limited to peripheral tissues such as saliva and white blood cells, and the relevance to brain physiology and pathology is uncertain. Obtaining any causal evidence or mechanistic insights linking DNA methylation alterations with physiological function is difficult in human studies, and even temporal relationships between exposure, DNA methylation, and phenotypic outcome mostly rely on cross-sectional design. The low absolute differences in methylation seen in most human behavioral EWAS raise questions about their biological significance.

Several approaches were used to overcome some but not all these inherent difficulties. First, studies used an evolutionary approach and examined whether epigenetic alterations in human post-mortem brain samples replicated observations in rodents and nonhuman primate experiments. For example, the region of the Nr3c1 gene that was differentially methylated by maternal care in rat hippocampus was also differentially methylated by child abuse in post-mortem hippocampi of humans. This overlap extended well beyond the Nr3c1 gene, and a broad evolutionary conserved response was observed across a syntenic locus that covers the Nr3c1 gene and the protocadherin alpha, beta, and gamma gene families.

Although post-mortem studies examine epigenetic programming in physiologically relevant tissues, they represent only a final and single stage that does not capture the dynamic evolution of environments and epigenetic programming in living humans. This is possible only in peripheral tissues such as blood and saliva. Studies have examined in the last decade alterations in DNA methylation in candidate genes in response to maternal stress, early life stress, and early life trauma including the NR3C1 exon 1f region, which corresponds to the region that is altered in hippocampi of rats, the proximal regulator of glucocorticoid receptor FKBP5, BDNF, OXTR, and the serotonin transporter SLC6A4. Low socioeconomic status in childhood was associated with increased methylation in AVP, FKBP5, and OXTR, and two inflammation-related genes CCL1 and CD1D. DNA methylation differences in CD1D and FKBP5 were associated with expression differences and these changes in expression were negatively correlated with changes in DNA methylation.

A third approach was to combine a human blood study with cross-species and cross-tissue (brain/blood) comparisons at multiple time points. Using this approach, the Morc1 gene was found to be differentially methylated in CD34+ cells from cord bloods from children who were exposed to prenatal stress compared with controls, in CD3+ T cells from newborn and adolescent monkeys and in the prefrontal cortex of adult rats. Morc1 was also associated with major depressive disorder (MDD) in a gene-set-based analysis of data from a genome-wide association study, possibly pointing to a link between epigenetic programming by early life stress and psychiatric disorders later in life.

A fourth approach was to combine blood/saliva DNA methylation with brain imaging. Methylation of the serotonin transporter gene SLC6A4 correlated with serotonin synthesis in the orbitofrontal cortex measured with positron emission tomography (PET) and childhood physical aggression.

Broad signature of early life experience in DNA methylation

The epigenetic response associated with early life stress has a broad footprint in DNA methylation in blood and brain. DNA methylation measured in 40 adults enrolled in
Perinatal stress and DNA methylation - Szyf

the 1958 British Birth Cohort showed association with early life socioeconomic status in multiple loci in blood. Socioeconomic status differences were associated with DNA methylation differences in nonhuman primates and humans in placenta at birth. Childhood abuse was associated with methylation in multiple loci in DNA and multiple changes in DNA methylation were observed in a study that compared people who suffered from post-traumatic stress disorder (PTSD) who were also exposed to childhood adversity with those exposed only to adult trauma. Prenatal maternal stress was associated with differential methylation in multiple loci in CD34+ cells from cord blood. However, the functional meaning of small epigenetic changes in methylation in blood in multiple loci is yet unclear.

In contrast to this line of studies that showed consistent but nevertheless small differences in DNA methylation in candidate genes and genome wide analyses, a recent study shed doubt on the association between childhood trauma and DNA methylation changes in blood. The study analyzed the largest number of samples to date and found no evidence for association of DNA methylation in blood with childhood trauma, even in candidate genes that were shown in multiple studies to associate with early life adversity, after correction for confounding factors such as cigarette smoking. The study compels us to replicate and revisit this question.

Natural disasters, a quasi-experimental design for examining causal relations between stress and epigenetic programming

The Quebec 1988 ice storm provided an opportunity to examine the impact of randomized objective stress of mothers on children who were born around the time of the storm. An epigenome-wide association study of T cells obtained from these children 13 years later revealed many changes in DNA methylation that correlated with maternal objective stress, while mothers’ cognitive appraisal of the stress associated with a different set of DNA methylation alterations. Analysis of the functional gene networks that were altered with perinatal maternal stress showed predominantly genes involved in immunity and inflammation, as well as the insulin pathway.

Consistent with the functional network analysis, DNA methylation was found to mediate the effects of prenatal maternal objective stress and mother’s cognitive appraisal on child’s C-peptide in adolescence (a measure of endogenous insulin secretion), as well as the effect of objective prenatal stress on cytokine production in the children. These data are consistent with a causal link between prenatal stress, DNA methylation in immune cells, and metabolic and immune system phenotypes.

What are the mechanisms that translate prenatal stress into DNA methylation alterations in multiple tissues?

A likely suspect to mediate a system-wide epigenetic response to stress is the glucocorticoid stress hormone. Glucocorticoids are released in response to social stress, are distributed systemically, have nuclear receptors that are distributed across the body, and are epigenetic modulators. Other endocrine molecules are also candidates.

In support of this hypothesis, prenatal synthetic glucocorticoid exposure in the guinea pig triggered broad alterations in DNA methylation, histone acetylation, and gene expression in the fetal hippocampus. Further evidence for the involvement of glucocorticoid pathway came from examining the impact of fetal hemizygous depletion of Nr3c1 on DNA methylation in placenta at birth in mice. Nr3c1 heterozygosity leads to altered DNA methylation in a sex-specific manner across multiple sites in the placenta; DNA methylation of several genes in the placenta correlated with anxiety-like behavior in adults, suggesting a temporal relationship between DNA methylation alterations and development of behavioral phenotypes.

A recent study provided evidence for this mechanism in human cells. Exposure of human hippocampal neuron progenitors to glucocorticoids resulted in long-lasting broad changes in DNA methylation, which were not associated with steady-state gene expression. However, re-exposure of the neurons to glucocorticoids resulted in an enhanced response. This is consistent with the idea that methylation defines the potential responsivity of the gene to future triggers. Differential methylation in glucocorticoid-treated hippocampal neurons overlapped with transcriptional response to glucocorticoids in peripheral blood and a polyepigenetic score computed from differentially methylated sites in neurons predicted exposure to prenatal glucocorticoids in newborn cord blood. These data further confirm
correspondence of brain blood DNA methylation responses to glucocorticoids.

**Summary and perspectives**

Animal studies have established plausible molecular pathways between perinatal social exposures, DNA methylation changes, and phenotype. Human studies have been criticized, however, for the small differences in DNA methylation, the small sample sizes, and confounding genetic heterogeneity and other common environmental exposures such as age, smoking, and alcohol. Indeed, a recent large epigenome-wide methylation study failed to confirm previously documented associations with early life trauma, casting doubt on previously described associations in peripheral tissues.67

One of the most criticized facets of studies of human early adversity epigenetics is the use of blood or white blood cells for studying behavior, a brain function. Inaccessibility of the brain in living humans precludes, except for post-mortem studies, examination of “functionally relevant” epigenetic profiles. Studies therefore examined blood or saliva DNA as a surrogate tissue. The assumption is that certain DNA methylation profiles show the same inter-individual differences in blood and brain or saliva and brain. Several studies have delineated such sites,76,77 and some studies use these comparisons as a guide. Methylation differences in “brain-specific genes” were reported in blood in several human studies. However, it is unclear whether corresponding changes exist in the brain.

Animal studies and experiments in human hippocampal neurons provide a plausible mechanism linking perinatal stress and changes in DNA methylation across several tissues. Involvement of the stress hormone in triggering these changes could explain how a stress sensed by the brain can have a systemic effect across several tissues and target overlapping sites in different tissues, since glucocorticoids have nuclear receptors in many tissues and overlapping glucocorticoid recognition elements in different tissues. It is possible then that DNA methylation changes only poise genes for expression,24 while the presence of a brain-specific transcription factor activates the gene in the brain. Therefore, although changes in methylation in brain specific genes in response to early life stress happen in several tissues, they will be functionally relevant only in the brain. Thus, absence of correlation between DNA methylation alterations and steady-state expression in peripheral tissues does not necessarily imply that the changes in DNA methylation are functionally irrelevant. In addition, even in the brain, DNA methylation alterations might be programming genes for response to future triggers such as glucocorticoids or neuronal activation, and this might not be reflected in steady-state transcription (Figure 2).

Questions remain on the functional meaning of the changes in DNA methylation for either brain function or peripheral physiological functions. These could be addressed by linking the genes affected to central phenotypes using imaging and mediation analyses in humans, as was described above, as well as pharmacology, targeted gene depletion, or epigenetic editing in animals or primary human hippocampal cultures. Studies of natural disasters provide quasi-experimental designs to draw a causal link between stress and changes in epigenetic states and mediation analyses define a causal link between early life stress, DNA methylation changes, and the phenotype. Although the Quebec ice storm study provides preliminary support for a causal link between perinatal stress, DNA methylation, and phenotypic changes in adults, further similar studies are needed. One of the main difficulties in relating DNA methylation alterations to phenotype is how to link DNA methylation changes in multiple genes with a specific phenotype, since current methods in genetics and pharmacology focus on specific targets.

Although the focus in the field is mainly on examining “brain relevant” DNA methylation marks in peripheral tissue, it is suggested here that the response to stress is systemic and that the immune system and brain are interactive partners in a coordinated developmental and physiological response. Moreover, early life stress is known to impact immune,78 cardiovascular, and metabolic disorders.79 DNA methylation alterations in the immune system are therefore an important component of the system-wide response to perinatal stress. Examining how peripheral changes in DNA methylation mediate changes in immunity and inflammatory systems and how they interact with the brain are critical for a comprehensive understanding of epigenetic programming by early life stress and its functional consequences.

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Original article

Transcriptional mechanisms of drug addiction

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Drugs of abuse can modify gene expression in brain reward and motivation centers, which contribute to the structural and functional remodeling of these circuits that impacts the emergence of a state of addiction. Our understanding of how addictive drugs induce transcriptomic plasticity in addiction-relevant brain regions, particularly in the striatum, has increased dramatically in recent years. Intracellular signaling machineries, transcription factors, chromatin modifications, and regulatory noncoding RNAs have all been implicated in the mechanisms through which addictive drugs act in the brain. Here, we briefly summarize some of the molecular mechanisms through which drugs of abuse can exert their transcriptional effects in the brain region, with an emphasis on the role for microRNAs in this process.

Keywords: cocaine; addiction; striatum; microRNA; MeCP2; BDNF; transcription factor

Introduction

Dorsal and ventral domains of the striatum receive extensive input from midbrain dopamine neurons, and this meso-accumbens dopamine system has been heavily implicated in addiction-relevant behavioral abnormalities. Dopamine triggers plastic responses in the so-called direct pathway (striatonigral) and indirect pathway (striatopallidal) medium spiny neurons (dMSNs and iMSNs, respectively) of the striatum. While drugs of abuse differ in their pharmacological mechanisms of action, chronic exposure can often result in common molecular adaptations in MSNs, likely related to the fact that all major addictive drugs stimulate dopamine. It has been hypothesized that regulation of gene expression by transcriptional and post-transcriptional mechanisms plays a key role in the long lasting changes in brain function by drugs of abuse that precipitate the emergence of the behavioral abnormalities that define addiction. To date, investigation of the transcriptional actions of drugs of abuse have focused on only a small fraction of the transcription factors that are likely to be involved. Beyond transcription, post-transcription mechanisms of gene regulation are increasingly recognized as important regulatory factors in addiction-relevant neuronal plasticity. Below, we briefly summarize findings on some of the most thoroughly explored transcription factors implicated in the transcriptional actions of addictive drugs in the striatum. In addition, we also summarize recent findings on the contribution of microRNAs, which are post-transcriptional regulators of gene expression, to the actions of addictive drugs.

FosB

ΔFosB is a member of the Fos family of transcription factors encoded by the fosB gene. ΔFosB can heterodimerize with the Jun family of proteins to form Activator Protein 1 (AP-1) complexes, which bind to AP-1 elements in the promoters of genes that contain AP-1 response elements to regulate their transcription. ΔFosB has a truncation on its C-terminal relative to other forms of FosB,
which renders it less sensitive to protein degradation and hence increases its relative stability compared with other Fos family proteins.\textsuperscript{3,4} Moreover, in vitro and in vivo studies have revealed that ΔFosB phosphorylation further stabilizes it.\textsuperscript{5,6} This makes it an important candidate for transcriptional regulation in the context of regulating addiction-related behaviors, as its relative stabilization and long biological half-life provides a molecular mechanism by which changes in gene expression can persist for weeks or even longer after drug consumption.

The Fos family proteins are expressed in response to exposure to all known drugs of abuse. Levels of ΔFosB are increased in various reward-related regions of the brain, especially the striatum,\textsuperscript{2,3,7} in response to repeated consumption of addictive drugs. Induction of ΔFosB in the ventral striatum (nucleus accumbens; NAc) and dorsal striatum by drugs of abuse is thought to occur exclusively in dMSNs,\textsuperscript{1,3} with this action linked to addiction-related behavioral abnormalities. Indeed, dMSN-specific overexpression of ΔFosB in NAc of mice increases sensitivity to the stimulant and reward-related properties of cocaine.\textsuperscript{8-10} Virus-mediated manipulation of ΔFosB in the striatum suggests that it plays a key role in many of the transcriptional changes that are triggered by addictive drugs. In addition to addictive drugs, natural rewards can also induce ΔFosB in dMSNs in NAc.\textsuperscript{3,10-12} Notably, ΔFosB has a long half-life compared with other members of the Fos family of proteins. Consequently, levels of ΔFosB accumulate in the NAc and other addiction-related brain regions in response to repeated drug use, resulting in greater levels after prolonged drug use compared with acute use. This provides a transcriptional mechanism through which chronic drug exposure has enduring actions on the function of brain reward systems that persists after the initial pharmacological actions of the drug have waned. Thus, ΔFosB is hypothesized to regulate transcriptional responses to natural rewards, with maladaptive recruitment of this transcription factor for prolonged time periods by drugs of abuse driving transcriptional plasticity that leads to addiction.

Significant progress has been made in identifying transcriptional targets through which ΔFosB influences behavioral responses to drug of abuse. These include genes related to dendritic spine structure such as synaptotagmin, activity-regulated cytoskeleton-associated protein (ARC), microtubule associated proteins (MAPs), cyclin-dependent kinase-5 (CDK5), and kinesin.\textsuperscript{13-15} There have been reports implicating ΔFosB in glutamatergic signaling via modulating AMPA receptors (AMPARs).\textsuperscript{9,16} This is consistent with the belief that ΔFosB mediates aspects of synaptic plasticity in MSNs after drug exposure.\textsuperscript{17,18} ΔFosB also serves as an important epigenetic modulator. More specifically, it can serve as a molecular switch to enhance (CDK5 expression in response to cocaine) or repress (fos in response to amphetamine) gene expression by binding to specific promoters and recruiting epigenetic modulators.\textsuperscript{2,19,20}

Taking all these studies together, it has been postulated that by regulating a number of transcriptional as well as epigenetic regulatory proteins, this transcription factor may serve as a master regulator of drug-induced changes in both structural and synaptic plasticity.\textsuperscript{17,21-23}

Cyclic AMP response element binding protein

The cyclic AMP response element (CRE)-binding protein (CREB) family of transcriptional activators plays important roles in the control of cellular metabolism, growth-factor-dependent cell survival, the function of brain circuits involved in the regulation of complex physiological processes such as learning and memory, and pathophysiological processes such as anxiety and drug addiction. Consistent with these diverse functions, CREB proteins are activated by phosphorylation at Ser133 in response to a number of signaling pathways, including mitogenic stimuli, cAMP, calcium and stress. Phosphorylation of CREB facilitates its nuclear translocation and subsequent binding to the scaffolding protein CREB-regulated transcription coactivator (CRTC), which plays a key role in recruiting other transcriptional factors to trigger plasticity-relevant gene expression in the striatum and other addiction-relevant brain sites has increased dramatically.
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components of the coactivator complex. This interaction between CREB and CRTC promotes expression of genes that express CRE-response elements through the recruitment of coactivators such as CREB-binding protein (CBP) and p300. CRTC can be deactivated by deacetylation by class III histone deacetylases (HDACs) such as sirtuin1 (SIRT1), which decreases CREB signaling.24

Perturbations in CREB function in the striatum are thought to be involved in addiction-related transcriptional plasticity. Recurring exposure to drugs such as cocaine increases the activity of the cAMP–PKA pathway in the NAc,25 which induces activation of CREB-mediated transcription of target genes.14,26 Work from the Nestler group has shown that CREB signaling, and major components of this signaling cascade, are important regulators of cocaine reward in the NAc and the transition to compulsive cocaine use mediated by the dorsal striatum, reflected by escalating levels of drug intake and consumption that persists despite negative consequences. Infusion of cAMP analogs that activate PKA into the NAc of rats causes a time-delayed increase in intravenous self-administration (IVSA) behavior and shift the cocaine dose-response curve to the right, consistent with decreased sensitivity to the rewarding properties of the drug. Furthermore, inhibition of PKA signaling in the NAc can shift the dose-response curve for intravenous cocaine infusions to the left, suggesting that PKA regulates the rewarding property of the drug.27

Evidence for a direct role for CREB came from their subsequent studies where viral mediated upregulation of CREB in rat accumbens resulted in reduction in the rewarding properties, while expression of a dominant negative CREB increased the rewarding properties of cocaine.28 In addition further studies from their group showed similar effects of CREB modulation on the rewarding properties of morphine.29 Similar observations were also observed in mouse models activating or inhibiting CREB function.

Extended (6 h) daily access to intravenous cocaine self-administration can precipitate a compulsive-like increase in consumption, a process termed escalation of intake. Rats that demonstrate escalation of cocaine intake show increased levels of phosphorylated CREB in the dorsal striatum. It has been shown that overexpression of the CREB coactivator CRTC1 in dorsal striatum blocks the emergence of escalated levels of cocaine intake in rats with extended daily access to the drug but does not alter the stable intake seen in rats with restricted (1 h) daily access.30 This may suggest that engagement of CREB/CRTC-mediated transcription in the dorsal striatum of rats with extended access to cocaine may serve as a “protective” homeostatic response that counters the rewarding and motivational actions of cocaine. This interpretation is consistent the fact that CREB signaling in the NAc has also been shown to oppose the rewarding and motivational effects of cocaine28 which likely reflects CREB-induced increases in the transcription of the anti-reward endogenous opioid dynorphin and related genes. In addition to CREB signaling, extended daily access to cocaine also increases levels of brain-derived neurotrophic factor (BDNF) and the transcriptional repressor methyl CpG-binding protein 2 (MeCP2) in the dorsal striatum.31 Moreover, virus-mediated knockdown of BDNF or MeCP2 in the striatum reduced cocaine intake in rats with extended but not restricted daily access to the drug.31 Since both BDNF and Mecp2 are CREB-responsive genes, these observations suggest that CREB, MeCP2, and BDNF may represent components of a larger transcriptional mechanism in the striatum that modulates the addiction-relevant actions of cocaine. These findings reflect the complexity of CREB signaling in the striatum, where it can stimulate the transcription of dynorphin and other anti-reward genes yet also promote the expression of BDNF and other genes that can enhance reward processes. Hence, the balance between CREB-induced increases in the expression of pro- and anti-addiction transcriptional programs likely plays a key role in determining vulnerability to, and the emergence of, drug addiction (Figure 1).

In addition to MeCP2 and BDNF, CREB regulates the expression of many other genes in the striatum that are involved in synaptic plasticity, neuronal growth, and cell adhesion. These include genes involved in transmission (syntaxin1A, dynorphin), intracellular signaling (adenylyl cyclase VI), and cell growth (BDNF), as well as other gene modulators such as c-Fos and Mef2.14 Many of these genes are very likely involved in regulating addiction-related plasticity in the striatum. For example, the genes encoding the subunits of NMDA receptors (GluN1 and GluN2B) contain a CREB-binding site, and it has been reported that in accumbens slice cultures, activation of CREB increases protein levels of both GluN1 and GluN2B subunits, but not GluN2A subunits.32,33 As additional molecular targets of CREB in the striatum and other addiction-related brain
areas are identified, characterization of their contribution to drug-related behaviors will further help us by providing information on how different drugs of abuse may reshape addiction-related circuits. Indeed, much work remains to be done to fully understand how CREB and related transcriptional regulators influence the emergence of drug addiction and their role in vulnerability versus resilience to this disorder.

**Myocyte enhancing factor-2**

The myocyte enhancing factor-2 (MEF2) family of transcription factors is comprised of four nuclear proteins, named MEF2A-D, that were originally identified in muscle tissue. Later, these transcription factors were shown to be expressed in neurons of various regions of the brain, including MSNs in the striatum. MEF2 regulates the tran-
scription of a wide range of genes. Flavell and colleagues used a genome-wide targeting strategy to identify activity-dependent MEF2 targets, including genes that regulate synapse development and function. These genes contribute to synapse weakening (eg, Homer 1a, kcnal, and kcn4 potassium channels) as well as synapse strengthening (eg, BDNF and adenylyl cyclase VIII), suggesting that MEF2 exerts a complex action in synaptic plasticity. The members of this family can form either homodimers or heterodimers to modulate gene expression. Depending on the protein recruited by these complexes, they can act either as activators or repressors.2,37

In a study by Pulipparachauvil and colleagues, cocaine was shown to suppress striatal MEF2 activity, with this action related to cocaine-induced activation of a D1 receptor–cAMP-dependent signaling event that resulted in inhibition of calcineurin, a Ca²⁺-dependent protein phosphatase. This reduction in MEF2 activity played a key role in cocaine-induced increases in MSN dendritic spine number, but appeared to oppose sensitization to the locomotor stimulatory actions of the drug.2,37 This finding also speaks to the current uncertainty about the role for drug-induced increases in the structural complexity of MSNs in response to drugs of abuse, with some studies suggesting that such plasticity contributes to addiction-related behavioral abnormalities and other studies suggesting that such plasticity protects against addiction. Cocaine increases MEF2C expression in rat cortex and striatum. Cocaine is thought to activate SILK1, which in turn phosphorylates and thereby inactivates HDAC5, which then de-represses MEF2 transcription.38 As MEF2 is known to play an important role in regulating structural and synaptic plasticity, cocaine-induced increases in MEF2 activity are likely involved in addiction-relevant transcriptional plasticity in response to cocaine exposure.

**Other transcription factors**

The transcription factors discussed above are those that have been most extensively investigated in the context of drug addiction. Others transcription factors, including NF-κB,39,40 glucocorticoid receptor (GR), nucleus accumbens 1 transcription factor (NAC1), and signal transducers and activators of transcription (STATs), have also been implicated in transcriptional responses to cocaine and other drugs of abuse. The role for GR in stress and psychiatric disorders is well characterized.31-44 Levels of the mRNA of NAC-1 are increased in the rat forebrain weeks after cocaine exposure.45,46 Virus-mediated overexpression of NAC-1 in the NAc of rats prevented the development (but not the expression) of locomotor sensitization in response to repeated administration of cocaine, suggesting that it may have a homeostatic, compensatory role similar to that described above for CREB. Thus, it has been suggested that elevated levels of NAC1 in the accumbens may influence expression of behaviors sometimes seen in drug addiction, particularly in those dependent on psychomotor stimulants, such as paranoia, by regulating gene transcription.47 More recently, a study by the Nestler group has implicated E2F3a in regulating cocaine action in accumbens.48 Some of the other transcription factors associated with addiction-like behaviors include Npas4,49 PGC1-α50 SMAD3,51 Egr3,52 and BRG1,53 to name but a few. With the advent of new sequencing technologies the list of transcriptional processes shown to be impacted by addictive drugs such as cocaine is likely to grow and further highlight the key importance of gene regulatory processes in addiction. In particular single-cell sequencing technologies are likely to reveal gene expression programs that are recruited by drugs of abuse in a cell type-specific manner to drive the emergence of addiction-related behavioral abnormalities.

**MicroRNAs**

MicroRNAs (miRNAs), which in their mature form are ~22 nucleotides long, are perhaps the best characterized class of functional non-protein coding regulatory RNA. miRNAs are important regulators of gene expression at the post-transcriptional level, and hundreds of miRNAs have been identified across mammalian species. The latest miRbase entry includes over 2500 human miRNAs,54 with more than 60% of human protein-coding genes thought to be under miRNA control.55 Therefore, miRNAs participate in the control of virtually all important physiological processes. The canonical mode of miRNA action is believed to be via binding of the miRNA through its so-called “seed sequence” to complementary sequence in the 3’ untranslated region (3’UTR) of gene transcripts, leading to mRNA degradation or translational repression.56,57 Most of the target prediction algorithms that predict transcripts regulated by miRNAs rely on conservation of putative target sequences to identify functional miRNA binding sites. A large number of such algorithms
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Several algorithms have been developed, including Targetscan, MiRanda, and TarBase. These algorithms can facilitate the identification of miRNA binding sites in the 3’UTRs of target genes and enable the discovery of functional miRNA-mRNA interactions. Importantly, other poorly characterized factors are likely to influence miRNA-mRNA interactions and search algorithms are likely to improve as these factors become better understood. As described below, miRNAs are emerging as important regulators of drug-induced plasticity in the brain.

MicroRNAs in addiction

One of the first studies to investigate the role for miRNAs in the addiction-related actions of cocaine was published by Schaefer and coworkers, who established that cocaine can induce robust alterations in the expression of a large number of miRNAs in the striatum. A subset of these cocaine-responsive miRNAs were shown to regulate the expression levels of genes implicated in addiction, including BDNF, FosB, and Cdk5r1. They showed that genetic ablation of Argonaute 2 (Ago2), which is the major catalytic unit of the RNA-induced silencing complex (RISC), in iMSN of the striatum dramatically reduced rewarding effects of cocaine in mice. This is important because the RISC complex plays an important role in regulating the suppressive effects of miRNAs on target transcripts and, in some cases, also regulates the maturation of miRNAs. The Ago2-iMSN mutant mice also demonstrated reduced levels of cocaine self-administration compared with their wild-type littermates across the entire dose-response curve, suggesting that the motivation to consume cocaine was decreased in the mutant animals. These findings suggest that Ago2, and the miRNAs that require Ago2 to function, play an important role in controlling the behavioral actions of cocaine.

miR-212/132 cluster in addiction

Previous studies from our group have shown that miR-212 plays a critical role in regulating compulsive cocaine intake in rats. We showed that miR-212 (and also miR-132) was upregulated in the dorsal striatum of rats with a history of cocaine consumption under extended (6 h) daily access conditions. By contrast, miR levels were unaltered in rats with restricted daily access to cocaine and in rats that received non-volitional cocaine infusions time-locked to rats that volitionally consumed cocaine during extended access conditions, compared with cocaine-naive control rats. Importantly, it is the extended access rats that show compulsive-like cocaine-taking behaviors. Virus-mediated overexpression of miR-212 in the dorsal striatum dramatically decreased cocaine intake in rats with extended daily access but had no effects in rats with restricted daily drug access. In addition, we detected a dramatic downward shift in the dose-response curve of cocaine infusions in the extended access rats in which miR-212 was overexpressed, suggesting that their motivation to consume cocaine was markedly decreased. Conversely, antisense oligonucleotide-mediated inhibition of miR-212 signaling in the striatum, increased cocaine intake in rats with extended but not in those with restricted daily access to the drug. These findings suggest that stri-
miR-212 signaling may protect against the actions of cocaine that drive the emergence of compulsive consumption of the drug in much the same way that striatal CREB signaling also protects against the addiction-related actions of cocaine (see above).30 (Figure 1).

As CREB opposes the motivational properties of cocaine,9,28 and CREB can stimulate increases in miR-212 expression30 (Figure 1), we tested the hypothesis that miR-212 signaling in the dorsal striatum may inhibit cocaine consumption under extended access conditions by recruiting and enhancing striatal CREB activity through a positive feedback loop. Indeed, we found that miR-212 dramatically enhances CREB signaling both in vitro and in vivo in the striatum.30 This suggests that miR-212 expression is induced in the striatum by cocaine, then subsequently sensitizes CREB signaling, which attenuates the motivational properties of the drug through expression of other CREB-responsive genes. Precisely how does miR-212 regulate CREB signaling? We found that miR-212 increases the activity of Raf1 kinase by repressing the Raf1 inhibitor SPREAD1 (Sprouty-related, EVH1 domain-containing protein 1). This results in enhanced Raf1 activity, which in turn phosphorylates and sensitizes the activity of adenyl cyclases in the striatum.30 These observations suggest that miR-212 controls cocaine intake in part by amplifying the CREB activity.

In a follow-up study, we investigated the mechanisms through which cocaine regulates miR-212 levels in the striatum.31 The miR-212 gene is located in a CpG-enriched region of the genome, suggesting that DNA methylation may play a role in its expression. MeCP2 binds to methylated DNA and can serve as a repressor of gene expression by recruiting transcriptional repressors.65 Therefore, we hypothesized that MeCP2 plays a role in cocaine-induced regulation of miR-212 expression in striatum. Consistent with this hypothesis, we found that knockdown of MeCP2 increased miR-212 expression in cultured cells.31 Similarly, RNA interference-mediated knockdown of MeCP2 transcripts in the striatum increased the stimulatory effects of cocaine on miR-212 expression and markedly reduced cocaine intake in rats with extended access to the drug. These data suggest that MeCP2 exerts an inhibitory effect on miR-212 expression and, as such, may promote the development of addiction by limiting the protective actions of miR-212 against cocaine-induced plasticity in the striatum31 (Figure 1). The miRNA miR-132 is closely related to miR-212, and they both share the same “seed” sequence, thought to be critical for target specification. Importantly, miR-132 was shown to inhibit MeCP2 expression by direct binding with the 3'UTR66 of MeCP2 transcripts in brain. Based on this observation, we hypothesized that miR-212 may also inhibit MeCP2. Consistent with this hypothesis, miR-212 was shown to exert a negative influence over MeCP2 expression in vitro and in the striatum in vivo.31 These findings suggest that a negative feedback loop exists between miR-212 and MeCP2 in which each inhibits the expression of the other. Based on this finding, we hypothesize that homeostatic interactions between miR-212 and MeCP2 may play a critical role in determining vulnerability to compulsive cocaine use addiction. Currently, little is known about the role for miR-212 in regulating other behavioral abnormalities associated with cocaine use, such as sensitization of locomotor activity, withdrawal-related symptoms, or vulnerability to relapse-like drug-seeking during periods of abstinence.

Summary

Over recent years, our understanding of how drugs of abuse recruit transcription factors to trigger plasticity-relevant gene expression in the striatum and other addiction-relevant brain sites has increased dramatically. We have also gained considerable insights into how noncoding RNAs and other post-transcriptional regulatory mechanisms control gene expression and thereby regulate the actions of drugs of abuse in the striatum. Emerging technologies, such as single-cell sequencing, are likely to further revolutionize our understanding of how addictive drugs remodel brain reward and motivation circuitries. Critical will be identification of approaches to leverage this information into the development of new therapeutics that can modulate or even reverse the transcriptional actions of abuse drugs in the striatum and elsewhere in the brain to facilitate cessation effects and combat addiction. New approaches to modulating transcription factors, RNAs, and the protein machineries through which they act, in a safe and effective manner, will be required to translate these important insights into new therapeutics to combat drug addiction.

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42. Ambroggi F, Turiault M, Milet A, et al. Stress and addiction: glucocorticoid receptor in dopami-
Epigenetics as a key link between psychosocial stress and aging: concepts, evidence, mechanisms

Anthony S. Zannas, MD, MSc, PhD

Psychosocial stress—especially when chronic, excessive, or occurring early in life—has been associated with accelerated aging and increased disease risk. With rapid aging of the world population, the need to elucidate the underlying mechanisms is pressing, now more so than ever. Among molecular mechanisms linking stress and aging, the present article reviews evidence on the role of epigenetics, biochemical processes that can be set into motion by stressors and in turn influence genomic function and complex phenotypes, including aging-related outcomes. The article further provides a conceptual mechanistic framework on how stress may drive epigenetic changes at susceptible genomic sites, thereby exerting systems-level effects on the aging epigenome while also regulating the expression of molecules implicated in aging-related processes. This emerging evidence, together with work examining related biological processes, begins to shed light on the epigenetic and, more broadly, molecular underpinnings of the long-hypothesized connection between stress and aging.

Keywords: aging; DNA methylation; epigenetics; inflammation; psychosocial stress; telomere

Introduction

In Hugo’s fictional work Les Misérables, an extreme stressor causes the main character, Jean Valjean, to undergo accelerated aging, depicted as rapid whitening of his hair. This dramatic depiction is just one among innumerable examples—found in literary works, movies, and folklore legends—of individuals whose “biological clocks” appear to tick fast in the face of life adversity. Beyond fiction, however, the connection between psychosocial stress and rate of biological aging is also seen in everyday life and clinical practice; for example, past US presidents notoriously exhibited signs of accelerated aging during their time in office, and individuals with stress-related psychiatric disorders often appear older than their stated age. Furthermore, several epidemiological studies have now grounded these observations on scientific evidence, linking psychosocial stress and related psychiatric conditions, such as major depressive and posttraumatic stress disorder, with increased risk for a number of aging-related disease states.

"It was he [Jean Valjean] in fact. The clerk's lamp illuminated his countenance. He was pale and he trembled slightly. His hair, which had still been gray at his arrival [to the court], was now entirely white; it had turned white during the hour he had sat there."

Victor Hugo, Les Misérables

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Together these observations raise important questions: Can the effects of stress on the aging process be quantified with biological measures? Do such biological effects shape health and disease outcomes? And could we, one day, prevent or even reverse stress-related aging for the benefit of individuals and societies?

The concept of accelerated biological and epigenetic aging: links with disease risk and stress burden

Aging is the single most important risk factor for several disease states that are currently the leading causes of morbidity and mortality, including cardiovascular disease, neurodegenerative disorders, and cancer.8 Yet individuals of the same age exhibit substantial variability in their propensity to develop aging-related disease.7 This variability could be in part explained by individual discrepancies between chronological age, the units of time elapsed since birth, and biological age, the wear-and-tear biological events thought to accrue throughout life and confer disease risk.10,11 The idea that biological aging may better reflect disease risk has spurred efforts to develop molecular measures of biological age using telomere length,12 epigenomic patterns,13,14 transcriptomic signatures,15 or proteomic profiles.16 Moreover, accelerated biological aging—i.e., having biological age more advanced than chronological age—has been hypothesized to confer disease risk beyond that associated with chronological age itself. This hypothesis has been vigorously tested using the so-called measures of epigenetic aging, composite markers that accurately predict age by combining in a statistical regression model the DNA methylation levels of multiple genomic sites. Such studies have indeed linked accelerated epigenetic aging with a host of aging-related disease outcomes, including all-cause mortality in late life,17 physical and cognitive impairment,18 cancer incidence,19 frailty in older ages,20 dementia,21 and others.22 Similar associations have been observed with other measures of accelerated biological aging, such as telomere shortening.23 Overall this work supports a model whereby variability in the rate of biological aging may underlie differences in disease risk across individuals.

Therefore, uncovering factors that explain interindividual variance in biological aging can yield novel insights into the pathogenesis of aging-related disease. As conceptually depicted on Figure 1, age predicted with biological measures, such as epigenetic age, generally correlates well with chronological age at the population level; yet the two can substantially differ for certain individuals, even when using the most accurate multi-tissue predictor of epigenetic age, developed by Steve Horvath.13 Individuals can thus be conceptualized to follow different trajectories, depending on whether their biological aging is accelerated (biological age > chronological age; orange continuous line) or decelerated (biological age < chronological age; blue continuous line). Such interindividual variance in biological aging may be explained by genetic factors that confer risk or resilience, environmental factors such as psychosocial stress, and their complex interplay. For illustrative purposes, the figure highlights two individuals with the same chronological age and either accelerated (orange circle) or decelerated biological aging (blue circle).
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Ology, contributing to negative outcomes. Given that stress is ubiquitous in modern fast-paced societies, gaining mechanistic insights into how it influences biological aging has important implications for prevention and treatment.

Among metrics of accelerated biological aging that may be influenced by stress exposure, the largest body of work to date has examined telomere length. Following the seminal work by Epel and colleagues linking chronic caregiver stress with telomere shortening, several studies found similar associations with other types of stress. A detailed account of these studies is beyond the scope of this article, but types and measures of stress examined to date in relation to telomere function include perceived stress, intraterine and early-life stress, work-related exhaustion, and major depression. Although these associations have not been replicated in all populations studied, meta-analyses have corroborated that higher stress burden and related phenotypes are associated with telomere attrition.

More recently, a number of studies have linked psychosocial stress with biological aging assessed using metrics of epigenetic age. In particular, acceleration in epigenetic aging has been observed with various types of early-life stress, including exposure to harsh parenting during childhood and adolescence, perceived racial discrimination during adolescence, childhood exposure to violence and threat, early-life socioeconomic disadvantage, and childhood abuse. Although lack of association with early-life stress has also been reported, a recent meta-analysis corroborated an overall positive but small relation between epigenetic age acceleration and childhood stress. Likewise, accelerated epigenetic aging has been associated with stress exposure in adulthood such as deployment to combat, financial hardship and socioeconomic disadvantage, and cumulative lifetime stress; however, lack of relation has been also reported with adulthood socioeconomic status and lifetime trauma. Lastly, some studies have found positive relations of epigenetic age acceleration with diagnosis of major depression and lifetime severity of posttraumatic stress disorder, whereas others noted no relation with major depression and nonsignificant or even inverse relations with posttraumatic stress diagnosis and symptoms. Such discrepancies across studies could result from heterogeneity in their design and methodology, including differences in the sociodemographic characteristics of the study participants, the definition of stress exposure and related outcomes, and the time lag between stress exposure and epigenetic assessment.

Additional work has linked stress with other measures of biological aging, such as oxidative stress, mitochondrial dysfunction, and N-glycosylation. Oxidative stress, a process that can impact aging through cumulative damage in macromolecules and cells, has been shown to increase with stress exposure and in stress-related psychiatric disorders. Mitochondrial dysfunction, determined with measures such as mitochondrial DNA levels and enzymatic activity, has been associated with both stress exposure and depressive symptoms. N-glycosylation, a marker that accumulates with advancing age, was found to increase in trauma-exposed individuals and persons with post-traumatic stress disorder. Taken together and notwithstanding discorndances across studies, this body of work links certain types of stress—most notably early-life, cumulative, chronic, and excessive stress—and related psychiatric disorders with accelerated aging determined using diverse molecular measures. This conclusion is important because it elucidates—at a deeper, molecular level—the previously known epidemiological connection between stress burden and risk for aging-related disease. It further supports the notion that future research endeavors should aim to determine the extent to which psychosocial and other environmental stressors as well supportive and nurturing environments contribute to interindividual variance in biological aging (Figure 1). While the multilayered associations reported above suggest a complex interplay among many molecular processes, the following section will discuss in more depth the role of epigenetics as a mechanistic link between stress and aging.

Epigenetic mechanisms linking psychosocial stress and aging

Epigenetics—a composite Greek word derived from the prefix “epi,” meaning “upon,” and “genetics”—encom
passes an ever-growing repertoire of biochemical processes that together regulate gene function without changing the genetic code itself. These processes include covalent DNA modifications, such as methylation of cytosine-guanine dinucleotides (CpG), posttranslational histone modifications, regulation of transcription by noncoding RNAs, and higher-order changes in chromatin conformation.\textsuperscript{55} These processes are known to not only direct cellular differentiation but to also respond to environmental triggers, including psychosocial stressors at various life stages,\textsuperscript{56} and to regulate genomic function, thereby acting as a molecular interface between environments and genomes. Epigenetic changes can thus be conceptualized as molecular switches or, better still, as rheostats that are set into motion by environmental stressors and can in turn fine-tune gene activity and cell function (Figure 2). While these “genomic rheostats” can respond dynamically to stressors,\textsuperscript{57} stress-related and other epigenetic signatures can also persist in time,\textsuperscript{56} having potentially lasting consequences on cell function and complex phenotypes. Epigenetic regulation has been implicated in many such phenotypes and is thought to represent a hallmark of the aging process.\textsuperscript{58} Although the various players of the epigenetic machinery act in concert, the rest of this section will largely focus on CpG methylation, which with the advent of microarray technology has become the most studied epigenetic modification in humans.

**Figure 2.** Simplified scheme that highlights epigenetic mechanisms linking psychosocial stress and aging. Stress exposure culminates in peripheral secretion of cortisol, the primary glucocorticoid in humans. The genomic effects of cortisol are largely driven by the glucocorticoid receptor (GR), a ligand-dependent transcription factor able to induce epigenetic modifications, such as DNA methylation changes, with potentially lasting impact on genomic function. These epigenetic modifications can thus be conceptualized as switches or rheostats that are set into motion by stress and can in turn fine-tune activity of susceptible genes. Stress-induced epigenetic changes can deregulate the expression of effector molecules, eg, FKBP5, and influence downstream biological pathways implicated in aging-related processes. Beyond such effects at selected gene loci, stress can also induce composite (systems level) effects on the aging epigenome, as captured, for example, by measures of epigenetic aging. Importantly, psychosocial stress and epigenetic mechanisms also influence other processes implicated in aging-related phenotypes, including inflammation, telomere function, oxidative stress, and mitochondrial function. Many of these relations are reciprocal and subject to regulation by feedback and feedforward loops. For simplicity, cortisol is depicted as a yellow hexagon and CpG methylation as an orange circle on a stick.
Psychosocial stressors trigger a set of behavioral, neural, hormonal, and molecular processes. Among such processes, previous work supports a key role for glucocorticoids, hormones secreted in the peripheral circulation upon exposure to stress. The genomic effects of glucocorticoids are largely driven by the glucocorticoid receptor (GR), a ligand-dependent transcription factor found in essentially every body tissue. These genomic effects can be mediated either by direct binding of the GR to glucocorticoid response elements (GRE)—thousands of conserved DNA sequences scattered throughout the genome—or by interactions with other transcription factors via GRE-dependent or -independent mechanisms. Notably, both stress and glucocorticoids can induce not only acute changes in gene transcription but also lasting epigenetic modifications, most notably changes in DNA methylation. These lasting effects are thought to represent a form of epigenetic memory that influences subsequent genomic function and stress-related outcomes.

The role of glucocorticoid signaling as a link between stress and epigenetic aging has been supported by two studies utilizing the Horvath measure of epigenetic age. In the first study, cumulative lifetime stress was positively associated with accelerated epigenetic aging in a highly traumatized cohort, and the CpGs comprising the Horvath measure were found to colocalize with GREs more often than expected by chance, indicating their susceptibility to stress and glucocorticoid exposure. Accordingly, treatment with a synthetic glucocorticoid induced DNA methylation changes at several epigenetic age CpGs as well as dynamic transcriptional changes in the majority of their neighboring genes. Corroborating these observations, another study found that greater total daily cortisol, the primary glucocorticoid in humans, is associated with accelerated epigenetic aging in a cohort of adolescent females. Notably, the glucocorticoid-regulated genes neighboring the epigenetic age CpGs were also associated with aging-related diseases, including coronary artery disease, arteriosclerosis, and leukemias. Together these findings support a model whereby stress and the associated increased glucocorticoid burden may accelerate epigenetic aging and contribute to disease risk.

Beyond such composite effects captured by measures of epigenetic aging, it is also relevant to interrogate selected gene loci that may be both susceptible to stress and implicated in aging-related disease. One such locus, the stress-responsive FKBP5 gene, was examined by a recent study combining large-scale measurements in human blood with mechanistic experiments in cells. The study found convergent evidence that stress—measured with childhood trauma and major depression questionnaires in humans and modeled with glucocorticoid exposure in cells—may synergize with aging to decrease DNA methylation at selected CpGs located near the FKBP5 transcription start site. This observation suggests that—similar to the notion that interindividual differences in stress exposure may in part explain variance in epigenetic aging—stress and glucocorticoids can shape the epigenetic state of selected sites as life advances. Notably, the aging/stress-related FKBP5 methylation signature enhanced the gene’s expression in immune cells, an effect that in turn activated the master immune regulator NF-κB and promoted chemotaxis and inflammation; thus, stress and aging can synergize to epigenetically deregulate effector molecules and downstream biological processes that are implicated in disease pathogenesis. In line with this hypothesis, the aging/stress-related FKBP5 methylation signature was present in individuals with a history of myocardial infarction, a disease state linked to inflammation.

Similar to these effects on inflammation, stress-induced epigenetic changes at selected sites could deregulate other downstream processes implicated in aging-related disease. Considering again the example of FKBP5, this versatile protein has been shown to influence not only NF-κB signaling and inflammation but also a host of other aging-related processes and disease phenotypes, including Akt signaling and cancer, tau degradation and neurodegeneration, autophagy, and telomere biology. Versatile effectors like FKBP5 could thus be viewed as “hub molecules” that may integrate pleiotropic effects of stress on aging-related processes. However, examining how stress-driven epigenetic changes regulates transcription of such effector molecules paints only part of the picture, given the complexity of regulatory loops and pathway crosstalk. For instance, higher NF-κB activity—which as discussed above can result from FKBP5 upregulation—can in turn trigger FKBP5 transcription through an NF-κB response element that is flanked and moderated by the age/stress-related FKBP5 methylation sites; this suggests a positive feedback loop of FKBP5-NF-κB signaling that may be prone to epigenetic deregulation.
by stress and aging. As another example, FKBP5 can decrease activity of DNA methyltransferase 1 (DNMT1), the mammalian enzyme that maintains DNA methylation, thereby reducing global and site-specific DNA methylation levels; thus, the epigenetic programming of *FKBP5* by stress and aging could have secondary effects on DNMT1 activity and in turn influence the epigenetic landscape in other parts of the genome.

While the evidence presented above supports a role for epigenetics as a link between stress and aging, this role should be put in broader context with other implicated processes—most notably telomere function, inflammation, and oxidative stress—as well as their complex interplay and reciprocal relations (Figure 2). Epigenetic processes can regulate telomere function, but they are also influenced by telomere regulators such as telomerase, the enzyme responsible for maintaining telomere length. Accelerated epigenetic aging and telomere shortening are associated with inflammation and oxidative stress, both processes that are not only influenced by psychosocial stressors but can themselves affect brain physiology and stress responses. These biological processes plausibly synergize and exert effects that may accumulate and eventually reach a critical threshold as life advances. In line with this “wear and tear” notion, cumulative life stress was found to have a greater effect on epigenetic aging in older as compared with younger individuals, though longitudinal studies to further support this association are lacking. Furthermore, it is currently unclear whether stress exposure simply influences the rate of aging-related epigenetic and other molecular changes or, vice versa, whether the aging process determines vulnerability or resilience of certain epigenetic sites and molecular pathways to stress. Elucidating the multifaceted mechanisms linking stress and aging will likely require orchestrated efforts that combine large-scale longitudinal studies in humans with experiments in cellular and animal model systems. Such efforts may ultimately yield deeper insights into the molecular events that shape stress-related disease along the human lifespan.

**Concluding remarks**

Gaining insights into the epigenetic and, more broadly, the molecular links between stress and aging can have major implications for developing novel prevention and treatment strategies. The field of epigenetics has generated much excitement because, unlike genetic mutations, epigenetic modifications are often acquired and, thus, potentially preventable and reversible. Accordingly, work in both rodents and humans suggests that prevention and reversal of stress-induced epigenetic modifications could be pursued by both behavioral modifications and medication management. Human studies show that supportive family environments and behavioral interventions targeting harsh parenting can ameliorate the accelerated epigenetic aging associated with early life stress. Rodent studies indicate that drugs targeting the epigenetic and transcriptional machinery can rescue DNA methylation changes associated with early life stress. Although the clinical significance of specifically modulating stress-induced epigenetic changes awaits to be tested, medications targeting the epigenetic machinery have been shown to improve aging-related disease trajectories, such as in certain types of cancer. Moreover, when manipulating the epigenome is not feasible or not desirable, interventions can instead target effector molecules that get aberrantly expressed as a result of stress-induced epigenetic changes. For example, treating cells with selective FKBP5 antagonists was shown to prevent the effect of glucocorticoid exposure and FKBP5 upregulation on NF-κB signaling. While the in vivo significance of this finding remains to be seen, it suggests FKBP5-NF-κB signaling as a tractable treatment candidate. Employing similar approaches in future studies may point to new avenues for prevention and treatment.

The need to elucidate the molecular underpinnings of stress and aging is pressing, now more so than ever. As populations age worldwide, aging-related diseases are expected to pose unprecedented challenges to individuals and societies. At the same time, surveys indicate that reported stress levels increase year by year and with successive generations. To face these challenges, it is critical to intervene early in the course of disease pathogenesis, ideally well before disease states set in. Molecular insights can guide the development of such interventions and their targeting to vulnerable stress-exposed individuals. Concurrently, social policies should strive to ameliorate or, when possible, prevent excessive stress in the first place.

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References

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Epigenetics and depression
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The risk for major depression is both genetically and environmentally determined. It has been proposed that epigenetic mechanisms could mediate the lasting increases in depression risk following exposure to adverse life events and provide a mechanistic framework within which genetic and environmental factors can be integrated. Epigenetics refers to processes affecting gene expression and translation that do not involve changes in the DNA sequence and include DNA methylation (DNAm) and microRNAs (miRNAs) as well as histone modifications. Here we review evidence for a role of epigenetics in the pathogenesis of depression from studies investigating DNAm, miRNAs, and histone modifications using different tissues and various experimental designs. From these studies, a model emerges where underlying genetic and environmental risk factors, and interactions between the two, could drive aberrant epigenetic mechanisms targeting stress response pathways, neuronal plasticity, and other behaviorally relevant pathways that have been implicated in major depression.

Introduction

Major depressive disorder (MDD) is currently the primary cause of disability worldwide \(^1\) and the World Health Organization predicts it will generate the greatest global burden by 2030.\(^2\) Despite these significant social and economic costs, the molecular mechanisms underlying MDD remain poorly understood.

It is well established that the risk for MDD is partially mediated by genetic factors. A meta-analysis quantified the increased risk of MDD as an odds ratio (OR) of 2.84 (95% CI = 2.31-3.49) for first-degree relatives of individuals with MDD.\(^3\) A large twin study in a Swedish population estimated the heritability of MDD to be approximately 37%, which is consistent with estimates made in previous studies.\(^4\) In more recent years, technological advances made in high-throughput genotyping have enabled researchers to move beyond family-based heritability studies of MDD to identify disease-associated susceptibility loci using genome-wide association studies (GWAS). What has become clear from these studies is that MDD is a polygenic disorder, with multiple loci being identified, but each with a small effect. The latest GWAS of MDD meta-analyzed data from 807,553 individuals and identified 102 loci that were associated with the disorder at genome-wide significance.\(^5\) Despite the strong evidence from both family-based heritability studies and GWAS indicating that genetic risk factors play an integral role in the pathogenesis of MDD, the heritability estimates (~40%) are less than those for other neuropsychiatric disorders, such as schizophrenia, bipolar disorder, and attention-deficit/hyperactivity disorder (all between 75% and 80%).\(^6,7\) This suggests that other factors also play a role in mediating the risk for MDD. Indeed, it is well established that environmental factors, especially stress and exposure to adverse life events, contribute to the risk.\(^8\) For example, a meta-analysis of 26 studies found that childhood trauma, especially neglect and emotional abuse, was strongly associated with depression in adulthood (OR 2.78 for neglect and 2.75 for emotional abuse).\(^9\)
Recently, it was proposed that epigenetic mechanisms could mediate the lasting increases in depression risk following exposure to an adverse life event and provide a mechanistic framework within which genetic and environmental factors can be integrated.\(^{11}\) Broadly, epigenetics refers to processes affecting gene expression and translation that do not involve changes in the DNA sequence. Epigenetic processes include DNA methylation (DNAm), small noncoding RNAs such as microRNAs (miRNAs), and histone modifications, among others. Epigenetic processes are integral in normal biological processes such as cellular differentiation, but have also been implicated in disease states. In the following, we will summarize evidence for the contribution of epigenetic processes in the pathogenesis of MDD. We include results from studies investigating DNAm, miRNAs, and histone modifications using different tissues and various experimental designs. This is summarized in Figure 1.

Evidence for epigenetic processes playing a role in depression: case/control differences in epigenetic modifications

**DNAm in peripheral tissue**

A number of studies have focused on whether there are differences in epigenetic modifications in various tissues of individuals with MDD versus controls. This has largely been centered on differences in the levels of DNAm, a process in which methyl groups are added to the 5’ position of cytosines in cytosine-phosphate-guanine dimucleotides (CpGs), which is generally associated with transcriptional repression.\(^{12}\) The majority of studies on DNAm in MDD have been performed in peripheral tissues. A recent systematic review of 67 publications,\(^{13}\) 61 of which were done in peripheral tissue, mainly blood cells, concluded there was evidence for DNAm differences between cases and controls at selected loci. Most consistently, candidate gene studies found that patients with MDD had hypermethylation in the loci encoding brain derived neurotrophic factor (BDNF), and SLC6A4, the serotonin transporter gene. However, even these findings are not consistent across all studies, highlighting the importance of sufficient cohort sizes, longitudinal study design, and robust experimental and statistical workflows.\(^{13}\) Genome-wide methylation approaches were also included, and although all studies found that DNAm was significantly associated with MDD at some loci, no consistent changes, either for direction or position, have been identified.\(^{13}\) Overall, there is very limited evidence for altered DNAm in peripheral blood in patients with MDD.

**DNAm in brain tissue**

Since DNAm is cell-type specific,\(^{12}\) and MDD is a neuropsychiatric disorder, aberrant DNAm patterns in brain tissue may provide more valuable insight into the molecular pathology of the disease than peripheral tissues. Studies performed in brain tissue, even when limited to smaller sample sizes, reveal some case/control differences. A recent methylome-wide association study in blood of MDD patients vs controls (n=1132) and in post-mortem brain (n=61) of patients who died by suicide vs psychiatrically healthy controls, found a number of significant MDD-associated regions in both brain and blood tissue.\(^{14}\) Interestingly, there was a significant overlap between the top MDD-associated differentially methylated regions (DMRs) in the blood with the top MDD-associated DMRs in Brodmann area (BA) 10 (\(P=5.4\times 10^{-5}\)). BDNF, one of the most robust findings from studies in peripheral tissue, was significantly associated with MDD in both blood and BA10. However, there was no significant overlap between the top DMRs in blood and those in another cortical region (BA25). In fact, only three loci overlapped in blood, BA10, and BA25. These three loci were replicated in an independent cohort, suggesting that despite the cell-specificity of DNAm, some DMRs have cross-tissue relevance. One of these three loci (GABBR2) encodes the metabotropic GABA\(_\text{B}\) receptor subunit, important for inhibitory neural activity, while the other two were located in RUFY3, encoding a protein involved in establishing neuronal polarity\(^{15}\) and axon growth.\(^{16}\) Both genes are important in normal brain development and function.

More studies have investigated MDD associated DNAm than the examples highlighted above, and they provide some evidence that case/control differences in DNAm exist in
peripheral and brain tissues. However, the factors driving these differences in epigenetic modifications have not yet been explored. Likely, a combination of both genetic and environmental factors contributes to these epigenetic changes in patients with MDD. These potential mechanisms will be explored in depth later in this review.

**Histone modifications**

Histone modifications have also been studied in MDD, albeit to a lesser degree than DNAm. Though various modifications of histones are possible, most studies have focused on histone acetylation and methylation. Histone acetylation is generally associated with transcriptional activation as it leads to chromatin decondensation, thus allowing transcriptional machinery access. The process is controlled by histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs), enzymes responsible for adding and removing acetyl groups from histone tails, respectively. Like with DNAm, case/control differences in depression have been observed. Global acetylation of the histone 3 at lysine 14 (H3K14ac), a modification shown to be dynamically regulated by social defeat in rodent models in various brain regions, was increased in the nucleus accumbens (NAc) taken from postmortem tissue of patients with MDD vs psychiatrically healthy controls. Correspondingly, mRNA levels of HDAC2 were observed in these patients. Another study of histone modifications in postmortem prefrontal cortex (PFC) of MDD patients reported enrichment of trimethylated H3K4—a modification generally associated with transcriptional activity—at the SYN1 promoter. SYN1 is a member of the synapsin vesicle family of neuronal phosphoproteins and plays a role in neurotransmitter release and synaptic plasticity. Ablortion H3K4me3 or H3K27me3—a repressive histone mark—has also been described in the promoter regions of OAZ1, TRKB, and BDNF in patients treated with antidepressants, in postmortem PFC.

In peripheral tissue, a limited number of studies have measured the expression of HDACs in peripheral blood cells from individuals with MDD compared with healthy controls. One study found elevated HDAC2 and HDAC5 levels in patients experiencing an acute depressive episode. Interestingly, upon remission, the levels normalized to those of healthy controls, highlighting the potential of HDAC levels to be used as a biomarker for disease monitoring.

Overall, very few studies have described case/control differences in histone acetylation and methylation, and they have not been performed on a genome-wide scale. Whether and how histone modifications are altered in depression thus remains to be seen.

**MicroRNAs**

Although miRNAs were discovered in the late 20th century, it has only been in recent years that they have been implicated in psychiatric disease. miRNAs are a type of short (typically 22 nt) noncoding RNA molecule that post-transcriptionally regulate gene expression for example via binding to mRNAs, causing their degradation and thus, translational repression. Not only do miRNAs play an important role in development and cellular differentiation by acting as a switch to silence appropriate gene groups, they also have a more nuanced role in dampening gene expression by decreasing, but not obliterating, mRNA transcripts of specific targets. Therefore, miRNAs may have a role in disease, via dysregulation of genes involved in specific disease relevant processes. Indeed, studies show altered levels of specific miRNAs in patients with psychiatric disorders, including MDD. In a recent review of 23 studies assessing miRNAs in peripheral tissues of patients with MDD, 178 different miRNAs were altered in cases versus controls. However, with the exception of miRNA-132 which was replicated in four independent studies, these miRNAs were not consistently altered across studies, highlighting the need for more robust studies with larger sample sizes, consistent methodologies, and more stringent diagnostic criteria.

There have also been studies focused on miRNAs in human brain tissue of patients with MDD. For example, miR-1202, a miRNA regulating a glutamate receptor (GRM4) was found to be downregulated in PFC of MDD patients who died by suicide compared with psychiatrically healthy controls. This was further replicated in two independent cohorts. Correspondingly, mRNA levels of GRM4 increased in both cohorts. Additionally, upon antidepressant treatment, peripheral levels of miR-1202 increased after 8 weeks of treatment, an example of how findings in human brain tissue could be applied for clinical use. Other studies of postmortem brain and peripheral tissues have identified altered levels of miRNAs in patients with MDD and after treatment with antidepressant medication (see ref 31 for a
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This highlights miRNAs’ potential as biomarkers for treatment response; however, further studies are needed.

**Challenges and solutions for overcoming cell-type specificity in epigenetic research in depression**

The above briefly described some of the evidence for altered epigenetic modifications in MDD both in brain and peripheral tissue. It is worth noting that in addition to the epigenetic modifications mentioned in this review, other epigenetic modifications have been observed in patients with MDD, such as RNA methylation; however, these are much less studied. Modifications in peripheral tissue have clinical potential to be used as biomarkers for diagnosis, treatment selection, and treatment monitoring. Although marks found in brain tissue are not suitable for biomarkers, they provide valuable insight into the pathological mechanisms, which is needed to develop a mechanistic understanding of disease and novel treatments. The cross-tissue relevance of findings in one tissue to another remains unclear and is most likely modification- and region-specific. Indeed, a recent study performed genome-wide methylation assays on blood, brain, saliva, and buccal samples and found that cross-tissue correlation strength was highly region-specific, with the correlation coefficient varying widely for different CpGs. Overall, the saliva-brain correlations ($r=0.9$) were higher than blood-brain and buccal-brain correlations, suggesting some peripheral tissues may be more useful than others. However, at the individual CpG level, correlations for all peripheral tissues were only nominally significantly correlated with brain tissue.

Given the cell-type specificity of epigenetic marks, even

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**Figure 1.** Examples of aberrant epigenetic modifications observed in patients with depression compared with healthy controls in brain and peripheral tissue.
drivers done using specific regions from postmortem brain are limited by the fact that tissue homogenates were used; these contain both neuronal and non-neuronal cell-types. Recent developments in single-cell technologies are circumventing this issue and allowing epigenetic modifications to be assessed on a single-cell level. However, to date, single-cell work in postmortem brain for MDD research is limited, and what exists is mainly limited to single-cell transcriptomics. A study published in 2018 analyzed single nuclei from the PFC of MDD patients and psychiatrically healthy controls. Twenty-six unique cellular clusters were identified, with the majority of the clusters showing case/control differences at the transcriptome level. Whether epigenetic modifications were involved in driving these transcriptional changes within the cellular clusters was not examined, but may be an indication of possible epigenetic modifications specific to certain cell types.

An alternative method to assess cell-type specific modifications relies on flow cytometry to sort cells into subtypes by using established cell-type specific markers, to which downstream epigenetic analyses are applied. Though not depression-specific, a recent study assessed various epigenetic modifications in postmortem brain sorted into excitatory glutamatergic neurons, medial ganglionic eminence-derived y-aminobutyric acid (GABA)-ergic inhibitory neurons, and oligodendrocytes. Downstream epigenetic analyses on specific cell types revealed patterns of epigenetic modifications exclusive to certain brain cell types. Most notably, there was an association between hydroxymethylation, an intermediate state in the reaction responsible for DNAm, and gene expression in the inhibitory neurons, but not in the other subtypes. The activity of these inhibitory neurons has previously been implicated in psychiatric disorders, providing an example of how studying a specific cell population may reveal unique disease relevant mechanisms that may have otherwise not been identified. Further cell-type specific studies may reveal other cell-type specific mechanisms and identify more robust epigenetic mechanisms involved in depression.

The abovementioned studies in single-cell transcriptomics and cell-specific epigenetic modifications have provided valuable insight into new cell populations that may be relevant in the molecular pathology of MDD, but more depression-focused studies on cell-type specific epigenetic modifications are required.

Drivers of aberrant epigenetic processes—genetic mechanisms

As described above, some evidence for case/control differences in epigenetic marks exist. However, the modifications themselves are only end-point observations, and most studies provide little insight into the factors driving these differences. Given the strong genetic component of MDD, genetically driven epigenetic mechanisms are a suitable starting point for further mechanistic studies. Drawing on research from neurodevelopmental disorders provides insight into how genetic factors can cause aberrant epigenetic processes. For example, there are monogenetic brain disorders in which the phenotype is driven by mutations in genes encoding epigenetic modifiers, such as Rett Syndrome (see ref 38 for review). Even though depression is clearly not a monogenetic disorder, variants in genes coding for epigenetic modifiers may still drive the epigenetic changes observed in patients with MDD. Indeed, one of the first single-nucleotide polymorphisms (SNPs; rs12413112) identified genome-wide to be associated with MDD is located next to SIRT1, which encodes a type III HDAC, an epigenetic modifier. A second SNP (rs10997875) in SIRT1 was found to be associated with MDD in a Japanese population. SIRT1 deacetylates histones and non-histone proteins involved in chromatin processes to modulate gene expression. It has been shown to modulate mood-related behaviors in rodent models, and to be necessary for normal neuronal excitability and synaptic functions.

A handful of studies have also identified depression risk variants in precursor miRNA encoding genes, variants located in or near miRNA target genes or variants located in genes involved in miRNA processing. In a study of both European-Americans and African-Americans, a variant (rs41305272), located in a predicted target site of the microRNA miR-330-3p in MAP2K5, was associated with MDD in the African-American population (OR=2.64, P=0.01; 427 cases), but not in the European-American population.

Another line of research on how genetic variants influence epigenetic modifications is methylation quantitative trait loci (mQTL) studies. An mQTL is a genetic variant that explains, or partly explains, variation of DNAm at a locus. A GWAS meta-analysis of MDD published in 2018 identified 44 depression-associated loci and integrated genetic
and mQTL data from two large studies to identify nine risk variants controlling local DNAm in blood. This overlap indicates that a subset of the risk variants for MDD could be responsible for the DMRs observed in patients with MDD. Interestingly, some of the nine mQTLs are located near genes encoding miRNAs; however, the function of these variants and DMRs must still be assessed.

Collectively, these studies indicate that the epigenetic differences observed in patients with MDD may have genetic correlates driving the aberrant epigenetic processes. However, these studies have not been followed up by directly measuring the epigenetic modifications themselves. Rather, they have identified genetic variants located in genes that encode epigenetic modifiers. As the power of GWAS continues to increase and more MDD risk variants are identified, more variants in genes encoding for epigenetic modifiers are likely to be discovered. To directly assess whether the genetic variants are driving the aberrant epigenetic modifications observed in case control studies of MDD, functional assays are required.

**Drivers of aberrant epigenetic processes—environmental mechanisms**

Since environmental factors mediate the risk for MDD, it is plausible that they too can drive the epigenetic differences observed in patients with MDD compared with healthy controls. Indeed, environmental factors have been shown to modulate epigenetic processes. Since stress and exposure to adverse life events are one of the most robust environmental risk factors for MDD, we will focus on them in this review. Studies from animal models demonstrate that stress, usually psychological stress via chronic social defeat paradigms, can induce changes in epigenetic marks. In a high-resolution genome-wide methylation study, O'Toole et al. identified striking changes in methylation patterns in the NAc of mice subjected to chronic social defeat stress compared with non-stressed controls. Hypermethylation at CpG sites was more prevalent than hypomethylation in the stressed versus control mouse. Genes (such as Est1, Caenac1, and Dec) associated with stress sensitivity and psychiatric disorders, including MDD in humans, were significantly differentially methylated. Network analysis of the DMRs showed involvement of β-catenin-related WNT/frizzled signalling, a pathway involved in neuroplasticity. β-catenin, one of the hub genes identified in the network, has previously been shown to modulate social stress. NAc-selective β-catenin knockout mice showed increased vulnerability to chronic stress, whereas an overexpression in the NAc increased stress resilience. Although transcriptomics were not performed in this particular study, other findings have identified widespread transcriptional changes in the NAc of chronically stressed mice. Collectively, this suggests that aberrant DNAm, specifically in the NAc of chronically stressed mice, may be a mechanism driving these transcriptional changes.

In human models, there is also evidence of environmental factors inducing epigenetic changes. In a model of stress using a human hippocampal progenitor cell line (HPCs), exposure to glucocorticoids (GCs) during differentiation and proliferation induced long-lasting DNAm changes. Subsequent post-differentiation exposure to GCs resulted in robust transcriptional changes in the HPCs that were initially treated during proliferation and differentiation, indicating that GC induced DNAm changes are long-lasting and prime the stress response for future exposure. Furthermore, the DMRs were enriched for the binding motif of the glucocorticoid receptor (GR), a key modulator of the hypothalamus-pituitary-adrenal (HPA) axis. The DMRs with GR binding motifs were more commonly demethylated than hypermethylated. Given that there is evidence that GRs can induce demethylation at glucocorticoid response elements (GREs), the authors postulate that the molecular mechanisms underlying the observed methylation changes in this study are GR-mediated. Further evidence of GR-mediated methylation changes will be discussed later in the review.

The effect of stress and exposure to adverse life events has also been well studied in human populations. Studies of adults with a history of child abuse, which is a strong risk factor for depression, have investigated whether childhood adversity is associated with aberrant DNAm patterns in brain and peripheral tissues. A genome-wide study of hippocampal tissue from 41 adult males found 362 differentially methylated gene promoter regions in individuals (n=25) who had experienced child abuse and completed suicide, compared with the psychiatrically healthy controls (n=16). These DMRs were most pronounced in the neuronal fraction of the brain tissue, and consistent with the findings in animal studies mentioned above, were enriched in genes involved in neuroplasticity. Other studies have also observed asso-
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ciations between childhood maltreatment and differences in DNAm in the brain, blood, and even gametes. However, well-powered studies have also reported negative results.

As with the case/control studies described previously, to date most work investigating how stress and exposure to adverse life events induce epigenetic modifications has focused on methylation. Nonetheless, a few studies have shown environmentally driven changes of the epigenetic landscape in other ways, such as via histone modifications and long noncoding RNAs. This has almost exclusively been done in animal models, with a few exceptions of studies in human cohorts. Overall, DNAm remains the most characterized epigenetic process that changes in response to exposure to adverse life events.

**Gene x environment mechanisms in depression**

Both genetic and environmental factors can drive epigenetic processes. However, these factors are not independent, and they have been shown to interact. Gene by environment (GxE) interactions refer to the influence of environmental and genetic factors on a measured phenotype, in this case, epigenetic modifications. However, in GxE interactions, the magnitude of the environmental influence on the phenotype depends on the genotype of the individual. For example, in a recent study of over 2000 newborns, the influence of the prenatal environment on genome-wide DNAm was examined. The prenatal environment (E), genotypes in cis (G), their additive (G+E) or interaction (GxE) effects on DNAm at DMRs in neonatal blood were assessed. The GxE and G+E models outperformed the models with G or E only in predicting DNAm at the DMRs, indicating a synergistic interaction between genetic and environmental factors. Notably, both models (G+E and GxE) were enriched for DMRs associated with GWAS of psychiatric disease, including MDD.

In another mechanistic study of GxE interactions driving epigenetic modifications, Klengel et al identified an interaction between a variant (rs1360780) located in an enhancer region in FK506 binding protein 5 gene (FKBP5) and childhood trauma on DNA methylation of FKBP5 in peripheral blood cells. FKBP5 is an important regulator of the HPA axis and is involved in a negative feedback loop to terminate the stress response. The authors found that methylation of FKBP5 was associated with childhood trauma in carriers of the risk allele, but not those with childhood trauma with the major allele, an example of gene x childhood trauma interaction. Importantly, this study identified not only an interaction, but also the mechanism by which it induces changes to DNAm. Using a series of experiments, the authors propose the following model: the risk allele of rs1360780 causes differential interactions between the enhancer and transcription start site upon GR activation induced via child abuse, resulting in transcriptional activation of FKBP5. Persistent overexpression of FKBP5 results in impaired termination of the stress response, including prolonged GR activation. The genotype dependent changes in chromatin structure, along with prolonged GR activity, cause DNA demethylation at CpGs located within and proximal of GREs that have been shown to increase FKBP5 mRNA levels. Highly dynamic GR-dependent changes in DNAm of CpGs in FKBP5 after acute GR activation have been shown to occur in adult peripheral blood cells, with de- and remethylation occurring within a period of 24 hours following GR activation. However, as shown in a human hippocampal progenitor cell line, GR-induced demethylation can become stable when initiated during certain early developmental periods. Such lasting changes in DNAm result in an altered set-point for transcriptional changes of FKBP5 upon subsequent GR activation. The exact mechanisms contributing to such stable changes have not been investigated so far, but likely involve developmental stage-specific functions of DNA methyltransferase, enzymes involved in active DNA de-methylation such as the ten-eleven translocation protein family as well as methyl-CpG binding proteins. Differences in the levels and dynamics of these proteins will have an impact on DNAm levels.

In summary, in the FKBP5 model, the genotype, via differential chromatin interactions, and environmental risk, via enhanced GR activation, converge to establish the long-term transcriptional changes of FKBP5 by lasting changes in DNAm of enhancer regions. Further studies have associated this interaction between rs1360780 and childhood abuse with psychiatric conditions later in life, including MDD, highlighting its clinical relevance.

**Future directions**

The risk for MDD is both genetically and environmentally determined. There is evidence from case/control studies that implicate epigenetic processes in MDD, and these
epigenetic processes are likely driven by both genetic and environmental factors. Collectively, a model emerges where underlying genetic and environmental factors, and interactions between the two, drive aberrant epigenetic mechanisms targeting stress response pathways, neuronal plasticity, and other behaviorally relevant pathways that have been implicated in MDD.

Yet, many gaps remain in our understanding of epigenetic processes in MDD. Most studies describing epigenetic modifications in patients with MDD have focused on DNAm, with fewer studies focused on histone modifications and long noncoding RNAs. Most studies have been done using peripheral tissue, and those that have used postmortem brain tissue are generally limited to smaller cohort sizes and brain tissue homogenates. These factors have contributed to the difficulty in validating depression-associated epigenetic modifications across cohorts. Other factors currently inherent to psychiatric research, such as the polygenic architecture of depression, symptom-based diagnoses, and retrospective recall of environmental exposures, have only added to the difficulty. However, recent advances have been made, such as consortium-based cohorts for GWAS, decreasing costs of next-generation sequencing technologies, and developments in single-cell methods. These developments, among others, can help to identify robust aberrant epigenetic mechanisms occurring in MDD, to increase our understanding of the molecular mechanisms governing this disease, and to guide future treatment.

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Use of the epigenetic toolbox to contextualize common variants associated with schizophrenia risk

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Schizophrenia is a debilitating psychiatric disorder with a complex genetic architecture and limited understanding of its neuropathology, reflected by the lack of diagnostic measures and effective pharmacological treatments. Geneticists have recently identified more than 145 risk loci comprising hundreds of common variants of small effect sizes, most of which lie in noncoding genomic regions. This review will discuss how the epigenetic toolbox can be applied to contextualize genetic findings in schizophrenia. Progress in next-generation sequencing, along with increasing methodological complexity, has led to the compilation of genome-wide maps of DNA methylation, histone modifications, RNA expression, and more. Integration of chromatin conformation datasets is one of the latest efforts in deciphering schizophrenia risk, allowing the identification of genes in contact with regulatory variants across 100s of kilobases. Large-scale multiomics studies will facilitate the prioritization of putative causal risk variants and gene networks that contribute to schizophrenia etiology, informing clinical diagnostics and treatment downstream.

Keywords: schizophrenia; epigenetics; higher-order chromatin; histone modification; multiomics; GWAS

Introduction

Schizophrenia (SZ) is a complex and debilitating neuropsychiatric illness that has approximately 1% average lifetime prevalence globally, although rates vary regionally up to fivefold.1,2 The disease is characterized by “positive” symptoms, consisting of recurrent psychosis, hallucinations, and disorganized speech; “negative” symptoms, including anhedonia, social withdrawal, and flattened affect; and broad cognitive dysfunction.3 Devastatingly, those diagnosed with SZ have a staggeringly high unemployment rate of 80% to 90%,4,5 and a life expectancy that is reduced by 10 to 20 years, with mortality often caused by suicide or cardiovascular conditions.4,6 An incredibly isolating disease on a personal level, SZ also poses a great economic burden in terms of health and social care.7 

SZ is thought to result from an intricate choreography of genetic and environmental factors throughout early brain development that predispose an individual to the disease. Heritability estimates range up to 80%, underlining the importance of genetic contributions to the calculation of SZ disease risk.7,9 The field has benefited greatly from the advent of next-generation sequencing and large-scale genome-wide association studies (GWAS) that have identified a combination of rare and common variants that are associated with SZ risk, spurring on the investigation of potential etiological mechanisms. Evidence from both genetic and epidemiological studies point to a disease of impaired early, potentially prenatal, brain development.10

A disease with heterogeneous clinical presentation, SZ’s non-Mendelian genetic risk architecture is equally hetero-
The field of SZ epigenetics has experienced a recent surge of discovery, in part facilitated by innovation in the technical capacities of epigenomic architecture mapping and high-throughput sequencing.

Profiling with genome-wide SNP genotyping in 166 human fetal brain samples (56 to 166 days post-conception) to find >16 000 methylation quantitative trait loci (mQTLs), instances of DNA methylation that can be influenced by variation in the sequence. Moreover, these fetal brain mQTLs, many of which are remarkably stable even in the adult brain, were significantly enriched amongst SZ risk loci, allowing for the homing in on discrete sites of methylation in the fetal brain that also harbor SZ risk variants.

Histone post-translational modifications (PTMs) are another layer of epigenetic control that contribute to higher-order chromatin regulation, such that combinatorial PTMs establish various chromatin states, including active or silenced transcription, regulatory sequences, etc. Like in early methylation studies of SZ, histone PTMs were assayed in single-gene fashion. For instance at the GAD1 locus, Huang et al discovered decreased histone 3 lysine 4 trimethylation (H3K4me3), associated with the transcriptional process, in the prefrontal cortex (PFC) of predominantly female patients with SZ when compared with controls at the 5’ end of the GAD1 gene, overlapping with a SZ risk locus. A later study detected decreased H3K9K14 acetylation, also marking transcriptionally active chromatin, at several candidate genes, including GAD1. Eventually, the chromatin immunoprecipitation (ChIP) assay used in these two studies was refined and made scalable to high-throughput library profiling with genome-wide SNP genotyping in 166 human fetal brain samples (56 to 166 days post-conception) to find >16 000 methylation quantitative trait loci (mQTLs), instances of DNA methylation that can be influenced by variation in the sequence. Moreover, these fetal brain mQTLs, many of which are remarkably stable even in the adult brain, were significantly enriched amongst SZ risk loci, allowing for the homing in on discrete sites of methylation in the fetal brain that also harbor SZ risk variants.

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generation in postmortem brain, taking on the form of ChIP with next-generation sequencing (ChIP-Seq) to query the whole genome, and not just at predetermined sites, in a cell type-specific fashion. Only recently has this approach been applied to the study of psychiatric disorders, specifically SZ. A study of 157 open chromatin-associated histone profile (H3K4me3 and H3K27ac) reference maps from PFC and anterior cingulate cortex (ACC) of sorted neuronal (NeuN+) and non-neuronal (NeuN-) cell populations revealed differing landscapes at loci based on cell type and brain region. Notably, the authors found a striking overrepresentation of risk variants for SZ that was highly specific to neuronal chromatin, as determined by partitioning heritability, highlighting the need to study epigenetic contributions to disease in a cell type-specific fashion. This is especially important when considering the fact that around half of the noncoding regions could have regulatory functions specific to a given tissue, cell type, or developmental stage. In the brain, this nuance is compounded by the enormous cellular heterogeneity and long developmental trajectories that occurs to a lesser degree in other tissues.

Given that the majority of SZ risk variants occur in these noncoding (ie, intronic or intergenic) regions of the genome, it is difficult to identify the associated causal gene, when considering that the variants could be in regulatory elements, such as enhancers or repressors, impacting the expression of distally located genes. Indeed, using heritability partitioning, the authors found that 16% of imputed SNPs overlapping DNase hypersensitivity sites—indicators of open chromatin and regulatory regions—explained an average of 79% of the SNP heritability spanning 11 diseases, including SZ. One approach is to leverage RNA-sequencing (RNA-seq) data with SNPs to identify genetic variants that are correlated with expression of genes, even if they are not immediately adjacent, called expression quantitative trait loci (eQTL). This is precisely what the CommonMind Consortium did, using expression data from dorsolateral PFC of people with SZ (N=258) and control subjects (N=279) and imputed SNP genotypes. They found that, of the 108 loci previously reported, 20 of them (~20%) had eQTL SNP-gene pairs within 1 megabase (Mb) of linear genomic distance (ie, cis eQTLs) that might contribute to altered gene expression and SZ liability. A recent study was able to expand the number of GWAS loci colocalized with eQTL signal from 20 to 40 by including conditional eQTLs (ie, not just one primary eQTL for each gene), beginning to consider epigenetic and cell states within which different SNPs might function.

Assay for transposase-accessible chromatin with sequencing (ATAC-seq) also offers glimpses into open chromatin regions (OCRs) along with footprints of DNA-binding factors, such as transcription factors. One study of sorted neuronal and non-neuronal cells from postmortem PFC identified cell type-specific OCRs, with neuronal OCRs more enriched for distal regulatory elements and evolutionarily conserved regions, as well as a subset of transcription factors highly enriched in SZ loci specific to neurons, providing a potential functional role. An expanded study assayed the same two cell types and 14 different brain regions in five individuals, reporting higher regional variability in neuronal chromatin and significant enrichment only for neuropsychiatric traits, including SZ, in cell- and region-specific fashion, again highlighting the need for approaches that take into account the many nuances of the brain. A case-control study of chromatin accessibility (142 SZ, 143 control) only identified three differentially accessible regions, suggesting that differences could be subtle between diseases and healthy PFC, although looking at cell type-specific maps may have improved the results. Taken together, such GWAS-guided ChIP-seq, eQTL, and ATAC-seq studies have begun to shed light on possible mechanisms in which SNPs located in noncoding portions of the human genome may exert a regulatory influence on gene expression, even distally, if they happen to coincide with functional elements such as enhancers or repressors.

Recently, the field has enjoyed a wave of “post-GWAS” analyses aimed at increasing the power to prioritize causal genes by performing gene-based, as opposed to single-variant-based, associations, with the underlying assumption that many genetic variants influence traits, like SZ, via transcriptional regulation. By focusing on the genetic component of expression, environmental factors are excluded from consideration, thereby increasing statistical power. One of these methods, called the transcriptome-wide association study (TWAS), has revealed 157 significant genes, 35 of which did not overlap a known GWAS locus. Of 157 genes, 42 genes were associated with specific chromatin phenotypes (ie, histone modification ChIP-seq peaks), suggesting putative regulatory mechanisms. Moreover, 105/157 TWAS-predicted risk genes overlapped with genes linked to index SNPs.
through chromatin interaction data from fetal brain. The method also revealed that the TWAS genes were more significantly associated with the trait in question than the nearest gene and had stronger eQTL effects at the index SNP, emphasizing the need to go beyond the linear genome. A similar method called PrediXcan has been applied to the largest transcriptomic imputation study in SZ to date, identifying 67 genic associations across 13 brain regions, 19 of which were novel. These genes were involved in new biological pathways such as hexaminidase-A and porphyrin metabolism, both of which have historic connections to SZ without much prior genetic evidence supporting them. Recently, this technique has again confirmed that most trait associations are tissue-specific, underscoring the necessity of studying homogeneous populations of specific cell types.

Large-scale functional genomic and epigenetic studies

Looking ahead, the most promising approaches could involve integration of multiple orthogonal datasets taking into account cell type contexts, particularly with larger sample sizes, in order to triangulate more functionally implicated genetic variants associated with SZ (Figure 1). Leveraging both RNA-seq and ATAC-seq data, one study identified 118 differentially transcribed enhancer RNAs (eRNAs), important for activity-dependent gene regulation in the human brain, in SZ compared with controls. The same study also found genetic variants that affect expression of approximately 1000 enhancers. A recent multiomics study of tissue and single-cell RNA-seq, histone modification landscapes, CTCF transcription factor binding sites, DNA methylation, and genotypes (1230 samples from 48 brains; 18 288 single cells/nuclei from 12 brains) aimed to comprehensively chart the human neurodevelopmental trajectory, from the embryonic stage to adulthood, of gene expression programs and their regulation in various brain regions and cellular contexts. With this integrative approach, the authors were able to identify a global transition in the transcriptomic profile during late fetal development where there was a sharp reduction in differences across brain regions coincident with increased signatures of expression of genes important for neuronal structure and activity.

Importantly, using partitioned linkage disequilibrium score regression analysis, they found that SZ SNP heritability was enriched in the dorsolateral PFC-specific regulatory elements as identified by H3K27ac peaks. With regard to cell type context, cortical excitatory neurons, embryonic/fetal progenitor cells, and adult cortical interneurons were enriched for expression of high-stringency genes associated with SZ, echoing another single-cell study that found similar cellular patterns. Analysis of gene coexpression modules revealed one particular module enriched for genes enriched in fetal and adult excitatory neurons associated with SZ, fetal enhancers, neuronal (as opposed to neural progenitor or glial) expression, and neuronal undermethylated sites. This module, consisting of 145 total genes, included MEF2C and SATB2, which have previously been associated with neurodevelopmental disorders in general and specifically SZ.

Another large study (over 2000 postmortem brain samples from individuals with SZ, bipolar disorder, and autism spectrum disorder as well as controls) looking at the transcript isoform level across SZ, bipolar disorder, and autism spectrum disorders, uncovered disease-specific differential splicing and expression and that isoform-level changes, as opposed to gene-level, showed the largest effect sizes and greatest disease specificity. Of note, approximately 700 noncoding RNAs (including 208 long intergenic noncoding RNAs, or lincRNAs), which are thought to have a transcriptional regulatory role, were differentially expressed in SZ, specifically in excitatory neurons. A TWAS performed on these data identified a stringent list of 64 significant genes, consistently prioritized by multiple methods, including downregulated lysine methyltransferases SETD6 and SETD8 along with brain-enriched lincRNA LINC00634, highlighting transcriptional and epigenetic regulation as important molecular mechanisms to consider in SZ risk. Taken together, these two studies testify to the power gained by leveraging large brain datasets across multiple modalities in order to identify the contributions of cell type-specific pathways and their regulation over time. Such “four-dimensional (4D) mapping,” including spatial and temporal components, will be vital to distinguish between the effects of direct genetic insult and its resulting indirect cascade of molecular events, as well as to identify when specifically in development intervention would be most ameliorative.

Schizophrenia risk-associated genome in 3D

Even with the identification of transcriptomic patterns and gene coexpression modules or the annotation of noncoding
variants in the context of SZ risk, led in large part by the
generation of brain-specific functional genomic datasets
by the PsychENCODE consortium,58 there is still a part of
the story that is missing. We cannot simply assume that
noncoding SZ variants, which make up most of the genetic
risk architecture, act on the genes that are closest to them
on the linear genome59,60 or are in linkage disequilibrium.61
Therefore, it is necessary to consider the genome in three
dimensions and map chromatin interactions that can bring
distal regions into close physical proximity, in many cases
for fine-tuning gene expression through regulatory elements
(eg, promoter-enhancer interactions (Figure 1)).62

The three dimensional conformation of chromatin and entire
chromosomes is referred to as the “3D genome” or 3DG.
Chromosome conformation capture methods combined with
deep sequencing, such as in situ Hi-C, are used to study the
3DG.63 Briefly, the technique involves crosslinking cells or
tissue, isolating intact nuclei, digesting the chromatin within
the nuclei with restriction enzymes, biotinylating cut ends,
ligating fragments in close proximity, shearing the DNA,
pulling down biotinylated chimeric fragments, and generat-
ing libraries for deep paired-end sequencing.63 Variants of
the technique include capture Hi-C to isolate fragments of
interest with baits,64,65 single cell Hi-C,66,67 Hi-C paired with
ChIP (HiChIP),69 and low input Hi-C,70-72 among others. This
cadre of methods has enumerated various principles of 3DG
organization (see Figure 2 for more details). Perhaps the
biggest layer of the 3DG involves the clustering of euchro-
matic (A) and heterochromatic (B) sequences into regions of
approximately ~5 megabases (Mb) called “compartments”73
(Figure 2), although recent reports say that they may be
at a smaller scale than previously envisioned (15-300kb),
often segregated by shared transcriptional states and histone
modification landscapes.63,74,75

The next component of the 3DG is topologically associating
domains (TADs), which are genomic sequences that pref-
erentially interact with themselves as opposed to regions outside of their boundaries.\textsuperscript{76,77} They act as insulators, preventing inappropriate interactions between elements such as promoters and enhancers, which may lead to abnormal levels of gene expression.\textsuperscript{76,78} Furthermore, TADs are thought to be more conserved across cell types, and even species, than are compartments.\textsuperscript{77,79-81} However, their insulation capacity and interconnections with other regions have been shown to be subject to cell type-specific changes, at least during lineage specification when primed perhaps by transcription factor binding.\textsuperscript{82} Additionally, TAD insulation is highly correlated with transcription, such that new borders are able to form at developmentally regulated gene promoters, suggesting that novel TAD landscapes may arise with certain contexts.\textsuperscript{83}

The spatial genome also includes chromatin loops or interactions, commonly defined as distinct pairwise contacts that, in Hi-C maps, sharply stand out from the surrounding “linear” genome background, often linking regulatory elements with distal gene promoters in a cell type-specific fashion.\textsuperscript{63} The anchors of many chromatin loops (65\% to 92\%) often have CTCF, which in this case acts as an architectural scaffolding protein, in an inward/convergent orientation at binding sites\textsuperscript{63,77,81,84} that, when experimentally inverted, could in some cases affect normal patterns of gene expression.\textsuperscript{85}

Because the majority of functional elements in noncoding portions of the human genome, such as enhancers and repressors, are not contacting the nearest TSS but instead are interacting with genes located elsewhere on the chromosome,\textsuperscript{86} it is unsurprising therefore that non-3DG based approaches relying on purely linear genomic distance, had only limited success in assigning specific target genes to risk loci.\textsuperscript{34} The inability to catalog reliably gene targets of SZ risk variants understandably hindered the downstream efforts to identify networks contributory to disease etiology. eQTLs, as discussed earlier, began to consider the impacts of common variants on distal genes; however, these are still mostly restricted to SNP-gene associations within 1 Mb and are computationally derived without using actual measurements of the 3DG space.\textsuperscript{37,87} In a pioneering study, Won and colleagues generated Hi-C chromosomal
conformations from fetal brain and mapped gene targets for schizophrenia GWAS noncoding variants; many of these genes were involved in disease-relevant pathways such as neurogenesis or cholinergic signaling. Another Hi-C study generating 3DG maps from fetal and adult human cortex to explore GWAS loci for multiple neuro-psychiatric disorders and traits found that chromatin interactions explained ~73% of genes implicated by eQTLs (2,292/3,121); strikingly, there are 4101 genes discovered psychiatric disorders and traits found that chromatin inter

Two recent studies addressed some of these technical shortcomings. First, Hi-C mapping of human induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (NPCs) and their isogenically differentiated excitatory neurons and glia increased the number of transcribed genes associated with SZ GWAS loci by approximately 2- to 3-fold (total N expressed genes connected to or located within an SZ GWAS locus ranged from 201-386, depending on cell type). Since neurons, together with NPCs, had the greatest number of cell type-specific interactions anchored in a risk locus (as compared with glia), one could conclude that the 3DG space corresponding to SZ risk disproportionately affects neurons, echoing other studies that report a neuronal burden in the SZ genetic architecture. Remarkably, the SZ-associated chromosomal connectome (ie, GWAS loci and their connected genes) specific to NPC or excitatory neurons was associated with coordinated gene expression “clusters” and protein-protein interactions, where one cluster strongly enriched for regulators of neuronal connectivity and synaptic plasticity, and another cluster for chromatin-associated proteins, including transcriptional regulators, similar to the results of Giusti-Rodriguez et al. Many interactions of interest between noncoding SZ variants and neurally relevant genes, such as pro-neuronal transcription factor ASCL1 or members of the clustered Protocadherin family, were functionally validated in NPCs with CRISPR epigenomic and genomic editing. Likewise, an expanded genome space involving higher-order chromatin interactions anchored in schizophrenia risk loci, prioritized with orthogonal CTCF and histone modification ChIP-seq datasets, has also been described for cultured primary sensory neurons from the olfactory neuroepithelium, also pointing to neurogenesis as an important gene set.

Furthermore, a massive multiomics study by the PsychENCODE consortium analyzed the open chromatin landscape, transcriptional histone marks, and the transcriptome from altogether 2000 postmortem brains, including hundreds of cases diagnosed with schizophrenia, and combined these “linear genome” profiles with Hi-C data from fetal and adult reference brains. The study mapped ~79 000 brain-active enhancers with their associated chromosomal contacts and TAD landscapes and identified a vast number of eQTLs and gene regulatory networks; perhaps most importantly, the investigators applied deep machine learning algorithms that, for the first time, were able to predict presence or absence of disease (ie, SZ) based on a subject’s brain transcriptome and chromatin profiles. The study approached disease prediction at a probability level of ~75%, reflecting a significant advancement over more conventional genomic approaches predicting disease only marginally above chance (50%).

Very recently, another study presented an integrative risk gene selector (iRIGS), a computational framework to integrate multiomics data, that predicted high-confidence risk genes that account for significantly enriched heritability, are expressed in brain tissues (especially prenatal), and are enriched for targets of already approved drugs, providing new opportunities to repurpose existing drugs for SZ.

Conclusions

The field of SZ epigenetics has experienced a recent surge of discovery, in part facilitated by innovation in the technical capacities of epigenomic architecture mapping and high-throughput sequencing. As a field, we have been able to progress from single candidate gene studies to probing the entire human genome, both the protein-coding and the hitherto enigmatic noncoding regions, in unbiased fashion across numerous modalities and cell types. However, the polygenic nature of SZ with its nuanced network of common variant influence requires more investigation to
decipher, through both large-scale consortium-led efforts as well as incisive functional and mechanistic studies on discrete, contributory pathways. The many studies enumerated here in the new wave of SZ epigenetics research utilized high-resolution genomic and epigenomic datasets from relevant tissue and cell types, taking into account the 3DG space and leveraging orthogonal approaches, in order to arrive at higher confidence gene sets and predictive capacity. In this vein, the polygenic risk score (PRS), a metric that summarizes the inherited common variant burden in an individual, is interesting from a clinical precision-medicine diagnostic perspective. While being in the top 10% of PRSs carries a greater than 10-fold increased risk of SZ, there is still a substantial overlap in the distribution of PRS between cases and controls, such that many controls are in the top decile and many cases in the lowest. Deep-learning algorithms such as those employed by Wang et al pave the way forward, combining different slices of information to construct a more holistic functional genomic picture that is missed when considering simply the genetic information, thereby enhancing disease prediction. Another benefit of compiling and integrating many brain datasets is the identification of high-fidelity risk variant-gene interactions with regulatory epigenomic landscapes. What genes are revealed as important in multiple approaches? Do they converge on pathways with pathogenic potential? Are there druggable targets for major regulators of these pathways or hubs? Questions like these can help focus the expeditions ahead as we try to understand the genetic, functional, and cellular/network architecture of SZ culminating in disease.

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A review of epigenetic contributions to post-traumatic stress disorder

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Post-traumatic stress disorder (PTSD) is a syndrome which serves as a classic example of psychiatric disorders that result from the intersection of nature and nurture, or gene and environment. By definition, PTSD requires the experience of a traumatic exposure, and yet data suggest that the risk for PTSD in the aftermath of trauma also has a heritable (genetic) component. Thus, PTSD appears to require both a biological (genetic) predisposition that differentially alters how the individual responds to or recovers from trauma exposure. Epigenetics is defined as the study of changes in organisms caused by modification of gene expression rather than alteration of the genetic code itself, and more recently it has come to refer to direct alteration of DNA regulation, but without altering the primary sequence of DNA, or the genetic code. With regards to PTSD, epigenetics provides one way for environmental exposure to be “written” upon the genome, as a direct result of gene and environment (trauma) interactions. This review provides an overview of the main currently understood types of epigenetic regulation, including DNA methylation, histone regulation of chromatin, and noncoding RNA regulation of gene expression. Furthermore, we examine recent literature related to how these methods of epigenetic regulation may be involved in differential risk and resilience for PTSD in the aftermath of trauma.

Keywords: epigenetics; genetics; DNA methylation; histone acetylation; noncoding RNA; trauma; post-traumatic stress disorder; childhood abuse

Introduction

Post-traumatic stress disorder (PTSD) is a *Diagnostic and Statistical Manual of Mental Disorders* 5th ed. (*DSM-5*) diagnosis marked by the development of stressor-related symptoms following one or more traumatic events.1 Outlined in the *DSM-5*, a traumatic event is defined as exposure to actual or threatened death, serious injury, or sexual violence that is experienced directly, witnessed, experienced vicariously through family or close friends, or experienced repeatedly or with extreme exposure to aversive details of the traumatic event. Diagnostic criteria and symptomology include the following: intrusive symptoms; avoidance of stimuli associated with the traumatic event; negative alterations in cognition and mood; alterations in arousal and reactivity associated with the traumatic event; and in some cases, dissociative symptoms.1

While around 50% to 60% of the population will experience traumatic stress over the course of their lifetime, the lifetime prevalence for PTSD using *DSM-IV* criteria has been estimated at around 8.7%.2,3 The disparity between trauma exposure and the development of trauma-related disorders has garnered much interest, and our understanding of what contributes to this susceptibility or resilience is still limited.4,5 The old debate of nature versus nurture sought answers in a single domain; however, as our understanding continues to evolve, it has become clear that—like many
other mental disorders—PTSD development is heavily influenced by an interplay between environmental factors and genetic predisposition or heritability. The study of epigenetics bridges both sides of this debate and focuses on the changes in gene expression that may be caused by our environment.

In our review on the epigenetics of PTSD, we will discuss the heritability of this disorder and give an overview of epigenetic mechanisms, targets, genome-wide association studies (GWAS), and epigenome-wide association studies (EWAS) conducted to date, and future directions for the field.

Heritability

Diagnosis of PTSD is reliant on environmental influence through a traumatic event. Given this fact, it may seem backwards then to study the heritability of a disorder that requires an outside event for its manifestation. Contrary to what may seem intuitive of a disorder with this diagnostic criterion, research suggests that genes do play a role, and perhaps a significant one, in the risk of developing PTSD.

Twin studies serve as an invaluable tool to parse out genetic and environmental factors and contribute in concert with newer molecular and genetic methods to help piece together a complete picture. In one twin study, heritable influences accounted for 46% of the variance in PTSD, and for 71% of variance in females. Another study suggested that exposure to assaultive trauma (robbery, sexual assault, and other life-threatening events) may not be entirely random and is influenced by individual and familiar risk factors. Indeed, it is known that parental post-traumatic stress can cause negative psychological outcomes and potential biological alterations in their offspring, with several studies indicating that severity of a parent’s PTSD symptoms may contribute to a child’s psychological difficulties—namely anxiety, depression, and behavior problems. Furthermore, childhood adversity has been strongly implicated in the development of many psychiatric disorders, and individuals who experience these early life adversities are at greater risk for PTSD in adulthood. The psychological collateral of trauma-related distress can percolate through the family unit, potentially exacerbating risk factors that may lead to the development of future psychological distress or disorder.

In addition, PTSD has common comorbidity with other mental disorders, namely major depression, substance abuse, and other anxiety disorders. While the DSM-5 is limited by its definitions and diagnostic criteria, genetic evidence suggests that these disorders may fall on a spectrum rather than being entirely independent entities.

Epigenetic approaches may afford novel targeted therapeutic approaches to enhance treatment and prevention of PTSD

Epigenetic mechanisms

Data from twin studies suggests that PTSD is at least partially heritable and, by definition, requires influence from environmental trauma. Epigenetics is defined as the study of changes in organisms caused by modification of gene expression rather than alteration of the genetic code itself, and more recently it has come to refer to direct alteration of DNA regulation, but without altering the primary sequence of DNA, or the genetic code. With regards to PTSD, epigenetics provides one way for environmental exposure to be “written” upon the genome, as a direct result of gene and environment (trauma) interactions. The epigenome is influenced by both genetic and environmental factors—the environment in effect is written onto the genes themselves. While epigenetics does not change the sequence of the DNA code, it does alter the expression of genes and may contribute to long-lasting—in some cases intergenerational—phenotypic effects. There are several mechanisms that drive this process, three of which have been widely studied.

Histone modification

Histone proteins (H2A, H2B, H3, H4) help organize DNA into structured units called nucleosomes. Nucleosomes are packaged units formed by spooling the DNA sequence of 200 nucleotide base pairs around eight histones (octamer), which help to compact the DNA. The nucleosomes can be thought of as “beads” and are connected by linker DNA forming a collection of “beads on a string” called chromatin. Chemical alteration or modification of histones—through acetylation or deacetylation—influences the structure of chromatin, remodeling it to either coil or uncoil and altering the ability of RNA polymerase to transcribe genes. Thus, histone acetylation or deacetylation regulates the extent to which a gene is expressed by altering chromatin structures. Histone regulation has been implicated in a number of activ-
ities in the brain related to emotion regulation, for example traumatic memory encoding and fear extinction,\textsuperscript{16} which is an important process that is dysregulated in PTSD.

Histone acetylation occurs when the enzyme histone acetyltransferase (HAT) interacts with the histone protein, adding an acetyl group to lysine residues in the N-terminal tail of the histone protein. Acetylation of histones by HAT causes the uncoiling or loosening of DNA, creating decondensed and “open” chromatin structure (euchromatin), which allows access to the DNA by proteins involved in the transcriptional machinery, copying the DNA sequence into RNA. Conversely, histone deacetylation occurs when histone deacetylase (HDAC) removes the acetyl group added by the HATs. Deacetylation of histones by HDAC causes the coiling of DNA, creating condensed and “closed” chromatin structure (heterochromatin) making it densely packed and more difficult to transcribe—in effect inhibiting or repressing gene expression. Histone methylation provides yet another mechanism of histone regulation, mediated by histone methyltransferases. Generally, histone methylation is thought to have an opposite effect to histone acetylation, generally consistent with condensing chromatin structure, though this is also dependent on which specific amino acid components of the histones are modified. In summary, without changing the DNA sequence or even directly modifying the DNA chemical structure, this process of histone modification allows genes to be turned on and off by making regions of DNA either accessible or inaccessible to the transcriptional machinery.

**DNA methylation**

DNA modification through direct methylation is one epigenetic process that has been widely studied in PTSD. Although there are more than 20 identified DNA modifications,\textsuperscript{17} 5-methylcytosine (5-mc) and 5-hydroxymethylcytosine (5-hmc)\textsuperscript{18} are two types of methylation-related modifications that are highly prevalent in neurons related to known processes involved in PTSD, such as learning and extinction of conditioned fear.\textsuperscript{19,21} DNA methylation changes within a gene can occur at any stage during the life cycle of a cell,\textsuperscript{22} and they have been characterized to make a long-term impact on transcriptional response due to different stress-related environmental factors, including early adverse life events.\textsuperscript{23,24}

There have been many reports of increased and decreased DNA methylation in response to exposure of stressful life events. Thus, it is important to understand the mechanism of both addition and removal of methyl groups. Methylation is mediated by DNA methyltransferase proteins, DNMT3a and DNMT3b, to add a methyl group to an unmethylated cytosine C5 position.\textsuperscript{25} The oxidation of 5-methylcytosine to 5-hydroxymethylcytosine is mediated by ten-eleven translocation proteins (TET1, TET2, and TET3); hydroxymethylcytosine is an intermediate step in DNA demethylation. DNA-binding proteins have also shown to be involved in active demethylation of DNA, and other proteins involved in dynamic transcriptional activation or repression can also “recruit” DNMT and TET proteins, leading to a longer-lasting alteration in DNA methylation status.\textsuperscript{26,27}

One example of DNA methylation findings are the role of differential methylation at the FKBP5 gene, which is further outlined below as a critical regulator of the HPA cortisol response. DNA demethylation at glucocorticoid receptor binding site (GREs) within the\textsuperscript{FKBP5} gene in peripheral blood cells and hippocampal progenitor cells was found to be associated with prior exposure to childhood abuse.\textsuperscript{23} Recent studies also suggest that epigenetic marks might be transmitted down to the next generation, influencing the risk of diseases in offspring,\textsuperscript{28,29} though these have typically been small or underpowered studies that require expansion and replication.

**Noncoding RNA**

Noncoding RNAs (ncRNAs) are transcripts from DNA, but unlike other RNAs, ncRNAs are not translated into a polypeptide or protein sequence. ncRNAs are functional and are involved in the processing and regulation of other RNAs such as messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA).\textsuperscript{30,31} Recent more detailed reviews highlight the role of different types of ncRNAs, such as micro RNA (miRNA), long noncoding RNA (IncRNA), and retrotransposons, that may become useful biomarkers for trauma-related brain disorders such as PTSD.\textsuperscript{32}

miRNAs are characterized by 21 to 24 nucleotides in length. They are thought to generally bind to the 3' untranslated region or other untranslated regions of their target mRNAs to regulate gene expression.\textsuperscript{33} Studies have reported the miRNA expression level changes in experimental stress models.\textsuperscript{34-36} One of those studies linked to hypothalamus-
pituitary-adrenal (HPA) axis pathways identified a specific miRNA, miR-34c, to be upregulated in a stress-dependent manner in mouse amygdala tissue.\textsuperscript{37} In rodents, miRNA also seem to be regulating glucocorticoid receptor (GR) function through post-transcriptional effects that are sensitive to stress exposure, thus influencing the regulation of GR-regulated downstream genes to alter the behavioral response to stress.\textsuperscript{34}

lncRNAs are characterized by longer than 200 nucleotides in length and are also involved in regulation of gene expression in PTSD.\textsuperscript{38} Although there are not many studies on the role of lncRNAs in PTSD, Guffanti et al previously identified a single nucleotide variant lncRNA- lncRNA-ALINC01090 (previously called AC068718.1) that reached genome-wide significance in a GWAS of PTSD.\textsuperscript{39}

**Candidate gene studies**

While twin studies suggest a genetic component to PTSD, candidate gene studies aid in identifying specific genes that may be associated with the disorder. Candidate gene designs examine the main effect of specific genes on expression of a disorder and typically focus on biological candidates that are selected using existing biological evidence.\textsuperscript{4,5} That said, there has been much pushback, even controversy, as to the use of candidate gene studies in recent years, as large-scale GWAS studies have been possible and have expanded markedly. Few if any prior candidate genes have been replicated in a well-powered GWAS, though the Million Veteran’s Program study appears to have found an HPA-related gene, corticotrophin releasing factor receptor (CRFR1), associated with hyperarousal in PTSD.\textsuperscript{40} Additionally, without well-defined pathophysiology of a disease—as is the case for psychiatric disorders—it is not always straightforward to define candidate genes. Overall, prior candidate gene studies need to be regarded with skepticism until larger-scale replications and extensions have more definitively demonstrated their involvement or lack thereof.

With those caveats in mind, we feel it is still worthwhile, even if for historical purposes, to mention the work to date. The current literature on the genetic markers for PTSD spans more than 100 studies published since 1991: for a detailed overview of this literature, we direct readers to several comprehensive reviews.\textsuperscript{4,5,41,44} The present review focuses on the HPA axis and FK506 binding protein 51 (FKBP5), discussing several of the more robust studies examining this target.

Repeated exposure to trauma alters endocrine mechanisms involved in the stress response. The hypothalamic-pituitary-adrenal (HPA) stress axis—in addition to filling many other roles such as immune and metabolic function—guides the endocrine response to stress.\textsuperscript{45} HPA axis dysregulation has been observed in both depression and PTSD, though each disorder appears to have unique manifestations.\textsuperscript{45} The effects of this dysregulation change the HPA axis function, altering its response to cortisol feedback. This is thought to be associated with physiological and psychological emotion dysregulation and stress hyper-responsiveness, both of which are implicated in PTSD.\textsuperscript{41} Evidence also suggests that early life adversity may contribute to epigenetic changes in the HPA axis which may impact the development of PTSD.\textsuperscript{45}

**FK506 binding protein 51 gene**

The FK506 binding protein 51 (FKBP5) gene is perhaps the most comprehensively studied candidate among genes related to the HPA axis. It is believed to be an important regulator of stress response through altering GR sensitivity.\textsuperscript{46} Through its role as both an inhibitor of GR translocation to the nucleus, but also an exquisitely stress- and GR-responsive gene, it is thought to act as a rapid, intracellular feedback regulator of GR sensitivity within the cell. FKBP5 has shown strong association with PTSD in conjunction with a history of childhood trauma/abuse when examined in gene-environment interaction (GxE) studies.\textsuperscript{23,47,48} One such study identified allele-specific, early trauma exposure-dependent demethylation of CpGs in FKBP5, which suggests a FKBP5 x child abuse interaction resulting in differential (upregulated) transcriptional activation of FKBP5 in response to childhood abuse.\textsuperscript{23} Wang et al systematically reviewed interactions between FKBP5, early life stress, and risk for PTSD. Results from this meta-analysis revealed a significant interaction between the T allele of rs1360780 and early life stress in those with PTSD. The C-allele of rs3800373 and the T-allele of rs9470080 also interacted with early life stress and predicted higher risk for PTSD.\textsuperscript{49} A second more recent meta-analysis reaffirmed these findings and adds to the mounting evidence of an overall effect of FKBP5 interacting with trauma exposure on PTSD.\textsuperscript{50} Although the precise mechanisms are still to be understood, a working
hypothesis is that exposure to prior trauma, particularly early life stress, interacts with stress-sensitive genotypes through long-lasting DNA methylation changes in FKBP5, leading to greater stress responsiveness later in life through altered GR sensitivity.

**Epigenetics of intergenerational transmission of stress**

Recent mechanistic studies using animal models have investigated the effects of stress on epigenetic machinery and how future generations may be affected. Rodent studies examining parental care influences revealed that differences in maternal phenotypes, namely grooming behavior, had effects on their pup’s development of behavioral and HPA responses to stress as adults.51 Further studies examining DNA methylation in high vs low maternal care parental behaviors revealed elevated DNA methylation levels in the offspring after low maternal care, and lower methylation in those with high maternal care—potentially contributing to reduced transcriptional activation of the GR in the low-maternal care offspring.23,51 In humans, Yehuda et al examined intergenerational effects of trauma in Holocaust survivors and their offspring through measuring cytosine methylation within the FKBP5 gene. Results revealed differential findings for survivors and their offspring, with higher levels of methylation in survivors compared with controls, and lower in offspring, further demonstrating the potential of trauma influences on epigenetic mechanisms to have intergenerational effects.52 While these studies have small sample sizes and need replication in much larger cohorts, as discussed below with genome-wide studies, they provide intriguing initial insight into gene regulation as a function of epigenetic alterations in trauma-related symptoms and syndromes.

**Genome-wide association studies**

The current most powerful and robust method to study the interplay between genetics and PTSD uses genome-wide association studies (GWAS) to provide an unbiased approach to identify loci in the genome that have association with PTSD. Large-scale GWAS compare hundreds of thousands of single-nucleotide polymorphisms (SNP) across the entire genome to identify variants that may have a causal effect on the disorder. Only in recent years has the fiscal feasibility of using this method become possible with a drastic 2000-fold reduction in cost per genotype in a 10-year period.44 A major challenge with this approach, however, is amassing a large enough sample to achieve the required statistical power to detect these loci—with a statistical P-value threshold of 5x10^-8 required for the multiple test correction after examining roughly a million SNPs per individual.44 The Psychiatric Genomic Consortium (PGC) was organized in 2007 to centralize the GWAS from around the world and to adequately power analyses.42 Subsequently they are now the largest collaboration in the history of psychiatry, with more than 250 000 subjects, and the inclusion of more than 500 scientists from 100 countries.42

Already other mental disorders such as schizophrenia, depression, and bipolar disorder have utilized GWAS successfully to identify genes and molecular pathways of interest, and only recently has focus turned to PTSD—with the first GWAS recently published in 2013.44 We have identified 12 successful GWAS in PTSD to-date (Table 1) from individual studies, which discovered several genes of interest due to their prior associations with stress or epigenetic regulation of neuronal function, including the following: LINC01090, BC036345, ZNRD1-ASI, and RORA.40,42,46,54,62 Most of these cohorts and many more have been combined for the meta-analytic approaches of the PGC, and are currently examining GWAS for PTSD in >150 000 subjects. Thus, it is still relatively early in the field and further research is required. GWAS are the first step in identifying these genes, and further studies using an array of molecular and clinical methods must still be employed to validate these findings.5 Given the multiple genes of small effect size that are found in large-scale GWAS, it is not yet entirely clear how these findings will lead to actionable interventions. There are several thoughts about this, which remain to be determined: (i) while any given SNP or gene may have small effects, “hub”genes or combined pathways may be triangulated and together have much larger effect size and serve as important targets representing additive risk from multiple genes and SNPs; (ii) while the common variant findings indeed have very small effects when the entire syndrome of PTSD is considered, there may be yet-to-be-determined biological subtypes of PTSD, each of which is determined by a smaller number of larger effect size variants with less biological heterogeneity; or iii) while the common variants and genes themselves in a causal fashion are of limited effect, their identification will lead to novel understanding of the biology of PTSD,
<table>
<thead>
<tr>
<th>STUDY</th>
<th>SAMPLE SIZE</th>
<th>COHORT</th>
<th>SNP(S)</th>
<th>GENE</th>
<th>P VALUE</th>
<th>HIGHLIGHTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logue et al, 2013</td>
<td>Discovery: N=491</td>
<td>Discovery: white European American military veterans</td>
<td>rs8042149</td>
<td>Retinoid-related orphan receptor alpha (RORA)</td>
<td>2.50E-08</td>
<td></td>
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<tr>
<td></td>
<td>Replication: N=600</td>
<td>Replication: African American military veterans</td>
<td></td>
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<tr>
<td>Xie et al, 2013</td>
<td>Discovery: N=4344</td>
<td>European American and African American</td>
<td>rs6812849</td>
<td>Tolloid-Like 1 (TLL 1)</td>
<td>3.10E-09</td>
<td></td>
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<tr>
<td></td>
<td>Replication: N=2643</td>
<td>European American and African American</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guffanti et al, 2013</td>
<td>Discovery: N=413</td>
<td>Discovery: DNHS women</td>
<td>rs10170218</td>
<td>LINC01090 (long noncoding RNA)</td>
<td>5.09E-08</td>
<td></td>
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<tr>
<td></td>
<td>Replication: N=2541</td>
<td>Replication: NHSII women</td>
<td></td>
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<tr>
<td>Wolf et al, 2014</td>
<td>Discovery: N=484</td>
<td>European American military veterans</td>
<td>rs263232</td>
<td>Adenylyl cyclase 8 (ADCY8)</td>
<td>6.12E-07</td>
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<tr>
<td>Nievergelt et al, 2015</td>
<td>Discovery: N=3494</td>
<td>MRS military veterans</td>
<td>rs6482463</td>
<td>Phosphoribosyl transferase domain containing 1 (PRTFDC1)</td>
<td>2.04E-09</td>
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<tr>
<td></td>
<td>Replication: N=491</td>
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<tr>
<td>Almil et al, 2015</td>
<td>Discovery: N=147</td>
<td>Discovery: Military veterans</td>
<td>rs717947</td>
<td>BC036345 (long noncoding RNA)</td>
<td>1.28E-08</td>
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<tr>
<td></td>
<td>Replication: N=2006</td>
<td>Replication: GTP Large urban community cohort</td>
<td></td>
<td></td>
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<tr>
<td>Study</td>
<td>Sample Size</td>
<td>Cohort</td>
<td>SNP(s)</td>
<td>Gene</td>
<td>p Value</td>
<td>Highlights</td>
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<td>Ashley-Koch et al, 2015</td>
<td>Discovery: N=1708</td>
<td>Non-Hispanic black (NHB) Non-Hispanic white (NHW)</td>
<td>rs7866350 (NHW Cohort)</td>
<td>TBC1 domain family member 2 (TBC1D2)</td>
<td>1.10E-06</td>
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<tr>
<td>Stein et al, 2016</td>
<td>Discovery: N=7774</td>
<td>American military veterans</td>
<td>rs159572</td>
<td>Ankyrin repeat domain 55 (ANKRD55)</td>
<td>2.34E-08</td>
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<td></td>
<td>Replication: N=5916</td>
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<td>rs11085374</td>
<td>Zinc finger prot. 626 (ZNF626)</td>
<td>4.59E-08</td>
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<tr>
<td>Kilaru et al, 2016</td>
<td>Discovery: N=3678</td>
<td>GTP Large urban community cohort</td>
<td>N/A</td>
<td>Neurolgin 1 (NLGN1)</td>
<td>minSNP: 1.00E-06</td>
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<td></td>
<td>Replication: N=205</td>
<td>DCHS pregnant south African women</td>
<td>N/A</td>
<td>ZNRD1-AS1 (long noncoding RNA)</td>
<td>VEGAS: 1.00E-06</td>
<td></td>
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<tr>
<td>Melroy-Greif et al, 2017</td>
<td>Discovery: N=512</td>
<td>Mexican Americans and American Indians</td>
<td>rs6681483 rs6667389 rs10888255 rs10888257</td>
<td>Olfactory receptor family 11 subfamily L Member 1 (OR11L1)</td>
<td>1.83E-06</td>
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</tr>
<tr>
<td>Duncan et al, 2017</td>
<td>Discovery: N=20730</td>
<td>Trauma exposed adults from 11 contributing studies</td>
<td>rs139558732 African American</td>
<td>Kelch-like protein 1 (KLHL1)</td>
<td>3.33E-08</td>
<td>PGC-PTSD</td>
</tr>
<tr>
<td>Morey et al, 2018</td>
<td>Discovery: N=157</td>
<td>European American and African American military veterans</td>
<td>rs6906714 rs17012755 rs76832471 rs9499406</td>
<td>LINC02571</td>
<td>5.99E-08</td>
<td>6.05E-08 6.51E-08 8.19E-08</td>
</tr>
<tr>
<td></td>
<td>Replication: N=133</td>
<td>GTP African American women</td>
<td></td>
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</tbody>
</table>

Table I. Genome-wide association studies in PTSD. GTP- Grady Trauma Project; DCHS- Drakenstein Child Health Study; NHSII- Nurses’ Health Study II.
<table>
<thead>
<tr>
<th>STUDY</th>
<th>SAMPLE SIZE</th>
<th>COHORT</th>
<th>SIGNIFICANT CPG SITE(S)</th>
<th>GENE</th>
<th>P VALUE</th>
<th>HIGHLIGHTS</th>
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</thead>
<tbody>
<tr>
<td>Uddin et al, 2010</td>
<td>N=100</td>
<td>DNHS</td>
<td>cg17709873, cg25831111</td>
<td>Retinoid-related orphan receptor alpha (RORA), Coenzyme A synthase (COASY)</td>
<td>3.00E-3</td>
<td>1.00E-3 (unadjusted)</td>
</tr>
<tr>
<td>Smith et al, 2011</td>
<td>N=110</td>
<td>African Americans</td>
<td>cg24577137, cg08081036, cg20098659, cg07967308, cg07759587</td>
<td>Translocated promoter region, nuclear basket protein (TPR), Annexin A2 (ANXA2), C-type lectin domain family 9 member a (CLEC9A), Acid phosphatase 5, Tartrate resistant (ACP5), TLR8 toll like receptor 8 (TLR8)</td>
<td>1.90E-06, 9.30E-06, 4.30E-06, 8.00E-06, 1.10E-05</td>
<td></td>
</tr>
<tr>
<td>Uddin et al, 2013</td>
<td>N=100</td>
<td>DNHS</td>
<td>118 CpG sites/116 genes</td>
<td></td>
<td>1.00E-2</td>
<td>(unadjusted)</td>
</tr>
<tr>
<td>Mehta et al, 2013</td>
<td>N=168</td>
<td>GTP</td>
<td>458 CpG sites/164 genes</td>
<td></td>
<td>&lt;5.00E-2</td>
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<td>Mehta et al, 2017</td>
<td>Discovery: N=211, Replication: N=115</td>
<td>Australian male Vietnamese war veterans, GTP males</td>
<td>cg26499155, cg02357741, cg09325682, cg17750109, cg16277944</td>
<td>Intergenic (43 kb from leucine-rich repeat containing 3B [LRRC3B]), BR Serine/threonine kinase 1 (BRSK1), Lipocalin 8 (LCN8), Nerve growth factor (NGF), Dedicator of cytokinesis 2 (DOCK2)</td>
<td>7.94E-07, 2.24E-06, 3.28E-06, 3.06E-06, 4.95E-06</td>
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<tr>
<td>Rutten et al, 2017</td>
<td>Discovery: N=93, Replication: N=98</td>
<td>Male Dutch military veterans, Male American military veterans</td>
<td>17 DMPs and 12 DMRs</td>
<td>Dual specificity phosphatase 22 (DUSP22), Histone cluster 1 H2A pseudogene 2 (HIS-T1H2APS2), Hook microtubule tethering Protein 2 (HOOK2), Ninjurin 2 (NINJ2), Paired box 8 (PAX8), Ring finger protein 39 (RNF39), Zinc finger protein 57 (ZFP57)</td>
<td>&lt;5.00E-2</td>
<td></td>
</tr>
</tbody>
</table>
### Table II. Epigenome-wide association studies in post-traumatic stress disorder.

<table>
<thead>
<tr>
<th>STUDY</th>
<th>SAMPLE SIZE</th>
<th>COHORT</th>
<th>SIGNIFICANT CPG SITE(S)</th>
<th>GENE</th>
<th>P VALUE</th>
<th>HIGHLIGHTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hammamieh et al, 2017</td>
<td>Training set: N=99 &lt;br&gt; Test set: N=60 &lt;br&gt; Merged: N=159</td>
<td>OEF/OIF Male American military veterans</td>
<td>5578 differentially methylated CpG islands</td>
<td>3662 DMGs &lt;br&gt; 3339 DMGs</td>
<td>&lt;5.00E-2 &lt;br&gt; (unadjusted)</td>
<td></td>
</tr>
<tr>
<td>Kuan et al, 2017</td>
<td>N=473</td>
<td>WTC</td>
<td>cg05693864 &lt;br&gt; cg06182923 &lt;br&gt; cg08696494 &lt;br&gt; cg25664402 &lt;br&gt; cg05569176 &lt;br&gt; cg09370982 &lt;br&gt; cg07654569</td>
<td>Zinc finger DHHC-type containing 11 (ZDHHC11) &lt;br&gt; CUB and sushi multiple domains 2 (CSMD2) &lt;br&gt; Collagen type IX alpha 3 chain (COL9A3) &lt;br&gt; Intergenic Programed cell death 6 Interacting protein (PDCD6IP) &lt;br&gt; TBC1 domain family member 24 (TBC1D24) &lt;br&gt; Family with sequence similarity 164, member A (FAM164A)</td>
<td>1.73E-06 &lt;br&gt; 4.73E-05 &lt;br&gt; 5.39E-05 &lt;br&gt; 5.80E-05 &lt;br&gt; 7.82E-05 &lt;br&gt; 8.97E-05 &lt;br&gt; 9.91E-05</td>
<td></td>
</tr>
<tr>
<td>Ratanathara-thorn et al, 2017</td>
<td>N=147</td>
<td>Four military cohorts (MRS, PRISMO, VA-M, and VA-NCPTSD) &lt;br&gt; Three civilian cohorts (DNHS, GTP, and WTC)</td>
<td>Proposed – Consortium Study Description</td>
<td>Planned meta-analysis</td>
<td></td>
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<tr>
<td>Uddin et al, 2018</td>
<td>N=545</td>
<td>Civilian trauma exposed cohorts</td>
<td>cg23637605 &lt;br&gt; cg19577098</td>
<td>Neuregulin 1 (NRG1) &lt;br&gt; Hepatocyte growth factor-regulated tyrosine kinase substrate (HGS)</td>
<td>4.66E-08 &lt;br&gt; 1.47E-07</td>
<td>Meta-analysis</td>
</tr>
</tbody>
</table>

*OEF/OIF-Operation Enduring/Iraqi Freedom; MRS- Marine Resiliency Study; PRISMO- Prospective Research in Stress-Related Military Operations; VA-M- Veterans Affairs’ Mental Illness Research, Education and Clinical Centers; VA-NCPTSD- National Center for PTSD; WTC- World Trade Center 9/11 First Responders study.*
leading to novel more powerful interventions. In summary, the field is hopeful that many robust GWAS gene candidates will be identified with these tools, potentially transforming our approach to the biology of PTSD.

Epigenome-wide association studies

Following a similar unbiased approach, epigenome-wide association studies (EWAS) offer a novel approach to finding candidate gene pathways through examining epigenetic mechanisms at a genome-wide level. EWAS studies to-date have focused primarily on DNA methylation, which has been the most cost-effective to examine in large data sets.30 We have identified 10 EWAS studies to date that examined DNA methylation (Table II).24,63-71 Recent EWAS focusing on combat veterans identified evidence for a relationship between combat trauma and PTSD symptoms, which may be mediated by longitudinal changes in DNA methylation.66-68 Analysis revealed a number of gene associations to PTSD symptom severity including the following: BRSK1, LCN8, NFG, DOCK2, ZFP57, and RNF39.66-68 The PGC-PTSD has also commissioned a work group to focus on building a data set to adequately power large-scale PTSD research examining DNA methylation and other epigenetic mechanisms.70 A recent publication by the PGC-PTSD outlines a framework for using meta-analysis with modest sample sizes to create well-powered epigenetic association.70 Further, Uddin et al conducted a meta-analysis using three civilian cohorts and identified NRG1 (cg23637605) and HGS (cg19577098) as biomarkers for PTSD.71

Conclusions and future directions

Epigenetics provides potentially the best approach for understanding the interaction of genetics with environmental exposure to trauma in PTSD. Here we have reviewed some recent progress in understanding DNA methylation, histone regulation, and noncoding RNA approaches to epigenetic regulation in PTSD. The most profound recent progress in the biology of PTSD has been the onset of large-scale collaborations to support enormous studies in the genetic architecture of PTSD through the Psychiatric Genomics Consortium and collaborative GWAS studies, allowing compilation of hundreds of thousands of samples. These consortia are also beginning to allow combined datasets examining DNA methylation arrays and RNA sequencing studies, which soon may also be very well-powered. Together such approaches offer great promise for determining the true genetic and epigenetic architecture of risk vs resilience in PTSD. Such progress may afford novel targeted therapeutic approaches to enhance treatment and prevention of PTSD in the aftermath of trauma.

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References

Epigenetics of PTSD - Howie et al


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| 1999 | Bipolar Disorders  
Depression in the Elderly  
Nosology and Nosography |
| 2000 | Posttraumatic Stress Disorder  
Alzheimer’s Disease  
From Research to Treatment in Clinical Neuroscience  
Schizophrenia: General Findings |
| 2001 | Genetic Approach to Neuropsychiatric Disorders  
Schizophrenia: Specific Topics  
Cerebral Aging  
New Perspectives in Chronic Psychoses |
| 2002 | Pathophysiology of Depression  
CNS Aspects of Reproductive Endocrinology  
Anxiety I  
Drug Development |
| 2003 | Dementia  
Psychiatric Disorders in Somatic Medicine  
Anxiety II  
Chronobiology and Mood Disorders |
| 2004 | Predictors of Response to Treatment in Neuropsychiatry  
Neuroplasticity  
Parkinson’s Disease  
Mild Cognitive Impairment |
| 2005 | Early Stages of Schizophrenia  
New Psychiatric Classification based on Endophenotypes  
Pharmacology of Mood Disorders  
Sleep Disorders, Neuropsychiatry, and Psychotropics |
| 2006 | Diagnosis and Management of Schizophrenic Disorders  
Depression in Medicine  
Drug Discovery and Proof of Concept  
Stress |
| 2007 | Neuropsychiatry and Cardiovascular Disease  
Neuropsychiatric Manifestations of Neurodegenerative Disease  
Chronobiology in Psychiatry  
Addictive Substances |
| 2008 | Epilepsy and Psychiatry  
Developments in Bipolar Disorder  
The Core of Depression  
Remission in Depression |
| 2009 | Child and Adolescent Psychiatry  
Alzheimer’s Disease and Mild Cognitive Impairment  
Neurotoxicity and Neuroprotection  
Personalized Medicine: Prediction, Prevention, Participation |
| 2010 | Genetics and Genomics  
Obsessive-Compulsive Spectrum Disorders  
Schizophrenia  
Neurocircuitry of Cognition, Emotion, and Behavior |
| 2011 | Medical and Physiological Aspects of Depression  
What Can We Learn From Naturalistic Vs Controlled Trials?  
Trauma, Brain Injury, and Post-traumatic Stress Disorder  
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