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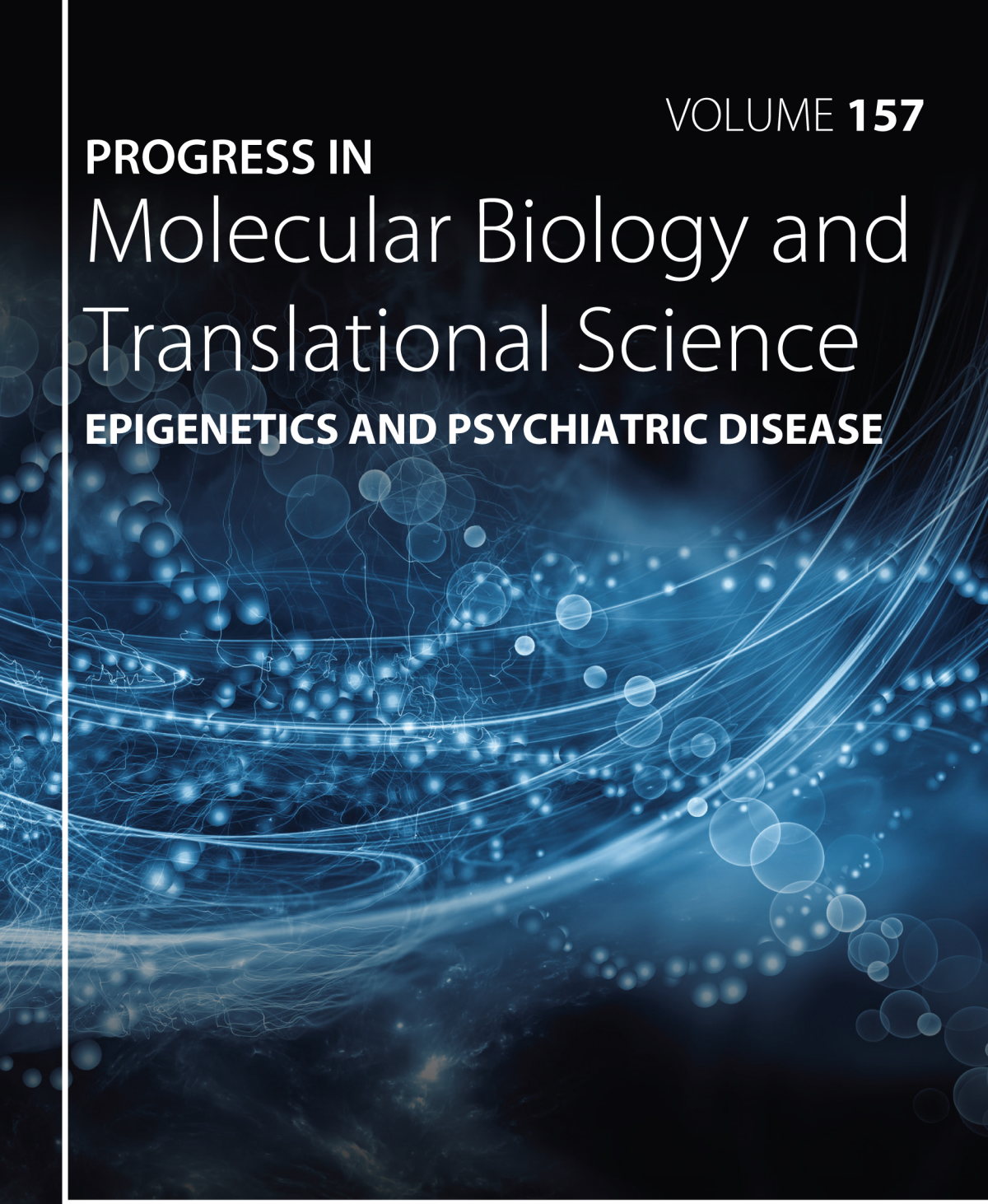

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CHAPTER NINE

DNA Methylation and Psychiatric Disorders

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Abstract

DNA methylation has been an important area of research in the study of molecular mechanism to psychiatric disorders. Recent evidence has suggested that abnormalities in global methylation, methylation of genes, and pathways could play a role in the etiology of many forms of mental illness. In this article, we review the mechanisms of

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DNA methylation, including the genetic and environmental factors affecting methylation changes. We report and discuss major findings regarding DNA methylation in psychiatric patients, both within the context of global methylation studies and gene-specific methylation studies. Finally, we discuss issues surrounding data quality improvement, the limitations of current methylation analysis methods, and the possibility of using DNA methylation-based treatment for psychiatric disorders in the future.

1. DNA METHYLATION IN HUMAN GENOME

DNA methylation is the epigenetic process by which methyl groups are added to DNA nucleotides, primarily cytosine and adenine. Methylation is thought to have a significant impact on the structure and functions of DNA through covalent chemical modification. This article focuses on DNA methylation in the human genome, specifically as it relates to psychiatric disorders.

1.1 Cytosine Methylation and Demethylation

In the human genome, DNA methylation predominantly occurs at cytosine nucleotides that are paired with guanine, resulting in what is termed CpG methylation. In addition, the majority of CpG sites (60%–90%) are methylated in mammals.[1] CpG methylation was once considered to be the only form of DNA methylation in the human genome, but recently, examples of non-CpG methylation have been discovered.

There are three major known forms of cytosine—cytosine, methylcytosine, and hydroxymethylcytosine, commonly symbolized as C, 5mC, and 5hmC, respectively, as the methylation and hydroxymethylation occur at the 5th carbon atom of the cytosine ring. 5mC is the most prevalent form of DNA methylation. 5hmC occurs more frequently in brain, heart, and sperm and less frequently in spleen, liver, and cancer cell lines.[2–4] 5hmC constitutes 0.6% of total nucleotides in Purkinje cells and 0.2% of total nucleotides in granule cells. In contrast, 5mC accounts for 1.5% of total nucleotides in Purkinje cells and 2% of granule cell nucleotides. About 0.5% of the total nucleotides in Purkinje and granule cells are native, unmodified C.[3]

5hmC is one of the intermediate products in mammalian DNA demethylation.[5] Ten-eleven translocation cytosine dioxygenases (TETs) oxidize 5mC to produce 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). These intermediate products can then

be removed by base excision repair (BER) proteins. These oxidized cytosines (5hmC, 5fC, and 5caC) may possess unique regulatory functions in the mammalian genome.[6] 5fC and 5caC are present at extremely low levels in the genome, and their levels of biological significance remain to be seen.

Both 5hmC and 5mC levels change over development. 5hmC levels were found to increase during neuronal differentiation in embryonic mouse brain[7]; 5hmC levels were also found to increase initially and then slowly decrease afterward in mouse embryonic stem cells. In contrast, 5mC levels were found to increase gradually over time in the same process.[2,3]

1.2 CpG and Non-CpG (CpH) Methylation

Even though CG dinucleotides are the primary sites for DNA methylation in mammals, DNA methylation at non-CpG sites (CpA, CpT, and CpC) have been discovered as well[8] and account for ~25% of DNA methylation in human and mouse brains.[9] Non-CpG methylation (denoted CpH) sites are most likely to be followed by a G; they are commonly seen in stem cells,[10] but have also been discovered in other tissues types, including skeletal muscle cell culture,[11] occyte,[12] and brain.[13] Most methylated CpH methylation sites are near highly methylated CpGs.[9] Neurons appear to have the highest level of CpH methylation.[13] Although CpG methylation typically exhibits a bimodal distribution of methylation levels, CpH shows intermediate methylation levels (mostly ~25%).[9]

1.3 Enzymes Involved in Cytosine Methylation and Demethylation

In mammals, there are three major DNA methyltransferases (DNMTs): DNMT1, DNMT3a, and DNMT3b. Typically, DNMT1 acts as a maintenance DNMT, whereas DNMT3a and 3b are de novo DNMTs that establish new CpG methylation. However, these functions are not entirely exclusive to one type of enzyme.[14,15] For example, DNMT3a has been found to be critical for maintaining CpH in postmitotic neurons after the CpH has been established during neuronal maturation.[9,16] DNA demethylation involves TET and BER.[5]

1.4 Adenine Methylation

Methylation of N^6-adenine (N6mA, or m6A) was reported in mammalian embryonic stem cells.[17] The gene family complex including methyltransferase like 3 (METTL3), methyltransferase like 14 (METTL14), and WTAP[18]

has been linked to methylation of N6mA.[19] The gene *Alkbh1* encodes a N6mA demethylase.[20] The METTL3–METTL14 complex methylates both DNA and RNA, with a preference for RNA.[21] Other N6Ma methyltransferases and demethylases might be discovered in the future, as METTL is a big gene family.

N6mA may perform many epigenetic regulatory functions, including suppressing gene expression and L1 element activity,[20] but much remains to be explored. A genome-wide sequencing method, DA-6mA-seq (DpnI-Assisted N^6-methylAdenine sequencing), has been developed to measure m6A in DNA.[22] Hopefully, this technology will lead to more studies of m6A in the human genome. Several reviews on N6mA are available for further reading.[23–26]

1.5 Functionality of DNA Methylation

Functions of CpG methylation: The primary function of DNA methylation has long been thought to be regulation of gene expression, particularly through suppression of gene expression, which is likely accomplished through the changing of transcription factor binding or chromatin organization. Unbiased genome-wide studies have revealed that DNA methylation levels of CpGs near transcription start sites frequently negatively correlate with expression levels of their corresponding genes, and methylation levels of gene body CpGs are often positively correlated with expression of their host genes. Moreover, both positive and negative correlations with gene expression occur at CpG sites across all kinds of genomic regions. Intergenic CpGs appear to correlate with gene expression less frequently. These phenomena have been observed in fibroblast cells and brain,[27,28] indicating that DNA methylation regulates gene expression differently according to its genomic context.

Altering transcription factor binding and changing chromatin organization are the two major ways in which DNA methylation may regulate gene expression.

Transcription factor binding: There is a possibility that some transcription factors are more sensitive to methylation status of DNA than others. For example, a study on CTCF showed that DNA methylation status has limited impact on CTCF binding patterns,[29] whereas a number of TFs, such as the ETS family and KLF4, have been found to be sensitive to methylated DNA.[30,31] EpiSELEX-seq[32] was recently developed to probe the sensitivity of transcription factor binding to DNA modifications in vitro. A catalog of DNA methylation-sensitive transcription factors will likely be needed for a better understanding of the impact of DNA methylation on gene expression.

Chromatin structure: DNA methylation established in early embryogenesis has been found to be critical to setting up chromatin structure, as measured by chromatin immunoprecipitation (ChIP) analysis.[33] More research is needed to resolve the relationship between DNA methylation and chromatin structure, though they may have a chicken–egg relationship: changes in methylation in a genomic region may lead to chromatin folding or unfolding, whereas, conversely, open chromatin could allow for binding of enzymes and changes in DNA methylation.[34]

However, a number of other biological effects, including gene expression, chromatin stability, and related mutation rates, could be products of chromatin changes,[35] genomic imprinting,[36] X chromosome inactivation,[37] and so on. The mutation rate was found to be much higher in medium (20%–60%) methylation level than in low (≤20%) methylation level and high (>80%) methylation level CpG sites within the human genome. Unmethylated CpGs had the lowest mutation rate.[35] X chromosome inactivation is highly correlated with methylation status at CpG islands.[37]

The functions of non-CpG methylation are still largely unknown. A few studies on promoter regions indicate that non-CpG methylation at gene promoters is associated with reduced gene expression, as in the case of the old model for CpG methylation.[38,39] Genome-wide assessment will be needed to get a complete picture of CpH methylation function, as has been done for CpG methylation.

1.6 Mitochondrial DNA Methylation

5mC and 5hmC were detected in mitochondrial DNA, where non-CpG methylation was found to account for ~50% of methylation within the D-loop region of mitochondria. The D-loop region controls mitochondrial DNA replication and transcription.[40] Therefore, it was suggested that all forms of DNA methylation (5mC, 5hmC, and non-CpG) may contribute to regulation of mitochondrial functions.

1.7 Techniques for Measuring DNA Methylation

A number of reviews have compared different methods for studying DNA methylation.[41–43] Global methylation level can be measured using ELISA, mass spectrometry,[44] and HPLC.[45] Measuring DNA methylation at the level of individual nucleotides in the genome requires methods such as microarray and sequencing.

Whole-genome bisulfite sequencing is the gold standard for measuring CpG and non-CpG methylation, but it is currently significantly more expensive than other methods. Choosing an ideal method requires a trade-off between costs and benefits. The best method for each experiment depends on the target methylation type (non-CpG, 5mhC, or 5mC), the coverage of the methylomic region being measured (candidate region vs. genome-wide), desired resolution (regional measure vs. single-base measure), input quantity (amount of DNA in experiment), and required sensitivity (extent of ability to detect small changes). For example, to quantify 5mhC, Tet-assisted bisulfite sequencing (TAB-Seq) is needed.[46] Some methods, such as methylated DNA immunoprecipitation (MeDIP), methyl-CpG-binding domain (MBD), and Cap-seq, only offer methylation measures of blocks of genomic regions, whereas other methods, such as bisulfite sequencing-based methods, have single-base resolution. Beyond this, data from different experimental platforms are associated with different costs and downstream analysis complexity. These factors should be considered when selecting methods for quantifying DNA methylation.

1.8 Materials for Studying DNA Methylation in Psychiatric Disorders

Tissues from live humans, postmortem specimens, and preserved clinical samples can be used to study DNA methylation. To study psychiatric disorders, we choose tissues based on a combination of accessibility and epigenetic similarities to brain. Because epigenetics is strongly tissue specific, we should know the pros and cons of all tissues for potential study. Brain is the most ideal tissue, though live brain tissue is only accessible via brain surgery, which is extremely rare for psychiatric patients today. For accessibility reasons, peripheral blood is the most commonly used tissue; saliva samples can also be used. Olfactory epithelium tissue and olfactory epithelium-derived cell lines have been proposed as promising surrogates for studying neuropsychiatric disorders[47]. However, harvesting olfactory epithelium is still a painful clinical procedure and so is less frequently used.

Brain data come with many confounding factors. What we can measure through postmortem tissue reveals only a snapshot of a human's life. Sex, age, life stress, brain injury, substance use, duration of illness, medication, cause of death, agonal state, and postmortem interval are all factors that could potentially bias findings and that therefore must be controlled for properly. The brain is a complex organ with many different regions composed of many

different cell types. Most previous and current studies have used bulk tissue, which measures the average of all cells sampled in each tissue dissection. The random composition of cell types could bias the results. Ideally, cell composition should be controlled using statistical methods. Cell sorting, laser capture microdissection, and microfluidics are experimental methods used to collect relatively homogeneous cells that can be used for either pooled-cell analysis or single-cell analysis.

Peripheral blood is valuable for developing biomarkers, even though it may be difficult to use blood as a proxy for brain to study CNS function. It is important to note that DNA methylation profiling in blood is very different from profiling in brain.[48] When searching for a proxy in blood to represent brain, it is important that the loci of interest have highly correlated methylation levels in blood and brain; the loci should also respond to environmental insult consistently in both tissues.

One DNA methylation study with paired blood and temporal lobe biopsy samples from 12 epilepsy patients detected 7.9% of CpG sites as being significantly correlated between blood and brain tissue.[48] A principal component analysis of DNA methylation in blood and brain from 17 subjects found a principal component associated with age independent of tissue type.[49] Based on this study, a web application, BECon (Blood–Brain Epigenetic Concordance; https://redgar598.shinyapps.io/BECon/), was developed to catalog the blood–brain methylation correlation[50] for CpG sites assayed on the Illumina 450 Methylation chip.

A study of blood, cortex, and hippocampus (HIP) from haloperidol-treated and untreated C57BL/6 mice detected highly correlated (correlation greater than 0.5) changes between blood and brain, showing there is hope for using blood to study haloperidol treatment-induced DNA methylation changes in brain.[51]

These results indicate the subset of CpG sites that could be analyzed from blood data to predict brain changes associated with disorders or treatments. In the future, it is quite possible that blood could regularly be used to study disease progression and treatment responses.

Cultured cells are another important category of biological material that we can use to study biological mechanisms, development, and drug response. Induced pluripotent stem cells (iPSC) carry potential for studying DNA methylation dynamics in early-stage development. iPSC can be induced from patient fibroblast, lymphocyte, or even urine cells for studying DNA methylation and its potential biological impact at early developmental stages.

Lymphoblastoid cell lines (LCLs), transformed from B cells by Epstein–Barr virus, can be preserved nearly immortally and serve as an almost unlimited source of material. LCLs have been widely used for case–control comparison, mostly as a way of studying biomarkers for drug effects. Due to its relatively homogeneous, controlled cultural environment, the LCL model is less influenced by environmental factors associated with human life, giving it unique power. The major catch of the LCL model is that the DNA methylation pattern in LCLs is significantly different from the DNA methylation pattern in whole blood.[52] Therefore, the results obtained from LCLs should be interpreted with caution.

Animal models will not be discussed in this article even though they are valuable in the study of general biology. DNA methylation in mouse brain cannot be compared with human brain at the single-base level. Besides this, animal behavior arguably differs significantly from human behavior.

2. FACTORS INFLUENCING DNA METHYLATION

DNA methylation is a product of interaction between genetic variants and environmental influence.

2.1 Genetic Factors

In the study of methylation analysis, the genetic factors governing methylation can be treated as quantitative traits. Mapping of methylation quantitative trait loci (mQTL) allows for analysis of association between SNP genotype and DNA methylation level, where a significant correlation indicates that the genomic elements regulate DNA methylation.[53] Like eQTL, genetic variants influence DNA methylation in a tissue-specific manner, making mQTL tissue specific.[54] A number of studies have identified mQTL in human brain.[53,55]

Allele-specific methylation (ASM) is another presentation of mQTL,[56,57] which focuses on *cis*-acting mQTL in relatively small genomic regions. A study of a four-generation family showed that ASM was pervasive at the nonimprinted loci and also present in somatic and germ cells.[58]

SNPs associated with mQTL or ASM may be useful in interpreting biological functions of SNPs and, further, in analyzing results of genetic associations for psychiatric disorders. mQTL SNPs have been found to be

enriched in GWAS signals for bipolar disorder[59] and schizophrenia[60] and have also been used to identify disease risk genes.[61]

Paternal age at time of birth has been related to the risk of developing psychiatric disorders, particularly schizophrenia and autism, possibly through increasing de novo DNA mutation rates.[62–64] In a mouse study, a genome-wide DNA methylation comparison revealed a significant loss of methylation in the older mice's sperm in regions regulating transcription.[65] This loss in DNA methylation in older sperm may contribute to de novo mutations, which could, in turn, increase disease risk in offspring.

2.2 Environmental Factors

DNA methylation status is not a constant like DNA sequence. It is dynamic, displaying effects of environmental changes at various times. Environmental factors can affect DNA prenatally or postnatally; some are relevant at a single point in life, whereas others are relevant to multiple stages of life.

2.2.1 Prenatal Exposures

The prenatal period is critical for fetal development and is particularly critical to brain development in the context of psychiatric disorders. Neurogenesis, neuron migration, maturation, and networking, as well as synaptic pruning, all require precise procedures that could be affected by environmental factors.

The fetus is, in general, well protected by the mother's body from biological and physical insults, though viral infection, malnutrition, and maternal substance and medication use pose potential biological threats. Many of these factors have been shown to increase risk of the fetus developing psychiatric disorders, and DNA methylation may play an important role in the process.[66] Even though a causal relationship has not been established,[67] much evidence connecting prenatal factors to DNA methylation changes are present in animal models.[68]

At least two studies have connected maternal malnutrition to the risk of schizophrenia.[69,70] Perinatal malnutrition was found to be linked to changes in DNA methylation, which may further lead to growth and metabolic disease.[71,72] DNA methylation is at its most dynamic period during early embryonic development; maternal intake of methyl-group donors such as folate, betaine, and folic acid was found to be associated with infant buccal cell DNA methylation, though only in the periconception period.[73]

Prenatal alcohol exposure was found to be associated with DNA methylation changes in genes related to protocadherins, glutamatergic synapses, and hippo signaling.[74]

Psychological stress experienced by the mothers may have an impact on the well-being of the fetus as well, possibly by affecting DNA methylation. A small study on pregnancy during the 1998 Quebec ice storm identified altered DNA methylation in blood and saliva of children 8 and 11 years old; the DNA methylation changes were correlated with objective maternal stress.[75] A new study of 121 subjects showed that even grand-maternal exposure to psychosocial stress during pregnancy had an effect on DNA methylation of the grandchildren.[76]

It is unlikely that DNA methylation is the only change maternal malnutrition and other stressors impose on fetal development and consequent risk of psychiatric and other disorders. Fetal neuroendocrine alteration[77] and inflammation reaction[78] could also lead to pathology risk.

2.2.2 Postnatal Exposures

Postnatal development and aging also involve neuronal changes, neuroplasticity, and degeneration. Moreover, biological and physical insult can lead to damage to neurons and their supporting systems.

Early life stress has been shown to have a significant adverse effect on psychiatric disease risk. Childhood abuse, neglect, poverty, and other forms of stressors can increase risk of adulthood psychiatric disorders, primarily mood and disorders such as depression, anxiety, and post-traumatic stress disorder. Mouse models indicate that at least some of these early life stressors could lead to sustained DNA hypomethylation in post-mitotic neurons.[79]

In adulthood, smoking, drinking, and substance use likely change DNA methylation levels. Studies have found that methamphetamine use can change Dnmt1, Dnmt2, and Reelin levels in rat brain[80,81]; nicotine use has been shown to decrease Dnmt1 levels in mouse GABAergic neurons and to increase expression of GAD67[82]; and DNMT-3a and DNMT-3b expression levels are reduced in alcoholic patients.[83] As DNMTs are methyltransferases, which are critical for DNA methylation, it is likely that their expression changes have an impact on DNA methylation. Indeed, alcohol use[84] and smoking[85] have been found to be associated with changes in DNA methylation in humans. Methamphetamine use was found to alter DNA methylation in a rodent model.[86] Studies of environmental factors' effects on DNA methylation changes in human brain or blood biomarkers have produced compelling results, though these require replication in large samples.

3. DNA METHYLATION IN MAJOR PSYCHIATRIC DISORDERS

Major psychiatric disorders such as schizophrenia (SZ), bipolar disorder (BD), major depressive disorder (MDD), autism spectrum disorder (ASD), eating disorders [including anorexia nervosa (AN) and bulimia nervosa (BN)], alcohol use disorder (AUD), and attention deficit hyperactivity disorder (ADHD) are characterized by complex symptoms and development of pathology determined by intricate underlying genetics and environmental factors.[87,88] Although many of these disorders are thought to be highly heritabile,[87] all psychiatric concordance rates are substantially below 100% for monozygotic twins (MZTs),[87] suggesting that environmental factors contribute significantly to the development of pathology. DNA methylation, a product of the interaction between genetics and environment, could play a significant role in the process. Here, we summarize the major studies on DNA methylation in psychiatric disorders within the realm of both global methylation and gene-specific methylation changes in candidate gene and epigenome-wide studies.

3.1 Global DNA Methylation Changes in Major Psychiatric Disorders

Global methylation changes have been studied for diseases and related traits. In the context of psychiatric disorders, global DNA methylation levels can be influenced by genetic variants,[89] specific disease, medication,[90–92] age of disease onset,[91] sex,[90,93,94] smoking status,[92] and brain region(s) being examined.[95]

Through the examination of *peripheral tissue* (blood and buccal cell) samples, many psychiatric conditions have been linked to either low or high global methylation levels. Studies have generally reported high global methylation levels in anxiety[96] and AUD.[97] In contrast, low global methylation levels have been found in smokers (vs. nonsmokers),[92] SZ,[90,91,94,98] BD,[99] MDD,[100] ASD,[101,102] and AN.[103,104] SZ patients on medication have higher global DNA methylation levels—almost as high as those of healthy controls—than drug-free patients.[90,91]

Five studies have investigated methylation level differences in brain. Measurement of methylation level in the frontal cortex (FC) revealed hypermethylation in AUD[97] and hypomethylation in BD,[95] whereas both hypermethylation[105] and hypomethylation[95] were associated with SZ. In anterior

cingulate cortex (ACC), hypermethylation was identified for SZ[95,105] and BD.[95] In HIP, hypermethylation was reported for SZ[105] and AUD.[97]

In summary, abnormal global DNA methylation levels have been detected for most psychiatric disorders, with some consistent results. In peripheral tissues and multiple brain regions, AUD patients have been recorded as having higher methylation levels than controls. In SZ and BD, increased methylation has mainly been detected in brain, whereas decreased methylation has mainly been detected in peripheral tissues. Other studies have reported negative results for methylation differences (Table 1).

3.2 Gene-Specific Methylation Studies in Psychiatric Disorders

Global methylation analysis does not capture changes of specific genes, biomarkers, and pathways. Gene-specific methylation is investigated in candidate gene studies and epigenome-wide association studies (EWASs). Neurotransmitter, neurodevelopment,[112,113] and immune system[114–116] have been hypothesized as mechanisms possibly underlying psychiatric disorders.[117,118] Results from candidate gene studies are summarized later.

3.2.1 DNA Methylation of Candidate Genes in Psychiatric Disorders

Current hypotheses in psychiatry pertaining to neurotransmitter systems include the overactivity of the dopaminergic system and hypofunction of the serotonergic system, the γ-aminobutyric acid (GABA)-ergic system and the glutamatergic system, primarily in SZ, BD, and MDD. The methylation changes consistently reported in candidate genes are plotted in the neurotransmitter systems in Fig. 1.

3.2.1.1 DNA Methylation of Dopaminergic System Genes in SZ, BD, MDD, AN, ADHD, and AUD

Dopamine (DA) overactivation has been strongly associated with SZ.[119] Antipsychotics that are antagonists of DRD2 are the most effective drugs for treating schizophrenia.[120] Methylation changes in DA system genes in major psychiatric disorders such as SZ, BD, MDD, AN, ADHD, and AUD have also been reported, in particular for the DA receptors (DRDs), catechol-*O*-methyltransferase (*COMT*), and DA transporter (*DAT*).

Five subtypes of DRD, namely, DRD1-DRD5, have been identified in mammals. Hypomethylation and high-expression of *DRD2*,[121–123] *DRD4*, and *DRD5* were identified in SZ.[123] Hypomethylation and high-expression of *DRD4* were also detected for ADHD.[124] However, in AN, hypermethylation of *DRD2* was reported.[125,126]

Table 1 Global DNA Methylation Studies of Psychiatric Disorders.

Studies	Samples	Tissues	Experimental Platforms	Major Findings
SZ and BD				
Shimabukuro *et al.* (2007)[94]	210 SZ, 237 C, Japanese	LCLs	HPLC	↓Male SZ
Dempster *et al.* (2011)[89]	22 discordant MZT pairs: 11 SZ, 11 BD	WB; B for	replication of TOP	HM27, EpiTYPER (Sequenom)
No systemic changes.				
Melas *et al.* (2012)[91]	177 SZ, 171 C	WB	LUMA; PCR	↓In SZ; ↑haloperidol (control-like); ↓early onset (a putative marker of SZ severity)
Bönsch *et al.* (2012)[90]	20 twin pairs discordant for SZ, 8 twin pairs concordant for SZ, 42 C twin pairs	WB	Methylation-sensitive restriction (HpaII) and PCR	↓In SZ twins compared with healthy C twins (males); ↑patients on medication (control-like) compared with patients not on medication
Nishioka *et al.* (2013)[98]	18 first-episode SZ, 15 C, Japanese	WB	HM27	↓In SZ
Xiao *et al.* (2014)[95]	5 SZ, 7 BD, 6 C	FC, ACC (BA9 and BA24)	MeDIP-Seq	↓SZ, BD in FC; ↑SZ, BD in ACC
Alelú-Paz *et al.* (2016)[105]	6 SZ DLPFC, 6 SZ HIP, 7 SZ ACC, 3 C	DLPFC, ACC, HIP	HM450	↑SZ

(*Continued*)

Table 1 Global DNA Methylation Studies of Psychiatric Disorders.—cont'd.

Studies	Samples	Tissues	Experimental Platforms	Major Findings
Bromberg *et al.* (2009)[106]	49 BD, 27 C	LCLs	Cytosine-extension assay	×BD
Rao *et al.* (2012)[107]	10 BD. 10 C	FC	ELISA-based assay	↑BD
Soeiro-de-Souza *et al.* (2013)[108]	50 BD, 50 C	WB	ELISA-based assay	↓5hmC levels in BD I patients; ×5mC
Burghardt *et al.* (2015)[92]	115 BD I	LCLs	LUMA	Average global methylation was significantly influenced by insulin resistance (↑), second-generation antipsychotic use (×), and smoking (↓)
Huzayyin *et al.* (2014)[99]	14 BD, 16 un-AR, 14 AR (BD or MDD), 16 C	TL	ELISA-based assay	↓In BD patients and their relatives; ↓in BD patients, with lithium treatment, not in relatives
MDD				
Tseng *et al.* (2014)[100]	49 MDD, 25 C	LCLs	Enzyme-linked immunosorbent assay-based method	Significant↓ in 5hmC and trends of↓ in 5mC, but only in the old group of MDD; 5mC level inversely correlated with disease severity
Byrne *et al.* (2013)[93]	12 MZT pairs discordant for MDD and 12 MZT pairs concordant for no MDD and low neuroticism	WB	HM450	× All; different in females
ASD				
	86 parents of cases, 200 mothers	WB		↓ASD

James *et al.* (2008)[102]			Enzymatic digestion and HPLC	
Melnyk *et al.* (2012)[101]	68 ASD, 54 C	WB	Enzymatic digestion and HPLC	↓ASD
Wong *et al.* (2014)[109]	50 MZT pairs discordant and concordant for ASD/ASD severity	WB	HM27	×ASD
Anxiety				
Murphy *et al.* (2015)[96]	25 anxious, 22 C	WB	RT-PCR, methylflash methylated DNA quantification kit	↑Anxiety
AN				
Frieling *et al.* (2007)[104]	22 AN, 24 BN, 30 C	WB	—	↓AN
Saffrey *et al.* (2013)[110]	10 AN, 10 C	Buccal cells	Sequenom mass array platform	×AN
Tremolizzo *et al.* (2014)[103]	32 AN, 13 C	WB	HpaII	↓AN
AUD				
Manzardo *et al.* (2012)[111]	10 AUD, 10 C	FC	Immunoprecipitation	×AUD
Otero *et al.* (2012)[97]	Rat	HIP, PFC	HpaII	↑AUD

ACC, Anterior cingulate cortex; *ADHD*, attention deficit-hyperactivity disorder; *AN*, anorexia nervosa; *ASD*, autism spectrum disorder; *AUD*, alcohol use disorder; *B*, brain; *BD*, bipolar disorder; *BN*, bulimia nervosa; *C*, controls; *DLPFC*, dorsolateral prefrontal cortex; *DNAm*, DNA methylation; *FC*, frontal cortex; *HIP*, hippocampus; *HM27*, Illumina Infinium HumanMethylation 27K; *HM450*, Illumina Infinium HumanMethylation 450K; *HPLC*, high-performance liquid chromatography; *LCLs*, leukocyte cell lines; *LUMA*, luminometric methylation assay; *MDD*, major depressive disorder; *SZ*, schizophrenia; *TL*, transformed lymphoblasts; *WB*, white blood cells; ↓↑ and × indicate reduced, increased, and unchanged global methylation, respectively.

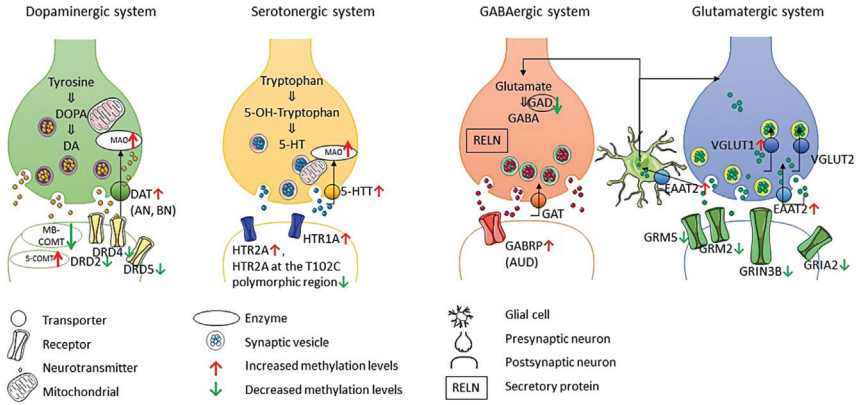

Fig. 1 Methylation changes of candidate genes related to the neurotransmitters in SZ and BD. Note: Two genes reported in non-SZ/BD are also plotted: DAT in Dopaminergic system for eating disorders and GABRP in GABAergic system for AUD because they play important roles in these neurotransmitter systems. Reports regarding RELN have been largely inconsistent.

COMT, one of the DA degradation enzymes, regulates the homeostatic levels of DA in the synapses. This gene is located on chromosome 22q11.21, a region genetically linked to SZ in several studies.[87,127] Two isoforms of *COMT* exist: the soluble short form (S-COMT) and the membrane-bound long form (MB-COMT); each isoform is believed to have distinct cellular distributions and roles. MB-COMT displays some preference for brain tissues, notably in the HIP, and primarily aids in terminating dopaminergic synaptic neurotransmission, whereas S-COMT mainly works to eliminate biologically active or toxic catechol in blood.[128]

No overall *COMT* methylation changes were identified for FC in SZ or BD.[117] Hypomethylation was found for MB-COMT in SZ and BD in both brain and saliva.[122,129,130] In brain, MB-COMT was found to be hypomethylated, especially in the left frontal lobe.[122,129,130] In blood, S-COMT hypermethylation was reported in SZ patients.[91] In the promoter of S-COMT, no methylation changes were found in SZ patients.[131]

Monoamine oxidase A (*MAOA*) encodes an enzyme that degrades DA, norepinephrine, and serotonin. Hypermethylation of *MAOA* at its promoter and exon1 was detected in SZ blood.[132]

DAT (also known as *SLC6A3*) is a DA transporter. Hypomethylation of *DAT* was detected in AUD,[133,134] and hypermethylation of *DAT* was found for AN and BN.[104]

Three main kinds of genes in dopaminergic system have been associated with methylation changes in psychiatric disorders: receptor genes (hypomethylation), transporter genes (hypermethylation), and degradation enzyme genes [both hypomethylation and hypermethylation (Fig. 1)]. These pieces of evidence support the idea that hyperactivity of DA is, to some extent, a product of methylation levels. Interestingly, the methylation changes in DA system genes for eating disorders (AN and BN) seem to be opposite to those in other disorders such as SZ and BD.

3.2.1.2 DNA Methylation of the Serotonergic System in SZ, BD, MDD, and ADHD

Serotonin, or 5-hydroxytryptamine (5-HT), a monoamine inhibitory neurotransmitter, regulates mood, appetite, and sleep. Serotonergic mechanisms play a role in the action of atypical antipsychotic drugs. Several EWASs have discovered signals possibly relevant to psychiatric illnesses in the serotonergic system pathways.[135]

Serotonin has several receptors. HT1A and HT2A, two of these receptors, are relevant to the symptoms of SZ. The *HTR1A* promoter is hypermethylated for SZ and BD in a study using LCLs.[136] In *HTR2A*, hypermethylation and low gene expression in SZ and BD were detected for FC samples,[137,138] but at its *T102C* polymorphic region, hypomethylation was reported in SZ and BD saliva.[139]

The serotonin transporter (*5-HTT*, also known as *SLC6A4*) encodes the sodium-dependent serotonin transporter. 5-HTT hypermethylation was reported in MDD[140,141] and ADHD.[140] No significant difference ($P = 0.15$) was reported between SZ and controls.[91] Hypermethylation of the *5-HTT* promoter is evident in SZ and BD.[142]

So far, serotonergic system gene methylation changes in receptor genes and transporter genes (Fig. 1) have been linked to major psychiatric disorders, suggesting that methylation could contribute to serotonin dysfunction. It is interesting that *5-HTT* was found to be mainly hypermethylated in SZ, BD, MDD, and ADHD.

3.2.1.3 DNA Methylation of Glutamatergic System in SZ and BD

Glutamate (Glu) plays a critical role in synaptic plasticity and is the most abundant fast excitatory neurotransmitter in the mammalian nervous system. Several findings have pointed to the activity in the Glu system as a partial determinant of psychosis.[89,117] Antagonists of NMDA receptors, such as ketamine, can produce a psychotic-like state in healthy subjects.

Chemical receptors of Glu were used to divide glutamate receptors into ionotropic and metabotropic. Ionotropic Glu receptors include AMPA, NMDA, and Kainate receptors. Metabotropic Glu receptors indirectly activate ion channels on the plasma membrane and include mGluR1 through mGluR8, which are encoded by genes *GRM1* through *GRM8*.

Hypomethylation of *GRM2* and *GRM5*, and no methylation irregularities for *GRM3* and *GRM8*, was identified in SZ.[143] Both glutamate ionotropic receptor AMPA-type subunit 2 (*GRIA2*) and glutamate ionotropic receptor NMDA-type subunit 3B (*GRIN3B*) were hypomethylated in male SZ patients.[117]

Glu transporters, which move Glu across the membrane, are composed of two families: the excitatory amino acid transporter (EAAT) family and the vesicular Glu transporter (VGLUT) family. VGLUT1 and VGLUT2 are complementarily expressed in brain. VGLUT1 is mainly concerned with cortico-cortical projections to relay information, with low fidelity, whereas VGLUT2 is concerned with thalamo-cortical projections, with higher fidelity.[144] VGLUT1 (encoded by *SLC17A7*) was found to be hypermethylated and has low expression in female SZ patients. VGLUT2 (encoded by *SLC17A6*), though it serves a similar function as VGLUT1, was found to be hypomethylated and highly expressed in female SZ patients.[117] This may be due to different modes of Glu release in VGLUT1 and VGLUT2. EAAT2 (encoded by *SLC1A2*) is hypermethylated in BD patients.[145] SCG2, a secretory protein located in neuronal vesicles, stimulates the release of Glu. Hypomethylation of SCG2 was detected in female SZ patients.[117]

In brief, Glu-related methylation changes have been found for SZ and BD but no other psychiatric disorders. In SZ and BD, methylation changes have been reported for glutamatergic receptor genes, secretory protein genes (hypomethylation), and transporter genes (Fig. 1).

3.2.1.4 DNA Methylation of GABAergic System in SZ, BD, and AUD

GABA acts as a potent inhibitory neurotransmitter in the adult human brain. Some EWASs have found that GABAergic neurotransmission may be involved in SZ,[117] Hypofunction of GABAergic interneurons appears to be important in the etiology of major psychosis. GABA can influence the development of neural progenitor cells via *brain-derived neurotrophic factor* (*BDNF*) expression.

Two general classes of GABA receptor are known: $GABA_A$ receptors, which are part of a ligand-gated ion channel complex, and $GABA_B$

receptors, which are metabotropic G protein-coupled receptors. *GABRP*, which encodes the $GABA_A$ receptor, was found to be hypermethylated in AUD.[146]

GAD1 (Glu decarboxylase 1, also known as GAD67), is an enzyme that synthesizes GABA from Glu. Brain region-specific results were observed in the differential methylation studies of *GAD1*. In the HIP, hypomethylation and high expression of *GAD1* have been identified in both SZ and BD.[147] In BA39-40 (parietal cortex) and cerebellar cortex (CBL), on the contrary, hypermethylation and low expression of *GAD1* were detected.[148] *GAD1* hypomethylation was identified in AUD in blood.[146]

Reelin (RELN), an extracellular matrix protein expressed by GABAergic interneurons of the cortex and glutamatergic cerebellar neurons,[149] is primarily related to neuronal migration, axonal branching, synaptogenesis, and cell signaling. The findings for methylation changes of *RELN* have been inconsistent. Hypermethylation and low expression were detected for SZ in FC[150,151] and occipital cortex[151] according to some studies, but other studies reported no significant methylation changes in SZ[117,152,153] or BD[117,153] in FC, forebrain,[153] or blood.[90] The brain studies detecting changes used small samples, lowering the reliability of the positive results.

Three genes, *GABRP*, *GAD1*, and *RELN* (Fig. 1), related to the GABAergic system, have been linked to methylation changes in major psychiatric disorders. *RELN* plays an important role in the neuron, but findings regarding the gene have been inconsistent. *GAD1* has shown brain region-specific changes.

3.2.1.5 DNA Methylation of Genes Associated With Immune System and Inflammation in SZ, BD, MDD, and PTSD

Cytokines, which can be proinflammatory or anti-inflammatory, are small secreted proteins important for communication between cells. They also act as important coordinators of immune and inflammatory responses.[154] Cytokines mobilize resources for handling physical and psychological stress by maintaining homeostasis. There has been increasing evidence for the role of inflammation in psychiatric disorders. Altered cytokine levels have been observed in multiple disorders, including SZ, MDD, OCD,[155] BD,[155,156] ASD,[157] PTSD, anxiety and panic disorder,[158,159] and possibly eating disorders.[160] Therefore, it is important to investigate immune-/inflammation-related methylation signatures for involvement in psychiatric disorders.

DNA methylation has been studied in immune-related candidate genes in PTSD, SZ, aggression, and also in treatment response to antidepressants.

Global methylation was found to be increased in PTSD patients. Differentially methylated CpG sites in PTSD mapped to genes that could be involved with or interact with inflammation pathways,[161] even though the methylation changes were not found for these cytokine genes per se. Another candidate gene study of PTSD in military service members detected DNA methylation changes at promoter regions of *H19* and *IL-18* in case–control and pre- to postdeployment comparison.[162] The HLA complex group *HCG9* gene shows consistently lower DNA methylation in brain, blood, and sperm of BD patients,[163] and also in another study of postmortem brains of SZ.[164] Differential DNA methylation of a number of interleukin genes and their transcription factor regulators are associated with chronic physical aggression.[165] IL-6 and C-reactive protein (*CRP*) were elevated in depressed patients. Furthermore, in depressive patients only, *IL-6* DNA methylation shows an inverse correlation with circulating *IL-6* and *CRP* in blood.[166] DNA methylation of the *IL-11* CpG island correlates with treatment effects of antidepressants, escitalopram, or nortriptyline in MDD patients.[167]

Glucocorticoids are a class of steroid hormones critical to the maintenance of basal and stress-related homeostasis; glucocorticoids also mediate stress and inflammation's relationship with immune response.[168] DNA methylation of the glucocorticoid receptor gene (*NR3C1*) has been frequently studied in relation to epigenetic impact of early life stress and regulation of hypothalamic–pituitary–adrenal (HPA) axis. Increased *NR3C1* promoter methylation was associated with higher cortisol responses to stress.[169]

Maternal stress can have an impact on the fetus in utero. Depressive mood in mothers correlates with *NR3C1* promoter methylation in newborn cord blood mononuclear cells.[169] In another study, *NR3C1* DNA methylation in children was shown to be related to the stress levels of their mothers during pregnancy.[170] Postnatal stress, like childhood sexual abuse, also affects this promoter's methylation level,[171] suggesting childhood maltreatment leads to permanent DNA methylation changes. DNA methylation of the *NR3C1* promoter is also found to be associated with BN,[172] and, along with interferon-gamma (*IFN-gamma*) and *IL*-6, associated with psychological traits.[173] Consequently, *NR3C1* promoter methylation has become one of the most promising epigenetic research targets in the immune–inflammation regulatory system, but whether it is involved in other psychiatric disorders such as SZ and BD remains to be seen.

Because glucocorticoids can modulate cytokine signaling,[174] the interaction between *NR3C1* and cytokines is an important topic for future epigenome-wide studies on psychiatric disorders that possibly involve immune–inflammation changes.

3.2.1.6 DNA Methylation of Other Candidate Genes

3.2.1.6.1 **BDNF in SZ, BD, and BN** *BDNF*, located on chromosome 11p14.1, has been found to be related to many biological functions, including NMDA receptor activity, synapse stability, dopaminergic, cholinergic, serotonergic, and GABAergic signaling, synaptogenesis, and dendritogenesis.

Several studies using blood samples have examined *BDNF* methylation. In *BDNF* promoter I, hypermethylation in SZ[175] is found, but *BDNF* promoter IV yields no significant results in SZ.[175] *BDNF* hypomethylation in drug-free SZ[176] was identified in another study, as was hypermethylation of *BDNF* in BN.[177] *BDNF* is associated with higher levels of methylation in BD II than in BD I.[178,179] Hypermethylation of *BDNF* is also detected in depressed patients compared with manic/mixed episode patients.[179] The respective methylation results for BD I and BD II patients seem congruent with the fact that mania is a clinical feature of BD I, whereas BD II only includes hypomania. In FC of SZ patients, *BDNF* hypomethylation is detected.[180]

3.2.1.6.2 **Candidate Genes From Genetic Studies** A number of candidate genes identified from genetic, linkage, and association studies have been further investigated for DNA methylation changes. These genes include *FOXP2*, *DTNBP1*, *AUTS2*, and *RAI1*.

FOXP2 (*forkhead box P2*), a transcription factor located on 7q31, was first found mutated in a large multigenerational family study, in association with a rare severe speech and language disorder; the gene was later implicated in SZ and MDD[181] by an association study. *FOXP2* is hypermethylated in the brains of SZ patients.[182]

DTNBP1 (*dystrobrevin-binding protein 1*), located on 6p22.3, encodes a neuronal protein that binds to beta-dystrobrevin, may be part of the dystrophin protein complex, and has been reported in genetic studies on SZ,[183–185] BD,[183,186,187] and MDD.[188] *DTNBP1* is hypermethylated in SZ and BD, according to a saliva study[189] and an FC study.[117]

AUTS2 (autism susceptibility candidate 2), located on 7q11, encodes a nuclear protein that is expressed primarily in the developing brain. It is mainly detected in ASD,[190] SZ,[191] and AUD.[192] *AUTS2* is found to be hypermethylated in SZ male FC samples.[117]

RAI1 (*retinoic acid induced 1*), located on 17p11.2, was identified in Smith–Magenis syndrome[193] and SZ[194] through genetic analyses. These genes are all hypermethylated in SZ and BD. RAI1 is located at a region that encodes a polymorphic polyglutamine tract associated with SZ and response to antipsychotic medication; it is found to be hypermethylated in the FC of SZ females.[117]

3.2.1.6.3 **Other Candidate Genes** Five genes located in two chromosomal regions, 1p36.22 and 11p15.5, were reported in several papers.

ANP (atrial natriuretic peptide), located in 1p36.22, is hypermethylated in BN[195]; *MTHFR* (*methylenetetrahydrofolatereductase*), which is also located in 1p36.22, is hypomethylated in ADHD.[124]

11p15.5 is enriched for imprinted genes and includes three relevant candidate genes, *IGF2*, *H19*, and *KCNQ1OT1*. *IGF2* is a paternally inherited member of the insulin family of polypeptide growth factors; *H19* is near *IGF2* and is only expressed from the maternally inherited chromosome; *KCNQ1OT1* is a maternally inherited long noncoding RNA gene located in an intron of *KCNQ1*. Hypomethylation of *IGF2*,[124,196] *H19*, and *KCNQ1OT1* was identified in ADHD.[124]

Table 2 summarizes all the candidate genes we found in the methylation studies associated with psychiatric disorders.

3.2.2 Epigenome-Wide Association Studies

EWASs are currently the best unbiased method for uncovering disease-related changes. Brain tissue is thought to be the tissue most linked to psychiatric disorders and is of great importance in the study of disease-related pathways and mechanisms. In addition, peripheral tissue studies have revealed some important findings, especially ones related to immune-related pathways and biomarkers. EWAS has examined data from both of these tissues; the findings are presented later.

3.2.2.1 DNA Methylation Changes in Brain Highlight the Importance of Early Brain Development

About 16 brain EWASs have been published so far. Alelú-Paz *et al.* found differentially methylated probes (DMPs) related to neurotransmission pathway in DLPFC, antigen processing and presentation in HIP, and protein kinase activity and signal transduction in ACC.[105] Mill *et al.*, using FC samples, found 51 genes in men and 37 genes in women that were differentially methylated in SZ patients in comparison to controls. These genes are enriched in brain development and mitochondrial functions.[117] Pathways

Table 2 DNA Methylation Studies of Candidate Genes for Psychiatric Disorders.

Gene	Locus	Function	Tissues	Major Findings	References
Dopaminergic system					
DRD2	11q23.2	DA receptor	B, WB	↓M and ↑E (SZ), ↑M (AN)	121–123,125,126,197
DRD4	11p15.5	DA receptor	B, WB	↓M and ↑E (SZ, ADHD)	123,124
DRD5	4p16.1	DA receptor	WB	↓M and ↑E (SZ)	123
MB-COMT	22q11.21	Regulates the homeostatic levels of DA in the synapses	B, S	↓M (especially in the left frontal lobe for SZ and BD)	122,129,130
S-COMT	22q11.21	Regulates the homeostatic levels of DA in the synapses	B, WB, S	↑M (SZ)	91,131
SLC6A3 (DAT)	5p15.33	DA transporter	WB	↑M (AN, BN), ↓M (AUD)	125,133,134
MAOA	Xp11.3	Degradation enzyme of DA, serotonin, and NE	WB	↑Male	132
DBH	9q34.2	DA beta-hydroxylase	WB	↓M (AUD)	146
Serotonergic system					
HTR1A	5q12.3	Serotonin receptor	WB	↑M (SZ and BD)	136
HTR2A	13q14.2	Serotonin receptor	B	↑M and ↓E (SZ)	137,138
HTR2A at the T102C	13q14.2	Serotonin receptor	S	↓M (SZ and BD)	139

(*Continued*)

Table 2 DNA Methylation Studies of Candidate Genes for Psychiatric Disorders.—cont'd.

Gene	Locus	Function	Tissues	Major Findings	References
polymorphic region					
SLC6A4 (5-HTT)	17q11.2	Serotonin transporter	B, WB, S	↑M (SZ and BD (antipsychotic-free, MZT), MDD, ADHD)	91,124,140–142,198
GABAergic system					
GAD1 (GAD67)	2q31.1	Enzyme that synthesizes GABA from Glu	B, WB	↓M (SZ, BD, AUD)	117,146–148
RELN	7q22.1	Cell positioning and neuronal migration during brain development	B, WB	↑M (SZ brain)	90,117,150–153
GABRP		GABA receptor	WB	↑M (AUD)	146
Glutamatergic system					
GRM2	3p21.2	Glu metabotropic receptor	B	↓M (SZ)	117
GRM5	11q14.2-q14.3	Glu metabotropic receptor	B	↓M (SZ)	117
GRIN3B	19p13.3	Located ~10 kb upstream of the NMDA-receptor-subunit	B	↓M (male SZ)	117
GRIA2	4q32.1	In the promoter of the AMPA-receptor-subunit	B	↓M and↑E (male SZ)	117
SCG2	2q36.1	A secretory protein located in neuronal vesicles that is known to stimulate the release of Glu	B	↓M (female SZ)	117
VGLUT1	19q13.33		B		117

		Packs Glu into synaptic vesicles		↑M and↓E (female SZ)	
VGLUT2	11p14.3	Glutamate transporter	B	↓M and↑E (female SZ)	117
SLC1A2 (EAAT2)	11p13	A member of the solute transporter proteins	WB	↑M (BD)	145
BDNF	11p14.1	Brain-derived neurotrophic factor; a principal regulator and developer, involved in function and plasticity of neurons, as well as in progression of pathology	B, WB	↑M (blood in BD II; blood in SZ at promoter I; blood in BN) ↓M (FC, B in drug-free SZ)	175–177,179,180,199
Immune and inflammation					
H19	2q33.2	HLA complex group	WB	↑M (PTSD)	162
IL18	11q23.1	Interleukin 18	WB	↑M (PTSD)	162
IL-6	7p15.3	Interleukin 6	WB	↑M (depression)	166
CRP	1q23.2	C-reactive protein	WB	↑M (depression)	166
IL11	19q13.42	Interleukin 11	WB	↑M (escitalopram), ↓M (nortriptyline)	167
HCG9	6p21.3	HLA complex group 9	B, WB,	sperm	↓M (BD, SZ)
163,164					
NR3C1	5q31.3	Glucocorticoid receptor	WB	↓M (ADHD)	124
NR3C1 promoter	5q31.3	Glucocorticoid receptor	WB, cord	blood	↑M (BN, stress, PTSD, BN)
169–172					

(*Continued*)

Table 2 DNA Methylation Studies of Candidate Genes for Psychiatric Disorders.—cont'd.

Gene	Locus	Function	Tissues	Major Findings	References
FKBP5	6p21.31	Immunoregulation	WB	↑M (BD)	200
Candidate genes from genetic studies					
FOXP2	7q31.1	Positive selection in human lineage and genes associated with SZ	B	↑M (SZ)	182
DTNBP1	6p22.3	Prenominated "psychosis-candidate gene"	B, S	↑M (SZ)	117,189
AUTS2	7q11.22	Spans a translocation breakpoint associated with mental retardation and autism	B	↑M (SZ)	117
RAI1	17p11.2	Polymorphic polyglutamine tract associated with SZ and response to antipsychotic medication	B	↑M (SZ)	117
1p36.22					
ANP	1p36.22	Atrial natriuretic peptide	WB	↑M (BN)	195
MTHFR	1p36.22	Methylenetetrahydrofolate reductase	WB	↓M (ADHD)	124
11p15.5					
IGF2DMR (IGF2)	11p15.5	Part of the insulin family of polypeptide GF, paternally inherited	WB, CB	↓M (ADHD)	124,196
H19	11p15.5	Near IGF2, maternally inherited	WB	↓M (ADHD)	124
KCNQ1OT1	11p15.5	LncRNA, maternally inherited	WB	↓M (ADHD)	124
Other candidate genes					
RPP21	6p22.1	A component of rib nuclease	B	↑M (SZ, female BD)	117
KEL	7q34	Part of the Kell blood group, metalloendopeptidase	B	↑M (SZ, female BD)	117
RPL39	Xq24	Ribosomal protein	B	↑M (female BD)	117

MECP2	Xq28	Chromosomal protein that binds to methylated DNA	FC	↑M and ↓E (ASD)	201
SNCA	4q22.1	Alpha synuclein	WB	↑M (AN, AUD) and ↓E	104,202
HERP	16q13	Homocysteine-induced endoplasmic reticulum protein	WB	↑M (AUD) and ↓E	203
PPM1G	2p23.3	Negative regulation of cell stress response	WB	↑M (AUD)	204
OXTR	3p25.3	Oxytocin receptor	BC	↑M (AN)	205
SSTR4	20p11.21	Somatostatin receptor 4	WB	↓M (AUD)	146
ALDH1L2	12q23.3	Aldehyde dehydrogenase 1 family member L2	WB	↓M (AUD)	146
ST6GALNAC1	17q25.1	Related "synthesis of substrates in *N*-glycan biosynthesis"	WB	↓M (BD)	89
KCNQ3	8q24.22	Regulation of neuronal excitability	B	↓M (BD)	206
COX-2	1q31.1	Prostaglandin biosynthesis	B	↓M (BD)	107
FAM63B	15q21.3	Removal of Lys-48-linked conjugated ubiquitin from proteins	WB	↓M (BD)	207

ADHD, Attention deficit-hyperactivity disorder; *AN*, anorexia nervosa; *AUD*, alcohol use disorder; *B*, brain; *BC*, buccal cells; *BD*, bipolar disorder; *BN*, bulimia nervosa; *CB*, cord blood; *DA*, dopamine; *GABA*, gamma-aminobutyric acid; *GF*, growth factors; *Glu*, glutamate; *LCLs*, leukocyte cell lines; *MDD*, major depressive disorder; *S*, saliva; *SZ*, schizophrenia; *TF*, transcription factor; *TL*, transformed lymphoblasts; *WB*, white blood cells.

for neuron development,[208–210] synaptic transmission,[105,209,211] nervous system differentiation,[95,210] embryo development,[210] calcium ion binding,[209] and cell fate commitment[210] (Table 3) are enriched in discovered DMPs and DMRs and are also identified in other studies. The neuron development pathway is frequently identified in brain EWASs, which indicates the importance of further studying the relationship between brain development methylation changes and psychiatric disorders.

Two papers have focused on the relationship between DNA methylation changes in fetal brain development and SZ. Jaffe *et al.*[210] found 2104 DMPs when comparing PFC samples of patients and controls. Furthermore, they found widespread DNA methylation changes when comparing prenatal to postnatal life; these changes were enriched for SZ-GWAS risk loci and related to age of onset.[210] Interestingly, Hannon *et al.*,[60] using fetal PFC, striatum, and CBL, found that, in fetal-specific brain mQTLs, there was an enrichment of SZ-GWAS loci.[60] DMPs at fetal and postnatal stages, especially CpGs with higher methylation in fetal PFC, were more likely to be enriched for GWAS loci than the remaining age-regulated CpGs. Jaffe *et al.*'s and Hannon *et al.*'s papers point to the important influence of prenatal brain development on SZ pathology.

Further EWAS results detected 1812 DMPs for males with AUD and zero DMPs for females with AUD, possibly because of the limited female sample sizes in this study.[208] For ASD, interestingly, decreased methylation probes were enriched in immune function, and increased methylation probes were related to synaptic membrane.[211]

3.2.2.2 DNA Methylation Changes in Peripheral Tissues Highlight the Relevance of Immune Function in Psychiatric Etiology

Cellular heterogeneity in the brain, cause of death, agonal state, postmortem interval, and other factors can contribute to inconsistent findings in postmortem brain studies such as those mentioned earlier. Peripheral tissues are better than postmortem brain tissues for studying the response to the environment because the peripheral tissues can be collected from living patients. Peripheral tissues, especially blood, play important roles in identifying biomarkers.

SZ and BD EWASs utilizing peripheral tissues have detected several DMPs. Of the DMPs, 65.4% were located in CpG islands (CGIs) and the regions flanking CGIs (CGI: 40.6%; CGI shore: 13.3%; CGI shelf: 11.5%). Of the 95 DMPs in the CGIs, most were located in the promoter regions (promoter: 75.8%; gene body: 14.7%; 3′-UTR: 2.1%).[218] Valproic acid and

Table 3 EWAS of Psychiatric Disorders in Brain.

Studies	Sample Sizes	Tissues	Platforms	Main Findings	Pathway Enrichment
SZ and BD					
Mill *et al.* (2008)[117]	35 **SZ**, 35 **BD**, 35 C	FC	Microarray	DMPs (51 genes in men and 37 genes in women)	Brain development, **mitochondrial** functions
Pidsley *et al.* (2014)[209]	43 PFC (20 SZ and 23 C), 44 CBL (21 **SZ** and 23 C)	PFC CBL	HM450	22 DMPs in PFC	Neuron development; synaptic transmission; calcium ion binding
Chen *et al.* (2014)[28]	39 **SZ**, 36 **BD**, 43 C	CBL	HM27	488 DMPs	—
Numata *et al.* (2014)[212]	106 **SZ**, 110 C	DLPFC	HM27	107 DMPs	—
Wockner *et al.* (2014)[213]	24 **SZ**, 24 C	FC	HM450	4641 DMPs	—
Xiao *et al.* (2014)[95]	5 **SZ**, 7 **BD**, 6 C	FC, ACC (BA9, BA24)	MeDIP-Seq	4985 DMRs in BA9; 3867 DMRs in BA24 for SZ, ↑hsa-mir-4266 in SZ	Guidance and signaling, multicellular organismal development and signaling, axon guidance, oligodendrocyte differentiation
Zhao *et al.* (2015)[214]	5 **SZ**, 7 **BD**, 6 C	BA9	MeDIP-Seq		—

(*Continued*)

Table 3 EWAS of Psychiatric Disorders in Brain.—cont'd.

Studies	Sample Sizes	Tissues	Platforms	Main Findings	Pathway Enrichment
				SZ: 10,961 (7,880↑ and 3,081↓) DMRs; BD: 16,599 (6,836↑ and 9,763↓)	
Ruzicka *et al.* (2015)[147]	8 **SZ**, 8 **BD**, 8 C	HIP	HM450	↑ GAD1, SZ and BD (family)	—
Alelú-Paz *et al.* (2016)[105]	6 **SZ** DLPFC, 6 **SZ** HIP, 7 **SZ** ACC, 3 C (DLPFC, HIP, ACC)	Several brain regions (DLPFC, ACC, HIP)	HM450	139 DMPs; DMPs may be enriched in CpG shelves and gene bodies; DLPFC: 66; HIP: 18; ACC: 55	DLPFC: neurotransmission; HIP: antigen processing and presentation; ACC: protein kinase activity and signal transduction
Jaffe *et al.* (2016)[210]	191 **SZ**, 335 C	PFC (DLPFC, BA46, and BA9)	HM450	2104 DMPs; DNAm differences between fetal and postnatal life were enriched for SZ risk loci and were associated with the age of illness onset	Embryo development, cell fate commitment, **nervous system differentiation**
MDD					
Sabunciyan *et al.* (2012)[215]	39 **MDD**, 26 C	FC	CHARM	224 DMPs	—
ASD					
	9 **ASD**, 9 C	BA19	HM27	×	—

Ginsberg *et al.* (2012)[216]					
Ladd-Acosta *et al.* (2014)[217]	19 **ASD**, 21 C	TC, PFC, CBL	HM450	4 significant DMPs (TC, CBL), 3 replicated	—
Nardone *et al.* (2014)[211]	23 **ASD**, 23 C	BA10, BA24	HM450	>5,000 DMPs in BA10, >10,000 DMPs in BA24	In BA10, ↓DMPs: **immune function**, ↑DMPs: **synaptic membrane**
AUD					
Wang *et al.* (2015)[208]	46 **AUD** PFC (16 male pairs, 7 female pairs), 46 C	PFC	HM450	1812 DMPs in male, 0 in female	**Neural development** and transcriptional regulation

ADHD, Attention deficit-hyperactivity disorder; *AN*, anorexia nervosa; *AR*, affected relatives; *AUD*, alcohol use disorder; *BC*, buccal cells; *BD*, bipolar disorder; *BN*, bulimia nervosa; *C*, control; *CBL*, cerebellum; *DMPs*, differential methylation probes; *DMRs*, differential methylation regions; *DNAm*, DNA methylation; *FC*, frontal cortex; *GABA*, gamma-aminobutyric acid; *HIP*, hippocampus; *HM27*, Illumina Infinium HumanMethylation 27K; *HM450*, Illumina Infinium HumanMethylation 450K; *MDD*, major depressive disorder; *MeDIP-Seq*, methylated DNA immunoprecipitation sequencing; *PFC*, prefrontal cortex; *SZ*, schizophrenia; *TC*, temporal cortex.

The bolded words indicate studies related to immunity and neurodevelopment.

quetiapine are significantly associated with altered methylation signatures after adjustment for drug-related changes of cell-type composition.[219] Interestingly, hypermethylated CGI genes are found to be enriched in neuroactive ligand–receptor interaction pathways and the Jak-STAT signaling pathway in SZ; hypomethylated CGI shores are enriched in infection; meanwhile, genes lacking CGIs are enriched in metabolism, cell adhesion, and axon guidance.[220] For MDD, most of the DMPs (85.7%) are located in CGIs, in the gene promoter regions.[221]

In analyzing the pathways in these studies, we found the DMPs identified from peripheral tissue EWAS are mainly related to stress,[222] immune function,[166,219,220,222–226] infection,[220] organismal injury and abnormalities,[227] neurogenesis,[219] neurodevelopment,[166,228] embryonic development,[219,227] neurological disease,[227] postnatal synaptic transmission,[229] modulation of monoamine and cholinergic neurotransmission,[118,223] mitochondrion organization,[98] calcium ion binding,[228] neuroactive ligand–receptor,[220] cholesterol storage and lipid transport,[118] and lipoprotein.[166] Interestingly, many studies on peripheral tissues were able to detect DNA methylation changes in pathways related to immune function in multiple disorders, including SZ, BD, ADHD, and AUD[166,220,222–226] (Table 4). Lipid-related pathways are detected in studies on AN and depression.[118,166]

In summary, EWASs have revealed that increased DMPs are likely more related to brain development, whereas decreased DMPs appear to be more related to immune function, according to both brain[211] and peripheral samples.[166,220]

3.2.3 Monozygotic Twin Studies on Psychiatric Disorders

MZTs that are discordant for psychiatric disorders bring unique value to the study of epigenetics. There is increasing evidence that epigenetic differences between MZTs are related to the etiology of psychiatric disorders. For example, in MZT studies, hypomethylation of *PPIEL*[233] in BP II and hypomethylation of *RELN* promoter in SZ[90] are detected. Furthermore, hypermethylation of *SOX10*,[90] *SLC6A*,[135] *RORA*,[234] and *ZBTB20*[235] are detected in MZT studies as candidate genes for SZ, BD, ASD, and MDD, respectively (as detailed in Section 3.1).

High correlations of global methylation levels based on blood samples have been calculated within discordant MZT pairs in SZ,[89] BD,[89] ASD,[109] and MDD,[93,236] indicating no systemic changes in genome-wide epigenetic programming. In addition, no differences are detected between discordant

Table 4 EWAS of Psychiatric Disorders in Peripheral Tissues.

Studies	Sample Sizes	Tissues	Platforms	Main Findings	Pathway Enrichments
SZ and BD					
Kinoshita *et al.* (2013)[218]	24 medication-free **SZ**, 23 C, 3 pairs MZT males	LCLs	HM450	234 DMPs	—
Kinoshita *et al.* (2014)[230]	63 **SZ**, 42 C	WB	HM450	2552 DMPs	Transcription-related functions
Liu *et al.* (2014)[226]	98 **SZ**, 108 C	LCLs	HM27	20 DMPs (16 validated, 11 of which were related to reality distortion symptoms)	**Inflammatory response**
van den Oord *et al.* (2016)[225]	712 **SZ**, 696 C	WB	MBD-seq	7 DMPs	**Immunity**
Montano *et al.* (2016)[227]	689 **SZ**, 645 C	WB	HM450	625 (68%) showed association in the same direction and 172 (19%) of these had a replication P value of less than 0.05	Neurological disease, organismal injury and abnormalities, and embryonic development.
Nishioka *et al.* (2013)[98]	18 first-episode **SZ**, 15 C; Japanese	WB	HM27	1429 CpG sites (1352 genes) in males and 173 CpG sites (172 genes) in females had significantly different DNA methylation statuses (compared to controls). Only 32 genes	Mitochondrion organization, the nuclear lumen, transcription factor binding, and nucleotide binding

(*Continued*)

Table 4 EWAS of Psychiatric Disorders in Peripheral Tissues.—cont'd.

Studies	Sample Sizes	Tissues	Platforms	Main Findings	Pathway Enrichments
				were found in common (between males and females)	
Sabunciyan *et al.* (2015)[224]	20 mania, 20 C	WB	HM450	↑*CYP11A1*	**Inflammatory markers in serum**
Houtepen *et al.* (2016)[219]	172 BD with 6 frequently used psychotropic drugs	WB	HM450 (50), HM27 (122)	DMPs	**Immunity**, neurogenesis, embryonic and regulatory functions
Walker *et al.* (2016)[228]	Scottish family with multiple cases of BD or MDD	WB	HM450	Altered methylation in carriers of a haplotype linked to BD and MDD	Neurodevelopment, calcium ion binding
Li *et al.* (2015)[220]	6 SZ, 3 BD, 1 C	WB	MeDIP-Seq	Thousands of DMPs located preferentially in promoters 3′-UTRs and 5′-UTRs of genes in SZ; 0 in BD	↑CGI genes: neuroactive ligand–receptor interactions and the Jak-STAT signaling pathway in SZ; ↓CGI shores: **infection**; genes lacking CGIs: metabolism, cell adhesion, and axon guidance
MDD					
Uddin *et al.* (2011)[166]	33 MDD, 67 C	WB	HM27	DMPs; IL-6 and CRP levels were elevated among those	↑: Brain development; ↓: **lipoprotein**

				with lifetime depression, and IL-6 methylation held an inverse correlation with circulating IL-6 and CRP among those with depression exclusively.	
Numata *et al.* (2015)[221]	20 medication-free MDD, 19 C; replication: 12 medication-free MDD, 12 C	LCLs	HM450	363 DMPs↓ in the discovery sets; most of these (85.7%) were located in CGIs in the gene promoter regions. Of the top 100 DMPs, 84 were replicated	—
Weder *et al.* (2014)[231]	94 maltreated children (35% with MDD), 96 C	Saliva	HM450	3 genome-wide significant predictors of depression: *ID3*, *GRIN1*, *TPPP*	*ID3*: stress response, *GRIN1*: neural plasticity, *TPPP*: neural circuitry development
ASD					
Berko *et al.* (2014)[229]	47 ASD, 48 C	Ectodermal cells	HM450	DNA methylation patterns are dysregulated in ectodermal cells in these individuals. *OR2L13*↓	Postnatal synaptic transmission
AN					
Booij *et al.* (2015)[118]	29 AN (10 with AN-restrictive type, 19 with AN-binge/purge type) and 15 C	LCLs	HM450	14 ↑DMPs; age of onset (development of the brain and spinal cord); chronicity of illness (synaptogenesis, neurocognitive deficits,	Histone acetylation, RNA modification, cholesterol storage and lipid transport, and dopamine and Glu signaling

(*Continued*)

Table 4 EWAS of Psychiatric Disorders in Peripheral Tissues.—cont'd.

Studies	Sample Sizes	Tissues	Platforms	Main Findings	Pathway Enrichments
				anxiety, altered social functioning, and bowel, kidney, liver, and immune function)	
ADHD					
Walton *et al.* (2017)[232]	1018 mother-offspring pairs (ADHD)	WB	HM450	13 DMPs, TOP: *SKI*, a gene related to transforming growth factor-beta signaling and neural tube development	Peroxisomal processes
Wilmot *et al.* (2015)[223]	46 ADHD, 46 C; Replication: 10 ADHD, 10 C	Saliva	HM450 and BS	*VIPR2* and *MYT1L*	**Inflammatory** processes and modulation of monoamine and cholinergic neurotransmission
AUD					
Zhang *et al.* (2013)[222]	63 AUD, 65 C (Chinese)	WB	HM27	DMPs	Stress, **immune response**, and signal transduction
Zhao *et al.* (2013)[146]	20 AUD (Chinese)	WB	HM450	865 ↓DMPs, 716 ↑DMPs; *SSTR4*↓, *ALDH1L2*↓, *GAD1*↓, *DBH*↓, and *GABRP*↑	AUD

ADHD, Attention deficit-hyperactivity disorder; *AN*, anorexia nervosa; *AUD*, alcohol use disorder; *BC*, buccal cells; *BD*, bipolar disorder; *BN*, bulimia nervosa; *CGI*, CpG islands; *DMPs*, differential methylation probes; *DMRs*, differential methylation regions; *DNAm*, DNA methylation; *GABA*, gamma-aminobutyric acid; *HM27*, Illumina Infinium HumanMethylation 27K; *HM450*, Illumina Infinium HumanMethylation 450K; *LCLs*, leukocyte cell lines; *MBD-seq*, methyl-CpG-binding domain protein-enriched genome sequencing; *MDD*, major depressive disorder; *MeDIP-Seq*, methylated DNA immunoprecipitation sequencing; *MTZ*, monozygotic twin; *SZ*, schizophrenia; *WB*, white blood cells.

The bolded words pertain to immunity and neurodevelopment.

MZT pairs and their matched concordant MZT pairs at any individual probe in a global methylation MDD study[93] using blood samples.

Numerous DMPs were detected for MZT pairs in BD,[89,237] SZ,[238] ASD,[234] and MDD.[93,236,239,240] The genes located via these DMPs are enriched for nervous system development and function,[89,236,237] fetal development,[234] morphogenesis,[234] apoptosis,[234] synaptic regulation,[234] steroid biosynthesis,[234] psychological disorders,[89,234,236,237] rapid glucocorticoid signaling,[239] interleukin-2-mediated signaling events,[239] cell proliferation on forebrain,[240] fat cell differentiation,[240] dopaminergic synapse,[89,237,239] and Glu signaling.[89,237,240] These pathways are similar to those found in EWAS (Section 3.2.2), which may be because the conventional EWAS having almost 1000 samples weakens some confounding influences (like genetics and placenta environment).

Because MZTs share unusually similar genetics, placentas, and (often) environments, identical twin studies, all other things being equal, should allow for more confidence in the true positivity of results when compared to basic case–control studies. However, the validity of twin study findings is frequently limited by small sample sizes and tissue specificity. No significant global methylation difference in psychiatric disorders is found for MZT pair blood samples, as opposed to in the other studies (described earlier), which identify global methylation differences between psychiatric cases and controls. It is possible that this discrepancy is due to the genetic and intrauterine influences shared by twins but not unrelated individuals; these factors may contribute heavily to global methylation differences between psychiatric disorder patients and controls (Table 5).

In synthesizing the results from global methylation studies, candidate gene studies, and EWAS in psychiatric disorders, we conclude the following:

(1) Global methylation levels mainly show increases in brain samples and decreases in peripheral tissue samples; the increased methylation probes detected in EWAS are enriched for neuron-related pathways, whereas the decreased methylation probes are enriched for immune-related pathways; the DMPs detected in EWAS with brain samples are mainly enriched for neurons, and the peripheral tissues are mainly enriched for immune-related pathways. These findings provide evidence for the role of the immune system in psychiatric disorders.

(2) Methylation levels may affect neurotransmitter systems, which may, in turn, contribute to risk of psychiatric disorders. Specifically, overactivity of dopaminergic and glutamatergic systems, as well as hypofunction of serotonergic and GABAergic systems, may be important to the etiology

Table 5 MZT Studies of Psychiatric Disorders.

Studies	Samples	Tissues	Platforms	Main Findings	Enrichments
Bönsch *et al.* (2012)[90]	20 twin pairs **discordant** for **SZ**, 8 twin pairs **concordant** for **SZ**, 42 C twin pairs (MZT and DZT)	WB	Methylation-sensitive restriction (HpaII) and PCR	Similar hypomethylation levels in the unaffected twins of discordant pairs and a mixed group of psychiatric controls	
Castellani *et al.* (2015)[237]	2 pairs of female MZT **discordant** for **SZ** and their parents	WB	MeDIP-Seq	27 genes	Cell death and survival, cellular movement, immune cell trafficking, nervous system development, and DRD4
Hannon *et al.* (2016)[238]	1714 individuals (353 **SZ**, 322 controls (phase 1); 414 **SZ**, 433 controls (phase 2); 96 MZT pairs (phase 3))	WB	HM450	76 DMPs from twin study	

Dempster *et al.* (2011)[89]	22 **discordant** MZT pairs: 11 **SZ**, 11 **BD**	WB; B for replication of TOP	HM27, EpiTYPER (Sequenom Inc., CA)	DMPs, TOP: *ST6GALNAC1*↓	**Nervous system development** and function, psychological disorders, DA and Glu receptor signaling, nervous system development
Sugawara *et al.* (2011)[135]	2 MZT pairs **discordant** for **BD** (male)	LCLs; B for replication of TOP	BS and PCR	↑*SLC6A*	Serotonin transporter
Kuratomi *et al.* (2008)[233]	1 MZT pair **discordant** for **BD** (male); replication: 16 **BD I**, 7 **BD II**, 18 C	Lymphoblastoid cells	BS and PS	*PPIEL* ↑E and ↓M in BP II	—
Byrne *et al.* (2013)[93]	12 MZT pairs **discordant** for **MDD** and 12 MZT pairs **concordant** for low neuroticism and absence of **MDD**	WB	HM450	DMPs were detected for affected vs. unaffected twins. × Discordant MZT pairs and their matched concordant MZT pairs	—

(*Continued*)

Table 5 MZT Studies of Psychiatric Disorders.—cont'd.

Studies	Samples	Tissues	Platforms	Main Findings	Enrichments
Davies *et al.* (2014)[235]	50 MZT pairs **discordant** for **MDD** (27 UK, 23 Australia); replication: 118 **MDD**, 236 C	WB	MeDIP-Seq	↑*ZBTB20*	—
Dempster *et al.* (2014)[236]	18 MZT pairs **discordant** for **MDD**; replication: 14 **MDD**, 15 C	MZT: BC, replication: CBL	HM450	Multiple DMPs, TOP: *STK32C*	Nervous system development and function, neurological disease and psychological disorders
Cordova-Palomera *et al.* (2015)[239]	17 MZT pairs (4 **concordant** for **MDD**, 6 **discordant**, 7 C)	WB	HM450	DMPs, *WDR26*	Rapid glucocorticoid signaling, DA synapse and *interleukin-2*-mediated signaling events

Oh *et al.* (2015)[240]	171 MZT pairs **MDD**	WB (100), PFC (71)	8.1 K CpG island microarrays and BS	PFC: 22 DMPs, *FOXD3*; WB: 44 DMPs	In cell proliferation on forebrain, fat cell differentiation, and Glu signaling pathway
Nguyen *et al.* (2010)[234]	3 MZT **discordant** for **ASD** (male), 2 unaffected siblings	LCLs	8.1 K CpG island microarray	73 DMPs, *RORA*	Synaptic regulation, fetal development, morphogenesis, apoptosis, inflammation, digestion, steroid biosynthesis, and mental deficiency
Wong *et al.* (2014)[109]	50 MZT pairs **discordant** and **concordant** for **ASD/ASD** severity	WB	HM27	DMPs	—

ASD, Autism spectrum disorder; *B*, brain; *BD*, bipolar disorder; *DA*, dopamine; *DZT*, dizygotic twin; *Glu*, glutamate; *HM27*, Illumina Infinium HumanMethylation 27K; *HM450*, Illumina Infinium HumanMethylation 450K; *LCLs*, leukocyte cell lines; *MDD*, major depressive disorder; *MZT*, monozygotic twin; *SZ*, schizophrenia; *WB*, white blood cells.
The bolded words pertain to immunity and neurodevelopment.

of major psychosis. Furthermore, hypomethylation of dopaminergic and glutamatergic system receptors and hypermethylation of serotonergic and GABAergic system receptors have been detected. These abnormal methylation levels may lead to receptor excitation or inhibition, giving rise to the functional changes of neurotransmission processes. Similar patterns were found for transporters and some enzymes.

(3) We listed several other candidate genes in Section 3.2.1. However, these alone may be less informative in the grand scheme of things because major psychiatric disorders are polygenic. It will be essential to detect additional related biomarkers to construct more complete models of relevant pathways and mechanisms.

(4) The biomarkers discovered in the EWAS using brain samples reveal important information regarding the importance of fetal brain development in SZ. Although a limited number of EWASs have looked at other psychiatric disorders due to differences in heterogeneity, it will be important to study the influence of fetal brain development on other disorders.

(5) Confounding factors such as smoking status, medication use, sex, age of disease onset, and varying research methods used in different studies could have an influence on any of the aforementioned results.

There are some results that cannot be explained by our current knowledge about biology and pathology. Inconsistent results could be the product of discrepancies in data quality, technical and analytical differences, and sample heterogeneity, the last of which is more likely to be a problematic factor in small sample sizes. These unresolved issues can inspire future scientific exploration.

4. FUTURE PERSPECTIVES AND CHALLENGES

In this article, we introduced DNA methylation and highlighted important findings on DNA methylation in major psychiatric disorders. Current studies still face several limitations and challenges. The major studies mentioned earlier focus on DNA methylation differences between cases and controls using bulk tissue samples. Both positive and negative results for differential methylation could be false because of confounding factors, including differences in cell composition in different tissues (e.g., brain vs. blood) used in different studies. Furthermore, more functional genomics data are still needed to help explain these epigenetic changes and their effects.

Innovative experimental and analytical approaches are expected to improve our understanding of DNA methylation and its relevance to psychiatric disorders in the coming years. Comethylation network analysis, integration of multidimensional omics data, and single-cell methylome and methylation editing techniques are a few such emerging approaches. In addition, studies on the association of DNA methylation with intermediate phenotypes other than disease diagnoses will produce more valuable findings. Lastly, DNA methylation-based therapy may serve as a future treatment for psychiatric disorders.

4.1 Network Analysis and Integration of Multidimensional Data

For a long time, DNA methylation studies have focused on individual loci or regions but failed to examine the relationships between these loci, though many methods for creating networks of loci exist. Coexpression network analysis uses gene expression correlations to construct networks; comethylation network analysis identifies CpGs with associated methylation when they are regulated by common mechanisms.[241–243] Using comethylation network analysis, Berko *et al.*[229] obtained two modules significantly associated with autism spectrum disorder case/control status after stringent Bonferroni correction.[229] Wang *et al.*[208] identified 21 AUD-associated comethylation modules in the prefrontal cortex (PFC), and found that the differentially methylated CpGs were overrepresented in two of the modules.[208] However, it should be noted that correlation does not necessarily indicate causality. Experimental validation is needed to clarify whether there is actually a regulatory relationship.

Findings on DNA methylation can be applied to various forms of methodology, as well as integrated into genetic and omics data. For example, haplotype-dependent allele-specific DNA methylation (hapASM) can help refine mQTL results by pinpointing DNA sequence variants that underlie genetic susceptibility to psychiatric disorders.[244] As gene expression regulation is considered to be the primary function of DNA methylation, many candidate gene methylation studies have focused on the expression–methylation relationship.[123,142,143,148] Systematic integration of transcriptome data and EWAS will be more effective and unbiased.[245] Finally, Hi-C,[246,247] ATAC-seq,[248] and DNase-seq,[249,250] which examine chromatin interaction and accessibility, could be used for investigating DNA methylation's role in regulating gene

expression.[251] Building networks involving genetic variants, chromatin structure, and DNA methylation may improve the quality of gene expression regulation predictions.

4.2 Single-Cell Methylome

The current strategies for studying DNA methylation in psychiatric disorders typically use bulk tissues. As mentioned earlier, cell-specific measurement of DNA methylation is more desirable, as it is less likely to yield confounding results.

Single-cell methylome methods, such as single-cell reduced representation bisulfite sequencing (scRRBS),[252] single-cell bisulfite sequencing (scBS-seq),[253] and single-nucleus methylcytosine sequencing (snmC-seq),[254] have been developed. snmC-seq has been applied to more than 6000 single neuronal nuclei from mouse and human FC. The results identified 37 neuronal subpopulations and showed that CG and non-CG methylation exhibited cell-type-specific distributions.[254]

In addition, novel methods have been developed to measure multiomics in a single cell: single-cell NOMe-seq (scNOMe-seq)[255] and single-cell multiomics sequencing (single-cell COOL-seq)[256] allow for the simultaneous measurements of DNA methylation and chromatin accessibility from the same cell. With these single-cell techniques, we will be able to evaluate disease pathology with better resolution and confidence.

4.3 DNA Methylation Signatures of Intermediate Phenotypes

Most previous publications on DNA methylation have targeted disorder diagnoses, but endophenotypes (or intermediate phenotypes) relevant to psychiatric disorders are getting increasingly more attention. Notably, alterations in methylation have been reported to be associated with cognitive functions,[105,257] reality distortion symptoms (possibility via immune response modulation),[226] and brain volume[258–260] in psychotic disorders. Using DNA methylation signatures to study these intermediate phenotypes could help dissect the complexity of the underlying biology of psychiatric disorders.

4.4 Methylation Editing and Functional Validation

Functional validation is an important step toward translating statistical correlation into causal biological function. Gene expression change can be

experimentally validated by gene editing, RNA interference silencing, and knock-in overexpression. However, validation of DNA methylation functionality through methylation editing approaches has only recently become feasible with the development of clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein systems (CRISPR-Cas).

Several different versions of DNA methylation editing methods have been developed. DNA methylation editing can be performed using the dCas9-based system, which lacks endonuclease activity.[261] dCas9 fused to the catalytic domain of the DNA demethylase TET1 was used to induce targeted DNA demethylation.[262–264] Similarly, a fusion of the catalytic domain of DNA methyltransferase 3A (DNMT3A) to dCas9 is capable of inducing de novo methylation of the targeted region.[264–267] The modification of DNA methylation by these systems leads to changed expression of the targeted genes and cell reprogramming.[264] Using the dCas9 methylation editing toolkit, we could study the function of DNA methylation.

4.5 DNA Methylation-Based Treatments

Given that DNA methylation likely contributes to the risk of psychiatric disorders, manipulation of methylation levels is a promising method for developing novel therapeutic approaches.

Specifically, modulation of enzymes such as DNMT and TET, which are related to DNA methylation and demethylation, may produce therapeutic effects. Many chemical compounds such as 5-aza-2′-deoxycytidine (AZA), doxorubicin (DOXO), and zebularine (ZEB) have been shown to decrease the methylation and increase the expression of candidate genes such as *GAD67* and *reelin* in in vitro and in vivo experiments.[268–271] Moreover, some existing FDA-approved drugs, such as Clozapine and Sulpiride, have demonstrated the ability to activate brain DNA demethylation.[271] The CRISPR-dCas9 system holds the hope of gene therapy via DNA methylation, as well. Still, the process of therapy through methylation modification will no doubt require much time and research to achieve substantial progress.

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