DNA Methylation and Hydroxymethylation and Behavior

Sarah R. Rustad, Ligia A. Papale, and Reid S. Alisch

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Abstract Environmentally sensitive molecular mechanisms in the brain, such as DNA methylation, have become a significant focus of neuroscience research because of mounting evidence indicating that they are critical in response to social situations, stress, threats, and behavior. The recent identification of 5-hydroxymethylcytosine (5hmC), which is enriched in the brain (tenfold over peripheral tissues), raises new questions as to the role of this base in mediating epigenetic effects in the brain. The development of genome-wide methods capable of distinguishing 5-methylcytosine (5mC) from 5hmC has revealed that a growing number of behaviors are linked to independent disruptions of 5mC and 5hmC levels, further emphasizing the unique importance of both of these modifications in the brain. Here, we review the recent

S. R. Rustad
Madison, WI, USA

L. A. Papale
Department of Neurological Surgery, University of Wisconsin, Madison, WI, USA

R. S. Alisch (✉)
Department of Neurological Surgery, University of Wisconsin, Madison, WI, USA

Department of Neurological Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA
e-mail: alisch@wisc.edu

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links that indicate DNA methylation (both 5mC and 5hmC) is highly dynamic and that perturbations in this modification may contribute to behaviors related to psychiatric disorders and hold clinical relevance.

Keywords 5-Hydroxymethylcytosine · 5-Methylcytosine · Behavior · DNA methylation · Epigenetics

1 Introduction

The most studied epigenetic modification in the mammalian genome is DNA methylation, which is the addition of a methyl group to the fifth carbon of cytosines (e.g., 5-methylcytosine (5mC)), and is predominantly found at cytosine-phosphate-guanine (CpG) dinucleotide sites (Fig. 1). This DNA modification is catalyzed by DNA methyltransferases (DNMTs), which utilize S-adenosyl-L-methionine as the methyl donor (Cheng 1995). Three active DNMTs have been identified in mammals, each displaying their own distinct functions. DNMT3a and DNMT3b initiate methylation, having an affinity toward unmethylated CpG sites, while DNMT1 preserves methylation, showing preference toward hemimethylated CpG sites (Okano et al. 1999). CpG-rich regions, known as CpG islands, and gene promoters have a significant reduction in 5mC levels, whereas the X chromosome has an overabundance of 5mC (Sharp et al. 2011; Ioshikhes and Zhang 2000). Generally speaking, this DNA modification functions in genomic imprinting, X-chromosome inactivation, chromatin structure, and gene silencing (Bird 2002; Sharma et al. 2010; Han et al. 2015; Suzuki and Bird 2008; Robertson 2005). Human studies support a role for 5mC in the development of behavior-related disorders, including bipolar disorder (BD), schizophrenia (SCZ), and major depressive disorder (MDD), often

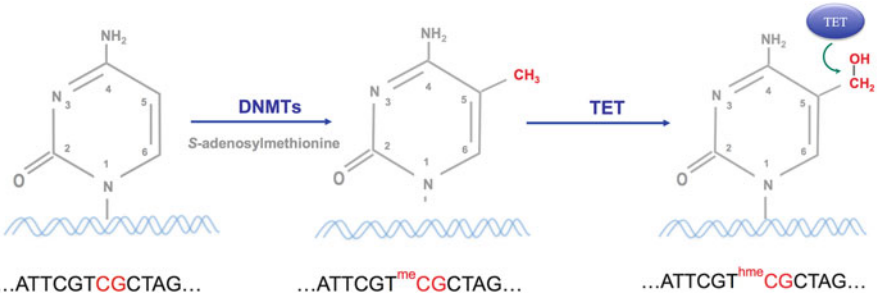

Fig. 1 The process of DNA methylation. Shown is the aromatic ring of cytosine. DNA methyltransferases (DNMTs) and S-adenosylmethionine facilitate the addition of a methyl group (CH_3) to the fifth position of cytosine in a CG (shown in red below; ^{me}CG) dinucleotide context. The ten-eleven translocation (TET) family of enzymes can oxidize the methyl group on cytosine, resulting in hydroxylation (OH; ^{hme}CG). Notably, the TET enzymes can further oxidize the methyl group, resulting in complete demethylation of the cytosine (not shown)

resulting in concomitant changes in gene expression (Abdolmaleky et al. 2006; Poulter et al. 2008; Kuratomi et al. 2008; Kappeler and Meaney 2010; Weaver et al. 2004). It was recently shown that 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC) and that this modification is environmental sensitive (Wu and Zhang 2011), stable (Penn et al. 1972), and highly enriched in the brain (Kriaucionis and Heintz 2009; Sun et al. 2014; Wang et al. 2014; He et al. 2011; Ito et al. 2010; Tahiliani et al. 2009). This oxidation process is catalyzed by the ten-eleven translocation (TET) family of enzymes and can contribute to active demethylation, whereby two mechanisms can convert 5hmC back into cytosine; iterative oxidation by TET enzymes, which continuously oxidize 5hmC; and deamination by activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex AID/APOBEC (Ito et al. 2011; Guo et al. 2014).

While the full functional potential of 5hmC is yet to be determined, data supporting several putative molecular mechanisms have been found, including the regulation of transcription factor (TF) binding (Li et al. 2016), sex-specific development (Gross et al. 2015), and transcript diversity through interactions with the spliceosome (Feng et al. 2015) (Fig. 2). It is noteworthy that traditional DNA methylation detection methods utilizing sodium bisulfite treatment cannot distinguish between the methylated and hydroxymethylated forms of cytosine, meaning that past studies using such methods report a composite of 5mC and 5hmC but have attributed any findings solely to 5mC. This chapter will provide evidence to support the independent importance of 5mC and 5hmC in mental health and the development of mental illness and present several putative molecular functions of DNA methylation that may shed light on its promising clinical relevance. With recent findings implicating dynamic and unique roles for 5mC and 5hmC in behavior, DNA methylation brings a new frontier to the field of psychiatry.

2 Total DNA Methylation (5mC + 5hmC) and Behavior

A growing body of evidence suggests that the methylome varies dramatically throughout life, as the result of extrinsic influences such as environmental and physical factors (Tammen et al. 2013; King-Batoon et al. 2008; Park et al. 2012). One such factor with robust connections to DNA methylation is psychological adversity though most of these links were found using animal models. For example, rodent mothers that performed enhanced licking, grooming, and arched back nursing altered their offspring's DNA methylation levels on the glucocorticoid receptor (*NR3C1*) gene in the hippocampus, which is a gene best known for its role in the stress response. These mother-to-offspring interactions affected the development of their offspring's hypothalamic-pituitary-adrenal (HPA) axis, which is a dynamic metabolic system that regulates homeostatic mechanisms, including future reactions to new environments. Similarly, an abusive caregiver leads to lifelong increases in offspring DNA methylation on the brain-derived neurotrophic factor (*Bdnf*) gene in the prefrontal cortex of both male and female rodents, which can persist to the next

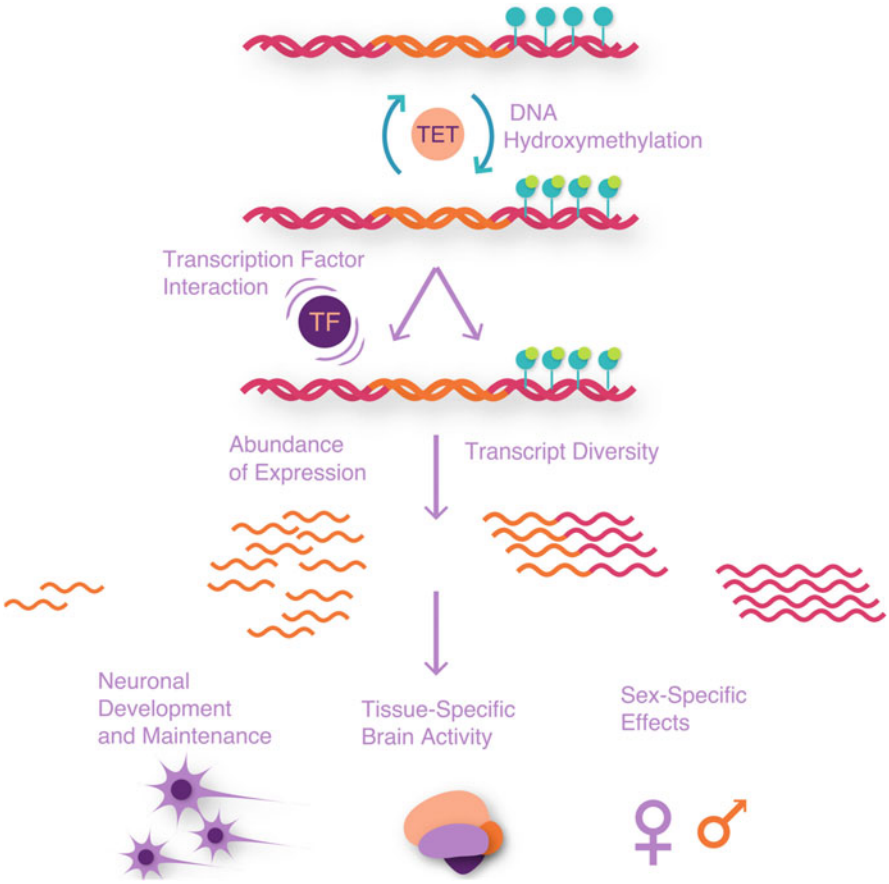

Fig. 2 Putative molecular mechanisms of 5hmC. Following oxidation by the TET enzymes, the 5hmC modification has been shown to interact with transcription factors (TF) and affect transcript abundance. In addition, 5hmC modifications have been implemented in the splicing process, resulting in transcript diversity. Ultimately, these changes in transcript expression and diversity can have lasting effects at the cellular and tissue level, in a sex-specific manner

generation (Roth et al. 2009). More recent work also found increased DNA methylation on *Bdnf* and concomitant decreased expression in the amygdala and hippocampus of rats exposed to prenatal stress (Boersma et al. 2014). Studies in young monkeys identified differentially methylated genes that are implicated as risk factors for developing anxiety and depressive disorders (Alisch et al. 2014). In addition, several other studies have shown evidence of altered DNA methylation and gene expression due to maternal separation or varied maternal care and prenatal stress (Beery et al. 2016; Murgatroyd et al. 2009; Mueller and Bale 2008; Novikova et al. 2008).

Human studies are obviously more difficult to conduct, due to limited access to brain tissue, making them slow to contribute to these findings. Nonetheless, more

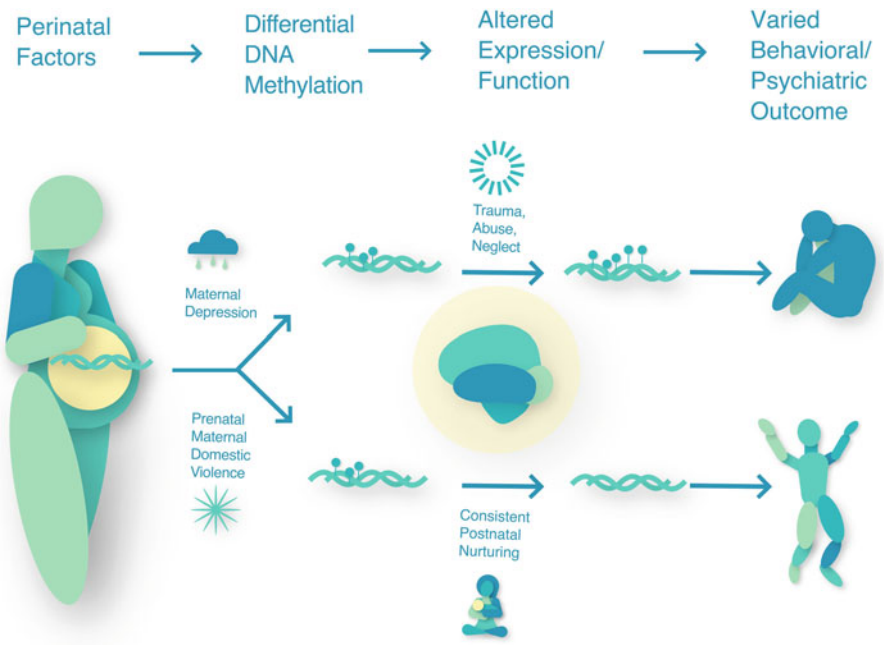

Fig. 3 Perinatal environmental adversities impact DNA methylation levels linked to human behavior. Perinatal exposure to maternal depression and negative experiences can alter DNA methylation in the perinatal brain, resulting in psychiatric disorders later in life. However, postnatal nurturing may reverse these effects and lead to healthier psychiatric outcomes

recent human studies also have shown that stress, anxiety, depression, neglect, socioeconomic status, violence, and maternal care all impact DNA methylation levels (Fig. 3) (Kim et al. 2016; Lam et al. 2012; King et al. 2015; Naumova et al. 2012), for example, suicide completers who were exposed to childhood abuse and neglect carried hypermethylation in the promoter regions of ribosomal RNAs and concomitant downregulated expression in the hippocampus (Mcgowan et al. 2008). However, aberrant DNA methylation patterns due to psychological influences were not restricted to promoter regions, as they also were linked to changes in gene bodies, intergenic regions, and 5′ and 3′ untranslated regions (UTRs) (Weder et al. 2014). Moreover, stress-dependent DNA methylation levels can be linked to genotype-specific gene transcription, resulting in a long-term dysregulation of the stress hormone system (Klengel et al. 2013). Together, these studies suggest that early-life adversities increase the risk of behavioral disturbances into adulthood and that these outcomes are governed by DNA methylation. Evidence of the environmental impact on DNA methylation patterns is often examined in the HPA axis. We start with *NR3C1*, the human glucocorticoid receptor gene, which is highly abundant in the HPA system.

2.1 The Perinatal Period

To examine the effects of offspring prenatal exposure to maternal psychological well-being on DNA methylation status at the promoter and exon 1F of the human *NR3C1* gene, cord blood from 82 newborn girls was collected, and maternal mood was measured using the Hamilton Rating Scale for Depression (HAM-D), Hamilton Rating Scale for Anxiety (HAM-A), and Edinburgh Postnatal Depression Scale (EPDS) (Oberlander et al. 2008). Increased scores in the third trimester for all three tests positively correlated with the DNA methylation levels on *NR3C1*, including sites containing the NGIF-A (transcription factor) binding site in exon 1F of *NR3C1*. Interestingly, saliva was collected from the offspring at 3 months of age, before and after exposure to negative visual stimuli, to measure cortisol stress response. Infants who responded with high cortisol levels had considerably higher *NR3C1* DNA methylation levels when compared with infants whose cortisol levels decreased in response to the stress assessment. These data suggest that maternal depression in the third trimester directly impacts DNA methylation on the *NR3C1* gene and alters the infant's stress response. Another study similarly found increased whole blood DNA methylation levels on exon 1F of *NR3C1* only in the offspring (Radtke et al. 2011). Notably, the tested offspring were between 10 and 19 years of age, indicating that exposure to maternal anxiety and stress in the intrauterine environment induced stable disruptions in DNA methylation. Similar to the licking and grooming studies conducted in rodents, maternal stroking in human infants leads to fluctuations of methylation of the *NR3C1* gene (Murgatroyd et al. 2015). In this study, DNA methylation levels were examined in saliva samples from 181 infants and showed that high maternal stroking of the infant face, back, abdomen, legs, and arms reduced *NR3C1* DNA methylation levels. This study highlighted the extreme vulnerability and sensitivity of the early postnatal period to extrinsic factors that may directly impact fetal development. While several groups have found links between maternal emotion, stress, childhood adversity, and the methylation status of exon 1F in the promoter of *NR3C1* (Mulligan et al. 2012; Hompes et al. 2013; Radtke et al. 2015; Braithwaite et al. 2015), sex-specific differences in *NR3C1* DNA methylation levels also have been shown, as male infants specifically exhibited elevated exon 1F methylation due to maternal depression during pregnancy (Braithwaite et al. 2015). This finding may shed mechanistic light on sex-specific behaviors, including psychological disorders.

Studies of extrinsic factors impacting development during the perinatal period also have focused on the neurotransmitter serotonin (*5-HT* or *SLC6A4*), which is linked with impulsive aggression as well as increased susceptibility for a lifetime risk of depression (Devlin et al. 2010; Feinn et al. 2005; Seo et al. 2008). A study of prenatal maternal depression in 82 pregnant women found that second trimester DNA methylation levels were lower on the *SLC6A4* promoter in mothers with higher depressed mood symptoms (Devlin et al. 2010). These decreased DNA methylation levels likely result in increased expression of *SLC6A4*, higher serotonin uptake, and decreased intrasynaptic serotonin, though such studies have not yet been conducted.

However, as serotonin modulates neuronal differentiation and growth, altered levels of the serotonin transporter during critical developmental periods likely affect brain development and may have long-term effects on subsequent child emotional development and susceptibility to affective disorders later in life (Ansorge et al. 2004, 2008; Gaspar et al. 2003).

Another mediator of neural function and plasticity is the brain-derived neurotrophic factor (*BDNF*), which is essential for neurogenesis and has been associated with a variety of affective disorders later in life (Mcewen 2007; Roth and Sweatt 2011a; Berton et al. 2006; Fuchikami et al. 2011; Keller et al. 2010; Post 2007). The genetic structure of the *BDNF* gene is complex, suggesting an intricate regulatory system. The gene consists of nine 5′ noncoding exons that are each linked to a distinct promoter that controls differential expression of exon-specific transcripts (Roth et al. 2009; Aid et al. 2007; Liu et al. 2006). Examination of *BDNF* DNA methylation levels related to prenatal maternal depression in 57 infants found reduced DNA methylation on the *BDNF* exon IV promoter. Interestingly, the reduced DNA methylation levels were located near the binding site of CREB, a transcription factor that regulates *BDNF* transcription via a DNA methylation-dependent mechanism, suggesting that prenatal maternal depression may affect this mechanism.

Oxytocin is a neuropeptide hormone that regulates reproductive physiology and contributes to maternal behaviors, pair bonding, and social interaction (Pedersen et al. 2006; Young and Wang 2004; Carter 2003; Winslow and Insel 2002; Carter et al. 1992; Pedersen and Boccia 2002; Popik and Van Ree 1991). An investigation into the potential interplay between oxytocin, DNA methylation, and the development of psychopathy examined the oxytocin receptor (*OXTR*) gene in blood from male children aged 4–16 years that had severe ratings of child conduct problems, as measured using the clinician rating scale called the Quality of Family Environment (Dadds et al. 2014; Rey et al. 1997). Interestingly, younger children (4–8 years) did not exhibit conduct-related DNA methylation levels on the *OXTR* gene; however, older children (9–16 years) had a positive correlation between conduct problems and DNA methylation levels on *OXTR*. This increased methylation in older children also had a direct impact on the level of circulating oxytocin, suggesting impairment of the entire oxytocin system, highlighted by the methylation-dependent downregulation of *OXTR* expression (Dadds et al. 2012, 2014). Indeed, childhood stress is linked to differential methylation of oxytocin (*OXT*) in the saliva of older girls (10–12 years) (Papale et al. 2018). Interestingly, a related study found that increased DNA methylation on the *OXTR* gene, and subsequent reduced expression, in the temporal cortex served as a valid indicator of autism spectrum disorder (ASD) (Gregory et al. 2009), differentiating between ASD and control subjects. Clearly the abundance of DNA methylation can be used to assess social behaviors in children.

While these aforementioned studies and many others were narrowly focused on a single promoter and gene, the use of genome-wide approaches has greatly improved our understanding of the impact of extrinsic factors on child susceptibility to affective disorders. One study collected saliva from 94 maltreated children and 96 controls (all 5–14 years of age) and found trauma-associated DNA methylation

levels across the genome, including some on well-known stress-related genes, such as *NR3C1*, *BDNF*, and FK506 binding protein 51 (*FKBP5*), as well as on biologically relevant genes, including *ID3* (DNA-binding protein inhibitor ID-3), *TPPP* (tubulin polymerization promoting protein), and *GRIN1* (glutamate (NMDA) receptor NR1 subunit) (Weder et al. 2014). Moreover, since *ID3* expression is increased following stress (Konishi et al. 2010), the variation in salivary cortisol can predict *ID3* methylation-related diurnal cortisol secretion. Another study that examined saliva from 11 girls that experienced extremely high levels and 11 girls with normative levels of early childhood stress exposure (all 9–12 years of age) also found trauma-related DNA methylation levels across the genome at more than 100 genes, including on stress-related genes like *OXT* and a serotonin receptor: *HTR3A* (Papale et al. 2018). Together, these data suggest that DNA methylation is stable long after trauma exposure and may hold predictive information of mental well-being and prognosis.

2.2 The Adult Period

Variations in DNA methylation levels due to early-life adversity can persist into adulthood. Studies have linked altered DNA methylation levels and gene expression of hippocampal *NR3C1* and psychological illnesses, including mood disorders and SCZ, which are associated with suicide in adults; indeed, often suicide relates to a history of childhood abuse (Heim and Nemeroff 2001; Webster et al. 2002). In one such study, postmortem hippocampal samples were obtained from suicide completers that had traumatic childhoods, suicide completers that did not have a history of abuse, and controls that had sudden accidental deaths and did not experience abuse (Mcgowan et al. 2009). The suicide completers who had traumatic childhoods showed higher DNA methylation in exon 1F of *NR3C1*, compared to both controls (i.e., suicide completers without a history of abuse and victims of sudden accidental death). As predicted, the increased DNA methylation levels interrupted NGIF-A binding to *NR3C1* and gene transcription.

Suicide victims also show decreased *BDNF* expression levels in the hippocampus and prefrontal cortex, suggesting that *BDNF* plays a critical role in pathophysiological aspects of suicidal tendencies. Examination of *BDNF* DNA methylation levels in postmortem brain samples (Wernicke's area of the brain) from 44 suicide completers and 33 controls revealed hypermethylation in suicide completers, which was shown in vitro to result in lower *BDNF* mRNA levels (Keller et al. 2010). This study showed that a gene-specific increase of DNA methylation downregulates its expression, which can account for the pathophysiology observed the suicidal brain.

Another study examined the blood of 101 women aged 30 and 41 years that had borderline personality disorder (BPD) and had experienced extreme childhood sexual, physical, and emotional traumas; 99 of the women had MDD and 15 had MDD and post-traumatic stress disorder (PTSD) (Perroud et al. 2011). A positive correlation was found between sexual abuse severity and *NR3C1* DNA methylation

levels. Similarly, physically and/or emotionally abused and neglected participants all had higher *NR3C1* DNA methylation than controls. In a related study, blood samples from 115 patients with BPD and 52 controls were collected for DNA methylation analysis, and the patients were found to exhibit higher *BDNF* DNA methylation compared to controls, which was positively correlated with childhood trauma such as sexual, physical, and emotional abuse and physical and emotional neglect (Perroud et al. 2013). Similarly, a positive association was observed between *BDNF* DNA methylation levels and hopelessness, depression severity, and impulsivity. Finding higher *BDNF* DNA methylation in BPD patients is consistent with the higher *BDNF* DNA methylation of suicide completers, and suicidal tendencies are common in BPD patients. Interestingly, these patients were subjected to intensive dialectical behavior therapy, which reduced *BDNF* DNA methylation and BPD symptoms in responding patients. While a similar increase in *BDNF* DNA methylation was observed in BD patients compared to controls, it was hypothesized that therapies such as antidepressants contributed to these elevated levels of *BDNF* DNA methylation. Together, these data suggested that *BDNF* DNA methylation levels might be a biomarker of BD therapy efficacy.

Childhood abuse also can stably alter DNA methylation levels that may contribute to the development of adult eating disorders, which are often comorbid with personality disorders. Examination of blood from 32 women with bulimia nervosa that had a history of childhood abuse, 32 women with bulimia nervosa that did not have a history of childhood abuse, and 32 controls revealed higher DNA methylation in exon 1C of *NR3C1* in women with bulimia nervosa and history of abuse compared to both controls (Steiger et al. 2013). While these findings do not definitively link DNA methylation to bulimia nervosa, they do suggest that the dysregulation of HPA activity, which contributes to the predisposition to psychological disorders observed in adults, originates early in life and is marked by stable changes in DNA methylation that are correlated to abuse severity. Other reports have corroborated these findings (Lawson et al. 2012; Maguire et al. 2013; Frieling et al. 2008; Toyokawa et al. 2012). In one such study, buccal cells from 15 anorexia nervosa patients and 36 controls were examined for DNA methylation levels on the *OXTR* gene (Kim et al. 2014). All participants were assessed using the Eating Disorder Examination Questionnaire (EDE-Q) (Fairburn and Beglin 1994), the Beck Depression Inventory (BDI), the Spielberger State and Trait Anxiety Inventory (STAI) (Speilberger et al. 1983), and the Autism Spectrum Quotient (ASQ) (Baron-Cohen et al. 2001), since eating disorders and autism have common traits including focus on detail, rigidity, and social cognition. *OXTR* DNA methylation levels were positively correlated with dietary restraint (EDE-Q), communication (ASQ), and depression and anxiety (BDI and STAI) and inversely correlated with body mass index (BMI). Notably, a linear regression of these data revealed that eating disorder psychopathology, BMI, and anxiety were the main determinants of the differential *OXTR* DNA methylation levels found in patients compared to controls. While it is not known whether DNA methylation levels in buccal cells truly reflect the levels in the brain, this study provides insight into a role for DNA methylation in eating disorder psychopathology.

One potential way to further link peripheral DNA methylation to brain function is by pairing DNA methylation data with functional magnetic resonance imaging (fMRI) data. One such study took this approach and showed that adverse childhood experiences can have long-lasting effects on DNA methylation-associated brain activity. This relationship was demonstrated in 25 mothers with interpersonal violence (IPV)-related PTSD, 9 with PTSD symptoms (non-IPV related), and 20 controls, revealing a positive correlation between *BDNF* DNA methylation and maternal brain activity in the ventromedial prefrontal cortex and anterior cingulate, which are regions associated with emotion regulation (Moser et al. 2015; Hu and Jiang 2014). Interestingly, there was a negative correlation in the right hippocampus and parahippocampus, left and right precuneus, left cerebellum, and right superior temporal gyrus. Together, these findings suggest that long-lasting changes in peripheral *BDNF* DNA methylation levels may reflect disrupted functions in many regions of the brain.

Using a similar approach, blood was collected from 42 participants that were subjected to social perception tasks during fMRI. These tasks recruit a network of brain structures that are vital to social perception and mentalizing abilities, such as the temporal parietal junction. A positive correlation was observed between *OXTR* DNA methylation levels and brain activity in the temporal parietal junction and dorsal anterior cingulate cortex (Jack et al. 2012). Moreover, the degree to which *OXTR* DNA methylation fluctuated was positively associated with BOLD activity (blood oxygenation level-dependent signal, which reflects neural activity) from the superior temporal gyrus into the supramarginal gyrus at the temporal parietal junction and the dorsal anterior cingulate cortex. The dorsal anterior cingulate cortex plays a central role in social and affective appraisals of motivationally salient stimuli. Functional impairment of this region is linked to emotional and social deficits in anxiety disorders and ASD (Etkin et al. 2011; Mcclure et al. 2007). Others also have shown that increased *OXTR* DNA methylation levels were concomitant with increased activity in the amygdala and high neural response, when observing negative facial expressions. Together, these studies highlighted that emotional and social perceptual processes involving the oxytocin system may be governed by DNA methylation.

Of course variations in DNA methylation also have been associated with psychological conditions in males, including anxiety, depression, hostility, happiness, and general life satisfaction. Examination of DNA methylation in blood of more than 500 men older than 70 years of age revealed psychological-related relationships to DNA methylation levels in several promoter regions of genes involved in immune/inflammatory processes related to atherosclerosis. For example, increased DNA methylation at the *ICAM-1* promoter was associated with increased anxiety, depression, and hostility, whereas happiness and life satisfaction showed an inverse correlation with DNA methylation. Notably, while higher promoter DNA methylation is expected to decrease *ICAM-1* expression, these psychological outcomes were not associated with lower serum *ICAM-1* levels. At the *TLR-2* promoter, DNA methylation levels were positively associated with hostility, while higher life satisfaction led to lower DNA methylation levels. At the coagulation factor III (*F3*)

promoter, DNA methylation levels were positively correlated with depression, whereas life satisfaction and happiness were inversely associated with *F3* DNA methylation. Finally, at the iNOS promoter, DNA methylation levels were inversely correlated with depression and anxiety, but happiness and life satisfaction positively correlated with DNA methylation. Notably, significant correlations were not found at the *NR3C1* promoter, which is in contrast with several other studies that have found associations with negative psychological factors and DNA methylation on the *NR3C1* promoter (Braithwaite et al. 2015; Perroud et al. 2011). This was one of the very few studies that associated an optimistic psychological factor (happiness) with DNA methylation levels, demonstrating a role for DNA methylation across the psychological spectrum.

To examine DNA methylation levels on the serotonin transporter gene (*SLC6A4*) in adults related to childhood aggressive behaviors, blood was collected from 25 healthy adult males (mean age of 27 years). Here, a positive correlation was found between childhood aggression and DNA methylation in monocytes and T cells (Wang et al. 2012a). This study also used positron emission tomography (PET) measures of brain serotonin synthesis and found inverse associations between mean *SLC6A4* DNA methylation and *SLC6A4* expression in the lateral left and right orbitofrontal cortex. Notably, a specific *SLC6A4* genotype, which is known to affect gene expression, was not correlated to DNA methylation levels. On the other hand, in vitro analysis confirmed that the aggressiveness-related DNA methylation levels decreased *SLC6A4* transcriptional activity, suggesting that peripheral DNA methylation might be used to screen for serotonin-related psychological and psychiatric disorders and allow for preventive and corrective interventions. A subsequent study found that more severe physical childhood abuse along with reduced hippocampal volume was associated with higher DNA methylation on the *SLC6A4* promoter (Booij et al. 2015). This finding is consistent with the fact that the hippocampus is richly innervated with serotonin and is central in regulation of stress (Frodl and O'Keane 2013). DNA methylation on the *SLC6A4* promoter also has been studied in adult monozygotic twins that are discordant for MDD or PTSD (Zhao et al. 2013). Here genomic DNA was extracted from blood leukocytes of 84 monozygotic twin pairs (168 total participants), revealing that increased *SLC6A4* promoter DNA methylation was associated with depressive symptoms. Finding associations between childhood abuse, peripheral *SLC6A4* DNA methylation, brain *SLC6A4* expression, and hippocampal volumes further supports that peripheral DNA methylation levels may serve as a valuable biomarker for serotonin-associated stress-related psychopathology.

Social anxiety disorder (SAD) is characterized by fear, anxiety, and evasion of social situations for fear of being negatively evaluated or scrutinized and rejected (Labuschagne et al. 2010; Stein and Stein 2008). To characterize the role of DNA methylation in SAD patients, blood was collected from 110 patients and 110 age- and sex-matched controls. The severity of social anxiety in both patients and controls were assessed using the Social Interaction Anxiety Scale and Social Phobia Scale (SIAS and SPS) (Stangier et al. 1999). Analysis of the *OXTR* promoter revealed significantly lower DNA methylation compared to controls and a negative

correlation between *OXTR* DNA methylation levels and SIAS and SPS scores (Ziegler et al. 2015). This finding was corroborated in independent saliva samples from 16 healthy females subjected to the Trier Social Stress Test that showed a negative correlation between *OXTR* DNA methylation and maximum salivary cortisol. An additional test on SAD patients using fMRI revealed increased amygdala activation and decreased *OXTR* DNA methylation during social phobia-related word processing. Together, this detailed study found that decreased *OXTR* DNA methylation was associated with SAD, stress-related cortisol levels, and heightened amygdala response, revealing the extensive role of DNA methylation in this chronic mental health condition.

Obsessive-compulsive disorder (OCD) is a severe disorder characterized by troubling thoughts and obsessions, such as the need for symmetry, exactness, and order and contamination fears and worries about harm to self and others (Barrett and Healy 2003; Lack 2012; Swedo et al. 1989). To investigate a role for DNA methylation in OCD, genomic DNA was obtained from the blood of 42 OCD patients that had Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) scores of $\geq$16 for the combination of compulsions and obsessions or $\geq$10 for compulsions and obsessions alone and 31 controls (all 18–65 years old) (Cappi et al. 2016; Goodman et al. 1989). *OXTR* DNA methylation levels were higher in OCD patients compared to controls and negatively correlated with depression severity (Cappi et al. 2016); this relation to depression contrasts the findings of other studies (Kim et al. 2014; Ziegler et al. 2015). It is unknown whether the OCD-related hypermethylation of *OXTR* influences *OXTR* expression. A similar study of psychosis recruited 167 men and women with a psychotic disorder, including SCZ, BD, and schizoaffective disorder, and 75 controls (Rubin et al. 2016). Here, females displayed higher *OXTR* DNA methylation that was linked to poorer recognition of emotional expressions. Moreover, while a positive association between *OXTR* DNA methylation levels and oxytocin levels were found in females, a negative association was found in males. Together, these sex-specific differences in *OXTR* DNA methylation and oxytocin levels may hold key information into more personalized therapeutic treatment of SCZ and BD.

Studies of DNA methylation levels in novel genes related to psychological conditions are being reported on a continual basis. For example, DNA methylation levels in the promoters of *ZNF266*, *AGTR1*, *ASPH*, *PLAC1L*, and numerous other genes have been linked to chronic physical aggression in both males and females (Guillemin et al. 2014; Provencal et al. 2013). SCZ patients exhibit differential DNA methylation in the first intron of *RELN* (Aberg et al. 2014) and in several glutamate receptor genes (*GRIA2*, *GRIA3*, *GMR2*, *GMR5*, *GMR8*) (Aberg et al. 2014; Kordi-Tamandani et al. 2013) when compared to controls. In addition, BD patients have higher DNA methylation in *PRIMA1* compared to controls. It is of great interest to confirm the role of the aberrant DNA methylation levels of these genes and determine if they can be used for novel diagnostic, prognostic, and modifiable therapeutic targets.

2.3 In the Pathogenesis of Neurodegeneration

Dynamic changes in DNA methylation levels have been linked with aging and memory loss, leading to neurodegeneration (Johnson et al. 2012; Day and Sweatt 2010; Day et al. 2013; Sanchez-Mut et al. 2016; Alisch et al. 2012). Indeed, several groups have shown locus-specific and/or global disruptions of DNA methylation abundance in postmortem brain tissue of Alzheimer's disease (AD) patients, though findings differ between studies, suggesting a complex involvement of DNA methylation in AD pathogenesis (Bakulski et al. 2012; Lunnon et al. 2014; De Jager et al. 2014; Watson et al. 2016; Coppieters et al. 2014). One such study isolated genomic DNA from four postmortem brain regions of 122 AD patients and premortem whole blood when available ($n = 57$) and measured genome-wide DNA methylation levels associated with Braak staging, a standardized measure of neurofibrillary tangle burden determined at autopsy (Braak and Braak 1991). This approach revealed that the DNA methylation levels in the ankyrin 1 (*ANK1*) gene were associated with neuropathology in the entorhinal cortex, superior temporal gyrus, and prefrontal cortex, but not in the cerebellum or whole blood of the same AD patients. These data suggest that AD-associated variation in DNA methylation is consistent across pathologically relevant regions of the brain. In addition, recent epigenome-wide association studies (EWAS) detected both cross-tissue and tissue-specific DNA methylation profiles in brain tissue, identifying differential methylation in candidate genes of AD and in genes previously unassociated with AD (Bakulski et al. 2012; Lunnon et al. 2014; De Jager et al. 2014; Watson et al. 2016). Together, these studies underscore the utility of EWAS in identifying novel genes and pathways associated with AD pathogenesis that may otherwise be overlooked. Notably, however, these previous studies have relied heavily on the accessibility of brain tissue. Shifting studies of DNA methylation to peripheral whole blood provides a potential tool that may be utilized clinically to improve diagnosis and guide personalized treatment of AD. Support for this approach came from a recent study that extracted genomic DNA from the whole blood of 45 late-onset AD (LOAD) patients and 39 matched controls and found differentially methylated positions (DMPs) that distinguish people with and without LOAD (Madrid et al. 2018). Interestingly, subsequent independent comparison using six continuous clinical LOAD phenotypes as variables, comprising RAVLT scores, and CSF t-tau and p-tau$_{181}$ levels, or t-tau/Aβ_{42}, p-tau$_{181}$/Aβ_{42}, or Aβ_{42}/Aβ_{40} ratios, yielded a unique set of 17 DMPs that were all hypomethylated in two genes, *B3GALT4* (beta-1,3-galatcosyltransferase 4) and *ZADH2* (prostaglandin reductase 3). Taken together, these data reinforce the use of blood as an accessible tissue of value in the identification of DMPs associated with dementia onset and progression.

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease in the elderly population. While the motor-related symptoms that characterize PD are bradykinesia, tremor, rigidity, and postural instability, the behavioral deficits include depression, anxiety, sleep disorders, and cognitive dysfunction. Collectively, these outcomes lead to severe impairment of the quality of life for

PD patients (Frucht 2004). The first genetic cause of PD was identified as a missense mutation in the alpha-synuclein (*SNCA*) gene (SNCAp.Ala53Thr), a locus that also was found to undergo duplications and triplications linked to PD (Polymeropoulos et al. 1997). *SNCA* gene dosage is critical for the development of PD, leading researcher to hypothesize that deregulation of *SNCA* may be a potential mechanism for PD. This hypothesis was confirmed when DNA methylation levels of *SNCA* were found to be reduced in the substantia nigra, putamen, and cortex of PD patients ($N = 12$) compared to controls ($N = 14$) and was linked to the increased expression of *SNCA* (Jowaed et al. 2010). In 2011, a comprehensive genomic study identified several PD risk loci in cerebellum and frontal cortex of PD brains, including *PARK16*, *GPNMB*, and *STX1B* genes, all of which were associated with differential DNA methylation at proximal CpG sites (International Parkinson's Disease Genomics C and Wellcome Trust Case Control C 2011). Examination of DNA methylation levels across the genome in the frontal cortex and blood leukocytes from the same PD patients ($N = 5$) and controls ($N = 6$) found a high concordance of genes differentially methylated in both tissues (Masliah et al. 2013). Taken together, these data reinforce the use of blood as an accessible tissue of value in the identification of differentially methylated sites associated with PD onset and progression and lend further support to the pathogenesis of PD, by providing novel diagnostic, prognostic, and modifiable therapeutic targets.

Huntington's disease (HD) is characterized by deficits in cognitive, psychiatric, and motor stability that is typically caused by a trinucleotide repeat (CAG) mutation in the *HTT* gene (Walker 2007). Expression of the disease protein, huntingtin, leads to extensive transcriptional dysregulation, and a growing body of evidence suggests that epigenetic modifications play a key role in HD pathogenesis (Lee et al. 2013; Reik et al. 1993). Although links between DNA methylation and HD are only beginning to emerge, several recent studies have reported HD-related changes in DNA methylation in rodents and humans. One such study targeted the *ADORA2A* gene, a G-protein-coupled receptor that decreases its expression in HD patients, and found increased levels of DNA methylation in the putamen brain tissue of HD patients ($N = 6$) compared to controls ($N = 5$) (Villar-Menendez et al. 2013). Interestingly, contradictory results were found when genome-wide approaches were employed to identify HD-related changes in DNA methylation throughout the genome. While one study found thousands of differentially methylated sites in a fibroblast cell line from an HD patient compared to control, another study failed to find differentially methylated sites in genomic DNA from the forebrain cortex brain tissue from seven HD patients and six controls (Jia et al. 2015; De Souza et al. 2016). Of course this latter study was more complex, profiling multiple cell types from both sexes, and the data was stringently normalized to account for this complexity, which may contribute for the lack of findings. Nonetheless, studies profiling rodent brain tissues have clearly shown that aberrant DNA methylation levels are associated with HD pathogenesis (Ng et al. 2013), indicating that larger human sample sizes are needed to definitely determine the role of DNA methylation in HD.

Finally, it was recently shown that patterns of DNA methylation correlate with chronologic age with high precision, thereby providing a "DNA methylation age"

(Horvath 2013); importantly, the gap between chronologic age and DNA methylation age widens with environmental exposures and clinical disease (Almen et al. 2014; Kananen et al. 2015; Beach et al. 2015). An accelerated DNA methylation age – that is, when estimated age is higher than expected (on the basis of chronological age) – predicts several age-related cognitive phenotypes. Notably, accelerated DNA methylation age has been associated with Alzheimer's, Parkinson's, and Huntington's disease (Horvath et al. 2016; Levine et al. 2015; Horvath and Ritz 2015; Chen et al. 2016). While it is currently unclear what these biomarkers can teach us about the biology of these disorders, their longitudinal investigation could help determine the impact of endogenous or exogenous stress factors on disease onset and progression. Perhaps the most exciting feature of identifying DNA methylation biomarkers is that epigenetic changes are reversible, raising the prospect that DNA methylation age estimates might be useful for identifying or validating interventions.

3 5hmC and Behavior

The elucidation of the link between 5hmC and behavior is still in its infancy, especially in humans. Aside from its relatively recent discovery in mammalian cells in 2009 (Kriaucionis and Heintz 2009; Tahiliani et al. 2009), the primary reason for this delay in humans, as compared to 5mC, is that 5hmC has a very low abundance in peripheral tissue, making access to brain tissue paramount for these studies. Nonetheless, in less than a decade, numerous studies in mice have led to the emergence of a putative role for 5hmC in behaviors related to psychiatric disorders. Accordingly, this section will feature more findings from rodent studies than humans, simply due to the limited number of human studies performed to date.

Studies have demonstrated an age-dependent accumulation of 5hmC in the brain throughout early-life development and into adulthood (Szulwach et al. 2011; Chen et al. 2012; Zampieri et al. 2015). Environmental stimuli can alter this age-associated accumulation. For example, calorically restricted mice exhibited an age-dependent reduction of 5hmC levels in hippocampal and cerebellar tissue (Chouliaras et al. 2012). In addition, mice exposed to an enriched environment had reduced 5hmC abundance in the hippocampus, primarily on genes involved in axon guidance (Irier et al. 2014). These alterations also were associated with increased learning and memory, suggesting that environmental enrichment might modulate the dynamics of 5hmC in the hippocampus and contribute to improved learning and memory. In contrast, mice subjected to a 30-min acute stress showed increased 5hmC levels on the glucocorticoid receptor gene (*Nr3c1*) in the hippocampus (Li et al. 2015). Genome-wide 5hmC analysis of these same mice revealed that short-term stress induced genome-wide disruptions of 5hmC and confirmed an overall increase in 5hmC following stress. Interestingly, altered 5hmC was found near several transcription factor (TF) binding sites of genes that were differentially expressed and have known roles in neurogenesis and neurological activities (Li et al. 2016),

suggesting that in response to stress, the function of 5hmC may be to influence TF binding to provide appropriate levels of gene expression needed to cope with the stress. The fact that 5hmC changes were found within 1 h of a short stress highlights the potential for rapid changes of 5hmC within the brain. It will be interesting to examine the long-term effects of short stress (i.e., more than 1 h after exposure) or how chronic stress alters 5hmC levels. Many believe 5hmC to be important in long-term consequences of mental health, yet these studies indicate that alterations in 5hmC can occur rapidly and may impact the expression of key genes related to the origins of mental illness.

Environmental stimuli can affect brain regions other than the hippocampus. For example, mice exposed to repeated administrations of cocaine have increased 5hmC in the nucleus accumbens, primarily in coding regions and enhancer sequences of genes involved in drug addiction (Feng et al. 2015). Notably, 5hmC changes persisted for a minimum of 1 month after cocaine exposure in only a small subset of loci, suggesting that these epigenetic changes are largely reversible. The modulation of 5hmC also may mediate behavioral adaptations. For example, fear extinction, a form of reverse learning, results in dramatic 5hmC changes in the prefrontal cortex of mice (Li et al. 2014). These studies also support unique molecular roles for the *Tet* enzymes, as *Tet3*, but not *Tet1*, mediated the increased gene expression that was associated with rapid behavioral adaptation. Interestingly, another group found that mice lacking the expression of *Tet1* exhibited impaired memory extinction, coupled with long-term synaptic depression and downregulation of neuronal activity-related genes (Rudenko et al. 2013). Thus, while *Tet3* may solely facilitate the accumulation of 5hmC in the prefrontal cortex of mice during rapid behavior adaptation in response to fear, *Tet1* governs alterations in 5hmC on synaptic plasticity genes during behavioral adaptation in response to stressful environmental exposures.

Taken together, these studies open up the possibility that 5hmC may function in the development of environmentally sensitive neuronal dysfunction. It will be of great interest to investigate the role of each *Tet* enzyme coupled with the rapid and stable dynamics of 5hmC at different developmental time points to understand its role in synaptic plasticity, neuronal development, the maintenance of mental health, and the onset of mental illness.

3.1 In the Origins of Psychiatric Disorders

Several studies indicate that early-life experiences have a profound impact on brain development and subsequent adult behavior (Oberlander et al. 2008; Mcgowan et al. 2009; Roth and Sweatt 2011b; Labonte et al. 2012). One such example involves rhesus macaques that were deprived of early-life maternal interactions. As adults, these monkeys have altered 5hmC in the prefrontal cortex on the promoter regions of genes related to neurological functions and psychological disorders (e.g., D_3 dopamine receptor (*DRD3*), serotonergic transporter (*5-HTT*), and GABAergic receptor (*GABRA2*)) (Massart et al. 2014). Since these 5hmC disruptions were detected

during adulthood, these findings suggest that early-life changes in 5hmC are stable throughout development and may represent the origins of developmental brain disorders such as SCZ, BD, and autism.

SCZ and BD are psychiatric disorders with shared and distinct clinical and genetic features; however, the majority of SCZ and BD cases *cannot* be explained by genetics alone. To investigate a role for 5hmC in SCZ and BD, genomic DNA and total RNA were obtained from the postmortem inferior parietal lobule (IPL) brain tissue of 10 SCZ patients, 9 BD patients, and 11 nonpsychiatric subjects, who had no history of psychiatric disorders. SCZ and BD patients exhibited increased 5hmC abundance and *TET1* expression, but not altered *TET2* or *TET3* expression (Dong et al. 2012). Remarkably, *TET1* was not altered in the cerebellum of these patients, suggesting that 5hmC may be involved in the development of psychosis through the inferior parietal lobule, but not the cerebellum, perhaps shedding light on the tissue-specific development of SCZ and BD. The increases of 5hmC in these patients were associated with reduced expression of biologically relevant genes including glutamic acid decarboxylase 67 (*GAD67*) and *APOBEC3A*, which plays a role in active DNA demethylation. Together, these findings suggest a common etiology in psychosis, one that includes genome-wide changes in 5hmC.

While associations between DNA methylation levels and MDD were described above, a recent study extracted genomic DNA from the inferior frontal gyrus of 19 clinically depressed suicide completers and 19 controls and measured genome-wide 5hmC levels. This approach revealed 550 differential hydroxymethylated sites in a plurality of genes, some of which also had differential expression, including myosin XVI (*MYO16*) and insulin-degrading enzyme (*IDE*), genes previously implicated in brain development and neurodegenerative disorders (Kurochkin and Goto 1994; Yui et al. 2015). These data shed light on an alternative molecular mechanism that may be involved in the development of MDD.

ASDs encompass a broad range of behaviorally related disorders with a high prevalence in children. Notably, only ~20% of ASD cases show a genetic etiology (Gaugler et al. 2014; Bulik-Sullivan et al. 2015). Prenatal factors shown to increase the risk of ASD in offspring include environmental influences such as multiple births, in vitro fertilization, and parental exposure to common drug treatments (e.g., antiepileptic drugs (e.g., valproate) or folic acid) (Gardener et al. 2009; Rogers 2008). Together, these findings effectively open the door for contributions from environmentally sensitive epigenetic modifications such as 5hmC to have an underlying role in the ASD etiology. Consistent with this hypothesis, genomic DNA extracted from one fetal and two human cerebellum brain tissues revealed that developmentally specific changes in 5hmC are highly enriched in known ASD genes (Wang et al. 2012b). In another study, the mRNA and 5hmC levels from the cerebellar cortex of ten ASD patients and ten controls were compared, and ASD patients had an enrichment of both *TET1* mRNA and 5hmC levels at the promoters of both *GAD67* and *RELN*, both candidate genes of ASD (Zhubi et al. 2014). More recently, genome-wide 5hmC levels were measured in cerebellum tissue from 17 ASD patients and 19 controls, revealing 797 age-dependent differentially hydroxymethylated regions (DhMRs) in the young group (age $\leq$ 18) and no

significant DhMRs in groups over 18 years of age, suggesting 5hmC may be affected across the spectrum early development and could contribute to the pathogenesis of ASD (Cheng et al. 2018). However, since all of these findings were observed post-symptomatically and in postmortem human brain tissue, it is unclear if the altered 5hmC represents a cause or a consequence of having autistic-like behaviors. Thus, these findings warrant a deeper investigation at pre-symptomatic developmental time points.

Finally, it is notable that rodent models exposed to prenatal stress exhibit long-lasting neurological, endocrinological, and behavioral changes that are thought to mirror the development of mental illness (Koenig et al. 2005). For example, prenatal stress increased anxiety-like behaviors and altered gene expression in mice heterozygous for the serotonin transporter (*5-HTT*) gene (Van Den Hove et al. 2011). Interestingly, these findings were more pronounced in female offspring, suggesting a sex-specific development of these altered behaviors. This study demonstrates a gene by environment interaction, and while this study did not examine 5hmC, the 5hmC-related data discussed in this review supports a 5hmC contribution to these altered behaviors and gene expression. If so, do these changes in 5hmC mirror those found in the homozygous mutants with advanced behavioral endophenotypes? This finding could shed light on the molecular mechanisms exacerbating early-life, stress-related behavioral outcomes in offspring that may have a genetic predisposition, a likely scenario for many mental disorders.

3.2 In Aging and Neurodegeneration

The emerging link between brain development and the accumulation of 5hmC with age has led researchers to examine this epigenetic mark in neurodegenerative diseases (Al-Mahdawi et al. 2014). Indeed, age-associated genes that acquire 5hmC are associated with pathways related to neurodegenerative diseases (Song et al. 2011). Human studies found that 5hmC was depleted in the hippocampus, cerebellum, and entorhinal cortex of ten patients suffering from AD, compared to ten controls (Chouliaras et al. 2013; Condliffe et al. 2014). On the other hand, studies of the middle frontal gyrus from 13 AD patients and the middle temporal gyrus from 29 AD patients revealed that an enrichment of 5hmC was positively correlated with hallmarks of AD, including neurofibrillary tangles (NFT), amyloid beta, and ubiquitin load (Coppieters et al. 2014). Together, these findings suggest that 5hmC may be driving the primary molecular components of AD progression within distinct regions of the brain. Notably, AD-associated levels of 5hmC also can be detected at preclinical stages of AD, as observed by the comparison of postmortem hippocampus and cerebellum brain tissues from five controls and five preclinical AD subjects, defined as having sufficient AD pathologic alterations at autopsy to meet intermediate or high NIA-RI criteria and moderate or frequent neuritic plaque scores according to the Consortium to Establish a Registry for AD (CERAD) with Braak scores of III–IV and antemortem psychometric test scores in the normal range when

corrected for age and education (Bradley-Whitman and Lovell 2013; Schmitt et al. 2000). These findings indicate that 5hmC may act as a viable biomarker of AD onset and progression. However, studies employing nuclear labeling or enzyme-linked immunosorbent assay (ELISA) were unable to find significant alterations in 5hmC associated with AD (Lashley et al. 2015), suggesting that higher-resolution methods are required to identify AD-associated changes in 5hmC. Connections between 5hmC and other neurodegenerative disorders including HD, ataxia-telangiectasia, and fragile X-associated tremor/ataxia syndrome have recently surfaced, and the link to 5hmC for each of these disorders is described below.

Recent epigenetic connections led to the investigation of the genome-wide distribution of 5hmC in a mouse model of HD, which found a deficiency of 5hmC in the striatum and cortex tissues (Wang et al. 2013). This altered distribution of 5hmC was generally associated with pathways involved in neuronal development/differentiation, axonal guidance, and neuronal function/survival. A specific example included a 5hmC reduction on the *ADORA2A* gene, a G-protein-coupled receptor that decreases its expression in HD patients ($N = 6$) compared to controls ($N = 5$), revealing that 5hmC may play a role in the pathological expression of *ADORA2A* found in HD patients (Villar-Menendez et al. 2013). Together, these results suggest that the loss of 5hmC explains the corresponding increase in total DNA methylation (5mC + 5hmC; see above) and provides a more complete understanding of a potential molecular mechanism underlying the cause of neuronal cell death associated with HD.

Ataxia-telangiectasia, a devastating neurodegenerative disorder that shows high cell specificity in its development, is caused by a mutation in the *ATM* gene (Mckinnon 2004). Despite this monogenic etiology, alterations in 5hmC were recently found in repeated sequences and regulatory elements in genomic DNA from *ATM*-deficient Purkinje cells of human patients ($N = 3$) compared to controls (Jiang et al. 2015). This 5hmC distribution is likely the result of TET1 responding to DNA damage, which is a hallmark of *ATM*-deficient neuronal cells. To test whether TET1 activity is related to Purkinje cell degeneration and behavioral deficits in mice, adenoviral particles encoding either human wild-type TET1 (TET1-WT) or kinase dead mutant TET1 (TET-KD) were infected into *ATM*-deficient mouse cerebellum slices and controls. Cerebellum samples lacking TET1 expression that were infected with TET1-WT showed no activation of *Caspase-3*, a cell death marker, while infection with TET1-KD activated *Caspase-3*. These findings suggest that TET1-mediated 5hmC production is essential for Purkinje cell viability and the prevention of ataxia-telangiectasia-like symptoms in mice, supporting the concept that vulnerability to neurodegeneration is linked to aberrant changes of 5hmC in neuronal cells.

Fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder, is one of the characterized fragile X disorders that are caused by a CGG expansion in the 5′UTR of the *FMR1* gene (Hagerman and Hagerman 2015; Santoro et al. 2012). Genome-wide analysis of 5hmC in the cerebellum of a FXTAS mouse model (rCGG mice) revealed an overall reduction in 5hmC at 16 weeks of age when compared to age-matched controls (Yao et al. 2014). Despite the overall reduction of 5hmC, these mice have an increase of 5hmC in repetitive sequences

as well as in cerebellum-specific enhancers. Differential 5hmC between the rCGG and control mice were predominantly found in transcription factor (TF) binding sites that are located in genes essential for neuronal development. Finally, ribosomal profiling revealed that the differential 5hmC-associated genes often exhibited altered ribosomal processing in the rCGG mice, suggesting that 5hmC may somehow influence translational changes. In summary, this study links 5hmC to the etiology of FXTAS and implicates a role for 5hmC in transcription factor binding and in regulating ribosomal processing of mature RNA transcripts.

Despite that the above disease-associated disruptions in 5hmC represent changes across age, these examples clearly demonstrate a role for 5hmC in development and in the onset and progression of neurodegenerative diseases. These developmental and neurodegenerative disease-associated changes in 5hmC often arise within distinct cell types and brain regions, supporting cell- and tissue-specific development of these diseases. Since these studies have been largely descriptive, it is imperative that future studies determine the functional mechanism(s) played by 5hmC if we are to modify it toward healthy outcomes.

4 Clinical Utilities of DNA Methylation

Diagnosis and risk assessment of psychiatric disorders are typically performed using family history, subjective symptom reports, and behavioral observations, which are vulnerable to the variability in accurate patient reporting and consistent clinician interpretations. Thus, molecular biomarkers are being sought because they represent objective measures that can more accurately diagnose and stratify behavior disorders. For example, suicide biomarkers could accurately distinguish between a pathological mood disorder and a normal symptomatic response to an acute stress (e.g., bereavement). These biomarkers also may provide insight into the underlying molecular mechanisms contributing to specific mood-related behaviors. As another example, a recent study examined the peripheral blood from PTSD patients with matched adult trauma exposure and found distinct PTSD-associated DNA methylation profiles, which were related to their different childhood adverse events ($N = 32$ and 29) (Mehta et al. 2013). These findings suggested that DNA methylation levels across the genome might reflect differences in the pathophysiology of PTSD. Ultimately, this information can provide much needed insight into treatment response and the development of novel therapeutics aimed at alleviating mood disorders.

DNA methylation provides a highly sensitive and dynamic biomarker in real time, immediately marking exposures to various environmental stimuli such as stress, cognitive behavioral therapy (CBT), electroconvulsive therapy (ECT), antidepressant treatment, and exercise. Moreover, it is notable that environmentally sensitive DNA methylation levels can be stable across generations. Thus, the methylome is a promising candidate to be an objective biomarker of human behavior following environmental stimuli. Recent support for using DNA methylation as an

objective biomarker of environmental stimuli came from a study that examined the genomic DNA from two cohorts of urban African Americans, from the Grady Trauma Project ($N = 421$) and the Johns Hopkins Center for Prevention Research Study ($N = 326$), and found an interaction between blood *SKA2* methylation and trauma scores (particularly childhood emotional abuse). This interaction was successful at predicting suicidal ideation and suicidal behavior with 71% and 73% accuracy, respectively (Clive et al. 2016). Notably, prediction of suicidal ideation and behavior from saliva samples also was promising, as *SKA2* methylation levels following childhood abuse had a 76% accuracy in predicting suicidal ideation. On the other hand, saliva *SKA2* methylation is linked to extreme anxiety and had 69% accuracy in predicting suicidal ideation, further supporting the sensitivity of DNA methylation to distinguish between experience-induced symptomatic responses. In the same study, the *SKA2* methylation prediction model was used to predict PTSD in the Grady Trauma Project cohorts. Interestingly, while *SKA2* methylation had 72% accuracy in predicting PTSD that was defined by the Child Trauma Questionnaire (CTQ) scores, *SKA2* methylation alone only had 55% accuracy in predicting PTSD. Together, these data suggested that combining DNA methylation information with rigorous behavioral assessments improves our power to diagnose mood disorders. Other researchers sought to further improve this suicide prediction model by determining if DNA methylation levels at other genes correlate with suicide. These studies revealed that the DNA methylation levels of three genes (*DDR1*, *ARHGEF10*, *SHP1*) correlated with suicide and *SKA2* methylation levels and together they could consistently predict suicide across all tested data sets, including youth at high risk for depression, pregnant women at risk for elevated postpartum depression, and middle-aged individuals with a high incidence trauma and PTSD. Together, these findings shed light on the possibility for standardized screening with a single objective test, perhaps without the need for a subjective behavioral assessment.

Another promising example of using DNA methylation to diagnose and stratify risk for behaviors includes recent work that investigated fear extinction in patients suffering from panic disorder. This study examined genomic DNA from blood of female patients that received CBT for 6 weeks ($N = 28$), panic disorder patients that did not receive CBT ($N = 20$), and controls ($N = 28$) and found that the DNA methylation on the monoamine oxidase A (*MAOA*) gene was inversely associated with panic disorder severity (as measured by either panic attacks or severity of agoraphobia symptoms). Perhaps most exciting, *MAOA* methylation levels responded to CBT, as the *MAOA* methylation in patients undergoing CBT matched the levels of controls. These findings indicate that *MAOA* methylation levels may be physiologically linked to the therapeutic effects of CBT, with the simplest interpretation being that increased *MAOA* methylation results in decreased *MAOA* gene expression and increased bioavailability of monoamines in the synaptic cleft. Clinically, this could allow for an objective screening tool for panic disorder symptoms and for monitoring the efficacy of panic disorder treatment.

The sensitivity of 5hmC levels following prenatal and/or acute stress underscores the potential for 5hmC as a novel biomarker in the diagnosis of mental health. In

addition, the presence/absence of these epigenetic modifications is reversible; thus, they may become relevant in therapeutic interventions (Szyf 2015), especially if methods to selectively modulate 5hmC in vivo are developed at the nucleotide level. Finally, 5hmC has sex-specific profiles, which is of interest because the development of several psychiatric disorders is seemingly sex-specific. For example, females show an increased risk in developing anxiety and depression, while males show a disposition to development of attention deficit hyperactivity disorder (Nolen-Hoeksema 1987; Wooten et al. 2004). A recent study found sex-specific 5hmC on genes with ontological terms correlating with organ morphogenesis, system development, and development of anatomical structures (Gross et al. 2015), suggesting that 5hmC may differentially influence the development of organs (e.g., the brain) in the sexes. Together, these factors must be considered for clinical application and treatment endeavors.

5 Conclusions

The findings described in this chapter highlight the research that has been conducted toward improving our understanding of the molecular events contributing to the development of mental health and illness. Importantly, these studies promote scientific questions that are less limited by the bounds of environment versus genetic, or categories of biologic versus psychiatric, and may spawn productive directions toward improved standard of care in psychiatry. Perhaps most promising is the quantitative nature of 5mC and 5hmC detection, indicating that these marks show promise to serve as unifying links between tissue-level activity and complex behavior. Surprisingly, the methylome is highly sensitive to environmental stimuli, showing alterations in as little as 30 min. Moreover, these marks are both stable and reversible – as shown in suicide completers and in cocaine addiction model, respectively. Harnessing the methods to control this reversibility is of great interest, and some examples were presented, which included decreases in methylation levels in patients with BPD following dialectic behavioral therapy or rodents in response to arched back nursing or enhanced maternal care. Fortunately, brain imaging methodologies such as BOLD and fMRI have confirmed that methylation levels in the blood are linked to tissue activity in the brain. This finding has enabled other studies to test a variety of tissues from which samples can be examined – expanding from brain tissue to blood and buccal swabs – allowing for more flexibility including the ability to obtain epigenetic data from living human subjects. While we have yet to fully understand the functional roles of DNA methylation and hydroxymethylation in behavior, we are beginning to recognize that environmentally sensitive alterations in the methylome are linked to gene expression changes governing behavior. The need for such understanding is especially clear in conditions where pathogenesis is not explained by genetics alone: as in autism, which is thought to be only ~25% explained by genetics; SCZ, MDD, and PTSD, for which monozygous twins can be discordant; and suicide, where predisposition is known to have a significant

interaction with environmental factors, such as life experiences and history of trauma. We hope that further understanding and study of the methylome will allow us to improve classification, detection, treatment, and prevention of psychiatric conditions.

Disease classification is an area of constant clinical and research interest. The *Diagnostic and Statistical Manual of Mental Disorders* (DSM-V) guides diagnosis of psychiatric conditions, almost exclusively using behavioral symptoms and signs. This manual, while carefully crafted and painstakingly edited longitudinally by experts in the field of psychiatry, remains vulnerable to subjectivity. It is well documented that many conditions can confound the clinical picture of others; for example, anxiety can share symptoms with ADHD, yet the treatment of one can exacerbate the other. In the future, we hope investigators seek objective biomarkers to aid in classification of mental health and believe that the methylome offers a potential means toward the goal of providing a more personalized diagnosis that will lead to more precise/effective treatments (Fig. 4). This scenario will eliminate the diagnostic odyssey that many mental illness patients endure and provide a rapid treatment strategy toward a healthy outcome.

We also anticipate that the early detection of mental illness will be an important clinical application of the methylome. An example where a molecular indicator, like the methylome, would be effective is in the "prodrome" phase of a disorder (e.g., SCZ), when a patient presents with a subtle abnormal behavior that does not indicate a clear diagnosis. In addition, early detection leads to early treatment, which can dramatically improve outcomes for most mental illness patients.

The current standard of care for the detection of a mental illness is limited, relying heavily on patient reporting and symptom recognition. Among future areas for research, we see an opportunity to identify novel quantitative indicators for risk, through identifying aberrations to the methylome which can be measured peripherally in a clinical setting, perhaps through leukocytes via blood draw or buccal swab. We already have observed a relationship between the methylation levels in the promoter region of the oxytocin receptor gene and autism and *BDNF* in suicide completers. However, much work remains to tease out correlations between genes in multiple disorders, such as why increased DNA methylation levels on the *OXTR* promoter region is associated with autism yet decreased DNA methylation levels on the *OXTR* promoter region is associated with social anxiety disorder – much like copy number variations? Can DNA methylation levels lead to different disorders based on too much or too little? We anticipate that improved understanding of the complete methylome may lead to clinically useful biomarkers for risk of a variety of psychiatric illnesses that span the lifetime. This knowledge of the methylome and its role in etiology of psychiatric conditions may allow us to identify unique physiologic targets for pharmacological intervention. We can likewise develop more targeted nonpharmacologic therapies and apply them with more specificity. In short, the methylome represents an exciting frontier that can lead to more effective collaboration between the individually developed fields of psychology, psychiatry, neuroscience, and genetics. This unification of understanding may potentially lead to more objective and precise disease classification, risk stratification, treatment, and ultimately improved quality of life for vulnerable individuals and populations.

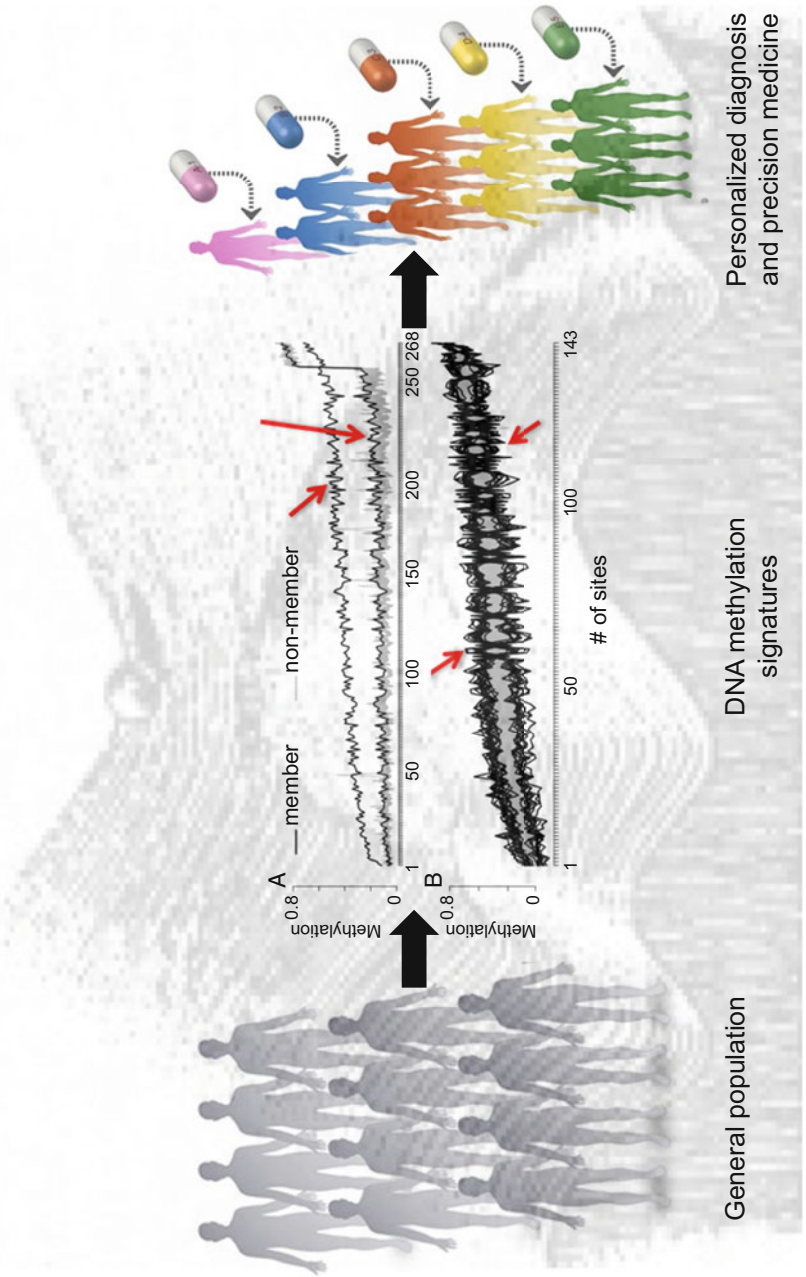

Fig. 4 Improving the standard of care in psychiatry. Genome-wide signatures of DNA methylation can be used to stratify the general population. This process can result in a personalized diagnosis that is more rapid and exact. Moreover, it will provide insight into the genes and pathways contributing to the individual diagnosis, which will guide more precise treatment for the individual

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