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DNA Methylation and Its Basic Function

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In the mammalian genome, DNA methylation is an epigenetic mechanism involving the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine. DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA. During development, the pattern of DNA methylation in the genome changes as a result of a dynamic process involving both *de novo* DNA methylation and demethylation. As a consequence, differentiated cells develop a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription. In this chapter, we will review the process of DNA methylation and demethylation in the nervous system. We will describe the DNA (de)methylation machinery and its association with other epigenetic mechanisms such as histone modifications and noncoding RNAs. Intriguingly, postmitotic neurons still express DNA methyltransferases and components involved in DNA demethylation. Moreover, neuronal activity can modulate their pattern of DNA methylation in response to physiological and environmental stimuli. The precise regulation of DNA methylation is essential for normal cognitive function. Indeed, when DNA methylation is altered as a result of developmental mutations or environmental risk factors, such as drug exposure and neural injury, mental impairment is a common side effect. The investigation into DNA methylation continues to show a rich and complex picture about epigenetic gene regulation in the central nervous system and provides possible therapeutic targets for the treatment of neuropsychiatric disorders.

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INTRODUCTION

Genetics is the study of heritable changes in gene activity or function due to the direct alteration of the DNA sequence. Such alterations include point mutations, deletions, insertions, and translocation. In contrast, epigenetics is the study of heritable changes in gene activity or function that is not associated with any change of the DNA sequence itself. Although virtually all cells in an organism contain the same genetic information, not all genes are expressed simultaneously by all cell types. In a broader sense, epigenetic mechanisms mediate the diversified gene expression profiles in a variety of cells and tissues in multicellular organisms.

In this chapter, we would introduce a major epigenetic mechanism involving direct chemical modification to the DNA called DNA methylation. Historically, DNA methylation was discovered in mammals as early as DNA was identified as the genetic material (Avery *et al*, 1944; McCarty and Avery, 1946). In 1948, Rollin Hotchkiss first discovered modified cytosine in a preparation of calf thymus using paper chromatography. Hotchkiss (1948) hypothesized that this fraction was 5-methylcytosine (5mC) because it separated from cytosine in a manner that was similar to the way that thymine (also known as methyluracil) separated from uracil, and he further suggested that this modified cytosine existed naturally in DNA. Although many researchers proposed that DNA methylation might regulate gene expression, it was not until the 1980s that several studies demonstrated that DNA methylation was involved in gene regulation and cell differentiation (Holliday and Pugh, 1975; Compere and Palmiter, 1981). It is now well recognized that DNA methylation, in concert with other regulators, is a major epigenetic factor influencing gene activities.

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that transfer a methyl group from *S*-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5mC (Figure 1). Dnmt3a and Dnmt3b can establish a new methylation pattern to unmodified DNA and are thus known as *de novo* Dnmt (Figure 1a). On the other hand, Dnmt1 functions during DNA replication to copy the DNA methylation pattern from

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the parental DNA strand onto the newly synthesized daughter strand (Figure 1b). All three Dnmts are extensively involved in the development of an embryo. By the time cells reach terminal differentiation, Dnmt expression is much reduced. This would seem to suggest that the DNA methylation pattern in postmitotic cells is stable. However, postmitotic neurons in the mature mammalian brain still express substantial levels of Dnmts, raising the possibility that Dnmts and DNA methylation may play a novel role in the brain (Goto *et al*, 1994; Feng *et al*, 2005).

Neurons react to the environment through patterns of depolarization that both relay information and encode a response. In recent years, it has become increasingly apparent that following depolarization, alterations in gene expression are accompanied by modifications of the epigenetic landscape that include alterations in the pattern of DNA methylation (Martinowich *et al*, 2003; Guo *et al*, 2011a). In order for the DNA methylation pattern to be altered, there must be both active DNA methylation and demethylation in the neuronal genome. However, no enzymes are known to cleave the methyl group directly from 5mC. As discussed below, the recent identification of 5-hydroxymethyl-cytosine (5hmC) in postmitotic neurons suggests that 5hmC serves as an intermediate in the DNA demethylation pathway. In this review, we will discuss the basic function of DNA methylation in epigenetic gene regulation, and further highlight its role in neural development and neurological disease.

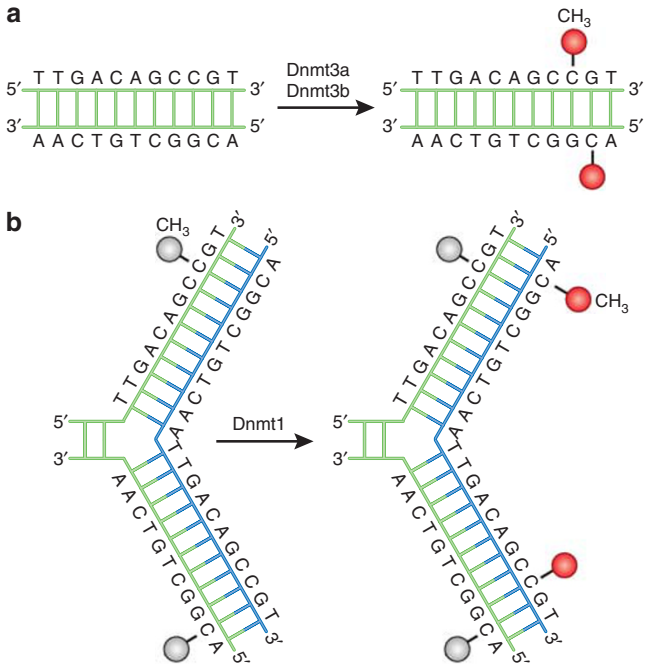

Figure 1. DNA methylation pathways. A family of DNA methyltransferases (Dnmts) catalyzes the transfer of a methyl group from *S*-adenyl methionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (5mC). (a) Dnmt3a and Dnmt3b are the *de novo* Dnmts and transfer methyl groups (red) onto naked DNA. (b) Dnmt1 is the maintenance Dnmt and maintains DNA methylation pattern during replication. When DNA undergoes semiconservative replication, the parental DNA stand retains the original DNA methylation pattern (gray). Dnmt1 associates at the replication foci and precisely replicates the original DNA methylation pattern by adding methyl groups (red) onto the newly formed daughter strand (blue).

LOCATION OF DNA METHYLATION

Although the brain contains some of the highest levels of DNA methylation of any tissue in the body, 5mC only accounts for ~1% of nucleic acids in the human genome (Ehrlich *et al*, 1982). The majority of DNA methylation occurs on cytosines that precede a guanine nucleotide or CpG sites. Overall, mammalian genomes are depleted of CpG sites that may result from the mutagenic potential of 5mC that can deaminate to thymine (Coulondre *et al*, 1978; Bird, 1980). The remaining CpG sites are spread out across the genome where they are heavily methylated with the exception of CpG islands (Bird *et al*, 1985). Interestingly, there is evidence of non-CpG methylation in mouse and human embryonic stem cells, however these methylation are lost in mature tissues (Ramsahoye *et al*, 2000; Lister *et al*, 2009). More thorough analysis of the murine frontal cortex has recently revealed that although the majority of methylation occurs within CpG sites, there is a significant percentage of methylated non-CpG sites (Xie *et al*, 2012). Because of its recent discovery, the role of non-CpG methylation is still unclear.

DNA methylation is essential for silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and X chromosome inactivation. Importantly, DNA methylation in different genomic regions may exert different influences on gene activities based on the underlying genetic sequence. In the following sections, we will further elaborate upon the role of DNA methylation in different genomic regions.

Intergenic Regions

Approximately 45% of the mammalian genome consists of transposable and viral elements that are silenced by bulk methylation (Schulz *et al*, 2006). The vast majority of these elements are inactivated by DNA methylation or by mutations acquired over time as the result of the deamination of 5mC (Walsh *et al*, 1998). If expressed, these elements are potentially harmful as their replication and insertion can lead to gene disruption and DNA mutation (Michaud *et al*, 1994; Wu *et al*, 1997; Kuster *et al*, 1997; Gwynn *et al*, 1998; Ukai *et al*, 2003). The intracisternal A particle (IAP) is one of most aggressive retroviruses in the mouse genome (Walsh *et al*, 1998). IAP is heavily methylated throughout life in gametogenesis, development, and adulthood (Walsh *et al*, 1998; Gaudet *et al*, 2004). Even within the embryo when the rest of the genome is relatively hypomethylated, Dnmt1 maintains the repression of IAP elements (Gaudet *et al*, 2004). When Dnmt1 is depleted by genetic mutations, leading to extensive hypomethylation, IAP elements are expressed (Walsh *et al*, 1998; Hutnick *et al*, 2010). This

demonstrates that within intergenic regions, one of the main roles of DNA methylation is to repress the expression of potentially harmful genetic elements.

CpG Islands

CpG islands are stretches of DNA roughly 1000 base pairs long that have a higher CpG density than the rest of the genome but often are not methylated (Bird *et al*, 1985). The majority of gene promoters, roughly 70%, reside within CpG islands (Saxonov *et al*, 2006). In particular, the promoters for housekeeping genes are often imbedded in CpG islands (Gardiner-Garden and Frommer, 1987). CpG islands, especially those associated with promoters, are highly conserved between mice and humans (Illingworth *et al*, 2010). The location and preservation of CpG islands throughout evolution implies that these regions possess a functional importance.

It appears that CpG islands have been evolutionarily conserved to promote gene expression by regulating the chromatin structure and transcription factor binding. DNA is regularly wrapped around histone proteins forming small, packaged sections called nucleosomes. The more tightly associated with histone proteins the DNA is, the less permissive it is for gene expression. One of the common features of CpG islands is that they contain less nucleosomes than other stretches of DNA (Tazi and Bird, 1990; Ramirez-Carrozzi *et al*, 2009; Choi, 2010). The few nucleosomes with which CpG islands are associated often contain histones with modifications involved in enhancing gene expression (Tazi and Bird, 1990; Mikkelsen *et al*, 2007). Although ~50% of CpG islands contain known transcription start sites, CpG islands are often devoid of common promoter elements such as TATA boxes (Carninci *et al*, 2006). As many transcription factor binding sites are GC rich, CpG islands are likely to enhance binding to transcriptional start sites. Despite their lack of common promoter elements, CpG islands enhance the accessibility of DNA and promote transcription factor binding.

The methylation of CpG islands results in stable silencing of gene expression (Mohn *et al*, 2008). During gametogenesis and early embryonic development, CpG islands undergo differential methylation (Wutz *et al*, 1997; Caspary *et al*, 1998; Zwart *et al*, 2001; Kantor *et al*, 2004). The ability of methylation to regulate gene expression through CpG islands is particularly important for establishing imprinting (Wutz *et al*, 1997; Caspary *et al*, 1998; Zwart *et al*, 2001; Choi *et al*, 2005). Imprinted genes are expressed from only one of the two inherited parental chromosomes and their expression is determined by the parent of inheritance. Beyond imprinted genes, DNA methylation of CpG islands regulates gene expression during development and differentiation (Shen *et al*, 2007; Weber *et al*, 2007; Fouse *et al*, 2008; Mohn *et al*, 2008; Meissner *et al*, 2008). As CpG islands are associated with the control of gene expression, it would be expected that CpG islands might display tissue-specific patterns of DNA methylation. Although CpG islands in intragenic and gene body regions can have tissue-specific patterns of methylation, CpG islands associated with transcription start sites rarely show tissue-specific methylation patterns (Rakyan *et al*, 2004; Eckhardt *et al*, 2006; Meissner *et al*, 2008; Illingworth *et al*, 2010; Maunakea *et al*, 2010). Instead, regions called CpG island shores, located as far as 2 kb from CpG islands, have highly conserved patterns of tissue-specific methylation (Irizarry *et al*, 2009). Like CpG islands, the methylation of CpG shores is highly correlated with reduced gene expression (Irizarry *et al*, 2009).

The role of CpG islands in regulating gene expression is still being uncovered. Methylation of CpG islands can impair transcription factor binding, recruit repressive methyl-binding proteins, and stably silence gene expression. However, CpG islands, especially those associated with gene promoters, are rarely methylated. Further studies are needed to determine to what degree DNA methylation of CpG islands regulates gene expression.

Gene Body

As the majority of CpG sites within the mammalian genome are methylated, the genes themselves must also contain methylation. The gene body is considered the region of the gene past the first exon because methylation of the first exon, like promoter methylation, leads to gene silencing (Brenet *et al*, 2011). Evidence suggests that DNA methylation of the gene body is associated with a higher level of gene expression in dividing cells (Hellman and Chess, 2007; Ball *et al*, 2009; Aran *et al*, 2011). However, in slowly dividing and nondividing cells such as the brain, gene body methylation is not associated with increased gene expression (Aran *et al*, 2011; Guo *et al*, 2011a, b; Xie *et al*, 2012). Furthermore, in the murine frontal cortex, methylation of non-CpG sites within gene bodies is negatively correlated with gene expression (Xie *et al*, 2012). How DNA methylation of the gene body contributes to gene regulation is still unclear.

BASIC MECHANISM OF DNA METHYLATION

The enzymes that establish, recognize, and remove DNA methylation are broken into three classes: writers, erasers, and readers. Writers are the enzymes that catalyze the addition of methyl groups onto cytosine residues. Erasers modify and remove the methyl group. Readers recognize and bind to methyl groups to ultimately influence gene expression. Thanks to the many years of research devoted to understanding how the epigenetic landscape is erased and reshaped during embryonic development, many of the proteins and mechanisms involved in DNA methylation have already been identified.

Writing DNA Methylation: the Dnmts

Three members of the Dnmt family directly catalyze the addition of methyl groups onto DNA: Dnmt1, Dnmt3a, and

Dnmt3b. Although these enzymes share a similar structure with a large N-terminal regulatory domain and a C-terminal catalytic domain, they have unique functions and expression patterns (Yen *et al*, 1992; Xie *et al*, 1999). Probably the best studied Dnmt, especially in the nervous system, is Dnmt1, which is highly expressed in mammalian tissues including the brain (Goto *et al*, 1994). Unlike the other Dnmts, Dnmt1 preferentially methylates hemimethylated DNA (Pradhan *et al*, 1999; Ramsahoye *et al*, 2000). During DNA replication, Dnmt1 localizes to the replication fork where newly synthesized hemimethylated DNA is formed (Leonhardt *et al*, 1992). Dnmt1 binds to the newly synthesized DNA and methylates it to precisely mimic the original methylation pattern present before DNA replication (Hermann *et al*, 2004) (Figure 1b). Additionally, Dnmt1 also has the ability to repair DNA methylation (Mortusewicz *et al*, 2005). For this reason, Dnmt1 is called the maintenance Dnmt because it maintains the original pattern of DNA methylation in a cell lineage. Knockout of *Dnmt1* in mice results in embryonic lethality between E8.0 and E10.5 (Li *et al*, 1992). At this time, knockout embryos exhibit a two-thirds loss of DNA methylation, in addition to numerous apoptotic cells in a variety of developing tissues including the brain. Interestingly, mouse embryonic stem cells lacking *Dnmt1* remain viable (Chen *et al*, 1998). However, *in vitro* differentiation results in massive cell death, recapitulating the phenotype observed in knockout embryos (Jackson-Grusby *et al*, 2001). These findings firmly establish that Dnmt1 plays a critical role in cellular differentiation as well as in dividing cells.

Dnmt3a and Dnmt3b are extremely similar in structure and function. Unlike Dnmt1, both Dnmt3a and Dnmt3b when overexpressed are capable of methylating both native and synthetic DNA with no preference for hemimethylated DNA (Okano *et al*, 1999). For this reason, Dnmt3a and Dnmt3b are referred to as *de novo* Dnmt because they can introduce methylation into naked DNA (Figure 1a). What primarily distinguishes Dnmt3a from Dnmt3b is its gene expression pattern. Although *Dnmt3a* is expressed relatively ubiquitously, *Dnmt3b* is poorly expressed by the majority of differentiated tissues with the exception of the thyroid, testes, and bone marrow (Xie *et al*, 1999). Similar to *Dnmt1*, the knockout of *Dnmt3b* in mice is embryonic lethal (Okano *et al*, 1999). On the other hand, *Dnmt3a* knockout mice are runted but survive to ~4 weeks after birth (Okano *et al*, 1999). From these results it appears that *Dnmt3b* is required during early development, whereas *Dnmt3a* is required for normal cellular differentiation.

A final member of the Dnmt family is Dnmt3L, a protein that lacks the catalytic domain present in other Dnmt enzymes (Aapola *et al*, 2000; Hata *et al*, 2002). Dnmt3L is mainly expressed in early development and is restricted to the germ cells and thymus in adulthood (Aapola *et al*, 2000, 2001). Although Dnmt3L has no catalytic function of its own, it associates with the Dnmt3a and Dnmt3b and stimulates their methyltransferase activity (Hata *et al*, 2002; Suetake *et al*, 2004; Jia *et al*, 2007). Consistent with its presence in early development and in germ cells, in mice, Dnmt3L is required for establishing both maternal and paternal genomic imprinting, for methylating retrotransposons, and for compaction of the X chromosome (Bourc'his *et al*, 2001; Hata *et al*, 2002; Kaneda *et al*, 2004; Bourc'his and Bestor, 2004; Webster *et al*, 2005; La Salle *et al*, 2007; Zamudio *et al*, 2011). Although Dnmt3L is expressed in the developing brain, Dnmt3L is downregulated during neuronal differentiation and is not observed in the brain postnatally (Lee *et al*, 2006; Kovacheva *et al*, 2007).

Writing DNA Methylation: Targeting *De Novo* DNA Methylation

(How the *de novo* Dnmts target specific genetic regions is still unclear). However, several mechanisms have been proposed. Dnmt3a and Dnmt3b can bind to DNA via a conserved PWWP domain (Ge *et al*, 2004); however, it is unclear how Dnmt3a and Dnmt3b target specific DNA sequences. One hypothesis suggests that RNA interference (RNAi) mechanisms target Dnmts to silence specific sequences of DNA (Morris *et al*, 2004). Although RNAi is clearly involved in DNA methylation in plant cells, the existing evidence is still very weak for a role of RNAi in DNA methylation in mammalian cells. The other theory is that transcription factors regulate *de novo* DNA methylation. Transcription factors can regulate DNA methylation by binding to specific DNA sequence to either recruit Dnmts for methylation or protect from DNA methylation. In some cases Dnmts bind to transcription factors or components of repressor complexes to target methylation to DNA (Brenner *et al*, 2005). In other cases, regardless of whether the gene is expressed, the transcription factor binding can help protect CpG sites from *de novo* methylation (Straussman *et al*, 2009; Gebhard *et al*, 2010; Lienert *et al*, 2011). CpG islands appear to primarily be protected from methylation by transcription factor binding (Brandeis *et al*, 1994; Macleod *et al*, 1994; Straussman *et al*, 2009; Gebhard *et al*, 2010). When transcription factor binding sites are mutated, CpG islands are unable to retain their unmethylated state (Brandeis *et al*, 1994; Macleod *et al*, 1994). Similarly, as differentiation induces the downregulation of transcription factors that bind to specific gene promoters, the now-exposed CpG sites can be targeted for DNA methylation (Lienert *et al*, 2011). These studies describe two mechanisms that likely function together to establish *de novo* DNA methylation. Dnmt3a and Dnmt3b can either be recruited to promoters by specific transcription factors or the *de novo* Dnmt may simply methylate all CpG sites across the genome that are not protected by a bound transcription factor.

Erasing DNA Methylation

DNA demethylation is characterized as either passive or active. Passive DNA demethylation occurs in dividing cells. As Dnmt1 actively maintains DNA methylation during cell

replication, its inhibition or dysfunction allows newly incorporated cytosine to remain unmethylated and consequently reduces the overall methylation level following each cell division. Active DNA demethylation can occur in both dividing and nondividing cells but the process requires enzymatic reactions to process the 5mC in order to revert it back to a naked cytosine (Mayer *et al*, 2000; Oswald *et al*, 2000; Paroush *et al*, 1990; Zhang *et al*, 2007). As of yet, there is no known mechanism in mammalian cells that can cleave the strong covalent carbon-to-carbon bond that connects cytosine to a methyl group. Instead, demethylation occurs through a series of chemical reactions that further modify 5mC, by deamination and/or oxidation reactions to a product that is recognized by the base excision repair (BER) pathway to replace the modified base with naked cytosine. Although it is generally agreed upon that the BER pathway is the final step in DNA demethylation, the specific enzymes and the chemical intermediates that are formed during DNA demethylation are still debated (Bhutani *et al*, 2011).

Several mechanisms of active DNA demethylation have been proposed (Figure 2). 5mC can be chemically modified at two sites, the amine group and the methyl group. Deamination of the amine to a carbonyl group by AID/APOBEC (activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex) effectively converts 5mC into thymine, thus creating a G/T mismatch and inducing the BER pathway to correct the base. Overexpression of AID/APOBEC promotes DNA demethylation in zebrafish (Rai *et al*, 2008), whereas knockdown or knockout inhibits the DNA demethylation of various genes necessary for cellular reprogramming and development (Bhutani *et al*, 2010; Muramatsu *et al*, 2000; Popp *et al*, 2010). Unlike the various *Dnmt* knockout mice, knockout *AID* mice are viable and fertile. If global DNA demethylation is as critical as DNA methylation in early development, then the knockout *AID* mice study raises the possibility that multiple mechanisms for active DNA demethylation exist and can compensate for one another.

In line with the multiple mechanisms hypothesis, another active DNA demethylation mechanism is found to be mediated by the ten–eleven translocation (Tet) enzymes Tet1, Tet2, and Tet3. Tet enzymes add a hydroxyl group onto the methyl group of 5mC to form 5hmC (Tahiliani *et al*, 2009; Ito *et al*, 2010). The developed brain contains significant 5hmC levels in multiple regions, ranging from 0.3 to 0.7%, which is approximately tenfold lower than the average abundance of 5mC (Kriaucionis and Heintz, 2009; Globisch *et al*, 2010). Once 5hmC is formed, two separate mechanisms can convert 5hmC back into cytosine in mammals. In the first, iterative oxidation by Tet enzymes continues to oxidize 5hmC first to 5-formyl-cytosine and then to 5-carboxy-cytosine (Ito *et al*, 2011). In the second, 5hmC is deaminated by AID/APOBEC to form 5-hydroxymethyl-uracil (Guo *et al*, 2011b). Consistent with the role of Tet in converting 5mC into 5hmC *in vivo*, *Tet1* knockout mouse embryonic stem cells have reduced levels of 5hmC that is accompanied by a subtle increase in 5mC at a global level (Dawlaty *et al*, 2011).

Whether 5hmC functions only as an intermediate in DNA demethylation is still unclear. Like methylation, 5hmC may regulate gene expression. In support of this theory, the conversion of 5mC to 5hmC impairs the binding of the repressive methyl-binding protein MeCP2 (Valinluck *et al*, 2004). But what is clear at this time is that 5hmC is found *in vivo* in mammalian tissue and may play an important role in regulating DNA demethylation and gene expression.

In all the mentioned mechanisms of active DNA demethylation, the BER pathway uses thymine DNA glycosylase (TDG) to cleave off the modified residue—thymine, 5-hydroxymethyl-uracil, 5-formyl-cytosine, and 5-carboxy-cytosine—and replace it with a naked cytosine (Cortellino *et al*, 2011; He *et al*, 2011). TDG is essential for DNA demethylation and is required for normal development. Knockout or inactivation of *TDG* leads to embryonic lethality in mice. Moreover, these mutant embryos exhibit hypermethylation, particularly in imprinting genes such as *Igf2* and *H19*, suggesting that active demethylation by TDG protects imprinted genes from spontaneous *de novo* methylation (Cortellino *et al*, 2011). Single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), another BER enzyme from the same uracil DNA glycosylase family as TDG, is also found to be involved in DNA demethylation (Cortellino *et al*, 2011; Guo *et al*, 2011a, b). In summary, active DNA demethylation arises from multiple pathways involving multiple enzymes and this complexity has likely contributed to much of the current scientific debate.

Reading DNA Methylation

Whereas DNA methylation may itself reduce gene expression by impairing the binding of transcriptional activators, a second class of proteins with a high affinity for 5mC inhibits transcription factor binding. DNA methylation is recognized by three separate families of proteins: the MBD proteins, the UHRF proteins, and the zinc-finger proteins. Of these families, the MBD was the first to be identified. MBD proteins contain a conserved methyl-CpG-binding domain (MBD) that confers a higher affinity for single methylated CpG sites (Nan *et al*, 1993). This family includes MeCP2, the first identified methyl-binding protein, along with MBD1, MBD2, MBD3, and MBD4 (Meehan *et al*, 1989; Lewis *et al*, 1992; Hendrich and Bird, 1998). MBDs are more highly expressed in the brain than in any other tissue, and many MBDs are important for normal neuronal development and function (Amir *et al*, 1999). Of the MBD family, MBD3 and MBD4 are unusual. For example, MBD3 is incapable of directly binding to DNA due to a mutation in its MBD domain (Hendrich and Bird, 1998). Although MBD4 binds to DNA normally, it preferentially recognizes when guanine is mismatched with a thymine, uracil, or 5-fluorouracil and associates with proteins involved in DNA mismatch repair (Bellacosa *et al*, 1999; Hendrich *et al*, 1999;

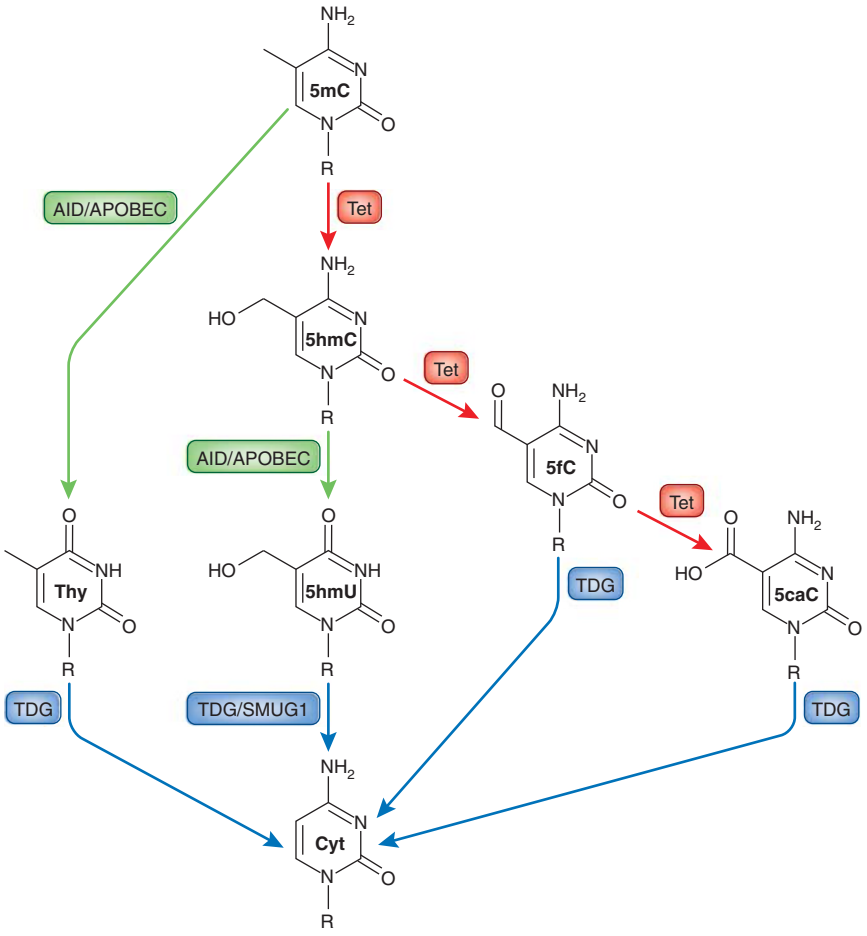

Figure 2. Active DNA demethylation pathways. 5-Methylcytosine (5mC) can be chemically modified at two sites: the amine group and the methyl group. The amine group of 5mC can be deaminated (green) by AID/APOBEC, converting 5mC into thymine (Thy). The methyl group of 5mC can be modified by the addition of a hydroxyl group mediated by Tet enzymes to generate 5-hydroxymethyl-cytosine (5hmC). 5hmC can also be chemically modified at two sites: the amine group and the hydroxymethyl group. AID/APOBEC can deaminate (green) 5hmC to produce 5-hydroxymethyl-uracil (5hmU). In another chemical pathway for 5hmC is that Tet can further oxidize (yellow) 5hmC to form 5-formyl-cytosine (5fC) and then 5-carboxy-cytosine (5caC). Eventually, the products of each pathway—Thy, 5hmU, 5fC, and 5caC—are recognized and cleaved off to replace with a naked cytosine mediated by TDG and/or SMUG1, both components of the base excision repair pathway (red).

Petronzelli *et al*, 2000; Millar *et al*, 2002; Wong *et al*, 2002). The remaining members of the MBD family have the ability to directly bind to methylated DNA and contain a transcriptional repression domain (TRD) that allows MBD proteins to bind to a variety of repressor complexes (Nan *et al*, 1998; Ng *et al*, 1999; Sarraf and Stancheva, 2004). In addition to its role as a transcriptional repressor, MeCP2 appears to have a unique role in the maintenance of DNA methylation. MeCP2 binds to Dnmt1 via its TRD and can recruit Dnmt1 to hemimethylated DNA to perform maintenance methylation (Kimura and Shiota, 2003). Although MBDs are the best studied class of methyl-binding proteins, they are not the only one.

The UHRF (ubiquitin-like, containing PHD and RING finger domain) proteins, including UHRF1 and UHRF2, are multidomain proteins that flip out and bind methylated cytosines via a SET- and RING-associated DNA-binding domain (Hashimoto *et al*, 2008, 2009). Unlike most methyl-binding proteins, the primary function of UHRF proteins is not to bind to DNA and repress transcription. The UHRF protein family first binds to Dnmt1 and then targets it to hemimethylated DNA in order to maintain DNA methylation, especially during DNA replication (Sharif *et al*, 2007; Bostick *et al*, 2007; Achour *et al*, 2008). UHRF1 appears to interact so closely with Dnmt1 that its deletion, like the deletion of Dnmt1, leads to embryonic lethality (Muto *et al*, 2002).

The last family of methyl-binding proteins binds to methylated DNA by a zinc-finger domain and is composed of Kaiso, ZBTB4, and ZBTB38 (Prokhortchouk *et al*, 2001; Filion *et al*, 2006). Although ZBTB4 and ZBTB38 have distinct tissue expression patterns, both are highly expressed in the brain and can bind to a single methylated CpG. The zinc-finger domain proteins are unusual. Despite their ability to recognize methylcytosine, both Kaiso and ZBTB4 have preferential binding for a sequence motif lacking a methylcytosine (Daniel *et al*, 2002; Sasai *et al*,

2010). Unlike other methyl-binding proteins, Kaiso preferentially binds to two consecutively methylated CpG sites (Daniel *et al*, 2002). Yet despite their differences, zinc-finger domain proteins, similar to the MBD family, repress transcription in a DNA methylation-dependent manner (Prokhortchouk *et al*, 2001; Yoon *et al*, 2003; Filion *et al*, 2006; Lopes *et al*, 2008).

Crosstalk of DNA Methylation and Other Epigenetic Mechanisms

DNA methylation works with histone modifications and microRNA (miRNA) to regulate transcription (Figure 3). In eukaryotes, DNA is associated with histone proteins that help to package the long strands of DNA into the small nuclear compartment. Chemical modifications that include methylation, acetylation, ubiquitination, and phosphorylation are added to three specific amino acids on the N-terminal histone tails. These modifications influence not only how DNA strands are packaged but also their transcriptional activity. Histone modifications that loosen DNA association with histones generally provide a permissive environment for transcription, whereas histone modifications that tightly package DNA and histones repress gene expression. Dnmts directly interact with enzymes that regulate histone modifications typically involved in gene repression (Figure 3). Both Dnmt1 and Dnmt3a are known to bind to the histone methyltransferase SUV39H1 that restricts gene expression by methylation on H3K9 (Fuks *et al*, 2003). Furthermore, Dnmt1 and Dnmt3b can both bind to histone deacetylases that remove acetylation from histones to make DNA pack more tightly and restrict access for transcription (Fuks *et al*, 2000; Geiman *et al*, 2004). In general, Dnmts cooperate with histone-modifying enzymes involved in adding and/or stripping histone markers in order to impose a repressive state on a gene region.

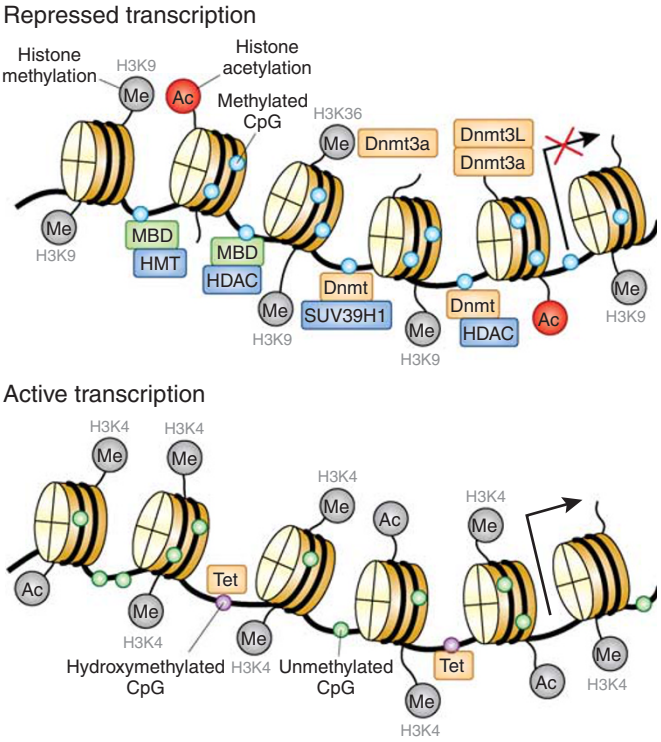

Figure 3. Epigenetic crosstalk. Transcription is ultimately regulated by the interaction of multiple epigenetic mechanisms that cooperate to activate or silence gene expression. Methylation is regulated by proteins such as Dnmt and Tet (purple) that are involved in the active addition or chemical modification (such as hydroxymethylation in red) of DNA methylation. To suppress gene expression, Dnmts target CpG sites and actively methylate DNA. For some Dnmts, their catalytic activity is enhanced by association with histone tails and with Dnmt3L. DNA methylation is recognized by methyl-binding proteins such as MBDs (yellow) that along with Dnmts recruit enzymes that modify the histone tails (orange) including histone deacetylases (HDACs), which remove acetylation (red), and histone methyltransferases (HMTs), which methylate histones (green) and in conjunction with DNA methylation serve to further repress gene expression. In regions of DNA with activate transcription, Tet removes DNA methylation, and histone tails in this region often contain H3K4me^3 that inhibits Dnmt binding to unmethylated CpG sites and maintains a permissive environment for transcription.

Histone modifications can also influence the DNA methylation pattern (Figure 3). Dnmt3L binds to H3 histone tails and recruits Dnmt3a and Dnmt3b to methylate DNA (Ooi *et al*, 2007). The direct binding of Dnmt3a to the H3 histone tail, sometimes facilitated by H3K36 trimethylation, a repressive histone mark, also stimulates its methyltransferase activity (Dhayalan *et al*, 2010; Li *et al*, 2011a). However, the presence of the active histone modification H3K4 trimethylation (H3K4me^3) impairs the binding of Dnmt3a, Dnmt3b, and Dnmt3L to H3 histone tails and prevents methylation (Ooi *et al*, 2007; Zhang *et al*, 2010). CpG islands contain particularly high levels of H3K4me^3 (Mikkelsen *et al*, 2007). Cfp1 is a component of the H3K4 methyltransferase complex that targets unmethylated CpG sites often found at murine CpG islands and may play a role in maintaining their hypomethylation (Lee and Skalnik, 2005; Thomson *et al*, 2010). Little is known regarding how the DNA demethylation machinery interacts with histone modifications, yet there is still evidence to suggest that they cooperate. For instance, elevated histone acetylation can trigger DNA demethylation (Cervoni and Szyf, 2001; D'Alessio *et al*, 2007). Tet1 contains a DNA-binding motif similar to Ctf1, suggesting that both proteins target similar sites, in this case CpG islands, to maintain DNA demethylation (Tahiliani *et al*, 2009). Although a direct connection between the two has yet to been shown, Tet1 does indeed localize to CpG islands and its depletion results in an increase in methylation within those CpG islands in mouse embryonic stem cell studies (Ficz *et al*, 2011; Wu and Zhang, 2011). Future studies are needed to further probe the interaction of Tet with histone modifications.

Methyl-binding proteins serve as the strongest link between DNA methylation and histone modification. Both the MBDs and the UHRF proteins interact with methylated DNA and histones to enhance gene repression (Figure 3) (Nan *et al*, 1998; Ng *et al*, 1999; Sarraf and Stancheva, 2004; Citterio *et al*, 2004; Karagianni *et al*, 2008). MeCP2 recruits histone deacetylases to remove active histone modifications

and repress gene transcription (Jones *et al*, 1998; Nan *et al*, 1998; Fuks *et al*, 2003). Furthermore, MeCP2 enhances the repressive chromatin state by recruiting histone methyltransferases that add repressive H3K9 methylation (Fuks *et al*, 2003). Overall, DNA methylation and histone modifications work closely together to regulate gene expression.

Recently, miRNAs have emerged as another important epigenetic mechanism that influences gene expression. Precursor miRNA forms a double-stranded RNA connected by a hairpin loop. Once transported into the cytoplasm precursor, miRNA is processed by Dicer to generate a 22–23 nucleotide miRNA:miRNA* duplex. Mature miRNAs associate with a miRNA-induced silencing complex (miRISC) that can bind to their target mRNA and repress gene expression by inhibiting translation or inducing RNA degradation (Berezikov, 2011). Like other sequences within the genome, DNA methylation can regulate the expression of miRNAs (Han *et al*, 2007; Lujambio *et al*, 2008). The loss of both *Dnmt1* and *Dnmt3b* in a colon cancer cell line revealed that ~10% of detected miRNAs are regulated by DNA methylation (Han *et al*, 2007). When Dnmts are inhibited, cancer cells reactivate some miRNAs that are initially silenced by hypermethylation of their CpG islands (Lujambio *et al*, 2008). Understood together, these studies demonstrate that DNA methylation regulates miRNA expression.

Conversely, miRNAs can also regulate histone modifications and Dnmt expression and, in so doing, regulate DNA methylation (Benetti *et al*, 2008; Sinkkonen *et al*, 2008). Knockout of *Dicer* in mouse embryonic stem cells results in depletion of miRNAs, one of which is miRNA-290, which indirectly regulates *Dnmt3a* and *Dnmt3b* expression (Benetti *et al*, 2008; Sinkkonen *et al*, 2008). This leads to a loss of DNA methylation and an increase in repressive histone methylation at H3K9. These studies provided evidence of a bidirectional influence between miRNA and DNA methylation.

DNA METHYLATION IN THE BRAIN

DNA Methylation in the Developing CNS

The precise temporal regulation of *de novo* methylation and demethylation is particularly important for the differentiation and maturation of the mammalian central nervous system (CNS). Multipotent neural progenitor cells (NPCs) sequentially undergo neurogenesis and astrogliogenesis (Qian *et al*, 2000; Sauvageot and Stiles, 2002). In particular, the differentiation switch of NPCs from neurogenesis to astrogliogenesis coincides with DNA methylation and demethylation events on the *glial fibrillary acidic protein* (*Gfap*) gene promoter region (Teter *et al*, 1994). Early in neurogenesis at E11.5, DNA methylation of the *Gfap* promoter represses its expression (Teter *et al*, 1996; Takizawa *et al*, 2001). The continual expression of *Dnmt1* in NPCs has been found to be important for the maintenance of the methylation pattern on the *Gfap* promoter through subsequent cell divisions (Fan *et al*, 2005). Interestingly, neurogenesis from E11.5 to E14.5 is the only time during neural development that *Dnmt3b* is strongly expressed before declining to nearly undetectable levels in the CNS (Fan *et al*, 2005). At E14.5, the *Gfap* promoter undergoes DNA demethylation to coincide with the differentiation of the astrocytic lineage (Teter *et al*, 1996). As development further progresses, the decline of *Dnmt3b* and the peak expression of *Dnmt3a* at 3 weeks postnatally coincide with remethylation and reduced transcription of the *Gfap* promoter (Fan *et al*, 2005; Nguyen *et al*, 2007). The coordinated expression of *Dnmts* and their ability to regulate the methylation pattern of the *Gfap* promoter organize and regulate neuronal development.

The importance of these coordinated events is highlighted by conditional knockout models of Dnmts during neural development (Fan *et al*, 2001, 2005; Golshani *et al*, 2005; Nguyen *et al*, 2007; Hutnick *et al*, 2009; Feng *et al*, 2010). Conditional knockout of *Dnmt1* between E8.5 and E13.5, a time period that coincides with neurogenesis, leads to hypomethylation of differentiating neurons and demethylation of the *Gfap* promoter in neural precursor cells, thus accelerating astrogliosis (Fan *et al*, 2001, 2005). Hypomethylated neurons are characterized by multiple maturation defects including dendritic arborization and impaired neuronal excitability (Fan *et al*, 2001; Golshani *et al*, 2005; Hutnick *et al*, 2009). These results are consistent with the conclusion that Dnmt1 has a critical role in neuronal differentiation and in maintaining the methylation of the *Gfap* promoter. Furthermore, these results suggest that DNA methylation is essential for neuronal maturation. If *Dnmt3a* is knocked out instead in the neural precursor cells, the majority of cortical neurons develop normally (Nguyen *et al*, 2007). In this case, the *Gfap* promoter that is normally remethylated in postnatal astrocytes remains hypomethylated in ~50% of cortical tissue, likely corresponding to the glial population (Nguyen *et al*, 2007). This is consistent with the idea that Dnmt3a is not required during differentiation or maturation by the majority of cortical neurons. Together, the knockout data confirm that precise regulation of DNA methylation is essential for differentiation and maturation of the CNS.

Like Dnmts, methyl-binding proteins are expressed in embryonic stem cells and in neural precursors, but unlike Dnmts their expression has little effect on neuronal or glial differentiation (Kishi and Macklis, 2004; Martin Caballero *et al*, 2009). Of the MBD proteins, MeCP2 is the best studied in the CNS because its mutation results in Rett Syndrome, one of the most common forms of mental retardation in females (Amir *et al*, 1999). During development, *MeCP2* is first expressed in the brainstem and thalamus, the most ancient regions of the brain, followed by a rostral progression of expression (LaSalle *et al*, 2001; Shahbazian *et al*, 2002). Like other methyl-binding proteins, MeCP2 associates with a variety of transcriptional repressors, including Dnmt1, and influences gene expression (Nan *et al*, 1998; Ng *et al*, 1999; Kimura and Shiota, 2003; Sarraf and Stancheva, 2004). Neural activity leads to phosphorylation of MeCP2, altering its ability to bind gene promoters and silence gene expression

(Zhou *et al*, 2006; Tao *et al*, 2009). MeCP2 is required for normal neuronal maturation and its loss or the loss of its ability to be phosphorylated results in aberrant dendritic arborization, synaptic function, and plasticity (Chen *et al*, 2001; Moretti *et al*, 2006; Asaka *et al*, 2006; Nelson *et al*, 2006; Cohen *et al*, 2011; Li *et al*, 2011b).

At the conclusion of neural development, the brain primarily consists of postmitotic neurons and glial cells with little proliferation potential. Although the expression of Dnmts normally declines in terminally differentiated cells, the brain appears to be an exception. Both *Dnmt1* and *Dnmt3a* are expressed by postmitotic neurons, whereas *Dnmt3b* expression is either low or virtually undetectable (Goto *et al*, 1994; Inano *et al*, 2000; Fan *et al*, 2005). This surprising discovery led researchers to investigate the role of active DNA methylation in postmitotic neurons of the adult brain.

DNA Methylation in the Adult Brain

To date, the majority of the DNA methylation mechanism has been characterized in embryonic stem cells. Although this *in vitro* model may predict the function of DNA methylation in a dividing cell, embryonic stem cells are an inadequate model for studying DNA methylation in a postmitotic cell. The fact that Dnmts are required for normal neuronal differentiation and maturation hinders the study of DNA methylation solely in postmitotic neurons. Despite these limitations, two models have emerged to study DNA methylation in postmitotic neurons.

The first model utilizes pharmacological inhibitors such as 5-aza-2′-deoxycytadine, zebularine, or RG108 injected into the brain to impair Dnmt activity (Levenson *et al*, 2006; Miller and Sweatt, 2007; Miller *et al*, 2010). Although pharmalogical inhibition of Dnmts has the advantage of inhibiting DNA methylation at the designated time point, the mechanism of action of Dnmt inhibitors in postmitotic neurons is still debated. Nucleoside inhibitors such as 5-aza-2′-deoxycytadine and zebularine must be first incorporated into DNA in order to inhibit Dnmts (Creusot *et al*, 1982). Once incorporated into the DNA, the nucleoside inhibitors trap Dnmts on the chromosome, which can lead to substantial cellular toxicity (Jüttermann *et al*, 1994; Zhou *et al*, 2002). Although it is possible that the BER pathway may be the source of nucleoside incorporation, it is still unclear how nucleoside inhibitors are incorporated into the DNA of a nondividing cell, like a postmitotic neuron. Because of the concerns surrounding the use of nucleoside inhibitors, new-generation Dnmt inhibitors, which include RG108, have been developed. After the discovery of the structure of the catalytic domain of Dnmt1, RG108 was identified in an *in silico* screen as a small molecule that could inhibit Dnmt1 without being incorporated into the DNA (Brueckner *et al*, 2005; Stresemann *et al*, 2006). RG108 has emerged as a promising Dnmt1 inhibitor with less cytotoxicity. In cell-free assays, RG108 is capable of impairing Dnmts with catalytic domains similar to Dnmt1. Although Dnmt3a and Dnmt3b share a highly conserved catalytic domain to Dnmt1, it is still unclear whether RG108 inhibits these methyltransferases (Brueckner *et al*, 2005).

To alleviate concerns of off-target effects and incomplete Dnmt inhibition, a second experimental model has emerged. In this model, *Dnmts* are conditionally knocked out using the cre/loxP system, with cre expressed by a brain-specific promoter (Fan *et al*, 2001; Golshani *et al*, 2005; Nguyen *et al*, 2007; Hutnick *et al*, 2009; Feng *et al*, 2010). Unlike pharmacological inhibition, this second method allows investigators to study the role of a specific Dnmt in subpopulations of neurons. Dnmts are required for normal neuronal differentiation. Therefore, to study the role of Dnmts in the adult brain, cre must be expressed by postmitotic brain-specific promoters such as *CamKIIα* (Fan *et al*, 2001; Golshani *et al*, 2005; Nguyen *et al*, 2007; Hutnick *et al*, 2009; Feng *et al*, 2010).

In postmitotic neurons, Dnmt1 and Dnmt3a appear to have overlapping roles. Neither knockout of *Dnmt1* nor of *Dnmt3a* in forebrain postmitotic neurons leads to any observable change in DNA methylation, gene expression, synaptic plasticity, or behavior (Feng *et al*, 2010). However, double knockouts have reduced DNA methylation that leads to deficits in synaptic plasticity in addition to learning and memory. Although research has yet to tease apart the role of Dnmt1 *vs* Dnmt3a in postmitotic neurons, DNA methylation has repeatedly been shown to play a role in learning and memory in the adult brain.

When neuronal activity is inhibited during fear conditioning, not only is memory formation prevented but so are changes in DNA methylation (Lubin *et al*, 2008). Early studies demonstrated that *in vitro* neuronal activity regulated the expression of *Bdnf* in an activity-dependent manner (Martinowich *et al*, 2003). Neuronal depolarization demethylates the *Bdnf* promoter, releasing the MeCP2 repressor complex from the promoter and increasing *Bdnf* expression (Martinowich *et al*, 2003). Persistent activity in neurons, as occurs during electroconvulsive stimulation or exercise, leads to active DNA methylation and demethylation across several genes within the brain. However, alterations in DNA methylation do not always correlate with the alterations in gene expression observed after heightened activity (Guo *et al*, 2011a). Hence, although both DNA methylation and demethylation are altered by neuronal activity, DNA methylation functions alongside other regulatory proteins and epigenetic mechanisms that determine gene expression.

Another class of proteins that work with DNA methylation to regulate gene expression in the CNS is the class of methyl-binding proteins. Methyl-binding proteins are continually expressed in the adult CNS and often act as repressors that recognize and bind to methylated cytosines (Nan *et al*, 1998; Ng *et al*, 1999; Sarraf and Stancheva, 2004). Hence, when methylation is removed as a result of neuronal activity, it is not surprising that MBDs are often released from promoters (Martinowich *et al*, 2003). However, the role of methyl-binding proteins is not this simple. Some

MBDs like MeCP2 undergo posttranslational modifications that alter their ability to bind to DNA (Zhou *et al*, 2006; Tao *et al*, 2009). Phosphorylation of MeCP2 is induced by neuronal activity and results in altered gene expression. When phosphorylation of MeCP2 is inhibited, synapse formation, synaptic plasticity, and learning and memory behavior are all affected (Cohen *et al*, 2011; Li *et al*, 2011a, b). As phosphorylation is normally a short-term modification, activity-dependent phosphorylation may temporarily release MeCP2 from promoters, making the gene sequence accessible for demethylation. On the other hand, DNA methylation and demethylation may be responsible for long-term changes in gene expression that regulate synaptic plasticity as well as learning and memory.

DNA Methylation in the Etiology of Neurological and Psychiatric Disorders

The pattern of DNA methylation established during development can be modulated by neural activity in order to encode learning and memory. When the mechanisms that establish and recognize the DNA methylation pattern are dysfunctional, problems with learning and memory frequently result. One of the most common forms of mental retardation, Rett Syndrome, is frequently caused by a mutation to the methyl-binding protein MeCP2 (Amir *et al*, 1999). The onset of symptoms at 6–18 months of age coincides with a time in early development when sensory experience is driving dendritic pruning and shaping connections in the brain (Samaco and Neul, 2011). Although *MeCP2* is expressed by the majority of cells, it is particularly important for normal neuronal function. In mice, loss of *MeCP2* in neurons is sufficient to recapitulate the majority of Rett symptoms (Chen *et al*, 2001; Guy *et al*, 2001). The phenotype of *MeCP2* mutant mice can be reversed by restoration of the *MeCP2* gene in postmitotic neurons (Luikenhuis *et al*, 2004; Giacometti *et al*, 2007; Guy *et al*, 2007). As previously mentioned, MeCP2 is regulated by neuronal activity and in turn regulates the expression of *BDNF*, which has enhanced expression following depolarization (Martinowich *et al*, 2003). The overexpression of *BDNF* in postmitotic neurons of MeCP2 mutant mice ameliorates their phenotype, suggesting that MeCP2 is critical for regulating the expression of genes like *BDNF* that are regulated by neuronal activity and essential for normal cognitive function (Chang *et al*, 2006). The role of MeCP2 in Rett Syndrome will be further discussed in later chapters.

One extremely rare neurodegenerative disease illustrates the importance of proper DNMT activity in the adult brain. Patients with hereditary sensory and autonomic neuropathy type 1 (HSAN1) develop dementia and hearing loss in adulthood that result from an autosomal-dominant mutation in the N-terminal regulatory domain of *DNMT1* (Klein *et al*, 2011). This mutation results in misfolding, impaired nuclear localization, and early degradation of DNMT1. However, the mutation does not affect the targeting of DNMT1 to the replication foci during cellular replication, but the DNMT1 association with heterochromatin beyond S phase is disrupted. This association may affect the maintenance of DNA methylation within these regions. Although there is only a modest 8% reduction of global DNA methylation level, neurodegeneration does result. The involvement of DNMT1 in the pathogenic mechanism of HSAN1 supports the necessity of DNMT1 in the adult brain.

Improper methylation of a single gene or a single allele can have drastic consequences within the brain. Fragile X Syndrome is caused by abnormal methylation of a trinucleotide repeat expansion in the *FMR1* gene on the X chromosome and is a common form of mental retardation (Verkerk *et al*, 1991; Turner *et al*, 1996). The hypermethylation in the repeat expansion of *FMR1* results in transcriptional silencing (Devys *et al*, 1993). Translation of the *FMR1* gene is regulated by neuronal activity (Weiler *et al*, 1997) and its protein product, FMRP, is involved in protein synthesis at the synapses following depolarization (for review, see Fatemi and Folsom, 2011). Similarly, improper methylation of a single imprinted allele, found in some disorders such as Prader–Willi Syndrome and Angelman Syndrome, can cause significant mental impairments (for review, see Buiting, 2010). As incorrect expression or loss of function of a single gene can have a dramatic effect in the brain, it is important to understand the mechanism of how DNA methylation affects gene expression.

DNA methylation can also be altered by repeated modulation of the microenvironment of the brain. In the case of recurrent seizures this microenvironment is repeatedly subject to unusual, synchronized neuronal activity. One way to mimic this unusual neural activity is by electric convulsive stimulation, which was found to result in genome-wide changes in the DNA methylation pattern (Ma *et al*, 2009; Guo *et al*, 2011a). Similarly, repeated drug usage modulates neuronal function as in the case of cocaine. Cocaine usage modulates *Dnmt3a* expression within the nucleus accumbens and enhances spine formation (LaPlant *et al*, 2010). Also, repeated cocaine usage increases *MeCP2* that, in turn, increases *Bdnf* expression (Im *et al*, 2010). Sometimes, drug exposure, like neural activity, can add posttranslational modifications to components of the methylation machinery such as MeCP2 (Deng *et al*, 2010; Hutchinson *et al*, 2012).

Although DNA methylation is clearly altered in the above disorders stemming from mutations, inappropriate methylation, or repeated modulation of the microenvironment, the role of DNA methylation in most psychiatric disorders is less clear. Yet, there is mounting evidence that altered patterns of DNA methylation are associated with many psychiatric disorders. For example, early-life stress in the form of maternal neglect was sufficient to alter DNA methylation in the brain of a rodent model (Weaver *et al*, 2004). Maternal neglect increased methylation within the promoter of the glucocorticoid receptor, thus reducing its expression. Surprisingly, this alteration in the DNA methylation pattern was retained into adulthood, leading to a heightened stress response. Similarly, in humans,

childhood abuse results in increased methylation of the promoter for the glucocorticoid receptor and a decrease in its expression, recapitulating the rodent model (McGowan *et al*, 2009). Furthermore, altered patterns of DNA methylation are observed in psychiatric patients diagnosed with schizophrenia and bipolar disorder (Mill *et al*, 2008).

FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

DNA methylation varies not only between tissues but also between brain regions, between gray matter and white matter, and possibly even between cells (Ladd-Acosta *et al*, 2007; Ghosh *et al*, 2010). Although current technology limits our ability to distinguish cell-specific methylation patterns, the advent of next-generation DNA sequencing has provided powerful tools to examine the genome-wide DNA methylation pattern with single-nucleotide resolution (Meissner *et al*, 2008; Lister *et al*, 2009; Popp *et al*, 2010). As technology improves, the cost of performing sequencing analysis will decline, thus making the technology more accessible. Recent technical developments have allowed for genome-wide DNA methylation analysis to be performed even with a sample amount as low as 150 ng (Popp *et al*, 2010). Aberrant DNA methylation patterns are observed in a wide variety of psychiatric and neurological illnesses. With declining costs and the ability to perform genome-wide methylation analysis on limited tissue quantities, it will soon be possible to map-out genome-wide DNA methylation patterns from distinct brain regions from patients with neurological and psychiatric disorders. The analysis of neural tissue from psychiatric patients will lead to new insights into the etiology of psychiatric illness and open up new avenues of drug discovery and targeted therapies.

Although current protocols enable scientists to precisely quantify DNA methylation at single-nucleotide resolution using progressively smaller tissue quantities, many of the most commonly used methods for profiling and quantification of DNA methylation, such as bisulfite sequencing and methylation-sensitive enzyme-based assays, are unable to distinguish between 5hmC and 5mC (Tahiliani *et al*, 2009; Huang *et al*, 2010). A few protocols are capable of distinguishing 5hmC from 5mC in the genome: CpG end-labeling followed by thin-layer chromatography (Tahiliani *et al*, 2009) and high-performance liquid chromatography (HPLC) with either UV detection (Liutkeviciute *et al*, 2009) or tandem mass spectrometry (Globisch *et al*, 2010; Le *et al*, 2011). Hydroxymethylated DNA can be enriched using antibodies that bind specifically to 5hmC or by biotinylation of modified 5hmC and precipitated sequences can be identified using microarray chips or by DNA sequencing (Szwagierczak *et al*, 2010; Ficz *et al*, 2011; Jin *et al*, 2011; Pastor *et al*, 2011; Wu and Zhang, 2011). Although these methods can quantify 5hmC and identify DNA sequences with which it is associated, single base-pair resolution has not been attained. In order to clarify the genomic distribution and the epigenetic role of 5hmC in the brain, a locus-specific method of identifying 5hmC will need to be developed.

As other high-throughput techniques, including RNA and chromatin immunoprecipitation (ChIP) sequencing, become more accessible to researchers, there is a growing need to integrate high-throughput data. Currently, DNA methylation, histone modification, and miRNA are studied in relative isolation. In order to fully understand how gene expression is regulated within the nervous system, future research must consider the epigenome as a whole. By dissecting the biological mechanisms that mediate crosstalk among these biological mechanisms and integrating high-throughput data, we can begin to study the epigenome as a whole. Finally, for a complete understanding of how the epigenome regulates gene expression, future research will have to uncover the biological mechanisms that mediate activity-dependent changes in the epigenomic landscape of the mammalian brain.

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DISCLOSURE

The authors declare no conflict of interest.

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