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Active DNA Demethylation in Post-Mitotic Neurons: A Reason for Optimism

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Abstract

Over the last several years proteins involved in base excision repair (BER) have been implicated in active DNA demethylation. We review the literature supporting BER as a means of active DNA demethylation, and explain how the various components function and cooperate to remove the potentially most enduring means of epigenetic gene regulation. Recent evidence indicates that the same pathways implicated during periods of widespread DNA demethylation, such as the erasure of methyl marks in the paternal pronucleus soon after fertilization, are operational in post-mitotic neurons. Neuronal functional identities, defined here as the result of a combination of neuronal subtype, location, and synaptic connections are largely maintained through DNA methylation. Chronic mental illnesses, such as schizophrenia, may be the result of both altered neurotransmitter levels and neurons that have assumed dysfunctional neuronal identities. A limitation of most current psychopharmacological agents is their focus on the former, while not addressing the more profound latter pathophysiological process. Previously, it was believed that active DNA demethylation in post-mitotic neurons was rare if not impossible. If this were the case, then reversing the factors that maintain neuronal identity, would be highly unlikely. The emergence of an active DNA demethylation pathway in the brain is a reason for great optimism in psychiatry as it provides a means by which previously pathological neurons may be reprogrammed into a more favorable role. Agents targeting epigenetic processes have shown much promise in this regard, and may lead to substantial gains over traditional pharmacological approaches.

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Key words: CpG methylation; 5-methylcytosine; DNA methyltransferase; 5-hydroxymethylcytosine; TET; GADD45

Abbreviations: 5CaC, 5-carboxylcytosine; 5FC, 5-formylcytosine; 5HMC; 5-hydroxymethylcytosine; 5HMU, 5-hydroxymethyluracil; 5MC, 5-methylcytosine; AICDA, activation-induced cytidine deaminase; APE1, Apurinic/apyrimidinic (AP) endonuclease; APOBEC, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like; BDNF, brain-derived neurotrophic factor; BER, base excision repair; COMT, Catechol-O-methyltransferase; CpG, cytosine-phosphate-guanine; DNMT, DNA methyltransferase; ECS, electroconvulsive seizure; FGF, fibroblast growth factor; G, guanine; GADD45, Growth Arrest and DNA Damage; H3K9me2, dimethylated lysine 9 of histone 3; H3K27, lysine 27 of histone 3; HDAC, histone deacetylase; LSD1, lysine-specific demethylase-1; KCl, potassium chloride; MBD, methyl-CpG binding domain; MeCP2, methyl CpG binding protein 2; NER, nucleotide excision repair; NMDA, N-Methyl-D-aspartic acid; NR2B, N-methyl D-aspartate receptor subtype 2B; PARP1, poly ADP ribose polymerase-1; PFC, prefrontal cortex; PGC, primordial germ cells; PP1, protein phosphatase 1; T, thymidine; TDG, thymine-DNA glycosylase; TET, ten-eleven translocation; SMUG1, single-strand selective monofunctional uracil-DNA glycosylase 1; XRCC1, X-ray Repair Cross-Complementing protein-1

1. Introduction

Approximately 70-80% of cytosines in CpG dinucleotides are methylated in human somatic cells. Generally, CpG methylation in gene promoter or enhancer regions is associated with repressed transcription (Razin et al., 1980), while gene body methylation is positively correlated with gene expression (Laurent et al., 2010; Lister et al., 2009; Rauch et al., 2009). This highly stable covalent modification to DNA in mammals produces potentially lifelong changes in gene expression making it integral to maintaining stable cellular identities When a CpG dinucleotide is not methylated on either DNA strand the de novo DNA methyltransferases, DNMT3a and DNMT3b, are primarily responsible for adding a methyl group. When one strand is already methylated the complementary strand is methylated via a maintenance DNMT, primarily DNMT1 (Sharma et al., 2010). While the factors that guide a DNMT to a specific CpG have not been fully elucidated, it is generally believed that DNMTs are recruited through their interactions with transcription factors, chromatin proteins (see section 3 below), as well as other proteins. In cultured cell lines, transcription factors shown to promote the targeting of DNMTs include DNMT1 and 3a by PML-RAR (Di Croce et al., 2002) and DNMT1 by Daxx (Muromoto et al., 2004) and E2F6 (Velasco et al., 2010).

DNA methylation gene silencing has been shown to be important not only for maintaining cellular subtypes, but also contributes to sustaining functional identities, including stabilizing neuronal interactions (Bird, 2002; Cortese et al., 2011; De Carvalho et al., 2010; Deaton et al., 2011; Iwamoto et al., 2011; Levenson et al., 2006). This is best exemplified by its contribution to long-term memory. DNA methylation silencing of the memory suppressor gene, protein phosphatase 1 (PP1), in combination with reduced

methylation of the synaptic plasticity gene reelin have been shown to be associated with new memory formation (Miller et al., 2007). The same group has also shown that cortical DNA methylation changes at specific genes are associated with consolidating and maintaining memory, and that established memories can be weakened using DNMT inhibitors (Miller et al., 2010). Similarly, DNMT1 and DNMT3a double knockout mice have been shown to have deficits in learning and memory (Feng et al., 2010), while restoration of DNMT3a2 levels in aged mice leads to improved memory (Oliveira et al., 2012). **Based on these studies it appears in general DNA methylation changes are important for memory formation. However, it has not yet been determined which methylation changes at specific genomic loci are functionally involved in memory formation and which are passive events perhaps not even affecting gene transcription.** Further, while neurotransmitters largely constitute the message neurons communicate to each other the neurons to which they communicate may be largely hard-wired using DNA methylation and histone modifications (Sharma et al., 2012b). With few exceptions all current psychopharmacological agents target neurotransmitter levels or their receptors and not these more profound factors. In mental disorders, such as schizophrenia, presumably there are abnormalities in both domains.

Without the existence of active DNA demethylation neurons once misprogrammed could never be reprogrammed as the DNA methyl mark can persist across a lifetime as well as be passed to subsequent generations (Anway et al., 2006; Heijmans et al., 2008; Morgan et al., 1999; Rakyan et al., 2003). The potential to pharmacologically alter this code would then be minimal due to the stability of the covalent bonds that link methyl groups to cytosines. The importance of recent insights

into the DNA demethylation pathway cannot be understated as this raises the possibility that neurons containing a gene expression profile that contributes to mental illness encoded by DNA methylation can be reprogrammed using epigenetic tools.

2. DNA demethylation

Until recently the existence of active DNA demethylation remained somewhat controversial (Ooi et al., 2008). While it was known that DNA can become demethylated passively as cells divide, when a maintenance DNMT does not act prior to cell division, active removal of methyl groups from cytosine or removal of the 5-methylcytosine (5MC) molecule itself was thought to be a rare occurrence (Ooi et al., 2008). However, there are several reasons, beyond recent direct evidence, that suggest the existence of a demethylation pathway. First, the abundance of DNMTs in nondividing cells, including neurons implies a DNA demethylation pathway (Sharma et al., 2008b). If not for active DNA demethylation in neurons high levels of DNMT1 and DNMT3a would be expected to lead to progressive accumulation of DNA methylation as animals age, which we now know is not the case (Numata et al., 2012). Secondly, genes silenced in nondividing cells through DNA methylation can be reactivated. For example, activity-dependent demethylation has been shown in neurons as has wide-spread non-mitotic dependent demethylation in early mammalian embryos (Chen et al., 2003; Martinowich et al., 2003; Reik et al., 2001). Finally, DNMT inhibitors or knockout of DNMTs in post-mitotic neurons have been shown to decrease DNA methylation (Feng et al., 2010; Levenson et al., 2006; Miller et al., 2007). In cultured mouse neurons 5-azacytidine was shown to reduce methylcytosine levels at the N-methyl D-aspartate receptor subtype 2B (NR2B)

and a Brain-Derived Neurotrophic Factor (BDNF) promoter (Marutha Ravindran et al., 2005; Nelson et al., 2008). In the latter study the authors demonstrate that this DNMT inhibitor-induced demethylation was dependent on basal excitatory synaptic activity (Nelson et al., 2008). In other words, while it is not clear whether DNMT inhibitors can induce demethylation in a completely inactive cell, in the brain where there is constant spontaneous neuronal electrical activity DNMT inhibitors are capable of reducing DNA methylation without training. **However, it should be stated that in the mouse brain neither the use of DNMT inhibitors nor in DNMT1 and DNMT3a double-knockouts is there an increase in reelin and PP1 expression** (Feng et al., 2010; Miller et al., 2007)**.** Considering the stability of the covalent bond linking a methyl group to a cytosine residue, simply inhibiting new 5MC formation would not be expected to significantly decrease its levels without the existence of a demethylation mechanism. Further, many DNMT inhibitors are cytosine analogs which act by incorporation into DNA where they trap DNMTs, thereby inhibiting their enzymatic activities (Liu et al., 2003; Schermelleh et al., 2005; Stresemann et al., 2008)**.** It was recently shown that cytosine analogs can be incorporated into the genome of non-dividing cells (Yamagata et al., 2012). The fact that cytosine analogs are incorporated into DNA in nondividing cells at a high enough rate to substantially trap DNMTs implies a mechanism of demethylation that involves replacing entire cytosine bases, such as a base excision repair (BER) process rather than removing methyl groups from cytosines. In the brain, DNA demethylation has been shown to be induced through activity-dependent processes, such as electroconvulsive seizure (ECS) and learning paradigms (Guo et al., 2011; Lubin et al.,

2008; Ma et al., 2009a). Hypermethylated GABAergic gene promoters can be reduced using histone deacetylase (HDAC) inhibitors (Dong et al., 2010).

Several mechanisms of DNA demethylation have been proposed over the last several decades. These include a pathway in which RNA is a key component, one in which the methyl group is removed from 5MC forming an unmethylated cytosine and methanol, and a nucleotide excision repair (NER) based pathway (Barreto et al., 2007; Bhattacharya et al., 1999; Zhu et al., 2000a; Zhu et al., 2000b). While controversies and lack of replication of earlier studies have slowed progress, there has been a consistent accumulation of studies implicating members of a BER pathway in DNA demethylation making this a promising pathway for further investigation (Jin et al., 2008; Wu et al., 2010).

3. DNA methylation and histone modifications

Restrictive epigenetic changes such as DNA methylation and histone modifications have been implicated in a variety of mental disorders. The process of chromatin condensation or 'heterochromatization' involves the accumulation of certain histone modifications, such as methylated lysines 9 (H3K9) and 27 (H3K27) of histone 3, and DNA CpG methylation at a gene promoter leading to reduced gene expression. These histone modifications and the enzymes that catalyze their formation are known to interact both directly and indirectly with DNA methyltransferases (DNMT) and methyl-CpG binding proteins (MBD), whereby the factors catalyzing the formation of restrictive histone marks promote increased DNA methylation and vice versa forming a heterochromatization positive feedback loop. The histone protein H1d recruits DNMT1 and 3b, the H3K9

histone methyltransferase (HMT) SUV39H1 recruits DNMT3a and another H3K9 HMT, G9a, recruits DNMT3a and 3b (Epsztejn-Litman et al., 2008; Fuks et al., 2003). Heterochromatin protein 1 (HP1) has been shown to be necessary for DNMT recruitment to specific loci as well (Fuks et al., 2003; Yang et al., 2013). On the other hand, MBDs MeCP2 and MBD2 are capable of recruiting histone deacetylases (HDAC) to a heavily DNA methylated region (Jones et al., 1998; Nan et al., 1998). This leads to a reduction of the 'open' histone acetyl mark. In addition, DNMT1 can directly interact with G9a to form more restrictive chromatin (Esteve et al., 2006). DNMTs can also bind to the H3K27 methyltransferases (Vire et al., 2006). Other direct interactions between DNMTs and histone modifying enzymes are known to exist as well (Fuks et al., 2003; Li et al., 2006).

While the process of forming 'constitutive' heterochromatin or the less transcriptionally repressive 'facultative' heterochromatin have been described in the literature there has been very little focus on the reversal of this state (Sharma et al., 2012b). Considering many mental disorders are characterized by local or global epigenetic abnormalities a better understanding of the components responsible for the alleviation of restrictive chromatin may indicate novel targets for promoting endogenous mediators that convert heterochromatin to transcriptionally facilitative euchromatin.

4. Base excision repair (BER)

In plants, active DNA demethylation is a well-characterized process in which the accepted mechanism involves BER (Gehring et al., 2009)**.** Over the last four years there is increasing support that a similar BER pathway is involved in active DNA

demethylation in animals as well. Recent studies indicate that in the adult mouse brain and in embryonic stem cells 5MC is first oxidized to form 5-hydroxymethylcytosine (5HMC) catalyzed by ten-eleven translocation (TET) enzymes (Guo et al., 2011; Ito et al., 2010; Koh et al., 2011). These TET enzymes may then further oxidize 5HMC to sequentially form 5-formylcytosine (5FC) and 5-carboxylcytosine (5CaC) (Ito et al., 2011). Alternatively to the formation of 5FC and 5CaC, 5MC or 5HMC can be deaminated by cytidine deaminases such as, activation-induced cytidine deaminase (AICDA) or apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like protein (APOBEC), forming either a thymidine (T) or a 5-hydroxymethyluracil (5HMU) base (Guo et al., 2011). The resultant mismatches, whether 5CaC:G, T:G or 5HMU:G are removed through a process involving thymidine or uracil glycosylases (Guo et al., 2011; He et al., 2011; Matsubara et al., 2004). However, it has also been suggested that 5FC and 5CaC may be converted directly back to unmethylated cytosine via the expulsion of formic acid or decarboxylation, respectively (**Fig. 1**) (Ito et al., 2011).

4.1. TET

TET participates in DNA demethylation by catalyzing the formation of 5HMC **(Fig. 2)** (Ito et al., 2010). Evidence for the role of TET in DNA demethylation has emerged from the developmental and neuroscience literature. Despite the fact that the TET family of enzymes can all convert 5MC to 5HMC the three isoforms share just 20% protein sequence identity. The differences in terms of function have yet to be fully elucidated. However, there is evidence that TET3 is more important in early embryonic development, as TET3 knockout mice are not viable (Gu et al., 2011). This is likely due

to the fact that TET3 is the only TET significantly expressed in the zygote (Wossidlo et al., 2010). TET1 and TET2 **knockout** mice are viable and fertile (Dawlaty et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011), although TET1-knockout mice have reduced body mass and smaller litter size (Dawlaty et al., 2011). There is also evidence that TET proteins may differ in the sequences they target. In chimeric B-cell-embryonic germ cells TET1 was necessary for reprogramming mature somatic cell imprinted control regions and TET2 was more broadly necessary for reactivating 'stemness' genes, such as OCT4 (Piccolo et al., 2013).

In the cortex it has been demonstrated that TET3 is the most highly expressed followed by TET2, although TET1 is also detectable (Hahn et al., 2013). In the hippocampus, mice with reduced levels of TET1 are incapable of demethylating BDNF and Fibroblast Growth Factor (FGF) following ECS (Guo et al., 2011), indicating the necessity of TET in activity-dependent DNA demethylation.

5HMC was first reported in 1952 in bacteriophages (WYATT et al., 1952). Its importance in neuroscience is indicated by the fact that in the brain 5HMC accounts for 10-40% of modified cytosines (Szulwach et al., 2011). Generally, 5HMC is enriched in exons, including in the brain (Jin et al., 2011). This differs from 5MC which is present at high levels globally, except at gene promoters and CpG islands (Lister et al., 2009). Experiments in vitro and using embryonic stem cells indicate that promoter 5HMC is associated with reduced expression and bivalent histone domains (Pastor et al., 2011; Robertson et al., 2011; Xu et al., 2011), while when located in the gene body there appears to be an association with increased expression (Pastor et al., 2011; Xu et al., 2011). In the brain, 5HMC enrichment at promoters was not associated with increased

transcription, but was when located in the gene body (Jin et al., 2011; Mellen et al., 2012). In various neuronal subtypes however, there may be differences in regards to the degree to which 5HMC is associated with increased expression. One factor to account for these differences among neurons has been proposed to be the amount of MeCP2 binding (Mellen et al., 2012). Prior in vitro data and experiments using embryonic stem cells indicated that 5HMC is incapable of binding certain MBD proteins, including MeCP2, leading to decondensed chromatin and increased gene expression (Ficz et al., 2011; Jin et al., 2010; Valinluck et al., 2004). However, Mellen et al. recently demonstrated that MeCP2 is the main 5HMC binding protein in neurons and when bound leads to increased transcription (Mellen et al., 2012). Further indications of this pro-transcriptional role is the fact that 5HMC is an intermediate step in the removal of 5MC (Guo et al., 2011).

4.2. Cytidine Deaminases

The cytidine deaminase family of related proteins that include AICDA (also known as AID) and APOBECs, were originally identified as RNA editors (Conticello, 2008). Cytidine deaminases are responsible for removing amine groups from both 5MC and 5HMC resulting in the formation of thymidine or 5HMU, respectively **(Fig. 2)** (Guo et al., 2011; Morgan et al., 2004)**.** In the adult mouse brain, APOBEC1 has an essential role in demethylating BDNF IXa and FGF1b following ECS (Guo et al., 2011). In the human parietal cortex, only APOBEC3A, APOBEC3C and APOBEC4 are detectable at significant levels (Dong et al., 2012). Whether these three enzymes are primarily responsible for deamination of 5HMC in the human cortex remains to be clarified.

In 2008 two studies were published indicating cyclical methylation/demethylation in part mediated by DNMTs, DNMT3a and DNMT3b (Kangaspeska et al., 2008; Metivier et al., 2008). Metivier et al. suggest that the role of DNMTs in DNA demethylation is as cytidine deaminases when donor methyl concentration is low. They further hypothesize that these enzymes promote demethylation when in association with TDG, but promote methylation under other conditions. However, the authors could not exclude that DNMT3a/3b recruit other deaminases and do not themselves participate directly in the demethylation process.

4.3. Thymidine glycosylases

The DNA glycosylases implicated in the BER pathway include MBD4, thymine-DNA glycosylase (TDG) and single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1) (Cortellino et al., 2011; Guo et al., 2011; Rai et al., 2008). Thymidine or uracil glycosylases are thought to participate in base excision repair DNA demethylation by removing thymidine or 5HMU from the T:G or 5HMU:G mismatch that results from deamination of 5MC or 5HMC, respectively **(Fig. 2)**. TDG, SMUG1, and MBD4 are all capable of removing 5HMU in a 5HMU:G mismatch (Cortellino et al., 2011; Hashimoto et al., 2012). However, it should be noted that SMUG1 knockouts are incapable of 5HMU removal in the brain (Kemmerich et al., 2012). In cultured cells lacking TDG, genes involved in embryogenesis and development are the most significantly misregulated (Cortazar et al., 2011). These findings may suggest that while TDG is more important developmentally, SMUG1 serves a more prominent role in demethylation in the adult brain.

4.4. GADD45s

The Growth Arrest and DNA Damage 45 (GADD45) family of proteins, GADD45a, b, and g, are a group of small acidic non-enzymatic proteins of ~18 kDa in size, with a 67% protein sequence homology. GADD45a and GADD45b have both been repeatedly implicated in DNA demethylation (Barreto et al., 2007; Cortellino et al., 2011; Ma et al., 2009b; Rai et al., 2008; Schmitz et al., 2009), while to our knowledge there have been no studies indicating whether GADD45g shares this ability. GADD45 proteins may be the lynchpins of the demethylation process in that they bind to and focus the enzymatic activities of cytidine deaminases and thymidine glycosylases to specific gene promoters. In co-immunoprecipitation experiments a direct physical interaction has been demonstrated between AICDA, TDG and GADD45a in cultured cells (Cortellino et al., 2011), while in zebrafish embryos a similar tripartite protein complex consisting of AICDA or APOBEC, MBD4 and GADD45a has been documented (Rai et al., 2008). Of note, GADD45 proteins are known to bind to nuclear hormone receptors (Yi et al., 2000) and preferentially to hyperacetylated nucleosomes (Carrier et al., 1999), suggesting that GADD45 proteins may also be involved in targeting promoters for demethylation.

In neuronal cultures GADD45b and GADD45g, but not GADD45a expression are increased after depolarization with either NMDA or KCl (Ma et al., 2009b; Sultan et al., 2012). In vivo, GADD45b is induced in the brain by seizures, whether kainite-induced or via ECS (Hevroni et al., 1998; Lemberger et al., 2008; Ma et al., 2009b; Nedivi et al., 1993), and after long-term potentiation (Hevroni et al., 1998). In behavioral models GADD45b and GADD45g, but again not GADD45a, are increased in the

amygdala after fear conditioning and in the hippocampus by both context exposure learning and fear conditioning (Keeley et al., 2006; Leach et al., 2012; Sultan et al., 2012).

Recently, two papers have been published in regards to the role of GADD45b in the hippocampus. Leach et al. published an article demonstrating that GADD45b knockout mice have deficits in hippocampus-dependent memory based on contextual fear conditioning experiments 24 hours, but not 1 hour after initial training (Leach et al., 2012). These findings are in accord with data indicating that GADD45b is a positive regulator of neurotrophic factors such as BDNF, FGF, and neurogenesis, in the hippocampus as a result of its role in active demethylation (Ma et al., 2009b). In contrast, Sultan et al. found that GADD45b deficient knockouts perform better on measures of hippocampus-dependent contextual fear conditioning after 24 hours that persisted at 28 days compared to wild-type littermates (Sultan et al., 2012). Further, their data suggest that this is not the result of compensation by other GADD45 proteins, as GADD45a and GADD45g are similarly expressed in knockout mice and increase comparably in neuronal cultures treated with KCl from wild-type and GADD45b knockouts. Their conclusion is that GADD45b may be a negative regulator of memory perhaps by demethylating memory suppressor gene promoter regions, thereby increasing the expression of genes such as PP1 and calcineurin (Sultan et al., 2012). The reasons for the opposing observations regarding GADD45b and hippocampus-dependent learning are not clear. Sultan et al. suggest several possibilities, such as background strain differences, differences in housing or training facility environments, or differences in training paradigms (Sultan et al., 2012).

4.5. Downstream BER Proteins

After the pyrimidine base (whether 5HMU or T) is excised by a thymidine glycosylase, Apurinic/apyrimidinic (AP) Endonuclease-1 (APE1, also known as APEX1), one of the major mammalian AP endonucleases cleaves the DNA backbone immediately 5' of the now abasic site (Madhusudan et al., 2005). The APE1 catalyzed DNA strand-break is a strong inducer of Poly ADP Ribose Polymerase-1 (PARP1), stimulating its enzymatic activity several hundred fold resulting in PARP1 dimerization at the break site (Jorgensen et al., 2009; Langelier et al., 2012; Mendoza-Alvarez et al., 1993). PARP1 plays several critical roles in DNA demethylation, including: 1) Inducing the expression of TET1; 2) Inhibiting DNMT1 expression; 3) Inhibiting DNMT1 enzymatic activity by preferentially binding PARP1 rather than DNA; 4) Catalyzing the addition of Poly(ADP-Ribose) polymers (PARs) groups to histone proteins (referred to as PARylation) thereby lessening their affinity for DNA; and 5) Recruiting the scaffold protein X-ray repair cross-complementing protein-1 (XRCC1) (Beneke, 2012; Ciccarone et al., 2012; Reale et al., 2005; Zardo et al., 2002). At the apyrimidinic site PARylation facilitates XRCC1 forming a complex with DNA ligase 3α, DNA polymerase β, and other proteins (Beneke, 2012). This complex replaces the missing nucleotide with an unmethylated cytosine.

PARP1 is abundantly present in neurons and glia (Strosznajder et al., 2010), and is required for long-term neuronal plasticity in Aplysia and mice (Cohen-Armon et al., 2004; Goldberg et al., 2009; Hernandez et al., 2009). The kinetics of the PARylation process are consistent with its role in activity-dependent processes in the brain. It was shown that ethanol can increase nuclear PAR group levels in cultured neurons within 15

minutes, its protein expression increases within 4 hours, and PARylation peaks at 6-8 hours (Cherian et al., 2008). Therefore, it is possible that PARP1 has a role in these rapid processes. It should be stated that of the four DNA demethylation mechanisms PARP1 participates in some would likely be rapid and some would be long-term ways of facilitating demethylation. For example, inducing TET1 or reducing DNMT1 expression would likely be more delayed effects while PARylating proteins is rapid. Despite the evidence that PARP1 has a role in DNA demethylation it has been reported that PARP1 inhibition does not prevent the KCl-induced increase in BDNF IV in cultured neurons (Chang et al., 2010). However, this is just one gene and may not be reflective of the overall role of PARP1 across the genome. Further studies aimed at testing whether PARP1 inhibition can prevent DNA demethylation in activity-dependent processes in neurons are necessary to support its role in this process.

5. Schizophrenia and DNA Demethylation

In a genomewide experiment abnormal DNA methylation networks have been demonstrated in psychotic patients (Mill et al., 2009). Also, a wide variety of DNA methylation alterations at individual gene promoters has been reported, including GAD1, reelin, BDNF, Catechol-O-methyltransferase (COMT), and SOX10 (Abdolmaleky et al., 2006; Guidotti et al., 2011; Iwamoto et al., 2005). We recently reported that the reduction of BDNF expression in the parietal cortex of schizophrenia patients was associated with an increase of 5HMC and 5MC at a BDNF promoter (Gavin et al., 2012). BDNF is a neurotrophic factor involved in synaptic plasticity inducible by activity dependent processes and is highly epigenetically regulated (Roth et al., 2009; Tian et al., 2010). The

reduction in the expression of this neuroplasticity gene in schizophrenia has been previously reported by several labs (Thompson Ray et al., 2011; Zhang et al., 2010). Furthermore, the schizophrenia candidate gene involved in synaptic plasticity, reelin, may be downregulated in schizophrenia perhaps via increased promoter DNA methylation (Abdolmaleky et al., 2005; Fatemi et al., 2005; Grayson et al., 2005). However, inconsistent results from postmortem studies (Dempster et al., 2006; Lintas et al., 2010; Mill et al., 2008; Tochigi et al., 2008) suggest that the DNA methylation status of a specific promoter is the result of a dynamic equilibrium resulting from the summation of DNMT activity and DNA demethylation factors (Dong et al., 2010; Szyf, 2010).

Many of the initial studies of epigenetic changes in psychosis and schizophrenia indicated an increase in either restrictive chromatin marks, such as DNA methylation or histone modifications, or increases in the enzymes that catalyze the reactions that lead to their formation. Increases in DNMTs have been reported in the prefrontal cortex (PFC) as well as patient peripheral blood cells (Guidotti et al., 2011; Veldic et al., 2005; Veldic et al., 2007; Zhubi et al., 2009). Similarly, increases in restrictive histone modifications have been reported in peripheral blood cells as well as increases in certain enzymes that catalyze their formation, such as HDACs in the brain (Benes et al., 2007; Gavin et al., 2008; Gavin et al., 2009a; Gavin et al., 2009b; Gavin et al., 2009c; Sharma et al., 2008a). Now that some of the components of the DNA demethylation pathway have begun to be discovered it is somewhat surprising that many of these components are elevated in psychosis.

GADD45b, GADD45g, MBD4, TET1, and APE1 have all been reported to be elevated in psychotic illnesses compared to nonpsychiatric controls. Elevated GADD45g was the first of these genes reported to be increased in the PFC of patients with schizophrenia (Arion et al., 2007). Subsequently, Benes et al. reported a significant increase in the expression of MBD4 in GABA cells in hippocampal regions CA3/2 and CA1 both in patients with schizophrenia and with bipolar disorder, and APE1 in CA1 of patients with bipolar disorder (Benes et al., 2009). More recently, two articles from our group specifically aimed at examining the DNA demethylation pathway in psychosis have indicated increased TET1 and GADD45b in parietal cortices, and GADD45b in the PFC (Dong et al., 2012; Gavin et al., 2012). In a reanalysis of publicly accessible datasets, the National Brain Databank: Brain Tissue Gene Expression Repository of Harvard Brain Tissue Resource Center (Harvard) and the Scripps Research Institute dataset (GEO accession GSE21138) (Scripps), we find a fairly consistent increase in this pathway as well. For example, comparing nonpsychiatric controls to patients with schizophrenia reveals an increase in GADD45b and GADD45g in both the Harvard and Scripps datasets in the PFC in schizophrenia, an increase in AICDA, APOBEC3A, APOBEC3F, and TET3 in the Harvard dataset in the PFC, and an increase in APOBEC3B, APOBEC3G, and MBD4 in the PFC in the Scripps dataset (**Fig. 3**). It should be stated that increases in demethylating genes in schizophrenia are not entirely consistent. For example, Benes et al. found a decrease in PARP1 expression in the hippocampus of bipolar patients, Dong et al. reported decreased APOBEC3A and 3C in the parietal cortex, and we did not find a difference in MBD4 expression in the parietal cortex of psychotic subjects from the Stanley Neuropathology Consortium (Benes et al.,

2006; Dong et al., 2012; Gavin et al., 2012). Regardless, the tendency for increases in these factors across several cohorts and laboratories indicates a potential pathway abnormality that warrants further investigation.

Several possibilities exist to explain the increase in BER gene expression. First, it is possible that select areas of the genome are hypomethylated in schizophrenia perhaps without an accompanying increase in gene expression. Indications of this come from the study of the GAD1 gene, a reduction of which is perhaps the most highly replicated finding in schizophrenia molecular biological research (Akbarian et al., 2006; Guidotti et al., 2000; Huang et al., 2007; Straub et al., 2007; Thompson Ray et al., 2011). Huang et al. documented that areas of the GAD1 promoter isolated based on the presence of a restrictive histone modification have decreased methylated DNA associated with decreased GAD1 expression (Huang et al., 2007). Second, the increase in the BER pathway may be in reaction to increases in DNA methylating enzymes that have been reported in psychosis, such as DNMTs. Third, this could be accounted for by an impediment in the DNA demethylation process. A functional failure is perhaps evidenced by fewer single strand DNA breaks, a hallmark of the BER process, in anterior cingulate cortices of patients with schizophrenia (Benes et al., 2003). It is possible that restrictive histone modifications are responsible for impeding access of GADD45b or other members of the BER pathway to DNA. This is supported by our observation that GADD45b is significantly less bound to the BDNF IX promoter and associated with increased promoter methylation in psychotic subjects compared to controls despite increased GADD45b expression (Gavin et al., 2012). Another possibility is based on the observation that many of the components of the BER pathway are reactive to cellular

stress outside of their role in DNA demethylation. Given the overall poorer health, and potential impacts of medications, neurons of psychotic subjects may be under increased genotoxic stress, which could increase expression of members of this pathway as well. Finally, Dong et al. reported that APOBEC3A and 3C are reduced in psychosis (Dong et al., 2012). If the block in this pathway lies in a cytidine deaminases deficiency, then this may induce the expression of other BER members through a feedback loop.

6. Utilizing DNA demethylation to Reprogram Neurons

Aberrant connections maintained by restrictive histone modifications and DNA methylation have been hypothesized to contribute to the maintenance of psychopathology (Guidotti et al., 2011; Sharma et al., 2012b). One means of reversing this state could be accomplished by inhibiting the participants of the transcription silencing process of heterochromatization (Sharma et al., 2012b). However, merely inhibiting the spread of restrictive chromatin cannot alone reactivate previously silenced genes. This is evidenced by the observation that inhibiting the enzyme that catalyzes the formation of the restrictive histone modification dimethylated lysine 9 of histone 3 (H3K9me2) using BIX-01294 increases not decreases DNA methylation in cultured fetal artery cells (Yang et al., 2012). This indicates that while the spreading and seeding of heterochromatin can be attenuated by preventing a particular restrictive chromatin mark it may not necessarily lead to the induction of an open chromatin state. Therefore, it becomes necessary to also understand the pathways responsible for reversing heterochromatin or 'euchromatization.' This opening of chromatin likely involves the cooperation of

proteins responsible for DNA demethylation in conjunction with other proteins capable of reversing repressive histone marks.

Although it remains unclear what the initial steps are for reversing restrictive chromatin the progression from heterochromatin to euchromatin can be inferred from several findings. Likely, one of the initial steps is the removal of methyl groups from histone lysine residues such as H3K9. This can be deduced from the fact that H3K9 methylation and acetylation are mutually exclusive. In vitro experiments indicate that H3K9 demethylases can completely remove methyl groups from synthetic histone peptides within 30 minutes (Horton et al., 2010). In neuronal culture studies depolarization was found to cause a significant reduction of H3K9me2 at 90 minutes. At this same time point there was a significant increase in the amount of H3K9 acetylation (Chen et al., 2003). Once this H3K9 methylation/acetylation switch is 'flipped' transcription factors are first able to bind (Thomassin et al., 2001). Transcription itself promotes DNA demethylation. This may be the result of transcription factors and nuclear hormone receptors recruiting GADD45 and TDG proteins to gene promoter regions (Cortellino et al., 2011; Yi et al., 2000). Histone acetylation also promotes DNA demethylation through several different mechanisms (Thomassin et al., 2001). First, in cell lines expressions of all three GADD45 genes are induced by increases in histone acetylation (Campanero et al., 2008; Chen et al., 2002; Della Ragione et al., 2001; Hirose et al., 2003), and GADD45 proteins have been shown to bind to acetylated histones more avidly than nonacetylated histones (Carrier et al., 1999). TDG has been shown to directly interact with histone acetyltransferases (Tini et al., 2002), and PARP1 is activated by histone acetylation (Hassa et al., 2005; Masson et al., 1997; Messner et al., 2009; Ryu et

al., 2010). Once activated PARP1 can PARylate histone protein amino acid residues such as H2AK13, H2BK30, H3K27, H3K37 and H4K16 (Messner et al., 2010). The combination of the charge neutralizing effects of histone acetylation with histone PARylation which does not merely neutralize the positive charge of the amino-acid side chain, but in fact reverses it into a negative charge weaken histone-DNA interactions thereby facilitating the DNA demethylation process (Ramanathan et al., 1989). Following DNA demethylation additional transcription factors are then able to bind. The demethylated site provides a memory of the original stimulus, allowing for subsequent induction of gene expression to progress stronger and more rapidly (Kress et al., 2001). The removal of 5MC also allows for H3K4 methyltransferase binding. The prerequisite of demethylated DNA is based on the observation that H3K4 methyltransferases, such as MLL proteins selectively bind unmethylated CpGs (Allen et al., 2006; Ayton et al., 2004; Birke et al., 2002). In addition to allowing for transcription, H3K4 methylation may immunize local DNA from being rapidly remethylated as DNMTs (specifically DNMT3L and DNMT3a) are repelled by H3K4 methylation (Ciccone et al., 2009; Nan et al., 1998; Ooi et al., 2007; Thomson et al., 2010). H3K4 methylation is facilitated by PARP1 which through PARylation inhibits the recruitment of one of its demethylases, KDM5B (Krishnakumar et al., 2010).

Clearly, several steps in euchromatization are not fully understood. For example, how TET can bind 5MC and convert it to 5HMC in association with bivalent histone marks which include methylated H3K4, when the H3K4 methyltransferase is only able to bind locations where there is no 5MC is still under active investigation (Matarese et al., 2011; Pastor et al., 2011). Moreover, the role of H3K27 demethylation in this process

has not been fully elucidated (McGarvey et al., 2006). Further studies to clarify the sequence of events are still required (**Fig. 4**).

Considering the bidirectional relationship between 'open' histone modifications and DNA demethylation the question becomes how this connection can be exploited to relieve the restrictive chromatin state that may be present in some psychiatric disorders. Normal reprogramming that takes place in vivo during the formation of primordial germ cells (PGCs) or in the early post-fertilization embryo is heavily reliant upon DNA demethylation (Hajkova et al., 2008; Kafri et al., 1992). Somatic cells can be reprogrammed into induced pluripotent stem cells through the overexpression of 'stemness' genes OCT4, SOX2, KLF4 and c-MYC (Takahashi et al., 2006). The overexpression of these four genes is known to lead to the DNA demethylation of other downstream stemness genes, such as NANOG and ESRRB (Doege et al., 2012; Hajkova et al., 2008; Kafri et al., 1992). In nondividing heterokaryons consisting of fused mouse embryonic stem cells with human fibroblasts rapid DNA demethylation occur at the OCT4 and NANOG promoters, accompanied by their transcriptional induction.

Although differentiated cell reprogramming can be accomplished using heterokaryons or transfected 'stemness' genes, these methods are clearly not practical in a clinical setting. Moreover, if one's intention is not induction of a toti- or pluripotent embryonic state, but a rearrangement of neuronal function to reverse a pathological state, then more subtle epigenetic manipulations may be required. Ideally, this intervention would be targeted to the diseased neuronal population that is the locus from which the pathology emanates. However, considering for most psychiatric disorders there is no known specific cell type or region in the brain primarily responsible for the condition this

is not possible. Therefore, this reprogramming could be considered a partial brain-wide 'reboot' whereby many of the entrenched epigenetic marks are erased with the potential to form new, non-pathogenic marks.

It has been shown that small molecule pharmacology that targets histone modifications and DNA methylation is capable of reactivating 'stemness' genes (Sharma et al., 2012a; Shi et al., 2008a; Shi et al., 2008b)**.** Moreover, DNA methylation inhibitors, such as 5-azacytidine, have been shown to be the most powerful agents in reprogramming differentiated cells such as mouse fibroblasts and B lymphocytes (Mikkelsen et al., 2008). Another option is to target histone modifications that indirectly affect DNA methylation. For example, HDAC inhibitors, such as valproic acid, are able to induce DNA demethylation in plasmids, endogenous DNA, and in nondividing cells such as neurons (Detich et al., 2002; Detich et al., 2003; Dong et al., 2010). It remains to be established whether methylated H3K4 increasing drugs, such as lysine-specific demethylase-1 (LSD1) inhibitor tranylcypromine can promote DNA demethylation, although one could presume this is the case based on the inability of DNMTs to bind methylated H3K4 (**Table 1)** (Allen et al., 2006; Lee et al., 2006) **.**

6. Conclusion

DNA methylation is the most stable of all epigenetic modifications, leading to the belief that it was irreversible. Recent evidence indicates that the BER pathway is the most likely method of active DNA demethylation. Mental disorders, including schizophrenia, have been shown to be characterized by a variety of epigenetic abnormalities. Some have reported these to be present at specific gene loci, while others have reported more

generalized abnormalities. The chronic nature of most psychiatric disorders implies that the abnormality is maintained through a highly stable means of gene regulation, such as DNA methylation. As such the discovery over the last several years regarding the mechanisms involved in the removal of this enduring mark provides a promising therapeutic target for reversing otherwise lifelong illnesses. We suggest that by pharmacologically manipulating DNA methylation it may become possible to reprogram neurons that have formed aberrant connections thereby allowing them to form new beneficial synapses.

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Figure Legends

Fig 1. Molecular Changes Leading to DNA Demethylation. 1) In an oxidation reaction catalyzed by ten-eleven translocation (TET) enzymes 5-methylcytosine (5MC) is converted into 5-hydroxymethylcytosine (5HMC). **2a)** Through a process involving deamination followed by base excision repair 5HMC is removed and replaced by an unmethylated cytosine. **2b)** Alternatively, 5HMC may be converted into 5-formylcytosine (5FC) in another oxidation reaction catalyzed by TET. **3a)** 5FC can be converted into 5-carboxylcytosine (5CaC) in another TET-catalyzed oxidation reaction. **3b)** 5FC hypothetically could be converted directly into unmethylated cytosine involving the expulsion of formic acid. **4a)** 5CaC can be removed via base excision repair. **4b)** 5CaC hypothetically could be converted directly into unmethylated cytosine in a reaction catalyzed by an as yet unidentified decarboxylase.

Fig 2. Base Excision Repair DNA Demethylation Pathway. 1. Following the TET catalyzed hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine (5HMC), 5HMC is deaminated by a Growth Arrest and DNA Damage (GADD45) protein recruited cytidine deaminase, such as AICDA or an APOBEC forming 5-hydroxymethyluracil (5HMU). **2.** GADD45 proteins also recruit a thymidine or uracil glycosylase, such as TDG, MBD4, or SMUG1, which then removes the 5HMU base. **3.** APE1 recognizes the now apyrimidinic site and creates a strand break 5' to the missing base. **4.** PARP1 is strongly induced by the resultant strand break. PARP1 dimerizes at the break and also adds Poly(ADP-Ribose) (PAR) groups to proteins such as scaffold protein XRCC1. The

addition of PAR groups facilitates protein-protein interactions between XRCC1 and DNA ligase and polymerase, which repair the break and replace the missing base.

Fig. 3. Increased expression of demethylating genes in schizophrenia vs. nonpsychiatric controls in two cohorts. We re-analyzed microarray data from the National Brain Databank established at Harvard Brain Tissue Resource Center (Harvard) obtained from the website (http://national_databank.mclean.harvard.edu/brainbank/Main) and Scripps Research Institute (Scripps) for genes involved in DNA demethylation. The Harvard study utilized the Affymetrix HG-U133A gene chip. Experimental result files had been generated using Affymetrix's MAS software. These include prefrontal cortical (PFC) samples from 25 normal control subjects and 19 with schizophrenia. Demographic details have been previously published (Sharma et al., 2008a). The result files were then merged using Affymetrix Expression Console Software with the annotation file and the annotated log2 results exported as a text file for third-party downstream analysis. The Scripps study utilized the Human Genome U133 Plus 2.0 array. We reanalyzed this post mortem expression data obtained from the Gene expression Omnibus (GEO accession GSE21138) based on diagnosis. The database includes PFC samples from 59 total subjects, 29 normal controls and 30 patients with schizophrenia. Demographic details have been previously published (Narayan et al., 2008). Expression was converted from log2 expression to linear expression as fold average of normal controls. Independent t-tests were used for group comparisons. All p-values are two-tailed. **A.** AICDA, APOBEC3A, and APOBEC3F are significantly increased in the Harvard study (*$P < 0.05$). **B.** APOBEC3B and APOBEC3G are increased in the Scripps dataset (*$P < 0.05$;

P < 0.01). **C & D. GADD45B and GADD45G are increased in both datasets. GADD45B has previously been shown to be elevated in the Stanley Medical research cohort as well (https://www.stanleygenomics.org/;(Gavin et al., 2012), while GADD45G has also been previously reported to be elevated in schizophrenia (Arion et al., 2007) (*P < 0.05). **E.** TET3 is increased in the Harvard dataset (*P < 0.05). **F.** MBD4 is significantly increased in the Scripps cohort. An increase in MBD4 in schizophrenia has previously been reported by Benes et al. (2006). Despite the consistent increase in DNA demethylating factors we do find a decrease in SMUG1 (*P < 0.05; ***P < 0.001). Mean+SEM.

Fig 4. Euchromatization. 1. The first step in the progression from restrictive heterochromatin to transcriptionally permissive euchromatin begins with the removal of histone marks such as, dimethylated lysine 9 of histone 3 (H3K9me2), and H3K9 acetylation. Restrictive histone modifications bar the DNA demethylation complex from accessing the underlying cytosine. **2.** H3K9 demethylation and acetylation allow for transcription factor binding as well as the binding of the mediators of DNA demethylation. Histone acetylation and the DNA demethylation process promote the addition of Poly(ADP-Ribose) polymers (PAR) to histone lysine residues such as H3K27. **3.** Once DNA is demethylated a H3K4 methyltransferase can bind. Methylated H3K4 prevents DNMT binding thereby maintaining the unmethylated DNA state.

Table 1. Pharmacological agents that promote euchromatization

Agent Name	Mechanism	Tested Clinically (Y=Yes; N=No)	FDA-Approved (Y=Yes; N=No)	Crosses Blood-Brain Barrier (Y=Yes; N=No; U=Unknown)	References
Decrease H3K9me2					
BIX-01294	Inhibits G9a/GLP	N	N	U	Vedadi, et al. 2011
UNC0638	Inhibits G9a/GLP	N	N	U	Vedadi, et al. 2011
UNC0224	Inhibits G9a/GLP	N	N	U	Vedadi, et al. 2011
Increase Histone 3 Acetylation					
SAHA (Vorinostat)	Inhibits HDACs 1-10	Y	Y	Y	Butler et al. 2002
VPA (Depakote)	Inhibits HDACs 1-10	Y	Y	Y	Gottlicher et al. 2001
FK228 (Romidepsin)	Inhibits HDAC1, 2, 3, and 8	Y	Y	U	Furumai et al. 2002
LBH589 (Panobinostat)	Inhibits HDACs 1-11	Y	N	U	Prince et al. 2009; Scuto et al. 2008
ITF2357 (Givinostat)	Inhibits HDACs 1-10	Y	N	Y	Leoni et al. 2005
PXD101 (Belinostat)	Inhibits HDACs 1-10	Y	N	N	Glaser 2007; Warren et al. 2008
MGCD0103 (Mocetinostat)	Inhibits HDAC1, 2, 3, and 8	Y	N	U	Buglio et al. 2010
PCI-34051	Inhibits HDAC8	Y	N	U	Balasubramanian et al. 2008
MS-275 (Entinostat)	Inhibits HDAC1, 2, 3, and 8	Y	N	N/Y[a]	Bracker et al. 2009
SB939 (Pracinostat)	Inhibits HDACs 1-11	Y	N	U	Novotny-Diermayr et al. 2010
4SC-201 (Resminostat)	Inhibits HDACs 1-10	Y	N	U	New et al. 2012
Decrease DNA Methylation					
5-azacytidine	Cytosine analog	Y	Y	N	Szyf 2001
5-aza-2′-deoxycytidine (Decitabine)	Cytosine analog	Y	Y	N	Szyf 2001
Procainamde (Pronestyl)	Reduces affinity DNMT for DNA	Y	Y	Y	Herken & Rietbrock 1969; Lee et al., 2005
(−)-epigallocatechin-3-gallate (EGCG)	Inhibits DNMT1	Y	N	Y	Lee et al. 2005
RG108	Inhibits DNMT1	N	N	U	Suzuki et al. 2010
Zebularine	Cytosine analog	N	N	N	Szyf 2001
Increase PARylation					
DEA	PARG Inhibitor	N	N	U	
ADP-HPD	PARG Inhibitor	N	N	U	Koh et al. 2003
Salicylanilide 6a	PARG Inhibitor	N	N	U	Steffen et al. 2011
RBPIs	PARG Inhibitor	N	N	U	Dunstan et al. 2012

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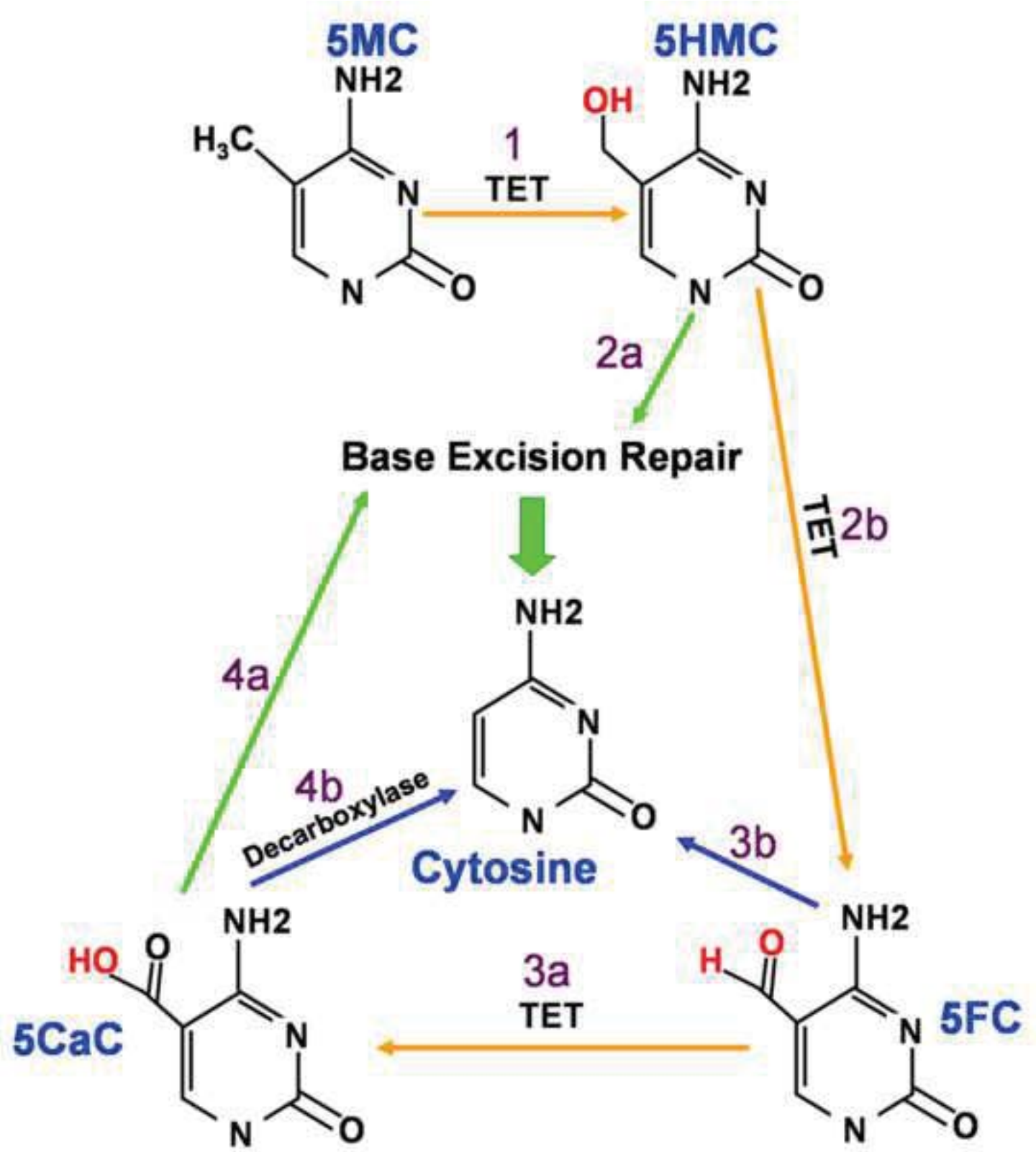

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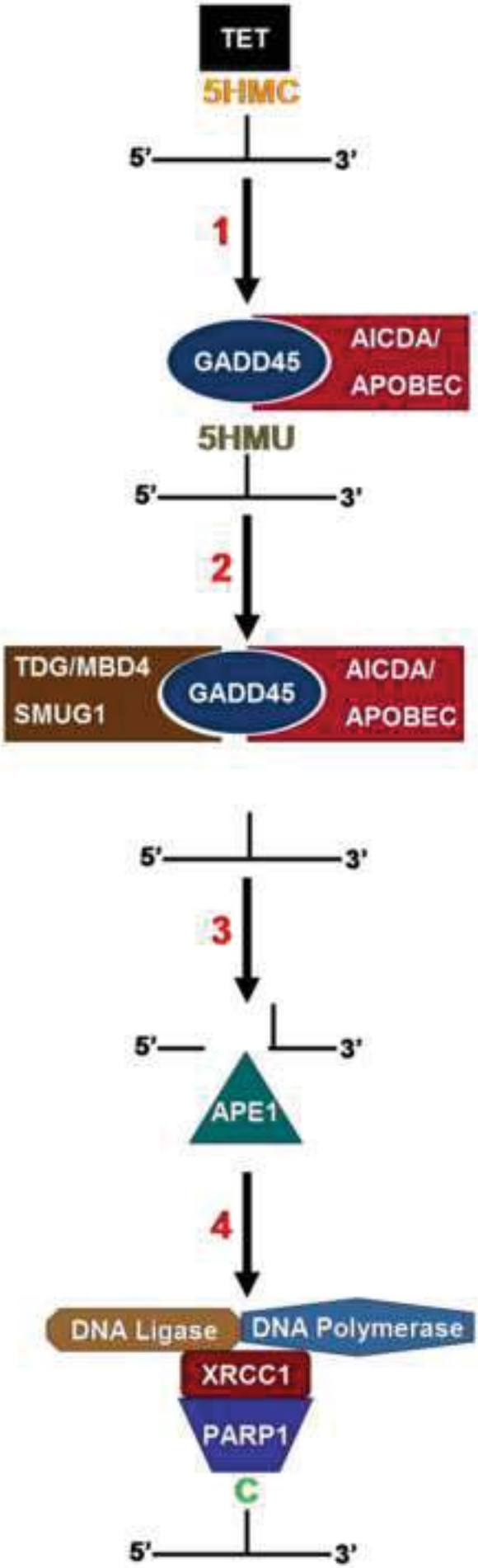

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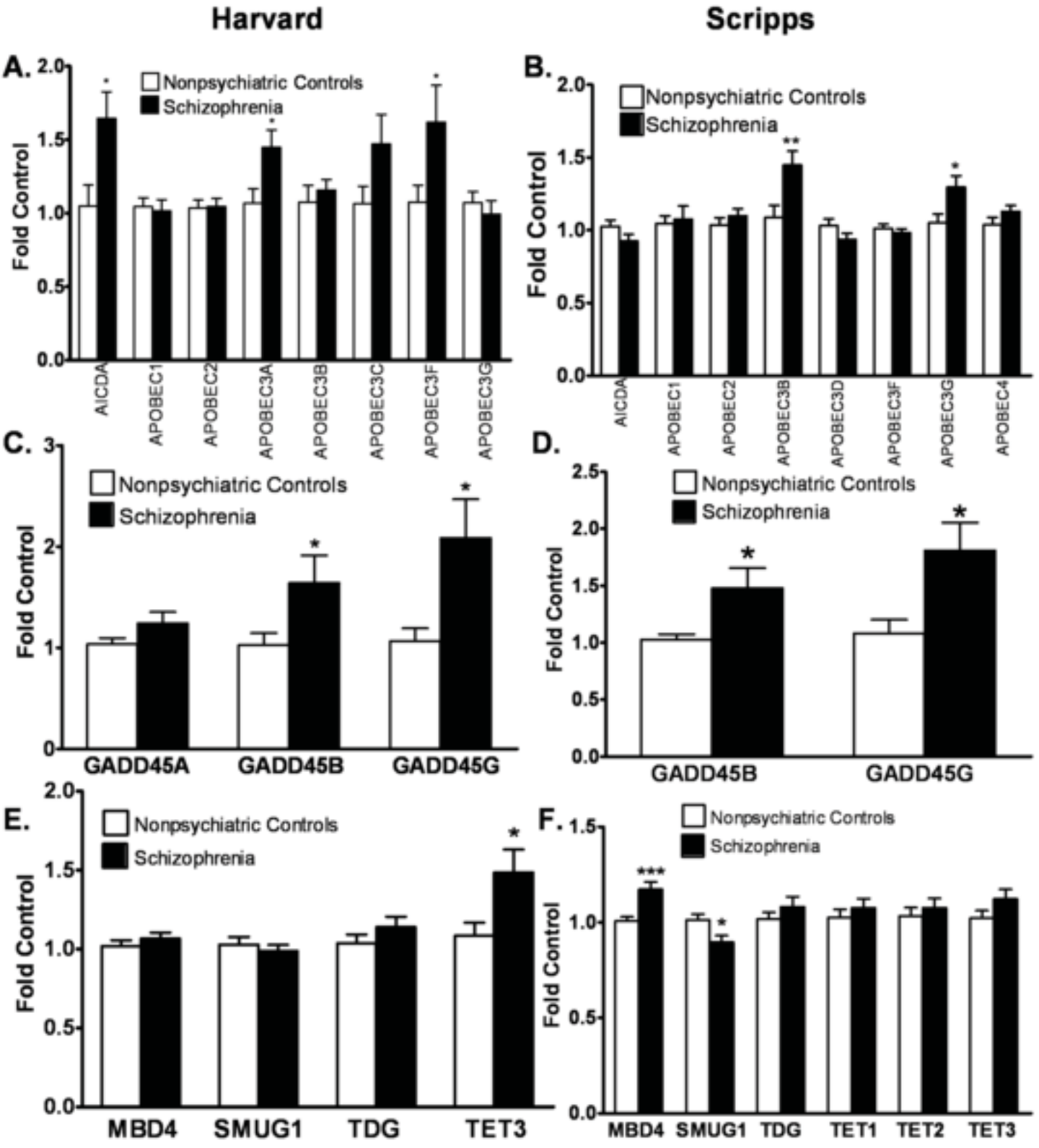

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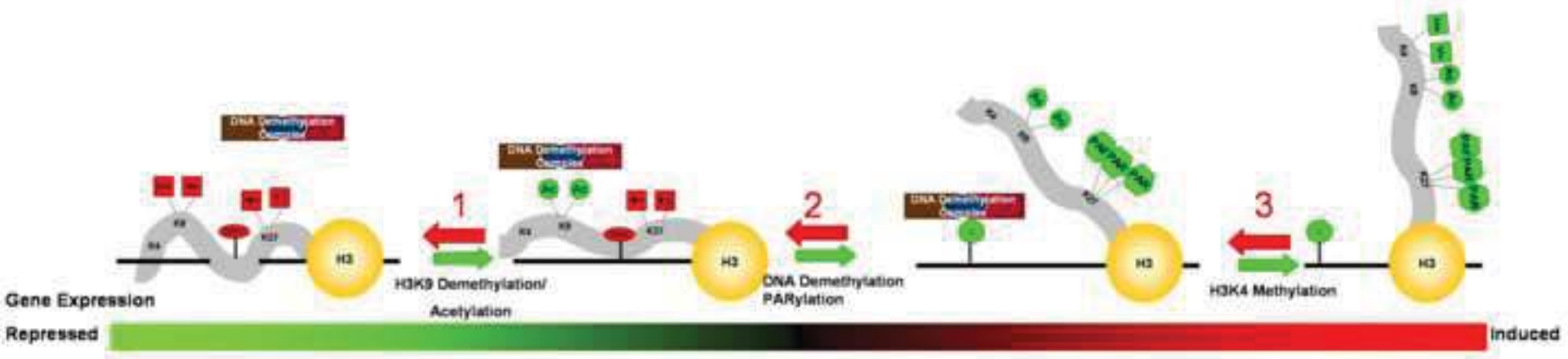

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Tables: 1