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Review Article

# Epigenetic mechanisms in Alzheimer's disease

Diego Mastroeni, Andrew Grover, Elaine Delvaux, Charisse Whiteside, Paul D. Coleman, Joseph Rogers*

*Banner Sun Health Research Institute, PO Box 1278, Sun City, AZ 85372, USA.*

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**Abstract**

Epigenetic modifications help orchestrate sweeping developmental, aging, and disease-causing changes in phenotype by altering transcriptional activity in multiple genes spanning multiple biologic pathways. Although previous epigenetic research has focused primarily on dividing cells, particularly in cancer, recent studies have shown rapid, dynamic, and persistent epigenetic modifications in neurons that have significant neuroendocrine, neurophysiologic, and neurodegenerative consequences. Here, we provide a review of the major mechanisms for epigenetic modification and how they are reportedly altered in aging and Alzheimer's disease (AD). Because of their reach across the genome, epigenetic mechanisms may provide a unique integrative framework for the pathologic diversity and complexity of AD.
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## 1. Introduction

Alzheimer's disease (AD) is a progressive, irreversible neurodegenerative disorder culminating in dementia. Its etiology and pathogenesis are complex, and encompass many genetic and environmental risk factors, changes in the expression of thousands of genes, and upregulation of multiple pathogenic pathways such as amyloid β peptide (Aβ) deposition, tau hyperphosphorylation, inflammation, oxidative stress, energy metabolism, and aberrant re-entry into the cell cycle/apoptosis. Moreover, with the exception of Aβ-inducing mutations, none of these molecular and genetic factors appears to have absolute penetrance in causing the disorder: many individuals may possess the most salient risk factors for AD, as well as express profuse Aβ and tau pathology, yet never develop the disorder (Lue et al., 1996). Indeed, even monozygotic twins can have dichotomous AD outcomes (Mastroeni et al., 2009; Räihä et al., 1997).

The emerging field of epigenetics has its roots in studies of the structure of chromatin and modifications to the structure of DNA, which extend back half a century or more (Felsenfeld, 2007). Although a unitary definition of epigenetics has yet to be reached, the many definitions that have been suggested all invoke heritability, lack of dependence on DNA sequence, and effects on transcription to produce diverse phenotypes. In particular, epigenetic modifications are capable of altering transcriptional activity in a coherent manner across thousands of genes and dozens of biological pathways, yet can do so differentially in monozygotic twins, the same individual at different developmental stages, or adjacent cells in the same organ, all of which share the same genetic code. Epigenetics also provides a means by which environmental factors such as diet, hazardous exposures, and life events can influence gene expression. As such, epigenetic mechanisms may provide a point of intersection for the diverse risk factors and pathophysiologic processes of AD.

The purpose of this review is to briefly describe the major epigenetic mechanisms, histone acetylation, DNA methylation,

* Corresponding author at: Banner Sun Health Research Institute, P.O. Box 1278, Sun City, AZ 85372, USA. Tel.: +1 623 876 5467; fax: +1 623 876 5461.

*E-mail address:* joseph.rogers@bannerhealth.com (J. Rogers).

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ribosomal DNA (rDNA), and microRNA (miRNA), and how they are reportedly altered in aging and AD.

## 2. Epigenetic regulation of gene expression

Epigenetic mechanisms modify heritable and nonheritable traits without necessarily altering the underlying DNA sequence. Thus, through epigenetic modification the diverse cellular phenotypes and functions needed by the body can be achieved using a single genetic code for all cells. These effects are typically accomplished by inhibition of transcriptional access to various genes, leading to their repression or silencing. Conversely, release from normal epigenetic repression can enhance gene expression (Bandyopadhyay and Medrano, 2003; Fraga and Esteller, 2007; Liu et al., 2003; Poulsen et al., 2007; Wilson and Jones, 1983). These modifications can occur at specific gene loci in specific cells to yield specific cellular phenotypes, or can encompass many genes in many cells, an orchestrating mechanism that is widely assumed to help drive such broad biological processes as development and aging (Bandyopadhyay and Medrano, 2003; Fraga and Esteller, 2007; Liu et al., 2003; Poulsen et al., 2007; Wilson and Jones, 1983).

Because phenotype and function are affected and effected by what genes are expressed and what genes are repressed, epigenetically regulated and dysregulated transcription states can give rise not only to different cell types and developmental stages, but also to favorable and unfavorable outcomes for specific cells within the same organ system. Thus, changes in epigenetic regulation can cause some cells to develop structural, physiologic, and metabolic abnormalities, while other cells of the same type remain normal. This is thought to occur, for example, in several hematologic malignancies after aberrant epigenetic silencing of genes that control proliferation (Mund et al., 2006).

In addition to direct regulation of gene expression, epigenetic modifications can mimic, exacerbate, or even cause genetic mutations. For example, epigenetic repression of tumor suppressor genes can mimic loss of function mutations of tumor suppressor genes, and both are highly associated with cancer development (Mund et al., 2006). Epigenetic silencing of the alpha subunit of the stimulatory G protein, a signaling peptide essential for the actions of parathyroid hormone, can cause pseudohypoparathyroidism, just as mutations to the alpha subunit do (Bastepe, 2008). Epigenetic modifications can exacerbate the effects of gene mutations, as occurs with adenomatous polyposis coli (APC) gene mutations in colorectal cancers (Colnot et al., 2004), E-cadherin mutations in gastric cancers (Strathdee, 2002), and mitochondrial DNA mutations in Leber's disease (Johns and Neufeld, 1993). Beyond these interactions, epigenetic repression of DNA repair genes may also induce gene mutations (Jacinto and Esteller, 2007).

Finally, epigenetic mechanisms provide a means by which environmental events can be translated to the cellular and molecular level. For example, ultraviolet ray exposure may induce epigenetic modifications in skin cells that culminate in cutaneous malignancies (Millington, 2008). Likewise, epigenetic changes induced by different environments, or simply by stochastic events, are thought to underlie the subtle phenotypic differences that emerge with time in monozygotic twins (Flanagan et al., 2006; Fraga et al., 2005).

### 2.1. Histone modifications

Epigenetic mechanisms typically involve changes in the micro- and macrostructure of chromatin, a complex of DNA, chromosome proteins, and histone proteins in which the histone proteins are tethered together in structures around which double-stranded DNA is wound. Conformational changes in histone proteins or modifications of the way in which DNA wraps around the histones may then differentially alter access of the transcriptional machinery to some genes while leaving access to other genes intact (Allfrey, 1966).

Although there are multiple mechanisms by which histones are modified, including methylation, phosphorylation, ubiquitination, sumoylation, citrullination, adenosine diphosphate (ADP)-ribosylation, and other posttranslational modifications of the amino acids that make up histone proteins, histone acetylation is one of the most ubiquitous and well studied. Here, histone acetyltransferases (HATs) catalyze the transfer of an acetyl group from acetyl-coenzyme A to lysine residues on the N-termini of histone proteins (Fig. 1). As a result of acetylation, the positive charge of the histone proteins is neutralized, decreasing interactions of the histone protein tails with negatively charged phosphate groups of associated DNA. This conformational relaxation of the chromatin permits access to and transcription of genes within the complex. Conversely, the histone deacetylases (HDACs) transfer acetyl groups from acetylated histone proteins back to coenzyme A, producing a more condensed chromatin state and decreased or silenced gene transcription.

### 2.2. DNA Methylation

DNA methylation provides a further means for histone modification, and is highly interactive with histone acetylation and the other histone-modifying mechanisms. For this reason, DNA methylation can, to some extent, be taken as a surrogate for histone modification profiles.

Beginning with seminal research in the late 1970s (Bird, 1978), it is now well established that adjacent cytosine-guanine (CpGs) dinucleotides within DNA can be methylated by the actions of the DNA methyltransferases, DNMT1, DNMT2, DNMT3a/b, and DNMT4. In mammals, DNMT1 appears to be primarily involved in maintenance methylation of hemimethylated DNA after DNA replication, whereas DNMT3a and DNMT3b are particularly important for de novo DNA methylation. DNMT2 is typically considered to be a ribonucleic acid (RNA) methyltransferase, although it also has 5-cytosine DNA methyltransferase activity (Tang et al., 2003) and

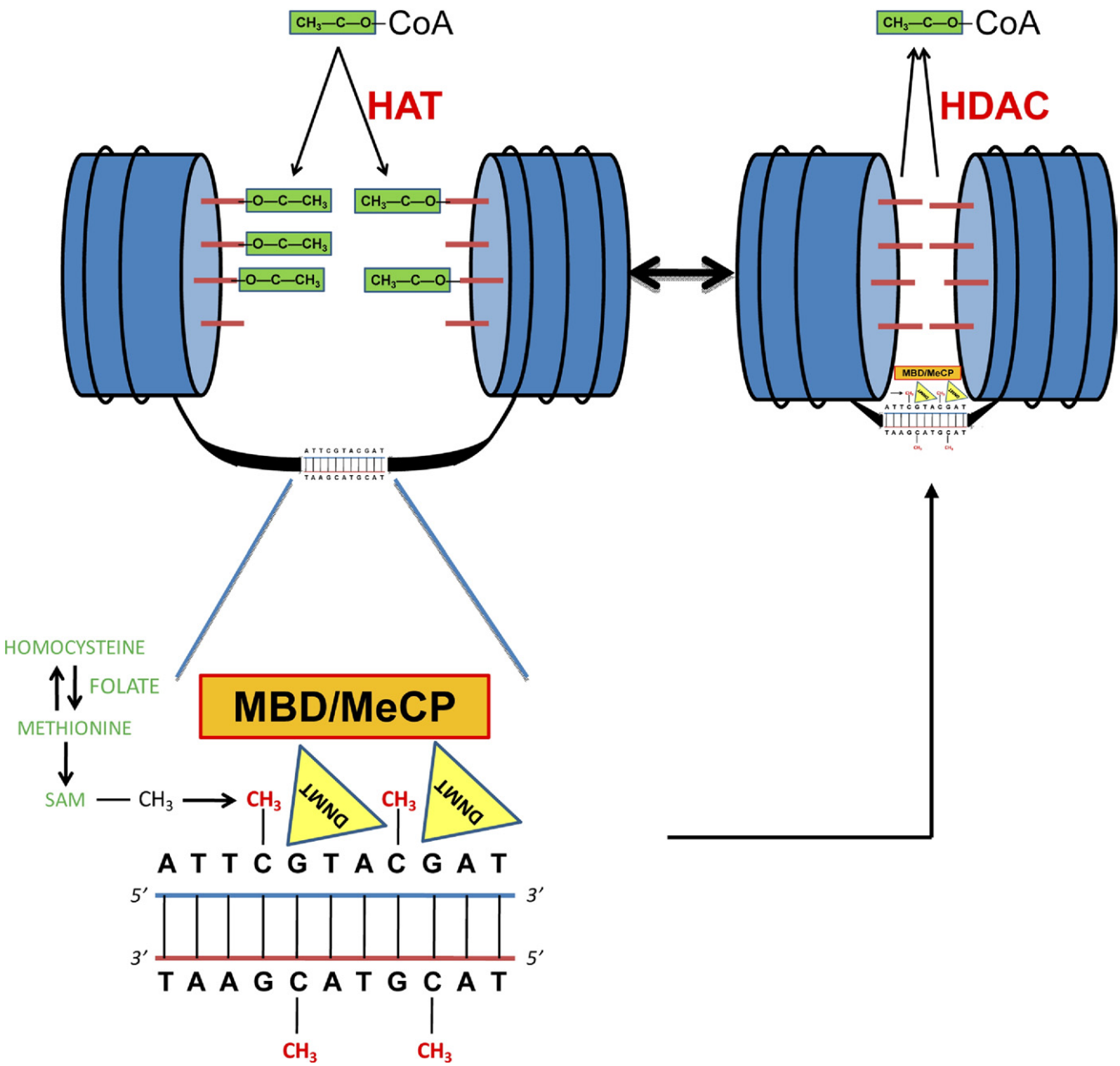


Fig. 1. Simplified schematic of histone acetylation and DNA methylation. (Upper left) In transcriptionally active genes the chromatin, made up of histones (blue cylinders) around which DNA is wrapped, is in a relaxed state, permitting transcriptional access to unwound DNA. This relaxed, euchromatin state is, in part, mediated by acetylation of histone tails (red rods) in which acetyl groups (green blocks) are transferred from acetyl-coenzyme A (acetyl-CoA) to the histone tails by histone acetyltransferases (HATs). (Bottom left) Within the DNA, the cytosines of adjacent C-G/G-C dinucleotides (CpGs) may be methylated. The methyl group ultimately derives from methyltetrahydrofolate in conjunction with the methionine/homocysteine cycle, and is transferred from S-adenosylmethionine (SAM) to the cytosine and incorporated into the genome by DNA methyltransferases (DNMTs). CpG-methyl-binding-domain proteins (MBDs) and methylation complex proteins (MeCPs) (which may contain MBDs) become associated with methylated CpGs, further inhibiting transcriptional access and repression of the gene. (Upper right) DNA methylation and histone modifications are integrally linked, because MBDs and MeCPs attract histone deacetylases (HDACs) that transfer acetyl groups on the histone tail back to CoA. Histone deacetylation, in turn, promotes the condensed, heterochromatin state characteristic of silenced or repressed genes.

forms denaturant-resistant complexes with DNA (Dong et al., 2001). The methyl group that is transferred to cytosine by the DNMTs ultimately derives from methyltetrahydrofolate through its interactions with S-adenosylmethionine (SAM) in the homocysteine-methionine cycle (Fig. 1).

Through these processes, approximately 70% of CpG dinucleotides within the human genome are methylated. Although DNA methylation can take place at any CpG site, whether in coding or noncoding regions, previous studies have often focused on CpG-rich stretches (CpG islands) within the

promoter region. Some 50,267 CpG islands exist in the human genome, with 28,890 in simple repeat and low complexity sequences that are masked (Bandyopadhyay and Medrano, 2003; Liu et al., 2003). Because CpG islands contain a high proportion of CpGs (and opportunities for CpG methylation), they are, perforce, among the most highly methylated regions of the genome. However, it has recently been shown that the percentage (rather than the absolute number) of methylated CpGs is relatively low in conventional CpG islands, and is actually higher in promoters with intermediate CpG densities (Eckhardt et al., 2006; Weber et al., 2007). Moreover, tissue-specific methylation patterns appear to be most pronounced not at CpG islands but at "CpG shores", regions within approximately 2 kb of CpG-enriched sequences. It has been suggested, in fact, that methylation patterns of CpG shores are "sufficiently conserved to completely discriminate tissue types regardless of species of origin" (Irizarry et al., 2009). Differential methylation of CpG shores in brain compared with liver has also been found to be highly correlated with differences in gene expression for these 2 organs (Irizarry et al., 2009).

Methylation of CpG sequences may alter gene expression by inducing histone modifications that inhibit access of the transcriptional machinery (Bird and Wolffe, 1999; Zhang et al., 2002) (Fig. 1). One would expect, then, that highly methylated genes would be repressed, and that hypomethylation of a gene would lead to enhanced expression or overexpression compared with the normally repressed, methylated state. Some notable exceptions to these expected states have been found, however. For example, the p16INK4a promoter is progressively hypermethylated with age (So et al., 2006), but expression of the p16INK4a gene appears to increase with age (Kim and Sharpless, 2006). More generally, Gius and colleagues (Gius et al., 2004) found that chemical hypomethylation of nearly half the genes they surveyed resulted in silencing rather than upregulation. Indeed, altering the methylation status of some CpG sites within a gene can be inconsequential compared with alterations at other sites (c.f. Murgatroyd et al., 2009; Yakovlev et al., 2010). Such findings indicate that it will be important to follow up genome-wide DNA methylation profiling, which can only survey a limited number of CpGs per gene, with more detailed DNA methylation maps that include shores and flanking regions. In addition, even highly-detailed DNA methylation profiles will require validation of functional effects at the gene expression and protein levels.

A second, linked mechanism by which DNA methylation may modify gene expression is through methyl-CpG-binding proteins (MeCPs) such as MeCP2. When bound to methylated DNA, MeCP2 has been shown to recruit HDACs, which, as noted earlier, may then induce a more condensed chromatin state and decreased or silenced gene transcription (Jones et al., 1998; Wade et al., 1998). Although previous studies assumed that MeCP2 required a methylated chromatin substrate for direct binding (Lewis et al., 1992), more recent research has demonstrated that MeCP2 can directly condense chromatin even in the absence of DNA methylation, histone deacetylase activity, or the cooperation of other transcriptional corepressors such as mSin3A (Wade, 2001). Mutations of the MeCP2 gene cause Rett's syndrome, with dysregulation of neural development, mental retardation, and motor dysfunction (Amir et al., 1999).

MeCP1, a macromolecule made up of some 10 different peptides, may also act as a mediator between DNA methylation and histone acetylation, recognizing and binding to CpG dinucleotides, recruiting HDACs, and inducing transcriptional repression (Feng and Zhang, 2001). Unlike MeCP2, however, MeCP1 does not bind directly to methylated DNA, but to a single methyl-CpG-binding domain protein, MBD-2. In addition to inducing histone modifications, MBD-2-bound MeCP1 helps maintain the DNA methylation status of CpGs by recruiting DNMT1. DNMT1 is then able to recognize and repair CpGs that have lost methyl groups on one DNA strand but not the other.

Recent studies have also revealed a further modification to methylated CpGs that may make them even more inaccessible to transcription. Here, the 5-methylcytosines are hydroxylated (oxidized) to form 5-hydroxymethylcytosine (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Valinluck and Sowers, 2007). Hydroxymethylated DNA has been observed in neurons (Kriaucionis and Heintz, 2009), and may occur as a result of oxidative damage and/or the actions of specific oxidative enzymes, particularly Ten-Eleven-Translocation-1 (TET1) (Tahiliani et al., 2009). Normally, 5-hydroxymethylcytosines are stripped from genomic DNA by glycosylases of the base excision repair (BER) system, permitting replacement with unmodified cytosines for subsequent de novo DNA methylation by the DNMTs. When left intact, however, 5-hydroxymethylcytosines reportedly reduce the interaction of DNA with DNA-binding proteins to an even greater extent than do 5-methylcytosines (Valinluck and Sowers, 2007; Valinluck et al., 2004). Because hydroxymethylated CpGs may go undetected by 5-methylcytosine antibodies, hydroxymethylation could lead to underreporting of DNA methylation, a possibility that the present authors are now investigating. Conversely, bisulfite sequencing may not discriminate normally methylated from hydroxymethylated CpGs.

### 2.3. RNA-related mechanisms

Epigenetic regulation also extends to mechanisms involving RNA such as microRNAs (miRNAs) and hereditable and cell cycle-maintained silencing of a portion of the ribosomal RNA (rRNA) genes. Repeated rRNA transcripts from repeated rRNA genes (rDNA) provide a structure and primary catalytic site for the eukaryotic ribosome, wherein gene expression culminates in protein synthesis. Different cell types exhibit different proportions of active rRNA genes, suggesting that the fraction of rRNA gene copies may be altered in development and differentiation. Epigenetic mechanisms are now known to play important roles in this process by silencing rRNAs, thereby providing a dynamic balance between active and in-

active rRNAs. DNA methylation appears to be one of these epigenetic mechanisms, and may be of particular importance given the unusually high frequency of CpG dinucleotides within the rRNA genes and their unusually high states of DNA methylation. For example, two studies have shown a correlation between DNA methylation status and activity of rRNA genes (Bird et al., 1981; Santoro and Grummt, 2001), and treatment with 5-aza-2-deoxycytidine (azacytidine), a hypomethylating agent, enhances expression by rDNA genes (Santoro and Grummt, 2001). For an excellent and comprehensive summary of rDNA methylation, as well as histone modifications and chromatin remodeling, which also play key roles in epigenetic regulation of rDNA, the reader is referred to the recent review of McStay and Grummt (2008).

The study of miRNAs represents an additional, critical area for epigenetics research. Often deriving from their own genes with their own promoter and regulatory elements, approximately 700–800 miRNAs have been identified in the human genome. These small (~22 nucleotide) RNAs regulate gene expression in a posttranscriptional manner by binding to their target mRNAs, inhibiting translation or, less often, inducing cleavage of the mRNAs (reviewed in Yang et al., 2007).

## 3. Dynamic epigenetic regulation in adult neurons

### 3.1. Histone modifications

Histone modifications have been implicated in broad neurobiological processes such as development of the central nervous system (CNS) (reviewed in MacDonald and Roskams, 2009), posttraumatic stress disorders (Sokolova et al., 2006), childhood abuse/suicide (McGowan et al., 2009; Meaney et al., 2007), memory formation (Gupta et al., 2010), and addiction (Impey, 2007); specific physiologic processes such as neuronal differentiation (Kular et al., 2009), regulation of choline acetyltransferase activity (Aizawa and Yamamuro, 2010), astrocyte glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) transcription (Wu et al., 2008c), microglial apoptosis (Chen et al., 2007), and axon pathfinding (Zinovyeva et al., 2006); and various neurologic disorders, including Parkinson's disease (Chen et al., 2007; Wu et al., 2008c), motor neuron disease (reviewed in Echaniz-Laguna et al., 2008), multiple sclerosis (reviewed in Gray and Dangond, 2006), X-linked mental retardation (Tahiliani et al., 2007), and stroke/cerebral palsy (Meisel et al., 2006). All of these histone-related processes occur in the context of the CNS, and many have been reported to function as dynamic regulatory mechanisms in postmitotic neurons.

### 3.2. miRNA

Like histone modifications, miRNA has been implicated in both broad and specific CNS processes and disorders. For example, miRNA-206 appears to promote neuromuscular synapse regeneration (Williams et al., 2009), and miR-329, miRNA-134, and miRNA-381, which are induced by neuronal activity, have been suggested to be essential for activity-dependent dendritic outgrowth of hippocampal neurons (Khudayberdiev et al., 2009). In turn, these and other miRNA-mediated mechanisms have been investigated in various CNS disorders, including human immunodeficiency virus (HIV) dementia (Witwer et al., 2010), amyotrophic lateral sclerosis (Williams et al., 2009), Tourette's syndrome (Abelson et al., 2005), AD (see section 5.2, below), and other neurologic conditions (reviewed in Maes et al., 2009).

Once again, these epigenetic processes have been demonstrated to occur in the context of postmitotic neurons. Indeed, it has been reported that the switch from a replicative state to the postmitotic state of neurons may itself be controlled by miRNA mechanisms (Yoo et al., 2009).

### 3.3. DNA methylation

Previously, DNA methylation reactions were considered primarily in the context of maintenance of the DNA methylation pattern across cell divisions. Why neurons and other postmitotic cells should express DNA methylation markers such as DNMT3 was therefore unclear. Likewise, how long term DNA methylation alterations might be accomplished and sustained in postmitotic cells was unknown. Recently, however, new studies have shown that hypo- and hypermethylation are dynamic events that can occur within cells (Kangaspeska et al., 2008; Métivier et al., 2008), including postmitotic neurons (Levenson et al., 2006; Murgatroyd et al., 2009), on the scale of tens of minutes. These findings open the door for long suspected, dynamic epigenetic mechanisms that may help mediate neuronal and synaptic plasticity (e.g., Arendt, 2005). For example, gene-specific hypomethylation of hippocampal neurons after DNMT inhibition blocks long term potentiation (Levenson et al., 2006) and fear conditioning (Miller and Sweatt, 2007). Weaver et al. (2005) have suggested that early life events — specifically, maternal care — alter adult stress responses through sustained DNA methylation changes in rat hippocampal neurons.

The role of DNA methylation in dynamic regulation of neuronal activity and function began to emerge with studies by Bredy and colleagues (Bredy et al., 2003), and has been vividly confirmed by recent work linking early life stress to enduring molecular, physiologic, memory, and behavioral changes in mice via epigenetic modifications to hypothalamic neurons (Murgatroyd et al., 2009). In particular, early exposure of mice to stress during the first 10 days of life has been found, as much as a year later, to be associated with impaired step-down avoidance learning, sustained hyperactivity of the hypothalamic-pituitary-adrenal axis, and corticosterone and pituitary adrenocorticotropin prohormone hypersecretion. These effects, in turn, were elegantly traced to persistent arginine vasopressin (AVP) overexpression by parvocellular neurons of the hypothalamic paraventricular nucleus, and to hypomethylation of specific CpG sites within the AVP gene. Notably, age-dependent increases in AVP gene hypomethylation at multiple CpG sites were observed in control mice in these studies, but hypomethylation of CpGs within a CpG island (CGI3) in the

AVP enhancer region approximately 0.5 kb downstream from the AVP gene itself appeared to be the primary determinant of AVP overexpression in early life stress mice. Further experiments went on to show that CGI3 hypomethylation was specific to the paraventricular nucleus compared with the supraoptic nucleus, and to provide a key mechanism for the epigenetic modifications that were observed: CGI3 CpG sequences serve as preferential and selective DNA-binding sites for MeCP2. Phosphorylation of MeCP2 by calmodulin-dependent protein kinase II as a result of neuronal membrane depolarization decreases MeCP2 occupancy of CGI3 CpGs, thereby enhancing transcription (Murgatroyd et al., 2009). This landmark study therefore shows that dynamic and long lasting DNA methylation changes can and do occur in postmitotic neurons.

Similarly, Yakovlev et al. (2010) have traced age-dependent downregulation of caspase-3 production in rat brain to significantly lower levels of histone 3 acetylated Lys14 and histone 4 acetylated Lys5, 8, 12, and 16, as well as to differential methylation of specific CpG sites within the caspase-3 promoter. These sites are in a region that is essential for caspase-3 promoter activity, and correspond to predicted binding sites for several transcription factors such as Ets-1 and Ets-2 that are known to help control caspase-3 synthesis and to play critical roles in neuronal differentiation, development, and death. Notably, Ets-1 and Ets-2 themselves did not show age-related decline in the study, highlighting the potential importance of interactions between DNA methylation modifications and transcription factor activity. That is, transcriptional control of a gene by its transcription factors may be significantly altered by differential methylation of the binding sites for those transcription factors.

New research has also shown that, in addition to their roles in DNA methylation, MBD-2 and DNMT3a/b may participate in dynamic demethylation processes. It has been reported, for example, that transient coexpression of MBD-2 and methylated promoters results in demethylation and activation of gene expression, whereas knockdown of MBD-2 inhibits replication-independent, active demethylation by valproate (reviewed in Szyf, 2009). Cyclical methylation/demethylation processes that mediate bouts of active and inactive transcription over periods of tens of minutes have also been demonstrated, and appear to help regulate the expression of multiple genes (Kangaspeska et al., 2008; Métivier et al., 2008). Here, DNMT3a/b, in concert with p68, bind and deaminate selective CpG sites, creating mismatches that are recognized by thymine-DNA glycosylase (TDG) and repaired by the BER. Because the repaired CpGs are no longer methylated, the local chromatin environment becomes poised for transcription. Following transcription, MBDs, MeCP2, and DNMTs are recruited and remethylate the CpG sites. Transcription is therefore turned off or retarded. The cycle may then begin again with DNMT3-mediated CpG deamination (Métivier et al., 2008). Although these dynamic MBD and DNMT demethylating mechanisms have, as yet, been examined only in non-CNS cells, there is no obvious reason why they should not occur in neurons. Similarly, a recent study has suggested that histone acetylation and deacetylation, like DNA methylation and demethylation, are dynamic, rapid turnover processes that can poise genes for transcription in peripheral cells (Clayton et al., 2006). As such, all these mechanisms may warrant significant attention in future neuroepigenetics research.

## 4. Epigenetic regulation of aging

Aging is universally considered to be one of the most salient risk factors for AD, with increasing risk for the disorder cumulating until at least the ninth decade of life (Gao et al., 1998; Kukull et al., 2002). Why aging should be a risk factor for AD (and other age-related disorders), however, is not well understood, particularly at a mechanistic level. Potentially deleterious changes in mitochondria/oxidative stress (Crouch et al., 2007), gonadotropins (Fuller et al., 2007), calcium (Thibault et al., 2007), glucocorticoids (Landfield et al., 2007), inflammation (Duenas-Gonzalez et al., 2008), trace metals (Brewer, 2007), insulin (Craft, 2005), cerebrovascular supply (Bailey et al., 2004), the cell cycle (Macaluso et al., 2005), A$\beta$ (Selkoe, 2003), tau (Maeda et al., 2006), and hundreds to thousands of genes (Berchtold et al., 2008; Parachikova et al., 2007) occur both in aging and AD, but a coherent explanation for why they occur and if their co-occurrence in aging and AD is coincidence or meaningful remains elusive.

DNA methylation and histone modifications have been widely implicated in the phenotypic alterations that occur during cellular senescence and the aging of various organisms (reviewed in Bandyopadhyay and Medrano, 2003; Fraga and Esteller, 2007; Liu et al., 2003; Poulsen et al., 2007; Wilson and Jones, 1983), and may provide a link between aging and AD. Histone acetylation mechanisms, particularly those involving the Sir2 family of histone deacetylases, have been linked to aging and senescence in yeast and invertebrates (reviewed in Bandyopadhyay and Medrano, 2003), but have yet to be investigated in mammals. By contrast, many studies have reported a genome-wide tendency to DNA hypomethylation with age in multiple vertebrate organs, including brain, liver, small intestine mucosa, heart, and spleen; multiple cell types, including fibroblasts and T lymphocytes; and multiple vertebrate species, including aging salmon, mice, rats, cows, and humans (Golbus et al., 1990; Richardson, 2002; Romanov and Vaniushin, 1980; Vanyushin et al., 1970, 1973; Wilson et al., 1987). Yu (2006) assayed human peripheral blood mononuclear cell DNA for the percentage of methylated to total cytosines and observed a 3% per decade decrease from the first to the tenth decade of life. In vitro, hypomethylation of human and mouse fibroblasts cultured to senescence has also been observed (Wilson and Jones, 1983). Likewise, progressive age-related decline in total genomic methylcytosine has been reported in various organisms (Mays-Hoopes, 1989). Because DNMT1, which is responsible for maintaining DNA methylation of CpG sites, is also progressively lost with age (Lopatina et al., 2002), it has been speculated that progressive, age-

related, genome-wide hypomethylation may be due to parallel DNMT1 deficits (Liu et al., 2003), and that the process overall may serve as a counting mechanism that triggers cellular senescence (Neumeister et al., 2002). Age-dependent increases in S-adenosyl-homocysteine (SAH) relative to SAM (Gharib et al., 1982; Varela-Moreiras et al., 1994) might also play a role, since SAH inhibits methylation reactions, including DNA methylation. In turn, overall decline in genomic methylation with age has been linked to specific age-related pathogenic processes such as aberrant cell cycle events (e.g., p53-dependent apoptosis) (Jackson-Grusby et al., 2001) and the increased inflammatory tone that occurs with advancing age (Wilson, 2008).

Hypomethylation of noncoding regions and other sites also occurs with age and has been suggested to be relevant to the aging process. For example, repetitive sequences (Mays-Hoopes et al., 1986; Rath and Kanungo, 1989; Romanov and Vanyushin, 1981), retrotransposons (Barbot et al., 2002), and endogenous retroviruses (Ono et al., 1989) that are normally repressed by DNA methylation can become hypomethylated with age, potentially promoting chromosome translocations, retrotransposon activation, and retrovirus emergence, respectively (reviewed in Richardson, 2002).

Age-dependent hypomethylation of a number of specific genes related to AD has been reported. For example, methylation of cytosines in the amyloid precursor protein (APP) promoter, particularly GC-rich elements from approximately −270 to −182, is significantly lower in autopsy cases ≥70 years old compared to cases <70 years old (Tohgi et al., 1999a). DNA methylation within the tau promoter reportedly declines overall with age, but with interesting variations at different transcription factor binding sites: binding sites for granulocyte chemotactic factor (GCF), which represses GC-rich promoters, become hypomethylated with age, whereas binding sites for specificity factor 1 (SP1), a transcriptional activator, become hypermethylated. These changes might therefore represent a double hit on tau gene transcriptional activity, causing decreased activity overall with age (Tohgi et al., 1999c). Promoter methylation of the receptor for advanced glycation end products (RAGE) gene exhibits similar complexity. Overall methylation of the promoter declines with age, but the change is manifest at cytosine residues other than CpG dinucleotides: CpC, CpA, and CTG sequences within activator protein 2 (AP2) and SP1 binding sites show significant hypomethylation with age (Tohgi et al., 1999b). Expression of the immune/inflammatory antigen CD11a increases with age (Pallis et al., 1993), an effect that appears to be linked to an age-related hypomethylation of flanking repeats some 1 kb 5′ to the CD11a promoter start site (Zhang et al., 2002).

Despite the trend to genome-wide and gene-specific DNA methylation with age, it should be emphasized that the trend is no more than that, as many genes exhibit age-related decreases in expression rather than the upregulation that is predicted by hypomethylation or histone acetylation. CpG islands on several specific genes undergo age-dependent hypermethylation (e.g., estrogen receptors, insulin-like growth factor 2 (Issa et al., 1994, 1996). Tumor suppressor genes appear particularly apt to show increasing methylation with age, providing a potential link to age-related cancers (Fraga and Esteller, 2007; Issa and Baylin, 1996; Issa et al., 1994; Jacinto and Esteller, 2007; Kim and Sharpless, 2006; Liu et al., 2003; Mays-Hoopes, 1989; Neumeister et al., 2002; Richardson, 2002; Romanov and Vaniushin, 1980; So et al., 2006). Many of these hypermethylation events could be due to age-related increases in DNMT3a/b expression (Liu et al., 2003; Lopatina et al., 2002), as these methyltransferases are responsible for de novo methylation of DNA. Alternatively, they have also been linked to demethylation processes (Kangaspeska et al., 2008; Métivier et al., 2008).

Further adding to the complexity of aging changes in DNA methylation, tissue-specific patterns should also be noted. For example, the tumor suppressor gene c-fos exhibits increasing CpG methylation with age in liver, but not brain or spleen (Ono et al., 1989). In brain, methylation profiles may differ substantially from one region to another (Ladd-Acosta et al., 2007) and even from one subregion to another (e.g., hippocampal dentate gyrus and CA fields) (Brown et al., 2008), underscoring the value of brain regional comparisons in epigenetic studies of aging and AD.

In addition to aging, epigenetics plays a major role in development (reviewed in Reik and Dean, 2001). These mechanisms could be relevant to AD because overt clinical symptoms of the disorder virtually never appear until after the developmental stages of infancy, childhood, and early adolescence have been completed, and this is true not simply in late-onset patients but in patients carrying APP, presenilin (PS)1, or PS2 mutations. Thus, epigenetic changes earlier in life might be a necessary but not sufficient step toward AD in susceptible individuals, a key concept in the latent early-life associated regulation ("LEARn") model of age-related neurologic disorders (Lahiri et al., 2007; Wu et al., 2008b). Support for this hypothesis comes from APP transgenic mouse research wherein earlier epigenetic manipulations appear to accelerate or delay the expression of A$\beta$ pathology (Fuso et al., 2008). Likewise, early exposure of monkeys to Pb reportedly decreases DNMT activity, increases APP, beta secretase (BACE), and SP1 expression, and alters levels and distribution of A$\beta$ in the animals in late life (Wu et al., 2008a).

## 5. Epigenetic alterations in AD

### 5.1. Histone modifications

Several reports have demonstrated alterations in histone proteins in AD. Phosphorylation of histone 3, a key step in the activation of the mitotic machinery, is increased to a hyperphosphorylated state in AD hippocampal neurons (Ogawa et al., 2003). A nonnuclear form of histone 1 ap-

pears to be upregulated in astrocytes and neurons in brain regions that are rich in AD pathology (Bolton et al., 1999). Linker histone H1, a vital component of chromatin, has been reported to preferentially bind A$\beta$-42, as well as A$\beta$-like structures of numerous proteins (Duce et al., 2006). In addition, the H1 molecule has been shown to be a major target for poly adenosine diphosphate (ADP)-ribosylation in areas of AD brain with ischemic brain injury (Love et al., 1999).

Manipulation of histone tail acetylation with HDAC inhibitors has been investigated in several animal models of AD. For example, it has been reported that after fear conditioning training in APP/PS1 mice, levels of hippocampal acetylated histone 4 (H4) were about 50% lower than in wild-type littermates. Treatment with the HDAC inhibitor trichostatin A increased the levels of acetylated H4 and contextual freezing performance to wild-type values (Francis et al., 2009). Treatment with HDAC inhibitors has also been shown to induce sprouting of dendrites, increase the number of synapses, and reinstate learning behavior and access to long term memories in CK-p25 transgenics (Fischer et al., 2007). In addition, valproic acid, which has HDAC1 inhibitor activity, has been shown to decrease A$\beta$ production and reduce plaque burden in the brains of PDAPP(APP(V717F)) transgenic mice (Su et al., 2004). Similarly, in the Tg2576 mouse model of AD, a daily dose of phenylbutyrate, another HDAC inhibitor, reversed spatial memory loss and normalized levels of phosphorylated tau in the hippocampus, but failed to alter A$\beta$ levels (Ricobaraza et al., 2009). Conversely, in a cortical neuron culture model, overexpression of APP resulted in a decrease in histone 3 and histone 4 acetylation, as well as a decrease in c-AMP response element (CREB) protein levels (Lonze and Ginty, 2002; Rouaux et al., 2003).

In summary, although it appears that histone modifications occur in AD, AD animal models, and AD culture models, the pattern of changes is complex and could entail both histone acetylation increases and decreases at specific loci that function disjointedly or in concert.

### 5.2. miRNAs

Global analyses of AD versus normal elderly control brains have revealed changes in the levels of several specific miRNAs that were concordant across two separate studies (Hébert et al., 2008; Nunez-Iglesias et al., 2010). Notably, however, both positive and negative relationships between levels of the miRNAs and levels of their targets were observed, suggesting the operation of upstream factors (Tsang et al., 2007). A third study has provided the additional caveat that significant AD changes in many miRNAs may be common to both pathologically vulnerable and pathologically spared brain regions. Other miRNAs, however, were specific both to AD and to areas of extensive AD pathology, and their targets had resonance with mainstream AD pathologic pathways (Cogswell et al., 2008).

In vitro experiments with HeLa, COS1, and HEK293 cells have shown that luciferase expression controlled by the APP 3′ untranslated region (UTR) can be regulated by miR-20a, miR-17-5p, and miR-106b miRNAs. Transient transfection of these miRNAs downregulated APP expression — in the case of miR-20a by inhibiting translation rather than by degradation of APP mRNA. Conversely, blocking expression of miR-20a increased endogenous APP levels by some 50%. Subsequent developmental studies of mouse brain revealed dramatic reductions in all three miRNAs that were significantly correlated with increased APP protein expression. The fact that APP mRNA levels remained stable under these conditions again suggested that the miRNAs have their effects on APP by inhibiting translation rather than promoting cleavage of APP mRNA. Finally, human pathology studies have shown that miR-106b is significantly decreased in AD cortex. Although all these findings indicate that APP may be targeted by miRNA mechanisms, levels of miR-20a, miR-17-5p, and miR-106b in AD cortex do not appear to correlate with levels of APP protein (Hebert et al., 2008).

Two studies have used computational analysis methods to reveal miRNA target sites in BACE mRNA that may be functionally relevant to AD pathogenesis. Wang et al. (2008) found multiple target sites for miR-107 in the 3′-untranslated region of BACE, and went on to show significant decreases in miR-107 that were apparent even in early AD cases, particularly in the large pyramidal cell cortical layers that may be especially vulnerable to AD pathology (Rogers and Morrison, 1985). Moreover, when assayed in the same cases BACE mRNA expression appeared to be negatively associated with miRNA-107 levels (Wang et al., 2008). An additional miRNA, miR-29a/b-1, also appears to target BACE mRNA and, like miR-107, is decreased in AD and inversely correlated with BACE — in this case, BACE protein levels (Hébert et al., 2008).

### 5.3. DNA methylation

#### 5.3.1. Genome-wide and multigene studies

Although an early analysis reported no significant difference in percent CCGG methylation of DNA in AD cortex, a number of caveats were given (Schwob et al., 1990), particularly the fact that CCGG methylation only covers approximately 20%–30% of CpG sites in the genome. Methylation status of 12 specific genes that have been implicated in AD pathology has also been reported to exhibit significant “epigenetic drift”, although the manner in which the data were analyzed makes it difficult to determine whether methylation was increased or decreased in AD. The study did note, however, that an age-specific epigenetic drift was observed in some of the CpG sites within the DNMT1 promoter (Wang et al., 2008).

From a genome-wide perspective, our laboratory has reported decreased immunoreactivity for some seven different markers of DNA methylation in AD compared with

matched, nondemented elderly control (ND) cortical neurons and glia (Figs. 2 and 3), whereas no such changes were observed in cerebellum, a brain region that is relatively spared in AD (Mastroeni et al., 2010). Highly similar results were subsequently obtained in a set of monozygotic twins discordant for AD (Mastroeni et al., 2009) (Fig. 4A), as well as in APP-overexpressing transgenic mice (Fig. 4B).

As previously noted, folate/methionine/homocysteine metabolism is critically linked to DNA methylation mechanisms. Folate deficiency in humans and in animal models, for example, typically results in global hypomethylation that is at least partially reversible with folate supplementation (reviewed in Choi and Friso, 2005; Choi and Mason, 2002; Fuso et al., 2005, 2008). Deficits in folate and alterations in the methionine/homocysteine cycle have been reported in aging and AD (reviewed in Smith, 2008), and may therefore provide a basis for the tendency to genome-wide hypomethylation summarized earlier in this review. Although one prospective study failed to find an association between dietary folate, vitamin B12, or vitamin B6 with incident AD (Morris et al., 2006), CSF folate has nonetheless been reported to be significantly decreased in AD (Serot et al., 2001), as has CSF and brain SAM and one of its synthesizing enzymes, methionine S-adenosyltransferase (Bottiglieri et al., 1990; Morrison et al., 1996). Increases in brain SAH (Kennedy et al., 2004) and plasma homocysteine (Clarke et al., 1998; Smith et al., 2008), which inhibit DNA methylation, have also been observed. Elevated plasma homocysteine has been reported to be a significant risk factor for AD in dementia-free cohorts of both the Framingham Study of Aging (Seshadri et al., 2002) and the Conselice Study of Brain Aging (Ravaglia et al., 2005). In fact, dietary effects of folate and homocysteine manipulation have been implicated in cognitive impairment generally, and in a wide range of neurologic conditions, including AD, Parkinson's disease, depression, corticobasal degeneration, multiple sclerosis, and frontotemporal dementia (Obeid et al., 2007).

### 5.3.2. Aβ-related genes

Epigenetic influences on Aβ-producing mutations have long been suspected based on the heterogeneity of clinical presentation by patients who share mutations in the same APP, BACE, or PS1 genes — sometimes in identical promoter sites (Larner and Doran, 2006). Consistent with this notion, the APP (Davidson et al., 1992; Mani and Thakur, 2006; West et al., 1995), BACE, and PS1 (Fuso et al., 2005) genes all contain manipulable, methylated CpG sites. A case study has reported complete demethylation of the APP gene in an AD postmortem cortical sample, but not in similar samples from a normal control or Pick's disease patient (West et al., 1995). In vitro, expression of the BACE and PS1 genes is enhanced after folate deprivation-induced hypomethylation, and restored to normal when folate deprivation is accompanied by SAM supplementation. Expression of TACE, ADAM10, and APP was unaffected by these manipulations (Fuso et al., 2005). In vivo, exposure of APP-overexpressing transgenic mice to a folate/$B_{12}$/$B_6$-deficient diet is associated with enhanced SAH relative to SAM, as well as PS1 and BACE upregulation, enhanced Aβ deposition, and an accelerated appearance of intraneuronal Aβ and cognitive deficits (although the latter was quite modest) (Fuso et al., 2008).

Finally, it has been demonstrated that Aβ itself may induce genome-wide hypomethylation in murine cerebral endothelial cell cultures while, at the same time, causing specific hypermethylation and repression of the gene for neprilysin, an Aβ degrading enzyme (Chen et al., 2009). Our laboratory has replicated the global hypomethylating effects of Aβ in human SK-N-BE2 neuroblastoma cells, and extended the results to show hypomethylation (as well as several instances of hypermethylation) of specific CpG islands in the BACE (Fig. 5) and caspase-3 genes (Grover et al., unpublished). Together with hypermethylation of neprilysin, these effects suggest the potential for a vicious cycle in which Aβ-induced methylation changes feed back to enhance Aβ production, further methylation changes, and further Aβ production. Moreover, if the overall trend to hypomethylation after Aβ exposure were to functionally impact other key AD genes, additional synergisms might occur. For example, tumor necrosis factor (TNF)-α (Wilson, 2008) and caspase-3 are upregulated when hypomethylated (Müerköster et al., 2008), and increased levels of TNF-α (Janelsins et al., 2008; McAlpine et al., 2009; Sommer et al., 2009) and caspases (Xie et al., 2008; Xiong et al., 2008) enhance Aβ expression, potentially generating new vicious cycles.

### 5.3.3. Tau

Methylation mechanisms with respect to tau and neurofibrillary tangle formation have also been explored. As previously noted, in normal adults the AP2 binding site of the tau promoter is not methylated, but the SP1 and GCF binding sites are. SP1, a transcriptional activator site, is increasingly methylated and GCF, a promoter repressor site, is increasingly demethylated with age, suggesting an overall downregulation of tau gene expression (Tohgi et al., 1999c). Although a corresponding age-related decrease in normal tau protein, particularly in frontal cortex and hippocampus, has been reported, there was no correlation with the modest neurofibrillary tangle pathology in the same subjects (Mukaetova-Ladinska et al., 1996).

Tau phosphorylation mechanisms are, however, subject to cytoplasmic methylation reactions, and have been the subject of several recent reports. Our studies, for example, revealed colocalized immunoreactivity for the methyl binding complex component p66α (as well as HDAC1) with paired helical filament 1 (PHF1)-positive neurofibrillary tangles (Mastroeni et al., 2010). Protein phosphatase 2A (PP2A) is an enzyme that can dephosphorylate phosphorylated tau, an action that may be potently activated by methylation of the PP2A catalytic subunit at its L309 site. In N2a cultures carrying the APP Swedish mutation (APPswe) and

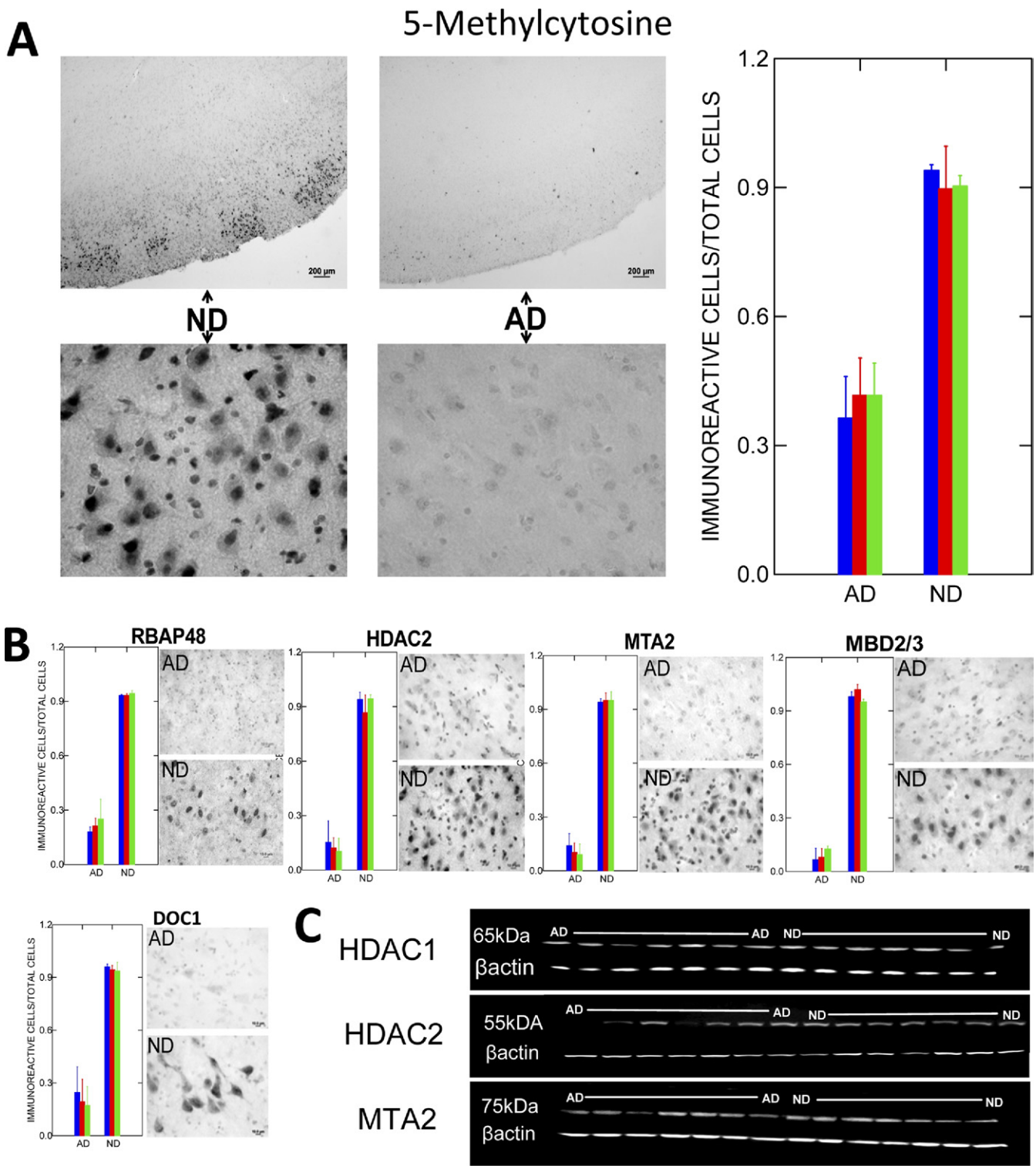


Fig. 2. DNA methylation markers in Alzheimer's disease (AD) and elderly control (ND) cortex. (A) Typical immunoreactivity for 5-methylcytosine, a global marker of DNA methylation, in AD and ND entorhinal cortex (from Mastroeni et al., 2008, with permission). Cases were well matched for age, gender, and postmortem intervals, which were all less than 3 hours, 15 minutes. Shaded bars represent means for different cases. Although glia and virtually all types of neurons exhibit 5-methylcytosine immunoreactivity, layer II "island" neurons, among the most vulnerable to AD pathology, exhibit particularly intense staining in ND cases, as shown in the upper left micrograph at low power. Such staining is weak to absent in AD cases (upper right micrograph). High power micrographs show the expected nuclear localization of immunoreactivity. Far right panel shows counts of immunoreactive neurons per total neurons per field. Normalizing to total neurons is important, as it helps to demonstrate loss of methylation within cell nuclei rather than loss of the methylated cell population itself. The significant decrement in AD ($p < 0.001$) is typical of dozens of AD and ND cases examined, with little to no overlap in any case. (B)

in APPswe/PS1 transgenic mice, levels of demethylated PP2A at L309 were significantly increased, corresponding with increases in tau phosphorylation at the tau-1 and PHF1 sites. Treatment with A$\beta$25-35 led to demethylation and enhanced tau phosphorylation (Zhou et al., 2008). Like treatment with A$\beta$25-35, exposure of rodent primary neuron cultures to methotrexate, a folate antagonist, also has been reported to result in demethylation of PP2A, with attendant enhancement of tau phosphorylation (as well as upregulation of APP and BACE) (Yoon et al., 2007). Consistent with all these findings, injections of homocysteine into rats for 2 weeks yielded decreased PP2A L309 methylation and PP2A activity, effects that were reversed by simultaneous administration of folate and vitamin B12. Hippocampal samples from the rats and from AD patients exhibited immunohistochemical colocalization of demethylated, but not methylated PP2A with hyperphosphorylated tau (Zhang et al., 2008).

#### 5.3.4. Aberrant cell cycle/apoptosis

A wide range of evidence suggests that attempted or aberrant re-entry into the cell cycle and/or apoptosis of neurons may be a common neurodegenerative mechanism in AD (reviewed in Neve and Mcphie, 2006). Many of the critical components of the cell cycle and apoptosis pathways are upregulated in degenerating AD neurons, and are subject to regulation by DNA methylation, including the P16, P21, P27, P53, RB1 (Moreira et al., 2009), cyclin B2 (Tschöp and Engeland, 2007), alternate open reading frame (ARF) protein product (Robertson and Jones, 1998), caspase 1 (Jee et al., 2005), caspase 3, caspase 7, caspase 8, and caspase 9 (Müerköster et al., 2008) genes. Hypomethylation of these genes would be expected to promote aberrant cell cycle events, and global hypomethylation has been reported to occur in cells as they move from the G(0) stage characteristic of postmitotic neurons to the G(1) stage characteristic of cell cycle re-entry (Brown et al., 2007). Indirect support for the role of hypomethylation in aberrant cell cycle events is provided by studies demonstrating apoptosis of cultured neurons when exposed to high homocysteine levels (Ho et al., 2002). Although many other biologic effects are possible, such treatment is known to hypomethylate DNA (Reynolds, 2006), and concurrent treatment with SAM antagonized the apoptotic effect (Ho et al., 2002). Interactions of the histone acetyltransferase Tip60 with the $\gamma$-secretase-generated APP C-terminal fragment APP-CT58, which translocates to the nucleus, also lead to apoptosis of human H4 neuroglioma cells (Kinoshita et al., 2002).

#### 5.3.5. Inflammation

Key genes at almost every level of the inflammatory response appear to be subject to DNA methylation influences. A highly abbreviated list of examples includes complement C3, factor B, interleukin (IL)-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-5, IL-6, IL-13, TNF-$\alpha$, TNFRI, interferon (INF)-$\gamma$, SOCS, S100A2, the chemokine receptors CXCR4 and CCR7, clusterin (ApoJ), and iNOS (Buslei et al., 2006; Mejía et al., 1995; Mi and Zeng, 2008; Mori et al., 2005; Nile et al., 2008; Parker et al., 2008; Pieper et al., 2008; Suuronen et al., 2007; Van Panhuys et al., 2008; Van Rietschoten et al., 2008). The TNF-$\alpha$ promoter, for example, contains 12 CpG-rich sites. Two of those sites ($-304$ and $-205$) are hypomethylated on exposure of macrophages to the classic inflammatory stimulus lipopolysaccharide, and their extent of hypomethylation is correlated with increasing expression of TNF-$\alpha$ (Wilson, 2008). Likewise, a single CpG site in the IL-6 promoter ($-1181$) is significantly hypomethylated in rheumatoid arthritis and this hypomethylation is correlated with IL-6 mRNA levels (Nile et al., 2008). Although virtually none of the many factors in inflammation has been investigated with respect to its DNA methylation status in AD, this could be a fertile area for investigation given the heightened expression of these molecules in the disorder. A recent Parkinson's disease study, for example, has demonstrated that the TNF-$\alpha$ promoter is hypomethylated in the substantia nigra relative to several other brain regions. Because DNA methylation of CpG sites in the TNF-$\alpha$ promoter inhibits SP1 and AP2 transcription factor binding and decreases TNF-$\alpha$ expression, it has been speculated that nigral TNF-$\alpha$ hypomethylation might explain the heightened vulnerability of nigral dopamine neurons to TNF-$\alpha$-mediated inflammatory reactions (Pieper et al., 2008).

#### 5.3.6. Apolipoproteins

Again, no specific studies have been done on the methylation status of apolipoprotein E (ApoE) in AD. Notably, however, Wang and colleagues (Wang et al., 2008) have reported that although the ApoE promoter is poorly methylated, the ApoE $\varepsilon$4 allele contains methylated CpG sequences that are not extant in the $\varepsilon$2 or $\varepsilon$3 alleles. Because not all $\varepsilon$4 carriers develop AD, it would be of great interest to know if methylation status at $\varepsilon$4 CpG sites is altered in $\varepsilon$4 carriers who develop (or do not develop) AD. Similarly, it might be useful to determine whether the significant but relatively low penetrance of ApoJ (clusterin) single nucleotide polymorphisms (SNPs) to AD risk (Harold et al., 2009; Lambert et al., 2009) might be explained by methyl-

Representative immunoreactivity and cell counts for various methyl-cytosine-guanine-binding protein (MeCP)1 components in AD and ND neocortex. Significant AD decrements ($p < 0.05$) were observed with all the markers — again with little to no overlap. (C) Western blots (normalized to $\beta$-actin) for these and other methylation markers exhibit immunoreactivity at appropriate molecular weights, with AD/ND differences similar to those observed by immunohistochemistry, suggesting that the latter are not due to cross-reactivity with other antigens.

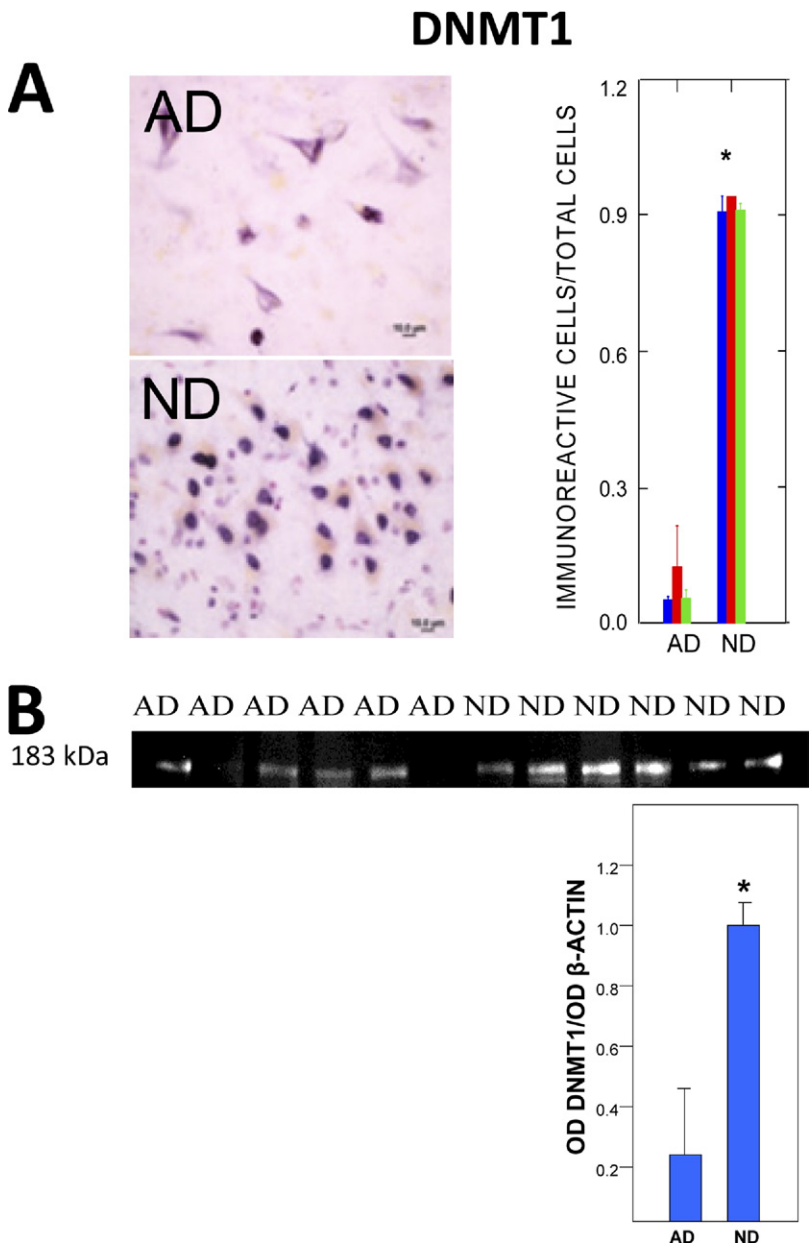


Fig. 3. Immunoreactivity for DNA methyltransferase (DNMT)1, the most prevalent methyltransferase in adult mammals. (A) Typical DNMT1 immunoreactivity and cell counts ($p < 0.001$) in Alzheimer's disease (AD) and elderly control (ND) neocortex. Shaded bars represent means for different cases. (B) Western blots (normalized to $\beta$-actin standards) show immunoreactive bands at appropriate molecular weights for DNMT1 and a significant ($p < 0.01$) decrement in AD cases.

ation changes, because the ApoJ promoter is rich in CpG sites and expression is increased on hypomethylation (Suuronen et al., 2007).

## 6. Conclusions

Global epigenetic changes, acting on a wide range of genes and biological pathways, appear to help orchestrate the cellular alterations that drive development, aging, and, in some cases, disease. Likewise, global epigenetic changes have been observed in pathologically vulnerable regions of the AD brain, and key genes in virtually every mainstream pathologic pathway in AD are known to be labile to such changes. The ability of epigenetic mechanisms to initiate an extremely wide range of pathogenic responses — an orchestrating capacity perhaps equaled only by transcription factors (which themselves often work together with epigenetic mechanisms to direct expression in specific sets of genes) (e.g., Agrawal et al., 2007; Ivascu et al., 2007; Yakovlev et al., 2010) — provides a relatively unique integrative framework for the diverse genetic factors and multifactorial pathology of AD, including A$\beta$, tau, inflammation, mitochondrial metabolism, oxidative stress, and aberrant cell cycle/

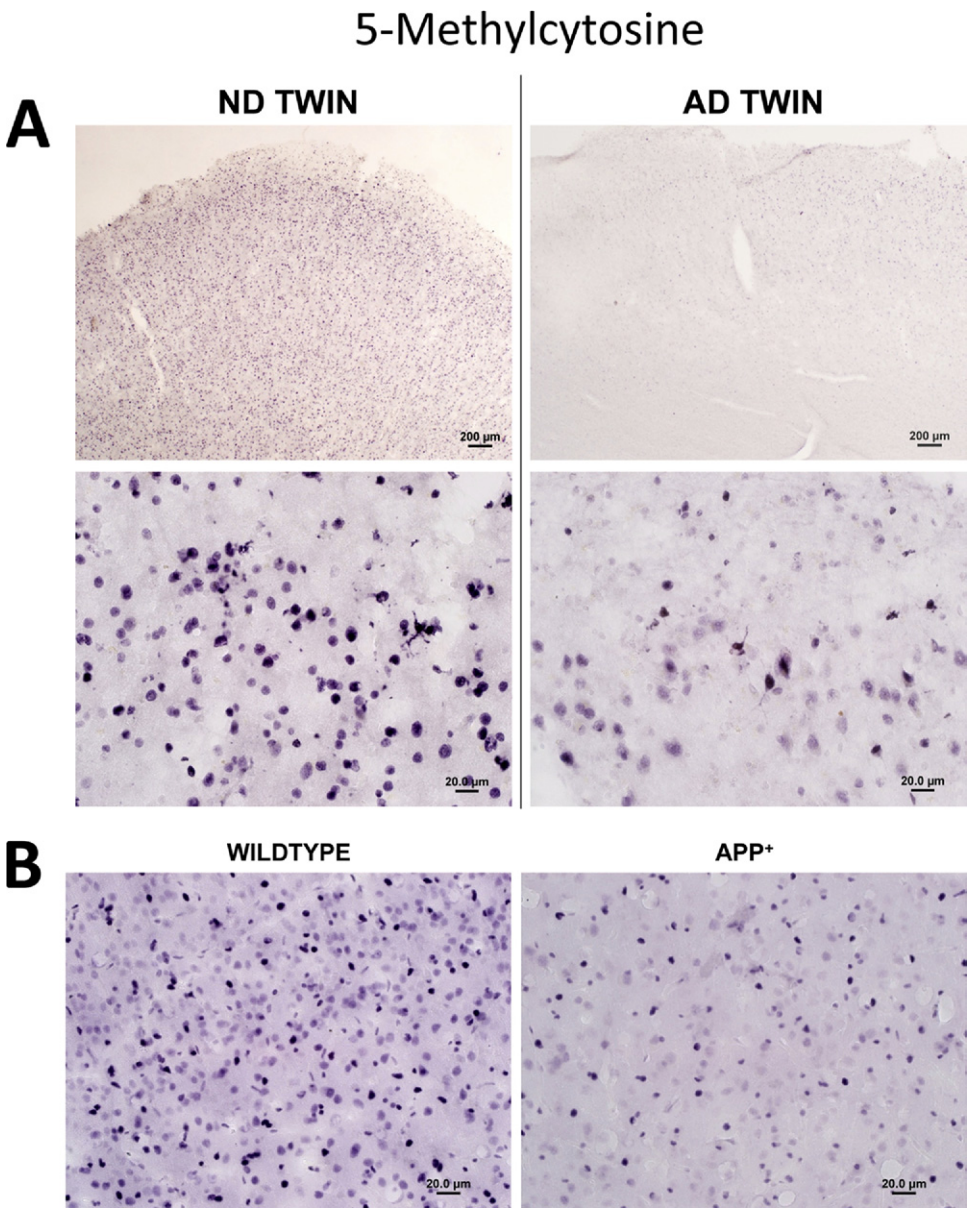


Fig. 4. Decreased overall DNA methylation in monozygotic twins discordant for Alzheimer's disease (AD). (A) Global hypomethylation (5-methylcytosine immunoreactivity) in an AD monozygotic twin compared with his normal sibling at low (upper micrographs) and high (bottom micrographs) power (Mastroeni et al., 2009) (courtesy of PLoS One). (B) Similar findings have recently been observed by our group in amyloid precursor protein (APP) transgenic mice compared with their wild type littermates.

apoptosis events. Moreover, the epigenetic modifications that have been reported in AD, particularly with respect to DNA methylation, typically resonate with similar trends in aging, and may therefore help explain not only the pathologic complexity of AD, but also the particular salience of aging as an AD risk factor.

Finally, whereas the field of epigenetics has previously emphasized mechanisms for preserving epigenetic profiles across generations of dividing cells, there is now ample precedent for active, dynamic epigenetic alterations in post-mitotic cells, including neurons, that play important roles in neuroendocrine, learning and memory, and apoptotic processes. These latter, landmark studies provide the tools for subsequent explorations of how the epigenetic modifications that have already been reported in AD occur, as well as a mechanistic underpinning for the AD genome-wide methylation profiling that is now in progress.

## 7. Future directions

At the level of basic research, DNA methylation profiling of aging and AD subjects is eagerly awaited in order to develop a better portrait of the normal methylation status of all genes across the AD genome, how that status may change in AD, and

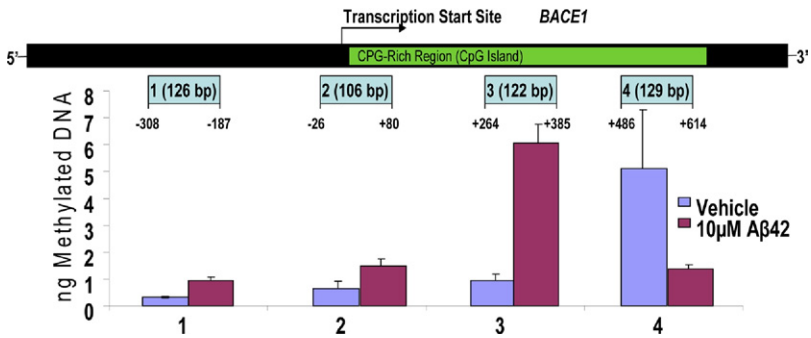


Fig. 5. MethylMiner methylation profile of selected flanking and initial *BACE* promoter sites after exposure of differentiated SK-N-BE(2) neuron-like cultures to 10 $\mu$M A$\beta$42. MethylMiner™ Methylated DNA Enrichment Kits (Invitrogen, Carlsbad, CA, USA) were employed to enrich and fractionate double-stranded DNA based on cytosine-phosphate-guanine (CpG) methylation density. The highly methylated region showed significant hypomethylation while poorly methylated regions exhibited hypermethylation (Grover et al., unpublished). Correlations with amyloid $\beta$ peptide (A$\beta$) production can help establish the functional relevance of methylation modifications at these and other CpG sites within the *BACE* gene.

whether or not such changes implicate AD-related proteins and pathogenetic processes. These studies would be especially significant if they were conducted in tandem with genome-wide gene expression arrays because the experiments would then provide validation of the functional effects of DNA methylation changes on gene expression. In addition, knowing the methylation states of genes containing putative AD SNPs could be useful. Many such SNPs, for example, remain controversial because their odds ratios for disease risk consistently hover at the statistical edges of significance or they fail to replicate in some studies but not others. Epigenetic regulation of the SNP genes could account for this variability. For example, a gain or decrease of function SNP could be compensated for by epigenetic downregulation or upregulation, respectively, of the gene's expression, so that some carriers might in effect possess the SNP with relative impunity. Finally, as valuable as large-scale epigenetic profiling of the AD genome will be, it will still not tell us why or how the profiles were altered, nor will it give us a detailed profile of each gene. Genome-wide methylation profiling is presently only able to sample a few of the CpG-rich sites within each gene, although the technology is rapidly expanding. Because both hyper- and hypomethylation can occur at different CpG sites in the same gene, with one but not the other causing functional changes in gene expression (e.g., Murgatroyd et al., 2009), follow-up studies giving detailed methylation maps of AD-relevant genes and concomitant changes in their expression will be essential. These same considerations may also apply to other neurologic conditions such as schizophrenia and bipolar disorders, where epigenetic mechanisms are being pursued (reviewed in Pidsley and Mill, 2011).

Of course, to hypothesize that epigenetic changes play a role in brain aging, AD, and other neurologic disorders still begs the question of what causes the epigenetic changes? The environment that cells and organisms are exposed to can have a profound influence on epigenetic mechanisms (Smith et al., 2008; Waterland and Michels, 2007), but simple stochastic processes may do so as well (Jaenisch and Bird, 2003). Whether as environmentally driven or randomly occurring events, however, the probability of epigenetic modifications must logically increase with time, and increased time is precisely what the advanced ages reached by many human beings may afford. Some of these modifications may be inconsequential, depending on the CpG site, the gene, or the organ. For example, inadvertent upregulation of an A$\beta$-synthesizing gene might have little to no impact on a muscle cell, whereas it could be highly significant in a pyramidal neuron. Similarly, the brain lacks several major defenses against inflammatory attack (Gasque et al., 2002; Zanjani et al., 2005), so that the inadvertent upregulation of a proinflammatory gene might be uniquely problematic there. Thus, the origin and organ-specific consequences of epigenetic modifications are important considerations for AD epigenetic studies, and will continue to be critical targets for AD basic research into epigenetic mechanisms.

At the clinical level, the initiation of AD trials with folate/B vitamins/SAM may constitute one means of testing an epigenetic orchestration hypothesis of AD, although it is becoming increasingly evident that reversing the course of human AD with any treatment may be an overambitious goal. For example, in a recent trial B vitamin treatment significantly slowed cognitive decline in mild AD, but was without effect in more advanced cases (Aisen et al., 2008). A trial in mild cognitive impairment patients might therefore be of great interest. A second impediment to successful treatment of epigenetic defects may be achieving sufficiently high levels of epigenetic therapeutics not only within cells, but within neuronal nuclei. Moreover, the many different biochemical pathways impacted by epigenetic mechanisms may make targeting specific disease processes difficult. Beyond folate and other such approaches, cancer chemotherapeutics that are directed at epigenetic mechanisms are available, but would need to be considered carefully. DNA demethylating agents such as 5-azacytidine and decitabine, for example, might actually prove harmful in AD given the profound global hypomethylation of AD neurons (Mastroeni et al., 2009, 2010). HDAC inhibitors such as val-

proate, by contrast, might counter many of the epigenetic changes that have been reported in AD, and have, in fact, been used successfully to improve cognitive outcome measures in AD transgenic mouse models (Fischer et al., 2007; Francis et al., 2009; Ricobaraza et al., 2009; Su et al., 2004). Treatment trials in human AD patients, however, have not been particularly encouraging, perhaps due to somnolence, agitation, and other side effects of the drug (Herrman et al., 2007; Profenno et al., 2005) or to lack of specificity of the drug to epigenetic mechanisms alone.

With respect to the development of new treatments for AD, a direct role for epigenetics would be the design and application of epigenetic therapeutics that have appropriate effects on specific epigenetic mechanisms in specific genes or sets of genes. As we have emphasized throughout, however, one of the defining hallmarks of epigenetic mechanisms is their ability to exert effects over many genes, and, accordingly, virtually all present epigenetic therapeutics also exert their effects over many genes. Unless a broad modifier such as an HDAC inhibitor can be found that just happens to impact the right genes, while sparing significant effects in others, the specificity requirements of an AD epigenetic therapeutic will be challenging. Nonetheless, elucidating specific genes that undergo significant epigenetic alterations in AD — as is now in progress in our laboratory and elsewhere — could, at the very least, help direct our attention to the most salient pathogenic elements of the disorder and to more conventional (e.g., agonist/antagonist) approaches to the protein products of the epigenetically-modified genes.

## Disclosure statement

The authors state that they have no actual or potential conflict of interest that could inappropriately influence this work.

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