Epigenetic dysregulation and the pathophysiology of Alzheimer's disease

Epigenetic Dysregulation and the Pathophysiology of Alzheimer's Disease

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Dedicated to my Grandfather
Giuseppe Abramo.

You may have forgotten my name, but I will hold on to yours for as long as I can.

Chapter 1

General Introduction

Alzheimer's disease (AD) is a progressive, irreversible neurodegenerative disorder characterized by progressive dementia that culminates in death. Its etiology and pathogenesis are complex, and encompass multiple genetic and environmental risk factors. Yet, with the exception of Aß-inducing mutations, none of these molecular and genetic factors appears to have absolute penetrance in causing the disorder. Moreover, many individuals may possess the most salient risk factors for AD, as well as express profuse Aß and tau pathology, without ever exhibiting the clinical symptoms of dementia[1]. In the face of such complexity and so many potential pathogenic mechanisms, the broad ability of epigenetics to coordinately alter gene expression and the many biological pathways that gene expression sub-serves could provide a critical, missing link.

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 consistent manner across thousands of genes and dozens of biological pathways, yet can do so differentially in monozygotic twins 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.

Gene expression in the AD brain has been shown to be altered in numerous reports [2-8], including a recent large-scale expression array study of single cell laser-captured entorhinal cortex layer II neurons [2.] Multiple pathways have been reported to be affected, from energy metabolism[5] to inflammation [6, 8]. The extent and breadth of these gene expression changes suggest a complex web of interacting effects on large numbers of transcription factors, and also raise the

possibility of more unitary mechanisms that are capable of modulating coordinate expression of large numbers of genes. In particular, there are a variety of epigenetic mechanisms that modulate the accessibility of genes to the transcription machinery, including histone modification, binding of non-histone proteins, and DNA methylation. [9, 10]

DNA methylation is a highly conserved process that has been implicated in many different modalities of gene expression. The factors responsible for the methylation process are a family of DNA methyltransferases that have been shown to catalyze the transfer of a methyl group to single-stranded DNA using S-adenosyl methionine (SAM) as the methyl donor. The recognition sequence for the mammalian DNA methyltransferase is relatively invariant, with nearly all cytosine methylations occurring on 5'-C-p-G-3' CpG 11, 12. There are four known active DNA methyltransferases in mammals, DNMT1, DNMT2, DNMT3A and DNMT3B. Of these, DNMT1 is the most abundant in mammalian cells. DNMT1 has been reported to be a key player in maintaining methylation in somatic cells, and loss of this enzyme has been shown to lead to nuclear disorganization, increased histone acetylation, and apoptosis [13-17].
Once methylation has occurred, methylation stability is maintained by the binding of specific complexes, MeCP1, to methylated regions of DNA. MeCP1 is not bound directly to methylated DNA, but rather to a single methyl-CpG-binding domain protein, MBD2. The resulting MeCP1/MBD2 complex is composed of 10 known proteins that include the complete nucleosome remodeling and histone deacetylase (NuRD) core, as well as MBD2. This group of proteins, in conjunction with CDK2AP1 (Doc1), make up a complex capable of nucleosome remodeling and histone deacetylation [18, 19.]
Methylation and methylation stabilizing factors can orchestrate changes in expression of a wide range of genes [9, 10, 18, 20-23].
Accordingly, one of the central hypotheses tested in this thesis is

that alterations in methylation and methylation stability might provide an over-arching mechanism that could explain expression differences in the thousands of genes that are reportedly altered in AD.

Current research has shown that 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 [24-26]). Deficits in folate and alterations in the methionine/homocysteine cycle have been reported in aging and AD (reviewed in[27]), and may therefore provide a basis for the tendency to genome-wide hypomethylation in aging and AD. Although one prospective study failed to find an association between dietary folate, vitamin B12, or vitamin B6 with incident AD[28], CSF folate has nonetheless been reported to be significantly decreased in AD [29], as has CSF and brain SAM and one of its synthesizing enzymes, methionine S-adenosyltransferase [29-31]. 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 [32] .

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 [33]. 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. 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.

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[34]. A non-nuclear form of histone 1 has been shown to be upregulated in astrocytes and neurons in brain regions that are rich in AD pathology[35]. Linker histone H1, a vital component of chromatin, has been reported to preferentially bind Aß42, as well as other Aß-like structures of numerous proteins[36]. 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[37]. Viewed more broadly, the present thesis research has demonstrated globally decreased nuclear IR for both HDAC1 and HDAC2 in AD entorhinal cortex[38].

In summary, the broad ability of epigenetic mechanisms to regulate gene expression suggests that the manifold and diverse

gene expression changes that have been reported in aging and AD could be caused by alterations in the epigenetic machinery, and many such alterations have been found in these conditions. The question then arises, however, what causes the epigenetic alterations? Normal cell function requires constant exchange of molecules between the nucleus and the cytoplasm. Virtually all nuclear proteins, for example, begin their existence in the cytoplasm, and their failure to be translocated back to the nucleus would be as catastrophic as their never having been synthesized at all. Dysregulation of nucleocytoplasmic transport might also account for the changes in epigenetic proteins that have been reported in aging and AD, particularly decreases in their nuclear levels and IR.

Collectively, future studies in nucleocytoplasmic transport dysfunction in AD may account for the diversity so often observed in the pathophysiology of the disease. Whether the many other nuclear proteins that rely on RAN-mediated nucleocytoplasmic transport follow this pattern remains to be determined. If they do, however, deficits in RAN followed by ectopic intracellular localization of epigenetic and other transcription-related molecules could well be a central event in the pathophysiology of AD, and could provide an overarching, integrative mechanism for the myriad pathogenic processes that occur in the disorder.

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Chapter 2

Epigenetic Mechanisms in Alzheimer's Disease.

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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.

Key Words: Epigenetics, DNA methylation, histone acetylation, rDNA, miRNA, genetics, gene expression, amyloid β peptide, inflammation, oxidative stress, cell cycle

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

2.0 Epigenetic regulation of gene expression

Epigenetic mechanisms modify heritable and non-heritable 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 (Wilson and Jones 1983; Bandyopadhyay and Medrano 2003; Liu et al. 2003; Fraga and Esteller 2007; Poulsen et al. 2007). 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 (Wilson and Jones 1983; Bandyopadhyay and Medrano 2003; Liu et al. 2003; Fraga and Esteller 2007; Poulsen et al. 2007).

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 (Siegmund 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 (Siegmund 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 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 et al. 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 (Fraga et al. 2005; Flanagan et al. 2006).

2.1 Histone modifications.

Epigenetic mechanisms typically involve changes in the micro- and macro-structure 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, ADP-ribosylation, and other post-translational 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 an RNA methyltransferase, although it also has 5-cytosine DNA methyltransferase activity (Tang et al., 2003) and 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 non-coding 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 to liver has also been found to be highly correlated with differences in gene expression for these two 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 to 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 (Figueiredo et al. 2006). More generally, Gius and colleagues (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 to alterations at other sites (c.f., Murgatroyd et al., 2009; Yakovlev et al., 2009). 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 (Robertson and Jones 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 co-repressors 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, MBD2. In addition to inducing histone modifications, MBD2-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 (Valinluck and Sowers 2007; Kriaucionis and Heintz 2009; Tahiliani et al. 2009). 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 TET1 (Tahiliani et al. 2009). Normally, 5-hydroxymethylcytosines are stripped from genomic DNA by glycosylases of the base excision repair (BER) system (Steen et al., 2008), 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 et al. 2004; Valinluck and Sowers 2007). Because hydroxymethylated

CpGs might go undetected by 5-methylcytosine antibodies or conventional bisulfite sequencing, it is also possible that hydroxymethylation could lead to under-reporting of DNA methylation, a possibility that the present authors are now investigating.

2.3 RNA-related mechanisms.

Epigenetic regulation also extends to mechanisms involving RNA such as micro-RNAs (miRNAs) and hereditable and cell cycle-maintained silencing of a portion of the ribosomal RNA genes. 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 inactive 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 & Grummt, 2001), and treatment with 5-aza-2-deoxycytidine (azacytidine), a hypomethylating agent, enhances expression by rDNA genes (Santoro & 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 post-transcriptional manner by binding to their target mRNAs, inhibiting translation or, less often, inducing cleavage of the mRNAs (reviewed in Yang, 2007).

3.0 Dynamic epigenetic regulation in adult neurons

3.1 Histone modifications.

Histone modifications have been implicated in broad neurobiological processes such as development of the CNS (reviewed in MacDonald and Roskams, 2009), post-traumatic stress disorders (Sokolova et al., 2006), childhood abuse/suicide (Meaney et al., 2007; McGowan et al., 2009), 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 GDNF and BDNF transcription (Wu et al., 2008), 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., 2008), 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, Fiore, and Schratt, 2009). In turn, these and other miRNA-mediated mechanisms have been investigated in various CNS disorders, including 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 DNMT1 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; Metivier 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 and co-workers (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 (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 pro-hormone 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 to 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, MBD2 and DNMT3a/b may participate in dynamic demethylation processes. It has been reported, for example, that transient co-expression of MBD2 and methylated promoters results in demethylation and activation of gene expression, whereas knockdown of MBD2 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 (Metivier et al., 2008; Kangaspeska

et al., 2008). Here, DNMT3a/b, in concert with p68, bind and deaminate selective CpG sites, creating mismatches that are recognized by 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 (Metivier 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.

4.0 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 (Blalock et al. 2004), 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. 2006), Aβ (Selkoe 2003), tau (Maeda et al. 2006), and hundreds to thousands of genes (Parachikova et al. 2007; Berchtold et al. 2008) 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 (Wilson and Jones 1983; Bandyopadhyay and Medrano 2003; Liu et al. 2003; Fraga and Esteller 2007; Poulsen et al. 2007), 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 (Romanov and Vaniushin 1980; Romanov and Vanyushin 1981; Wilson et al. 1987; Golbus et al. 1990; Lu et al. 2006). Yu and colleagues (Guo et al. 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 (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., p-53-dependent apoptosis) (Jackson-Grusby et al. 2001) and the increased inflammatory tone that occurs with advancing age (Wilson 2008).

Hypomethylation of non-coding regions and other sites also occurs with age and has been suggested to be relevant to the aging process. For example, repetitive sequences (Romanov and Vanyushin 1981; Mays-Hoopes et al. 1986; Rath and Kanungo 1989), 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 (Lu et al. 2006).

Age-dependent hypomethylation of a number of specific genes related to AD has been reported. For example, methylation of cytosines in the 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. 1999). DNA methylation within the tau promoter reportedly declines overall with age, but with interesting variations at different transcription factor binding sites: binding sites for GCF, which represses GC-rich promoters, become hypomethylated with age, whereas binding sites for 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. 1999). 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 AP2 and SP1 binding sites show significant hypomethylation with age (Tohgi et al. 1999). 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; Issa et al. 1996). Tumor suppressor genes appear particularly apt to show increasing methylation with age, providing a potential link to age-related cancers (Romanov and Vaniushin 1980; Mays-Hoopes 1989; Issa et al. 1994; Issa and Baylin 1996; Neumeister et al. 2002; Liu et al. 2003; Figueiredo et al. 2006; Lu et al. 2006; So et al. 2006; Fraga and Esteller 2007; Jacinto et al. 2007). Many of these hypermethylation events could be due to age-related increases in DNMT3a/b expression (Lopatina et al. 2002; Liu et al. 2003), as these methyltransferases are responsible for de novo methylation of DNA. Alternatively, they have also been linked to demethylation processes (Metivier et al., 2008; Kangaspeska 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 et al. 2001). These mechanisms could be relevant to AD since 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, PS1, 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 "LEARn" (latent early-life associated regulation) model of age-related neurologic disorders (Lahiri et al. 2007; Wu et al. 2008a). Support for this hypothesis comes from APP transgenic mouse research wherein earlier epigenetic manipulations appear to accelerate or delay the expression of Aβ pathology (Fuso et al. 2008). Likewise, early exposure of monkeys to Pb reportedly decreases DNMT activity, increases APP, BACE, and SP1 expression, and alters levels and distribution of Aβ in the animals in late life (Wu et al. 2008b).

5.0 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 non-nuclear form of histone 1 appears 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 Aβ-42, as well as Aβ–like structures of numerous proteins (Duce et al. 2006). In addition, the H1 molecule has been shown to be a major target for poly (ADP-ribosyl)ation 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 (Chessell et al. 1994). 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 Aβ 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 Aβ 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 CREB-binding 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 (Hebert 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, Zhu, and van Oudenaarden, 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'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., 2009).

Two studies have used computational analysis methods to reveal miRNA target sites in BACE mRNA that may be functionally relevant to AD pathogenesis. Wang and colleagues (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 (Hebert et al., 2008).

5.3 DNA methylation.

5.3.1 Genome-wide and multi-gene 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 to matched, non-demented elderly control (ND) cortical neurons and glia (Figs. 2, 3), whereas no such changes were observed in cerebellum, a brain region that is relatively spared in AD (Mastroeni et al. 2008). 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 Mason 2002; Choi et al. 2005). 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), 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, cortico-basal degeneration, multiple sclerosis, and fronto-temporal 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, recent reports have established that the genes encoding APP (West et al. 1995; Miller et al. 2004; Mani and Thakur 2006), BACE, and PS1 are methylated at CpG sites under normal conditions, whereas TACE and ADAM10 are not (Fuso et al. 2005). 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 exposure of cultured neurons to folate/B12-deficient medium results in hypomethylation of PS1 and BACE, increased PS1 and BACE expression, and enhanced Aβ production (Fuso et al. 2005). Similarly, exposure of APP-overexpressing transgenic mice to a folate/B12/B6-deficient diet is associated with enhanced SAH relative to SAM, 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). By contrast, exposure to exogenous SAM inhibits the hypomethylation of PS1 and decreases Aβ secretion in neuroblastoma cell lines (Fuso et al. 2005).

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, TNF-α (Wilson, 2008) and caspase-3 are upregulated when hypomethylated (Muerkoster 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. 1999). 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 co-localized immunoreactivity for the methyl binding complex component p66α (as well as HDAC1) with PHF1-positive neurofibrillary tangles (Mastroeni et al. 2008). 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 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 PHF-1 sites. Treatment with Aβ25-35 led to demethylation and enhanced tau phosphorylation (Zhou et al. 2008). Like treatment with Aβ25-35, 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 two 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 co-localization of demethylated, but not methylated PP2A with hyperphosphorylated tau (Poleshko 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 (Corneveaux et al. 2010). 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 (Honda et al. 2005), cyclin B2 (Tschop and Engeland 2007), ARF (Robertson and Jones 1998), caspase 1 (Jee et al. 2005), caspase 3, caspase 7, caspase 8, and caspase 9 (Muerkoster 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 γ-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, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-13, TNF-α, TNFRI, INF-γ, SOCS, S100A2, the chemokine receptors CXCR4 and CCR7, clusterin (apoJ), and iNOS (Mejia et al. 1995; Li et al. 2005; Buslei et al. 2006; Suuronen et al. 2007; Mi and Zeng 2008; Nile et al. 2008; Parker et al. 2008; Pieper et al. 2008; van Panhuys et al. 2008; van Rietschoten et al. 2008). The TNF-α 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-α (Wilson 2008). Likewise, a single CpG site in the IL-6 promoter (-1,181) 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-α promoter is hypomethylated in the substantia nigra relative to several other brain regions. Since DNA methylation of CpG sites in the TNF-α promoter inhibits SP1 and AP-2 transcription factor binding and decreases TNF-α expression, it has been speculated that nigral TNF-α hypomethylation might explain the heightened vulnerability of nigral dopamine neurons to TNF-α-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 (2008) have reported that although the ApoE promoter is poorly methylated, the ApoE ε4 allele contains methylated CpG sequences that are not extant in the ε2 or ε3 alleles. Because not all ε4 carriers develop AD, it would be of great interest to know if methylation status at ε4 CpG sites is altered in ε4 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) SNPs to AD risk (Harold et al. 2009; Lambert et al. 2009) might be explained by methylation changes, since the ApoJ

promoter is rich in CpG sites and expression is increased on hypomethylation (Suuronen et al. 2007).

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)—provides a relatively unique integrative framework for the diverse genetic factors and multifactorial pathology of AD, including Aβ, tau, inflammation, mitochondrial metabolism, oxidative stress, and aberrant cell cycle/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 postmitotic 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.

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 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. 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, 2010).

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 (Waterland and Michels., 2007; Smith and Kim, 2008), 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 Aβ-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 pro-inflammatory 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 over-ambitious 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 succesful 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., 2008, 2009). HDAC inhibitors such as valproate, 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 (Su et al., 2004; Fischer et al., 2007; Francis et al., 2009; Ricobaraza et al., 2009; Guan et al., 2009). Treatment trials in human AD patients, however, have not been particularly encouraging, perhaps due to somnolence, agitation, and other side effects of the drug (Profenno et al., 2005; Herrman et al., 2007) 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.

Acknowledgements

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Figures

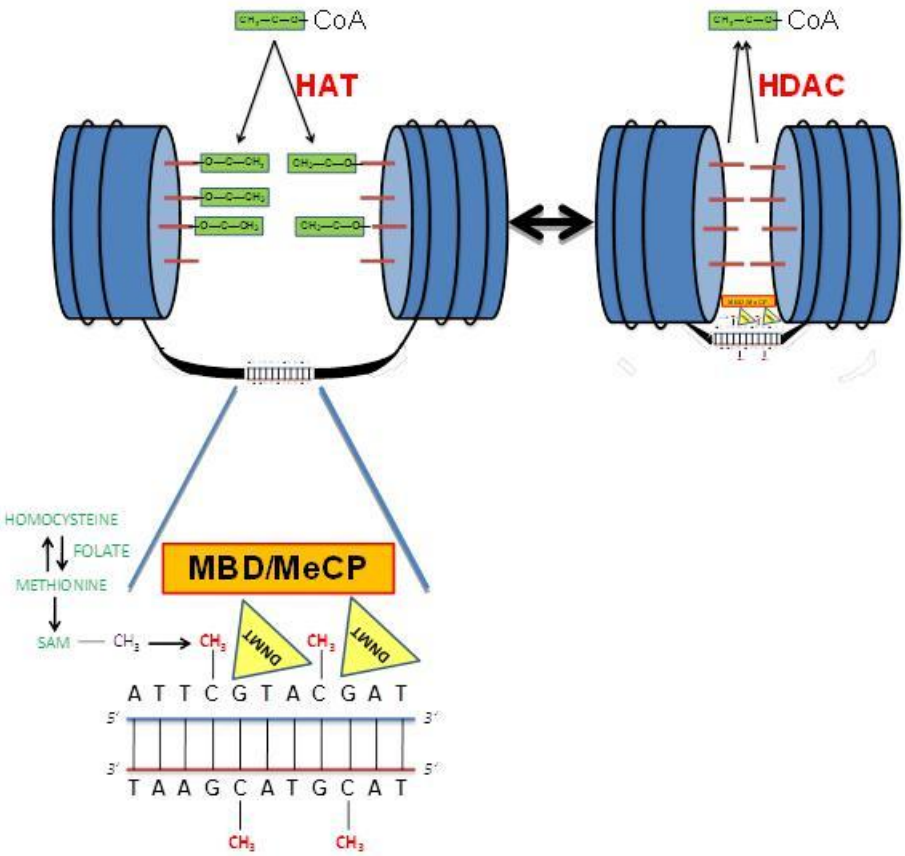
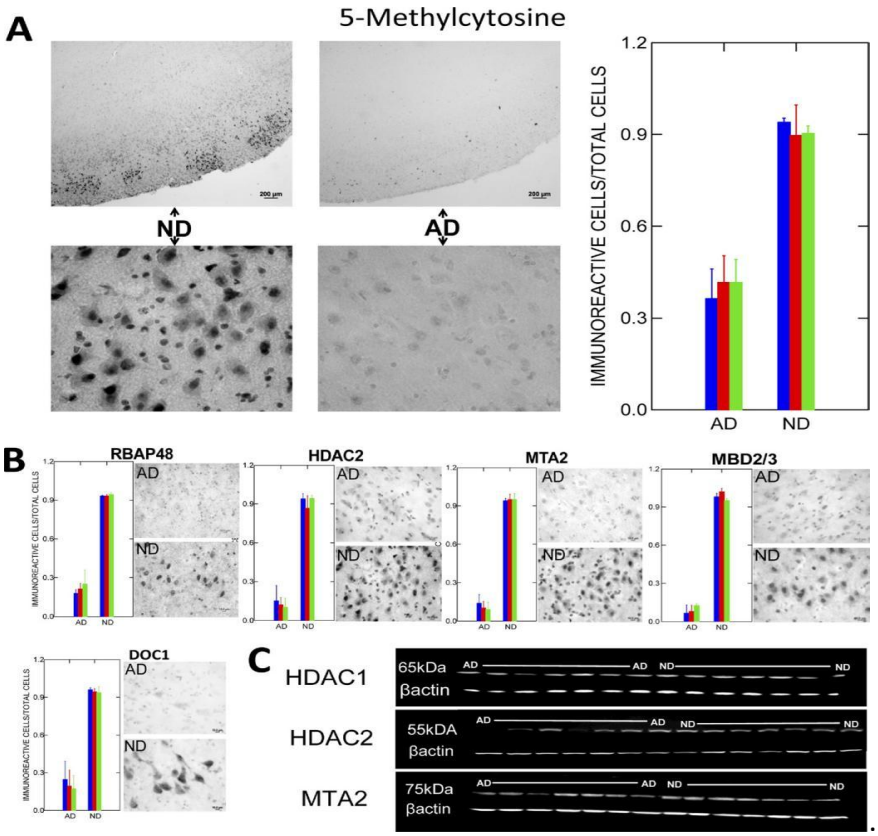


Fig. 1. Simplified schematic of histone acetylation and DNA methylation. (Upper Left) In transcriptionally active genes the

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.

Fig. 2. DNA methylation markers in AD and 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)

Representative immunoreactivity and cell counts for various MeCP1 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 β-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.

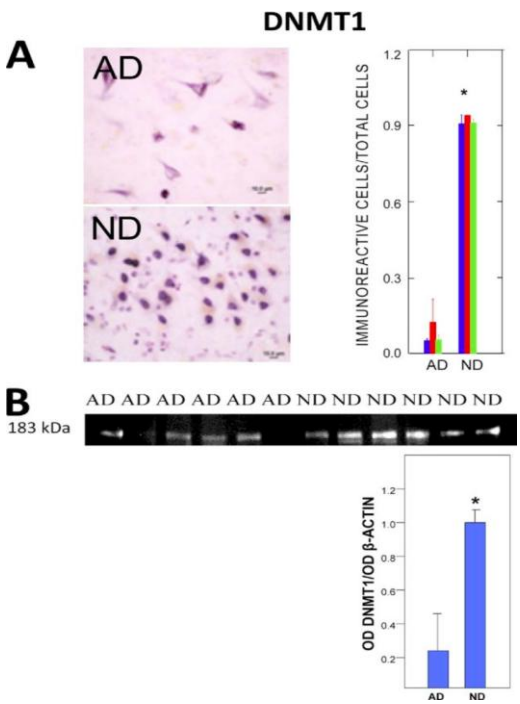


Fig. 3. Immunoreactivity for DNMT1, the most prevalent methyltransferase in adult mammals. A) Typical DNMT1 immunoreactivity and cell counts ($P < 0.001$) in AD and ND neocortex. Shaded bars represent means for different cases. B) Western blots (normalized to β-actin standards) show immunoreactive bands at appropriate molecular weights for DNMT1 and a significant ($P < 0.01$) decrement in AD cases.

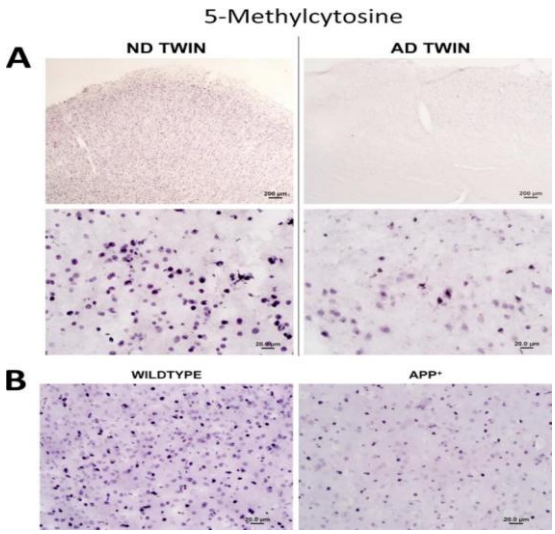
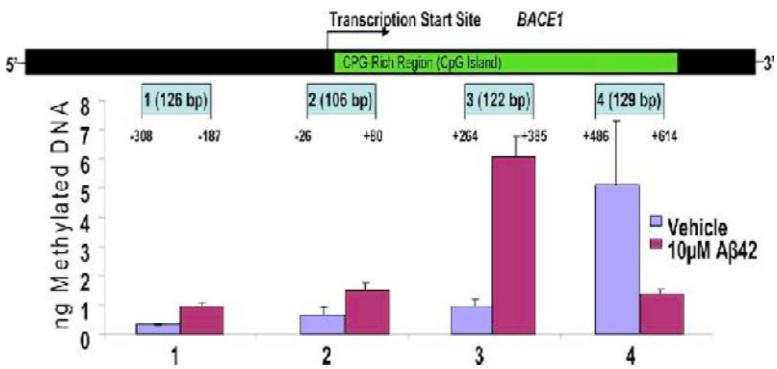


Fig. 4. Decreased overall DNA methylation in monozygotic twins discordant for AD. A) Global hypomethylation (5-methylcytosine immunoreactivity) in an AD monozygotic twin compared to his normal sibling at low (upper micrographs) and high (bottom micrographs) power (Mastroeni et al. 2009) (courtesy of PLoS1). B) Similar findings have recently been observed by our group in APP transgenic mice compared to their wildtype littermates.

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 μM Aβ42. MethylMiner™ Methylated DNA Enrichment Kits (Invitrogen, Carlsbad, CA) were employed to enrich and fractionate double-stranded DNA based on

CpG methylation density. The highly methylated region showed significant hypomethylation while poorly methylated regions exhibited hypermethylation (Grover et al., unpublished). Correlations with Aβ production can help establish the functional relevance of methylation modifications at these and other CpG sites within the BACE gene 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.

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Chapter 3

Epigenetic changes in Alzheimer's Disease: Decrements in DNA methylation.

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Abstract

DNA methylation is a vital component of the epigenetic machinery that orchestrates changes in multiple genes and helps regulate gene expression in all known vertebrates. We evaluated immunoreactivity for two markers of DNA methylation and eight methylation maintenance factors in entorhinal cortex layer II, a region exhibiting substantial Alzheimer's disease (AD) pathology in which expression changes have been reported for a wide variety of genes. We show, for the first time, neuronal immunoreactivity for all 10 of the epigenetic markers and factors, with highly significant decrements in AD cases. These decrements were particularly marked in PHF1/PS396 immunoreactive, neurofibrillary tangle-bearing neurons. In addition, two of the DNA methylation maintenance factors, DNMT1 and MBD2, have been reported also to interact with ribosomal RNAs and ribosome synthesis. Consistent with these findings, DNMT1 and MBD2, as well as p66 , exhibited punctate cytoplasmic immunoreactivity that co-localized with the ribosome markers RPL26 and 5.8 s rRNA in ND neurons. By contrast, AD neurons generally lacked such staining, and there was a qualitative decrease in RPL26 and 5.8 s rRNA immunoreactivity. Collectively, these findings suggest epigenetic dysfunction in AD-vulnerable neurons.

Keywords: Epigenetics; DNA methylation; Alzheimer's disease; Neuron; Ribosome

Introduction

Gene expression in the Alzheimer's disease (AD) brain has been shown to be altered in a wide variety of reports (Robinson et al., 1994; Loring et al., 2001; Dunckley et al., 2006; Weeraratna et al., 2007; Liang et al., 2008a,b,c), including a recent large-scale expression array study of sin- gle cell laser-captured entorhinal cortex layer II neurons (Dunckley et al., 2006). Multiple physiologic and molecular pathways are affected, including energy metabolism (Liang et al., 2008c) inflammation (Loring et al., 2001; Weeraratna et al., 2007) and aberrant cell cycle events (Arendt, 2000; Bowser and Smith, 2002), among others. Although individual pathogenic factors such as amyloid peptide (A) and tau phosphorylation are clearly critical links, no over-arching principle to explain the consistency, extent, and breadth of the gene expression, physiologic, and molecular changes reported in AD has received consensus acceptance. Epige- netic mechanisms such as histone modification (Mclachlan et al., 1984), binding of non-histone proteins, and DNA methylation (Adcock et al., 2007; Suzuki and Bird, 2008) are capable of modulating coordinate expression of large numbers of genes across many different pathways, and may therefore warrant investigation for their potential role in AD pathogenesis.

DNA methylation is a highly conserved process that has been implicated in many different modalities of gene expression. The factors responsible for the methylation process are a family of DNA methyltransferases that have been shown to catalyze the transfer of a methyl group to single-stranded DNA using S-adenosyl methionine as the methyl donor. The recognition sequence for the mammalian DNA methyl- transferase is relatively invariant, with nearly all cytosine methylations occurring on 5∗-C-p-G-3∗ (CpG) (Bird, 1986,1992). There are four known active DNA methyltransferases

in mammals, DNMT1, DNMT2, DNMT3A and DNMT3B. Of these, DNMT1 is the most abundant in mammalian cells. DNMT1 has been reported to be a key player in maintaining methylation in somatic cells, and loss of this enzyme has been shown to lead to nuclear disorganization, increased histone acetylation, and apoptosis (Chan et al., 2001; Fan et al., 2001; Jackson et al., 2004; Milutinovic et al., 2004; Espada et al.,2007).

Once methylation has occurred, methylation stability is maintained by the binding of specific complexes, MeCP1, to methylated regions of DNA. MeCP1 is not bound directly to methylated DNA, but rather to a single methyl-CpG-binding domain protein, MBD2. The resulting MeCP1/MBD2 com- plex is composed of 10 known proteins that include the complete nucleosome remodeling and histone deacetylase (NuRD) core, as well as MBD2. This group of proteins, in conjunction with CDK2AP1 (Doc1), make up a complex capable of nucleosome remodeling and histone deacetylation (Feng and Zhang, 2001, 2003).

Because methylation and methylation maintenance fac- tors can orchestrate changes in expression of a wide range of genes (Ashraf and Ip, 1998; Nan et al., 1998; Fujita et al., 1999; Ng et al., 1999; Feng and Zhang, 2001; Adcock et al., 2007; Suzuki and Bird, 2008), we hypothesized that alterations in methylation and methylation stability might provide an over-arching mechanism that could help explain expression differences in the thousands of genes that are reportedly altered in AD (Robinson et al., 1994; Loring et al., 2001; Dunckley et al., 2006; Liang et al., 2007, 2008a,b,c; Weeraratna et al., 2007). Here, we report highly significant decrements in immunoreactivity for two markers of DNA methylation and eight DNA methylation maintenance fac- tors in AD neurons of entorhinal cortex layer II, one of the most consistently vulnerable brain regions to AD pathology (Braak et al., 1993; Kordower et al., 2001).

2. Methods

2.1. Subjects and brain samples

Brain tissue was obtained through the Sun Health Research Institute Brain and Body Donation Program (Sun City, AZ). Specimens were collected under IRB-approved protocols and informed consents that permitted use of the samples for research by the investigators. Cases included in the study had received antemortem evaluation by board- certified neurologists and neuropsychologists, as well as postmortem evaluation by a board-certified neuropatholo- gist. Evaluations and diagnostic criteria followed consensus guidelines for National Institute on Aging Alzheimer's Dis- ease Centers. Diagnoses of patient condition included AD (N = 20) and cognitively and neurologically normal for age (ND) (N = 20). AD patients taking conventional AD therapeu- tics were not excluded from the study. At expiration, subject ages ranged from 60 to 97 years, with a mean of 79.9 ± 1.3 years. Postmortem intervals for the subjects averaged 2 h 40 min ± 12 min. Subject age, gender, and postmortem inter- val were well matched between the experimental groups, and there were no significant between-groups differences on any of these variables. Because neurons of entorhinal cortex layer II, which form clusters or "islands", are among the earliest and most consistently impacted neurons in AD brain (26), this brain region was the focus for all experiments.

2.2. Immunohistochemistry

Temporal cortex was sliced axially into 1-cm thick slabs, immersion fixed for 48 h in buffered 4% paraformaldehyde at 4 ◦C, washed extensively in phosphate buffer (PB), and cry- oprotected in ethylene

glycol and glycerol. The slabs were then sectioned at 40 m a freezing cryostat. Free-floating sections at the level of the entorhinal cortex were stored in freezing solution (glycol/glycerol/PB) at −20 ◦C until required for experiments. Tissue sections used for bright field microscopy were immunoreacted using the avidin–biotin complex/diaminobenzidine (DAB) method. Briefly, tissues were washed 2×, blocked in 1% hydrogen peroxide for 45 min, washed 3×, blocked in 3% bovine serum albumin (BSA) for 1 h, washed 2×, and incubated at 4 ◦Covernight in primary antibody solutions containing 0.25% BSA. Unless otherwise stated, all washes were with 1× PBS Triton (PBST). Available information about the anti-bodies is given in Table 1. After incubation with primary antibody, sections were washed 3×, incubated in biotiny- lated, species-specific secondary antibodies (Vector) for 2 h, washed 3×, and incubated in avidin–biotin complex (Pierce) for 1 h. Following incubation with secondary antibody, the sections were washed 3×, once in PBST and twice in 0.05 M Tris buffer, then exposed to DAB solution containing 125 l of 5 mg/ml DAB (Sigma), 11.125 ml 50 mM Tris buffer pH 7.6, and 500 l saturated nickel ammonium sulfate. Incuba- tions during chromagen development were no longer than 10 min, and were followed by two quick rinses in 50 mM Tris to stop the reaction. Finally, the sections were dried, taken through graded alcohols, de-fatted in Neoclear (EMD), and mounted with Permount (Pierce). AD and ND sec- tions were immunoreacted simultaneously using netwells in well-less plates. For fluorescence microscopy, the sec-tions were washed 3×, blocked with either 3% normal goat serum or 3% BSA, and incubated for 2 h. The sections were then washed 2×, incubated in primary antibody in 0.25% BSA at 4 ◦C overnight, washed again, and incubated in species-specific, fluorophore-conjugated secondary antibod- ies (Molecular Probes) at room temperature for 2 h. After a final wash, the sections were mounted, taken through Sudan Black to reduce autofluorescence, and

coverslipped with Vectashield mounting media (Vector). Deletion of primary antibody or incubation with pre-immune serum resulted in abolition of specific immunoreactivity in all cases (data not shown). Adjacent serial sections were stained with cresyl vio- let for cell layer identification and verification that the island neurons of layer II were intact. For some sections, nuclei were counterstained with 4∗,6∗-diamidino-2-phenylindole (DAPI) (Invitrogen) before mounting.

Double-label immunohistochemistry was also employed to evaluate possible associations of epigenetic factors with ribosomes and neurofibrillary tangles. Briefly, sections were washed, blocked with either 3% normal goat serum or 3% BSA, and incubated for 1–2 h. Sections were then washed 2×, incubated in primary antibodies raised in different species in 0.25% BSA/PBST at 4 ◦C overnight. After primary incubation sections were washed 3× in PBST, and incubated in species–specific, fluorophore-conjugated secondary antibodies (Molecular Probes) at room temperature for 2 h. After a final wash, the sections were mounted, taken through Sudan Black to reduce autofluorescence, and coverslipped with Vectashield mounting media (Vector). Immunostained tis- sue sections were examined on Olympus IX51 and Olympus IX70 microscopes equipped with epifluorescence illumina- tion or with confocal laser scanning using argon and krypton lasers (Olympus IX70). The findings were documented pho- tographically with an Olympus DP-71 color digital camera or, for confocal microscopy, by Fluoview software (Olympus).

2.3. Cell quantification and statistical analysis

For cell quantification, number-coded sections from three AD and three ND subjects were randomly selected fromthe pool of 20 AD and 20 ND subjects for each of the methylation factors (i.e., samples from different or overlap- ping subjects were evaluated for each factor). Measurements were made by an investigator blind to the

subjects' exper- imental condition. At 40× magnification, the total number of neurons, which were readily discriminated from glia by their large nuclei, within each of three randomly selected entorhinal cortex layer II islands, and the total number of neurons within each of those islands that exhibited clear immunoreactivity for the antibody being tested was recorded. A conventional two-tailed t-test of the percentage of cells labeled per island was then conducted to analyze the results.

3. Results

3.1. Immunoreactivity for markers of DNA methylation

Nuclear labeling with 5-methylcytosine and 5- methylcytidine has been used to assess methylation status in many reports (e.g., Halle et al., 1995; Havlis and Trbusek, 2002). In addition, both of these markers have also been reported to be associated with ribosomal RNA (rRNA) (Dunn, 1960; Tantravahi et al., 1981; Obara et al., 1982; Negre et al., 1989).

3.1.1. Nuclear staining

Nuclear immunoreactivity for 5-methylcytosine and 5-methylcytidine was clearly evident in entorhinal cortex layer II neurons in ND cases, but was markedly decreased in AD cases (Fig. 1). The percentage of neurons showing positive 5-methylcytosine nuclear immunoreactivity was 91.3% ± 1.3% in ND and 39.9% ± 3.4% in AD subjects ($t = 514.0$, $P < 0.0001$). Positive 5-methylcytidine nuclear immunoreactivity was observed in 90.1% ± 1.3% of ND neurons, but only 51.8% ± 6.1% of AD neurons ($t = 383.1$, $P < 0.0001$). There was no overlap in these data for ND and AD cases. By contrast, in pathologically spared regions of brain such as cerebellum, immunoreactivity for 5-methylcytosine and 5-

methylcytidine was similar in ND and AD sections (Fig. 1D and Supplementary Fig. 1). Staining of entorhinal cortex glia could be discerned at high power in ND cases, but was dramatically decreased or not seen in AD cases (Fig. 2). Deletion of primary antibody or pre-absorption with primary antigen completely abolished staining with 5-methylcytosine, 5-methylcytidine, and all other antibodies employed in this study.

3.1.2. Cytoplasmic staining

In ND neurons, cytoplasmic 5-methylcytidine and 5- methylcytosine immunoreactivity was evident at the light level (Fig. 3A1 and C1). With confocal microscopy, the immunoreactivity had a punctate morphology (Fig. 3A2, and C2), and was highly co-localized with immunoreactiv ity for the ribosomal protein RPL26 (Fig. 3A3 and C3). By contrast, 5-methylcytosine and 5-methylcytidine stain- ing was faint to absent in the cytoplasm of AD neurons (Fig. 3B1 and D1), and where it could be discerned, there was no co-localization with RPL26 (Fig. 3B2, B3, D2, D3).

3.2. Immunoreactivity for DNA methylation stabilizing factors

To investigate mechanisms that might underlie the changes observed in neuronal DNA methylation, we next examined a wide range of proteins that methylate CpG sites or help maintain normal methylation status.

3.2.1. Nuclear staining

Nuclear immunoreactivity for DNMT1, a major methyl- transferase (Mortusewicz et al., 2005), and six different components of the MeCP1/MBD2 methylation complex, including MTA2, HDAC1, HDAC2, p66 , RbAp48, and MBD2/3, was significantly diminished in entorhinal cortex layer II neurons of AD compared to ND subjects (Fig. 4) (see also Supplementary Fig. 2). Mean percent of

immunopositive neurons (± SEM) and comparisons by t-test were: DNMT1—ND (91.9% ± 1.0%), AD (7.6% ± 2.4%) (t = 33.0, P < 0.0001); HDAC1—ND (65.1% ± 4.2%), AD (17.0% ± 4.2%) (t = 8.1, P < 0.0001); HDAC2—ND (91.7% ± 2.7%), AD (12.7% ± 3.3%) (t = 18.6, P < 0.0001); p66 —ND (79.8% ± 4.6%), AD (12.7% ± 3.0%) (t = 12.3, P < 0.0001); MTA2—ND (94.7% ± 1.5%), AD (11.1% ± 2.4%) (t= 29.8, P < 0.0001); RbAp48—ND (93.7% ± 0.4%), AD (21.4% ± 2.8%) (t = 25.1, P < 0.0001); and MBD2/3—ND (98.3% ± 1.4%), AD (9.1% ± 2.0%), (t = 37.1, P < 0.0001). By contrast, when these factors were evaluated in cerebellum, a region that exhibits little to no AD pathology, immunoreactivity in ND and AD sections was again equivalent (Supplementary Fig. 1).

3.2.2. Cytoplasmic staining

Methylation of pre-rRNA is necessary for correct mat- uration of rRNA, and three of the methylation factors that were evaluated, DNMT1, HDAC1, and MBD2, have pre- viously been reported to interact with rRNA as important components of ribosome synthesis (Ghoshal et al., 2004; Zhou and Grummt, 2005). Consistent with these findings in peripheral cells, DNMT1, MBD2, as well as p66 , another member of the MeCP1/MBD2 complex, exhibited punctate cytoplasmic immunoreactivity and co-localization with the ribosome markers RPL26 and 5.8 s rRNA in ND neurons (Fig. 5). HDAC1, however, showed only diffuse cytoplasmic reactivity without a punctate appearance and without RPL26 or 5.8 s rRNA co-localization (not shown). In AD neurons, by contrast, ribosome-associated cytoplasmic immunoreactivity for DNMT1, MBD2, and p66 was weak or not observed (Fig. 5). Moreover, consistent with the important role of these methylation factors in the synthesis of ribosomes, there was a noticeable decrease in immunoreactivity for the ribosome markers themselves in AD neurons (Fig. 5). Compromised expression of rRNA was also suggested by the

absence in AD neurons of punctate nuclear immunoreactivity for SNF2H, a component of NoRC, which is integral to rRNA synthe- sis, whereas punctate nuclear SNF2H immunoreactivity was clearly evident in ND neurons (see Supplementary Fig. 4).

3.3. Relationship of methylation markers to neurofibrillary tangles

Even in many ND cases, entorhinal cortex layer II is char- acterized by high densities of neurons with neurofibrillary tangles. Interestingly, there appeared to be an inverse relation- ship of entorhinal cortex layer II nuclear immunoreactivity for DNA methylation markers and markers for late-stage tan- gles (Fig. 6). This was especially true for 5-methylcytosine methylation in AD cases, where it was extremely difficult to find any PHF1 or PS396 immunopositive tangle-bearing neu- rons that also exhibited discernable nuclear 5-methylcytosine immunoreactivity (Fig. 6A). Similar but less extreme results were seen for MeCP1 complex components (Fig. 6C, D and supplementary Fig. 3). Even in ND cases, the majority of layer II PHF1 and PS396 immunopositive neurons showed little to no co-localized MeCP1 component immunoreactiv- ity, and where such co-localization was seen the MeCP1 component staining appeared to be cytoplasmic, not nuclear (Fig. 6D, arrow).

Discussion

Perhaps because epigenetics is itself a relatively new field and has been primarily investigated in the context of oncology, epigenetic changes in AD are just beginning to be explored (Scarpa et al., 2003; Siegmund et al., 2007; Silva et al., 2008; Spremo-Potparevic et al., 2008; Wang et al., 2008; Wu et al., 2008). However, the changes in mani- fold individual genes in multiple cellular pathways that are

emerging from large-scale gene expression array studies of AD (Robinson et al., 1994; Loring et al., 2001; Dunckley et al., 2006; Liang et al., 2007, 2008a,b,c; Weeraratna et al.,2007) suggest that epigenetic mechanisms could play a role in the disorder because of the ability of such mechanisms to modulate the coordinate expression of large numbers of genes. Here we provide, for the first time in human brain, immunohistochemical evidence that a wide range of epi- genetic markers and regulators are expressed in neurons from control entorhinal cortex layer II and are dramatically decreased in AD patients, consistent with the high vulnerability of this brain region to AD pathology. In addition, we show co-localization of methylation stabilizing factors with rRNA (5.8 s rRNA) and the ribosomal protein RPL26, a decrement in AD samples, and an apparent decrease in AD ribosomes themselves. We also show loss of SNF2H, a core component of the NoRC that is directly involved in rRNA expression. The present findings suggest that global DNA and RNA methylation status in entorhinal cortex layer II neurons is significantly diminished in AD. A complete loss of methylation is, of course, unlikely in any cell, and, indeed, extensive digital enhancement of superficially blank fields in AD cases often revealed faint nuclear and cytoplasmic immunoreactivity for most of the methylation markers. Several previous studies have examined methylation at specific gene loci in AD cortex. One report examined methy- lation status of 12 specific genes that have been implicated in AD pathology. Significant “drifts” in status were observed in some of the loci, but the manner in which the data were analyzed makes it difficult to determine whether methylation was increased or decreased in AD (Wang et al., 2008). A second paper found AD cytosine methylation changes in two genes, one of which, S100A2, exhibited a significant decrease in methylation. Cytidine methyla-tion was not examined (Siegmund et al., 2007). Scarpa et al. (2003) have reported loss of methylation control of the BACE and

presenilin 1 genes and its potential to cause overexpression of APP in AD. A very early, more global analysis found no significant difference in percent CCGG methylation of DNA in AD cortex, but a num- ber of caveats were given (Schwob et al., 1990). CCGG methylation, for example, would only cover approximately 20–30% of CpG sites. Because only DNA extracts were assayed, alterations in cytoplasmic ribosomal methylation would also have been missed. Data on postmortem delay for the samples, which could have a substantial effect on methylation integrity, were unavailable. By contrast, post- mortem intervals for the present study averaged 2 h 40 min, and were well matched among AD and ND cases. Moreover, as the authors of the previous research themselves noted, "Degenerating neurons, the cells most likely to reflect the molecular derangements of AD, probably contributed little to the bulk-extracted DNA from brain slices from patients with AD." Our analysis, on the other hand, included all cells on the entorhinal cortex sections. Finally, in lympho- cyte preparations, Silva et al. (2008) did not observe AD methylation changes in the SIRT3, SMARCA5, or CDH1 genes, but did report significant hypermethylation of the HTERT gene. The lymphocyte HTERT gene, however, is unusual in that its hypermethylation is believed to result in increased expression rather than the more usual silencing (Silva et al., 2008). AD decrements in methylation status, as assayed by 5-methylcytosine and 5-methylcytidine immunoreactivity, were accompanied by decrements in all eight of the methy- lation stabilizing factors examined. These factors are critical components of MeCp1, NoRC, and NuRd, which, in turn, are important to maintenance of normal methylation state. The loss of many of these factors may be interlinked. For example, DNMT1 is recruited by HDAC1, and both are recruited by NoRC (Zhou et al., 2002; Zhou and Grummt, 2005). Functionally, improper maintenance of methylation in somatic cells leads to nuclear disorganiza- tion, de-

differentiation, upregulation of tumor suppressor proteins, and an increase in histone acetylation (Chen et al., 1998; Santos et al., 2002; Zhu and Otterson, 2003; Hoffmann and Schulz, 2005). That methylation deficits in the present study were functionally associated with neurodegeneration is also suggested by their co-occurrence with PHF1 and PS396 immunoreactivity, which are considered markers for the late stages of neurofibrillary tangle forma- tion.

Methylation is involved in many aspects of cellular regulation, including ribosomal control and function. In addition to nuclear changes, we also report, for the first time, loss of cytoplasmic immunoreactivity for 5-methylcytidine and 5-methylcytosine, markers of methylation, and three methy- lation stabilizing factors, DNMT1, MBD2, and p66, in AD entorhinal cortex island neurons. Genes for rRNA are heavily methylated in both transcribed and non-transcribed regions of the repeat unit (Bird, 1978), leading to the suggestion that changes in epigenetic factors may disrupt processing and function of rRNA (Espada et al., 2007). Our findings are wholly consistent with this hypothesis. (1) Loss of rRNA methylation alters rRNA post-transcriptional modification (Swaminathan et al., 2007), and AD neurons showed a near absence of the two key methylation markers 5-methylcytosine and 5-methylcytidine. (2) DNMT1 deficits, as reported here for AD neurons, have been previously demonstrated to disrupt DNA methylation and increase acetylation levels of lysine 16 histone H4 on rRNA genes (Espada et al., 2007). (3) The normal association of methylation stabilizing factors such as DNMT1, MBD2, and p66 with rRNA was nearly absent in entorhinal cortex layer II AD neurons. (4) SNF2H, a component of NoRC that is integral to rRNA synthesis, appeared to be lost in AD neurons. Indeed, we observed qualitative decreases in immunoreactivity for two markers of ribosomes themselves, RPL26 and 5.8 s rRNA, in these neurons. It

will therefore be of interest in future studies to test the hypothesis that loss of methylation markers and methylation stabilizers in AD neurons may cause deficits in rRNA gene expression, rRNAs, ribosomes, and ribosomal protein synthesis.

A final point that may warrant further research follows from the ability of epigenetic shifts to orchestrate entry or re-entry into the cell cycle (reviewed in (Golubnitschaja, 2007), a process that would presumably lead to apoptosis in postmitotic neurons (Nagy et al., 1998; Arendt, 2000; Nagy, 2000; Copani et al., 2001; Bowser and Smith, 2002; Herrup and Arendt, 2002). Many reports have suggested a derangement of cell cycle events in AD, including the observation that premature centromere division of the X chromosome is increased in AD (Spremo-Potparevic et al., 2008). Treatment of terminally differentiated PC12 neurons with the demethylating agent 5-azacytodine, for example, causes the cells to retract their processes, express cell cycle pro- teins, and apoptose (Hossain et al., 1997). Similarly, loss of methylation and DNMT1 in cultured neurons has been shown to lead to increased expression of cell cycle-related genes in vitro (Mattson, 2003). Thus, the decrements in methylation and methylation stabilization observed in the present study could provide an explanation for the many previous reports that have suggested an aberrant re-entry of AD neurons into the cell cycle and/or their apoptosis. Development and aging are processes that have been consistently linked to epigenetic orchestration (reviewed in (Wu et al., 2008), and aging remains one of the most salient risk factors for AD. Epigenetic shifts have also been considered as major causative factors in disease states, par- ticularly cancer (reviewed in (Esteller, 2008; Gonzalo et al., 2008; Vucic et al., 2008). Thousands of genes have been reported to increase or decrease their expression in AD neu- rons (Liang et al., 2008a), suggesting that there may be some over-arching, but presently unknown principle under- lying these massive genomic

changes. It will therefore be of great interest to explore, in prospective studies, interactions of AD pathology with the epigenetic mechanisms and factors that are highlighted by the present descriptive findings.

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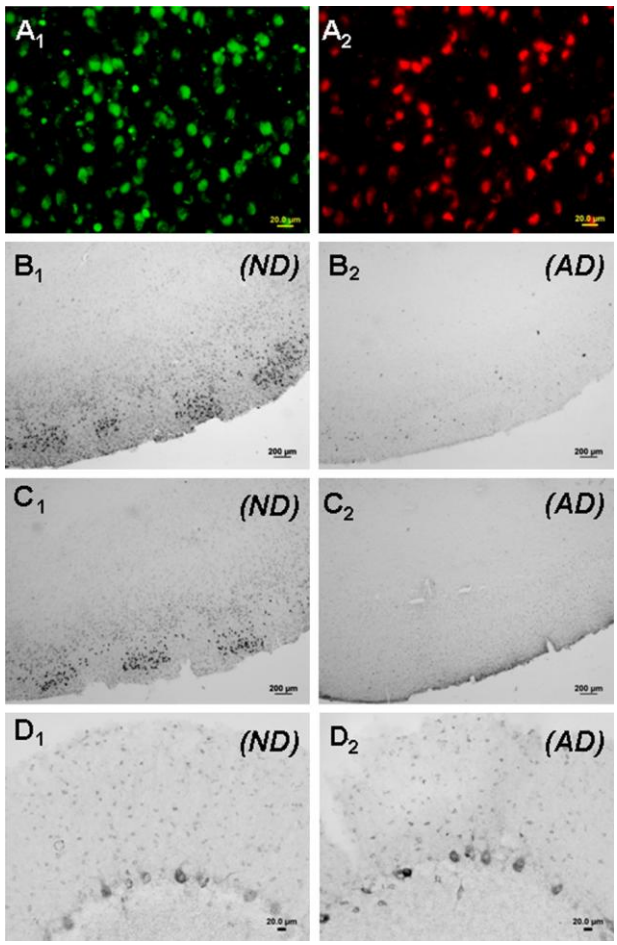
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.
2008.12.005.

Table 1
Antibodies.

Antibody	Host/type	Dilution	Cata#	Antigen/epitope
NSE	Chicken polyclonal	1:500	Chemicon/AB9698	Synthetic peptides from rat and neuron specific enolase
NeuN	Mouse monoclonal/biotin	1:1000	Chemicon/MAB377B	Uncharacterized vertebrate neuron specific nuclear protein
GFAP	Rabbit polyclonal	1:1000	Chemicon/ab5804	Bovine glial fibrillary acidic protein
RAC1	Lectin	1:1000	Vector/B-1085	Ricinus communis agglutinin I
pS396	Rabbit polyclonal	1:1000	Invitrogen/44-752G	Phosphopeptide serine 396 tau
PHF1	Mouse monoclonal	1:1000	Gift of Dr. P. Davies	Human tau, phosphorylated serine 396 and 404 regions
MBD3	Mouse monoclonal	1:400	Abcam/ab45027	Human MBD3/CKAFMVTDEDIRKQEE
DOC1	Rabbit polyclonal	1:400	Abcam/ab31794	Human DOC1/TSSQYRQLLSDYGPPS
DNMT1	Rabbit polyclonal	1:400	Abcam/ab19905	Human DNMT1/Within residues 100-200
5-Methylcytidine	Mouse monoclonal	1:500	Genway/20-783-71663	5-Methylcytidine
RPL26	Rabbit polyclonal	1:500	Abcam/ab59567	Human RPL26/MKFNPFVTSDRSKNRKR
HDAC1	Rabbit polyclonal	1:400	Abcam/ab19845	Human HDAC1/Within residues 450 to the C-terminus
P66 alpha	Rabbit polyclonal	1:400	Abcam/ab13714	Human P66 /amino acids 137-150
MTA2	Rabbit polyclonal	1:400	Abcam/ab8106	Human MTA2/amino acids 652-668
HDAC2	Rabbit monoclonal	1:400	Abcam/ab32117	Human HDAC2/residues within C-terminal
rRNA 5.8S	Mouse monoclonal	1:500	Novus/NB100-662	5.8S protein associated with rRNA
5-Methylcytosine	Mouse Monoclonal	1:500	Genway/20-003-40005	5-Methylcytosine
RbAp48	Mouse monoclonal	1:400	Abcam/ab488	RbAp48/amino acids 1-425
MBD2	Goat polyclonal	1:400	Abcam/ab58241	MBD2/RNDPLNQNKGKPDLN

Fig.1. Representative micrographs of 5-methylcytosine and 5-methylcytidine immunoreactivity in entorhinal cortex layer II and cerebellar neurons from AD and ND cases. (A1) High power micrograph of entorhinal cortex labeled with an antibody to 5-methylcytosine. (A2) Same field labeled with an antibody to neuron-specific enolase to show co-localization (ND case). (B1) Low power micrograph of 5-methylcytosine immunoreactivity in entorhinal cortex of an ND case to show general staining pattern. (B2) 5-methylcytosine, AD case, entorhinal cortex. (C1) 5-methylcytidine, ND case, entorhinal cortex. (C2) 5-methylcytidine, AD case, entorhinal cortex. (D1) 5-methylcytosine, ND case, cerebellum. (D2) 5-methylcytosine, AD case, cerebellum. Staining with 5-methylcytidine in AD and ND cerebellum was virtually indistinguishable from that with 5-methylcytosine (see Supplementary Fig. 1).



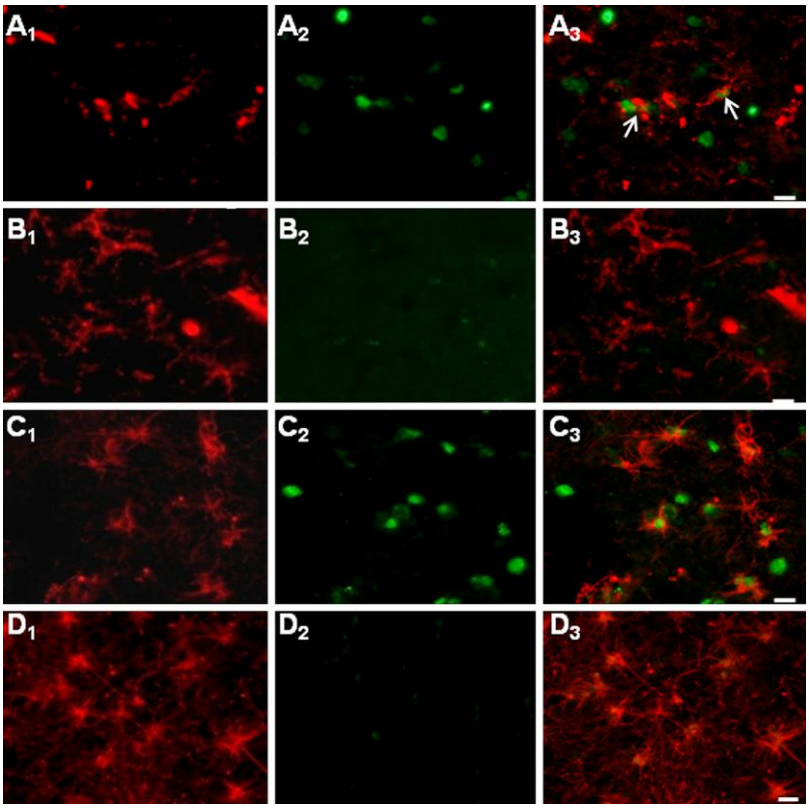
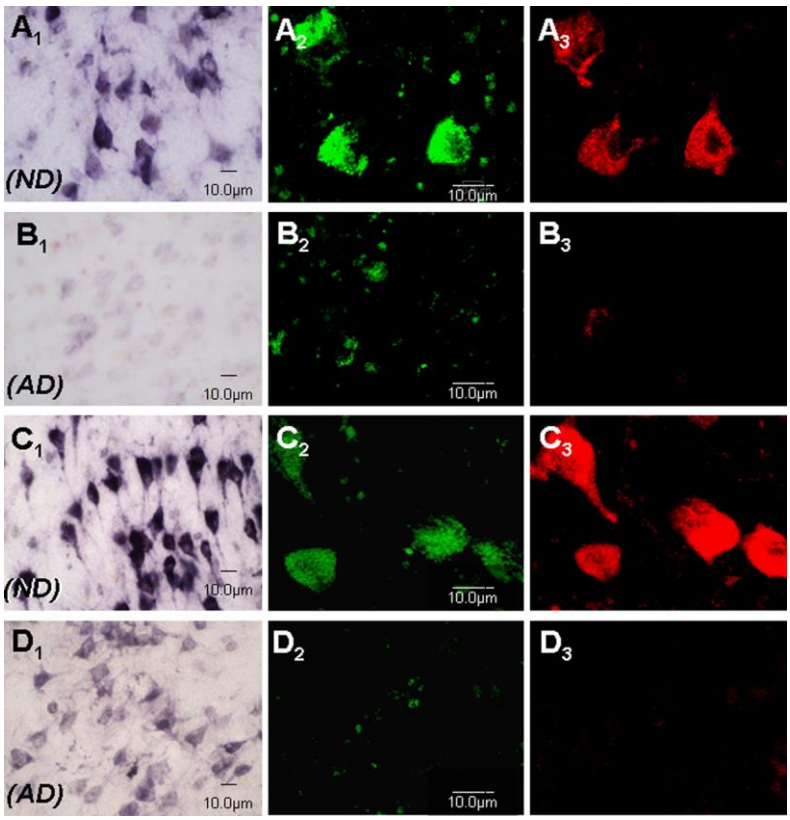


Fig. 2. Representative high power micrographs of 5-methylcytosine immunoreactivity in entorhinal cortex microglia and astrocytes. (A1) Immunoreactivity for the microglial marker RAC1 in an ND case. (A2) Same field labeled with 5-methylcytosine. (A3) Merged image to show co-localization (arrows). (B1) RAC1 immunoreactivity in an AD case, where microglia were typically more abundant than in AD cases. (B2) Same field labeled with 5-methylcytosine. (B3) Merged image. (C1) Immunoreactivity for the astrocyte marker GFAP in an ND case. (C2) Same field labeled with 5-methylcytosine. (C3) Merged image. (D1) GFAP immunoreactivity in an AD case. (D2) Same field

labeled with 5 methylcytosine. (D3) Merged image. (Calibration bars in all fields equal 20 um).

Fig. 3. Representative micrographs of cytoplasmic immunoreactivity for 5-methylcytosine and 5-methylcytidine (green fluorophore in confocal micrographs) and their co-localization with



immunoreactivity for the ribosomal protein RPL26 (red fluorophore in confocal micrographs) in AD and ND entorhinal cortex layer II neurons. Confocal planes were chosen that emphasized cytoplasmic rather than nuclear staining. (A1) 5-methylcytosine, ND case, bright field. (A2) 5-methylcytosine, ND case, confocal microscopy. (A3) Same field as A2 for RPL26. (B1) 5-methylcytosine, AD case, bright

field. (B2) 5-methylcytosine, AD case, confocal microscopy. (B3) Same field as B2 for RPL26. (C1) 5-methylcytidine, ND case, bright field. (C2) 5-methylcytidine, ND case, confocal microscopy. (C3) Same field as C2 for RPL26. (D1) 5-methylcytidine, AD case, bright field. (D2) 5-methylcytidine, AD case, confocal microscopy. (D3) Same field as D2 for RPL26.

Fig. 4. Representative micrographs of immunoreactivity for selected com- ponents of the MeCP1/MBD2 complex in entorhinal cortex layer II neurons. (A1) MTA2, ND case. (A2) MTA2, AD case. (B1) HDAC2, ND case. (B2) HDAC2, AD case. (C1) RBAP, ND case. (C2) RBAP, AD case. Similar results were obtained for all eight of the factors evaluated(see Supplementary Fig. 2).

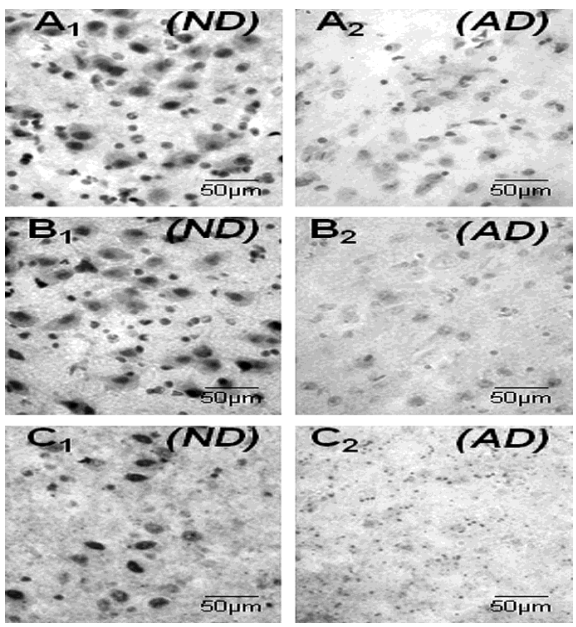


Fig. 5. Representative micrographs of cytoplasmic immunoreactivity for p66 (green fluorophore), its co-localization with 5.8 s rRNA (red fluorophore), and AD decrements in 5.8 s rRNA and the ribosomal protein RPL26 in entorhinal cortex layer II neurons. (A1) 5.8 s rRNA, ND case, confocal microscopy. (A2) Same field as A1 for p66 . (B1) 5.8 s rRNA, AD case, confocal microscopy. (B2) Same field as B1 for p66 . Similar results were observed for MBD2 and DNMT1. (C1) 5.8 s rRNA, ND case, bright field. (C2) 5.8 s rRNA, AD case, bright field. (D1) RPL26, ND case, bright field. (D2) RPL26, AD case, bright field.

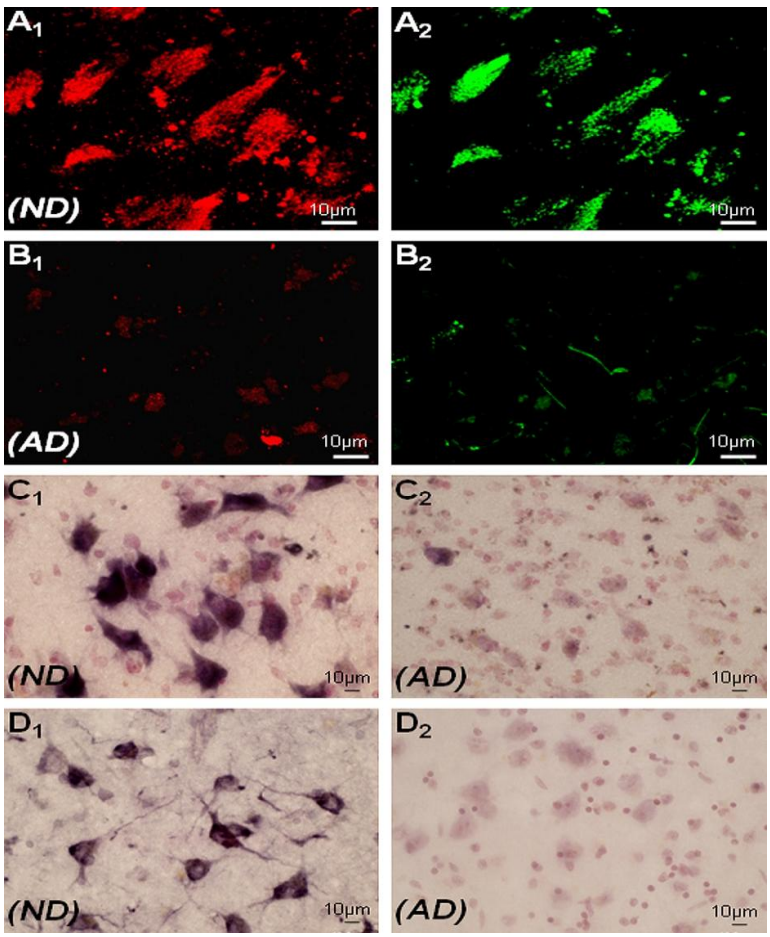
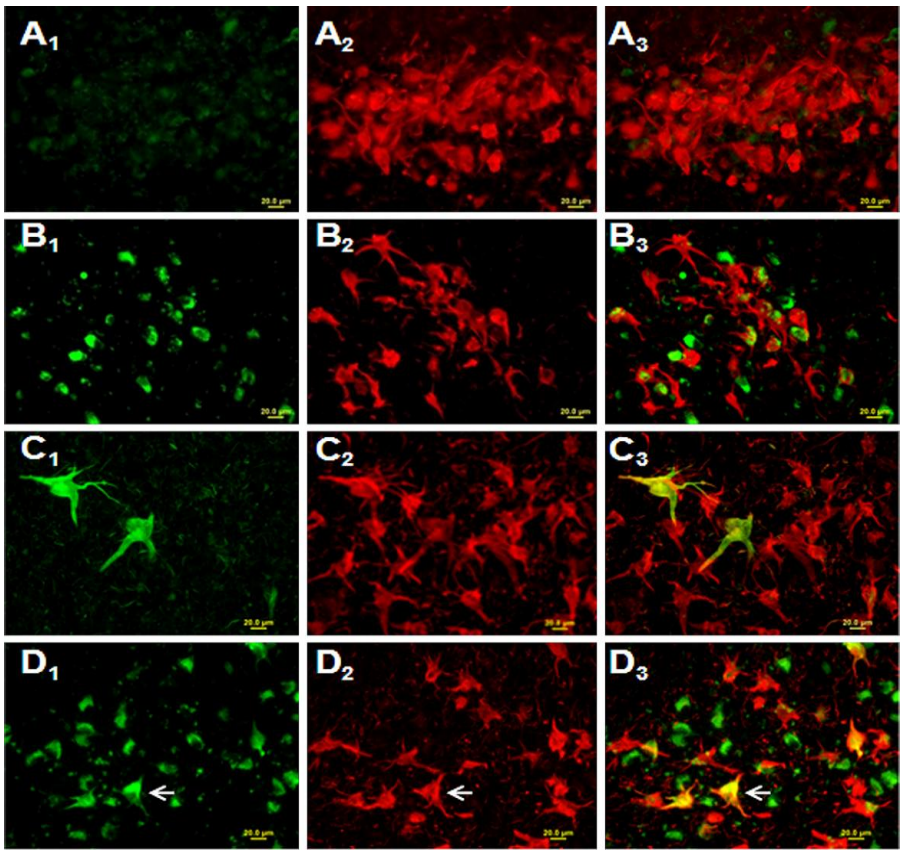


Fig. 6. Decreased 5-methylcytosine and p66 nuclear immunoreactivity in entorhinal cortex layer II island neurons that were immunoreactive for the late-stage tangle markers PS396 and PHF1. (A1) 5-methylcytosine immunoreactivity, AD case. (A2) Same field, PS396 immunoreactivity. (A3) Merged image. (B1) 5-methylcytosine immunoreactivity, ND case. (B2) Same field, PS396 immunoreactivity. B3) Merged image. (C1) p66 immunoreactivity, AD case. (C2) Same field, PHF1 immunoreactivity.



(C3) Merged image. (D1) p66 immunoreactivity, ND case. (D2) Same field, PHF1 immunoreactivity. (D3) Merged image. See Supplementary Fig. 3 for HDAC1/PHF-1 co-localization.

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Chapter 4

Epigenetic Differences in Cortical Neurons from a Pair of Monozygotic Twins Discordant for Alzheimer's Disease.

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Abstract

DNA methylation (Adcock et al. 2007; Suzuki and Bird 2008) is capable of modulating coordinate expression of large numbers of genes across many different pathways, and may therefore warrant investigation for their potential role between genes and disease phenotype. In a rare set of monozygotic twins discordant for Alzheimer's disease (AD), significantly reduced levels of DNA methylation were observed in temporal neocortex neuronal nuclei of the AD twin. These findings are consistent with the hypothesis that epigenetic mechanisms may mediate at the molecular level the effects of life events on AD risk, and provide, for the first time, a potential explanation for AD discordance despite genetic similarities.

Introduction

Wide varieties of studies have examined candidate genes for associations with Alzheimer's disease (AD). Although such associations have been found, they are probabilistic rather than inevitable, with the exception of familial AD. A potential explanation of the probabilistic nature of the association between specific genes and disease may be the apparent genetic complexity of AD. However, the existence of rare monozygotic twins discordant for AD offers the opportunity to examine other factors that may be contributing to the probabilistic nature of the association between genes and AD, specifically epigenetic mechanisms. Since epigenetic modifications contribute to the phenotypic differences that emerge in monozygotic twins, including discordant disease states (Poulsen et al. 2007), we examined DNA methylation, an important epigenetic mechanism, in monozygotic twins discordant for Alzheimer's disease

(AD). As has been described in sporadic AD cases (Mastroeni et al. 2008), neurons of the AD twin exhibited dramatic decrements across multiple DNA methylation markers compared to the non-AD twin. Epigenetics may therefore, constitute a basic molecular genetic mechanism in the pathophysiology of AD.

Methods

*__Ethics Statement:__

Written informed consent for autopsy was obtained for both cases in compliance with institutional guidelines of Boston University. The Boston University Institutional Review Board approved this study including recruitment, enrollment, and autopsy procedures. Both twins and their respective next-of-kin consented to brain autopsy for the purpose of research analysis as participants in the Boston University Alzheimer's Disease Center. The human brain tissue used in this manuscript was from routine existing autopsies, which fully qualifies for 4C exemption by NIH guidelines. In addition samples were analyzed anonymously (e.g. twin 1 and twin 2) throughout the experimental process.

Subjects and brain samples.

Using standard protocols of NIH AD Centers, both twins were thoroughly evaluated antemortem and postmortem by board-certified neurologists and a neuropathologist who determined their respective diagnoses as AD and neurologically normal, non-demented (ND). The AD twin was a white male chemical engineer who had extensive pesticide contact in his work. He developed AD symptoms at age 60, first manifest as the inability to read maps, followed by progressive loss of memory and intellect over 16 years

until his death at age 76. His identical twin, also a chemical engineer with an identical education but different work environment, died at 79 years from prostate cancer. At the time of his death, he was cognitively intact. The twins were autopsied at the same facility using the same tissue processing protocols. Post mortem delay for the control twin was 3h 10 minutes and 7h 20 minutes for the AD twin. Both subjects were immediately snap frozen on aluminum plates cooled to -80ºC on dry ice and immediately transferred to -80ºC freezer for long term storage. In the AD twin post-mortem examination confirmed severe AD (NIA-Reagan: high, CERAD plaque: frequent, Braak: VI). In the non-demented twin post-mortem examination revealed sparse neuritic plaques and entorhinal and transentorhinal NFTs (NIA-Reagan: low, CERAD: sparse, Braak: II). The AD twin showed dense plaques and neurofibrillary tangles (NFT) in the anterior temporal cortex, while these stigmata were remarkably rare in the non-demented twin (Figure 1).

Immunohistochemistry.

Temporal neo-cortex was sliced axially into 1-cm thick slabs, immersion fixed for 48h in buffered 4% paraformaldehyde at 4°C, washed extensively in phosphate buffer (PB), and cryoprotected in ethylene glycol and glycerol. The slabs were then sectioned at 40 µm on a freezing cryostat. Free-floating sections were stored in freezing solution (glycol/glycerol/PB) at -20°C until required for experiments. Tissue sections used for bright field microscopy were immunoreacted using the avidin-biotin complex/diaminobenzidine (DAB) method. Briefly, tissues were washed 2X, blocked in 1% hydrogen peroxide for 45 min, washed 3X, blocked in 3% bovine serum albumin (BSA) for 1 h, washed 2X, and incubated at 4ºC overnight in primary antibody solutions containing 0.25% BSA. Unless otherwise stated, all washes were with 1X PBS Triton (PBST). Available information about the antibodies is given in (Table 1). After

incubation with primary antibody, sections were washed 3X, incubated in biotinylated, species-specific secondary antibodies (Vector) for 2 h, washed 3X, and incubated in avidin-biotin complex (Pierce) for 1 h. Following incubation with secondary antibody, the sections were washed 3X, once in PBST and twice in 0.05M Tris buffer, then exposed to DAB solution containing 125 µl of 5 mg/ml DAB (Sigma), 11.125 ml 50 mM Tris buffer pH 7.6, and 500 µl saturated nickel ammonium sulfate. Incubations during chromagen development were no longer than 10 min, and were followed by two quick rinses in 50 mM Tris to stop the reaction. Finally, the sections were dried, taken through graded alcohols, de-fatted in Neoclear (EMD), and mounted with Permount (Pierce). AD and ND sections were immunoreacted simultaneously using netwells in well-less plates. For fluorescence microscopy, the sections were washed 3X, blocked with either 3% normal goat serum or 3% BSA, and incubated for 2 h. The sections were then washed 2X, incubated in primary antibody in 0.25% BSA at 4°C overnight, washed again, and incubated in species–specific, fluorophore-conjugated secondary antibodies (Molecular Probes) at room temperature for 2 h. After a final wash, the sections were mounted, taken through Sudan Black to reduce autofluorescence, and coverslipped with Vectashield mounting media (Vector). Deletion of primary antibody or incubation with pre-immune serum resulted in abolition of specific immunoreactivity in all cases (data not shown). Adjacent serial sections were stained with cresyl violet for cell layer identification and verification that the island neurons of layer II were intact. For some sections, nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen) before mounting.

Double-label immunohistochemistry

was also employed to evaluate the associations of epigenetic factors with specific neuronal, and glial cell types. Briefly, sections

were washed, blocked with either 3% normal goat serum or 3% BSA, and incubated for 1-2 h. Sections were then washed 2X, incubated in primary antibodies raised in different species in 0.25% BSA/PBST at 4°C overnight. After primary incubation sections were washed 3X in PBST, and incubated in species–specific, fluorophore-conjugated secondary antibodies (Molecular Probes) at room temperature for 2 h. After a final wash, the sections were mounted, taken through Sudan Black to reduce autofluorescence, and coverslipped with Vectashield mounting media (Vector). Immunostained tissue sections were examined on Olympus IX51 and Olympus IX70 microscopes equipped with epifluorescence illumination or with confocal laser scanning using argon and krypton lasers (Olympus IX70). The findings were documented photographically with an Olympus DP-71 color digital camera or, for confocal microscopy, by Fluoview software (Olympus).

Cell Quantification and statistical analysis.

Using the Fluoview software, integrated fluorescence intensities for 5-methylcytosine of layer II and layer III of the anterior temporal neocortex from each individual were analyzed. Briefly, sections were processed in parallel using identical antibody concentrations. DAPI + Nuclei of NSE+ cells were randomly selected in coded slides. The detection bandwidth was set at 488nm for 5-methylcytosine indicator, and fluorescence intensity of the nucleus was recorded (N=20 per layer per case).

Results

DNA methylation Immunoreactivity. Figure2a shows representative immunohistochemistry of anterior temporal neocortex for 5-methylcytosine, a marker of methylated CpG sites on DNA. In the AD twin, decreased immunoreactivity relative to the ND twin was

readily apparent in the anterior temporal neocortex, a region severely affected in AD. Similar results were obtained in the pathologically-vulnerable superior frontal gyrus (Figure S1). To develop quantitative data, integrated fluorescence intensities of immunoreactivity for 5-methylcytosine were recorded for layer II and layer III nuclei of DAPI-and neuron specific enolase positive cells from coded slides. By two-tailed t-test, all markers exhibited highly significant ($P < 0.0001$) decrements in immunoreactivity in the AD twin compared to the ND twin (Figure 2b). These findings are consistent with our previous results for non-twin AD and ND subjects (Mastroeni et al. 2008).

To show that the results obtained in neocortex were not due to differences in tissue handling, storage time, or quality, DNA methylation markers were also evaluated in cerebellum, a brain region that is largely spared from AD pathology. As in our previous assessment of AD and control cases (Mastroeni et al. 2008), the cerebella of both twins exhibited virtually identical staining patterns and intensity for 5-methylcytosine (Figure 2a), as well as for another methylation marker, 5-methylcytidine (Figure S1) and several methylation stabilizing factors (MBD2/3 and HDAC2; Figure S2)

Relationship of DNA methylation marker 5-metylcytosine to neurons and Glial cells. In Figure 3 we examine co-localization of the DNA methylation marker, 5-methylcytosine, with markers for neurons (neuron specific enolase), for reactive astrocytes (GFAP) and for microglia (RCA1). These figures show that DNA methylation is present in all three cell types in non-demented brain and that the decrement in DNA methylation seen in AD extends to all three cell types.

Discussion

Large-scale expression array studies have reported significant up- or downregulation of thousands of genes in AD (Liang et al. 2008a; Liang et al. 2008b). These alterations in gene expression span multiple pathogenic pathways, including amyloid β peptide (Aβ) processing, tau hyperphosphorylation, and inflammation, among others. Because many genes across the genome have methylation sites in their promoters (Suzuki and Bird 2008), extensive hypomethylation in AD may provide an over-arching principle that accounts for significant aspects of the molecular and pathogenic complexity of the disorder. For example, amyloid precursor protein (APP), the Aβ precursor, has been shown to be normally methylated, and hypomethylated with age (Tohgi et al. 1999) which apparently enhances Aβ production (West et al. 1995). However, more recent data indicates no difference in methylation of the APP gene in AD (Wang et al. 2008). Furthermore, inducible nitric oxide synthase, interleukin 1, and tumor necrosis factor-α are all increased as part of the inflammatory response in AD cortex (Akiyama et al. 2000); all their respective genes are methylated; and all show enhanced secretion with hypomethylation (Kovacs et al. 1987; Chan et al. 2005; Sato and Mitchell 2006). At the protein level, protein phosphatase 2A is methylated (probably by peptidylarginine methyltransferases or lysine methyltransferases), and its hypomethylation results in tau hyperphosphorylation (Sontag et al. 2007).

The present findings indicate that epigenetic mechanisms may provide a molecular basis for the effect of life events, including exposure to hazardous substances, on AD risk. More specifically, they may provide a rationale for the consistent epidemiologic and neuropathologic association of AD with homocysteine elevation and

folate deficiencies (Seshadri et al. 2002; Luchsinger et al. 2008), since folate ultimately provides the methyl group for DNA methylation. Maintaining adequate dietary folate (and B12) or increasing S-adenosylmethionine levels might therefore be useful, inexpensive strategies to decrease risk for AD. Since epigenetic patterns can be passed on to subsequent generations, epigenetics may also constitute a mechanism by which AD in a first degree relative confers increased risk of "sporadic" disease.

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Figures / Tables

Table 1: Antibody source/sequence and dilution

Antibody	*Host*	*Dilution*	*Source/Catalogue#*	*Recognition / sequence*
NSE	Chicken plyclonal	1:500	Chemicon/AB9698	Neuron Specific Enolase
GFAP	Rabbit polyclonal	1:1000	Chemicon/ab5804	class-III intermediate filament
RAC1	lectin	1:1000	Vector/B-1085	Ricinus communis agglutinin I
pS396	Rabbit polyclonal	1:1000	Invitrogen/44-752G	Serine 396
PHF1	Mouse monoclonal	1:1000	Gift of Dr. P. Davies	Paired Helical Filament 1
MBD3	Mouse monoclonal	1:400	Abcam/ab45027	CKAFMVTDEDIRKQEE
5-methylcytidine	Mouse monoclonal	1:1000	Genway/20-783-71663	Methylation
MTA2	Rabbit polyclonal	1:500	Abcam/ab8106	Amino acids 652-668
HDAC2	Rabbit monoclonal	1:500	Abcam/ab32117	Reidues within C-terminal end
5-methylcytosine	Mouse Monoclonal	1:1000	Genway/20-003-40005	Methylation

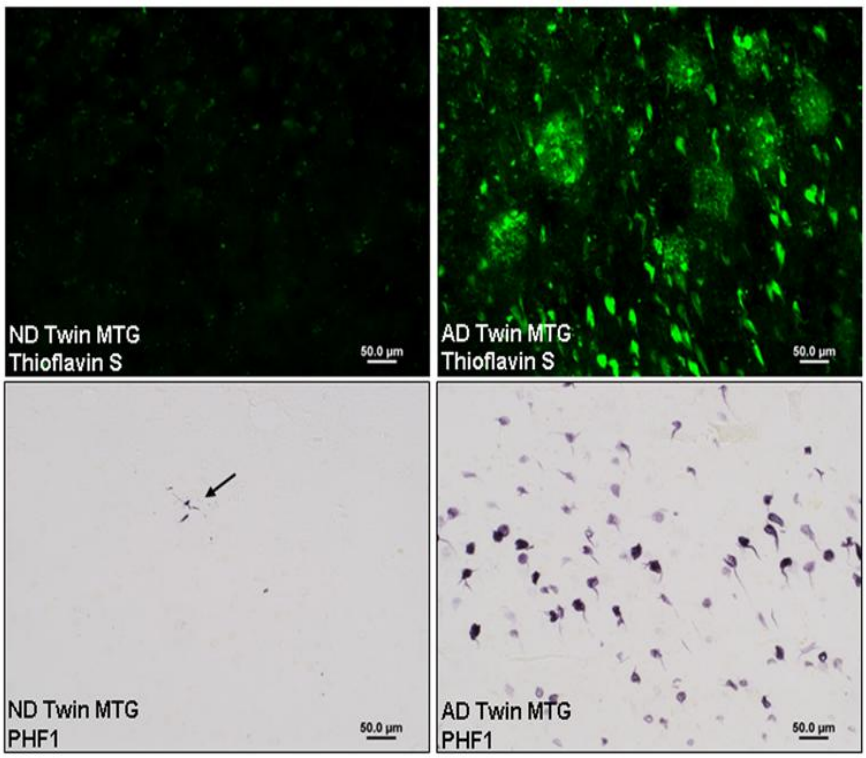


Figure 1. Thioflavin S plaques and PHF1 neurofibrillary tangle pathology in middle temporal gyrus (MTG) of non-demented and AD twin. AD pathology was remarkably rare in the non-demented twin and abundant in the AD twin.

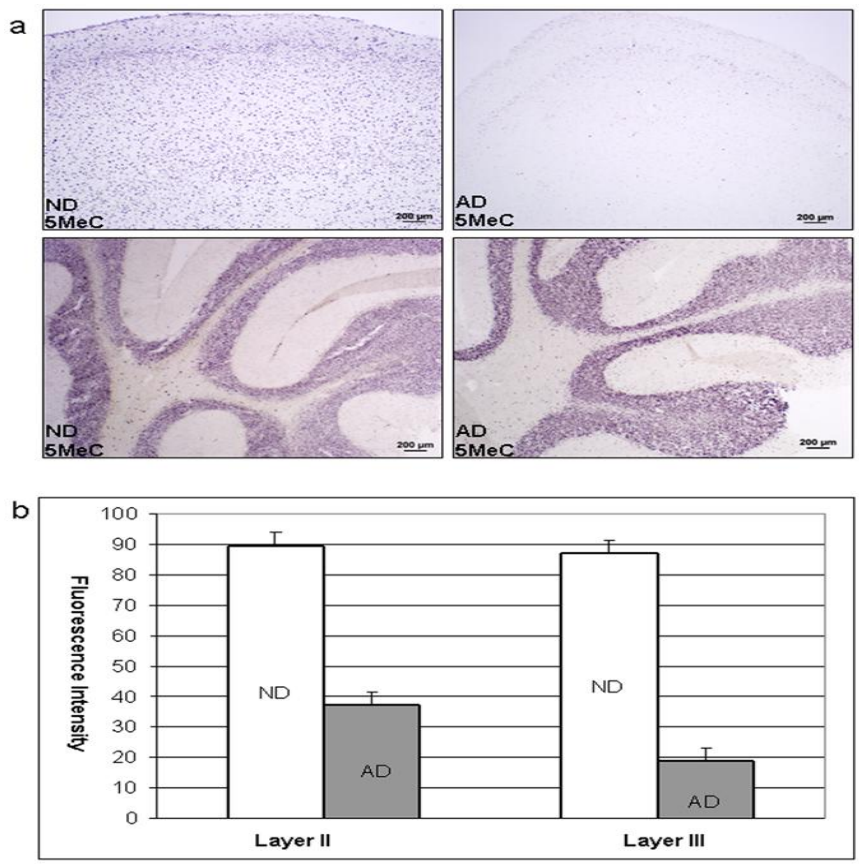
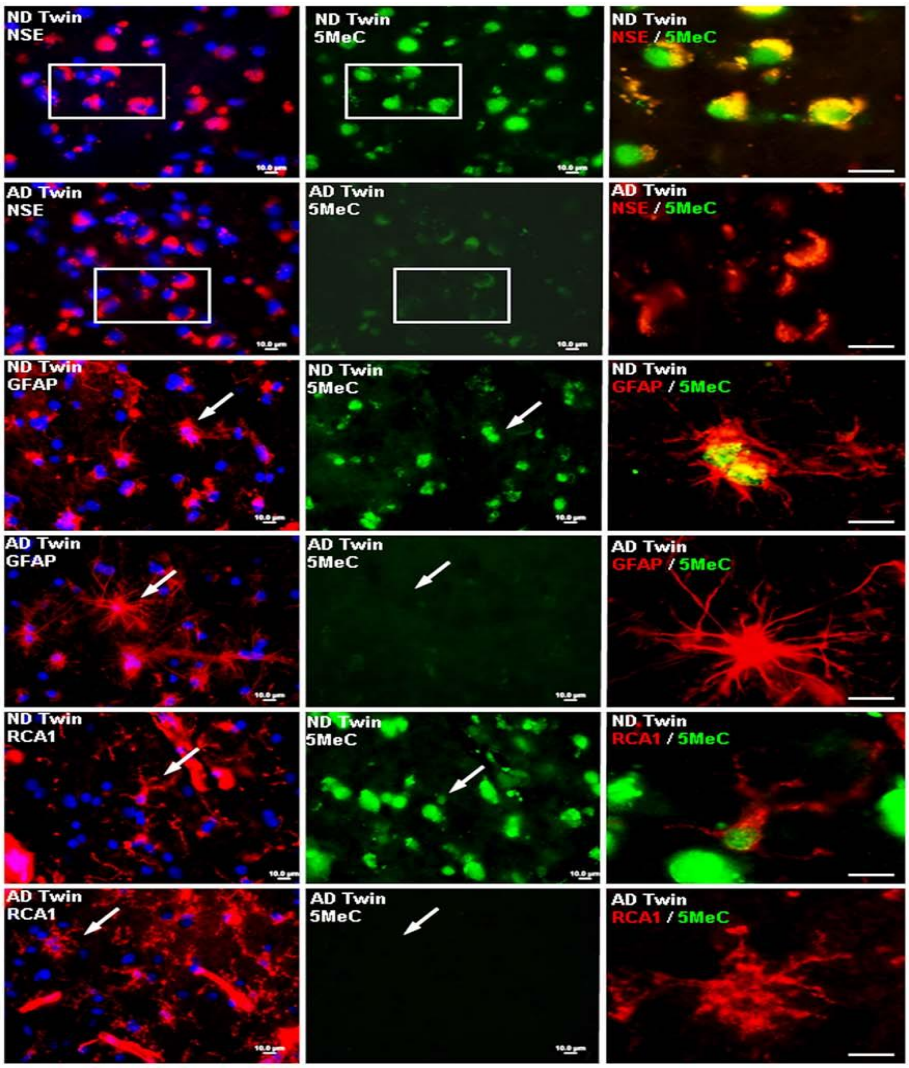


Figure 2. a) Anterior temporal neocortex (left and right top panels) and cerebellum (left and right bottom panels) immunoreactivity for DNA methylation marker in a pair of monozygotic twins discordant for Alzheimer's disease. Sections were processed in parallel using identical antibody concentrations to 5-methylcytosine (1:1000) with nickel intensification. Note similarity of immunoreactivity in cerebellar granule cell layer of both twins in this brain region that is relatively unaffected in Alzheimer's disease. b) Integrated fluorescence intensities of DAPI-counterstained anterior temporal neocortex layer II and layer III neuronal nuclei in a set of monozygotic twins discordant for AD. Sections were processed at the same time using identical immunohistochemical methods [4] and antibody concentrations.

Nuclei of NSE+ cells (N = 20/brain) were randomly selected and traced from coded slides by an investigator blind to subject condition. The appropriate detection fluorescence bandwidth (488 nm) for 5- methylcytosine indicator was then set, and the integrated fluorescence intensity within the traced area was taken. Highly significant decrements in the pathologically-vulnerable entorhinal cortex was observed in the AD twin (p,3.51E-08 for layer II and p,3.71E-06 for layer III), whereas readings for the cerebellum were nearly identical in the AD and ND twin (data not shown).

Figure 3. Co-localization of immunoreactivity for 5-methylcytosine with neuron specific enolase, GFAP, RCA1 (microglial marker) in non-demented and Alzheimer's disease twin. Note extensive co-localization of 5-methylcytosine in ND twin with all three cell specific markers, and the lack of co-localization with 5-methylcytosine in the AD twin.



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Chapter 5

Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients.

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Abstract

Epigenetic dysregulation of gene expression is thought to be critically involved in the pathophysiology of Alzheimer's disease (AD). Recent studies indicate that DNA methylation and DNA hydroxymethylation are two important epigenetic mechanisms which regulate gene expression in the aging brain, yet, very little is known about the levels of markers of DNA methylation and hydroxymethylation in the brains of patients with AD, the cell-type-specificity of putative AD-related alterations in these markers, as well as the link between epigenetic alterations and the gross pathology of AD. The present quantitative immunohistochemical study investigated the levels of the two most important markers of DNA methylation and hydroxymethylation, i.e. 5-methylcytidine (5-mC) and 5- hydroxymethylcytidine (5-hmC), in the hippocampus of AD patients (n=10) and compared these to non- demented, age-matched controls (n=10). In addition, the levels of 5-hmC in the hippocampus of the two individuals of a monozygotic twin pair discordant for AD were assessed. The levels of 5-mC and 5-hmC were furthermore analyzed in a cell-type and hippocampal subregion-specific manner, and were correlated with amyloid plaque load and neurofibrillary tangle load. The results showed robust decreases in the hippocampal levels of 5-mC and 5-hmC in AD patients (19.6% and 20.2%, respectively). Similar results were obtained for the twin with AD when compared to the non-demented co-twin. Moreover, levels of 5-mC as well as the levels of 5-hmC showed a significant negative correlation with amyloid plaque load in the hippocampus (r_p=-0.539, p=0.021 for 5-mC and r_p=-0.558, p=0.016 for 5-hmC). These human postmortem results thus strengthen the notion that AD is associated with alterations in DNA methylation and hydroxymethylation, and provide a basis for further

epigenetic studies identifying the exact genetic loci with aberrant epigenetic signatures.

Keywords: Epigenetic, 5-methylcytidine (5-mC), 5-hydroxymethylcytidine (5-hmC), twins
Introduction

Introduction

The biological mechanisms that underlie age-related dysfunction and the pathophysiology of Alzheimer's disease (AD) are still largely unknown. Recent findings have suggested that aging and AD are associated with profound changes in the epigenetic regulation of gene expression, especially at the level of DNA methylation (Chouliaras et al. , 2010, Mastroeni et al. , 2011). DNA methylation, one of the fundamental epigenetic mechanisms, regulates gene transcription and can result in long-term changes in cellular function (Jaenisch and Bird, 2003). Methylation of CpG di-nucleotides is catalyzed by DNA methyltransferases (Dnmts), disrupts the binding of transcription factors and recruits proteins known as methyl-CpG-binding domain proteins (MBDs) that are associated with chromatin compaction and gene silencing (Cedar and Bergman, 2009). DNA hydroxymethylation is a newly described epigenetic modification derived from the oxidation of methylated cytosines by ten-eleven translocation (TET) enzymes (Kriaucionis and Heintz, 2009, Tahiliani et al. , 2009). Recent evidence suggested that the biological role of DNA hydroxymethylation might be different from DNA methylation, while it is highly abundant in the brain compared to other tissues (Jin et al. , 2011, Munzel et al. , 2010, Valinluck and Sowers, 2007, Valinluck et al. , 2004).

Next to genetic mutations and the Apolipoprotein E4 genotype, aging is the most important risk factor for developing AD and is associated with aberrant DNA methylation patterns. In particular, aging has been linked with global DNA hypomethylation and hypermethylation of CpG islands in various bran regions, including the frontal and temporal cortex (Christensen et al. , 2009, Hernandez et al. , 2011, Siegmund et al. , 2007). Other recent reports have also shown alterations in the DNA hydroxymethylation marker 5-hydroxymethylcytidine (5-hmC) in the aging mouse brain (Chouliaras et al. , 2012, Song et al. , 2011, Szulwach et al. , 2011)

Evidence from genetic and immunohistochemical studies on AD cases further supports the notion that aberrant DNA methylation and hydroxymethylation is involved in the pathophysiology of AD. A genetic association study reported an increased risk of AD for carriers of certain genetic variants in TET1 (Morgan et al. , 2008). Immunohistochemical studies showed decreased levels of the DNA methylation marker 5-methylcytidine (5-mC) and a variety of enzymes and proteins involved in the process of DNA methylation, such as Dnmt1, and methyl-CpG binding protein 2 (MeCP2), in the entorhinal cortex of AD patients, (Mastroeni et al. , 2010), while similar findings of decreased 5-mC were observed in the frontal cortex of a monozygotic twin pair discordant for AD (Mastroeni et al. 2009) — the same twin pair used in the present study. Recent epigenetic studies furthermore indicate gene-specific alterations in DNA methylation in the brain, peripheral lymphocytes and transgenic mouse models of AD neuropathology (Bakulski et al. , 2012, Bihaqi et al. , 2011, Bollati et al. , 2011, Fuso et al. , 2012, Wang et al. , 2008); for review see (Chouliaras et al. , 2010). For example, Bakulski et al. (2012) have found disease-associated methylation differences in 948 out of 27,578 CpG sites examined using a genome-wide DNA methylation array in DNA derived from the human prefrontal cortex (Bakulski et al. , 2012),

while Fuso and colleagues (2012) have reported a decrease in the methylation of the presenilin 1 gene (PSEN1) promoter induced by vitamin B deficiency in cell lines and transgenic mouse models of AD, carrying familial mutations of the amyloid precursor protein (APP) gene (Fuso et al. , 2011).

Thus, accumulating evidence suggests a role for DNA methylation and hydroxymethylation in AD. However, it is currently unknown whether the previously reported AD-related decrements in DNA methylation and associated molecules in the entorhinal cortex are also present in the hippocampus, while alterations in DNA hydroxymethylation have not been investigated so far in AD. Furthermore, given the fact that most investigations were carried out on whole-tissue homogenates, it remains unclear whether the various hippocampal subregions and hippocampal cell types show selective vulnerability for alterations in epigenetic markers, and whether hippocampal alterations in epigenetic markers correlate with hippocampal amyloid plaque and/or neurofibrillary tangle load.

Materials and Methods

Human subjects and tissue processing

Postmortem brain materials were collected through the Banner Sun Health Research Institute Brain and Body Donation Program (Sun City, AZ). Blocks of fixed, paraffin-embedded hippocampal tissue (at the level of the lateral geniculate nucleus) from 10 AD cases and 10 age-matched controls were obtained from the donated brains. All tissue samples were collected under Institutional Review Board (IRB)-approved protocols and informed consents, according to the Declaration of Helsinki (BMJ 1991; 302: 1194), permitting the use of samples for research purposes. Antemortem evaluation by board-

certified neurologists and neuropsychologists and postmortem evaluation by a board-certified neuropathologist was performed for all cases as described previously (Beach et al. , 2012). The diagnostic criteria followed consensus guidelines for National Institute on Aging Alzheimer's Disease Centers. Age, gender and postmortem interval were well matched between the groups (see Table 1). The hippocampal tissue was sliced axially into 1-cm thick blocks, immersed to 4% parafolmaldehyde fixative solution for 48h at 4oC and embedded in paraffin. Subsequently, the paraffin blocks were cut into 6µm thick slices on a rotary microtome (Leica, Wetzlar, Germany), placed on microscope slides (VWR, Batavia, IL, USA) and stored in a dark at room temperature until further use.

The tissue from the monozygotic twin pair was obtained from the Boston University Alzheimer's Disease Center. Written informed consent for autopsy was obtained for both cases in compliance with institutional guidelines of Boston University, according to the Declaration of Helsinki (BMJ 1991; 302: 1194). Details regarding this monozygotic twin pair discordant for AD have been described previously (Mastroeni et al. , 2009). In brief, antemortem and postmortem evaluations were performed by board certified neurologists and a neuropathologist who determined the diagnosis according to the standard NIH AD centers protocols. The AD twin died at 76 years of age and the non-demented control (ND) twin at 79 years of age. Postmortem delay was 7.3 hours for the AD twin and 3.1 for the ND twin. They were autopsied at the same facility using the same tissue processing protocols. Due to limitations in hippocampal tissue availability only sections containing the CA1 region were available (see protocols below). In brief, the sections were sliced axially into 1-cm thick slabs, immersion fixed for 48 h in 4% parafolmaldehyde at 4°C, washed in phosphate buffer (PB), and cryoprotected in ethylene glycol and glycerol. The slabs were then sectioned at 40 µm on a cryostat. Free-floating sections were stored

in freezing solution (glycol/glycerol/PB) at −20°C until further use (Mastroeni et al. , 2009).

Immunohistochemistry and image analysis

For each marker, the sections were immunoreacted simultaneously using standard immunohistochemical procedures. In case of paraffin-embedded sections, deparaffination in two series of xylene, and rehydration in series of 100%, 96%, and 80% ethanol, and distilled water were performed before the immunohistochemical procedure. Briefly, the sections were further rinsed in PBS- T, followed by antigen retrieval using 10 mM citrate buffer at 95oC for 20 minutes, after which the sections were rinsed again with PBS-T and incubated in 1% H_2O_2 for 30 minutes to quench endogenous peroxidase. Subsequently, the sections were rinsed in PBS-T blocked in 3% bovine serum albumin (BSA) and incubated with the primary antibodies (5-mC, diluted 1:1000, Genway Biotech, San Diego CA, USA; 5-hmC, diluted 1:5000, Active Motif, Carlsbad, CA, USA) overnight at room temperature. After primary antibody incubation the sections were washed again and incubated for 2 hours with the secondary antibody (goat anti-mouse alexa 488 for 5-mC and goat anti-rabbit alexa 488 for 5-hmC, 1:5000, Invitrogen, Grand Island, NY, USA). Following washing in PBS-T, the sections were counterstained with Neurotrace red fluorescent stain (Invitrogen), washed again in PBS-T, taken through Sudan Black B (Sigma Aldrich, St Louis, MO, USA) to reduce autofluorescence, air-dried overnight and coverslipped using Vectashield mounting medium (Vector Labs, Burlingame, CA, USA). The Neurotrace counterstain was performed to employ neuron-specific analyses based on the characteristic morphology and size of neuronal cells when stained with this counterstain. The specificity of the 5-mC and 5-hmC primary antibodies was confirmed by using methylated or hydroxymethylated nucleotides (10 nM 5-mC dCTP,

10 nM 5-hmC dCTP; Zymoresearch, Irvine, CA, USA), which decreased the IR signal when pre-incubated with the primary antibodies (data not shown).

An Olympus IX51 microscope (Olympus, Tokyo, Japan) was used for all imaging procedures. Images were taken with the 40x objective using a green fluorescence filter for each staining, while their corresponding Neurotrace-counterstained images were taken using a red fluorescence filter. All images were taken using identical camera, microscope lens and light settings. One hippocampal section was used per each staining, where images were taken from five sites in the DG, five sites in the CA3, and five sites in the CA1 per subject (total of 15 images per subject for each staining concomitant with 15 corresponding Neutrorace-counterstained images). Fluorescence intensity analysis was performed using ImageJ software (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, imagej.nih.gov/ij/). The intensity measurements were corrected for background differences by dividing the measured intensities with the average intensity of a cell-free region, such as the hippocampal white-matter, in each section. For each image, the fluorescence intensity of 15 individual neurons and 15 individual cells of non-neuronal origin were analyzed by delineating the nucleus of each cell and measuring the mean intensity value. Thus, the fluorescence intensity of a total of 450 cells per individual was analyzed per staining. The discrimination between cells of neuronal and non-neuronal origin was done based on their characteristic morphology and size of neurons when using the Neurotrace counterstain (Sekirnjak et al. , 2003). According to these criteria, a clear differentiation between neurons and glia in the DG was not possible and thus no cell-type specific analyses were carried on in this subregion. Of note the measurements of fluorescence intensities were calculated in arbitrary units and do not represent absolute levels of these markers (Figure 1).

Statistical analysis

A multilevel linear mixed model was used for the statistical analyses. For each marker, the fluorescence intensities were used as the dependent variable and the AD diagnosis as the independent variable. Measurements of individual cells (level 1) were clustered in the different subjects (level 2). Statistical significance was set at $\alpha = 0.05$. Following the multilevel analyses, stratified analyses for neuronal cells and cells of non-neuronal origin (glial cells) were performed for each hippocampal subregion (CA3, CA1-2) or just for neuronal cells (DG) separately using the same statistical model. Correlation analyses were carried out by calculating the Pearson's correlation coefficient (rp). All statistical calculations were performed using STATA 11 (StataCorp, Texas, USA) or the Statistical Package for Social Science (SPSS 17, SPSS Inc., Chicago, IL, USA). Graphs were built in GraphPad Prism (Version 4, GraphPad Software, San Diego, USA).

Results

Qualitative analysis and quantitative analyses of 5-mC and 5-hmC IR in the hippocampus

5-mC and 5-hmC IR was detected in all cells, and was primarily observed as punctate IR in the nucleus. Microscopic inspection suggested a striking decrease in IR of both markers in the AD hippocampus when compared to controls (Figures 2, 3), and a similar pattern was observed for 5-hmC IR in the hippocampus of the AD twin when compared to the ND co-twin. Quantitative image analyses were further carried on to confirm these findings. Multilevel linear model analysis revealed a decrease of 5-mC IR in

the hippocampus of AD patients when compared to ND controls (-19.6%, p = 0.006). Similarly a significant decrease of 5-hmC IR (-20.2%, p = 0.012) was observed in the AD hippocampus when compared to ND controls.

5-mC and 5-hmC in glial cells and neurons of the hippocampal subregions

Analysis of 5-mC IR in neurons and glial cells in the three hippocampal subregions revealed that AD patients had a significantly decreased level of 5-mC in glial cells in the CA3 (-26.9%, p = 0.016), neurons in the CA1 (-21.1%, p = 0.01) and glial cells in the CA1 (-25.7%, p = 0.003), but no statistically significant differences for the DG or the CA3 neurons. Analysis of 5-hmC IR in neurons and glial cells in the three hippocampal subregions revealed that AD patients had a significantly decreased level of 5-hmC in cells of the DG (-16.1%, p = 0.042) and glial cells in the CA3 (-34.2%, p = 0.011), while tendencies towards statistical significance were observed for neurons within the CA3 (-18.4%, p = 0.064) and the neurons in the CA1 (-17.8%, p =0.083). No statistically significant differences for glia cells in the CA1 were found.

5-hmC in the monozygotic twins discordant for AD

Analysis of 5-hmC fluorescence intensity in the twins discordant for AD revealed a 31.4% reduction of 5-hmC IR in the CA1 of the hippocampus of the AD twin. Stratified analyses showed a reduction of 39.6% in the neurons and 20.7% in the glial cells of the CA1 subregion of the AD twin compared to the ND twin.

Correlation analyses

Linear correlation analyses revealed significant negative correlations between 5-mC IR and hippocampal amyloid plaque load (rp= -0.539,

p = 0.021), as well as 5-hmC IR and hippocampal amyloid plaque load (rp= -0.558, p = 0.016). Similarly, a trend towards statistical significance for a
negative correlation was observed between hippocampal 5-mC IR and neurofibrillary tangle load (rp= -0.461, p = 0.054).

Discussion

Qualitative and quantitative assessment of the sections of the human hippocampus revealed an AD- related decrease of both the DNA methylation marker 5-mC and the DNA hydroxymethylation marker 5- hmC. Moreover, similar patterns of decreased 5-hmC IR where observed in the CA1 hippocampal subregion in the affected twin of a monozygotic twin pair discordant for AD.

AD is associated with decreased hippocampal 5-mC IR

In the present study, we report that 5-mC IR, as a marker for global DNA methylation, is decreased in the hippocampus of AD patients when compared to ND controls. Pooled data from all cell types and all regions also revealed a decrease of 5-hmC IR, reflecting global DNA hydroxymethylation. Stratification of the data according to cell type and subregions reveals that the observed differences are particularly pronounced in the CA1 neurons and glia and in the glial cells residing in the CA3, while no differences were observed in the DG. The observed differences in 5-mC are in agreement with previous reports of a global loss of methylation in the entorhinal cortex of AD patients (Mastroeni et al. , 2010) and can further be linked to aberrant methylation patterns previously associated with AD (Bakulski et al. , 2012, Siegmund et al. , 2007, Wang et al. , 2008). Although the exact reason of the observed methylation changes is

not yet clear, this might be related to AD-specific deficits in the nuclear transport of epigenetic molecules, such as Dnmt1, which is responsible for maintenance of DNA methylation (Mastroeni et al. submitted). This disturbed nucleocytoplasmic transport has been linked with the downregulation of the Ras-related nuclear protein (RAN), which plays an important role in the transport of molecules into and out of the cell nucleus (Mastroeni et al.,2013).

AD is associated with decreased hippocampal 5-hmC IR

Besides DNA methylation marker 5-mC, which reflects a well-known epigenetic modification, the present study also addressed 5-hmC, which represents a recently described epigenetic modification. To our knowledge, this is the first study to investigate changes of 5-hmC in human AD. Pooled analyses showed a decrease of 5-hmC IR in the hippocampus of AD patients compared to ND controls, while stratified analyses showed that the observed changes were more pronounced in the CA3 and the DG regions. As with 5-mC IR, an AD-associated decrease was the main effect, in both neurons and glial cells. Because 5-hmC is an exclusive product of 5-mC, it could be speculated that the observed changes in 5-hmC are closely associated with the decrease of 5-mC. Interestingly, while age-associated increases of hippocampal 5-hmC have been reported (Chouliaras et al. , 2012, Song et al. ,2011) in normal aging our recent work in transgenic APPswe/PS1ΔE9 mice suggests a misbalance of hippocampal 5-mC and 5-hmC in APPswe/Ps1ΔE9 when compared to wild-type mice (Chouliaras et al, submitted), In parallel, another study reported that a genetic variant of TET1, one of the enzymes that catalyze DNA hydroxymethylation, is associated with an increased risk of developing AD (Morgan et al., 2008). Such findings suggest that 5-hmC and an altered methylation/hydroxymethylation potential might be linked with AD pathology (Van den Hove et al. , 2012). The exact consequences of

the observed changes of 5-hmC are unknown, but as with 5-mC, could be associated with gene expression alterations and cell-cycle reprogramming of cells in AD (Blalock et al. , 2004, Busser et al. , 1998, Nagy et al. , 1998), considering that 5-hmC is known to play an important role in regulating stem cell function (Haffner et al. , 2011, Pastor et al. , 2011, Szulwach et al. , 2011).

Twins

Similar to the comparison between AD cases and controls, 5-hmC IR was diminished in the hippocampus of the AD twin compared to the ND sibling. Unfortunately, due to limitations associated with the availability of brain materials from monozygotic twins discordant for AD, we were only able to examine 5-hmC IR in the CA1 hippocampal subregion. Previous reports on the same cases have already shown decrements of DNA methylation and associated markers in the temporal neocortex of this twin pair (Mastroeni et al. , 2009). We now report that the hippocampal 5-hmC changes are comparable to the general cohort, with an AD-associated decrease of hippocampal 5-hmC IR. Notably, the occupation-related environmental exposures of the AD twin were different from those of the ND twin and this could be associated with the development of AD (Mastroeni et al. , 2009).

Negative correlations between hippocampal AD pathology and DNA (hydroxy) methylation markers

There were negative correlations between hippocampal 5-mC and 5-hmC IR and amyloid plaque pathology. Moreover, a trend towards significance pointed to a negative correlation between 5-mC IR and hippocampal neurofibrillary tangle load. Such findings suggest that AD pathology is associated with epigenetic changes. However, it is not yet clear whether the observed epigenetic changes are the cause or the consequence of AD pathology and future studies should

therefore assess whether the observed epigenetic changes precede AD pathology.

Conclusions and future directions

Future studies should identify the gene-specific epigenetic changes that are associated with AD. Most of the techniques developed to date require large amounts of input DNA, and whole-tissue homogenates are mainly used for that purpose. However, cell-type- and subregion-specific changes, and neuronal loss occur in AD, which might alter the neurons/glia ratio when comparing AD brains to controls (Morrison and Hof, 2002, Torres-Munoz et al. , 2004). Thus, novel epigenetic approaches utilizing cell-sorting or laser-capture microdissected tissues and requiring less input material should be further developed.

Interestingly, as epigenetic changes are responsive to environmental stimuli, it should be examined whether brain-related profiles correlate with peripheral profiles, and whether changes of epigenetic markers in accessible tissue types may be used as a biomarker for AD. Epigenetic mechanisms are influenced by environmental exposures, and the data on discordant monozygotic twins further supports this notion. Specific environmental exposures that might be associated with AD, such as previous reports on metal-lead exposure, related to the latent early-life associated regulation (LEARn) model hypothesis, appear promising in this aspect (Bihaqi and Zawia, 2012, Lahiri et al. , 2009). Furthermore, specific gene-environment interactions should be investigated, as it is known that the effect of certain environmental exposures is highly dependent on individual genetic susceptibility (Chouliaras et al. ,2010).

Future research needs also investigate the exact genomic localization and downstream effects of the observed AD-associated epigenetic changes, such as the effects on histone modifications, and gene and protein expression. While it is not yet clear whether epigenetic dysregulation is a cause or a consequence of the disease, the fact that similar effects have also been observed in transgenic APPswe/PS1ΔE9 mice (Chouliaras et al, submitted) suggest that the observed DNA methylation and hydroxymethylation changes might be triggered by the Aβ pathology and may be associated with disturbances of nuclear-cytoplasmic transport of epigenetic molecules (Mastroeni et al., 2013). Given the limitations of the current technologies and the limited availability of brain tissue, examination of peripheral blood in cohort studies or animal studies could possibly answer these certain and related questions. Furthermore, examination of correlations with markers associated with the disease, such as synaptic deficits and neuronal loss, is of high importance (Coleman and Yao, 2003, Rutten et al. , 2005, Selkoe, 2002). Furthermore, the exact consequences of the observed epigenetic changes are unknown, but considering the role of epigenetic programming in the development and differentiation of cells, it could be speculated that the observed epigenetic changes induce gene expression changes, the accumulation of which may result in neuronal loss. As such, it will be crucial to clarify the involvement of epigenetic changes in the pathophysiology of AD. The present findings suggest that AD is associated with global changes of DNA methylation and hydroxymethylation and further show negative correlations between these epigenetic markers and hippocampal AD pathology.

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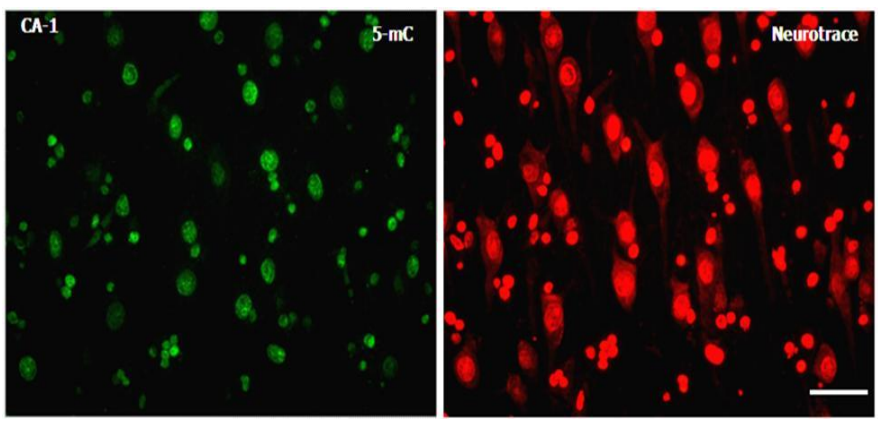
Table / Figures

Table 1 Descriptive statistics

Subject	Non-demented (ND) controls	Alzheimer's disease (AD) cases
Age	Mean = 77.91, s.d.= 4.1	Mean = 75.36, s.d. =5.5
Gender	W = 3, M = 7	W = 3, M = 7
Postmortem interval	Mean = 2.77, s.d.= 0.61	Mean = 2.98, s.d. =0.93
Braak Staging	I-III	V-VI

Figure-1. Hippocampal 5-methylcytidine (5-mC) immunoreactivity (IR) and Neurotrace counterstain. Representative photomicrograph of 5-mC IR (green) and the corresponding Neurotrace image (red) taken from the CA1 hippocampal subregion. Based on the Neurotrace morphology, a total of 15 neurons and 15 glial cells were delineated within each photomicrograph and their fluorescence intensity was analyzed using the



ImageJ software (see text for more details). A total of 15 images per subject for each staining concomitant with 15 corresponding Neutrorace-counterstained images were analyzed. Scale bar = 50 µm.

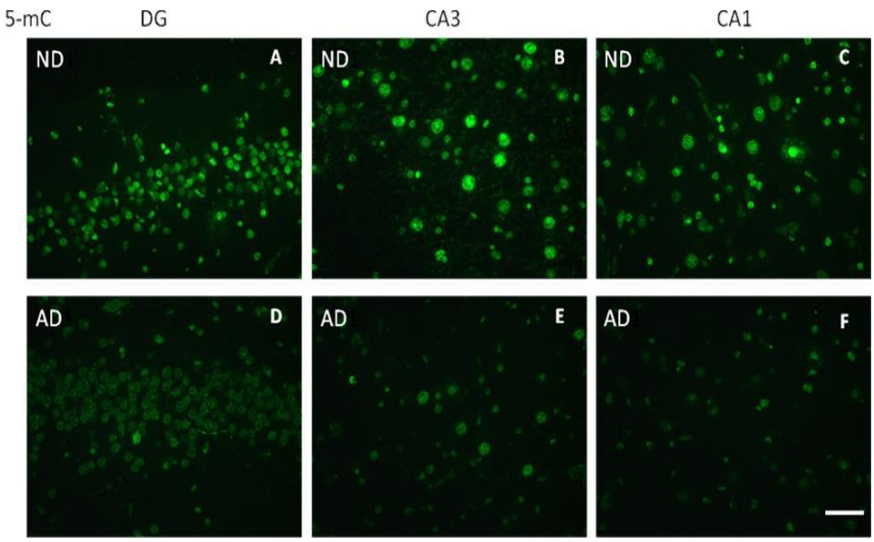


Figure-2. Representative photomicrorapgs of 5-mC IR. High magnification representative photomicrographs of the hippocampal DG, CA3, and CA1-2 regions. A-C represent non-demented control cases (ND), and D-F Alzheimer's disease cases (AD). Note: A loss of 5-mC IR is observed in AD cases when compared to ND controls in all three hippocampal subregions. The images were taken with a 40x objective. Scale bar = 50 µm.

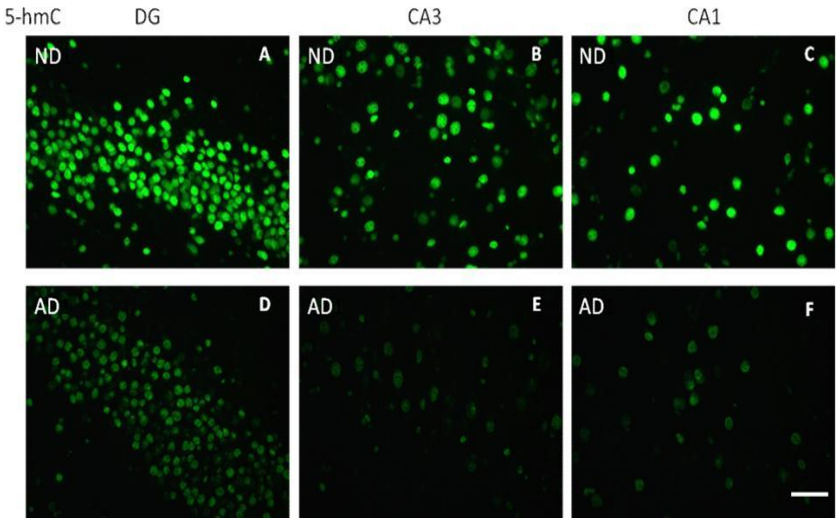


Figure-3. Representative photomicrographs of 5-hydroxymethylcytosine (5-hmC) IR. High magnification representative photomicrographs of the hippocampal DG, CA3, and CA1-2 regions. A-C represent non- demented control cases (ND), and D-F Alzheimer's disease cases (AD). Note: A decrease of 5-hmC IR is observed in AD cases when compared to ND controls in all three hippocampal subregions. The images were taken with a 40x objective. Scale bar = 50 µm.

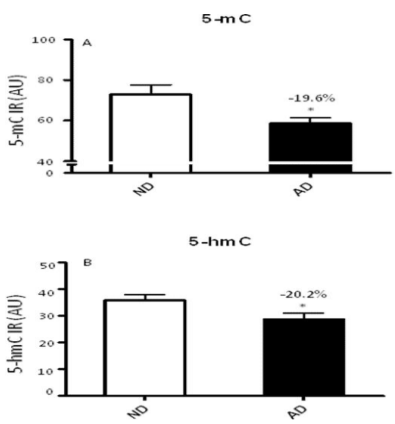


Figure-4. 5-mC and 5-hmC hippocampal fluorescence intensities. Mean and standard error of the mean of fluorescence intensity measurements of 5-mC (A) amd 5-hmC (B) IR. Pooled data from the three hippocampal subregions of ND cases in white bars and AD cases in black bars. The percentage of

decrease in each analysis and the significant effects ($p < 0.05$ in all cases) are indicated with an asterisk in each graph. AU= arbitrary units.

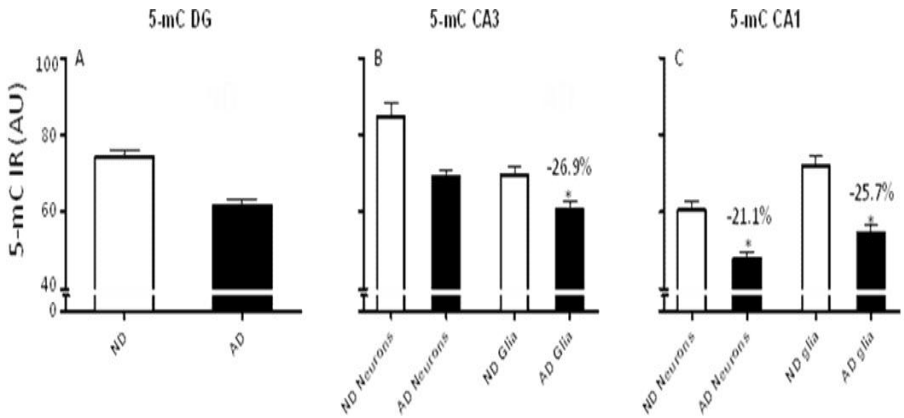


Figure-5. Stratified hippocampal 5-mC fluorescence intensities. Mean and standard error of the mean of fluorescence intensity measurements of 5-mC IR (A-C). Pooled data from the ND (white bars) and AD cases (black bars) are represented separately for the DG (A), CA3 (B) and CA1-2 (C). The percentage of decrease in each analysis and the significant effects ($p < 0.05$ in all cases) are indicated with an asterisk in each graph. AU= arbitrary units.

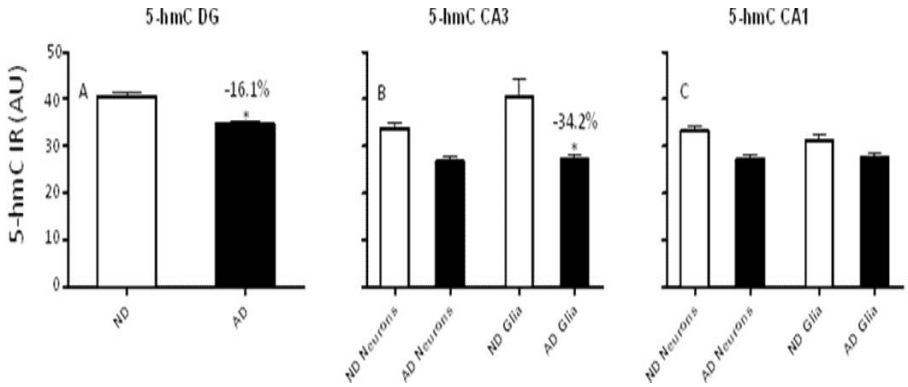


Figure-6. Stratified hippocampal 5-hmC fluorescence intensities. Mean and standard error of the mean of fluorescence intensity

measurements of 5-hmC IR (A-C). Pooled data from the ND (white bars) and AD cases (black bars) are represented separately for the DG (A), CA3 (B), and CA1-2 (C). The percentage of decrease in each analysis and the significant effects ($p < 0.05$ in all cases) are indicated with an asterisk in each graph. AU= arbitrary units.

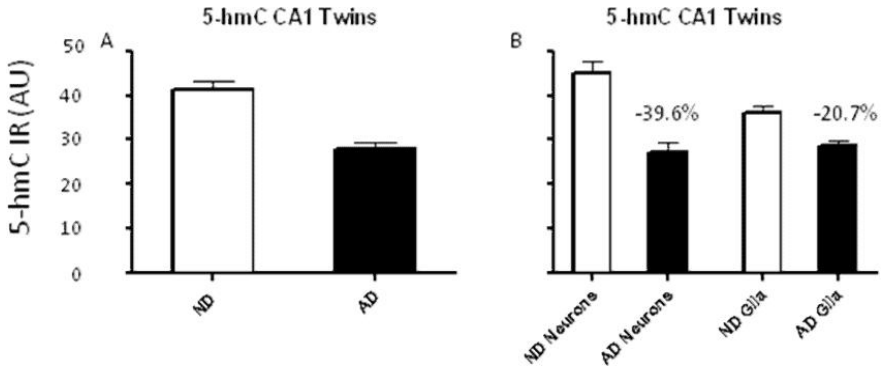
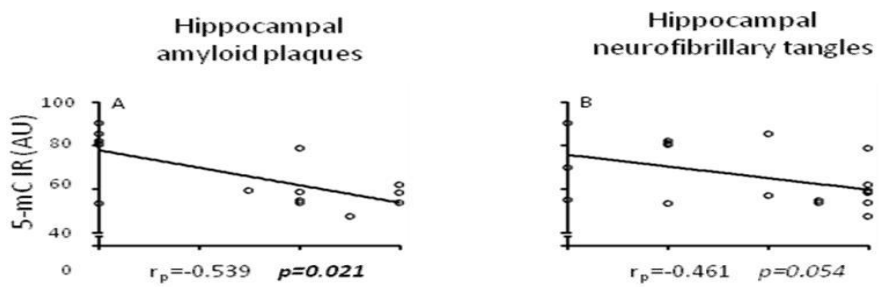


Figure 7. Hippocampal 5-hmC fluorescence intensities in the monozygotic twin pair discordant for AD. Mean and standard error of the mean of fluorescence intensity measurements of 5-hmC IR (A-B). Pooled data (A) and stratified data from neurons and glial cells (B) from the ND (white bars) and AD twin (black bars) are represented for the CA1 hippocampal subregion. The percentages of decrease in each analysis are indicated in each graph. AU= arbitrary units



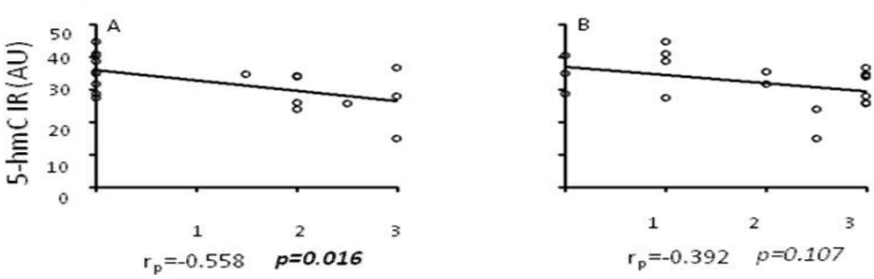


Figure 8. Correlation analysis between hippocampal 5-mC, 5-hmC IR and amyloid plaque and neurofibrillary tangle loads. Spearman's correlation coefficients and p values are noted in the bottom of each graph.

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Chapter 6

Age-related disturbances in DNA methylation and hydroxymethylation in the hippocampus of transgenic APPswe/PS1ΔE9 mice.

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Abstract

Brain aging has been associated with aberrant DNA methylation patterns, and a global loss of methylation and related markers has been observed in the entorhinal cortex of Alzheimer's disease (AD) patients. DNA hydroxymethylation, however, has been sparsely investigated in aging and AD. We have recently reported that the levels of the DNA methylation marker 5-methylcytidine (5-mC) and the DNA hydroxymethylation marker 5-hydroxymethylcytidine (5-hmC) increase in the mouse hippocampus during aging from 12 to 24 months, while caloric restriction attenuated these changes. We have also reported robust decreases in 5-mC and 5-hmC in the hippocampus of AD patients compared to non demented controls. In the present study, we investigated 3- and 9-month-old APPswe/PS1ΔE9 transgenic and wild-type mice for possible age-related alterations in 5-mC and 5-hmC levels in three hippocampal subregions (dentate gyrus, CA3, CA1-2) using quantitative immunohistochemistry. While age-related increases in levels of both 5-mC and 5-hmC were found in wild-type mice, APPswe/PS1ΔE9 mice showed decreased levels of 5-mC and no age-related changes in 5-hmC throughout the hippocampus. Altogether, these findings suggest that aberrant amyloid processing impact on the balance between DNA methylation and hydroxymethylation in the hippocampus during aging of mice.

Keywords

Aging; Alzheimer's Disease (AD); APPswe/PS1ΔE9; Epigenesis; Epigenetics; DNA methylation; 5-methylcytidine (5-mC); DNA hydroxymethylation; 5-hydroxymethylcytidine (5-hmC).

Introduction

Alzheimer's disease (AD) is the most common form of dementia and its neuropathology is known to be associated with altered gene expression, in a hippocampal subregion-specific manner (Dunckley et al., 2006; Liang et al., 2010). Mutations in the genes encoding amyloid precursor protein (APP) and presenilin 1 (PS1) have been identified in autosomal dominant cases of AD and are known to affect brain function by altering APP processing during aging, and producing toxic amyloid beta (Aβ) protein species (Hardy, 1999, 2009; Tanzi and Bertram, 2001, 2005).

Recent evidence suggests that epigenetic regulation of gene expression is critically involved in the pathophysiology of AD (Chouliaras et al., 2010a; Mastroeni et al., 2011). Decreased levels of the DNA methylation marker 5-methylcytidine (5-mC) have been observed in the entorhinal cortex of AD patients when compared to age-matched controls (Mastroeni et al., 2010). This loss of DNA methylation is further associated with reduced levels of DNA methyltransferase (Dnmt) 1, methyl-binding protein (MBD) 2, and histone deacetylase (HDAC) 2 (Mastroeni et al., 2010). Similar findings were further observed in a monozygotic twin pair discordant for AD (Mastroeni et al., 2009). Interestingly, alterations of various global and gene-specific methylation profiles have been observed in post-mortem brain material of AD patients, in peripheral lymphocytes of AD patients and also in animal and cell culture models of AD neuropathology (Bollati et al., 2011; Chen et al., 2009; Fuso et al., 2009; Siegmund et al., 2007; Wang et al., 2008; West et al., 1995; Wu et al., 2008) (for review see (Chouliaras et al., 2010a)). Further implication of epigenetic dysregulation in AD is supported by

studies showing that treatment with HDAC inhibitors (HDACi), drugs that target the epigenetic machinery, reversed contextual memory impairment in APPswe/PS1 mice (Kilgore et al., 2010).

Advances in the field of epigenetics have recently revealed that hydroxymethylation of DNA, which yields 5-hydroxymethylcytosine (5-hmC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009), represents an intermediate, previously unknown product in the pathway of active DNA demethylation (Guo et al., 2011) and that 5-hmC is particularly abundant in the brain. Thus, one could speculate that 5-hmC may be crucially involved the dynamic regulation of gene expression in the brain during aging and in neurodegenerative disorders such as AD. To date, little is known on the role of DNA hydroxymethylation in AD (Van den Hove et al., 2012). Recent studies on the mouse hippocampus and cerebellum reported dynamic changes of 5-hmC during neurodevelopment and aging, such as an age-associated increase of hydroxymethylation of genes related to amyloid processing, (Song et al., 2011; Szulwach et al., 2011). Interestingly, a genetic association between AD and one of the enzymes that catalyze the 5-mC to 5-hmC conversion, TET1, has been reported (Morgan et al., 2008), suggesting that the conversion from 5-mC to 5-hmC might be particularly important in AD. Furthermore, we have recently reported a robust reduction of the DNA methylation marker 5-mC and the DNA methylation marker 5-hmC in the hippocampus of AD patients compared to matched controls (Chouliaras et al,2013), as well as an AD-associated decrease in 5-hmC in the hippocampus of a monozygotic twin pair discordant for AD (Chouliaras et al, 2013).

Aging is the most important risk factor for AD and age-related changes in epigenetic mechanisms in AD-vulnerable brain regions may thus be involved in setting the stage for the development of AD (Chouliaras et al., 2010a; Hernandez et al., 2011). We have previously shown in large cohorts of wild-type (WT)

mice that aging is associated with increased levels of 5-methylcytidine (5-mC) (Chouliaras et al., 2011b), as well as increased levels of the DNA hydroxymethylation marker 5-hmC in the various hippocampal cell layers (Chouliaras et al., 2012). However, no experimental animal studies to date have investigated whether age-related aberrations in APP and Aβ processing impact on the balance between 5mC and 5hmC in the brain. As such, it remains unknown whether the recent reports of global epigenetic changes in the AD brain are closely associated with aberrant amyloid processing.

Based on the postulated disbalance between DNA methylation and DNA hydroxymethylation in the pathophysiology of AD (Van den Hove et al., 2012) and our recent findings in the hippocampus of AD patients and controls, the aim of the current study was to investigate age-related changes in the hippocampal levels of the DNA methylation marker 5-mC, and the DNA hydroxymethylation marker 5-hmC, in a mouse model carrying familial mutations of AD. For that purpose, 5-mC and 5-hmC immunoreactivity (IR) was assessed in three hippocampal subregions, the dentate gyrus (DG), CA3, and CA1-2 of 3-month-old and 9-month-old wild-type (WT) mice and transgenic APPswe/PS1ΔE9 mice. In the present study, transgenic APPswe/PS1ΔE9 mice were used, which express high levels of mutated APP in neurons and develop AD-like amyloid pathology by 4 months of age (Jankowsky et al., 2001; Jankowsky et al., 2003; Van Tijn et al., 2012). Furthermore, hippocampal amyloid plaque loads were analyzed in these mice to verify Aβ deposition.

Materials and Methods

2.1. Animals

The present study used 11 male WT C57bl6J and 9 male transgenic APPswe/PS1ΔE9 mice on a C57BL6 background. The double transgenic APPswe/PS1ΔE9 line 85, originally described by

(Jankowsky et al., 2004), carries a co-integrate of 1) chimeric mouse/human APP695 carrying the Swedish mutation (K594M/N595L) and 2) human PS1 with deletion of exon 9 (Jankowsky et al., 2001), each under control of a mouse prion protein promoter. Line 85 was back-crossed to a C57Bl6 background for more than seven generations. PCR amplification of genomic DNA, isolated from the mouse tails, was performed in order to determine the presence of the APP and PS1 mutations and verify the genotype of the mice used in the present study as described previously (Van Tijn et al., 2012). All mice were housed in groups of 2, with ad libitum access to food and water, kept on a 12/12 hours light:dark circle and under standard temperature, humidity and specified pathogen free (SPF) conditions. All experiments were approved by the Animals Ethics Board of Maastricht University.

2.2. Experimental Design

Mice were sacrificed at ages of 3 and 9 months for immunohistochemical analysis. Thus, 4 experimental groups were generated based on age and genotype: 1) 3-month-old-WT mice (n = 5), 2) 9-month-old WT mice (n = 6), 3) 3-month-old APPswe/PS1ΔE9 mice (n = 4), and 4) 9-month-old APPswe/PS1ΔE9 mice (n = 5).

2.3. Tissue processing

The mice were anesthetized and transcardially perfused with tyrode solution and fixative solution (4% parafolmaldehyde, 0.9% NaCl, 1% acetic acid). Subsequently, the brains were removed and stored in a 1% sodium azide TBS solution (at 4o C). They were then embedded in 10% gelatin and cut serially in 50 µm-thick free-floating sagittal sections using a vibratome (VT 1200S, Leica, Wetzlar, Germany), yielding 10 subseries of every 10th section and stored again in 1% sodium azide TBS solution at 4o C until further histological processing.

2.4. Immunohistochemical detection of 5-mC and 5-hmC

For each immunohistochemical procedure, a series of sections was stained using standard immunohistochemical protocols as previously described (Chouliaras et al., 2011a). Briefly, the sections were rinsed in TBS and TBS-T, after which antigen retrieval was performed with 10 mM citrate buffer (PH 6.0) at 95o C for 20 minutes. The sections were then rinsed again, and incubated with 1% H2O2 for 1 hour to quench endogenous peroxidase activity. Subsequently, the sections were rinsed and blocked with 3% normal donkey serum and incubated overnight at room temperature with the primary antibodies (see below). The sections were then rinsed again with TBS and TBS-T, followed by incubation with the secondary antibodies (see below), After rinsing with TBS and TBS-T the sections were incubated with avidin–biotine–peroxidase complex (diluted 1:400; Vector laboratories, Burlingame, CA, USA) for another 2 hours. To visualize the horseradish peroxidase reaction product, the sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (1:1 DAB:Tris–HCl, 0.3% H2O2) (Sigma, Uithoorn, The Netherlands). The reaction was stopped after 10 min by rinsing with TBS. Subsequently, the sections were mounted on gelatin-coated glasses, dehydrated, and coverslipped using Pertex (Histolab Products AB, Göteborg, Sweden). Mouse monoclonal anti-5-mC (dilution 1:500, Genway Biotech, San Diego, CA, USA) was used as a primary antibody for 5-mC, and a biotinylated donkey anti-mouse (dilution 1:200; Jackson Westgrove, PA, USA) as the secondary antibody. For the detection of 5-hmC, a rabbit polyclonal anti-5-hmC antiserum (dilution 1:25,000; Active Motif, Rixensart, Belgium) was used as a primary antibody and a biotinylated donkey anti-rabbit (dilution 1:200; Jackson Westgrove, PA, USA) as the secondary antibody.

2.5. Analysis of 5-mC and 5-hmC IR

Mean intensity and surface area of 5-mC and 5-hmC IR were analyzed. For each of the two markers, 2 images from the CA1-2 region, 2 images from the CA3 pyramidal layer and 4 images from the granule cell layer of the DG were taken (Fig. 1) at 4 different lateral levels (2.525 mm, 1.95 mm, 1.35 mm and 0.675 mm according to the Allen Brain Atlas (www.mouse.brain-map.org), using a 40x objective. Thus, a total of 32 images were taken for every animal for each marker, with a digital camera (F-view; Olympus, Tokyo, Japan) connected to an Olympus AX70 brightfield microscope (AnalySIS; Imaging System, Münster, Germany). Mean intensities and surface area measurements of each image were obtained using the ImageJ software program (version 1.42q, Wayne Rasband, National Institutes of Health, Bestheda, Maryland, USA), after delineating the regions of interest and correcting for background variation by setting minimum thresholds. Surface area measurements might be affected by volume changes and could be corrected for that (Chouliaras et al., 2011a). However, in the present study no significant hippocampal volume changes were detected (data not shown) and thus corrections for volumes were not performed.

2.6. Hippocampal Aβ plaque load IR

Hippocampal amyloid plaque loads were determined in a series of sections from the same mice used for 5-mC and 5-hmC IR analyses, with a triple fluorescence immunohistochemical analysis. An antibody against the N-terminal of Aβ (Amyloid β [N] IBL international, Hamburg, Germany) was used to stain all Aβ, thioflavin S (Sigma Aldrich, Zwijndrecht, The Netherlands) to stain highly dense-core plaques and DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma Aldrich, Zwijndrecht, The Netherlands) was used as a counterstain. Briefly, antigen retrieval was performed with

10 mM citric buffer pH 6.0 at 95oC. Then, the sections were with TBS and TBS-T, incubated with H2O2 to quench endogenous peroxidase, washed again with TBS and TBS-T and blocked with 3% normal donkey serum (NDS) in TBS-T before overnight incubation with the primary antibody (Amyloid β [N]; IBL international, Hamburg, Germany; diluted 1:1000 in 0.3% NDS). The next day, the sections were rinsed again with TBS and TBS-T, incubated with the secondary antibody (donkey anti-mouse Alexa 594 Invitrogen, Grand Island, NY, USA), rinsed again with TBS and TBS-T, incubated with Thioflavin S (0.0075%) for 10 minutes and then rinsed for 5 minutes in 70% ethanol. Subsequently, the sections were washed again with TBS and TB-T and counterstained with DAPI. Accordingly, the sections were mounted on gelatin-coated microscope glasses, taken through sudan black (Sigma Aldrich, St Louis, MO, USA) to reduce autofluorescence and coverslipped using 80% glycerol in TBS.

2.7. Analysis of Hippocampal Aβ plaque load densities

All measurements were performed using a modified BX50 bright field microscope (Olympus, Tokyo, Japan), Olympus UPlanApo objectives (Olympus, Tokyo, Japan), a three-axis high-accuracy computer-controlled stepping motor specimen stage for automatic sampling (4x4 Grid Encoded Stage; Ludl Electronics, Hawthorne, NY, USA), a linear z-axis position encoder (Ludl), a MBF-CX9000 CCD color camera (1200x1800 pixels; CX9000; MBF Bioscience) and controlling software (Stereo Investigator, MBF Bioscience, Williston, VT, USA). Plaque density analysis was performed in all the hippocampal sections stained for Aβ. Delineations of the total hippocampus and the cell layers of the DG, the CA3 and the CA1-2 were performed using the 20x objective. Within the delineated areas, the plaque area was measured with the Area Fraction Fractionator probe of the Stereo Investigator software using a 40x objective (oil, NA=1.00). Thioflavin S staining indicated the presence

of dense-core plaques, while antibody Aβ [N] could demonstrate the less fibrillar plaque load. The plaque area was compared to the total hippocampal area of that respective section to obtain an estimate of the plaque load.

2.8. Statistical Analysis

All data are presented as mean and standard error of the mean (S.E.M.). The general linear model univariate analysis of variance (GLM) was used for comparisons between groups, accounting for the main and interactive effects of age, and genotype. Statistical significance was set at $\alpha = 0.05$. Pair-wise comparisons were performed with a Bonferroni post-hoc test. In the absence of a significant interaction, main effects of age were analyzed by an additional stratified analysis per genotype, in order to assess whether overall effects of age were more pronounced in a particular genotype group. All statistical calculations were performed using the Statistical Package for the Social Sciences, (SPSS 17, SPSS Inc., Chicago, IL, USA). Graphs were built in GraphPad Prism (Version 4, GraphPad Software, San Diego, USA).

Results

3.1. Qualitative analysis of 5-mC and 5-hmC IR

Nuclear IR of both 5-mC and 5-hmC was observed in the hippocampal subregions (Figs 2, 3). 5-mC IR consisted mainly of compact condensations, while 5-hmC IR was spread throughout the nucleus (Figs 2, 3). Qualitative microscopic inspection indicated marked increases of both 5-mC and 5-hmC IR in all 3 hippocampal subregions of 9-month-old as compared to 3-month-old WT mice (Figs 2A-F, 3A-F). In contrast, an age-related decrease of 5-mC IR (Fig. 2G-L) was observed in the 9-month-old as compared to 3-

month-old APPswe/PS1ΔE9, while no apparent differences in 5-hmC IR (Fig. 3G-L) were observed in these same mice.

3.2. 5-mC intensity

GLM revealed a main effect of genotype in the DG ($p < 0.001$) and CA1-2 ($p = 0.008$) regions and a main effect of age in the DG ($p = 0.02$) (Fig. 4A-C). Furthermore, significant age x genotype interactions were observed in all hippocampal subregions ($p = 0.001$ for DG, $p = 0.002$ for CA3, and $p = 0.004$ for CA1-2; Fig. 5A-C). Pairwise Bonferroni post-hoc comparisons showed that age was associated with a significant increase in the intensity of 5-mC IR in the DG (+8,6%, $p = 0.001$) and the CA3 (+6,5 %, $p = 0.044$) regions of WT mice. Moreover, Bonferroni post-hoc comparisons revealed a decrease of 5-mC IR intensity in the 9-month-old APPswe/PS1ΔE9 mice compared to 9-month-old WT mice in all hippocampal subregions (-11,3%, $p < 0.001$ for DG; -9,1%, $p = 0.004$ for CA3; and -10,8%, $p = 0.001$ for CA1-2). Thus, age was associated with significantly higher levels of 5-mC IR in WT mice, whereas the APPswe/PS1ΔE9 genotype with lower levels of 5-mC IR in 9-month-old mice.

3.3. 5-mC surface area

GLM for surface area measurements of 5-mC IR showed a significant age x diet interaction in the DG ($p = 0.031$) (Fig. 4D), but not in CA3 ($p = 0.079$) or CA1-2 ($p = 0.104$). Pair wise post-hoc comparisons using the Bonferroni correction showed that the observed changes were not specific for any individual experimental groups.

3.4. 5-hmC intensity

GLM revealed a tendency towards statistical significance for the main effect of age in the DG ($p = 0.053$) and the CA3 ($p = 0.099$) subregions (Fig. 5A-C), and no effect in the CA1-2 region ($p = 0.149$).

Stratified analyses per genotype showed an increase of mean intensity of 5-hmC IR associated with aging from 3- to 9-month-old mice in all hippocampal subregions ($p = 0.022$ for DG, $p = 0.016$ for CA3, and $p = 0.044$ for CA1-2) in the WT mice, but not in the APP/PS1 mice ($p = 0.565$ for DG, $p = 0.688$ for CA3, and $p = 0.726$ for CA1-2).

3.5. 5-hmC surface area

GLM revealed only non-significant trends for increased 5-hmC surface area IR by age in the DG ($p = 0.074$) and the CA3 ($p = 0.054$) subregions, and no effect in CA1-2 ($p =0.249$).

3.6. Hippocampal Aβ plaque load densities and correlation with 5-mC and 5-hmC IR

Amyloid plaque analysis demonstrated an age-dependent deposition from 3-9 to month-old mice (Supplementary Fig.1).

Discussion

Qualitative and quantitative assessment of the various hippocampal subregions revealed that aging in WT mice was associated with increased levels of 5-mC and 5-hmC. APP/PS1 mice showed however lower levels of 5-mC IR at 9 months of age and no significant alterations in 5-hmC levels. Altogether, these findings indicate that mutations in APP and/or PS1 impact on the balance between DNA methylation and hydroxymethylation in the aging hippocampus

4.1. Age-related increase of hippocampal 5-mC and 5-hmC IR in WT mice

In the present study, 5-mC IR was increased in 9-month-old compared to 3-month-old WT mice. As the anti-5-mC antibody particularly detects CpG-rich loci with higher sensitivity, the observed increase of 5-mC IR likely reflects increased methylation of CpG islands (Chouliaras et al., 2011b). These findings are in agreement with recent studies reporting that aging is associated with aberrant DNA methylation patterns, such as hypermethylation of CpG islands and loss of methylation of loci outside CpG islands (Christensen et al., 2009; Hernandez et al., 2011; Siegmund et al., 2007). Altered DNA methylation of the Arc gene has also been observed in the hippocampus of aging rats (Penner et al., 2010), while an age-related increase in global DNA methylation has been observed in brains of rodents (Rath and Kanungo, 1989; Ryu et al., 2011). Our current findings of increased 5-mC IR from 3- to 9-month-old WT mice are in accordance with our earlier findings of age-associated increases in levels of 5-mC (Chouliaras et al., 2011b) and DNA methylation-associated markers like Dnmt3a (Chouliaras et al., 2011a), as well as HDAC2 (Chouliaras et al, submitted), in 24-month-old mice compared to 12-month-old C57Bl6 mice. Similar to the findings on 5-mC IR in WT mice, 5-hmC IR in hippocampal subregions showed an age-related increase from 3- to 9-month-old in WT mice. 5-hmC has recently gained increased attention as it was described to be particularly abundant in the brain (Kriaucionis and Heintz, 2009; Munzel et al., 2010). A number of studies have shown that 5-hmC, which is a product of 5-mC, might have a different role in the regulation of gene expression when compared to 5-mC (Ito et al., 2010; Jin et al., 2010; Jin et al., 2011; Valinluck and Sowers, 2007; Valinluck et al., 2004). In fact, 5-hmC has also been identified as an intermediate step in active DNA demethylation (Guo et al., 2011).

Our finding of an age-associated increase of 5-hmC IR in WT mice parallels a recent report showing increases in 5-hmC in the aging mouse cerebellum (Song et al., 2011). Interestingly, the increases in

DNA hydroxymethylation in that study were particularly pronounced in genes related to neurodegeneration, including those encoding presenilins and secretases (Song et al., 2011). Our current findings are furthermore in agreement with our previous observations of increased 5-hmC IR in the hippocampus of 12- to 24-month-old mice (Chouliaras et al., 2012).

4.2. Disturbed age-related alterations of 5-mC and 5-hmC in APP/PS1 mice

Striking differences were observed in the temporal patterns of age-related changes in 5-mC IR in WT as compared with APPswe/PS1ΔE9 mice: 5-mC IR increased with aging from 3 to 9 months of age in WT mice, while aging from 3 to 9 months of age in APPswe/PS1ΔE9 mice was associated with lower levels of5-mC IR. A similar, but less pronounced, opposition of age-related patterns was found for 5-hmC IR in the hippocampus: 5-hmC IR increased with aging from 3 to 9 months of age in WT mice, but did not show significant age-related changes in APPswe/PS1ΔE9 mice. Thus, our findings indicate that mutant APP and/or PS1 induce alterations in the molecular cascade of DNA methylation and hydroxymethylation during aging in the mouse hippocampus, and suggest that aberrant amyloid processing may be associated with DNA (hydroxy)methylation changes also observed in the human AD brain (Chouliaras et al. submitted). The observed negative correlation between 5-mC IR and hippocampal amyloid plaque load in the CA1-2 subregion further supports this notion.

The age-related disturbances in 5-mC and 5-hmC in the APPswe/PS1ΔE9 from 3 to 9 months of age coincide with the development of AD-like pathology and behavioral deficits in this model (Garcia-Alloza et al., 2006; Ruan et al., 2009; Savonenko et al., 2005). Although our findings indicate that mutant APP and/or PS1 impact on age-related changes of DNA methylation and

hydroxymethylation, it remains to be answered whether age-related changes of DNA methylation and hydroxymethylation are primarily causal or compensatory. In this context, other studies have documented that intervening in the DNA methylation processes has consequences on amyloid processing. For example, dietary disturbances of the one-carbon metabolism, which regulates the methylation potential, induced promoter hypomethylation of the PS1 gene and increased amyloid plaque deposition in mice (Fuso et al., 2008; Fuso et al., 2011; Scarpa et al., 2006). Clearly, the interrelations between aging, amyloid processing, and DNA methylation are complex and further work in this area is needed. The present findings can be linked to our previous observations of decreased 5-mC and 5-hmC in the hippocampus of AD patients and controls (Chouliaras et al. submitted), and suggest that the presence of amyloid pathology is closely related to robust changes in global DNA methylation. In line with our present findings, various reports have shown alterations in DNA methylation in certain brain regions as well as in lymphocytes of AD patients when compared to controls (for review see (Chouliaras et al., 2010a)). For example, Mastroeni and colleagues have shown a robust loss of 5-mC IR and DNA methylation-stabilizing factors, like Dnmt1, MeCP2 and HDAC2 in the entorhinal cortex of AD patients when compared to controls (Mastroeni et al., 2010), while these findings were also replicated in a monozygotic twin pair discordant for AD (Mastroeni et al., 2009). Studies focusing on methylation changes in specific genes, repetitive elements and ribosomal DNA have furthermore reported significant changes in AD cases as compared to controls (Balazs et al., 2011; Bihaqi et al., 2011; Bollati et al., 2011; Mastroeni et al., 2011; Pietrzak et al., 2011; Wang et al., 2008; Wu et al., 2008).

It is important to mention that most of the current techniques used so far for the detection of DNA methylation, and thus applied in AD research, either cannot detect 5-hmC or cannot discriminate it

reliably from 5-mC (Huang et al., 2010; Jin et al., 2011; Nestor et al., 2010). Thus, the importance of 5-hmC might be underrepresented in current epigenetics research on aging and AD (Van den Hove et al., 2012).

4.4. Future prospects and conclusions

While the current immunohistochemical approach allows for the detection of subregion-specific changes and gives an estimate of total levels of 5-mC and 5-hmC, it would be interesting to investigate gene-specific changes of the two epigenetic markers and identify potential target pathways that are specifically affected by aging and by mutated APP and PS1. Evidently, the appropriate methods for the precise detection of each modification should be selected. Furthermore, the functional impact of epigenetic changes in DNA (hydroxy)methylation on gene expression, associated AD-like pathology, and behavioral correlates should be further examined. Moreover, whereas the present study focused solely on the hippocampus of male mice, investigation of potential sex effects and examination of other brain regions that are either heavily affected by aging and AD or are resilient are equally of high importance. The fact that 5-mC but not 5-hmC decrease in correspondence with Aβ plaque deposition in these mice suggests that mutant APP and/or PS1 disturb the balance between these 2 epigenetic processes during aging. Clearly, the exact implications of this disturbance and its possible role in the pathophysiology of AD remain to be elucidated. The current study design involved WT and double transgenic APP/PS1 mice. As such, it cannot be explained whether the findings of this study are due to genetic variation in the APP gene, the PS1 gene or the combination of both. While the observed epigenetic changes might be associated with the presence of AD pathology in the transgenic mice and the stochastic events that occur with aging, it should be noted that epigenetic mechanisms are

dynamic processes that are subject to environmental exposures and thus the effects of the environment and its impact on the epigenome should be more thoroughly investigated in the context of AD (Chouliaras et al., 2010a; Chouliaras et al., 2010b; Mill, 2011)
In conclusion, the present findings reveal differential age-associated changes of DNA methylation and hydroxymethylation in the hippocampus of WT and transgenic APP/PS1 mice and suggest that an altered DNA methylation/hydroxymethylation balance is likely implicated in the pathophysiology of AD.

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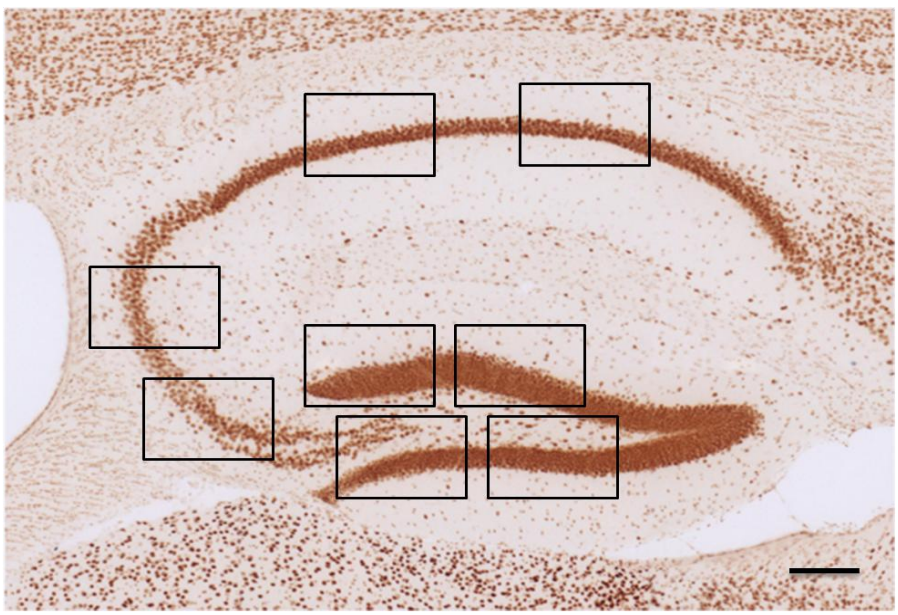
Figures

Figure 1. Hippocampal 5-hydroxymethylcytidine (5-hmC) immunoreactivity (IR). Representative photomicrograph of a hippocampal section stained for 5-hmC (lateral level 1.35 mm). The black boxes indicate where the high-magnification photomicrograph were taken for both the 5-hmC and the 5-methylcytidine (5-mC) analysis. A total of 32 photomicrographs per animal (4 photomicrographs in the dentate gyrus (DG), 2 in the CA3, and 2 in the CA1-2 regions, at 4 different lateral levels) were taken for each staining (see text for more details). Scale bar = 200 µm



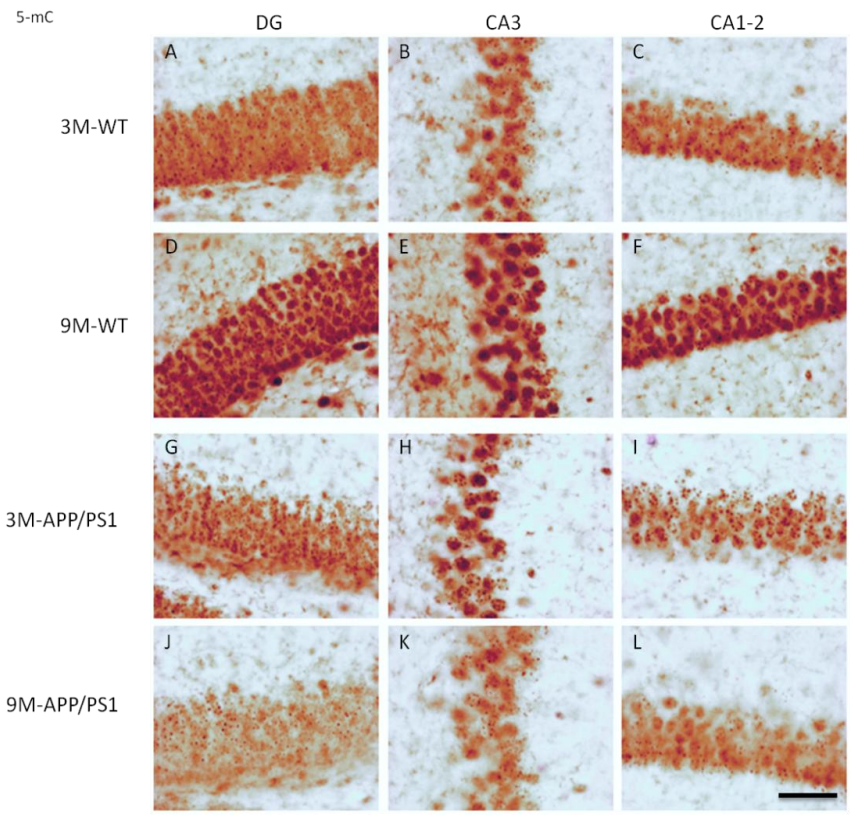


Figure 2. Representative photomicrographs of 5-mC IR. High-magnification representative fields of the hippocampal DG, CA3, and CA1-2 regions. A-C represent a 3-month-old wild-type (WT) mouse, D- F represent a 9-month-old WT mouse. G-I, represent a 3-month-old APPswe/PS1dE9 (APP/PS1) mouse, and J-L a 9-month-old APP/PS1 mouse. Note that an increase in 5-mC IR is observed from 3 to 9 months, in WT mice while a loss of 5-mC IR is observed in from 3- to 9-month-old APP/PS1 mice in all three hippocampal subregions. The photomicrographs were taken with a 40x objective. Scale bar = 50 µm.

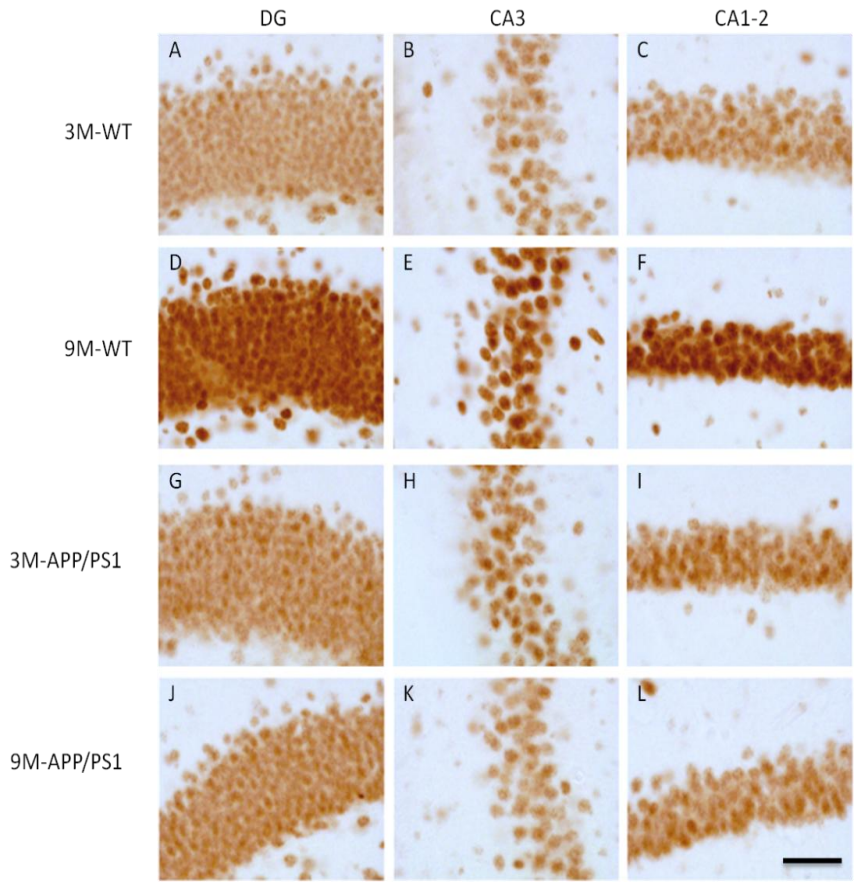


Figure (3). Representative photomicrographs of 5-hmC IR. High magnification representative photomicrographs of the hippocampal DG, CA3, and CA1-2 regions. A-C represent a 3-month-old WT mouse, D- F represent a 9-month-old WT mouse. G-I, represent a 3-month-old APP/PS1 mouse, and J-L a 9-month-old APP/PS1 mouse. Note that an increase in 5-hmC IR is observed from 3 to 9 months in WT mice while no apparent differences are observed in the APP/PS1 mice in all three hippocampal subregions. The images were taken with a 40x objective. Scale bar = 50 µm.

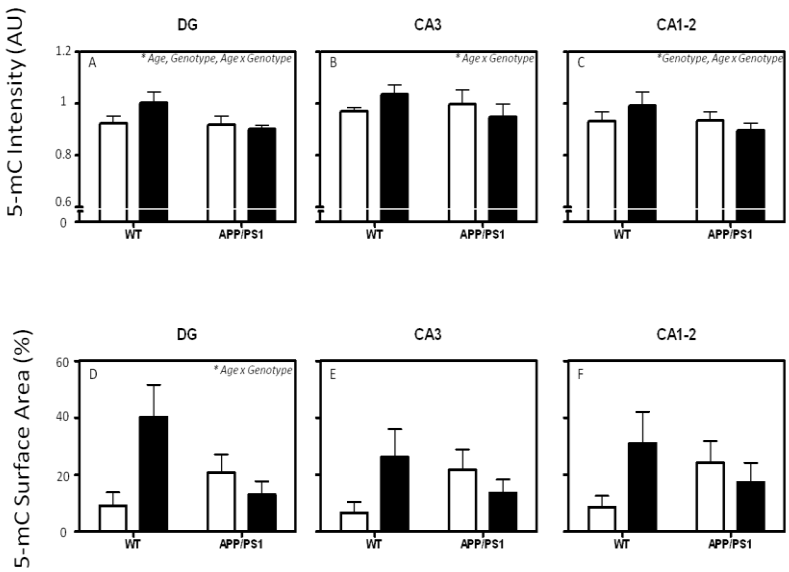


Figure (4). 5-mC intensity and surface area. Mean and standard error of means of mean intensity value measurements of 5-mC IR (A-F). Pooled data from the 4 groups of 3-month-old (white bars) and 9-month-old WT and APP/PS1 mice (black bars) are represented separately for the DG (A, D), CA3 (B, E), and CA1-2 (C, F). Significant effects ($p < 0.05$ in all cases) in each analysis are indicated with an asterisk in the top right corner of each graph. AU= arbitrary units.

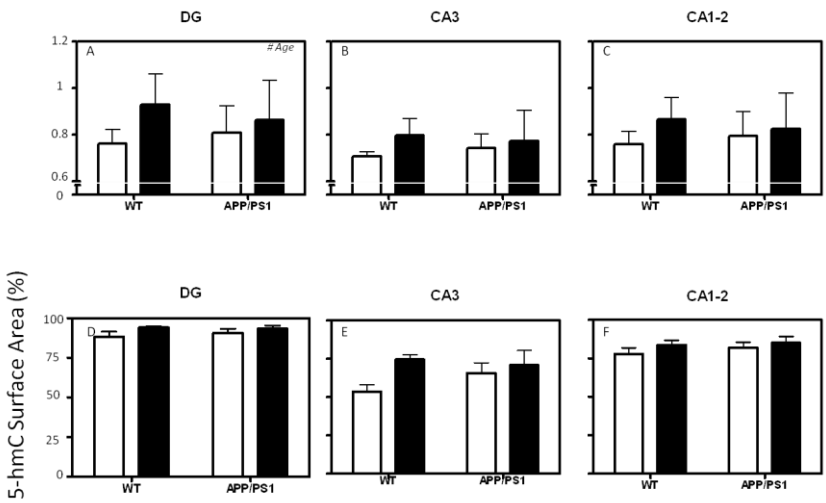


Figure (5). 5-hmC intensity and surface area. Mean and standard error of means of mean intensity value measurements of 5-hmC IR (A-F). Pooled data from the 4 groups of 3-month-old (white bars) and 9-month-old (black bars) WT and APP/PS1 mice are represented separately for the DG (A, D), CA3 (B, E), and CA1-2 (C, F). Significant effects ($p < 0.05$ in all cases) in each analysis are indicated with an asterisk in the top right corner of each graph. AU= arbitrary units.

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Chapter 7

Reduced RAN expression and disrupted transport between cytoplasm and nucleus; a key event in Alzheimer's disease pathophysiology.

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Abstract

Transcription of DNA is essential for cell maintenance and survival; inappropriate localization of proteins that are involved in transcription would be catastrophic. In Alzheimer's disease brains, and *in vitro* studies, we have found qualitative and quantitative deficits in transport into the nucleus of DNA methyltransferase 1 (DNMT1) and RNA polymerase II (RNA pol II), accompanied by their abnormal sequestration in the cytoplasm. RAN (*RA*s-related *N*uclear protein) knockdown, by siRNA and oligomeric Aβ42 treatment in neurons, replicate human data which indicate that transport disruption in AD may be mechanistically linked to reduced expression of RAN, a pivotal molecule in nucleocytoplasmic transport. *In vitro* studies also indicate a significant role for oligomeric Aβ42 in the observed phenomena. We propose a model in which reduced transcription regulators in the nucleus and their increased presence in the cytoplasm may lead to many of the cellular manifestations of Alzheimer's disease.

Introduction

Normal cell function requires constant exchange of molecules between the nucleus and the cytoplasm. Classically, RNA from the nucleus is packaged and transported to the cytoplasm for translation, and the resulting, newly-synthesized molecules either remain in the cytoplasmic compartment or are transported back into the nucleus to participate in a variety of functions. Virtually all

nuclear proteins begin their existence in the cytoplasm, and their failure to be translocated back to the nucleus could be as catastrophic as their failure to be synthesized. Nucleocytoplasmic transport in both directions is mediated by transport proteins and macromolecules that carry molecules through the nuclear pore complex, a process that depends on the active participation of the pivotal molecule RAN[1].

Epigenetic and other highly-conserved transcription-related factors are among important proteins that depend on RAN-mediated transport for appropriate nuclear localization[2,3]. DNA methylation by DNA methyltransferases (DNMT1 for example), can alter binding of transcription factors to their target genes, as well as recruit methyl-CpG-binding domain (MBD) proteins to the locus[4]. This, in turn, may recruit other chromatin remodeling proteins such as histone deacetylase 1 (HDAC1), thereby modifying histone proteins and transcriptional access[5]. Likewise, RNA pol II, which is essential for transcribing RNA also requires RAN-mediated transport directly, or indirectly, in order to fulfill its functional roles[6,7].

Several previous studies have suggested deficits in Alzheimer disease (AD) of molecules related to transport between the nucleus and the cytoplasm [8-10]. These reports led us to hypothesize disrupted transport of transcription-related molecules between the cytoplasm and the nucleus in AD. In view of the emerging evidence of the importance of epigenetic molecules in regulating gene expression in AD[11-13], our tests of this hypothesis concentrated on a fundamental epigenetic molecule, DNMT1, as well as one other molecule representative of an additional aspect of regulation of transcription, RNA pol II. Examination of human, and *in vitro* samples using immunohistochemistry, Western blots, *in situ* hybridization, gene expression microarrays and siRNA revealed a potentially catastrophic failure of transport into the

nucleus of DNMT1 and RNA pol II in pathologically-vulnerable AD neurons. We show, for the first time, decreased appearance of these molecules in the nucleus and their cytoplasmic sequestering. Our data also indicate that this phenomenon may be causally linked to reduced protein and mRNA expression of RAN, a pivotal component in the transport of molecules between the cytoplasm and nucleus, and that oligomeric Aβ42 plays a significant role in the phenomena we describe.

Materials and methods:

***Ethics Statement:**

Written informed consent for autopsy was obtained for all cases in compliance with institutional guidelines of Banner Sun Health Research Institute. Banner Sun Health Research Institute review board approved this study including recruitment, enrollment, and autopsy procedures. All donors and their respective next-of-kin consented to brain autopsy for the purpose of research analysis as participants in the Banner Sun Health Research Institute autopsy program. The human brain tissue used in this manuscript was from routine existing autopsies, which fully qualifies for 4C exemption by NIH guidelines.

1.1. Subjects, brain samples and cells.

Samples of human limbic cortex, and cerebellum were secured from AD and ND brains obtained at autopsy at the Banner Sun Health Research Institute Tissue Bank. Cognitive status of all cases was evaluated antemortem by board-certified neurologists, and postmortem examination by a board-certified neuropathologist

resulting in a consensus diagnosis using standard NIH Alzheimer's Center criteria for AD or neurologically normal, non-demented elderly control (ND). The AD and ND groups were well matched for age (AD: 83 +/- 3.3 years; ND: 84 ± 2.4 years), gender (3 females and 2 males in each group), and postmortem interval (PMI) (AD: 3 hours 12 min +/- 9 min; ND: 3 hours 48 minutes +/- 8 min). For ***in vitro* studies**, SK-N-BE(2) neuroblastoma cells (ATCC) were reconstituted and maintained following ATCC guidelines. Cells were maintained in a humidified 37ºC incubator with 5% CO_2, and were supplied with complete DMEM (500 ml DMEM with high glucose, minus phenol red (Invitrogen-Gibco)50 ml FBS (Gemini Bio-Products; West Sacramento, CA), 10 ml HEPES (Irvine Scientific, Santa Ana, CA.), 5 ml sodium pyruvate (Mediatech Cellgro), 5 ml penicillin/streptomycin (Invitrogen-Gibco), and 0.5 ml gentamycin (Irvine Scientific) every three days until experiments were performed (approx. 1 week after initial platting The general health of the neurons before and after treatment was tested using both calcein AM (Invitrogen) and LDH assays (Invitrogen) (supplementary fig 1), which showed excellent health.

Affymetrix Array studies:

RNA was isolated from laser captured pyramidal single neurons from postmortem human samples of CA1 of the hippocampus, superior frontal gyrus, and visual cortex. Gene expression was analyzed using Affymetrix Human Genome U133 plus 2.0 microarrays. The AD (n=10) and ND (n=10) groups were well matched for age (AD: 77.8 +/- 4 years; ND: 81.5 +/- 1.2 years), gender (AD: 3 females and 7 males, ND: 4 females and 6 males). See section 1.4. for further details.

1.2. Immunohistochemistry/Immunocytochemistry.

Brain tissue or cells were fixed in PFA, washed in phosphate

buffer (PB). Tissue samples were sectioned at 40µm on a cryostat, and stored in a cryoprotectant solution of 33% glycol/33% glycerol/33% PB at -20°C until required for experiments. Sections were washed, incubated in 10mM citrate buffer (antigen retrieval) for 10 min at 95°C, washed, blocked in 1% hydrogen peroxide followed by 1h incubation in 3% bovine serum albumin (BSA), and incubated at 4ºC overnight in primary antibody solutions containing 0.25% BSA. Sources and dilutions for antibodies are given in Table 1. After incubation, the sections were washed, incubated in biotinylated, species-specific secondary antibodies (Vector), and incubated in avidin-biotin complex (Pierce). Following incubation with secondary antibodies, the sections were washed and immersed in DAB solution for no longer than 10 min, followed by two quick rinses in 50 mM Tris to stop the reaction. AD and ND sections were immunoreacted simultaneously using netwells in well-less plates. Sections were mounted with Permount (Pierce). For fluorescence microscopy, the sections were washed 3X, blocked with either 3% normal goat serum or 3% BSA, and incubated for 1h. After further washing, sections were incubated in primary antibody, washed again, and incubated in species–specific, fluorophore-conjugated secondary antibodies (Molecular Probes). After a final wash, the sections were mounted, taken through Sudan Black to reduce autofluorescence, and coverslipped with Vectashield (Vector). Deletion of primary antibody or incubation with pre-immune serum resulted in abolition of specific immunoreactivity in all cases (data not shown). Adjacent serial sections were stained with cresyl violet, or within sections with neutral red for structural visualization. For some sections, nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen), a nuclear counterstain, before mounting.

Immunostained tissue sections were examined on Olympus IX51 and Olympus IX70 microscopes equipped with epifluorescence illumination or with confocal laser scanning using argon and krypton lasers (Olympus IX70). The findings were documented photographically with an Olympus DP-71 color digital camera or, for confocal microscopy, by Fluoview software (Olympus). Following photographical imaging, cellular localization was quantified using Image J software.

1.3. Western blot analysis.

For Western blots, tissue was cut into 1cm cubed pieces and white matter was carefully removed under a dissecting microscope, reserving the grey matter only for processing. After dissection, tissue was lysed in a solution containing 20mM Tris pH7.5, 0.5% Nonident (Sigma), 1mM EDTA (Sigma), 0.1M NaCl (Sigma), 1mM PMSF (Sigma), Sigma protease inhibitors 1, 2, and complete protease inhibitor cocktail (Roche). Protein concentrations were determined by BCA assay (Pierce) using bovine serum albumin as the standard. A total of 20ug of sample protein was combined with Laemmli sample buffer for separation by SDS-PAGE, followed by transfer to PVDF membrane (Bio-Rad). Membranes were blocked using 5% non-fat dry milk and probed with primary antibodies (Table 1). After incubation with primary antibody, membranes were washed, incubated with secondary antibody, washed again, reacted with chemiluminescence substrate (Pierce), imaged on an Alpha Ease detection system, and analyzed using AlphaEaseFC software (Alpha Innotech).

1.4. RAN and RAN Binding Proteins in Gene Expression Microarray

To extend the RAN data to selected RAN related transcripts, we mined a recent, collaborative, gene expression microarray study in which AD and ND cortical and hippocampal neurons were evaluated

after laser capture microdissection. Detailed methods for the study, which employed autopsy specimens from our Institution's brain bank and the brain bank at Washington University, have been previously published[14]. Briefly, RNA was isolated from approximately 500 laser-captured pyramidal neurons from the superior frontal gyrus (BA 10 and 11), visual cortex (BA 17), and CA1 of the hippocampus. Following amplification 10µg of fragmented cRNA was hybridized to an Affymetrix Human Genome U133 plus 2.0 Array. All genes that did not meet a 10% present call threshold were removed by Genespring GX 7.3 Expression Analysis software (Agilent, technologies: Palo Alto, CA).

1.5. RAN mRNA probe construction and *In situ* Hybridization:

Probe Construction:

RAN DNA oligonucleotide template was designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Palindromic sequences were omitted using palindromic sequence finder (http://biophp.org/minitools/find_ palindromes/demo.php), and sequence combinations stretching across exon-exon boundaries were omitted. The resulting templates were 5'-TGGTTGGTGATGGTGGTACTGGAAAAACGACCTTCGTGAAACGTCA TTTGACTGGTGAATTTGAGAAGAAGTATGTAGCCACCTTGGGTGTTGAG GTTCAT-3' and 5'-AAGAAGAATC TTCAGTACTACGACATT-3' (Sigma). Both sequences required the addition of an eight nucleotide sequence to the 3' end of the oligonucleotide (5'-CCTGTCTC-3"). Sense probes were constructed as negative controls. The mRNA probes for sense and anti-sense were constructed following the manufacturer's instructions, using the mirVana miRNA Probe Construction Kit (Applied Biosystems).

***In situ* Hybridization:**

20µm hippocampal AD (n=3) and ND (n=3) brain sections were mounted onto plus slides and allowed to dry overnight, immersed in a 0.007% pre-warmed 600 mAU/ml solution of proteinase K (Qiagen), and washed in DEPC ddh20 followed by the addition of 125µl acetic anhydride in 0.1M TEA. After incubating for 15min at RT, sections were washed in 2x SSC (saline sodium citrate), incubated in pre-hybridization solution, rinsed, and reacted with RAN-specific biotinylated mRNA probe in hybridization solution. Tissue was then incubated for 16h at 42°C in a humid chamber. After incubation, slides were washed, incubated with 50% formamide, rinsed in 2X SSC, incubated in 100µg/ml Rnase A solution, washed, and incubated in a series of SSC buffer. For staining, samples were treated as stated in section 1.2., with the exception that Streptavidin-conjugated secondary antibodies were employed to detect the biotinylated probes.

1.6. siRNA RAN knockdown:

SK-N-BE(2) neuroblastoma cells were seeded at a density of 15,000 cells/well in 12-well plates using antibiotic-free medium. The next day, cell culture medium was removed, replaced with Opti-MEM (Invitrogen), and cultures were placed in a 37ºC incubator with 5% CO_2. ON-TARGETplus Non-targeting Pool negative control siRNA (Dharmacon) and ON-TARGETplus SMARTpool siRNA to human RAN (Dharmacon) and cells were resuspended in nuclease free H_2O and diluted in Opti-MEM to various concentrations. The manufacture's protocol for Lipofectamine 2000 (Invitrogen) RNAi transfection mix was employed throughout, except for the Lipofectamine 2000 volume, which was increased to 2µL. Opti-MEM was then removed from the SK-N-BE(2) cells and each siRNA transfection complex was added to a specific well of cells. The cells were rocked every 10min for 0.5h in a 37ºC incubator with 5% CO_2. After 0.5h the cells were

overlaid with 900µL Opti-MEM for a final volume of 1mL. Final concentrations of negative control siRNA and RAN siRNA are indicated in the Figure legend. After 6h the Opti-MEM medium was removed and replaced with DMEM (Gibco) supplemented with 10% fetal bovine serum. Post transfection of RAN and control siRNA took place for 48h in a 37ºC incubator with 5% CO_2. Following experimental manipulation, cells were immunoreacted against antibodies (Table 1), following the methods stated in section 1.2. Cells were examined using epi-fluorescences (IX-71) microscopy. Experiments were performed in triplicate.

1.7. *In vitro* Amyloid beta 42 Treatment.

SK-N-BE(2) neuroblastoma cells were treated as described in section 1.1. prior to the addition of higher oligomeric or lower oligomeric amyloid beta 42 (Bachem). Amyloid beta 42 peptides were reconstituted in 0.5% ammonium hydroxide, and were immediately frozen down (lower molecular weight oligomeric Aβ42), or allowed to sit at room temperature for 96 hours (higher molecular weight oligomeric Aβ42). Western blot analysis using oligomeric antibody A11 (Sigma), revealed the presence of oligomers in both preparations, with higher weight oligomers in lane 1 (96 hour aggregation), compared to lane 2 (immediately frozen down) (Figure 3g). Amyloid beta 42 peptides were diluted in serum free Opti-Mem(Gibco) at an experimentally determined concentration of 1uM. Cells were incubated for 36 hours in triplicate and immunoreacted against antibodies (Table 1), following the methods stated in section 1.2. Cells were examined using confocal and epi-fluorescences microscopy and quantified using image J software.

1.8. Statistical analysis:

Tissue and Cells:

Fluorescence and bright field intensity analysis was performed using the Image J software. The intensity measurements were corrected for background differences by dividing the measured intensities with the average intensity of a region that showed no reactivity. Per image, the fluorescence intensity of 20 individual neurons per sample per condition, totaling 100 neurons from AD and 100 neurons from ND were analyzed. Neurons were evaluated by delineating the nucleus (dashed circle) from cytoplasm (solid black line, see figure 1), of each cell and measuring the mean intensity value of each area (i.e. nuclear and cytoplasmic). Analysis of fluorescence intensities were calculated in arbitrary units and do not represent the absolute quantity of these markers. Significance was determined using a two-tailed, paired student's t-test and declared significant at a p-value < .05. Correlation analysis was determined by using Pearson product-moment correlation coefficient analysis. Significance was set at the .05 level.

Array

A two-tailed unpaired t-test, assuming unequal variance (using multiple testing corrections by Benjamini and Hochberg False Discovery Rate), was applied to identify genes whose expression was significantly different in AD and ND. Fold changes were calculated by taking the ratio of the average expression signal for a particular gene across all samples (ND and AD) divided by the average expression signal for the same gene in ND samples (see [14]for further details).

Results

Reduced nuclear localization and cytoplasmic sequestration of DNMT1 and RNA pol II in CA1 neurons of human AD hippocampus.

Both epigenetic and transcription-related nuclear proteins evaluated, DNMT1, and RNA pol II exhibited markedly reduced nuclear immunoreactivity in AD hippocampal CA1 neurons compared to matched, cognitively normal ND cases. In AD, only very faint, punctate, nuclear immunoreactivity could be observed, whereas cytoplasmic staining was readily apparent. The cytoplasmic accumulation of nuclear proteins was largely contained within the cell soma, with some extension to first order dendrites and less extension to distal dendrites (Figure 1, a2, b2). ND neurons, by contrast, showed profuse immunoreactivity for both DNMT1 and RNA pol II within the nuclear compartment, with only modest cytoplasmic accumulation (Figure 1, a1, b1 respectively). Pathologically spared cerebellum was virtually identical in nuclear immunoreactivity in both ND and AD samples (Figure 1, c1-c2, d1-d2). Quantitation of AD neurons within CA1 (Figure 1e) showed significant decrease in DNMT1 nuclear immunoreactivity p= 8.5e-7 (t-test), and for RNA pol II p= 1.2e-4 (t-test). Cytoplasmic immunoreactivity for DNMT1 was significantly increased in AD, p= 1.8e-10 (t-test), and for RNA pol II, p= .001 (t-test). Moreover, correlation analysis between nuclear and cytoplasmic immunoreactivity revealed a significant negative correlation (r= -.45, p= .006 for DNMT1, and r= -.27, p=.012 for RNApol II) in AD neurons. Thus, as nuclear immunoreactivity decreases, cytoplasmic immunoreactivity increases. Similar regression analysis of control samples showed no significant correlation for DNMT1 (r=.12, p=.234) or RNA pol II (r=.22, p=.19). Array analysis of transcript expression of ten Alzheimer's cases and ten well matched controls

showed decreased expression of both DNMT1 and RNA pol II (Figure 1f). Although both mRNA probes show decreased expression in the CA1 of hippocampus, RNA pol II was the only probe to reach significance p=.0029 (t-test).

Decreased neuronal RAN mRNA and protein in AD CA1 of human hippocampus.

To investigate potential mechanisms that might underlie reduced nuclear and increased cytoplasmic localization in AD neurons, we assessed the expression of the key nucleocytoplasmic transport molecule, RAN. In ND hippocampus, RAN immunoreactivity was abundant, and localized throughout the nuclear compartment and cell soma, extending into the apical dendrites (Figure 2a). This is the appropriate distribution for RAN [15], a molecule whose function requires it to be transiently present in the cytoplasm or nucleus depending on the direction of transport. In AD cases, however, RAN immunoreactivity was qualitatively decreased, with the limited amount of staining being primarily localized to the nucleus (Figure 2b). Western blots of AD and ND cortical grey matter from temporal neo cortex quantitatively confirmed reduced protein expression, showing significant RAN decreases (p =0.0001) in AD compared to ND samples (Figure 2e). RAN deficits in AD were negligible in the pathologically-spared cerebellum, although an occasionally void in immunoreactivity could be seen in the larger Purkinje neurons in AD (see asterisk, Figure 2c, 2d).

At the transcript level, analysis of Affymetrix Human Genome U133 Plus 2.0 microarray data showed significant differences in RAN mRNA expression in AD compared to ND in laser captured neurons from CA1 of hippocampus, superior frontal gyrus, and primary visual cortex (Figure 2 h and i). Fold changes were similar in the heavily-impacted hippocampus and superior frontal gyrus (-4.6 and -5.0, respectively, for AD relative to ND case), and were substantially less

(-0.25) in the primary visual cortex, a cortical region with only modest AD pathology[16] (Figure 2 j and k). In addition to RAN mRNA, transcript expression for several key RAN binding proteins was analyzed and found to exhibit significant AD deficits (Table 2).

Reduced RAN transcript in AD was also demonstrated by fluorescence-label *in situ* hybridization. Signal for RAN mRNA was strongly localized to the nucleus, nucleolus, and cytoplasm of ND hippocampal CA1 neurons (Figure 2f), but was negligible in AD neurons (Figure 2g). Although the reactivity was weak in AD, the most prominent labeling could be observed within the nuclear compartment, with limited cytoplasmic signal that was consistent with the findings from immunohistochemistry (c.f., Figure 2b). Both AD and ND samples were incubated with sense probes and showed no obvious immunoreactivity (data not shown).

Reduced nuclear localization of epigenetic and other transcription-related molecules in oligomeric Aβ42 treated SK-N-BE(2) neuroblastoma cells.

Since disrupted nuclear localization was observed in AD in which amyloid precursor protein is expressed in excess, we confirmed the relationship between Abeta and RAN expression experimentally by treating cells with Abeta *in vitro*. Experimental treatment of neuroblastoma cells with 1µm oligomeric Aβ42 corroborated our hypothesis that amyloid is involved in reduced expression of RAN and consequent aberrant cytoplasmic sequestering of DNMT1 and RNA pol II. Figure 3, a1 shows the normal distribution of RAN protein in untreated cells and the aberrant loss of nuclear and cytoplasmic RAN with higher MW oligomeric Aβ42 (Figure 3, a2) and lower MW oligomeric Aβ42 treatment (Figure 3, a3). Although both species of Aβ42 were found to reduce RAN protein, higher MW oligomers was found to reduce RAN 19% more than lower MW oligomers (Figure 3d). Both epigenetic and transcription-related

nuclear proteins evaluated, DNMT1, and RNA pol II, showed qualitative and quantitative decreases in nuclear immunoreactivity and increased cytoplasmic sequestering when treated with both lower and higher MW oligomeric Aβ42 (Figure 3e and 3f). All experiments were performed in triplicate.

Knock down of RAN message by SMARTpool siRNA in vitro induces cytoplasmic sequestering of nuclear proteins similar to that observed in situ in the AD brain.

To further validate a link between aberrant localization of nuclear proteins in AD neurons and RAN deficits, we conducted RAN knockdown experiments in SK-N-BE(2) neuroblastoma cells using an experimentally-determined optimum concentration of RAN siRNA of 100nM. After knock down, Western blots of RAN protein showed a mean RAN reduction of 71% (Figure 4a). Prior to exposure, SK-N-BE(2) exhibited normal nuclear localization of RNA pol II and DNMT1 (Figure 4, c1 and d1 respectively), but this pattern was disrupted after 48 hours of RAN siRNA treatment (Figure 4, c2 and d2). Nuclear localization of RNA pol II, for example, was reduced, whereas abnormal accumulation in the cytoplasm was readily apparent, similar to the pattern observed in AD neurons (compare Figure 4 c2, with Figure 1, b2).

Discussion

Our data demonstrate in Alzheimer's disease greatly diminished nuclear localization and increased cytoplasmic localization of two molecules representative of two different classes of mechanisms in the regulation of expression: DNMT1, an epigenetic molecule modulating DNA methylation, and RNA pol II, central to transcription. The diminished nuclear localization is demonstrated

in brain regions conventionally affected in AD but not in regions traditionally less affected in AD. We show this altered nuclear/cytoplasmic ratio associated with reduced expression of RAN. We also demonstrate reduced expression of RAN and altered nuclear/cytoplasmic localization in a mouse model of AD. Since both human AD and the mouse model of AD are associated with expression of Abeta, we demonstrate that *in* vitro treatment with Abeta results in both reduced expression of RAN and aberrant nuclear and cytoplasmic localization of DNMT1 and RNA pol II. In order to separate the effect of reduced RAN expression from effects of Abeta we used siRNA to knock down of RAN expression *in vitro* and showed that this resulted in reduced nuclear and increased cytoplasmic localization of DNMT1 and RNA pol II.

Miss-localization of transcriptional regulators

Failure of nuclear proteins to reach the sites at *which* they act would have many pathogenic consequences. For example, nuclear loss and cytoplasmic sequestering of DNMT1 has been linked to increased α-synuclein in Parkinson's disease, Lewy body disease, and α-synuclein transgenic mice[18], and has been suggested to underlie global DNA hypomethylation in affected neurons similar to that which we previously reported in AD[12,19]. More globally, inadequate transport of epigenetic and other nuclear proteins would be expected to disrupt chromatin structure, which could lead to large-scale changes in gene expression such as have been described in microarray studies of AD brain where thousands of genes mediating dozens of essential cell functions are altered [14,18,20-22].

In addition to the pathogenic potential of reduced presence of transcriptional regulators in the nucleus, their aberrant presence in the cytoplasm may also have deleterious consequences on cytoskeletal elements, mitochondria, and other organelles. For example, Husseman and colleagues observed that cytoplasmic

phosphorylated RNA polII co-localized with cdc2, a cell cycle enzyme that phosphorylates and inhibits RNA pol2, an event that was suggested to precede neurofibrillary tangle formation [23]. Other reports have shown that other epigenetic factors such as extracellular HDAC1 disrupts axonal transport by interacting with motor proteins, leading to axonal loss [24]

The involvement of amyloid

The role of Aβ42 in disrupted nuclear transport suggested by the abundance of amyloid pathology in the vulnereable CA1 of the hippocampus, is further supported by our data showing that Aβ42 treatment of neurons mimics aspects of our human data in spite of the time course differences in Aβ42 exposure. We certainly do not suggest that APP is the only potential modulator of nuclear transport, as it is known, for example, that reactive oxygen species may also modulate nuclear transport [9]. The relative contributions of APP and/or its fragments, reactive oxygen species as well as other modulators of transport on the AD cellular phenotype remain to be tested in detail. Our study however, demonstrates a relationship among Aβ42 treatment, loss of RAN, and cytoplasmic sequestering of nuclear proteins. Although it remains to be determined what portion of APP may be responsible for the effects reported *in vivo*, our data implicate oligomeric Aβ42 in knocking down RAN protein and subsequently reducing nuclear localization.

An additional aspect of reduced expression of RAN may be its effect on the synapse through activation of caspase 3. *In vitro* analysis of RAN knockdown has shown been shown to activate caspase 3 [25], both a potent activator of apoptosis [26] and also a trigger of early synaptic dysfunction [27]. Activated caspase 3 has been implicated in multiple effects seen in AD, including GGA3 cleavage, which is required for BACE lysosomal degradation[28], the PI3k-Akt/mTOR pathway which regulates Aβ oligomer induced

neuronal cell cycle events[29] and tau hyperphosphorylation, which cleaves tau and initiates/or accelerates tau pathology[30]. Microarray data indicate a strong positive correlation of gene expression between RAN and synaptophysin, r = 0.85 (, p=0.000005) for AD and r= 0.82 p=0 .00001) for age matched controls. Collectively, these data suggest a cascade of events which includes the down-regulation of RAN by Aβ42 oligomers (shown here), the release of caspase 3 in response [25], and resulting degeneration of synapses [27]. Whether the many other nuclear proteins that rely on RAN-mediated nucleocytoplasmic transport follow similar pattern we observed in DNMT1 and RNA pol II remains to be determined. If they do, however, deficits in RAN followed by ectopic intracellular localization of epigenetic and other transcription-related molecules could well be a central event in the pathophysiology of AD, and could provide an overarching, integrative mechanism for the myriad pathogenic processes that occur in the disorder. Moreover, it is important to note, that the current study focused solely on neuronal populations and non-neuronal cells (i.e. glia) could prove to be similarly affected.

In closing, our data suggest a model in which ectopic intracellular localization of epigenetic molecules may be a central event in the pathophysiology of AD. This model, driven by Aβ42 resulting in RAN depletion, has several components that suggest broader implications. These components are: 1) Failure of epigenetic molecules to translocate to the nucleus in affected neurons in AD, with consequential effects on chromatin structure and, as a result, gene expression. 2) Increased epigenetic molecules in the cytoplasm which may lead to detrimental interactions with cytoskeletal elements, an insult in transport along neuronal processes, mitochondria and other organelle dysfunction, affecting structural and functional cellular properties.

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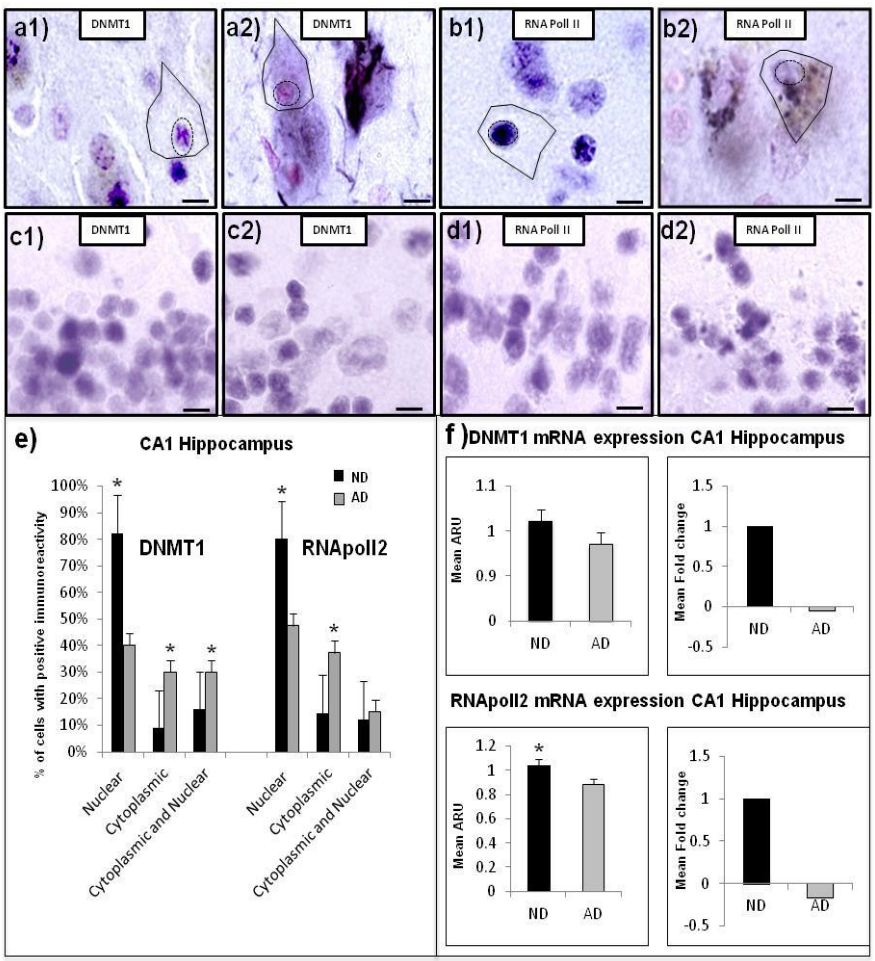
Figures / Tables

Figure 1. Cellular localization of epigenetic and other transcription-related molecules in CA1 of the hippocampus from AD and ND cases. All tissue samples were counterstained with neutral red for cell layer and cell landmark verification. (a1) high power micrograph of CA1 labeled with an antibody to DNMT1 in a typical ND case,



showing appropriate cellular distribution. Comparable field in a typical AD case shows cytoplasmic accumulation and nuclear loss of DNMT1 immunoreactivity (a2). Figures (c1) and (c2) show normal nuclear immunoreactivity for both ND and AD respectively in the pathologically spared cerebellum. Similar patterns of immunoreactivity were observed for RNA pol2 in ND (b1) and AD (b2) in CA1 of hippocampus and ND (d1) and AD (d2) in cerebellum. (Scale bars = 15µM). (e) Quantification of the cellular distribution of immunoreactivity in CA1 of the hippocampus in 5 AD and 5 ND samples. 100 neurons from CA1 of AD and ND cases were quantified and the mean signal/group/area (i.e. signal intensity/ AD or ND/ nuclear, cytoplasmic or both) was analyzed. Neurons were evaluated by delineating the nucleus (dashed circle) from cytoplasm (solid black line). (f) DNMT1 and RNA pol II affymetrix array data from AD (n=10) and ND (n=10) hippocampal CA1 neurons (500 neurons/case). * Indicates significant difference compared to control, 2-tailed t-test p= <.05.

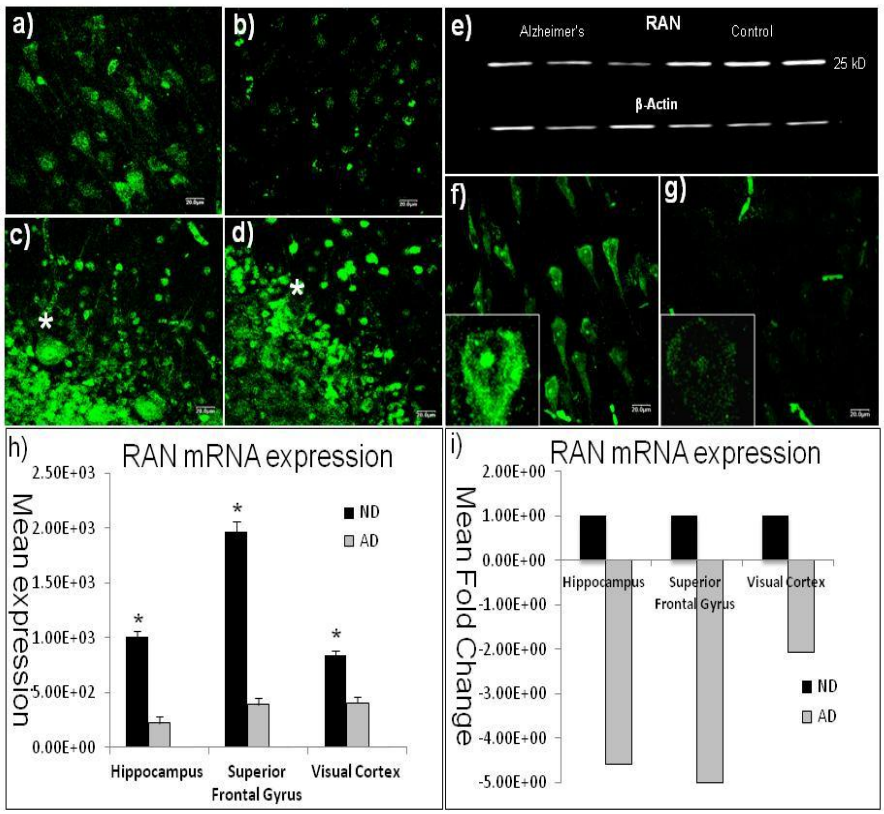


Figure 2. Decreased Ran protein and message in CA1 of AD hippocampus. Confocal micrographs (488 nm bandwidth, green fluorphore) reveals robust nuclear and cytoplasmic RAN immunoreactivity in ND hippocampal CA1 neurons (a), compared to AD (b). Pathologically spared cerebellum show similar patterns of immunoreactivity in both ND (c) and AD (d) samples in all but the larger Purkinje neurons (asterisks). (e) Significant AD decrements in RAN protein were confirmed in Western blot analyses ($p<0.0001$). (f) 40X micrographs show normal cytoplasmic and nuclear distributions of RAN message by fluorescence *in situ* hybridization in ND. By contrast, there was a significant decrease ($p=0.00001$) in

overall signal in comparable fields from AD, with limited reactivity in the cytoplasm and slightly greater reactivity in the nucleus (g). . (h) Mean RAN expression, and fold change (i) from Affymetrix array data looking at AD (n=10) and ND (n=10) hippocampal CA1 neurons (500neurons/case). * Indicates significant difference between AD and control at p<0.05. Scale bars = 15µm.

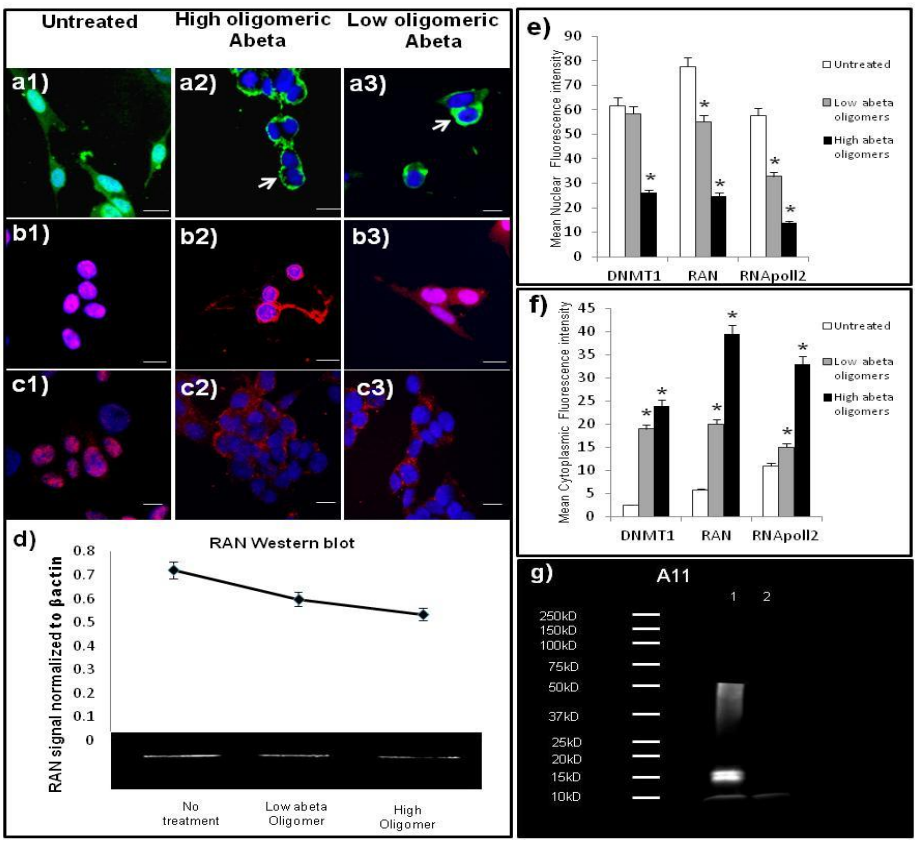


Figure 3. ***In vitro*** **Aβ42 treatment replicates human neuronal distributions of nuclear proteins.** Micrographs of DNMT1 and RNA pol II in neurons (SK-N-Be(2)) treated with higher molecular weight (MW) oligomers of Aβ42 (1uM) or lower MW oligomers of Aβ42 (1uM) for 36 hours. High power micrograph (40X) of cultures

labeled with an antibody to RAN before (a1) and after treatment with higher MW oligomeric Aβ42 (a2) or lower MW oligomeric Aβ42 (a3); shows nuclear and cytoplasmic loss with nuclear envelope accumulation (arrows), similar to that seen *in vitro*. Western blot analysis confirms these data of an overall reduction in basal RAN protein levels when treated with oligomeric abeta (d). Normal distributions for nuclear molecules DNMT1 (b1), and RNA pol II (c1) was readily apparent in the nucleus of untreated neurons, but translocation to the cytoplasm is seen in both molecules when treated with high or lower MW oligomeric Aβ42; Dnmt1 (b2, b3), and RNA pol II (c2, c3). e) Mean nuclear fluorescence intensity and mean cytoplasmic fluorescence intensity (f) of nerve cells treated with either low MW Aβ42 oligomers, or high MW Aβ42 oligomers. Asterisk (*) signifies a significant difference compared to control samples ($p<0.05$). Data are presented as mean +/- S.E.M. (g) Western blot analysis using oligomeric antibody A11, revealed the presence of oligomers in both preparations, with higher MW oligomers in lane 1 (96 hour aggregation), compared to lane 2 (immediately frozen). (Scale bars = 15um).

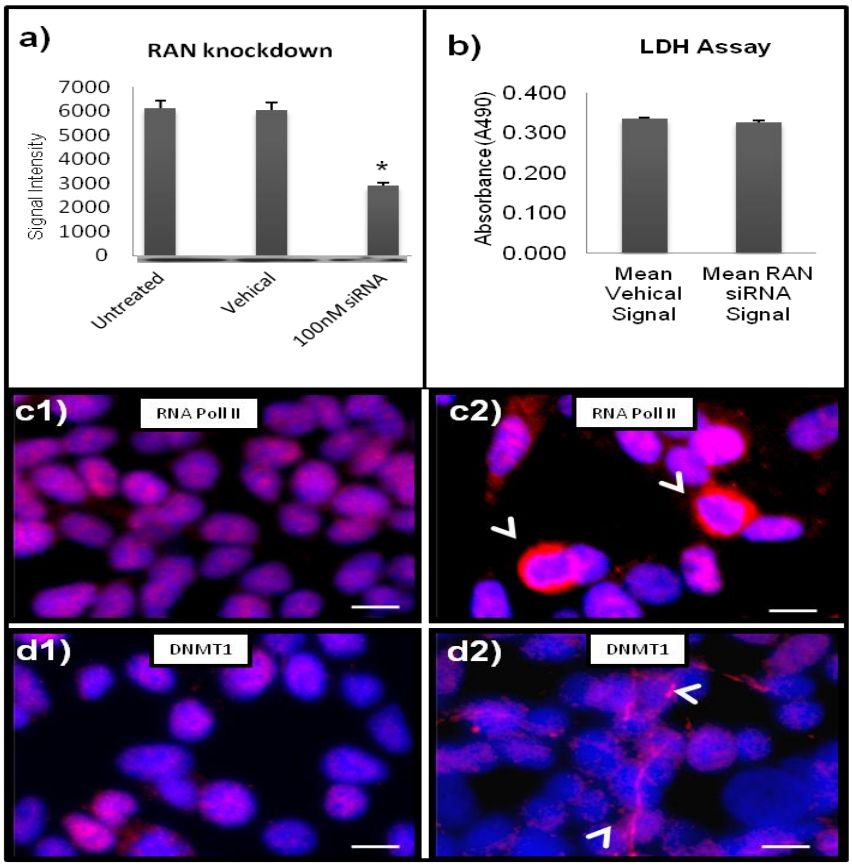


Figure 4. RAN knock down *in vitro* replicates neuronal distribution of nuclear proteins. Neuroblastoma cells were treated with 100nM RAN SNArpol siRNA, a concentration that reduced RAN levels by 71% (a), but did not materially affect cell viability (b). Untreated, tranfection reagent only (c1, d1) and treated, RAN siRNA (c2-d2) cells were immunoreacted for RNA polII (c1, c2), and DNMT1 (d1, d2). After 48 hours, RAN knockdown induced cytoplasmic accumulation and nuclear losses in both target proteins, recapitulating *in vivo* observations in AD brain. DNMT1 IR after siRNA treatment was largely localized in axons (d2, arrows), RNA pol2 immunoreactivity was chiefly located in the cytosol (c1, arrows).

Supplementary Figure 1. LDH and Calcein AM assays to determine cytotoxicity levels in culture. The general health of neurons in culture was analyzed using two tests. 1) Cultures were treated with Calcein AM to determine relative cell viability. 2) LDH assay was performed to analyze cellular toxicity and membrane integrity.

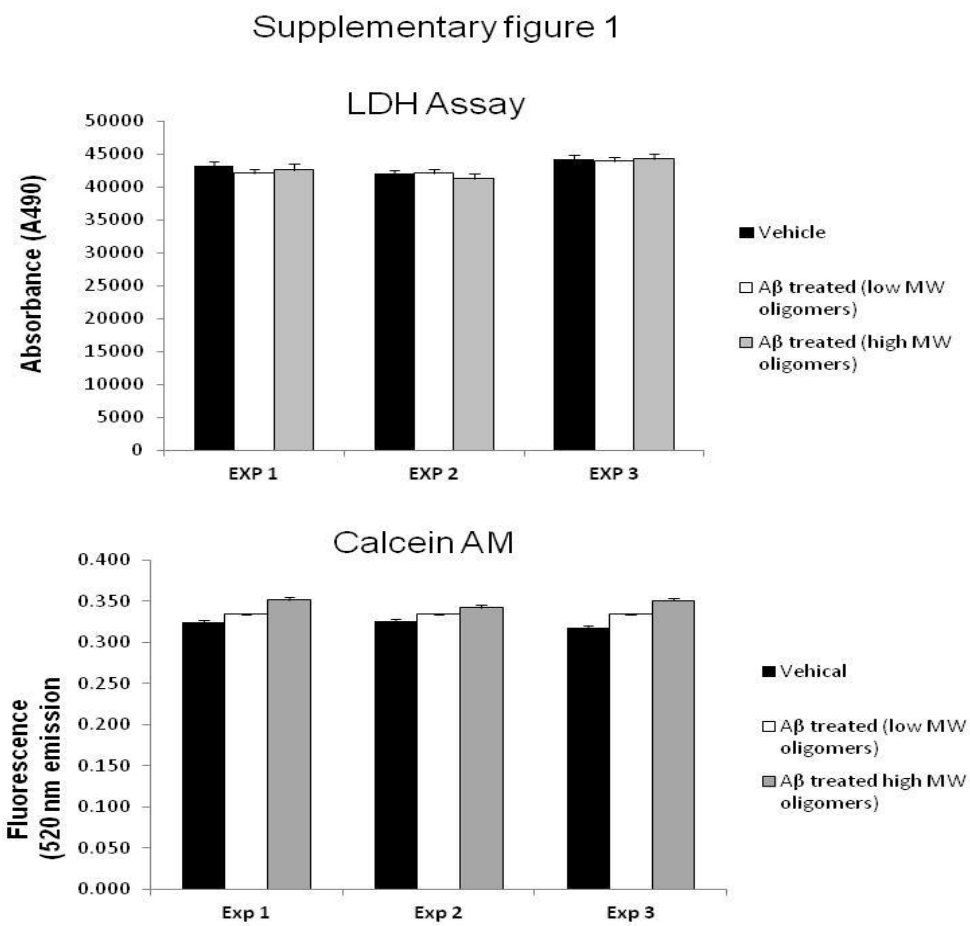


Table 1: The antibodies used, sources, dilutions and recognition motif.

Antibody	*Host*	*Dilution*	*Source/Cat#*	*Recognition sequence*
Anti-DNMT1	*Rabbit*	*1:500*	*Abcam ab19905*	*Within reidues 100-200*
Anti-RNA poll II	*Rabbit*	*1:1000*	*Millipore ABE30*	*KLH-conjugated linear peptide corresponding to human RNA polymerase II subunit B1.*
Anti-RAN	*Rabbit*	*1:1000*	*Abcam 53775*	*Synthetic peptide derived Human Ran*
Anti-Oligomer	*Rabbit*	*1:10,000*	*Invitrogen AHB0052*	*Recognizes oligomers*

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Brain Region	Gene Name	Description	p-value	Fold Change	Mean ND	Mean AD
	RAN	RAN, member RAS oncogene family				
Hippocampus			5.82E-05	-4.60E+00	1.01E+03	2.20E+02
Superior Frontal Gyrus			1.32E-02	-5.01E+00	1.96E+03	3.91E+02
Visual Cortex			1.97E-02	-2.08E+00	8.43E+02	4.05E+02
	RANBP1	RAN binding protein 1				
Hippocampus			2.35E-03	-2.47E+00	3.02E+02	1.22E+02
Superior Frontal Gyrus			2.74E-02	-2.57E+00	3.83E+02	1.49E+02
Visual Cortex			3.85E-01	-1.28E+00	1.90E+02	1.48E+02
	RANBP2	RAN binding protein 2				
Hippocampus			7.14E-03	-1.74E+00	7.96E+02	4.57E+02
Superior Frontal Gyrus			6.12E-02	-1.91E+00	5.83E+02	3.06E+02
Visual Cortex			4.15E-02	-1.22E+00	3.73E+02	3.06E+02
	RANBP5	RAN binding protein 5				
Hippocampus			4.21E-02	-1.49E+00	1.69E+02	1.14E+02
Superior Frontal Gyrus			2.26E-02	-2.86E+00	2.65E+02	9.26E+01
Visual Cortex			8.82E-04	-1.79E+00	1.62E+02	9.07E+01
	RANBP6	RAN binding protein 6				
Hippocampus			8.21E+00	1.03E+00	1.15E+03	1.19E+03
Superior Frontal Gyrus			2.05E-02	-3.02E+00	3.05E+03	1.01E+03
Visual Cortex			3.94E-02	-1.47E+00	1.38E+03	9.39E+02
	RANBP9	RAN binding protein 9				
Hippocampus			6.26E-05	-3.06E+00	1.89E+02	6.18E+01
Superior Frontal Gyrus			1.57E-02	-2.62E+00	1.98E+02	7.56E+01
Visual Cortex			1.71E-01	-1.45E+00	1.05E+02	7.29E+01
	RANBP10	RAN binding protein 10				
Hippocampus			1.74E-02	-2.07E+00	1.42E+02	6.86E+01
Superior Frontal Gyrus			1.64E+00	-1.36E+00	1.11E+02	8.14E+01
Visual Cortex			1.14E+00	-1.19E+00	8.79E+01	7.37E+01

Table 2: mRNA expression from laser captured neurons in the CA1 of the hippocampus, superior frontal gyrus, and visual cortex

using Affymetrix Human Genome U133 plus 2.0 microarrays. mRNA data from all three brain regions show a significant decreases in RAN and RAN binding proteins, with lesser amounts in the visual cortex, an area with only modest AD pathology [16]. With the exception of RANBP6 in the hippocampus, all other RAN binding proteins were significantly down in AD compared to controls.

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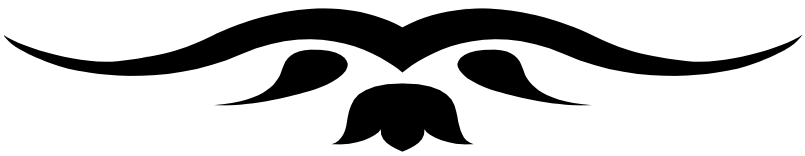
Chapter 8



Discussion
&
Future Perspectives

"The Human Genome Project provided the blueprint for life, but the epigenome will tell us how these genes are excuted"-Alexander Olek

Discussion

Conventional insight into human health and disease is dominated by gene-based theories that often fail to consider the environment in which genes reside. The milieu inside the cell, however, is dynamic, an ever-changing microenvironment capable of altering gene expression. Genetics and the cellular environment are therefore intertwined, and the vehicle by which they are intertwined is epigenetics. Epigenetics is, essentially, the interfacing software between the cell environment and the genome. Without epigenetics, neither could function nor survive. Decades of research has revealed significant gene expression changes in AD, in dozens of major pathologic pathways, including tau phosphorylation[1], Aβ deposition[2], inflammation[3], oxidative stress[4], and mitochondrial metabolism[5], as well as consistent changes in the expression of thousands of genes[6, 7]. Moreover, these alterations are evident in some neurons but not adjacent neighboring neurons[8], despite the fact that they all share the same genetic code, genetic risk factors, and local microenvironment. This bewildering complexity and pathologic diversity strongly suggests that investigations into broad, integrative mechanisms such as epigenetics and nuclear transport at the level of the single cell could be a fruitful strategy compared to the studies of restricted molecular events that now characterize AD research.

The present thesis therefore addresses an integrative, epigenetic mechanism for AD pathogenesis and provides a deeper look into how epigenetic changes might arise in AD neurons. At the level of basic research, it well established that methylation and methylation stabilizing factors can orchestrate changes in expression of a wide range of genes [9-15]. Accordingly, one of the central hypotheses tested in this thesis is that alterations in DNA

methylation and DNA methylation stability might provide an over-arching mechanism that could explain expression differences in the thousands of genes that are reportedly altered in AD (chapter 3). From a genome-wide perspective, the present thesis research demonstrates decreased nuclear immunoreactivity (IR) for seven different markers of DNA methylation in AD compared to matched, non-demented elderly control (ND) cortical neurons and glia, whereas no such changes were observed in cerebellum, a brain region that is relatively spared in AD.[1]

Moreover, highly similar results were subsequently obtained in a set of monozygotic twins discordant for AD[17] (Chapter 4), which indicated for the first time that epigenetic mechanisms might provide a molecular basis for the effect of life events, including exposure to hazardous substances, on AD risk. Since epigenetic patterns can be passed on to subsequent generations, epigenetics may also constitute a mechanism by which AD in a first degree relative confers increased risk of 'sporadic'' disease. These findings are consistent with the hypothesis that epigenetic mechanisms may mediate, at the molecular level, the effects of life events on AD risk, and provide, for the first time, a potential explanation for AD discordance despite genetic similarities.

Furthermore, these effects were recapitulated in a detailed study analyzing nuclear IR for both 5-methylcytosine and 5-hydroxymethylcytosine in CA1 of the hippocampus (chapter's 5-6). Similar to the highly impacted entorhinal cortex, the CA1 of the hippocampus showed significant decrements in methylation and hydroxymethylation in AD compared to normal aging individuals. In this comprehensive study an encouraging association between plaque load and methylation status was made, indicating that amyloid accumulation may account for the aforementioned loss of global methylation/hydroxymethylation.

The compelling research in chapters 3-6 begs the question, why is this happening? Where are the enzymes that maintain and methylate DNA? In going back and reviewing chapter 3 we realized the answer was in plain sight, the methylation maintenance molecule DNMT1 is not lost, but miss-localized, and sequestered in the cytoplasm in AD brains. Accordingly, a key hypothesis of the present thesis research is that a major disruption of transport between the cytoplasm and the nucleus occurs in AD, and that this may account for many of the alterations in DNA methylation and, perhaps histone modification that have been reported in the disorder (chapter 7). Through the examination of human, mouse model (data not shown), and *in vitro* preparations, the research in chapter 7 reveals an important role for the failure of RAN- mediated transport which prevents key epigenetic regulators from reaching their nuclear sites resulting in altered chromatin structure and consequently altered transcription as observed in AD[6, 8, 18, 19]. We show that cytoplasmic sequestering of epigenetic proteins also lead to their abnormal localization to axonal and dendritic compartments, which impairs transport and yields a beading pattern (chapter 6). Similarly, beading patterns within axons/dendrites has been shown to increase Ca2+[20], which activates calpain[21] and impairs axonal transport[20]. Collectively, these data suggest a cascade of events that includes the down-regulation of RAN by Aβ oligomers (chapter 7), the release of caspase 3 in response[22], and resulting degeneration of synapses[23].

Moreover, current research in our lab links the deficiencies in energy metabolism that have been shown to be a central event in Alzheimer's disease pathophysiology [7](Appendix). Epigenomic modification of nuclear DNA (nDNA) requires a constant consumption of calories. Mitochondrial oxidative phosphorylation is a key factor, which drives an organism's energetic demands. This

system converts environmental calories into ATP, acetyl-Coenzyme A (acetyl-CoA), S- adenosylmethionine (SAM) and reduced NAD+ [24]. When calories are abundant the potential energy generated by ATP and acetyl-CoA drives epigenetic mechanisms to phosphorylate and acetylate chromatin, resulting in active nDNA transcription, as well as maintenance of the methylation status on those genes that require suppression (i.e. imprinting genes). When calories are less abundant, as seen in AD [25], phosphorylation and acetylation mechanisms are suppressed and methylation mechanisms altered, as we have shown in AD brains (chapter 2), and in monozygotic twins discordant for AD (chapter 3) [16, 17]. It is well established that amyloid is a major player in AD pathophysiology, but recent evidence implicates the more oligomeric forms of Aβ as the more toxic species [26]. Furthermore, recent work has demonstrated in a mouse model of AD, that oligomeric Aβ is toxic to mitochondria both at the level of the synapse and in the cytosol, an insult that precedes synaptic degeneration [27]. Because bioenergetics provides the link between the environment and the epigenome these data will reveal the importance of bioenergetics in regulating the epigenome, and may provide the framework for future therapeutic intervention (Mastroeni 2013)

Future Perspectives

DNA methylation/hydroxymethylation 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 normal genome, how that status may change in AD, and 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, for the experiments would then provide validation of the functional effects of DNA methylation changes on gene expression. 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[28], 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 [29]. 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 [30, 31] but simple stochastic processes may do so as well[32]. Whether as environmentally driven or randomly occurring events, however, the probability of epigenetic changes 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 up-regulation of an Aβ-synthesizing gene might have little to no impact on a fibroblast, whereas it could be highly significant in a pyramidal neuron. 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.

Although it is well established that DNA methylation and histone modification/chromatin remodeling are at the forefront of epigenetic research, these modifications are the result of a series of

biochemical processes. In fact, molecules like DNMT's, HDAC's, and complexes like MeCP's are ultimately the true regulators of methylation and histone patterns. Future directions in epigenetic research will always include specific site methylation analysis, as AD will always include plaque burden, but methylation and hydroxymethylation profiling across the entire genome will be fundamental in future studies. Although the technology to analyze the entire genome is still illusive, resent collaborations with University of Southern California, looking at 450,000 methylation sites per sample at single-nucleotide resolution of 200 controls and 200 AD samples in mid temporal gyrus (MTG) and cerebellum has begun a new era of epigenetic research, one which holds much promise. Although only covering a small percentage of the total methylation sites/gene, very interesting global data has already emerged. For example, using a principle component analysis, comparing all organs outside of the brain (i.e. heart, kidney, liver etc.) show a strong clustering pattern with much overlap, where the brain shows a much different story. Both MTG and cerebellum are separate from the other organs outside the brain and furthermore are separated from each other, revealing a very different pattern of methylation within the same organ (Coleman 2013). These data indicate that organs other than the brain are more stable, and perhaps may have remained the same for thousands of years, where the brain is constantly evolving, or is a multi-organ in itself; just another dimension we must consider when analyzing methylation patterns in aging and AD brains.

Although our recent analysis of genome-wide DNA methylation has revealed a large number of significant changes in Alzheimer's disease, the magnitude of these statistically significant changes is uniformly extremely small. In view of the fact that many changes in gene expression in AD are fairly large; the finding of very small changes in DNA methylation in AD raises a number of issues

regarding the relationship between DNA methylation and gene expression. One hypothesis regarding the relationship between DNA methylation and gene expression is that small changes in DNA methylation are able to cause fairly large changes in gene expression, either directly, or through recruitment of intermediate molecules. Ultimately, single gene analyses of every cpg site are mandatory in determining the specific patterns of methylation, and ultimately the functional relevance of these specific marks. We may find that methylation sites upstream/downstream from the regulatory region may have more influence on expression than methylation in the regulatory region itself. Currently, we are in the process of analyzing AD disease specific genes, which will be of great interest and may indicate new future directions in AD research.

The field of epigenetics is now poised to be at the forefront of AD research. The rise of epigenetics marks a new field of discovery. Epigenetics is now a dynamic discipline, challenging and revising conventional paradigms of inheritance laid out by Darwin, Lamarck and Mendel. The future of epigenetics will reveal the complexities of genetic regulation and one day answer many of the bewildering questions presented by Alois Alzheimer a hundred years ago, which still elude us today.

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Appendix

Alzheimer's disease is associated with reduced expression of energy metabolism genes in posterior cingulate neurons.

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Abstract

Alzheimer's disease (AD) is associated with regional reductions in fluorodeoxyglucose positron emission tomography (FDG PET) mea¬surements of the cerebral metabolic rate for glucose, which may begin long before the onset of histopathological or clinical fea¬tures, especially in carriers of a common AD susceptibility gene. Molecular evaluation of cells from metabolically affected brain regions could provide new information about the pathogenesis of AD and new targets at which to aim disease-slowing and preven¬tion therapies. Data from a genome-wide transcriptomic study were used to compare the expression of 80 metabolically relevant nuclear genes from laser-capture microdissected non-tangle-bear-ing neurons from autopsy brains of AD cases and normal controls in posterior cingulate cortex, which is metabolically affected in the earliest stages; other brain regions metabolically affected in PET studies of AD or normal aging; and visual cortex, which is relatively spared. Compared with controls, AD cases had significantly lower expression of 70% of the nuclear genes encoding subunits of the mitochondrial electron transport chain in posterior cingulate cor¬tex, 65% of those in the middle temporal gyrus, 61% of those in

hippocampal CA1, 23% of those in entorhinal cortex, 16% of those in visual cortex, and 5% of those in the superior frontal gyrus. Western blots confirmed underexpression of those complex I–V subunits assessed at the protein level. Cerebral metabolic rate for glucose abnormalities in FDG PET studies of AD may be associated with reduced neuronal expression of nuclear genes encoding subunits of the mitochondrial electron transport chain.

Key Words
Gene expression I Affymetrix microarrays I laser capture micro-dissection

Introduction

Alzheimer's disease (AD) is associated with characteristic and progressive reductions in regional positron emission tomography (PET) measurements of the cerebral metabolic rate for glucose (CMRgl). These CMRgl reductions have been reported in the posterior cingulate, parietal, and temporal cortex, and in the frontal cortex and whole brain in more severely affected patients (1–5). Other studies have reported CMRgl reductions in anatomically well characterized hippocampal and entorhinal cortical regions of interest (6–10). The posterior cingulate cortex (PCC) and the neighboring precuneus are metabolically affected in the earliest clinical and preclinical stages of AD (4, 11), and the primary visual cortex is relatively spared (4, 11). In an ongoing series of studies, we have detected CMRgl reductions in cognitively normal carriers of the apoli¬poprotein E (APOE) 64 allele (11–15), a common late-onset AD susceptibility gene (16–18). CMRgl reductions in AD-affected areas

were correlated with APOE 64 gene dose (i.e., three levels of genetic risk for AD) and were progressive in late-middle-aged persons (19). These reductions were also apparent in young adult APOE 64 heterozygotes (13), more than four decades before the anticipated median onset of dementia, years before the expected onset of the major histopathological features of AD (neurofi¬brillary tangles and amyloid plaques), and, indeed, in anticipat¬ing the initial regional appearance of fibrillar amyloid deposi¬tion. (20, 21).

AD-related CMRgl reductions could reflect reductions in the density or activity of terminal neuronal fields or peri-synaptic glial cells (22, 23), a metabolic dysfunction in neurons or glial cells not related to neuronal activity (24, 25), or a combination of these factors. These changes do not appear to be solely attributable to the combined effects of atrophy and partial-volume averaging (26). In a postmortem histochemistry study, we previously found that AD cases had lower cytochrome c oxidase activity than controls in the PCC, and that this reduction was significantly greater than that in primary motor cortex (27), another region that is relatively spared. Based on this observa¬tion, we proposed that AD might be related to an impairment in neuronal metabolism.

Molecular evaluation of the cells from metabolically affected brain regions could provide new information about the pathogenesis of AD and new targets at which to aim disease slowing and prevention therapies. Data from a genome-wide transcriptomic study were used to compare, in each of our sampled brain regions, the expression of metabolically relevant nuclear genes from laser-capture microdissected non-tangle bearing neurons of expired cases with clinically characterized and histopathologically verified AD and expired controls who did meet clinical criteria for dementia and histopathological criteria for AD.

In particular, the data were used to compare cases and controls in the expression of 80 nuclear genes encoding mitochondrial electron

transport chain (ETC) subunits along with translocases of the inner and outer mitochondrial membranes(TIMMs and TOMMs, respectively), in six brain regions. These nuclear genes included 39 complex I genes coding for NADH dehydrogenase, all 4 nuclear-encoded complex II genes coding for succinate dehydrogenase, 9 complex III genes coding for ubiquinolcytochrome c reductase, 13 complex IV genes coding for cytochrome c oxidase, and 15 complex V genes coding for ATP synthase, as well as 11 TIMMs and 6 TOMMs, which regulate the transport of nuclear-encoded electron transport subunits into the mitochondria. These ETC complexes and translocases are illustrated in Fig. 1. The six brain regions included the PCC, which is associated with unusually early CMRgl reductions in AD (4, 28); the middle temporal gyrus (MTG); the hippocampal field CA1 (HIP) and entorhinal cortex (EC), which are also metabolically affected in AD (1, 20); the superior frontal gyrus (SFG), which is associated with preferential CMRgl reductions in normal aging (29–33); and the primary visual cortex (VC), which is relatively spared from CMRgl reductions in both aging and AD (4, 11).

We initially tested the hypothesis that AD would be associated with reduced neuronal expression of metabolically relevant nuclear genes in the PCC and that these AD-related reductions would be significantly greater than those in VC. (Findings from our survey of 55,000 neuronal transcripts have been published in ref. 54.) AD-related reductions in the posterior cingulate neu¬ronal expression of several of the implicated complexes I–IV and ATP synthase (complex V) subunits were subsequently validated at the protein level, suggesting that the reductions in the neuronal expression of metabolically relevant nuclear genes might be associated with the CMRgl reductions found in fluorodeoxyglucose positron emission tomography (FDG PET) studies of AD.

Results

Fold change values and P values for the 80 ETC and translocase genes in each of the sampled regions are posted at www.tgen.org/ neurogenomics/pcc, and additional expression results have been reported in ref. 54. The proportion of significantly underex¬pressed ETC and translocase genes in each of the sampled brain regions ($P < 0.01$ after correction for multiple comparisons) is shown in Table 1. In comparison with controls, AD cases had significantly lower expression of 70% of the nuclear genes encoding mitochondrial ETC subunits in PCC pyramidal neu¬rons, 65% of those in MTG neurons, 61% in HIP neurons, 23% in EC layer II stellate neurons, 16% in VC neurons, and 5% in SFG neurons. By comparison, AD cases had significantly higher expression of only 4% of the nuclear genes encoding mitochon¬drial ETC subunits in PCC pyramidal neurons, 13% of those in MTG neurons, 8% of HIP neurons, 1% of EC neurons, 4% of VC neurons, and 0% of SFG neurons (data for these genes are listed on the supplementary data site). Additional factors that displayed statistically significant underexpression in the PCC, and which may influence mitochondrial energy metabolism, include the TIMMs and TOMMs (Table 1; individual P values and fold changes are located on the supplementary data site), which are required for the transportation of proteins, including ETC components, from the cytoplasm to the inner mitochon¬drial membrane and different compartments in the mitochon¬dria (34–37). Overall, 35% of these translocases demonstrated underexpression in the PCC neurons, 47% in the MTG neurons, 41% in the HIP neurons, 35% in the EC neurons, 6% in the VC neurons, and 24% in the SFG neurons. By comparison, only 6% of the translocases showed overexpression in the PCC and HIP; 12% in the VC; and 0% in the MTG, EC, and SFG.

In a post hoc analysis, we compared the proportion of all genes underexpressed in the neurons of AD cases and controls. AD was associated with underexpression of 61% of statistically signifi¬cant ($P < 0.01$, corrected for multiple comparisons) genes in the PCC, 43% of those in the MTG, 56% of those in the HIP, 69% of those in the EC, 81% of those in the SFG, and 58% of those in the VC. These findings suggest that although metabolism genes may not be disproportionately affected in AD, the regional pattern of underexpressed metabolic genes (PCC > SFG and VC) more closely reflects the pattern of metabolic reductions observed in PET studies.

To validate our findings of underexpression of energy meta¬bolic factors, we performed Western blots on the posterior cingulate in controls and AD cases. Concurrent with expression findings, statistically significant decreases were found in the protein levels of each enzyme complex subunit assessed using two-tailed t tests, $P < 0.01$): complex I (mitochondrially encoded subunit ND6), 41.9 ± 7.0% (mean ± 95% CI) of control, $P < 0.006$; complex II, 70.6 ± 9.5% of control, $P < 0.000005$; complex III, 45.3 ± 7.8% of control, $P < 0.008$; complex IV (mitochon¬drially encoded subunit COXII), 59.1 ± 9.3% of control, $P < 0.002$; and complex V, 72.4 ± 11.1% of controls, $P < 0.000008$ (Fig. 2; human heart was used as positive control)

Discussion

This study provides transcriptomic and protein evidence of neuronal metabolic impairments in AD brains, complementing previously established PET evidence of metabolic impairments in per¬sons afflicted by or at risk for this disorder. In our analysis of the nuclear genes influencing mitochondrial energy metabolism (i.e., 80 nuclear genes encoding ETC subunits and 17 nuclear genes encoding mitochondrial translocases responsible for the entry of ETC subunits into the mitochondria), the largest proportion of underexpressed

genes was in the PCC, a region which PET studies find to be metabolically affected in the earliest stages of AD. The proportion of underexpressed genes was significantly greater than those in the VC, which is relatively spared in PET studies of AD. The MTG, EC, and HIP, which are also affected in PET studies of AD, had proportions of underexpressed genes in between those in the PCC and VC.

Because our analysis was confined to laser-capture microdissected neurons, our findings suggest that the CMRgI reductions observed in PET studies of AD are at least partly related to molecular processes in neurons themselves, and are thus not solely attributable to a reduction in the activity of perisynaptic glial cells known to influence CMRgI (23). They also suggest that the CMRgI reductions could be at least partly related to molecular processes in neuronal cell bodies (i.e., changes in nuclear gene expression), even though alterations in PET CM¬RgI measurements have been suggested to be more strongly influenced by the activity of terminal neuronal fields (22). Furthermore, this differential pattern of ETC and metabolic involvement across the cortex highlights the differential vulner¬ability of various regions to the pathophysiology of AD. Our current transcriptomic findings and previous PET studies raise the possibility that the molecular processes involved in neuronal energy metabolism may be involved in the earliest pathogenesis of AD, possibly preceding the onset of neuritic plaques.

Earlier gene expression and functional activity studies provide confirmatory evidence of this regional pattern of ETC changes. Underexpression was found for mitochondrially-encoded and nuclear encoded ETC subunits in the middle temporal cortex, but not in motor cortex, of AD patients (38, 39). In a laminar analysis, the PCC also showed the most prominent and signifi¬cant functional declines across each cortical layer in complex IV activity, whereas the motor cortex was relatively spared (27). Middle

temporal, superior temporal, and inferior parietal cortices showed significant layer III activity declines, in agreement with our gene expression data, with the midfrontal cortex showing the smallest decline (40). Further, as the layer III cell bodies from which the expression data were taken project apical dendrites (containing the highest metabolic demand) into the superficial layers of the cortex, these layers (I and II) showed globally, albeit not always significantly, reduced ETC activity that could result from decreased subunit expression in the layer III soma (40).

The regional pattern of underexpression in the genes encoding ETC subunits, which have been validated here with Western blots, corresponds to the regional pattern of CMRgl reductions observed in PET studies of patients with AD (28, 41–45) such that the PCC and precuneus are preferentially affected in AD (11, 16, 17, 19). Because PET studies have also identified the PCC as showing the earliest metabolic changes in cognitively normal carriers of the APOE 64 allele (19), these changes may precede downstream development of AD pathologies including amyloid plaque and neurofibrillary tangle formation. Metabolic alterations in the PCC have also been implicated in maintaining the brain's "default"state when it is not engaged in the performance of specific tasks (46) or episodic memory tasks (20), as well as in the predisposition to initial hypometabolic (11–13) and subsequent fibrillar amyloid changes (20, 47, 48) associated with AD. The current study provides new information about the neuronal processes involved in these normal and pathological human behaviors.

This study capitalized on the genome-wide evaluation of gene transcripts from laser-capture microdissected neurons from ex¬tremely high-quality brain tissue [mean postmortem interval (PMI) = 2.5 h] in clinically and neuropathologically well-characterized AD cases and age-matched controls, and on theanalysis of neurons from brain regions preferentially affected or spared in PET studies of

AD. Although this study provides clues about the molecular processes related to the CMRgI reductions observed in persons afflicted by and at genetic risk for AD, it has several limitations. First, given the descriptive nature of our findings, it remains to be clarified whether the changes in the nuclear expression of mitochondrial metabolism genes cause reductions in the density or activity of terminal neuronal fields or instead are a consequence of the reduced metabolic demands associated with terminal neuronal changes in AD. Second, it also remains to be clarified whether or not AD is also associated with underexpression of mitochondrially encoded genes from the same neurons (a technically challenging question to address in laser-capture microdissected cells) or in the nuclear or mitochondrial genes of perisynaptic glial cells. Third, although we controlled for mean age at death and gender, there is a small possibility that other group differences in the agonal state preceding death contributed to some of the AD related reductions in neuronal gene expression observed in this study. Finally, findings from our study suggest that a high proportion of genes are underexpressed in AD, whether or not they are known to directly influence neuronal metabolism. However, the regional pattern of underexpressed genes regulating mitochondrial metabolism appear to correspond to the pattern of CMRgI changes in AD.

Noting the pattern of metabolic changes in our PET studies of AD, Roses et al. have suggested that insulin sensitizers may be helpful in the treatment and prevention of AD (49, 50). Findings from our neurotranscriptomic study could provide clues about the molecular mechanisms that may be involved in the earliest pathogenesis of this disorder and potential targets at which to aim new disease-slowing and prevention therapies

Conclusion

This study provides transcriptomic and protein evidence that the neuronal nuclear genes influencing mitochondrial energy

metabolism are underexpressed in AD, particularly in brain regions like the PCC, which are found to be preferentially affected in PET studies of AD patients and cognitively normal persons at genetic risk for this disorder. In doing so, this study provides information about the molecular processes involved in the pathogenesis of AD and potential therapeutic targets at which to aim disease-slowing and prevention therapies.

Materials and Methods

Tissue Collection.

Brain samples were collected at three Alzheimer's Disease Centers (Washington University, Duke University, and Sun Health Research Institute) from clinically and neuropathologically classified late-onset AD-afflicted individuals (15 males and 18 females) with a mean age of 79.9 ± 6.9 years. Tissue collection of healthy elderly controls was published in a previous report (51); Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuritic plaque densitywas infrequent and Braak stages ranged from Ito II. All individuals were Caucasian and were matched as closely as possible for gender and mean age at death. Subjects in the AD group had a Braak stage of V or VI (47) with a CERAD score of moderate or frequent (52). Samples were collected (mean PMI of 2.5 h) from six brain regionsthat are either histopatho¬logically or metabolically relevant to AD. These include the EC (BA 28 and 34), SFG (BA 10 and 11), HIP, VC (BA 17), MTG (BA 21 and 37), and PCC (BA 23 and 31). The EC and HIP are preferentially affected by intracellular neurofibrillary tangles, the MTG and PCC are preferentially affected by metabolism and extracellular p-amyloid plaques, the SFG is preferentially affected by aging, and the VC is relatively spared from both aging and AD

pathologies. After dissection, samples were frozen, sectioned (10 m), and mounted on glass slides.

As previously described (53), brain sections were stained with a combination of thioflavin S (Sigma) and 1% neutral red (Fisher Scientific), pyramidal neuronswere identified bytheir characteristic size, shape, and location within the region of interest, and tangles were identified by the bright green fluorescence of thioflavin-S staining. In the EC, the large stellate neurons lacking thioflavin S staining were collected from layer II, and pyramidal cells lacking thioflavin-S staining were collected from CA1 of the HIP. The CA1 region was selected for study because this area is the most, and earliest, affected region in terms of tangle formation, and it has already been expres¬sion profiled in neurologically healthy elderly individuals. In all other regions, cortical layer III pyramidal neurons lacking thioflavin-S staining were collected (for all collected neurons, cell bodies were extracted). For each individual, 500 histopathologically normal pyramidal neurons were collected from the six brain regions by using laser-capture microdissection (Veritas automated laser-capture microdissection system; Arcturus). Cells were collected onto Arcturus CapSure Macro LCM caps and extracted according to the manufacturer's instructions. Total RNA was isolated from the cell lysate by using the Arcturus PicoPure RNA isolation kit with DNase I treatment using the Qiagen RNase-free DNase set.

Expression Profiling.

Expression profiling was performed as previously de¬scribed (51). Isolated total RNA was double-round amplified, cleaned, and biotin labeled with the AffymetrixGeneChip two-cycle target labeling kitwith a T7 promoter and the Ambion MEGAscript T7 high-yield transcription kit according to the manufacturer's instructions. Amplified and labeled cRNAwas quantitated on a

spectrophotometer and run on a 1% TAE gel to check for an evenly distributed range of transcript sizes. Twenty micrograms of cRNA was fragmented to 35–200 bp by alkaline treatment (200 mM Tris-acetate, pH 8.2; 500 mM KOAc; 150 mM MgOAc) and run on a 1% TAE gel to verify fragmentation. Separate hybridization cocktails were made by using 15 g of fragmented cRNA from each sample according to Affymetrix's instructions.

Microarray Analysis.

Two hundred microliters of each mixture was separately hybridized to an Affymetrix Human Genome U133 Plus 2.0 array for 16 hat 45°C in the Hybridization Oven 640. The Affymetrix human genome arrays measure the expression of over 47,000 transcripts and variants, including 38,500 characterized human genes. Hybridization cocktails for nine EC samples (normal neurons from AD brains) previously collected following the same profiling methodology used in this project (53) were reanalyzed on the U133 Plus 2.0 array to be evaluated in this study; a 10th EC sample was also separately processed for this sample group. Arrays are washed on the Af¬fymetrix upgraded GeneChip Fluidics Station 450 by using a primary strepta¬vidin phycoerythrin (SAPE) stain, subsequent biotinylated antibody stain, and secondarySAPE stain. Arrays are scanned on the Affymetrix GeneChip Scanner 3000 7G with AutoLoader. Scanned images obtained by the Affymetrix Ge¬neChip Operating Software (GCOS) v1.2 were used to extract raw signal intensity values per probe set on the array and calculate detection calls (absent, marginal, or present). Assignment of detection calls is based on probe-pair intensities for which one probe is a perfect match of the reference sequence and the other is a mismatch probe for which the 13th base (of the 25-oligonucleotide reference sequence) is changed. All raw chip data were scaled to 150 in GCOS to normalize signal intensities for interarray compari¬sons. Reports

generated by GCOS were reviewed for quality control; we looked for at least 20% present calls, a maximum 3'/5' GAPDH ratio of 30, and a scaling factor <10. Twenty arrays that failed to pass these standards were not included in further analyses.

Pyramidal Cell Quality Control.

To ensure neuronal cell purity in the samples, we evaluated expression of GFAP, an astrocyte cell marker. Fourteen samples that had GFAP expression greater than one standard deviation from the mean were removed from statistical analyses.

Statistical Analysis.

Data for samplesfrom neurologically healthy elderly controls were generated in a previous study (51). Microarray data files of the normal samples are available on the Gene Expression Omnibus site at www.ncbi.nlm. nih.gov/geo/query/acc.cgi?acc = GSE5281 (project accession no. GSE5281), and regional analyses are posted at www.tgen.org/neurogenomics/data/private3.

We compared AD cases and controls in the neuronal expression of 80 nuclear genes encoding subunits of the mitochondrial ETC in each of our sampled brain regions. The nuclear genes included 39 complex I genes coding for NADH dehy¬drogenase (NDUFA1–A13, NDUFAF1, NDUFAB1, NDUFB1–B11, NDUFC1 and -C2, NDUFS1–S8, and NDUFV1–V3), all 4 complex II genes coding for succinate dehy-drogenase (SDHA, SDHB, SDHC, and SDHD), 9 complex III genes coding for ubiquinol-cytochrome c reductase (UCRC, UQCR, UQCRB, UQCRC1, UQCRC2, UQCRFS1, UQCRH, CYC1, and CYCS), 13 complex IV genes coding for cytochrome c oxidase (COX4I1, COX5A, COX5B, COX6A1, COX6A2, COX6B1, COX6C, COX7A2L, COX7B, COX7B2, COX7C, COX8A, and COX8C), and 15 complex V genes coding for ATP synthase (ATP5A1, ATP5B, ATP5C1, ATP5D, ATP5E, ATP5F1, ATP5G1–G3, ATP5H, ATP5I, ATP5J, ATP5J2, ATP5L, and ATP5O). TIMM and

TOMM expression was also evaluated (TIMM8A, TIMM8B, TIMM9, TIMM10, TIMM13, TIMM17A, TIMM17B, TIMM22, TIMM23, TIMM44, TIMM50, TOMM7, TOMM20, TOMM22, TOMM34, TOMM40, and TOMM70A). Mitochondrial DNA-encoded subunits were not assessed in this study.

Direct comparisons between neurologically healthy and AD-afflicted brains were performed in all six regions to analyze expression differences in the above listed ETC subunits. For each analysis, genes that did not demonstrate at least 10% present calls across all transcripts profiled for each region-specific com-parison were removed by using Genespring GX 7.3 Expression Analysis software (AgilentTechnologies). A two-tailed unpaired ttest, assuming unequal variances (with a multiple testing correction using the Benjamini and Hochberg false discovery rate), wasapplied to each comparison forall genesthat passedthe 10% present-call criterion to locate genes that were statistically significant in differ¬entiating expression between healthy brains and AD brains. (After the present-call filter, 32,153 genes in the PCC were used for P value correction, 30,897 genes in the EC, 32,265 genes in the MTG, 31,496 genes in the HIP, 32,482 genes in the VC, and 32,118 genes in the SFG.) For each analysis comparing AD expression levels with control levels, genes that had a corrected P value ~ 0.01 were collected, and those genes whose average AD signal and average control signal were both below a threshold of 150 were removed. Fold-change valuesweredetermined by calculating the ratio between the averages called expression signal (for all affected samples) for a gene from the AD sample region and the average scaled expression signal for the same gene from the normal samples across all regional comparisons.

Using this approach, we evaluated ETC and translocase genes that were differentially expressed in the AD cases versus controls for each of the six brain regions of study. Proportions of ETC and

translocase genes demonstrating under-and overexpression were determined by calculating the number of subunits (at least 10% present calls with corrected P < 0.01) showing such changes over the total number of nuclear-encoded subunits (at least 10% present calls with corrected P < 0.01) on the Affymetrix human genome array. Evaluation of all human genes demonstrating underexpression in each region focused on only those genes with statistical significance (P < 0.01 with multiple testing correc¬tions, after present-call filters) for each regional AD versus controls comparison; underexpression percentages were calculated based on a ratio of underex¬pressed statistically significant genes over all genes.

Data Posting.

Minimum information about a microarray experiment (MIAME)-compliant microarray data files for control samples are located on the Gene Expression Omnibus (GEO) site at www.ncbi.nlm.nih.gov/geo/query/ acc.cgi?acc=GSE5281 (project accession no. GSE5281). Fold changes and cor¬rected and uncorrected P values for all ETC and translocase genes on the Af¬fymetrix Human Genome Array for each of the six regional comparisons are available online at www.tgen.org/neurogenomics/pcc. Posted lists show respective genes that have at least 10% present calls across regional samples (no fold change thresholds have been applied on these lists). Additional expression results will be published in a separate report.

Western Blotting Validation.

Based on availability, 10 profiled AD PCC cases and 5 profiled healthy control cases were collected for Western blot validation. Blotswere run by researchers blind to subject condition. Sectionswerewashed from slides by using PBS, and 35 g of protein per lane was loaded into 10–20% 1.5 mm Tris-glycine 15-well minigels (Invitrogen) with

Novex Tris¬glycine SDS 2x sample buffer. Gels were run in Novex minicell (Invitrogen) for 90 min at constant 150 V with Novex Tris-glycine SDS running buffer. Proteins were transferred to Immobilon P (Millipore) PVDF membrane, fully sub¬merged in CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-propane sul¬fonic acid, pH 11 with NaOH, 10% methanol) by using constant 150 mA for 2 h. Membranes were blocked overnight in 5% Carnation dry milk in PBS at 4°C and then probed with the total OXPHOS detection kit (MS601; 1:1,000 in 1% dry milk; Mitosciences) for 2 h at room temperature with gentle rocking. This five-antibody mixture marks the 20-kDa subunit of complex I (ND6; mitochon¬drially encoded), the 30-kDa iron–sulfur subunit of complex II, the core 2 subunit of complex III, COX II of complex IV(mitochondriallyencoded), and the F1a subunit of ATP synthase.

Goat anti-mouse alkaline phosphatase (AP)- conjugated secondary antibody (1:10,000 in 1% dry milk; Santa Cruz Biotech¬nology) was used (2 h at room temperature with rocking), followed by application of an AP substrate detection kit (Bio-Rad), according to the manufacturer's instructions. Membranes were dried overnight, then simulta¬neously scanned on a Mustek A3 EP flatbed scanner. All blots were performed in duplicate. The image was imported into Optimas image analysis software (Media Cybernetics), and average OD measures of each band were taken by using a sampling window of constant size. Groups were compared by using Student's two-tailed t tests, uncorrected for multiple comparisons, and 95% confidence intervals for each complex were calculated in Excel.

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Tables / Figures

Table 1. The proportion of underexpressed metabolism-related genes in each of the sample brain *regions*

Brain region	*Complex I*		*Complex II*		*Complex III*		*Complex IV*		*Complex V*		TIMMs		TOMMs	
	No. of subunits	Fold	No. of subunits	F	No. of subunits	F	No. of subunits	F	No. of subunits	Fold	No. of subunits	Fold	No. of subunits	Fold
PCC	28/39(72)	3.0	2/4(50)	2.8	8/9(89)		3.6		7/13(54)	2.8	11/15(73)	3.8	3/11	
MTG	27/39(69)	2.5	2/4(50)	2.5	6/9(67)		2.3		8/13(62)	2.4	9/15(60)	3.3	3/11	
HIP	25/39(64)	2.5	2/4(50)	2.7	5/9(56)		2.8		7/13(54)	2.2	10/15(67)	2.7	3/11(27)	
EC	9/39(23) 1/6(17)	4.5 2.8	0/4 (0)		1/9(11)	3.7	3/13(23)	4.5	5/15(33)	4.3	5/11(45)	3.7		
VC	7/39(18)	1.8	0/4 (0)		1/9(11)	2.0	1/13 (8)	1.5	4/15(27)	2.1	0/11 (0)		1/6(17)	2.
SFG	4/39(10) 2/6(33)	3.9 2.4	0/4 (0)		0/9 (0)		0/13 (0)		0/15 (0)		2/11(18)	3.6		

The numerator indicates the number of subunits showing statistically significant ($P < 0.01$ with multiple testing corrections) underexpression and the denominator indicates the total number of nuclear-encoded subunits for the complex/translocase. This ratio is expressed as a percentage in parentheses. PCC

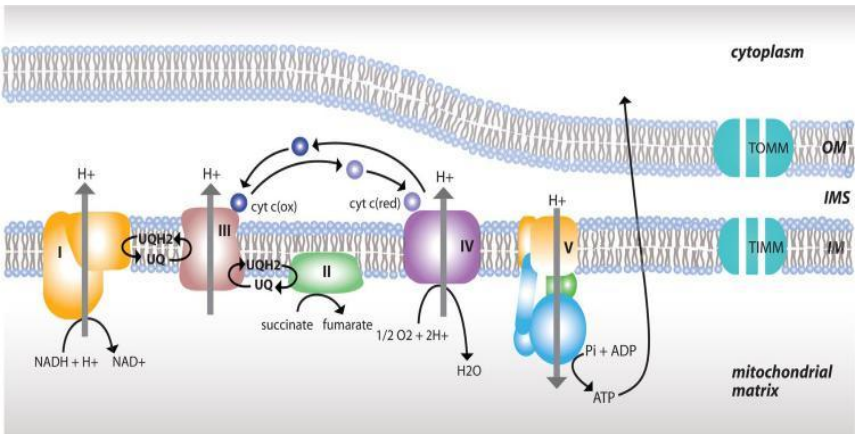

Fig. 1. Altered expression of mitochondrial energy metabolism elements. Energy metabolism-relevant elements showing statistically significant underex¬pression in the PCC are shown. These elements include the five complexes of the ETC and TIMMs and TOMMs. OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner mitochondrial membrane.

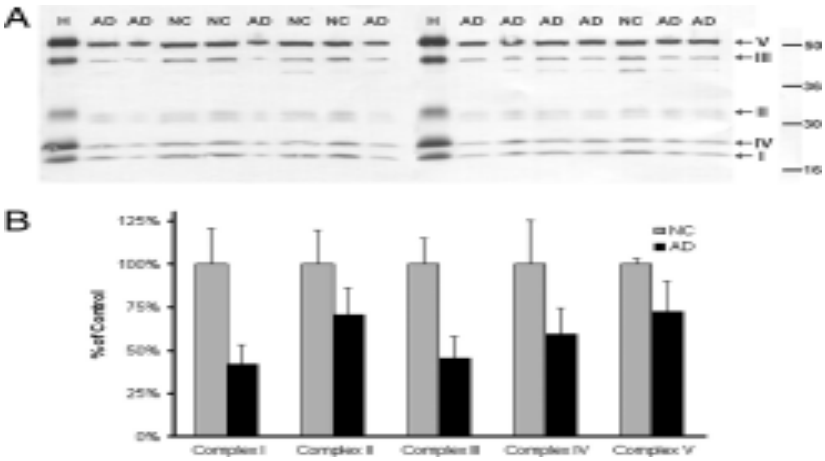

Fig. 2. Western blot validation. (A) Western blots using a five-antibody mixture labeling a subunit of each mitochondrial enzyme complex from PCC whole-brain extract. H, human heart for positive control; AD, Alzheimer's patient; NC, normal control. Apparent molecular mass scale is indicated on the right. From highest to lowest are complex V alpha subunit, complex III core 2 subunit, complex II 30-kDa subunit, complex IV subunit II, and complex I 20-kDA subunit. Results are representative of duplicate blots. (B) Western blot-band optical density, expressed as percentage of normal-aged control. Subunit protein expression was significantly lower (two-tailed t test, $P < 0.01$) for each subunit tested. n = 10 AD; n = 5 NC.

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Curriculum Vitae

Diego Fernando Mastroeni was born on September 28th 1978 in a small rural community, nestled in the majestic mountains of Mendoza, Argentina. At the age of two, Diego and his family packed up and migrated to Phoenix Arizona, a metropolitan city in the United States of America. It was here Diego learned how to speak English as a second language and attended primary, and secondary school. Upon graduating high school with honors, Diego received a scholarship to attend Northern Arizona University in the fall of 1997. Diego spent the next several years focusing on the discipline of microbiology. After his third year Diego opted to extend his major to include a secondary degree in chemistry, Spanish and an emphasis in Medical technology. After completing his Bachelors of Science in fall of 2004, Diego landed his first job at the Sun Health Research Institute. Since then Diego has maintained his stay at the same institute for nine years. During this time, Diego attended Johns Hopkins University Graduate School until an opportunity arose which allowed him to finish up his work at Maastricht University. Diego has had the opportunity to work with some of the greatest minds in Alzheimer's research, which, in turn, has sparked many creative scientific opportunities for both Diego and the laboratory. During his stint here at the now Banner Sun Health Research Institute; he has directed the laboratory into two facets of Alzheimer's research that would have been either overlooked or uncovered by other laboratories.

Firstly, based on Diego's work, the laboratory is now able to consistently isolate and maintain neuronal progenitor cells in culture from human adult biopsies and rapid postmortem autopsies of the subventricular zone. Characterization studies that he has performed demonstrate that the progenitors give rise primarily to nerve cells

and astrocytes. A summary of this research can be found on page 250, publication number 9.

Secondly, in addition to the neuronal progenitor work, Diego's most current and ongoing contribution has been the discovery of epigenetic alterations in Alzheimer's disease. This finding has led the laboratory to six publications, an NIH RO1 roadmap grant, an AARC grant and most importantly the premise of this Thesis.

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9. Leonard BW, ***Mastroeni D (Joint first Author)***, Grover A, Liu Q, Yang K, Gao M, Wu J, Pootrakul D, van den Berge SA, Hol EM, Rogers J (2008) ***Subventricular zone neural progenitors from rapid brain autopsies of elderly subjects with and without neurodegenerative disease***. J Comp Neurol 515:269-294.

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