DNA methylation alterations in Alzheimer’s disease

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Abstract

The observation that Alzheimer’s disease (AD) patients with similar and even identical genetic backgrounds often present with heterogeneous pathologies has prompted the hypothesis that epigenetics may contribute to AD. While the study of epigenetics encompasses a variety of modifications including histone modifications and non-coding RNAs, much of the research on how epigenetics might impact AD pathology has been focused on DNA methylation. To this end, several studies have characterized DNA methylation alterations in various brain regions of individuals with AD, with conflicting results. This review examines the results of studies analyzing both global and gene-specific DNA methylation changes in AD and also assesses the results of studies analyzing DNA hydroxymethylation in patients with AD.

Key words: DNA methylation; Alzheimer’s disease; epigenetics; dementia; DNA hydroxymethylation

Introduction to dementia and Alzheimer’s disease

Dementia, an age-related neurodegenerative disorder characterized by progressive cognitive decline, affects ~35.6 million people worldwide and is becoming an increasingly relevant concern as the population ages [1, 2]. It is estimated that the prevalence of dementia is expected to double every 20 years such that 115.4 million individuals will have dementia by 2050 [2]. In terms of its economic burden, the total estimated cost of dementia worldwide was $604 billion U.S. dollars in 2010 [3]; in the United States alone, the annual societal cost of dementia was between $159 billion and $215 billion [1]. Moreover, predictive modeling suggests that there will be a cost increase of almost 80% per adult by 2040 [1]. In light of its substantial societal and economic burden, there has been a strong push within the research community to better understand the etiology of dementia.

Dementia is a broad term encompassing a wide span of neurodegenerative disorders including Alzheimer’s disease (AD), vascular dementia and dementia with Lewy bodies [4]. AD is the most common type of dementia, accounting for 50–80% of all dementia cases [5–7]. Currently, AD affects ~5.4 million Americans, yet this number is expected to increase to 13.8 million by 2050 as the population ages [5]. As of 2013, AD was the sixth leading cause of death in the United States behind heart disease, cancer, lower respiratory disease, unintentional injury and stroke [8]. Strikingly, between 2000 and 2013, deaths from stroke and heart disease decreased by 23 and 14%, respectively, whereas deaths from AD increased by 71%, highlighting the necessity to better understand the etiology and pathogenesis of AD [5]. Because AD is the most common type of dementia, this review will focus specifically on AD.

AD is characterized by cerebral extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) [9]. The sequential cleavage of amyloid precursor protein (APP) by the
membrane-bound proteins β-secretase and γ-secretase generate amyloidogenic Aβ peptides, which accumulate within the extracellular space to form insoluble Aβ plaques [10, 11]. In addition, APP also can be cleaved by α-secretase and then again by γ-secretase, resulting in the production of soluble, nonamyloidogenic Aβ peptides. Besides Aβ plaques, intracellular NFTs are the other primary pathological hallmark of AD. These tangles are created by hyperphosphorylation of microtubule-associated protein tau (MAPT), and the number of NFTs in the neocortex is positively correlated with the severity of dementia [12]. The presence and severity of Aβ plaques and NFTs within the brain are often used for postmortem neuropathological assessment of individuals diagnosed with AD [13].

AD is classified as early-onset AD (EOAD) or late-onset AD (LOAD) [14, 15]. EOAD occurs in individuals younger than age 65 but may manifest as early as age 40 or 50 [16]. In addition, if EOAD coincides with genetic mutations in APP, presenilin-1 (PSEN1) or presenilin-2 (PSEN2), the latter two of which code for proteins that make up the main catalytic component of γ-secretase, the patient is diagnosed with familial AD. In this case, multiple generations within a family can be affected. EOAD is relatively rare and accounts for <10% of all AD cases [17]. LOAD, on the other hand, which occurs in individuals older than age 65, is more common yet has no clear genetic association or cause [18]. Despite its substantial socioeconomic burden, the etiology of LOAD has not been elucidated in part due to its complex pathogenesis and presentation.

Introduction to epigenetics

Recent research suggests that epigenetic factors often have an impact on chronic diseases including cancer and AD [19–22]. Although there is no single, universally accepted definition of epigenetics, many definitions touch upon the following components [23–25]; (i) heritability, defined as the ability of a dividing cell to pass epigenetic marks to its daughter cells; (ii) preservation of DNA sequence, in which the 5’ to 3’ order of nucleotides remains unchanged; (iii) transcriptional regulation, whereby epigenetic changes influence the transcription of genes and therefore the phenotype of the cell; and (iv) stability, defined as the maintenance of epigenetic modifications over time [26].

The number of studies examining epigenetic mechanisms has risen dramatically as big-data genomics has gained popularity. With this increased focus, the question of the stability of epigenetic modifications has become a highly debated topic. It was long thought that epigenetic marks were stable throughout a lifetime [27]. However, it is now known that epigenetic modifications are dynamic, and as a result, research has begun to focus on how environmental exposures can alter the epigenetic landscape [24, 28–31]. Although epigenetics is a broad term encompassing DNA methylation, histone modifications and non-coding RNA, this review will focus specifically on DNA methylation in AD; histone modifications and non-coding RNA have been reviewed in detail elsewhere [32–36].

DNA methylation

DNA methyltransferase (DNMT) enzymes, of which DNMT1, DNMT3A and DNMT3B are best characterized [37, 38], catalyze the transfer of a methyl group from S-adenosylmethionine to DNA. The product of this reaction, S-adenosylhomocysteine, is then converted back to S-adenosylmethionine through a series of reactions as part of the one-carbon metabolism cycle [39]. Importantly, an elevation in plasma homocysteine, an intermediate in this cycle, is associated with a greater risk for developing dementia and AD [40–42], thus implicating DNA methylation and alterations in one-carbon metabolism with AD pathogenesis [43–47].

In mammals, DNA methylation primarily occurs on cytosine residues of cytosine-guanine (CpG) dinucleotides and functions to modulate gene expression [22, 48]. Canoically, DNA methylation is associated with reduced gene expression [49], yet recent evidence suggests that the impact of DNA methylation on gene expression is dependent upon its context within the genome [50]. Though 5-methylcytosine (5mC) is the most abundant modified base in the mammalian genome, recent studies have identified additional modified bases such as N6-methyladenine (N6mA) and 5-formylcytosine (5fC). N6mA, though prevalent in prokaryotes, was thought to be absent in eukaryotes. In 2015, researchers identified N6mA in three eukaryotic species: green algae [51] (Chlamydomonas reinhardtii), nematodes [52] (Caenorhabditis elegans) and fruit flies [53] (Drosophila melanogaster), and in 2016, researchers characterized the existence of N6mA in mouse embryonic stem cells [54]. The results of these studies show that there is large variation in both the prevalence of this modification and its effect on gene expression across eukaryotic species. 5fC was originally thought to be an intermediate in enzyme-mediated DNA demethylation. Recent studies suggest, however, that 5fC can be a stable DNA modification that affects gene expression by altering the structure of the DNA double helix [55–57]. Despite the progress gained by these pioneering studies, knowledge of these modified bases is still incomplete, and more research is needed to further characterize their distribution and function.

For the purposes of this review, the term “global” DNA methylation describes the average percent methylation across the entire genome. The term “gene-specific” DNA methylation, on the other hand, refers to the average percent methylation within a specific gene. Global DNA methylation, although useful in that it provides an over-arching picture of methylation status in a sample, is sometimes misleading as the proportion of genes with significant alterations in DNA methylation to genes with insignificant DNA methylation differences is generally very small [58, 59]. Gene-specific DNA methylation, however, is able to detect these hidden significant DNA methylation differences. In studies on DNA methylation in AD, global DNA methylation is often assessed via antibody-based methods such as immunohistochemistry, while gene-specific DNA methylation is analyzed using array-based methods such as Illumina’s Infinium HumanMethylation450 BeadChip array. High-throughput techniques utilizing bisulfite conversion are used to measure both global and gene-specific DNA methylation.

DNA hydroxymethylation

The recent surge of interest in DNA hydroxymethylation began with the publication of two papers in 2009 characterizing this modification in separate systems: cerebellar purkinje neurons and mouse embryonic stem cells [60, 61]. In contrast with the relatively constant tissue distribution of 5mC, there is substantial variation in the tissue distribution of 5-hydroxymethylcytosine (5hmC). It is most abundant within the central nervous system, with the highest percentage of 5hmC found within the cerebral cortex, followed closely by the brainstem, spinal cord and cerebellum [62]. In addition to the central nervous system, 5hmC is also present at lower levels in the heart, kidney, liver, muscle and lung [62]. Ten-eleven translocation enzymes oxidize the 5-methyl group on methylated cytosines to a
S-hydroxymethyl group [63]. Despite a growing number of studies on the subject, the function of 5hmC is currently unknown. It is hypothesized to either be an intermediate in active DNA demethylation or be involved in gene regulation. More specifically, 5hmC within gene bodies is positively correlated with gene expression. Although it is still unclear what the mechanism behind this correlation is, it is thought to be due to the association of 5hmC with histone marks H3K4me1 and H3K27ac, both of which are markers of active promoters and enhancers [64–66].

RNA modifications

Of the various RNA-related chemical modifications found in eukaryotic cells, most have been identified in rRNAs and tRNAs. They have been shown to play a role in altering the structure and function of mature RNAs to influence gene expression [67, 68]. Reversible mRNA modifications, on the other hand, have not been seriously studied until recently [69]. N6-methyladenosine (m6A), the most abundant of these modifications, was the first to be discovered [70–72]. Initial studies in mammalian cells using RNA immunoprecipitation followed by high-throughput sequencing demonstrate that m6A is highly enriched around stop codons and within long internal exons and may function to affect gene expression [73, 74]. Since the discovery of m6A, other dynamic RNA modifications such as pseudouridine and N4-methyladenosine have been identified, though more research is necessary to determine the mechanisms by which these modifications influence gene expression [75–79]. The information garnered thus far on RNA modifications is summarized in numerous review articles [67, 68].

Importantly, epigenetic mechanisms do not work in isolation [80, 81]. Rather, they often work together to bring about changes in gene expression and therefore phenotype. For example, methyl-CpG-binding domain proteins (MBD2) bind to methylated cytosines and recruit methyl-CpG binding protein 1 (MeCP1) complexes. Among other proteins, the MeCP1 complex includes a nucleosome remodeling protein with a histone deacetylase core [82]. Together, these proteins act to silence that particular region of the genome. Recent studies suggest that there are various types of epigenetic alterations associated with the pathophysiology of AD, including alterations in DNA methylation [17, 83, 84]. This review will focus on evidence concerning the association between DNA methylation alterations and AD.

Global DNA methylation alterations in AD

Due to the heterogeneous clinical presentation of AD in patients with very similar and sometimes identical genetic backgrounds, it has been hypothesized that epigenetics may contribute to the pathology of AD. The study of identical twins has classically been used to separate the effect of genetics and environment. In 2009, Mastroeni et al. [85] analyzed DNA methylation in a pair of monozygotic twins discordant for AD. Using immunohistochemical methods to detect 5mC, the authors found that global DNA methylation within the anterior temporal neocortex and the superior frontal gyrus was significantly decreased in the twin with AD compared with the neurologically normal, nondemented twin. Interestingly, although both twins were chemical engineers with similar levels of education, the AD twin worked extensively with pesticides whereas the non-demented twin did not, suggesting that work-related pesticide exposure could have contributed to the development of AD. Thus, the results of this study indicate that not only genetic mutations but also environmental influences can affect AD presentation.

Although the study of identical twins is often preferred to separate genetic and environmental factors, it is extremely rare. More commonly, research on DNA methylation alterations in AD involves unrelated participants discordant for AD. Both prior to and since Mastroeni’s study in 2009, other groups have analyzed global DNA methylation in various brain regions of unrelated individuals discordant for AD.

Temporal cortex

As a follow-up to their twin study, Mastroeni et al. [82] used immunohistochemistry to study DNA methylation in the entorhinal cortex of a sample population with and without AD. Like the conclusions drawn from their previous study, the authors found that there was a significant decrease in global DNA methylation in individuals with AD compared to individuals without AD. Importantly, immunoreactivity for 5mC was not significantly different in relatively AD-spared regions such as the cerebellum, suggesting that DNA methylation alterations in AD are region-specific. Although the results of this study in combination with the results from the 2009 twin study might lead readers to surmise that individuals with AD have lower levels of DNA methylation in the temporal cortex than individuals without AD, results from other studies using similar antibody-based methods do not support this conclusion. In contrast, Coppieters et al. [17] showed that there was an increase in the global DNA methylation of brain tissue samples derived from the middle temporal gyrus of subjects with AD compared with age-matched, cognitively normal controls. Lashley et al. [86] found that there was no significant difference in global DNA methylation in the entorhinal cortex of individuals with and without AD [86].

Phipps et al. [87], recognizing that perhaps DNA methylation could be cell-type specific, used immunohistochemistry to analyze 5mC and 5hmC in neuronal and glial cell types in the inferior temporal gyrus of human AD cases and age-matched controls. Results of their study suggest that extranuclear 5mC in neurofilmament-labeled pyramidal neurons, which are particularly vulnerable to AD pathology [88–90], is significantly decreased in AD cases compared with controls. This same trend was seen in astrocytes. Interestingly, AD-spared calretinin interneurons and microglia did not have significant alterations in 5mC or 5hmC, which supports the hypothesis that DNA methylation alterations in AD are dependent upon the particular cell type studied.

Frontal cortex

The frontal cortex is a site of significant synapse loss in AD [91, 92], and as such, it is often analyzed in AD studies. As mentioned previously, Mastroeni’s twin study found decrements in DNA methylation in the frontal cortex of the twin with AD compared with the cognitively-normal twin [85]. Since then, however, other studies using similar antibody-based methods have found that individuals with AD tend to have a higher level of DNA methylation than individuals without AD. Coppieters et al. [93], for example, found that DNA methylation was higher in the middle frontal gyrus of individuals with AD than in the same region of age-matched controls. Similarly, Rao et al. [94] also found increased global DNA methylation in the frontal cortex of AD patients compared with cognitively-normal controls.
Hippocampus
One of the hallmark pathologies of AD is cerebral atrophy, and atrophy of the hippocampus, a region involved in memory formation, is particularly severe. Thus, researchers have long been analyzing the hippocampus as a brain region significantly impacted by AD. Like the results of studies on DNA methylation within the temporal cortex and frontal cortex, the results of studies analyzing DNA methylation alterations within the hippocampus are inconclusive. A study by Chouliaras et al. [95] found a significant decrease in hippocampal DNA methylation in AD cases compared to unrelated, age-matched controls. In addition, the authors found that glial cell DNA methylation was significantly different in the CA1 and CA3 subregions, whereas neuronal DNA methylation was significantly different only in the CA1 subregion, suggesting that cell type specific alterations in DNA methylation vary depending on hippocampal subregion. The same year that Chouliaras et al. published these results, Bradley-Whitman et al. [96] also published a study analyzing DNA methylation within the hippocampus. Interestingly, they found the opposite: individuals with AD had higher levels of DNA methylation in the hippocampus than individuals without AD.

Gene-specific DNA methylation alterations in AD
To date, there have been several studies analyzing gene-specific DNA methylation in brain tissue of patients with and without AD [86, 94, 97–100]. Much like the results from studies analyzing global DNA methylation, these studies have been inconclusive. However, a number of common DNA methylation alterations in specific genes have been observed across studies, providing relatively strong evidence that methylation within these genes may be altered in AD [98, 101, 102].

Genes related to AD pathology
Initially, studies of gene-specific DNA methylation differences in AD focused on genes associated with AD pathology such as APP, PSEN1, MAPT and apolipoprotein E (APOE). Barrachina et al. [103] analyzed DNA methylation of APP, PSEN1 and MAPT in the frontal cortex and hippocampus of non-demented control individuals and individuals in various stages of AD and found no significant differences in the DNA methylation in any of the three genes studied. Iwata et al. [104] used pyrosequencing to analyze DNA methylation of various CpG sites of AD-related genes in the inferior temporal lobe, the superior parietal lobe and the cerebellum in AD subjects and non-demented control subjects. They found significant differences in DNA methylation profiles of APP, MAPT and GSK3B, but not of PSEN1, BACE1 or APOE. Moreover, the authors demonstrated that these alterations in DNA methylation translated into changes in gene expression, which provides a potential mechanism by which DNA methylation can impact the AD phenotype in these subjects.

APOE is an apolipoprotein that associates with lipoproteins in the plasma as a component of systemic lipid metabolism. In addition, APOE is widely expressed in the central nervous system and functions as the primary cholesterol carrier necessary for the maintenance, growth and repair of neurons [105]. Importantly, APOE is reduced in the hippocampus of patients with AD and is thought to enhance proteolytic degradation of amyloidogenic Aβ [105–107]. Moreover, a specific allele of APOE, APOE-ε4, is significantly less effective at degrading Aβ than the other alleles [106]. APOE-ε4 is a risk factor for AD [108]; individuals homozygous for the APOE-ε4 allele are eight times as likely to develop AD as individuals without the APOE-ε4 allele [108]. Using MALDI-TOF mass spectrometry in postmortem brain tissue samples derived from the prefrontal cortex of individuals with AD and matched controls, Wang et al. [109] found that the promoter region of APOE, but not APP, was hypermethylated in individuals with AD.

In addition to the genes classically associated with AD, numerous genome-wide association studies have identified genetic variants associated with increased AD susceptibility, including but not limited to sortilin-related receptor, low-density lipoprotein receptor class A repeat-containing protein (SORL1), ATP-binding cassette, sub-family A, member 7 (ABCA7), and bridging integrator 1 (BIN1) [110–113]. Yu et al. [113] analyzed DNA methylation in the dorsolateral prefrontal cortex of subjects with and without AD at 28 gene loci associated with AD pathology. Of the 28 gene loci, DNA methylation of five genes (SORL1, ABCA7, HLA-DRB5, SLC2A4 and BIN1) was significantly associated with pathological AD. SORL1 regulates recycling of APP back into the cell, and in its absence, APP is instead directed towards endosomal secretase cleavage pathways that generate Aβ [114, 115]. ABCA7 is primarily known for its role in lipid efflux from cells to lipoprotein particles, but it has also been shown to regulate APP processing, secretion and clearance [116–118]. Although there is relatively little known about the role of BIN1 in AD pathogenesis, it is hypothesized to primarily affect tau pathology. APP endocytosis and intracellular trafficking, neuro-inflammation and calcium transients [119]. Results from studies such as those mentioned here suggest that alterations in DNA methylation of AD-related genes are significantly associated with AD pathology.

Genes related to neuroplasticity, memory formation and neuroinflammation
Multiple genes involved in neuroplasticity and memory formation are differentially methylated between individuals with AD and individuals without AD. Sanchez-Mut et al. [84] used a customized Illumina VeraCode GoldenGate DNA Methylation array to study DNA methylation of genes related to sensory perception, cognition, neuroplasticity, brain physiology and neurological diseases in the frontal cortex of two transgenic mouse models of AD (APP/PSEN1 and 3xTg-AD). The authors validated the results of the array by pyrosequencing and found significant DNA hypermethylation in various genes, including thromboxane A2 receptor (Tbxa2r) and sorbin and SH3 domain containing 3 (Sorbs3). Tbxa2r encodes a G-protein coupled receptor that modulates cAMP response-element binding protein (CREB), which in turn is a transcription factor involved in neuronal plasticity, long-term memory formation and neuroprotection [120]. The protein product of Sorbs3 is a scaffold protein that, among other functions, modulates cell survival by regulating epidermis growth factor-induced activation of c-Jun N-terminal kinase (JNK) [121, 122]. The results of Sanchez-Mut’s study were similar to those of an earlier study that found hypermethylation within SORBS3 in the temporal cortex of AD patients [31].

Mendioroz et al. [123] analyzed gene expression and methylation of CREB-regulated transcription factor 1 (CRTC1), a coactivator of the CREB-dependent gene transcription pathway involved in synaptic plasticity and long-term memory formation that is highly expressed in hippocampal neurons. CRTC1 was significantly hypomethylated in the hippocampus of individuals with AD compared with controls, and furthermore,
methylation within CRTC1 was inversely correlated with Aβ and phosphorylated tau expression. Also, the expression of CRTC1 mRNA was significantly lower in AD cases compared with controls, and downstream target genes of CRTC1 were down regulated, demonstrating that DNA hypomethylation within CRTC1 translated functionally into decreased transcriptional expression and downstream effects.

Neuroinflammation is commonly associated with AD [124–126]. There is evidence that expression of IL-1β and IL-6 peak in the early stages of AD but return to normal levels in the later stages [127]. Since both IL-1β and IL-6 are modulated by DNA methylation in various chronic diseases [128, 129], Nicolla et al. [130] sought to determine whether or not DNA methylation in the promoter region of these genes contributes to the fluctuation in expression levels seen across AD stages. After analyzing DNA methylation in the frontal cortex of patients with AD and middle-aged controls using techniques based on bisulfite modification, the authors found evidence of hypomethylation in the IL-1β promoter in the early stages of AD that returned to middle-aged control levels in later stages. In contrast, they found that IL-6 methylation decreased with AD progression.

DNA methylation in peripheral blood

Abernath methylation in the peripheral blood is correlated with AD disease status [131, 132], so some argue that peripheral blood is a good indicator for AD-associated methylation changes when brain tissue is not available. However, evidence concerning DNA methylation differences in peripheral blood is inconclusive [133, 134]. As an example, brain-derived neurotrophic factor (BDNF), a protein involved in neuronal survival, differentiation and plasticity, is often reduced in AD and is commonly analyzed in AD studies [135–137]. Carboni et al. [138] found no significant differences in DNA methylation in the promoter region of BDNF in peripheral blood. On the other hand, Chang et al. [139] showed an elevation of promoter BDNF DNA methylation in peripheral blood samples of AD patients compared with gender- and age-matched controls. Similarly, Nagata et al. [140] found that DNA methylation of the BDNF promoter in peripheral blood mononuclear cells of patients with AD was significantly higher than that of age-matched cognitively normal controls. Importantly, percent methylation of certain CpG sites within the BDNF promoter correlated negatively and significantly with neuropsychological test scores, suggesting that BDNF promoter methylation is associated with clinical manifestations of AD.

DNA hydroxymethylation in AD

Alteration of global 5hmC has been shown in a variety of neurological disorders such as Rett syndrome, autism spectrum disorder and Huntington’s disease [141–143]. It is not surprising, then, that differences in 5hmC occur in AD as well, although the differences appear to be region-specific. For instance, Condliffe et al. [144] showed that there is a significant decrease in 5hmC in the entorhinal cortex and cerebellum of individuals with AD compared with their age-matched controls. Bradley-Whitman et al. [96], who studied hydroxymethylation within the hippocampus and cerebellum, demonstrated that there is a significant increase in 5hmC in the hippocampus, but not in the cerebellum. In contrast to these results, Chouliaras et al. [95] used immunohistochemistry to show that there was a 20.2% reduction in 5hmC immunoreactivity in the hippocampus of AD patients when compared with non-demented, age-matched controls. Similar results were obtained when analyzing monozygotic twins discordant for AD; 5hmC immunoreactivity was 31.4% lower in the CA1 region of the hippocampus of the AD twin compared with the non-demented twin. Coppieters et al. [93] found that there was a significant increase in 5hmC in the mid-frontal gyrus and mid-temporal gyrus of patients with AD and further showed that 5hmC was relatively low in astrocytes and microglia, but high in neurons.

Evidence suggests that neurogenesis, which is associated with alterations in hydroxymethylation, is altered in AD [145]. The subventricular zone (SVZ) and the subgranular zone are the primary sites of neurogenesis in the aging brain [146]. It has been hypothesized that neurogenesis decreases in the early stages of AD but increases during later stages as part of a compensatory mechanism [145, 147, 148]. Mastroeni et al. [149] found an increase of 5hmC in the SVZ of AD patients compared with non-diseased controls in vivo (brain tissue) and in vitro (primary SVZ cultures) using antibody-based methodologies. They concluded that the compensatory increase in cell proliferation in AD is linked to an increase in 5hmC.

Due to technological advancements in the area of 5hmC high-throughput sequencing, studies have recently begun to analyze gene-specific alterations in 5hmC in AD patients. Bernstein et al. [150] analyzed genome-wide 5hmC in the prefrontal cortex of AD cases and then correlated this with transcriptional changes using RNA-sequencing. They identified 325 genes containing differentially hydroxymethylated loci, of which 140 displayed concomitant changes in gene expression.

The type of starting material used may impact the distribution of 5hmC. A recent study by Shu et al. [65] using adult neural stem cells shows that treatment with Aβ decreased global 5hmC, suggesting that Aβ itself may have a direct impact on methylation. Moreover, when comparing 5hmC levels in HEK293ft cells, adult neural stem cells and neuronal tissue, they demonstrated that 5hmC is significantly higher in tissues than in cells. This suggests that the type of starting material itself has an impact on DNA hydroxymethylation. In addition, the authors characterized changes in DNA hydroxymethylation associated with the interaction between aging and AD by studying 5hmC levels in various brain regions of wildtype mice and a transgenic AD mouse model at 12 weeks of age and at 67 weeks of age. Although there were no significant differences in DNA hydroxymethylation in either the cortex or cerebellum with aging or with AD, there was a significant difference between transgenic mice and wildtype mice in the hippocampus at 67 weeks. It would appear, then, that brain region is important when analyzing DNA hydroxymethylation levels. Finally, the authors characterized the location of differentially methylated 5hmC regions within the genome. These regions were primarily located in gene bodies associated with genes involved in neuronal development and function. More studies like the one described above will further support a complete understanding of 5hmC distribution and characterization.

Conclusions

Despite the surge of interest in DNA methylation and AD, evidence is still inconclusive as to whether or not DNA methylation or DNA hydroxymethylation is altered, particularly on a global scale. Conflicting results have been reported for both types of modifications. Additional studies measuring DNA methylation and DNA hydroxymethylation both accurately and reproducibly are needed before any conclusions can be reached. Numerous limitations exist that impede progress in studying DNA methylation in AD. For example, most studies on this topic predominately use immunohistochemical approaches and/or microarray-based...
Studies have shown, however, that DNA methylation is well important for three reasons. First, it is extremely difficult to col-
ries on global methylation in AD are done in humans, which is 
could be due to any number of reasons. For instance, most stud-
has been difficult, with studies often giving conflicting results. 
WGBS data. For these reasons, studying DNA methylation in AD 
Furthermore, there is no standardized method for analyzing 
For. Thus, the inconsistencies seen here could be due to any number of reasons. For instance, most stud-
ies on global methylation in AD are done in humans, which is 
important for three reasons. First, it is extremely difficult to col-
lect the brain from a human subject exactly at the time of death, 
and there is concern that the sample degrades during the time 
between death and sample collection (postmortem interval). 
Studies have shown, however, that DNA methylation is well 
preserved in the postmortem brain for up to 48 h or even 72 h 
[103, 151]. As the postmortem intervals recorded in most studies 
are well below 48 h, sample degradation due to a long delay 
between death and tissue processing does not account for the 
variable results described here. Second, there is a naturally large 
interindividual variation across human subjects, which can veil 
anything but the most significant of differences. To further com-
plicate the matter, most human subject studies concerning DNA 
methylation in AD use a relatively small sample size. The inher-
ently large interindividual variation across humans coupled with 
the use of small sample sizes may bias the results of studies. 
Third, AD is a complex, multifactorial disease that often presents 
with heterogeneous pathologies. Hence, DNA methylation in AD 
may depend largely on AD stage and pathological presentation. 
Thus, conclusive results from human subject studies may be dif-
cult to acquire, particularly in studies with small sample sizes.

In addition, the inconsistencies seen when analyzing DNA 
methylation in AD could be due to a failure to recognize and 
appreciate the complexity of the brain as an organ; the brain is 
comprised of multiple regions and subregions, each with its 
own function and unique DNA methylation profile [152]. In AD, 
pathologies are often dependent upon brain region (e.g. the cer-
bellum is relatively spared in AD pathogenesis). Thus, it would 
be tempting to conclude that variation seen across studies could 
be due to a lack of consistency in DNA methylation alterations 
in AD across brain regions. As demonstrated in the studies pre-
presented in this review, however, there are differences even 
between studies analyzing the same brain region. Importantly, 
not only does the brain consist of numerous distinct regions, 
each of these regions is also composed of numerous different 
cell types. Cells are specialized with their own unique gene 
expression and methylation signatures. Therefore, it is possible 
that significant differences between methylation profiles could 
be lost when cell types are analyzed together.

Finally, methodological differences could contribute to the 
inconsistencies seen across studies. Earlier studies on DNA 
methylation in AD use immunohistochemistry, a semi-
quantitative method that does not provide an accurate, quanti-
tative measurement of methylation or hydroxymethylation. 
In addition, immunohistochemistry relies heavily on the 
particular antibody probe being used; concerns about antibody 
specificity must always be taken into account when comparing 
studies using this method. More recent studies use methods 
based on bisulfite conversion, in which unmethylated cytosines 
are converted to uracils. Pyrosequencing and WGBS, both of 
which are based on the process of bisulfite conversion, are con-
sidered gold standards for quantifying DNA methylation due to 
their ability to measure DNA methylation at single-nucleotide 
resolution. Despite this advantage, however, bisulfite conver-
sion does not distinguish between 5mC and 5hmC. This is a sig-
ificant problem for studying DNA methylation in AD, as 5hmC 
is particularly abundant in the brain. In addition, because these 
bisulfite-based methods are relatively new, standardized and 
user-friendly software for analyzing bisulfite sequencing data 
has only recently been developed. Thus, post-collection analy-
sis of data is different across studies, which could contribute to 
the inconsistencies reported here. For more information regard-
ing the advantages and disadvantages of DNA methylation 
analysis techniques, the reader is referred to excellent reviews 
by Laird [153] and Kurdyukov et al. [154].

It is apparent that more research is needed to provide con-
clusive results. As the cost of WGBS decreases and the bioinform-
atics software associated with WGBS analysis becomes more 
user-friendly and widely available, it should become easier to 
carry out large-scale studies on DNA methylation in the brain 
and its association with AD. With further advancements in cell-
sorting and DNA methylation technology using small quantities 
of sample, we should begin to see an increase in studies looking 
at DNA methylation in specific cell types. In addition, studies 
should focus on the interaction between the methylome, tran-
scriptome and the plethora of histone marks and non-coding 
RNA in AD, as epigenetic modifications often work together to 
achieve a particular phenotype [155]. Finally, the field should 
consider: (i) using a combination of standard techniques such as 
immunohistochemistry coupled with bisulfite sequencing 
and (ii) comparing the results of genome-wide studies to focus 
on common genes to improve comparability across studies. In 
essence, although results are inconclusive, it is evident that the 
environment, through its effect on DNA methylation, has an 
impact on AD pathology. Thus, studies of epigenetic alterations 
in AD will continue to be a topic of immense interest within the 
research field.

Funding
This study was supported by the Richard A. and Nora Eccles 
Harrison Endowed Chair in Diabetes Research Fund (to J.C.R.) 
and the National Institutes of Health through the following 
grants: the National Institute on Aging Grant AG039094 (to J.C.R.) 
and the National Institute of Diabetes and Digestive 
and Kidney Diseases Grants U24 DK092993-05S1 (to J.C.R.), 
U24 DK092993 (to Kent Lloyd), R01DK104770-01A1 (to V.M.) 
and R03DK099427-02 (to V.M.). J.C.R. was partially funded 
through Grant U24 DK092993, which was awarded to Kent 
Lloyd. Kent Lloyd did not contribute to this manuscript.

Conflict of interest statement. None declared.

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