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Liqi Shu April 15, 2015

Genome-wide alteration of 5-hydroxymethylcytosine in a mouse model of Alzheimer's disease

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Abstract

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Alzheimer's disease (AD) is the most common form of neurodegenerative disorder that leads to a decline in cognitive function. In AD, aggregates of amyloid β peptide precede the accumulation of neurofibrillary tangles, both of which are hallmarks of the disease. Strong evidences have indicated the implication of epigenetic modifications, including histone modification and DNA methylation, in AD. Recent studies revealed that 5-hydroxymethylcytosine (5hmC)-mediated DNA demethylation is dynamically regulated during neurodevelopment and aging. Here we show that amyloid peptide 1-42 (Aβ1-42) could significantly reduce the global level of 5hmC in cell cultures. We found that the global level of 5hmC displayed differential response to the pathogenesis in different brain regions, including the cortex, cerebellum, and hippocampus of APP-PSEN1 double transgenic (DTg) mice. We observed a significant decrease of overall 5hmC in hippocampus, but not in cortex and cerebellum, as the DTg mice aged. Our genome-wide profiling study identified differential hydroxymethylation regions (DhMRs) in DTg mice, which were highly enriched almost everywhere, for example, introns, exons and intergenic regions. Gene ontology analysis indicated that DhMR-associated genes are highly enriched in multiple signaling pathways involving neuronal development/differentiation and neuronal function/survival. Our results strongly argue that 5hmC-mediated epigenetic regulation could contribute to the pathogenesis of AD.

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Table of Contents

Table of Figures and Table

Introduction

Epigenetics

In the early 19th century, genetics and developmental biology were considered to be two separate disciplines (1). Conrad Waddington first coined the term "epigenetics" in the middle of the twentieth century to describe the molecular events involved in early undifferentiated embryonic development, linking the two important fields together (2). The current definition of epigenetics is the study of heritable changes of gene expression and function that do not alter DNA sequence (3, 4).

Epigenetic regulation often results in chromatin remodeling. In the traditional DNA model, DNA strands are wrapped around an octamer of histone proteins to form a nucleoprotein complex, chromatin. Therefore, in order to execute DNA-templated processes such as gene expression, DNA replication and repair, DNA strands must assume an "open" configuration to access the RNA polymerase II complex, the replication machinery and DNA repair systems (5). There are currently two major epigenetic mechanisms, cytosine modifications, histone modifications, and ATP-dependent chromatin remodeling (6, 7). These modifications result in charge disruption and steric hindrance that induce chromatin remodeling, which gives rise to altered levels of mRNA transcription and gene expression (5). Additionally, epigenetic modifications have been shown to be dynamic and can change due to various environmental factors, including diet, infection, oxidative stress and drug exposure (8).

Alzheimer's Disease

One hundred years ago, Alois Alzheimer presented the first case of Alzheimer's disease (AD) at a congress in Tübingen, Germany. Alzheimer described typical clinical characteristics with memory disturbances and instrumental signs, and the neuropathological picture with miliary bodies (plaques) and dense bundles of fibrils (tangles), which we today consider as the hallmarks of AD (9).

Alzheimer's disease, the most common cause of dementia, is a progressive neurodegenerative disease involving multiple pathologic processes and is characterized by the deposition of amyloid beta (Aβ) peptide, neurofibrillary tangles (NFTs) composed of hyperphosphorylated protein tau, and neuronal cell loss (10, 11). Familial Alzheimer's disease (FAD) is a very rare autosomal dominant disease with early onset, caused by mutations in the amyloid precursor protein (APP) and presenilin genes (PSEN), which are related to Aβ metabolism. Contrasting to FAD, sporadic Alzheimer's disease is heterogeneous and very common with more than 15 million people affected worldwide. However, the cause of the sporadic AD is unknown, probably caused by a complex interaction of both genetic and environmental risk factors (12).

Recent studies indicate that epigenetic pathways could be involved in the pathogenesis of AD (13-16). DNA methylation (5-methylcytosine, 5mC) plays important roles in regulating gene expression and is involved in multiple neurodevelopmental and neurodegenerative disorders (7, 17, 18).

DNA methylation

One of the most well studied epigenetic modifications is the methylation of the 5th carbon on the cytosine nucleotide, which gives rise to 5-methylcytosine (5mC), catalyzed by DNA methyltransferases (DNMTs) (19). DNA methylation is the only mechanism that directly imposes on DNA (20), and was first proposed to play an important role in long-term memory formation and remained the major DNA covalent modification to influence transcriptional states, and ultimately cellular identity (21).

Typically 5mC occurs in the regions that contain a high frequency of CG dinucleotides in the mammalian genome and plays pivotal roles in the regulation of gene expression, chromatin structure, gene imprinting, X-chromosome inactivation, and genomic stability (22-24).

Changes in 5mC at the global level or at specific loci are seen in the brain tissues of AD model mice, as well as AD patients (13, 16, 25-28). Although some regions and loci show hypermethylation (13), global DNA hypomethylation has been observed in the entorhinal cortex of some AD patients (14), suggesting DNA methylation is differentially affected in a region- and loci-specific manner. Previous studies also found that the promoter regions of amyloid precursor protein (APP) and presenilin 1 (PSEN1) displayed age-dependent hypomethylation (26, 29-31). Furthermore, *in vitro* hypomethylation of PSEN1 increased the cleavage of APP and the production of Aβ in a neuroblastoma cell line (32). Recently, two large-scale epigenome-wide association studies uncovered site-specific altered methylation in the brains of AD patients (27, 28). These results imply DNA methylation could play important roles in the pathogenesis of AD.

5-Hydroxymethylcytosine

Recently, another cytosine modification, 5-hydroxymethylcytosine (5hmC), was identified and found to be highly abundant in the neuronal system (33-35). Ten-eleven translocation (TET) family proteins, including Tet1, Tet2, and Tet3, are known to catalyze the hydroxylation of 5mC to 5hmC (33, 36-38). Recent studies strongly indicate that 5hmC not only serves as an intermediate of DNA demethylation, but can also perform as a stable epigenetic marker.

Oxidation of 5mC to 5hmC by Tet enzymes is proposed as an intermediate step in active DNA demethylation. Studies have shown that Tet proteins can oxidize 5mC to 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), and that 5caC can be excised further to cytosine by a thymine-DNA glycosylase (TDG)–mediated base excision repair mechanism (37-40).

In humans, although the levels of 5mC are comparable throughout the major organs, 5hmC is ~10-fold more enriched in neurons than other cell types, and it is acquired globally and exhibits dynamic features and region-specific patterns during postnatal development and aging of the neuronal system (34, 41, 42). Genome-wide studies reveal that 5hmC can be enriched in distinct genomic regions, such as gene bodies, promoters, and distal regulatory regions, suggesting that the enrichment of 5hmC could be positively correlated with transcriptional level, which might be achieved via interaction with histone modifications (34, 41).

The alteration of global 5hmC and differentially hydroxymethylated regions (DhMRs) are seen in several neurodevelopmental diseases, including Rett syndrome, autism, and neurodegenerative diseases like Huntington's disease and fragile X-associated

tremor/ataxia syndrome (FXTAS), suggesting 5hmC could play important roles in neurological diseases (34, 41, 43-45). Determining the AD-specific acquisition of 5hmC or any AD-specific alterations in 5hmC levels (or genomic distribution) may reveal specific loci that are most affected in AD patients. It is possible that distinct modifications on the 5hmC may provide an effective biomarker for AD risk screening. We believe the high concentration of Aβ serves as an external stress, which alters the epigenetic modifications of neuronal cells, including the global level of 5hmC.

Differentially Hydroxymethylated Regions

Despite the clear alteration of DNA methylation observed in AD, whether and how 5hmC is involved in AD pathogenesis still remain largely unknown. Using an immunostaining method, Condliffe and his colleagues found a significant decrease of global 5hmC in the cortex and cerebellum of AD patients (46). In contrast, using the same technology, other studies reported an increase of global 5hmC in both AD mouse model and patients samples (47-49). To study the alteration of 5hmC in AD and explore the potential role(s) of 5hmC-mediated epigenetic regulation in the pathogenesis of AD, here we investigated the effect of Aβ on 5hmC in cell cultures and found Aβ treatment could significantly decrease the global level of 5hmC in a dose-dependent pattern. Furthermore, we found that 5hmC levels displayed an age-related decrease in the hippocampus, but not in the cortex and cerebellum, of APP-PSEN1 double-transgenic (DTg) mice. Using a chemical-labeling 5hmC enrichment technology, we performed genome-wide profiling of 5hmC. We found that, although AD pathogenesis did not change the overall distribution of 5hmC, there were differentially hydroxymethylated

regions (DhMRs) in DTg mice. The DhMRs identified are involved in a number of neuronal signaling pathways, indicating a 5hmC-mediated epigenetic pathway could play important roles in the pathogenesis of AD.

Methods

Animals

Twelve- and 67-week-old wild-type (WT) and mutant amyloid precursor protein and presenilin-1 (APP/PS1) double transgenic littermate mice were used in this study (50). Mice were maintained at ambient temperature (22-24°C) on a 12:12 light/dark cycle with free access to food and water.

Cell culture and Aβ treatment

Human Embryonic Kidney 293ft (HEK293ft) cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 U penicillin–streptomycin at 37°C in a humidified incubator containing 5% CO_2. Past research implemented Aβ(1-42) at either 0.50 or 1.00-μM concentration to induce neurotoxicity in cell cultures (51-53). Therefore, cells were treated with Aβ1-42 peptide (54A9810) at a concentration of 0.00 (DMSO), 0.25, 0.5, 0.75, or 1.00 μM for 48 h. Adult neural stem cells (aNSCs) were treated with Aβ1-42 peptide at a concentration of 1.00μM for 48 h.

Genomic DNA isolation and 5hmC dot-blot

Genomic DNA was extracted as described previously (41). Briefly, the dissected brain samples or cells were homogenized in lysis buffer (5 mM EDTA, 0.2% SDS, 200 mM NaCl in 100 mM Tris-HCl, pH 8.5), treated with proteinase K, and kept at 56°C overnight. The second day, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, P-3803, Sigma) was added, mixed completely, and centrifuged at 14,000 rpm

for 10 min. An equal volume of isopropanol was added to the supernatant to precipitate DNA, which was dissolved with 10 mM Tris-HCl (pH 8.0).

5hmC dot-blot was performed as before (34). In brief, genomic DNA was spotted on an Amersham Hybond-N+ membrane (GE Healthcare), followed by baking at 80°C for 30 min. The membrane was incubated with polyclonal 5hmC antibody (Active Motif, #39769) overnight at 4°C. The second day, a horseradish-peroxidase-conjugated secondary antibody against rabbit was used to probe, and the film was developed with a Biorad ChemiDoc XRS. 5hmC level were quantified with Photoshop software, and data were analyzed with Graphpad software (Mean ± SE, unpaired *t*-test). This highly sensitive and selective dot-blot technique has been tested during development and was able to detect single 5hmC in oligo controls (42).

5hmC-specific enrichment and high-throughput sequencing

Chemical labeling-based 5hmC enrichment was described previously (42). Briefly, DNA was sonicated to fragments with 100-500 bp size, and then mixed with 100 μl solution containing 50 mM HEPES buffer (pH 7.9), 25 mM $MgCl_2$, 250 μM UDP-6-N3-Glu, and 2.25 μM β-glucosyltransferase for 1 h at 37°C. DNA substrates were purified via Qiagen DNA purification kit. 150 μM dibenzocyclooctyne-modified biotin was then added to the purified DNA, and the labeling reaction was performed for 2 h at 37°C. The biotin-labeled DNA was enriched by Streptavidin-coupled Dynabeads (Dynabeads® MyOne™ Streptavidin T1, Life Technologies) and purified.

5hmC libraries were generated with 25 ng input or 5hmC-captured DNA according to the manufacturer's protocol (NEBNext ChIP-Seq Library Prep Reagent Set

for Illumina). DNA fragments between 150 and 300 bp were gel-purified after the adapter ligation step. An Agilent 2100 BioAnalyzer was used to quantify the amplified DNA. 20 pM diluted libraries were eventually used for sequencing.

Chemical labeling-based 5hmC enrichment is efficient and selective with high yielding (~90%) (42). However, two 5hmC targeted methodologies serve different purposes: the immunolabeling technique helps to quantify the global 5hmC level in the whole genome, whereas the chemical labeling technique captures 5hmC containing DNA fragments to perform high-throughput sequencing.

Sequence alignment and mapped reads annotation

FASTQ sequence files were aligned to mouse NCBI37v1/mm9 references using Bowtie 0.12.9. Each unique mapped read with no more than two mismatches in the first 25 bp was concatenated to achieve combined wild-type and APP/PS1 mice 5hmC sequence. Association of mapped reads with genomic features was performed by overlapping reads files with known genomic features obtained from UCSC Tables for NCBI37v1/mm9. Unique 5hmC mapped reads were plotted to various genomic regions using an R program package termed ngsplot (https://code.google.com/p/ngsplot/).

DhMR identification, annotation, and motif analysis

Model-based Analysis of ChIP-Seq (MACS) software (55) was adopted to identify DhMRs between wild-type and APP/PS1 mice by directly comparing one to the other, rather than comparing to the input. The effective genome size = 1.87×10^{9}, tag size = 38, bandwidth = 200, *P*-value cutoff = 1.00×10^{-5}. Identified wild-type- and APP/PS1-

specific DhMRs were annotated to various genomic regions and associated genes by HOMER software (56). DhMR-associated genes were extracted, and enrichment analysis was performed with WebGestalt (57).

Results

Aβ reduces the global level of 5-hydroxymethylcytosine in cell cultures

Aβ deposition is one of the hallmarks of AD pathogenesis and is known to induce neuronal cell death. To study the roles of 5-hydroxymethylcytosine (5hmC)-mediated epigenetic modification in AD pathogenesis, we first studied the effect of Aβ(1-42), a toxic form of peptide associated with AD, on the global level of 5hmC in cell cultures. After being treated with Aβ for 48 h, the overall level of 5hmC in HEK293ft cells decreased, and Aβ peptide at a 1-μM concentration had a greater effect (Fig1).

Considering cognitive function is severely impaired and the roles of adult neurogenesis in learning and memory, we further tested the effect of Aβ peptide on 5hmC levels in adult neural stem cells (aNSCs). ANSCs harbor in specific regions, subventricular zone of lateral ventricle and subgranular zone of dentate gyrus of adult mammalian brain, and are involved in neurological disorders including AD (58). After treated with Aβ peptide at a 1-μM concentration for 48 h, the level of 5hmC was also significantly decreased in the culture aNSCs (Fig1). Taken together, these results indicate that Aβ peptide could significantly affect the global level of 5hmC.

5-hydroxymethylcytosine level decreases during aging in an AD mouse model

Previous studies have indicated that 5hmC could be acquired in the brain during postnatal development and aging (34, 41). To examine whether the level of 5hmC is affected during AD pathogenesis, we dissected multiple brain regions, including cortex, cerebellum, and hippocampus from 12-week-old (adult) and 67-week-old (aged) wild-type (WT) and APP-PSEN1 double transgenic (DTg) mice. Consistent with our previous

results, from 12 weeks to 67 weeks, 5hmC exhibits no or slight acquisition in the cortex and cerebellum of WT and DTg mice (Fig2). Quantification results showed no significant difference between WT and DTg mice (Fig2). In hippocampus, there was no significant change in 5hmC in WT mice during aging; however, at the 67-week time point, the 5hmC level of DTg mice hippocampus decreased significantly compared to age-matched WT control (Fig2). Collectively, these results indicate that the global level of 5hmC is affected in specific brain regions during AD pathogenesis.

Acquisition of 5hmC on gene bodies is altered in aged AD mice

To explore whether the distribution features of 5hmC in the genome are altered during AD pathogenesis, we employed a previously established 5hmC chemical labeling and affinity purification method (42) and performed 5hmC genome-wide profiling. Based on dot-blot results, our subsequent study focused on hippocampus. To perform genome-wide sequencing of 5hmC, hippocampus tissues were dissected from three adult (12-week) DTg mice and three littermate WT mice; at the 67-week time point (aged), hippocampus tissues were dissected from two DTg mice and two WT littermate mice. Through deep-sequencing, 11-23 million total reads and 9-18 million monoclonal reads were generated from each sample (Table 1). Sequence data were analyzed using our established pipeline (34), and peaks were identified by MACS software (55). 6115 and 8335 5hmC peaks were called from adult WT and DTg mice biological replicates, respectively (Fig3). At the 67-week time point, 39,606 and 19,977 peaks were identified from WT and DTg mice biological duplicates, respectively (Figure 3A). The number of total peaks decreased significantly more in aged DTg mice compared to WT littermates.

During the aging process, AD pathogenesis did not significantly affect the shared peaks between adult and aged mice of each genotype: 5518 peaks were shared between adult and aged WT mice, and 5289 peaks were shared between adult and aged DTg mice (Fig3).

Differential hydroxymethylated regions (DhMRs) associated with AD

The partial overlapping of adult and aged 5hmC peaks in WT and DTg mice suggested differential hydroxymethylation (Fig3). We next sought to identify differential hydroxymethylation regions (DhMRs) in the genome. Compared to age-matched WT mice, 5324 and 4975 specific DhMRs were identified in adult and aged DTg mice, respectively (Fig4). Among them, 244 specific DhMRs were shared between adult and aged DTg mice, which did not appear in either adult or aged WT mice (Fig4). The DhMRs identified in adult and aged DTg mice displayed similar distribution trend: abundantly enriching in intron, exon and intergenic regions (Fig4), suggesting a high conservation during AD progress. DTg specific 5hmC peaks (shared in adult and aged DTg mice) highly enriched in exons, introns, and intergenic regions (Fig4). Furthermore, 5hmC peaks also displayed enrichment on DNA repeat elements, such as long interspersed elements (LINE), short interspersed nuclear elements (SINE) and long terminal repeat retrotransposons (LTR) (Fig4).

To further reveal the biological function of identified DhMRs in DTg mice, the genes associated with these DhMRs were extracted for enrichment analysis. Out of 193 pathways in the Wikipathway database from July 2013, 22 signaling pathways were highly enriched ($p<0.05$, hypergeometric test), including the Wnt and ErbB pathways,

which play important roles in the neuronal system. The top nine pathways with lowest p values are presented in Figure 4. Taken together, these results suggest that the specific enrichment of 5hmC could play some role(s) in regulating gene expression and be involved in neuronal function.

Two recent large-scale studies identified alterations of DNA methylation in some loci of AD patients (11, 12), suggesting DNA demethylation might be involved in this process. Next, we examined whether those loci displayed altered DNA demethylation. An Integrative Genomics Viewer (IGV) image showed the overall reduction of 5hmC peaks in aged DTg mice compared to age matched WT mice (Fig5). We next examined the 5hmC distribution profile of two AD-associated genes, Dip2a and TMX4, and found the enrichment of 5hmC peaks in some regions of these two genes decreased, suggesting hampered DNA demethylation (Fig5).

Previous studies also noted that the enrichment of 5hmC in gene bodies might be positively correlated with gene expression (29). In our AD model mice, two AD associated genes, APP and PSEN1 were over-expressed. We found 5hmC peaks highly enriched in APP gene bodies, especially in exons, in both adult and aged DTg mice compared to WT mice (Fig5). It is of interest to note that we did not see significant difference of 5hmC distribution in PSEN1. These data indicated that 5hmC enrichment could be one of the mechanisms promoting gene expression.

Discussion

In the present study, we performed cell cultures and live mouse model studies to characterize the alterations of 5hmC in a mouse model of AD. We found that treatment with the AD pathogenic protein amyloid peptide led to a decrease of global 5hmC in cell cultures. Our mouse model study also found the level of 5hmC decreased in one specific brain region, i.e., the hippocampus, but not all brain regions of AD mice. Though we did not find global 5hmC decrease in the cortex and cerebellum as Condliffe and his colleagues reported, we found that the global 5hmC is decreased in the mouse hippocampus. Our AD mouse model behaves similar to FAD, whereas Condliffe and his colleagues used generic AD patient brains, most of which are likely sporadic Ads due to its high occurrence. Different types of AD might have different disease mechanisms and thus different 5hmC levels in brains regions have been found. Our genome-wide profiling study revealed that the distribution of 5hmC was also altered in distinct genomic regions. Differentially hydroxymethylated regions (DhMRs) were also identified in the hippocampus of AD model mice. DhMR-associated genes displayed high enrichment of multiple signaling pathways that are related to neuronal development and neuronal function. Our study therefore uncovered new roles for 5hmC-mediated epigenetic modification in neurologic disorders and revealed a new layer of the pathogenic mechanism of AD.

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease and the most common cause of dementia. The deposition of Aβ in the brain is one of the hallmarks of the disease (59), and amyloid plaques play key roles in the

development of AD (60). Previous studies revealed that epigenetic mechanisms, such as histone deacetylases, could influence the progression and outcome of AD (61, 62). In our present study, Aβ treatment led to a significant decrease in global 5hmC level, pointing out an interaction between amyloid accumulation and DNA modification. Recently, global and site-specific alterations of DNA methylation were identified in AD (26, 29, 63-66), and very recently, epigenome-wide association with AD revealed the relationship between differential methylation of CpGs and the expression of nearby genes, some of which are connected to a known AD susceptibility network (27, 28). Our present studies also uncovered altered demethylation in these identified loci, which further points to roles for DNA methylation and demethylation in AD.

Global and site-specific alterations of DNA methylation had been identified in AD (10, 14, 15, 35-37). Epigenome-wide association with AD revealed the relationship between differential methylation of CpGs and the expression of nearby genes, some of which are connected to a known AD susceptibility network (11, 12). Previous studies had conflicting reports about the direction of alteration of DNA demethylation (30-33). In our present study, Aβ treatment led to a significant decrease in global 5hmC level of different types of cells. The significant alteration of demethylation was only observed in hippocampus but not in cortex and cerebellum during the ageing of AD model mice. Our present studies also uncovered altered demethylation in these identified loci, which further points to roles for DNA methylation and demethylation in AD. Together with previous studies, it supports the idea that dysregulation of DNA demethylation is age-

and region/loci specific, indicating an interaction between amyloid accumulation and DNA modification.

The accumulation of amyloid peptide plays essential roles in the pathogenesis of AD. Aβ leads to neuronal cell death, the pathogenic hall marker of AD, via diverse mechanisms including activating apoptosis pathways and inducing oxidative stress. Interestingly, 5mC has also been linked to cell death and increased 5mC levels precede the apoptosis of motor neurons (67). Furthermore, 5hmC also is enriched in the intragenic regions of genes involved in cell death (35). Indeed, the enrichment of 5hmC in gene bodies is positively correlated with gene expression (68-70). These studies support a concept that cytosine modification, particularly 5mC and 5hmC, is involved in the pathogenesis of AD through regulating the expression of apoptosis related genes.

Previous studies have found 5hmC is highly enriched in the neuronal system (33, 42), and the enrichment is enhanced and displays dynamic features during postnatal development and aging of the brain (34, 71), indicating 5hmC could be important for brain function. Subsequent studies found that 5hmC-mediated epigenetic modification is involved in multiple neurological disorders, including autism spectrum disorders, Huntington's disease, and FXTAS (34, 41, 44, 45). Moreover, the Rett syndrome protein MeCP2 could bind to 5hmC, and its dosage is negatively correlated with 5hmC level (34, 43). Compare to the DhMRs in 12-week, those in 67-week is more important, because AD actually developed in aged mice, whereas in the 12-wk, AD haven't developed yet. However, we still analyzed the shared DhMRs between 12-week and 67-week because

the Aβ might already show some effects at 12-week although it did not show significant pathological changes yet. Our present studies identified some DhMR-associated genes enriched in multiple signaling pathways that are related to neuronal function and neurological disorders (34, 41, 44, 45). These results suggest a potential mechanism to explain how 5hmC-mediated epigenetic modification functions.

Overall, our results indicated that not only the global level but also the distribution features of 5hmC was altered in AD model mice. Aβ treatment decreased 5hmC both in cell model and in live mouse model. Alterations of 5hmC will lead to aberrant gene expressions (72). The DhMR-associated genes identified in AD mice are specifically related to some signaling pathways that play a role in neuronal function and neurological disorders. Furthermore, those altered gene expressions could be involved in the pathogenesis of AD. Taken together, our present results argue that 5hmC-mediated epigenetic modification could be involved in the initiation and development of AD.

Future Directions

In this study, as we have found hundreds of DhMR-associated genes identified in AD mice, we want to further narrow down the gene candidates that are most important. We will use Drosophila to screen the massive gene lists we got from our genetic profiling and analysis. To test some of these genes, we will cross those knockdown gene Drosophila models with Alzheimer's Drosophila model, such as Aβ and tau transgenic flies. The effect of the knockdown gene interaction will be observed via eye phenotype. The quantitative and qualitative data from the rescued or enhanced eye phenotype of

Alzheimer's Drosophila model will enable us to select genes with the greatest correlation with Alzheimer's disease, such that we can perform further investigations with these few genes on using a mouse model. These genes could be potential targets to treat Alzheimer's disease.

Figures and Tables

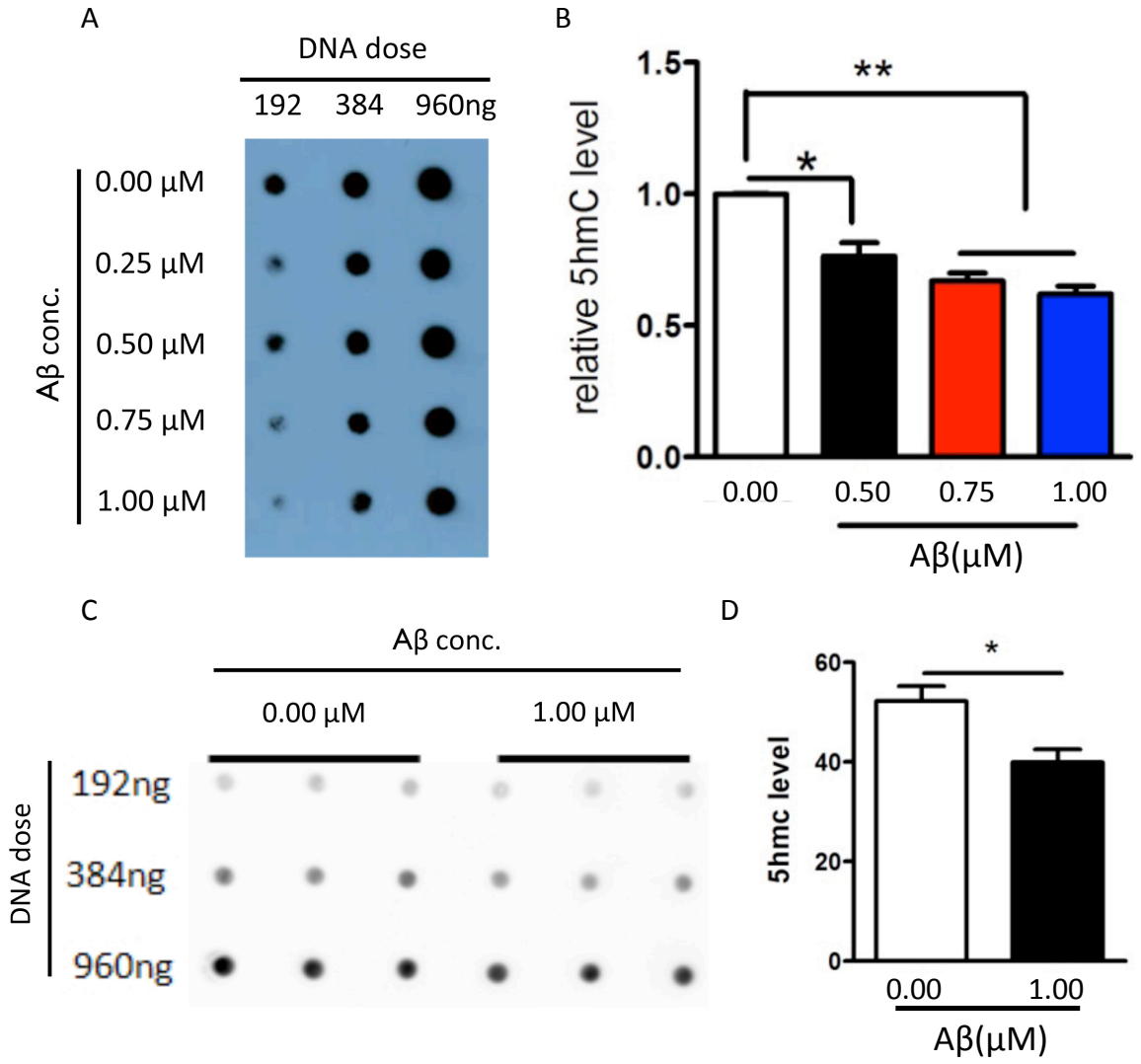

Figure 1. Aβ(1-42) peptide reduces 5hmC abundance in cell cultures. (A) Dot-blot assay shows Aβ treatment significantly decreased total 5hmC levels in a dose-dependent manner in HEK293ft cells (n=3 for each Aβ concentration). (B) The quantification result indicates Aβ at 1-µM concentration is more effective than lower concentrations at decreasing 5hmC levels (*p<0.05; ** p<0.01, unpaired t-test). (C) The representative images of dot-blot assay of Aβ treatment on 5hmC level in aNSCs (n=3). (D) The quantification result indicates Aβ at 1-µM concentration significantly decreases 5hmC level in aNSCs (*p<0.05; ** p<0.01, unpaired t-test).

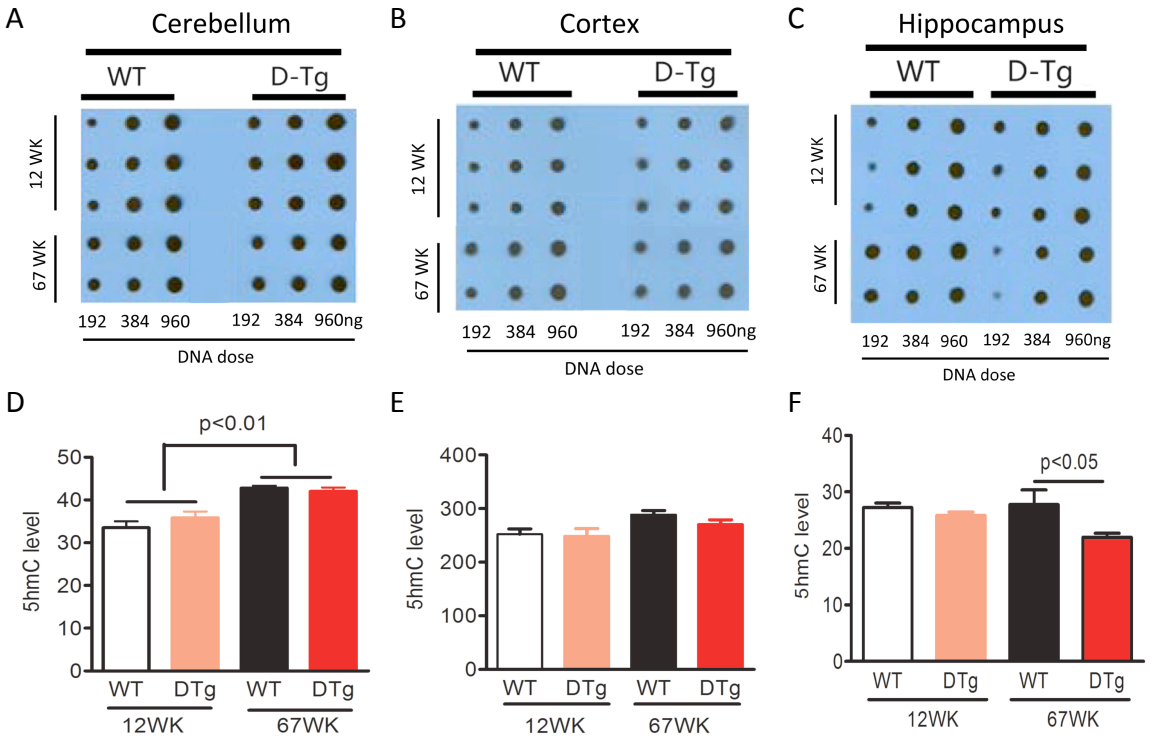

Figure 2. Reduced 5hmC at selective brain regions in a mouse model of AD. (A-C) Representative images of 5hmC dot-blot assay of 12- and 67-week-old WT and DTg mice cortex (A, n=3), cerebellum (B, n=3), and hippocampus (C, n=3). (D-F) The quantitative results indicated that the global levels of 5hmC did not show significant difference in cortex and cerebellum of WT and DTg mice (D, E). In hippocampus, the overall abundance of 5hmC was significantly decreased in DTg mice compared to WT mice at 67-week stage while it did not show observable difference between WT and DTg mice at 12-week stage (F). (ANOVA post Bonferroni's Multiple Comparison Test, mean±s.e.m. **p*<0.05, ***p*<0.01).

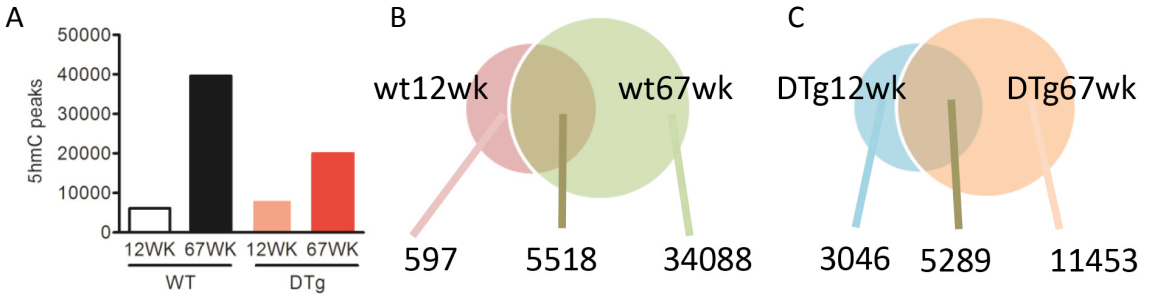

Figure 3. Genomic features of 5hmC peaks in hippocampus between WT and DTg mice. (A) 6115, 39,606, 8335, and 19,977 of 5hmC peaks were called from 12- and 67-week-old WT and age-matched DTg mice biological replicates, respectively. (B) 5518

peaks overlapped between 12- and 67-week-old WT mice. (C) 5289 peaks overlapped between 12- and 67-week-old DTg mice.

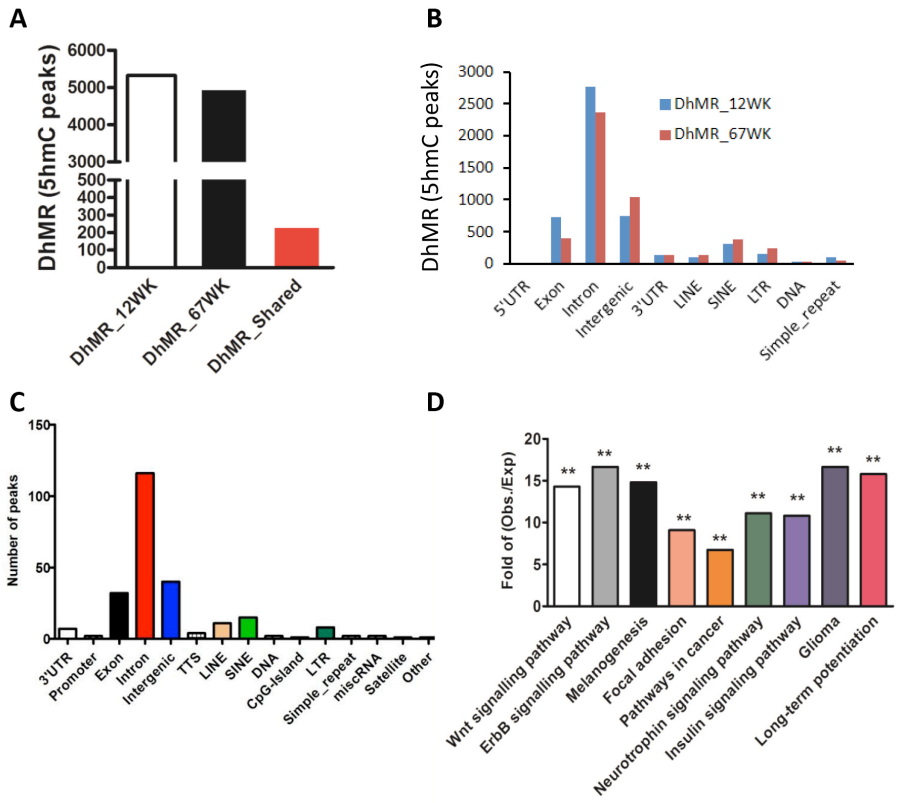

Figure 4. Identification and characterization of DhMRs in AD mouse model. (A) Compared to age-matched WT mice, 5324 of adult-specific DhMRs and 4975 of DTg-specific DhMRs were identified. In addition, 244 specific DhMRs were shared between adult and aged DTg mice, which did not appear in either adult or aged WT mice. (B) The distribution features of DhMRs identified in adult and aged DTg mice, respectively. DhMRs highly enrich in introns, exons and intergenic regions. (C) The distribution of shared DhMRs identified in both adult and aged DTg mice. (D) Gene ontology assay shows that fold of observed and expected DhMR-associated genes are highly enriched in multiple neuronal signaling pathways.

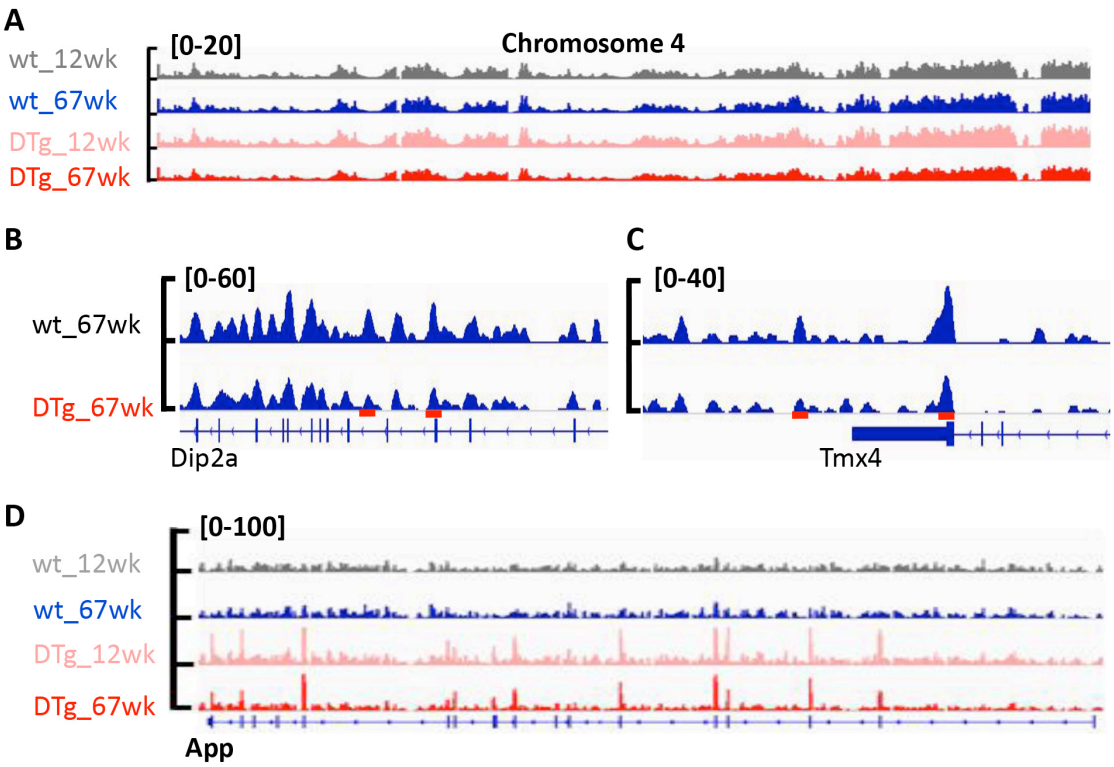

Figure 5. Identification and characterization of DhMRs in AD mouse model. (A) A representative IGV image shows the reduction of overall 5hmC in aged DTg mice compared to WT mice and adult DTg mice. (B-C) Representative IGV images show the decrease of 5hmC in some genomic regions of thioredoxin-related transmembrane protein 4 (TMX4) and DIP2 disco-interacting protein 2 homolog A (Dip2a), which display dysreguation of DNA methylation in AD. (D) 5hmC highly enriched in the gene body of APP gene, which is overexpressed in the used AD model mice.

Table 1. The sequencing reads of 5-hmC.

Sample Name	Genotype	Age	Total Reads	Monoclonal Reads	Mapping Uniquely (%)
Sample_APP330	WT	12-WK	15,788,115	10,684,116	67.67%
Sample_APP331	WT	12-WK	15,806,370	11,665,944	73.81%
Sample_APP333	WT	12-WK	12,869,500	9,798,842	76.14%
Sample_APP332	D-Tg	12-WK	17,194,469	12,744,811	74.12%
Sample_APP334	D-Tg	12-WK	11,803,291	8,883,787	75.27%
Sample_APP335	D-Tg	12-WK	23,618,473	18,292,639	77.45%
Sample_APP115	WT	67-WK	21,613,283	16,344,440	75.62%
Sample_APP147	WT	67-WK	21,613,264	15,376,207	71.14%
Sample_APP150	D-Tg	67-WK	14,604,176	10,764,531	73.71%
Sample_APP118	D-Tg	67-WK	18,879,820	13,464,932	71.32%

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