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# Single-base resolution mapping of 5-hydroxymethylcytosine modifications in hippocampus of Alzheimer's disease subjects

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## Abstract

Epigenetic modifications to cytosine have been shown to regulate transcription in cancer, embryonic development, and recently neurodegeneration. While cytosine methylation studies are now common in neurodegenerative research, hydroxymethylation studies are rare, particularly genome-wide mapping studies. As an initial study to analyze 5-hydroxymethylcytosine (5-hmC) in the Alzheimer's disease (AD) genome, reduced representation hydroxymethylation profiling (RRHP) was used to analyze more than 2 million sites of possible modification in hippocampal DNA of sporadic AD and normal control subjects. Genes with differentially hydroxymethylated regions were filtered based on previously published microarray data for altered gene expression in hippocampal DNA of AD subjects. Our data show significant pathways for altered levels of 5-hmC in the hippocampus of AD subjects compared to age-matched normal controls involved in signaling, energy metabolism, cell function, gene expression, protein degradation, and cell structure and stabilization. Overall, our data suggest a possible role for the dysregulation of epigenetic modifications to cytosine in late stage AD.

### Keywords

5-hydroxymethylcytosine; Alzheimer's disease; hippocampus; epigenetics

## Introduction

Alzheimer's disease (AD) is the sixth leading cause of death in the United States, and diagnosis of AD is projected to exceed 13 million patients by 2050 (Fargo 2014). AD, the most common type of neurodegeneration in the elderly, is characterized by memory loss, decreased cognition, and specific pathologic markers including amyloid plaques, composed of the amyloid beta (Aβ) peptide; and neurofibrillary tangles (NFT) composed of hyperphosphorylated microtubule associated protein tau (Walsh & Selkoe 2004). A genetic component has been linked to familial AD, e.g. mutations in amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) (Selkoe 1997), however, in sporadic/late-

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onset AD (LOAD), a clear mechanism of dysfunction and neurodegeneration remains elusive. Several susceptibility genes, most commonly *APOE*, have been identified in large genome-wide association studies, but exactly how these genes influence AD etiology is not well understood (reviewed by (Karch & Goate 2015).

Non-genetic factors, such as oxidative damage and epigenetic modifications, could potentially contribute to the complexity of AD dysfunction. Although oxidative damage plays a role in AD (Zhao & Zhao 2013), the effects of epigenetic modification remain unclear. With the discovery of several oxidative derivatives of 5-methylcytosine (5-mC), the most common epigenetic mark to the genome, generated by the ten-eleven translocase (TET) family of enzymes, the field of epigenetics as it relates to neurodegeneration has advanced steadily.

5-mC is oxidized by TET to generate 5-hydroxymethylcytosine (5-hmC), which is further oxidized by TET to form 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (Tahiliani *et al.* 2009). These derivatives are thought to play a role in regulating gene transcription, although their exact function remains unclear. Methylation of cytosine can deregulate transcription (Nabel *et al.* 2012), but hydroxymethylation modifications show varied effects on gene expression. While 5-hmC modifications of introns and exons are generally thought to provide an increase in gene expression, the presence of this epigenetic mark within promoters has shown conflicting results (Nabel et al. 2012). Boundary locations of modification can alter gene expression as well as individual marks of 5-hmC at key genomic locations (transcription start and stop sites, enhancer regions, etc.) that could potentially be more important than overall distribution of modifications within genomic regions (Wen *et al.* 2014, Wen & Tang 2014). Further research is needed to elucidate how locations of 5-hmC modifications affect gene expression.

Whether epigenetic marks are altered in AD compared to normal aging is controversial, as global levels of 5-mC and 5-hmC have been reported with seemingly conflicting results, possibly attributed to variations in quantitative/semi-quantitative analytical techniques (Bradley-Whitman & Lovell 2013, Chouliaras *et al.* 2013, Condliffe *et al.* 2014, Coppieters *et al.* 2014, Lashley *et al.* 2014, Mastroeni 2016). Recently, our lab analyzed DNA of brain tissue specimens from cognitively normal and demented subjects using gas chromatography/mass spectrometry, and showed altered global levels of 5-mC and 5-hmC, indicating epigenetic modifications to cytosine may play a role in early stages of AD, including preclinical AD and mild cognitive impairment, in brain regions susceptible to plaque and tangle formation (Ellison *et al.* 2017).

While measurement of bulk levels of epigenetic modification provides valuable insight into regions of the brain susceptible to epigenetic changes throughout AD, determination of loci-specific levels of modification throughout the genome could enhance our understanding of how these epigenetic markers affect specific gene targets, as well as gene transcription changes and essential biological pathways and processes deregulated in AD. Genome-wide methylation mapping using bisulfite conversion has been used to analyze differentially methylated regions throughout the genome, providing gene targets relevant to AD progression that may be epigenetically regulated (Bakulski *et al.* 2012) (De Jager *et al.* 2014)

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(Lunnon *et al.* 2014). Although bisulfite conversion has been the technique of choice to study genome-wide methylation of cytosine in the past, this technique is unable to distinguish between 5-mC and 5- hmC (Huang *et al.* 2010). Recently, three sequencing techniques, oxBS-seq (oxidative bisulfite sequencing), TAB-seq (TET-assisted bisulfite sequencing), and reduced representation hydroxymethylation profiling (RRHP), have advanced the field by specifically quantifying 5- hmC throughout the genome (Booth *et al.* 2012) (Petterson *et al.* 2014) (Schuler & Miller 2012). RRHP is suggested to be the most sensitive technique, utilizing specific restriction endonuclease enzymes to detect 5-hmC modifications throughout the genome without the use of harsh chemical labeling techniques (Petterson et al. 2014).

As a follow up to our global study of altered 5-mC and 5-hmC levels throughout the progression of AD (Ellison et al. 2017), our lab sought to analyze loci-specific levels of 5-hmC modifications in DNA isolated from the hippocampus/parahippocampal gyrus (HPG) of late onset AD subjects compared to NC. In this study, we hypothesize that biological pathways with altered hydroxymethylomes relevant to AD will be determined, providing future gene targets to study 5-hmC modification and the potential role these modifications play in AD. Using an in- house RRHP procedure by Zymo Research Corporation (CA, US), millions of sites of potential hydroxymethylation were identified. To find meaningful statistical relationships between counts of 5-hmC between the subject groups, a negative binomial distribution analysis was used. Biological relevance to pathways with altered 5-hmC modifications were analyzed using the Cytoscape plugin app, Reactome Functional Interaction (FI) network. Pathways related to signaling, energy metabolism, gene expression, protein degradation, cell function, and cell structure and stabilization were significantly enriched in genes with altered 5-hmC levels according to our analysis, giving insight into the potential role epigenetic modifications to cytosine play in Alzheimer's disease and neurodegeneration.

## Materials and methods

### AD diagnosis

Tissue specimens were obtained from short postmortem interval (PMI) autopsies of three LOAD and two age-matched normal control (NC) subjects through the Neuropathology Core of the University of Kentucky Alzheimer's Disease Center (UK-ADC), in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and IRB approved protocols. All subjects in the UK-ADC are followed longitudinally, where annual neuropsychological testing and biannual physical examinations are completed (Schmitt *et al.* 2000). All AD subjects met the criteria for the clinical diagnosis of AD based on the AD and Consortium to Establish a Registry for Alzheimer's disease (CERAD) and the National Institute of Aging-Reagan Institute (NIA-RI) neuropathology criteria (McKhann *et al.* 1984, Mirra *et al.* 1991, Hyman & Trojanowski 1997). Postmortem scores of AD-related pathologic markers using Braak stage scoring and diffuse and neuritic plaques were determined as described previously (Braak & Braak 1995, Nelson *et al.* 2007). Cognitive function was determined based on the Mini Mental State Examination (MMSE) (Folstein *et al.* 1975) for AD and NC subjects. At autopsy, specimens of hippocampus/

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parahippocampal gyrus (HPG) were flash frozen in liquid nitrogen and stored at −80°C until processed for analysis.

## DNA isolation

DNA isolation was performed as previously described (Mecocci *et al.* 1993, Wang *et al.* 2006, Bradley-Whitman & Lovell 2013). Briefly, 250 mg of frozen tissue was digested using a 5 mg proteinase-K/10 mL digestion buffer (0.5% sodium dodecyl sulfate, 0.05 M Tris, 0.1 M EDTA, 0.1 M $CaCl_2$, pH 7.5) solution and centrifuged at 7,000 × *g* for 5 min. Nuclear DNA (nDNA) was extracted using 25:24:1 phenol: chloroform: isoamyl alcohol and was precipitated overnight at −20°C in 80 μL of 5 M NaCl and 1.5 mL cold ethanol solution. After three washes in 60% ethanol, the nDNA pellet was dried and resuspended in $ddH_2O$. Concentration and purity were measured by UV absorption analysis using a NanoDrop spectrophotometer (Thermo Fisher Scientific, NY, US).

## 5-hmC whole genome profiling

To analyze sites of 5-hmC modification at single base resolution, 1 μg of nDNA was submitted to Zymo Research Corporation (ZRC) (CA, US) for analysis by reduced representation hydroxymethylation profiling (RRHP), which utilizes specific restriction endonuclease enzymes to analyze CCGG sequences throughout the genome (Petterson et al. 2014). As described previously by Petterson *et al.* (Petterson et al. 2014), the specific restriction endonuclease MspI cuts between the CC bases in the CCGG sequence. If the internal cytosine (C<u>C</u>GG) is modified as 5-hmC, glucosylation of this site protects it from MspI cleavage and the epigenetic marker can be evaluated at each CCGG site. RRHP library construction and sequencing, as well as alignment to the human genome and count reads were performed by ZRC, resulting in more than 2 million sites of possible 5-hmC modification across the genomes of three LOAD and two NC subjects. Data files were downloaded from ZRC.

## Data analysis

Data *BAM* files from ZRC were converted to *BED* format using a bedtools PERL script *bamtobed* (Quinlan & Hall 2010), freely available from the Comprehensive Perl Archive Network (cpan.org). *BED* files were analyzed using an exact negative binomial test (Anders & Huber 2010) which utilizes a sliding window approach in diffReps v1.55.4 (Shen *et al.* 2013), a free PERL script initially designed for analysis of CHIP-Seq count data. In diffReps, the window size was set to 200 base pairs (bp) with a slide of 20 bp. Normalization of read counts within 200 bp windows was performed in diffReps, where raw read counts were linearly scaled by the geometric mean of all the reads in the specified window for each sample. Only windows that pass an initial uncorrected p value ($p < 1e^{-04}$) cutoff were retained. Windows that passed this cutoff were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini & Hochberg 1995). To annotate windows to the hg19 human genome, a free PYTHON script *regionanalysis.py*, a now standalone script initially written for diffReps, was used. All scripts were run using command line calling in Terminal v2.6.1 (Apple, Inc., Cupertino, CA, USA).

Due to the small sample size of subjects in this analysis, a conservative cutoff was used in which only windows with an adjusted FDR p value ($p \quad 1e^{-04}$) were retained for downstream analysis. Sites that aligned to promoter or genebody locations were filtered based on a published microarray of differentially expressed genes in the hippocampus of AD subjects (Blalock *et al.* 2004). This particular hippocampal AD microarray was chosen because the cohort came from the UK-ADC, where enrollment criteria and PMI were the same as the subjects in the current study. This list of 329 overlapping genes was generated for pathway enrichment analysis.

### Functional Interaction Network Analysis

Using the Reactome FI (RFI) plugin app available through Cytoscape3.0, a gene network of functionally related genes was created. With the use of linker genes provided by RFI, 322/329 genes with transcription and hydroxymethylation changes in the hippocampus of AD subjects compared to NC were used to create the network. The gene network was then clustered into six modules using spectral partition clustering (Newman 2006) and enrichment scores for module pathways were determined, excluding linker genes to prevent bias and only give pathways relevant to the 322 target genes. Statistical significance for pathway enrichment was determined in RFI using a binomial test (Huang da *et al.* 2009), while multiple comparisons were corrected for using a permutation test to give FDR p values (Vandin *et al.* 2011, Vandin *et al.* 2012). Only pathways with adjusted FDR ($p < 0.05$) were retained.

### Statistical Analysis

To determine significant differences between LOAD and NC demographic subject data, all normally distributed values were analyzed using Student's t test, while non-normally distributed data were analyzed using the nonparametric Mann-Whitney U test. All subject demographic significance tests were performed in SigmaPlot v.13 (Systat Software, Inc., San Jose, CA, USA).

## Results

As an initial study to determine differences in site-specific levels of 5-hmC in the HPG of LOAD subjects, the epigenome of three LOAD and two NC subjects were compared. No significant differences were found between groups in PMI or neurofibrillary tangle (NFT) burden in the CA1 using Student's *t* test ($p < 0.05$) (Table 1). The NFT burden in the subiculum (Sub) of AD subjects was significantly higher compared to NC ($p = 0.02$, *t*-test). Although epigenetic landscapes have been suggested to change with increasing age (Teschendorff *et al.* 2013, Issa 2014, Shah *et al.* 2014), the ages of LOAD and NC subjects were not significantly different, theoretically correcting for changes in epigenetic mechanisms associated with age. While there was a clear difference in the median Braak stage scores and MMSE between the LOAD and NC groups, the small number of subjects prevented a significant *p* value ($p$=0.2, Mann-Whitney U).

To analyze 5-hmC across the genome of LOAD subjects, Zymo Research Corporation's RRHP sequencing technique was utilized. By optimizing the relationship between specific



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restriction endonucleases and cut sites, 5-hmC can be mapped throughout the genome at the single base resolution. RRHP sequencing identified more than 2 million potential sites of 5-hmC modification at CCGG sequences throughout the genome from 3 LOAD and 2 NC subjects and read counts of 5-hmC at each site were determined by ZRC (Online Resource File 1). Aligned *BAM* files were downloaded from ZRC and analyzed using a sliding window approach to analyze count data across the genome. Due to the complexity of the data set, (i.e. genome wide counts of 5-hmC, likely over-dispersion within the data sets, and biological replicates for each group), a negative binomial distribution was applied.

The window size was set to 200 bp with a slide of 20 bp. The diffReps program uses a default initial statistical cutoff of $p < 1e^{-04}$, which included $3.29e^{08}$ windows in our analysis. To correct for multiple comparisons, FDR adjusted values were calculated, resulting in $1.65e^{06}$ windows, which were annotated to the hg19 genome. The statistical cutoff predefined in diffReps for the negative binomial test has an estimated FDR of 0.2%, however, due to the small sample size in our analysis, we chose a conservative adjusted significance p value ($p$ $1e^{-04}$) cutoff which included 43,112 windows (Online Resource File 2). Annotation of windows included Ensembl gene and transcript IDs, where single windows mapped to several splice variants for a single gene. After removing duplicated windows annotated to multiple transcript IDs for a single gene, 15,158 unique windows or differentially hydroxymethylated regions (DhmRs) remained. These regions were sorted into increased and decreased levels of 5-hmC in LOAD vs NC subjects (Figure 1). The majority of these DhmRs show increased levels of 5-hmC in the AD genome, 64% overall, mainly confined to promoter and genebody (including intron and exon regions) genomic regions. This overall increase in 5-hmC in AD in intragenic regions is consistent with previously reported genome wide studies of 5-mC/5-hmC in AD (Bernstein *et al.* 2016, Zhao *et al.* 2017).

Epigenetic modifications to cytosine are believed to be a mechanism for gene activation and regulation (Breiling & Lyko 2015). To analyze the 5-hmC modification sites relevant to AD gene expression changes, the DhmRs were sorted to only include sites within genes with altered gene expression in sporadic AD in the hippocampus according to a published microarray (Blalock et al. 2004). This narrowed our list to 329 genes with altered expression as well as altered levels of 5-hmC (Table 2).

To determine biologically relevant pathways in AD containing the 329 genes from our analysis, Reactome FI was used to create a gene network of likely interactions. The curated network, containing 322 target genes, was clustered into six modules and pathway enrichment analysis was performed on each module, excluding linker genes included in the network build by RFI (Figure 2). Only pathways with an adjusted FDR ($p < 0.05$) were retained. To simplify results, significantly enriched pathways within the modules relevant to AD and neurodegeneration are discussed below (for complete list of significantly enriched pathways and genes identified, see Online Resource Files 3a-b).

Enriched pathways highlighted in the current study for the signaling module include calcium signaling, Wnt signaling, synapse signaling, and long-term depression. In the energy metabolism module, the TCA cycle and electron transport, several neurodegenerative

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diseases, cGMP-PKG signaling, and axon guidance mechanisms show significant alterations in 5-hmC levels. The cell cycle module included significant enriched pathways related to BER, transcription pathways, notch signaling, and endocytosis. For the gene expression module, RNA polymerase and mitochondrial transcription, as well as processing of pre-mRNA pathways were significantly enriched. Additionally, protein degradation pathways, like ubiquitin mediated proteolysis and degradation of cell cycle proteins, as well as cell structure and stabilization pathways, like focal adhesion, extracellular matrix organization, actin cytoskeleton regulation, and NCAM signaling for neurite out-growth, show significant alterations in 5-hmC (Figure 2b).

Significantly enriched pathways with similar genes, including signaling pathways of four types of neurons (cholinergic, dopaminergic, glutamatergic, and serotonergic neurons) and three neurodegenerative diseases (Huntington's disease, Parkinson's disease, and Alzheimer's disease) were combined and the largest adjusted p value for the combined pathways was reported (denoted with ╪ in Figure 2). For specific genes within each enriched pathway, please refer to Online Resource File 3b.

## Discussion

While there have been great advances in our understanding of AD genes and pathways that lead to neuron dysfunction and degeneration, the mechanism of how sporadic AD begins still remains unanswered. To date, no cure or treatment plan has shown success in halting or slowing the aggressive and destructive progression of this disease. In the absence of genetic mutations to account for loss of function in the sporadic form of AD, environmental factors may hold the key to causal mechanisms. Epigenetic modifications in the disease could potentially be causal to the shift in gene transcription evident in AD progression.

Epigenetic modifications to cytosine are known to play a role in the regulation of transcription although the exact mechanism is not well understood. To filter our list of genes with significantly altered 5-hmC levels in the hippocampus of sporadic AD subjects, a previously published microarray of differentially expressed genes in mild, moderate, and severe cases of sporadic AD compared to age-matched NC subjects was used (Blalock et al. 2004). The resulting list of 329 genes with AD related transcriptional and epigenetic alterations was then subjected to network analysis software to find meaningful biologically relevant pathways related to AD and neurodegeneration. Reactome FI (RFI), a Cytoscape (Shannon *et al.* 2003) plugin app, combines gene ontology and biological pathways curated from online sources including CellMap (Krogan *et al.* 2015), Reactome (Milacic *et al.* 2012, Croft *et al.* 2014), KEGG pathways (Kanehisa & Goto 2000, Kanehisa *et al.* 2016, Kanehisa *et al.* 2017), NCI PID (Schaefer *et al.* 2009), Panther (Thomas *et al.* 2003), and BioCarta (Nishimura 2004). From our list of target genes, the curated network, including 322 genes, was clustered into modules containing genes related to signaling, energy metabolism, cell function processes, gene expression, protein degradation, and cell structure and stabilization (Figure 2). The relevance of significantly enriched pathways within each module to AD, as well as specific genes within them, are discussed below.

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Several genes identified within significantly enriched pathways in the signaling and cell cycle modules of our network gene ontology analysis have also been implicated as genes that play a role in the pathophysiology of sporadic AD, including complement receptor 1 (*CR1*), bridging integrator 1 (*BIN1*), and clusterin (*CLU*). These genes have continuously appeared as target genes in genome-wide association studies of AD subjects (Lambert *et al.* 2009, Seshadri *et al.* 2010) as well as genome-wide methylation mapping studies (De Jager et al. 2014, Yu *et al.* 2015, Watson *et al.* 2016) for their roles in endocytosis, immune function, lipid processing, and clearance mechanisms (Tan *et al.* 2013). While not as strongly associated with AD genome-wide associate studies as the aforementioned genes, *DLGAP1*, a postsynaptic density regulator, is a suggested gene with AD relevant SNPs and likely plays a role in sporadic AD (Bertram *et al.* 2008).

Deregulation of cellular signaling, particularly at the synapse, is a common feature in neurodegeneration and neuropsychological disorders. Our network analysis showed numerous pathways within the signaling module with altered regions of 5-hmC and expression in the hippocampus. Genes encoding for G proteins (eg. *GNAO1, GNA14, GNAL, GNAS, GNAZ, GNB1*), as well as phospholipase C (eg. *PLCB1, PLCE1, PLCG2*), and adenylate cyclase (eg. *ADCY2, ADCY3, ADCY7, ADCY9*) proteins within synapse and calcium signaling cascades regulate gene expression, exocytosis, and long-term depression within neurons according to curated KEGG pathways. Synaptic signaling pathways specific to cholinergic, serotonergic, dopaminergic, and glutamatergic neurons show altered levels of 5-hmC in our study and have been gene targets in neurodegeneration and neuropsychological disorder studies. Protein levels of *SHANK2* (SH3 and Multiple Ankyrin Repeat Domains 2) were elevated at the postsynaptic density of AD subjects where glutamate receptor dysfunction leads to destruction of the Shank- postsynaptic platform likely contributing to synapse loss in AD (Gong *et al.* 2009). Epigenetic dysregulation of *HTR2A*, 5-Hydroxytryptamine Receptor 2A, occurs in brain regions of early onset schizophrenia and bipolar disorder patients (Abdolmaleky *et al.* 2011), and loss of *HTR2A* receptors in AD brain correlates to rate of cognitive decline (Lai *et al.* 2005). Genes within these signaling pathways also regulate sodium (*GRIA3*), calcium (*CACNA1*), and potassium (eg. *KCNJ14, KCNQ2*) ion channels, including *KCNA6*, a potassium channel gene thought to play a role in insulin secretion and neurotransmitter release (Etcheberrigaray *et al.* 1994). *KCNA6* was also identified as a "hub gene" in a recent 5-hmC mapping study of prefrontal cortex AD brain, associating hydroxymethylation and neuropathological burden (Zhao et al. 2017).

Wnt signaling plays a critical role in many cellular processes including adhesion, survival, proliferation, differentiation, and apoptotic pathways (Clevers & Nusse 2012, Nusse 2012). Many genes within Wnt signaling pathways identified in our analysis have been implicated in AD. Genetic variations in *LRP6* (LDL Receptor Related Protein 6) have been shown to lead to AD progression (De Ferrari *et al.* 2007). One of the susceptibility genes identified for late onset AD, *CLU*, regulates β-amyloid toxicity in the non-canonical Wnt/PCP pathway known to contribute to tau phosphorylation and cognitive decline (Killick *et al.* 2014). A recent study of an APP/PS1 transgenic mouse model of AD showed downregulation of *WNT7B*, involved in synapse organization according to gene ontology analysis (Orre *et al.* 2014a), as well as altered expression of *WNT7B* in hippocampal tissue of AD subjects

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(Riise *et al.* 2015). Several studies show that alterations in Wnt signaling pathways are involved in the regulation of synaptic development and likely play a role in the progression of AD (Vargas *et al.* 2014).

Deregulation of energy metabolism processing and mitochondrial function is a major hallmark across neurodegenerative diseases, including Huntington's disease, Parkinson's disease, and AD (Godoy *et al.* 2014). Key mechanisms in the production of energy needed for healthy cellular function include the TCA cycle, electron transport chain processes, and the regulation of insulin signaling processes. Genes involved in regulating the TCA cycle (eg. *OGDH*) show decreased expression in AD brain and in AD mouse models (Bubber *et al.* 2005, Ciavardelli *et al.* 2010). Genes regulating mitochondrial complexes I-V, i.e. the electron transport chain, including NADH dehydrogenases (eg. *NDUFA3, NDUFA10, NDUFB3*) ATP synthase (eg. *ATP5J, ATP5B*) succinate dehydrogenase (*SDHD*), and ubiquinol-cytochrome c reductase (*UQCRC2*), also show transcription alterations across neurological dysfunction disorders (Liang *et al.* 2008, Ferreira *et al.* 2010, Zhang *et al.* 2015). Genes relevant to insulin processing mechanisms (eg *INSR*, *IRS2*) necessary for learning and synaptic plasticity (Horwood *et al.* 2006) also modulate neurotransmission in glutamate receptors and recruitment of GABA receptors to postsynaptic sites (Wan *et al.* 1997). While there is no definitive link between diabetes and AD, overlapping pathways regulated by insulin suggest a possible role in altered energy metabolism and neurodegeneration (Akter *et al.* 2011). The deregulation of energy metabolism pathways plays key roles in neuron stabilization and axon guidance and development. Plexins and semaphorins, a family of axon guidance cues, have also been shown to facilitate downstream pathways leading to phosphorylation of tau, ultimately causing microtubule destabilization (Uchida *et al.* 2005). In a gliomal brain cancer model, *PLXNB2* was shown to be an activator of Rho-GTPase pathways, potentially causing downstream affects in dendrite formation and maintenance (Le *et al.* 2015), while genes in the SEMA3 family of proteins (eg. *SEMA3B*, *SEMA3F*) have been shown to facilitate attractive and repulsive axon guidance mechanisms (Falk *et al.* 2005). Axon initial segments require *ANK3* (ankryin 3 or ankryin G), a scaffolding protein, to form and are disrupted by Aβ plaques in a mouse model of AD, reducing the length of axon segments and overall neuron excitability (Marin *et al.* 2016).

Alterations in cell function pathways are key features of many diseases, including cancer and neurodegeneration (Klein & Ackerman 2003, Maddika *et al.* 2007). These pathways include base excision repair mechanisms, endocytosis pathways, as well as NOTCH signaling, transcription and gene expression. Oxidative damage to DNA is an established feature of neurodegeneration throughout the progression of AD (Markesbery 1997, Lovell & Markesbery 2007b, Lovell & Markesbery 2007a), and genes involved in base excision repair (BER) mechanisms appear to play a role in epigenetic modifications to cytosine (Weber *et al.* 2016). In our analysis, BER genes (*OGG1*, *TDG*, and *UNG)* show altered levels of 5-hmC in the hippocampus and show decreased expression throughout the progression of AD (Weissman *et al.* 2007, Lovell *et al.* 2011). Genes within endocytosis mechanisms, including *FGFR3* and *TGFB1*, show altered levels of expression in AD mouse models (Salins *et al.* 2008, Orre *et al.* 2014b), and *TGFB1* levels were increased in CSF of probable AD patients (Rota *et al.* 2006). *BIN1*, a recently discovered risk factor gene for sporadic AD, not only

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regulates endocytosis and other cell function mechanisms, but also may aid in tau pathology development in AD (Tan et al. 2013). Signaling cascades involving the NOTCH family of proteins, regulators of presenilin pathways, and genes with NOTCH signaling pathways, likely play significant roles in amyloid production and plaque formation. AD associated SNPs in *NOTCH4* were discovered in a northern Israeli population study, a population with an abnormally high prevalence of sporadic AD (Sherva *et al.* 2011). *NCOR2*, a transcriptional regulatory hub gene, affects memory formation in AD brain (Acquaah-Mensah & Taylor 2016), while *TCF3* showed increased expression in the hippocampus of AD subjects, along with several integral Wnt signaling genes (Riise et al. 2015). Dysfunction in transcriptional regulatory mechanisms, including regulator genes *CREBBP* and *NR2F6*, lead to neuronal apoptosis (Rouaux *et al.* 2003) and deficits in early memory and learning in an AD mouse model (Kummer *et al.* 2014). Gene expression mechanisms involving RNA polymerase also regulate splicing pathways (eg. *PTBP1, PRPF8, CDC5L*), and when deregulated, these genes can alter neuron differentiation pathways, repress pre-mRNA splicing mechanisms, and deregulate cell proliferation (Makeyev *et al.* 2007, Kurtovic-Kozaric *et al.* 2015). *CDK7*, a regulator of cell cycle pathways and a member of a transcription factor complex that activates RNA Polymerase II, is upregulated in the hippocampus of AD subjects (Zhu *et al.* 2000).

Protein degradation and clearance mechanisms are essential to healthy cellular function, while the disruption of proper clearance and degradation pathways, like the ubiquitin-proteasome system, lead to the accumulation of intracellular aggregate-prone proteins ultimately causing cell loss and neurodegeneration (Popovic *et al.* 2014, Zheng *et al.* 2016). Not only in degradation pathways, but the ubiquitination of proteins is also essential for endocytosis, trafficking, signaling, and DNA repair mechanisms (Grabbe *et al.* 2011, Kulathu & Komander 2012, Rieser *et al.* 2013). *PSMA1*, a proteasome protein, showed increased levels in the serum of mild cognitive impairment and probable AD vs cognitively normal subjects (Kang *et al.* 2016). Genes that regulate ubiquitination mechanisms are also involved in Wnt-signaling including *BTRC* and *FZR1*. *BTRC* regulates phosphorylated β-catenin (Hart *et al.* 1999), a Wnt-pathway gene involved in regulating gene transcription, where increased levels of phosphorylated β-catenin lead to proteasome dysfunction (Ghanevati & Miller 2005). While ubiquitin mediated protein degradation appears to play a significant role in the progression of neurodegeneration and in several pathways critical to healthy cellular function, the exact mechanism is not well understood (Zheng et al. 2016).

The maintenance and regulation of pathways involved in cell structure and stabilization play important roles in neuronal function and healthy synapse formation. Focal adhesions are structures that form between membrane receptors and the actin cytoskeleton, signaling molecules that result in the reorganization of the actin cytoskeleton, and can regulate gene expression via growth factor mediated signaling (Iskratsch *et al.* 2014). Focal adhesion genes, including integrin receptors (eg. *ITGB4, ITGB5, ITGB8*) and collagen proteins (eg. *COL4A1, COL4A3, COL6A2*) regulate extracellular matrix organization mechanisms and lie upstream of actin polymerization and Wnt-signaling pathways. The proper regulation of actin is essential for healthy cell homeostasis. The phosphorylation of LIM domain kinase (eg. *LIMK2*) leads to phosphorylation of cofilin, an actin severing protein, rendering it inactive, and allowing for actin cytoskeleton reorganization (Sumi *et al.* 1999). Cofilin is

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activated by the dephosphorylation of slingshot, which is activated by the phosphorylation by p21 activated protein kinase 4 (*PAK4*) (Soosairajah *et al.* 2005). Activation of cofilin leads to actin filament turnover, while the inactivation of cofilin leads to actin cytoskeleton reorganization. Current studies show that cofilin is deregulated in AD (Barone *et al.* 2014). *NRXN1*, a presynaptic ligand linked to autism, is thought to be a component of the synaptic regulatory pathway, where dysregulation of neurexins and neuroligins can lead to alterations in dendritic spine morphology (Sudhof 2008, Penzes *et al.* 2011).

The precise role of epigenetic modifications to cytosine in neurodegeneration remains to be determined, however it is clear that levels of 5-mC and 5-hmC are altered in AD and other neurodegenerative diseases on global and loci-specific scales (Sanchez-Mut *et al.* 2016, Ellison et al. 2017). While this initial study of loci-specific levels of 5-hmC in the hippocampus of LOAD subjects compared to NC was limited by sample size, to obtain a better understanding of changes to epigenetic marks in AD, larger sample sizes should be evaluated. Advances in loci- specific techniques to study whole epigenomes have increased greatly in the past several years, however the cost of this new technology is high, limiting the number of subjects per analysis. Although samples sizes were small, two previously published studies of differentially hydroxymethylated regions of the AD genome in prefrontal cortex tissue specimens (Bernstein et al. 2016, Zhao et al. 2017) showed several overlapping genes with altered hydroxymethylation in our gene list. One gene common to all three genome-wide 5-hmC studies in AD brain, myelin transcription factor 1-like (*MYT1L*), was used to produce induced neuronal cells and has been described as a neuronal fate inducing factor (Vierbuchen *et al.* 2010), however the function of *MYT1L* in AD brain has yet to be determined. The overlap of genes within these studies, despite variation in brain regions analyzed (hippocampus and prefrontal cortex) and discrepancy in sequencing techniques (5-hmC single base resolution mapping and 5-hmC enrichment analysis), strengthens the validity of this initial study of genes within biologically relevant pathways that show alterations in 5-hmC levels as well as alteration in gene expression in the hippocampus of LOAD subjects.

It should also be noted that genes with DhmRs in this analysis represent a mixture of cell types, including neuronal and glial cells. While 5-hmC levels are cell-type specific (Nestor *et al.* 2012), the current study lacks the specificity to identify whether 5-hmC alterations in AD are representative of glia, neurons, or a combination of both. Significant changes to levels of 5-mC and 5-hmC have been shown in both neuronal and glial cells in previous AD studies (Mastroeni *et al.* 2010, Chouliaras et al. 2013), however further research is needed to clarify the specific roles both neuronal and glial cytosine modifications play in AD.

Finally, the gender distribution of the subjects, including one male subject in the LOAD subject pool, should be considered. Exclusion of this subject, resulting in two female LOAD and two female NC subjects, gave 231 of the original 329 genes using the conservative FDR cutoff ($p$ $1e^{-04}$). Enrichment analysis using RFI gave similar results, including significant enrichment (FDR, $p < 0.05$) in pathways involved in signaling, energy metabolism, cell function, gene expression, degradation, and cell structure and stabilization (Online Resource File 4). However, in future studies, similar distributions in gender should be analyzed to

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prevent gender bias and achieve a more complete picture of how epigenetic modifications affect gene expression in AD.

## Conclusion

It remains unclear if epigenetic modifications are upstream or downstream of neurodegeneration associated with the progression of AD, but it is highly likely that epigenetics play a role in the disease. Whether these changes instigate pathology or are an attempt to reestablish homeostasis still remains to be determined. To analyze the exact role, changes in early stages of AD may give clearer insights into the mechanism of dysfunction rather than levels of markers in the final stage of the disease. However, suggested pathways that seem to be deregulated in late stage AD, both changes to transcriptional states as well as epigenetic modifications, could give insight into mechanisms that are altered in AD, providing possible therapeutic targets to study earlier in disease progression.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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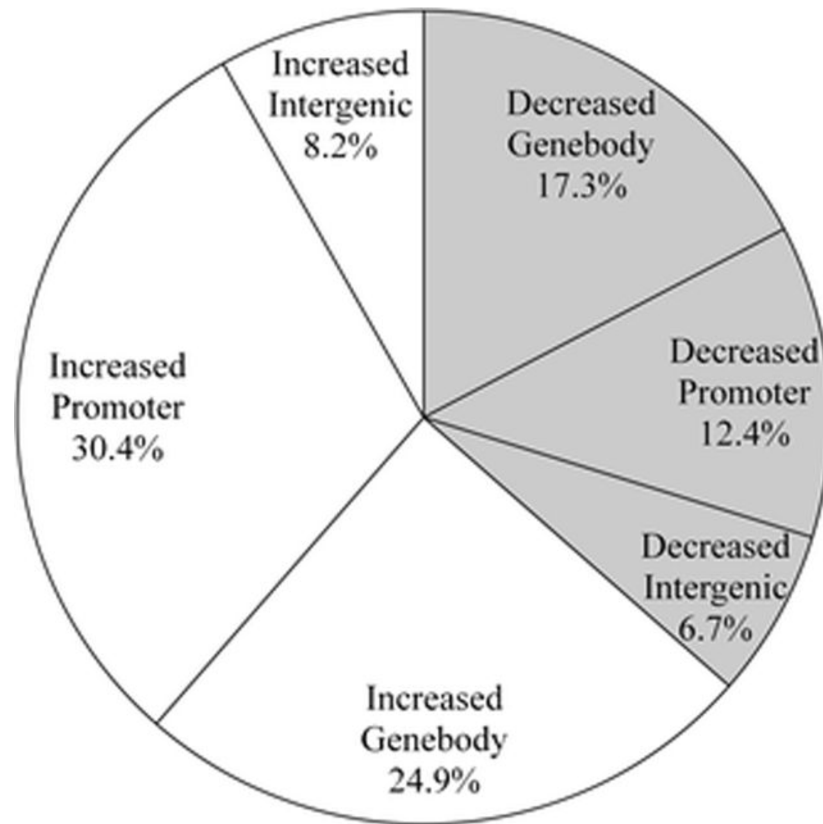
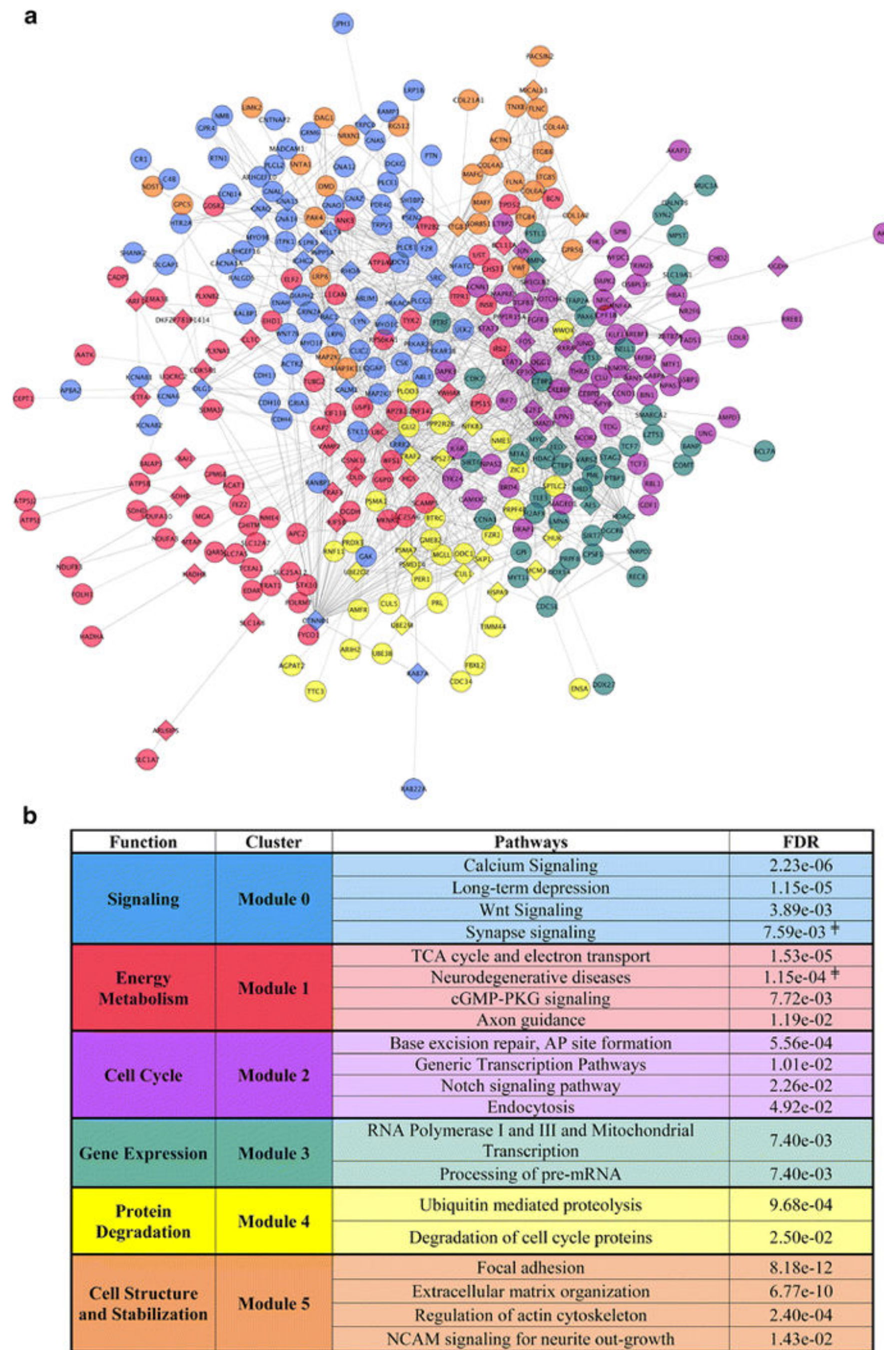



**Figure 1. 5-hmC distribution**

Overall distribution of 15,158 DhmRs in intragenic and intergenic genomic locations. Intragenic locations, including promoter and genebody (exons and introns) regions, show the majority of DhmRs. Distribution of genomic regions for increased (white) and decreased (gray) DhmRs passing FDR cutoff ($p$ $1e^{-04}$) are labeled.



| Function | Cluster | Pathways | FDR |
|---|---|---|---|
| Signaling | Module 0 | Calcium Signaling | 2.23e-06 |
| | | Long-term depression | 1.15e-05 |
| | | Wnt Signaling | 3.89e-03 |
| | | Synapse signaling | 7.59e-03 ‡ |
| Energy Metabolism | Module 1 | TCA cycle and electron transport | 1.53e-05 |
| | | Neurodegenerative diseases | 1.15e-04 ‡ |
| | | cGMP-PKG signaling | 7.72e-03 |
| | | Axon guidance | 1.19e-02 |
| Cell Cycle | Module 2 | Base excision repair, AP site formation | 5.56e-04 |
| | | Generic Transcription Pathways | 1.01e-02 |
| | | Notch signaling pathway | 2.26e-02 |
| | | Endocytosis | 4.92e-02 |
| Gene Expression | Module 3 | RNA Polymerase I and III and Mitochondrial Transcription | 7.40e-03 |
| | | Processing of pre-mRNA | 7.40e-03 |
| Protein Degradation | Module 4 | Ubiquitin mediated proteolysis | 9.68e-04 |
| | | Degradation of cell cycle proteins | 2.50e-02 |
| Cell Structure and Stabilization | Module 5 | Focal adhesion | 8.18e-12 |
| | | Extracellular matrix organization | 6.77e-10 |
| | | Regulation of actin cytoskeleton | 2.40e-04 |
| | | NCAM signaling for neurite out-growth | 1.43e-02 |

**Figure 2. Functional Interactive network**

Using Reactome FI, a gene network was created using 322 genes with significant alterations in 5-hmC. Linker genes are shown as diamonds, while the 322 target genes are depicted with circles (a). The network was clustered into six modules and pathway enrichment analysis was performed. To correct for multiple comparisons, false discovery rate p values were calculated ($p < 0.05$). Significantly enriched pathways within each module are listed with p values (b). Like terms were combined (*Neurodegenerative disease*: Huntington disease,

Parkinson's disease, and AD; *Synapse signaling*: dopaminergic, serotoninergic, cholinergic, and glutamatergic synapse signaling) and the least significant FDR was reported (╪).

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**Table 1**

Demographic data of LOAD and NC subjects.

| Demographics | NC | LOAD |
|---|---|---|
| Gender | 2F | 2F/1M |
| Age (y) | 84.5 ± 0.7 | 85.3 ± 4.0 |
| PMI (h) | 2.5 | 3.0 ± 0.7 |
| MMSE | 29 | 5 |
| Braak Stage | 0 | VI |
| NFT (CA1) | 0 | 66.5 ± 38.9 |
| NFT (Sub) | 0 | 109.5 ± 30.6 * |

Age, postmortem interval (PMI), Braak stage score, MMSE and neurofibrillary tangle (NFT) burden were compared. NFT burden values were determined for CA1 and subiculum (Sub) HPG regions. Statistical significance was determined using Student's *t*-test or Mann-Whitney U (MWU) analysis

* $p < 0.05$.

Age, PMI, and NFT counts are given as mean ± SD, while MMSE and Braak Stage are median scores for each subject group.

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## Table 2

Modules and genes from Reactome FI network analysis.

| RFI Network Module | Gene List |
|---|---|
| Module 0: Signaling | *ABL1, ABLIM1, ACTR2, ADCY2, APBA2, ARHGEF10, ARHGEF16, C4B, CACNA1A, CDH10, CDH13, CDH4, CLIC2, CNTNAP2, CR1, CSK, DGKG, DIAPH2, DLGAP1, ENAH, F2R, GAK, GNA12, GNA14, GNAL, GNAO1, GNAS, GNAZ, GPR4, GRIA3, GRIN2A, GRM6, HTR2A, IQGAP1, ITPK1, JPH3, KCNA6, KCNAB1, KCNAB2, KCNJ14, LRP1B, LRP6, MADCAM1, MAP2K3, MYO1C, MYO1F, MYO9B, NFATC1, NMB, PDE4C, PLCB1, PLCE1, PLCG2, PLCL2, PRKAR1B, PRKAR2B, PTN, RAB22A, RALBP1, RALGDS, RAMP1, RANBP3, RTN1, SH3BP2, SHANK2, STK11, TRPV1, ULK2, WNT7B* |
| Module 1: Energy Metabolism | *AATK, ACAT1, ANK3, AP2B1, APC2, ATP1A2, ATP2B2, ATP5B, ATP5J, ATP5J2, BAIAP3, BGN, CADPS, CAP2, CEPT1, CHST3, CSNK1E, EDAR, EHD1, ELF2, EPS15, FEZ2, FOLH1, FRAT1, FYCO1, G6PD, GHITM, GOSR2, GPM6B, HADHA, INSR, IRS2, ITPR1, KIF13B, L1CAM, MGA, MKNK2, NDUFA10, NDUFA3, NDUFB3, NME4, OGDH, PLXNA1, PLXNB2, POLRMT, QARS, RPS6KA1, SCAMP3, SDHD, SEMA3B, SEMA3F, SLC12A7, SLC1A7, SLC25A12, SLC25A6, SLC7A5, STK10, TCEAL1, TPD52, TUBG2, TYK2, UQCRC2, USP3, UST, WFS1, ZNF142* |
| Module 2: Cell Function | *AK5, AKAP12, AMPD3, BIN1, BRD4, CAMKK2, CCND3, CEBPD, CHD2, CLU, CPT1B, CREBBP, DAPK2, DAPK3, DRAP1, FADS1, FGFR3, GDF1, HBA1, IL6R, IRF7, JUND, KCNN3, KLF1, LDLR, LPIN1, LTBP2, MAGED1, MAPRE3, MTF1, NCOR2, NFIC, NOTCH4, NPAS2, NPAS3, NR2F6, OGG1, OSBPL10, PKNOX2, PPP1R15A, RBL1, RREB1, SH3GLB2, SPIB, SREBF1, SREBF2, SSBP1, STK24, TCF3, TDG, TGFB1, THRA, TRIM26, UNG, WFDC1* |
| Module 3: Gene Expression | *BANP, BCL7A, CCNA1, CDC5L, CDK7, COMT, CPSF1, CTBP1, CTBP2, DDX27, DGCR6, FSTL1, GPI, H2AFX, HDAC4, LMNA, LZTS1, MBD3, MPST, MTA1, MUC3A, MYT1L, NELL1, PAX6, PML, PRPF8, PTBP1, PTRF, REC8, SIRT6, SIRT7, SLC19A1, SMARCA2, SNRPD2, STAG2, SYN2, TCF7, TFAP2A, TLE3, VARS2* |
| Module 4: Protein degradation | *AGPAT2, AMFR, ARIH2, BTRC, CDC34, CUL5, ENSA, FBXL2, FZR1, GLI2, GMEB2, MGLL, NME3, ODC1, PER1, PLOD3, PPP2R2B, PRDX3, PRL, PRPF4B, PSMA1, PSMD14, RNF11, SPTLC2, TIMM44, TTC3, UBE3B, WWOX, ZIC1* |
| Module 5: Cell Structure and Stabilization | *ACTN1, COL21A1, COL4A1, COL4A3, COL6A2, DAG1, DMD, FLNA, FLNC, GPC5, GPR56, ITGB4, ITGB5, ITGB8, LIMK2, LRP8, MAFF, MAFG, MAP2K7, MAP3K11, NDST1, NRXN1, PACSIN2, PAK4, RGS12, SNTA1, SORBS1, TNXB, VWF* |

Genes used to build RFI network included 322 out of the 329 genes with altered 5-hmC levels according to our analysis, as well as differentially expressed in the HPG of AD subjects (Blalock et al, 2004). Pathway enrichment analysis was performed on each module containing the genes above.