UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of DNA Methylation in Mediating the Microglial Inflammatory Response in Alzheimer's Disease

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Aaron Creswell

Committee in charge:

Professor Paula Desplats, Chair
Professor Susan Ackerman, Co-chair
Professor Keefe Reuther

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The thesis of Aaron Creswell is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I'd first like to thank Dr. Paula Desplats for granting me with this extraordinary research opportunity, and for the selfless and inspiring mentorship she has provided throughout this process.

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LIST OF ABBREVIATIONS

5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
DNMT	DNA methyltransferase
TET	Ten-eleven Translocation Methylcytosine Deoxygenase
LPS	Lipopolysaccharide
Aβ	Amyloid-beta
AD	Alzheimer's Disease
RRBS	Reduced Representation Bisulfate Sequencing
RRHP	Reduced Representation Hydroxymethylcytosine Profiling
DM	Differentially Methylated
DEG	Differentially Expressed Gene
DAM	Disease-Activated Microglia
ROS	Reactive Oxygen Species
GO	Gene Ontology
CRH	Corticotropin Releasing Hormone
LTP/LTD	Long Term Potentiation/Depression
IHC	Immunohistochemistry
FDR	False Discovery Rate

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ABSTRACT OF THE THESIS

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Microglia, the brain's resident macrophage, display a diverse array of phenotypes in the adult brain. Alzheimer's Disease (AD) is associated with a unique subtype of these cells which transmit excessive inflammatory signals resulting in both synapse and cell loss. To investigate the role of DNA methylation in driving these responses, I profiled genome-wide methylation levels with single-nucleotide resolution in human microglia following *in vitro* exposure to either lipopolysaccharide or amyloid-beta. I

defined specific changes in the methylation landscape and established an important role for both 5-methylcytosine and 5-hydroxymethylcytosine in directing inflammatory and phagocytic processes in response to different stimuli, thus implicating differential methylation as a possible mechanism by which microglia convert to an AD-associated neurotoxic phenotype. Furthermore, I provide preliminary evidence that DNA methylation changes are integral to mounting an inflammatory response *in vivo*.

Introduction

Alzheimer's Disease

Alzheimer's Disease (AD), first described in 1906 by Dr. Alois Alzheimer, has long been known as the leading cause of dementia across the globe. As of a census conducted in 2010, there were 4.7 million Americans over the age of 65 suffering from this disease [1], however this number is growing rapidly. It is estimated that 5.7 million Americans of all ages had AD in 2016, and by 2050 around 13.8 million Americans will be diagnosed, in part due to the large baby boomer generation reaching the higher risk ages of over 65 years [2]. Not only does this disease have high prevalence and a rapidly increasing incidence, but the symptoms are also exceedingly draining for those responsible for patient care, both financially and emotionally. In addition to the prototypical memory impairment, AD will often cause a general cognitive decline affecting functions such as language, judgement, problem solving skills, and mood or personality. While symptoms vary widely between individuals, patients may withdraw from work or social activities, become confused, agitated, anxious, apathetic or depressed, and could have increasing difficulties completing simple familiar tasks. In later stages, many sufferers of AD cannot complete simple tasks like eating or bathing, and could even become bed-ridden with limited motor function [2]. This set of symptoms can be tragic to observe, as the patient is stripped of their defining traits and may no longer remember the faces of their loved ones. The combination of agitation and impaired reasoning also demands many hours of care; in 2015, family members and other unpaid caregivers provided a combined 18 billion hours of care for patients of AD and other dementias, a commodity estimated to be valued at over $200 billion. In 2016, the total payments for health care and caretakers for sufferers of dementia over the age of 65 was over $230 billion. Much of this care is not covered by Medicare or other insurances, which places a massive financial burden on the patient's families [2].

Molecular Pathology of AD

1. Amyloid

There are two major pathological hallmarks that define AD: the oligomerization of amyloid beta (Aβ) which forms extracellular plaques, and the accumulation of hyper-phosphorylated Tau protein which creates intracellular neurofibrillary tangles (NFTs). Despite $3 billion of annual federal funding [3], however, it is hitherto unresolved how these aberrant protein chemistries are related, thus generating two general schools of thought in AD research: the amyloid hypothesis and the tau hypothesis. As both are supported by convincing evidence, the merits of both will be discussed here, beginning with that of the amyloid hypothesis. The idea that amyloid pathology plays a causative role in disease progression was first documented by Glenner and Wong in 1984. With the intent of developing a diagnostic biomarker, they were able to isolate amyloid fibrils from the brains of human patients and discovered a uniquely folded subset of amyloid present only in AD patients: Aβ. They also reasoned that the previously established link between Down's Syndrome and AD was likely due to the extra copy (and therefore overproduction) of the Amyloid Precursor Protein (APP) gene, from which Aβ is cleaved [4]. This concept that AD results from a buildup of Aβ is further supported by other genetic mutations later linked to the onset of AD. Sequence alterations in the genes encoding Presenilin1 and Presenilin 2 (PSEN1 and PSEN2) are now known to be major risk factors for inherited AD, and these proteins are directly responsible for the enzymatic production of Aβ from APP [5-6]. In addition to these high-risk genotypes, there is also a neuroprotective allele of APP. Donned the "Icelandic" allele due to the geographic origin of its holders, this allele was found to reduce the production of Aβ by 40% *in vitro* and consequently diminish the incidence of AD in this population [7]. These findings began to suggest that changes in amyloid processing, either the overproduction of Aβ or impairments in its clearance, are directly responsible for the development of symptoms, at least in those cases with a clearly defined genetic basis. But how does an accumulation of Aβ contribute to neurodegeneration? While the presence of Aβ plaques is required for a formal diagnosis, it is now more widely acknowledged that the formation of these hallmarks correlates poorly with the onset and severity of symptoms. These conglomerates are comprised

of oligomers of various sizes, which can be either soluble or insoluble in aqueous solutions. Soluble oligomers of smaller size have in fact been shown to be more bioactive than their larger counterparts [8], and are more robust indicators of disease severity [9]. Because these proteins accumulate extracellularly, it is generally thought that they bind to cell surface receptors to facilitate their effects. Increases in soluble fraction levels can directly weaken synapses [10-11] and are highly associated with synapse loss [12], which itself has been identified as the primary correlate of AD-related cognitive impairment [13]. Soluble oligomers have also been shown to impair the glutamate-reuptake machinery in excitatory synapses [14] and in astrocytes specifically [15]. This impairment contributes to excitotoxicity: a phenomena whereby excessive synaptic glutamate produces a sustained activation of its receptors, causing ion imbalance, mitochondrial disfunction, and eventually cell death. In addition to perturbing neuronal communication, soluble amyloid has been associated with oxidative damage [16-17]. Oxidative stress occurs when mitochondrial processes are disturbed and oxygen is converted to reactive oxygen species (ROS), or a free electron may be released. Disruptions in the cellular mechanisms for rectifying these errors may also contribute to the accumulation of these byproducts, which are extremely reactive and neurotoxic, able to damage DNA, protein, or lipids. Not only can aqueous Aβ generate free radicals even without the presence of cells [18], it can, through various mechanisms that are not yet fully resolved, facilitate other pro-oxidative effects which were found to reduce neuronal projections and increase cell loss [19-20]. Aβ has also been shown to disrupt insulin signaling, earning AD the label of 'type-3 diabetes'. Soluble oligomers have been shown to reduce the cell-surface expression of insulin receptors [21], and can disrupt many related intracellular processes [22-23] which may further contribute to memory impairments. Lastly, Aβ fibrils and oligomers are known to activate glial cells and induce chronic neuroinflammation, which will be discussed below in much greater detail.

2. Tau

Despite the wealth of evidence which frames Aβ as a key driver of AD pathology, there are also some findings which question its significance. Firstly, while imaging studies date the initiation of

amyloid plaque formation earlier in time than tau tangle formation, modern neuropathology analysis clarified that the onset of aberrant Tau chemistry precedes the deposition of Aβ plaques, in some cases appearing in the brain stem as young as 20 years old [24]. This finding flipped the traditional view that Aβ may play a causal role in the development of NFTs, and thus questioned the notion that Aβ drives AD pathogenesis. It was also found that total amyloid volume in the hippocampus was a worse predictor of cognitive status [25] and even grey matter loss [26] than total NFT counts, meaning that by these measures, tauopathy is better correlated with symptom severity than Aβ. This idea is further supported by the limited success of treatments which reduce brain amyloid [27], as well as the discovery of a unique APOE mutation which allowed its host to display unusually high levels of amyloid while remaining devoid of tauopathy, neurodegeneration, or symptoms of dementia [28]. Certain forms of dementia are driven by tauopathy in the absence of Aβ, rejecting the concept that Aβ is necessary for the onset of symptoms [29]. It has even been hypothesized that in late-onset AD, the formation of NFTs can indirectly influence the cleavage of Aβ from APP [30-31] by impairing vesicle transport and 'trapping' APP in endosomes to be cleaved. But what exactly is tauopathy, and how does it contribute to neurodegeneration? In contrast to APP, the endogenous function of the tau protein is well defined. It binds to and stabilizes microtubules, which are a critical component of the cytoskeleton and provide structure for intracellular cargo transport. As microtubules are canonically elongated and shortened, the binding of these stabilization proteins is robustly regulated through phosphorylation. When these regulatory processes are disturbed as in AD, tau protein becomes hyper-phosphorylated and dissociates from the microtubules, then self-assembles into filaments which comprise the larger NFTs. These intracellular tangles have been demonstrated to spread from cell to cell in a prion-like fashion [32], and fragments are also known to be recognized by microglia and further propagate inflammation [33].

Microglia

A third characteristic of AD pathophysiology that has received increased attention in recent years is a prolonged and toxic neuroinflammation mediated by glial cells, predominately microglia. However,

before I discuss microglia in the context of AD, it is first important to describe these cells' role in a healthy brain. While the presence of this cell type has been acknowledged for quite some time, even by Dr. Alzheimer himself, their properties in physiology and in disease have only recently begun to be elucidated. Microglia originate from primitive macrophages that enter the CNS very early in embryonic development, after which there is little to no migration into or out of the brain [34]. Though they are best known as the brain's resident macrophage, they have many diverse functions in neurodevelopment and homeostasis. As the developing brain matures, the over-abundance of neuronal connections must be pruned to retain only the most integral; a job undertaken primarily by phagocytic microglia [35]. Even in a mature brain, neuronal plasticity demands synapses be destroyed as well as created, processes which also largely depend on microglia. In addition to synapses, microglia can also engulf entire cells in the context of disease, apoptosis, or in the construction of neuronal circuits [36-37]. These cells are also continuously monitoring their environment for perturbations [38]. They are responsive to neuronal activity and have been shown to reduce spontaneous and evoked activity of highly active neurons through cell-cell contact [39-40]. They can also remodel the extracellular matrix, an ability shared with their developmental relatives in the periphery [41]. Thus, by surveilling and reacting to their surroundings, they help maintain the homeostatic balance of ions, metabolites, trophic factors, etc. that are crucial for neuronal health and function [42]. Microglia also direct the brain's immune response to invading pathogens. They can propagate inflammatory signals from the periphery as well as in the CNS, and once activated they release a cocktail of pro-inflammatory cytokines including tumor necrosis factor alpha (TNFa), IL-6, and IL-1b. These cytokines induce the release of various secondary inflammatory signals, which together coordinate sickness behaviors [43]. This inflammatory immune response is generally self-limiting and is thus terminated once the tissue damage is repaired or the pathogen is cleared [44]. The high level of microglial reactivity makes them rather difficult to study, however. Even within an individual brain, the high heterogeneity of microglia can mask disease-related processes when observing the cell population as a whole. Therefore, many researchers have turned to a single cell approach for the ability to distinguish between distinct subsets of microglia that appear in different physiological

conditions. Single cell analysis of morphology, intracellular anatomy, transcriptomics, proteomics, and other measures have demonstrated the dependence of microglial phenotypes on brain regions and disease states [45-47], and have begun to provide a basis for targeted therapeutic approaches.

Microglia in AD

Microgliosis was known to be a hallmark of AD very early in disease characterization, as the cells were found in 1907 to display an activated morphology and surround Aβ plaques [48]. Genome wide association studies have also implicated microglial genes APOE and TREM2 as major risk factors for AD. Due to their now well-established role in injury protection and pathogen defense, however, it was long debated whether AD-associated gliosis was beneficial, neutral, or detrimental to pathology. Not only do they represent an important Aβ clearance mechanism, but recent evidence also suggests that they form a protective barrier around the plaques to sequester the toxic soluble Aβ oligomers into insoluble fibrils and prevent them from traveling to nearby cells [49]; a process that relies on both APOE [50] and TREM2 [51]. In fact, disrupting this barrier increases axonal degradation and tau pathology. In addition, genetic studies identified several mutations which impaired the neuroprotective effects of microglia, in turn hastening the progression of the disease [51]. Despite these proposed benefits, hyper-activated microglia have repeatedly been shown to be detrimental to nervous system function. Many studies show that silencing the complement system or depleting microglia is neuroprotective by rescuing the synapse and neuron loss associated with Aβ plaques [52-53]. Complement system and microglial activation has also been linked to the spread of Tau pathology, and while the mechanisms are unclear, it is likely that microglia uptake tau and release it in vesicles, enhancing its spread through the brain [54-55]. Furthermore, dying neurons and protein aggregates directly activate microglia through various receptors to promote the release of inflammatory signals [56]. Chronic high levels of these factors can exacerbate the tissue damage and result in unnecessary cell death. Experimental inhibition of the involved receptor pathways reduces both Aβ deposition into plaques and cognitive impairments [57], which further

implicates the general inflammatory pathway in driving AD pathology. However, as alluded to above, it is not nearly as simple as either 'resting' or 'activated' microglia (scheme 1).

Single cell experiments have characterized a unique microglial subtype present in AD and in other neurodegenerative diseases. In 2016, detailed electron microscopy (EM) analysis yielded the description of 'dark' microglia, associated with chronic stress, aging, and an APP-PS1 mouse model of AD, which possess pathogenic mutations in the aforementioned APP and PSEN1 genes [58]. The name refers to their dark appearance under EM, which is indicative of oxidative stress and dysfunctional mitochondria. These cells were also found to be overly phagocytic, engulfing entire synapses as well as pre and post-synaptic components. Notably, nuclear chromatin remodeling was also observed, which suggests active epigenetic regulatory processes. Shortly after, Keren-Shaul et. al published the discovery a unique microglial subtype associated with AD, termed disease activated microglia (DAM) [59]. This team isolated immune cells from neural tissue of 5XFAD mice, an AD mouse model, and used single-cell RNA-sequencing to cluster subpopulations based on their transcriptional signatures. This approach elucidated a cluster of microglia not observed in wildtype mice, characterized by decreased expression of homeostatic genes, an upregulation of known AD risk factors like APOE, and an enrichment in phagocytic and lipid metabolism pathways. Importantly, they also found the proportion of DAMs increased along the disease trajectory, and that these cells were localized around Aβ plaques, which implicates Aβ as a key initiator of the conversion to a DAM phenotype.

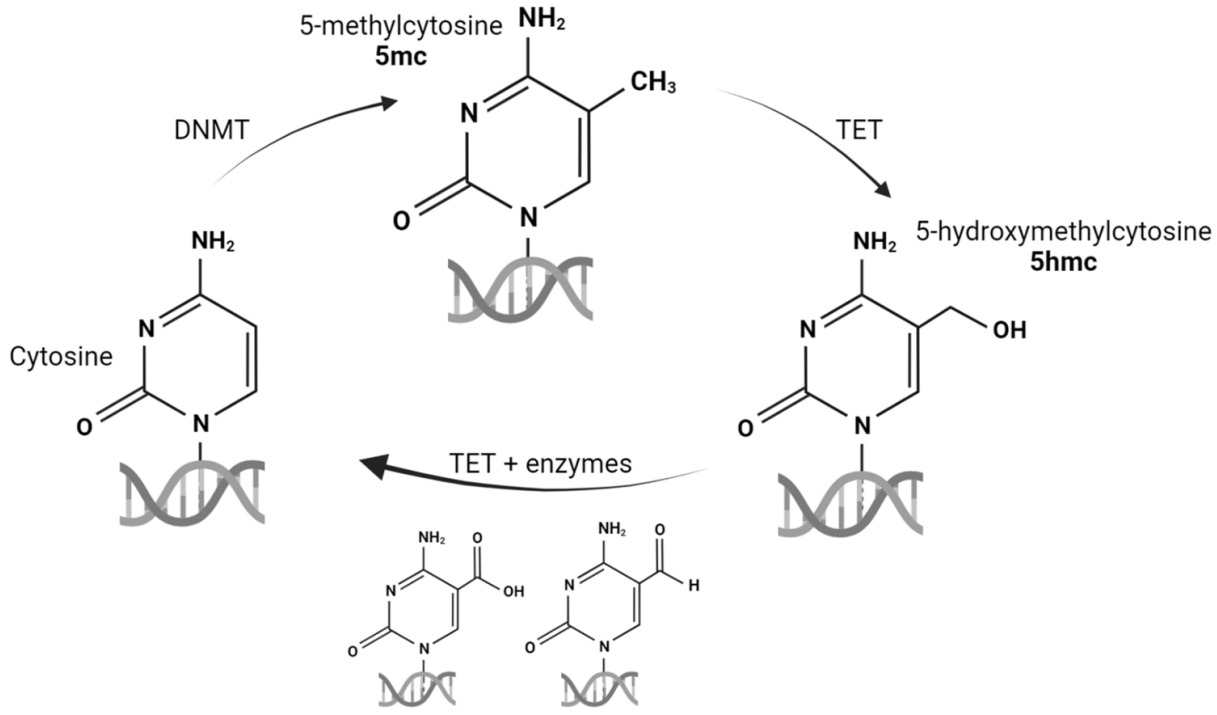
Epigenetics in AD

As epigenetics are responsible for the diversity of all cell types that stem from an individual's genome, it is logical that these processes must also be involved in generating the heterogenous population of microglia observed in health and disease. Indeed, chromatin remodeling, post-translational histone modifications, non-coding RNAs, and DNA methylation have already been implicated in many forms of neurodegeneration [60] and to some extent microglial processes [61-62], though I shall focus here on DNA methylation which is likely the best characterized of these mechanisms. This regulatory process

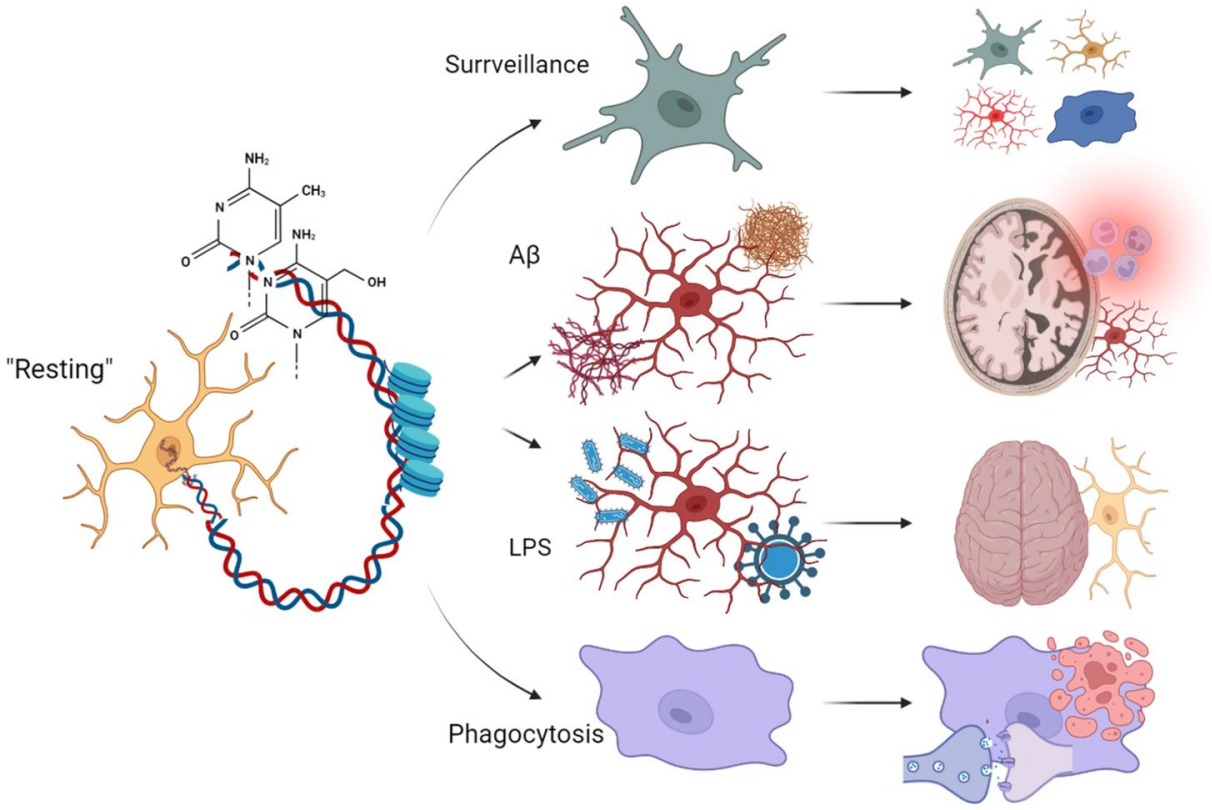
involves the addition of a methyl group to the nucleotide base cytosine in a CpG dinucleotide, by the DNA-methyl transferase (DNMT) enzyme family, resulting in 5-methyl-cytosine, or 5mC (scheme 2). There is also recent evidence of adenine and guanine methylation [63], however the effects of these modifications are less clear and will be disregarded in this report. The formation of 5mC occurs in the context of DNA replication or DNA damage repair, in which case DNMT1 ensures the methylome of the parent strand is faithfully copied to the new daughter strand. Notably, however, DNMT1 is also expressed in post-mitotic cells like neurons, suggesting alternative roles for this enzyme. There is also *de novo* methylation, in which DNMT3a or DNMT3b enhances existing DNA with a new methyl group. Although the effects of 5mC are highly dependent on its precise genomic location [64], its presence in gene promoters is generally associated with repression of gene expression, accomplished by interfering with the binding of transcriptional machinery [65], or by recruiting histone modification pathways to condense the chromatin [66]. 5mC in the gene body is involved in alternative splicing of introns and exons, and is also correlated with an enhancement of gene expression through yet-unknown mechanisms [67]. To remove the methyl group, the TET family of enzymes oxidizes the 5mC to form 5-hydroxymethylcytosine, or 5hmC. 5hmC is further converted by the TET enzymes to 5-formylcytosine (5fc), 5-carboxylcytosine (5cc), and finally back to cytosine. Besides occupying an intermediate state between 5mC and unmethylated cytosine, 5hmC has been shown to function as an independent epigenetic modulator, and its concentration is highly enriched in the brain [69]. Unlike 5mC, the presence of 5hmC is associated with active gene expression, regardless of its location within the gene [70].

Changes in 5mC and 5hmC have previously been associated with AD, as well as differences in the expression of related machinery. In 2010, Mastroeni et al. reported a decrease in 5mC immunoreactivity in human postmortem AD tissue compared to non-AD cases [70]. These results were later contradicted by Lashley et al., however, who reported no changes in 5mC or 5hmC in the entorhinal cortex (EC), an area known to be particularly susceptible to AD pathology [71]. As these conflicting results began to suggest a brain-region or cell-type dependence of methylation changes, Phillips et al.

examined 5mC and 5hmC signal in various cell types, and discovered pyramidal neurons in human AD tissue to be deficient in 5mC, fewer astrocytes to be positive for 5mC or 5hmC, while no changes in either marker were observed in microglia [72]. While studies examining global 5mC remain conflicting and somewhat inconclusive, it is notable that the promoters of general microglial inflammatory factors IL-6 and IL-1b were reported to become hypomethylated along the progression of AD [73]. 5hmC has also been observed to change in AD brains, however the data is similarly conflicting. Condliffe et al. observed a decrease in global 5hmC in the EC of postmortem AD tissue [74], while increases in hippocampal 5hmC and 5mC were reported by Bradley-Whittman et al [75]. This is in direct contrast to the work of Chaoliaras et al., who demonstrated robust decreases in both 5mC and 5hmC in the hippocampi of AD patients [76]. The lack of consensus on global 5mC and 5hmC changes highlight the need for more gene-specific analysis.



Scheme 1: The methylation cycle of cytosine nucleotides and the associated enzymes. Cytosine is converted to 5-methylcytosine by DNMTs, which is further converted to 5-hydroxymethylcytosine by TETs. Cytosine is reformed by TETs and decarboxylation enzymes.



Scheme 2: A diagram representing the role of epigenetics in producing the wide variety of microglial phenotypes observed *in vivo*. Upon activation by various stimuli, surveilling microglia adopt a variety of phenotypes to execute their many functions in the brain. Our hypothesis is that epigenetic mechanisms govern these transitions.

While the role of histone modifications in directing microglial processes are relatively well documented, fewer studies have investigated the role of DNA methylation in regulating microglial activation in disease states. Lee et al. found that by knocking down TET2, they were able to rescue cell death associated with a Parkinson's disease mouse model. This attenuation was also associated with stunting microglial activation, even following systemic injection of LPS [77]. In support of these results, Carillo-Jimenez et al. demonstrated an upregulation of TET2 in microglia stimulated with LPS, and found that it directs downstream transcriptional changes without altering the methylation profile of its target genes.

Previous work in Desplats lab

Previous research in the Desplats lab has demonstrated an inverse relationship between DNA methylation and the microglial inflammatory response *in vitro*, using an SV40 immortalized human microglial cell line. Pharmacological inhibition of the DNMT family resulted in a global decrease in 5mC and prompted the cells to propagate a pro-inflammatory response, releasing cytokines like TNFa and IL-6. Conversely, by viral overexpression of DNMT1, an increase in global 5mC was observed, and the inflammatory response was stunted even in the presence of stimulants like LPS or Aβ. Interestingly, there were also fluctuations in the expression level of some DNMTs and TETs following LPS exposure, which was not recapitulated by treatment with Aβ. These results suggest that global hypermethylation may be neuroprotective in the context of AD, however they also beg several major questions. First, was it an increase in 5mC that directed these effects, or was it the subsequent conversion to 5hmC? Next, the hyper-methylation of which specific genes were most responsible for the observed effects? Finally, in which way does the methylome canonically change following inflammatory stimuli?

To answer these questions, I conducted the current study by treating human immortalized microglia cells with LPS, Aβ, or vehicle, and used isolated DNA to conduct reduced representation bisulfate sequencing (RRBS) and reduced representation hydroxymethylcytosine profiling (RRHP), which provide measures of 5mC and 5hmC reads for each methylated site, enabling single-base resolution and high genomic coverage. Our comprehensive data analysis, including gene ontology, gene set enrichment and RNA-sequencing analysis, indicates that differential DNA methylation of both 5mC and 5hmC is highly involved in directing microglial-specific programs in response to different stimuli. I also report preliminary evidence that methylation provides a mechanism by which microglia activate either DAM-specific programs or canonical pro-inflammatory signaling networks. These data are also supported by an *in vivo* model of inflammation, in which differential expression of DNA-methylation machinery may contribute to the observed alterations in the microglial methylome.

Methods

Cell culture and treatment

A human microglial cell line immortalized with SV40 was obtained from Applied Biological Materials. Cells were cultured in PriGrow III media (Applied Biological Materials) with 10% fetal bovine serum and 1% penicillin-streptomycin, and incubated in a 5% CO_2 environment at 37ºC. Culture dishes were coated with Extracellular Matrix also from Applied Biological Materials, diluted 1:2 in PBS, and cells were dissociated and collected using TrypLe Express detachment reagent from Gibco.

Aβ 1-42 fragments were purchased from rPeptide and dissolved in DMSO, then diluted in culture media to a final concentration of 2.5 µM. LPS was obtained from Sigma Aldrich and resuspended in PBS, and final concentration of 100ng/ml in culture media was used for treatment. All samples were treated for 24 hours, and were run in biological duplicates.

Reduced Representation Bisulfate Sequencing (RRBS) library construction and sequencing

Cells were first exposed to LPS, Aβ, or the appropriate vehicle, then collected according to the methods above. 200-500ng of Genomic DNA was extracted using the DNA Clean & Concentrator-5 kit from Zymo Research, and was digested with 30 units of Mspl (NEB) to isolate CpG-rich fragments. According to Illumina's guidelines, these fragments were ligated to pre-annealed adapters containing 5mC as opposed to cytosine, and recovered using the same DNA Clean & Concentrator-5 kit. To convert all unmethylated cytosines into uracil, the ligated fragments were treated with bisulfate using the EZ DNA Methylation-Lightning Kit from Zymo Research, then amplified using customized PCR. The products were sequenced on an Illumina HiSeq, from which reads were identified and analyzed in a proprietary pipeline from Zymo Research. Essentially, the fragments were aligned to a reference genome and examined for the presence of a thymine in the amplified fragment, which indicates an unmethylated cytosine in the original DNA. For each site that had coverage of at least five reads, a methylation ratio was calculated as the number of methylated cytosines out of the total number of cytosines covered at that

site. The methylation difference (meth diff) refers to the difference in methylation ratio at that site between the two treatment groups. A Fisher's exact test was also performed on the reads to provide a p-value that describes the statistical significance of the difference in methylation levels between treated and control cells. The sites were also mapped to genes if applicable, and annotated as promoter, intron, or exon.

Data analysis of RRBS

DNA from cells treated with LPS, Aβ, or vehicle was sequenced to obtain a measure of the methylation level of each covered site. Any site mapping to either the X or Y chromosome were removed from the analysis, as methylation is sex-specific and would confound our analysis. A $p<0.05$ threshold was applied to define sites which display significantly different 5mC levels compared to control cells, and these sites were subsequently separated by the genomic location to which they mapped (figures 2, 6). To understand the biological relevance of these changes, I also determined the genes associated with these differentially methylated (DM) sites. As many genes contained multiple DM sites, often with changes in opposing directions, I therefore assessed the size of the methylation differences and assigned increase or decrease categories based on the larger occurrence across all DM sites in the same gene. For pathway analysis, I inverted the associated methylation difference metric for DM sites mapped to promoters, as 5mC exclusively in these regions has an inverse relationship with gene expression.

Reducted Representation Hydroxymethylcytosine Profiling (RRHP) library preparation

Genomic DNA from cells treated with LPS, Aβ, or vehicle was digested with Mspl, an enzyme insensitive to hydoxymethyl groups, to restrict the representation to genomic locations rich in CpGs. It was then purified and ligated using T4 DNA ligase to Modified TruSeq P5 and P7 adapters from Illumina, which contain specialized 5'-CG overhangs. The strands were then extended using Taq DNA polymerase, and the 5hmC bases were tagged via glucosylation by treatment with β-glucosyltransferase. The following overnight digestion with Mspl therefore refrained from cutting any fragments containing a

glucosyl-5hmC site due to the augmentation. Using the ZymoClean Gel Recovery Kit from Zymo Research, fragments were size-selected for 100-500bp and amplified via PCR. Fragments not containing glucosyl-5hmC sites were cleaved and therefore not amplified, which allowed positive selection of 5hmC-modified DNA. Low quality bases, as well as the P7 adapter, were trimmed from these fragments, and they were then aligned to the reference genome. The number of reads containing the Mspl tag were then quantified and reported as a read count for each site. A read count ratio was also established which provides a proportion of change between the control and treated cells.

RRHP data analysis

DNA from cells treated with LPS, Aβ, or vehicle was profiled for site-specific 5hmC content. I first removed from our analysis any site mapping to either the X or Y chromosome. Any site with a read count between one and five was considered below the detection threshold and was also disregarded. A two-tailed student's t-test was then applied, comparing the reads from the LPS or Aβ treated cells to those treated with vehicle. A p-value<0.01 significance filter was applied to identify the DM sites described by figures 2 and 6.

Analysis of RNA-seq data

The same cells used for methylation analysis were also subjected to transcriptional profiling via RNA-sequencing. RNA was extracted from treated cells using the RNeasy MiniKit (Qiagen) including DNase treatment. Total RNA was quantified by Qubit and submitted for library preparation and sequencing to the UCLA genomics core. Reads were processed for QC analysis, base trimming, and alignment. Differential expression was determined with DESEQ2 after normalization. A false discovery rate FDR<0.2 threshold was applied comparing vehicle-treated cells to those treated with Aβ to define differentially expressed genes (DEGs) (figure 12).

Pathway analysis of differentially methylated genes and differentially expressed genes

Pathway analysis of DM genes and DEGs was performed using Ingenuity Pathway Analysis (Qiagen). Reference pathways were restricted by species to homo sapiens, and to processes associated with the immune system, the central nervous system (CNS), or immune or CNS cell lines. The report of enriched pathways, networks, or upstream regulators was confined to p-value<0.05.

In vivo studies in mouse

C57BL/6J wild-type mice (4-month-old males, n=3) were injected intraperitoneally (i.p) with 1mg/kg LPS (Sigma Aldrich L8274) or saline once a day for seven days. Mice were sacrificed on day seven, two hours after the last injection. Brains were collected and one hemibrain was stored in RNA-later (Sigma Aldrich) while the other was fixed with 4% paraformaldehyde (PFA) for immunohistochemistry.

Immunohistochemistry

Fixed brains were cut to 20µm using a 5100mz vibrating microtome from Campden Instruments. Sections were first pretreated for 20 minutes with 10% H_2O_2, 0.1% Triton-x in PBS, then blocked in 10% normal serum from the host species of the secondary antibody. All primary antibodies were incubated overnight at 4 ºC diluted in PBS. DNMT1 (Abcam ab188453) was used at a concentration of 1:100, Iba1 (Sigma SAB2702364) at 1:500, TET2 and TET3 (A-1701 and A70559, Epigentek) at 1:100. The sections were then washed and incubated in biotinylated antibodies at a concentration of 1:200 in PBS, then developed using a 3, 3'-diaminobenzidine (DAB) kit from Vector Laboratories according to the manufacturer instructions. IHC images taken on a Hamamatsu Nanozoomer were quantified using ImageJ software. Representative images (figure 15) were taken on a Zeiss Axio Imager2.

Real-time PCR

The hippocampus was isolated and RNA was extracted using the RNeasy Lipid Tissue Mini kit from Qiagen. The RNA was quantified with a nanodrop spectrophotometer (DeNovix) at 260nm, and 1µg was converted to cDNA using the high-capacity cDNA reverse transcription kit from Thermoscientific. Real-time PCR reactions were then conducted using Taqman fast primers, Taqman Fast Advanced master

mix, and the StepOne Plus Real-Time PCR system from Applied Biosystems. Relative RNA abundance was then calculated using the comparative Delta Ct method normalized to beta-actin.

Results

To investigate the role of DNA methylation in modulating the microglial inflammatory response, we designed an experimental assay employing human immortalized microglial cells. Microglia were exposed to LPS (100ng/ml) as a model of canonical inflammation, amyloid-beta (2.5 µM) as a model of AD, or the corresponding vehicle as a control. Cells were collected after 24 hours, and both DNA and RNA were extracted for downstream analysis, including genome-wide profiling of 5mC levels by RRBS, 5hmC levels by RRHP, and transcriptomic analysis using RNA-seq. The sequence coverage and depth were sufficient and consistent across experimental replicates (figure 1).

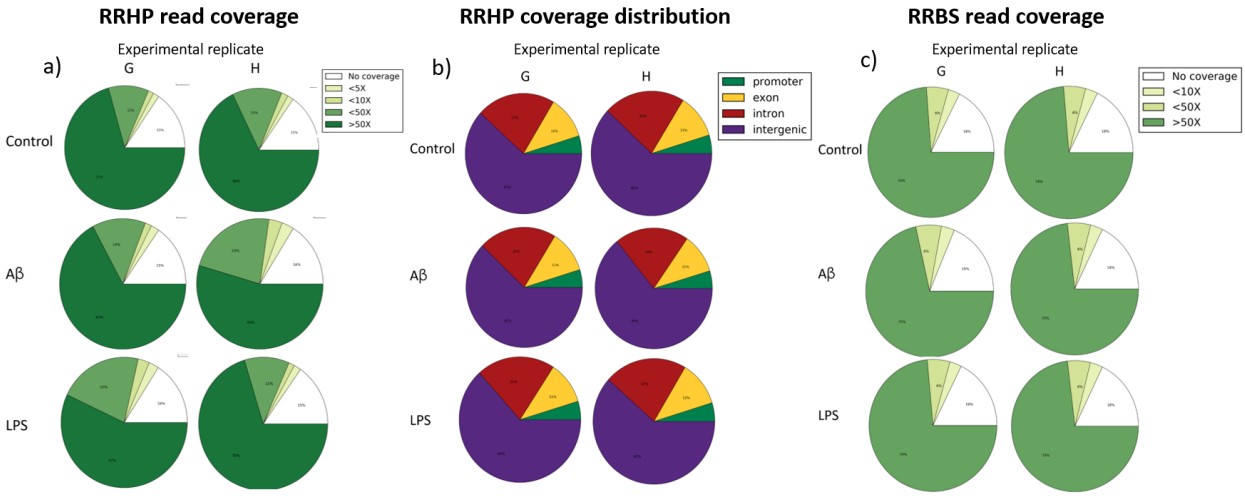


Figure 1: Quality Control of Reduced Representation Bisulfate Sequencing (RRBS) and Reduced Representation Hydroxymethylcytosine Profiling (RRHP). Read coverage (a) and distribution (b) of RRHP, and read coverage of RRBS (c)

Profiling DNA alterations in microglia during canonical inflammatory responses

To understand the role of DNA methylation in mediating inflammatory signals in microglia, I first set out to define the epigenetic changes that occur during a canonical inflammatory response. Immortalized human microglia were exposed to either LPS or vehicle, and isolated DNA was sequenced

to determine site-specific levels of both 5mC and 5hmC. I then evaluated the distribution of sites presenting significant changes in 5mC and/or 5hmC in LPS compared to vehicle-treated control cells.

Genome-wide remodeling of 5mC in LPS treated microglia

I applied Reduced Representation Bisulfate Sequencing (RRBS) to obtain genome-wide and site-specific measures of 5mC sites in both LPS- and vehicle-treated cells. Following bioinformatics steps including quality control, base trimming, and alignment, I obtained the quantity of 5mC reads per site and determined differences in 5mC levels between LPS treated cells and control cells (figure 2). I found well over 200,000 sites that exhibited significant (p-value<0.05) changes in 5mC following LPS exposure, and notably almost 90% of these differentially methylated (DM) sites displayed decreases in 5mC levels, ranging from 10-100% difference in methylation. The distribution of these sites appears relatively uniform across all genomic locations, though introns and intergenic regions contain the highest quantity of DM sites (figure 2). I discovered over 17,000 total genes to be affected by these changes, with over 11,000 more genes containing DM sites within the gene body than in the promoter region. I combined the DM sites from introns and exons for this analysis, as the presence of 5mC in these two areas is unlikely to influence gene expression or alternative splicing through differing mechanisms. Using the direction of the observed difference in methylation, I also defined whether these genes were hyper-methylated or hypo-methylated in LPS-treated cells compared to controls to better understand the biological effects of these methylation changes.

Cellular processes affected by differential 5mC in LPS treated microglia

The differentially methylated (DM) genes in LPS-exposed cells were submitted to Ingenuity Pathway Analysis (IPA) for gene ontology analysis to determine which cellular functions may be impacted by changes in 5mC. For this analysis, I ran two types of queries: a) I combined genes containing DM sites in any genomic location (total n=2341) using stringent significance thresholds (p-value<$1X10^{7}$ and methylation difference (meth diff)>0.50); b) I also submitted genes with DM sites specific to either

promoters (total n=2372, $p<0.05$) or gene bodies (total n=2809, $p<0.05$ and meth diff>0.40). As expected, following LPS exposure, microglial genes with differential 5mC were found to contribute to general inflammatory response networks (table 1.1), clustering in both inflammatory and neurological diseases (figure 5). Specific canonical pathways include CCR5 signaling in macrophages (-log(p)=4.35), CXCR4 signaling (-log(p)=2.33), IL-15 production (-log(p)=2.58), RhoGDI signaling (-log(p)=2.1), and RhoA signaling (-log(p)=4.35). Phagosome formation was also highly enriched (-log(p-value)=4.5), which indicates a role for DNA methylation for guiding the phagocytic functions of microglia in addition to the release of pro-inflammatory factors. Many processes involved in synapse formation or reconstruction were also enriched, such as axonal guidance (-log(p)=8.43), CREB signaling (-log(p)=9.83), long term depression (-log(p)=5.62) and potentiation (-log(p)=3.29) (LTD, LTP), and synaptogenesis (-log(p)=2.34). In addition, DM genes clustered to several other general signaling pathways like opioid (-log(p)=5.66), GABA (-log(p)=6.78), serotonin (-log(p)=2.84), glutamate (-log(p)=1.97), and corticotropin releasing hormone (CRH) signaling (-log(p)=6.45), suggesting a fundamental role for differential 5mC in mediating the phenotypic responses to canonical inflammatory stimuli.

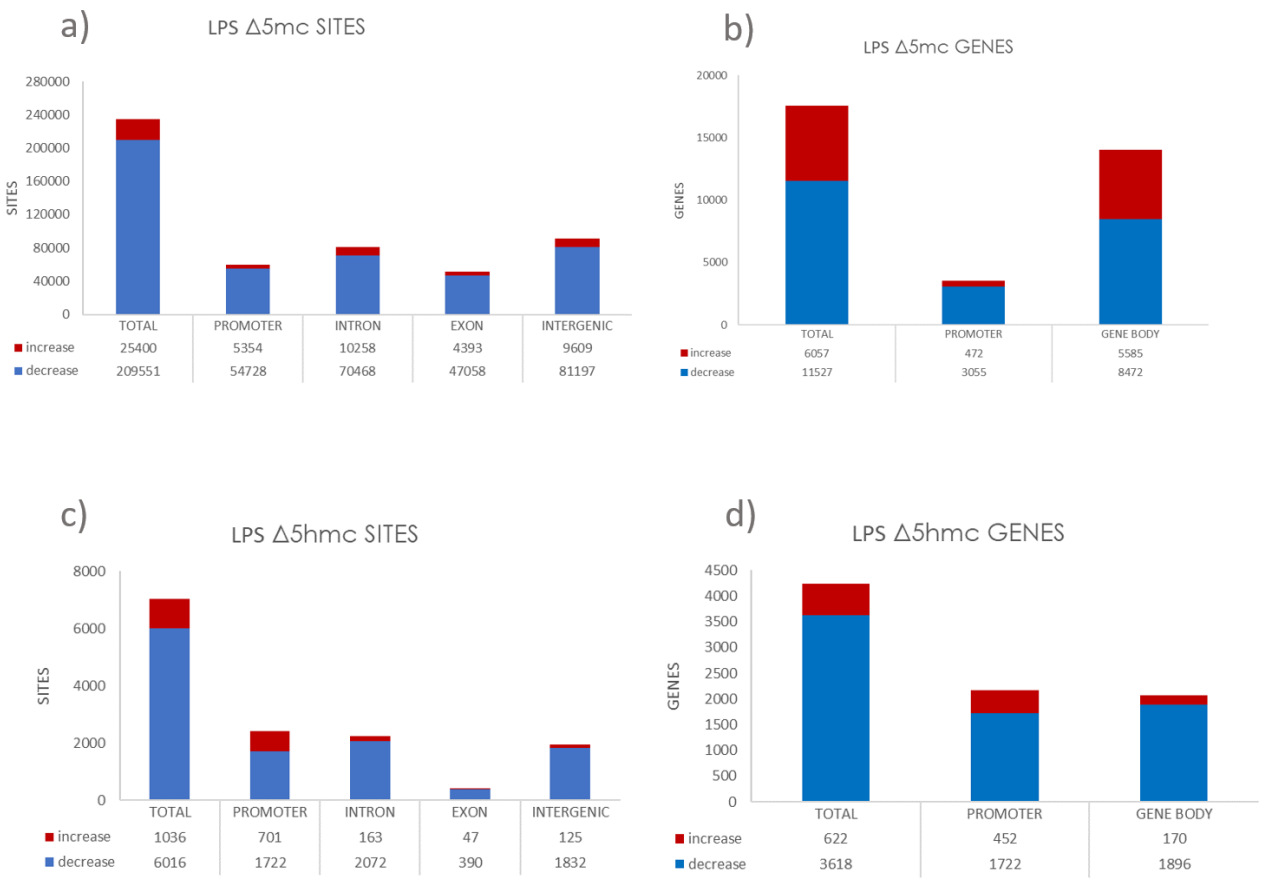


Figure 2: The distribution of sites (a,c) and genes (b,d) displaying differential 5mC (a-b) and 5hmC (c-d) following treatment with LPS.

Importantly, many AD-related processes were also enriched in DM genes after LPS exposure, including Amyloidosis (p=0.04), Tauopathy (p=0.04), degenerative dementia (p=0.01), and even AD explicitly (p=0.02), with the latter two boasting almost 80 contributing DM genes. This supports the notion that 5mC changes can thus also contribute to disease pathways in conjunction with canonical cellular programs. Notably, I discovered APOE to be an enriched upstream regulator within these gene sets, along with cytokine CSF3, lipid receptor LRP1, and transcriptional regulators EZH2 and MeCP2. In all, these results validate our *in vitro* model and experimental design, and unravel an important function of methylome remodeling as an upstream event in microglial activation.

Genome-wide remodeling of 5hmC in LPS treated microglia

The cycle of DNA methylation progresses from 5mC to 5hmC upon oxidation of the methyl group, catalyzed by the action of TET enzymes. Importantly, 5hmC is the most abundant modification in the adult human brain and functions as a unique transcriptional modulator. To produce a comprehensive map of all methylation changes associated with canonical microglial activation, I next analyzed genome-wide 5hmC coverage using Reduced Representation Hydroxymethylcytosine Profiling (RRHP).

Using precisely the same cellular model of canonical inflammation, I evaluated the quantity and distribution of sites that exhibit significant changes in 5hmC following LPS treatment, compared to vehicle treated cells (figure 6). I discovered 7052 DM sites (p-value<0.01), with 85% of these displaying a decrease in 5hmC abundance upon LPS exposure. Much like that observed in 5mC, the distribution of differential 5hmC sites appears fairly homogeneous across genomic regions, though in this case exons contain about 1500 fewer DM sites than other regions. I then determined the genes mapping to these sites, a total of 4240, again categorizing each gene as either hyper- or hypo-hydroxymethylated according to the direction of difference in 5hmC levels. Interestingly, due to the scarcity of differential 5hmC in exons (437 total sites), I found similar numbers of genes to be differentially hydroxymethylated in promoters and gene bodies.

Table 1.1: Top canonical pathways enriched in genes differentially methylated in 5mC in LPS-exposed cells

LPS Δ5mc	Canonical Pathways	-log(p-value)	Overlap
TOTAL	CREB Signaling in Neurons	9.83	115/574
	Axonal Guidance Signaling	8.43	97/483
	GABA Receptor Signaling	6.78	36/131
	Corticotropin Releasing Hormone Signaling	6.45	38/146
	G-Protein Coupled Receptor Signaling	6.28	316/668
	Circadian Rhythm Signaling	6.24	56/258
	Endocannabinoid Neuronal Synapse Pathway	5.91	37/147
	Opioid Signaling Pathway	5.66	57/275
	Synaptic Long Term Depression	5.62	73/187
	Phagosome Formation	4.45	104/639
	CCR5 Signaling in Macrophages	4.35	28/115
	Calcium Signaling	4.27	42/204
	Insulin Secretion Signaling Pathway	3.22	47/262
	IL-15 Production	2.58	20/115
	Synaptogenesis Signaling Pathway	2.34	70/331
	RHOGDI Signaling	2.1	35/208
	Signaling by Rho Family GTPases	1.98	42/264
	Glutamate Receptor Signaling	1.97	
	RHOA Signaling	1.83	22/122
PROMOTER	Type II Diabetes Mellitus Signaling	4.54	36/145
	nNOS Signaling in Neurons	2.62	13/45
	CXCR4 Signaling	2.33	35/161
	Role of JAK2 in Hormone-like Cytokine Signaling	2.17	10/32
	eNOS Signaling	1.96	34/148
GENE BODY	Actin Cytoskeleton Signaling	3.35	87/228
	Synaptic Long Term Potentiation	3.29	49/125
	Serotonin Receptor Signaling	2.84	23/43
	Melatonin Signaling	1.82	30/69

Table 1.2: Top disease pathways enriched in genes differentially methylated in 5mC in LPS-exposed cells

LPS Δ5mc	Disease pathways	p-value	# molecules
TOTAL	Inflammation of absolute anatomical region	0.00254	13
	Inflammation of body cavity	0.00293	12
	Inflammation of organ	0.00897	21
	Apoptosis of neuroglia	0.0193	5
	Apoptosis	0.0351	58
	Amyloidosis	0.0413	43
	Tauopathy	0.045	41
	Degenerative brain disorder	0.0467	8
PROMOTER	Sporadic neurological disorder	0.0276	6
	Brain damage	0.0382	4
	Psychosis	0.0392	5
GENE BODY	Guidance of axons	4.06E-06	39
	Degenerative dementia	0.0165	79
	Severe psychological disorder	0.0198	103
	Alzheimer disease	0.0212	77
	Cell death of neuroglia	0.032	9

Table 1.3: Top upstream regulators (c) enriched in genes differentially methylated in 5mC in LPS-exposed cells

LPS Δ5mc	Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	# targets
TOTAL	EZH2	transcription regulator		0.000325	11
	MBD2	transcription regulator		0.00138	3
	RAG2	enzyme	1.807	0.00235	15
PROMOTER	APOE	transporter		0.00439	22
	KIT	transmembrane receptor		0.00968	4
	EP300	transcription regulator		0.0176	3
	MECP2	transcription regulator		0.0176	3
GENE BODY	LRP1	transmembrane receptor	0.896	0.000797	6
	CSF3	cytokine	-0.816	0.00621	6

Table 2.1: Top canonical pathways enriched in genes differentially methylated in 5hmC in LPS-exposed cells

LPS Δ5hmc	Canonical Pathways	-log(p-value)	Overlap
TOTAL	D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	2.78	9/26
	AMPK Signaling	2.4	92/293
	Axonal Guidance Signaling	2.3	74/483
	G-Protein Coupled Receptor Signaling	2.28	118/688
	Triacylglycerol Biosynthesis	2.27	12/47
	Superpathway of Methionine Degradation	2.24	10/36
	Circadian Rhythm Signaling	2.16	43/258
	Phosphatidylglycerol Biosynthesis II (Non-plastidic)	2.07	8/27
	Huntington's Disease Signaling	2.06	45/276
	Insulin Secretion Signaling Pathway	2.04	43/262
	Senescence Pathway	1.89	46/290
	Netrin Signaling	1.89	41/71
	Endocannabinoid Neuronal Synapse Pathway	1.82	26/147
	Synaptogenesis Signaling Pathway	1.74	48/311
	Corticotropin Releasing Hormone Signaling	1.61	25/146
	Synaptic Long Term Potentiation	1.52	22/127
	IL-7 Signaling Pathway	1.5	14/72
	Gαq Signaling	1.47	27/165
	Type II Diabetes Mellitus Signaling	1.38	19/146
	CREB Signaling in Neurons	1.36	379/574
PROMOTER	Adipogenesis pathway	1.8	23/131
	Amyloid Processing	1.42	10/49
	IL-22 Signaling	1.38	6/24
GENE BODY	Melatonin Signaling	4.15	16/69
	Opioid Signaling Pathway	4.14	35/264
	Synaptogenesis Signaling Pathway	3.56	42/305
	G Protein Signaling Mediated by Tubby	2.89	10/42
	Actin Cytoskeleton Signaling	2.7	31/228
	nNOS Signaling in Neurons	2.65	10/45
	CCR5 Signaling in Macrophages	2.46	18/114
	Signaling by Rho Family GTPases	2.4	33/258
	RHOGDI Signaling	2.29	27/203
	Chemokine Signaling	2.22	13/76
	RAR Activation	2.11	25/190
	Glutamate Receptor Signaling	1.92	31/65
	Fcγ Receptor-mediated Phagocytosis in Macrophag	1.9	14/92
	GABA Receptor Signaling	1.87	13/130
	Androgen Signaling	1.73	17/164
	Calcium Signaling	1.59	24/200

Table 2.2: Top disease pathways enriched in genes differentially methylated in 5hmC in LPS-exposed cells

LPS Δ5hmc	Disease	p-value	#molecules
TOTAL	Cellular homeostasis	0.000883	10
	Infection of phagocytes	0.00618	3
	Alzheimer disease	0.0327	8
	Apoptosis	0.0477	10
PROMOTER	Tauopathy	0.000532	49
	Degenerative dementia	0.000629	49
	Alzheimer disease	0.000752	48
GENE BODY	Efflux of cholesterol	0.0202	3
	Guidance of axons	0.00784	14
	Cell spreading	0.0198	6
	Rett Syndrome	0.0386	14

Table 2.3: Top upstream regulators enriched in genes differentially methylated in 5hmC in LPS-exposed cells

LPS Δ5hmc	Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	# targets
TOTAL	E2F6	transcription regulator	0.447	0.0161	5
	IKZF3	transcription regulator		0.0362	2
	PRKCD	kinase	-1.264	0.0397	4
PROMOTER	E2F6	transcription regulator	-0.447	0.0139	5
	IKZF3	transcription regulator		0.0338	2
GENE BODY	APOE	transporter		0.0278	11
	MECP2	transcription regulator		0.0337	2

Cellular processes affected by differential 5hmC in LPS treated cells

I then sought to ascertain which microglial functions may be impacted by the observed differential 5hmC. Similar to our analysis of 5mC, I performed gene ontology on a combined list of all DM genes (total n=2282) with a significance threshold of p-value<0.001, as well as separate analysis for genes with DM promoters (total n=2101) and DM gene bodies (total n=1442) both with a threshold of p-value<0.01. While I found that changes in 5hmC affect networks associated with both immunological and neurological diseases, similar to that observed in 5mC, strikingly genes DM with 5hmC clustered in distinct biological pathways (table 2.1-2.3)

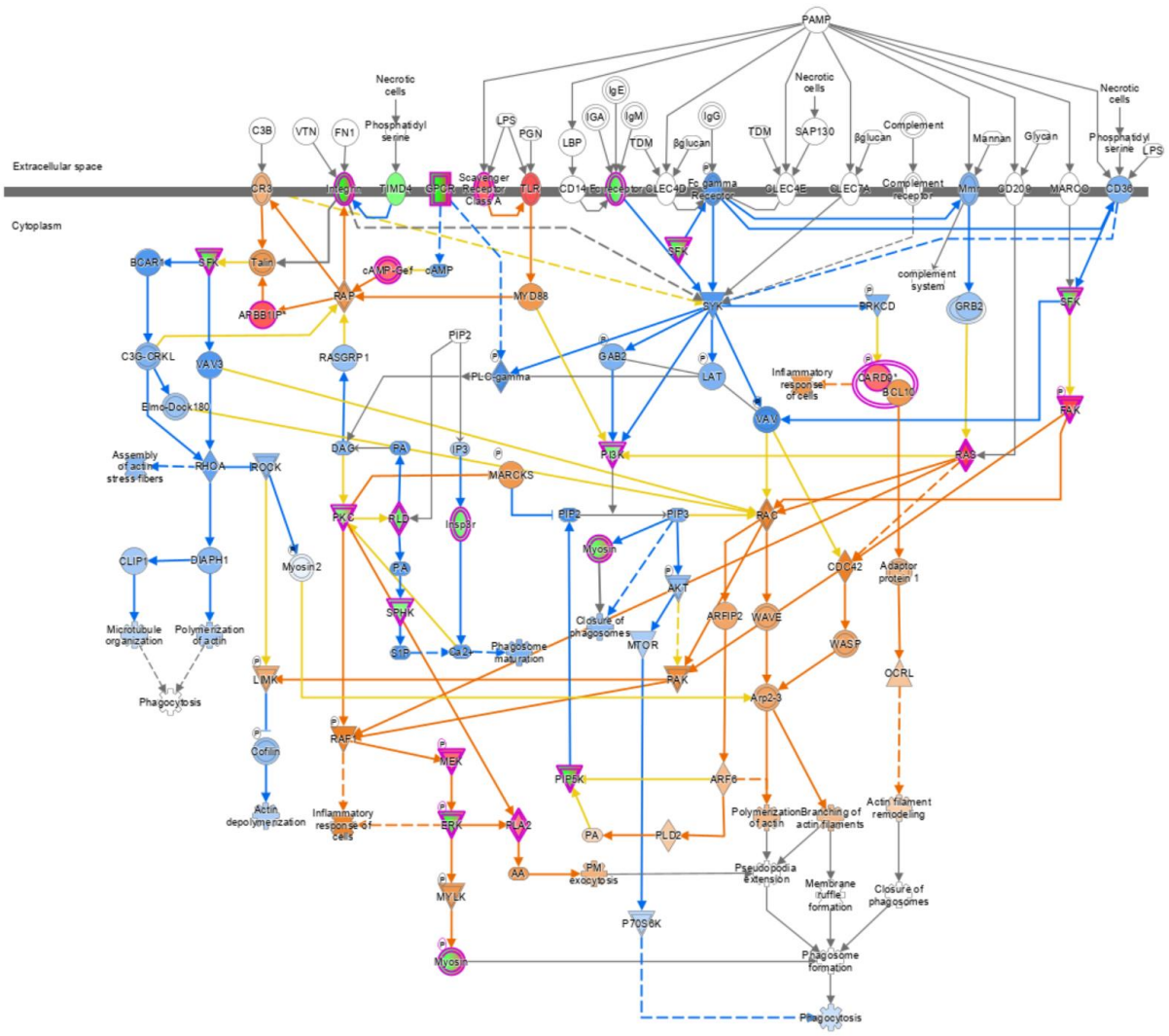


Figure 3: The roles of differential 5mC on phagosome formation in LPS exposed cells

For example, I found enrichment in the senescence pathway (-log(p)=1.89), chemokine signaling (-log(p)=2.22), and amyloid processing (-log(p)=1.42), which implies differential 5hmC is not only a by-product of 5mC changes but also acts as a major mediator of specific microglial processes in the inflammatory response. I also observed enrichment in synapse modulation pathways like synaptogenesis (-log(p)=3.56) and LTP (-log(p)=1.52), as well as in general signaling mechanisms such as CRH (-log(p)=1.61), opioid (-log(p)=4.14), GABA (-log(p)=1.87), and glutamate receptor signaling (-log(p)=1.92). In addition, I discovered specific disease pathways like infection of phagocytes, as well as

Tauopathy (p=0.0005), degenerative dementia (p=0.0006), and AD (p=0.03). Thus, differential 5hmC appears to also be involved in activating disease-related networks including neurodegeneration. Interestingly, APOE and MeCP2 were enriched as upstream regulators, which suggest these mechanisms may be influenced by changes in both 5mC and 5hmC.

Profiling DNA methylation changes in microglia in the context of Alzheimer's Disease

As our data supported a major role for DNA methylation in directing many functions of canonically activated microglia, I next investigated the role of this epigenetic mechanism in the response to amyloid beta as a model of AD. Isolated DNA from Aβ or vehicle exposed cells was then profiled for site-specific 5mC and 5hmC content in the same manner as above.

Genome-wide remodeling of 5mC in Aβ treated microglia

RRBS in Aβ- and vehicle-treated cells provided measures of 5mC levels at each methylated site (figure 6). Compared to our control cells, I discovered 31,592 sites that exhibited significant ($p<0.05$) changes in 5mC following treatment with Aβ, with methylation differences ranging from 10-100%. In stark contrast to what I observed in cells treated with LPS, I identified approximately the same number of these sites showing increases as decreases in 5mC, with no specific clustering in any genomic region. Following quantification of the genes affected by these changes, a total of 10637, I discovered 2700 more genes DM in the gene body than DM in the promoter region.

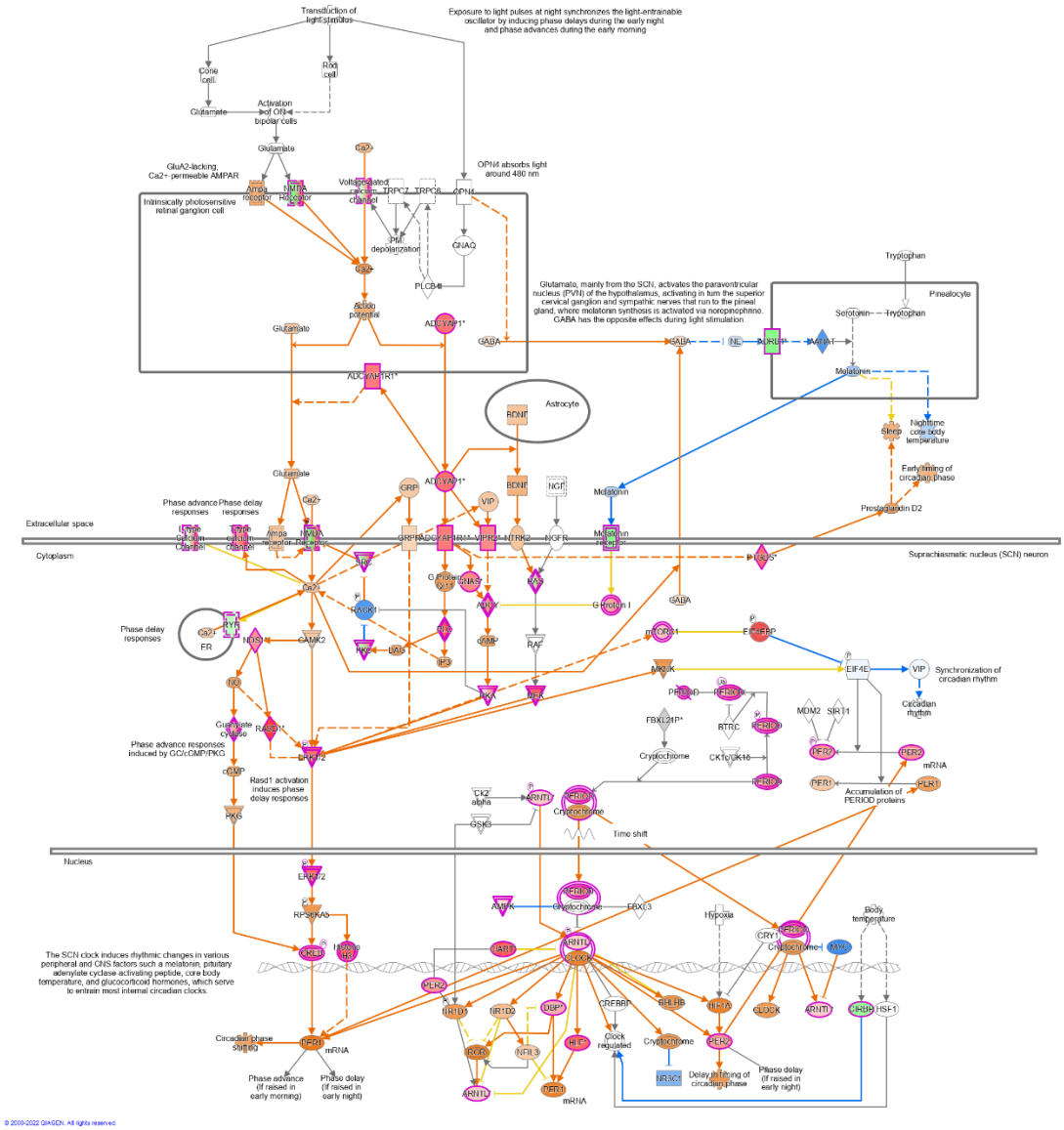


Figure 4: Changes in 5mC within gene promoters following LPS treatment affects genes involved in circadian rhythm signaling

Cellular processes affected by differential 5mC in Aβ treated microglia

I then assessed which microglial functions may be modified by the differential 5mC observed following Aβ exposure. I conducted gene ontology analysis on a list of all DM genes (total n=2600) with a significance threshold of $p<0.005$, as well as genes with DM promoters (total n=3221, $p<0.05$) and DM gene bodies (total n=1624, $p<0.05$, meth diff>0.40) separately. In Aβ -treated cells, much like those

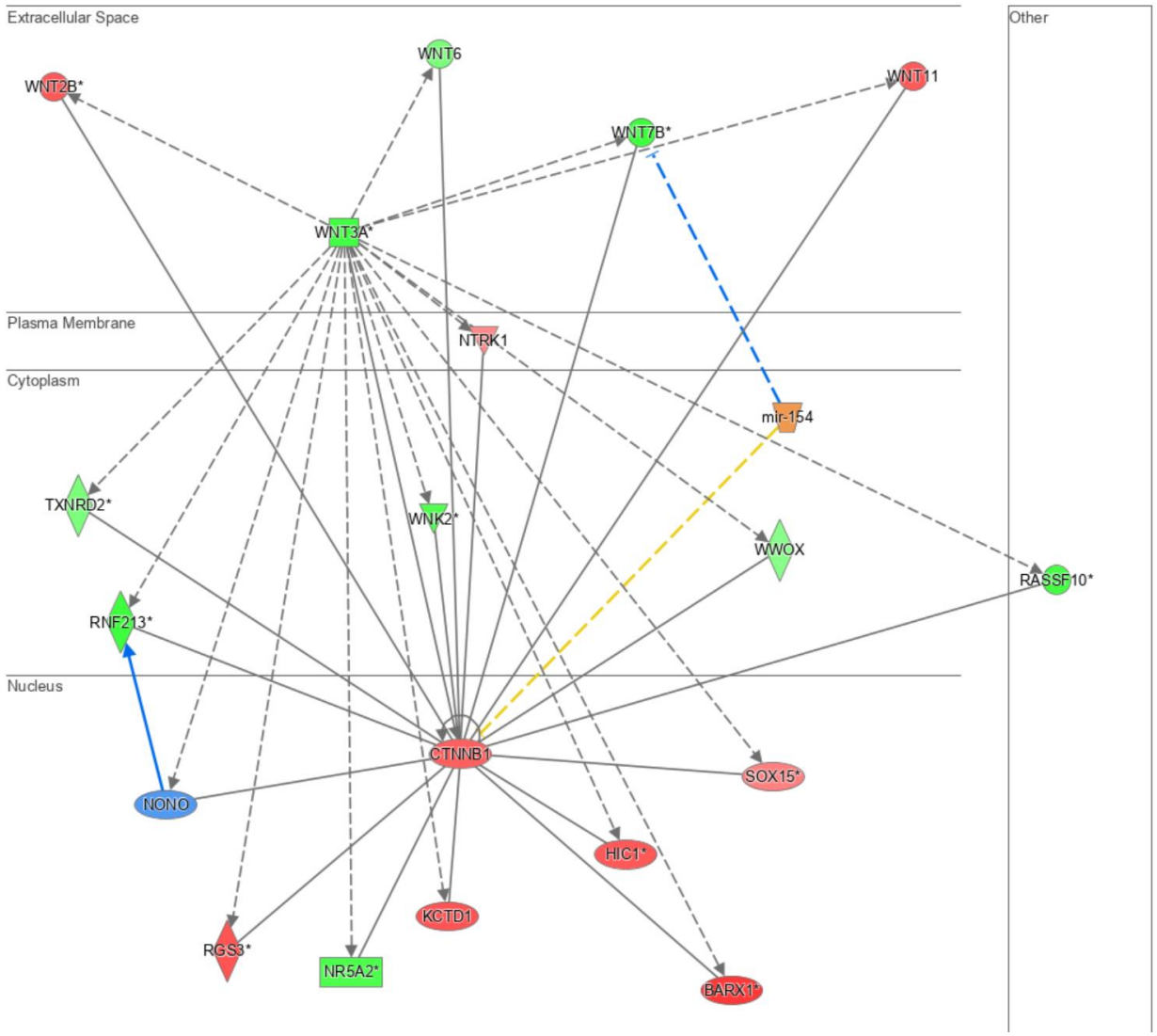


Figure 5: Alterations in microglial 5mC following LPS exposure impact signaling networks associated with inflammatory responses and diseases. This represents the most significantly enriched network relevant to microglial function.

treated with LPS, genes with differential 5mC were found to cluster in pathways associated with both metabolic and neurological diseases (tables 3.1-3.3). In addition, DM genes were also involved in cellular morphology, movement, and survival networks, which did not appear in our analysis of LPS inflammation (figure 9). Interestingly, calcium signaling was the most highly enriched pathway (-log(p)=5.21) and was followed by other general signaling systems like GABA (-log(p)=4.82) and

glutamate (-log(p)=4.58) that were also enriched following LPS exposure. These results suggest 5mC changes can impact both inflammatory programs and inter-cellular signaling in microglia exposed to Aβ, just as in cells stimulated by bacterial pathogens. Unique to Aβ treatment, however, was modulation of neurovascular coupling signaling (-log(p)=2.05), as well as disease pathways like DNA breakage (p=0.007), size of cells (p=0.02), and microtubule dynamics (p=0.04), all of which are implicated in AD pathology. In addition, I observed many pathways directly related to AD such as the formation of senile plaques (p=0.007) and neurodegeneration (p=0.02), as well as pathways also enriched in LPS-treated cells like amyloidosis (p=0.01) and AD (p=0.007). Also in common with LPS cells was enrichment for upstream regulators APOE (p=0.01), methyl-binding protein MeCP2 (p=0.04), methyltransferase EZH2 (p=0.04), and cytokine CSF3 (p=0.03).

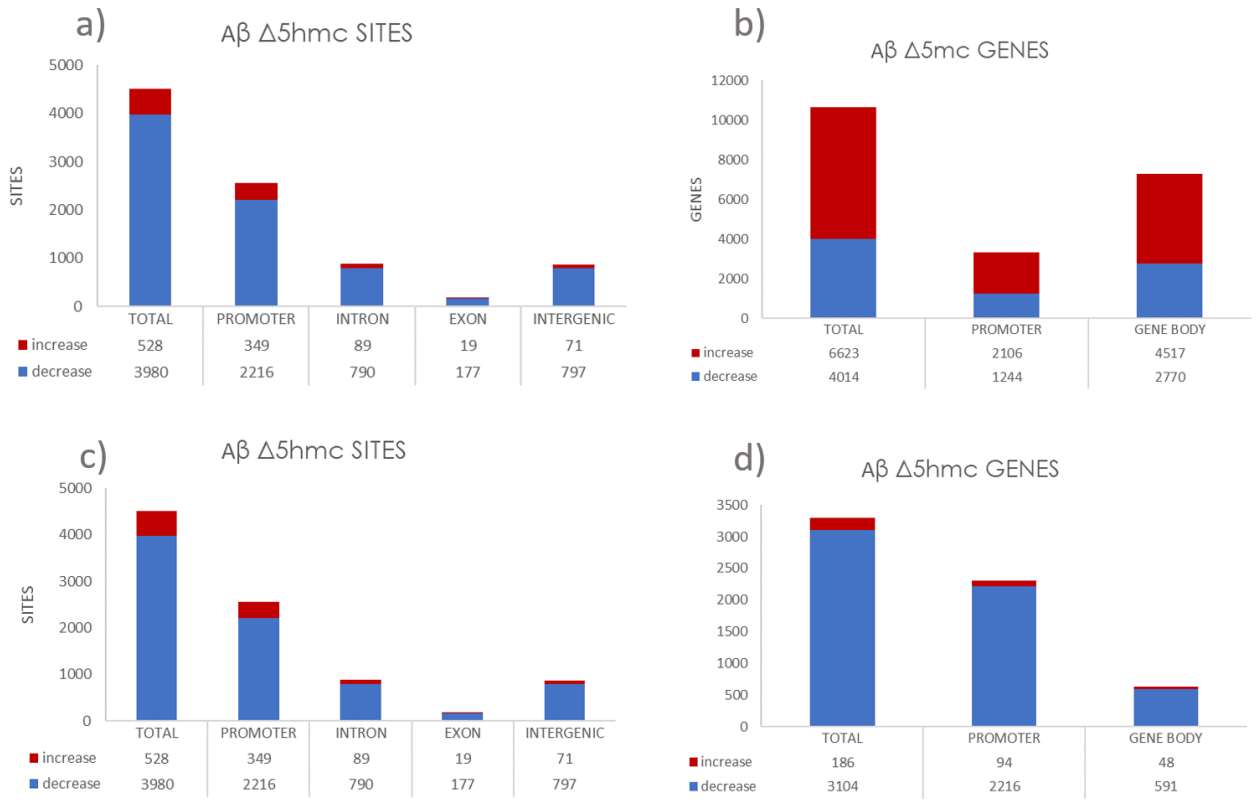


Figure 6: The distribution of sites (a,c) and genes (b,d) displaying differential 5mC (a-b) and 5hmC (c-d) following treatment with Aβ.

Table 3.1: Top canonical pathways enriched in genes differentially methylated in 5mC in Aβ-exposed cells

AB Δ5mc	canonical pathways	-log(p-value)	z score	overlap
TOTAL	Calcium Signaling	5.21	0.962250449	37/198
	GABA Receptor Signaling	4.82		27/130
	Glutamate Receptor Signaling	4.58	0.333333333	67/165
	CREB Signaling in Neurons	4.22	1.324244384	76/562
	Axonal Guidance Signaling	3.65		64/473
	G-Protein Coupled Receptor Signaling	3.11	0.447213595	80/649
	Circadian Rhythm Signaling	3.05		37/250
	Endocannabinoid Neuronal Synapse Pathway	2.82	0.426401433	24/145
	Synaptogenesis Signaling Pathway	2.12	0.666666667	99/302
	Serotonin Receptor Signaling	1.99		9/43
	RHOGDI Signaling	1.78	0.229415734	27/203
	CCR5 Signaling in Macrophages	1.73		17/114
	Corticotropin Releasing Hormone Signaling	1.66	0.229415734	20/143
	Synaptic Long Term Depression	1.42	-0.62554324	23/180
PROMOTER				
GENE BODY	Synaptogenesis Signaling Pathway	2.98	0.780868809	153/305
	Opioid Signaling Pathway	2.12	0.685994341	138/269
	Neurovascular Coupling Signaling Pathway	2.05	0.538815906	177/285

Table 3.2: Top disease pathways enriched in genes differentially methylated in 5mC in Aβ-exposed cells

AB Δ5mc	Disease	p-value	# molecules
TOTAL	Guidance of axons	0.003	16
	Breakage of double-stranded DNA	0.0075	2
	Formation of senile plaques	0.0075	2
	Alzheimer disease	0.00776	36
	Amyloidosis	0.0117	37
	Size of cells	0.0277	4
	Cell death of neuroglia	0.0305	5
PROMOTER			
GENE BODY	Accumulation of vesicles	0.00948	2
	Neurodegeneration of brain	0.0239	3
	Microtubule dynamics	0.0462	22

Table 3.3: Top upstream regulators enriched in genes differentially methylated in 5mC in Aβ-exposed cells

AB Δ5mc	Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	# targets
TOTAL	CSF3	cytokine		0.0371	3
	MECP2	transcription regulator		0.0404	2
	EZH2	transcription regulator		0.0482	6
PROMOTER					
GENE BODY	APOE	transporter		0.0104	14
	IL13	cytokine	1.807	0.472	15

Table 4.1: Top canonical pathways enriched in genes differentially methylated in 5hmC in Aβ-exposed cells

AB Δ5hmc	canonical pathways	-log(p-value)	z score	overlap
TOTAL	Estrogen Receptor Signaling	4.71	-2.13808994	17/385
	Oxytocin Signaling Pathway	3.66	-1.73205081	12/260
	Corticotropin Releasing Hormone Signaling	3.14	-1.88982237	8/143
	Opioid Signaling Pathway	2.99	-0.63245553	11/267
	Type II Diabetes Mellitus Signaling	2.45	-1	7/145
	Axonal Guidance Signaling	2.3		14/473
	mTOR Signaling	2.3	-1.13389342	8/195
	IL-7 Signaling Pathway	1.79	-1	4/72
	IL-8 Signaling	1.71	-1.13389342	7/201
	IL-3 Signaling	1.68	-1	4/78
	Autophagy	1.64	-1.13389342	7/208
	Androgen Signaling	1.62	-1	6/164
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.46	-0.4472136	6/179
	Synaptic Long Term Depression	1.45	-0.81649658	6/180
	Senescence Pathway	1.38	-1.13389342	8/288
PROMOTER	D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	2.82	-2.33333333	9/25
	Triacylglycerol Biosynthesis	2.32	-3.31662479	12/45
	Endocannabinoid Neuronal Synapse Pathway	1.98	-2.85773803	27/145
	Circadian Rhythm Signaling	1.7		41/252
	CXCR4 Signaling	1.65	-3.41121146	28/161
	Endoplasmic Reticulum Stress Pathway	1.62	-2.44948974	6/20
	Cysteine Biosynthesis III (mammalia)	1.43	-2.44948974	6/22
	D-myo-inositol-5-phosphate Metabolism	1.31	-4.81125224	30/187
GENE BODY	Dopamine Receptor Signaling	2.41	-2	9/77
	Insulin Secretion Signaling Pathway	2.26	-3.63803438	17/257
	Actin Cytoskeleton Signaling	1.7	-1.15470054	9/228
	Amyloid Processing	1.65		44/49
	eNOS Signaling	1.59	-2.52982213	10/148
	Signaling by Rho Family GTPases	1.59	-0.90453403	15/258
	GABA Receptor Signaling	1.54		9/130
	IL-13 Signaling Pathway	1.53	-1.13389342	7/90
	Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.48	-1.13389342	7/92
	Gαi Signaling	1.39	-0.37796447	9/138

Genome-wide remodeling of 5hmC in Aβ treated microglia

In the same cells used for 5mC sequencing, I also profiled 5hmC content with single nucleotide resolution in both Aβ and vehicle treated cells (figure 6). Compared to control microglia, those treated with Aβ displayed 4500 sites that significantly altered in 5hmC levels ($p<0.01$), with read-count ratios ranging from 0.00006 to 4.66.

Table 4.2: Top disease pathways enriched in genes differentially methylated in 5hmC in Aβ-exposed cells

AB Δ5hmc	disease	p-value	#molecules
TOTAL	Infection of phagocytes	0.00618	3
PROMOTER	Formation of cytoskeleton	0.000931	5
	Formation of actin stress fibers	0.00163	3
	Degenerative dementia	0.00727	47
	Alzheimer disease	0.00835	46
	Amyloidosis	0.00993	48
	Neuronal cell death	0.0123	17
	Lewy body disease	0.0321	4
	Senescence of cells	0.0324	5
	Cell death of brain	0.0389	10
	Apoptosis of brain cells	0.0416	5
	Concentration of lipid	0.0474	7
GENE BODY	Autophagy of cells	0.00437	6
	Microtubule dynamics	0.00554	12
	Cellular homeostasis	0.00729	15

Table 4.3: Top upstream regulators enriched in genes differentially methylated in 5hmC in Aβ-exposed cells

AB Δ5hmc	Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	# targets
TOTAL	IL15	cytokine	-0.132	0.0383	4
PROMOTER					
GENE BODY	CSF1	cytokine		0.0102	3
	APOE	transporter		0.0108	7
	TNFSF11	cytokine		0.0342	2

88% of these DM sites decreased in 5hmC following the Aβ treatment, as indicated by the read-count ratio. A distinctive feature of 5hmC genomic distribution following Aβ treatment is enrichment for promoter regions, which contained 57% of all DM sites, while only 196 DM sites (4%) were mapped to exons. In line with our previous analysis, I then established the genes associated with these DM sites (total n=3290), and classified them as either increasing or decreasing in 5hmC after Aβ exposure.

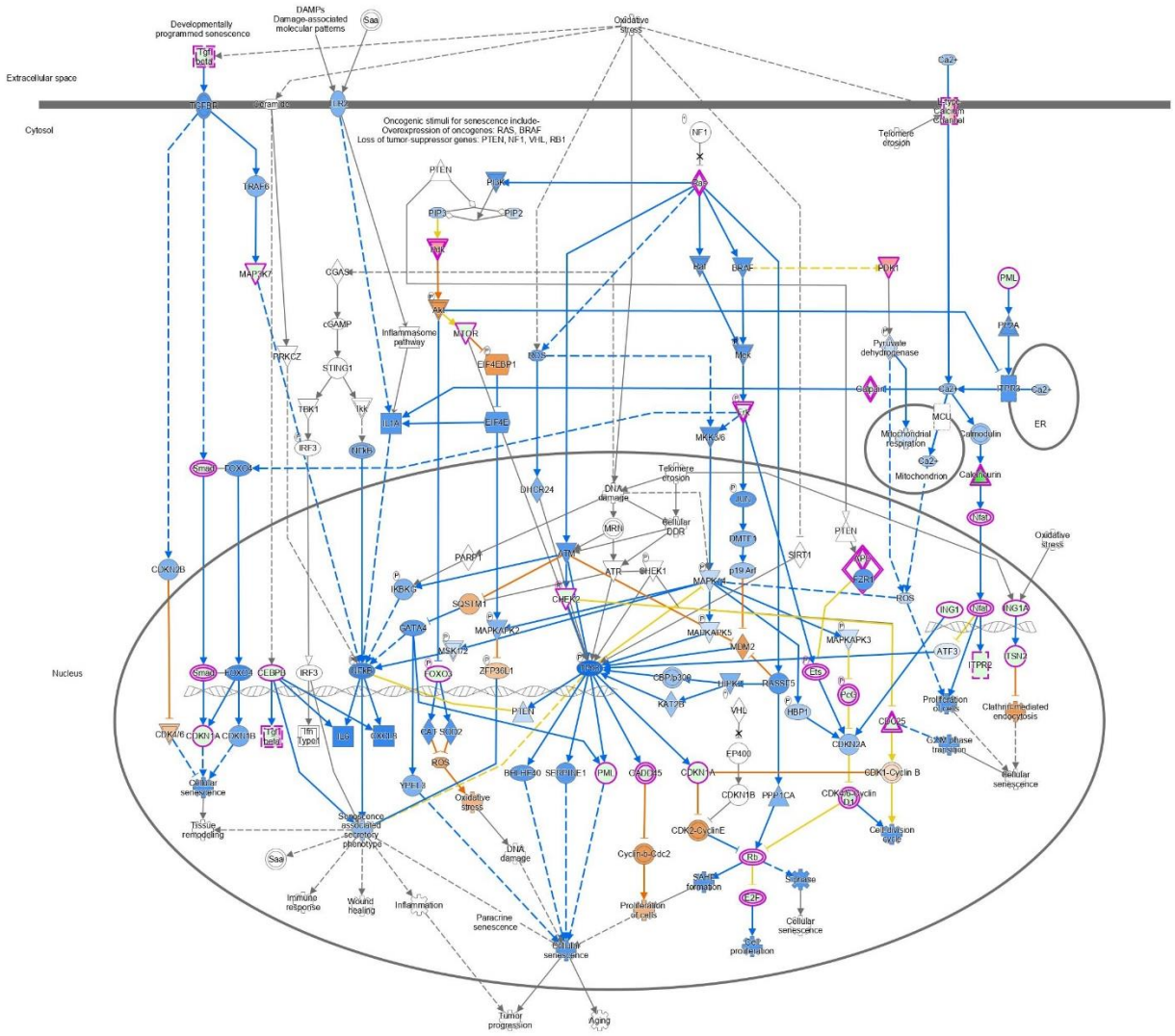


Figure 7: 5hmC changes in microglia following Aβ treatment mediate cellular senescence.

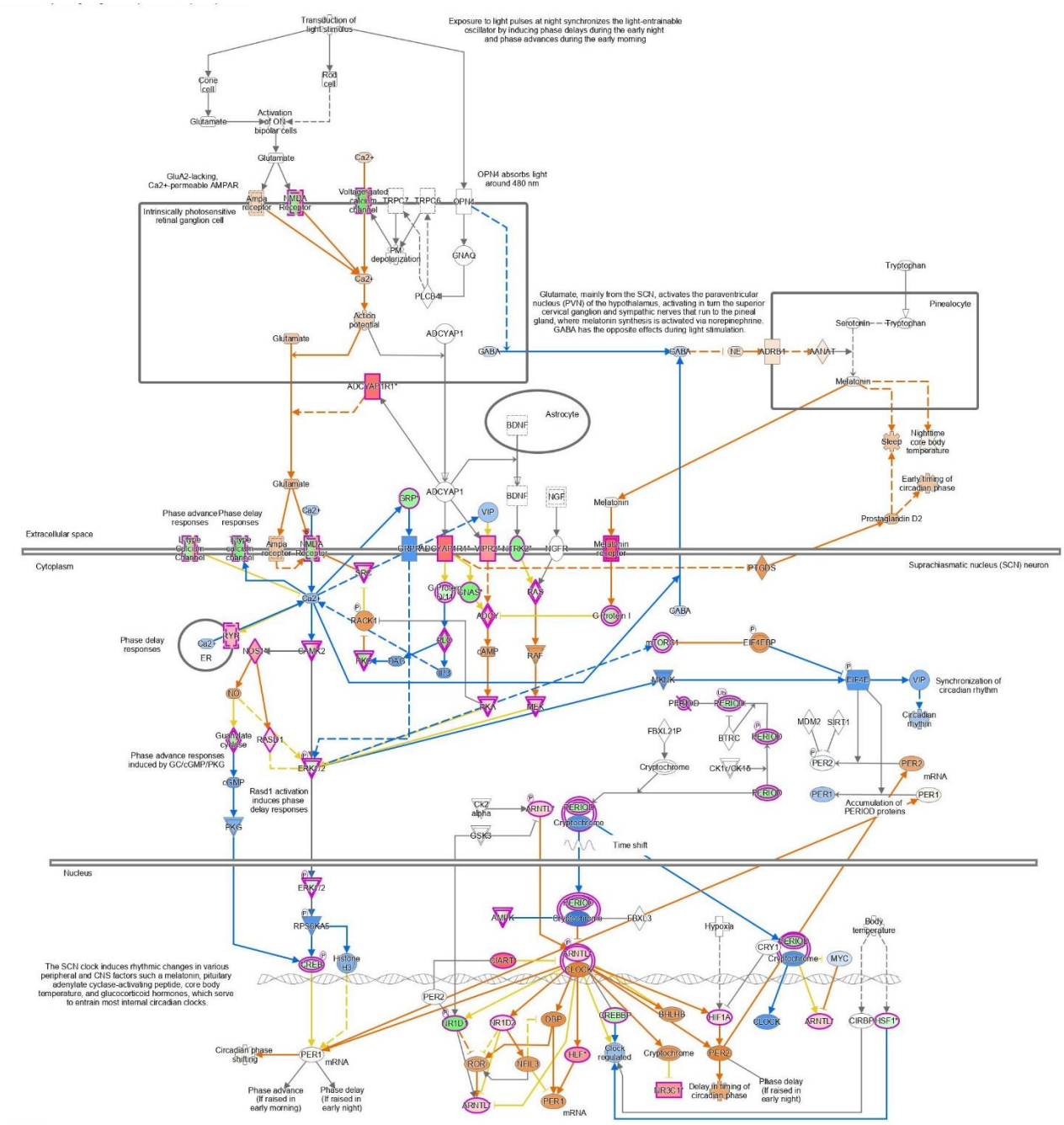


Figure 8: Differential 5mC in gene promoters following Aβ exposure affect circadian rhythm signaling.

Cellular processes affected by differential 5hmC in Aβ treated microglia

To determine the potential cellular effects of these changes in 5hmC, I assessed our lists of DM genes for pathway enrichment (tables 4.1-4.3). As before, I used a master list of genes from differential

5hmC sites in all genomic locations (total n=2185, $p<0.001$), as well as separate analysis for DM promoters (n=2235) and DM gene bodies (n=621) both with a threshold of $p<0.01$. Importantly, I identified unique canonical pathways enriched in this analysis, with autophagy (-log(p)=1.64), production of nitric oxide and reactive oxygen species in macrophages (-log(p)=1.46), and amyloid processing (-log(p)=1.66) standing out as processes highly relevant to AD pathology. Changes in 5hmC were also found to affect dopamine signaling (-log(p)=2.41), which I found to be unique to Aβ exposure. Metabolic functions also appear to be highly regulated by dynamic changes in 5hmC, as D-myo-inositol biosynthesis (-log(p)=2.82), triacylglycerol biosynthesis (-log(p)=2.32), as well as mTOR signaling (-log(p)=2.3), were all enriched in these gene sets. Aberrant lipid metabolism is a well-defined characteristic of AD-associated microglia, and these data propose an important role for 5hmC in these processes. Unique disease pathways affected by differential 5hmC include neuronal cell death (p=0.01), Senescence of cells (p=0.03), and concentration of lipid (p=0.04), though many disease pathways already mentioned in previous analysis were also enriched. Cytokines IL-15 (p=0.03), CSF1 (p=0.01), and TNFSF11 (p=0.03) were uniquely enriched as upstream regulators, along with APOE (p=0.01), which strikingly was affected by both 5mC and 5hmC changes in both LPS and Aβ treated cells.

Dissecting specific pathways impacted by differential methylation in Aβ exposed cells

The parallel analysis of a canonical inflammatory response and an AD-specific microglial response enabled us to define common and unique pathways in each process, with the goal of understanding how epigenetic remodeling contributes to AD neuroinflammation. I therefore identified genes displaying DM after exposure to both LPS and Aβ treatment, which I reasoned represent the core inflammatory response, in addition to those responding specifically to either LPS or Aβ (figure 10). I expected to identify disease-associated genes in Aβ group, but also to unravel some genes in the LPS group that are important for the resolution of inflammation that are aberrantly regulated in the Aβ group and thus contribute to the prolonged inflammation in AD. Using a p-value<0.05, I obtained a group of 1878 genes with differential 5mC in the common response, with overall methylation changes ranging

from 80% to -80%. The group of genes uniquely responding to LPS consisted of 1632 genes, and 1437 genes

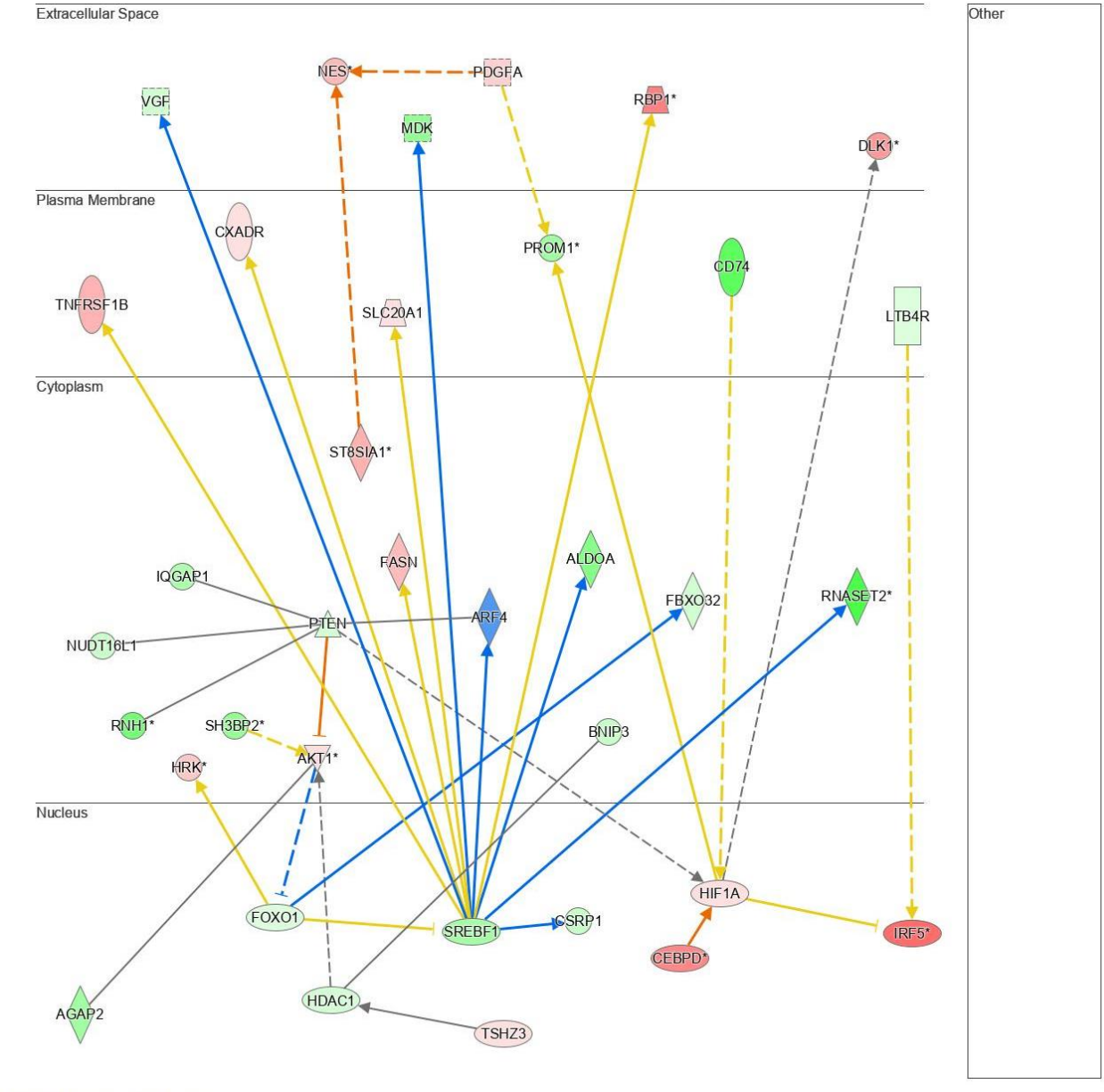


Figure 9: Signaling network involved in organismal injury and neurological diseases is enriched in genes exhibiting promoter 5mC changes in Aβ exposed cells. This represents the most significantly enriched network relevant to microglial function.

uniquely responded to Aβ. I conducted the same analysis in genes with differential 5hmC using a p-value<0.01. 1159 genes were involved in the common response to both stimuli, and exhibited read-count ratios ranging from 0.00006 to 3.33. LPS treated cells contained 1015 DM genes that were unique to this treatment, while 1151 DM genes were specific to Aβ exposure.

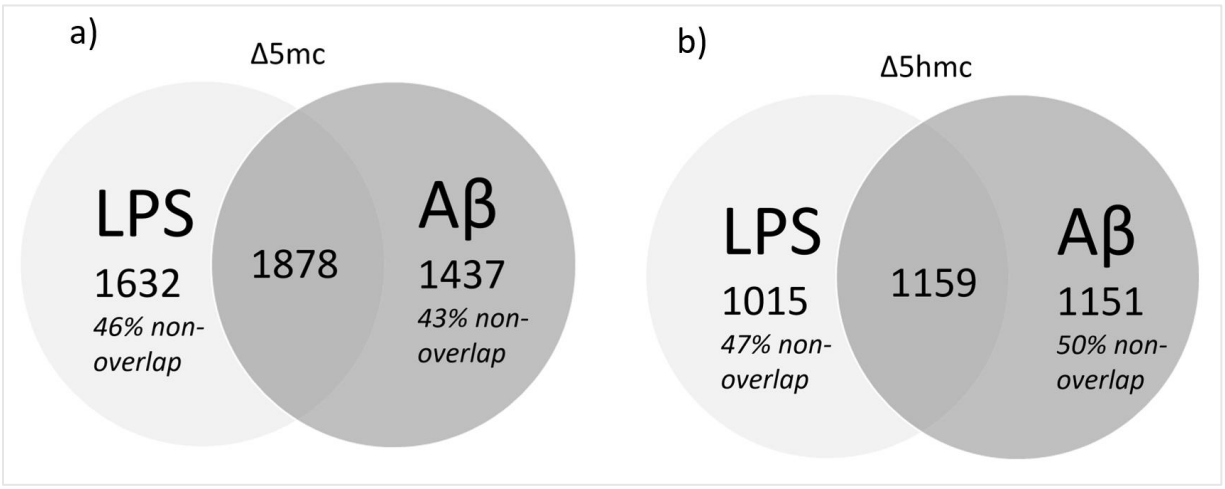


Figure 10: Overlap comparison of the differential methylation in 5mC (a) and 5hmC (b) observed following LPS and Aβ exposure

Analysis of genes exhibiting differential methylation in response to both stimuli confirmed much of the overlap observed in the previous analysis (table 5) : Circadian Rhythm signaling (-log(p)=6.47), GABA receptor signaling (-log(p)=26.35), phagosome formation (-log(p)=5.66), IL-13 signaling (-log(p)=1.5), as well as Insulin secretion (-log(p)=2.25) and type-II diabetes Mellitus signaling (-log(p)=1.97) were all enriched by 5mC changes. Differential 5hmC affected the senescence pathway (-log(p)=2.77), circadian rhythm signaling (-log(p)=1.54), and CXCR4 signaling pathways (-log(p)=1.48) following both stimuli, and degenerative dementia (p=0.001) and AD (p=0.001) were shared disease networks. Therefore, methylation appears as an important modulator of the core microglial response.

I next analyzed these new gene sets for gene ontology, to unravel the role of methylation in distinguishing between the response to the two stimuli (table 6). LPS-specific 5mC changes affected about 30 genes associated with amyloidosis (p=0.02) and AD (p=0.01). Importantly, I observed

significant enrichment (p=0.03) in the hypersensitivity response pathway, with four contributing molecules. This pathway is defined as an acquired immune response, in which second exposure to a particular stimulus produces an exaggerated and deleterious reaction resulting in tissue damage. In addition, genes showing differential 5hmC specific to LPS treatment were associated inflammatory response and infectious disease networks, with IL-7 (-log(p)=2.03), IL-10 (-log(p)=1.64), IL-22 (-log(p)=1.53), and CD40 (-log(p)=1.78) noted as specifically enriched pathways.

Table 5: Canonical and disease pathways impacted by differential methylation in 5mC (a) and 5hmC (b) that occurs following both LPS and Aβ exposure

a)

SHARED Δ5mc	
Canonical Pathways	**-log(p-value)**
Circadian Rhythm Signaling	6.47
GABA Receptor Signaling	6.35
Phagosome Formation	5.66
Endocannabinoid Neuronal Synapse Pathway	5.19
Synaptic Long Term Depression	4.5
Insulin Secretion Signaling Pathway	2.25
Type II Diabetes Mellitus Signaling	1.97
IL-13 Signaling Pathway	1.5

SHARED Δ5mc		
Disease pathways	**p-value**	**# molecules**
Migration of neuroglia	0.0476	2

b)

SHARED Δ5hmc	
Canonical Pathways	**-log(p-value)**
Senescence Pathway	2.77
Triacylglycerol Biosynthesis	2.27
Sphingosine-1-phosphate Signaling	2.15
Circadian Rhythm Signaling	1.54
CXCR4 Signaling	1.48

SHARED Δ5hmc		
Disease pathways	**p-value**	**# molecules**
Degenerative dementia	0.00129	30
Alzheimer disease	0.00197	29
Concentration of lipid	0.0247	5

In contrast, Aβ-specific 5mC changes affected genes involved in the senescence pathway (-log(p)=2.77) and LTP (-log(p)=1.58), as well as epoxysqualene biosynthesis pathway (-log(p)=2.26), a specific lipid metabolism pathway reported to be perturbed in AD brains [78] and also enriched by DEGs in our AD model. On the other hand, 5hmC changes unique to Aβ exposure affected IL-8 (-log(p)=1.31) and apoptosis signaling (-log(p)=1.55), as well as disease networks like the infection and migration of neuroglia (p=0.003) and the release of lipids (p=0.008). Thus, while LPS-induced differential methylation impacts general inflammatory signaling, changes in 5mC and 5hmC associated with Aβ exposure influence apoptosis, senescence, and lipid processing.

Table 6: Canonical and disease pathways associated with differential methylation specific to each immune challenge. a) differential 5mC unique to LPS. b) differential 5hmC unique to LPS. c) differential 5mC unique to Aβ. d) differential 5hmC unique to Aβ.

a)

LPS-SPECIFIC Δ5mc	
Canonical Pathways	**-log(p-value)**
Role of JAK2 in Hormone-like Cytokine Signaling	1.47

LPS-SPECIFIC Δ5mc		
Disease pathways	**p-value**	**# molecules**
Quantity of phosphatidylinositol-3,4,5-triphosphate	0.000411	3
Oxidation of fatty acid	0.00553	2
Quantity of phosphatidylinositol 3,4-diphosphate	0.00553	2
Alzheimer disease	0.0171	31
Amyloidosis	0.0222	32

b)

LPS-SPECIFIC Δ5hmc	
Canonical Pathways	**-log(p-value)**
IL-7 Signaling Pathway	2.03
CD40 Signaling	1.78
IL-10 Signaling	1.64
IL-22 Signaling	1.53
Phagosome Maturation	1.49

LPS-SPECIFIC Δ5hmc		
Disease pathways	**p-value**	**# molecules**
Quantity of lysosome	0.00249	2
Accumulation of phagocytes	0.0327	2
Tauopathy	0.046	21

c)

Aβ-SPECIFIC Δ5mc	
Canonical Pathways	**-log(p-value)**
Phosphatidylethanolamine Biosynthesis II	2.78
Senescence Pathway	2.77
Epoxysqualene Biosynthesis	2.26
Adipogenesis pathway	2.08
Synaptic Long Term Potentiation	1.58

d)

Aβ-SPECIFIC Δ5hmc	
Canonical Pathways	**-log(p-value)**
Apoptosis Signaling	1.55
IL-8 Signaling	1.31

Aβ-SPECIFIC Δ5hmc		
Disease pathways	**p-value**	**# molecules**
Infection of neuroglia	0.0033	2
Release of lipid	0.00852	3
Formation of microtubules	0.00952	2
Migration of neuroglia	0.0183	2
Priming of monocytes	0.0183	2

Investigating the role of DNA methylation in defining disease-associated microglial subtypes

DAMs are defined as a distinct subpopulation of microglia that appear in several neurodegenerative conditions and are associated with a chronic over-activation that results in excessive inflammation and cell death. In AD, DAMs have been shown to cluster around amyloid plaques, which suggests that Aβ is important player in the conversion to a DAM phenotype, and thus that our cellular model of AD may share similar characteristics. As DAMs are characterized by a unique transcriptional signature, I sought to evaluate whether specific genes dysregulated in DAMs (compared to homeostatic microglia) overlap with genes displaying alterations in DNA methylation in our *in vitro* model. Keren-Shaul et al. reported a distinct subtype of microglia isolated from the 5XFAD mice model of AD that was not observed in wild-type mice, providing a comprehensive list of genes that distinguish these DAMs from resting microglia. I restricted our analysis to the top 568 of these differentially expressed genes (DEGs) (p-value<0.0001) and assessed 5mC and 5hmC values for those genes across our experimental

data derived from Aβ exposed cells (figure 11). I identified circa 2% of DM genes were also differentially expressed in DAMs, totaling 37 genes changing in 5mC ($p<0.05$) and 4 genes changing in 5hmC ($p<0.001$, read count ratio >0.30)

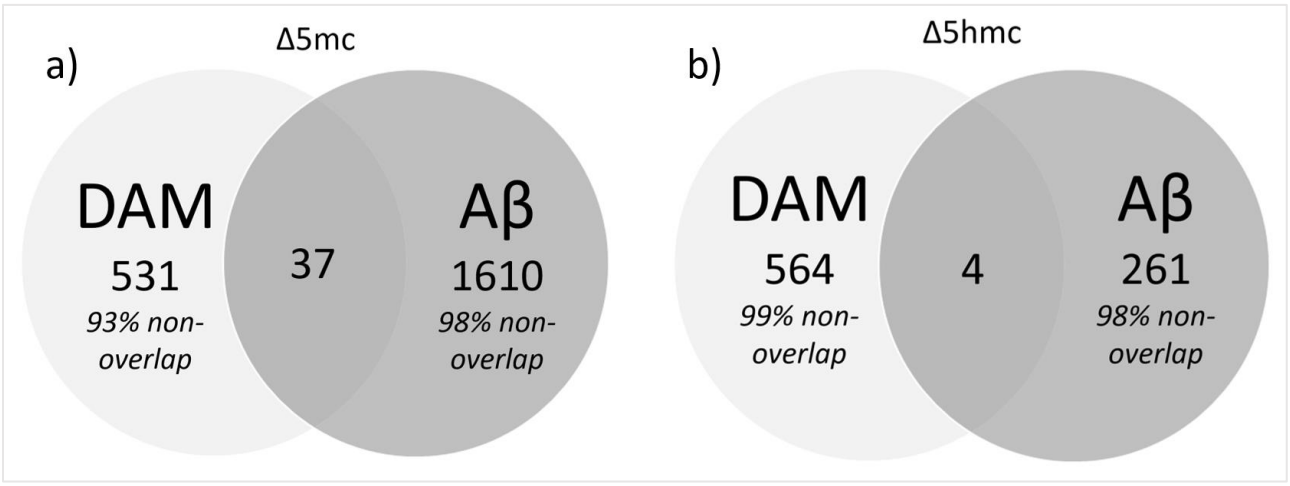


Figure 11: Overlap comparison between genes differentially expressed in an *in vivo* AD model of disease-activated microglia and genes from our *in vitro* model of AD exhibiting differential methylation in 5mC (a) and 5hmC (b)

The role of DNA methylation in directing transcriptomic changes in AD microglia

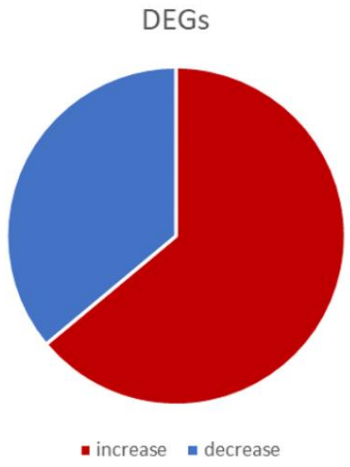
In order to elucidate the extent to which differential 5mC and 5hmC influences gene expression, I next conducted a robust analysis of the microglial transcriptome in the same Aβ- and vehicle-treated cells used for methylation profiling. Evaluation of RNA-sequencing data identified 59 genes with significantly differential expression (FDR<0.2) following Aβ treatment in comparison to control cells (figure 12). Interestingly, I found three DEGs (5% overlap) that were also reported as dysregulated in 5XFAD DAMs, thus partially validating our *in vitro* model. These genes are ATP5J2 and ATP5E, which are mitochondrial ATP synthase enzymes, and SEPP1. I observed a low correlation between methylation and transcription, however, with only four genes showing concomitant changes in 5mC. These include RNF213, ARHGAP19, SCHIP1, and DYC1C1.

I also performed GO analysis on a larger set of DEGs responding to Aβ exposure using a more relaxed criteria (p-value<0.001, n=199) (figure 12). Oxidative phosphorylation (-log(p)=1.97) and mitochondrial dysfunction (-log(p)=1.34) appeared as the top canonical pathways, among epoxysqualene biosynthesis (-log(p)=1.79) which was also enriched in genes with differential 5hmC after treatment with Aβ. Though I report minimal overlap between DEGs and DM genes, it is important to note that this metabolic pathway in addition to mitochondrial dynamics are also enriched in the analysis of differential methylation following Aβ exposure. Importantly, microglial receptors TREM2 (p=0.002) and TREM1 (p=0.049) appear as enriched upstream regulators within DEGs, with 6 and 1 target molecules, respectively, along with cytokine CCL5 (p=0.0002)

Preliminary evaluation of epigenetic mechanisms that modulate neuroinflammation *in vivo*

I finally sought to validate our results in an *in vivo* model by examining changes in the expression of methylation enzymes at the RNA and protein levels in the mouse brain. To this end, we administered LPS (1mk/kg) or saline to C57BL6/J wild-type mice via daily i.p. injection for seven days, and collected brain tissue two hours after the final injection to model acute inflammation. We isolated the hippocampus from one hemisphere for RNA extraction, and sectioned the opposing hemisphere fixed in PFA to perform immunohistochemical (IHC) analysis. Mice injected with LPS exhibited a significant increase in Il-1β transcription in the hippocampus when compared to saline-injected controls (figure 13). I observed moderate increases in Tnfα and Il-6, however these did not reach statistical significance.

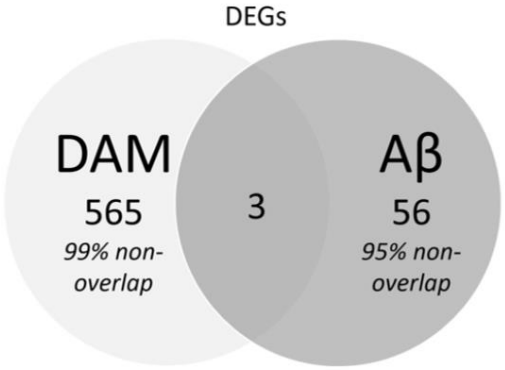
a)



b)

Gene_symbol	Fold change
NUDT4P1	1283.165
ABHD14A	394.187
SYNJ2BP	242.302
FAM101A	187.611
CSNK2A3	-176.900
PTGES3L	163.526
HIST1H4J	-145.220
TRIM6	133.405
PRR5	-108.876
ANKRD20A2	70.306

c)



d)

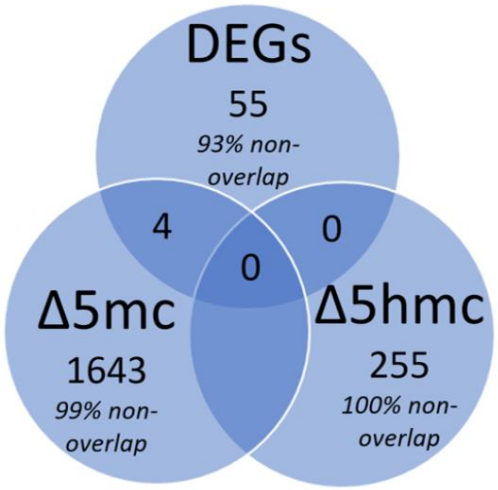


Figure 12: Analysis of differentially expressed genes (DEGs) in microglia stimulated by Aβ.
a) proportion of genes increasing and decreasing in transcript expression in Aβ -treated cells. b) top 10 changing genes ranked by the magnitude of the fold change. c) overlap comparison between genes dysregulated in an *in vivo* AD model of disease-activated microglia and genes differentially expressed in Aβ -treated cells. d) overlap comparison of DEGs and differentially methylated genes.

Importantly, I also detected a trend towards decreased (albeit not significant) transcription of methyl transferases Dnmt1, Dnmt3a, and Dnmt3b, as well as Tet1 enzymes. I used IHC detection of ionized calcium binding adaptor molecule 1 (Iba1), a specific microglial marker, to document morphology changes in different brain areas indicative of microglial activation, including engrossed cell bodies and degree of branching (figure 15). Quantification of the area covered by activated microglia showed significant increases in the hippocampus and the frontal cortex upon repeated LPS exposure (figure 14).

This measure reflects both an increased cell-count due to proliferation and the increased branching and coverage of each individual cell.

Table 7.1: Canonical pathways enriched in differentially expressed genes in Aβ treated cells.

Aβ DEGs	Canonical Pathways	-log(p-value)	Overlap
	IL-17A Signaling in Fibroblasts	2.44	3/38
	Parkinson's Signaling	2.13	2/16
	Oxidative Phosphorylation	1.97	4/105
	Epoxysqualene Biosynthesis	1.79	1/2
	DNA Methylation and Transcriptional Repression Signaling	1.4	2/37
	Mitochondrial Dysfunction	1.34	4/160

Table 7.2: Disease pathways enriched in differentially expressed genes in Aβ treated cells.

Aβ DEGs	Disease pathways	p-value	# molecules
	Progressive neurological disorder	0.00853	9
	Huntington Disease	0.00943	7
	Parkinson's disease	0.0122	4

Table 7.3: Upstream regulators enriched in differentially expressed genes in Aβ treated cells.

Aβ DEGs	Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	# targets
	SRF	transcription regulator		0.000000947	4
	CCL5	cytokine	1	0.00025	4
	MAP2K1	kinase		0.00102	2
	TREM1	transmembrane receptor	2.449	0.00282	6
	TREM2	transmembrane receptor		0.0491	1

I also investigated the expression of methylation enzymes at the protein level using IHC (figure 13). Notably, and despite inter-animal variability, I uncovered a significant decrease in the quantity of Dnmt1-positive nuclei in the frontal cortex region. Finally, our preliminary data show trends towards increases in the number of Tet2-positive cells and decreases in the quantity of Tet3-positive cells in the frontal cortex. These intriguing results extend our *in vitro* observations and support the notion that the inflammatory response requires modulation of DNA methylation *in vivo*.

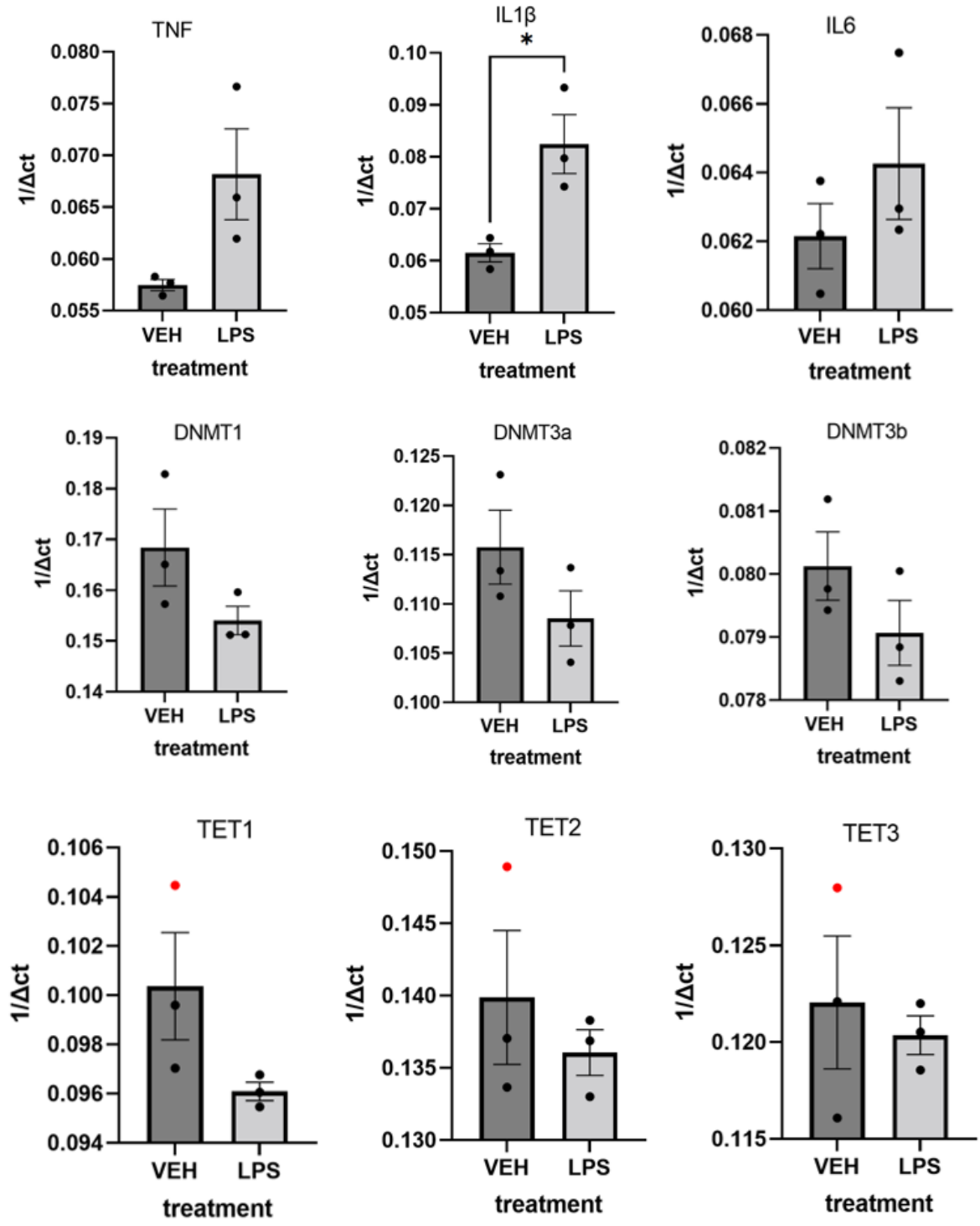


Figure 13: Changes in hippocampal RNA following repeated i.p. injections of LPS *in vivo*. Error bars represent mean+/-standard error of the mean. Red data points indicate an individual mouse across experiments

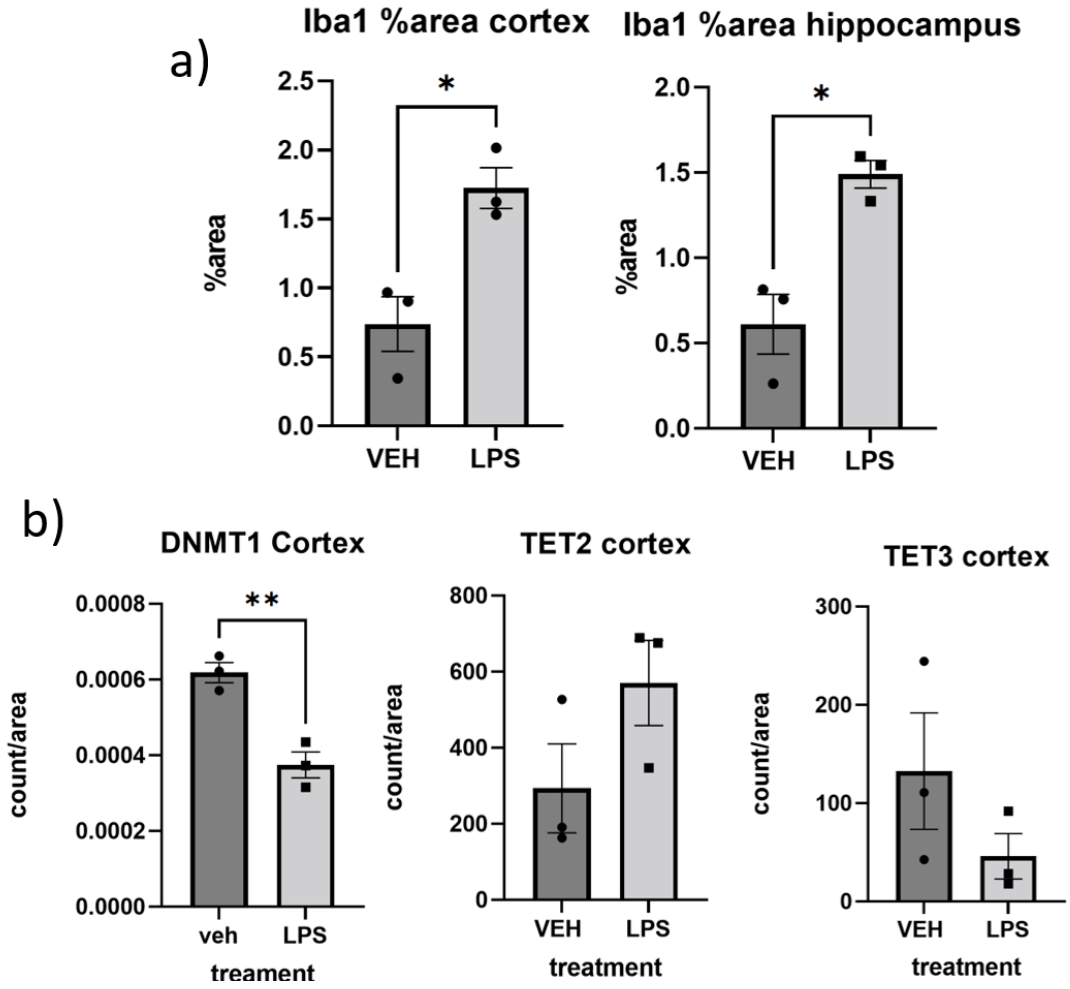


Figure 14: IHC analysis of *in vivo* LPS model of canonical inflammation. (a) the percent area occupied by ramified microglia in the hippocampus and cortex of animals injected with LPS or vehicle. (b) the number of nuclei positive for each marker in the frontal cortex of animals injected with LPS or vehicle. Error bars represent mean+/-standard error of the mean.

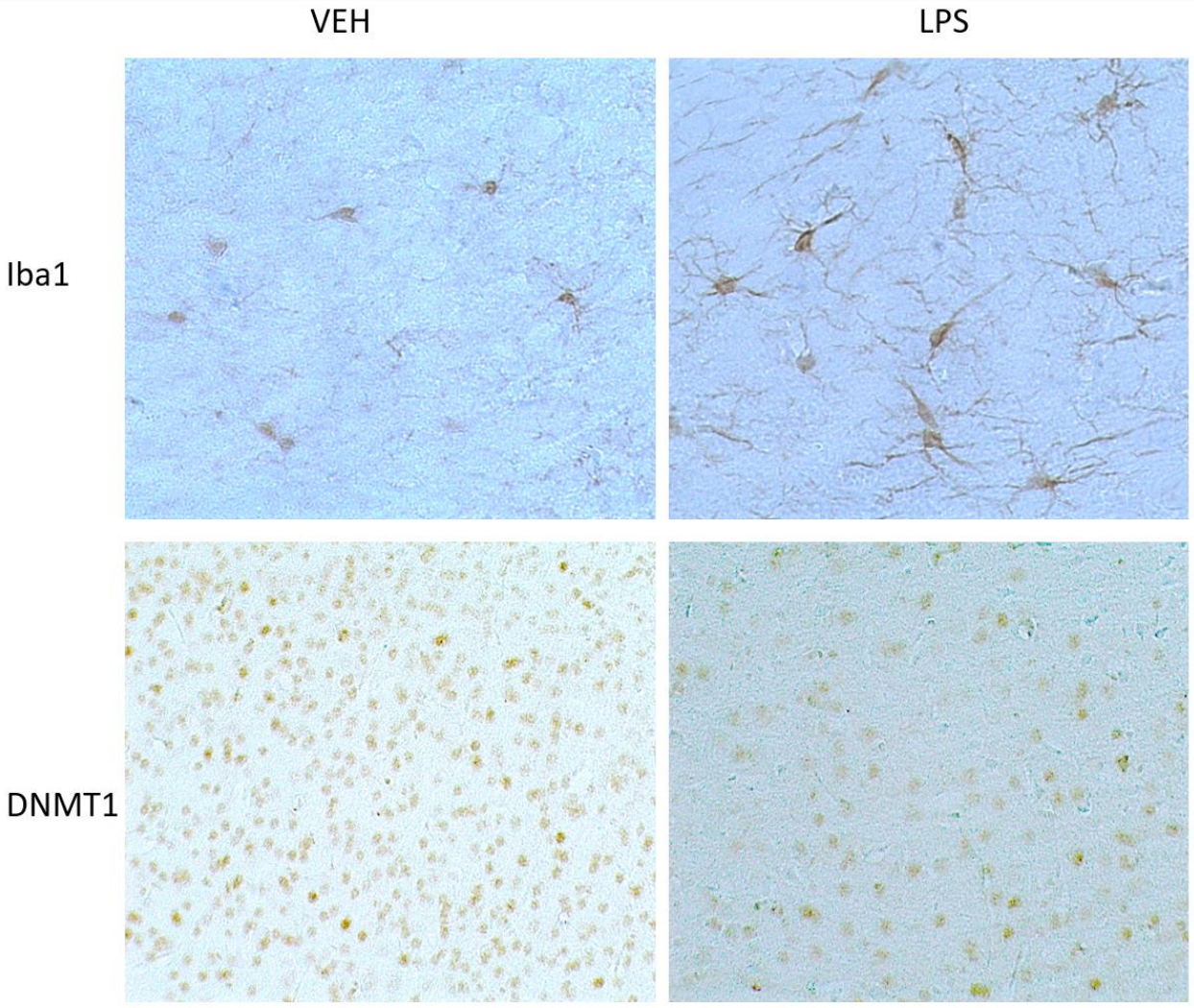


Figure 15: Representative images of IHC of Iba1 (top) and DNMT1 (bottom) in the frontal cortex of mice injected with vehicle (right) or LPS (left).

Discussion

By profiling genome-wide 5mC and 5hmC levels in LPS- and vehicle- exposed cells, I were able to identify site-specific methylation changes involved in the canonical inflammatory response. I have shown that differences in both 5mC and 5hmC contribute to specific processes involved in the propagation of inflammatory signals as well as phagocytic functions. Furthermore, by running a parallel analysis in cells treated with amyloid-beta, I have demonstrated the role of DNA methylation in differentiating between canonical inflammation and that associated with Alzheimer's Disease, providing preliminary evidence that changes in 5mC and 5hmC may contribute to the transition to a disease-activated microglial phenotype.

Differential methylation in LPS exposed cells

The idea behind using LPS-induced inflammation as a reference was to observe the methylation changes occurring during a canonical or stereotypical inflammatory response that typically concludes when the pathogen is cleared and is not exceedingly neurotoxic. I hoped to tease apart what makes amyloid-induced inflammation uniquely prolonged and deleterious from the perspective of epigenetics, while noting shared mechanisms as well as the genes or pathways that are specific to the LPS response which allow it to resolve without resulting in excessive damage.

To therefore consider LPS exposure first, a large majority of DM sites showed a decrease in 5mC or 5hmC following stimulation. These data suggest a location-specific recruitment of TET enzymes to convert both these modifications to unmethylated-cytosines, possibly in concert with decreased activity of DNMTs. In addition, I observed many more DM sites in LPS exposed cells compared to those treated with Aβ, and there were more of these DM sites per gene, which was true for both 5mC and 5hmC. This could indicate a more prominent role of DNA methylation changes in modulating transcriptomic changes in response to canonical pro-inflammatory stimuli, modeled by LPS exposure. Importantly, DM genes were enriched for general inflammation pathways, including several interleukins, CD40, and cytokine

signaling, as well as related functions like phagosome formation, cell death of neuroglia, and cellular senescence. These results illustrate the important role of DNA methylation in activating the well-described inflammatory programs associated with pathogen defense. Strikingly, many AD-related pathways were also enriched, such as amyloid processing and tauopathy. While this could stem from the longer list of genes that was submitted relative to that of Aβ-affected genes, which allows for greater overlap with more pathways, it is also likely that microglia-mediated inflammation may rely on the much of the same machinery regardless of the stimulus. Enrichment in inflammation pathways therefore prompts AD-associated GO outputs no matter the context, as these networks are known to be fundamental to AD pathology. Furthermore, while LPS is a widely used stimulant to induce canonical inflammation, it is an imperfect model of a non-pathogenic inflammatory response. Not only is it hindered by the blood-brain barrier and is thus unlikely to directly bind to microglia *in vivo* [79], with chronic administration it can also be used to model brain disorders like neurodegeneration or depression [80]. LPS may therefore activate some programs thought to be unique to AD-associated DAMs and consequently generate enrichment in neurodegenerative networks.

Differential methylation in Aβ exposed cells

Similar to the effects observed upon exposure of microglia to LPS, most sites with aberrant 5hmC profiles following treatment with Aβ also showed a decrease in methylation, a reaction likely catalyzed by TET enzymes. In contrast to LPS, however, Aβ-treated cells exhibited a larger proportion of genomic sites with increases in 5mC levels, potentially implying either an increased involvement of the DNMT family, or an inhibition of TET enzymes that prevents the subsequent conversion of 5mC to 5hmC. Genes mapped to these DM sites contribute to the inflammatory response including many interleukin signaling pathways, as well as to both neurological and metabolic diseases. Notably, there were many DM networks directly related to AD, including amyloidosis, neurodegenerative dementia, formation of senile plaques, and AD explicitly. Importantly, pathways differentially methylated upon Aβ exposure overlapped with mitochondria-related processes, as significant networks such as production of ROS, mitochondrial fission

and dysfunction were enriched here but not in LPS-affected gene sets. Toxic mitochondrial by-products are highly reactive and contribute to both protein and DNA damage, and strikingly I found differentially methylated genes to partially overlap with DNA damage repair processes as well. Oxidative stress and high levels of ROS in the brain [81] as well as in AD-associated microglia [58] are well-established mediators of AD pathology, with demonstrated links to aberrant lipid and cholesterol metabolism. Interestingly, I saw enrichment in many metabolic processes, lipid and inositol metabolism in particular, which were further confirmed by our transcriptional analysis in the same cells. Keren-Shaul et al. reported enrichment in lipid metabolism networks when first characterizing DAMs, and inositol is known to participate in APOE-related pathways in AD, increasing in concentration prior to the onset of AD symptoms [82]. The combination of these results highlights the role of DNA methylation in differentiating between canonical microglial programs and DAMs associated with chronic neuroinflammation.

I also found some general signaling systems to be exclusively enriched by DM following Aβ exposure. Estrogen signaling, which was highly affected by 5hmC changes, is involved in directing microglial programs and is generally associated with the resolution of activation [83]. Oxytocin signaling was similarly enriched by Aβ exposure, and has been demonstrated to attenuate the microglial response to LPS *in vivo* [84]. Finally, dopamine receptor signaling was also influenced by differential 5hmC in gene bodies, and is implicated in neuro-immune crosstalk in the context of neurodegeneration [85]. Though I cannot infer whether these signaling pathways were enhanced or repressed by the observed changes in 5hmC, it is feasible that differential methylation induced by Aβ inhibits the conclusion of inflammation by interfering with these signaling cascades.

By evaluating the transcriptional changes occurring in the same cellular model used for our methylation analysis, I was able to postulate the extent to which differential methylation is involved in influencing gene expression. While the overlap of differentially methylated (DM) genes and differentially expressed genes (DEGs) was actually quite small, I discovered enrichment in many of the same cellular

pathways. Critically, these pathways are the major differentiators between canonically activated microglia and dysfunctional neurotoxic microglia. DEGs were heavily involved in mitochondrial functions like oxidative phosphorylation, and were even enriched for the use of DNA methylation as a transcriptional regulator. These results further support DNA methylation as a previously unreported mechanism by which microglia transition to a DAM phenotype.

Differential methylation in common between LPS and Aβ exposed microglia

As expected, I observed differential methylation in many overlapping genes between the microglial response to both LPS and Aβ. The common pathways populated by these DM genes correspond to the core functional response of microglia to all pro-inflammatory stimuli. For example, CXCR4 and CCR5, which direct signaling cascades enriched in both our LPS and Aβ data, are chemokine receptors expressed in macrophages including microglia. A physical relationship has been established between the pair, along with dysregulated expression across several neurodegenerative diseases [86]. In addition, IL-8 and IL-13 are involved in proinflammatory and inflammatory signaling, respectively, and mediate pathways that were enriched in several of our gene sets. Cellular senescence was also highly enriched in many of our analysis, albeit more saliently in Aβ exposed cells. This cellular fate is elicited by many challenges like aging, stress, DNA damage, as well as AD, and is associated with an arrest of the cell cycle as well as the propagation of pro-inflammatory signals. In fact, senescent microglia share many of the same features as DAMs, and represent another distinct microglial phenotype associated with chronic neuroinflammation and cognitive decline [87]. Finally, genes differentially methylated following both LPS and Aβ exposure were enriched in many synaptic remodeling processes like LTP, LTD, and synaptogenesis. Microglial over-activation in AD is thought to result in excessive engulfment of synapses, contributing to the pathological synapse loss that drives cognitive deficits. As the reference pathways used in our GO analysis included all CNS tissue, however, it is unclear the extent to which these pathways represent microglia-specific processes or neuronal mechanisms. By implicating

DNA methylation in directing these processes, I provide a potential therapeutic avenue for rescuing critical features of pathology.

Some networks enriched by DM following both LPS and Aβ exposure are related to AD more indirectly. Melatonin and Circadian rhythm signaling, for example, are highly intertwined with neurodegeneration, as alluded to by the above overview of AD symptoms. While the pathway I reference in our results is executed in the master circadian regulatory center, the suprachiasmatic nucleus (SCN), there are also intracellular rhythms that coordinate periods of high energy catabolism with periods of energy consumption and anabolism. While disturbances in this cycle throughout the brain contribute to neurodegeneration and vice versa, the expression of microglial inflammatory factors is also reportedly cyclic [88], a process that our data suggests may depend on differential DNA methylation as well. Furthermore, while this signaling pathway was enriched by DM following both LPS and Aβ stimulation, these two challenges did not perturb circadian rhythm signaling in precisely the same way. Other intriguing results from our GO analysis is the enrichment in insulin secretion signaling and type II diabetes mellitus in both LPS and Aβ gene sets. AD is known to result in an acquired insulin resistance in the brain due to disturbances in receptor expression and intracellular signaling cascades, and our present data suggest that exposure to Aβ or LPS induces changes in the 5mC and 5hmC landscape that may contribute to the differential expression of these receptors and downstream signals.

Also of special interest are the upstream regulators enriched in our data sets, many of which were in common between LPS and Aβ affected genes. The most notable of which is APOE, which was discussed above as the highest known risk factor for the development of AD and a major mediator of lipid signaling. It is also recognized as an integral regulator of the microglial response to Aβ, directing phagocytic functions like Aβ clearance [89]. Furthermore, the APOE pathway together with TREM2 is thought to mediate the switch from homeostatic to dysfunctional neurodegenerative microglial phenotype [90]. LRP1 is also involved in APOE signaling cascades, and is known to be a major constituent in the direction of inflammatory processes [91]. Several cytokines were also noted as upstream regulators, such

as CSF3, TNFSF11, IL-13 and IL-15. The finding that these crucial regulatory mechanisms are influenced by differential methylation is paramount, and highlights the need to unravel the mechanisms that catalyze locus-specific methylation changes. It was therefore notable to find enrichment of MBD2 and MeCP2, which are methyl-binding proteins that actuate the regulatory effects of differentially methylated DNA, as well as EZH2, a methyltransferase that mediates chromatin remodeling. Not only do these results provide a proof-of-concept that DM genes are associated with appropriate regulatory mechanisms, they also yield specific targets that are involved in coordinating cellular processes affected by dynamic methylation.

I was also intrigued by the generic signaling systems that were enriched in our cells following both the immune challenges. For example, GABA and glutamate receptor signaling were similarly enriched upon LPS and Aβ treatment. Though they are highly abundant neurotransmitters, these receptors are also expressed in microglia, and their activation has been demonstrated to mediate the release of pro-inflammatory factors like the interleukins. Importantly, Aβ can even directly activate some isoforms of microglial glutamate receptors [92]. Serotonin signaling and CRH were also affected by dynamic methylation changes in both LPS and Aβ treatment groups. Microglial serotonin receptors are integral for directing neurodevelopmental processes, and may also be involved in phagocytic functions as well as inflammatory responses [93]. CRH signaling, which manages organismal and cellular responses to stress, represents the critical entanglement of chronic stress and neurodegeneration. Stress is associated with over-activation of the hypothalamic-pituitary axis (HPA), which in turn increases CRH signaling. Stimulation of this pathway is known to trigger microglia into adopting a pro-inflammatory phenotype, and is associated with an increase in ROS as well as neurodegeneration like AD. I also observed enrichment in Rho GTPase signaling, including RhoGDI. These molecules belong to a large family of GTPases, which catalyze many cellular processes including morphology, migration, and cell death. The role of these enzymes in neurodevelopment is well defined, though mounting evidence suggests that disruptions in these signaling cascades may also contribute to neurodegeneration. Interestingly, RhoA has

been demonstrated to play an important role in mediating microglial activation, influencing phagocytic functions as well as the production and release of both pro-inflammatory factors and ROS [94].

Limitations of the study

Of course, there are some shortcomings of this study, the most fundamental of which is that when microglia are removed from the brain to be studied *in vitro*, they have repeatedly been shown to behave differently than when they reside in the brain. As these cells are constantly monitoring fluctuations in the composition of the extracellular matrix as well as integrating signals from surrounding cells, it is not surprising that they perform differently when isolated in culture media. The high overlap of our DM genes with published DAM-associated genes profiled *in vivo*, however, provides some validation to our AD model. Our experimental paradigm posed another limitation, in the time at which the DNA and RNA samples were collected. As the peak induction of inflammatory RNA occurs at about 4 and 12 hours for *in vitro* LPS and Aβ exposure respectively, DNA and RNA were collected 24 hours after exposure to focus on mechanisms of inflammation resolution. This may have overlooked some of the transient fluctuations in RNA, as well as the temporary addition or removal of 5mC or 5hmC that may mediate them. Another, albeit small, technical limitation is that our gene ontology programs were based on packages designed for transcriptomics, and there are yet no specific pipelines that infer gene networks from methylation data. One consequence is that the multiple DM sites mapping onto a single gene had to be compressed into one overall methylation change to project into gene-specific analysis. While this caused us to overlook the significance of differentially methylated regions in close proximity, which are an important feature of epigenetic regulation and which will be investigated in the future, the high specificity of the networks generated by our analysis, the high overlap of these with DAMs profiles obtained *in vivo*, and the correlation between methylation and gene expression support the validity of our approach.

Microglial memory stored in differential DNA methylation

Risk of developing AD is significantly exacerbated by stress or infection early in life, a well-characterized phenomena thought to rely in part on epigenetic imprinting [95]. The variations in methyl markers documented in the present study may be preserved past the peak of inflammation, and therefore represent a form of epigenetic memory that can be transmitted to cellular progeny. This perspective allows another plausible explanation for why AD-related pathways appeared in the GO analysis of LPS-stimulated cells: these networks were not necessarily activated during the cells' reaction to LPS, as alterations in the methylome do not always translate to revisions in gene expression. Therefore, pathways like amyloid processing or tauopathy may have been epigenetically primed by the exposure to LPS, to then be differentially activated if the cell were to encounter an AD-related stimulus later in life. This idea is supported by the observed enrichment of the hypersensitivity response in our LPS exposed cells, which provides preliminary evidence of a DNA methylation-based priming mechanism. To test this hypothesis in a future study, a mouse model of AD, along with appropriate controls, will be injected with LPS or saline at a young age, to then be followed until after the onset of AD pathology. I will measure the influence of LPS on exacerbating pathology, by measuring plaque-load or cognitive impairments in transgenic versus non-transgenic mice. I would also examine how LPS primes microglia by comparing the activation of LPS-injected mice to those injected with saline. To unravel the dependence of this immune priming on differential methylation, I will then isolate the microglia from brain tissue and apply the same methods used in this study to analyze changes in the methylome.

***In vivo* changes in methylation machinery**

Our cellular model was further validated by our findings *in vivo*. Following repeated i.p. injections of LPS, I detected activated microglia morphologies throughout the brain combined with increases in the expression of microglial pro-inflammatory factors IL-1b, TNF, and IL-6 in the mouse hippocampus. As I analyzed RNA from whole hippocampal tissue, these results may have been diluted by RNA from neurons, and thus could be further unraveled by isolating microglia. Additionally, I discovered

moderate decreases in the expression of all DNMT enzymes in the hippocampus at the RNA level, and significant decreases in the number of DNMT1-positive cells in the frontal cortex at the protein level. Though this decrease in cell count may represent cell death, a decrease in DNMT expression or activity could contribute to the decrease in both 5mC and 5hmC that I report in my *in vitro* findings. To determine the extent to which these changes in protein expression localize in microglia in future studies, I will employ immunofluorescence to double-stain these enzymes with microglial-specific markers to restrict our analysis to only our cells of interest.

Concluding remarks

In conclusion, the results from the present study strongly implicate DNA methylation as a means by which microglia mount an inflammatory and phagocytic response to different stimuli, and may provide a mechanistic basis for the heterogenous microglial population observed *in vivo*. I have shown that specific microglial cell processes involved in both general inflammation and AD-related DAM responses are associated with dynamic changes in the methylome, which allow the activation of specific programs in response to particular immune challenges.

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