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Epigenetic Regulation of Gene Expression in Temporal Lobe Epilepsy

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EPIGENETIC REGULATION OF GENE EXPRESSION IN TEMPORAL LOBE EPILEPSY

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GENETICS, GENOMICS, AND BIOINFORMATICS

ABSTRACT

Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy and is characterized by seizures originating from the temporal lobe of the brain. A frequent seizure focus in TLE is the hippocampus, a structure located within the temporal lobe crucial for its role in memory formation. Seizures are an excess of synchronous excitation in the brain caused by a surplus of excitatory neurotransmission and a lack of sufficient inhibitory transmission. Coordinating the process of neuronal transmission is differential expression of genes likely resulting in an increase or decrease in proteins responsible for neural signaling such as neurotransmitters, ion channels, and critical factors such as BDNF (brain-derived neurotrophic factor). These genes are actively regulated through epigenetic mechanisms such as DNA modifications, histone modifications, and non-coding RNAs rapidly turning the expression of genes on and off in response to inputs from the neuronal network. In this dissertation, I investigate the distribution of DNA Hydroxymethylation (5-hmC) in the epileptic rodent hippocampus. Through hmeDIP-sequencing, we discover that 5-hmC is lost to a greater extent across the genome than the more regularly studied DNA 5-methylcytosine methylation (5-mC) and create a map of epigenetic 5-hmC loss. We find that the majority of 5-hmC loss occurs at intergenic

regions, and identify differential methylation of genes involved in several epilepsy-associated pathways. In addition, we investigate the potential of epileptic differential DNA Hydroxymethylation (DhM) in the regulation of epileptic gene expression. We find that both hyper- and hypo- 5-hmC genes exhibit loss of gene expression, implicating the involvement of multiple gene regulatory mechanisms and cell types in 5-hmC control of epilepsy. We also investigate the histone H3k9me2 methyltransferase G9a in seizures. We find that conditional neuronal loss of G9a in the dentate gyrus leads to reduced seizure threshold in fl/fl KO animals and in fl/wt KD animals. This adds to evidence of the involvement of the H3k9me2 gene regulation pathway in seizure development and adds support to further investigation of G9a as a crucial seizure regulator.

Keywords: epilepsy, epigenetics, hydroxymethylation, G9a, seizure, sequencing

DEDICATION

I dedicate this dissertation to my family.

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THE EPIGENETICS OF EPILEPSY AND ITS PROGRESSION

LIST OF ABBREVIATIONS

5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
AAV	Adeno-associated virus
AED	Anti-epileptic drug
AMPK	5’adenosine monophosphate activated protein kinase
AP	Anteroposterior
CA	Cornu ammonis
CGI	CpG Island
ChIP	Chromatin immunoprecipitation
CpG	Cytosine-phospho-guanine
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTCF	CCCTC-binding factor
DALYs	Disability-adjusted life years
DAPI	4’,6-diamidino-2-phenylindole; nuclei stain
DEG	Differentially expressed gene
DG	Dentate gyrus
DhM	Differentially hydroxymethylated
DhMR	Differentially hydroxymethylated regions
DMNT	DNA methyltransferase
DMR	Differentially methylated region

DREADDs	Designer receptors exclusively activated by designer drugs
DNA	Deoxyribonucleic acid
DV	Dorsoventral
EC	Entorhinal cortex
EEG	Electroencephalogram
FDR	False discovery rate
FISH	Florescent in situ hybridization
FL	Floxed
GABA	Gamma-aminobutyric acid
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein; astrocyte marker
GFP	Green Fluorescent Protein
GLP	G9a-like protein
GO	Gene Ontology
HAT	Histone acetyltransferase
HDAC	Histone Deacetylase
Het	Heterozygous
HS	Hippocampal sclerosis
hSyn	Human synapsin
IACUC	Institutional animal care and use committee
IEG	Immediate early gene
IHC	Immunohistochemistry
ILAE	International league against epilepsy

IP	Intraperitoneal
iPSCs	Induced pluripotent stem cells
KA	Kainic acid (Kainate)
KD	Knockdown
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knock out
lncRNA	Long non-coding RNA
LTD	Long-term depression
LTP	Long-term potentiation
meDIP	methylated DNA immunoprecipitation
meDIP-seq	Methylated DNA immunoprecipitation sequencing
mIPSC	Miniature inhibitory postsynaptic currents
miRNA	MicroRNA
ML	Mediolateral
mRNA	Messenger RNA
NeuN	Neuronal nuclei; neuron marker
ncRNA	Non-coding RNA
NIH	National Institute of Health
NIMH	National Institute of Mental Health
NINDS	National Institute of Neurological Disorders and Stroke
PBS	Phosphate-buffered saline solution
Pilo	Pilocarpine
PRC2	Polycomb repressive complex 2

PTZ	Pentylenetetrazol
PV	Parvalbumin
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
scRNA-seq	Single cell RNA sequencing
SE	Status epilepticus
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
snRNA-seq	single nuclei RNA sequencing
SUDEP	Sudden unexpected death with epilepsy
TAD	Topologically associated domain
TBI	Traumatic brain injury
TET	Ten-eleven translocation enzyme
TLE	Temporal lope Epilepsy
TSS	Transcription start site
VPA	Valproic acid
WB	Western blot
WT	Wildtype

INTRODUCTION

Introduction to Epilepsy

Epilepsy

Epilepsy is a neurological disorder characterized by multiple unprovoked seizures. Epilepsy is the most common neurological disorder in the world affecting more than 50 million people worldwide. These seizures are caused by an excess of excitatory synchronous neural activity and can greatly affect patient quality of life. Seizures can broadly be grouped into two categories depending on where seizures occur and originate. Generalized seizures happen across the entire brain and frequently manifest in grand mal seizures characterized by violent uncontrollable muscle contractions. Focal seizures occur in an isolated region of the brain, and the physical manifestation can vary depending on the affected structures. It is important to note that seizure activity can spread throughout the brain, and a seizure originating in one region can then grow to affect nearby structures or the whole brain. Despite its prevalence, around one-third of people with epilepsy do not have control of their seizures using existing medication. Pharmacoresistant patients may undergo surgery to remove seizure origin sites (Engel, 2014).

Seizures can develop into Status Epilepticus (SE), defined as a severe prolonged seizure event lasting more than 5 minutes or multiple seizures without complete regain of consciousness between them. In the most severe cases, a SE event lasting at least 30 minutes causes damage to the brain (Trinka et al., 2015). In addition to being a primary neurological disorder, epilepsy often occurs alongside other conditions. Autism,

Alzheimer's disease, and traumatic brain injury (TBI) are three conditions associated with seizures as a possible symptom. About 5% of people with epilepsy developed epilepsy following a TBI (Agrawal et al., 2006). 10-22% of people with Alzheimer's Disease will have at least one seizure (Mendez & Lim, 2003), and patients with dementia will experience seizures at a rate about 10 times more than the general population (Hauser et al., 1986). Around 20% of people with autism also experience epilepsy, with occurrence higher in patients also exhibiting intellectual disability, and many genes have been associated with autism and genetic epilepsies (Besag, 2017). Combined, epilepsy and epilepsy related disorders present a significant burden on the world's healthcare system ranking fourth in disability-adjusted live years (DALYs) (Whiteford et al., 2015).

Comorbidities

Around half of the individuals with epilepsy experience at least one comorbidity (Keezer et al., 2016). Many patients with temporal lobe epilepsy experience memory loss as a comorbidity, especially patients with temporal lobe epilepsy where the hippocampal formation is often affected. Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy and describes epilepsy with focal seizures originating from the temporal lobe of the brain. Seizures in these individuals may manifest as staring, confusion, and loss of awareness. Many patients describe odd feelings such as deja vu or strange smells or tastes during a temporal lobe seizure, and patients may experience memory loss or difficulty speaking following the seizure (MayoClinic.org, 2021).

The hippocampus is a region crucial for memory formation located within the temporal lobe that is frequently affected in patients with temporal lobe epilepsy.

Memory loss and impaired cognition are frequently listed as a top complaint of epileptic patients affecting 40-60% of individuals (Fisher et al., 2000). Bjørke et al recently have shown that evidence of cognitive impairment at epilepsy diagnosis (Bjørke et al., 2021). In addition to memory impairment due to epilepsy, it has been reported that AEDs can also impair cognition (Kwan & Brodie, 2001).

Anxiety and depression are reported more frequently in people with epilepsy. People with epilepsy can often experience fear over the next seizure occurrence, and many patients express feelings that their seizures limit their independence and quality of life (Fisher et al., 2000). Depression and anxiety are highest among individuals with uncontrolled seizures. This can lead to an increase in suicidal ideation and one population study reported the risk of suicide was 3 times higher than the general population in people with epilepsy (Christensen et al., 2007; Kwon & Park, 2014).

With recurrent seizures, some people can experience damage to the brain. In the case of TLE, some people will develop hippocampal sclerosis. Patients with hippocampal sclerosis are frequently drug resistant. Hippocampal sclerosis consists of gliosis of the hippocampus and neuronal death. Gliosis can spread to surrounding structures such as the amygdala causing further damage (Yilmazer-Hanke et al., 2000). Gliosis is caused by an abundance of reactive glia occupying the tissue. Hippocampal sclerosis can be divided into 3 types defined by the International League Against Epilepsy (ILAE). Types 1-3 define types of cell death within the hippocampus, with type 1 affecting mostly CA4 and CA1 pyramidal neurons without gliosis, type 2 affecting CA1 pyramidal neurons with gliosis, and type 3 affecting mostly CA4 neurons with gliosis. In addition, roughly 20% of patients who undergo surgical resection of their hippocampus as treatment for

refractory epilepsy will have tissue gliosis but no neuronal death (Blümcke et al., 2013). Classification of hippocampal sclerosis in the resected tissue can predict surgical outcomes with around 70% of patients free of seizures 2 years following surgery with type 1 sclerosis and 40% for type 2 sclerosis (Thom et al., 2010; Walker, 2015).

Sudden unexpected death with epilepsy (SUDEP) is a risk in people with epilepsy. SUDEP is defined as deaths of people with epilepsy with no apparent cause. SUDEP is a rare outcome in patients with epilepsy, affecting roughly 1 per 1000 children with epilepsy per year, and up to 6 per 1000 adults per year, and the risk of SUDEP is highest in individuals with generalized tonic clonic seizures (Wicker & Cole, 2021)

Animal Models of Epilepsy

Several animal models are used to study epilepsy in the lab. Some genetic epilepsies are studied through the use of transgenic mice (Yang & Frankel, 2004). A common way to study epilepsy is using acquired epilepsy models such as electrical or chemical kindling and traumatic brain injury. Electrical kindling is caused by electrical stimulation of the brain to promote seizures. Chemical kindling refers administration of a chemo convulsant to induce seizures, this can be administered either systemically or injected into the brain.

Kainic acid (KA) is a glutamate analog and injection intraperitoneally (IP) into rats induces SE. Pilocarpine (Pilo) is a muscarinic cholinergic agonist that when injected IP will induce SE. Following SE the animals will enter a silent period where they experience no seizures. After several weeks, the animals develop epilepsy and begin spontaneously seizing. Rats and mice are among the most common model organisms

used in epilepsy research. Zebrafish have recently gained popularity in epilepsy research for genetic epilepsies and for their ease of use in high-throughput drug screening. In addition, zebrafish embryos are transparent allowing visualization of brain structures and activity using current imaging techniques (Griffin et al., 2016; Scharfman, 2019).

Epileptogenesis

Seizures can be triggered by a variety of events including fever, infection, and trauma. Not everyone who experiences a seizure will develop epilepsy, however, some will go on to have recurrent seizures and be diagnosed with epilepsy. This process of epilepsy development is referred to as epileptogenesis. A critical timepoint for epilepsy intervention is after the initial seizure or insult prior to epilepsy development. By further understanding the process of epileptogenesis, we may be able to prevent epilepsy development through novel therapeutics (Lubin, 2012).

Epigenetics in Epilepsy

Introduction to Epigenetics

Epigenetics broadly refers to anything altering DNA without changing the DNA sequence. These modifications can be acquired or inherited and are crucial in controlling gene expression by regulating gene transcription. Altered gene expression through variations in epigenetic marks on the DNA determine cell identity and function. The epigenetic landscape of DNA is constantly changing as a normal part of cellular function. DNA increases in methylation as a part of the aging process, and rapid alterations in epigenetic marks are required for processes such as neuronal signaling necessary for tasks

like memory formation (Butler et al., 2019; Irwin et al., 2021; Jarome et al., 2021; Webb et al., 2020). Epigenetic modifications can also be altered in disease states, causing cells to function in an abnormal way. Symptoms can be worsened or alleviated by targeting specific epigenetic modifiers, for example Li et al showed improvement of memory in a transgenic mouse model of Alzheimer's disease by increasing expression of the enzyme Tet2 responsible for oxidating DNA methylation. By decreasing expression of Tet2, they impaired memory (Li et al., 2021).

DNA is organized in the nucleus in tightly compressed chromosomes. DNA is wrapped around a core histone complex called a nucleosome. Several nucleosomes on a string of DNA or "beads on a string" make up chromatin which is then tightly wound into chromosomes (**fig. 1**). The core histones exist in octamers consisting of histone H2A, H2B, H3, and H4 (**fig. 2 bottom)**. Chromatin can be generally classified as heterochromatin or euchromatin. Heterochromatin is tightly compacted, making it difficult for RNA polymerase to access the DNA resulting in gene silencing. Euchromatin is more loosely compacted, more easily accessed by polymerase, and is associated with gene activation. Proteins can bind to epigenetic modifications recruiting repressive or enhancing complexes to alter gene regulation (Hauser et al., 2017; Irwin et al., 2021).

DNA methylation

DNA methylation (5-mC) was the first epigenetic modification discovered (Hotchkiss, 1948). DNA can be methylated at both cytosine and adenosine bases, resulting in DNA 5-methylcytosine and N6-methyladenine (Beemon & Keith, 1977;

Hotchkiss, 1948). DNA methylation is most frequently found in the genome at c-5 position on cytosine bases, and for the purposes of this dissertation DNA methylation will refer to DNA 5-methylcytosine (5-mC) and DNA hydroxymethylation will refer to DNA 5-hydroxymethylcytosine (5-hmC) (Dhar et al., 2021; Kumar et al., 2018; Moore et al., 2013). DNA methylation is commonly found in heterochromatin and is often associated with transcriptional silencing of the affected gene, especially when located in regulatory regions such as the gene promoter. However, DNA methylation can also result in enhancement of gene transcription depending on the activity of the methyl binding proteins that attach to the methylated DNA. For example, the SUVH proteins have been shown to recruit gene activating complexes in *Arabidopsis thalina* (Dhar et al., 2021; Harris et al., 2018). DNA is methylated at cytosine residues through the activity of DNA methyltransferases (DNMTs). DNA 5-mC can be oxidized by ten-eleven translocation enzymes (TETs) to form DNA Hydroxymethylation (5-hmC). Further oxidation of 5-hmC results in DNA 5-fmC and 5-caC (5-formyl cytosine and 5-carboxylcytosine) as intermediates in the process of DNA methylation removal (**fig 2** top). 5-hmC can also be a stable epigenetic mark, and in contrast to DNA 5-mC, 5-hmC is frequently associated with increased gene expression (Bachman et al., 2014; Irwin et al., 2021).

DNA 5-mC methylation has been shown to be altered in the brain and blood of people with epilepsy, as well as in several animal models of epilepsy. (Bahabry & Lubin, 2020; Berger et al., 2020; de Nijs et al., 2019; Dębski et al., 2016; Liu et al., 2016; Long et al., 2017; Miller-Delaney et al., 2015; Miller-Delaney et al., 2012; Ozdemir et al., 2019; Parrish et al., 2015; Ryley Parrish et al., 2013; Sen et al., 2019; Tao et al., 2021; Zhang et al., 2021). DNMT isoforms are differentially expressed in TLE with DNMT3a2

decreased in the hippocampus and DNMT1 and DNMT3a1 increased in the neocortex (de Nijs et al., 2019). DNA methylation regulates expression of seizure associated genes such as *Bdnf* (Brain-derived neurotrophic factor). *Bdnf* exon IX has decreased DNA methylation correlating to increasing BDNF protein and gene expression in the KA rat model of TLE (Ryley Parrish et al., 2013). DNA methylation patterns appear to be specific to seizure origin (Dębski et al., 2016; Mohandas et al., 2019). Recently, DNA 5-hmC has been implicated in epilepsy. 5-hmC is decreased in the pilocarpine rat model of epilepsy, and TET2 is increased in temporal cortex in patients in drug resistant epilepsy (Kong et al., 2021; Shen et al., 2020).

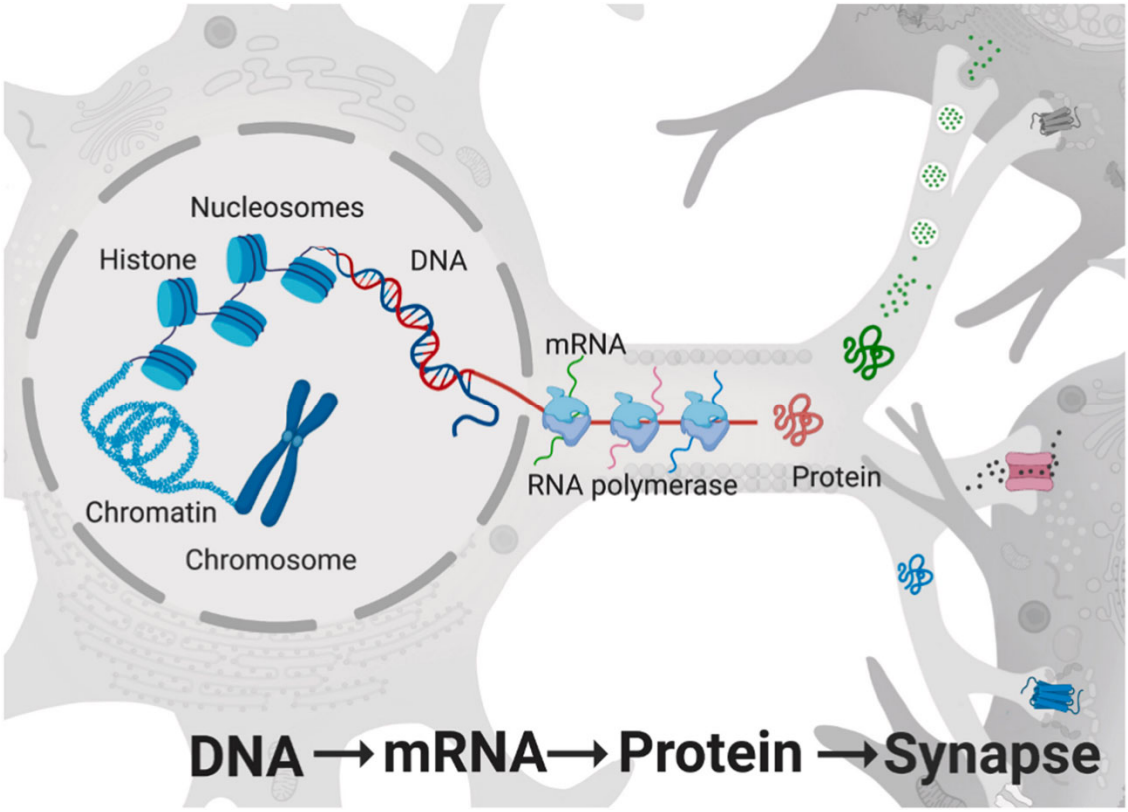

Figure 1. Illustration of the central dogma of molecular biology showing DNA transcription into RNA and RNA translation into protein. In the case of neurons, these proteins can either be released at the synapse or can join with the cell membrane in the case of neurotransmitter receptors and ion channels.

(Irwin et al., 2021)
Note: From "A putative role for lncRNAs in epigenetic regulation of memory" by A.B. Irwin, R. Bahabry, and F.D. Lubin, 2021, *Neurochemistry International*, *150*, pg. 105184. Copyright 2021 by Elsevier. Reprinted with permission.

Histone Modifications

Histone proteins can be covalently modified, promoting open or closed chromatin states. The effect of histone methylation on gene expression varies depending on the histone and residue methylated. H3K9me2 and H3K27me3 are considered repressive histone marks, H3K4me3. Gene silencing or activation is primarily determined by binding of protein complexes to the methylated histone. Hp1 binds to H3k9me2 recruiting DNMT3a/b to the DNA to repress transcription (El Gazzar et al., 2008). In

addition the G9a/GLP histone methyltransferases responsible for addition of H3k9me2 interacts with the Polycomb Repressive Complex3 (PRC2) which binds H3k27me3 (Mozzetta et al., 2014). Noches et al show increased H3k9me2/3 and decreased H3K4me2/3 in the hippocampus of the mouse pilocarpine model of TLE (Noches et al., 2021). In addition, a role for Ezh2 in epileptogenesis has been shown in the KA mouse model of TLE and the Pilo rat model of SE. Ezh2 expression is transiently increased following SE, but returns to baseline expression in epileptic animals (Khan et al., 2019).

Histone acetylation is typically promotes euchromatin formation and acts as a gene activating mark. Histone H4 acetylation was lost at GluR2 promoter but increased at Bdnf 3 hours following pilocarpine induced SE (Huang et al., 2002). HDACs (Histone de acetyltransferase) are a group of enzymes that remove acetylation from histone protens. This group is differentially regulated at varies stages in epileptogenesis and epilepsy in the unilateral KA intrahippocampal mouse model of epilepsy, treating with HDAC inhibitor SAHA improved seizure severity in the KA rat model of TLE (Hu & Mao, 2016; Jagirdar et al., 2015).

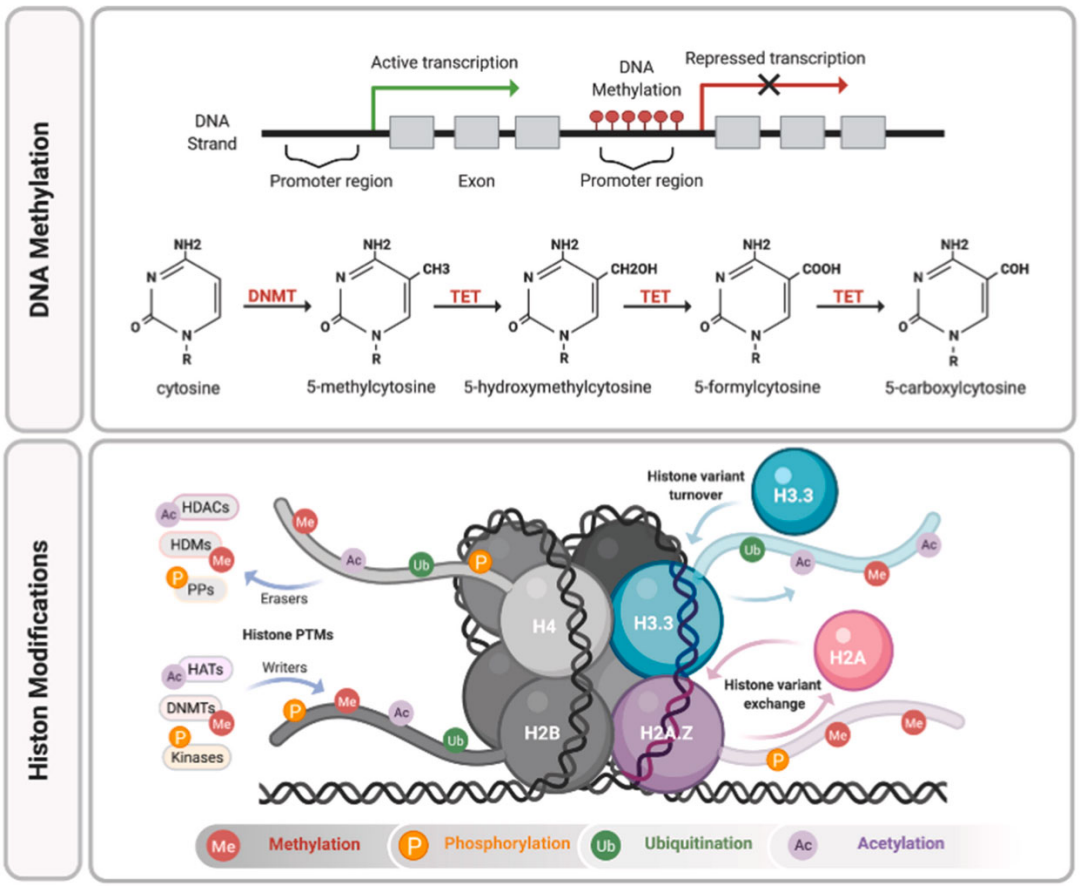

Figure 2. Illustration of (top) DNA methylation regulation of gene transcription and modified cytosine residues in the process of methylation and subsequent oxidation ultimately resulting in DNA methylation removal. (bottom) Illustration of a nucleosome with core histone post translational modifications including methylation, phosphorylation, ubiquitination, and acetylation.

(Irwin et al., 2021)
Note: From "A putative role for lncRNAs in epigenetic regulation of memory" by A.B. Irwin, R. Bahabry, and F.D. Lubin, 2021, *Neurochemistry International*, *150*, pg. 105184. Copyright 2021 by Elsevier. Reprinted with permission.

Non-coding RNAs

Non-coding RNAs are any RNAs that do not encode for a protein. The most commonly studied RNAs are long non-coding RNAs (lncRNAs) and micro RNAs (miRNAs). ncRNAs alter gene expression through a variety of mechanisms including binding to DNA to enhance or impair gene transcription, regulate gene splice variants,

and alteration in chromatin structure (**fig 3**) (Irwin et al., 2021). ncRNAs are not only found within cells, but they can be circulating through the blood in structures called exosomes (Wei et al., 2020). miRNAs can be altered with disease state and have been gaining popularity for their potential as use for early biomarkers of disease. In the past 2 years alone, many papers show differential expression of miRNA in the brain and serum of patients with epilepsy and animal models of epilepsy (Baloun et al., 2020; Bohosova et al., 2021; Brennan & Henshall, 2020; Martins-Ferreira et al., 2020; Pagni et al.; Zheng et al., 2016). An online database, epimiRBase has been created to keep track of all differentially expressed miRNAs in epilepsy (Mooney et al., 2016). Unfortunately, there does not yet appear to be a clear consensus in commonly differentially expressed miRNAs for TLE at this time.

Overall, epigenetic mechanisms are being discovered to play an important role in epilepsy development, with implications for novel anti-epileptic medications and biomarker identification. Future studies are investigating the cell-type specificity of these changes, as common epigenetic changes as opposed to model specific changes.

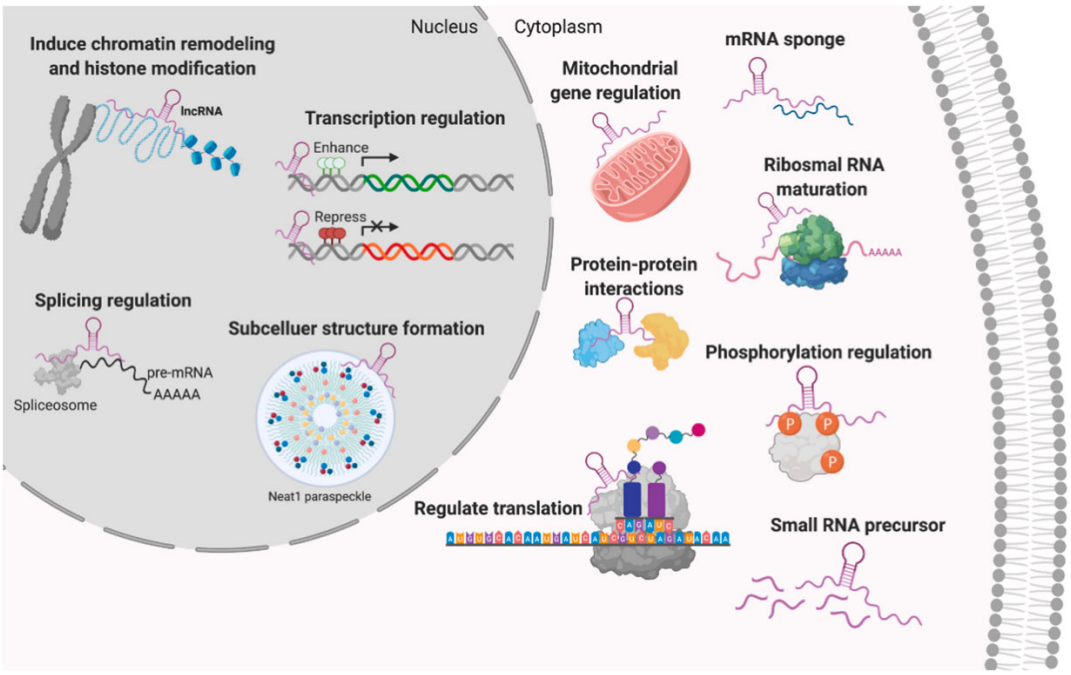

Figure 3. Illustration of multiple ways that lncRNAs can regulate cellular activity including modulation of gene transcription, chromatin structure, and protein binding.

(Irwin et al., 2021)
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CHARACTERIZATION OF 5-HMC DNA METHYLATION PATTERNS IN THE HIPPOCAMPUS OF AN EXPERIMENTAL MODEL OF REFRACTORY EPILEPSY

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Abstract

Temporal lobe epilepsy (TLE) is a type of focal epilepsy with recurrent seizures originating from the temporal lobe, and frequently the seizures originate from the hippocampus. The progression of epilepsy is controlled by alterations in gene expression resulting in a hyperexcitable network, and epigenetic mechanisms play an important role in the regulation of these genes. Previously, several studies have investigated the potential of DNA 5-methylcytosine methylation (5-mC) as a regulator of epilepsy development; however, the contribution of 5-hydroxymethylcytosine (5-hmC) has been understudied. 5-hmC is abundant in the brain and acts as a stable epigenetic mark altering gene expression through several mechanisms. Here, we show consistently in both human temporal lobe epilepsy and the kainic acid rat model of epilepsy that there is an overall loss of DNA 5-hmC but not 5-mC in the hippocampus. Using 5-hmC meDIP-sequencing, we characterize the distribution of epileptic 5-hmC loss and investigate its potential for the regulation of epilepsy-associated gene expression. We identify DNA 5-hmC association with several critical epilepsy-associated pathways and differential methylation at genes enriched in multiple cell types, showing 5-hmC has the potential to be a crucial epigenetic regulator of epilepsy pathology.

Introduction

Temporal lobe epilepsy (TLE) is a neurological disorder characterized by recurrent unprovoked seizures arising from the temporal lobe, frequently in the hippocampus. Epigenetic mechanisms play a crucial role in the regulation of the epileptic state. Particularly, DNA cytosine methylation is significantly altered in several models of epilepsy in both the brain and blood (Bahabry & Lubin, 2020; Berger et al., 2020; Caramaschi et al., 2020; Dębski et al., 2016; Kobow et al., 2013; Liu et al., 2016; Long et al., 2017; Miller-Delaney et al., 2015; Miller-Delaney et al., 2012; Mohandas et al., 2019; Ozdemir et al., 2019; Parrish et al., 2015; Ryley Parrish et al., 2013; Sen et al., 2019; Tao et al., 2021; Zhang et al., 2021). However, DNA methylation patterns differ by the epilepsy model used, showing that seizure origins are crucial in the consideration of epileptic DNA methylation patterns (Dębski et al., 2016). Previous studies investigating DNA methylation in epilepsy are limited by using mainly on the traditional bisulfite sequencing method, which cannot differentiate between two forms of methylated DNA, 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) (Huang et al., 2010). This study addresses the contribution of 5-hmC to the epileptic methylome using 5-hydroxymethylcytosine methylated DNA immunoprecipitation sequencing (hMeDIP-seq).

DNA can be methylated through the activity of dna methyl transferases (DNMTs) at cytosine residues, and methylated DNA (5-mC) typically represses transcription of the associated gene (Bird & Wolffe, 1999; Hsieh, 1999; Okano et al., 1999). 5-mC can be oxidized by TET (Ten-Eleven-Translocation) enzymes to form DNA hydroxymethylation (5-hmC) (Ito et al., 2010; Tahiliani et al., 2009). This oxidation both acts as an

intermediate step in active DNA demethylation and as a stable hydroxymethyl mark added to the DNA (Bachman et al., 2014; He et al., 2011; Weber et al., 2016). In contrast to 5-mC, which is typically associated with gene repression, 5-hmC is mostly associated with gene activation. 5-hmC is enriched at transcriptionally active sites such as gene bodies and poised and active enhancers (Shi et al., 2017; Song et al., 2011). DNA 5-hmC can act by preventing repressive proteins with methyl binding domains (MBDs) from binding to and repressing gene expression, therefore increasing gene expression (Jin et al., 2010). 5-hmC also can act as a *cis*-element regulating gene expression through the binding of transcription factors (TFs) to 5-hmC at gene regulatory regions. In addition, 5-hmC is negatively correlated with H3K27me3-marked and H3K9me3-marked repressive genomic regions suggesting that 5-hmC may contribute to gene expression in a histone dependent manner (Hahn et al., 2013; Leung et al., 2014; Tillo et al., 2017). In addition, 5-hmC at exon/intron junctions is thought to play a role in gene splicing (Khare et al., 2012).

5-hmC is more abundant in the brain than in other tissue types by approximately ten-fold, while 5-mC is more consistently enriched throughout tissue types (Globisch et al., 2010; Kriaucionis & Heintz, 2009). It is thought that active DNA methylation/demethylation is necessary for the regulation of neuronal activity. Previous studies have shown that 5-hmC is crucial for neuronal maturation and function, with 5-hmC levels increasing up to ten-fold over the course of development and enriched at genes with synapse-related functions (Hahn et al., 2013; Szulwach et al., 2011). 5-hmC and TET enzymes have a crucial role in normal brain functions such as memory and memory acquisition (Biergans et al., 2015; Chen et al., 2017; Gontier et al., 2018; Greer

et al., 2021; Kaas et al., 2013; Kumar et al., 2015; Parrish et al., 2015; Rudenko et al., 2013; Webb et al., 2017). In addition, 5-hmC also has been associated with numerous neurological disorders, including Rett syndrome, Alzheimer's disease, autism, and Huntington's disease (Cheng et al., 2018; James et al., 2014; Kuehner et al., 2021; Mellén et al., 2012; Papale et al., 2015; Shu et al., 2016; Wang et al., 2013; Zhang et al., 2020; Zhubi et al., 2017). Consequently, the methyl-binding protein MeCP2 is abundant in the brain and dysregulated in Rett syndrome. MeCP2 acts to regulate gene expression through interactions with methylated DNA (Mellén et al., 2012). Also interestingly, MeCP2 binds to both 5-mC and 5-hmC, and MeCP2 binding appears to prevent conversion of 5-mC to 5-hmC (Ludwig et al., 2016; Mellén et al., 2012). This suggests unique roles for methyl binding proteins in genome and epigenome regulation dependent upon underlying DNA methylation type.

Recently, both 5-hmC and TET enzymes have been found to be altered in the brain both in epilepsy models and following status epilepticus, a prolonged seizure event (de Nijs et al., 2019; Kaas et al., 2013; Ryley Parrish et al., 2013; Shen et al., 2020). Due to 5-hmC's abundance in the brain and its role in regulating neuronal activity, it is crucial to consider the contribution of 5-hmC when interpreting previously reported bisulfite sequencing data. In this study, we sought to map 5-hmC across the epileptic epigenome. We observe that hippocampal tissue samples from epileptic humans and rodents with TLE exhibit genome-wide loss of 5-hmC with no significant changes to global 5-mC levels. However, this trend is not reflective of the 5-hmC at the gene-specific level. The majority of gene-associated 5-hmC loss or gain occurs within gene bodies. The results from this study takes a closer look at DNA hydroxymethylation in epilepsy and further

supports the importance of DNA 5-hmC in the brain and as a critical epigenetic regulator in epilepsy worthy of further studies.

Materials and methods

Animals and Epilepsy Model

Male Sprague-Dawley rats received from Harlan weighing 125-150g at time of arrival were used for these experiments. Animals were housed in pairs in plastic cages and had access to water and NIH-31 lab rat diet ad libitum with a 12:12hr light/dark cycle. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and done in accordance with the National Institute of Health and ethical guidelines. Animals were handled by investigators for one week after arrival. Half of the animals were intraperitoneally (IP) injected with saline (vehicle), and the other half with 10mg/kg of kainic acid (KA) (0222, Tocris, Minneapolis, MN, USA). Behavioral seizure severity was measured using the Racine scale. 1: mouth and face clonus and head nodding; 2: clonic jerks of one forelimb; 3: bilateral forelimb clonus; 4: forelimb clonus and rearing; 5: forelimb clonus with rearing and falling (Lüttjohann et al., 2009; Racine, 1972). The onset of status epilepticus (SE) was defined as the time from KA injection to the start of continuous seizure activity with scores of 4 or 5 in the Racine scale. The animals providing samples for mass spectrometry analysis were sacrificed at 8 weeks post KA. The cohort used for hydroxymethylated DNA immunoprecipitation (hMeDIP-seq) were administered IP injections of saline once a day for two weeks after 2 months post KA. Animals were

euthanized by rapid decapitation 24 hours following the last injection, and their hippocampi were removed and subdissected in ice-cold oxygenated artificial cerebral spinal fluid (ACSF). The cornu ammonis (CA) region was collected and frozen on dry ice. Mass spectrometry analysis was performed as previously described (Kaas et al., 2013).

Hydroxymethylated DNA immunoprecipitation (hMeDIP) and Library Prep

Dissected CA samples were homogenized in Tris/EDTA (TE) buffer made from 1% 1M Tris pH 8.1 and 0.2% containing 1% SDS and 100µg of proteinase K (EO492, ThermoFisher Scientific) and incubated for 2hrs at 55°C. DNA was extracted through phenol/chloroform/isoamyl alcohol and ethanol precipitation. 400ng of DNA were used for hMeDIP assays. Samples were sheared to ~300bp using 25 cycles on a Bioruptor (Bioruptor XL, Diagenode, Lome, AT) at high power, denatured at 95°C for 15min, and diluted with IP buffer (16mM Tris, 1.2mM EDTA, 167mM NaCl, 1% SDS, 1% Triton). DNA was then added to MagnaChip magnetic protein A/G beads (16-663, Millipore, Burlington, USA) overnight that were incubated with either 5-hmC, or IgG the day before (2µg of anti –IgG (ab171870, Abcam) and 2µg of 5-hmC (39791, Active Motif, Carlsbad, USA)). The DNA bead complexes were washed with low salt buffer (0.1% SDS, 1% Triton X-100, 0.4% 0.5M EDTA, 2% 1M Tris-HCl pH 8.1, 0.875% w/v NaCl in DDH2O), high salt buffer (0.1% SDS, 1% Triton X-100, 0.4% 0.5M EDTA, 2% 1M Tris-HCl pH 8.1, 2.74% w/v NaCl in DDH2O), LiCl buffer (1.06% w/v LiCl, 0.1% NP-40, 1% Sodium Deoxycholate, 0.2% 0.5M EDTA, 1% 1M Tris-HCl pH8.1 in DDH2O), and TE buffer. Samples were then extracted with TE buffer containing 1% SDS and

100µg of proteinase K for 2hrs at 55°C and 10 minutes at 95°C. DNA was extracted and purified by phenol/chloroform/isoamyl alcohol and then ethanol precipitation. Library preps of the immunoprecipitated DNA samples were made using the ThruPLEX DNA-seq Kit (R400427, Rubicon Genomics, Ann Arbor, MI, USA), and sequencing was performed at UAB's Heflin Genomics core at ~15 million single end 75bp reads.

hMeDIP-seq bioinformatics

The FASTQ files were uploaded to the UAB High Performance Computer cluster for bioinformatics analysis with the following pipeline built in the Snakemake workflow system (v5.2.2): first, quality and control of the reads were assessed using FastQC, and trimming of the bases with quality scores of less than 20 and adapters were performed with Trim_Galore! (v0.4.5). Following trimming, the reads were aligned with Bowtie2 (Langmead & Salzberg, 2012)(v2.3.4.2, with option `--very-sensitive-local` set) to the University of California Santa Cruz (UCSC) rat genome (rn6), which resulted in an average mapping rate of 96.20%. BAM files were sorted and indexed with SAMtools(v1.6) and logs of reports were summarized and visualized using MultiQC (v1.6). BAM files were imported into a local RStudio session (R version: 3.5.0) for the identification of differentially methylated regions (DMRs) with MEDIPS (v.1.34.0)(Lienhard et al., 2014) across two DMR criteria: 1) the entire promoter (defined as 5000 bp upstream of the gene's transcriptional start site (TSS) and the entire gene-body 2) genome-wide quantification using windows of 500 bp for visualization purposes of regions outside of gene-bodies and promoters). The `BSgenome.Rnorvegicus.UCSC.rn6` (v.1.4.1) library was used as the reference. For the

quantification of DMRs across promoters and gene-bodies, reference `bed` files (from UCSC) of each region were implemented as the region of interest (ROI) in MEDIPS. Thus, MEDIPS datasets were created with the following parameters to the ` MEDIPS.createROIset` function: ROI=<promoter or gene-body references>, extend=0, shift=0, uniq= 1e-3 (to remove duplicates), paired=FALSE.

Computation of differential methylation was executed with the `MEDIPS.meth` function using the following parameters: p.adj="BH" (Benjamini-Hochberg method for p-value adjustment), diff.method="edgeR", MeDIP=TRUE, CNV=FALSE, minRowSum=10, diffnorm="quantile", CSet=CS (created with `MEDIPS.couplingVector(pattern = "CG")` with one of the control samples provided to `refObj`), and MSet1 represented the ES group while MSet2 represented the CS group. Promoters and gene-bodies were called to be statistically significant if they contained an adjusted p-value < 0.05 and minimum mean count of 10 in one of the two groups. To generate genome-wide DMRs, MEDIPS datasets were created with the following parameters: extend = 300, shift = 0, uniq = 1e-3, window size = 500 and paired = FALSE. Differential methylation was computed in the same manner as promoters and gene bodies.

The circos plot was generated by Circos (v0.69-5) (Krzywinski et al., 2009) using the rn6 ideogram obtained from the UCSC genome browser. The genome profile plots across the genic (gene-body), promoters, intergenic and CpG islands regions were generated with deeptools (v3.5.1) (Ramírez et al., 2016). The input data for the profile plots were bigwig files generated from the genome-wide quantification analysis with a coverage cutoff of minimum mean count of 10 in one of the two groups (chromosome M

was excluded). No statistical cutoff was applied for the generation of genome profile plots. The reference for intergenic region was created with BEDtools (v2.26.0) with `bedtools complement` to generate a bed file of regions across the genome without genic and promoter regions. The CpG islands reference was acquired from UCSC, and region references were provided to deeptools' `computeMatrix` function via the -R parameter. Non-default arguments included ` --skipZeros -bs 50`. For the CpG islands plot, upstream and downstream areas were extended by 4000bp. CpG shores and shelves were labeled as up to 2000 bp and 2000-4000bp up- and downstream of CGI respectively.

Gene ontology information was collected using WebGestalt(Liao et al., 2019). Genes inputted were genes with an adjusted p value of ≤ 0.05 and a log2FC of at least |0.75|. Seizure and epilepsy associated genes were taken from DisGeNET RDF v7.0 (Piñero et al., 2019) using search terms "seizure" and "epilepsy." For candidate genes an adjusted p value ≤ 0.05 and a log2FC of at least |0.9| was used excluding chrM. Volcano plots were created using EnhancedVolcano (v1.12.0).

Average cell type gene expression data was collected using the Allen Brain Map whole cortex & hippocampus – 10X genomes (2020) with 10X – smart-seq taxonomy (2021) scRNA-seq dataset accessed November 3, 2021. The mean trimmed mean expression from cells and the metadata of class labels "Glutamatergic" and "GABAergic" were used to calculate the mean of GABAergic neuron and glutamatergic neuron gene expression. The mean trimmed mean from cells with the metadata subclass labels "Astro," "Oligo," and "Endo" were used to calculate the mean of gene expression in astrocytes, oligodendrocytes, and endothelial cells(Yao et al., 2021). Graphical figures

were created with BioRender.com. GraphPad Prism version 9.2.0 was used to create bar charts and heat maps. Gene maps were created using IGV version 2.11.2.

Quantitative RT-PCR

Real-time PCR amplification of CA cDNA was performed on the Biorad CFX-96 Real-time system using TaqMan® Fast Advanced Master Mix and TaqMan® Gene expression assay using the following protocol: UNG activation at 50.0°C for 2 min, then polymerase activation at 95.0°C for 20 s, denature at 95.0°C for 3 s, followed by an Anneal/Extend at 60°C for 40 cycles. *Hprt1* expression was used to normalize gene expression. Cycle threshold (Ct) values were analyzed using comparative Ct method to calculate differences in gene expression between samples. Primers are as specified: *Hprt1* assay ID: Rn01527840_m1 VIC-MGB, *Sv2a* assay ID: Rn00589491_m1 FAM-MBG, *GAL* assay ID: Rn00583681_m1 FAM-MBG, *Kcnj11* assay ID: Rn01764077_s1 FAM-MBG, *BDNF* assay ID: Rn02531967_s1 FAM-MBG, *TLR4* assay ID: Rn00569848_m1 FAM-MBG, *GAD2* assay ID: Rn00561244_m1 FAM-MBG.

Human resected samples

Human samples were obtained from patients with medically intractable epilepsy undergoing elective neurosurgical resection of an epileptogenic hippocampus. All patients gave their informed consent, before surgery, for the use of the resected brain tissue for scientific studies. Human samples used for mass spectrometry were provided by Kristen O. Riley, MD from the Department of Neurological Surgery and G Yancy

Gillespie, MD from the Wallace Tumor Institute at UAB, and from Tore Eid M.D. from Yale School of Medicine. Rosalinda Roberts, Ph.D. provided non-epileptic post-mortem samples. Patient demographics and pharmacological history were previously described in supplementary table 2 (Sánchez et al., 2019).

Statistical analyses

Data is presented as a mean and standard error of the mean (SEM) where appropriate. Experiments were analyzed using unpaired t-tests performed by GraphPad Prism version 9.2.0. Adjusted p values for sequencing data were calculated using edgeR (Chen et al., 2016). Gene ontology FDRs were obtained from WebGestalt (Liao et al., 2019).

Results

5-hmC but not 5-mC is lost with epilepsy

To determine if 5-mC or 5-hmC changes globally in the epileptic hippocampus, we investigated total 5-hmC and 5-mC levels in both resected epileptic human and rodent hippocampus through mass spectrometry **(Figure 1c)**. Analysis of epileptic human tissue showed no overall changes in levels of DNA 5-mC **(Figure 1d)**, yet it revealed a global loss of 5-hmC **(Figure 1e)**. To determine if the change in DNA hydroxymethylation existed in rodent models of epilepsy, rats were injected intraperitoneally with kainic acid to induce status epilepticus. Eight weeks following status epilepticus the animals were fully epileptic, and the Cornu Ammonis (CA) region of the hippocampus was collected

and analyzed for DNA methylation using mass spectrometry **(Figure 1f)**. Mirroring the human data, we observed no change in DNA 5-mC levels **(Figure 1g)** and a decrease in total levels of DNA 5-hmC **(Figure 1h).**

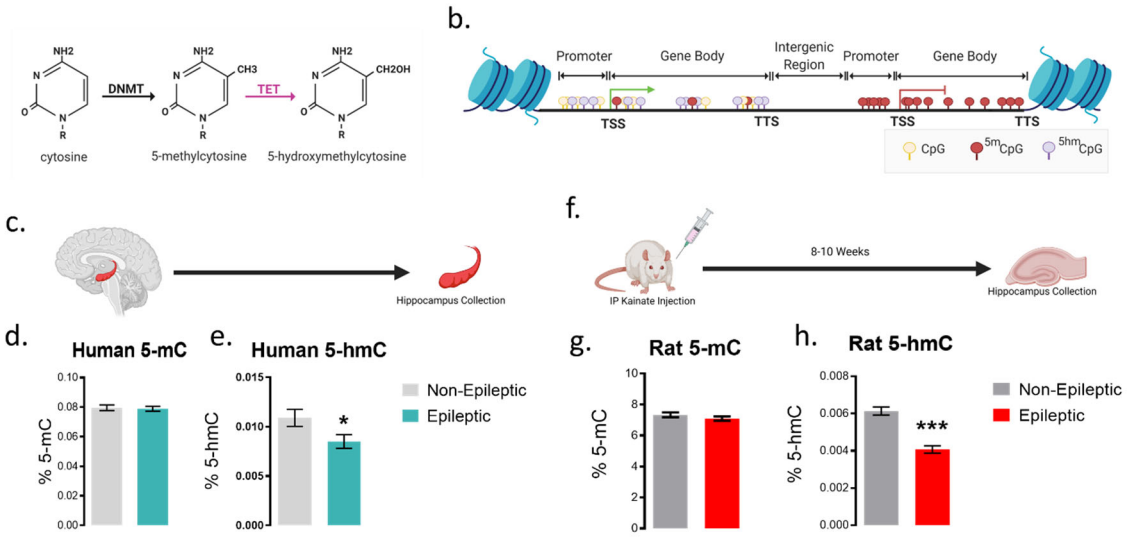


Figure 1. Total 5-hmC but not 5-mC is Lost with Epilepsy. a) DNMTs methylate cytosine residues on DNA which can then be hydroxymethylated by TETs. b) Illustration of the relationship of 5-mC and 5-hmC to gene expression. 5-hmC at the gene promoter and gene body promote transcription, while DNA 5-mC at the promoter and gene body inhibit transcription. c) Human hippocampi were collected from epilepsy patients undergoing surgical resection of their hippocampus and from control post-mortem tissue. d) Mass spectrometry analysis showing total percentage of DNA 5-mC in epilepsy and e)5-hmC percentage from resected epileptic human hippocampus and post-mortem controls. f) Timeline of epilepsy rat model. Kainic acid is injected IP into rats to induce status epilepticus. After 8-10 weeks the animals develop spontaneous seizures and the CA region of the hippocampus is collected. g) Mass spectrometry analysis total DNA 5-mC and h) 5-hmC percentage from epileptic rat hippocampus. *p<0.05, **p<0.01, ***p<0.001 unpaired t-test.

Epileptic loss of 5-hmC occurs primarily at intergenic regions

While our mass spec data showed global changes in 5-hmc, we wanted to determine the genomic distribution of 5-hmC in epileptic animals, the CA region of the hippocampus in control and epileptic rats was used for 5-hmC meDIP-sequencing. The genome was split into 500bp windows, of which were 5517 hypermethylated and were 6485 hypomethylated. Agreeing with our mass spectrometry data, epileptic rats had less DNA 5-hmC overall levels. Excluding mitochondrial DNA, 290, 2455, and 2772 windows were hypermethylated in gene promoters, bodies, and intergenic regions respectively. 300, 2567, and 3618 windows were hypomethylated in gene promoters, gene bodies and intergenic regions respectively. The intergenic windows, annotated gene bodies, and annotated gene promoters were then mapped across the genome. **(Figure 2)**. When examining only differentially hydroxymethylated (DhM) gene bodies, no general trend towards loss or gain of 5-hmC is noticeable **(Figure 2a).** Similarly, gene promoter regions also show no clear bias towards loss or gain of 5-hmC except for chromosome X, which shows no hyper-hydroxymethylated promoters **(Figure 2a).** Interestingly, the majority of 5-hmC loss is seen within intergenic regions **(Figure 2a).**

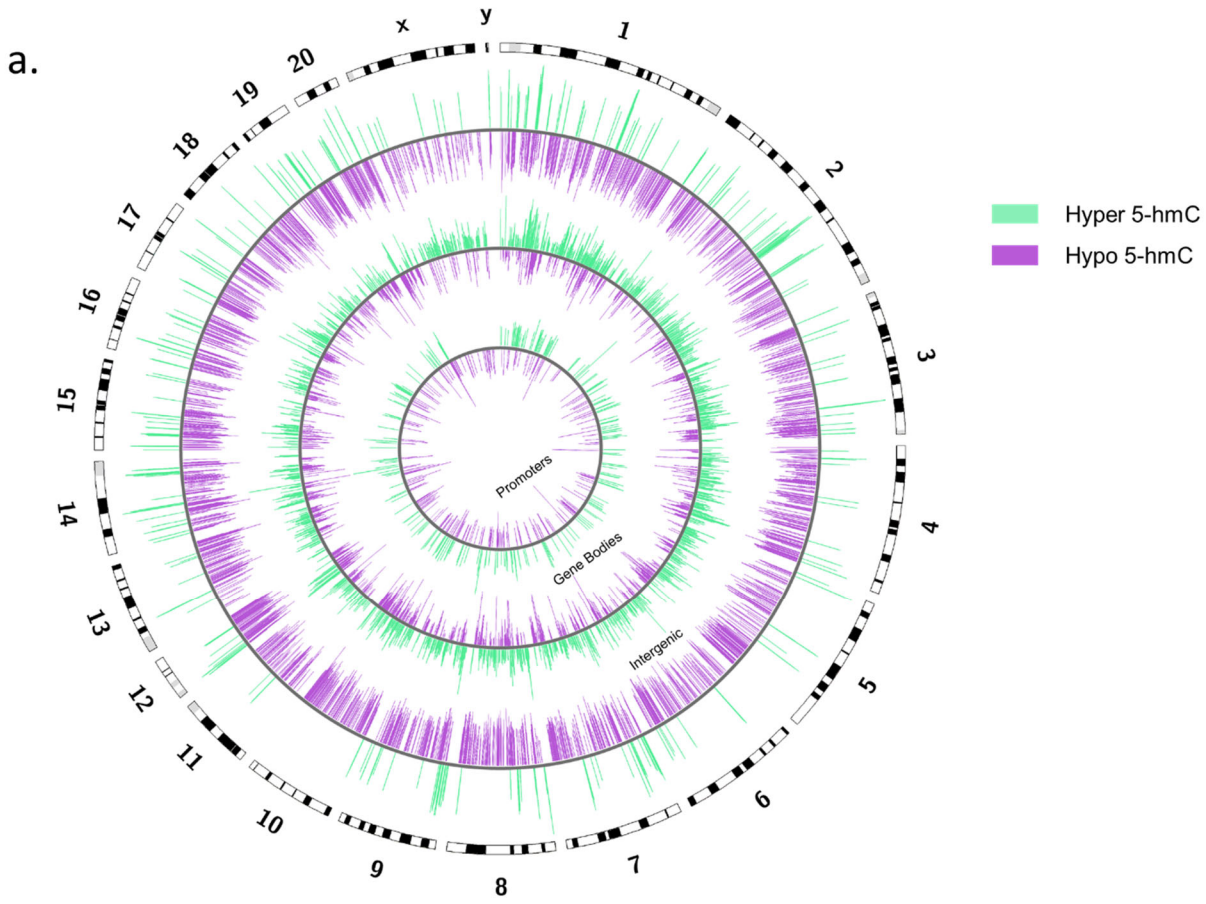


Figure 2. Differential 5-hmC Windows Across Genome Regions. Circos plots of gene promoter and gene body regions of interest and intergenic 500bp windows 5-hmC showing Log2FC of DhMRs from epileptic rats relative to non-epileptic animals. Purple indicates regions with reduced 5-hmC relative to non-epileptic animals and green regions with increased 5-hmC.

Epilepsy alters 5-hmC DNA methylation at gene bodies and gene promoters

To identify genes regulated by 5-hmC in epilepsy, we looked specifically at gene bodies and gene promoters with significant increases or decreases of 5-hmC. The majority of gene-associated DNA 5-hmC changes were seen at the gene bodies with 2531 genes identified as hypomethylated and 2455 genes hypermethylated in epileptic rats relative to non-epileptic controls **(Figure 3d)**. In addition, we found 298 hypomethylated gene promoters and 290 hypermethylated promoters in epileptic rats **(Figure 3e)**. Across

the average, promoter, gene body, and intergenic region, there is a consistent decrease in 5-hmC occupancy in epileptic rats **(Figure 3a-c)**. Relative to CpG islands (CGI), the majority of differentially hydroxymethylated regions (DHMRs) were found at CpG shores agreeing with past CpG methylation studies **(Figure 3g)** (Irizarry et al., 2009; Mitsumori et al., 2020), epileptic rats showed less 5-hmC than control animals with enrichment at CGI regions and a relatively even distribution of 5-hmC across CpG shores and shelves **(Figure 3f, g).**

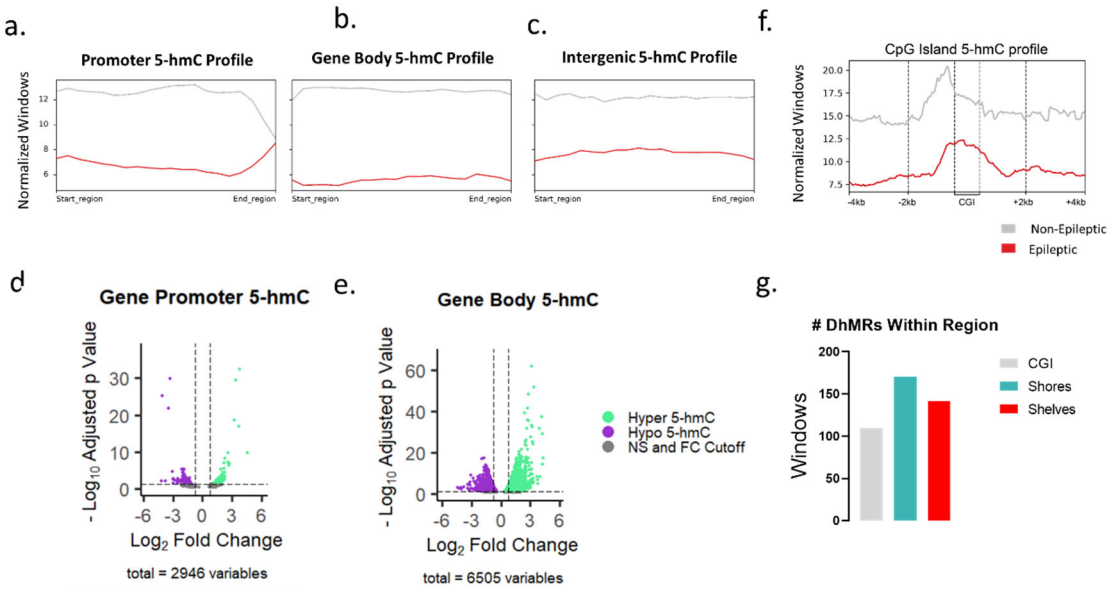
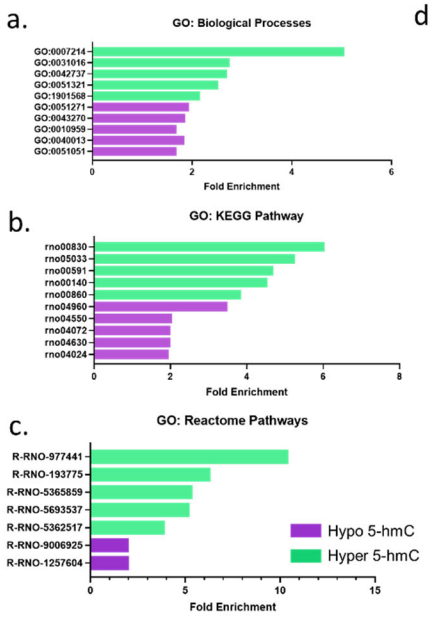


Figure 3. 5-hmC Distribution. a) Profile plots of average 5-hmC distribution across gene promoters b) gene bodies, c) intergenic regions, and f) CpG islands. d) Volcano plot showing hypo (purple) and hyper (green) hydroxymethylated gene promoters and e) Gene bodies. Adjusted p value cutoff of 0.05 and fold change cutoff of Log2FC 0.75 are indicated by dotted lines. Genes with an adjusted p value of more than 0.05 or a Log2FC 0.75 less than 0.75 are indicated in gray. g) Total number of differentially methylated 500bp windows of epileptic relative to control rats located within CGI (CpG Islands), CGI shores, and CGI shelves

Differentially hydroxymethylated genes are involved in several epilepsy-associated pathways

To address what pathways differentially methylated genes are involved in, the most significantly hypo- and hypermethylated gene bodies with a log2 fold change absolute value of at least 0.75 were submitted for gene ontology analysis (**Figure 4)**. We found that these genes were involved in several pathways, including GABA signaling as the top hit for hypermethylated gene bodies and several ion transport pathways in hypomethylated genes. GABA signaling is known to be important in epilepsy, with loss of GABA promoting seizure activity and increasing GABA having an antiseizure effect. In addition, multiple GABA receptor mutations result in clinical epilepsy (Akyuz et al., 2021; Hernandez & Macdonald, 2019; Maljevic et al., 2019; Qaiser et al., 2020). Ion channels are also critical for a variety of neuronal functions such as action potential propagation, resting membrane potential, and intercellular signaling and mutations in several channel genes cause genetic epilepsy (Burke & Bender, 2019; Lerche et al., 2013; Oyrer et al., 2018). KEGG pathway analysis showed enrichment of genes involved in retinol metabolism and nicotine addiction in hypermethylated genes. Retinoic acid acts as an anti-seizure therapeutic, and nicotine can be a pro-convulsant but has anti-convulsant effects in treating certain epilepsies (Dworetzky et al., 2010; Rosiles-Abonce et al., 2021; Woolf et al., 1996). Hypomethylated genes were associated with several signaling pathways such as sodium reabsorption regulated by aldosterone, JAK-STAT signaling, and cAMP signaling. Loss of sodium causing hyponatremia can result in seizures, and hyperaldosteronism can result in seizures (Nardone et al., 2016; Young, 2019). Both the JAK-STAT and cAMP signaling pathways are implicated in seizures (Grabenstatter et

al., 2014; Han et al., 2018; Lund et al., 2008; Martínez-Levy et al., 2018; Mertz et al., 2020). Associated Reactome pathways of hypermethylated genes include GABA-A receptor activation and Retinoic Acid synthesis and pathways, and hypomethylated genes were enriched in PIP3 activation of AKT signaling and intracellular signaling. The PIP3 pathway has also been associated with epilepsy progression with seizures causing a reduction in PIP3 (Chang et al., 2014). Overall, enrichment analysis by GO terms, KEGG pathway, and Reactome pathways show overlapping patterns with DhMR enrichment in genes associated with GABA receptors, retinoic acid metabolism, and intracellular signaling. This indicates that 5-hmC might be involved in the regulation of multiple important epilepsy-associated pathways and that 5-hmC could serve as a critical regulator of epilepsy development.



d.

Gene Set	Description	Enrichment Ratio	FDR	genes/gene set
GO:0007214	gamma-aminobutyric acid signaling pathway	5.0498	0.001447	10/21
GO:0031016	pancreas development	2.7572	0.000559	13/50
GO:0042737	drug catabolic process	2.6993	0.00023	28/110
GO:0051321	meiotic cell cycle	2.5214	0.000181	34/143
GO:0051271	negative regulation of cellular component movement	1.9393	0.01038	37/198
GO:0043270	positive regulation of ion transport	1.8615	0.01038	40/223
GO:0040013	negative regulation of locomotion	1.846	0.015687	37/208
GO:0010959	regulation of metal ion transport	1.6943	0.015687	48/294
GO:0051051	negative regulation of transport	1.6901	0.01038	57/350
rno00830	Retinol metabolism	6.031	0	39/85
rno05033	Nicotine addiction	5.2578	9.41E-07	16/40
rno00591	Linoleic acid metabolism	4.6944	1.29E-05	15/42
rno00140	Steroid hormone biosynthesis	4.5379	1.70E-10	29/84
rno00860	Porphyrin and chlorophyll metabolism	3.8471	0.001225	12/41
rno04960	Aldosterone-regulated sodium reabsorption	3.4928	0.018303	11/39
rno04550	Signaling pathways regulating pluripotency of stem cells	2.0344	0.036929	23/140
rno04630	JAK-STAT signaling pathway	1.9973	0.036929	25/155
rno04072	Phospholipase D signaling pathway	1.9947	0.036929	24/149
rno04024	cAMP signaling pathway	1.9487	0.018303	31/197
R-RNO-977441	GABA A receptor activation	10.436	2.82E-05	8/9
R-RNO-193775	Synthesis of bile acids and bile salts via 24-hydroxycholesterol	6.3219	0.006236	7/13
R-RNO-5365859	RA biosynthesis pathway	5.3812	0.000892	11/24
R-RNO-5693537	Resolution of D-Loop Structures	5.2181	0.006841	8/18
R-RNO-5362517	Signaling by Retinoic Acid	3.9136	0.002363	13/39
R-RNO-1257604	PIP3 activates AKT signaling	2.0342	0.021057	35/193
R-RNO-9006925	Intracellular signaling by second messengers	2.0201	0.021057	38/211

Figure 4. Pathway Enrichment of Differentially Hydroxymethylated Gene Bodies. a) Fold enrichment of gene ontology pathways for hyper (green) and hypo (purple) hydroxymethylated genes for KEGG pathways, b) Biological Processes, c) and Reactome Pathways for gene bodies with an adjusted p value <0.05 and a Log2FC of at least 0.75. d) Table describing gene set ids, descriptions, enrichment ratios, and FDR of gene ontology graphs in a, b, and c.

DhMRs affect genes in a variety of cell types

To develop a list of candidate genes for 5-hmC regulation in epilepsy, we filtered our list of differentially methylated genes for genes known to be associated with epilepsy or seizures. From this, 69 genes were identified. This was further narrowed by the genes with the largest fold change in hydroxymethylation compared to non-epileptic animals to generate a list of 26 candidate genes **(Figure 5a, b, c)**. Multiple cell types are involved in the hippocampal pathology of TLE. Seizures result from an overabundance of excitatory glutamatergic transmission and a loss of GABAergic inhibition. In epilepsy with hippocampal sclerosis, there is gliosis of the hippocampus resulting in the increase of hippocampal astrocytes. Astrocytes regulate glutamate in the brain as part of the tripartite synapse and the glutamate-glutamine cycle through uptake of glutamine and conversion to glutamate (Hertz & Rothman, 2017). As a crucial component of the myelin sheath, oligodendrocytes are essential regulators of neuronal transmission. In epilepsy, myelination abnormalities and damaged myelin sheaths have been reported (Isackson et al., 1991; Liu et al., 2021; Scholl et al., 2017; You et al., 2011; Zhong et al., 2019). Endothelial cells line blood vessels and play a vital role in maintaining the blood-brain barrier, which is disrupted in epilepsy (Gorter et al., 2019). Using the Allen brain institute's scRNA-seq cell type database (Yao et al., 2021), we compared our list of candidate genes to the average expression in the following cell types: astrocytes, glutamatergic neurons, GABAergic neurons, oligodendrocytes, and endothelial cells. Of the top 26 hits, 7 genes are highly expressed in glutamatergic neurons, 6 in GABAergic neurons, 1 in astrocytes, 1 in endothelial cells and 13 genes had no to low average expression in the cell types examined **(Figure 5d).**

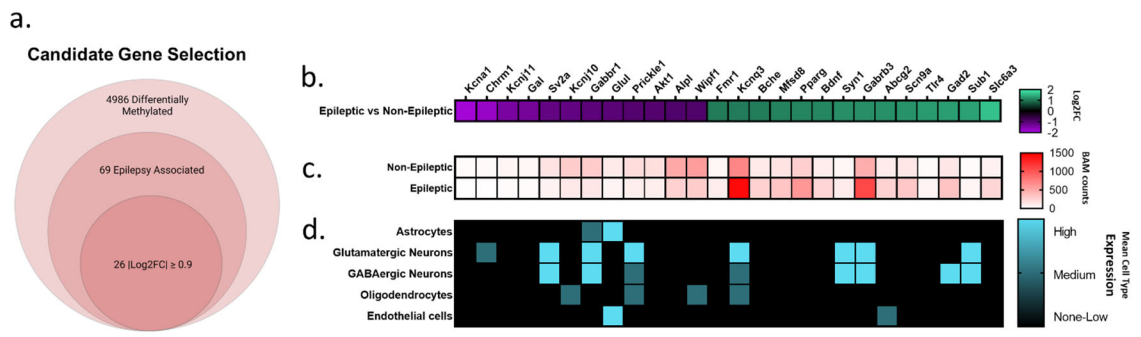


Figure 5. Candidate Gene Selection and Putative Cell Types. a) Schematic illustrating candidate gene selection. Significantly hypo- or hydroxymethylated genes with an adjusted p value <0.05 were filtered for genes known to be associated with epilepsy or seizures. The epilepsy/seizure associated genes with the largest fold change were filtered by a Log2FC of at least 0.9 to generate a list of 26 genes candidate genes for regulation by 5-hmC in epilepsy. b) Log2FC candidate genes in epilepsy relative to non-epileptic animals. c) BAM counts of candidate genes in epileptic and non-epileptic rats. d) Relative mean expression of candidate genes in astrocytes, glutamatergic neurons, gabaergic neurons, oligodendrocytes, and endothelial cells. Mean cell type expression values from the Allen Brain cell types database was collected and sorted into 3 bins for high, medium, or low average gene expression.

Epileptic gene regulation by DhMRs

To examine if epileptic 5-hmC resulted in functional changes, we chose 3 hypo- and hypermethylated genes from our candidate genes to measure gene expression via qPCR: *Sv2a, Gal, Kcnj11* **(Figure 6)**, *Bdnf, Tlr4*, and *Gad2* **(Figure 7)**. *Sv2a* encodes synaptic vesicle protein 2a, which is the binding site for the antiepileptic drug levetiracetam. Galanin (G*al*) is an endogenous neuropeptide that acts to inhibit seizures (Guipponi et al., 2015; Lerner et al., 2008). Mutations in the potassium channel subunit Kir6.2 (K*cnj11*) are known to result in genetic epilepsy (Gloyn et al., 2006; Villa & Combi, 2016). Brain derived Neurotrophic factor (*Bdnf*) has antiseizure effects, however infusion of BDNF can also lead to seizures (Ernfors & Persson, 1991; Isackson et al.,

1991; Larmet et al., 1995; Osehobo et al., 1999; Scharfman et al., 2002). Toll-like receptor 4 (*Tlr4*) activation increases inflammation which can lead to seizures (Abdelsalam et al., 2020; De Simoni et al., 2000; Henneberger & Steinhäuser, 2016; Kleen & Holmes, 2010; Vezzani et al., 2011). Glutamate decarboxylase 2 (*Gad2*) converts glutamate into GABA, and loss of expression and activity of GAD2 is associated with seizure activity (Daif et al., 2018; Kakizaki et al., 2021; Zhang et al., 2017). Typically, 5-hmC correlates with an increase in gene expression. All 3 hypomethylated genes had reduced gene expression in the hippocampus **(Figure 6a-i)**. Interestingly, all the hypermethylated genes tested also showed a significant reduction in gene expression **(Figure 7a-i)**. This could potentially be explained by the heterogeneity of cell types in the tissue sent for meDIP-seq, regulation of these genes through other epigenetic mechanisms, or involvement of intergenic 5-hmC regulation.

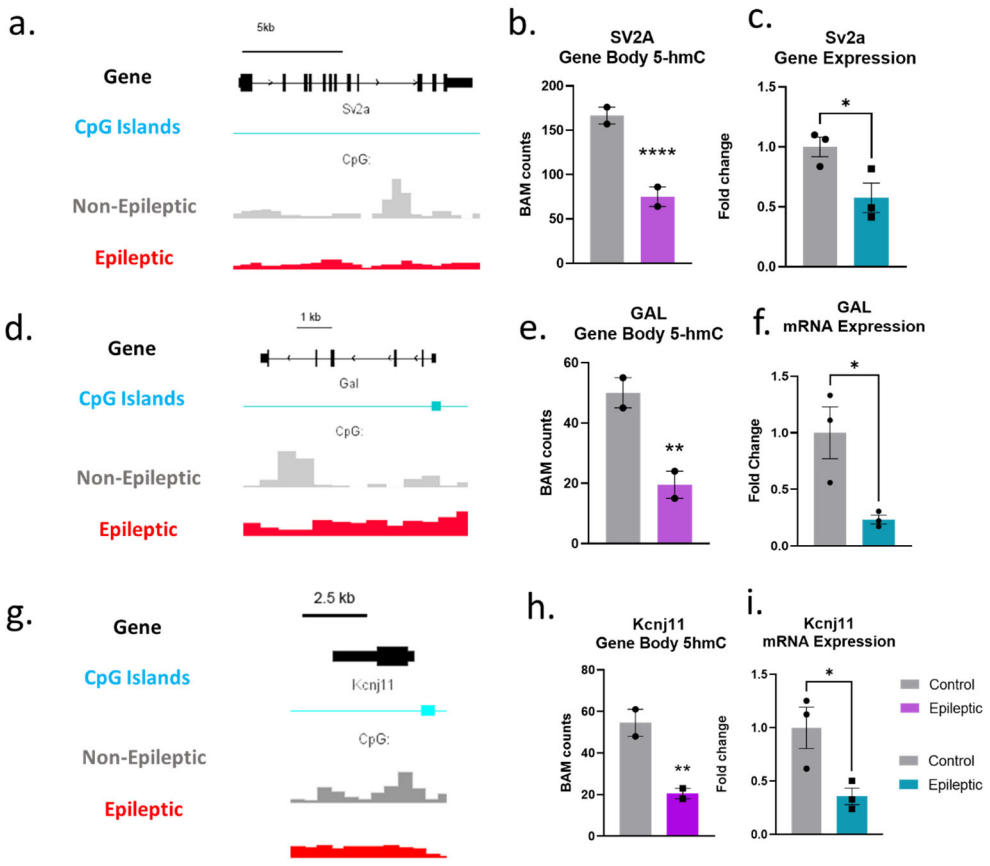


Figure 6. Gene Expression of Hypohydroxymethylated Genes. a) Gene profile map of SV2a. Non-epileptic 5-hmC profiles are shown in gray, epileptic profiles are red. CpG islands are shown in blue, and the gene body exons and introns are shown in black. b) BAM counts of SV2a 5-hmC in epileptic (purple) and non-epileptic (gray) animals. c) qPCR gene expression of Sv2a in epileptic (blue) and non-epileptic (gray) animals. d) Average gene profile of gal 5-hmC in epileptic and non-epileptic animals. e) BAM counts of gal 5-hmC in epileptic and non-epileptic rats. f) mRNA expression of gal in epileptic rats. g) Profile of the kcnj11 gene's 5-hmC distribution. h) BAM counts of kcnj11 5-hmC in epileptic and non-epileptic animals. i)mRNA expression of kcnj11 in epileptic rats. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001 unpaired t-test (mRNA expression graphs), edgeR adjusted p value (BAM counts).

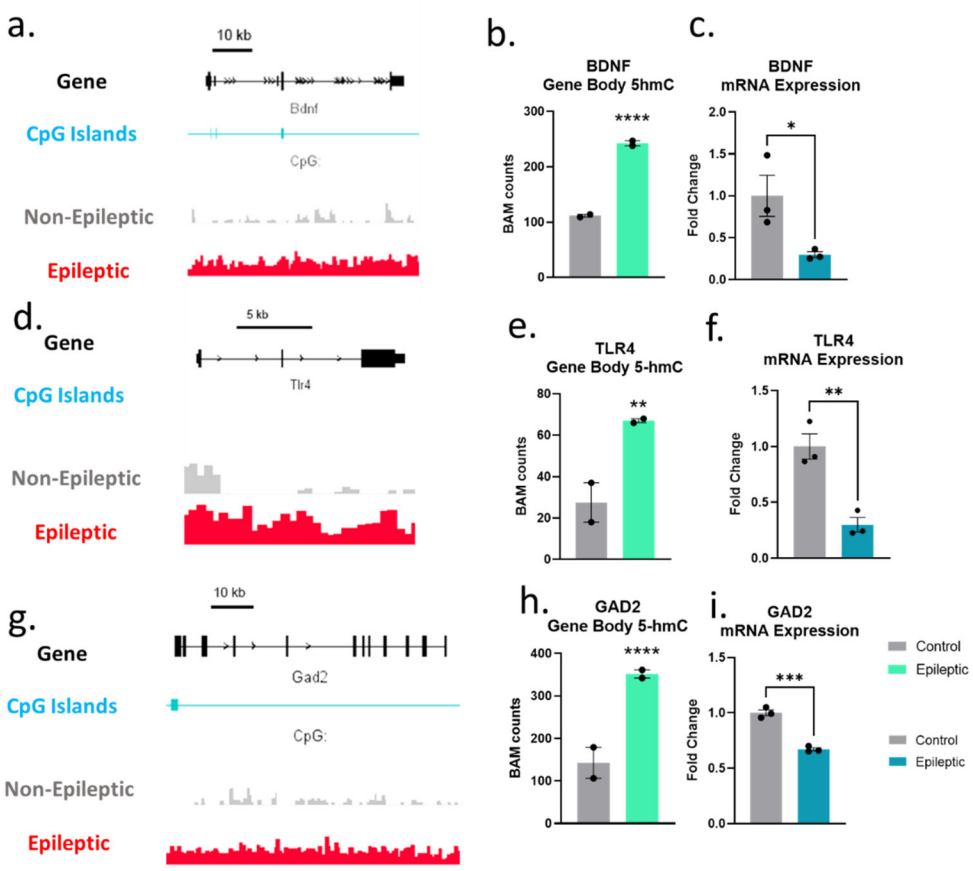


Figure 7. Gene Expression of Hyperhydroxymethylated Genes. a) Gene profile map of bdnf. Non-epileptic 5-hmC profiles are shown in gray, epileptic profiles are red. CpG islands are shown in blue, and the gene body exons and introns are shown in black. b) BAM counts of bdnf 5-hmC in epileptic (green) and non-epileptic (gray) animals. c) qPCR gene expression of bdnf in epileptic (blue) and non-epileptic (gray) animals. d) Average gene profile of tlr4 5-hmC in epileptic and non-epileptic animals. e) BAM counts of tlr4 5-hmC in epileptic and non-epileptic rats. f) mRNA expression of tlr4 in epileptic rats. g) Profile of the gad2 gene's 5-hmC distribution. h) BAM counts of gad2 5-hmC in epileptic and non-epileptic animals. i) mRNA expression of gad2 in epileptic rats. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 unpaired t-test (mRNA expression graphs), edgeR adjusted p value (BAM counts).

Discussion

In this study, we performed the first DNA 5-hmC meDIP-sequencing in an epilepsy model, established a 5-hmC map of epilepsy, and showed that overall changes in epileptic DNA methylation is largely driven by 5-hmC instead of 5-mC. This study shows that DNA 5-hmC should be considered when examining DNA methylation of epilepsy and provides insight that can help us interpret previous bisulfite methylation sequencing results. We observed loss of DNA 5-hmC and no change in total 5-mC in human TLE hippocampus and the kainic acid rat model of epilepsy. Total loss of 5-hmC is also described in TLE by de Nijs et al (de Nijs et al., 2019), the electrical kindling model of epilepsy by (Shen et al., 2020), and 24 hours following flurothyl induced seizures (Kaas et al., 2013), suggesting that DNA 5-hmC loss in the hippocampus might be a consistent hallmark of epilepsy pathology, and that 5-hmC could be more important than 5-mC as a crucial epigenetic regulator of epilepsy.

We characterized the distribution of genomic 5-hmC in epilepsy and identified several candidate genes for the regulation of DNA 5-hmC in epilepsy. Several of these genes are highly expressed in various cell types implying that alterations in 5-hmC could affect multiple cells within the hippocampus. We examined gene expression of three candidate genes implicated in epilepsy with significantly hypomethylated gene bodies: *Sv2a, Gal,* and *Kcnj11*. These genes show decreased mRNA expression in the hippocampus of epileptic rats. We also examined the expression of three hypermethylated genes: *Bdnf, Tlr4,* and *Gad2*. All of the hypermethylated genes tested showed a significant loss in gene expression contrary to the expected increase with increased 5-hmC occupancy at the gene body. Interestingly, several studies have shown an increase in

Bdnf gene and protein expression (Ernfors & Persson, 1991; Isackson et al., 1991; Parrish et al., 2015; Ryley Parrish et al., 2013). *Bdnf* has several splice variants that are differentially expressed in response to neuronal activity such as with and epilepsy and memory formation (Chiaruttini et al., 2008; Lubin et al., 2008; Martínez-Levy et al., 2016; Martínez-Levy et al., 2018; Notaras & van den Buuse, 2020; Parrish et al., 2015; Ryley Parrish et al., 2013). For the purposes of this study, total *Bdnf* mRNA (exon IX) was evaluated due to differential hydroxymethylatation analysis covering the whole gene body, and the variance by isoform specific expression was not accounted for. *Tlr4* signaling pathways have been shown to be increased with seizures (Henneberger & Steinhäuser, 2016; Kleen & Holmes, 2010). Loss of *Gad2* activity promote seizures, *Gad2* knockout mice develop epilepsy, and humans with anti-GAD antibodies present with drug-resistant epilepsy(Daif et al., 2018; Kakizaki et al., 2021; Kash et al., 1997; Qi et al., 2018). However, *Gad2* has also been reported to be increased in GABAergic neurons in epileptic rodents (Esclapez & Houser, 1999) showing the importance of cell type on gene expression. The inverse effect of 5-hmC on mRNA expression for these genes could be explained by heterogeneity of cell types in the tissue, the contribution of other transcriptional regulators, or due to a regulatory effect of intergenic 5-hmC.

Many studies have shown enrichment of intergenic 5-hmC, associating with both decreases and increases in gene expression of the nearest TSS (Kamdar et al., 2016; Uribe-Lewis et al., 2020)**.** Several studies have reported 5-hmC association with H3k4me1 and enhancers, resulting in increased expression of the associated gene (Cui et al., 2020; Hu et al., 2017; Kołodziej-Wojnar et al., 2020). In addition, intergenic 5-hmC has been shown to interact with CTCF sites, TADs, and transposable elements (Cheng et

al., 2018; Kołodziej-Wojnar et al., 2020). Overall, intergenic 5-hmC can affect gene expression in multiple ways depending on the genomic context and cell type, and future research into the regulatory potential of 5-hmC in epilepsy is needed to fully understand the effects of 5-hmC on epileptic gene expression and if there are cell types more majorly affected by 5-hmC in epilepsy than others. In addition, the role of intergenic 5-hmC remains to be completely understood. Since the majority loss of 5-hmC loss is in intergenic regions, the mechanisms of intergenic 5-hmC on gene expression and cell activity are crucial to understanding how 5-hmC loss affects the epileptic brain. DhMRs were associated with several epilepsy-associated GO terms, including GABA signaling regulation of ion transport suggest that 5-hmC is involved in regulating these pathways. However, it remains to be known if 5-hmC loss is sufficient to drive seizures or if it is a result of recurrent seizures.

The role of TET in epilepsy requires further understanding. TET catalyzes the transformation of 5-mC to 5-hmC. TET1 protein activity and expression remain to be studied in an epilepsy model. Parrish et al show no changes in *Tet1* mRNA expression in the kainic acid model of temporal lobe epilepsy, however a loss of *Tet1* is observed in area CA3 and the dentate gyrus following status epilepticus, while 5-hmC is lost in area CA3 following status epilepticus and the dentate gyrus of epileptic animals (Ryley Parrish et al., 2013). This data suggests that TET1 and 5-hmC is regulating neuronal activity differently across the timeline of epileptogenesis as well as hippocampal subfields. Recently, it has been reported that *Tet2* is increased in the brains of patients with drug resistant epilepsy (Kong et al., 2021). Further evidence for the importance of

TET enzymes in epilepsy, humans with *TET3* mutations resulting in *TET3* deficiency can experience seizures (Beck et al., 2020; Levy et al., 2021; Seyama et al., 2021).

We show that drug-resistant TLE patients have a loss of total 5-hmC in their hippocampi. Targeting 5-hmC directly or through modulation of TET activity could be a target for antiepileptic therapeutics in these patients. In addition, there are no drugs to date that treat the comorbidities of epilepsy such as memory loss. Previous studies have shown that Tet1 and DNA 5-hmC are crucial for learning and memory. *Tet1* isoforms differentially regulate fear memory (Greer et al., 2021), *Tet1* KO mice have deficits in fear memory extinction and LTD (Rudenko et al., 2013), *Tet1* overexpression impairs long term memory formation (Kaas et al., 2013). *Tet2* overexpression in aged animals enhances fear memory (Gontier et al., 2018), and in a mouse model of Alzheimer's disease *Tet2* knockdown impairs cognition while *Tet2* overexpression improves memory (Li et al., 2021). In addition, global DNA 5-hmC is increased in area CA1 of the hippocampus following fear memory retrieval (Webb et al., 2017). Targeting epigenetic pathways with pharmaceutical intervention is growing in interest for the treatment of several diseases including cancer (Jin et al., 2021; Topper et al., 2020), and in epilepsy, recent studies have investigated the potential of histone deacetylase (HDAC) inhibitors in animal models of epilepsy with some success (Citraro et al., 2017; Hu & Mao, 2016; Sint Jago & Lubin, 2020). Targeting the TET/5-hmC pathway in epilepsy could have the potential to be a novel therapeutic that can treat both seizures and memory deficits.

Declarations

Ethics approval

All animal procedures were approved by the University of Alabama at Birmingham's institutional animal care and use committee (IACUC).

Competing Interests

The authors declare no competing interests.

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G9A LOSS REDUCES SEIZURE THRESHOLD AND INCREASES NEURONAL ACTIVITY IN VIVO

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Format adapted for dissertation

Abstract

Epilepsy is the most common neurological condition in the world. Lately, epigenetic mechanisms in the brain have been recognized as crucial regulators of epilepsy development through the modulation of gene transcription. However, the precise pathways acted upon to induce seizures are still being discovered. Much of the focus of epigenetics in epilepsy has been on DNA methylation, histone acetylation, and non-coding RNAs. Histone methylation is a major regulator of gene expression and has been shown to be crucial in normal brain operations such as memory formation. H3k9me2 is a repressive histone mark that is added by the G9a and GLP methyltransferases. Humans with mutations in the GLP gene, *ehmt1*, have Kleefstra syndrome, a developmental disorder marked by intellectual disability. These patients commonly experience seizures as a symptom of the disorder, implicating the GLP methyltransferase and H3k9me2 pathway in seizure development. However, not much is known about the role of G9a in seizures. In this study, we utilize a transgenic mouse model to investigate the role of G9a loss in seizures. We discover that loss of G9a in dentate gyrus neurons leads to altered seizure threshold and increased *cfos* activated cells in the hippocampus. This study is crucial for understanding the role of G9a in seizure development and the H3k9me2 pathway in epilepsy.

Introduction

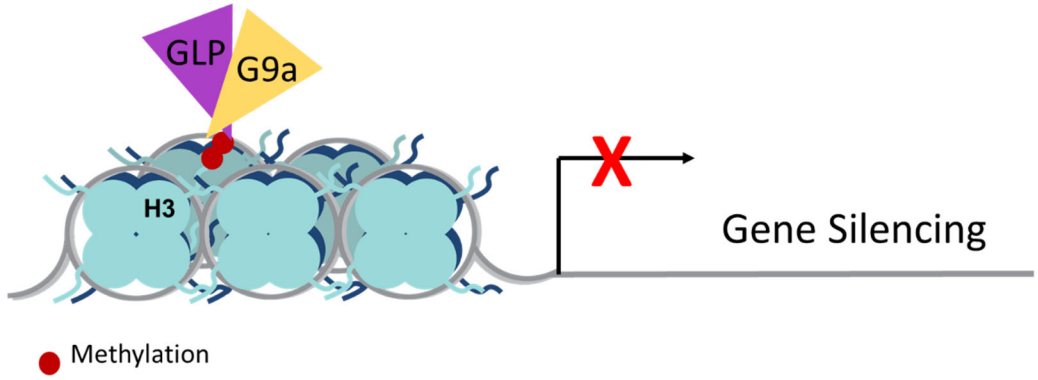
Epilepsy is the most common neurological disorder and affects roughly 50 million people worldwide. Despite its wide spread, around 30% of patients are resistant to current medication (Sheng et al., 2018). To decrease the number of patients without control of their seizures, it is crucial to understand the mechanisms involved in seizures and epilepsy formation. Epigenetic mechanisms such as DNA methylation have been implicated in the pathology of temporal lobe epilepsy (Berger et al., 2020; de Nijs et al., 2019; Kobow et al., 2013; Mohandas et al., 2019; Parrish et al., 2015; Shen et al., 2020). Histone methylation is a major epigenetic mechanism that has been less studied in the context of TLE (Hauser et al., 2017; Reichard & Zimmer-Bensch, 2021).

The H39me2 pathway has been implicated in seizures in humans through G9a's partner protein GLP. The histone methyltransferase G9a works as a dimer with GLP to dimethylate lysine 9 of histone H3 (H3k9me2). H3k9me2 is abundantly found in heterochromatin and is associated with gene repression (**fig 1**).Humans with mutations in the GLP encoding gene *ehmt1* present with Kleefstra syndrome. Kleefstra syndrome patients have developmental delays and intellectual disability. Many of these patients also experience epilepsy as a result of the mutation (Kleefstra & de Leeuw, 1993). Both GLP and G9a can form hyperactive networks *in vitro.* Loss of GLP through shRNA administration causes increased excitation in primary neuron cultures, but haploinsufficiency of G9a promotes hyperactive networks in iPSCs from an individual with autism (Deneault et al., 2019; Frega et al., 2020). In the context of epilepsy, H3K9me2 has been recently implicated in status epilepticus (SE) by Noches et al who

showed an increase in global H3K9me2 in the dentate gyrus (DG) 24 hours following pilocarpine (pilo) -induced SE in mice and primary neuronal cultures (Noches et al., 2021). Zhang et al show in pilo treated rats 5 hours following SE H39me2 and G9a were decreased in the hippocampus, and H3k9me2 was lost in the promoter region of the potassium channel gene *Kcnj10* (Zhang et al., 2018).

G9a and GLP have both been implicated as crucial for memory formation (Gupta-Agarwal et al., 2012; Kramer et al., 2011; Schaefer et al., 2009). In addition, G9a has been shown to regulate the expression of *bdnf* a well-known gene important in epilepsy development and memory formation (Gupta-Agarwal et al., 2012; Gupta et al., 2010; Isackson et al., 1991; Lubin et al., 2008; Martínez-Levy et al., 2016; Martínez-Levy et al., 2018; Notaras & van den Buuse, 2020; Parrish et al., 2015; Ryley Parrish et al., 2013). This implicates that G9a could contribute to epilepsy in converging pathways, and might be important for regulation of both seizures and memory loss, a common comorbidity in patients with temporal lobe epilepsy.

Here, we investigate the role of neuronal G9a loss in the dentate gyrus in seizures. The dentate gyrus is a structure within the hippocampus often referred to as the gate of hippocampal transmission. The hippocampus transmits signals in a trisynaptic circuit. Inputs are received from the entorhinal cortex (EC) which must pass through the dentate gyrus before propagating through the CA regions of the hippocampus and exiting through the EC (Knierim, 2015). For seizures to occur within the hippocampus, seizures must progress through the abundance of GABAergic inhibition in the DG. For this reason, the DG is frequently targeted in the study of epilepsy to stop the aberrant epileptiform activity causing seizures (Dengler & Coulter, 2016; Hsu, 2007; Scharfman, 2019).



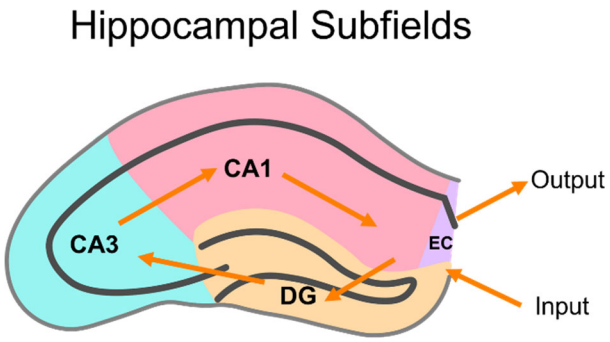


Figure 1. Gene Repression by G9a and the Hippocampus. (top) Schematic of gene silencing by H3k9me2 and G9a. (bottom) schematic of hippocampal subfields. DG = Dentate gyrus; EC = Entorhinal cortex; simplified for the purposes of this dissertation to 4 subfields.

Materials and Methods

Animal Model

Transgenic male *ehmt2* floxed mice on a c57bl/6 background were used, as described in (Schaefer et al., 2009) and (Sampath et al., 2007). For AAV injection, mice were injected at 6-8 weeks old. The virus was allowed to express for 2 weeks. Animals were then anesthetized using isoflurane and euthanized by cervical dislocation. Brains were frozen on dry ice and stored at -80ºC.

Surgical Injections of AAV

Mice were injected with 200nL of either AAV9.hSyn.eGFP.WPRE.bGH or AAV9.hSyn.HI.eGFP-Cre.WPRE.SV40 received from the Penn Vector core (Addgene plasmids #105539 and #105540 respectively) into each brain hemisphere targeting the dorsal dentate gyrus (AP -0.22, DV -0.20, ML +/-0.13 relative to bregma) using a stereotaxic frame and glass pipette microinjector. Animals were placed under isoflurane for surgical procedures.

Immunohistochemistry

Slides were removed from storage at -80ºC. Brains were embedded in Tissue-Tek OCT compound and sliced into 10μM thick sections using a cryostat before mounting on glass slides. Slides were stored at -80ºC before beginning the protocol. Slides were removed from the freezer and tissue was fixed on the slide using 10% formalin solution

for 10 minutes at room temperature. Slides were washed 3 times for 5 minutes each in PBS. Antigens were retrieved by placing slides in boiling sodium citrate buffer for 30 minutes. Slides were washed for 5 minutes in PBS 3 times. Blocking buffer (5% normal donkey serum, 5% normal goat serum, 0.3% Triton X in PBS) was added to the slides for 1 hour at room temperature in a humid chamber. Blocking buffer was removed using a kimwipe and primary antibody was added to the slides in antibody dilutant (1% normal donkey serum, 1% normal goat serum, 0.3% Triton X in PBS). Slides were incubated overnight at 4ºC in a humid chambrer. Slides were washed with PBS 3 times fo 5 minutes each and dried with a kimwipe. In a dark room, secondary antibodies (in blocking buffer) was added and left to incubate for 2 hours at room temperature in a humid chamber. Slides were washed 3 times with PBS solution for 5 minutes each, dried with a kimwipe and stained using Invitrogen ProLong Glass Antifade Mountant with NucBlue. Slides were left to dry at room temperature for 30 minutes, then were imaged using a florescent microscope. Primary antibodies used: Millipore NeuN MAB377 1:500, Cell Signaling GFAP 3670S 1:300. Secondary antibody: Jackson Immuno TRITC Donkey αMs 715-025-150 1:200.

Fluorescent in situ hybridization (FISH)

The *Ehmt2* FISH was done with a custom BaseScope probe from ACDbio. The probe is BA-Mm-Ehmt2-2EJ, a 2ZZ probe targeting 3243-3369 of NM_145830.2. The probe covers the exon junctions between exon 23/exon 24 and exon 24/exon 25. *cfos* FISH was done with RNAscope from ACDbio using Probe-Mm-Fos catalog number

316921 following manufacturer protocol. Images were taken using a fluorescence microscope.

Seizure Threshold

Mice were injected intraperitoneally with Pentylenetetrazol (PTZ) 60mg/kg to induce seizures. Animals were monitored for 30 minutes following injection for signs of seizures using a combined racine/pinel scale (**fig 7a)**. Animals were euthanized by isoflurane and cervical dislocation before brain collection. Brains were frozen on dry ice and stored at -80ºC

Statistical Analyses

2-way ANOVA was used to analyze FISH data. Seizure latency data was analyzed using repeated-measures ANOVA, and maximum seizure score was analyzed using an unpaired t-test. All statistical tests were performed in GraphPad Prism v9.2.0.

Results

Conditional Knockout of G9a

To study G9a in seizures we used transgenic G9a fl/fl mice (**fig 2d)** injected with hSynGFP-Cre AAV or hSynGFP AAV in the dorsal dentate gyrus (**fig 2a-c)**. The hSyn promoter targets neuronal cell types, conditionally knocking out expression of G9a in

dorsal dentate gyrus neurons. Expression of the virus construct were contained to the DG region of the hippocampus (**fig 2b)**.

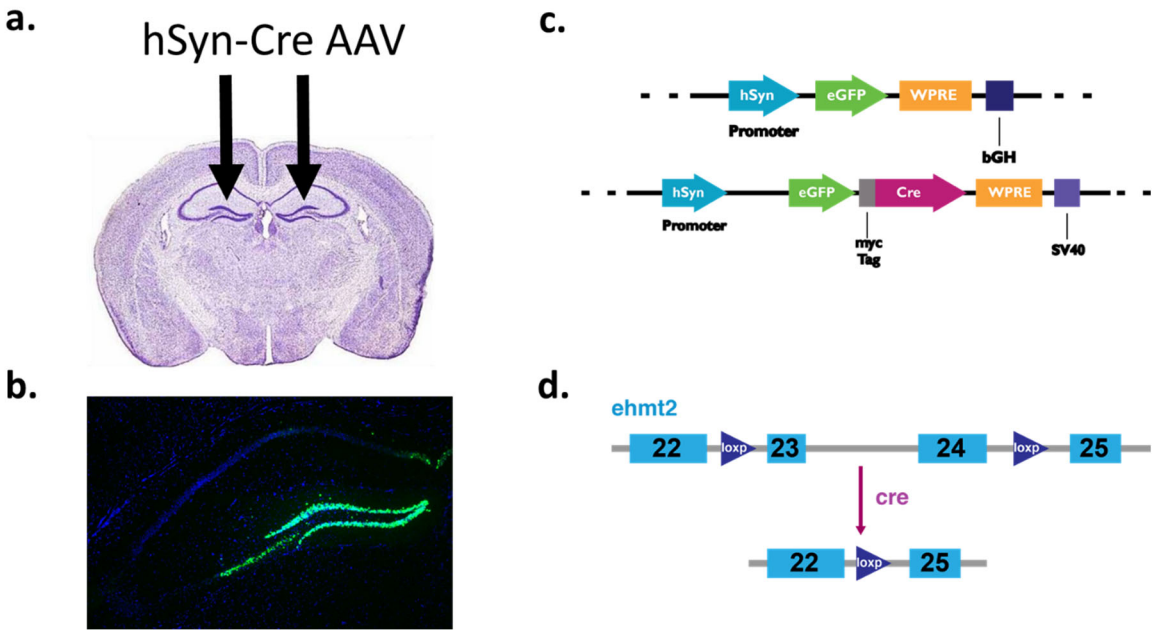


Figure 2. Conditional Knockout of G9a. a) diagram of injection into mouse dorsal hippocampus b) representative image of hSyn-Cre AAV expression (green) in the dentate. DAPI (blue) c) diagrams of vector constructs for AAVs d) diagram of transgenic G9a floxed mouse transgene.

To validate our model, conditional knock out animals were probed for G9a (*Ehmt2)* mRNA expression using a conditional FISH probe targeting the floxed region of the transgene, exons 23 and 24. Mean pixel density analysis of G9a FISH shows that G9a fl/fl cre injected mice exhibit a loss of G9a expression compared to wt cre injected and fl/fl gfp-only injected mice (**fig 3a, b)**.

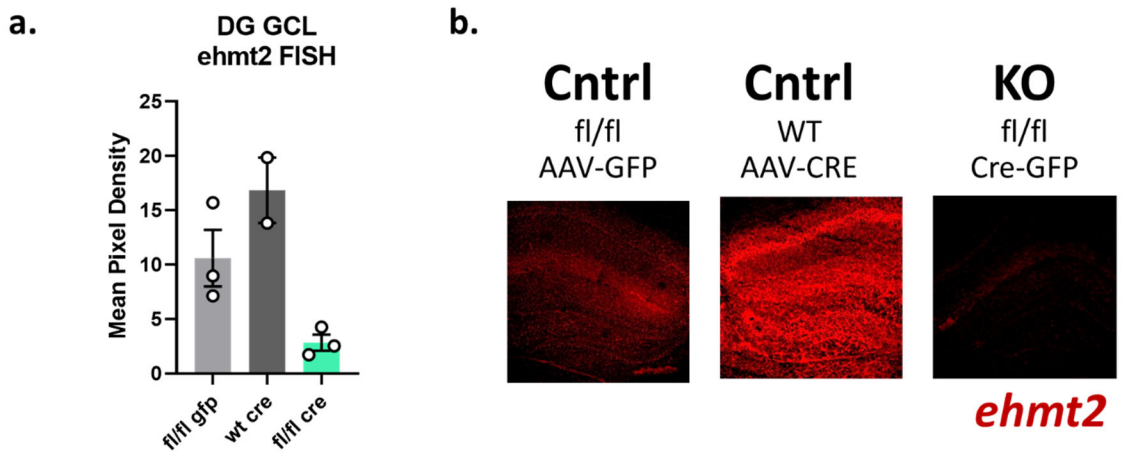


Figure 3. Confirmation of G9a Knockout. a) Mean pixel density analysis of *Ehmt2* FISH staining in fl/fl or mice injected with GFP or Cre-GFP AAV in the granule cell layer of the hippocampus. b) representative images. *Ehmt2* (red).

Knockdown of G9a Alters Hippocampal Morphology

Heterozygous animals injected with cre-AAV or G9a KD animals interestingly exibit enlargement of ventricle size (**fig 4a**) compared to normal mice (**fig 4b**). These animals also exhibit NeuN positve neuron loss particularly in area CA1 of the hippocampus. (**fig 5a**) KD mice also exhibited gliosis of the tissue, frequently seen in response to injury or seizures (**fig 5b**). Interestingly, in epilepsy with hippocampal sclerosis neuron loss and gliosis are also seen (Walker, 2015). KO mice exhibited no structureal abnormalities. Previous KO studies using the G9a fl/fl mice crossed the animals with CamKII-cre mice, knocking out G9a in excitatory neurons in the whole brain. These animals also exhibited no structural abnormalities (Schaefer et al., 2009).

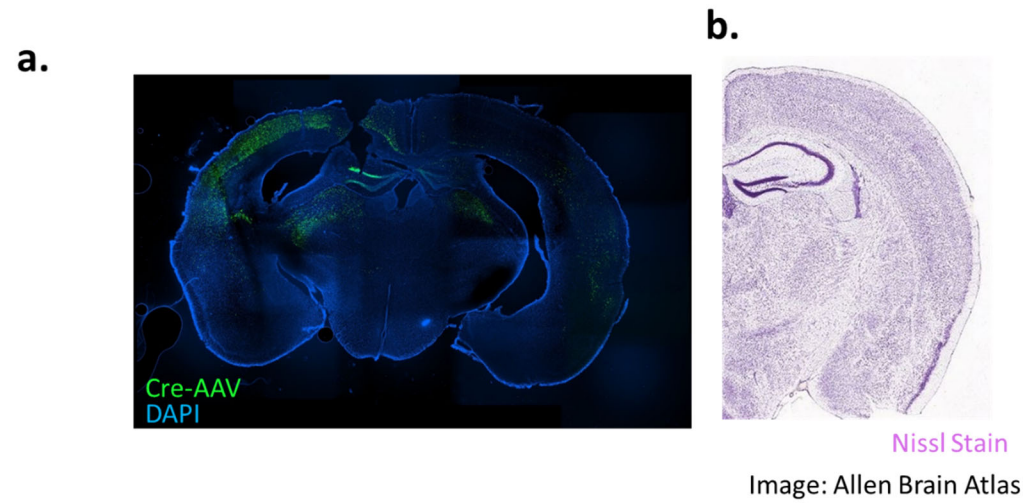


Figure 4. Ventricle Enlargement with G9a KD. a) image of fl/wt mouse injected with cre-GFP AAV (green) DAPI (blue) b) Nissl stained image from the Allen Brain Atlas of the same region in 4a.

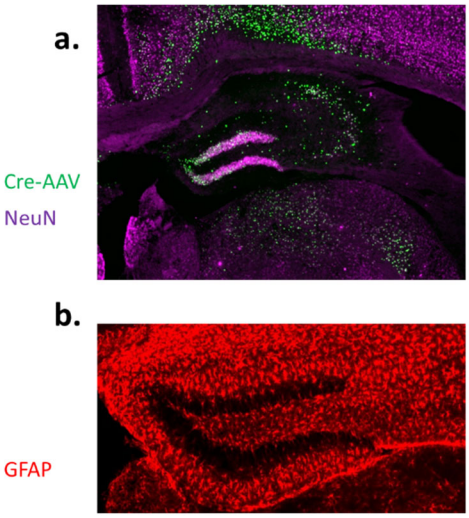


Figure 5. Cell Loss and Gliosis with G9a KD. a) fl/wt mouse injected with cre-GFP AAV (green) stained for NeuN (magenta) and b) GFAP (red).

cFos Signaling with G9a Loss

To determine if there is an increase in neuronal activity in our KO animals, we counted the number of *cfos* positive cells in the dentate gyrus (**fig 6a**). *cfos* is an imeadiate early gene (IEG) and is expressed by neurons quickly following neuronal firing or activation. In our fl/fl KO mice, we observe and increase in *cfos* positive cells indicating a more active neuronal network (**fig 6b**). These results are similar to an *in vitro* study in which iPSCs haploinsufficient in *ehmt2* form a hyperexcitable network (Deneault et al., 2019). However, this study disagrees with Frega et al where *ehmt2* was knocked down using shRNA in primary rat cortical neuron cultures. This study saw an increase in neuronal firing rate when *ehmt1* or GLP was knocked down but not G9 (Frega et al., 2020).

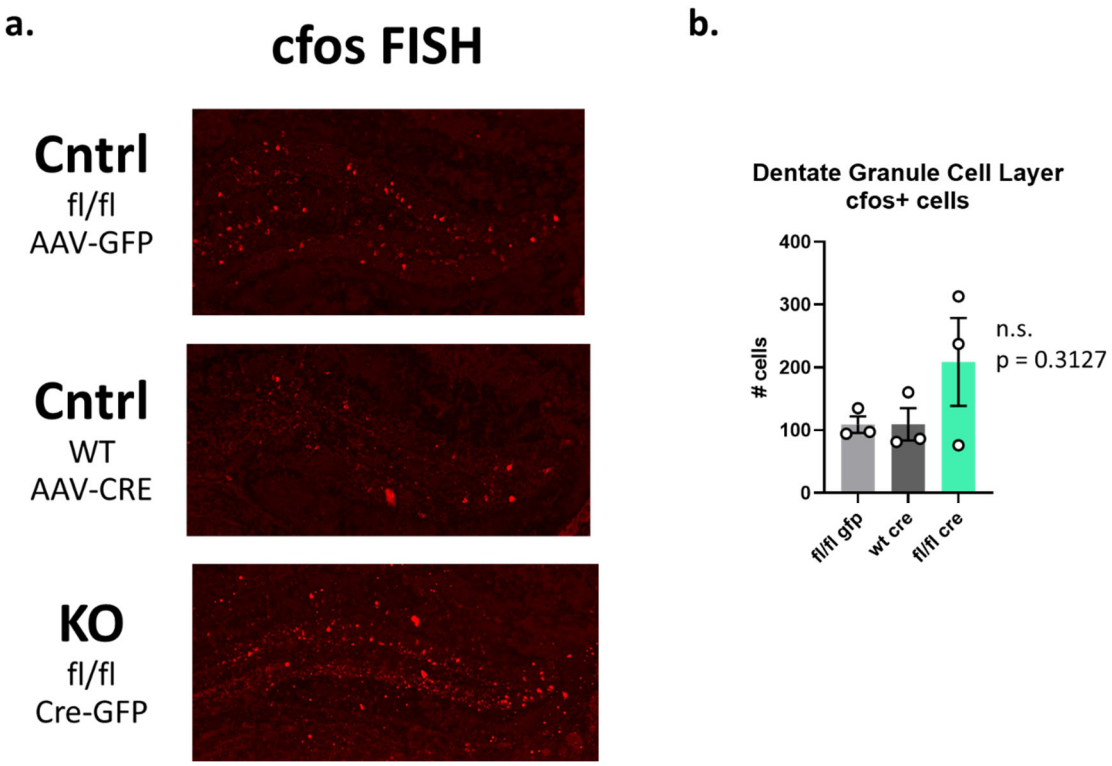


Figure 6. *cfos* Signaling with G9a KO. a) FISH fos (red) staining in the dentate gyrus of fl/fl and wt mice injected with GFP AAV or Cre-GFP AAV and b) count of fos positive cells in the granule cell layer of the dentate gyrus

Loss of G9a Alters Seizure Threshold

To determine if neuronal loss of G9a in the dentate gyrus alters seizure threshold, we challenged our conditional KO and KD mice with PTZ to induce SE. Animals were monitored for seizure scores based on a combined Racine and Pinel scale (**fig 7**). Evaluating seizure threshold refers to examining the amount of brain activity required to induce a seizure. We found that KO and KD mice in general reached higher maximum seizure scores compared to controls (**fig 8 a-d)**. Interestingly, both KO and KD mice exhibited altered seizure latency however KO mice reached maximum seizure scores more quickly than control animals, and KD mice reached maximum seizure scores more

slowly than controls. This implies that different pathways are involved in lowering the seizure threshold in KO and KD mice.

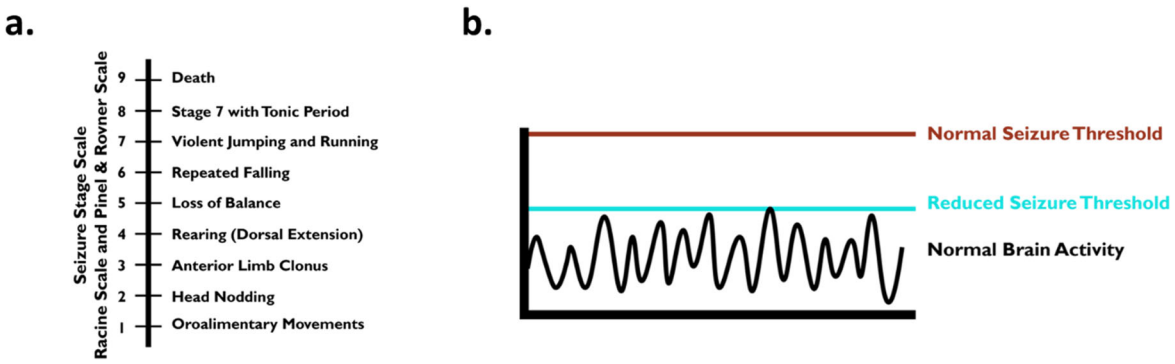


Figure 7. Seizure Threshold Schematic. a) Combined Racine-Pinel seizure score scale. B) seizure threshold diagram.

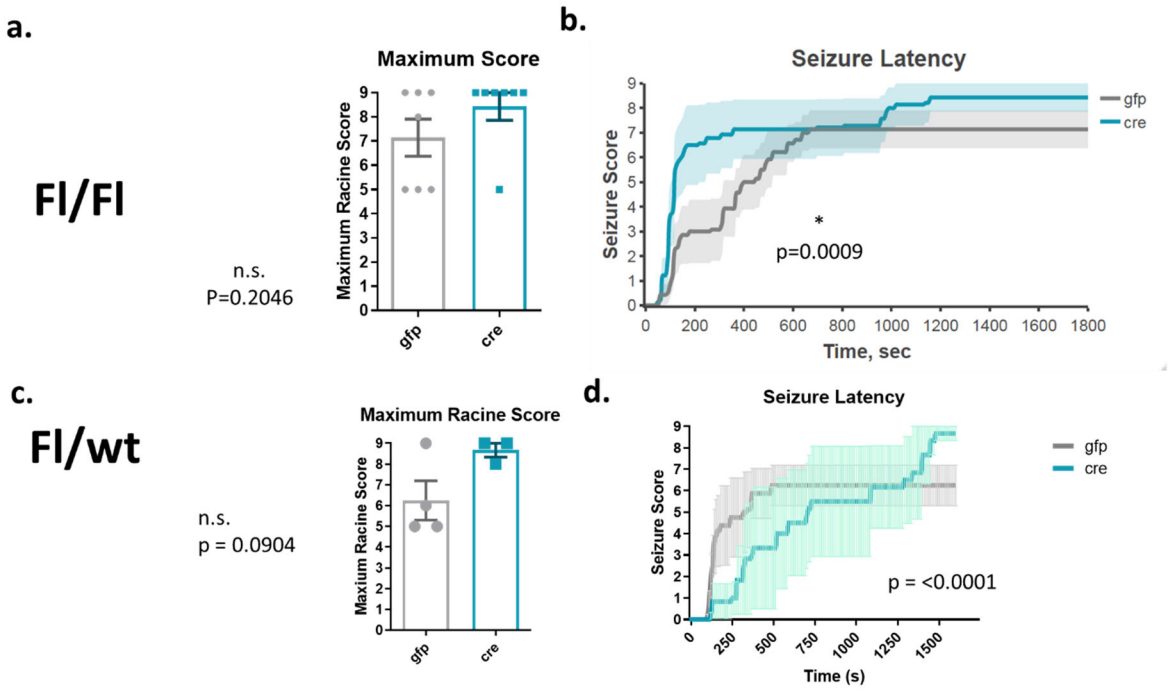


Figure 8. Seizure Threshold with Loss of G9a. a) maximum seizure score of fl/fl GFP AAV injected with GFP AAV and cre-GFP AAV and their b) latency to seizure scores. C) maximum seizure score of fl/wt GFP AAV or cre-GRP AAV injected mice and their d) latency to seizure scores.

Discussion

Recently, there has been increasing evidence for the H3k9me2 pathway and G9a/GLP complex in the regulation of seizures in both human mutations and research models. Interestingly, we have seen conflicting results in supporting the role of G9a specifically in existing studies. Noches et al show no increase in cellular activity with knockdown of G9a but Deneault et al showing hyperactivity in *ehmt2* +/- iPSCs (Deneault et al., 2019; Frega et al., 2019; Noches et al., 2021; Zhang et al., 2018). In our study, we see differing results in KD and KO animals. With KD animals showing

increased seizure latency and KO animals showing reduced seizure latency, though both groups ultimately reached high seizure scores.

In addition, our KD animals showed a physical neuronal phenotype suggestive of injury not seen in our KO animals or in past neuronal knockout studies using G9a fl/fl mice (Schaefer et al., 2009). It is important to note that total germline knockout of *ehmt2* is embryonic lethal, so humans with mutations in *ehmt2* would likely be haploinsufficient and possibly more similar to our KD mice or Deneault's iPSCs (Deneault et al., 2019; Walker, 2015). It is likely that G9a is acting in a dose specific manor. G9a can methylate iself resulting in a regulatory loop (Chin et al., 2007). This property can sometimes result in counterintuitive results such as loss of *ehmt1* increasing levels of H3k9me2 (Iacono et al., 2018).

In addition, G9a can act through alternate mechanisms such as through differing activity of splice variants and methylation of non-histone proteins (Deimling et al., 2017; Fiszbein et al., 2016). Therefore it is possible that G9a is acting through different mechanism to regulate seizure threshold in KO and KD mice. G9a could also be acting in a cell type and region specific manner. Schaefer et al knocked out G9a in all camkii excitatory neurons in the brain, while our conditional KO model targets both excitatory and inhibitory neurons in only the DG. It is possible that loss of G9a in inhibitory neurons is necessary to see the pathology exhibited in our KD mice, or alternatively effects are only seen when KD occurs only in the dorsal DG.. Also, KO mice born of a transgenic cre cross develop with the mutation, giving more time for the possibility of compensatory mechanisms to overcome the loss of G9a. Our animals experience KO or KD as adults, which could result in a more severe phenotype.

With the increase in *cfos* activation with G9a KO, there appears to be more neuronal activity at baseline with these animals. It is unknown if these animals experience any abnormal transmission. No behavioral seizures are observed in these mice, and future studies will use depth electrodes to monitor hippocampal activity to determine if there are any abnormalities (**fig s1**). This study increases the knowledge of the role of G9a in seizures which could ultimately lead to novel anti-epileptic therapeutics targeting these mechanisms, and with the known role of G9a in memory formation, it is possible that targeting G9a in epilepsy could improve memory deficits experienced by patients.

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THE EPIGENETICS OF EPILEPSY AND ITS PROGRESSION

by

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Abstract

Epilepsy is a common and devastating neurological disorder characterized by recurrent and unprovoked spontaneous seizures. One leading hypothesis for the development and progression of epilepsy is that large-scale changes in gene transcription and protein expression contribute to aberrant network restructuring and hyperexcitability, resulting in the genesis of repeated seizures. Current research shows that epigenetic mechanisms, including posttranslational alterations to the proteins around which DNA is coiled, chemical modifications to DNA, and the activity of various noncoding RNA molecules exert important influences on these gene networks in experimental epilepsy. Key findings from animal models have been replicated in humans using brain tissue obtained from living patients at the time of neurosurgical resection for pharmacoresistant epilepsy. These findings have spurred efforts to target epigenetic processes to disrupt or modify epilepsy in experimental models with varying degrees of success. In this review, we will (1) summarize the epigenetic mechanisms implicated in epileptogenesis and epilepsy, (2) explore the influence of metabolic factors on epigenetic mechanisms, and (3) assess the potential of using epigenetic markers to support diagnosis and prognosis. Translation of these findings may guide the development of molecular biomarkers and novel therapeutics for prevention or modification of epileptic disorders.

Introduction

Epilepsy is a prevalent neurological disorder affecting more than 50 million people worldwide (Ngugi and others 2010). Patients suffer recurrent seizures, which are a result of aberrant, excessive, and synchronous firings of groups of neurons within the brain. Seizures originating from within a specific brain region are focal or localization-related, whereas generalized seizures occur simultaneously within both hemispheres of the brain. Seizures are dampened or reduced in many epileptic patients through treatment with antiepileptic drugs (AEDs). There are more than 20 AEDs in common use, each working to dampen brain excitability by boosting inhibitory or attenuating excitatory neurotransmission. However, in approximately 30% of epileptic patients, many AEDs fail to control seizures, and no current treatment options are disease-modifying or are known to prevent epilepsy in at-risk patients (Kwan and Brodie 2000).

The cause of many epileptic disorders is unknown in an estimated 60% of patients. There are monogenic forms of epilepsy, often due to a mutation in an ion channel or component of excitatory or inhibitory neurotransmission. However, these cases are relatively rare, and the contribution of genetics in the majority of patients is less clear (Thomas and Berkovic 2014). Other forms of epileptic disorders can develop as a result of an earlier insult to the brain such as trauma or infection including temporal lobe epilepsy (TLE), the most common focal epilepsy in adults which is characterized by seizures frequently originating from the hippocampus and is often associated with select neuronal loss and gliosis termed hippocampal sclerosis (Blumcke and others 2013; Falconer 1974). Most TLE patients are pharmacoresistant to AEDs and as a result, likely

to undergo surgical resection as a treatment option for their seizures (Wiebe and others 2001). Furthermore, beyond debilitating seizures, common comorbidities of this epileptic disorder include cognitive deficits, anxiety, and depression, making the search for the underlying cause and more effective treatments increasingly more urgent to improve patients' quality of life (Perrine and others 1995). Still, the molecular causes leading to such impairments are poorly understood, and effective treatments are slow to emerge. Recently, epigenetic mechanisms have been linked to epilepsy and cognitive function. Since most of the work on uncovering epigenetic processes in epilepsy has been performed in the context of TLE, this review will focus on this epileptic disorder. However, it is likely that epigenetics also plays a role in various forms of epileptic disorders.

Epigenetic control of gene transcription has gained attention for its potential in the persistent and dynamic regulation of gene expression in the brain. Previously thought to be static in non-dividing, terminally differentiated cells, epigenetic mechanisms have been shown to remain dynamic and active in the mature human brain, and are necessary for the maintenance of essential neuronal function. Those same epigenetic modifications responsible for the regulation of normal brain activity are gaining attention as possible dysregulated systems within neuronal disorders including epilepsy. These biochemical signatures may represent potential biomarkers of epileptogenesis and epilepsy progression or serve as drug targets for the prevention or mitigation of epilepsy.

Epigenetics originally referred to heritable changes to the genome that did not involve direct changes to the genetic code. Now, epigenetics more broadly describes cellular processes that affect the medium to long-term readability and accessibility of the

genome to transcription, thus influencing gene expression outside of direct changes made to the DNA sequence. Epigenetic mechanisms include DNA methylation at 5-methylcytosine (5-mC), hydroxymethylation at 5-hydroxymethylcytosine (5-hmC), changes to chromatin structure facilitated by posttranslational modification of histone proteins, and chromatin structural regulation by noncoding RNAs. Each of these categories of epigenetic processes has been shown to be altered in epilepsy. Efforts to determine whether these changes contribute to the development of the disease or are a response to the seizures themselves is still not fully known, and research has largely been driven by animal models of epilepsy. Epilepsy is, however, unusual among neurological disorders in having brain tissue available to study from living patients as a result of neurosurgical resections. Thus, researchers have been able to compare epigenetic changes observed in animal models of epilepsy to findings in human tissue. Animal modeling approaches have provided important corroborating evidence of epigenetic mechanisms while being a powerful validation of the available animal models. Nevertheless, individual models suffer limitations of their own, and a key challenge has been to cross-compare findings. Thus, epilepsy is perhaps uniquely positioned to gain clinically relevant insights into the influence of epigenetics on gene expression through the use of multiple models and human tissue from living patients.

Epigenetic mechanisms have also been proposed to explain the development of pharmacoresistance in epilepsy patients, influencing the sustained patterns of gene expression that regulate AED uptake and mechanism of action. In the future, drugs inhibiting DNA methyltransferases (DNMTs) and histone lysine deacetylases (KDACs) could provide new treatments for patients unaffected by currently available anti-epilepsy

medications. Not only are drugs acting on epigenetic processes currently available and in development for a variety of human diseases, but there is some evidence that one or more commonly used AEDs may act, in part, through epigenetic mechanisms. Most of the work identifying epigenetic changes in epilepsy has focused on DNA methylation. Recently, there has been exceptional interest in the role of microRNAs, especially where attention has also focused on their potential for use as biomarkers to support early diagnosis and prognosis in the clinic. In contrast, other aspects, such as histone modifications and long noncoding RNAs, have been less studied.

To determine if an epigenetic modification is causal to the development of epilepsy or a result of repeated seizures, it is most crucial to look at time points soon after the first status epilepticus event. Status epilepticus is the initiating injury event in many models of TLE characterized by a long convulsive seizure event. Following the initial seizure, both humans and rodents enter a latent period where they experience no seizures before developing epilepsy. However, not all patients will develop epilepsy after the initial seizure event. This latent period between status epilepticus and recurrent seizures is crucial to understanding the alterations in brain connectivity necessary to make a brain epileptic. Epigenetics is being discovered to be increasingly more important during this crucial time point before epileptogenesis. Epigenetic changes seen after status epilepticus but before prolonged epilepsy may have roles in making the switch to an epileptic brain state.

Epilepsy is associated with large changes in gene transcription that are exacerbated by altered metabolism. Network homeostatic regulation is achieved through a careful balance between excitatory and inhibitory synaptic inputs regulated through

various intracellular metabolic processes, including glycolysis and interwoven methionine and adenosine–related pathways. Disorders, such as epilepsy, with aberrant cell metabolism will ultimately lead to changes in the overall epigenetic landscape of the cell, as metabolites fundamentally provide the donor substrates necessary for epigenetic modification (reviewed in Xu and others 2016). DNA and histone methylation are reliant on availability of metabolically produced methyl donors. Through this and several other pathways, metabolism can signal to the cell to control the underlying epigenetic landscape (Figure 1).

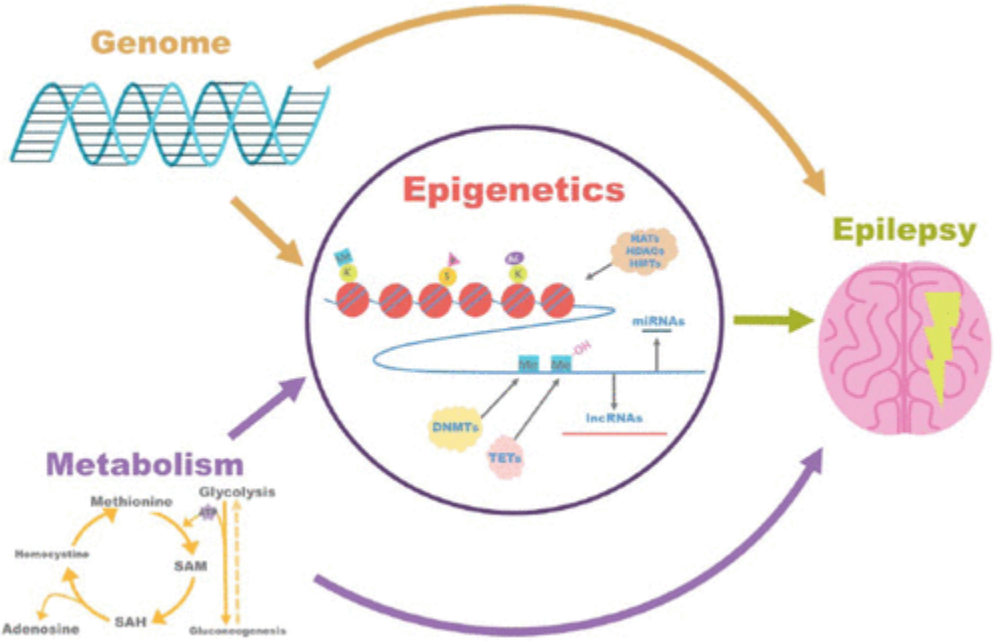

Figure 1. This schematic figure illustrates how epigenetic mechanisms are influenced by both genome and metabolism leading to their role in epilepsy.

Epigenetic Mechanisms Implicated in Epilepsy

DNA Methylation

DNA methylation is one of the best understood epigenetic modifications. It occurs primarily in regions of DNA containing CpG sites, defined as cytosine residues directly next to guanine residues within the underlying DNA sequence. Methyl groups are added to DNA bases by enzymes known as DNMTs, which have been shown to have diverse and specific functions. DNMT3a and DNMT3b are largely associated with the addition of new methylation marks, whereas DNMT1 is primarily associated with maintenance of DNA methylation (Okano and others 1999). Typically, after a region of DNA has been methylated, the transcription of the associated gene is repressed. Gene repression through DNA methylation is achieved by providing binding sites for transcription regulating proteins or preventing transcription factor binding. These factors will either promote compaction or opening of the DNA, making the corresponding sequence either more or less accessible to transcription by RNA polymerase. DNA methylation can be found anywhere within the genome, but methylation at promoter regions is especially interesting because of the regulatory potential for robust changes in transcription of the associated gene. Previously considered to be a stable mark, it is now known that DNA methylation is dynamic, and methyl groups can be modified or removed from DNA residues through the activity of certain enzymes including GADD45B (Ma and others 2009).

Alterations in DNA methylation has been observed in TLE, with several genome-wide studies supporting evidence that epilepsy progression is accompanied by large-scale methylome changes. Accompanying genome-wide methylation changes is evidence supporting dynamic protein expression of DNA modifying enzymes in TLE human tissue. For example, evidence that DNMT3a and DNMT1 are up-regulated in TLE hippocampal tissue and are especially prevalent in neuronal cells, suggests that de novo DNA methylation and its maintenance are involved in human TLE (Zhu and others 2012). Injuries associated with inciting TLE have been reported to promote a lowered state of methylation, suggesting a switch toward increased gene transcription. In contrast, research evidence shows that the chronic epileptic state associated with overall global hypermethylation of the genome. An initial study of DNA methylation in a rat pilocarpine model demonstrated that global increases in methylation inversely correlated with a decrease in the expression of some genes (Kobow and others 2013b). Intriguingly, an amygdala stimulation model of TLE showed a decrease in promoter methylation that correlated with an increase in gene expression (Debski and others 2016). These studies underscore the importance of distinguishing global DNA methylation and promoter methylation changes as it relates to gene transcription silencing and the role of DNA methylation in epilepsy development and progression.

Another interesting aspect of the DNA methylation study performed by Debski and colleagues compared the pilocarpine, amygdala stimulation, and traumatic brain injury models of epilepsy and found no commonly differentially methylated genes or enrichment of similar gene ontologies. However, the three epilepsy models did share seven similarly up-regulated genes (Debski and others 2016). Interestingly, a genome-

wide methylation study in the kainate model of TLE identified 321 differentially methylated genes, predominately hypomethylation events, with an inverse correlation between decreased DNA methylation and increased gene expression (Miller-Delaney and others 2012). Of note, there was little overlap among the studies described above in the specific genes reported undergoing changes. Collectively, these findings indicate a strong influence of etiology and model-specific regulation of methylation.

DNA methylation has been found to be altered for 146 genes in the hippocampus of TLE patients defining hippocampal sclerosis, most of which were found to be hypermethylation events. Gene ontology analysis revealed that these genes were involved in neural development, differentiation, remodeling, and maturation. Notably, there appeared to be a weak association between methylation state and expression of the affected gene (Miller-Delaney and others 2015). Interestingly, methylation at microRNA gene regions was also investigated, suggesting that expression of several microRNAs were highly sensitive to methylation at the transcriptional start site and putative promoter regions (Miller-Delaney and others 2015).

DNA methylation at 5-mC can be further modified to 5-hydroxymethylcytosine (5-hmC) by ten-eleven translocation (TET) enzymes which mediate the oxidation of 5-mC marks to 5-hmC (Tahiliani and others 2009). The role of 5-hmc in the brain is of exceptional interest from a neuroscience perspective because this mark is abundantly present in the brain, indicating a likely prevalent role as a major epigenetic regulator of neuronal activity (Kriaucionis and Heintz 2009; Munzel and others 2010). Genome wide analysis shows that 5-hmC can be present in areas of both transcriptional repression and activation, though this mark is typically associated with enhanced gene transcription

(Wen and others 2014; Wu and others 2011). Hydroxymethylated regions can serve as binding sites for proteins acting as regulators of transcription (Spruijt and others 2013). Notably, within the brain, the methyl binding protein MeCP2 was shown to preferentially bind to 5-hmC marks, supporting a major role for MeCP2 in the regulation of the neural transcriptome (Mellen and others 2012).

DNA hydroxymethylation remains understudied in the context of epilepsy in comparison to DNA methylation. This discrepancy was, however, recently addressed. Global levels of 5-hmC were found to be decreased in the hippocampus in areas CA3 and the dentate gyrus in a rodent kainate model of status epilepticus (Parrish and others 2013). Additionally, TET1, the protein responsible for the addition of hydroxymethylation, was found to be down-regulated in the hippocampus following acute seizures (Kaas and others 2013). Though a global role for 5-hmC and its regulatory enzyme TET1 has been shown in the epileptic hippocampus, at present, DNA hydroxymethylation has not been mapped at a gene-specific level within epilepsy. Thus, it is uncertain how dynamic these changes are and whether demethylation events affect the same or different regions of the genome as methylation in epilepsy. Moreover, using current technologies, it is often difficult to differentiate 5-mC and 5-hmC marks, meaning it is very likely then that 5-hmC levels are also responsively changing in bisulfite sequencing studies showing alterations in 5-mC with epilepsy (Huang and others 2010; Jin and others 2010).

As DNA methylation and hydroxymethylation regulate the genome through either allowance or prevention of regulatory protein binding, the role of methyl binding proteins is thought to modulate epilepsy progression in addition to basal methylation changes (Li

and others 2015). Methyl binding proteins bind to regions of methylated DNA and can aide in either transcriptional repression or activation of the bound sequence. The influence of dysregulated or dysfunctional methyl binding proteins on seizure activity is perhaps most obvious in epilepsy-related neurological disorders such as Rett syndrome. Rett syndrome is caused by genetic mutations in the methyl binding protein MeCP2, and patients with Rett syndrome commonly experience epileptic seizures as a comorbidity of the disorder. One of the effects of reduced MeCP2 is bidirectional alterations in expression of genes involved in neurotransmission and various neuromorphological phenotypes including reduced dendritic complexity which likely promote the excessive excitatory input to neurons that can result in seizures (reviewed in Lyst and Bird 2015).

MeCP2 is known to be associated with hypermethylation at the RELN promoter, a region with increased methylation in TLE. The RELN gene encodes a protein (reelin) secreted from Cajal-Retzius cells critical to cell positioning, including the densely-packed granule cell layer of the dentate gyrus. This suggests the role of methyl binding proteins in the dysregulation of this extracellular matrix protein encoding gene associated with epilepsy (Kundakovic and others 2007). In addition to MeCP2, the methyl binding protein MBD2 was found to be implicated in epilepsy through binding to specific CpG sites involved in the up-regulation of the sodium channel protein coding gene Scn3a. As sodium channels help in the regulation of neuronal excitation, this suggests a possible mechanism for Scn3a dysregulation seen in epilepsy through interactions of DNA demethylation and MBD2 (Li and others 2015).

Posttranslational Modifications of Histones

DNA is condensed into chromatin within cell nuclei and wrapped around proteins called histones. The single unit of chromatin is called the nucleosome, and consists of approximately 146 nucleotides wrapped around a histone core. The histone core is an octamer composed of 4 histones, typically H2A, H2B, H3, and H4. Histones can be modified on their N-terminus, commonly on lysine or arginine residues, and these histone tail modifications can promote the transformation of chromatin conformation into a readily accessible and heavily transcribed configuration called euchromatin, or they can condense the chromatin into tightly wound heterochromatin typically associated with areas of transcriptional repression. The change from hetero- to euchromatin depends on both the type of modification being added, what histone is being modified, and the location of that modification on the N-terminal histone tail. Typically, the addition of acetyl or phosphate groups physically alters the structure of chromatin, making the DNA more easily accessible to transcription by polymerase. Alternately, methylation marks act as modulators of transcription through permitting the binding of proteins that either repress or activate transcription of the bound DNA via either polymerase recruitment or exclusion (reviewed in Lubin 2012).

It is noteworthy that there are AEDs in use that target histone modifications. This includes valproic acid, one of the most commonly prescribed drugs for the treatment of epilepsy. Valproic acid is known to have lysine deacetylase (KDAC) inhibiting properties, and interestingly valproic acid also affects DNA methylation at specific genes within the hippocampus (Aizawa and Yamamuro 2015; Detich and others 2003;

Gottlicher and others 2001; Milutinovic and others 2007). Because of the drug's known KDAC inhibiting properties, histone modification changes were accordingly targeted early on in the study of epilepsy epigenetics, most notably with the study of histone acetylation marks. Acetylation of histone H4 was shown to be altered in the hippocampus of rats at the BDNF and GluR2 genes following status epilepticus, associated with regulation of the resulting mRNA expression (Huang and others 2002). Since then, the study of altered histone modifications in TLE has expanded to include changes in histone H3 phosphorylation (Crosio and others 2003; Sng and others 2006; Zybura-Broda and others 2016). However, other tested KDAC inhibitors do not appear to have anticonvulsive effects suggesting that this property is probably not important for the seizure-suppressive effects of valproic acid (Hoffmann and others 2008). While there is limited functional evidence that histone acetylation is required for epileptogenesis, one study suggests that loss of the lysine deacetylase KDAC4 results in the development of later-life epilepsy in mice (Rajan and others 2009).

Epilepsy is a disorder characterized by altered neuronal excitability, including alterations in glutamate receptors following status epilepticus. This process is known to be epigenetically controlled through a decrease in histone H4 acetylation at the glutamate receptor encoding gene GluR2, lowering total mRNA expression. Likewise, acetylation regulates several processes relevant to the progression of epilepsy, including increased H4 acetylation at the BDNF P2 promoter following status epilepticus (Huang and others 2002), and H4 acetylation-driven activation of c-fos and c-jun expression during epileptogenesis within the hippocampus (Sng and others 2006). Along with histone acetylation, histone phosphorylation plays a role in the transcriptional regulation of

epilepsy-associated genes. Coupled to H4 acetylation, H3S10Ph was found to be initiating up-regulation of c-fos in the dentate gyrus (Sng and others 2006; Taniura and others 2006), and H3S10Ph has been implicated in the regulation of hippocampal Mmp-9, a transcript encoding an extracellular matrix protein, during epileptogenesis (Zybura-Broda and others 2016).

As opposed to histone acetylation, which is an activator of gene transcription, histone methylation can either enhance or repress gene transcription, depending on the location and number of modifications per amino acid residue. Histone lysine methylation, in particular, has been widely studied and annotated as a part of efforts to understand the histone code. Histone methylation changes have not yet been thoroughly explored in the context of epilepsy. However, a recent study examining the polycomb repressive complexes 1 and 2 (PRC1 and PRC2) which regulate trimethylation at histone H3 at lysine 27 (H3K27me3), a well-annotated posttranslational histone modification corresponding with transcriptional repression, found H3K27me3 influenced the expression of matrix metalloproteinase-9 during epileptogenesis (Zybura-Broda and others 2016).

Histone methylation has been shown to play critical roles in neuronal function and is known to be highly regulated in processes such as learning and memory (Gupta and others 2010; Gupta-Agarwal and others 2012). Since histone modifications are crucial in the regulation of chromatin structure, and therefore the regulation of gene expression, these dynamic changes could play an important role in epilepsy progression. As environmental cues change, so too can histones be rapidly modified to better adapt the cell to a new environment. Histone modifications show promise in the regulation of

chromatin structure and gene expression during epilepsy (Table 1). However, this area requires further study.

Table 1. Summary of epigenetic mechanisms regulated in epilepsy.

Model	Tissue	Target	DNA Methylation	Histone Modifications	Source
Rat					
	CA3	*BDNF*	n/a	H4 acetylation increase	(Huang and others 2002)
	CA3	*GluR2*	n/a	H3 and H4 acetylation decrease	(Huang and others 2002)
	Hippocampus	n/a	n/a	H4 acetylation increase	(Sng and others 2006)
	DG	n/a	n/a	H3 phosphorylation increase	(Sng and others 2006)
	CA1/CA3	*Grin2b*/*Nr2b*	Increase	n/a	(Parrish and others 2013)
	CA1/CA3	n/a	Global increase	n/a	(Parrish and others 2013)
	CA1	Total 5-hmc	Global increase	n/a	(Parrish and others 2013)
	DG	Total 5-hmc	Global decrease	n/a	(Parrish and others 2013)
	DG	n/a	Global decrease	n/a	(Parrish and others 2013)
	CA1/CA3/DG	*BDNF*	Decrease	n/a	(Parrish and others 2013) (Parrish and others 2015)
	Hippocampus	n/a	Global increase	n/a	(Kobow and others 2013a)
	Hippocampus	*Gria2*	Increase	n/a	(Machnes and others 2013)
	Hippocampus	*Mmp-9*	n/a	H3S10Ph increase	(Zybura-Broda and others 2016)
	Hippocampus	n/a	n/a	H3K27me3 decrease	(Zybura-Broda and others 2016)
	Hippocampus	n/a	n/a	H2AK119ub1 increase	(Zybura-Broda and others 2016)
	CA3/DG	n/a	Global decrease (PILO and amygdala stimulation)	n/a	(Debski and others 2016)
	CA3/DG	n/a	Global increase (TBI)	n/a	(Debski and others 2016)
Mouse					
	Hippocampus	n/a	n/a	H3Ser10ph increase	(Crosio and others 2003)
	CA3	n/a	Global decrease	n/a	(Miller-Delaney and others 2012)
	Hippocampus	Scn3a	Decrease -39C site	n/a	(Li and others 2015)
Human					
	Resected TLE brain tissue	*RELN*	Increase with granule cell dispersion	n/a	(Kobow and others 2009)
	Resected TLE brain tissue	Increased DNMT1	n/a	n/a	(Zhu and others 2012)
	Resected TLE brain tissue	Increased DNMT3a	n/a	n/a	(Zhu and others 2012)
	Peripheral blood leukocytes	*CPA6*	Increase	n/a	(Belhedi and others 2014)
	TLE resected hippocampus	n/a	146 differentially methylated genes	n/a	(Miller-Delaney and others 2015)
	Temporal neocortex refractory epilepsy	*TUBB2B*	Increase	n/a	(Wang and others 2016b)
	Temporal neocortex refractory epilepsy	*ATPGD1*	Increase	n/a	(Wang and others 2016b)
	Temporal neocortex refractory epilepsy	*HTR6*	Decrease	n/a	(Wang and others 2016b)
	Brain tissue refractory epilepsy patients	n/a	62 differentially expressed genes	n/a	(Liu and others 2016)
	Resected epileptic hippocampus	*MMP-9*	Decrease	n/a	(Zybura-Broda and others 2016)
	Peripheral blood	n/a	Increase at lncRNA and miRNA promoters	n/a	(Xiao and others 2017)
	Peripheral blood	n/a	Global increase	n/a	(Long and others 2017)

CA1 = *cornu ammonis* region 1; CA3 = *cornu ammonis* region 3; DG = dentate gyrus; n/a = not applicable; PILO = pilocarpine; TBI = traumatic brain injury.

Table 1. Summary of epigenetic mechanisms regulated in epilepsy

Non-coding RNAs

Non-coding RNAs comprise a diverse class of regulatory molecules that broadly includes small noncoding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) including circular RNAs, long interspersed elements, and pseudogenes (reviewed in St Laurent and others 2015). MicroRNAs have gained particular interest in epilepsy as various miRNAs are dysregulated in human TLE. Functional studies have reported powerful seizure-regulating effects of at least a dozen different miRNAs to date. Biofluids from patients with epilepsy have also been reported to contain altered levels of certain miRNAs supporting their potential use as putative biomarkers of epilepsy. For a recent review on this topic, the reviewer is referred elsewhere (Henshall and others 2016), and interested readers can search EpimiRBase for a comprehensive list of miRNAs associated with epilepsy (Mooney and others 2016).

Unlike miRNAs, long non-coding RNAs (lncRNAs) have not yet gained much attention within the epilepsy field, and they require further study. LncRNAs are around 200 nucleotides or more in length, typically do not encode for protein, and act through a variety of mechanisms to impact epigenetic modifiers and gene transcription. LncRNAs are gaining attention in the regulation of several neuronal mechanisms including the regulation of cognition and memory (reviewed in Butler and others 2016). A genome-wide lncRNA profiling study found differential regulation of lncRNAs in both the pilocarpine and kainate acid models of TLE, suggesting lncRNAs act as regulators of inflammation and neuronal differentiation pathways in the epileptic brain (Lee and others 2015). Interestingly, the nuclear lncRNA Neat1 is down-regulated following seizure

activity and aids in the regulation of neuronal excitation, notably through interactions with potassium channels (Barry and others 2017). Supporting the involvement of lncRNAs in the maintenance of neuronal excitation, the lncRNA BC1 comprises the balance of neuronal excitation and repression through transcriptional inhibitory mechanisms with seizures (Zhong and others 2009), and Malat1 has been reported to aid in synaptogenesis (Bernard and others 2010).

With regard to the methylation hypothesis of epilepsy progression, a recent study has looked at the methylation of non-coding RNA genes in the blood of patients with TLE. LncRNA promoters were found to be mostly hypermethylated, and these lncRNAs targeted genes in pathways relevant to pharmacoresistance and neuronal signaling (Xiao and others 2017). This idea complements the differential methylation of miRNA and lncRNA genes found in epileptic human hippocampal tissue (Miller-Delaney and others 2015). These studies show the importance of furthering the investigation of this transcriptional regulatory mechanism in epilepsy. In addition to elucidating potential involvement in molecular mechanisms of epilepsy development, lncRNAs represent potential drug targets for the treatment of epilepsy. This potential gains further support as administration of an antagoNAT resulted in up-regulation of the sodium channel protein SCN1A and reduced seizures by targeting endogenous NAT (natural antisense transcript) RNAs in a model of the epilepsy-related disorder Dravet syndrome (Hsiao and others 2016) (Table 2, Figure 2).

Table 2. Summary of Recent In Vivo Studies Demonstrating Anti-Seizure Effects of Manipulating Noncoding RNAs in Animal Models of Epilepsy.

Noncoding RNA	Model (Species)	Finding	Target and/or Mechanism	Reference
MicroRNA				
miR-22-3p	KA (mouse)	Mimic (agomir) reduced spontaneous seizures	P2X7 receptor (ATP), inflammation	(Jimenez-Mateos and others 2015a)
miR-23b-3p	KA (mouse)	Mimic (agomir) reduced seizures	Not studied	(Zhan and others 2016)
miR-124	PTZ and pilocarpine (rat)	Mimic (agomir) reduced/delayed seizures	CREB, transcription of excitatory neurotransmission genes	(Wang and others 2016c)
miR-134	PILO (mouse) PTZ (mouse) PPS (rat)	Inhibition (antagomirs) reduced evoked and spontaneous seizures	LIM kinase 1, neuronal microstructure	(Jimenez-Mateos and others 2015b) (Reschke and others 2017)
miR-146a	KA (mouse)	Mimic (agomir) reduced spontaneous seizures	Inflammatory signaling (IL1β/TLR4)	(Iori and others 2017)
miR-199a-5p:	PILO (rat)	Inhibition (antagomirs) reduced seizures	SIRT1	(Wang and others 2016a)
miR-203	PILO (mouse)	Inhibition reduced spontaneous seizures	Glycine receptor-β/ inhibitory transmission	(Lee and others 2016)
miR-219	KA (mouse)	Mimic (agomir) reduced seizures	NR1 (NMDA receptor)/ excitatory transmission	(Zheng and others 2016)
miR-324-5p	KA (mouse)	Inhibition (antagomirs) reduced seizures	Kv4.2 potassium channel, neurotransmission	(Gross and others 2016)
Long non-coding RNA				
Neat1	Resected human tissue and PILO (rat)	Up-regulated with epilepsy	KCNAB2 and KCNIP1, excitability	(Barry and others 2017)
Antisense non-coding RNA (SCN1ANAT)	Scn1a haploinsufficient mice (model of Dravet)	Increased (heat induced) seizure threshold	Sodium channel function/ neurotransmission	(Hsiao and others 2016)

CREB = cyclic AMP response element binding protein; IL1β = interleukin 1β; KA = kainic acid; PILO = pilocarpine; PTZ = pentylenetetrazol; SIRT1 = Sirtuin; TLR = Toll-like receptor.

Table 2. Summary of recent in vivo studies demonstrating anti-seizure effects of manipulating noncoding RNAs in animal models of epilepsy

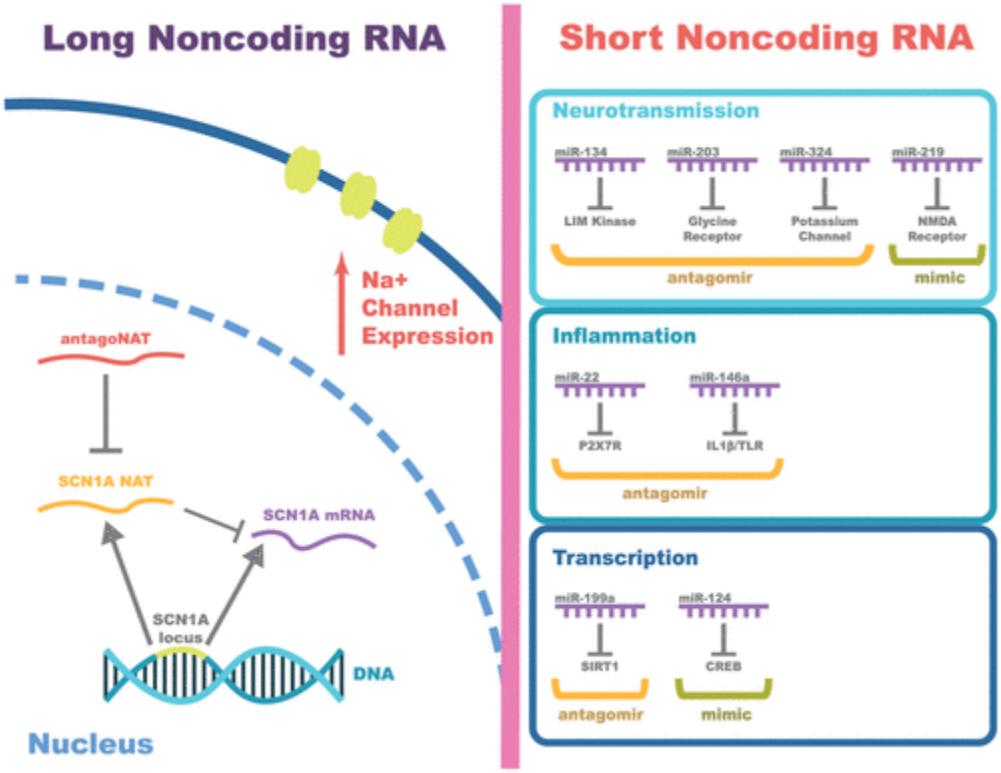

Figure 2. Illustration showing mechanisms by which non-coding RNAs affect seizures and the impact of their targeting. Highlighted here are the proposed targets of a candidate long non-coding RNA implicated in epilepsy (Hsiao and others 2016) and several microRNAs (Gross and others 2016; Iori and others 2017; Jimenez-Mateos and others 2015a; Jimenez-Mateos and others 2015b; Lee and others 2016; Reschke and others 2017; Wang and others 2016a; Wang and others 2016c; Zheng and others 2016).

The Role of Metabolism in the Epigenetic Regulation of Epilepsy

Glucose Utilization in Epilepsy

It is well-established that metabolism plays an important role in neuronal network stability and that metabolic dysfunction is a strong influence in certain epilepsy syndromes. Glucose is the brain's primary source of energy, and treatments targeting glucose metabolism show promise in epilepsy. The ketogenic diet is designed to resemble fasting and results in ketosis, an increase of ketone body metabolites. This provides

neurons with an alternate energy source to glucose. The ketogenic diet has long been used to help reduce seizures in pharmacoresistant patients and continues to be popular for refractory epilepsy in children. Likewise, the ketogenic diet is known to effectively treat GLUT1 deficiency syndrome, a disorder characterized by decreased GLUT1 transporter expression and is accompanied by seizures (Klepper and others 2005).

The mechanism by which the ketogenic diet works is not fully understood. It has been shown that ketones themselves do not have an antiepileptic effect in studies using direct administration of ketones to hippocampal slices; however, in vivo administration results in seizure reduction. It has been suggested that the ketones could act by alteration of glutamate signaling pathways and that the effect of the ketogenic diet is linked to extracellular glucose levels (Kawamura and others 2014; Kawamura and others 2016). Inhibition of the glycolytic pathway through the administration of 2-deoxy-d-glucose shows an anticonvulsant effect (Stafstrom and others 2008; Stafstrom and others 2009). These findings suggest that dysregulated glucose and its metabolic intermediates play a crucial role in epilepsy progression. Glycolysis feeds into the citric acid cycle to ultimately produce glutamate and other important neurotransmitters (Olsen and Sonnewald 2015). Alterations in the way neurons metabolize glucose and harvest this energy could result in the large changes in gene expression that lead to the sudden, spontaneous electrical firings seen with epileptic seizures.

Methionine

Methionine metabolism results in the production of the methyl-donor S-adenosyl methionine (SAM), making it an important regulatory metabolite in the pathway of DNA methylation. The reaction responsible for the addition of SAM's methyl group to another molecule results in the cycling of SAM to S-adenosyl-l-homocysteine (SAH). An increase in the concentration of intracellular SAM can promote DNA and histone methylation, and in turn, high SAH levels prevent further DNA and histone methylation (reviewed in Berger and Sassone-Corsi 2016).

Within the study of epilepsy, SAM has been shown to have antiepileptic properties (Dhediya and others 2016). SAM metabolism results in the production of neuroprotective adenosine, possibly contributing to these anti-seizure effects. However, SAM can also act through involvement in transmethylation pathways, serving as a methyl donor for DNA and histones. Direct methionine (met) infusion results in a global increase in methylation, and artificial overexpression of DNA methylation through methionine injection can act as a valuable tool in the study of DNA methylation interactions in epilepsy (Parrish and others 2015).

The hippocampus, a brain region important for memory formation, is frequently the site of pathology and ictogenesis in TLE. Accordingly, patients often experience cognition and memory difficulties as a comorbidity of the disorder. The BDNF gene has been known to be important in memory formation and dysregulated with epilepsy (Lubin and others 2008; Martinez-Levy and others 2016). Parrish and others observed an increase in activity-dependent memory formation associated with increased levels of BDNF in the rat kainate model of TLE. Supplementation with methionine both decreased

BDNF transcript and improved the memory impairment in epileptic rats, suggesting a role for DNA methylation in epilepsy associated memory decline (Parrish and others 2015).

Methionine is a component of methionine sulfoximine (MSO), a chemical that has been known to induce seizures for decades; however, its mechanism of action has not been described. MSO is molecularly similar to glutamate, suggesting action through inhibition of glutamate receptors (Campbell and others 1951; Cloix and Hevor 2009). Alternatively, it has been reported that MSO inhibits glutamine synthetase, leading to neuronal activation through disruption of normal glutamine metabolism (Albright and others 2016; Richard and Hevor 1995).

Adenosine

Adenosine acts as an endogenous anticonvulsant, and adenosine is a well-defined inhibitory neuromodulator within the brain acting on A1 receptors at synapses. Inhibition at epileptic synapses through adenosine administration suggests that network excitability is controlled by adenosine cycling (reviewed in Boison 2016). Adenosine is involved in several pathways at an intracellular level being an essential component of ATP/ADP, the primary energy unit of cells, and involved in reduction and oxidation as a component of NADH. Interestingly, adenosine aids in the regulation of DNA methylation through modulation of SAM/SAH cycling, and augmentation with adenosine is known to both prevent epileptogenesis and decrease global DNA methylation in the hippocampus (Williams-Karnesky and others 2013). Since methylation changes with epileptogenesis

have been well documented, it is possible that adenosine's anticonvulsant properties are at least in part due to the regulation of this epigenetic pathway.

Maintenance of adenosine is achieved through regulation by adenosine kinase, and the balance of adenosine and adenosine kinase is a crucial factor in epilepsy development. This regulation is especially relevant to epilepsy within astrocytes as adenosine kinase is abundant in astrocytes. Astrogliosis is commonly seen in epileptic tissue, suggesting a strong role for astrocytic metabolism in epilepsy (Li and others 2008).

The biochemical interactions between the pathways of adenosine and methionine metabolism conceptually elucidate the contribution of maladaptive changes in brain biochemistry as a major contributing factor to epilepsy development. In association with epilepsy and alterations in adenosine and methionine pathways, there is evidence for dynamic changes in cofactors utilized by epigenetic modifiers and epigenetic substrates. However, whether aberrant metabolism is the root source of the sudden seizures, or instead the result of epileptic activity and the underlying continuous editing of the epigenome is still uncertain. Because of the defined interwoven nature of metabolism and epigenetics, the role of metabolism in the study of epilepsy epigenetics cannot be ignored.

Epigenetic and Metabolic Control of Glutamate Transmission in Epilepsy

Glutamate is an important neurotransmitter, and regulation of glutamate and its metabolic intermediates is crucial to epilepsy progression through their involvement in

the control of neuronal excitability. In addition to glutamate, the regulation of glutamate receptors has a strong role in epilepsy. The glutamate receptor encoding genes Gria2 and GluR2 have been shown to be associated with epileptic tissue. Furthermore, these genes are known to be epigenetically regulated in the context of epilepsy through both histone acetylation and DNA methylation changes (Huang and others 2002; Machnes and others 2013).

In TLE, glutamine synthetase (GS), the molecule responsible for the synthesis of the neurotransmitter glutamate, has been shown to be down-regulated in hippocampal astrocytes (Dhaher and others 2015). As astrogliosis is a common hallmark of epileptic foci in the brain, this suggests that GS dysregulation in these cells may contribute to dysregulation of glutamate homeostasis. Further supporting the importance of glutamine synthetase regulation's contribution to epilepsy progression, haploinsufficiency of GS increases susceptibility to febrile seizures in a mouse model (van Gassen and others 2009). It is clear that a balance of GS is crucial to the maintenance of healthy brain activity, and regulation of this pathway could provide an important link between epigenetic processes and cellular metabolism.

Putative Epigenetic Biomarkers for Early Diagnosis of Epilepsy

There are currently no well-annotated molecular biomarkers for epilepsy to aid in the early diagnosis of abnormal epileptic associated activity within the brain. Such a biomarker would be transformational since epilepsy is associated with high misdiagnosis rates and the best diagnostic tool—electroencephalography (EEG)—is slow and

technically demanding. Moreover, many patients with epilepsy have a normal EEG and patients without epilepsy can have an abnormal EEG. Although EEGs are capable of monitoring the abnormal activity seen with epilepsy, the patient must be experiencing epileptic activity at the time of reading to make it a useful tool in the diagnosis of epilepsy. Currently, a reliable biomarker could help in getting patients treatment before further progression of the disorder does irreversible damage to the brain. The detection of biomarkers could lead to the development and use of anti-epileptogenic drugs that will target the underlying cause of epilepsy before patients succumb to recurrent seizures.

In the search for new biomarkers, it is important to keep in mind accessibility in the clinic. The biomarkers should be easily read with common clinical lab equipment, and ideally, could be screened for during routine tests such as blood or urine samples. Because of this, molecular mechanisms underlying epileptogenesis, including epigenetic mechanisms, are then key focal points in the study and identification of epileptic biomarkers because they can change with the onset of the disorder and can be present in biofluids collected from patients.

MicroRNAs are the leading candidates in the search for epigenetic biomarkers for the early detection epilepsy due to their differential expression and ready accessibility in blood samples. Recently, mir-134 levels in serum have been shown to have promise for easy detection in epilepsy patients (Spain and others 2015). Likewise, serum mir-4521 could be a potential biomarker for patients with refractory epilepsy (Wang and others 2016d). Several miRNAs, notably mir-301a-3p, are shown to be differentially expressed in serum between drug resistant and drug responsive patients (Wang and others 2015).

Therefore, miRNAs in serum could be used in the clinic to detect both epilepsy and likelihood of drug resistance early.

DNA methylation is proving to be a key molecular mechanism of epileptogenesis and could be invaluable as a means to detect epilepsy progression. Furthermore, the DNA methylation status in TLE patients can easily be assessed, as it has been demonstrated to be differentially regulated in TLE patient blood (Long and others 2017; Xiao and others 2017). Therefore, as testing in the clinic improves, it is possible that DNA methylation levels of certain genomic regions can be tested for as a biomarker of epilepsy progression and could potentially be used to inform clinicians of the most effective anti-epileptogenic drugs to use to halt the further development of epilepsy.

Therapeutic Prospects for the Future

One of the challenges facing treatment options for epileptic disorders is determining the optimal time window for intervention or preventative care. For example, if reliable biomarkers are discovered for the detection of epilepsy onset prior to structural neuronal damage due to chronic seizures, this would be an ideal time window to begin intervening treatments. However, the availability of this optimal time window is often not a reality, as most patients diagnosed with epilepsy have already experienced chronic seizure episodes and damage to neuronal networks. Thus, the goal to identify new biomarkers early on to prevent further epilepsy progression and to lessen seizure severity and frequency remains a challenge. The epigenetics field represents the potential for the

discovery of new molecular biomarkers and anti-epileptogenic drug targets, and uncovering mechanisms underlying epileptogenesis.

With the development of new technology, researchers and physicians can more accurately evaluate epigenetic mechanisms that make studying the role of epigenetics in epilepsy more attainable. There are new molecular, bioinformatic, and surgical tools developed to allow us to increase our understanding of the underlying mechanisms involved in the development of epilepsy. One such tool is the EpiMiRbase database, which will prove to be increasingly relevant with the number of miRNAs associated with epilepsy rising (Mooney and others 2016). The development of new molecular tools such as CRISPR, optogenetics, and DREADDS can also improve epilepsy research by allowing the targeting of specific genes, epigenetic modifications, cell types, and receptors in rodent models of epilepsy. Likewise, new surgical tools are improving the diagnosis of seizures. Utah arrays are surgically implanted into the brain and help to pinpoint the source of focal epilepsies to allow physicians to treat the pharmacoresistant patients more specifically through surgical resection (Fernández and others 2014). As new techniques and technologies are developed to improve the diagnosis and study the progression of epilepsy, our understanding of this disorder improves, and we come closer to the development of new treatments for patients.

Conclusions

The study of epigenetics in epilepsy is a new field with opportunities for researchers and physicians alike. Through the use of human tissue and animal models, it

has been shown that epigenetic mechanisms are dynamically regulated during the progression of epilepsy. Genome-wide DNA methylation changes are observed in epileptic tissue, with the potential for altering transcription of genes important for the switch to an epileptic state (Debski and others 2016; Kobow and others 2013b; Miller-Delaney and others 2012). Chromatin structure is altered in epilepsy through changes in histone modifications, notably histone acetylation. Because of the global shift in DNA methylation, it is likely that histone methylation is dynamically changing as well, and histone methylation remains an area warranting further study in the context of epilepsy. LncRNAs and miRNAs involved in the regulation of the chromatin state surrounding gene regions are beginning to show a promising role in the development of epilepsy. Excitingly, this translates into the likelihood that ncRNAs hold promise for use as potential biomarkers for early detection of epilepsy in the clinic.

Epilepsy is a disorder that is highly interwoven with aberrant metabolism. Because of the fundamental nature of metabolites in providing donor substrates for epigenetic modifications, it is inevitable that epileptic changes in metabolism regulate the epigenetic landscape of the brain. Methionine and adenosine metabolic pathways play considerable roles in the development of epigenetic marks with epilepsy. The role of methionine in epilepsy has been shown through the pro-convulsant effects of MSO administration. Notably, methionine metabolism is crucial in the regulation of intracellular SAM levels, affecting DNA methylation through the regulation of methyl donation. In contrast to methionine, adenosine administration has been shown to have an anticonvulsant effect. However, adenosine also aids in the regulation of SAM through

feedback into SAM/SAH cycling. Metabolism of both these molecules has been shown to affect epilepsy progression and aid in the regulation of global methylation within the cell.

As epilepsy is a disorder characterized by hyperexcitability, dysregulation of the excitatory neurotransmitter glutamate and glutamate receptors is critical to the development of epilepsy. Not only are glutamate receptors differentially expressed in TLE, but this regulation has been shown to be epigenetically driven (Huang and others 2002). In the absence of glucose, neurons can use ketones as an energy source. Glucose metabolism is highly linked to epilepsy, and switching to ketone utilization through the ketogenic diet is shown to have an anti-seizure effect in some epilepsy-related disorders. Likewise, supplementation with the glycolysis derivative 2-deoxyglucose has some anti-epileptic properties suggesting that glucose metabolism pathways could serve as new targets for future AEDs (Stafstrom and others 2008).

Many patients are resistant to current AEDs. Therefore, it is critical that new drugs with the potential to benefit pharmacoresistant patients are developed. Epigenetic pathways are strong candidates for the future of AED targets, as several epigenetic pathways are altered in epilepsy. Drugs targeting epigenetic mechanisms are already in use, and they are of high interest for potential treatments for several diseases and disorders including epilepsy. DNMT and KDAC targeting drugs could show potential in epilepsy treatment as DNA methylation and histone acetylation show robust changes with epilepsy. Likewise, ncRNA targeting drugs have the potential to target several dysregulated pathways at once.

Beyond better treatments for epilepsy, the discovery of biomarkers could serve in the diagnosis of epilepsy. There are currently no biomarkers in use for early identification

of epilepsy or pharmacoresistance. However, miRNAs show strong promise for use as biomarkers because of their availability in biofluids and differential expression in epilepsy, and they could soon be used for early detection and aided diagnosis in the clinic. As we further elucidate the epigenetic regulatory mechanisms underlying epilepsy, there is great potential for translational applications for patients.

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SUMMARY AND CONCLUSIONS

5-hmC in Epilepsy

My study adds to the scientific literature of DNA Hydroxymethylation in epilepsy by characterizing epileptic loss of 5-hmC across the epileptic genome. Through both mass spectrometry analysis and 5-hmC meDIP-sequencing we show that 5-hmC is lost in human and rodent temporal lobe epilepsy, however we see no change in global levels in 5-mC DNA methylations. The majority of 5-hmC loss occurs intergenically, with no clear bias towards hypo- or hyper- methylation at gene promoters or bodies, however the majority of differential methylation at gene associated regions occurs within the gene body. The average profile of 5-hmC over gene promoters, bodies, and intergenic regions stays relatively consistent loss of 5-hmC occupation. Relative to CpG islands we see enrichment of 5-hmC at CpG islands, with consistent loss of 5-hmC in epilepsy across all regions. Agreeing with other studies, the majority of differentially methylated windows occur in CpG shores (Irizarry et al., 2009; Mitsumori et al., 2020).

Gene Ontology analysis of significantly differentially hydroxymethylated gene bodies showed enrichment of epilepsy associated pathways including GABAergic signaling, ion channels, and cAMP signaling. Several candidate genes for 5-hmC gene regulation were identified, with seven and six genes highly enriched in Glutamatergic and GABAergic neurons respectively, 1 gene highly enriched in astrocytes, and 1 in endothelial cells. This suggests that 5-hmC could be regulating several cell types in the hippocampus. Three significantly hypo- and hyper-methylated genes were chosen for gene expression analysis through qPCR. Interestingly, all hypo-methylated genes showed

decreases in gene expression as expected but the hyper-methylated also showed decreases in gene expression. This could indicate the involvement of other transcriptional regulation processes in epilepsy, be a consequence of the heterogeneity of cell types examined, or potentially through alternate DNA 5-hmC regulation in the intergenic regions such as through association with enhancer region or prevention of DNA binding proteins from binding to DNA.

This study is one of multiple indicating a clear involvement of DNA hydroxymethylation in temporal lobe epilepsy and seizures. Decreases in overall levels of 5-hmC is seen in humans with epilepsy as well as several rodent models, suggesting that 5-hmC loss could be a hallmark of epilepsy pathology and a critical regulator of epilepsy development (de Nijs et al., 2019; Kaas et al., 2013; Ryley Parrish et al., 2013; Shen et al., 2020). This sequencing study serves as a map to help investigators in further elucidating the role of 5-hmC in epilepsy.

Future Directions

This study gives a glimpse into 5-hmC gene regulation in epilepsy, however a RNA-seq study from a cohort of epileptic rats would allow us to gain a clearer picture of genes involved in epilepsy controlled by 5-hmC. To date, no study has looked at loss of Tet1 protein in epilepsy. It is unknown if loss of Tet1 leading to a decrease in global 5-hmC would be sufficient to cause seizures. Tet1 could be knocked down in the hippocampus by injecting siRNA to silence gene expressions. Depth electrode recordings could be done in these animals to determine if behavioral or EEG seizures occur.

In our rat model of epilepsy, AAVs could be injected into the hippocampus to increase Tet1 expression and increase DNA 5-hmC in the hippocampus of epileptic rats to determine if overexpression of Tet1 improves seizures. In addition, since Tet1 is known to be important for memory formation, behavioral tests such as contextual fear conditioning and the Barnes maze could see if modulation of Tet1 can also improve memory in epilepsy (Chen et al., 2017; Greer et al., 2021; Kaas et al., 2013; Kumar et al., 2015; Rudenko et al., 2013).

The Role of G9a in Seizure Threshold

Our study further implicates the involvement of G9a in seizures. We show that we can conditionally knockout or knockdown G9a in dentate gyrus neurons using a transgenic mouse model and a hSyn-cre-GFP AAV. We show that KO mice exhibit a decreased seizure threshold compared to control hSyn-GFP AAV injected animals. KO mice ultimately reached a higher maximum seizure score than controls, and KO mice had a reduced latency to maximum seizure score. Interestingly our KD (G9a fl/wt hSyn-cre AAV injected animals) mice appeared to show a more severe phenotype than KO animals. G9a KD mice also and a reduced seizure threshold compared to controls through achievement of a higher maximum seizure score, however KD mice had a reduced latency to maximum seizure score. This implies that different pathways regulating seizures are being affected with KO than with KD.

Interestingly, KD mice but not KO mice experience neuronal loss and gliosis of the hippocampus. Previous studies with G9a fl/fl mice crossed with CaMKii-cre mice show no structural abnormalities (Schaefer et al., 2009). An interesting explanation for

this is the hSyn promoter targets both inhibitory and excitatory neurons, while CaMKii only targets excitatory neurons. To date, there are no studies in loss of G9a specifically in inhibitory neurons. However, haploinsufficiency of G9a's partner protein GLP delays Parvalbumin -positive (PV+) interneurons in the barrel subfield of the primary somatosensory cortex, primary auditory cortex, and the primary visual cortex during development with reduction of PV+ markers in S1 layer 4 in the somatosensory cortex into adulthood. mIPSC recordings reduced in frequency by not amplitude, indicating less GABAergic transmission. The hippocampus of these animals is not described. This indicates reduction in GLP (+/-) in the development of PV+ inhibitory neurons and GABA signaling (Negwer et al., 2020). Therefore, it is possible that loss of G9a could result in a similar phenotype. Loss of inhibitory GABAergic transmission could also explain the increase in *cfos* staining in the KO mice.

Future Directions

The amount of *Ehmt2* mRNA and *cfos* staining has not yet been done in our KD mice. This is needed to determine the extent of *Ehmt2* loss and to determine if KD of G9a has an effect on the baseline *cfos* cell activation in the DG compared to controls and knockout KO animals. I would anticipate that KD mice would have more *cfos* positive cells than controls. I speculate that the cell loss and gliosis seen in KD mice would increase baseline neuronal activity greater than that of KO mice.

To determine if loss of G9a in inhibitory neurons is responsible, KO and KD of G9a should be done using either a GABAergic targeting cre-AAV injection or a interneuron targeting cre mouse line cross. Due to evidence of the H3k9me2 pathway

lessening GABAergic transmission and delaying PV+ neuron development, a *Pvalb* promoter could be used to induce loss of G9a specifically in PV+ neurons. A CaMKii-cre AAV KO and KD could be done as a control to determine if loss G9a in our hands in these cell types is sufficient for the effects seen in the hSyn mice.

In vivo hippocampal recordings could be done at baseline in KO and KD mice to determine if loss of G9a results in any abnormalities in hippocampal activity. Recordings could also be done during seizure threshold test to determine if there are any differences in traces from KO and KD mice at seizure onset. Since G9a's primary role is addition of the H3K9me2 epigenetic mark which silences gene expression. H3k9me2 ChIP-sequencing and RNA-sequencing could be done in these animals to determine where loss of H3K9me2 is occurring and what genes are increased in expression due to G9a loss. These genes could then be cross-referenced with genes known to be important in epilepsy development, and H3K9me2 ChIP-qPCR could be performed in resected tissue from TLE tissue to determine if patients with TLE also have H3k9me2 regulation of genes identified in the sequencing studies.

Looking Forward: Targeting Epigenetics in Epilepsy

Treatments

Traditional treatment targeting epigenetic mechanisms include drugs such as HDAC inhibitors, methyltransferase inhibitors, and DNA methylation inhibitors. These treatments do not target a specific gene or pathway, but they work to regulate the overall levels of their respective epigenetic modifications. Zebularine, the DNA methylation inhibitor was investigated in the rat KA model of temporal lobe epilepsy. Treatment

decreased levels of DNA methylation globally and at epilepsy associated genes, however, was not sufficient to reduce seizures and lowered seizure threshold (Ryley Parrish et al., 2013). Recent investigations have shown that currently used anti-seizure medications also affect epigenetic mechanisms, and could be working in part through this modulation. The AED diazepam through targeting 5'adenosine monophosphate activated protein kinase (AMPK) alters activity of HATs, HDACs, and DNMTs (Pathak et al., 2021). Rats chronically treated with diazepam experience increased expression of HDAC1 and HDAC2 as well as loss of histone acetylation in the cortex (Auta et al., 2018). The AED valproic acid (VPA) is a HDAC inhibitor that also induces demethylation of DNA as well as histone acetylation (Detich et al., 2003).

ncRNAs could be used as small molecule anti-epileptic therapies through specific targeting of dysregulated genes in the brain contributing to epilepsy. miR-219 was found to be decreased in the brain of the low dose KA-mouse model of TLE and in serum from epilepsy patients (Zheng et al., 2016). By increasing miR-219 through injection of its agomir (an artificial double stranded miRNA) reduced seizure severity. Interestingly, inhibition of miR-219 induced seizures. This shows feasibility that miRNAs can be critical regulators of epilepsy and has the potential to be utilized as a supplement for treatment of TLE (Zheng et al., 2016).

miRNAs can be encapsulated by exosomes for circulation throughout the body. These exosomes can cross the blood-brain barrier resulting in differential disease-associated miRNAs to pass from the brain to the blood (Reynolds & Mahajan, 2020). Exosomes can also carry mRNAs, proteins, and liposomes and are crucial for cell communication. These exosomes can be utilized as a vector to package miRNAs

dysregulated with epilepsy to specifically treat and target the brain through systemic administration. Injection of mesenchymal stem-cell derived exosomes intravenously was able to decrease inflammation in mice with KA induced seizures as well as improve memory following SE (Wei et al., 2020; Xian et al., 2019).

With the emergence of gene therapy, specific genes can be targeted to control seizures. Gene therapy allows deliverance of RNA through a vector to specifically increase or decrease expression of target genes. By directly affecting specific targets, patients with refractory epilepsy could see an improvement in seizures by targeting mechanisms difficult to reach with traditional drugs. In addition, by targeting epigenetic mechanisms it might be possible to prevent the development of epilepsy, something that cannot be done using current AEDs. With further advancement of gene therapy and an increase in gene therapy clinical trials, gene therapy could be used to express DREADDs or optogenetic channels to modulate neuronal activity and prevent seizures in difficult to treat epilepsies (Zhang & Wang, 2021).

Biomarkers

miRNAs show promise in serving as biomarkers of epilepsy. miRNAs are stable in serum meaning they would be easy to collect through a blood draw. By differentiating between drug resistant and non-resistant patients through the differential expression of miRNAs in serum. This could help in choosing the best treatment course for each patient. In addition, currently there is no way to determine hippocampal sclerosis (HS) type of refractory epilepsy patients until after the surgery. Since HS type can be predictive to seizure control following surgical resection, patients could be pre-screened to determine

if they were likely to respond well to the surgery (Thom et al., 2010). The availability of reliable biomarkers could also be used to monitor seizure treatment and predict the severity of epilepsy progression.

Several studies have shown differential miRNA expression in epilepsy. Wang et al showed feasibility of use of miRNAs as biomarkers of drug resistant epilepsy. Five miRNAs were found differentially expressed in the serum of drug resistant and non-resistant epilepsy patients. In this study, miRNA expression was able to predict refractory epilepsy with roughly 80% specificity and sensitivity with expression of miR-301a-3p observed to increase with seizure severity (Wang et al., 2015). Differentially expressed miRNAs differ based on epilepsy model, age of seizure onset, and type of epilepsy (Baloun et al., 2020)

The identification of novel biomarker candidates has significantly increased over the past few years. However, work still needs to be done in larger patient cohorts to validify candidates to be diagnostic. Using a panel of miRNAs as a biomarker has been suggested. Martins-Ferreira et al used a panel of miR-146a, miR-155, and miR-132 to test serum from patients with genetic generalized epilepsy. The panel was able to differentiate between epileptic and non-epileptic serum with a specificity of 80% and sensitivity of 73% (Asadi-Pooya et al., 2021; Martins-Ferreira et al., 2020). With improvement in technology and identification of common consistently altered miRNAs, miRNAs could be used as epilepsy biomarkers in the clinic someday.

Conclusion and Final Statements

Epigenetic regulation of gene transcription has been proven to be crucial in epilepsy development. This field is rapidly progressing and has the promise to discover novel molecular mechanisms underlying epilepsy development, which could be utilized either as anti-seizure targets or biomarkers of epilepsy. Through the work in my dissertation, I have characterized 5-hmC DNA loss in epilepsy and implicated G9a loss with a reduction in seizure threshold, however further research is needed to determine if these pathways would be valid targets for new anti-seizure therapeutics.

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APPENDIX A

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 06-Mar-2020

TO: Lubin, Farah D

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 06-Mar-2020.

Protocol PI:	Lubin, Farah D
Title:	Epigenetic Mechanisms in Epilepsy-Related Memory Decline
Sponsor:	National Institute of Neurological Disorders and Stroke/NIH/DHHS
Animal Project Number (APN):	IACUC-10190

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 05-Mar-2023.

Institutional Animal Care and Use Committee (IACUC)

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 30-Sep-2021

TO: Lubin, Farah D

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 30-Sep-2021.

Protocol PI:	Lubin, Farah D
Title:	Epigenetic Mechanisms in Epilepsy-Related Memory Formation
Sponsor:	National Institute of Neurological Disorders and Stroke/NIH/DHHS
Animal Project Number (APN):	IACUC-20662

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 15-Dec-2022.

Institutional Animal Care and Use Committee (IACUC)

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 27-Oct-2021

TO: Lubin, Farah D

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 27-Oct-2021.

Protocol PI:	Lubin, Farah D
Title:	Chromatin Remodeling Mechanisms of Gene Transcription in Memory
Sponsor:	National Institute of Mental Health/NIH/DHHS
Animal Project Number (APN):	IACUC-09652

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 15-Jun-2023.

Institutional Animal Care and Use Committee (IACUC)

403 Community Health on 19th | 933 19th Street South

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