The role of HBP1 in Genetic and Idiopathic Epilepsy

A thesis submitted by

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Abstract

Epilepsy is one of the most prevalent neurological diseases which results from abnormal neuronal firing, leading to the development of spontaneous recurrent seizures. Despite the development of multiple anti-epileptic medications in the last decade, about one third of the patients eventually develop resistant to anti-epileptic drugs. One of the most important reasons behind the drug resistance is the inability to identify a clear mechanistic pathway to be targeted for treatment. Existing pharmaceutical treatments function primarily to reduce hyperexcitability thus providing only symptomatic control. Identifying alternative mechanistic targets for anti-epileptic treatment to prevent the progression of chronic epilepsy has been the main research focus recently. The Yee lab has shown that the Wnt/β-catenin pathway is aberrantly activated in both acute and chronic epilepsy, driving a metabolic reprogramming of aerobic glycolysis (Warburg-like metabolism) resulting in elevated mTOR signaling. Key to these changes was phosphorylation-dependent inhibition of Pyruvate Dehydrogenase (PDH) by the Wnt target gene PDK4.

One of the known inhibitors of Wnt signaling, HMG box protein 1 (HBP1), was shown to decline in the epileptogenic period, suggesting a mechanism for Wnt activation in epileptogenesis. As a test of this mechanism, HBP1 knockout mice had elevated Wnt signaling in the hippocampus and recapitulated the metabolic reprogramming to aerobic glycolysis (Warburg-like metabolism) and elevated mTOR signaling. In-depth analysis of gene and protein expression, bioinformatics and confocal microscopy suggested additional changes. Among these, we observed a reduction in multiple GABA receptors and increased glutamate receptor expression along with altered glutamate transport. These results were consistent with a remarkably altered susceptibility to seizure induction along with occasional

spontaneous seizures in HBP1$^{-/-}$ mice. Accompanying the seizure phenotype we observed gliosis, which is consistent with seizure and the altered signaling and metabolic alterations. Collectively, these data provide evidence that HBP1 alterations/Wnt signaling may determine the threshold of acquired seizure susceptibility.

Consistent with the mouse results, several genome-wide studies suggest a disruption of Wnt/β-catenin in epilepsy patients. Moreover, multiple case reports were published highlighting deletions within the genomic region of the HBP1 and its possible correlation with neurological diseases including autism, intellectual disability and epilepsy. Human genetic sequencing data were obtained from 1600 patients and family members in the Epi4K database yielded 8 HBP1 variants and a near-significant association with Lennox-Gastault epilepsy tied to a specific variant (R375P). Mutagenesis experiments to evaluate the functional impact of these variants on HBP1 protein function demonstrated that R375P was disruptive, resulting in HBP1^{R375P} failing to repress Wnt activity. These data suggest that loss of HBP1 function through deletion or mutation results in susceptibility to epilepsy.

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List of Abbreviations

Ca+: Calcium
CACNA1A: Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit
D97G: Aspartic acid 97 Glycine
DG: Dentate gyrus
DNMT1: DNA methyltransferase 1
E55G: Glutamic acid 55 Glycine
EE: Epileptic encephalopathy
EEG: Electroencephalogram
Epi4K: Epilepsy 4000 epilepsy consortium
EZH2 : Enhancer of zeste 2 polycomb repressive complex 2
FOXG1: Forkhead Box G1
FZD :Frizzled Receptors
GABA: g-amino butyric acid.
GABRB3: Gamma aminobutyric type A receptor subunit beta 3
GAD: L-glutamic acid decarboxylase.
GCL: Granule cortical layer.
GFAP: Glial fibrillary acidic protein.
GGE: Genetic Generalized Epilepsy
GLS: Glutaminase.
GLUD1: Glutamate Dehydrogenase.
GLUL: Glutamate ammonia ligases
GLUT1: Glucose transporter 1
GRIN2A: Glutamate ionotropic receptor NMDA subunit type 2A
GS : Glutamine synthetase
GSK3β: Glycogen synthase kinase 3 β
GWAS: Genome wide association study
HDAC1: Histone deacetylase 1
HK2: Hexokinase-2
HMG-box: High mobility group box.
IDH: Isocitrate dehydrogenase.
ILAE: International League Against Epilepsy
K^+: Potassium
KCNA1: Potassium voltage gated channel subfamily A member 1
KD: Ketogenic Diet.
KO: Knockout.
LDHA: lactate dehydrogenase A
LEF: lymphoid enhance factor
LRP5/6: Low density lipoprotein receptor 5/6
mGluR : Metabotropic glutamate receptors
mTOR: Mammalian target of Rapamycin.
N447K: Asparagine 477 Lysine
Na^+: Sodium
NMDAR: N-methyl-D-aspartate receptor
P175S: Proline 175 Serine
PBS: Phosphate Buffered Saline
PDH: Pyruvate dehydrogenase enzyme
PDK4: Pyruvate dehydrogenase lipoamide kinase isozyme 4
PKM2: Pyruvate Kinase M2

PL/Pilo: Pilocarpine.
Q367P: Glutamine 376 Proline
Q511del: Glutamine 511 deletion
R375P: Arginine 375 Proline
S409F: Serine 409 Phenylalanine
S422R: Serine 422 Arginine
SCN1A: Sodium voltage -gated channel alpha subunit 1
SFRP1: secreted frizzled related protein 1
SLC1A1: Excitatory amino acid transporter 3
SLC1A2: Excitatory amino acid transporter 2
SRS: Spontaneous Recurrent Seizures.
TBI: Traumatic brain injury
TCA: Tricarboxylic acid cycle
TCF: T-cell factor
TLE: Temporal lobe epilepsy
TSC: Tuber Sclerosis Complex.
VGLUT: Vesicular glutamate transporters
Wnt: Wnigless/Integrated.

Chapter 1: Introduction

1.1 Epilepsy

1.1.1 Introduction

The International League against Epilepsy (ILAE) define an epileptic seizure as a temporary occurrence of symptoms due to an abnormal neuronal activity in the brain. Moreover, the epileptic brain has the tendency to develop recurrent, unprovoked seizures due to the alteration of neurologic function caused by excessive, hypersynchronous discharge of the neurons in the brain. Seizures are characterized by brief episodes of involuntary shaking that may involve a part or the entire body and sometimes are accompanied by loss of consciousness and loss of bowel or bladder control. An epileptic seizure results from transient abnormal synchronization of neurons in the brain that disrupts normal patterns of neuronal communication and electrical discharges in the EEG (Steinlein, 2004). Furthermore, the ILAE have clear criteria for clinical epileptic seizure in which it will be either clinically manifested by having 2 or more unprovoked seizures with more than 24 hours period apart or having an increased risk of more than 60% for developing a seizure within the next 10 years (Falco-Walter, 2020).

Epilepsy, one of the most common brain conditions, affects over 70 million people worldwide (Myers & Mefford, 2015). It is one of the most common disabling neurological conditions which interfere with the quality of life of the affected individuals. Furthermore, about one-third of the patients suffer from epilepsy refractory to treatment. Hence, due to the tendency of the affected individuals to have epileptic seizures , they tend to suffer from health concerns related to seizure. Mostly, an increased risk of mortality and disability which include cognitive, psychological and social consequences. Epilepsies may result

from primary genetic abnormalities or secondary to well-defined structural or metabolic disorders, of which, some also have genetic causes. Genetic epilepsy can be either causative or susceptibility genes. The majority of the epilepsies especially among pediatric patients are due to genetic reasons. Metabolic or structural factors which include stroke, brain tumor, immune or infectious agents which are capable of causing seizures these account for about 30% of the cases and these etiologies are most common among adults without the genetic predisposition. Lastly, unknown causes for epilepsy which are also called idiopathic (Moshé, Perucca, Ryvlin, & Tomson, 2015). Epilepsy is known to be a complex disease with the previously mentioned etiologies which will be manifested by different outcomes. Hence, multiple factors are considered during the process of epilepsy diagnosis including the age of onset, the severity or the progression of the disease and the overall health status of the affected individual which include the presence of co-morbidities (Myers & Mefford, 2015).

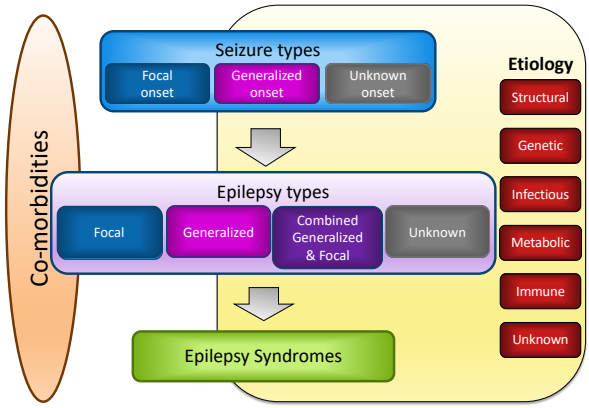


Figure 1.1: ILAE classification of the epilepsies. Reprinted with permission from Scheffer et al ., ILAE classification of the epilepsies: position paper of the ILAE Commission for Classification and Terminology. Epilepsia , 2017. 58(4): p. 515.

1.1.2 Genetic Epilepsy

Due to the complex nature of epilepsy which include different manifestation, different electroencephalogram pattern and the age of onset, epilepsy is classified into different classes. Genetic Generalized Epilepsy (GGE) which was previously identified as Idiopathic generalized epilepsy, focal epilepsy and epileptic encephalopathy. GGE have an early onset and usually they start in childhood and the seizure development involve both brain hemispheres are affected. An example for GGE is juvenile myoclonic epilepsy. Mostly, GGE are associate with normal physical and intellectual development (Guler et al., 2017).

Temporal lobe epilepsy is an example for focal epilepsy which usually initiated in one hemisphere (Blumcke et al, 2001). The most severe form of epilepsy is epileptic encephalopathy (EE) it is characterized by early onset, refractory seizure. Moreover, EE is usually associated with developmental delay and an example for that is Dravet syndrome (Myers & Mefford, 2015). Over the past decade the advances in the genetic technologies have helped in understanding different causes of epilepsy, especially genetics involvement. A possible way to classify the epilepsies according to genetic contribution to their etiology is to divide them into monogenic epilepsies and complex epilepsies. The former are caused by mutation in a single gene; the latter are multifactorial in origin, caused by the interaction of a few or multiple genetic variants, and possibly further influenced by environmental factors.

The hypothesis of genetics linked to epilepsy existed decades ago. The hypothesis originated from the epidemiological studies which showed that there is an increased incidence of epilepsy among relatives and that incidence is even higher among twins especially monozygotic twins. The first mutations identified were ion channelopathies including the sodium voltage gated channel alpha subunit 1 (SCN1A)

in Dravet syndrome (Myers & Mefford, 2015). Chromosome microarray and next generation sequencing are all technologies which helped identify the increasing number of epilepsy associated genes, variants within the genes or linking specific chromosomal deletion with epilepsy and revolutionized the diagnostic approach for patients with epilepsy.

Furthermore, gene wide associated studies (GWAS) have identified genetic variants associated with epilepsy. However, in the beginning, GWAS could not convey the clinical importance of genetic variants in seizure development either due to small sample size or the GWAS results was not reproducible. Several genetic association studies have shown point mutations associated with seizure development. These include genes linked to voltage gated sodium channel (SCN1A), potassium channels (KCNA1), GABA and glutamate receptors (GABRB and GRIN2A) (Chen, Giri, Xia, Subedi, & Li, 2017) .

Epi4K is an international collaboration for epilepsy genomics. This consortium was founded in 2011 and it was established to use as a tool to study the genetics and genomics of human epilepsy. The goal for that consortium was to use next generation sequencing technology to sequence the blood samples for at least 4000 individuals with epilepsy since the majority of the epilepsy etiologies remain idiopathic. Studying the involvement of the genetics causes of epilepsy becomes essential for epilepsy diagnosis. Moreover, this should help to identify the appropriate treatment for the affected individual. Multiple de novo mutations were identified as a causative mutation for epilepsy.

GABRB3 was one of the missense mutations which was identified through Epi4K and it was linked to different types of epilepsy which included infantile spasm , Lennox–Gastaut syndrome (Epi, 2012). Furthermore, multiple studies have analyzed

the sequencing data from the Epi4K to identify rare variants which contribute to familial epilepsy and multiple genes were significantly enriched in the Epi4K patients. These genes are GRIN2A, PCDH11, LG11, DEPDC5 and SCN1A (Allen et al., 2017). Additionally, Epi4K aimed to identify the de novo mutations which are linked to epilepsy and a well-established example for that is SCN1A which is the major cause for Dravet syndrome in about 90% of the patients (Claes et al., 2001).

1.2 Cellular & Molecular Alterations in Epilepsy

1.2.1 GABA & Glutamate Alterations in Epilepsy

The pathophysiology of epilepsy has been linked to multiple causes and the main theory behind seizure development is the existence of hyperexcitation and reduce in the inhibitory neurotransmitters. Glutamate is the major neurotransmitter for excitations, and it plays other role in synaptic plasticity, memory, learning and central nervous system development. Glutamate is non-essential amino acid which lacks the ability to cross the blood brain barrier and it has to be synthesized in the neurons using the local precursor glutamine (Alcoreza, Patel, Tewari, & Sontheimer, 2021).

The state of overexcitation which accompanied the release of excess glutamate have been proposed to be the main drive behind the pathogenic mechanism for several neurologic and neurodegenerative diseases including: epilepsy, Alzheimer disease and multiple sclerosis due to neurotoxicity (Lau & Tymianski, 2010). There are several types for glutamate receptors exist which reflect its functional diversity. The first type is N-methyl-D-aspartate receptor (NMDAR), AMPA and Kiante receptors and they all represent the same category of ionotropic glutamate receptors which are responsible for the fast synaptic transmission.

NMDA receptors activation require various ligands for activation. Moreover, NMDAR allow the passage of different cations including sodium (Na^+), potassium (K^+) and calcium (Ca^+) which eventually lead to an increase in the intracellular calcium and activation of signaling cascade which can be either favorable or pathological. Another method for the NMDA receptor activation is via the removal of Mg+ which act as a physiological block for the passage of cations (Lau & Tymianski, 2010). It is composed of two subunits GluN1 and GluN2, the different subtypes of NMDA receptors show a different level of importance when it comes to its regulatory role.

GluN1 is expressed throughout the brain, whereas GLUN2 and its subtype (A-D) has different pattern of expression, and their expression varies with age (Gao et al., 2017). For instance, GluN2A and GluN2C start their expression after birth and their expression increases with age (Gao et al., 2017). In contrast, GluN2B and GluN2D expressed at early age and their expression decreases with age. Recently, multiple mutations have been found within the NMDAR receptors and have been linked to epilepsy (Gao et al., 2017; Xu et al., 2018).

These includes but not limited to GRIN2A which encodes the subunit GluN2A and GRIN2B which encodes for GluN2B subunits. In both cases, it was reported that mutations within these genes which can cause either loss of function or gain of function and they are linked to different types of epilepsy in human patients (Gao et al., 2017). Metabotropic glutamate receptors are another other type of glutamate receptors. They are a seven transmembrane spanning proteins linked to intracellular G proteins and they play a vital role in the mammalian CNS. These functions include regulation of calcium channels , mediate slow excitation, regulation of NMDA, regulation of neuronal development and involvement in the formation of memory.

Due to their various functions, they are thought to be part of different neurological disorders including seizure development (Ferraguti & Shigemoto, 2006). They consist of 8 different types (mGluR 1-8) which are further categorized into 3 groups (group I, II and III). mGluR1 and mGluR5 are part of the group I metabotropic glutamate receptors and they mostly localized at postsynaptic neurons. Group I of metabotropic receptors are activated by increasing the production of phospholipase C, which as consequent will lead to an increase in the production of inositol triphosphate. Eventually, that will lead to an increase in the intracellular calcium concentration (Lau & Tymianski, 2010).

Group II metabotropic glutamate receptors include mGluR2 and mGluR3. They localize at both pre and postsynaptic neurons and on glial cells. They exert their neurotransmission function by causing a reduction in adenylyl cyclase and that leads to an inhibition of the voltage gated calcium channels. Lastly, metabotropic glutamate receptors group III which consist of mGluR4, mGluR6, mGluR7 and mGluR8 and they both share similar function similar to group II (Ure, Baudry, & Perassolo, 2006). In addition, there have been several studies which showed the involvement of metabotropic glutamate receptors in epileptogenesis or in seizure development. For instance, an increased in the production of TGF-B with an upregulation in the expression of mGluR5 and mGluR3 was detected in a rat model of mesial temporal epilepsy (Aronica et al., 2000) .

Gamma-aminobutyric acid (GABA) has had a central role in the inhibitory neural transmission and it dysregulation is associated with multiple neurological diseases including epilepsy (Petroff, 2002). GABA receptors are widely distributed in the brain and they have different types $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_A$ and $GABA_C$ are both ligand gated chloride channels. Hyperpolarization follows the

opening of theses receptors and the passing of the chloride ion into the neurons. $GABA_B$ receptors can mediate pre and post synaptic inhibition and it does that by indirectly activating the potassium channels leading to hyperpolarization by causing the potassium ions to flow out of the neurons.

Moreover, $GABA_A$ have different subunits which include (a1-6) among other subunits. While, $GABA_B$ have 2 subunits which include $GABA_B$R1 and $GABA_B$ R2. Multiple studies have investigated the change in the expression level of these receptors upon seizure induction. Kindling and electrical induction for status epilepticus led to a reduction in the gene expression of $GABA_A$ subunits (Nishimura et al., 2005). Furthermore, GABRA3 loss of function genetic variant was associated with epileptic encephalopathy in human patients (Niturad et al., 2017).

Under pathophysiological condition glutamate/GABA cycle is perturbed, which can contribute to epileptogenesis and seizure progression. Microdialysis studies have shown an increase in the extracellular glutamate concentration in epileptic hippocampus. That was an evidence of the physiological significance of glutamate transporters, in which they mainly handle the removal of the glutamate in the synaptic cleft and they play a role in the glutamatergic signaling (Verhoog, Holtman, Aronica, & van Vliet, 2020).

Excitatory amino acid transporters (EAAT) consist of 5 transporters with different expression in neurons and glia in the CNS. EAAT1 and EAAT2 are mainly astrocytic glutamate transporters, and their downregulation was confirmed in an animal model of epilepsy (Bauer et al., 2010 , Takahashi et al., 2015). EAAT3 is another glutamate transporter, and it is mostly expressed in the glutamatergic and GABAergic neurons and it is enriched in the hippocampus among other regions of the brain (Bianchi, Bardelli, Chiu, & Bussolati, 2014). Mature astrocytes do not express

this transporter. However, EAAT3 was found to be expressed in human glioma cell line (Palos et al., 1996). Moreover, EAAT3 gene expression was upregulated in the brains of patients with TLE (Crino et al., 2002).

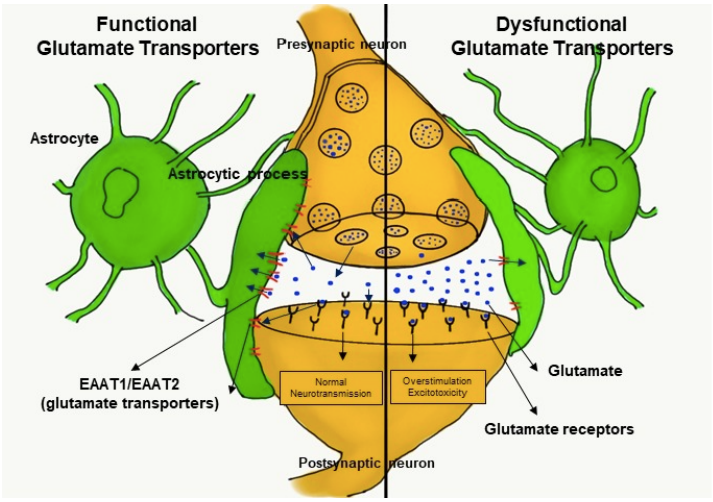


Figure 1.2: Role of astrocytic glutamate transporters in the central nervous system (CNS) and neurological disorders. Reprinted with permission Pajarillo et al., The role of astrocytic glutamate transporters GLT-1 and GLAST in neurological disorders: Potential targets for neurotherapeutics. Neuropharmacology, 2019 . 161 (107559): p. 2.

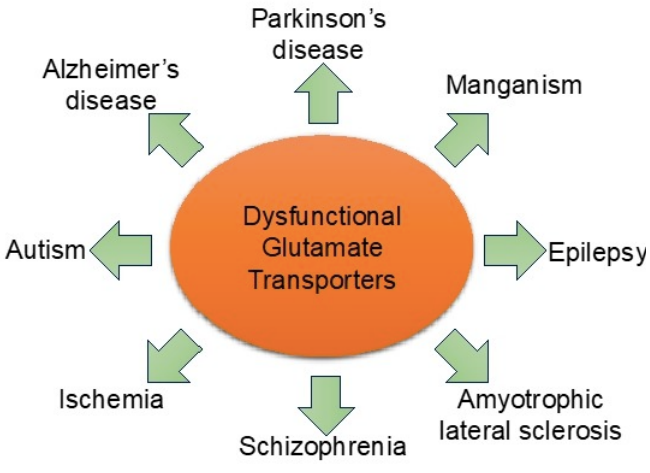


Figure 1.3: Dysfunctional glutamate transporters have been implicated in various neurological disorders such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, schizophrenia, epilepsy, and autism. Reprinted with permission from Pajarillo et al., The role of astrocytic glutamate transporters GLT-1 and GLAST in neurological disorders: Potential targets for neurotherapeutics. Neuropharmacology, 2019 . 161 (107559): p. 2.

1.2.2 Morphological, Cellular and Metabolic Alterations In epilepsy

The pathogenesis of epilepsy is complex process mechanism in which the persistence hyperexcitation in the brain can contribute to structural, cellular and morphological changes in the epileptic brain. The most prominent phenomenon of an epileptic brain is the development of sclerotic hippocampi characterized by gliosis , neuronal cell loss specifically in the CA1 region of the hippocampus (Alonso-Nanclares & DeFelipe, 2014). The reactive gliosis or astrogliosis is a function of astrocytes which is abundant in the CNS. Astrocytes are considered as a key player in the pathogenesis of epilepsy due to its diverse biological functions in the CNS. Regulation of neurotransmitter release of glutamate and GABA, maintain the structure of the blood-brain barrier and provide the metabolic support for neurons are all the functions of astrocytes (Vezzani et al., 2022).

A features of astrocytes reactivity is the upregulation of the intermediate filament glial fibrillary acidic protein(GFAP), which is common among patients with epilepsy specifically patients with mesial temporal lobe epilepsy (MTLE) (Robel & Sontheimer, 2016). Moreover, astrocytes reactivity is a hallmark of epileptic brain in the human and experimental animals (Verhoog et al., 2020). Henceforth, GFAP and s100b are common biomarkers of astrocytes reactivity detectable in the plasma and cerebrospinal fluid of the patients with epilepsy (Gurnett, Landt, & Wong, 2003).

Furthermore, the astrocytes harbor multiple metabolic enzymes, as well as specialized enzymes which include glutamine synthetase (GS). Glutamate ammonia ligases (GLUL) is the gene which encode glutamine synthetase. GS is found in the cytoplasm of the astrocytes and it catalyzes the conversion of glutamate to glutamine in the brain (Robel & Sontheimer, 2016). The importance of this reaction rely on the

biological significance of glutamine as precursor used by the neurons for glutamate and GABA synthesis (Coulter & Steinhauser, 2015).

Multiple studies have indicated the GS activity in the brain is reduced in several neurological disorders, including Alzheimer disease and MTLE (Anlauf & Derouiche, 2013). Case reports have identified loss of function mutation of GS was identified in human and it led to sever brain malformation and eventually neonatal death (Haberle et al., 2005). Furthermore, GFAP specific GS Knockout mice died at postnatal day 3 (He et al., 2010). Adult neurogenesis in which a newborn neurons in the hippocampus contribute to the neural circuitry is a characteristic of an epileptic brain. Moreover, rodent model of epilepsy showed an increase in the hippocampal neurogenesis and confirmed its negative consequences (Ammothumkandy et al., 2022).

The neuronal hyperactivity is partially due to the generation of the newborn neurons. Furthermore, multiple studies have shown the effect of silencing the newborn neurons in reducing seizure activity (Lybrand et al., 2021). Moreover, Nestin which is an intermediate filament protein and used as a marker for neuronal precursor (Liu et al., 2018). It was found to be upregulated transiently in the proliferating neuronal cells after brain insult or injury and its expression level was increased in epilepsy cases (Blumcke et al., 2001).

Epilepsy is a complex disease with alteration at cellular, morphological and metabolic levels. The metabolic alterations in epilepsy are poorly understood, yet may yield treatable targets. Metabolic deficiency was the main etiology for the genetic epilepsy glucose transporter 1 (GLUT1) (Mongin, Mezouar, Dodet, Vidailhet, & Roze, 2016). Moreover, GLUT1 patients usually diagnosed with microcephaly and they may have intellectual disability or developmental delay (Koch & Weber, 2019).

Prior work done in the Yee laboratory has revealed another cell signaling pathway associated with metabolic dysfunction and epilepsy. Specifically, the elevated Wnt signaling was linked to an increase in the Warburg metabolism in the brain and an alteration in the Tricarboxylic acid (TCA) cycle during epileptogenesis (Alqurashi et al., 2021). Moreover, the same changes were recapitulated using a genetic model for elevated Wnt signaling. High mobility group box protein 1 (HBP1) is a transcriptional repressor for Wnt/β-catenin signaling. $HBP1^{-/-}$ was heavily investigated to identify the metabolic changes in the brain (Alqurashi et al., 2021). The finding has confirmed the Warburg like metabolism in the hippocampus of $HBP1^{-/-}$ mice. Pyruvate dehydrogenase kinase 4 (PDK4) is a Wnt target gene and it regulates the Pyruvate dehydrogenase (PDH) activity via phosphorylation , the phosphorylation of PDH lead to its inactivation and reducing the metabolism of pyruvate into acetyl-CoA and the increased in the cellular pyruvate is a feature of aerobic glycolysis which identified as a metabolic alteration during epileptogenesis and in a genetic model for elevated Wnt signaling (Alqurashi et al., 2021).

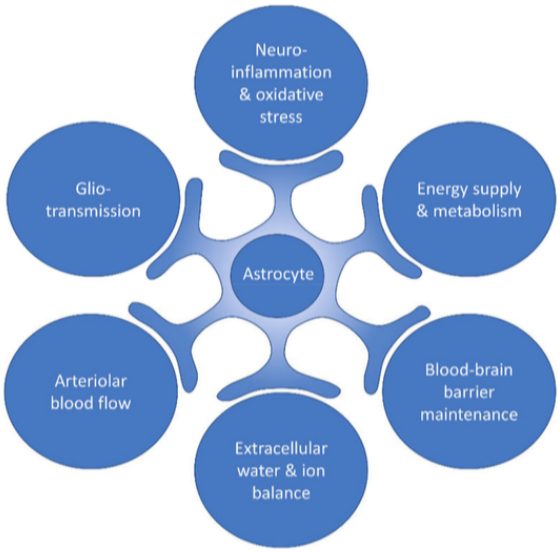


Figure 1.4: Processes within the brain in which astrocytes are involved.
Reprinted with permission from Verhoog et al., Astrocytes as Guardians of Neuronal Excitability: Mechanisms Underlying Epileptogenesis. Front Neurol, 2020. 11(591690.): p. 2.

1.3 Wnt / β-catenin Signaling

1.3.1 Introduction

The Wnt pathway is an ancient pathway and it is found in many species including Hydra, Caenorhabditis elegans, Drosophila, mouse and human. Wnt signaling is a highly conserved signaling pathway and they have a prime role in in a wide range of physiological processes. The variety of physiological processes which are governed by this transduction cascade includes embryonic development, cellular homeostasis, cellular differentiation and tissue regeneration and the dysregulation of this pathway is visible in developmental defects, cancer and degenerative disorder (Parsons, Tammela, & Dow, 2021). Moreover, alteration in the expression of Wnt signaling pathway has been linked to malignant transformation, tumor resistance to treatment and overall activated Wnt is associated with poor prognosis for cancer (Galluzzi, Spranger, Fuchs, & Lopez-Soto, 2019) .

Wnt signaling pathway compromised of 19 glycoproteins which contribute to the above-mentioned physiological processes. Wnt signaling transduction is separated into Canonical (β -catenin dependent) and non-canonical Wnt (β-catenin-independent). The activation for the canonical Wnt pathway is done by the binding of Wnt ligands which are lipid modified glycoprotein and have a conserved rich cysteine protein to Frizzled (FZD), which are 7 transmembrane with long amino terminal extension termed cystine-rich domain(CRD) and low-density lipoprotein receptor (LRP5/6) receptor in vertebrate both of them are required for Wnt to be function.

The schematic diagram of Canonical Wnt signaling pathway is shown in Figure (1.5). The whole notion for Wnt activity is related to the stabilization of β-catenin in the cytoplasm which eventually will translocate into the nucleus activating Wnt target gene which are usually identified by the presence of the cysteine pattern with other

conserved residues. In the absence of Wnt ligands the destruction complex which consist of adenomatous polyposis coli (APC), axin 1(AXIN1), casein kinase 1 alpha (CSNK1A), and glycogen synthase kinase 3 (GSK3) phosphorylate β-catenin which leads to its ubiquitination. However, when Wnt ligands binds to FZD or LRP5/6 that will results in the involvement of disheveled segment polarity protein 1 (DVL1) or DVL2 which is an upstream component of the transduction cascade to bind to the destruction complex and deactivate its ability to ubiquitinate β-catenin. Then, nuclear translocation of β-catenin and complex formation with T cell factor / lymphoid enhance factor family (TCF/LEF) transcription factors will follow and that will influence Wnt regulated genes (Galluzzi et al., 2019).

HBP1 inhibits the WNT/ β-catenin pathway, which plays a crucial role in the cell cycle, in addition to directly regulating genes (Sampson et al., 2001). This signaling pathway causes β-catenin to accumulate in the nucleus where it binds to TCF/LEF proteins and activates genes including c-Myc and cyclin D1. HBP1 binds to TCF4 and blocks WNT target gene regulation (Elfert et al., 2013).T-cell factor 4 (TCF4), together with β-catenin serves as the primary transcriptional mediator of the canonical Wnt signaling pathway. The 192–400 amino acid region of HBP1 has been identified as the essential functional region for interacting with TCF4, which contains the repression domain. The functional inhibition correlated with a physical interaction between HBP1 and TCF4 (Sampson et al., 2001). Several studies have demonstrated that HBP1 inhibits the WNT pathway, which in turn causes c-Myc (a Wnt target gene) to be downregulated. The transcriptional activity of the c-Myc and N-Myc proteins can also be inhibited by direct interactions between HBP1 and these proteins (Escamilla et al., 2010; Claeys et al., 2018).

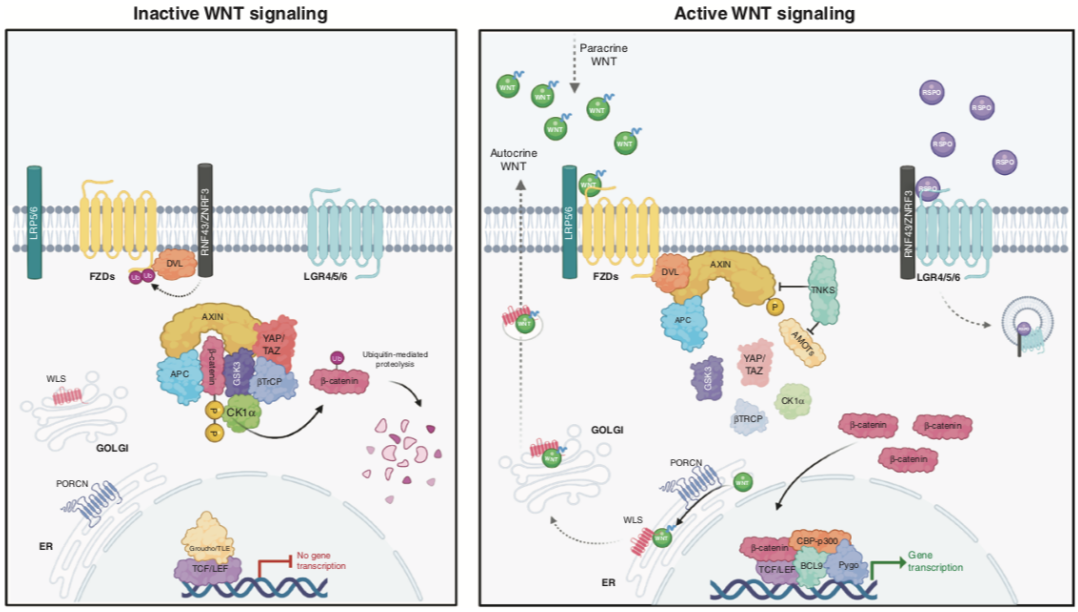


Figure 1.5: Wnt/β-catenin signaling pathway in inactive (A) and active (B) state. Reprinted permission Parsons et al., WNT as a Driver and Dependency in Cancer. Cancer Discovery, 2021. 11 (10): p. 2414.

1.3.2. Wnt/ β-catenin Signaling in Epilepsy

Wnt signaling pathway is a major and well conserved pathway, in which it plays an essential role in CNS development. Moreover, an increasing number of evidence has shown the involvement of Wnt/β-catenin signaling in brain developments which include synaptogenesis, contribute to neural stem cells proliferation and differentiation, participate in synaptic plasticity and neural patterning (Mulligan & Cheyette, 2012). Furthermore, a dysregulation of Wnt/β-catenin pathway was studied in multiple neurological disorder including Autism, Schizophrenia, Alzheimer disease and epilepsy (Purro, Dickins, & Salinas, 2012).

Moreover, multiple studies have shown the involvement of Wnt/β-catenin signaling in seizure development and epilepsy. Recently, a genetic knockout model for HBP1 demonstrated its role in Wnt suppression and epilepsy (Alquarshi et al., 2021). Further, the findings were consistent when two different seizure-inducing agents, pilocarpine and kainic acid, were used on two different mouse backgrounds

(FVBN and C57BL6/J), the upregulation of Wnt components, including β-catenin, and reduction of Wnt inhibitors HBP1 and sFRP1 was confirmed in both studies (Alqurashi et al., 2021). Elevation of Wnt signaling was identified in the hippocampus of an animal model of TLE. β-catenin among other key regulators of Wnt signaling were upregulated during epileptogenesis (Qu et al., 2017).

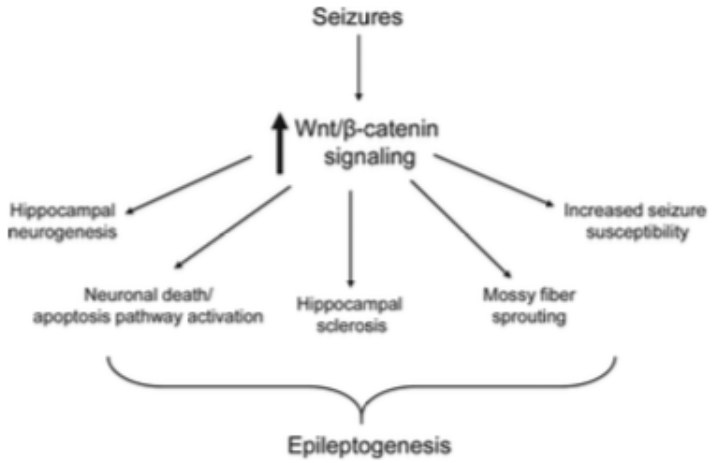


Figure 1.6: Relationship between seizures, Wnt/β-catenin signaling, and epileptogenesis. Reprinted with permission Hodges & Lugo., Wnt/β-catenin signaling as a potential target for novel epilepsy therapies. Epilepsy Research, 2018. 146 (2018) : p.12.

1.4 HBP1 (HMG BOX Transcription factor 1)

1.4.1 Introduction

High mobility group box protein 1 (HBP1) is highly conserved in vertebrates and it acts as a transcription factor which involved in multiple cellular functions including proliferation, differentiation and senescence (Chan et al., 2020). Furthermore, it acts as tumor suppressor and as transcriptional inhibitors and activators for multiple genes. HBP1 structure composed of a transcriptional repressor region and it includes ataxin homology domain (de Chiara et al., 2003). HBP1 inhibits the WNT/ β-catenin pathway, which plays a crucial role in the cell cycle, in addition to directly regulating genes (Sampson et al., 2001). This signaling pathway causes β-catenin to accumulate in the nucleus where it binds to TCF/LEF proteins and activates

genes including c-Myc and cyclin D1. HBP1 binds to TCF4 and blocks WNT target gene regulation (Elfert et al., 2013). Several studies have demonstrated that HBP1 inhibits the WNT pathway, which in turn causes c-Myc to be downregulated. The transcriptional activity of the c-Myc and N-Myc proteins can also be inhibited by direct interactions between HBP1 and these proteins (Escamilla et al., 2010; Claeys et al., 2018). Moreover, the same region of HBP1 interacts with histone deacetylases (HDAC4) and it contributes to the repression function of HBP1 (Wang, Pan, Chen, Huang, & Zhang, 2012). Additionally, HBP1 play a major role in multiple posttranslational modifications which include ubiquitination, phosphorylation and acetylation. All of these processes can majorly impact the HBP1 function and either by altering its transcriptional activity or affect stability of the HBP1 protein. The retinoblastoma tumor suppressor family (RB, p130 and p107) has diverse functions in cellular pathway and it includes cell cycle regulation (Wang et al., 2012).Furthermore, HBP1 contains two RB-binding sites which were identified through mutagenesis experiments . High affinity binding site near to the N-terminal region (LxCxE) and low affinity binding site which locate within the repression domain and both site required to be mutated to have an effect on HBP1 function (Tevosian et al., al.,1996).

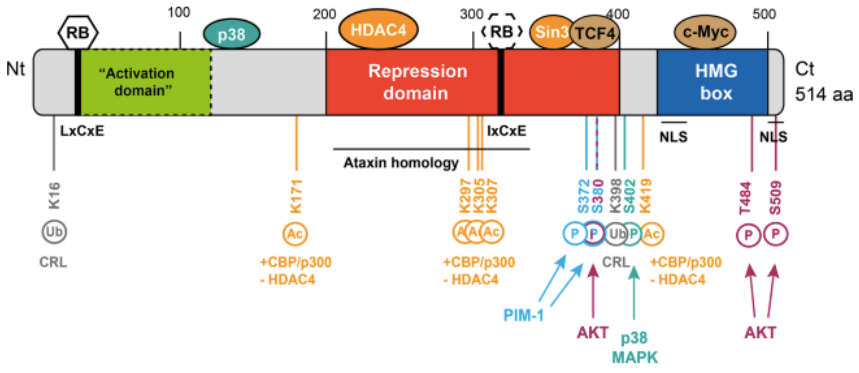


Figure 1.7: HBP1 structure, posttranslational modifications and interactions. Reprinted with permission Bollaert et al., The HMG box transcription factor HBP1: a cell cycle inhibitor at the crossroads of cancer signaling pathways. Cellular and Molecular Life Sciences, 2019. 76(8): p. 1530.

1.4.2 HBP1 Function and Target Genes

The transcriptional regulatory effect of the HBP1 gene exerted via multiple mechanisms either by inhibition of transcription factor binding which include MYC and TCF4 (Bollaert, de Rocca Serra, & Demoulin, 2019). Additionally, HBP1 can directly repress the expression of specific genes such as DNMT1 and MYCN. The regulation oh DNMT1 gene by HBP1 emphasize the importance of HBP1 gene in epigenetic regulation . Hence, DNMT1 is a DNA methyltransferase and its repression via HBP1 lead to a global hypomethylation and senescence (Pan et al., 2013). EZH2 is another gene which is repressed by HBP1 and it is involved in senescence.

This has an important implication especially in cancer where epigenetic regulation is linked to the pathogenesis. Additionally, HBP1 is plays a crucial role in Ras-induced premature senescence to inhibit the tumorigenesis process (Li et al., 2010). Another oncogenic pathway tends to overcome HBP1 activity is PI3K -AKT signaling pathway. Additionally, HBP1 inhibits the Wnt/β-catenin signaling pathway which is known to play multiple physiological roles and its dysregulation has been linked to cancer and tumorigenesis (Sampson et al., 2001).

Figure 1.8 HBP1 target genes. Reprinted with permission, Bollaert et al. The HMG box transcription factor HBP1: a cell cycle inhibitor at the crossroads of cancer signaling pathways. Cellular and Molecular Life Sciences, 2019. 76(8): p. 1532.

1.4.3 The role of HBP1 in Diseases

The diversity of the biological functions of the HBP1 gene highlight its possible involvement with the progression of multiple human diseases. Molecular investigation shown earlier a possible correlation between a deletion of the HBP1 chromosomal region (7q22-31) and the developments of different cancers which included breast cancer and different types of myeloid neoplasms in adults and children (Driouch et al., 1998).

Previous work was conducted by Paulson et al, has shown the reduction of HBP1 mRNA level was associated with an invasive of breast cancer (Paulson et al., 2007). Moreover, HBP1 is a potential candidate gene for controlling invasive breast cancer.

The molecular studies of HBP1 function are consistent with a role for HBP1 in breast cancer and indicate that it may also work to inhibit tumorigenicity and invasiveness. Increased invasiveness and tumorigenic proliferation, which are hallmarks of invasive and recurring breast cancer, are associated with HBP1 abrogation or lower levels. Similarly, future clinical relapse appears to be associated with decreased expression of HBP1 in the initial breast tumor (Paulson et al., 2007).

Furthermore, recent study have identified the involvement of same genomic location of 7q22 in the increase risk of cardiovascular disease which included coronary artery disease and large artery ischemic stroke. That region was described as cardiovascular susceptibility loci (van der Laan et al., 2018). Additionally, multiple clinical feature was reported via different case reports linked a variation within the same genomic region to multiple developmental and neurological diseases which included mental retardation, seizure development and microcephaly (Young et al. 1984 ; (Al-Hassnan et al., 2011). All the previously mentioned clinical data emphasize the possible involvement of the disruption of the genomic regions of HBP1 with multiple diseases.

Chapter 2: Genetic Deletion of HBP1 in Mice Results in Altered Gene Expression of Gabaergic Receptors, GABA/Glutamate Metabolism and Seizure Susceptibility.

Alrubiaan, S; Alqurashi, R; Malone, T; Paulson, E; Yee, A. To be submitted to *PLOSONE.*

2.1 Introduction

Epilepsy is a complex of multiple chronic neurological diseases in which the cumulative incidence is about 1-3% of the population worldwide (Falco-Walter et al., 2020). The epilepsies are characterized by recurrent spontaneous seizures and commonly associated co-morbidities (Myers & Mefford, 2015). About 20-30% of epilepsy cases are termed idiopathic, and most of these are due to acquired causes which include stroke, head trauma, head injury or brain tumor (Chambers et. al., 2016). However, the majority of the cases are believed to arise as a result of one or more genetic factors (Moshé et al., 2015).

Many genetic mutations have been linked to epilepsy using classic human family study approaches. Several mutations have been shown to cause epilepsy such as CACNA1A loss-of-function mutations. CACNA1A gene encodes the alpha1 subunit of the $Ca_V2.1$ P/Q-type voltage-gated calcium channel. Mutations in this gene cause familial hemiplegic migraine type 1 (FHM1), episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 and epilepsy. (Ophoff et al., 1994; Damaj et al., 2015). Furthermore, Xu et al., have identified a heterozygous mutation of GRIN2A (p.N447K) in a patient with Rolandic epilepsy, in which the functional assay has revealed N447K variant was linked to an enhanced glutamate potency. These results suggest that the N447K is a gain of function mutation (Xu et al., 2018).

Further, the pathogenetic mechanisms and potential therapeutic targets of epilepsy can be examined using rodent models that exhibit spontaneous seizures that resemble the symptoms of human epilepsy. The role of mouse systems such as knock-out or knock-in of specific alterations have been valuable tools for understanding the function and context of mutations that cause epilepsy. For example, mutations in components of the Mammalian Target of Rapamycin (mTOR) signaling pathway are

causal in a number of genetic epilepsies (Ostendorf & Wong, 2015). Other components of the mTOR pathway have also been identified in human genetic epilepsies (Dibbens et al., 2013). For instance, the GATOR1 protein complex which is comprised of DEPDC5, NPRL3, NPRL2 and plays a pivotal role in regulating mTOR signaling (Iffland, Carson, Bordey, & Crino, 2019). Further, DEPDC5, NPRL3, or NPRL2 mutations are linked to focal cortical dysplasia and seizures (Baldassari et al., 2019).

As defined by the experiments and analyses in multiple mouse models, synaptic plasticity, neurogenesis, dendritic morphology, and axonal sprouting are all influenced by mTOR signaling (Ostendorf & Wong, 2015). Rapamycin, which is an mTOR inhibitor was effective in reducing seizure frequency, neurological comorbidities and neuronal and glial pathology in numerous epilepsy models, including the TSC genetic mouse model and post-traumatic epilepsy (Guo, Zeng, Brody, & Wong, 2013). Furthermore, the use of Everolimus, which is an mTOR inhibitor analogue was effective in reducing seizures and increasing seizure free rate in patients with TSC associated seizures (Mizuguchi et al., 2019). These results demonstrate the importance of animal models of epilepsy in understanding the pathophysiology and behavioral changes associated with the disease, as well as pioneering treatment modalities.

The idiopathic class of epilepsies represent essentially the entirety of the unknown causes of epilepsy. Experimental models in mice include excessive receptor stimulation and postnatal traumatic brain injury. Each of these models has proved useful in understanding the pathophysiology of idiopathic epilepsies that are acquired outside of genetic causes. Seizure induction by receptor agonists such as Pilocarpine (cholinergic agonist, Cavalheiro, 1995) and Kainic acid (a class of glutamate receptor

agonist, Levesque & Avoli., 2013) have been a mainstay of basic mechanisms of epilepsy research for decades.

These models of seizure induction have been used to understand some of the molecular alterations that occur during epileptogenesis, which then develops into TLE in mice (Wang et al., 2022). The chemical-inductive models for TLE can reliably and quickly cause acute seizures termed status epilepticus in rodents. Recovery from induced seizures typically takes place over approximately two weeks. This latent period, termed epileptogenesis, is then followed by the development of spontaneous recurrent seizures, i.e. epilepsy. Indeed, my colleague and prior student in the Yee lab showed that induction of Wnt signaling is a primary cause of downstream mTOR activation during the epileptogenic period in these excitatory models (Alquarshi et al., 2021).

Both Kainic acid (KA) and pilocarpine (PILO) produce seizures in a pattern that is similar to those found in patients with mesial temporal sclerosis or temporal lobe epilepsy. These lesions frequently involve pyramidal cell death in the CA3 and CA1 hippocampal layers as well as GABAergic interneuron loss in the dentate hilus (Best et al., 1993, 1994; Morin et al., 1998). Because these models are well-defined and characterized, they can be used to study the neuro-pathological alterations and evaluate therapeutic intervention for both acute and chronic seizures (Sarkisian., 2001). Studies of both normal development as well as disruptions in development or injury models continue to inform the pathophysiology that results epilepsy.

Neural stem/progenitor cells (NSCs/NPCs), which can produce various types of neurons and glial cells, multiply and differentiate to form a functional central nervous system (CNS) (Inestrosa & Arenas., 2010). To keep NSC homeostasis and functional neuronal circuits, external niche cues and intrinsic transcriptional factors must

cooperate (Bond et al., 2015). The essential elements that control NSC homeostasis have been the subject of significant research, including nuclear receptors, extracellular matrix components, and cytoplasmic factors (Herrgen & Akerman, 2016; Gkikas et al., 2017). Wnt/β-catenin signaling was initially identified as a crucial regulator of mammary tumors, but it is now widely recognized as a crucial regulator of NSC homeostasis during development and in adulthood, including cortical precursor cells and dopaminergic precursor cells (L'Episcopo et al., 2014; Mishra et al., 2019).

Wnt signaling is a conserved cell signaling mechanism that is assumed to be involved in a variety of processes in the developing and adult brain, including NSC self-renewal, neurogenesis, embryonic patterning, and homeostatic tissue regeneration (Bond et al., 2015; Clevers et al., 2014; Liu et al., 2017; Tiwari et al., 2014; Zhao et al., 2019). A homeostatic equilibrium between progenitor proliferation and neural differentiation is needed to maintain a functional neural circuit, and this balance is largely dependent on the physiological activation of the Wnt pathway (Lie et al., 2005). Moreover, several mutations in Wnt pathway could lead to brain defects and malformations (Brault et al., 2001). Furthermore, the dysregulation of the WNT pathway has also been implicated in multiple neurological diseases, including schizophrenia, autism, Alzheimer's and epilepsy (Purro et al., 2012).

The role of Wnt signaling in seizure development was identified by the elevation of β-catenin level in rats hippocampi after using kainic acid for seizure induction that model was used to simulate TLE and it was confirmed that β-catenin among other key regulator of Wnt signaling were upregulated during epileptogenesis (Qu et al., 2017). Work from Yee lab showed the involvement of Wnt signaling activation in the coordination of a Warburg-like metabolism and altered TCA cycle in the

hippocampus during epileptogenesis (Alqurashi et al., 2021). The main mechanism is the regulation of pyruvate dehydrogenase (PDH) through its kinase and Wnt target gene PDK4. Interestingly, PDH is causative gene in certain genetic epilepsies. The Warburg-like metabolism in early epileptogenesis is orchestrated between neurons and astrocytes, in which the constitutive Wnt signaling was responsible for that variation. Further, that study provided the molecular basis governing mTOR activation in early epileptogenesis to include Wnt and metabolic signaling via AMPK and implicate new potential mechanisms for therapeutic targeting in epileptogenesis (Alqurashi et al., 2021).

During epileptogenesis a decline in Wnt suppressor HBP1 was concomitant with Wnt signaling activation (Alquarshi et al., 2021). Therefore, Alqurashi et al. analyzed the role of HBP1 in maintaining a barrier to high Wnt signaling in the hippocampus using a mouse knockout of HBP1. $HBP1^{-/-}$ mice were created by insertion of a β-galactosidase gene trap in intron 1. The gene trap provided a further tool to use β-galactosidase as a representative marker for HBP1 mRNA expressing cells. As shown in (Figure 2.1), both CA1 and dentate gyrus (DG) stained positively for β-galactosidase activity, suggesting that the HBP1 gene is expressed in those regions. Additional regions such as Purkinje cells in the cerebellum also showed β-galactosidase expression, but are not discussed here. Hippocampal expression of HBP1 is relevant due to the fact that the majority of the neuropathologic alterations which are concomitant with seizure development in mouse models and human studies occur in the hippocampus (Majores, Schoch, Lie, & Becker, 2007). Lastly, Alqurashi et al. attempted to directly inhibit Wnt signaling in the brain to block the Wnt-AMPK-mTOR signaling during epileptogenesis using Porcupine inhibitor XAV939. However, that attempt failed to decrease Wnt/β-catenin or mTOR activity in the

epileptogenic brain, likely due to the inability of the compound to cross the blood brain barrier (Alqurashi et al., 2021). In this chapter we hypothesized that Wnt signaling inhibitor HBP1 may contribute to a genetic susceptibility to epilepsy, rather than act as a dominant genetic epilepsy such as TSC2 mutants. This investigation was motivated by two observations:1) HBP1 knockout mice, which appeared otherwise normal, had rare spontaneous seizures and a susceptibility to seizure induction, and 2) a fellow Ph.D. student, Roaya Alqurashi, showed that Wnt signaling was required to initiate a program of molecular changes during epileptogenesis that resulted in the subsequent development of chronic seizures i.e. the hallmark of epilepsy.

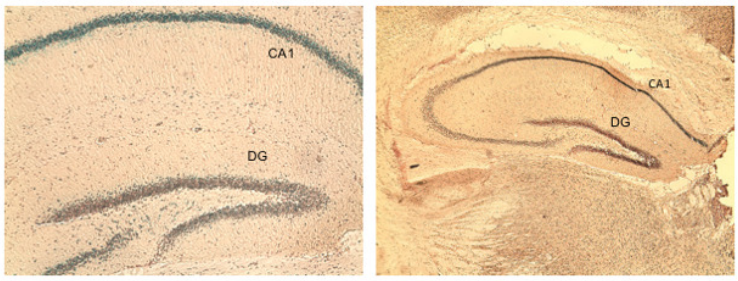


Figure 2.1: HBP1 is Expressed in CA1 and DG of the hippocampus. HBP1 gene is expressed in the CA1 and DG regions. The expression pattern of the HBP1 gene was determined in the HBP1 KO mouse through the inserted β-galactosidase gene. Note that the HBP1 KO mouse was generated by insertion of the β-galactosidase gene into intron 1 of the HBP1 genomic loci . Thus, β-galactosidase activity is driven by the HBP1 promoter and is a reporter of the HBP1 promoter activity. Representative slides of HBP1 KO adult mouse hippocampus with β-galactosidase gene inserted into intron 1 of HBP1 gene. Adapted with permission from Alquarshi et al., A Warburg-like metabolic program coordinates Wnt, AMPK, and mTOR signaling pathways in epileptogenesis. Plosone, 2021. 16 (8): p.18. Changes include (The figure is cropped from the original figure 7A).

2.2 Results

2.2.1 Spontaneous Seizure and Seizure Susceptibility in HBP1$^{-/-}$ Mice.

The phenotype which was observed in HBP1 deficient mice was episodic spontaneous seizures. Based on observations made by the animal facility and subsequent monitoring, HBP1$^{-/-}$ mice were confirmed to have spontaneous seizures (work of K.E. Paulson, T. Malone and M.W. Tam). Specifically, over a two-week

period, WT and KO mice were monitored by video-EEG. A total of three KO mice (out of five analyzed) were observed to have the seizure activity. Two of the KO mice had tonic-clonic seizures and the EEG profile for both mice were accompanied by visually apparent seizure (Figure. 2.2) (Racine 1972). No evidence of spontaneous seizures were observed in WT mice. Moreover, the HBP1 KO mice showed an abnormal EEG pattern which was concomitant with hyperactive behavior which include compulsive circling and continuous jumping (data not shown)**.** Lastly**,** HBP1$^{-/-}$ mice were confirmed to have seizure susceptibility relative to WT mice in response to receptor activation by pilocarpine and reduced overall survival rate after seizure induction (Figure. 2.3 A-B) (Alquarshi et al., 2021).

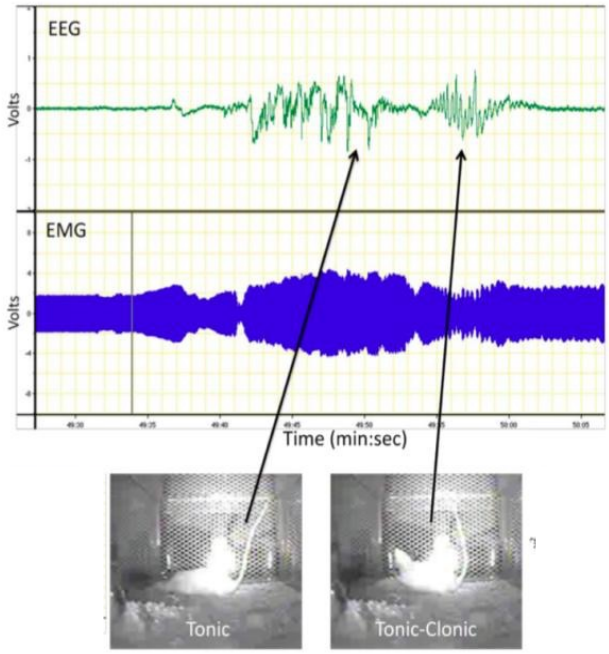


Figure 2.2: EEG data from an HBP1$^{-/-}$ mouse spontaneous seizure. Mouse showed a spontaneous seizure event mid sleep cycle. The sizing episode which affected the mouse started with a tonic seizure characterized by a high amplitude waveform, which is also manifested behaviorally as the rigid extension of the tail. The mouse then developed a tonic-clonic seizure with a more regular high amplitude waveform, which can be seen behaviorally as the rearing of the mouse and "piano playing" with its paws in a non-grooming fashion (bottom) (Racine 1972).

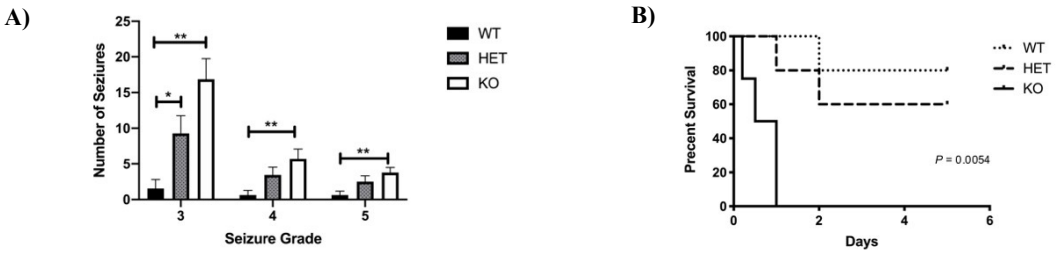


Figure 2.3: HBP1 gene dosage alters seizure susceptibility. A) Seizure susceptibility for the different HBP1 genotypes were tested ($HBP1^{+/+}$, $HBP1^{-/+}$ and $HBP1^{-/-}$) is shown by inducing seizure using pilocarpine for FVBN strain. HBP1 KO had increased seizure sensitivity. Results based on the number and severity of seizures on a 5-point modified Racine scale [. (n = 11–17 mice for each group). Data are represented as mean ± SEM. *P <0.05, **P < 0.01 by unpaired student's t-test using Prism 9.0 (Graphpad). **B)** Decreased survival after seizure induction in HBP1 KO mice. Survival curves were made for the first 5 days after seizure induction. (n = 5 mice for each group). Data are represented as mean ± SEM. *P <0.05 by **P < 0.01, *** P < 0.001 by unpaired student's t-test using Prism 9.0 (Graphpad). Adapted with permission from: Alquarshi et al., A Warburg-like metabolic program coordinates Wnt, AMPK, and mTOR signaling pathways in epileptogenesis. Plosone, 2021. 16 (8): p.18. Changes include (The figure is cropped from the original figure 7J and 7K).

2.2.2 Decreased HBP1 Expression Recapitulates the Wnt-Driven Epileptogenic Hippocampus.

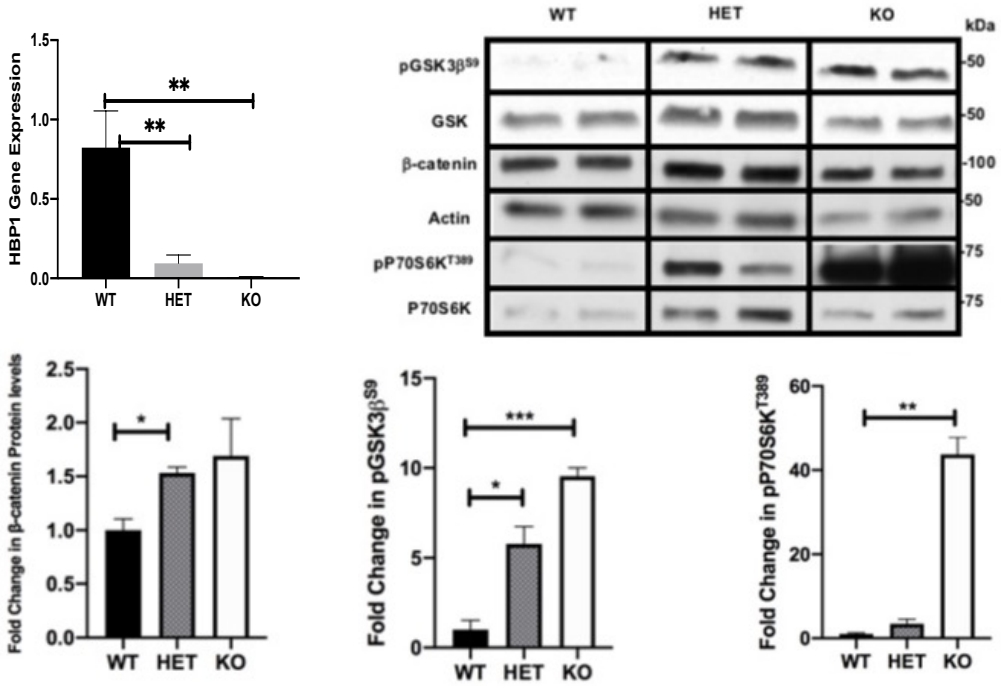
Activation of Wnt signaling during epileptogenesis was identified in a TLE mouse model (Alqurashi et al., 2021). The consequence of Wnt signaling was the induction of Wnt metabolic targets including glycolytic genes resulting in elevated lactate, as well as the Wnt target pyruvate dehydrogenase kinase 4 (PDK4) and the phosphorylation of pyruvate dehydrogenase. The elevated glucose metabolism consequently inhibited AMPK activity, resulting in decreased phosphorylation of downstream AMPK target TSC2 and elevated mTOR activity. In the same paper from Yee lab, a similar molecular framework of constitutively elevated Wnt signaling and increased mTOR activity was observed in a model of constitutive Wnt activity, $HBP1^{-/-}$ mice (Alqurashi et al., 2021). Figure (2.4 A) shows a clear HBP1 dose-dependence on the constitutive protein expression of β-catenin and serine 9 phosphorylation of

pGSK3β (pGSK3β^{S9}), both measures of Wnt signaling activity. Further, Alqurashi et.al. investigated whether the hippocampal Wnt signaling is altered in the knockout mice, and to detect if the alteration of hippocampal Wnt signaling has a gene dosage effect (Alquarshi et al., 2021). It was essential to examine the level of expressions of β-catenin and pGSK3B in all HBP1 genotypes. Figure 2.4 A showed a significant increase in the protein expression of β-catenin and pGSK3β. Moreover, phosphorylation of P70S6K (pP70S6K^{T389}), which is a downstream marker for mTOR activity, showed a similar HBP1 dose dependence, with clear differences between HBP1$^{+/+}$, HBP1$^{+/-}$ and HBP1$^{-/-}$ mice.

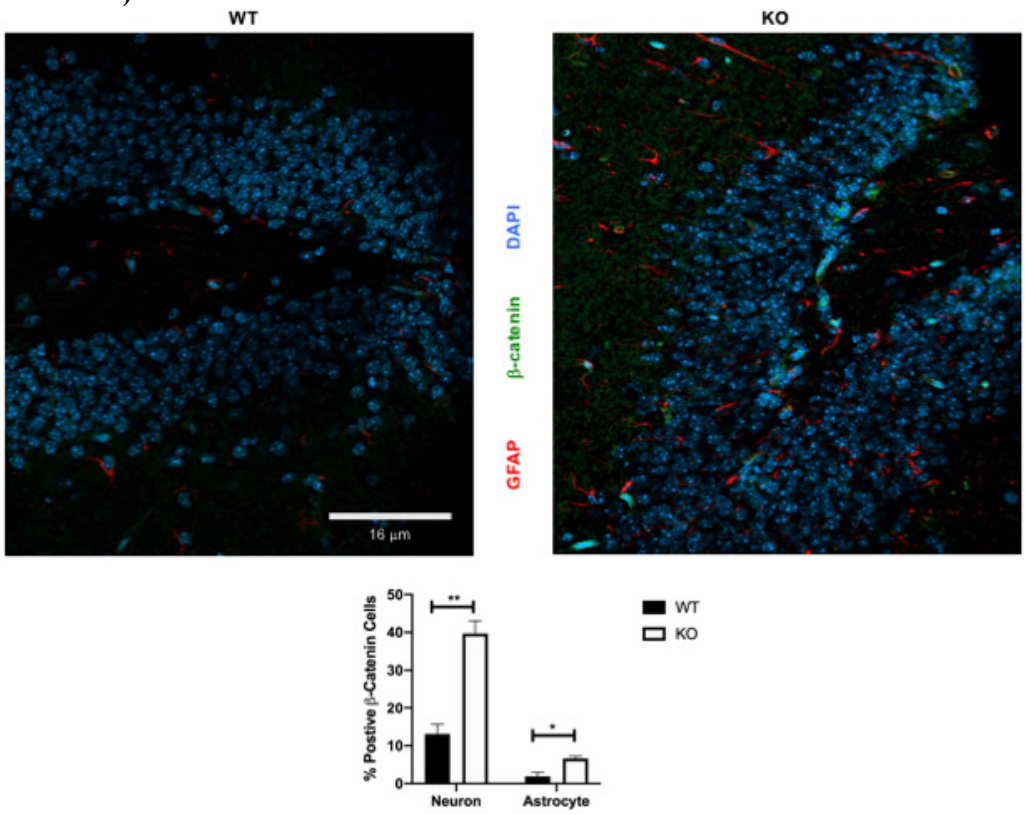
Additionally, cellular localization of Wnt activity markers β-catenin, pGSK3B^{S9} were consistent with high Wnt signaling in the brain of HBP1$^{-/-}$ mice. As shown in Figure 2.4 B, β-catenin expression was highest in the subventricular zone and hilus, which is consistent with the location of active neuronal stem cells. In contrast, we only observe an occasional β-catenin positive cells in the subventricular zone or hilus in WT mice. Similar, although not identical results are shown for the cellular localization of pGSK3β^{S9} (Figure 2.4 C). Like β-catenin, pGSK3β^{S9} is prominently expressed in the hilus, but is more broadly expressed in what appears to be a random set of neurons throughout the dentate gyrus. Finally, and consistent with the western blot data, mTOR activity as measured by pP70S6K^{T389} expression is present in most neurons of the dentate gyrus (Figure 2.4D). We also examined the level of another Wnt inhibitor sFRP1, which inhibits Wnt signaling by sequestering extracellular Wnt ligands (Matsuda, Schlange, Oakeley, Boulay, & Hynes, 2009). Previous work from the Yee lab showed a positive correlation between HBP1 and sFRP1 expression via the epigenetic regulation of sFRP1 (Paulson et al., 2007). As shown in Figure 2.4 E, sFRP1 expression declined in tandem with HBP1 expression,

confirming the mechanism previously published, but also the coordinate loss of Wnt signaling inhibition as a result of altered HBP1 gene dosage. Finally, the Wnt inhibitor FOXG1which also declines during epileptogenesis in TLE model (Alquarshi et al., 2021), did not appear to be altered as a result of HBP1 gene dosage. These initial observations for HBP1$^{-/-}$ mice recapitulate the epileptogenic state of constitutive increase in Wnt signaling, mTOR activity, increased seizure susceptibility and spontaneous seizures. Consequently, HBP1$^{-/-}$ mice likely have further broad metabolic and molecular alterations in response to and possibly as compensation for constitutive Wnt signaling.

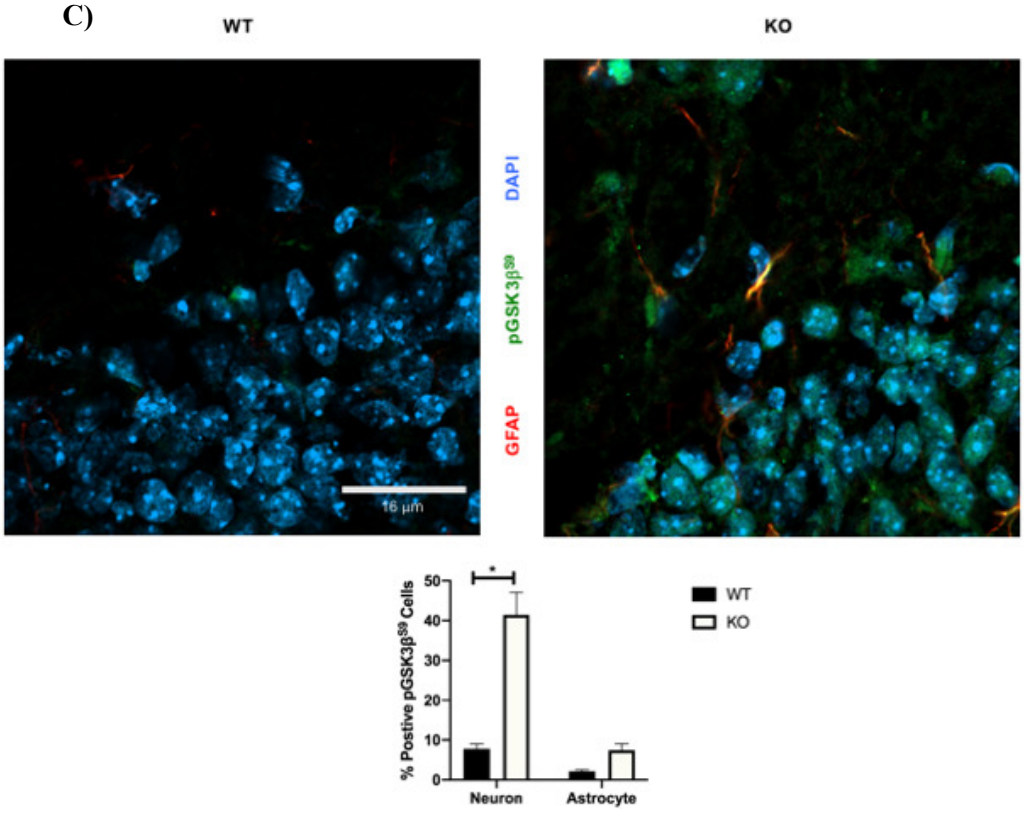
A)



B)


WT
KO
DAPI
β-catenin
GFAP
16 µm
% Postive β-Catenin Cells
50
40
30
20
10
0
**
*
Neuron
Astrocyte
WT
KO

C)


WT
KO
DAPI
pGSK3β^{S9}
GFAP
16 µm
% Postive pGSK3β^{S9} Cells
50
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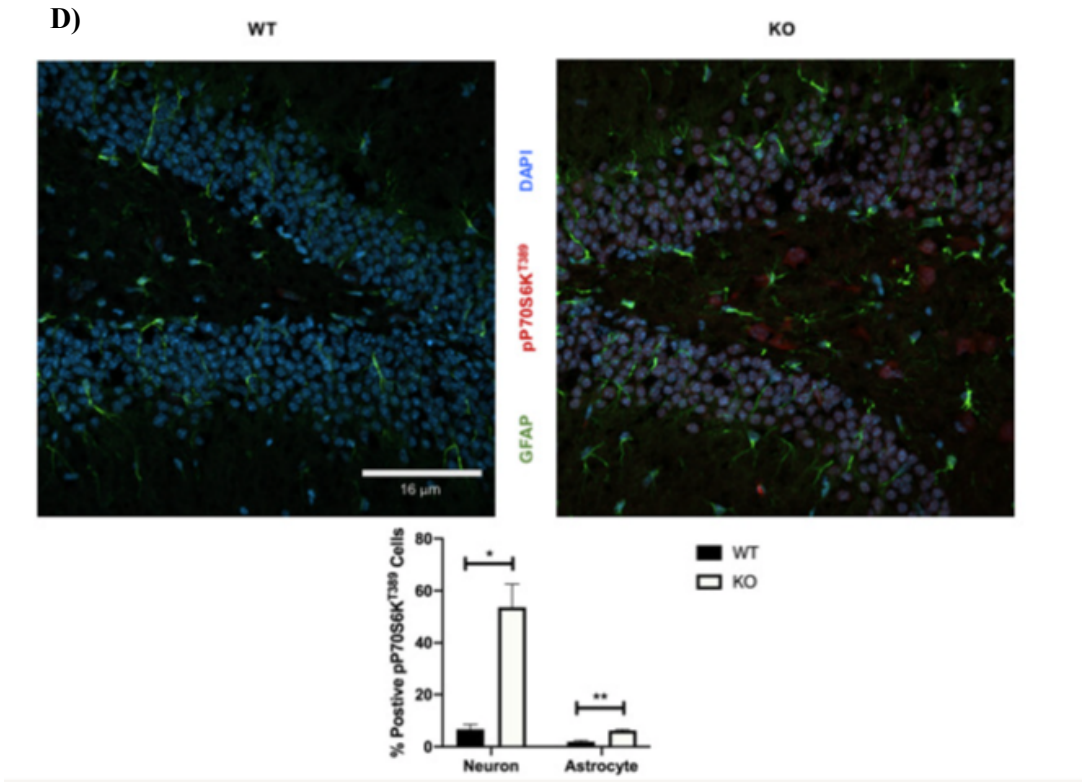
Neuron
Astrocyte
WT
KO



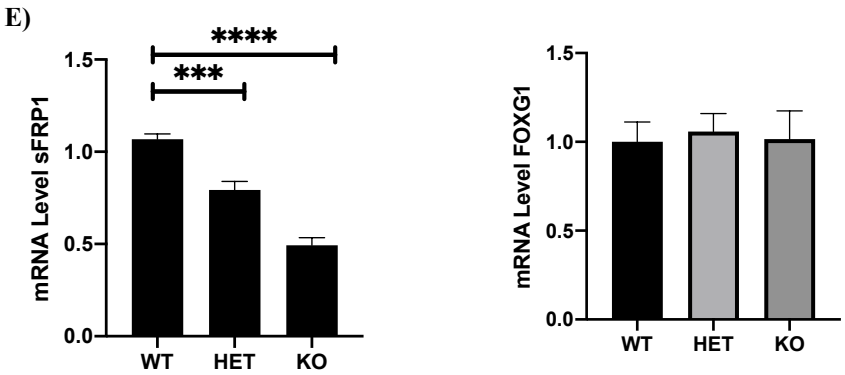


Figure 2.4: Decreased HBP1 expression recapitulates the Wnt-driven epileptogenic hippocampus. A) Activation of Wnt and mTOR signaling in HBP1 WT, HET and KO mice. Representative western blot with quantified data of baseline levels of Wnt (pGSK3β and β-catenin) and mTOR signaling marker (pP70s6K) in FVBN mouse strain were increased in KO and HET mice when compared to the WT. (**B-D**) Localization of activated Wnt and mTOR signaling in HBP1 WT and KO mice by confocal microscopy. Representative confocal sections with quantified analysis of positive pGSK3βS9 (green; x63 in C), β-catenin (green; x20 in B), and pP70S6KT389 (red; x20 in D) neuronal cells are increased in the DG region of KO when compared to WT. [GFAP (red in B and C; green in D) and Dapi (blue)]. (n = 3–5 mice for each group). Data are represented as mean ± SEM. *P < 0.05, **P < 0.01, *** P < 0.001 by unpaired student's t-test using Prism 9.0. **E)** Gene expression levels for Wnt inhibitor sFRP1 was significantly downregulated in the HBP1 KO mice while FOXG1 was not changed. Figures. A-D Adapted with permission from: Alquarshi et al., A Warburg-like metabolic program coordinates Wnt, AMPK, and mTOR

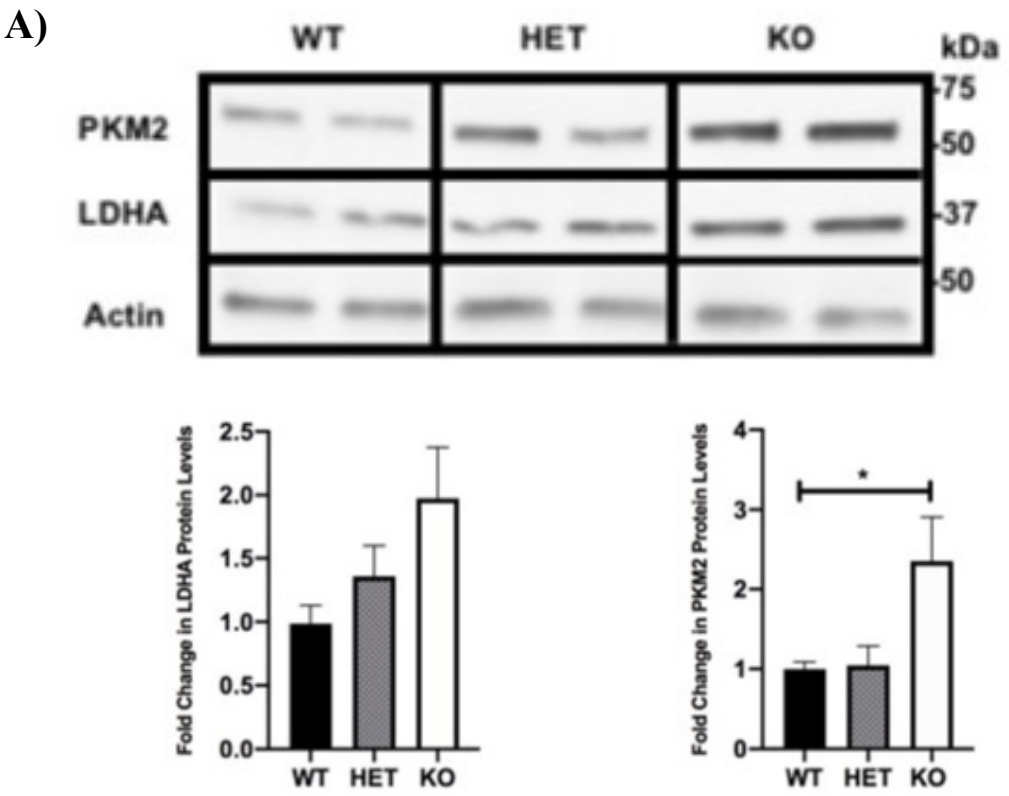
signaling pathways in epileptogenesis. Plosone, 2021. 16 (8): p.18. Changes include (Figure A HBP1 gene expression was added to the original figure 7B, Figures C-E are cropped from the original figure 7 C-E).

2.2.3 Wnt-driven Metabolic Alterations of the HBP1$^{-/-}$ Mice

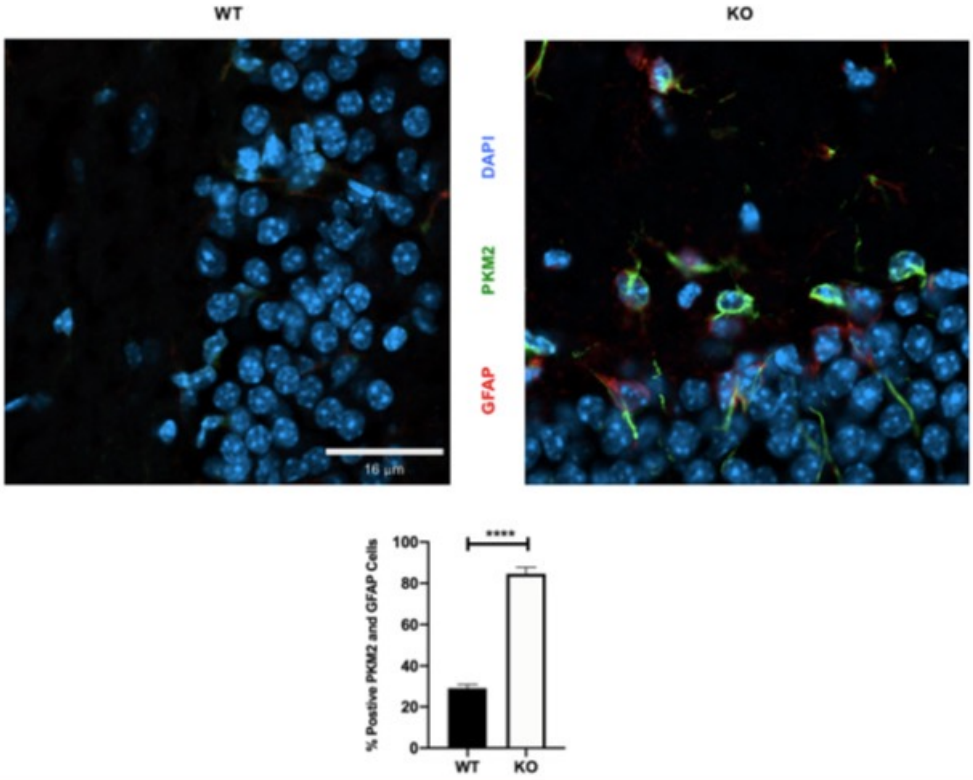
In cancer, Wnt signaling is linked to Warburg metabolic re-programming. The tumor cells will have an altered metabolism, which is characterized by increased glucose uptake and usage to provide energy for the sustained proliferation (Pate et al., 2014; Thompson, 2014). Furthermore, previous work published from Yee lab detected metabolic shift in the hippocampus during epilpetogenesis. We hypothesized that similar metabolic shifts could be existing in the hippocampus of the HBP1 knockout mice. Moreover, expanding the characterization of the HBP1$^{-/-}$ mice, to identify the metabolic consequences of high Wnt signaling in the brain and characterize potential resemblance of metabolic changes which was confirmed in an epileptic brain. Therefore, we have investigated the hippocampal metabolic enzymes which is consistent with a metabolic change to aerobic glycolysis. Concomitant with increased Wnt signaling, we observed an increase in the protein expression of Warburg glycolytic enzymes pyruvate kinase isoform M2 (PKM2) and lactate dehydrogenase isoform (LDHA) (Figure 2.5A). In the brain, there is unique and complex arrangement of overall metabolism between neuron and astrocyte, in which astrocytes export lactate for neuronal metabolic use (Magistretti, Sorg, Yu, Martin, & Pellerin, 1993), although, neurons can also utilize glucose for metabolic purposes (Diaz-Garcia et al., 2017; Yellen, 2018).

Therefore, identifying the overall expression of Warburg enzymes was crucial. Furthermore, the cellular localization for the Warburg enzymes is also significant to determine the metabolic shift consequences at a cellular level. Figure (2.5 B) showed that a large subset of hippocampal astrocytes expressed PKM2 in the knockout mice.

One of the Wnt target genes identified in Wnt driven Warburg effect is pyruvate dehydrogenase kinase 4 (PDK4) (Pate et al., 2014). The PDK enzymes phosphorylate and PDH at serine 293, inhibiting PDH activity. PDH inhibition leads to an increase in pyruvate, which is subsequently converted to lactate, a characteristic of Warburg metabolism. In HBP1 KO mice, PDK4 gene expression and $pPDH^{S293}$ increases significantly (Figure 2.5 C), consistent with observed Wnt activation. (Figure 2.5 C-D). Lastly, in HBP1 KO mice PDH^{S293} phosphorylation was observed to be predominately in neurons of the hippocampus (Figure 2.5 D). These findings are consistent with observed Warburg metabolic alterations during epileptogensis (Alqurashi et al., 2021).



B)


WT
KO
DAPI
PKM2
GFAP
16 µm
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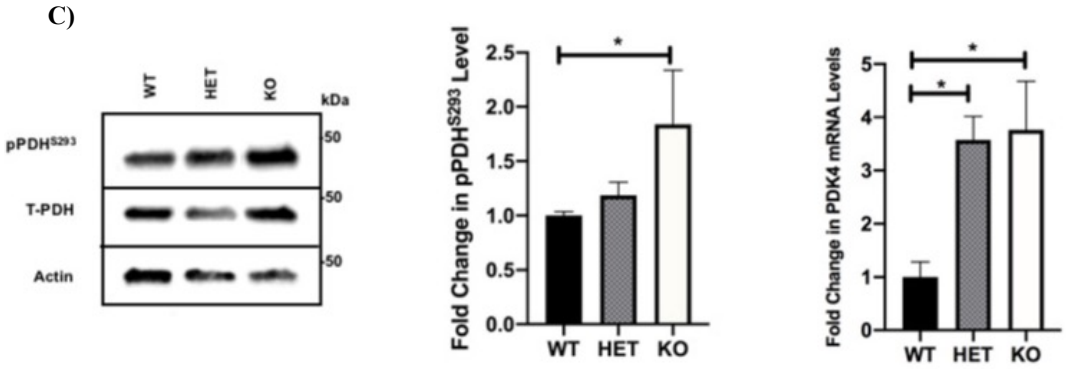
% Positive PKM2 and GFAP Cells
100
80
60
40
20
0
WT
KO

C)


WT
HET
KO
kDa
pPDH^{S293}
T-PDH
Actin
50
50
50
*
Fold Change in pPDH^{S293} Level
2.5
2.0
1.5
1.0
0.5
0.0
WT
HET
KO
*
*
Fold Change in PDK4 mRNA Levels
5
4
3
2
1
0
WT
HET
KO

D)

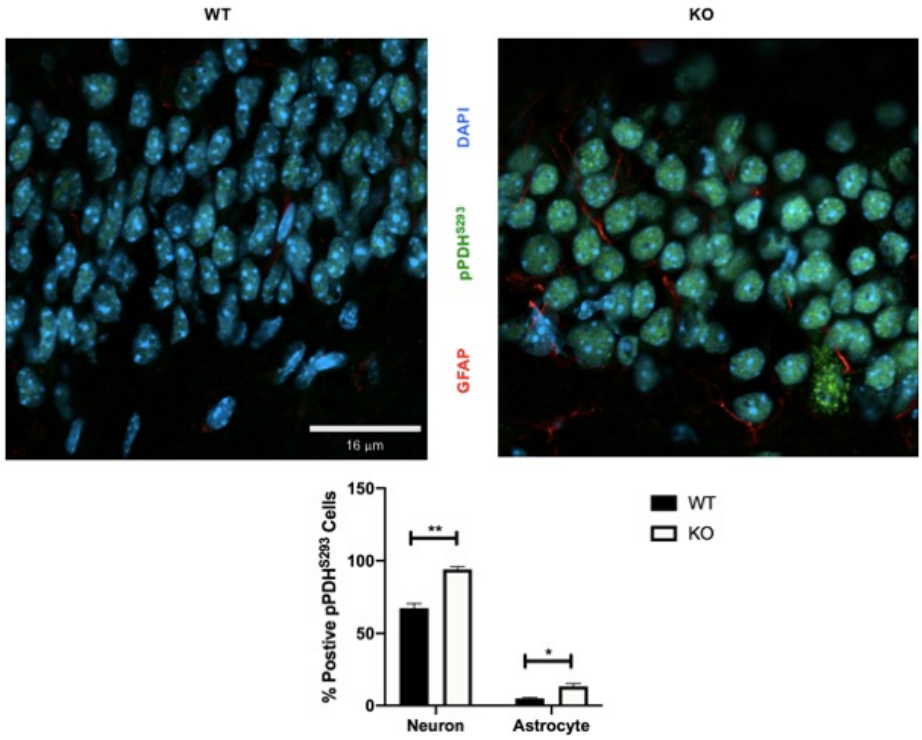


Figure 2.5 : Wnt-driven metabolic alterations of the HBP1 KO mice. A) Protein quantification for glycolytic enzymes PKM2 and LDHA and both were significantly increased in the HBP1 KO mice. **B)** Confocal images for PKM2(green) and GFAP (red) 63X showing an increased expression of the PKM2 in the HBP1 KO mice. **C)** Protein quantification and of pPDH^{s293} and PDK4 mRNA level both were significantly increased in the HBP1 KO mice. **D)** Confocal images for pPDH^{s293} (green) and GFAP (red) showing a higher localization with neurons compared to the WT. Data are represented as mean ± SEM. * $P < 0.05$, **$P < 0.01$, *** $P < 0.001$ by student t- test using Prism 9.0. Adapted with permission from: Alquarshi et al., A Warburg-like metabolic program coordinates Wnt, AMPK, and mTOR signaling pathways in epileptogenesis. Plosone, 2021. 16 (8): p.18. Changes include (Figures A-D are cropped and rearranged from the original figures 7 F,G,H and I).

2.2.4 Bioinformatic Analysis Reveals Multiple Neuro-Pathological Alterations Associated with HBP1 Loss.

The HBP1$^{-/-}$ model is a genetic model for an elevated Wnt signaling. Moreover, these mice suffer from spontaneous seizures and have increased seizure susceptibility using chemical agent to induce seizure. These observations recapitulate many of the features of the epileptogenic period, yet are unlikely to be the only molecular

alterations. Therefore, we performed an unbiased analysis of the hippocampal transcriptome using RNA-seq and Ingenuity Pathway Analysis to identify potential gene expression differences between WT and KO mice.

One of the ways IPA's bibliomic analysis functions is to group genes known to be dysregulated in specific diseases or disorders and determine if there is a disruption comparing two groups. This analysis suggested HBP1 KO mice may have increased likelihood of multiple neurological disorders (Table 2.1). One suggested clustering of disorders included brain tumors. Given the known involvement of decreased HBP1 in invasive and triple negative breast cancer, this suggestion is certainly plausible. Furthermore, HBP1 functions as a proliferation barrier and consistent with this, confocal microscopy showed a significant increase in the proliferation marker Ki67 in the HBP1$^{-/-}$ mice (Figure 2.6 E). Furthermore, the confocal images showed an increase in Ki67 started at 4 weeks, however, it was not statistically significant (Figure 2.6 F).

Furthermore, CD133 is a membrane bound glycoprotein encoded by the PROM1 gene, that may function in cell differentiation, epithelial to mesenchymal transition, and is a marker for human neural stem cells (Shmelkov, St Clair, Lyden, & Rafii, 2005). CD133 has been used primarily as a marker to identify cancer stem cells in multiple human cancers including brain tumors (Ludwig & Kornblum, 2017). In the HBP1 deficient mice, CD133 gene expression was significantly increased (Figure 2.6 D). However, in experiments done in the Yee lab in which they aged a cohort of WT and KO mice, no brain tumors were observed.

A second type of clustered disorder suggested altered coordination. While we have not done any classical mouse studies, we do observe a high frequency of HBP1 KO mice that run in their cage all day. Similarly, when young HBP1 KO mice have a

hyperactivity phenotype with significant spontaneous jumping. While these may not necessarily be coordination traits, they could also be consistent with the learning gene grouping pathology identified by IPA. Clearly, the IPA disorder analysis suggests multiple avenues of animal behavior or function tests that may help identify more fully some of the possible pathologies associated with altered HBP1 expression (Table 2.1).

Another of IPA's bibliomic analysis functions is to analyze genes regulated by specific signaling pathways, again determining if there is a significant difference between two groups. This analysis brought fresh insight into the difference between WT and HBP1 KO mice in that both glutamate receptor signaling, CREB signaling and CNTF were all altered significantly in HBP1$^{-/-}$ mice. Ciliary neurotrophic factor (CNTF), it is released by astrocytes and stimulates the survival of developing neurons (Escartin et al., 2006). Further, CNTF activation of astrocytes in vivo is associated with noticeable phenotypic and molecular changes leading to a better handling of increased levels of extracellular glutamate (Escartin et al., 2006). IPA analysis has shown a reduction in the CNTF in the HBP1 knockout mice which might be contributing to the epileptic phenotype HBP1 deficient mice are exhibiting (Table 2.2). Additionally, we noted an increase in astrocytic genes and functions in HBP1 KO mice, which would have the potential to alter normal neurotransmitter function (Table 2.2). Finally, this result was consistent with our observation that HBP1 KO mice had significant gliosis, similar to what we and others have observed a week following status epilepticus in mice (Alquarshi et al., 2021). Gliosis and neuronal cell death are among other pathologic alterations which are frequent in patients with TLE (Alonso-Nanclares & DeFelipe, 2014).

The HBP1 gene dosage effect on gliosis was observed by immunofluorescence staining using an astrocytic marker (GFAP) to identify the number of astrocytes in all HBP1 genotypes. Confocal microscopy showed a clear increase in the number of GFAP positive cells in the dentate gyrus as an inverse function of HBP1 expression level: $HBP1^{+/+}$ mice few GFAP positive astrocytes, $HBP1^{+/-}$ mice had more GFAP positive astrocytes while $HBP1^{-/-}$ mice had a nearly 7-fold increase in GFAP positive astrocytes relative to $HBP1^{+/+}$ mice (Figure 2.6A). As shown in Figure 2.6 B, GFAP mRNA levels in the hippocampus, measured using qRT-PCR, increased significantly. Further, s100B gene, which is an astrocytic gene and function as a calcium binding protein, it is highly expressed in the astroglial compartment of the brain and an increase in its level has been linked to CNS damage (Gerlach et al., 2006). qRT-PCR result showed a significant increase in s100B mRNA level in the $HBP1^{-/-}$ mice (Fig. 2.6 B).

Among the multiple physiological functions of astrocytes, also they play an important function in synaptic transmission and metabolism (Binder & Steinhauser, 2006). Alqurashi et.al, showed a number of changes in glutamine-glutamate homeostasis in epileptogenesis (Alquarshi et al., 2021), and given the susceptibility to seizures in $HBP1^{-/-}$ mice, we wondered if some of those changes were recapitulated in as a result of the loss of HBP1 expression. One of the function for astrocytes is to maintain glutamate-glutamine homeostasis (Dvorzhak, Melnick, & Grantyn, 2018). Glutamine synthase (GS) converts glutamate captured from synapses to glutamine, which is then harmlessly exported from astrocytes to be recaptured by neurons and converted back to glutamate. Many epilepsies show a decline in GS despite the increase in astrocytes, therefore we examined GS levels. As shown in (Figure 2.6 B), GS levels declined as HBP1 gene dosage declined.

With increased gliosis, we observed multiple astrocytic and neuronal genes expression was altered significantly in the RNA-seq (Table 2.3). Next, we investigated whether the HBP1 loss will lead to a detectable change in neuron numbers or if it will affect their maturation. The RNA-seq analysis showed a significant reduction in the intermediate differentiation marker NeuroD1 (Table 2.3), however, the qRT-PCR did not confirm these result. Therefore, we explored this further by examining the expression of the post-mitotic terminal differentiation marker NeuN using immunohistochemistry. Similar to our observation with NeuroD1 gene expression, NeuN positive cells declined slightly but insignificantly in $HBP1^{-/-}$ hippocampi in the DG, suggesting that HBP1 deletion does not alter the number of the mature neurons (Figure 2.6 C). Given the decline in astrocytic GS, we wondered if there was a compensating increase in glutamine-dependent production of glutamine in neurons. An essential enzyme for the production of glutamate in neurons is phosphate-activated glutaminase (PAG), which catalyzes the conversion of glutamine to glutamate. Patients with TLE have elevated PAG in hippocampal neurons in the CA1 and CA3 as well as in the polymorphic layer of the dentate gyrus (Eid et al., 2006). PAG was significantly upregulated in the $HBP1^{-/-}$ mice, suggesting a compensating alteration of metabolism had occurred given then alteration in GS levels in astrocytes. Further, it appears that glutamate homeostasis is clearly altered in HBP1 deficient mice (Figure 2.6 D). Additionally, knowing the function of the HBP1 and its relevancy to the proliferation ability of the cells it was necessary to identify the expression level of an early neuronal marker. Nestin expression levels were significantly upregulated in the $HBP1^{-/-}$ mice when compared to the $HBP1^{+/+}$and $HBP1^{-/+}$ Figure (2.6D). Moreover, upregulation of nestin is a finding which was confirmed in both pilocarpine induced seizures and TLE (Blumcke et al., 2001).

Collectively, the cellular and the morphological changes in the HBP1$^{-/-}$ mice are suggestive for a potential epileptic phenotype.

Table 2.1: IPA analysis showing the potential neuro-pathological impact of HBP1 gene loss. Functional annotation of genes that are significantly and uniquely changes in the hippocampus of HBP1$^{-/-}$ mice, showing an increased risk of multiple neurological diseases.

Categories	Diseases or Functions Annotation	p-value	Predicted Activation State	Activation z-score
Neurological Disease, Organismal Injury and Abnormalities	Brain lesion	1.87E-09	Increased	2.847
Neurological Disease	Motor dysfunction or movement disorder	5.17E-28	Increased	2.679
Neurological Disease	Movement Disorders	2.9E-27	Increased	2.634
Cancer, Neurological Disease, Organismal Injury and Abnormalities	Central nervous system solid tumor	5.29E-13	Increased	2.591
Cancer, Neurological Disease, Organismal Injury and Abnormalities	Central nervous system tumor	5.43E-13	Increased	2.591
Cancer, Neurological Disease, Organismal Injury and Abnormalities	Brain tumor	8.62E-08	Increased	2.449
Cancer, Organismal Injury and Abnormalities	Tumor in nervous system	4.71E-13	Increased	2.401
Cancer, Organismal Injury and Abnormalities	Cancer of head	1.41E-15	Increased	2.238
Cancer, Neurological Disease, Organismal Injury and Abnormalities	Central nervous system cancer	5.32E-17	Increased	2.07
Behavior, Nervous System Development and Function	Memory	6.91E-11	Decreased	-2.333
Behavior	Learning	6.89E-19	Decreased	-2.36

Nervous System Development and Function	Coordination	5.68E-11	Decreased	-2.765

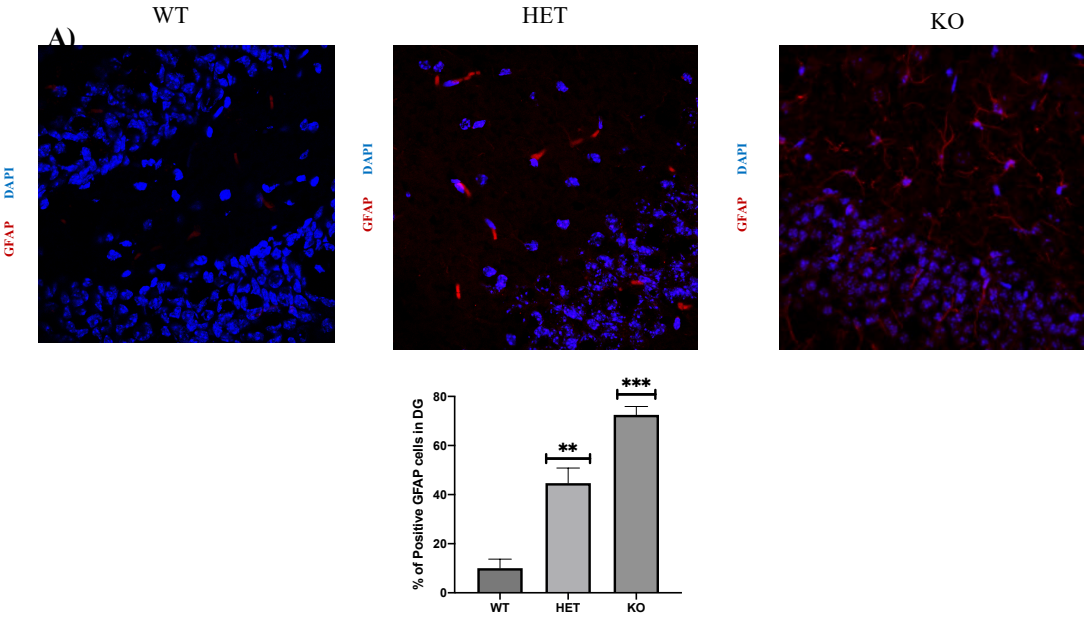
Table 2.2: Multiple signaling pathways are disrupted due to HBP1 loss. Top canonical pathways significantly altered in the hippocampus of HBP1$^{-/-}$ mice.

Ingenuity Canonical Pathways	**-log (p-value)**	**Ratio**	**z-score**	**Downregulated**	**Upregulated**
Neuropathic Pain Signaling In Dorsal Horn Neurons	8.02	0.304	-4.226	30/115 (26%)	5/115 (4%)
CREB Signaling in Neurons	7.87	0.245	-3.795	39/212 (18%)	13/212 (6%)
Dopamine-DARPP32 Feedback in cAMP Signaling	3.11	0.195	-3.78	24/164 (15%)	8/164 (5%)
Gαq Signaling	3.26	0.199	-3.528	23/161 (14%)	9/161 (6%)
Calcium Signaling	2.54	0.175	-3.413	29/206 (14%)	7/206 (3%)
Synaptic Long Term Potentiation	5.77	0.262	-2.828	22/122 (18%)	10/122 (8%)
Amyotrophic Lateral Sclerosis Signaling	6.25	0.279	-2.746	22/111 (20%)	9/111 (8%)

Glioma Invasiveness Signaling	2.52	0.229	-2.5	13/70 (19%)	3/70 (4%)
Synaptic Long Term Depression	2.67	0.184	-2.475	25/174 (14%)	7/174 (4%)
Glutamate Receptor Signaling	2.57	0.246	-2.333	12/57 (21%)	2/57 (4%)
CNTF Signaling	3.43	0.266	-2.183	13/64 (20%)	4/64 (6%)
Opioid Signaling Pathway	5.5	0.21	-2.143	36/243 (15%)	15/243 (6%)

Table 2.3: RNA sequencing data showing a change in the gene expression for multiple astrocytic genes. Multiple astrocytic genes were upregulated in the KO mice.

Gene	Sample 1	Sample 2	Value 1	Value 2	Fold change	P value	Significant
GFAP	Control	KO	98.4205	154.191	0.647691	5.00E-05	Yes
S100b	Control	KO	65.4116	75.3796	0.20462	0.03595	Yes
Aqp1	Control	KO	1.59658	4.07176	1.35066	0.00205757	Yes
Aqp4	Control	KO	28.3319	44.3961	5.00E-05	0.00205757	Yes
Neurod1	Control	KO	16.851	12.1684	-0.469686	5.00E-05	Yes


A)
WT
HET
KO
GFAP DAPI
GFAP DAPI
GFAP DAPI
% of Positive GFAP cells in DG
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60
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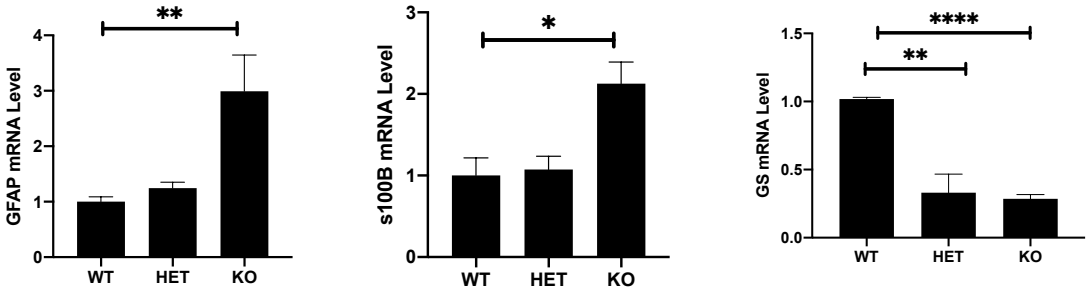
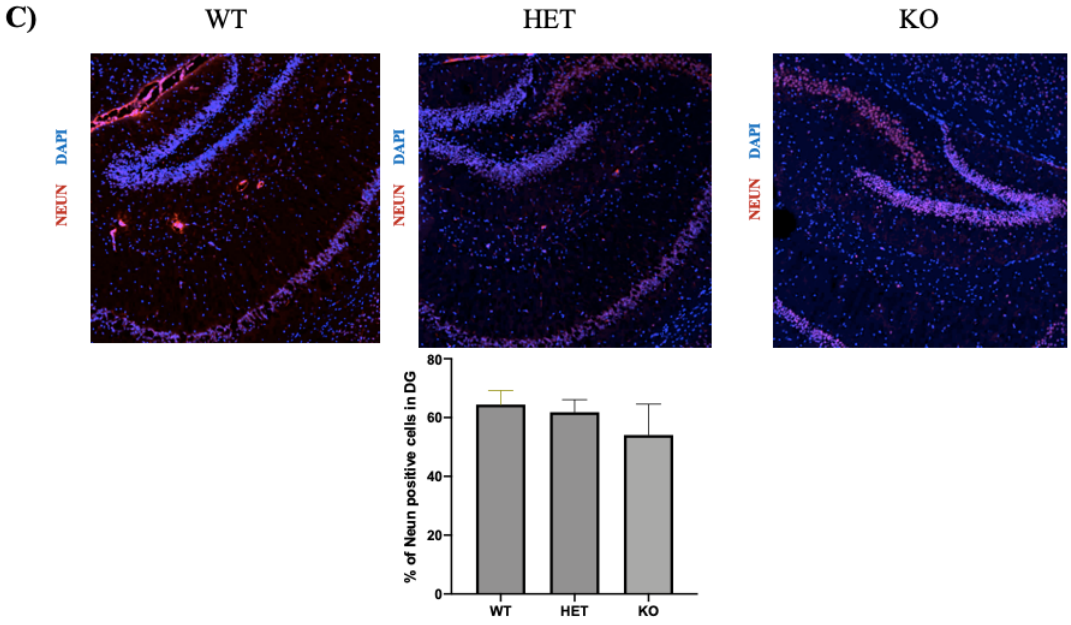
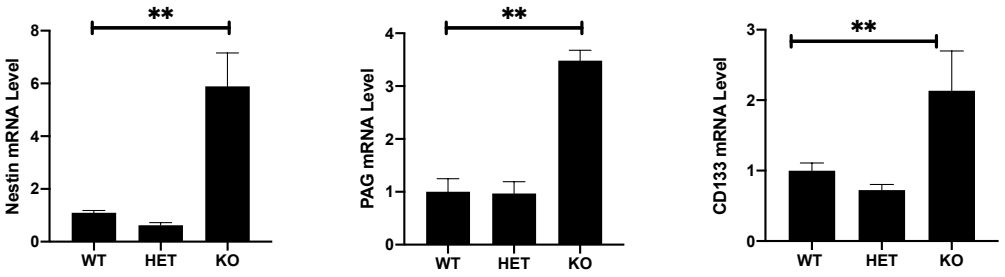
***
WT
HET
KO


B)
GFAP mRNA Level
0
1
2
3
4
**
WT
HET
KO
s100B mRNA Level
0
1
2
3
*
WT
HET
KO
GS mRNA Level
0.0
0.5
1.0
1.5
****
**
WT
HET
KO


C)
WT
HET
KO
NEUN DAPI
NEUN DAPI
NEUN DAPI
% of Neun positive cells in DG
0
20
40
60
80
WT
HET
KO

D)


**
Nestin mRNA Level
0
2
4
6
8
WT
HET
KO
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PAG mRNA Level
0
1
2
3
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WT
HET
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CD133 mRNA Level
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1
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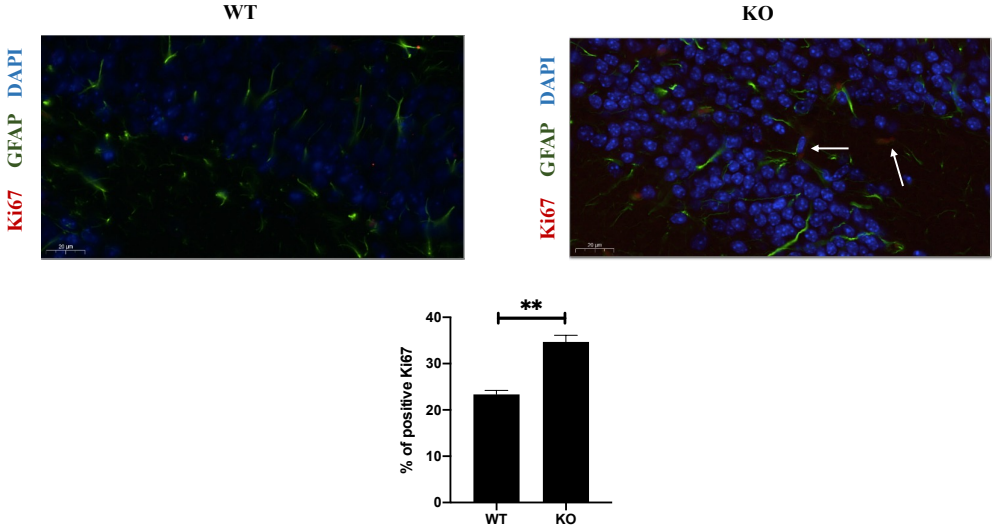
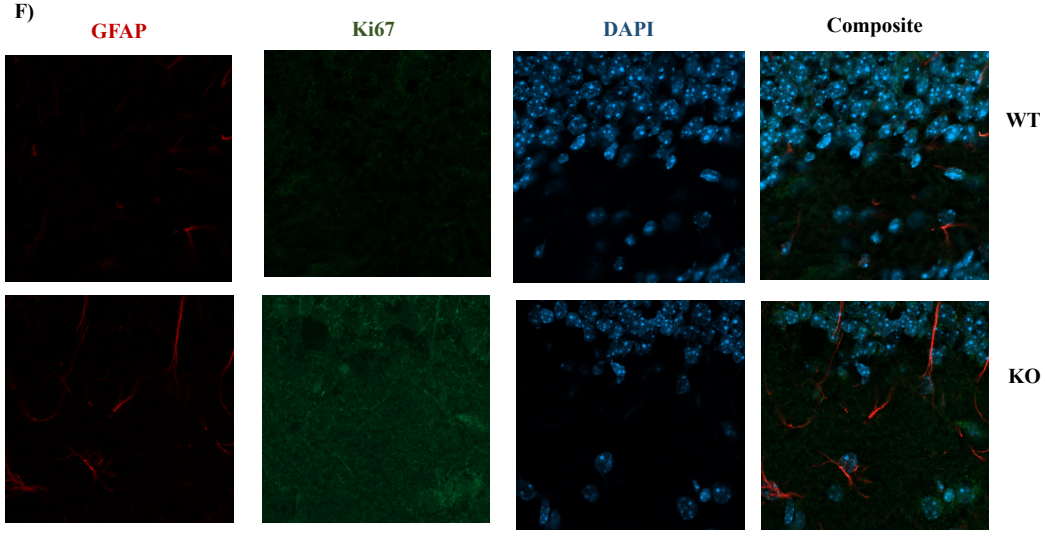
WT
HET
KO

E)


WT
KO
DAPI
GFAP
Ki67
DAPI
GFAP
Ki67
**
% of positive Ki67
0
10
20
30
40
WT
KO

F)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Ki67
DAPI
Composite
WT
KO

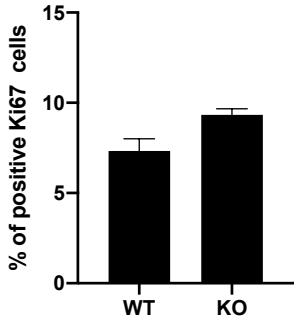


Figure 2.6: Multiple neuro-pathological alterations associated with HBP1 loss. A) Representative confocal images (63X) for GFAP expression (Red) and Dapi (Blue) in the different HBP1 genotype (WT, HET and KO). Data are represented as mean ± SEM. * $P < 0.05$, **$P < 0.01$, *** $P < 0.001$ by student t-test using Prism 9.0**. B)** Quantitative RT-PCR of astrocytic genes GFAP, s100B and GS were and presented as fold change for (WT, HET and KO). **C)** Representative confocal images (20X) Neun expression(red) and Dapi (blue) for the different HBP1 genotype (WT, HET and KO). (n = 3–5 mice for each group). Data are represented as mean ± SEM. * $P < 0.05$, **$P < 0.01$, *** $P < 0.001$ by student t-test using Prism 9.0 (Graphpad). **D)** Gene expression levels for neuronal marker Nestin and PAG enzyme and CD133 for all HBP1 genotypes. **E)** A representative confocal images (20X) for GFAP expression(Green) and Ki67 (Red) and Dapi (blue) for WT and KO mice for 7-8 weeks old mice. **F)** A representative confocal image (63X) for GFAP (Red), Ki67 (Green) and Dapi (Blue) for WT and KO for 4 weeks old mice. Data are represented as mean ± SEM. * $P < 0.05$, **$P < 0.01$, *** $P < 0.001$ by student t-test using Prism 9.0 (n = 3–5 mice for each group).

2.2.5 A Broad Molecular Rearrangement in GABA/Glutamate Receptor Function and Metabolism in the HBP$^{-/-}$ mice.

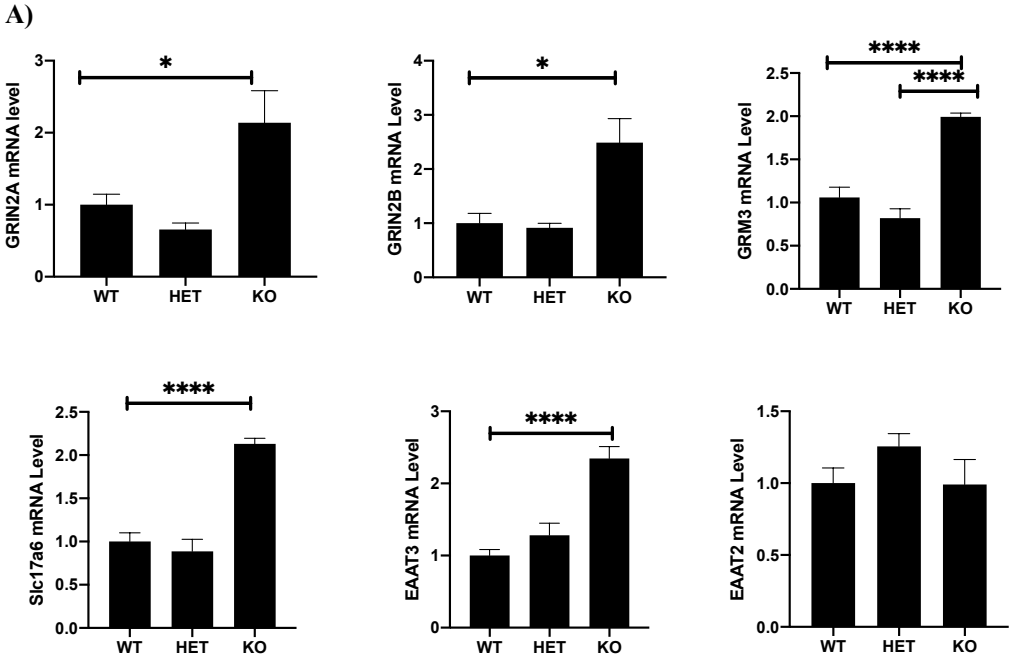
Previously, Alqurashi et.al. demonstrated an increase in astrocytic Warburg glycolysis in both pilocarpine-induced epileptogenesis as well as HBP1$^{-/-}$ mice with susceptibility to seizure induction. This was beautifully exemplified by staining with the Warburg glycolytic enzyme PKM2 (Alqurashi, et.al.2021). I extended these observations to glutamine-glutamate homeostasis (Figure 2.7). Because glutamate functions as an excitatory neurotransmitter, an energy source, a nitrogen source, and the metabolic precursor for the inhibitory neurotransmitter GABA (Schousboe, Bak, & Waagepetersen, 2013), we hypothesized the metabolic shift due to high Wnt signaling in HBP1$^{-/-}$ mice could have an impact on the expression of glutamate and GABA receptors. This suggestion was bolstered by the results of the RNA-seq

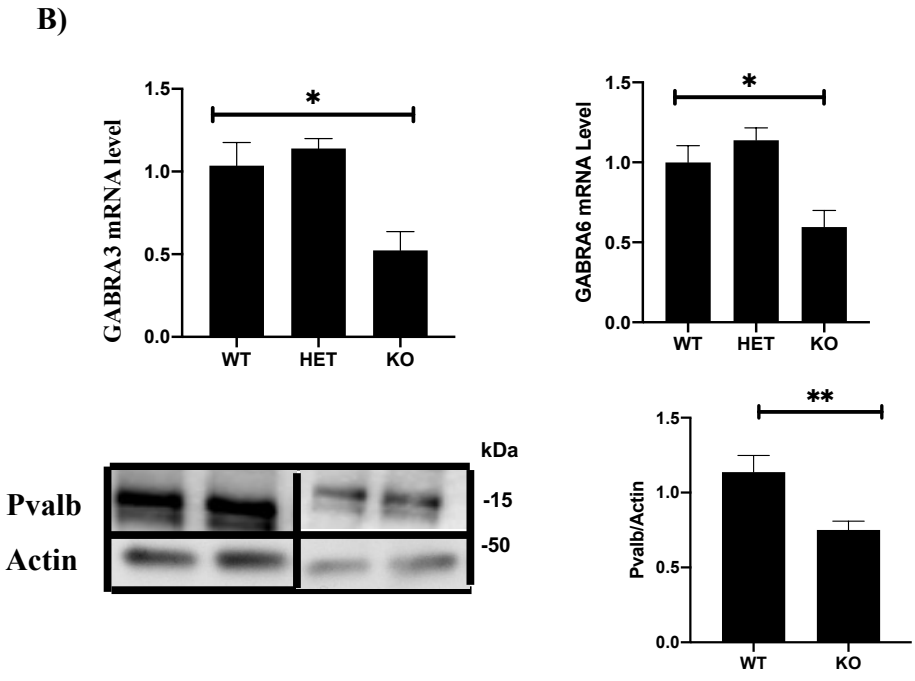
analysis (Table 2.2), which clearly showed multiple alterations in different canonical pathways. Changes included upregulation in GRIN2A and GRIN2B, which are both part of ionotropic glutamate receptors, which are ligand-gated ion channels that mediate excitatory synaptic transmission throughout the central nervous system. The RNA-seq results were confirmed by qRT-PCR analysis (Figure 2.7 A). Interestingly, gain of function mutations in GRIN2A and GRIN2B have been reported in patients with epilepsy (Gao et al., 2017). Metabotropic glutamate receptors (mGLUR) are G protein-coupled glutamate receptors and are comprised of different types. Additionally, disruption of mGluR gene expression can lead to reduced synaptic plasticity, augmented glutamate concentrations, and overexcitation in epilepsy (Celli et al., 2019). Furthermore, a study by Aronica et al. found upregulation of mGluR3 (GRM3) expression in astrocytes of rats in a model of TLE (Aronica et al., 2000). GRM3, was significantly upregulated in RNA seq and qRT-PCR analysis confirmed the increase (Figure 2.7 A).

In the brain there are several glutamate transporters and each is expressed in specific cell types (Alcoreza et al., 2021). Glutamate transporters are thought to be crucial for preventing accumulation of neurotoxic levels of extracellular glutamate. These transporters were also verified by qRT-PCR to identify any alteration in their expression in the HBP1$^{-/-}$ mice. Excitatory amino acid transporter 2 (EAAT2), which is a major glutamate transporter expressed mainly in the astroglia cells showed no significant change (Figure 2.7 A). However, excitatory amino acid transporter 3 (EAAT3) which is mainly expressed by the neuronal cells was significantly upregulated in the knockout mice (Figure 2.7 A). The SLC17 gene family consists of nine members (Reimer, 2013). Slc17a6 are predominantly expressed in glutamatergic neurons of the central nervous system (CNS). The upregulation of SLC17a6

(vGLUT2) immunoreactivities, was described by Kang et al., and others (Kang et al., 2005; Harrington et al., 2007; Touret et al., 2007), may all be causally or consequently related to the hyperexcitability and/or presence of seizures in animal models. Additionally, SLC17a6, which plays a role in excitatory neurotransmission, was significantly upregulated in HBP1 KO compared to the WT and HET hippocampus (Figure 2.7 A). Collectively, these data suggest that glutamate homeostasis is being altered by HBP1 gene loss.

Finally, the RNA-seq analysis suggested several GABA receptors were downregulated in the knockout mice, and mRNA measurements confirmed these data for Gabra3 and Gabra6 receptors (Figure2.7 B). Interestingly, the protein expression for parvalbumin, which is expressed by inhibitory interneurons, was significantly downregulated in the $HBP1^{-/-}$ mice in the DG (Figure 2.7 B). These data were confirmed by immunofluorescence staining for parvalbumin in HBP1 WT and KO DG of the hippocampus (Figure 2.7C). Reduced parvalbumin protein expression has been observed in other animal models of TLE (Hussain et al, 2019), suggesting again that $HBP1^{-/-}$ mice are consistent with a seizure phenotype. Collectively, RNA seq and expression analysis for several GABA and glutamate receptors in $HBP1^{-/-}$ mice points to a potential imbalance in the process of excitation and inhibition. These changes may contribute to the spontaneous seizures and increased seizure susceptibility.


A)
GRIN2A mRNA level
GRIN2B mRNA Level
GRM3 mRNA Level
Slc17a6 mRNA Level
EAAT3 mRNA Level
EAAT2 mRNA Level
WT
HET
KO
*
****


B)
GABRA3 mRNA level
GABRA6 mRNA Level
Pvalb
Actin
kDa
-15
-50
Pvalb/Actin
WT
HET
KO
*
**

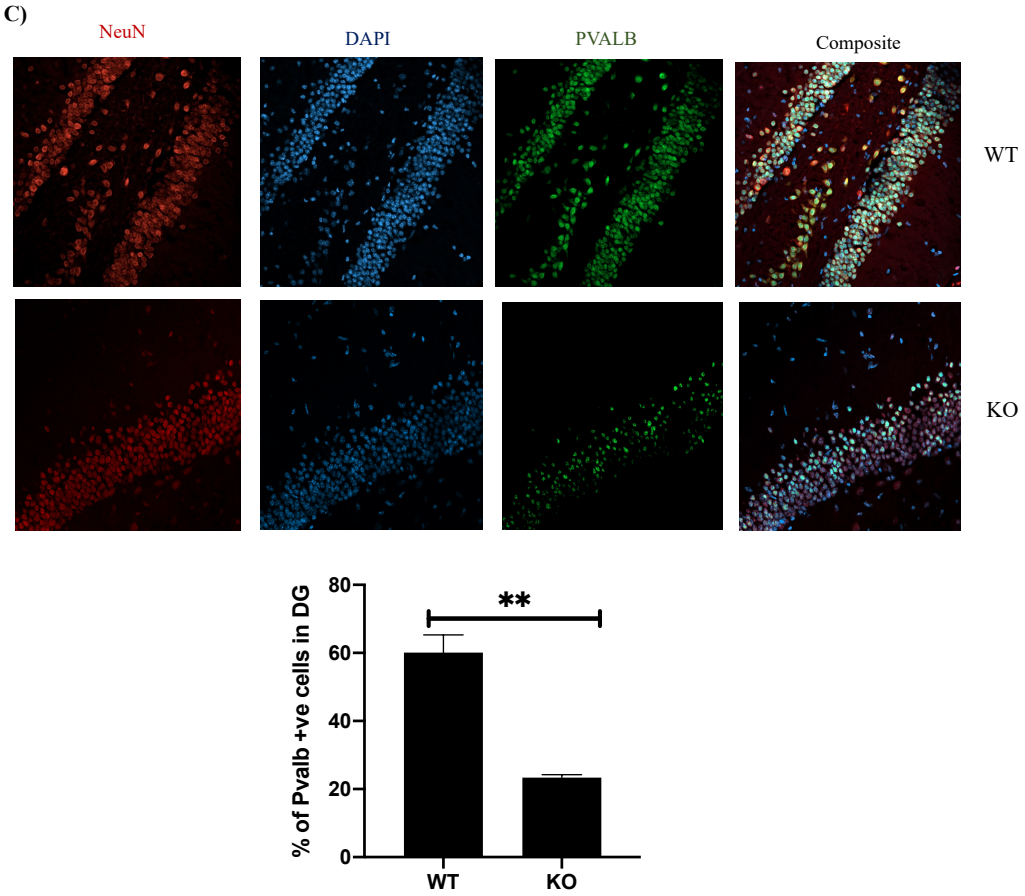


Figure 2.7: A broad molecular rearrangement in GABA/glutamate receptor function and metabolism. A) Glutamate receptor gene expression was conducted using qRT-PCR and it showed GRIN2A , GRIN2B and GRM3, Slc17a6 and EAAT3 were all significantly upregulated in the HBP1$^{-/-}$ mice**. B)** The gene expression of Gabra3, Gabra6 were significantly downregulated in the HBP1 KO mice. In addition, the protein expression showed a significant downregulation in the inhibitory interneuron parvalbumin in the KO mice. **C)** Immunofluorescence of Parvalbumin in the DG declined in the HBP1 KO mice. Data are represented as mean ± SEM. * P < 0.05, **P < 0.01, *** P < 0.001 by student t- test using Prism 9.0 .

2.2.6 Schematic Representation for The Molecular And Cellular Alterations Associated With High Wnt Signaling In The Brain.

In this figure we summarized the cellular and molecular alterations, which were identified in the HBP1$^{-/-}$ mice. In this study we investigated the molecular alterations in the hippocampus of mouse model of high Wnt signaling. Several studies confirmed the upregulation of Wnt signaling in epileptic brain. However, the consequences of high Wnt signaling at cellular and molecular level in epilepsy is understudied. In our study, we elaborated on the molecular mechanism for the

contribution of Wnt signaling in epileptic brain which can be used to evaluate multiple therapeutic interventions. Previous work published by Yee lab has confirmed the upregulation of Wnt and mTOR signaling during epileptogenesis and illustrated the metabolic and cellular consequences for that. In conclusion, HBP1$^{-/-}$ mice share similar neuro-pathological findings when compared to TLE mouse model (Alqurashi et al., 2021).

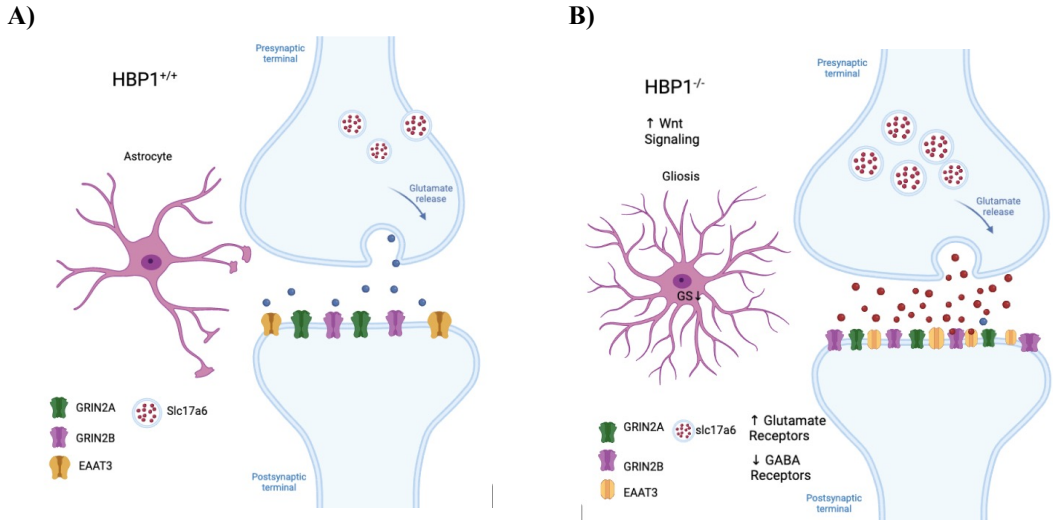


Figure 2.8: Schematic representation for the molecular and cellular alterations associated with high Wnt signaling in the brain. A) Represent the molecular findings for the HBP1$^{+/+}$ mice. B) Summarize the cellular and molecular changes in the HBP$^{-/-}$ mice. This figure was created using Biorender.

2.3 Discussion

2.3.1 Summary of the Results

The Wnt/β-catenin signaling pathway may play a role in dysfunctional neurogenesis and underlying changes in the hippocampus, these molecular changes alter the hippocampus in case of epilepsy. Several molecular components in the canonical Wnt signaling pathway, such as Dkk-1, GSK-3b, and β-catenin, serve as potential targets for regulating Wnt signaling. However, the specific mechanisms of canonical Wnt signaling in epilepsy is not fully understood. Thus, further studies of Wnt signaling and its dysfunction in epileptic brain are needed. Further, drugs that

modulate Wnt activity should be considered as therapeutic candidates for epilepsy. Moreover, understanding the detailed molecular and metabolic alterations in the HBP1$^{-/-}$ mice will provide us with enough knowledge to study the involvement of Wnt activity in seizure susceptibility and potentially characterize HBP1 gene as susceptibility gene for seizure development. In this project, we illuminate a mechanism by which high Wnt signaling triggers a complex metabolic reprogramming with downstream consequences which include mTOR activation and change in the GABA/Glutamate neurotransmitter balance. This complicated molecular mechanism suggests possible new therapies to avoid the development of recurrent seizures. In the HBP1$^{-/-}$ mouse model, we documented an increase in Wnt and mTOR signaling in the hippocampus. Furthermore, a gene dosage effect of HBP1 loss was linked to the level of mTOR and Wnt signaling expressed in the hippocampus. The mechanism for an induction of Wnt signaling is either a decrease or complete loss of Wnt pathway inhibitor (HBP1 and sFRP1) (Figure 2.4 A and 2.4 E). The increased Wnt signaling in the HBP1$^{-/-}$ mouse was confirmed by β-catenin induction (Figure 2.4 A). Several studies have linked Warburg metabolism with the induction of Wnt signaling especially in cancer. The tumor cells utilize the increased glucose to sustain their proliferation. Further, HBP1$^{-/-}$ mice represent a model of high Wnt signaling. We investigated the metabolic alterations in the hippocampus of HBP1$^{-/-}$ mice. PDK4, is a Wnt target gene was activated in the knockout mice. The upregulation in the PDK4 gene expression has metabolic consequences. It included the change in the phosphorylation status of PDH provided evidence to the significant contribution of altered metabolism and the suggestive predisposed machinery which may contribute to the seizure development. Furthermore, confocal microscopy demonstrated that the Warburg re-programming in the hippocampus of HBP1$^{-/-}$ mice

is split between neurons and astrocytes (Figure 2.5D). PKM2, the Warburg isoform of pyruvate kinase, was induced exclusively in astrocytes (Figure 2.5 B), suggesting that astrocytes are playing a crucial role in the aerobic glycolysis. On the other hand, the majority of neurons has alteration in the phosphorylation status of PDH^{S293} via PDK4, while small fraction of astrocytes showed the same result (Figure 2.5 D). Collectively, the previous results suggest that there is an increase in lactate production from astrocytes, and that lactate is being utilized by the neurons to meet its energetic need. This hypothesis was discussed previously in multiple studies (Yellen et al., 2018). Thus, metabolic reprogramming was confirmed in the $HBP1^{-/-}$ mice which suggests this influences the cellular changes and the seizure susceptibility observed.

The pathophysiological mechanism of epilepsy has been linked to either reduced GABA-inhibitory postsynaptic potential or activation of postsynaptic glutamate receptors in the brain (Engelborghs et al., 2000; Stafstrom and Carmant, 2015). Glial fibrillary acidic protein (GFAP) is an inter- mediate filament protein expressed in astrocytes and its expression level could be affected by neuronal injury (Cotter et al., 2001; Diaz- Arrastia et al., 2014). Reactive gliosis is a prominent morphological feature of TLE can be demonstrated by the expression of GFAP (do Nascimento et al., 2012). Experimental studies have confirmed up-regulation of GFAP expression in hippocampus in different neurological diseases including epilepsy (Glushakova et al., 2012). Furthermore, GFAP was significant upregulated in the $HBP1^{-/-}$ mice (Figure 2.6 B) and that increase was noticed starting at 4 weeks of age (data not shown). In epilepsy, the increased expression of the excitatory glutamate neurotransmitter leads to seizure activity by binding on glutamate receptor in the brain (Aronica et al., 1997).

Metabotropic glutamate receptor (GRM3) in hippocampal astrocytes has been reported to be altered in a rat model of epilepsy (Aronica et al., 2000). In addition, GRM3 was upregulated in pilocarpine rat model of epilepsy with a history of prolonged febrile seizure (Alese & Mabandla, 2019). Further, the same receptors were significantly increased in HBP1$^{-/-}$ mice (Figure 2.7 A). Additionally, other glutamate receptors genes were upregulated in the knockout mice (Figure 2.7 A). Moreover, GABA receptors were also affected in the HBP1$^{-/-}$ mice. Multiple GABA receptors have reduced expression, first identified first by RNAseq and later validated by qRT-PCR (Figure 2.7 B). The imbalance in the inhibitory/excitatory receptors and transporters is a potential mechanism for decreased seizure threshold and the increased seizure susceptibility in the HBP1 knockout mice.

2.3.2 The Pathological Consequence of High Wnt Signaling in the Brain.

The Wnt signaling pathway is well conserved among species and it is critical for embryonic and central nervous system (CNS) development. Several studies have shown that canonical Wnt/β-catenin activity is essential for processes involved in early brain development, including cell migration, cell polarity, apoptosis, synapse development, and plasticity (Brault et al., 2001, Mulligan and Cheyette, 2012). Mutations resulting in elevated Wnt signaling have been linked to developmental defects and overall brain malformation (Brault et al., 2001).

The Yee lab established a new molecular mechanism which target cellular, molecular and metabolic alterations in the HBP1$^{-/-}$ mouse model. This mechanism will provide the opportunity for various therapeutic interventions and consequently reduce the seizure susceptibility and probably reduce or reverse some of the neuro-pathological changes in the hippocampus. Among other pathways which were activated in the knockout mice is the mTOR pathway. Genetically, mTOR activation

was associated with multiple forms of epilepsy (Pun et al., 2012). Our study suggests that high Wnt signaling initiates metabolic alterations that may contribute to neuro-pathological features associated with epileptic brain.

HBP1 deficient mice showed a neuro-pathological alterations associated with epileptic brain. For instance, reactive gliosis, high expression of glutamate receptors and the reduction in multiple GABA receptors were all confirmed in the HBP1$^{-/-}$ mice. However, it is unknown if these finding are solely due to high Wnt signaling or if mTOR activation is contributing to these pathogenic alterations. Further work is required to identify the effect of the activation of each signaling pathway and profile the changes for each signaling pathway independently.

In adult brain, Wnt/β-catenin is restricted to astrocyte-like neural stem cells in the subventricular zone and the dentate gyrus of hippocampus (Faigle and Song, 2013). Several studies have found an increased Wnt signaling to be associated with increased neurogenesis and neuronal death commonly observed after seizures (Madsen et al., 2003, Yang et al., 2016). Furthermore, a study conducted by Yang et al, confirmed the upregulation of Wnt/B-catenin signaling in reactive astrocytes after ischemia (Yang et al., 2016). Aquaporins (AQPs) are a group of selective transmembrane channels which are expressed in every major organ group in the human body (Verkman, Anderson, & Papadopoulos, 2014). Aquaporin-4 (AQP4) is the main water channel protein expressed in the central nervous system (CNS). AQP4 is heavily expressed in astrocyte end-feet and is an important factor in CNS water and potassium homeostasis (Cheng et al., 2020). Changes in AQP4 activity and expression have been implicated in several CNS disorders, including epilepsy (Devinsky et al., 2013). Moreover, the RNA-seq of the HBP1$^{-/-}$ showed the

upregulation of AQP4 (Table 2.3). All of these molecular findings suggest astrocyte function might be influenced by high Wnt signaling.

A study conducted by Theilhaber et al, demonstrated the alterations in the hippocampal gene transcription profile one week following an initial series of seizures induced by mild hypoxia at post-natal day 10 (P10). The hypoxic seizure response was found to be enriched for PI3K/mTOR and Wnt signaling pathways (Theilhaber et al., 2013). Further, glutamatergic, synaptic and axonal processes were also enriched, possibly regulated as a downstream consequence of activation of these pathways (Theilhaber et al., 2013).

WW domain containing oxidoreductase (WWOX) was identified as Wnt suppressor gene. Homozygous deletion of WWOX exons has been found in different cancer cell lines (Paige et al., 2001), and hypermethylation of the WWOX promoter has been detected in both cancer cell lines and primary tumors (Kuroki et al., 2004). Furthermore, the association of WW domain-containing oxidoreductase WWOX gene loss of function with central nervous system (CNS) related pathologies is well documented (Chang et al., 2014). WWOX-related epileptic encephalopathy was reported in familial cases of WWOX homozygous loss of function (Mignot et al., 2015). Furthermore, Hussain et al investigated the neuro-pathological alterations associated with WWOX gene loss in an animal model. A reduction in GABAergic interneurons parvalbumin and astrogliosis was identified in the WWOX deficient mice (Hussain et al., 2019). Interestingly, these findings are similar to the HBP1$^{-/-}$ mice. The resemblance of these findings suggests that Wnt signaling influences the expression of GABAergic receptors and it may have an impact on astrocyte function.

To conclude, HBP1 deficient mice represent a preclinical model for elevated Wnt signaling. Moreover, it provides the opportunity to study multiple therapeutic

interventions. Due to the variety of physiological functions performed by Wnt signaling, its disruption will have multiple pathological consequences. For instance, the Warburg metabolism which was identified in the hippocampus of HBP1 deficient mice might be contributing to the pathogenesis and could possibly be targeted by ketogenetic diet. Furthermore, mTOR inhibitors could also be used and the molecular alterations can be re-evaluated after the intervention.

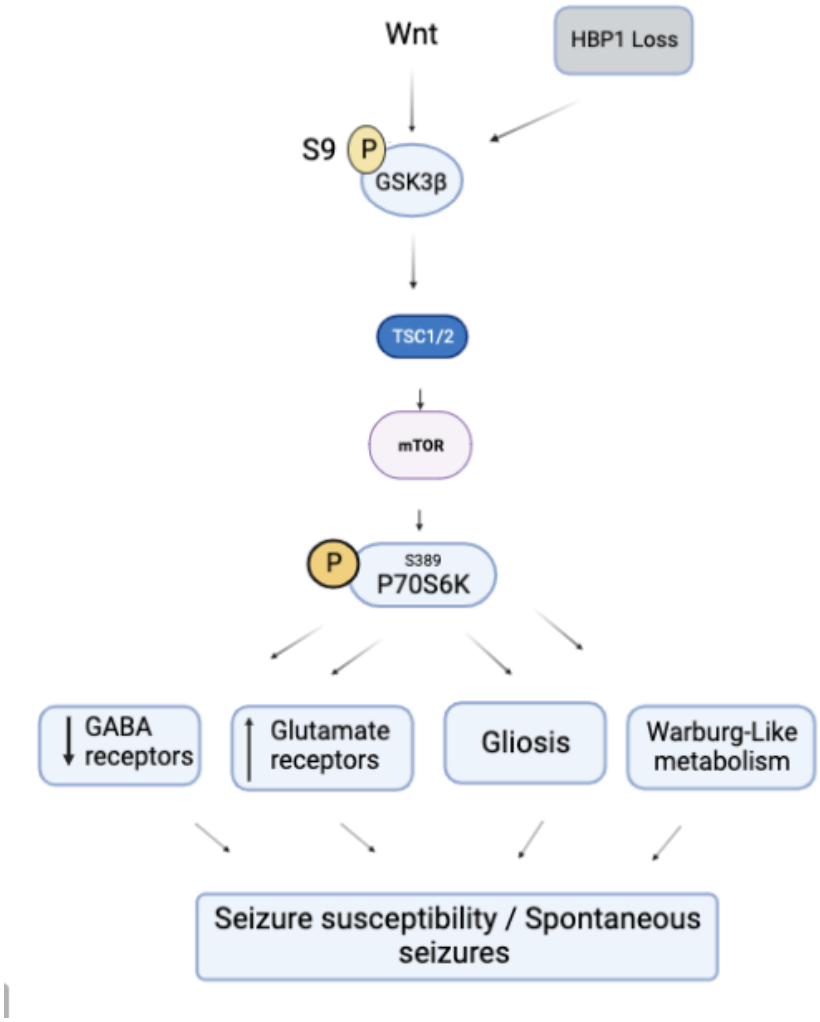


Figure 2.9: Summary for the functional connection between Wnt sand mTOR signaling and the neuro-pathological alterations associated with their activation. This figure was created using Biorender.

2.4 Materials and methods

2.4.1 Status Epilepticus Induction and Seizure Grading

All animal experiments were carried out in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at Tufts University (Boston, MA). 7-week old male FVBN (The Jackson laboratory) mice were injected intraperitoneally with injection of pilocarpine in the FVBN strain and kainate in the C57 strain. Pilocarpine initially was injected at150 mg/kg, then at 135 mg/kg to minimize the death rate. Kainate was injected at 30 mg/kg (Milestone PharmTech). Control mice were injected with PBS in order to obtain baseline levels of brain biochemistry.

SE induced mice were graded for seizure activity until seizure activity had subsided, which corresponds to roughly three hours. Seizures were graded from 1-5 based on the Racine scale (Racine 1972). Seizures of a grade 3 or higher could be reliably assessed by eye, so these were recorded. Grade 3 seizures are equivalent to rearing, grade 4 equivalent to rearing and falling, and grade 5 equivalent to convulsions while on its side and unable to right itself. These are roughly based on human seizure intensities with a five equating to a grand mal seizure. Survival curves and their significance values were determined using the software PRISM and its associated statistical programs. Seizure frequency analysis was performed using SPSS (IBM). After induction or control injection, mice were maintained for 5 days, and then were sacrificed using cervical dislocation. Three samples were taken from each half of the brain, frozen on dry ice, and finally stored at -80° C. until taken out for analysis. These samples were a piece of the frontal cortex, a piece of the cerebellum, and the entire hippocampus.

2.4.2 EEG Recording

EEG was recorded at 1000 Hz over a two week period with two EEG channels and one EMG channel through mounted cortical electrodes. Video feed was also taken over this period. Currently, 3 WT and 3 KO mice have been analyzed. Manual curation of the data was performed to look for seizure events in both the EEG data and video feed. Power analysis was performed to find the frequency makeup of the wave function. Lab Chart (ADInstruments) was used for both the scanning and analysis of data.

2.4.3Total RNA Extraction and cDNA Synthesis

For in vitro experiment: cells were harvested for total RNA extraction after transfection. For in vivo experiment: Hippocampal samples was used for total RNA isolation. RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen) and was eluted with Rnase-free water. Nanodrop spectrophotometer (Thermo Scientific) was used to measure RNA concentration by measuring absorbance at 260nm. After determining RNA concentration, 500 μg of RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad).

2.4.4 Quantitative RT-PCR

The primers used here are detailed in the Supplemental Material. Total hippocampal RNA was extracted using RNAeasy Mini kits (Qiagen) and the reverse transcribed with random hexamer mix (iScript, Bio-Rad). The resulting cDNA was used for real-time qPCR performed using an iCycler (BioRad). SYBR-Green master mix was purchased from Bio-Rad. All quantifications were normalized to an endogenous 18 S RNA control. The relative quantitative value for each target gene compared to the calibrator for the target is expressed as comparative Ct (2-(DCt-Cc)) method (Ct and Cc are the mean threshold cycle differences after normalizing to 18S).

Quantitative RT-PCR experiments were performed in triplicate. PCR efficiency with given primers was between 95 to 105%.

2.4.5 Western Blotting

Whole tissue lysates were prepared from frozen tissue by extraction for 30 min at 40C in lysis buffer (50mM HEPES, pH7.5, 150 mM NaCl, 1.5mM MgCl2, 1MM EDTA, 10% glycine, 1% Triton X-100, 20mM β-glycerophosphate, 0.1 mM sodium vanadate, 0.1% SDS, 1% deoxycholate, 1 mg/ml leupeptin, 200 mM PMSF, 1 mg/ml pepstatin). All lysates were clarified by centrifugation for 15 min at 14000 rpm and frozen at –800C. The supernatants were quantified by using Quant-It Assay protocol from Thermo Scientific. 30 μg of total protein samples in each group were subjected to SDS-PAGE (Bio-RadMini-PROTEAN TGX gel), then transferred to PVDF membrane. The membranes were incubated with primary antibodies overnight at 4ºC and secondary antibody for 1 hour at room temperature. The antibodies used were provided in Supplemental Material. The western blot results were developed with Thermo Fisher Supersignal West Femto Chemiluminescent substrate and quantitated by the Bio-Rad Chemidoc MP Imaging System.

2.4.6 Immunofluorescence Staining

7-week old male C57BL/6 or FVBN wild type mice were sacrificed after 5-days following pilocarpine injection and induced status epilepticus (see above). The mice were anesthetized with isoflurane 4% and perfused with cold 4% PFA (pH 7.4). The brains were surgically removed and fixed in cold 4% PFA for at least four hours or overnight at 4ºC. The fixed brains were cryoprotected in 30% Sucrose overnight at 4ºC. Hippocampal coronal sections (12 μm) were cut using a Leica Biosystem cryostat. The coronal sections were stored at -20oC for later immunostaining. Before immunostaining, the sections were pretreated with 3% hydrogen peroxide in methanol

for 5 min and washed with PBS for another 5 min. Thereafter, the coronal sections were incubated with primary antibodies 1:100, as listed in Supplemental Material, diluted in 10% donkey serum (Abcam), 5% Non-fat dry milk, 4% BSA (Thermo Fisher), and 0.1% Trion X-100 (Sigma Aldrich) overnight at 4ºC in moisturized chamber. The next day, the sections were washed with PBS for 5 minutes three times and incubated with secondary antibody 1:150; as listed in Supplemental Material, diluted in donkey blocking buffer at room temperature for one hour. Afterwards, the sections washed three times with PBS for 5 minutes and then mounted with flourished mounted DAPI (Abcam) for confocal imaging.

2.4.7 Confocal Imaging

Stained sections were imaged on a LSM800 laser scanning confocal microscope (ZEN 2.3-blue edition) in multi-tracking mode. The images were obtained with optimal pinhole a 63X, 1.4 NA, plan-apochromat oil immersion objective, 0.8 micron per optical sections, at a scan speed of 3, averaging of 4 with an overall pixel dwell time of ~12 µsec. All image processing and analysis was performed in Fiji. In all images, only balance, contrast, and evenness of the illumination were adjusted. Final immunolabeling quantification results were reported as percentage of positive cells (neuronal or astrocytes)/ total cells (neuronal or astrocytes). A minimum of 4 photographic fields were quantified per mouse (n = 3-4 mice for each group) using ImageJ.

2.4.8 Statistical Analysis

Statistical analysis was performed using Student's unpaired t-test. $P < 0.05$ was considered statistically significant. All western blot, qRT-PCR and cell quantification data were analyzed using GraphPad Prism version 9 softwar

2.5 Supplemental Materials:

2.5.1. Oligonucleotides

Table 2.4: Mouse Primers Sequences

Gene	Forward	Reverse
18s	GCCCGAAGCGTTTACTTTG	CTTAATCATGGCCTCAGTTCC
Axin-2	GAC AGT AGC GTA GAT GGA GTC C	CGG CTT TCC AGC TCC AGT TT
CD133	TGAATTAAGTGGAAGGAG CCCA	GGAAACTCAAAGGAAAT GGGT
EAAT2	GGGGAAGATGGGTGAACA GG	GAGAAAGATGCCCCCGT GAA
EAAT3	ATTGCTGTTGACTGGCTCC T	AAGGCAAAGGGGTTCAC GAT
FOXG1	GCT TTG AGC CAG TGA GGA TT	ATG GTTTGGTGG GGA GGTTG
GABRA 3	GGTCCTGAAGAGAGAAGT AAGCA	GCTAGTGGTTCCAGGGA GAA
GABRA 6	GATGGTCTTGCTTCT CCCCT	TTACAGCACCCCCAAATC CT
GAD67	TAG CCT GGA AGA GAA GAG TCG	GTT TGC TCC TCC CCG TTC TT
GFAP	ACCGCATCACCATTCCTGT A	AAGGGAGAGCTGGCAGG
GRIN2A	CAGTGTCCCCTCAAGCAA AC	GTTTGTAAGGGTCCGAG GGA
GRIN2B	CAAGCGAGATTCGGTCAG TG	GAAGTTGCCCCCAGACC TAT
GRM3	GGCCATTTCAGGGGCAAA AT	CGTCACTGAATTCCTCGC AC
GS	CAG GGT GAG AAA GTC CAA GC	CTC GAA ACA TGG CAA CAG GA
HBP1	GGA AGA CTT TGC TAG AGC CG	CAG TGA GCA AGC CAT CTT CT
IDH1	GTCGCTGTTACCGTATGTG T	TCCACCACAGAACCTCCT TG

Gene	Forward	Reverse
MCT1	CCTCATCATCGCGGGTATCT	ACTGTGCAGCTTTCATCGTC
NeuroD1	CAATCTTCTCTTCCGGTGCC	CTGTACGCACAGTGGATTCG
Nestin	GGGGAAGAATCTTTTCAGATGTGG	GCCAGCGCTGATCGAC
PAG	GGGTCAAGGAAGGACGGT TT	ATG GTG TCC AAA GTG TAGTGC TT
PDK4	AAAGATGCTCTGCGACCAGT	GGGTCAAGGAAGGACGGT TT
S100B	CATCCTTACACCTGCTTGCC	AAGGGTGTAGGCGATCAGTC
SLC17A6	ATGTTGTCTGCAGTCCCTCA	AGATGGCCACCCCTCTAGTA
SFRP1	ACT GGC CCG AGA TGC TCA AA	CAT CCT CAG TGC AAA CTC GCT
Wnt8b	CCGTGTGCGTTCTTCTAGTCA	TTCCAACGGTCCCAAGCA AA

Table 2.5: Antibodies used for Western blot and Immunofluorescence

Reagent	Source	Identifier
actin	Sigma-Aldrich	Cat. # A2228
Alexa 488 Donkey Anti-Goat	Jackson Immunoresearch	Cat. # 705-546-147
Alexa 488 Donkey Anti-Mouse	Jackson Immunoresearch	Cat. # 715-586-150
Alexa 488 Donkey Anti-Rabbit	Jackson Immunoresearch	Cat. # 711-546-152
Alexa 594 Donkey Anti-Goat	Jackson Immunoresearch	Cat. # 705-586-147
Alexa 594 Donkey Anti-Mouse	Jackson Immunoresearch	Cat. # 715-546-150
Alexa 594 Donkey Anti-Rabbit	Jackson Immunoresearch	Cat. # 711-586-152
beta-catenin	Millipore	Cat. # 06-734
DAPI	Abcam	Cat. # ab104139
GFAP	Abcam	Cat. #

		ab53554
NeuN	Millipore	Cat. # mab377
pGSK	Cell Signaling	Cat. # 9323S
pP70S6K (IF staining)	Millipore	Cat. # MABS82
pP70S6K (Western Blot)	Cell Signaling	Cat. # 9234S
TP70S6K (Western Blot)	Cell Signaling	Cat. # 2708S
pPDH	Abcam	Cat. # ab92696
TPDH	Abcam	Cat. # ab168379
PKM2	Cell signaling	Cat. # 4053S

2.6 Authors Contribution

EP & TM Performed Figure 2.1 and 2.2 and 2.3. SA, RA and TM performed Figure 2.4 A. RA participated in Figure 2.4(B) and 2.5(A). EP conducted the bioinformatic analysis table 2.1. SA performed western blots and qRT-PCR experiments and analyzed data for Figures 2.4, 2.5, 2.6, 2.7 and performed all the immunostaining experiments and confocal microscopy.

Chapter 3: HBP1 in Genetic and Idiopathic Epilepsy

Alrubiaan, S: Malone, T; Batrosky, R; Paulson, E; Yee, A. To be submitted to *PLOSONE.*

3.1 Introduction

A set of debilitating illnesses known as severe epilepsies of infancy and childhood are characterized by recurrent epileptic seizures linked to developmental delay or regression and they are defined as the developmental and epileptic encephalopathies (DEEs) (Berg et al., 2010). These conditions cover a broad range of diseases known as epileptic encephalopathies, in which the infant or child frequently experiences multiple seizure types, exhibits significant epileptiform activity on the EEG, and may experience developmental slowing or regression after a seizure begins or worsens. Epileptic encephalopathies may start out with normal or delayed development in the background.

Comorbidities in epilepsy are frequent, and the results are frequently negative. Examples include autism spectrum disorder and behavioral issues. One in 2,000 infants had severe epilepsies that start before the age of 18 months, according to a study on a larger cohort of these cases (Banerjee, Filippi, & Allen Hauser, 2009). Infantile spasms, Dravet, Lennox-Gastaut, and West syndromes are a few of the DEEs that have been significantly investigated (McTague, Howell, Cross, Kurian, & Scheffer, 2016).

Epilepsy development is also linked to genetic risk factors. For example, the risk is multiplied by two to four in first-degree relatives of those who have epilepsy of unknown etiology (formerly known as idiopathic generalized epilepsies (IGEs), (Annegers et al., 1982; Ottman et al., 1996 a,b). Furthermore, human genetics and genomics research over the past ten years has advanced with the development of new technologies such as next-generation sequencing (NGS), resulting in a surge in the identification of genes linked with various human diseases. Over 50 additional genes associated with epilepsy have been identified in the last three years, which emphasize

the importance of NGS in identifying the susceptibility genes for complex diseases such as epilepsy (Bamshad, Nickerson, & Chong, 2019). For example, the Epi4K study included families with two or more affected individuals for whole genome sequencing (WGS) to detect genomic variation that affects the risk for common types of epilepsy. Several mutations were identified through Epi4K and were clinically and statistically associated with epilepsy including GABRB3 (Epi et al., 2013).

In addition, one of the most studied genetic mutations associated with epilepsy is tuberous sclerosis complex (TSC) mutations. TSC is a multi-system disorder caused by inactivating mutations in either TSC1 or TSC2, resulting in constitutive activation of the mTOR signaling pathway (Salussolia, Klonowska, Kwiatkowski, & Sahin, 2019). About 80% of TSC newborns experience focal seizures and infantile spasms, making TSC a major contributor to severe, drug-resistant epilepsy (Overwater et al., 2015). Autism and other neurodevelopmental comorbidities, such as intellectual disability, are also frequent in TSC patients (Overwater et al., 2015). Additionally, the Yee lab has shown that altered phosphorylation of TSC2 and concomitant activation of mTOR signaling occurs in the pilocarpine model of epileptogenesis, driven by Wnt/β-catenin signaling (Alquarshi et al., 2021).

A non-hypothesis driven strategy to finding novel disease genes and a better understanding of the overall contribution of ultra-rare genetic variation to disease are provided by exome sequencing of large cohorts followed by genome-wide association analysis (Berkovic et al., 2013). Several genetic mutations have been identified as a direct cause for epilepsy via GWAS studies. The contribution which was made by the availability of the sequencing data has helped in improving the therapeutic outcome for drug- resistant epileptic patients. Several genetic mutations have been linked to epilepsy in patients including TSC and PDH mutations (Prasad et al., 2011;

Overwater et al., 2015). Channelopathies are also well studied and characterized in epileptic patients. Deleterious variants have been identified in multiple ion gated channels including SCN1A which is characterized as a direct causative gene for Dravet syndrome (Lagae, 2021). However, studying the involvement of different signaling pathways in the pathogenesis of epilepsy is crucial to identify mechanistic targets which can be used for therapeutic intervention in epilepsy.

Wnt/β-catenin signaling is crucial for controlling neurogenesis, neuronal differentiation, synapse development, and plasticity in the central nervous system (Liebner et al., 2008). Previously, Wnt signaling has been known as important regulator of neurological development (Qu et al., 2017). Recently, several studies have been published showing the involvement of Wnt signaling in neurophysiology and neuropathology (Alquarshi et al., 2021; Qu et al., 2017). An example for the involvement of Wnt activation in epilepsy is a mutation of Forkhead box G1 (FOXG1), a Wnt transcription suppressor, which normally plays a role in forebrain and telencephalon development (Danesin et al., 2009; Cargnin et al., 2018). Furthermore, the telencephalon is involved in the coordination of multiple neurological functions including learning and cognition. FOXG1 mutations are linked to different types of epilepsies including Lennox-Gastaut syndrome, and infantile spasms (Paciorkowski, Thio, & Dobyns, 2011; Terrone et al., 2014).

In this thesis, we investigated the clinical relevancy of HBP1 mutations with seizure development/epilepsy. HBP1 is a repressor of Wnt signaling by binding to and forming a heterodimer with TCF, inhibiting the transcription of Wnt target genes. Many studies have shown a role for HBP1 in maintaining cell differentiation, including in cortex development (Bollaert, de Rocca Serra, & Demoulin, 2019; Kim et al., 2006; Sampson et al., 2001).

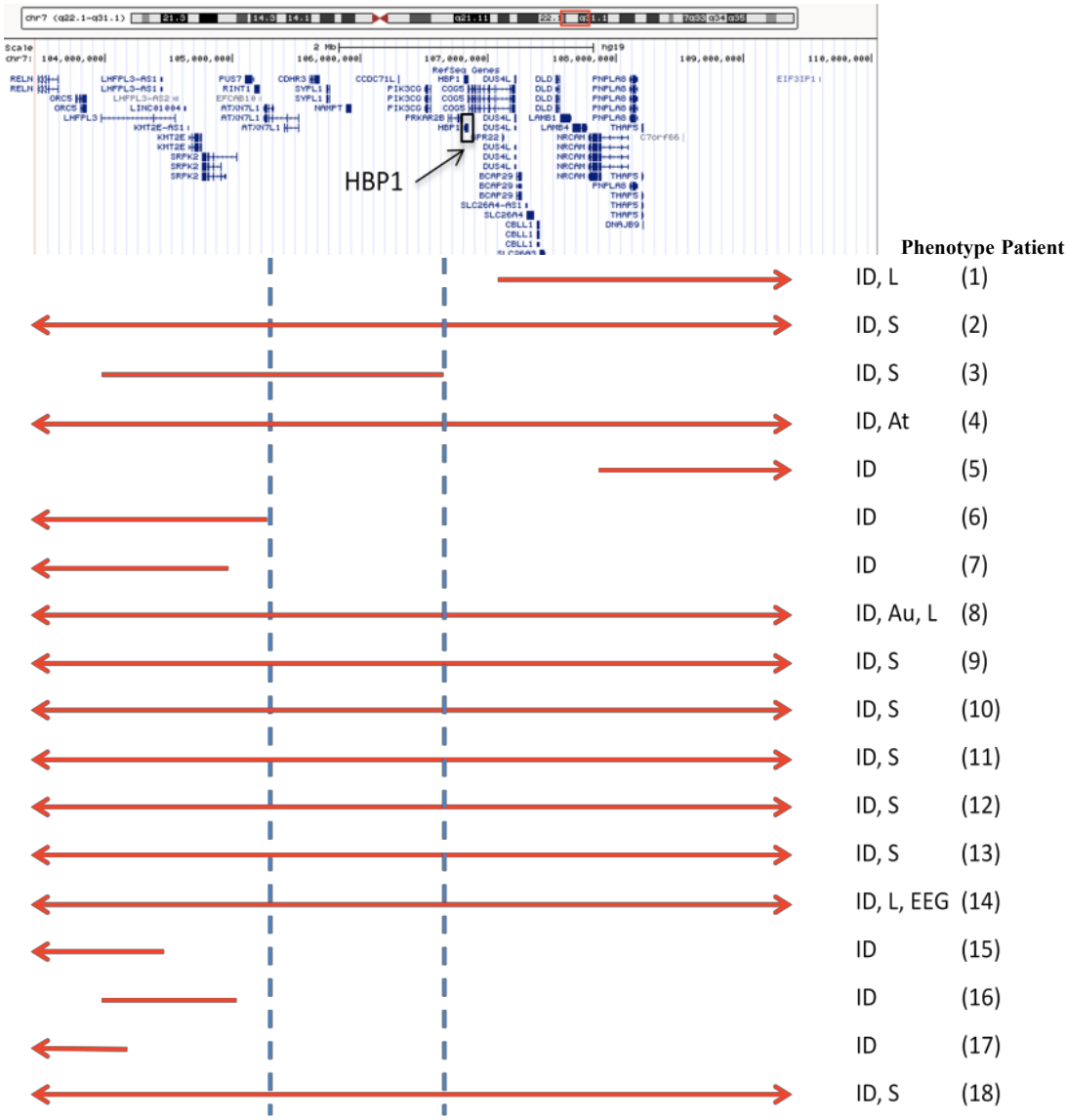
Previous data from our lab has confirmed the existence sporadic seizures and severe sensitivity to seizure induction through neurotransmitter activation in a genetic knockout mouse model of HBP1 (Alquarshi et al., 2021). Finally, several clinical cases deletion of the HBP1 genomic region at 7q22.3 is associated with seizures in pediatric patients (i.e. Uliana et al., 2010). In the present study, we investigated the HBP1 variants identified from an epilepsy specific database (Epi4K). In attempt to further understand the potential impact of HBP1 variants in the pathogenesis of epilepsy.

3.2 Results

3.2.1 The Gene Block Containing HBP1 Is Positively Correlated with Increased Frequency of Seizure Development or EEG abnormality.

To identify the clinical relevancy of HBP1 deletion with the epilepsy phenotype, a literature search was conducted to identify studies that show correlation between HBP1 genomic region deletion and seizure development. In one study, patients were identified with a heterozygous deletion in long arm of chromosome 7 where the HBP1 gene is located among 15 genes. That deletion caused patients to develop intellectual disability, physical abnormality and seizures (Figure 3.1 A). This analysis showed a positive correlation between a region of 7q22.3 containing the HBP1 gene and an increased frequency for seizure development. Figure 3.1B shows that over 50 percent of patients with deletions within HBP1 region suffered from seizures or had an abnormal EEG, which is significantly higher than the 12% of patients with a deletion outside of the gene block who developed seizures or an abnormal EEG (Fisher's Exact Test, $p= 0.00216$). This was the first initiative to study HBP1 correlation to seizure development.

A



Phenotype

ID – Intellectual Disability Au – Autism At – Ataxia L – Language Disability
EEG – Abnormal EEG S – Seizure

Patient

1. DECIPHER 801
2. DECIPHER 253694
3. DECIPHER 4470, Uliana, 2010
4. DECIPHER 255298
5. DECIPHER 258652
6. DECIPHER 625
7. DECIPHER 4433
8. DECIPHER 800
9. DECIPHER 265003
10. Higginson, 1976
11. Franceshini, 1978
12. Abuelo, 1982
13. Young, 1984
14. Klep-De Pater, 1979
15. Al-Hassnan, 2011
16. Talseth-Palmer, 2009
17. Krepischi-Santos, 2006
18. Fagan, 1989

B

		Seizures/Abnormal EEG		Total
		YES	NO	
HBP1 deletion	YES	10	8	18
	No	4	29	33
Total		14	37	51

Fisher's Exact Test: p=0.00216

Figure 3.1: The gene block containing HBP1 is positively correlated with increased frequency of seizure development or EEG abnormality. A) Schematic representation for chromosome 7 q22.3. The region of 7q22.3 containing HBP1 is expanded from the UCSC Genome Browser and HBP1 is identified by an arrow pointing to a small box. The patient-specific chromosomal deletions are represented

by a solid red line if encompassed entirely within the 7q22.3 region. If the deletion encompasses the entire region (i.e. patient 2), the deletion is represented by a red double arrow. If the deletion begins within 7q22.3 and continues into other parts of 7q, the deletion is represented by a directional arrow (i.e. patient 1). The dotted red lines represent the smallest common deleted region and contains HBP1 and other 8 genes (termed HBP1 gene block). **B)** Deletion within HBP1 gene block was positively correlated with seizure development or EEG abnormality using Fisher's Exact Test.

3.3.2 Identifying Variants in the HBP1 Gene from Epi4K

The previous analysis was conducted using information from Decipher as well as published clinical case reports could only narrow the region to 10-15 genes surrounding the HBP1 gene. However, we were able to suggest the clinical relationship of a phenotype pattern for HBP1 loss. In addition, the presence of HBP1 loss-of-function mouse model with a neuro-pathological alterations consistent with an epileptic brain provided the incentive for further study of HBP1 gene in epileptic patients. Considering that previous analysis has many caveats, which included lack of sufficient gene specificity to establish and support strong correlation between HBP1 loss and seizure development. Next, we attempted to examine the genetics of the HBP1 gene itself and whether it as correlated to genetic epilepsies.

Further, to support and expand research to determine clinical validity of HBP1 gene loss of function with seizure development, we aimed to build a specific and coherent method of analysis of sequencing data from epileptic patients by using an epilepsy specific database. Epi4K, is an epilepsy specific database which aims to address the genetics of rare and severe childhood epilepsy. In this paper, the bioinformatic analysis was conducted by our collaborator Dr. Rebecca Batrosky. The analysis was aimed toward finding a mutation within the HBP1 gene. Over 1600 patient exons and introns were scanned for mutations within HBP1 which resulted in over 200 variants in total. We then selected exon specific variants that altered amino acids which resulted in 8 variants (Table3.1) in 46 patients.

The data extracted from Epi4k were used to study the clinical relevancy of the HBP1 gene in epilepsy. We proposed to functionally analyze the eight different HBP1 variants and determine whether the variants have compromised repression function for Wnt signaling. We created these variants an performed functional assays to determine if the eight HBP1 variants have a potential impact on the HBP1 protein function in Wnt suppression. Such a result would support the role of HBP1 in epilepsy.

Most of these variants located in a region of the gene where it can affect the protein function. Furthermore, our collaborator, Rebecca Batorsky, conducted two more analyses to identify the existence of other epilepsy causing genes. A list of known direct epilepsy causing genes was obtained from GeneDx and it was used in the analysis. Moreover, a whole exome analysis was conducted for the 46 human subjects who have variants within the HBP1 gene to determine the existence of an epilepsy associated genes which may contribute to seizure development. The result for this analysis showed 2 out of the 46 patients had SNPs (single nucleotide polymorphism) in 2 epileptic genes. One patient had a PLOG variant and another patient had an STXBP1 variant. Both of these variants are known to be pathogenic and they are direct causing genes for epilepsy (Table 3.2). These mutations existed in a total of three patients, all of whom had R375P variant. These patients were excluded from further analysis. Two of these patients were diagnosed with infantile spasm and the third patient had no diagnosis for epilepsy (Table 3.2). To test our hypothesis, we further analyzed the cohort for familial inheritance of HBP1 variants and to correlate variants with disease phenotype. The clinical data for the identified patients was examined for their specific epilepsy phenotypes. The clinical information showed two of the HBP1 variants were heritable; D97G and R375P. The sequential identification

number for the study participants was given to the participated families (Table 3.3). In that analysis only one patient had R375P variant and no other mutations and was diagnosed with LGS. One patient had D97G variant and two other mutations identified on Clinvar database, however, these mutations are not direct causing mutations for epilepsy. That patient was diagnosed with infantile spasm (Table 3.3).

The R375P variant existed at a higher frequency in the Epi4K relative to its overall frequency in the human population about 1% . Therefore, we investigated if that specific variant might be associated with specific epilepsy phenotype. Based on the clinical data obtained from Epi4K we observed that the R375P variant is usually associated with LGS diagnosis, especially in the probands (Table 3.4). The majority of these patients had multiple genetic variants in other genes, none of which are known to be related to epilepsy. Five out of 38 R375P study participants were diagnosed with LGS providing near significant association via Fisher's exact test. (Table 3.4).

Table 3.1: Identified variants within the HBP1 gene. The illustrated data were obtained from an epilepsy specific database (Epi4K) contain Whole genome sequencing data. The original analysis resulted in 200 mutations and the data were filtered to the variants located on the HBP1 exons**.**

Gene	Genomic Position	cDNA position	Protein Change	Allele Frequency	Consequences
HBP1	106820502	c.350A>G	p.Glu55Gly	6.20E-04	missense variant
HBP1	106822938	c.476A>G	p.Asp97Gly	6.20E-04	missense variant
HBP1	106826370	c.709C>T	p.Pro175Ser	3.10E-04	missense variant
HBP1	106836335	c.1310G>C	p.Arg375Pro	0.013	missense variant
HBP1	106836338	c.1313A>C	p.Gln376Pro	3.10E-04	missense variant
HBP1	106836437	c.1412C>T	p.Ser409Phe	3.10E-04	missense variant

HBP1	106836477	c.1452C>G	p.Ser422Arg	3.10E-04	missense variant
HBP1	106841863	c.1719-1721	p. 511-512	3.10E-04	inframe_deletion

Table 3.2: Scanning the exome of the 46 HBP1 study participant for the presence of epilepsy causing genetic mutations. Two patients with clinically relevant variants which were associated with seizure or progressive sclerosing poliodystrophy were found. Both variants were registered in ClinVar as pathogenic/likely pathogenic based on multiple submission from different institutions. These patients were excluded from further analysis.

Patient ID	**Gene**	**Type**	**VEP-Conse-quence**	**Ami-no acid**	**ClinVar Interpretation**	**HBP1 Variant**	**Epilepsy diagnosis**
SRR997689	STXBP1	SNP	Stop-gained	R/*	Pathogenic	R375P	Infantile spasm
SRR997522	PLOG	SNP	Missense variant	G/R	Pathogenic/Likely pathogenic	R375P	Infantile spasm
SRR997651	PLOG	SNP	Missense variant	G/R	Pathogenic/Likely pathogenic	R375P	No epilepsy diagnosis was provided

Table 3.3:Transmissible HBP1 variants and the epilepsy diagnosis associated with it. This table illustrates the inherited HBP1 variants and the epilepsy phenotype associated with it.

Patient ID	**Participant type**	**Age at enrollment**	**Genomic Coordinate**	**Protein change**	**Epilepsy type**	**Other mutation**
SRR997866	Proband	20	Chr7:g106836335G>C	p.Arg375Pro	LGS	None
SRR997867	Parent	48	Chr7:g106836335G>C	p.Arg375Pro	None	MYBPC3
SRR997408	Proband	4	Chr7:g106822938A>G	p.Asp97Gly	IS	OCA2 , LPL

SRR997407	Parent	36	Chr7:g106822938A>G	p.Asp97Gly	None	LPL
SRR997520	Parent	46	Chr7:g106836335G>C	p.Arg375Pro	None	AK7
SRR997522	Proband	5	Chr7:g106836335G>C	p.Arg375Pro	IS	POLG

Table 3.4: Epi4K participant diagnosed with LGS. Five proband participants with R375P variants are diagnosed with LGS.

Participant Type	Age At Enrollment	Genomic Coordinate	Protein Change	Epilepsy Type
Proband	20	Chr7:g106836335G>C	p.Arg375Pro	LGS
Proband	15	Chr7:g106836335G>C	p.Arg375Pro	LGS
Proband	21	Chr7:g106836335G>C	p.Arg375Pro	LGS
Proband	24	Chr7:g106836335G>C	p.Arg375Pro	LGS
Proband	10	Chr7:g106836335G>C	p.Arg375Pro	LGS

3.3.3 Location of the Variants in the HBP1 Protein

As shown in Figure. 3.2, the location of the variants and the deletion on HBP1 gene were present along the entire length of the protein. Interestingly, a cluster of variants are present on the 3' end of the repression domain in a region that has previously been shown to be regulated by phosphorylation. The Yee lab first showed that HBP1 protein stability was regulated by p38 MAP kinase phosphorylation at serine 402 (Xiu et al., 2003). The elevation of Wnt signaling leads to the accumulation of cytoplasmic β-catenin and its translocation into the nucleus. In the nucleus β-catenin will form a complex with TCF4 leading to activation of Wnt target genes. HBP1 could also bind TCF4, an important transcription factor in Wnt/β-catenin signaling, through the repression domain and inhibit its transcriptional

activity. Other post-translational modifications in the region include PIM-1 phosphorylation at Ser372 and Ser380, promoting its accumulation and transcriptional activation (Wang et al., 2017). In contrast, HBP1 expression is unaffected by AKT's phosphorylation at three locations, Ser380, Thr484, and Ser509, but its transcriptional activity is decreased (Bollaert et al., 2018). Furthermore, acetylation also regulates HBP1 function. The acetyltransferase p300/CBP acetylates HBP1 on five lysine residues(Lyss171, Lys297, Lys305,Lys307 and Lys 419), promoting its transcriptional activity (Wang et al., 2012). Further, HBP1 is polyubiquitinated by ubiquitin ligases, the cullin–RING ligases (CRL) 3 and 4 were reported to interact with HBP1 and ubiquitinate Lys16 and Lys398 (Emanuele et al., 2011 ; Wagner et al., 2011).

Given the number of post-translational modification sites affecting protein stability in the region between the repression domain and the DNA binding domain, we predicted that one or more of the variants might have an effect on the functionality of the HBP1 protein (Bollaert et al., 2018).

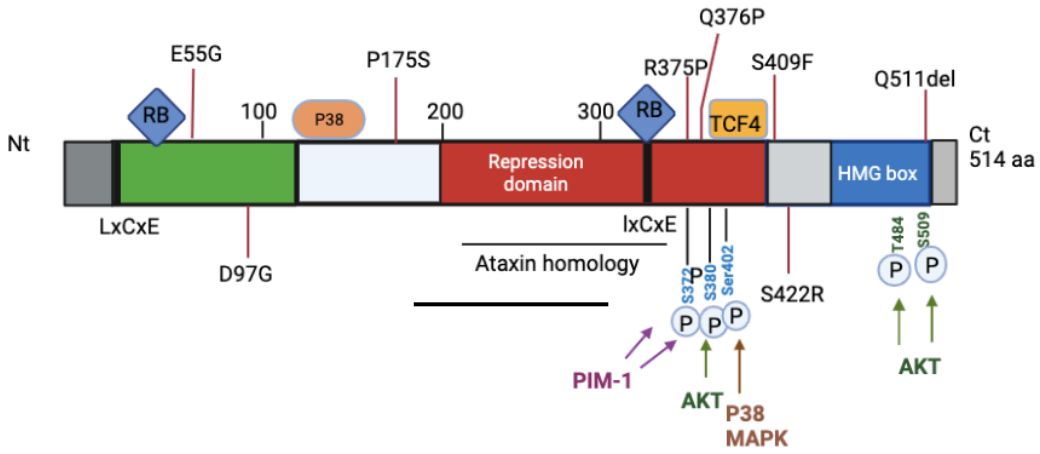


Figure 3.2: Location of the variants and the deletion on HBP1 protein. Schematic representation for the location of HBP1 variants and the locations of previously identified functional domains, repression domain, phosphorylation sites and RB interacting sites. This figure was created using Biorender.

3.3.4 HBP1 Variants Are Defective in Repressing Wnt Reporter Activity.

HBP1 has several functions which include cell cycle inhibition, activation or repression of different cell cycle genes and it acts as a suppressor of the Wnt/β-catenin pathway. Any alterations in variant HBP1 function may help us understand its relevance to epilepsy and seizure development. Because HBP1 is an important inhibitor of the Wnt signaling pathway, we predicted that one of the variants between the repression domain and the HMG box would be defective in Wnt repression. Any variant dysfunction would thus provide a potential mechanism of clinical phenotype and cement the potential role of Wnt activity in seizure development. To determine the functional impact of these eight different HBP1 variants, I engineered the mutations using site-directed mutagenesis. The cDNA sequence of human HBP1, either wild-type or variant, was cloned into p-CMV mammalian HBP1 vector with C-terminal Myc-DDK tag.

Wnt/β-catenin activation results in the stabilization and accumulation of cytosolic β-catenin, a GSK-3ß substrate, which when phosphorylated by GSK-3ß, is targeted for ubiquitin-mediated proteolysis. β-catenin then translocates to the nucleus, where in complexes with members of the Tcf/LEF family of transcription regulators, activates transcription of Tcf-responsive genes. TOPFLASH is a synthetic TCF/LEF reporter plasmid containing TCF motifs upstream of a minimal c-fos promoter and luciferase gene as a reporter. TOPFLASH is highly sensitive to induction of Wnt signaling, for example by LiCl. A change in the transcriptional repression function of HBP1 to suppress this Wnt-induced reporter gene expression would indicate HBP1 malfunction. By identifying the presence of the HBP1 variants located at different regions of HBP1 gene, we used LiCl-activated TOPFLASH expression to evaluate if these HBP1 variants disrupted HBP1 protein function for Wnt repression.

Specifically, the location for several variants near important regions of HBP1 function/regulation were predicted to have an impact either on HBP1 protein function or stability. All of the variants showed a high degree of repression except for R375P, which was almost completely defective for reporter gene repression (Figure 3.3 A-B). Next, I investigated the homology sequence of the arginine residue in 5 different species (Figure 3.4 B). That R residue was highly conserved which imply a functional importance for that region. Furthermore, all of the Epi4K HBP1 variants were located in a highly conserved regions of the HBP1 protein across multiple species. The highly conservered sequence around R375 is illustrated in Figure 3.4 A.

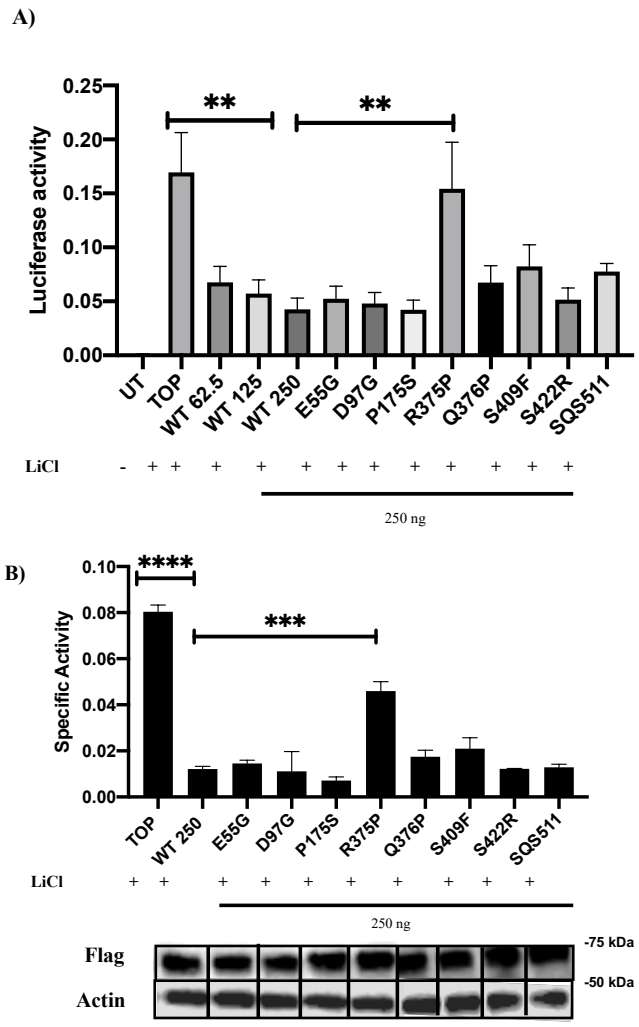


Figure 3.3: HBP1 Variants Are Defective in Repressing Wnt Reporter Activity. **A)** Represent the fold change of the induced Wnt activation via lithium chloride. HEK293T cells were transfected titrated amount of WT. The ratio of luciferase

(TOPFLASH) plasmid to Renilla was 10:1 and 250 ng of the HBP1 variants were used in the co-transfection. Cells were harvested 48h after transfection. The results are expressed as a relative ratio of luciferase to Renilla activity. The data were represented as mean ± SEM using unpaired student's t-test and the comparison was made to WT 250 ng. (Prism 9.0, Graphpad.*P<0.05 , **P < 0.01). **B)** HBP1 WT and variants identified from Epi4K were transfected into HEK 293T cells with titrated amount of WT. The ratio of 10:1 of TOPFLASH to Renilla and 250 ng of each of the HBP1 variants was used in the co-transfection. Lithium chloride was used for Wnt activation. Cells were harvested 48h after transfection. The specific activity is expressed as relative ratio of luciferase activity to the protein expression. The ability of HBP1 to repress Wnt signaling was lost with R375P variant has reduced ability for the suppression, Lithium chloride was used for Wnt activation. The data were represented as mean ± SEM using unpaired student's t-test and the comparison was made to WT 250 ng. (Prism 9.0, Graphpad.*P<0.05 , **P < 0.01 and *** P < 0.001). (Bottom Panel) Western Blot for the Flag tag expression in the co-transfection experiment.

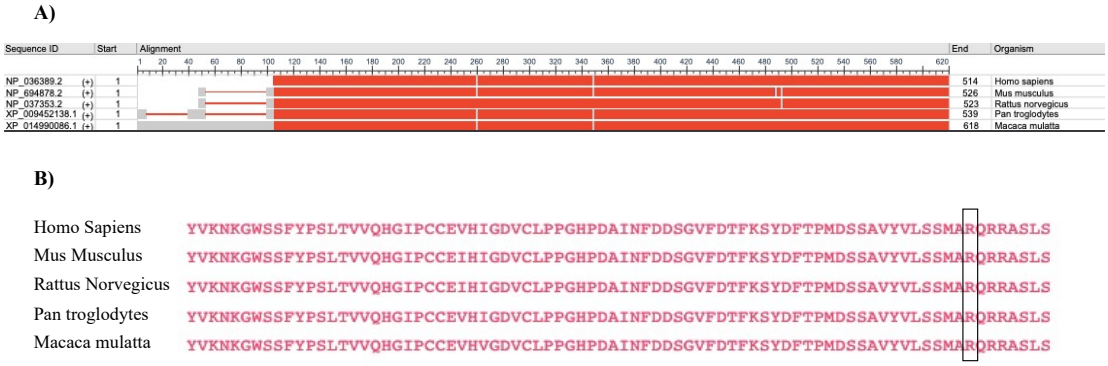


Figure 3.4: HBP1 protein variants and interspecies conservation of the amino acids. A) HBP1 protein alignment for 5 species has shown the regions where HBP1 variants extracted from the Epi4K are located within conserved region in 5 different species. B) Amino acid sequence homology shows that arginine R at residue 375 is highly conserved in HBP1 in human and across multiple species.

3.3 Discussion

As noted in the introduction to this chapter, epilepsy is collection of conditions with the single unifying feature of brain seizures. In many ways epilepsy is like cancer in its complexity and with multiple mechanisms that can arrive at the same outcome that being seizure. Like cancer, epilepsy research has been aided by the identification of genetic causes and contributions to disease development, which has helped to improve and aid diagnosis and treatment modalities.

My studies are based on the Yee lab's initial discovery that the Wnt/β-catenin signaling pathway is implicated in epileptogenesis in the pilocarpine and kainate epileptic animal models (Alquarshi et al., 2021). In that paper, Wnt inhibitor HBP1 declined during the period of epileptogenesis. Further, HBP1 knockout mice had rare spontaneous seizures and were more susceptible to kainate and pilocarpine induced seizures (Alquarshi et al., 2021). In Chapter 2, I expanded those initial studies to show that decreased HBP1 leads to a significant alteration in neuronal genes involved in GABA and glutamate signaling and metabolism. Here, we expanded our research to identify the involvement of HBP1 gene among epileptic patients.

Dr. Paulson and T. Malone in the Yee lab showed a genetic correlation for deletion of the HBP1 gene genomic locus with abnormal EEG and seizure epilepsy by analyzing multiple clinical case reports (Figure 3.1). With the aid of Rebecca Batorsky (Tufts biostatistician), I surveyed the Epi4K genomic sequence database of patients and families with epilepsy and found possible deleterious variants of the HBP1 gene to test its possible contribution in epileptic patients by analyzing sequencing data from an epilepsy specific database (Epi4K). Among all of the variants which were identified and tested, only the R375P variant caused loss of repression of HBP1 protein when tested in vitro.

The Wnt reporter assay (TOPFLASH) was performed and used to evaluate the efficiency of the HBP1 variants in Wnt repression. Most of the variants were able to repress Wnt activity except for the R375P, suggesting HBP1 protein malfunction. In addition, genetic scoring algorithms for variant pathogenicity such as CADD and Polyphne-2 (Rentzsch, Witten, Cooper, Shendure, & Kircher, 2019) showed R375P was predicted to be pathogenic. Moreover, after obtaining the clinical information for parents and probands in our study cohort containing HBP1 variants, demographic and

clinical data was obtained from Epi4K including, patients age, age at enrollment, the epilepsy type, the existence of psychiatric symptoms, the localization of the epileptogenic zone (bilateral, multifocal, etc.), and the affected family members.

As observed in Table 3.5, LGS is present in a near-significant number of R375P patients. LGS is a severe form of childhood epilepsy characterized by generalized multiple-type seizures, intellectual disability, and a particular EEG abnormality termed a spike-and-wave. Children that are affected may have had infantile spasms or an underlying brain condition in the past, although the cause may be idiopathic (Abu Saleh & Stephen, 2008). Moreover, the list of identifiable etiologies for Lennox-Gastaut syndrome include brain injury, tuberous sclerosis, prenatal infections, genetic metabolic illnesses, brain malformations, and brain injury have all been implicated as causes for LGS. Furthermore, LGS can be acquired, and the causes include birth asphyxia, encephalitis, meningitis or head injuries (Asadi-Pooya, 2018). In addition, de novo mutations in several genes, including GABRB3, ALG13, SCN8A, STXBP1, and DNM1, have been found in patients with LGS who have no history of infantile spasm (IS) and no known etiology (Epi., 2013).

Additional evidence that Wnt signaling may play a role in genetic epilepsy comes from the role of FOXG1 in epilepsy. FOXG1 functions as transcriptional repressor for Wnt signaling is associated with LGS. A case report showed 8-year-old child with this mutation in FOXG1 had intellectual disability, severe postnatal microcephaly and Rett-like features in a case report (Terrone et al., 2014). The Forkhead DNA-binding domain (FHD) in this patient's FOXG1 (amino acids 181-275) had an A to G substitution at position 559 (c.559A>G; p.Asn187Asp). Asn187 is very conserved across species (Homo sapiens, Bos taurus, Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio) suggesting its disruption in the patient had

severe consequences, although there was no demonstrated disruption in Wnt signaling.

By exploring the patient clinical information, LGS was a common diagnosis associated with R375P variant. The majority of the R375P variant patients had other mutations which might be contributing to their epilepsy. Moreover, this clinical observation will require further work to identify if R375P is a susceptibility factor for LGS among pediatric patients. The R375P variant is located in the repression domain of the HBP1 gene. Functionally, that area is required for the TCF4 binding with HBP1 and repress Wnt activity. It is possible that R375P might disrupt that binding site and abolishing the HBP1 protein ability to repress Wnt signaling. Immunoprecipitation assay for the R375P with TCF4 plasmid might demonstrate if the TCF4 binding site on HBP1 is being disrupted due to the presence of that variant. Creating a knock-in mouse for the R375P variant would definitively determine a role for this variant in seizure development, and might even recapitulate the LGS symptoms which include early and severe epileptic seizures starts, perhjaps as early as postnatal day 10 in mice.

The dysfunctionality of the HBP1 protein resulted from R375P variant might be due to two reasons. The site where that variant is located is a well conserved area among multiple species including Homo sapiens, and Mus musculus, which imply functional importance of that region. First, R375P is located in an area in which protein stability is regulated by phosphorylation, including p38MAPK (Xiu et al., 2003). Second, AKT phosphorylates multiple sites in this region including Ser380 which reduces HBP1 transcriptional activity (Bollaert et al., 2018). Both these phosphorylations alter HBP1 protein stability, although we did not investigate that in our work.

In conclusion, the above-mentioned results showed a potential clinical significance of the HBP1 variants. The variants were extracted from an epilepsy specific database, in which the clinical information for the study cohort has a confirmed epilepsy diagnosis. However, larger study size and obtaining full and detailed clinical and demographic for the participants data is required to establish an accurate statistical and clinical significance.

3.4 Material and Method

3.4.1 Plasmids

Mammalian expression vectors used for transient transfection include tagged protein with the C-terminal Myc-DDK tags. The human HBP1 expression vector was provided by Origene (Figure 3.4) . HBP1 variants were obtained from Epi4K. TopFlash 8x and FopFlash plasmids were obtained from addgene (Figure 3.5-3.6).

3.4.2 Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin. The cells were maintained in a humidified, 5% CO2 atmosphere at 37 °C. HEK-293T using Lipofectamine 3000, 48 h post transfection, cells were harvested , pelleted and protein was extracted for Western blotting . Protein concentrations for the pellet were determined by a bicinchoninic acid (BCA) assay (Biorad).

3.4.3 Site-directed mutagenesis

All the mutants were generated by using the Site direct mutagenesis kit from *New England Biolab* according to the manufacturer's protocol. The different mutations were verified by DNA sequencing. Mutagenic primers were synthesized by TUCF and are listed in

3.3.4 Reporter assays

Cells were seeded in 48 wells plates (1×10^5 cells/well)), then cells were co-transfected with reporter constructs containing the luciferase using lipofectamine 3000 , 24 hour post-transfection cells were treated with 7.5mM Lithium chloride for Wnt activation. Then, 48 hours post transfection , the media was removed and cells washed with 100 ul of PBS, then 100 ul of Promega passive lysis buffer were used, which was supplemented with the kit . The cell lysates were rocked at 4°Cfor 30min at room temperature . Luciferase reagent II was prepared by supplementing the lyophilized luciferase assay substrate with 10 ml of luciferase buffer provided with the kit. Luminescence activity was measured using Dual Luciferase Reporter Assay Kit (Promega) following the manufacturer protocol. The Firefly luciferase activity measurement was standardized to the same sample's Renilla luciferase activity.

3.3.5 Statistical Analysis

Statistical analysis was performed using Student's unpaired t-test. $P < 0.05$ was considered statistically significant. All western blot, qRT-PCR and cell quantification data were analyzed using GraphPad Prism version 9 software.

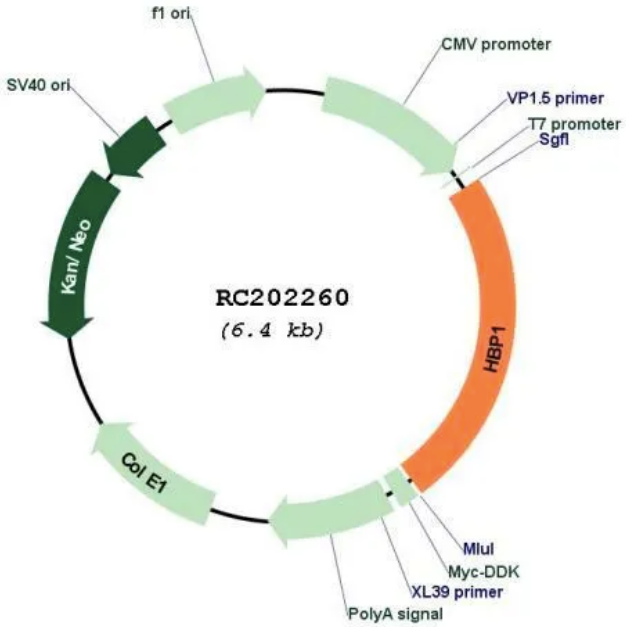


Figure 3.5 : HBP1 expression vector used for engineering the HBP1 variants used for mutagenesis Experiment.

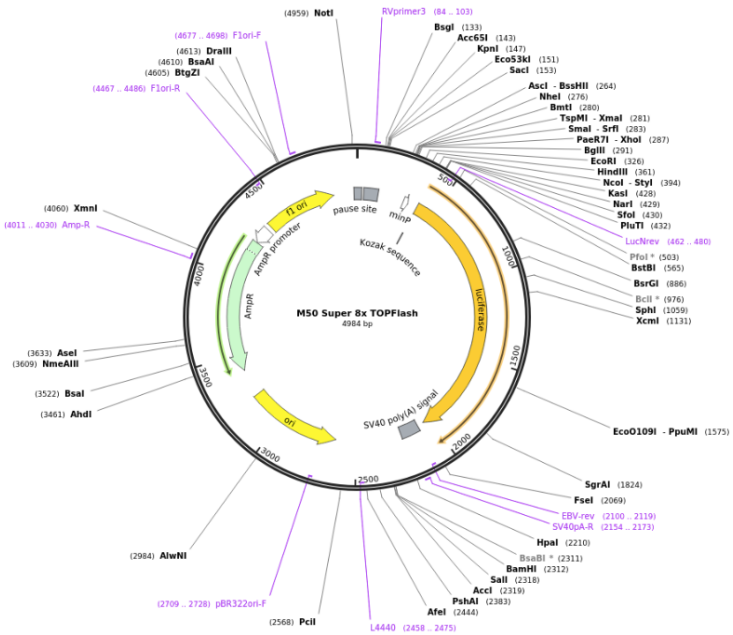


Figure 3.6: Super8X TOPFlash a luciferase reporter of beta-catenin-mediated transcriptional activation

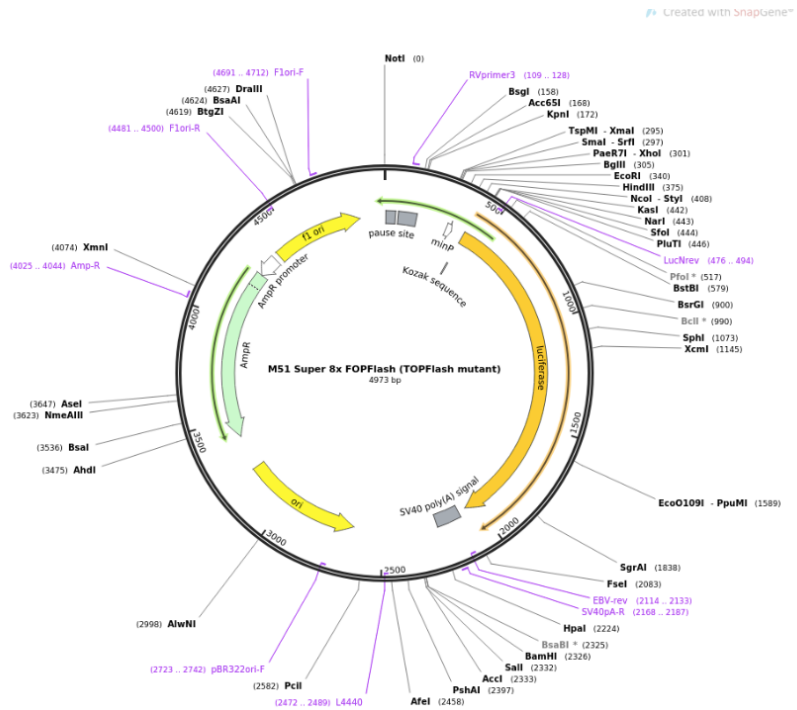


Figure 3.7: Super8X FOPFlash a luciferase reporter, which has a mutant TCF/LEF sites

Table 3.5: Human primers used for the mutagenesis experiment.

HBP1 Variants	Forward	Reverse
E55G	GAACACATGGGGCTTGATGAC	GACCTATATTGAGGACACTA
D97G	AATACCTCAGGCATACCAGAAAC	GTTCTAGTAGAACCTTGGTT
P175S	GGAGGAAACATCAGTAAGACAC	GGGAGTAGTGGTAACCTT
R375P	AGTATGGCTCCCCAGCGTCGT	CTAAGAAGACGTCAAATACACAATTCA
Q376P	ATGGCTCGCCCGCGTCGTGCA	ACTACTTAACACATAAACTGCAGAGAATCCATAG
S409F	TCACAGCTCTTTTCCAATTCTTTGTA	ACACCGAGTGGACCTAGT
S422R	AAAACCACAGGTCAGGGACTG	GAAACATACGATTTCGACAGT
SQS 511	ACAACATACGCGTACGCG	GAGCCTGAATTGGTTCTTTTCC
HBP1	GGCGACGGGTTTGTCAGAG	TGCCAGATTGGGTAGGATCAC

3.5 Author contributions

EP and TM performed the experiment in Figure 3.1. The analysis in Table 3.1 and 3.2 was conducted by RB. The initial tables was then filtered and re adjusted by EP and SA. SA performed all the mutagenesis experiment and Wnt reporter assay Figure 3.3 and analyzed the data for the same figure.

Chapter 4: Summary/ Future direction

4.1 Summary of thesis result

Epilepsy is a debilitating neurological disorder which occurs in all age groups. It affects approximately 1% of the global population (over 70 million people) hence producing a tremendous societal and eco- nomic burden (Englot and Chang, 2014; Devinsky et al., 2018) Epilepsies affect 1% of the population and occur most frequently in childhood (Berg, Shinnar, Levy, & Testa, 1999; Pitkanen & Sutula, 2002). Yet, the pathogenesis for the diseases remains poorly understood. A complete understanding for the disease pathology is required to design and develop therapeutic intervention which will aid in slowing the disease progression and improving the quality of life for the affected individuals. The neuro-pathological mechanism for disease development becomes more clinically significant especially when genetics is influencing the disease progression. Several factors has been linked to the pathophysiology of epilepsy which include a reduction in the GABA-inhibitory postsynaptic potential and activation of postsynaptic glutamate receptors (Engelborghs et al., 2000; Stafstrom and Carmant, 2015).

Another alteration usually associated with epilepsy in both human and animal model of epilepsy is an upregulation of GFAP an intermediate filament protein called glial fibrillary acidic protein (GFAP). GFAP is almost exclusively produced in astrocytes and may be released in response to neuronal injury (Cotter et al., 2001; Diaz- Arrastia et al., 2014). Expression of GFAP can show reactive gliosis, a significant morphological characteristic of TLE (do Nascimento et al., 2012). There is evidence to support the idea that seizures cause astrocytic hypertrophy, which

explains why there is a substantial increase in GFAP expression in astrocytes (Chmielewska et al., 2018).

In addition, the Wnt signaling pathway has drawn a lot of interest recently due to its critical function in controlling embryonic development, cell growth, and adult tissue maintenance in a range of cell types. It has been demonstrated to influence the central nervous system's (CNS) neurogenesis, neuronal differentiation, synapse development, and plasticity. A number of CNS illnesses, including Alzheimer's disease, schizophrenia, and mood disorders, are also linked to wnt signaling (Hodges & Lugo, 2018; Jia, Pina-Crespo, & Li, 2019). Recent research on human data and animal models of epilepsy emphasizes the significance of the Wnt/β-catenin signaling pathway in epilepsy. This work has shown a dysregulation of Wnt signaling has a cellular, molecular and metabolic consequences. Moreover, Wnt signaling activation leads to an epileptic phenotype of the mice hippocampi. Characterizing the HBP1 knockout mice as a seizure prone model can be used to study the involvement of Wnt signaling in the neuro-pathological alterations associated with epilepsy. Alquarshi et al, demonstrated that molecular, cellular and metabolic changes alterations in the $HBP1^{-/-}$ mice resembled those observed during epileptogenesis (Alquarshi et al., 2021). GWAS has helped in identifying deleterious genetic mutations associated with epilepsy. The main genetic cause of epileptic encephalopathies has been identified as de novo mutagenesis, and whole exome sequencing has helped in identifying the deleterious genetic mutations associated with epilepsy (Claes et al., 2001; Epi4K Consortium et al., 2013; Euro Epinomics et al., 2014). An example of a well-studied genetic mutation which is causative for epilepsy is TSC. When one of the TSC genes is inactivated (TSC1/TSC2), the mTOR (mechanistic target of rapamycin) pathway becomes overactive and leads to the growth of benign tumors or hamartomas in a

variety of organ systems, including the skin, brain, eyes, heart, and kidneys. Developmental delay, epilepsy, and neurobehavioral or neuropsychiatric diseases such as autism spectrum disorder (ASD) and epilepsy are frequently caused by TSC mutations, with the brain frequently being the organ system that is most severely affected (Overwater et al., 2015). Previously, Yee lab has confirmed the involvement of over activated Wnt signaling in epileptogenesis using an animal model of TLE (Alquarshi et al ., 2021).

FOXG1 mutations have been linked to developmental delay, microcephaly and epilepsy (Wong et al., 2019). FOXG1 is a member of the Forkhead box transcription factor family that functions in both transcriptional activation and transcriptional repression, also known as brain factor-1 (Dong et a., 2021). FOXG1, which functions as a Wnt repressor and it is highly expressed in the cerebral cortex, is essential for the CNS's development.

We hypothesized that HBP1 gene is a susceptibility gene for epilepsy. That was suggested by the neuro-pathological alterations of the HBP1 knockout genetic mouse model. Then, we investigated whether Wnt signaling might be altered in patients with epilepsy. To investigate that hypothesis, we conducted multiple analysis. Started by gathering and analyzing multiple case reports which showed a deletion of the HBP1 genomic region was positively corelated with neurological abnormality including epilepsy. However, one of the caveat for that analysis that it was too broad and not specific. Next, we investigated human patients for HBP1 mutation or variants in an epilepsy specific database (Epi4K). A total of eight variants were identified within the HBP1 gene. R375P was our designated variant in which the functional assay confirmed its disruption to the HBP1 protein function for Wnt suppression.

4.1.1 Summary of chapter 2 findings

The data presented in chapter 2 presents the characterization of the neuro-pathological alterations associated with a hyperactive Wnt signaling in the brain, in which the HBP1 knockout model exemplify a model for seizure susceptibility. The two hallmarks of Wnt signaling, b-catenin and pGSK3bS9, were induced in the hippocampus and they were localized in neuronal cells and subset of astrocytes. Consistent with Wnt activation, there also decreased pathway inhibitor (sFRP1).

Unexpected lactate production was seen, along with additional signs of a Warburg-like effect and concomitant glutamate-GABA change achieving equilibrium causes GABA levels to drop sharply. Then, we noticed the induction of the PKM2 is one of the glycolytic enzyme isoforms linked to the Warburg effect and appeared to be specific to astrocytes. We then investigated a crucial Wnt-regulated metabolic enzyme. PDH, which is associated with epilepsy and the Warburg-like effect. A mechanism known as aerobic glycolysis occurs when PDH is inhibited by phosphorylation. This limits the amount of glucose that may enter the TCA cycle and directs glycolytic metabolites to other fates like lactate. The split Warburg effect between astrocytes and neurons was further demonstrated by the finding that neuronal PDH activity was likely inhibited by raising the expression of PDK4, a negative regulator of PDH and a Wnt target that was localized in neuronal cells and a fraction of astrocytes. The significance of PDK4 and an uncommon temporary metabolic plasticity in early epileptogenesis are highlighted by these mechanistic observations. This crucial finding clearly implies that Wnt signaling is what drives the altered metabolic alterations in the HBP1 knockout mice. A schematic figure 2.8 summarizes the neuropathological alterations associated with an over activated Wnt signaling in the brain. This model predicts the metabolic and potential neuro-pathologic

consequences associated with high Wnt signaling. Furthermore, this model can be used to test whether early therapeutic intervention would be beneficial in reversing the pathological changes associated with high Wnt signaling. mTOR inhibitors and ketogenic diet are examples for therapeutic interventions which could be studied in the HBP1 deficient mice.. One of the caveat for this study is that the analysis was conducted using a global knockout mice. Notably, most of the changes in the HBP1 deficient mice were astrocytic. Generating an astrocyte specific HBP1 knockout and investigate if the previous neuro-pathological findings would be replicated in this model.

4.1.2 Summary of chapter 3 findings

The third chapter of this thesis investigated the genetic component of Wnt signaling and its involvement in epilepsy among epileptic patients. Analyzing the raw genetic data which were obtained from Epi4K database by our collaborator Dr. Rebecca Batrosky. Detecting variants within our gene of interest and filtering the variants to their location on the HBP1 exome. The bioinformatic analysis motivated us to conduct the mutagenesis experiments and the functional assays. We aimed to identify which of the variants will simulate our animal model, in which it will lead to loss of function of HBP1. Furthermore, obtaining the clinical data was as important as the functional assay to provide a clinical validity of the HBP1 variants. From the 8 variants which were identified by the Epi4K database, only R375P was the variants provide functional deficient in the ability of HBP1 protein to suppress Wnt signaling. Simulating a total loss of function of the HBP1 gene to prove the preclinical study significance and to confirm the clinical validity of the HBP1 gene as a potential susceptibility gene for epilepsy. Furthermore, a clinical observation was identified by scanning the clinical data for our study cohort was the association of R375P variants

with Lennox-Gastaut syndrome (LGS) especially among pediatric patients. Moreover, in our bioinformatic analysis only two other epilepsy genes were identified in in three patients. These data solidify the potential role of HBP1 gene in epilepsy. In conclusion, HBP1 gene preclinical studies suggested its possible contribution in epilepsy and the human genetic data are consistent with the clinical validity of HBP1 gene as a susceptibility gene for epilepsy.

4.2 Future direction

Anti-epileptic drugs (AEDs) and/or surgery can partially or completely control seizures in two-thirds of patients with epilepsy, however all patients still struggle with the disease. In this thesis, we uncovered a few of the basic processes associated with the progression of epilepsy. In particular, we identified unique metabolic reprogramming and Wnt signaling with implications for epilepsies. We identified Wnt signaling as a potential target for epilepsy. Strengthening the knowledge regarding the genetic involvement of Wnt signaling in genetic epilepsy is crucial. Multiple animal studies have confirmed its involvement of hyperactivated Wnt signaling in epileptogenesis. Furthermore, Wnt signaling transcription factor (FOXG1) was identified as a causative gene for epileptic encephalopathy

4.2.1 Pharmaco-genomics in epilepsy

With the aid of next-generation sequencing, the area of epilepsy genetics has progressed towards precision medicine. In the previous ten years, neurologists were only aware of 20 different gene types that were connected to epilepsy; now, with the advent of commercial NGS panels, this number has increased to over 300 (Myers, Johnstone, & Dyment, 2019). For instance, in TSC-mutation individuals, the application of precision medicine to a particular gene was successful, since the administration of everolimus to the affected children prevented the onset of epilepsy

(Samueli et al., 2016). If a particular gene mutation in epilepsy results in a functional change in the physiological systems that regulate brain excitability, a sensible therapeutic plan may ideally be to attempt to correct or avoid the malfunction. Identifying underlying genetic mutations in epilepsy patients is required for the treatment strategy in precision medicine and it can improve the therapeutic outcome for the affected patients.

The management of GLUT-1 deficiency, a genetic metabolic encephalopathy caused by mutations in the SLC2A1 gene, which encodes the glucose type I transporter (GLUT-1), is a well-known example of the application of the precision medicine concept. This condition is caused by impaired glucose transport across the blood-brain barrier. Since, AEDs frequently fail to control seizures, GLUT-1 deficiency exhibits a wide range of phenotypic pleiotropy, including intellectual disability, movement dysfunction and drug-resistant epilepsy. The ketogenic diet is the gold standard for treating neuroglycopenia symptoms because it lowers glucose and offers ketones as an alternate fuel for cerebral metabolism. Although the benefit on neurodevelopment is debatable, early diagnosis and introduction of the ketogenic diet are essential to fuel the brain and control seizures (Pong et al ., 2012). Due to the pleiotropic symptoms of numerous mutant genes, it is challenging to consider GLUT-1 deficiency as a potential diagnosis. This obstacle can be overcome by using gene panels or more thorough sequencing (exome or genome).

The creation of innovative anti-epileptogenic treatments will probably depend on focusing on different mechanisms of action in addition to correcting the excitatory-inhibitory imbalance. Multiple indirect factors that impair the neuronal connections and communication lowering the seizure threshold for patients (Campos et al., 2004; Huang et al., 2015; Köhling, 2014; Lucke-Wold et al., 2015). For instance,

dysregulated mTOR existed in genetic and acquired epilepsy in both humans and animal models of epilepsy (Ostendorf and Wong, 2015; Wong, 2010; Zeng et al., 2009). In mice models of epilepsy, pharmacological inhibition of mTOR has been shown to decrease neuronal death, lower seizures overall, and decrease the pathological changes associated with epilepsy (Brewster et al., 2013; Buckmaster et al., 2009; Guo et al., 2016; Huang et al., 2010; Sunnen et al., 2011; van Vliet et al., 2012).

Furthermore, HBP1 knockout model can be used to study multiple therapeutic intervention and demonstrate its effectiveness. For instance, hyperactivated Wnt signaling has a metabolic consequences which was demonstrated earlier. Using ketogenic diet to study its efficacy in reducing the spontaneous seizures and potentially reverse the neuro-pathologic features associated with high Wnt signaling. Furthermore, using an mTOR analogue to study whether the inhibition of mTOR signaling will be enough to reduce the seizure susceptibility and reverse the neuro-pathological alterations in HBP1 knockout mice. Finally, the observations reported here fill a gap of knowledge providing clues to start understanding the consequences and potential mechanisms at play in the CNS of patients affected by HBP1 loss of function conditions.

Furthermore, the clinical data suggests an association between R375P and loss of HBP1 protein function in pediatric patients diagnosed with LGS. Further work is required to determine whether HBP1 loss of function and activated Wnt signaling is linked to the pathogenies of LGS in pediatric patients. Future studies with the mutation-bearing mouse model and patient-derived neuronal and astrocytic cells will further our understanding of how the variations in HBP1 give rise to epilepsy. Create a conditional HBP1 deletion in the hippocampus and assess for the spontaneous and

induced seizures. We have an HBP1 $^{fl/fl}$ mouse and create a conditional deletion by crossing GFAP-Cre mouse with the HBP1$^{fl/fl}$ mouse. To insure conditional knockout, the HBP1 levels in the GFAP cre expressing cells will be assessed by qPCR. Next, validate if that mouse model will recapitulate the molecular alterations and seizure susceptibility which existed in the global HBP1 knockout mice and investigate whether the flox mice will have increased β-catenin levels, mTOR activation, gliosis, glutamate and GABAergic neuronal cell dysregulation. Further, create HBP1$^{R375P/+}$ heterozygous knock-in mice could be created with GFAP Cre and investigate for the seizure susceptibility and if these mice will recapitulate multiple features of human LGS. Moreover, conducting RNA-seq to investigate the disrupted signaling pathways resulted from that variant in the in vivo study.

4.2.2 Tumor associated epilepsy

Clinically seizures are frequently the primary sign of various malignancies, such as gliomas and meningiomas (Kirschstein & Kohling, 2016). HBP1 is classified as a tumor suppressor protein, known to regulate proliferation and senescence, also it function as Wnt suppressor. Further, WNT signaling is often overactived in glioblastoma (GBM) and brain tumors. The infrequent spontaneous seizures of the HBP1$^{-/-}$ mice was the primary observation which lead to investigating the underlying mechanism behind it. The IPA analysis included brain tumor and brain cancer as part of the potential neurological disorders in HBP1 deficient mice. Furthermore, the metabolic profile for the HBP1 KO mice is similar to GBM which included lactic acid induction and Warburg- like metabolism (Duan et al., 2018).

Our experiment did not include evaluation of the brain morphology. However, we could identify neuro-pathological changes which can be linked to tumor. The upregulation of Ki67 which was confirmed by the confocal microscopy and the

increase in the mRNA expression of CD133. CD133 has been shown to be an important stem cell factor that promotes glioma progression. However, the mechanism for CD133-mediated glioma progression has yet to be fully elucidated (Li et al., 2017). HBP1 deficient mice should be evaluated and further studied regrading brain tumorigenesis especially, that molecular, morphological, and metabolic findings suggest its plausibility.

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