Alterations in Brain Metabolism in Pilocarpine-Induced Epilepsy and Potential Treatment Modalities

A thesis submitted by

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Abstract

Epilepsy is one of the most common chronic neurological disorders and is manifested by abnormal hypersynchronized neuronal discharge. A cascade of molecular, neuronal and metabolic alterations are involved in the process of developing epilepsy (epileptogenesis). The exact mechanisms underlying epileptogenesis are still unclear. In order to expand our knowledge and improve our understanding of epilepsy, we have investigated two main objectives. The first objective is identifying the role of HBP1 in epilepsy. HBP1 acts as Wnt repressor, and epileptogenesis occurs when Wnt signaling is up-regulated in an HBP1 knockout mouse. Importantly, deletion of 9 genes in the HBP1 chromosomal region in human populations (7q22.3) is associated with seizure or abnormal EEG, indicating the mouse model may recapitulate human disease. Utilizing ^{1}H and ^{13}C NMR spectroscopy, the metabolic profile of HBP1 knock out and wild type has been quantified in hippocampus, but no significant differences were detected. The second objective in this project was testing the efficacy of the EGCG/DAC combination (a HBP1 inducer) and observing its effect on the metabolism of normal and epileptogenic brain. The EGCG/DAC is proposed to be protective against the development of epilepsy. Using NMR the metabolite concentrations were obtained. A comparison between pilocarpine induced status epilepticus and control mice revealed a series of significant metabolite alterations. In hippocampus, GABA, AMP, NAD+, were significantly decreased in the pilocarpine group compared to control mice. In contrast, concentrations of IMP, lactate, UMP and myo-inositol were significantly increased in hippocampus. Glutamate and NAA were significantly decreased in pilocarpine group. On the other hand, glutamine

was significantly up regulated in pilocarpine mice in both brain regions. Also, comparisons between pilocarpine and control in the background of being treated with the drugs versus pilocarpine and control groups respectively, showed significant increase of fumarate and UDP-glucuronate in treated control and no significant change was detected in the treated pilocarpine mice. Additionally, a comparison between treated pilocarpine and treated control groups displayed no significant difference, except for the significant increase of the O-phosphoethanolamine in treated control in contrast to ethanolamine which increased significantly in treated pilocarpine group. In conclusion, besides investigating the essential signaling pathway Wnt/β-catenin and HBP1, we have examined the effect of EGCG/DAC in epilepsy in order to develop a paradigm shift in treating and preventing epilepsy disorders.

Acknowledgement

I would like to express my sincere thanks to my advisor Dr. Jim Baleja for the continuous support, patience, motivation and immense knowledge. His guidance and assistance helped me during my work on thesis. Beside my advisor, I would like to thank the Pharmacology and drug development program for their support and for giving me this great opportunity which during I have gained massive laboratory skills and knowledge. Last but not the least, nobody has been more important to me in the pursuit of this project than the members of my family. I wish to thank my parents, whose love and guidance are with me in whatever I pursue they are the ultimate role models. Most importantly, I would like to thank my loving and supportive husband, Abdulrahman, and my wonderful daughter Nawara, for their unending inspiration and love.

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1. MacDonald, B.T., K. Tamai, and X. He, Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell, 2009. 17(1): p. 9-26.
2. Kim, J., et al., Suppression of Wnt signaling by the green tea compound (-)-epigallocatechin 3-gallate (EGCG) in invasive breast cancer cells. Requirement of the transcriptional repressor HBP1. J Biol Chem, 2006. 281(16): p. 10865-75.

List of Abbreviations

AEDs: Antiepileptic drugs.
AML: Acute myeloid leukemia.
AMPA: α-amino-3- hydroxy-5-methyl-4-isoxazole propionic acid.
AMP: Adenosine monophosphate.
APC: adenomatous polyposis coli gene.
CNS: Central nervous system.
CK1: Casein kinase 1.
DAC: 5-aza-2′-deoxycytidine.
2-DG: 2-Deoxy glucose.
EGCG: Epigallocatechin-3-gallate.
EP: Pilocarpine treated EGCG/DAC.
ES: Saline EGCG/DAC treated group.
FDA: Food and drug administration.
GABA: γ-amino butyric acid.
GAD: L-glutamic acid decarboxylase.
GSK3: Glycogen synthase kinase 3.
GTP: Guanosine triphosphate.
HET: Heterozygous.
HMG-box: High mobility group box.
HMDB: Human metabolome database.
IMP: Inosine monophosphate.
KO: Knockout.
LEF-1: Lymphoid enhancer factor-1.
LRP6: Lipoprotein receptor-related protein 6.
LRP5: Lipoprotein receptor-related protein 5.
NAA: N-acetylaspartate.
NMDA: N-methyl-D- aspartate.
NMR: Nuclear magnetic resonance.
NAD+: Nicotinamide adenine dinucleotide.
PDH: Pyruvate dehydrogenase.
PL: Pilocarpine.
PKC: protein kinase C.
PC: Pyruvate carboxylase enzyme.
S: Saline or control group.
SE: Status epilepticus.
SD: Standard deviation.
SGZ: Subgranular zone.
TCA: Tricarboxylic acid cycle.
TCF: T-cell factor.
TNBC: Triple-negative breast cancer.
UDP-glucose: Uridine diphosphate.
UMP: Uridine monophosphate.
WT: Wild type.

Chapter 1: Introduction

1.1. Epileptogenesis

Epilepsy, a disorder characterized by recurrent seizures, is estimated to affect at least 65 million people worldwide. Epilepsy is one of the most common chronic neurological disorders and is manifested by abnormal hypersynchronized neuronal discharge. The neuronal discharge is caused mainly by an imbalance between excitation and inhibition conductance. The increase in excitatory signaling pathway is considered as a main feature of epileptogenic activity [1-3]. A cascade of molecular, cellular, neuronal and metabolic alterations that are involved in the process of developing epilepsy called epileptogenesis [1]. In epileptogenesis, alterations in neuronal network appear during latent phase lead to development of spontaneous seizures and subsequently become chronic epilepsy [1]. The latent phase of epileptogenesis is characterized by no seizure activity after the initial seizure and is considered a crucial time for treatment interference to prevent the development of spontaneous seizures associated with epilepsy [1]. The exact mechanisms underlie epileptogenesis are still unclear. An explanation of epilepsy mechanisms and the underlying causes, could aid in finding a potential therapeutic approach to treat and prevent epilepsy disorders.

The first experiments in humans to investigate anti-epileptogenic interventions started more than 60 years ago [4]. Several antiepileptic drugs (AEDs) such as phenobarbital, carbamazepine, and valproate have since been used [4]. Although AEDs provide remission for approximately 70-80 % of epileptic patients, 20-30 % of patients do not respond. Furthermore, none of these AEDs prevent the development of seizure before the occurrence of the first attack nor development of drug resistant epilepsy [5, 6]. By

improving our understanding of the molecular and cellular mechanisms underlying epileptogenesis, new novel targets could be determined yielding more promising and efficacious therapeutic approaches leading to prevention of epilepsy [1].

Altered neurotransmitter balance has been a major hypothesis for the development of epilepsy. The principal excitatory neurotransmitters include glutamate, aspartate, serotonin, noradrenaline and adrenaline, and the principal inhibitory neurotransmitters include γ-amino butyric acid (GABA) and glycine [3]. GABA and glutamate are considered the most relevant neurotransmitters in epileptogenesis [3].

GABA is mainly synthesized from glutamate by L-glutamic acid decarboxylase (GAD) and interacts with two receptor subtypes: $GABA_A$ and $GABA_B$. In the Post-synapse, where $GABA_A$ receptors are found, the hyperpolarization is mediating the opening of ion channels to permit either influx of Cl^- or efflux of K^+, GABA ultimately mediating the inhibitory effect in the brain [3, 7, 8]. On the other hand, $GABA_B$ receptors are located both pre and post synaptically, resulting in an efflux of K^+ by coupling to G proteins and finally leading to hyperpolarization [3, 7] . The influx of Na^+ and outflow of K^+ are mediating the membrane depolarization and leading to the generation of action potential [9, 10]. Altered properties of hyperpolarization and sustained depolarization current are associated with pro-epileptogenic mechanisms [9, 10].

Glutamate has an essential role in fast excitatory synaptic conductance in cortex and hippocampus regions of the brain [11]. The glutamate receptors are divided into two classes, the ionotropic and the metabotropic receptors. The ionotropic class includes NMDA, AMPA and kainate receptors, which are named after the agonists that activate them: NMDA (N-methyl-D- aspartate), AMPA (α-amino-3- hydroxy-5-methyl-4-

isoxazole propionic acid) and kainic acid [12]. All ionotropic glutamate receptors are permeable to Na^+ and K^+ [12]. After the release of glutamate in extracellular fluid of neurons, glutamate is taken up by astrocytes and converted into glutamine by glutamine synthetase. Glutamine is then moved back to neurons and deaminated into glutamate. Glutamate in turn can be converted into α-ketoglutarate and acts as a substrate for the tricarboxylic acid cycle (TCA) [11, 13].

Increasing evidence demonstrate that epilepsy is a multifactorial disorder [14, 15]. Although the brain represents only 2% of body weight, it consumes around 25% of the total body glucose which is considered the main source of brain energy [3, 14]. Factors such as glucose hypometabolism, reduced level of neuronal transduction and disturbances in amino acid neurotransmitters are all playing an important role in developing epileptogenesis [14, 15]. Investigating such factors is an important step to improve our knowledge about epilepsy disorders and potential advances in expanding the available therapeutic options. Signaling pathways have distinct roles in epilepsy and many other neurological diseases, offering a new hope in treating and preventing epilepsy disorders [16].

1.2. Wnt/β-catenin Signaling Pathway and HBP1 gene

The Wnt/β-catenin signaling pathway has roles in modulating neurogenesis, neural differentiation, synapse development, and plasticity in the central nervous system (CNS) and thus, has an essential role as a regulator in the body [2, 16, 17]. When up-regulated, the Wnt signaling pathway results in abnormal neurogenesis and epileptogenesis [2]. The Wnt signaling pathway is involved in normal neurogenesis, especially in the subgranular

zone (SGZ) of hippocampal dentate gyrus [2]. Induction of epilepsy by pilocarpine results in neuronal damage as a result of abnormal neurogenesis and is characterized by elevated mossy fiber sprouting and activated Wnt/β-catenin signaling [2]. Binding of Wnt ligand to a seven transmembrane Frizzled receptor and its complex with low-density lipoprotein receptor-related protein 6 (LRP6), or its close relative LRP5, leads to activation of the Wnt/β-catenin pathway (Figure 1.1). This activation results in phosphorylation of LRP5/6 and activation of the Axin complex which is composed of adenomatous polyposis coli gene product (APC), casein kinase-1 (CK1), and glycogen synthase kinase 3 (GSK3). Eventually, β-catenin phosphorylation mediated by Axin complex will be inhibited leading ultimately to stabilization of β-catenin and then translocation into the nucleus to associate with lymphoid enhancer factor-1 (LEF-1) and T-cell factor (TCF) to activate the expression of Wnt target genes. When Wnt signaling is inactive, CK1 and GSK3 phosphorylate β-catenin, resulting in its degradation and prevents β-catenin from reaching the nucleus and forming a complex with LEF/ TCF. Thereby the Wnt target genes are suppressed by LEF/ TCF [16].

Another influential factor on Wnt signaling pathway is the HBP1 transcriptional repressor. HBP1 is a high mobility group box containing transcription factor (HMG-box). Many studies have also revealed the HBP1 as a regulator of cell differentiation [18, 19]. HBP1 suppresses Wnt/ β-catenin signaling by interacting with LEF/TCF proteins (without affecting β-catenin level) (Figure 2.2). Down regulation of HBP1 leads to Wnt signaling increase and ultimately occurrence of epilepsy [18, 19]. The Yee lab has shown that HBP1 is expressed in adult mouse hippocampus, and that HBP1 transiently declines in the epileptogenic period. This suggests that HBP1 may be a contributing factor in

developing epileptogenesis [18, 19]. Testing the role of HBP1 in epilepsy and its effect on metabolism in the brain could lead to identify a potential therapeutic strategy in the field of epilepsy.

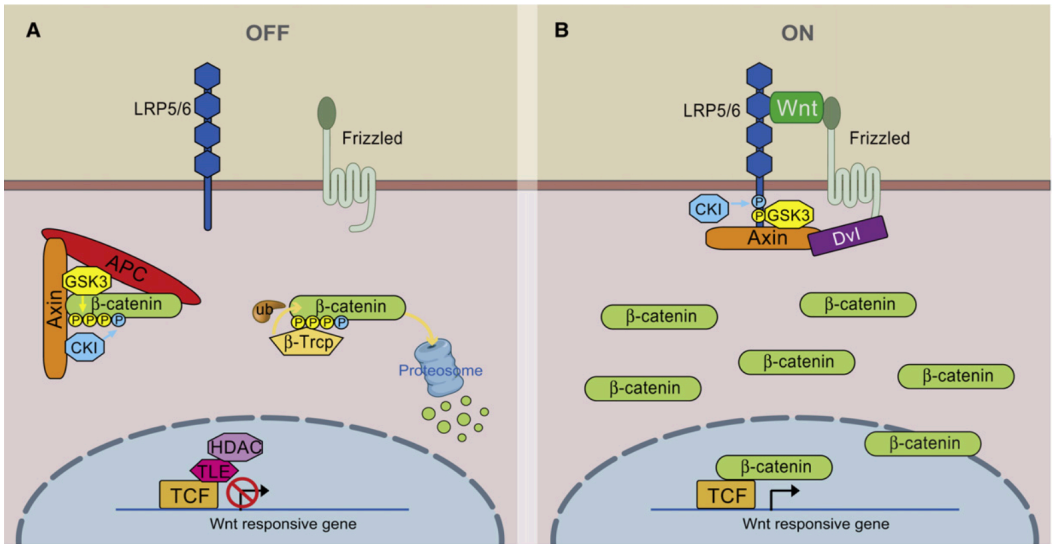


Figure 1.1: Wnt/ β-catenin signaling pathway. (A) Inactive and (B) Active Wnt / β-catenin signaling pathway [16]. Reprinted with permission from [MacDonald, B.T., K. Tamai, and X. He, Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell, 2009. 17(1): p. 9-26].

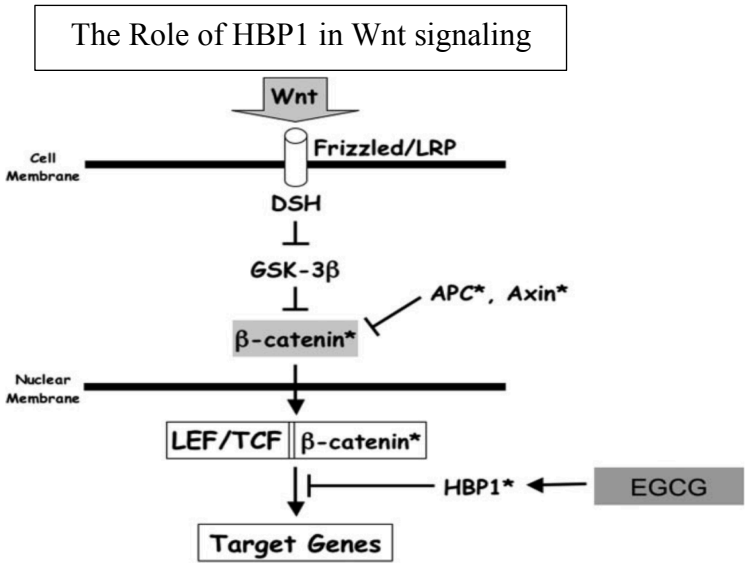


Figure 1.2: The Role of HBP1 in Wnt/ β-catenin pathway. HBP1 inhibits Wnt/ β-catenin pathway by inhibiting LEF/TCF. EGCG stabilizes the HBP1 and blocks the Wnt signaling pathway [18]. Adapted with permission from [Kim, J., et al., Suppression of Wnt signaling by the green tea compound (-)-epigallocatechin 3-gallate (EGCG) in invasive breast cancer cells. Requirement of the transcriptional repressor HBP1. J Biol Chem, 2006. 281(16): p. 10865-75]. The figure was adapted by removal of a box showing the effect of LiCL or SB415286 on GSK-3β.

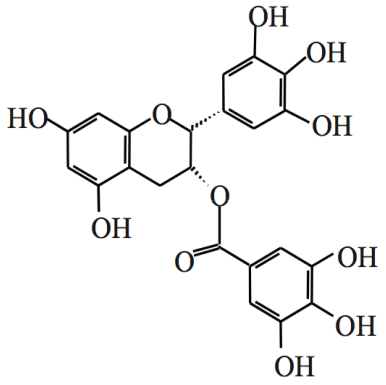
1.3. EGCG/DAC treatment

A strategy being explored in the Yee lab is a combination of EGCG and DAC. Epigallocatechin-3-gallate (EGCG) (Figure 1.3A), the main catechin in green tea, has revealed many beneficial effects in different diseases including neurodegenerative disorders [20]. A study published by the Yee lab shows that EGCG blocked Wnt signaling through stabilizing the HBP1 transcriptional repressor [19]. Also, many studies have illustrated the neuroprotective effect of EGCG by activating protein kinase C (PKC) and facilitating the glutamate release by enhancement of Ca^{2+} entry in the cerebrocortical nerve terminal [20-22]. Thus EGCG may prevent the decrease of

glutamate release that has been suggested to be involved in the pathogenesis of age-related neurodegenerative disorders [20].

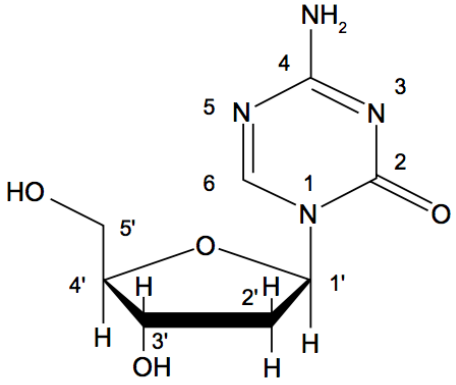
Decitabine (DAC) 5-aza-2′-deoxycytidine (Figure 1.3B), a DNA-hypomethylating agent is approved by the FDA for the treatment of myelodysplastic syndrome and acute myeloid leukemia (AML) [23]. The Yee lab experiments have demonstrated that the combination of DAC causes a synergistic effect with EGCG. DAC is used in treating many diseases that involve the DNA hypermethylation mechanism in their pathogenesis [24]. The combination of EGCG and DAC can cross blood brain barriers [21, 23], and could represent a promising therapy in treating and preventing epilepsy. The synergistic effect of the combination of DAC with EGCG results in reducing the growth of triple-negative breast cancer (TNBC) or lieomyosarcoma tumors by the induction of HBP1 expression in mouse xenograft model (unpublished results). Also, the Yee lab revealed that the number of seizure attacks was lowered with the administration of EGCG/DAC five days after inducing SE with pilocarpine, when compared with the control group (manuscript in preparation). In this thesis, we are interested in understanding the altered metabolism associated with Wnt signaling in epilepsy [18, 19].

(A)



(-)-Epigallocatechin-3-gallate

(B)



5-aza-2'-deoxycytidine

Figure 1.3: Chemical structures of EGCG and DAC [19, 20]. (A) EGCG is the main catechin in green tea. (B) Decitabine is an FDA approved drug for the treatment of myelodysplastic syndrome and AML.

1.4. Nuclear Magnetic Resonance (NMR) Spectroscopy and Metabolomics

NMR based metabolomics is a powerful and sensitive method to assess metabolism. NMR can be used to assess the biochemical irregularities and differences in metabolic states between various conditions. NMR method is used to identify compounds and the relative concentrations of those compounds. One of the most interesting applications of

NMR is the broad variety of biological samples that can be investigated using this method such as blood, urine, cells, isolated perfused organs, and tissues in vivo. The basic idea of NMR spectroscopy is that when nuclei are placed in a magnetic field and then excited, signals are produced. Nuclei in different chemical groups will resonate at different frequencies in an effect known as the chemical shift. The basic idea of NMR chemical shift is that by sharing electrons, nuclei in different chemical structures are bonding together. As a result, different chemical groups are surrounded by different electrons, these electrons have associated magnetic fields depending on their numbers. The multiple signals corresponding to all of the distinct chemical groups are detected in the NMR spectrum. After obtaining NMR spectra, databases that contain the approximate peak positions of common organic compounds are used to match known substances with the experimentally obtained peaks [25]. In this thesis, we used NMR to investigate the effects of altering Wnt signaling on the metabolism in a model of epileptogenic brain. Also, we aimed to determine the efficacy and molecular mechanism of EGCG/DAC treatment on altered metabolic pathways during epileptogenesis.

Chapter 2: Materials and Methods

2.1. Animal model

Much of the improved understanding of epilepsy disorders has been gained from appropriate animal models. A great variety of animal models of epilepsy has played a major role in studying the pathophysiology of human epilepsies [26]. The pilocarpine model is now widely used, and it has been modified in a number of laboratories [26]. The Pilocarpine to induces SE by acting as an agonist of the M1 muscarinic receptor subtype. Pilocarpine causes an imbalance between excitatory and inhibitory transmission resulting subsequently in the generation of SE [27]. A detailed study has compared the pilocarpine model in three different mouse strains FVB/N, C57BL/6 and CD-1 [27]. The mouse strain FVB/N demonstrated the best response in terms of developing SE although with higher mortality [27]. Generally, FVB/N mice presented larger hippocampal damage and a stronger reactive gliosis [27]. In this thesis, we used 7 weeks old FVB/N mice weighing 25g for experiments (the mouse model was generated by the Yee lab). The FVB/N mice strain were bred to include an HBP1 knock out allele caused by the insertion of the B-galactosidase gene into exon 2 (the first coding exon) of HBP1. Mice could be bred to be WT, heterozygous (HET), or knockout (KO) mice were obtained. To limit individual variation, only male mice were used for these experiments.

2.2. Status epilepticus (SE) induction and EGCG/DAC model

SE was induced by the Yee lab through intraperitoneal injection of pilocarpine (Milestone PharmTech). The initial pilocarpine dose used in HBP1 KO mice was 150 mg/kg, then 135 mg/kg to minimize death rate. For HBP1 WT mice, 220 mg/kg dose of

pilocarpine was used. For the EGCG/DAC treated groups the Yee lab treated the mice with 16.5mg/kg of EGCG and 0.5 mg/kg of DAC continuously for 7 days. An (S) group which represents the control mice were injected with saline. An (ES) group that represents the negative control received EGCG/DAC without the induction of SE by pilocarpine. An (PL) group is obtained as a result of SE induction by pilocarpine. An (EP) group represents the treated samples with the combination of EGCG and DAC after inducing SE by pilocarpine and obtaining the altered metabolism. SE was considered started when the mouse developed stage 5 seizures according to Racine's scale (Racine, 1972). After induction of seizure or control injection, mice were maintained for 5 days, and then were sacrificed. The Yee lab has observed that by 3 days post-SE induction there was a modest enhancement of GSK protein phosphorylation and it becomes maximal by the fifth day post-SE with Wnt signaling activation and β-catenin expression, and declining to control level by day 7. We therefore assumed that changes in metabolism would follow the same time course as the level of protein. Two samples, one frontal cortex and one hippocampus were taken from each half of the brain, frozen on dry ice, and stored at (-80° C) until analysis.

2.3. Tissue Extraction

After 5 days of Pilocarpine induced-SE, mice were injected intraperitoneally with 125μL of ^{13}C-glucose at 5mg/mL in Saline. Mouse brain was harvested 30 minutes after injection, samples were dissected from each half of the brain frontal cortex and the hippocampus on ice, and then flash-frozen and finally stored at (-80° C). All these procedures were done by the Yee Lab.

The tissue extraction protocol was adapted from the "two-step" procedure described by Wu et al [28]. First, we added 0.4 mL of cold 100% methanol (-20 ºC) and 0.085 mL cold water (4 ºC). Then, tissue was minced in a 1.5 mL Eppendorf tube until chunks are less than 1 mm. After that, tissue was sonicated while keeping on ice as much as possible until homogenous, we used continuous duty and amplitude at 40 using a microtip sonicator probe. Sample was kept on ice when we added a few beads of Chelex-100, since the samples may contain paramagnetic metal ions such as Fe^{2+}. Paramagnetic impurities can affect the NMR spectra by increasing the relaxation rate which causes broadening of resonances [29]. After adding the Chelex-100 we added 0.4 mL of cold chloroform (-20 ºC) and 0.085 mL cold water (4 ºC). Then we vortexed the samples for 60 seconds, left them on ice for 10 minutes and after that samples were centrifuged for 5 minutes at 2000g at (4 ºC) to separate out layers. The upper polar (methanol/water) layer was removed to a fresh tube, and the lower nonpolar (chloroform) layer was removed to another fresh tube. All samples were dried down using the speed-vacuum overnight. Dried samples were stored at (-20 ºC) until NMR analysis.

2.4. Samples

In this project, we have tested multiple sets of samples. To investigate the role of HBP1 in epilepsy, we have tested a total of six ^{13}C glucose labeled Hippocampus Brain tissue samples (Table 2.1). Those samples are divided into two groups, Wild type (WT) and HBP1 knockout (KO) groups. HET group was kept in reserve and would be used if we found significant difference between the HBP1 KO and WT groups. Each group is composed of three samples. In order to investigate the EGCG/DAC efficacy we tested

twenty-eight ^{13}C glucose labeled samples. Samples were divided into two groups according to the tissue region in the brain. The first group is the Hippocampus and the second group is the cerebral cortex, each group is divided into four subgroups (Table 2.2). First subgroup is the Saline or control (S), this set is composed of four samples labeled as S#1, S#2, S#4 and S#5. Second set is Saline treated EGCG/DAC subgroup (ES), this set is composed of three samples ES#1, ES#2 and ES#4. Third set is the Pilocarpine (PL) subgroup, this set composed of four samples P#1, P#2, P#3 and P#5. The final set is the Pilocarpine treated EGCG/DAC (EP) subgroup, this set is composed of three samples EP#3, EP#5 and EP#6.

WT Samples	**HBP1 KO Samples**
3554	3553
3557	3555
3571	3556

Table 2.1: Wild type (WT) and HBP1 KO samples.

Hippocampus			
Saline(S)	Saline + EGCG/DAC	Pilocarpine	Pilocarpine + EGCG/DAC
S#1	ES#1	PL#1	EP#3
S#2	ES#2	PL#2	EP#5
S#4	ES#4	PL#3	EP#6
S#5		PL#5	
Cerebral Cortex			
Saline(S)	Saline+ EGCG/DAC	Pilocarpine	Pilocarpine+ EGCG/DAC
S#1	ES#1	PL#1	EP#3
S#2	ES#2	PL#2	EP#5
S#4	ES#4	PL#3	EP#6
S#5		PL#5	

Table 2.2: Hippocampus and Cerebral cortex samples. Top Represents samples in the hippocampus and bottom represents samples in cerebral cortex.

2.5. ^{1}H and ^{13}C NMR spectroscopy

In our ^{1}H NMR experiments, we used two choices of tubes. The standard 5 mm NMR tubes were used with WT and HBP1 KO samples, and Shigemi tubes were used with S, ES, PL and EP samples. The standard 5 mm NMR tubes is slightly easier to obtain ^{1}H NMR spectra with narrower resonances and thus better NMR data. In order to prepare the WT and HBP1 KO samples for analysis, the dried samples were re-suspended in 550μL of 50 mM PO_4 and 0.5 mM of NMR standard (deuterated DSS-d6) in D_2O. Before

placing the samples in NMR tube, samples were vortexed and then centrifuged in room temperature for two minutes. 530μL was placed in the NMR tube to be analyzed. The number of scans for the ^{1}H was 128 scans and 0.5 mM concentration of DSS-d6 was set as the standard for each ^{1}H NMR spectrum,

Using Shigemi tubes, samples were re-suspended in 300 μL with the same NMR standard compound, vortexed and centrifuged for two minutes in room temperature. Then, the whole sample was placed in the Shigemi tube for analysis. Shigemi tubes use less volume and therefore it provides more concentrated samples, and provide higher quality data of ^{13}C NMR. After the samples placed in tubes, they were analyzed by NMR and produce a spectrum of metabolites. Typically, the number of scans was for ^{13}C was 12,000 to 20,000 scans, and the 5μM concentration was set for ^{13}C NMR spectrum (^{13}C at 1% abundance in DSS, 0.5x0.01= 5μM). NMR analysis of samples was done using the Bruker Avance III 600 spectrometer. ^{1}H and ^{13}C spectra were recorded at 25°C. Usually, the detected metabolite contains one or more hydrogens, and each of these hydrogens produces one or more peaks. The number of peaks generated by a metabolite, as well as their location and ratio of heights, are reproducible and uniquely determined by the chemical structure of the molecule [30].

2.6. Metabolite quantification

We quantified amounts of metabolites in hippocampus for WT and HBP1 KO samples using ^{1}H and ^{13}C NMR spectroscopy. In S, ES, PL, EP groups the metabolite concentrations were quantified using ^{1}H NMR in hippocampus and cortex. Relevant peaks of the ^{1}H NMR spectra were analyzed using CHENOMX program. CHENOMX is

a program which allows identification of compounds by matching spectra of individual compound in its database to the spectra. Once NMR spectra are acquired, their analysis involves many steps starting with processing data, phase and baseline correction, peak linewidth adjustment, spectral fitting, identification and quantification of metabolites, normalization and ending with statistical analysis of data. The normalization was done by dividing observed concentration by the sum of all observed metabolites. The ^{13}C NMR data were processed using the BRUKER TopSpin (version 3.5) software and we used the reference library human metabolome database (HMDB) for metabolites identification. The acquired metabolite levels were in arbitrary units. The results of the analysis were exported into Excel files where average values, and significant changes using T-test were determined. $P<0.05$ was considered to indicate a statistically significant difference.

Chapter 3: Results

3.1. The Effect of HBP1 on Metabolism in Epilepsy

We hypothesized that HBP1 transcriptional repressor is a causative factor in developing epileptogenesis, as illustrated by many studies that HBP1 is a Wnt suppressor [18, 19]. When Wnt signaling is up regulated, the epileptogenic activity will occur. To test this hypothesis, we used an experimental model of mice lacking the HBP1 gene ($HBP1^{-/-}$), and WT mice which used as control to obtain the metabolic profile in hippocampus. The Yee lab has shown that HBP1 in the adult mice is highly expressed in CA1 region of hippocampus compared to other regions (unpublished results). After the fifth day post-PL SE induction, both groups were examined at the steady state level ^{1}H NMR and at the non-steady state level following a pulse of ^{13}C glucose.

3.1.1. Metabolism by ^{1}H NMR spectroscopy

The ^{1}H NMR analysis conducted here produced spectra as depicted in (Figure 3.1). These spectra were analyzed in order to determine concentrations compared to the DSS-d6 standard, which can be seen as the peak at 0 ppm. By analyzing the spectra using Chenomx, we obtained the concentrations of various metabolites in hippocampus (Figure 3.2). Metabolite levels showed no significant differences between the HBP1 knockout group and the wild type group (Table 3.1) as all P values were greater than 0.05.

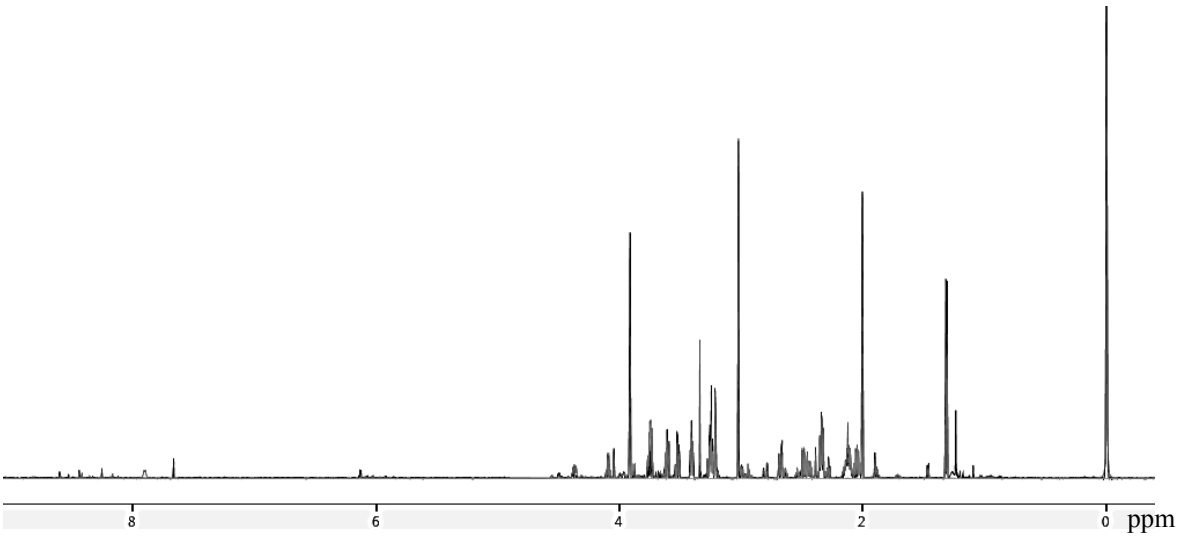


Figure 3.1 Representative of 600 MHz 1H NMR spectrum of hippocampus. The peak at 0 ppm is the DSS-d6 standard.

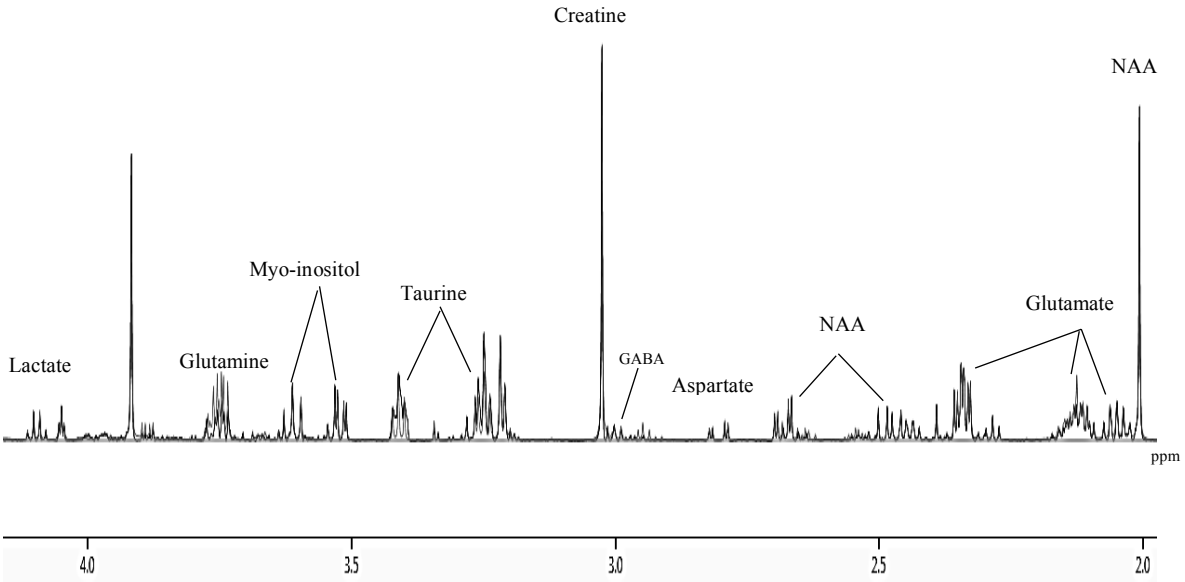


Figure 3.2: Close up of metabolites found between 2 and 4.3 ppm.

Metabolites	3571 Wt	3554 Wt	3557 Wt	3555 KO	3553 KO	3556 KO	Fold change	TTEST
4-Aminobutyrate	2.20	2.23	2.09	2.14	2.43	2.20	1.04	0.47
ADP	0.70	0.72	0.61	0.64	0.77	0.70	1.04	0.62
AMP	2.20	2.01	2.41	2.20	1.81	2.20	0.94	0.48
Acetate	0.24	0.17	0.16	0.25	0.20	0.25	1.25	0.20
Adenosine	0.22	0.26	0.30	0.33	0.22	0.22	0.98	0.89
Alanine	0.82	0.94	0.66	0.81	0.92	0.82	1.05	0.68
Ascorbate	4.17	3.64	4.32	3.92	3.87	3.92	0.97	0.57
Aspartate	2.19	2.09	2.27	2.11	2.81	2.28	1.10	0.41
Choline	0.07	0.09	0.10	0.08	0.06	0.07	0.81	0.25
Creatine	12.15	11.70	12.30	12.17	11.10	12.14	0.98	0.58
Creatine phosphate	0.10	0.09	0.18	0.11	0.14	0.10	0.95	0.84
Ethanolamine	0.16	0.28	0.33	0.13	0.16	0.15	0.57	0.16
Formate	0.26	0.22	0.21	0.25	0.25	0.26	1.09	0.29
Fumarate	0.03	0.03	0.03	0.03	0.02	0.03	0.97	0.82
GTP	0.28	0.22	0.24	0.27	0.26	0.27	1.08	0.36
Glutamate	14.87	15.50	14.28	14.88	15.86	14.73	1.02	0.61
Glutamine	5.28	5.21	3.81	4.26	4.20	5.28	0.96	0.77
Glutathione	1.19	1.23	1.41	1.22	1.22	1.15	0.94	0.35
Glycine	0.87	0.87	0.72	0.67	0.85	0.91	0.99	0.91
Histidine	0.09	0.09	0.09	0.08	0.11	0.09	1.00	0.97
IMP	0.17	0.14	0.26	0.21	0.12	0.17	0.86	0.60
Isoleucine	0.13	0.06	0.08	0.06	0.06	0.13	0.92	0.82
Lactate	8.29	8.16	7.64	7.81	9.15	8.31	1.05	0.44
Leucine	0.26	0.18	0.18	0.20	0.22	0.25	1.08	0.60
Methionine	0.29	0.23	0.41	0.34	0.33	0.27	1.01	0.95
N-Acetylaspartate	8.96	8.89	9.19	9.58	8.70	9.31	1.02	0.57
NAD+	0.43	0.55	0.53	0.45	0.49	0.43	0.91	0.38
O-Phosphocholine	0.61	0.69	0.84	0.63	0.61	0.60	0.86	0.28
O-Phosphoethanolamine	1.81	1.54	0.47	1.91	2.22	1.83	1.56	0.22
Oxypurinol	3.62	3.94	4.19	4.64	3.10	3.53	0.96	0.77
Phenylalanine	0.06	0.04	0.12	0.04	0.04	0.07	0.71	0.50
Pyruvate	0.05	0.05	0.05	0.05	0.06	0.03	0.94	0.75
Serine	0.90	0.97	0.55	0.51	1.35	0.84	1.11	0.76
Succinate	0.54	0.56	0.47	0.58	0.54	0.54	1.06	0.37
Taurine	16.85	16.97	17.68	17.25	16.07	16.85	0.97	0.37
Threonine	0.31	0.42	0.16	0.14	0.46	0.33	1.04	0.92
Tyrosine	0.14	0.15	0.22	0.15	0.15	0.16	0.90	0.57
UDP-galactose	0.08	0.04	0.06	0.04	0.05	0.05	0.77	0.31
UDP-glucose	0.12	0.07	0.14	0.11	0.11	0.11	0.98	0.93
UDP-glucuronate	0.03	0.03	0.05	0.01	0.02	0.05	0.71	0.38
UMP	0.14	0.12	0.20	0.19	0.14	0.13	1.00	1.00
Valine	0.17	0.21	0.19	0.14	0.19	0.17	0.87	0.23
myo-Inositol	6.86	7.15	8.32	7.17	7.30	6.96	0.96	0.57
sn-Glycero-3-phosphocholine	1.09	1.24	1.49	1.26	1.27	1.12	0.96	0.69

Table 3.1: Steady state 5-Day post-PL induced-SE Hippocampal metabolism in both WT and HBP1 KO groups. Polar metabolites were identified and quantified by[1H] NMR showing no significant differences between the HBP1 KO and the control group.

3.1.2. Metabolism by ^{13}C NMR spectroscopy

In this thesis, mice were injected with ^{13}C glucose to obtain information about the metabolic flux in epilepsy. Metabolic flux provides detailed information and establishment of which metabolites are produced or consumed using ^{13}C isotopes labeled [31]. The formed labeled metabolites in HBP1 KO and control groups were analyzed

using TopSpin to derive information on the metabolic pathways and fluxes of hippocampus.

To elucidate the ^{13}C results, it is important to explain and analyze the metabolism of glucose. Uniformly ^{13}C-labeled Glucose is metabolized to [U-^{13}C] pyruvate, which can be converted to different metabolites. [U-^{13}C] Pyruvate could be converted into [U-^{13}C] alanine, [U-^{13}C] lactate, or metabolized by pyruvate dehydrogenase (PDH) and enter the tricarboxylic acid (TCA) cycle as [1,2-^{13}C] acetyl-CoA. Metabolism of [1,2-^{13}C] acetyl-CoA in the TCA cycle results in production of [4,5-^{13}C] α-ketoglutarate, which is a precursor for [4,5-^{13}C] glutamate. Subsequently, [4,5-^{13}C] glutamate may be converted to [4,5-^{13}C] glutamine by the enzyme glutamine synthetase or to [1,2-^{13}C] GABA in GABAergic neurons. Pyruvate could lead also to the production of [2,3-^{13}C] α-ketoglutarate by pyruvate carboxylase enzyme (PC) and ultimately formation of astrocytic [2,3-^{13}C] glutamate, [2,3-^{13}C] glutamine and [3,4-^{13}C] GABA [15]. Because of tissue specificity of the enzymes, pyruvate when metabolized by PC enzyme eventually leads to the formation of astrocytic glutamate, but when metabolized by PDH this is indicative that the formed glutamate is in the neurons.

Since the sample 3553 of the HBP1 KO group was unlabeled, we compared three samples of the WT group to two samples of the HBP1 KO group. To detect the differences between the HBP1 KO and WT groups, we calculated the average value and the standard deviation (SD) of lactate compound. No significant differences were detected between the two groups ($P \geq 0.05$) (Figure 3.3).

The imbalance leading to epileptogenesis is characterized by decrease in inhibitory neurotransmitter GABA and increase in excitatory neurotransmitter glutamate. Therefore, we were interested in investigating the ratio of GABA to glutamate in samples of both groups. No significant changes were detected between the samples (P≥0.05) (Figure 3.4).

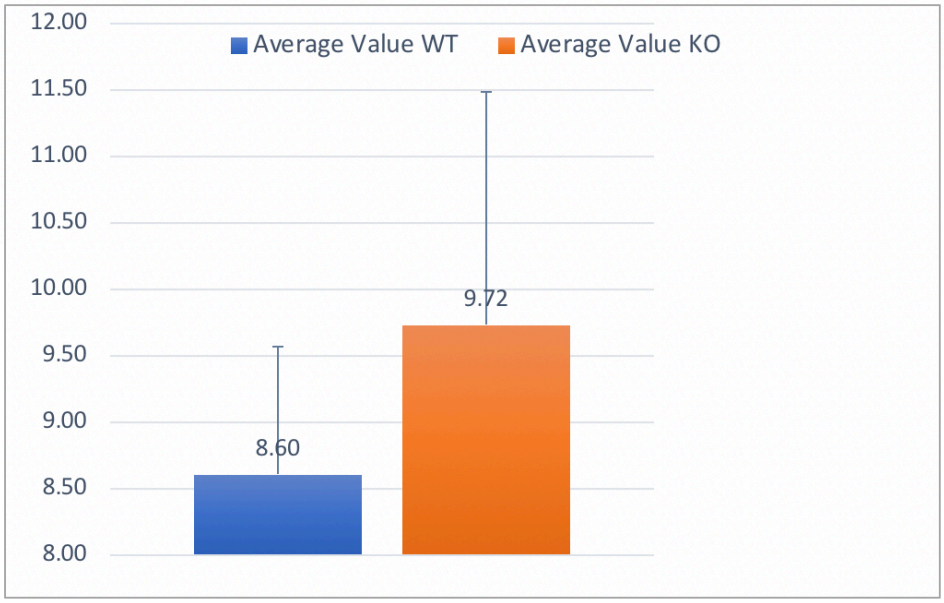


Figure 3.3: Average value of lactate in WT and HBP1 KO groups. No significant difference was detected (P≥05), the error bars indicate the standard deviation.

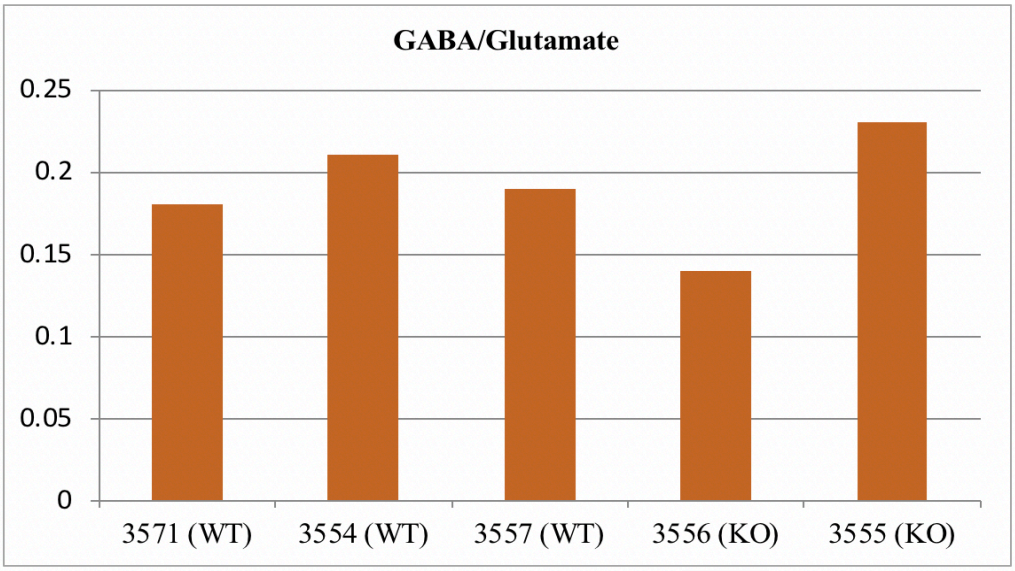


Figure 3.4: GABA to glutamate ratio of WT and HBP1 groups. No significant differences were detected between the inhibitory and excitatory neurotransmitters in both groups.

No significant results have been shown with glutamine to glutamate ratio in WT and HBP1 KO groups (Figure 3.5). To identify whether the formed glutamate was in the neurons or astrocytes, we compared the [4,5- ^{13}C] glutamate which labeled via PDH to [2,3- ^{13}C] glutamate which labeled via PC. However, besides the unlabeled ^{13}C glucose sample 3553 HBP1 KO, we saw no peaks indicating ^{13}C-^{13}C splitting in sample 3556 HBP1 KO therefore we could not measure [4,5- ^{13}C] glutamate to [2,3- ^{13}C] glutamate ratio (Figure 3.6). Despite the sample 3556 HBP1 KO was clearly ^{13}C glucose labeled because the third lactate carbon (C3) which showed ^{13}C-^{13}C splitting and thus was neighbored by other ^{13}C carbons, the glutamate displayed by one peak only which represents the β carbon which make the glutamate ^{13}C labeling undetectable. To explain the reason behind the untraceable sample, usually the detectable glutamate compound during analysis is represented by multiple peaks (Table 3.2A) (Figure 3.7A). Those peaks

arise from the glutamate structure which is consistent of 5 carbons that resonate at 183.91, 177.29, 57.57, 36.23 and 29.7 ppm. These carbons include α, β and γ carbons which appear on carbon number 2,3 and 4 respectively. The β carbon at 29.7 ppm, which represent the middle peak illustrates the attachment of the ^{12}C-^{13}C and neighbored by peaks illustrating ^{13}C-^{13}C coupling (Figure 3.7A). Sample 3556 HBP1KO have produced and represented the glutamate only by the β carbon peak which illustrate the ^{13}C that is attached to ^{12}C only (Table 3.2B) (Figure 3.7B). Thus, we could not calculate or detect any statistically significant difference of [4,5- ^{13}C] glutamate to [2,3- ^{13}C] glutamate ratio between the WT group and HBP1 KO group. Because of 3556 HBP1 KO sample was represented only by the β carbon peak and the ^{13}C unlabeled 3553 HBP1 KO sample, two of three samples of HBP1 KO group were not eligible to be included in the analysis. So, a complete analysis and comparison between the HBP1 KO samples and WT could not be done.

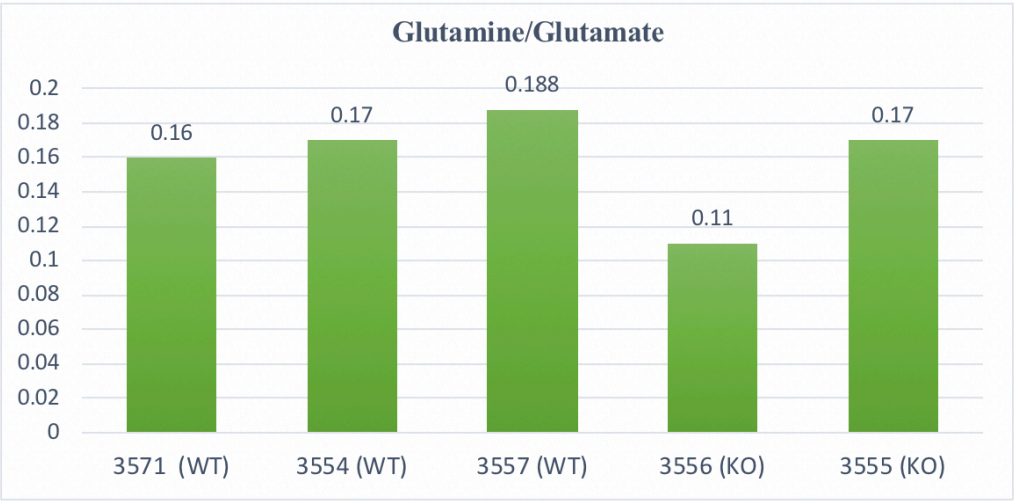


Figure 3.5: Glutamine to glutamate ratio. No significant difference between the samples in both groups WT and HBP1 KO was detected.

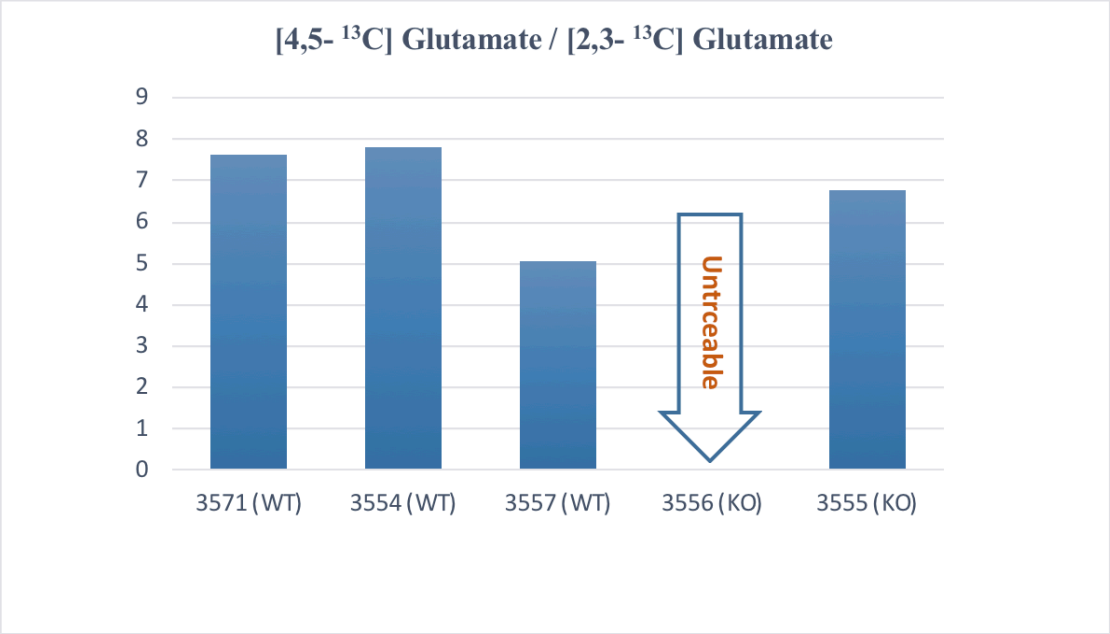


Figure 3.6: Pyruvate metabolism. Represents [4,5- ^{13}C] Glutamate to [2,3- ^{13}C] Glutamate ratios in all samples of WT and HBP1 KO groups, the results could not be detected since the 3553 HBP1 KO sample was unlabeled and the 3556 sample was not traceable.

(A)

Peaks	ppm	Intensity [abs]
1	29.7782	156159.00
2	29.6758	1043946.41
3	29.5495	203596.46

(B)

Peak	ppm	Intensity [abs]
1	29.7286	756017.9141

Table 3.2: Identified peaks of glutamate compound. (A) showing peak number that representing the traceable glutamate compound during analysis and their intensities. (B) showing the one peak representing the untraceable glutamate compound and its intensity.

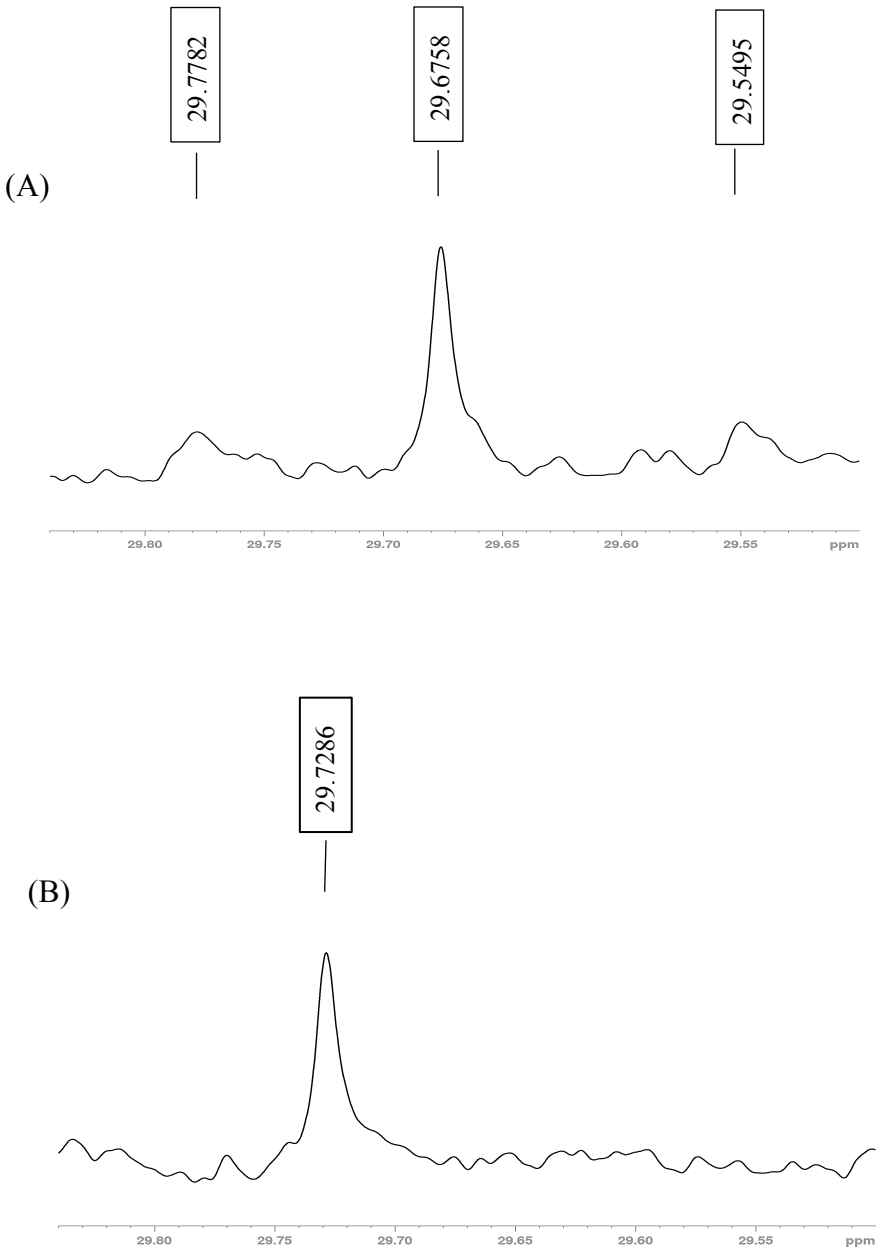


Figure 3.7: Assessment of ^{13}C labeling in glutamate. (A) The detectable β carbon in glutamate compound represented by multiple peaks as indicated by the arrows (B) The β

carbon in untraceable glutamate compound represented as one peak only as indicated with the arrow.

3.2. The effect of EGCG/DAC on metabolism in epileptogenic brain

Our interest to understand the effect of EGCG/DAC combination in altered metabolism in epilepsy was raised after the promising results of the combination in reducing the growth of triple- negative breast cancer (TNBC), and the decreased number of seizure attacks in the fifth-day post-Pilocarpine inducing SE (Yee lab, unpublished observations).

Metabolite contents of cerebral cortex and hippocampus were analyzed to investigate the energy and the brain metabolism of various metabolites in epileptogenic mouse brain in comparison with control group. Some samples after the analysis were ^{13}C glucose unlabeled (Table 3.3), possibly owing to an error in the injection site of ^{13}C glucose such as the abdominal cavity [32]. Since many samples were ^{13}C unlabeled, amounts of metabolites in hippocampus and cerebral cortex of (S), (ES), (PL) and (EP) groups were quantified using ^{1}H NMR spectroscopy only. We noticed that the carbon number three in lactate compound in some samples was not neighbored by any other carbons. When carbon three in lactate is not neighbored by other carbons, and appeared as one peak, this is an indication of unlabeled samples (Figure 3.8A). In labeled samples, lactate carbon number three will be adjacent to other carbons and during analysis will appear as three peaks (Figure 3.8B). To assess the effect of EGCG/DAC in altered metabolism of epileptogenic brain, we obtained the concentrations of different metabolites in cortex and hippocampus utilizing ^{1}H NMR spectroscopy.

Samples	^{13}C glucose Labeling
S#1	Labeled
S#2	Unlabeled
S#4	Unlabeled
S#5	Unlabeled
ES#1	Labeled
ES#2	Labeled
ES#4	Labeled
PL#1	Labeled
PL#2	Labeled
PL#3	Labeled
PL#5	Unlabeled
EP#3	Labeled
EP#5	Unlabeled
EP#6	Labeled

Table 3.3: ^{13}C glucose labeled and Unlabeled samples. Labeled and unlabeled ^{13}C glucose samples of (S), (ES), (PL) and (EP) groups that were identified after analysis due to possible error in the injection site.

First, we assessed metabolites alterations in Pilocarpine induced-SE (PL) group in comparison with control (S) group in both hippocampus (Table 3.4) and cortex (Table 3.5). Both hippocampus and cortex showed significant metabolite alterations.

In hippocampus, GABA, AMP, Adenosine, Creatine, NAD+, serine and taurine were significantly decreased in (PL) compared to Control (S) mice ($P<0.05$), but were not altered in cortex. Glutamate and N-acetylaspartate were significantly decreased in (PL) mice compared to (S) in both brain regions ($P<0.05$). On the other hand, glutamine was significantly increased in the (PL) group in both hippocampus and cortex. We found that the concentrations of IMP, lactate, leucine, lysine, UDP-glucose, UMP and myo-inositol were significantly increased in the (PL) group ($P<0.05$) in hippocampus. Furthermore, acetate, GTP and glycine levels were significantly increased in the (S) group in contrast to ascorbate, ethanolamine and glutathione concentrations which were significantly increased in the (PL) mice in cortex.

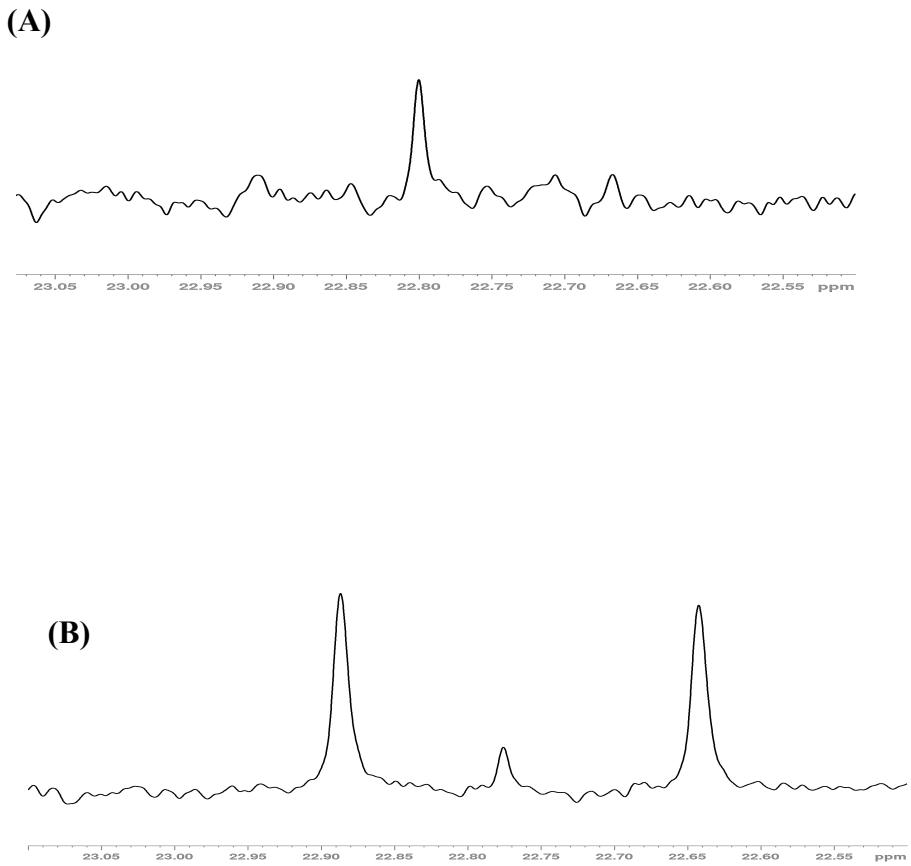


Figure 3.8: Assessment of ^{13}C labeling. The third carbon in the lactate compound indicating the ^{13}C labeling and unlabeling, (A) The third carbon in the lactate compound is not neighbored by other carbons which indicates unlabeled sample. (B) The third carbon in the lactate compound is neighbored by other carbons which indicate that the sample is ^{13}C glucose labeled.

Metabolites	PL#1	PL#2	PL#3	PL#5	S#1	S#2	S#4	S#5	Fold change	TTEST
4-Aminobutyrate	**1.99**	**2.19**	**1.88**	**1.89**	**2.57**	**2.46**	**2.54**	**2.69**	**0.78**	**0.00**
ADP	0.15	0.16	0.24	0.19	0.17	0.21	0.13	0.15	1.11	0.49
AMP	**1.81**	**1.73**	**1.97**	**1.86**	**2.11**	**2.19**	**1.99**	**2.14**	**0.88**	**0.01**
Acetate	0.21	0.21	0.21	0.18	0.18	0.23	0.32	0.34	0.76	0.19
Adenosine	**0.21**	**0.19**	**0.17**	**0.15**	**0.34**	**0.27**	**0.24**	**0.26**	**0.65**	**0.01**
Alanine	0.93	0.86	0.77	0.87	0.85	0.80	0.86	0.93	1.00	0.97
Arginine	0.12	0.39	0.13	0.47	0.29	0.15	0.63	0.17	0.90	0.84
Ascorbate	3.22	2.98	2.85	2.91	3.48	3.08	3.00	2.55	0.99	0.85
Aspartate	3.09	3.04	2.62	3.12	3.13	3.34	3.05	3.33	0.92	0.13
Choline	0.16	0.19	0.21	0.16	0.18	0.13	0.13	0.13	1.25	0.09
Creatine	**11.39**	**10.70**	**11.19**	**10.92**	**11.66**	**12.03**	**11.42**	**11.89**	**0.94**	**0.01**
Creatine phosphate	0.33	0.04	0.02	0.16	0.04	0.00	0.11	0.00	3.68	0.26
Ethanolamine	0.18	0.19	0.26	0.18	0.16	0.04	0.15	0.18	1.51	0.13
Formate	0.39	0.40	0.55	0.38	0.30	0.53	0.74	0.63	0.79	0.32
Fumarate	0.09	0.09	0.08	0.08	0.09	0.11	0.12	0.10	0.84	0.06
GTP	0.19	0.19	0.20	0.20	0.10	0.23	0.23	0.23	0.99	0.94
Glutamate	**14.71**	**12.29**	**13.00**	**13.98**	**15.70**	**15.78**	**16.47**	**16.62**	**0.84**	**0.01**
Glutamine	**7.23**	**7.65**	**7.13**	**9.25**	**5.50**	**5.52**	**5.03**	**4.96**	**1.49**	**0.01**
Glutathione	2.51	2.34	3.59	3.08	3.52	2.41	2.89	1.71	1.09	0.62
Glycine	0.79	0.95	0.83	0.65	0.82	0.82	0.88	0.78	0.98	0.80
Histidine	0.10	0.14	0.13	0.16	0.08	0.09	0.08	0.08	1.65	0.03
IMP	**0.31**	**0.39**	**0.60**	**0.49**	**0.23**	**0.24**	**0.19**	**0.30**	**1.89**	**0.04**
Isoleucine	0.05	0.06	0.06	0.04	0.04	0.03	0.05	0.06	1.17	0.38
Lactate	**13.68**	**15.90**	**14.24**	**13.57**	**11.10**	**11.85**	**11.29**	**11.58**	**1.25**	**0.01**
Leucine	**0.14**	**0.21**	**0.17**	**0.16**	**0.12**	**0.09**	**0.13**	**0.14**	**1.41**	**0.04**
Lysine	**0.64**	**0.63**	**0.48**	**0.45**	**0.22**	**0.20**	**0.07**	**0.32**	**2.70**	**0.00**
Methionine	0.23	0.30	0.34	0.28	0.16	0.28	0.37	0.20	1.15	0.52
N-Acetylaspartate	**6.01**	**4.20**	**5.27**	**5.05**	**8.10**	**8.24**	**8.00**	**8.52**	**0.62**	**0.00**
NAD+	**0.11**	**0.12**	**0.13**	**0.10**	**0.17**	**0.17**	**0.13**	**0.14**	**0.75**	**0.02**
O-Phosphocholine	0.58	0.67	0.73	0.57	0.74	0.66	0.65	0.67	0.94	0.36
O-Phosphoethanolamine	1.67	1.83	1.69	1.59	1.96	1.74	2.31	1.73	0.88	0.18
Phenylalanine	0.11	0.13	0.13	0.13	0.08	0.06	0.09	0.12	1.41	0.06
Pyruvate	0.09	0.05	0.06	0.06	0.04	0.03	0.10	0.01	1.43	0.44
Serine	**1.20**	**1.22**	**1.08**	**1.01**	**0.89**	**0.86**	**0.79**	**0.91**	**1.31**	**0.01**
Succinate	0.34	0.34	0.32	0.35	0.28	0.29	0.33	0.34	1.10	0.13
Taurine	**13.81**	**12.95**	**12.63**	**13.31**	**14.07**	**15.12**	**15.14**	**15.66**	**0.88**	**0.01**
Tyrosine	0.10	0.11	0.12	0.10	0.08	0.08	0.12	0.12	1.06	0.66
UDP-galactose	0.13	0.28	0.28	0.21	0.15	0.14	0.08	0.13	1.82	0.05
UDP-glucose	**0.20**	**0.22**	**0.23**	**0.21**	**0.12**	**0.17**	**0.13**	**0.19**	**1.39**	**0.03**
UDP-glucuronate	0.04	0.11	0.12	0.07	0.03	0.05	0.04	0.03	2.15	0.09
UMP	**0.25**	**0.33**	**0.41**	**0.35**	**0.15**	**0.20**	**0.14**	**0.16**	**2.08**	**0.01**
Valine	0.19	0.21	0.20	0.15	0.18	0.16	0.16	0.17	1.11	0.28
myo-Inositol	**8.81**	**10.57**	**10.67**	**9.39**	**8.44**	**7.66**	**7.42**	**7.39**	**1.28**	**0.01**
sn-Glycero-3-phosphocholine	1.48	2.23	2.02	1.53	1.38	1.25	1.22	1.25	1.42	0.06

Table 3.4: PL Vs. S metabolites in hippocampus. Various metabolites (bold) have shown significant alteration in (PL) control group in comparison with (S) group. Data represents 4 controls and 4 PL induced-SE samples.

Metabolites	PL#1	PL#2	PL#3	PL#5	S#1	S#2	S#4	S#5	fold change	TTEST
4-Aminobutyrate	1.73	2.02	1.02	1.70	1.88	1.86	2.10	2.11	1.23	0.18
ADP	0.06	0.09	0.09	0.09	0.02	0.08	0.10	0.10	0.94	0.81
AMP	2.30	2.19	1.89	2.16	2.68	2.54	0.39	0.35	0.70	0.39
Acetate	**0.23**	**0.22**	**0.16**	**0.31**	**0.31**	**0.37**	**0.59**	**0.43**	**1.84**	**0.04**
Adenosine	0.18	0.20	0.18	0.21	0.30	0.24	0.19	0.22	1.21	0.17
Alanine	1.10	1.29	0.42	0.88	0.82	0.74	0.89	0.91	0.91	0.68
Arginine	0.18	0.17	0.13	0.32	0.21	0.02	0.94	0.17	1.71	0.55
Ascorbate	3.56	3.27	3.45	2.98	1.63	2.74	0.96	2.53	0.59	0.04
Aspartate	4.15	4.26	3.12	4.62	4.69	4.93	5.44	4.85	1.23	0.05
Choline	0.12	0.12	0.06	0.13	0.12	0.10	0.09	0.09	0.95	0.78
Creatine	10.30	9.99	16.54	10.54	10.07	9.86	11.96	10.42	0.89	0.49
Creatine phosphate	0.11	0.00	0.09	0.00	0.00	0.00	0.16	0.00	0.81	0.85
Ethanolamine	**0.17**	**0.22**	**0.26**	**0.18**	**0.15**	**0.08**	**0.06**	**0.14**	**0.52**	**0.02**
Formate	0.42	0.47	0.30	0.62	0.48	0.92	1.15	1.49	2.24	0.07
Fumarate	0.02	0.03	0.01	0.04	0.03	0.05	0.04	0.06	1.77	0.11
GTP	**0.23**	**0.27**	**0.21**	**0.22**	**0.31**	**0.35**	**0.31**	**0.37**	**1.44**	**0.00**
Glutamate	**16.08**	**16.46**	**16.01**	**16.82**	**19.40**	**18.59**	**22.16**	**21.55**	**1.25**	**0.01**
Glutamine	**7.25**	**7.72**	**7.99**	**9.28**	**5.56**	**5.69**	**5.70**	**5.96**	**0.71**	**0.01**
Glutathione	**3.49**	**3.55**	**3.82**	**2.71**	**1.94**	**2.59**	**2.08**	**2.10**	**0.64**	**0.01**
Glycine	**0.45**	**0.55**	**0.58**	**0.46**	**0.70**	**0.63**	**0.69**	**0.61**	**1.28**	**0.01**
Histidine	0.05	0.11	0.02	0.12	0.08	0.07	0.04	0.14	1.11	0.82
IMP	0.25	0.17	0.37	0.51	0.25	0.24	0.10	0.15	0.57	0.16
Isoleucine	0.04	0.04	0.03	0.05	0.05	0.05	0.07	0.10	1.78	0.07
Lactate	9.60	10.72	8.45	10.72	8.24	8.33	9.11	7.79	0.85	0.06
Leucine	0.10	0.12	0.09	0.13	0.11	0.09	0.16	0.15	1.17	0.39
Lysine	0.32	0.27	0.19	0.29	0.22	0.00	1.07	1.19	2.32	0.32
Methionine	0.13	0.14	0.10	0.30	0.20	0.46	1.17	0.82	3.95	0.10
N-Acetylaspartate	**8.57**	**8.30**	**8.98**	**7.26**	**10.49**	**10.54**	**11.51**	**11.32**	**1.32**	**0.00**
NAD+	0.39	0.37	0.50	0.42	0.47	0.39	0.69	0.51	1.23	0.23
O-Phosphocholine	0.52	0.43	0.73	0.56	0.64	0.56	0.62	0.69	1.12	0.38
O-Phosphoethanolamine	1.69	2.03	0.41	0.83	2.57	2.64	1.50	1.50	1.66	0.15
Phenylalanine	0.10	0.09	0.08	0.13	0.08	0.14	0.20	0.29	1.80	0.16
Serine	1.58	1.25	0.19	1.09	0.91	0.84	0.25	0.65	0.65	0.33
Succinate	0.55	0.54	0.46	0.49	0.52	0.50	0.72	0.67	1.19	0.18
Taurine	15.07	13.78	12.17	13.52	14.92	15.30	7.87	11.34	0.91	0.53
Tyrosine	0.03	0.09	0.05	0.04	0.09	0.03	0.09	0.17	1.88	0.24
UDP-galactose	0.10	0.18	0.11	0.11	0.11	0.15	0.12	0.17	1.11	0.53
UDP-glucose	0.14	0.17	0.15	0.17	0.10	0.20	0.15	0.25	1.13	0.58
UDP-glucuronate	0.03	0.03	0.02	0.00	0.01	0.00	0.05	0.05	1.30	0.71
UMP	0.20	0.22	0.14	0.31	0.21	0.17	0.15	0.14	0.76	0.23
Valine	0.14	0.13	0.12	0.11	0.15	0.09	0.19	0.15	1.14	0.46
myo-Inositol	6.52	5.96	7.90	6.79	6.57	5.43	6.58	5.85	0.90	0.22
sn-Glycero-3-phosphocholi	1.73	1.74	2.41	1.79	1.68	1.38	1.60	1.45	0.80	0.10

Table 3.5: PL Vs. S metabolites in cortex. Various metabolites (bold) have shown significant alteration in (PL) control group in comparison with (S) group. Data represents 4 controls and 4 PL induced-SE samples.

To investigate the efficacy of EGCG and DAC combination therapy on unaltered metabolism in control mice, we compared the metabolite concentrations in treated control (ES) with untreated control group (S) in both brain regions. Only fumarate and UDP-glucuronate showed significant changes in hippocampus (Table 3.6). Both metabolites show significant decrease in treated control (ES) compared to control group.

Fumarate is an easy measurable compound, but it is hard to measure UDP-glucuronate, so we should be cautious with this result. Anyway, at this point we do not understand the significance of this observation and is not discussed further in this thesis. Additionally, Glutamate and NAD+ were increased non- significantly in ES group compared to S group in hippocampus (Table 3.6). However, lactate level showed non-significant decrease in ES mice compared to control group. Generally, except for the significant inhibition of fumarate and UDP-glucuronate in hippocampus in ES mice , both brain regions have shown no significant alterations of the EGCG/DAC treated saline in comparison to the untreated control (Table 3.7).

Metabolites	ES#1	ES#2	ES#4 S	S#1	S#2	S#4	S#5	Fold change	TTEST
4-Aminobutyrate	2.48	2.61	2.34	2.57	2.46	2.54	2.69	0.97	0.39
ADP	0.19	0.14	0.16	0.17	0.21	0.13	0.15	1.00	0.98
AMP	2.06	2.23	2.20	2.11	2.19	1.99	2.14	1.03	0.42
Acetate	0.18	0.21	0.23	0.18	0.23	0.32	0.34	0.80	0.25
Adenosine	0.30	0.31	0.24	0.34	0.27	0.24	0.26	1.01	0.96
Alanine	1.04	0.93	0.90	0.85	0.80	0.86	0.93	1.11	0.13
Arginine	0.23	0.25	0.17	0.29	0.15	0.63	0.17	0.70	0.47
Ascorbate	3.66	3.32	3.85	3.48	3.08	3.00	2.55	1.19	0.07
Aspartate	2.63	2.98	2.91	3.13	3.34	3.05	3.33	0.88	0.05
Choline	0.08	0.15	0.13	0.18	0.13	0.13	0.13	0.83	0.35
Creatine	11.68	11.77	11.96	11.66	12.03	11.42	11.89	1.00	0.76
Creatine phosphate	0.00	0.02	0.03	0.04	0.00	0.11	0.00	0.42	0.47
Ethanolamine	0.09	0.17	0.11	0.16	0.04	0.15	0.18	0.92	0.80
Formate	0.39	0.26	0.37	0.30	0.53	0.74	0.63	0.62	0.11
Fumarate	0.07	0.07	0.08	0.09	0.11	0.12	0.10	0.71	0.01
GTP	0.17	0.20	0.20	0.10	0.23	0.23	0.23	0.95	0.78
Glutamate	16.93	17.39	16.55	15.70	15.78	16.47	16.62	1.05	0.06
Glutamine	5.45	5.73	5.65	5.50	5.52	5.03	4.96	1.07	0.10
Glutathione	2.84	2.10	2.93	3.52	2.41	2.89	1.71	1.00	0.98
Glycine	0.77	0.80	0.80	0.82	0.82	0.88	0.78	0.96	0.20
Histidine	0.07	0.07	0.06	0.08	0.09	0.08	0.08	0.83	0.06
IMP	0.16	0.23	0.39	0.23	0.24	0.19	0.30	1.09	0.78
Isoleucine	0.05	0.04	0.05	0.04	0.03	0.05	0.06	1.06	0.70
Lactate	10.74	9.27	10.08	11.10	11.85	11.29	11.58	0.88	0.06
Leucine	0.16	0.16	0.14	0.12	0.09	0.13	0.14	1.24	0.07
Lysine	0.16	0.21	0.19	0.22	0.20	0.07	0.32	0.92	0.79
Methionine	0.15	0.15	0.21	0.16	0.28	0.37	0.20	0.67	0.19
N-Acetylaspartate	8.21	8.63	8.77	8.10	8.24	8.00	8.52	1.04	0.19
NAD+	0.14	0.11	0.11	0.17	0.17	0.13	0.14	0.79	0.05
O-Phosphocholine	0.67	0.75	0.71	0.74	0.66	0.65	0.67	1.04	0.40
O-Phosphoethanolamine	2.04	2.45	2.35	1.96	1.74	2.31	1.73	1.18	0.12
Phenylalanine	0.09	0.08	0.10	0.08	0.06	0.09	0.12	0.99	0.96
Pyruvate	0.05	0.04	0.04	0.04	0.03	0.10	0.01	0.97	0.95
Serine	0.86	0.79	0.84	0.89	0.86	0.79	0.91	0.96	0.35
Succinate	0.53	0.35	0.36	0.28	0.29	0.33	0.34	1.33	0.21
Taurine	14.85	14.73	14.98	14.07	15.12	15.14	15.66	0.99	0.70
Tyrosine	0.07	0.07	0.05	0.08	0.08	0.12	0.12	0.66	0.06
UDP-galactose	0.12	0.13	0.10	0.15	0.14	0.08	0.13	0.95	0.76
UDP-glucose	0.16	0.12	0.07	0.12	0.17	0.13	0.19	0.75	0.28
UDP-glucuronate	0.02	0.01	0.01	0.03	0.05	0.04	0.03	0.33	0.02
UMP	0.15	0.18	0.15	0.15	0.20	0.14	0.16	0.98	0.82
Valine	0.19	0.20	0.16	0.18	0.16	0.16	0.17	1.08	0.34
myo-Inositol	7.89	8.20	7.10	8.44	7.66	7.42	7.39	1.00	1.00
sn-Glycero-3-phosphocholine	1.24	1.38	1.18	1.38	1.25	1.22	1.25	0.99	0.91

Table 3.6: ES Vs. S metabolites in hippocampus. Data represents 3 treated EGCG/DAC and 4 control mice. No significant difference was identified between the two groups of metabolites.

Metabolites	ES#1	ES#2	ES#4	S#1	S#2	S#4	S#5	Fold change	TTEST
4-Aminobutyrate	1.80	1.86	1.56	1.88	1.86	2.10	2.11	0.88	0.10
ADP	0.05	0.09	0.07	0.02	0.08	0.10	0.10	0.94	0.85
AMP	2.26	2.58	2.57	2.68	2.54	0.39	0.35	1.66	0.23
Acetate	0.29	0.27	0.35	0.31	0.37	0.59	0.43	0.71	0.12
Adenosine	0.29	0.28	0.20	0.30	0.24	0.19	0.22	1.10	0.55
Alanine	1.01	0.90	0.93	0.82	0.74	0.89	0.91	1.13	0.09
Arginine	0.00	0.17	0.00	0.21	0.02	0.94	0.17	0.17	0.27
Ascorbate	2.51	3.11	3.10	1.63	2.74	0.96	2.53	1.48	0.11
Aspartate	3.67	4.34	4.40	4.69	4.93	5.44	4.85	0.83	0.05
Choline	0.08	0.12	0.08	0.12	0.10	0.09	0.09	0.95	0.78
Creatine	9.79	9.65	11.07	10.07	9.86	11.96	10.42	0.96	0.56
Creatine phosphate	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.39
Ethanolamine	0.09	0.09	0.06	0.15	0.08	0.06	0.14	0.72	0.27
Formate	0.40	0.46	0.54	0.48	0.92	1.15	1.49	0.46	0.08
Fumarate	0.03	0.03	0.03	0.03	0.05	0.04	0.06	0.77	0.29
GTP	0.30	0.23	0.31	0.31	0.35	0.31	0.37	0.84	0.13
Glutamate	21.20	19.06	18.34	19.40	18.59	22.16	21.55	0.96	0.49
Glutamine	5.69	5.73	5.44	5.56	5.69	5.70	5.96	0.98	0.45
Glutathione	1.52	3.07	3.06	1.94	2.59	2.08	2.10	1.17	0.55
Glycine	0.51	0.58	0.70	0.70	0.63	0.69	0.61	0.91	0.39
Histidine	0.06	0.06	0.04	0.08	0.07	0.04	0.14	0.68	0.31
IMP	0.18	0.40	0.26	0.25	0.24	0.10	0.15	1.51	0.29
Isoleucine	0.04	0.04	0.05	0.05	0.05	0.07	0.10	0.61	0.09
Lactate	9.76	8.58	7.96	8.24	8.33	9.11	7.79	1.05	0.55
Leucine	0.12	0.14	0.11	0.11	0.09	0.16	0.15	0.98	0.89
Lysine	0.00	0.30	0.15	0.22	0.00	1.07	1.19	0.24	0.22
Methionine	0.13	0.16	0.18	0.20	0.46	1.17	0.82	0.24	0.10
N-Acetylaspartate	10.12	10.55	11.39	10.49	10.54	11.51	11.32	0.97	0.58
NAD+	0.63	0.46	0.55	0.47	0.39	0.69	0.51	1.06	0.72
O-Phosphocholine	0.54	0.56	0.64	0.64	0.56	0.62	0.69	0.93	0.32
O-Phosphoethanolamine	1.58	2.53	1.14	2.57	2.64	1.50	1.50	0.85	0.59
Phenylalanine	0.10	0.06	0.09	0.08	0.14	0.20	0.29	0.46	0.11
Serine	0.78	0.86	0.69	0.91	0.84	0.25	0.65	1.17	0.52
Succinate	0.64	0.57	0.58	0.52	0.50	0.72	0.67	0.99	0.91
Taurine	14.97	14.51	15.77	14.92	15.30	7.87	11.34	1.22	0.22
Tyrosine	0.06	0.06	0.05	0.09	0.03	0.09	0.17	0.60	0.29
UDP-galactose	0.15	0.13	0.12	0.11	0.15	0.12	0.17	0.99	0.90
UDP-glucose	0.16	0.15	0.12	0.10	0.20	0.15	0.25	0.81	0.38
UDP-glucuronate	0.03	0.00	0.06	0.01	0.00	0.05	0.05	1.12	0.89
UMP	0.13	0.19	0.17	0.21	0.17	0.15	0.14	0.98	0.88
Valine	0.16	0.16	0.16	0.15	0.09	0.19	0.15	1.10	0.52
myo-Inositol	6.54	5.52	5.47	6.57	5.43	6.58	5.85	0.96	0.58
sn-Glycero-3-phosphocholine	1.58	1.34	1.41	1.68	1.38	1.60	1.45	0.94	0.42

Table 3.7: ES Vs. S metabolites in cortex. Data represents 3 treated EGCG/DAC and 4 control mice. No significant difference was identified between the two groups of metabolites.

Additionally, we evaluated the metabolite concentrations in pilocarpine induced-SE (PL) group and compared it to the EGCG/DAC treated pilocarpine induced-SE mice (EP) in both brain regions. We could not detect any significant differences between the (EP) and (PL) groups in either brain regions (Table 3.8) (Table 3.9). Moreover, we have examined and compared the outcomes of EP and ES mice to indicate the efficacy of EGCG/DAC combination on altered metabolism of (PL) group in comparison to normal metabolism of control group in hippocampus and cortex.

Metabolites	EP#3	EP#5	EP#6	PL#1	PL#2	PL#3	PL#5	Fold change	TTEST
4-Aminobutyrate	1.42	2.15	1.97	1.73	1.87	1.62	1.66	0.93	0.63
ADP	0.09	0.22	0.20	0.13	0.14	0.20	0.17	0.95	0.86
AMP	1.81	1.92	1.56	1.57	1.48	1.70	1.64	0.91	0.26
Acetate	0.17	0.20	0.24	0.21	0.25	0.23	0.22	1.11	0.37
Adenosine	0.15	0.24	0.17	0.18	0.16	0.15	0.14	0.85	0.39
Alanine	0.73	0.83	0.64	0.81	0.74	0.66	0.76	1.01	0.88
Arginine	0.52	0.19	1.08	0.55	1.57	1.12	1.36	1.94	0.17
Aspartate	2.71	2.44	2.32	2.68	2.60	2.26	2.74	1.03	0.64
Choline	0.10	0.10	0.15	0.14	0.16	0.18	0.14	1.36	0.12
Creatine	9.86	10.57	9.68	9.90	9.14	9.66	9.59	0.95	0.22
Creatine phosphate	0.15	0.02	0.13	0.28	0.03	0.01	0.14	1.19	0.81
Ethanolamine	0.14	0.06	0.13	0.15	0.17	0.23	0.16	1.60	0.09
Formate	0.34	0.31	0.70	0.33	0.34	0.48	0.34	0.83	0.61
Fumarate	0.03	0.07	0.05	0.08	0.08	0.07	0.07	1.50	0.12
GTP	0.20	0.16	0.15	0.16	0.16	0.17	0.18	0.99	0.96
Glutamate	14.91	13.16	10.71	12.77	10.49	11.22	12.27	0.90	0.43
Glutamine	9.58	4.53	8.64	6.21	6.53	6.31	8.08	0.89	0.66
Glutathione	2.81	2.25	2.21	2.18	2.00	3.10	2.70	1.03	0.83
Glycine	0.57	0.81	0.76	0.69	0.82	0.71	0.57	0.98	0.86
Histidine	0.11	0.10	0.14	0.08	0.12	0.12	0.14	0.98	0.89
IMP	0.25	0.27	0.57	0.27	0.34	0.52	0.43	1.07	0.84
Isoleucine	0.04	0.04	0.05	0.05	0.05	0.06	0.03	1.05	0.82
Lactate	9.28	9.40	12.53	11.88	13.58	12.29	11.92	1.19	0.19
Leucine	0.11	0.11	0.13	0.12	0.18	0.18	0.14	1.35	0.07
Methanol	0.82	2.50	2.51	1.13	2.30	2.03	0.83	0.81	0.61
Methionine	0.14	0.13	0.13	0.13	0.13	0.09	0.11	0.88	0.17
N-Acetylaspartate	5.83	7.53	4.10	5.53	3.85	4.75	4.56	0.80	0.37
NAD+	0.22	0.07	0.10	0.10	0.10	0.12	0.09	0.76	0.55
O-Phosphocholine	0.57	0.58	0.53	0.50	0.57	0.63	0.50	0.98	0.74
O-Phosphoethanolamine	1.31	1.65	1.41	1.45	1.57	1.46	1.39	1.01	0.92
Oxypurinol	3.05	4.20	3.67	3.50	3.13	4.22	3.73	1.00	0.99
Phenylalanine	0.12	0.10	0.14	0.10	0.11	0.11	0.11	0.91	0.49
Pyruvate	0.02	0.03	0.04	0.08	0.04	0.05	0.04	1.63	0.13
Serine	0.51	0.77	0.94	1.04	1.04	0.93	0.99	1.35	0.17
Succinate	0.36	0.30	0.38	0.29	0.29	0.27	0.30	0.83	0.11
Taurine	11.30	12.91	6.36	12.00	11.06	10.90	11.68	1.12	0.60
Tyrosine	0.09	0.07	0.10	0.08	0.09	0.10	0.09	1.05	0.66
UDP-galactose	0.21	0.08	0.21	0.12	0.24	0.26	0.19	1.20	0.57
UDP-glucose	0.20	0.11	0.22	0.18	0.19	0.20	0.18	1.04	0.84
UDP-glucuronate	0.09	0.02	0.08	0.03	0.09	0.10	0.06	1.15	0.76
UMP	0.27	0.17	0.32	0.22	0.28	0.36	0.31	1.14	0.52
Valine	0.11	0.14	0.20	0.17	0.18	0.17	0.13	1.10	0.64
myo-Inositol	8.47	5.98	9.82	7.65	9.03	9.21	8.24	1.05	0.74
sn-Glycero-3-phosphocholine	1.25	0.96	1.51	1.29	1.90	1.74	1.34	1.26	0.20

Table 3.8: EP Vs. PL metabolite amounts in hippocampus. No significant change has been detected between the two groups. Data comparing 3 EGCG/DAC treated PL induced SE to 4 untreated PL induced-SE mice.

Table 3.9: EP Vs. PL metabolite level in cortex. No significant change has been detected between the two groups. Data comparing 3 EGCG/DAC treated PL induced SE to 4 untreated PL

Metabolites	EP#3	EP#5	EP#6	PL#1	PL#2	PL#3	PL#5	Fold change	TTEST
4-Aminobutyrate	2.09	1.88	1.41	1.73	2.02	1.02	1.70	1.11	0.57
ADP	0.07	0.08	0.14	0.06	0.09	0.09	0.09	1.19	0.57
AMP	2.37	2.17	2.20	2.30	2.19	1.89	2.16	1.05	0.33
Acetate	0.19	0.25	0.23	0.23	0.22	0.16	0.31	0.98	0.88
Alanine	0.89	0.93	0.86	1.10	1.29	0.42	0.88	0.97	0.88
Arginine	0.06	0.29	0.18	0.18	0.17	0.13	0.32	0.89	0.80
Ascorbate	2.64	3.60	3.26	3.56	3.27	3.45	2.98	0.95	0.66
Aspartate	4.12	4.32	4.16	4.15	4.26	3.12	4.62	1.04	0.65
Choline	0.11	0.12	0.12	0.12	0.12	0.06	0.13	1.11	0.55
Creatine	10.07	11.28	10.55	10.30	9.99	16.54	10.54	0.90	0.50
Creatine phosphate	0.00	0.10	0.00	0.11	0.00	0.09	0.00	0.67	0.72
Ethanolamine	0.15	0.19	0.13	0.17	0.22	0.26	0.18	0.77	0.15
Formate	0.37	0.61	0.50	0.42	0.47	0.30	0.62	1.10	0.67
Fumarate	0.04	0.05	0.04	0.02	0.03	0.01	0.04	1.81	0.07
GTP	0.27	0.29	0.26	0.23	0.27	0.21	0.22	1.17	0.05
Glutamate	20.06	17.46	16.10	16.08	16.46	16.01	16.82	1.09	0.32
Glutamine	5.51	10.21	9.47	7.25	7.72	7.99	9.28	1.04	0.84
Glutathione	3.00	3.85	3.45	3.49	3.55	3.82	2.71	1.01	0.91
Glycine	0.58	0.69	0.47	0.45	0.55	0.58	0.46	1.14	0.39
Histidine	0.08	0.08	0.09	0.05	0.11	0.02	0.12	1.11	0.77
IMP	0.19	0.30	0.61	0.25	0.17	0.37	0.51	1.13	0.79
Isoleucine	0.04	0.03	0.04	0.04	0.04	0.03	0.05	0.97	0.87
Lactate	7.76	10.06	9.55	9.60	10.72	8.45	10.72	0.92	0.45
Leucine	0.13	0.12	0.09	0.10	0.12	0.09	0.13	1.04	0.79
Lysine	0.20	0.57	0.27	0.32	0.27	0.19	0.29	1.29	0.56
Methionine	0.13	0.30	0.14	0.13	0.14	0.10	0.30	1.13	0.76
N-Acetylaspartate	10.21	8.04	9.28	8.57	8.30	8.98	7.26	1.11	0.29
NAD+	0.49	0.39	0.33	0.39	0.37	0.50	0.42	0.96	0.77
O-Phosphocholine	0.65	0.49	0.60	0.52	0.43	0.73	0.56	1.04	0.80
O-Phosphoethanolamine	2.52	2.04	1.45	1.69	2.03	0.41	0.83	1.62	0.18
Phenylalanine	0.10	0.10	0.09	0.10	0.09	0.08	0.13	1.00	0.99
Pyruvate	0.11	0.03	0.03	0.03	0.05	0.01	0.02	2.19	0.35
Serine	0.96	1.21	0.77	1.58	1.25	0.19	1.09	0.96	0.90
Succinate	0.52	0.44	0.41	0.55	0.54	0.46	0.49	0.90	0.26
Taurine	15.12	7.56	13.70	15.07	13.78	12.17	13.52	0.89	0.59
Tyrosine	0.07	0.06	0.08	0.03	0.09	0.05	0.04	1.41	0.22
UDP-galactose	0.13	0.09	0.14	0.10	0.18	0.11	0.11	0.97	0.88
UDP-glucose	0.12	0.14	0.17	0.14	0.17	0.15	0.17	0.94	0.57
UDP-glucuronate	0.03	0.04	0.04	0.03	0.03	0.02	0.00	1.72	0.12
UMP	0.12	0.23	0.26	0.20	0.22	0.14	0.31	0.94	0.81
Valine	0.14	0.15	0.12	0.14	0.13	0.12	0.11	1.10	0.37
myo-Inositol	5.82	7.39	6.48	6.52	5.96	7.90	6.79	0.97	0.72
sn-Glycero-3-phosphocholine	1.54	1.53	1.45	1.73	1.74	2.41	1.79	0.79	0.09

Opposed to O-phosphoethanolamine in hippocampus (Table 3.10), ethanolamine showed a significant increase in EP group in cortex compared to ES group (Table 3.11). Generally, no other significant changes have been detected in either brain regions. Generally, even with presence of drug combination there were no significant changes in PL induced-SE mice compared to control mice in term of metabolites by analyzing both brain regions.

Metabolites	EP#6	EP#3	EP#5	ES#1	ES#2	ES#4	fold change	TTEST
4-Aminobutyrate	1.56	2.52	2.34	2.48	2.61	2.34	0.86	0.37
ADP	0.10	0.25	0.23	0.19	0.14	0.16	1.19	0.59
AMP	2.02	2.26	1.85	2.06	2.23	2.20	0.95	0.43
Acetate	0.16	0.21	0.22	0.18	0.21	0.23	0.93	0.59
Adenosine	0.17	0.28	0.20	0.30	0.31	0.24	0.77	0.18
Alanine	0.81	0.97	0.76	1.04	0.93	0.90	0.89	0.24
Arginine	0.20	0.15	0.59	0.23	0.25	0.17	1.46	0.54
Ascorbate	3.28	3.60	3.49	3.66	3.32	3.85	0.96	0.45
Aspartate	3.04	2.87	2.76	2.63	2.98	2.91	1.02	0.74
Choline	0.11	0.11	0.18	0.08	0.15	0.13	1.12	0.65
Creatine	11.05	12.40	11.51	11.68	11.77	11.96	0.99	0.74
Creatine phosphate	0.17	0.02	0.15	0.00	0.02	0.03	7.31	0.17
Ethanolamine	0.19	0.07	0.15	0.09	0.17	0.11	1.10	0.78
Formate	0.38	0.36	0.83	0.39	0.26	0.37	1.55	0.35
Fumarate	0.04	0.08	0.06	0.07	0.07	0.08	0.79	0.33
GTP	0.22	0.18	0.18	0.17	0.20	0.20	1.05	0.64
Glutamate	16.80	15.53	12.79	16.93	17.39	16.55	0.89	0.24
Glutamine	10.42	5.84	10.27	5.45	5.73	5.65	1.58	0.16
Glutathione	3.15	2.64	2.63	2.84	2.10	2.93	1.07	0.60
Glycine	0.64	0.95	0.91	0.77	0.80	0.80	1.05	0.71
Histidine	0.12	0.12	0.17	0.07	0.07	0.06	2.04	0.05
IMP	0.28	0.32	0.68	0.16	0.23	0.39	1.64	0.33
Isoleucine	0.04	0.05	0.06	0.05	0.04	0.05	0.99	0.97
Lactate	10.38	11.03	14.62	10.74	9.27	10.08	1.20	0.27
Leucine	0.12	0.13	0.16	0.16	0.16	0.14	0.90	0.34
Lysine	0.36	0.26	0.49	0.16	0.21	0.19	1.98	0.10
Methionine	0.19	0.12	0.42	0.15	0.15	0.21	1.44	0.51
N-Acetylaspartate	6.13	8.57	4.55	8.21	8.63	8.77	0.75	0.21
NAD+	0.24	0.08	0.12	0.14	0.11	0.11	1.23	0.64
O-Phosphocholine	0.64	0.68	0.64	0.67	0.75	0.71	0.92	0.12
O-Phosphoethanolamine	**1.47**	**1.94**	**1.68**	**2.04**	**2.45**	**2.35**	**0.74**	**0.03**
Phenylalanine	0.14	0.11	0.17	0.09	0.08	0.10	1.56	0.07
Pyruvate	0.04	0.04	0.05	0.05	0.04	0.04	1.02	0.85
Serine	0.57	0.90	1.27	0.86	0.79	0.84	1.10	0.72
Succinate	0.41	0.36	0.45	0.53	0.35	0.36	0.99	0.93
Taurine	12.32	15.14	7.56	14.85	14.73	14.98	0.79	0.29
Tyrosine	0.10	0.08	0.12	0.07	0.07	0.05	1.53	0.05
UDP-galactose	0.23	0.10	0.26	0.12	0.13	0.10	1.62	0.27
UDP-glucose	0.22	0.13	0.26	0.16	0.12	0.07	1.76	0.13
UDP-glucuronate	0.10	0.02	0.10	0.02	0.01	0.01	5.63	0.16
UMP	0.30	0.20	0.38	0.15	0.18	0.15	1.87	0.11
Valine	0.12	0.16	0.23	0.19	0.20	0.16	0.93	0.72
myo-Inositol	9.57	7.02	11.68	7.89	8.20	7.10	1.22	0.34
sn-Glycero-3-phosphocholine	1.40	1.13	1.80	1.24	1.38	1.18	1.14	0.46

Table 3.10: EP Vs. ES metabolite level in hippocampus. O-phosphoethanolamine increased significantly in ES group. Data representing 3 EGCG/DAC treated PL induced SE to 3 EGCG/DAC treated control mice.

Metabolites	**EP#3**	**EP#5**	**EP#6**	**ES#1**	**ES#2**	**ES#4**	fold change	TTEST
4-Aminobutyrate	2.09	1.88	1.41	1.80	1.86	1.56	1.03	0.83
ADP	0.07	0.08	0.14	0.05	0.09	0.07	1.35	0.40
AMP	2.37	2.17	2.20	2.26	2.58	2.57	0.91	0.16
Acetate	0.19	0.25	0.23	0.29	0.27	0.35	0.75	0.07
Adenosine	0.22	0.21	0.23	0.29	0.28	0.20	0.85	0.30
Alanine	0.89	0.93	0.86	1.01	0.90	0.93	0.94	0.26
Arginine	0.06	0.29	0.18	0.00	0.17	0.00	3.14	0.24
Ascorbate	2.64	3.60	3.26	2.51	3.11	3.10	1.09	0.50
Aspartate	4.12	4.32	4.16	3.67	4.34	4.40	1.02	0.82
Choline	0.11	0.12	0.12	0.08	0.12	0.08	1.24	0.28
Creatine	10.07	11.28	10.55	9.79	9.65	11.07	1.05	0.46
Ethanolamine	**0.15**	**0.19**	**0.13**	**0.09**	**0.09**	**0.06**	**2.07**	**0.02**
Formate	0.37	0.61	0.50	0.40	0.46	0.54	1.06	0.75
Fumarate	0.04	0.05	0.04	0.03	0.03	0.03	1.32	0.17
GTP	0.27	0.29	0.26	0.30	0.23	0.31	0.98	0.81
Glutamate	20.06	17.46	16.10	21.20	19.06	18.34	0.92	0.32
Glutamine	5.51	10.21	9.47	5.69	5.73	5.44	1.49	0.20
Glutathione	3.00	3.85	3.45	1.52	3.07	3.06	1.35	0.22
Glycine	0.58	0.69	0.47	0.51	0.58	0.70	0.98	0.87
Histidine	0.08	0.08	0.09	0.06	0.06	0.04	1.47	0.03
IMP	0.19	0.30	0.61	0.18	0.40	0.26	1.31	0.58
Isoleucine	0.04	0.03	0.04	0.04	0.04	0.05	0.89	0.40
Lactate	7.76	10.06	9.55	9.76	8.58	7.96	1.04	0.70
Leucine	0.13	0.12	0.09	0.12	0.14	0.11	0.91	0.51
Lysine	0.20	0.57	0.27	0.00	0.30	0.15	2.29	0.25
Methionine	0.13	0.30	0.14	0.13	0.16	0.18	1.20	0.61
N-Acetylaspartate	10.21	8.04	9.28	10.12	10.55	11.39	0.86	0.12
NAD+	0.49	0.39	0.33	0.63	0.46	0.55	0.74	0.10
O-Phosphocholine	0.65	0.49	0.60	0.54	0.56	0.64	1.00	0.97
O-Phosphoethanolamine	2.52	2.04	1.45	1.58	2.53	1.14	1.14	0.65
Phenylalanine	0.10	0.10	0.09	0.10	0.06	0.09	1.20	0.25
Pyruvate	0.11	0.03	0.03	0.02	0.01	0.03	3.26	0.26
Serine	0.96	1.21	0.77	0.78	0.86	0.69	1.26	0.24
Succinate	0.52	0.44	0.41	0.64	0.57	0.58	0.76	0.03
Taurine	15.12	7.56	13.70	14.97	14.51	15.77	0.80	0.33
Tyrosine	0.07	0.06	0.08	0.06	0.06	0.05	1.25	0.22
UDP-galactose	0.13	0.09	0.14	0.15	0.13	0.12	0.89	0.43
UDP-glucose	0.12	0.14	0.17	0.16	0.15	0.12	1.03	0.85
UDP-glucuronate	0.03	0.04	0.04	0.03	0.00	0.06	1.18	0.77
UMP	0.12	0.23	0.26	0.13	0.19	0.17	1.25	0.46
Valine	0.14	0.15	0.12	0.16	0.16	0.16	0.87	0.16
myo-Inositol	5.82	7.39	6.48	6.54	5.52	5.47	1.12	0.28
sn-Glycero-3-phosphocholine	1.54	1.53	1.45	1.58	1.34	1.41	1.04	0.47

Table 3.11: EP Vs. ES metabolite level in cortex. Ethanolamine increased significantly in EP group. Data representing 3 EGCG/DAC treated PL induced SE to 3 EGCG/DAC treated control mice.

Finally, by comparing the total metabolite concentrations in labeled and unlabeled samples in both hippocampus and cortex, the ^{13}C glucose labeled samples showed higher concentrations than unlabeled ones. The mean ± SD of the ^{13}C labeled and unlabeled samples in the hippocampus were (6.0 ± 1.0, 4.2 ±1.3) respectively and in the cortex, they were (6.64 ± 1.4, 2.1 ± 1.1) respectively. There might be a difference in metabolite concentrations between hippocampus and cortex in the different conditions, but no enough data have been obtained to support this assumption.

Sample	^{13}C glucose Labeling	Sum of metabolite concentrations in Hippocampus	Sum of metabolite concentrations in Cortex
S#1	Labeled	6.93	4.63
S#2	Unlabeled	3.92	2.05
S#4	Unlabeled	3.12	.80
S#5	Unlabeled	3.43	1.15
ES#1	Labeled	6.26	7.15
ES#2	Labeled	7.89	5.85
ES#4	Labeled	6.24	5.16
PL#1	Labeled	5.13	7.45
PL#2	Labeled	5.17	6.51
PL#3	Labeled	6.50	9.26
PL#5	Unlabeled	6.30	3.14
EP#3	Labeled	5.23	7.07
EP#5	Unlabeled	4.47	3.18
EP#6	Labeled	4.90	6.64

Table 3.12: Assessment of the total metabolite concentrations depending on ^{13}C labeling in hippocampus and cortex.

Chapter 4: Discussion and Future Directions

The metabolic profile of different samples was discussed in this thesis. A comparison between HBP1 KO and WT groups was done. The aim of the comparison was to investigate the effect of HBP1 transcriptional repressor on the metabolism of epileptogenic brain and its role in development of epileptogenesis. Other comparisons between control (S), EGCG/DAC treated control (ES), pilocarpine induced-SE (PL) and EGCG/DAC treated pilocarpine-induced-SE (EP) groups were made which aimed to examine the efficacy of EGCG/DAC as a potential preventive treatment of epilepsy disorders.

Using the FVB/N mouse strain generated by the Yee lab, HBP1 KO mouse was bred and utilized to investigate the role of HBP1 in epileptogenesis. In this experiment, no significant difference was detected between HBP1 KO and WT mice after ^{1}H data analysis (Table 3.1). Upon the ^{13}C analysis of HBP1 KO and WT samples, no significant results were obtained after analyzing the lactate average value, GABA to glutamate, glutamine to glutamate and [4,5-^{13}C] glutamate to [2,3-^{13}C] glutamate ratios. Plus the small size of samples, one of the HBP1 KO samples was not ^{13}C glucose labeled, another sample 3556 belongs to the same group (HBP1 KO) showed only one peak that representing the β carbon of glutamate and resulted in undetectable level of ^{13}C labeled in the sample (Figure 3.6). Avoiding all these limitations could change the result obtained from the metabolic flux analysis of these samples.

Using ^{13}C glucose, it is possible to distinguish between the astrocytic and neuronal glutamate. ^{13}C glucose is metabolized to pyruvate and involved in the neuronal TCA cycle via PDH metabolizing enzyme to produce [1,2-^{13}C] acetyl-CoA or in the astrocytic

TCA cycle via PC metabolizing enzyme to produce oxaloacetate. [1,2-^{13}C] acetyl-CoA results in the formation of neuronal [4,5-^{13}C] glutamate, and oxaloacetate leads to the formation of astrocytic [2,3-^{13}C] glutamate. [4,5- ^{13}C] glutamate to [2,3- ^{13}C] glutamate ratio could not be determined in sample 3556 HBP1 KO. We could not detect any difference between HBP1 KO and control group samples mostly because the sample 3553 HBP1 KO was not ^{13}C labeled.

The data from the Yee lab indicates that the combination of EGCG and DAC increases the stability of the HBP1 gene, thus suppressing Wnt signaling activity. To assess the efficacy of EGCG/DAC combination and its effect on metabolic alterations in epileptogenesis, we evaluated the ^{1}H NMR data collected for EP, ES, PL, S groups. No ^{13}C glucose labeled data was analyzed, since too many of the samples were unlabeled in ^{13}C (Table 3.3). Metabolite concentrations were quantified in both hippocampus and cerebral cortex brain regions in order to analyze the epilepsy metabolomics in EP, ES, PL, S groups. By comparing the PL group with control in both brain regions, various metabolites revealed significant alterations. In hippocampus, GABA, AMP and NAD+ were significantly decreased in PL group compared to control (Table 3.4) but were not altered in the cortex (Table 3.5). Furthermore, both glutamate and N-acetylaspartate were significantly down regulated in PL group in both cortex and hippocampus in comparison with S mice ($P<0.05$). Similarly, studies on CD1 mice models of temporal lobe epilepsy (TLE) have shown reduction in the concentration of glutamate and NAA in hippocampus as well as a decrease in the content of GABA [15]. Numerous studies suggested that the decrease in NAA and glutamate contents might reflect neuronal loss [13, 33]. However, no recent studies have succeeded to show a direct correlation between the reduction in

NAA and glutamate concentrations with neuronal loss [13, 33]. On the other hand, glutamine level was significantly increased in both hippocampus and cortex in PL induced-SE group. Lactate and myo-inositol in hippocampus showed significant increase in PL group compared to control. These results are consistent with the finding of various studies of pilocarpine model of SE in mice and rats [15, 34]. A metabolite analysis of ES and EP groups compared to S and P groups respectively in both cortex (Table 3.7) (Table 3.9) and hippocampus (Table 3.6) (Table 3.8) showed a significant reduction of fumarate and perhaps UDP-glucuronate in the ES group in hippocampus. No significant change was detected between ES and S in cortex. Also, no significant alterations were detected between EP and PL groups in both brain regions. The comparison between EP and ES metabolites has indicated a statistically significant increase of ethanolamine in EP group in cortex, while the level of O-phosphoethanolamine was increased significantly in hippocampus in ES group.

Several factors and limitations have emerged during experiments and analysis, these factors have affected the outcomes. Again, small sample size in each group was a potential influencing factor on the results. Another factor that could affect the results is some mice received glucose while others did not. The total metabolite concentrations of ^{13}C labeled compared to unlabeled samples showed an increase in metabolite concentrations in the labeled ones (Table 3.3). The mice that were injected intraperitoneally received a bolus of glucose whereas those who did not show labeling probably did not receive glucose. Therefore, the mice with glucose might have different metabolism than those who did not. Thus, future studies will require that all mice should be treated equally.

Another logical proposed step is to use ^{13}C glutamine tracer. Glutamine is one of the most abundant nutrients to support energy production and acts as precursor for macromolecular synthesis. Also, Glutamine serves as transporter of carbon and nitrogen and considered as the major source of nitrogen for nonessential amino acids such as asparagine and glutamate [35, 36]. Two major routes are involved in glutamine metabolism, glutaminolysis and reductive carboxylation. Both routes begin with the deamination of glutamine to glutamate and the conversion of glutamate into α-ketoglutarate which oxidized via TCA cycle and decarboxylated to pyruvate [37]. So, using the ^{13}C glutamine labeled tracer could be effective in evaluating the total contribution of glutamine and other metabolites in the TCA cycle and could be a useful step toward expanding our knowledge and improving the current therapeutic approaches in epilepsy disorders.

Applying the strategy of the combinational therapy of EGCG/DAC on another of pilocarpine model of epilepsy such as CD1 or HET mouse strains and compare the result with the obtained data from the FVB/N strain could aid in improving our understanding of epilepsy and compare if there is any difference in the response to the EGCG/DAC combination treatment.

Improving the therapeutic outcomes and preventing the development of epilepsy is the main aim of this work. Future experiment testing the effects of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) on the metabolism in epileptogenic brain serves in reaching our aim. 2-DG is a non metabolizable glucose analog which does not participate in glycolysis. It differs from glucose by the absence of one oxygen atom at 2-position. Many

studies have illustrated the anticonvulsant effect of 2-DG in both rats and mice beside its neuroprotective action [38, 39]. Moreover, by using Western blot to test the metabolic enzymes the Yee lab showed that 2-DG as a metabolic block is sufficient to reduce metabolic energy generation and interrupt the metabolic programming in PL induced-SE model (Yee lab, unpublished results). Testing of 2-DG using NMR is an important step in understanding its effect on metabolic changes in epilepsy. Investigating and understanding 2-DG effect could lead to the development of a novel therapeutic target that help in treating and preventing of epilepsy disorders

In conclusion, we investigated an essential signaling pathway besides investigating the effect of the pharmacological intervention of EGCG/DAC in epilepsy. No significant changes have been detected between HBP1 KO and WT groups. Also, direct comparisons between the NMR metabolomics of epileptic brain and control were reported in this thesis. The comparison between PL and control mice have illustrated many metabolite alterations. However, no metabolic alterations between pilocarpine induced-SE and treated EGCG/DAC pilocarpine induced-SE have been detected. Fumarate and UDP-glucuronate showed significant decrease in SE group compared to S group in hippocampus without knowing the exact reason underlying this significance. No significant changes between EP and ES were observed. The obtained results from these experiments showed no significant alterations which contradict our previous assumption that the metabolism alterations would follow the protein level at the fifth day post pilocarpine SE induction, and the proteins level that were observed are not rate limiting. Currently, Baleja's lab utilizing NMR spectroscopy is investigating the 2-DG effect on

metabolic changes in post pilocarpine induced-SE model. Our work and the proposed future analysis will aid in the development of treating and preventing epilepsy disorders.

Chapter 5: Bibliography

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