AWARD NUMBER: W81XWH-10-1-0381

TITLE: EPILEPSY AND THE WNT SIGNALING PATHWAY

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REPORT DATE: August 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE	Form Approved OMB No. 0704-0188 1

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2016	Final	1JUN2010 - 31MAY2016

4. TITLE AND SUBTITLE

Epilepsy and the Wnt Signaling Pathway

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0381

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Epileptogenesis is a gradual process by which normal brain transforms into one that sustains seizures. It is instigated by an inciting event (e.g. prolonged seizure called status epilepticus (SE), head injury, infection or stroke). This is followed by a variable (months to years in humans) “latent period” followed by the emergence of spontaneous seizures, with potential for later mood and learning disabilities. While the latent period is a time during which behavioral seizures are not observed, it is a period of tissue and cellular remodeling that sets up the development of chronic seizure activity, or epilepsy. In this grant, we have drawn expertise from other fields to discover new mechanistic insights into epileptogenesis. In the past year, we have expanded our understanding of molecular mechanisms, specifically Wnt signaling and an altered metabolism in the hippocampus of mice undergoing early epileptogenesis. In doing so, we have uncovered a new therapeutic drug combination that we had been developing for breast cancer treatment aimed at Wnt signaling. Surprisingly, the combination attenuates seizures in two different models of acute seizures as well as in a model of chronic seizure onset. This combination may have efficacy for preventing the onset of chronic seizures that define epileptic progression.

15. SUBJECT TERMS

Status Epilepticus, Wnt Signaling, Epileptogenesis

16. SECURITY CLASSIFICATION OF: U			**17. LIMITATION**	**18. NUMBER**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	16	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)

Table of Contents

INTRODUCTION: An important aspect to advancing therapeutic tools to more fully understand a particular disease process is the availability of experimental models to molecularly dissect a disease sub-type. In this grant, we will investigate the mechanisms of Status Epilepticus (SE) and the ensuing latent period in animal models of temporal lobe epilepsy (TLE), a disease subtype that afflicts about 40% of epilepsy patients. A well-established model of SE and chronic epilepsy is the kainate-treatment[8]; in which rats or mice undergo an inciting event of SE with a latent period of 2 weeks to 3 months, and then develop chronic epilepsy characterized by partial complex and secondarily generalized motor seizures [9-15]. This model also recapitulates many pathologic alterations seen in patients with temporal lobe epilepsy and allows investigation of compounds during different stages epileptogenic progression.

While epilepsy research has traditionally focused upon in the imbalance of excitatory (NMDA, AMPA,) and inhibitory (GABA) neurotransmitter systems, several signaling pathways are under investigation for modulating epilepsy. Some of the cells signaling pathways include mTOR [12-14,19], Jak/Stat[21] and ERK pathways[22]. Recent work has compared cancer and epileptic progression[23]. An objective of this grant is to capitalize on the molecular and therapeutic developments in cancer to develop new signaling and therapeutic paradigms for epileptogenesis. In our partnership, we have been investigating the possible role of Wnt signaling in SE and now have excellent evidence that Wnt signaling is elevated in both the rat and mouse models of temporal lobe epilepsy. Capitalizing on our respective cancer and epilepsy expertise, we will address the molecular circuitry of Wnt signaling and test an unexpected pre-clinical regimen for suppression of SE and epileptogenesis.

BODY: Our collaborative grant has three specific aims. As the partnering PI, we have emphasized Aims 1 and 3.

Aim1. Generate time course of Wnt activation following a prolonged seizure, status epilepticus (SE).

Task 1A. Develop time course for Wnt activation following kainate-induced SE

Task 1B: To confirm if Wnt activation is model dependent vs. status epilepticus dependent, develop time course for Wnt activation following pilocarpine-induced SE

Task 1C: Utilizing results from a, b, use real time PCR to determine Wnt target gene expression following SE.

Aim2. To identify the role of Wnt signaling in two potential mechanisms of early epileptogenesis following SE.

Task 2A. To identify if changes in Wnt signaling alters **early epileptiform activity** in CA3 bursting, an in-vitro model of seizure propensity in control vs. animals which have undergone SE.

a. Obtain in-vitro slices from control animals. Examine the role of bath-applied Wnt activators and inhibitors on CA3 burst frequency, a measure of seizure propensity. Obtain in-vitro slices from SE animals. Examine the role of bath-applied Wnt activators and inhibitors on CA3 burst frequency, a measure of seizure.

Aim3. Animal Clinical Trial: Determine if Wnt inhibition modulates cell death and delays the onset to epilepsy in a whole animal model of SE

Task 3A. Develop a delivery method for Wnt inhibitor, F8CDFr in CNS.

Task 3B. Develop antibody assay for measurement of F8CDFr in serum.

Task 3C. Develop time course for administration of F8CDFr into animals following SE.

Task 3D. Optimize dosing regimen for F8CDFr into animals following SE.

Task 3E. Examine if F8CDFr application alters cell death 7 days after SE

Task 3F. Examine if F8CDFr application alters onset to epilepsy using Neurophysiology Core.

Aim 1. Wnt signaling. Our collaborative studies have identified Wnt signaling at the time of SE and again in the early epileptogenic period. For practical reasons described below, we will focus on the epileptogenic period.

At SE. We have been delineating a time course for Wnt signaling in mouse to complement Audrey Yee's studies in the rat. We have complementary evidence for the increase in Wnt target genes (MYC, LEF, and Axin 2 genes) in the period from 1-24 hours after SE. In the mice, we are also utilizing Bat-GAL mice in which there is an integrated reporter gene for detecting Wnt signaling. A representative experiment is depicted in Figure 1.

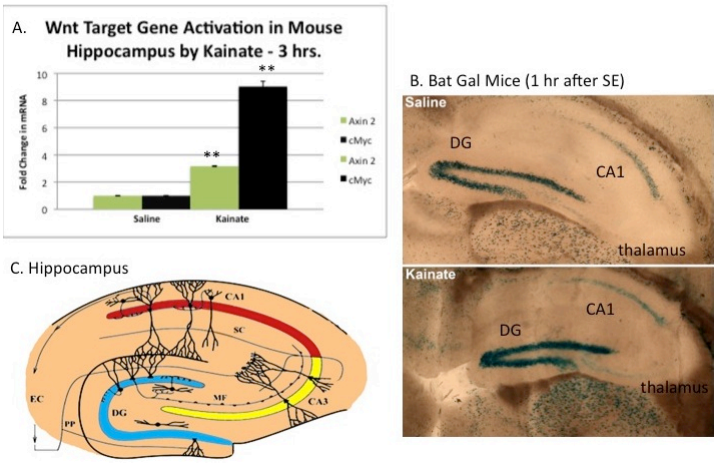


Figure 1 . Wnt signaling is activated in mouse following KA-induced SE. A. Wnt target genes cMyc and Axin2 mRNA level are increased in mouse hippocampus 3 hrs. after SE. RNA from the indicated brain regions (from 5 animals) was analyzed by qRT-PCR to quantify the mRNA levels. The p-value for Axin2 and MYC activation were determined by (Student's T-test with Welch correction ($p<0.015$ and $p<0.032$, respectively). B. **Wnt signaling is increased at 1 hr. after SE in the hippocampus in BAT-GAL mice, a Wnt pathway reporter strain.** KA was used to induce SE and the mice were sacrificed at 1 hour. Xgal was used to detect Wnt activity encoded by the Wnt reporter transgene. C. Schematic of the hippocampus. DG = dentate gyrus

At Epileptogenesis. Epilepsy is defined as two recurrent, spontaneous seizures, that is, an initiating seizure and a subsequent event. Key considerations for therapeutic development are interventions into the epileptogenic period. Practically, a patient would seek treatment after the first seizure to prevent a second seizure, thus placing the therapeutic windows of opportunity into the early epileptogenic period. All the experiments below featured either kainate or pilocarpine treatment at day 0 and then the early epileptogenic period from day 1 to 7 was analyzed. In Figure 2, we found that Wnt signaling occurred in the early epileptogenic period from day 3 to 7, with a maximum at day 5. The first analysis used the BAT Gal mouse, in which there is an integrated Wnt signaling reporter gene. That is, any region with active Wnt signaling will stain blue with the X-gal dye due to expression of the β-galactosidase gene upon Wnt pathway activation. Figure 2A shows that induction of seizures with kainate induction at day 0 results in Wnt signaling at day 5 of epileptogenesis and in the dentate gyrus of the hipppcampal region, cortex, thalamus (not shown). Biochemical analysis of Wnt signaling extended the in vivo Bat gal mice results by showing induction of β-catenin and P-GSK3β in day 5 of epileptogenesis (Fig. 2B). This observation is supported by biochemical and molecular analysis showing that markers of Wnt signaling (β-catenin levels) and Wnt target genes (e.g. axin2) are elevated. Using a Wnt signaling array, Axin 2 and a subset of other

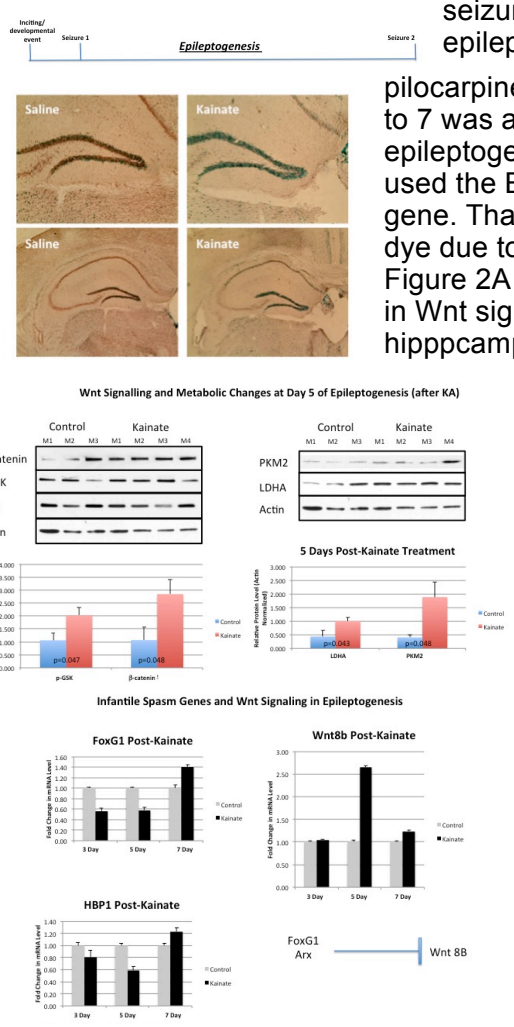


Figure 2. Wnt signaling is activated in the early epileptogenic period. A. Schematic diagram of early epileptogenesis. B. Wnt Signaling at day 5 after KA-induced SE. The hippocampus from BAT-GAL mice at day 5 of epileptogenesis was stained with X-gal to visualize regions of Wnt signaling. Both the DG and cortex show increased Wnt signaling. C. Wnt and metabolic signaling changes at day 5. The indicated markers of Wnt signaling were increased at day 5, co-incident with the increased reporter activity. D. Elevation of a specific Wnt gene and decline of Wnt pathway inhibitors in early epileptogenesis. Three genes associated with early brain development and/or Wnt signaling are elevated in early epileptogenesis at day 5 with the peak of Wnt signaling. Fox G1 is a repressor of Wnt signaling that specifies forebrain development. The primary target is Wnt 8b, which is elevated in this period [7]. Fox G1 is also genetically associated with infantile spasms [16]. Finally, our lab showed that the HBP1 was a repressor of Wnt signaling [17,18] and recent studies implicate a role in seizure onset [20]

Wnt signaling are also increased. An interesting finding is that all Wnt signaling genes are not increased. The implications of the subset of genes are under current investigation (Figure 2, and not shown).

We next wanted to investigate how Wnt signaling might be elevated in the early epileptogenic period. We hypothesized that the periodic elevation might be a combination of decreases in pathway inhibitors, elevation of Wnt ligands, or both. Building on the genetics of epilepsy and infantile spasms, we found that both mechanisms appeared to contribute. Two of the three genes came from our observation that several genes involved in infantile spasms encode regulators of Wnt signaling. To date, several genes have been associated with spasms, several encode regulators of Wnt signaling, including Fox G1[16]. During early epileptogenesis, we found that Fox G1 was decreased, whereas Wnt 8B was elevated (Figure 2). FoxG1-Wnt 8B axis that has been recently shown to be critical for development of the forebrain, that gives rise to the cortex and hippocampus, regions that are critical for epilepsies and likely developmental defective in the infantile spasms. Recent studies have shown that Fox G1 is a repressor of Wnt 8B, whose expression pattern is critical for determining development of forebrain regions or the retinal regions[7].

Second, the HBP1 gene is also decreased and we have previously characterized the HBP1 gene as an inhibitor of Wnt signaling. Our data shows that decreases in HBP1 results in heightened Wnt signaling. A recent case study reports that HBP1 is a candidate gene in a small deletion in a patient with developmental delays and seizures [20]. Thus, the decrease in HBP1 expression is consistent with the heightened Wnt signaling in Fig 2. Lastly, in the rat model, Wnt 3A is also elevated in the same critical period. Together, the elevation of Wnt signaling in early epileptogenesis is a likely composite and complex input of elevated Wnt ligands and diminished Wnt inhibitors, some of which are based in the genetics and developmental abnormalities that lead to infantile spasms and childhood epilepsies.

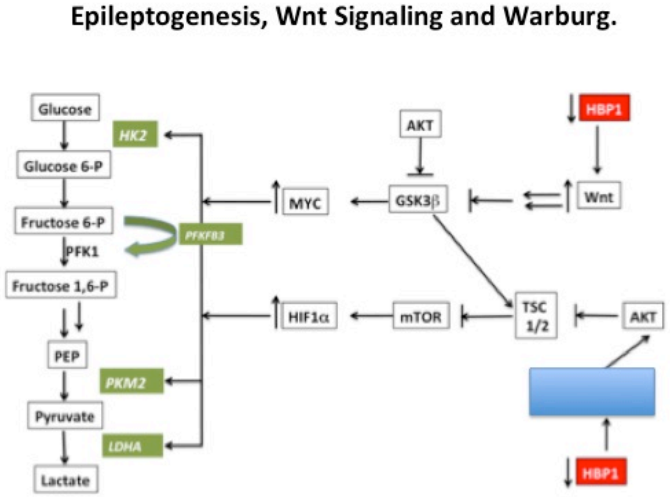
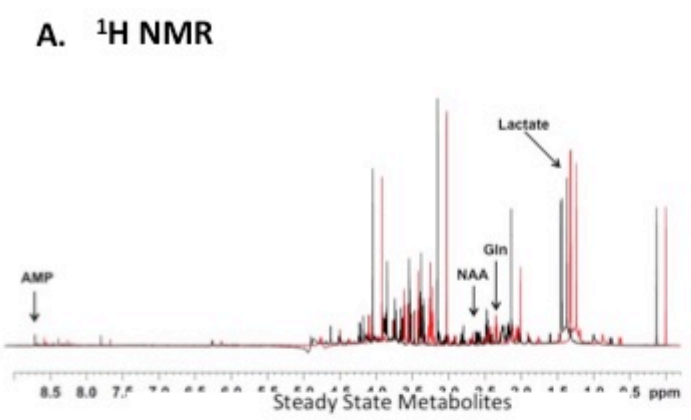


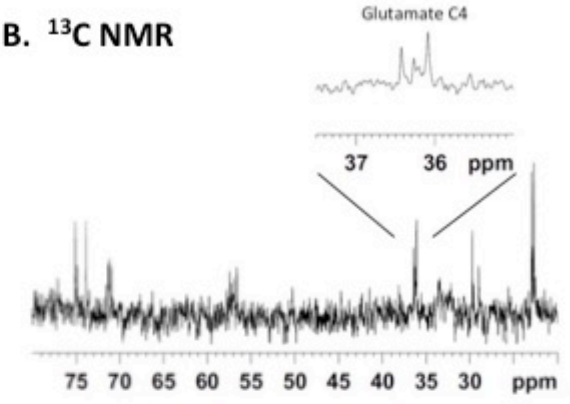
Figure 3: A Hypothesis for Metabolic Signaling. We postulate that the Wnt signaling and EGFR/mTOR signaling increase the MYC and HIF1α transcription factors and to increase metabolic re-programming through some of the following genes. Some of these transitions may be mediated through the HBP1 gene (described below).
HK2. Hexokinases catalyze the conversion of glucose to glucose 6-P and is the first committed step in glycolysis. HK2 is increased in several cancer cells and in other tissues exhibiting a Warburg effect. GSK3β inhibition in Wnt signaling is associated with HK2[1], which is regulated by MYC and HIF1α[2,3].
PFKFB3. PFKFB3 is an isoform of phosphofructose kinase 2, which catalyzes the synthesis of fructose 2,6 bisphosphate (F2, 6P), an allosteric activator of phosphofructose kinase 1 (PFK1), the rate-limiting step of glycolysis. Thus, elevated PFKFB3 activity should increase PFK1 activity and overall glycolysis. The PFKFB3 isoform is elevated in tumor or proliferating cells.
PKM2. Pyruvate Kinase M2 is a proliferation-associated isoform of pyruvate kinase that catalyzes the conversion of phosphoenolpyruvate to pyruvate in glycolysis. PKM2 was the first glycolytic enzyme to be associated with the Warburg effect in cancer cells and is an alternative splice form of pyruvate kinase[4]. PKM2 also promotes the interaction of β-catenin with TCF4, an essential step of Wnt signaling[5].
LDHA. Lactate Dehydrogenase A is an isoform of lactate dehydrogenase, which catalyzes the conversion of pyruvate to lactate. LDHA is expressed in cancer cells directly regulated by the MYC and HIF1α[6].

Wnt Signaling, Metabolic Reprogramming and Epilepsy? We next sought to understand how a pathologically elevated Wnt signaling might contribute to epileptogenesis. By understanding the molecular consequences, we hoped to identify therapeutic targets for attenuating epileptogenesis and thus preventing the onset of the chronic seizures that define epilepsy. Proliferation, differentiation, rearrangements in channel function and pathological remodeling in to an eventual epileptic brain mark the period in early epileptogenesis. Wnt signaling has been recently linked to metabolic re-arrangements know as a Warburg effect in tumor cells [24,25]. First discovered in cancers, the Warburg effect is a phenomenon of metabolic re-programming that converts a cell/tissue for biosynthesis, rather than catabolic oxidative metabolism. The Warburg effect has been largely associated with tumor cells in which there is a sustained and rapid proliferative response and is now one of the established new hallmarks of cancer [4,26]. Recent studies in numerous non-neuronal tissues have highlighted that widespread metabolic changes contribute to cycles of proliferation and differentiation, yet metabolic regulation remains largely unexplored in the brain and in non-cancerous tissues and is an emerging area. A recent Science paper has highlighted a role for lactate dehydrogenase in epilepsy[27].

Building on the work in tumor cells with Wnt signaling [24,25] , we investigated whether there was a Warburg-like metabolic rearrangement during an epileptogenic time window in which Wnt signaling is maximal. Fig 2C shows that PKM2, HK2, and LDHA are induced at day 5 of epileptogenesis. These genes are targets of MYC, which itself is induced by Wnt signaling. The elevation of these genes is a signature of ~~a~~ metabolic re-programming phenomenon known as the Warburg effect. During a Warburg reprogramming, the cells and tissue undergo a metabolic reprogramming in which oxidative catabolism is decreased and glycolysis and other pathways are switched towards biosynthesis and preservation of intermediates in order to meet the increase metabolic needs. PKM2 is an embryonic and splice variant of pyruvate kinase, a gatekeeper of glycolysis. The action of PKM2 contributes to accumulation of glycolytic intermediates for biosynthetic reaction and diminishes the catabolic processes. LDHA is an isoform that converts pyruvate to lactate, a signature step in a Warburg effect, also known as aerobic glycolysis. Lastly, Hexokinase 2 (HK2), another signature isoform in metabolic re-programming[28] , is elevated. It is remarkable that several isoforms alone in the glycolytic part of the Warburg effect are coincidentally elevated in two different models of induced epileptogenesis. We are currently assessing the localization of the HK2 and other signals. Previous studies have attributed glycolysis to the glial cells and an absence in neurons.

Next, we used NMR-based metabolomics to assess the metabolic consequences of heightened Wnt signaling in early epileptogenesis. We examined the complement of metabolites at the steady state level (^{1}H NMR) and at the non-steady state level following a pulse of ^{13}C-glucose. The configuration of the ^{13}C NMR and ^{1}H experiments provides kinetic and steady state "snapshots" of metabolism at day 5 of epileptogenesis after pilocarpine induction. The data from multiple dissected hippocampi from control and epileptogenic mice were analyzed by Chenomix and Metaboanalyst software with embedded statistics packages. Figure 4 summarize the data. The embedded tables summarizes the metabolites detected by Chenomix analysis of the NMR data. We focused on metabolite changes with $p<0.05$. Principal Component analyses (PCA) highlighted that distinct metabolite populations were evident in the control and epileptogenic mice in both types of NMR experiments.





Steady State Metabolites
5 Days Post-SE

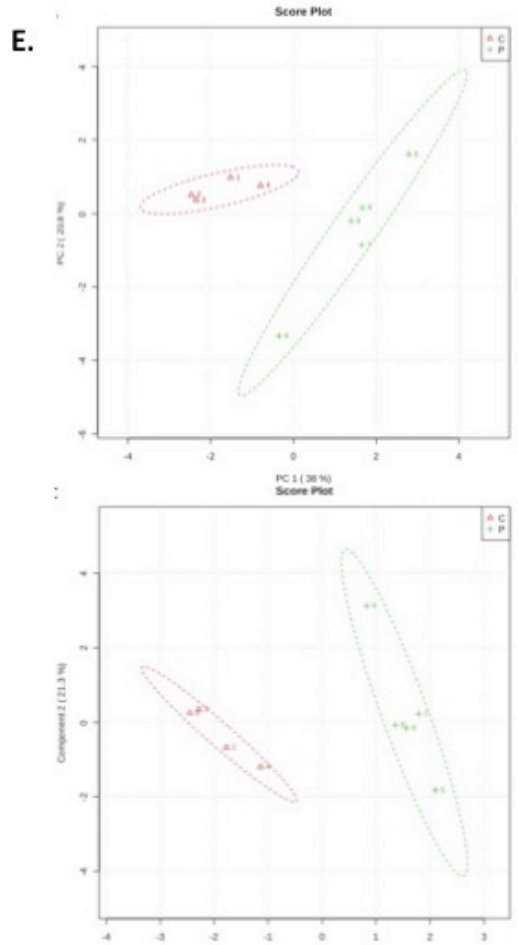
C.

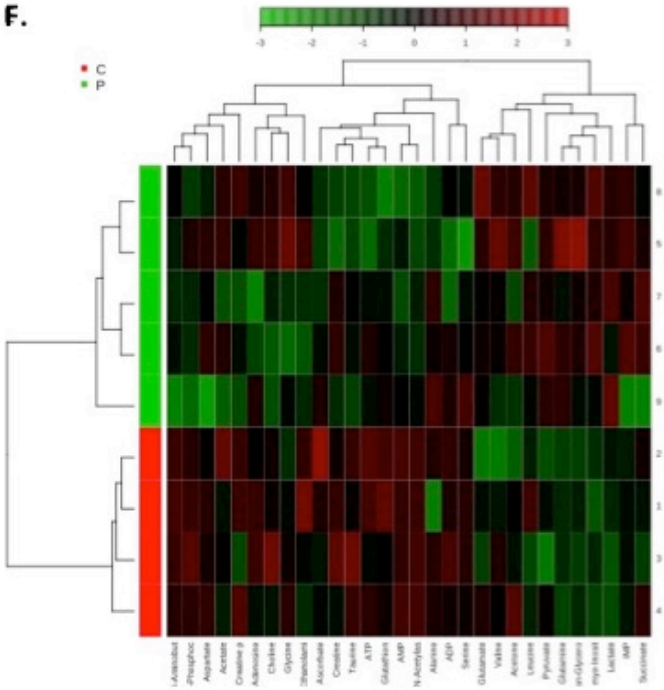
Metabolite	Control Average	Pilocarpine Average	Percent Change	TTEST
4-Aminobutyrate	0.0291	0.0235	-19.23	**0.0011**
Acetate	0.0048	0.0047	-1.19	0.9005
Acetone	0.0038	0.0041	7.90	0.6563
Adenosine	0.0046	0.0042	-9.61	0.5145
ADP	0.0031	0.0019	-40.01	**0.0562**
Alanine	0.0102	0.0109	6.80	0.4341
AMP	0.0185	0.0140	-24.25	**0.0003**
Ascorbate	0.0387	0.0332	-14.15	0.2308
Aspartate	0.0265	0.0234	-11.72	0.3934
ATP	0.0055	0.0048	-11.94	**0.0694**
Choline	0.0022	0.0020	-10.35	0.4421
Creatine	0.1327	0.1319	-0.59	0.7649
Creatine phosphate	0.0074	0.0056	-24.84	0.2623
Ethanol	0.0044	0.0035	-19.95	0.2626
Ethanolamine	0.0054	0.0039	-27.07	0.3270
Glutamate	0.1387	0.1209	-12.78	**0.0034**
Glutamine	0.0516	0.0765	48.23	**0.0001**
Glutathione	0.0137	0.0131	-4.91	0.2444
Glycine	0.0125	0.0132	5.26	0.6024
IMP	0.0059	0.0081	37.16	0.1905
Lactate	0.1117	0.1507	34.94	**0.0003**
Leucine	0.0018	0.0022	18.39	0.3469
myo-Inositol	0.0835	0.1073	28.56	**0.0008**
N-Acetylaspartate	0.0727	0.0444	-38.87	**0.0001**
Niacinamide	0.0030	0.0024	-19.95	0.2626
O-Phosphocholine	0.0076	0.0066	-12.96	**0.0258**
Serine	0.0237	0.0200	-15.66	0.3786
sn-Glycero-3-phosphocholine	0.0117	0.0164	40.67	**0.0402**
Succinate	0.0024	0.0037	54.76	**0.0901**
Taurine	0.1612	0.1411	-12.43	**0.0019**
Valine	0.0015	0.0018	17.45	0.1761

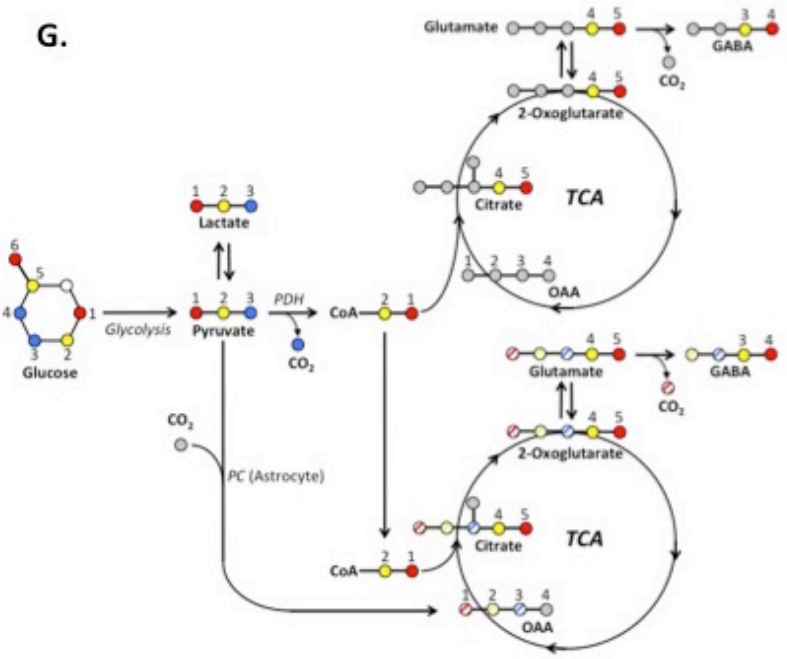
D.

Non-Steady State Metabolites
5 Days Post-SE

Metabolite	Control Average	Pilocarpine Average	Percent Change	TTEST
Ala CH3	0.0118	0.0152	28.668	0.005
GABA-alpha	0.0200	0.0089	-55.659	0.042
GABA-beta	0.0142	0.0049	-65.319	0.011
GABA-gamma	0.0272	0.0181	-33.625	0.023
Glu beta	0.1292	0.0900	-30.352	0.079
lactate 2	0.0678	0.0991	46.268	0.023
lactate ch3	0.1648	0.2310	40.159	0.020
Myo3	0.0117	0.0186	58.404	0.004
Myo4	0.0098	0.0157	60.695	0.026







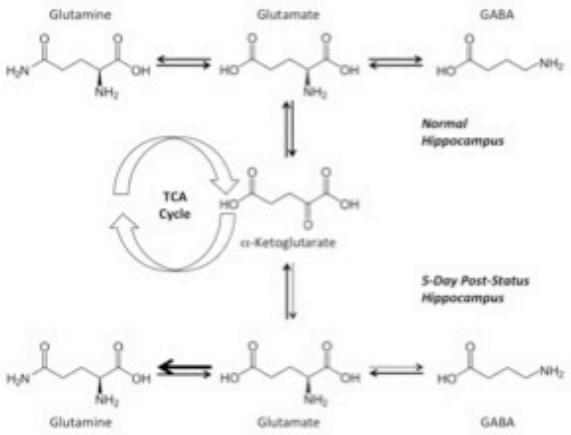


Fig. 4. **Steady-State Analysis of 5-Day Post Status Epilepticus Hippocampal Metabolism**. Polar metabolites extracted from 4-5 hippocampi were analyzed by NMR. Data were quantitated with Chenomx software and analyzed using Metaboanalyst. A. NMR data for ^{1}H NMR (steady state). (B) ^{13}Cglucose (pulse, non steady state) (C) Table of Metabolites for ^{1}H NMR. (D) Table of Metabolites for ^{13}C NMR Principal Component Analysis. (E) Principal Component Analysis. The metabolite principal components showed significant separation (>95% confidence) between control mice (red triangles) and pilocarpine-treated mice (green +). Upper: ^{1}H NMR; Lower ^{13}C NMR. (F) Hierarchical Cluster Analysis of Metabolites. Metabolites were segregated using the Pearson Clustering Method in Metaboanalyst. Control and Pilocarpine-treated metabolites self-segregated into two clusters with clear up- and down-changes in metabolites between control and pilocarpine-treated mice. G. Schematic depiction of the fate of labeled carbons for the ^{13}C glucose pulse experiment in the context of glycolysis and TCA cycles. H. Schematic depiction of the glutamate-glutamine-GABA (GLU-GLN) cycle in brain in relation to the TCA cycle.

The metabolite profiles are consistent with a high flux through glycolysis and a marked shift in the metabolism of glutamate, which is an intermediate in the synthesis of GABA, a critical neurotransmitter in epilepsy. First, there appeared to be a high flux through glycolysis with a steady state and kinetic accumulation of lactate in both the ^{1}H-NMR and ^{13}C-NMR analyses The accumulation of lactate is the signature hallmark of a Warburg effect in cancer [4,26] and is consistent with the elevation of LDHA, which converts pyruvate to lactate. In addition, there is an accumulation of alanine in the steady state measurement. Alanine is derived from pyruvate, which is a glycolytic intermediate. Excess pyruvate would be consistent with the accumulation of both alanine and lactate. Second, both the steady state and kinetic indicate a decrease in GABA levels with a concomitant increase in steady-state glutamine levels at day 5 of epileptogenesis. These observations have some implications in the context of known epilepsy facts and in terms of the apparent altered Warburg metabolism. The decrease in GABA levels is consistent with epileptogenesis and elevation of GABA is a frequent therapeutic strategy. By assessing glutamate, GABA, and glutamine together in the epileptogenic hippocampus, these data additionally suggest that the decrease in GABA may be due to a decrease in GABA synthesis (mediated by the GAD67 enzyme) and/or a shift towards glutamine synthesis (mediated by glutamine synthetase enzyme). Studies to assess GAD67 and glutamine synthetase are now underway. How a Warburg-like metabolism relates to the glutamate-glutamine cycle in the CNS is not known.

Together, the data in Figs. 2-4 suggest that the heightened Wnt signaling in epileptogenesis triggers a Warburg-like metabolic rearrangement increased flux through glycolysis, manifesting as increased lactate production. It is important to note that the Warburg effect is not simply a lactate effect, but a global re-arrangement of metabolism. Increases in GLN are frequent in tumors with a Warburg metabolism and are considered to be part of the anaplerotic reactions that sustain the TCA cycle [4,29]. In brain, the GLN-GLU cycle that is known to be operational in the CNS for synthesis of the neurotransmitter GLU and GABA [30,31]. It is not currently known how metabolic state influences the GLN-GLU cycle in the CNS, but basic biochemistry clearly indicates that a net increase in the TCA intermediate 2-oxoglutarate (or α-ketoglutarate) is necessary for GLU and GLN synthesis.

Together the studies in Figures 2-4 have several important therapeutic implications and perspectives for future work on epileptogenesis and for a surprisingly understudied angle into metabolism. Our awareness of the cancer field directly impacted our ability to make a surprising new set of observations for Wnt signaling and the metabolic state of the epileptogenic hippocampus. The apparent presence of a Warburg-like effect may explain some longstanding and new observations in the epilepsy field. First, the efficacy of a ketogenic diet in treating some forms of drug-intractable epilepsy already suggest a role for glucose metabolism in the etiology of certain epilepsies. The basis of the ketogenic diet is the switch of intermediary metabolism to the use of ketone bodies, rather than glucose. Brain is normally depending on glucose for its energy needs, but can use ketone bodies. Thus, the ketogenic diet reduces the use of glucose in the brain. A recent study has indicated the LDHA is associated with the etiology of seizures in some pre-clinical models and is also consistent with the notion that the conversion of pyruvate to lactate might contribute to the etiology of seizures. Thus, the heightened glucose metabolism that we detected in epileptogeneiss is consistent with this notion that a Warburg-like effect may contribute to epileptogenesis. Second, our metabolomic studies suggest that the metabolic factors may regulate the GLN-GLU cycle in the brain that is responsible for neurotransmitter synthesis. In cancer cells, the Warburg metabolism is accompanied by changes in glutamine. In our studies, a sharp increase in lactate a signature metabolite of the Warburg effect, accompanied a definable decrease in GABA, a hallmark of epilepsies. Lastly, we chose NMR for our metabolomics analysis because of the potential to develop MRI-based markers to enhance imaging of epileptic brain. Magnetic Resonance Spectroscopy is an MRI-based tool that can be used to non-invasively visualize the chemistry of a limited tissue region (denoted a voxel) and can be programmed into a routine MRI. The challenge is delineating the informative spectral regions in which to focus in the voxel. By rigorously defining the critical metabolites, it is possible that lactate may become an MRS-MRI-based biomarker for an epileptogenic brain region and perhaps for a patient that might benefit from ketogenic diet or LDH inhibitor intervention to prevent the epileptogenesis from progressing to chronic seizures that define epilepsy. A current gap in the field is a dearth of molecular and noninvasive criteria to define when specific anti-epilepsy treatments should be applied.

Unexpected Findings. Wnt signaling and spontaneous seizures? Building upon the observation that the HBP1 gene is decreased in epileptogenesis and has been linked to developmental seizures [20], we directly asked if the HBP1 gene is a potential gatekeeper of seizure susceptibility. My lab has reported HBP1 to be an inhibitor of Wnt signaling and had developed these mice for the breast cancer studies. We carefully developed strain-specific KO in C57Bl6 and FVBN. These strains are also the background used for induced seizure models by kainate and pilocarpine, respectively. The data in Figure 2 suggested that HBP1 may be a barrier to heightened Wnt signaling in epileptogenesis. A striking observation is that the HBP1 KO mice have greatly

increased seizure susceptibility, resulting in death at doses in which the normal control mice exhibit modest seizures. Notably, kainate and pilocarpine work by two different mechanisms, so the results are not a consequence of the chemical inducer, rather are due to the seizure induction process itself.

We next examined the seizure induction using behavioral observation and video-EEG methods. Preliminary behavioral observation studies indicate that the HBP1 KO mice exhibit spontaneous low-grade seizures of Racine scale 2 but with characteristics of absence seizures. We have just begun with video-eeg monitoring and are processing the eeg data. The initial characterization shows abnormal electrical activity (not observed in the wild type) with periods of additional absence-like seizures with freezing-like behaviors. An additional abnormal eeg pattern is accompanied by hyperactive circling behavior. This unusual pattern of eeg abnormalities and circling behavior have been reported in mice with deletions of the β3 subunit of the Gaba A receptor (GABRB3; [32]). This GABRB3 gene was also identified in the Epi4K project as a gene associated with de novo epilepsies and in Angelman's syndrome, a developmental syndrome in which patients have seizures [33,34] . We have been consulting with Dr. Kevin Staley, an expert on EEG analysis in animal models and an expert neurologist, and the chief of pediatric neurology at MGH.

The next objective was to investigate the role of HBP1 in de novo epilepsy. Using a public database of genetic disease from the Sanger Institute (DECIPHER), we discovered that deletions through a region containing the HBP1 appear to be associated with abnormal EEG/seizures, as well as other developmental abnormalities. HBP1 is located at 7q22.3. Monosomy 7 has been associated with disease such as leukemias, but del 7 mutations have been associated with developmental abnormalities. The DECIPHER database correlates mapped by array CGH with the clinical phenotypes provided by the clinicians. All deletions were mapped by array CGH on blood from the patient and thus must be present in all tissues. Using DECIPHER and the literature, we found 18 patients with deletions through the HBP1 region, of which 10 exhibited seizures and/or abnormal eeg. In one case, the deletion encompassed 3.2mb with 15 genes, including HBP1 [20]. Importantly, we examined 33 deletions that were adjacent to the HBP1 gene, only 4 patients reported seizures. Thus, these data suggest a statistically significant association of the HBP1 region with seizures and abnormal EEGs in patients (Fisher's exact test $p<0.0019$). Together, the animal and human genetic data suggest that HBP1 is an excellent candidate gene for seizure susceptibility and for de novo epilepsies.

It is useful to compare the HBP1$^{-/-}$ phenotypes with other genetic models in the field. There are few genetic models of de novo epilepsies. In fact the discovery/delineation of such models is a 2014 NINDS benchmark. To date, the SCN1a mutations and Dravet's syndrome represent the best example of de novo epilepsy with a genetic basis [35-37]. SCN1a represents the gene encoding the tetrodotoxin (TTX)-sensitive sodium channel. Patients with Dravet's syndrome, also known as Severe Myoclonic Epilepsy of Infancy, is a rare and catastrophic epilepsy that is often intractable to standard treatment and with numerous cognitive and developmental co-morbidities. Some of the epilepsy changes have been modeled in mice (SCN1a +/-) with heterozygous deletion or by specific knock-in of human mutations. While the basis of the TTX-sensitive sodium channel in disease still remains unclear, aberrant GABA-ergic signaling contributes to the phenotype in mice. Remarkably, the SCN1a mice also exhibit the same unusual circling behavior and absence-like seizures that were reported for the GABRB3-/- mice [32,38].

Together, these HBP1KO mice may be new genetic model of seizure susceptibility that may recapitulate clinical disease and our observations prompt several investigational possibilities for future studies. Suffice to say, the unique environment of the epilepsy studies converge onto potentially new insights into epileptogenesis with some common underpinnings in the fundamental mechanisms of unrelated diseases such as breast cancer. By carefully analyzing our observations through multidisciplinary lenses of biochemistry and genetics, this project is on its way to advancing a new genetic model of disease that is rooted in Wnt signaling and in human genetics. Furthermore, the HBP1-/- mouse has exciting potential for future anti-epileptogenic drug development, based on the human genetics. In principle, the susceptible patient population should be identifiable through testing of the blood. The HBP1 $^{-/-}$ mouse bears a mutation in every tissue, akin to the human patients profiled in the DECIPHER database in which a blood sample was analyzed by array CGH for mutations for mutations. Thus, the HBP1$^{-/-}$ mouse maybe a tremendous tool for the discovery and testing of anti-epileptogenic therapies and especially for future trials by identifying a patient population that might best benefit from therapies developed in this new and novel pre-clinical model.

Conclusion. Together, we report that this period of epileptogenesis is marked by elevation of Wnt signaling and with changes that sustain the elevated Wnt signaling that are re-capitulated form earlier brain development. Such observations underscore the proliferation and differentiation in epileptogenesis as a time of aberrant differentiation and development to set up an altered brain that may sustain repeated seizures.

Aim 3. Testing Therapeutics. The original focus of this aim was to use a potential recombinant inhibitor F8CDFr for Wnt signaling to determine if treatment attenuated seizures. F8CDFr is an analog of SFRP1, a naturally occurring Wnt inhibitor[39]. Because this drug has not entered clinical trials, we requested a different direction in the previous period. In the course of other work in the lab, we found that a combination of EGCG and Decitabine was effective at inducing Wnt pathway inhibitors in other tissues. In surprising new data, the combination of EGCG and Decitabine attenuated SE and chronic seizures (Figure 5-6).

Background. Green tea and EGCG. Green tea is the world's second most popular beverage after water and is under development in numerous clinical trials [40]. The prevalence of clinical trials provides an ideal opportunity to develop EGCG in epilepsy). The green tea compound EGCG [(-) epigallocatechin gallate] is the main catechin component in dry green tea (about 30%). Green tea is about 0.1% EGCG solution (w/v), or 2 mm Green tea and EGCG (4~8 U.S. cups/day) has no appreciable side effects in humans [41,42 43]. We showed that EGCG blocks Wnt signaling by increasing the mRNA stability of the HBP1 transcriptional repressor, which we have described as an inhibitor of Wnt signaling. [44] EGCG appears to function by increasing HBP1 mRNA stability[44]. The preliminary studies show that the increases in HBP1 also elevate SFRP1 (a Wnt inhibitor). Several animal studies demonstrate efficacy for EGCG in cancer models [45,46 47,48]. Thus, our work shows that EGCG is effective in blocking constitutive Wnt signaling involving HBP1 and SFRP1. While EGCG has been investigated for cancer therapies and has bioavailability to the brain[40], few studies have used neuronal models [49-51], with none in epilepsy.

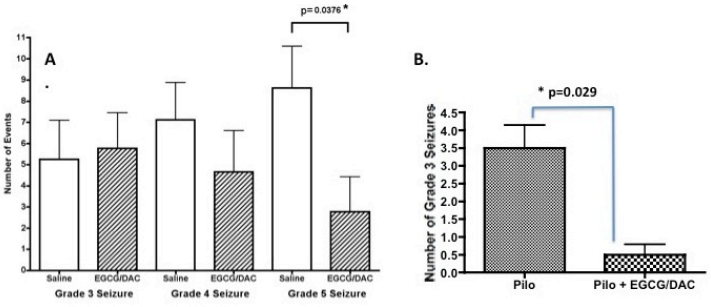


Figure 5. EGCG/DAC treatment decreases SE induced by Kainate (KA) or Pilocarpine (PILO). A. Seizure Severity (KA). 9 mice were pre-treated with 3 doses (EGCG (16.5 mg/kg, IP) and DAC (0.5 mg/kg, IP) on alternate days, then treated with KA (30 mg/kg, IP) on the seventh day. Control mice were pre-treated with saline (vehicle). Seizures occurred within 30 minutes were scored using the Racine scale. B. Seizure Severity (Pilo). 2 groups of 5 FVBN mice were treated with saline or EGCG/DAC and then treated were treated with a low dose Pilo (10 mg/kg). Only grade 3 seizures were observed within 10-30 minutes were scored using the Racine scale.

Decitabine. DNA hypermethylation has emerged as a major mechanism for gene silencing[52] and is known to regulate SFRP1 and other Wnt pathway components. Epigenetic regulation, of which DNA methylation is one type, is prevalent in many diseases. An established epigenetics-based agent is 5-aza-dc (Decitabine, DAC), whose principal action is to inhibit DNA methyltranferases to induce gene hypomethylation[37]. Decitabine is FDA-approved for the treatment of myelodysplastic disorders and is used to treat various leukemias. In this preclinical analysis, the dose will be 0.5 mg/kg, which are less that then FDA-recommended dose. Potential side effects at the higher therapeutic dose can be nausea, neutropenia, and myelosuppression, but are fully manageable in current oncology practice. Using combinations will decrease the manageable side effects. There are no reports on the use of Dectabine for treatment of epilepsy or for any neurological disorders, despite brain bioavailability[53,54].

EGCG/DAC attenuated SE in two mouse models. The initial goal is to determine whether SE is attenuated by EGCG/DAC treatment. We utilized either kainate or pilocarpine to induce SE. The significance is that pilocarpine and kainate induce SE through two modes—muscarinic ACHR and kainate receptor, respectively. Yet, both have a common mechanism for induction of Wnt signaling and in mTOR signaling [12-14,19,55]. In these experiments, mice were pre-treated with EGCG/DAC with doses defined in other studies in our lab and Figure 5 shows a decrease in SE.

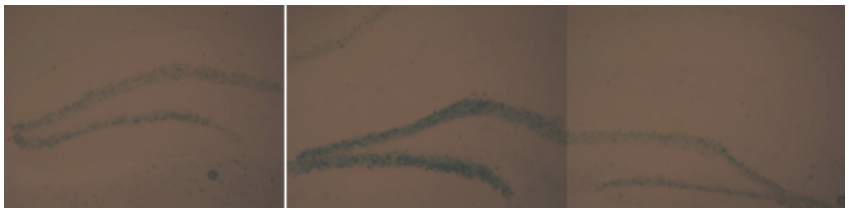


Figure 6. Seizure induction is coincident with Wnt signaling; EGCG/DAC returns Wnt signaling to control levels. The BAT-GAL reporter mouse, containing an integrated TCF B-gal reporter gene, was induced for seizures (kainate injection). The depicted picture shows the day 5 hippocampal dentate gyrus upon X-gal staining. Left: control; middle; kainate; right; after EGCG/DAC treatment

Wnt Signaling and EGCG/DAC in epileptogenic period. The next set of experiments examined the impact of EGCG/DAC treatment on the Wnt signaling in the epileptogenic period (Figure 6). The treatment protocol was the delivery of EGCG/DAC within 6 hours of seizure induction and continuous delivery throughout the early epileptogenic period. We chose this protocol to mimic clinical conditions in which a patient undergoing a first seizure might seek treatment. Using the Bat-Gal Mice, we found that kainate induced Wnt signaling (consistent

with Figure 1) maximally at day 5 and that treatment with EGCG/DAC diminished the elevation of Wnt signaling to control levels. Biochemical analysis akin to Figure 2 is now underway to better elaborate the molecular consequences of EGCG/DAC treatment in epileptogenesis.

This experiment provides a potentially very useful insight. Note that EGCG/DAC treatment reverts the degree of Wnt signaling from the aberrant and pathological levels, back to the control levels. Because Wnt signaling is likely to be important for to-be-determined basal functions of the brain, the lack of complete inhibition is likely to be a benefit when considering side-effects and the impact to normal tissues, not affected by epileptogenic transitions. Notably, control cells are also not reduced and the EGCG/DAC treatment appears to target those cells with aberrant Wnt signaling.

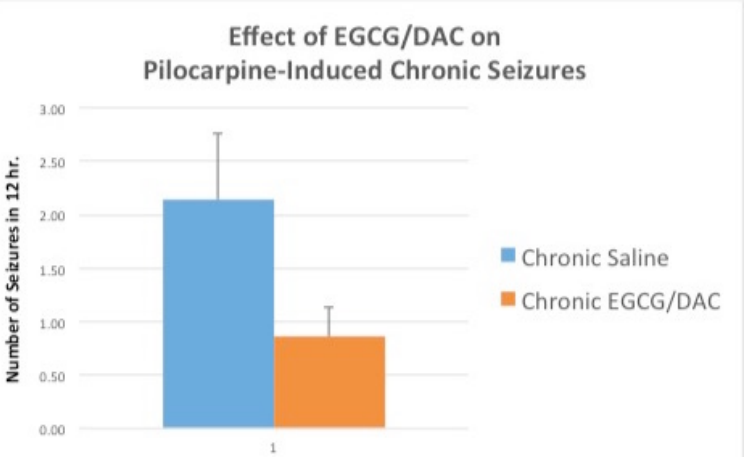


Figure 7. EGCG/DAC treatment decreases chronic seizures induced by pilocarpine (PILO). Two groups of 9 FVBN mice were treated with a low dose Pilo (10 mg/kg) and all mice exhibited SE. Within 6 hours of observing SE, one group of 9 mice was treated with saline whereas a second group was treated with EGCG (16.5 mg/kg, IP) and DAC (0.5 mg/kg, IP) on alternate days for 3 weeks. 8 saline-treated mice and 9 ECG/DAC treated mice were monitored by continuous videotaping. 155 hours and 150 hours were analyzed for the saline and EGCG/DAC treated mice—all of which had initially documented SE induction by pilocarpine. The mean seizure rate per 12 hours of recorded time was calculated. The saline treated mice had a mean seizure rate of 2.14 +/- 0.61, whereas the EGCG/DAC treated mice had a mean seizure rate of 0.86 +- 0.27 ($p<0.05$ by Student's t test (Graphpad Prism).

. While EGCG/DAC could attenuate acute seizures induced by either kainate or by pilocarpine, the real potential for therapeutic intervention lies in the ablity to influence epileptogenesis, the process that can occur after an intial seizure to remodel for sustaining chronic seizures. Thus, we tested whether EGCG/DAC treatment could diminish the prevalence of chronic seizres. We utilised the pilocarpine model of chronic seizure onset in which multiple seizures occures within 2-3 weeks of the initial SE triggers by pilocarpine. We then delivered EGCG/DAC at the doses in Figure 7, but continuous for the next 2-3 weeks. As an initial analysis, we monitored the control and treated mice by video analysis as a prelude to future EEG analysis. As shown in Figure 7, the mean detected seizure rate decreased upon EGCG/DAC treatment to 0.86 from 2.14 for the saline-treated control mice ($p<0.05$). Anecdotaly, the EGCG/DAC treated mice exhibited normal behaviors (for mice) while the saline treated mice appeared more skittish and difficult to handle. Nonetheles, the objective seizure quantitation data highlight that EGCG/DAC treatment appears to decrease the prevalence of chronic seizures, when delivered after the initaitaing SE.

These analyses suggest that EGCG/DAC may be disease modifying in preventing the onset of chronic seizures. A future analysis will use the full video-EEG to discern the impact of EGCG/DAC on chronic seizure onset. Together, the data in Figure 5-7 suggest that EGCG/DAC reduces Wnt siganling and decreases the onset of chronic seizures.

KEY RESEARCH ACCOMPLISHMENTS:

- Elaboration of a complex Wnt signaling network in SE and in epileptogenesis based upon principles of Wnt signaling and the genetics of infantile spasms and childhood epilepsies.
- Discovery and elaboration of a novel metabolic re-programming framework in epileptogenesis.
- Discovery of a potentially new genetic model of spontaneous seizures.
- Discovery of EGCG/DAC as a potential new drug for the acute and chronic seizures in the pre-clinical setting.

REPORTABLE OUTCOMES:

Abstracts: American Epilepsy Society Meeting 2011, 2012, 2013. Gordon Conference 2014.
Funding applied: DOD grants, CURE foundation grants (not funded).

Successful funding of CURE foundation dream team grant on Infantile Spasms:
Some preliminary data went into a successful multi-investigator CURE foundation grant on infantile spasms.

Our discovery of Wnt signaling increases in epileptogenesis set the stage for the new research project. The team is Prof. Chris Dulla, Amy Yee, and Chris Dulla (PI). Dr. Audrey Yee had a key early role. Dr. Audrey Yee correctly identified Aicard's like syndromes in a cAPCKO mice, predicted to have elevated Wnt signaling (owned by Dr. Michele Jacob). Drs. Amy Yee, Dulla and Jacob worked as a team to develop the project and investigate APC and Wnt signaling as an etiology for IS. Dr. Amy Yee created an elegant Infantile and Wnt signaling framework on which to evaluate and develop the multidisciplinary results. The working hypothesis incorporated the genetics of IS and numerous concepts of Wnt signaling gleaned from the cancer and developmental biology field. Remarkably, Dr. Yee noticed that several genes genetically linked to IS were directly linked to different aspects of Wnt pathway function, leading us to hypothesize that interference with Wnt signaling functions may be an excellent therapeutic strategy. Dr. Chris Dulla is analyzing the in vitro electrophysiology and in vivo EEG underlying the development of infantile spasms in the cAPCKO mice.

Manuscripts in preparation: Wnt signaling and metabolic reprogramming.

CONCLUSION. These studies establish Wnt signaling and its metabolic network as a new set of molecular and therapeutic targets for the etiology of SE and potentially for epilepsy. The scientific discoveries underscore the importance of this expanded network and begin to advance the notion that the early period of epileptogenesis may recapitulate aspect s of brain development. The recapitulation to a period in earlier development has been a hallmark of diseases such as cancer. Our data would support models in the field in which epilepsy is a result of excessive stem cell proliferation and then abnormal differentiation—which together, set up a pathological environment that sustains seizures. Our observations that excessive glucose usage may contribute suggests that novel interventions such as the ketogenic diet may attenuating effects,

Our studies also define a novel regiment of drugs in clinical uses that block Wnt signaling attenuates SE induced by two distinct means. These observations underscore the generality of Wnt signaling and provide proof-of-principle that intervening in Wnt signaling may be efficacious for modifying the course of epileptogenesis to prevent recurrent seizures. The Wnt pathway is under intense therapeutic development for cancer and other diseases. By defining the pre-clinical frameworks for epilepsy, this provides an ideal future opportunity to test drugs that attenuate Wnt signaling for their efficacy in disease modification for epilepsy.

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, study questionnaires, and surveys, etc.

N/a

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

These are incorporated into the text.