On the Role of the HBP1 Transcription Factor in Epileptogenesis

An honors thesis for the Department of Biology

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Abstract

Epilepsy is a disease that is characterized by increased neural activity resulting in recurrent seizures. The development of epilepsy occurs during a period of gradual brain alterations called epileptogenesis, which can result from a precipitating event such as head trauma or an initial seizure event called status epilepticus (SE) or it can occur naturally due to genetic predisposition. Our lab previously identified a correlation between epileptogenesis after chemically induced SE and increased Wnt signaling in the hippocampus, a primary region implicated in temporal lobe epilepsies. The additional observation that expression of HBP1, a Wnt repressor, decreased during this period led us to hypothesize that HBP1 may be a causal agent in epileptogenesis. A literature search revealed a gene block containing HBP1 and eight other genes that when deleted was significantly correlated with increased chance of developing seizures. We created an HBP1 knockout strain of mice, compared its phenotype to that of the clinical patients, and found that the mouse model preserved the increased seizure susceptibility, incomplete penetrance, and development of spontaneous seizures. This data suggests that an HBP1 deletion might be the cause of seizures in these patients. To test if this model could be extended further, we looked at the molecular pathways altered in the knockout mice and found that both the mTor and Wnt signaling pathways were upregulated, which is consistent with alterations observed during epileptogenesis in models of temporal lobe epilepsies. If further investigation reveals that this model is in fact a more general model for epilepsy, it can be used to aid in both the treatment and diagnosis of temporal lobe epilepsies.

Introduction

Epilepsy is a disease characterized by increased activity of neural connections in the brain, which results in physical convulsions, or seizures. Two types of epilepsy exist, genetic and acquired. Genetic epilepsy develops as a result of genetic and environmental influences. While some of the genetic influences of innate epilepsy have been well characterized, considerable gaps remain in a complete understanding of all the genetic factors that can lead to epilepsy (Lerche 2013). Acquired epilepsy is the result of an initial seizure event called status epilepticus (SE), which is usually the result of head trauma or other head injury. After SE, the brain undergoes a latent period of gradual remodeling called epileptogenesis, which typically leads to increased seizure susceptibly and, eventually, chronic epilepsy (Williams 2007).

Several challenges exist in the treatment and diagnosis of epilepsy. Because the mechanisms of epileptogenesis remain largely elusive, drugs that target this period have been largely unsuccessful. Instead, the currently available drugs attempt to prevent seizures from occurring once epilepsy has already developed. However, a treatment that focused on the epileptogenic period would attempt to prevent the development of epilepsy, effectively treating the underlying cause of the seizures rather than the seizures themselves (Loscher 2013). This lack of viable drug options is further limited by the inability of many drugs to cross the blood-brain barrier (Pinzon-Daza 2013). Together, these limitations result in a very limited supply of drugs available for the treatment of epilepsy. Further elucidation of the underlying pathways of epileptogenesis and epilepsy has the potential to greatly improve the treatment of epilepsy by increasing the number of potential drug targets (Loscher 2013). There is also room for significant improvement in the diagnosis of acquired epilepsy. At present, a diagnosis can only be obtained by witnessing seizure events and using MRI to look for signature patterns left by seizure activity

on the brain. This use of MRI can provide diagnostic information for selection of treatment, but has limited effectiveness during the epileptogenic period. It is unable to characterize the progression of epileptogenesis because seizures are absent and neither they nor their effects can be observed (Prado 2012). This limitation can prevent diagnosis until after the condition has developed into chronic epilepsy, by which point any drugs attempting to prevent the development of epilepsy have become irrelevant.

Two of the molecular pathways correlated with epileptogenesis and epilepsy are the Wnt signaling pathway and the mTor signaling pathway (Figure 1) (Berdichevsky 2013, Caraci 2008). Both pathways are involved in the regulation of cell growth and proliferation in many cell types throughout the body (Niehrs 2012). However, they have both been found to play a role in neuron development as well (Bateup 2013, Parks 2012). Wnt signaling has been implicated in the development of neuron polarity and axon guidance, as well as in the regulation of synapse formation, differentiation, and plasticity. Due to the complexity of Wnt signaling, it can have either a promoting or an inhibitory affect on both pre and post synaptic formation. Wnt signaling can also regulate the degree of excitatory or inhibitory function of particular synapses. This final function is especially important in the hippocampus where Wnt signaling acts as a positive regulator of LTP (Parks 2012).

The mTor signaling pathway also has important functions in the brain, regulating homeostasis between excitatory and inhibitory elements in synapses (Bateup 2013). One of the major components is the Tuber sclerosis complex (TSC), which acts as a negative regulator of mTor signaling (Galanopoulou 2012). Mutations in TSC have been associated with several neurodevelopment diseases, including autism and epilepsy. These mutations create a misregulation of the excitatory/inhibitory balance in synapses and result in hyperexcitability of

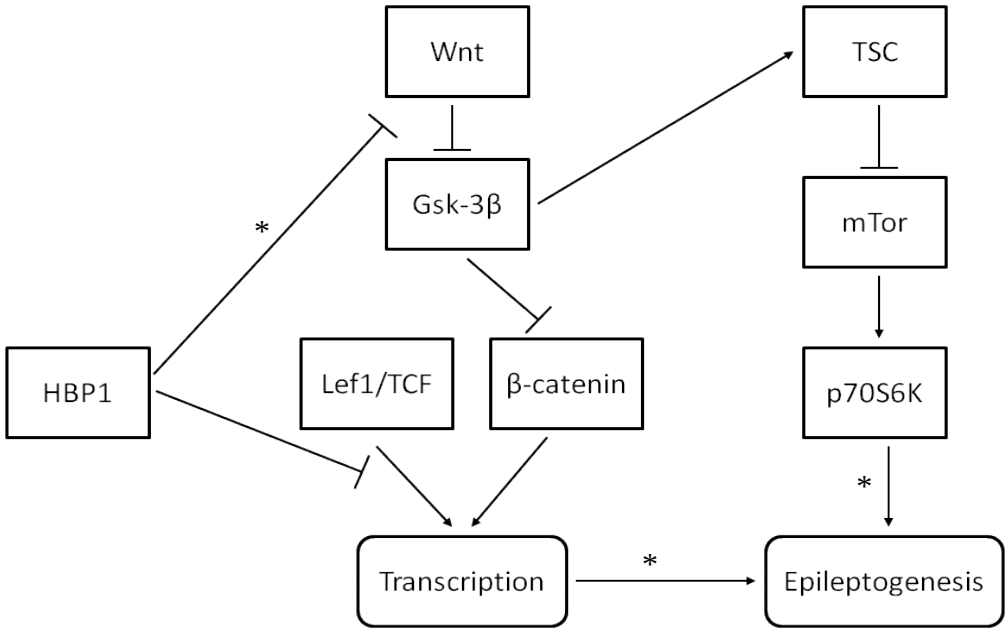


Figure 1. Working model of the Wnt signaling and mTor pathway and their relation to epileptogenesis. The Wnt ligand initiates a signaling cascade through binding to a Frizzled/LRP receptor in the extracellular membrane, which ultimately inactivates the β-catenin destruction complex, including Gsk-3β. This increases β-catenin levels in the cell, which allows β-catenin to be transported into the nucleus where it binds to the transcription factors LEF1/TCF, signaling transcription of Wnt targets (Marchetti 2013). In addition to prevention of β-catenin degradation, inactivated Gsk-3β can no longer activate the Tuber sclerosis complex subunit II, part of the mTor pathway (Buller 2008). TSC1/2 is one of the hubs for control, as its function as a repressor of mTor signaling is regulated by phosphorylation from several different proteins (Song 2012). HBP1 acts as a downstream repressor of Wnt signaling by binding to the LEF1/TCF complex (Sampson 2001). * signifies an interaction that is conjecture. We do not know if HBP1has an upstream target or what that target would be, but the existence of such a target is consistent with the upregulation of mTor markers (phospho-p70S6K) shown in this study in HBP1knockout mice (Song 2012). We also do not know what upstream changes are directly causing epileptogenesis. The two interactions shown are place markers, and the direct cause of epileptogenesis could be any response to increases in either of these pathways.

the hippocampus (Bateup 2013). Again, we see the hippocampus as a central figure in the normal functioning of these pathways.

Therefore, when looking at how these pathways might be involved in epileptogenesis we continued to focus on the hippocampus. In particular, we used mouse models of temporal lobe epilepsy in our experiments because the hippocampus is the primary region implicated in these epilepsies (Berdichevsky 2013). Wild type mice of these model systems will undergo SE after

injection with pilocarpine or kainate. They then undergo a latent period of epileptogenesis for a couple weeks before they begin to have spontaneous recurrent seizures (Williams 2007).

Another interesting component of the Wnt signaling pathway is the HMG-box transcription factor 1 (HBP1), which binds to the Lef1/TCF complex and prevents its function as a transcriptional activator (Figure 1) (Sampson 2001). HBP1 also acts as a repressor in several other pathways, including the epidermal growth factor receptor pathway and the Ras and p38 MAPK-mediated senescence pathway. Generally speaking, HBP1 acts as an inhibitor of the cell cycle and proliferation through transcriptional repression (Zhang 2006). Interestingly, it does not bind directly to DNA in its repression of Wnt signaling, but binds directly to the DNA binding domain of the Lef1/TCF complex, while it does bind to DNA in its other repressive functions (Sampson 2001). Due to its role in regulating the cell cycle, HBP1 has important roles as a tumor suppressor gene, and HBP1 mutations have been implicated in several cancers (Kim 2006).

Preliminary results from our lab showed that while Wnt signaling increases during epileptogenesis, HBP1 levels decrease (Figure 2). This suggests that Wnt signaling is a possible candidate for the driving force behind epileptogenesis and that HBP1 might be the causative agent of this increase through its inability to maintain its repression of Wnt signaling when its expression decreases. Our results show that in adult mice HBP1 is normally expressed almost exclusively in the CA1 region of the hippocampus with minimal co-expression in the dentate gyrus (Figure 2), which further supports HBP1 acting as a causative agent in epileptogenesis because it again implicates the hippocampus.

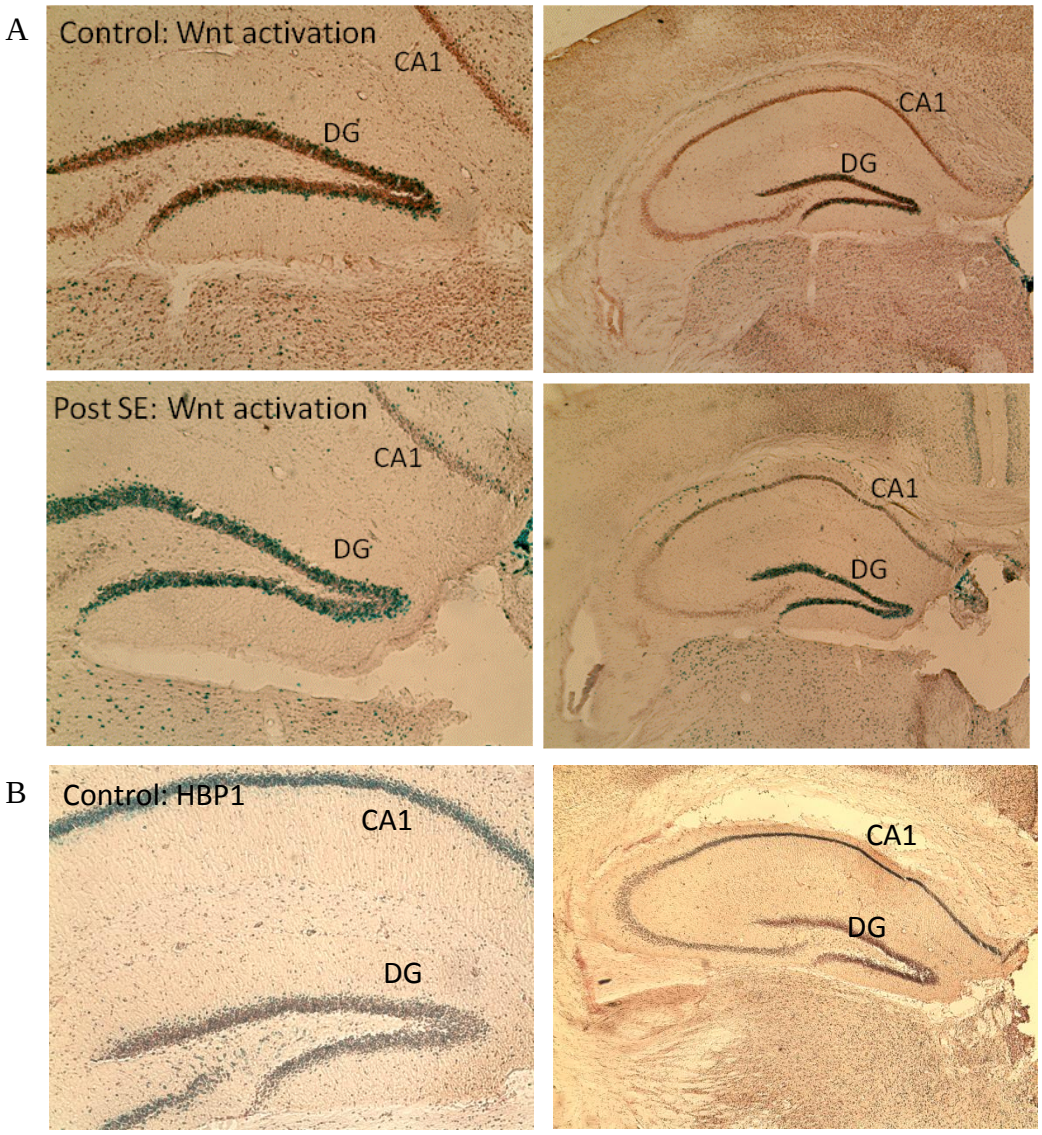
To test this possibility, SE was induced in wild type (WT), HBP1 heterozygous (HET), and HBP1 knockout (KO) mice of two strains that model temporal lobe epilepsy. The KO mice lack the repressor of Wnt signaling, so they would be predicted to have increased Wnt signaling

and the associated increase in cell proliferation. If Wnt signaling were an essential aspect of epileptogenesis, then it would be predicted that the KO mice would have more severe epileptogenesis and thus increased seizure susceptibility relative to the WT mice. Mice were tested for their seizure susceptibility and several other characteristics, which were compared to a clinical phenotype of patients with a deletion in the area of HBP1. Biochemical analysis of mouse hippocampuses at a baseline level in WT, HET, and KO mice was performed to test the association of temporal lobe epilepsy with Wnt signaling and mTor signaling, and the mechanism by which this association arises.

Methods

Case Study Analysis: Clinical cases were found using a literature search and through use of the Decipher database (Firth 2009). Deletions from Decipher that did not contain HBP1 were included in the analysis if they were "close" to HBP1 (~4 MB). Most deletions from other sources that did not include HBP1 were not included in analysis because these were either based on karyotyping which only detects massive deletions or the distances from HBP1 were extremely large, minimizing the relevance of these studies. Studies that used genetic methods and identified deletions close to HBP1 were included. Cases were categorized according to if they had an HBP1 deletion and if the patient in the study presented with abnormal EEG's or with seizures. These results were tabulated in SPSS (IBM) and contingency analysis was run using Fisher's Exact Test.

Figure 2. Wnt signaling and HBP1 expression is localized to the hippocampus in the brain. A) Wnt signaling increases in the dentate gyrus of the hippocampus after induction of SE. Slides of the mouse hippocampus were taken before (top) and after (bottom) SE in mice with β-galactosidase expression at a triple Lef1/TCF targeted minimal promoter, which is highly activated by the presence of β-catenin, indicating increased Wnt signaling (blue). B) HBP1 expression in the brain is exclusive to the CA1 region of the hippocampus. Slides of adult mouse hippocampus with β-galactosidase gene inserted into exon two of HBP1 (blue). CA1 and dentate gyrus (DG) are



Mouse Strains: The two mouse strains used in these experiments were pure background FVBN and C57EL6 strains. Each strain was bred to include an HBP1 knockout allele caused by the insertion of the B-galactosidase gene into exon 2 of HBP1. Mice could be bred to be wild type (WT), heterozygous (HET), or knockout (KO). By mating HET mice, a Mendelian spread of all

three genotypes was obtained. To limit individual variation, only male mice were used for these experiments.

Status Epilepticus Induction and Seizure Grading: SE was induced through intraperitoneal injection of pilocarpine in the FVBN strain and kainate in the C57 strain. Pilocarpine initially was injected at150 mg/kg, then at 135 mg/kg to minimize the death rate. Kainate was injected at 30 mg/kg (Milestone PharmTech). Control mice were injected with PBS in order to obtain baseline levels of brain biochemistry.

SE induced mice were graded for seizure activity until seizure activity had subsided, which corresponds to roughly three hours. Seizures were graded from 1-5 based on the Racine scale (Racine 1972). Seizures of a grade 3 or higher could be reliably assessed by eye, so these were recorded. Grade 3 seizures are equivalent to rearing, grade 4 equivalent to rearing and falling, and grade 5 equivalent to convulsions while on its side and unable to right itself. These are roughly based on human seizure intensities with a five equating to a grand mal seizure. Survival curves and their significance values were determined using the software PRISM and its associated statistical programs. Seizure frequency analysis was performed using SPSS (IBM).

After induction or control injection, mice were maintained for 5 days, and then were sacrificed using cervical dislocation. Three samples were taken from each half of the brain, frozen on dry ice, and finally stored at -80° C. until taken out for analysis. These samples were a piece of the frontal cortex, a piece of the cerebellum, and the entire hippocampus.

EEG Recording: EEG was recorded at 1000 Hz over a two week period with two EEG channels and one EMG channel through mounted cortical electrodes. Video feed was also taken over this

period. Currently, 3 WT and 3 KO mice have been analyzed. Manual curation of the data was performed to look for seizure events in both the EEG data and video feed. Power analysis was performed to find the frequency makeup of the wave function. Lab Chart (ADInstruments) was used for both the scanning and analysis of data.

Western Blotting: Protein expression levels in the mouse hippocampus samples were analyzed using western blotting. Samples were thawed on ice and protein was extracted. Extraction procedure involved crushing sample in a 250µL solution of RIPA buffer, phosphatase inhibitor cocktail, and EDTA. Samples were kept on ice for 15 minutes and vortexed periodically. Samples were then centrifuged at 4° C for 10 minutes at 10,000 rpm. The supernatant was saved as final protein sample and stored at -80° C. The concentration of protein was determined using a Quant-iT protein assay kit (Life Technologies). Western blots were run using 50 ug of protein. Primary antibodies used were Actin (abcam 20272), β-Catenin (Millipore 2037638), phospho-p70S6K (Cell Signaling 9205), p70S6K (Cell Signaling 2708), phospho-AKT (Cell Signaling), AKT (Cell Signaling), phospho-Gsk3β (Cell Signaling), Gsk3β (Cell Signaling), and cMyc (Cell Signaling #5605). Femto maximum sensitivity substrate was used during western blot development. Results were quantified using imajeJ (NIH) and normalized relative to Actin levels.

Real-time PCR: RNA extraction was performed using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. An added step was to run the sample through a shredder column (QIAGEN) before beginning the protocol. RNA was stored at -80° C. 500 ng RNA was converted to cDNA using a reverse transcription kit (Promega). RT-PCR was performed using

primers for NKCC1, KCC2, and 18S ribosomal RNA. 18S was used to normalize the results of the other two primers. The ratio of the two primers (KCC2:NKCC1) was taken and then a student's t-test was performed to determine significance.

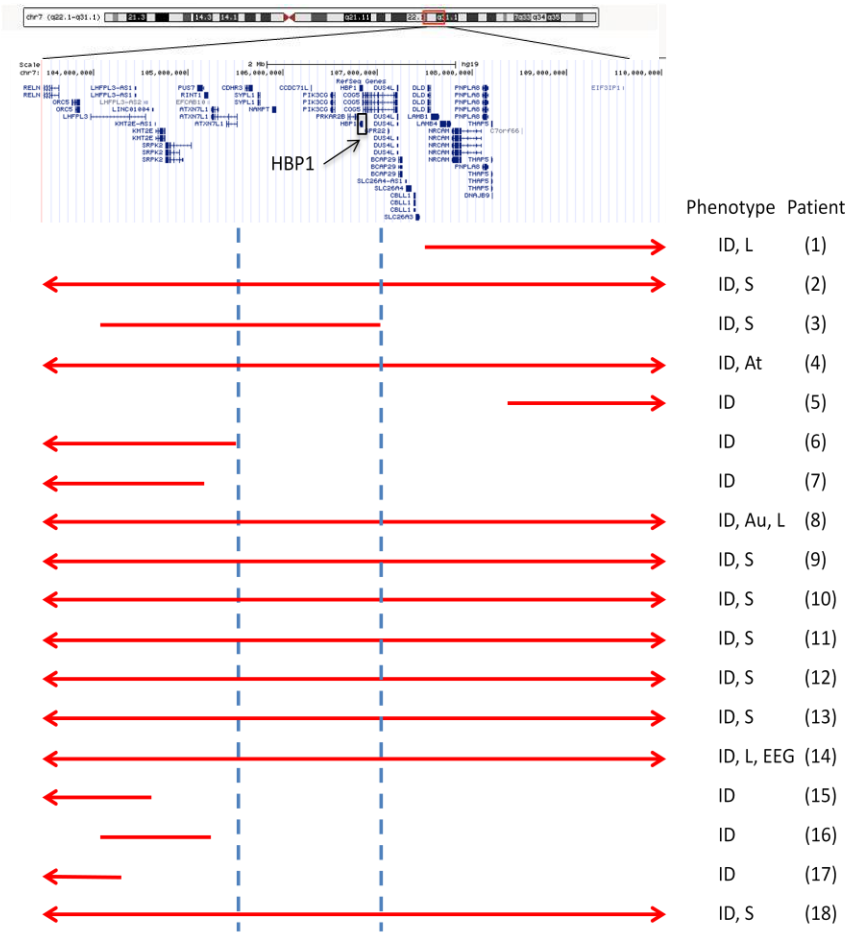
Methods for preliminary experiments are not in the scope of thesis project.

Results

Clinical phenotype of HBP1 deletion

The initial motivation for looking at HBP1 in epilepsy was a case study in which a patient possessed a microdeletion in the region of HBP1 (Uliana 2010). In this study, 15 genes, including HBP1, were deleted on the long arm of chromosome 7. The patient developed an intellectual disability, physical abnormalities, and seizures. A literature search revealed several similar case studies in which patients with deletions in this region developed seizures or an abnormal EEG. Comparing the specific regions in which these deletions occur narrowed the region whose deletion was highly correlated with developing seizures to a nine gene region, still including HBP1. Patients with deletions in this and surrounding regions presented several other abnormal phenotypes, but only the gene block containing HBP1 was positively correlated with an increased frequency of developing seizures (Figure 3). 56 percent of patients with deletions including this gene block developed seizures or an abnormal EEG, which is significantly higher than the 12% of patients with a deletion outside of the gene block who developed seizures or an abnormal EEG (Fisher's Exact Test, $p= 0.00216$). A complete listing of the case studies analyzed can be found in Appendix 1.

A



Phenotype

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

Patient

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

Figure 3. The gene block containing HBP1 is positively correlated with increased frequency of developing seizures or an abnormal EEG. A) Schematic of HBP1 on chromosome 7. Top: red box indicates location of the inset below on chromosome 7. Inset identifies the location of HBP1 on chromosome 7. Bottom: Representative sample of case studies looking at deletions in the HBP1 region of chromosome 7. Includes deletion location relative to inset, the phenotype observed, and reference. Arrowheads indicate that a given deletion extends beyond the inset. The blue lines indicate the smallest common region that consistently displays a seizure phenotype. This gene block contains HBP1 and 8 other genes. B) Summary of all case studies with deletions in the area of HBP1. Indicates the number of patients who possessed a deletion of the HBP1 containing gene block and the number who presented with seizures or an abnormal EEG. The presence of an HBP1 deletion was positively correlated with the presentation of seizures or an abnormal EEG (Fisher's exact test, p=0.0022).

B

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

Fisher's Exact Test: p=0.00216

Seizure susceptibility of HBP1 deficient mice

Our preliminary results (Figure 2) indicate that the epileptogenic period of induced temporal lobe epilepsy in mice is correlated with increased levels of Wnt signaling. Because HBP1 acts a repressor of Wnt signaling, we thought it possible that the deletion of HBP1 in these

patients might be the cause of some of their abnormal phenotypes through an inability to regulate Wnt signaling properly. To this end, we tested the seizure response of mice with an HBP1 deletion. Status epilepticus is induced by giving the mice an intraperitoneal injection of either pilocarpine or kainate, depending on the strain of the mouse. FVBN mice were induced with pilocarpine, while C57 mice were induced with kainate. The inducement of status epilepticus using these drugs models temporal lobe epilepsy (Reddy 2013). After injection, mice will generally have an initial seizure event after about 20 minutes and then periodically seize for about 3 hours. The number and severity of seizures (on a 5-point scale) were the quantitative measure of seizure response. Two strains were used for measurements to ensure that the results were not strain specific and were a function of the deletion itself.

Seizure induction and measurements were performed on mice of all three HBP1 genotypes (Fig 4). In the FVBN strain, there was a notable drop in the number of seizures of increasing grade with mice having the most grade 3 seizures and the least grade 5 (Fig. 4A, E). There was a significant difference between the number of seizures WT mice had compared to KO mice ($p \leq 0.006$). The HET mice were intermediate at every grade. In C57 mice, there were more grade 3 seizures than grade 4 or 5, but there was no clear difference in the number of grade 4 or 5 seizures (Fig. 4C). There was no significant difference between the number of seizures WT mice and HET mice had at any grade, but KO mice had significantly more grade 4 and 5 seizures than both WT and HET mice ($p \leq 0.005$).

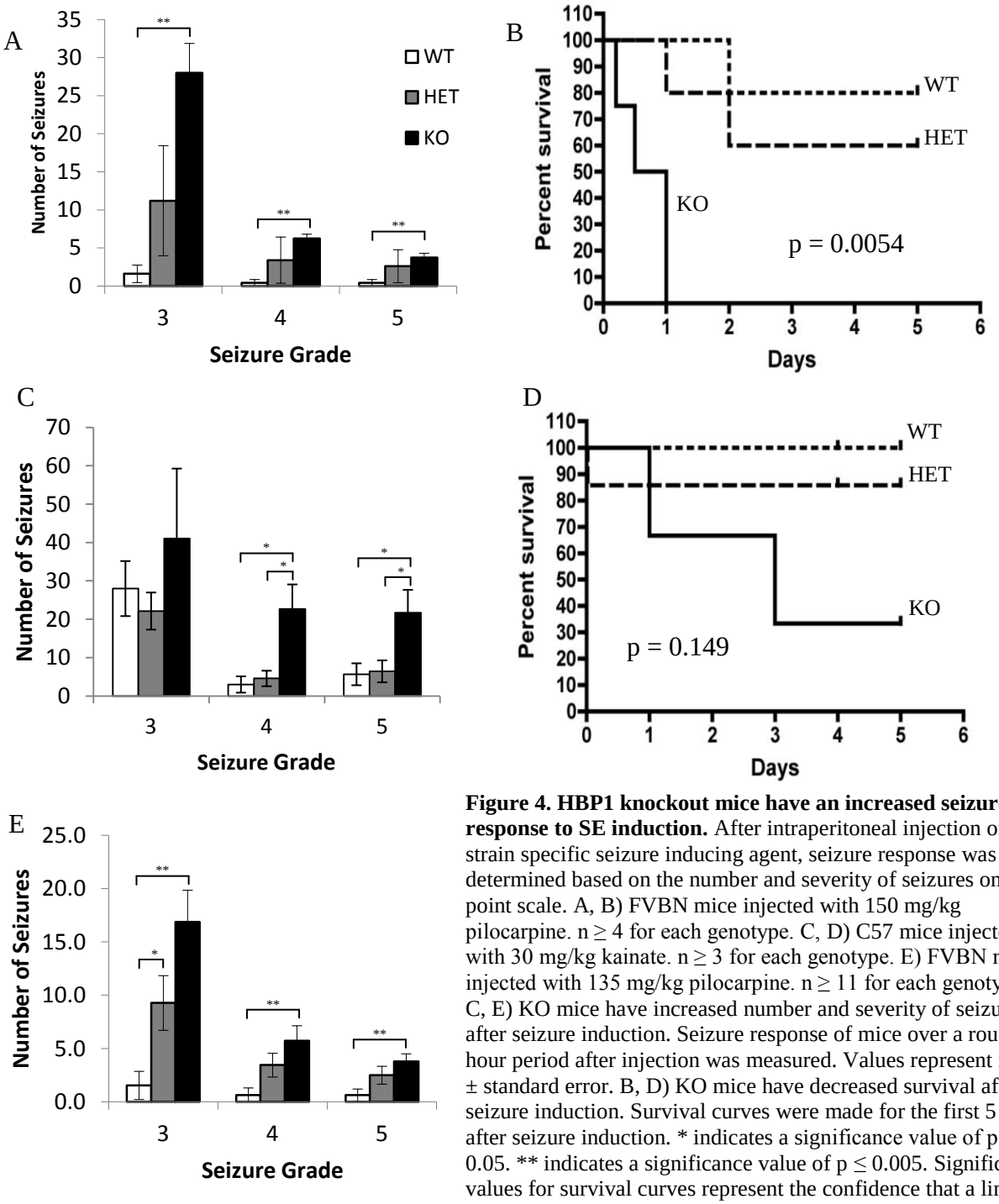


Figure 4. HBP1 knockout mice have an increased seizure response to SE induction. After intraperitoneal injection of a strain specific seizure inducing agent, seizure response was determined based on the number and severity of seizures on a 5 point scale. A, B) FVBN mice injected with 150 mg/kg pilocarpine. $n \geq 4$ for each genotype. C, D) C57 mice injected with 30 mg/kg kainate. $n \geq 3$ for each genotype. E) FVBN mice injected with 135 mg/kg pilocarpine. $n \geq 11$ for each genotype. A, C, E) KO mice have increased number and severity of seizures after seizure induction. Seizure response of mice over a roughly 3 hour period after injection was measured. Values represent means ± standard error. B, D) KO mice have decreased survival after seizure induction. Survival curves were made for the first 5 days after seizure induction. * indicates a significance value of $p \leq 0.05$. ** indicates a significance value of $p \leq 0.005$. Significance values for survival curves represent the confidence that a linear trend exists in death rate across genotypes.

In FVBN mice, the initial dosage of pilocarpine used was 150 mg/kg, but this led to death of many knockout mice within several days of induction (Fig 4B). A similar trend of increased death occurred after the 30 mg/kg dosage of kainate in C57 mice. An analysis of the survival

curves of mice after seizure induction reveals a trend that parallels the increase in seizure severity (Fig. 4B, D). In FVBN mice, the number of mice surviving five days after seizure induction decreased linearly from WT to HET to KO (p=0.0054). Most WT and HET mice survived, but no KO mouse survived the duration. In C57 mice, a similar trend emerged, with all WT mice and all but one HET mouse surviving 5 days while only one KO mouse survived five days (p=0.149). FVBN mice induced with an adjusted of dosage of 135 mg/kg, a level at which KO mice no longer died after induction, showed a similar trend in seizure response across genotypes as the higher dosage (Fig 4E).

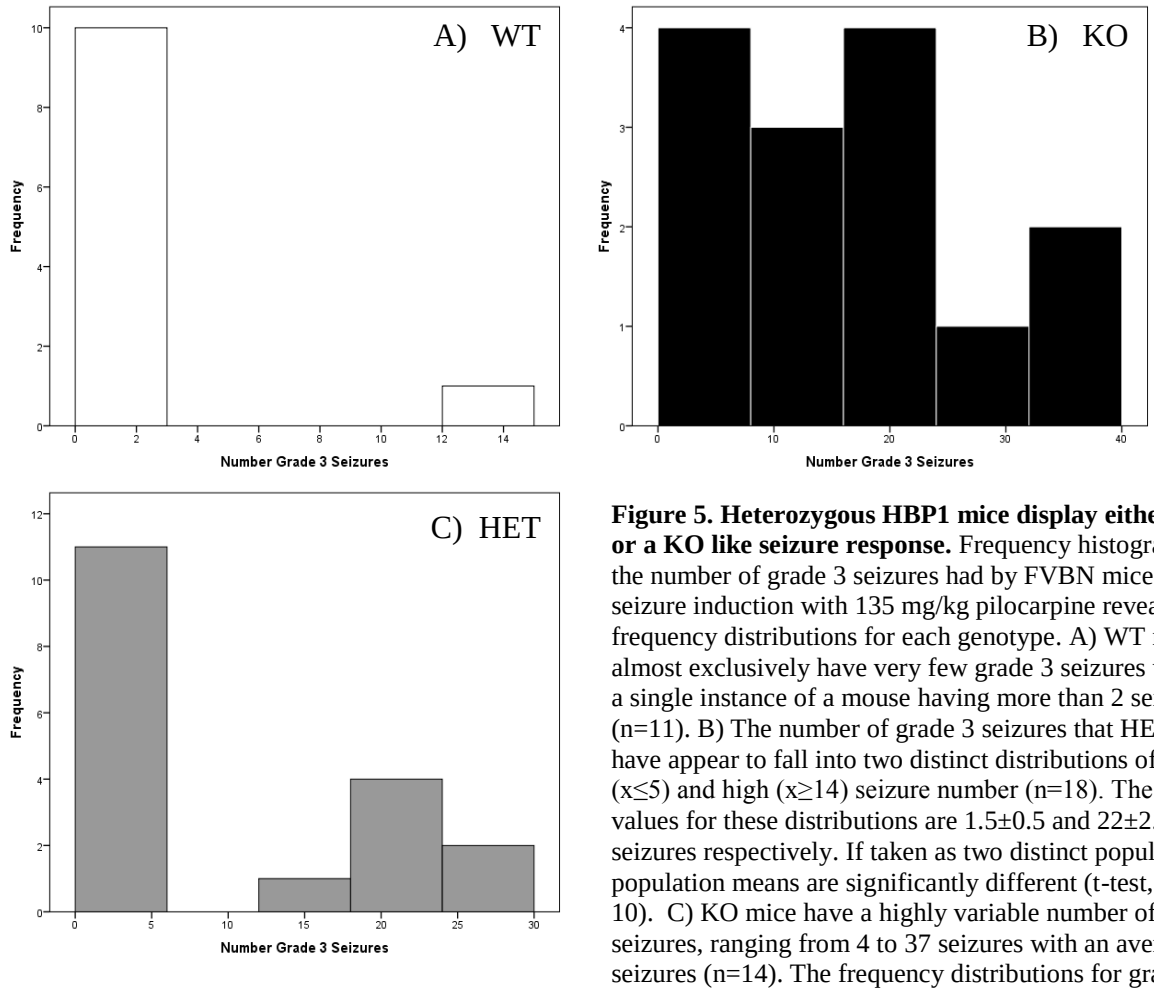


Figure 5. Heterozygous HBP1 mice display either a WT or a KO like seizure response. Frequency histograms for the number of grade 3 seizures had by FVBN mice after seizure induction with 135 mg/kg pilocarpine reveal different frequency distributions for each genotype. A) WT mice almost exclusively have very few grade 3 seizures with only a single instance of a mouse having more than 2 seizures (n=11). B) The number of grade 3 seizures that HET mice have appear to fall into two distinct distributions of low ($x \leq 5$) and high ($x \geq 14$) seizure number (n=18). The average values for these distributions are 1.5±0.5 and 22±2.0 (±SE) seizures respectively. If taken as two distinct populations, the population means are significantly different (t-test, p=8.5E-10). C) KO mice have a highly variable number of grade 3 seizures, ranging from 4 to 37 seizures with an average of 17 seizures (n=14). The frequency distributions for grade 4 and grade 5 seizures display similar trends (data not shown).

Incomplete penetrance of seizure phenotype

Most additional experiments will focus on the WT and KO genotypes, as this comparison best reveals the genetic role of HBP1 in causing the increased seizure susceptibility. However, it is worth briefly examining the seizure response of the HET mice because these mice best parallel the clinical condition of a single HBP1 deletion in a patient (Fig 5). Interestingly, individual HET mice either acted like WT or KO mice and not an intermediate. Either they had a large number of seizures or they had very few seizures, resulting in the high variance observed. This parallels the incomplete penetrance seen in the seizure phenotype of clinical patients.

Spontaneous seizures in HBP1 knockout mice

Another aspect of the clinical phenotype that an ideal model system would retain is the development of spontaneous seizures, so we looked for evidence of this in the HBP1 deficient mice. Thus far, we have found evidence of three mice having spontaneous seizure activity. The most severe of these was actually a neonate HET mouse, which had a prolonged grand mal seizure and died as a result. The other two mice with evidence of spontaneous seizures were found as the result of EEG/video recordings. WT and KO mice were recorded continuously over a two week period and the results were analyzed for evidence of seizure activity. Of the three KO mice analyzed, two mice had seizure activity, which showed a typical EEG profile for both the tonic and tonic-clonic portions of the seizures and were associated with expected behavioral activity (Figure 6) (Racine 1972). No evidence of spontaneous seizures was observed in WT mice using the EEG/video data or behaviorally while working with the WT mice.

Abnormal EEG activity was recorded in several KO mice, which was associated with abnormal hyperactive behavior. These behaviors were also observed in several other KO mice

throughout working with them. Typical examples of this hyperactive behavior include compulsive circling and continuous jumping (jumping not observed during EEG recordings). This type of abnormal behavior has been associated with seizure activity in other mouse models and in some human patients with epilepsy, suggesting possible seizure activity in these HBP1 KO mice as well (DeLorey 1998, Gastaut 1986). None of these abnormal EEG readings or behaviors was observed in WT mice during EEG recording or otherwise.

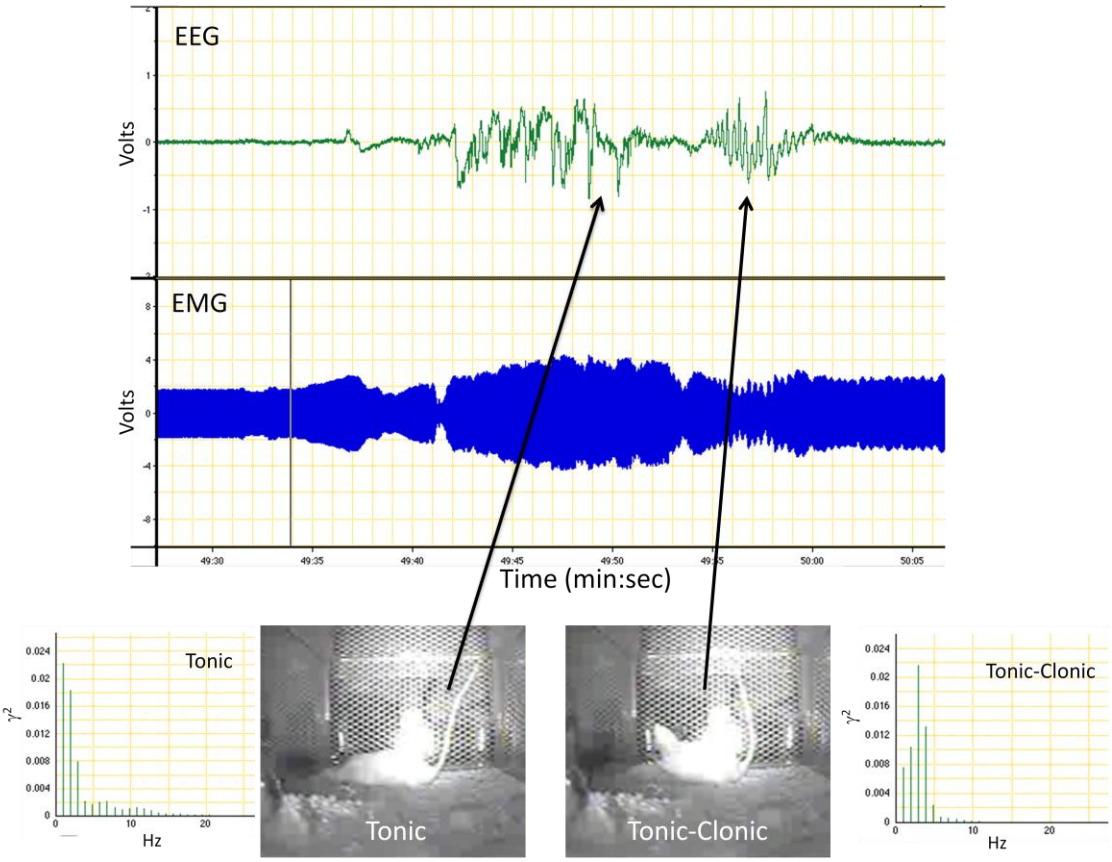


Figure 6. HBP1 KO mice have spontaneous seizures. Selection of EEG data from a KO mouse without seizure induction. EEG was recorded at 1000 Hz over a two week period with two EEG channels and one EMG channel through mounted cortical electrodes (top). Mouse exhibited a spontaneous seizure event mid sleep cycle. The mouse initially exhibited a tonic seizure with a chaotic high amplitude waveform, which can be seen behaviorally as the rigid extension of the tail. The mouse then progressed to a tonic-clonic seizure with a more regular high amplitude waveform, which can be seen behaviorally as the rearing of the mouse and "piano playing" with its paws in a non-grooming fashion (bottom) (Racine 1972).

Wnt and mTor signaling in HBP1 knockout

To understand how the HBP1 knockout is causing increased seizure susceptibility, the baseline expression levels for various proteins involved in the Wnt signaling and the mTor pathway were analyzed (Figure 7). The analysis was performed on hippocampus extracts of five day post-injection mice. The expectation is that the baseline expression profile of the KO mice will be similar to that of WT mice after induction because both are periods of increased seizure response over the WT baseline. To this end, expression levels of markers for increased Wnt signaling (phospho-GSK3β, β-catenin, and cMyc) and increased mTor signaling (phospho-P7S6K, phospho-AKT) were measured because these are hallmarks of the epileptogenesis period in WT mice (Berdichevsky 2013, Caraci 2008). Although the data is not entirely consistent between strains, for each marker at least one strain shows a tentative increase in its expression, and each strain has at least one upregulated marker per pathway. The most striking result is the increase in phosphorylation of p70S6K and GSK3β. The p70S6Kresult in particular shows an massive increase across strains suggesting a strong upregulation of mTor signaling (Song 2012). Work is currently being performed to determine the exact mechanism by which HPB1's absence affects these expression levels.

Downstream changes in HBP1 knockout

Finally, we wanted to look at the downstream effects that might be resulting in the increased seizure susceptibility in the HBP1 knockout mice. Specifically, we looked at the expression of two cation-chloride cotransporters (NKCC1 and KCC2). The ratio of these two proteins determines whether chloride concentrations are greater inside or outside the cell, which

in turn determines whether GABA receptors are activating or inactivating at synapses (Benarroch 2013). Our preliminary results looking at RNA expression of these genes in FVBN mice indicate

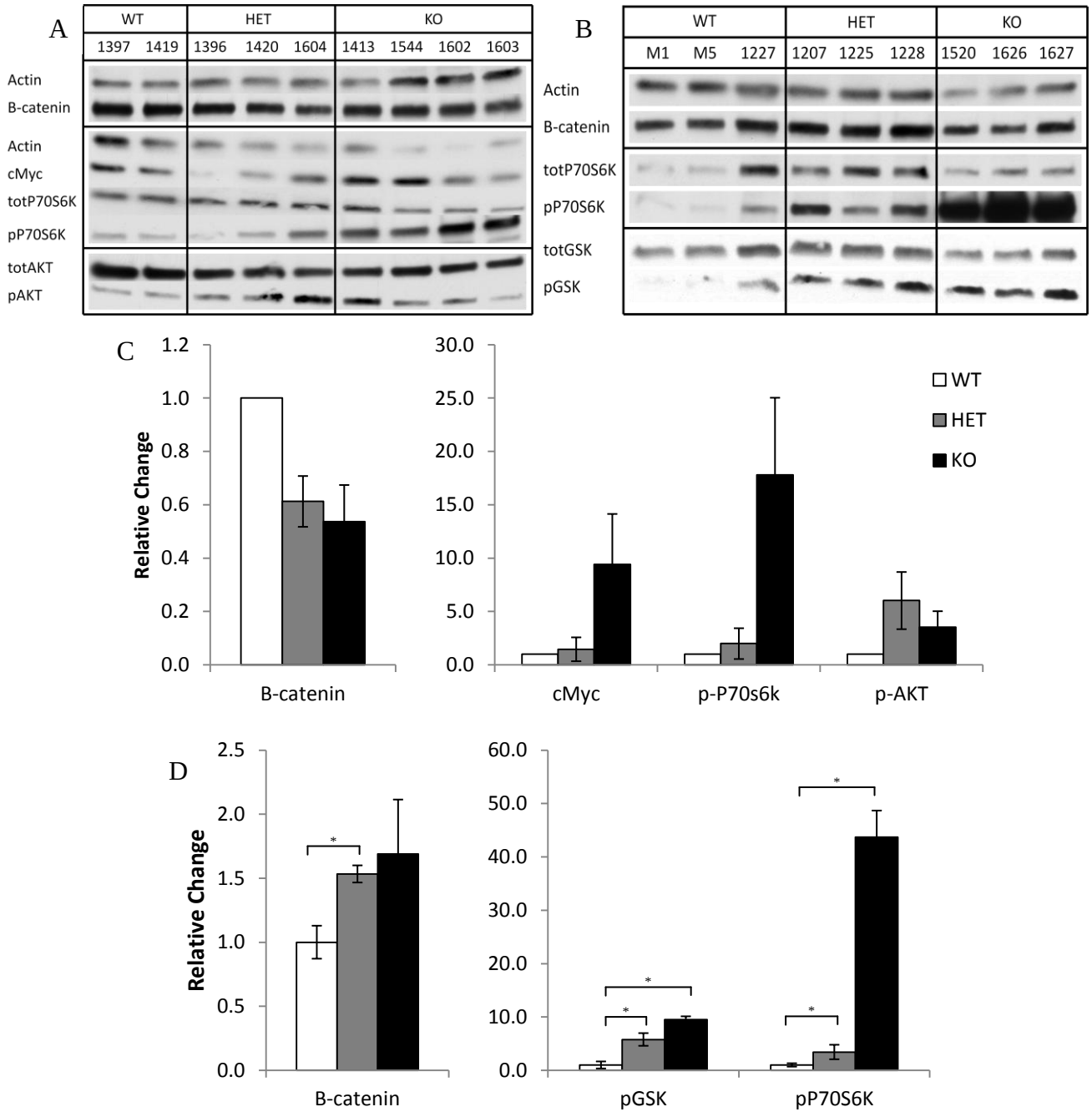


Figure 7. Baseline levels of Wnt and mTor signaling markers are increased in HBP1 KO mice. Protein expression levels were determined by Western blot analysis on hippocampus extracts from mice sacrificed 5 days after a control injection of PBS solution in WT, HET, and KO mice. Both the FVBN strain (A,C) and C57 strain (B,D) were analyzed. Quantification was performed using ImajeJ (NIH) using Actin levels for normalization (C,D). Results reveal a strain independent increase in phosphoP70S6K, an mTor signaling marker, and strain specific increases in phosphoGSK-3β, cMyc, β-catenin, and phospoAKT, markers for mTor and Wnt signaling. * indicates a significance value of $p \leq 0.05$.

a possible, although insignificant, shift toward NKCC1 dominance in HBP1 knockout mice (Figure 8). However, these results did not appear to translate to the protein level or the C57 strain.

Discussion

HBP1 knockout models clinical phenotype

These results present a deletion of HBP1 as a possible cause of seizure activity in clinical patients with microdeletions on chromosome 7q. Patients with a deletion in a nine-gene region containing HBP1 had an increased risk of developing seizures or an abnormal EEG relative to patients with deletions outside this region. While these patients presented several other abnormal phenotypes, these were not highly correlated with the region containing HBP1. Two lines of evidence led us to propose that HBP1 might be the driving force behind this clinical phenotype. First, HBP1expression in the mouse brain is exclusive to the hippocampus. This is the primary region of the brain implicated in epileptogenesis of temporal lobe epilepsies (Berdichevsky 2013). Second, increased Wnt signaling was seen in the mouse hippocampus during epileptogenesis after induced SE. This increase in Wnt signaling was correlated with a decrease in HBP1 expression. Together, these lines of evidence led us to propose that HBP1 is normally expressed in the hippocampus where it functions to limit Wnt signaling in these cells. However, as a result of SE induction, HBP1 expression is lowered, which triggers an upregulation of Wnt signaling. This increased Wnt signaling activity triggers some portion of the epileptogenic pathway, which eventually leads to the development of epilepsy. An extension of this hypothesis is that if HBP1 were absent from the hippocampus, as in a KO mouse or a patient with an HBP1 deletion, an aberrant increase in Wnt signaling would occur, which would spontaneously trigger

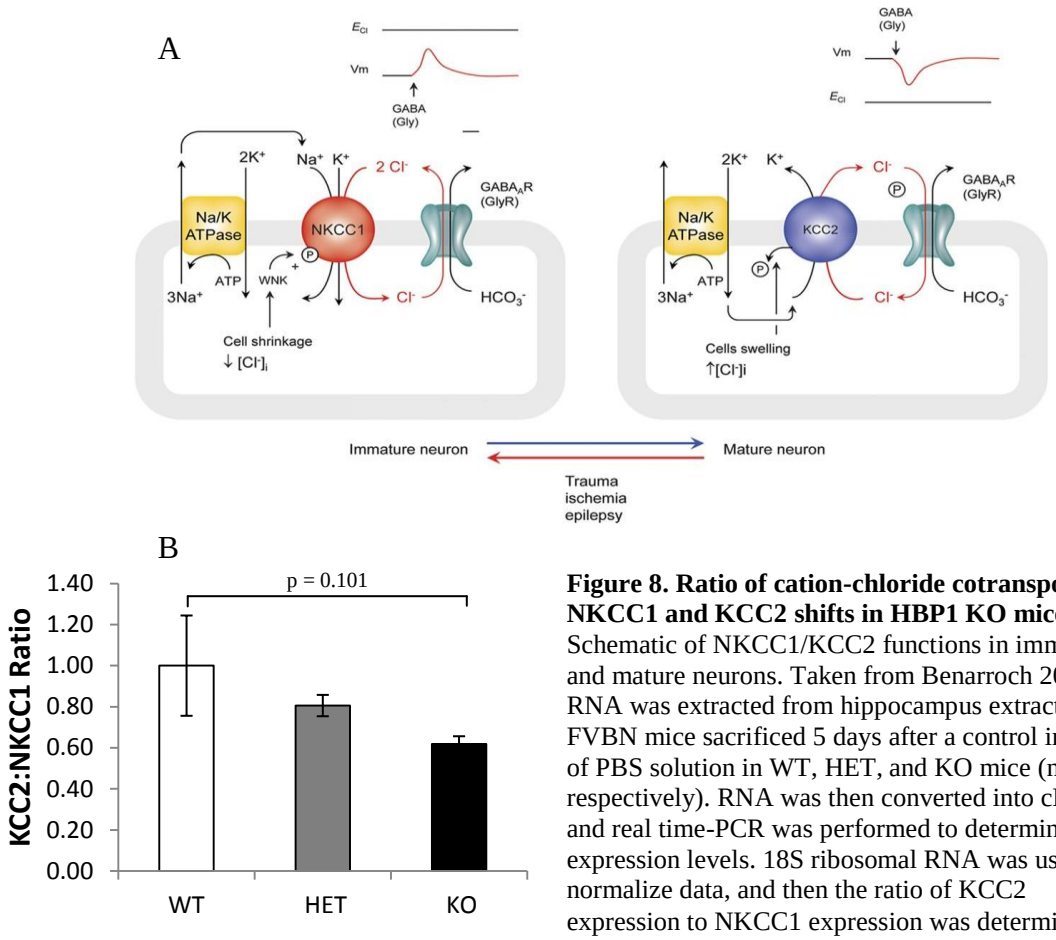


Figure 8. Ratio of cation-chloride cotransporters NKCC1 and KCC2 shifts in HBP1 KO mice. A) Schematic of NKCC1/KCC2 functions in immature and mature neurons. Taken from Benarroch 2013. B) RNA was extracted from hippocampus extracts of FVBN mice sacrificed 5 days after a control injection of PBS solution in WT, HET, and KO mice (n=5,2,4 respectively). RNA was then converted into cDNA and real time-PCR was performed to determine RNA expression levels. 18S ribosomal RNA was used to normalize data, and then the ratio of KCC2 expression to NKCC1 expression was determined.

epileptogenesis without the occurrence of SE. This spontaneous epileptogenesis would progress similarly to standard epileptogenesis and act to increase seizure susceptibility and possibly result in spontaneous seizure activity. If true, this would explain the clinical phenotypes seen in patients with deletions of the HBP1 gene block.

To test the validity of this hypothesis, we investigated whether HBP1 KO mice developed similar phenotypes to the clinical patients. Specifically, we looked at three phenotypes expected to develop in HBP1 deficient mice if HBP1 deletions in humans were the cause of seizure development. These were increased seizure susceptibility, incomplete penetrance of seizures, and spontaneous seizure activity. Seizure susceptibility and incomplete penetrance were tested in two mouse strains by induction of SE using pilocarpine or kainate as models of temporal lobe

epilepsy. After seizure induction, KO mice had a significantly worse seizure response than WT mice as measured by number of seizures, severity of seizures, and mortality rate during the five day period after SE. HBP1 HET mice showed incomplete penetrance in seizure susceptibility after SE induction. Two populations of HET mice existed in the population; roughly half had responses similar to WT mice with very few seizures while the others had responses similar to KO mice with a large number of seizures. These results clearly show that the HBP1 gene is somehow involved in suppressing seizure susceptibility in the mouse model because in its absence, seizure susceptibility is greatly increased in mice. While these are two characteristics predicted for a genetic model based on the clinical cases, one must be careful in comparing the results of these experiments to the clinical cases. These results come from a model of seizure susceptibility based on induced SE, while the clinical patients develop spontaneous seizures without any chemical or physical insult. Therefore, while this data is consistent with the hypothesis that an HBP1 deletion is the cause of the clinical phenotype and that our HBP1 KO mice are a genetic model for this phenotype, it is by no means conclusive evidence.

To further test the validity of our model, we looked for evidence of spontaneous seizure activity, which is much more relevant to the clinical phenotype. A combination of observational data and EEG recordings has revealed several mice with spontaneous seizures. The most severe of these was a grand mal seizure in a HET neonate, which resulted in the death of the mouse. The EEG recordings have thus far revealed spontaneous seizures in two out of three KO mice analyzed, while no WT mice presented with spontaneous seizures. This more concrete link between the KO mice characteristics and the clinical phenotype is currently being analyzed further in an attempt to quantify the penetrance of spontaneous seizures development in the KO mice.

This evidence further supports HBP1 as being the causative agent of a seizure phenotype in clinical patients, but the eight other genes present in the gene block must be ruled out as possible contributors to the phenotype before anything conclusive can be determined. Not all of the genes in the block have been well characterized, but the characteristics of deficiencies of six out of the eight genes have been looked at in some way (Table 1). None of these genes seems to have a particular link to a seizure phenotype, which supports but does not prove that none of them is involved. Thus, while the current study presents substantial evidence that HBP1 is the causative agent of the clinical seizure phenotype seen in patients with deletions of the HBP1 gene block, further characterization of knockout models for each of these genes must be performed to show this conclusively.

Possible expansions of the HBP1 model

While having a model system for the specific clinical phenotype resulting from microdeletions of HBP1 might be extremely useful in determining how specific antiepileptic

Table 1: Phenotypes for deficiencies of the eight genes in the HBP1 gene block

Gene	Phenotype	Reference
ATXN7L1	Case study: Interruption by inversion causes developmental delay (PDE1C also interrupted)	Uliana 2010, Gamage 2013
CDHR3	no characterization	
SYPL1	Theoretical: Possible implication in psychomotor delay	Uliana 2010
NAMPT	Tumor cells: inhibition blocks lipid synthesis and decreases proliferation while triggering cell death	Thakur 2012, Bowlby 2012
CCDC71L	no characterization	
PIK3CG	Mouse micromass culture: inhibition leads to a delay in chondrocyte differentiation and reduced bone growth	Ulici 2008
PRKAR2B	Mouse: knockout reduces obesity and white adipose tissue	Czyzyk 2008
COG5	Case study: mild psychomotor retardation with delayed motor and language development	Paesbold-Burda 2009

drugs (AEDs) will affect these patients, extending the model farther would be even more useful (Loscher 2013). It is possible that the molecular mechanisms by which this mutation leads to epilepsy are unique, but it is also possible to imagine that these same molecular mechanisms convert a healthy brain into a seizure prone brain in acquired or genetic temporal lobe epilepsies.

To establish whether HBP1 deletions represent a widely applicable epilepsy model, molecular data must be used to show that the baseline molecular pathways active in HBP1 KO mice correspond to those pathways that are activated in epileptogenesis by SE. This paper attempts to provide preliminary evidence supporting this claim. The most striking molecular evidence found is a strain independent, massive increase in the phosphorylation level of p70S6K in HBP1 KO mice. This indicates a baseline increase in mTor signaling. Both the phosphorylation of p70S6K and increased mTor signaling are well characterized aspects of the epileptogenic period (Berdichevsky 2013). The changes in markers for Wnt signaling and other markers for mTor signaling (phospho-GSK3β, β-catenin, cMyc, phospho-AKT) were less pronounced and were not consistent between stains, but the current data suggests that both Wnt and mTor signaling are increased in the hippocampus of HBP1 KO mice (Berdichevsky 2013, Caraci 2008). Direct comparison and localization studies comparing these mice to mice undergoing SE induced epileptogenesis are still required to validate that this is an accurate model for the changes in expression seen during epileptogenesis (See Figure 1 for summarized predictions of current model).

To validate the HBP1 knockout system as a model of epilepsy further, one would need to show the downstream effects that led to the increased seizure susceptibility and spontaneous seizures. In this study, we show only a preliminary analysis of what these downstream affects

might be. We show that at the RNA level, HBP1 knockout mice appear to revert from KCC2 dominance to NKCC1 dominance in cation-chloride cotransporter expression. Normally KKC2 is the dominant protein expressed in neurons, which transports chloride out of the cell to create hyperpolarizing and thus inhibitory GABA receptors. However, in developing neurons NKCC1 is dominant, which transports chloride into cell causing GABA receptors to be depolarizing and thus activating (Figure 8) (Benarroch 2013). The shift to KCC2 dominance in mature neurons normally happens shortly before birth in humans, but a reversion to NKCC1 dominance has been implicated in several diseases, including temporal lobe epilepsies (Dzhala 2005, Benarroch 2013). Therefore, the shift seen in the HBP1 knockout mice is consistent with an epilepsy phenotype. However, we were unable to confirm this shift in the C57 strain or at the protein level. This does not necessarily mean that the expression levels do not change because they might be localized changes, but it does not support a change. Currently, we are pursuing additional downstream targets that might cause the shift in seizure susceptibility and looking into mechanisms that might link these with the upstream changes in Wnt and mTor signaling. These include the expression levels of various GABA receptor subunits as well as other targets identified using RNA sequencing data.

Potential Applications

While we are far from demonstrating that HBP1 deletions represent a model for multiple epilepsies, if this turns out to be the case it could provide useful information for both the treatment and diagnosis of these epilepsies. By revealing a key molecular pathway in epileptogenesis, it could provide new drug targets to use in developing treatment. More importantly, it could reveal drug targets that would allow the attenuation of the epileptogenic

process itself, which would be a major improvement over current treatments that only work to prevent seizures once they are already occurring (Locher 2013). We are currently looking into the potential benefits of one possible drug combination, EGCG/DAC, which acts to repress Wnt signaling by increasing levels of HBP1 (Kim 2006). Many drugs with similar targets cannot be used to treat epilepsy because they do not cross the blood brain barrier, but EGCG/DAC does (Pinzon-Daza 2013). Therefore, it might be possible to use ECGC/DAC to counteract the effect of increased Wnt signaling present during epileptogenesis. Such a treatment would greatly aid the field of epilepsy treatment by preventing epilepsy rather than just managing the symptoms (Locher 2013).

Early diagnosis of epilepsies during the epileptogenic period could also be improved using an HBP1 model. One characteristic of epileptogenesis is an altered metabolism including a lactate buildup possibly due to increased cell growth and proliferation present during that period (Pittau 2014). If true, one should be able to identify a metabolic profile of epileptogenesis using NMR spectroscopy of hippocampus extracts from HBP1 knockout mice or from other mouse models of epileptogenesis. It may even be possible to detect differences in this metabolic profile between different types or severities of epilepsy. Once these metabolic profiles existed, they could be used to diagnose patients using MRS technology. This modified version of an MRI allows detection of the concentrations of specific metabolites. Thus detecting the specific metabolic profile associated epilepsy the patient can be used diagnostically (Pittau 2014). This would be especially useful for patients with acquired epilepsy because after SE, MRS could be used to determine if the patient is exhibiting characteristic signs of epileptogenesis, and treatment could be given accordingly.

References

1. Abuelo, D. & Padre-Mendoza, T. Cat-like cry and mental retardation owing to 7q interstitial deletion (7q22-+7q32). *J Med Genet* **19**, 473-476 (1982).

2. Al-Hassnan, Z., Al-Bakheet, A., Abu-Dheim, N. & Kaya, N. A Novel Interstitial Microdeletion of 7q22.1-7q22.3 Detected by Array Comparative Genomic Hybridization. *Am J Med Genet Part A* **155**, 3128-3131 (2011).

3. Bateup, H. *et al*. Excitatory/Inhibitory Synaptic Imbalance Leads to Hippocampal Hyperexcitability in Mouse Models of Tuberous Sclerosis. *Neuron* **78**, 510-522 (2013).

4. Benarroch, E. Cation-chloride cotransporters in the nervous system. *Neurology* **80**, 756-763 (2013).

5. Berdichevsky, Y. *et al*. PI3K-Akt Signaling Activates mTOR-Mediated Epileptogenesis in Organotypic Hippocampal Culture Model of Post-Traumatic Epilepsy. *J Neurosci* **33**, 9056-9067 (2013).

6. Bowlby, S., Thomas, M., D'Agostino, R. & Kridel, S. Nicotinamide Phosphoribosyl Transferase Nampt)Is Required for De Novo Lipogenesis in Tumor Cells. *PLoS One* **7**, e40195 (2012).

7. Buller, C., Loberg, R., Fan, M., Zhu, Q. & Brosius, F. A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. *Am J Physiol Cell Physiol* **295**, C836-C843 (2008).

8. Caraci, F., Busceti, C., Biagioni, F., Aronica, E. & Nicoletti, F. The Wnt Antagonist, Dickkopf-1, as a Target for the Treatment of Neurodegenerative Disorders. *Neurochem Res* **33**, 2401-2406 (2008).

9. Czyzyk, T., Sikorski, M., Yang, L. & McKnight, S. Disruption of the RIIbeta subunit of the PKA reverses the obesity syndrome of Agouti lethal yellow mice
. *Proc Natl Acad Sci USA* **105**, 276-281 (2008).

10. DeLorey, T., Handforth, A., Anagnostaras, S., Homanics, G. & Olsen, R. Mice Lacking the b3 Subunit of the GABAA Receptor Have the Epilepsy Phenotype and Many of the Behavioral Characteristics of Angelman Syndrome. *J Neurosci* **18**, 8505-8514 (1998).

11. Dzhala, V., Talos, D., Sdrulla, D., Brumback, A. & Staley, K. NKCC1 transporter facilitates seizures in the developing brain. *Nature Methods* **11**, 1205-1213 (2005).

12. Fagan, K., Gill, A., Henry, R., Wilkinson, I. & Carey, B. A summary of 7q interstitial deletions and exclusion mapping of the gene for -glucuronidase. *J Med Genet* **26**, 619-625 (1989).

13. Feuk, L. *et al*. Absence of a Paternally Inherited FOXP2 Gene in Developmental Verbal Dyspraxia. *Am. J. Hum. Genet.* **79**, 965--972 (2006;79:965).

14. Firth, H., Richards, S., Bevan, A. & Carter, N. DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources. *Am J Hum Genet* **84**, 524-533 (2009).

15. Franceschini, P. *et al*. Interstitial Deletion of the Long Arm of Chromosome 7 46,XX,del(7)(pter-q2200 : : q3200-qter). *Hum. Genet.* **1978**, 345-348 (44).

16. Galanopoulou, A., Gorter, J. & Cepeda, C. Finding a better drug for epilepsy: The mTOR pathway as an antiepileptogenic target. *Epilepsia* **53**, 1119-1130 (2012).

17. Gamage, T., Misceo, D., Fannemel, M. & Frengen, E. A balanced de novo inv(7)(p14.3q22.3) disrupting PDE1C and ATXN7L1 in a 14-year old developmentally delayed boy. *Eur J Med Genet* **56**, 361-364 (2013).

18. Gastaut, H., Aguglia, U. & Tinuper, P. Benign Versive or Circling Epilepsy with Bilateral 3-cps Spike-and-Wave Discharges in Late Childhood. *Ann Neurol* **19**, 301-303 (1986).

19. Higginson, G., Weaver, D., Magenis, R. & Hepburn, D. Interstitial deletion of the long arm of chromosome no. 7 (7q-) in an infant with multiple anomalies. *Clin Genet* **10**, 307-312 (1976).

20. Kim, J. *et al*. Suppression of Wnt Signaling by the Green Tea Compound -Epigallocatechin 3-Gallate EGCG in Invasive Breast Cancer Cells. *J Biol Chem* **281**, 10865-10875 (2006).

21. Klep-De Pater, J., Bijlsma, J., Bleeker-Wagemakers, E., France, H. & Vries-Ekkers, C. Two cases with different deletions of the long arm of chromosome 7. *J Med Genet* **16**, 151-154 (1979).

22. Krepischi-Santos, A., Vianna-Morgante, A., Jehee, F., Passos-Bueno, M. & Rosenberg, C. Whole-genome array-CGH screening in undiagnosed syndromic patients: old syndromes revisited and new alterations.*Cytogenet Genome Res* **115**, 254-261 (2006).

23. Lerche, H. *et al*. Ion channels in genetic and acquired forms of epilepsy. *J Physiol* **591**, 753-764 (2013).

24. Löscher, W., Klitgaard, H., Twyman, R. & Schmidt, D. New avenues for anti-epileptic drug discovery and development. *Nat Rev Drug Discov* **12**, 757-776 (2013).

25. Marchetti, B. & Pluchino, S. Wnt your brain be inflamed? Yes, it Wnt! *Trends Mol Med* **19**, 144-156 (2013).

26. Martínez-Jacobo, L. *et al*. Delineation of a de novo 7q21.3q31.1 Deletion by CGH-SNP Arrays in a Girl with Multiple Congenital Anomalies Including Severe Glaucoma. *Mol Syndromol* **4**, 285-291 (2013).

27. Montgomery, T., Wyllie, J. & Oley, C. Ectrodactyly and glaucoma associated with a 7q21.2-q31.2 interstitial deletion. *Clin Dysmorphol* **9**, 235-239 (2000).

28. Morey, M. & Higgins, R. Ectro-amelia Syndrome Associated With an Interstitial Deletion of 7q. *Am J Med Genet* **35**, 95-99 (1990).

29. Niehrs, C. & Acebron, S. Mitotic and mitogenic Wnt signalling. *EMBO J* **31**, 2705-2713 (2012).

30. Paesold-Burda, P. *et al*. Deficiency in COG5 causes a moderate form of congenital disorders of glycosylation. *Hum Mol Genet* **18**, 4350-4356 (2009).

31. Pinzon-Daza, M. *et al*. Nanoparticle- and Liposome-carried Drugs: New Strategies for Active Targeting and Drug Delivery Across Blood-brain Barrier. *Curr Drug Metab* **14**, 625-640 (2013).

32. Pittau, F. *et al*. The role of functional neuroimaging in pre-surgical epilepsy evaluation. *Front Neurol* **5** (2014).

33. Prado, J. Structural magnetic resonance imaging in epilepsy. *Radiología* **54**, 9-20 (2012).

34. Racine, R. Modification of seizure activity by electrical stimulation: ii. Motor seizure. *Electroencephalogr Clin Neurophysiol* **32**, 281-295 (1972).

35. Reddy, D. & Kuruba, R. Experimental Models of Status Epilepticus and Neuronal Injury for Evaluation of Therapeutic Interventions. *Int. J. Mol. Sci.* **14**, 18284-18318 (2013).

36. Sampson, E. *et al*.
Negative regulation of the Wnt-beta-catenin pathway by the transcriptional repressor HBP1. *EMBO J* **20**, 4500-4511 (2001).

37. Serup, L. Interstitial Deletion of the Long Arm of Chromosome 7. *Hum. Genet.* **54**, 19-23 (1980).

38. Song, M., Salmena, L. & Pandolfi, P. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* **13**, 283-296 (2012).

39. Talseth-Palmer, B., Bowden, N., Meldrum, C., Nicholl, J. & Scott, R. A 1q44 deletion, paternal UPD of chromosome 2 and a deletion due to a complex translocation detected in children with abnormal phenotypes using new SNP array technology. *Cytogenet Genome Res* **124**, 94-101 (2009).

40. Thakur, B., Dittrich, T., Chandra, P., Becker, A. & Welte, K. Inhibition of NAMPT pathway by FK866 activates the function of p53 in HEK293T cells. *Biochem Biophys Res Commun* **424**, 371-377 (2012).

41. Uliana, V., Grosso, S., Cioni, M. & Renieri, A. 3.2 Mb microdeletion in chromosome 7 bands q22.2eq22.3 associated with overgrowth and delayed bone age. *Eur J Med Genet* **53**, 168-170 (2010).

42. Ulici, V., Hoenselaar, K., Gillespie, J. & Beier, F. The PI3K pathway regulates endochondral bone growth through control of hypertrophic chondrocyte differentiation. *BMC Dev Biol* **8** (2008).

43. Williams, P., Hellier, J., White, A., Staley, K. & Dudek, F. Development of Spontaneous Seizures after Experimental Status Epilepticus: Implications for Understanding Epileptogenesis. *Epilepsia* **48**, 157-163 (2007).

44. Young, R. *et al*. Terminal and Interstitial Deletions of the Long Arm of Chromosome 7: A Review With Five New Cases. *Am J Med Genet* **17**, 437-450 (1984).

45. Zhang, X., Kim, J., Ruthazer, R., McDevitt, M. & Yee, A. The HBP1 Transcriptional Repressor Participates in RAS-Induced Premature Senescence. *Mol Cell Biol* **26**, 8252-8266 (2006).

Appendix 1: Genetic Analysis of Deletions in the Region of HBP1

	HBP1 deletion	Seizure/Abnormal EEG
Decipher 1303	YES	NO
Decipher 255298	YES	NO
Decipher 263745	YES	NO
Decipher 800	YES	NO
Feuk 2006	YES	NO
Martinez 2013	YES	NO
Montgomery 2000	YES	NO
Morey 1990	YES	NO
Abuelo 1982	YES	YES
Decipher 253694	YES	YES
Decipher 265003	YES	YES
Decipher 4470/Uliana 2010	YES	YES
Fagan 1989	YES	YES
Franceschini 1978	YES	YES
Higginson 1976	YES	YES
Klep de Pater 1979	YES	YES
Serup 1980	YES	YES
Young 1984	YES	YES
Al-hassnan 2011	NO	NO
Decipher 1301	NO	NO
Decipher 1621	NO	NO
Decipher 2391	NO	NO
Decipher 249280	NO	NO
Decipher 250108	NO	NO
Decipher 251096	NO	NO
Decipher 252368	NO	NO
Decipher 253184	NO	NO
Decipher 253185	NO	NO
Decipher 254957	NO	NO
Decipher 257739	NO	NO
Decipher 258645	NO	NO
Decipher 258652	NO	NO
Decipher 262086	NO	NO
Decipher 262064	NO	NO
Decipher 265270	NO	NO
Decipher 274898	NO	NO
Decipher 275974	NO	NO
Decipher 277945	NO	NO
Decipher 281462	NO	NO

Decipher 282074	NO	NO
Decipher 4433	NO	NO
Decipher 625	NO	NO
Decipher 801	NO	NO
Decipher 813	NO	NO
Decipher 966	NO	NO
Krepischi-Santos, 2006	NO	NO
Talseth-Palmer, 2009	NO	NO
Decipher 1297	NO	YES
Decipher 263273	NO	YES
Decipher 269744	NO	YES
Decipher 281826	NO	YES