ABSTRACT

Superimposing Status Epilepticus on NS-PTEN Haploinsufficient and Wild Type Mice Results in Long-term Changes in Behavior

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Autism Spectrum Disorder (ASD) is a neurodevelopment disorder with a prevalence rate of approximately 1% in children. Epilepsy is another neurodevelopmental disorder with similar 1% prevalence in the US population. Approximately 1 in 10 individuals will have a seizure within their lifetime. Recent research has shown evidence of a high comorbidity between autism and epilepsy. However, the mechanism underscoring this comorbidity is not well understood. In the present study, we evaluated the effects of superimposing seizures on a genetic mutation (NS-PTEN heterozygous mice) that has been shown to be involved in both autism and epilepsy. We then measured mouse activity levels, anxiety, social behavior, repetitive behavior, and learning in a battery of behavioral tests.

NS-PTEN heterozygous (HT) and wildtype (WT) adult mice received injections of kainic acid (20 mg/kg; intraperitoneal) to induce status epilepticus (continuous seizures) or received injections of the vehicle (0.9% physiological saline). They received pentobarbital (20 mg/kg intraperitoneal) to terminate seizures one hour after the first

injection, vehicle mice also received the pentobarbital. Following a few days of recovery, they received a battery of behavioral tasks in order to evaluate activity levels, anxiety, repetitive-stereotyped behavior, social behavior, learning and memory.

Following the battery of behavioral tests the hippocampus from the mice was collected. A series of western blotting was conducted on the hippocampal samples in order to determine any changes in the PI3K/AKT/mTOR pathway in which PTEN is involved, as well as several synaptic and other related proteins.

In the open field task, we found that after seizures HT mice showed a significant increase in total activity and total distance in the surround region of the open field. Following seizures HT mice also displayed increased total distance and velocity as compared to HT mice that did not undergo seizures and WT controls in the elevated plus maze. In the social chamber test, we found the HT mice after seizures displayed an impairment in social behavior. These findings demonstrate that superimposing seizures on a genetic mutation can result in long-term alterations in activity and social behavior in mice.

Superimposing Status Epilepticus on NS-PTEN Haploinsufficient and Wild Type Mice Results in Long-term Changes in Behavior

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AED	Anti-epileptic drug
ASD	Autism spectrum disorder
CS	Conditioned stimulus
EPM	Elevated plus maze
FXS	Fragile X syndrome
HET/HT	Heterozygous
IP	Intraperitoneal
ITI	Inter-trial interval
kDa	Kilodalton
KO	Knockout
mTOR	Mammalian target of rapamycin
MWM	Morris water maze
PD	Postnatal Day
PTEN	Phosphatase and tensin homolog
SE	Status epilepticus
TLE	Temporal lobe epilepsy
US	Unconditioned stimulus
USV	Ultrasonic vocalization
WT	Wild type

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CHAPTER ONE

Introduction

It is well established that individuals with a history of seizures have higher incidence rates for other comorbidities. There is increasing evidence for a strong relationship between epilepsy and autism, with estimates of prevalence of epilepsy in children with autism spectrum disorder (ASD) ranging from 5-40% (El Achkar & Spence, 2015; Levisohn, 2007; Tuchman & Rapin, 2002). In one study with a cohort of almost 15,000 people, approximately 19% of those with epilepsy were also found to have ASD, while the controls were only found to have a 2% rate of ASD (Kohane et al., 2012). Although the variety of findings across studies may be due to seizure classification, ASD classification, study populations, and study methods, there is substantial support for the relationship between autism and epilepsy.

There are several mechanisms that may underlie the relationship between ASD and epilepsy. One possibility is a common neurobiological vulnerability, such as brain lesions during development or genetic susceptibilities leading to abnormal brain development, subsequently resulting in ASD and epilepsy. Another possible mechanism could be the abnormal brain circuitry underlying ASD conveys a higher sensitivity to seizures. Overlap between these two mechanisms may also exist. Several genetic developmental disorders have been associated with an increased risk of developing epilepsy, including both fragile X syndrome (FXS) (Cohen et al., 1991; Tuchman & Rapin, 2002) and tuberous sclerosis (Smalley, 1998; Tuchman & Rapin, 2002).

Genetic knockout models have provided insight into the neural mechanisms involved in autism and epilepsy, though it has been difficult to differentiate the effects of seizures from the genetic condition. One genetic knockout model that may be useful in examining these effects may be the *PTEN* deletion model. Phosphatase and tensin homolog (PTEN) acts as a negative repressor of the PI3K/AKT/mTOR intracellular signaling pathway (Endersby & Baker, 2008). Mouse models with a deletion of *PTEN* show learning and memory problems, autistic-like behavioral deficits (Kwon et al., 2006; Lugo, Smith, Morrison, & White, 2013), and spontaneous seizures (Backman et al., 2001). In Neuron-subset specific Pten knockout (NS-PTEN KO) mice, onset of seizure activity occurs at 4 weeks of age, with progressive seizure intensity as the animal continues to develop (Ljungberg, Sunnen, Lugo, Anderson, & D'Arcangelo, 2009). The autistic-like behavioral deficits in NS-PTEN KO mice occur primarily at 6-8 weeks of age (Lugo, Smith, et al., 2014). Given the staggered onset of seizure and behavioral phenotypes, it is not clear whether seizures are the cause of the behavioral deficits or are co-occurring with the behavioral deficits. Mice with germline haploinsufficiency in *PTEN* have social behavior deficits, however do not exhibit spontaneous seizures (Clipperton-Allen & Page, 2015). Therefore, mice that are *PTEN* haploinsufficient may be useful for characterizing the effects of induced seizures on the magnitude of behavioral deficits.

Seizure induction has been shown to result in long-term autistic-like behavioral deficits in mice (Bernard et al., 2013; Lugo, Swann, & Anderson, 2014). For this study, we attempted to characterize the impact of seizures on autistic-like behavioral phenotypes expressed by NS-Pten haploinsufficient mice. To establish typical ASD behaviors in

mice, performance was observed in an array of behavioral tests known reliably to model the characteristic symptoms of ASD exemplified in human patients. Tests included the open field test and elevated plus maze to characterize general exploratory behaviors, including activity and anxiety levels. Social interactions were examined using the three chambered social test. Hippocampal dependent learning and memory was tested using the Morris water maze and trace fear conditioning (Crawley, 2007; Morris, Garrud, Rawlins, & O'Keefe, 1982; Wiltgen, Sanders, Ferguson, Homanics, & Fanselow, 2005). Lastly, the marble burying test was used to investigate repetitive behaviors (Hoeffer et al., 2008).

In addition to behavior, protein expression levels were examined using western blotting analyses. In animal models of Fragile X syndrome, tuberous sclerosis, and *PTEN* deletion, aberrant changes have been found in the PI3K/AKT/mTOR pathway (Bhattacharya et al., 2012; Endersby & Baker, 2008; Kwon et al., 2006; Meikle et al., 2007; Sharma et al., 2010). In humans, disruptions in the PI3K/AKT mTOR pathway have been shown to occur in approximately 14% of individuals diagnosed with ASD (de Vries, 2010; Kelleher & Bear, 2008). Further, disruption of *PTEN* occurs in approximately 8.3% of children with ASD (Varga, Pastore, Prior, Herman, & McBride, 2009) and 12.2% of children with developmental delay/mental retardation (Varga et al., 2009). Therefore, we examined several proteins with known involvement in the PI3K/AKT/mTOR signaling pathway, as well as others that are known to be altered in animal models of Fragile X Syndrome, tuberous sclerosis, and *PTEN* deficiency.

CHAPTER TWO

Literature Review

Epilepsy

According to the World Health Organization (WHO), approximately 50 million people worldwide, or about 4 to 10 people in 1000, suffer from epilepsy. Epilepsy is a disorder characterized by the presence of recurrent seizures. In 2005, epilepsy was conceptually defined as a neurological disorder characterized by the occurrence of at least one unprovoked epileptic seizure (Fisher et al., 2014). This initial report resulted in a nearly simultaneous rebuttal from another prominent epilepsy researcher in the field. In their rebuttal letter they noted that the definition would include those who have had one febrile seizure, but who do not have another seizure for the rest of their life (Beghi et al., 2005). Thus, this wider inclusion could broaden the epilepsy definition too much. A more recent definition of epilepsy includes those who have had one unprovoked seizure, but require the inclusion of other associated factors (Fisher et al., 2014). The struggle to define epilepsy has led some to view epilepsy as more of a spectrum disorder, with variability in causes, types of seizures and severity among individual patients (Jensen, 2011).

Currently, there are several treatments for epilepsy ranging from pharmacological interventions (anti-epileptic/anti-seizure medications), surgical manipulation of neural structures, device implantation, like vagus nerve stimulator (VNS), and even diet changes (Besag, 2006; Jensen, 2009, 2011; Klinger & Mittal, 2016). There are several types of

epilepsies that do not respond to any treatment, with approximately 30% of epilepsy patients resistant to any drug treatment (Klinger & Mittal, 2016; Kwan & Brodie, 2000). This study investigates some of the basic effects seizures have on the brain in hopes of improving the overall understanding of neural mechanisms that are affected in the epilepsy phenotype.

Autism Spectrum Disorder

A recent survey by the CDC's Autism and Developmental Disabilities Monitoring Network (ADDM) estimate the prevalence of autism spectrum disorder (ASD) among children at 8 years of age to be about 1 in 68 children (Wingate et al., 2014). These statistics are reported from parents who live within 11 ADDM sites. The surveillance consists of 2 phases, which first consists of gathering evaluations from professional providers in the community. The second phase involves trained clinicians to evaluate all evaluations to confirm that the diagnosis of ASD is consistent with the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) diagnostic criteria. The diagnostic criteria is to include those with autistic disorder, pervasive developmental disorder-not otherwise specified (including atypical autism), or Asperger disorder. The prevalence rate of 1 in 68 children is then broken down with 1 in 42 boys and 1 in 189 girls living within the 11 ADDM Network communities having the diagnosis of ASD. These results are reported for the 2010 period. It should be noted that these data do not provide a representative sample of the entire United States. The rate of 1 in 68 does not imply that all children aged 8 years in the United States have a 1 in 68 chance of having ASD. One recent report that reviewed the consistency of the prevalence rate by the ADDM network, applied the ADDM methodology to children in South

Carolina (Nicholas et al., 2012). The investigators found a concordance rate of 82% for children between 8 and 15 years of age, which suggests a high reliability for the use of the ADDM methodology to this age group. However, even though the concordance rate may be high, some caution should be taken when generalizing the results from 11 sites in the ADDM network to the entire US population.

The symptoms of ASD generally include social impairment, communication deficiencies, and repetitive and stereotyped behaviors (Bozdagi et al., 2010; Cheh et al., 2006; Clarke et al., 2005; Lewis, Tanimura, Lee, & Bodfish, 2007; Nadler et al., 2004; Niemeyer et al., 2010; Young, Schenk, Yang, Jan, & Jan, 2010). There is also a higher than normal prevalence of cognitive deficits in learning and memory in those with ASD (Cheh et al., 2006). The results from the CDC's ADDM network found that among seven sites 31% of children with ASD had IQ scores in the range of intellectual disability (IQ < 70), 23% in the borderline range (IQ=71-85), and 46% in the average or above average range (IQ = > 85) (Wingate et al., 2014). The exact cause or disease mechanisms of autism are not yet fully understood, but are thought to be a combination of genetic (Ebert & Greenberg, 2013; Lo-Castro & Curatolo, 2014; Rutter, 2005), epigenetic (Hogart, Wu, LaSalle, & Schanen, 2010) and environmental factors (Engel & Daniels, 2011).

Comorbidities

Approximately 30-50% of patients with epilepsy have psychiatric and neurological comorbidities (Gaitatzis, Carroll, Majeed, & J, 2004). Mood and anxiety disorders are some of the most frequently reported comorbidities with epilepsy, with a lifetime prevalence rate of 30-35% (Gaitatzis et al., 2004; Tellez-Zenteno, Patten, Jette, Williams, & Wiebe, 2007). ADHD has been reported to have a prevalence rate of 13-

50% of pediatric populations (Dunn, Austin, Harezlak, & Ambrosius, 2003; Lindsay, Ounsted, & Richards, 1979).

There is also a high comorbidity of autism with epilepsy (Clarke et al., 2005; Meidenbauer, Mantis, & Seyfried, 2011; Niemeyer et al., 2010) with approximately a third of patients on the autistic spectrum having experienced at least two epileptic seizures by the end of adolescence (Niemeyer et al., 2010) and about 30% of epileptic patients diagnosed with autism spectrum disorder (Clarke et al., 2005). However, the rates of this relationship varies greatly across studies. One study reported a prevalence of epilepsy in patients with ASD at 2.4% (Amiet et al., 2013). This study examined the number of individuals (N=2644) with epilepsy in a cohort of children from the Simons Simplex Collection. In a different study, where investigators investigated the incidence of epilepsy in autism by the use of EEGs, they found that 46% of children with autism had epilepsy (N=78) (Hughes & Melyn, 2005).

One of the key shared risk factors between epilepsy and autism appears to be overall cognitive function (Tuchman, Cuccaro, & Alessandri, 2010). In one study that used a meta-analysis approach, researchers analyzed the relationship between intellectual disability, ASD, and epilepsy by grouping the subjects by IQ (<40, 40-50, 50-70, >70) (Amiet et al., 2008). This study found that as the rate of epilepsy increased there was a decrease in IQ, suggesting an inversely proportional relationship. In addition, there is normally a relationship of 3.5:1 male to female ratio in non-epilepsy ASD, while in the epilepsy group there is a ratio of 2:1 of males to females.

The impact of comorbidities is not often accounted for when developing a comprehensive treatment plan for those with epilepsy. There was a long-held belief that

psychoactive drugs had pro-convulsant effects. This belief changed after a study where investigators compared the incidence of seizures among randomly assigned patients and those who received a placebo drug (Alper, Schwartz, Kolts, & Khan, 2007). The incidence in individuals with seizures who were given antidepressants were significantly lower than those who received a placebo. In addition, there is evidence that AEDs can have a positive or a negative impact on comorbidities (Kanner, 2016). Therefore, additional knowledge of the types of comorbidities in epilepsy can assist in selecting the best AED to use in patients with epilepsy.

Animal Models in Epilepsy and Comorbidities

Animal models of epilepsy have provided significant insight into the comorbidities associated with epilepsy. One of the most commonly used animal models of epilepsy is administration of chemoconvulsants, such as pilocarpine or kainic acid. Pilocarpine is a muscarinic agonist, while kainic acid primarily works through glutamatergic kainate receptors (Pearson, Schulz, & Patel, 2014; Scorza et al., 2009). Both chemoconvulsants produce continuous seizures, also known as status epilepticus, which can last from 30 minutes to several hours.

There have been several studies that found that induction of status epilepticus during the early postnatal period result in long-term deficits in learning and memory. One study found working memory deficits in the radial arm maze (RAM) when Sprague Dawley rats were given kainate on PD1, 7, or 14 (Lynch, Sayin, Bownds, Janumpalli, & Sutula, 2000). Induction of status epilepticus through kainate was found to result in learning and memory deficits when induced on PD10, 18, 25, or 35 (Bolanos et al., 1998; de Feo, Mecarelli, Palladini, & Ricci, 1986). Studies that examined the effect of kainate

in young adulthood also found impairment in rats in the Morris Water Maze, RAM, and novel object recognition (Inostroza et al., 2011). Similar learning deficits were found when kainate was given in adulthood (Gobbo & O'Mara, 2004; Jessberger et al., 2007).

The induction of SE through pilocarpine has provided similar results to the kainate model. Learning and memory impairments were found when pilocarpine was presented to PD16, PD20, PD34, and PD45 rodents (Cilio et al., 2003; Faverjon et al., 2002; Inostroza et al., 2011; Liu, Gatt, Werner, Mikati, & Holmes, 1994; Rutten et al., 2002). Similar learning and memory impairments have been reported when pilocarpine is used to induce SE during young adulthood or adulthood (Chauviere et al., 2009; Harrigan, Peredery, & Persinger, 1991; Hort, Brozek, Komarek, Langmeier, & Mares, 2000; Hort, Brozek, Mares, Langmeier, & Komarek, 1999; Mohajeri et al., 2003; Rice, Floyd, Lyeth, Hamm, & DeLorenzo, 1998; Sroubek, Hort, Komarek, Langmeier, & Brozek, 2001).

Even though the focus of many of the animal model studies was to examine learning and memory deficits, many also reported alterations in anxiety behavior. One study found a consistent increase in anxiety behavior, as measured by the elevated plus maze, when the rats were presented with kainic acid during different periods of early postnatal period (Sayin, Sutula, & Stafstrom, 2004). A separate study found impairments in anxiety behavior when rats on PD18 and PD25 were given kainic acid (Mikulecka, Krsek, & Mares, 2000). Another study examined the differences in anxiety behavior across two strains of rats when exposed to the chemoconvulsant kainic acid or pilocarpine during young adulthood (Inostroza et al., 2011). Wistar rats exhibit no change in anxiety behavior when presented with kainic acid, however are less anxious

when pilocarpine was used to induce the seizures. Sprague Dawley rats are less anxious in the elevated plus maze test when the seizure induction method is through kainic acid or through pilocarpine.

There is also mounting evidence that chemoconvulsants during different periods of development can result in changes in autistic-like behaviors in adulthood. Autistic-like behaviors include alterations in social behavior, repetitive behavior, and communication. One study by Krsek et al. administered a non-convulsive dose of pilocarpine to PD90 Wistar rats and found the rats to have a significant reduction in social interaction with juvenile rats. The pilocarpine treated animals are also known to have motor learning impairments (Krsek et al., 2004). Further, similar results in autistic-like behaviors have been observed in rodents with early-life seizures. Pilocarpine induced early-life seizures on PD7 results in a decrease in social behavior in rats (Bernard et al., 2013). Flurothyl seizures on PD7-11 result in a decrease in social behavior without any changes in repetitive behavior or anxiety (Lugo, Swann, et al., 2014). However, pilocarpine induced early-life seizures on PD7 does result in a decrease in marble burying behavior and social behavior, without changes in anxiety (Bernard, Castano, Beitzel, Carlson, & Benke, 2015). There has been less investigation into the communication deficits after seizures. One study used pilocarpine-induced status epilepticus on PD14 Wistar rats and found a suppression of ultrasonic vocalizations (USVs) in male rats (Lopez-Meraz et al., 2014). Another study observed that febrile-induced seizures on PD7 results in an increase in USVs in male rats on PD12 (Keller, Saucier, Sheerin, & Yager, 2004). These studies demonstrate that there are long-term effects of seizures on autistic behaviors; however,

additional studies are needed to examine the neural mechanisms that underlie these behavioral deficits.

Animal models of epilepsy have allowed investigators to understand the pathophysiology of epilepsy comorbidities. The investigator can control for the developmental time window to induce seizures, the latency between the induction of seizures and behavior testing, and the ability to investigate specific behaviors. Furthermore, animal models can be used to examine the effects of therapeutic interventions on epilepsy and associated comorbidities (Blumenfeld et al., 2008; Russo et al., 2011). One of the goals of the current study was to expand our knowledge of the role of genetics in modifying the impacts of seizures on behavioral comorbidities.

Possible Links

The prenatal environment is known to have significant influence on developmental trajectory. Therefore, perturbations in this environment may provide a link to these two developmental disorders. Insufficient micronutrient availability *in utero* may be a potential link for epilepsy and ASD comorbidity. Previous studies suggest that vitamin D and zinc deficiencies, as well as copper toxicity, during embryonic development are capable of leading to both epilepsy and ASD diagnoses (S. Johnson, 2001; Kočovská, Fernell, Billstedt, Minnis, & Gillberg, 2012). In further support of this possible link, vitamin D deficiency is relatively common among pregnant women (D. D. Johnson et al., 2011). It is also known that neurotoxic prenatal exposure to industrial chemicals, heavy metals, and organophosphates are associated with developmental disorders (De Felice, Scattoni, Ricceri, & Calamandrei, 2015; Grandjean & Landrigan,

2006; Hill et al., 2015). Therefore, exposure to these chemicals may contribute to the comorbidity of epilepsy and ASD.

Several genetic links underscore the comorbidity between epilepsy and autism. Given that pathological findings in epilepsy and ASD include excitatory-inhibitory imbalance, candidate genes underscoring this comorbidity may encode ion channels. Previous studies have shown that alterations in K^+-channel genes may represent a link between the two disorders, including those which encode for Kv4.2, Kv7.3, $K_{Ca}1.1$, $K_{ir}2.1$, and $K_{ir}4.1$ channel proteins (Ambrosini et al., 2014; Guglielmi et al., 2015). Previous studies have also identified mutations in the *SCN1A* and *SCN2A* Na^+-channel genes in the emergence of Generalized Epilepsy with Febrile Seizures Plus (GEFS+) in familial autism spectrum disorder (Weiss et al., 2003). Ca^{2+}- and Cl^--channels have also been suggested as possible links between epilepsy and ASD comorbidity. Further, (Jabbari & Nurnberg, 2016) recently investigated human brain-specific genome and the postsynaptic proteome for candidate genes underlying epilepsy and ASD comorbidities. These authors concluded that candidate genes underlying this comorbidity tend to be large, AT-rich gene transcripts. Given that numerous genes have been suggested as contributors of epilepsy and ASD comorbidity, it may be possible to use these findings as selection criteria to narrow the pool of genes for investigation.

It has also been suggested that seizure pathogenesis in individuals with ASD may arise from aberrant neuroinflammatory responses. It has been hypothesized that allergies, environmental-stimuli, and stress may trigger the neural mast cell cascade, causing focal disruption of the blood-brain barrier (BBB) and subsequent seizure activity in individuals with ASD (Theoharides & Zhang, 2011). In support of this link, it has been shown that

individuals with ASD may have constitutively active neuroinflammatory signaling (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). The Akt-mTOR pathway is known to play a central role in ASD and seizures, and may also participate in neuroinflammatory cytokine release (Vargas et al., 2005). Therefore, building the link between Akt-mTOR signaling and neuroinflammatory markers may yield significant insights into the comorbidities between ASD and epilepsy.

Molecular Mechanisms

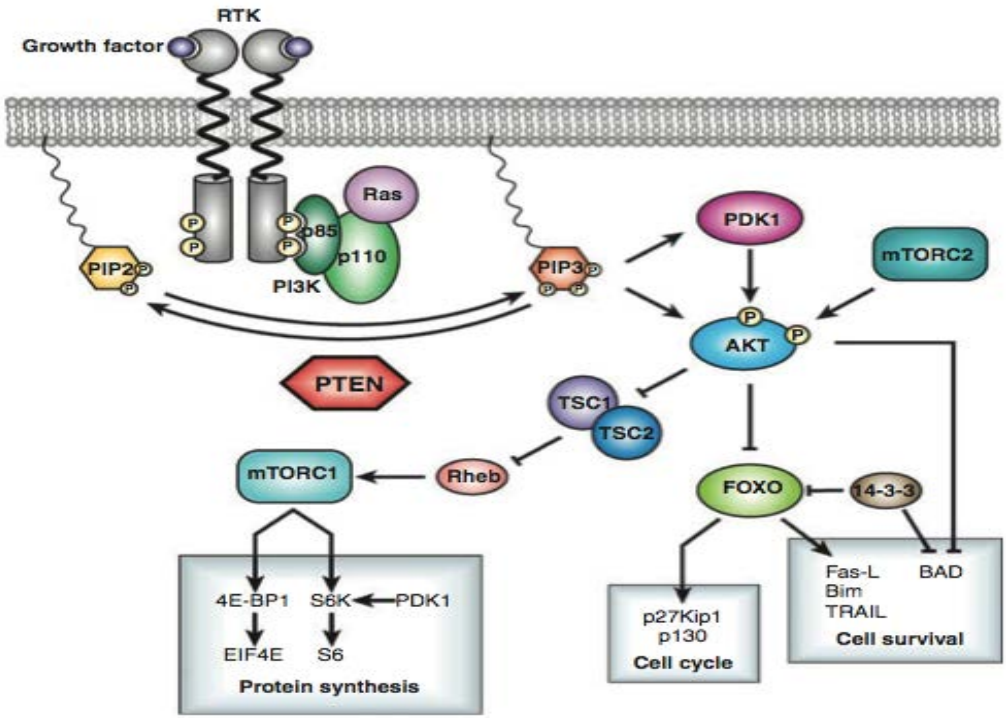

Figure 1- A schematic overview of the mTOR and PI3K/AKT signaling pathway (Endersby & Baker, 2008).

mTOR

A portion of this project was to determine changes in multiple components of the PI3K/AKT signaling pathway and mTOR of PTEN haploinsufficient and wildtype mice by western blot analysis. This project also investigated changes in synaptic proteins,

scaffolding proteins, and ion channels downstream of the PI3K/AKT signaling pathway. Investigation of these proteins is important because PTEN interacts directly with the PI3K/AKT pathway and mTOR (Figure 1).

PI3K/Akt Signaling Pathway

Following activation of cell surface receptors, Phosphoinositide 3 Kinase (PI3K) catalyzes the conversion of Phosphatidylinositol 4,5-bisphosphate (PIP_2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Cantley, 2002). PIP_3 subsequently activates AKT protein kinase via PDK1-mediated phospholipid binding at the threonine 308 site and direct phosphorylation at the serine 473 site (Alessi et al., 1996). AKT then activates numerous downstream targets for the purpose of promoting cell growth and survival (Franke, Kaplan, & Cantley, 1997). The most relevant of these targets is the mammalian target of rapamycin (mTOR), a major contributor to protein synthesis, cell cycle regulation, cell survival, and overall cell growth (Endersby & Baker, 2008). AKT-mediated action on mTOR occurs directly by phosphorylative activation at the RAPTOR subunit and indirectly by phosphorylative inhibition of its inhibitor tuberous sclerosis complex 2 (TSC2) (Inoki, Li, Zhu, Wu, & Guan, 2002; Manning, Tee, Logsdon, Blenis, & Cantley, 2002; Nave, Ouwens, Withers, Alessi, & Shepherd, 1999). In the hippocampus, PI3K/AKT signaling pathway and mTOR activity may play an important role in the behavior and social deficits associated with ASD and its related disorders (Lugo, Smith, et al., 2014; Lugo et al., 2013; Sharma et al., 2010). These pathways are also highly characterized in epileptogenesis (Berdichevsky et al., 2013; Meng, Yu, Song, Chi, & Tan, 2013; Theilhaber et al., 2013; M. Wong, 2013).

PTEN

The Phosphotase and Tensin homolog (PTEN) is a lipid phosphatase which antagonizes PI3K-mediated conversion of Phosphatidylinositol 4,5-bisphosphate (PIP_2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) in the PI3K/Akt signaling pathway (Huang et al., 2012). As a signaling molecule PIP_3 perpetuates further activation of the PI3K/AKT signaling and regulates multiple processes at the plasma membrane, including endocytosis, exocytosis, and cytoskeleton organization (Czech, 2000). Disruption of PTEN leads to hyperactivation of the PI3K/AKT signaling and is closely associated with disorganized neural circuitry, spontaneous seizures, macrocephaly, developmental delays, and ASD (Backman et al., 2001; J. Zhou & L. F. Parada, 2012).

S6 Ribosomal Protein

Ribosomes are large, complex molecular machines responsible for all intracellular protein synthesis. These are composed of one large 60S subunit and one small 40S subunit (Ruvinsky & Meyuhas, 2006). Their even smaller ribosomal protein constituents determine the function of each subunit. The S6 ribosomal protein is both a constituent of the small 40S subunit and a target of the PI3K/AKT signaling pathway. Activation of this pathway triggers subsequent S6 phosphorylation at conserved serine 235, 236, 240, and 244 sites via p70 S6 kinase (p70S6K) (Pullen & Thomas, 1997). When phosphorylated at any of these sites, S6 influences translational action to generate proteins that regulate cell cycle progression, as well as new ribosomal proteins and elongation factors associated with translation (Jefferies et al., 1997; Peterson & Schreiber, 1998). Given that S6 phosphorylation status is dependent on PI3K/AKT signaling, it can be hypothesized that hyperactivation secondary to PTEN disruption may cause increased S6 phosphorylation.

Therefore, it was relevant for this study to investigate changes in p70S6K, S235/236-phosphorylated S6, and S240/244-phosphorylated S6.

HCN

Hyperpolarization-Activated Cation (HCN) channels are a family of cation channels which mediate the integration of signaling networks and provide pacemaker depolarization during rhythmic oscillatory activity (Pape, 1996). Given that generalized seizures are known to result from aberrant synchronization of hyperexcitability (Avanzini, de Curtis, Pape, & Spreafico, 1999; Blumenfeld, 2005) and the adequate stimulus of HCN channels is hyperexcitability, these channels have received much attention in epileptogenesis (Tang, Sander, Craven, Hempelmann, & Escayg, 2008). Since PTEN disruption commonly causes PI3K/AKT-mediated hyperexcitability leading to seizure activity, it is within the scope of this study to evaluate select HCN channel expression. The HCN1 channel subtype is selectively localized to brain areas involved in epileptogenesis, including the hippocampus, cerebellum, and superior colliculus. The other three HCN channels are present diffusely throughout the brain and are not localized to any specific tissue (Moosmang, Biel, Hofmann, & Ludwig, 1999). Therefore, only HCN1 was selected for investigation in this study.

FMRP

Fragile-X Mental Retardation Protein (FMRP) is a known suppressor of numerous target mRNAs, which function downstream of the PI3K/AKT pathway (Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001). The localization of FMRP-mediated action is primarily dependent on its phosphorylation status, with

unphosphorylated FMRP associating with actively translating polyribosomes and phosphorylated FMRP associating with stalled polyribosomes (Ceman et al., 2003). Several previous studies suggest that disruption of PTEN leads to a downstream increase in total and S499-phosphorylated FMRP (Lugo, Smith, et al., 2014; Lugo et al., 2013). Interestingly, it has been hypothesized that FMRP may prevent PI3K/AKT hyperactivation by suppressing the PI3K enhancer, PIKE-S, however a definitive mechanism remains unclear (Sharma et al., 2010). Because FMRP has also been reported to suppress translation of the mRNA for nearly 842 genes (Steinberg & Webber, 2013), downstream synaptic and ion channel proteins regulated by FMRP were also investigated. By further elucidating the interactions between PTEN and FMRP on PI3K/AKT signaling physiology, development of a novel pathway may be investigated for its role in both autism and epilepsy.

Ankyrin

Ankyrins are a family of adaptor proteins responsible for linking integral membrane proteins to the intracellular cytoskeleton and cell adhesion molecules (Cunha & Mohler, 2009). More specifically, in neurons, ankyrins mediate the localization of voltage gated sodium channels to the axonal initial segment (Garrido et al., 2003; Lemaillet, Walker B Fau - Lambert, & Lambert, 2003). Previous studies indicate that these adaptor proteins are involved in the pathogenesis of epilepsy and therefore (Chen et al., 2009; Nashef, Hindocha, & Makoff, 2007) were selected for investigation in this study.

Kv4.2 & PSD-95

In the brain, voltage-gated potassium channel subunit 4.2 (Kv4.2) associate into homotetrameric, A-type voltage gated potassium channels, which are known to mediate the excitability of cortical pyramidal neurons and hippocampal dendrites (Birnbaum et al.; Carrasquillo, Burkhalter A Fau - Nerbonne, & Nerbonne, 2012; Guglielmi et al., 2015). Activation of A-type currents typically occurs during the post-hyperpolarization phase when the membrane potential is sufficiently negative. By this mechanism, A-type voltage gated potassium channels delay depolarization and regulate the overall rate of neuronal firing (Amberg, Koh, Imaizumi, Ohya, & Sanders, 2003). As would be expected, disruption of the Kv4.2 subunit is known to be associated with epilepsy (Monaghan, Menegola, Vacher, Rhodes, & Trimmer, 2008; Singh et al., 2006). Similar disruption is also observed in autism spectrum disorder (Guglielmi et al., 2015). Interestingly, previous studies indicate that localization and trafficking of the Kv4.2 subunits is significantly dependent upon anchoring to the membrane associated guanylate kinase protein, PSD-95 (W. Wong, Fau, Jugloff, Jones, & Schlichter, 2002; W. Wong & Schlichter, 2003). Given the indicated role of Kv4.2 in epilepsy and autism, as well as dependence on PSD-95, both of these were selected for investigation in this study.

GluR1

A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are ionotropic transmembrane glutamate receptor proteins (Honoré, Lauridsen, & Krogsgaard-Larsen, 1982). These receptors are heterotetrameric proteins, which consist of multiple subunits including GluR1, GluR2, GluR3, and GluR4. These receptors mediate fast excitatory synaptic transmission between neurons in the central nervous

system due to their cation permeability (Palmer, Cotton L Fau - Henley, & Henley). These receptors are found abundantly in brain regions associated with epileptogenesis and learning and memory (Lynd-Balta, Pilcher Wh Fau - Joseph, & Joseph, 1996; O'Leary, Bernard, Castano, & Benke, 2015; Sommer, Roth Su Fau - Kiessling, & Kiessling, 2001). Many previous studies have found dysregulation of AMPA receptors in both epilepsy (Rogawski & Donevan, 1999) and autism (Carlson, 2012). Because the GluR1 subunit has received the most extensive previous investigation, only this subunit was selected for the purposes of the present study.

mGluR1/5

Metabotropic glutamate receptors 1 and 5 (mGluR1/5) are G-protein coupled receptors found abundantly throughout the brain. Activation by the excitatory neurotransmitter, glutamate, subsequently triggers activation of the phosphatidylinositol signaling cascades via phospholipase C and adenylyl cyclase (Hermans & Challiss, 2001; Niswender & Conn, 2010; Pin, Joly, Heinemann, & Bockaert, 1994). Both mGluR1/5 play significant roles in long term potentiation and depression within the hippocampus (Gladding, Fitzjohn Sm Fau - Molnar, & Molnar, 2009). Given that these learning processes are found to be dysregulated in autism (Jung et al., 2013) and epilepsy (Meador, 2007), the mGluR1/5 receptors were selected for investigation in this study.

Pan Shank

SHANKs are a family of proline-rich postsynaptic scaffolding proteins, which include SHANK1, SHANK2, and SHANK3. During early brain development this family of proteins localize to the postsynaptic densities of excitatory synapses, where they

participate in early cytoskeletal organization within the synapse (Naisbitt et al., 1999; Sheng & Kim, 2000). Several previous studies suggest that impaired function of these proteins may result in autistic-like phenotypes, as well as spontaneous seizures (Jiang & Ehlers; Yoo, Bakes, Bradley, Collingridge, & Kaang, 2014). Therefore, we elected to use a Pan-SHANK antibody for investigation of the entire SHANK family in this study.

Pan SAPAP

SAP90/PSD95-Associated Proteins (SAPAPs) are a family of proteins which are highly expressed in the postsynaptic densities of glutamatergic cells. These proteins participate in synaptic plasticity by interacting directly with SAP90 and PSD95 scaffolding proteins to maintain and organize neuronal postsynaptic densities (Takeuchi et al., 1997; Welch, Wang D Fau - Feng, & Feng, 2004). Given that disorganized neural circuitry is observed in both autism and epilepsy (Farias, Low Sq Fau - Peterson, Peterson Gm Fau - Ribak, & Ribak, 1992; Isaeva, Isaev, & Holmes, 2013; O'Connor, Bariselli, & Bellone, 2014; Rudie et al., 2013), we elected to investigate whether the SAPAP protein family was disrupted in this study.

Hippocampus

In humans the hippocampus is a brain structure in the limbic system that is involved in learning and memory (Hayman et al., 1998; J Thammaroj, 2005; Per Andersen, 2007; Turgut & Turgut, 2011). The limbic system is involved in emotion and learning and memory, (Per Andersen, 2007). Research has shown that one of the major functions of the hippocampus is to regulate spatial learning and navigation. Single cells within the hippocampus fire only during exploration of specific locations within an

environment and hippocampal lesions are known to lead to impairment in spatial navigation tasks (Morris et al., 1982; Moser, Kropff, & Moser, 2008). Research has also shown that the hippocampus plays an important role in memory processing (J Thammaroj, 2005) and bilateral hippocampal lesions can lead to the inability to form new memories (Hayman et al., 1998). Research has also shown that reduction in GABA inhibitory receptors in the dentate gyrus facilitate trace fear conditioning, which is dependent on hippocampal learning (Wiltgen et al., 2005).

Seizure Induction

Seizures will be induced using kainic acid, which is a glutamate receptor agonist that causes activation of post synaptic neurons (Huettner, 2001). Over activation with IP injections of kainic acid results in generalized seizures and will act as the seizure model for this project (Leite, Garcia-Cairasco, & Cavalheiro, 2002).

Experiments Planned

Activity Level

Overall activity levels can be measured as part of the open field test and elevated plus maze. As part of both tests, total distance and velocity will be measured, as well as several other exploratory behaviors, such has rearing and head dips in the plus maze and grooming and rearing in the open field.

Anxiety

Anxiety levels are measured in the elevated plus maze, determined by amount of time spent in the open vs. closed arms of the maze. Lower anxiety is exemplified by an

increase in time in the open arms, verses higher anxiety is represented by more time spent in the closed arms compared to controls (Walf & Frye, 2007). Anxiety levels will also be determined by comparing time spent in the center (decreased anxiety) versus the surround (increased anxiety) in the open field test.

Learning and Memory

Learning and memory, specifically hippocampal based learning and memory will be evaluated using both trace fear conditioning and the Morris water maze. In Morris water maze spatial hippocampal based learning is investigated by the mice's ability to learn the location of a hidden platform based on distal visual cues (Morris et al., 1982). In trace fear conditioning, learning is determined by an association of a conditioned stimulus (CS), a tone, to an unconditioned stimulus (US), a mild foot shock, when the CS and US are separated by a 20 second trace period (Raybuck & Lattal, 2011; Wiltgen et al., 2005). Additionally, an association between the US and the environment can be established. The strength of the association is determined based on the amount of time spent freezing during subsequent presentation of the tone in a novel environment/context and time spent freezing when placed back in the original context. Freezing is a fear response in mice and can be used to determine strength of learning (Bolles & Collier, 1976).

Social Behaviors

Social interactions will be measured using the three chamber social interaction test. As part of the test, the mice will be introduced to a novel stranger mouse and time

spent interacting with the novel mouse will be a measure of social behaviors (Nadler et al., 2004).

Repetitive Behaviors

Repetitive behavior will be determined using the marble burying test and open field test. An increase in marble burying represent an increase in repetitive behavior (Hoeffer et al., 2008). In open field, repetitive behavior is measured by behaviors, such as grooming time, and clockwise and counter-clockwise rotations or spins.

Innovation

There are many different possible interactions that could lead to the autism-epilepsy comorbidity. There could be underlying neural or genetic causes that lead to both disorders, seizures seen in epilepsy could lead to the autistic symptoms, or even the anti-epileptics taken by epileptic patients could contribute to ASD symptoms.

The innovation of this project comes from looking at both the genetic basis of ASD and epilepsy and the effects of superimposed seizures simultaneously. Many studies have been conducted looking at one or the other, (Betancur, Sakurai, & Buxbaum, 2009; Bourgeron, 2009; Kelleher & Bear, 2008; Jing Zhou & Luis F. Parada, 2012) but looking at both at the same time would be an innovative research direction.

This project is based on determining the mechanism by which seizures affect the brain and result in behavioral and learning abnormalities. By taking an established animal model of epilepsy and inducing seizures in mice to determining if their behavior and learning deficits fall in the ASD, the causes of comorbidity between autism and epilepsy can be explored and mapped out. From there, understanding the underlying

mechanisms that leads to ASD can be determined with the long term goal being the development of therapies that could reverse the damage caused by epileptic seizures, treat the symptoms of or even cure ASD in human patients.

CHAPTER THREE

Materials and Methods

General Outline of Experiments

We utilized a rapid interval testing approach that has previously been shown to avoid the confounding effect of repeated-testing on behavioral performance (Paylor, Spencer, Yuva-Paylor, & Pieke-Dahl, 2006). In addition, the testing order has been constructed to prevent test order effects (McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001). All behavior testing was conducted over a 4 week period. Week 1 included open field testing followed by at least one day off and then elevated plus maze testing. Week 2 of testing began with marble burying followed by at least one day off, then social chamber testing. Trace fear conditioning was tested over 4 days during week 3 and Morris water maze was performed over 5 days during week 4. Following behavioral testing all mice were sacrificed and brains were removed and dissected to isolate the hippocampus. The hippocampi were then stored at -80º C until the tissue could be processed and used in western blotting analysis.

Experimental Groups

For this study male neuron subset-specific Pten (NS-Pten) conditional mice, previously described as GFAP-Cre; $Pten^{loxP/loxP}$ (Backman et al., 2001; Kwon et al., 2001) were utilized. They were on a FVB-based mixed background strain that had been bred for more than 10 generations. We bred NS-$Pten^{loxP/+}$ heterozygote parents to produce

NS-Pten$^{+/+}$ wild type (WT), Pten^{loxP+} heterozygous (HT), and Pten$^{loxP/loxP}$ knockout (KO) mice (not used in this study). Mice were all generated and housed at Baylor University, on a 14 hour light 10 hour dark diurnal cycle, at an ambient temperature of 22º C. All mice were housed with their male littermates following weaning. Mice that displayed aggressive and continuous fighting behaviors were separated and housed individually for the duration of testing. All mice were given *ad libitum* access to both food and water. All testing and housing procedures were in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and all protocols were approved by Baylor University Animal Care and Use Committee.

Seizure Induction

Seizure induction with adult mice began on PD60. For each genotype group there were sham injected (saline) mice and seizure induction mice, creating four experimental groups. The four experimental groups for this study were: wild type control/saline injected mice (WT-Saline), wild type mice with seizure induction (WT-Seizure), heterozygous control/saline injected mice (HT-Saline), and heterozygous mice with seizure induction (HT-seizure). Seizures were induced using kainic acid (Catalog # 0222; Tocris, Bristol, UK) that was suspended in 0.9% saline and administered by intraperitoneal (IP) injection; the dosage was determined such that status epilepticus (continuous seizures) was induced, but with a dose resulting in a low mortality rate. The presence of behavioral status epilepticus was determined using previously described methods (Barnwell et al., 2009). Based on pilot studies the dosing was set to 20 mg/kg. Initially, any mice that did not show any behavioral seizure activity were given a second 10 mg/kg dose of kainic acid, however mice that received the second dose did not survive

to be tested. Therefore any mouse that did not show any behavioral seizure activity after the first injection of kainic acid were removed and not used in this study.

At approximately 1 hour following either saline or kainic acid injections all mice received a 20 mg/kg injection of pentobarbital to terminate seizure activity. If mice continued to show behavioral seizure activity 1 hour after pentobarbital injections, a second 10 mg/kg dose was administered. Mice that continued seizure activity following the second dose of pentobarbital were administered additional doses of pentobarbital every hour, however prolonged seizure activity following multiple doses of pentobarbital tended to result in the death of the mouse prior to behavior testing.

All seizures were induced in the mice while they were held in isolation. Seizure activity was monitored through continuous behavioral observation (described below) for at least 2 hours following kainic acid injections, and then regularly checked on for several hours, until all mice showed recovery. Mice were considered recovered when they no longer showed any seizure activity and began to display normal grooming and exploratory behaviors. Following recovery, mice were returned to home cages with their littermates. Mice that displayed continuous aggression and fighting behaviors to the point of injury were separated and singly housed for the duration of testing.

Monitored behavioral signs of seizures included either or both hindlimb or forelimb clonus, rearing and falling, continuous jumping, and uncontrolled rolling. Limb clonus was determined when the mouse began to rapidly contract and relax the muscles in that limb. Clonus could be observed in either one limb or a set of limbs or all four limbs at once. Rearing and falling was considered when a mouse would rear on its hindlimbs with a stiff body and then fall over in an uncontrolled manner. Continuous

jumping was recorded when the mice would repeatedly jump for a prolonged period of time. When mice were observed to have clonus in all four limbs at once, either with or without rolling, the mice were considered to be in status epilepticus. Time post injection for each onset of these events was recorded. Several of these events were observed in each of the mice.

The WT-saline group will function as the control group for this study. The HT-saline group will function as a secondary control group for comparing the differential effects of seizures on the two different genetic variants.

Behavioral Testing

Following seizure induction or saline injections all mice were given a 1 week recovery period before behavioral testing was conducted. For all behavior testing mice were transported from the animal housing facility to the testing rooms in their home cages. Following transportation mice were marked on their tails for identification and weighed (once a week) and then given at least 30 minutes to acclimate to the testing room while being held in their home cages before testing began. All testing rooms have a constant background noise level and consistent light level. Following testing, mice were held in separate holding cages until all testing of mice from a given home cage was concluded then mice were returned to their home cages. Once testing for the day was finished, mice were returned back to the animal housing facility. All testing apparatuses were cleaned using 30% isopropanol, unless otherwise noted, in between each test subject.

The first behavioral test was the open field test to determine locomotor activity, exploratory behaviors, and anxiety. In the open field test mice were allowed to explore

an open field arena for 30 minutes. The various behavioral measures recorded during open field testing included: total horizontal activity (total distance travel), vertical activity (rearing), time spent in center and surround areas of the arena, as well as other behaviors such as spinning, stereotypy (grooming), and number of fecal boli.

For open field testing two 40 cm x 40 cm x 30 cm clear acrylic open boxes were used, allowing for two mice to be tested simultaneously in separate fields. All activity was recorded using an optical recording system controlled by Fusion (Omnitech Electronics, Inc., Columbus, OH).

Elevated plus maze was used as a test for anxiety levels. The elevated plus maze consists of an elevated plus platform with two enclosed arms with tall walls and two open arms without walls. The mice were placed in the center of the maze with their head facing an open arm and allowed to freely explore the maze for 10 minutes. Total distance and time spent in each of the arms was recorded. More time spent in the closed arms vs. open arms is indicative of a higher anxiety level (Walf & Frye, 2007). Additional observations were made via video scoring, including number of rearings in the closed and open arms, number of head dips over the edge of the open arms, and total number of fecal boli. The additional measures were used to compare overall activity and exploratory behaviors.

The elevated plus maze is made from black opaque acrylic and consist of two open arms opposite of each other and two closed arms running perpendicular to the open arms. All arms are 30 cm long by 5 cm wide connected by a 5 cm x 5 cm open central square. The walls of the closed arms are 15 cm tall. The maze is raised 40 cm off of the ground by acrylic dowels. Mouse activity and movements were tracked using the video

tracking software Noldus (Ethovision, Netherlands). Simultaneous video recording was done using video capturing software Dazzle video creator plus HD (Corel, Canada).

Marble burying was used to test repetitive behaviors. For marble burying, test cages (same pan as home cages) were set up with extra deep bedding (approximately 8 cm) and a 4 x 5 array of 20 black 20 mm opaque glass marbles. Prior to placing the mice in the testing cage, a small amount of bedding from the mouse's home cage was sprinkled into the testing cage to add a familiar smell. The mice were then placed into the test cage and left for 30 minutes. At the end of the 30 minute test period the mice were removed and the number of marbles buried at various depths (50%, 75%, 100%, and completely buried) were counted. Completely buried marbles were marbles that were unseen when viewed from above. The 100% marbles included the completely buried marbles and the ones that were buried up to the full height of the marble with some bedding on top of the marble, but the marble itself was still visible. The 75% marbles were all marbles that were buried up to a least 75% of the height of the marble and the above two categories. Finally, the 50% group were all marbles that were buried up to at least 50% of the height of the marble and all marbles that were included in the other three higher categories. The more marbles buried indicated higher repetitive behavior tendencies (Hoeffer et al., 2008).

Social behaviors were tested using a three chamber apparatus. The test is divided into two phases. In the first phase a mouse was placed in a three chambered apparatus, described below, where they can explore the testing arena, all three chambers, and both cups for a total of 10 minutes. Each of the side chambers of the arena contained an empty wire mesh cup. The time spent interacting with each of the cups and the time

spent in each of the chambers was recorded. Following phase 1 the mouse was returned to the central chamber and the doors to the adjacent chambers were blocked. A novel or stranger mouse (same-sex, weight and age) was placed in one cup and a novel object, small object roughly the same size and color as a mouse, was placed in the other cup. All stranger mice were male wild type mice from the C57BL/6J strain. Stranger mice were habituated to the cup by spending 1 hour a day in the cup on both of the two days prior to testing. During phase 2 the mice were again allowed to freely explore the testing arena for 10 minutes. Time spent in each chamber and time spent interacting with each of the cups was recorded again. The sides of the arena in which the stranger mouse and object was placed was varied between testing subjects. The amount of time spent interacting with the novel mouse is a measure of sociability (Nadler et al., 2004). A deficit in social interaction would be spending less time interacting with the novel mouse when compared to the control group.

The three chamber testing apparatus consisted of a clear acrylic box measuring 60 cm x 40.5 cm x 22.5 cm. The apparatus was split into three different chambers by a 0.25 cm thick clear acrylic wall, the two outer chambers measured 20.5 cm x 40.5 cm and the middle chamber measured 18.5 cm x 40.5 cm. In the center of each of the dividing walls was a door that was 10 cm x 5 cm. In the far corner of each of the two outer chambers an inverted wire mesh cup was placed. On top of the wire cups a large water bottle was placed to prevent the moving of the cup and to prevent the test mouse from climbing on top of the wire cup. The video was captured using Dazzle video creator plus HD (Corel, Canada), for scoring at a later time.

The main tests used to determine hippocampal learning and memory were the Morris water maze and trace fear conditioning tests. Trace fear conditioning is a four day test that is used to determine hippocampal dependent learning, previously described by (Wiltgen et al., 2005). On day 1, the mice were placed into the testing chamber (described below) and introduced to the context. The mice are left in the chamber for a total of 720 seconds. On day 2 the mice were again placed back into the testing chamber and after a 240 second acclimation period were presented with a 20 second white noise tone at 70 dB. Following the end of the tone there was a 20 second trace period that ends with a 2 second mild foot shock at 0.5 mA. After a 60 second inter-trial interval (ITI) another tone trace/shock pairing is presented. A total of 6 pairings were presented followed by 40 second recovery period. The mice are in the chamber for a total of 840 seconds. On test day 3 the mice were returned the test chamber, this time under novel contextual cues (see below for description). After a 180 second baseline period the mice were again present with the 20 second white noise tone at 70 dB followed by an 80 second ITI. A total of 4 tone presentations were given and the mice were in the test chamber for a total of 580 seconds. On test day 4 the mice were again placed in the test chamber under the original contextual setup, this time without any stimuli and were left in the chamber for a total of 180 seconds. The percentage of time spent freezing was recorded throughout all 4 days of testing and later was broken down into important periods of testing for each day. A reduction in time spent freezing compared to the controls would demonstrate a deficit in learning the association of the tone to the shock or the context to the shock.

The testing chamber consisted of two clear acrylic sides and two metal sides with a metal bar floor that can receive an electrical current. The chamber was 26 cm x 22 cm x 18 cm and was placed in a sound attenuated outer chamber to control for background noise and light. On novel contextual testing days the floor of the chamber was replaced by a white foam pad under a clear acrylic square, and an additional clear acrylic wall was added diagonally across the chamber giving the mice access to half the chamber in a triangular form. An additional house light and fan were turned on in the testing chamber to alter the background light and noise levels. The apparatus was cleaned with 70% ethanol instead of 30% isopropanol, and a tray of vanilla extract was placed in the chamber to alter the smell. The testing room lights were dimmed and the bedding in the transfer cages was replaced with shredded paper to alter the context of transferring the mice from the holding room to the testing chamber. Two identical chambers were set up, in order to allow for testing of two different mice simultaneously. The freezing behaviors of the mice were recorded using Freeze Frame monitoring system (Coulbourn, Ohio, USA).

It has been shown that Morris water maze can be used to determine hippocampal dependent spatial learning (Morris et al., 1982). The procedure is based on those described previously (Lugo, Brewster, Spencer, & Anderson, 2012) and consists of training the mice to find a hidden platform in the water maze with 2 blocks of training per day for 4 days. Each block had four trials. Each trail was continued until the mouse reached the platform or 1 min of swimming occurred. After each trial the mouse was held on the platform for about 10 seconds before starting the next trial. If the mouse was not on the platform at the end of the 1 min trial it was picked up and placed onto the

platform. All trials that did not result in the mouse finding the platform were scored with an escape latency of 1 minute. The latency to escape, swim distance, and swim speeds were recorded. The last block, on day 4, was followed by a probe trial in which the hidden platform was removed and the mice were placed in the maze for 1 minute. For the probe trial the time spent in each quadrant and number of platform crossings (area where the platform was previously located) was recorded. On day 5 a visible platform test was given to make sure the vision and swimming ability of the mice was not a factor in longer escape latencies. For visible platform training a total of 2 blocks of 2 trials were administered. Longer escape latency to the hidden platform over the eight trials and equal time spent in the target zone vs. non-target zones and/or fewer platform crossings during the probe trial demonstrates a deficit in spatial learning.

The testing apparatus for Morris water maze consisted of a black plastic 130 cm diameter x 60 cm tall round pool. The pool was filled with water to a point at about 18 cm from the top and made opaque by adding 400 ml of black water soluble paint. The hidden platform was 14.5 cm x 14.5 cm and was submerged 2 cm below the water level. The visible platform was also 14.5 cm x 14.5 cm and was also submerged 2 cm below the water level but had an additional 14.5 cm x 14.5 cm acrylic square attached to a central column that was 9.5 cm above the lower platform. The room the maze was placed in was held at constant light levels, temperature, and background noise level. The water maze was filled at least one day prior to testing to allow for enough time so that the water temperature was equal to room temperature. The testing room had additional geometric shapes placed on the walls to provide distal visual navigations cues. The testing was recorded using the video tracking software Noldus (Ethovision, Netherlands).

Western Blotting

One week after the completion of behavior testing, the mice were sacrificed and the hippocampus was rapidly dissected out of the brain, rinsed in 1X PBS on ice, and then placed in dry ice prior to storing at -80° C. Tissue was processed, as previously described by (Lugo et al., 2008), by first homogenizing the whole hippocampus in homogenizing buffer (0.32 M sucrose, 1 mM EDTA, 5 mM Hepes) containing protease inhibitor cocktail (Sigma, St. Louis, MO). The samples were then given a quick 1 min spin at 1000 g at 4° C. A portion of the sample was taken for total homogenate samples, the remainder of the supernatant was pipetted into another centrifuge tube then spun at 800 g at 4° C for 10 minutes. The supernatant was then transferred to another tube and spun for 15 minutes at 7100 g at 4° C to produce a crude synaptosome from the pellet. This pellet was then resuspended with homogenizing buffer with protease inhibitor cocktail. Sample buffer (4X: 0.25 M Tris, pH 6.8, 6% SDS, 40% Sucrose, 0.04% Bromophenol Blue, 200 mM Dithiothreitol) was then added to the total homogenate and synaposome for western blotting analysis. Samples were run though 8-12 % SDS-PAGE gels and then proteins were transferred to Hybond-P polyvinyl difluoride membranes (GE Helthcare, Piscataway, NJ). Blocking solution was prepared [5% non-fat milk in 1X Tris Saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) with 0.1% Tween (1X TTBS) and 1 mM Na3VO4], and the membranes were incubated in the blocking solution for 1 hour at room temperature. Following blocking, membranes were incubated over night at 4° C with primary antibodies in blocking solution (see table 1 for antibody specifics). Following primary antibody incubation, membranes were washed 3 times for 5 minutes in TTBS, then incubated for 1 hour at room temperature with horseradish peroxidase

labeled secondary antibodies in the milk solution at a concentration of 1:20,000. After another three 5 minute washes in TTBS, membranes were incubated for 5 minutes at room temperature in GE ECL Prime (GE Healthcare, Piscataway, NJ). The chemiluminescent immunoreactive bands were imaged using a digital western blot imaging system (ProteinSimple, Santa Clara, CA).

We used the ProteinSimple Alphaview software to determine the optical density of each of the western blotting bands. All samples were normalized to loading control (actin or mortalin) within its own lane, and then normalized to the control group (WT-saline). For antibodies that have weaker signals, the blot was first probed for the primary antibody then reprobed for the loading constant. For stronger signaled antibodies the primary and loading constant were probed simultaneously. All phosphorylated proteins were normalized to the total concentration of that protein (within sample) and expressed as a percentage of the total. For all steps that involved the membranes to be placed in a milk solution (blocking, primary and secondary antibody incubations) 20% Sodium Orthovanadate at a concentration of 1:100 was added to the milk solution for all phospho antibodies. All antibodies were obtained from either Cell Signaling (Cell Signaling Technology, Boston, MA), NeuroMab (NeuroMab, Davis, CA) or Abcam (Abcam, Cambridge, MA) (see table 1 for antibody specifics).

Statistical Analysis

Single measurement comparisons were analyzed using independent samples t-tests or similar nonparametric tests (Mann-Whitney), when necessary to account for unequal variances. For repeated measure tests, data were analyzed using two-way

ANOVA. All statistical and data analyses were conducting using Prism 6 (GraphPad Software, La Jolla, CA).

Table 1: Details on antibodies usage

Primary Antibody	Sample Used	Conc.	Manufacturer	Loading constant	Pairing	Secondary
AKT	Homogenate	1:500	Cell Signaling	Actin	Together	Rabbit
phospho-AKT (s473)	Homogenate	1:500	Cell Signaling	Actin	Together	Rabbit
S6	Homogenate	1:500	Cell Signaling	Actin	Together	Rabbit
phospho-S6 (s235/236)	Homogenate	1:500	Cell Signaling	Actin	Together	Rabbit
phospho-S6 (s240/244)	Homogenate	1:500	Cell Signaling	Actin	Together	Rabbit
p70 S6 kinase	Homogenate	1:500	Cell Signaling	Actin	Separate	Rabbit
Ankyrin	Synaptosome	1:500	NeuroMab	Actin	Together	Mouse
FMRP	Synaptosome	1:500	Cell Signaling	Actin	Separate	Rabbit
phospho-FMRP (s499)	Synaptosome	1:500	Abcam	Actin	Separate	Rabbit
GluR1	Synaptosome	1:500	NeuroMab	Actin	Together	Mouse
HCN1	Synaptosome	1:500	NeuroMab	Actin	Together	Mouse
Kv 4.2	Synaptosome	1:500	NeuroMab	Actin	Together	Mouse
mGluR1/5	Synaptosome	1:500	NeuroMab	Actin	Together	Mouse
Pan Shank	Synaptosome	1:500	NeuroMab	Mortalin	Together	Mouse
Pan SAPAP	Synaptosome	1:500	NeuroMab	Actin	Together	Mouse
PSD 95	Synaptosome	1:4000	NeuroMab	Actin	Together	Mouse
Actin	Both	1:1000	Cell Signaling	-	-	Rabbit
Mortalin	Both	1:1000	NeuroMab	-	-	Mouse

CHAPTER FOUR

Results

Seizure Induction

The first observations were made during seizure induction. Only mice that showed seizure activity were included in this study, and all mice that showed any amount of seizure activity eventually reached status epilepticus. The typical seizure would begin with some form of limb clonus in either forelimbs or both hind and forelimbs. Limb clonus would usually only last for a short bout of time (<10 seconds) but multiple episodes would occur. Hindlimb clonus was not typically seen on its own and was typically only seen when both hindlimb and forelimb clonus occurred at the same time resulting in status epilepticus. The repetitive jumping and rolling were only seen in a few mice throughout the study. The typical seizure progression would start with at least one episode of limb clonus followed by multiple rearing and fallings then eventually reach full status epilepticus.

A total of 59 mice survived and were used in behavior testing. WT-Saline group had 18 mice, Het-Saline group had 12 mice, WT-Seizure group had 15 mice, and Het-Seizure group had 14 mice. There were no differences observed between the WT and Het mice when it came to typical type of seizures seen during induction. Of the 21 WT mice injected, 3 (14.3%) died, 3 were removed because they did not go into status epilepticus (14.3%), and the average onset to first seizure was 19.65 seconds. Of the 16 Het mice injected, 2 (12.5%) died with an average onset to first seizure of 19.68 seconds.

Behavior Results

Open Field

Status epilepticus in the HT group resulted in hyperactivity in the open field test. There was a significant difference between groups in total distance $K(3,53) = 13.68$, $p < 0.01$ (Figure 2A). Dunn's post hoc tests revealed that the HT-seizure group mice traveled significantly greater distances when compared to the WT-control and HT-control groups. Nonparametric tests were used to account for unequal variances between groups. There was no difference in the number or total duration spent performing stereotypy actions $F(3,53) = 2.3$, $p = 0.09$ (Figure 2B), which is a measure of self-grooming events, and no difference between groups in number of stereotypy events (Figure 2C). However, there was a significant difference in the number of rearing events $K(3,53) = 8.1$, $p < 0.05$ (Figure 1D). There was also a marginal effect in the number of clockwise rotations between groups $K(4,57) = 7.31$, $p = 0.06$ (Figure 2E), but no significant differences in the number of counter clockwise rotations amongst the groups $K(3,53) = 5.45$, $p = 0.14$ (Figure 1F). All open field analysis data was lost for two mice due to computer error following testing, including one from the WT-saline group and one from WT-seizure group.

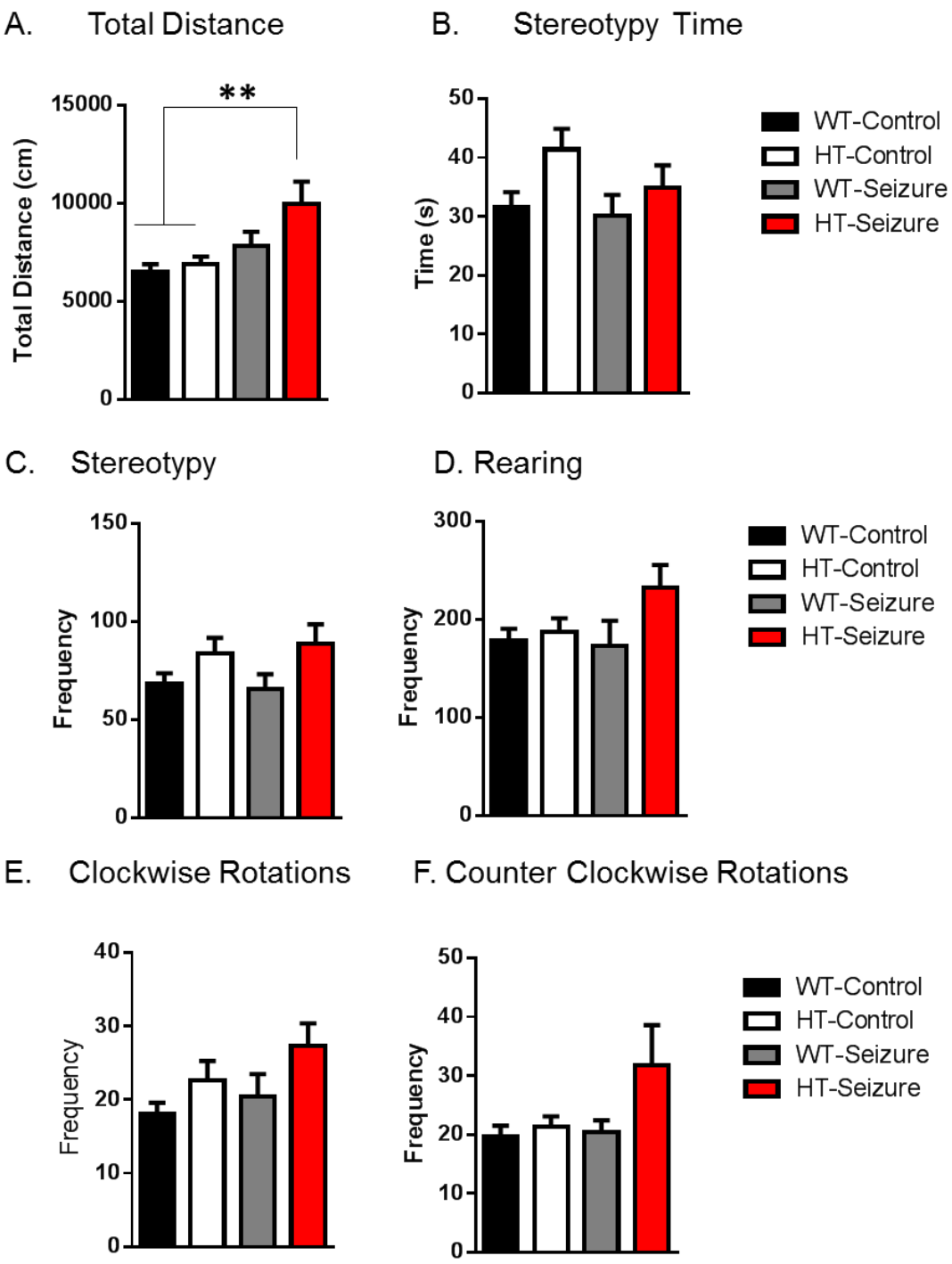

Figure 2. Locomotor activity and stereotypy behavior in the open field test in Pten wildtype and heterozygous mice after status epilepticus. The mice were placed in an open field test for thirty minutes and several measurements were made of their locomotor behavior. (A) Total distance, (B) time performing stereotypy behavior, (C) frequency of stereotypy events, (D) number of rearing events, (E) number of clockwise rotations, and (F) number of counter clockwise rotations. Data are shown as mean ± standard error of the mean. ** = $P < 0.01$.

When comparing total distance traveled in the surround vs. center of the open field test, there was a marginal group effect in the center area $K(3,53) = 7.6$, $p = 0.056$ (Figure 3A Left Graph). There was a significant difference between groups in distance traveled in the surround area $K(3,53) = 13.71$, $p < 0.01$ (Figure 3A Right Graph). Using Dunn's multiple comparison test we found significant differences between the WT-Control and HT-Control, as compared to the HT-Seizure group. There was no significant differences between the groups in distance traveled in the center or in the amount of time spent in the center vs. the surround (Figure 3B). There was no significant differences in fecal bolus production between groups $F(3,55) = 0.57$, $p = 0.63$ (Figure 3C).

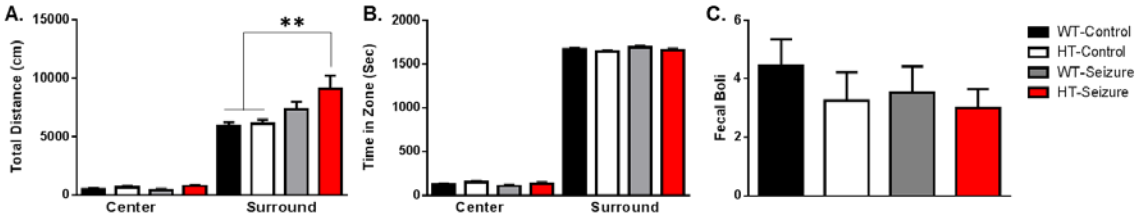

Figure 3. Anxiety data in the open field test. The data were then reanalyzed by center and surround regions of the open field apparatus. (A) Total distance in the center and surround region of the open field test. (B) Total time spent in the center and surround region of the open field test. (C) Fecal bolus production counts. Data are shown as mean ± standard error of the mean. ** = $P < 0.01$.

Elevated Plus Maze

Induction of status epilepticus in the HT group resulted in hyperactivity without changes in anxiety in the elevated plus maze test. There was a significant effect in total distance traveled by mice in the elevated plus maze $K(3,55) = 8.1$, $p < 0.05$ (Figure 4A). Dunn's post hoc tests revealed a significant difference in distance between the WT-

Control and HT-Seizure groups. Similar differences were observed in reference to velocity in the maze $K(3,55) = 9.5$, $p < 0.05$ (Figure 4B). Dunn's post hoc tests revealed a significant difference in velocity between the WT-Control and HT-Seizure groups.

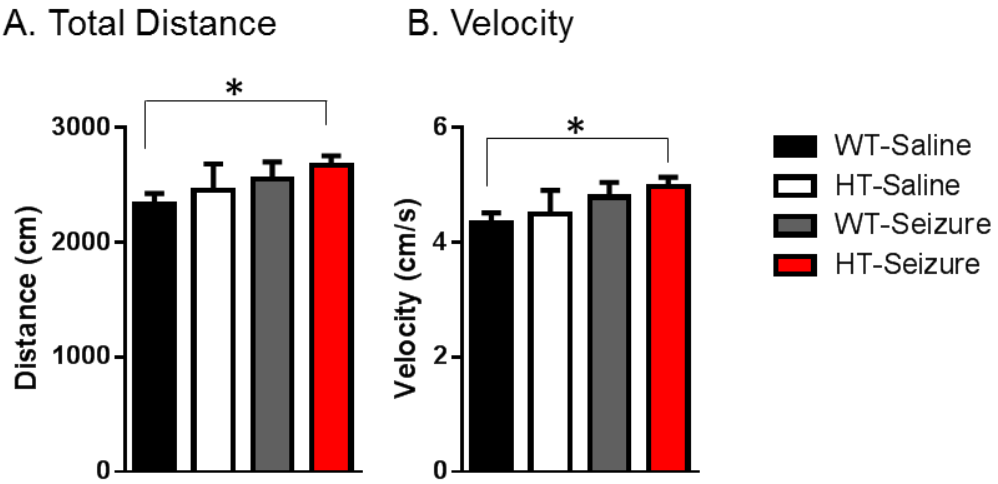

Figure 4. General activity data for the elevated plus maze. A. Total distance traveled during elevated plus maze testing. B. Average velocity of movement during elevated plus maze testing. Data are shown as mean ± standard error of the mean. * = $P < 0.05$.

For analysis of the open versus closed arms, the total time spent and number of entries into each type of arm was grouped together based on type. Center refers to the central section where all of the arms meet. There were no differences in time spent in the open arms $F(3,55) = 0.12$, $p = 0.95$; center $F(3,55) = 1.01$, $p = 0.39$; or in the closed $F(3,55) = 0.41$, $p = 0.74$ (Figure 5A). Similar results were found in the frequency of visits to each arm for the open $F(3,55) = 1.1$, $p = 0.37$; center $F(3,55) = 2.6$, $p = 0.06$; and closed $F(3,55) = 1.6$, $p = 0.19$ (Figure 5B). When comparing the amount of time spent in open vs. closed arms within groups, all of the groups show the same pattern of spending more time in the closed arms than in the open arms (Figure 5C), all but the Het-saline group show the same pattern for number of arm entries, Het-saline group show no

significant difference in number of arm entries between open and closed arms (Figure 5D).

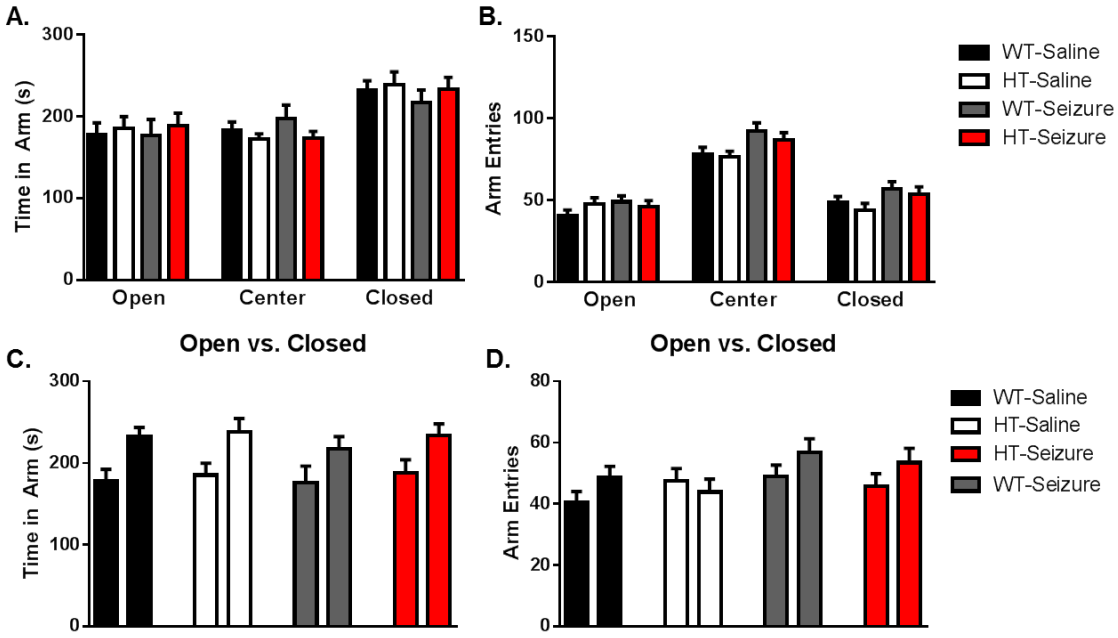

Figure 5. Anxiety measures for elevated plus maze. A. Time spent in each of the different sections of the elevated plus maze. B. Number of entries into each type of arm. C. Comparison of type spent in open vs. closed arms for each group. D. Number of entries into open vs. closed arms for each group. Data are shown as mean ± standard error of the mean.

In reference to general exploratory behaviors during the elevated plus maze task, the number of head dips in the open arm and number of rearings in the closed arms were compared across the groups. There were no significant differences in head dipping frequency $F(3,55) = 1.9$, $p = 0.13$ (Figure 6A) or duration $F(3,55) = 1.6$, $p = 0.18$ (Figure 6B). There were no differences in the duration of rearing in the closed arms $F(3,55) = 0.81$, $p = 0.5$ (Figure 6C). However, there was a marginal effect in the number of rearing events in the closed arms $F(3,55) = 2.5$, $p = 0.068$ (Figure 6D).

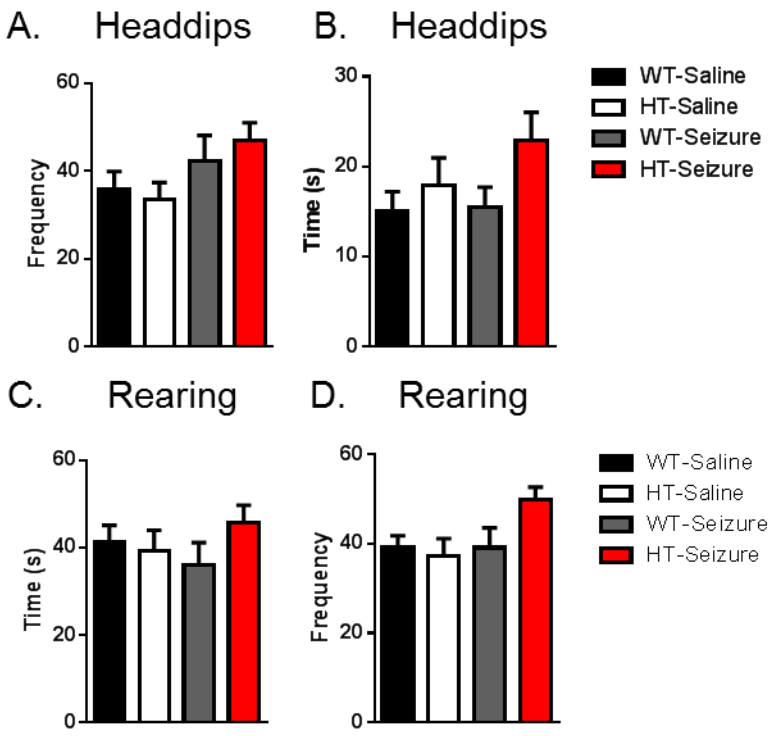

Figure 6. Exploratory behavioral measurements for the elevated plus maze. A. Average number of head dips in open arms during elevated plus maze testing. B. Average total amount of time spent performing head dips by each mouse. C. Average total amount of time spent rearing by each mouse in the closed arms. D. Average number of rearing events in closed arms. Data are shown as mean ± standard error of the mean.

Marble Burying

There were no significant differences in the number of marbles buried at various depths between groups at any depth. There were no difference at the 50% $F(3,55) = 1.3$, $p = 0.27$; 75% $F(3,55) = 1.6$, $p = 0.20$; 100% $F(3,55) = 1.6$, $p = 0.18$; or completely buried marbles $F(3,55) = 1.6$, $p = 0.20$ (Figure 7).

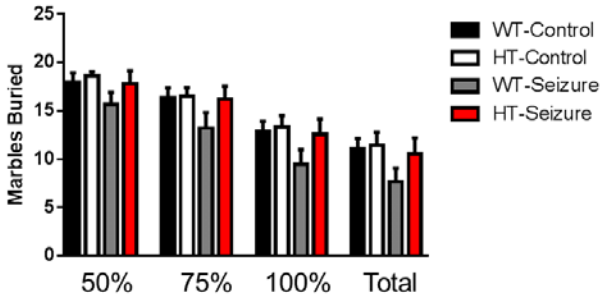

Figure 7. Repetitive behavior in Pten wildtype and heterozygous mice after status epilepticus. There were no changes in the number of marbles buried in a 30 minute test. The number of marbles buried were examined at different levels: 50%, 75%, 100%, and completely buried. Data are shown as mean ± standard error of the mean.

Social Chamber

The first variable looked at in social chamber is a measure of overall activity of the mice during the social chamber testing. For both phases A and B there was no significant difference in the total distance traveled during the 10 minute social chamber test (Figure 8).

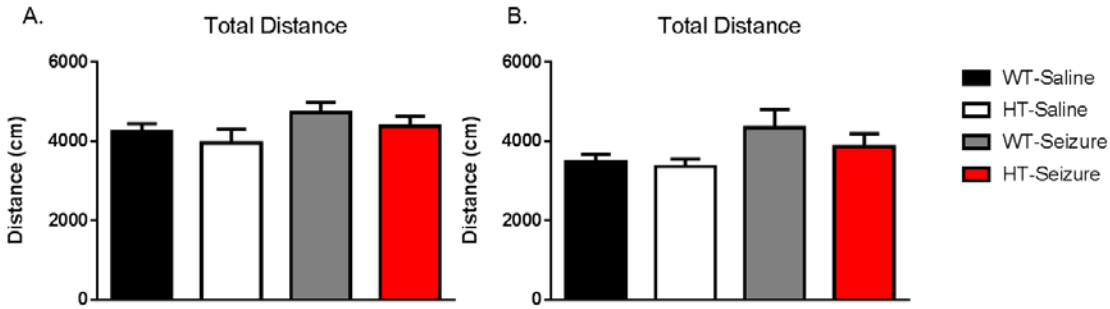

Figure 8. Data on overall activity level during social chamber testing. A. Average total distance traveled during the phase A of testing. B. Average total distance travel during phase B of testing. Data are shown as mean ± standard error of the mean.

For all phase A analyses, chambers and cups are referred to as left and right, where in phase B all sides are referred to as either mouse or object based on what was

placed in the cup on that side. There were no significant differences between groups in duration of time in the chambers $F(3,55) = 0.12$, $p = 0.85$, but there was a significant difference across chambers $F(2,110) = 91.4$, $p < 0.001$ (Figure 9A). There were no significant differences in duration of visit at the empty cups between groups $F(3,55) = 0.4$, $p = 0.75$ (Figure 9B), as well as no differences in time spent at the cups $F(1,55) = 1.2$, $p = 0.26$.

In phase B, there were no differences between the groups in the time spent between the chambers $F(3,55) = 1.2$, $p = 0.317$, but there was a difference across chambers $F(2,110) = 103.0$, $p < 0.001$ (Figure 9C) and a significant interaction between groups across chambers $F(6,110) = 2.6$, $p < 0.05$. Separate One-Way ANOVA tests revealed a significant difference in the total time spent with the object $F(3,55) = 3.4$, $p < 0.05$, with Tukey *post hoc* tests revealing a significant difference between HT-Control and WT-Seizure groups. There was a marginal effect in total time spent at the chamber that housed the mouse $F(3,55) = 2.4$, $p = 0.081$.

In Phase B when we analyzed duration at the cups housing the mice, there were no differences between groups in time spent at the cup $F(3,55) = 2.1$, $p = 0.11$ (Figure 9D). There was a difference in the time spent at each cup $F(1,55) = 74.6$, $p < 0.001$. There was a significant interaction between the groups across the cups $F(3,55) = 3.6$, $p < 0.05$. Separate one way ANOVA tests revealed a significant main effect of group in the time spent at the cup with the mouse $F(3,55) = 3.2$, $p < 0.05$. There were marginal *Tukey* post-hoc tests at the duration of time spent at the mouse cup with WT-Control compared to WT-Seizure and HT-Seizure ($p = 0.06$). We also measured the mean number of visits for the cup that housed the mouse and objects. There was no main effect of group

$F(3,55) = 1.5$, $p = 0.22$ (Figure 9E). However, there was a difference in mean time spent per cup $F(1,55) = 59.3$, $p < 0.001$. There was also a significant interaction of group x cup $F(3,55) = 3.2$, $p < 0.05$. There were no statistically significant differences between groups with a One-Way ANOVA. However, separate paired t-tests revealed a significant preference for the cup with the mouse over the object in all groups WT-Vehicle $t(1,17) = 5.5$, $p < 0.001$; HT-Vehicle $t(1,11) = 4.8$, $p < 0.001$; WT-Seizure $t(1,14) = 3.6$, $p < 0.01$; except the HT-Seizure $t(1,13) = 1.5$, $p = 0.16$. The paired t-test detected that the HT-Seizure group did not show a preference between the mouse and the object, revealing a social behavior deficit in the HT-Seizure group.

Trace Fear Conditioning

For day one of fear conditioning when the mice are placed in the testing chamber with no additional stimuli, there was no significant difference between the groups in amount of time spent freezing. On day two during the shock and tone presentations, there was no significant difference between the groups in time spent freezing under all of the conditions; baseline, tone presentation, tracer period, and inter trial interval.

Status epilepticus in the HT group resulted in enhanced learning in the trace fear conditioning test during test day three. On the third day of testing, mice were placed in a novel context and a conditioned stimulus tone was presented 4 different times. The freezing levels of the mice were recorded during baseline, the conditioned stimulus, the trace period, and all inter-trial intervals. Results demonstrated a main effect of group $F(3,55) = 4.0$, $p < 0.05$ and a main effect of time $F(3,165) = 54.9$, $p < 0.001$ (Figure 10A). Tukey post hoc tests revealed a significant difference between the WT-saline group compared to the HT-seizure group. The HT-seizure group displayed an increase in

freezing across the entire testing period. Furthermore, there was a marginal interaction between group and time $F(9, 165) = 1.8$, $p = 0.078$. On the fourth day, we tested the groups in the original context for three minutes. There was no main effect of group over the context test period $F(3,55) = 2.3$, $p = 0.089$, and no main effect of time $F(6,110) = 0.75$, $p = 0.61$ (Figure 10B). There was also no interaction between group over time $F(6,110) = 0.75$, $p = 0.61$.

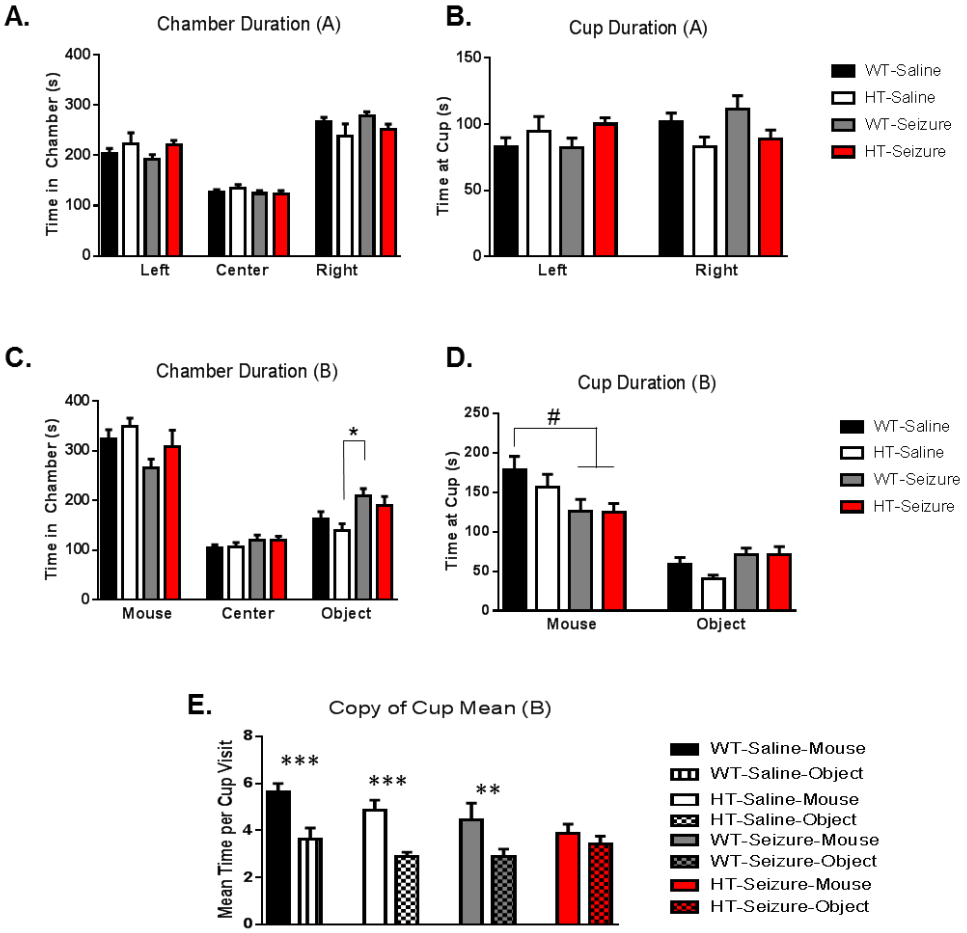

Figure 9. Social behavior measurements in the three chamber social test for Pten wildtype and heterozygous mice after status epilepticus.(A) Time in the left, center, and right chamber in phase A. (B) Duration of interaction at the cups within the chamber in phase A. (C) Time in the chamber with the mouse, center, and chamber that housed the novel object. (D) Time spent with the cup that housed the mouse and object in the three chamber social behavior test. (E) Mean time interacting with the mouse compared to the novel object across groups. The values represent the mean ± SEM. # = $P = 0.06$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

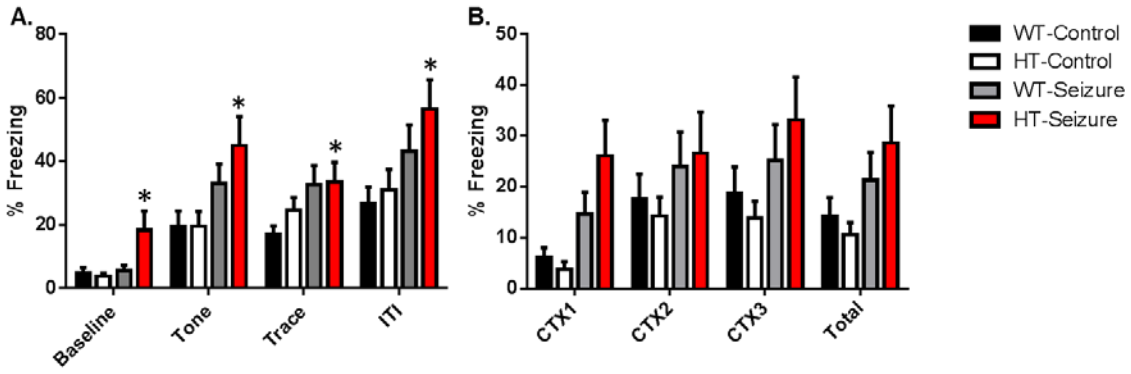

Figure 10. Freezing levels of Pten wildtype and heterozygous mice after status epilepticus in Trace Fear Conditioning. Status epilepticus results in enhancement of learning. Mice were examined for differences in learning and memory through trace fear conditioning test. (A) The HT mice with status epilepticus had more freezing across all trials. (B) No differences were observed in contextual conditioning a day later. Data are shown as mean ± standard error of the mean. * = $P < 0.05$.

Morris Water Maze

Status epilepticus in the HT mice did not result in spatial learning and memory deficits. There were no difference between groups in path length $F(3,55) = 0.67$, $p = 0.57$ (Figure 11A), time to find hidden platform (escape time) $F(3,55) = 0.78$, $p = 0.51$ (Figure 11B), and swim velocity $F(3,55) = 0.7$, $p = 0.56$ (data not shown) across the 8 blocks (4 trials per block per mouse) of training for Morris water maze. All groups showed learning and improvement across training blocks. There was a significant main effect of time for path length, $F(7,385)= 11.39$, $p < 0.0001$, escape time, $F(7,385)= 19.02$, $p < 0.0001$, and swim velocity, $F(7,385)= 14.84$, $p < 0.0001$. There were no significant interactions between group and trial in any of the measures.

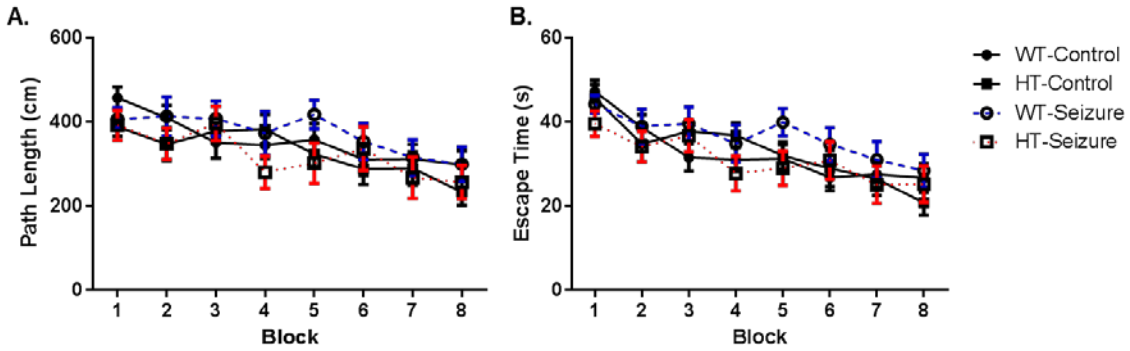

Figure 11. Spatial learning acquisition for Morris Water Maze. A. Average path length per block (4 trial per mouse per block) during training. B. Average escape time per block during training. Data are shown as mean ± standard error of the mean.

The probe trial was run following the last training block on day 4. There was no difference in the duration of time in each quadrant between groups $F(3,55) = 0.53$, $p = 0.66$, but there was a significant difference across quadrants $F(3,165) = 9.3$, $p < 0.001$ (Figure 12A). There was a marginal effect of number of times in the zone where the hidden platform was located $F(3,55) = 2.5$, $p = 0.073$ (Figure 12B). There was a main effect across zones $F(3,165) = 14.7$, $p < 0.001$. There were no significant interactions found between groups across the four quadrants.

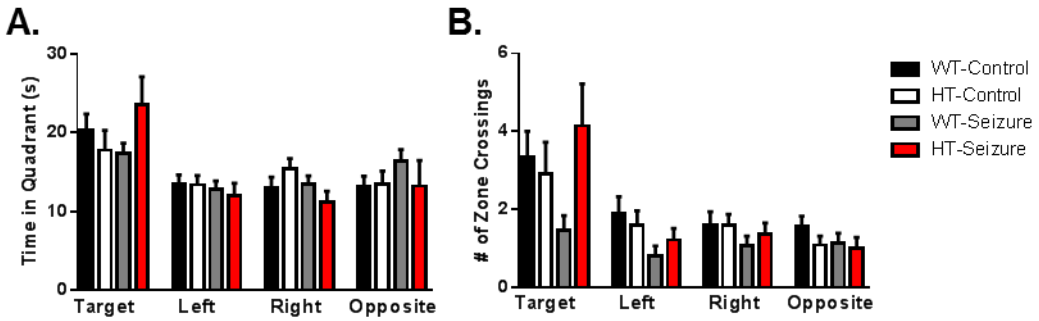

Figure 12. Probe trial data for Morris Water Maze. There were no differences in the amount of time in each quadrant (A) or in the number of crossings in each zone (B). Data are shown as mean ± standard error of the mean.

On the fifth day of testing, the platform was made visible and two blocks of two trials were run. During the visible platform trials, no significant difference was found between groups in path length $F(3,55) = 0.853$, $p = 0.47$ (Figure 13A) or in average escape time $F(3,55) = 1.02$, $p = 0.39$ (Figure 13B).

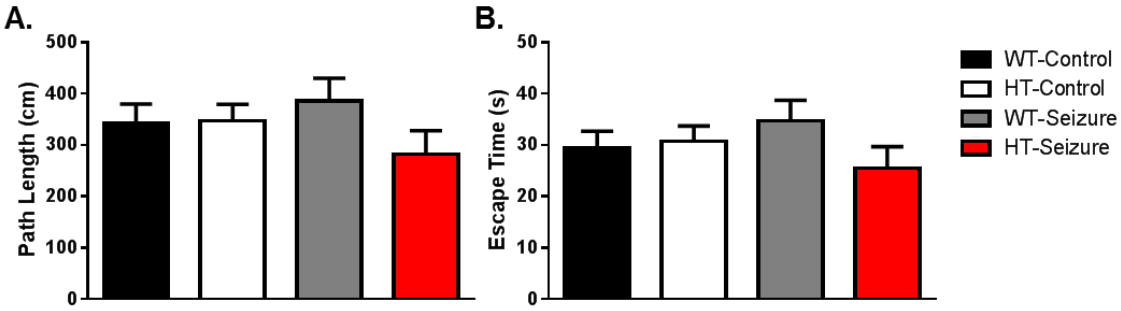

Figure 13. Visible platform data for Morris Water Maze. A. Average path length taken to the platform over the 4 visible platform trials. B. The average escape time over the 4 visible platform trials. Data are shown as mean ± standard error of the mean.

Western Blotting Analysis

Two different sets of westerns were run for each of the different proteins. The values from each set of westerns were combined for analysis. The total number of sample for each group was as follows; WT-control n=8, HET-control n=10, WT-seizure n=8, and HET-seizure n=10. Figure 14 contains all of the analyses for the hippocampal total homogenate samples and figures 15 and 16 contain the analyses for the hippocampal P2 (rough synaptosome) samples.

For AKT there were no significant differences between groups $F(3,32) = 1.264$, $p = 0.106$ (Figure 14A). There were no significant differences between groups for the ratio of pAKT (s473) to AKT $F(3,32) = 0.5375$, $p = 0.660$ (Figure 14B). For S6, no significant difference was observed between groups $F(3,32) = 0.3247$, $p = 0.808$ (Figure 14C). For the ratio of pS6 (s235/236) to S6 no significant difference was seen between

groups $F(3,32) = 0.3595$, $p = 0.783$ (Figure 14D). For the ratio of pS6 (s240/244) to S6 there were no significant differences between groups $F(3,32) = 1.348$, $p = 0.276$ (Figure 14E). For p70 S6K, no significant difference between groups was observed $F(3,32) = 0.4701$, $p = 0.705$ (Figure 14F).

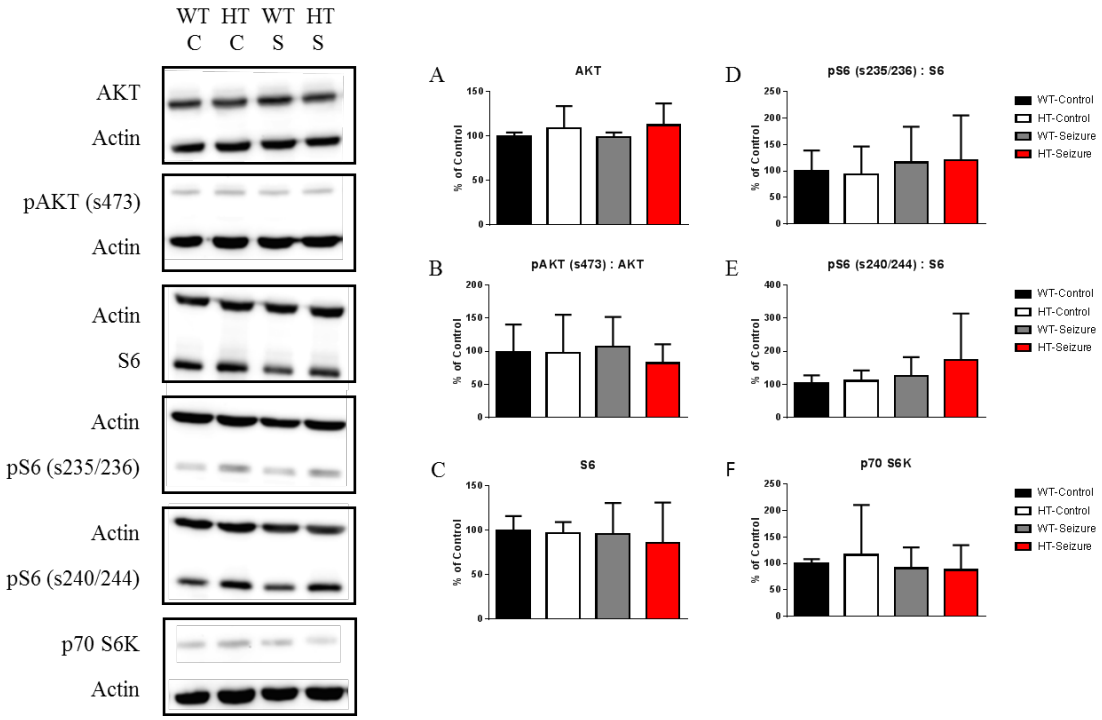

Figure 14. Western blotting data for hippocampal total homogenate samples. Left: Image of the western blot. Right: Relative changes in protein concentration normalized to the loading constant and expressed as a percentage of the control group. All phosphorylated proteins are expressed as a percentage of phosphorylated protein to total protein concentration. A. AKT. B. pAKT (s473). C. S6. D. pS6 (s235/236). E. pS6 (s240/244). F. p70 S6K.

When looking at GluR1 there were no significant differences between groups $F(3,32) = 0.2163$, $p = 0.884$ (Figure 15A). For mGluR1/5 no significant differences between groups was found $F(3,32) = 1.004$, $p = 0.404$ (Figure 15B). No significant difference were detected between groups for HCN1 $F(3,32) = 0.5942$, $p = 0.6234$ (Figure 15C). There were no significant differences between groups for Kv4.2 $F(3,32) = 1.274$, p

= 0.300 (Figure l5D). For FMRP, no significant differences between groups were found $F(3,32) = 1.308$, $p = 0.289$ (Figure 15E). For the ratio of pFMRP (s499) to FMRP there were also no significant differences between groups $F(3,32) = 1.004$, $p = 0.404$ (Figure 15F).

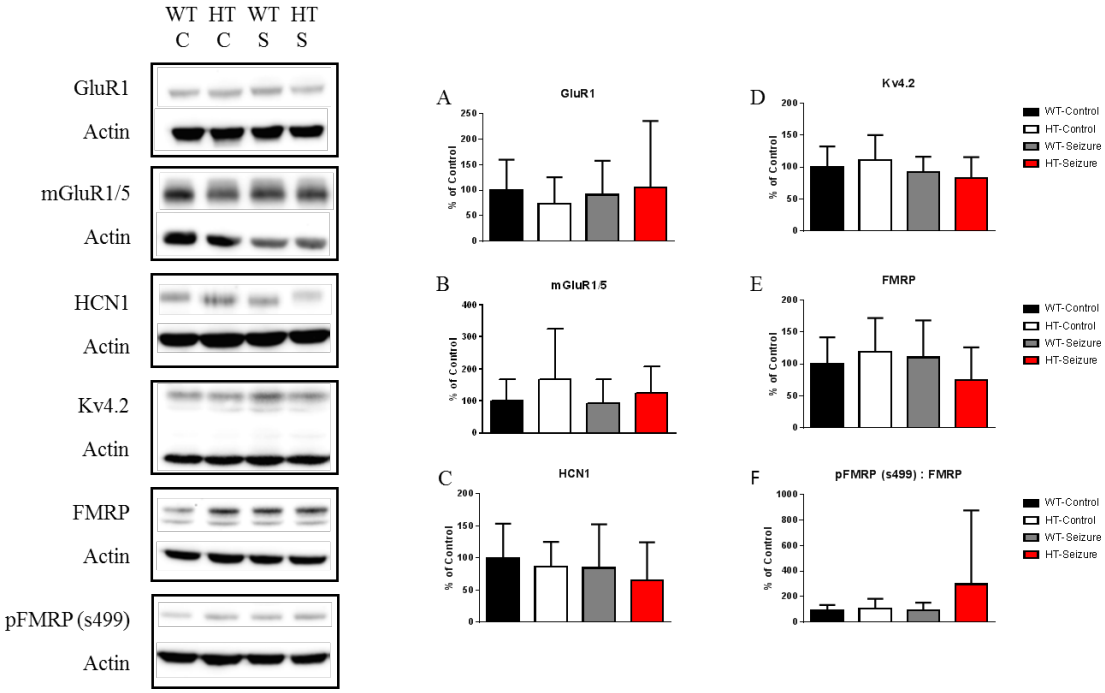

Figure 15. Western blotting data for hippocampal P2 samples. Left: Image of the western blot. Right: Relative changes in protein concentration normalized to the loading constant and expressed as a percentage of the control group. All phosphorylated proteins are expressed as a percentage of phosphorylated protein to total protein concentration. A GluR1. B. mGluR1/5. C. HCN1. D. Kv4.2. E. FMRP. F. pFMRP (s499).

For Ankyrin, no significant differences were observed between groups $F(3,32) = 0.4715$, $p = 0.704$ (Figure 16A). When looking at PSD-95 no significant differences between groups were seen $F(3,32) = 1.102$, $p = 0.363$ (Figure 16B). No significant differences were detected between groups for Pan Shank $F(3,32) = 1.429$, $p = 0.252$ (Figure 16C). For both the bands of Pan SAPAP (110 kDa and 120 kDa) no significant

differences were detected between the groups, for the 110 kDa band $F(3,32) = 1.009$, $p = 0.402$ (Figure 16D) and for the 120 kDa band $F(3,32) = 0.5317$, $p = 0.664$ (Figure 16E).

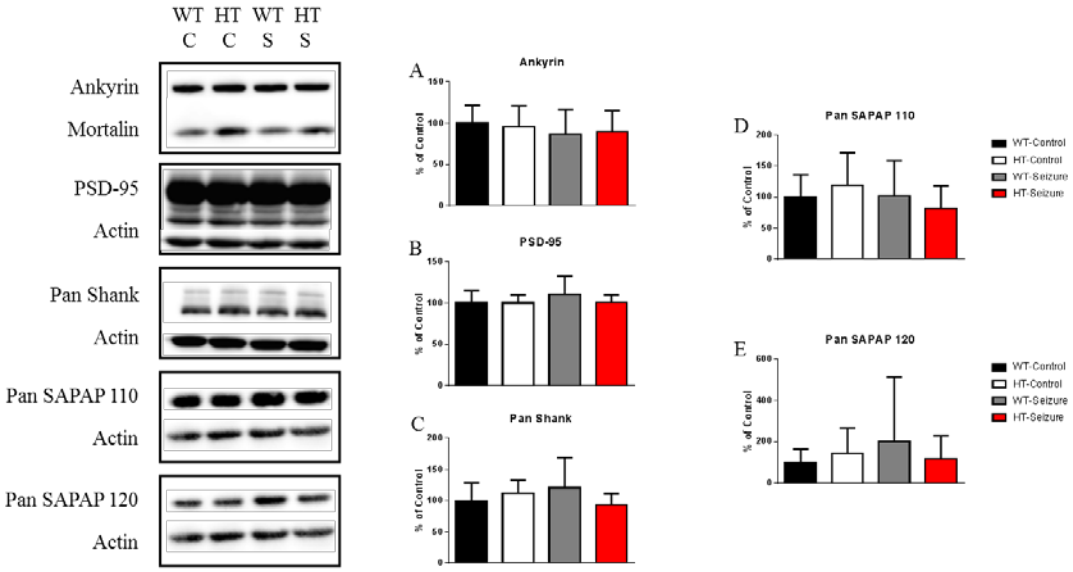

Figure 16. Western blotting data for hippocampal P2 samples. Left: Image of the western blot. Right: Relative changes in protein concentration normalized to the loading constant and expressed as a percentage of the control group. A. Ankyrin. B. PSD-95. C. Pan Shank. D. Pan SAPAP 110 kDa. E. Pan SAPAP 120.

The analysis of all of the westerns showed no significant difference in protein levels for any of the proteins tested from the hippocampus of the test mice. The mean and standard error of the means for the four groups are presented in Table 2. The table also includes the statistical information for comparisons of the four groups.

Table 2: Western blotting analysis, summary of results (Values are mean±SEM).

Protein	WT-Saline	HT-Saline	WT-Seizure	HT-Seizure	Sig. Change
AKT	100.0±1.469, n=8	108.5±9.035, n=8	98.83±1.685, n=10	112.4±7.758, n=10	-
phospho-AKT (s473)	100.0±15.87, n=8	101.5±19.34, n=8	105.2±14.16, n=10	92.19±11.33, n=10	-
% Total phospho-AKT (s473)	98.87±14.78, n=8	97.47±20.51, n=8	106.7±14.29, n=10	82.31±9.047, n=10	-
S6	100.0±5.705, n=8	96.50±4.582, n=8	96.04±4.582, n=10	86.19±14.32, n=10	-
phospho-S6 (s235/236)	100.0±15.71, n=8	91.98±19.25, n=8	105.4±20.03, n=10	78.87±16.51, n=10	-
phospho-S6 (s240/244)	100.0±2.592, n=8	104.7±9.164, n=8	107.0±9.600, n=10	96.67±3.830, n=10	-
% Total phospho- S6 (s235/236)	99.30±14.03, n=8	93.51±18.69, n=8	116.2±21.33, n=10	120.8±26.66, n=10	-
% Total phospho- S6 (s240/244)	103.4±8.695, n=8	110.7±11.18, n=8	125.4±18.21, n=10	173.5±44.61, n=10	-
p70 S6 kinase	100.0±2.891, n=8	116.3±33.31, n=8	91.02±12.45, n=10	87.77±14.86, n=10	-
Ankyrin	100.0±7.670, n=8	95.17±9.111, n=8	86.32±9.657, n=10	89.23±8.224, n=10	-
FMPR	100.0±14.83, n=8	119.6±18.63, n=8	110.5±18.34, n=10	74.83±16.26, n=10	-
phospho-FMRP (s499)	100.0±22.12, n=8	109.6±19.21, n=8	110.0±38.88, n=10	80.18±15.73, n=10	-
% Total phospho-FMRP (s499)	94.53±14.28, n=8	106.3±27.25, n=8	91.32±19.48, n=10	297.5±182.9, n=10	-
GluR1	100.0±21.17, n=8	73.97±18.24, n=8	91.15±21.11, n=10	105.2±41.33, n=10	-
HCN1	100.0±18.97, n=8	86.28±13.72, n=8	84.47±21.47, n=10	64.53±19.00, n=10	-
Kv 4.2	100.0±11.52, n=8	111.6±13.65, n=8	92.18±7.640, n=10	82.94±10.36, n=10	-
mGluR1/5	100.0±24.13, n=8	169.0±54.93, n=8	93.47±23.34, n=10	123.8±26.61, n=10	-
Pan Shank	100.0±10.08, n=8	112.1±7.318, n=8	120.6±15.24, n=10	93.17±5.78, n=10	-
Pan SAPAP 110	100.0±12.75, n=8	119.7±18.47, n=8	101.7±18.07, n=10	81.27±11.76, n=10	-
Pan SAPAP 120	100.0±22.78, n=8	144.1±42.82, n=8	200.9±98.67, n=10	115.3±35.63, n=10	-
PSD 95	100.0±5.247, n=8	99.96±3.576, n=8	110.4±6.926, n=10	100.2±3.096, n=10	-

CHAPTER FIVE

Discussion

The most consistent outcome we found in our study was hyperactivity of the Pten haploinsufficient mice that received status epilepticus (SE). The Pten haploinsufficient mice that received SE during adulthood had an increase in total activity in the open field test, an increase in total distance in the surround region of the open field, and an increase in total distance and velocity in the elevated plus maze task. Previous clinical and animal model studies provide support for the comorbidity of hyperactivity in epilepsy. Attention-deficit hyperactivity disorder (ADHD) has a prevalence rate of 7 to 9% in children and 2.5-4% in adults and there is evidence that individuals with epilepsy have 2-3 times higher rates of ADHD (Williams, Giust, Kronenberger, & Dunn, 2016).

Animal models of epilepsy provide additional support for the comorbidity between epilepsy and hyperactivity. In one study, investigators induced SE in adult male Wistar rats with pilocarpine and found that half of the subjects displayed inattention and impulsivity (Pineda et al., 2014). Interestingly, the other half of the subjects showed a significant increase in time of immobility in the forced swim test. The forced swim test is commonly used to measure depression-like behavior in rodents. The authors of the study suggested that an epileptogenic insult may result in an increase in hyperactivity or an increase in depression-like behavior. For our experiments we only evaluated locomotor activity in the open field and elevated plus maze tests. Additional studies could include a lateralized reaction-time task and other tests of impulsivity to examine changes in the

subjects' impulsivity and attention. Future studies could also include the forced swim test or tail-suspension test to measure changes in depression-like behavior in mice after status epilepticus.

The Pten haploinsufficient mice that received SE during adulthood also displayed an impairment of social behavior in the three chamber social test. Impairment in social behavior is one of the three core features of ASD. The other two behavioral features of ASD include repetitive behavior and communication deficits. During the second phase of the three chamber social behavior test, Pten haploinsufficient mice with SE did not show a preference between the cup that housed the mouse compared to the cup with the novel object. The other groups showed a significant preference for the mouse. Other animal models of epilepsy have found that seizures during early development and during adulthood can result in social behavior deficits (Bernard et al., 2015; Bernard et al., 2013; Lugo, Swann, et al., 2014). In addition, we found conditional Pten knockout mice to have social behavior deficits (Kwon et al., 2006; Lugo, Smith, et al., 2014). Therefore, it appears that social behavior deficits may be a sensitive behavioral measure of seizures.

Contrary to previous studies, we did not observe deficits in social behavior for the Pten haploinsufficient mice that received a vehicle injection during adulthood. Existing studies using Pten germline haploinsufficient mice have found them to have social behavior deficits in the three chamber social behavior test (Clipperton-Allen & Page, 2014), as well as, decreased aggression and an increase in repetitive behavior (Clipperton-Allen & Page, 2015). The male Pten germline haploinsufficient did not show a preference in chamber time in either social approach or social novelty compared to the wildtype mice. In a separate social recognition test where the same stimulus mouse is

presented four times to measure social habituation, the Pten germline haploinsufficient demonstrated dishabituation but no change in habituation compared to wildtype mice.

One difference between the Pten haploinsufficient mice in these studies compared to the present study is that the Pten mouse they used had a germline mutation, while the mice used in our studies are instead a neuron-subset specific deletion of Pten using the Cre-loxP recombination system. The germline Pten haploinsufficient mice have a significant increase in the anterior and posterior regions of the cerebral cortex, an increase in cerebellum size, and a nearly 25% increase in overall brain mass compared to the Pten wildtype males. While the NS-Pten HT mice have only selective localized increases in brain overgrowth (Kwon et al., 2001). In addition, male mice with germline heterozygous deletion of Pten also have alterations in repetitive behavior and mood/anxiety (Clipperton-Allen & Page, 2014). For instance, the germline Pten HT mice have been found to bury more marbles in the marble burying test than the Pten WT mice. These Pten HT mice displayed a higher preference for the dark chamber over the light chamber in the light/dark test, which indicates a higher level of anxiety. In addition, Pten HT mice have been found to be more immobile than WT in the tail suspension test and forced swim test. It may be that a constitutional heterozygous mutation in *PTEN* is necessary to influence social behavior.

There doesn't appear to be any difference in anxiety levels in the mice following seizure induction. Both when looking at time spent in open vs. closed arms in elevated plus maze and time spent in the center vs. surround in open field, no alterations in anxiety were evident. Several studies have shown that following a single early-life seizure can lead to lasting increases in anxiety (Moreira et al., 2011; Sayin et al., 2004), however that

increase in anxiety is not always seen following seizures (Cornejo, Mesches, & Benke, 2008). The lack of changes in anxiety levels in this study may be due to strain or species, such as mouse vs. rat differences.

Additional behavioral measures examined in the open field were clockwise and counter-clockwise rotations. The spinning behaviors can be used as a crude measure of repetitive behaviors. The HT seizure mice in this study showed a significant increase in clockwise rotation but only trended towards significance in counter-clockwise rotation. However when directly testing for repetitive behaviors using the marble burying test (Hoeffer et al., 2008), no significant differences were found between the groups. One caveat in the method we used to score marble burying may result in a less sensitive measure of the repetitive behavior. We measured marble burying at one time point after 30 minutes. A more sensitive method may be to video record the behavior and score it at a later time. Bernard et al., 2015 video recorded the marble burying behavior in the rats after early-life seizures. They measured the number of marbles buried (>50%) and the time the animal spent attending to the marbles. When the experimenters later scored the behavior they observed that several rats displayed several instances of head movements. Bernard et al., 2015 reported that rats that had early-life seizures buried fewer marbles and moved fewer marbles compared to controls. Future studies could determine whether the mice are burying, attending to the marbles, reburying the marbles, and repetitive head movements. This would result in a more complete picture of the behavior compared to the one time point measurement (Bernard et al., 2015).

One surprising result was the increase in freezing behavior displayed by the Pten HT-Seizure mice in the trace fear conditioning test. The increase in freezing during tone,

trace, and the ITI periods all indicated that Pten HT-Seizure mice demonstrated enhanced learning in the trace fear conditioning test. Even though we hypothesized that seizures in the Pten haploinsufficient mice would result in learning deficits, previous studies have shown that seizures can instead lead to enhanced learning. Clinical research has found that school-aged children who experienced febrile seizures have significantly better learning, memory retrieval, consolidation, and delayed recognition compared to age-matched control children (Chang, Guo, Huang, Wang, & Tsai, 2000; Chang, Guo, Wang, Huang, & Tsai, 2001). It has also been shown that adult mice that experienced febrile seizures on PD14 showed enhanced memory performance in contextual fear memory (Tao, Ichikawa, Matsuki, Ikegaya, & Koyama, 2016). These mice have an increase in large mossy fiber terminals in the dentate gyrus of the hippocampus, which was suggested to correspond to the enhanced learning in these mice. A separate study found that febrile seizures induced in PD10 rat pups results in an increase in dendritic complexity of newborn dentate granule cells (Raijmakers et al., 2016). The most significant deletion of Pten in the NS-PTEN mice is in the dentate gyrus. It is possible that seizures are enhancing some of the connections in the dentate gyrus in these mice. Future studies could further examine the alterations within the dentate gyrus of Pten mice.

We performed western blotting studies on a number of proteins in the hippocampus of WT and HT mice with and without seizures. We found no differences between groups in the protein levels we measured in the hippocampus. We hypothesized that we would find hyperactivation in the PI3K/AKT/mTOR signaling pathway, given that this effect has been shown in other animal models after seizures (Shima et al., 2015;

Zeng, Rensing, & Wong, 2009; Zhang & Wong, 2012). However, an important consideration is that we did not observe spontaneous seizures in our mice. One study found that using pentylenetetrazole, which induces acute seizures and does not result in later spontaneous seizures, only results in temporary hyperactivation of the mTOR pathway (Zhang & Wong, 2012). The investigators injected six-week old Sprague-Dawley rats with PTZ (75 mg/kg) to induce generalized clonic or tonic seizures. They then examined the mTOR pathway at time intervals from 5 minutes to 3 weeks after the seizure onset. There was a significant increase in phosphorylated S6 at 5 min, 30 min, 1h, 3h, 6h, but not at the 16h and later time points in the hippocampus and neocortex. They found similar effects in phosphorylated AKT in both brain regions.

The persistent long-term increase in mTOR activation occurs when there is confirmation of epileptogenesis in the animal model (Zeng et al., 2009). In Zeng et al., 2009 they observed an increase in phosphorylated S6 at 3 days, a peak at days 5-10, then sustained hyperactivation for several weeks. The investigators also observed a significant increase in seizure frequency 6 days after the induction of status epilepticus. The persistent hyperactivation in mTOR was an important contributor to epileptogenesis because inhibition of the mTOR pathway by pretreatment of rapamycin (inhibitor of mTOR) results in a significant suppression of seizures. In our study we induced SE for 30 minutes because of high mortality rates when extending the period to over 1 hour (preliminary results). It may be that a longer duration of status epilepticus is required to result in chronic hyperactivation of mTOR, which would then lead to epileptogenesis. In addition, the increase in mortality due to kainic acid may be unique to the FVB strain that we used for our Pten WT and HT mice. Future studies may use intrahippocampal

injections of kainate, as this method results in epileptogenesis and overall decreases the mortality rate in mice (Shima et al., 2015).

Our study provides support to existing evidence that seizures superimposed on a genetic condition can result in behavioral comorbidities. We found that the Pten haploinsufficient mice with SE were hyperactive and showed some degree of impairment in social behavior. Several studies have used a "two-hit" model to examine how seizure induction during early development can increase susceptibility to seizures and behavioral comorbidities later in life (Hoffmann, Zhao, & Holmes, 2004; Koh, Storey, Santos, Mian, & Cole, 1999). In Hoffman, Zhao, and Holmes (2004) they induced SE by pilocarpine on PD11. The status group then received 25 flurothyl-induced seizures on PD12-16. They found that the group that had both seizure insults were impaired in the MWM test but did not have any histological changes. Koh et al. (1999) induced SE by kainic acid on PD15 and then induced SE again on PD45. The rats with the initial SE insult were more susceptible to seizures in the second hit. The group that received two bouts of SE had increased spatial learning impairments in the MWM. An additional observation in both papers is that there was no overt cellular or structural injury.

Only recently has the "two-hit" model included a genetic deletion along with an induction of seizures. In one study, investigators administered electrical kindling in the Tg2576 mouse of model of Alzheimer's disease (Chan, Jones, Bush, O'Brien, & Kwan, 2015). The rationale behind their experiments is that individuals with Alzheimer's disease are 10 times more likely to develop epilepsy when compared to other age-matched individuals (Hauser, Morris, Heston, & Anderson, 1986). They used the widely used Tg2576 mouse model of Alzheimer's disease for their studies. This mouse model

expresses human amyloid precursor protein with the Swedish mutation (K670N/M671L) driven by the hamster prion protein. The mice show increased Aβ1-42 and Aβ1-40 around 7 months, which is then followed by significant accumulation of insoluble Aβ. By 8 months of age the mice show diffuse and cored amyloid plaques. They found that the Tg2576 mice at 12-14 months are more susceptible to electrically evoked seizures and more vulnerable to kindling. The mice also had a significant increase in mossy fiber sprouting in the dentate gyrus of the hippocampus, which is commonly believed to later lead to epileptogenesis. Even though the authors did not examine behavioral comorbidities, they provide evidence that a "two-hit" exposure to seizures can result in additional neurological deficits. Future studies could further examine the impact of seizures superimposed on genetic mutations in mice that develop spontaneous seizures or demonstrate susceptibility to seizures.

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