ABSTRACT

The Effect of Early-life Status Epilepticus on Ultrasonic Vocalizations in Mice

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The purpose of this study is to investigate the effects of kainate-induced early life seizures on vocalization behavior and intracellular signaling pathways involved in nervous system development. On postnatal day 10, male and female 129SvEvTac mice received a single intraperitoneal injection of kainic acid 2.5 mg/kg to induce 1-2 hours of status epilepticus. On postnatal days 11 and 12, mice were removed from the home cage and isolation-induced ultrasonic vocalizations (USVs) were recorded using the UltraVox software. The PI3K-Akt-mTOR and Canonical Wnt intracellular signaling pathways in the brains of pups on postnatal day 12 were investigated by western blotting analysis. There was significant suppression of USV quantity and total duration at the 50kHz frequency on postnatal day 12 following seizures. These results were both sex-specific and associated with changes in the PI3K-Akt-mTOR intracellular signaling pathway. These findings support the growing body of evidence for USV behavior as an early behavioral marker of neural deficits.

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THE EFFECT OF EARLY-LIFE STATUS EPILEPTICUS ON ULTRASONIC VOCALIZATIONS IN MICE

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CHAPTER ONE

1.1 Introduction

The prevalence rate of neurodevelopmental disorders in the United States recently increased 12.84% to 15.04% in children ages 3-17 from 1997-2008, and is projected to continue[4]. Early intervention has been shown to improve the long-term behavioral outcomes for children with Autism Spectrum Disorder[14], and may provide therapeutic benefit to those with other neurodevelopmental disorders. However, early diagnoses are often missed by newborn disease screenings that are limited to conditions with known and reliable interventions. The U.S. Department of Health and Human Services has recently instituted new recommendations which expand the scope newborn disease screening to include several heritable disorders. Despite these advances, only a small fraction of the affected population is addressed, in part due to a lack of reliable indicators[2]. New diagnostic methods are needed for earlier recognition of neurodevelopmental disorders.

Social communication deficits are commonly identified in children with neurodevelopmental disorders. One form of prelingual communication in humans may be neonatal vocalization behavior. This behavior is characterized as a series of innate calling patterns emitted by neonates, which are intended to orient parental attention towards the fulfillment of specific needs required for optimal growth and survival [26; 33]. Neonatal vocalizations are also highly conserved among many non-human species, including birds, rodents, and primates[30]. Given that neonatal vocalizations are readily

observable, acoustic analysis of these calls may have significant potential as an early behavioral marker for neurodevelopmental disorders.

Due to the strong genetic components involved in neurodevelopmental disorders, many studies have utilized mouse models to investigate neonatal vocalization behavior as a possible early behavior marker of neural deficits[17; 37; 38; 41]. Neonatal mice vocalizations are whistle-like sounds emitted at ultrasonic frequencies between 30kHz—90kHz[5]. Previous studies have shown abnormal ultrasonic vocalizations (USVs) in mouse models of neurodevelopmental syndromes such as Fragile X Syndrome[38], Rett Syndrome[37], Down Syndrome[17], as well as in perturbations of *FoxP2*, a gene highly associated with language development[41]. Though significant progress has been made in understanding this behavior and its viability as an early behavioral marker in the context of genetic neurobehavioral syndromes, very few studies have investigated the effects of early life seizures on USVs[21; 27].

1.1.1 Aim #1

The first aim of this study was to investigate the effect of a single kainate-induced seizure on neonatal vocalization behavior in mice. In mice, seizures during early development are known to result in long-term deficits in social behavior and in learning and memory[6; 29; 35; 42]. In humans, the rate of epilepsy affects approximately 1% of the population according to the CDC (2012)[1] and shares high comorbidities with other neurodevelopmental disorders[36]. Therefore, understanding this relationship has potential to improve the lives of many children. If neonatal vocalization behavior is an early

behavioral marker of neural deficits after neonatal seizures, then we hypothesize vocalizations should be altered in mice after seizures.

1.1.2 Aim #2

This second aim of this study was to examine the influence of early life seizures on two intracellular signaling pathways implicated in early neural development and epileptogenesis. The first targeted pathway was the phosphoinositide 3-kinase|serine/threonine kinase|mammalian target of rapamycin (PI3K-Akt-mTOR) intracellular signaling pathway, which has been well characterized. During early life this pathway supports the developing central nervous system by mediating neuronal survival, as it preserves appropriately wired synaptic connections and prunes aberrant ones[8]. In adulthood this pathway is correlated with cell proliferation, survival, neural plasticity, learning, and memory[15; 16]. The second targeted pathway was the Canonical Wnt intracellular signaling pathway, which is primarily active during pre- and postnatal development. During early development, this pathway supports neurogenesis by mediating embryonic cell proliferation, differentiation, formation of anteroposterior-dorsoventral axes and body patterning[34; 44]. In adulthood the activity of this pathway persists primarily in the neural stem cell microdomains, such as the subventricular zone of the lateral ventricle and subgranular zone of the hippocampal dentate gyrus. In these regions, canonical Wnt signaling maintains the pluripotency of neural stem cells required for adult neurogenesis[25; 43].

Neonatal seizures are known to cause both acute pathological changes in the hippocampus, including extensive cell loss in the CA1 and CA3 subfields, and hilus

region[13]. These seizures are also associated with long-term impairments in hippocampal plasticity, working memory, and excitatory-inhibitory balance[11; 31]. Previous studies indicate that the PI3K-Akt-mTOR and Canonical Wnt intracellular signaling pathways are significantly affected by acute seizures in adult rodents[6; 9; 47], but few similar studies have examined the impact of acute seizures on these pathways during early development[42]. Given the importance of hippocampus in both neurodevelopment and epileptogenesis, we selected this structure as the focus for investigating molecular signaling changes. It is expected that changes in USV behavior may be associated with changes in one or both of these pathways.

CHAPTER TWO

2.1 Materials & Methods

2.1.1 Subjects

Male and female 129 SvEvTac mice (n=62) were generated at Baylor University for this study. All mice were subsequently housed with parents and littermates at an ambient temperature of 22°C, with a 14-hour light and 10-hour dark diurnal cycle. Mice were also given *ad libitum* access to food and water. All procedures involving mice were conducted in compliance with Baylor University Institutional Animal Care and Use Committee and the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

2.1.2 Seizure Induction

On postnatal day 10 (PD10), pups were randomly assigned to seizure and control groups. Pups were then weighed and administered intraperitoneal injections of either 0.5% kainic acid (2.5mg/kg) (Tocris, USA), or equivalent doses of 0.9% physiological saline. Pups from both treatment groups were placed into individual containers with clean bedding, which was kept warm with a heating pad throughout the seizure induction period. The seizure group entered status epilepticus within 30 minutes of kainic acid administration, which was characterized by continuous tonic-clonic seizures persisting for approximately 1-2 hours. This group was then monitored until cessation of all seizure

activity. Upon full recovery, pups from both treatment groups were returned to their home cages.

2.1.3 Ultrasonic Vocalizations

It has previously been shown that pups emit USVs upon separation from their home cages in order to parental signal retrieval[5]. To determine whether mice develop deficits in USV behavior following early life seizures, we examined isolation-induced USV in seizure and control group pups. All cohorts of animals were tested on postnatal days 11 and 12. Prior to testing, mice were placed in a housing pan with clean bedding, which was warmed with a heating pad to ambient nesting temperature. During testing all mice were placed individually into an acrylic, sound-attenuating chamber with USV detectors mounted in each upper corner and set to 50, 60, 70, and 80 kHz (Mini-3 Detector, Ultra Sound Advice, United Kingdom). These frequencies were selected because they collectively span the critical mass of vocalizations emitted by mice. Quantity and average duration of USV emissions at each frequency was measured over a 5-minute period by automatic detection software (Ultravox Software by Noldus, Netherlands). Following testing, mice were placed back into the warm housing pan. After all mice were tested they were returned to their home cage. No more than 6 mice were tested at a time, so time separated from the mother was 35 minutes or less. All groups were tested approximately 24 and 48 hours after receiving injections.

2.1.4 Western Blot Analysis

A separate cohort of mice was used for the western blotting experiments to avoid any effects due to repeated behavioral testing. Mice were sacrificed on postnatal day 12 and hippocampi were rapidly dissected. The resulting samples were then rinsed in 1X phosphate buffer solution, placed on dry ice, and stored at -80°C until used. Both hippocampi were homogenized in ice-cold homogenization buffer (0.32M sucrose, 1mM EDTA, 5mM Hepes) containing protease inhibitor cocktail (P3840, Sigma, USA) and processed for western blotting as previously described[28]. Through this procedure we produced crude synaptosomes and total homogenate samples. Total homogenate samples were used for total Akt, S473-phosphorylated Akt, T308-phosphorlated Akt, total S6, S235/236-phosphorylated S6, S240/244-phosphorylated S6, p70S6k, and FMRP. The crude synaptosomes were used for FMRP, S499-phosphorylated FMRP, GSK3α, GSK3β, Axin1, Dvl2, Dvl3, and Naked2. Equivalent protein concentrations were confirmed using the Bradford Protein Assay (Bio Rad, Hercules, CA, USA) and diluted in Laemmli loading buffer (4X: 0.25M Tris, pH 6.8, 6% SDS, 40% Glycerol, 0.04% Bromophenol Blue, 200mM Dithiothreitol). Following SDS-PAGE, proteins were transferred to Hybond-P polyvinyl difluoride membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were then incubated for 1 hour at room temperature in blocking solution [5% non-fat milk diluted in 1X Tris Buffered Saline (50mM Tris-HCl, pH 7.4, 150mM NaCl) with 0.1% Tween (1X TBS-T) and 1mM Na_3VO_4]. The membranes were then incubated overnight at 4°C with the following primary antibodies in 5% milk in TTBS: Akt, S473-phosphorylated Akt, S6, S235/236-phosphorylated S6, S240/244-phosphorylated S6, p70S6k, FMRP, S499-phosphorylated FMRP, GSK3α GSK3β, Axin1, Dvl2, Dvl3,

Naked2, and actin (Table 2.1). After the incubation period membranes were washed in 1X TTBS (3 x 5min). Membranes were then incubated with horseradish peroxidase labeled secondary antibody, anti-rabbit IgG. Following a final wash cycle in 1X TBS-T, membranes were then incubated with GE ECL Prime (GE Healthcare, Piscataway, NJ, USA). Immunoreactive bands were captured by a ProteinSimple western blot imaging system (ProteinSimple, Santa Clara, CA, USA).

ProteinSimple AlphaView software was then used to measure the optical density of resulting immunoreactive bands. Measurements obtained from all bands of interest were normalized to actin levels within the same lane. All experimental points represent a single mouse each (n=1). All groups were normalized to the average of the control group per blot. All values from control mice represent biological replicates and were collected from control littermates.

Table 2.1. Antibody Specifications

Primary Antibody	Sample	Concentration	Manufacturer	Loading Constant	Pairing	Secondary
Total AKT	Homogenate	1:500	Cell Signaling (# 9272S)	Actin	Together	Rabbit
Phosopho-AKT (S473)	Homogenate	1:500	Cell Signaling (#9271)	Actin	Together	Rabbit/Mouse
Phospho-AKT (T308)	Homogenate	1:250	Cell Signaling (#9275)	Actin	Separate	Rabbit
Total S6	Homogenate	1:500	Cell Signaling (#2217)	Actin	Together	Rabbit
Phospho-S6 (S235/236)	Homogenate	1:500	Cell Signaling (#4858)	Actin	Together	Rabbit
Phospho-S6 (S240/244)	Homogenate	1:500	Cell Signaling (#2215)	Actin	Together	Rabbit
P70 S6 kinase	Homogenate	1:500	Cell Signaling (#2708)	Actin	Separate	Rabbit
GFAP	Homogenate	1:500	NeuroMab (P14136)	Actin	Together	Rabbit/Mouse
FMRP	Synaptosome	1:500	Cell Signaling (#4317)	Actin	Separate	Rabbit
Phospho-FMRP (S499)	Synaptosome	1:500	PhosphoSolutions (p1125-499)	Actin	Together	Rabbit
GSK3α	Synaptosome	1:500	Cell Signaling (#5676)	Mortalin	Together	Rabbit/Mouse
GSK3β	Synaptosome	1:500	Cell Signaling (#5676)	Mortalin	Together	Rabbit/Mouse
Axin1	Synaptosome	1:500	Cell Signaling (#2087)	Actin	Separate	Rabbit
Dvl2	Synaptosome	1:500	Cell Signaling (#3216)	Actin	Separate	Rabbit
Dvl3	Synaptosome	1:500	Cell Signaling (#3218)	Actin	Separate	Rabbit
Naked2	Synaptosome	1:500	Cell Signaling (#2073)	Actin	Together	Rabbit
LRP6	Synaptosome	1:500	Cell Signaling (#2560)	Mortalin	Separate	Rabbit/Mouse
Phospho-LRP6 (S1490)	Synaptosome	1:500	Cell Signaling (#2568)	Mortalin	Separate	Rabbit/Mouse
Wnt5a/b	Synaptosome	1:500	Cell Signaling (#2530)	Mortalin	Separate	Rabbit/Mouse

2.1.5 Statistical Analyses

All data were analyzed by using SPSS 21.0 for PC (SPSS, Chicago, IL) or by Graphpad Prism 6 for PC (San Diego, CA). For all comparisons, the level of significance was set at $p < 0.05$. We used a two-way repeated-measures design for the ultrasonic vocalization day 11 and 12 data. We examined main effects and interactions. If we found an interaction we then performed an independent samples t-test on each day or a repeated measures t-test per group across days. In order to keep the results section manageable we only included significant statistical effects.

CHAPTER THREE

3.1 Results

3.1.1 Suppression of ultrasonic vocalization behavior after seizures

We first examined whether seizures elicited changes in the quantity of USVs at each frequency. In our evaluation we did not find a main group effect for change in the quantity of 50kHz calls from PD11 to 12 between groups $F(1,58) = 0.187$, $p = 0.67$ (Figure 3.1A-B). However, there was a significant interaction between both time and group $F(1, 58) = 4.1$, $p < 0.05$, as well as time x group x sex for the quantity of 50kHz calls $F(1,58) = 6.5$, $p < 0.05$. An independent samples t-test on PD11 found no significant differences between the groups $t(1,60) = 0.9$, $p = 0.37$. However, comparisons on PD12 revealed a marginal suppression of 50kHz calls in the seizure group $t(1,60) = 2.0$, $p = 0.054$, with seizure pups vocalizing fewer times than control pups. We found no other significant main effects or interactions at any other call frequency between the seizure and control groups.

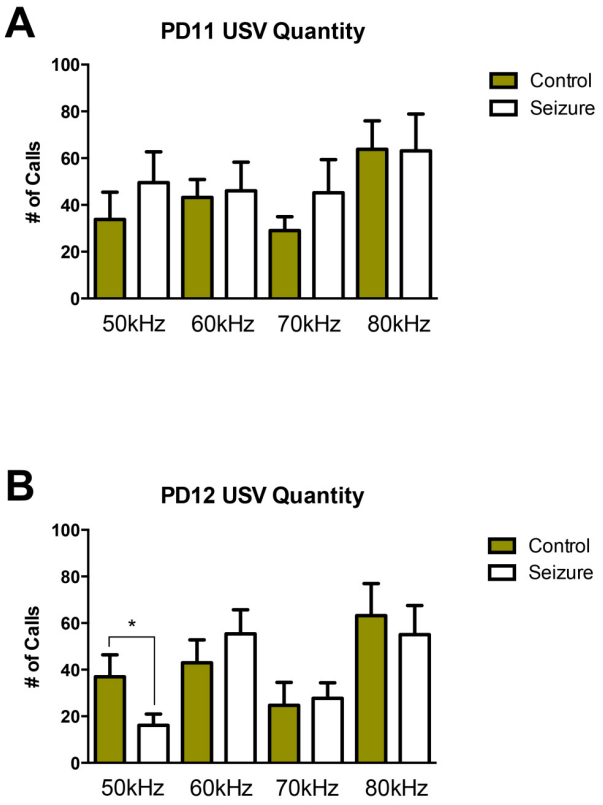

Figure 3.1. The effect of status epilepticus on ultrasonic vocalization quantity on postnatal days 11 and 12. (A) Seizures on PD10 do not affect USV quantity on PD11, **(B)** but cause suppression of USV quantity at the 50kHz frequency on PD12. * = $p < 0.05$. Bars represent the mean and the error bars represent the standard error of the mean.

We then examined whether seizures elicited changes in the mean and total duration of USVs at each frequency. We did not find a main effect of group or sex from PD11 to 12. However, there was a significant interaction of time and group $F(1,58) = 5.1$, $p < 0.05$ for the total duration of 50kHz calls (Figure 3.2D). Upon separating the postnatal days to investigate this interaction, we found no main effect of seizure-induction on the mean or total duration of USVs at any frequency on PD11 (Figure 3.2A-B). However, comparisons on PD12 revealed a significant difference between groups on

PD12 $t(1,60) = 2.26$, $p < 0.05$, with seizure pups spending less total time vocalizing than control pups (Figure 3.2D). There were no other significant main effects or interactions at any other call frequency between the seizure and control groups (Figure 3.2C-D). These results suggest that early life seizures suppress neonatal vocalization behavior, with the strongest effect observable in 50kHz calls on PD12.

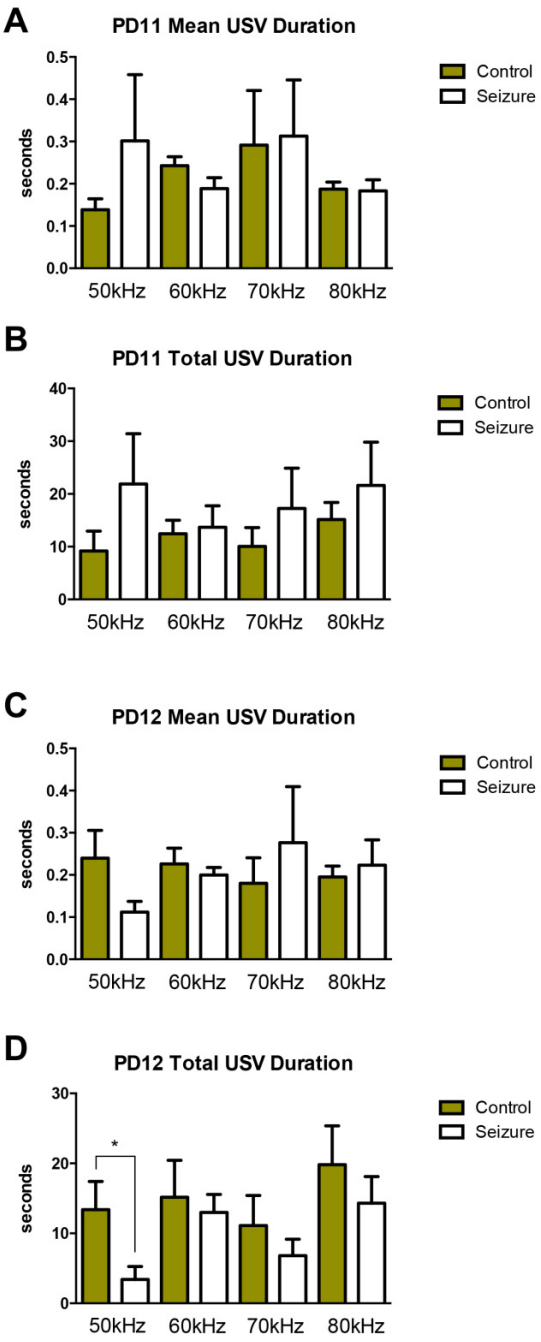

Figure 3.2. The effect of status epilepticus on ultrasonic vocalization mean duration and total duration on postnatal days 11 and 12. (A-B) Seizures on PD10 do not affect the mean or total duration of USVs on PD11, **(C)** nor affect mean duration of USVs on PD12. **(D)** However, they do lead to suppression of total duration of USVs at the 50kHz frequency on PD12. * = $p < 0.05$. Bars represent the mean and the error bars represent the standard error of the mean.

3.1.2 Sex-specific suppression of ultrasonic vocalization behavior after seizures

We next wanted to determine whether the seizure-induced USV suppression was equivalent between sexes. Since we initially found a significant group x time x sex interaction, we analyzed the total quantity of USVs emitted at each frequency by sex. There was a significant interaction between time and group only in males $F(1,28) = 8.3$, $p < 0.01$ (Figure 3.3C). Upon separating the postnatal days to investigate this interaction we found significant suppression in the quantity of 50kHz calls in seizure group males PD12 $t(1,28) = 2.4$, $p < 0.05$, with male seizure pups vocalizing fewer times than male control pups. A repeated measures t-test also revealed a significant decrease in the quantity of 50kHz calls from PD11 to 12 in seizure group males $t(1,17) = 2.6$, $p < 0.05$. There were no significant changes in USV quantity for male seizure or control animals at any other call frequency. There were also no significant changes in USV quantity for female seizure or control animals at any call frequency.

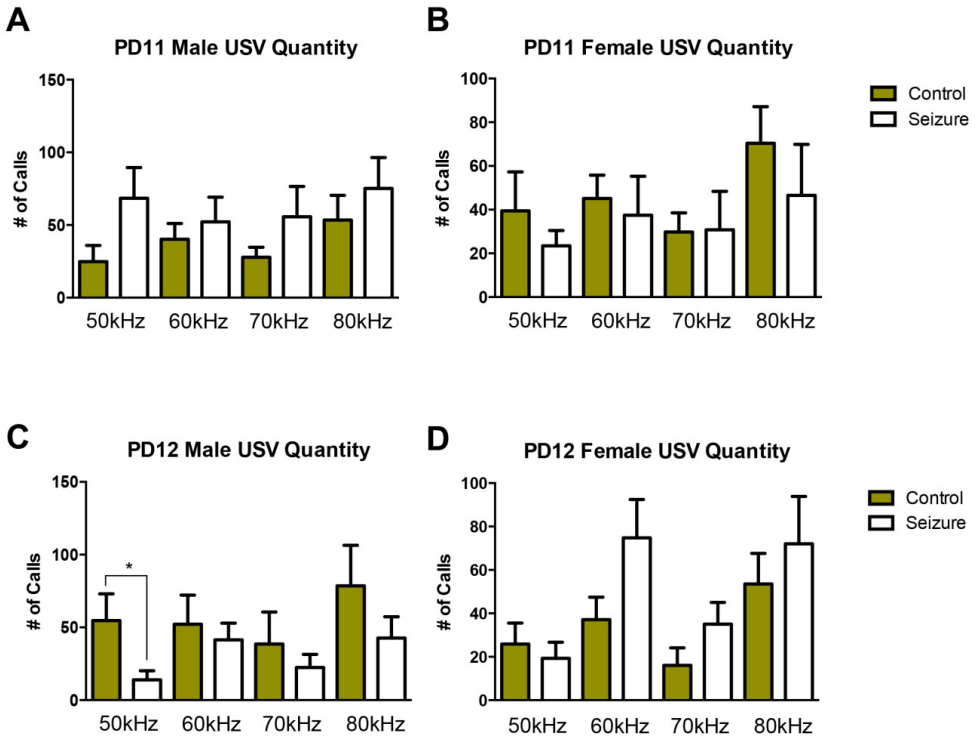

Figure 3.3. The sex-specific effect of status epilepticus on ultrasonic vocalization quantity on postnatal days 11 and 12. (A-B) Seizures on PD10 do not affect the USV quantity in males or females on PD11. **(C)** However, there was large suppression of USVs quantity at the 50kHz frequency in males on PD12. **(D)** USV quantity was not affected in females on PD12. * = $p < 0.05$. Bars represent the mean and the error bars represent the standard error of the mean.

There was no main effect of seizure-induction on the mean or total duration of USVs for male or female pups at any frequency on PD11 (Figure 3.4A-D). On PD12 there was no main effect of seizure-induction on the mean USV duration for male or female pups at any frequency (Figure 3.4E-F). However, comparisons did reveal a suppression in the total duration of 50kHz calls only in male pups $t(1,28) = 2.4$, $p < 0.05$, with seizure pups spending less total time vocalizing than control pups (Figure 4G). There were no significant changes in total USV duration for male seizure or control

animals at any other frequency. There were also no significant changes in total USV duration for female seizure or control animals at any frequency on PD12 (Figure 3.4H). These results suggest that males display an increased sensitivity to seizure-induced suppression of neonatal vocalization behavior.

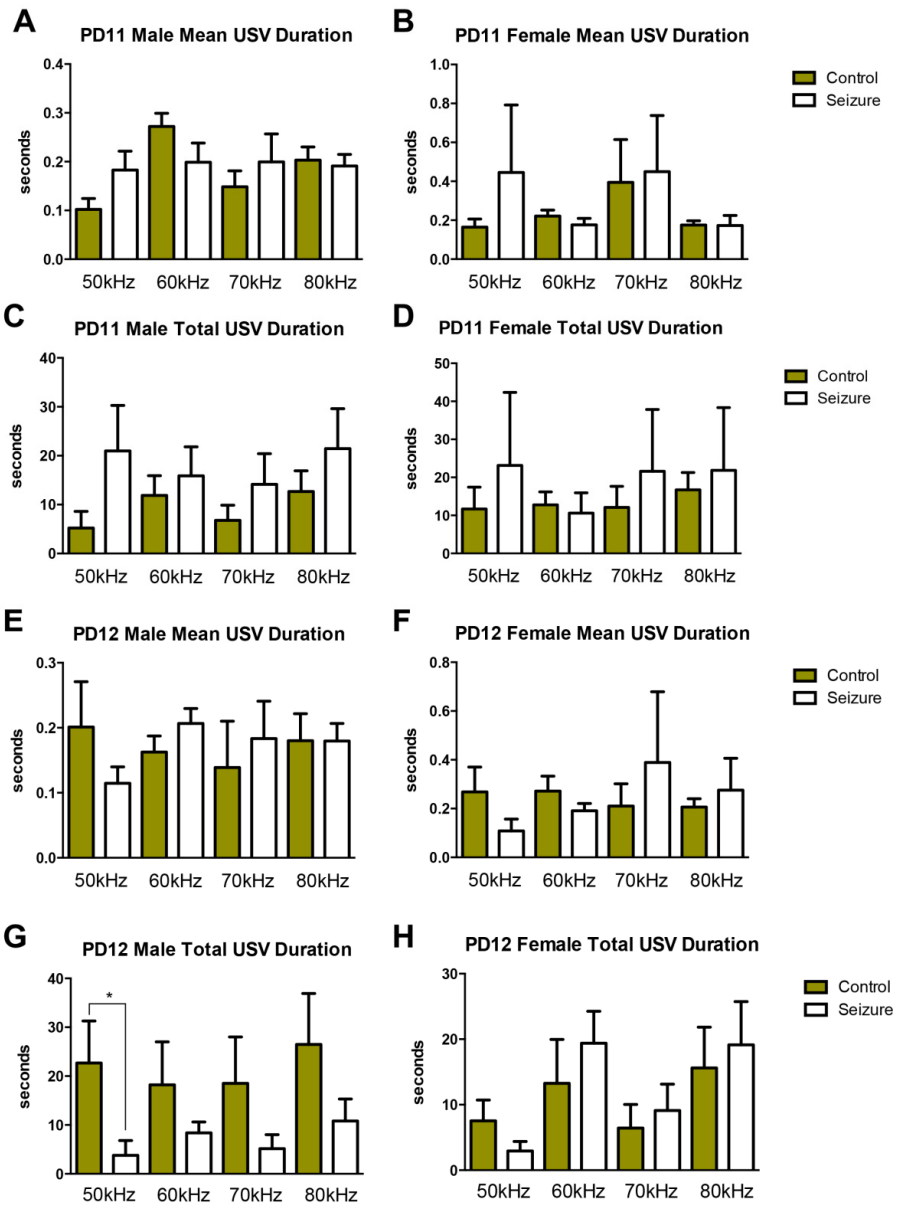

Figure 3.4. The sex specific effect of status epilepticus on ultrasonic vocalization mean duration and total duration on postnatal days 11 and 12. (A-D) Seizures on PD10 do not affect the mean or total duration of USVs in males or females on PD11, **(E-F)** nor affect mean duration of USVs in males or females on PD12. **(G)** However, there was a large suppression of total duration of USVs at the 50kHz frequency in males on PD12. **(H)** Total duration of USVs were not affected in females on PD12. * = $p < 0.05$. Bars represent the mean and the error bars represent the standard error of the mean.

3.1.3 Seizure-induced suppression of neonatal vocalization behavior is associated with sex-specific changes in PI3K-Akt-mTOR signaling

Because changes in USV behavior were only observed on PD12, only this time point was selected for subsequent western blot analysis of hippocampal tissue. In our comparison of the PI3K-Akt-mTOR pathway between these groups, we found no differences in total Akt, S473-phosphorylated Akt, the ratio of S473-phosphorylated Akt/ total Akt, T308-phosphorylated Akt or T308-phosphorylated Akt/ total Akt in either sex (Table 3.1 & Table 3.2). We also found no differences in total S6, S240/244-phosphorylated S6, the ratio of S240/244-phosphorylated S6/ total S6, or 235/236-phosphorylated S6 in either sex (Table 3.1 & Table 3.2). However, there was a significant increase in the ratio of S235/236-phosphorylated S6/ total S6 in males $t(1,16)=2.367$, $p<0.05$, with seizure pups expressing a much larger ratio than control pups (Figure 3.5A & Table 3.1). This effect was not reflected in female pups. (Table 3.2) We also found no difference in total FMRP in either sex (Table 3.1 & Table 3.2), but observed a male-specific suppression of S499-phosphorylated FMRP $t(1,16)=2.34$, $p< 0.05$ and the ratio of S499-phosphorylated FMRP/total FMRP ratio $t(1,16)=2.18$, $p< 0.05$ in seizure pups compared to control pups (Figure 3.5B-C & Table 3.1). This effect was also not reflected in female pups (Table 3.2).

In our evaluation of the Canonical Wnt pathway in PD12 male mice, we found no changes in Axin1, Dvl2, Dvl3, GSK3a, GSK3b, total LRP6, S1490-phosphorylated LRP6, the ratio of S1490-phosphorylated LRP6/ total LRP6, Wnt5a/b, or Naked2 in either sex (Table 3.1 & Table 3.2).

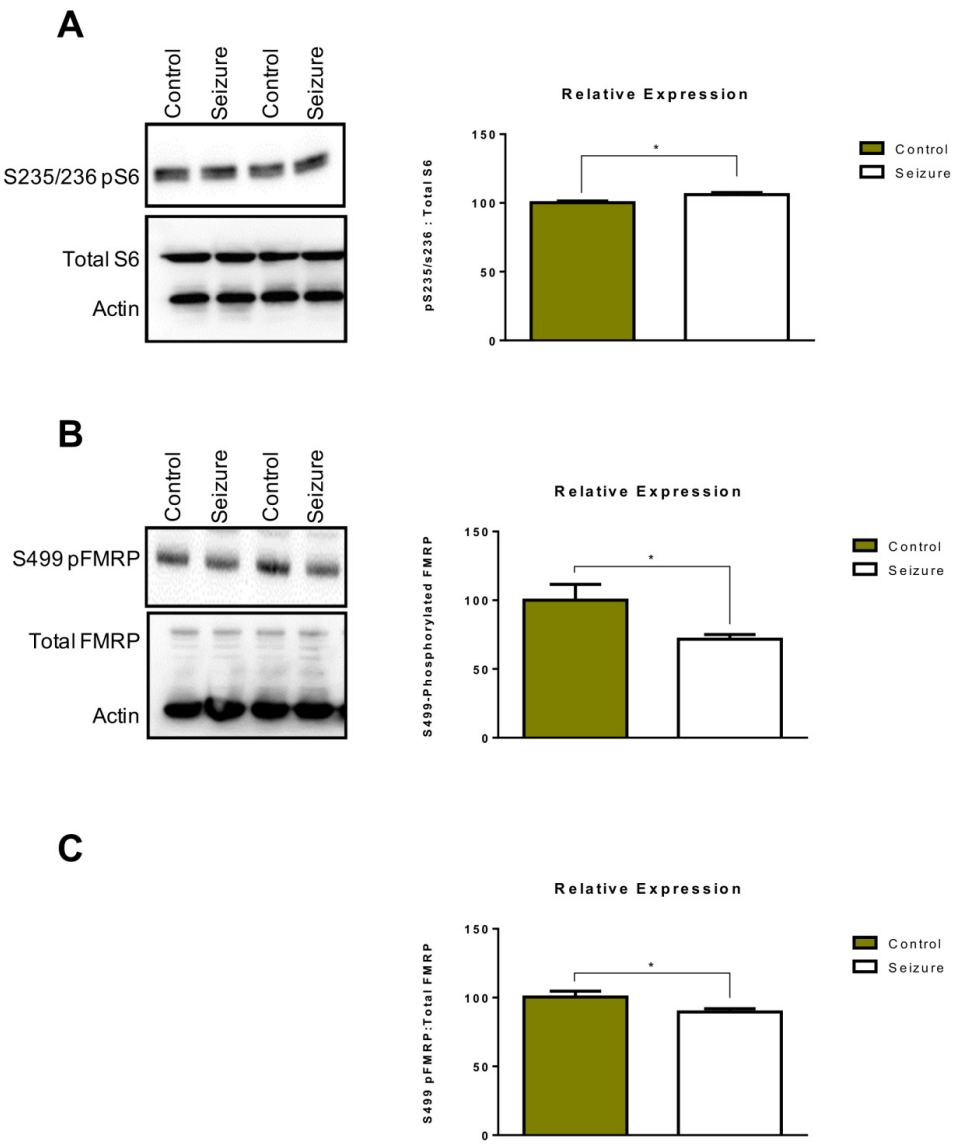

Figure 3.5. The effect of status epilepticus on phosphorylated Serine S6 kinase, phosphorylated FMRP, and total FMRP on postnatal days 12 in the hippocampus. Seizures on PD10 lead to suppression of **(A)** the ratio of S235/236-phosphorylated S6/ total S6 **(B)** S499-phosphorylated FMRP, and **(C)** the ratio of S499-phosphorylated FMRP/total FMRP.

Table 3.1 Western Blotting Analysis Summary for Males

Protein	Control	Seizure	Change	Significance
Total AKT	100.0 ± 3.146, n=9	96.28 ± 4.916, n=9	-	-
Phospho-AKT (S473)	100.0 ± 5.706, n=9	99.37 ± 5.249, n=9	-	-
% Total Phospho-AKT (S473)	99.50 ± 3.554, n=9	104.1 ± 5.331, n=9	-	-
Total AKT	100.0 ± 4.053, n=9	100.0 ± 4.305, n=9	-	-
Phospho-AKT (T308)	100.0 ± 12.68, n=9	92.57 ± 9.266, n=9	-	-
% Total Phospho-AKT (T308)	100.4 ± 12.50, n=9	92.29 ± 8.006, n=9	-	-
Total S6	100.0 ± 3.225, n=9	96.64 ± 2.386, n=9	-	-
Phospho-S6 (S235/236)	100.0 ± 3.105, n=9	102.4 ± 2.699, n=9	-	-
% Total Phospho-S6 (S235/236)	100.1 ± 1.674, n=9	106.1 ± 1.886, n=9	↑	*
Total S6	100.0 ± 3.883, n=9	98.38 ± 1.681, n=9	-	-
Phospho-S6 (S240/244)	100.0 ± 3.624, n=9	98.60 ± 1.892, n=9	-	-
% Total Phospho-S6 (S240/244)	100.1 ± 0.5248, n=9	100.2 ± 0.8164, n=9	-	-
P70 S6 kinase	100.0 ± 15.94, n=9	80.63 ± 13.41, n=9	-	-
GFAP	100.0 ± 2.371, n=9	103.5 ± 4.328, n=9	-	-
FMRP	100.0 ± 10.98, n=9	80.50 ± 4.724, n=9	-	-
Phospho-FMRP (S499)	100.0 ± 11.61, n=9	71.64 ± 3.478, n=9	↓	*
% Total Phospho-FMRP (S499)	100.4 ± 4.350, n=9	89.56 ± 2.393, n=9	↓	*
GSK3α	100.0 ± 24.03, n=9	104.1 ± 37.28, n=9	-	-
GSK3β	100.0 ± 28.75, n=9	95.33 ± 38.64, n=9	-	-
Axin1	100.0 ± 17.43, n=9	98.24 ± 21.48, n=9	-	-
Dvl2	100.0 ± 4.389, n=9	100.4 ± 8.847, n=9	-	-
Dvl3	100.0 ± 4.733, n=9	100.7 ± 6.201, n=9	-	-
Naked2	100.0 ± 10.37, n=9	106.1 ± 10.81, n=9	-	-
LRP6	100.0 ± 20.76, n=9	109.3 ± 19.95, n=9	-	-
Phospho-LRP6 (S1490)	100.0 ± 12.94, n=9	90.14 ± 12.33, n=9	-	-
% Total Phospho-LRP6 (S1490)	214.7 ± 84.29, n=9	107.5 ± 21.01, n=9	-	-
Wnt5a/b	100.0 ± 4.630, n=9	98.58 ± 10.13, n=9	-	-

Table 3.2 Western Blotting Analysis Summary for Females

Protein	Control	Seizure	Change	Significance
Total AKT	100.0 ± 5.646, n=9	108.8 ± 8.535, n=9	-	-
Phospho-AKT (S473)	100.0 ± 5.148, n=9	106.3 ± 8.542, n=9	-	-
% Total Phospho-AKT (S473)	102.3 ± 7.722, n=9	100.2 ± 8.807, n=9	-	-
Total AKT	100.0 ± 7.406, n=9	105.0 ± 18.99, n=9	-	-
Phospho-AKT (T308)	100.0 ± 35.99, n=9	86.74 ± 29.04, n=9	-	-
% Total Phospho-AKT (T308)	102.5 ± 37.29, n=9	87.18 ± 23.03, n=9	-	-
Total S6	100.0 ± 3.485, n=9	100.7 ± 4.994, n=9	-	-
Phospho-S6 (S235/236)	100.0 ± 3.615, n=9	97.59 ± 3.592, n=9	-	-
% Total Phospho-S6 (S235/236)	101.4 ± 6.052, n=9	98.77 ± 5.632, n=9	-	-
Total S6	100.0 ± 3.485, n=9	100.7 ± 4.994, n=9	-	-
Phospho-S6 (S240/244)	100.0 ± 1.377, n=9	101.5 ± 2.175, n=9	-	-
% Total Phospho-S6 (S240/244)	100.9 ± 3.367, n=9	103.4 ± 6.665, n=9	-	-
P70 S6 kinase	100.0 ± 9.935, n=9	113.0 ± 12.04, n=9	-	-
GFAP	100.0 ± 6.518, n=9	100.8 ± 6.688, n=9	-	-
FMRP	100.0 ± 13.18, n=9	88.82 ± 15.80, n=9	-	-
Phospho-FMRP (S499)	100.0 ± 9.856, n=9	101.7 ± 10.22, n=9	-	-
% Total Phospho-FMRP (S499)	105.3 ± 7.169, n=9	160.8 ± 39.61, n=9	-	-
GSK3α	100.0 ± 6.486, n=9	93.16 ± 7.322, n=9	-	-
GSK3β	100.0 ± 7.836, n=9	95.66 ± 8.770, n=9	-	-
Axin1	100.0 ± 6.683, n=9	92.40 ± 8.053, n=9	-	-
Dvl2	100.0 ± 9.099, n=9	100.7 ± 7.136, n=9	-	-
Dvl3	100.0 ± 8.009, n=9	85.93 ± 7.202, n=9	-	-
Naked2	100.0 ± 12.22, n=9	81.88 ± 8.164, n=9	-	-
LRP6	100.0 ± 20.76, n=9	109.3 ± 19.95, n=9	-	-
Phospho-LRP6 (S1490)	100.0 ± 9.782, n=9	97.69 ± 12.77, n=9	-	-
% Total Phospho-LRP6 (S1490)	99.67 ± 6.788, n=9	98.62 ± 6.244, n=9	-	-
Wnt5a/b	100.0 ± 26.27, n=9	100.3 ± 30.42, n=9	-	-

CHAPTER FOUR

4.1 Discussion

Though there has been significant progress in understanding neonatal USVs in the context of neurodevelopmental disorders, there has been little investigation into whether they are effected by early life seizures. The objective of the present study was to determine the effect of one episode of kainate-induced status epilepticus on ultrasonic vocalization behavior in neonatal 129 SvEvTac mice. Further, given that the effect of neonatal seizures on neurodevelopmental protein signaling is poorly understood, we also sought to characterize changes in several components of the PI3K-Akt-mTOR and Canonical Wnt signaling pathways. Through a series of experiments it was demonstrated that seizures on PD10 lead to sex-specific suppression of neonatal vocalization behavior at the 50-kHz frequency. This effect was found to be associated with changes in specific components of the PI3K-Akt-mTOR pathway, but not the Canonical Wnt pathway.

There are very few pre-existing studies that examine neonatal USV behavior after seizures. One previous study by López-Meraz et al (2014)[27] used pilocarpine-induced status epilepticus on PD14 Wistar rats. Although pilocarpine and kainic acid diverge in their mechanisms of eliciting status epilepticus in the brain[12; 24], previous studies indicate that they produce seizures of similar severity[18]. In isolation-induced USVs on PD15, these authors observed a similar suppression of USV duration and a decrease in latency to vocalize. Interestingly, they also found decrease in USV latency only in male rats after seizures. Similar sex-specific effects were found in a separate study where investigators induced febrile seizures on PD7 they found an increase in USVs in male rats on PD 12 [21].

Even though the seizure induction methods were different across all three studies, all studies have found that males USVs are altered after early life seizures.

The cause for differential effects of seizures between sexes in these three studies is unclear. The results of these studies are in line with several population-based epidemiological studies which suggest the incidence of seizures is greater in males[19; 32]. It has been proposed that sex hormones may play a key role in the incidence and outcome of seizures; however the exact mechanisms have yet to be elucidated.

The functional significance of 50-kHz calls has not been extensively investigated in mice, or other rodents during the neonatal periods. However, these calls are well characterized in previous studies using rat models. In adolescence and adulthood, these calls are associated with the anticipation or presence of pleasurable stimuli, as well as a variety of social contexts[7; 22; 45; 46]. In a previous study, Wöhr et al (2008)[45] recorded the ultrasonic vocalization behavior of 12 week old Wistar rats. These animals selectively emitted 50-kHz calls when isolated from cage mates into a separate housing pan, as well as during open field and elevated plus maze testing. Given that rats produced 50-kHz calls exclusively when separated from cage mates, Wöhr et al (2008)[45] posit that such calls must serve a social-coordinating function. In support of this hypothesis, another study by Wohr et al. (2007)[46] demonstrated that playback of 50-kHz calls induce behavioral activation and increased exploratory behavior in the radial arm maze test. These animals were also observed to emit 50-kHz calls during intervals between playbacks, even further suggesting their role in social coordination behavior. Although the functional significance of 50-kHz calls during the neonatal period has not yet been established, it may be reasonable to conclude that calls at this frequency are essential for

inducing parental-orienting behavior. In this case, 50-kHz calls would be essential in communicative function neonatal vocalization behavior.

Previous studies indicate that early life seizures lead to long-term deficits in social behavior [6; 29; 35; 42]. The present study found that seizures on PD10 lead to sex-specific suppression of neonatal vocalization behavior. It is possible that these results reflect an early detection of acute impairments in social behavior. Future studies may seek to correlate seizure-induced suppression of neonatal vocalizations with long-term changes in social behavior.

The present study found changes in specific components of the PI3K-Akt-mTOR pathway to be associated with suppression of USV behavior in male mice after PD10 seizures, namely S235/236-phosphorylated S6 and S499-phosphorylated FMRP. The S6 ribosomal protein is a component of the small 40S ribosomal subunit. In conjunction with the large 60S subunit, these compose the intracellular ribosome machinery necessary for all protein synthesis[39]. The function of the small 40S subunit is highly regulated by the phosphorylation status of the S6 ribosomal protein. When phosphorylated, this protein influences transcriptional processes which generate proteins to regulate cell cycle progression, as well as new ribosomal proteins and elongation factors to aid in transcription[20]. Although we did not find changes in overall levels of total or phosphorylated S6, there was a significantly elevated ratio of S235/236-phosphorylated S6/ total S6. This would seem to indicate a qualitative, rather than quantitative, change in overall ribosomal function. Further supporting this assumption, we also found suppressed levels of S499-phosphorylated FMRP, a phospho-specific protein known to antagonize ribosomal function by degrading target mRNA[23]. These changes were

corroborated by Talos et al. (2012)[42] and Bernard et al (2013)[3], who induced kainic acid seizures in rats during neonatal development. Given that seizures are known to cause widespread damage across multiple brain areas[12; 24], we hypothesize that this elevated ribosomal function may indicate a compensatory role in neuroregenerative processes.

We did not observe changes in Canonical Wnt signaling following neonatal seizures. This finding is in contrast with a previous study by Busceti et al. (2007)[10], which found acute kainic acid-induced seizures in Sprague-Dawley rats to cause elevation of Dkk-1, an inhibitor of the Canonical Wnt signaling pathway. The divergence in results obtained between this study and ours may be due to seizure induction at different time periods. Busceti et al. (2007) used rats that were 150-170 g. In addition, they found the increase in Dkk-1 only in the hippocampus of rats that had neuronal damage. They did not find a change in Dkk-1 in "low" responders, which were rats that had received kainate but did not have neuronal damage. Unfortunately, there is a lack of previous studies inducing early life seizures and observing the Canonical Wnt signaling pathway. Future studies could include induction of seizures at multiple developmental time points, as well as utilize different chemoconvulsant agents.

Taken together the results of this study confirm the hypothesis that early life seizures cause sex-specific suppression of USV behavior in neonatal mice, and suggest this effect may be secondary to changes in PI3K-Akt-mTOR signaling proteins. These findings support the growing body of evidence that USV behavior may serve as an early behavioral marker of neurodevelopmental disorders. Future directions for neonatal USV behavior will involve much more detailed qualitative analyses of acoustic parameters, using spectrographic techniques to determine specific call-type deficits. Several recent

studies conducting spectrographic analyses of neonatal USVs have observed altered calling patterns in models of neurodevelopmental disorders[38; 40]. However, none of these studies have correlated their results with subsequent long-term behavioral analyses. Such studies could identify whether specific acoustic parameters of neonatal vocalization behavior are reliable, early predictors of neurodevelopmental disorder onset. This technology would enhance existing newborn disease screening protocols, and aid clinicians in earlier diagnoses for the growing population of children afflicted with these disorders.

WORKS REFERENCED

1. Prevalence of autism spectrum disorders--Autism and Developmental Disabilities Monitoring Network, 14 sites, United States, 2008. *MMWR Surveill Summ* 2012;61:1-19.

2. ACHDNC. Recommended Uniform Screening Panel Core Conditions. U.S. Department of Health and Human Services; 2015.

3. Bernard PB, Castano AM, O'Leary H, et al. Phosphorylation of FMRP and alterations of FMRP complex underlie enhanced mLTD in adult rats triggered by early life seizures. *Neurobiology of Disease* 2013;59:1-17.

4. Boyle CA, Boulet S, Schieve LA, et al. Trends in the prevalence of developmental disabilities in US children, 1997-2008. *Pediatrics* 2011;127:1034-1042.

5. Branchi I, Santucci D, Alleva E. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. *Behav Brain Res* 2001;125:49-56.

6. Brewster AL, Lugo JN, Patil VV, et al. Rapamycin reverses status epilepticus-induced memory deficits and dendritic damage. *PLoS One* 2013;8:e57808.

7. Brudzynski SM. Ultrasonic calls of rats as indicator variables of negative or positive states: Acetylcholine–dopamine interaction and acoustic coding. *Behav Brain Res* 2007;182:261-273.

8. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* 2001;11:297-305.

9. Busceti CL, Biagioni F, Aronica E, et al. Induction of the Wnt inhibitor, Dickkopf-1, is associated with neurodegeneration related to temporal lobe epilepsy. *Epilepsia* 2007;48:694-705.

10. Busceti CL, Biagioni F, Aronica E, et al. Induction of the Wnt Inhibitor, Dickkopf-1, Is Associated with Neurodegeneration Related to Temporal Lobe Epilepsy. *Epilepsia* 2007;48:694-705.

11. Cornejo BJ, Mesches Mh Fau - Coultrap S, Coultrap S Fau - Browning MD, et al. A single episode of neonatal seizures permanently alters glutamatergic synapses. *Annals of Neurology* 2007;61:411-426.

12. Curia G, Longo D, Biagini G, et al. The pilocarpine model of temporal lobe epilepsy. *J Neurosci Methods* 2008;172:143-157.

13. Dunleavy M, Shinoda S, Schindler C, et al. Experimental Neonatal Status Epilepticus and the Development of Temporal Lobe Epilepsy with Unilateral Hippocampal Sclerosis. *The American Journal of Pathology* 2010;176:330-342.

14. Eikeseth S, Klintwall L, Jahr E, et al. Outcome for children with autism receiving early and intensive behavioral intervention in mainstream preschool and kindergarten settings. *Research in Autism Spectrum Disorders* 2012;6:829-835.

15. Graber TE, McCamphill PK, Sossin WS. A recollection of mTOR signaling in learning and memory. *Learn Mem* 2013;20:518-530.

16. Hoeffer CA, Klann E. mTOR signaling: at the crossroads of plasticity, memory and disease. *Trends Neurosci* 2010;33:67-75.

17. Holtzman DM, Santucci D, Kilbridge J, et al. Developmental abnormalities and age-related neurodegeneration in a mouse model of Down syndrome. *Proc Natl Acad Sci U S A* 1996;93:13333-13338.

18. Inostroza M, Cid E, Brotons-Mas J, et al. Hippocampal-dependent spatial memory in the water maze is preserved in an experimental model of temporal lobe epilepsy in rats. *PLoS One* 2011;6:e22372.

19. Jallon P, Smadja D, Cabre P, et al. EPIMART: prospective incidence study of epileptic seizures in newly referred patients in a French Carribean island (Martinique). *Epilepsia* 1999;40:1103-1109.

20. Jefferies HB, Fumagalli S, Dennis PB, et al. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *Embo j* 1997;16:3693-3704.

21. Keller A, Saucier D, Sheerin A, et al. Febrile convulsions affect ultrasonic vocalizations in the rat pup. *Epilepsy Behav* 2004;5:649-654.

22. Knutson B, Burgdorf J, Panksepp J. Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *Journal of Comparative Psychology* 1998;112:65-73.

23. Laggerbauer B, Ostareck D, Keidel EM, et al. Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum Mol Genet* 2001;10:329-338.

24. Levesque M, Avoli M. The kainic acid model of temporal lobe epilepsy. *Neurosci Biobehav Rev* 2013;37:2887-2899.

25. Lie DC, Colamarino SA, Song HJ, et al. Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 2005;437:1370-1375.

26. Lingle S, Wyman MT, Kotrba R, et al. What makes a cry a cry? A review of infant distress vocalizations. *Current Zoology* 2012;58:698-726.

27. Lopez-Meraz ML, Medel-Matus JS, Morgado-Valle C, et al. Effect of lithium-pilocarpine-induced status epilepticus on ultrasonic vocalizations in the infant rat pup. *Epilepsy Behav* 2014;31:263-266.

28. Lugo JN, Barnwell LF, Ren Y, et al. Altered phosphorylation and localization of the A-type channel, Kv4.2 in status epilepticus. *J Neurochem* 2008;106:1929-1940.

29. Lugo JN, Swann JW, Anderson AE. Early-life seizures result in deficits in social behavior and learning. *Experimental Neurology* 2014;256:74-80.

30. Lummaa V, Vuorisalo T, Barr RG, et al. Why Cry? Adaptive Significance of Intensive Crying in Human Infants. *Evolution and Human Behavior* 1998;19:193-202.

31. Lynch M, Sayin U Fau - Bownds J, Bownds J Fau - Janumpalli S, et al. Long-term consequences of early postnatal seizures on hippocampal learning and plasticity. *The European Journal of Neuroscience* 2000;12:2252-2264.

32. McHugh JC, Delanty N. Epidemiology and classification of epilepsy: gender comparisons. *Int Rev Neurobiol* 2008;83:11-26.

33. Michelsson K, Michelsson O. Phonation in the newborn, infant cry. *Int J Pediatr Otorhinolaryngol* 1999;49 Suppl 1:S297-301.

34. Niehrs C. On growth and form: a Cartesian coordinate system of Wnt and BMP signaling specifies bilaterian body axes. *Development* 2010;137:845-857.

35. Nishimura M, Gu X Fau - Swann JW, Swann JW. Seizures in early life suppress hippocampal dendrite growth while impairing spatial learning. *Neurobiology of Disease* 2011;44:205-214.

36. Persad V, Thompson MD, Percy ME. Epilepsy and Developmental Disability Part I: Developmental Disorders in Which Epilepsy May be Comorbid. *Journal on Developmental Disabilities* 2003;10:123-152.

37. Picker JD, Yang R, Ricceri L, et al. An altered neonatal behavioral phenotype in Mecp2 mutant mice. *Neuroreport* 2006;17:541-544.

38. Roy S, Watkins N, Heck D. Comprehensive analysis of ultrasonic vocalizations in a mouse model of fragile X syndrome reveals limited, call type specific deficits. *PLoS One* 2012;7:e44816.

39. Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem Sci* 2006;31:342-348.

40. Scattoni ML, Gandhy SU, Ricceri L, et al. Unusual Repertoire of Vocalizations in the BTBR T+tf/J Mouse Model of Autism. *PLoS One* 2008;3.

41. Shu W, Cho JY, Jiang Y, et al. Altered ultrasonic vocalization in mice with a disruption in the Foxp2 gene. *Proc Natl Acad Sci U S A* 2005;102:9643-9648.

42. Talos DM, Sun H, Zhou X, et al. The Interaction between Early Life Epilepsy and Autistic-Like Behavioral Consequences: A Role for the Mammalian Target of Rapamycin (mTOR) Pathway. *PLoS ONE* 2012;7:e35885.

43. Varela-Nallar L, Inestrosa NC. Wnt signaling in the regulation of adult hippocampal neurogenesis. *Front Cell Neurosci* 2013;7:100.

44. Wang J, Sinha T, Wynshaw-Boris A. Wnt signaling in mammalian development: lessons from mouse genetics. *Cold Spring Harb Perspect Biol* 2012;4.

45. Wöhr M, Houx B, Schwarting RKW, et al. Effects of experience and context on 50-kHz vocalizations in rats. *Physiology & Behavior* 2008;93:766-776.

46. Wöhr M, Schwarting RKW. Ultrasonic Communication in Rats: Can Playback of 50-kHz Calls Induce Approach Behavior? *PLoS One* 2007;2:e1365.

47. Zeng LH, Rensing NR, Wong M. The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. *J Neurosci* 2009;29:6964-6972.