ABSTRACT

Impact of Neuroinflammatory and mTOR Signaling Inhibition Following Flurothyl Seizures on the Acute Development of Autistic-like Behavior in C57BL/6 Mice

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Epilepsy is a common neurological disorder, with individuals having an increased susceptibility of seizures in the first few years of life. Children with epilepsy are at risk of developing a multitude of cognitive and behavioral comorbidities throughout development. In addition, approximately one third of individuals with epilepsy are resistant to anti-seizure drugs, emphasizing the need for therapeutics that extend beyond manipulation of neuronal transmission and target alternative mechanisms. The present study examined the role of PI3K/Akt/mTOR pathway activity and neuroinflammatory signaling in the development of autistic-like behavior following seizures in the neonatal period. Male and female C57BL/6 mice were administered 3 flurothyl seizures on postnatal (PD) 10, followed by administration of minocycline, the mTOR inhibitor rapamycin, or a combined treatment of both therapeutics. On PD12, isolation-induced ultrasonic vocalizations (USVs) of mice were examined to determine the impact of seizures and treatment on communicative behaviors, a component of the autistic-like phenotype. Hippocampal tissue was collected on PD12 to examine proinflammatory

cytokine expression with qRT-PCR, along with western blotting to examine mTOR protein expression and astrocyte and microglial reactivity. Seizures on PD10 increased the quantity of USVs in female mice and reduced the amount of complex call types emitted in males compared to controls. Inhibition of mTOR with rapamycin significantly reduced the quantity and duration of USVs in both sexes. Changes in USVs were associated with increases in mTOR activity and astrocyte reactivity in male mice, however, three PD10 seizures did not result in enhanced proinflammatory cytokine expression in either sex. Rapamycin treatment significantly reduced % total pS6(235,236) and % total pS6(240,244) expression on PD12, however, minocycline did not impact any of the examined proteins. These findings emphasize the importance of differences that may exist across preclinical seizure models, as three flurothyl seizures did not induce as drastic of changes in mTOR activity or inflammation as observed in other models. Early-life seizures can have a profound impact on the developing brain, and thus it is critical to continue investigating potential therapeutics that target the underlying pathology of seizures and could prevent the development of cognitive and behavioral comorbidities.

Impact of Neuroinflammatory and mTOR Signaling Inhibition Following Flurothyl Seizures on the Acute Development of Autistic-like Behavior in C57BL/6 Mice

by

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CHAPTER ONE

Introduction

Epilepsy is a chronic neurological disorder that affects 1 to 4% of the general population (Berg et al., 2010; Hauser, 1995). It is characterized by recurrent, unprovoked seizures and can be the result of diverse genetic and acquired etiologies. Epilepsy is associated with numerous neurobiological consequences, especially long-term impairments in cognition and behavioral abnormalities (Stafstrom, 2002; Vingerhoets, 2006). Despite the multitude of available pharmacological therapies, approximately one third of individuals with epilepsy are resistant to anti-epileptic treatments and continue to suffer from seizures (Lerche, 2015; Ventola, 2014). In addition, the majority of currently available treatments are symptomatic and function to primarily suppress seizures by decreasing neuronal excitability (Goldenberg, 2010; Rogawski & Loscher, 2004). The development of novel therapies most likely relies on targeting alternative mechanisms in order to modulate the underlying pathological changes caused by seizures and to prevent epileptogenesis.

The incidence of seizures is significantly higher in the first few years of life, making these children at risk for developing a multitude of cognitive and behavioral comorbidities (Baca, Vickrey, Caplan, Vassar, & Berg, 2011; Hauser, 1994; Kramer, 1999). One of the most common comorbid disorders diagnosed in children with epilepsy is Autism spectrum disorder (ASD), which occurs in approximately 30% of children with epilepsy (Talos et al., 2012; Tuchman, Moshe, & Rapin, 2009; Tuchman & Rapin, 2002).

The core behavioral impairments associated with ASD, including social and communication deficits, along with repetitive or stereotypical behavior, have been studied extensively in rodent models of epilepsy (Bernard & Benke, 2015; Keller, Basta, Salerno, & Elia, 2017). While previous studies have provided evidence for how early-life seizures can impact long-term behavioral outcomes in adulthood, few studies have examined how seizures could impact the development of autistic-like behaviors in an acute time period.

There has been substantial evidence that epilepsy is a disorder beyond solely neuronal excitation, and that other components such as glial cells and the release of inflammatory signals may play a critical role in the pathogenesis of seizures (van Vliet, Aronica, Vezzani, & Ravizza, 2017; Vezzani, French, Bartfai, & Baram, 2011). Several cytokines and inflammatory molecules are released following seizures and can initiate cascades that ultimately increase neuronal excitability and decrease the threshold for subsequent seizures (Shimada, Takemiya, Sugiura, & Yamagata, 2014; Turrin & Rivest, 2004; Viviani, Gardoni, & Marinovich, 2007). Pharmacological therapies that reduce seizure-induced neuroinflammation have shown promising results for the treatment of epilepsy in both humans and rodent models (Aronica et al., 2017; van Vliet et al., 2017). While inhibiting inflammatory processes post-seizures is one promising therapeutic target to prevent epileptogenesis, it is most likely not the only factor in what converts the brain into a chronic epileptic state.

Components of the PI3K/Akt/mTOR pathway have also been shown to influence epileptogenesis and neuronal excitability in the brain following seizures (Ostendorf & Wong, 2015). Dysregulation of mammalian target of rapamycin (mTOR) signaling

activity is evident in several genetic and acquired epilepsies, and inhibiting mTOR with rapamycin has demonstrated beneficial effects in reducing seizure frequency and associated seizure-induced neurological damage (Wong, 2013). For example, administration of rapamycin can suppress seizures and delay epileptogenesis in rodents, in part by decreasing mossy fiber sprouting and neuronal death (Buckmaster, Ingram, & Wen, 2009; Sunnen et al., 2011; Zeng, Rensing, & Wong, 2009). In addition, inhibition has shown to protect against seizure-induced learning and memory impairments in rats (Brewster et al., 2013). Dysregulated mTOR signaling activity is also associated with autistic-like phenotypes, as mutations in certain components of the pathway serve as monogenic causes of ASD (Onore, Yang, Van de Water, & Ashwood, 2017; Sato, 2016).

Neuroinflammatory signaling, specifically proinflammatory cytokines, and PI3K/Akt/mTOR signaling are known to interact to influence disease states (Dello Russo, Lisi, Tringali, & Navarra, 2009). However, it is unknown whether seizure-induced neuroinflammatory processes interact with mTOR signaling following early-life seizures to result in behavioral impairments characteristic of the autistic-like phenotype. In the present study, we examined the impact of inhibiting neuroinflammation and mTOR pathway activity separately, as well as with a combined treatment, following an early-life flurothyl seizure paradigm on postnatal day (PD) 10 in mice. To determine whether concomitant inhibition of these factors could prevent the development of autistic-like behaviors, we examined communication via measuring ultrasonic vocalizations on PD12. Future therapeutics aimed at targeting the pathological brain abnormalities that underlie epileptogenesis can hopefully reduce the percentage of individuals with medically

refractory epilepsy, as well as minimize commonly associated comorbid behavioral and cognitive impairments.

CHAPTER TWO

Literature Review

Sections of this chapter are based on the published article: Hodges, S., Lugo, J. (2020). "Therapeutic role of targeting mTOR signaling and neuroinflammation in epilepsy." *Epilepsy Research,* (In Press).

Etiology of Epilepsy

Epilepsy is a neurological disorder of the central nervous system (CNS) that affects more than 65 million people worldwide (Singh & Trevick, 2016). It is estimated that 1.2% of the United States population have epilepsy, accounting for approximately 3.4 million people (Zack & Kobau, 2017). The incidence of the disorder is higher in developing countries and in regions with low socioeconomic status, such as that approximately 80% of people diagnosed live in under-developed countries (Ngugi et al., 2011). Another important factor is age, as epilepsy is known to effect mainly children and elderly individuals (Hauser, 1992). The disorder is characterized by spontaneous, recurrent seizures that are unprovoked and result in uncontrolled neural excitation in the brain. According to the International League Against Epilepsy, epilepsy is typically diagnosed under three circumstances (Fisher et al., 2014). The first and most recognized diagnostic criteria is when an individual has at least 2 unprovoked or reflex seizures, occurring more than 24 hours apart. A diagnosis can also be made when someone experiences one unprovoked or reflex seizure, with the probability of having another seizure similar to the reoccurrence risk of having two unprovoked seizures (approximately 60%) over the next 10 years. Lastly, diagnoses can be made when

individuals have an underlying genetic cause and are diagnosed with a defined epilepsy syndrome. There has been debate surrounding the criteria for appropriate diagnosis of epilepsy, however, most definitions focus on having one or more unprovoked seizures (Falco-Walter, Scheffer, & Fisher, 2018).

There are many diverse classifications of seizures, usually characterized as being focal, generalized, or of unknown onset (Falco-Walter et al., 2018). Focal is synonymous with previous terminology commonly used including partial, simple, or localized, and occur when the seizures originate and are limited to one hemisphere of the brain. Generalized seizures are often termed tonic-clonic seizures, and the onset includes activation of both hemispheres, and usually multiple brain structures. Focal seizures may also develop into generalized seizures, termed secondary generalization or more recently termed "focal to bilateral tonic-clonic seizures" (Falco-Walter et al., 2018; Tomlinson & Venkataraman, 2017). In other circumstances, the onset location may be unknown, yet other manifestations are known such as tonic-clonic motor activity or non-motor (absence) activity. Seizures can further be subdivided based on a multitude of clinical criteria, such as degree of uncontrolled limb movement and loss of consciousness. A few of the most common generalized seizures include absence, myoclonic, clonic, tonic, tonic-clonic, and atonic seizures (Goldenberg, 2010). The length of seizures can also vary across types, such as in status epilepticus (SE), which is a continuous seizure in which uninterrupted seizure activity lasts longer than 30 minutes and is associated with high rates of mortality in individuals (Boggs, 2004; Seinfeld, Goodkin, & Shinnar, 2016).

Seizures can also be subdivided based on proposed etiology, however, understanding the root of seizures is often difficult due to them being attributed to several

mechanisms. Seizures are considered to be a result of an imbalance between excitation and inhibition due to aberrant neuronal firing in the brain (Žiburkus, Cressman, & Schiff, 2013). However, this excitatory/inhibitory (E/I) imbalance can stem from a variety of conditions. When the direct underlying cause is unknown, the seizures are often due to a genetic abnormality and is specified to be idiopathic. Many genetic mutations have been associated with specific epilepsy syndromes, specifically single-gene mutations in subunits of voltage or ligand-gated ion channels, or could also be due to a combination of unknown genetic polymorphisms or copy number variants (Lerche et al., 2013). For example, many childhood epilepsies are due to mutations in subunits of voltage-gated sodium channels which are responsible for the depolarization of the action potential in neuronal conduction (Lerche et al., 2013). These types of epilepsy are known as channelopathies, because the only defect is a single genetic mutation encoding a protein critical for the formation and proper functioning of an ion channel (George, 2004). Seizures can also be associated with other genetic conditions, such as mutations in the *FMR1* gene resulting in Fragile X syndrome or a mutation in *TSC1* or *TSC2* in Tuberous sclerosis syndrome (Hagerman & Stafstrom, 2009; Holmes, Stafstrom, & Group, 2007; Zeng et al., 2011). Other forms of epilepsy are associated with neurodevelopmental disorders in which a genetic linkage is unknown. For instance, approximately one third of individuals diagnosed with ASD will also experience seizures at some point in their lifetime (Talos et al., 2012; Tuchman et al., 2009).

Other causes of epilepsy include structural abnormalities, metabolic conditions, infectious agents, and aberrant immune responses. Structural etiologies may include tumors, cysts, vascular malformations, stroke, and traumatic brain injuries (Englot,

Chang, & Vecht, 2016; Ettinger, 1994). Metabolic conditions, such as cerebral folate deficiency or electrolyte abnormalities, can also result in seizures (Al-Baradie & Chaudhary, 2014; Lin Lin Lee et al., 2018; Nardone, Brigo, & Trinka, 2016). Infectious etiologies may be exemplified by a number of bacterial infections, such as acute bacterial meningitis or tuberculosis, as well as viral stimuli such as human herpes virus-type 6 (Vezzani et al., 2016). A more recent etiology category are immune causes, including various auto-immune diseases, such as antibody-mediated limbic encephalitis (Bien & Holtkamp, 2017). Seizures themselves also induce chronic neuroinflammatory responses in the brain, leading to recurrent seizures and often a decreased seizure threshold later in life (Vezzani et al., 2011). The role of inflammatory mechanisms in epilepsy will be discussed in detail later in the review.

Anti-epileptic Treatments for Epilepsy

There are numerous pharmacological and non-pharmacological options available to treat epilepsy. Pharmacological anti-epileptic medications (AEDs) are usually the first line of treatment for most individuals. However, approximately 30% of individuals have medically refractory epilepsy and have continued seizures despite previous AED therapy (Liu, Slater, & Perkins, 2017). For these individuals, surgical interventions are often necessary. Resection of the seizure focus in the brain has shown to decrease the frequency of, and sometimes eliminate, seizures along with improve quality of life (Jette, Reid, & Wiebe, 2014). However, cognitive deficits are common after surgery, and are dependent on the specific resection area (Gül et al., 2017; Hamberger & Drake, 2006; Luerding, Boesebeck, & Ebner, 2004). Other non-pharmacological options also exist, such as dietary therapies including the high-fat, low-carbohydrate ketogenic diet (Bough

& Rho, 2007). Vagus nerve stimulation is also an option for those resistant to AEDs and in which surgery is not an option (Ben-Menachem, 2002). This section of the review will be focused on the pharmacological options and mechanisms of AEDs, as these are the primary treatment options for individuals with epilepsy.

The overall mechanism of AEDs is to suppress the excessive and rapid neuronal firing of seizures, along with preventing the spread of excitation throughout the brain. The primary mechanism of first generation AEDs included blockade of sodium (Na^{+}) or calcium (Ca^{++}) channels, or potentiation of GABA transmission (Brodie, 2010). However, due to potent side effects, in the early 1990's a new generation of AEDs were developed that have decreased rates of toxicity, are more tolerable, and involve less complex drug-drug interactions (French & Gazzola, 2011). These new generation AEDs include more specific binding of previously targeted mechanisms, as well as new pharmacologic targets. The mechanisms of these AEDs can be classified into four main groups: (1) modulation of voltage-gated ion channels, (2) enhancement of synaptic inhibition via GABA mechanisms, (3) modulation of synaptic release through effects on cell release machinery mechanisms, and (4) inhibition of synaptic excitation via glutamate blockade (Rogawski & Loscher, 2004).

Some of the common voltage-gated ion channel targets include Na^{+} channels, in which there are 9 types ($Na_v1.1$ – $Na_v1.9$), with $Na_v1.2$ predominately expressed in CNS neurons. Blockade of Na^{+} channels inhibit high-frequency, repetitive spike firing and can protect against generalized tonic-clonic and partial seizures. T-type voltage-gated Ca^{++} channels, with primarily 3 subtypes ($Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$), play a role in thalamocortical oscillations and are important targets in controlling absence seizures.

Lastly, K_v7 voltage-gated K^+ channels are common targets, for instance ezogabine is effective in the treatment of partial onset seizures by enhancing inhibitory type slow M-current. Enhancement of synaptic inhibition often involves agonism of $GABA_A$ receptors, including the common drug groups barbiturates and benzodiazepines. Synaptic inhibition can also be enhanced by inhibiting GAT-1 GABA transporters, preventing uptake of GABA into presynaptic terminals, thereby raising synaptic GABA levels and prolonging inhibitory postsynaptic potentials. Some AEDs target components of synaptic release machinery within cells, such as SV2A, in which the mechanistic function is poorly defined, yet is the target of the common AED levetiracetam (Keppra). Lastly, AEDs function to inhibit synaptic excitation by blocking glutamate receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate receptor (NMDA), and kainate receptors.

Despite the multitude of available AEDs that have diverse mechanistic targets, approximately 30% of individuals with epilepsy are resistant to AEDs and continue to experience seizures following treatment (Liu et al., 2017). One potential reason for this high unresponsive rate is that current AEDs are symptomatic, functioning to block or reduce seizures, yet do not target the underlying pathological cause of seizures in the brain. Therefore, many individuals suffer numerous side effects of the drug treatment, and often develop a number of comorbid cognitive and psychiatric conditions (Holmes, 2015; Srinivas & Shah, 2017). These comorbidities can be a result of the direct effect of seizures on the brain, as well as the side effects associated with the medication (Eddy, Rickards, & Cavanna, 2011; Park & Kwon, 2008). Recently, efforts have been focused on developing drugs that instead target the underlying cause of seizures, with hopes to

prevent the development of chronic epilepsy. These novel therapeutics are termed anti-epileptogenic therapies, as they are attempting to prevent the development of chronic epilepsy, or epileptogenesis (Aronica et al., 2017; D'Ambrosio, Eastman, Fattore, & Perucca, 2013; Lerche, 2015). By targeting the underlying root of seizures, this should not only prevent and stop seizure progression, but attenuate side effects and comorbid conditions associated with epilepsy. Possible anti-epileptogenic targets are cell signaling cascades that are disrupted following seizures, such as the mammalian target of rapamycin (mTOR), or PI3K/Akt/mTOR, pathway. Another potential target that underlies epileptogenesis is seizure-induced neuroinflammation. The role of both PI3K/Akt/mTOR signaling and neuroinflammation following seizures will be discussed at length later in the literature review.

Behavioral and Molecular Effects of Early-life Seizures on the Developing Brain

Seizures can result in profound neurological and structural brain changes. The widespread consequences of seizures are often dependent on multiple factors such as the region in which the seizure originated, the degree to which the uncontrolled electrical activity spread, duration of the seizure, and age of seizure onset. One of the most susceptible periods in life to experience seizures is early in life or in the neonatal period (Hauser, 1994, 1995; Kramer, 1999). Experiencing seizures in this critical period of development can have long-term effects on the developmental trajectory of the child. Many studies have found that epilepsy in children is linked to several comorbid cognitive and behavioral impairments later in life, including higher rates of attention-deficit-hyperactivity disorders, specific learning disorders, and an overall gradual loss of mental abilities over time (Fastenau, Jianzhao, Dunn, & Austin, 2008; Karlsson & Dunn, 2014;

Vingerhoets, 2006). One of the most common comorbid behavioral phenotypes associated with early-life seizures is the development of autistic-like behavior (Jeste & Tuchman, 2015; Stafstrom & Benke, 2015). It has been shown that approximately 30% of individuals with epilepsy will be diagnosed with ASD at some point in their life time, as well as 30% of those with ASD will experience seizures (Tuchman & Rapin, 2002; Velíšková, Silverman, Benson, & Lenck-Santini, 2018). The most significant predictor of whether individuals with epilepsy will develop ASD is whether the child also has low cognitive functioning and intellectual disability (Berg & Plioplys, 2012; Stafstrom & Benke, 2015; Tuchman & Cuccaro, 2011). During critical periods of development, the immature brain is thought to react differently to the long-term effects of seizures than does the mature brain. In order to study the relationship between epilepsy and autism, and more specifically how early-life seizures can lead to the development of autistic-like behavior, several rodent models of epilepsy have been utilized.

There are several methodologies utilized to induce seizures in the brain of rodents. Some of these induction methods include chemoconvulsants that mimic temporal lobe epilepsy (TLE), electrical stimulation models (electroshock-induced seizures, afterdischarges model, kindling, etc.), hyperthermic seizures to mimic febrile seizures, and hypoxia in neonatal rodents (Kandratavicius et al., 2014). Specifically in the neonatal brain, generalized tonic-clonic seizures are often induced with the chemoconvulsant kainic acid (KA) or pilocarpine (Curia, Longo, Biagini, Jones, & Avoli, 2008; Levesque & Avoli, 2013). When KA or pilocarpine is administered it is usually via a single intraperitoneal injection that results in SE. Another method that involves multiple administrations to mimic recurrent generalized seizures in the neonatal brain is with the

inhalant flurothyl (Ferland, 2017). Flurothyl acts as a $GABA_A$ antagonist that functions to block inhibition, thereby increasing excitation and resulting in a brief generalized seizure. Administration of flurothyl has many advantages over other methods of seizure induction in neonatal rodents, as there is minimal stress to the animals as no injection is required, it is rapidly eliminated unmetabolized through the lungs, animals recover very quickly following the seizures, and there is a low mortality rate (Ferland, 2017; Kadiyala et al., 2016). Animals subjected to recurrent flurothyl seizures also present with elevated seizure susceptibility in adulthood, but do not have spontaneous seizures (Holmes, Gairsa, Chevassus-Au-Louis, & Ben-Ari, 1998; Huang et al., 1999).

Long-term Impact of Early-life Seizures on Behavioral Outcomes

There is substantial evidence demonstrating how early-life seizures in rodents can lead to the development of cognitive and behavioral abnormalities in adulthood, specifically in regard to autistic-like behavior (Akman, Moshe, & Galanopoulou, 2014; Bernard & Benke, 2015; Holmes, 2016). Preclinical rodent models have served as important tools to delineate whether rodents display characteristics of the ASD phenotype. According to the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) there are two core clusters that comprise the diagnostic criteria for ASD. These include: 1) impairments in reciprocal social communication and 2) repetitive behaviors, restricted interests, and behavioral inflexibility (Grzadzinski, Huerta, & Lord, 2013). These clusters comprising the diagnostic criteria can be examined in rodent models with a variety of behavioral paradigms. Rodent behavioral paradigms examine the autistic-like phenotype by primarily focusing on social behavior, repetitive or stereotypical behavior, and communication measured via ultrasonic vocalizations (USVs).

Regarding social behavior, studies often find that early-life seizures result in reduced sociability in adulthood. For instance, when C57BL/6 (C57) mice were administered 15 flurothyl seizures, across PD7 to PD11, they displayed decreased social interaction in adulthood in a social partition and social chamber task (Lugo, Swann, & Anderson, 2014). Similarly, when rats received a single injection of KA on PD7, they exhibited enhanced social anxiety observed by reduced grooming in the presence of an unfamiliar rat, as well as abnormal social interactions in a social approach task in adulthood at PD60 (Bernard, Castano, Beitzel, Carlson, & Benke, 2015; Bernard et al., 2013). In another study, rats given a single injection of pilocarpine on PD9 displayed reduced social novelty at PD60 (Castelhano, Cassane Gdos, Scorza, & Cysneiros, 2013). Hypoxic seizures on PD10 in rats has also led to a lack of social novelty in adulthood (Talos et al., 2012). Rats that received KA injections on PD7 and PD14 displayed an active avoidance of any contact with a social partner in an open field apparatus (termed social evade), as well as reduced social exploration and contact with the partner in adulthood (Waltereit, Japs, Schneider, de Vries, & Bartsch, 2011). Sociability is the most characterized component of the autistic-like phenotype that has shown to be affected by early-life seizures, specifically with seizures producing long-term impairments in social behavior.

Evidence has demonstrated variable findings regarding whether early-life seizures result in repetitive or stereotypical behaviors in adulthood in rodents. For example, pilocarpine seizures in PD9 rats resulted in increased self-grooming in an anxiogenic environment in adulthood, a measure indicative of increased stereotypy (Castelhano et al., 2013). However, in contrast, rats given a single KA seizure on PD7 exhibited reduced

marble burying, suggestive of restricted interests, as well as reduced grooming in adulthood (Bernard et al., 2015). In addition, early-life flurothyl seizures from PD7 to PD11 in C57 mice resulted in no change in nosepoke or marble burying in adulthood (Lugo et al., 2014). These variable findings across studies are most likely due to differences in the seizure induction method, timeline of induction, and rodent species and strain utilized. Additional studies are needed to further explore the association between early-life seizures and stereotypical behavior to elucidate whether this could be a long-term behavioral phenotype resultant from early-life seizures.

The third component of the autistic-like phenotype that can be studied in rodents is communicative behaviors, measured via USVs. Ultrasonic vocalizations are whistle-like sounds that range from 30 to 90kHz frequencies and are emitted in response to being isolated from the dam in neonatal periods (Branchi, Santucci, & Alleva, 2001). These vocalizations are important in the maternal response, including searching and retrieval of the pups, and have shown to be important for the physiological and behavioral development of the pup (Branchi et al., 2001; Fischer & Hammerschmidt, 2011; Portfors & Perkel, 2014). Many studies have examined the impact of the deletion of genes associated with neurodevelopmental conditions on USV behavior (Fragile X syndrome, Rett syndrome, Down syndrome), but few studies have examined how USVs are altered following early-life seizures (Binder & Lugo, 2017; Holtzman et al., 1996; Picker, Yang, Ricceri, & Berger-Sweeney, 2006; Reynolds, Nolan, Jefferson, & Lugo, 2016a; Roy, Watkins, & Heck, 2012). To examine the effect of febrile convulsions on vocalizations, hyperthermia was induced in rat pups on PD7, with USV behavioral testing on PD10 and PD12. Keller et al. (2004) found that pups that convulsed vocalized a significantly greater

amount than sham-treated pups, yet in a less efficient and complex manner (Keller, Saucier, Sheerin, & Yager, 2004). Despite calling more, seizure female pups received the lowest amount of maternal care suggesting that dams differentially respond to their pups based on whether they experienced the convulsions (Keller et al., 2004). When SE was induced by lithium-pilocarpine in PD14 rats, there was no difference in the quantity of USVs between seizure and control pups, but the latency to emit the first USV was significantly decreased in male SE pups, and the average duration was significantly reduced in female SE pups compared to controls (Lopez-Meraz et al., 2014).

Our lab has also found USVs to be altered following early-life seizures, utilizing a variety of seizure induction methods and across different mouse strains (Nolan et al., 2019; Reynolds, Nolan, Huebschman, Hodges, & Lugo, 2017; Reynolds, Smith, Jefferson, & Lugo, 2016b). In 129SvEvTac mice, we found that KA seizures on PD10 suppressed the quantity and total duration of 50kHz calls on PD12 following seizures (Reynolds et al., 2016b). Utilizing the same seizure paradigm in C57 mice, we found that PD10 KA seizures similarly suppressed USV quantity and total duration compared to control mice (Reynolds et al., 2017). The Reynolds et al. (2017) study used a broad spectrum ultrasonic microphone (Avisoft Bioacoustics), capable of identifying spectrographic properties of vocalizations, compared to the UltraVox recording equipment utilized in Reynolds et al. (2016) which only determines the quantity of calls emitted at a set number of frequencies (Reynolds et al., 2017; Reynolds et al., 2016b). Following manual identification of all vocalizations into 10 categories based on an established call type classification schema, we found that C57 pups administered seizures

on PD10 emitted fewer complex calls than controls (Reynolds et al., 2017; Scattoni, Gandhy, Ricceri, & Crawley, 2008).

Recently, we examined the impact of seizure load on vocalization development in C57BL/6J pups. While most studies to date have reported the effect of early-life seizures using chemoconvulsants that produce SE or single continuous seizures, few have determined whether recurrent seizures or the quantity of seizure load differentially effects USV behavior and the autistic-like phenotype of mice. Utilizing two different seizure paradigms, we found that a high seizure load comprised of 15 flurothyl seizures across PD7 to PD11 resulted in increased average duration and cumulative duration of USVs across both sexes compared to controls (Nolan et al., 2019). However, a low seizure load, with only 3 flurothyl seizures administered on PD10, resulted in no change in spectral or temporal features of USVs. Interestingly, both seizure load paradigms resulted in a reduced number of complex calls emitted from seizure pups compared to controls. The reduction in complex calls appears to be a consistent finding in the qualitative aspect of USVs across early-life seizure and genetic ASD models (Binder & Lugo, 2017; Nolan et al., 2019; Reynolds et al., 2017; Takahashi et al., 2016). While our lab and others have gained insight into the effect early-life seizures can have on vocalization development, further research is critical to begin to understand the underlying meaning of the quantitative and qualitative seizure-induced changes in USVs.

Molecular Mechanisms Mediating the Epilepsy and Autism Comorbidity

The underlying mechanism in which early-life seizures can result in long-term behavioral impairments is attributed to several diverse mechanisms. Specifically, there are many molecular aberrations that are similar in epilepsy and ASD, thus serving as

potential links mediating the high comorbidity rate between the two disorders. Seizure-induced changes in brain development can be diverse, based on etiology of the seizures, age of onset, and the origin and type of seizures (Haut, Veliskova, & Moshe, 2004; Nickels, Zaccariello, Hamiwka, & Wirrell, 2016). Despite these factors, the mechanism in which seizures can lead to autistic-like behavioral phenotypes later in life usually stems from a combination of the following factors: imbalances in excitation and inhibition (E/I imbalance), genetic abnormalities, chronic molecular changes in synaptic architecture, altered cell signaling activity, and enhanced neuroinflammation in the brain. These factors are most likely not mutually exclusive, and rather they are often intertwined and it is most likely a combinatory effect of diverse mechanisms that produces the behavioral phenotype and presentation of both epilepsy and ASD.

The E/I imbalance is based on the assumption that normal brain functions relies on a homeostatic balance between excitatory and inhibitory inputs to the brain. This balance of neurotransmission can go awry, resulting in too much excitation and too little inhibition leading to hyperexcitability of the network and subsequent seizures (Berg & Plioplys, 2012). Specifically, when seizures occur early in development, these seizures produce neuropathological changes that can alter intrinsic neuronal properties and neurotransmitter systems, resulting in behavioral and cognitive deficits throughout the lifespan (Velíšková et al., 2018). In rodent models, induction of seizures can lead to long-term changes in ion channels and receptor densities, rewiring of neuronal networks, and cell death (Rigas et al., 2018; Sayin, Hutchinson, Meyerand, & Sutula, 2015; Velíšková et al., 2018). For example, the synaptic densities of AMPA and NMDA receptors, the main receptors for the primary excitatory neurotransmitter glutamate, have found to be

altered following early-life seizures (Lippman-Bell, Zhou, Sun, Feske, & Jensen, 2016; O'Leary, Bernard, Castano, & Benke, 2016; Schidlitzki et al., 2017). Following a single early-life seizure in rats, there is evidence for loss of long-term potentiation (LTP) and elevated long-term depression (LTD) in adulthood (Bernard et al., 2013; Cornejo, Mesches, Coultrap, Browning, & Benke, 2007). Separately, imbalances in E/I circuitry have also been found in individuals with autism and mouse models of ASD. Individuals with ASD have found to have reduced GABA levels, the primary inhibitory neurotransmitter, in various cortical regions of the brain, as well as decreased $GABA_A$ receptor binding in the frontal cortex (Puts et al., 2017; Zurcher, Bhanot, McDougle, & Hooker, 2015). Postmortem studies have also found a reduction in parvalbumin interneurons in the medial prefrontal cortex of those with ASD (Ariza, Rogers, Hashemi, Noctor, & Martinez-Cerdeno, 2018; Hashemi, Ariza, Rogers, Noctor, & Martinez-Cerdeno, 2017). ASD has also been associated with increased excitation in regions of the brain, including primarily enhanced principal cell excitability of glutamatergic signaling (Nelson & Valakh, 2015). Various genetic abnormalities that are known to alter the E/I balance have been identified in individuals with both epilepsy and ASD.

Numerous single gene mutations that result in genetic conditions and abnormal excitability have been linked to both epilepsy and ASD diagnoses. For example, mutations in the Fragile X mental retardation (*Fmr1*) gene are the largest genetic contributor to ASD, and result in a diagnosis of the neurodevelopmental disorder known as Fragile X syndrome (FXS) (Ciaccio et al., 2017; Hagerman, 2006). Approximately 10 to 20% of individuals with FXS experience seizures, as well as many have abnormal electroencephalograph (EEG) recordings associated subclinical seizure activity (Berry-

Kravis, 2002; Berry-Kravis et al., 2010; Hagerman & Stafstrom, 2009; Musumeci et al., 1999). The presence of seizures in individuals with FXS has also been associated with behavioral outcomes. For example, identification of EEG endophenotypes within FXS populations demonstrated that the incidence and degree of epileptic EEG is correlated with worsened behavioral symptoms in children with FXS and seizures, specifically with impairments in attention (Cowley, Kirjanen, Partanen, & Castrén, 2016). Seizures in FXS may be due to elevated neuronal excitability, stemming from reduced translational inhibition typically provided by FMRP and elevated glutamatergic stimulation and signaling (Bear, Huber, & Warren, 2004; Bianchi, Chuang, Zhao, Young, & Wong, 2009; Contractor, Klyachko, & Portera-Cailliau, 2015; Gross, Yao, Pong, Jeromin, & Bassell, 2011). Other genetic conditions that are associated with both autistic and epileptic phenotypes include Rett syndrome (*Mecp2* mutation), tuberous sclerosis syndrome (*Tsc1/Tsc2* mutation), and Cowden syndrome (*Pten* mutation) (Adachi, Takigawa, Nomura, Watanabe, & Kowa, 2018; Brooks-Kayal, 2010; Caumes et al., 2014; Dani et al., 2005; Marchese et al., 2014). In addition to these single genetic mutations being associated with disruption in the E/I balance in the brain, many of them also intersect at the molecular signaling level and loss of their respective proteins can produce similar downstream alterations in synaptic architecture.

Expression of proteins associated with synaptic plasticity and neuronal wiring of the brain throughout development have been found to be altered following early-life seizures and in ASD. Abnormalities can arise from diverse mechanisms, including disruption in signaling molecules and changes in the synaptic densities of receptor subtypes. In addition, disruption of processes involved in synaptic plasticity can

predispose the brain to developing behavioral and cognitive impairments later in life (Zoghbi & Bear, 2012). Some of these mechanistic processes include alterations in structural proteins involved in synaptic vesicle release, anchor synaptic machinery, and those that control the migration of neurons and overall organization of network connectivity (Bartholome et al., 2017; Ebrahimi-Fakhari & Sahin, 2015; Upreti et al., 2012; Volk, Chiu, Sharma, & Huganir, 2015). For example, a single early-life KA seizure in rats has found to enhance expression of synaptic scaffolding protein PSD-95 and reduce total NMDA receptor 2A expression in adulthood (Cornejo et al., 2007). In addition, synaptic membrane proteins such as neuroligins and SHANKs proteins have shown to be altered following early-life seizures and have been associated with ASD phenotypes in rodent models (Fang et al., 2016; Han et al., 2013; Monteiro & Feng, 2017; Nakanishi et al., 2017). Additionally, many of these synaptic alterations are linked to disruption of molecular signaling cascades in both epilepsy and ASD.

Several molecular signaling pathways are disrupted following seizures and have shown to contribute to the development of behavioral and cognitive impairments later in life. These signaling pathways are involved in numerous processes, including synaptic plasticity, neuronal death or apoptosis, neurogenesis, and more broadly translation of genes involved in critical processes throughout development (Bozzi, Dunleavy, & Henshall, 2011; Hodges & Lugo, 2018; Meng, Yu, Song, Chi, & Tan, 2013). Many signaling cascades are activated post-seizures to restore homeostatic processes that may have gone awry, however, other times signaling mechanisms become hyperactive or quiescent and have detrimental effects on brain development. For example, canonical Wnt signaling has found to be hyperactive following seizures, and is associated with both

the elevated neurogenesis and neuronal death commonly observed after seizures (Hodges & Lugo, 2018; Madsen, Newton, Eaton, Russell, & Duman, 2003; Rubio et al., 2017; Theilhaber et al., 2013; Yang et al., 2016). Additionally, modulation of Wnt signaling with pharmaceuticals and novel small molecule inhibitors have found to protect against seizure-induced neuronal damage (Jung et al., 2006; Li et al., 2010; Qu et al., 2017). Another signaling pathway critical for early brain development is the mTOR signaling pathway. Components of this pathway have shown to be disrupted following early-life seizures, as well as are altered in models of ASD and are linked to core behavioral impairments in ASD. The involvement of mTOR signaling in early-life seizures will be discussed in length later in the review of literature.

Lastly, enhanced inflammatory signaling in the brain is thought to be another potential link mediating the high comorbidity rate between epilepsy and ASD. Various inflammatory molecules and signaling pathways have shown to be hyperactive following seizures in the brain, as well as inflammatory insults early in life are thought to contribute to the mechanisms of epileptogenesis (Aronica, Ravizza, Zurolo, & Vezzani, 2012; Vezzani, Aronica, Mazarati, & Pittman, 2013a; Vezzani, Friedman, & Dingledine, 2013b). There are also increased levels of proinflammatory cytokines and molecules in individuals with chronic epilepsy, suggesting that heightened inflammatory processes could be involved in the development of chronic epilepsy. In addition, individuals with ASD often have dysregulated immune function, displaying an overall proinflammatory phenotype (Meltzer & Van de Water, 2017).

Neuroinflammation and Epilepsy

An increasing body of clinical and experimental evidence suggests that the immune system plays an important role in epilepsy. Seizures induce a cascade of biological events, characterized by the release of inflammatory molecules, such as cytokines and attractant molecules known as chemokines (Rana & Musto, 2018). In normal physiological conditions, cells of the immune system will activate receptors following injury in order to stimulate a variety of pathways that function to repair damage to the brain and provide protection from any additional injury. However, if these processes are dysregulated following an insult, it can lead to sustained neuroinflammatory signaling that results in neuronal and glial damage. The damage from heightened neuroinflammatory cascades can manifest clinically, dependent on the region of the brain effected (Alam, Hana, Jin, Suen, & Ma, 2018; Zhao et al., 2019). Evidence has suggested that dysregulation of the immune system could be what potentiates the hyperexcitability following seizures and underlies epileptogenesis in the brain (Vezzani et al., 2013b).

Cytokines are small, secreted and membrane-bound proteins that act as chemical messengers and regulate inflammation and several other cellular activities including the growth, survival, and differentiation of cells (Deverman & Patterson, 2009). They can be produced by a number of cells in the brain, such as glial cells including microglia and astrocytes, as well as neurons and the endothelial cells that make up the blood-brain barrier (BBB) (Galic, Riazi, & Pittman, 2012). These cell types also express receptors for their respective cytokines (toll-like receptors [TLR], i.e.) which can initiate downstream inflammatory signaling events that activate transcription factors (NF-kβ, i.e.) and initiate

production of additional immune molecules (Kawasaki & Kawai, 2014). The activation of these inflammatory signaling cascades can result in propagation of the inflammatory response to other regions of the brain. In healthy brains, cytokines are secreted in order to produce an inflammatory environment conducive to recruiting cells to fight infection and aid in tissue repair and recovery. Cytokines are considered to be primary anti-inflammatory (IL-10, IL-4) or proinflammatory (IL-1β, IL-6, TNFα), however, some can also have dual functions dependent on the physiological situation (Cavaillon, 2001; Shohami, Ginis, & Hallenbeck, 1999; Tanaka, Narazaki, & Kishimoto, 2014). Early in development, the release of cytokines from microglia also play a key role in synaptic and neural development, such as the pruning and refining of synapses (Paolicelli et al., 2011; Schafer et al., 2012). They can also initiate signaling cascades and activate target genes that are involved in the survival, proliferation, and differentiation of cells (Mousa & Bakhiet, 2013). However, in disease-states, such as following seizures, the sustained release of cytokines produces a chronic inflammatory state that results in cellular toxicity and has detrimental effects on the brain.

There is substantial evidence that epilepsy is a disorder beyond solely neuronal excitation, and that other components such as glial cells in the brain and the release of inflammatory signals play a critical role in the pathogenesis of seizures. Many components of immune function are dysregulated in both epilepsy and ASD, including cytokine expression and release, microglial and astrocyte activation, and aspects of adaptive immunity (Di Marco, Bonaccorso, Aloisi, D'Antoni, & Catania, 2016; Gupta et al., 2014; Vezzani et al., 2011). Cytokines are normally expressed at low levels in the brain, however, following seizures a variety of cytokines (IL-1, IL-6, TNFα, IFNγ) and

chemokines (MCP-1/CCL2, CCL3, CCL5) are rapidly produced and released in various brain regions (Arisi, Foresti, Katki, & Shapiro, 2015; Kosonowska, Janeczko, & Setkowicz, 2015; Li et al., 2011; Uludag et al., 2015; Yu et al., 2012). When cytokines bind to their respective receptors post-seizures, this can lead to several downstream signaling events. For instance, it can result in increased expression of adhesion molecules on endothelial cells lining the BBB, resulting in enhanced permeability of the BBB (Broekaart et al., 2018; van Vliet et al., 2007). This allows the recruitment of peripheral immune cells, involved in both innate and adaptive immune responses, to cross the BBB. The invasion of leukocytes into the CNS is guided by the chemoattractant properties of chemokines and will contribute to the upregulation in cytokine production and release, thereby resulting in a chronic inflammatory environment in the brain. Additionally, this can lead to several downstream effects that ultimately increase neuronal excitability and decrease the threshold for subsequent seizures (Galic et al., 2012; Iori, Frigerio, & Vezzani, 2016). For example, cytokine release can result in an influx of neuronal calcium (Ca^{++}), as well as increased glutamate release from astrocytes and decreased K^{+} and glutamate uptake into astrocytes (Shimada et al., 2014; Tian et al., 2005). Cytokines can also promote the release of toxic mediators such as reactive oxygen species and glutamate that will activate excitatory NMDA and AMPA receptors (Stellwagen, Beattie, Seo, & Malenka, 2005; Terrone, Frigerio, Balosso, Ravizza, & Vezzani, 2019; Ye & Sontheimer, 1996). Overall, there is an exaggeration of the immune response which can lead to neuronal and glial toxicity, along with disrupted neural signaling that can have detrimental effects on behavior and cognition post-seizures. In addition, sustained inflammatory processes can lead to the reoccurrence of seizures and the development of

refractory epilepsy that is resistant to anti-seizure treatment. Due to the substantial evidence of a heightened neuroinflammatory state following seizures, anti-inflammatory and immunosuppressive drugs have shown to be promising treatments for epilepsy in both humans and rodent models (Aronica et al., 2017; D'Ambrosio et al., 2013).

Many anti-inflammatory therapies have been utilized in rodent models and clinical trials in an attempt to prevent the development of chronic epilepsy. Some of these promising targets include cyclooxygenase-2 (COX-2) and prostaglandin EP2 receptor signaling, high mobility group box 1 (HMGB1)/TLR signaling, and IL-1/IL-1R signaling pathways (Jiang et al., 2013; Jung et al., 2006; Maroso et al., 2010; Serrano et al., 2011). Many studies have also found that a multi-target strategy is most effective in controlling the pathologic inflammation following seizures (Aronica et al., 2017). Inhibition of these signaling pathways has shown to prevent seizure-induced neuronal damage in various regions of the brain, in addition to reducing the incidence, frequency, and duration of spontaneous seizures in rodent models of epilepsy. However, translating these potential epileptogenic targets into effective therapies in clinical populations has been a major challenge. In contrast to entirely novel molecular targets, therapies that function to reduce pathologic inflammation and are already FDA-approved and used safely in clinical populations would be an alternative avenue for future anti-epileptogenic therapies.

One potential therapy could be minocycline, a tetracycline-class antibiotic that has potent anti-inflammatory properties (Moller et al., 2016). It has the ability to cross the BBB and has a direct effect on the proinflammatory subtype M1-type microglia by inhibiting the secretion of proinflammatory cytokines (Elewa, Hilali, Hess, Machado, & Fagan, 2006). Minocycline has been shown to delay disease progression primarily by

preventing cell death and inhibiting chronic neuroinflammation in rodent models (Noble, Garwood, & Hanger, 2009). Specifically in rodent seizure models, it has shown to have anti-convulsant effects including reducing microglial activation and decreasing hippocampal damage post-seizures (Abraham, Fox, Condello, Bartolini, & Koh, 2012; Heo et al., 2006; Wang, Englot, Garcia, Lawton, & Young, 2012). While used for several diverse clinical applications, only one case study to date has utilized minocycline to reduce seizures. Specifically, it has been found to reduce seizure frequency in a patient with severe symptomatic epilepsy due to an astrocytoma (Nowak et al., 2012). However, beyond that single piece of evidence there is little investigation into whether it could be a potential therapeutic to prevent the development of chronic epilepsy in individuals.

PI3K/Akt/mTOR Signaling and Epilepsy

Signaling via the PI3K/Akt/mTOR (mTOR) pathway is critical for neural development early in life. It regulates several processes, including cell growth, proliferation, protein synthesis, neuronal morphology, cortical development, and immune responses (Laplante & Sabatini, 2012). Beyond these classical roles of mTOR, activation of the pathway can also influence neuronal signaling and excitability, such as axonal and dendritic morphology, neurotransmitter expression, synaptic plasticity, and cognition and behavior (Bekinschtein et al., 2007; Jaworski, Spangler, Seeburg, Hoogenraad, & Sheng, 2005; Tang et al., 2002). The pathway can be activated by diverse mechanisms, including neurotransmitters (ex. glutamate binding to NMDA receptors), neurotrophins (ex. BDNF binding to TrkB receptors), intracellular signals (ex. nutrient and energy status, stress), and immune molecules (ex. cytokines and other ligands binding to TLRs) (Laplante & Sabatini, 2012; Page et al., 2006; Takei & Nawa, 2014).

In normal physiological conditions, the phosphoinositide 3-kinase (PI3K) will be activated by receptor tyrosine kinases following binding of some molecule. PI3K functions to convert phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Through the kinase PDK1, Akt will then be phosphorylated and activated and proceed to phosphorylate and inactivate the tuberous sclerosis (TSC1/TSC2) complex. When Akt phosphorylates the TSC1/TSC2 dimer, it inhibits TSC1/TSC2's GTPase-activating protein (GAP) activity allowing the G protein Rheb to maintain an active GTP-bound form. The active form of Rheb will proceed to activate the mammalian target of rapamycin (mTOR) 1 complex. mTOR is a conserved serine/threonine protein kinase that is in a complex with other regulatory proteins including raptor, mLST8, and inhibitory protein PRAS40. mTOR will directly phosphorylate the p70S6 kinase (p70S6K), which itself phosphorylates many targets. For example, activated p70S6K phosphorylates ribosomal protein S6, which promotes production of ribosomal proteins and elongation factors to increase translation of several genes. P70S6K also phosphorylates and inhibits eukaryotic elongation factor 2 kinase (eEF2K), which endogenously inhibits eEF2 and slows down the elongation step of translation. Thus, phosphorylation and inhibition of eEF2K by p70S6K ultimately allows translation elongation to proceed. In addition, phosphorylation of p70S6K leads to activation of eIF4B, which is an RNA-binding protein that potentiates ribosome recruitment and stimulates translation of mRNAs. mTOR can also phosphorylate and inhibit the activity of eukaryotic 4E - binding protein 1 (4E-BP1). Inhibition of 4E-BP1 causes it to dissociate from eukaryotic initiation factor 4E (eIF4E), allowing eIF4E to complex with other initiation factors to initiate mRNA translation (Fig. 1).

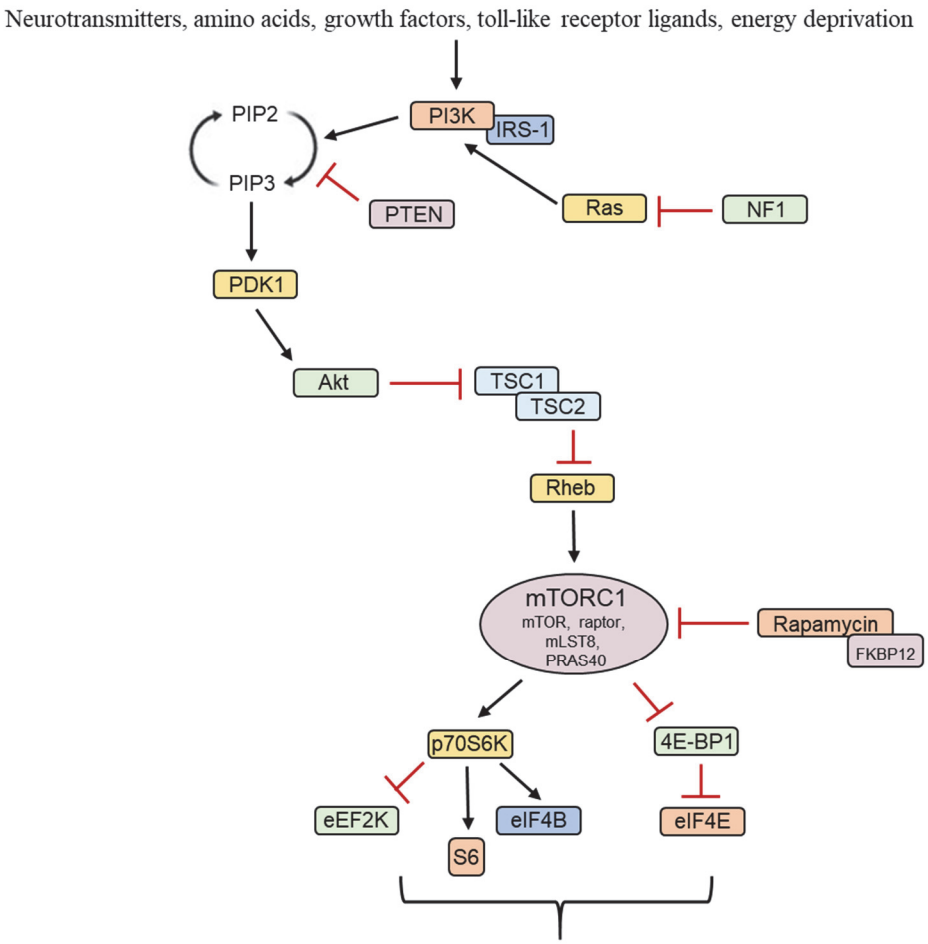


Figure 1. The PI3K/Akt/mTOR signaling pathway. The mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase, which forms two complexes, rapamycin-sensitive mTORC1 and relatively rapamycin-insensitive mTORC2 (not shown). The mTOR pathway can be activated by several extracellular (neurotransmitters, amino acids, growth factors, toll-like receptor ligands) and intracellular stimuli (energy deprivation), resulting in activation of a series of downstream effectors. Signaling via the mTOR pathway is initiated with activation of PI3K by receptor tyrosine kinases, which leads to conversion of PIP_2 to PIP3. Akt is phosphorylated by PDK1, which leads to phosphorylation and inhibition of the TSC1/TSC2 complex. This allows the active form of GTP-binding protein Rheb to activate mTORC1. mTORC1 is comprised of mTOR, in addition to regulatory protein raptor, mLST8, and inhibitory protein PRAS40. mTORC1 activation leads to phosphorylation of several targets involved in ribosomal biogenesis (p70S6K, S6) and protein translation (eEF2K, eIF4B, 4E-BP, eIF4E). Beyond protein translation, mTOR pathway activity regulates cell proliferation, cell growth, synaptic plasticity, ion channel expression, and apoptosis. The mTOR pathway can be negatively regulated by stimuli, such as PTEN, which antagonizes pathway activity by dephosphorylating PIP3 to PIP2. In addition, rapamycin binds FKBP12 to inhibit mTORC1 activity. PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphatidylinositol-dependent kinase 1; TSC, tuberous sclerosis complex; mLST8, mammalian lethal with SEC13 protein 8; PRAS40, proline-rich Akt substrate of 40 kDa; eEF2K, eukaryotic elongation factor-2 kinase; eIF4B, eukaryotic translation initiation factor 4B; eIF4E, eukaryotic translation initiation factor 4E; 4E-BP1, (eIF4E)-binding protein 1; PTEN; phosphatase and tensin homolog on chromosome 10; FKBP12, 12-kDa FK506 and rapamycin-binding protein; IRS-1, insulin receptor substrate 1; NF1, neurofibromatosis type I.

There is evidence for dysregulated mTOR signaling in individuals with genetic and acquired epilepsies, as well as in animal models of epilepsy (Ostendorf & Wong, 2015; Wong, 2010; Zeng et al., 2009). For example, mutations in components of the mTOR pathway have been associated with several types of epilepsies, as well as serve as monogenic causes of ASDs. Specifically, mutations in *TSC1/TSC2* and *PTEN* result in epileptic phenotypes and lead to diagnoses of Tuberous sclerosis syndrome and Cowden syndrome, respectively (Conti et al., 2011; Marchese et al., 2014; Rosset, Netto, & Ashton-Prolla, 2017). Both TSC1/TSC2 and PTEN function as endogenous inhibitors of the mTOR pathway, and thus mutations or deletions of these genes results in hyperactivation of mTOR signaling activity. In addition, seizures that are due to some neurological insult such as traumatic brain injury or infection have also been linked to heightened mTOR signaling (Guo, Zeng, Brody, & Wong, 2013; Wang et al., 2018). Evidence regarding the role of mTOR signaling in epilepsy has stemmed primarily from studies that examine how inhibition of pathway activity impacts molecular and behavioral phenotypes.

One of the most common ways to modulate mTOR signaling following seizures is with the administration of rapamycin. Rapamycin is a direct inhibitor of the mTOR1 complex and how mammalian target of rapamycin received its name from an experiment in yeast that discovered rapamycin as a target of TOR. The compound rapamycin was found in the soil of Easter Island and is produced by the bacterium *Streptomyces hygroscopicus* (Ballou & Lin, 2008; Kunz et al., 1993). Rapamycin is usually known for its potent immunosuppressive and anti-tumor effects, and has shown to influence both innate and adaptive immune responses (Dai et al., 2012; Weichhart, Hengstschlager, &

Linke, 2015). It is currently an FDA-approved treatment for Tuberous sclerosis complex, lung disease (lymphangioleiomyomatosis), and for organ transplant rejections. In addition, the rapamycin analog Everolimus has shown to be effective in reducing seizure frequency in individuals with Tuberous sclerosis complex (Cardamone et al., 2014; Krueger et al., 2013).

In rodent models, by downregulating seizure-induced mTOR hyperactivity, rapamycin has demonstrated anti-seizure effects by suppressing seizures and preventing epileptogenesis. Specifically, it has shown to delay epileptogenesis by decreasing mossy fiber sprouting, inhibiting seizure-induced neuronal death, and reducing dendritic and astrocytic injury post-seizures (Brewster et al., 2013; Buckmaster et al., 2009; Guo et al., 2016; Huang et al., 2010; Sunnen et al., 2011). For example, when rats were administered pilocarpine seizures at PD34, they exhibit elevated mTOR signaling shown by an increased ratio of phosphorylated S6 to total S6 at 24 hours and 7 days post-SE (Buckmaster et al., 2009). Continuous infusion of rapamycin inhibited mTOR hyperactivation and mossy fiber sprouting in the hippocampus, however, suppression of pathway activity and sprouting required continual treatment (Buckmaster et al., 2009). In another study, rats underwent SE and then received rapamycin treatment once daily for 7 days, and then once every other day until brain tissue was taken 6 weeks after SE (van Vliet et al., 2012). Treatment with rapamycin did not alter the severity or duration of seizures, but treated rats developed minimal or no seizures during the 6-week treatment regimen whereas controls showed progressive increases in the number of seizures following a single bout of SE. The rapamycin-treated SE rats also had significantly reduced cell loss and mossy fiber sprouting that would have normally occurred following

SE (van Vliet et al., 2012). While there is considerable evidence regarding the benefits of rapamycin in preventing seizure-induced injury, little is known regarding how rapamycin may prevent the development of behavioral impairments, specifically autistic-like behavior. One study has found that following pilocarpine-induced seizures in adult rats, rapamycin treatment starting 2 weeks post-SE and continued every other day for 4 treatments was able to improve hippocampal-dependent spatial learning and memory in a Morris water maze task and recognition memory in a novel object recognition task (Brewster et al., 2013). Additional studies examining the impact of rapamycin treatment on the development of autistic-like behavior are critical.

Interaction Between Neuroinflammation and mTOR Signaling in Epilepsy

Neuroinflammatory signaling and mTOR pathway activity have been previously shown to interact to influence disease states (Aronica et al., 2017; Dello Russo et al., 2009; Drion et al., 2018). Toll-like receptor ligands, such as a variety of cytokines and bacterial components (ex. lipopolysaccharide), can directly stimulate the pathway (Ribeiro et al., 2018; Schmitz et al., 2008; Xiao, Peng, Yang, Kong, & Yin, 2015). The upregulation and secretion of cytokines post-seizures most likely exacerbates elevated mTOR signaling activity by directly activating the pathway. Activation of the mTOR pathway is also important for regulation of cytokine production downstream, as well as inhibition of signaling activity can have diverse effects on innate immune function (Fortin et al., 2011; Xie et al., 2014). In addition, activation of the pathway is required for development of the immune system in normal physiological conditions, especially in regard to dendritic cell development, a key immune cell subtype involved in the adaptive immune response (Sukhbaatar, Hengstschläger, & Weichhart, 2016).

A few studies have examined the interaction between inflammation and mTOR activity following seizures, however, no study has investigated this yet in the neonatal period in rodents. In adulthood, inhibition of mTOR activity with rapamycin has shown to have immunosuppressive effects following seizures. Specifically, adult rats that underwent SE and were then treated with rapamycin 4 hours post-SE, daily for 7 days, and then continued every other day until they were sacrificed at 6 weeks of age had elevated immunoproteasome expression in neurons and astrocytes in the hippocampus and piriform cortex (Broekaart et al., 2017). Expression of immunoproteasome components was reduced by rapamycin treatment, as well as treatment significantly reduced the quantity of spontaneous seizures in rats (Broekaart et al., 2017). Another study utilizing a similar seizure and rapamycin treatment paradigm found that the treatment restored integrity of the BBB in seizure rats (van Vliet et al., 2012). In addition, rapamycin treatment has shown to reduce activated microglia and astrogliosis in rats that received KA seizures (van Vliet et al., 2016). Furthermore, both pre-treatment and post-treatment with rapamycin after seizures has shown to reduce astrocyte injury (Guo, Zou, & Wong, 2017). A detailed schematic outlining the relationship between neuroinflammation and hyperactive mTOR signaling after seizures is shown in Figure 2.

It is known that inflammatory signaling can intersect with mTOR pathway activity specifically in disease states, and that the interaction could potentially exacerbate the pathologic damage following injury in the brain. However, while there are a few pieces of evidence investigating this interaction following seizures, it is unknown whether inflammation and mTOR signaling could be contributing to the development of cognitive and behavioral impairments following seizures in the neonatal period. In the

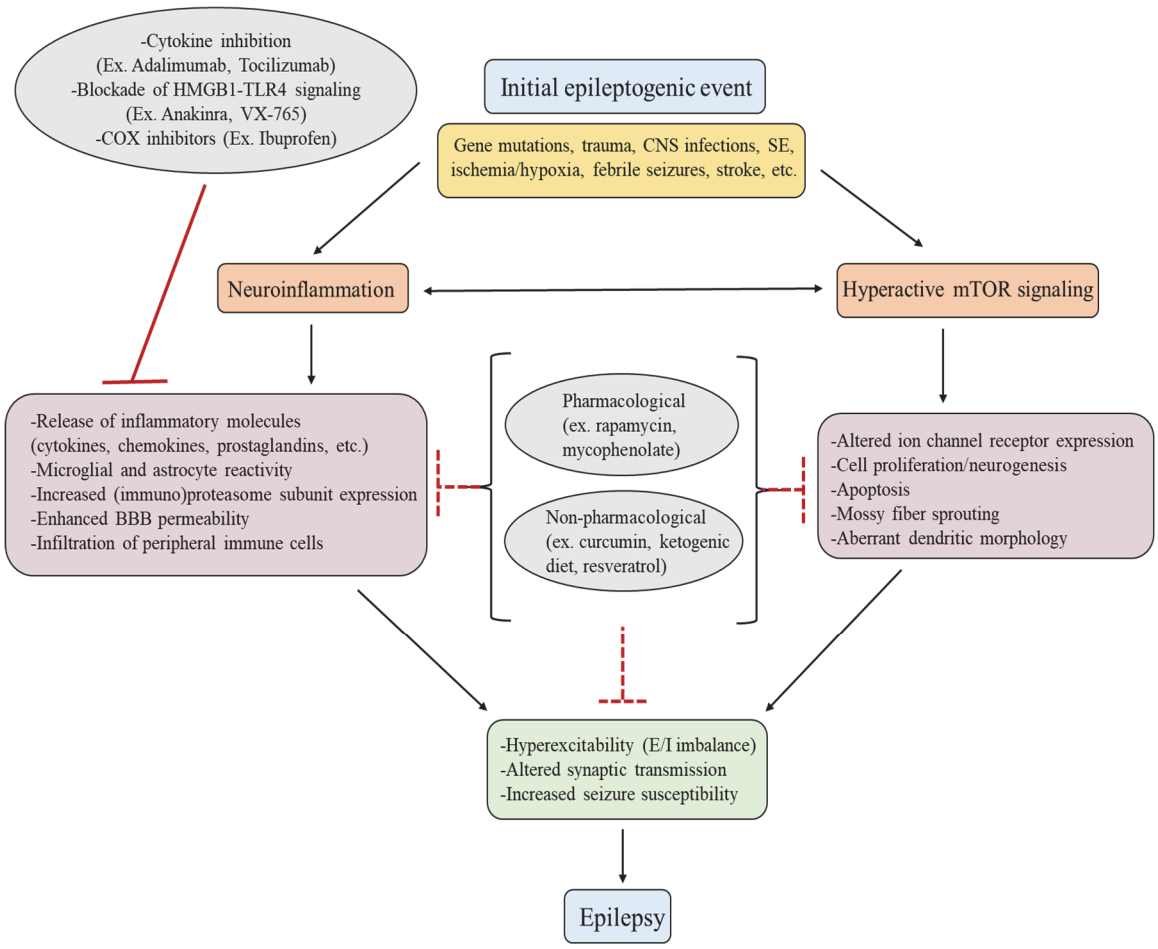


Figure 2. Relationship between epilepsy, neuroinflammation, and hyperactive mTOR signaling. Epileptic events in the brain can be caused by several etiologies, including gene mutations, trauma, CNS infections, SE, ischemia and hypoxia, febrile seizures, and stroke. Seizures are associated with both neuroinflammation and hyperactive mTOR signaling, which can lead to a cascade of downstream pathophysiological effects. These changes can result in hyperexcitability, altered synaptic transmission, and increased seizure susceptibility, which may underlie the development of chronic epilepsy. Many pharmacological (ex. rapamycin, mycophenolate) and non-pharmacological (ex. curcumin, ketogenic diet, resveratrol) compounds have effects on both neuroinflammatory signaling and mTOR pathway activity. These therapies could be beneficial in inhibiting two key processes that potentially underlie epileptogenesis. In addition, there are several anti-inflammatory treatments that have shown efficacy in reducing seizures and associated pathological damage, such as cytokine inhibitors, HMGB1-TLR4 signaling cascade inhibitors, and COX inhibitors. CNS, central nervous system; SE, status epilepticus; mTOR, mammalian target of rapamycin; HMGB1, high-mobility group box protein 1; TLR4, toll-like receptor 4; COX, cyclooxygenase; BBB, blood brain barrier; E/I, excitatory/inhibitory.

present study, we investigated whether inhibiting neuroinflammation and hyperactive mTOR signaling separately, as well as simultaneously, in the neonatal period could be effective in attenuating seizure-induced behavioral impairments, specifically autistic-like communicative behaviors. Restoring proper signaling activity, along with reducing inflammation, could serve as a potential therapeutic avenue for future treatments aimed at reducing comorbid behavioral impairments in individuals with epilepsy.

CHAPTER THREE

Methods and Materials

Animals

Subject mice included male and female C57BL/6 pups (PD10-12). All mice were bred and group housed at Baylor University in standard laboratory conditions at an ambient temperature of 22°C (12-hour light/12-hour dark diurnal cycles). Mice were provided with food and water *ad libitum*. All procedures were conducted in compliance with the Baylor University Institutional Animal Care and Use Committee and the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.*

Seizure Induction

On PD10, pups were randomly assigned to receive either seizure or control procedures. Prior to the first seizure on PD10, toes of all pups were clipped for identity purposes throughout the experiment and placed back into the home cage for 30 min prior to seizure induction. Seizure mice received 3 seizures, each 2 hours apart, on PD10 for a total of 3 seizures. For each seizure, mice were placed in groups of 2 or 3 littermates in a clear acrylic inhalation chamber (29 cm x 16 cm x 15 cm) located within a fume hood. The chemoconvulsant flurothyl (bis-2,2,2-trifluroethyl ether) obtained from Sigma-Aldrich (St. Louis, MO, USA) was used to induce seizures. Flurothyl was administered at a rate of 50µl/minute, using a Harvard Apparatus syringe pump (Model 11 Plus), until all mice experienced a generalized tonic-clonic seizure, displayed by tonic extension of the forelimbs and hindlimbs. Pups receiving the control procedure were handled similarly

and placed in an identical acrylic chamber placed on a lab bench outside the fume hood, with no syringe pump inserted or infusion of flurothyl administered. Following seizure or control procedures, pups were placed with their same treatment counterpart(s) in individual containers containing clean bedding, warmed with a heating pad to approximately 35°C. All pups were monitored and kept in the warmed containers until 15 min following the end of drug administration (1 hour following 3rd seizure) on PD10. Following the 1st and 3rd seizures, both seizure and control pups received a subcutaneous injection between the shoulder blades of 0.1mL 0.9% physiological saline in order to account for time when pups were unable to suckle in the home cage. The seizure and control chambers were cleaned with a 30% isopropanol solution in between each seizure or control administration.

Treatment Administration

One hour following the third (final) seizure on PD10, mice were randomly assigned to receive 1 of 4 treatments. All mice received two intraperitoneal (i.p.) injections of a combination of 0.9% physiological saline, minocycline (Sigma-Aldrich #M9511, St. Louis, MO, USA), or rapamycin (LC Laboratories, Woburn, MA, USA). The treatment groups were as follows: saline/saline, minocycline/saline, rapamycin/saline, minocycline/rapamycin. With the combined administration of both minocycline and rapamycin, the drug that was given first alternated between each mouse in order to counterbalance injection location. Minocycline was dissolved and diluted in saline for a concentration of 12.5mg/mL and administered at a dose of 50mg/kg. For the rapamycin preparation, a vehicle solution containing 5% polyethylene glycol 400 (Sigma-Aldrich) and 5% Tween 80 (Sigma-Aldrich) was first dissolved in saline.

Rapamycin was then dissolved in 4% ethanol and the vehicle solution for a final concentration of 0.75mg/mL and was administered at a dose of 3mg/kg. Minocycline and rapamycin were administered so each pup received approximatively 0.02mL per injection. Saline was administered at a similar dose, with each mouse also receiving approximately 0.02mL per saline injection. Following administration of all treatments, pups were left in their respective containers on the warmed heating pad (~35°C) to be monitored for 15 min prior to being returned to the home cage.

Ultrasonic Vocalization Recording Paradigm

We examined ultrasonic vocalizations (USVs) on PD12 using an isolation-induced paradigm to assess changes in vocalization production following seizure and treatment administration on PD10. Prior to the recording phase, all pups were transferred to a new housing cage with fresh bedding that was warmed with a heating pad to an ambient temperature of ~35°C. During recording, one at a time pups were placed into a separate housing pan placed within an acrylic sound-attenuating chamber. Vocalizations were recorded for 2 min for each pup. The recording apparatus consisted of a condenser microphone (CM16/CMPA, Avisoft Bioacoustics, Germany) which was connected to an ultrasound-recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics) and recorded all USVs on a continuous spectrum from 0 to 125kHz. Following recording, pups were placed back into the warmed housing pan with littermates. This procedure was repeated in sequence until all pups were recorded, and upon completion all pups were returned to their original home cage. The total number of recording sessions allowed for pups to not be separated from their home cage and mothers for longer than 30 minutes.

Ultrasonic Vocalization Analysis

Avisoft SASLab Pro software (Avisoft Bioacoustics, Germany) was utilized to convert all USV files into spectrograms, by automatically detecting calls via threshold-based algorithms and programmed hold time mechanisms. Spectrograms were created using a fast Fourier transformation (FFT) with the following parameters held constant for all USV files: FFT length = 1024, time window overlap = 75% (100% Frame, Hamming Window), frequency resolution = 488 Hz, time resolution = 1 ms. Duration, peak frequency, fundamental frequency, and amplitude were collected and analyzed for each group of mice. All calls were also manually identified by an experimenter blind to group using a previously described classification scheme (Scattoni et al., 2008). Calls were designated as 1 of 10 distinct types based on internal pitch changes, lengths, and shapes of individual calls (complex, harmonics, two-syllable, upward, downward, chevron, shorts, composite, frequency steps, flat).

RNA Isolation and qRT-PCR for Cytokine Analysis

Hippocampal expression of cytokines was examined by isolating RNA and conducting quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Following euthanization via rapid decapitation on PD12 (4 hours following USV testing), the hippocampus was dissected from the brain, rinsed in 1X PBS, placed on dry ice, and stored at -80°C until processed. Left hippocampal samples from each animal were individually homogenized and total RNA was isolated from samples using the RNeasy Mini Kit according to established protocols (Qiagen, Germany). Samples were run on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) to ensure adequate concentration (> 40ng/uL) and purity (260/280 = 1.9-2.1) of all samples.

Isolated RNA was reverse transcribed into single-stranded complementary DNA using the High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems, Carlsbad, CA). The thermocycler protocol was as follows: 25˚C for 10 min, 37˚ for 120 min, 85˚C for 5 min, and held at a temperature of 4˚C until further processing. Gene expression was determined by qRT-PCR utilizing TaqMan probe and primer chemistry on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Gene-specific measurements of each cDNA sample were run in triplicate in a 384-well plate, along with the endogenous control gene (β-actin) used for normalization. Males and females were analyzed separately. All groups were compared to control/saline-saline animals. The relative expression levels of each gene (IL-1β, IL-6, TNFα) were quantified using the comparative threshold ($2^{-\Delta\Delta Ct}$) method of quantification.

Western Blotting Analysis

Western blotting was performed to examine the effect of seizures and subsequent treatment administration on inflammatory markers and mTOR pathway protein expression. Animals were euthanized via rapid decapitation on PD12 and the hippocampus dissected with identical procedures as described for qRT-PCR. The right hippocampus from each animal was utilized for all western blotting. Tissue was processed as previously described to extract whole homogenate samples and crude synaptosomes (Lugo et al., 2008). Briefly, individual right hippocampal samples were homogenized in ice-cold homogenization buffer containing 0.32M sucrose, 1mM EDTA, 5mM HEPES, and a protease inhibitor cocktail (P8340, Sigma, USA). Total homogenate or crude synaptosomes were run through 8-12% SDS-PAGE gels and transferred

overnight to Hybond-P polyvinyl difluoride membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were incubated for 1 h at room temperature in a blocking solution consisting of 5% nonfat milk diluted in 1X Tris buffered saline (50mM Tris-HCl, pH = 7.4, 150mM NaCl) with 0.1% Tween (1X TTBS) and 1mM Na_3VO_4. Membranes were then incubated overnight at 4ºC on a Hoeffer rocker II with primary antibodies (phosphorylated Akt, Akt, p70S6K, S6, phosphorylated S6[s235/236], phosphorylated S6[s240/244], ionized calcium binding adaptor molecule 1 [Iba1], glial fibrillary acidic protein [GFAP]) in 5% milk in 1X TTBS (See Table 1 for antibody specifics). Following incubation in primary antibodies, membranes were washed in 1X TTBS 3 times (5 min each wash), and then incubated with horseradish peroxidase-labeled secondary antibodies in a milk solution (1:20,000) for 1hr. Secondary antibodies were either anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Cell Signaling Technology, Boston, MA, USA). Membranes were washed again (3 x 5 min) in 1X TTBS, and then incubated in GE ECL Prime (GE Healthcare, Piscataway, NJ, USA) for 5 min at room temperature.

Chemiluminescent immunoreactive bands were imaged with a digital western blot imaging system (ProteinSimple, Santa Clara, CA, USA) and the optical density of immunoreactive bands were subsequently measured with ProteinSimple AlphaView software. Measurements from protein bands of interest were normalized to endogenous actin levels for each tissue sample, with all groups being normalized to the control group per blot (control/saline-saline group). To obtain measurements of % total phosphorylated S6 (pS6[235,236], pS6[240,244]), the ratio of pS6 at each phosphorylation site in relation to total hippocampal S6 protein levels was quantified.

Table 1. Antibody specifics for western blotting.

Sample Preparation	Primary Antibody	Concentration/ Manufacturer	Secondary Antibody	Concentration/ Manufacturer	Concentration/ Loading Standard
Total Hippocampal Homogenate	Akt	1:500 Cell Signaling	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
	pAkt (s473)	1:500 Cell Signaling	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
	p70S6K	1:500 Cell Signaling	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
	S6	1:500 Cell Signaling	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
	pS6 (s235,236)	1:500 Cell Signaling	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
	pS6 (s240,244)	1:500 Cell Signaling	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
	Iba1	1:250 Wako Chemicals	Rabbit IgG	1:20000 Cell Signaling	1:1000 Actin
Hippocampal Synaptosome	GFAP	1:500 Cell Signaling	Mouse IgG	1:20000 Cell Signaling	1:1000 Actin

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7 software (La Jolla, CA, USA) or SPSS 25.0 (IBM, USA). The male and female sample sizes for USVs, qRT-PCR, and western blotting are in Table 2 and 3, respectively. Changes in weight from PD10 until the time of tissue collection on PD12 was analyzed with a repeated-measures analysis of variance (ANOVA) with a within subject factor of timepoint (PD10, PD12). Quantitative USV results were evaluated using a two-way (Seizure administration [control, seizure] x Treatment [saline-saline, minocycline-saline, rapamycin-saline, minocycline-rapamycin]) ANOVA. Qualitative differences in the types of USVs emitted between all male and female groups was analyzed using a Pearson Chi-Square, followed by individual *z*-tests to examine individual group differences between the control and seizure groups within each treatment group. For analysis of cytokines with qRT-PCR, the

comparative threshold method of quantification was utilized to determine relative gene expression levels, with all groups normalized to the control/saline-saline group followed by analysis with two-way ANOVAs.

Table 2. Sample sizes for behavioral testing, qRT-PCR, and western blotting in male mice. Mino., minocycline; Rapa., rapamycin.

Measure	Gene/ protein of interest	Control				Seizure			
		Saline	Mino.	Rapa.	Mino. + Rapa.	Saline	Mino.	Rapa.	Mino. + Rapa.
Ultrasonic vocalizations	-	19	19	19	20	18	20	15	19
qRT-PCR	IL-1β	7	8	7	8	7	7	7	7
	IL-6	8	8	8	8	8	8	8	8
	TNFα	8	8	8	8	8	8	8	8
Western blotting	p70S6K	8	8	8	8	8	8	8	6
	S6	8	8	8	8	8	8	8	7
	pS6(235,236)	8	8	8	8	8	8	8	7
	% total pS6(235,236)	8	8	8	8	8	8	8	7
	pS6(240,244)	8	8	8	8	8	8	8	7
	% total pS6(240,244)	8	8	8	8	8	8	8	7
	Akt	8	8	8	8	8	8	8	7
	pAkt	8	8	8	8	8	8	8	7
	% total pAkt	8	8	8	8	8	8	8	7
	Iba1	7	7	7	7	7	7	7	7
	GFAP	8	8	8	8	8	8	8	8

For western blotting, two-way ANOVAs were utilized, with all groups being normalized to the control/saline-saline group average per blot. For USV, gene, and protein analysis,

males and females were analyzed separately. Any significant interactions were followed by creating unique group identifiers for each group combination and examined using Tukey's HSD *post-hoc* comparisons. Differences in lettering on all graphs indicate significance between groups at the level of at least $p < 0.05$ for all comparisons. All data are expressed as mean ± standard error of the mean (SEM).

Table 3. Sample sizes for behavioral testing, qRT-PCR, and western blotting in female mice. Mino., minocycline; Rapa., rapamycin.

Measure	Gene/ protein of interest	Control				Seizure			
		Saline	Mino.	Rapa.	Mino. + Rapa.	Saline	Mino.	Rapa.	Mino. + Rapa.
Ultrasonic vocalizations	-	23	24	22	24	21	20	22	19
qRT-PCR	IL-1β	8	8	8	8	8	8	8	8
	IL-6	8	8	8	8	8	8	8	8
	TNFα	8	8	8	8	8	8	8	8
Western blotting	p70S6K	7	7	7	7	7	7	7	7
	S6	8	8	8	8	8	8	8	7
	pS6(235,236)	8	8	8	8	8	8	8	7
	% total pS6(235,236)	8	8	8	8	8	8	8	7
	pS6(240,244)	8	8	8	8	8	8	8	7
	% total pS6(240,244)	8	8	8	8	8	8	8	7
	Akt	8	8	8	8	8	8	8	7
	pAkt	8	8	8	8	8	8	8	7
	% total pAkt	8	8	8	8	8	8	8	7
	Iba1	6	6	6	6	6	6	6	6
	GFAP	8	8	8	8	8	8	8	7

CHAPTER FOUR

Results

Animal Weights

Measurements of weight were obtained at the time of injection on postnatal day (PD) 10 and prior to tissue collection on PD12. A repeated-measures analysis of variance (ANOVA) with a within subject factor of "timepoint" (PD10, PD12) was utilized to analyze weight, with analyses divided by sex. In males, there was a significant within subject effect of timepoint, with mice weighing more at the time of tissue collection on PD12 ($F[1,72] = 261.75$, $p < 0.001$). In addition, treatment significantly interacted with timepoint ($F[3,72] = 22.71$, $p < 0.001$). Tukey's *post-hoc* analyses demonstrated that treatment significantly impacted weight measurements on PD12, specifically with mice treated with rapamycin or the combined treatment having reduced weight compared to saline-treated mice, as well as rapamycin-treated mice having reduced weight compared to minocycline-treated mice ($p < 0.05$). Seizure administration also interacted with timepoint ($F[1,72] = 22.54$, $p < 0.001$), however, Tukey's *post-hoc* analyses revealed no discernable effects on weight at either timepoint. No three-way interaction between treatment, seizure administration, and timepoint was detected ($F[3,72] = 1.40$, $p = 0.25$). Between-subjects effects revealed a significant main effect of treatment on weight ($F[3,72] = 3.33$, $p < 0.05$). Tukey's *post-hoc* analyses demonstrated that mice treated with rapamycin or the combined treatment had reduced weight compared to saline-treated mice, as well as rapamycin-treated mice had reduced weight compared to minocycline-

treated mice ($p < 0.05$). There was no significant between-subjects main effect of seizure administration on weight ($F[1,72] = 0.87$, $p = 0.36$). A two-way interaction between treatment and seizure administration was detected ($F[3,72] = 3.15$, $p < 0.05$). Given the significant interaction, animals were subdivided into 8 groups for *post-hoc* analyses: control/saline-saline, control/minocycline-saline, control/rapamycin-saline, control/minocycline-rapamycin, seizure/saline-saline, seizure/minocycline-saline, seizure/rapamycin-saline, seizure/minocycline-rapamycin. Tukey's *post-hoc* analyses revealed that control/saline-saline male mice had significantly increased weight compared to several groups (seizure/saline-saline, control/rapamycin-saline, seizure/rapamycin-saline, control/minocycline-rapamycin) (Fig. 3A).

In female mice, there was also a significant within subject effect of timepoint, with mice weighing more at the time of tissue collection on PD12 ($F[1,94] = 355.09$, $p < 0.001$). In addition, treatment significantly interacted with timepoint ($F[3,94] = 30.12$, $p < 0.001$). Tukey's *post-hoc* analyses demonstrated that treatment significantly impacted weight measurements on PD12, specifically with female mice given the combined treatment weighing less than saline-treated female mice ($p < 0.05$). Seizure administration also interacted with timepoint in female mice ($F[1,94] = 15.52$, $p < 0.001$), however, Tukey's *post-hoc* analyses revealed no discernable effects on weight at either timepoint. There was no three-way interaction between treatment, seizure administration, and timepoint for female mice ($F[3,94] = 1.31$, $p = 0.28$). No between-subjects effect of treatment ($F[3,94] = 1.07$, $p = 0.37$), seizure administration ($F[1,94] = 0.73$, $p = 0.39$), or a two-way interaction between treatment and seizure administration ($F[3,94] = 1.17$, $p = 0.33$) were detected in female mice (Fig. 3B).

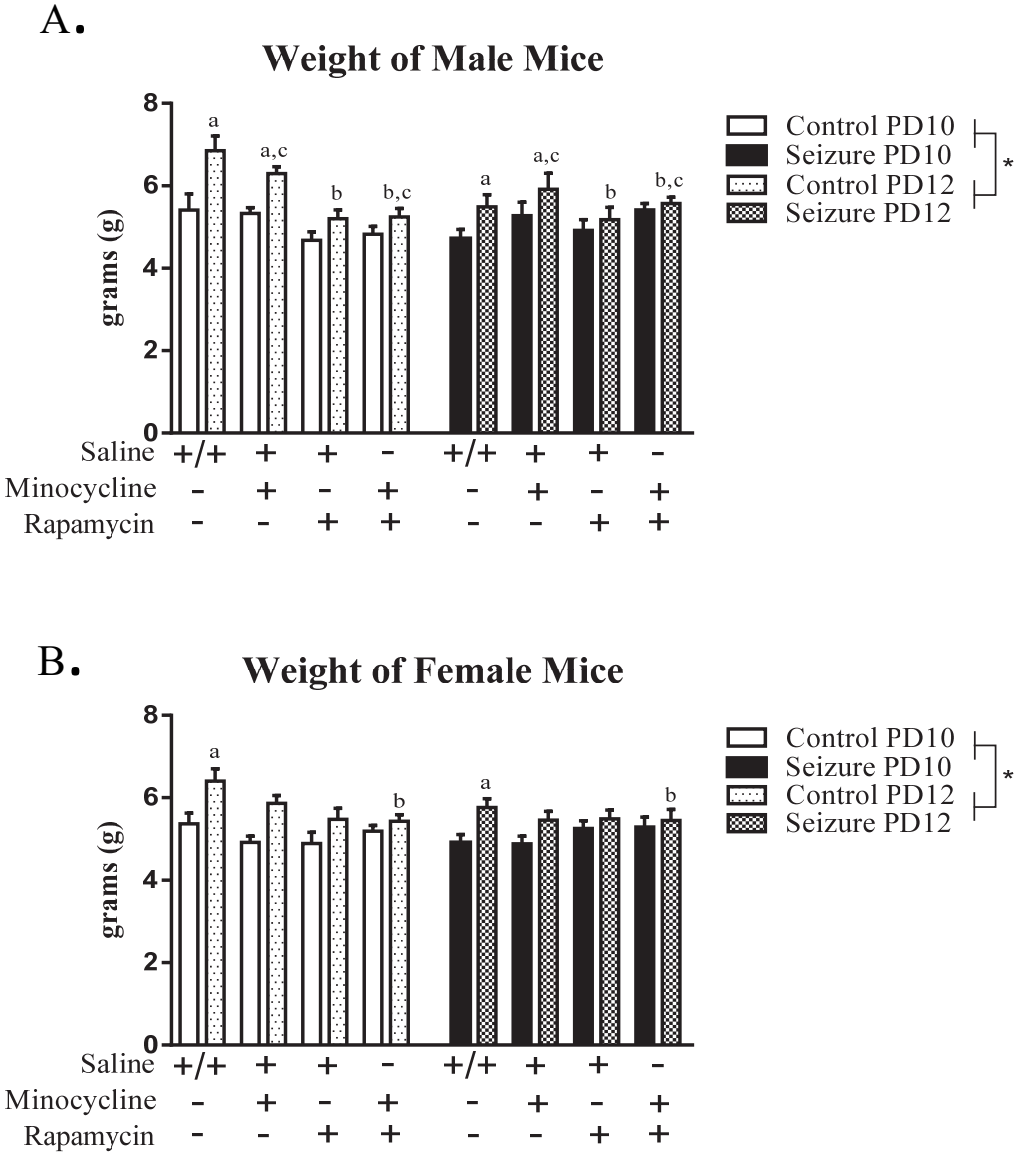


Figure 3. Weights of male and female mice. At the time of tissue collection on PD12, male mice that were administered rapamycin or the combined treatment weighed less than saline-treated mice, as well as rapamycin-treated mice had reduced weight compared to minocycline-treated male mice (A). In female mice, those given the combined treatment weighed significantly less than saline-treated mice on PD12 (B). There was a significant effect of time in both sexes, as both male and female mice gained weight from PD10 to PD12 (A,B). Data are expressed as mean ± standard error of the mean (SEM), * $p < 0.05$. Differences in lettering indicate significance between groups at the level of $p < 0.05$.

Ultrasonic Vocalization Results

Quantitative USV Parameters in Male Mice

Following seizures and treatment administration on PD10, ultrasonic vocalizations (USVs) of all mice were recorded on PD12 to identify potential deficits in early-life communicative behaviors. A total of 8 male mice did not vocalize and were removed from all USV analysis. The male mice that did not vocalize belonged to the following groups: control/saline-saline: n = 2; control/minocycline-saline: n = 1; seizure/saline-saline: n = 1; seizure/minocycline-saline: n = 1; seizure/rapamycin-saline: n = 2; seizure/minocycline-rapamycin: n = 1. A two-way ANOVA was utilized to examine the impact of seizure and treatment administration on quantitative parameters of the USVs. The USVs emitted from male and female mice were analyzed separately to parallel how gene and protein expression levels were examined and quantified. In male mice, a two-way ANOVA did not detect a significant main effect of seizure administration ($F[1,141] = 0.33$, $p = 0.57$) on the quantity of calls emitted. There was a significant effect of treatment on quantity of calls emitted in male mice ($F[3,141] = 5.72$, $p < 0.05$), with rapamycin-treated mice emitting significantly reduced calls compared to minocycline-treated mice and those that received the combined treatment of minocycline and rapamycin ($p < 0.05$) (Fig. 4A). No interaction was detected between seizure administration and treatment for quantity of calls in male mice ($F[3,141] = 0.34$, $p = 0.80$).

In addition to the quantity of USVs emitted, quantitative spectral properties of calls were examined. A two-way ANOVA did not detect a significant effect of seizure administration on average duration of USVs ($F[1,141] = 0.03$, $p = 0.85$). However, there

was a significant main effect of treatment (F[3,141] = 3.01, $p < 0.05$), with rapamycin-treated mice emitting USVs of significantly reduced duration compared to minocycline-treated mice and those that received the combined treatment ($p < 0.05$) (Fig. 4B). There was not a significant interaction between seizure administration and treatment for the duration of USVs (F[3,141] = 0.44, $p = 0.72$). No significant main effects for seizure administration (F[1,141] = 0.53, $p = 0.47$) or treatment (F[3,141] = 1.94, $p = 0.13$) were detected in the average peak frequency of USVs emitted in male mice. There was also no significant interaction between seizure administration and treatment for average peak frequency of calls (F[3,141] = 1.72, $p = 0.17$) (Fig. 4C). We also examined the average fundamental frequency of USVs and found that seizure administration similarly had no effect in male mice (F[1,141] = 0.02, $p = 0.90$). However, there was a significant main effect of treatment for average fundamental frequency (F[3,141] = 3.09, $p = 0.03$), with rapamycin-treated mice emitting USVs of significantly reduced fundamental frequency compared to mice given minocycline ($p < 0.05$) (Fig. 4D). No interaction between seizure administration and treatment was detected in the fundamental frequency of USVs in male mice (F[3,141] = 2.00, $p = 0.12$). There were also no significant main effects for seizure administration (F[1,141] = 1.69, $p = 0.20$) or treatment (F[3,141] = 0.91, $p = 0.44$) in the average amplitude of USVs emitted by male mice. There was a significant interaction between seizure administration and treatment for average amplitude (F[3,141] = 3.92, $p < 0.05$). Given the significant interaction, male mice were subdivided into 8 groups for *post-hoc* analysis. Tukey's *post-hoc* analyses showed that male control mice that received rapamycin treatment had significantly increased amplitude compared to male control mice that received saline ($p < 0.05$) (Fig. 4E).

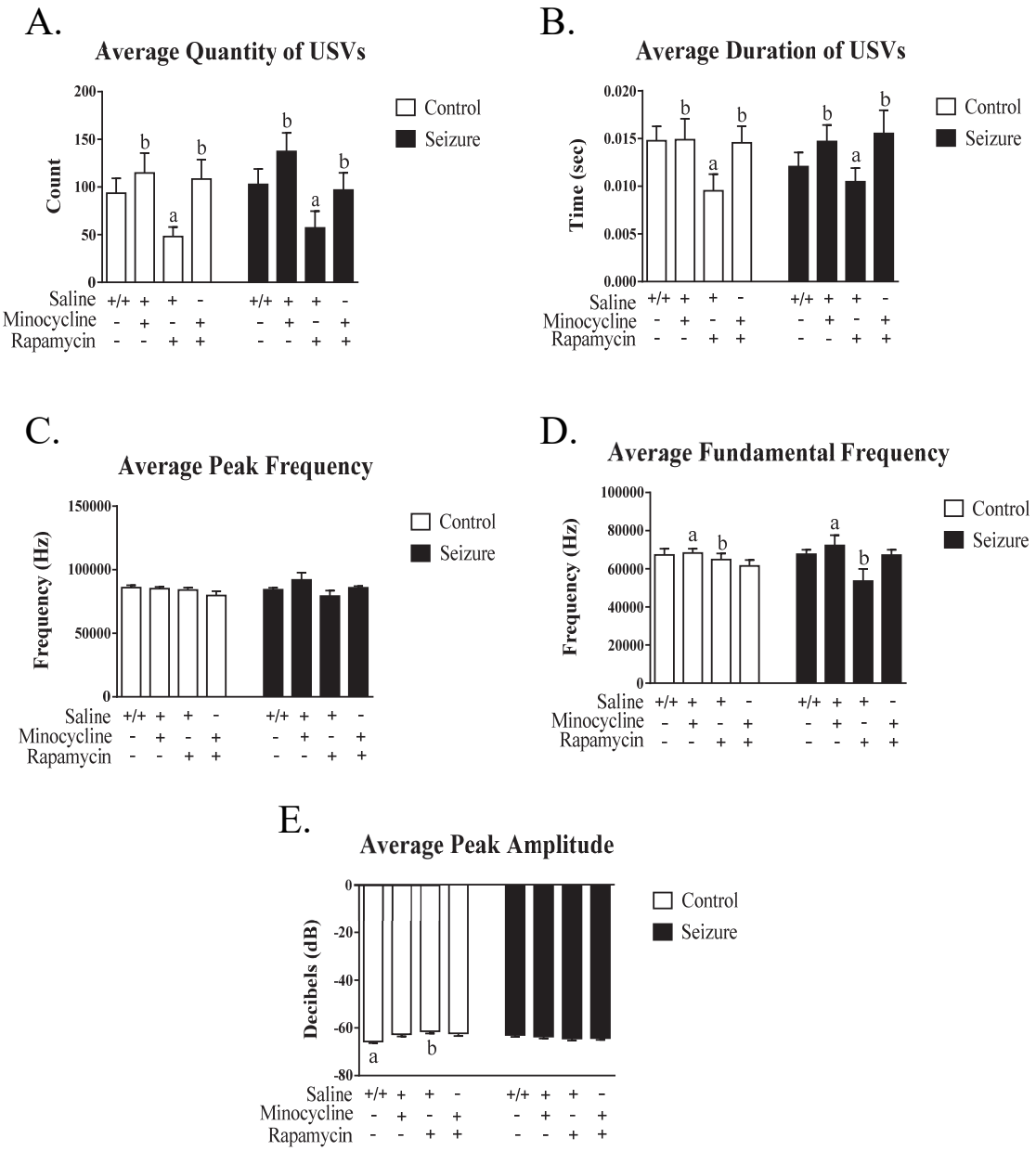


Figure 4. Quantitative parameters of ultrasonic vocalizations (USVs) in male mice. Flurothyl seizures did not impact the quantity of USVs emitted in male mice, however, rapamycin-treated male mice emitted significantly less USVs compared to minocycline-treated mice and those that received the combined treatment (A). Rapamycin-treated mice also emitted USVs of significantly reduced duration compared to minocycline-treated mice and those that received the combined treatment (B). No significant effects were detected in the average peak frequency of USVs in male mice (C). Male mice that were treated with rapamycin emitted USVs with significantly reduced fundamental frequency compared to minocycline-treated mice (D). Control mice that received rapamycin emitted calls of significantly increased amplitude compared to control saline-treated mice (E). Data are expressed as mean ± standard error of the mean (SEM). Differences in lettering indicate significance between groups at the level of $p < 0.05$.

Quantitative USV Parameters in Female Mice

A total of 7 female mice did not vocalize and were removed from all USV analysis. The female mice that did not vocalize belonged to the following groups: control/rapamycin-saline: n = 1; control/minocycline-rapamycin: n = 2; seizure/saline-saline: n = 3; seizure/rapamycin-saline: n = 1. In female mice, a two-way ANOVA revealed a significant main effect of seizure administration (F[1,167] = 3.93, $p < 0.05$), with female seizure mice emitting significantly more USVs than female control mice ($p < 0.05$). There was also a main effect of treatment for quantity of calls (F[3,167] = 8.66, $p < 0.001$), with rapamycin-treated mice emitting significantly less calls than all other groups ($p < 0.05$). There was no interaction between seizure administration and treatment for quantity of USVs emitted in female mice (F[3,167] = 0.45, $p = 0.72$) (Fig. 5A).

When examining the average duration of USVs in female mice, there was no significant effect of seizure administration (F[1,167] = 1.97, $p = 0.16$). There was a significant effect of treatment (F[3,167] = 2.89, $p < 0.05$), with rapamycin-treated mice emitting USVs of significantly reduced average duration compared to minocycline-treated mice ($p < 0.05$) (Fig. 5B). No significant interaction between seizure administration and treatment was detected for average duration of USVs in female mice (F[3,167] = 0.02, $p = 1.00$). For the average peak frequency of USVs, there was no significant main effect of seizure administration (F[1,167] = 0.54, $p = 0.47$), treatment, (F[3,167] = 0.81, $p = 0.49$), or a significant interaction between the two factors in female mice (F[3,167] = 0.81, $p = 0.49$) (Fig. 5C). There were also no significant main effects for average fundamental frequency of USVs for seizure administration (F[1,167] = 0.07, $p = 0.79$), treatment (F[3,167] = 1.81, $p = 0.15$), or an interaction between the two factors

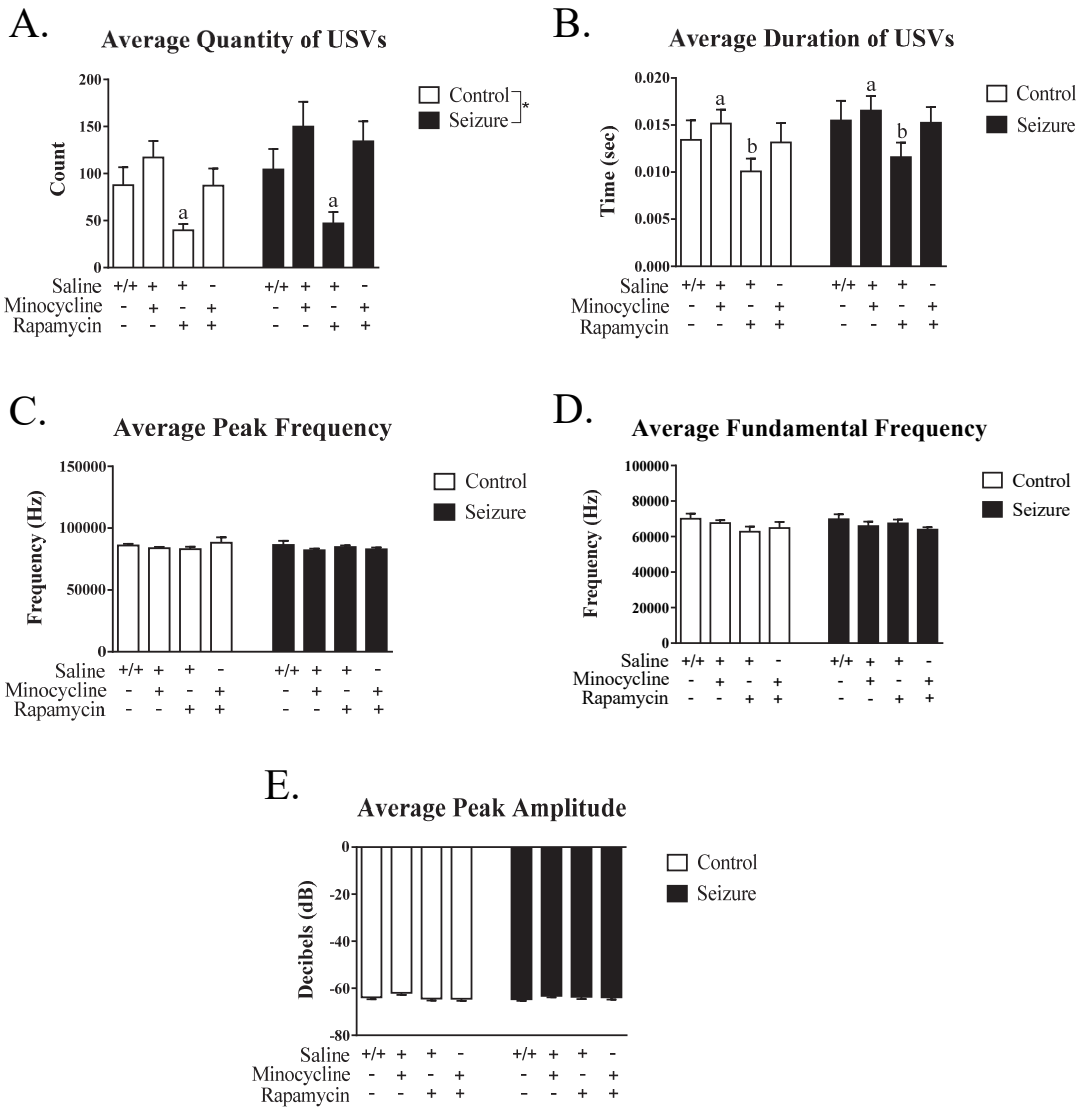


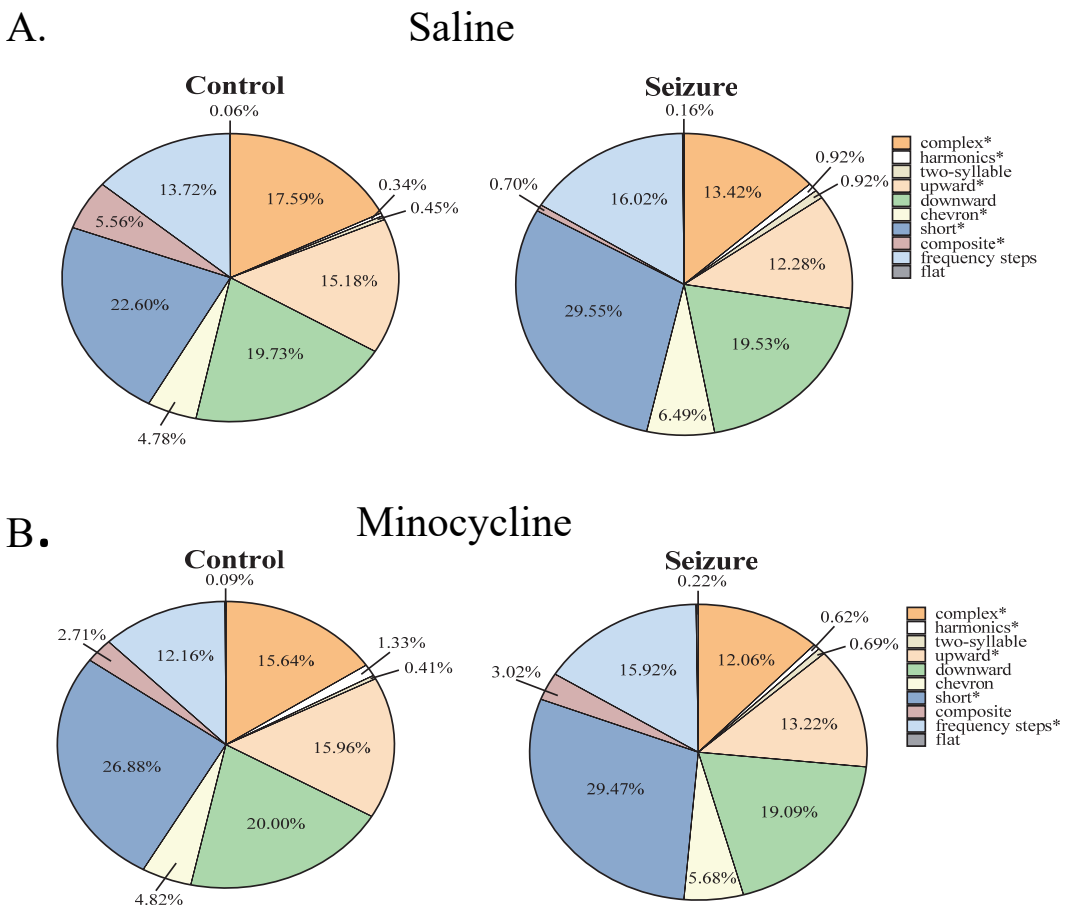
Figure 5. Quantitative parameters of ultrasonic vocalizations (USVs) in female mice. Administration of flurothyl seizures significantly increased USV quantity compared to control mice. In addition, rapamycin treatment in female mice resulted in significantly reduced USV quantity when compared to all other treatment groups (A). Seizures had no effect on the average duration of USVs, however, rapamycin-treated male mice emitted USVs of significantly reduced duration compared to minocycline-treated mice (B). No significant effects were detected in the average peak frequency (C), average fundamental frequency (D), or average peak amplitude (E) of USVs in female mice. Data are expressed as mean ± standard error of the mean (SEM), * $p < 0.05$. Differences in lettering indicate significance between groups at the level of $p < 0.05$.

(F[3,167] = 0.63, p = 0.60) (Fig. 5D). For the average amplitude of USVs in female mice, there was also no significant main effect of seizure administration (F[1,167] = 0.08, p = 0.78), treatment (F[3,167] = 1.64, p = 0.18), or an interaction between seizure administration and treatment (F[3,167] = 0.66, p = 0.58) (Fig. 5E).

Qualitative USV Parameters in Male Mice

In addition to quantitative parameters of USVs, qualitative differences in the types of calls emitted by each group was also examined. Ultrasonic vocalizations were identified as 1 of 10 distinct types based on changes in call length, pitch, and the shapes of individual calls (Scattoni et al., 2008). A Pearson Chi-Square revealed a significant population difference between the types of calls emitted between all 8 groups of male mice (X^2[63, N = 14,328] = 467.80, $p < 0.001$). Individual group differences were examined by performing separate z-tests to analyze the number of calls emitted between male control and seizure mice within each treatment group: control treatment (saline-saline), minocycline-treated (minocycline-saline), rapamycin-treated (rapamycin-saline), combined treatment (minocycline-rapamycin). In the male control treatment group, seizure mice emitted significantly reduced complex, upward, and composite calls and a significantly increased number of harmonics, chevron, and short calls compared to control male mice ($p < 0.05$) (Fig. 6A). In minocycline-treated male mice, seizure mice emitted significantly reduced complex and harmonic calls, and an increased amount of upward, short, and frequency step calls compared to control minocycline-treated mice ($p < 0.05$) (Fig. 6B). In rapamycin-treated male mice, seizure mice emitted a decreased amount of upward and downward calls, and an increased amount of complex, chevron, and frequency step calls compared to control rapamycin-treated mice ($p < 0.05$) (Fig.

6C). Lastly, in male mice that received the combined treatment of minocycline and rapamycin, seizure mice emitted reduced upward, downward, and chevron call types, as well as a significantly increased amount of short call types compared to control mice that received the combined treatment ($p < 0.05$) (Fig. 6D).



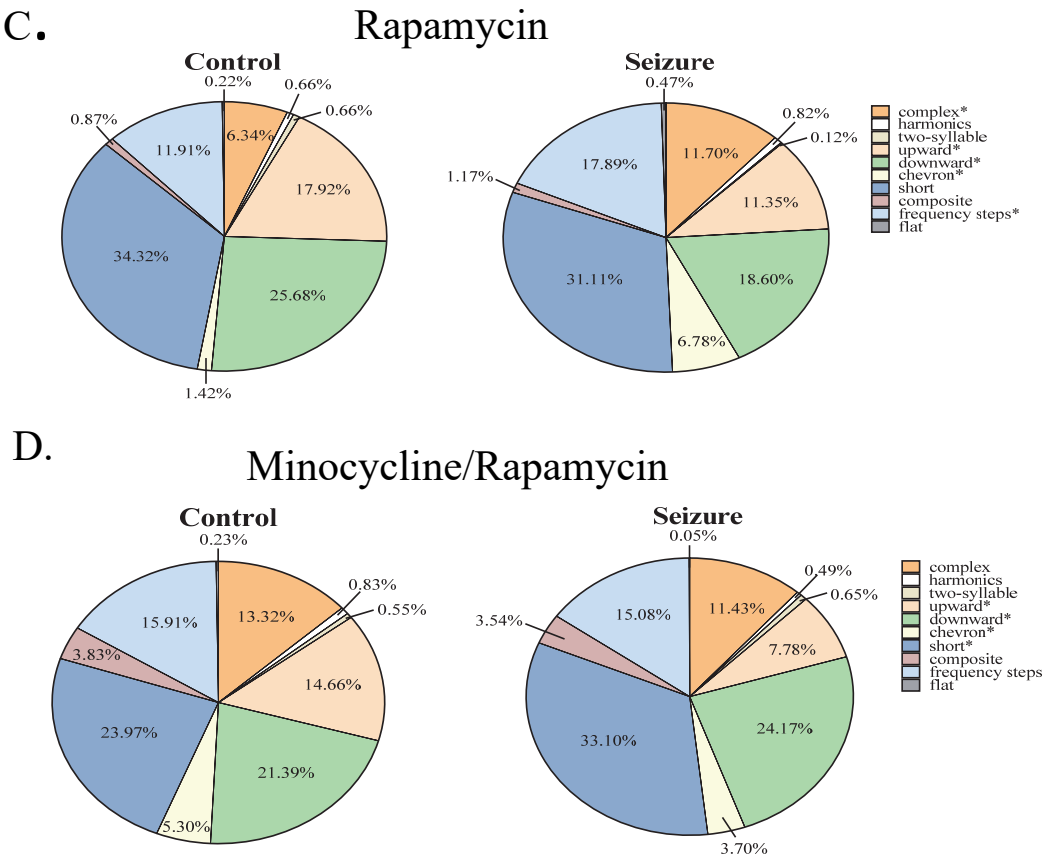
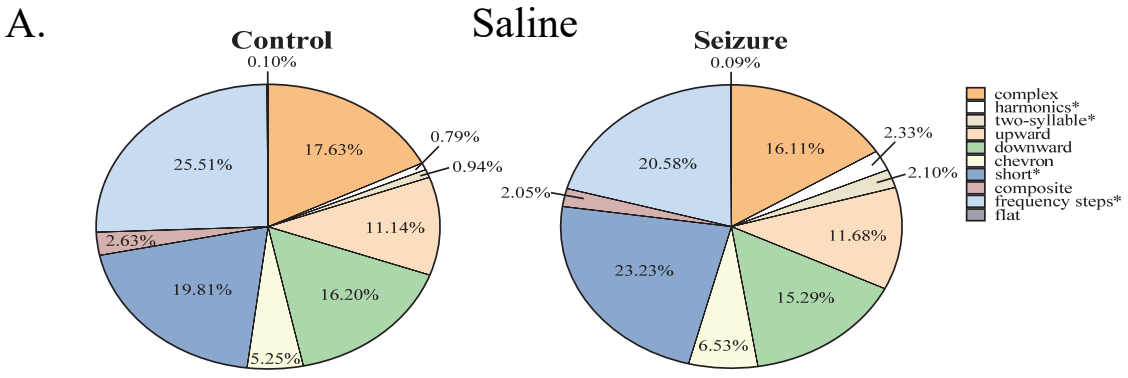


Figure 6. Call type utilization patterns in male mice. The types of calls emitted different significantly across all 8 groups of male mice (X^2[63, N = 14,328] = 467.80, $p < 0.001$). Differences in call type patterns between seizure and control mice were compared between each treatment group: saline-treated (A), minocycline-treated (B), rapamycin-treated (C), and those that received the combined treatment (minocycline/rapamycin) (D). Significant differences between control and seizure groups at the level of $p < 0.05$ are denoted by an asterisk (*).

Qualitative USV Parameters in Female Mice

A Pearson Chi-Square also revealed a significant population difference between the types of calls emitted between all 8 groups of female mice (X^2[63, N = 16,558] = 770.51, $p < 0.001$). Similar to the analyses performed in male mice, separate *z*-tests were conducted to analyze the number of calls emitted between female control and seizure

mice within each treatment group: control treatment (saline-saline), minocycline-treated (minocycline-saline), rapamycin-treated (rapamycin-saline), combined treatment (minocycline-rapamycin). In the female control treatment group, seizure mice emitted a significantly reduced amount of frequency step calls, as well as an increased number of harmonics, two-syllable, and short call types compared to female control mice in the control treatment group ($p < 0.05$) (Fig. 7A). In female minocycline-treated mice, seizure mice emitted a reduced number of downward and short calls, and an increased amount of complex, harmonics, two-syllable, chevron, and frequency step calls compared to minocycline-treated control mice ($p < 0.05$) (Fig. 7B). In female rapamycin-treated mice, seizure mice emitted a significantly reduced amount of short and composite calls, along with an increased number of complex and harmonic call types compared to control rapamycin-treated mice ($p < 0.05$) (Fig. 7C). In female mice that received the combined treatment, seizure mice had a significantly decreased amount of complex and composite call types, as well as emitted a significantly increased amount of two-syllable, chevron, and frequency step calls compared to control female mice that received the combined treatment ($p < 0.05$) (Fig. 7D).



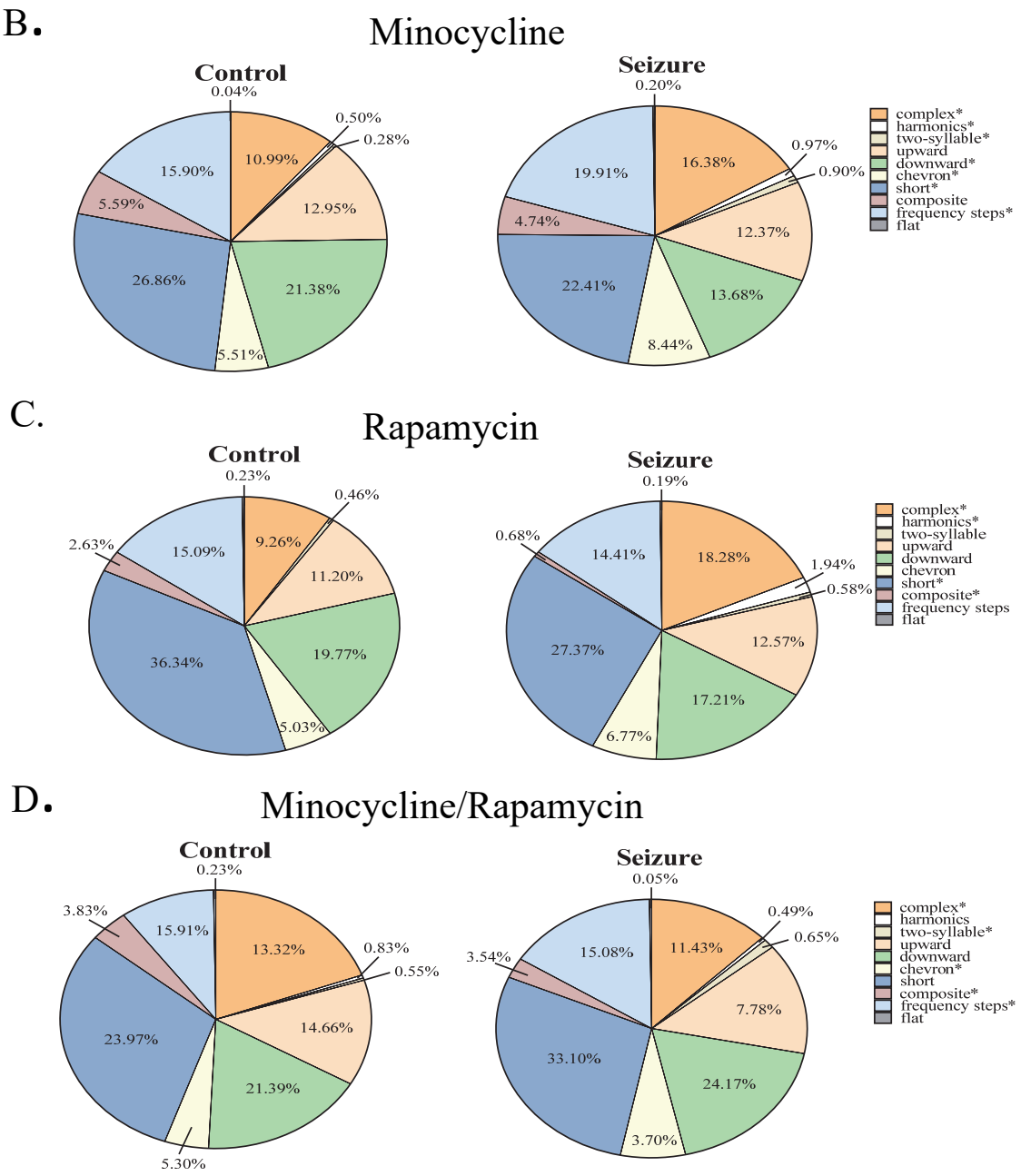


Figure 7. Call type utilization patterns in female mice. The types of calls emitted different significantly across all 8 groups of female mice ($X^2[63, N = 16,558] = 770.51$, $p < 0.001$). Differences in call type patterns between seizure and control mice were compared between each treatment group: saline-treated (A), minocycline-treated (B), rapamycin-treated (C), and those that received the combined treatment (minocycline/rapamycin) (D). Significant differences between control and seizure groups at the level of $p < 0.05$ are denoted by an asterisk (*).

Cytokine Expression Results

In male mice, seizures on PD10 did not significantly increase hippocampal gene expression of interleukin-1β (IL-1β) ($F[1,50] = 0.42$, $p = 0.52$), interleukin-6 (IL-6) ($F[1,50] = 0.48$, $p = 0.49$), or tumor necrosis factor-α (TNFα) ($F[1,50] = 0.28$, $p = 0.60$) on PD12. However, there was a significant main effect of treatment for IL-1β ($F[3,50] = 7.83$, $p < 0.001$), IL-6 ($F[3,50] = 7.10$, $p < 0.001$), and TNFα ($F[3,50] = 3.75$, $p < 0.05$) hippocampal gene expression levels in male mice (Fig. 8A-C). Mice that received minocycline or the combined treatment of minocycline and rapamycin had significantly increased hippocampal IL-1β expression compared to mice that received rapamycin or saline ($p < 0.05$) (Fig. 8A). No differences were detected in IL-β between the minocycline and the combined treatment group, or between the saline and rapamycin groups ($p > 0.05$). For IL-6, male mice that received minocycline or the combined treatment had significantly higher expression levels compared to saline-treated mice ($p < 0.05$). In addition, mice that received rapamycin had significantly decreased IL-6 expression compared to mice that received the combined treatment ($p < 0.05$) (Fig. 8B). *Post-hoc* analyses revealed no discernable effects between treatment groups for TNFα, apart from a trending increase in mice that received that combined treatment compared to rapamycin-treated mice ($p = 0.07$) (Fig. 8C). No significant interactions were detected between seizure administration and treatment for IL-1β ($F[3,50] = 0.55$, $p = 0.65$), IL-6 ($F[3,50]$ 0.19, $p = 0.90$), or TNFα ($F[3,50] = 0.06$, $p = 0.98$) hippocampal gene expression levels in male mice.

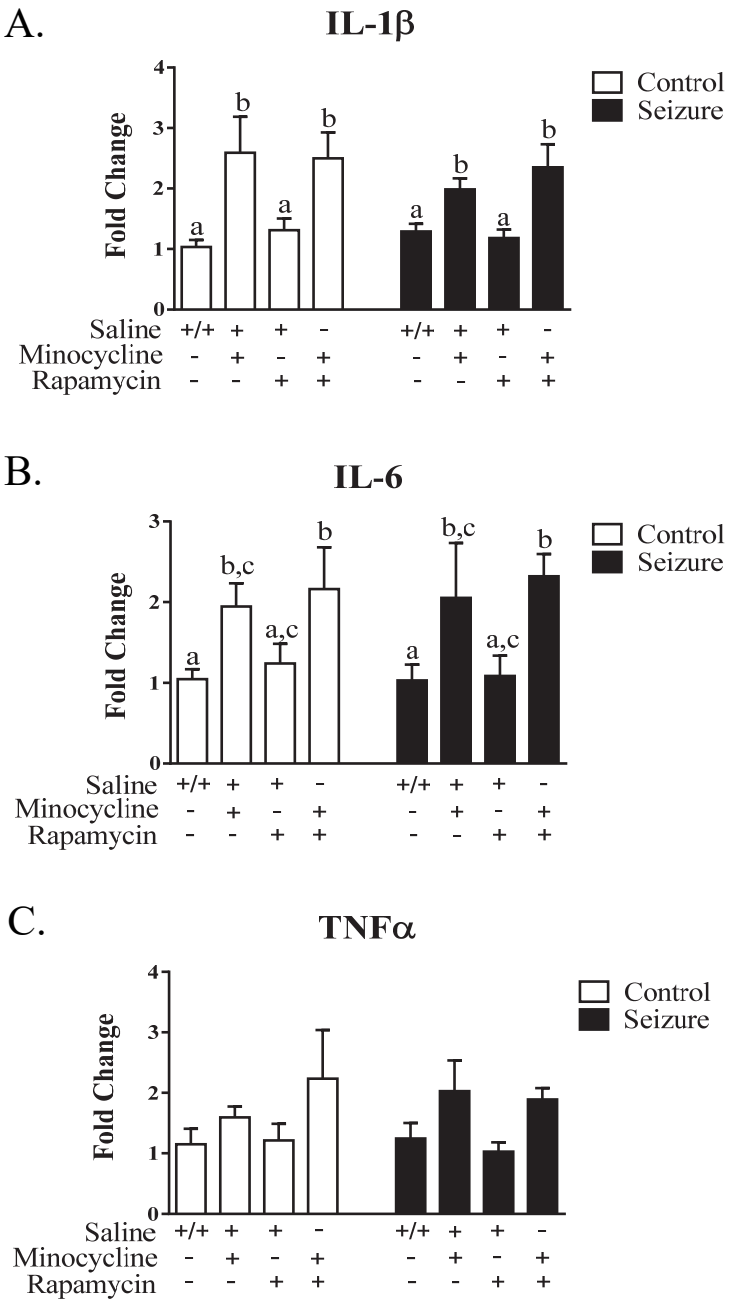
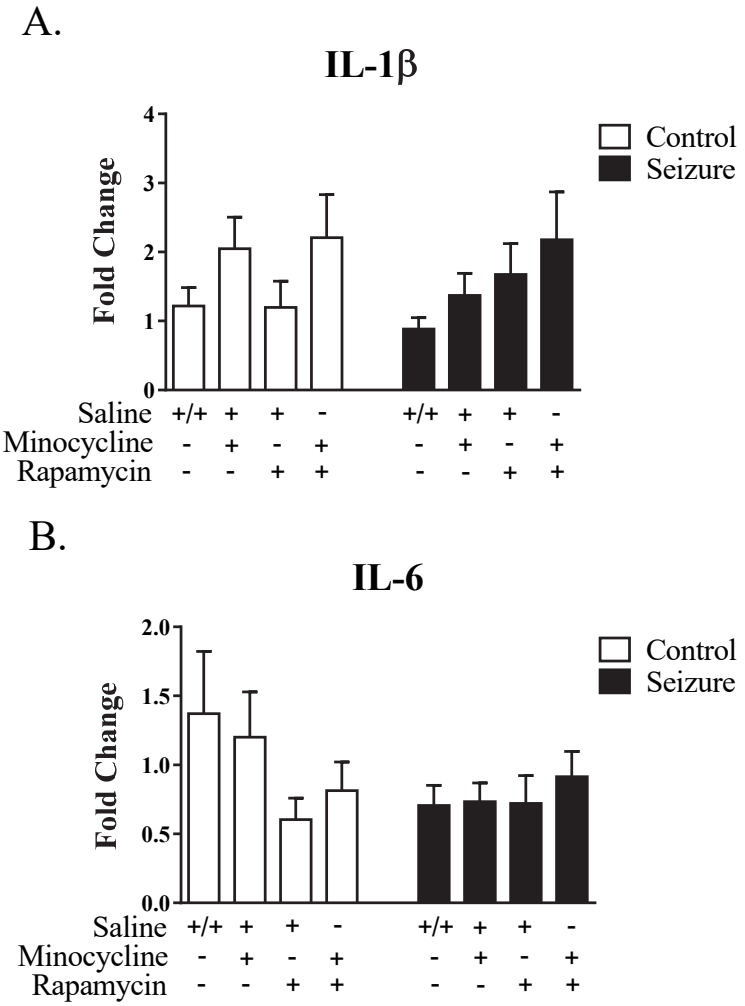


Figure 8. Hippocampal proinflammatory cytokine expression in male mice. Seizures did not significantly increase the expression of IL-1β, IL-6, or TNFα in male mice (A-C). A significant effect of treatment was detected for IL-1β, with minocycline-treated mice or those that received the combined treatment having significantly increased expression compared to mice that received rapamycin or saline (A). A significant effect of treatment was detected for IL-6, with mice that received minocycline or the combined treatment having significantly higher expression levels compared to saline-treated mice, along with rapamycin-treated mice having reduced IL-6 expression compared to mice that received the combined treatment (B). A significant effect of treatment was also detected for TNFα, however, no individual treatment group differences were detected (C). Gene expression measurements on individual samples were performed in triplicate. Data are expressed as mean ± standard error of the mean (SEM). Differences in lettering indicate significance between groups at the level of $p < 0.05$.

In female mice, seizures similarly did not result in enhanced hippocampal gene expression of IL-1β (F[1,56] = 0.20, p = 0.66), IL-6 (F[1,56] = 1.71, p = 0.20), or TNFα (F[1,56] = 1.10, p = 0.30). There were also no significant effects of treatment for IL-1β (F[3,56] = 2.27, p = 0.09), IL-6 (F[3,56] = 0.87, p = 0.46), or TNFα (F[3,56] = 1.30, p = 0.28) expression levels in female mice. No interactions were detected between seizure administration and treatment for IL-1β (F[3,56] = 0.59, p = 0.62), IL-6 (F[3,56] = 1.29, p = 0.29), or TNFα (F[3,56] = 0.53, p = 0.67) hippocampal expression levels in female mice (Fig. 9A-C).



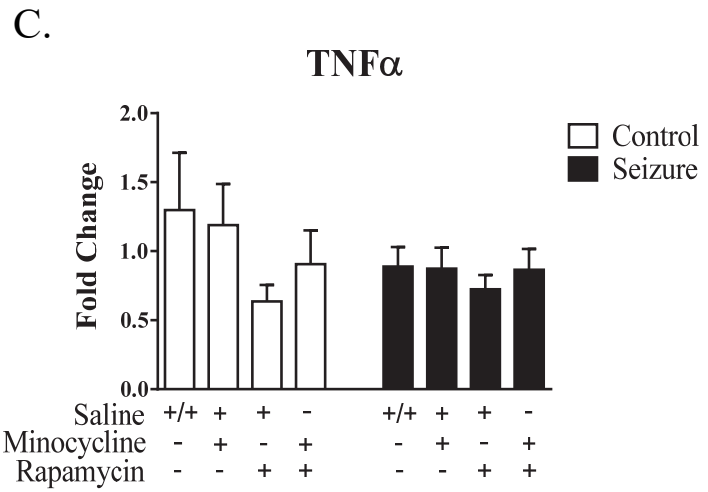


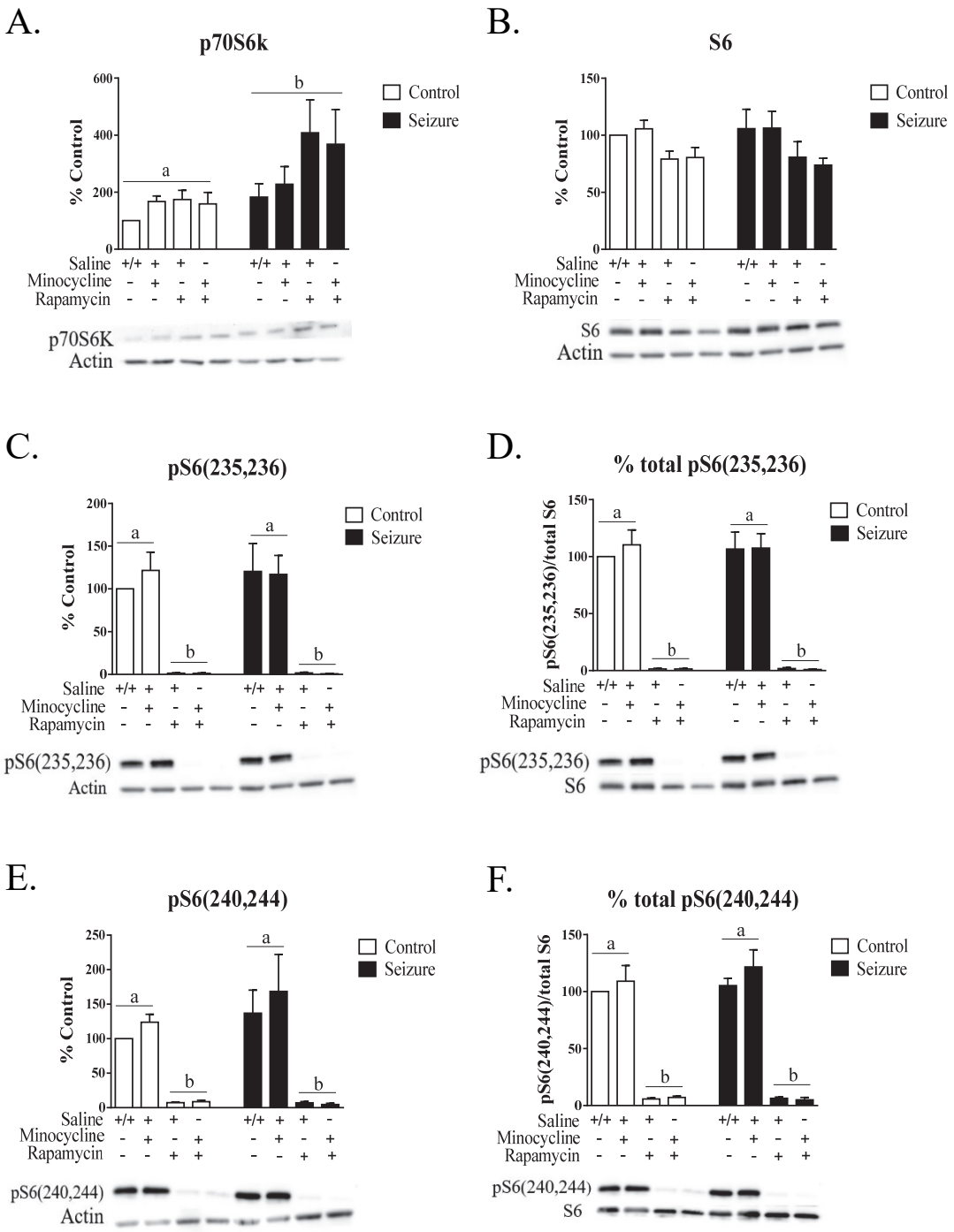
Figure 9. Hippocampal proinflammatory cytokine expression in female mice. Seizures and treatment administration did not result in any changes in IL-1β (A), IL-6 (B), or TNFα (C) expression. Gene expression measurements on individual samples were performed in triplicate. Data are expressed as mean ± standard error of the mean (SEM).

Western Blotting Results

Hippocampal Expression of mTOR Signaling Proteins

Western blotting was utilized to examine protein expression in components of the PI3K/Akt/mTOR pathway on PD12, two days after seizure and treatment administration. A two-way ANOVA revealed that seizures in male mice had significantly increased p70S6K expression ($F[1,46] = 13.48$, $p < 0.05$) (Fig. 10A). No other significant main effects for seizure administration were detected, including for S6($F[1,46] = 0.23$, $p = 0.63$), pS6(235,236) ($F[1,46] = 0.12$, $p = 0.74$), % total pS6(235,236) ($F[1,46] = 0.00$, $p = 1.00$), pS6(240,244) ($F[1,46] = 1.23$, $p = 0.27$), % total pS6(240,244) ($F[1,46] = 0.99$, $p = 0.33$), Akt ($F[1,46] = 0.07$, $p = 0.79$), pAkt ($F[1,46] = 3.61$, $p = 0.06$), and % total pAkt ($F[1,46] = 0.09$, $p = 0.76$) hippocampal protein expression (Fig. 10B-I). There was a significant main effect of treatment for S6 ($F[3,46] = 4.53$, $p < 0.05$), with rapamycin-

treatment mice having a trending decrease in S6 expression levels compared to mice administered minocycline ($p = 0.07$) (Fig. 10B). There was also a significant main effect of treatment for pS6(235,236) ($F[3,46] = 47.38$, $p < 0.001$) and % total pS6(235,236) ($F[3,46] = 91.57$, $p < 0.001$), with mice administered rapamycin or the combined treatment having significantly reduced expression levels compared to all other groups ($p < 0.001$) (Fig. 10C,D). In addition, there was a significant main effect of treatment for pS6(240,244) ($F[3,46] = 14.49$, $p < 0.001$) and % total pS6(240,244) ($F[3,46] = 88.74$, $p < 0.001$), with mice administered rapamycin or the combined treatment having significantly reduced expression levels compared to all other groups ($p < 0.001$) (Fig. 10E,F). There was also a significant main effect of treatment for pAkt ($F[3,46] = 12.15$ $p < 0.001$), with those administered the combined treatment having significantly decreased expression levels compared to saline-treated and minocycline-treated mice ($p < 0.05$). In addition, rapamycin-treated mice had significantly reduced pAkt expression compared to minocycline mice ($p < 0.001$) (Fig. 10H). No significant main effects of treatment were detected for p70S6K ($F[3,46] = 2.77$, $p = 0.05$), Akt ($F[3,46] = 1.29$, $p = 0.29$), or % total pAkt ($F[3,46] = 1.34$, $p = 0.27$) (Fig. 10A, G, I).


A.
p70S6k
% Control
Control
Seizure
Saline
Minocycline
Rapamycin
p70S6K
Actin
B.
S6
S6
Actin
C.
pS6(235,236)
pS6(235,236)
Actin
D.
% total pS6(235,236)
pS6(235,236)/total S6
pS6(235,236)
S6
E.
pS6(240,244)
pS6(240,244)
Actin
F.
% total pS6(240,244)
pS6(240,244)/total S6
pS6(240,244)
S6

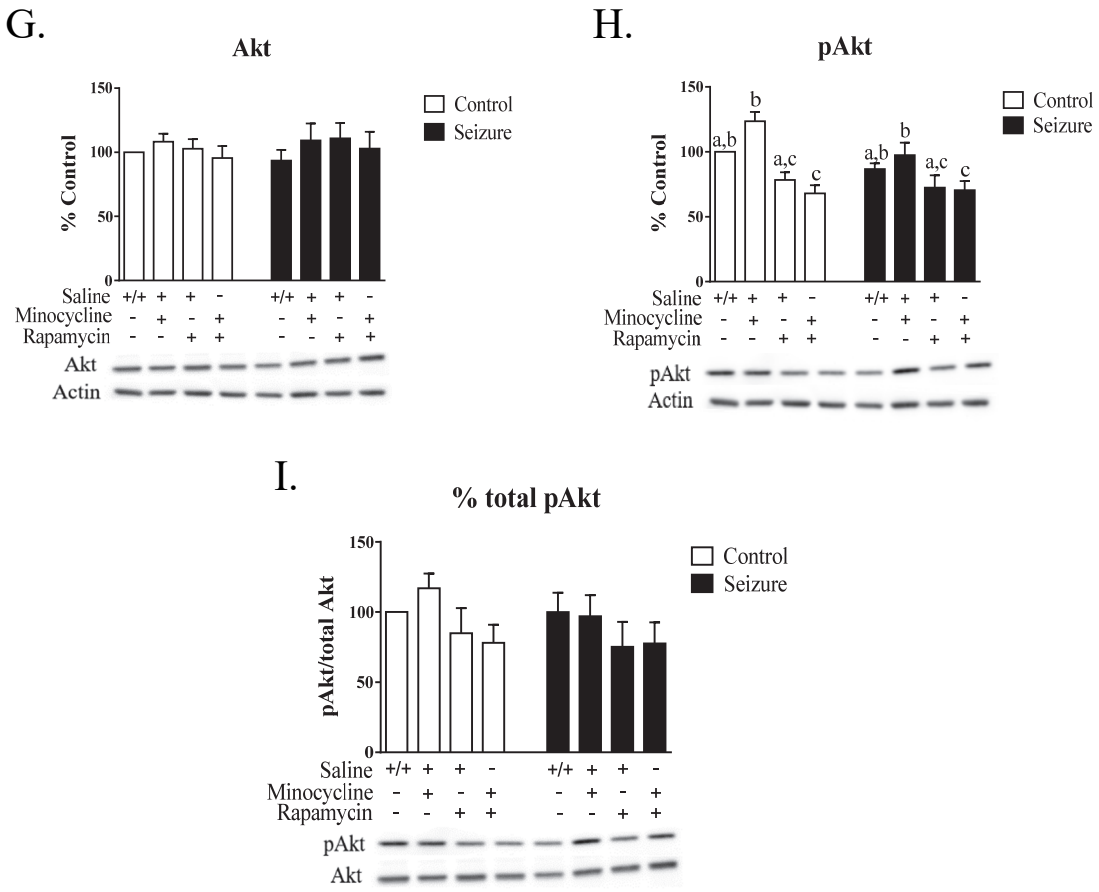


Figure 10. Hippocampal expression of mTOR signaling proteins in male mice. Seizures significantly increased p70S6K expression levels compared to controls (A). No effect of seizure administration or treatment was detected for S6 (B). Mice that received rapamycin or the combined treatment had significantly downregulated levels of pS6(235,236) (C), % total pS6(235,236) (D), pS6(240,244) (E), or % total pS6(240,244) (F). There was no effect of seizures or treatment on Akt (G) or % total pAkt (I) expression levels. There was a significant effect of treatment for pAkt, with those administered the combined treatment having significantly decreased expression levels compared to saline-treated and minocycline-treated mice, and rapamycin-treated mice having reduced expression compared to minocycline-treated mice (H). Data are expressed as mean ± standard error of the mean (SEM). Differences in lettering indicate significance between groups at the level of $p < 0.05$.

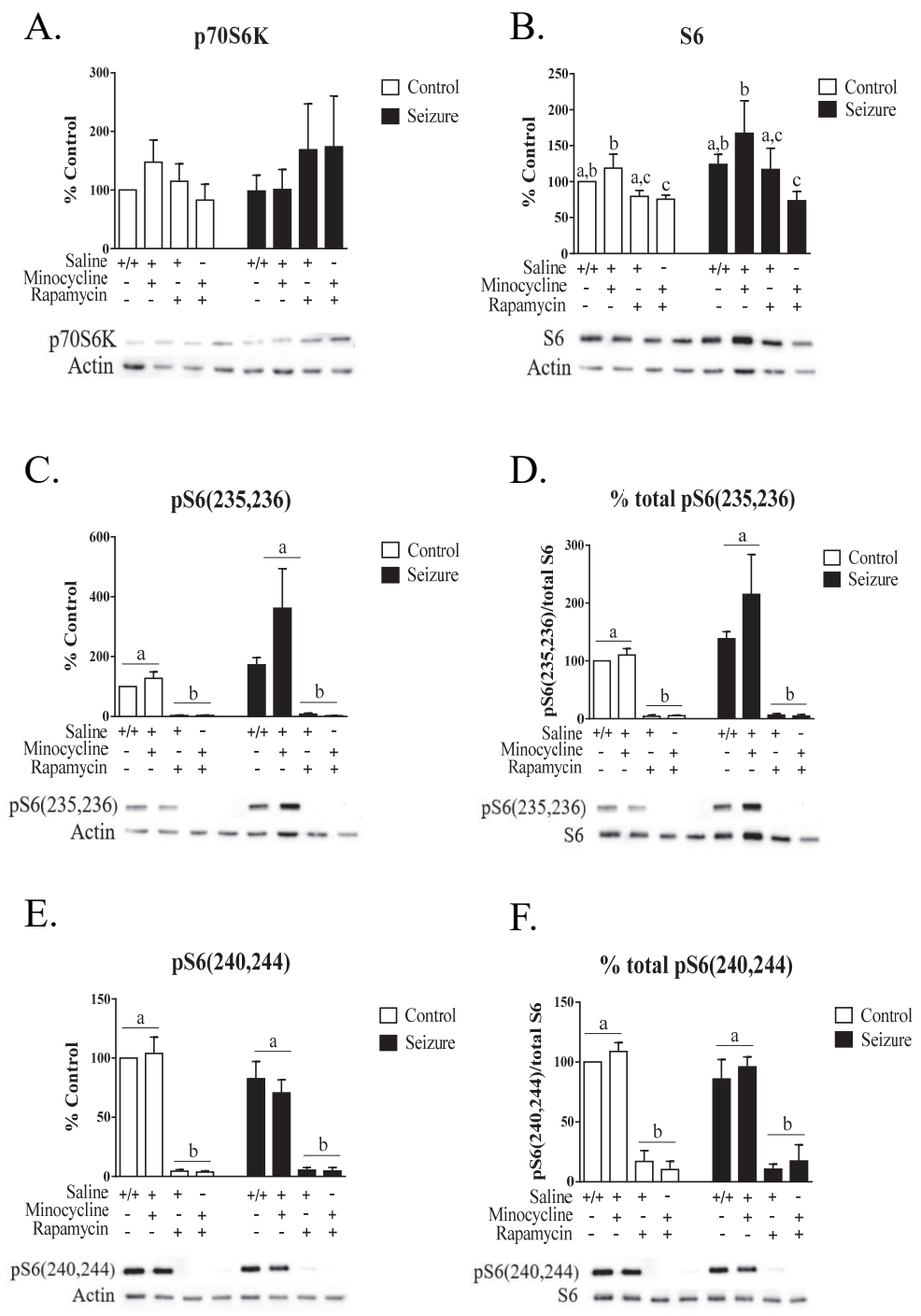
There were also no significant interactions between seizure administration and treatment for any of the examined proteins in male mice: p70S6K (F[3,46] = 1.16, p = 0.34), S6 (F[3,46] = 0.06, p = 0.98), pS6(235,236) (F[3,46] = 0.05, p = 0.99), % total

pS6(235,236) (F[3,46] = 0.05, p = 0.99), pS6(240,244) (F[3,46] = 0.55, p = 0.65), % total pS6(240,244) (F[3,46] = 0.64, p = 0.59), Akt (F[3,46] = 0.38, p = 0.77), pAkt (F[3,46] = 2.24, p = 0.10), % total pAkt (F[3,46] = 0.29, p = 0.83). A summary of the means and SEM for western blotting analyses in male mice can be found in Table 4.

Table 4. Summary of western blotting analyses in male mice. Results reported as mean ± SEM. Mino., minocycline; Rapa., rapamycin.

	Control				Seizure			
Protein	Saline	Mino.	Rapa.	Mino. + Rapa.	Saline	Mino.	Rapa.	Mino. + Rapa.
Akt	100 ± 0	108.4 ± 6.12	102.8 ± 7.48	95.6 ± 9.27	93.5 ± 8.48	109.3 ± 13.17	110.9 ± 11.85	102.9 ± 13.17
pAkt (s473)	100 ± 0	123.6 ± 7.13	78.3 ± 6.00	68.1 ± 6.20	86.7 ± 4.53	97.5 ± 9.54	72.4 ± 9.50	70.5 ± 6.94
% Total pAkt (s473)	100 ± 0	116.9 ± 10.54	84.9 ± 17.92	78.1 ± 12.81	99.9 ± 13.90	97.1 ± 14.98	75.2 ± 17.87	77.8 ± 14.94
p70S6K	100 ± 0	167.5 ± 19.50	174.5 ± 32.44	158.8 ± 40.56	182.9 ± 47.00	227.5 ± 62.42	408.1 ± 116.30	368.5 ± 121.10
S6	100 ± 0	105.6 ± 7.39	79.2 ± 6.95	80.5 ± 8.77	105.8 ± 17.05	106.3 ± 14.75	80.8 ± 13.62	73.8 ± 6.08
pS6 (s235/236)	100 ± 0	121.6 ± 21.58	1.4 ± 0.71	1.3 ± 0.60	120.4 ± 32.71	117.0 ± 22.21	1.7 ± 0.96	0.81 ± 0.43
% Total pS6 (s235/236)	100 ± 0	110.3 ± 13.21	1.5 ± 0.63	1.5 ± 0.51	106.6 ± 14.95	107.6 ± 12.59	1.8 ± 0.87	0.9 ± 0.51
pS6 (s240/244)	100 ± 0	123.9 ± 11.33	7.2 ± 1.13	8.7 ± 1.92	137.1 ± 33.29	168.5 ± 53.39	7.0 ± 1.82	4.7 ± 1.80
% Total pS6 (s240/244)	100 ± 0	109.0 ± 13.95	5.8 ± 0.98	7.2 ± 1.23	105.3 ± 6.25	121.7 ± 15.02	6.4 ± 1.17	5.08 ± 2.15
Iba1	100 ± 0	120.0 ± 19.94	83.1 ± 12.73	110.8 ± 16.64	147.7 ± 28.94	104.9 ± 15.58	110.2 ± 27.72	158.8 ± 52.35
GFAP	100 ± 0	109.1 ± 10.66	93.8 ± 7.80	98.4 ± 7.17	151.3 ± 20.62	128.2 ± 17.48	103.3 ± 8.58	128.1 ± 11.47

In female mice, seizures did not significantly alter the expression of any of the examined proteins. Specifically, seizures did not produce changes in p70S6K (F[1,32] = 0.10, p = 0.75), S6 (F[1,32] = 1.21, p = 0.28), pS6(235,236) (F[1,32] = 2.76, p = 0.11), % total pS6(235,236) (F[1,32] = 1.87, p = 0.18), pS6(240,244) (F[1,32] = 0.27, p = 0.61), % total pS6(240,244) (F[1,32] = 0.04, p = 0.85), Akt (F[1,32] = 0.15, p = 0.70), pAkt (F[1,32] = 0.002, p = 0.97), or % total pAkt (F[1,32] = 0.77, p = 0.39) (Fig. 11A-I). There was a significant main effect of treatment for S6 protein levels in female mice (F[3,32] = 7.51, $p < 0.05$), with mice that were administered the combined treatment having significantly decreased expression levels compared to saline-treated and minocycline-treated mice ($p < 0.05$). In addition, rapamycin-treated mice had significantly reduced S6 expression compared to minocycline-treated mice ($p < 0.05$) (Fig. 11B). There was also a significant main effect of treatment for pS6(235,236) (F[3,32] = 7.95, $p < 0.001$) and % total pS6(235,236) (F[3,32] = 7.77, $p < 0.001$), with mice administered rapamycin or the combined treatment having significantly reduced expression levels compared to all other groups ($p < 0.05$) (Fig. 11C,D). In addition, there was a significant main effect of treatment for pS6(240,244) (F[3,32] = 59.79 $p < 0.001$) and % total pS6(240,244) (F[3,32] = 29.42, $p < 0.001$), with mice administered rapamycin or the combined treatment having significantly reduced expression levels compared to all other groups ($p < 0.001$) (Fig. 11E,F). No significant main effects for treatment were detected in female mice for p70S6K (F[3,32] = 0.28, p = 0.84), Akt (F[3,32] = 0.21, p = 0.89), pAkt (F[3,32] = 0.80, p = 0.50), and % total pAkt (F[3,32] = 1.15, p = 0.35) (Fig. 11A,G-I).


A.
p70S6K
% Control
Control
Seizure
Saline
Minocycline
Rapamycin
p70S6K
Actin
B.
S6
% Control
Control
Seizure
Saline
Minocycline
Rapamycin
S6
Actin
C.
pS6(235,236)
% Control
Control
Seizure
Saline
Minocycline
Rapamycin
pS6(235,236)
Actin
D.
% total pS6(235,236)
pS6(235,236)/total S6
Control
Seizure
Saline
Minocycline
Rapamycin
pS6(235,236)
S6
E.
pS6(240,244)
% Control
Control
Seizure
Saline
Minocycline
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pS6(240,244)
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F.
% total pS6(240,244)
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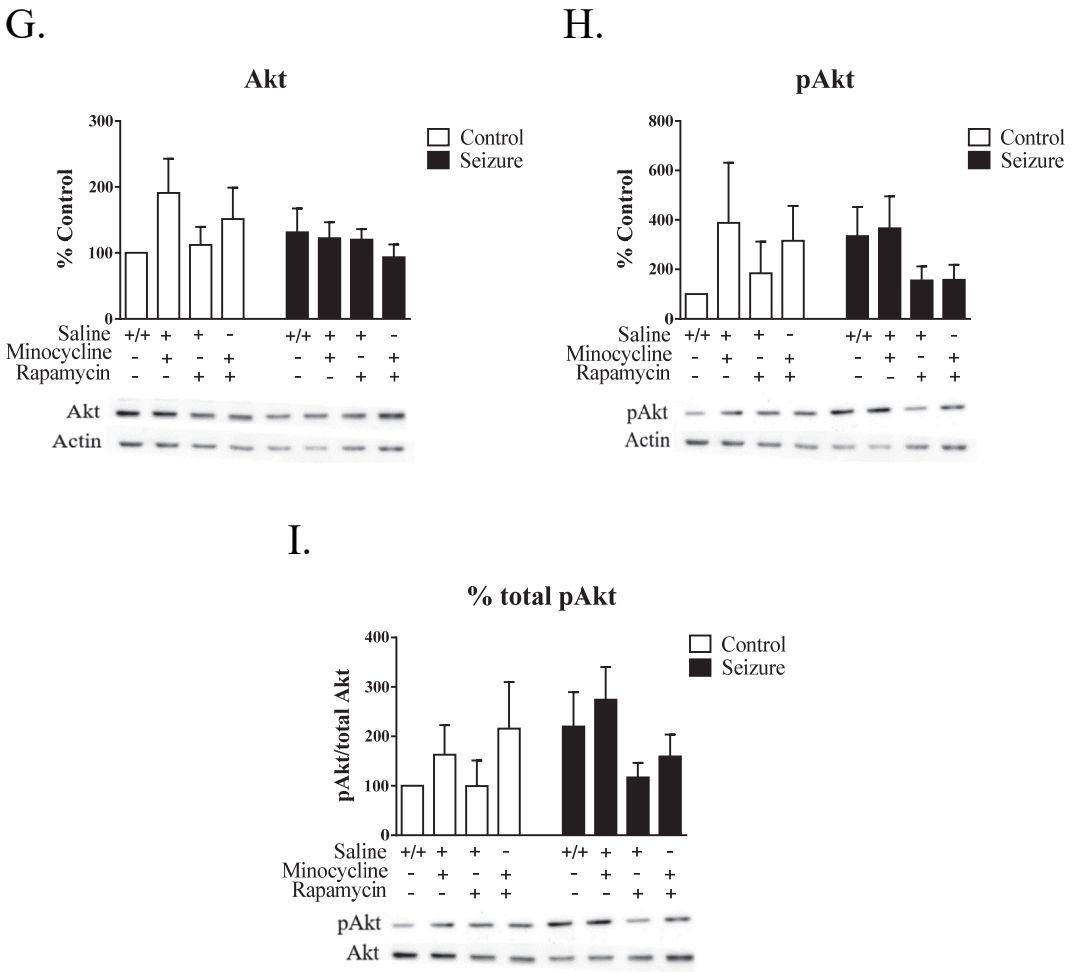

Figure 11. Hippocampal expression of mTOR signaling proteins in female mice. No effect of seizure administration or treatment was detected for p70S6K expression levels (A). There was a significant effect of treatment for S6, with female mice that received the combined treatment having significantly decreased expression levels compared to saline-treated and minocycline-treated mice, and rapamycin-treated mice having reduced expression compared to minocycline-treated mice (B). Mice that received rapamycin or the combined treatment had significantly downregulated levels of pS6(235,236) (C), % total pS6(235,236) (D), pS6(240,244) (E), or % total pS6(240,244) (F). No effect of seizure administration or treatment was detected for Akt (G), pAkt (H), or % total pAkt (I). Data are expressed as mean ± standard error of the mean (SEM). Differences in lettering indicate significance between groups at the level of $p < 0.05$.

There were also no significant interactions between seizure administration and treatment for any of the examined proteins in female mice: p70S6K ($F[3,32] = 0.41$, $p = 0.75$), S6 ($F[3,32] = 0.67$, $p = 0.58$), pS6(235,236) ($F[3,32] = 1.26$, $p = 0.31$), % total

pS6(235,236) (F[3,32] = 1.23, p = 0.32), pS6(240,244) (F[3,32] = 0.98, p = 0.42), % total pS6(240,244) (F[3,32] = 0.19, p = 0.90), Akt (F[3,32] = 1.23, p = 0.31), pAkt (F[3,32] = 1.31, p = 0.29), % total pAkt (F[3,32] = 2.82, p = 0.05). A summary of the means, standard error of the means (SEM), and sample sizes for western blotting analyses in female mice can be found in Table 5.

Table 5. Summary of western blotting analyses in female mice. Results reported as mean ± SEM. Mino., minocycline; Rapa., rapamycin.

	Control				Seizure			
Protein	Saline	Mino.	Rapa.	Mino. + Rapa.	Saline	Mino.	Rapa.	Mino. + Rapa.
Akt	100 ± 0	190.8 ± 52.24	112.1 ± 27.52	151.5 ± 47.26	131.5 ± 35.97	122.3 ± 24.19	120.2 ± 15.93	93.4 ± 19.38
pAkt (s473)	100 ± 0	388.5 ± 243.20	184.6 ± 127.50	315.7 ± 141.30	334.2 ± 118.40	366.1 ± 129.30	155.6 ± 56.51	157.9 ± 60.82
% Total pAkt (s473)	100 ± 0	162.8 ± 60.06	99.5 ± 51.66	216.0 ± 93.89	219.8 ± 69.65	274.1 ± 65.90	117.3 ± 29.32	159.6 ± 44.19
p70S6K	100 ± 0	147.6 ± 37.71	114.8 ± 29.80	82.7 ± 27.18	98.4 ± 27.05	100.9 ± 34.27	168.7 ± 78.10	174.0 ± 86.71
S6	100 ± 0	118.9 ± 19.25	79.4 ± 8.42	75.4 ± 5.93	123.8 ± 13.93	167.1 ± 45.33	116.9 ± 29.51	73.3 ± 13.12
pS6 (s235/236)	100 ± 0	127.3 ± 21.61	3.2 ± 1.56	3.9 ± 0.66	172.5 ± 24.00	361.7 ± 132.4	8.0 ± 3.42	2.64 ± 0.80
% Total pS6 (s235/236)	100 ± 0	110.0 ± 11.26	4.4 ± 2.23	5.3 ± 1.01	138.5 ± 11.94	215.2 ± 68.86	6.4 ± 2.55	5.0 ± 2.32
pS6 (s240/244)	100 ± 0	104.0 ± 13.81	4.6 ± 1.20	3.6 ± 1.15	82.6 ± 14.53	70.7 ± 11.07	5.5 ± 2.04	4.7 ± 2.92
% Total pS6 (s240/244)	100 ± 0	108.8 ± 7.34	16.9 ± 9.08	10.3 ± 6.77	86.0 ± 16.09	96.0 ± 8.38	10.7 ± 4.11	17.4 ± 13.62
Iba1	100.0 ± 0	91.8 ± 30.78	68.5 ± 10.96	70.2.8 ± 13.17	75.3 ± 11.70	97.7 ± 14.83	76.8 ± 6.40	53.0 ± 15.42
GFAP	100 ± 0	148.9 ± 16.56	107.0 ± 9.94	103.4 ± 9.54	140.1 ± 21.88	136.3 ± 27.07	120.0 ± 21.43	165.8 ± 46.60

Hippocampal Expression of Neuroinflammatory Proteins

In male mice, seizures resulted in significantly upregulated GFAP expression (F[1,46] = 6.22, $p < 0.05$), indicative of increased astrocyte reactivity following seizures on PD10 (Fig. 12B). However, there was no effect of treatment (F[3,46] = 1.66, $p = 0.19$) or an interaction between the two factors for GFAP expression (F[3,46] = 1.25, $p = 0.30$). No significant effect of seizure administration (F[1,46] = 2.94, $p = 0.09$), treatment (F[3,46] = 1.13, $p = 0.35$), or an interaction (F[3,46] = 0.90, $p = 0.45$), was detected for Iba1 expression levels in male mice (Fig. 12A).

In female mice, no significant effects of seizure administration (F[1,32] = 0.64, $p = 0.43$), treatment (F[3,32] = 1.89, $p = 0.15$), or an interaction (F[3,32] = 0.78, $p = 0.52$) was detected for Iba1 expression levels (Fig. 12C). In addition, there was no significant effect of seizure administration (F[1,32] = 0.81, $p = 0.37$), treatment (F[3,32] = 0.51, $p = 0.68$), or an interaction (F[3,32] = 0.59, $p = 0.62$) for GFAP expression levels in female mice (Fig. 12D).

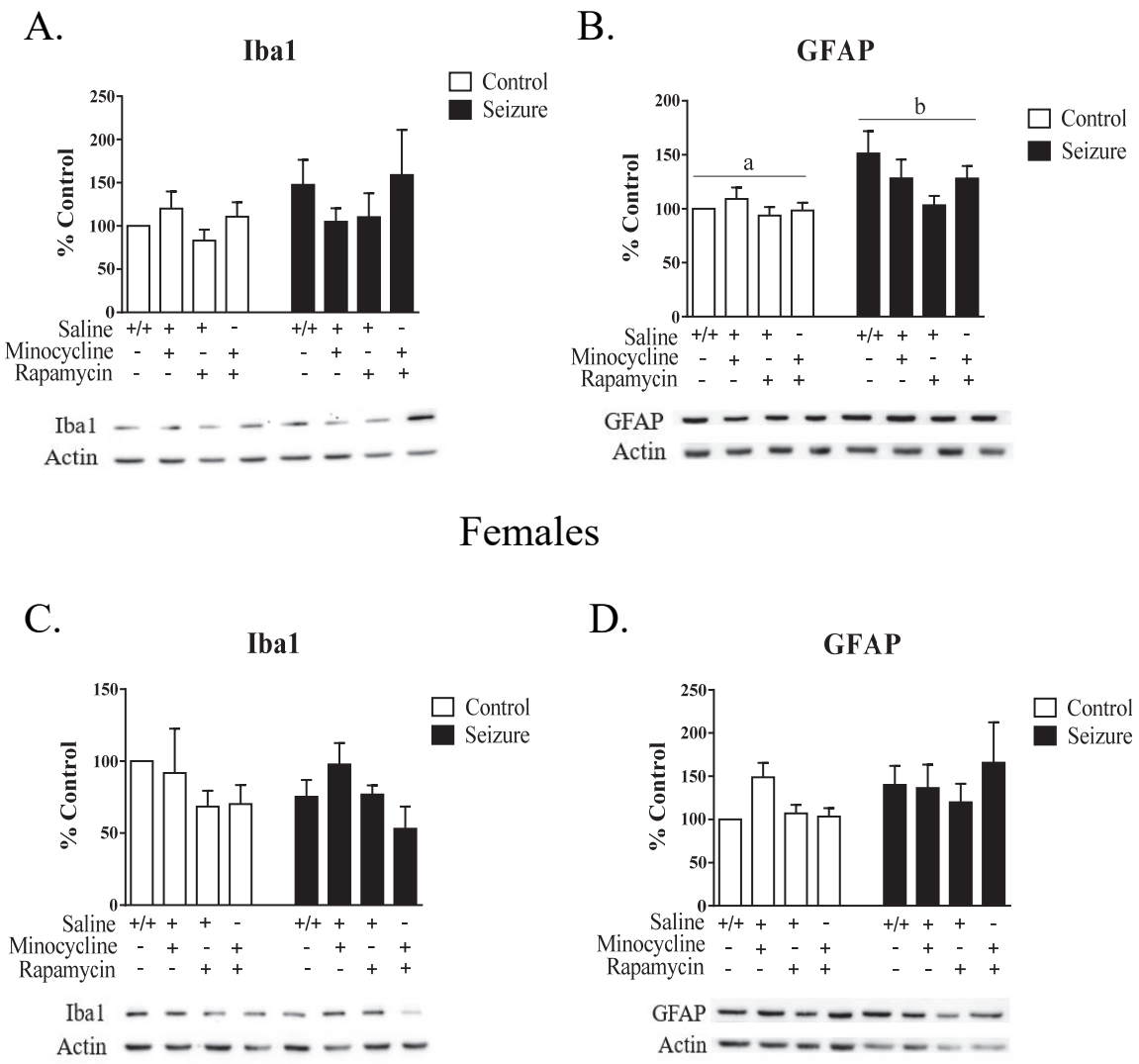

Figure 12. Hippocampal expression of microglial (Iba1) and astrocyte (GFAP) reactivity in male and female mice. In male mice, no effect of seizure administration or treatment was detected for Iba1 (A). However, seizures significantly increased GFAP levels in male mice (B). In female mice, there was no effect of seizure administration or treatment on Iba1 (C) or GFAP (D) hippocampal expression levels. Data are expressed as mean ± standard error of the mean (SEM). Differences in lettering indicate significance between groups at the level of $p < 0.05$.

CHAPTER FIVE

Discussion

Early-life seizures can have profound effects on the developing brain and have been associated with both acute and long-term impairments in cognition and behavior (Bernard & Benke, 2015; Lugo et al., 2014; Velíšková et al., 2018). Seizures can induce several pathophysiological changes in the brain that can lead to the development of chronic epilepsy in individuals. Hyperactivity of the mTOR pathway and neuroinflammation are two potential factors that are thought to underlie epileptogenesis and could serve as promising therapeutic targets in order to prevent seizure-induced neurological damage. The present study investigated whether administration of the anti-inflammatory drug minocycline and the mTOR inhibitor rapamycin following seizures on postnatal day (PD) 10 could attenuate deficits in ultrasonic vocalizations (USVs) on PD12. By examining quantitative and qualitative aspects of USV behavior, we were able to investigate whether these therapeutics could prevent seizure-induced deficits in communicative behaviors, a component of the autistic-like phenotype.

Overall, three flurothyl seizures on PD10 resulted in minimal quantitative differences in USV behavior. Seizures resulted in females emitting a significantly greater amount of USVs, however, other quantitative parameters of USVs (duration, fundamental and peak frequency, amplitude) were not impacted by seizures. Qualitatively, male mice that received seizures emitted a reduced amount of complex call types compared to control mice, suggesting that their calls were less spectrographically diverse and complicated. Behavioral changes were associated with increased astrocyte reactivity and

p70S6K expression in male mice that received seizures, however, these changes were not detected in female mice. Minocycline or rapamycin treatment alone, or when combined, did not attenuate these changes in male mice, nor did they have beneficial effects on USV behavior.

The behavioral and molecular changes following seizures early in life may be heavily dependent on seizure load, or the quantity of seizures experienced in a critical period of development (Hermann et al., 2002; Nolan et al., 2019). In children, seizures can increase the risk of developing cognitive, behavioral, and psychiatric conditions later in life, however, the magnitude of cognitive impairment could be due to several factors. For instance, earlier age at seizure onset, lifetime seizure frequency, and longer duration of seizures has been related to the magnitude of executive functioning and memory deficits in children (Berg, Zelko, Levy, & Testa, 2012; Black et al., 2010; Hermann et al., 2002). Similarly, in rodent models, the extent of behavioral deficits could be associated with a multitude of factors, including timing of seizure induction, seizure load, and method of seizure induction.

In the current study, C57BL/6 mice only received 3 flurothyl seizures on PD10, and it is possible that increasing the seizure quantity across several days could have resulted in more profound molecular and behavioral changes. For instance, when administering 15 flurothyl seizures across PD7 to PD11, seizures resulted in increased average and cumulative duration of USVs in both male and female C57BL/6 mice, as well as induced significant changes in call type utilization patterns (Nolan et al., 2019). In addition to the seizure load, the impact of seizures on communicative behaviors may also be affected by the seizure induction method. For example, a single kainic acid seizure on

PD10 resulted in reduced USV quantity and average call duration in both 129 SvEvTac and C57BL/6 male mice (Reynolds et al., 2017; Reynolds et al., 2016b). In other early-life seizure models, febrile seizures on PD7 in rats led to increases in USV quantity on PD10 and PD12 (Keller et al., 2004). In rats that received lithium-pilocarpine seizures on PD14, seizures did not have an effect on the number of USVs, but decreased latency to first USV emitted and the duration of USVs when measured on PD15, PD16, and PD21 (Lopez-Meraz et al., 2014).

Beyond examination of quantitative parameters of USVs, qualitative aspects of calling behavior are also indicative of changes in early-life communication. Several studies utilizing both seizure and ASD models have found significant changes in call type production patterns (Binder & Lugo, 2017; Nolan et al., 2019; Reynolds et al., 2017; Scattoni et al., 2008; Takahashi et al., 2016). While the significance of the different call types is not entirely elucidated, specific patterns that have shown to be consistent across studies suggest that altered call type utilization may be a better indicator of autistic-like communicative deficits in mice in comparison to quantitative changes in USVs. For instance, we found that seizure male mice emitted significantly fewer complex calls compared to control male mice. Complex call types contain one syllable with two or more directional changes in pitch, each being greater than 6.25kHz (Scattoni et al., 2008). In line with our findings, other studies have found that complex calls are altered following seizures, as well as in several rodent models of ASD. For example, C57Bl/6 mice that received kainic acid seizures on PD10, as well as C57Bl/6 mice that received 15 flurothyl seizures across PD7 to PD11 also emitted reduced complex calls on PD12 (Nolan et al., 2019; Reynolds et al., 2017). Complex calls have also been shown to be

reduced in mouse models of ASD, including BTBR T+tf/J mice on PD8, neuronal subset-specific *Pten* KO mice on PD8 and PD11, and in *Tbx1* heterozygous mice on PD8 (Binder & Lugo, 2017; Hiramoto et al., 2011; Scattoni et al., 2008; Takahashi et al., 2016).

A reduction in the complexity of USVs has been associated with less maternal retrieval in mice (Takahashi et al., 2016). This atypical pattern of USV emission could therefore impact social communication between pups and mothers and influence long-term neurodevelopmental outcomes in seizure pups. Evidence of altered USVs in mouse models of ASD has also been related to atypical crying patterns in infants with ASD (Esposito, Valenzi, Islam, & Bornstein, 2015; Esposito & Venuti, 2009). For example, the crying patterns of those with ASD were more difficult to understand which could negatively impact the quality of care by caregivers (Esposito, Hiroi, & Scattoni, 2017; Sheinkopf, Iverson, Rinaldi, & Lester, 2012).

Several preclinical and clinical studies have demonstrated a role for mTOR signaling in both genetic and acquired models of epilepsy (Wong, 2013). However, despite considerable evidence suggesting that mTOR activity would be increased following seizures, we did not detect significant changes in hippocampal protein expression of components of the pathway (Citraro, Leo, Constanti, Russo, & De Sarro, 2016; Ostendorf & Wong, 2015). In males, seizures significantly increased p70S6K, however, this seizure effect appears to be driven by the impact of treatment in addition to seizures rather than solely a seizure-induced change on its own. In neurodevelopmental conditions with epileptic phenotypes that are associated with genetic mutations in components of the mTOR pathway, such as with *TSC1/TSC2* or *Pten* mutations,

rapamycin has shown to reduce pathway activation and has significant seizure-suppressing effects (Way et al., 2012; Zeng, Xu, Gutmann, & Wong, 2008). In addition to suppressing seizures, rapamycin in these genetic models has shown to be protective against seizure-induced dendritic abnormalities, aberrant mossy fiber sprouting, and cellular hypertrophy (Ljungberg, Sunnen, Lugo, Anderson, & D'Arcangelo, 2009; Nguyen et al., 2015; Nguyen, Mahadeo, & Bordey, 2019).

However, in contrast to the strong evidence for mTOR hyperactivity in genetic models, other studies in addition to ours have demonstrated variable mTOR hyperactivation in acquired models, especially in the neonatal period. One study that examined acute changes in mTOR activity following hypoxia-induced seizures in rats on PD10, found the downstream mTOR target p4E-BP1 (Thr37/46) and p70S6K to be increased in the hippocampus and cortex 12hrs. post-seizures, followed by elevated pS6 levels in the same regions at 24hrs. (Talos et al., 2012). However, these changes returned to baseline by 48hrs. post-seizures on PD12 (Talos et al., 2012). If we had collected tissue at an earlier timepoint prior to PD12, it is possible we could have found similar findings following flurothyl seizures. Another study utilizing a multiple-hit rat model of infantile spasms, found that phosphorylated S6 expression levels in the cortex were elevated during the spasms (PD4 and PD6-7), however, pS6 levels returned to baseline by PD13 (Raffo, Coppola, Ono, Briggs, & Galanopoulou, 2011). In contrast to the neonatal period, in adulthood many studies have shown evidence for sustained mTOR hyperactivity in preclinical acquired models and in clinical populations with TLE (Talos et al., 2018; van Vliet et al., 2012; Zeng et al., 2009; Zhu et al., 2017). It is possible that different mechanisms regulate mTOR pathway activity following seizures in the neonatal

period compared to in adulthood. For example, recent evidence has shown that mTOR signaling is suppressed during early neuronal differentiation in humans, and that this is critical for normal neurogenesis and gliogenesis (Blair, Hockemeyer, & Bateup, 2018). In addition to age of seizure onset, other factors such as the type of seizure model utilized, could play a role in the variation of mTOR signaling activity following seizures in preclinical animal models.

In line with the substantial evidence of mTOR hyperactivity in genetic models, and more variable findings in acquired models, the efficacy of rapamycin treatment in reducing seizure frequency exhibits a similar pattern. Specifically, rapamycin exerts significant seizure-suppressing effects in genetic models such as with mutations in *Pten* or *Tsc1/Tsc2*, however, treatment is less consistent across preclinical TLE models and any beneficial effects often requires continual treatment (Buckmaster et al., 2009; Buckmaster & Lew, 2011; Hartman, Santos, Dolce, & Hardwick, 2012; Ljungberg et al., 2009; Zeng et al., 2008). One potential reason for this could stem from the mechanism in which rapamycin exerts its' seizure-suppressing effects. Evidence has shown that rapamycin has minimal impact on neuronal excitability, unlike other traditional anti-seizure medications (Hartman et al., 2012; Rogawski & Loscher, 2004). Instead, rapamycin most likely exerts its effects on the electrical activity of neurons indirectly, by modulating neurotransmitter expression and voltage-gated ion channel activity (Huang, McMahon, Yang, Shin, & Huang, 2012; Niere & Raab-Graham, 2017; Tyan et al., 2010). Therefore, the effects of rapamycin could also be highly dependent on the type of seizure induction utilized, as different models differ in the degree of neuropathological and molecular changes following seizures.

Flurothyl seizures in rodents mimic recurrent generalized seizures in the neonatal period and does not induce significant neuronal loss or damage acutely or long-term (Riviello, de Rogalski Landrot, & Holmes, 2002). However, recurrent flurothyl seizures in the neonatal period can lead to a reduction in newly born granule cells of the dentate gyrus, or when induced in adulthood can lead to increases in mossy fiber sprouting and changes in intrinsic membrane properties of CA1 pyramidal neurons (Holmes, Sarkisian, Ben-Ari, & Chevassus-Au-Louis, 1999; McCabe et al., 2001). Utilizing other chemoconvulsants in the neonatal period, such as with kainic acid or pilocarpine, can have more profound effects and result in neuronal injury in the amygdala, hippocampus, and thalamus, in addition to mossy fiber sprouting (Cilio et al., 2003; Cross & Cavazos, 2007; Kubová et al., 2001; Sankar et al., 1998). A future study comparing the developmental time course of mTOR hyperactivation, along with impact of rapamycin treatment, using various neonatal seizure induction paradigms would be beneficial to determine the optimal paradigm to identify the impacts of rapamycin.

While the therapeutic potential of rapamycin has demonstrated efficacy in treating seizures in both preclinical and clinical populations, the side effects resultant from continual treatment is of concern. Some of the more common side effects of rapamycin treatment include chronic immunosuppression, mucositis, skin reactions, and enhanced cholesterol and triglyceride levels (McDaniel & Wong, 2011; Tsang, Qi, Liu, & Zheng, 2007). In addition to these observed side effects in clinical populations, the impact of rapamycin on behavioral phenotypes in mice is variable. For example, rapamycin treatment has shown to be protective against seizure-induced impairments in hippocampal-dependent learning and memory tasks, as well as against long-term social

deficits in adulthood (Brewster et al., 2013; Talos et al., 2012). However, chronic rapamycin treatment in a model of Fragile X syndrome (*Fmr1* KO mice) did not reverse the majority of autistic-like behavioral impairments observed in KO mice (Sare et al., 2017). In addition, treatment had an adverse effect on social behavior and sleep duration in both *Fmr1* KO and WT mice, as well as increased anxiety in control mice (Sare et al., 2017).

In the present study, we found that rapamycin treatment significantly reduced the quantity and duration of vocalizations emitted from both male and female seizure and control mice. In addition, control mice that received rapamycin emitted significantly fewer complex calls when compared to saline-treated control mice. This was the first study to examine the impact of mTOR inhibition on communicative behaviors in a mouse model. The significant impact that rapamycin treatment had on USV production in neonatal mice is concerning, in that it could potentially lead to long-term deficits in communication, a component of the autistic-like phenotype (Ferhat et al., 2016; Mody & Belliveau, 2013). It is unknown how mTOR inhibition could impact vocalizations on a mechanistic level, however, one potential mechanism could be due to overall reduced protein translation due to mTOR inhibition (McDaniel & Wong, 2011). Rapamycin forms a complex with 12-kDA FK506-binding protein (FKBP12), which will bind to the FRB (FKBP12/rapamycin-binding) domain of mTOR to selectively inhibit the phosphorylation of mTORC1, leading to reduced ribosomal protein recruitment and translation (Li, Kim, & Blenis, 2014). While specific proteins regulated by downstream mTOR signaling that could be linked to vocalization behavior have yet to be identified, this would be an interesting finding if such proteins exist. In addition, another reported

side effect with the rapamycin analog Everolimus is “loss of voice” which could potentially be tied to the lack of vocalizations in rapamycin-treated mice. However, several additional studies are required to determine how exactly rapamycin is associated with the production of USVs and communicative abilities in both humans and mice. With Everolimus being FDA approved to treat partial epilepsy in individuals with TSC, in addition to several other ongoing trials with mTOR inhibitors, it is critical to investigate whether these deficits in USV emission persist beyond the acute time period following treatment and could impact communication in adulthood (Krueger et al., 2013; Krueger et al., 2016).

There is substantial evidence for enhanced inflammatory signaling following seizures, which has shown to contribute to hyperexcitability and potentially underlie epileptogenesis in the brain (Vezzani, Balosso, & Ravizza, 2019). We found that seizures in male mice resulted in enhanced GFAP expression, indicative of enhanced astrocyte reactivity. Increased astrocyte reactivity can contribute to further disruption of the BBB and release of inflammatory molecules (Choi & Koh, 2008; Robel et al., 2015). Specifically, reactive astrocytes undergo extensive physiological changes that can alter neurotransmitter homeostasis, such as increased release of glutamate which can further contribute to the excitability underlying seizures (Bezzi et al., 2001; Shimada et al., 2014). Interestingly, we only found increased astrocyte reactivity in male mice, suggesting that sex may play a role in the degree of inflammatory response following seizures.

In addition, we did not detect any differences in Iba1 expression on PD12 in either sex, suggesting that three flurothyl seizures on PD10 did not result in enhanced

microglial activation. Studies have found that microglial activation often precedes astrocyte reactivity after seizures, suggesting that if we had examined Iba1 expression at an earlier timepoint such as at 24hrs. post-seizures we may have found the expected increase (Sano et al., 2019; Shapiro, Wang, & Ribak, 2008; Vargas-Sánchez et al., 2018). Microglial reactivity has found to be model-dependent, in that differences in M1 (proinflammatory) versus M2-type (anti-inflammatory) microglia phenotypes were observed between pilocarpine and kainic acid SE models, suggesting that the time course of reactivity may also differ in the flurothyl model (Benson, Manzanero, & Borges, 2015). In addition, seizures induce changes in the morphological phenotypes of microglia, such that future studies should perform immunohistochemistry to examine potential differences in the state of microglia after seizures in this model (Wyatt-Johnson, Herr, & Brewster, 2017). While western blotting provides us with a qualitative measurement of the degree of reactive Iba1-positive microglia, we are unable to determine their phenotypic state and function, including ramified versus amoeboid microglia (Dubbelaar, Kracht, Eggen, & Boddeke, 2018). However, this is the first study to examine microglial reactivity in a neonatal flurothyl seizure model in mice, and it could be that flurothyl does not induce as drastic changes in the neuroinflammatory response as is detected in other models.

Several studies have found that seizures are associated with increases in the release of proinflammatory molecules, including cytokine and chemokine production and release (Arisi et al., 2015; Li et al., 2011; Uludag et al., 2015). Cytokines have typically low concentrations in the brain and can be released from a variety of cells, including microglia, astrocytes, neurons, and endothelial cells (Galic et al., 2012). Following

seizures, microglia have been shown to contribute significantly to the acute upregulation in secretion of proinflammatory cytokines (Eyo, Murugan, & Wu, 2017; Hiragi, Ikegaya, & Koyama, 2018). The lack of microglial reactivity observed could be a potential reason we did not detect the expected increases in hippocampal proinflammatory cytokine expression levels of IL-1β, IL-6, and TNFα following flurothyl seizures. However, since initial microglia activation can induce astrocyte reactivity, it could be that there is an increase in specifically astrocyte-secreted cytokines that could be detected at a later timepoint (Sano et al., 2019). Furthermore, minocycline primarily targets M1-type proinflammatory microglia and inhibits the secretion of cytokines from these activated microglia (Elewa et al., 2006). This provides evidence for why minocycline did not have a significant effect on reducing enhanced GFAP expression in male seizure mice. If proinflammatory cytokines were elevated post-seizures in this flurothyl model, we believe minocycline would have been an efficacious treatment in reducing cytokine levels post-seizures.

Minocycline has previously been shown to have anti-convulsant effects in rodent models of epilepsy, by reducing microglial activation and decreasing hippocampal damage post-seizures (Abraham et al., 2012; Heo et al., 2006; Wang et al., 2012). In a case study of an individual with severe epilepsy due to an astrocytoma, minocycline reduced seizure frequency (Nowak et al., 2012). One of the primary reasons in which we chose this anti-inflammatory treatment was due to its' known effects on communication and language abilities in both preclinical models and clinical populations. For example, in a mouse model of Fragile X syndrome (FXS), minocycline treatment was able to normalize USV deficits in adult *Fmr1* KO mice, specifically increasing the USV call rate

to WT levels (Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012). In children with FXS, treatment with minocycline for at least 2 weeks increased the use of expressive language and improved social communication (Utari et al., 2010). Mutations in *Fmr1* are the largest genetic cause of ASD, suggesting that anti-inflammatory treatments could also help ameliorate communicative deficits in other models of autistic-like behavior (Mila, Alvarez-Mora, Madrigal, & Rodriguez-Revenga, 2018). These beneficial effects could be related to the ability of minocycline to promote dendritic spine maturation and enhance NMDA-receptor dependent plasticity in the hippocampus (Bilousova et al., 2009; Yau et al., 2018). However, the exact mechanism in which minocycline impacts communicative behaviors in rodent models and in humans is unknown and requires further investigation.

While minocycline has shown to be efficacious in reducing inflammation in disease models, our findings suggest that the anti-inflammatory treatment may have diverse effects when administered in the neonatal period versus in adulthood (Elewa et al., 2006; Heo et al., 2006; Wang et al., 2012). Male mice that received minocycline or the combined treatment on PD10 had significantly increased IL-1β and IL-6 levels compared to saline-treated and rapamycin-treated mice. This suggests that a 50mg/kg dose may have been too high, resulting in toxicity and instead enhanced the neuroinflammatory response in male mice. Interestingly, minocycline did not increase proinflammatory cytokine expression in female mice. Another study has found that minocycline treatment (45mg/kg) in perinatal female mice from embryonic day 18 to PD1 resulted in significantly increased cell death in pups in several brain regions (primary sensory cortex, septum, hippocampus, hypothalamus), in addition to increasing Iba1 labeling of microglia (Strahan, Walker, Montgomery, & Forger, 2017). Increased

cell death was also observed in both male and female pups when they were administered a total of 5 minocycline treatments from PD3 to PD5 (Strahan et al., 2017). These findings, along with our own, suggest there may be a developmental switch in how neonatal rodents respond to minocycline, which could potentially be due to age-related changes in cell populations early in life. Extrapolating the effects of minocycline across developmental periods is of concern, especially because it is currently being prescribed to treat infections in young children and is in several clinical trials for pediatric disorders (Grieco et al., 2014; Kaneko et al., 2012; Okada et al., 2012; Pardo et al., 2013).

Early-life seizures can have a profound impact on the developing brain, producing long-lasting behavioral and cognitive effects (Bernard & Benke, 2015; Holmes, 2016). Several pathophysiological changes occur following seizures that can contribute to the persistent hyperexcitability that underlies the development of chronic epilepsy. The present study emphasizes that the seizure paradigm and model utilized can significantly impact the degree of seizure-induced brain changes. While three flurothyl seizures on PD10 only produced minor increases in mTOR hyperactivity and astrocyte reactivity on PD12, it is possible that increasing the seizure load could induce the more drastic changes other preclinical models have found. However, when determining the best model to use it is critical to consider how it parallels the human condition, specifically the frequency of seizures that are typically experienced in the neonatal period. Flurothyl has been used as a chemoconvulsant in numerous studies, yet the question of the optimal number of seizures and time period of induction still requires investigation.

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