ABSTRACT

Multiple Early-life Seizures Alter Neonatal Communicative Behavior in *Fmr1* Knockout Mice

Dexter Nguyen

Director: Joaquin Lugo, Ph.D.

Fragile X syndrome (FXS) is the leading monogenic cause of intellectual disability and a significant contributor to Autism spectrum disorder. In addition to autistic-like phenotypes, individuals with FXS are subject to developing numerous comorbidities, one of the most prevalent being seizures. In the present study, we investigated how FMR1 germline mutation impacts neonatal communicative behavior in the FXS mouse model, both with and without early-life seizures (ELSs). On postnatal day (PD) 7 through PD11, we administered 3 flurothyl seizures to both *Fmr1* KO and wild-type mice. On PD12, all pups were temporarily isolated from their home cage and USVs were recorded. Significant alterations were found in both spectral and temporal measurements across seizure groups. We found that induction of seizures across PD7–11 resulted in increased frequency of USVs produced ($P < 0.05$), longer duration ($P < 0.05$), and cumulative duration ($P < 0.05$) in both genotypes. Overall, this study provides evidence that magnitude of communication impairment in FXS mice is significantly impacted by seizure frequency load early in development.

APPROVED BY DIRECTOR OF HONORS THESIS

_____

Dr. Joaquin Lugo, Department of Psychology and Neuroscience

APPROVED BY THE HONORS PROGRAM

_____

Dr. Andrew Wiseley, Interim Director

DATE: _____

MULTIPLE EARLY-LIFE SEIZURES ALTER NEONATAL COMMUNICATIVE BEHAVIOR IN FMR1 KNOCKOUT MICE

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Dexter Nguyen

Waco, Texas

May 2021

TABLE OF CONTENTS

LIST OF TABLE AND FIGURES

ACKNOWLEDGMENTS

This thesis project would not have been possible without the assistance and support of a number of faculty and students.

I would like to thank Paige Womble in the Department of Psychology and Neuroscience for teaching me how to perform the ultrasonic vocalization recording. I also would like to thank David Narvaiz in the Department of Psychology and Neuroscience for helping me understand the statistic and the results of the experiment. Additionally, I would like to thank the Honors Program for the constant encouragements and assistance throughout the project. I would like to give a special thanks to my defense committee members, Dr. Hwa and Dr. Pitts, for agreeing to be a part of my defense pane and for reading my thesis and providing helpful feedbacks.

Finally, I would like to personally thank Dr. Joaquin Lugo, my thesis mentor, for allowing me to work in his lab for the past year and a half on such a short notice, following the departure of Dr. Sekeres. The completion of this thesis would have been impossible without his guidance, commitment, and assistance, especially through the pandemic. Dr. Lugo made this experience educational and positive. I could not have completed this thesis without Dr. Lugo's devotion and generous help along the way.

CHAPTER ONE

Introduction

Fragile X Syndrome

Fragile X syndrome (FXS) is an X-linked neurodevelopmental disorder and the most common cause of intellectual disability (Hagerman et al., 2008). FXS is caused by an expansion of a >200 repeats CGG trinucleotide repeat sequence within the promoter region of the FMR1 gene, resulting in an absence in the production of the fragile X mental retardation protein (FMRP). In a healthy individual, FMRP is an mRNA-binding protein that serves to represses the translation of metabotropic glutamate receptors (Bear et al., 2004).

Clinical symptoms of FXS can include impaired cognition, heightened anxiety, and unusual physical features (Bagni et al., 2012, Kazdoba et al., 2014). Due to the X-linked nature of the offspring, FXS phenotypes vary considerably between males and females. It is estimated that FXS affects 1 in 2,500 to 5,000 men and 1 in 4,000 to 6,000 women (Bagni et al., 2012). Men with FXS display varying degrees of symptoms ranging from mild to severe. Since females have 2 X chromosomes, they usually only have FMR1 mutation, only 1 in 3 female carriers experience the full mutation and the majority have normal IQ with a few cognitive and emotional problems (Bagni et al., 2012).

Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by social interaction, communication impairments, repetitive or stereotyped

behaviors, and unusual behaviors or interests (APA, 2013). In some cases, ASD is also accompanied by intellectual disability. A recent study from the Center for Disease Control and Prevention estimated the prevalence of ASD one in 54 children, and the male-to-female ratio is 4.3:1 (Maenner, 2020). Among ASD children who had IQ information, 33% of ASD children met the category for intellectual disability (IQ ≤ 70), and 24% had an IQ in the borderline range (IQ 71–85) (Maenner, 2020). Since it is described as a spectrum disorder, ASD manifests and affects different children to a different degree. Since there is no biomarker for diagnosis, the diagnosis of ASD is primarily based on clinical observations, behavioral evaluation, and the use of the autism criteria in the *Diagnostic and Statistical Manual of Mental Disorder (*DSM-5).

Epilepsy and Seizure

Epilepsy is one of the most common neurological disorders, along with stroke migraines, meningitis, and Alzheimer's, and other dementias. It is a chronic disorder characterized by recurrent (two or more), unprovoked seizures which affect an individual's mental and physical functions (Stafstrom & Carmant, 2015). Multiple seizures within the 24-h period or an episode of status epilepticus (SE) are considered a single event. It is estimated that epilepsy has impacted 50 million individuals worldwide with approximately 75% epilepsy cases begin during childhood, thus showing heightened susceptibility for epilepsy in the pediatric population (Banerjee et al., 2009; Stafstrom & Carmant, 2015).

A seizure is a burst of uncontrolled electrical activity between neurons in the brain, resulting in temporary abnormalities in muscle ton, behaviors, and consciousness (Stafstrom & Carmant, 2015). Seizures can be divided into two major categories by the

International League Against Epilepsy (ILAE): generalized seizure, and focal seizure. Focal seizures are limited to part of a cerebral hemisphere, whereas generalized seizures affect distributed neuronal networks bilaterally (Banerjee et al., 2009).. The types of seizure/epilepsy can be diagnosed using a comprehensive history of the patient, electroencephalography (EEG) abnormalities, and supporting information (Stafstrom & Carmant, 2015).

Purpose of this Study

Previous investigation in our lab has sufficiently detected the impact of a single episode of status epilepticus (SE) on vocalization behavior in both *Fmr1* KO and wild type (WT) during the sensitive period (Huebschman et al., 2020). However, it is unclear how seizure frequency load impact the magnitude of these communication alterations. The current study has two main purposes. The first is to add to the existing literature regarding ultrasonic vocalization (USVs) in FXS by examining the difference in communicative behavior between *Fmr1* KO and WT pups via the isolation-induced vocalization paradigm. The second is to observe the impact of multiple early-life seizures (ELSs) on separation-induced neonatal ultrasonic vocalization in both *Fmr1* KO and WT mice. The discussion, in addition to characterizing the superimposing effect of seizure on the germline mutation of *Fmr1* in mice, provides further support for the potential use of vocalization and neonatal communicative behaviors as an early diagnosis tool for FXS.

CHAPTER TWO

Review of Literature

This section discusses the literature in support of the comorbidity between fragile X syndrome (FXS) and epilepsy, along with discussing the communication development in FXS human and rodents.

FXS and Autism comorbidity

Autism spectrum disorder (ASD) is a common comorbid condition in patients with FXS. Mutation in the FMR1 gene is the most common known monogenic contributor to ASD, accounting for about 2-6% of all cases of ASD (Hagerman et al, 2008). As a result, FXS and ASD share multiple behavioral phenotypes, such as hyperactivity, impaired social interaction and communication, and stereotypical or repetitive behaviors, such as hand flapping, poor eye contact, and perseverative speech. (Niu et al, 2017). For these reasons, *Fmr1* KO mouse is typically regarded as an appropriate model for studying both autistic phenotypes and FXS. It is common to diagnose for both ASD and FXS separately with many children diagnosed with autism before they are diagnosed with FXS. It is estimated that around 25 to 50% of all individuals with FXS also meet the DSM criteria for ASD (Abbeduto et al.,2014; Kaufman et al., 2017). According to a national parent survey, 30% to 43% males met diagnostic criteria for ASD, and 16% to 20% females met diagnostic criteria for autism (Kaufman et al., 2017).

Communication Development in FXS and ASD individuals

Diagnosis of FXS typically begins in children who showed delayed or absent speech during their sensitive period around the age of 3 (Bagnit et al., 2012). Social communication impairments and delay in language development in FXS individuals have been well characterized across multiple domains, such as vocalization, verbal/nonverbal, gestural, and symbolic communications (Brady et al., 2006; Finestack et al., 2009). While most FXS children eventually learn to speak, it was reported that the onset of speech was significantly delayed, with receptive language developed faster than expressive language. In a study of 55 FXS children, age ranging from 18 months to 3 years old, it was reported that over half of the children were nonverbal and still communicate using prelinguistic communications, such as vocalizations, or gestures (Brady et al., 2006). Furthermore, it was reported that children with both FXS and ASD scored lower in receptive language and expressive language compared to FXS children who did not have autism (Philofsky et al., 2004).

In general, FXS patient's symptoms increase in severity when comorbid with ASD. In the study involving adolescents and adults either diagnosed with ASD only, FXS only, or both, individuals with both FXS and ASD were more impaired in communication and social interaction than individuals with FXS alone (Smith et al., 2012). In addition, this study and other studies have shown that individuals diagnosed with both FXS and ASD experience a greater deficit in communication impairments and other autistic-phenotypic behaviors, such as repetitive behaviors, compared to their ASD only or FXS only counterparts (Kaufmann et al., 2004; Smith et al., 2012).

Early-Life Seizures

Individuals diagnosed with FXS are also at a higher risk for experiencing early-life seizures (ELS). Approximately 10% to 20% of individuals with FXS also experience seizures, and many have abnormal EEG activity without experiencing seizures (Berry-Kravis et al., 2010; Hagerman and Stafstrom, 2009; Incorpora et al., 2002). The average age of seizure onset in FXS individuals ranges from 6-month of age to 4 years of age (Incorpora et al., 2002). In most cases, childhood epilepsy ceases in adolescence; however, there are a few cases epilepsy persists into adulthood (Sabaratnam et al., 2001). In addition, the prevalence of seizures is higher in patients diagnosed with both FXS and ASD, compared to the FXS only individual (20.7% vs 7.6%) (Kaufman et al., 2017). Cooccurrence between FXS and childhood epilepsy creates a variety of cognitive and behavioral impairments that vary across individual (Wouters et al., 2006).

Seizure Loads and Magnitude of Impairment

Early-life seizure increased the risk of experiencing psychiatric and behavior comorbidities (Baca et al., 2011) and cognitive deficits (Elger et al., 2004). The magnitude of these impairments depends upon different factors like age of seizure onset, duration of seizure onset, or frequency of seizures in a lifetime (Black et al., 2010; Herman, Seidenberg, & Bell, 2002). In a study involving patients with either confirmed temporal lobe epilepsy or psychogenic nonepileptic seizures, the result suggests that age of seizure onset is a strongest predictor of cognitive impairment, indicating that earlier onset of seizure during childhood is significantly correlated to more severe cognitive impairment in adulthood (Black et al., 2010). In addition, this study and others have

reported a strong correlation between lifetime seizure load and deficits in both intellectual and memory measures (Herman, Seidenberg, & Bell, 2002).

Similar to cognitive impairment, children who experienced onset of seizures within the first year of life are predisposed to a higher risk for autism and likelihood of developing speech disorder (Saemundsen et al., 2007; Sillanpää, 1992). A study from our lab conducted using animal model reported similar findings where multiple early-life seizures during critical period of development produced significant long-term deficits in memory tasks and social behaviors in adult mice (Lugo, Swann, & Anderson, 2014), suggesting the role of recurrent seizures in explaining the autistic-like phenotypes. Data from another animal study targeting communication, the other aspect of autistic-like phenotype, supports the notion that experiencing high frequency seizure load (15 seizures over 5 days) during sensitive period produced significant changes in vocalization behaviors that were not seen under the low frequency seizure load (3 seizures in one day) (Nolan et al., 2019). A recent investigation by Huebschman et al. (2020) characterized the interaction between FXS and ELS on vocalization, using *Fmr1* KO mice. In the Hueschman et al. study (2020), a status epilepticus (an episode of continuous seizure lasting over 30 minutes, or two or more seizures without full recovery of consciousness between any of them) was induced on neonatal pups on PD10 using kainic acid, followed by an isolation-induced vocalization paradigm during PD12. While this study detailed the superimposing impacts of one episode SE on vocalization behavior in FXS model mice, it is unclear how the frequency of seizure onset, or seizure loads, would impact communicative behavior, similar to evidence for cognitive deficits.

EEG Patterns and Seizure Types

EEG patterns and seizures types across FXS individuals are often multifactorial and complicated, with varying degrees in severity. In a study conducted by Musumeci et al. (1999) involving 192 FXS male patients to characterize the EEG abnormalities of FXS, while only 35 out 192 individuals experienced overt seizure, the majority of the young patients with FXS demonstrated an EEG pattern of paroxysmal discharges, that was similar to recordings of individuals with benign childhood epilepsy with centrotemporal spikes (Musumeci et al., 1999). In the Berry-Kravis et al., 2010 study, out of 16 children with FXS and epilepsy, 12 children exhibited focal epilepsy, and 10 out of the 12 displayed EEG anomalies with the pattern of paroxysmal discharges, often characteristic of centrotemporal spikes. In addition, 23% of the children EEG displayed centrotemporal spikes without experiencing seizure (Berry-Kravis et al., 2010). The most common type of seizures observed in FXS are complex focal seizures, and less frequently, generalized seizures (Berry-Kravis et al., 2010, Incorpora et al., 2002, Musumeci et al., 1999).

Cause of Seizure in FXS patients

The underlying mechanism explaining epileptogenesis in FXS individuals is not currently well understood, as the literature varies across studies. However, it is thought that the increased frequency of seizures may be due to the increase in excitatory synapses and neuronal hyperexcitability, stemming from the silencing of the FMRP and enhanced activation of metabotropic glutamate receptor (mGluR) (Bear et al., 2004; Hagerman & Stafstrom, 2009).

One hypothesis targeted the role of mGluR5 and its heightened stimulation in the absence of the FMRP protein leading to alteration of neuronal synaptic connectivity. In the study conducted in Huber et al. 2002, they observed that mice lacking FMRP displayed enhanced long-term depression (LTD) in the hippocampus and that this depression was dependent on protein synthesis (Huber et al., 2000a; Huber et al., 2000b). Further examination determined that this process of LTD can be blocked using 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a mGluR5 antagonist (Gaspani et al., 1999). Coined "the mGluR theory of fragile X mental retardation", this hypothesis can be used to explain many phenotypes in FXS patients, including improved seizure activity, as shown in the past in a *Fmr1* KO mouse model with audiogenic seizure (Hagerman & Stafstrom, 2009).

While the mGluR model explains many of the phenotypes in FXS individuals, weakened postsynaptic connectivity is not sufficient to explain neuronal hyperexcitability and EEG patterns. In the recent investigation, several studies pointed to the synergistic activations between mGluR5 and mGluR1 as the potential mediator for epileptogenesis in FXS patients (Bianchi et al., 2009; Chuang et al., 2001; Chuang et al., 2001). Bianchi et al. (2009) demonstrated that stimulating both mGluR1 and mGluR5 with the agonist (*S*)-dihydroxyphenylglycine (DHPG) led to persistent activation of a voltage-gated inward current, and prolonged epileptiform discharges that lasted over an hour in the hippocampal CA3 region, even with the washout of the agonist. Interestingly, the same synergistic stimulation of glutaminergic synapses in wild-type mice produced no significant effect, indicating the role of activation of mGluR5 in multiple synapses with the absence of FMRP in the neuronal hyperexcitability phenotype in FXS individual.

Fmr1 knockout mice as a model

Fmr1 knockout (KO) mice display similar phenotypic physiologies, behaviors, and EEG patterns that are often observed in FXS patients. Similar to humans, mice with deletion of FMR1 gene lack FMRP protein (Kazdoba et al., 2014). Since it is first created and characterized, the *Fmr1* KO mouse model has been extensively used to further understanding the role of FMRP, and how it relates to FXS clinical symptoms.

Fmr1 KO mice display similar morphological phenotypes compared with humans with FXS. Male patients with FXS tend to have dysmorphic features, such as narrow faces, loose joints, smooth skin, and macroorchidism (enlarged testes). *Fmr1* KO mice have significantly heavier testes compared to wild-type mice, but otherwise normal body structures. At the cellular level, *Fmr1* KO mice have an excess density of immature dendritic spines and show a similar deficit in spine numbers when compared to human cortical tissue in individuals with FXS (Berry-Kravis et al., 2010; Kazdoba et al., 2014).

Some behavioral symptoms of FXS patients include attention deficits, heightened anxiety, impaired social interaction, and stereotypic/repetitive behaviors (Bagni et al., 2012). Behavioral studies using *Fmr1* KO mice model demonstrated similar behavioral deficit when compared to FXS individuals. For instance, *Fmr1* KO mice buried more marbles in the marble-burying test (Spencer et al., 2011), indicating signs of repetitive behaviors. Furthermore, on the social chamber approach task testing for sociability in mice, *Fmr1* KO mice were found to have no preference for a novel mouse over an object (Dahlhaus & El-Husseini, 2010) and have reduced sniffing duration of the novel mouse

when compared to wildtypes (McNaughton et al., 2008). The results in the social chamber test suggests deficits in social behavior.

Several studies demonstrated that knockout mice display EEG phenotypes similar to those observed in FXS individuals (Lovelace et al., 2018; Wang et al., 2017). Previous EEG studies in humans with FXS revealed cortical oscillation deficits, enhanced resting-state EEG gamma power (30-80 Hz), and reduced evoked gamma responses (Wang et al., 2017). Lovelace et al. (2018) reported similar EEG patterns in *Fmr1* KO mice with higher resting-state EEG gamma power in both the auditory and frontal cortex and reduced evoked gamma synchronization. Gamma activity is thought to correlate to the activity in the cortex, thus, this finding was in line with sensory hypersensitivity in FXS individuals.

CHAPTER THREE

Methods and Materials

The experimental portion of this study examined potential communication behaviors in *Fmr1* knockout mice through the implementation of two experimental phases: the seizure induction phase, and the ultrasonic vocalization recording phase.

The experiment utilized a double-hit model to include subjects of seizure-induced *Fmr1* knockout mice, *Fmr1* knockout control mice, seizure-induced wild-type mice, and wild-type control mice. The mice were randomly assigned to each group. The experimenter was blind to the subject genotype. Subjects included male *Fmr1* KO mice and WT mice, bred on a C57BL/6J strain. Male mice were bred at Baylor University for this experiment. All mice were housed in individual cages with parents and littermates in standard controlled environments with temperature held at and a 12-hour light, 12-hour dark cycle. Mice have access to food and water ad libitum. WT males and heterozygous dams were crossed to produce male *Fmr1* KO pups and WT littermate control. This breeding scheme resulted in only KO and WT offspring since *Fmr1* is inherited through the X chromosome.

Seizure Induction

Flurothyl (bis-2,2,2-trifluoroethyl ether), a non-competitive receptor antagonist, is a potent volatile convulsant and isused as a model for inducing neonatal generalized tonic-clonic seizures. In adult mice, the first sign of behavioral signs of flurothyl exposure is motor arrest, followed by a clonic seizure. In younger mice (until postnatal

day (PD) 15), the pups do not experience motor arrest, but rather experience either clonic or tonic-clonic seizures. This shows the higher susceptibility of younger animals to seizures and convulsive agents (Auvin and Nehlig, 2017).

There are three primary advantages of using flurothyl to induce seizures. First, it eliminates the need for injection, thus minimizing stressors placed on the pups, since flurothyl is highly volatile and is inhaled. Second, flurothyl-induced seizure durations are short (*e.g., e.g.,* typically around 15-60 sec depending on the expressed seizure types), thus allowing for induction of multiple seizures in a day, which is critical for this project. Lastly, flurothyl is rapidly eliminated unmetabolized through the lungs, thus eliminating potential confounds of residual convulsant remaining in the body (Ferland, 2017).

Procedure

Prior to their first day of seizure, mice were randomly assigned into two groups, receiving either seizures or control procedure, and toes were clipped to maintain the mice's identity throughout the experiment. After the toes were clipped, mice are returned to the home cage for 30 minutes prior to the first seizure.

For the first seizure in PD7, pups were isolated from home cage and placed into clear plexiglass inhalation chamber (29 cm x 16 cm x 15 cm), inside a laboratory fume hood (Kewaunee ® Scientific Corp., NC, USA). Seizure was induced via pumping flurothyl (Sigma-Aldrich, St. Louis, USA) through a gas chamber at a rate of 50 µL/ min, using a syringe pump (Model: 11 Plus, Harvard Apparatus). Following seizure induction, mice were placed into a separate container with other same-treatment counterpart for monitoring. These containers were filled with clean bedding and warmed to ambient nesting temperature (~ 35℃) with a heating pad. Following the first and last seizure

induction of each day, the mice received a subcutaneous injection at the nape of the neck of 1.0 mL of 0.9% saline solution, to reduce the effects of dehydration (Nolan et al., 2019).

For the control animals, similar procedures were performed, including placing animals in an identical chamber outside of the fume hood for an identical amount of time and similar injections of saline to prevent dehydration. Each seizure animal received 3 seizures per day from PD7 – PD11, with 2-hour interval between each episode of seizure. Thus, each seizure was scheduled to receive up to a total of 15 episodes of seizure per animal.

Ultrasonic Vocalization

Ultrasonic vocalization (USVs) is often used extensively as a model for studying early communicative behavior (Branchi et al., 2006). Neonatal rodents produced USVs with frequencies between 30 and 90 kHz, often upon separation from the dam, littermates, and home cage, to elicit maternal approach and retrieval. These USVs are a one of the few behavioral phenotypes that can be quantitatively studied and analyzed from an early postnatal age.

Procedure

All ultrasonic vocalization recordings occurred between 1 pm to 5 pm, approximately 24-hour after the last seizure for the seizure group. To assess alterations in USV behavior following seizures, we examined USV recording on PD12 using the isolation-induced USV paradigm previously described (Reynold et al., 2017; Nolan et al., 2019).

Litters were allowed to acclimate for approximately 45 minutes in the testing room. All tested pups were then transferred to a holding cage with fresh bedding, warmed by an electronic heating pad to ambient nesting temperature (~35℃).

To begin the test, the pup was transferred into its testing cage and placed within an acrylic sound-attenuating chamber (40 cm x 40 cm x 30 cm). The cage was covered by a styrofoam lid with the microphone attached. USV was recorded using a condenser microphone (CM16/CMPA, Avisoft Bioacoustic, Germany) connected to an ultrasound-recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics), which allowed recording of all USVs within the range from 0 to 125 kHz. Each pup's USVs were recorded for 2 minutes.

After each trial, the pup was weighed and labeled, before were placed back into the holding cage with their littermates. This procedure was repeated until each pup in the litter was tested. The experimenter remained in the testing room during all recordings. At the conclusion of the experiment, pups were returned to the home cage.

Analysis

At the conclusion of all testing, all files were downloaded and analyzed across both genotypes and treatment conditions MATLAB DeepSqueak software was used on all recorded audio files to convert them into spectrograms using a fast Fourier transformation procedure (Coffey et al., 2019) (Figure 1). The following parameters for our lab have been set to maintain consistency between data: Short Rat Call neural network, total analysis length = 0, analysis chunk length = 6 s, overlap = .0001 s. Frequency range was set between 40 kHz and 140 kHz. USVs detected by DeepSqueak were manually reviewed and categorized either as noise or USV. A second trained

researcher also reviewed a set of USV for interrater reliability. USV acoustic parameters included in the analysis and their operational definitions are summarized in Table 1. The total call duration, average call duration, average fundamental frequency, mean power, total number of USVs, and latency to first call identified on these spectrograms were used to examine the potential difference in communicative behaviors between groups. Additionally, call-types were manually scored by an experimenter blinded to the group identity, using a previously described classification scheme. These call types included complex, two-syllable, upward, downward, chevron, short, composite, frequency steps, flat, and harmonic (Figure 2) (Scattoni et al., 2008). Harmonic call types were excluded from analysis since there was no call emitted that fit this call-type categorization. A new call-type, unstructured, was introduced to observe whether its appearance was a characteristic of either early life seizures, or the germline deletion of FMR1 gene, or an interaction of both.

Table 1. The definitions of acoustic dependent variables of USV detected by DeepSqueak (all definitions taken form DeepSqueak documentation) (Coffey et al., 2019).

Acoustic Dependent Variables (unit of measurement)	**Definitions**
USV production per recording condition (#)	Number of USVs per recording condition per individual mice
Duration (ms)	Duration of USV
Principal fundamental frequency (kHz)	Median frequency of the frequencies of the call contour
Mean Power (dB/Hz)	Average power spectral density of the call contour. By using the call contour this measurement of intensity is not influenced by background noise

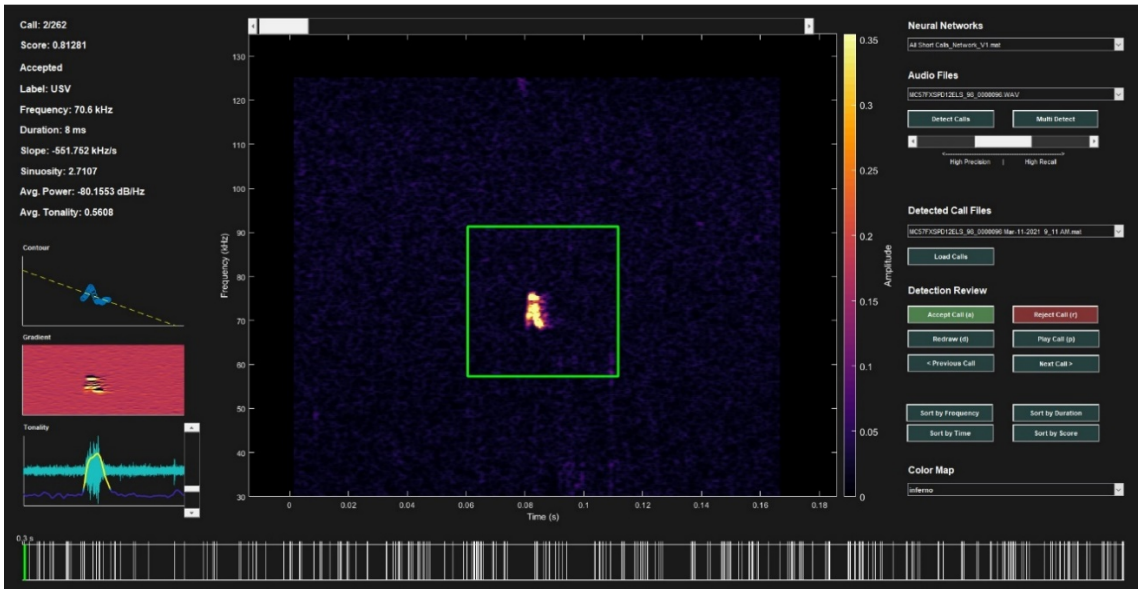

Figure 1. Example of a spectrogram of detected USV by DeepSqueak. The spectrogram contains the detected USV within the detection box, with the intensity labeled by the heat color gradient bar on the right.

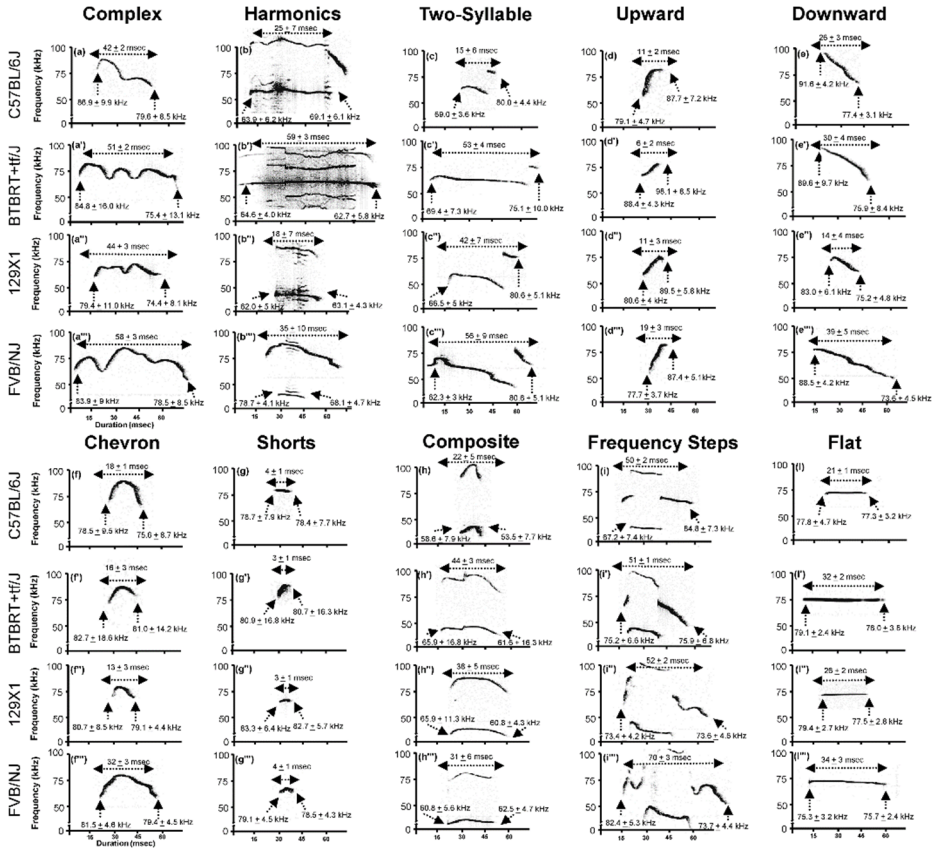

Figure 2. Typical sonograms of ultrasonic vocalization (Scattoni et al., 2008)

CHAPTER FOUR

Results and Conclusion

Results and Data Analysis

Animals

A total of 64 mice were initially designated for these experiments. Nine mice did not undergo the complete seizure or control procedure, therefore were excluded from subsequent analysis. Three mice did not vocalize during USV recording (2 WT seizure and 1 KO control) and were also excluded from subsequent analysis, leaving the sample sizes as follow: $n_{WT\ control} = 9, n_{WT\ seizure} = 22, n_{KO\ control} = 11, n_{KO\ seizure} = 10$.

Duration and Counts of USV

During ultrasonic vocalization recording, the average call duration, total call duration, latency to first call, average fundamental frequency, mean power, and total number of calls were recorded. A two-way (Treatment [control, seizure] x Genotype [wild-type, knockout]) analysis of variance (ANOVA) were used to evaluate these parameters. Significant interactions were followed with appropriate post hoc analysis using Bonferroni correction, at the level of $P < 0.05$. The results are presented in Figure 3.

We first analyzed the total amount of time spent vocalizing by a given animal in the two-minute testing session. Results for the average duration of calling behavior showed that there was a main effect of treatment where pups that received seizures produce longer calls than control pups, $F(1,48) = 9.16$, $p < 0.01$, though there was no

main effect of genotype, $F(1,48) = 0.04$, $p = 0.85$, nor significant interaction between genotype and treatment, $F(1,48) = 0.23$, $p = 0.63$ (Figure 3A). The cumulative duration of vocalization was also increased for pups the received seizures, $F(1,48) = 8.40$, $p < 0.01$, and this variable also not interact significantly with genotype, $F(1,48) = 1.04$, $p = 0.78$ (Figure 3B).

We next analyzed the total number of USV produced per animal regardless of call types during the two-minute testing session. A two-way ANOVA demonstrated a significant main effect of treatment, $F(1,48) = 6.20$, $p < 0.05$, with seizure mice emitting higher numbers of call than control pups (Figure 3C). There was no significant main effect of genotype, $F(1,48) = 0.002$, $p = .97$, nor significant interaction between genotype and treatment, $F(1,48) = 0.83$, $p = 0.37$.

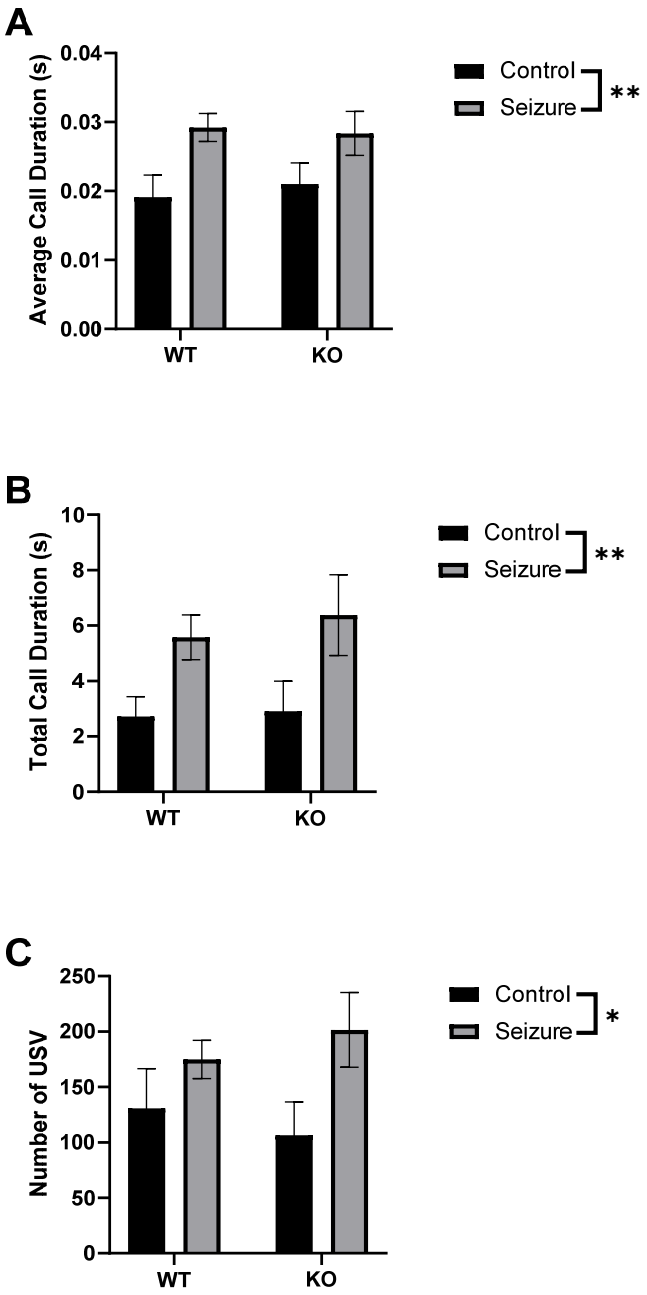

Figure 3. Changes in durations and counts of USV produced by wild-type (WT) and *Fmr1* knockout (KO) mice on postnatal day 12 (PD 12) with and without seizure induction on PD7–11. **A.** The average call duration was significantly increased in animals that experienced seizures. **B.** The total time spent vocalizing was also significantly increased in animals that experience seizures. **C.** The number of USV produced was significantly increased in animals that experience seizures. * = $P < 0.05$; ** = $P < 0.01$. The bar graphs represent the mean, and the error bars represent the standard error of the mean.

Spectral features of USV

To further explore the effect of early life seizure on neonatal communicative behavior, we analyzed the spectral features of USVs produced by individual mice during the two-minute testing session.

First, we explored the differences in latency to begin vocalizing, utilizing the two-way ANOVA. There was a significant main effect of treatment, $F(1,48) = 7.22$, $p < 0.05$, but not genotype, $F(1,48) = 0.40$, $p = 0.531$, nor interaction between both variables, $F(1,48) = 1.22$, $p = 0.27$ (Figure 4A). Post hoc analysis revealed that ELS significantly reduced the time interval when pups were first placed in the testing cage and their first call.

We next analyzed the differences in mean power or amplitude of the calls made by individual pups. A two-way ANOVA revealed a significant main effect of treatment, $F(1,48) = 5.88$, $p < 0.05$; however, there was neither main effect of genotype, $F(1,48) = 0.02$, $p = 0.89$, nor interaction between treatment and genotype, $F(1,48) = 0.04$, $p = 0.85$ (Figure 4C). Post hoc analysis showed a basal difference between ELS pups and control pups, with ELS pups producing USV with significantly higher power or amplitude. There was no significant difference in power between *Fmr1* KO pups and WT pups.

Lastly, we analyzed the difference in average fundamental frequency, or the median frequency of the USVs produced by individual pup during the testing session. A two-way analysis revealed no significant main effect of treatment, $F(1,48) = 1.02$, $p = 0.32$, nor main effect of genotype, $F(1,48) = 1.56$, $p = 0.22$ (Figure 4B). There was also no significant interaction between two factors, $F(1,48) = 0.25$, $p = 0.62$.

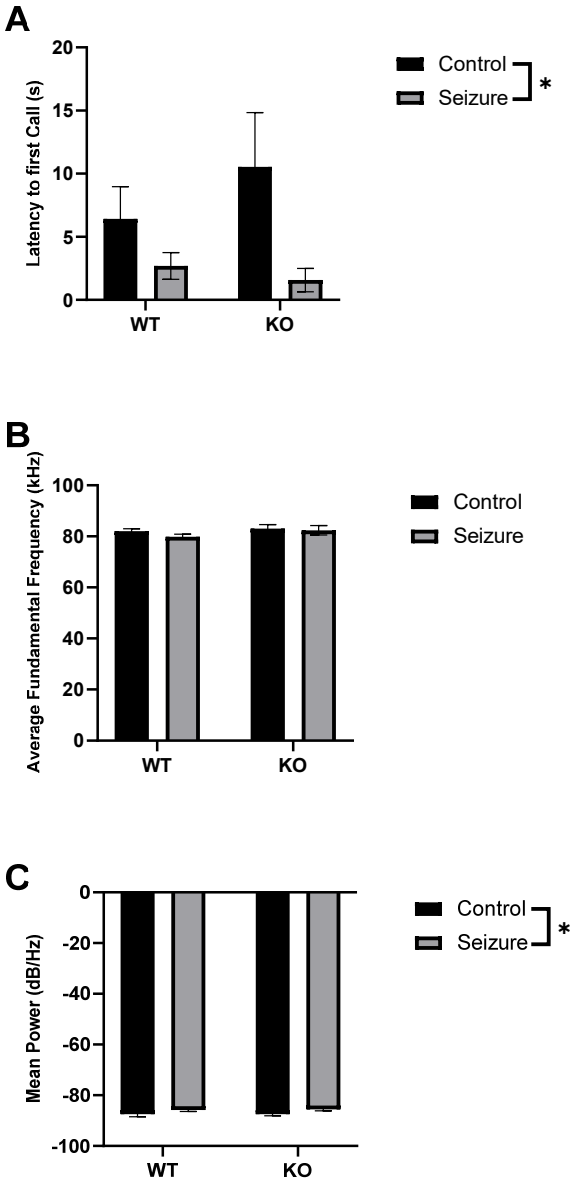

Figure 4. Changes in spectral aspects of USV produced by wild-type (WT) and *Fmr1* knockout (KO) mice one postnatal day 12 (PD 12) with and without seizure induction on PD7–11. **A.** Seizure had a significant effect on latency to begin vocalizing, as seizure animals begin to call earlier than control mice. **B.** There was no significant effect of treatment nor genotype on the average frequency of USV produced. **C.** The mean power, or amplitude, was significantly increased in USV produced by animals that experience seizures. * = $P < 0.05$; ** = $P < 0.01$. The bar graphs represent the mean, and the error bars represent the standard error of the mean.

Call-types

In addition to the quantitative properties of the USV, all recorded USVs were manually identified as a specific call-type based on their spectral features (Figure 2). (Scattoni et al., 2008). In order to evaluate differences in distributions of these call types between experimental groups, we utilized two-way ANOVA for each individual call classification to evaluate the main effects. Significant interactions were followed up with Bonferroni's post hoc analysis for each call-type. The relative frequency distribution of specific call-types for each experimental group are listed in figure 6.

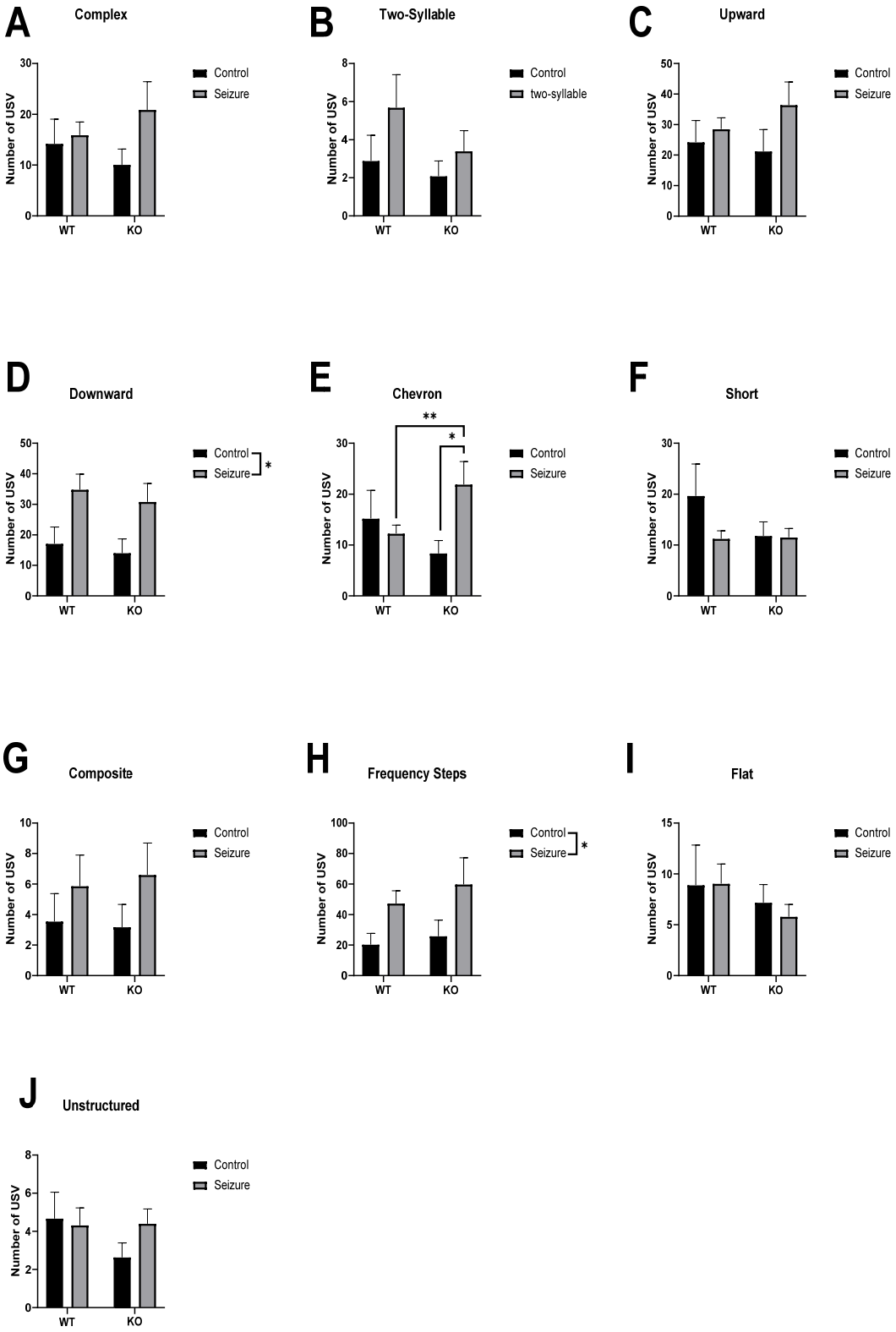
A
Complex
Number of USV
Control
Seizure
WT
KO
B
Two-Syllable
Number of USV
Control
two-syllable
WT
KO
C
Upward
Number of USV
Control
Seizure
WT
KO
D
Downward
Number of USV
Control
Seizure
*
WT
KO
E
Chevron
**
*
Number of USV
Control
Seizure
WT
KO
F
Short
Number of USV
Control
Seizure
WT
KO
G
Composite
Number of USV
Control
Seizure
WT
KO
H
Frequency Steps
Number of USV
Control
Seizure
*
WT
KO
I
Flat
Number of USV
Control
Seizure
WT
KO
J
Unstructured
Number of USV
Control
Seizure
WT
KO

Figure 5. Changes in qualitative aspects of vocalization behavior following exposure to seizures from PD7–11. **A**. There was no significant effect of genotype, treatment, nor interaction for complex call-type. **B.** There was no significant effect of genotype, treatment, nor interaction for two-syllable call-type. **C.** There was no significant effect of genotype, treatment, nor interaction for upward call-type. **D.** Males pups experienced seizures produced more downward call-type. **E**. There was an interaction between genotype and treatment for chevron call-type, as KO mice that experienced seizures produced more chevron call-type than KO control mice and WT mice that experience seizures. **F.** There was no significant effect of genotype, treatment, nor interaction for short call-type. **G**. There was no significant effect of genotype, treatment, nor interaction for composite call-type. **H.** Males pups experienced seizures produced more frequency step call-type. **I.** There was no significant effect of genotype, treatment, nor interaction for flat call-type. **J.** There was no significant effect of genotype, treatment, nor interaction for unstructured call-type. * = $P < 0.05$; ** = $P < 0.01$. The bar graphs represent the mean, and the error bars represent the standard error of the mean.

We observed a significant interaction between treatment and genotype for chevron, $F(1,48) = 6.30$, $p < 0.05$ (Figure 5E). The post hoc analysis revealed a significant increase in the frequency of chevron call-type emitted by *Fmr1* KO mice after seizure, but not in WT mice, resulting in a significant deficit of this call-type in WT/Seizure group compared with the KO/Seizure group.

Two-way ANOVA also revealed a significant main effect of treatment for downward, $F(1,48) = 8.35$, $p < 0.01$, and frequency steps, $F(1,48)$, $p < 0.05$, call-types, with seizure mice producing more of both call types (Figure 5. D, H). However, there was no significant main effect of genotype for downward call-type, $F(1,48) = 0.36$, $p = 0.55$, nor frequency steps call-type, $F(1,48) = 0.60$, $p = 0.44$. Similarly, there was no significant interaction between the two variables for both downward, $F(1,48) = .01$, $p = 0.94$, and frequency steps, $F(1,48) = 0.09$, $p = 0.77$, call-types.

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 2.54$, $p = 0.12$, nor main effect of genotype, $F(1,48) = 0.01$, $p = 0.91$, nor interaction between both variables, $F(1,48) = 1.35$, $p = 0.25$, for complex call-type (Figure 5A).

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 1.38$, $p = 0.25$, nor main effect of genotype, $F(1,48) = 0.78$, $p = 0.38$, nor interaction between both variables, $F(1,48) = 0.18$, $p = 0.67$, for two-syllable call-type (Figure 5B).

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 2.52$, $p = 0.12$, nor main effect of genotype, $F(1,48) = 0.16$, $p = 0.69$, nor interaction between both variables, $F(1,48) = 0.79$, $p = 0.38$, for upward call-type (Figure 5C).

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 2.10$, $p = 0.21$, nor main effect of genotype, $F(1,48) = 0.01$, $p = 0.94$, nor interaction between both variables, $F(1,48) = 0.06$, $p = 0.81$, for short call-type (Figure 5F).

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 1.62$, $p = 0.25$, nor main effect of genotype, $F(1,48) = 0.78$, $p = 0.38$, nor interaction between both variables, $F(1,48) = 0.18$, $p = 0.67$, for composite call-type (Figure 5G).

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 0.63$, $p = 0.80$, nor main effect of genotype, $F(1,48) = 1.03$, $p = 0.32$, nor interaction between both variables, $F(1,48) = 0.10$, $p = 0.75$, for flat call-type (Figure 5I).

Two-way ANOVA revealed no significant effect of treatment, $F(1, 48) = 0.43$, $p = 0.51$, nor main effect of genotype, $F(1,48) = 0.82$, $p = 0.37$, nor interaction between both variables, $F(1,48) = 0.97$, $p = 0.33$, for unstructured call-type (Figure 5J).

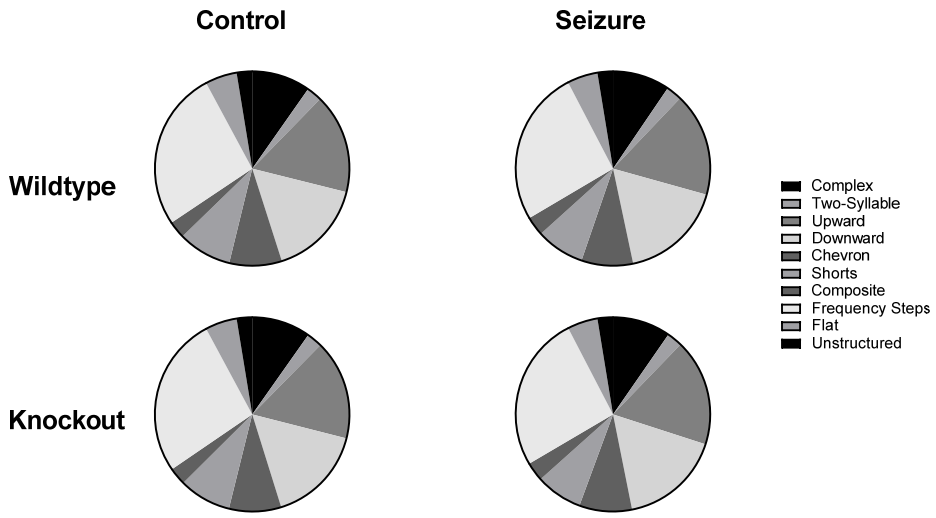

Figure 6. Relative frequency distribution of specific call-types for each experimental group

Discussion

It is well established that both childhood epilepsy and germline mutation of the *Fmr1* gene can independently lead to persistent communication impairments in both rodents and humans. The present study builds upon findings of previous investigations from our lab that characterized the impact of a single episode of SE during sensitive period of development on communicative behavior in *Fmr1* knockout mice (Huebschman et al., 2020). Despite the effect of ELS on FXS mice model, it is unclear whether seizure frequency load contributes toward the magnitude of these communication alterations. In the present study, we hypothesized that similar to the relationship of cognitive impairments and seizure load, a greater seizure load during the sensitive period would correspond to a greater magnitude of autistic-like communication impairments. We found that experiencing a high seizure load (15 seizures over 5 days) led to an increase in call duration, total number of USVs, mean power, and shorter latency to first call on

isolation-induced vocalization behavior over the testing window on PD12 in both WT and *Fmr1* KO mice.

In the current study, we found that call duration was increased in seizure-induced animals compared to the controls. These results spanned both cumulative and average call durations, regardless of the numbers of USV produced per individual pups. Call duration has been critical in understanding maternal behavior, as longer-duration calls seem to elicit stronger maternal retrieval (Smith, 1976). Increased call durations over the testing window of PD12 have been reported across different models of neurodevelopmental disorders, including FXS (Reynold et al., 2017), and ELS (Nolan et al., 2019). However, a previous investigation from our lab utilizing the same two-hit impact of ELS on a preexisting genetic deletion of the *Fmr1* gene reported that ELS decreased the mean duration of USV in WT/seizure animals (Huebschman et al., 2020). It is, however, important to note that the Huebschman et al. study was conducted in FVB/NJ mice, whereas in the present study, mice were on a C57BL/6J background strain. The contradicting result could be accounted for by the strain difference between studies, as previous investigation has reported strain-dependent differences in vocalization behaviors in mice (Scattoni et al., 2008).

We also observed a significant increase in the total number of USVs produced by seizure animals compared to the controls, which is similar to previous studies that examined vocalization behavior on PD12 after seizure induction in rodents (Heubschman et al., 2020; Keller et al., 2004). However, previous investigation from our lab utilizing the same seizure paradigm reported nonsignificant changes in the number of USVs made (Nolan et al. 2019). Furthermore, multiple studies reported a significant suppression in

vocalization behavior in rodents following seizures (Lopez-Meraz et al., 2014; Reynolds et al., 2017). In the Lopez-Meraz et al., 2014 study, they found a suppression in the number of USVs in PD14 rats (Lopez-Meraz et al., 2014). A similar suppression was found in the Reynolds et al., 2017 study, which was conducted in mice (Reynolds et al., 2017). One key difference between the present study and previous studies is that they used chemoconvulsants pilocarpine and kainic acid to induce SE in a single day. Therefore, the differences in results between these studies and the current study could be accounted for by the types of seizure induction, the numbers of seizure induction in a single day, or the time frame of seizure induction. Several studies have shown different neuronal effects between flurothyl and kainic acid/pilocarpine toward the developing brain. Pilocarpine and kainic acid result in neuronal damages in various brain regions like CA1, amygdala, or the mediodorsal thalamic nucleus (Kubová et al., 2001; Sankar et al., 1998). However, flurothyl, which was used to mimic recurrent generalized tonic-clonic seizures, does not show similar neuronal damage but rather results in greater numbers of dentate granule cells and aberrant sprouting of mossy fibers in the CA3 region (Holmes, 2005). Thus, these studies could suggest that different types of chemoconvulsant affect the developing brain in a different way compared to one another.

The results of the present study are in line with a previous investigation from our lab utilizing the same seizure-inducing paradigm in male WT pups on PD12 (Nolan et al., 2019). In addition to showing increased USV average, cumulative call durations, and mean amplitude, our quantitative findings also showed an increase in the total number of USV produced and reduced latency to begin vocalizing. The finding of reduced latency to begin vocalizing in male pups is largely in agreement with one previous study, which

examined USVs following pilocarpine-induced SE in neonatal rats (Lopez-Meraz et al., 2014). Reduced latency to begin vocalizing could reflect an increase in the emotional reactivity of the pups, but further investigations examining the meaning behind this quantitative assessment are necessary to determine whether this is a phenotypic trait of seizure animals.

One primary difference between the USV analysis of this study in comparison to the previous studies was the use of DeepSqueak software, instead of the traditional USV analysis completed using SASLab Pro (Avisoft Bioacoustics). While previous published studies have provided several different call classification schemes, we selected the Scattoni et al. classification scheme for the current study because of its application in the ASD rodent field. During the analysis of the USV spectrograms, we noticed an irregular call-type detected only on DeepSqueak (Figure 7). One drawback of using DeepSqueak is the inability to manually add/breakdown USV, thus, we have introduced unstructured call-type, a new call-type categorization that does not follow the previously described classification scheme (Scattoni et al., 2008). We found that there was no significant main effects of treatment and genotype, or interaction between the two, thus suggesting that the presence of unstructured call-type could be attributed to methodological factor, including the analysis software used. Furthermore, using the Scattoni et al. classification scheme, we failed to observe significant main effects of treatment and genotype or a significant interaction between the two variables for complex call-type. While distributions of different call-types can vary according to background strain, reduced complex call-type has been consistent in ASD rodent models across multiple background strains (Nolan et al., 2019; Scattoni et al., 2008). It is important to note that DeepSqueak neural network

was trained using rat model, rather using a mice model. Prior to the beginning of the experiment, our lab compared and found a high correlation (R>0.9) in quantitative properties, such as total numbers of USV produced and durations, between the two systems. Compared to the traditional Avisoft, DeepSqueak provides a more time-efficient way to analyze vocalization behavior. Furthermore, the results of the present study corroborate with previous investigations from our lab, thus providing support for the utilization of DeepSqueak as a potential software to analyze vocalization behavior in the future. Currently, there is a lack of a consensus classification scheme and interpretation of USV subtypes using the DeepSqueak software. Future studies should target to (1) determine the meaning of the different call-types, and (2) create a consensus classification scheme utilizing the DeepSqueak software.

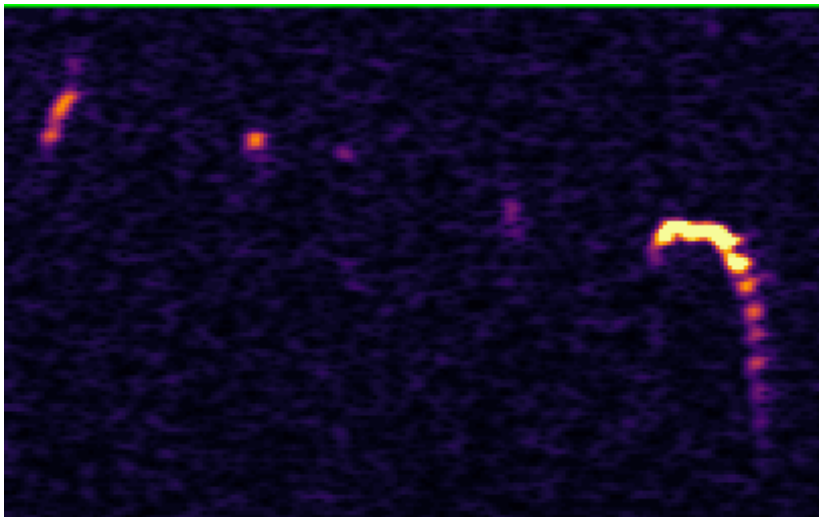

Figure 7. An example of unstructured call-type that only detected on DeepSqueak.

Conclusion

FXS individuals with epilepsy can suffer from a number of behavioral comorbidities, including communication deficits. The effect of multiple ELS on wild-type mice has been investigated in an earlier study (Nolan et al., 2019). However, the results of the present study corroborate and extend previous findings, demonstrating the superimposing effect between FMR1 germline mutation and recurrent seizures during a critical period of language development.

Male pups that experienced seizures, not only produced longer individual USV and had longer call times, but also produced a higher number of USVs, louder USVs, and begin to vocalize at a shorter time. This difference in findings could be attributed to a superimposing effect with the introduction of the FMR1 germline mutation as an interacting variable. However, it could also be attributed to the methodological difference between the study with the difference in spectrogram analysis software. Nevertheless, these studies highlight the importance of utilizing double-hit models to understand how comorbid conditions individually alter behavioral phenotypes and provide support for the inclusion of double-hit models in neurodevelopmental research.

BIBLIOGRAPHY

Abbeduto, L., McDuffie, A., & Thurman, AJ. (2014). The Fragile X Syndrome–Autism Comorbidity: What Do We Really Know? *Frontiers in Genetics*. *Vol. 5.* 355

Auvin, S., & Nehlig, A. (2017). Chapter 38 - Models of Seizures and Status Epilepticus Early in Life. In A. P. Pitkänen, P. S. Buckmaster, A. S. Galanopoulou, & S. L. Moshé (Eds.), *Models of Seizures and Epilepsy* (2nd ed., pp. 569–86). Academic Press.

Bagni, C., Tassone, F., Neri, G., & Hagerman, R. (2012). Fragile X Syndrome: Causes, Diagnosis, Mechanisms, and Therapeutics. *The Journal of Clinical Investigation Vol. 122 (12*), 4314–22.

Bear, M. F., Huber, K.M., & Warren S.T. (2004). The MGluR Theory of Fragile X Mental Retardation. *Trends in Neurosciences Vol 27(7),* 370–77.

Berry-Kravis, E., Raspa, M., Loggin-Hester, L., Bishop, E., Holiday, D., & Bailey Jr., D.B. (2010). Seizures in Fragile X Syndrome: Characteristics and Comorbid Diagnoses. *American Journal on Intellectual and Developmental Disabilities Vol 115(6),* 461–72.

Bianchi, R., Chuang, S-C., Zhao, W., Young, S.R, & Wong, R.K.S. (2009). Cellular Plasticity for Group I MGluR-Mediated Epileptogenesis. *Journal of Neuroscience Vol 29 (11),* 3497–3507.

Black, L.C., Schefft, B.K., Howe, S. R., Szaflarski, J.P., Yeh, H-s, and Privitera, M.D. (2010). The Effect of Seizures on Working Memory and Executive Functioning Performance. *Epilepsy & Behavior: E&B Vol. 17 (3),* 412–19.

Brady, N., Skinner, D., Roberts, J., & Hennon, ,E. (2006). Communication in Young Children with Fragile X Syndrome: A Qualitative Study of Mothers' Perspectives. *American Journal of Speech-Language Pathology Vol. 15 (4)*, 353–64.

Branchi, I., Santucci, D., & Alleva, E., (2006). Analysis of Ultrasonic Vocalizations Emitted by Infant Rodents. *Current Protocols in Toxicology Vol. 30 (1),* 13.12.1-13.12.14.

Chuang, S-C., Bianchi, R., Kim, D., Shin, H-S., & Wong, R.K.S. (2001). Group I Metabotropic Glutamate Receptors Elicit Epileptiform Discharges in the Hippocampus through PLCβ1 Signaling. *Journal of Neuroscience Vol. 21(16),* 6387–94.

Chuang, S-C., Zhao, W., Bauchwitz, R., Yan, O., Bianchi, R., & Wong, R.K.S. (2005). Prolonged Epileptiform Discharges Induced by Altered Group I Metabotropic

Glutamate Receptor-Mediated Synaptic Responses in Hippocampal Slices of a Fragile X Mouse Model. *Journal of Neuroscience Vol. 25 (35),* 8048–55.

Coffey, K.R., Marx, R.G., & Neumaier, J.F. (2019). DeepSqueak: A Deep Learning-Based System for Detection and Analysis of Ultrasonic Vocalizations. *Neuropsychopharmacology Vol. 44 (5),* 859–68.

Dahlhaus, R., & El-Husseini, A. (2010). Altered Neuroligin Expression Is Involved in Social Deficits in a Mouse Model of the Fragile X Syndrome."*Behavioural Brain Research Vol. 208 (1),* 96–105.

Elger, CE., Helmstaedter, C., & Kurthen, M. (2004). Chronic Epilepsy and Cognition. The *Lancet Neurology Vol 3(11),* 663-672.

Ferland, R.J. (2017). The Repeated Flurothyl Seizure Model in Mice. *Bio-Protocol Vol. 7 (11).*

Finestack, LH., Richmond, EK., & Abbeduto, L. (2009). Language Development in Individuals with Fragile X Syndrome. *Topics in Language Disorders Vol. 29 (2),* 133–48.

Gibson, JR., Bartley, AF., Hays,SA., & Huber, KM. (2008). Imbalance of Neocortical Excitation and Inhibition and Altered UP States Reflect Network Hyperexcitability in the Mouse Model of Fragile X Syndrome. *Journal of Neurophysiology Vol. 100 (5),* 2615–26.

Hagerman, PJ., & Stafstrom, CE. (2009). Origins of Epilepsy in Fragile X Syndrome. *Epilepsy Currents Vol. 9(4),* 108–12.

Hagerman, RJ., Rivera, SM., & Hagerman, PJ. (2008). The Fragile X Family of Disorders: A Model for Autism and Targeted Treatments. *Current Pediatric Reviews Vol. 4 (1),* 40–52.

Hermann, BP., Seidenberg, M., & Bell, B. (2002). The Neurodevelopmental Impact of Childhood Onset Temporal Lobe Epilepsy on Brain Structure and Function and the Risk of Progressive Cognitive Effects. In *Progress in Brain Research*, *Vol. 135*, 429–38. Do Seizures Damage the Brain. Elsevier,

Holmes, GL. (2005). Effects of seizures on brain development: lessons from the laboratory. *Pediatr Neurol Vol 33(1),* 1-11.

Huber, KM., Gallagher, SM., Warren, ST., & Bear, MF. (2002). Altered Synaptic Plasticity in a Mouse Model of Fragile X Mental Retardation. *Proceedings of the National Academy of Sciences Vol. 99 (11),* 7746–50.

Huber, KM., Kayser, MS., & Bear, MF. (2000). Role for Rapid Dendritic Protein Synthesis in Hippocampal MGluR-Dependent Long-Term Depression. *Science Vol. 288, (5469),* 1254–56.

Huebschman, JL., Hodges, SL., Reynolds, CD., Nolan, SO., & Lugo, JN. (2020). A Single Episode of Early-Life Status Epilepticus Impacts Neonatal Ultrasonic Vocalization Behavior in the Fmr1 Knockout Mouse. *Epilepsy & Behavior* Vol. 111, 107279.

Incorpora, G., Sorge, G., Sorge, A., & Pavone, L. Epilepsy in Fragile X Syndrome. *Brain and Development Vol. 24 (8*), 766–69..

Kaufmann, WE., Cortell, R., Kau, ASM., Bukelis, I., Tierney, E., Gray, RM., Cox, C., Capone, GT., & Standard, P. (2004). Autism Spectrum Disorder in Fragile X Syndrome: Communication, Social Interaction, and Specific Behaviors. *American Journal of Medical Genetics Part A Vol. 129A (3),* 225–34.

Kaufmann, WE., Kidd, SA., Andrews, HF., Budimirovic, DB., Esler, A., Haas-Givler, B., Stackhouse, T., et al. (2017). Autism Spectrum Disorder in Fragile X Syndrome: Cooccurring Conditions and Current Treatment. *Pediatrics Vol. 139 (3)* S194–206.

Kazdoba, TM., Leach, PT., Silverman, JL., & Crawley, JN. Modeling Fragile X Syndrome in the Fmr1 Knockout Mouse. *Intractable & Rare Diseases Research* Vol. 3 (4), 118–33.

Keller, A., Saucier, D., Sheerin, A., & Yager, J. (2004). Febrile Convulsions Affect Ultrasonic Vocalizations in the Rat Pup. *Epilepsy & Behavior Vol. 5 (5),* 649–54.

López-Meraz, M-L, Medel-Matus, J-S., Morgado-Valle, C., Beltrán-Parrazal, L., Pérez-Estudillo, C., & Manzo, J. (2014). Effect of Lithium-Pilocarpine-Induced Status Epilepticus on Ultrasonic Vocalizations in the Infant Rat Pup. *Epilepsy & Behavior Vol. 31*, 263–66.

Maenner, MJ (2020). Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2016. *MMWR. Surveillance Summaries Vol. 69.*

Niu, M., Han, Y., Dy, ABC., Du, J., Jin, H., Qin, J., Zhang, J., Li, Q., & Hagerman, RJ. (2017). Autism Symptoms in Fragile X Syndrome. *Journal of Child Neurology Vol. 32 (10),* 903–9.

Nolan, SO., Hodges, SL., Condon, SM, Muhammed, IDA., Tomac, LA., Binder, MS., Reynolds, CD., & Lugo, JN. (2019). High Seizure Load during Sensitive Periods of Development Leads to Broad Shifts in Ultrasonic Vocalization Behavior in Neonatal Male and Female C57BL/6J Mice. *Epilepsy & Behavior Vol. 95*, 26–33.

Philofsky, A., Hepburn, SL., Hayes, A., Hagerman, R., & Rogers, SJ. (2004). Linguistic and Cognitive Functioning and Autism Symptoms in Young Children WithFragile X Syndrome. Edited by Leonard Abbeduto. *American Journal on Mental Retardation Vol. 109(3),* 208–18.

Reynolds, CD., Nolan, SO., Huebschman, JL., Hodges, SL., and Lugo, JN. (2017). Early-Life Status Epilepticus Acutely Impacts Select Quantitative and Qualitative Features of Neonatal Vocalization Behavior: Spectrographic and Temporal Characterizations in C57BL/6 Mice. *Epilepsy & Behavior Vol.72*, 58–62.

Reynolds, CD., Nolan, SO., Jefferson, T., & Lugo, JN. (2016). Sex- & Genotype-Specific Differences in Vocalization Development in FMR1 Knockout Mice. *Neuroreport* Vol. 27(18), 1331–35.

Sabaratnam, M., Vroegop, P.G, & Gangadharan, SK. (2001). Epilepsy and EEG Findings in 18 Males with Fragile X Syndrome. *Seizure Vol. 10 (1),* 60–63.

Saemundsen, E., Ludvigsson, P., Hilmarsdottir, I., & Rafnsson, V. (2007). Autism Spectrum Disorders in Children with Seizures in the First Year of Life—A Population-Based Study. *Epilepsia Vol. 48 (9*), 1724–30.

Scattoni, ML, Gandhy, SU., Ricceri, L., & Crawley, JN. (2008). Unusual Repertoire of Vocalizations in the BTBR T+tf/J Mouse Model of Autism. *PLOS ONE Vol. 3 (8),* e3067.

Smith, JC. Responses of Adult Mice to Models of Infant Calls. *Journal of Comparative and Physiological Psychology Vol. 90 (12),* 1105–15.

Smith, LE., Barker, ET., Seltzer, MM., Abbeduto, L., & Greenberg, JS. (2012). Behavioral Phenotype of Fragile X Syndrome in Adolescence and Adulthood. *American Journal on Intellectual and Developmental Disabilities Vol. 117 (1),* 1–17.

Spencer, CM., Alekseyenko, O., Hamilton, SM., Thomas, AM., Serysheva, E., Yuva-Paylor, LA., & Paylor, R. (2011). Modifying Behavioral Phenotypes in Fmr1 KO Mice: Genetic Background Differences Reveal Autistic-Like Responses. *Autism Research : Official Journal of the International Society for Autism Research Vol. 4 (1)* 40–56.

Stafstrom, CE., & Carmant, L. (2015). Seizures and Epilepsy: An Overview for Neuroscientists. *Cold Spring Harbor Perspectives in Medicine Vol. 5 (6).*

Wang, J, Ethridge, LE., Mosconi, MW., White, SP., Binder, DK., Pedapati, EV., Erickson, CA., Byerly, MJ., & Sweeney, JA. (2017). A Resting EEG Study of Neocortical Hyperexcitability and Altered Functional Connectivity in Fragile X Syndrome. *Journal of Neurodevelopmental Disorders Vol. 9 (1),* 11.

Wouters, H, Fonteyne, A., & Stiers, P. (2006). Specific Memory Impairment in a Multiple Disabled Male with Fragile X Syndrome and Temporal Lobe Epilepsy. *Developmental Medicine & Child Neurology Vol. 48 (5),* 378–82.