ABSTRACT

Ultrasonic Vocalization Behavior in –Fmr1 – Knockout Mice Following Early Life Seizures

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Fragile X Syndrome (FXS) is a genetic disorder caused by an expansion mutation of the CGG triplet in the –fmr-1 gene on the X chromosome. This disorder is characterized by hyperactivity, increased anxiety, repetitive-stereotyped behaviors, and impaired language development. Many children diagnosed with FXS also experience seizures during their lifetime. Previous studies estimate the comorbidity between FXS and epilepsy to be approximately 20%. However, the underlying etiology of this relationship is not fully understood. Ultrasonic vocalizations (UVs) are one tool that may be used to measure early behavioral changes in mice pups. In the present study we used neonatal UVs to analyze early communicative behaviors in a mouse model of FXS, both with and without early life seizures. On postnatal day (PD) 10, we administered 2.5 mg/kg of kainic acid via intraperitoneal injections to male FXS knockout (KO) and wild type (WT) mice to induce continuous seizures (status epilepticus). On PD 12, pups from all groups were temporarily isolated from their dam and ultrasonic vocalizations were recorded. We found a several alterations in number and duration of certain type of calls emitted in the KO seizure mice when compared to the WT seizure mice. In particular there were differences in the chevron, complex, composite, short, and downward types of vocalizations. There was an overall decrease in the number of calls made by the KO seizure group, $p<.05$. Our results provide support that early-life seizures and Fmr1 knockout can impact the communication aspect of behavior in mice during early development.

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ULTRASONIC VOCALIZATION BEHAVIOR IN –*FMR1*- KNOCKOUT MICE FOLLOWING EARLY LIFE SEIZURES

A Thesis Submitted to the Faculty of

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CHAPTER ONE

Introduction

Fragile X Syndrome (FXS) is an X chromosome linked disorder characterized by delayed or impaired language development, increased anxiety, repetitive behaviors, and hyperactivity (Bagni, Tassone, Neri, & Hagerman, 2012). Individuals with FXS may also have a comorbid diagnosis of epilepsy, with 14% of males and 6% of females reporting seizures in a national survey (Berry-Kravis, 2002). The survey also indicated that the FXS seizure population was more likely to have a diagnosis of autism, experience increased anxiety levels, display increased aggression, and have poor verbal abilities compared to the seizure free FXS population.

Diagnosis of FXS often occurs around the time of language development, when abnormalities become more apparent (Bagni et al., 2012). Speech in young FXS individuals is typically characterized by shorter utterances with fewer pauses between them than healthy age matched individuals (Roy,Watkins, & Heck, 2012). Young mice will produce ultrasonic vocalizations (UVs) when separated from their mother, and these calls can be classified into distinct categories based on frequency, duration, and pitch changes (Roy et al., 2012; Scattoni, Gandhy, Ricceri, & Crawley., 2008). Previous studies have compared UVs produced by wild type (WT) mice and those produced by the FXS model –*Fmr1*- knock out (KO) mice, but have found various results. Some studies found an increased number of vocalizations in the KO mice, some found no difference, and others found a decreased number of vocalizations (Kazdoba, Leach, Silverman, &

Crawley, 2014). In one study, there was no significant difference in the average number of calls emitted between –*Fmr1*- KO mice and WT mice when separated from their mother on post-natal day 8 (PD8) (Roy et al., 2012). However, a different study found an increased number of calls emitted by the KO group compared to the WT on PD7, but not on PD4 or PD10, suggesting that differences in vocalization behavior may be dependent on pup age (Lai, Sobala-Drozdowski, Zhou, Doering, & Faure, 2014). In addition, both studies found a change in specific call type vocalizations between the KO and WT groups, indicating that the key differences may be in the types of calls made rather than the number of calls (Roy et al., 2012; Lai et al., 2014).

The current study has two key objectives. The first is to add to the existing literature regarding ultrasonic vocalizations in –*Fmr1*- KO pups by examining the differences in isolation induced vocalization behavior between KO pups and healthy wild type (WT) pups. Specifically, we will look for differences in the overall number of calls made and the quantity and duration of each call type emitted. The second aim of this study is to examine UVs in –*Fmr1*- KO mice who have experienced early life seizures, comparing these calls to those made by WT seizure models and –*Fmr1*- KO mice without seizure experience. Consistent differences in early life vocalization behavior amongst these populations can establish early communicative behavior, such as cries in newborns, as a behavioral marker for neurodevelopmental disorders such as Fragile X Syndrome. This may provide a means of earlier detection and intervention, which can enhance treatment effectiveness.

CHAPTER TWO

Review of Literature

Fragile X Syndrome

Fragile X Syndrome (FXS) was first described by Martin and Bell (1943), though the mental defect did not yet have a name. They studied an extended family with eleven known sons affected by a mental defect that slowed their development of language and social skills. The authors were able to create a pedigree for the family, and concluded that the disorder was likely caused by a sex-linked recessive gene (Martin & Bell, 1943). Further studies supported Martin and Bell's conclusion; the disorder does not follow a standard Mendelian pattern of inheritance (Yaron, Musci, & Cuckle, 2013). Rather, FXS is an X-linked genetic disorder. Because of this, it is more prevalent in males: 1 in 2,500 to 5,000 males are affected by a full mutation versus 1 in 4,000 to 6,000 women (Bagni et al., 2012). In women with a full mutation on one chromosome, the second un-mutated X chromosome provides compensation; only 1 in 3 females with a full mutation will be affected by Fragile X Syndrome, and those who are typically experience fewer cognitive affects (Hagerman, Au, & Hagerman, 2011).

Causes

Fragile X Syndrome is caused by an expansion of a CGG triplet on the *FMR1* gene. The gene was discovered in 1991 by positional cloning (Verkerk et al., 1991). The CGG triplet may be repeated between 5 and 54 times in a normal allele and 55 to 200 times in a premutation allele (Bagni et al., 2012). These premutation alleles are fairly

common in the general population, found in 1 in 130 to 250 women and in 1 in 250 to 810 men (Hagerman et al., 2011). Individuals with a premutation may exhibit fewer and less severe FXS symptoms. During maternal transmission these premutation alleles tend to expand further; alleles with as few as 56 repeats have potential to expand to a full mutation and premutation alleles with more than 99 repeats have close to a 100% expansion risk. Nolin et al. (2003) evaluated 1,338 genetic transmissions from 936 mothers to confirm the positive correlation between the number of CGG repeats in the premutation allele and the risk of full mutation expansion in the next generation. The authors also found that premutation individuals with AGG triplets interspersed in the CGG region decreases the chance of expansion into a full mutation. Individuals with more than 200 CGG repeats are considered to have full mutation alleles, and are likely to display the phenotypic qualities of Fragile X Syndrome.

The un-mutated "healthy" *FMR1* gene is methylated in the promoter region of the gene, further upstream than the CGG repeat sequences (Bagni et al., 2012). These normal alleles are not methylated near the actual repeat sequence, so this area is thought of as a boundary that prevents methylation from spreading. In FXS alleles, this boundary is not present and the area directly upstream of the repeat sequence becomes methylated. This usually occurs around the thirteenth week of embryonic development. This methylation stops gene transcription, meaning that no protein product (FMRP) is generated from this gene. In a healthy individual, FMRP acts as an RNA binding protein and a transport protein (Hagerman et al., 2011). It acts as an inhibitor of mRNA functioning, suppressing protein formation; in the knock out (KO) FXS mouse model a 20% increase in hippocampal protein production can be observed. FMRP also regulates

presynaptic and postsynaptic proteins, so its absence can lead to synaptic dysregulation. In addition, in FXS individuals the glutaminergic mGluR5 pathway is upregulated, and the GABA system is downregulated. These physiological changes cause disruptions in synaptic plasticity, adult neurogenesis, and neuronal activity levels.

Physiological Changes

Fragile X Syndrome is known for causing physiological changes in the dendritic spines found on the cell bodies of neurons. In healthy individuals, metabotropic glutamate receptor (mGluR) activation leads to an increased localization of FMRP and its target *Fmr1* mRNA in the dendrites (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004). FMRP acts as a repressor, preventing transcription of the mRNA into synaptic proteins. In both FXS patients and FVB mouse models, studies have shown that there is an increase in the number of long, thin dendritic spines and overall spine density after development (Beckel-Mitchener & Greenough, 2004). Long, thin dendritic spines are characteristic of immature tissue, suggesting that the disruption of FMRP in FXS causes a disruption in dendritic development. In addition, the increased spine density in FXS individuals suggests a disruption in the pruning process (Antar et al., 2004).

Detection and Diagnosis

With modern technology, it is possible to screen for FXS both prenatally and in newborns. However, this area tends to be controversial. Yaron et al. (2012) argues that prenatal diagnosis of FXS can be largely beneficial. The screening can predict the chances of a premutation expanding into a full mutation during transmission based on the

number of CGG repeats and the presence of interspersed AGG triplets in the mother's premutation allele. The World Health Organization states that prenatal screenings may be used for diseases that cause significant health problems when there is a natural history of the disease; there must also be a suitable, economically balanced, test for identifying the disease. The prenatal screening test for FXS meets all of these requirements, but as of 2012, it is typically reserved only for at risk individuals (i.e. individuals with family history of FXS or autism). However, the authors also demonstrate a stand against universal prenatal FXS screening. They state that the FXS screening is unique in that it not only identifies potential health conditions in the fetus, but also provides information on health hazards to the mother. These include FXS-associated primary ovarian insufficiency and FXS associated tremor and ataxia syndrome, both of which have a late onset and may affect carriers of a premutation. In addition, they state that the screening presents a challenge in reporting results, primarily because the number of CGG repeats cannot completely predict the resulting phenotype. The controversy regarding this prenatal screening for FXS is not entirely resolved (Yaron et al., 2012).

Postnatal detection usually occurs as development progresses and the symptoms of the disorder become apparent. In most cases, this occurs with the development of motor coordination and language around 2 and 3 years of age (Bagni et al., 2012). In some cases, a late diagnosis of FXS can be made in patients with less severe symptoms or in individuals who may have been misdiagnosed, especially before the discovery of the FMR1 gene (Verkerk et al., 1991).

Symptoms

Children usually begin displaying clear symptoms around the age of 2 or 3 (Bagni, 2012). These children may display impaired speech development, delayed motor skills, hand flapping, poor eye contact, or irritability. Symptoms similar to those of autism spectrum disorder (ASD) may lead to an initial misdiagnosis until the distinction is made clear; around 30% of males with FXS also meet the diagnostic criteria for ASD. Individuals with FXS are also at a higher risk for experiencing seizures, especially during childhood (Hagerman et al., 2009). Males are 13-18% more likely to experience early life seizures, and females are about 5% more likely; individuals with FXS typically display abnormal electroencephalographic findings, even without experiencing epileptic seizures. As FXS children develop, symptoms are largely marked by general hyperactivity. In addition, FXS children may at first avoid social interactions, but can develop out of this behavior later on (Hagerman et al., 2011). Adult males with FXS have an average IQ in the 40s, but this may vary with the level of methylation present in the *FRM1* gene; cognitive abilities in FXS individuals are positively correlated with FMRP levels. FXS is also associated with increased anxiety levels. Males with FXS also seem to deal with more behavioral issues, such as hyperactivity and aggression, while females with FXS seem to deal with more emotional issues, such as depression and anxiety (Valdovinos, 2007).

Animal Model Characteristics

-*Fmr1*- Knockout (KO) mice display behaviors that translate to those typically observed in FXS patients, and have been used as an animal model of FXS for research.

These KO mice have a disruption in the *FMR1* gene, and therefore lack FMRP protein (Kazdoba et al., 2014). Behavioral activities of the *–Fmr1-* KO model have been characterized, and consistently find increased movement and hyperactivity in open field tests (Kazdoba et al., 2014; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005). Studies examining anxiety levels in these mice vary across the literature, depending on the lab and the behavioral test used (Kazdoba et al., 2014). For example, in one study, mice placed on an elevated plus maze tended to make more entries into the open arms during the first minute than the WT control mice (Eadie et al., 2009). This indicates decreased anxiety in the KO mice, which differs from the increased anxiety typically observed in FXS patients. However, mirrored chamber experiments performed by Spencer et al. (2005) may indicate increased anxiety in *–Fmr1-* KO mice. Social tests, including novel object and social partition tests, have generally indicated normal sociability in the genotype, but some groups have observed deficits (Kazdoba et al., 2014). Characterization of anxiety and social behaviors in the *FMR1* KO mouse vary across mouse strains and studies.

-Fmr1-KO pups can also be characterized by the ultrasonic vocalizations made when separated from the mother (Scattoni et al, 2008). These results are varied, with some studies finding more vocalizations in the KO mice, some finding no difference, and some finding less vocalizations in KO mice (Kazdoba et al., 2014). In numerous studies there have been various differences in the frequency of certain call types and characteristics in the KO mice (Lai et al., 2014; Roy et al., 2012).

Treatment

Treatment of Fragile X Syndrome can be approached in a variety of ways. Phenotypic behavioral symptoms can be treated by using medications available for other disorders resulting in similar phenotypes (Hagerman et al., 2009). For example, stimulants used to treat ADHD can be effective in treating irritability and hyperactivity in FXS individuals. Likewise, selective serotonin reuptake inhibitors (SSRIs) typically used to treat mood disorders like depression may be beneficial in treating anxiety in FXS patients. Other forms of treatment examine the neurobiology that results from the *FMR1* mutation. It appears that hippocampal and cerebellar pathways regulated by mGluR5 are enhanced in *FMR1* knockout models, so mGluR5 antagonists are being examined as potential treatments for FXS. The increased activation of mGluR5 results in long term depression (LTD) of the pathway, and increased protein synthesis preparing the synapse for the next signal (Liu & Smith, 2014). The synthesis of FMRP is proposed to act as a brake when the appropriate number of proteins have been made. In the absence of FMRP, there is exaggerated mGluR5 and protein synthesis activity. Lithium also plays a role in this protein synthesis regulation pathway and has seemed to be beneficial in both fruit fly and mouse models of FXS. Current studies are being conducted to evaluate the efficacy and safety of lithium in human FXS patients. The research is moving from symptom based treatment to neurobiological based treatment.

Early Life Seizures in Fragile X Syndrome

As mentioned previously, individuals with Fragile X Syndrome are at a higher risk for early life seizures than a healthy individual (Hagerman et al., 2009). In most cases, epilepsy will disappear in adolescence, however there are a few cases of it persisting into adulthood (Tondo et al., 2011). The average age of seizure onset in FXS patients is between 6 months and 4 years of age (Incorpora, Sorge, Sorge, & Pavone, 2002). Studies have shown as high as a 20% seizure and EEG abnormality rate in FXS individuals. The combination of epilepsy and FXS can have additional behavioral impacts, which vary across individuals (Wouters, Fonteyne, Lagae, & Stiers, 2006).

EEG Patterns and Seizure Types

EEG recordings and seizure types tend to vary across FXS patients. In 1991, Musumeci et al. found similarities in the EEGs of FXS seizure patients to EEG recordings of individuals with benign childhood epilepsy with centrotemporal spikes (BCECTS). However, Rees et al. (1993) found that there was no association between BCETS and FXS gene regions using linkage analysis. The literature in more recent years varies, with some, but not all, individuals displaying EEG patterns similar to those observed in BCETS. One study that closely examined seizure FXS patients found the seizures to be either partial complex or secondary generalized seizures, with EEG recordings resembling BCECTS recordings in 10% of patients observed (Incorpora et al., 2002). Other EEG recordings have been observed in FXS patients that resemble those of individuals with childhood epilepsy with occipital paroxysms, Landau-Kleffner

Syndrome, partial frontal epilepsy with favorable evolution, and status epilepticus during sleep. The persistence of seizures into adulthood seems to be related to the type of seizures and EEG recordings observed in childhood. A focal, frontal, rhythmic, slowing pattern and poor seizure control seem to be indicators of persisting epilepsy in FXS individuals (Hagerman et al., 2009).

Treatment of Seizures in FXS

Gauthey, Polini, Ramelli, Roulet-Perez, and Korff. (2010) examined the seizure behavior of 5 individuals whose initial seizure was status epilepticus. They found that the seizure activity seemed to decrease as the patients grew older, with one individual, given no treatment, never experiencing an additional seizure. Incorpora et al. (2002) showed individuals with a variety of EEG recordings who also responded differently to treatment. Of six patients with full convulsive seizures and abnormal EEG readings, two individuals' seizures disappeared completely by age 8, two continued to experience sporadic seizures, and two experienced frequent, severe seizures. These last two individuals were unresponsive to various attempts at treatment, including phenobarbital, valproic acid, and clonazepam. The other individuals improved with either phenobarbital or valproic acid treatment.

Further studies support the findings that seizures in most FXS patients can be treated with a single anticonvulsant (Hagerman et al., 2009). The difficulty is determining which anticonvulsant is best suited for each patient in an effort to avoid the aversive side effects of medications on top of existing behavioral and cognitive deficits in FXS individuals. Valproic acid and carbamazepine are typically work well, but

additional medications may be used to monitor persistent seizures. It also has been determined that phenobarbital and gabapentin should be avoided because of their tendency to further increase behavioral problems such as hyperactivity. Future studies in seizure treatment in FXS individuals should focus on the specific biological effects of FMRP deficiency.

Cause of Seizures in FXS Patients

The underlying mechanism for seizures in FXS patients is not currently well understood, and the literature varies across studies. Generally speaking, it is thought that the increase in excitatory synapses and cortical activity may account for some of the seizure activity seen in FXS individuals (Valdovinos, 2007). However, it is not clear what additional factors are necessary to trigger seizures in some individuals and not others.

One hypothesis evaluates the role of brain derived neurotrophic factor (BDNF) on neuronal activity levels (Louhivuori et al., 2009). Excess BDNF due to mutations in the pathways can lead to hyper-excitability of neurons, and has been tied to epilepsy in some cases. Louhivuori et al. (2009) evaluated the occurrence of a Val66Met mutation in the BDNF coding region in FXS patients with seizures. They found that in 10% of 27 FXS patients experience seizures, and that every one of these individuals had a Val66Met polymorphism, indicating that this BDNF related mutation may predispose FXS individuals for epilepsy. However, Tondo et al. (2011) found no statistically significant difference in the presence of Val66Met and epilepsy in FXS patients.

Another area of research examining the connection between FXS and epilepsy focuses on premutation carrier mothers. There is a significant increase in occurrence of epilepsy in FXS individuals whose mothers have an autoimmune disease (Chonchaiya et al., 2010). It is hypothesized that the interaction between the down regulation of GABA receptors and an increased expression of glutamic acid decarboxylase (GAD) in FXS KO mice may be influenced by the presence of an autoimmune disease in the mother and results in a lower threshold for seizures. The role of autoimmune disease in carrier mothers is an area still being explored.

CHAPTER THREE

Methods

Subjects

Fifty-five FVB/NJ mice were used in this study. Of these, 29 mice were Fmr1 knockout (KO) mice and 26 were wild type (WT). All mice were housed in a controlled environment, with temperatures held to 22^0 C and a 14-hour light/10-hour dark diurnal cycles. Mice were also given full access to food and water. All procedures were carried out in compliance with Baylor University Institutional Animal Care and Use Committee and the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Seizure Induction Method

On postnatal day ten (PD10) both KO and WT mice were randomly assigned to either the control group of the experimental group, with an approximately equal number of KO and WT in each group. The KO control group consisted of 14 mice, and the seizure group consisted of 15 mice. The WT control group had 14 mice and the seizure group had 12 mice. The control group received an intraperitoneal (IP) injection of saline, while the experimental group received an IP injection of kainic acid (kainate). IP injections are a standard method of administration in mice and allow for more accurate and less stressful injections than intravenous injections. Each mouse received 2.5 mg/kg of either 0.9% saline or kainic acid. Kainic acid is a chemoconvulsant that activates ionotropic receptors that respond to the excitatory neurotransmitter glutamate. IP

injections of kainic acid serve as an agonist to this pathway, and cause excitotoxicity resulting in seizures. All mice were monitored for 1-2 hours after injection, and seizures were recorded following the procedure outlined below.

All mice were monitored for signs of seizure activity following PI injections. A timer was started at the time of injection, and the time was noted at which a mouse entered one of the stages of seizures. Forelimb clonus, usually the first sign of seizure activity, typically occurred around 20 minutes after injection and is observable as stiff, extended forelimbs. Hindlimb clonus, usually accompanied by continued forelimb clonus, is observable as stiff, extended hindlimbs. The presence of both forelimb and hindlimb clonus is indicative of generalized seizures affecting most areas of the brain. The final stage noted was status epilepticus. Status epilepticus is identified by consistent convulsions and the forelimbs moving medial to bring the paws together. If a mouse in the experimental group had not begun seizing by 30 minutes after the initial injection, they were given an additional injection with a dose 1/3 of the original. After mice entered status epilepticus, they seized for a period of time as the kainic acid was metabolized. Mice were returned to their home environment after a full recovery was made. Control mice were run in parallel under similar conditions.

Ultrasonic Vocalizations

Mice pups will typically vocalize (isolation-induced USVs) when separated from the mother and other littermates (Bronchi et al., 2006). Ultrasonic vocalization tests were conducted for all mice on postnatal day 12 (PD12). In preparation for testing, mice were removed from the home cage and placed into a housing pan with clean bedding. The

temporary housing pan was warmed to nesting temperature with a heating pad. For testing, mice were individually placed into an acrylic, sound-attenuating chamber while isolation-induced vocalizations were recorded. The recording apparatus consisted of a condenser ultrasonic microphone (CM16/CMPA, Avisoft Bioacoustics, Germany, part #40011) connected to an ultrasound-recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics, part # 41161/41162). After the 2-minute period, mice were returned to the heated housing pan. After all mice were tested, they were returned to their home cage. Testing groups consisted of no more than 6 mice, so time separated from the mother was 20 minutes or less.

Vocalization Analysis

The frequency and duration of vocalizations was recorded for each mouse. Fast Fourier transformation (FFT) was performed on all recorded audio files using Avisoft SASLab Pro software. All spectrograms were then generated with an FFT-length of 1024 points, a time window overlap 75% (100% Frame, Hamming Window), frequency resolution of 488 Hz, and a time resolution of 1 ms. Each call was then analyzed and labeled using Scattoni et al.'s (2008) findings of typical ultrasonic vocalizations emitted by FVB/NJ mice (see Appendix). The call frequency, duration, and call types were then compared across the four groups using a 1-way ANOVA multiple comparisons test. Differences between each group were analyzed using an unpaired t-test.

CHAPTER FOUR

Results

Spectrogram Analysis

After the initial manual analysis, spectrograms of each 2-minute vocalization period were saved. These spectrograms display the various call types described in Scattoni et al. (2008). A random spectrogram from our sample is provided in Figure 1.

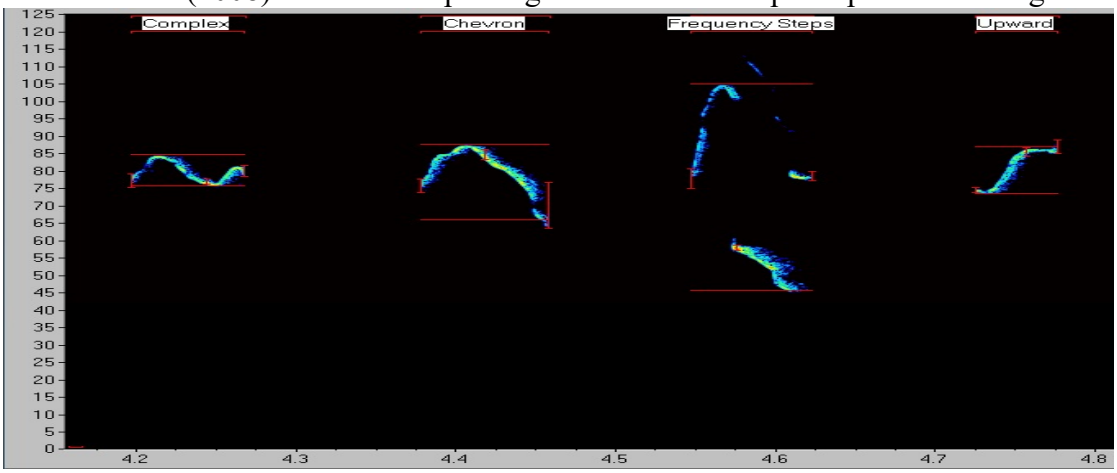


Figure 1. Sample Spectrogram. A section from the spectrogram of a random mouse in this study. This figure shows complex, chevron, frequency steps, and upward call types.

Vocalization Behavior across Groups

Overall, the WT-Seizure group made significantly more downward calls than the KO-Control and the KO-Seizure groups $F(3,51) = 4.806$, $p \leq 0.01$ (Fig. 2A). The WT-Seizure group spent significantly more time making these downward calls than the KO-Seizure group $F(3,51) = 6.837$, $p \leq 0.001$ (Fig. 2B). In addition, the WT-Seizure group made significantly more short calls than the other groups $F(3,51) = 3.84$, $p \leq 0\ .05$ (Fig. 2C).

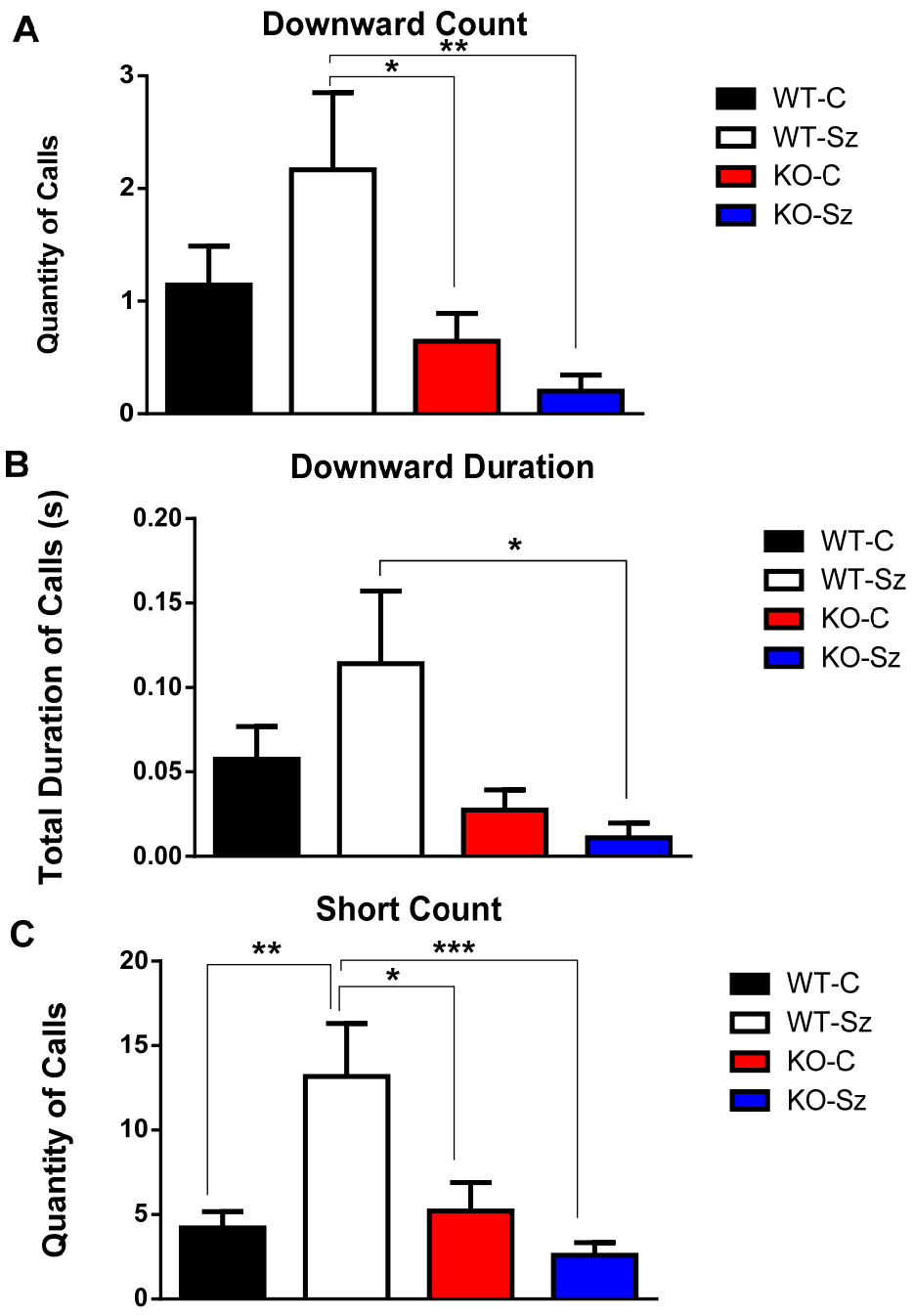


*Figure 2. Vocalization Behavior Across Groups. Significant differences were observed in the quantity and duration of downward calls, and the quantity of short calls. * = p<0.05, ** = p<0.01, *** = p<0.005*

Vocalization Behavior between Groups

Overall the KO-Control group animals made shorter flat calls than the WT-Control group $t(26) = 2.067$, $p \leq 0.05$ (Fig.3). There were no other differences in vocalization behavior between the KO-Control and the WT-Control.

The WT-Seizure group made significantly more short calls $t(24) = 2.904$, $p \leq 0.01$, and spent more time producing short calls $t(24) = 2.581$, $p \leq 0.05$, than the WT-Control group (Fig. 4). The other call types did not differ between the WT-Seizure and WT-Control groups. There were no differences in vocalization behavior between the KO-Seizure and KO-Control groups.

Chevron, short, downward, and complex call types differed between the WT-Seizure and the KO- Seizure groups. Overall, the KO-Seizure group made fewer chevron calls $t(25) = 2.417$, $p \leq 0.05$, and spent less time making chevron calls, $t(25) = 2.274$, $p \leq 0.05$, than the WT-Seizure group (Fig.5A, B). The KO-Seizure group also made significantly fewer short calls, $t(25) = 3.618$, $p \leq 0.01$ (Fig. 5C). The quantity of downward calls $t(25) = 3.124$, $p \leq 0.01$, the total duration of all downward calls $t(25) = 2.603$, $p \leq 0.05$, and the average length of each downward call $t(25) = 2.476$, $p \leq 0.05$, were all significantly lower in the KO-Seizure group as well (Fig. 5D-E). Likewise, the KO-Seizure group made fewer complex calls $t(25) = 2.758$, $p \leq 0.01$, and spent less time making complex calls than the WT-Seizure group $t(25) = 2.443$, $p \leq 0.05$ (Fig. 5G, H). Finally, the KO-Seizure group made significantly fewer calls, regardless of call-type, than the WT-Seizure group $t(25) = 2.510$, $p \leq 0.05$ (Fig. 5I).

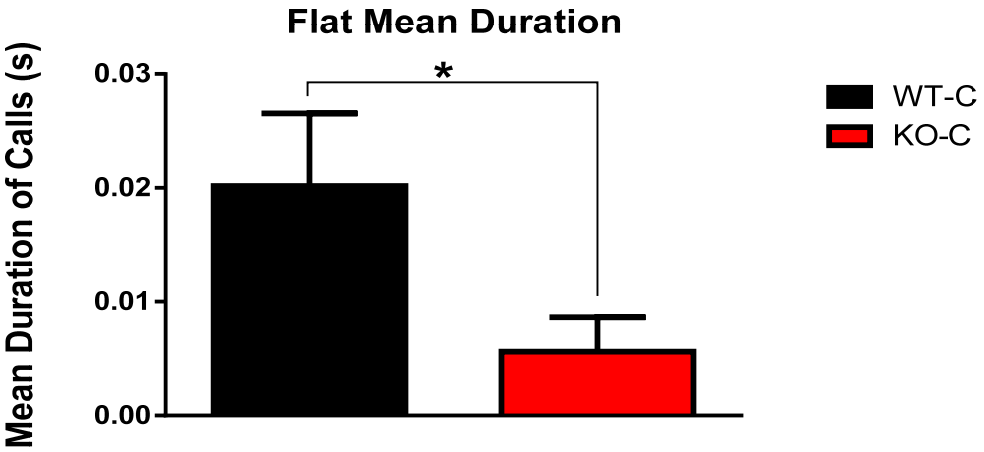


*Figure 3. WT-Control v. KO-Control. WT-Control animals vocalized significantly more than KO-Control animals. * = p<0.05*

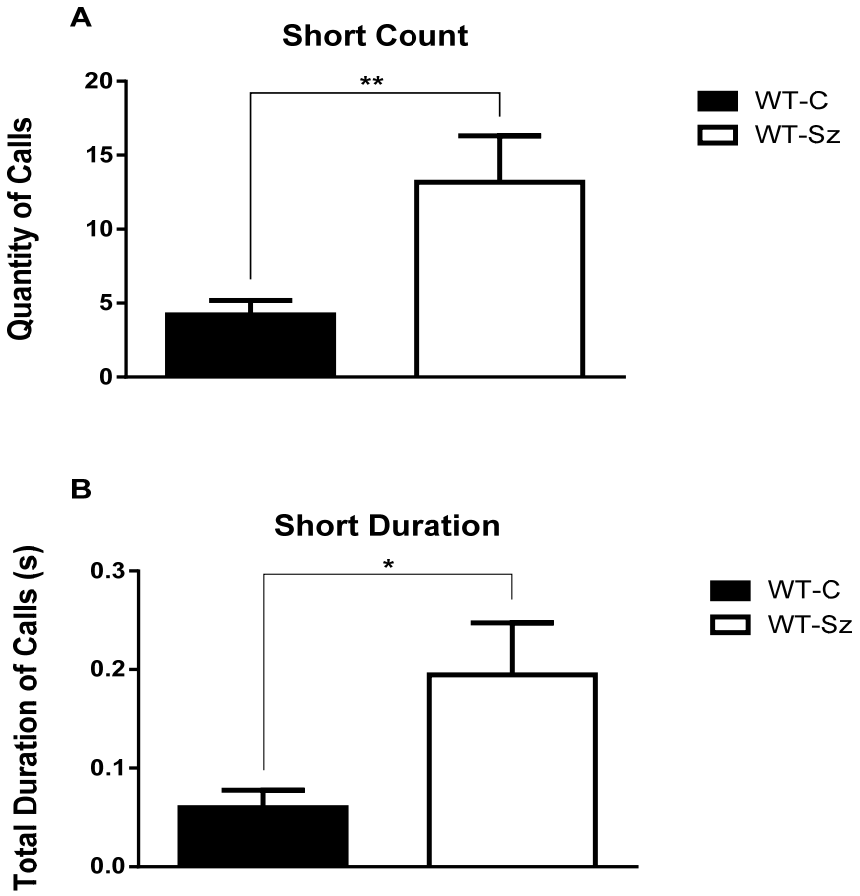


*Figure 4. WT-Control v. WT-Seizure. WT animals displayed increased short call behavior after early life seizure. * = p<0.05, ** = p<0.01*

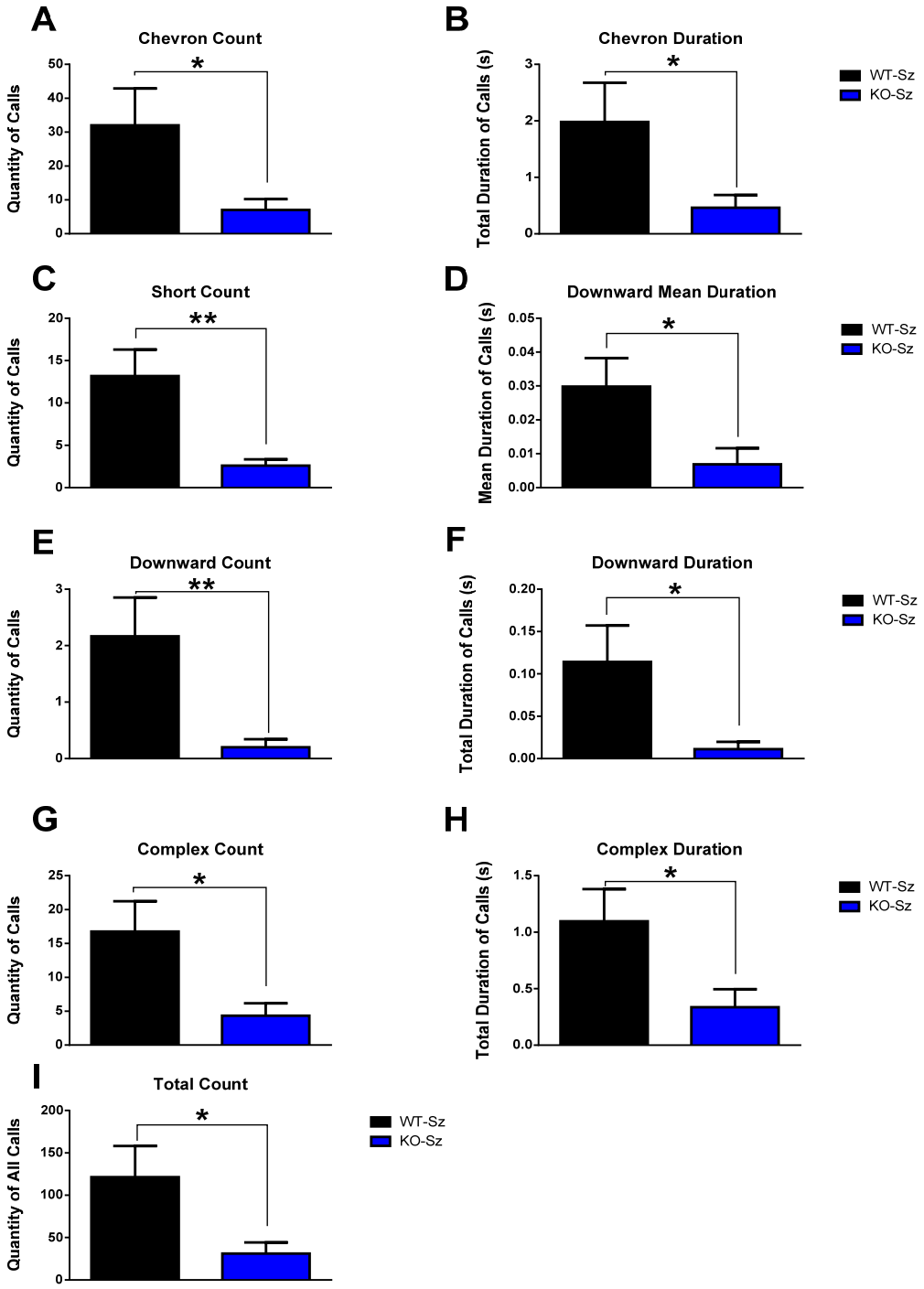


*Figure 5. WT-Seizure v. KO-Seizure. The KO-Seizure mice displayed decreased vocalization behavior in chevron, short, downward, and complex call types. KO-Seizure mice also made significantly fewer total calls than WT-Seizure mice. * = p<0.05, ** = p<0.01*

CHAPTER FIVE

Discussion

Early life seizure activity causes significant changes in call-type specific vocalization behavior. In WT mice, we observed a significant increase after seizure experience in both the quantity of short calls made and the total time spent making short calls in the two minute period. We also saw a significant difference between the WT-Control group and the WT-Seizure group average short call count when we looked for differences across all of the groups. Few studies have examined the effects of early life seizures on ultrasonic vocalization behavior, but our data supports the previous findings that seizure experience alters communicative behavior in rodents (Keller, Saucier, Sheerin, & Yager, 2004; Lopez-Meraz et al.,2014). However, we found a call-type specific increase in vocalizations, while previous studies observed a suppression in USV activity with seizure experience. Lopez-Meraz et al. (2014), examined vocalization behavior in male rats, using UV detectors picking up calls in the 40 kHz range, after pilocarpine-induced seizures. Keller et al. (2004) also examined vocalization behavior in rats after seizure induction, detecting calls made in the 20-30 kHz range. However, rodent pups can emit vocalizations with frequencies anywhere between 30 and 90 kHz (Branchi, Santucci, & Alleva, 2006). Our study is unique because we were able to examine calls over a frequency range of 0-125 kHz using the Avisoft microphones and software, allowing us to detect all vocalizations emitted during the 2-minute testing period.

Previous studies report altered vocalization behavior in the –*Fmr1*- KO model as well (Lai et al., 2014; Roy et al., 2012). Our results suggest that this alteration may be call-type specific, with the KO-Control group producing significantly less flat calls than their WT counterparts. We found no difference in the average number of total calls made. Some previous studies found no differences in the average number of calls made between groups, while others did report significant differences (Laie et al., 2014; Roy et al., 2012). The discrepancy in these studies may be due to the various ages at which vocalizations were recorded. One study reporting significantly more vocalizations in KO groups found differences only on PD7, but not PD4 or PD10 (Lai et al., 2014). Other studies have reported no significant differences in call behavior recorded on PD8, and our study found no significant differences on PD12 recordings (Roy et al., 2012). Future studies might examine the potential time dependent nature of vocalization behavior effects, focusing on the time between PD5 and PD7. Perhaps KO mice exhibit increased vocalization behavior only during certain times of development.

It is interesting to note that the differences in call behavior between WT and KO mice extend to additional call types after early life seizures. In the absence of early life seizures, there is only a significant difference in the average duration of flat calls. After early life seizure experience, four call types (chevron, complex, short, downward) are significantly different between the WT and KO groups. Overall, the quantity and duration of all four call types decreased in the KO-Seizure animals. In fact, KO-Seizure animals made significantly less vocalizations overall than the WT-Seizure group did. Our data does not provide a clear explanation for this difference. As no significant differences were observed between the KO-Control and KO-Seizure groups, it does not

seem to be caused by any effect the early life seizure may have had on KO vocalization behavior. We did observe some increased call specific vocalization behavior in the WT mice following early life seizure, and this increase could be the basis for the significant differences that exist between the WT-Seizure and the KO-Seizure groups. Vocalization behavior appears to increase in some manner in WT animals after early life seizure activity, but there is no data suggesting that a similar increase occurs in the KO animals. It may be that early life seizure experience acts through different mechanisms, resulting in different vocalization behavior. Further studies might chose to examine molecular differences that exist between these four groups in an attempt to better understand the different effects of early life seizures on WT and KO animals.

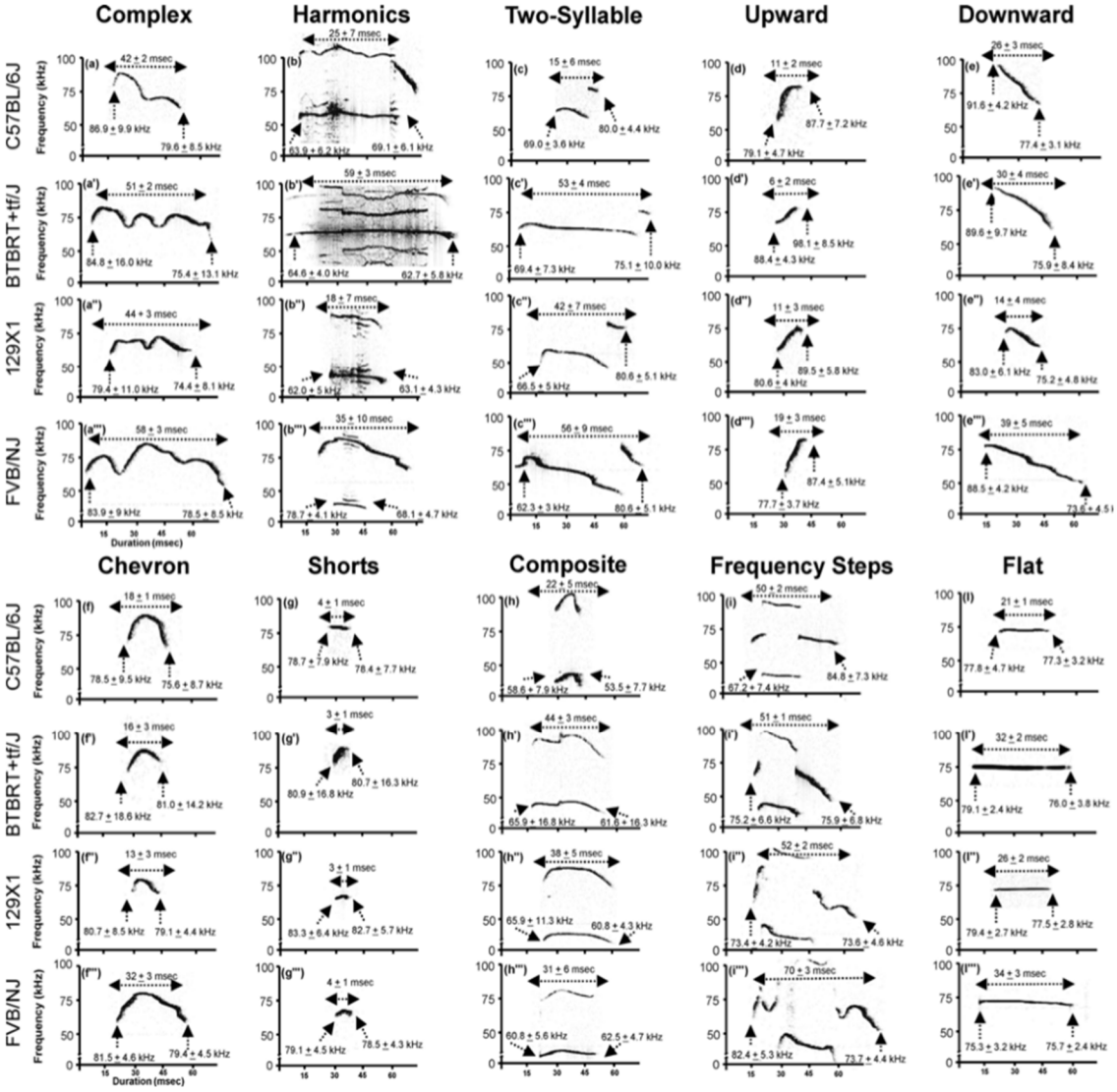
This study offers further characterization of ultrasonic vocalization communicative behavior in model mice systems, emphasizing the differences in vocalizations following early life seizures and in the –*Fmr1*- KO model. Early communicative behavior deficits can be characterized by call-types and used as early behavioral markers in mouse models. Neonatal communicative behavior may be analyzed and used as an early diagnostic tool for neurodevelopmental disorders such as FXS. Future directions for ultrasonic vocalization behavior will involve looking at vocalization behavior at different time points in development, as well as a deeper analysis of specific call-type patterns. Consistency in call-type patterns would provide further evidence for altered neonatal communicative behavior as an indicator for neurodevelopmental disorders, allowing for earlier diagnosis and earlier treatment.

APPENDIX

APPENDIX

Ultrasonic Vocalization Call-Type Classifications

Established by Scattoni et al. (2008)



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