Published in final edited form as:

Epilepsy Behav. 2020 October ; 111: 107279. doi:10.1016/j.yebeh.2020.107279.

A single episode of early-life status epilepticus impacts neonatal ultrasonic vocalization behavior in the *Fmr1* knockout mouse

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Abstract

Fragile X Syndrome (FXS) is a genetic disorder caused by a trinucleotide (CGG) expansion mutation in the *Fmr1* gene located on the X chromosome. FXS is characterized by hyperactivity, increased anxiety, repetitive-stereotyped behaviors, and impaired language development. Many children diagnosed with FXS also experience seizures during their lifetime. However, the underlying etiology of the relationship between FXS and epilepsy is not fully understood. Ultrasonic vocalizations (UVs) are one tool that may be used to measure early behavioral changes in mouse pups. In the present study, neonatal UVs were analyzed as a measure of communicative behavior in a mouse model of FXS, both with and without early life seizures. On postnatal day (PD) 10, status epilepticus was induced via intraperitoneal injections of 0.5% kainic acid (2.0 mg/kg) in male *Fmr1* knockout (KO) and wild type (WT) mice. On PD 12, all pups were temporarily isolated from their dam and UVs were recorded. Significant alterations were found in both spectral and temporal measures across genotype and seizure groups. Early-life seizure experience resulted in a significant increase in the quantity of UVs only in WT animals ($p < 0.05$). We also found that while there was no difference between genotypes in the total number of vocalizations made, calls produced by *Fmr1* KO mice were significantly shorter and had a higher peak frequency compared to WT mice. Overall, these findings support the use of vocalization behavior as an early phenotypic marker and highlight the importance of utilizing double-hit models to better understand comorbid disorders.

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Graphical Abstract

Keywords

fragile X syndrome; early-life seizure; ultrasonic vocalization; double-hit model

1. Introduction

Fragile X syndrome (FXS) is an X-linked neurodevelopmental disorder and the leading monogenic cause of autism and intellectual disability, characterized by hyperactivity, anxiety, impaired social development, repetitive behaviors, and changes in synaptic function and plasticity [1]. FXS is caused by a trinucleotide (CGG) expansion mutation in the 5'-untranslated region of the *Fmr1* gene, functionally silencing its expression and production of the fragile X mental retardation protein (FMRP) [2]. FMRP associates with polyribosomes to regulate the translation of hundreds of brain RNAs, many of which are known to have important synaptic functions [3, 4]. Germline disruption of FMRP causes long-term deficits in neurogenesis, synaptic plasticity, and neuronal transmission which can lead to persistent changes in cognitive function and behavior, as observed in FXS [1]. Social communication deficiency and impaired language development have been extensively characterized among children with FXS and suggest deficits across several domains including gestural, verbal/nonverbal, and symbolic communication [1, 5–7].

Ultrasonic vocalizations (UVs) with frequencies between 30–90 kHz are produced by neonatal rodents and have been used extensively as a model for studying early communicative behavior [8, 9]. These UVs, often emitted upon separation from the dam, littermates, and home cage, elicit maternal approach and retrieval, and are one of few behavioral outputs which can be quantitatively analyzed from a very early postnatal age [10, 11]. Throughout postnatal development and into adulthood, mice will continue to emit UVs in response to appetitive events, including various mating and social encounters [12, 13]. UVs can be further classified into distinct call-type categories based on frequency, duration, and pitch changes, providing a robust, quantifiable approach to examining communicative behavior in rodents [14, 15]. Investigation from our lab and others has identified select alterations in UV behavior of the *Fmr1* KO mouse at various time points in development and adulthood [14, 16–20].

Approximately 10–20% of individuals with FXS also experience seizures and those with a comorbid epileptic phenotype have increased anxiety, hyper-aggression, and decreased verbal communication compared to typical FXS patients [21]. This subset of individuals with FXS and seizures are also more likely to meet DSM criteria for Autism Spectrum Disorder (ASD) [22]. The highest incidence of seizures in FXS is during early childhood, which typically resolve by late adolescence [22]. In addition, early life seizures (ELSs) in

mice are known to directly result in long-term communication and social deficits and have previously been linked to autism-related deficits [23–25]. However, despite the relationship between seizure experience and FXS, the impact of ELSs on neonatal vocalization behavior in FXS is largely unknown.

In the present study, we examined the impact of a single ELS on separation-induced neonatal UVs in both *Fmr1* knockout (KO) and wildtype (WT) mice. In addition to characterizing the effect of superimposing seizures on a germline deletion of *Fmr1* in mice, our results provide further support for the potential use of neonatal communicative behavior as an early diagnostic tool for FXS and highlight the unique impact of ELSs on previously established alterations in the *Fmr1* knockout. Given the highest incidence of seizures in FXS individuals occurs during early life, it is crucial to understand how seizures can influence behavioral impairments associated with FXS. Further, understanding the effects of this comorbidity is critical to proper early diagnosis and therapeutic intervention, and may provide valuable insight into underlying neural mechanisms of FXS.

2. Materials and methods

2.1 Animals

Subjects included male *Fmr1* knockout (KO) and wild type (WT) mice on a FVB/NJ background strain (Jackson Labs Stock No: 004624). Given that the *Fmr1* gene is located on the X chromosome and FXS is therefore significantly more prevalent in males, only male mice were included in our experiments. Mice were bred and group housed in standard controlled conditions at Baylor University with temperatures held at 22°C and a 12-hour light, 12-hour dark cycle. Mice had access to food and water *ad libitum*. WT males and heterozygous dams were crossed to produce male *Fmr1* KO pups and WT littermate controls. This breeding scheme produces only KO and WT offspring since *Fmr1* is only inherited through the X chromosome. 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*. Our animal welfare number is A3948–01 and our IACUC approval number is 493338–5.

2.2 Seizure Induction

On postnatal day ten (PD10), *Fmr1* KO and WT pups were randomly assigned to either treatment or control groups, with approximately half of the males in any given litter selected for each group in order to minimize the influence of any single litter. At least 30 minutes prior to injection, mice were given unique toe clips for future identification throughout the duration of the experiment. Treatment mice received a single intraperitoneal injection of 0.5% kainate (2.0 mg/kg) and control mice received an equivalent amount of 0.9% physiological saline. Mice were then placed into individual containers with clean bedding on a heating pad (~35°C) for the remainder of the induction period to be monitored. Mice in the treatment group reached status epilepticus (SE) approximately 30 minutes after kainate administration, which was characterized by continuous tonic-clonic seizure activity, as measured by the Racine scale, lasting 1–2 hours [26]. Mice were monitored until cessation of all seizure activity, at which point both treatment and control mice were returned to their

home cages. Mice that did not enter full SE were excluded from behavioral analysis to ensure that all test mice had a similar severity of early-life seizure experience. The final sample sizes for vocalization testing were as follows: WT/control – 18; KO/control – 8; WT/seizure – 8; KO/seizure – 15.

2.3 Ultrasonic Vocalization Recordings

Isolation-induced ultrasonic vocalizations (UVs) were recorded from all mice on PD12 as a measure of neonatal communication behaviors. The recording chamber consisted of an acrylic, sound-attenuating chamber with a condenser ultrasonic microphone (CM16/CMPA, Avisoft Bioacoustics, Germany, part #40011) connected to an ultrasound-recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics, part # 41161/41162). Prior to testing, pups were removed from the home cage and placed into a housing pan with clean bedding on a heating pad warmed to ~35°C. For testing, pups were then individually placed into the recording chamber for two minutes and isolation induced UVs were recorded. Following the two-minute recording period, pups were returned to the temporary housing pan. The experimenter was blind to treatment conditions and pups were selected for testing in a random manner. After all pups completed testing, the testing group was returned to their home cage. Testing groups consisted of no more than 6 pups to keep total time separated from the dam under 20 minutes.

2.4 Ultrasonic Vocalization Analysis

In total, 3,429 calls were analyzed across both genotypes and treatment conditions. Fast Fourier transformation (FFT) was performed on all recorded audio files using Avisoft SASLab Pro software. Spectrograms were 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 millisecond. The duration, peak frequency, and maximum amplitude of individual calls identified on these spectrograms were used to investigate potential differences in communicative behavior between groups. Each UV was also manually identified as a specific call-type, per Scattoni et al's (2008) classification scheme [15]. These call-types included two-syllable, composite, flat, harmonic, chevron, complex, downward, frequency steps, short, and upward.

2.5 Statistical Analysis

Data were analyzed using GraphPad Prism 7 software (San Diego, CA, USA) and SPSS 21.0 (IBM, USA). Two-way analysis of variance (ANOVA) (genotype [wild type, knockout] x treatment [control, seizure]) were used to evaluate quantity, average and total duration, peak frequency, amplitude, and types of calls emitted. Significant interactions were followed with appropriate post hoc analysis, correcting for multiple comparisons (Tukey's HSD). Specifics on each analysis can be found within the respective results section. For all comparisons, the level of significance was set at $p < 0.05$.

3. Results

3.1 Seizure induction

A total of 56 mice were initially designated for these experiments and underwent seizure induction or control treatment. Following kainic acid injection, 3 WT mice (27.27%) and 1 *Fmr1* knockout (KO) mouse (5.26%) died. A Pearson Chi-Square revealed no significant difference in mortality rate between the genotypes following kainic acid injection ($\chi^2[1, n = 30] = 2.921, p = 0.087$). All 8 (100%) of the surviving WT mice injected with kainic acid reached status epilepticus (SE) and underwent later vocalization testing. Of the 18 surviving *Fmr1* KO mice injected with kainic acid, 3 (16.7%) did not go into SE. A Pearson Chi-Square revealed no significant difference in the proportion of mice from each genotype that underwent SE ($\chi^2[1, n = 26] = 1.507, p = 0.22$). To ensure mice were in the same category of seizure severity, only those who entered full SE were included in vocalization analyses. Of the mice that underwent SE, we observed a difference in the latency to reach SE between genotypes, with KO mice (30.04 ± 4.70 min) reaching SE more quickly than their WT counterparts (45.2 ± 4.349 min) ($t(21) = 2.104, p < 0.05$).

3.2 ELS increases total vocalization behavior in WT, but not *Fmr1* KO, mice

We first analyzed the total number of UVs made by a given animal during the two-minute testing session, regardless of call-type. A two-way ANOVA detected a significant main effect of genotype, $F(1,45) = 8.97, p < 0.01$, and a significant interaction of genotype and treatment, $F(1,45) = 4.75, p < 0.05$, with KO mice producing fewer UVs than WT. There was no significant main effect of treatment, $F(1, 45) = 2.6, p = 0.12$. Given the significant interaction, additional Tukey's post-hoc analysis revealed that seizures increased the total number of UVs made by WT mice compared to their saline controls ($p < 0.05$), but had no effect on number of UVs made by *Fmr1* KO mice (Fig. 1A). These results indicate that ELS experience differentially affects UV production in WT and *Fmr1* KO mice, resulting in a significant difference in the total number of UVs produced by each genotype after seizure experience (Fig. 1A).

We next analyzed the total amount of time spent vocalizing by a given animal during the two-minute testing session. A two-way ANOVA showed a significant main effect of genotype, $F(1,45) = 6.29, p < 0.05$, with *Fmr1* KO mice spending less time vocalizing compared to WT mice (Fig. 1B). There was no main effect of treatment, $F(1,45) = 1.95, p = 0.17$, and the interaction between genotype and treatment was not significant, $F(1,45) = 1.96, p = 0.17$.

A few animals did not make any UVs during the two-minute testing session (WT/Control – 2; KO/Control – 2; WT/Seizure – 0; KO/Seizure – 2). These animals were included in the analysis of total number and duration of UVs described above. However, these animals were excluded from additional analyses exploring features of specific UVs (average duration, frequency, amplitude) and specific call-type distributions.

3.3 ELS results in genotype-specific changes in average call duration and peak frequency, but not maximum amplitude

To further investigate the effects of ELS on aspects of neonatal communicative behavior, we analyzed spectral features of individual UVs made during the two-minute testing session. All UVs recorded were included in analysis, resulting in the following sample sizes: WT/Control – 1,234; KO/Control – 379; WT/Seizure – 1,332; KO/Seizure – 485.

Differences in the average duration of individual calls were analyzed using a two-way ANOVA. There was a significant main effect of treatment, $F(1, 3426) = 12.61, p < 0.01$, but not genotype, $F(1, 3426) = 0.05, p = 0.82$. There was a significant interaction between treatment and genotype, $F(1,3426) = 40.31, p < 0.0001$. Tukey post-hoc analysis revealed a significant baseline difference in call duration between WT and *Fmr1* KO mice, with KO mice making shorter calls. In addition, ELS experience resulted in a significant decrease in duration of calls in WT animals, but a significant increase in duration of calls produced by *Fmr1* KO animals. There was also a significant increase in call duration in the KO/Seizure group compared to the WT/Seizure group (Fig. 2A).

We next investigated differences in the peak frequency of individual UVs produced by each group. A two-way ANOVA revealed significant main effects of genotype, $F(1, 3426) = 25.61, p < 0.001$ and treatment, $F(1, 3426) = 39.13, p < 0.001$, and a significant interaction between treatment and genotype, $F(1,3426) = 35.32, p < 0.001$. Post-hoc analysis showed a basal difference between WT and *Fmr1* KO animals, with UVs produced by KO animals having a significantly higher peak frequency. While seizure experience had no effect on the peak frequency of WT UVs, *Fmr1* KO animals showed a significant decrease in peak frequency after ELS. There was no significant difference between WT and KO animals who received seizures (Fig. 2B).

A two-way ANOVA was utilized to analyze maximum amplitude of each call, an indicator of the relative loudness. There was no significant main effect of either genotype, $F(1, 3426) = 0.37, p = 0.5418$, or treatment, $F(1, 3426) = 0.03, p = 0.85$, nor any significant interaction between the two variables, $F(1, 3426) = 1.13, p = 0.29$ (Fig. 2C). It is important to note that UV amplitude measurements may be impacted by the pup's orientation towards the microphone and our findings should be considered with this in mind.

3.4 ELS results in genotype-specific changes in call-types

All recorded UVs were manually identified as a specific call-type (two-syllable, composite, flat, harmonic, chevron, complex, downward, frequency steps, short, or upward) based on their spectral features [15]. The relative frequency distribution of these call-types for each group is displayed in Figure 3. To evaluate differences in the frequency of each call-type between experimental groups, we utilized a two-way ANOVA to evaluate significant main effects. Significant interactions were followed up with Tukey's post-hoc analysis as appropriate for each call-type.

We observed a significant interaction between treatment and genotype for composite, $F(1,45) = 4.11, p < 0.05$, flat, $F(1,45) = 5.14, p < 0.05$, chevron, $F(1,45) = 4.50, p < 0.05$, downward, $F(1,45) = 4.30, p < 0.04$, short, $F(1,45) = 16.38, p < 0.001$, and upward, $F(1,45)$

= 6.40, $p < 0.05$, call-types. The post-hoc analysis revealed a significant increase in the frequency of composite, flat, and short call-types in WT animals after ELS, but not in *Fmr1* KO animals, resulting in a significant deficit in these call-types in the KO/Seizure group compared to the WT/Seizure group (Fig 4A–C). For chevron, downward, and upward call-types there was also significant deficit in the quantity emitted by the KO/Seizure group compared to the WT/Seizure group, despite no basal differences for these call-types in the non-seizure groups (Fig. 4D–F).

Two-way ANOVA revealed a significant main effect of genotype for the quantity of two-syllable, $F(1,45) = 5.10$, $p < 0.05$, and complex, $F(1,45) = 7.11$, $p < 0.05$, calls, with KO mice making fewer of both call types (Fig. 4G–H). There were no significant interactions or main effects of treatment and genotype for harmonic or frequency steps call-types (Fig. 4I–J).

4. Discussion

It has been well established that both ELS and mutations in the *Fmr1* gene can independently result in abnormal communication behavior in humans, as well as abnormal UVs in rodent models [19, 27, 28]. However, despite the high comorbidity rate between ELS and FXS, it is unclear to what extent communicative behavior may be altered in this double-hit scenario. We found that a single episode of kainic acid-induced status epilepticus on PD 10 had differential effects on isolation-induced vocalization behavior on PD12 in WT and *Fmr1* KO mice.

We observed a significant increase in the total number of UVs made by WT/Seizure animals during the testing period compared to control WT mice, which is in line with a previous study examining vocalization behavior in the 20–30 kHz range on PD 12 after seizure induction in rats [27]. However, another study reported a significant decrease in vocalization behavior in rats on PD 15, 16, and 21 in the 40 kHz range following pilocarpine-induced seizures [28]. We have also previously reported a suppression in vocalization behavior in mice with prior seizures, using the same seizure paradigm that was utilized in the present study [29]. However, it is important to note that the Reynolds et al. (2017) study was conducted in C57BL/6 mice, whereas in the current study mice were on a FVB/NJ background strain. Previous studies have found significant strain dependent differences in vocalization behavior, which may account for some of the variation in reported results across the literature [15, 30]. Our results provide further evidence that seizures during early life significantly impact vocalization behavior in mice.

Much of the previous literature has focused on UVs within a relatively narrow frequency range (10–20 kHz range), but rodent pups can emit calls with frequencies anywhere between 30 and 90 kHz [8]. By using broad-spectrum microphones, we were able to detect calls over a frequency range of 0 to 125 kHz and thus analyze all potential UVs made during the testing period. Differences in the frequency range of recorded UVs, as well as method of seizure induction, seizure duration, and animal age on both seizure and testing day may also account for the conflicting findings across previous studies. Animal age, in particular, plays a key role in spectrotemporal features of UVs. For example, from birth until PD 17 isolation

induced UVs continuously become shorter in duration and increase in frequency; as the juvenile animal (PD 17 – PD 49) matures into adulthood, courtship UVs begin to increase in duration, stabilizing around PD 50 [31].

We hypothesized that the two-hit impact of ELS on a preexisting genetic condition like FXS would result in additional deficits in communicative behavior. We found that, unlike in WT animals, seizure exposure did not change the total number of UVs made by *Fmr1* KO animals. We also found that ELS decreased the mean duration of individual UVs in WT animals, and significantly increased the duration of UVs produced by KO animals. This increase in call duration has previously been described in models of neurodevelopmental disorders, including both FXS [32] and ELS [33]. The longer calls may influence the interaction between pups and the mother, as maternal approach is more strongly elicited by longer duration calls [34]. It may be that these increases in UV duration are an adaptive response to increase maternal approach, retrieval, and care, but further studies more closely examining maternal behavior in response to altered UVs in these models are necessary to determine if this phenotype is advantageous. We also found that ELS resulted in a significant decrease in the peak frequency of UVs only in KO mice. There were genotype-specific changes in the quantity of specific call types, with increases in composite, flat, and short call-types produced by WT animals after ELS. Abnormalities in the frequency of specific call-types, whether as a result of genotype or ELS, likely results in abnormal UV sequences which are critical for optimal maternal approach. Indeed, a previous study demonstrates that WT pups utilize diverse UV sequences containing simple and complicated call-types, and randomizing the call order within such sequences or altering the frequency of a given call-type within the sequence negatively impacts maternal approach behavior [35]. The genotype-specific changes we observe highlight the importance of including double-hit models when trying to understand comorbid diseases, as ELS experience may affect individuals with FXS differently.

Rodent UV behavior changes significantly throughout development and into early adulthood, creating a developmental profile for this behavior and emphasizing the importance of evaluating UVs at various time points during development [31]. In the current study, examining UVs on PD 12, post-hoc analysis of a significant interaction between genotype and seizure experience revealed no significant difference between control WT and *Fmr1* KO mice in the total number of UVs made during the testing period, which is line with a previous study examining *Fmr1* KO vocalization behavior on PD 8 [14]. *Fmr1* KO pups produce more than WT mice on PD 7 [19], while numerous studies examining vocalization behavior in adult *Fmr1* KO mice report no differences in UV quantity [18, 36]. Together these findings suggest that increased vocalization behavior early in development may normalize over time and future studies examining vocalization behavior in FXS models should keep this in mind. Even though WT and *Fmr1* KO mice made the same number of UVs on PD 12, we observed abnormalities in the duration and frequency of calls made by KO pups. These UVs were significantly shorter in duration and had a higher peak frequency than those made by WT mice. These temporal and spectrographic alterations indicate a significant change in communicative behavior and provide support for the use of neonatal vocalization analysis as an early diagnostic tool for neurodevelopmental disorders, such as FXS.

ELScan have lasting impacts, increasing future seizure susceptibility and altering behavioral phenotypes well into adulthood [37, 38]. However, despite the high comorbidity of seizures with other neurodevelopmental disorders, very few studies have examined the impact of this experience on underlying genetic mutations. A recent study found that repeated short seizures induced by hyperthermia in a *Scn1a*^{RH/+} mouse model of Dravet syndrome at PD21 exacerbated disease phenotypes in adulthood, including increased susceptibility to additional seizures, alterations in novelty-associated locomotor activity, increased stereotyped behavior, and impaired sociability [39]. In addition, recent work from our lab, utilizing the same double-hit model described here, found that a single ELS effected adult behavior differently in *Fmr1* KO mice compared to WT controls, with KO animals showing fewer behavioral changes as a result of ELS [38]. Admittedly, given the short time span between ELS and UV analyses in the current study, it is possible that our findings are due to more acute effects of SE (e.g. muscular and metabolic fatigue), rather than the chronic damage caused by ELS. In either case, the differential impact of ELS on WT and *Fmr1* KO UV behavior highlights the importance of utilizing double-hit models to examine the impact of common comorbidities.

5. Conclusion

Our study described here is, to the best of our knowledge, the first to examine how just a single seizure can impact the underlying *Fmr1* mutation in an acute time period, a critical period of development when changes in communication could impact later developmental outcomes. Together, these studies highlight the importance of utilizing double-hit models to understand how comorbid conditions differentially alter behavioral phenotypes associated with underlying genetic disorders and provide support for the inclusion of double-hit models in neurodevelopmental research.

Acknowledgements

We would like to thank David Narvaiz and Paige Womble for their critical review of the paper. The authors do not have any conflicts of interest to declare.

Funding Sources

This work was supported by the National Institutes of Health grant R15S088776 and through an Undergraduate Research Scholarly Award grant from Baylor University to Joaquin Lugo.

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Highlights

- Early-life seizure experience increases vocalization behavior in WT mouse pups
- Seizure experience leads to genotype-specific changes in call duration, frequency
- Chevron, downward, and upward call-types increased in WT pups after seizures
- *Fmr1* KO pups make fewer two-syllable and complex calls than WT

Figure 1.

Total number of vocalizations made by wildtype (WT) and *Fmr1* knockout (KO) mice on postnatal day 12 (PD12) with and without seizure experience on PD10 (A). WT animals with an early life seizure experience made significantly more vocalizations than WT control animals, though treatment had no effect on number of vocalizations produced by KO animals. WT seizure mice also spent significantly more time vocalizing than KO seizure mice (B). * $p < 0.05$, ** $p < 0.01$; data shown are mean $\pm$ S.E.M.

Figure 2.

Characteristics of vocalizations produced by wildtype (WT) and *Fmr1* knockout (KO) mice on postnatal day 12 (PD12) with and without seizure experience on PD10. Seizure experience resulted in a significant decrease in vocalization duration in WT mice, but a significant increase in duration of vocalizations produced by KO mice (A). Vocalizations produced by KO mice had a higher maximum peak frequency than those produced by WT, and seizure experience significantly reduced max peak frequency only of vocalizations made by KO mice (B). There were no significant differences in the maximum amplitude of vocalizations between any groups (C). * $p < 0.05$, **** $p < 0.0001$; data shown are mean $\pm$ S.E.M.

Figure 3.
Relative frequency distribution of specific call-types for each experimental group.

Figure 4.

Frequency of each call-type emitted by wildtype (WT) and *Fmr1* knockout (KO) mice. WT/SZ mice made significantly more composite (A), flat (B), and short (C) calls than WT/SAL or KO/SZ mice, and significantly more chevron (D), downward (E), and upward (F) calls than their KO/SZ counterparts. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; data shown are mean $\pm$ S.E.M.