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High seizure load during sensitive periods of development leads to broad shifts in ultrasonic vocalization behavior in neonatal male and female C57BL/6J mice

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Abstract

There is increasing evidence that seizures during early development can impact ultrasonic vocalizations emitted from neonatal mice. However, most of the effects of early-life seizures have been reported using chemoconvulsants that produce continuous seizures (status epilepticus). In the present study, we evaluated the impact of different seizure frequency loads during early-life vocalization development in C57BL/6J male and female mice. For the high seizure load (HSL) paradigm, we administered 3 flurothyl-induced seizures to mice on PD7 through PD11, and recorded USVs on PD12. We found that the induction of seizures across PD7 – 11 resulted in increased average duration ($P < 0.05$) and cumulative duration ($P < 0.05$) of USVs across both sexes. Call-type analyses indicated several call-type changes, including reduced production of complex call-types from males HSL condition. For the low seizure load (LSL) paradigm, we induced 3 flurothyl seizures only on PD10 and recorded USVs on PD12. We found no change in any spectral or temporal features of USVs. However, call-type production analyses indicated that both male and female animals from the LSL paradigm also produced changes in call-types. This study provides evidence that the magnitude of communication impairment following seizures is significantly impacted by seizure frequency load early in development.

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Author contributions

JNL, SN, and SH were involved in project design. SN, SH, SC, IM, LT, MB collected the data. IM, LT, SC, and SN analyzed the data. SN wrote the manuscript. All authors revised the article critically and approved the final version for submission.

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Declaration of interest

None.

Keywords

Critical period; Autism; Flurothyl; Developmental disorder; Spectrogram

1. Introduction

Epilepsy is a neurological disorder impacting an estimated 50 million individuals worldwide [1], with particularly high incidence in the pediatric population [2]. Children with epilepsy experience an increased risk of psychiatric and behavioral comorbidities [3] and cognitive deficits [4, 5]. It is estimated that half of all individuals with epilepsy have some form of cognitive or psychiatric condition [6, 7]. The magnitude of these cognitive impairments can be related to any number of variables. For example, results from large cohort studies indicate that lifetime seizure load can affect working memory and executive functioning performance [8, 9]. Similarly, the timing of the seizure onset is a potent predictor of the magnitude of cognitive dysfunction, as earlier onset of seizures during childhood is significantly related to more severe cognitive impairments later in life [8, 10]. This early childhood vulnerability is potentially due to developmental processes occurring during this sensitive period, as synaptic plasticity mechanisms implicated in learning and memory are occurring at a high rate [11].

Similar to cognitive impairments, children who experience a seizure in the first year of life also have a higher risk for autism than the general population [12]. Data from animal models supports the notion that experiencing high seizure load during this sensitive period may produce lasting disruptions in the development of sociability, suggesting a role for recurrent seizures in the etiology of autistic-like phenotypes [13]. However, less is known about the relationship between seizure load during sensitive periods of brain development and another aspect of autistic-like behavior, communication. Clinical data indicates the presence of seizure-induced communication impairments, as childhood epilepsy has been shown to result in an increased likelihood of having speech disorders [14].

Previous studies in both mouse and rat models have reinforced the relationship between seizures and communication deficits. A single instance of status epilepticus (SE) can affect qualitative and quantitative aspects of neonatal ultrasonic vocalization (USV) behavior [15–18]. Our lab has reported male-specific suppression in the quantity and total duration of 50 kHz calls on postnatal day 12 in 129SvEvTac mice following exposure to kainic-acid (KA) induced SE on postnatal day 10 [16]. Using the same KA seizure paradigm and a broader vocalization spectrum recording system, total USV quantity and duration were found to be suppressed on PD12 following SE on PD10 in C57BL/6 mice, as well as changes in call-type production patterns [15]. These findings suggest that a single episode of SE on PD10 is sufficient to produce changes in communication behavior during development.

While the previous findings have sufficiently detailed the impact of one episode of SE on vocalization behavior during a sensitive period of development, it is unclear if the magnitude of these communication alterations may be related to the seizure frequency load, similar to the evidence for seizure-induced cognitive impairments. To address this question, we conducted two separate experiments. In the first experiment, we exposed male and female

C57BL/6J pups to three seizures per day, each day across PD7 – PD11 using the chemoconvulsant flurothyl, designated as the “high seizure load” (HSL) paradigm. One day following exposure to the seizure paradigm, USVs were collected on PD12 using the Avisoft recording system. Quantitative and spectrographic analyses were conducted to detect changes in aspects of this behavior following exposure to seizures. In the second experiment, we exposed mice to three flurothyl seizures on PD10, designated as the “low seizure load” (LSL) paradigm, and recorded and analyzed PD12 USVs in the same manner. We hypothesized that the magnitude of seizure load would be related to the magnitude of changes in aspects of ultrasonic vocalizations.

2. Materials and methods

2.1 Animals

Male and female C57BL/6J pups were bred at Baylor University for this experiment. All pups were housed in individual cages with parents and littermates. The light cycle was kept at 12 hr light/dark, and the colony room was kept at an ambient temperature of 22° C. Animals had *ad libitum* access to food and water. All procedures performed were in accordance with Baylor University Institutional Animal Care and Use Committee, as well as the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All testing was conducted during the light cycle, specifically between 8 am and 5 pm.

2.2 Flurothyl Seizure Induction

Prior to the first seizure on PD7, animals were randomly assigned to receive either seizures or control procedures, and toes were clipped to maintain animal identity throughout the experiment. After toes were clipped, animals were placed back into the home cage for 30 minutes before the first seizure. For the first seizure on PD7, pups were isolated from the home cage and placed in a clear plexiglass inhalation chamber (29 cm × 16 cm × 15 cm), inside a laboratory fume hood (Kewaunee ® Scientific Corp., NC, USA). Flurothyl (bis-2,2,2-trifluoroethyl ether) (Sigma-Aldrich, St. Louis, MO, USA) was pumped into the chamber at a rate of 50 µL per minute, using a syringe pump (Model: 11 Plus, Harvard Apparatus). Following seizure induction, animals were placed in separate containers with a same-treatment counterpart and monitored. These containers were filled with clean bedding and warmed with a heating pad to ambient nesting temperature (~35°C). Following the first and last seizures each day, the animals received a subcutaneous injection at the nape of the neck of 1.0 mL 0.9% saline solution, to reduce the effects of dehydration. Control procedures included placing the animals in an identical induction chamber outside the fume hood for an identical amount of time and similar injections of saline to prevent dehydration. To test the impact of seizure load on our outcome measures, we had two seizure paradigms. The high seizure load paradigm (HSL) was administered as follows: each seizure animal received 3 seizures per day from PD7—PD11, with 2 hours between each seizure episode, for a total of 15 seizures per animal. The low seizure load (LSL) paradigm was administered as follows: each seizure animal received 3 seizures on PD10, with 2 hours between each episode.

2.3 Ultrasonic Vocalization Recording

To assess changes in USV behavior following seizures, we examined USVs on PD12 using the isolation-induced USV paradigm previously described [15, 16]. All vocalizations were recorded in the afternoon (between 1 and 5 pm), approximately 24 hours after the last seizure for the HSL paradigm and 48 hours later for the LSL paradigm. Briefly, all pups were weighed and transferred to a holding cage with fresh bedding, warmed by an electronic heating pad to ambient nesting temperature ($\sim 35^{\circ}\text{C}$). Ultrasonic vocalizations were recorded using a condenser microphone (CM16/COMPA, Avisoft Bioacoustics, Germany) connected to an ultrasound-recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics), which allowed assessment of all USVs on a continuous spectrum from 0–125 kilohertz (kHz). Each pup was individually placed into another housing pan, within an acrylic sound-attenuating chamber ($40\text{ cm} \times 40\text{ cm} \times 30\text{ cm}$) where USVs were recorded for 2 minutes. Following recording, the pups were placed back into the holding cage with their littermates. This procedure was repeated until each pup in the litter was tested. An experimenter remained in the room during all recordings. At the conclusion of testing, pups were returned to the home cage.

2.4 Ultrasonic Vocalization Analyses

Following the conclusion of all testing, all files were downloaded and Avisoft SASLab Pro software (Avisoft Bioacoustics, Germany) was used to convert all USV files (.wav) into spectrograms using a Fast Fourier transformation procedure. The following parameters for our lab have been set to maintain consistency between experiments: FFT length = 1024, time window overlap = 75% (100% Frame, Hamming window), time resolution = 1ms. Sampling frequency was set at 22050. Additionally, call-types were manually identified by an experimenter blinded to treatment identity, using previously described classification scheme [15]. For HSL paradigm, all animals vocalized during the recording period and none were excluded from analysis ($n_{\text{male control}} = 7$, $n_{\text{female control}} = 12$, $n_{\text{male seizure}} = 8$, $n_{\text{female seizure}} = 10$). For LSL paradigm, 3 subjects did not vocalize (2 female control and 1 female seizure) and were excluded from subsequent analysis, leaving the final sample sizes as follows: $n_{\text{male control}} = 8$, $n_{\text{female control}} = 8$, $n_{\text{male seizure}} = 10$, $n_{\text{female seizure}} = 10$).

2.5 Statistical Analysis

All data were analyzed using IBM SPSS Statistics 23 (Aramonk, NY). Results were evaluated using a two-way (Treatment [control, seizure] $\times$ Sex [male, female]) analysis of variance (ANOVA) for each time point examined (seizures on PD7 – PD11 and seizures only on PD10). Figures were created using GraphPad Prism Software 7.0 (San Diego, CA). Any significant interactions were followed by creating a unique group identifier (Group) for each of the treatment combinations (i.e. male seizure) and examined using Tukey's LSD post-hoc, at the level of $P < 0.05$.

3. Results

3.1 High seizure load early in development results in increased cumulative and average call duration as well as changes to the call-type production distribution

A two-way ANOVA was conducted that examined the effect of treatment and sex on the aforementioned parameters for USVs recording during the testing window. There was no interaction of treatment and sex, $F(1, 33) = 3.56$, $P = 0.07$, nor a treatment effect on the number of vocalizations produced, $F(1, 33) = 1.42$, $P = 0.24$ (Figure 1a). Results for the average duration of calling behavior indicated that the average length of the vocalizations produced was significantly increased in pups that received the seizures, $F(1, 33) = 4.58$, $P < 0.05$, though this variable didn't significantly interact with sex, $F(1, 33) = 0.24$, $P = 0.63$ (Figure 1b). The cumulative duration of vocalizations was also increased in animals that experienced seizures, $F(1, 33) = 6.00$, $P < 0.05$, and this wasn't dependent on the sex of the subject, $F(1, 33) = 2.45$, $P = 0.13$ (Figure 1c). Fundamental frequency, or pitch, was not significantly affected by treatment, $F(1, 33) = 0.11$, $P = 0.75$, even across the sexes, $F(1, 33) = 3.53$, $P = 0.07$ (Figure 1d). Results also indicated that seizures increased the average peak amplitude of the vocalizations, $F(1, 33) = 6.63$, $P < 0.05$, regardless of the sex, $F(1, 33) = 1.66$, $P = 0.21$ (Figure 1e). Average peak frequency was not affected by the treatment, $F(1, 33) = 2.81$, $P = 0.10$, regardless of sex, $F(1, 33) = 3.04$, $P = 0.09$ (Figure 1f).

3.2 High seizure load early in development results in changes in call-type production patterns

Using previously defined schemas, aspects of calling behavior were examined and USVs were categorized by call-type [19]. A chi-square analysis revealed that the distribution of call-types produced between pups that experienced seizures and controls were significantly different, $\chi^2(7) = 121.64$, $P < 0.001$. Subsequent Z -tests for column proportions indicated that production of several specific call-types differed from each other at the level of $P < 0.05$. Animals that received seizures produced proportionally fewer upward, complex, and short call-types, while producing proportionally more frequency steps, two-syllable, and composite call-types (Figure 1g). Flat-type calls were not included in the analysis as they were not produced by any subjects.

Moreover, when these analyses were split by sex, differences appeared. Call-type production was significantly different between males who received seizures and controls, $\chi^2(7) = 109.8$, $P < 0.001$. Subsequent Z -tests for column proportions indicated production of several specific call-types differed from each other at the level of $P < 0.05$. Specifically, male pups that received the seizures produced fewer complex calls, and more frequency steps and two syllable call-types compared to male control pups (Figure 2a). Female pups that received seizures also produced a significantly different call-type production profile compared to female control pups, $\chi^2(7) = 97.4$, $P < 0.001$. Subsequent Z -tests for column proportions indicated that several proportions significantly differed at the level of $P < 0.05$. Specifically, female pups that received seizures produced fewer upward and short, as well as more frequency steps, two-syllable, and composite call-types compared female control pups (Figure 2b).

3.3 Low seizure load elicits no changes in quantitative aspects of ultrasonic vocalization behavior on PD12

Results from the two-way ANOVA indicated that no changes in the following variables were noted after experiencing three seizures: number of vocalizations, $F(1, 32) = 1.93$, $P = 0.17$ (Figure 3a), average duration, $F(1, 32) = 0.33$, $P = 0.57$ (Figure 3b), cumulative duration, $F(1, 32) = 1.32$, $P = 0.26$ (Figure 3c), average fundamental frequency, $F(1, 32) = 0.004$, $P = 0.95$ (Figure 3d), average peak amplitude, $F(1, 32) = 0.11$, $P = 0.74$ (Figure 3e), and average peak frequency, $F(1, 32) = 0.14$, $P = 0.71$ (Figure 3f). Moreover, sex did not interact with treatment on any variable of interest: number of vocalizations, $F(1, 32) = 0.01$, $P = 0.92$, average duration, $F(1, 32) = 0.01$, $P = 0.91$, cumulative duration, $F(1, 32) = 0.007$, $P = 0.93$, average fundamental frequency, $F(1, 32) = 0.001$, $P = 0.98$, average peak amplitude, $F(1, 32) = 0.09$, $P = 0.77$, and average peak frequency, $F(1, 32) = 2.37$, $P = 0.13$.

3.4 Low seizure load early in development alters call-type production patterns

Using previously defined schemas, aspects of calling behavior were examined and USVs were categorized by call-type. Chi-square analysis revealed that call-type production proportions differed between the seizure and control groups, $\chi^2(8) = 66.83$, $P < 0.001$. Z -tests for column proportions indicated that several call-type proportions were significantly different between groups at the level of $P < 0.05$. Namely, both male and female animals that received three seizures on PD10 produced proportionally fewer chevron and complex call-types, and proportionally more composite, downward and frequency steps call-types than male and female control pups (Figure 3g).

Moreover, when these analyses were split by sex, results were different from the effect of treatment across the two sexes. Call-type production was significantly different between males who received seizures, $\chi^2(7) = 97.0$, $P < 0.001$. Subsequent Z -tests for column proportions indicated that several proportions significantly differed at the level of $P < 0.05$. Specifically, male pups that received the seizures produced more composite, downward, and frequency steps call-types, as well as fewer short call-types compared male control pups (Figure 4a). Female pups that received seizures also produced a significantly different call-type production profile compared to female control pups, $\chi^2(8) = 73.6$, $P < 0.001$. Subsequent Z -tests for column proportions indicated that several proportions significantly differed at the level of $P < 0.05$. Specifically, female pups that received seizures produced fewer chevron and upward, as well as more composite and short call-types than female control pups (Figure 4b).

4. Discussion

Childhood epilepsy can lead to persistent communication impairments [14]. Previous rodent studies have supported this relationship, demonstrating that experiencing early life seizures results in impairments in communication behavior in rodent models. The present study hypothesized that comparable to the relationship of cognitive impairments and seizure load, a higher seizure load during a developmentally sensitive window results in a greater magnitude of autistic-like communication impairments. The findings of the present study strongly demonstrate that experiencing a high seizure load (15 seizures over 5 days), but not

a low seizure load (3 seizures in one day), during early development increases both average call duration, cumulative call duration and peak amplitude over the testing window on PD12. Moreover, exposure to both high seizure load, and low seizure load changes the distribution of call-types produced, specifically reducing the proportion of complex call-types produced in male subjects.

In the current study, call duration was increased in the high seizure load seizure group compared to controls. These findings spanned both average and cumulative duration, unrelated to the number of calls produced. An increase in call duration more strongly elicits maternal approach [20]. Increased call duration has been reported across many mouse models of ASD, including the BTBR strain [19], the tuberous sclerosis complex 2 (*TSC2*) knockout [21], and the Fragile X mental retardation (*Fmr1*) knockout [22]. One future study could be to determine whether the increase in call duration from the seizure group functions to increase retrieval of the pup. The disruption in USVs due to the high seizure load may be an early indicator of autistic-like behavioral deficits. We previously found that the 15 seizures induced from PD7–11 result in social behavior deficits during adulthood [13]. Therefore, it appears that one of the consequences of the high seizure load during early development may be autistic-like behavioral deficits across the lifetime of the mice.

Our results are different from previous studies that described a pattern of decreased call duration in both rat and mouse pups [15, 16, 18]. In the Lopez-Meraz et al., 2014 study, they found a suppression in the number of USVs in PD14 rats. A similar suppression of USVs was reported in the other two studies, which were conducted in mice [15, 16]. One key difference between these studies and the current study is that they used the chemoconvulsant pilocarpine or kainic acid to induce status epilepticus on a single day. Other differences between these studies and the current study could be due to the type of seizure induction, the number of seizures induced on a single day, or the day the seizures were induced. Kainic acid and pilocarpine result in acute neuronal injury across various regions of the brain [23, 24]. However, exposure to recurrent flurothyl seizures, used to mimic recurrent generalized tonic-clonic seizures, does not show the same acute damage [25]. Thus, it is possible that the different types of seizure induction could be impacting the developing brain in different ways.

Early life seizures are known to cause widespread damage and persistent neurological dysfunction. The sex of the animal is considered a major mediator of this seizure-induced injury, as the trajectory of brain development differs between the two sexes, particularly early in life, rendering males more vulnerable to damage. This male-specific vulnerability extends to some behavioral consequences after early life seizures, such as social play impairments and cognitive impairments [26, 27]. Similar to previous studies, the current study found exposure to a high seizure load during this sensitive period reduced the proportion of complex call-types produced in male subjects, but not in female subjects [15]. In conjunction with previous literature, this suggests that males are particularly vulnerable to call-type changes. Overall, our findings add more evidence to the observation that sex differences may exist in the response to seizure activity during this sensitive period.

We initially hypothesized that we would find an interaction of sex and treatment in the features of USVs. However, most of our findings did not detect that sex mediated seizure-induced changes in the number of calls or their duration. Previous work from our lab has demonstrated significant, male-specific suppression in the quantity and total duration of 50 kHz calls following status-epilepticus [16]. This discrepancy could be attributed to a multitude of methodological factors, including the recording system used. For example, the previous study in our lab used the Ultravox recording system. We recently demonstrated that the Ultravox system is less sensitive than the Avisoft system used in the current study [28]. We used the more sensitive Avisoft recording software and found that suppression of total USV quantity and duration on PD12 following KA-induced status epilepticus on PD10 in male C57BL/6 mice [15].

The present study hypothesized that the impact of a high seizure load during this critical window would be related to a greater impact on vocalization production, similar to previous studies showing that lifetime seizure frequency is related to a greater risk of cognitive impairment in later life [8, 9]. Cognitive impairments are a fundamental concern following seizures and are considered one of the largest comorbidities in epilepsy [29, 30]. Another common comorbidity for individuals with epilepsy is ASD, but this relationship is less clear [12]. Ultrasonic vocalizations are not typically considered an expression of cognitive behavior, but rather their disruption indicates changes in autistic-like social communication [14]. Thus, the findings of the present study support that similar to the relationship of lifetime seizure load and cognitive dysfunction, higher seizure load is related to increased communication dysfunction in the present rodent model.

In the current study we found qualitative and quantitative changes to ultrasonic vocalizations following multiple generalized seizures early in development. This study also provides support for future studies determining the meaning of the different call types. These call-types are defined based on duration, spectrographic shape, and frequency changes, and their proportions vary significantly based on environmental context, background strain and sex of the mouse [19, 31]. While previously published studies have also utilized other call-type categorization schemas, we selected this one for the present study due to its application in the ASD rodent field. Previous studies in ASD mouse models have similarly found reduced complex call types during the early postnatal period [19, 32, 33]. While the distribution of call-type percentages can be modulated by background strain alone, decreased complex calls in ASD models appears consistent across background strains, specifically C57BL6/J, BTBR, and FVB.129, suggesting that this finding occurs at levels above chance. Moreover, in another monogenic model of ASD, representative sequences from the mutant pups, containing fewer complex calls, were less likely to elicit maternal approach [33]. Combined with the findings in the ASD literature, we believe that this supports that this suppression of complex call production as an indicator of an ASD-like communication deficit. Altogether, these findings suggest a need for subsequent studies to elucidate the value communicated by the different call-types.

5. Conclusions

Individuals with epilepsy can suffer from a number of behavioral comorbidities, including communication deficits. The results of the present study corroborate and extend previous findings, demonstrating that experiencing a high seizure load, but not low seizure load, early in development elicits significant changes in vocalization behavior, including increased average call duration and decreased complex call production. Moreover, these results support the conclusion that sex differences may exist in response to developmental insults such as seizures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Early-life HSL increased average and cumulative vocalization duration
- Sex did not have an impact on seizure-induced increased vocalization duration
- Early-life HSL decreased complex call-type production in males
- Early-life LSL did not impact spectral or temporal features of vocalizations

Figure 1.

Changes in quantitative aspects of vocalization behavior following exposure to high seizure load. A. No changes were found in the number of vocalizations produced between the two groups. B. The average call duration was significantly increased in animals that experienced high seizure load. C. The total time spent vocalizing was also significantly increased in animals that experienced high seizure load. D. No changes were found to average fundamental frequency across the groups. E. Average peak amplitude was significantly increased in animals that experienced seizures. F. No changes were noted in the average peak frequency across all calls produced between the two groups. G. Across the sexes, pups that high seizure load on PD7 – PD11 produced a significantly different call profile compared to controls. Specifically, they produced fewer upward, complex and short call-

types, as well as more frequency steps, two-syllable, and composite call-types. * = $p < 0.05$. The bar graphs represent the mean and the error bars represent the standard error of the mean.

Figure 2.

Changes in qualitative aspects of vocalization behavior following exposure to high seizure load. A. Male pups that experienced high seizure load produced fewer complex, and more two-syllable and frequency steps call-types, compared to males that did not experience multiple seizures. B. Female pups that experienced seizures produced fewer upward and short call types, as well as more frequency steps, two-syllable and composite call types, compared to female pups who did not experience seizures. * = $p < 0.05$.

Figure 3.

No changes to quantitative aspects of calling behavior on PD12 after low seizure load. A. No changes were detected in the number of vocalizations produced. B. No changes were noted in the average duration of calling behavior. C. No changes were noted in the cumulative duration of calling behavior during the testing window. D. No changes were noted in the average fundamental frequency of all calls made between groups. E. No changes were noted in the average amplitude of calls produced between groups. F. No changes were noted in the average peak frequency of calls produced between groups. G. Across the sexes, pups that received low seizure load on PD10 alone produced a significantly different call profile compared to controls. Specifically, they produced proportionally fewer chevron and complex

call-types, as well as more composite, downward and frequency steps call-types. The bar graphs represent the mean and the error bars represent the standard error of the mean.

Figure 4.

Changes in qualitative aspects of vocalization behavior following exposure to low seizure load. A. Male pups that experienced low seizure load produced more composite, downward and frequency steps call types, as well as fewer short call types on PD12, compared to male pups that experienced no seizures. B. Female pups that received low seizure load produced fewer chevron and upward, as well as more composite and short call types, compared to females that did not receive seizures.