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Morgan McNair April 3, 2017

Acoustic Properties of Early Life Ultrasonic Vocalizations are Altered in the Valproic Acid Rat Model of Autism

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Abstract

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by communication delays, social and emotional deficits, and restrictive, repetitive behaviors and interests. While many studies have assessed parallel social deficits in various rat models of ASD, few studies have examined parallel communication delays and no studies have analyzed patterns of USV acoustic properties in an ASD rat model. The present study investigated potential developmental differences of early life vocalizations in rats exposed to valproic acid (VPA) in utero. Dams were gavaged with VPA dosages on embryonic days 12, 13, and 14 and recordings of pups in isolation were collected on postnatal days (P) 7, P11, and P14. We examined USV number, duration, frequency, and structure in VPA rat pups of each postnatal day, emphasizing not only a developmental approach to USV analysis but also a comprehensive examination of USV acoustic properties within the VPA ASD model. The findings of the present study confirm that prenatal exposure to VPA alters the acoustic properties of rat pup USVs during the first two weeks of life, resulting in decreased USV emission, increased higher frequency USVs, and dissimilar USV structural patterns. Moreover, while prenatal VPA exposure does not alter USV durations, the pattern of USV durations for both VPA and saline controls do change from P7 to P11 to P14. These data suggest that several acoustic properties of early life vocalizations are altered by prenatal VPA exposure and that these communication abnormalities may have decreased functionality, impact maternal behavior differently than control USVs, and serve as a potential biomarker of future autistic-like symptomatology. The findings of this study also emphasize, through an animal model, that early life vocalization is an area in which the field should focus on as a potential early detection method of ASD in infants.

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ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by communication delays, social and emotional deficits, and restrictive, repetitive behaviors and interests. While many studies have assessed parallel social deficits in various rat models of ASD, few studies have examined parallel communication delays and no studies have analyzed patterns of USV acoustic properties in an ASD rat model. The present study investigated potential developmental differences of early life vocalizations in rats exposed to valproic acid (VPA) in utero. Dams were gavaged with VPA dosages on embryonic days 12, 13, and 14 and recordings of pups in isolation were collected on postnatal days (P) 7, P11, and P14. We examined USV number, duration, frequency, and structure in VPA rat pups of each postnatal day, emphasizing not only a developmental approach to USV analysis but also a comprehensive examination of USV acoustic properties within the VPA ASD model. The findings of the present study confirm that prenatal exposure to VPA alters the acoustic properties of rat pup USVs during the first two weeks of life, resulting in decreased USV emission, increased higher frequency USVs, and dissimilar USV structural patterns. Moreover, while prenatal VPA exposure does not alter USV durations, the pattern of USV durations for both VPA and saline controls do change from P7 to P11 to P14. These data suggest that several acoustic properties of early life vocalizations are altered by prenatal VPA exposure and that these communication abnormalities may have decreased functionality, impact maternal behavior differently than control USVs, and serve as a potential biomarker of future autistic-like symptomatology. The findings of this study also emphasize, through an animal model, that early life vocalization is an area in which the field should focus on as a potential early detection method of ASD in infants.

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by communication delays, social and emotional deficits, and restrictive, repetitive behaviors and interests. Development of ASD symptomology occurs within the first few years of life and hinders normal functioning in everyday tasks. While the cause of ASD remains unclear, the disorder appears to be highly polygenetic, with only 15% of cases being associated with a known genetic mutation (DSM-5, 2013). However, evidence suggests that nonspecific environmental risk factors such as advanced parental age or fetal exposure to teratogenic agents may increase the risk of ASD (Sandin et al., 2016; Christensen et al., 2013).

ASD has a wide dimensional presentation, varying greatly in both severity and impairment. The DSM-5 delineates ASD severity into three levels: Level 3 requiring very substantial support, Level 2 requiring substantial support, and Level 1 requiring support (DSM-5, 2013). These ASD classifications are measured by both level of necessary support and impairment from deficits in social communication and restricted, repetitive behaviors. Level of impairment, however, can be highly variable despite severity of deficits, which poses some concerns for ASD diagnosis and categorization (Masi et al., 2017).

In addition to the dimensional presentation of ASD symptomatology, there is a male bias in ASD prevalence. The Center for Disease Control and Prevention (CDC) reports that ASD is 4.5 times more common in males than in females (2012). Multiple theories, such as the ‘extreme male brain’ or the ‘female protective model,’ have been proposed to elucidate this gender distribution. The ‘extreme male brain’ hypothesis states that ASD may be an overexaggerated presentation of the typically developing male cognitive profile. This over-exaggeration may be due to increased levels of fetal sex steroids in the amniotic fluid of males who are later diagnosed with ASD (Baron-

Cohen, 2002). In contrast, the ‘female protective model’ proposes that females are more resilient to genetic insults than males; however, females tend to present with more severe cases of neurodevelopmental genetic mutations even when their male counterparts have the same disorder (Jacquemont et al., 2014). Werling & Geschwind (2013) reported additional evidence for differential genetic liability in males and females: siblings of females with ASD present with more autistic deficits and impairments than siblings of boys with ASD. This model may explain why females with ASD are not as common yet they present with more severe symptomatology (Jacquemont et al., 2014). Another theory proposed to explain male bias in ASD prevalence is that females may be underdiagnosed. Because of societal expectations and stereotypes of females and feminine behavior, some scientists believe autistic-like symptoms and difficulties may be mislabeled or missed entirely in this population (Bargiela, Steward, & Mandy, 2016). Given the spectrum of autism severity, onset of symptoms, and gender presentation, countless studies have investigated these varying phenotypes as potential ways to diagnose ASD and begin intervention earlier in life.

Early detection and intervention treatment services are crucial for the improvement of a child’s development. According to the CDC, a certified professional can make a reliable ASD diagnosis by the age of two; however, most children do not receive a proper diagnosis until preschool or kindergarten age, resulting in delayed intervention services these children need (2012). Multiple current studies investigating early detection methods target differences in vocalizations, particularly pre-speech development, between infants who develop ASD and their typically developing (TD) peers. High risk infants, i.e. younger siblings of children with ASD, produce significantly more non-speech vocalizations, fewer consonant types, and less canonical syllable shapes at 6, 9, and 12 months of age than infants with no family history of ASD (Paul et

al., 2011; Patten et al., 2014). Infants who go on to develop ASD tend to fall on the lagging end of each pre-speech developmental phase, exhibiting slower transitions from one phase to the next as well as slower transitions away from non-speech vocalizations (Paul et al., 2011).

In the first year of life, TD infants vocalize with reduplicated babbling, which is simple, repeated syllables formed by a consonant and a vowel [*bobo*, *gaga*]. Reduplicated babbling then graduates into two syllables with different vowels [*bogo*, *mada*], and this next babbling stage serves as a bridge to word development (Chericoni et al., 2016). Also during the first year, TD infants develop joint attention, responsivity to language, use of eye gaze to communicate, verbal and motor imitation, and communicative gestures. Any absence of these characteristics of the paralinguistic phase of language development serve as predictive factors for future social and communication deficits (Saulnier & Ventola, 2012). Children who go on to develop ASD tend to have delayed onset of babbling, decreased two-syllable babbling, and later production of first words (Chericoni et al., 2016). Additionally, pre-ASD children exhibit decreased joint attention and imitation, which are skills that typically have a positive correlation with receptive and expressive language development. That is, deficits in joint attention and imitation are associated with lower levels of receptive and expressive language (Charman et al., 2003; Landa, Holman, & Garrett-Mayer, 2007; Manwaring et al., 2017). While language deficits become harder to deny as children age, they seem to be present from very early in development, as a result of either their genetics or teratogen exposure in or around the first trimester.

Notably, exposure to sodium valproate (VPA, valproic acid, Depakote), an antiepileptic drug, in utero may increase the risk of ASD and other neurodevelopmental disorders in children. Fetal Valproate Syndrome, caused by use of VPA during pregnancy, is characterized by various major and minor congenital malformations, cognitive developmental deficits, and dysmorphic

features (Bromley et al., 2013; Mutlu-Albayrak, Cahide & Caksen, 2016). Multiple studies have found a significant association between VPA use during pregnancy, particularly during the first trimester, and an increased prevalence of ASD, ADHD, and dyspraxia (Bromley et al., 2013; Christensen et al., 2013; Mutlu-Albayrak et al., 2016). Significantly, children exposed to VPA monotherapy or polytherapy in utero were 6 to 10 times more likely to be diagnosed with ASD compared to controls who exhibited an ASD prevalence (1.87%) consistent with the general population (1%) (Bromley et al., 2013). Importantly, severity of neurodevelopmental disorder increases with dosage exposure and high doses of VPA have been shown to be negatively associated with IQ, verbal ability, non-verbal ability, memory, and executive functioning in children at 6 years of age (Bromley et al., 2013; Meador et al., 2013).

In both mice and rats, in utero exposure to VPA also results in autistic-like behaviors such as increased anxiety, repetitive and stereotyped behaviors, decreased social interaction, and developmental delays (Christensen et al., 2013; Roullet et al., 2010; Roullet, Lai & Foster, 2013; Schneider & Przewlocki, 2005). Schneider & Przewlocki (2005) tested the behavioral validity of VPA exposure as a model for ASD in rats and found that the VPA rats displayed decreased frequency of pinning during social play, lowered social and non-social exploratory behavior, longer latency to bedding as pups, and repetitive, stereotyped behavior. Subsequent studies have shown that VPA rodent models also emit fewer ultrasonic vocalizations (USVs) than controls, suggesting parallel communication delays with those seen in humans with ASD (Gandal et al., 2010; Raza et al., 2015; Wellmann, Varlinskaya & Mooney, 2014). Additionally, the type of USVs from VPA pups differ from controls (Wellmann et al., 2014). Wellmann (2014) found that untreated, 21-day-old, VPA-exposed rat pups emitted more atypical (opposed to simple, harmonic, or frequency modulated) calls than their d-cycloserine treated counterparts (controls), suggesting

that call structure may be more important in terms of information processing in dams rather than USV number.

USVs are context- and state-dependent vocalizations emitted by rodent pups and are typically above 20 KHz (Portfors, 2007). Infant rodents are deaf and blind for the first one to two weeks of postnatal life but use USVs as a form of communication (Ehret, 2005), such that rat pups isolated from their mothers emit USVs. Moreover, adult rats exposed to prolonged, stressful situations emit ultrasonic alarm calls, mirroring the biological function of isolation calls from pups. While the meaning or semiotic content of these calls cannot be interpreted, rat USVs are reactogenic, i.e. can modify the recipient's, typically the dam's, behavior (Brudzynski, 2005). Unlike adult USVs, pup calls are highly variable in acoustic properties (number, frequency, single-call duration, and sonographic structure) and contain features including abrupt call beginnings and ends, variable durations (80-150ms), and frequency-modulated sweeps (30 to 65 kHz) that facilitate sound localization (Brudzynski, 2005; McNair, (unpublished), Portfors, 2007; Schwarting & Wöhr, 2012).

Both mouse and rat dams respond to pup USVs with search behavior and pup-retrieval. Zippelius & Schleidt (1956) found that mouse dams, when retrieving scattered pups, would only retrieve pups that vocalized and not anesthetized or sacrificed pups, suggesting that USVs play an important role in mother search behavior and pup-retrieval. Sewell (1970) investigated how USV recordings from mouse pups influenced maternal search behavior. By placing a loudspeaker on either side of a "T" partition at the end of the cage, Sewell observed correct (positive) and incorrect (negative) search behavior from the lactating mothers, finding that the mouse dams were correct 79% of the time. The mothers did not respond to background noise or artificial 45 KHz pulses, indicating that USVs hold communication value and influence maternal behavior. Uematsu et al.

(2007) conducted a similar experiment, using hypothermic pups, recordings of pup USVs, silent (anesthetized) pups, and other ultrasound recordings. The researchers found that mothers would approach the live pups and the recordings of pup USVs but would not approach other ultrasound recordings and silent pups.

Both Farrell & Alberts (2002) and Smotherman et al. (1974) investigated maternal approach and search behavior in mice and rats, finding the same results as Zippelius & Schleidt (1956), yet their studies also concluded that olfactory cues enhanced maternal search behavior and correct responses. In the Farrell & Alberts (2002) study, the team examined the relationship between olfaction and maternal responsiveness to USVs by inducing a temporary anosmia. By perfusing the dams' nasal cavities with ZnSO4 solution, Farrell & Alberts (2002) were able to eliminate olfactory cues as a factor. The temporary anosmia attenuated mothers' responses to vocalizing pups, demonstrating olfaction enhances mother search behavior and pup-retrieval. Nevertheless, Smotherman et al. (1974) found that olfactory cues alone, while they serve an important purpose in pup-retrieval, do not provide directionality. The lactating females would choose a vocalizing pup (both olfactory and auditory cues) over silent pup (olfactory cue only) 77.5% of the time.

Several studies have suggested that USVs are an epiphenomenon of hypothermia (Brunelli, Shair, & Hofer, 1994; Uematsu et al., 2007), however recent evidence suggests that this premise is most likely incomplete (Shair et al., 2003). The Rainnie Lab at Yerkes National Primate Research Center has data suggesting call reduction is not due to wheezing from hypothermia but rather seems to be related to maternal care. Barrett et al. (In Preparation) recorded pup USVs as they approach maternal bedding. If the hypothermia hypothesis were true, then pups would emit more USVs towards the end of the testing period. Our findings, however, contradict this concept

such that the number of USVs emitted from pups as they approach maternal bedding decrease, suggesting maternal presence, not lack of warmth, regulates USVs.

Early vocalizations and infant voice quality have been a research area of interest for early detection of ASD, as vocal quality difference may be a possible predictive factor of ASD (Paul et al., 2011). Many studies have investigated social and behavioral impairments in animal models of ASD while few studies have examined parallel communication deficits. In rodents, previous studies have used the VPA-exposed rat model to investigate autistic-like behaviors, ASD genetics, potential drug treatments, and USVs (Foley et al., 2014; Schneider & Przewlocki, 2005; Wellmann et al., 2014). Of the studies that looked at USVs from VPA-exposed rat pups, only call number and type of USV were analyzed (Wellmann et al., 2014).

To our knowledge, no previous study to date has investigated the acoustic properties and structures of USVs in the VPA rat model of ASD. Through this study, we aim to explore the differences in USVs between VPA-exposed and control pups and demonstrate communication deficits in this rat model of ASD that parallel those observed in ASD infants. We hypothesize that VPA-exposed rat pups exhibit developmental delays in USV communication, not only emitting fewer USVs when isolated from dams but also expressing stunted acoustic development that mirrors the pre-speech developmental phases delays seen in high risk, pre-ASD children.

METHODS

All procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Emory University Institutional Animal Care and Use Committee.

Subjects

The subjects of this study included 105 – 149 pups (VPA-exposed n=25 – 39, Control n=24 – 40 pups per round; see Table 1) born from 42 time-mated Sprague Dawley rat dams (VPA-gavaged n=22, Saline-gavaged n=20). Dams and pups lived in group housing with enrichment items and had ad libitum access to food and water. VPA exposure was comprised of three separate gavage administrations of VPA (500 mg/kg dissolved in 0.05M of phosphate buffered saline (PBS)) on embryonic days 12, 13, and 14. Control dams received vehicle, 0.05M PBS, by gavage on embryonic days 12, 13, and 14.

USV Collection from Pup Isolation

On postnatal days (P) 7, 11, and 14, simultaneous USV recordings (two pups per session) were collected. The pups were removed from dams and placed in separate cages with sound eliminating padding around the sides, absorbent lining on the bottom of the cage, and a mesh cover before being transported to the testing room. The cages were placed in one of two compartments of a sound-attenuating chamber. Flaps to the sound proof chambers were sealed closed and the USVs were recorded using the Sonotrack USV recording equipment from Metris B.V. (Hoofddorp, Holland) for five minutes. After the recording session, the cages were removed from the chambers and the pups received a small, identifying ear punch on the top or bottom of their right ears,

signifying which compartment they were in in the recording apparatus. Each cage was cleaned before and after every session and the absorbent liner replaced.

USV Acoustic Analysis

USV data were imported into Metris Sonotrack Manual Analysis 2.3.0 (Sonotrack). USV detector parameters were set to 17.13 V for Amplitude, 4 dB for noise threshold, and 8 ms for discrimination factor. Within Sonotrack, every call was manually checked to remove any non-call sounds picked up during recording sessions. Calls for each session were organized by duration and frequency into 40 millisecond and 5 KHz bins, respectively, and then imported into R for graphing.

USV Structural Analysis

USV data were was imported into Sonotrack Clustering Tool (Trial 2.1). Parameters were set to distinguish the type of call emitted. Calls classified as "Short" were set to ≤ 25ms; One component calls ("Chevron/U-form" or "Up/Down") were set to ± 3 KHz; Two component calls ("One After Another") were set to ± 3 KHz; Tseohree component calls ("Step Up/Step Down") were set to ± 10 KHz. Calls that were less than 5ms in duration were not analyzed. Calls 4ms and/or 2 KHz apart were merged together and counted as one call. The frequency range for calls detected was set between 20 and 98 KHz. Call structures were classified into 11 different types: Short, Flat, Up, Down, Chevron, U-Form, One After Another, Step Down, Step Up, Split, Other (see Table 2). USV data from P7 pups were analyzed, and the relative proportions ([sum of percentages for each call structure type] /[total number of pups]) of call structure types for both VPA and control groups were calculated.

Statistical Analysis

USV numerical data were analyzed using SPSS Statistical Software. A two-way univariate analysis of variance (ANOVA) was used to test the interaction between sex and drug. Finding no interaction, a Student's t-test was used to analyze the effect of drug on USV number.

In R, statistical analysis on duration and frequency data were performed using repeated measure ANOVA to account for within subject observations across bins and age groups. For duration, this test aimed to determine if there were statistically significant differences in the mean durations of USVs within 40 millisecond bins between 0 and 320 ms. Post hoc analysis was performed using a Student's t-test to test for interaction between drug and age group. For frequency, the repeated measure ANOVA test aimed to determine if there were statistically significant differences in the mean frequencies of USVs within 5 KHz bins between 30 and 74 KHz. Post hoc analyses were performed using two-way repeated measures ANOVA to test for drug and frequency bin interaction for each age group. Seeing that the 45-49 KHz bin of P11 was the only instance in which VPA pups called more than saline controls, a Student's t-test was performed for that specific frequency range at P11.

In SPSS, USV structure data were analyzed using a two-way multivariate ANOVA to test the interaction between drug and sex for each call type. Finding only one interaction between drug and sex for Up calls, Student's t-tests were performed to analyze the effect of drug on type of call for males and females separately.

RESULTS

VPA-exposed rat pups have reduced number of calls during the first two weeks of life.

The total number of calls emitted from VPA and control pups during 5 minutes of isolation is displayed in Figure 1. On P7, VPA pups emitted fewer calls than control pups, across both males (VPA $\bar{x}$ = 332.59, control $\bar{x}$ = 513.58) and females (VPA $\bar{x}$ = 338.14, control $\bar{x}$ = 487.43). On P11, VPA pups continued to emit fewer calls than controls (VPA male $\bar{x}$ = 386.97, VPA female $\bar{x}$ = 410.21, control male $\bar{x}$ = 566.38, control female $\bar{x}$ = 503.32), but both treatment groups called more, on average, at P11 than at P7. On P14, there were no differences in number of calls between VPA (VPA male $\bar{x}$ = 158.66, VPA female $\bar{x}$ = 127.84) and control groups (control male $\bar{x}$ = 171, control female $\bar{x}$ = 152.21).

Results of a two-way univariate ANOVA test revealed no interaction between drug and sex for P7 ($F_{1,148}$= 0.070, p=0.792), P11 ($F_{1,137}$=0.170, p=0.681), or P14 ($F_{1,101}$=0.604, p=0.439). We also did not see a main effect of sex. Because we did not see an interaction between drug and sex, we combined males and females and performed a Student's t-test to compare the effect of drug. A Student t-test revealed VPA pups, compared to saline controls, called significantly less on P7 (t_{150}=4.308, p<0.001) and P11 (t_{139}=2.879, p<0.005) but showed no significant difference on P14 (t_{103}=0.562, p=0.576).

General USV duration patterns change in the first two weeks of life but VPA does not alter USV duration patterns in rat pups.

The average duration of calls emitted from VPA and control pups during 5 minutes of isolation is displayed in Figure 2. For descriptive purposes, plots were created to visualize the effects of treatment, duration groups, sex, and age. Based on inspecting these plots, we saw no

evidence of sex difference. Across P7 and P11, the calls emitted by VPA pups were reduced but showed the same duration pattern as saline controls. At P14, VPA and saline pups both vocalize less and are indistinguishable in duration pattern. To statistically test if the overall reduction in number of calls differ across age, we ran a two-way repeated measure ANOVA with drug as a between subject factor and age as a within subject factor. This model showed a significant main effect of both drug ($F_{1,313}$=22.17, $p < 0.001$) and age ($F_{1,313}$=19.96, $p < 0.001$) as well as the interaction effect between drug and age ($F_{1,313}$=9.30, p=0.002). For post hoc testing we used a one way repeated measure ANOVA to account for within subject effect. These tests showed a significant effect of drug at P7 ($F_{1,146}$=17.4, $p < 0.001$) and P11 ($F_{1,139}$=8.51, p=0.004). At P14, the effect of drug was not significant ($F_{1,103}$=0.33, p=0.57).

VPA-exposed rat pups emit more high frequency USVs.

The mean frequency of calls emitted from VPA and control pups during 5 minutes of isolation is displayed in Figure 3. For descriptive purposes, plots were created to visualize the effects of treatment, duration groups, sex, and age. Based on inspecting these plots, we saw no evidence of sex difference. Across all ages, the calls emitted by VPA pups, compared to saline controls, were not only reduced in number but also shifted right in frequency pattern. However, at P14 the rightward shift was less pronounced.

To statistically test if the effect of treatment differs across frequency bins as well as age, we ran a three-way repeated measures ANOVA and found a significant three-way interaction between treatment, age, and frequency bin ($F_{8, 3230}$=6.0, $p < 0.001$). Post hoc ANOVAs showed the interaction between treatment and frequency bin is significant for P7 ($F_{8,1177}$=7.18, $p < 0.001$), P11 ($F_{8,1112}$=13.08, $p < 0.001$), and P14 ($F_{8,824}$=4.89, $p < 0.001$). By looking at the F values, it is clear

the effect is strongest at P11 and weakest at P14. Through plot observation, we saw the largest shift to the right in frequency occurred in VPA P11 pups. To statistically test the relationship between USV number and specific frequency bins within P11, we ran a Student's t-test and found a significant difference (t_{139}=2.72, p=0.007) at bin 45-49 KHz, indicating VPA pups vocalize significantly more at 45 – 49 KHz on P11.

VPA-exposed rat pups express a different distribution of USV structure at P7.

The type of call structures emitted from VPA and control pups during 5 minutes of isolation are displayed in Table 2. The relative proportions of call structures at P7 are displayed in Figure 4. To test the interaction between drug and sex for each call type, we performed a two-way multivariate ANOVA. We found a significant interaction between drug and sex for only Up calls ($F_{1,35}$=864.808, p=0.01). Post hoc analysis of Up calls using a Student's t-test showed a significant interaction between drug and call type in males ($t_{10.99}$=2.194, p=0.05) but not in females. Next, we separately analyzed the effect of drug on type of call in males and females using Student's t-tests. For females, significant interaction between drug and call type was seen in only Short structures ($t_{8.411}$=2.932, p=0.01). For males, significant interaction was seen between drug and call type for Short ($t_{10.412}$=2.351, p=0.04), Flat ($t_{16.465}$=2.775, p=0.01), Up ($t_{10.99}$=2.194, p=0.05), and Down structures ($t_{10.633}$=3.95, p=0.002). In males, there were no significant interactions between drug and call type for all other calls.

DISCUSSION

The present findings collectively demonstrate that prenatal exposure to VPA impacts early life vocalizations, and potentially, communication development in rat pups. Our results showed that, compared to controls, VPA pups emit not only fewer USVs but also express a different pattern of call frequencies and structures during the first two weeks of life. However, contradictory to our original hypothesis, VPA and control groups did not differ in call duration pattern, suggesting that prenatal VPA exposure alters specific USV acoustic properties.

To our knowledge, this is the first study to look at developmental differences of early life vocalizations in the VPA ASD rat model. Multiple studies have investigated the difference in USV number for both VPA rat pups and adults (Gandal et al., 2010; Raza et al., 2015; Wellmann et al., 2014), while only one study, Wellmann et al. (2014), has analyzed call structural differences in this model, doing so with juvenile, 21-day-old VPA rats. We examined USV number, duration, frequency, and structure in VPA rat pups at P7, P11, and P14, emphasizing not only a developmental approach to USV analysis but also a comprehensive examination of USV acoustic properties within the VPA ASD model.

It has been reported that children exposed to VPA in utero develop language impairments. Indeed, Meador et al. (2013) found that children who had in utero VPA exposure performed poorly on both verbal and non-verbal ability tests compared to their typically developing peers. Similarly, Shallcross et al. (2014) investigated language development in children born to women taking either levetiracetam (LEV) or VPA for epilepsy and found that children of women taking VPA scored significantly lower on both comprehension and expressive language abilities than children exposed to LEV or unexposed children. Given the high comorbidity rate of ASD with Fetal Valproate Syndrome and the prominent language deficits observed in infants with either developmental

disability, our study explored vocalization differences in VPA rat pups to draw potential parallels to language impairments in an animal model.

In normally developing rats, pups begin emitting USVs between P3 and P5, peaking in number of calls between P5 and P10, and then decreasing gradually until P21 (Schwarting & Wohr, 2005). We found that VPA pups emit significantly fewer calls than controls on both P7 and P11 but were indistinguishable in call number from controls by P14 (Figure 1). In both VPA and controls, call numbers peaked at P11 and significantly reduced in number by P14. These findings are in agreement with Gandal et al. (2010) who found that VPA mouse pups, on average, emit fewer USVs than saline controls. Prior studies have also shown that adult VPA rats call less than adult controls (Raza et al., 2015; Wellmann et al., 2014). In the Rainnie Lab, we analyzed call number in VPA rat pups at P7, P11, and P14 and found similar results: decreased USVs from VPA pups and significant call reduction at P14 for both saline controls and VPA pups.

Decreased USVs in VPA pups may serve as a parallel to absent or delayed language in children with ASD. Johnson (2008) wrote that one of the most common signs of children with ASD is absent or delayed speech. Similarly, Zablotsky et al. (2017) found, through survey data, that 88.8% of parents of children with ASD listed their child's verbal communication abilities as one of their primary concerns before seeking an assessment. Our study's findings coincide with not only prior ASD rodent model literature but also the findings in human infant studies on language and ASD, thus providing further support that prenatal VPA exposure in rat pups results in an autistic-like phenotype even in communication.

In addition to number of USVs, our study also analyzed the pattern of call duration in VPA pups over the first two weeks of life. While it is known that rat pups, on average, emit calls within the 80 – 150 ms range (Schwarting & Wohr, 2005), no studies to date have analyzed their call

duration patterns. Scattoni et al. (2008) investigated mean durations in the BTBR T+tf/J mouse model of ASD and found that mouse pups produce longer calls than controls. Although the study used mice, not rats, the findings of Scattoni et al. (2008) pose an interesting juxtaposition to our findings. At P7, P11, and P14, we found no difference in pattern of call durations between VPA and control groups (Figure 2). On average, VPA pups emitted similar call durations to control pups but were decreased in overall number of calls. While these contradictory findings may be due to species differences, we believe that our analysis, looking at mean duration of calls within 40 ms bins between 0 and 320 ms, is a better representation of duration pattern than mean duration of total calls, as examined in Scattoni et al. (2008). Through our analytic approach, we found that the USV duration patterns across ages for both VPA and saline controls pups were significantly different. The call duration pattern at P7 ranges from 5 – 240 ms and has one main peak of calls at 81 – 120 ms. At P11, call durations also range from 5 – 240 ms but have two peaks, the first at 1 – 40 ms and the second at 121 – 160 ms. The call duration pattern at P14 looks similar to P11 in duration peaks but extends to 280 ms in duration range. To our knowledge, no previous studies have analyzed data on VPA rat pup call duration or pup call duration patterns. Our study brings to light the development of this acoustic property within normally developing rat pups and indicates that prenatal exposure to VPA alters this development.

In normally developing rat pups, the cries pups emit when they are isolated from their mother fall within the 30 – 65 KHz range (Hashimoto et al., 2004; Portfors, 2007; Schwarting & Wohr, 2012). Similarly, in our study, we found that both VPA and saline control pups emit calls within the 30 – 70 KHz range (Figure 3). In looking at the pattern difference between treatment groups, the call frequency pattern in VPA pups across all ages shifts right compared to controls, i.e. VPA pups emit more high frequency calls. Of particular interest were the calls emitted by VPA

pups at P11 in the 45 – 49 KHz range. This frequency bin was the only range in which VPA pups called significantly more than controls. This finding not only further supports the rightward shift we see in VPA USV frequencies but also suggests that looking at call differences within this specific frequency range may serve as a biomarker of autistic-like symptoms later in life. While there are no studies that have analyzed frequency patterns in VPA rat pups, Scattoni et al. (2008) looked at peak frequencies in the BTBR T+f/J mouse model of ASD and found that BTBR T+f/J pups emit lower frequency calls, on average, than controls. Our results conflict with Scattoni et al. (2008) but these contradictory findings may be due to either species differences or analysis approach. We believe our method of frequency analysis not only clearly conveys average USV frequency but also better illustrates the differences in USV frequency distribution of both VPA and saline control pup calls.

Similarly, infants who are later diagnosed with ASD show differences in their cries compared to TD infants. The cries of infants with ASD are of a higher fundamental frequency (f0) and have shorter pauses between cries (Bornstein et al., 2016; Esposito et al., 2012). Typical f0 falls between 300-600 Hz and a higher f0, evolutionarily, indicates seriously ill or low-fitness infants (Furlow, 1997). Our findings in the VPA rat pup model align with these infant studies in that VPA pups also emit more high frequency calls than controls. Additionally, Bornstein et al. (2016) investigated mother response time to the cries of TD infants, infants with ASD, non-human animals, and non-cry environmental noises. The researchers found mothers identified the cries of infants with ASD slower than the cries of TD infants, animal cries, and unrelated environmental noises, suggesting that mothers may have trouble perceiving and processing the cries of infants with ASD. Because both VPA rat pups and infants who are later diagnosed with ASD similarly present with decreased vocalization and higher frequency cries, the findings of Bornstein et al.

(2016) pose the question of whether the maternal behavior of rat dams is also influenced by the altered vocalizations of VPA rat pups.

A limitation of the current study is the inability to decipher meaning from rat pup USVs, as doing so would impose human language upon rat communication. While we cannot conclude the meaning of their calls, the different USV acoustic patterns that VPA pups produce may imply a functional difference in their calls that influence the behavior of dams differently than control USVs, like the mothers in Bornstein et al. (2016). Various studies (Farrell & Alberts, 2002; Sewell, 1970; Smotherman et al., 1974; Uematsu et al., 2007) have investigated maternal search behavior in response to USVs but none to date have examined this paradigm using an ASD model. Our findings suggest prenatal VPA exposure alters early life vocalizations and warrants further investigation as to how these USV differences influence dam search behavior, pup retrieval, and general maternal care. Previous studies have looked into maternal behavior in response to USV playbacks through recording equipment and future studies should emulate these experimental models using VPA pup USVs, as well. If VPA pups' calls are functionally different from the calls of controls and rat dams are simply responding differently to these altered calls, this information could provide animal model support to further negate the "refrigerator mother" theory of ASD.

A caveat to duration and frequency is USV structure. In this study, we have been working with Metris to test the Sonotrack Clustering tool trial versions and finesse USV structure detection, data output, and ease of use. While the software currently allows users to run USV data through it and produces a chart with the call structure proportions [number of specific USV structure/ total number of USVs detected] for each pup, we are in the process of applying these patterns onto duration and frequency data to align our findings on acoustic properties with structural data.

In our study, we found that VPA and saline control pups have different distributions of USVs structure at P7. In both males and females, VPA pups produce more Short structure (≤ 25 ms) calls than controls (Figure 4). In males, we saw VPA pups emitted significantly fewer Flat and Down structured calls but significantly more Up structured calls than controls. While females VPA pups also showed differences, the differences were not statistically significant for any call structure other than Short.

Multiple studies have investigated mouse USV structures and structural development (Fischer & Hammerschmidt, 2011; Grimsley et al., 2011; Scattoni et al., 2008), while very few studies have looked into rat USV structures. Hashimoto et al. (2004) examined rat pup USV structures, finding four different wave forms which they labeled R-I, R-II, R-III, and R-IV. In comparing their structures to ours, R-I looked similar to a Flat or U-form call, R-II and R-IV resembled a Step Down structure, and R-III looked like a Short and/or Down call (Table 2). Wellmann et al. (2014) was the only study to investigate USV structure in VPA rats, and they showed that juvenile rats emit four types of calls: simple, harmonic, frequency modulate, and atypical. Additionally, Wellmann et al. (2014) found that VPA rats produced increased simple and atypical calls and had decreased harmonic and frequency modulated calls. “Simple” calls in Wellmann et al. (2014) look like Short, Flat, Up, and Down structured calls. “Harmonic” calls look like narrow Chevron structures. “Frequency Modulated” looks similar to Step Up/Step Down calls, and “Atypical” calls seem most like Other calls. While simple and atypical calls are relatively unspecific terms compared to our 11 structural categories, our findings build off Wellmann et al. (2014) and provide a further in depth analysis of rat USV structure for both saline controls and the VPA ASD model.

Studies have shown that infants who are high risk for ASD present with atypical babbling and vocalizations. The structure of this atypical babbling differs in syllables, vowels, and inflection (Chericoni et al., 2016; Johnson, 2008). Children with ASD sometimes present with echolalia (echoing words, sentences, or scripts), ritualistic speech, and non-functional language. These vocalizations can mask deficits in functional speech and make children appear to have more advanced language than they actually have (Johnson, 2008). While children with these language abnormalities may learn phrases, names of objects, numbers, and colors, they typically lack the ability to generate these words or phrases in novel sentences.

The findings of this study also suggest that early life vocalizations could serve as a possible biomarker of autistic-like symptoms. A number of clinical studies have investigated voice quality and differences in language development in infants who are at high risk for developing ASD as a potential early detection method (Bornstein et al., 2016; Charman et al., 2003, Chericoni et al., 2016; Patten et al., 2014; Paul et al., 2011). Our study found that VPA pups produce fewer and acoustically different USVs than control pups, emphasizing, through an animal model, that early life vocalizations is an area in which the field should focus on as an early detection method for ASD. Given the large role communication deficits play in ASD symptomatology, future studies should continue to use the VPA rat model to investigate the developmental trajectories of these pups and examine if early call differences predict autistic-like behaviors later in life. Additionally, rats are typically highly social animals and continue to emit USVs during social interactions later in life. Our findings lay the groundwork for future studies to investigate how these altered USV acoustic properties in the VPA ASD model impact rat socialization with conspecifics, as these potential communication deficits may further exacerbate prosocial development.

There is a strong effect of prenatal VPA exposure on acoustic properties of rat pup USVs during the first two weeks of life, USV number, duration, and frequency patterns across postnatal ages, and subtle sex differences in USV structure. In our call structure analysis, male VPA pups were more severely affected than VPA females. Lack of significant difference for VPA females, however, does not mean that these pups were not affected by prenatal VPA exposure. The VPA female pups expressed a similar pattern of call structures to VPA males but had smaller effects, which possibly contributed to the lack of significance found. Given a larger n value, we may have seen significant differences. The subtleties of the female phenotype in USV structure mirrors the sex specific difference seen in humans and suggests that early life vocalizations may demonstrate these differences, as well. Our study lays a foundation for USV acoustic property analysis as a predictive measure of autistic-like symptomatology in the VPA rat model of ASD, and through this study, we hope to use these USV differences to predict genetic alterations in VPA rats as well as identify critical periods to implement potential pharmacotherapeutic drugs as early intervention for social and communication deficits.

TABLES AND FIGURES

Postnatal Day (P)	Total Number of Pups	VPA-Exposed	Controls
P7	149	35 Females 39 Males	35 Females 40 Males
P11	141	33 Females 37 Males	34 Females 37 Males
P14	105	25 Females 29 Males	24 Females 27 Males

Table 1. Distribution of Rat Pups

The pups listed in the table were the pups used for USV experiments. Pups were collected from 42 different litters, 22 VPA-exposed and 20 controls. The number of pups decreased from postnatal day (P) 7 to P14 because of either death or, specifically for P14, recordings for one round of litters were not collected for that day.

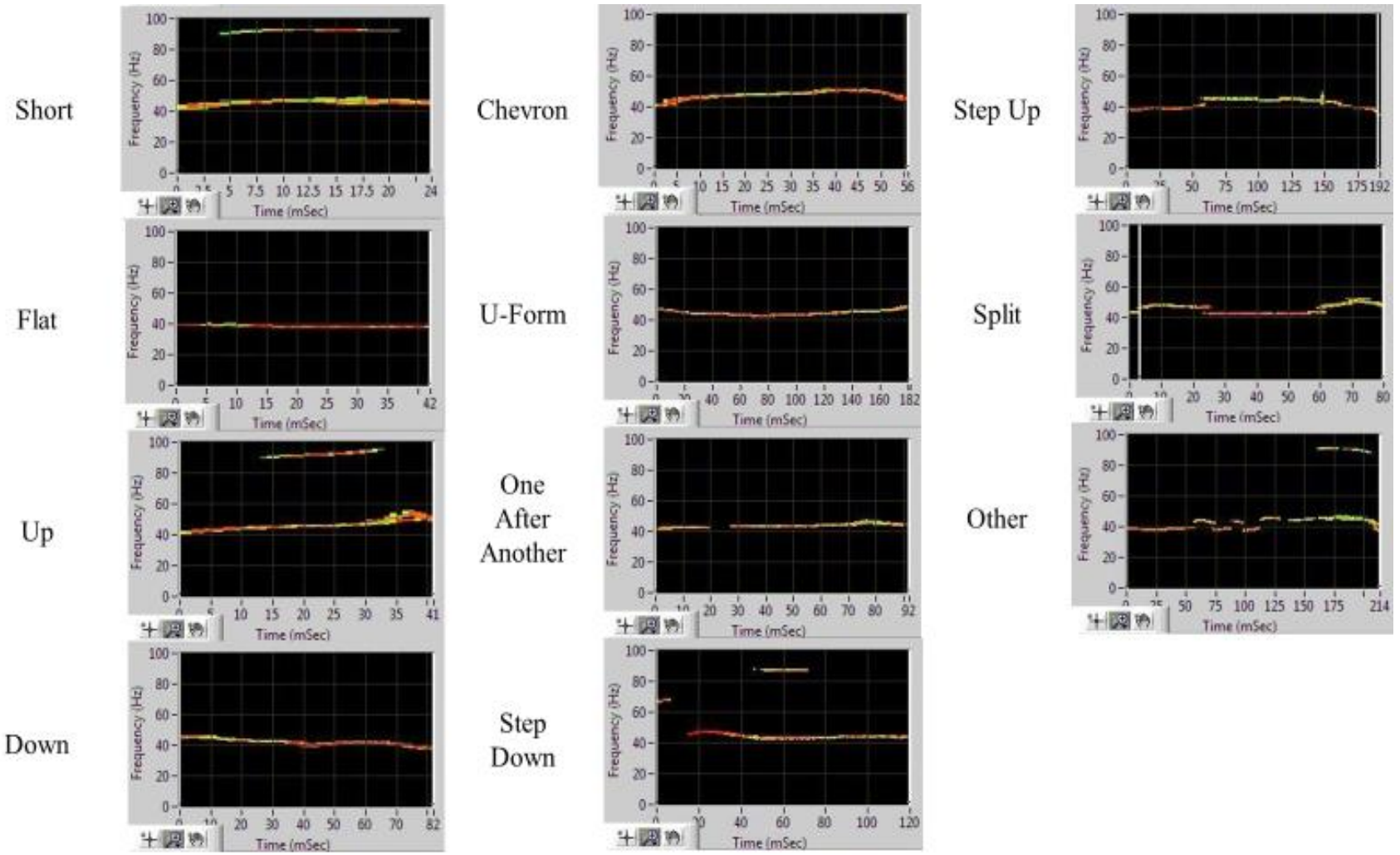

Table 2. Examples of USV Structures

Sonotrack Clustering (Trail 2.1) identified eleven different types of USV structures in P7 VPA and control pups. Visual examples were taken from different P7 sessions.

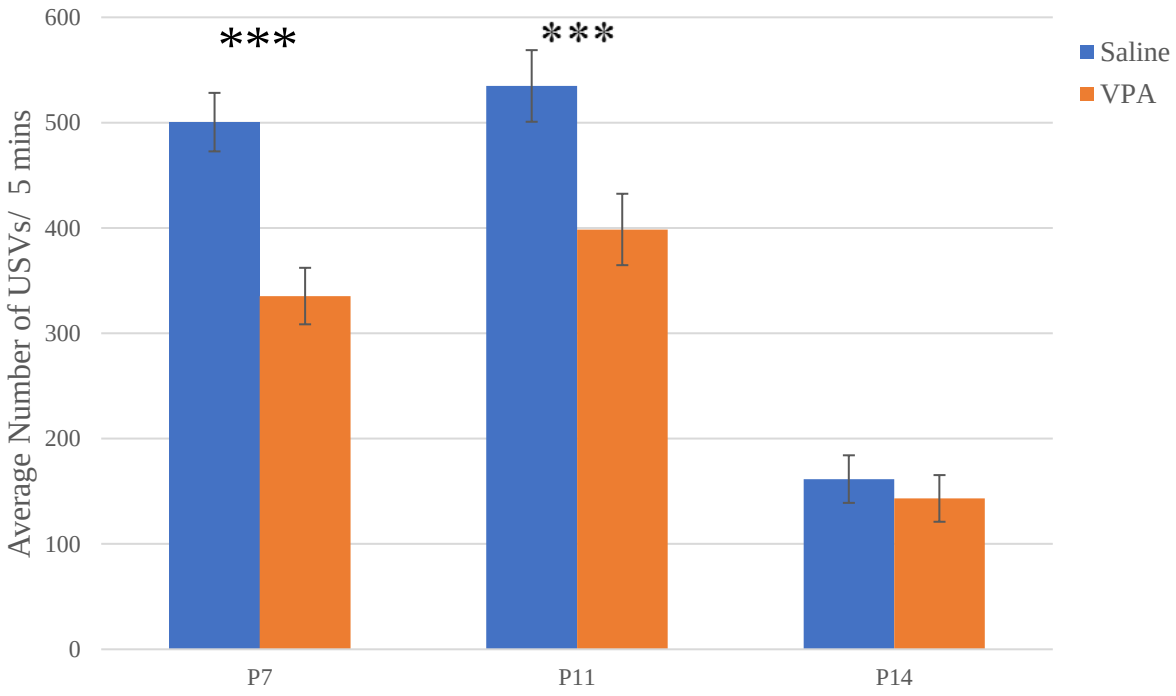

Figure 1. VPA-exposed rat pups have reduced number of calls in the first two weeks of life.

The figure above shows the number of USVs from both VPA and control pups collected on P7, P11, and P14 during 5 minutes of isolation in recording chambers. Results of a two-way univariate ANOVA test revealed no interaction between drug and sex for all ages. There were significant differences in the average number of calls on P7 ($p<0.001$) and P11 ($p<0.005$) but no significant difference in number of calls between VPA and saline controls on P14 ($p=0.576$). Error bars represent the standard error of the mean. ***P < 0.005

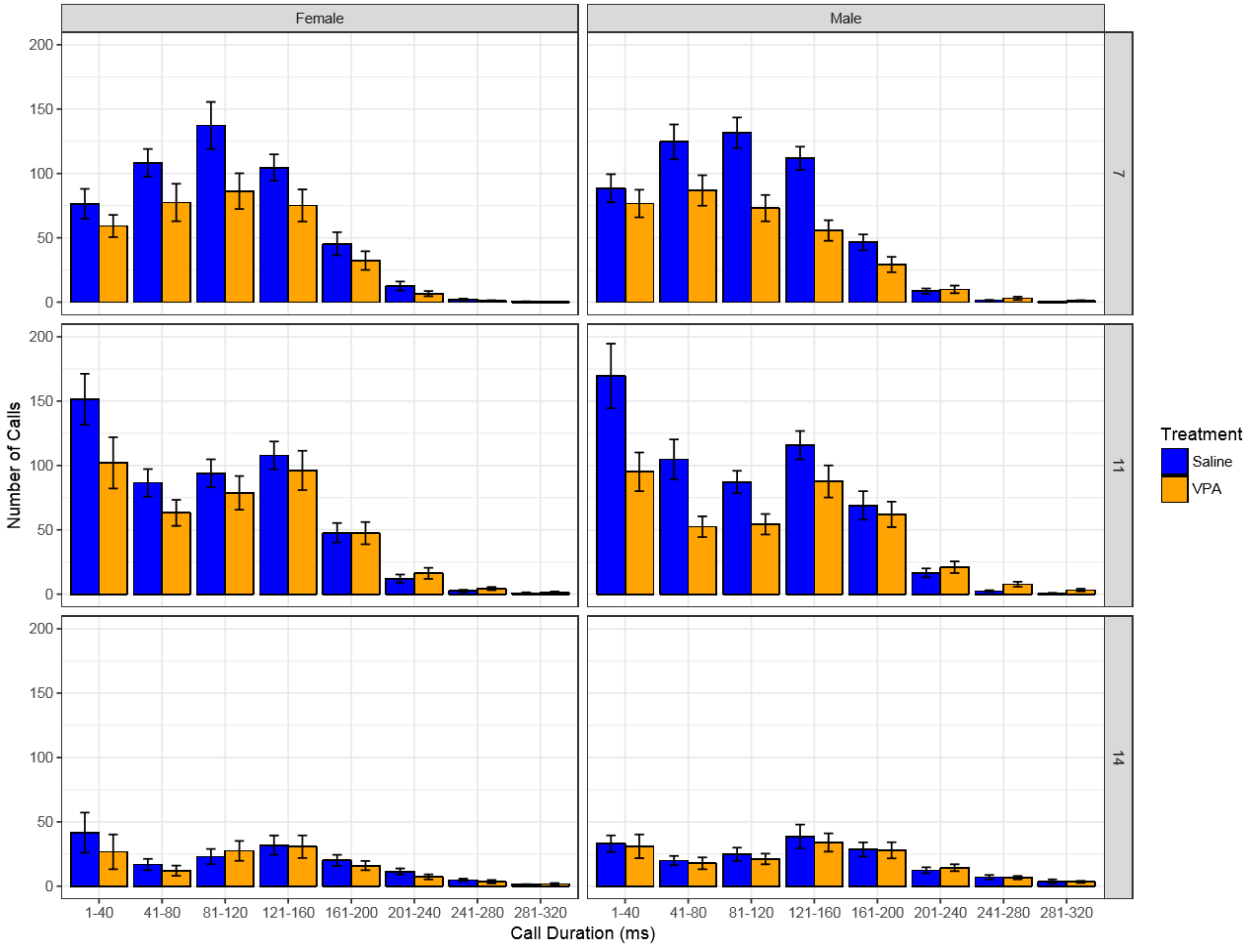

Figure 2. General USV duration patterns change in the first two weeks of life but VPA does not alter USV duration patterns in rat pups.

USV duration data was collected from VPA and control pup recordings during 5-minute isolation periods on P7, P11, and P14. The figure above shows the average number of USVs in 40 ms duration bins from 0 to 320 ms. A two-way repeated measure ANOVA showed a significant main effect of both drug ($p < 0.001$) and age ($p < 0.001$) as well as the interaction effect between drug and age ($p=0.002$). No evidence of sex difference was found. Post hoc tests using one way repeated measures ANOVA showed a significant effect of drug at P7 ($p < 0.001$) and P11 ($p=0.004$). At P14, the effect of drug was not significant ($p=0.57$). Error bars represent the standard error of the mean.

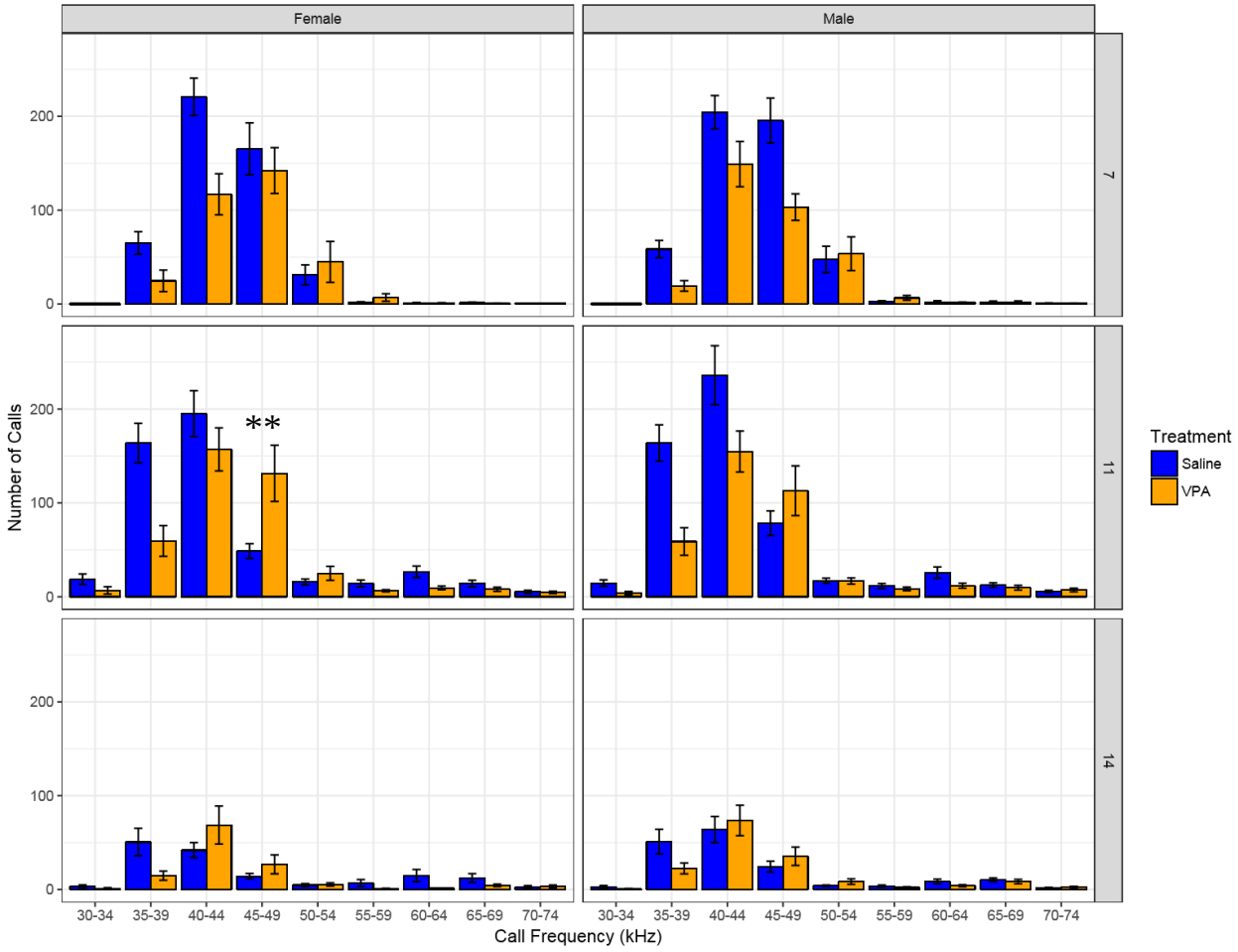

Figure 3. VPA-exposed rat pups emit more higher frequency USVs.

USV frequency data was collected from VPA and control pup recordings during 5-minute isolation periods on P7, P11, and P14. The figure above shows the average number of USVs in 5 KHz frequency bins from 30 to 74 KHz. A three-way repeated measures ANOVA found a significant three-way interaction between treatment, age, and frequency bin ($p < 0.001$). Post hoc ANOVAs showed a significant interaction between treatment and frequency bin at P7 ($p < 0.001$), P11 ($p < 0.001$), and P14 ($p < 0.001$). Plot observations revealed the largest rightward shift in frequency occurred in VPA P11 pups. A Student's t-test showed a significant difference ($p=0.007$) at bin 45-49 KHz, indicating VPA pups vocalize significantly more at 45 – 49 KHz on P11. Error bars represent the standard error of the mean. **P < 0.01.

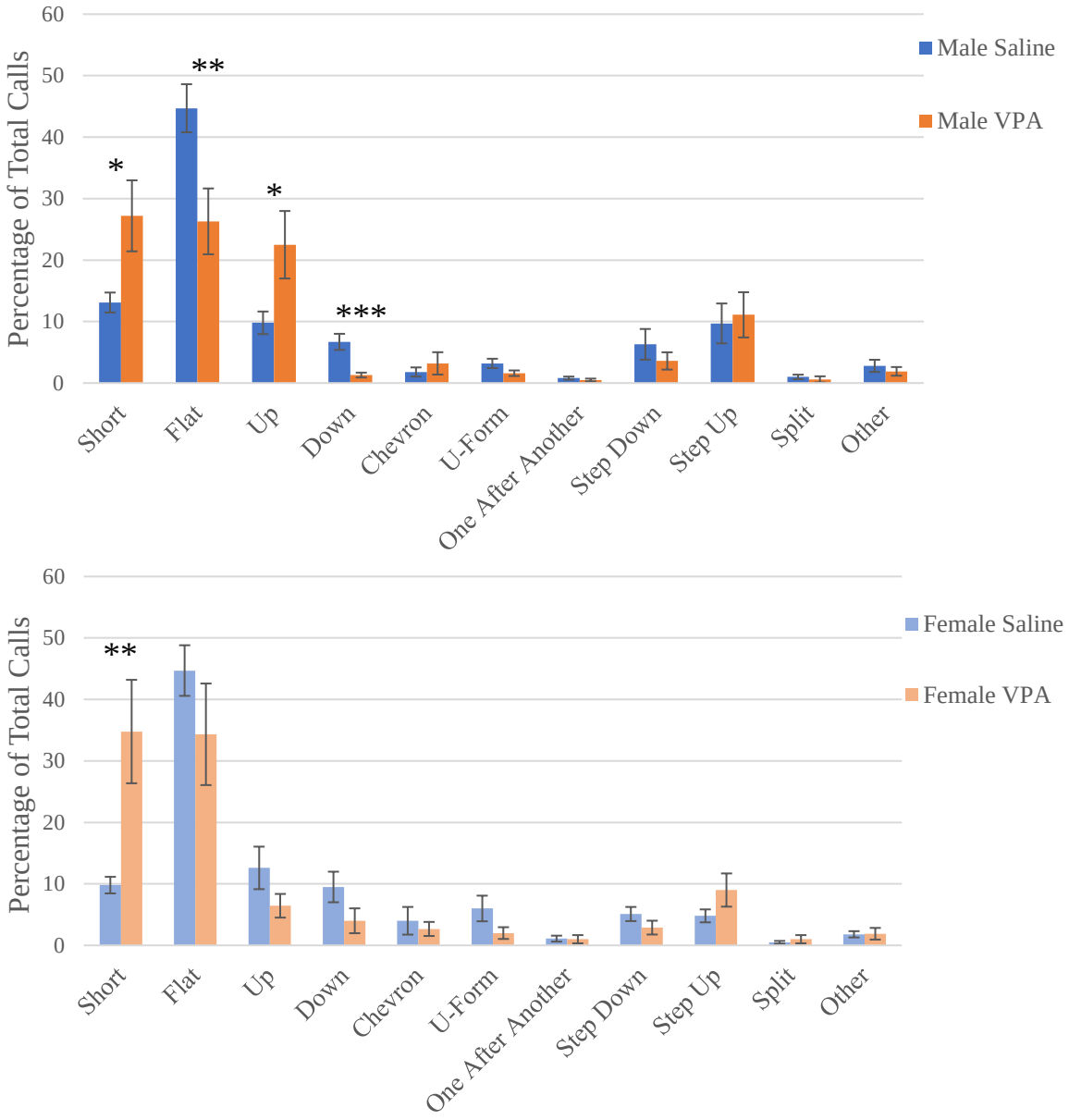

Figure 4. VPA-exposed rat pups express a different USV structure type distribution at P7.

The figure above shows the relative proportions of USV structures out of total number of calls collected from VPA and control pup on P7. Results of a two-way multivariate ANOVA test showed a significant interaction for Up calls (p=0.01). Post hoc Student's t-tests revealed a significant difference in proportions of Short structures (p=0.01) in females and Short (p=0.04), Flat (p=0.01), Up (p=0.05), and Down structures (p=0.002) in males. Error bars represent the standard error of the mean. *P<0.05, **P<0.01, ***P<0.005.

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