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Title	Effects of prenatal ethanol exposure on acoustic characteristics of ultrasonic vocalizations in rat pups
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Citation	Neurotoxicology, 69, 29-36 https://doi.org/10.1016/j.neuro.2018.08.006
Issue Date	2018-12
Doc URL	https://hdl.handle.net/2115/76233
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Type	journal article
File Information	Effects of prenatal ethanol.pdf

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Title

Effects of prenatal ethanol exposure on acoustic characteristics of ultrasonic vocalizations in rat pups

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Abstract

Rat pups produce ultrasonic vocalizations (USVs) on isolation from their dam. Ultrasonic vocalization is a sensitive tool for evaluating social behavior between pups and their dam. Prenatal ethanol-exposure leads to a reduction in USVs and have the potential of inducing difficulties in social behavior between pups and their dam. However, effects of prenatal ethanol-exposure on the acoustic characteristics of USVs remain unclear. In this study, we recorded USVs produced by rat pups that were prenatally exposed to ethanol and examined their acoustic characteristics. Ethanol was administered to 13 pregnant rats in three stages by gradually increasing concentrations between gestational days (GDs) 8–20. From GDs 14–20, ethanol-containing tap water at concentrations of 30% and 15% (v/v) was administered to the high- and low-ethanol groups, respectively. Tap water without ethanol was given to the control group. On postnatal days (PNDs) 4, 8, 12, and 16, individual newly-born pups were isolated from their dam and littermates and USVs produced by them were recorded for 5 min. The number of USVs in the high-ethanol group was greater than that in both low-ethanol and control groups on PND 12. The mean, minimum, and maximum fundamental frequencies of USVs were elevated in the high-ethanol group compared with that in both low-ethanol and control groups. Higher amplitudes of USVs were produced by male pups in the high-ethanol group than in those in both low-ethanol and control groups on PND 12. These results suggest that prenatal ethanol exposure changed emotionality and accordingly, the high-ethanol group produced more USVs as distress calls.

Keywords**:** emotionality, ethanol, maternal isolation, rat pup, ultrasonic vocalization

1. Introduction

Alcohol is a popular beverage worldwide. Alcohol intake can elicit feelings of euphoria but can also lead to various health problems, such as damages to internal organs, alcohol-dependency, and Korsakoff's syndrome. Fetal alcohol syndrome is the most important disorder associated with maternal alcohol consumption during pregnancy. Fetal alcohol syndrome is a permanent birth defect characterized by prenatal and postnatal growth deficiency, central nervous system damages/dysfunctions, and a unique cluster of facial anomalies (Jones et al., 1973; Astley & Clarren, 2000; Bertrand et al., 2005; Chudley et al., 2005; Hoyme et al., 2005). Although any amount of alcohol ingestion during the prenatal period can adversely affect infant development, high or moderate levels of alcohol ingestion can lead to long-term impacts on cognitive and social domains of brain functions (Streissguth et al., 1991; Mattson et al., 1999; Green et al., 2009). Atypical attachment behavior and impairments in state regulation are observed in infants that are prenatally exposed to alcohol (Kelly et al., 2000). In accordance with these findings, prenatal alcohol exposure is considered as one of the most common causes of developmental insults (Thomas et al., 1998; Day et al., 2002; Green et al., 2009).

The literatures on the most severe human form of prenatal alcohol exposure report that offspring of mothers who consume alcohol during pregnancy are known to suffer from developmental delays and/or numerous behavioral changes in a dose-dependent manner. Alcohol consumption during pregnancy, regardless of the amount, has been associated with an increase in infantile anomalies, attachment insecurity, distinctive patterns of crying, attention deficit hyperactivity disorder, slight intellectual deficiency, difficulties in initiating attention and encoding information and so on (Lester et al., 2002; O'Connor et al., 2002; Kable & Coles, 2004; O'Callaghan et al., 2007; Ornoy & Ergaz, 2010; Chen, 2012). Consequences of consuming alcohol have the potential to adversely affect attachment in

infants if proper maternal care is not received during critical perinatal periods (Kim et al., 2006; Gershon et al., 2013). Infants with heavy prenatal alcohol-exposure demonstrated more negative emotionality, such as whining, shouting, crying, and gesturing, than those exposed to lower levels of alcohol (O'Connor, 2001). Moreover, these developmental and behavioral difficulties persist into adolescence (Streissguth et al., 1999). Therefore, studying effects of prenatal alcohol ingestion on infant–maternal interactions is critically important.

However, human studies are often complicated by co-ingestion of substances such as tobacco, marijuana, or psychotropic drugs, and other uncontrollable factors, like nutritional status, postnatal care, socioeconomic status, and genetic variability. Animal model studies, therefore, are important and effective means to examine effects of prenatal ethanol ingestion on pup–dam interactions during developmental periods, allowing strict control of factors such as genetic variation, nutritional status, and multidrug use.

In animal model studies, disruption of the behavior in the pup–dam interaction is known to have a critical impact on the socio-emotional behavior of the pup (Meaney, 2001; Champagne & Meaney, 2007). Alterations caused by ethanol-exposure in rodents are dependent on the timing of exposure, age, and sex of the animal tested. In rats, prenatal ethanol-exposure alters aggression, social interaction, social recognition and communication, maternal behavior, and sexual behavior (Kelly et al., 2000). Prenatal ethanol-exposure can also result in a longer latency for pups breastfeeding and shorter durations to breastfeed (Rockwood & Riley, 1990; Barron et al., 1992; Subramanian, 1992). Another example of social interactions between pups and their dam is that the pups prenatally exposed to ethanol are unable to elicit retrieval behavior from their dam as quickly as the control pups (Ness & Franchina, 1990). Similar to results of reports on human studies, ethanol-exposure during development disturbs sleep and feeding rhythms (Hilakivi, 1986; Hilakivi et al., 1987; Subramanian, 1992; Stone et al., 1996) and the development of abilities to regulate body

temperature (Zimmerberg et al., 1987; Zimmerberg et al., 1993) in rat pups by disrupting the process of psychobiological attunement that may underlie attachment (Field, 1996). Thus, ethanol-induced alterations in early pup–dam interactions suggest the likelihood of cascade-like effects (Ness & Franchina, 1990) such that deficits in pups could result in altered dam behavior, which in turn could result in even more aberrant behavior in the pups (Kelly et al., 2000).

It is well known that rodents produce ultrasonic vocalizations (USVs) and use them as a communication tool in social context (Brudzynski, 2010). For example, rat pups emit USVs with an approximate average frequency of 40 kHz when they are isolated from their dam. This signals the dam to locate her pups and retrieve them back to the nest (Portfors, 2007; Ise & Ohta, 2009; Schwarting & Wohr, 2012). USVs with an approximate average frequency of 40 kHz have important roles in the pup–dam communications.

Ethanol affects the production of USVs in rat pups. Prenatal exposure to ethanol has reduced the number of USVs (Kehoe & Shoemaker, 1991; Wellmann et al., 2015) and postnatal exposure has also decreased USVs (Barron et al., 2000). If ethanol-exposure reduces pups' USVs, these signals will not be noticeable to their dam and this may negatively impact the survival of pups in wild life where the pups are unable to receive necessary maternal care.

Apart from USV productions, USV acoustic characteristics, such as durations and frequencies, have crucial roles in pup–dam interactions because this information is used by the dam for identifying each pup (Hahn et al., 2000). Nevertheless, effects of ethanol on acoustic characteristics of USVs have not been ascertained. In this study, we recorded USVs emitted by rat pups prenatally exposed to ethanol and examined the acoustic characteristics, such as the number, duration, frequency, and amplitude. We predicted that there would be acoustic alterations in USVs in pups exposed to ethanol.

2. Materials & methods

2.1. Subjects

Thirteen pregnant Wistar rats, at gestational day (GD) 7, were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were randomly assigned into three groups: high-ethanol (n = 4), low-ethanol (n = 5), and control (n = 4) groups. They were housed in individual cages and were provided the certified rat chow MF (Oriental Yeast Ltd., Sapporo, Japan) and tap water ad libitum. Laboratory ethanol (purity = 99.5%; Kanto Chemical Co., Inc., Tokyo, Japan) was dissolved in tap water and administered to the animals as drinking water from GDs 8–20. Ethanol-containing water was administered to the animals in three stages by gradually increasing the concentration between GDs 8–20. From GDs 8–10, 10% and 5%, from GDs 11–13, 20% and 10%, and from GDs 14–20, 30% and 15% ethanol-containing water (v/v) was administered to the high- and low-ethanol groups, respectively. Blood ethanol levels from GDs 8–20 were estimated to reach at 250–350 mg/dl and 100–150 mg/dl for the high- and low-ethanol groups, respectively (Ness & Franchina, 1990; Marino et al., 2002; Lugo Jr. et al., 2003; Lawrence et al., 2008). Tap water without added ethanol was given to the control group throughout the study period. Before and after the ethanol-exposure periods, ordinary tap water was given to all groups as drinking water.

We administered ethanol to the animals via drinking water (Nio et al., 1991; Salami et al., 2004) because this was a simple and stress-free method. The idea of gradually increasing ethanol-concentrations was adopted from studies by several investigators (Jones et al., 1981; Sandberg et al., 1982; Ludena et al., 1983; Marquis et al., 1984; Testar et al., 1986; Raul et al., 1987; Macieira et al., 1997; Garcia-Moreno & Cimadevilla, 2012; Wellmann et al., 2015).

The day of birth of the pups was designated as postnatal day (PND) 0. On PND 4, each litter was culled to four male and four female pups to ensure uniformity in the rate of growth of the pups through the availability of breast milk in litters. Four pups (two males and

two females) were randomly sampled from each litter as subjects. The remaining pups were used as subjects in subsequent studies during young adulthood. Thus, eight male and eight female pups from the high-ethanol and control groups and another 10 male and 10 female pups from the low-ethanol group, were sampled. These pups were repeatedly monitored for USV recording on PNDs 4, 8, 12, and 16.

The temperature of the breeding room was maintained at 22°C ± 2°C with relative humidity of 50% ± 10%. Animals were subjected to a 12-h light/dark cycle (light: 20:00–08:00 h and dark: 08:00–20:00 h). This research design was approved by the animal ethics committee of Hokkaido University, and all experimental conditions were compliant with guidelines for the Care and Use of Laboratory Animals, Hokkaido University.

2.2. Apparatus

An ultrasonic microphone and the Sonotrack system version 2.4.0 (Metris, Hoofddorp, The Netherlands) were used to record and analyze USVs of the pups. Sonotrack software was installed on a personal computer and was run on MS Windows 7 Ultimate. To avoid interference from external sound and light, the ultrasonic microphone was placed in a sound-proof box.

2.3. Recording of USVs

USVs were recorded on PNDs 4, 8, 12, and 16. Each pup was individually isolated from the dam and littermates in the breeding room and placed in a translucent cup with a 13-cm bottom diameter, 15-cm top diameter, and 15-cm height, and taken to the experiment room to record USVs. The pup was left alone in the sound-proof box. The first 5 min was the period of habituation for the pup, followed by another 5 min of USV recording. The ultrasonic microphone was positioned at a height of 20 cm from the bottom of the translucent cup. After recording, the body weight of the pup was measured, and the pup was returned to

the dam and littermates. Thus, the pup was isolated from the dam and littermates for 10 min on each day of the experiment.

The temperature of the experimental room was maintained at 19°C–21°C, and the relative humidity was 61%–81%. USVs were recorded during the dark period of the light-dark cycle. After each session of the experiment, the used translucent cups were cleaned with ethanol and water.

2.4. Statistical analyses

Recorded USVs were analyzed by the Sonotrack system on selection of the automatic mode setting of the device. The time of resolution was 1 ms, and to reduce background noise, low and high cut-off frequencies for analyzing were set to 30 and 90 kHz, respectively. The Sonotrack system calculated the lowest frequency in a periodic waveform of USV at every 1 ms interval and obtained fundamental frequencies. Where the fundamental frequency at the start and end points of USV was < 30 and ≥ 70 kHz, USV was re-analyzed with the manual selection mode. USVs that satisfied all of the following criteria were selected for statistical analyses (Reno et al., 2013; Wada, 2017).

i) Fundamental frequencies at both start and end points of USV were ≥30 and <70 kHz.

ii) The mean fundamental frequency of USV was <90 kHz.

iii) The bandwidth between the maximum and minimum fundamental frequencies of USV was <60 kHz.

iv) Duration of USV was ≥20 ms.

Acoustic characteristics included the number, duration, fundamental frequency, and amplitude of USVs; fundamental frequency difference; bandwidth; and percentage of frequency-modulated USVs. The root mean square (RMS) of USV amplitudes was calculated to indicate the magnitude of amplitudes because USV amplitudes periodically alternated between +V and −V. The difference in the fundamental frequency was calculated as

fundamental frequency at the end point of USV minus the fundamental frequency at the first point of USV. The bandwidth was defined as the range between maximum and minimum fundamental frequencies of USV. The percentage of frequency-modulated USVs was calculated as (total number of frequency-modulated USVs/total number of USVs) × 100. The frequency-modulated USV was defined as USV with a bandwidth ≥ 5 kHz (Reno et al., 2013).

Considering the small litter size, data of two male pups within the litter were averaged and taken as one for the purpose of data analysis. Similarly, data of two female pups within the litter were averaged and taken as one for statistical analyses. Therefore, the sample size for the high-ethanol, low-ethanol, and control groups were n = 8, 10, and 8, respectively. The acoustic characteristics of USV data were analyzed using a three-factor analysis of variance (ANOVA) comprising two between subject variables, concentration (high/low/control) and sex (male/female), and one within subject variable, age (PNDs 4/8/12/16).

USV data on PND 16 were analyzed only for the number of USV recordings but not for other acoustic characteristics. Because no USVs were produced by many pups on PND 16, regardless of ethanol administration, it was impossible to run ANOVA on these data except the number of USVs. Body weights were analyzed using a three-factor ANOVA comprising two between subject variables of concentration and sex, and one within subject variable of age. Ethanol intake was analyzed using a one-factor ANOVA comprising one between subject variable of concentration. Where a variable was found to be significant, multiple comparisons were performed using Ryan's method. Statistical analyses were conducted using ANOVA 4 (http://www.hju.ac.jp/_kiriki/anova4/ about.html).

3. Results

3.1. Ethanol intake

As shown in Fig. 1, the volume of ethanol intake from GDs 14–20 was 3.47 and 2.41 ml/day for the high- and low-ethanol groups, respectively. Ethanol intake by dams

significantly varied across the groups [F (1, 7) = 10.041, $p < 0.05$]. Ethanol intake in the high-ethanol group was significantly higher than that in the low-ethanol group ($p < 0.05$). Volume of ethanol-containing water consumed by the high- and low-ethanol groups from GDs 14-20 was 11.57 and 16.06 ml/day respectively showing a gradual increase in consumption for both groups on each gestational day.

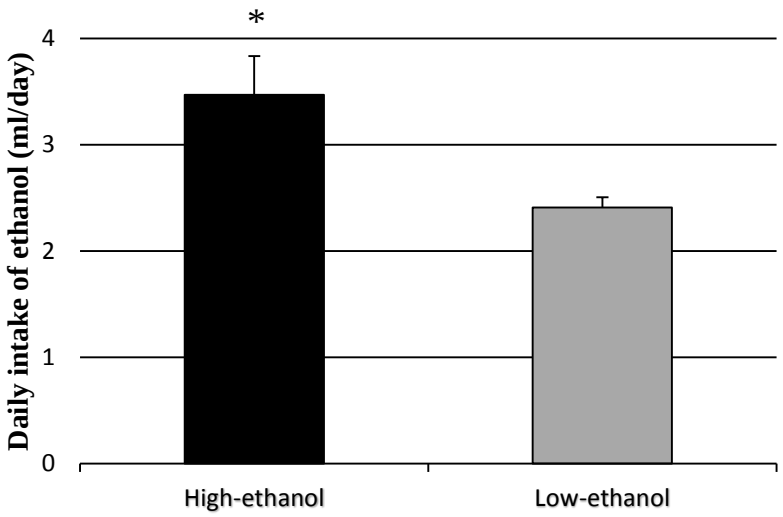


Fig. 1. Daily intake of ethanol in dams from GDs 14-20

Data are mean and SEM; *$p < 0.05$ compared with that in the low-ethanol group.

3.2. Body weights

Neither ethanol nor sex had statistically significant effects on body weights of rat pups. Age-dependent variations in body weights were observed in all three groups [F (3, 60) = 2665.001, $p < 0.001$; Fig. 2].

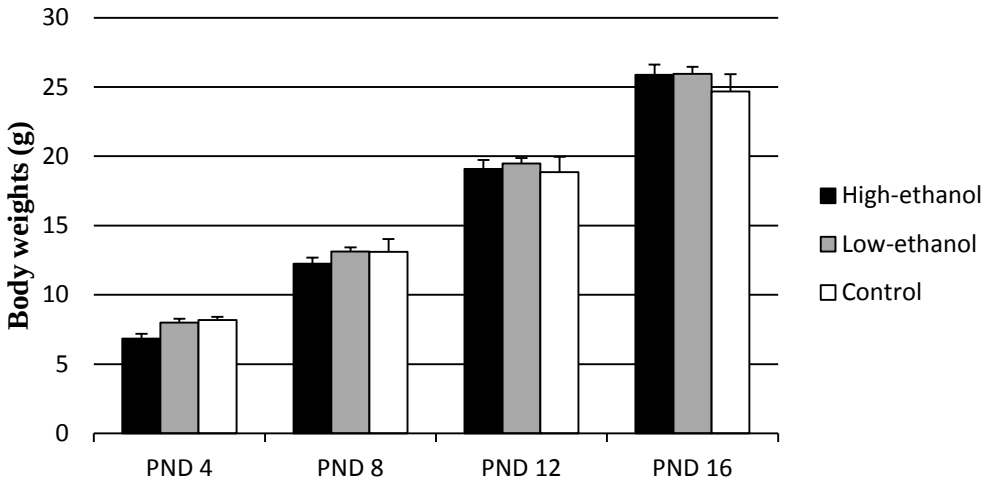


Fig. 2. Effects of prenatal ethanol-exposure on body weights of rat pups

Data are the mean and SEM.

3.3. Number of USVs

The relationship between ethanol and age was significant [F (6, 60) = 3.338, $p < 0.01$; Fig. 3]. On PND 12, the high-ethanol group produced greater number of USVs than both low-ethanol and control groups ($p < 0.05$). The age of the pups had a significant effect on the number of USVs produced [F (3, 60) = 96.223, $p < 0.001$], and the highest number of USVs was recorded on PND 8 followed by that on PNDs 4, 12, and 16 ($p < 0.05$).

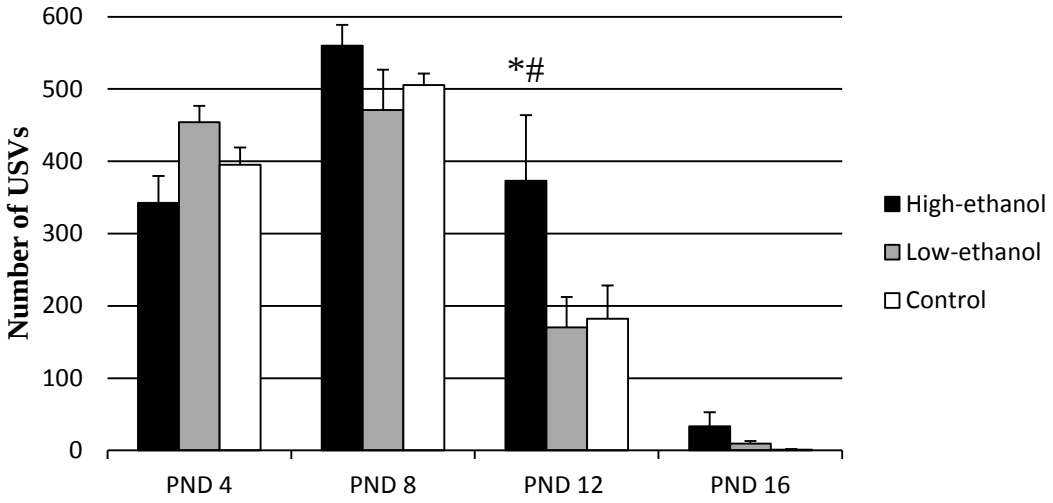


Fig. 3. Effects of prenatal ethanol-exposure on the number of USVs in rat pups

Data are mean and SEM; *p < 0.05 compared with that in the control group, #p < 0.05 compared with that in the low-ethanol group.

We averaged the USV numbers of two male pups within the litter and similarly, averaged the USV numbers of two female pups within the litter. These unified data might affect the variability of USV numbers. Thus, we re-analyzed the USV numbers using individual pup's data and found that the relationship between ethanol and age was significant [F (6, 147) = 5.381, p < 0.001; Table 1]. On PND 12, the high-ethanol group produced greater USVs than both the low-ethanol and control groups (p < 0.05).

Table 1 Effects of prenatal ethanol exposure on the number of USVs produced by rat pups

Group	n	PND 4	PND 8	PND 12	PND 16
High-ethanol	16	342.6 ± 32.7	559.8 ± 27.2	373.0 ± 66.5*	33.6 ± 14.4
Low-ethanol	20	454.0 ± 17.2	471.0 ± 43.0	170.3 ± 36.3	9.3 ± 3.3
Control	16	395.0 ± 19.0	505.4 ± 27.0	182.4 ± 35.1	1.1 ± 0.8

Data are mean and SEM; *p < 0.05 compared with that in the low-ethanol and control groups.

3.4. Duration of USVs

The relationship between durations of USV and ethanol and sex was not significant. However, the relationship between durations of USV and age of the pups was significant [F (2, 38) = 9.562, p < 0.001]. Data analysis further revealed that the duration of USVs increased as the pups aged, showing significantly longer durations on PND 12 than those on PNDs 4 and 8 (p < 0.05).

3.5. USV bandwidth

Figure 4 shows USV bandwidth, which was defined as the range between the maximum and minimum fundamental frequencies of USV. Effects of ethanol were significant for both maximum and minimum fundamental frequencies of USVs [F (2, 19) = 5.478, p < 0.05 and F

(2, 19) = 7.163, p < 0.005]. Both maximum and minimum fundamental frequencies increased as the concentration of ethanol increased, showing higher frequencies in the high-ethanol group (p < 0.05). Main effects of age on maximum and minimum fundamental frequencies were also significant [F (2, 38) = 7.112, p < 0.005 and F (2, 38) = 21.214, p < 0.001]. Both maximum and minimum fundamental frequencies decreased as the age of the pups increased. Decreased maximum fundamental frequencies were observed on PND 12 compared with those observed on PNDs 4 and 8 (p < 0.05). Similarly, lower minimum fundamental frequencies were observed on PNDs 8 and 12 compared with those observed on PND 4 (p < 0.05).

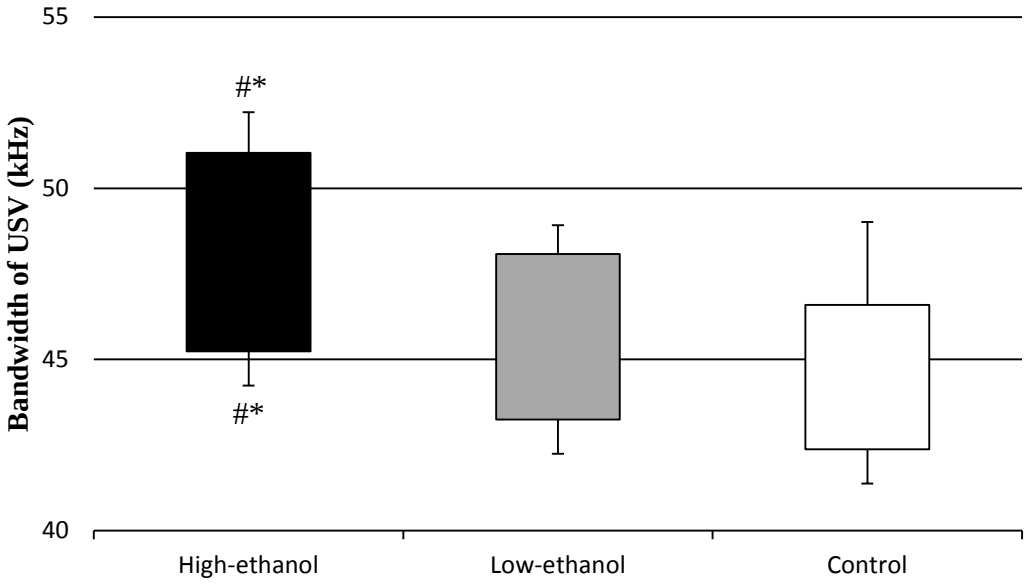


Fig. 4. Effects of prenatal ethanol-exposure on the bandwidth of USVs in rat pups

Data are mean and SEM; *p < 0.05 compared with that in the control group, #p < 0.05 compared with that in the low-ethanol group.

3.6. Mean fundamental frequency of USVs

Ethanol significantly affected the mean fundamental frequency of USVs [F (2, 19) = 8.343, p < 0.005]. The mean fundamental frequency was higher in the high-ethanol group than in both low-ethanol and control groups (p < 0.05; Fig. 5). In addition, mean fundamental

frequencies significantly decreased [F (2, 38) = 20.606, p < 0.001] as the age of the pups increased. Higher mean fundamental frequencies were observed on PNDs 4 and 8 than those on PND 12 (p < 0.05).

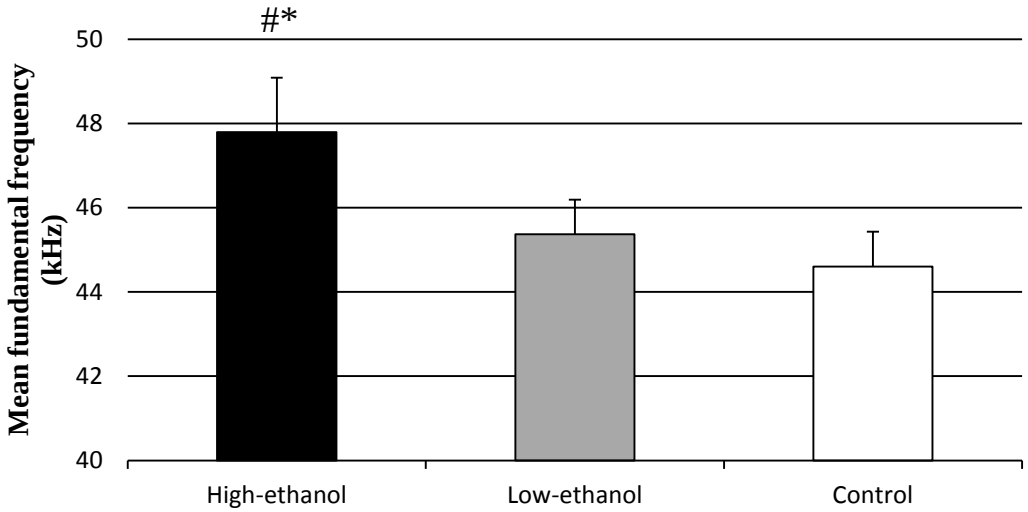


Fig. 5. Effects of prenatal ethanol-exposure on the mean fundamental frequency of USVs in rat pups

Data are the mean and SEM; *p < 0.05 compared with that in the control group, #p < 0.05 compared with that in the low-ethanol group.

3.7. Frequency-modulated USV

Effects of ethanol and sex were not significant on the percentage of frequency-modulated USVs. Effects of age were significant [F (2, 38) = 28.429, p < 0.001], and the percentage of frequency-modulated USVs displayed elevations on PNDs 8 and 12 compared with that on PND 4 (p < 0.05).

3.8. Difference in the fundamental frequency of USVs

Difference in the fundamental frequency was calculated by subtracting the fundamental frequency at the starting point of USV from that at the end point. A positive value for this difference means that there is a higher frequency at the end point indicating an upward USV, whereas a negative value for this difference means a higher frequency at the starting point indicating a downward USV. Age-related differences were significant [F (2, 38) = 6.368, p < 0.005], and negative values for this fundamental frequency difference were

obtained on PNDs 4, 8, and 12. Downward USVs were observed in all three groups. Effects of ethanol and sex on the fundamental frequency difference of USVs were not significant.

3.9. Amplitude of USVs

The relationship among ethanol, sex, and age were significant [$F(4, 38) = 2.666$, $p < 0.05$; Fig. 6]. Male pups in the high-ethanol group showed greater RMS of USV amplitudes than those in both low-ethanol and control groups on PND 12 ($p < 0.05$). Significant effects of age were observed [$F(2, 38) = 44.736$, $p < 0.001$], indicating greater RMSs of USV amplitudes on PNDs 8 and 12 than those on PND 4 ($p < 0.05$).

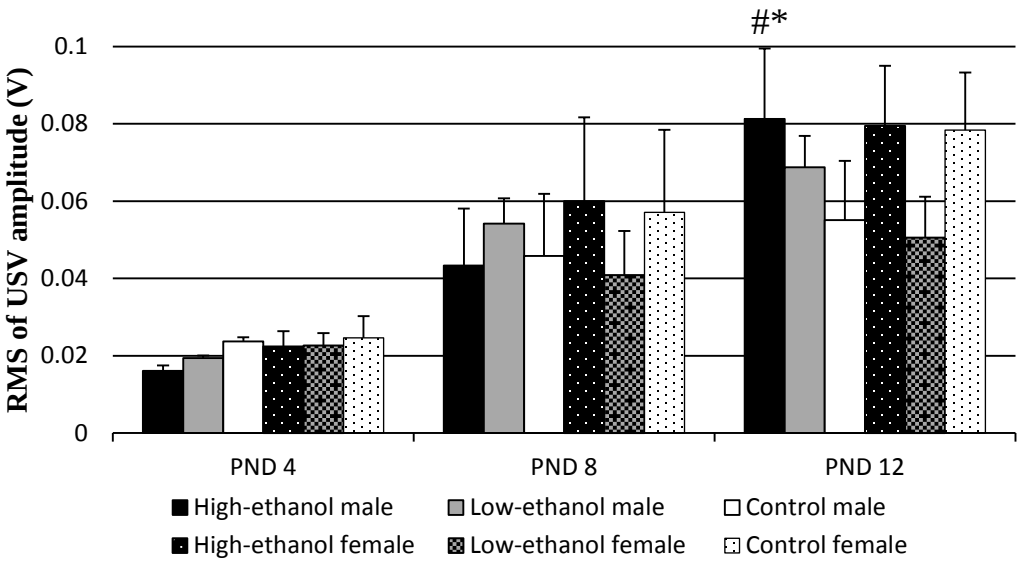


Fig. 6. Effects of prenatal ethanol-exposure on RMS of USV amplitude in rat pups

Data are the mean and SEM; *$p < 0.05$ compared with that in males in the control group on PND 12, #$p < 0.05$ compared with that in males in the low-ethanol group on PND 12.

4. Discussion

Previous studies have elucidated the developmental changes in the acoustic characteristics of USV emitted by rat pups upon maternal isolation (Kehoe & Harris, 1989; Elsner et al., 1990; Brudzynski et al., 1999; Schwarting & Wohr, 2012; Kromkhun et al., 2013; Wada, 2017) and reported that the number of USVs increases on PNDs 3–5 to reach a

peak on PNDs 5–11 (Brudzynski et al., 1999; Schwarting & Wohr, 2012). USV duration exhibits an age-dependent increase, whereas USV frequency exhibits an age-dependent decrease (Elsner et al., 1990; Kromkhun et al., 2013). Subsequently, as they age, the pups become less dependent on their dam because they are covered with body hair and can regulate their body temperature. Body muscles develop enabling the pups to walk around. Their eyes open and their teeth start developing. USVs gradually decrease and completely disappear at approximately PNDs 20–21, i.e., by weaning age (Kehoe & Harris, 1989; Schwarting & Wohr, 2012).

Rat pups' USVs vary widely in terms of number, duration, and frequency. The USV repetition rate is considerably influenced by the surrounding circumstances but usually does not exceed 80–90 USVs/min (Elsner et al., 1990; Goodwin et al., 1994; Brunelli et al., 1996). USV duration varies between 80 and 150 ms, and the sound frequency ranges from 30 to 65 kHz with an approximate average frequency of 40 kHz (Portfors, 2007; Ise & Ohta, 2009; Schwarting & Wohr, 2012).

The findings of the present study are consistent with those of previous studies. The number of USVs produced by rat pups in our study exhibited an age-dependent increase, reaching a peak on PND 8 and then exhibited a drastic decline on PND 16, which is consistent with previous studies (Kehoe & Harris, 1989; Schwarting & Wohr, 2012). The call duration and mean fundamental frequency indicated age-dependent increases and decreases, respectively (Elsner et al., 1990; Kromkhun et al., 2013). Moreover, on PND 12, pups in the high-ethanol group produced more USVs compared with that produced by the pups in the low-ethanol and control groups. This finding is consistent with that reported in Marino et al. (2002), who found that rat pups prenatally exposed to high-dose ethanol (4.5 g/kg daily) produced an increased number of USVs during early postnatal period. Conversely, other studies have reported that prenatal exposure to ethanol reduces the number of USVs (Kehoe

& Shoemaker, 1991; Wellmann et al., 2015), possibly due to the daily low-dose exposure to 5% ethanol from GDs 7–20 (Kehoe & Shoemaker, 1991) or to daily gradual low-to-moderate dose exposures of 2.1%, 3.8%, and 6.3% ethanol from GDs 6–7, 8–10, and 11–21, respectively (Wellmann et al., 2015). In the present study, high-dose exposure to ethanol (approximately 30%, v/v) during critical prenatal periods was associated with an increase of USVs in rat pups.

4.1. Effects of ethanol on the central network of vocalization

When isolated from their dam, rat pups develop hypothermia, which threatens their survival. This causes the pups to emit signals in the form of USVs to their dam to rescue and take them back into the nest (Ness & Franchina, 1990; Blumberg et al., 2002; Schwarting & Wohr, 2012). Longer isolation periods increase USV frequency, prompting search and retrieval behavior from the dam (Portfors, 2007; Ise & Ohta, 2009; Schwarting & Wohr, 2012). Therefore, USVs emitted by pups are considered as distress calls (Brudzynski et al., 1999) expressing negative emotionality, such as anxiety (Portfors, 2007).

In our study, the pups in the high-ethanol group exhibited an increased number and amplitude of USVs compared with those in both the low-ethanol and control groups. The maximum, minimum, and mean fundamental frequencies were also increased in the high-ethanol group. These results suggest that the pups in the high-ethanol group relatively experienced more negative emotionality.

In rats, USVs induced by negative emotionality are mainly initiated by the activities of the core limbic system and tegmental structures (Brudzynski, 2007). The thalamic and hypothalamic sites of the core limbic system maintain a reciprocal anatomical interaction with the periaqueductal gray (PAG), which serves as a bottleneck-like structure receiving negative USV-related inputs from the diencephalon and sending relevant action commands into the medullary motoneurons (Schwarting & Wohr, 2012), which in turn regulate the

peripheral vocalization-related organs. More importantly, the tegmental structures in the midbrain are considered as the direct initiators of USVs in rats (Brudzynski et al., 1998; Brudzynski, 2007, 2010, 2013). Negative emotionality is strongly associated with enhanced neuronal activity in the laterodorsal tegmental nucleus (LDT) (Brudzynski et al., 2011). In rat pups, LDT neurons play a functional role in USV generation upon maternal isolation (Kehoe et al., 2001; Middlemis-Brown et al., 2005) and can reach functional maturity between GDs 12 and 16 (Semba & Fibiger, 1988).

It is well established that the neural systems originating from the laterodorsal and ventral tegmental areas are activated during ethanol administration (Larsson et al., 2005; Valenta et al., 2013) and undergo synaptic changes upon ethanol exposure (Zhang et al., 2006; Bernier et al., 2011). The core limbic system and/or tegmental structures in rat pups were activated due to prenatal ethanol-exposure and the subsequently changed emotionality might cause increases in terms of the number, amplitude, and fundamental frequency of USVs in the pups in the high-ethanol group.

4.2. Effects of ethanol on peripheral vocalization-related organs

In rat pups, some USV characteristics develop as a simple function of maturation of peripheral vocalization-related organs. USVs are generated by high-pressure air flow from the thorax through a small orifice located in the closed vocal folds (Brudzynski & Fletcher, 2010). An increase in the rate of development of the size and length of the larynx and vocal tract would be expected to produce lower fundamental frequencies of USVs, which has been previously reported to occur as rat pups age (Elsner et al., 1990; Wada, 2017).

In the present study, the high-ethanol group exhibited an increase in the mean, maximum, and minimum fundamental frequencies compared with those exhibited by the pups in both the low-ethanol and control groups. However, all the groups underwent normal growth regardless of ethanol administration, reaching the same levels of body weight gains.

Therefore, it is reasonable to assume that peripheral vocalization-related organs such as the larynx and vocal tract properly matured.

4.3. Effects of other factors on the acoustic characteristics of USVs

It may be argued that ethanol exposure changed emotionality in dams and led to inappropriate maternal behavior, which in turn may have caused the pups to increase the number, amplitude, and fundamental frequency of USVs. However, the dams were exposed to ethanol from GDs 8 to 20, which did not include the lactation period. Moreover, all the pups were normally weaned on PND 21 regardless of ethanol exposure, indicating that prenatal ethanol exposure did not affect maternal behavior.

Prenatal experience with ethanol has potential to modulate rat pups' responsiveness to ethanol. Youngentob & Glendinning (2009) demonstrated that gestational ethanol exposure via dam's diet increases postnatal ethanol acceptability in rats. Because we cleaned the used translucent cups with ethanol and water, residual ethanol odor might affect pups' emotionality. However, pups' USVs are considered as distress calls reflecting negative emotionality such as anxiety (Portfors, 2007) and residual ethanol odor may not induce negative emotionality in animals with increased ethanol acceptance.

Another possibility of the increased number, amplitude and fundamental frequencies of USVs in the high-ethanol group could be because the pups in the high-ethanol group did not have their eyes open but those in the other groups did. It is well documented that prenatal ethanol exposure delays the eye opening in rat pups with the delayed appearance of other developmental landmarks including body muscles, body weight, incisor eruption and ear opening (Lopez-Tejero et al., 1986; Gottesfeld & Silverman, 1990). The high-ethanol group before eye opening might increase USV numbers and amplitudes to call their dam. In our study, eye opening of pups in all groups was observed on PND 15 except some pups of the high-ethanol group whose eyes were opened on PND 16. No pups' eyes were opened on PND

12. Thus, altered USVs on PND12 were not attributed to the delayed eye opening in the high-ethanol group.

No significant differences were observed in any of the acoustic USV characteristics based on the sex of the pup. Brunelli et al. (1996) and Zimmerberg et al. (2003) reported that the sex does not affect the number of USVs induced by maternal isolation. In contrast, Naito & Tonoue (1987) and Bowers et al. (2013) revealed that isolated male rat pups emitted substantially more USV calls, which were characterized by a substantially lower frequency and amplitude compared with that produced by female rat pups. Differentiation of USVs based on sex may not be distinctive in the postnatal periods because of sexual immaturity.

Our findings suggest that prenatal exposure to ethanol caused acoustic alterations in the number, fundamental frequency, and amplitude of USVs. However, all pups could be weaned on PND 21 and they survived afterward regardless of the ethanol administration. The social behavior among the pups and their dam may be robust and independent of acoustic alterations in the USVs emitted by the pups.

Neurobehavioral studies on pups are challenging because they are immobile due to muscle immaturity. We measured neurobehavioral development in rat pups by introducing USV analyses as a well-suited tool and studied the effects of prenatal ethanol-exposure on the social relationship between the pups and their dam. USV analysis is a highly sensitive tool that can be applied to various research areas.

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