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Title

Acoustic alterations of ultrasonic vocalization in rat pups induced by perinatal hypothyroidism

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Abstract

Perinatal hypothyroidism causes serious damage to auditory functions that are essential for vocalization development. In rat pups, perinatal hypothyroidism potentially affects the development of ultrasonic vocalization (USV) as a result of hearing deficits. This study examined the effect of perinatal hypothyroidism on the development of USVs in rat pups. Twelve pregnant rats were divided into three groups and treated with the anti-thyroid drug methimazole (MMI) via drinking water, from gestational day 15 to postnatal day (PND) 21. The MMI concentration (w/v) was 0% (control group), 0.01% (low-dose group), or 0.015% (high-dose group). After birth, the pups were individually separated from the dam and littermates on PNDs 5, 10, 15, and 20, and their USVs were recorded for 5 min. On PNDs 5 and 10, compared with the control group, the low- and high-dose groups exhibited reductions of both frequency-modulated and downward USVs. On PND 15, however, the low- and high-dose groups displayed increases in number, duration, and amplitude of USVs compared with those in the control group. Lower body weights were observed for the low- and high-dose groups than for the control group. Total thyroxine concentrations in plasma were dose-dependently reduced. The onset of auditory functions appeared on PNDs 11–14. Thus, the rat pups were unable to hear externally produced USVs before PND 11. USVs emitted on PNDs 5 and

10 might have been spontaneous and independent of the pups' own or littermate-emitted USVs. The developmental retardation of vocalization-related organs or muscles might underlie the acoustic alterations of USVs on PNDs 5 and 10. The greater number, duration, and amplitude of USVs on PND 15, after which the hearing onset occurred, suggested that the elevation of auditory thresholds occurred as a result of hearing deficits in the low- and high-dose groups. Perinatal hypothyroidism appears to have caused acoustic alterations in the USV development.

Keywords: auditory function; hearing deficits; hypothyroidism; methimazole; ultrasonic vocalization; rat pup

1 Introduction

Auditory functions in rats normally appear on postnatal days (PNDs) 12–14 (Brunjes & Alberts, 1981; Gabriele et al., 2000). The external acoustic meatus opens and auditory startle responses are observed on PNDs 11–14 for the first time (Brunjes & Alberts, 1981). However, perinatal hypothyroidism appears to delay the onset of auditory startle responses (Comer & Norton, 1982; Schneider & Golden, 1986). In addition, the amplitude of startle responses is reduced (Goldey et al., 1995; Henck et al., 1996). Irreversible damage to auditory functions was demonstrated (Wada et al., 2013); severe hearing deficits were detected over a wide range of tone frequencies from 1 to 40 kHz (Goldey et al., 1995). Hearing deficits make it difficult for rats to hear their own vocalizations and those of their conspecifics. Auditory functions are indispensable for vocalization development.

Vocalization has been investigated, particularly in rodents such as rats and mice, and the communicative functions of vocalization have been increasingly elucidated upon (Brudzynski, 2010). For example, rat pups emit ultrasonic vocalizations (USV) when they are separated from the dam (Portfors, 2007; Ise & Ohta, 2009; Schwarting & Wöhr, 2012). The dam approaches the pups in response to their USVs, retrieves them, and returns them to the nest (Schwarting & Wöhr, 2012). In contrast to this, the pups

suppress USVs upon contact with an unknown male adult rat (Takahashi, 1992; Shair et al., 1997; Wiedenmayer et al., 2003), possibly because adult male rats often attack and kill pups in order to mate with their dam (Hofer, 2010).

USVs emitted by rat pups were shown to increase on PNDs 3–5 and reach a maximum on PNDs 5–10. Subsequently, USVs decreased and then completely disappeared around PND 21, when pups were able to wean (Schwarting & Wöhr, 2012). USVs in rat pups have been shown to be at frequencies of approximately 40 kHz and durations of approximately 80–150 ms (Portfors, 2007; Ise & Ohta, 2009; Schwarting & Wöhr, 2012). Because 40-kHz USVs in rat pups were demonstrated to be emitted upon maternal separation, USVs were considered to express the pups' distress state (Brudzynski et al., 1999; Portfors, 2007).

Rat pups seem to hear 40-kHz USVs emitted by themselves and their littermates after PNDs 13–15 because auditory startle responses can be induced by means of a 40-kHz ultrasonic tone on PNDs 13–15 (Brunjes & Alberts, 1981). However, perinatal hypothyroidism has been shown to cause morphological abnormalities in the cochlea (Uziel et al., 1980, 1981, 1985), and hearing deficits may be extended to the ultrasonic frequency band of 40 kHz (Goldey et al., 1995). Therefore, perinatal hypothyroidism potentially affects the development of rat pup's USVs as a result of hearing deficits.

Nevertheless, little is known about the effects of hypothyroidism on the development of USVs.

In this study, pregnant rats were treated with the anti-thyroid drug methimazole (MMI), and USVs emitted by the pups from control and treated mothers were recorded upon the separation from the dam. The acoustic characteristics of the USVs were analyzed and examined to determine whether perinatal hypothyroidism affected the development of USVs during the lactation period. Acoustic alterations were predicted to be caused in hypothyroid pups as a result of hearing deficits.

2 Materials and Methods

2.1 Subject

Twelve pregnant Wistar rats at gestational day (GD) 12 were purchased from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed in individual cages; supplied with the certified rat chow MF (Oriental Yeast Ltd., Sapporo, Japan) and tap water *ad libitum*; and randomly assigned to either a control group (n = 4), a low-dose group (n = 4), or a high-dose group (n = 4). MMI (Sigma Aldrich Co., Mo, USA) was dissolved in distilled water and administered to the animals via drinking water starting on GD 15 and extending to PND 21. The following concentrations of MMI (w/v) were

used: 1) 0% (control), 2) 0.01% (low-dose), and 3) 0.015% (high-dose). These concentrations were selected for two reasons. First, the 0.01% MMI was the lowest concentration that causes hearing deficits in rats (Comer & Norton, 1982; Albee et al., 1989). Second, the 0.01% and 0.015% MMI treatments induced dose-dependent reduction of T4 (shown in Fig. 4). Dose-dependent alterations were expected in the acoustic parameters of the USVs. Because fetal thyroid functions in rats begin at approximately GD 17 (Gilbert & Zoeller, 2010), the MMI administration started on GD 15. MMI is able to pass through the placenta and reach the fetuses during gestation (Marchant et al., 1977; De Escobar et al., 1988; Sack et al., 1995), and it is excreted into breast milk and taken by pups on PNDs (Johansen et al., 1982; Cooper, 1984). The date of birth was designated PND 0. On PND 4, each litter was culled to four males and four females. Two pups (one male and one female) were randomly sampled from each litter as subjects. Thus, four male and four female pups were chosen from each MMI treatment group. The same pups were repeatedly tested for USV recording at each age.

The temperature of the breeding room was maintained at 22°C ± 2°C with a relative humidity of 50% ± 10%. The dams and pups were subjected to a 12-h light/dark cycle (light: 20:00–08:00 h; dark: 08:00–20:00 h). This experimental protocol was approved by the Animal Ethics Committee of Hokkaido University; all experimental

conditions were compliant with the Guide for the Care and Use of Laboratory Animals, Hokkaido University.

2.2 Apparatus

USVs were recorded and analyzed using an ultrasonic microphone and the Sonotrack system version 2.1.5 (Metris, Hoofddorp, the Netherlands). The software of the Sonotrack system was installed on a personal computer and run on MS Windows XP Professional. The ultrasonic microphone was positioned at a height of 16 cm from the bottom of a translucent cup with a 13-cm bottom diameter, 15-cm top diameter, and 15-cm height. The ultrasonic microphone and translucent cup were placed in a sound-insulated box in order to attenuate external sound and light.

2.3 Recording of USVs

USVs were recorded on PNDs 5, 10, 15, and 20 as described in this section. A pup was individually separated from the dam and littermates in the breeding room, put into the translucent cup described above and brought to the experimental room. The pup was left alone in the sound-insulated box for a 5-min period of habituation, followed by 5 min of USV recording. Thus, the total duration of separation from the dam was 10

min. After the recording, the body weight of the pup was measured, and the pup was returned to the dam and littermates. The temperature of the experimental room was 20°C–23°C, and the relative humidity was 50%–70%. USVs were recorded during the dark period. The translucent cup was cleaned with ethanol and water.

2.4 Determination of thyroid hormone concentrations

Additional 24 pregnant Wistar rats were prepared for either a control group (n = 8), a low-dose group (n = 8), or a high-dose group (n = 8) and treated with MMI using the same procedure. Two pups (one male and one female) were sampled from each litter, and eight male pups and eight female pups per group were served for thyroid hormone determinations. Whole blood was collected from the abdominal aorta of ether-anesthetized pups on PNDs 20–21, centrifuged at 3000 rpm for 10 min, and the plasma was stored in a micro-tube. Both the total triiodothyronine (T3) and thyroxine (T4) concentrations were determined using the ACS-FT3 II and LKFT41 kits, respectively (Siemens Healthcare Diagnostics Co., Tokyo, Japan). Thyroid-stimulating hormone (TSH) concentrations were determined using the rat TSH ELISA kit (R-type) (Shibayagi Co., Shibukawa, Japan). All assays were performed at Mitsubishi Chemical Medience (Tokyo, Japan).

2.5 Data analyses

USVs were analyzed with the automatic selection mode of the Sonotrack system. The time resolution was 1 ms, and to reduce background noise, the low and high cut-off frequencies for the recording were set to 30 kHz and 90 kHz, respectively. The Sonotrack system calculated the lowest frequency in a periodic waveform of USV at every 1 ms time step and obtained fundamental frequencies. If the fundamental frequency at either the start or end point of an USV was out of the 30–70 kHz range, the 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).

(i) The fundamental frequencies at both the start and end points of the USV were $\geq$30 and <70 kHz.

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

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

(iv) The duration of the USV was $\geq$20 ms.

Acoustic characteristics included the number, duration, fundamental frequency, and amplitude of the USVs; the fundamental frequency difference between the start and

end points of an USV; the bandwidth (i.e., difference between maximum and minimum fundamental frequencies of an USV); and the percentage of frequency-modulated USVs (i.e., bandwidth was $\geq$5 kHz (Reno et al., 2013)). The root mean square (RMS) of USV amplitudes was calculated to indicate the magnitude of amplitudes, because the USV amplitudes changed periodically between +V and –V.

In consideration of the small litter size, the data of one male and one female pup within the same litter were averaged and unified for statistical analyses. Therefore, the sample size per group was the same as the litter size (n = 4). Sex affected neither the USVs nor body weight in the data. The acoustic characteristics of USVs were analyzed using a two-factor analysis of variance (ANOVA) between subject variables of MMI concentrations and within subject variables of age. USV data on PND 20 were not analyzed because low numbers of USVs were obtained independent of the MMI concentrations. The mean and SEM were 4.167 and 2.685, respectively. Body weights were analyzed with a two-factor ANOVA between subject variables of MMI concentrations and within subject variables of age. Thyroid hormone concentrations were analyzed with a one-factor ANOVA between subject variables of MMI concentrations. Sex did not affect thyroid hormone concentrations, and accordingly, male and female data were pooled and analyzed with ANOVA. When a primary effect

was found to be significant, multiple comparisons were performed by using Ryan's method. These statistical analyses were executed using ANOVA 4 on the Web (http://www.hju.ac.jp/˜kiriki/anova4/about.html).

3 Results

3.1 Number of USVs

The number of USVs is shown in Fig. 1a. The effects of MMI were significant [$F(2, 9) = 8.157$, $p < 0.01$], and the low- and high-dose groups displayed greater numbers of USVs than the control group ($p < 0.05$). The effects of age were also significant [$F(2, 18) = 40.158$, $p < 0.001$]. The number of USVs exhibited age-dependent decreases ($p < 0.05$). Interaction was found between MMI and age [$F(4, 18) = 3.518$, $p < 0.05$]. The high-dose group exhibited a greater number of USVs than the control group on PND 10 ($p < 0.05$), and the low- and high-dose groups showed increases in USVs compared with the control group on PND 15 ($p < 0.05$).

3.2 Duration of USVs

Figure 1b shows the duration of the USVs. The effects of age were significant [$F(2, 18) = 32.760$, $p < 0.001$], and age-dependent increases of the USV duration were

observed. The interaction between MMI and age was significant [$F(4, 18) = 5.103$, $p < 0.01$]. The low- and high-dose groups showed longer USV durations than the control group on PND 15 ($p < 0.05$).

3.3 Fundamental frequency of USVs

Age affected the mean fundamental frequency of the USVs [$F(2, 18) = 6.039$, $p < 0.01$]. The mean fundamental frequency was reduced in an age-dependent manner. Neither MMI nor the interaction between MMI and age was shown to be significant (data not shown).

3.4 Amplitude of USVs

The RMSs of the amplitudes are exhibited in Fig. 1c. The effects of age were significant [$F(2, 18) = 8.102$, $p < 0.005$], and the RMSs of amplitudes were greater on PNDs 10 and 15 than on PND 5 ($p < 0.05$). The interaction between MMI and age was also significant [$F(4, 18) = 4.509$, $p < 0.05$]. The low- and high-dose groups displayed greater RMSs of amplitudes than the control group on PND 15 ($p < 0.05$).

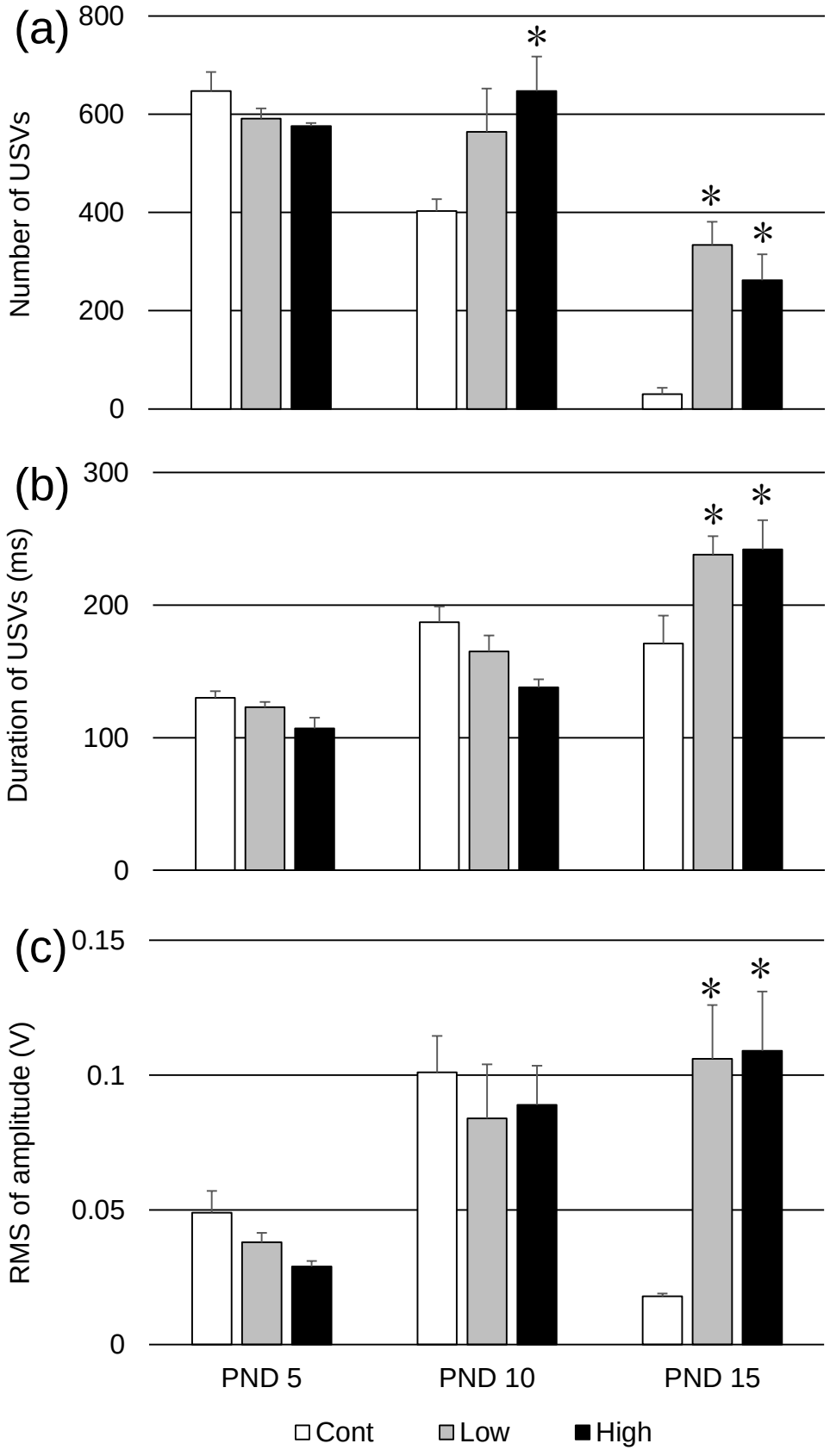

(a)
800
600
400
200
0
Number of USVs
*
*
*
(b)
300
200
100
0
Duration of USVs (ms)
*
*
(c)
0.15
0.1
0.05
0
RMS of amplitude (V)
*
*
PND 5
PND 10
PND 15
Cont
Low
High

Fig. 1 Effects of perinatal hypothyroidism on the acoustic characteristics of USVs in rat pups.

Number, duration, and RMSs of amplitude are displayed in panels a, b, and c, respectively. Male and female data within the same litter were averaged and unified in consideration of the small litter size. Hence, the sample size per group was equal to the litter size (n = 4). Sex did not have any significant effect. Cont, Low, and High are abbreviations of the control, low-dose, and high-dose groups, respectively. Data are the mean and standard error. *$p < 0.05$ compared with that in the control group.

3.5 Frequency-modulated USVs

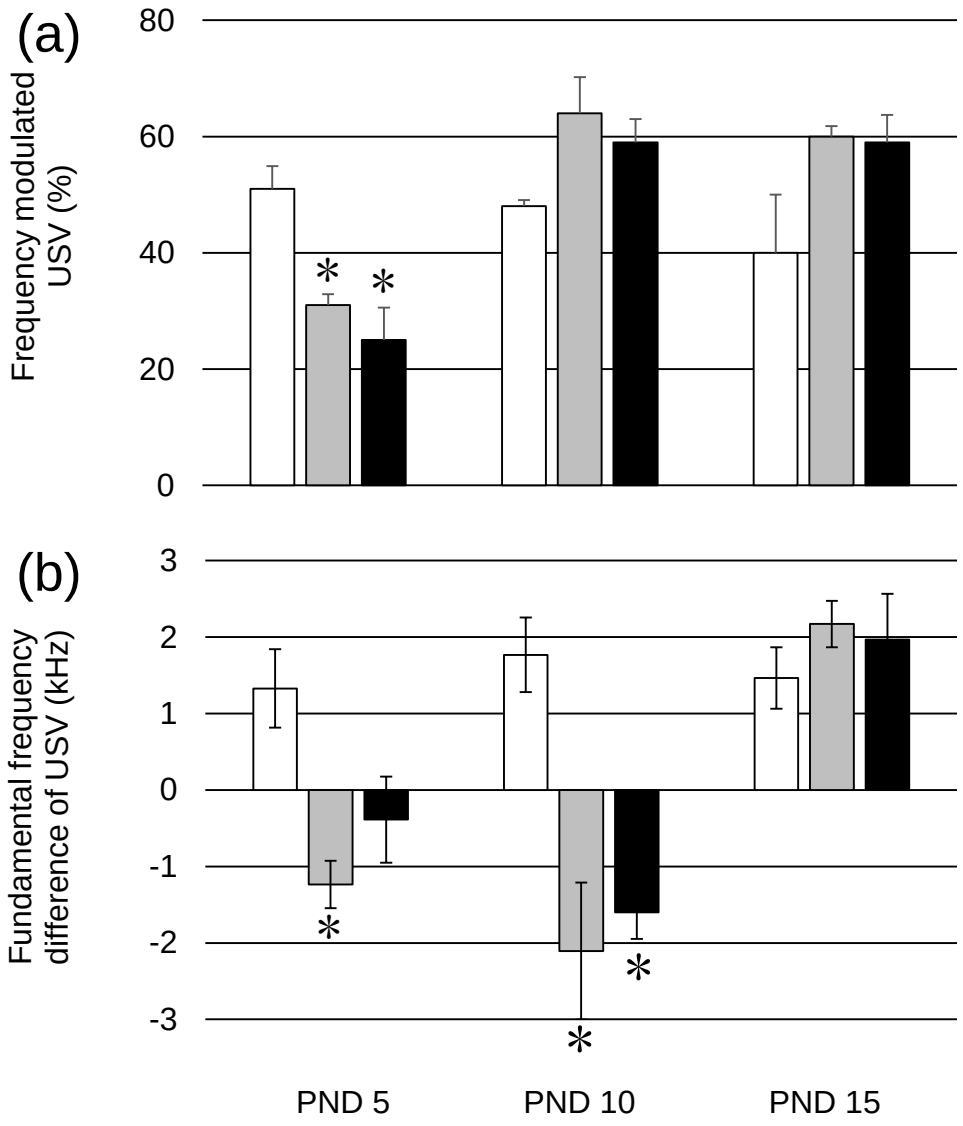
Figure 2a shows the percentage of frequency-modulated USVs. The percentage was calculated as (total number of frequency-modulated USVs/total number of USVs) × 100. The effects of age were significant [$F (2, 18) = 8.649$, $p < 0.005$], and the percentage displayed elevations on both PNDs 10 and 15 compared with PND 5 ($p < 0.05$). The interaction was significant between MMI and age [$F (4, 18) = 4.223$, $p < 0.05$]. On PND 5, a reduction of frequency-modulated USV percentages was observed in the low- and high-dose groups compared with the control group ($p < 0.05$), although the number of USVs showed no differences among the three groups (Fig. 1a).

3.6 Fundamental frequency difference of USVs

The fundamental frequency difference of USVs is shown in Fig. 2b. The fundamental frequency difference was calculated by subtracting the fundamental frequency at the end point of the USV from that at the start point. The effects of MMI were significant [$F(2, 9) = 7.201$, $p < 0.05$]. The control group displayed a positive value. This meant that the control group emitted USVs with greater fundamental frequencies at the start point than those at the end point, indicating downward USVs. In contrast, a negative value was obtained in the low- and high-dose groups. The low- and high-dose groups emitted USVs with greater fundamental frequencies at the end point than those at the start point, indicating upward USVs. Age was significant [$F(2, 18) = 15.821$, $p < 0.001$]. The interaction was also significant between MMI and age [$F(4, 18) = 4.838$, $p < 0.01$]. Compared with the control group, the low-dose group demonstrated a significant difference on PND 5 ($p < 0.05$). On PND 10, the low- and high-dose groups exhibited significant differences compared with the control group ($p < 0.05$). However, all three groups showed a positive value on PND 15, indicating downward USVs.

3.7 USV Bandwidth

Figure 2c shows USV bandwidth, which was defined as the range between the maximum and minimum USV fundamental frequencies. MMI concentrations were significant [$F(2, 9) = 5.230$, $p < 0.05$]. The maximum fundamental frequency of the bandwidth was significantly lowered in the low- and high-dose groups compared with the control groups ($p < 0.05$), while the minimum fundamental frequency of the bandwidth was not changed.


(a)
Frequency modulated USV (%)
80
60
40
20
0
(b)
Fundamental frequency difference of USV (kHz)
3
2
1
0
-1
-2
-3
PND 5
PND 10
PND 15

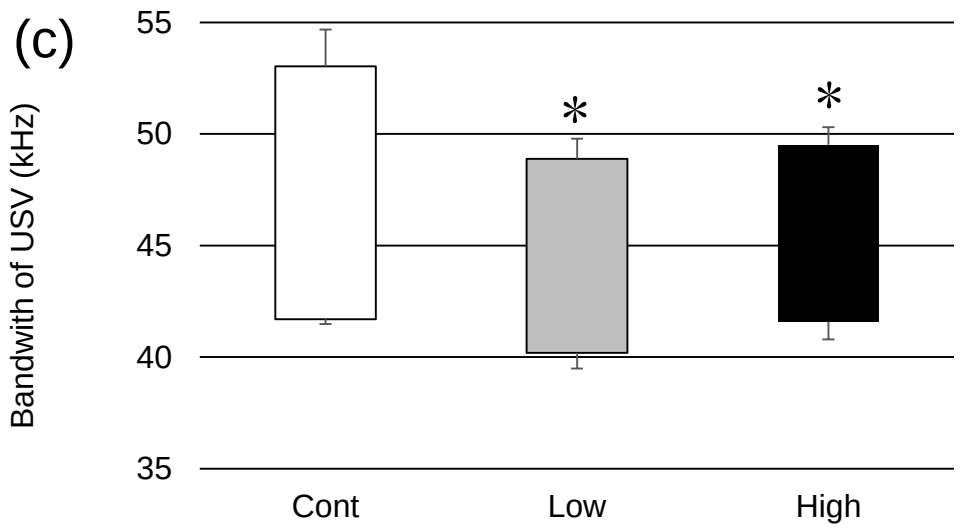


Fig. 2 Effects of perinatal hypothyroidism on the acoustic characteristics of USVs in rat pups.

The percentages of frequency-modulated USVs, fundamental frequency differences between the start and end points of USVs, and bandwidth between maximum and minimum USV fundamental frequencies are shown in panels a, b, and c, respectively. Male and female data within the same litter were averaged and unified in consideration of the small litter size. Hence, the sample size per group was equal to the litter size (n = 4). Sex had no significant effect. Cont, Low, and High are abbreviations of the control, low-dose, and high-dose groups, respectively. Data are the mean and standard error. *p < 0.05 compared with that in the control group.

3.8 Body weights

Body weights are shown in Fig. 3. The effects of MMI and age were significant [$F(2, 9) = 31.636$, $p < 0.001$: $F(3, 27) = 1608.112$, $p < 0.001$]. Although age-dependent elevations of body weights were displayed in all three dose groups, the low- and high-dose groups showed lower body weights than the control group ($p < 0.05$). The interaction between MMI and age was also significant [$F(6, 27) = 31.801$, $p < 0.001$]. The low- and high-dose groups indicated lower body weights than the control group on both PNDs 10 and 15 ($p < 0.05$). Dose-dependent reductions in body weights were observed on PND 20 ($p < 0.05$).

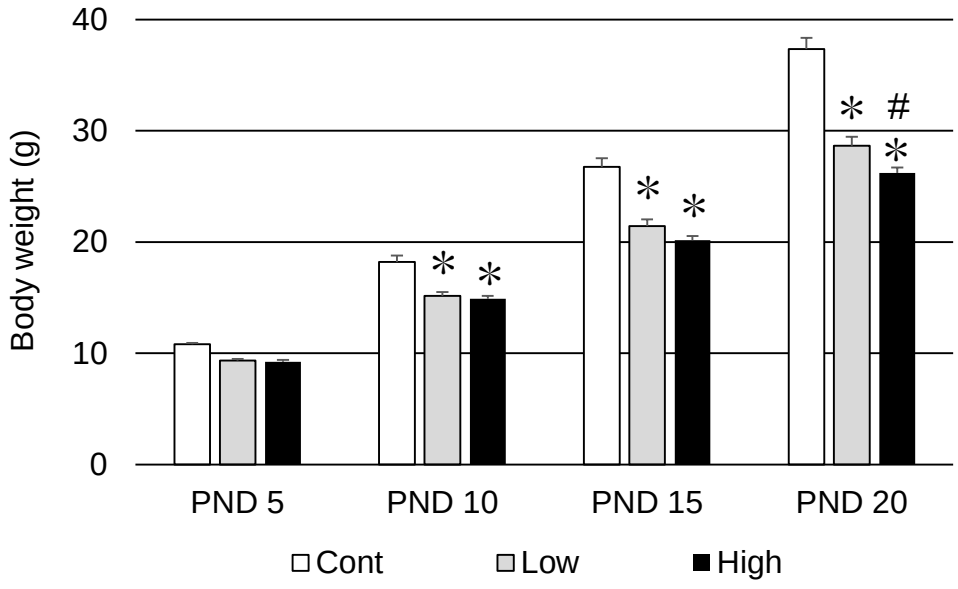


Fig. 3 Effects of perinatal hypothyroidism on rat pups' body weights.

Male and female data within the same litter were averaged and unified in consideration of the small litter size. Hence, the sample size per group was equal to the litter size (n = 4). Sex had no significant effect. Cont, Low, and High are abbreviations of the control, low-dose, and high-dose groups, respectively. Data are the mean and standard error. *p < 0.05 compared with that in the control group, #p < 0.05 compared with that in the low-dose group.

3.9 Landmark of physical development

The landmarks of physical development are displayed in Table 1. The low- and high-dose groups showed a one-day delay in body hair growth and eye opening. However, incisor eruption was observed on PND 9 for all three groups.

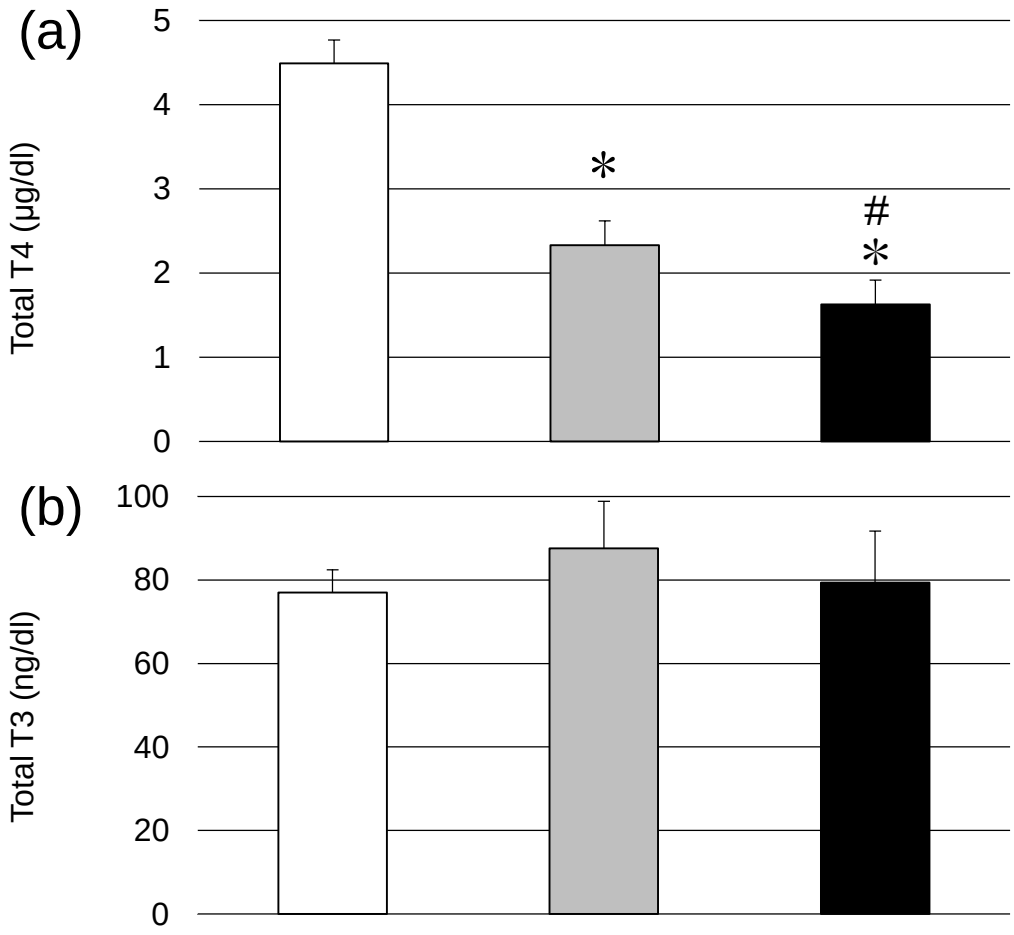
Table 1 Landmarks of physical development.

Group	Incisor eruption	Body hair	Eye opening
Cont	PND 9	PND 7	PND 16
Low	PND 9	PND 8	PND 17
High	PND 9	PND 8	PND 17

Cont, Low, and High are abbreviations of the control, low-dose, and high-dose groups, respectively. The sample size per group is (n = 8). Each PND indicates the day when the onset of the landmark appeared on all pups within the group.

3.10 Thyroid hormone concentrations

Figure 4 shows the thyroid hormone concentrations in plasma. MMI significantly affected total T4 concentrations [$F(2, 45) = 54.45$, $p < 0.001$] (Fig. 4a), and a dose-dependent reduction was revealed ($p < 0.05$). However, no effects of MMI on either total T3 or TSH concentration were observed (Fig. 4b and c).



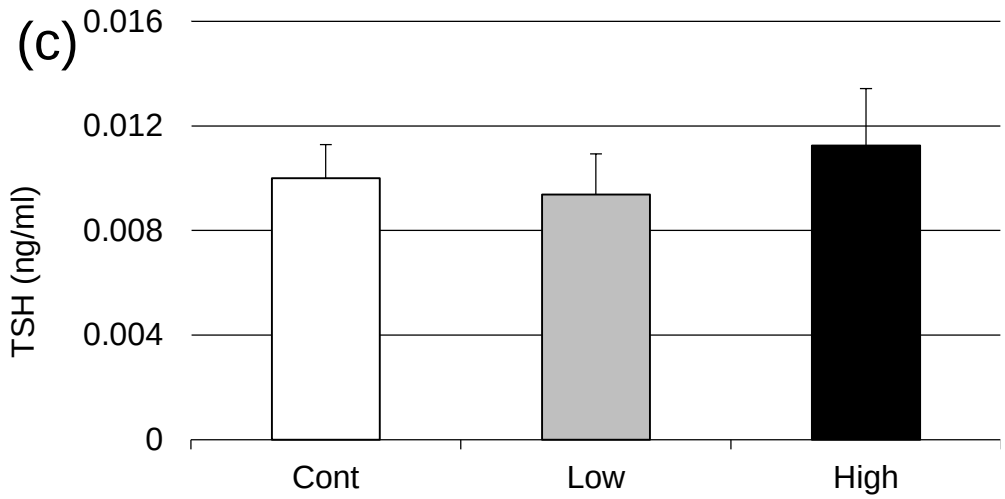


Fig. 4 Effects of perinatal hypothyroidism on thyroid hormone concentrations in rat pups.

Plasma concentrations of total T4 and T3 as well as TSH are displayed in panels a, b, and c, respectively. Male and female data were pooled because sex did not have any significant effects on thyroid hormone concentrations. Each group consisted of eight male pups and eight female pups. Accordingly, the sample size per group was (n = 16). Cont, Low, and High are abbreviations of the control, low-dose, and high-dose groups, respectively. Data are the mean and standard error. *$p < 0.05$ compared with that in the control group, #$p < 0.05$ compared with that in the low-dose group.

4 Discussion

Humans and animals develop vocalization by hearing their own and conspecifics' vocalizations. Hence, auditory functions are indispensable for the development of vocalization. The onset of auditory functions in rats has been shown to be PNDs 11–14 (Brunjes & Alberts, 1981; Gabriele et al., 2000). Brunjes and Alberts (1981) reported that auditory stimuli with frequencies of 1 kHz, 4 kHz, and 16 kHz induced startle responses in rat pups on PND 11, 11, and 12, respectively. Schneider and Golden (1986; 1987) also indicated that startle responses were detected on PND 12 in response to auditory stimuli with frequency ranges of 1–8 kHz. However, an auditory stimulus with a frequency of 40 kHz, which had similar frequencies to pups' USVs, did not induce startle responses before PND 14 (Brunjes & Alberts, 1981). Therefore, it appears that rat pups are unable to hear their own and littermates-emitted 40-kHz USVs before PND 14. This suggests that USVs before PND 14 may be spontaneously produced and are independent of their own and littermates' USVs.

4.1 Before auditory function onset

The present study detected 40-kHz USVs on PNDs 5 and 10, and the low- and high-dose groups revealed several acoustic alterations compared with the control group.

First, the low- and high-dose groups showed a reduction in the percentage of frequency-modulated USVs on PND 5. Second, the control group emitted downward USVs with greater fundamental frequencies at the start point than at the end point, whereas the low- and high-dose groups emitted upward USVs with greater fundamental frequencies at the end point than at the start point on PNDs 5 and 10. Third, the low- and high-dose groups had lower maximum fundamental frequencies of the bandwidth than the control group throughout the USV recording ages. Regardless of various alterations in USVs, dose-dependent alterations were not revealed. The two concentrations of MMI appeared to have similar effects on most of the acoustic parameters of the USVs throughout the recording ages. Brudzynski et al. (1999) described that, when compared to flat frequency USVs (i.e., bandwidth was <5 kHz (Reno et al., 2013)), frequency-modulated USVs were more crucial for communication between pups and their dam because frequency-modulated USVs were easily detected by the dam. USVs with wider bandwidths also increased the chance of being detected by the dam (Brudzynski et al. 1999). Moreover, downward USVs might stand out because they start with higher frequencies. On PNDs 5 and 10, the rat pups were unable to move or regulate their body temperature in the absence of the dam or littermates (Alberts, 1978). This suggests a higher dependence on maternal care during the early

lactation period. Reductions of frequency-modulated USVs, downward USVs, and bandwidths may not be advantageous for pup survival.

Auditory functions were not responsible for the USV acoustic alterations manifested on PNDs 5 and 10 because auditory functions are not set until PNDs 12–14. USV generation originates from the thorax and vocal folds. High-pressure air flow from the thorax has been shown to create USVs by passing through a small orifice of the closed vocal folds (Brudzynski & Fletcher, 2010). Hypothyroidism has been shown to inhibit body weight gain and delay physical development in this and other studies (Schneider & Golden, 1987; Albee et al., 1989; Henck et al., 1996; Wada et al., 2013). Lower body weights could cause a reduction in the volume of thorax and breast muscles necessary to create high-pressure air flow. USV-related organs such as the thorax, vocal folds, vocal tract, or larynx might be immature to regulate high pressure air flow and result in acoustic alterations of USVs. The retardation of physical development might underlie the acoustic alterations of USVs emitted by the MMI-treated groups before the onset of auditory functions. However, these alterations disappeared on PND 15. Moreover, the mean fundamental frequencies of USVs were age-dependently reduced in all groups. Because the frequency of USVs decreased as the vocalization-related organs

developed (Kromkhun et al., 2013), those organs in the low- and high-dose groups seemed to mature, to some extent, until PND 15.

4.2 After auditory function onset

On PND 15, auditory functions were set; the control group exhibited a drastic reduction of USVs, and by PND 20, they showed a complete lack of USVs (data not shown). In contrast, the low- and high-dose groups demonstrated higher numbers of USVs on PNDs 10 and 15, but the USVs had almost completely disappeared by PND 20. Furthermore, on PND 15, the low- and high-dose groups displayed longer durations of USVs than the control group. The control group drastically declined RMS of USV amplitudes on PND 15 and reached the minimum. In contrast, the low- and high-dose groups maintained much greater RMS of amplitudes over PND 10 and reached the maximum on PND 15. The low- and high-dose groups emitted greater numbers of USVs with longer durations and stronger amplitudes on PND 15 than the control group.

Animal model studies demonstrated irreversible damage to auditory functions in rats as a result of perinatal hypothyroidism (Comer & Norton, 1982; Albee et al., 1989; Knipper et al., 2000; Wada et al., 2013). Comer and Norton (1982) treated 0.1 mg/ml MMI in drinking water. They gave it to pregnant rats from GD 17 to PND 10 and

revealed a delay in auditory startle response acquisition for the resultant pups. The control group acquired startle responses on PND 12, whereas the MMI-treated group displayed them on PND 18. Albee et al. (1989) also administered 0.1 mg/ml MMI to pregnant rats via drinking water, starting on GD 17 and extending to PND 10. Auditory functions were evaluated by using click tone–induced auditory brainstem responses (ABRs). The offspring exhibited the waveforms of ABRs with slowed latency, decreased amplitude, and altered shape. The concentration of MMI in these two studies was 0.1 mg/ml in drinking water, which was the same concentration of MMI in the present low-dose group. Therefore, hearing deficits were fairly well predictable in the present low- and high-dose groups.

Knipper et al. (2000) discussed thyroid hormone deficiency and hearing deficits in rats. Slowed latency of ABRs might be caused by dysmyelination of the auditory nerves due to perinatal hypothyroidism. Decreased amplitude indicated an elevation of thresholds to induce ABRs, suggesting malfunctions of the cochlea for the mechanoelectrical transduction of tone stimuli (Knipper et al., 2000). Uziel et al. (1981; 1983) revealed malfunctions of the cochlea, which were the distortion of the tectorial membrane and loss of outer hair cells caused by perinatal hypothyroidism. Severe hearing deficits were shown to expand into wide frequency bands from 1–40 kHz

(Goldey et al., 1995). Because rat pups emit USVs with frequencies of approximately 40 kHz upon separation from the dam, hypothyroid pups may be potentially unable to hear their own USVs or those emitted by littermates due to hearing deficits. The occurrence of hearing deficits as a result of hypothyroidism might make it difficult to control their emission of USVs and result in the greater number of USVs with longer durations and stronger amplitudes observed in the low- and high-dose groups on PND 15.

4.3 Other effects on vocalization

Perinatal hypothyroidism causes retardation of physical development. Lower body weights, delay of incisor eruption and eye opening, and low ability of thermoregulation have been reported (Schneider & Golden, 1987; Albee et al., 1989; Henck et al., 1996). The low- and high-dose groups might be compromised in their ability to counter a hypothermic challenge caused by maternal separation. Subsequently, they emitted a greater number of USVs with longer durations and stronger amplitudes as distress calls even on PND 15. However, the increased USVs had almost completely disappeared by PND 20. Body hair growth and incisor eruption were observed in all groups by PNDs 8 and 9, respectively. Eye opening was on PND 16 for the control

group and on PND 17 for the low- and high-dose groups. The reduction of T4 was revealed in the low- and high-dose groups but the reduction of T3 and elevation of TSH were not observed. The degree of hypothyroidism seemed to be milder than that anticipated from other studies with MMI. Animal strains, body size of dams, and intake of MMI-contained water may contribute to the absence of T3 and TSH changes. The total duration of separation from the dam was 10 min. It could be insufficient to promote a hypothermic response in the pups. According to the above-mentioned reasons, the effects of hypothermia or retardation of physical development seemed to be less severe to induce alterations of USV on PND 15.

Differences in sex were not detected in any of the acoustic parameters of the USVs. Brunelli et al. (1996) and Zimmerberg et al. (2003) reported that sex did not affect the number of USVs induced by maternal separation. In contrast, Naito and Tonoue (1987) and Bowers et al. (2013) revealed that male pups emitted USVs more vigorously with lower frequencies and amplitudes compared with female pups. Sex differences of USVs in rat pups were controversial. Nevertheless, adult rats indicate clear sex differences of USVs in frequency and duration. Female rats emitted more frequent and longer USVs to potential threats such as cat odor. More frequent production of USVs is adaptive for female rats to protect their pups from threats (Litvin

et al., 2010). Male rats emitted 50 kHz USVs to sexually receptive female rats (Inagaki et al., 2013) and 22 kHz USVs after ejaculation (Barfield & Geyer, 1972). Sex differentiation of USVs may be distinctive in adulthood.

USVs are generated by the neural network in the brainstem including the periaqueductal gray, lateral parabrachial region, nucleus retroambiguus, lateral pontine, and lateral reticular formation (Hage, 2010). This network regulates the laryngeal, respiratory, and articulatory components of vocalization. Since hypothyroidism alters gene expressions related to myelination or neural network formation (Barradas et al., 2000; 2001; Kobayashi et al., 2009; 2005), it is possible that the brainstem neural network was impaired and, accordingly, the low- and high-dose groups altered their USVs. This possibility should be investigated in future studies.

Despite the USV acoustic alterations described above, all groups were able to wean on PND 21 and the pups survived after weaning. Maternal behavior may be independent of acoustic alterations of pups' USVs.

Recently, USV analyses have been applied to the mouse model studies for neurodevelopmental disorders such as autism (Moy & Nadler, 2008; Scattoni et al., 2009). USV alterations are considered to indicate communication deficits or social interaction impairments. The present study applied USV analyses to developmental

neurotoxicology of hypothyroidism in rat pups and provides evidence of acoustic alterations in USVs. Thyroid hormone systems are essential for the development of brain functions but vulnerable to the effects of environmental chemicals such as polychlorinated biphenyls (PCBs) and dioxins (Porterfield, 1994; 2000). Flame retardants such as polybrominated diphenyl ethers (PBDEs) also interfere with thyroid hormone systems (Bansal et al., 2014; Hu et al., 2014; Xu et al., 2014; Yu et al., 2015; Zhao et al., 2015). This study suggests that hypothyroidism induced by environmental chemicals may cause alterations of USV. Although further research is necessary, the spontaneous emission and non-invasive detection of USV may provide a new tool to detect and characterize the impact of thyroid disrupting chemicals on neurodevelopment.

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