Decabromodiphenyl Ether (BDE 209) and Ultrasonic Vocalization in Rat Pups

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Introduction

Polybrominated diphenyl ethers (PBDEs) are commonly used as flame-retardants because they can make plastic and rubber goods nonflammable. However, PBDEs affect the thyroid hormone systems in animals [1-4] as well as humans [5, 6]. Because thyroid hormones are essential for the proper development of brain functioning, PBDEs may cause developmental impacts on various brain functions in both animals and humans.

Recently, we reported on acoustic alterations of ultrasonic vocalizations (USVs) induced by perinatal hypothyroidism in rat pups [7]. Rat pups produce USVs at frequencies of 40–50 kHz when they are separated from the dam. In response to their USVs, the dam will approach the pups and bring them to the nest [8]. Thus, USVs are considered to function as a form of communication in rodents [9]. However, when the acoustically altered USVs go unnoticed by nearby dams the survival of pups may be subsequently threatened. Because PBDEs are known to affect the thyroid hormone system, this represents a potential way of causing acoustic alterations of USVs.

In this study we focused on decabromodiphenyl ether (BDE-209), because it is the major environmental contaminant of PBDEs. Pregnant rats were exposed to BDE-209 and the USVs in their pups were recorded upon maternal separation. We hypothesized that perinatal exposure to BDE-209 generates the acoustic alterations of USVs.

Materials and methods

Seven pregnant Wistar rats at gestational day (GD) 13 were purchased from Japan SLC Inc. (Hamamatsu, Japan). These rats were housed in individual cages and randomly assigned to the control, low-dose, or high-dose groups. BDE-209 (purity ≥ 97.0%; Merck, Darmstadt, Germany) was administered daily to the pregnant rats from GD 15 to postnatal day (PND) 21 at the following doses: 0 mg/kg (control group), 500 mg/kg (low-dose group), or 1000 mg/kg (high-dose group). BDE-209 was mixed with sweetened condensed milk (Megmilk Snow Brand Co., Ltd., Tokyo, Japan). The pregnant rats freely and voluntarily ate the sweetened condensed milk containing BDE-209. The volume of sweetened condensed milk used was 10 g/kg. The control group ate sweetened condensed milk only. The birth date of the litter was designated PND 0, and on PND 4 the pups were culled to four males and four females per litter. The recording of pup USVs was done on PNDs 4, 7, 10, 13, 16, 19, and 22 using an ultrasonic microphone and the Sonotrack system v.2.4.0. (Metris, Hoofddorp, The Netherlands). Each pup was individually separated from the dam and littermates and left alone below the ultrasonic microphone in a sound-insulated box for a 5-min period of habituation, followed by a 5-min USV recording, after which the pup was returned to the dam and littermates.

The temperature of the sound-insulated box was maintained at 19–22°C, and the relative humidity was kept at 40–60%. Both 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), and the USVs were recorded during the dark period. The dams were supplied with rat chow and tap water *ad libitum*. The experimental protocol was approved by the Animal Ethics Committee of Hokkaido University, and all the experimental conditions complied with the Guide for the Care and Use of Laboratory Animals (Hokkaido University).

The collected USV data recordings were processed via automatic analyses in the Sonotrack system. A three-factor ANOVA tested the main and interactive effects of BDE-209 dose, sex of the pups, and age on the number, duration, and mean frequency of the USV calls by pups and their body weight gain. When a main effect was found to be significant, multiple pair-wise comparisons of group means were performed using Ryan's method. These statistical analyses were executed using the ANOVA 4 software (http://www.hju.ac.jp/~kiriki/anova4/about.html). Low numbers of USV calls were obtained on PNDs 19 and 22, regardless of the treatment group. Three female pups in the high-dose group, and one male and female pup in the low-dose group, had died; on PND 16, one male pup in the low-dose and control groups each produced no USV calls. Hence, the USV data from these pups were excluded from the final data analyses.

Results and discussion

The body weight gains of the rat pups are shown in Figure 1. Dose was a significant factor ($F_{(2, 44)} = 5.334$, $P < 0.01$), as the low-dose group had gained more body weight than the high-dose group ($P < 0.05$). The interaction between dose and age was also significant ($F_{(12, 264)} = 4.809$, $P < 0.001$) such that the high-dose group had a lower body weight than did the low-dose group on PNDs 13, 16, 19, and 22 (Ps < 0.05). The control group also had a lower body weight than the low-dose group on PNDs 16, 19, and 22 ($P < 0.05$ for all). No significant difference in mean body weight was found between the high-dose and control groups. Sex was also a significant factor ($F_{(1, 44)} = 7.159$, $P < 0.05$) in that the male pups gained more body weight than the female pups. Age-dependent increases in body

weight were also observed ($F_{(6, 264)} = 3175.307$, $P < 0.001$).

The mean number of USV calls is shown in Figure 2. Dose was not a significant factor, but sex was ($F_{(1, 42)} = 6.883$, $P < 0.05$) as the male pups produced more USV calls than the female pups ($P < 0.05$). The USV calls reached their peak levels on PNDs 7 and 10 and subsequently decreased in an age-dependent manner. The mean frequency of USV calls is shown in Figure 3. Again, dose did not have a significant effect. Sex, however, was significant ($F_{(1, 42)} = 8.548$, $P < 0.05$). The female pups produced USV calls having higher frequencies than the male pups ($P < 0.05$). Age-dependent decreases in the mean frequencies were also revealed ($F_{(4, 168)} = 99.390$, $P < 0.001$). In contrast to these results, the duration of USV calls was significantly affected by dose ($F_{(2, 42)} = 8.217$, $P < 0.001$) (Fig. 4), which reduced the USV call durations in both the low- and high-dose groups when compared with the control group ($P < 0.05$ for all). The interaction between dose and age was also significant ($F_{(8, 168)} = 2.632$, $P < 0.05$). Specifically, the low-dose group displayed shorter USV call durations than the control group, but only on PND 7 ($P < 0.05$), while the high-dose group displayed shorter call durations than the low-dose and control groups on PND 10, and the call durations of both the high- and low-dose groups were shorter than those of the control group on PND 13 ($P < 0.05$ for all). Sex was not a significant factor in this ANOVA. Age-dependent increases in durations were observed except for PND 16 ($F_{(4, 168)} = 55.830$, $P < 0.001$).

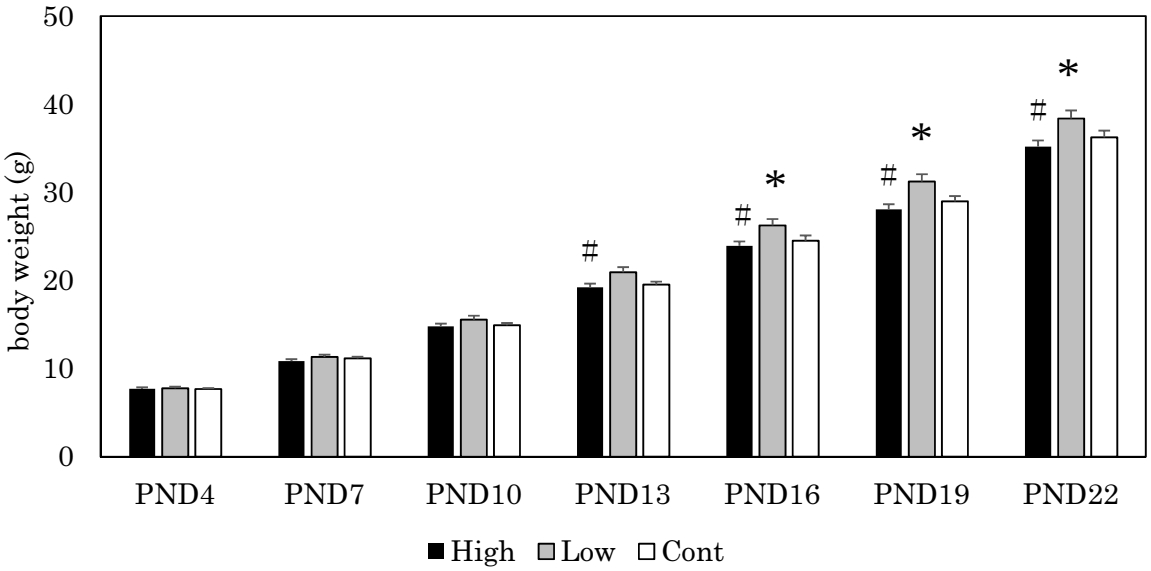

Fig. 1. Body weight gains in rat pups on the USV-recording days. Bars represent the mean and SEM. #, $P < 0.05$ when compared with the low-dose group; *, $P < 0.05$ when compared with the control group.

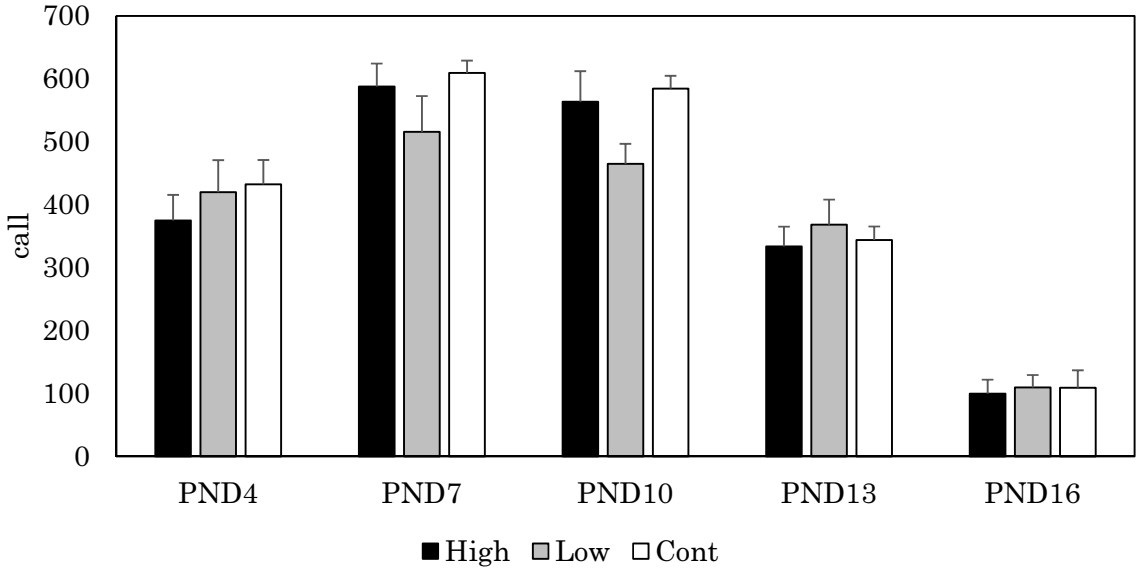

Fig. 2. Number of USV calls by rat pups recorded in a 5-min period after their separation from the dam and littermates on PNDs 4, 7, 10, 13, and 16. Bars represent the mean and SEM.

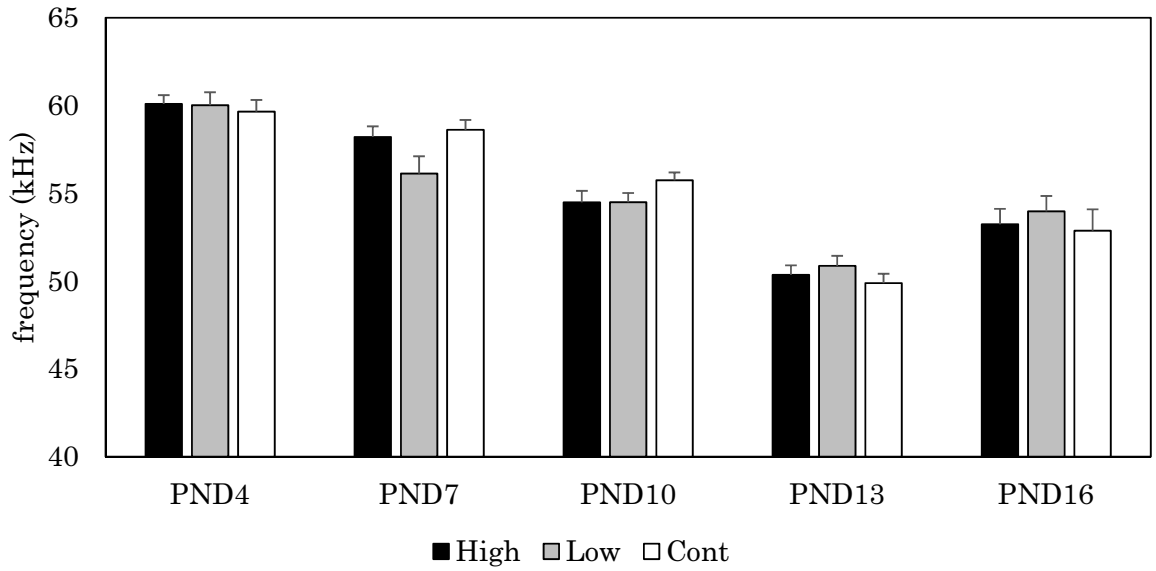

Fig. 3. Frequency of USV calls by rat pups recorded in a 5-min period after their separation from the dam and littermates on PNDs 4, 7, 10, 13, and 16. Bars represent the mean and SEM.

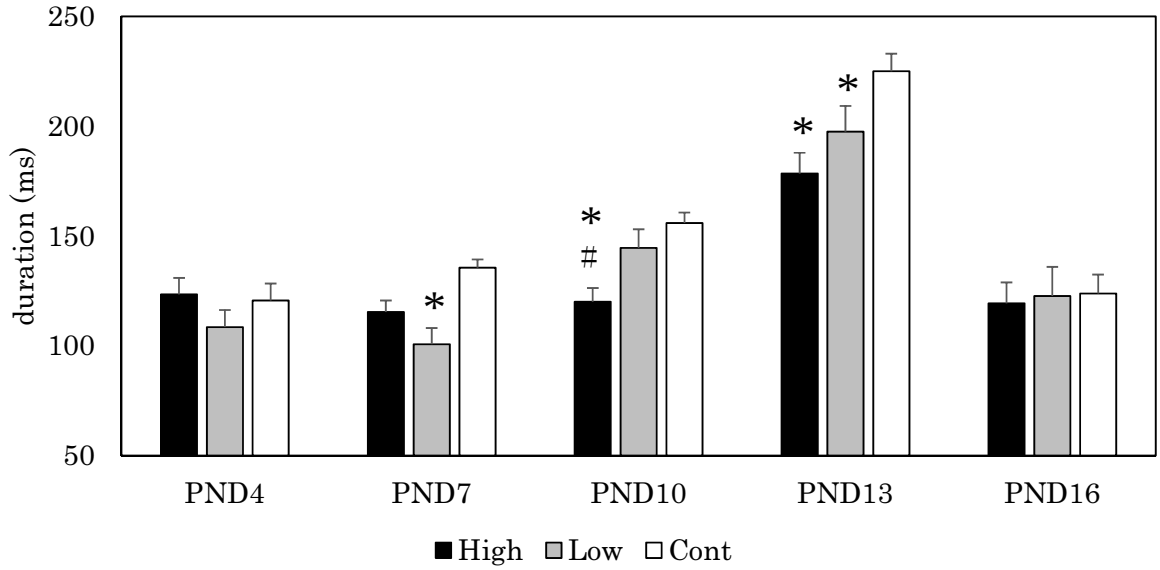

Fig. 4. Duration of USV calls by rat pups recorded in a 5-min period after their separation from the dam and littermates on PNDs 4, 7, 10, 13, and 16. Bars represents the mean and SEM. *, $P < 0.05$ when compared with the control group; #, $P < 0.05$ when compared with the low-dose group.

The compound BDE-209 causes thyroid hormone deficiencies in animals and humans. In animal model studies, the dosage of 320 mg/kg BDE-209 from GD 6 to GD 18 induces the reduction of thyroxine (T4) in the female rat pups and an elevation of the thyroid-stimulating hormone (TSH) in both male and female rat pups [10]. A thyroid hormone deficiency occurred in adult rats treated with 1000, 2000, and 4000 mg/kg for 28 days such that triiodothyronine (T3) reduction was caused by the 1000 mg/kg dosage and T4 reduction by all three dosages tested [3]. PBDEs are considered to compete with T4 to bind to the transthyretin transport protein [11]. Epidemiological studies have also reported on thyroid hormone deficiency related to PBDEs. In children aged 4–6 years, negative correlations between PBDEs and free T3 and positive correlations between PBDEs and TSH have been reported [6]. Considering the total PBDE burden, 70% of it is attributed to BDE-209. Because a thyroid hormone deficiency can cause acoustic alterations of USVs in rat pups [7], we hypothesized that BDE-209 was repsonsible for inducing these acoustic alterations.

In this study, we found that both low- and high-dose groups exhibited reductions in USV durations on PNDs 7, 10, and 13. Rat pups will produce USVs and call their dam when their body temperature falls. Here, we had separated pups from dam and littermates for a 5-min period of habituation plus a 5-min period of USV recording. The

maternal separation generated hypothermia in the pups and so they emitted the USVs as a distress call. However, the BDE-209-exposed groups produced shorter USVs than the control group. In the natural world, however, the dams may not notice these USVs of shorter duration, causing a threat to the pup's survival.

Neither the number nor the mean frequency of USV calls was affected by the BDE-209 dose throughout the USV-recording days. Growing rat pups are able to slightly regulate their body temperature after PND 15 [12]. At this age, the pups no longer need to call the dam to mitigate hypothermia. This likely explains why the number and duration of USV calls diminished on PND 16, and the lack of a significant difference among the three tested dose groups. This further indicates that the pups in the BDE-209-exposed groups—as well as in the control group—grew up normally and were successfully regulating their body temperature after PND 16.

The USVs are generated from the vocal organs of the thorax and the vocal folds. As they grow up, rat pups begin to emit USVs with lower frequencies as the vocal organs develop completely. All of the three dose groups in our study exhibited age-dependent reductions in the mean frequencies, thus indicating the normal development of the vocal organs. In addition, the body weight gains in the BDE-209-exposed groups were not lower than those in the control group. Hence, perinatal exposure to BDE-209 up to 1000 mg/kg may not affect the physical development of rat pups. A dosage of 1000 mg/kg BDE-209 from GD 6 to PND 21—a dose level similar to that of our study but administered for a longer period than us—did not reveal treatment-related neuropathological or morphometric alterations [13]. The absorption rate of BDE-209 is very low, less than 2% of the administered doses [14, 15]. Such a low absorption rate may be one reason for why any BDE-209-related neuropathological or morphometric alterations were not revealed in that study. Nevertheless, in our present study, the BDE-209-exposed female pups had slightly lower survival rates compared with the BDE-209-exposed male pups. Three female pups in the high-dose group and one female pup in the low-dose group were dead, whereas only one male pup in the high-dose group died. Because female pups display not only decreased thyroid hormone levels but also decreased estradiol levels [10], female pups may have more adverse effects due to BDE-209 exposure.

Our study has several limitations. The litter sizes were small and the acoustic alterations of USVs are not the same as the results of hypothyroidism [7]. The determination of thyroid hormones was not conducted. However, we did apply the USV analyses to the developmental neurotoxicology of BDE-209 in rat pups. Further, we provided evidence of acoustic alterations in the USVs. Although spontaneously emitted by rat pups, their USVs are easily recorded in non-invasive ways. As such, USV-based analyses represent potential new tools to detect and characterize the impacts of PDBEs on neurodevelopment.

Acknowledgements

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