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Adult Rats Exposed to Low-Doses of Di-*n*-Butyl Phthalate During Gestation Exhibit Decreased Grooming Behavior

Hideo Hoshi · Teruya Ohtsuka

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Abstract Developmental neurotoxicity of low-dose di-*n*-butyl phthalate (DBP) to rats was studied. Pregnant rats were orally given DBP at doses less than 1.0 mg/kg/day during gestation period. The body weight of all dams and their offspring as well as the offspring's motor function showed no significant adverse effect. At 21 weeks, behaviors of male rats were examined by placing into a test cage. The rats born from dams exposed to 10 μg DBP/kg/day exhibited a significant decrease of grooming. This indicates low-dose DBP adversely affects emotional stability in a novel environment.

Keywords DBP · Low-dose effect · Grooming · Rat

Chemicals known as endocrine disruptor can be found in our environment but at much lower than harmful doses (Kavlock et al. 2002; McKee et al. 2004). Bisphenol A, the most well-known endocrine disruptor, has been found to produce adverse effects on the developing endocrine system when exposed to low doses during gestation, generally defined as less than 'no observed adverse effect level' (NOAEL) (vom Saal and Hughes 2005). Adverse effects to rodents have also been found in maternal behavior (Palanza et al. 2002) and the hyperactivity (Xu et al. 2007). Another endocrine disruptor, di-*n*-butyl phthalate (DBP) commonly used as plasticizers and solvents, has also been shown to have adverse estrogenic effects; NOAEL of DBP to rats by oral gavage is 50 mg/kg/day (Mylchreest et al. 2000; McKee et al. 2004; Zhang et al. 2004). To humans, the NOAEL of DBP is 125 mg/kg/day (Dibutyl phthalate CASRN 2009) while DBP concentrations in the environment are about 1 μg/L in drinking water and 0.1–1.5 mg/kg in various foods (Kavlock et al. 2002). Although this amount of DBP showed no adverse effect on the reproductive organs (Mylchreest et al. 2000), little is known about the effect of neuronal development when exposed to low-dose DBP during gestation (Coecke et al. 2007). In this study, we gave DBP to pregnant rats at less than 1/50 of the NOAEL and then investigated any adverse effects on the reproductive organs and the motor activity of their pups. Then we further studied the behaviors of these pups at 21 weeks of age.

Stereotyped behaviors have been studied in rats in response to various drugs and exogenous hormones (D'Aquila et al. 2000; Diaz Heijtz and Castellanos 2006; Matell et al. 2006). Among several behaviors, the grooming is believed to be related to emotional stability, and has been studied by short-term observation. As opposed to the study of acute drug application using established behavior examination techniques, it would be difficult to detect any adverse effects from low-dose exposures. In the present study, we developed a new, inexpensive device for recording the 24-h activity of rats for more than a week. In the actogram of this device, we identified several stereotyped behaviors, as well as measured the duration and frequency of grooming activity. The aim of this study is to assess the adverse effect of low dose DBP during gestation period by simple behavioral measurements.

H. Hoshi
Department of Ophthalmology and Visual Science,
University of Texas at Houston, Houston, TX 77030, USA

T. Ohtsuka (✉)
Neurobiology Laboratory, Biology Research Division,
Graduate School of Sciences, Toho University, 2-2-1
Miyama, Funabashi, Chiba 274-8510, Japan
e-mail: ohtsuka@bio.sci.toho-u.ac.jp

Materials and Methods

Sprague-Dawley (S-D) rats at gestation day (GD) 7 were purchased from Clea Japan (Tokyo). Each pregnant rat was housed in a standard polycarbonate cage (34 × 38 × 18 cm height). The housing room was maintained at 23°C on a 12-h light-dark cycle, with light between 8:00 and 20:00. The animals were allowed to eat and drink distilled water ad libitum. At the end of the behavioral experiment, all animals were anesthetized using diethyl ether inhalation and killed by decapitation; all animals were treated according to the NIH Guide for Care and Use of Laboratory Animals.

The NOAEL of DBP on fertility and reproductive development of S-D rats has been reported as 50 mg/kg/day by gavage (Mylchreest et al. 2000; Zhang et al. 2004). For reducing the stress to pregnant rats, we gave DBP by food. DBP (#021-06936, Wako) was dissolved into sesame oil (#8008-74-0, Sigma) and infiltrated into food pellets (Certified diet MF, Oriental Yeast Corp). Two dams were treated with 10 μg/kg/day (1/5,000 NOAEL), two with 1.0 mg/kg/day (1/50 NOAEL), and two dams received only the sesame oil as a control. All three groups showed no avoidance to the DBP-contained pellets and the vehicle oil. When some leftovers were found in the morning, the amount of new pellets was adjusted to the correct daily amount of DBP. The rats were given the pellets with or without DBP for 2 weeks starting from GD 8 to the date of birth. After birth, the DBP-containing pellets were replaced with untreated ones.

The anogenital distance (AGD), an indicator of feminization, has been measured as a hormonally sensitive development in rodents (Jiang et al. 2007), and thus is one of the testing guidelines for prenatal exposure of phthalate. Both the body weight and AGD of male pups were measured at 4 and 21 weeks of age. Motor function, as well as the vestibular maturation, of all pups was studied during postnatal day (PD) 4–9 by measuring the latency of the righting reflex, the time it took to return to a prone position from a supine position (Palanza et al. 2002). This test was performed once a day for each pup to prevent any adapting or learning effects. After the lactation period of 3 weeks, we randomly chose five male pups born from each dam for further studies.

We developed a device that can record stereotyped rat behaviors including minute grooming activity, 24 h a day for more than a week. This device consists of two parts: a detecting board for vertical movement attached to a connecting lever with a rotational position transducer for isotonic measurement (PK-051, Unicom) and a personal computer (Edi Cube, Epson) for acquiring the data. A rat was placed in a new clean test cage, the same as the housing one, with a water bottle and food pellets, and then the cage was placed on the detecting board. The detecting board was suspended from rubber bands at each of the four corners while the opposite end of the bands was fixed to the top of an iron frame, the test cage on the board was suspended at about 2 cm from the desk. The vertical displacement was calibrated by a known weight placed on the board; a 200 g weight sank the board about 3 mm. By adjusting the elasticity of the rubber bands, the minute deflection caused by animal behavior, such as grooming, could be detected and transferred to the rotational transducer by a connecting lever. We set up six sets of the device and recorded the behavior of six rats randomly chosen from dams exposed to 0, 10 μg, and 1.0 mg DBP/kg/day for a week.

The displacement of each board was transduced into the output voltage of six rotational transducers, which were digitized with the multi-channel A/D board (ADM-682PCI, Microscience), with a sampling frequency of 10 Hz, and stored in a hard disk. The program for A/D conversion and data acquisition was written in Quick BASIC (ver. 4.5, Microsoft). The waveforms caused by rat behaviors were visualized by Excel 2000 (Microsoft). We analyzed grooming behavior of a total of 30 adult male rats of three DBP-treated groups. Each male rat was 21–24 weeks of age and their body weights ranged from 500 to 600 g.

Prior to the quantitative analysis of grooming behavior, we studied the various waveforms on the actogram initiated by different animal behaviors, recorded simultaneously on the videotape. An infrared (IR) video camera (TCD5241BD, Toshiba) was used under IR-LED (SLR-931A, λmax = 945 nm, Sanyo) illumination during the night. We could record a maximum or 27 h of behavior by using a 9 h videotape in each of 3 VCRs controlled sequentially. Later, we studied the relationship between specific waveforms on the actogram and the stereotyped behavior on the videotape. Among the several distinct behaviors, a syntactic chain of grooming showed a thick wave lasting longer than 30 s (Matell et al. 2006). Therefore, we used the thick wave on the actogram lasting longer than 30 s as an indicator of single grooming bout, which was used as an indicator of emotional stability for further analysis.

Data were presented as mean ± standard deviation (SD) all through the text. DBP effects were analyzed by ANOVA followed by post-hoc Dunnett's test. Significance was set less than 0.05.

Results and Discussion

Body weights of the three DBP-treated groups of dams showed no significant difference (Table 1). They consumed almost the same amount of food, about 10% of their

Table 1 Body weight of maternal rats exposed to different doses of DBP (upper table)

DBP (/kg bw/day)	0		10 μg		1.0 mg	
Dam	#1	#2	#3	#4	#5	#6
Body weight (g)						
GD 8	267	242	285	278	257	278
GD 21	408	387	415	396	381	415
Litter male/female	5/8	5/9	7/8	7/6	7/7	8/10

Males (n = 10) from dam	#1 & #2	#3 & #4	#5 & #6
Body weight (g)			
4 weeks	68.6 ± 5.60	75.0 ± 4.40	62.0 ± 15.7
21 weeks	588 ± 39.3	566 ± 29.4	523 ± 28.4
AGD (mm)			
4 weeks	26.6 ± 1.10	24.0 ± 1.13	22.4 ± 1.32
21 weeks	54.5 ± 2.63	53.6 ± 2.02	53.7 ± 1.28

Body weight and AGD of male offspring, five males randomly selected from each dam on the day of weaning (lower table)

body weight every day. The wide range of gender ratios between male and female pups seemed to be caused by a small number of animals. The body weight of male pups showed no significant difference, while the AGD decreased in a dose-dependent manner at 4 weeks. However, when AGD was normalized by the cube root of the body weight (Lee et al. 2006), exposure to DBP did not affect AGD in male neonates. The anti-androgenic effect, the shortening of the AGD, has been shown in male S-D rats born from dams exposed to 500 mg DBP/kg/day, a 10 times higher concentration than the NOAEL (Mylchreest et al. 2000). In the present study, this adverse effect could not be found.

Motor function, as well as vestibular maturation, of all the pups was studied by measuring the latency of the righting reflex. Three groups of pups exposed to 0, 10 μg and 1.0 mg DBP/kg/day showed a similar latency curve among the postnatal days. Some pups exposed to 1.0 mg DBP/kg/day did show a long latency of more than 20 s from PD 4–7, but at PD 8 the latency of these pups matched that of the other two groups. Similar to our results, mouse pups born from dams exposed to low-doses of bisphenol A showed an elongation of latency at an early age (Palanza et al. 2002). This is believed to be related to a decrease in maternal behaviors such as licking or grooming her pups. In our preliminary experiment, we also recognized that one of the specific maternal behaviors, crouching for collecting her pups under the belly, observed during PD 1 to PD 10 decreased 20% in the dam exposed to 1.0 mg DBP/kg/day, or 1/50 NOAEL. We need to further study the maternal behavior from dams exposed to low-doses of DBP and the neuronal development of their pups should be clarified.

Rats placed in a new environment start to explore for about 20–30 min until they stay in one place and start a series of grooming (D'Aquila et al. 2000). Figure 1 shows the actogram of a normal rat during the second day in the test cage. The rat placed in a test cage 1 day prior to the experiment to exclude the initial hyperactivity showed several specific types of waveforms. They were categorized by comparing the distinct behaviors simultaneously recorded with the videotapes. During the daytime, a relatively flat line in the actogram, waveform, indicates that the rat rested for most of the time (Fig. 1A). Occasionally, small deflecting waveforms were made by a slight change of posture, rolling the neck or the body at the same resting position. A thick wave lasting for a minute indicates a grooming bout; a series of scratching the head or the body by the fore- or hind-leg at a frequency of 2–3 Hz. This fast scratching gave a characteristic thick wave on the actogram. Contrarily, during the nighttime, rats moved vigorously just after the cessation of room light at 20:00 and kept moving around (Fig. 1B). Several distinct stereotyped behaviors could be identified in the actogram: moving around, eating the food pellet, drinking water, exploration, and grooming.

An exploration behavior in rodents indicates a 'lack of emotion' (D'Aquila et al. 2000). In the actogram, an exploration behavior often came out with other stereotyped behaviors and a large deflection covered other specific waveforms. Therefore, we studied another emotional indicator, the grooming, which has been used more often in recent studies (Champagne et al. 2004; Kalueff and Tuohimaa 2005; Matell et al. 2006). Ordinarily, a rat stops moving and stays in one place, and then starts grooming for more than 30 s without interruption. Grooming, therefore,

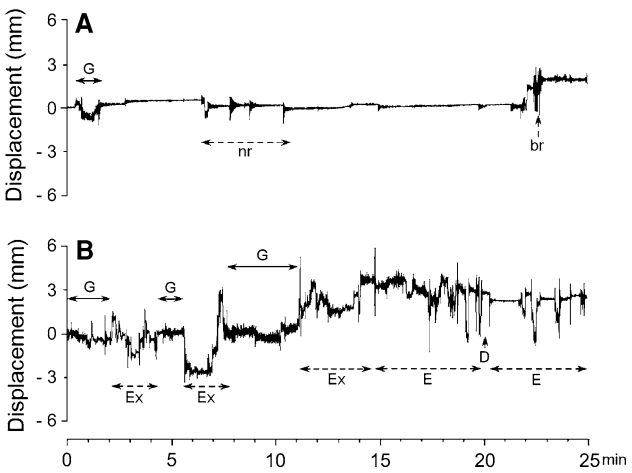

Fig. 1 Actograms of a rat without DBP during the daytime (**A**) and nighttime (**B**). Several waveforms caused by stereotyped behaviors were marked. *D* drinking water, *E* eating food pellets, *Ex* exploration, *G* grooming, *nr* neck-rolling, *br* body-rolling. The ordinate indicates vertical displacement of test cage

exhibits a characteristic long-lasting thick wave without a large displacement (Fig. 1B). Thus, we measured 'the duration of single grooming bout' and counted the number of each bout as 'the frequency of single grooming bout' during the daytime or nighttime.

The transition of emotional behaviors, either by exploration or grooming, of rodents has been analyzed in various studies, including the effects of narcotics and other drugs (D'Aquila et al. 2000; Champagne et al. 2004; Kalueff and Tuohimaa 2005). Grooming, consisting of several microstructures of scratching behavior, could be observed by the naked eye (Matell et al. 2006). Different from these acute drug applications, it would be difficult to study any low-dose effect using a similarly limited observation time. IR cameras can assist the long-term recording of rat behaviors, especially during the nighttime; however, a minute scratching by a hind-leg done behind the body is often difficult to observe. In the present study, we developed a simple recording system to detect especially small but long-lasting vibrations caused by a series of grooming behaviors on the actogram.

Just after the light went on, at 8:00, we placed a male rat from one of the DBP-treated groups in a newly prepared test cage. Figure 2 shows the total time of activity, defined by any deflection in the actogram, during the daytime and nighttime of the rats exposed to different doses of DBP at first 3 days. During the nighttime, the rats, nocturnal animals, kept moving 2–3 times longer than during the daytime. This diurnal difference was consistent for the 5 days of testing. We found that there was no significant difference between the total activity during daytime or nighttime. Any hyperactivity shown in the recent study of low-dose bisphenol A (Xu et al. 2007) was not found.

Once the rats started grooming, the chain of different grooming activities described above lasted about 3 min. The duration of each single grooming bout was a little longer during the nighttime than daytime in all three DBP-treated groups (Fig. 3A–C). We confirmed that the duration of a single grooming bout was unaltered when the rat was placed in the novel environment (Kalueff and Tuohimaa 2005). However, the frequency of individual grooming bouts of all the rats gradually decreased during the first 3 days (Fig. 3D–F). The frequency of individual grooming bouts of the rats exposed to 10 μg DBP/kg/day during gestation was significantly lower, by 25%, at the first day during nighttime (Fig. 3D). The significant decrease, 30%, was also found at the second day (Fig. 3E). Unexpectedly, the rats exposed to the higher concentration of DBP,

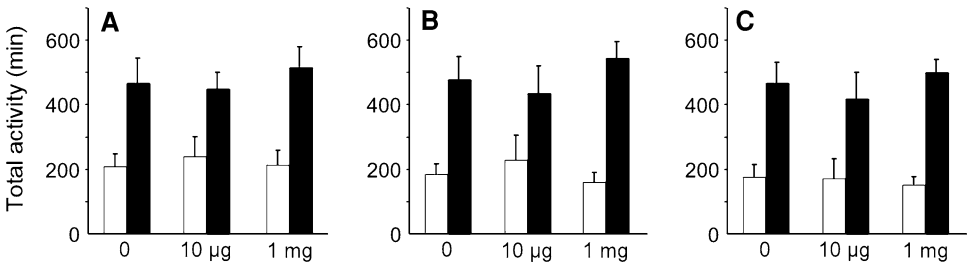

Fig. 2 Total activity of ten adult male rats from dams exposed to different doses of DBP. **A–C** The first to third day after the rat was placed in a test cage. Each *bar* represents the total activity during the daytime (*open bar*) or nighttime (*filled bar*)

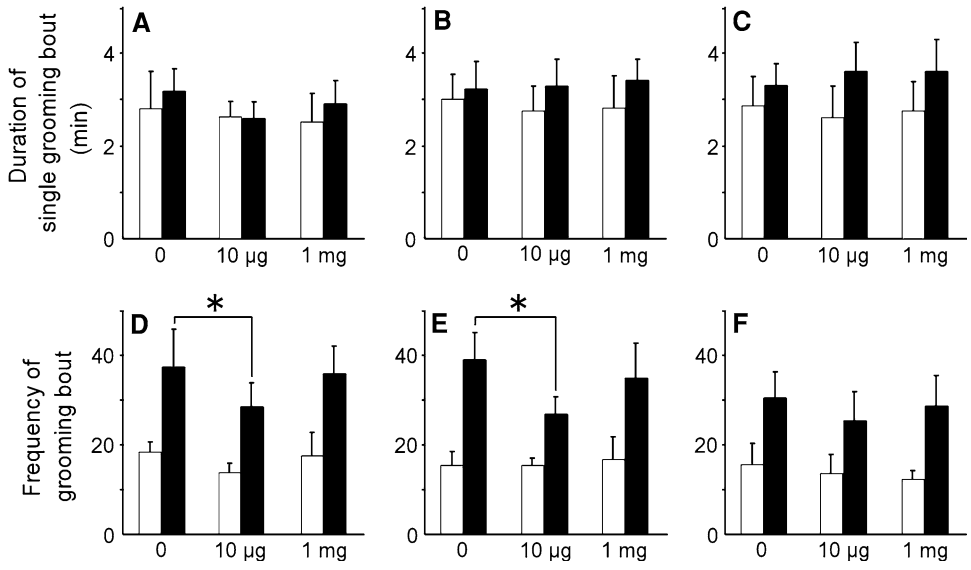

Fig. 3 Grooming behavior of ten adult male rats from dams exposed to different doses of DBP. Each *bar* represents the result during daytime (*open bar*) or nighttime (*filled bar*). The duration of a single grooming bout on the first day (**A**), the second (**B**), and the third (**C**). The frequency of individual grooming bouts, the total number of individual grooming bouts, on the first day (**D**), the second (**E**), and the third (**F**). Symbol * represents significance $p < 0.05$

1.0 mg/kg/day, during gestation showed no decrease in the frequency of individual grooming bouts.

The present study reveals a significant decrease in the frequency of single grooming bouts in rats born from dams exposed to only 1/5,000 of the NOAEL of DBP, however, not to 1/50 of the NOAEL of DBP. This inverted-U dose-response relationship has been shown in the study of prostate weight in response to exposure to both estradiol and diethylstilbestrol as well as in sperm production of male rats born from dams exposed to low-doses of bisphenol A (vom Saal and Hughes 2005). Little is known whether the low-dose of DBP affects on the developmental neurotoxicity (Coecke et al. 2007). It has been shown that dopamine D_2 receptor agonist reduces the grooming time in rats (D'Aquila et al. 2000). While the mesolimbic dopaminergic activity modulated by the dopamine uptake inhibitor seemed to be associated with the pup licking/grooming of lactating rats (Champagne et al. 2004). These studies suggested that low-dose DBP might affect the dopamine D_2 receptor or modulate the maternal behavior which would result the pup's emotional responses.

We found the significant decrease in the frequency of single grooming bouts during the first 2 days after the rat was transferred to a novel environment, specifically a new cage. Therefore, present analysis using the actogram recorded for several days seems to be an appropriate tool for studying the adverse effect on the neuronal development in rats exposed to low-dose of DBP.

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