

胎生期・授乳期のビスフェノールA暴露が マウスの情動行動に及ぼす影響

Behavioral effects of bisphenol A exposure during the embryonic stage and lactation period in C57BL/6J mice

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【日本語要約】

胎児期および乳児期における医薬品や化学物質の暴露が子どもの発達に悪影響を与えることが広く知られている。内分泌機能に影響を及ぼし得る化学合成物質は内分泌かく乱物質(EDCs)と呼ばれており、ビスフェノールA (BPA)は代表的なEDCsの一つである。BPAはポリカーボネートやエポキシ樹脂の原料として利用されているが、プラスチック製品から溶出して体内のエストロゲン受容体に結合し、ホルモン類似作用を生起することが報告されてきた。医薬品のみならず化学物質の生体に対する影響は、容量のみならず暴露される経路や時期、期間など多様な要因の影響を受けるものと考えられる。したがって、単なる影響の有無の検討では、安全な用法・用量あるいは規制に向けた十分な検討が困難である。そこで、EDCsに特化した評価系とともに、他の薬物・医薬品などと比較可能な評価系が必要である。本研究では、胎生期および授乳期におけるBPAの低用量暴露が情動行動に及ぼす影響について、医薬品暴露の評価系と同一の方法で検証を加えた。その結果、BPA暴露条件では仔マウスの体重が有意に増加していた。これは先行研究の結果と一致しており、本研究における暴露方法の妥当性と信頼性を示す結果である。乳幼児マウスにおいては、BPA暴露条件において新奇性テストにおいて若干の変化が見られた。また、成長後はオープンフィールド試験・高架式十字迷路試験・聴覚性驚愕反応および情動記憶テストにおいて変化が見られた。

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*Behavioral effects of bisphenol A exposure during the embryonic stage
and lactation period in C57BL/6J mice*

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Abstract

It is widely known that exposure to some medicines and/or chemical substances during the embryonic stage and lactating period may have adverse effects on offspring development. Environmental pollution due to chemical substances has grown more serious, leading to extensive research. Chemical substances that impact endocrine function are called endocrine disrupting chemicals (EDCs). Bisphenol A (BPA) is an EDC used to produce polycarbonate plastics and epoxy resins. BPA reportedly has hormone-like actions when released from plastics and bound to estrogen receptors. In the present study, the behavioral effects of exposure to BPA during the embryonic stage and lactating period were investigated using a mouse model that was developed to analyze the effects of drug exposure during early development. BPA-exposed mouse pups showed a significant increase in body weight, similar to previous studies. This result indicates the validity and reliability of the mouse model for the present study. BPA-exposed pups exhibited a slight emotional change in the novel object test compared with vehicle-exposed pups. In adulthood, BPA-exposed mice showed some emotional changes in the open field, elevated plus-maze, auditory startle response, and fear conditioning tests. This study showed that low-dose exposure to BPA during early development affects emotional behavior in offspring, but the observed adverse effects were limited.

Key Words: endocrine disrupting chemicals, BPA exposure, emotional behavior, C57BL/6J mice, behavioral toxicity

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I . Introduction

Exposure to some medicines and chemical substances during the embryonic stage and lactating period has adverse effects on the development and behavior of animals, including humans [1-4]. Environmental pollution by chemical substances, such as pesticides, has grown more serious, leading to extensive research [5-7]. Chemical substances that have the capacity to impact endocrine function are called endocrine disrupting chemicals (EDCs) [6, 7]. Bisphenol A (BPA), which is used to produce polycarbonate plastics and epoxy resins, reportedly has hormone-like actions when released from plastics and bound to estrogen receptors [6-9]. Since BPA is an endocrine disruptor, many biochemical investigations based on large-scale epidemiological surveys have shown its adverse effects on the reproductive system [10, 11] and the metabolic function of offspring [9, 12] exposed to BPA *via* the placenta and breast milk. Additionally, many studies have reported that BPA alters cognitive and emotional development and behavior of infants in both laboratory animal experiments [13-16] and human surveys [17-19]. BPA interferes with synaptic remodeling, and thus may cause some mental disorders, such as depression and schizophrenia [20]. Furthermore, EDCs like BPA may adversely affect organisms that are exposed to a much lower concentration than the predicted safe dose, known as the low-dose effect [6, 8].

Despite accumulating evidence showing the adverse effects of BPA, negative results have also been presented regarding reproduction [21], metabolic diseases [22], neural development and behavior [23, 24], and the low-dose effect [23, 25]. Inconsistency among the research results is, to some extent, attributable to the complex interaction among many chemical compounds, as well as the dosage and timing of exposure; the absence of common optimal assays is also a factor. Studies concerning the relationship between BPA exposure and cognitive and emotional development, as well as behavior, have been conducted using an exploratory research strategy with various animal strains (e.g., mice [26], rats [23, 24], zebrafish [27, 28], and rhesus macaque [29]), and a wide range of behaviors [13, 23, 30, 31]. An appropriate assessment, using an identical analysis for comparison with other known hazardous substances, should be required for safety and regulatory purposes.

The present study was conducted in order to elucidate two aims. First, to utilize an established method, which revealed adverse behavioral effects in mice from early exposure to antidepressants [32, 33], to assess the behavioral effects of BPA. Second, to explain the extent to which BPA exposure, during the embryonic stage and lactating period, leads to behavioral changes in infants and adults using a comparable method.

II . Materials and methods

Animals: Six-week-old male (n=24) and female (n=48) C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were acclimated to the breeding room for one week, and then mating was conducted for 10 days (one male and two females per cage). Mouse pairs were randomly divided into two groups: BPA and vehicle. On the afternoon of the 11th day of the mating period, male mice were separated from female mice. Following parturition, mouse pups were randomly assigned into two experimental groups: infant and adult. Mouse pups for the adult experiments were not used in any infant experiments; they were weaned at four weeks of age, and then housed with three to four littermates per cage. At eight weeks, the mice for the adult experiment were individually housed to start the home-cage activity measurement. The schedule for the breeding and the experiments is summarized in Figure 1. The breeding and experiment rooms were air-conditioned ($22 \pm 1^\circ\text{C}$, 50–60% humidity), with a 12 h light-dark cycle (lights on at 0800). Food and water were freely available in the breeding cages. All behavioral experiments, except home-cage activity measurements, were conducted during the light cycle between 1300 and 1700. All experimental procedures were performed in strict accordance with the guidelines of the Institute of Physical and Chemical Research (RIKEN), and were approved by the institute's Animal Investigation Committee.

Drug treatment: For the BPA exposure group, BPA (CAS No. 80-05-7; Wako, Osaka, Japan) was first diluted in ethanol, and then adjusted to 1 ppm (ethanol: 0.01% v/v) by distilled water (Otsuka, Tokyo, Japan). For the vehicle group, 0.01% ethanol (v/v) was added in distilled water. This concentration of BPA (approximately 0.5 to 1.5 $\mu\text{g d}^{-1}$) was comparable to the research investigating “low-dose effect” in a human study [8]. To avoid the EDC contamination from plastic laboratory containers, treatment solutions were prepared using glass containers and supplied through water carriers made of glass and stainless steel. Vehicle solution and BPA solution were presented all day, and solutions and containers were changed daily. Exposure to BPA and vehicle was conducted until weaning (28 days after parturition). The experimental schedule is summarized in Figure 1.

Developmental and behavioral assessment of infants: Developmental and behavioral assessments were started on postnatal day (PD) 5. Each pup was weighed and verified incisor eruption and eye-opening daily. Righting reflex and grasp reflex were verified on PD5. In the righting reflex test, mouse pups were individually placed on their backs with their limbs pointing upwards and the time needed to turn over and resume their normal prone position was recorded (maximum time was set at 30 s) [34]. For the grasp reflex test, the pup’s forelimb was stimulated with a thin wire, and whether the pup grasped it immediately was observed [34]. Click startle reflex was assessed on PD8. An individual pup was placed on the floor of a clear plastic arena (20 × 20 × 20 cm), administered a sharp metallic sound using a dog clicker, and whether pups responded to the stimulus or not was observed [35]. The open field test was conducted on PD13 using an open plastic arena (20 × 20 × 20 cm) with black walls and a white floor. Pups were individually introduced into the center of the arena; their behavior was recorded for three minutes with a charge-coupled device (CCD) camera placed above the arena. The novel object test was conducted on PD18 using the same arena as the open field test. In the novel object test, a small object made of plastic (3 cm diameter × 6 cm tall) was placed in the center of the arena. Pups were individually introduced into one of the four corners of the arena facing the wall; their behavior was recorded for three minutes with the CCD camera (Figure 2). The image data for the open field and novel object tests were analyzed using ANY-maze software (Stoelting, IL, USA).

Behavioral assessment of adults: The groups of mice used in the adult behavior assessment were independent from those used in the infant experiment. The behavioral assessment was started when the mice were eight weeks old. Behavior tasks consisted of home-cage activity measurement and open field, light-dark box, elevated plus-maze, auditory startle response (ASR) and prepulse inhibition (PPI), and classical fear conditioning tests. The methods for these tests were identical to previous studies [32, 33].

Home-cage activity measurement was conducted using a 24-channel activity measurement system (O’Hara, Tokyo, Japan). Image data were collected and analyzed using Image OF4 (open field test), Image LD4 (light-dark box test), Image EPM (elevated plus-maze test), and Image FZC (classical fear conditioning test) (O’Hara, Tokyo, Japan). Mouse Startle (O’Hara, Tokyo, Japan) was used to analyze ASR and PPI test data.

Statistical Methods: Statistical analyses were conducted using SPSSTM version 19 statistical software (Japan IBM, Tokyo, Japan) and Excel Statistics version 5.0 statistical software for non-parametric statistics (Esumi, Tokyo, Japan). A Student’s *t*-test was used to compare the continuous data of the two groups, and a Mann-Whitney U-test to analyze the ratio data. Repeated testing paradigms were analyzed using a repeated measures analysis of variance (ANOVA; general linear model [GLM]). Differences with a $p < 0.05$ were deemed statistically significant.

III. Results

In the present study, 48 females were mated and a total of 167 pups were obtained. Since the C57BL/6J strain of mice were used, litter sizes for both vehicle- and BPA-exposed mouse dams were relatively small (four to 10 pups per mouse dam) [36]. However, there was no abnormality in parturition and nursing behavior in BPA-exposed dams compared with vehicle-exposed dams. Litter size, sex ratio, and gross appearance of BPA-exposed pups did not differ from vehicle-exposed pups. The results of the infant behavioral assessments are summarized in Table 1. Body weight of BPA-exposed pups was significantly higher than vehicle-exposed pups (main effect of drug: $F(1, 49) = 66.178, p < 0.001$; main effect of sex: $F(1, 49) = 0.440, p = 0.510$); *drug* \times *sex* interaction: $F(1, 49) = 1.100, p = 0.299$; male: $t = -5.576, p < 0.001$; female: $t = -5.862, p < 0.001$) (Figure 3A). However, righting reflex ($\chi^2 = 1.856, p = 0.173$), grasp reflex ($\chi^2 = 1.142, p = 0.285$), and click startle response ($\chi^2 = 1.654, p = 0.179$) in BPA-exposed pups were not different from vehicle-exposed pups. Although BPA-exposed pups showed earlier eye-opening than vehicle-exposed pups ($\chi^2 = 26.133, p < 0.001$), no differences were observed in the open field and novel object tests (Table 1). As behavior might be affected by handling and experimental operations [37], the novel object test was replicated with another batch of mice (both male and female, vehicle: $n = 15$; BPA: $n = 24$). For this replicate, the handling procedure was limited to daily rearing management by the institutional animal keeper. Again, there were no significant differences between vehicle-exposed pups and BPA-exposed pups in any indices except jumping. BPA-treated mice showed a statistically decreased number of jumps ($U = 122, p < 0.05$), and smaller number of jumping pups ($\chi^2 = 4.103, p < 0.05$) compared to the vehicle-treated mice (Table 1: novel object test (PD18) replication).

At eight weeks old, the body weight of BPA-exposed mice was still greater than vehicle-exposed mice (main effect of drug: $F(1, 29) = 6.718, p < 0.05$; main effect of sex: $F(1, 29) = 75.021, p < 0.001$; *drug* \times *sex* interaction: $F(1, 29) = 0.340, p = 0.564$; male: $t = -1.388, p = 0.188$; female: $t = -2.318, p < 0.05$) (Figure 3B). The results of adult behavioral tests are summarized in Table 2. There were no remarkable differences between vehicle-exposed and BPA-exposed mice in home-cage activity measurement. In the open field test, BPA-exposed females spent a significantly shorter time in the center than vehicle-exposed females ($t = 3.040, p < 0.01$, Figure 4). On the other hand, BPA-exposed males in the elevated plus-maze test spent a significantly shorter time in open arms than vehicle-exposed males ($U = 6, p < 0.01$). BPA-exposed mice exhibited enhanced ASR (Figure 5A: main effect of drug: $F(1, 29) = 7.636, p < 0.05$; main effect of sex: $F(1, 29) = 22.087, p < 0.001$; *drug* \times *sex* interaction: $F(1, 29) = 1.771, p = 0.589$; male: $t = -4.734, p < 0.001$; female: $t = -0.590, p = 0.563$). However, there were no significant differences in PPI between vehicle-exposed and BPA-exposed mice (main effect of drugs: $F(1, 29) = 0.112, p = 0.740$). In the classical fear conditioning test, BPA-exposed male mice showed enhanced fear response in the context test (Figure 5B: $U = 7, p < 0.05$), but no difference in the cued test ($U = 15, n.s.$).

IV. Discussion

In the present study, the effect of maternal exposure to BPA during the embryonic stage and lactation period on cognitive and emotional behavior of offspring was examined during infancy and adulthood. The amount of residual BPA in the mothers' and offspring's bodies was not confirmed in this study. However, the amount BPA consumed by mothers was estimated approximately 0.5 to $1.5 \mu\text{g d}^{-1}$ (about 15 to $40 \mu\text{g kg}^{-1} \text{d}^{-1}$), and comparable to the dose that may cause "low-dose effect" in human studies [8]. The body weight of BPA-exposed pups was statistically higher than vehicle-exposed pups both in infancy and adulthood (Figure 3), which indicates that the method of exposure and the dose of BPA was effective in this experimental protocol [9, 12].

As in the previous study [24], no obvious developmental abnormalities and disturbance in infant reflexes were observed in BPA-exposed pups in the present study. Although eye-opening of BPA-exposed pups was

earlier than that of vehicle-exposed pups (PD13, Table 1), the relationship between early eye-opening and BPA exposure is unknown at present. The timing of eye-opening could affect visual development [38], and thus may affect exploration and cognitive ability. Therefore, the causal relationship between BPA exposure and early eye-opening should be carefully examined.

In the present study, a systematic behavioral assessment was conducted during infancy. The protocol was comparable to those for adult mice. No obvious behavioral abnormalities in BPA-exposed pups were observed in the open field and novel object tests (Table 1). It is widely known that emotionality of animals should be affected by prior experience [37]. In the present study, mouse pups were repeatedly handled in daily breeding management and experimental operation, and thus the effects of BPA exposure on emotional and/or exploratory behaviors might be concealed under those handling effects. Therefore, the novel object test was replicated using independent groups in which handling procedures were limited to daily rearing management by the institutional animal keeper. In the replication, BPA-exposed pups exhibited a slightly decreased number of jumps and of pups that exhibited jumping behavior compared to vehicle-exposed pups (Table 1: novel object test (PD18) replication). Jumping behavior against the wall of an open field arena is a kind of exploratory and/or stereotypic behavior [39, 40]. Therefore, decreased jumping behavior suggests that BPA exposure leads to emotional disturbance in infancy. Previous studies reported that BPA exposure during the embryonic stage and lactation period leads to behavioral abnormalities like attention deficit/hyperactivity disorder (ADHD) in both animals [31] and human children [17, 18]. The pathology of ADHD is largely divided into two categories: 1) inattention and hyperactivity, and 2) impulsivity [41]. Although BPA-exposed mouse pups did not show hyperactivity in the present study, decreased approaching behavior to the novel object suggests impaired attention in the BPA exposure group. Therefore, the results of the present study might provide new evidence about the relationship between BPA exposure and ADHD. More extensive research using laboratory animals [42], as well as human epidemiological studies, is needed to explain the relationship between prenatal/perinatal exposure to BPA and ADHD.

On the other hand, adult behavioral assessments revealed some changes in anxiety in the open field and elevated plus-maze tests (Table 2). Changes in anxiety have been reported in previous studies, both in animal experiments [13] and human observations [19]. Elevated response to an auditory stimulus was also shown in the ASR test (Table 2). Enhanced startle response has also been previously reported in BPA-exposed animals [14]. In addition, BPA-exposed male mice showed elevated fear response in the classical fear conditioning test (Table 2). These behavioral changes were also observed in studies that exposed infant mice to antidepressants [32, 33], thus early BPA exposure may have a comparable effect to these previous studies.

Some studies reported that there is sexual difference in the effect of BPA exposure on behavior. Decreased exploration, and increased anxiety [13], light/sound aversion, startle response [14], hyperactivity, and spatial memory impairment [15] have been reported in BPA-exposed male rats. On the other hand, sexually dimorphic behaviors such as exploration, anxiety, and response to a novel stimulus were not observed in BPA-exposed female mice [16]. The literature shows that the absence of sexually dimorphic behaviors is attributable to masculinization of females by BPA exposure; however, the effects on emotional and/or cognitive function cannot be fully explained by female masculinization. In the present study, anxiety tended to be elevated in both males (significantly shorter time in open arms in the elevated plus-maze test) and females (significantly less time in the center area in the open field test) exposed to BPA. Thus, early exposure to BPA may affect emotionality, or at least anxiety, which is a sexually independent trait.

In the present study, BPA-exposed males exhibited elevated context fear response in the classical fear conditioning test. A stronger freezing response to conditioned stimuli suggests higher learning or memory capability in animals. However, BPA-exposed males showed an almost identical fear response to vehicle-

exposed males in the cued test (auditory stimulus conditioned to electric foot shock). It is plausible that BPA-exposed male mice elevated their responsiveness to the noxious stimulus, as in the ASR test, rather than learning or memory enhancement.

V. Conclusions

The effects of BPA exposure depend on dosage, timing, animal strains, and other factors [11, 21]; therefore, the discrepancy between previous studies and the present study might be attributable to methodological differences. Thus, the results of the present study are limited and do not necessarily contradict the results from previous studies. This study on BPA used an experimental system that was developed to study the behavioral effects of drug exposure during early development. Importantly, the results of the present study showed the validity of using this experimental system for studying the effects of EDCs even at very low doses. However, the experimental system needs to be refined to verify its reliability and validity. Ultimately, we need more studies that compare the behavioral toxicity of BPA with other drugs or medicines using the same methods.

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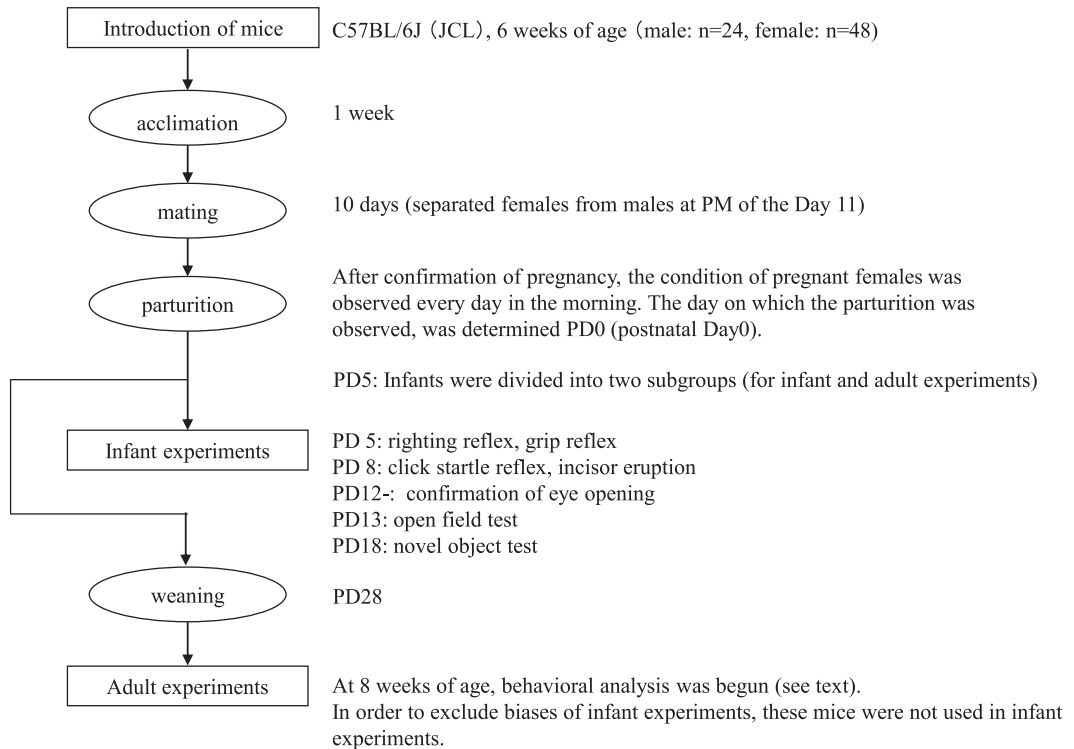


Figure 1 Summary of the experimental schedule

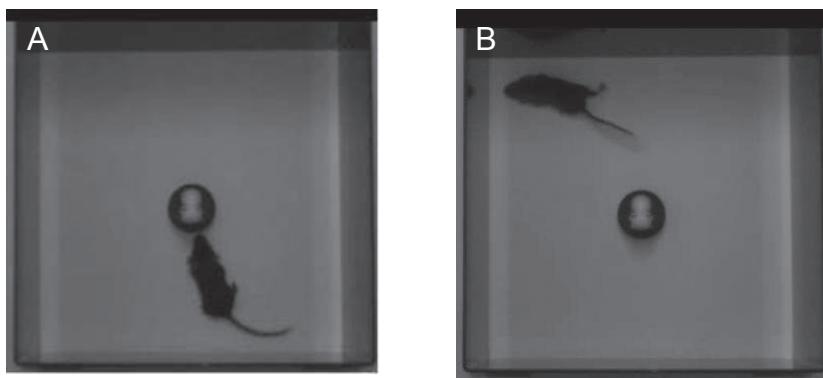


Figure 2 Novel object test in mouse pups (PD18). A small plastic object was placed on the center of the arena. A: Representative approaching and contacting behavior to the object. B: Exploratory running along the wall and preparing to jump against the wall.

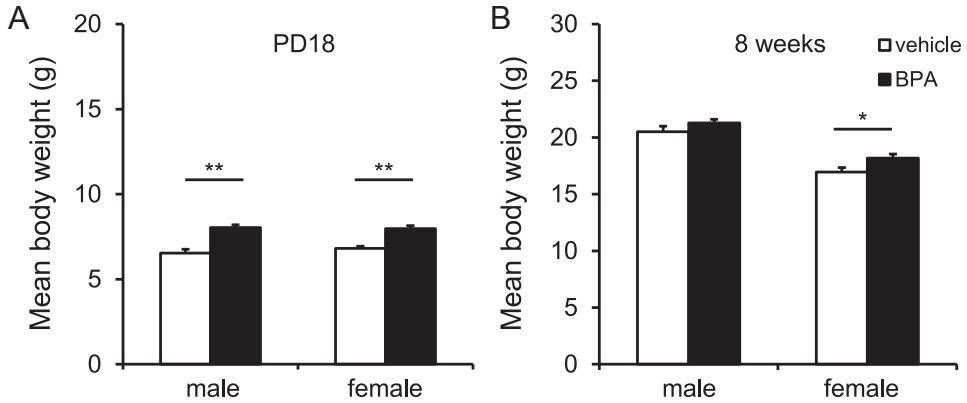


Figure 3 Mean body weight of vehicle- and BPA-exposed mouse pups on PD18 (A), and at 8 weeks of age (B). Data represent mean + SEM. **: $p < 0.01$, *: $p < 0.05$.

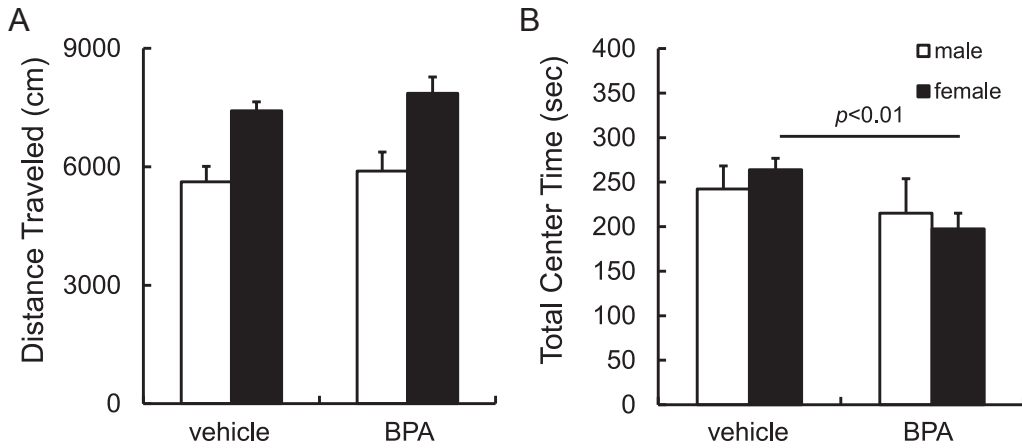


Figure 4 Results of the open field test. Distance traveled (A), and total time spent in center area (B). Data represent mean + SEM.

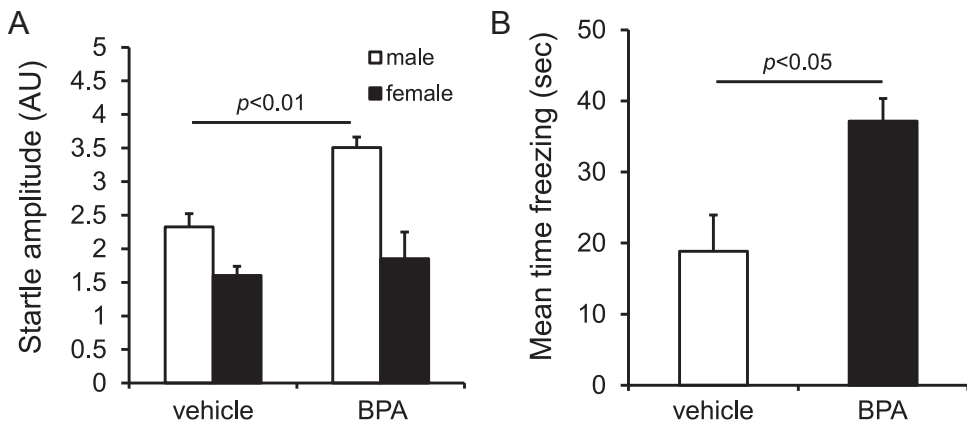


Figure 5 Results of the auditory startle response test and classical fear conditioning test. A: Mean startle amplitude in the auditory startle response test (startle stimulus = 120 dB). B: Mean time freezing in the context test of the classical fear conditioning test. Data represent mean + SEM.

Table 1 Summary of results of infant experiment

category/task	result	statistical value	P-value
<i>infant (-PD18)</i>			
body weight (PD18)	vehicle < BPA	F(1, 4)=66.178	<0.001
	male	t=-5.504	<0.001
	female	t=-5.666	<0.001
righting reflex (PD5)	n.s.	$\chi^2 = 1.856$	0.766
grasp reflex (PD5)	n.s.	$\chi^2 = 1.142$	0.285
click startle response (PD8)	n.s.	$\chi^2 = 1.654$	0.297
eye opening (PD13)	vehicle < BPA	$\chi^2 = 26.133$	<0.001
open field (PD13)			
Distance Traveled	n.s.	t=-0.024	0.981
Time Immobile	n.s.	t=-0.982	0.338
Time Peripheral Zone	n.s.	t=-1.222	0.249
Time Center Zone	n.s.	t=1.222	0.249
novel object test (PD18)			
Time Immobile	n.s.	t=1.204	0.244
Time Head in the Object Zone	n.s.	t=-0.094	0.926
No. in the Object Zone	n.s.	t=-0.659	0.519
1 st Latency to Enter the object Zone	n.s.	t=0.7506	0.619
novel object test (PD18) replication			
mean No. of jumping	vehicle > BPA	U=122	<0.05
No. of pups exhibited jumping	vehicle > BPA	$\chi^2 = 4.103$	<0.05

Table 2 Summary of results of adult test

category/task	result	statistical value	P-value
<i>adult (8 weeks-)</i>			
body weight (8 weeks)	vehicle < BPA	F(1, 29)=6.718	<0.05
	male	t=-1.354	0.204
	female	t=-2.318	<0.05
HC activity open field	n.s.	F(1, 29)=0.378	0.543
Distance Traveled	n.s.	F(1, 29)=0.860	0.361
Time Center Zone	n.s.	F(1, 29)=3.542	0.070
	male	t=0.584	0.570
	female	t=3.040	<0.01
L-D box			
%Distance in Open arena	n.s.	F(1, 19)=0.034	0.855
%Time in Open Arena	n.s.	F(1, 29)=0.202	0.657
Number of Transition	n.s.	F(1, 29)=0.924	0.344
elevated plus maze			
Distance Traveled	n.s.	F(1, 28)=2.288	0.142
%Time in Open Arms	n.s.	F(1, 28)=3.274	0.081
	male	U=6	<0.01
	female	U=26	0.200
%Number Open Arm Entry	n.s.	F(1, 28)=3.451	0.074
auditory startle &PPI			
Auditory Startle Response	main effects of drug	F(1, 29)=7.636	<0.05
	male	t=-4.684	<0.01
	female	t=-0.590	0.569
Prepulse Inhibition	n.s.	F(1, 29)=0.112	0.740
fear conditioning (male only)			
context test	vehicle < BPA	U=7	<0.05
cued test	n.s.	U=15	0.092

