Altered social interactions in male juvenile cynomolgus monkeys prenatally exposed to bisphenol A

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Abstract

Bisphenol A (BPA) is a widespread environmental contaminant, and humans are routinely exposed to BPA. We investigated whether prenatal exposure to BPA influences behavioral development in juvenile cynomolgus monkeys (*Macaca fascicularis*). Pregnant cynomolgus monkeys were implanted with subcutaneous pumps and exposed to 10 µg/kg/day BPA or vehicle (control) from gestational day 20 to 132. Both BPA-exposed and control juvenile monkeys (aged 1–2 years) were assessed using the peer-encounter test that was conducted to evaluate behaviors in social interaction with a same-sex, same-treatment peer. In the encounter test, prenatal BPA exposure significantly reduced *environmental exploration* and *presenting*, a gesture related to sexual reproduction, and increased *visual exploration*, but only in males; furthermore, it significantly reduced the typical sexual dimorphism of the aforementioned behaviors normally observed between male and female juvenile cynomolgus monkeys. This study demonstrates that prenatal BPA exposure affects behavioral development during adolescence and results in the demasculinization of key sexually dimorphic behaviors in male juvenile monkeys.

Key words: bisphenol A; cynomolgus monkey; social behavior; sexual differentiation

1. *Introduction*

Bisphenol A (BPA) is used to make polycarbonate and epoxy resins, resulting in widespread environmental contamination and human exposure. Furthermore, BPA has been detected in the majority of the human population [11,13,54,69]. Several studies have described adverse effects of BPA exposure on the reproductive system [47,67,71], progression of physical puberty [29], adult behaviors [16,40], enhanced risks of obesity [45,64], type 2 diabetes mellitus [4], and tumor development [8]. In addition, adverse effects of pre- and perinatal BPA exposure on brain development [20,26,32,33,42,58,66] and neurobehavioral development [1,2,21,25,35,50,51,74] are of growing concern, among which the disturbance of monoaminergic system development [1,32,35,50] and the lack of brain and/or behavioral sexual dimorphism [21,33,42,58] should be particularly noted. However, some studies argued that there are minimal or no adverse effects of BPA [31,34,60], which leaves room for discussion regarding the risk of BPA exposure on human health and development.

Nonhuman primates such as macaque monkeys have a close evolutionary relationship with humans and have been successfully used in numerous biomedical fields, including research on prenatal exposure to environmental chemicals. The findings obtained with nonhuman primates are expected to fill the gaps in data between rodents and humans. The nonhuman primate model has been used to assess the effects of environmental contaminants, such as dioxin [52], polychlorinated biphenyls (PCBs) [39,48,57,63], methylmercury [12,24,55], and lead [56], on behavioral developments. Moreover, a recent study reported that prenatal BPA exposure has adverse effects on the midbrain dopamine neurons and hippocampus spine synapses in rhesus monkeys [19].

We previously reported that male infant cynomolgus monkeys (aged 2–3 months) prenatally exposed to BPA exhibit female-like behaviors during mother–infant interactions [49], but it is

uncertain whether these behavioral alterations are transient or long lasting. In the present study, animals from the prenatal exposure cohort were tested in the behavioral peer-encounter test that was conducted to evaluate behaviors in social interaction with a same-sex, same-treatment peer [52] as juveniles (aged 1–2 years) to evaluate the long-term effects of developmental BPA exposure on social and nonsocial behaviors.

2. Materials and Methods

2.1 Animals

In this study, the cynomolgus monkeys (*Macaca fascicularis*) were the offspring of 37 hematologically and serologically normal adult females (body weight: 2.5–4.0 kg; age: 5–13 years) (China National Scientific Instruments and Materials Import/Export Corporation, China) who were the same monkeys used in our previous study [49] and were exposed to BPA throughout pregnancy. Animal breeding, mating, and behavioral experiments were performed at the Shin Nippon Biomedical Laboratories (SNBL), Ltd., Japan. All animals were individually housed in stainless cages constructed according to the NIH guidelines (69 × 61 × 75 cm). The environment was maintained at 26 ± 2 °C with humidity of $50 \pm 10\%$ under a 12-h light–dark cycle. All animals received 108 g of food pellets (12 g × 9 pellets) (Teklad Global Certified 25% Protein Primate Diet, Harlan Sprague Dawley, Inc., IN, USA) once daily and water *ad libitum*. All animal cages used in this study were washed daily. Each female monkey with normal menstrual cycles (20–32 days) was caged with a healthy male monkey randomly selected from the breeding colony in SNBL for 3 days during the expected period for ovulation (11–15 days after menstruation) (one male and one female in a cage) and then housed individually. Consequently, no female monkey copulated with the same male monkey. A trained observer confirmed copulation or presence of intravaginal sperm. Gestational day 0 was defined as the median day of the 3-day mating period. The mothers and their infants [delivery day was designated as postnatal day (PND) 0] housed together until weaning at 7–8 months of age. After weaning, juvenile monkeys were housed individually with only visual access to other monkeys. At PND 0 and 28, body weight of offspring was measured after separation from its mother under anesthesia. At PND 196, 360, and 720, body weights of juvenile monkeys were measured under brief restraint in a small transfer cage. It should be noted that the juvenile monkeys used

in this study were the same in our previous study [49] reporting behavioral alterations by prenatal BPA exposure in mother–infant interactions. Details regarding the breeding and outcomes are available in our previous publication [49].

2.2 Chemical treatment

The schedule of BPA administration is shown in Figure 1A. In the present study, BPA exposure began on gestational day 20. Alzet® osmotic pumps (DURECT Corporation, CA, USA), which release a fixed volume of solution (6 μ L/day) for 28 days, were surgically implanted into the dorsal subcutaneous tissue of the mother under light anesthesia (ketamine, 5 mg/kg, im). Eighteen pregnant monkeys received BPA (Tokyo Chemical Industry Corporation, Ltd., Japan) dissolved in a vehicle consisting of N,N-dimethylacetamide (Wako Pure Chemical Industries, Ltd., Japan) and polyethylene glycol (1:1) (Wako Pure Chemical Industries) through the osmotic pumps, and 19 received the vehicle. To administer 10 μ g/kg/day BPA to pregnant monkeys, BPA concentration in the osmotic pumps was calculated before implantation using the following formula: body weight (kg) $\times$ 10 (μ g/kg/day)/6 (μ L). Pumps were replaced every 28 days [on gestational days 48, 76, 104, and 132 (extirpation)] under anesthesia with ketamine. The normal gestational period for cynomolgus monkeys is 160–170 days. Our previous study revealed route dependence [oral vs. subcutaneous (s.c.) administration] and remarkable interspecies differences (rats vs. cynomolgus monkeys) in the bioavailability of BPA [area under the curve for 24 h (AUC_{24h})] [68], in which the ratio of AUC_{24h} in cynomolgus monkeys exposed to BPA at 10 mg/kg by oral gavage to that in rat exposed to BPA in a similar manner was 5.9, and the ratio of AUC_{24h} in monkeys exposed to BPA by s.c. injection of dosage 10 mg/kg to that in monkeys exposed to the same dose of BPA orally was 443.6. In brief, this study indicated that the ratio of the bioavailability in monkeys exposed to BPA subcutaneously to that in rats exposed to BPA orally would be 2000 to 3000 [AUC_{24h} (monkey, s.c., 10 mg/kg)/ AUC_{24h} (rat, p.o., 10

mg/kg)], which indicated that approximately a dosage of 10 μ g/kg/day BPA administered by s.c. injection to monkeys was equivalent to an oral dosage of approximately 5 mg/kg/day in rats. To date, no-observed-adverse-effect-level (NOAEL) of BPA based on the results of studies using rats, which were conducted according to standardized toxicity test guidelines, was 5 mg BPA/kg/day by oral gavage [70]. This dose was used as a cutoff dose for low-dose effects in the National Toxicology Program's report of the endocrine disruptors low-dose peer review [44]. This was the reason why we selected 10 μ g/kg/day BPA in the present study. All experiments were humanely performed in accordance with the guidelines for animal experimentation of SNBL and were approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, University of Tokyo.

2.3 Encounter test

For the encounter test, four male juvenile monkeys from the control group and four male age-matched juveniles from the BPA-exposed group were selected. In a similar manner, five female control and eight female age-matched BPA-exposed juvenile females were selected. The other monkeys born to the vehicle- or BPA-exposed females could not be tested because of age differences. Monkeys were individually housed except for the trial in the encounter test and lived in the same housing area; this allowed them to have visual access. Two monkeys of the same-sex and -treatment group were selected and evaluated on the encounter tests using all possible combinations (a round-robin tournament) at 1 and 2 years of age as previously described [52] with some modifications. Thus, each monkey encountered all other monkeys in turns, both at 1 and 2 years of age. Each monkey experienced the encounter test once a day. The experimental cage for the encounter test ($69 \times 122 \times 75$ cm) (Fig. 2A) consisted of two rooms separated by two overlying removable walls, one made of stainless steel and the other of transparent acrylic. Two monkeys were placed in the cage, one monkey per room. The walls

prevented the monkeys from seeing each other. After a 10-min acclimation period, the stainless steel wall was removed, allowing the monkeys to see each other through the acrylic wall. After 5 min the acrylic wall was also removed, followed by a 10-min encounter. Each monkey's behavior during the test was recorded using a video camera. Two trained observers blinded to the treatment group independently scored the videotapes using Observer 5.0 software (Noldus Information Technology, The Netherlands). Behaviors were classified into the following 17 categories: *aggression*, *contact cling*, *environmental exploration*, *fear grimace*, *inaction*, *lip smacking*, *locomotion*, *outward looking*, *passive contact*, *proximity*, *presenting*, *retreat*, *self-directed behavior*, *social exploration*, *threat*, *visual exploration*, and *vocalization* (Supplementary Table 1). The frequency of each behavior was noted using the one-zero sampling method [5,61]. Briefly, 5 s of each video footage was analyzed and scored (0 = behavior absent; 1 = behavior present), and the mean frequencies calculated by each observer were averaged to yield the final trial scores. The arithmetic mean frequency of each behavior displayed by an individual monkey over a series of encounter tests was used in statistical analyses.

2.4 Statistical analyses

2.4.1 Body weight of offspring

Body weights of offspring were analyzed using a three-way repeated measures analysis of variance (ANOVA) with drug (BPA exposure vs. vehicle) and sex as between-subjects factors and age as a within-subjects factor (Supplementary Table 2).

2.4.2 Encounter test

2.4.2.1 Univariate analysis

The frequencies of the 17 target behaviors (Supplementary Table 1) were first separately analyzed using three-way repeated measures ANOVA (drug and sex as between-subjects factors

and age as a within-subjects factor), where a P -value of $<0.003 [1 - (1 - 0.05)^{1/17}]$ was regarded as the level of significance for each behavior to ensure that the α level of each was 0.05 (Supplementary Table 3). When a significant interaction was observed, an appropriate simple main effect test (two-way interaction) or simple interaction test (three-way interaction) followed by the simple–simple main effect test was performed to further analyze each level (Supplementary Table 3–6).

2.4.2.2 *Multivariate analysis*

Discriminant analysis was performed to reveal the comprehensive effects of BPA exposure on the behaviors identified in the encounter test. Discriminant analysis can find linear combinations of independent variables that maximize the differences among groups. Before performing discriminant analysis, principal component analysis (PCA) was used to determine independent variables [principal components (PCs)] because several of the behaviors observed significantly correlated (Supplementary Table 7), and thereby unsuitable for discriminant analysis. PCA determined six PCs (eigenvalues >1.00) that explained 73.1% of the variance observed in the original data (Table 1 and Supplementary Table 8). In the discriminant analysis, three PCs (PC1, 2, and 3; explaining 51.1% of the variance) were used because the number of variables cannot exceed the size of the smallest group ($n = 4$ for BPA-exposed male offspring). Discriminant analyses provided three discriminant functions (Table 2), but only the first function could be effectively used for discrimination. Finally, the discriminant scores of the first function were analyzed using three-way repeated measures ANOVA and appropriate following tests (simple interaction tests and simple-simple main effect tests) (Supplementary Table 9). All multivariate analyses were performed using SPSS (SPSS Japan Inc., Japan) and StatView® (HULINKS Inc., Japan) software.

3. Results

3.1. Long-term effects of BPA on weight and species-typical behaviors

In the present study, pregnant cynomolgus monkeys were exposed to 10 µg/kg/day BPA or vehicle from gestational day 20 to 132 (Fig. 1A) using subcutaneously implanted pumps. BPA exposure was stopped at 28 days after the final pump was implanted, i.e., gestational day 132. Therefore, offspring were likely exposed to a relatively constant circulating BPA concentration *in utero*. Prenatal BPA exposure did not affect the body weight gain during the suckling period, but it significantly increased body weight of male monkeys at 720 days after birth (aged: 2 years) (Fig. 1B and Supplementary Table 2).

In the encounter test, each monkey encountered another monkey of the same-age, -sex, and -drug treatment group in an experimental cage specifically designed for this test (Fig. 2A). Among the 17 typical behaviors examined (Supplementary Table 1), significant effects of prenatal BPA exposure ($P < 0.003$) were observed in the frequencies of *environmental exploration*, *presenting*, and *visual exploration* (Fig. 2B) (Supplementary Table 3–6). The significant effect for *environmental exploration* was a three-way interaction of BPA exposure $\times$ sex $\times$ age; the significant effects for *presenting* and *visual exploration* were two-way interactions of BPA exposure $\times$ sex (Supplementary Table 3). The frequencies of all other behaviors analyzed are shown in Supplementary Figure 1. The frequency of *environmental exploration* was significantly reduced by prenatal BPA exposure only in males aged 2 years. In addition, the normal difference in the frequency of *environmental exploration* between control males and females aged 2 years [higher in males ($P < 0.003$)] was reversed in BPA-exposed monkeys [lower in males than females ($P < 0.003$)]. Although the frequency of *presenting* was very low when compared with other behaviors, it was significantly reduced in male monkeys ($P < 0.003$) by prenatal BPA exposure. All animals other than control male monkeys hardly showed

presenting. The significant difference in frequency of *presenting* observed between control male and female juveniles ($P < 0.003$) was absent in BPA-exposed monkeys, and this was not related to age. Conversely, the frequency of *visual exploration* in male monkeys was significantly increased by prenatal BPA exposure ($P < 0.003$), and the significant difference between male and female controls ($P < 0.003$) was absent in BPA-exposed monkeys, which was not related to age. To further analyze the behaviors observed in the encounter test, discriminant analysis, a form of multivariate analysis, was applied. As expected, there were moderate, but significant, correlations between some pairs of behaviors such as *social exploration* and *proximity* (positive correlation) and *locomotion* and *inaction* (negative correlation) (Supplementary Table 7). Before performing the discriminant analysis, PCA was performed. PCA produced six PCs (PC1–PC6) that were independent of each other (Table 1 and Supplementary Table 8). The three highest-ranking PCs (PC1–PC3) were used in the discriminant analysis, and three discriminant functions were produced (Table 2). The scores of the first discriminant function were determined to be significantly effective for discrimination ($P < 0.0001$) and these scores were clearly affected by prenatal BPA exposure (Fig. 2C). Discriminant scores were significantly ($P < 0.05$) decreased by prenatal BPA exposure in male monkeys aged 1 and 2 years, and it increased in female monkeys aged 2 years. In addition, the significant ($P < 0.05$) differences in discriminant scores between control male and female monkeys were absent in BPA-exposed monkeys aged 1 year and reversed in BPA-exposed monkeys aged 2 years.

4. Discussion

In the present study, the offspring of pregnant cynomolgus monkeys exposed to BPA throughout gestation were evaluated on a social encounter test as juveniles. We administered BPA in pregnant females on gestational day 20 at 10 µg/kg/day through pumps implanted to produce an exposure regimen with a steady level of circulating BPA without the stress of daily restraint essential for oral administration. As a result, pregnant animals had to be anesthetized four times across pregnancy; although the anesthetization of pregnant dams is not ideal in fetal exposure studies, both control and BPA-exposed monkeys received an identical schedule of sedations. We attempted to measure the circulating BPA concentration in pregnant monkeys on gestational day 50 (exposed to BPA for 30 days) using LC-MS/MS as described previously [68], in which the quantitative limit was 0.1 ng/mL. However, we could not detect BPA, even in BPA-exposed samples in quantitative ranges. Thus, we concluded that maternal plasma BPA levels were as low as 0.1 ng/mL in BPA-exposed monkeys and that those of the offspring would be further lower. If possible, more sensitive methods should be applied to detect BPA of biological samples obtained from the present study. Although no special procedure was undertaken to minimize background contamination of BPA in the present study, the issue of background contamination in the animals, feed, housing quarters, and supplies should be considered carefully in future studies. However, a previous study reported that maternal levels of unconjugated BPA ranged between 0.5 and 22.3 ng/mL in southeastern Michigan mothers [54]. Our data suggested that the monkeys used in this study may be exposed to levels of BPA that were lower than human exposure. As previously reported [49], gross physical abnormalities associated with BPA exposure were not observed during the nursing period. However, at the age of 720 days, prenatal BPA exposure slightly increased the body weight of male juvenile monkeys. Some previous studies reported increased body weight of female mice [29], female

rats [59], and male and female mice [45] after BPA exposure. In this study, because the sample size was relatively small when compared with that of previous studies using rodents, BPA-induced increases in body weight of male juvenile monkeys should have been handled with caution or considered as preliminary data until more large-scale studies are conducted.

In the encounter test, changes in the frequencies of *environmental exploration*, *presenting*, and *visual exploration* were observed in BPA-exposed male monkeys. Although it was almost impossible to clearly characterize behavioral/psychological alterations in BPA-exposed male monkeys at the age of 2 years, the decreased *environmental exploration* (brachial, oral, or pedal manipulation of the physical environment), decreased *presenting*, and increased *visual exploration* in BPA-exposed male monkeys may mean reduced motivation to do something involving physical movements in the encounter test. On the other hand, the expected behavioral differences between males and female juveniles were lacking in BPA-exposed monkeys. It is noteworthy that BPA-exposed males showed female-like behavioral patterns rather than BPA-induced abnormal behaviors in the encounter test.

Multivariate analysis also revealed statistically significant alterations in the behavioral patterns of BPA-exposed male monkeys. Our present and our previous study [49], which reported female-like mother–infant behaviors in BPA-exposed male infant monkeys, provides preliminary but compelling evidence for long-term behavioral effects following prenatal BPA exposure.

In the present study, we used the one–zero sampling method for analyzing typical behaviors expressed in the encounter tests, which counts only the absence (0) or presence (1) of each behavior during 5 s of each video footage and yields only the frequencies (per 120 5-s samples) of behaviors during 600 s (10 min). We also understand that analyzing the duration of each behavior was important. However, in this test, in some cases (or frequently), it was

difficult to define the time of start and end of each behavior because two monkeys were observed from only one direction. Although it is rather troublesome to define the duration objectively for each behavior expressed in this test, further analyses of the duration would be very important in future studies.

Previous studies reported the impairment of sexual differentiation in exploratory behaviors [21] and performance in both the elevated-plus maze test and the forced swim test [30] in BPA-exposed rats. In addition, the normal sex-related differences in the number of corticotropin-releasing hormone-immunoreactive neurons in rat [22], the size of the rat locus coeruleus [32], and the number of tyrosine hydroxylase-positive neurons in mouse brain [58] were lost. More recent studies reported perturbed social behaviors in neonatally BPA-exposed mice [53], and altered anxiety- and depression-like behaviors in perinatally BPA-exposed mice [73]. With the publication of the data described in this study, it is now apparent that BPA disrupts behavioral development across mammals, from rodents to nonhuman primates. In the present study, although social behaviors in BPA-exposed juvenile monkeys *in utero* were investigated, cognitive functions and other important behaviors were not assessed. It is important that those behaviors should also be examined in future studies.

The monkeys used in the encounter test were weaned at the age of 7–8 months and individually-housed after weaning; therefore, they had no previous direct contact with conspecifics (although they could see others in the same room). Weaning and isolation from the mother has potential influence on the neurobehavioral development of juveniles [7,14,62] because maternal care could affect the development of offspring in diverse ways. Group housing is preferable for captive and laboratory monkeys [6] and social interactions, such as tactile contacts [36], are important for normal behavioral development in nonhuman primates [23,41]. Notwithstanding, individual housing was selected for the following two reasons: one was to

avoid physical injuries in the home cage that would alter social behavior and second was to minimize dominant/submissive relationships among the tested monkeys, which would strongly influence social behaviors in the encounter test. It is important to consider the possibility of alteration in behavioral development by the lack of adequate socialization and establishment of dominance hierarchies even in partial social isolation conditions without physical contact.

During the prenatal period, testosterone and estradiol (aromatized from testosterone in the brain) induces cellular responses in neurons that result in sexual dimorphism (i.e., the masculinization of the male brain). In male rhesus monkeys, blocking testosterone accelerates pubertal development [28] and strengthens both click-evoked otoacoustic emissions [43] and spatial memory [27], traits that are considerably greater in females than in males. We suggest that prenatal BPA exposure disrupts normal monkey brain development by anti-estrogenic or anti-androgenic actions, resulting in feminized behaviors in BPA-exposed males. Although BPA has weak estrogenicity *in vitro* and *in vivo* through the classical nuclear receptor pathway [10], other studies have revealed effects of BPA through alternative pathways. BPA has anti-estrogenic [9,76], anti-androgenic [37], and anti-thyroid hormone-like activities [46,75], and it binds strongly to estrogen-related receptor gamma [65]. In addition, recent studies have proposed nongenomic actions of BPA such as rapid extracellular signal-regulated kinase activation in the rat cerebellum *in vivo* [76] and in human breast cancer cells [18] and rapid impairment of intracellular calcium homeostasis in pancreatic α cells [3]. Epigenetic influences as BPA-induced abnormal DNA methylations have also been reported [17,72]. These multifunctional properties of BPA should be considered with regard to any observed adverse effects of BPA exposure *in vivo*.

Although information regarding the effects of BPA exposure on nonhuman primates is limited, a recent study reported the prevention of estradiol-induced synaptogenesis by BPA

exposure in the hippocampus and prefrontal cortex of ovariectomized African green monkeys [38]. Furthermore, prenatal BPA exposure exerted some detrimental impact on the midbrain dopamine neurons and hippocampal spine synapses in rhesus monkeys [19]. To the best of our knowledge, our present and previous study [49] are the only two studies that have assessed the potential effects of BPA exposure on behavioral development in nonhuman primates. As mentioned earlier, juvenile monkeys in the encounter test were the same monkeys used in our previous study [49]. We supplementarily performed correlation analyses between the discriminant score in the encounter test and that in the mother-infant interaction (Supplementary Table 10) and between the behaviors affected by prenatal BPA exposure in the two tests (Supplementary Table 11). Correlation analysis between the discriminant scores in these two tests indicated significant and strong correlations. In addition, significant correlations between the behaviors in the encounter test and those in the mother–infant interaction were observed. For example, *visual exploration* in the encounter test and *outward looking* in the mother–infant interaction showed strong positive correlation (Supplementary Table 11). These analyses suggest long-term behavioral effects following prenatal BPA exposure, and some behavioral characteristics of infant (in the mother–infant interaction) may predict those of the juvenile (in the encounter test). On the other hand, these findings and our previous study simultaneously implied the possibility that BPA directly alters maternal behaviors [15], which influences behaviors in the mother–infant interaction and subsequently those in the encounter test. Further investigations on the effects of prenatal BPA exposure on adult male sexual behaviors are essential.

To increase the reliability of our findings, a large-scale study using experimental monkeys will be necessary. Until such a study is performed, the current results should be interpreted with caution. However, the consistency in the findings of our present and previous study [49]

suggests that the cynomolgus monkey is a useful model for assessing the risk of developmental BPA exposure in humans.

3. *Conclusion*

This study demonstrates that prenatal BPA exposure could affect behavioral development and implied behavioral demasculinization by BPA in male juvenile monkeys.

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Figure Legends

Fig. 1.

Prenatal BPA exposure in cynomolgus monkeys. (A) The schedule of BPA administration and behavioral tests performed on the offspring. (B) Developmental increases in body weights of offspring (mean $\pm$ SD, n = 4/group).

Fig. 2.

Prenatal BPA exposure affected the behaviors of cynomolgus monkeys in the encounter test. (A) The experimental cage for the encounter test. (B) The frequencies of three behaviors (mean $\pm$ SD) (*environmental exploration*, *presenting*, and *visual exploration*) among the 17 identified behaviors that were significantly ($P < 0.003$) affected by prenatal BPA exposure. *: $P < 0.003$. (C) The discriminant scores calculated from PC scores were significantly ($P < 0.05$) affected by prenatal BPA exposure. *: $P < 0.05$.

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Table 1. Principal component analysis synthesized from behaviors in encounter tests.

Principal component (PC)	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	4.105	2.763	1.819	1.559	1.136	1.051
Proportion rate (%)	24.1	16.3	10.7	9.2	6.7	6.2
Cumulative proportion rate (%)	24.1	40.4	51.1	60.3	67.0	73.1

Table 2. Results of discriminant analysis.

	Eigenvalue	PR ^(a) (%)	CPR (%) ^(b)	Wilk's Lambda	df	<i>P</i>	Weighing coefficient in a discriminant function		
							PC1	PC2	PC3
Discriminant function									
#1	3.695	92.3	92.3	0.162	21	0.0000	0.894	0.264	0.426
#2	0.278	6.9	99.2	0.758	12	0.6311	-0.135	0.585	0.088
#3	0.032	0.8	100.0	0.969	5	0.9532	-0.115	-0.103	0.643

^(a) PR: proportion rate of variance

^(b) CPR: cumulative proportion rate of variance

Negishi et al.

Fig. 1.

Fig. 2.