

4, the AGD was measured for pups in each group using calipers with a reproductive precision of 0.01 mm. On PND 21, all pups were weaned and half of the pups in each group (2M = 13, 1M = 37, 0M = 11, 2F = 14, 1F = 43, 0F = 12) were subjected to necropsy, and the testes, epididymides, and prostates with seminal vesicles (fluid was not removed and all lobes were included) in males, and uteri and ovaries in females, were weighed. For the remaining male and female pups in each litter (2M = 21, 1M = 32, 0M = 30, 2F = 23, 1F = 36, 0F = 26), as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 28), and preputial separation for males (beginning on PND 35), were assessed, and each rat was weighed when these criteria were achieved.

2.2.2.2. Postweaning tests of behavior, evaluation of estrous cycle, and histological observation of reproductive organs.

One male and one female were randomly selected from each litter in each group (number of rats examined: 2M = 18; 1M = 27; 0M = 25; 2F = 17; 1F = 27; 0F = 25), and were subjected to an open field test and wheel cage activity test to assess the emotionality and regulatory running activity, respectively. At 4 weeks of age, the rats were placed into a circular area (140 cm in diameter) surrounded by a wall (40 cm in height). The light and noise levels averaged 500 lx and 50 dB, respectively, at the center of the circular area. Rearing, grooming, defecation, and urination were counted, and ambulation was recorded automatically on a computer (Unicom, Inc., Japan), during a 3-min trial between 13:00 and 16:00 h on one day. At 7 weeks of age, the rats were placed into a wheel cage (Nippon Cage, Inc., Japan), 32 cm in diameter and 10 cm in width, as a measure of spontaneous activity. Each rat was kept within the wheel for 24 h with free access to food (NIH-07-PLD) and distilled water in the same animal room. The number of revolutions was automatically recorded with a 20-channel digital counter (Seiko Denki, Inc., Japan).

Each morning (9:00–10:00 h), from 6 to 10 weeks of age, all females in each group were subjected to vaginal lavage. The lavage fluid was applied to a glass slide, air-dried, and stained with Wright–Giemsa stain. Cytology was evaluated and the stage of the estrous cycle was determined using the method of Everett [19].

At 10 weeks of age, 3–5 males in each group were weighed, and anesthetized. Transcardial perfusions were carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the prostate gland was sampled, rinsed three times in phosphate buffer, postfixed for 2 h at 4 °C in 2% osmium tetroxide, and dehydrated in alcohol; the prostate gland was embedded in epoxy resin. Ultrathin sections of the prostates were stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7100, Hitachi, Japan). The remaining males in each group (2M = 18, 1M = 27, 0M = 25) were weighed and subjected to necropsy, and the testes, epididymides, ventral prostate, and

dorsal prostates with seminal vesicles, were weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. All females (2F = 17, 1F = 27, 0F = 25) were weighed and subjected to necropsy when the stage of the estrous cycle was diestrus. The ovaries and uteri were then weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. These reproductive organs were embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.3. Experiment II (examination of low-dose in utero effects of 17 β -estradiol in mice)

The objective of this experiment was to determine whether the intrauterine position of male fetuses, which is related to background levels of estradiol (elevated in males located between two female fetuses) and testosterone (elevated in males located between two male fetuses), would influence the response of the developing prostate to low dose 17 β -estradiol. In addition, we examined whether the intrauterine position of male and female fetuses would affect the postnatal growth of other reproductive organs and sexual maturation.

2.3.1. Administration, cesarean delivery and fostering

Thirty female mice at 9 weeks of age were administered 17 β -estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μ g/kg per day for 7 days before mating, during a mating period of 7 days at the longest, and on day 0 through 17 of gestation. In a preliminary study, the offspring of the ICR pregnant females exposed to 17 β -estradiol at 0.05 μ g/kg per day on day 0 through 17 of gestation showed no changes in weight and histological morphology of reproductive organs in adulthood. However, the offspring of dams exposed to 17 β -estradiol at 0.1 μ g/kg per day on these gestational days showed changes in the parameters in adulthood (data not shown). In the present study, 30 control females were administered corn oil (Nacalai Tesque, Co., Tokyo). After the administration for 7 days before mating, female mice were caged with untreated males overnight and examined for a vaginal plug the next morning. The day on which a plug was found was termed day 0 of gestation. In this study, 30 female mice in the 17 β -estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO₂ asphyxiation, and subjected to cesarean sectioning. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed, and then the AGD was measured. The fetuses obtained by cesarean delivery were fostered to 60 dams that had just given birth naturally (one litter to each female). The day of cesarean section was considered as PND 0. Pup body weights were recorded on PND 21 (day of weaning), and at 5, 7, and 10 weeks of age.

Neonates from 30 pregnant females exposed to corn oil and 30 pregnant females exposed to 17 β -estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17 β -estradiol exposed group and the control group: 38 and 41) and 0M (33 and 32), and 2F (41 and 32) and 0F (28 and 37). Fetuses adjacent to dead embryos, and fetuses that were closest to each ovary or the cervix, were discarded. In this experiment, fetuses of two intrauterine positions, 1M and 1F, were also discarded.

2.3.2. Observations of postnatal growth

2.3.2.1. Evaluation of sexual maturation. On PND 21, all male and female pups (2M, 0M, 2F, 0F) in each litter were weaned. For all male and female mice in each litter, as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 25), and preputial separation for males (beginning on PND 30), were assessed, and each pup was weighed when these criteria were achieved.

At 10 weeks of age, five males in each group were weighed and processed to the transcatheter perfusion to observe the histological alteration of the prostate by electron microscope. The remaining males in each group were weighed and subjected to necropsy, and the testes, epididymides and seminal vesicles, were weighed. All females were weighed and subjected to necropsy. The ovaries were then weighed. These reproductive organs including prostates and uteri were fixed in 0.1 M phosphate-buffered 10% formalin solution and embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.4. Data analyses

Statistical analysis of the data for the offspring (AGD, body weight and organ weight, organ/body weight ratios, timing of vaginal opening and preputial separation) was per-

formed using the litter as the unit [20,21]. The AGD, body weight and organ weight, organ/body weight ratios (relative organ weight), timing of vaginal opening and preputial separation, were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect the significances among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, or there was a group whose variance was zero, Kruskal–Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. Comparisons between groups were made using $P \leq 0.05$ as the level of significance.

3. Results

3.1. Experiment I

3.1.1. AGD and body weights of fetuses at cesarean section and pups at PND 4

Table 1 shows the AGD, body weight, AGD/body weight (AGDI: anogenital distance index), and AGD/ $\sqrt[3]{\text{body weight}}$ of fetuses at various intrauterine positions and pups at PND 4. It is reasonable to anticipate that the AGD might vary with body weight of fetus or pup. It has been proposed that the relationship between AGD and body weight should be more properly evaluated using the cube root of the body weight [22–25]. If it is desirable to normalize AGD to body weight, the AGD/ $\sqrt[3]{\text{body weight}}$ seems to provide a more appropriate adjustment.

There were no statistically significant differences in any parameter evaluated at cesarean section (PND 0) or PND 4

Table 1
Effects of prior intrauterine position on anogenital distance in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
AGD of fetuses at cesarean section						
No. of litters	19	27	24	18	29	27
No. of pups	36	73	43	38	83	41
Body weight (g)	5.6 \pm 0.4 ^a	5.6 \pm 0.3	5.7 \pm 0.4	5.2 \pm 0.3	5.4 \pm 0.3	5.3 \pm 0.4
AGD	2.43 \pm 0.22	2.42 \pm 0.22	2.42 \pm 0.28	1.21 \pm 0.20	1.23 \pm 0.19	1.22 \pm 0.24
AGD/body weight	0.43 \pm 0.04	0.42 \pm 0.04	0.42 \pm 0.05	0.23 \pm 0.02	0.22 \pm 0.02	0.23 \pm 0.02
AGD/ $\sqrt[3]{\text{body weight}}$	1.36 \pm 0.12	1.36 \pm 0.14	1.35 \pm 0.18	0.69 \pm 0.08	0.70 \pm 0.07	0.70 \pm 0.08
AGD of pups on PND 4						
No. of litters	19	27	24	18	29	27
No. of pups	34	69	41	37	79	38
Body weight (g)	10.9 \pm 1.5	11.2 \pm 1.5	10.8 \pm 1.1	10.4 \pm 1.4	10.3 \pm 1.1	10.4 \pm 1.3
AGD	4.57 \pm 0.54	4.41 \pm 0.48	4.43 \pm 0.51	2.00 \pm 0.22	1.99 \pm 0.19	2.00 \pm 0.21
AGD/body weight	0.42 \pm 0.06	0.40 \pm 0.05	0.41 \pm 0.03	0.19 \pm 0.04	0.19 \pm 0.03	0.19 \pm 0.04
AGD/ $\sqrt[3]{\text{body weight}}$	2.06 \pm 0.22	1.99 \pm 0.19	1.99 \pm 0.19	0.92 \pm 0.12	0.91 \pm 0.11	0.92 \pm 0.13

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean \pm S.D.

Table 2
Effects of prior intrauterine position on reproductive organs before maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight on PND 21						
No. of litters	18	27	24	17	27	25
No. of offspring	13	37	11	14	43	12
Body weight (g)	40.9 ± 6.3 ^a	40.5 ± 6.5	40.2 ± 9.6	38.8 ± 6.3	38.2 ± 7.0	40.7 ± 7.0
Testes (mg) ^b	169.3 ± 27.5	172.2 ± 22.2	164.9 ± 26.1			
Testes ^c	416.3 ± 48.4	429.2 ± 40.3	418.2 ± 46.7			
Epididymides (mg) ^b	23.3 ± 3.1	23.5 ± 4.8	21.9 ± 4.4			
Epididymides ^c	60.5 ± 10.8	58.2 ± 7.8	55.2 ± 7.0			
Prostate + SV (mg) ^{b,d}	47.2 ± 9.9	46.7 ± 10.3	45.9 ± 7.9			
Prostate + SV ^{c,d}	115.6 ± 18.0	115.7 ± 19.0	117.2 ± 19.5			
Ovaries (mg) ^b				24.3 ± 4.0	22.9 ± 3.9	24.8 ± 3.6
Ovaries ^c				63.5 ± 10.1	60.8 ± 9.5	61.5 ± 7.3
Uterus (mg) ^b				10.2 ± 2.0	11.2 ± 3.7	11.8 ± 2.9
Uterus ^c				26.4 ± 4.5	28.8 ± 6.8	29.1 ± 6.0

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

^b Absolute weight.

^c Relative weight (g or mg per 100 g body weight).

^d Seminal vesicle.

between groups 2M, 1M and 0M in males, or groups 2F, 1F and 0F in females.

No significant differences in viability of fetuses at cesarean section (PND 0), or that from PND 0 to PND 4 (the number of pups died; 2M = 2, 1M = 4, 0M = 2, 2F = 1, 1F = 4, 0F = 3), were detected between the groups. In addition, there were no statistically significant differences in body weight at PND 0 and 4.

3.1.2. Body weight and reproductive organ weight of offspring at PND 21

The absolute and relative weights of testes, epididymides, and prostates with seminal vesicles in males, and ovaries and uteri in females, as well as body weight of offspring at PND 21 are shown in Table 2. Irrespective of the intrauterine position, no significant differences were

detected between the groups in absolute or relative reproductive organ weights, or body weights of male and female weanlings, suggesting that the intrauterine position did not affect postnatal growth before weaning in rats.

3.1.3. Sexual maturation and estrous cycle of offspring

Table 3 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups. The estrous cycle of female offspring from 6 to 10 weeks of age is shown in Table 4. No significant differences were detected between the groups in mean estrous cycle length, or the frequency of females showing each stage of estrous cycle.

Table 3
Effects of prior intrauterine position on sexual maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
No. of litters	18	27	24	17	27	25
No. of offspring	21	32	30	23	36	26
Day of preputial separation	43.3 ± 1.3 ^a	43.4 ± 1.2	44.0 ± 1.8			
Body weight (g) ^b	211.8 ± 5.5	212.1 ± 4.3	212.9 ± 5.2			
Day of vaginal opening				33.8 ± 2.2	33.8 ± 1.8	34.1 ± 1.7
Body weight (g) ^b				125.6 ± 4.1	124.6 ± 4.4	126.1 ± 3.9

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

^b Body weight when the criterion was achieved.

Table 4
Effects of prior intrauterine position on estrous cycle in Sprague–Dawley rats

	Group		
	2F	1F	0F
No. of litters	17	27	25
No. of female offspring	23	36	26
Mean estrous cycle length (day) ^a	4.16 ± 0.29 ^a	4.08 ± 0.30	4.20 ± 0.42
No. of females showing Regular cycle (%)	18 (78.3)	28 (77.8)	21 (80.8)
No. of females showing Irregular cycle (%)	5 (21.7)	8 (22.2)	5 (19.2)

2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses. No significant differences were observed between groups.

^a Mean ± S.D.

3.1.4. Behavior and locomotor activity of offspring

Table 5 shows the results of an open field test at 4 weeks of age, and spontaneous activity within the wheel for 24 h at 7 weeks of age, for male and female offspring. There were no significant differences between groups 2M, 1M and 0M in latency, ambulation, rearing, grooming, defecation and urination, or number of revolutions for 24 h in a wheel cage. In the females, urination in group 0F was significantly increased as compared with that in group 2F, whereas other behavioral parameters, including the number of revolutions in a wheel cage were comparable between groups 2F, 1F and 0F.

3.1.5. Weights and histology of reproductive organs of offspring in adulthood

Table 6 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed

in any of the reproductive organs, including the prostates, of the offspring.

3.2. Experiment II

3.2.1. AGD and body weights of fetuses at cesarean section

Table 7 shows the body weight, AGD, AGD/body weight, and AGD/ $\sqrt[3]{\text{body weight}}$, of embryonic day 18 (PND 0) fetuses exposed to corn oil or 17 β -estradiol. There were no significant differences in any of the parameters between the groups. No significant differences in viability of fetuses at cesarean section, or that from PND 0 to PND 21 were detected between the groups (the number of pups died from PND 0 to PND 21: see Tables 7 and 8).

3.2.2. Sexual maturation of offspring

Table 8 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups.

Table 5
Effects of prior intrauterine position on postnatal behavior in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Open field						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Latency (s)	20.4 ± 40.8 ^a	17.9 ± 16.9	15.3 ± 16.2	12.0 ± 9.4	13.8 ± 12.3	16.9 ± 36.1
Ambulation (cm)	676.3 ± 411.3	627.1 ± 417.2	659.0 ± 501.9	940.6 ± 538.1	1039.8 ± 436.3	970.7 ± 449.8
No. of rearing	2.3 ± 3.1	3.0 ± 3.2	1.5 ± 1.4	3.5 ± 2.1	4.5 ± 3.4	3.8 ± 2.3
No. of grooming	0.6 ± 0.9	0.7 ± 0.7	1.1 ± 1.2	0.8 ± 0.9	0.4 ± 0.5	0.8 ± 0.8
No. of defecation	2.8 ± 1.9	2.1 ± 1.5	3.3 ± 2.2	1.9 ± 1.9	1.7 ± 1.8	1.8 ± 1.9
No. of urination	0.4 ± 0.6	0.4 ± 0.6	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.7 ± 0.6 ^{**}
Spontaneous activity						
Count/24 h	1547 ± 467	1789 ± 697	1559 ± 638	4107 ± 1140	4429 ± 1501	4746 ± 1831

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

^{**}Significantly different from group 2F, $P < 0.01$ (by multiple comparison and Student t -test).

^a Mean ± S.D.

Table 6
Effects of prior intrauterine position on reproductive organs after maturation in Sprague-Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight at 10 weeks old						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Body weight (g)	417.2 ± 31.6 ^a	416.1 ± 34.4	413.6 ± 36.9	270.0 ± 23.2	271.8 ± 28.9	273.6 ± 29.1
Testes (mg) ^b	3.00 ± 0.20	2.98 ± 0.15	3.00 ± 0.17			–
Testes ^c	0.72 ± 0.05	0.72 ± 0.06	0.73 ± 0.07			–
Epididymides (mg) ^b	0.77 ± 0.05	0.78 ± 0.07	0.76 ± 0.06			–
Epididymides ^c	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.02			–
Ventral prostate (g) ^b	0.46 ± 0.08	0.44 ± 0.08	0.43 ± 0.10			–
Ventral prostate ^c	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.03			–
Dorsal prostate (g) + SV ^{b,d}	1.53 ± 0.28	1.56 ± 0.24	1.52 ± 0.27			–
Dorsal prostate + SV ^{c,d}	0.37 ± 0.07	0.38 ± 0.05	0.37 ± 0.07			–
Ovaries (mg) ^b				92.6 ± 13.3	91.8 ± 13.7	95.4 ± 16.9
Ovaries ^c				34.3 ± 3.6	33.8 ± 3.4	35.0 ± 5.8
Uterus (g) ^b				0.36 ± 0.06	0.38 ± 0.06	0.38 ± 0.05
Uterus ^c				0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.02

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

^b Absolute weight.

^c Relative weight (g or mg per 100 g body weight).

^d Seminal vesicle.

Table 7
Effects of prior intrauterine position on anogenital distance in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	41	32	32	37	38	33	41	28
Body weight (g)	1.41 ± 0.08 ^a	1.42 ± 0.05	1.32 ± 0.05	1.33 ± 0.04	1.42 ± 0.09	1.41 ± 0.10	1.32 ± 0.07	1.30 ± 0.11
AGD	1.92 ± 0.07	1.90 ± 0.06	0.95 ± 0.02	0.95 ± 0.03	1.92 ± 0.08	1.93 ± 0.06	0.93 ± 0.09	0.95 ± 0.05
AGD/body weight	1.36 ± 0.09	1.35 ± 0.10	0.75 ± 0.03	0.73 ± 0.05	1.40 ± 0.09	1.38 ± 0.10	0.75 ± 0.05	0.74 ± 0.07
AGD/ $\sqrt[3]{\text{body weight}}$	1.71 ± 0.07	1.70 ± 0.10	0.88 ± 0.03	0.89 ± 0.05	1.70 ± 0.09	1.72 ± 0.11	0.89 ± 0.07	0.88 ± 0.08

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

Table 8
Effects of prior intrauterine position on sexual maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	39	30	31	35	37	31	39	28
Day of preputial separation	27.2 ± 1.5 ^a	27.3 ± 1.3			27.0 ± 1.8	26.9 ± 2.0		
Body weight (g)	30.3 ± 1.9	31.1 ± 1.5			30.0 ± 2.1	31.3 ± 1.8		
Day of vaginal opening			24.5 ± 1.6	25.1 ± 1.5			24.4 ± 1.7	24.9 ± 1.6
Body weight (g)			21.5 ± 0.9	21.6 ± 1.2			21.6 ± 1.1	22.0 ± 1.5

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

Table 9
Effects of prior intrauterine position on reproductive organs after maturation in ICR mice exposed to 17 β -estradiol

Treatment and intrauterine position	Corn oil				17 β -Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	34	25	26	30	32	26	34	23
Terminal body weight (g)	51.3 \pm 4.2 ^a	53.6 \pm 4.4	40.3 \pm 2.7	41.2 \pm 3.4	55.1 \pm 5.1	53.1 \pm 6.2	41.8 \pm 2.1	42.1 \pm 3.1
Testes (g)	257.9 \pm 28.4 ^b	266.5 \pm 23.5			259.3 \pm 25.5	260.3 \pm 19.9		
	486.3 \pm 76.3 ^c	484.2 \pm 71.3			488.3 \pm 62.3	479.1 \pm 60.9		
Epididymides (mg)	89.5 \pm 8.7	92.2 \pm 9.3			94.6 \pm 7.1	93.1 \pm 7.1		
	175.2 \pm 21.5	161.9 \pm 16.9			161.2 \pm 13.6	173.6 \pm 4.9		
Seminal vesicle (mg)	413.9 \pm 30.6	452.2 \pm 13.9			431.3 \pm 18.2	454.8 \pm 21.0		
	812.8 \pm 56.9	802.6 \pm 44.2			811.5 \pm 42.3	809.3 \pm 33.5		
Ovary (mg)			15.3 \pm 3.3	14.6 \pm 4.2			15.5 \pm 3.9	15.3 \pm 4.5
			35.6 \pm 7.5	33.2 \pm 4.6			34.1 \pm 6.9	33.6 \pm 5.1

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.

Five males in each group were processed to the transcardial perfusion. Male pups shown here were subjected to necropsy.

No significant differences were observed between groups.

^a Mean \pm S.D.

^b Absolute weight.

^c Relative weight (mg per 100 g body weight).

3.2.3. Weights and histology of reproductive organs of offspring in adulthood

Table 9 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed.

4. Discussion

4.1. Anogenital distance

The AGD of newborn rats and mice is longer in males than in females, and it has been demonstrated that the AGD varies as a function of the intrauterine position of the animals [1–4]. The AGD is commonly regarded as a hormonally sensitive developmental measure in rodents [26], and it has been reported that a longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus [27,28]. Evidence supports the hypothesis that exposure to testosterone and estrogen in utero are critical components of the intrauterine position effect [29]. Female mouse fetuses located between two males have significantly higher serum testosterone levels and lower estradiol levels than their sisters that were located between two females. Male mice located between two females have significantly higher levels of estradiol and lower levels of testosterone than males located between two

males [4,12]. The mechanism for these intrauterine position effects can be traced to amniotic fluid transport between adjacent fetuses in uterus [30,31]. However, our data were not consistent with previous reports showing a significant effect of intrauterine position on AGD in rats and mice [2,32–34].

A failure to replicate the effects of intrauterine position on AGD may have potentially arisen for a number of methodological reasons. A set of potential problems revolves around possible errors in the measurement of the AGD. One possibility was that our calipers were not accurate enough to detect small mean differences between females located in various positions in the uterus, found by other investigators [2,32–34]. However, as the calipers could be read to an accuracy of 0.01 mm, they were clearly accurate enough to detect differences of this magnitude. Another possibility is that of human error. Given the short distances being measured, it was absolutely essential that all fetuses or pups be oriented in exactly the same fashion, as even a slight arching of the animal's back could significantly distort the AGD measurements. Two attempts were made to minimize these sorts of errors: (i) efforts were made to orient all fetuses or pups in exactly the same fashion when measuring, and (ii) two independent measurements were taken for each fetus or pup and averaged to obtain the value used. In most cases, the different measurements were highly similar for the same animal.

Simon and Cologer-Clifford [35] reported an absence of an intrauterine position effect on AGD in CF-1 mice. Their finding is only the second study to examine AGD in CF-1 mice, and the original report was more than 10 years old [2]. Therefore, it is possible that either genetic drift, or differences in the source of the CF-1 breeding stock, may

underlie the discrepant findings. In this context, Jubilan and Nyby [6] also found no effect of intrauterine position on the AGD/body weight (AGDI) in CF-1 offspring, using stock from the same supplier employed by the Simon and Cologer-Clifford [35] report.

4.2. Sexual maturation and estrous cycle

Since prenatal exposure of females to testosterone delays vaginal opening [36,37], it was predicted that females situated proximate to males in utero would display vaginal opening later than females not proximate to males during gestation. However, in the present study in rats and mice, there were no significant differences in days of vaginal opening or preputial separation between the groups (see Tables 3 and 8), suggesting that intrauterine position did not influence the sexual maturation in males and females. vom Saal [4] reported that 2F and OF mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than OF (see categorization of the different intrauterine positions shown in Section 2).

Female mouse fetuses occupying an intrauterine position between male fetuses exhibit longer estrous cycles in adulthood than females formerly residing in utero next to other female fetuses [11,27]. Prior intrauterine position is therefore a source of individual variation in the production of, and sensitivity to, cues that modulate the timing of puberty and the length of subsequent estrous cycles in female mice, suggesting that prenatally androgenized females occupying an intrauterine position between male fetuses may have a reproductive advantage over other females at high population densities [4]. In the present study of rats, however, OF and 2F did not differ significantly in the estrous cycle length, although the estrous cycle length of 2F (4.16 ± 0.29) tended to be shorter than that of OF (4.20 ± 0.42). Prior studies have shown that, in the absence of males, vaginal estrus does not correlate with ovulation in peripubertal CF-1 female mice [38,39]. Further studies in which ovulation is confirmed by the presence of corpora lutea and tubal ova is thus required.

4.3. Behavior

Kinsley et al. [40] demonstrated that female mice located in utero between two female fetuses exhibited higher levels of regulatory running activity (locomotor activity) in adulthood than females located between two male fetuses. Male mice, which were less active than females, were also influenced by intrauterine contiguity, indicating that intrauterine position influences the behaviors involved in the maintenance of metabolic homeostasis. Previous work has shown that female rats and mice display higher levels of regulatory running activity than males, and that perinatal testosterone is responsible for this sex difference [41–43]. The present study also showed female rats displayed higher levels of running activity than males.

In the present study of rats, however, there were no significant differences in spontaneous activity in the wheel cage, or in ambulation in the circular area, as well as the frequency of rearing, grooming and defecation between the groups in both sexes, suggesting no intrauterine position effects on locomotor activity in rats. Interestingly, the frequency of urination for females that developed in utero between male fetuses was significantly increased, more than in females that developed in utero between female fetuses (see Table 5). Females that were located between female fetuses in utero were found to urine mark at higher rates than females that were located between male fetuses, in adulthood in CF-1 mice [2]. The frequency of urination in the circular area, observed in the present study, would relate to the emotionality of the animals when placed in a novel environment, and differ from urine marking. Female urine marking may play an important role in communication between female mice, as well as in inter-sexual communication. It has been suggested that in natural populations of mice, females urine mark to advertise their dominant breeding status to other females; urine-marking appears to be dependent on female social/reproductive status [44]. Taken together, these observations suggest that the intrauterine position did not affect behavior as evaluated by the open field test and the wheel cage.

4.4. Prostate development

Growth and differentiation of the prostate is primarily under the control of androgen. Expression of the androgen metabolizing enzyme, 5α -reductase, within prostatic mesenchyme cells is also necessary for normal development of the prostate [45]. The possibility that estrogen might be involved in modulating the effects of androgen on prostatic development during early life has been the subject of speculation for over 60 years [46–49]. Timms et al. [50] demonstrated that development of the urogenital system in male and female rat fetuses is influenced by their intrauterine proximity to fetuses of the same or opposite sex, and suggested that exposure to supplemental estradiol (due to being positioned between two female fetuses) induces prostatic bud development in females, and enhances the growth of prostatic buds in both males and females. An enlarged prostate in males located between two female fetuses was hypothesized to be mediated by an elevated level of serum estradiol, relative to males located between two males, due to the transport of estradiol from adjacent female fetuses [4,30]. This hypothesis was confirmed in a study in which estradiol was experimentally elevated by 50% in male mouse fetuses (via maternal administration), and the estrogen-treated males showed both a significant increase in prostatic glandular buds and significantly larger buds during fetal life, as well as enlarged prostates in adulthood [46].

In the present study, however, the weights of the prostates (with seminal vesicles) of the rats at PND 21, and the ventral and dorsal prostates (with seminal vesicles) of the rats at 10

weeks of age, were not significantly different between the groups. In addition, morphological observation of prostates in the rats and mice, in weanlings or adulthood, by light and electron microscope revealed no alteration in males located in any uterine position.

4.5. Developmental exposure to 17 β -estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17 β -estradiol administration to pregnant mice on the early development of the prostate in male mouse fetuses, with attention being paid to the intrauterine position of the males. Timms et al. [17] reported that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly reduced serum estradiol in males located between two females, but not males located between two males, and also significantly interfered with initial budding and subsequent growth of the prostate in males located between two females or two males. In sharp contrast, the seminal vesicles were larger in the control males located between two males than in control males located between two females, similar to prior findings in mice [29], and TCDD only decreased the size of the seminal vesicles in males located between two males. Taken together, the findings of Timms et al. [17] demonstrate that in utero exposure to TCDD disrupts the development of the prostate, but this disruption depends on an interaction with background levels of estradiol. Howdeshell and vom Saal [16] reported that fetal mouse exposure via the mother to an estrogen-mimicking chemical, bisphenol A, increased the rate of postnatal growth in males and females, and also advanced the timing of puberty in females. They also demonstrated that the greatest response to bisphenol A occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A treatment, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development.

In the present study, however, mouse fetal exposure via the mother to low-dose 17 β -estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. Therefore, we concluded that exposure to low-dose estrogenic endocrine disrupting chemicals during fetal life does not contribute to the intrauterine position.

5. General discussion

We are at a loss to explain why we were unable to replicate the effects of intrauterine position on AGD, or to find intrauterine position effects upon sexual maturation, and the estrous cycle. However, we know the difficulty in demonstrating intrauterine position effects upon morphology and

behavior [35]. In addition, in contrast to earlier work [11] which examined blood androgen titers in mouse fetuses, Baum et al. [51] reported that whole-body androgen levels in female rat fetuses did not vary as a function of intrauterine position, and suggested that intrauterine position effects upon rodent morphology and behavior may not have the robust generality that is generally assumed.

Howdeshell and vom Saal [16] demonstrated that one source of variability in the response of both male and female mouse fetuses to an estrogen-mimicking chemical, bisphenol A, is their background levels of endogenous sex hormones. They suggested that a very small increase in the level of endogenous estradiol may substantially increase the susceptibility of fetuses to endocrine disrupting chemicals consumed or absorbed through the skin or lungs by pregnant animals and humans.

Contiguous [1,52], caudal [53,54], and no effect [51,55–57], due to intrauterine position, have been reported. Hotchkiss et al. [55] in a study with Sprague–Dawley rats examined the effect of intrauterine position on concentrations of testosterone in several different tissues. No effect of either contiguous or caudal intrauterine position on testosterone concentration was detected in fetal carcasses, reproductive tracts, or amniotic fluid. Furthermore, no correlation was found between masculinization due to intrauterine position and increasing anogenital distance. It is unclear at this time why there is such a discrepancy between the previous findings and the present results in rats and mice. However, varied strains of rats and mice, multiple uncontrolled variables, and different criteria for defining the effects of intrauterine positioning, may all contribute to this uncertainty. In addition, the discrepancies in the data may be attributed to such factors as the dietary influences (such as background levels of phytoestrogens and caloric intake), caging (steel versus polycarbonate), bedding, housing (group versus individual), and seasonal variation, as well as differences among the studies in control body and prostate weights [58,59].

The results of the present study clearly showed that intrauterine position of embryos/fetuses did not influence postnatal development, including sexual maturation and behavior.

Acknowledgments

The technical support provided by the reproductive and developmental biology group was essential to this study and was greatly appreciated. This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan.

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R00077638_RTX_5582



Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect

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Received 15 January 2006; received in revised form 25 March 2006; accepted 8 April 2006

Abstract

Bisphenol-A has been extensively evaluated for toxicity in a variety of tests as the most common environmental endocrine disruptors. In the previous study, we reported that prenatal and neonatal exposure to high-dose of bisphenol-A affects the development of central dopaminergic system in the mouse limbic area. The present study was then undertaken to investigate whether prenatal and neonatal exposure to lower dose of bisphenol-A could change the morphine-induced several pharmacological actions such as rewarding effect and hyperlocomotion in mice. Prenatal and neonatal exposure to low-dose of bisphenol-A enhanced the morphine-induced hyperlocomotion and rewarding effect. Additionally, the treatment with bisphenol-A produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain, which is thought to play a critical role for hyperlocomotion and rewarding effects by drugs of abuse. These findings suggest that prenatal and neonatal exposure to low-dose of bisphenol-A can potentiate the central dopamine receptor-dependent neurotransmission, resulting in the supersensitivity of the morphine-induced hyperlocomotion and rewarding effects in the mouse.

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Keywords: Bisphenol-A; Dopamine; Morphine; Rewarding effect; Hyperlocomotion; Endocrine disruptor

Bisphenol-A is an environmental endocrine-disrupting chemical that affects reproduction in wildlife [4,5]. Bisphenol-A is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in the food cans and found as a contaminant not only in the liquid of the preserved foods, but also in the water autoclaved in the cans [1,7]. This chemical is also released from polycarbonate flasks during autoclaving [9]. Moreover, it has been reported that significant amounts of bisphenol-A are detected in the saliva of dental patients treated with fissure sealants [15].

On the endocrine-disrupting chemical problems, the low-dose actions of the endocrine-disrupting chemicals are serious problems. However, little is known about its action on the central nervous system induced by low-dose of bisphenol-A. The aim of the present study was then undertaken to investigate whether prenatal and neonatal exposure to low-dose of bisphenol-A in mice could affect the rewarding effect and locomotor-enhancing effects induced by morphine.

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

All experiments were performed using 7 weeks old male ddY mice (Tokyo Animal Science Co., Tokyo, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 3×10^{-2} , 3×10^{-1} , 3×10^2 , 2×10^3 μg bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, animals did not show weight loss and disruption of maternal behaviors.

The place conditioning paradigm has been known to the method to evaluate the motivational properties as the self-administration paradigm [13,18]. The apparatus was a shuttle box ($15 \times 30 \times 15$ cm: $w \times l \times h$) which was made of acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is

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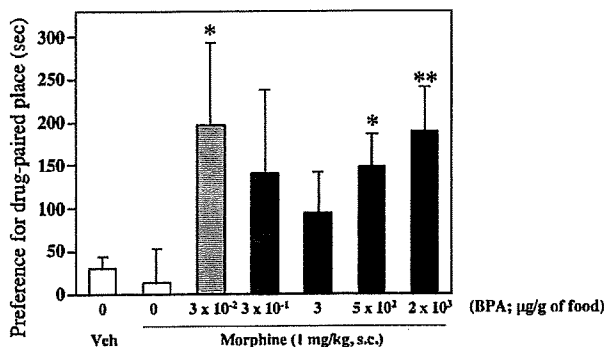


Fig. 1. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine (1 mg/kg, s.c.)-induced rewarding effect in mice. Each column represents the mean conditioning score with S.E.M. of 6–14 mice. * $p < 0.05$, ** $p < 0.01$ vs. morphine-treated control group.

black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injection. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. A day after these conditioning sessions, the animal is placed in the test apparatus without any confinements. At the dose of 1 mg/kg (s.c.), morphine produced neither place preference nor place aversion in control mice (Fig. 1). However, treatment with 1 mg/kg (s.c.) of morphine produced a significant place preference in the mice chronically treated with low- and high-dose of bisphenol-A, but not middle-dose of bisphenol-A, from mating to weaning (Fig. 1). The locomotor activity of mice was measured by an ambulator as described previously [14]. Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 h following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). Total activity was counted for 3 h after the treatment. Treatment with 10 mg/kg (s.c.) of morphine produced a locomotor-enhancing effect in

all groups (Fig. 2). In mice chronically treated with low- and high-dose of bisphenol-A, but not middle-dose of bisphenol-A, from mating to weaning, the hyperlocomotion induced by morphine was dramatically potentiated as compared to that in control (Fig. 2). These findings suggest that prenatal and neonatal exposure to low- and high-dose of bisphenol-A lead to the supersensitivity of morphine-induced pharmacological actions. It should be mentioned that prenatal and neonatal exposure to bisphenol-A shows the biphasic effect on the supersensitivity of morphine-induced pharmacological actions. Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors [2,3]. We have already confirmed that acute administration of bisphenol-A with adult mice could not affect the dopamine-related behaviors (data not shown). On the other hand, the behavioral abnormalities are induced by prenatal and neonatal exposure to bisphenol-A [11,12,19,20]. These findings indicate that prenatal and neonatal exposure to bisphenol-A may cause the neuronal toxicity specifically in the developmental process. We next investigated the influence of prenatal and neonatal exposure to bisphenol-A in the development of central dopaminergic function using [35 S]GTP γ S binding assay as described previously [20]. In the membrane preparation, mice were killed by decapitation and the limbic forebrain including the nucleus accumbens was then dissected as described previously [17]. The limbic forebrain was rapidly excised at 4 °C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris–Mg $^{2+}$ buffer containing 50 mM Tris–HCl (pH 7.4), MgCl $_2$ and 1 mM EGTA for the [35 S]GTP γ S binding assay. The homogenate was centrifuged at 4 °C for 10 min at 48,000 \times g. The pellet was resuspended in ice-cold Tris buffer of [35 S]GTP γ S binding assay buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl $_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4 °C for 10 min at 48,000 \times g. The resultant pellet was resuspended in ice-cold Tris buffer or [35 S]GTP γ S binding assay buffer with 10 μ M dopamine, 30 μ M guanosine-5'-diphosphate

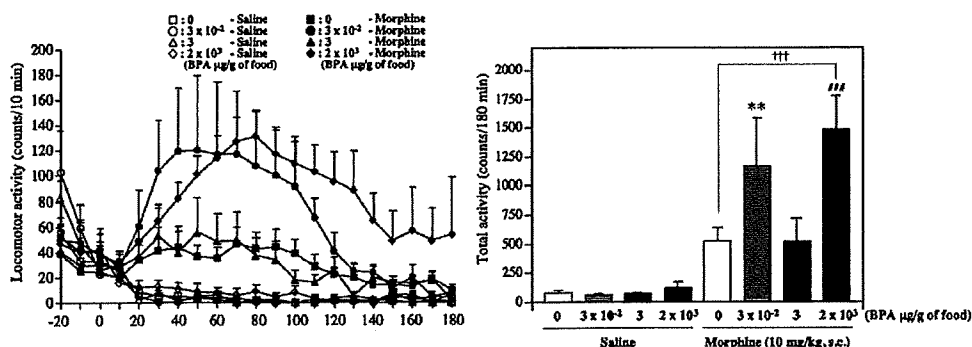


Fig. 2. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine-induced hyperlocomotion in mice. (A) Time course changes in the morphine-induced hyperlocomotion in mice. Each point represents the mean activity counts for 10 min with S.E.M. of 5–15 mice. (B) Total locomotor activity of morphine-induced locomotor enhancing effect in mice. Each column represent the mean activity for 180 min with S.E.M. of 5–15 mice. ** $p < 0.01$ vs. saline-treated 3×10^{-2} BPA μ g/g of food-treated group. ### $p < 0.001$ vs. saline-treated 2×10^3 BPA μ g/g of food-treated group. ††† $p < 0.001$ vs. morphine-treated control group.

(GDP) and 50 pM [35 S]GTP γ S (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂ at 4 °C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris-HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyser. Non-specific binding was measured in the presence of 10 μ M unlabeled GTP γ S. Comparable results were obtained from at least three independent sets of experiments. Dopamine (10 μ M) produced an increase in [35 S]GTP γ S binding to membranes from the limbic forebrain including the nucleus accumbens of control mice. Under these conditions, the stimulation of [35 S]GTP γ S binding induced by dopamine was potentiated in mice chronically treated with wide range of concentrations of bisphenol-A from mating to weaning. Especially, the enhancement of the stimulation of [35 S]GTP γ S binding induced by dopamine in mice chronically treated with low- and high dose of bisphenol-A was noted, which suggests that bisphenol-A shows the biphasic effect on the regulation of dopamine receptor function in the limbic forebrain (Fig. 3). Taken together, these findings suggest that the supersensitivity of morphine-induced pharmacological actions following prenatal and neonatal exposure to especially low- and high dose of bisphenol-A may result from a drastic up-regulation of dopamine receptor function in the limbic forebrain (see Fig. 4).

All of data represent the mean counts with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnnett's test.

As mentioned above, humans might be orally exposed to bisphenol-A in daily life. In the previous study, we chronically treated female mice with bisphenol-A-admixed powder food containing 2×10^3 μ g of bisphenol-A/g of food, and this

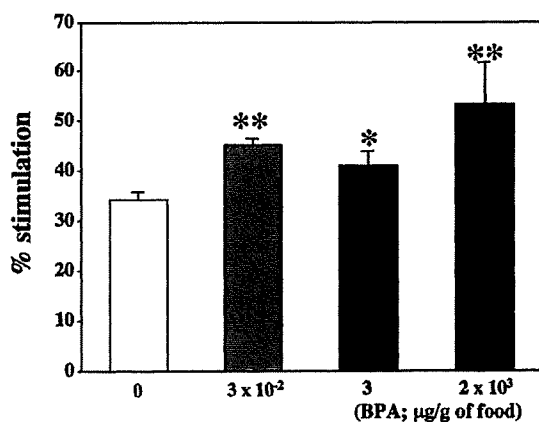


Fig. 3. Comparison of the stimulation of [35 S]GTP γ S binding to membranes from the limbic forebrain by dopamine between control and a wide range of concentrations of bisphenol-A-treated mice. Membranes were incubated with [35 S]GTP γ S (50 pM) and GDP (30 μ M) with dopamine. The data are shown as the percentage of basal [35 S]GTP γ S binding measured in the presence of GDP and absence of dopamine. Each column represents the mean with S.E.M. of three samples. ** $p < 0.01$ vs. control group.

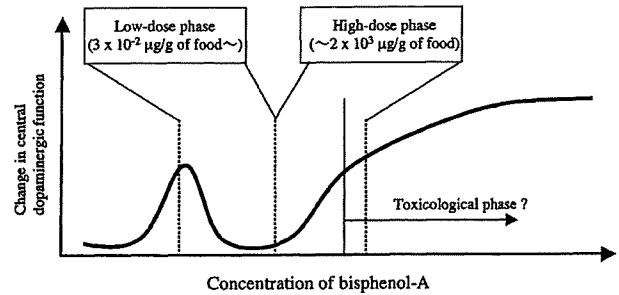


Fig. 4. A schematic drawing of the biphasic effect on the central dopaminergic function by prenatal and neonatal exposure to bisphenol-A. In the previous study, we reported that prenatal and neonatal exposure to high dose ($2\text{--}2 \times 10^3$ μ g/g of food) of bisphenol-A change the central dopaminergic function. Here, our findings suggest that supersensitivity of morphine-induced pharmacological actions following prenatal and neonatal exposure to low dose ($3 \times 10^{-2}\text{--}3$ μ g/g of food) of bisphenol-A may result from an up-regulation of dopamine receptor function in the limbic forebrain.

enhanced the rewarding effect induced by drugs of abuse in their pups [11,12,20]. Under these conditions, the blood level of bisphenol-A in their pups was approximately 10 ng/ml, which is considered to be more than 30 times higher than the level for healthy human exposure [20]. On the other hand, vom Saal et al. estimated that humans are exposed to bisphenol-A at a dose of 2–20 μ g/kg/day [21]. Based on these reports, we here ascertained the effects of low dose of exposure to bisphenol-A. Adult female mice were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 3×10^{-2} , 3×10^{-1} , 3×10^2 , 5×10^2 , 2×10^3 μ g bisphenol-A/g of food from mating to weaning.

Bisphenol-A and alkylphenols have been reported to have estrogenic activity [8]. Recent molecular studies have suggested the transcriptional activation of the human dopamine D₁ receptor gene by estrogen [10]. Since bisphenol-A has only very weak estrogenic effects [23], it does not seem likely that low dose of bisphenol-A is accompanied by a classical estrogenic activity. It was reported that the binding of bisphenol-A to the non-classical membrane-bound estrogen receptor activates a guanylyl cyclase, protein kinase G and closing K_{ATP} channels [16]. Additionally, low dose of bisphenol-A can activate the transcription factor, cAMP-responsive element binding protein (CREB). Phosphorylated CREB has been shown to be increased after only a 5 min application of bisphenol-A in a calcium-dependent manner [16]. Therefore, the supersensitivity of morphine-induced pharmacological actions caused by prenatal and neonatal exposure to low dose of bisphenol-A may be mediated by non-classical membrane-bound estrogen receptors.

On the other hand, the animal model for hyperactivity was produced by Shaywitz et al., who demonstrated that rat pups treated with 6-hydroxydopamine (6-OHDA) via intracisternal administration at 5 days of age developed increased motor activity caused by the reduction of tyrosine hydroxylase (TH)-sensitive dopamine, leading to cognitive difficulties in shuttle-box learning between 2 and 4 weeks of age [17]. Additionally, Ishido et al. have reported that high dose of bisphenol-A affects the central dopaminergic system, resulting in hyperactivity due most likely to a large reduction TH activity in the midbrain

[6]. Based on these reports, we hypothesize that prenatal and neonatal exposure to high dose of bisphenol-A may cause the dopamine depletion for the limited time period and in turn induce the long-lasting supersensitivity of dopamine receptor-related action following chronic treatment with morphine.

It is very difficult to explain the fact that the prenatal and neonatal exposure to middle-dose of bisphenol-A have only weak effect on the disruption of functional changes in the dopaminergic transmission. Although the mechanisms of the weak effect by the prenatal and neonatal exposure to middle-dose of bisphenol-A remain unclear, one possibility is that the potentiation of the central dopaminergic transmission caused by the prenatal and neonatal exposure to low dose of bisphenol-A can be offset by the middle-dose of bisphenol-A through the negative feedback regulation. It is also likely that prenatal and neonatal exposure to high dose of bisphenol-A may potentiate the dopamine receptor function following a dramatic deletion of TH-sensitive dopamine and/or dysfunction of negative feedback mechanism against dopamine receptor function following the overshooting of its negative feedback.

On the endocrine-disrupting chemical problems, the low dose actions are serious problems. As well as described in the present study, it was recently reported that there were effects caused by exposure to low doses of bisphenol-A on rate of growth and sexual maturation, hormone levels in blood, reproductive organ function, fertility, immune function, enzyme activity, and brain structure, brain chemistry and behavior [22]. Therefore, our findings warn that prenatal and postnatal exposure to low- and high doses of bisphenol-A may dramatically change the neuronal transmission including dopaminergic transmission in the adult brain. This phenomenon could explain the aggravation of the development of dependence on drugs of abuse.

Acknowledgments

This work was supported in part by grants from the Ministry of Health, Labour and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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RESEARCH ARTICLE

Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice

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Abstract

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been evaluated extensively for toxicity and carcinogenicity. However, little is still known about its action on the central nervous system (CNS). In the previous study, we found that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effect induced by morphine. Here we found that prenatal and neonatal exposure to BPA resulted in the attenuation of dopamine D3 receptor-mediated G-protein activation by 7-OH-DPAT in the mouse limbic forebrain. This treatment also caused a significant decrease in the B_{max} value of [3 H]DPD128907, a dopamine D3 receptor ligand, in this area. Under these conditions, no change in dopamine D3 receptor mRNA expression in the limbic forebrain and lower midbrain was observed by prenatal and neonatal exposure to BPA. The present data provide further evidence that prenatal and neonatal exposure to BPA leads to the reduction of functional dopamine D3 receptors without affecting the new synthesis of dopamine D3 receptors in the mouse limbic forebrain.

Introduction

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. It has been reported that administration of bisphenol-A (BPA) to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of human produces significant changes in the postnatal growth rate and brings on early puberty in these mice.¹ These results imply that BPA may cause the unpleasant toxicity in the developmental

process. In contrast, little is known about the neuronal toxicity induced by BPA in the central nervous system (CNS). More recently, we have reported that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced rewarding effect and central dopamine D1 receptor function in mice.²

The dopamine D3 receptor cloned by Sokoloff and colleagues has been characterized extensively.³ The dopamine D3 receptor shows a distinct distribution in limbic areas of the brain, including the nucleus accumbens and olfactory

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Received for publication 11th November 2003. Accepted 27th December 2003.

tubercle.³ Several pharmacological studies with dopamine D₃ receptor-preferring agonists such as 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) suggest that the dopamine D₃ receptor regulates the inhibitory effect to produce hyperlocomotion in rodents.^{4,5} In addition, we found that the morphine-induced rewarding effect and hyperlocomotion were markedly enhanced in mice lacking dopamine D₃ receptor gene.⁶ These findings suggest that the dopamine D₃ receptor plays a critical role in the psychoeffective functions of dopamine neurotransmission.

The present study was then designed to investigate the changes in the function of dopamine D₃ receptors by prenatal and neonatal exposure to BPA in mice.

Materials and method

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering.

Animals

All experiments were performed using male ddY mice (8–12 weeks old) that had been exposed prenatally and neonatally to BPA. Prenatal and neonatal BPA exposure was conducted as described previously.² Adult female mice were chronically treated with BPA-admixed powder food containing 0 (B0) and 2 (B2) mg BPA/g of food from mating to weaning. Their pups were prenatally (about 3 weeks) and neonatally (about 2 weeks) exposed to the respective concentration of BPA from their mothers. During the treatment with BPA, animals did not show either weight loss or disrupted maternal behaviours.

Membrane preparations

In the membrane preparation, mice were killed by decapitation and the limbic forebrain was then dissected as described previously.⁷ The limbic forebrain was rapidly excised at 4°C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20

volumes (w/v) of ice-cold Tris-Mg²⁺ buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂ and 1 mM EGTA for the [³⁵S]GTPγS binding assay. The homogenate was centrifuged at 4°C for 10 min at 48,000 g. The pellet was resuspended in ice-cold Tris buffer or [³⁵S]GTPγS binding assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4°C for 10 min at 48,000 g. The resultant pellet was resuspended in ice-cold Tris buffer or [³⁵S]GTPγS binding assay buffer and stored at -70°C until used.

[³⁵S]GTPγS binding assay

The membrane homogenate (3–8 μg protein/assay) was incubated at 25°C for 2 h in 1 ml of assay buffer with 10 μM 7-OH-DPAT or a dopamine D₂ receptor agonist N-propylnorapomorphine (NPA), 30 μM guanosine-5'-diphosphate (GDP) and 50 pM [³⁵S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂ at 4°C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris-HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyser. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Dopamine D₃ receptor binding assay

The dopamine D₃ receptor binding assays were carried out in duplicate with [³H] PD128907 (specific activity, 111 Ci/mmol; Amersham, Arlington Heights, IL) at 0.039 to 5 nM in a final volume of 1.0 ml which contained 50 mM Tris-HCl buffer (pH 7.4), 100 μM guanosine-5'-[β,γ-imido]triphosphate (Gpp(NH)p) and 0.1 ml of the homogenated membrane fraction. The amount of membrane proteins used in each assay was in the range of 90 to 140 μg, as determined by the method of Bradford.⁸ The test tubes were incubated for 2 h at 25°C. The specific binding was defined as the difference in bindings observed in the absence and

presence of 10 μM unlabeled PD128907. The incubation was terminated by collecting the membranes on Whatman GF/B filters using a Brandel cell harvester. The filters were then washed three times with 5 ml Tris-HCl buffer (pH 7.4) at 4°C and transferred to scintillation vials. Then, 0.5 ml of Soluene-350 (Packard Instrument Company, Inc, Meriden, CT) and 4 ml of Hionic Fluor Cocktail (Packard Instrument Company) were added to the vials. After a 12 h equilibration period, the radioactivity in the samples was determined in liquid scintillation analyzer.

RT-PCR assay

Total RNA in the limbic forebrain and lower midbrain were extracted using SV Total RNA Isolation System (Promega, Madison, WI). To prepare first strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM DTT, 2.5 mM MgCl_2 , dNTP mix, 200 U of reverse transcriptase II (Gibco-BRL, Grand Island, NY) and 0.1 mM oligo (dt)₁₂₋₁₈ (Gibco-BRL). The dopamine D₃ receptor was amplified in a 50 μl PCR solution containing 0.8 mM MgCl_2 , dNTP mix and DNA polymerase with synthesized primers: a sense primer of dopamine D₃ receptor, which is at position 391–407 (5'-

GCA GTG GTC ATG CCA GTT CAC TAT CAG-3') of the receptor, and an antisense primer at position 498 to 526 (5'-CCT GTT GTG TTG AAA CCA AAG AGG AGA GG-3'), which were designed according to sequence Accession nos U26915 in GenBankTM.

Samples were heated to 94°C for 2 min, 55°C for 2 min and 72°C for 3 min, and cycled 35 times through 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. The final incubation was 72°C for 7 min. The mixture was run on 1% agarose gel electrophoresis with the indicated markers and primers of the internal standard GADPH. Three independent experiments were performed in this study. Semi-quantitation of the intensity of the bands for dopamine D₃ receptor/GADPH was conducted by using a NIH image.

Results

The attenuation of dopamine D₃ receptor-mediated G-protein activation in mice prenatally and neonatally exposed to BPA

The dopamine D₃ receptor agonist 7-OH-DPAT (0.001–10 μM) produced a concentration-dependent increase in [³⁵S]GTP γ S binding to membranes obtained from the limbic forebrain including the nucleus accumbens of B0 mice.

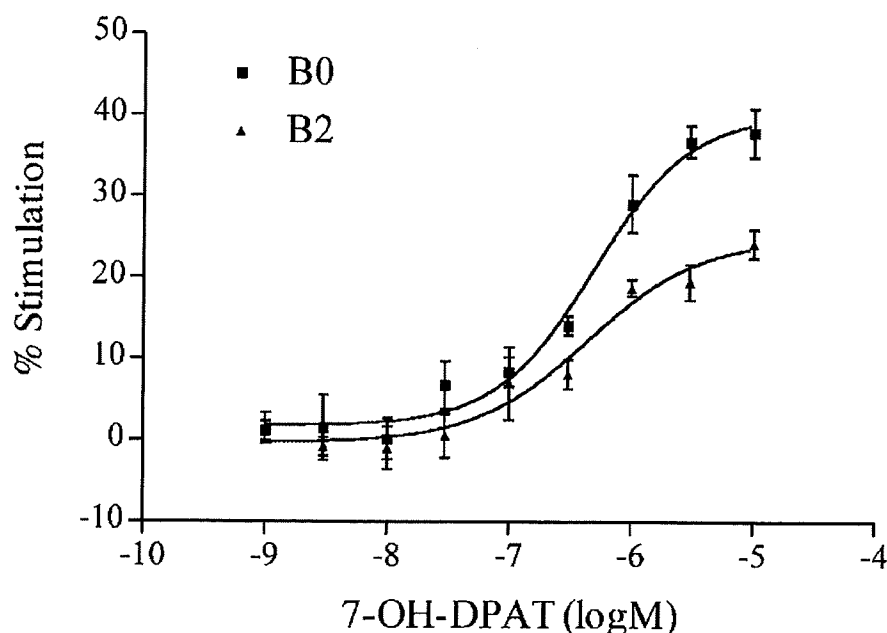


Figure 1. Comparison of the stimulation of [³⁵S]GTP γ S binding by dopamine D₃ receptor agonist 7-OH-DPAT to membranes of the limbic forebrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with [³⁵S]GTP γ S (50 pM) and GDP (30 μM) with 7-OH-DPAT (10^{-9} – 10^{-5} M). The values are expressed as percentage increase of the value in B0 mice. Data are expressed as the mean \pm SEM of three independent samples.

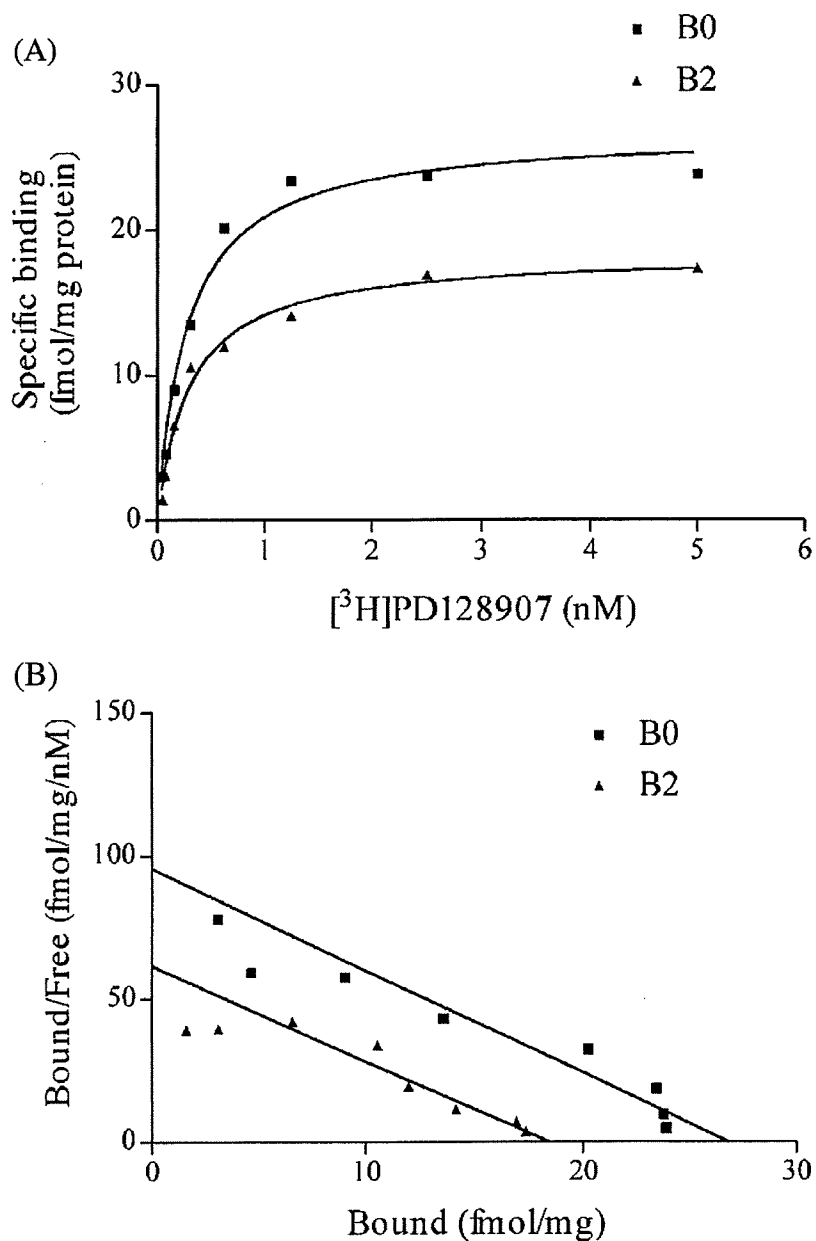


Figure 2. Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally exposed to BPA. Saturation binding (A) and Scatchard analysis (B) were performed by receptor binding assay using [^3H]PD128907. Membranes were incubated with [^3H]PD128907 (0.039–5.0 nM) and Gpp(NH)p (100 μM).

The key finding of the present study was that the stimulation of [^{35}S]GTP γ S binding induced by 7-OH-DPAT was attenuated markedly in B2 mice ($F_{1, 174} = 30.45$, $p < 0.001$; Fig. 1). Under these conditions, the G-protein activation induced by the selective dopamine D₂ receptor agonist N-propylnorapomorphine in the limbic forebrain was not affected by prenatal and neonatal exposure to BPA (only 3% increase; figure not shown).

Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally exposed to BPA

To evaluate the population of dopamine D3 receptors in the mouse brain, we performed the saturation-binding analysis using [^3H]PD128907. The saturation-binding curve and Scatchard analysis of [^3H]PD128907 are shown in Fig. 2. The B_{max} and apparent K_d value are shown in Table 1. We found that prenatal and

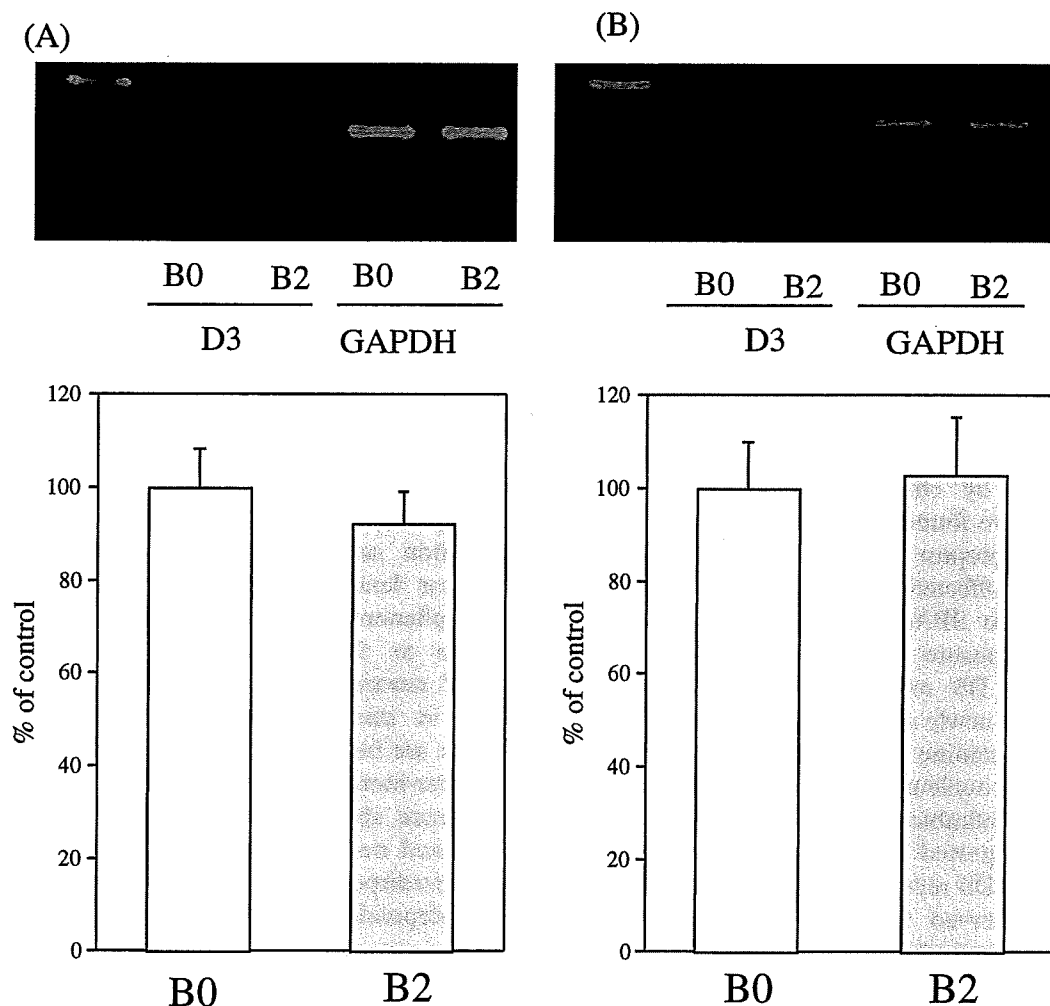


Figure 3. No changes in the expression of dopamine D3 receptor mRNAs in the (A) limbic forebrain and (B) lower midbrain obtained from B0 and B2 mice. Upper panel: representative reverse transcription-polymerase chain reaction for dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from B0 and B2 mice. Lower panel: no changes in the expression of dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from B2 mice (hatched bar) compared to that from B0 mice (open bar). The values are expressed as a percentage of the value in B0 mice. Each column represents the mean \pm SEM of three independent samples.

Table 1. Dopamine D₃ receptor density and affinity performed by [³H]PD128907 binding to the mouse brain membrane preparation from control (B0) and bisphenol A treated (B2) mice

	Bmax (fmol/mg of protein)	Kd (nM)
B0	28.24 \pm 1.57	0.28 \pm 0.04
B2	18.60 \pm 0.22*	0.29 \pm 0.03

Each value represents the mean with SEM of three samples. **p* < 0.05 vs. B0 group

neonatal exposure to BPA caused the decrease of dopamine D3 receptor density in the mouse limbic forebrain.

No change in the expression of dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from mice prenatally and neonatally exposed to BPA

We also found that the expression of dopamine D3 receptor mRNA in the limbic forebrain and lower midbrain was not affected by prenatal and neonatal exposure to BPA (Fig. 3).

Discussion

In the previous study, we demonstrated that prenatal and neonatal exposure to BPA enhanced the methamphetamine-induced rewarding effect associated with the moderate upregulation of

central dopamine D1 receptor function in mice.² In the present study, we found that the stimulation of [³⁵S]GTP γ S binding induced by 7-OH-DPAT was markedly attenuated in B2 mice. Under these conditions, the G-protein activation induced by the selective dopamine D₂ receptor agonist N-propylnorapomorphine in the limbic forebrain was not affected by prenatal exposure to BPA.

To evaluate the population of dopamine D3 receptors in the membrane surface of the mouse brain, we performed a saturation-binding analysis using [³H]PD128907. In the present study, the density of dopamine D3 receptor was significantly decreased by chronic exposure to BPA. In addition, we found that the expression of dopamine D3 receptor mRNA in the limbic forebrain was not affected by prenatal and neonatal exposure to BPA. These findings suggest that chronic exposure to BPA caused the increase in dopamine D3 receptor turnover without any changes in newly synthesized receptors.

The dopamine D3 receptor is highly distributed in the nucleus accumbens, the terminal sites of the mesolimbic dopaminergic system.^{3,9} This can be supported by the present finding that the dopamine D3 receptor expression was predominantly observed in the limbic forebrain. The limbic system-selective expression of the dopamine D3 receptor has led to particular interest in this receptor as a potential mediator of some of the psychoeffective functions of dopamine neurotransmission.¹⁰⁻¹³ In the previous study, we demonstrated that the dopamine D3 receptor knockout mice exhibit the enhancement of morphine-induced rewarding effect and hyperlocomotion.⁵ Furthermore, prenatal and neonatal exposure to BPA enhances the morphine-induced rewarding effect and hyperlocomotion without direct changes in the μ -opioid receptor function.¹⁴ Thus, these findings provide the possibility that the dramatic suppression of postsynaptic dopamine D₃ receptor function in the nucleus accumbens may be, at least in part, involved in the enhancement of morphine-induced rewarding effect followed by chronic exposure to BPA.

The desensitization of G-protein-coupled receptors is mediated by the phosphorylation of serine and threonine residues within the intracellular domains of receptors.¹⁵ Both second messenger-dependent kinases, such as protein kinase A and protein kinase C, and G-protein-coupled receptor kinases (GRKs) have been shown to

contribute to the desensitization and internalization of activated dopamine receptors.¹⁶⁻¹⁸ It has been reported recently that dopamine D3 receptor desensitization can be regulated by GRK3.¹⁹ In the present study, we clearly observed the increase in dopamine D3 receptor turnover without any changes in newly synthesized receptors after chronic treatment with BPA. Taken together, a hypothesis would be advanced that, although further study is required, prenatal and neonatal exposure to BPA may lead to the changes in activities of these kinases associated with the turnover of dopamine D3 receptors.

In conclusion, the present study suggests that chronic exposure to BPA causes the inhibition of dopamine D3 receptor-mediated G-protein activation associated with the decrease in its receptor density in the limbic forebrain of mice. This phenomenon may, at least in part, contribute to the enhancement of morphine-induced rewarding effect by chronic exposure to BPA in mice. Public attention and research efforts are being driven by an understanding of the ever-increasing problems and magnitude of substance abuse. Our findings warn that prenatal and neonatal exposure to BPA in females may predispose their children to the psychological dependence of morphine.

Acknowledgements

This work was supported in part by grants from the Ministry of Health, Labour and Welfare and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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