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Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice

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Abstract

In rodents, steroid hormones are thought to be transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses may have higher serum levels of estradiol, and lower serum levels of testosterone, relative to siblings of the same sex that develop between two male fetuses. The consequence in the variation of postnatal growth, development, and function in the intrauterine position, using various parameters such as anogenital distance, preputial separation and vaginal opening, estrous cycle, locomotor activity, and growth of reproductive organs, were examined in Sprague–Dawley rats. ICR mice were treated with 17β-estradiol before copulation and during pregnancy to address the interaction with endogenous estradiol during pregnancy. In rats, no evidence of effects of prior intrauterine position was observed for any of the parameters examined. Mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. The results of this study suggested that the intrauterine position of the embryos/fetuses did not affect the postnatal growth of the reproductive organs, sexual maturation, or behavior in rats and mice.

Keywords: Intrauterine position; Postnatal growth; Sexual maturation; Behavior; Anogenital distance; Rats; Mice

1. Introduction

The development of sexually differentiated phenotypes depends upon the hormonal environment during a critical period of growth [1]. Testosterone secretion by the fetal testis causes a longer anogenital distance (AGD), seen in neonatal males, relative to females. The AGD of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position of the animals [1–4]. 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. Females with a male fetus on only one side are immediate [4].

In all litter-bearing species that have been examined to date, the intrauterine position that a fetus occupies relative to fetuses of the same or opposite sex has profound effects on its reproductive, behavioral, and morphological traits measured during adult life [4–7]. Gerbil males and females that developed in utero between two female fetuses or two male fetuses, respectively, did not differ in relative hippocampal size [8].

The effects of intrauterine position are apparently not the result of the position itself, but rather of the movement of steroid hormones between the fetuses, and variations in the hormonal environment relative to the proximity of an individual fetus to other fetuses of the same or opposite sex [9]. Male rats located between two females had elevated serum estradiol and larger prostates than males located between two males, which had elevated serum testosterone and larger seminal vesicles [10]. The effect of intrauterine position in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity [11,12].

Recently, intrauterine position has been the focus of discussions in the toxicology community because of its potential to alter the susceptibility of fetuses to endogenous hormones and endocrine disrupting chemicals [13,14]. In this regard, failure to account for intrauterine position in endocrine disrupting chemical toxicology studies could lead to false negative results, especially when adverse alterations

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are produced by low doses in fetuses from only one intrauterine position [14,15]. This possibility has been raised because of investigations into estrogenic compounds in mice. In rats, consistent effects due to intrauterine position on testosterone concentrations, and therefore potential interactions with endocrine disrupting chemicals, have not been found. Howdeshell and vom Saal [16] demonstrated that the greatest response to the estrogenic chemical, 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 within the range of human exposure, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development. It has been demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin interacted with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses [17].

The objectives of this study were to determine the effects of intrauterine position, under normal physiological conditions, on the development of rat offspring, as well as sexual maturation, estrous cycle, behavior, and reproductive organ development. Another objective of this study was to determine whether the intrauterine position of mouse fetuses, which is related to background levels of estradiol and testosterone, would influence the response of the postnatal growth of gonads, including sexual maturation, to low dose 17β-estradiol.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Crj:CD, IGS), and ICR mice (Crj:CD-1) were purchased from Charles River, Laboratories, Inc. (Atsugi, Japan). Twenty-seven male rats (9 weeks of age), 84 female rats (8 weeks of age), 130 male mice (9 weeks of age), and 130 female mice (8 weeks of age), were used. The rats and mice arrived with mean weights of 301.1 ± 7.9 g for males and 216.2 ± 8.1 g for females, and 37.2 ± 1.2 g for males and 29.1 ± 0.9 g for females (mean ± S.D.), respectively. The animals were acclimated to the laboratory for 7–14 days prior to the start of the experiments to evaluate weight gain and any gross signs of disease or injury. The animals were housed individually in stainless steel, wire-mesh cages in a room with controlled temperature (22–25 °C) and humidity (50–65%), with lights on from 07:00 to 19:00 h daily. The animals were given access to food (NIH-07-PLD: phytoestrogen low diet, Oriental Yeast Co., Japan) and tap water through metal pipes (distilled water, Wako Pure Chem., Japan) ad libitum. In a few instances, the temperature and humidity were outside the standard ranges, but the magnitude and duration of these incidents were minimal and judged to be of no consequence. The contents of genistein and daidzein in the diet and wood bedding (ALPHA-dri, Shepherd Specialty Paper, USA) used in the present study were determined. Neither genistein nor daidzein were not detected in the diet or wood bedding (detection limit: 0.5 mg/100 g in each individual phytoestrogen, by HPLC).

Animal care and use conformed to published guidelines [18].

2.2. Experiment I (examination of intrauterine position effect on postnatal growth in rats)

2.2.1. Cesarean delivery and fostering

Estrous female rats at 10–11 weeks of age were cohabited overnight with a single male to obtain 66 pregnant females within 4 days. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Thirty-three pregnant females were killed by CO_2 asphyxiation and cervical dislocation, and subjected to cesarean sectioning on day 21 of gestation. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed. Anogenital distance (AGD) was measured with a digital micrometer (reproductive precision of 0.01 mm, Digimatic caliper CD-15C, Mitutoyo Co., Kanagawa, Japan) under an Olympus dissecting microscope for each fetus, and the average was taken. The subject was held steady and in the same position during measurement. Measurements were made without knowledge of intrauterine position by one person. The AGD was measured from the center of the phallus to the center of the anus. The fetuses obtained by cesarean delivery were fostered to 33 dams that had just given birth naturally (one litter to each female). The original littermates remained together when cross-fostered. The litter sizes were similar for each cross-fostered dam. The day of cesarean section was considered as postnatal day (PND) 0. Pup body weights were recorded on PND 21 (day of weaning). Following weaning, and until 10 weeks of age, offspring were weighed once a week.

Neonates from 33 pregnant females were categorized as occupying six different intrauterine positions: 2M (male fetus located between two male fetuses; number of pups and litters on PND 0 = 36 and 19); 1M (male fetus that located between a male fetus and a female fetus; $n = 73$ and 27); 0M (male fetus located between two female fetuses; $n = 45$ and 24); 2F (female fetus located between two female fetuses; $n = 38$ and 18); 1F (female fetus located between a female fetus and a male fetus; $n = 83$ and 29); 0F (female fetus located between two male fetuses; $n = 41$ and 27). Fetuses adjacent to dead embryos (resorptions or macerated fetuses), and fetuses that were closest to each ovary or the cervix, were discarded from further analyses.

2.2.2. Observations of postnatal growth

2.2.2.1. Measurement of AGD and reproductive organ weights, and evaluation of sexual maturation. On PND

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 17β-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 17β-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 17β-estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μg/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 17β-estradiol at 0.05 μg/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 17β-estradiol at 0.1 μg/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 17β-estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO_2 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 17β-estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17β-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 transcardial 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 performed 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 ± 0.4[a]	5.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4
AGD	2.43 ± 0.22	2.42 ± 0.22	2.42 ± 0.28	1.21 ± 0.20	1.23 ± 0.19	1.22 ± 0.24
AGD/body weight	0.43 ± 0.04	0.42 ± 0.04	0.42 ± 0.05	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
AGD/$\sqrt[3]{\text{body weight}}$	1.36 ± 0.12	1.36 ± 0.14	1.35 ± 0.18	0.69 ± 0.08	0.70 ± 0.07	0.70 ± 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 ± 1.5	11.2 ± 1.5	10.8 ± 1.1	10.4 ± 1.4	10.3 ± 1.1	10.4 ± 1.3
AGD	4.57 ± 0.54	4.41 ± 0.48	4.43 ± 0.51	2.00 ± 0.22	1.99 ± 0.19	2.00 ± 0.21
AGD/body weight	0.42 ± 0.06	0.40 ± 0.05	0.41 ± 0.03	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.04
AGD/$\sqrt[3]{\text{body weight}}$	2.06 ± 0.22	1.99 ± 0.19	1.99 ± 0.19	0.92 ± 0.12	0.91 ± 0.11	0.92 ± 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 ± 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 17β-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 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	34	25	26	30	32	26	34	23
Terminal body weight (g)	51.5 ± 4.2[a]	53.6 ± 4.4	40.3 ± 2.7	41.2 ± 3.4	55.1 ± 5.1	53.1 ± 6.2	41.8 ± 2.1	42.1 ± 3.1
Testes (g)	257.9 ± 28.4[b]	266.5 ± 23.5			259.3 ± 25.5	260.3 ± 19.9		
	486.3 ± 76.3[c]	484.2 ± 71.3			488.3 ± 62.3	479.1 ± 60.9		
Epididymides (mg)	89.5 ± 8.7	92.2 ± 9.3			94.6 ± 7.1	93.1 ± 7.1		
	175.2 ± 21.5	161.9 ± 16.9			161.2 ± 13.6	173.6 ± 4.9		
Seminal vesicle (mg)	413.9 ± 30.6	452.2 ± 13.9			431.3 ± 18.2	454.8 ± 21.0		
	812.8 ± 56.9	802.6 ± 44.2			811.5 ± 42.3	809.3 ± 33.5		
Ovary (mg)			15.3 ± 3.3	14.6 ± 4.2			15.5 ± 3.9	15.3 ± 4.5
			35.6 ± 7.5	33.2 ± 4.6			34.1 ± 6.9	33.6 ± 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 ± 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 0F mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than 0F (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, 0F 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 0F (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 17β-estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17β-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 17β-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.

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

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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.

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, 5×10^{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 × 30 × 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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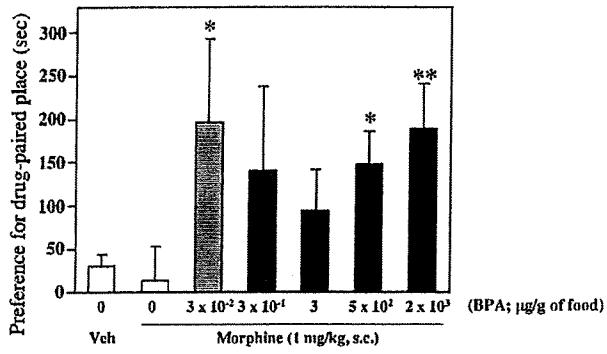

Fig. 1. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine (1 mg/kg, s.c.) -induced rewarding effect in mice. Each column represents the mean conditioning score with S.E.M. of 6–14 mice. $^{*}p<0.05$, $^{**}p<0.01$ vs. morphine-treated control group.

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 ambulometer 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]GTPγS 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]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 of [^{35}S]GTPγS 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 × *g*. The resultant pellet was resuspended in ice-cold Tris buffer or [^{35}S]GTPγS binding assay buffer and stored at −70 °C until used. 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 dopamine, 30 μM guanosine-5′-diphosphate

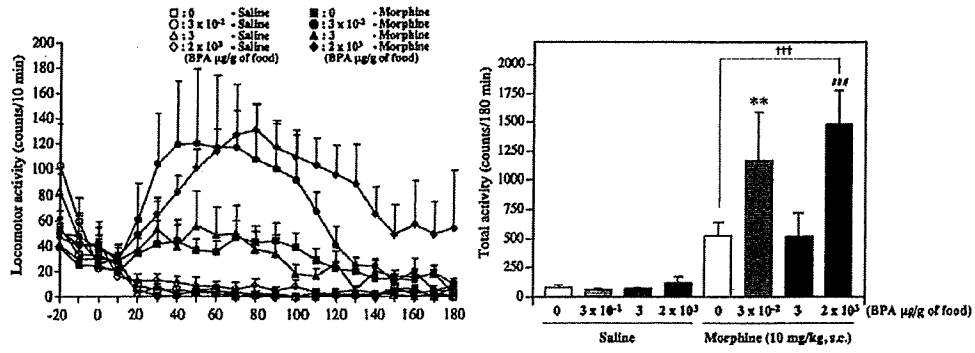

Fig. 2. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine-induced hyperlocomotion in mice. (A) Time course changes in the morphine-induced hyperlocomotion in mice. Each point represents the mean activity counts for 10 min with S.E.M. of 5–15 mice.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

(GDP) and 50 pM [^{35}S]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 $MgCl_2$ 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 (10 μM) produced an increase in [^{35}S]GTPγS binding to membranes from the limbic forebrain including the nucleus accumbens of control mice. Under these conditions, the stimulation of [^{35}S]GTPγS 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]GTPγS binding induced by dopamine in mice chronically treated with low- and high dose of bisphenol-A was noted, witch 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/Dunnett'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 μg of bisphenol-A/g of food, and this

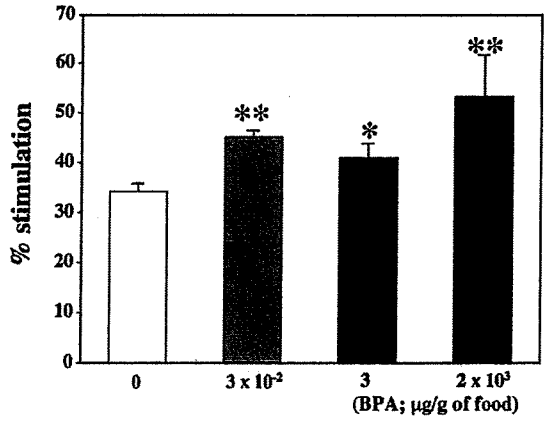

Fig. 3. Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and a wide range of concentrations of bisphenol-A-treated mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS 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.

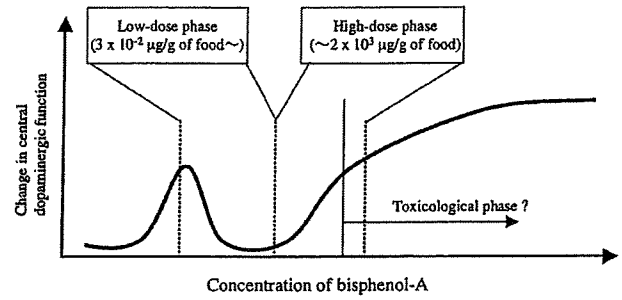

Fig. 4. A schematic drawing of the biphasic effect on the central dopaminergic function by prenatal and neonatal exposure to bisphenol-A. In the previous study, we reported that prenatal and neonatal exposure to high dose ($2–2 \times 10^3$ μg/g of food) of bisphenol-A change the central dopaminergic function. Here, our findings suggest that supersensitivity of morphine-induced pharmcological actions following prenatal and neonatal exposure to low dose (3×10^{-2}–3 μg/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 μg/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 μg 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 D_1 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.

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Dynamic Changes in Dopaminergic Neurotransmission Induced by a Low Concentration of Bisphenol-A in Neurones and Astrocytes

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Key words: bisphenol-A, neurone, astrocyte, dopamine, 17β-oestradiol, drug abuse.

Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA). We previously reported that prenatal and postnatal exposure to BPA potentiated central dopaminergic neurotransmission, resulting in supersensitivity to psychostimulant-induced pharmacological actions. Many recent findings have supported the idea that astrocytes, which are a subpopulation of glial cells, play a critical role in neuronal transmission in the central nervous system. The present study aimed to investigate the role of neurone–astrocyte communication in the enhancement of dopaminergic neurotransmission induced by BPA. We found that treatment of mouse purified astrocytes and neurone/glia cocultures with BPA *in vitro* caused the activation of astrocytes, as detected by a stellate morphology and an increase in levels of glial fibrillary acidic protein. A low concentration of BPA significantly enhanced the Ca^{2+} responses to dopamine in both neurones and astrocytes. Furthermore, a high concentration of BPA markedly induced the activation of caspase-3, which is a marker of neuronal apoptotic cell death in mouse midbrain neurone/glia cocultures. By contrast, treatment with 17β-oestradiol (E_2) had no such effects. Prenatal and neonatal exposure to BPA led to an enhancement of the dopamine-dependent rewarding effect induced by morphine. These findings provide evidence that BPA alters dopamine responsiveness in neurones and astrocytes and that, at least in part, it may contribute to potentiate the development of psychological dependence on drugs of abuse.

The foetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA), which is used in the manufacture of many types of products. These include polycarbonate plastic food storage containers (i.e. baby bottles and water carboys), the lining of food or beverage cans (1, 2), dental composites and sealants and a bioactive bone cement, indicating the potential for human exposure to BPA in daily life.

Our recent studies suggest that exposure to BPA during prenantal and postnatal development has long-lasting effects on central dopaminergic systems linked with behavioural rewarding effects, as well as drug addiction and the reward induced by drugs of abuse (3, 4). The dopamine projection most often linked with a behavioural-rewarding effect is the mesolimbic dopamine system, which originates from the ventral tegmental area (VTA) and terminates at the nucleus accumbens.

We previously demonstrated that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effects induced by drugs of abuse, such as methamphetamine (5) and morphine (6). We also demonstrated that, in adult mice, prenatal and neonatal exposure to BPA enhanced function mediated by central D_1 receptors, which play a substantial role in the rewarding effect of drugs of abuse (5). These treatments also attenuated dopamine function mediated by the D_3 receptor subtype that contributes to the inhibitory modulation of D_1/D_2 receptor-mediated signalling (7, 8). These findings indicate that exposure to BPA during development alters dopaminergic neurotransmission in the central nervous system (CNS), which results in enhancement of the psychological dependence on drugs of abuse. However, the mechanisms underlying these enduring effects of BPA are unknown.

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Many toxic stimuli activate astrocytes, as determined by morphological changes and by an increase in the levels of glial fibrillary acidic protein (GFAP), which is a marker of astrocytes (9, 10). The activation of astrocytes may control the structural and functional plasticity of synapses in the CNS. However, long-term exposure to drugs of abuse can induce neuronal plasticity (11, 12), and we have shown that treatment of mouse cortical neurone/glia cocultures with drugs of abuse, such as methamphetamine and morphine, caused morphological changes in astrocytes (13). Moreover, treatment with methamphetamine increased the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (13). Together, these findings indicate that astrocytes may play an important role in the development of dependence on drugs of abuse.

Although BPA may affect dopaminergic signalling in the CNS, little is known about the role of BPA in neurone-astrocyte communication. The present study aimed to clarify the effect of BPA in neurone-glia communication. We used mouse midbrain neurone/glia cocultures and purified astrocytes to determine the effects of BPA in the mesolimbic dopamine system.

Because the sex steroid hormones (oestrogens and androgens) have been shown to exert profound effects on brain differentiation, neural plasticity and central neurotransmission (14, 15) and BPA has an affinity for oestrogen receptors, albeit 1 : 2000 that of 17β-oestradiol (E_2) (1), we also investigated the effect of E_2 on astrocytic and neuronal responses and the rewarding effect induced by morphine.

Materials and methods

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.

Tissue processing

Purified midbrain astrocytes

Midbrains were dissected from ICR mice at postnatal 1 day (Tokyo Laboratory Animals Science, Tokyo, Japan), minced, and treated with trypsin (0.025%; Invitrogen, Grand Island, NY, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02% L-cysteine monohydrate (Sigma-Aldrich, St Louis, MO, USA), 0.5% glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02% bovine serum albumin (Wako Pure Chemicals). After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (20 min, 1000 *g*). After centrifugation, cells were plated in a flask (75 cm^2 culture flask; Corning Inc., Corning, NY, USA). Seven days after seeding in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% precolostrum newborn calf serum (FBS, Invitrogen), 5% heat-inactivated (56 °C, 30 min) horse serum (HS, Invitrogen), 10 U/ml penicillin and 10 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, the flask was shaken for 12 h at 37 °C to remove nonastrocytic cells. Seven days after seeding, the cells were seeded at a density of 1×10^5 cells/cm^3, and maintained for 7 days in DMEM supplemented with 5% FBS, 5% HS, 10 U/ml penicillin and 10^{-5} g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Midbrain neurone/glia cocultures

Midbrains were obtained from newborn ICR mice at postnatal 1 day, minced, and treated with papain (9 U/ml; Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (10 min, 1000 *g*). The cells were then seeded at a density of 2×10^6 cells/cm^3. The cells were maintained for 7 days in DMEM supplemented with 10% FBS, 10 U/ml penicillin and 10 μg/ml streptomycin. Eight days after seeding, the cells were treated with drugs. In this culture condition, NeuN-positive neurones are surrounded by astrocytes (16).

Drug treatment and immunohistochemistry

Eight days after seeding, *in vitro*, the cells were treated with either normal medium or medium containing bisphenol A (BPA; 10 fM to 1 μM; Wako Pure Chemicals) or 1,3,5[10]-estratriene-3,17β-diol (E_2, 10 fM to 1 μM; Sigma-Aldrich) for 24 h. To explore role for steroid hormone receptors in mediating the effects of BPA (1 pM or 1 μM, 24 h), cells were pretreated with either an oestrogen receptor antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3.17-diol (ICI182,780; 100 nM, 1 μM or 2 μM; Tocris-Cookson, Ellisville, MO, USA), an oestrogen receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM; Sigma-Aldrich), a progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM; Sigma-Aldrich) or an androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM; Sigma-Aldrich) for 24 h. Cells were then treated with normal medium or BPA (1 pM, 1 mM) with or without these steroid hormone receptor ligands for an additional 24 h. Glial cells were then identified by immunofluorescence using mouse anti-glial fibrillary acidic protein antibody (GFAP, dilution 1 : 1000; Chemicon Inc., Temecula, CA, U.S.A), rabbit anti-GFAP (dilution 1 : 1000, Chemicon) or mouse anti-neuronal nuclei (Neu-N) antibody (dilution 1 : 1000, Chemicon) followed by incubation with Alexa 488-conjugated goat anti-mouse IgG (dilution 1 : 4000), Alexa 488-conjugated goat anti-rabbit IgG (dilution 1 : 4000) or Alexa 546-conjugated goat anti-mouse IgG (dilution 1 : 4000). Images were collected using a Radiance 2000 laser-scanning microscope (Radiance 2000; Bio-Rad, Carlsbad, CA, USA).

The intensity of GFAP-like immunoreacivity was measured with a computer-assisted system (NIH Image, Bethesda MD, USA). The upper and lower threshold intensity ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and nonimmunoreactive material as white pixels. The area and intensity of pixels within the threshold value representing immunoreactivity were calculated. We randomly chose 10 areas (80 × 80 pixels) for calculation of GFAP-like immunoreactivity in each image (512 × 512 pixels). The experiments were repeatedly performed by at least three independent culture preparations. The intensity of GFAP-like immunoreactivity was expressed as a percent increase (mean ± SEM) with respect to that in control cells, which were seeded on the same plate.

To evaluate the apoptotic neuronal cell death, mouse midbrain neurone/glia cocultures were treated with normal medium, BPA (1 pM, 1 nM or 1 μM) or E_2 (1 pM, 1 nm or 1 μM) for 24 h. The cells were then identified by immunofluorescence, using rabbit-anticleaved caspase-3 antibody (dilution 1 : 100; Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488 conjugated goat anti-rabbit IgG (dilution 1 : 10000). Images were collected using a Radiance 2000 laser-scanning microscope.

Confocal Ca^{2+} imaging

Confocal Ca^{2+} imaging was conducted as previously described (13, 16). Mouse midbrain neurone/glia cocultures or purified astrocytes were incubated for 24 h with normal medium or medium containing BPA (1 pM, 1 nM or 1 μM). Cells were then loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Kumamoto, Japan) during a 90-min incubation at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the cells which seeded on coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000). Fluo-3 was excited with the 488-nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cell was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (ratio: $R_s = F/F_0$). The amplitude was determined by subtracting the average of baseline fluorescence ratio (F_{basal}/F_0) from the maximum of fluorescence ratio after a drug treatment (F_{max}/F_0). Dopamine (1, 10 or 100 μM; Sigma-Aldrich) was perfused via a plastic tube for 30 s at 5 ml/min at room temperature in cultured cortical neurones or astrocytes followed by superfusion of basal salt solution (BSS,

