

### Delayed effects of neonatal oral exposure to ethynylestradiol

in the both experiments if possible. When the numbers of female neonates in dams were less than 8, male offspring were placed under the dams to adjust the size. There were two exceptions in the litter size; 10 female neonates were placed under one dam in the main experiment, and 13 female neonates were placed under one dam in the satellite experiment until blood collection on PND 2.

In the main experiment, EE was orally administered for 5 days from PND 1 at a dose level of 0 (corn oil 10 mL/kg), 0.4, or 2 µg/kg using an intubation tube prepared according to Watanabe *et al.* (2003). In the satellite experiment, EE was orally administered at PND 1 or for 5 days from PND 1 using the same method as that used in the main study at a dose level of 2 µg/kg. The body weight of the neonates was individually measured daily during the administration period in the both experiments and weekly measured from PND 7 and on the day of necropsy in the main experiment. Their general condition was observed as well.

In the satellite experiment, blood was collected from the hearts or abdominal vessels at 24 hr after the first dosing at PND 1 or at 6 or 24 hr after the final dosing at PND 5, respectively, from 8-14 animals. The collected blood was centrifuged at 2,500 rpm for 25 min at 4°C, following which serum was collected and kept at -50°C until determination of the EE concentration.

In the main experiment, at PND 10, 4-5 animals from each group under 2 dams were killed by decapitation. Then, their ovaries were collected. The collected ovaries were fixed in Bouin's solution to determine the number of primordial follicles. Animals under the other dams were determined for the age at eyelid opening, and were weaned at PND 21. From PND 28, the animals were observed for vaginal opening. For animals found to have a vaginal opening, the body weight was measured as well. From postnatal week (PNW) 8, the animals in each group were periodically monitored for the estrous cycle by observing the vaginal cytology for 2 weeks at 2-week intervals until PNW 21.

Necropsy was performed at PNW 22-23 on the day of estrus if at all possible. For necropsy, animals were killed by bleeding from the abdominal aorta under anesthesia with sodium pentobarbital (Somnopentyl; Schering-Plough Animal Health, Osaka, Japan). Following necropsy, the pituitary gland, mammary glands, and major thoracic and abdominal organs or tissues were dissected. Among these, mammary glands were fixed in a phosphate-buffered 10% formalin solution, and the ovaries were fixed in Bouin's solution. Oviducts were collected to determine ovulation using the method of Burdick and Whitney (Burdick and Whitney, 1941). These tis-

ues were dehydrated and embedded in paraffin for further examination.

#### Determination of serum EE concentrations

The serum EE concentration was determined by ultra-fast liquid chromatography (Prominence; Shimadzu Scientific Instruments, Kyoto, Japan) and triple quadrupole mass spectrometry (API 5000; AB Sciex Ltd., Framingham, MA, USA) at Sumika Chemical Analysis Service, Ltd. (Osaka, Japan) according to the methods described by Borgers *et al.* (2009). Prior to analysis, serum samples collected from 3-5 animals after the initial treatment or from 2-3 animals after the final treatment were pooled to 1 sample (300 µL), and 10 µL of internal standard, 50 ng/mL of ethynylestradiol-*d*<sub>4</sub> solution, and 150 µL of 0.1 N hydrochloride were added to each sample. These samples were mixed with 4 mL of *tert*-butyl ether, following which the organic phase of the mixture was transferred into a glass tube. The tubes were evaporated to dryness and were then mixed with 150 µL of dansylchloride (1 mg/mL in acetone) and 150 µL of carbonate pH standard solution (pH 10.01) to be derivatives. They were incubated for 30 min at 60°C, following which the derivatives were extracted with 3 mL of hexane. The organic phase was evaporated to dryness, and the residue was dissolved in 200 µL of acetonitrile and water mixture (1:1 v/v). The samples were applied to the system after ultrafiltration (Centricut Ultramini; Kurabo, Osaka, Japan). Chromatography and mass spectrometry conditions are shown in Table 1. In the present analysis, the lowest and highest quantification limits were 3 pg/mL and 30 ng/mL, respectively.

#### Classification of estrous cycle

Estrous cycles were categorized into 3 types: 4-5-day estrous cycle, persistent estrus, and irregular cycle. That is, animals in which the estrus day revolved at 4- or 5-day intervals were classified into the 4-5-day estrous cycle, those showing no consecutive diestrus/metestrus day were classified into persistent estrus, and those not classified into either of these 2 patterns were classified into the irregular cycle. In addition, the cumulative estrus or proestrus days and the cumulative metestrus or diestrus days were calculated for each period.

#### Determination of the number of primordial follicles and morphological analysis in the ovary

The paraffin-embedded ovarian tissues were serially sectioned at 5 µm thickness and were stained with hematoxylin and eosin. The number of primordial follicles was determined for ovaries from 4-5 neonates at PND 10 and

**Table 1.** Chromatography and mass spectrometry conditions in the determination of serum 17 $\alpha$ -ethynylestradiol (EE) concentration.

Ultra-fast liquid chromatography condition							
Analytical column	Synergi 4 $\mu$ Polar-RP 80A, 2.0 nm I.D. x 50 mm L., Phenomenex						
Column temperature	40°C						
Mobile phase	A: 0.1% formic acid B: 0.1% formic acid containing acetonitrile						
Gradient condition	Time (min)	0.00	0.50	4.00	5.00	5.01	8.00
	Mobile phase (%)	60	60	90	90	60	stop
Flow rate	0.3 mL/min						
Triple quadrupole mass spectrometry condition							
Collision gas	Set at 6 arbitrary unit, nitrogen						
Scan mode	Multiple reaction monitoring mode						
Monitored ions							
Dansyl-ethynylestradiol	Precursor ion: m/z 530; Production ion: m/z 171						
I.S. (dansyl-ethynylestradiol- $d_4$ )	Precursor ion: m/z 534; Production ion: m/z 171						

I.S., internal standard

**Table 2.** Serum 17 $\alpha$ -ethynylestradiol (EE) concentration of female neonates after oral treatment with 2  $\mu$ g/kg/day of EE for 5 days from postnatal day (PND) 1.

Time after treatment	Number of samples	EE (pg/mL)
At 24 hr after the initial treatment	4	12.8 $\pm$ 4.5 <sup>a</sup>
At 6 hr after the final treatment	3	47.3 $\pm$ 5.2 <sup>b</sup>
At 24 hr after the final treatment	3	6.7 $\pm$ 5.2 <sup>a</sup>

Values represent mean  $\pm$  S.E.M. Each sample consists of serum from 3-5 neonates at 24 hr after the initial treatment and from 2 or 3 neonates at 6 and 24 hr after the final treatment. Different characters shown in the serum EE concentration represent significantly different at  $P < 0.05$  by the Tukey-Kramer honest significant difference (HSD) test.

from 3 animals at PNW 22-23 in each group according to a previously reported method (Shirota *et al.*, 2003). Ovarian tissues showing the presence of cystic follicles and corpus luteum were observed on the serial sections in all of the animals.

#### Immunohistochemistry of mammary gland

To define the localization of mammary acini and ducts in mammary tissues from nulliparous animals,  $\alpha$ -smooth muscle actin (SMA)-expressing cells (i.e., myoepithelial cells surrounding the acini and ducts) were detected by immunohistochemistry with anti-SMA antibody (clone 1A4; Dako A/S, Glostrup, Denmark) as described previously (Yasuno *et al.*, 2013). The mammary tissue specimens were also stained with hematoxylin and eosin.

#### Statistical analysis

Statistical analysis was performed using JMP Statistical Analysis Software (SAS Institute, Cary, NC, USA). Initially, all data were analyzed using a one-way analysis of variance (ANOVA). Differences between the control group and any group receiving EE were analyzed by Dunnett's test. A  $P$  value of less than 0.05 was considered statistically significant.

## RESULTS

#### Serum concentration of EE after oral administration

The serum concentration of EE in the neonates after oral administration of 2  $\mu$ g/kg of EE is summarized in Table 2. Each time point consists of 3-4 pooled serum

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**Table 3.** Body weight changes of female rats orally administered 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

PND	EE ( $\mu\text{g}/\text{kg}/\text{day}$ )		
	0	0.4	2
1	6.7 $\pm$ 0.2 (15)	6.8 $\pm$ 0.2 (15)	6.7 $\pm$ 0.2 (15)
2	7.5 $\pm$ 0.3 (15)	7.4 $\pm$ 0.2 (15)	7.5 $\pm$ 0.2 (15)
3	8.6 $\pm$ 0.3 (14) <sup>a</sup>	8.3 $\pm$ 0.3 (15)	8.2 $\pm$ 0.2 (15)
4	9.7 $\pm$ 0.3 (14)	9.2 $\pm$ 0.3 (15)	9.4 $\pm$ 0.2 (15)
5	10.8 $\pm$ 0.3 (14)	10.4 $\pm$ 0.4 (15)	10.6 $\pm$ 0.2 (15)
7	16.2 $\pm$ 0.5 (14)	15.5 $\pm$ 0.6 (15)	15.7 $\pm$ 0.4 (15)
10	25.0 $\pm$ 0.7 (14) <sup>b</sup>	23.0 $\pm$ 1.0 (15) <sup>b</sup>	23.3 $\pm$ 0.4 (15) <sup>b</sup>
14	35.8 $\pm$ 0.9 (10)	34.7 $\pm$ 0.9 (9)	34.2 $\pm$ 0.5 (10)
21	60.0 $\pm$ 2.0 (10)	58.0 $\pm$ 2.0 (9)	56.5 $\pm$ 1.4 (10)
28	96.2 $\pm$ 3.1 (10)	91.2 $\pm$ 3.0 (9)	90.9 $\pm$ 2.5 (10)
35	137.8 $\pm$ 4.4 (10)	133.2 $\pm$ 4.5 (9)	136.1 $\pm$ 4.2 (10)
42	174.8 $\pm$ 5.2 (10)	170.8 $\pm$ 4.9 (9)	176.9 $\pm$ 3.6 (10)
49	205.0 $\pm$ 5.6 (10)	198.8 $\pm$ 5.2 (9)	205.2 $\pm$ 5.2 (10)
56	228.1 $\pm$ 6.0 (10)	219.3 $\pm$ 6.3 (9)	227.7 $\pm$ 4.1 (10)
63	247.4 $\pm$ 6.8 (10)	239.6 $\pm$ 6.9 (9)	246.7 $\pm$ 4.0 (10)
70	264.5 $\pm$ 8.2 (10)	254.0 $\pm$ 8.3 (9)	263.9 $\pm$ 5.2 (10)
77	280.7 $\pm$ 9.4 (10)	266.6 $\pm$ 8.8 (9)	277.7 $\pm$ 5.2 (10)
84	293.7 $\pm$ 9.1 (10)	281.9 $\pm$ 9.1 (9)	292.5 $\pm$ 6.0 (10)
91	299.9 $\pm$ 9.0 (10)	291.6 $\pm$ 9.6 (9)	306.4 $\pm$ 6.6 (10)
98	310.4 $\pm$ 11.0 (10)	299.2 $\pm$ 10.5 (9)	314.2 $\pm$ 7.8 (10)
105	319.3 $\pm$ 10.8 (10)	308.3 $\pm$ 11.3 (9)	324.2 $\pm$ 9.0 (10)
112	328.4 $\pm$ 11.1 (10)	322.7 $\pm$ 11.4 (9)	340.3 $\pm$ 9.1 (10)
119	331.6 $\pm$ 11.4 (10)	308.3 $\pm$ 12.3 (9)	324.2 $\pm$ 9.9 (10)
126	336.1 $\pm$ 12.7 (10)	328.0 $\pm$ 13.6 (9)	344.9 $\pm$ 10.5 (10)
133	342.7 $\pm$ 13.6 (10)	335.3 $\pm$ 14.7 (9)	352.9 $\pm$ 11.7 (10)
140	344.3 $\pm$ 12.3 (10)	342.3 $\pm$ 15.3 (9)	359.4 $\pm$ 11.6 (10)
147	347.9 $\pm$ 13.1 (10)	347.6 $\pm$ 15.5 (9)	367.2 $\pm$ 12.6 (10)
154	352.2 $\pm$ 13.5 (10)	352.6 $\pm$ 16.2 (9)	373.4 $\pm$ 14.0 (10)

<sup>a</sup> Died by intubation error. <sup>b</sup> Parts of animals were sacrificed at PND 10 to collect ovaries. Values represent mean (g)  $\pm$  S.E.M., and numbers in parentheses indicate the number of animals examined.

samples collected from 2-3 neonates. The highest serum level among all the time points was observed at 6 hr after the final administration. The value was significantly different from those at 24 hr after the initial and the final administration; however, no significant difference was observed between those at 24 hr after the initial and the final administration.

### General condition, physical growth, and timing of vaginal opening

There was no abnormal general condition related to the EE treatment during the experimental period. No significant difference was observed in the body weight (Table 3), whereas body weight gain during PNW 12-13 in the 2  $\mu\text{g}/\text{kg}$ -treated group was significantly greater than that in the control group (data not shown).

Table 4 summarizes the timing of eyelid opening and

**Table 4.** Ages and body weights at eyelid opening and vaginal opening of female rats orally administered 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

Dose of EE ( $\mu\text{g}/\text{kg}/\text{day}$ )	0	0.4	2
Number of animals examined	10	9	10
Ages at eyelid opening (day)	13.5 $\pm$ 0.2	13.8 $\pm$ 0.2	13.7 $\pm$ 0.2
BW at eyelid opening (g)	34.8 $\pm$ 0.8	34.2 $\pm$ 0.9	33.4 $\pm$ 0.8
Ages at vaginal opening (day)	33.0 $\pm$ 0.8	33.1 $\pm$ 0.8	31.9 $\pm$ 0.8
BW at vaginal opening (g)	125 $\pm$ 3	121 $\pm$ 3	115 $\pm$ 3

Values represent mean  $\pm$  S.E.M.

vaginal opening of the treated animals. Eyelid opening, one of the landmarks of physical development, was confirmed at identical ages among the groups. The age at vaginal opening was slightly advanced in the 2  $\mu\text{g}/\text{kg}$ -treated group; however, no significant difference was observed among the groups.

#### Effects on estrous cycle

Distributions of animals showing respective patterns of the estrous cycle and the ratio of cumulative number of estrus or proestrus days and that of metestrus or diestrus days are illustrated in Figs. 1 and 2, respectively. The number of estrous cycles revolved and the cumulative number of estrus or proestrus days during each observation period are summarized in Table 5. In the control group, the distribution and the cumulative number of estrus or proestrus days and that of metestrus or diestrus or days were not significantly different between those at PNW 8-9 and the rest of the periods, and only 1 animal showed persistent estrus from PNW 20. In the 0.4  $\mu\text{g}/\text{kg}$ -treated group, no significant differences were observed in comparison with those parameters in the control group until PNW 9. From PNW 12, however, animals in this group began to show persistent estrus (Fig. 2), and the number of estrous cycles revolved during PNW 12-13 or older ages was significantly smaller than that in the control (Table 5). By PNW 21, none of the animals showed a normal estrous cycle. In the 2  $\mu\text{g}/\text{kg}$ -treated group, none of the animals showed a normal estrous cycle at the beginning of the monitoring, and the number of estrous cycles revolved was significantly reduced at PNW 8-9.

#### Ovulation and reproductive organ weights at terminal necropsy

Ovulation and weights of the ovaries and uterus are summarized in Table 6. In the control group, 3 animals failed to ovulate on the day of vaginal estrus. Among these, 1 animal showed a normal estrous cycle and the

other 2 showed persistent estrus or an irregular cycle by the day of necropsy. In contrast, none of the animals in the EE-treated groups showed ovulation at necropsy, and the uterine weight in the 2  $\mu\text{g}/\text{kg}$ -treated group was significantly lower than that in the control group that failed to ovulate. Ovarian weights in the EE-treated groups were lower than those in the control group; however, no significant difference was observed in comparison with that in control animals that failed to ovulate.

#### Number of primordial follicles

As shown in Fig. 3, the numbers of primordial follicles counted at PNW 22-23 were significantly lesser than those counted at PND 10 in respective groups; however, no significant difference was observed among the groups at any age.

#### Ovarian histology

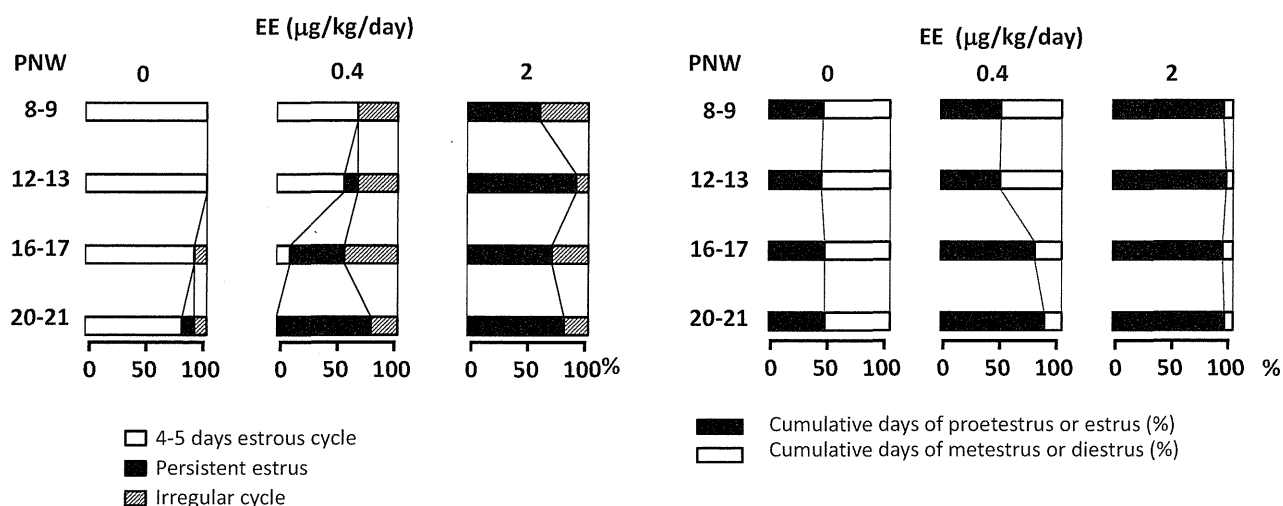
The summary of histological evaluation of ovaries and representative histology are shown in Table 7 and Fig. 4, respectively.

Among the ovaries of the control group (Fig. 4A), those from 2 animals that failed to ovulate lacked corpus luteum, and those from 5 animals developed cystic follicles. In the EE-treated groups (Fig. 4B), all the ovaries lacked corpus luteum, except that from 1 animal in the 0.4  $\mu\text{g}/\text{kg}$ -treated group, and all developed cystic follicles.

#### Mammary glands

Spotted or macular accumulations of milky solution were observed beneath the skin in 2 out of 9 and 6 out of 10 animals in the 0.4  $\mu\text{g}/\text{kg}$ - and 2  $\mu\text{g}/\text{kg}$ -treated groups, respectively, whereas none of the animals in the control group showed such changes. Immunohistochemistry with anti-SMA antibody clearly showed myoepithelial cells around the lactiferous ducts and secretory acini of the mammary gland. In the control group, lactiferous

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**Fig. 1.** Distributions of animals showing a 4-5-day cycle, irregular cycle, or persistent estrus in each observation period in the animals administered 0, 0.4 or 2 µg/kg of 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

**Fig. 2.** Proportions of days judged as estrus or proestrus (estrus/proestrus days) and those judged as diestrus or metestrus (metestrus/diestrus days) during each observation period in the animals administered 0, 0.4, or 2 µg/kg of 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

**Table 5.** Effects of neonatal 17 $\alpha$ -ethynylestradiol (EE) exposure on the number of estrous cycles revolved and the number of estrus/proestrus days for each observation period in the female rats orally administered EE for 5 days from postnatal day (PND) 1.

Dose of EE (µg/kg/day)	0	0.4	2
Number of animals examined	10	9	10
Postnatal week (PNW)	The number of estrous cycle revolved		
8-9	2.4 ± 0.2	1.9 ± 0.2	0.1 ± 0.2**
12-13	2.3 ± 0.2	1.6 ± 0.2**	0.3 ± 0.2**
16-17	2.2 ± 0.2	0.8 ± 0.2**	0.2 ± 0.2**
20-21	2.1 ± 0.3	0.7 ± 0.3**	0.2 ± 0.3**
	The number of estrus/proestrus days		
8-9	6.3 ± 0.5	7.0 ± 0.5	12.9 ± 0.5**
12-13	6.1 ± 0.5	6.9 ± 0.5	13.2 ± 0.5**
16-17	6.5 ± 0.7	10.9 ± 0.7**	12.8 ± 0.7**
20-21	6.5 ± 0.9	12.0 ± 0.9**	13.0 ± 0.9**

Values represent mean ± S.E.M. \* and \*\*, significantly different from control at  $P < 0.05$  and 0.01, respectively.

ducts without development of acini were scattered in the fat tissue (Fig. 5A). On the other hand, increased numbers of lactiferous ducts with developed acini were distinct in the EE-treated mammary gland (Figs. 5A and B), indicating mammary lobular hyperplasia which seems to actively secrete milk (Fig. 5C).

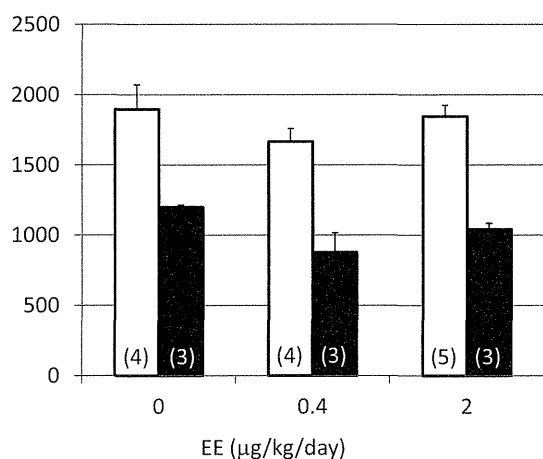
### Non-reproductive organ weights

Weights of non-reproductive organs are summarized in Table 8. Pituitary weight was significantly increased in the groups administered 0.4 µg/kg or more, and liver weight was significantly increased in the group administered 2 µg/kg. Adrenal weight was also increased in the 2 µg/kg-treated group; however, no statistical difference

**Table 6.** Weights of reproductive organs of female rats orally administered 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

Dose of EE ( $\mu\text{g}/\text{kg}/\text{day}$ )	0	0.4	2
Number of animals examined	10	9	10
Number of animals found ovulation	7	0	0
Body weight (g)	353 $\pm$ 13		
Number of oocytes	12.7 $\pm$ 1.0 <sup>a</sup>		
Ovaries (mg)	85 $\pm$ 3		
Uterus (mg)	610 $\pm$ 27		
Number of animals found no ovulation	3	9	10
Body weight (g)	358 $\pm$ 27	363 $\pm$ 16	379 $\pm$ 15
Ovaries (mg)	63 $\pm$ 9	59 $\pm$ 5	45 $\pm$ 5
Uterus (mg)	640 $\pm$ 61	584 $\pm$ 35	465 $\pm$ 33 <sup>**</sup>

Values represent mean  $\pm$  S.E.M. \*\*, significantly different from control at  $P < 0.01$ . <sup>a</sup>Number of oocytes could be determined for 6 animals.



**Fig. 3.** Number of primordial follicles counted at postnatal day (PND) 10 or postnatal week (PNW) 22-23 in the ovaries from animals orally administered 17 $\alpha$ -ethynylestradiol (EE) for 5 days from PND 1. Open columns represent the average number at PND 10, and closed columns represent that at PNW 22-23. Vertical bars indicate S.E.M., and parentheses indicate the number of animals examined.

was observed from that in the control group.

## DISCUSSION

This study clearly demonstrated that neonatal oral exposure to EE arrests the estrous cycle even at a dose level of 0.4  $\mu\text{g}/\text{kg}/\text{day}$ . The treatment increased the circulating EE levels 6 hr after the final dosing at that of estradiol-17 $\beta$  on the day of proestrus (Smith *et al.*, 1975;

Asai *et al.*, 2002; Nozawa *et al.*, 2014; Usuda *et al.*, 2014) even at 2  $\mu\text{g}/\text{kg}/\text{day}$  of EE. Because the relative *in vitro* estrogenic activity of EE was found to be 90-250% of estradiol-17 $\beta$  (Soto *et al.*, 1995; Coldham *et al.*, 1997; Fang *et al.*, 2000; Nishihara *et al.*, 2000; Sanseverino *et al.*, 2009), estrogenic activity of the circulating EE after the administration of 0.4  $\mu\text{g}/\text{kg}/\text{day}$  seemed to be equivalent to or lower than basal estradiol-17 $\beta$  levels in the cyclic rats. Thus, very slight estrogenic stimulus during the neonatal period exerts irreversible effects on the animals.

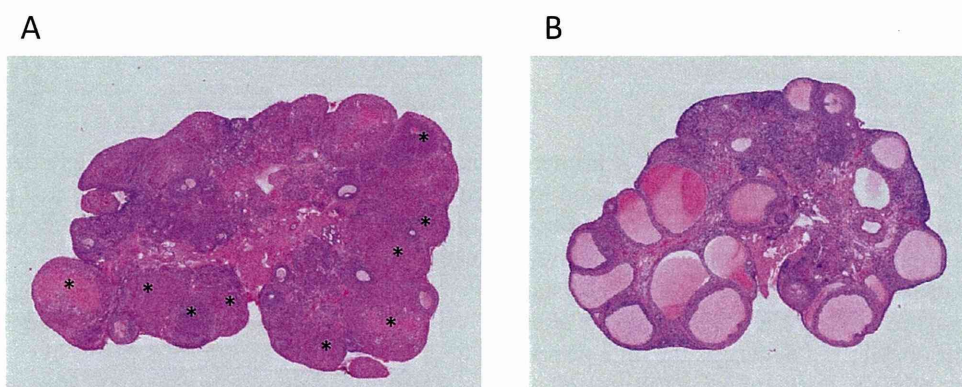
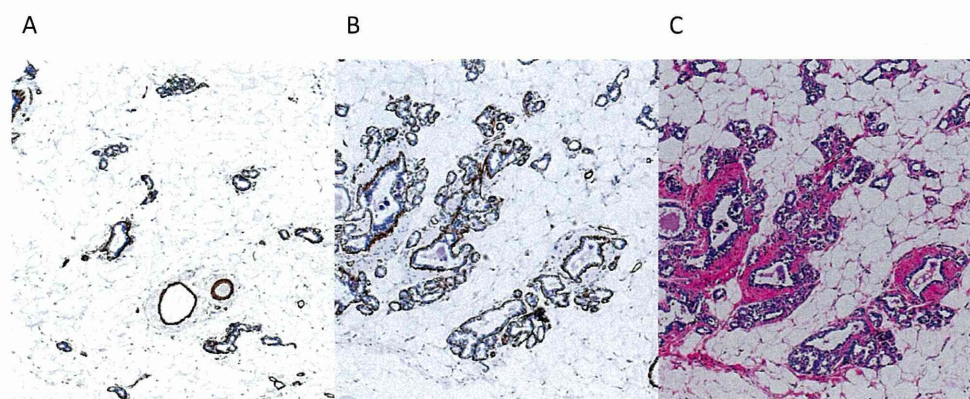
In the present study, we showed that repeated administration of EE at a dose level of 0.4  $\mu\text{g}/\text{kg}/\text{day}$  arrests the estrous cycle from PNW 12 to 13. We have previously confirmed that a single s.c. injection of EE at PND 1 arrested the estrous cycle from PNW 20 even at a dose level of 2  $\mu\text{g}/\text{kg}$ . Similar results were also reported in the study with EE doses ranging from 0.02 to 200  $\mu\text{g}/\text{kg}$  at PND 0, and more than 20  $\mu\text{g}/\text{kg}$  of EE affected the estrous cycle from PNW 12 (Takahashi *et al.*, 2013). Because we confirmed that orally administered EE was cleared within 24 hr, repeated estrogenic stimulus during the neonatal period may exert greater effects on revolution of the estrous cycle. Gene expression of ER $\alpha$ , by which EE exerts its effects, increases in the hypothalamus during the perinatal period in female mice (Mogi *et al.*, 2015) and female rats (Walker *et al.*, 2012). Therefore, daily estrogenic stimulus may have a greater effect on the hypothalamus than a single administration.

The ovarian histology of EE-treated animals lacked corpus luteum and formed cystic follicles; these findings are frequently observed in the ovaries of middle-

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**Table 7.** Incidence of animals showing absence of corpus luteum and formation of cystic follicles in ovaries and white spots in mammary glands at terminal necropsy from female rats orally administered 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

Dose of EE ( $\mu\text{g}/\text{kg}/\text{day}$ )	0	0.4	2
Number of animals examined	10	9	10
Absence of corpus luteum	2	8	10
Presence of cystic follicles	5	8	10

**Fig. 4.** Representative histology of ovaries collected at postnatal week (PNW) 22-23 from animals orally treated with corn oil (A) or 17 $\alpha$ -ethynylestradiol (EE; B) for 5 days from postnatal day (PND) 1. In the ovary from the EE-treated animals, large cystic follicles share ovarian tissue, and corpus luteum was not confirmed in the tissue. Asterisks indicate corpus luteum formed at recent ovulations. Hematoxylin and eosin staining.**Fig. 5.** Representative histology of mammary glands at postnatal week (PNW) 22-23 from animals orally treated with corn oil (A) or 2  $\mu\text{g}/\text{kg}$  of 17 $\alpha$ -ethynylestradiol (B and C) for 5 days from postnatal day (PND) 1. (A, B) Immunohistochemistry of  $\alpha$ -smooth muscle actin; (C) hematoxylin and eosin staining.

aged rats (Bukovsky *et al.*, 2000). Some ovaries from animals in the control group developed cystic follicles and lacked corpus luteum. In the EE-treated group, however, the instances of animals showing these findings were increased in a dose-dependent manner. Because none of the animals in the EE-treated groups failed to ovulate on

the day of estrus, the treatment may cause long-term anovulation, probably because of incomplete or abolished gonadotropin surges, as reported in middle-aged animals (Downs and Wise, 2009) and in animals treated with EE s.c. at PND 0 (Usuda *et al.*, 2014; Nozawa *et al.*, 2014; Ichimura *et al.*, 2015).

**Table 8.** Weights of nonreproductive organs of female rats orally administered 17 $\alpha$ -ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

Dose of EE ( $\mu$ g/kg/day)	0	0.4	2
Number of animals examined	10	9	10
BW at necropsy (g)	354 $\pm$ 14	363 $\pm$ 15	379 $\pm$ 14
Pituitary (mg)	15.2 $\pm$ 1.0	22.7 $\pm$ 1.9*	22.2 $\pm$ 2.7*
Liver (g)	11.1 $\pm$ 0.3	12.4 $\pm$ 0.5	13.6 $\pm$ 0.5**
Kidneys (g)	2.1 $\pm$ 0.1	2.2 $\pm$ 0.1	2.4 $\pm$ 0.1*
Adrenal glands (mg)	75.3 $\pm$ 2.6	79.0 $\pm$ 4.6	82.7 $\pm$ 4.7

Values represent mean  $\pm$  S.E.M. \* and \*\*, significantly different from control at  $P < 0.05$  and  $0.01$ , respectively.

Despite long-term anovulation, the number of primordial follicles, a stockpile of oocytes, was decreased by aging in the EE-treated ovaries, as observed in the control ovaries. It has been reported that s.c. injection of diethylstilbestrol (DES) or bisphenol A (BPA) to female Wistar rats at 48-hr intervals from PND 1 to PND 7 does not alter the total number of oocytes but decreases the ratio of primordial follicles at PND 8. Furthermore, DES treatment increases multiple organ failure (MOF) in rats (Rodríguez *et al.*, 2010), as found in mice treated with various ER agonists (Iguchi *et al.*, 1990; Chen *et al.*, 2009; Cimafranca *et al.*, 2010). Under the present experimental condition, however, MOF was not increased in the EE-treated group, and their primordial follicle stockpile may supply to the growing phase.

In addition to effects on the reproductive organs, neonatal rats receiving EE treatment developed spotted or macular accumulations of milky solution beneath the skin. Histopathological observation revealed mammary lobular hyperplasia, which was found in the animals administered EE s.c. during the neonatal period (Shirota *et al.*, 2012; Takahashi *et al.*, 2013). Similar macroscopic findings have also been reported in a study in which DES was orally administered to neonatal rats (Ohta *et al.*, 2012). However, all these previous reports confirmed the findings at elder ages than those in the present study. Repeated oral exposure to EE may advance the development of mammary alveolar hyperplasia in nulliparous rats. Because neonatal exposure to estrogenic compounds can directly cause morphological or molecular alterations in the mammary gland (Umekita *et al.*, 2011; Betancourt *et al.*, 2010; Ayyanan *et al.*, 2011; Moral *et al.*, 2011), exposure to EE during the neonatal period may exert direct effects on undifferentiating mammary tissue or may exert indirect effects via endocrinological alterations.

In conclusion, the present study clearly indicated that a slight increase in circulating estrogens during the neo-

natal period exerts irreversible delayed effects on females and that repeated oral exposure (the most probable route of exposure of humans and wildlife to xenoestrogens) exerts such effects.

## ACKNOWLEDGMENTS

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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# Effects of sulpiride and ethylene glycol monomethyl ether on endometrial carcinogenicity in Donryu rats

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**ABSTRACT:** Sulpiride and ethylene glycol monomethyl ether (EGME) are known ovarian toxicants that stimulate prolactin (PRL) secretion, resulting in hypertrophy of the corpora lutea and increased progesterone (P4) production. The purpose of the present study was to investigate how the PRL stimulatory agents affected uterine carcinogenesis and to clarify the effects of PRL on endometrial adenocarcinoma progression in rats. Ten-week-old female Donryu rats were treated once with *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (20 mg kg<sup>-1</sup>), followed by treatment with sulpiride (200 ppm) or EGME (1250 ppm) from 11 weeks of age to 12 months of age. Sulpiride treatment inhibited the incidence of uterine adenocarcinoma and precancerous lesions of atypical endometrial hyperplasia, whereas EGME had no effect on uterine carcinogenesis. Sulpiride markedly prevented the onset of persistent estrus throughout the study period, and EGME delayed and inhibited the onset of persistent estrus. Moreover, sulpiride-treated animals showed high PRL and P4 serum levels without changes in the levels of estradiol-17 $\beta$ , low uterine weights and histological luteal cell hypertrophy. EGME did not affect serum PRL and P4 levels. These results suggest that the prolonged low estradiol-17 $\beta$  to P4 ratio accompanied by persistent estrous cycle abnormalities secondary to the luteal stimulatory effects of PRL may explain the inhibitory effects of sulpiride on uterine carcinogenesis in rats. Copyright © 2015 John Wiley & Sons, Ltd.

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**Keywords:** prolactin; endometrial adenocarcinoma; sulpiride; ethylene glycol monomethyl ether; Donryu rats

## Introduction

Endometrial cancer is one of the most common malignancies of the female genital tract, with the majority of cases arising in post-menopausal women (Persson, 2000). Ovarian steroid hormones have critical roles in uterine carcinogenesis, and an increase in the ratio of estradiol-17 $\beta$  (E<sub>2</sub>) to progesterone (P4) in the blood is associated with a high risk of uterine cancer in women and rodents (Maekawa *et al.*, 1999). However, the effects of other pituitary or ovarian hormones on uterine carcinogenicity are not well known.

Prolactin (PRL) has important roles in genital function, including lactation and pregnancy, in both humans and rodents (Ben-Jonathan *et al.*, 2008). PRL is produced by endometrial stromal cells and the pituitary gland and regulates stromal proliferation and differentiation by the autocrine/paracrine system (Ben-Jonathan *et al.*, 2002; Tseng and Mazella, 1999). However, little is known about the role of PRL in uterine carcinogenicity.

Some drugs and chemicals have been shown to disrupt the hypothalamic–pituitary–gonadal system and induce carcinogenicity in female genital organs (Soto and Sonnenschein, 2010; Yuan and Foley, 2002). Sulpiride and ethylene glycol monomethyl ether (EGME) are known ovarian toxicants that induce luteal hypertrophy and increase PRL and P4 secretions following repeated administration (Davis *et al.*, 1997; Dodo *et al.*, 2009; Taketa *et al.*, 2011a, b, 2013). Sulpiride, a dopamine D2 antagonist, is used clinically as an atypical antipsychotic drug (Lacruz *et al.*, 2000). In rats, D2 antagonists block the inhibitory effects of dopamine on PRL release, resulting in the preservation of functional corpora lutea (CL). The

resulting serum P4 elevation produces a pseudopregnant state and disrupts the estrous cycle (Rehm *et al.*, 2007). EGME, which is widely used in various industrial products such as detergents, adversely affects both the male and female genital systems (Johanson, 2000; Welsch, 2005). In female rats, EGME induces PRL secretion (Taketa *et al.*, 2011b). EGME and its active metabolite, 2-methoxy acetic acid, are also known to induce the hypersecretion of P4 from luteal cells (Almekinder *et al.*, 1997; Davis *et al.*, 1997).

In women as well as rodents, atypical endometrial hyperplasias have been considered precancerous changes for endometrial carcinomas (Nagaoka *et al.*, 1994). Aged Donryu rats exhibit a high incidence of spontaneous uterine endometrial adenocarcinomas, which are similar to human cases as follows: (1) multistep development of uterine lesions from atypical hyperplasias to adenocarcinomas; (2) ovarian hormonal imbalance, in particular,

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elevation of the serum E<sub>2</sub> level relative to P4; and (3) morphologic similarities to endometrioid adenocarcinomas in humans (Maekawa *et al.*, 1986). Thus, Donryu rats are a useful model for assessing endometrial adenocarcinoma in the corpus in women, particularly for endometrioid adenocarcinoma (Yoshida *et al.*, 2009).

The purpose of the present study was to investigate how the PRL stimulatory agents, sulpiride and EGME, affected the development of endometrial adenocarcinoma and to clarify the effects of PRL on the mechanism of endometrial adenocarcinoma progression in the Donryu rat endometrial carcinogenesis model.

## Materials and methods

### Animals

Adult female and male Crj:Donryu rats were purchased from Charles River Japan (Kanagawa, Japan). Females were mated with males on proestrus day, and plugs and sperm in the vagina were used to judge pregnancy. Dams with offspring were housed in plastic cages until the pups were weaned. After weaning, females in the same treatment group were selected and housed in cages (three or four animals per cage). Animals were maintained at 23–25 °C with a relative humidity of 50–60% and a 12 h light/dark cycle. Commercial rodent chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were available *ad libitum*. The stage of the estrous cycle was determined each weekday morning by vaginal smears every week or every other week (Yuan and Foley, 2002). The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

### Chemicals

Sulpiride and EGME were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) was purchased from Nacalai Tesque Inc. (Tokyo, Japan).

### Experimental design

Examination of the long-term effects of sulpiride or EGME on uterine carcinogenicity was performed modifying the Donryu rat initiation–promotion assay model for uterine corpus cancer (Ando-Lu *et al.*, 1994; Yoshida *et al.*, 2009). Briefly, at 10 weeks of age, all female pups were administered a single dose of 20 mg kg<sup>-1</sup> ENNG into the uterine horn using a stainless steel catheter via the vagina. After 1 week, 72 rats (24 in each group) were assigned to three groups: control (basal diet), sulpiride (200 ppm in the diet) and EGME (1250 ppm in drinking water). The effective doses in genital organs were selected and calculated based on relevant literature (Dodo *et al.*, 2009; Ishii *et al.*, 2009; Taketa *et al.*, 2011b).

At 12 months of age, all surviving animals were killed by decapitation, blood serum samples were collected and animals were subjected to necropsy. Animals found dead or moribund during the dosing period were not evaluated. At necropsy, the uterus and ovaries were weighed, and the ovaries, uterus, vagina, pituitary gland, mammary glands, adrenal glands and thyroid glands were fixed in 10% neutral buffered formalin and then processed for routine paraffin-embedded sections stained with hematoxylin and eosin to evaluate the development of proliferative lesions.

### Histological examination

In the uterine examination, the upper, middle and lower parts of the uterine horn and cervix were cut into three pieces each in cross-sections. Endometrial preneoplastic and neoplastic lesions were classified into three degrees of atypical glandular hyperplasia (slight, moderate or severe) and endometrial adenocarcinoma (Fig. 1) according to the current International Harmonization of Nomenclature and Diagnostic Criteria nomenclature and previous study (Dixon *et al.*, 2014; Yoshida *et al.*, 2011). Slight/moderate endometrial hyperplasia refers to increased numbers of glands with slight to moderately atypical cells in focal and/or segmental areas of the endometrium. Severe hyperplasia was composed of diffuse irregular proliferation of atypical glands. Lesions composed of glandular-structured epithelial cells with atypia showing invasive proliferation to the muscle layer or serosa were diagnosed as endometrial adenocarcinomas. Uterine atypical glandular hyperplasia and adenocarcinoma were evaluated according to the following two parameters, *i.e.*, (1) the number of animals bearing the most serious lesions, and (2) the total number of these lesions in three parts of uterine horns and cervix per animal. Data for these two parameters were expressed as the incidence and multiplicity, respectively. Both ovaries from each animal were dissected, and the maximum transverse sections were examined. The vagina, pituitary gland, mammary glands, adrenal glands and thyroid glands were also dissected and examined.

### Hormone assays

The serum concentrations of PRL, P4, E<sub>2</sub>, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and inhibin (INH) were determined using double antibody radioimmunoassays and <sup>125</sup>I-labeled radioligands. P4 and E<sub>2</sub> were measured as described by Taya *et al.* (1985). National Institute of Diabetes and Digestive and Kidney Disease radioimmunoassay kits were employed for measurement of rat PRL, LH and FSH (NIAMDD, NIH, Bethesda, MD, USA) (Taya *et al.*, 1983). Iodinated bovine INH and rabbit anti-bovine INH antibodies (TNDH-1) were used for measurement of immunoreactive serum INH (Hamada *et al.*, 1989).

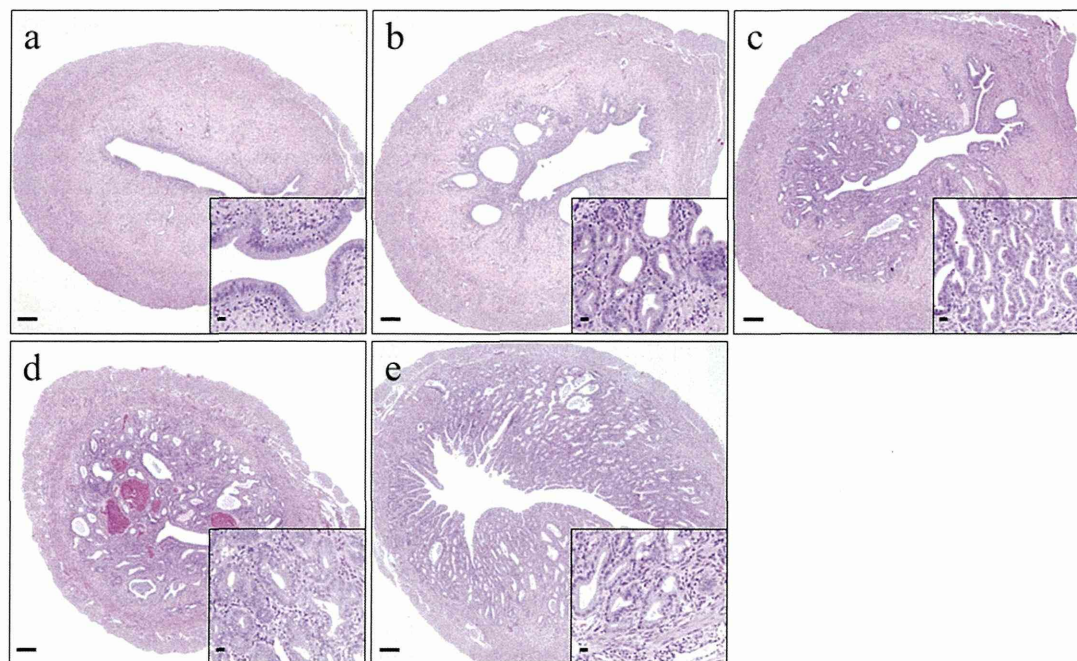
### Statistical analysis

Following Bartlett's test, variance in data for body and organ weights, multiplicity of uterine glandular hyperplasia and hormone assays were compared between treatment and control groups by one-way analysis of variance. When statistically significant differences were detected, Dunnett's multiple comparison test was employed for comparison between the control group and treatment groups. The incidence of histological findings and percentage of persistent estrus was compared using Fisher's exact probability test. In these tests, the level of significance was set at 0.05 or 0.01.

## Results

### Body and organ weights

The final body and organ weights are summarized in Table 1. The average body weight of rats in the sulpiride group was significantly higher than that in controls ( $P < 0.01$ ). Moreover, the absolute and relative uterine weights were significantly lower in the sulpiride group than in the control group ( $P < 0.01$ ). The absolute and



**Figure 1.** Representative histological pictures of endometrial preneoplastic and neoplastic lesions. Normal uterus at 12 months of age showed no endometrial proliferation (a). Atypical glandular hyperplasia was classified into 3 degrees (slight, moderate or severe) as below. Slight, increased numbers of glands with slight atypical cells in focal areas of the endometrium (b); moderate, increased numbers of glands with moderately atypical cells in segmental areas of the endometrium (c); severe, diffuse irregular proliferation of atypical glands (d). Endometrial adenocarcinoma showed invasive glandular proliferation to the muscle layer (e). Hematoxylin and eosin staining. Scale bars = 200  $\mu$ m; inset scale bars = 20  $\mu$ m.

relative ovarian weights in the sulpiride and EGME groups were significantly higher than in the control group (sulpiride,  $P < 0.01$ ; EGME,  $P < 0.05$ ). Body and uterus weights in the EGME group were not changed compared to the control group.

### Estrous cyclicity

The sequential change in the incidence of persistent estrus (showing estrus every day), which is commonly observed in the aging rodent estrous cycle is shown in Fig. 2. In the control group, all animals showed regular 4- or 5-day estrous cycles until 4 months of age, at which point the number of animals showing persistent estrus increased gradually, reaching about 92% at 12 months of age. In contrast, few animals showed persistent estrus throughout the study in the sulpiride group (about 5% at 12 months of age), and most animals showed irregular vaginal smears consistent with persistent diestrus, occasionally containing

mucous fluid, throughout the dosing period. The EGME group showed a tendency for delayed onset of persistent estrus, and significantly lower incidences were observed at 10 and 12 months of age (about 57% and 70%, respectively) compared to that in the control group (about 88% and 92%, respectively) ( $P < 0.05$ ). EGME-treated animals also showed irregular estrous cycles characterized by continuous estrus (2–3 days) or persistent diestrus (more than 3 days) before the onset of persistent estrus.

### Changes in uterine histology

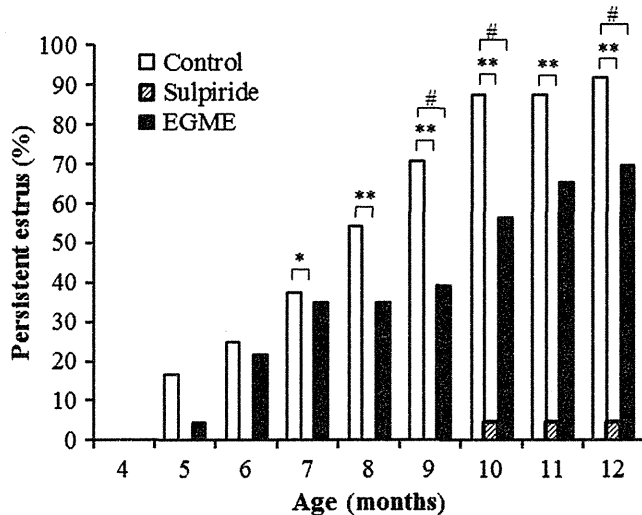
Uterine histological findings are shown in Table 2. In the control group, 83% of animals showed atypical glandular hyperplasia with 3.13 degrees of multiplicity, and 13% of animals showed endometrial adenocarcinoma. In the sulpiride group, the incidence of atypical glandular hyperplasia (36%) was significantly lower than that in the control group ( $P < 0.01$ ), and no endometrial

**Table 1.** Body and organ weights

	Control	Sulpiride	EGME
No. of animals examined	24	22	24
Body weight (g)	309.5 $\pm$ 31.7	356.0 $\pm$ 42.8**	302.6 $\pm$ 26.2
Uterus			
Weight (g)	0.95 $\pm$ 0.32	0.68 $\pm$ 0.24**	0.97 $\pm$ 0.22
g% relative to body weight	0.31 $\pm$ 0.10	0.19 $\pm$ 0.08**	0.32 $\pm$ 0.08
Ovaries			
Weight (mg)	18.6 $\pm$ 5.5	37.3 $\pm$ 8.8**	24.3 $\pm$ 8.9*
mg% relative to body weight	6.0 $\pm$ 1.8	10.6 $\pm$ 2.6**	8.1 $\pm$ 2.8*

\*\*  $P < 0.01$ , significantly different from the control group (Dunnett's multiple comparison test).

\*  $P < 0.05$ , significantly different from the control group (Dunnett's multiple comparison test).



**Figure 2.** Sequential change in the incidence of persistent estrus by vaginal cytology. The percentage of the persistent estrus represents the percentage of animals showing estrus every day. White, diagonal and black columns indicate the control, sulpiride and EGME groups, respectively. \*\* $P < 0.01$  and \* $P < 0.05$ : significant differences between the sulpiride group and control group. # $P < 0.05$ : significant differences between the EGME group and control group. EGME, ethylene glycol monomethyl ether.

adenocarcinoma was observed; however, deciduoma was observed in 14% of animals. The multiplicity of uterine neoplastic lesions in the sulpiride group was 0.5 and significantly lower than that in the control group ( $P < 0.01$ ). In the EGME group, no significant changes in uterine histological carcinogenicity parameters were observed. As non-preneoplastic lesion, diffuse endometrial hyperplasia was observed in several animals only in the sulpiride group.

**Histological changes in the ovaries, vagina and other endocrine organs**

Histological findings in the ovaries and vagina are shown in Table 2. In the ovaries, absence of CL, which suggests persistent anovulation, was observed in almost all animals (96%) in the control group (Fig. 3a). In contrast, sulpiride-treated animals showed a significantly lower incidence of absence of CL (5%) compared with the control group ( $P < 0.01$ ), although 36% of animals showed luteal cell hypertrophy (Fig. 3b). The EGME group showed a significantly lower incidence of absence of CL (67%) than the control group ( $P < 0.05$ ) (Fig. 3c). In the vagina, stromal hyperplasia was observed in half of the control animals (Supplementary Fig. 1), whereas no or few animals showed stromal hyperplasia in the sulpiride and EGME groups. In the sulpiride group, 77% of animals showed mucification/mucinous degeneration; this change was not observed in control animals.

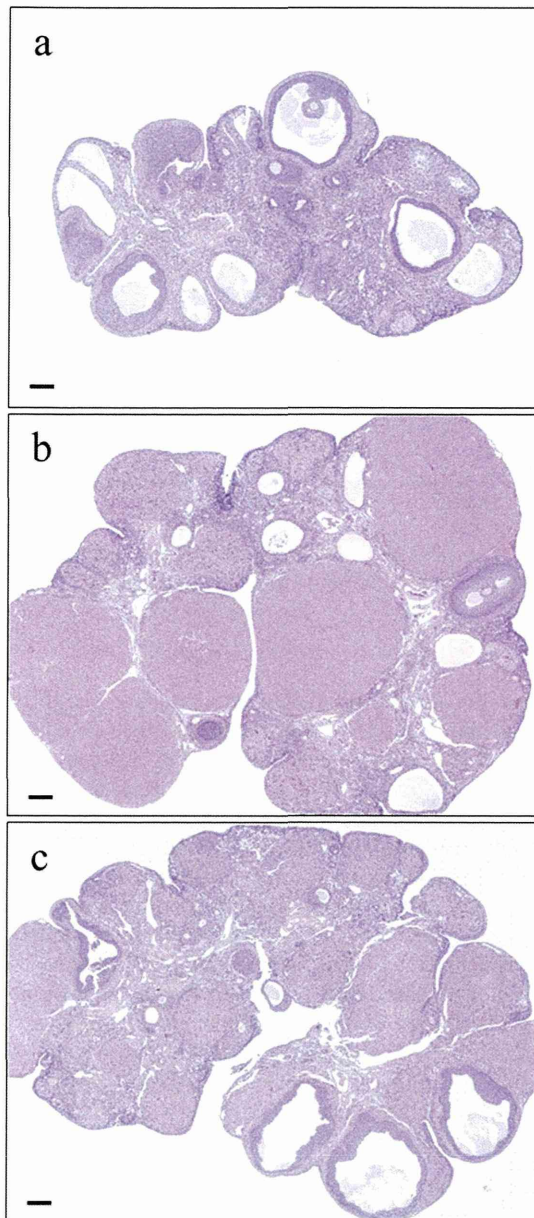
In other endocrine organs, adrenomedullary cell hyperplasia tended to be increased in EGME-treated animals compared with controls. There was no significant change in the incidence of proliferative lesions in the pituitary gland, mammary glands and thyroid glands in the sulpiride and EGME groups and in the adrenal glands in the sulpiride group compared to the control group (data not shown). In the sulpiride group, mammary gland enlargement was observed in almost all animals.

**Changes in levels of sex-related hormones**

Serum hormone levels, determined at necropsy at 12 months of age, are shown in Fig. 4. PRL and P4 levels were significantly higher in the sulpiride group than in the control group ( $P < 0.01$ ). The levels of other hormones (i.e.,  $E_2$ , LH, FSH and INH) in the sulpiride group and the levels of all hormones in the EGME group were not altered compared with those in the control group. Serum hormone

	Control	Sulpiride	EGME
No. of animals examined	24	22	24
Uterus			
Proliferative lesions			
Atypical glandular hyperplasia	20 (83%)	8 (36%)**	18 (75%)
Slight	8	7	6
Moderate	5	1	8
Severe	7	0	4
Endometrial adenocarcinoma	3 (13%)	0	2 (8%)
Deciduoma	0	3 (14%)	0
Non-proliferative lesions			
Diffuse endometrial hyperplasia	0	3 (14%)	0
Multiplicity of hyperplasia and adenocarcinoma	3.13 ± 1.45	0.5 ± 0.74 <sup>††</sup>	2.67 ± 1.83
Ovaries			
Absence of corpora lutea	23 (96%)	1 (5%)**	16 (67%)*
Luteal cell hypertrophy	0	8 (36%)**	1 (4%)
Vagina			
Stromal hyperplasia	12 (50%)	0**	2 (8%)**
Mucification/mucinous degeneration	0	17 (77%)**	1 (4%)

Multiplicity of hyperplasia and adenocarcinoma: mean ± SD.  
 \*\*  $P < 0.01$ , significantly different from the control group (Fisher's exact probability test).  
 \*  $P < 0.05$ , significantly different from the control group (Fisher's exact probability test).  
 ††  $P < 0.01$ , significantly different from the control group (Dunnett's multiple comparison test).



**Figure 3.** Representative histological pictures of the ovaries. In the control animals, absence of the corpora lutea was observed (a). The sulpiride-treated animals showed luteal cell hypertrophy (b). Third part of ethylene glycol monomethyl ether-treated animals retained the corpora lutea (c). Hematoxylin and eosin staining. Scale bars = 200  $\mu$ m.

ratios among  $E_2$ , P4 and PRL are shown in Fig. 5. The  $E_2$  to P4 ratio was significantly lower in the sulpiride group than in the control group ( $P < 0.01$ ). The  $E_2$  to PRL ratio in the EGME group and the P4 to PRL ratio in the sulpiride and EGME groups were significantly higher than in the control group (sulpiride,  $P < 0.01$ ; EGME,  $P < 0.05$ ).

## Discussion

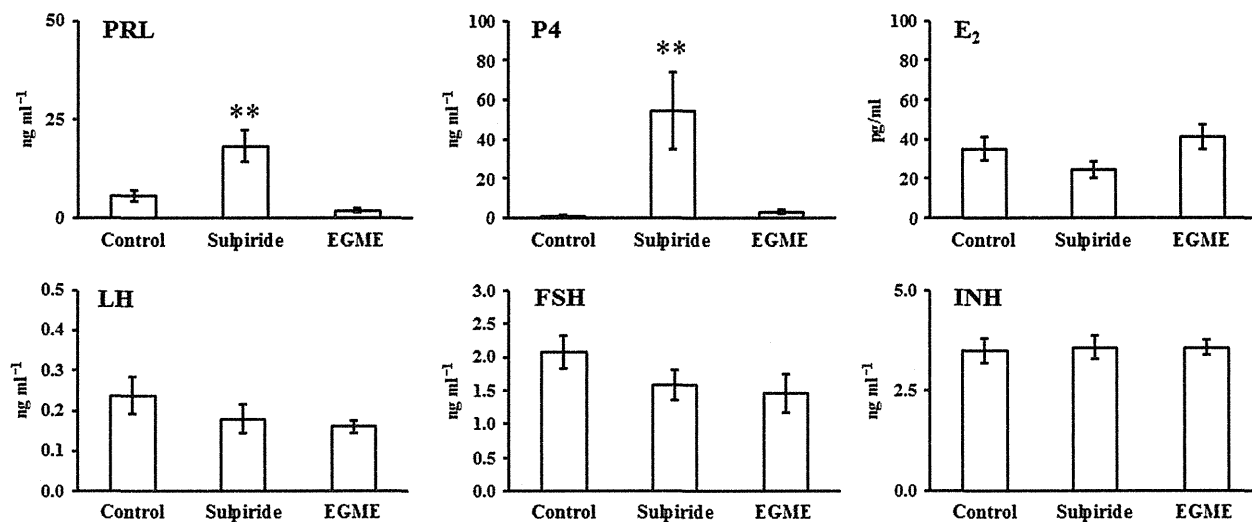
The present study investigated the long-term effects of sulpiride and EGME, known ovarian toxicants affecting PRL release, on uterine carcinogenesis using the Donryu rat endometrial carcinogenesis model. We showed that sulpiride treatment but not EGME

treatment induced a marked decrease in the incidence in uterine proliferative lesions. These results indicated that these two chemicals exerted different effects on uterine tumorigenesis, with sulpiride exhibiting potential inhibitory effects.

Regarding the low incidence of uterine proliferative lesions observed following sulpiride treatment, a marked decrease in the incidence of persistent estrus was observed throughout the study period. Additionally, high blood levels of PRL and P4, lower uterine weights and high ovarian weights were observed in sulpiride-treated animals compared with control animals, and histological luteal cell hypertrophy was concomitantly observed at the termination of the study. Our previous study showed that repeated administration of sulpiride resulted in disturbance of the estrous cycle, characterized by persistent diestrus, histological luteal cell hypertrophy and vaginal mucification (Taketa *et al.*, 2011b). D2 antagonists, such as sulpiride, have an inhibitory effect on dopamine secretion and promote PRL release in the pituitary gland, resulting in the preservation of functional CL, as PRL has stimulatory effects on luteal cells. The resulting serum P4 elevation produces a pseudopregnant state and disrupts the estrous cycle in rats (Rehm *et al.*, 2007). From these reports, the high ovarian weights induced by sulpiride treatment in the present study corresponded to histological luteal cell hypertrophy, indicating the maintenance of functional CL. The lower uterine weights in this group seemed to reflect the low incidence of uterine proliferative lesions as the number of endometrial glands and thicknesses of epithelium were increased in the proliferative lesions that resulted in a higher uterine weight. Taken together, our data demonstrated sustained functional CL and continuous serum PRL elevation in response to sulpiride treatment throughout the dosing period.

There are three hypotheses of the mode of action of rat uterine carcinogenesis: (1) estrogens; (2) increased ratio of  $E_2$  to P4; and (3) modulation of estrogen metabolism (Yoshida and Maekawa, 2005). A prolonged increase in the  $E_2$  to P4 ratio is regarded as an important factor for the development of endometrial carcinoma in humans and rodents, including the Donryu rat model (Ando-Lu *et al.*, 1994; Modan *et al.*, 1998; Nagaoka *et al.*, 1990; Niwa *et al.*, 1991). Considering the present data demonstrating a decrease in the  $E_2$  to P4 ratio and luteal cell hypertrophy in the sulpiride group at termination of the study, sulpiride may induce a prolonged decrease in the  $E_2$  to P4 ratio due to elevated serum P4 levels concomitant with high PRL levels throughout the dosing period. Therefore, the main cause of the inhibitory effects of sulpiride on uterine proliferative lesions may be the prolonged low  $E_2$  to P4 ratio accompanied by abnormal persistent diestrus secondary to the luteal stimulatory effects of PRL. In contrast, there was no clear correlation between the incidence of uterine proliferative lesions and the  $E_2$  to PRL or P4 to PRL ratio among three groups. Thus, these ratios do not seem to be critical for the development of uterine proliferative lesions in this model. The present study evaluated the serum hormone levels only single time point at termination and time course of hormonal changes were undetermined. Further investigations for the prolonged effect of each hormone level on uterine carcinogenicity should be required.

Another possible explanation is the direct inhibitory effect of PRL on uterine carcinogenicity. PRL has been shown to inhibit endometrial cell growth in humans and rodents (Gunin *et al.*, 2002; Tseng and Mazella, 1999). Moreover, PRL has been shown negatively to regulate the extent of differentiation (decidualization) of human uterine cells (Eyal *et al.*, 2007). These



**Figure 4.** Serum sex-related hormone levels at 12-month necropsies. Data are shown as the mean  $\pm$  SEM. \*\* $P < 0.01$  and \* $P < 0.05$ ; significant differences compared to the control group. E<sub>2</sub>, estradiol-17 $\beta$ ; EGME, ethylene glycol monomethyl ether; FSH, follicle-stimulating hormone; INH, inhibin; LH, luteinizing hormone; P4, progesterone; PRL, prolactin.

reports support the present results describing the inhibitory effects of PRL on uterine carcinogenicity. On the other hand, Forsberg and Breistein (1976, 1979) have reported the synergistic stimulatory effects of PRL and E<sub>2</sub> on uterine carcinogenesis in rodents. Additionally, another study suggested that PRL might contribute to uterine carcinogenicity through its roles in stromal proliferation and differentiation (Yurkovetsky *et al.*, 2007) and through predominant uterine expression of the PRL receptor in rats (Nagano and Killy, 1994). Indeed, the sulpiride-dependent increase in the incidence of decidualoma in the present study seemed to support that PRL affects stromal differentiation. Although the reason for this discrepancy between the present results and previous reports is unknown, further studies are required to determine the direct effects of PRL on uterine carcinogenicity.

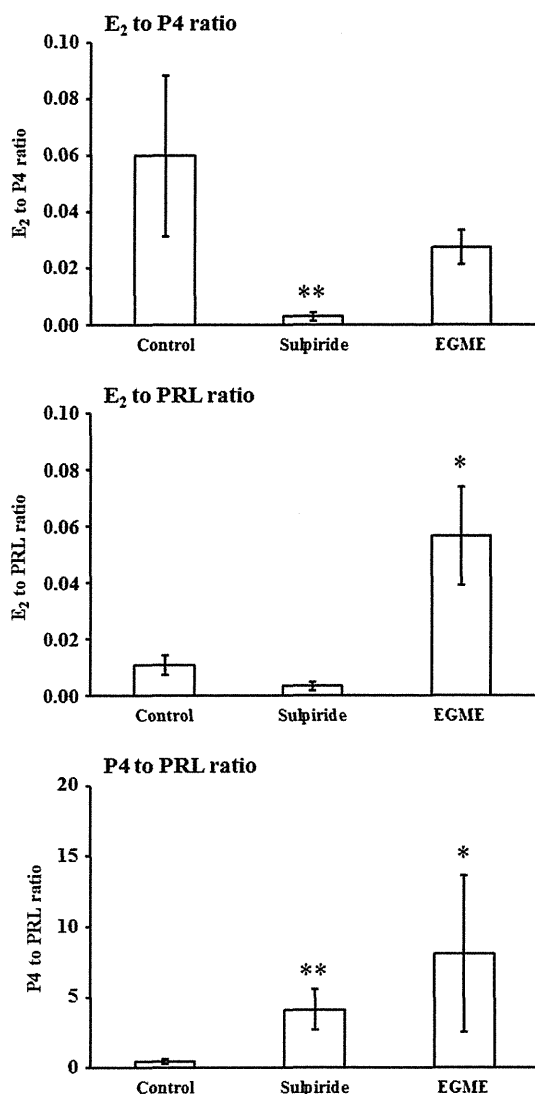
Although we observed abnormalities in the estrous cycle and reduced incidence of persistent estrus, along with increased ovarian weights in the EGME group compared with the control group, no significant changes in serum hormone levels or the incidence of uterine proliferative lesions were observed in the EGME group compared with the control group when the study was terminated. Higher ovarian weights in the EGME group than the control group were considered due to retention of CL in the animals that did not show persistent estrus. This retention of CL would be correlated with extension of the estrous cycle interval and resulted in the delay of gonadal senescence by EGME treatment. The differences between the effects of EGME and sulpiride may have been because EGME at the dose used in this study was not sufficient to stimulate PRL secretion and affect luteal function throughout the dosing period. Although the present dose level of EGME may not have been sufficient to determine the effects of PRL on uterine carcinogenicity, it is clear that EGME had no effects on uterine carcinogenesis under the present conditions. Further studies using higher doses of EGME are needed to evaluate the effects of EGME on uterine carcinogenicity.

Regarding the other changes observed in this study, histological enlargement of mammary glands in the sulpiride group indicated sustained high levels of PRL in these animals, whereas the incidence of proliferative lesions in the endocrine organs,

including the mammary glands and pituitary gland, did not change in this group. This result indicated that PRL did not affect endocrine organ carcinogenicity in Donryu rats. The observation of diffuse endometrial hyperplasia in several sulpiride-treated animals may have been caused by prolonged high levels of P4 (Ohtake *et al.*, 2009). Additionally, the increase in body weight following sulpiride treatment can be explained by its known effects in promoting appetite (Baptista *et al.*, 2002; Lacruz *et al.*, 2000). The increased incidence of adrenomedullary cell hyperplasia in the EGME group indicated the possibility that EGME promoted the development of pheochromocytoma in rats. Alteration of calcium homeostasis might contribute to the present result because some agents, including vitamin D3 and retinoids, have been known to induce adrenomedullary proliferative lesions via altered calcium homeostasis (Rosol *et al.*, 2001; Tischler *et al.*, 1999). As few data are available concerning the carcinogenicity of EGME (Multigner *et al.*, 2005), further investigations are required. Finally, we observed vaginal stromal hyperplasia in the control group, and the incidence of the abnormality was decreased in the treatment groups; however, no reports have described such lesions in aged rats. Considering the histological features of vaginal stromal hyperplasia were similar to those in the cervix (Dixon *et al.*, 2014) and cervical stromal proliferative lesions were induced by estrogenic stimulation in rodents (McLachlan *et al.*, 1980), it may be associated with ENNG treatment and the prolonged increase in the E<sub>2</sub> to P4 ratio.

In conclusion, sulpiride treatment but not EGME treatment decreased the incidence of uterine neoplastic lesions, dramatically prevented the onset of persistent estrus, and increased serum PRL and P4 levels without affecting E<sub>2</sub> levels. These results indicated the possibility that prolonged lowering of the E<sub>2</sub> to P4 ratio accompanied by persistent abnormalities in the estrous cycle was the main cause of uterine neoplastic lesions. Alternatively, PRL may have a direct inhibitory effect on uterine carcinogenesis. To our knowledge, this is the first report to evaluate the effects of PRL on uterine carcinogenicity in Donryu rats, which is a useful model for human risk assessment for endometrial cancer.





**Figure 5.** Serum E<sub>2</sub> to P<sub>4</sub>, E<sub>2</sub> to PRL and P<sub>4</sub> to PRL ratios at 12-month necropsies. Data are shown as the mean ± SEM. \*\**P* < 0.01 and \**P* < 0.05: significant differences compared to the control group. E<sub>2</sub>, estradiol-17β; EGME, ethylene glycol monomethyl ether; P<sub>4</sub>, progesterone; PRL, prolactin.

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### Conflict of interest

The authors did not report any conflict of interest.

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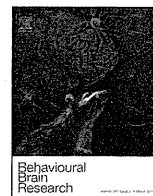
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## Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.



## Research report

# Higher detection sensitivity of anxiolytic effects of diazepam by ledge-free open arm with opaque walled closed arm elevated plus maze in male rats

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## HIGHLIGHTS

- Arm structure of the elevated plus maze alters detection sensitivity for anxiety.
- Open arm ledges increase open arm exploratory behavior.
- Closed arm wall opacity partially alters open arm exploratory behavior.
- No-Ledges/Opaque EPM shows high detection sensitivity for anxiolytic drug diazepam.

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## ABSTRACT

The elevated plus maze (EPM) is an established method for testing animal anxiety. However, EPM apparatuses and their features can differ among laboratories, most notably in the presence/absence of ledges on the open arm and/or the transparency/opaque of walls on the closed arm. The combined effects of these variable arm features on EPM behavior are not yet fully understood. In the present study, we prepared four types of EPM apparatus – open arms with (0.5 cm) or without (0 cm) ledges × closed arms with transparent or opaque walls – and compared the maze-exploration behavior of male Sprague–Dawley rats. We found that the presence of open arm ledges significantly increased the incidence of open arm exploration. Furthermore, time spent in the distal segment of the open arm was shortest in the apparatus that had open arms with no ledges and opaque closed arms (No-Ledges/Opaque), and was longest in the apparatus that had open arms with ledges and transparent closed arms (Ledges/Transparent). Additionally, the No-Ledges/Opaque apparatus could detect the effect of 0.5 mg/kg diazepam, an anxiolytic drug, whereas the Ledges/Transparent apparatus could not. These results indicate that arm structure (features of both open and closed arms) significantly influences maze-exploratory behavior in rats, and that No-Ledges/Opaque apparatuses have higher detection sensitivity for anxiolytic effects of diazepam than that of Ledges/Transparent apparatuses.

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## 1. Introduction

The number of patients suffering from mood disorder, including anxiety-disorder and depression, is increasing. According to the 2005 National Comorbidity Survey-Replication study, around 20.9 million American adults, or 9.5% of the population ages 18 and older, have mood disorders. Although the development of these disor-

ders involves both genetic and environmental factors, the detailed pathogenic mechanisms that underlie mood disorders are not yet fully understood [1]. Therefore, further research needs to focus on uncovering the underlying mechanisms of mood disorders, as well as the development of effective drugs that have fewer side effects. Researchers also need to understand basic features of the experimental devices that have been adopted to evaluate mood disorders in order to provide reliable, precise outcomes in the laboratory.

The elevated plus maze (EPM), which was originally established to examine emotional reactivity in rodents [2,3], is widely used in laboratory research to investigate anxiety. In this maze, animals are free to explore the apparatus, which consists of two open and

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two closed arms, and is usually placed 50 cm from the floor. Because mice and rats prefer enclosed spaces, animals experiencing anxiety tend to avoid open arm exploration [3]. The incidence of staying in closed arms can be increased and reduced by anxiogenic and anxiolytic drugs, respectively. It is also known that the EPM induces an increase in freezing behavior, fecal boli, and plasma glucocorticoid levels [3]. Thus, the EPM can be applied to test animal anxiety and to perform preclinical drug screening of potential treatments for human mental diseases, such as anxiety-disorder. The EPM also offers a way to measure the effects of anxiety-inducing and anxiety-relieving drugs.

There are several structural differences between EPM apparatuses used in different laboratories [4,5]. For example, there are two types of open arms, namely those with or without ledges along the side. Similarly, there are two types of closed arms, namely those with transparent or opaque walls. The use of open arms with ledges prevents animals from falling off the apparatus [6] and the use of transparent walled closed arms facilitates the observation of behavior [7,8]. Although there are a few studies that have examined how these arm differences may differ in their ability to detect animal anxiety [4,7–10], the combined effects of open (with/without a ledge) and closed (transparent/opaque walls) arm features on maze-exploratory behaviors have not yet been investigated. Therefore, in the present study, we studied the combined effects of open/closed arm features on rat EPM behaviors in the first experiment. In the second experiment, we compared the detection sensitivity of selected pairs of open/closed arm combinations for the anxiolytic effects of diazepam. We discuss the combined effects of the arm features on rat EPM behaviors, sensitivity of the EPM apparatus to detect anxiolytic drug, and other findings from these experiments. We believe that our findings may enhance the basic understanding of EPM features and ultimately contribute to the broader field of anxiety research.

## 2. Materials and methods

### 2.1. Experimental design

Two experiments were performed. In Experiment 1, we prepared four types of EPM apparatus: open arms with ledges and closed arms with transparent walls (Ledges/Transparent), open arms with ledges and closed arms with opaque walls (Ledges/Opaque), open arms without ledges and closed arms with transparent walls (No-Ledges/Transparent), and open arms without ledges and closed arms with opaque walls (No-Ledges/Opaque). Rat behaviors in each of the four EPM apparatuses were then compared. In Experiment 2, we tested the effect of diazepam, an anxiolytic drug, on the expression of anxiety-related behaviors in the two apparatuses that were found to be the most and least anxiety-inducing in the first experiment. This allowed us to compare the detection sensitivity between the apparatuses.

### 2.2. Animals

In Experiment 1, 64 male Sprague–Dawley rats at 4 weeks-old were purchased from Charles River Japan (Shiga, Japan) and randomly assigned to four experimental groups (see experimental design). In Experiment 2, 63 male Sprague–Dawley rats at 4 weeks-old were purchased, and randomly divided into two apparatus groups (see experimental design). In these two apparatus groups, animals were further subdivided into three treatment groups (vehicle, low-dose, or high-dose diazepam treatments). All animals underwent at least 1 week of habituation to the animal housing

conditions at Meiji University before the experiment. The animal housing room was maintained at standard ambient conditions of light (12:12 light/dark schedule with lights on at 10:00 h), room temperature ( $25.0 \pm 0.5^\circ\text{C}$ ), and humidity ( $55 \pm 10\%$ ), and animals had *ad libitum* access to food and water. All experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Meiji University.

### 2.3. Drug preparation

In order to determine the detection sensitivity of the EPM apparatus to animal anxiety in Experiment 2, low-dose diazepam (0.25 mg/kg, intraperitoneal (i.p.), Taiyo Pharmaceutical Industry, Aichi, Japan), high-dose diazepam (0.50 mg/kg, i.p.) dissolved in 0.9% saline with 10% Tween 80 (Wako Pure Chemical Industries, Osaka, Japan), or vehicle was administered 30 min prior to the EPM test.

### 2.4. EPM test

The EPM apparatus was located in a handmade container (W150 × D150 × H200 cm), which was elevated to a height of 50 cm from the floor, and its two open and two closed arms merged at a central square (10 × 10 cm) to form a plus shape. Illumination on the surface of the apparatus was adjusted to approximately 100 lux. For the present study, we prepared four types of EPM apparatus with the following arm structure combinations: open arms (W50 × D10 cm) with (H0.5 cm) or without (H0 cm) short ledges, and closed arms (W50 × D10 × H45 cm) with black opaque or transparent walls (W50 × H45 cm). Each rat was placed on the central square facing an open arm and was allowed to freely explore the maze for 5 min. Behavior was recorded by a digital video camera, and the time rats spent within each arm, as well as the number of entries into these arms, was measured by three independent observers using stopwatches. An 'entry' into one of the four arms was defined as instances in which all four paws of the animal were on an arm [11]. With these data, the percentage of time spent in open arms ( $\text{time spent in open arms} / (\text{time spent in open arms} + \text{time spent in closed arms}) \times 100$ ) and the percentage of entries into open arms ( $\text{number of entries into open arms} / (\text{number of entries into open arms} + \text{number of entries into closed arms}) \times 100$ ) were calculated. Furthermore, risk-assessment behavior, defined as the frequency of stretched-back posture toward an open arm while being backed into a closed arm on the central square was also manually observed. The traveling distance, time spent in the three equal segments of the open arm (proximal, middle, and distal segments from the central square), and a minute-by-minute analysis for time spent in open and closed arms were measured by EthoVision XT version 8.0 software (Noldus, Wageningen, Netherlands). This software allows the animal to be tracked via its contrast with the background field; the animal, which is assigned a fixed size, is identified as the brighter object and locomotion of its center point can thus be used for animal tracing. The relative value of time spent in proximal and distal segments over the time spent in the middle segment was calculated in order to gain a more detailed understanding of the open arm exploratory pattern for each of the four apparatuses. The apparatus was cleaned with 70% ethanol solution and wiped with distilled water before each test. During the EPM tests in Experiment 2, two animals (one from the vehicle-injection group and one from the low-dose diazepam injection group) from the No-Ledges/Opaque apparatus, and three animals (from the high-dose diazepam injection group) from the Ledges/Transparent apparatus fell off of the apparatus. Thus, their data were not included in the final analysis.