### 2.5. Data analysis

All data were expressed as means ± SEM for each group. In Experiment 1, a two-way analysis of variance (ANOVA) with the type of open arm (Ledges/No-Ledges) and closed arm (Transparent/Opaque) as experimental factors was performed, and multiple comparisons were carried out using the Bonferroni multiple comparisons test. In Experiment 2, the Bonferroni multiple comparisons was performed for each EPM apparatus group. For minute-by-minute analyses, a repeated measures ANOVA, with type of treatment (vehicle, low- and high-dose diazepam) and time-lapse (1–5 min) as experimental factors, was performed for each EPM apparatus group.

A $P$ value of less than 0.05 was considered statistically significant when a two-way ANOVA or repeated-measures ANOVA was applied. For Bonferroni multiple comparisons, a $P$ value less than 0.05/number of comparisons was considered statistically significant. Thus, $P$ values were set to 0.0167 and 0.0125 when comparing three and four experimental groups, respectively. All data analysis was performed using GraphPad Prism software (GraphPad Prism software Inc., CA, USA).

## 3. Results

### 3.1. Characteristics of each apparatus on rat EPM behaviors

A two-way ANOVA revealed a significant effect of open arm structures on the time spent in open arm ($F_{[1,60]} = 13.49$, $P = 0.0005$) (Fig. 1A), percentage of time spent in open arm ($F_{[1,60]} = 12.43$, $P = 0.0008$) (Fig. 1B), frequency of open arm entry ($F_{[1,60]} = 11.17$, $P = 0.0014$) (Fig. 1C), and percentage of open arm entry ($F_{[1,60]} = 15.12$, $P = 0.0003$) (Fig. 1D). However, no significant main effect of closed arm structure on these measures was found, nor was there a significant interaction between open and closed arm structures.

In contrast, a two-way ANOVA revealed a significant effect of closed arm structure on the amount of time spent in central square ($F_{[1,60]} = 8.524$, $P = 0.0352$) (Fig. 1E), frequency of risk-assessment behaviors ($F_{[1,60]} = 4.03$, $P = 0.0492$) (Fig. 1F) and traveling distance ($F_{[1,60]} = 6.279$, $P = 0.0149$) (Fig. 1G). There was no main effect of open arm structure and there were no interactions in either time spent in the central square (Fig. 1E), frequency of risk-assessment behaviors (Fig. 1F), and traveling distance (Fig. 1G).

### 3.2. Comparison of open arm exploration patterns in Experiment 1

It has been reported that longer exploration into the distal segment of open arm reflects a reduced anxiety-like status of animals [12]. In consideration of this, we compared the time spent in distal segment of open arms across the four groups (Fig. 2A). A two-way ANOVA with the type of open arm (with/without ledges) and type of closed arm (transparent/opaque) structures as experimental factors revealed a significant main effect of open arm features on time spent in the distal segments of open arms ($F_{[1,60]} = 2.82$, $P = 0.0064$). Furthermore, we found that closed arm features showed a tendency to affect the time spent in the distal segments of open arms ($F_{[1,60]} = 8.00$, $P = 0.0983$). A multiple comparison test showed that the amount of time spent in the distal segment of open arm was significantly lower in the No-Ledges/Opaque group (2.70 ± 1.5 s, $n = 16$) compared to the Ledges/Transparent group (26.28 ± 7.2 s, $n = 16$, $P = 0.0031$) (Fig. 2A). On the other hand, the amount of time spent in distal segment of the open arms for both the Ledges/Opaque group (17.96 ± 5.9 s, $n = 16$) and No-Ledges/Transparent group (11.95 ± 4.6 s, $n = 16$) were not significantly different from any other experimental group (Fig. 2A).

To further understand exploration patterns within the open arms, relative values of the time spent in proximal and distal segments over that of the middle segment were compared (Fig. 2B–E). Multiple comparison tests indicated that in the Ledges/Transparent group, animals spent significantly longer time in proximal segments (3.64 ± 1.0, $n = 16$) than middle segments (1.00 ± 0.2, $n = 16$, $P = 0.0142$) (Fig. 2B). There was no significant difference between the relative value of time spent in distal (3.02 ± 0.2, $n = 16$) and middle segments (Fig. 2B). Similarly, the Ledges/Opaque group spent significantly longer in proximal segment (3.83 ± 0.7, $n = 16$) than the middle (1.00 ± 0.3, $n = 16$, $P = 0.0007$) but not distal segment (2.36 ± 0.8, $n = 16$) (Fig. 2C). In the No- Ledges/Transparent group, rats spent significantly longer in proximal segment (5.08 ± 1.1, $n = 16$) than that of middle segment (1.00 ± 0.3, $n = 16$, $P = 0.0009$), but not longer than in distal segment (3.15 ± 1.2, $n = 16$) (Fig. 2D). In contrast to the other groups, the No-Ledges/Opaque group spent significantly longer in proximal segment (5.30 ± 1.0, $n = 16$) than the middle (1.00 ± 0.5, $n = 16$, $P = 0.0006$) and distal segments (0.81 ± 0.5, $n = 16$, $P = 0.0003$) (Fig. 2E).

### 3.3. Comparison of detection sensitivity for anxiolytic effect of diazepam between No-Ledges/Opaque and Ledges/Transparent EPM apparatus

The first experiment revealed that No-Ledges/Opaque and Ledges/Transparent apparatus induced the highest and lowest anxiety-related behavior, respectively (Fig. 2A). Based on these findings, we compared the detection sensitivity for anxiety-related behavior of these two apparatuses using rats that were treated with an anxiolytic drug, diazepam. The total time and percentage of time that high-dose diazepam-, low-dose diazepam-, and no dose (vehicle)-treated rats spent in these two apparatuses will first be reported, followed by their entries into the open arms, time spent in the central square, and frequency of risk-assessment behavior.

A multiple comparison tests revealed that in the No-Ledges/Opaque apparatus (associated with the greatest anxiety-related behavior in Experiment 1), rats that received a high-dose treatment of diazepam spent significantly longer amounts of time in open arms (99.05 ± 19.9 s, $n = 11$) than did vehicle-injected (17.82 ± 7.9 s, $n = 9$, $P = 0.0026$) and low-dose treated (31.76 ± 10.9 s, $n = 10$, $P = 0.0096$) rats. There was no difference between vehicle-injected and low-dose treated rats in amounts of time in open arms (Fig. 3A). Similarly, high-dose treated (45.59 ± 8.0%, $n = 11$) rats spent a significantly greater percentage of time in open arms compared to vehicle-injected (10.47 ± 4.6%, $n = 9$, $P = 0.0023$) and low-dose treated rats (17.81 ± 6.5%, $n = 10$, $P = 0.0159$) (Fig. 3C). Further, there was no difference between vehicle-injected and low-dose treated rats (Fig. 3C). In contrast to the No-Ledges/Opaque apparatus, no significant effect of diazepam treatment was found in the Ledges/Transparent apparatus (associated with the least anxiety-related behavior in Experiment 1). This was true in terms of the total time spent in open arms, as well as the percentage of time spent in the open arms in high-dose (time in open arms, 124.9 ± 29.7 s; percentage of time in open arms, 56.28 ± 10.3%, $n = 8$) or low-dose (time in open arms, 57.74 ± 14.7 s; percentage of time spent in open arms, 28.24 ± 7.1%, $n = 10$) treated rats compared to the vehicle-injected group (time in open arm, 54.83 ± 14.0 s; percentage of time spent in open arms, 27.47 ± 7.0%, $n = 10$) (Fig. 3B and D).

In addition to differences in duration and percentage of time, rats in the No-Ledges/Opaque apparatus that received high-dose treatment (6.82 ± 1.0, $n = 11$, $P = 0.0019$), but not those that received low-dose treatment (3.57 ± 1.3, $n = 10$), exhibited a significantly

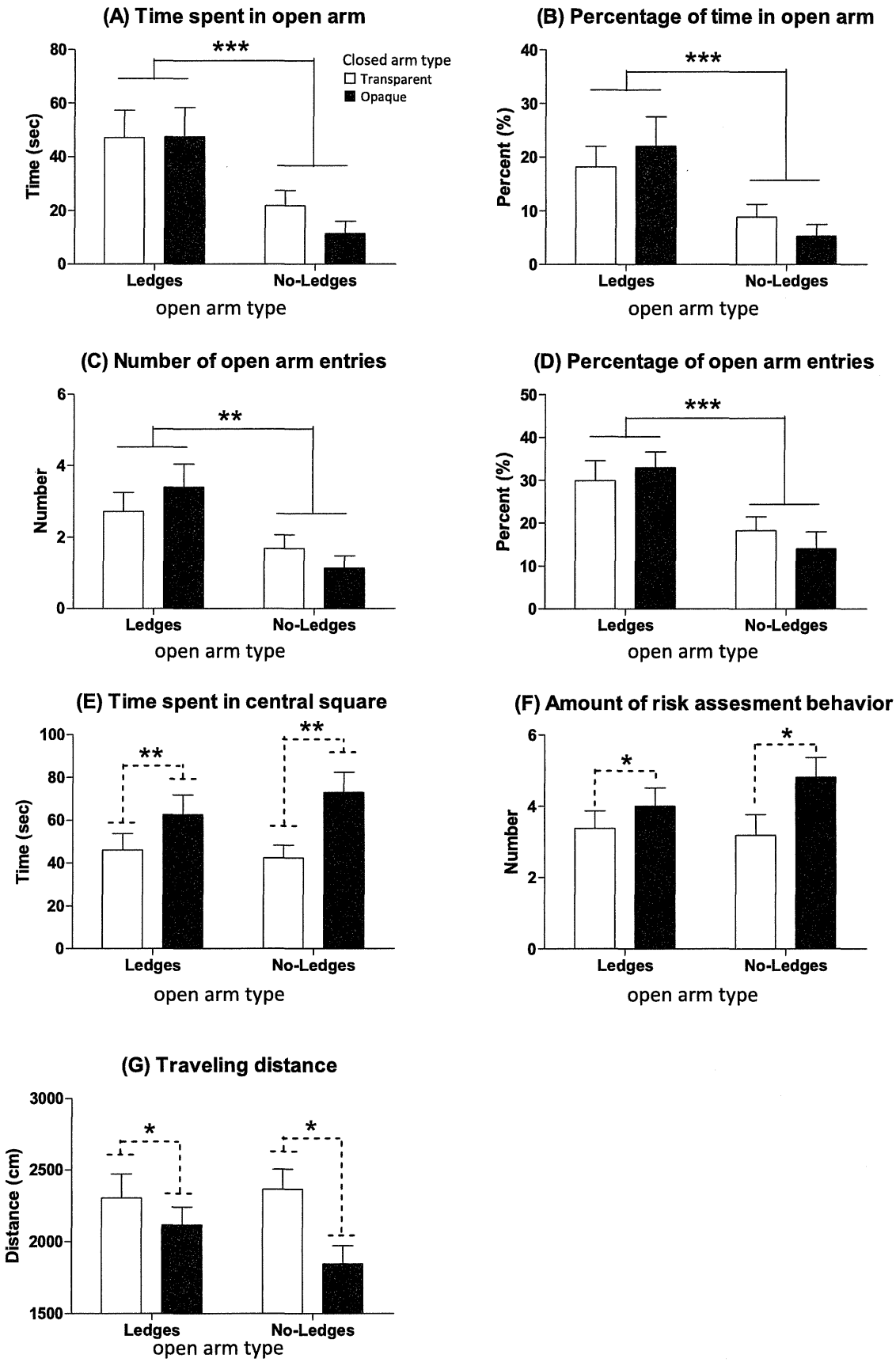


**Fig. 1.** Comparison of rat behaviors among each combination of EPM open and closed arm features.
Panels A and C show the amount of time spent in, and entries into the open arms, respectively. Panels B and D show the percentage of time spent in, and entries into the open arms, respectively. Panels E, F, and G show the time spent in the central square, amount of risk-assessment behavior in the central square, and total distance traveled, respectively. Solid and dashed lines indicate significant main effects of open and closed arm structures, respectively (Two-way ANOVA, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

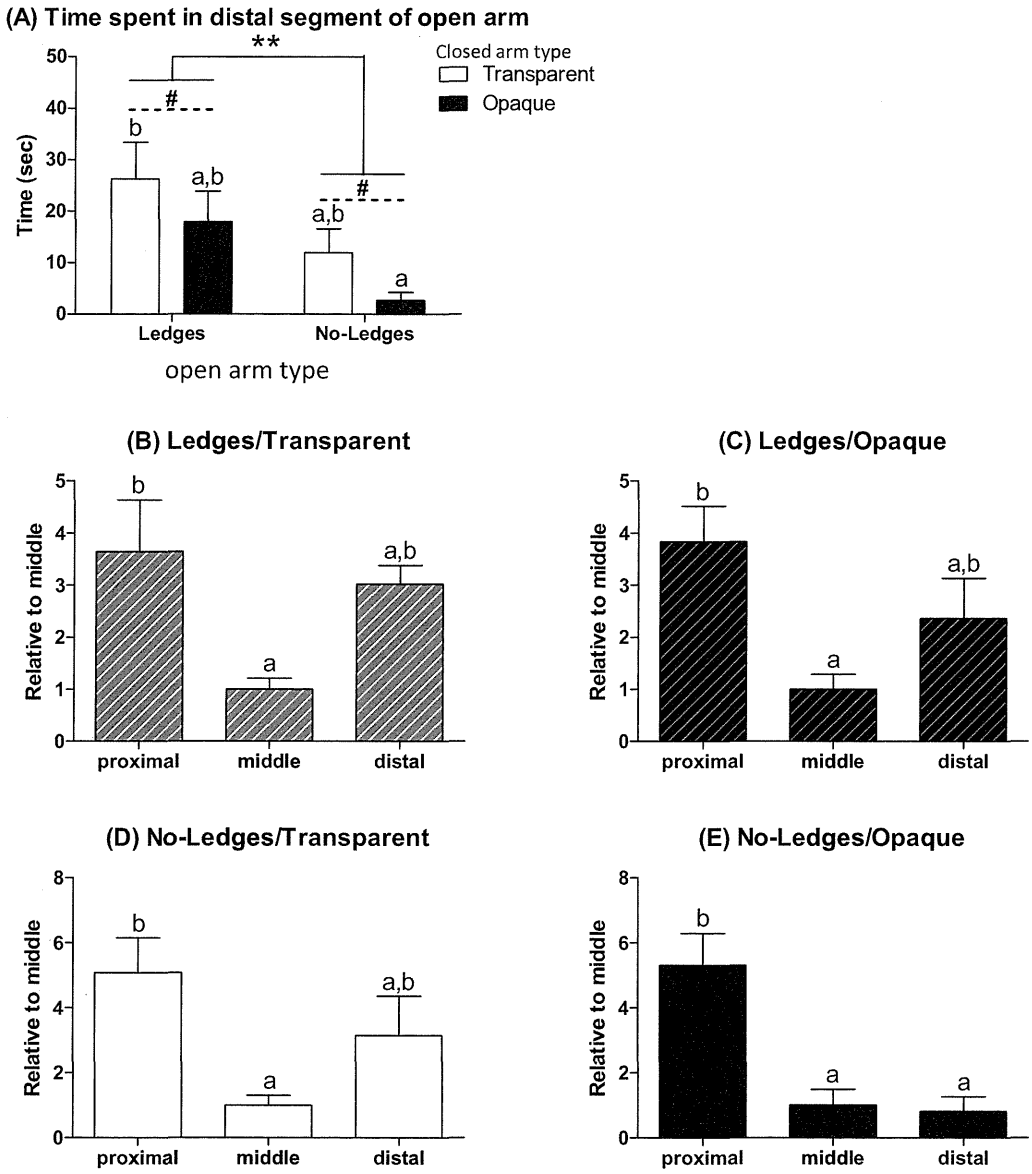


**Fig. 2.** Comparison of exploratory behaviors in distal segments of the open arms among each of the EPM apparatuses.
Panel A shows the time spent in distal segments of the open arms in different arm feature combinations. Panels B to E show the relative time spent in proximal or distal segments of open arms over time spent in the middle segment, and in each feature combination of apparatuses. Solid and dashed lines indicate effects of open and closed arm structures, respectively (Two-way ANOVA, $^{\#}P<0.1$, $^{**}P<0.001$). Different letters beside the bars indicate a significant difference (Bonferroni multiple comparison test, $a<b$, $P<0.0125$ for panel A and $P<0.0167$ for panels B–E).

greater number of entries into the open arms than vehicle-injected rats ($1.69 \pm 0.8$, $n=9$) (Fig. 3E). The number of entries into open arms made by low-dose-treated rats did not significantly differ from the number of entries made by rats in either of the other treatment groups (Fig. 3E). Similarly, the percentage of entries into the open arms was significantly higher in the high-dose- treated group ($40.47 \pm 5.8\%$, $n=11$) when compared to that in the vehicle-injected group ($13.01 \pm 4.9\%$, $n=9$, $P=0.0034$), but not the low-dose treated group ($22.33 \pm 6.7\%$, $n=10$) (Fig. 3G). In contrast to the No-Ledges/Opaque apparatus, no significant effects of diazepam on either entries into the open arms or percentage of entries into the open arm were observed for the Ledges/Transparent apparatus in either high-dose (entries into open arm, $9.69 \pm 2.3$; percentage of entries into open arm, $51.98 \pm 7.8\%$, $n=8$) or low-dose (entries into open arm, $5.05 \pm 1.1$; percentage of entries into open arm, $38.12 \pm 6.0\%$, $n=10$) treatment groups when compared to the vehicle-injection group (entries into open arm, $4.32 \pm 0.9$; percentage of entry frequency into open arm, $29.96 \pm 4.9\%$, $n=10$) (Fig. 3F and H).

Finally, there were no effects of diazepam treatment on the time spent in the central square, as well as the frequency of risk-assessment behaviors for both the No-Ledges/Opaque and Ledges/Transparent apparatuses (Fig. 3I and J).

### 3.4. Comparison of the effect of diazepam treatment on minute-by-minute profiles of open arm exploratory behavior between No-Ledges/Opaque and Ledges/Transparent apparatuses

The minute-by-minute analysis of the amount of time and percentage of time spent in open arms for Experiment 2 was

performed to further characterize the effect of diazepam treatment on open arm exploratory behavior. A repeated measures ANOVA for the No-Ledges/Opaque apparatus group revealed significant main effects of diazepam treatment ($F_{[2,108]} = 3.82$, $P = 0.0345$) and time-lapse ($F_{[4,108]} = 4.77$, $P = 0.0014$) on time spent in the open arm (Fig. 4A), while no significant interaction between these experimental factors was found (Fig. 4A). Similarly, a repeated measures ANOVA revealed a significant main effect of diazepam treatment ($F_{[2,108]} = 4.51$, $P = 0.0204$) and time-lapse ($F_{[4,108]} = 5.25$, $P = 0.0007$) on the percentage of time rats spent in the open arms of the No-Ledges/Opaque apparatus (Fig. 4C), and no significant interaction between these experimental factors was observed (Fig. 4C). On the other hand, a repeated measures ANOVA for the Ledges/Transparent apparatus group revealed a significant main effect of time-lapse ($F_{[4,100]} = 8.21$, $P < 0.0001$) and a significant interaction between diazepam treatment and time-lapse ($F_{[8,100]} = 2.31$, $P = 0.0255$) on time spent in the open arms (Fig. 4B). However, no significant main effect of diazepam treatment on time spent in the open arms for this apparatus group was observed (Fig. 4B). In terms of the percentage of time spent in the open arms of the Ledges/Transparent apparatus, a repeated measures ANOVA revealed a significant main effect of time-lapse ($F_{[4,100]} = 8.73$, $P < 0.0001$) but no main effect of diazepam treatment (Fig. 4D). Moreover, there was no significant interaction between these experimental factors (Fig. 4D).

## 4. Discussion

The present study suggests that presence/absence of open arm ledges and opaqueness/transparency of closed arm walls alters detection sensitivity of anxiety-related behavior of rats in the EPM.

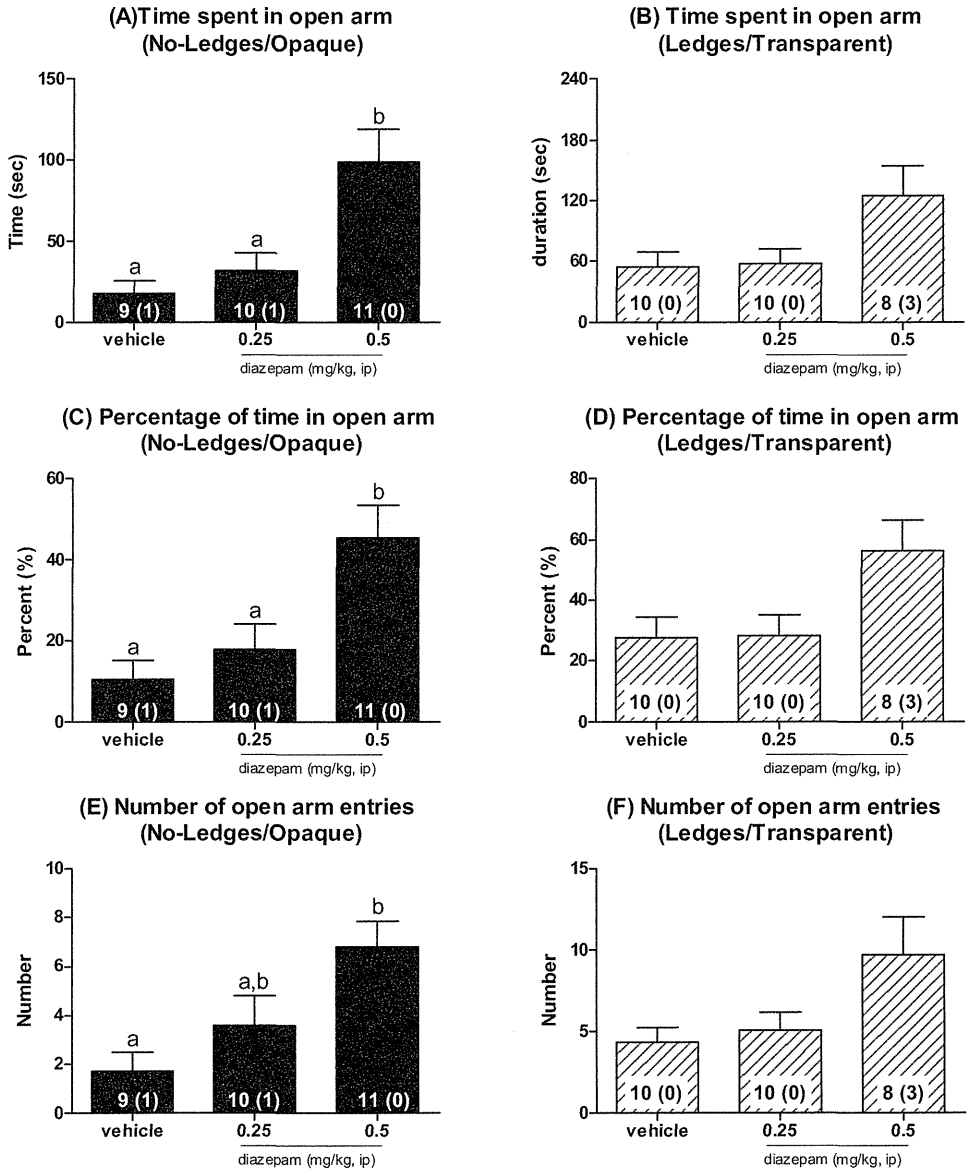


**Fig. 3.** Comparison of detection sensitivity of anxiolytic effect of diazepam between two types of EPM apparatus.
Panels A, C, E, G, I, and K show the amount of time spent in open arms, percentage of time spent in open arms, entries into open arms, percentage of entries into open arms, time spent in the central square, and amount of risk-assessment behavior of the No-Ledges/Opaque apparatus, respectively. Panels B, D, F, H, J, and L show the amount of time spent in open arms, percentage of time spent in open arms, entries into open arms, percentage of entries into open arms, time spent in the central square, and amount of risk-assessment behavior of the Ledges/Transparent apparatus, respectively. Diazepam was injected 30 min prior to the test (0.25 or 0.50 mg/kg, i.p.). Numbers in the column indicate the number of animals used, and numerals in parentheses indicate the number of animals that dropped off the apparatus. Different letters beside bars indicate significant differences (Bonferroni multiple comparison test, $a < b$, $P < 0.0167$).

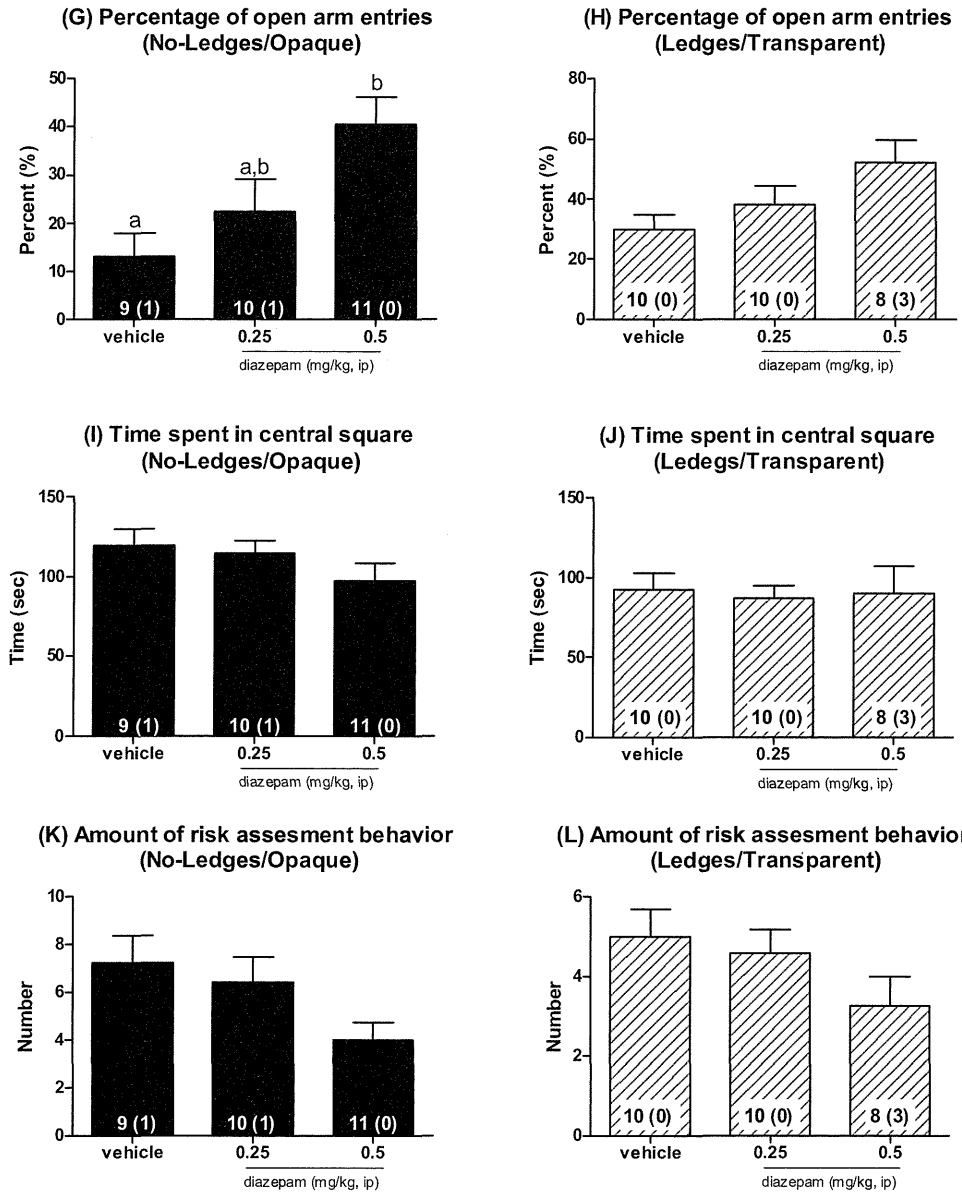


**Fig. 3.** (*Continued*).

Our findings also highlight the importance of understanding the detailed characteristics of the EPM apparatus before starting an experiment. These points will be discussed in-detail in the following sections.

### 4.1. The effect of open arm features on rat EPM behavior

The results of Experiment 1 showed that the presence of ledges on open arms increased exploration onto open arm, as indicated by the overall duration and percentage of time spent in open arms, as well as the overall number and percentage of open arm entries.

Although it has been reported that behaviors on the EPM are influenced by arm structures and/or environmental conditions, such as testing room size or illuminant levels [4–10], until now, no studies have directly investigated the combined effects of the presence/absence of ledges on open arms and the transparency/opaqueness of walls on closed arms. It was thus our aim to investigate the relationship between combinations of open and closed arm features under the same environmental conditions. Although there have been at least two reports on the effect of ledges on open arms combined with opaque closed arms [6,10], ours is the first to report greater open arm exploratory behavior in the presence of ledges. For example, while Martinez et al. [6] studied the effect of a series of ledges (5, 10, and 20 cm high) on open arm exploration compared to a control of 1 cm-high ledges, they did not compare these effect with those of open arm s without ledges (0 cm high). Fernandes and File [10] reported that the presence of 0.5 cm ledges on open arms induces an anxiolytic effect when testing chlordiazepoxide (7.5 mg/kg, i.p.); however, there was no effect of the presence/absence of ledges in their vehicle group. Thus, the present study may be the first to report an anxiolytic effect of open arms with ledges on male rats in the EPM test.

### 4.2. The effect of closed arm wall features on rat EPM behavior

As well as the effect of the presence/absence of open arm ledges, closed arm wall features had significant effects on rat EPM behaviors, such as the time spent in the central square, frequency of

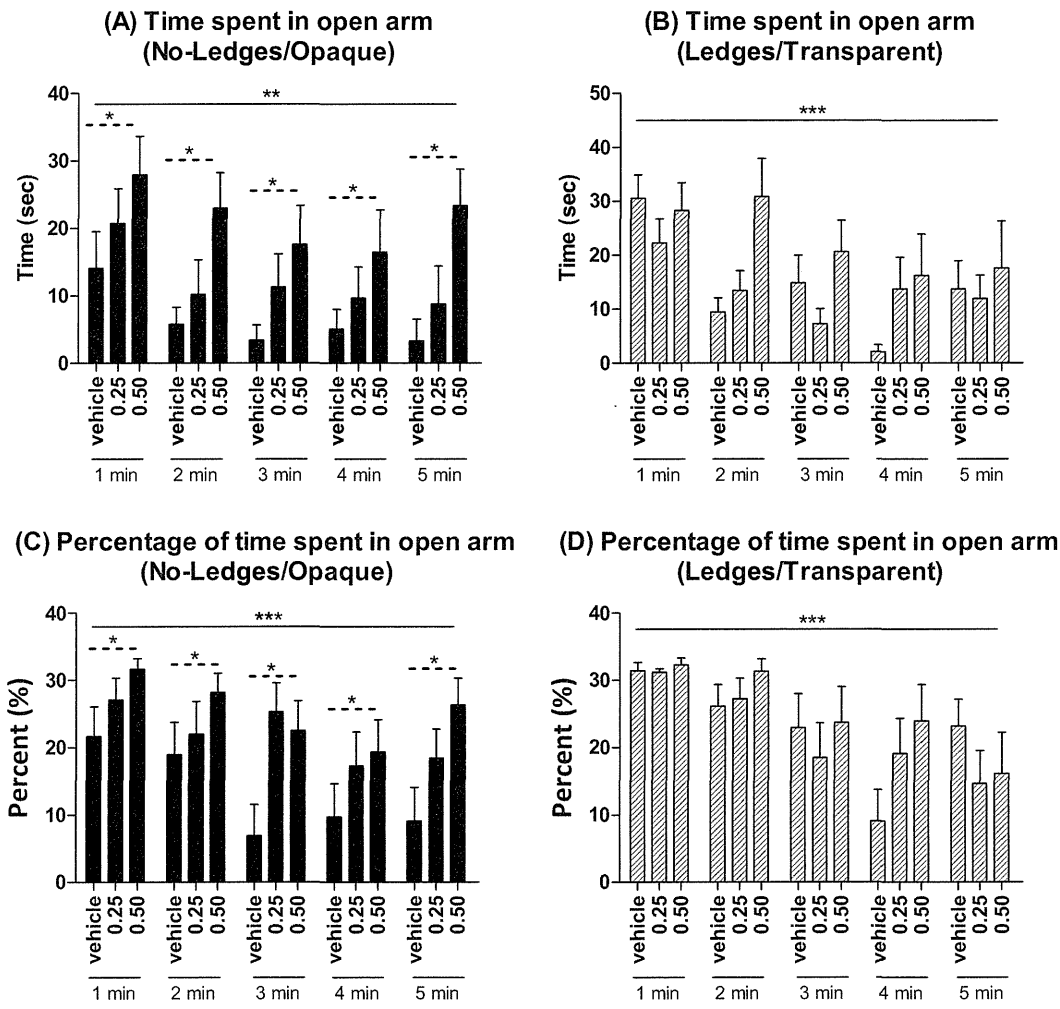


**Fig. 4.** Comparison of open arm exploratory behaviors by minute-by-minute analysis.
Panels A (time spent in open arms) and C (percentage of time spent in open arms) show changes in rat open arm exploratory behaviors across the time-lapse after different dosages of diazepam on the No-Ledges/Opaque apparatus. Panels B (time spent in open arms) and D (percentage of time spent in open arms) show changes in rat open arm exploratory behaviors across the time-lapse after different dosages of diazepam in the Ledges/Transparent apparatus. Vehicle or diazepam (0.25 or 0.50 i.p.) was injected 30 min prior to the test. Solid and dashed lines indicate significant main effects of the time-lapse and diazepam treatments, respectively (repeated measures ANOVA, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

risk-assessment behaviors, and traveling distance in Experiment 1.

It has previously been reported that open arm exploration is decreased in EPMs with opaque-walled closed arms compared to those with transparent walls [4,7–9]. Although such an effect was not observed in the present study, the time that rats spent in the distal segments of open arms tended to be shorter when the walls of the closed arms were transparent (Fig. 2A). Opaque closed arms, on the other hand, decreased the total number of entries when compared to transparent closed arms in Violle et al.'s [4] study. Similarly, we found that traveling distance was decreased in EPMs with opaque closed arms compared to those with transparent closed arms, which suggests that the opaqueness of closed arms mainly decreased animal's total activity and partially affected open arm exploratory behaviors.

This study presents evidence to show, for the first time, that time spent in the central square, as well as the frequency of risk-assessment behavior, may be affected by closed arm opaqueness, as demonstrated in Experiment 1. Indeed, it seems that time spent in the central square is dependent on the contrast between open and closed arm features [13–15]. For example, Lee and Rodgers [16] and Rodgers et al. [17] have observed risk-assessment behavior in the central square and consider this area of the EPM a place for a decision-making (also see [18]). If this is indeed the case, then it can be speculated that the opaqueness of the closed arm may delay decision latency, since animals in a transparent closed arm have access to information beyond the closed wall, whereas animals in opaque closed arm EPM do not. Thus, less information in opaque closed arm conditions may increase the time needed for risk-assessments and delay decision-making latency.

### 4.3. Comparison of detection sensitivity of anxiolytic drug between No-Ledges/Opaque and Ledges/Transparent EPM apparatuses

Since exploration into the distal segment of the open arm is taken as an index of reduced anxiety in animals [12], we measured and compared the time spent in the distal segment of the open arms in our apparatuses. Using this measure, we determined that time spent in distal segment of the open arms was the shortest and longest in No-Ledges/Opaque and Ledges/Transparent EPM

apparatuses, respectively. Therefore, among the four tested EPM apparatuses, we considered that the former and latter induced the highest and lowest anxiety-related behavior, respectively. Furthermore, we compared exploration patterns within the open arms. The results clearly indicated that rats exposed to the No-Ledges/Opaque apparatus significantly avoided exploration into distal segments of the open arms, suggesting that this specific apparatus induced the greatest anxiety-related behavior among the four EPM apparatuses. Based on these results, we hypothesized that No- Ledges/Opaque and Ledges/Transparent apparatuses would differ in their sensitivity to detect anxiety. To test this, Experiment 2 used rats treated with an anxiolytic drug, diazepam, which is commonly used in pharmacological studies on anxiety. As expected, the results showed that the No-Ledges/Opaque apparatus was more sensitive to anxiety-related behaviors than the Ledges/Transparent apparatus.

Relatively few studies have compared detection sensitivity of different EPM apparatuses using certain anxiolytic drugs. In one study, Violle et al. compared the sensitivity of EPM apparatuses with different closed arm features using diazepam-treated rats, and showed that apparatuses with opaque walls provided greater sensitivity than those with transparent walls [4]. Conversely, the remaining studies have reported that the use of transparent-walled closed arms did not reduce the sensitivity to detect the anxiolytic effects of various benzodiazepines when compared to opaque-walled closed arms [7–9]. Previous studies that have employed anxiolytic drugs have therefore produced mixed results. However, none of these studies investigated the combined effects of open and closed arm features under the same environmental conditions. Thus, in an effort to elucidate their effects on the sensitivity of detecting anxiety-related behavior, Experiment 2 of the present study investigated this for the first time.

To further understand the difference in the sensitivity of No-Ledges/Opaque and Ledges/Transparent apparatuses to detect anxiolytic behavior, we performed a minute-by-minute analysis. Our results clearly demonstrated that open arm exploration was greatest at the first initial minute and declined gradually across the time-lapse in all EPM apparatuses regardless of diazepam treatment. The decline across the time-lapse has been observed in previous reports [19,20], and it has been suggested that animals avoid open arm exploration after an initial overall exploration of the maze [20]. These findings indicate that our EPM procedure and minute-by-minute analysis are reliable. With this analysis, we found that the No-Ledges/Opaque apparatus could show a dose-dependent effect of diazepam treatment on both time, and the percentage of time spent in open arms, whereas the Ledges/Transparent apparatus could not. This difference may be due to increased basal activity of open arm exploratory behavior in the vehicle-treated group of the Ledges/Transparent apparatus. More specifically, it seems that the effect of diazepam reached a plateau in that the percentage of time rats spent in the open arms of both apparatuses per minute did not exceed around 35%; however, due to the increase in basal activity of vehicle-treated animals on the Ledges/Transparent apparatus, the effect of drug treatment was not detected. Thus, we considered that the No-Ledges/Opaque apparatus had an advantage in detecting effects of diazepam-induced anxiolytic behavior in male rats.

### 4.4. The effect of diazepam treatment on rat EPM behaviors

Thus far, the discussion has mainly focused on the relationship between EPM arm features and their sensitivity in detecting anxiolytic effects of diazepam. In addition to these points, we will address a few further findings below.

First, we would like to discuss the effect of diazepam treatment on time spent in the EPM central square. It has been reported that anxiolytic drugs influence the time that animals spend in the central square of EPM apparatuses. For example, pentylenetetrazole, which induces anxiety in rodents [3], decreases the time spent in the central square [21]. Similarly, 7-hydroxydipropylaminotetralin, a dopamine receptor agonist known for its anxiolytic properties [22], increases the time spent in the central square [17]. These reports suggest that elevated anxiety levels result in shorter amounts of time spent in the central square. However, the present study found no significant effect of diazepam treatment on time spent in the central square. Similar to our results, other reports have also failed to find a significant effect of diazepam on the amount of time spent in the central square in both mice [23] and rats [4]. Therefore, it may be that the effect a drug has on time spent in the central square may differ depending on the drug treatment. Moreover, clarification of the meaning of central square behavior may contribute to the understanding of different aspects of drug action.

Interestingly, we found that more animals fell off of the Ledges/Transparent apparatus than the No-Ledges/Opaque apparatus, although it was mentioned earlier that one advantage to using ledged open arms is to prevent animals falling from the apparatus [6]; however, none of the previous studies investigated the relationship between anxiolytic drug treatment and animal drop-off with or without ledges. Thus, we speculated that this finding might have been due to the mixed effects of having ledges on open arms and the administration of an anxiolytic drug; both open arms with ledges and diazepam treatment reduce animal anxiety. Thus, these factors may have acted synergistically to increase the opportunity for animals to fall off the apparatus, because open arms with ledges increase rodent exploratory behavior and diazepam treatment reduces animal anxiety. Taken together, these findings suggest that the use of EPM apparatuses with ledges on open arms might not prevent animals from falling when combined with anxiolytic drug administration.

### 4.5. Conclusions

The present study demonstrated, for the first time, that the expression of anxiety-related behaviors in the EPM is significantly affected by the presence or absence of ledges on open arm. In addition, we showed that the transparency/opaqueness of walls on the closed arms mainly influenced total activity, time spent in the central square, and risk-assessment behavior, and partially affected open-arm exploratory behaviors. Furthermore, the minute-by-minute analysis revealed the existence of a ceiling effect for diazepam, as well as an increase in basal activity for open arm exploratory behavior in the Ledges/Transparent apparatus. Perhaps, these differences derived from open/closed arm features resulted in the higher sensitivity of the No-Ledges/Opaque apparatus to detect the anxiolytic effect of diazepam when compared to the Ledges/Transparent apparatus.

Using a No-Ledges/Opaque EPM apparatus (different from that used in the present study), we previously reported that an inbred strain of male Hatano high-active-avoidance rats show higher anxiety-related behaviors than low-active-avoidance rats [24]. However, this strain difference became unclear when tested in an apparatus that had ledges with open arms (H0.5 cm) with transparent-walled closed arms (i.e., the same model that was used in the present study); almost twice as many animals were required to attain a statistically significant difference in the Ledges/Transparent apparatus experiment, and open arm exploratory behavior was also greater than that of the previous experiment. While we hypothesized that these No-Ledges/Opaque and Ledges/Transparent apparatuses may have relatively large dif-

ferences in their ability to detect anxiety-related behavior, these two experiments were also performed in different testing rooms. We thus aimed to test this hypothesis using the same testing room size, illuminant levels, and other environmental conditions. Therefore, the present results support our hypothesis that the No-Ledges/Opaque apparatus may have higher sensitivity in detecting anxiety-related behavior in untreated intact animals.

However, whether or not the higher sensitivity of the No-Ledges/Opaque apparatus would be true for other anxiolytic drugs has yet to be seen. Further research would be required before making the more generalized conclusions about the sensitivity of No-Ledges/Opaque apparatuses in detecting anxiety-related behavior in rats.

Taken together, the present study suggests that it is vital for researchers to understand the characteristics of their EPM apparatus according to their experimental purpose. Failing to do so could result in false-negative results when testing potential anxiolytic drugs with inappropriate combinations of EPM arm features. Thus, this study contributes to all research that employs the EPM.

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*Original Article*

# Early indicators of delayed adverse effects in female reproductive organs in rats receiving neonatal exposure to 17alpha-ethynylestradiol

**Miwa Takahashi[1], Kaoru Inoue[1], Tomomi Morikawa[1], Saori Matsuo[1], Seigo Hayashi[1], Kei Tamura[1], Gen Watanabe[2], Kazuyoshi Taya[2] and Midori Yoshida[1]**

*[1]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
*[2]Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan*

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**ABSTRACT** — We previously reported that neonatal exposure to 17α-ethynylestradiol (EE) led to delayed adverse effects in which age-related anovulation after sexual maturation was accelerated. To identify early indicators of these adverse effects, female Wistar Hannover GALAS rats received a single EE injection (0, 0.02, 0.2, 2, 20, or 200 μg/kg) within 24 hr of birth. Histopathological changes in ovarian and uterine development were investigated from postnatal day (PND) 14 to 10 weeks of age. Immunohistochemical expression of estrogen receptor alpha (ERα) in the uterus, serum levels of sex-related hormones and gene expression in the hypothalamus were examined. Although neonatal exposure to EE did not affect body growth or ovarian development, serum FSH tended to decrease at doses ≥ 2 μg/kg, and *Kiss1* mRNA level in the whole hypothalamus was significantly decreased in all EE-treated groups at PND14. The number of uterine glands at PND21 was suppressed at doses ≥ 20 μg/kg, and ERα expression in the uterine epithelium at estrus stage decreased in a dose-dependent manner at 10 weeks of age. These results demonstrated that the various identified changes that occurred before the appearance of delayed adverse effects could be candidate early indicators.

**Key words:** 17α-ethynylestradiol, Neonatal exposure, Delayed effects, ERα, Rat, Female

## INTRODUCTION

In mammals, sexual differentiation of the brain occurs during prenatal and early postnatal development. These organizational processes are critically important for the attainment and maintenance of adult reproductive function (Dickerson *et al.*, 2011). The exposure of animals to chemicals with estrogenic activity during the susceptible period of development (late embryonic to early postnatal age in rodents) reportedly causes reproductive deficits (Dickerson *et al.*, 2011; Gore *et al.*, 2011). In some cases, increased carcinogenic risk and impaired reproductive function are apparent later in life as delayed adverse effects in rodents as well as in humans, even though normal development until maturation (Newbold *et al.*, 1990; Newbold, 2011; Swan, 2000).

Previously, we investigated the long-term effects of neonatal exposure to various doses of diethylstilbestrol (DES) or 17α-ethynylestradiol (EE) on the female reproductive tract using rats (Yoshida *et al.*, 2011; Takahashi *et al.*, 2013). These studies demonstrated that neonatal exposure to DES or EE, which exert estrogenic activity *in vivo*, induced early onset of age-related anovulation in a dose-dependent fashion after sexual maturation. Estrous cyclicity is a precise indicator of delayed adverse effects on the female reproductive tract (Yoshida *et al.*, 2011; Takahashi *et al.*, 2013). Additionally, it was suggested that the early onset of anovulation leading to prolonged exposure to relatively elevated estrogen might be a risk factor for uterine carcinogenesis. Dysfunction of the ovulation center in the hypothalamus is presumed to be a possible mechanism underlying the early onset of anovulation based on the lack of abnormalities in the remaining follicles and pituitary hormones, although the precise mechanism has not been delineated.

Persistent estrus status resulting from the early onset

Correspondence: Miwa Takahashi (E-mail: mtakahashi@nihs.go.jp)

of anovulation is a useful indicator for delayed effects on female reproductive organs. However, it takes a protracted time to detect the effects caused by neonatal exposure to estrogenic compounds, the onset age being 22 weeks of age at the lowest dose tested (Takahashi *et al.*, 2013). For risk assessment of chemicals, delayed adverse effects have become a serious issue because such effects might be overlooked by existing reproductive toxicity or developmental toxicity studies in accordance with authorized guidelines due to limited observation periods. Thus, toxicologic indicators applicable to early detection of delayed adverse effects are needed for risk assessment of offspring toxicity. The present study was performed to examine early events following neonatal exposure to EE with a view to finding early indicators for subsequent delayed adverse effects. Using rats that received a single EE injection during the neonatal period at doses capable of inducing delayed adverse effects, we conducted histopathological observations of ovarian and uterine development between postnatal day (PND) 14 to 10 weeks of age. In addition, immunohistochemical expression of estrogen receptor alpha (ERα) in the uterus, serum levels of sex-related hormones and gene expression in the whole hypothalamus were examined.

## MATERIALS AND METHODS

### Animals and chemicals

Pregnant Wistar Hannover GALAS rats (n = 47) were obtained from CLEA Japan, Inc. (Tokyo, Japan) at gestational day 14. The rats were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature, 24 ± 1°C; relative humidity, 55 ± 5%) with a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water available *ad libitum*. Light cycle was set as follows: light on, 5:00-17:00; light off, 17:00-5:00 (12 hr light/dark cycle). The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

EE was purchased from Sigma (CAS No. 57-63-6; St. Louis, MO, USA) with purity > 98%. EE was stirred in a small amount of sesame oil overnight and then used after dilution.

### Experimental design

Dams were assigned to 6 groups before delivery: 2 to 5 dams/group for autopsy at PND14 and 21; 3 to 5 dams/group for autopsy at PND34 and 10 weeks of age. All of the pups received a single subcutaneous injection of EE (0, 0.02, 0.2, 2, 20, or 200 μg/kg of body weight) dissolved in sesame oil (5 mL/kg of body weight) within 24 hr of birth. Litters were culled randomly to preserve 8 pups, with a female predominance on PND3, and then the female offspring were weaned on PND 21 and separated from males. From PND 25, we daily checked the vaginal opening. Estrous cyclicity was continuously monitored by vaginal smear from 7 weeks of age.

On PND 14, 21, and 34, 5 randomly selected female pups per group were autopsied. Additionally, 5 animals at estrus were autopsied at 10 weeks of age. The animals were decapitated, and blood samples were collected for hormone assays. Then, the ovaries, uteri, vagina, adrenals, liver, pituitary, thymus, brain, thyroid, mammary glands and sites with macroscopic abnormalities were removed. At PND34 and 10 weeks of age, weights of the ovaries and uteri were measured. The hypothalamus was dissected out, as described in detail elsewhere (Roa *et al.*, 2006), by a horizontal cut of about 2 mm in depth with the following limits: 1 mm anteriorly from the optic chiasm, the posterior border of mammillary bodies, and the hypothalamic fissures. Hypothalamic samples were immediately removed upon decapitation, frozen in liquid nitrogen, and stored at -80°C until processing for RNA analysis. We excluded 1 animal per group that underwent transcardial perfusion from blood and hypothalamic sampling and measurement of organ weights. The autopsy procedures (including decapitation and blood collection) were conducted in a room separate from the animal room at 10:00 -12:00.

All organs except for the brain were fixed in 10% neutral buffered formalin. Tissues were routinely processed and stained with hematoxylin and eosin (HE) for histopathological examination. The left uterine horns were cut in cross-section at 5 mm intervals. To elucidate the development of uterine glands, the number of uterine glands per section was measured. Using transverse sections of the uterus obtained at PND14, 21 and 34, the number of uterine glands located away from the endometrium was counted, and the number of uterine glands per section per animal was calculated by dividing the number of sections observed. Histological pattern of estrous cycle stage was checked using the ovary, uterus and vagina (Westwood, 2008).

### Hormone assays

Serum samples obtained after decapitation were stored at -80°C until assay. The serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined using double-antibody radioimmunoassays and $^{125}$I-labeled radio-ligands. National Digestive and Kidney Disease (NIDDK) radioimmunoassay

kits were used for rat FSH and LH (NIAMDD, NIH, Bethesda, MD, USA), as described previously (Taya *et al.*, 1983). As for the serum samples at PND34 and 10 weeks of age, estradiol-17β (E2) and progesterone (P4) were measured by radioimmunoassay as described by Taya *et al.* (1985).

### Real-time RT-PCR

Total RNA was isolated from whole hypothalamus using ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan). Two μg of total RNA was used for reverse transcription (RT) with a high-capacity cDNA Archive Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Then, quantitative real-time RT-PCR was performed with an ABI Prism 7900HT (Applied Biosystems Japan Ltd.). Taqman® Gene Expression Assay (Applied Biosystems Japan Ltd.) was used to measure mRNA levels of *Kiss1* metastasis-suppressor (*Kiss1*, Rn00710914_ml), KISS1 receptor (*Kiss1r*, Rn00576940_ml), gonadotropin-releasing hormone 1 (*Gnrh1*, Rn00562754_ml), estrogen receptor alpha (*Esr1*, Rn01640372_ml), estrogen receptor beta (*Esr2*, Rn00562610_ml) and cytochrome P450, family 19, subfamily a (aromatase, *Cyp19a1*, Rn00567222_ml). Expression values were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), as *Gapdh* does not change during development in the hypothalamus of rat (Walker *et al.*, 2009). The expression level in the 0 μg/kg group was expressed as 1, and relative levels in the EE-treated groups were calculated.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded uterine sections (n = 4/group, except for 1 rat that underwent transcardial perfusion) were subjected to immunohistochemistry for ERα and proliferating cell nuclear antigen (PCNA). Rabbit polyclonal antibody against ERα (c-7207, dilution at × 50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal antibody against PCNA (M0879, dilution at × 100, Dako Japan Inc., Tokyo, Japan) were used as primary antibody (Yoshida *et al.*, 2011, 2012). Immunodetection was conducted using Histofine® Simple Stain MAX PO (Nichirei Biosciences Inc., Tokyo, Japan) with 3, 3'-diaminobenzidine/$H_2O_2$ as the chromogen. For antigen retrieval, the sections were heated in citrate buffer solution (pH 7) by microwave for 10 min at 98°C and 90°C before incubation with the ERα and PCNA antibodies, respectively.

The percentage of ERα and PCNA positive cells was assessed at 400-fold magnification separately in endometrial epithelial cells, uterine glands and stromal cells and scored as follows: 0, negative; 1, slightly positive (< 10%); 2, partly positive (10-30%); 3, positive in about half (30-70%); 4, mostly positive (> 70%). The average score was calculated by observation of 6 randomly selected areas from the upper, middle and lower uterine horns.

### Statistical analysis

Because EE was not injected into the dams but into each pup after birth, we analyzed the data by individual pups. A recent report in which pups treated with various doses of EE at PND1 were allocated to foster dams demonstrated that differences between litters have little influence on delayed adverse effect (Shirota *et al.*, 2012). However, litter size has a large effect on body growth until sexual maturation. Therefore, the data for body and organ weights were checked by individual pups as well as litters.

Following Bartlett's test, the variance in data for body and organ weights, the number of uterine glands per section, serum hormones, and gene expression levels were compared with the 0 μg/kg group by one-way analysis of variance or the Kruskal-Wallis test. When statistically significant differences were detected, Dunnett's multiple comparison test was employed for comparison between the 0 μg/kg group and the treatment groups.

## RESULTS

### Body and organ development

Before weaning, no deaths or abnormal clinical signs caused by EE treatment were observed, and body weight gain per animal or per litter was similar among the groups (data not shown). In animals autopsied at PND21, body weight per animals was significantly elevated at 200 μg/kg compared with the 0 μg/kg group (Fig. 1). At PND34, a significant increase of relative brain weight in the 0.02 μg/kg group was found, but without dose-dependency (Table 1). There was no intergroup difference in body or organ weight at 10 weeks of age.

With regard to animals autopsied at PND34 and 10 weeks of age, the average day of vaginal opening was PND30 or 31 (Table 2). No intergroup difference was found when analyzed by individual pups or by litters. As shown in Table 2, although different estrous stage existed at PND34, there was no particular bias among the groups. All animals autopsied at 10 weeks of age demonstrated normal estrous cyclicity in the examination of vaginal smear.

### Histopathological examination of the female reproductive organs

In the ovaries, follicular development was normal both

**Table 1.** Organ weights at postnatal day 34 and 10 weeks of age

| | | EE (μg/kg) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 0.02 | 0.2 | 2 | 20 | 200 |
| Postnatal day 34 | | | | | | | |
| No. of animals (No. of litter) | | 4 (4) | 4 (2) | 4 (4) | 4 (4) | 4 (3) | 4 (4) |
| Brain | (g) | 1.59 ± 0.02[a] | 1.63 ± 0.05 | 1.62 ± 0.06 | 1.60 ± 0.10 | 1.56 ± 0.05 | 1.62 ± 0.05 |
| | (g%) | 1.35 ± 0.01 | 1.53 ± 0.06 * | 1.43 ± 0.10 | 1.41 ± 0.11 | 1.37 ± 0.07 | 1.43 ± 0.07 |
| Ovaries | (mg) | 40.0 ± 8.1 | 33.3 ± 8.0 | 31.8 ± 8.5 | 35.8 ± 4.0 | 37.0 ± 6.2 | 42.5 ± 5.0 |
| | (mg%) | 33.9 ± 6.9 | 31.0 ± 6.2 | 28.0 ± 8.4 | 31.5 ± 1.9 | 32.6 ± 6.4 | 37.3 ± 3.7 |
| Uterus | (g) | 0.17 ± 0.03 | 0.16 ± 0.05 | 0.18 ± 0.09 | 0.18 ± 0.03 | 0.26 ± 0.17 | 0.13 ± 0.02 |
| | (g%) | 0.14 ± 0.02 | 0.15 ± 0.05 | 0.16 ± 0.08 | 0.16 ± 0.04 | 0.23 ± 0.16 | 0.12 ± 0.01 |
| 10 weeks of age | | | | | | | |
| No. of animals (No. of litter) | | 4 (4) | 4 (4) | 4 (4) | 4 (4) | 4 (3) | 4 (4) |
| Brain | (g) | 1.74 ± 0.08 | 1.76 ± 0.04 | 1.90 ± 0.10 | 1.79 ± 0.06 | 1.80 ± 0.07 | 1.77 ± 0.07 |
| | (g%) | 0.84 ± 0.10 | 0.78 ± 0.02 | 0.88 ± 0.05 | 0.87 ± 0.02 | 0.82 ± 0.06 | 0.90 ± 0.10 |
| Ovaries | (mg) | 90.0 ± 5.5 | 92.8 ± 10.6 | 81.8 ± 14.7 | 81.0 ± 8.2 | 82.0 ± 18.3 | 79.8 ± 13.7 |
| | (mg%) | 43.4 ± 2.8 | 41.0 ± 4.5 | 37.9 ± 6.4 | 39.4 ± 4.6 | 37.6 ± 9.2 | 39.7 ± 2.1 |
| Uterus | (g) | 0.45 ± 0.06 | 0.51 ± 0.03 | 0.48 ± 0.06 | 0.57 ± 0.20 | 0.65 ± 0.12 | 0.59 ± 0.41 |
| | (g%) | 0.22 ± 0.05 | 0.23 ± 0.01 | 0.22 ± 0.02 | 0.28 ± 0.11 | 0.29 ± 0.06 | 0.29 ± 0.20 |

[a]: mean ± S.D.

*, significantly different from 0 μg/kg group at $p < 0.05$.

**Table 2.** Mean days of vaginal opening and estrous cycle stage at postnatal day 34 in rats exposed to EE during the neonatal period

| EE (μg/kg) | Vaginal opening | | | | Estrous cycle stage at PND34[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Per animal | (n) | Per litter | (n) | P | E | M | D |
| 0 | 31.0 ± 0.7 [a] | (10) | 31.0 ± 0.6 | (5) | 0 | 1 | 2 | 2 |
| 0.02 | 31.8 ± 1.4 | (10) | 31.6 ± 1.3 | (7) | 2 | 0 | 1 | 2 |
| 0.2 | 30.8 ± 1.1 | (10) | 30.8 ± 0.9 | (5) | 0 | 1 | 2 | 2 |
| 2 | 31.3 ± 1.1 | (10) | 31.3 ± 0.6 | (5) | 0 | 2 | 2 | 1 |
| 20 | 30.8 ± 0.8 | (10) | 30.8 ± 0.6 | (5) | 1 | 1 | 3 | 0 |
| 200 | 30.7 ± 0.8 | (10) | 30.7 ± 0.7 | (5) | 0 | 0 | 4 | 1 |

[a]: mean ± S.D.

[b]: estrous cycle stage was checked by histopathological examination of the ovary, uterus and vagina.

P: proestus, E: estrus, M: metestrus, D: diestrus

PND, postnatal day.

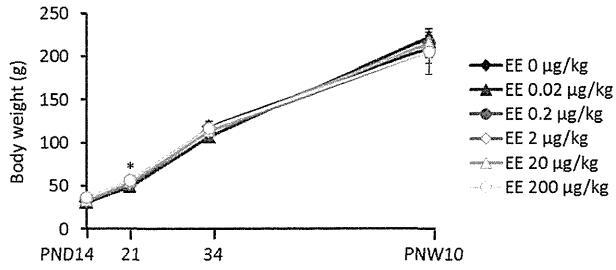


**Fig. 1.** Body weight curves for rats subjected to neonatal exposure to EE. Data are represent means ± S.D. n = 5 per group. *, significantly different from 0 μg/kg group at $p < 0.05$. PND, postnatal day; PNW, postnatal week.

in the 0 μg/kg and EE-treated groups. Polyovular follicles were evenly found in small number in all groups.

In the 20 and 200 μg/kg groups, the number of uterine glands per section showed a tendency to decrease at PND21 (Fig. 2). At PND34, the number of uterine glands varied widely between individuals. Clear correlation with estrous cycle was not detected and there was no intergroup difference in the number of uterine glands. At 10 weeks of age, significant changes were not seen in the number of uterine glands in the histopathological examination. A few animals from different dams of the 20 and 200 μg/kg group showed some abnormalities in the histology of the ovary, uterus and vagina. Their histology could

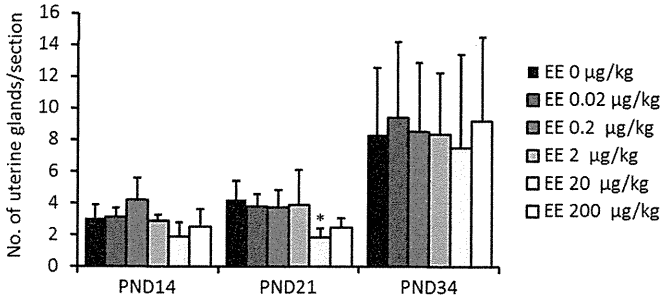


**Fig. 2.** Number of uterine glands per section from postnatal days 14, 21 and 34. Data represent means ± S.D. n = 5 per group. *, significantly different from 0 μg/kg group at $p < 0.05$.

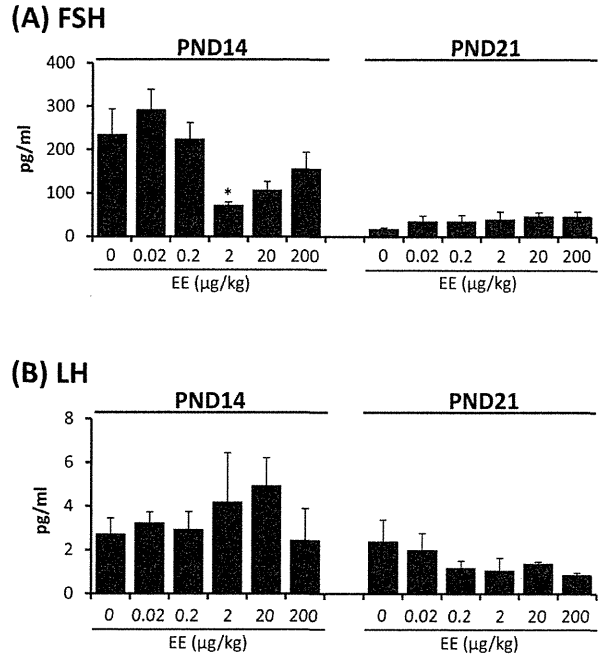


**Fig. 3.** Serum levels of FSH (A) and LH (B) at postnatal days 14 and 21. Data are means ± S.E. n = 4 per group. *, significantly different from 0 μg/kg group at $p < 0.05$.

not be classified into any estrus cycle stage. In the ovary, newly formed corpora lutea with small basophilic cells, a feature of corpora lutea at estrus, was not found, and the number of recent corpora lutea was decreased. Although there were some large antral follicles, the histology was different from both proestrus and estrus. The lumen of the uterus mildly dilated, and the degeneration/apoptosis of epithelial cells characteristic to estrus was not found. In their vagina, the epithelium showed incomplete keratinization, unlike typical of estrus stage. Treatment-related abnormalities were not found in other organs such as the pituitary, mammary glands, liver, adrenal glands, or the thymus.

## Sex-related hormones

The levels of serum FSH and LH at PND14 and 21 are shown in Fig. 3. Serum FSH in the groups treated with 2 μg/kg or more showed a tendency to decrease at PND14. In contrast, the level of LH did not differ among the groups. At PND21, significant changes related to EE treatment were not detected in either FSH or LH. Since the hormone levels widely fluctuated due to mixed estrous stage, intergroup differences were not found in FSH, LH, E2 or P4 at PND34 (data not shown). At 10 weeks of age, level of E2 tended to be high in the animals showing abnormal histology of the ovary, uterus and vagina. The average levels of FSH, LH, E2 or P4 at estrus stage were not statistically different among the groups (Fig. 4).

## Expression of Kiss1 and related genes

In the whole hypothalamus, the level of *Kiss1* mRNA was significantly decreased in all of the EE-treated groups at PND14 (Fig. 5). However, *Kiss1* mRNA did not fluctuate at PND21, 34, or 10 weeks of age. No treatment-related change in the expression of *Kiss1r* (Fig. 5), *Esr1*, *Esr2*, *Gnrh* or *Cyp19a1* (data not shown) was found at any time point examined, although a few statistical differences were noted without dose-dependency.

## Expression of ERα and PCNA during uterine development

ERα and PCNA were strongly expressed in the nuclei of glandular cells and stromal cells at PND14 in both the 0 μg/kg and the EE-treated groups. Similarly, the expression of ERα or PCNA was mainly found in the glandular cells and stromal cells at PND21 (Fig. 6). Although expression patterns of ERα and PCNA changed depending on the estrous cycle at PND34, there was no difference among the groups. At 10 weeks of age, most of the endometrial epithelial cells expressed ERα in the 0 μg/kg group. In contrast, ERα-positive epithelial cells decreased in a dose-dependent manner, and there were few epithelial cells expressing ERα in the 200 μg/kg group (Fig. 7). The scores for expression of ERα in the glandular cells and stromal cells did not differ among the groups. No intergroup difference was found in the expression of PCNA in the endometrial epithelial cells, uterine glands and strom-

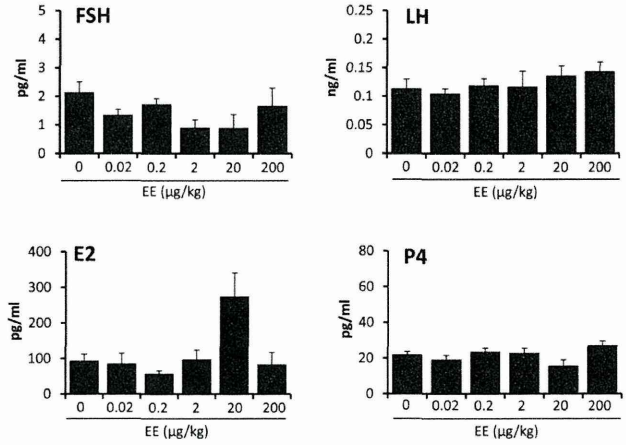


**Fig. 4.** Serum levels of sex-related hormones at 10 weeks of age. Data are means ± S.E. n = 4 per group.

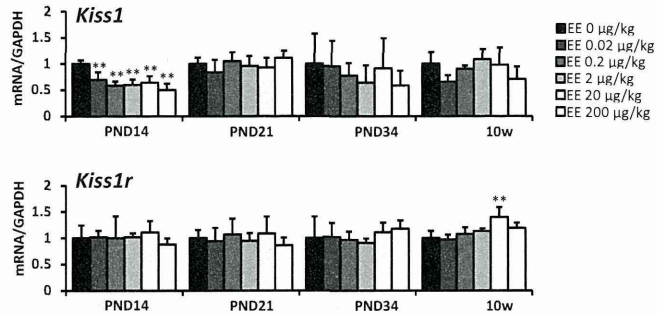


**Fig. 5.** Hypothalamic mRNA levels of rats subjected to neonatal exposure to EE. Data are means ± S.D. n = 4 per group. **, significantly different from the 0 μg/kg group at $p < 0.01$.

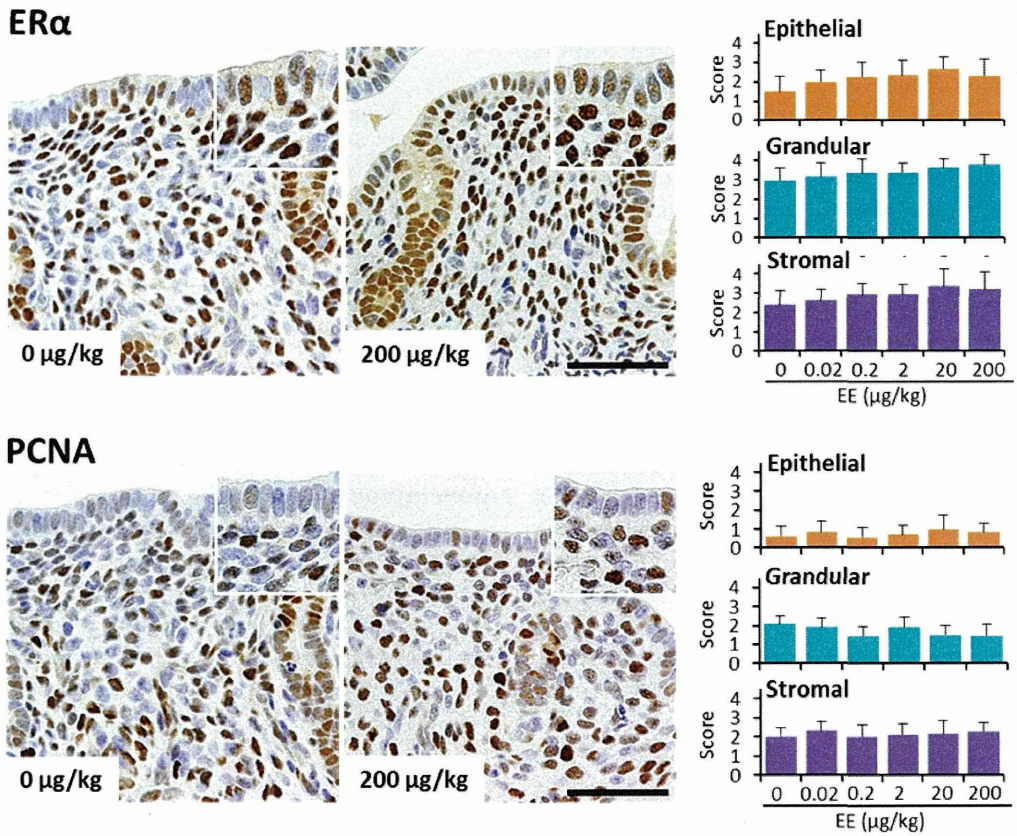


**Fig. 6.** Immunohistochemical expression of ERα and PCNA in the uterus at postnatal day 21. Representative photomicrographs and scores of positive cells in endometrial epithelial cells, uterine glands and stromal cells. ERα (upper) and PCNA (lower) were strongly expressed in the nuclei of glandular cells and stromal cells both in the 0 μg/kg and EE-treated groups. Upper right corner in each photo shows high-power field of ERα- and PCNA-positive nuclei. Bars = 50 μm. Score: 0, negative; 1, slightly positive (< 10%); 2, partly positive (10-30%); 3, positive in about half (30-70%); 4, mostly positive (> 70%). Data are means ± S.D.

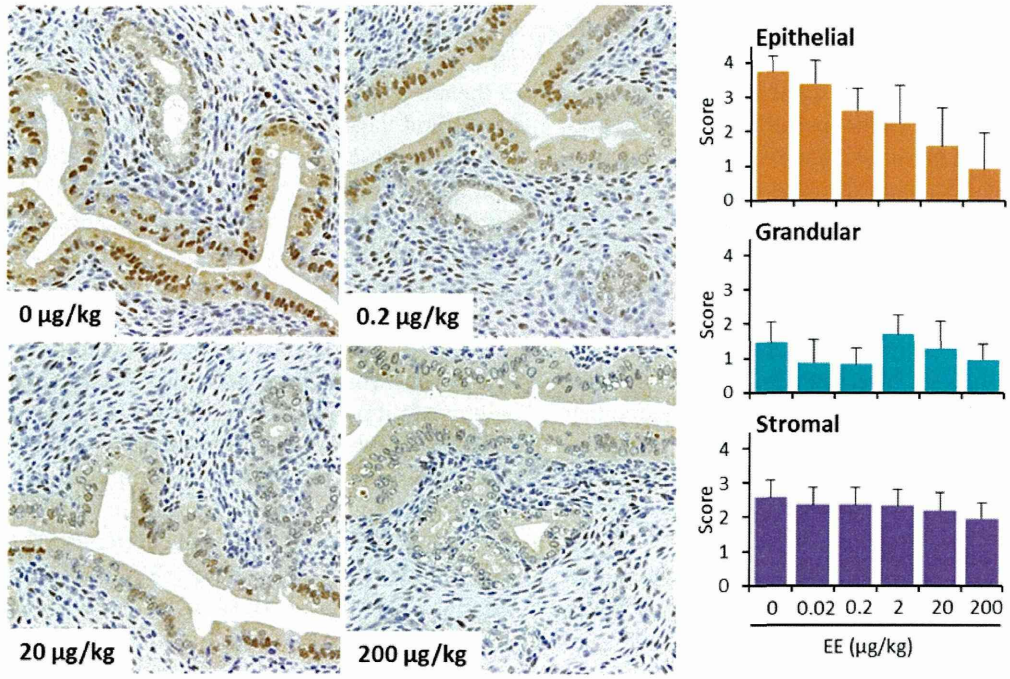


**Fig. 7.** Immunohistochemical expression of ERα in the uterus at 10 weeks of age. Representative photomicrographs and scores of positive cells in endometrial epithelial cells, uterine glands and stromal cells. In the 0 μg/kg group, most of the endometrial epithelial cells expressed ERα. ERα-positive epithelial cells decreased in a dose-dependent manner and there were few epithelial cells expressing ERα in the 200 μg/kg group. Bars = 50 μm. Score: 0, negative; 1, slightly positive (< 10%); 2, partly positive (10-30%); 3, positive in about half (30-70%); 4, mostly positive (> 70%). Data are means ± S.D.

al cells at 10 weeks of age.

## DISCUSSION

A significant increase of body weight was observed in the 200 μg/kg group at PND 21. This finding was considered incidental due to litter size because there were no intergroup differences in the body weight gain of total pups prior to weaning. Although relative brain weight in the 0.02 μg/kg group significantly increased at PND34, it is not regarded as toxicologically significant, because dose-dependency was not found. Therefore, under the present conditions, neonatal exposure to EE did not affect body growth.

In the histopathological examination, there was no effect of EE exposure on ovarian development. It has been reported that the incidence of polyovular follicles was increased by neonatal DES exposure in mice (Iguchi *et al.*, 1990). In the present study, polyovular follicles were found in small numbers distributed evenly across all groups, and unrelated to neonatal EE exposure. At 10 weeks of age, although all animals exhibited normal estrous cycle in the examination of the vaginal smear, decrease of recent corpora lutea was observed in a few animals at 20 and 200 μg/kg, suggesting irregular ovulation during recent cycle.

In the uterus, there was a tendency for a decrease in the number of uterine glands per section in the 20 and 200 μg/kg groups at PND21. Additionally, immunohistochemical staining revealed that the expression of ERα by endometrial epithelial cells decreased in a dose-dependent fashion at 10 weeks of age. It is known that early postnatal exposure to estrogenic compounds can suppress uterine gland genesis and expression of estrogen receptors, and can alter the uterine response to estrogen (Branham *et al.*, 1985, 1988; Yoshida *et al.*, 1999; Katsuda *et al.*, 2000; Newbold *et al.*, 2004). Therefore, it is likely that these findings were caused by neonatal exposure to EE. Because the doses that showed significant decrease were different between the number of uterine glands at PND21 and the levels of serum FSH and *Kiss1* mRNA at PND14, it is considered that EE directly affected the uterine glands, without through hypothalamus. In our previous study investigating long-term effects

of neonatal exposure to EE (Takahashi *et al.*, 2013), the incidence of adenomyosis was decreased in the high dose groups at 10 months of age. Adenomyosis has been reported as a common lesion in aged rats (Dixon *et al.*, 1999). It is likely that hormonal perturbations are important for the development of adenomyosis similar to mice, although studies of adenomyosis using rats are very limited (Greaves and White, 2006). Because presence of high P4 level is known to be one of a factor to develop adenomyosis, lowered P4 level at 10 months of age might contribute decreased incidence. However, the possibilities that suppression of uterine gland genesis and alteration of ERα expression affected adenomyosis in later life cannot be excluded. In mice, it has been reported that neonatal exposure to bisphenol A increased adenomyosis (Newbold *et al.*, 2007). Therefore, further investigation is required to clarify the early and long-term effects of neonatal exposure to EE on the uterus in rats.

Although incomplete keratinization of vaginal epithelium was observed only in a few animals of the 20 μg/kg group at 10 weeks of age, no abnormality was observed in development or morphology of the vagina in the histopathological examination. In the mammary glands, an increase in acini exhibiting oxyphilic and hypertrophic changes (virilization) was observed at ≥ 0.2 μg/kg in a dose-dependent manner at 10 months of age in our previous report (Takahashi *et al.*, 2013). However, treatment-related changes were not found until 10 weeks of age. There are some reports that perinatal exposure to hormonal agents, such as genistein and bisphenol A, leads to abnormal development and morphology of the mammary gland around puberty in rats (Durando *et al.*, 2007; El Sheikh Saad *et al.*, 2011). Therefore, more detailed examination including quantitative methods might be necessary to clarify whether neonatal exposure to EE affects development of mammary glands or not.

At PND 14, the serum level of FSH exhibited a tendency to decrease at ≥ 2 μg/kg. In rats, the FSH level in blood is low at PND10 and subsequently reaches a peak around PND15, followed by a remarkable reduction at PND25 - 30. This transient increase of FSH occurs independently of inhibin regulation (Herath *et al.*, 2001). It has been reported that the peak of FSH is delayed or decreased in androgenized female rats, but the difference disappeared at PND20 - 25 (Cheng and Johnson, 1974; Chiappa and Fink, 1977). Also, chemical exposure during the perinatal period affects the level of FSH in infantile female rats (Wilson and Handa, 1997; Katsuda *et al.*, 2000). Therefore, a decrease of FSH level at PND14 might be involved in neonatal EE exposure. However, further study is required to confirm that the peak of FSH is decreased or delayed. On the other hand, there were no intergroup differences in serum hormones at PND34 and 10 weeks of age, although level of E2 tended to be high in the animals showing abnormal histology of the ovary, uterus and vagina at 10 weeks of age. In both our previous and present studies, there was no difference in the average day of vaginal opening (Takahashi *et al.*, 2013). Therefore, it is probable that EE exposure in the neonatal period has little impact on ovarian development and sexual maturation.

Dysfunction of the hypothalamus is suspected as one cause of delayed adverse effects (Takahashi *et al.*, 2013). Kisspeptin, which is expressed in specific neurons in the anteroventral periventricular (AVPV) nucleus and arcuate (ARC) nucleus of the hypothalamus, is widely recognized as playing a critical role in female reproductive function, including regulation of ovulation and estrous cyclicity (Uenoyama *et al.*, 2009; Roa *et al.*, 2011). Therefore, we examined the expression of *Kiss1* mRNA in the whole hypothalamus from PND14 to 10 weeks of age. In addition, *Kiss1* related genes such as *Kiss1r*, *Esr1*, *Esr2*, *Gnrh* and *Cyp19a1* were examined using the same samples. Only *Kiss1* showed a significant decrease in all of the EE-treated groups at PND14. Neonatal injection of estradiol benzoate to male and female rats results in a dose-dependent decrease in hypothalamic *Kiss1* mRNA levels in the prepubertal stage (Navarro *et al.*, 2009). Therefore, a significant decrease of *Kiss1* mRNA at PND14 might be induced by neonatal exposure to EE. The expression patterns of *Kiss1* in the AVPV and ARC during the postnatal period are different (Takumi *et al.*, 2011). At PND14, because the expression level in the ARC is dominant, a decrease of *Kiss1* mRNA is likely to be caused by suppressed expression in the ARC. However, it is known that the AVPV but not the ARC regulates ovulation (Adachi *et al.*, 2007). In our study, because we used whole hypothalamus to examine *Kiss1* and its related genes, the expression patterns in the AVPV and ARC were combined. Since the relation between *Kiss1* expression and gonadotropin secretion during postnatal period is not fully understood, the association with the decrease of serum FSH at PND14 or unchanged LH remains uncertain. It is known that the expression levels of *Kiss1* mRNA fluctuates in association with estrous cycle, and its expressions becomes highest in the proestrous afternoon in AVPV to induce LH surge (Adachi *et al.*, 2007). The level of *Kiss1* mRNA at PND34 widely fluctuated due to mixed estrous stage in the present study, and further analysis of unified estrous stage will be necessary. In addition to estrus, the expression specific to proestrus should be examined.

In summary, delayed adverse effects have been report-

ed to appear only after maturation so far. However, animals neonatally exposed to EE demonstrated various changes, such as suppressed development of the uterine glands, decreased ERα expression in the uterine epithelium, lowered FSH level, and reduced expression of *Kiss1* mRNA in the hypothalamus prior to the appearance of delayed adverse effects by detailed investigations. Although the association between the above changes and the acceleration of age-related anovulation must be clarified, it is suggested that these changes or their combination might be candidate indicators for the early detection of delayed adverse effects. Particularly, *Kiss1* is expected as the key molecule, because the change was detected from lowest dose and it is directly involved in regulation of ovulation. In order to clarify the relevance between early onset of anovulation and *Kiss1* expression, hypothalamic region and estrous stage specific analysis is currently progress in our laboratory.

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

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# Inhibitory Potential of Postnatal Treatment with Cyclopamine, a Hedgehog Signaling Inhibitor, on Medulloblastoma Development in Ptch1 Heterozygous Mice

Saori Matsuo[1,2], Miwa Takahashi[1], Kaoru Inoue[1], Kei Tamura[1], Kaoru Irie[1], Yukio Kodama[3], Akiyoshi Nishikawa[2,4], and Midori Yoshida[1]

[1]*Division of Pathology, National Institute of Health Sciences, Tokyo, Japan*
[2]*Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan*
[3]*Division of Toxicology, National Institute of Health Sciences, Tokyo, Japan*
[4]*Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan*

Abstract

Medulloblastomas (MBs) are thought to be derived from granular cell precursors in the external granular layer (EGL) of the developing cerebellum. Heterozygous patched1 (Ptch1) knockout mice develop MBs that resemble those in humans when the sonic hedgehog (Shh) signaling pathway is activated. The present study was conducted to evaluate postnatal effects of a Shh signaling inhibitor, cyclopamine, on the development of MBs in Ptch1 mice. Ptch1 and wild-type mice were treated daily with subcutaneous cyclopamine at 40 mg/kg or vehicle from postnatal day (PND) 1 to PND14, and the subsequent development of MBs and preneoplastic lesions was examined up to week 12 (W12). Proliferative lesions in the cerebellum, MBs, and preneoplastic lesions were only detected in Ptch1 mice. Cyclopamine treatment resulted in a statistically significant reduction in the incidence and/or area of proliferative lesions at PND14 and 21. The trend of decreasing preneoplastic lesions persisted up to W12. At PND7, cyclopamine treatment reduced the width and proliferation of the EGL regardless of genotype. These results indicate that inhibition of Shh signaling during cerebellar development has prolonged inhibitory potential on MB development in Ptch1 mice. This inhibitory potential might be related to inhibition of EGL proliferation, including preneoplastic MB cells.

*Keywords:* cyclopamine; cerebellum; medulloblastoma; patched1; preneoplastic lesion; smoothened inhibitor; sonic hedgehog inhibitor.

## Introduction

Medulloblastoma (MB) is the most common malignant brain tumor in children (Bartlett, Kortmann, and Saran 2013; Dhall 2009; Hatten and Roussel 2011; Jones et al. 2012). Although exposure to environmental compounds and radiation during the developmental period and early life stages has been thought to be critically involved in the causation of tumors, little is known about the etiology of childhood brain tumors (Bunin et al. 2006; Birnbaum and Fenton 2003; Dietrich et al. 2005; Mckean-Cowdin et al. 2003; Norman, Holly, and Preston-Martin 1996; Takahashi et al. 2012).

Molecular analysis of sporadic human MBs revealed activation of the sonic hedgehog (Shh) signaling pathway caused by the loss of *patched1* (*Ptch1*) and mutations in other components of the Shh pathway. *Ptch1* encodes a receptor for Shh, Ptch1, and is one of the key genes related to MB formation in humans (Dhall 2009; Raffel 2004). Pathway activation is triggered by binding of Shh to Ptch1; in the absence of Shh, the activity of Smoothened (Smo) is suppressed. Shh binding to Ptch1 or mutational inactivation of Ptch1 relieves the inhibition on Smo culminating in the activation of one or more of the Gli1 transcription factors that regulate the expression of downstream targets (Hahn et al. 1999; Huse and Holland 2010; Roussel and Hatten 2011).

Heterozygous *Ptch1* knockout mice (Ptch1 mice) have been used as a valuable model of MB due to the high incidence of MBs (14–30%) and the morphological and molecular similarities to human MBs (Corcoran and Scott 2001; Goodrich et al. 1997; Hahn et al. 1999; Pazzaglia 2006; Raffel 2004; Wetmore, Eberhart, and Curran 2000). Moreover, it has been reported that Shh signaling is activated in MBs of Ptch1 mice (Dyer 2004; Goodrich et al. 1997; Oliver et al. 2005; Wetmore, Eberhart, and Curran 2000).

MBs in humans and Ptch1 mice are thought to be derived from residual granule cell precursors (GCPs) located in the external granule cell or external granular (germinal) layer (EGL) of the cerebellum (Behesti and Marino 2009; Roussel

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Address correspondence to: M. Yoshida, Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; e-mail: midoriy@nihs.go.jp.

Abbreviations: EGL, external granular layer; GCP, granule cell precursor; HGF, hepatocyte growth factor; MB, medulloblastoma; PND, postnatal day; Ptch1, patched1; Shh, sonic hedgehog; Smo, smoothened; W12, postnatal week 12.

and Hatten 2011). During normal cerebellar development in mice, GCPs proliferate postnatally, and the proliferative period peaks between postnatal days (PND) 4 and 8 (Behesti and Marino 2009; Roussel and Hatten 2011). For GCP proliferation, Shh signaling is required (Lewis et al. 2004; Raffel 2004; Roussel and Hatten 2011; Vaillant and Monard 2009; Wallace 1999).

While Ptch1 mice have been accepted as a useful MB model, it takes an extended amount of time to detect the efficacy of treatments using end points such as the clinical signs of increased intracranial pressure due to MB or death after the long latent period of 9 to over 12 months (Ayrault et al. 2009; Briggs et al. 2008; Ecke et al. 2008; Farioli-Vecchioli et al. 2007; Kimura et al. 2005; Pazzaglia et al. 2006; Pazzaglia et al. 2009; Pogoriler et al. 2006; Uziel et al. 2005; Wetmore, Eberhart, and Curran 2001).

Recently, we found that the earliest signs of MBs and their preneoplastic lesions in Ptch1 mice were morphologically detectable within 2 weeks after birth (Matsuo et al. 2013). Changes in these indicators are thought to be early novel end points for assessment of the modifying effects of chemicals and/or agents on MB development in studies using Ptch1 mice.

Cyclopamine is a naturally occurring alkaloid of the corn lily *Veratrum californicum* that causes cyclopia in sheep by blocking the Shh/Ptc/Smo signaling pathway (Ecke et al. 2008; Heretsch et al. 2010a and b; Lipinski et al. 2008). The inhibitory effects of cyclopamine on the Shh pathway have been reported in a number of *in vitro* and *in vivo* studies (Ecke et al. 2008; Scales and Sauvage 2009; Stecca and Ruiz i Altaba 2002). Furthermore, cyclopamine was shown to inhibit the *in vitro* growth of human MB cell lines (Berman et al. 2002), and other studies have examined the effect of cyclopamine *in vivo* on spontaneously developing Hedgehog (Hh)-dependent tumors including MBs (Sanchez and Ruiz i Altaba 2005; Ecke et al. 2008; Fan et al. 2011; Coon et al. 2010). However, the effects of developmental exposure to cyclopamine using Ptch1 mice have not been reported. Here, we used the Ptch1 mouse model of MB to test if cyclopamine treatment from PND 1 to 14 is able to inhibit tumor growth and to affect cerebellar development.

## Materials and Methods

### *Animals*

Heterozygous ptch1 knockout mice (Ptch1 mice) maintained on a mixed C57B1/6 × 129Sv background were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in our facility in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature 24°C ± 1°C, relative humidity 55% ± 5%, 12-hr light–dark cycle) with a basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water available *ad libitum*. Animals were genotyped by polymerase chain reaction amplification of genomic DNA extracted from tails (Matsuo et al. 2013). The experimental protocol regarding animal use was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

### *Cyclopamine Treatment*

Cyclopamine (CAS No. 4449-51-8, purity >99%, LC Laboratories, Woburn, MA) was dissolved in ethanol then suspended in triolein (Wako, Osaka, Japan). Animals were weighed just prior to each injection, and cyclopamine (40 mg/kg/day) or vehicle (triolein: ethanol, 4:1 vol/vol) was injected subcutaneously from PND1 to PND14. The dosing volume, dose of cyclopamine and vehicle, and route of administration were selected based on previous studies (Berman et al. 2002; Lipinski et al. 2008) and our preliminary study. The administration period, from PND1 to PND14, was chosen to match the developmental period in the cerebellum in which GCP proliferation is prominent (Behesti and Marino 2009; Haldipur et al. 2012; Vaillant and Monard 2009). This duration also covers the highly susceptible period of Ptch1 mice to X-ray irradiation and carcinogens (Pazzaglia et al. 2002; Takahashi et al. 2012). The number of mice in each group at each time point is listed in Table 1. At least 5 wild-type mice and 9 Ptch1 mice from 3 to 9 dams were allocated to each group.

### *Necropsy*

To examine the effects of cyclopamine on early cerebellar development, Ptch1 and wild-type mice at PND7 were subjected to necropsies. To examine the effect of cyclopamine on MB development, necropsy was performed at PND14 and PND21. In addition, at postnatal week 12 (W12), Ptch1 and wild-type mice were examined to determine whether the effects of cyclopamine treatment on the cerebellum during the developmental period persist after the maturation of the cerebellum. At necropsy, all mice were euthanized under deep anesthesia with isoflurane.

### *Tissue Processing and Histopathology*

After necropsy, brains were removed and weighed before fixation in 10% neutral buffered formalin. Midsagittal (right hemisphere) and cross (left hemisphere) sections of the cerebella were routinely processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin. The prepared histopathological specimens were examined by light microscopy. To examine the effect of cyclopamine on MB development, we counted preneoplastic lesions such as thickenings of the EGL at PND14 and Ki-67-positive foci at PND21 and W12 in addition to MBs. MBs were divided into 2 types according to the previous study (Matsuo et al. 2013): a focal MB occupying 1 to 2 lobules of the cerebellum was defined as a small MB and an advanced MB spreading over 3 or more lobules was defined as a large MB.

### *Immunohistochemistry*

Antibodies used for immunohistochemistry included monoclonal rat anti-mouse Ki-67 (Clone TEC-3, Dako Cytomation,