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ORIGINAL ARTICLE

Prenatal exposure to bisphenol A impairs predator odor-induced fear behavior in young rat offspring

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Abstract

OBJECTIVES: This study evaluated the changes in behaviors and the endocrine system in rat offspring at postnatal day 20 following prenatal exposure to bisphenol A (BPA), a major environmental endocrine disruptor.
DESIGN: Using A predator odor (2,4,5-trimethylthiazoline [TMT]) as a stressor, I evaluate behavioral and endocrine responses to check whether the normal stress response is affected by BPA.
MATERIALS AND METHODS: A low-dose group (BPA-L; 0.015 mg/kg/day) and a high-dose group (BPA-H; 1.5 mg/kg/day) were compared to assess dose dependency. The control group was not exposed to BPA. Spontaneous behaviors (rearing, ambulation, grooming, and freezing) were assessed in the presence or absence of TMT odor.
RESULTS: In the control group, TMT odor increased freezing but not grooming behaviors. Conversely, in the BPA-H group, freezing was unchanged, but grooming behavior increased; however, increased freezing and grooming behaviors were observed following TMT odor exposure in the BPA-L group. In addition, blood corticosterone levels increased following TMT odor exposure in all three groups, but there was no difference between the BPA-exposure groups and the control group. Therefore, in the BPA-H group, despite the activation of the hypothalamus-pituitary-adrenal axis by TMT, freezing behavior did not increase, suggesting the absence of defensive behaviors.
CONCLUSION: These findings suggest that prenatal exposure to high-dose BPA causes habituation to stress induced by the predator odor and alters the normal stress response in young rat offspring.

Abbreviations:

ANOVA	- Analysis of variance
CNS	- Central nervous system
EPA	- Environmental Protection Agency
FST	- Forced swimming test
HPA	- Hypothalamic-pituitary-adrenal

INTRODUCTION

Bisphenol A (BPA) is widely used in the synthesis of plastics such as polycarbonate and epoxy resin. For example, sunglasses, compact discs, food containers, baby bottles, and certain dental materials contain BPA (Krishnan *et al.* 1993; Geens *et al.* 2012; Valentino *et al.* 2016). BPA is an endocrine-disrupting substance, and its role in the induction of changes in the central nervous

system (CNS) has been well studied. Rodent behavioral studies have investigated the effects of BPA on the CNS. Our previous studies demonstrated that perinatal BPA exposure induces sex-dependent changes in locomotor activity, exploratory behavior, and the size of the locus coeruleus in rats (Kubo *et al.* 2001; Kubo *et al.* 2003; Fujimoto *et al.* 2007). Furthermore, we also showed that prenatal and postnatal BPA exposure induce the loss of sex differences in exploratory behavior in the open-field test and prolong the immobility time in the forced swimming test (FST) (Fujimoto *et al.* 2006; 2013). In addition, activity in the hypothalamic-pituitary-adrenal (HPA) axis is reportedly altered by BPA (Poimenova *et al.* 2010; Panagiotidou *et al.* 2014). This finding suggests that BPA exposure induces certain changes in the neural mechanisms of the brain that regulate the stress response.

Most *in vivo* studies were conducted in animals at the adolescent to adult stages. Recently, there has been an increased demand for the development of medications for children; therefore, young animals are increasingly used for safety testing during drug development. In a study on BPA, Stump *et al.* (2010) continuously measured the locomotor activity of rat offspring at postnatal days (PNDs) 13, 17, and 21, over 60 min, using an automated measuring device. Similarly, Ferguson *et al.* (2011) evaluated the righting reflex of PND 3–6 rats and slant board behavior of PND 8–11 rats. Notably, both studies failed to show any clear effects of BPA. However, Wang *et al.* (2014) found that BPA-exposed PND 21 rats exhibited decreased rearing behavior and locomotor activity, while Komada *et al.* (2014) found that prenatal BPA-exposed 1-day-old offspring (PND 1) exhibited hyperactivity during a 6-min observation period. However, the effects of BPA on other animal behaviors, such as stress-related behaviors, have not been well-investigated.

Stress is highly associated with fear- and anxiety-related behaviors. In studies on pre-weaning young rats, electric shock stress was found to increase fear-related behaviors and stress hormone levels (Takahashi *et al.* 1990; 1991). Furthermore, young rats exhibit increased freezing when exposed to cat odor (Kabitzke *et al.* 2011) or to adult male rats (Wiedenmayer and Barr, 2003). In addition, 2,4,5-trimethylthiazoline (TMT), a substance found in fox feces, has been used extensively in animal studies to induce predator odor stress; however, most studies were performed in adult animals (Vernet-Maury *et al.* 1984; Morrow *et al.* 2000; Fendt *et al.* 2005). Notably, our previous study was the first to use TMT to examine behavioral changes induced by BPA exposure and found that BPA-exposed rats exhibited increased avoidance behavior in response to the TMT odor (Fujimoto *et al.* 2015). However, in that study, we only used adult offspring and did not measure freezing behavior or stress hormone levels.

In this study, I hypothesized that prenatal exposure to BPA affects the neural mechanisms in the brain associated with stress responses, leading to changes in behavior and endocrine responses in PND 20 rat offspring. To test this hypothesis, I investigated the effects of prenatal BPA exposure on behavioral (including freezing, rearing, grooming, and locomotor activity) and endocrine (stress hormone levels) responses to predator-odor stress in PND 20 rat offspring.

MATERIALS AND METHODS

Animals

Twenty-four pregnant rats (SLC: Wistar, gestational day [GD] 11) were purchased from Japan SLC (Hamamatsu, Japan), and eight animals were randomly assigned to each of the following three groups: a control group (CON), a low-dose BPA exposure group (BPA-L), and a high-dose BPA exposure group (BPA-H). Animals were housed in the animal room (temperature, 23 ± 1°C; humidity, 60 ± 10%) with a 12 h/12 h light/dark cycle (light on at 08:00) at the Osaka Dental University. Animals were allowed to freely consume laboratory chow (CE-2, CLEA Japan, Tokyo, Japan) and tap water during the study. All experiments were approved by the Osaka Dental University Animal Research Committee (13-02017 and 14-02011), and this study conformed to the EC Directive 86/609/EEC for animal experiments.

BPA treatment

BPA was purchased from Sigma-Aldrich (St. Louis, MO) and was administered to pregnant dams via drinking water in glass bottles. At GD 14, eight dams were provided with a 0.1 ppm BPA solution (BPA-L group), eight were provided with a 10 ppm BPA solution (BPA-H group), and eight were provided with distilled water as the vehicle control (CON group). Exposure to BPA or vehicle continued until the day of birth (PND 0). The average BPA intake over 7 days (calculated based on body weight and water intake) was 0.015 mg/kg/day in the BPA-L group and 1.5 mg/kg/day in the BPA-H group. These doses were markedly lower than the no-observed-adverse-effect level (NOAEL; 50 mg/kg/day) based on a previous study (Cagen *et al.* 1999). In the BPA-L group, the dose was lower than the reference dose (0.05 mg/kg/day) according to the U.S. Environmental Protection Agency (EPA) (https://www.bisphenol-a.org/). On PND 1, the litters were balanced such that each litter consisted of four male pups and four female pups.

Behavioral tests

A total of 186 rat offspring at PND 20 were subjected to behavioral tests (29 CON males, 32 CON females; 32 BPA-L males, 30 BPA-L females; 31 BPA-H males, and 32 BPA-H females). All behavioral experiments were recorded with a digital video camera (HDC-HS9, Matsushita Electric Industrial Co., Ltd., Osaka, Japan), and each behavioral parameter was scored manually

upon later viewing of the video. Behavioral tests were conducted using a testing apparatus consisting of a long, black acrylic observation box (6 cm in width, 44 cm in length, and 15 cm in height, with no bottom or ceiling). Four testing boxes were arranged in parallel, with the video camera mounted overhead such that the behaviors of four animals could be recorded simultaneously. Papers with line markers, spaced 5 cm apart, were placed beneath each apparatus and were changed between each test. During testing, each of the four same-sex littermates was placed in the center of each apparatus and allowed to move freely for 3 min. Rearing frequency, ambulation, grooming (total duration), and freezing (total duration) behaviors were assessed for each animal. Ambulation was defined as the number of times the animal crossed a 5 cm marker line plus the number of direction changes for each rat. Freezing was defined as a state in which the rats were immobile, with the exception of minor movements related to breathing. In the odor-free session, the procedure was performed as described above, but nothing was placed in the apparatus. Subsequently, the same animals were subjected to testing in the TMT odor session. The TMT odor source was placed at one end of the apparatus and was hidden beneath the line paper under the boxes. At the opposite end, odor-free oil was similarly placed. For counterbalancing, the orientation of this arrangement was changed for each measurement. The TMT odor source was prepared by placing a piece of filter paper in a microtube, and 30 μL of 0.3% TMT was dropped onto the filter paper. TMT was purchased from Contech Inc. (Victoria, BC, Canada). Triethyl citrate (an odor-free oil) was used for dilution, and the odor-free solution was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The interval between the odor-free session and the TMT odor session for each animal was approximately 90 min. All tests were conducted between 14:00 and 18:00.

Serum corticosterone measurement

At PND 24, 38 pups from five dams in the CON group, 40 pups from five dams in the BPA-L group, and 39 pups from five dams in the BPA-H group were used for the measurement of blood corticosterone levels. Four males and four females from one dam were divided into the following two groups: two males and two females were used for the odor-free session, and the remaining two males and two females were used for the TMT odor session. In the odor-free session, the animals were anesthetized immediately after removal from the breeding cage using pentobarbital sodium (40 mg/kg, intraperitoneal injection; Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). Blood was collected by cardiac puncture and was allowed to coagulate; it was then centrifuged to isolate the serum, which was collected and stored at -30°C until analysis. After the odor-free session, the animals were subjected to the TMT odor test session. The TMT odor microtube was prepared and placed in the chamber as described in the "Behavioral tests" section. Animals removed from the breeding cage were transferred to the chamber and exposed to the odor for approximately 20 min; they were then anesthetized following the procedure described in the odor-free session, and blood was collected. A common mouse breeding cage was used as the anesthetic induction chamber. All procedures were performed between 14:00 and 18:00. Serum corticosterone levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Assaypro, St. Charles, MO, USA).

Statistical analyses

For the behavioral tests, the mean values of the data obtained from eight pups (four males and four females) from each dam were calculated. Statistical analysis was performed with the average value for each dam as n = 1. Therefore, the total sample size was n = 8 in each of the CON, BPA-L, and BPA-H groups, which is the same as the total number of dams. For each of the TMT and odor-free sessions, a one-way analysis of variance (ANOVA) was used to compare the three groups (CON, BPA-L, and BPA-H). In addition, the difference between the scores of both sessions was calculated (TMT odor session minus the odor-free session), and a one-way ANOVA was conducted to compare the three groups. Comparisons between both sessions within groups were performed using paired t-tests.

To compare corticosterone levels, the offspring from five dams were used in all three groups. In both sessions, the mean values were calculated using the data of four pups (two males and two females) from each dam. Statistical analysis was performed to compare the mean values obtained from the offspring from each dam as n = 1. Therefore, the total sample size was n = 5 for each group. For each session (odor-free and TMT odor), a one-way ANOVA was used to compare the three groups. Within-group comparisons between both sessions were performed using student's t-tests.

Significant differences were observed using a one-way ANOVA (p-values of <0.05) and Fisher's least significant difference test was further performed for post-hoc comparisons in each group. All data were analyzed using BellCurve for Excel in Windows (ver. 3.00, Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Behavioral tests

No significant differences in rearing behavior were observed among the three groups for the odor-free session (Fig. 1a) and for the TMT odor session (Fig. 1b). In addition, no significant differences were found among the three groups in terms of the differences between the TMT odor and odor-free testing scores (Fig. 1c). Comparisons between sessions indicated decreased scores in the TMT odor exposure

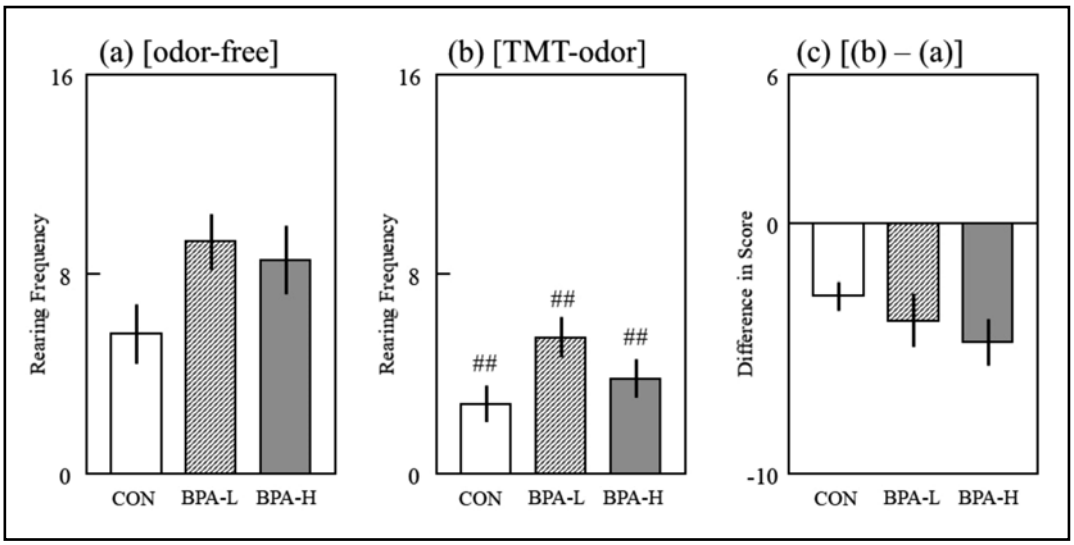

Fig. 1. Quantification of rearing behavior
(a) Odor-free session; (b) 2,3,5-trimethyl-3-thiazoline (TMT) odor session; (c) differences in scores between test sessions (TMT odor session minus odor-free session). The solid white column represents the control (CON) group, the hatched-line column represents the low-dose bisphenol A (BPA-L) group, and the gray column represents the high-dose BPA (BPA-H) group. Data are presented as the mean ± standard error of the mean (n=8 per group). ## $p<0.01$ vs. the odor-free session (paired t-test).

conditions compared with the odor-free conditions in all three groups (Fig. 1). Similarly, in ambulation scores, no significant differences were observed among the three groups for the odor-free session (Fig. 2a), for the TMT odor session (Fig. 2b), and for the differences in scores between the TMT odor and odor-free conditions (Fig. 2c). Comparisons between sessions revealed decreased scores in the TMT odor exposure conditions compared with the odor-free conditions in all three groups (Fig. 2). Both vertical and horizontal locomotor activities (rearing and ambulation) were significantly reduced following TMT odor exposure, but BPA exposure had no effects on these activities.

Freezing behavior significantly differed in both sessions among the three groups ($F_{(2,21)} = 4.45$, $p<0.05$ for the odor-free session, Fig. 3a; $F_{(2,21)} = 14.43$, $p<0.001$ for the TMT odor session, Fig. 3b). Post-hoc tests revealed lower freezing behavior scores in the odor-free session in the BPA-L group than in the CON group. In the presence of TMT odor, lower freezing scores were

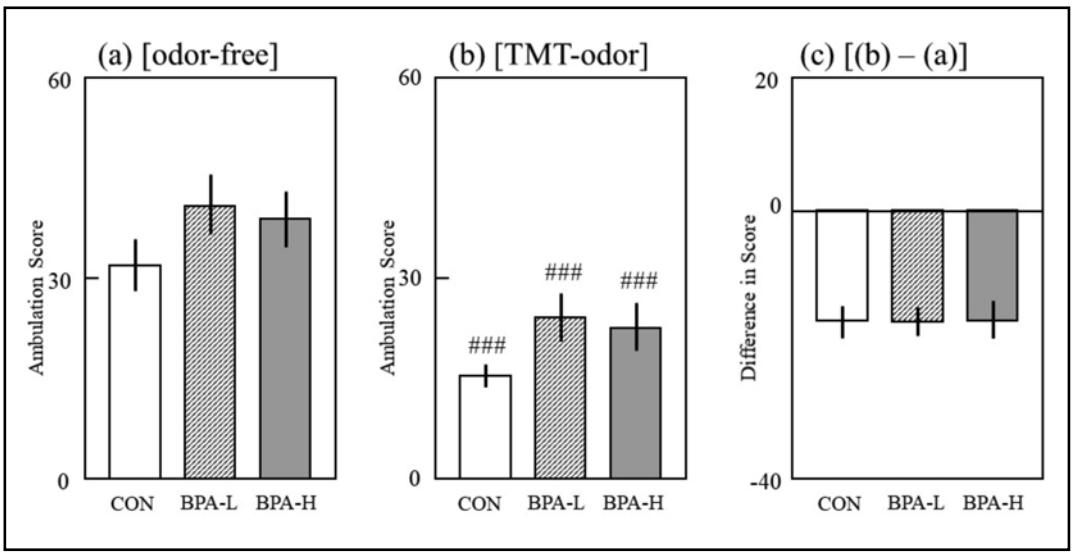

Fig. 2. Ambulation scoring
(a) Odor-free session; (b) 2,3,5-trimethyl-3-thiazoline (TMT) odor session; (c) differences in scores between test sessions (TMT odor session minus odor-free session). The solid white column represents the control (CON) group, the hatched-line column represents the low-dose bisphenol A (BPA-L) group, and the gray column represents the high-dose BPA (BPA-H) group. Data are presented as the mean ± standard error of the mean (n=8 per group). ### $p<0.001$ vs. the odor-free session (paired t-test).

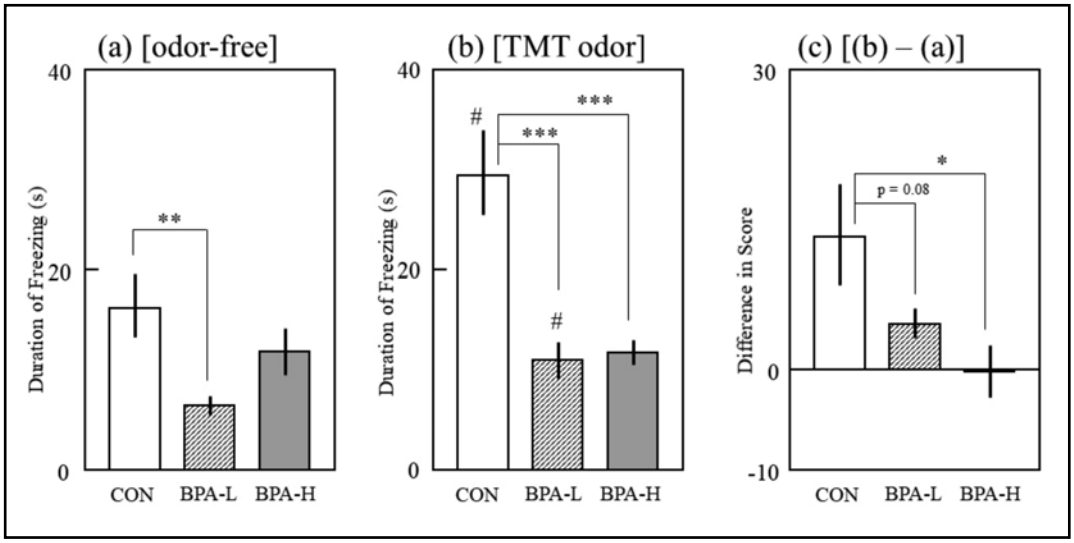

Fig. 3. Freezing duration
(a) Odor-free session; (b) 2,3,5-trimethyl-3-thiazoline (TMT) odor session; (c) differences in scores between test sessions (TMT odor session minus odor-free session). The open column represents the control (CON) group, the hatched-line column represents the low-dose bisphenol A (BPA-L) group, and the gray column represents the high-dose BPA (BPA-H) group. Each column represents the mean ± standard error of the mean (n=8 per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. the control group (Fisher's least significant difference test after one-way analysis of variance); # $p<0.05$ vs. the odor-free session (paired t-test).

observed in both the BPA-L and BPA-H groups than in the CON group. In addition, the differences in scores between the TMT odor and odor-free conditions significantly differed among the three groups ($F_{(2,21)} = 4.04$, $p<0.05$, Fig. 3c). A significantly lower freezing score was found in the BPA-H group than in the CON group; a similar trend was observed in the BPA-L group, but the difference was not significant ($p = 0.08$). Comparisons between sessions revealed reduced freezing scores in the presence of the TMT odor in both the CON and BPA-L groups, but not in the BPA-H group (Fig. 3). The findings indicate that prenatal exposure to BPA inhibits the fear behavior (freezing) caused by predator odor-induced stress.

Grooming behavior did not significantly differ in either session among the three groups for the odor-free session (Fig. 4a) and for the TMT odor session (Fig. 4b). However, the differences in scores between the TMT odor and odor-free conditions significantly differed among the three groups ($F_{(2,21)} = 5.02$, $p<0.05$; Fig. 4c).

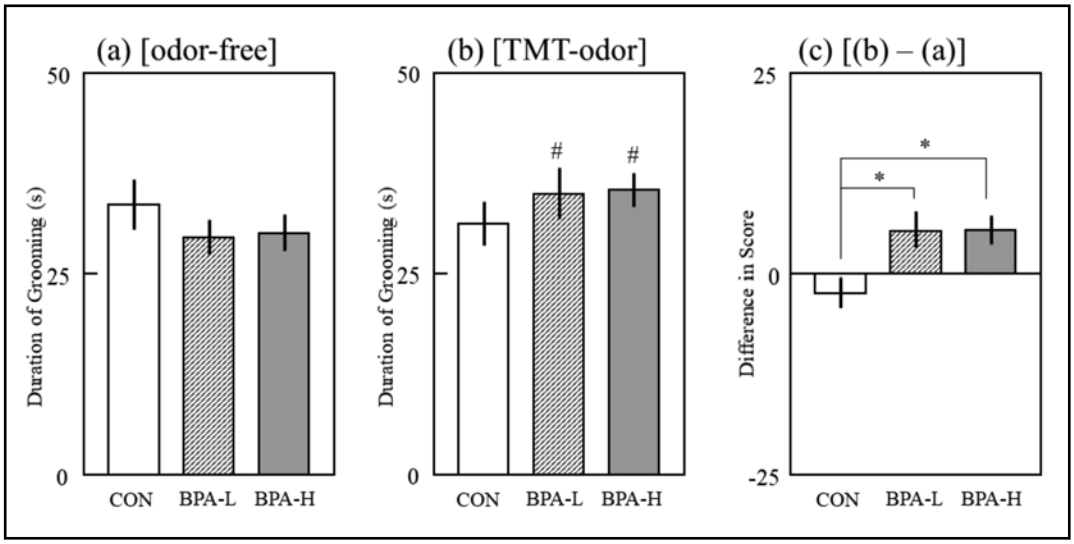

Fig. 4. Duration of grooming behaviors
(a) Odor-free session; (b) 2,3,5-trimethyl-3-thiazoline (TMT) odor session; (c) differences in scores between test sessions (TMT odor session minus odor-free session). The solid white column represents the control (CON) group, the hatched-line column represents the low-dose bisphenol A (BPA-L) group, and the gray column represents the high-dose BPA (BPA-H) group. Data are presented as the mean ± standard error of the mean (n=8 per group). * $p<0.05$ vs. the control group (Fisher's least significant difference test after one-way analysis of variance). # $p<0.05$ vs. the odor-free session (paired t-test).

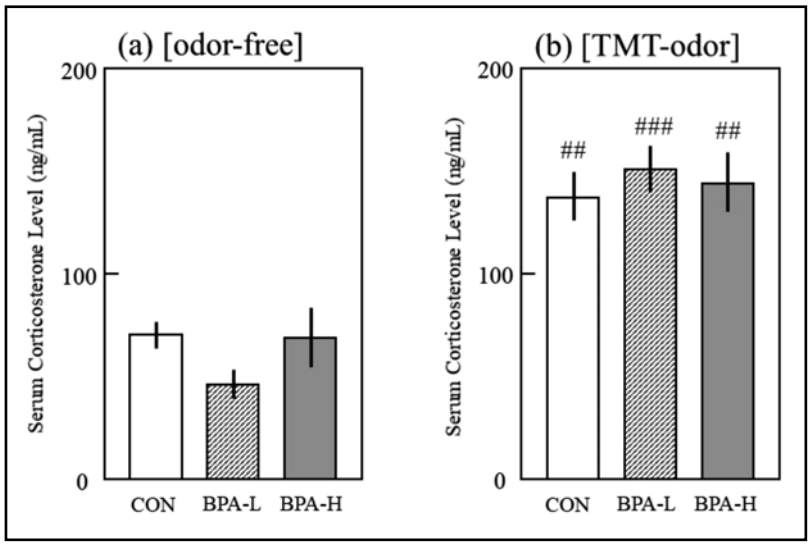

Fig. 5. Serum corticosterone levels
(a) Odor-free session; (b) 2,3,5-trimethyl-3-thiazoline (TMT) odor session. The solid white column represents the control (CON) group, the hatched-line column represents the low-dose bisphenol A (BPA-L) group, and the gray column represents the high-dose BPA (BPA-H) group. Data are presented as the mean ± standard error of the mean (n=5 per group). ## $p<0.01$, ### $p<0.001$ vs. the odor-free session (student's t-test).

Both the BPA-L and BPA-H groups achieved significantly higher scores than the CON group. Comparisons between sessions revealed increased grooming scores in the TMT odor condition compared with the odor-free condition in both the BPA-L and BPA-H groups (Fig. 4). These findings suggest that prenatal BPA exposure increases grooming behavior caused by predator odor-induced stress.

Serum corticosterone measurement

Corticosterone levels did not significantly differ among the three groups, in each session (the odor-free session (Fig. 5a) and the TMT odor session (Fig. 5b)). However, comparisons between sessions indicated significantly higher corticosterone levels in the TMT odor conditions than in the odor-free conditions in all three groups (Fig. 5). Thus, the HPA system is activated by exposure to the predator odor, regardless of BPA exposure.

DISCUSSION

In this study, I evaluated changes in stress- and fear-related behaviors and corticosterone levels in the young offspring (PND 20) of rats exposed to BPA during pregnancy.

Multiple studies using adult animals have demonstrated that TMT odor increases the levels of stress hormones in rats (Morrow *et al.* 2000; 2002; Day *et al.* 2004; Nikaido and Nakashima, 2009). In this study, I aimed to assess its effect in young rats, and the concentration of the stress hormone corticosterone was measured in the blood to determine the response of the HPA axis to predator odor-induced stress. The results showed that the TMT odor activated stress responses and increased corticosterone levels in all three groups, but there was no difference among the three groups (Fig. 5). An important consideration is that the blood sampling in the odor-free session was performed immediately after the animals were removed from their breeding cages, while blood sampling in the TMT odor session was performed following exposure to the odor in another chamber. I did not assess whether there were changes in hormone levels due to only the novel environment (the chamber). However, some studies have shown that activation of the HPA axis is mainly due to TMT odor rather than the chamber novelty (Morrow et al. 2000; Nikaido and Nakashima, 2009). In any case, the results show no difference between the CON, BPA-L, and BPA-H groups, with no effect of BPA exposure.

Multiple studies demonstrate that freezing is not always increased after TMT odor exposure. For example, Morrow *et al.* (2000) found that TMT odor did not alter grooming, rearing, ambulation, or immobility (freezing), despite increasing corticosterone levels. Using two types of open-field apparatuses (a low-anxiety type and a high-anxiety type), Morrow *et al.* (2002) observed increased immobility caused by TMT odor only in the high-anxiety type environment. These findings suggest that the environment of the high-anxiety-type open-field apparatus, which had a larger size and was more brightly lit, lowers the threshold for the expression of fear behavior. In contrast, Wallace and Rosen (2000) showed that TMT odor exposure increased freezing in both large- and small-sized open-field apparatuses, and freezing increased in an odor intensity-dependent manner; Nikaido and Nakashima (2009) observed that freezing was increased by TMT odor exposure in familiar and unfamiliar home cages, but

the increase was more significant in the familiar home cage. The discrepancy between the aforementioned findings could be due to differences in environmental factors. Indeed, fear behavior is influenced by various environmental factors, such as the experimental devices used for testing, odor intensity, and anxiety-related factors; thus, the threshold for immobility (freezing) is likely to fluctuate based on these confounding factors. In this study, after TMT odor exposure under the same environmental conditions in all three groups, freezing increased in the CON group, slightly increased in the BPA-L group, and remained unchanged in the BPA-H group. This finding suggests that prenatal exposure to high doses of BPA may have increased the threshold for fear-related behavior, although the exact neural mechanisms of this remain unknown.

Rodents perform self-grooming for cleaning or maintaining their skin or hair. Self-grooming has been reported to account for 40% of the rodents' waking time spent in the home cage; it is a common behavior, even in situations unrelated to skincare (Bolles, 1960). In addition, grooming behavior is increased by exposure to a novel environment, such as an open-field apparatus, which is commonly used to assess changes induced by mild stress (Dunn *et al.* 1979; Jolles *et al.* 1979; Escorihuela *et al.* 1999). In contrast, grooming is suppressed when animals are exposed to aversive stimuli, such as an electric foot shock (Hannigan & Isaacson, 1981; Estanislau *et al.* 2013; Fernández-Teruel & Estanislau, 2016). Growing evidence indicates no increase in grooming behavior following TMT odor exposure (Morrow *et al.* 2000; 2002; Wallace and Rosen, 2000; Nikaido and Nakashima, 2009; Horii *et al.* 2010). Consistently, in our study, grooming was not changed by TMT odor exposure in the CON group; however, both the BPA-L and BPA-H groups exhibited increased grooming behavior (Fig. 4). Exposure to TMT odor reduced motor activity in all three groups (Figs. 1, 2), increased freezing in the CON group (Fig. 3), and increased grooming in both BPA groups (Fig. 4). In addition, Brenes *et al.* (2009) suggested that grooming behavior is a good indicator of habituation. Based on these results, we speculate that BPA exposure induces a habituation-like effect on the stress caused by the TMT odor. When animals are placed in a novel environment, they initially move rapidly and exhibit frequent rearing; however, with time, this locomotor activity decreases, but grooming increases. The transition between these behavioral patterns might result from habituation to the novel environment (Woods, 1962; Varty *et al.* 2000; Brenes *et al.* 2009). Therefore, the offspring of rats exposed to BPA during pregnancy in this study might have quickly habituated to the TMT odor, resulting in increased grooming and unchanged freezing behavior.

I found that the HPA axis was similarly activated by TMT odor, regardless of prior BPA exposure. In the BPA-H group, fear-like behavior was suppressed, despite the activation of HPA-related stress responses in the brain. Further investigation is needed to identify the cellular and molecular mechanisms underlying alterations in the neural stress responses and behavioral control systems. Steroid hormone receptors are widely expressed throughout the brain and can be targets for the endocrine-disrupting effects of BPA (Simerly *et al.* 1990; Shughrue *et al.* 1997). The amygdala plays an important role in mediating fear-related behaviors (Takahashi *et al.* 2005). Several brain lesion studies have identified the role of the medial amygdala (MeA) in modulating fear behaviors induced by TMT odor exposure (Li *et al.* 2004; Muller and Fendt, 2006). Interestingly, the MeA has recently been shown to be closely involved in regulating stress-induced self-grooming behaviors (Hong *et al.* 2014; Kalueff *et al.* 2016). I have previously investigated the odor-induced responses of MeA neurons using an electrophysiological method (Fujimoto & Aou, 2018). Rats exposed to BPA exhibited high responses to TMT odor, suggesting that BPA causes functional changes in this area of the brain. Thus, BPA exposure may have some effect on MeA neurons via hormone receptors, thereby affecting MeA-related behaviors such as freezing and grooming.

It remains unclear whether BPA promotes habituation to stress and induces a positive effect in animals. Habituation to general stress can certainly lead to positive outcomes; however, habituation to predator odors is not advantageous to an organism. Freezing is categorized as a defensive behavior, along with the "fight-or-flight" response (Blanchard & Blanchard, 1989). Impairment of defensive behavior increases predation risks in wildlife. Previous studies have demonstrated that exposure to a herbicide (haloxyfop-P-methyl ester), as well as ZnO nanoparticles, reduced defensive behaviors in mice, suggesting that these chemicals might affect population dynamics (De Oliveira Mendes *et al.* 2018; Da Luz *et al.* 2020). In the present study, animals in the BPA-H group exhibited a clear deficit in defensive behaviors. The dose of BPA administered in this group was only 1.5 mg/kg/day, which is well below the NOAEL (50 mg/kg/day) but approximately 30-fold higher than the reference dose of the EPA (0.05 mg/kg/day). The results in the BPA-L group, in which BPA was administered at a dose of 0.015 mg/kg/day, were similar to those in the BPA-H group, but the magnitude of the effect of BPA was considerably weaker in the BPA-L group than the BPA-H group. These results suggest that prenatal exposure to higher doses of BPA could lead to impaired defensive behaviors, thus increasing predation risks.

My previous study examined behavioral changes induced by exposure to TMT odor in adult offspring; however, in that study, we performed only a limited number of experiments, and freezing, grooming, and corticosterone levels were not measured (Fujimoto *et al.* 2015). In addition, rats in the BPA group (at the same dose as in the BPA-L group in this study) displayed an enhanced avoidance response to TMT, suggesting that

BPA induces increased defensive behavior. Although this observed trend is opposite to that in the young rat offspring, further studies, using the same methodology, are needed to directly compare the changes induced by prenatal BPA exposure between adult rats and young PND 20 rats. A reduction in defensive behavior induced by BPA exposure may occur only during early stages of life. My study results will help to deepen our understanding of the mechanism underlying the susceptibility of young animals to chemicals. I also have previously reported that 9-week-old rats exposed to low-dose BPA exhibited an elongated immobility time in the FST (Fujimoto *et al.* 2006; 2013). Attention has recently been afforded to the possibility that vulnerability to stress is deeply related to the onset of mental illness such as depression (Weger and Sandi 2018). BPA may have caused some modifications to the neural base in the brain that addresses stress. The direct relationship between these data and the current result (a deficit of defensive behavior in young rats) remains unknown. However, an impairment of the anti-stress behavior at a young age may have some effects on the elongation of the immobility time in adulthood. The detailed elucidation is an interesting point that should be addressed in the future.

In conclusion, PND 20 rats exhibited stress-related responses against the predator odor. Stress hormone levels were increased by a predator-odor stressor, but no differences were observed in stress hormone levels between the BPA-exposed groups and the control group. In response to the predator odor, increased freezing behavior was not observed, but grooming behavior was increased in the offspring of rats exposed to high-dose BPA during gestation. The present findings indicate increased habituation to predator odors and a lack of defensive behavior in these PND 20 offspring rats. Further studies are needed to better understand how prenatal BPA exposure alters stress-related brain mechanisms in young offspring and how this leads to stress-related behavioral changes.

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CONFLICT OF INTEREST STATEMENT

I have no financial interests in this manuscript and no affiliations (relationships) to disclose.

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