Effects of maternal bisphenol A diglycidyl ether exposure during gestation and lactation on behavior and brain development of the offspring

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Running head: Behavioral and histological change by BADGE exposure

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1. Introduction

Advances in food storage technology have greatly contributed to social development. Canned or packaged foods are very convenient because they require little work and have shortened the time to prepare a meal. The metallic packaging is commonly coated with a thin polymeric film on the inner surface to prevent corrosion and food contamination. The most commonly used materials for coating are epoxy resins. Despite the advantages of convenience, unfortunately these packaging can be a source of contamination in the stored food. Previous studies have reported that components of the epoxy resins used for lining commercial cans leach out of the coating, which increases the risk of adverse health outcomes, including genotoxic effects (Calafat et al., 2005; Golub et al., 2010; La Merrill et al., 2020; Lang et al., 2008; Russo et al., 2019).

Bisphenol A diglycidyl ether (BADGE) is an epoxy resin and synthesized by reacting bisphenol A (BPA) and epichlorohydrin (Poole et al., 2004). BADGE has been used for inner coating of canned food and beverages for more than half a century (Poole et al., 2004). Various studies have demonstrated that BADGE can easily percolate from the container during the process of sterilization and long-term preservation, and become a contaminant (Cabado et al., 2008; Kudlak et al., 2019; Marqueno et al., 2019). BPA is well documented as an endocrine-disrupting chemical (EDC), which reacts even at low concentrations with endogenous receptors such as androgenic, estrogenic, and glucocorticoid receptors (Russo et al., 2019) and interferes with hormone action (La Merrill et al., 2020; Stoker et al., 2020). In addition, previous studies have reported that prenatal exposure to BPA induced acceleration of cortical

neurogenesis, abnormal layer structure formation, and hyperactivity in newborn mice (Komada et al., 2012; Komada et al., 2014). While the toxicity of BPA has been widely studied, information about the toxic effect of BADGE, particularly the neurological effects of this compound, is still lacking.

The purpose of this study is to examine the effects of BADGE exposure to the dams on the behavioral abnormality, cortical development, and neurogenesis in the offspring. The European Food Safety Authority (EFSA) has established the tolerable daily intake (TDI) of BADGE at 0.15 mg/kg/day to avoid its carcinogenicity and genotoxicity (EFSA, 2004). Therefore, we selected the doses of BADGE as 0.15 mg/kg/day and 1.5 mg/kg/day (ten-fold of TDI) in this study. Female pregnant mice were fed with a diet containing BADGE during gestation and lactation periods. Behavioral abnormality of the offspring at 5–8 weeks old were evaluated by an open field test and an elevated plus maze test. We also performed the histological analyses using brain slices of mice at postnatal day (PD) 1 delivered from control- or BADGE (1.5 mg/kg/day)-treated dams. Furthermore, we examined the effects of direct BADGE exposure on the neurite outgrowth using primary cultured cortical neurons.

2. Materials and Methods

2.1. Animals and Experimental Design

All the experimental procedures were conducted in accordance with the NIH Guide for the Care and Use of Experimental Animals, the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and the Policy on the Care and Use of the Laboratory Animals of Okayama University, and

were approved by the Animal Care and Use Committee of Okayama University (approval reference number OKU-2017133) that is certified by The Japanese Association Laboratory Animal Facilities of National University Corporations.

Adult female and male Crl:CD1 (ICR) mice (7 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan). Animals were housed with a 12-h light/dark cycle at a constant temperature (23 °C), and an *ad libitum* access to food and water. Seventeen female and male ICR mice were paired (1:1) for overnight. Mating was verified by the presence of a vaginal plug, which was considered gestational day (GD) 0. After mating, 9 pregnant females were housed singly and divided randomly into three experimental treatment groups (n = 3 pregnant mice per group): control (fed with normal diet), BADGE Low dose (fed with 0.0011 mg/g diet), and BADGE High dose (fed with 0.011 mg/g diet). BADGE was purchased from Sigma-Aldrich (St. Louis, MO, USA) and sent to Oriental Yeast Co., Ltd. (Tokyo, Japan) for incorporation into the diet. We selected these doses of BADGE based on the preliminary experiments of measuring daily food consumption and body weight of dams during gestation and lactation. BADGE-containing diet (0.0011 mg/g diet) corresponds to 0.15 mg/kg/day BADGE exposure (Low dose), which is reported as TDI of BADGE (EFSA, 2004). We also prepared BADGE-containing diet (0.011 mg/g diet) corresponding to 1.5 mg/kg/day BADGE exposure (High dose). Pregnant mice were fed with a BADGE-containing diet during the whole gestation period (from GD 0 to 20) followed by the lactation period (from lactation day (LD) 0 to 14). Dams were returned to taking a normal diet at LD 14 from BADGE-containing diet, because some offspring started to eat the food by themselves. Offspring were remained with

their dams until PD 21 (3 weeks old), at which time they were group-housed by litter and sex and continued on the control diet. Offspring at 5–8 weeks old were used for the behavioral assessment. For the histological analysis of offspring brains at PD 1 from control- or BADGE High-dose-treated dams, mice at PD 1 (3 pups/dam, total 9 pups) were decapitated, and the brains were fixed with 4% paraformaldehyde (PFA). A graphical presentation of the experimental design is shown in Figure 1.

2.2. Record of food consumption and body weight

The individual food consumption and body weight of dams were recorded throughout the BADGE treatment period: food consumption on GD 2–17 and LD 0–14; body weight on GD 2, 4, 6, 8, 10, 13, 16 and LD 3, 5, 7, 14, 21. Doses of BADGE were calculated based on their food consumption and average body weight during gestation and lactation (Table 1). Body weight of offspring was recorded on PD 3, 7, 14, 21 and 5, 6, 7, and 8 weeks old.

2.3. Behavioral Assessment

Behavioral assessment including the open field test and the elevated plus maze test were performed on offspring at 5–8 weeks old delivered from control- or BADGE-exposed dams. The behavioral tests were conducted from 11:00 to 16:00 under lower lighting conditions. Mice were habituated to the testing room at least for 2 h prior to the behavioral assessment. Experimental areas were cleaned with 70% ethanol and wiped dry before setting the next animal. All data were analyzed using the video tracking system (LimeLight, Neuroscience, Inc., Tokyo, Japan).

2.3.1. Open field test

Locomotor activity and anxiety-related behavior were measured using the open field test. Each mouse was placed in the center of the open field apparatus (50 × 50 × 35 cm). Mice were allowed to move freely and the movement of mice was recorded for 5 min (10 sec after setting until 310 sec). Total distance moved and time spent in the corner, wall, or center area was measured.

2.3.2. Elevated plus maze test

The apparatus used for the elevated plus maze test comprised of two open arms (29 × 7.5 × 0.25 cm), two closed arms (29 × 7.5 × 23 cm) and a center platform (7.5 × 7.5 × 0.25 cm), perpendicular to each other. The platform was elevated 41 cm above the floor. Each mouse was placed onto the central square area at the beginning of the test, and its behavior was recorded for 5 min (10 sec after setting until 310 sec). The time spent in the open or the closed arm or center area was then measured.

2.4. Histological analysis of offspring brains at postnatal day 1

Offspring were sacrificed on PD 1, and the brains were removed from the skull and washed with ice-cold saline. The brains were cut in half lengthwise at the longitudinal fissure and then fixed with 4% PFA for 24 h. After fixation, the brains were embedded in paraffin and cut into 4-μm thick sagittal sections serially. To identify the structural alterations of the cortical area, the brain slices were deparaffinized in xylene, rehydrated in graded ethanol solutions, and then Nissl-

stained with 0.1% Cresyl violet. For immunohistochemistry, the brain slices were deparaffinized in xylene, rehydrated in graded alcohol solutions, and subjected to antigen retrieval by autoclave in 10 mM sodium citrate (pH 6.0) for 5 min at 121 °C. After return to room temperature (RT), the sections were incubated in 1% normal goat serum for 30 min at RT, and then reacted with primary antibody diluted in 10 mM phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (0.2% PBST) for 18 h at 4 °C: mouse anti-Cut-like homeobox 1 (Cutl1) (1:500, Abcam, Cambridge, UK), rabbit anti-Forkhead box P2 (Foxp2) (1:500, Abcam), rat anti-COUP-TF interacting protein 2 (Ctip2) (1:1000, Abcam), mouse anti-nestin (1:500, Abcam) or rabbit anti-T-box transcription factor 2 (Tbr2) (1:500, Abcam) antibodies. After washing in 10 mM PBS (pH 7.4; 3 × 10 min), slices were reacted with the appropriate goat anti-mouse IgG conjugated to Alexa Fluor 594, goat anti-rabbit IgG conjugated to Alexa Fluor 488 or goat anti-rat IgG conjugated to Alexa Fluor 488 secondary antibodies (1:500, Invitrogen, San Diego, CA, USA) for 2 h, at RT. The slices were counterstained with Hoechst 33342 nuclear stain (10 μg/mL) for 2 min and washed prior to mounting with Fluorescence Mounting Medium (DakoCytomation, Glostrup, Denmark).

All slides were analyzed under a microscope (Olympus BX53, Tokyo, Japan) using the cellSens software imaging system (Olympus). Immunofluorescent-positive signals were detected using a mercury lamp through 360–370 nm, 470–495 nm, or 530–550 nm band-pass filters to excite Hoechst 33342, Alexa Fluor 488, or Alexa Fluor 594, respectively. Light emission from Hoechst 33342, Alexa Fluor 488, or Alexa Fluor 594 was collected through a 420 nm long-pass filter, a 510–550 nm band-pass filter, or a 590 nm long-pass filter, respectively.

2.5. Primary neuronal culture

Primary cultured neurons were prepared from the cortex of Sprague–Dawley (SD) rat embryos at 15 days of gestation using the method described previously (Miyazaki et al., 2019). Briefly, to prepare the cortical neuronal cultures, the cortex was dissected and cut into small pieces with scissors. Next, the tissue was incubated for 15 min in 0.125% trypsin-EDTA at 37 °C and then centrifuged (1500 g, 3 min). The resulting cell pellet was treated with 0.004% DNase I solution containing 0.003% trypsin inhibitor for 7 min at 37 °C then centrifuged (1500 g, 3 min) again. Following this, the resulting cell pellet was gently re-suspended in a small volume of Dulbecco's modified Eagle's medium (DMEM) with high glucose 4.5 g/L D-glucose (Invitrogen) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, and 60 mg/L kanamycin sulfate, and plated in the same medium at a density of 2×10^5 cells/cm^2 in the 4-chamber culture slides coated with poly-D-lysine (Falcon, Corning, NY, USA). The next day, the medium was replaced with a fresh medium containing BADGE (1, 10, or 100 pM) supplemented with 2 μM cytosine-β-D-arabinofuranoside (Ara-C) to inhibit glial cell replication, followed by incubation for 48 h. Cell cultures were maintained at 37 °C in a 5%-95% CO_2-air gas mixture.

2.6. Immunocytochemistry

Cortical neurons on the chamber slides were fixed with 4% PFA for 20 min at RT, blocked with 2.5% normal goat serum for 20 min, then reacted with mouse anti-Microtubule-associated protein 2 (MAP2) (1:5000, Sigma-Aldrich) primary antibody

diluted in 0.1% PBST for 18 h at 4 °C. After washing, the cells were reacted with goat anti-mouse IgG conjugated to Alexa Fluor 594 secondary antibodies (1:500, Invitrogen) for 1.5 h, at RT. Finally, cells were counterstained with Hoechst 33342 nuclear stain (10 μg/mL) for 2 min. Slides were analyzed under a fluorescence microscope (Olympus BX53) using a cellSens software imaging system (Olympus) using a mercury lamp through 530–550 nm or 360–370 nm band-pass filters to excite Alexa Fluor 594 or Hoechst dye, respectively. Light emission from Alexa Fluor 594 or Hoechst was collected through a 590 nm long-pass filter or 420 nm long-pass filter, respectively. Axon length of MAP2-positive neurons was measured in randomly chosen fields under ×400 magnification (8–10/chamber × 4 chambers) using cellSens software imaging system.

2.7. Statistical Analysis

All statistical analyses were performed using KaleidaGraph v4.0 software (HULINKS Inc., Tokyo, Japan). Data were presented as mean ± SEM. For comparisons between multiple groups, we used one-way ANOVA followed by *post-hoc* Fisher's least significant difference (LSD) test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of BADGE on food consumption and body weight of dams

Individual food consumption of dams was recorded every day throughout the BADGE treatment period (GD 2–17 and LD 0–14) (Fig. 2A and B). We also recorded

the body weights of dams during gestation (GD 2, 4, 6, 8, 10, 13 and 16) and lactation (LD 3, 5, 7, 14 and 21) (Fig. 2C and D). There is no difference in the food consumption and body weight among the control, BADGE Low and High dose-treated groups during gestation and lactation period, suggesting no toxic effect of BADGE on dams. The food consumption of dams was increased slightly in the latter half of gestation. In addition, in the lactation period, the diet increases furthermore compared to the gestation period. According to the growth of the embryos, the body weight during gestation (GD 10–16) increases in all groups. Doses of BADGE were calculated based on their food consumption and average body weight during gestation and lactation. In the gestation period, exposure doses of BADGE Low and High dose-treated dams are almost the same level as 0.15 mg/kg/day and 1.5 mg/kg/day, respectively. In the lactation period, dams are exposed to higher levels (> two-fold) of BADGE compared to the gestation period (Table 1).

3.2. Body weight of offspring delivered from control- or BADGE-exposed dams

Body weight of postnatal offspring (PD 3–21) increases in proportion to their growth, and BADGE does not inhibit the growth of pups during PD 3–21 (Fig. 3A). Body weight of female, but not male, offspring (5–7 weeks old) from BADGE Low dose-treated dams is significantly heavier than the control group (Fig. 3B and C).

3.3. Effects of BADGE exposure during embryonic and pre-weaning period on behavior of offspring

First, to examine the effect of BADGE exposure on the locomotor activity of

offspring, total distance moved was measured by an open field test. The total distance of both male and female mice does not change by BADGE exposure as seen in any week-old pups when compared to the control pups (Fig. 4). To evaluate the anxiety-like behavior, we separated the field into a corner, wall, and center area, and measured the time spent in each area (Fig. 5 and 6). Almost all mice prefer staying in the corner or wall area, compared to the center area. The percentage of time spent in the corner area of the male mice of the BADGE High dose-exposed group at 5 weeks old is significantly higher than that of the control group (Fig. 5B). Contrarily, no significant effect of BADGE exposure is observed on the time spent in each area by female mice (Fig. 6). The results from the elevated plus maze test of offspring are shown in Fig. 7 and 8. No change is observed in the time spent in either closed arm or open arm by male and female offspring among control and BADGE-exposed groups (Fig. 7 and 8).

3.4. Effects of BADGE High-dose exposure during embryonic period on cortical structure of neonatal mice at PD1

Because male offspring at 5 weeks old delivered from the BADGE High dose-exposed dams exhibit anxiety-like behavior in motor assessment, we examined the effects of high-dose BADGE exposure on brain development by histological analysis using brain slices of neonatal mice at PD1. We visualized the cortical structures by Nissl staining (Fig. 9). The Nissl-positive signals are strong and dense especially in the shallow layer in the control mice. We cannot identify the layer structure in the cortex of control mice at PD1. Conversely, the layer structure of the neonatal cortex of the BADGE High dose-exposed group is clearer than that of the control group.

Next, we tried to identify the cortical layer structure by immunohistochemistry using several specific markers for cortical layers (Fig. 10). Cutl1-positive signals, marker for cortical layers 2–4 (Cubelos and Nieto, 2010; Nieto et al., 2004), is distributed diffusely throughout the whole cortical area. There is no apparent difference in the signal density and distribution between the control and BADGE High dose groups. Foxp2-positive signals, marker for the cortical layer 4 (Sakakibara et al., 2012), are weak and there is no specific distribution in the cortical layer in the control mice. In contrast, the Foxp2-positive signals are clear and apparently localized in the middle layer of cortex in the BADGE-exposed group (Fig. 10A and B). In addition, Ctip2-positive signals, a marker of the cortical layers 5 and 6 (Chang and Kawai, 2018; Lickiss et al., 2012; Saito et al., 2011), are restricted in the lower layers in the cortex of the BADGE-exposed mice, but not the control group (Fig. 10C and D).

3.5. Effects of BADGE High-dose exposure during embryonic period on neurogenesis in the subventricular zone of neonatal mice at PD1

To examine the effects of BADGE exposure on neurogenesis in the subventricular zone (SVZ) of neonatal mice at PD1, we performed the double immunostaining of nestin, a marker for fiber of radial glia (Komada et al., 2012), and Tbr2, a marker for intermediate progenitor (Englund et al., 2005) (Fig. 11). In control mice at PD1, we can observe strong nestin-positive fibers in the SVZ and many Tbr2-positive progenitors in the inner SVZ. In contrast, nestin- and Tbr2-positive signals are dramatically decreased in the SVZ and inner SVZ of the BADGE-exposed mice.

3.6. Effects of BADGE exposure on neuronal differentiation in cultured cortical neurons

To examine the effects of direct BADGE exposure on the differentiation of cortical neurons, we prepared primary cultured cortical neurons, and these cells were treated with BADGE (1–100 pM) for 48 h. We observed that BADGE exposure (10, 100 pM) promotes neurite outgrowth followed by neuronal connection (Fig. 12A). Neurite length also significantly increases by BADGE (10, 100 pM) treatment (Fig. 12B).

4. Discussion

BPA and its synthesis product BADGE are used for inner coating of canned food, beverage containers, baby bottles and dental composites, and their food contamination is anxious about health hazard (Cabado et al., 2008; Gorecki et al., 2017; Lestido Cardama et al., 2019; Mertens et al., 2016). There are similarities between the two compounds in not only chemical structure but also endocrine disrupting effects and toxicokinetics. BADGE and BPA exhibited anti-androgenic properties through binding to androgen receptor (Satoh et al., 2004) and decreased testosterone levels in the adult human testis (Desdoits-Lethimonier et al., 2017). To date, various studies demonstrated occurrence and distribution of BPA, BAGDE and its derivatives in human specimens such as urine, adipose tissue and placenta (Calafat et al., 2005; Liu et al., 2019; Rocha et al., 2018; Wang et al., 2012; Wang et al., 2015). Wang et al. (Wang et al., 2012) reported that the concentration of BADGEs (i.e.,

BADGE and its derivatives) in human urine from the United States were 3- to 4-fold higher than the corresponding concentrations of BPA. They also measured concentration of BPA and BADGEs in human adipose fat and blood plasma samples, and demonstrated a significant positive correlation between concentration of BPA and BADGEs in plasma samples (Wang et al., 2015). In addition, it is reported that BADGE may generate BPA endogenously (Hanaoka et al., 2002). Furthermore, Chen et al. (Chen et al., 2016) recently demonstrated occurrence and maternal transfer of chlorinated BPA in pregnant women and their embryos. Therefore, examination of effects of maternal exposure to epoxy resin on offspring has been focused (Golub et al., 2010). Indeed, numerous experiments using animal models have reported that gestational BPA exposure induces behavioral alteration: hyperactivity, anxiety-and depression-like behaviors (Braun et al., 2011; Cox et al., 2010; Fan et al., 2018; Komada et al., 2014; Luo et al., 2013; Nakamura et al., 2012). Causality of early BPA exposure and behavioral change has been supported by observations in humans (Braun et al., 2011; Braun et al., 2009; Evans et al., 2014; Perera et al., 2012). Cohort studies have reported that prenatal BPA exposure is associated with child behavioral problems: externalizing behavior, aggression, oppositional/defiant disorder traits, anxiety and depressive symptoms, and attention deficit/hyperactivity disorder symptoms (Perera et al., 2012). As mentioned, BPA is the most famous EDC; therefore, the effects of BPA exposure on child behaviors are well documented. However, the effects of BADGE on behavioral alteration have not been fully examined.

In this study, we first confirmed that the BADGE diet does not affect food consumption and causes any increment in the body weight of dams. Dramatical increase in the body weight of dams during GD 10–16 suggest that maternal BADGE exposure does not inhibit the growth of embryos. At postnatal weeks 5–8, the body weight of female offspring from BADGE Low dose-exposed dams is significantly higher than that of the control group. Although the mechanism of increasing effect of low-dose BADGE on body weight is unclear, we suppose that BADGE, which is also identified as an antagonist of peroxisome proliferator–activated receptor gamma (PPARγ) (Wright et al., 2000), could affect lipid metabolism, and thus, body weight (Yee et al., 2012). To examine the effects of maternal BADGE exposure during gestation and lactation periods on behavioral alteration of the offspring, we explored locomotor activity and anxiety-like behavior of offspring delivered from control-, BADGE Low and High dose-treated dams at postnatal weeks 5–8 by an open field test and elevated plus maze test. Although maternal BADGE exposure had no effect on pup locomotor activity, the time spent in the corner area of male mice of the BADGE High dose-exposed group at 5 weeks old is significantly extended. On the other hand, we could not observe any change by an elevated plus maze test. Taken together with these results, it is suggested that maternal high-dose BADGE exposure could induce anxiety-like symptom in young male offspring, but the effect is weaker than the observation of BPA studies (Cox et al., 2010; Fan et al., 2018; Luo et al., 2013). In our study, maternal exposure to low-dose BADGE (corresponding to TDI of BADGE) does not induce any behavioral alteration in offspring. Moreover, we performed an

open field test and an elevated plus maze test on control- and BADGE-exposed dams: BADGE exposure does not show any effect on the behavior of dams (data not shown).

Brain is a target of developmental disruption induced by environmental EDCs (Itoh et al., 2012). Fetal and/or newborn exposure to EDCs via placental transport or lactation induced rapid growth of the brain with neurogenesis, neuronal differentiation and migration (Chen et al., 2016; Nakamura et al., 2006; Wan et al., 2010). It is well known that BPA can influence the timing and duration of neurogenesis specifically by altering the neural stem cell proliferation and differentiation (Itoh et al., 2012). Nakamura et al. reported that a low-dose BPA exposure may disrupt neocortical development by accelerating neuronal differentiation/migration (Nakamura et al., 2006). BADGE is also an endocrine disruptor. Based on these observations, it is suggested that BADGE can affect neuronal differentiation or neurogenesis in a developing brain. In our behavioral assessment, the BADGE High dose-exposed group, but not the Low dose-exposed group, exhibited anxiety-like behavior. So, we examined the effects of maternal BADGE exposure during gestation on cortical development by histological analysis using brain slices of PD1 mice delivered from control or BADGE High dose-exposed dams. This study found evidence that high dose BADGE treatment could accelerate neuronal differentiation during the brain development in neonatal mice. There are some clear differences in the distribution and number of positive signals of Nissl staining or certain markers that indicate neurogenesis and neurodevelopment in the cortex and SVZ. Foxp2 is an important factor for neurodevelopment, which participates in neurogenesis (Tsui et al., 2013) and neurite growth (Vernes et al., 2011) in developing brain. The High-dose treated

mice, but not the control group mice, show an increase and abnormal accumulation of Foxp2-positive cells in the middle layer of the cortex. Cutl1 expressed in the pyramidal neurons in the layers 2–4 of the cortex (Cubelos et al., 2010; Nieto et al., 2004) showed no significant changes between the BADGE doses and control groups, which indicates little or no effect on the pyramidal neurons in the upper layers. In addition, we observed a restriction of Ctip2 in layers 5 and 6 in the cortex of the BADGE High dose group, but not the control group. These findings suggest that high-dose BADGE exposure accelerates neuronal development. Furthermore, we examined the effects of BADGE exposure on neurogenesis in newborn mice at PD1 using neuronal lineage markers. Nestin is an intermediate filament protein and is expressed in neuroepithelial stem cells and radial glia (Fisher, 1997; Frederiksen and McKay, 1988). Radial glia spans the width from ventricular to pial surface, and divides asymmetrically to produce radial glia and intermediate progenitor cells, which expresses a transcription factor Tbr2 (Bifari et al., 2017; Englund et al., 2005; Molnar et al., 2011). Intermediate progenitor cells differentiate into post-mitotic immature neurons, which migrate to their destination and integrate into the neural network (Dwyer et al., 2016). In this study, we observed the presence of nestin-positive radial glial fibers with apical-basal polarity and many Tbr2-positive intermediate progenitors in the inner SVZ. Conversely, BADGE-exposed group show broken apical-basal polarity of the fibers of radial glia and reduced Tbr2-positive signals. In embryogenesis and at birth, nestin levels are high and expressed in multipotent neural stem cells such as radial glial cells. According to development, differentiation of these progenitor cells into neurons and glial cells replaces nestin with cell-specific proteins.

Therefore, nestin levels decrease over the process of differentiation. A previous study has reported that nestin-positive radial fibers in the SVZ were shortened in BPA-exposed mice, suggesting an acceleration of neurogenesis (Komada et al., 2012). In addition, it is reported that the number of neurons in the cerebral cortex increases between PD 3 and 7, then decrease until PD 15 (Bandeira et al., 2009). Furthermore, we demonstrated that low-dose BADGE (10, 100 pM) exposure promotes neurite outgrowth followed by neuronal connection in cultured cortical neurons. Taken together with these observations and our results of *in vivo* and *in vitro*, we suggest that maternal BADGE exposure induces acceleration of process of neuronal differentiation in fetuses, although the mechanism of acceleration of neuronal differentiation is unclear.

In the present study, we selected the doses of BADGE as 0.15 mg/kg/day based on TDI of BADGE (EFSA, 2004) and 1.5 mg/kg/day (ten-fold of TDI). It is reported that maximum consumer exposure to BADGE and its derivatives ranges from 0.0028 mg to 0.14 mg/day equivalent approximately to 0.05–2.3 μg/kg/day (EFSA, 2004). Compared to that, the doses in our experiments seem extremely high. EFSA explains background of TDI establishment of BADGE (0.15 mg/kg/day); TDI was established considering the No-Observed-Adverse-Effect-Level (NOAEL) of 15 mg/kg/day derived from the oral chronic toxicity/carcinogenicity study in rat with BADGE, and applying an uncertainty factor of 100 from rat to human (EFSA, 2004). Based on the above, BADGE High dose 1.5 mg/kg/day in our experiments using mice is thought to be equivalent to 0.015 mg/kg/day in human. In addition, in the previous toxicological studies, the dose levels range from 50 to 1000 mg/kg/day (EFSA, 2004;

Hyoung et al., 2007). Moreover, Punt et al. (Punt et al., 2019) performed quantitative *in vitro*-to-*in vivo* extrapolation and estimated the plasma concentration of BADGE in human. The peak free plasma concentration ($C_{max, free}$) of BADGE was 0.00001 μM (= 10 pM). In our study, we observed that BADGE exposure (10, 100 pM) promoted neurite outgrowth followed by neuronal connection in cultured cortical neurons. Taken together, the doses of BADGE in our current study are not high and could be reproducible in human evaluation. This study underscores the importance of recognizing that maternal BADGE exposure, especially in pregnancy, could induce abnormal brain development of fetus. Our findings can contribute to review of TDI of BADGE considering not only carcinogenicity and teratogenic effects but also neurological effects in the central nervous system.

Acknowledgments: This work was supported by Health and Labour Sciences Research Grants for Research on Regulatory Science of Pharmaceuticals and Medical Devices (to M.A.) from the Japanese Ministry of Health, Labour and Welfare, and by a Research Grant from the Okayama Medical Foundation (to I.M.). We would like to thank Editage (www.editage.com) for English language editing.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure legends

Fig. 1. Schematic illustration of the experimental protocol. After mating, pregnant female mice were fed with a diet containing BADGE during the whole gestation period followed by a lactation period (from LD 0 to 14). Dams were returned to taking normal diet at LD 14. Offspring were kept with their dams until PD 21 (3 weeks old) and continued on the control diet. Offspring at 5–8 weeks old were used for behavioral assessment. Neonatal brains at PD 1 were used or histological analysis.

Fig. 2. Changes in food consumption and body weight of dams during gestation and lactation periods. (A, B) Food consumption (g/day/mouse) of dams during gestation (A) and lactation (B) days. (C, D) Body weight (g) of dams during gestation (C) and lactation (D) days. Data are mean ± SEM. Control, n = 3; BADGE Low dose, n = 3; BADGE High dose, n = 3.

Fig. 3. Changes in body weight of offspring. (A) Body weight (g) of offspring at PD 3-21. Data are mean ± SEM. Control, n = 12; BADGE Low dose, n = 14; BADGE High dose, n = 8. (B, C) Body weight (g) of male (B) and female (C) at postnatal weeks 5–8. Data are mean ± SEM. Male offspring: control, n = 6; BADGE Low dose, n = 9; BADGE High dose, n = 3. Female offspring: control, n = 6; BADGE Low dose, n = 5; BADGE High dose, n = 5. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control group at each time point.

Fig. 4. Effects of BADGE exposure during embryonic and pre-weaning period on locomotor activity of offspring assessed by an open field test. Total distance (cm) of males (A, C, E, G) and females (B, D, F, H) for 5 min was measured at postnatal weeks 5 (A, B), 6 (C, D), 7 (E, F), and 8 (G, H). Data are mean ± SEM. Male offspring: control, n = 6; BADGE Low dose, n = 9; BADGE High dose, n = 3. Female offspring: control, n = 6; BADGE Low dose, n = 5; BADGE High dose, n = 5.

Fig. 5. Effects of BADGE exposure during embryonic and pre-weaning period on anxiety-like behavior of male offspring assessed by an open field test. The field was separated into a corner, wall, and center area (A). Time spent in the corner (B, E, H, K), wall (C, F, I, L), or center (D, G, J, M) was measured at postnatal week 5 (B, C, D), 6 (E, F, G), 7 (H, I, J), and 8 (K, L, M). Data are mean ± SEM. Control, n = 6; BADGE Low dose, n = 9; BADGE High dose, n = 3. *$p < 0.05$ vs. control group.

Fig. 6. Effects of BADGE exposure during embryonic and pre-weaning period on anxiety-like behavior of female offspring assessed by open field test. The field was separated into a corner, wall, and center area (A). Time spent in the corner (B, E, H, K), wall (C, F, I, L), or center (D, G, J, M) was measured at postnatal week 5 (B, C, D), 6 (E, F, G), 7 (H, I, J), and 8 (K, L, M). Data are mean ± SEM. Control, n = 6; BADGE Low dose, n = 5; BADGE High dose, n = 5.

Fig. 7. Effects of BADGE exposure during embryonic and pre-weaning period on anxiety-like behavior of male offspring assessed by an elevated plus-maze test. The

apparatus used for the elevated plus-maze test comprises two open arms, two closed arms, and a center platform (A). Time spent in the closed arm (B, E, H, K), open arm (C, F, I, L), or center area (D, G, J, M) was measured at postnatal week 5 (B, C, D), 6 (E, F, G), 7 (H, I, J), and 8 (K, L, M). Data are mean ± SEM. Control, n = 6; BADGE Low dose, n = 9; BADGE High dose, n = 3.

Fig. 8. Effects of BADGE exposure during embryonic and pre-weaning period on anxiety-like behavior of female offspring assessed by an elevated plus maze test. The apparatus used for the elevated plus maze test comprises two open arms, two closed arms, and a center platform (A). Time spent in the closed arm (B, E, H, K), open arm (C, F, I, L), or center area (D, G, J, M) was measured at postnatal week 5 (B, C, D), 6 (E, F, G), 7 (H, I, J), and 8 (K, L, M). Data are mean ± SEM. Control, n = 6; BADGE Low dose, n = 5; BADGE High dose, n = 5.

Fig. 9. Representative photomicrographs of Nissl staining using brain slices of offspring at PD1 delivered from control- or BADGE High dose-exposed dams. (A) Low magnification images of the cortical area. Scale bar: 100 μm. (B) High magnification images of (A). Scale bar: 50 μm.

Fig. 10. Representative photomicrographs of immunohistochemistry of markers for the cortical layers using brain slices of offspring at PD1 delivered from control- or BADGE High dose-exposed dams. (A, B) Cutl1 (red) and Foxp2 (green) double immunostaining. Brain slices were nuclear stained with Hoechst 33342. (A) Low

magnification images of the cortical area. Scale bar: 100 μm. (B) High magnification images of (A). Scale bar: 50 μm. (C, D) Ctip2 (green) immunostaining. Brain slices were nuclear stained with Hoechst 33342. (C) Low magnification images of the cortical area. Scale bar: 100 μm. (D) High magnification images of (C). Scale bar: 50 μm.

Fig. 11. Representative photomicrographs of nestin and Tbr2 double immunostaining using brain slices of offspring at PD1 delivered from control or BADGE High dose-exposed dams. (A) Low magnification images of nestin (red) and Tbr2 (green) staining in the VS and SVZ. Brain slices were nuclear stained with Hoechst 33342. Scale bar: 100 μm. (B) High magnification images of (A). Scale bar: 100 μm.

Fig. 12. Effects of BADGE exposure on neuronal differentiation in the cultured cortical neurons. Primary cultured cortical neurons were treated with BADGE (1–100 pM) for 48 h. (A) Representative photomicrographs of MAP2 (red) immunostaining. Cells were nuclear stained with Hoechst 33342. Scale bar: 50 μm. (B) Neurite length (μm) of MAP2-positive neurons 48 h after treatment with BADGE (1–100 pM). Data are mean ± SEM (n = 4/group). *$p < 0.05$, ***$p < 0.001$ vs. control group.