Original Article

Prenatal and postnatal bisphenol A exposure inhibits postnatal neurogenesis in the hippocampal dentate gyrus

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ABSTRACT — Bisphenol A (BPA), an endocrine disruptor with estrogenic effects, is widely used as a raw material for manufacturing polycarbonate plastic and epoxy resins. Prenatal and postnatal exposure to BPA affects brain morphogenesis. However, the effects of prenatal and postnatal BPA exposure on postnatal neurogenesis in mice are poorly understood. In this study, we developed a mouse model of prenatal and postnatal BPA exposure and analyzed its effects on hippocampal neurogenesis. The hippocampal dentate gyrus is vulnerable to chemical exposure, as neurogenesis continues in this region even after birth. Our results showed that in mice, prenatal and postnatal BPA exposure decreased the number of type-1, 2a, 2b, and 3 neural progenitor cells, as well as in granule cells, in the hippocampal dentate gyrus on postnatal days 16 and 70. The effect of prenatal and postnatal BPA exposure on neural progenitors were affected at all differentiation stages. In addition, prenatal and postnatal BPA exposure affects the maintenance of long-term memory on postnatal day 70. Our results suggest that neurodevelopmental toxicity due to prenatal and postnatal BPA exposure might affect postnatal morphogenesis and functional development of the hippocampal dentate gyrus.

Key words: Bisphenol A, Dentate gyrus, Neurogenesis, Neural stem cells, Granule cells

INTRODUCTION

Toxicity-mediated functional and morphological changes in the prenatal and postnatal central nervous system (CNS) often produce adverse effects in adulthood (Bearer, 2000; Komada *et al.*, 2010). Bisphenol A (BPA) is widely used in food packaging industries for the manufacture of polycarbonate bottles and food can linings. BPA is also used in dentistry as a hardener for sealants and dental resins. Humans are exposed to BPA through contact with these products, which leach BPA under normal use conditions. Exposure to BPA can occur through dietary intake, inhalation, or dermal exposure. Studies suggest that humans are exposed to detectable levels of BPA through environmental exposure. Due to its high lipophilicity, BPA can cross the blood-brain barrier and placenta (Sun *et al.*, 2002). Prenatal BPA exposure is associated with abnormal behaviors and cognitive disabilities in young children (Braun, 2017). Moreover, BPA exposure during pregnancy induces adolescent neurobehavioral problems, such as depression, anxiety, and autism spectrum disorders (Miodovnik *et al.*, 2011;

Perera *et al.*, 2016).

BPA has well-known endocrine-disrupting estrogenic activities (Braun, 2017; Gould *et al.*, 1998; Krishnan, *et al.*, 1993; Santangeli *et al.*, 2017; Steinmetz *et al.*, 1997; vom Saal *et al.*, 2007; Wetherill *et al.*, 2007; Wolstenholme *et al.*, 2011), and a previous study showed that BPA exposure induces abnormal DNA methylation in the estrogen receptor (ER) α gene and disrupts sexually dimorphic behaviors in mice (Kundakovic *et al.*, 2013). Moreover, studies have shown that prenatal BPA exposure leads to hyperactivity, learning deficits, anxiety, and disruption of sexually dimorphic behaviors in newborn and adult rodents (Komada *et al.*, 2014; Nakamura *et al.*, 2012). Additionally, BPA induces abnormal neurogenesis and hyperplasia of the cortical plate in the dorsal telencephalon (Itoh *et al.*, 2012; Komada *et al.*, 2012, 2014; Nakamura *et al.*, 2006). It has been reported that prenatal exposure to BPA abolishes the brain's sexual differentiation and developing hypothalamus neurogenesis (Funabashi *et al.*, 2004; Kinch *et al.*, 2015; Rubin *et al.*, 2006; Wolstenholme *et al.*, 2011).

The dentate gyrus (DG) is an important structure with-

in the hippocampus and plays critical roles in consolidation of information from short-term memory to long-term memory, as well as spatial learning. Neural stem/progenitor cells, which undergo neurogenesis, are present in the sub-granular zone (SGZ) of the DG, which contains pools of neural stem cells that generate new neurons during postnatal life (Kempermann *et al.*, 2004). Neural stem cells in the SGZ, which differentiate into granular cells, are anchored within the granular layer of the DG, and following differentiation establish synaptic formation with neighboring neurons and maintain the function of the hippocampus. Adult neurogenesis in the SGZ is a highly regulated process starting from type-1 neural stem cells, which produce proliferative progenitor cells in the order of type-2a, type-2b, and type-3. This process has been shown to start from self-renewal of type-1 neural stem cells, exhibiting morphology typical of radial glia, to produce intermediate progenitor cells (IPCs), a type of transit amplifying cells (Hodge *et al.*, 2008; Kempermann *et al.*, 2004). Type-2a and type-2b IPCs divide to produce neuronal committed type-3 IPCs (Kempermann *et al.*, 2004). Type-3 IPCs differentiate into post-mitotic immature granule cells and finally into mature granule cells that populate the granule cell layer (GCL) (Hodge *et al.*, 2008).

This study aimed to evaluate the effects of prenatal and postnatal BPA exposure on postnatal brain development in mice. As the hippocampus is involved in memory formation and learning and is one of the sites of adult neurogenesis, the impact of prenatal and postnatal BPA exposure on hippocampal neurogenesis and neural stem cells was evaluated by immunohistochemical and behavioral methods.

MATERIALS AND METHODS

Animals and housing

Eight-week-old Institute of Cancer Research (ICR) mice purchased from SLC Inc. (Osaka, Japan) were acclimatized for 2 weeks to the animal facility before experimentation. Mice were kept under specific pathogen-free conditions and housed in polycarbonate cages in a room under controlled temperature ($24 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) with a 12 hr light/dark cycle (lights on at 7 a.m.). To avoid stress, noise levels were kept minimum within the room, as well as in adjacent areas. Food (Certified Rodent Chow CE-2, CLEA, Osaka, Japan) and drinking water were available *ad libitum*. A certificate of analysis for each lot of food was provided by the manufacturer. To avoid the possible effects of variations in food content on our experiments, the same lot was used

throughout the experiments. Distilled water was provided in glass bottles with Teflon seals. Mice were housed individually throughout the study in polypropylene plastic tubs with stainless steel lids and corncob bedding. Ten- or 11-week-old mice were housed in a cage at a 1:1 female to male ratio and allowed to copulate overnight. Females were checked the next morning for the presence of vaginal plugs, indicating conception; females with vaginal plugs were separated. The presence of a plug represented embryonic day (E) 0. Pregnant mice could give birth and nurse their pups until postnatal day (PND) 16. The day of birth was designated as PND 0. At PND 4, the number of pups in each litter was adjusted to 5-7 males to cull pups.

All animal experiments were conducted in compliance with the "Guidelines of Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of Kindai University.

BPA treatment

BPA [2, 2-bis (4-hydroxyphenyl) propane; CAS no. 80-05-7, Sigma-Aldrich, Tokyo, Japan] was suspended in corn oil and administered by oral gavage from E6 to PND 15. This administration period was selected since the hippocampus formation in mice begins at E11, and neurogenesis in the SGZ of the DG is active between PND 3 and PND 14 (Reznikov, 1991). Fresh BPA solution was prepared every 5 days and analyzed before dosing. The BPA concentration was confirmed to be within $\pm 10\%$ of the target concentration. The drug was administered at the same time every day (12:00 p.m. - 12:15 p.m.). Based on our preliminary results, 200 μg BPA/kg/day was selected as our experimental dose in this study. For dose standardization, BPA at 0, 2, 20, and 200 μg /kg/day was administered orally from E0 to PND 15; 200 μg /kg/day BPA administration significantly decreased the percentage of NeuN-positive cells in the hippocampal DG compared to controls' administration at PND 16. Mice administered with diethylstilbestrol (DES, CAS no. 56-53-1, Sigma-Aldrich; 0.5 μg /kg/day) or corn oil (5 mL/kg/day) were used as positive and negative controls, respectively. Twelve pregnant mice in each group received BPA at 20 or 200 μg /kg/day. Nineteen pregnant mice were treated with corn oil as controls.

Tissue preparation and immunohistochemistry

At PND 16, nine male offspring from five dams in each group were deeply anesthetized using CO_2/O_2 and perfused transcardially with phosphate-buffered saline, followed by periodate lysine paraformaldehyde (PLP) at a flow rate of 10 mL/min. Male offspring of average weight

were randomly selected. As neurogenesis is influenced by the levels of circulating steroid hormones during the estrous cycle (Pawluski *et al.*, 2009), only male offspring were used in this study. Brains were removed, weighed, and post-fixed in PLP overnight at 4°C. Brains were then embedded in paraffin and sectioned at a thickness of 5 µm for histological and immunohistochemical analysis. Immunohistochemistry was performed as described previously (Komada *et al.*, 2008, 2012, 2014). The remaining male offspring were maintained until PND 70. At PND 70, nine male offspring from five dams per group were perfused for immunohistochemistry at a flow rate of 35 mL/min using the above-mentioned protocol.

The following primary antibodies were used: mouse anti-NeuN (1:100; Abcam, Cambridge, UK, AB177487; pan-neuronal marker), rabbit monoclonal anti-Ki67 (1:200; Lab Vision, SP6; cell proliferation marker), rabbit anti-doublecortin (DCX; 1:1,000; Abcam ab18723; immature granule cell marker), rabbit anti-Sox2 (1:2000; Abcam ab97959; type-1, type-2a, type-2b neural progenitor cell marker), rabbit anti-GFAP (1:400; Abcam ab49874; type-1 neural progenitor cell marker), rabbit anti-calbindin (1:1,000; Abcam ab11426; mature granule cell marker), rat monoclonal anti-BrdU (for CldU immunostaining; 1:50; AbD Serotec BU1/75, Kidlington, UK), and mouse monoclonal anti-BrdU (for IdU immunostaining; 1:50; BD Biosciences B44, NJ, USA).

Nissl staining

Sections were stained with cresyl violet solution according to previously described methods (Smith *et al.*, 2011). Three coronal sections from each offspring were analyzed to estimate the total number of cresyl violet-positive neurons (cell bodies) in the whole of DG.

Evaluation of immunoreactive cells

Immunofluorescence staining was quantified by manually counting the number of immunoreactive cells and the total number of cells [stained by 4',6-diamidino-2-phenylindole (DAPI)] in the whole DG (Sup. Fig. 1 and 2). Three anatomically matched sections from each offspring were analyzed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA). The percentage of immunoreactive cells [for NeuN, Ki67, CldU, and IdU immunostaining; (number of immunoreactive cells/total number of DAPI-stained cells) × 100 (Sup. Fig. 2)] and that of the stained area (for DCX, GFAP immunostaining; expression area per frame area × 100, Sup. Fig. 2) were calculated. The number of Sox2- and calbindin-positive cells in each section (number of positive cells per 10,000 pixels of DG, Sup Fig. 2) was also calculated. Cell counting

and quantitative evaluation were performed as previously described (Komada *et al.*, 2008, 2017). The analysis was performed in a double-blinded manner.

CldU and IdU incorporation assays

For *in vivo* labeling of the cells in the S-phase, PND 16 and 70 mice were intraperitoneally injected with CldU (50 mg/kg body weight; MP Biomedicals, Santa Ana, CA, USA) and IdU (50 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) 1 hr (CldU, for proliferation) and 24 hr (IdU, for cell cycle exit), respectively, before sampling at each developmental stage. The CldU positive cells represented the actively proliferating cells, while IdU+/Ki67- cells represented the post-mitotic cells that have exited the cell cycle (Chenn and Walsh, 2002).

Step-through passive avoidance test

We performed a step-through passive avoidance test to evaluate learning and memory in mice, as described previously (Park *et al.*, 2000). In brief, in the initial session on day 1, mice were placed in the light compartment of a passive avoidance apparatus (MPB-M001; Melquest, Toyama, Japan), and the latency to enter the dark compartment was measured. The animals received a mild foot shock (0.05 mA, 2 sec) by a shock generator (SG-100, Melquest). After 24 hr, a memory-retention session was carried out to measure the latency to enter the dark compartment in the same apparatus. If the animal did not enter the dark compartment within 3 min, it was given a score of 180 sec. Twenty-one male offspring from nine dams per group underwent passive avoidance test at PND 70.

Statistical analysis

Each litter was considered an independent experimental unit. In this study, one-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used to analyze data for the number of immunoreactive cells. $p < 0.05$ was considered statistically significant.

RESULTS

Clinical signs/reproductive performance of pregnant mice exposed to BPA

None of the pregnant females in the BPA-treated, DES-treated, or control group showed abnormal clinical signs or abnormal parturition during the experimental period, and all dams gave birth on E19. In addition, no lethal effect of BPA or DES on fetuses or neonates was detected, estimated by the number of live newborns at PND 1 and PND 16. The body weight (A) of male and female newborns in the BPA-treated group (male: 7.10 ± 1.01 g, female:

6.54 $\pm$ 1.32 g) and that of females in the DES-treated group (7.14 $\pm$ 0.83 g) was significantly lower than that of controls (male: 9.20 $\pm$ 1.85 g, female: 9.04 $\pm$ 1.73 g), whereas no significant differences in absolute brain weight (B) of both sexes were detected between the treated groups and controls. The relative brain weight (B/A) of male and female offspring in the BPA- and DES-treated groups was also comparable with that of the controls. In addition, we analyzed the TUNEL assay in the hippocampal DG of BPA- and DES-treated mice (unpublished data). At PND 16, the number of TUNEL positive cells in the DG of BPA- and DES-treated mice did not significantly differ from that of the control mice.

BPA exposure decreases the number of cells within the DG

We analyzed the histological abnormalities in the hippocampal DG of BPA- and DES-treated mice using Nissl staining (Fig. 1A, Sup. Fig. 1). At PND 16, the number of cells in the DG of BPA- and DES-treated mice was significantly lower than that in control mice (Fig. 1B). However, no significant difference was observed in the number of DG cells among groups (Fig. 1B). Moreover, at PND 70, no significant difference was observed in the total number of cells within the DG among groups (Fig. 1A, B). These results suggest that prenatal and postnatal exposure to BPA or DES affects the development and maintenance of the hippocampal DG.

BPA exposure affects neurons at all differentiation stages

To investigate whether BPA exposure affects neurons at a specific differentiation stage, we performed immunostaining using neuronal markers of different stages [anti-GFAP: type 1 (Fig. 2A, Sup. Fig. 2) and astrocytes; anti-Sox2: type 1, 2a, 2b (Sup. Fig. 3A); anti-Ki67: type 2a, 2b (Fig. 3A); anti-DCX: type 3, immature neurons (Sup. Fig. 4A); anti-NeuN: immature and mature neurons (Fig. 4A); anti-calbindin: mature neurons (Fig. 5A)]. Results showed that GFAP expression was significantly lower in the DG of BPA- and DES-treated mice than that of control mice at PND 16 and 70 (Fig. 2A, B). No significant difference was observed in the number of Sox2-positive cells within the DG of BPA-treated mice compared to that of control mice at PND 16 and 70 (Sup. Fig. 3A, B). At PND 16, the percentage of Ki67-positive cells was significantly lower in the DG of BPA- and DES-treated mice than that of control mice (Fig. 3A, B). However, at PND 70, no significant difference was observed in the percentage of Ki67 positive cells among groups (Fig. 3A, B). Moreover, at PND 16, no significant dif-

ference in DCX expression was observed between BPA-treated and control mice (Sup. Fig. 2A, B). However, at PND 70, DCX expression was found to be higher in the DG of BPA- and DES-treated mice than that of control mice (Sup. Fig. 4A, B). At PND 16, the percentage of NeuN positive cells was significantly lower in the DG of BPA- and DES-treated mice than that of the control mice (Fig. 4A, B); however, no difference was observed at PND 70 (Fig. 4A, B). The number of calbindin positive cells was also significantly lower in the DG of BPA- and DES-treated mice than that of the control mice at both PND 16 and 70 (Fig. 5A, B). These results suggest that the expression of several markers indicative of neuronal differentiation stages decreased in the DG following BPA exposure, especially at PND 16.

Effects of BPA exposure on neurogenesis and neural stem cell proliferation

We investigated the effect of BPA on neural stem cell proliferation and neurogenesis by thymidine analogue (CldU and IdU) incorporation analysis. First, we evaluated the actively proliferating cells in the hippocampal DG. Mice were injected with CldU 1 hr prior to being sacrificed, and CldU incorporation in the hippocampal DG was analyzed using the anti-CldU antibody. Results showed that the number of CldU-positive cells was significantly lower in the DG of BPA- and DES-treated mice than that of control mice at PND 16 and 70 (Fig. 6A, B).

Next, we investigated neurogenesis by examining cells that had permanently exited the cell cycle. At 24 hr post IdU injection, animals were sacrificed, and their brains analyzed using anti-Ki67 and anti-IdU antibodies. The ratio of IdU-positive/Ki67-negative cells to the total number of cells represented the cells that had exited the cell cycle. Our results revealed that the number of cells in the DG that had permanently exited the cell cycle was significantly lower in BPA- and DES-treated mice than that in control mice at PND 16 and 70 (Fig. 7A, B). These results suggest that prenatal BPA exposure affects the proliferation of neural stem cells and neurogenesis during the postnatal period.

BPA exposure affects the long-term memory maintenance

To determine whether the reduced neurogenesis in the hippocampal dentate gyrus due to prenatal and postnatal BPA exposure was involved in maintaining memory, we performed a step-through passive avoidance test at PND 70. BPA administration significantly altered long-term memory (24 hr) in mice challenged with the passive avoidance test. In the short-term memory test (45

Effects of prenatal and postnatal BPA exposure on neurogenesis

Fig. 1. Histological analysis of the hippocampal dentate gyrus (DG). (A) Nissl staining was performed on sagittal sections of the mice brain obtained at postnatal day (PND) 16 and PND 70 for control, bisphenol A (BPA)-exposed, and, diethylstilbestrol (DES)-exposed groups. (B) Graphs depicting the total number of cells in the DG. Scale bar = 200 μ m. Bars represent mean $\pm$ SD. * p < 0.05 compared to the control group using one-way analysis of variance and Bonferroni post-hoc test.

min), there was no significant difference in the retention time between BPA-treated and control groups. However, during a long-term memory test (24 hr), the retention time in the BPA-treated group (186.58 ± 120.4 sec) was shorter than that in the control group (250.77 ± 75.3 sec, $p = 0.0358$; Fig. 8). These data indicate that prenatal and postnatal BPA exposure affects the maintenance of long-term memory, as analyzed by the step-through passive avoidance test.

DISCUSSION

The present study aimed to evaluate the developmental neurotoxicity caused by prenatal and postnatal exposure of mice to BPA. Developmental toxicity due to BPA exposure was evaluated at PND 16 and 70 by analyzing

Fig. 2. GFAP-positive cells in the hippocampal dentate gyrus (DG) are reduced in the bisphenol A (BPA)-exposed group. (A) Immunostaining using anti-GFAP antibody on sagittal sections of mouse brains obtained at postnatal day (PND) 16 and 70 from all groups. (B) Graph depicting the percentage of the GFAP-positive area in the hippocampal DG. Scale bar = 100 μ m. Bars represent mean $\pm$ SD. * p < 0.05 and ** p < 0.01 compared to the control group using analysis of variance and Bonferroni post-hoc test.

the number of neural stem cells and neurogenesis in the hippocampal DG. Our results showed that prenatal and postnatal BPA exposure reduces the number of type 1 neural progenitor cells at both stages. Moreover, BPA exposure suppressed the differentiation of type 2a and 2b neural progenitor cells in the hippocampal DG at PND 16,

Fig. 3. Ki67-positive type-2a and 2b cells in the hippocampal dentate gyrus (DG) are reduced at postnatal day (PND) 16 in the bisphenol A (BPA)-exposed group. (A) Ki67 immunostaining of sagittal brain sections obtained at PND 16 and 70 from BPA- and diethylstilbestrol (DES)-treated mice. (B) Graph represents the percentage of Ki67-positive cells in the hippocampal DG. Scale bar = 200 μ m. Bars represent mean $\pm$ SD. * p < 0.05 and ** p < 0.01 compared to the control group using analysis of variance and Bonferroni post-hoc test.

Fig. 4. NeuN-positive immature and mature granule cells in the hippocampal dentate gyrus (DG) are reduced at postnatal day (PND) 16 in the bisphenol A (BPA)-exposed group. (A) NeuN immunostaining of sagittal brain sections obtained at PND 16 and 70 from BPA- and diethylstilbestrol (DES)-exposed mice. (B) Graph represents the percentage of NeuN-positive cells in the hippocampal DG. Scale bar = 100 μ m. Bars represent mean $\pm$ SD. * p < 0.05 compared to the control group using analysis of variance and Bonferroni post-hoc test.

however, no difference was observed at PND 70. Upon exiting the cell cycle, cells differentiate into type 3 neural stem cells in the hippocampal DG. We observed that the number of cells in the hippocampal DG that had permanently exited the cell cycle was significantly lower in the BPA-exposed group than in the control group, indicating that type 3 neural stem cell differentiation is suppressed upon BPA exposure. BPA exposure also reduced the number of immature and mature granule neurons in the hippocampal DG, suggesting compromised neurogenesis, and impaired neuronal migration. The total number of cells in the hippocampal DG was also reduced in the

BPA-exposed mice, suggesting that prenatal and postnatal BPA exposure affects hippocampal DG neurogenesis without stage specificity. There was neither difference in the number of Sox2-expressing cells within the DG between the two groups at PND 16 and PND 70, nor in the total number of cells at PND 70. These results suggest that prenatal and postnatal BPA exposure suppresses neuronal differentiation during early adulthood; however, normal neurogenesis is restored as the hippocampal DG develops (Fig. 9). In mice, morphogenesis in the hippocampal DG begins at early postnatal stages and is completed at around 8 weeks postnatally (Reznikov, 1991). At

Effects of prenatal and postnatal BPA exposure on neurogenesis

Fig. 5. Bisphenol A (BPA) exposure reduces the number of calbindin-positive mature granular cells in the hippocampal dentate gyrus (DG) at postnatal day (PND) 16 and 70. (A) Representative images of sagittal sections stained with anti-calbindin antibody at PND 16 and 70. (B) Graph represents the percentage of the calbindin-positive area in the hippocampal DG. Scale bar = 100 μ m. Bars represent mean $\pm$ SD. * p < 0.05 and ** p < 0.01 compared to the control group using analysis of variance and Bonferroni post-hoc test.

PND 16, the hippocampal DG is still undergoing morphogenesis; therefore, the detected abnormalities in the hippocampal DG might be corrected with development.

The present study demonstrates that prenatal and postnatal BPA exposure impairs learning and memory in male mice, as assessed by the step-through passive avoidance test. This test measures learning and memory in mice,

Fig. 6. Prenatal and postnatal bisphenol A (BPA) exposure reduces neural stem cell proliferation in the hippocampal dentate gyrus (DG). (A) Representative images of parasagittal sections stained with anti-CldU antibody. (B) Graph depicting the percentage of CldU-positive cells in the hippocampal DG. Scale bar = 200 μ m. Bars represent mean $\pm$ SD. * p < 0.05 and ** p < 0.01 compared to the control group using analysis of variance and Bonferroni post-hoc test.

based on their natural aversion to well-lit places. A shorter latency before entering the dark chamber in the retention trial suggests impairment of fear-motivated learning and memory (Park *et al.*, 2000). Male mice exposed to BPA had significantly shorter latencies than control mice in the long-term test (24 hr). Our data suggest that prenatal and postnatal BPA exposure impairs inhibitory avoidance memory in male mice. Gonçalves *et al.* (2010) reported that prenatal exposure to low-dose BPA (40 μ g/kg/day) impairs both short-term and long-term memory in the inhibitory avoidance test. However, other reports have shown that perinatal BPA exposure does not affect inhibitory avoidance memory in male mice (Xu *et al.*, 2010)

Fig. 7. Prenatal and postnatal bisphenol A (BPA) exposure decreases the number of post-mitotic cells in the hippocampal dentate gyrus (DG). (A) Representative images of parasagittal brain sections of E15.5 embryos stained with anti-Ki67 and anti-IdU antibodies 24 hr after IdU pulse-labeling in control, BPA-treated, and diethylstilbestrol (DES)-treated mice. (B) Graph represents the percentage of IdU/Ki67+ cells 24 hr after labeling. Scale bar = 200 μm. Data are shown as means ± SD. * $p < 0.05$ compared to the control group using analysis of variance and Bonferroni post-hoc test.

and male rats (Fujimoto *et al.*, 2006; Kuwahara *et al.*, 2013). In addition, passive avoidance learning depends on multiple cortical and sub-cortical structures, including both dorsal and ventral striatum as well as hippocampus and amygdala (Pittenger *et al.*, 2006). This could explain why we could not detect the effects of prenatal and postnatal exposure to BPA on learning and memory in male mice, in this study. Moreover, no abnormalities were observed in long-term memory during the passive avoidance test using the DES-exposed group as a positive control. This might be due to differences in the neurode-

Fig. 8. Prenatal and postnatal bisphenol A (BPA) exposure impairs long-term memory maintenance assessed by step-through passive avoidance test. The effects of BPA exposure on the latency to enter the dark chamber in the step-through passive avoidance test in postnatal day (PND) 70 male mice. Data are shown as means ± SD. * $p < 0.05$ compared to the control group using analysis of variance and Bonferroni post-hoc test.

Fig. 9. Illustration depicting a result's summary. Prenatal and postnatal exposure to bisphenol A (BPA) suppresses neural stem cell proliferation and neurogenesis in the hippocampal dentate gyrus. BPA-mediated toxicity is not stage specific since neurons at all differentiation stages are affected. However, the developmental abnormalities are more pronounced at postnatal day (PND) 16 than at PND 70.

velopmental toxicity mechanism of DES and BPA.

Pre- and postnatal exposure of BPA inhibits neural stem cell proliferation and differentiation in rat brains that are dependent on the Wnt/beta-catenin pathway (Tiwari *et al.*, 2015). In addition, pre- and postnatal BPA administration has no effects on adult neurogenesis in the dentate

gyrus of 8-week old mice (Kim *et al.*, 2009). Our histological data showed that pre and postnatal BPA exposure may suppress neurogenesis in the hippocampal dentate gyrus, which is not completely consistent with results of previous studies. We hypothesize that discrepancies are due to timing (developmental stages), route of administration (intraperitoneal or oral), and dose (low or high) of BPA exposure. BPA accumulates in the body mostly during the prenatal and postnatal period than during the adulthood because its metabolic and excretion pathways are immature. In addition, BPA has a different binding affinity for each receptor, and its action differs in a concentration-dependent manner (Kim *et al.*, 2009). For example, BPA concentration-dependent exposure decreases the proliferation of murine-derived multipotent neural progenitor cells, while high concentration exposure produces cytotoxicity. Moreover, low BPA concentrations, which possess estrogenic activity, stimulate differentiation of neural progenitor cells into a neuronal phenotype *in vitro* (Kim *et al.*, 2009).

Signaling pathways that are disrupted by BPA, such as estrogen (Denley *et al.*, 2018) and thyroid hormone pathways (Bernal, 2007), have different roles according to the developmental stage. Thyroid hormones are known to affect brain development, and several environmental chemicals interfere with their function. Studies have shown that, in adult rats, a decrease in thyroid hormone levels reduces the proliferation and survival of hippocampal progenitors, while adult hippocampal progenitors exhibit enhanced proliferation and survival in response to thyroid hormones (Ambrogini *et al.*, 2005; Desouza *et al.*, 2005; Montero-Pedrazuela *et al.*, 2006). Thyroid hormone receptors (TRs) are expressed on neural stem cells and involved in regulating the proliferation, survival, and differentiation of progenitors in the hippocampal DG (Kapoor *et al.*, 2011, 2010). Studies have shown that BPA can bind to the rat TR and act as a thyroid hormone antagonist *in vitro* (Moriyama *et al.*, 2002) and *in vivo* (Zoeller *et al.*, 2005). In addition, BPA competitively inhibits the binding of protein disulfide isomerase to thyroid hormone 3,3',5-triiodo-L-thyronine (Hiroi *et al.*, 2006). Thus, BPA-mediated modulation of thyroid hormones function might underlie the effects of prenatal and postnatal BPA exposure on neurogenesis and neural stem cell proliferation in the DG.

Estrogen is secreted primarily by the follicular cells of the ovary but are also produced in the testis, placenta, and adrenal glands. Previous studies in rodents have shown that estrogens regulate neurogenesis in the adult hippocampus (Galea *et al.*, 2013; Pawluski *et al.*, 2009). Estradiol binds to the classical ERs, ER α and ER β , and

the G protein-coupled estrogen receptor (GPER) to exert its physiological effects (McEwen *et al.*, 2012; Prossnitz *et al.*, 2008; Vasudevan and Pfaff, 2008). As both, ER α and ER β are expressed on hippocampus stem cells, these cells constitute important targets of estrogen (Brailoiu *et al.*, 2007; Hazell *et al.*, 2009; Towart *et al.*, 2003). In addition, the ER α agonist propyl-pyrazole triol and the ER β agonist diarylpropionitrile significantly enhance cell proliferation in the DG of female rats (Mazzucco *et al.*, 2006). In this study, prenatal and postnatal exposure to BPA suppressed the proliferation and neuronal differentiation of neural stem cells in the DG. In addition, in rodents, cells in or near the dentate granule cell layer transiently express high levels of estrogen binding and ER α protein in the nucleus during the first two postnatal weeks (O'Keefe *et al.*, 1995; Solum and Handa, 2001). In adult rats and mice, ER β mRNA and protein are found in the perikarya of granule cells, as well as in cells of the dentate sub-granular layer (Li *et al.*, 1997; Milner *et al.*, 2005; Mitra *et al.*, 2003; Shughrue *et al.*, 1997). In this study, prenatal and postnatal exposure to BPA induced the neurodevelopmental toxicity of the hippocampal dentate gyrus observed, possibly through estrogen signaling.

In conclusion, prenatal and postnatal BPA exposure has subacute and delayed effects on neurogenesis in the hippocampal DG. As the number of neural stem cells and neurons at each differentiation stage was reduced, it is possible that BPA delays or reduces neurogenesis. A more severe phenotype was also observed in the positive control DES, suggesting that estrogen signaling mediates neurodevelopmental toxicity. However, in order to clarify the effects of BPA exposure on the morphogenesis of the hippocampal DG, it is necessary to study BPA at multiple doses and analyze its concentration dependence.

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

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Effects of prenatal and postnatal BPA exposure on neurogenesis

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