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Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses

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ABSTRACT

Bisphenol A (BPA), an endocrine-disruptor, is widely used in the production of plastics and resins. Human perinatal exposure to this chemical has been proposed to be a potential risk to public health. Animal studies indicate that postnatal exposure to BPA may affect neocortex development in embryos by accelerated neurogenesis and causing neuronal migration defects. The detailed phenotypes and pathogenetic mechanisms, especially with regard to the proliferation and differentiation of neural stem/progenitor cells, however, have not been clarified. C57BL/6J pregnant mice were orally administered BPA at 200 μg/kg from embryonic day (E) 8.5 to 13.5, and the fetuses were observed histologically at E14.5. To clarify the histological changes, especially in terms of neurogenesis, proliferation and cell cycle, we performed histological analysis using specific markers of neurons/neural stem cells and cell cycle-specific labeling experiments using thymidine-analog substances. Cortical plate was hyperplastic and the number of neural stem/progenitor cells was decreased after the exposure to BPA. In particular, the maternal BPA oral dosing related to the effects on intermediate progenitor cells (IPCs, neural progenitor cells) in the subventricular zone (SVZ) of dorsal telencephalon. Exposure to BPA associated the promotion of the cell cycle exit in radial glial cells (RGCs, neural stem cells) and IPCs, and decreased the proliferation resulting from the prolong cell cycle length of IPCs in the SVZ. Our data show that maternal oral exposure to BPA related to the disruption of the cell cycle in IPCs and the effects of neurogenesis in the developing neocortex.

1. Introduction

Endocrine-disrupting industrial chemicals are released into the environment and interfere with normal endocrine function. Bisphenol A (BPA; 2,2-bis(4-hydroxy-phenyl) propane) is known to be one of the endocrine-disrupting chemicals because of its weak estrogenic, androgenic and thyroid hormone-like activity (Hiroi et al., 2006; Krishnan et al., 1993; Takayanagi et al., 2006; Xu et al., 2005). BPA is used in polycarbonate plastics, epoxy resins, and dental resin-based composites (Howe and Borodinsky, 1998; Pulgar et al., 2000; Sasaki et al., 2005). BPA has been detected in the serum of pregnant women (1–2 ng/ml), fetus serum (0.2–9.2 ng/ml), amniotic fluid (8.3–8.7 ng/ml), placental tissue (1.0–104.9 ng/ml), and breast milk (1.1 ng/ml) (Ikezuki et al., 2002; Schonfelder et al., 2002; Ye et al., 2005), suggesting that the human fetus is exposed to this compound during pre- and post-natal development. Several studies reported some adverse effects of high- and low-dose BPA on various organs during the prenatal period (Golub et al., 2010; Kundakovic and Champagne, 2011). During fetal development, there is a sensitive period during which environmental exposure may cause persistent damage to the developing brain. Maternal exposure to low-dose BPA (20 μg/kg/day, intraperitoneally) has been shown to affect cortical development in embryos by accelerated neurogenesis and neuronal migration during the mid-gestational period in mice (Nakamura et al., 2006). In addition, BPA exposure results in abnormal neuronal positioning and aberrant neuronal network formation between the thalamus and the cortex in the mature brain exposed prenatally (Nakamura et al., 2007). However, no findings have been added to these reports about the morphological changes of the neocortex induced by low-dose BPA exposure.

During neocortical development, neural stem/progenitor cells sequentially pass through phases of expansion, neurogenesis and gliogenesis. Radial glial cells (RGCs) in the ventricular zone (VZ), neural stem cells, expand their population by symmetric

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division that produces two RGCs and simultaneously self-renew and generate more differentiated cells through asymmetric cell division (Gotz and Huttner, 2005). The daughter cells of ventricular RGCs are often neuronal progenitor cells and can migrate superficially into the subventricular zone (SVZ) to divide (Haubensak et al., 2004; Noctor et al., 2004). RGC divisions are asymmetric and associated with self-renewal, while the daughter progenitor cells (intermediate progenitor cells: IPCs) usually undergo one terminal symmetric division that produces two neurons and depletes the progenitor cells (Noctor et al., 2004). The majority of layer II/III neuron-producing cell divisions are by IPCs during the late neurogenesis stages (Kowalczyk et al., 2009). The number of symmetric IPC divisions could also affect neuron number and be a determinant of neocortical size. A human genetic study pinpointed the disease-causing gene in a family exhibiting congenital microcephaly to the homozygous silencing of Tbr2 (Baala et al., 2007), a transcriptional factor shown in rodent studies to be a selective marker for IPCs (Englund et al., 2005). These reports suggest the overall importance of IPCs of SVZ in the control of neocortical size.

The purposes of this study are to clarify the mechanisms underlying the abnormal proliferation and differentiation of neural stem cells in fetuses exposed to BPA. Observation of the BPA-treated fetuses revealed the accelerated neurogenesis and a reduced number of neural stem/progenitor cells, especially IPCs, in the dorsal telencephalon. Our data suggested that these aberrations of developing neocortex result in disruption of the cell cycle in IPCs.

2. Materials and methods

2.1. Animals and housing

Eight-week-old male and female C57BL/6J mice were purchased from Japan SLC Inc. to be used as breeder animals in this study. These mice were quarantined and habituated for 2 weeks. These animals were kept under SPF conditions and a constant light–dark cycle (dark period from 7:00 pm to 7:00 am) at 24 ± 1 °C and 55 ± 5% relative humidity. To avoid the possibility of stressing the animals, noise levels were kept to a minimum both within the room and in the adjacent areas. Diet food (Certified Rodent Chow CE-2, CLEA Japan) and drinking water were available as libitum. Certification analysis of each lot of diet was performed by the manufacturer. The same lots of diet were provided to animals from control and BPA-treated groups and at the same times, in order to control across groups for possible variation in the content of the diet. Water was available via glass bottles with Teflon seals during the exposure period. Pregnant females were housed individually throughout the study in polypropylene plastic tubs with stainless steel lids and corncob bedding. Mice in all experiments were humanely treated according to the guidelines of the Animal Research Committee of Kinki University. Ten-week-old mice were allowed to copulate overnight at a 2:1 or 1:1 female to male ratio. Females were checked at 12 h intervals for the presence of vaginal plugs, indicating copulation, and were separated from the male if a plug was present. The presence of a plug represented embryonic day (E) 0.

2.2. Test substance and treatment regimen

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane 4,4′-isopropylidenediphenol, CAS no. 80-05-7, Sigma Aldrich) was suspended in corn oil and administered by oral gavage from E8.5 to E13.5. The dose solution was prepared once per 5 days and analyzed prior to dosing. The BPA concentration was confirmed to be within ±10% of targeted concentration. Administration occurred at a defined time (12:00 pm). In a preliminary study, three dams per group were exposed orally to BPA at 20 or 200 μg/kg from E8.5 through E13.5, and killed on E14.5 to collect the fetuses. Nine female fetuses in each group were examined histologically in terms of the developing brain (Sup. Table 1). No histological abnormality, such as hyperplasia of cortical plate and the acceleration of cell cycle exit (Sup. Fig. 1, Tables 1 and 2), were detected in the fetuses exposed to BPA at 20 μg/kg. In the fetuses exposed to BPA at 200 μg/kg, hyperplasia of cortical plate and the promotion of neurogenesis were identified. On the basis of these results, BPA dose used in the present study was chosen as 200 μg/kg/day, which revealed clear effects on the development of neocortex. Control animals received an equal volume of corn oil. At this dose of BPA (200 μg/kg/day) we calculate intake to be approximately 3 μg of BPA daily; body weight of mice used was approximately 30–40 g during the administration period. In the previous study, when rat dam was orally administered BPA at 6 μg/kg/day, 4–5 μg/L BPA was detected in embryonic serum (Yoshida et al., 2004). In addition, prenatal exposure to BPA at 200 μg/kg/day BPA induced changes of embryonic body weight and total number of embryos born per litter (Cagen et al., 1999). These data indicate that the embryos were exposed to BPA in utero in the experimental conditions of the present study.

2.3. Body weight measurement and tissue preparation

Pregnant mice were humanely killed and underwent cesarean section on E14.5, and the fetuses were observed histologically. Fetuses were sampled out of the ostium of the uterus, because there were shown to be no differences in the postnatal growth of the reproductive and endocrine systems, sexual maturation and estrous cycle, or behavior depending on the embryo position in utero (Nagao et al., 2004). For body weight measurement, the body weight before fixation was measured for E14.5 mouse fetuses. Fourteen males and 17 female fetuses from 6 litters in the BPA-treated group and 9 males and 12 females from 6 litters in the control group were used for head size and body weight measurement (Sup. Table 1). Fetuses were fixed for 3 h in periodate lysin paraformaldehyde (PLP) at 4 °C, and washed in phosphate-buffered saline (PBS). For paraffin sections, fetuses were embedded in paraffin and sectioned at 5 μm for histological and immunohistochemical observation. For frozen sections, fetuses were embedded in 30% sucrose/PBS for cryoprotection, and sectioned at 10 μm for immunohistochemical observation. Nine female fetuses from 3 litters in both BPA-treated and control groups were sampled on E14.5 for HE staining and immunostaining (Sup. Table 1). Fetal body weight and brain measurement data were analyzed employing Student's *t*-test.

2.4. Immunohistochemistry

The following antibodies were used: mouse monoclonal anti-neural class III β-tubulin (Tuj1, 1:500; Covance); pan-neuronal marker, rabbit monoclonal anti-Ki67 (1:200; Lab Vision, SP6); a proliferative cell marker, rat monoclonal anti-BrdU (CldU) (1:50; Oxford Biotechnology, BU1/75), mouse monoclonal anti-BrdU (IdU) (1:50; Becton Dickinson, B44), rabbit polyclonal anti-Tbr2 (1:200, Abcam); transcriptional factor and IPC marker, rabbit polyclonal anti-Pax6 (1:200, Covance); transcriptional factor and RGC marker, mouse monoclonal anti-Nestin (1:200, BD Pharmingen); marker of radial fiber in RGC. Secondary antibodies were conjugated with Alexa 568 and 488 (1:200; Invitrogen). The nuclei were counterstained with DAPI in mounting medium (Vector Labs). Immunohistochemistry was performed as described previously (Komada et al., 2008), and standard immunostaining procedures were used in E14.5 fetuses. Nine female fetuses from 3 litters in the BPA-treated group and 9 female fetuses from 3 litters in the control group were sampled on E14.5 for immunostaining (Sup. Table 1).

2.5. CldU and IdU incorporation

For in vivo labeling of S-phase cells (thymidine analog incorporation), one injection of CldU (105478, MP Biomedicals Inc.) and IdU (I7125, Sigma) was made 24 h and 1 h, respectively, prior to cesarean sectioning on E14.5. Fetuses were allowed to develop to E14.5 and then sacrificed and processed for CldU and IdU immunohistochemistry. The quantification of positive cells and their distribution within the cortical layer were analyzed according to the methods of Komada et al. (2008), with 2 anatomically matched sections from each fetus (9 BPA-treated and 9 control female embryos).

2.6. Cell cycle kinetics (analysis of cell cycle length and cell cycle exit)

For estimation of cell cycle length, we counted the number of IPCs labeled by 1 h pulse of IdU in Tbr2-positive cells. The population of IdU/Tbr2 double-positive cells among all Tbr2-positive cells enables estimation of the cell cycle length of IPCs. A smaller population of IdU-positive cells among Tbr2-positive cells indicates a greater cell cycle length (Chenn and Walsh, 2002).

Cell cycle exit was estimated from the ratio of CldU-positive cells/Ki67, Pax6, or Tbr2-negative (postmitotic and/or differentiation) cells to all CldU-positive cells at E14.5 in the dorsal telencephalon (Ki67) or upper SVZ (Pax6 and Tbr2) (Chenn and Walsh, 2002). One injection of CldU was made 24 h prior (at E13.5) to sampling (at E14.5).

2.7. Quantification of cell number for cell cycle exit, proliferation, RGC, IPC, and cell cycle length

For quantification of data obtained using immunofluorescent staining, the counterpart areas in the dorsal telencephalic region of brain of the control and BPA-treated fetuses were selected, and the total number of cells (DAPI-stained) and cells stained with each antibody were manually counted in two 100 μm-wide sampling box (indicated by open boxes in Figs. 2–6) with 2 anatomically matched sections from each fetus (9 BPA-treated and 9 control female embryos) using Adobe photoshop CS4 (Adobe). We performed the counting and quantitative procedures by the method reported previously (Komada et al., 2008).

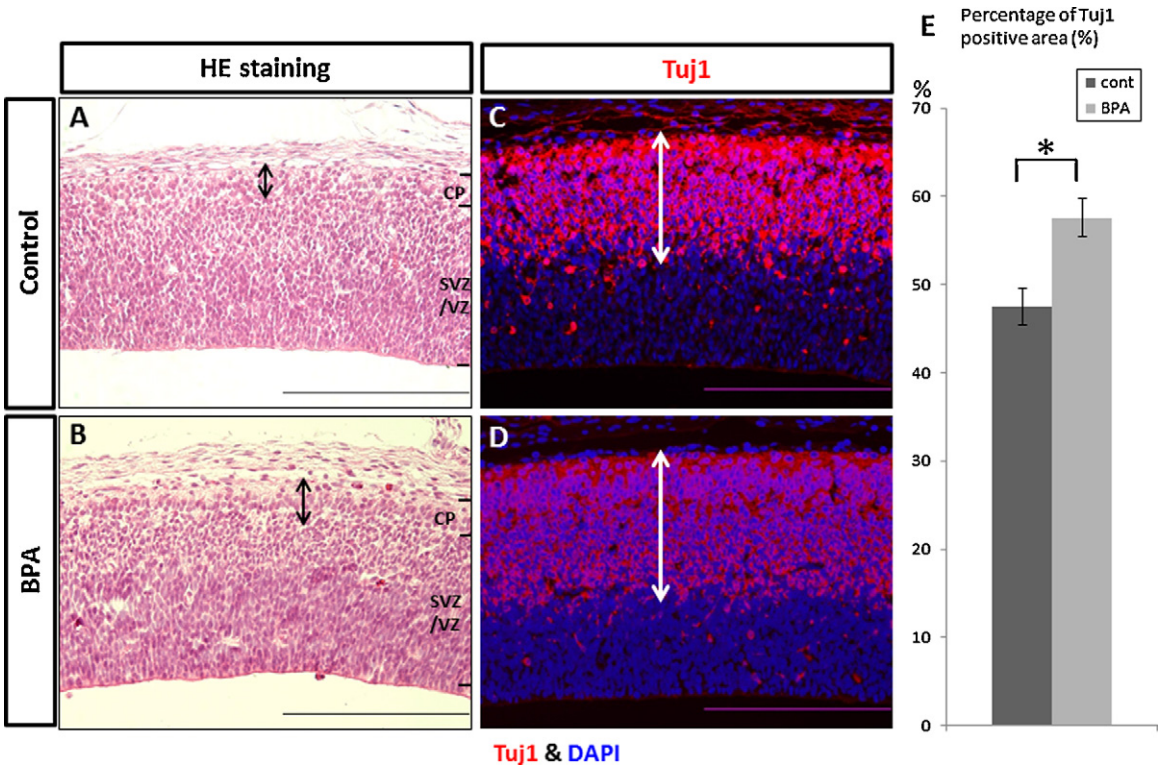

Fig. 1. Maternal BPA oral dosing induces hyperplasia of cortical plate and promotes neurogenesis. Parasagittal sections of the telencephalons of the control fetuses (A) and BPA-treated fetuses (B) at E14.5 were stained with HE. These panels illustrated that the size of the cortical plate was reduced in BPA-treated fetuses (black arrow). Parasagittal sections of E14.5 dorsal telencephalon were immunostained with anti-neuron-specific class III β-tubulin (Tuj1) antibody (C, D) and expressing regions were measured on the sections (white arrow). (E) Tuj1-stained regions were significantly increased in the dorsal telencephalon of BPA-treated fetuses ($57.6 \pm 2.11\%$, $n = 9$, $^*P < 0.05$) compared with those of control fetuses ($47.5 \pm 2.09\%$, $n = 9$). Scale bar: 200 μm.

3. Results

3.1. Hyperplasia of cortical plate in BPA-treated fetuses

In order to clarify the effects of BPA exposure on the development of neocortex, pregnant mice were treated continuously with BPA at a dose of 200 μg/kg from E8.5 (this day is the start point of mouse neocortex organogenesis). Fourteen male and 17 female fetuses from 6 litters in the BPA-treated group and 9 male and 12 female fetuses from 6 litters in the control group were sampled at E14.5 (Sup. Table 1). These fetuses were weighed and we measured the rostral-caudal length and width of the head region. The body weight and head length and width of male and female fetuses treated with BPA were comparable to those of control fetuses at E14.5 (date not shown). In addition, there was no fetal lethal effect of BPA when evaluated at E14.5 (date not shown).

As for the histopathological observation of fetuses treated with BPA, hyperplasia of the CP in the dorsal telencephalon was found in the HE staining of the parasagittal sections (Fig. 1A and B). To determine the difference of neurogenesis in fetuses treated with BPA, the Tuj1 (neuron-specific class III β-tubulin; pan-neuronal marker) expression regions were quantified by measuring the total thickness of the dorsal telencephalon and the thickness of CP (Tuj1-expression region) (Fig. 1C and D). There was a significant increase in the thickness of CP in BPA-treated fetuses ($57.6 \pm 2.11\%$, $P < 0.05$) compared with that of control fetuses ($47.5 \pm 2.09\%$) (Fig. 1E). However, hyperplasia of the CP and the expansion of Tuj1-expression area in the dorsal telencephalon were not found in the HE staining (Sup. Fig. 1A–C′) and immunostaining of Tuj1 (Sup. Fig. 1D–F′, Sup. Table 2) in the 20 μg/kg/day BPA-treated fetuses. Nakamura et al. reported that low-dose BPA might disrupt normal neocortical development by accelerated neuronal differentiation/migration (Nakamura et al., 2006). Our results coincided with these previous data and indicated that the maternal BPA oral dosing associated with the accelerated neurogenesis and hyperplasia of CP during the development of telencephalon.

3.2. Acceleration of cell cycle exit in the neural stem/progenitor cells of the dorsal telencephalon

An adequately controlled cell cycle is important for proper neurogenesis during corticogenesis in the neocortex. To investigate the cause of neurogenesis promotion, we examined cell cycle exit of neural stem/progenitor cells. We performed double immunostaining using anti-Ki67 (nucleoprotein, expressed in proliferative cells) and anti-CldU (thymidine analog) antibodies after 24 h CldU labeling (Fig. 2A and B) (Chenn and Walsh, 2002). Cell cycle exit was determined as the ratio of cells that exited the cell cycle (red, CldU+/Ki67−, indicating cells no longer dividing) to all cells labeled with CldU (red and yellow) after 24 h labeling. Quantification of the experiment showed significantly increased cell cycle exit in BPA-treated fetuses ($44.1 \pm 3.8\%$, $P < 0.01$) compared with that in the control fetuses ($34.3 \pm 4.7\%$) (Fig. 2E). However, in the 20 μg/kg/day BPA-treated fetuses, the significant increase in rate of cell cycle exit was not observed in the dorsal telencephalon (Sup. Fig. 1G–I, Sup. Table 2). These data indicated that the hyperplasia of CP was caused by the accelerated neurogenesis of neural stem/progenitor cells in the dorsal telencephalon. Ki67-expression region was decreased and the distribution of double positive cells (Ki67+ and CldU+, yellow cells) was abnormal, remaining in a deep position of Ki67-positive SVZ/VZ, compared with that in control fetuses (Fig. 2A and B). We hypothesized that these phenotypes resulted from the aberration of interkinetic nuclear migration in the SVZ/VZ, in which their nuclei migrate using radial fibers between the apical surface

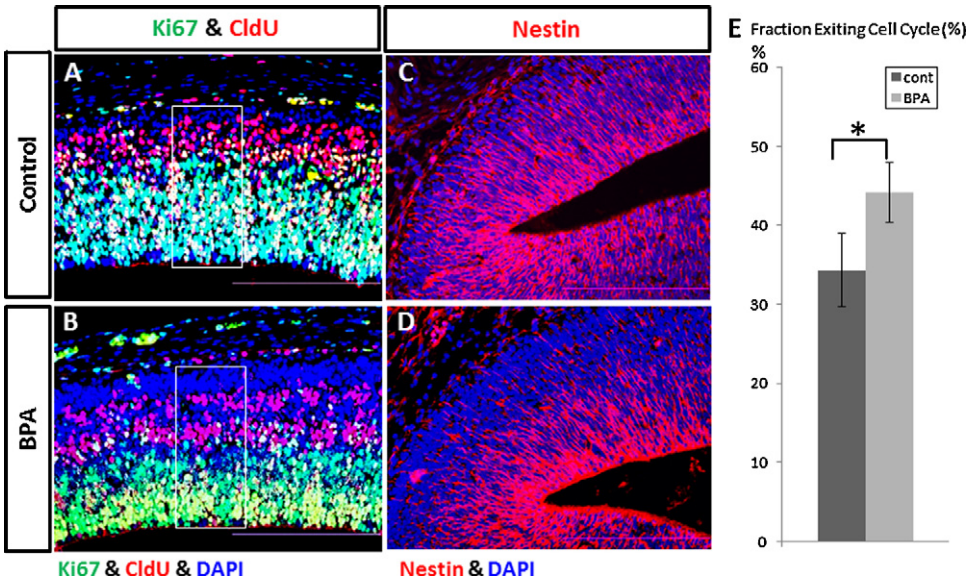

Fig. 2. The maternal oral exposure to BPA leads to acceleration of cell cycle exit and decreases the number of neural stem/progenitor cells. To estimate cell cycle exit of neural stem/progenitor cells, sections were stained with anti-Ki67 and anti-CldU antibodies 24 h after CldU pulse labeling at E14.5 in the control (A) and BPA-treated (B) fetuses. Parasagittal sections of E14.5 dorsal telencephalon were immunostained with anti-Nestin antibody. In BPA-treated fetuses, Nestin-stained radial fibers were shorter than those of the controls (C, D). (E) Cell cycle exit was determined as ratio of cells that exited the cell cycle (red, CldU+/Ki67−, no longer dividing) to all cells labeled with CldU (red + yellow) after 24 h labeling. Cells were counted in 100-μm-wide sampling boxes (white boxes in A and B). In the BPA-treated fetuses, the ratio was significantly increased at E14.5 (control fetuses: 34.3 ± 4.7%, $n = 9$, BPA-treated fetuses: 44.1 ± 3.8%, $n = 9$, $^*P < 0.01$). Scale bar: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and the basal part of the SVZ/VZ in synchrony with the cell cycle. Thus, we performed immunostaining using anti-Nestin (the marker of radial fibers of RGCs) antibody in the parasagittal sections. In the BPA-treated fetuses, the Nestin-positive radial fibers were shorter than control ones at E14.5 (Fig. 2C and D). These data suggested that the short radial fibers resulting from the abnormal interkinetic nuclear migration in the SVZ/VZ and abnormal positioning of Ki67+ cells and double-positive (Ki67+ and CldU+) cells.

3.3. Reduction of proliferative neural stem/progenitor cells in the dorsal telencephalon

Accelerated neurogenesis might impact on the proliferation of neural stem/progenitor cells in the dorsal telencephalon. To examine whether cell proliferation was affected in the BPA-treated fetuses, we performed immunostaining using anti-Ki67 antibodies (Fig. 3A and B). At E14.5, the Ki67-immunopositive cell index (Ki67-positive cells/DAPI-stained cells) was significantly decreased in the dorsal telencephalon of BPA-treated fetuses (41.6 ± 1.0%, $P < 0.01$) compared with that in the control fetuses (47.2 ± 1.0%) (Fig. 3C). These data suggested that the maternal BPA oral dosing related to the reduction of neural stem/progenitor cells as a result of the promotion of neurogenesis in the dorsal telencephalon.

3.4. BPA specifically affected the intermediate progenitor cells

During the corticogenesis of neocortex, three different types of neural stem/progenitor cells (RGCs, IPCs, and outer radial glial cells) exist in the SVZ/VZ of the dorsal telencephalon (Molnar et al., 2011). These neural stem/progenitor cells are distinguished by their distribution, self-renewal ability, capacity for neurogenesis and expression of transcriptional factors (Molnar et al., 2011). To determine which cell population (RGCs or IPCs) was affected by BPA exposure, we performed immunostaining using anti-Pax6 (transcriptional factor, the marker of RGCs) and anti-Tbr2 (transcriptional factor, the marker of IPCs) (Fig. 4A–D). Pax6-positive cells represent RGCs in VZ and Tbr2-positive cells are IPCs in SVZ. There was no significant difference in the number of Pax6-positive cells between the BPA-treated (54.1 ± 3.1%) and control fetuses (55.1 ± 3.8%) (Fig. 4E). However, the number of Tbr2-positive cells was significantly decreased in the BPA-treated fetuses

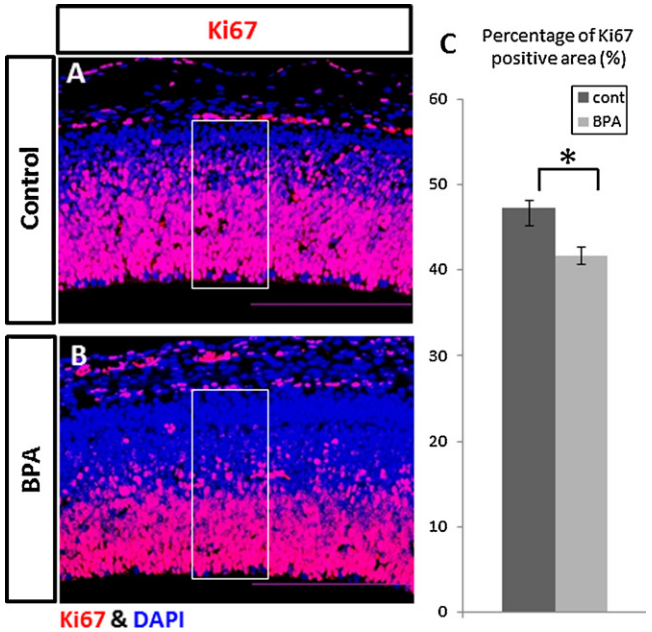

Fig. 3. Proliferation of stem/progenitor cells in the dorsal telencephalon is reduced by maternal BPA exposure. Immunostaining with Ki67 (red) of sagittal sections of E14.5 (A, B) fetal brains treated with BPA. Ki67 was expressed in the proliferative cells in the SVZ/VZ of dorsal telencephalon. Cells were counted in 100-μm-wide sampling boxes (white box). Examples of anti-Ki67 labeling of the dorsal telencephalon of the control (A) or BPA-treated fetuses (B). C: BPA-treated fetuses showed significant decrease in the ratio of Ki67+ cells at E14.5 (control fetuses: 47.2 ± 1.0%, $n = 9$, BPA-treated fetuses: 41.6 ± 1.0%, $n = 9$, $^*P < 0.01$). Scale bar: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

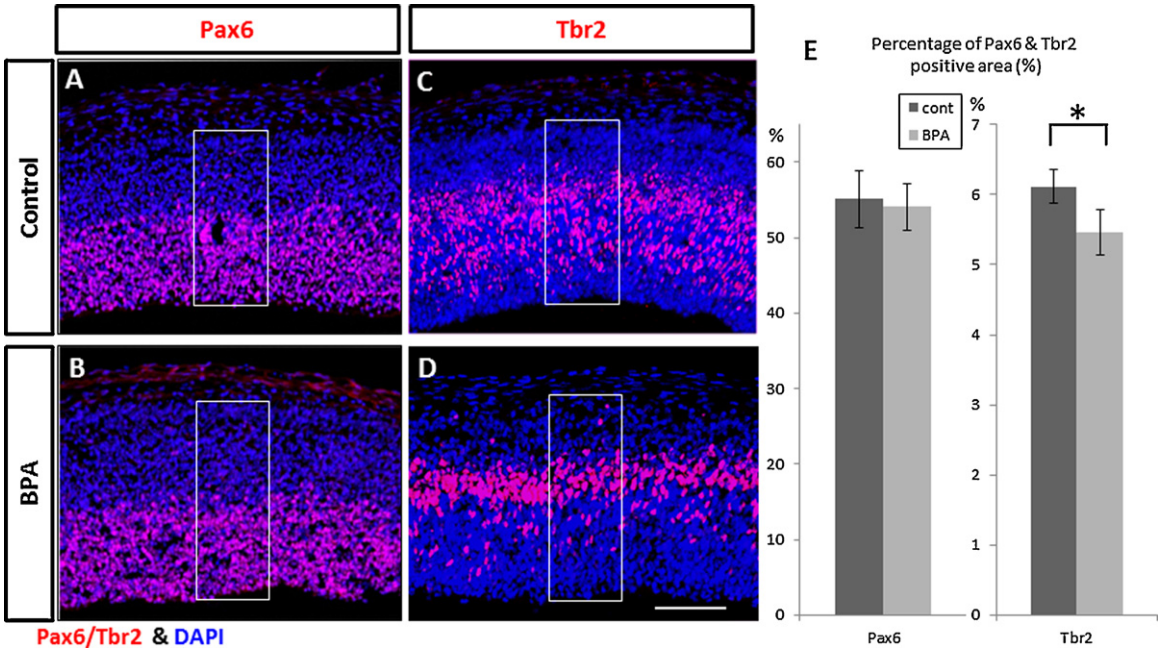

Fig. 4. Maternal BPA oral dosing induces the reduction of intermediate progenitor cells and does not affect radial glial cells. Immunostaining on coronal sections of E14.5 dorsal telencephalon with anti-Pax6 (A, B) and anti-Tbr2 (C, D). The Pax6-positive cells were considered as RGCs in VZ of the control (A) and BPA-treated fetuses (B) at E14.5. Cells were counted in 100-μm-wide sampling boxes (white box). There was no significant difference in Pax6-positive RGC number between the control and BPA-treated groups (E: control fetuses: 55.1 ± 3.8%, $n = 9$, BPA-treated fetuses: 54.1 ± 3.1%, $n = 9$). The Tbr2-positive cells were identified as intermediate progenitor cells (IPCs) in SVZ of the control (C) and the BPA-treated fetuses (D). (E) Tbr2-positive IPCs of the BPA-treated fetuses (6.12 ± 0.24%, $n = 9$, $^*P < 0.05$) showed significant reduction compared with those in the control fetuses (5.46 ± 0.32%, $n = 9$). Scale bar, 100 μm.

(5.46 ± 0.32%, $P < 0.05$) compared with that in the control fetuses (6.12 ± 0.24%) (Fig. 4E). These data suggested that the maternal BPA oral dosing specifically associated with the maintenance of IPCs in the SVZ of dorsal telencephalon.

3.5. The acceleration of RGCs and IPCs differentiation by BPA exposure

During the corticogenesis of neocortex, RGCs differentiate to IPCs or neurons and IPCs differentiate to neurons. IPCs mainly produce the projection neurons in layer II/III of neocortex. In evolution, layer II/III is among the most highly evolved regions and IPCs might have important roles in the progression of neocortex. Thus, to determine which cell (RGCs or IPCs) differentiation was affected by maternal BPA exposure, we performed cell cycle exit analysis using thymidine analog labeling with each cell. RGCs and IPCs are proliferative cells labeled by CldU in the SVZ/VZ. In the CP and upper SVZ after 24 h of CldU injection, CldU positive and Pax6/Tbr2 negative cells are defined as postomitotic (differentiated) cells from RGCs/IPCs, respectively. Differentiated RGCs and IPCs were identified by double-immunostaining using anti-Pax6/anti-CldU and anti-Tbr2/anti-CldU antibodies, respectively (Fig. 5A–D). The quantification of these experiments showed that the cell cycle exits of RGCs (37.4 ± 2.4%, $P < 0.01$) and IPCs (35.7 ± 1.2%, $P < 0.01$) in the BPA-exposed fetuses were significantly promoted compared with those in the control fetuses (RGCs: 32.8 ± 2.8%, IPC: 30.7 ± 3.7%) at E14.5 (Fig. 5E). These results indicated that the maternal BPA treatment related to the acceleration of neurogenesis of IPCs in the SVZ and differentiation of RGCs in the VZ of dorsal telencephalon.

3.6. BPA leads to prolongation of cell cycle of IPC

To investigate the cause of reduction of IPCs (Fig. 4), we examined the cell cycle length of IPCs. We hypothesized that prolongation of the cell cycle results in decreased proliferation of IPCs in the SVZ of dorsal telencephalon. We performed double immunostaining using anti-Tbr2 and anti-IdU antibodies (Fig. 6A and B). Cell cycle length of IPC was calculated as the rate of the total number of IPCs (Tbr2-positive cell) and the number of S-phase IPCs for 1 h (Tbr2/IdU double-positive cell). A smaller population of IdU-labeled cells among Tbr2-positive cells indicates a greater cell cycle length (Chenn and Walsh, 2002). In the BPA-treated fetuses, the cell cycle was significantly prolonged (8.4 ± 0.6%, $P < 0.01$) compared with that in the control fetuses (16.5 ± 4.3%) at E14.5 (Fig. 6C). These data indicated that the extension of the cell cycle length of IPCs induced the reduction of self-renewal, resulting in the decreasing number of IPCs.

4. Discussion

In previous studies, exposure to BPA (20 μg/kg/day) induced the accelerated neuronal differentiation/migration in ICR mice (Nakamura et al., 2006). Our data indicated that treatment with BPA at the dose of 20 μg/kg/day showed no differences in the morphogenesis of CP (Sup. Fig. 1A–C′) and neurogenesis of neural stem/precursor cells (Sup. Fig. 1D–I, Sup. Table 2) at E14.5 between the BPA-treated and control fetuses in C57BL/6J mice (see the materials and methods). We hypothesized that the difference of phenotype resulted from the different administration routes (intraperitoneally or orally) or mouse strains used, with variation in sensitivity to BPA. In a previous study involving the maternal BPA oral dosing, number of fetuses born per litter and embryonic body weight were significantly lower in the group exposed to BPA at 200 μg/kg/day than those in the control group. However, no difference was detected between the group exposed to BPA at 20 μg/kg/day and the control group (Cagen et al., 1999). Our data also showed that histological changes (cell cycle, proliferation, and differentiation changes) were related to BPA at 200 μg/kg/day. However, these were not found after oral exposure to BPA at 20 μg/kg/day (Sup. Fig. 1, Sup. Table 2).

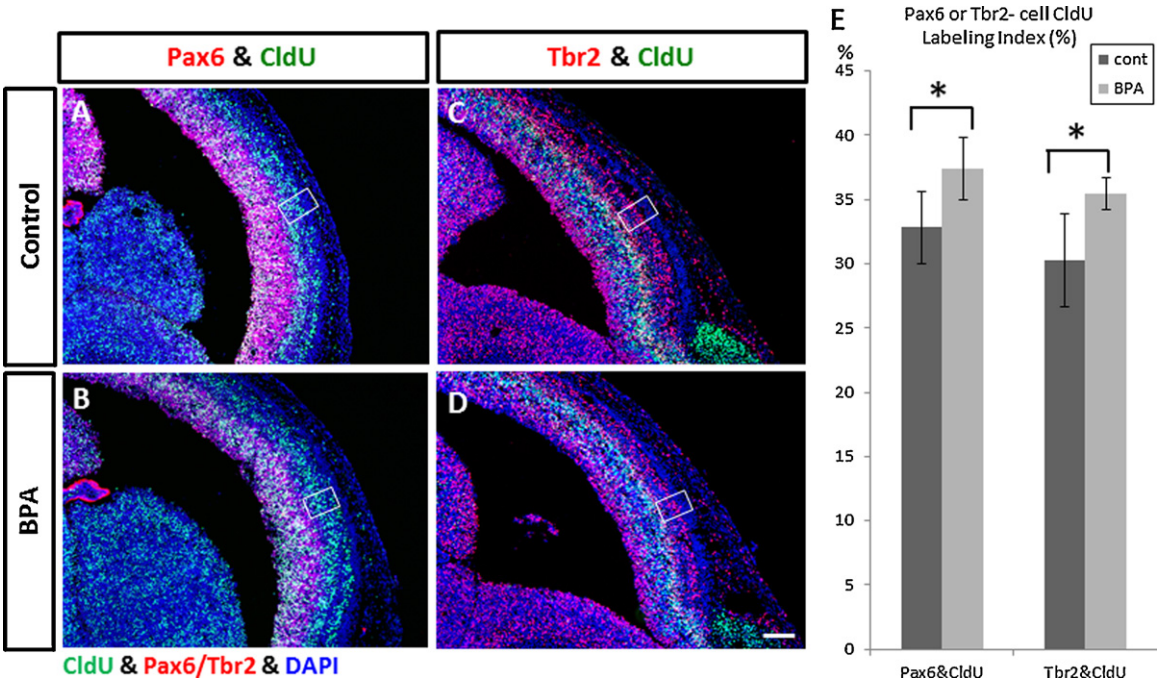

Fig. 5. Differentiation of radial glial cells and intermediate progenitor cells is promoted by maternal BPA exposure. To estimate the differentiation of RGCs and IPCs, sagittal sections were stained with anti-Pax6 or -Tbr2 and anti-CldU antibodies 24 h after CldU pulse labeling at E14.5 in the control (A, C) and the BPA-treated (B, D) fetuses. Differentiation was determined as the ratio of cells that differentiated from RGCs (green, CldU+/Pax6−, A, B) and IPCs (green, CldU+/Tbr2−, C, D) to all cells (blue: DAPI) after 24 h labeling. Cells were counted in 100-μm-wide sampling boxes (white box) in the SVZ (RGC, A, B) or upper SVZ (IPC, C, D). (E) In the BPA-treated fetuses, the ratio of differentiated cells from RGCs was significantly increased at E14.5 (control fetuses: 32.8 ± 2.8%, $n = 9$, BPA-treated fetuses: 37.4 ± 2.4%, $n = 9$, $^*P < 0.01$) and the ratio of differentiated cells from IPCs was also significantly increased at E14.5 (control fetuses: 30.3 ± 3.7%, $n = 9$, BPA-treated fetuses: 35.7 ± 1.2%, $n = 9$, $^*P < 0.01$). Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

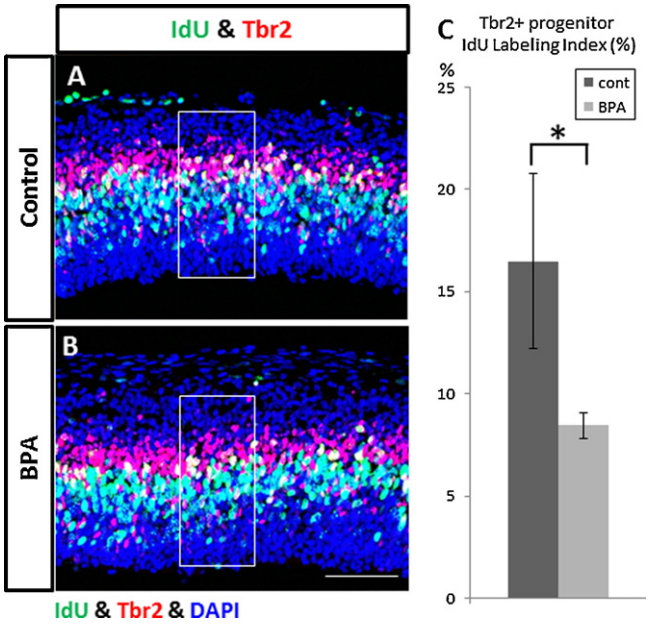

Fig. 6. The maternal exposure to BPA leads to prolonged cell cycle of intermediate progenitor cells. After 1 h pulse labeling of IdU, immunostaining of coronal sections was performed with anti-Tbr2 (red) and anti-IdU (green) antibodies at E14.5 (A, B). Cell cycle length was estimated as percentage of Tbr2 and IdU double-positive cells among all Tbr2-positive cells. Smaller percentage represents longer cell cycle. Cells were counted in 100-μm-wide sampling boxes (white box). (C) The BPA-treated fetuses showed significantly prolonged cycle length of IPC (8.4 ± 0.6%, $n = 9$, $^*P < 0.01$) compared with control fetuses (16.5 ± 4.3%, $n = 9$) at E14.5. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

BPA, an endocrine disruptor, induces the promotion of neurogenesis and regulation of proliferation in neural stem/precursor cells. BPA is known as an estrogenic chemical that interacts with human estrogen receptor (ER) (Krishnan et al., 1993), acts as an antagonist for human androgen receptor (Xu et al., 2005) and strongly binds to human estrogen-related receptor gamma (Takayanagi et al., 2006). ERs and AR are expressed in many areas of the developing brain in rodents (Matsuda et al., 2008; Zsarnovszky and Belcher, 2001). In the development of CNS, estrogens have neurotrophic and differentiation-promoting effects. ERα and ERβ are crucial for the functions during a critical period of brain development (Beyer, 1999). In analysis using ERβ knockout mice, ERβ was shown to play important roles in the neuronal migration, differentiation and survival in the developing brain (Wang et al., 2003). In addition, BPA increases ERα expression in the brain (Bromer et al., 2010). BPA may also enhance estrogenic activity through the up-regulation of ERs and up- or down-regulation of estrogenic target gene expression in the target tissues. Presently, we are examining whether the estrogen and/or ER are related to these phenotypes and attempting to identify the impaired expression of estrogenic target genes after BPA exposure using our BPA exposure model fetuses.

In addition, BPA binds to protein disulfide isomerase (PDI), the same binding site as thyroid hormone 3,3′,5-triodo-L-thyronine (T3) (Hiroi et al., 2006). Thyroid hormone is essential for proper brain development. In the neocortex, thyroid hormone deficiency during development results in abnormal cytoarchitecture and neuronal network formation (Cuevas et al., 2005). Thyroid hormone receptor alpha and beta were expressed in the cerebral cortex and hippocampus of rat brain (Di Liegro, 2008). The results of the present study suggest that BPA has possibilities of interaction with each factor (receptors and/or enzymes) during the development of neocortex and can induce excessive signaling of these factors.

Our data suggested that BPA exposure inhibited the proliferation and promoted the differentiation of RGC (Pax6-positive cells) and IPC (Tbr2-positive cells) in the SVZ/VZ of dorsal telencephalon. ERβ is detected in the deep layer of the dorsal telencephalon at E16.5 (Fan et al., 2006), indicating that RGC and/or IPC in the SVZ/VZ expressed the ERβ. In addition, the estrogen signaling might maintain the stem cell niche of RGC and IPC and regulate neurogenesis (Wang et al., 2003). We hypothesized that BPA acts as an agonist of ER and induces excessive estrogen signaling in the dorsal telencephalon, resulting in the promotion of neurogenesis.

A recent study based on cellular behavior, morphology and gene expression pattern identified three similar progenitors during the development of neocortex: outer radial glial cells, RGCs and IPCs. RGCs span the width from ventricular to pial surface, performing self-renewal from symmetric divisions and producing neurons from asymmetric divisions (Molnar et al., 2011). IPCs are derived from asymmetric divisions of RGCs. IPCs express the transcriptional factor Tbr2 and divide within the SVZ (Noctor et al., 2004). IPCs mainly produce projection neurons in layer II/III of the neocortex during the later neurogenesis and are very important for the evolutional process of the human brain, upgrading and becoming enormous in the neocortex. In the BPA-treated model, our data indicated that the abnormality of IPC resulted in neurogenesis defect and aberrant corticogenesis. Accordingly, it is reasonable to suggest that the detection of IPC defects is essential for the risk assessment of chemical exposure in the development of human brain.

BPA exposure induced the alteration of gene expression associated with neurogenesis. Basic helix-loop-helix (bHLH) genes have two types, repressor type and activator type. Repressor types, such as Hes1 and Hes5, maintain the neural stem cells and promote gliogenesis; activator types, Mash1, Mash2 and Ngn2, accelerate neurogenesis. Nakamura et al. reported that Mash1 and Ngn2 were significantly up-regulated in BPA-treated embryos at E14.5 (Nakamura et al., 2006). In addition, we also indicated that Mash1 was up-regulated at E14.5 in BPA-treated embryos (data not shown). These data suggested that BPA affected the expression of activator-type bHLH gene, Mash1 or Ngn2, causing accelerated neurogenesis.

5. Conclusion

In our studies, the maternal BPA oral dosing related to hyperplasia of CP during the development of telencephalon (Fig. 1) and shortened the radial fibers of RGCs in the SVZ/VZ (Fig. 2). The phenotypes were induced by the accelerated neurogenesis of neural stem/progenitor cells in the dorsal telencephalon (Fig. 2). In addition, BPA associated with the reduction of neural stem/progenitor cells in the SVZ/VZ as a result of the promotion of neurogenesis in the dorsal telencephalon (Fig. 3). In particular, BPA related to the maintenance and neurogenesis of IPCs in the SVZ of dorsal telencephalon (Figs. 4 and 5). These phenotypes (accelerated neurogenesis and the reduction of IPC number) were induced by the extension of the cell cycle length of IPCs in the SVZ (Fig. 6). In the near future, we will clarify whether these morphological defects in the embryonic brain persists, and result in the functional aberrations after birth. The maternal BPA dosing associated with the disruption of cell cycle in IPCs and related to the effects on neurogenesis in the development of neocortex of fetuses.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2012.02.013.

References

Baala, L., Briault, S., Etchevers, H.C., Laumonnier, F., Natiq, A., Amiel, J., Boddaert, N., Picard, C., Sbiti, A., Asermouh, A., Attie-Bitach, T., Encha-Razavi, F., Munnich, A., Sefiani, A., Lyonnet, S., 2007. Homozygous silencing of T-box transcription factor EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis. Nat. Genet. 39, 454–456.

Beyer, C., 1999. Estrogen and the developing mammalian brain. Anat. Embryol. (Berl.) 199, 379–390.

Bromer, J.G., Zhou, Y., Taylor, M.B., Doherty, L., Taylor, H.S., 2010. Bisphenol-A exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response. FASEB J. 24, 2273–2280.

Cagen, S.Z., Waechter Jr., J.M., Dimond, S.S., Breslin, W.J., Butala, J.H., Jekat, F.W., Joiner, R.L., Shiotsuka, R.N., Veenstra, G.E., Harris, L.R., 1999. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol. Sci. 50, 36–44.

Chenn, A., Walsh, C.A., 2002. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 297, 365–369.

Cuevas, E., Auso, E., Telefont, M., Morreale de Escobar, G., Sotelo, C., Berbel, P., 2005. Transient maternal hypothyroxinemia at onset of corticogenesis alters tangential migration of medial ganglionic eminence-derived neurons. Eur. J. Neurosci. 22, 541–551.

Di Liegro, I., 2008. Thyroid hormones and the central nervous system of mammals. Mol. Med. Report 1, 279–295 (Review).

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A., Bulfone, A., Kowalczyk, T., Hevner, R.F., 2005. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J. Neurosci. 25, 247–251.

Fan, X., Warner, M., Gustafsson, J.A., 2006. Estrogen receptor beta expression in the embryonic brain regulates development of calretinin-immunoreactive GABAergic interneurons. Proc. Natl. Acad. Sci. U. S. A. 103, 19338–19343.

Golub, M.S., Wu, K.L., Kaufman, F.L., Li, L.H., Moran-Messen, F., Zeise, L., Alexeeff, G.V., Donald, J.M., 2010. Bisphenol A: developmental toxicity from early prenatal exposure. Birth Defects Res. B Dev. Reprod. Toxicol. 89, 441–466.

Gotz, M., Huttner, W.B., 2005. The cell biology of neurogenesis. Nat. Rev. Mol. Cell Biol. 6, 777–788.

Haubensak, W., Attardo, A., Denk, W., Huttner, W.B., 2004. Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc. Natl. Acad. Sci. U. S. A. 101, 3196–3201.

Hiroi, T., Okada, K., Imaoka, S., Osada, M., Funae, Y., 2006. Bisphenol A binds to protein disulfide isomerase and inhibits its enzymatic and hormone-binding activities. Endocrinology 147, 2773–2780.

Howe, S.R., Borodinsky, L., 1998. Potential exposure to bisphenol A from food-contact use of polycarbonate resins. Food Addit. Contam. 15, 370–375.

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y., Taketani, Y., 2002. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum. Reprod. 17, 2839–2841.

Komada, M., Saitsu, H., Kinboshi, M., Miura, T., Shiota, K., Ishibashi, M., 2008. Hedgehog signaling is involved in development of the neocortex. Development 135, 2717–2727.

Kowalczyk, T., Pontious, A., Englund, C., Daza, R.A., Bedogni, F., Hodge, R., Attardo, A., Bell, C., Huttner, W.B., Hevner, R.F., 2009. Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. Cereb. Cortex 19, 2439–2450.

Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., Feldman, D., 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132, 2279–2286.

Kundakovic, M., Champagne, F.A., 2011. Epigenetic perspective on the developmental effects of bisphenol A. Brain Behav. Immun. 25, 1084–1093.

Matsuda, K., Sakamoto, H., Kawata, M., 2008. Androgen action in the brain and spinal cord for the regulation of male sexual behaviors. Curr. Opin. Pharmacol. 8, 747–751.

Molnar, Z., Vasistha, N.A., Garcia-Moreno, F., 2011. Hanging by the tail: progenitor populations proliferate. Nat. Neurosci. 14, 538–540.

Nagao, T., Wada, K., Kuwagata, M., Nakagomi, M., Watanabe, C., Yoshimura, S., Saito, Y., Usumi, K., Kanno, J., 2004. Intrauterine position and postnatal growth in Sprague-Dawley rats and ICR mice. Reprod. Toxicol. 18, 109–120.

Nakamura, K., Itoh, K., Sugimoto, T., Fushiki, S., 2007. Prenatal exposure to bisphenol A affects adult murine neocortical structure. Neurosci. Lett. 420, 100–105.

Nakamura, K., Itoh, K., Yaoi, T., Fujiwara, Y., Sugimoto, T., Fushiki, S., 2006. Murine neocortical histogenesis is perturbed by prenatal exposure to low doses of bisphenol A. J. Neurosci. Res. 84, 1197–1205.

Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., Kriegstein, A.R., 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat. Neurosci. 7, 136–144.

Pulgar, R., Olea-Serrano, M.F., Novillo-Fertrell, A., Rivas, A., Pazos, P., Pedraza, V., Navajas, J.M., Olea, N., 2000. Determination of bisphenol A and related aromatic compounds released from bis-GMA-based composites and sealants by high performance liquid chromatography. Environ. Health Perspect. 108, 21–27.

Sasaki, N., Okuda, K., Kato, T., Kakishima, H., Okuma, H., Abe, K., Tachino, H., Tuchida, K., Kubono, K., 2005. Salivary bisphenol-A levels detected by ELISA after restoration with composite resin. J. Mater. Sci. Mater. Med. 16, 297–300.

Schonfelder, G., Wittfoht, W., Hopp, H., Talsness, C.E., Paul, M., Chahoud, I., 2002. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ. Health Perspect. 110, A703–A707.

Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., Shimohigashi, Y., 2006. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. Toxicol. Lett. 167, 95–105.

Wang, L., Andersson, S., Warner, M., Gustafsson, J.A., 2003. Estrogen receptor (ER)beta knockout mice reveal a role for ERbeta in migration of cortical neurons in the developing brain. Proc. Natl. Acad. Sci. U. S. A. 100, 703–708.

Xu, L.C., Sun, H., Chen, J.F., Bian, Q., Qian, J., Song, L., Wang, X.R., 2005. Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. Toxicology 216, 197–203.

Ye, X., Kuklenyik, Z., Needham, L.L., Calafat, A.M., 2005. Quantification of urinary conjugates of bisphenol A, 2,5-dichlorophenol, and 2-hydroxy-4-methoxybenzophenone in humans by online solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 383, 638–644.

Yoshida, M., Shimomoto, T., Katashima, S., Watanabe, G., Taya, K., Maekawa, A., 2004. Maternal exposure to low doses of bisphenol a has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. J. Reprod. Dev. 50, 349–360.

Zsarnovszky, A., Belcher, S.M., 2001. Identification of a developmental gradient of estrogen receptor expression and cellular localization in the developing and adult female rat primary somatosensory cortex. Brain Res. Dev. Brain Res. 129, 39–46.