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Regular Article

Low Doses of Bisphenol A Disrupt Neuronal Differentiation of Human Neuronal Stem/Progenitor Cells

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Bisphenol A (BPA) is an endocrine disrupting chemical. Human epidemiological studies have suggested that adverse neurobehavioral outcomes are induced by fetal exposure to BPA. The remarkable differences in the corticogenesis between human and agyrencephalic mammals are an increase in the intermediate progenitor cells (IPCs) and a following increase in the subplate thickness. It is uncertain whether low doses of BPA (low-BPA) affect human early corticogenesis when basal progenitor cells (BPs) produce IPCs resulting in amplified neurogenesis. In this study, human-derived neuronal stem/progenitor cells were exposed to low-BPA or the vehicle only, and the resultant cell type-specific molecular changes and morphology were analyzed. We focused on stem cells immunoreactive for SOX2, BPs for NHLH1, and immature neurons for DCX. SOX2-positive cells significantly decreased at day *in vitro* (DIV) 4 and 7, whereas NHLH1-positive cells tended to be higher, while DCX-positive cells significantly increased at DIV7 when exposed to 100 nM of BPA compared with the vehicle. Morphologically DCX-positive cells showed a decrease in unipolar cells and an increase in multipolar cells when exposed to 100 nM of BPA compared with the vehicle. These results provide insights into the *in vivo* effect of low-BPA on neuronal differentiation in the human fetal corticogenesis.

Key words: endocrine disrupting chemicals, Bisphenol A (BPA), neural stem/progenitor cells, corticogenesis, differentiation

I. Introduction

Bisphenol A (BPA; 2,2-bis (4-hydroxy-phenyl) propane) is a well-known endocrine disrupting chemical [6] widely used in various industries and the field of dentistry [15]. In spite of the international regulations such as the Tolerance Daily Intake (TDI) of BPA [1], BPA has been detected in the maternal urine, blood, amniotic fluid, or children's urine, which suggests human fetuses, infants, and children are still exposed to BPA regardless of the amount [27, 30]. On the other hand, children, especially in boys, who are diagnosed with autism spectrum disorder or attention deficit/hyperactivity disorder (ADHD) have been increasing recently [1]. Some reviews pointed out that fetal exposure to BPA could be the cause of this phenomenon [22]. Human epidemiological prospective cohort studies have suggested the relationship between maternal BPA-exposure and adverse neurobehavioral outcomes [5]. This study suggests that prenatal BPA exposures, especially those that occur during early pregnancy, are associated with the later development of behavioral problems in children [31]. However, as it is difficult to study human fetuses experimentally, the etiology or the pathology is unknown [12]. Recently, based on its study the European Food Safety Authority (EFSA) proposed reducing by a factor of 100,000 the tolerable daily intake of BPA [28]. In their remarkable studies, a variety of tissues were analyzed for

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the effect of BPA in rats. There were no significant findings reported for changes in brain weights, clinical observations related to behavior, or increases in histopathological findings in any BPA dose group [10]. In this regard, there seems to be a difference between human epidemiological and animal studies.

Since studies in humans are ethically and practically challenging, species-specific *in vitro* models could provide a complementary approach to animal studies for understanding the evolution and complexity of the human brain [8]. *In vitro* models have seen significant progress in recent years, from the reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) [29] to organogenesis in a dish, including the generation of neural organoids and cortical spheroids [18]. For example, forebrain organoids derived from human iPSCs were developed for modeling Zika virus exposure [25] and human midbrain organoids applied for a fully automated high-throughput screens for the assessments of drug effects [26]. Regarding the assessment of BPA developmental neurotoxicity, the European Commission Joint Research Centre has recently evaluated the developmental neurotoxicity induced by chemical mixtures using human iPSCs derived neuronal and glial cultures [24] and introduced a concept regrading adverse outcome pathways to identify chemical mixtures which could potentially cause neuronal dysfunctions. Their study was mainly focused on mature neurons as the treatment with chemicals was conducted after DIV7 [24]. It is uncertain if low doses BPA affect the earlier stage of human neuronal differentiation, which could lead to ASD/ADHD, accompanied with underlying molecular mechanisms.

In this study, human neural stem/progenitor cells (NSPCs) derived from the fetal dorsal forebrains were exposed to low dose BPA or the vehicle in the differentiation medium. The aim was to evaluate whether low dose BPA affects human early corticogenesis with special references to the neural cell types and the timing during development. The stemness marker (SOX2)-positive cells significantly decreased, whereas the basal progenitor marker (NHLH1)-positive cells and DCX-positive cells tended to be higher when exposed to 100 nM of BPA for 7 days, suggesting that low doses of BPA might disrupt the neuronal differentiation from neuronal stem/progenitor cells to immature neurons in human cerebral development process.

II. Materials and Methods

2-1. Chemicals

BPA (2,2-bis (4-hydroxy-phenyl) propane (CH3)2C(C6H4OH)2) (FUJIFILM Wako Chemical co., Osaka, Japan) was dissolved in ethanol (99.5%vol.) (CH3Ch2OH) (Merck KGaA, Darmstadt, Germany) and wad diluted into 0.1, 1, 10, and 100 nM each as the final concentrations. The ethanol concentration used was 3.41 nM.

2-2. Cell culture and exposure to BPA

Human NSPCs were generated from fetal dorsal forebrains aged 7–10 gestational weeks (a gift from Kanemura *et al.* [16]). NSPCs were cultured in a growth medium composed of DMEM/Ham's F-12 (Nacalai Tesque INC., Kyoto, Japan), Heparin sodium salt (Merck KGaA, Darmstadt, Germany), Antibiotic-Antimycotic (Thermo Fisher Scientific Inc., MA, USA), MACS NeuroBrew-21 (B21) (Miltenyi Biotec, Bergisch Gladbach, Germany), 20 ng/mL rhEGF (Pepro Tech, NJ, USA), 20 ng/mL rhbFGF (Pepro Tech, NJ, USA) and rhLIF (Oriental Yeast co., Tokyo, Japan) in T-75 flasks and formed spheres. The spheres were separated into single cells with Accutase (Nacalai Tesque INC., Kyoto, Japan) and single NSPCs were seeded onto Poly-L-lysine hydrobromide (mol wt 70,000–150,000) (Merck KGaA, Darmstadt, Germany) coated coverglass with a density of 2.1×10^4 cells/cm^2. When the seeding was conducted, the medium was changed into the differentiation medium, composed of Neurobasal medium (Gibco, Thermo Fisher Scientific Inc., MA, USA), L-glutamine, B21, and Antibiotic-Antimycotic. In 3 hours, BPA or ethanol, as the vehicle control, were added at the final concentration as described above (2-1. Chemicals).

In order to analyze dose dependent effects of BPA on NSPCs proliferation and differentiation, we cultured NSPCs with BPA at concentration of 0.1, 1, 10 and 100 nM or the vehicle control for 7 days and fixed cells for immunohistochemistry (Figs. 1, 2, Supplementary result 1). For evaluation of phenotypes of the immature neurons or more mature neurons we cultured NSPCs with BPA at concentration of 100 nM or the vehicle control for 7 and 12 days and fixed cells for immunohistochemistry (Figs. 3, 4). In order to evaluate temporal expression of cell marker proteins, we cultured NSPCs with BPA at concentration of 100 nM or the vehicle control for 2, 4 and 7 days and fixed cells for immunohistochemistry (Figs. 5–9).

2-3. Immunocytochemistry

Cell nuclei were visualized with Hoechst (Thermo Fisher Scientific Inc., MA, USA) before the NSPCs were fixed by 4% PFA at each time point (DIV, 2, 4, 7 or 12 depending on the experiments). Cells were washed with Dulbecco's Phosphate-Buffered Saline (D-PBS) at room temperature in all of the washing procedures. Blocking procedure was conducted at room temperature for 1 hr. The primary antibody reaction was conducted at 4°C overnight; DCX (ab18723, abcam, rabbit polyclonal, Cambridge, UK, 1:300), SOX2 (AB5603, rabbit polyclonal, Merck millipore, MA, USA, 1:300), Tuj1 (MAB1195, mouse monoclonal IgG2A, R&D systems, MN, USA, 1:300), Nestin (sc-23927, mouse monoclonal IgG1, Santa Cruz biotechnology, TX, USA, 1:200), GFAP (ab53554, abcam, goat polyclonal, Cambridge, UK, 1:500), NHLH1 (HPA01794, rabbit polyclonal, Atlas Antibodies, Bromma, Sweden, 1:200), and TH (MAB5280, mouse monoclonal, clone 2/40/15, Merck millipore, MA, USA, 1:300). The sec-

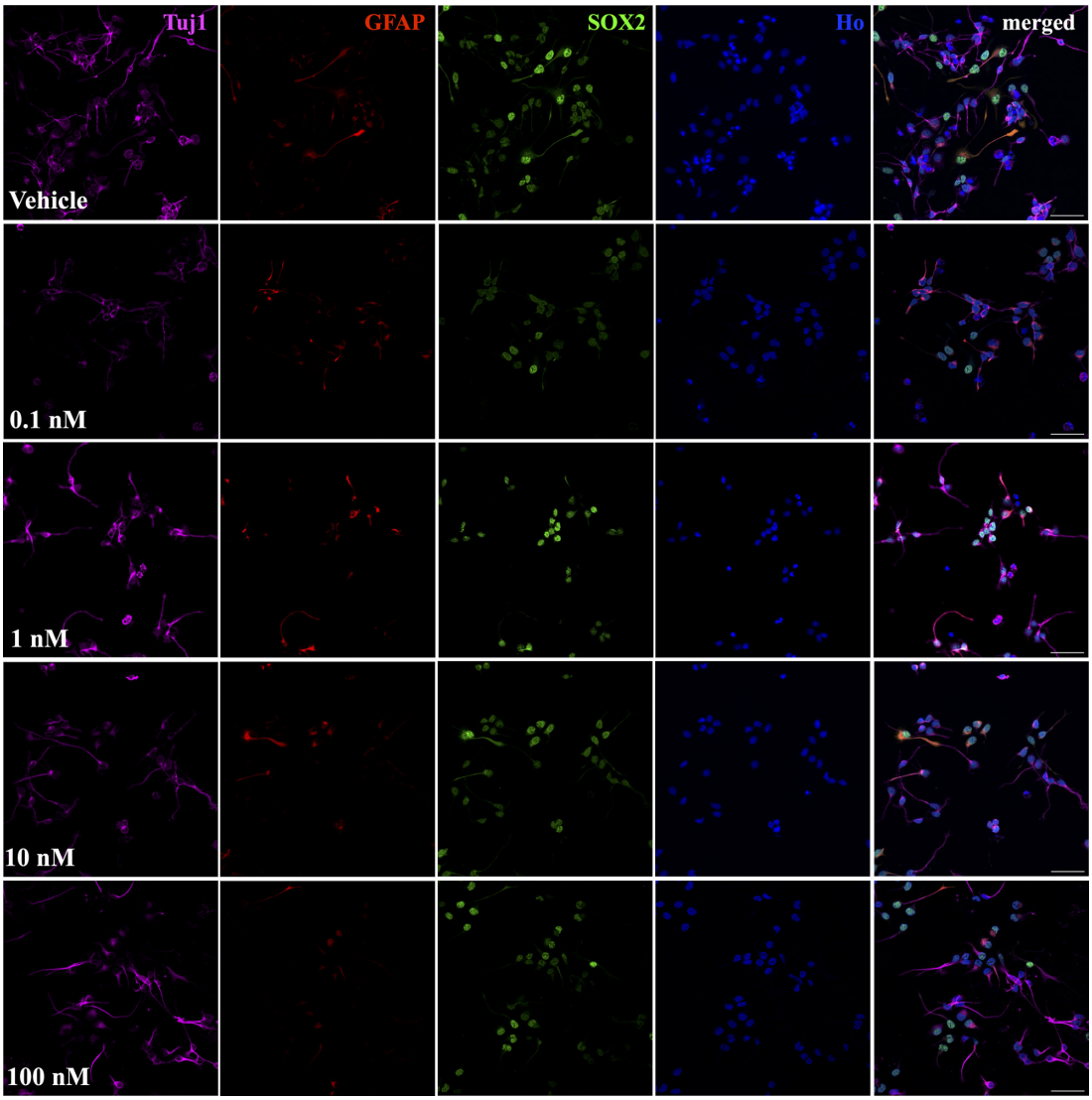

Fig. 1. Effects of BPA on NSPCs differentiation. The immunofluorescence analysis of the immature neuron marker Tuj1 (magenta), the radial glia marker GFAP (red), the stemness marker SOX2 (green), nuclei (Hoechst 33342, blue) and merged images in the right column. Top; vehicle control, the 2nd row; 0.1 nM, the 3rd row; 1 nM, the 4th row; 10 nM, the bottom; 100 nM of BPA. Bar = 50 μm (Original magnification, ×200). Abbreviation: GFAP; glial fibrillary acidic protein, Ho; Hoechst 33342.

ondary antibody reactions were conducted in the dark at room temperature for 1 hour; Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody (Alexa Fluor 555™, 1:1000), Goat anti-Rabbit IgG(H+L) Cross-Adsorbed Secondary Antibody (Alexa Fluor™ 488, 1:1000), Goat anti-Rabbit IgG(H+L) Highly Cross-Adsorbed Secondary Antibody (Alexa Fluor™ 568, 1:500), Goat anti-Mouse Cross-Adsorbed Secondary Antibody, (Alexa Fluor™ 568, 1:1000), Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Alexa Fluor™ 647, 1:1000), and Goat anti-Rabbit IgG(H+L) Highly Cross-Adsorbed Secondary Antibody (Alexa Fluor™ 647, 1:1000). All of the 2nd antibodies were purchased from Life Technologies Japan (Thermo Fisher Scientific Inc., MA, USA).

Cells on the coverslips were mounted in SlowFade™ Gold Antifade Mountant (cat.36936: without DAPI) (Thermo Fisher Scientific Inc., MA, USA) onto slide glass. Control cells were incubated in blocking buffer without any primary antibodies as a negative control. Imaging and analysis were conducted using IN Cell Analyzer 2200 (Global Life Sciences Technologies Japan, Tokyo, Japan).

2-4. Cell count methods

In all of the experiments, a cell culture was prepared with duplicates regarding each condition and one experiment was conducted independently three times. The quality of cell culture after seeding was checked with the

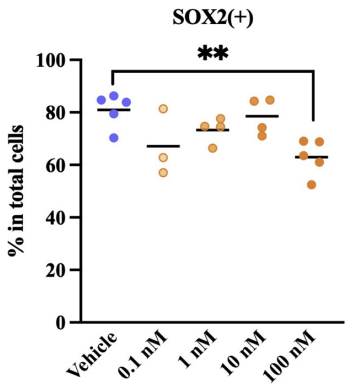

Fig. 2. The quantification of the immunofluorescence of stem cells marker SOX2. The results show percentage of SOX2-positive cells by total cells (i.e. the number of Hoechst 33342-positive cells, approximately 14,000 cells each). Each bar indicates the mean. ** $P < 0.005$, three independent experiments.

immunofluorescence of immature neuron marker DCX and glial marker GFAP at DIV7.

IN Cell Analyzer 2200 was used for automated image acquisition and automated cell count. Imaging data was acquired from 137 fileds (137 snaps) which covered about 80% of whole area of a coverslip. The acquired image data (137 snaps each) was analyzed using IN Cell Analyzer 2200 software which calculated the number of total cells (Hoechst positive cells, overall 14,000 cells from each coverslip) and the number of immunoreactive cells using appropriate protocol for the detection of signal. Each protocol was optimized using biological negative controls. Every result (e.g. the number of SOX2-positive cells was divided by the Hoechst-positive cells) was plotted onto the graph as points or represented by a bar graph. The results as percentage of interest protein-positive cells were applied for the statistical analysis.

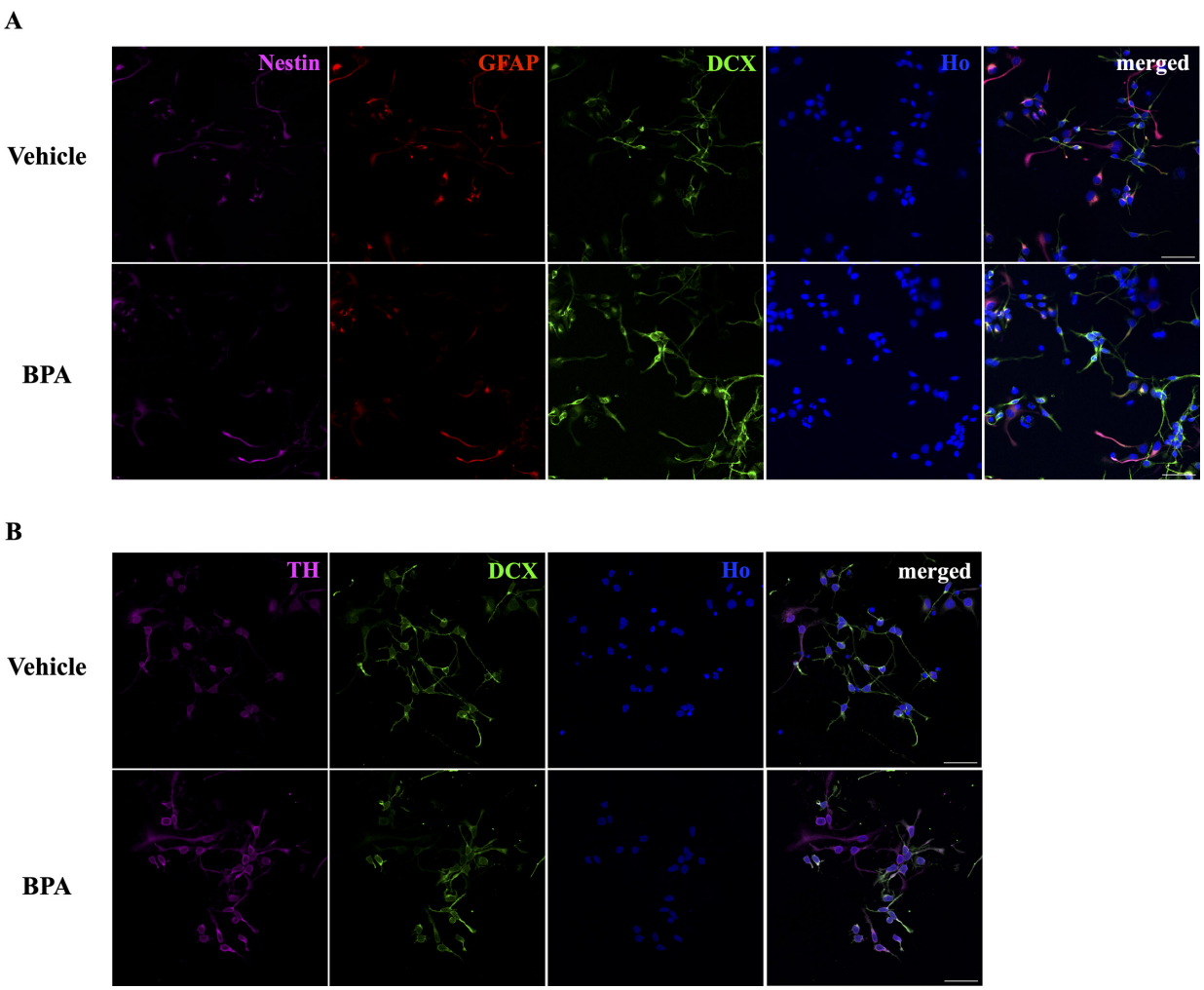

Fig. 3. The effects of low doses of BPA on NSPCs-neuronal differentiation. (**A**) The immunofluorescence analysis of the radial glia marker Nestin (magenta), the GFAP (red), the immature neuron marker DCX (green), nuclei (Hoechst 33342, blue) and merged data in the right column. Upper; vehicle control, lower; 100 nM of BPA. (**B**) The immunofluorescence analysis of the mature neuronal marker TH (magenta), the immature marker DCX (green), nuclei visualised by Hoechst 33342 (blue) and merged images in the right column. Upper; vehicle control, lower; 100 nM of BPA. Bar = 50 μm (Original magnification, ×200). Abbreviation: DCX; doublecortin, TH; tyrosine hydroxylase, Ho; Hoechst 33342.

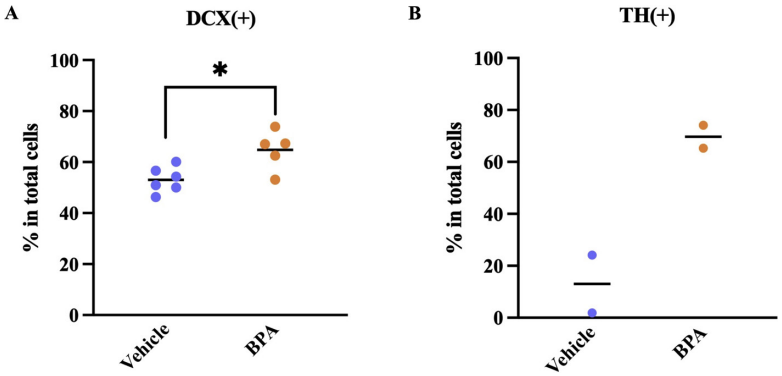

Fig. 4. The quantification of the immunofluorescence of neuronal marker protein. (**A**) The results show percentage of DCX-positive cells by total cells (i.e. the number of Hoechst 33342-positive cells, approximately 14,000 cells each). (**B**) The results show percentage of TH-positive cells by total cells (i.e. the number of Hoechst 33342-positive cells, approximately 8,000 cells each). Each bar indicates the mean. **P* < 0.05, three independent experiments.

2-5. Morphology analysis

Raw image data was acquired using IN Cell Analyzer 2200 with a ×20 objective at DIV 7. Data-sets from each group were prepared for the morphology analysis. The data was manually chosen from the raw image data with the following two factors, first, there was the Hoechst 33342-positive cell number/ROI (i.e. 70–90 cells), the other factor considered was the percentage of the DCX-positive cells in the ROI (i.e. 50–60%). The former was for the purpose of avoiding misdetection due to cell aggregation, the latter was in order to select the area showing neuronal differentiation in each sample. The longest neurite length, the total neurite length, and the neurite number per cell were analyzed using Fiji/ImageJ software [4].

2-6. Imaging

In order to confirm the localization of the SOX2, NHLH1, and DCX, images were acquired using a confocal microscope (ZEISS LSM 900, Carl Zeiss Co. Ltd, Tokyo Japan).

2-7. Statistical analysis

Statistical analyses were performed using Graphpad Prism 9 (MDF Co., Ltd., Tokyo, Japan). Bonferroni's multiple comparison, Unpaired and Welch's-test, or the Mann-Whitney U test were also employed. A p-value <0.05 was considered to be statistically significant.

III. Results

3-1. Dose dependent effects of BPA on NSPCs differentiation

The low doses of BPA to be studied were determined by preliminary cell cultures and proliferation analysis (Supplementary result 1). In order to analyse whether low doses of BPA affect neuronal differentiation in a dose dependent manner, NSPCs were exposed to low doses of BPA (0.1, 1, 10, and 100 nM) or the control vehicle for 7 days. The SOX2-positive cells (% in Hoechst33342-positive cells) significantly decreased on exposure to 100 nM BPA, compared to the vehicle control (P = 0.0016, Bonferroni's multiple comparison) (Figs. 1, 2). There were no significant differences in the other BPA-exposure groups. (P > 0.05, Bonferroni's multiple comparison) (Fig. 2). 100 nM of BPA was therefore selected as the effective concentration of BPA to affect neuronal differentiation and used in subsequent experiments. In this first experiment, 100 nM of BPA led to a significant decrease in SOX2-positive cells (%), implying that BPA induced abnormal neuronal differentiation.

3-2. The effects of low doses of BPA on NSPCs neuronal differentiation

Since the decrease in the percentage of SOX2-positive cells found in Figs. 1 and 2, implied that neuronal differentiation was disrupted, NSPCs were cultured for 7 or 12 days in order to analyze the phenotypes of the immature neurons or more mature neurons, respectively. In order to study the effect on the early stage of neuronal differentiation, the immature neuronal marker, doublecortin (DCX)-positive cell population were analyzed. A significant increase in DCX-positive cells (% in Hoechst 33342-positive cells) was observed in 100 nM concentration of BPA compared to that in the vehicle control (P = 0.0232, Welch's t test) (Figs. 3A, 4A). The tyrosine hydroxylase (TH)-positive population was analyzed to detect the dopaminergic neurons, based upon the fact that TH is known to be the earliest mature neuronal marker in the culture of mouse embryonic stem cells [32]. TH-positive cells showed an increase (%) in 100 nM of BPA compared to that in the vehicle control, but the difference did not reach significance (P = 0.0889, Welch's t test, n = 2, one experiment) (Figs. 3B, 4B). These results suggest that 100 nM of BPA might disrupt the normal neuronal differentiation of NSPCs.

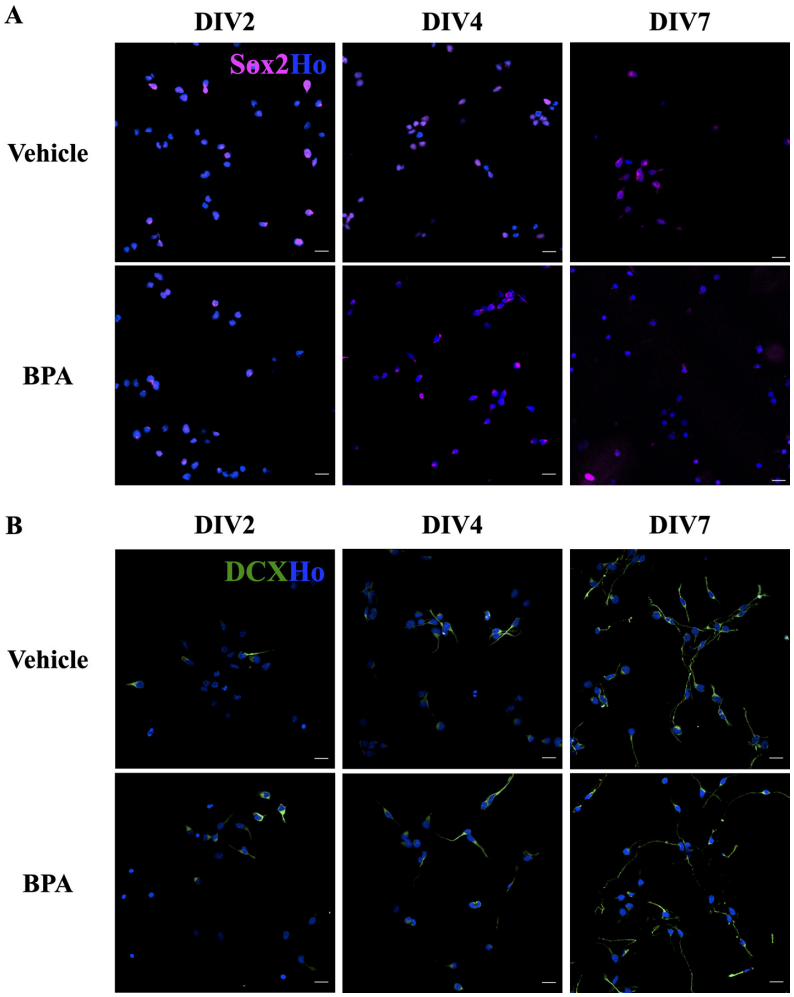

Fig. 5. Temporal expression of the stem cells marker SOX2 and the immature neuron marker DCX. (**A**) The immunofluorescence analysis of the stemness marker Sox2 (magenta) and nuclei (Hoechst 33342, blue). (**B**) The immunofluorescence analysis of immature neuron marker DCX (green) and nuclei (Hoechst 33342, blue). Upper; vehicle control, lower; 100 nM BPA. DIV 2, DIV 4 and DIV 7 are shown. Bar = 20 µm (Original magnification, ×200). Abbreviation: DCX; doublecortin, Ho; Hoechst 33342.

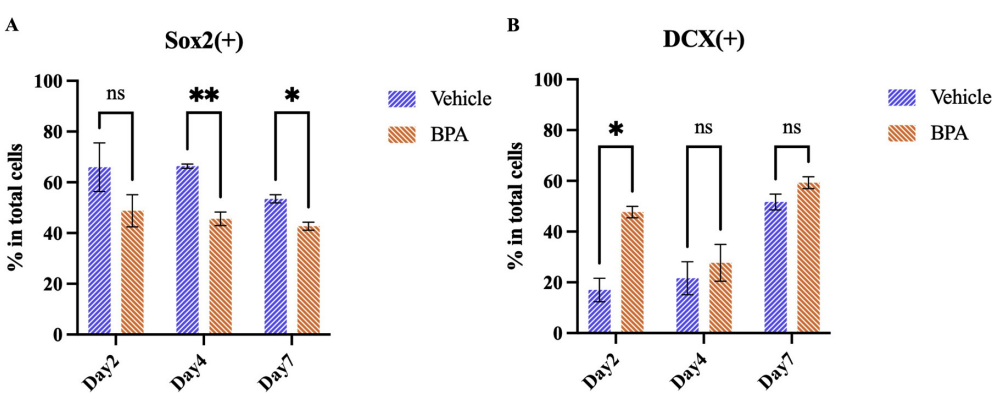

Fig. 6. The quantification of the immunofluorescence of the temporal expression of Sox2 and DCX. (**A**) The results show percentage of SOX2-positive cells by total cells (i.e. the number of Hoechst 33342-positive cells, approximately 8,000 cells at DIV2 and 14,000 cells at DIV4 and 7) (mean ± SEM). (**B**) The results show percentage of DCX-positive cells by total cells (i.e. the number of Hoechst 33342-positive cells, approximately 8,000 cells at DIV2 and 14,000 cells at DIV4 and 7). (mean ± SEM). $^{**}P < 0.005$, $^{*}P < 0.05$, three independent experiments.

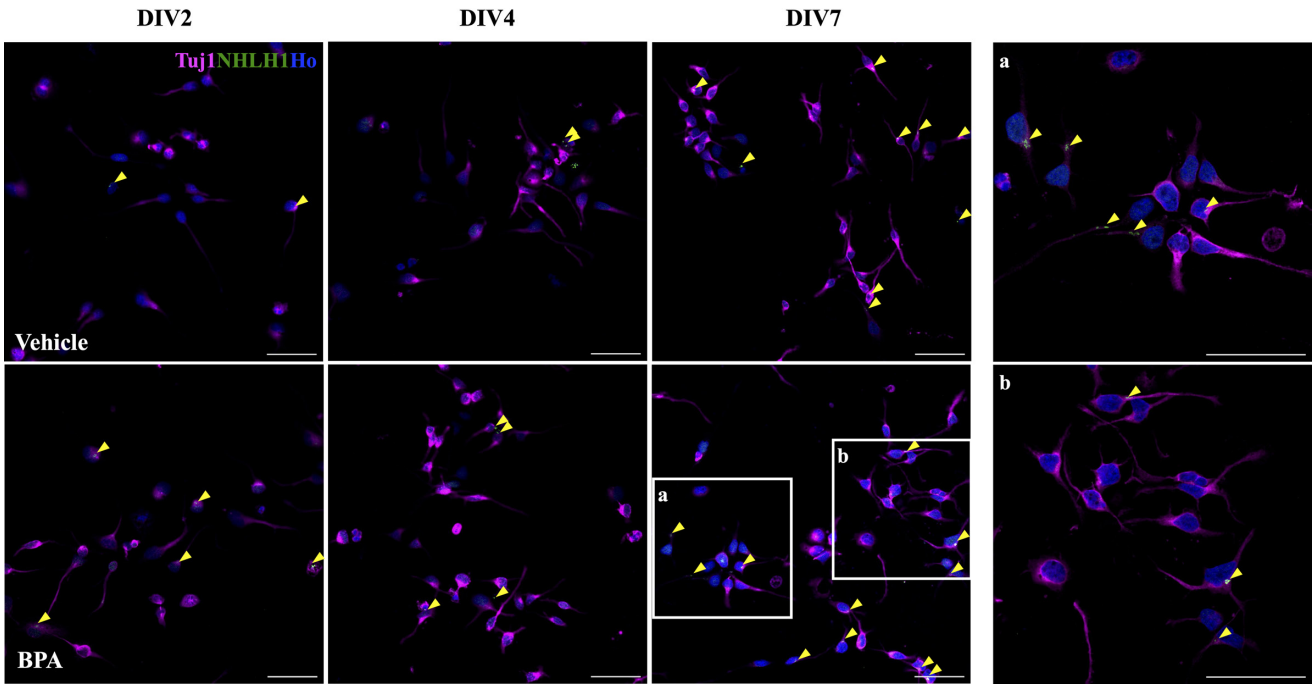

Fig. 7. Temporal expression of the basal progenitor marker-NHLH1. The immunofluorescence analysis of the immature neuronal marker Tuj1 (magenta), the basal progenitor cell marker NHLH1 (green, indicated by a yellow arrowhead) and nuclei (Hoechst 33342, blue). Upper; vehicle control, lower; 100 nM BPA. DIV 2, DIV 4, and DIV 7 are shown. Bar = 50 μm (Original magnification, ×200). (**a, b**) Cropped and magnified images of boxed areas. Bar = 50 μm (Original magnification, ×400).

3-3. Temporal expression of cell marker proteins

Since 7 days exposure to 100 nM of BPA led to an increase in immature neurons, the time course of these expressions were analyzed at DIV (day *in vitro*), DIV 2, DIV 4, and DIV 7. The SOX2-positive population (% in Hoechst33342-positive cells) significantly decreased at DIV 4 and DIV 7 in the 100 nM of BPA condition, compared to the control ($P > 0.05$, $P = 0.0028$, $P = 0.024$ at Day 2, 4, 7 respectively, multiple unpaired Welch t-test) (Figs. 5A, 6A). The immature neuronal marker, DCX-positive population (%) was significantly increased in the 100 nM of BPA, compared to the control at Day 2 ($P = 0.012$, $P > 0.05$, $P > 0.05$ at Day 2, 4, and 7, respectively, multiple unpaired Welch t-test) (Figs. 5B, 6B). The Basal progenitor cells marker, NHLH1 is expressed highly in the proliferative BPs [7, 34]. NHLH1-positive cells tend to increase after treatment with 100 nM BPA but there was no significance at all the timepoints ($P > 0.05$, $P > 0.05$, $P > 0.05$ at Day 2, 4, and 7, respectively, multiple unpaired Welch t-test) (Figs. 7, 8). NHLH1 is also known to be expressed in the Golgi apparatus. Consistent with this knowledge, in this study too, it was localized in the Golgi apparatus and in neurites (Fig. 7). These results indicated that 100 nM BPA led to a decrease in stem cells and showed a tendency for an increase in basal progenitor cells.

3-4. Morphological analysis

In Figs. 1–8, the effects of low doses of BPA were analysed on cell-type population during neuronal differentiation, using marker proteins. Results indicate that exposure to 100 nM BPA led to a decrease in stem cells and showed a tendency for an increase in basal progenitor cells. It is well documented that BPA has effects on morphological features during neural development, especially on neuronal outgrowth. These include decreased number of branch points for 14 days and an increased number of neurites for 3 days (12.74 μM on human iPSCs-derived NSCs and astroglia coculture, by β-III-tubulin [24]), decreased neurite length (1 nM on NSCs derived from human embryonic stem cells H9 for 16 days, was observed by β-III-tubulin [19]). We analysed cell types and the neurite length in

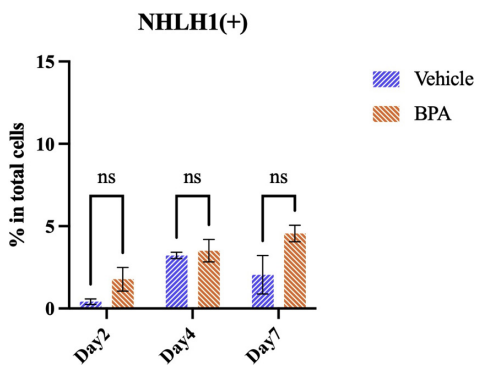

Fig. 8. The quantification of the immunofluorescence of NHLH1. The results show percentage of NHLH1-positive cells by total cells (i.e. the number of Hoechst 33342-positive cells, approximately 8,000 cells at DIV2 and 14,000 cells at DIV4 and 7) (mean ± SEM). Three independent experiments.

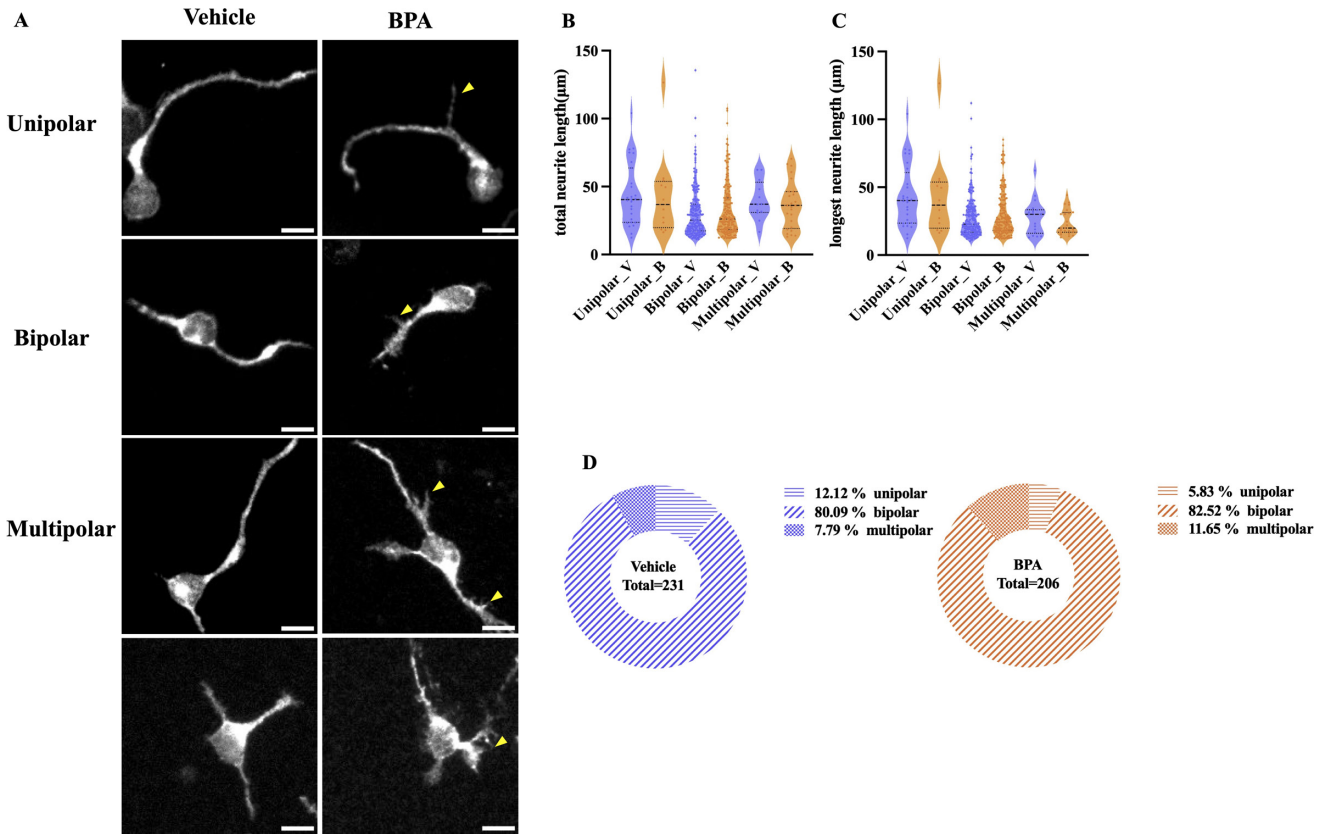

Fig. 9. Morphological analysis. (**A**) Extracted image examples after the analysis. Left; vehicle, right; BPA. Upper; unipolar, middle; bipolar and lower; multipolar. Bar = 10 μm. Note that DCX-positive cells in BPA had a neurite with short branches (yellow arrowhead). (**B, C**) Quantification of neurite morphology. (**B**) Total neurite length according to the neurite number (unipolar, bipolar or multipolar) and (**C**) longest neurite length according to the neurite number (unipolar, bipolar or multipolar). (**D**) The distribution of unipolar cells, bipolar cells, and multipolar cells (100 nM BPA vs vehicle control, $P = 0.0045$, Chi-square test, three independent experiments).

DCX-positive cells at DIV 7 to clarify whether BPA affects these morphological features. Representative analyzed images and their classifications (unipolar, bipolar, or multipolar) are shown in Fig. 9A. Neurites in the 100 nM BPA group frequently had branches and they were shorter than the neurites in the control (Fig. 9A, yellow arrowhead). For the quantification, the longest neurite length, the total neurite length, and the neurite number per cell were analyzed. Unipolar DCX-positive cells (% in DCX-positive cells) decreased and multipolar DCX-positive cells (%) increased when treated with 100 nM BPA compared with that in the vehicle control (100 nM BPA vs vehicle control, $P = 0.0045$, Chi-square test, three independent experiments) (Fig. 9D). There were no differences in the total neurite length ($P > 0.05$, Mann-Whitney test) (Fig. 9B) and in the longest neurite length ($P > 0.05$, Mann-Whitney test) (Fig. 9C) at DIV 7. These results suggest that exposure to 100 nM BPA for 7 days induced multipolar DCX-positive cells.

IV. Discussion

This study describes the effect of low doses of BPA on human early corticogenesis using human Neuronal Stem/Progenitor Cells (NSPCs) *in vitro*. The concentration of BPA was determined carefully so that our experimental methods could mimic human fetal exposure to BPA. Specifically, our results indicate that low doses of BPA could induce the decrease in stem cells, increase in basal progenitor cells and an increase in neurons with morphological phenotype. The following aspects will be discussed. (I) SOX2 related pathway and morphology, and (II) low doses of BPA on human corticogenesis.

(I) The SOX2 related pathway and morphology

SOX2 plays an important role in the embryonic development [33]. As a transcription factor, SOX2 recognizes and binds to the promoter of various target genes and thus regulates various physiological processes [33]. SOX2 cooperates with Wnt/β-catenin signaling pathway and may be expressed with the regulation of Wnt signaling activity. For example, the activation of Wnt/β-catenin signaling suppresses SOX2 expression and maintains osteosarcoma cells in a differentiated osteoblast-like state [3]. On the other hand, the Wnt signaling stimulates dendrites morphogenesis [9]. In this study, exposure to 100 nM BPA led to a significant decrease in SOX2-positive cells and to an increase

in multipolar DCX-positive cells. Unipolar cells in the 100 nM concentration frequently had a branch, bipolar cells in the 100 nM concentration had shorter neurites, and the neurites of multipolar cells in the 100 nM concentration often had small branches (Fig. 9A, yellow arrowhead). Thus, Wnt signaling might be involved in the mechanisms of decreased SOX2-positive cells and the increased multipolar-cells.

(II) low doses of BPA on human corticogenesis

Basal progenitor cells (BPs) give rise to neurons by way of intermediate progenitor cells, unlike apical progenitor cells, which produce neurons directly [2, 23]. The increase of BPs in number and subtypes are important in human corticogenesis [13, 17]. In this study, human NSPCs were exposed to low doses of BPA. Regardless of the level of significance, there were always subtle differences in neuronal differentiation for 7 days, regarding the number of stem cells and BP cells. Although we could not determine the situation in regard to the IPCs, these increased proliferative BPs might hypothetically give rise to an increased number of neurons at a later or final stage. Furthermore, TH-positive cells, detected as the earliest mature neurons, developed to a greater extent when exposed to 100 nM BPA for 12 days (Figs. 3B, 4B). According to previous studies, an increase in neuronal cell numbers was identified in the prefrontal cortex of autistic children (about 67%) compared with healthy control children [11] and neurons derived from autistic children presented impaired neurite morphology, with shorter and fewer-branched neurites [20, 21]. Above all, it could be assumed that those subtle changes at DIV 7, induced by exposure to the 100 nM BPA led to an abnormal increase of mature neurons at DIV 12, which might reflect one aspect of aberrant corticogenesis, such as in ASD or ADHD.

The EPA plan to end animal testing by 2023 [14], or some fundamental changes to experimental methods are desirable, such as a tissue or an iPSCs bank to continue to study these phenomena. Some reports suggested that human organoids derived from iPSCs better reflect the situation than animal tests, especially regarding neuronal behavior tests. In this regard, our current study provides the first findings about abnormal human early corticogeneis induced by low doses of BPA, specifically, the decrease in stem cells and the increase in immature abnormal neurons, using human NSPCs as an *in vitro* model.

V. Ethical statement

All of the procedures performed in the present study involving human cells were conducted in accordance with the ethical standards of the institutional and national research committee (ERB-G-22-4, Kyoto Prefectural University of Medicine).

VI. Conflicts of Interest

The authors declare no conflicts of interest.

VII. Consent for Publication

Not applicable.

VIII. Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

IX. Author Contributions

K.F. conceived the present study and performed the experiments. T.Y. and T.F. analyzed the data. K.F. and K.I. contributed to the conception of the present study and wrote the draft.

X. Acknowledgments

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