Effects of estrogens on proliferation and differentiation of neural stem/progenitor cells

Makiko Okada¹, Koichi Murase¹, Akihisa Makino¹, Mitsunari Nakajima², Teppei Kaku², Shoei Furukawa¹ and Yoshiko Furukawa¹,²
¹ Laboratory of Molecular Biology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585 and ² Department of Pharmaceutical Pharmacology, College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan

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ABSTRACT

We investigated the effect of the female hormone 17β-estradiol (E2) and the hormone mimic bisphenol A (BPA) on the proliferation and differentiation of rat neural stem/progenitors cells (NS/PCs) cultured from the telencephalon of embryonic day-15 rats. Basic fibroblast growth factor (FGF-2) is a potent mitogen of early generated NS/PCs, and is used for the proliferation of NS/PCs in vitro. Administration of E2 or BPA alone to the NS/PCs stimulated their proliferation in the absence but not in the presence of FGF-2. E2- or BPA-treatment increased the ratio of the oligodendrocytes generated from the NS/PCs to total cells; however, this ratio did not change when the cells were stimulated with platelet-derived growth factor (PDGF), a mitogen for oligodendrocyte precursors, or with neurotrophin-3, an oligogenic factor for glial progenitor cells. These results suggest that estrogens would influence the fate of NS/PCs when the cells are poorly supplied with mitogens or differentiation factors during the early stages of neurogenesis.

Numerous lines of recent evidence have indicated that female estrogenic hormones such as 17β-estradiol (E2), affect the function of developing and adult brains by regulating cognitive function, pain, and susceptibility to seizure (12). Most of these studies have examined neurons in vivo as well as in vitro (16,18). However, neural stem cells (NSCs) are likely to be the best cells in order to examine the effect of estrogens on the early developmental stage of central nervous system (CNS), because these cells are the most progenitor cells in the CNS, and are capable of self-renewal (proliferation by symmetric division) and are multipotent (differentiating into progenitors of neurons and glial cells) (11). Furthermore, their differentiation has been shown to be tightly regulated by environmental signals including secreted factors as well as cell-autonomous signaling (4, 14) during CNS development, survival and proliferation (21). Brännvall et al. (2) showed that E2-treatment of NSCs cultured from rat striatal tissue of embryonic day (E) 20, resulted in increased proliferation and neural differentiation of these cells. NSCs are known to propagate in vitro as neurospheres, which are round free-floating aggregates, in the presence of a mitogen such as basic fibroblast growth factor (FGF-2) or epidermal growth factor (EGF) (19). Early NSCs are responsive only to FGF-2, whereas late ones are responsive to both FGF-2 and EGF; and thus responsiveness depends on the gestational age at which they were isolated (7). As the cells from E 20 rats used in the previous study of Brännvall et al. (2) responded to EGF, in the present study we investigated whether NSCs cultured from rats of a younger gestational age, i.e., E 15, which would be responsive to FGF-2, would also be affected by E2.

It has been reported that bisphenol A (BPA), a
MATERIALS AND METHODS

Reagents. E2, BPA, EGF, 5-bromo-2′-deoxyuridine (BrdU), and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, USA). Other agents and their sources were the following: FGF-2, from R&D Systems (Minneapolis, MN, USA); platelet-derived growth factor (PDGF)-BB, from Genzyme/Technogen (Cambridge, MA, USA); and neurotrophin (NT)-3, from PeproTech Inc. (Rocky hill, NJ, USA). Brain-derived neurotrophic factor (BDNF) was kindly donated by Sumitomo Pharmaceutical, Inc. (Osaka, Japan).

Primary Cultures. NSCs were isolated from the telencephalon of Wistar rats (Nippon SLC, Shizuoka, Japan) at E15, and cultured as described previously (5). The proliferation medium consisted of Dulbecco’s modified Eagle’s minimum essential medium nutrient mixture F-12 HAM (DMEM/F12) supplemented with insulin (25 μg/mL), apo-transferrin (100 μg/mL), progesterone (20 nM), putrescine (100 μM), sodium selenite (30 nM), penicillin (100 U/mL), and streptomycin (100 μg/mL). The required amount of FGF-2 for a final concentration of 10 ng/mL was added to the original medium every day. The daily addition of FGF-2 for 7 days resulted in undifferentiated NSCs forming neurospheres (Fig. 1A); whereas when EGF was added instead of FGF-2 as a mitogen, the number or size of neurospheres was rather smaller even after daily addition of the growth factor (data not shown). After 7 days, the cells were mechanically dissociated from the spheres in Hank’s balanced salts modified solution.

We isolated FGF-2-responsive cells from the rat telencephalon of E15. The cells after plating retained their ability to proliferate in response to FGF-2, and thus presumed to be NSCs, but some of them differentiated into progenitor cells. Therefore, hereinafter we refer to the cells used in the present experiments as neural stem/progenitor cells (NS/PCs). As the present study aimed to address whether estrogens are involved in the environmental signals that regulate the fate of NS/PCs, we investigated the stimulatory effects of estrogens on the proliferation and differentiation of NS/PCs in the presence or absence of mitogens or differentiation factors, respectively.

The results showed that both E2 and BPA stimulated their proliferation and interestingly also did their differentiation into oligodendrocytes only in the absence of mitogen or differentiation factor.

**Fig. 1** Expression of mRNA of nuclear ERs in the FGF-2-responsive NS/PCs cultured from an E15 rat telencephalon. Cells were cultured for 7 days in non-adherent dishes in the presence of 10 ng/mL FGF, and formed neurospheres (A). The cells were transferred to poly-L-ornithine-coated dishes, and then cultured for another 3 days with FGF-2 prior to preparation of total RNA.
(HBSS), suspended in proliferation medium, and plated onto poly-L-ornithine-coated plates or dishes. These cultures were incubated for 3 days in medium containing FGF-2, with daily addition of the factor, and then offered for the experiments. Rat cortical neurons were cultured as described before (13).

**RT-PCR.** Cells plated on 6-well plates (1 × 10^4 cells/cm²) were cultured for 3 days in the proliferation medium. The mRNA levels of estrogen receptor (ER)α and ERβ were examined by means of RT-PCR as described earlier (6). The following primers were used, with the expected PCR product length given in terms of base pair (bp): β-actin 5'-gtgggcgcctagggcagccacag-3' and 5'-ctcttaatgtcagccaggtat-3', 542 bp; ERα 5'-ccgccaagggaggagaagttg-3' and 5'-caggacttctcccgaggttg-3', 438 bp; ERβ 5'-tggatgcgtaggcagcaca-3' and 5'-caggagcttccccgggtgtt-3', 407 bp. The numbers of PCR cycles and specific annealing temperatures were 22 cycles and 63°C for β-actin, 37 cycles and 51°C for ERα, and 36 cycles and 53°C for ERβ. Reaction products were electrophoresed on 2% agarose gels and stained with ethidium bromide solution.

**BrdU labeling.** Cells were plated on micro slide/cover glasses (Matsunami Glass Inc. Ltd., Osaka, Japan) in 24-well plates (6 × 10^4 cells/well) and cultured for 3 days in the proliferation medium, and then in medium containing the test compound for another 1 day. BrdU (final concentration, 10 μM) was then added to the culture medium. Two hours later the number of BrdU-positive cells was determined by an immunocytochemical technique with anti-BrdU mouse antibody (Sigma) and rhodamine-labelled anti-mouse IgG antibodies (Chemicon, Temecula, CA, USA). The total number of cells and the number of BrdU-positive cells were counted in 7 arbitrarily selected fields of each well. For each treatment, 4 ~ 8 wells were analyzed; and the experiments were repeated more than 3 times. For statistical analysis, Student’s t-test was used.

**MTT assay.** Cells were plated (2 × 10^4 cells/well) in 96-well plates and cultured for 3 days in FGF-2-free medium, and then for an additional day in FGF-2-free medium containing the test compound. Thereafter, 0.5 mg/mL of MTT was added to each well, and the plates were incubated for 4 h at 37°C to obtain blue formazan crystals. After the crystals had been dissolved in HCl/isopropanol, the absorbance at 570 nm was determined by using a micro plate reader (Bio-Rad, model 550). The cell number compared with that of the ethanol-treated control group was presented as a fold-increase. The total MTT converted to formazan by the cells (i.e., total cell number) in all of the wells had an absorbance that ranged from 0.2 to 0.75 at 570 nm.

**Immunocytochemistry.** Cells were plated on micro slide/cover glasses that had been placed in 24-well plates filled with the proliferation medium (4 × 10^4 cells/well), and cultured for 3 days. They were then reacted for another 5 days with the test compound prepared in FGF-2-free medium and thereafter washed with phosphate-buffered saline (PBS) and fixed for 10 min with 4% paraformaldehyde (PFA). As described before (5), the primary antibodies included anti-glial fibrillary acidic protein (GFAP) rabbit antibody (DAKO, Copenhagen, Denmark), anti-βIII tubulin (Tuj-1) mouse antibody (Promega, Madison, WI, USA), anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) mouse antibody (Sigma), anti-Ng2 chondroitin sulfate proteoglycan (Ng2) rabbit antibody (Chemicon), and anti-nestin mouse antibody (Chemicon). The total number of cells and the number of immunoreactive cells were counted in 7 arbitrarily selected fields of each well. For each treatment, 4 ~ 8 different wells were analyzed, and the experiments were repeated more than 3 times. For statistical analysis, Student’s t-test was used.

**RESULTS AND DISCUSSION**

**Effect of E2 or BPA on proliferation of NSCs**
We determined by RT-PCR whether ERα and/or ERβ were expressed in the FGF-2-responsive NS/PCs used in this study. As shown in Fig. 1B, FGF-2-responsive NS/PCs expressed predominantly ERβ mRNA rather than the ERα mRNA that was predominant in neurons. As NS/PCs were shown to express ERs, we then studied whether the proliferation of FGF-2-responsive NS/PCs would be influenced by estrogens. In a previous study, Brännvall et al. (2) used EGF-free medium containing E2 when they plated the cells for proliferation experiments. Namely, they initiated E2 treatment simultaneously with the start of the subculture. When we adopted the same conditions, our cells failed to proliferate even in the presence of FGF-2. Therefore we plated the cells for the experiments in medium containing FGF-2, cultured them for 3 days with FGF-2, and then initiated the treatment with estrogens in FGF-2-free medium.

The cells were cultured for 24 h in FGF-2-free
medium containing various concentrations ($10^{-11} \sim 10^{-3}$ M) of E2, and then their numbers were determined by use of the MTT assay. Dose-response analysis showed that a wide range of concentrations of E2 from $10^{-10}$ to $10^{-3}$ M resulted in a statistically significant increase in the number of viable cells (Fig. 2A, closed circles), the extent being the same as that obtained with FGF-2 (10 ng/mL, triangle). We thus treated NS/PCs with $10^{-8} \sim 10^{-7}$ M E2 in subsequent experiments, which are concentrations reported previously to be effective (2, 22). Next we treated NS/PCs with BPA of various concentrations ($10^{-11} \sim 10^{-3}$ M) in FGF-2-free medium for 24 h and conducted the MTT assay. Exposure of the cells to BPA over a wide range of concentrations ($10^{-11} \sim 10^{-4}$ M) significantly increased the cell number (Fig. 2A, open squares), but a toxic effect of BPA was observed at $10^{-3}$ M or higher concentrations. These results indicate that BPA as well as E2 was able to increase the number of FGF-2-responsive NS/PCs and that the concentration of BPA suitable for subsequent experiments was $10^{-5}$ M, which is considered to be the responsive concentration for rat cortical neurons (10).

**Fig. 2** Effect of E2 or BPA on cell proliferation of NS/PCs. (A) The cells treated with various concentrations of E2 or BPA in FGF-2-free medium for 24 h were subjected to the MTT assay. The ratios of the value for the estrogen-treated cells to that value for the control cells (ethanol-treated cells) were calculated, and are shown on the ordinate. Values are presented as the mean ± SEM ($n = 4 \sim 8$, different cultures). (B) The cells on poly-L-ornithine-coated cover glasses were cultured with $10^{-7}$ M E2 or $10^{-5}$ M BPA in FGF-2-free or -containing medium for 24 h and then for another 2 h in the presence of BrdU. Thereafter, they were immunocytochemically examined by using anti-BrdU antibody and rhodamine-labeled anti-mouse IgG antibodies (scale bar = 50 µm). (C) The percentage of BrdU-positive cells in the various cultures was calculated, and is shown on the ordinate. Values are presented as the mean ± SEM ($n = 4 \sim 8$, different cultures). Significance, *p < 0.05, **p < 0.01, ***p < 0.001 versus control.
In order to confirm the cell proliferation, we determined the percentages of the cells in the S phase of the cell cycle by measuring the incorporation of the thymidine analogue BrdU (Fig. 2B). When NS/PCs maintained in FGF-2-free medium were exposed for 24 h to $10^{-7}$ M E2 (Fig. 2C-a, closed bar) or $10^{-5}$ M BPA (Fig. 2C-a, shaded bar), 37 ± 2.5% and 41 ± 3.4%, respectively, of cells were labeled by BrdU; whereas 23 ± 2.6% of the cells incorporated BrdU in the non-treated control group (Fig. 2C-a, open bar). These results strongly suggest that E2 and BPA had the ability to stimulate the proliferation of FGF-2-responsive NS/PCs. The number of BrdU-positive cells was 35 ± 1.6% in the presence of FGF-2 (Fig. 2C-b, open bar), indicating that the stimulatory effect of the estrogens was similar to that of FGF-2.

When NS/PCs maintained in FGF-2-containing medium were exposed for 24 h to $10^{-7}$ M E2 (Fig. 2C-b, closed bar) or $10^{-5}$ M BPA (Fig. 2C-b, shaded bar), the number of BrdU-positive cells decreased slightly (29 ± 4.1% with E2 + FGF-2, closed bar; 31 ± 1.6% with BPA + FGF-2, shaded bar), but there was no significant difference across these 3 FGF-2-treatment conditions (only FGF-2; FGF-2 + E2; BPA + FGF-2). These results demonstrate that neither E2 nor BPA influenced the mitogenic activity of FGF-2.

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**Fig. 3** Effect of E2 or BPA on differentiation of NS/PCs. (A) The cells on poly-L-ornithine-coated cover glasses were treated with $10^{-8}$ M E2 or $10^{-6}$ M BPA in FGF-2-free medium for 5 days, and then examined by immunocytochemistry using anti-nestin, anti-Tuj-1, anti-GFAP or anti-CNPase antibodies (scale bar = 50 µm). (B) The percentage of cells positive for each marker was calculated, and is shown on the ordinate. Values represent mean ± SEM (n = 4 - 16, different cultures). Significance, *p < 0.05 versus control.
The results of the previous study using EGF-responsive NSCs (2) showed that E2 significantly stimulated the proliferation of embryonic cells, but only by 7%, and had no effect on adult cells. Our present results using FGF-2-responsive NSCs show that estrogens stimulated more than 1.5-fold the proliferation of these cells in the absence of FGF-2. These findings suggest the possibility that the stimulatory effect of E2 on proliferation of NS/PCs decreases age-dependently, although further study is needed to identify the critical time points.

The earlier study by Brännvall et al. (2) showed that E2 slightly stimulated the proliferation of EGF-responsive NSCs, that EGF obviously stimulated their proliferation (4.5-fold), and that about 13% of this increase was decreased by E2. Our present results using FGF-2-responsive NS/PCs show that estrogen treatment did not modulate the stimulatory effect of FGF-2 on the proliferation of FGF-2-responsive NS/PCs. As far as we have studied, the modulatory effect of estrogens on mitogen-induced proliferation of NS/PCs is likely to be less powerful during early development; but the proliferation of NS/PCs absolutely depends upon mitogens. When the activity of mitogen is weakened, the effect of estrogens might not be negligible.

**Effect of E2 or BPA on differentiation of NSCs**

The ‘neuron-first’ ‘glia-second’ differentiation theme in vivo can be recapitulated in culture. After mitogen withdrawal upon plating, NSCs in vitro were shown to give rise to neurons after short-term culturing, and then they switched from being neurogenic to gliogenic over time (17).

As Brännvall et al. (2) indicated that E2 increased the ratio of neurons to glia cells derived from EGF-responsive NS/PCs, we inspected the fate or differentiation of FGF-2-responsive NS/PCs after a 5-day treatment with $10^{-8}$ M E2 or $10^{-5}$ M BPA. Using an immunocytochemical technique, we detected nestin-positive NSCs, Tuj-1-positive neurons, GFAP-positive astrocytes, and CNPase-positive oligodendrocytes in the NS/PC cultures (Fig. 3A). About half of the control cells (45 ± 5.4%) expressed nestin,
and approximately 20% of the control cells were positive for Tuj-1 (26 ± 4.2%) or GFAP (19 ± 3.9%); whereas only 10 ± 1.5% of them were CNPase-positive (open bars in Fig. 3B). When the cells were treated with E2 (solid bars in Fig. 3B) or BPA (shaded bars in Fig. 3B), the proportion of nestin-positive cells, Tuj-1-positive cells or GFAP-positive cells did not change significantly, but the percentage of CNPase-positive cells was significantly increased to 17 ± 0.9% (1.7-fold) by E2 and 18 ± 1.4% (1.8-fold) by BPA. To confirm the effect of E2 and BPA on the glial differentiation of NS/PCs, we also detected NG2-positive oligodendrocyte precursor cells in the NS/PC cultures (Fig. 4A). As shown in Fig. 4B-a, the percentage of NG2-positive cells was significantly increased by E2 (27 ± 2.9%; 1.6-fold, solid bar) and BPA (28 ± 3.0%; 1.6-fold, shaded bar) compared with that for the non-treated cells (17 ± 2.2%, open bar).

The results of a previous study using EGF-responsive NSCs (2) showed that E2 could increase the relative proportion of neurons to astroglial cells present in embryonic NSC cultures but not in cultures of adult NSCs. Our present results using FGF-2-responsive NSCs show that estrogens increased the proportion of oligodendrocytes in FGF-2-responsive NS/PCs. These findings suggest the possibility that the stimulatory effect of E2 on the differentiation of NSCs into oligodendrocytes decreases age-dependently. As far as we have studied, the modulatory effect of estrogens on the differentiation of NS/PCs is likely to be more powerful during early development; and NS/PCs exposed to estrogens differentiate directly into oligodendrocytes rather than into astrocytes that then differentiate into oligodendrocytes.

Next we investigated whether estrogens would modulate the effect of PDGF, a mitogen for oligodendrocyte precursor cells (3). We found that the number of NG2-positive cell was 42 ± 3.0% in the presence of PDGF (Fig. 4B-b, open bar); that is to say, PDGF by itself increased the percentage of NG2-positive cells about 2.5-fold compared with that for the control cultures (Fig. 4B-a, open bar). As shown in Fig. 4B-b, there was no significant difference in the percentage of NG2-positive cells across the 3 groups: treatment with PDGF (open bar, 42 ± 3.0%), PDGF plus E2 (closed bar; 42 ± 3.6%) and PDGF plus BPA (shaded bar; 46 ± 4.4%). These results suggest that estrogens do not disturb the stimulatory effect of PDGF on the proliferation of oligodendrocyte progenitors.

We also tested whether estrogens would modulate the stimulatory effect of NT-3 on oligodendroglial differentiation (Fig. 4B-c). NT-3, a member of the neurotrophin family of neurotrophic factors, was reported to have a crucial role in oligodendrocyte development in vitro (1). To determine whether E2 or BPA could influence NT-3-induced differentiation of FGF-2-responsive NS/PCs into oligodendrocytes, we cultured the cells in the presence of NT-3 with or without estrogens, and then performed immunocytochemical analysis to examine the percentage of NG2-positive cells. Fig. 4B-c shows that NT-3 by itself (open bar) increased the percentage of NG2-positive cells in the cultures about 2-fold (34 ± 3.4%) compared with that for the control cultures (17 ± 2.2%). There was no significant difference in the percentage of NG2-positive cells in the NT-3/E2-treated group (closed bar; 34 ± 3.4%) or NT-3/BPA treated group (shaded bar; 38 ± 3.9%) compared with that for the group treated with only NT-3 (34 ± 3.4%).

We previously showed that BDNF enhanced the neuronal differentiation of NSCs in the presence or absence of FGF-2 (6). When the cells were exposed to E2 or BPA in the presence of BDNF for 5 days, immunohistochemical analysis showed that the percentage of Tuj-1-positive cells did not change compared with that for the BDNF-treated group (data not shown), indicating that the estrogens did not affect the BDNF-induced neuronal differentiation.

In conclusion, this is the first report to show that the effect of various proliferation factors or differentiation factors on FGF-2-responsive NSCs might take priority over the effect of estrogen during early developmental stages, if these physiological secretory factors are fully present and act normally. But the effect of estrogen might not be negligible if the activity of these factors is weakened.

For many years sex steroids including estrogen have been considered to be synthesized in peripheral tissues (prostate, ovary, adrenal cortex, etc.), reaching the brain through the blood circulation and, once there, controlling sex performance or stress responses. Recently it has become evident that estrogen is synthesized in rat brain hippocampal neurons (9) and that it exerts a broad spectrum of actions including neuroprotective effects and a stimulatory effect on cognitive function (20). In early developmental stages, the site of estrogen synthesis is unknown. It is important to clarify whether NSCs themselves might synthesize estrogens, whereby an autocrine mechanism might be at play.
REFERENCES


