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Estradiol promotes proliferation of dopaminergic precursors resulting in a higher proportion of dopamine neurons derived from mouse embryonic stem cells

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Abstract

Estradiol protects dopamine neurons of the *substantia nigra* from toxic insults. Such neurons succumb in Parkinson's disease; one strategy for restoring dopamine deficiency is cell therapy with neurons differentiated from embryonic stem cells. We investigated the effects of 17 β -estradiol on dopaminergic induction of embryonic stem cells using the 5-stage protocol. Cells were incubated with different steroid concentrations during the proliferation (stage 4) or differentiation (stage 5) phases. Estradiol added at nM concentrations only during stage 4 increases the proliferation of dopaminergic precursors expressing Lmx1a, inducing a higher proportion of dopamine neurons at stage 5. These actions were mediated by activation of estrogen receptors, because co-incubation of cells with estradiol and ICI 182,780 completely abolished the positive effect on both proliferation of committed precursors, and subsequent differentiation to dopaminergic neurons. Our results suggest that estradiol should be useful to produce higher proportions of dopamine neurons from embryonic stem cells aimed for treating Parkinson's disease.

Keywords

Dopamine neurons; Embryonic stem cells; Estradiol; Lmx1a; Tyrosine hydroxylase

1. Introduction

There is evidence indicating that estrogens can induce the development and protection of nigrostriatal dopamine (DA) neurons *in vivo* (Kipp et al., 2006; Liu and Dluzen, 2007). Parkinson's disease (PD) is a neurodegenerative disorder characterized by a progressive and selective loss of midbrain DA neurons of the *substantia nigra pars compacta*. This decrease of DA neurons results in a dopamine depletion in the striatum, which impairs normal motor functions. Epidemiological studies show that PD is 1.5 to 2 times more common in men than in women (Baldereschi et al., 2000; Czlonkowska et al., 2006; Haaxma et al., 2007); recently,

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delayed onset and more benevolent progress have been reported for women when compared to men patients with PD, and these differences could be due to estrogens action (Haaxma et al., 2007). Furthermore, in experimental animal models, numerous studies demonstrated that estrogens administration protects against neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (reviewed in (Morissette et al., 2008), methamphetamine (Dluzen and McDermott, 2002) and 6-hydroxy-DA (reviewed in Dluzen, 1997) that selectively destroy DA neurons of the *substantia nigra*. All these findings imply that estrogens play a protective role for DA neurons. In addition to this well-described action, in recent years a proliferative action of estradiol (E₂) has been reported for neural precursor cells (Brannvall et al., 2002; Martinez-Cerdeno et al., 2006; Wang et al., 2008).

Embryonic stem cells (ESC) are pluripotent cell lines derived from the inner cell mass of pre-implantation embryos. Because of their ability to self-renew, proliferate undifferentiated *in vitro*, while maintaining the potential to differentiate into most cell types, ESC are useful to dissect critical steps of cell commitment. Furthermore, they also represent a potential source for cell replacement therapy (McKay, 2000). In this context, it has been shown that midbrain DA neurons can be induced *in vitro* from mouse ESC with the 5-stage method and that these DA neurons have improved parkinsonism in rodents for over 32 weeks after grafting (Díaz et al., 2007; Kim et al., 2002; Rodríguez-Gómez et al., 2007). We have previously reported the expression of sex steroid hormone receptors during this protocol. The last two phases of this procedure produce nestin-positive cells (stage 4, neural precursors) that respond to the application of Fibroblast growth factor (FGF) 2, FGF 8 and Sonic hedgehog (SHH) to produce terminally differentiated DA neurons (stage 5). We found (Díaz et al., 2007) that both pluripotent ESC and DA neurons (stage 1 and stage 5 cells respectively), expressed estrogen receptor α (ER- α). Nineteen percent of DA neurons co-expressed ER- α and tyrosine hydroxylase (TH, the ratelimiting enzyme for DA biosynthesis). These results suggest that the activation of these receptors by E₂ might participate in the induction of dopaminergic cells, and that this hormone could be used to modulate DA neuron production from ESC.

2. Experimental procedures

2.1. ESC differentiation to DA neurons

We used male R1 mouse ESC from Dr. Nagy's laboratory (Nagy et al., 1993), which have been proved to produce DA neurons (Díaz et al., 2007; Kim et al., 2002; Rodríguez-Gómez et al., 2007). The differentiation procedure was performed as reported (Díaz et al., 2007). Briefly, undifferentiated ESC (stage 1) were grown on gelatin-coated tissue culture plates in the presence of 1000 U/ml of Leukemia inhibitory factor (Chemicon, USA) in medium supplemented with ESC-tested fetal calf serum (Wisent, Canada). To induce formation of floating embryoid bodies (stage 2), cells were dissociated into cell suspension with trypsin and plated onto bacterial dishes at a density of 2×10^6 cells/57 cm² in the presence of Leukemia inhibitory factor. Embryoid bodies were cultured for 4 days and then plated onto adhesive tissue culture surface. Enrichment of nestin-positive cells (stage 3) was initiated in serum-free insulin / transferrin / selenite / fibronectin (ITSFn) medium. After 9–11 days of culture, cells were dissociated with trypsin and plated at a concentration of 1.5×10^5 cell/cm² in serum-free N2 medium (DMEM/F12 supplemented with 25 μ g/ml insulin, 100 μ g/ml apotransferrin, 100 μ M putrescine, 30 nM selenite and 20 nM progesterone) with or without phenol red. These neural stem cells were plated on dishes pre-coated with poly-L-ornithine and 1 μ g/ml mouse laminin (Becton Dickinson, USA), treated with 10 ng/ml FGF 2, 100 ng/ml FGF 8b and 100 ng/ml of human SHH (growth factors from R&D Systems and Peprotech, USA) for 4 days to expand/instruct DA precursors (stage 4). Differentiation (stage 5) was induced by growth factors withdrawal and feeding with the corresponding N2 medium with 200 μ M ascorbic acid

for 6 days. This differentiation protocol produces only DA neurons and not other catecholaminergic neurons (Lee et al., 2000).

2.2. Estradiol and pharmacological treatments

To determine the effects of E₂ on final stages of the protocol to differentiate ESC to DA neurons, cells were treated with different steroid concentrations at the period of proliferation (stage 4) or differentiation (stage 5). Water soluble 17 β -E₂ (cyclodextrin-encapsulated; Sigma, St. Louis, MO, USA) was used at several concentrations ranging from 1 nM to 10 μ M. During proliferation, E₂ was applied in the first and third days of this stage in N2 medium. For the experiments in the phase of differentiation, E₂ was added every other day, starting at day one until day five. Controls received 30 nM water soluble cyclodextrin (Sigma), which is equivalent to the amount applied with micromolar E₂ treatments.

In another series of experiments, we employed the ER antagonist ICI 182,780 at 2 μ M (Tocris Cookson Inc, Ellisville, MO) to determine whether the effect of E₂ was mediated through their nuclear receptors. The following treatments of hormone and its antagonist were applied in the stage 4 of the protocol in N2 medium in the presence of phenol red: 1) Control (cyclodextrin). 2) 2 μ M ICI. 3) 10 nM E₂. 4) 10 nM E₂ + 2 μ M ICI. 5) 10 μ M E₂. 6) 10 μ M E₂ + 2 μ M ICI. For N2 medium without phenol red, we repeated conditions 1 – 4 described above.

2.3. Immunofluorescence

Immunofluorescence procedures were carried out using described standard protocols (Díaz et al., 2007; Molina-Hernandez and Velasco, 2008; Velasco et al., 2003). After fixing cells with 4% paraformaldehyde, primary antibodies were applied as follows: rabbit anti-TH antibody, 1:1000 (Pel-Freeze, USA), mouse anti- β Tubulin III (a neuronal marker) monoclonal antibody, 1:1000 (Covance, USA), rabbit anti-Lmx1a (a DA precursor marker; a kind donation from Dr. Michael German), 1:2000, and mouse anti-Ki67 (a nuclear antigen expressed in all proliferating cells; Novocastra, U.K.), 1:200. Appropriate fluorescently-labeled secondary antibodies (Molecular Probes, USA) were used. Nuclei were stained with 1 ng/ml of Hoechst 33258 (Sigma). Negative controls consisted of cells in which the primary antibody was omitted. These experiments did not produce any staining (data not shown). As previously reported, all TH-positive cells also expressed β Tubulin III (Díaz et al., 2007).

2.4. RNA extraction and RT-PCR

Assays were performed as described (Molina-Hernandez and Velasco, 2008). Total RNA was isolated from differentiated (stage 5) cultures, using TRIZOL (Invitrogen). RNA (1 μ g) was reverse transcribed with random hexamers and 2 μ l from the reaction were used in PCR containing 2 U of Taq polymerase (Invitrogen), 20 pmol of specific primers (Sigma), 500 μ M deoxynucleoside triphosphates and 1.5 mM MgCl₂. For cDNA amplification, the following reported (Barberi et al., 2003; Kim et al., 2002) forward (F) and reverse (R) primer sequences were used: DA transporter (DAT), F:5'-GGACCAATGTCTTCAGTGGTGGC-3', R: 5'-GGATCCATGGGAGGTCCATgg-3'; aromatic L-amino acid decarboxylase (AADC), F: 5'-CCTACTGGCTGCTCGGACTAA-3', R: 5'-GCGTACCAGTGACTCAAACCTC-3'; Engrailed-1 (En-1), F: 5'-TCAAGACTGACTCACAGCAACCCC-3', R: 5'-CTTTGTCCTGAACCGTGGTGGTAG-3'; Lmx1b, F:5'-CCTCAGCGTGGTGTGGTC-3', R: 5'-AGCAGTCGCTGAGGCTGGTG-3'; Nurr 1, F:5'-TGAAGAGAGCGGAGAAGGAGATC-3', R: 5'-TCTGGAGTTAAGAAATCGGAGCTG; Ptx 3, F:5'-AGGACGGCTCTCTGAAGAA-3', R5'-TTGACCGAGTTGAAGGCGAA-3'; c-RET, F:5'-GCGCCCCGAGTGTGAGGAATGTGG-3', R: 5'-GCTGATGCAATGGGCGGCTTGTGC-3'; β Tubulin III, F: 5'-TCAGCGATGAGCACGGCATA-3', R:5'-CACTCTTCCGCACGACATC-3' and Glyceraldehyde phosphate dehydrogenase (GADPH), F:5'-

ATCACCATCTTCCAGGAGCG-3', R:5'-CCTGCTTCACCACCTTCTTG-3'. Cycling parameters were as follows: denaturalization at 94°C for 1 min, annealing at 57–62°C for 1 min depending on the primer, and elongation at 72°C for 1 min. The number of cycles varied between 25 and 35, depending on the gene of interest. Final extension at 74°C for 10 min was terminated by rapid cooling at 4°C. PCR products were analyzed in 2% agarose gel electrophoresis, and the size of products was determined by comparison with molecular weight standards after ethidium bromide staining. As a negative control for PCR amplification, reactions with RNA in the absence of retrotranscription were included.

2.5. Cell counting

At the final stage of differentiation, cell counts from immunofluorescence experiments were performed from pictures taken with a Nikon (Tokyo, Japan) digital camera DMX1200 F and the Nikon ACT-1 imaging software. Analysis of cultures was performed by counting in duplicate the number of TH+ and β Tubulin III+ cells in 8 random fields taken at 400X, from 3–6 independent experiments. In the case of staining for Lmx1a and Ki67, cells were fixed at the end of stage 4. In these cultures, we quantified the total number of cells by counting the Hoechst-stained nuclei. Some images were acquired in a FV1000 confocal microscope (Olympus, Tokyo, Japan) using a super apochromat objective (N.A. 0.40, Olympus), to detect Alexa 488, Alexa 568 and Hoechst fluorescence in a sequential fashion, by exciting with different lasers. To establish co-expression of the used markers, merged images were generated. The examiner was unaware of the treatment condition of cells.

2.6. Electrophoresis and Western blot

Assays were performed as described (Díaz et al., 2007). Briefly, cells of each stage of differentiation were homogenized in lysis buffer (10 mM Tris-HCl, 1 mM dithiothreitol, 30% glycerol, 1% Triton X-100, 15 mM sodium azide, 1 mM EDTA, 4 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Proteins were obtained by centrifugation at 15,000 rpm at 4 °C for 15 min, and supernatant was quantified by a modified Bradford assay (BioRad, Germany). Proteins (30 μ g) were resolved on 8% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Bioscience, USA) which were blocked with 5% non-fat dry milk and incubated overnight with primary antibodies (diluted 1:1000). Pre-stained markers (BioRad) were included for size determination. The following antibodies were used: rabbit anti-TH (Pel-Freez, USA) and mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology). Membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology; diluted 1:5000). Immunoreactive bands were detected using a semi-quantitative enhanced chemiluminescence method (Millipore). To correct for differences in the amount of total protein loaded in each lane, TH protein content was normalized to that of GAPDH. After TH detection, blots were stripped with glycine (0.1 M, pH 2.5, 0.5% SDS) at 37°C for 30 min and re-probed with anti-GAPDH. The intensity of the bands was quantified by densitometry using a Hewlett-Packard scanner for capturing data and the Chemiimager Image Analysis software for densitometric comparisons.

2.7. Statistical analysis

Data are expressed as mean $\pm$ SEM of at least 3 independent experiments performed by duplicate. Statistical analysis was made by ANOVA followed by Fisher's test. GB-STAT Version 7.0 program (Dynamic Microsystem, USA) was used for calculating probability values. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of 17 β -E₂ during stages 4 or 5 on DA neuron differentiation of ESC

To establish the effects of 17 β -E₂ during the proliferation stage of the method, we performed a set of experiments with several hormone concentrations in N2 medium together with FGF 2, FGF 8 and SHH. All cultures started at the same initial density, were incubated for 4 days in the presence of cytokines, with or without 17 β -E₂, and then allowed to differentiate for 4–6 days in N2 medium with ascorbic acid. Representative pictures of differentiated (day 6 of stage 5) cultures treated with 17 β -E₂ only during stage 4 are shown to document that this hormone at nM concentration is beneficial, and at μ M doses deleterious, for dopaminergic induction (Fig. 1). We quantified the number of neurons (β Tubulin III-positive cells) and DA (TH-positive) neurons by performing double immunodetection of these markers in control and hormone-treated cultures. We found a significant 43 % increase in the number of β Tubulin III-positive cells with the dose of 10 nM of 17 β -E₂ as compared with control (Fig. 2A). We also observed statistically significant increases (76–96%) in the number of TH-positive neurons with 1, 10 and 100 nM 17 β -E₂, relative to control (Fig. 2A). When we used 10 μ M 17 β -E₂, a significant 66 % decrease in the number of TH+ neurons was observed (Fig. 2A) in absence of apparent variations in total cell number after nuclei staining with Hoechst (first column of Fig. 1). Accordingly, we found statistically significant increases in the percentage of TH-positive neurons (i.e. # of TH-positive / # β Tubulin III-positive cells X 100) with 10 (46 ± 4 %) and 100 nM (47 ± 2 %) E₂, and a decrease with 10 μ M (15 ± 2 %) E₂, in comparison with control conditions (34 ± 3 %; Fig. 2C). Cells treated during stage 5 with 10 nM 17 β -E₂ showed augmented numbers of β Tubulin III-positive neurons (Fig. 2B). However, no significant differences were found in TH-positive cell number nor the percentage of TH-positive cells with the tested concentrations of 17 β -E₂ at this stage (Fig. 2B and 2D).

3.2. Estrogen receptor antagonism inhibits the increase in DA neurons measured at stage 5 in N2 medium with or without phenol red

Once we established the effects of E₂ in the number of DA neurons differentiated from ESC, we wanted to find out if such effects were mediated by activation of ER. We incubated cells in stage 4 with 10 nM or 10 μ M E₂ in the presence or absence of 2 μ M of the ER antagonist ICI 182,780. In this set of experiments, we observed that 10 nM E₂ increased the numbers of β Tubulin III- and TH-positive cells relative to control conditions at the end of stage 5. Co-incubations of E₂ with ICI abolished the increase in the number of DA neurons caused by 17 β -E₂ (Fig. 3A). We also found significant differences in percentages of DA neurons between control- and E₂-treated cells and such difference was reverted by the ER blocker ICI (Fig. 3C). When we used 10 μ M E₂, we observed diminished numbers and percentages of DA neurons, but incubation with ICI brought both parameters closer to values of the control group (Fig. 3A and 3C). ICI alone did not modify the number or the % of TH positive cells, as compared with control conditions.

Because the standard N2 medium contains phenol red, and this molecule bears a structural resemblance to some non-steroidal estrogens that could exhibit estrogenic activity (Berthois et al., 1986), we performed a series of experiments with N2 medium lacking phenol red. When we incubated cells in stage 4 with 10 nM E₂, we observed again significant increases in the number of β Tubulin III (25%) and TH (74 %) positive cells, as well as in the percentage of DA neurons (Fig. 3B and 3D) at stage 5. Co-incubation of E₂ with ICI blocks the increases in total neurons, DA neurons and in the percentage of dopaminergic cells. ICI alone did not modify control behavior. All subsequent experiments were made in medium lacking phenol red.

3.3. Estradiol treatment during stage 4 increases TH content at stage 5

To have a complementary approach that allows us to establish if the increase in TH⁺ cells was present throughout the culture, we decided to perform immunoblots with protein extracted from control or E₂-treated cells in medium without phenol red. We estimated TH content relative to the house keeping protein GAPDH. We found that E₂ treatment significantly augmented TH content relative to control levels, reaching a significant increase of 68 % at the end of stage 5 (Fig. 4A and 4B).

3.4. Estradiol-treated cells express dopaminergic molecular markers

To further characterize if the TH-positive neurons produced after E₂ treatment express dopaminergic neuron markers, semi-quantitative polymerase chain reaction after mRNA reverse transcription (RT-PCR) was used to evaluate the expression of genes specifically involved in dopamine neuron function at stage 5. We found similar expression levels of AADC, En-1, Lmx1b, and Nurr1 in control and E₂-treated cells. Other markers such as β Tubulin III, DAT (which in vertebrates is exclusively found in dopamine neurons), Ptx3 (uniquely found in midbrain dopamine neurons) and c-Ret were all up-regulated in differentiated ESC treated with 10 nM of E₂ when compared with ESC without 17 β -E₂ treatment (Fig. 5). These results suggest that E₂ did not modify the molecular features of mesencephalic DA neurons.

3.5. Estradiol increases the number of proliferating Lmx1a-positive cells

Since estradiol has been reported to modify proliferation of several cell types (Heldring et al., 2007), we performed immunostaining of neural precursors at stage 4 with Ki67, an antigen present in cells with active cell cycle (Scholzen and Gerdes, 2000). At this stage, cells are evenly distributed, allowing quantification of total nuclei after Hoechst staining (first column of Fig. 6). Total cell numbers were not modified by 10 nM E₂ (507 ± 40), ICI (406 ± 17), or E₂ + ICI (382 ± 42) relative to controls (488 ± 43). To establish if E₂ was affecting the proportion of proliferating cells, we calculated the percentage (i.e. number of Ki67-expressing / total cells X 100) at the end of stage 4, and found that all tested conditions were similar in terms of the proportion of Ki67-positive cells: control: 18 ± 2 %; ICI: 17 ± 3 ; 10 nM E₂: 22 ± 6 ; 10 nM E₂ + ICI: 16 ± 3 . In agreement, the mean number of cells expressing Ki67 per microscopic field was not significantly changed by the tested treatments (Fig. 7A).

To gain insight into the possible mechanism involved in DA neuron induction by E₂, we investigated the effect of this hormone on the number of cells positive for the marker of dopaminergic progenitors Lmx1a (Fig 6). We incubated cells in stage 4 with 10 nM E₂ with or without ICI in medium without phenol red. When neural precursors were exposed to E₂, we observed a significant increase in the Lmx1a⁺ cell population as compared with control (Fig. 7A). This change was also sensitive to ICI, since cultures incubated with E₂ and the ER antagonist showed reduced numbers of Lmx1a-positive cells. When we analyzed the percentage of DA precursors (Lmx1a-positive) that have active cell cycle (Ki67-positive) we found that E₂ statistically increased the number of double-positive cells and ICI antagonized this rise (Fig. 7B). These results show that E₂ specifically increases the number of proliferating Lmx1a-positive cells (DA precursors), and it does not have a general proliferative effect on the total population of neural precursors.

4. Discussion

In the present study, we examined the effect of E₂ in the last stages of differentiation of mouse ESC to DA neurons *in vitro*. We found significant increases in the number and the percentage of TH-positive neurons at the end of differentiation with nM concentrations of E₂, but only when cells were incubated with this hormone during stage 4. The increase in DA neurons was correlated with higher numbers of proliferating cells positive to Lmx1a, a protein necessary

for the induction of a DA phenotype (Andersson et al., 2006; Cai et al., 2008). Both effects of E₂ were mediated by ER, since they were blocked by co-incubation of E₂ with the antagonist ICI 182,780. These effects of the hormone appears to be lineage-specific (Lmx1a population), because we did not find an overall increase in total cell number nor in the proportion of Ki67 + population.

Growth factors and estrogens are both necessary for the regulation of developmental process and for controlling the function of midbrain DA neurons (Beyer, 1999; McEwen and Alves, 1999). Such steroid/growth factor interactions may stimulate the synthesis of proteins required for neural proliferation, differentiation, survival and maintenance of DA neurons functions. In this regard, it has been demonstrated that the exposure to estrogens of cultured mesencephalic DA neurons enhanced a number of developmental characteristics, such as neurite growth, elongation, branching pattern and TH mRNA/protein levels (Ivanova and Beyer, 2003). These effects were reversible with the withdrawal of the hormone (Beyer and Karolczak, 2000). Our results are in line with these studies, since the application of E₂ in the proliferative phase increased the number of TH-positive cells and the hormone had no effects in the differentiation stage. Due to the fact that in stage 4 we added SHH and FGF 8, factors that promote the DA lineage, our results suggest that E₂ differentiating effects are produced in conjunction with others growth factors. In addition, it has been demonstrated that 17β-E₂ promotes the proliferation of undifferentiated mouse ESC (Han et al., 2006), rat embryonic cortical progenitors (Martinez-Cerdeno et al., 2006) and fetal striatal neural stem cells (Brannvall et al., 2002). A proliferative effect mediated by ER-β has also been reported for human cortical neural stem cells exposed to nM concentrations of E₂ (Wang et al., 2008). Both ER subtypes (α and β) are expressed at significant levels in DA neurons in the developing and adult rodent brain (Kuppers and Beyer, 1999; Mitra et al., 2003; Raab et al., 1999). There is evidence that estrogens regulate the expression of key parameters of dopaminergic functions and its neuroprotection; however, it has been proposed that ER α is the most active ER in this system (Kipp et al., 2006). Furthermore, ER α knock-out animals have diminished TH mRNA expression and lower TH protein content in the ventral midbrain, when compared with wild-type mice (Kuppers et al., 2008). Although the estrogen receptor antagonist ICI 182,780 does not discriminate between which ER subtype is mediating E₂ effects, these data suggest that ER α activation could be responsible for the effects of the hormone in our conditions.

As already mentioned, E₂ has neuroprotective roles for DA neurons. Recently it was reported that in murine midbrain neuronal cultures, E₂ in the range of 1 – 100 nM increased 25–50% the number of TH-positive neurons over control levels, decreased the number of glial cells, and enhanced neurite development. This increase in DA neurons could be due to survival of differentiated DA neurons, but neurogenesis was suggested as an alternative explanation (Rodriguez-Navarro et al., 2008). Other studies have clearly shown that undifferentiated neural (Brannvall et al., 2002) or ESC (Murashov et al., 2004) exposed to nM concentrations of E₂ differentiated to neurons. Furthermore, in human mesencephalic neural stem cells, 10 nM 17β-E₂ increased the number of Microtubule associated protein 2-positive cells (a marker of mature neurons) and also augmented the number of TH cells at 10 nM and 100 nM (Kishi et al., 2005). In our studies, the only concentration that increased the number of β Tubulin III-positive cells was 10 nM of 17β-E₂, and a broad range of 17β-E₂ doses (1–100 nM) increased the number of DA neurons; only 10 and 100 nM E₂ increased the percentage of DA neurons. All the abovementioned studies are in accordance with our findings, and the described results underline the similarities between ESC-derived neural precursors and those isolated from the developing midbrain, suggesting that both populations possess similar mechanisms to differentiate into DA neurons under the influence of 17β-E₂.

To rule out estrogenic interference of phenol red in our experimental system, we tested whether the TH-promoting effects of E₂ during stage 4 were present in medium without phenol red.

We found that the dopaminergic induction exerted by 10 nM E₂ was present in either medium. Furthermore, in both media we observed that E₂ effects were mediated through E₂-ER binding, since the antagonist ICI 182,780 was completely effective in blocking E₂ actions. To further validate the differences we are reporting, immunoblots were performed to estimate TH induction by 10 nM E₂ in the absence of phenol red. Increases of around 70 % were observed in cell counting and immunoblots (Fig. 4C and Fig. 5).

We found that the highest dose of 17 β -E₂ (10 μ M) reduced the number and percentage of TH-positive cells when applied in stage 4, and this effect was also partially prevented by ICI. It is of interest to observe that 17 β -E₂ is generally considered a proliferative factor in mammary and uterine tissue (Heldring et al., 2007), but at high concentrations might have opposite effects in cells of neural origin. In agreement with this result, it has been reported that 10–20 μ M E₂ reduce the number of TH positive cells in mice midbrain neuronal cultures (Rodríguez-Navarro et al., 2008). In the same line, 17 β -E₂ can induce entry of human neuroblastoma cells (SK-ER3) to the G0 phase of the cell cycle (Agrati et al., 1997; Ma et al., 1993). Also, nM concentrations of E₂ increase cell number in undifferentiated ESC, but 1 μ M E₂ treatment caused a decrease in the amount of cells found in the culture (Han et al., 2006). The mechanism underlying the effect of the highest dose of E₂ used here might deserve further research, but in this work we decided to explore the mechanisms by which E₂ increased DA number.

In order to test whether E₂ was in fact critical to the commitment of neural precursor cells derived from ESC, we evaluated the number of DA precursors by quantifying Lmx1a-positive cells at the end of stage 4. We found that E₂ increased the number of Lmx1a+ cells, and this effect was blocked by ICI (Fig. 6A). Furthermore, the number of proliferating dopaminergic precursors (i.e. Lmx1a/Ki67 double-positive cells) presented the same behavior (Fig. 7B). Lmx1a is specifically expressed in DA progenitor cells and its expression is maintained over the period of DA neuron generation; it has been reported that Lmx1a acts as a specific transcriptional activator of downstream mesencephalic DA phenotypic markers such as Ptx3, Nurr1, Aldh1a1, TH, DAT and Girk2 (Cai et al., 2008). Additionally, over-expression of Lmx1a in mouse ESC produced more TH-positive neurons with an authentic midbrain identity, with cells expressing late markers of mature DA neurons, including DA and vesicular monoamine transporters, providing additional evidence that these cells differentiate into *bona fide* midbrain DA neurons (Andersson et al., 2006). Neural progenitors from different brain regions, that normally do not generate DA neurons, do not express Lmx1a and this lack of expression is correlated with an incapacity to produce mesencephalic DA-specified neurons, even when they are placed in an *in vitro* or *in vivo* environment which promotes the development of DA traits (Cai et al., 2008). We also demonstrated that treatment with 17 β -E₂ induced TH+ neurons with classic molecular markers of DA cells such as Nurr1, c-Ret, Lmx1b, DAT, En1, and A9 DA neurons identifiers such as Ptx3 (Fig. 5). Finally, the fact that the hormone selectively increased the proliferation of cells commitment to TH lineage (Lmx1a) and has not effect in the entire population (as indicated by the number of Hoechst- and Ki67-positive cells) imply a specific effect in this population. Taken together, these results support the notion that E₂ increase the number and proliferating Lmx1a+ cells that will become mesencephalic DA neurons. In summary, we have found that 17 β -E₂ induced a higher number of differentiated DA neurons when applied during the proliferation phase of ESC-derived neural stem cells. This observation should be useful for producing higher numbers and increased proportions of DA neurons from mouse ESC (and probably human ESC as well), which can then be used for transplantation studies in PD models. This strategy could have the advantage of adding these hormones *in vitro* to produce DA neurons, avoiding the collateral effects of *in vivo* hormonal therapies.

Abbreviations

DA, dopamine; E₂, estradiol; ER, estrogen receptor; ESC, embryonic stem cells; FGF, Fibroblast growth factor; GAPDH, Glyceraldehyde phosphate dehydrogenase; PD, Parkinson's disease; SHH, Sonic hedgehog; TH, Tyrosine hydroxylase.

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Figure 1.

Effects of 17β -E₂, added only in the phase of proliferation in N2 medium with FGF 2, FGF 8 and SHH of neural precursors derived from ESC, on DA neuron number analyzed at end of stage 5. Representative confocal images of immunofluorescence detection for β -Tubulin III (green), TH (red) and co-localization of both markers. Hoechst fluorescence to show the total number of nuclei is shown in blue. Ten nM 17β -E₂ caused an increase and 10 μ M 17β -E₂ diminished the number of DA neurons. The control group was incubated only with cyclodextrin. Scale bar applies to all pictures and represents 100 μ m.

Figure 2.

Effects of 17β-E₂ added during stage 4 (proliferation) in N2 medium with FGF 2, FGF 8 and SHH or stage 5 (differentiation) on DA neuron numbers analyzed at end of stage 5.

Quantification of the total number (A and B) of β-Tubulin III and TH-positive cells and the percentage of TH-positive neurons (C and D) from 8 fields from 3–6 independent experiments made in duplicate. Cells were incubated in N2 medium, with different E₂ concentrations during proliferation (A and C) or differentiation stages (B and D). Results are expressed as mean ± SEM. * P < 0.05 and ** P < 0.001 as compared with corresponding (cyclodextrin) group determined by ANOVA followed by Fisher's test for 3–6 independent experiments.

Figure 3.

Effects of 17β-E₂ added at stage 4, in N2 medium in the presence of FGF 2, FGF 8 and SHH with or without phenol red, on DA neuron number, and its antagonism by ICI 182,780. Quantification of the total number (**A** and **B**) of β-Tubulin III and TH-positive cells and the percentage of TH-positive neurons (**C** and **D**) from 8 fields from 3–5 independent experiments made in duplicate. Cells were incubated in N2 medium with (**A** and **C**) or without (**B** and **D**) phenol red, at the indicated E₂ concentrations during proliferation stage. ICI 182,780 was added at 2 μM where indicated. Results are expressed as mean ± SEM. * P < 0.05 and ** P < 0.001 as compared with control (cyclodextrin) group. & P < 0.05 relative to 10 nM E₂ determined by ANOVA followed by Fisher's test for 3–6 independent experiments.

Figure 4.

Estradiol added during stage 4 in N2 medium without phenol red with FGF 2, FGF 8 and SHH increases TH content at the end of stage 5 in cultures derived from ESC. Cells in stage 4 were treated with cyclodextrin (control), or with 10 nM of 17 β -E₂. Cells were lysed and 30 μ g of protein were separated by electrophoresis on 8% SDS-PAGE. Gels were transferred to nitrocellulose membranes and incubated with anti-TH or anti-GADPH antibodies as described in Experimental methods. The protein-antibody complexes were detected by enhanced chemiluminescence. TH bands were quantified by densitometric analysis and corrected using GADPH protein content. A representative assay is shown in A. Results from 5 experiments

were expressed as mean $\pm$ SEM and are shown in **B**. * $P < 0.05$ relative to control (cyclodextrin) group determined by ANOVA for 3 independent experiments.

Figure 5.

Effects of estradiol added at stage 4 on expression of genes characteristic of midbrain DA neurons. Genes involved in DA neuron function were analyzed by RT-PCR at the end of stage 5. Note that the neuronal marker β -Tubulin III the dopamine-specific gene *DA transporter* (*DAT*) and the DA nigral marker *Ptx3* and *c-Ret* are expressed at higher levels after estradiol exposure in the presence and *SHH* / FGF 8 / FGF 2 in N2 medium without phenol red during stage 4, whereas the other genes have similar expression with or without E₂ treatment. RNA without retrotranscription (-RT) produced no signal. The size of each band is indicated in base pairs (bp). n = 3 independent experiments.

Figure 6.

Estradiol increases the number of Lmx1a+ cells at the end of stage 4 and ICI antagonizes this effect. Cells were incubated in the presence of FGF 2, FGF 8 and SHH, fixed at the end of stage 4 and immunostained. Representative confocal images of immunofluorescence detection for Lmx1a (green), Ki67 (red) and co-localization of both markers. Total nuclei are shown in blue after Hoechst staining. Treatment with 10 nM 17 β -E₂ increased the number of Lmx1a cells. This effect was blocked with 2 μ M ICI 182,780. The control groups were incubated with cyclodextrin or with 2 μ M ICI. In the last column, double-positive cells are marked with arrows. Scale bar applies to all pictures and represents 100 μ m.

A

B

Figure 7.

Increased numbers of proliferating DA precursors after treatment with estradiol, and its reversion by ICI co-incubation. Cells were incubated in N2 medium without phenol red in the presence of FGF 2, FGF 8 and SHH, fixed at the end of stage 4 and immunostained. Quantification of the total number of Lmx1a- and Ki67-positive cells (**A**) and the percentage of Lmx1a/Ki67 double positive cells (**B**) from 8 fields evaluated in 3 independent experiments made in duplicate. Where indicated, 2 μ M ICI 182,780 was present. Results are expressed as mean $\pm$ SEM. * $P < 0.05$ as compared with control (cyclodextrin) group. & $P < 0.05$ relative to 10 nM E₂ determined by ANOVA followed by Fisher's test for 3 independent experiments.