Published in final edited form as:

Brain Res Bull. 2007 June 15; 73(1-3): 75–80.

CHANGES IN THE CONTENT OF ESTROGEN α AND PROGESTERONE RECEPTORS DURING DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS TO DOPAMINE NEURONS

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Abstract

Embryonic stem cells (ESC) can differentiate to derivatives of the three embryonic germ layers. Dopamine neurons have been produced from mouse and human ESC. This *in vitro* induction mimics the developmental program followed by dopaminergic cells *in vivo*. Production of dopamine neurons might have clinical applications for Parkinson's disease, which has a higher incidence in men than in women, suggesting a protective role for sex hormones, particularly progesterone and estradiol. These hormones exert many of their effects through the interaction with their nuclear receptors. In this study, we used a described 5-stage protocol for dopamine neuron differentiation of ESC, allowing neuronal commitment as evidenced by specific markers and by behavioural recovery of hemiparkinsonian rats after grafting. We studied the expression of steroid hormone receptors by immunoblot during this procedure and found an increase in the content of both A and B isoforms of progesterone receptor (PR) and a decrease in estrogen receptor α (ER- α) when cells were at the neural/neuronal stages, when compared with the amount found in initial pluripotent conditions. We also found the same pattern of PR and ER- α expression by immunocytochemistry. Ninety-two percent of dopamine neurons expressed progesterone receptors and only 19% of these neurons co-expressed tyrosine hydroxylase and ER- α . These results show a differential expression pattern of ER- α and PR isoforms during neuronal differentiation of ESC.

Keywords

Sex hormone receptors; Tyrosine Hydroxylase; Nestin; Oct-4; Graft; Estradiol

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Conflict of interest The authors declare no conflict of interest.

1. Introduction

Embryonic stem cells (ESC) are derived from the inner cell mass of mammalian blastocysts. Because of their ability to proliferate indefinitely *in vitro* while maintaining an undifferentiated state, preserving their developmental potential to differentiate into most cell types, ESC are not only useful to analyze critical steps of cell development, but also represent a potential source for cell replacement therapy [1,2].

Midbrain dopamine (DA) neurons can be efficiently induced *in vitro* from mouse ESC with the 5-stage method [3-6]. ESC-derived DA neurons obtained by this and other protocols have improved Parkinsonism in rodents after grafting [3,7,8].

On the other hand, sex steroid hormones, estradiol (E) and progesterone (P), play major roles in development, metabolism and reproduction in mammals. The diverse biological effects of these hormones are primarily mediated by their binding to specific nuclear receptors which act as transcription factors, inducing changes in gene expression. Sex steroid hormone actions are not restricted to tissues involved in reproductive functions, but also influence brain physiology [9]. P and E could also have a beneficial role in neurodegenerative diseases, since incidence of Parkinson's disease is higher in men than in women [10,11]. In fact, several groups have shown that striatal DA depletion by neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [12], methamphetamine [13] and 6-hydroxy-dopamine (6-OHDA) [14] was lower when rodents were pre-treated with estrogens.

There is also evidence that P protects dopaminergic neurons against degeneration induced by MPTP [12] and methamphetamine [15] in rodents. These data suggest that E and P play a role in maintenance of DA neurons. The present study investigated the expression pattern of P receptors (PR) and E receptors (ER)- α at protein level by Western blot during the 5-stage protocol of DA neuron differentiation of mouse ESC.

2. Materials and Methods

In vitro differentiation of ESC to DA neurons

We used R1 mouse ESC from Dr. Nagy's laboratory [16], which have been proved to produce DA neurons [3,8]. The differentiation procedure was performed as reported [3,4]. Briefly, undifferentiated ESC (stage 1) were grown on gelatin-coated tissue culture plates in the presence of 1000 U/ml of leukaemia inhibitory factor (LIF; Chemicon, USA) in medium supplemented with ESC-tested fetal calf serum (Wisent, Canada). To induce formation of floating embryoid bodies (EB, stage 2), cells were dissociated into a single-cell suspension with trypsin and plated onto bacterial dishes in the presence of LIF. EB 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 ITSFn medium. After 9-11 days of culture, cells were dissociated with trypsin and plated in N2 medium, which contained 10 nM P. These neural stem cells were plated on dishes or glass coverslips pre-coated with poly-L-ornithine and 1 μ g/ml mouse laminin (Becton Dickinson, USA), treated with 10 ng/ml Fibroblast Growth Factor-2, 100 ng/ml Fibroblast Growth Factor-8b and 100 ng/ml of human Sonic Hedgehog (growth factors from R & D Systems, USA) for 4 days to expand/instruct DA precursors (stage 4). Differentiation (stage 5) was induced by growth factors withdrawal and feeding with N2 medium with 200 μ M ascorbic acid for 6-8 days.

Immunocytochemistry

Immunocytochemical procedures were carried out using described standard protocols [3,17]. After fixing the cells with 4% paraformaldehyde, primary antibodies were applied as follows: mouse anti-Oct3/4 antibody, 1:1000 (BD Biosciences Pharmingen, USA); rabbit anti-tyrosine

hydroxylase (TH) antibody, 1:1000 (Pel-Freeze, USA); mouse anti- β Tubulin III monoclonal antibody, 1:1000 (Covance, USA); rabbit anti-Nestin, 1:100 (a kind gift from Dr. Ron McKay, NIH). Appropriate fluorescently-labelled secondary antibodies (Molecular Probes, USA) were used alone or in combination, and nuclear detection with Hoechst 33258 (Sigma, USA) is presented in some cases.

For immunocytochemical detection of PR and ER- α , antigen retrieval was performed with 10 mM sodium citrate (Sigma) solution, pH 6.0. The cells were heated in a microwave oven at 1000 W for 3 cycles of 5 minutes, and were cooled between microwave irradiations for 3 minutes, as described previously [18]. They were washed twice with PBS, pH 7.4, and incubated successively with 0.5% triton X-100 in PBS for 30 minutes and 1% normal goat serum in PBS for 30 minutes. Primary antibodies were diluted in PBS, 0.3% triton X-100 and 1% gelatin and incubated for 72 hours at 4° C. Rabbit anti-ER- α (HC-20) antibody (Santa Cruz Biotechnology, USA) was used at 1:50 and rabbit PR Ab-13 (NeoMarkers Inc., USA) was used at 1:100 dilution. This antibody recognizes both PR isoforms. Fluorescent secondary antibodies were used for performing the double immunostaining technique. The negative control consisted of omitting the primary antibody in the incubations. These procedures did not result in any staining (data not shown).

Acquisition of confocal images to study the colocalization of hormone receptors and cell markers

ESC attached to coverslips were tested for Oct-4, Nestin, β Tubulin III/TH, Oct-4/PR, Oct-4/ER- α , TH/PR and TH/ER- α expression by immunostaining, and these preparations were visualized by using a FV1000 confocal microscope (Olympus, Japan), to detect Alexa 488, Alexa 568 and Hoechst fluorescence in a sequential fashion by exciting with different lasers. The same cells were analyzed by Nomarski technique. Individual digital images for each fluorochrome were captured using the Super Apochromat objective 40X (N.A. 1.3, Olympus). To establish co-expression of hormone receptors with others proteins, merged images were generated.

Electrophoresis and Western blot

Assays were performed as described [19]. Briefly, cells of each stage of differentiation were homogenized in lysis buffer (Roche, Germany) supplemented with protease inhibitors. 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: mouse anti-PR monoclonal antibody (Santa Cruz Biotechnology, USA); mouse anti-ER- α monoclonal antibody (Santa Cruz Biotechnology); mouse anti-Oct 3/4 monoclonal antibody (BD Biosciences Pharmingen); rabbit anti-TH (Pel-Freez, USA) and mouse anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Santa Cruz Biotechnology). Membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology; diluted 1:15,000). Immunoreactive bands were detected using a semi-quantitative enhanced chemiluminescence method (Amersham). When needed, membranes were stripped using a commercial solution (Chemicon). The intensity of the protein bands was quantified by densitometry using a Hewlett-Packard Scanner for capturing data and the KODAK 1D Image Analysis Software for densitometric analysis. The density of each band was normalized by GAPDH intensity. To have a positive control for TH, we used 15 μ g of rat *substantia nigra* homogenates and for PR, 20 μ g of T47D breast cancer cells (data not shown).

6-OHDA lesion, transplantation and behavioural testing

The lesion, transplantation and behavioural evaluation was made as described [3]. Surgical procedures were approved by the local animal care and use committee and complied Mexican and NIH guidelines. Briefly, adult female Wistar rats (230-250 g) were injected with 8 µg of 6-OHDA (Sigma) in the left medial forebrain bundle (-1.0 mm anteroposterior, 1.5 mediolateral and -7.5 mm dorsoventral). Fifteen days after injection, apomorphine-induced rotations were quantified during 60 min, and animals were classified as lesioned when they had more than 7 contralateral turns per min. ESC were trypsinized at day 3 of stage 5, and re-suspended at a density of 133,000 viable cells per µl. Three microlitres of the cell suspension were grafted into the lesioned striatum of 4 hemiparkinsonian rats (0.0 mm anteroposterior, 3.0 mm mediolateral and - 5.5 mm dorsoventral). In 3 sham animals, 3 µl of N2 medium was injected. All animals were immunosuppressed daily with cyclosporine A (Neoral, France), starting 24 h before grafting. Animals were evaluated 45 days after sham/graft surgeries by apomorphine administration (1 mg/kg).

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis was made by ANOVA followed by Fisher's test. GB-STAT Version 7.0 program (Dynamic Microsystems, USA) was used for calculating probability values.

3. Results

Stage 1 ESC grew as multicellular colonies characteristic of undifferentiated cells (Fig. 1A). To further confirm that our ESC were in a pluripotent state, we performed immunocytochemistry to detect Oct-4. We found that this marker was present in 92% of the cells (Fig. 1B). In stage 4, cells showed the typical morphology of neural precursors, including rosettes (Fig. 1C). To verify the neural precursor state, we stained for Nestin and found that cells expressed this intermediate filament protein in a high proportion (94%; Fig. 1D). After *in vitro* differentiation to stage 5, neuronal cells are readily observed (Fig. 1E). Dopaminergic differentiation was evidenced by β Tubulin III and TH co-expression in 26% of neurons (Fig. 1F).

Western blot analysis showed that Oct-4 was expressed in stage 1, indicating that ESC were undifferentiated at beginning of the protocol (Fig. 2). At stage 5, we detected TH, the rate-limiting enzyme for the biosynthesis of DA (Fig. 2).

We then analyzed PR isoforms content. In all stages, two bands of 83 and 115 kDa were detected, corresponding to PR-A and PR-B, respectively (Fig. 2). We found that the content of PR-A and PR-B increased significantly in stage 5 to 235% and 313% respectively, in comparison to stage 1 (Fig. 3).

ER- α was detected in all stages as a band of 66 kDa (Fig. 2). In contrast to PR, a higher content of ER- α was present in the first stage and, as differentiation proceeded, its content significantly diminished to 28-29% of its initial value in the last two stages (Fig. 3).

To study the cellular localization of ER- α and PR in undifferentiated ESC and terminally differentiated DA neurons, we performed double immunocytochemistry in stage 1 and 5 cultures. We found that stage 1 cells co-expressed Oct-4 and ER- α in a high proportion (Fig. 4A). In contrast, only a few cells expressed PR at this stage (Fig. 4B). In stage 5, we observed that 19% of TH-positive cells expressed ER- α (Fig. 4C), and PR was present in 92% of all DA neurons (Fig. 4D).

To demonstrate the functionality of DA-producing TH-positive neurons, we grafted stage 5 cells in 4 hemiparkinsonian rats. Animals lesioned with 6-OHDA received either N2 medium (sham, $n = 3$) or a graft of 5×10^5 cells dissociated in day 3 of stage 5. We have used female rats to study the functionality of DA neurons derived from ESC. Although female rats show a lower behavioural dysfunction and reduced DA neuron loss when compared to males after 6-OH-DA injection [20], the selected animals were confirmed to have at least 7 turns per minute after apomorphine administration, before inclusion in our study. As demonstrated before [8] such lesions in female rats are stable for over 8 months without DA grafts. We tested the rotational behaviour after apomorphine injection of sham and grafted animals. Pre-graft rotational values were 601 ± 51 for sham and 586 ± 41 for to-be grafted animals. After 6 weeks, the sham group increased to 920 ± 81 turnings. In contrast, animals grafted with DA neurons derived from ESC, showed a decrease in rotation behaviour (332 ± 59 ; $P < 0.001$ relative to sham). Finally, sham-grafted animals showed no TH-positive elements in the ipsilateral *substantia nigra* or striatum. Animals grafted with ESC had TH-positive neurons in the dorsal striatum (data not shown). No teratomas were observed in these ESC transplantation studies.

4. Discussion

We utilized the 5-stage protocol to study the expression pattern of sex steroid hormone receptors during differentiation events from ESC to DA neurons. We showed an inverse pattern in the content of PR and ER- α during DA differentiation both at cell population and cellular levels. An increase in the content of PR had been reported during the differentiation of mouse ESC to EB formation [21]. This is the first demonstration that the content of PR increased during the generation of neurons, and in contrast, ER- α content was diminished. Our method generated DA neurons as noticed by the presence of reported markers and by reversion of parkinsonian symptoms *in vivo*. All this data confirm the functionality of DA neurons derived from ESC.

E and P have been found to affect dopaminergic function [22]: they enhance striatal DA release in ovariectomized animals [23,24]. P can also rapidly increase DA release in the striatum of rats when measured behaviourally and biochemically *in vivo* [25]. Striatal DA release [26], and DA receptor binding is modified [27] depending on the day of the estrous cycle.

We have reported that the content of PR, ER and their cofactors changes during the estrous cycle in a tissue-specific manner in the brain, and such changes might regulate diverse physiological functions [19,28,29]. Additionally, E and P differentially regulate PR isoforms expression in several tissues. For example, P down-regulates PR in most target tissues [30], but in the brain, P can either decrease PR content or have not effects on it [19]. Interestingly, we have found a higher content of both PR isoforms in stages 4 and 5. In these stages, cells are cultured in medium N2 supplemented with 10 nM P, suggesting that in neural precursors and neurons, PR isoforms are not down-regulated by its ligand. The regulation of PR expression by P in these cells deserves further research. It has been shown that 30-100 μM DA could mediate the activation of the human PR *in vitro* [31]. In this context, it is possible that a cross-talk between DA receptors and PR is taking place, and PR are playing a role in the differentiation of DA neurons.

With regard to the effects of sex steroid hormones in differentiation of neurons, 17 β -estradiol promotes the differentiation and maturation of neuronal cells derived from mouse ESC [32] and from rat neural stem cells [33]. E promoted differentiation and survival of DA neurons differentiated from human neural stem cells, and accordingly with our results, only a subset of the TH-positive neurons co-expressed ER- α [34]. These differentiating and trophic effects of E are probably mediated through the initial activation of membrane ER- α , and intracellular pathways involving calcium elevations and kinases activation, both in neuronal DA precursors

and in astrocytes [35]. Although E can have direct effects on neuronal precursors, stimulation of cytokine secretion by astrocytes is mediated by membrane ER, and is thought to be important for the survival of DA neurons. It is possible that in our differentiating cultures, activation of membrane ER- α is playing a role in DA neuron differentiation or survival.

The expression of ER- α and PR observed in the first stages of development are in accordance with the results obtained by other groups: Hong, et al. (2004) have shown the presence of mRNA for various steroid hormone receptors in undifferentiated human ESC and their EB, as well as the influence of E treatment on the differentiation of these ESC to endodermal lineages [36]. Han et al. (2006) demonstrated that E stimulated proliferation of mouse ESC and that this action can be blocked by the anti-estrogen tamoxifen [37].

The expression of PR seems to be dispensable for the generation of DA neurons, since PR KO mice do have morphologically normal *substantia nigra* and ventral tegmental area [38]. However, PR presence might be helpful since PR KO animals have less TH neurons in the *substantia nigra*. This suggests that the increased PR expression during our differentiation protocol could facilitate DA neuron generation. Recently, addition of a dopaminergic D1 receptor agonist resulted in a ligand-independent activation of ER, which in turn lead to increased PR levels [39]. This novel mechanism was not analyzed in our experiments, but remains as a possible explanation for the increased PR expression. DA release in the absence of estrogen could participate in PR regulation in our differentiating cultures.

In conclusion, our results show a differential regulation of sex steroid hormone receptors during neuronal differentiation of ESC and constitute a starting point to study in detail the functional significance of P and E, and hormone receptor expression during DA neuron production from ESC.

Acknowledgements

This research was supported by Impulsa and DGAPA (IN226703) from Universidad Nacional Autónoma de México, Fundación Alemán and NINDS (1 R01 NS057850-0101A1 to I.V.). N.F. Díaz was supported by a postdoctoral fellowship from DGAPA, UNAM and N.E. Diaz-Martinez was supported by a Conacyt graduate fellowship. We acknowledge Dr. Jorge Aceves and Mr. Arturo Sierra for letting us use their rotometers, and Dr. Araceli Patron and B.Sc. Gabriel Orozco for confocal microscope operation.

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Figure 1.

Differentiation of ESC to DA neurons. **A**, Nomarski Differential Interference Contrast (DIC) of ESC in day 4 of stage 1, growing on gelatin. **B**, Confocal image of immunocytochemical detection of Oct-4 (red) in ESC in the same field shown on panel A. **C**, Nomarski DIC of cells in day 4 of stage 4. **D**, Nestin (green) is expressed in over 90% of cells, indicating its neural precursor state. **E**, Nomarski DIC of cells in day 6 of stage 5. **F**, ESC progeny at stage 5 differentiated into tyrosine hydroxylase (TH)-positive (green) neurons (labelled with β Tubulin III in red). All TH-positive cells co-express β Tubulin III, and therefore they are yellow. Nuclei are shown in blue by Hoechst staining in fluorescence images. Stainings are representative of 4 independent experiments. Scale bar in B applies to all pictures and represent 50 μ m.

Figure 2. Sex steroid receptors and differentiation markers during differentiation of ESC to DA neurons. PR-A and PR-B isoforms, ER- α , Oct-4 and TH protein content during differentiation in the 5-stage method. A representative assay of 3 Western blot experiments, performed as indicated in Methods section is shown. *Substantia nigra* (sn) served as a TH-positive control and GAPDH was used to correct differences in the amount of total loaded protein.

Figure 3.

Densitometric analysis of sex steroid receptors and differentiation markers during differentiation of ESC to DA neurons. PR-A and PR-B isoforms, ER- α , Oct-4 and TH protein contents were quantified by Western blot in 3 independent experiments and normalized by GAPDH signal. Results are expressed as mean $\pm$ SEM. * $P < 0.05$ compared with stages 1-4. ** $P < 0.05$ when compared with stages 1-2. # $P < 0.05$ when compared to stage 1.

Figure 4.

Co-expression of ER- α and PR with Oct-4 or TH in cells at stages 1 and 5, respectively. The left side of each pair shows Nomarski Differential Interference Contrast and the right picture show the merged immunofluorescence images. ESC in day 4 of stage 1 are positive for the pluripotent marker Oct-4, express ER- α in a high proportion (**A**) and PR in only a few cells (**B**). Dopaminergic neurons in day 6 of stage 5 can be identified by TH expression. Some of these DA neurons express ER- α (**C**). In contrast, all TH-positive neurons are also positive for PR (**D**). Stainings are representative of 3 independent experiments. Scale bar in A applies to all pictures and represent 50 μm .