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Changes in estrogen receptor-alpha mRNA in the mouse cortex during development

Amanda K. Prewitt and Melinda E. Wilson

Department of Physiology, College of Medicine, University of Kentucky, Lexington, KY 40536

Abstract

Estrogen plays a critical role in brain development and is responsible for generating sex differences in cognition and emotion. Studies in rodent models have shown high levels of estrogen binding in non-reproductive areas of the brain during development, including the cortex and hippocampus, yet binding is diminished in the same areas of the adult brain. These binding studies demonstrated that estrogen receptors decline in the cortex during development but did not identify which of the two estrogen receptors was present. In the current study, we examined the expression of estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) in the mouse cortex during the first month of life. Messenger RNA was isolated from cortical tissue taken from C57BL/6 mice on postnatal day (PND) 1, 4, 10, 18 and 25 and expression levels were determined by real-time PCR. ER α mRNA expression in the mouse cortex at PND 25 was significantly reduced as compared to PND 1 ($p < 0.01$). ER β mRNA expression at PND25 was significantly increased as compared to PND 1 ($p < 0.05$). Although the increase in ER β mRNA was statistically significant, the ER β levels were extremely low in the isocortex compared to ER α mRNA levels, suggesting that ER α may play a more critical role in the developmental decrease of estradiol binding than ER β . Additionally, we measured ER α mRNA expression in organotypic explant cultures of cortex taken from PND 3 mice. Explants were maintained *in vitro* for 3 weeks. mRNA was isolated at several time points and ER α and ER β mRNA was measured by real-time RT-PCR. ER α and ER β mRNA levels reflected a similar pattern *in vitro* and *in vivo*, suggesting that signals outside the cortex are not needed for this developmental change. This study lays the groundwork for an understanding of the mechanisms of the developmental regulation of ER α mRNA.

1. INTRODUCTION

While estrogen is often thought of as being primarily involved in controlling various aspects of reproduction, it also performs a vital role in regulating many aspects of brain function. Estrogen has been implicated in several non-reproductive brain functions, which include involvement in memory and learning [26], the maintenance of dendritic spine density in the hippocampus [18, 35], modulation of mood [1], and protection against brain injury [3, 9, 15, 33]. Recent studies in postmenopausal humans and aged rats, however, suggest that estrogen may have complex and contradictory actions in the brain in terms of neuroprotection [12, 29].

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Address correspondence and reprint requests to: Dr. Melinda E. Wilson, Department of Physiology, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536, Phone: (859) 323-9618, Fax: (859) 323-1070, melinda.wilson@uky.edu.

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Sex differences in structure and function of the brain are observed throughout the animal kingdom [19]. A complex cascade of events is initiated by gonadal hormones and results in an extensive array of effects associated with sexual differentiation of the brain. Exposure to estrogen during development is believed to result in the development of a phenotypic male brain. The absence of such a hormone milieu results in a shift in development towards a phenotypic female brain [20]. In addition to generating sex differences in structural changes in the brain, estrogen is also responsible for the generation of sex differences in cognition and emotion [2, 6, 13, 21].

Receptor binding studies have demonstrated that early in postnatal life of male and female rodents, estrogen binds not only in reproductive areas of the brain, such as the hypothalamus, but also shows a unique binding pattern in the cortex [28, 30]. In early postnatal development there are high levels of estradiol binding in the cortex and hippocampus and as the animal approaches puberty, this diminishes. In rats, ER α mRNA expression was shown to correlate with the changes in estrogen binding within specific brain regions, including the hippocampus [22, 23, 27, 32]. The expression of ER α or ER β mRNA in the mouse cortex during postnatal development has not been investigated. In the present study we have determined the developmental changes in ER α mRNA and ER β mRNA in mice and have begun to investigate potential mechanisms of this developmental regulation.

2. RESULTS

ER α mRNA levels decrease during development in the male and female mouse cortex

C57BL/6 male and female mice were killed on postnatal days (PND) 1, 4, 10, 18 and 25, with day of birth defined as PND 0. Total RNA was isolated from isocortex and reverse transcribed using oligo-dT primers. The resulting cDNA was subjected to quantitative real-time PCR using ER α specific primers. These primers have previously been shown to produce a single PCR product corresponding to the DNA binding region of the ER α gene [16,11]. Histone 3.1 was included as a housekeeping control gene and all data normalized to its expression as previously described [34]. ER α mRNA levels significantly declined by PND 10 in male mice (Figure 1). There was a ten-fold decrease in ER α mRNA expression in the male between PND 1 and PND 25 ($F_{[4,19]} = 43.82$, $p < 0.001$). Females also demonstrated a ten-fold decrease in ER α mRNA expression between PND 1 and PND 25 ($F_{[4,19]} = 87.61$, $p < 0.001$). There was no statistically significant effect of sex ($p = 0.708$).

ER β mRNA levels increase during development in the mouse cortex

To quantify ER β mRNA expression, C57BL/6 male and female mice were killed on postnatal days (PND) 1, 4, 10, 18 and 25, with day of birth defined as PND 0. Real time PCR for ER β was performed as described for ER α with primers specific to ligand binding domain of the ER β gene [11, 16]. ER β mRNA levels increased in the cortex of the developing male mouse ($F_{[4,19]} = 12.40$, $p = 0.039$) (Figure 2). A similar increase was observed in females ($F_{[4,19]} = 7.644$, $p = 0.0009$). There was no statistically significant difference in ER β mRNA expression between male and female mice ($p = 0.88$).

ER α mRNA levels decrease over developmental time points in organotypic explant cultures of the cortex

To determine if the changes in estrogen receptor expression occur in the absence of inputs from other regions of the brain, ER α and ER β mRNA expression were examined in organotypic explant cultures. Organotypic explant slices contain both neurons and glia and maintain local cytoarchitecture yet are independent of synaptic input from other regions of the brain. The explants also express both types of estrogen receptors, thus providing an outstanding *in vitro* system for studying the regulation of ER α mRNA. Explants were

isolated from C57BL/6 male and female pups at 3 days of age and placed in dissection medium. Media was changed every 3-4 days. The explants were kept in culture for varying lengths of time and 3 explants were pooled for each time point to obtain adequate quantities of total RNA. Total RNA was isolated and reverse transcription and quantitative real-time PCR was performed as described above. ER α mRNA levels were quantified and the data was normalized to the expression of the housekeeping gene Histone 3.1, which was constant throughout the length of time in culture. All time points were expressed relative to Day 1 *in vitro*. ER α mRNA expression levels decreased five-fold in male explants at time points corresponding to PND 11, 18 and 25 ($F_{[5,17]}=13.70$, $p<0.001$) (Figure 3A). Female explants also exhibited a five-fold decrease in ER α mRNA expression at the time points corresponding to PND 11, 18 and 25 ($F_{[5,17]}=27.50$, $p<0.001$) (Figure 3A). There was no statistically significant effect of gender ($p=0.49$). ER β mRNA levels were also examined by means of real-time PCR utilizing the ER β specific primers. ER β mRNA levels were quantified and the data was normalized to the expression of the housekeeping gene Histone 3.1 and expressed relative to Day 1 *in vitro*. ER β mRNA expression increased in explants from both male ($F_{[5,17]}=16.89$, $p<0.001$) and female ($F_{[5,17]}=75.76$, $p<0.001$) mice and with significant increases seen at Day 18 and 14 respectively (Figure 3B). There was no statistical difference between males and females ($p=0.054$). This pattern of ER α and ER β mRNA expression *in vitro* follow the same trends that occur *in vivo*, suggesting that ER α and ER β mRNA expression in the developing mouse cortex is independent of synaptic input from other areas of the brain.

To control for the possible changes in the cellular composition in the explants over time in culture, quantitative real-time PCR utilizing glial fibrillary acidic protein (GFAP) specific primers [7] was performed on the resulting cDNA from the explants. GFAP mRNA levels were quantified and the data was normalized to the expression of the housekeeping gene Histone 3.1 (Figure 4A). Data is expressed relative to Day 1 *in vitro*. No change in relative expression in GFAP mRNA expression in males ($F_{[5,17]}=0.84$, $p=0.549$) or females ($F_{[5,17]}=0.26$, $p=0.922$) suggests that the decrease in ER α mRNA expression in the organotypic explant cultures is not a result of excessive astrocyte growth. Additionally, the overall neuronal content throughout the experiment was measured by assessing the neural marker microtubule-associated protein 2 (MtAP-2) expression (Figure 4B). Quantitative real-time PCR for MtAP-2 showed no significant change across time (males $F_{[5,17]}=0.57$, $p=0.725$ and females $F_{[5,17]}=0.39$, $p=0.840$). Finally, aside from initial cell death that occurs immediately after dissection, there was no significant difference in cell death in the cultures throughout the experiment as measured by propidium iodide uptake (Figure 4C). In addition, explants were fixed (4% paraformaldehyde, 10 min) and processed for immunohistochemistry to examine activated Caspase 3 at a dilution of 1:1000 as previously described in Wilson, 2002. Only an occasional cell positive for activated Caspase 3 was observed (data not shown). Together these results indicate the decrease in ER α mRNA likely results from decreased expression in neurons and not due to cell death or a decline in neuronal population.

ER α protein levels decrease over developmental time points

To confirm that the changes in ER α mRNA reflect changes in protein during development, we assayed ER α protein levels using immunohistochemistry. C57BL/6 male and female mice were killed on PND 7 and PND 42, brains were then removed and flash frozen. Resulting brains were then sliced in 20-micron sections taken from approximately Bregma 1.2 to -0.34 mm, to obtain a rostral-caudal sampling of cortical tissue. The sections were then incubated with a donkey anti-ER α antibody (Upstate C1355) at a dilution of 1:2000, followed by an anti-rabbit conjugated secondary antibody (Jackson Immuno Laboratories) at 1:50. The sections were then developed in diaminobenzidine (DAB) for 5–30 minutes and mounted.

The number and amount of ER α positive cells was decreased at adulthood as compared to PND7 (Figure 5), correlating with the developmental decrease of ER α mRNA expression.

3. DISCUSSION

In the present study we have examined the expression levels of ER α and ER β mRNA in the developing mouse cortex. Estrogen binding studies were performed prior to the discovery of ER β , thus making it difficult to determine if ER α and/or ER β were responsible for the decrease in estrogen binding in the cortex [28]. This study indicates that the developmental decrease in estradiol binding in the mouse cortex is likely a result of a decline ER α mRNA and not ER β mRNA.

In early postnatal development of the mouse, estrogen binding is high in the cortex and diminishes as the animal approaches puberty [28, 30]. This correlates with the drastic decrease in ER α mRNA expression shown in the current study. The current study also shows an increase in ER β mRNA levels, although it is important to remember that they are normalized to PND1 as well. So although the change appears large, the relative levels of ER β mRNA are very low in the cortex at PND 25, thus suggesting that ER α mRNA levels likely plays a larger role in the decrease of estrogen binding in the mouse cortex than ER β mRNA levels.

During development, exposure of the brain to steroid hormones regulates sexual differentiation of the brain. In the developing male brain, testosterone is aromatized to estrogen and this exposure to estrogen early on results in the development of a phenotypic male brain, while its absence results in a shift in development towards a phenotypic female brain [5, 20]. Female brains appear to be deprived from estrogen due to the inability of it to cross the blood brain barrier due to α -fetoprotein [4, 21]. Other data suggests that estrogen-bound α -fetoprotein accumulates in the brain and is the source of estrogens in the female brain [8, 31]. In either case, there was no difference in estradiol binding activity between male and female mice [28]. Similarly, we found that the pattern of ER α and ER β mRNA expression in the developing mouse cortex shows no difference between males and females, suggesting that its regulation is not due to estrogen influences. It remains to be seen if other hormones such as endogenous neurosteroids are involved.

ER α mRNA levels declined with age in the organotypic explant cultures, corresponding with *in vivo* developmental time points. Since this developmental ER α mRNA decrease occurs both *in vivo* and *in vitro*, it appears to be programmed within the cells of the cortex. This developmental decrease in ER α mRNA has been suggested to be intrinsic to the cells that express estrogen receptors in the rat cortex. Studies utilizing grafts of neonatal cortical regions were transplanted into a host 7 days older, showed estradiol binding patterns similar to that of the donor rather than that of the host, suggesting an intrinsic developmental decrease in estrogen receptor expression [24]. Our study correlates with this observation that the decrease in ER α mRNA expression is independent of synaptic input from other areas of the brain. Our data, however, cannot completely rule out the possibility that removal of cortical targets result in a loss of a regulatory signal.

The mechanism of ER α mRNA down regulation is not known. One possible mechanism of ER α mRNA regulation in the cortex may be by epigenetic modification of the ER α promoter. The suppression of ER α mRNA expression by methylation has been observed in breast cancer cells [17]. Normal breast epithelial cells express the ER, while breast cancer cells can be either ER-positive or ER-negative. Studies have shown that in the ER negative cells the ER α promoter is methylated while in ER positive cells the ER α promoter is unmethylated. To date, this possibility has not been studied in detail.

Additionally, ER α mRNA may also be regulated by variations in transcription factor, repressor and/or co-activator expression. A developmental decrease in transcription factors and co-activators would decrease their availability and would thus result in a decrease in the transcriptional machinery necessary for ER α mRNA expression. Transcriptional repressors could also play a potentially important role in regulating ER α mRNA expression. A developmental increase in transcriptional repressors could decrease access of the transcriptional machinery to the ER α promoters, thus resulting in a decrease in ER α mRNA expression. A detailed analysis of transcription factors that regulate ER α expression in the brain have not been performed, although numerous transcription factors undergo developmental regulation in the cortex [25].

The current study establishes the temporal expression of ER α and ER β mRNA in the neonatal mouse. Understanding the expression patterns of ER α mRNA will allow for the basis for thoroughly exploring the regulation of ER α mRNA. As reactivation of ER α expression is required for estrogen in the neuroprotection of the adult cortex following brain injury [10, 11] an understanding of the regulation of ER α mRNA expression in the cortex is not only important to the understanding of basic developmental biology, but also has potential future applications in understanding the role of ER α mediating neuroprotective actions of estrogen.

4. METHODS

Animals

All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facilities at the University of Kentucky. C57BL/6 mice were bred for the purpose of these studies. Animals were maintained in constant temperature conditions on a 14:10 light/dark cycle and were provided food and water *ad libitum*.

Organotypic Explant Cultures

Organotypic cortical explants were isolated from pups at 3 days of age and placed in dissection medium containing 0.2M MgCl₂, 0.75% glucose in Gey's balanced salt solution on ice. Using a Vibratome, 300-micron thick coronal slices were taken from approximately Bregma 1.2 to -0.38 mm [14]. The cortical tissue was dissected on ice dorsal to the corpus callosum from the midline lateral to the secondary somatosensory cortex. Individual cortical slices were then placed on Millicell-CM membranes in 50% 1X BME, 22.5% Hanks BSS, 25% heat-inactivated charcoal-stripped gelding serum, 0.75% glucose, 2 mM glutamine, and phenol red-free media. Organotypic explants were grown in culture for varying lengths of time. Media was changed every 3-4 days. For experiments examining cell death, propidium iodide (5ng/ml) was incubated with the explants for 30 minutes and cells were examined by epifluorescent microscopy.

RNA Extraction

The isocortex from approximately Bregma 1.2 to -0.38 mm was dissected from the nucleus accumbens, dorsal to the corpus callosum and lateral to the beginning of the hippocampal formation male and female mice on postnatal day 1 (PND 1), PND 4, PND 10, PND 18 and PND 25 (PND 0 being day of birth). 50-100 mg of tissue was homogenized in 1 ml of TRIZOL Reagent. The RNA pellet was briefly air dried and suspended in 50 μ l RNase-free water. The suspended RNA was incubated at 56°C for 10 minutes. The RNA was stored at -80°C.

Reverse Transcription

1µg of total RNA for each sample and the appropriate amount of DEPC H₂O was added to bring the volume to 10µl. 1µl of Invitrogen Random Primers and 1 µl of 10mM dNTP's were added to each reaction. The samples were incubated at 65°C for 5 minutes and then chilled on ice. 4µl of 5X first strand buffer, 2µl 0.1M DTT, 1µl RNasin, and 1µl Superscript RT. Samples were then incubated at room temperature for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes.

Real Time PCR

Each reaction contained 21.25 µl of DEPC H₂O, 25µl of 2X SYBRGreen Brilliant Master Mix (Stratagene), 1 µl of upstream primer, 1 µl of downstream primer, 0.75 µl of Reference Dye (diluted 1:500) (Stratagene) and 1µl of appropriate cDNA. Primer specific concentrations were previously optimized for each gene and result in a single DNA RCR product with no primer-dimer formation [7, 11, 34]. Each 96 well plate contained a non-template control and each sample was run in triplicate. The primers for ERα and ERβ are described below. Cycling parameters were as follows: 1 cycle at 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, annealing temperature for 1 minute, 72°C for 30 seconds, and 1 cycle of 95°C for 1 minute and 55°C for 30 seconds. Real time fluorescent measurements were taken at every cycle and change in threshold cycle (ΔCt) was calculated. The ΔCt for each sample was normalized to PND 1 for that gender. All data was normalized to the housekeeping gene Histone 3.1. The primers used for each gene were: ERα [16] 5' AATTCTGACAATCGACGCCAG (+472 relative to ATG), 3'GTGCTTCAACATTCTCCCTCCTC (+794 relative to ATG); ERβ [16] 5'TTCCCGGCAGCACCAGTAACC (+38 relative to ATG), 3'TCCCTCTTTGCGTTTGACTA (+279 relative to ATG); GFAP [7] 5'ACCGCATCACCATTCTGTAC, 3'TGGCCTTCTGACACGGATT; Histone 3.1 [34] 5' GCAAGAGTGCGCCCTCTACTG, 3' GGCCTCACTTGCCCTCCTGCAA. MtAP-2 primers were purchased from Superarray and have been optimized to produce DNA products specific toMtAP-2 and are suitable for quantitative real-time PCR.

Immunohistochemistry

C57BL/6 female mice were killed on PND 7 and adult. The brains were removed and rapidly frozen in -20°C 2-methylbutane and stored at -80°C. They were then sectioned on a cryostat in 20µm. Sections taken from Bregma 1.98 to -2.12 were then placed on slides and fixed by 4% paraformaldehyde in PBS for 30 minutes. After blocking endogenous peroxidase activity with 0.30% peroxidase and non-specific binding 10% Normal Donkey Serum (Jackson Laboratories) the sections were then incubated with a donkey anti-ERα antibody (Upstate C1355) at a dilution of 1:2000 overnight at 4°C, followed by incubation in an anti-rabbit conjugated secondary antibody (Jackson Immuno Laboratories) at 1:50 for 1 hour at room temperature. The sections were then developed in diaminobenzidine (DAB) for 5 –30 minutes and then mounted using Cytoseal (Stephens Scientific).

Statistics

Four animals were analyzed for each time point using two-way analysis of variance (ANOVA) (age × sex). One-way ANOVA and Student Neuman-Keuls post-hoc analysis were performed where appropriate. Results were considered significant at a p <0.05.

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Figure 1.

ERα mRNA expression in mouse cortex decreases during development in both male and female mice. Quantitative real-time PCR was performed on RNA isolated from PND1, PND4, PND10, PND18 and PND25 mouse cortex. Data was normalized to the housekeeping gene Histone 3.1 and expressed relative to PND1. Bars represent the mean $\pm$ SEM, $n=4$. * = significantly different from PND1.

Figure 2.

ERβ mRNA expression in the mouse cortex increases during postnatal development in both male and female mice. Quantitative real-time PCR was performed on RNA isolated from PND1, PND4, PND10, PND18 and PND25 mouse cortex. Data was normalized to the housekeeping gene Histone 3.1 and expressed relative to PND1. Bars represent the mean $\pm$ SEM, n=4. * = significantly different from PND1.

Figure 3.

ER α and ER β mRNA expression *in vitro* follows *in vivo* trends across developmental time points. Quantitative Real-time PCR utilizing ER α specific primers was performed on RNA isolated from explants from PND3 males and females kept in culture for varying lengths of time (A). Data was normalized to the housekeeping gene Histone 3.1 and expressed relative to PND1. (B) Quantitative Real-time PCR utilizing ER β specific primers was performed on

RNA isolated from explants from PND3 males and females kept in culture for varying lengths of time. Data was normalized to the housekeeping gene Histone 3.1 and expressed relative to PND1. Three explants were pooled at each time point. Bars represent the mean $\pm$ SEM, n=3. * = significantly different from PND4.

Figure 4.

Control gene expression in explant cultures. (A) GFAP mRNA expression remains consistent across age of organotypic explant cultures. Quantitative real-time PCR was performed on the RNA isolated from the organotypic explant cultures of both males and females using mouse GFAP specific primers. Bars represent the mean $\pm$ SEM, $n=3$. (B) Neuronal content during the experiment was assessed by quantitative real-time PCR of MtAP-2 mRNA levels. Data was normalized to the housekeeping gene Histone 3.1 Bars represent the mean $\pm$ SEM, $n=3$. (C) Cell death in the cultures was monitored by propidium iodide uptake, which is indicative of cell death. The number of dead cells per six random fields (200X) were counted and averaged. Bars represent the mean $\pm$ SEM, $n=3$.

Figure 5.

ERα protein expression in the cortex decreases during development. ERα specific immunohistochemistry was performed on slices from PND7 (A,C) and Adult (B,D) animals. A representative micrograph of cortex (A, B) and medial preoptic area (C, D) is shown (magnification = 100X). Dark spots represent immunopositive nuclei.