Published in final edited form as:

Horm Behav. 2011 March ; 59(3): 353–357. doi:10.1016/j.yhbeh.2010.08.004.

Estrogen receptor-alpha gene expression in the cortex: Sex differences during development and in adulthood

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Abstract

17 β -estradiol is a hormone with far-reaching organizational, activational and protective actions in both male and female brains. The organizational effects of early estrogen exposure are essential for long-lasting behavioral and cognitive functions. Estradiol mediates many of its effects through the intracellular receptors, estrogen receptor-alpha (ER α) and estrogen receptor-beta (ER β). In the rodent cerebral cortex, estrogen receptor expression is high early in postnatal life and declines dramatically as the animal approaches puberty. This decline is accompanied by decreased expression of ER α mRNA. This change in expression is the same in both males and females in the developing isocortex and hippocampus. An understanding of the molecular mechanisms involved in the regulation of estrogen receptor alpha (ER α) gene expression is critical for understanding the developmental, as well as changes in postpubertal expression of the estrogen receptor. One mechanism of suppressing gene expression is by the epigenetic modification of the promoter regions by DNA methylation that results in gene silencing. The decrease in ER α mRNA expression during development is accompanied by an increase in promoter methylation. Another example of regulation of ER α gene expression in the adult cortex is the changes that occur following neuronal injury. Many animal studies have demonstrated that the endogenous estrogen, 17 β -estradiol, is neuroprotective. Specifically, low levels of estradiol protect the cortex from neuronal death following middle cerebral artery occlusion (MCAO). In females, this protection is mediated through an ER α -dependent mechanism. ER α expression is rapidly increased following MCAO in females, but not in males. This increase is accompanied by a decrease in methylation of the promoter suggesting a return to the developmental program of gene expression within neurons. Taken together, during development and in adulthood, regulation of ER α gene expression in the cortex can occur by DNA methylation and in a sex-dependent fashion in the adult brain.

Introduction

Estrogens have long been known to play a crucial role in coordinating many neuroendocrine events that control sexual development, sexual behaviour and reproduction. 17 β -estradiol is the primary biologically active form of estrogen. In rodents, estradiol is critical for sexual differentiation of the brain (see review by (McCarthy, 2008)). For example, estradiol organizes neural circuits and regulates apoptosis of neurons leading to long-term differences in the male and female brain (Anderson et al., 1986; Rhees et al., 1990; Toran-Allerand,

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1976). In addition to its role in development, estradiol modulates numerous facets of brain function in the adult brain (for review see (McEwen and Alves, 1999) and (Simpkins and Singh, 2008)). Estradiol prevents neuronal cell death in a variety of brain injury models, modulates learning and memory and promotes the formation of synapses (Li et al., 2004; Sherwin, 1994; Simpkins et al., 1994; Wise et al., 2001; Woolley and McEwen, 1993).

The physiological effects resulting from estradiol actions in target tissues are mediated primarily by two intracellular receptors, ER α and ER β (Green et al., 1986; Koike et al., 1987; Kuiper et al., 1996; Mosselman et al., 1996; White et al., 1987). Both ER α and ER β have been observed in neurons and glia in the brain (Chaban et al., 2004; Donahue et al., 2000), and both are expressed throughout the brain with distinct patterns in different brain regions and with differing levels of expression during development (Gonzalez et al., 2007; Osterlund et al., 2000a; Osterlund et al., 2000b; Prewitt, 2007; Shughrue et al., 1997).

The ER α gene is highly conserved between human and rodents, containing similar gene and protein structures. ER α is preceded by multiple promoters that generate several mRNA splice variants (Hirata et al., 2001; Kos et al., 2001; Monje et al., 2007). Alternative splicing occurs at the first exon, which is then spliced to a common splice acceptor site upstream of the translational initiation codon in Exon 1. This alternative promoter splicing results in mRNA splice variants that differ only in their 5' untranslated region encoding the same protein. These different mRNAs may be important in regulating stability or processing of the mRNA (Kos et al., 2001). Differences in the 5'UTRs may also provide for areas of different epigenetic modifications that can then modulate changes in gene expression. The regulatory binding sites of transcription factors that control gene expression are also likely located in the regions surrounding these promoters. To date, relatively little is known about the molecular composition of the regulatory elements that control ER α gene expression.

The regulation of gene expression by epigenetic modification is an emerging mechanism for controlling neuronal gene expression. Epigenetic modification of chromatin involves changes to DNA bases and the associated proteins (for review see (Wolffe and Matzke, 1999) and (Klose and Bird, 2006)) in the absence of changes in the DNA sequence. Epigenetic modifications include histone acetylation, histone methylation and DNA methylation (Bird and Wolffe, 1999; Cooper and Krawczak, 1989). The first step in DNA methylation results in the enzymatic transfer of a methyl group to the 5'-position of the pyrimidine ring of a cytosine residue followed by a guanine (CpG dinucleotides). The modification of the cytosines in CpG residues are carried out initially by the enzyme DNA methyltransferase 3A (DNMT3A) and maintained by DNMT1 (Klose and Bird, 2006). CpG residues are often found upstream or downstream of the transcriptional start site.

The methylated CpGs are stabilized by methyl-CpG-binding proteins. The family of methyl-CpG-binding proteins contains several members including methyl binding domain (MBD) proteins 1, 2, 3, 4 and MeCP2 (Nan et al., 1998; Ng et al., 2000; Ng et al., 1999). These proteins bind to the methylated CpG residues potentially disrupting transcription. These proteins can also associate with co-repressor protein complexes of promoters that include histone deacetylases (HDAC) (Ballestar and Wolffe, 2001; Wade, 2001). Together, these complexes suppress transcription of genes with methylated promoter DNA.

Epigenetic modification of chromatin in neurons has been shown to play an important role in regulating gene expression during neuronal development and in learning and memory (Kiefer, 2007; Levenson and Sweatt, 2005). MeCP2 gene mutations are also the cause of some cases of Rett syndrome, a progressive neurological developmental disorder that appears during early childhood when sensory experience is driving the synaptic reorganization required for creating mature circuits in the brain (Zoghbi, 2003) (Guy et al.,

2001). Furthermore, MeCP2 is present in high levels in mature neurons (Meehan et al., 1992), and studies suggest that the MeCP2 protein plays a role in forming synapses between neurons (Zhou et al., 2006). Additionally, MeCP2 is differentially expressed in the hypothalamus during a critical time in sexual differentiation of the brain (Kurian et al., 2008). Furthermore, DNMT3 expression has also been shown to be dynamically regulated in the developing brain as well as in the adult cortex (Feng et al., 2005; Siegmund et al., 2007).

The ER α gene undergoes changes in promoter methylation under normal and pathological conditions. For example, methylation of the ER α promoter has been reported to occur in the colon during aging (Issa et al., 1994) and changes in the expression of ER α have been associated with the progression of numerous types of cancerous tissues including breast and lung (Issa et al., 1994; Issa et al., 1996; Lapidus et al., 1998; O'Doherty et al., 2002; Oh et al., 2001; Ottaviano et al., 1994; Sasaki et al., 2002). Although evidence exists for multiple mechanisms of epigenetic modification of the ER α gene, DNA methylation has been the most widely described epigenetic phenomenon, and is the first epigenetic mechanism we have investigated.

Developmental regulation of estrogen receptor-alpha mRNA

ER α protein and mRNA levels change dramatically during postnatal brain development (Shughrue et al., 1997; Simerly et al., 1990; Toran-Allerand et al., 1992). High levels of estradiol binding in non-hypothalamic regions such as the cortex and hippocampus during the first two weeks of life have been identified by receptor autoradiography (Pfaff and Keiner, 1973; Sheridan, 1979; Shughrue et al., 1990). This expression declines as animals approach puberty. In later studies in rats and mice, ER α mRNA expression was shown to correlate with the changes in estrogen binding in the hippocampus and cortex (O'Keefe et al., 1995; Prewitt, 2007). While estrogen receptors can be expressed in both neurons and glial cells, studies using *in situ* hybridization have demonstrated that the expression in the cortex is primarily in neurons. We examined ER α mRNA expression in the isocortex during postnatal development by quantitative real-time RT-PCR and determined that the decline in ER α mRNA expression begins during the second week of life (Prewitt, 2007) (Figure 1). We also examined ER α mRNA expression in the prefrontal cortex as this area may be responsible for cognitive processes potentially modified by estradiol. We saw a similar decline in ER α mRNA expression, however, with a significant sex difference on PND10. The result of this difference at this age is not known in rodents, but may play a role in sex differences and hormonal influences observed in cognitive behaviors associated with the prefrontal cortex (Galea et al., 2008; Overman, 2004; Welborn et al., 2009). Consistent with other studies, we have found that ER α mRNA in the whole hippocampus declines during development as well (Figure 1) replicating what others have shown (Shughrue et al., 1990 and O'Keefe, 1995 #179).

To determine the mechanisms by which this developmental regulation of ER α mRNA occurs, several experiments have been performed to test whether the signals are intrinsic to the neurons themselves. When either fetal rat hippocampal or cortical tissue was transplanted to the brain of a neonatal animal, the developmental profile of ER α mRNA expression in the transplants continued with the profile of the age of the donor (O'Keefe et al., 1993). This suggests that the control of ER α gene expression is programmed in the developing tissue and does not rely on cues from the surrounding tissue. Similar results were observed with transplants from the hypothalamus transplanted to animals treated with different hormonal paradigms (Paden et al., 1985), suggesting that hormonal signals do not regulate ER α expression at this age.

The molecular factors that control ER α mRNA expression at the transcriptional levels are not well known. One example, however, includes the transcription factor Stat5 (Champagne

et al., 2006). Stat5 is associated with the JAK2-Stat5 signaling pathway and has shown to be activated by prolactin (Frasor et al., 2001). The rat promoter contains the consensus sequence for Stat5 (Frasor and Gibori, 2003). Following prolactin treatment, ER α mRNA expression is increased in the uterus and in the medial preoptic area of the hypothalamus. Furthermore, inhibition of Stat5 binding abolishes the ability of prolactin to increase ER α gene expression indicating its role in the hypothalamus (Champagne et al., 2006). While the possibility of this factor regulating ER α mRNA in the cortex exists, the expression of Stat1 and Stat5 actually increase during postnatal development (Claudio et al., 1998), suggesting it is likely that additional mechanisms of ER α mRNA regulation in the brain will be found.

To investigate such additional potential mechanisms regulating this decline in ER α mRNA expression across early postnatal development, we examined the methylation status of the ER α promoter (Prewitt et al., 2007). Genomic DNA was isolated from cortical tissue and the methylation status of the ER α promoters was analyzed by methylation specific PCR (MSP) followed by pyrosequencing of the product (Westberry et al., 2010). This technique examines the methylation status of the cytosines in CpG regions of the promoter. Using this technique, we have observed that several of the promoters of the mouse ER α gene become progressively methylated beginning at postnatal day 10 (Westberry et al., 2010). This age corresponds with the beginning of the decline in ER α mRNA expression in the cortex. Furthermore, chromatin precipitation assays determined that the methyl-DNA binding protein, MeCP2, is associated with this promoter at the same time it becomes methylated. These observations suggest that methylation may play a role in the suppression of ER α mRNA in the developing brain and that the ER α promoter is a target of MeCP2 activity. Matching the mRNA expression, no sex difference was observed. Additionally we examined the expression pattern of the *de novo* DNMT, DNMT3A, in the isocortex, prefrontal cortex and hippocampus. A gradual increase with age was observed in DNMT3A mRNA expression and no sex difference was seen (Wilson, 2009). However, DNMT1 mRNA was significantly lower in the adult male prefrontal cortex and hippocampus. Suggesting potential sex differences in modulation of genes by methylation in the adult.

Regulation of estrogen receptor-alpha mRNA following stroke

In addition to the developmental changes in expression in ER α mRNA widely described, ER α mRNA expression is also dramatically regulated following brain injury. Middle cerebral artery occlusion (MCAO) is a well-established model of focal ischemia. Studies in rats and mice have demonstrated a gender difference in neuronal cell death following MCAO. Female brains are consistently protected against cell death (Alkayed et al., 1998; Rusa et al., 1999; Simpkins et al., 1997). Pretreatment with low doses of estradiol prevents apoptosis in the overlying penumbral cortex of both female and male rats (Dubal et al., 1998; Rusa et al., 1999; Toung et al., 1998). In this model of brain injury the ability of estradiol to act as a neuroprotectant is dependent on the presence of ER α (Dubal et al., 2001; Rau et al., 2001). As discussed above, ER α mRNA expression is very low in the adult cortex, however, following unilateral MCAO, the ER α gene in the cortex is rapidly increased on the injured side of the brain (Dubal et al., 2006). This activation is independent of estradiol treatment, although some differences are observed in the timecourse of expression. Furthermore, this increase is critical for estradiol neuroprotection because in female ER α knockout mice, estradiol is no longer protective (Dubal et al., 1999). We have extended these studies and performed MCAO in males and females to examine the regulation of ER α gene expression. In this model of injury, males have a much larger MCAO-induced cell loss than females and estradiol is also neuroprotective (Alkayed et al., 1998; Toung et al., 1998). We determined that the increase in ER α mRNA expression only occurs in females, as males do not show a similar increase (Westberry et al., 2008a). Furthermore, estradiol treatment had no effect in females or males on the induction of ER α

mRNA expression following MCAO. Interestingly, in gonadectomized males ER α mRNA expression is increased as in females suggesting a suppressive effect of androgens on the ability of the ER α gene to be activated following MCAO (Westberry et al., 2009). The fact that E2 can be protective in males but we failed to show a change in ER α mRNA expression, suggests that the mechanisms of neuroprotection by estradiol is different in males and females and remains to be determined. Additionally, alterations in the regulation of DNA methylation of the ER α promoter resulting in changes in mRNA expression could contribute to the observation that estradiol is not neuroprotective in reproductively senescent females (Selvamani and Sohrabji, 2008). Although ER α receptor levels were not examined in this study, overall DNA methylation levels increase in the aging brain (Richardson, 2003) and we have observed effects of age on the methylation of the ER β promoter in the aging rat (Westberry et al., 2008b).

We have also examined methylation status of the ER α promoter in intact male and female rats following MCAO (Westberry et al., 2008a). Genomic DNA was isolated from micropunches from the cortex adjacent to the area of the injury or on the corresponding areas on the control side of the brain 24 hours after injury and individual CpG residues analyzed by pyrosequencing. We found that ischemia decreased methylation in the ischemic cortex of females, while there was no change in methylation in the male. Interestingly some CpGs are never methylated, some are always methylated, and others appear susceptible to changes after injury. More CpG islands are methylated on the control side of the brain as compared to the injured side. Furthermore, the overall level of methylation of the ER α gene is lower in the injured side of the brain.

The association of MeCP2 with the ER α promoter using chromatin immunoprecipitation assays was also examined in intact male and female rats after MCAO. MeCP2 was associated with the ER α promoter on the uninjured side of the brain of both male and female rats, but dissociated from the ER α promoter following injury only in female rats, corresponding with the methylation status of the promoter (Westberry et al., 2008a). Together these data demonstrate a difference in the regulation of ER α mRNA expression in response to MCAO between male and female rats and this difference is correlated with altered DNA methylation. Additionally, the male gonadal hormones may influence the changes in DNA methylation and gene expression following a neuronal injury.

Taken together these data suggest that following a brain injury such as stroke, the gene expression mechanisms within neurons are altered, particularly in females, and resemble those observed early in development, potentially as a compensatory mechanism to prevent cell death or as a potential recovery mechanism. Previously changes in expression in proteins responsible for neurodevelopment including growth-associated-protein-43 and microtubule-associated protein 1B have been shown to be regulated following a neuronal injury (Emery et al., 2003) leading to the hypothesis that neurons revert back to an early neurodevelopment pattern of gene expression when challenged with an injury. The data discussed here with the reversible epigenetic regulation of the ER α gene would lend further support to this hypothesis.

Summary

Estrogens mediate many diverse and critical actions in the brain. Most of these actions require the presence of classical estrogen receptors. Thus, coordinated regulation of the estrogen receptor genes is critical for mediating these responses to estrogens in an age, gender, and brain region-specific manner. Alterations in the normal regulation either during development, disease or aging could potentially interfere with estradiol action. We have begun to identify numerous physiological influences that can influence the expression of the estrogen receptor gene by reversible epigenetic mechanisms. Furthermore, some of these

mechanisms are differentially regulated in the male and female brain, particularly in the adult. A deeper understanding of these mechanisms will ultimately lead to our understanding of the molecular mechanisms underlying differences in the male and female brain, and importantly, differences in how the male and female brain may be able to respond to neuronal insults encountered with injury, neurodegeneration and normal aging.

Acknowledgments

This work cited from our laboratory was supported by the National Science Foundation (NSF IOS0919944), COBRE grant P20 RR15592 from the National Center for Research Resources (NCRR), and R01 HL073693 (MEW). All opinions, findings and conclusions expressed in this material are those of the authors and not those necessarily of NSF or NCRR.

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Figure 1. ERα mRNA expression during early postnatal development

RNA was extracted from brain tissue taken from female and male mice at PND 4, 10, 18 and 25. RNA was reverse-transcribed and the resulting cDNA was used for real time PCR using ERα-specific primers. **(A.)** ERα mRNA expression was high in both the male and female isocortex at PND 4. ERα was significantly decreased in both male and female cortex at PND 10, 18 and 25 (Prewitt, 2007). **(B.)** ERα mRNA expression was high in both the male and female PFC at PND 4. ERα was significantly increased in the female, but not male PFC at PND 10. ERα was significantly decreased in both male and female PFC at PND 18 and 25. **(C.)** ERα mRNA expression was high in both the male and female hippocampus at PND 4. ERα was significantly decreased in both male and female hippocampus at PND 10, 18 and 25. Data was normalized to Histone 3.1 and compared to PND 4. Samples were run in triplicate. In all three brain regions, two-way ANOVA revealed a significant overall effect of age ($p < 0.01$). Asterisks on the graph indicate significant differences from PND 4 ($p < 0.05$, $n = 6-8$ per timepoint).

Estrogen Receptor- α Promoter Methylation		
	Sexual	Adult/Child Ratio
Insular	No sex difference	No
Prefrontal Cortex	Sex difference in temporal profile	Yes
Hippocampus	No sex difference	N/D

Figure 2. Summary of sex differences in ER α promoter methylation in the brain
N/D not determined.