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SHORT COMMUNICATION

Estrogen receptor alpha (*Esr1*) regulates aromatase (*Cyp19a1*) expression in the mouse brain

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Abstract

OBJECTIVES: Local estrogen production in the brain regulates critical functions including neuronal development, gonadotropin secretion and sexual behavior. In the mouse brain, a 36 kb distal promoter (l.f) regulates the *Cyp19a1* gene that encodes aromatase, the key enzyme for estrogen biosynthesis. *In vitro*, promoter l.f interacts with estrogen receptor alpha (*Esr1*) to mediate *Cyp19a1* mRNA expression and enzyme activity in mouse hypothalamic neuronal cell lines. The *in vivo* mechanisms that control mammalian brain aromatase expression during fetal and adult development, however, are not thoroughly understood. Our aim was to elucidate the basis of the in vivo connection between *Esr1* and *Cyp19a1*.
METHODS: Pregnant mice were sacrificed at gestational days 9, 11, 13, 15, 16, 19, 21 and the brain tissues of the fetuses were harvested along with five newborns at the age of postnatal day 2. *Esr1*KO (female) were also sacrificed and their hypothalamus were excised out. Then both fetuses and adults RNA were isolated, reverse transcribed and amplified employing primers specific for *Esr1* and *Cyp19a1* with Real time PCR.
RESULTS: In the fetal mouse brain, *Cyp19a1* mRNA levels are inversely correlated with estrogen receptor alpha (*Esr1*) mRNA levels in a temporal manner. Moreover, *Cyp19a1* mRNA levels increased in the hypothalamus of estrogen receptor-alpha knockout female mice (*Esr1*KO).
CONCLUSION: Taken together, our findings might indicate that *Esr1* has crucial roles in the in vivo regulation of aromatase expression in the brain during fetal and adult life.

INTRODUCTION

Sex steroids influence most aspects of cellular organization and mammalian development including shaping neural functions and reproductive behaviors (Turgeon *et al.* 2006). Sex steroids are primarily synthesized by male and female reproductive organs and the adrenal cortex, but they can also be locally synthesized by various tissues and organs including the brain (Wrobel & Karasek 2008).

An important member of sex steroids is estrogens, which are synthesized in a series of six main enzymatic steps that begin with the entry of cholesterol into the mitochondria. The most important step of sex steroid synthesis the conversion of C_{19} steroids to estradiol (E_2) is catalyzed by an enzyme called aromatase, which is the key enzyme in steroidogenesis (Bulun *et al.* 2005).

Aromatase is encoded by the *CYP19A1* gene in humans, which spans approximately 123 kb on chromosome 15q21.2. The ATG translational start site is located in coding exon II, and the coding region of aromatase protein is found within 30 kb of the 3'-end and contains nine exons (II–X). The 93-kb 5'-flanking region upstream of the coding region contains a number of alternative untranslated first exons, the expression of which is driven by multiple tissue-specific promoters. Transcription initiated by use of each promoter gives rise to a transcript with a unique 5'-untranslated end that contains the sequence encoded in the first exon immediately downstream of this particular promoter. Therefore, the 5'-untranslated region of aromatase mRNA is promoter-specific and may be viewed as a signature of the particular promoter used. It should be emphasized that all of these 5'-ends are spliced onto a common junction 38 bp upstream of the ATG translation start site. Therefore, the sequence encoding the open reading frame is identical in each case. Thus, the expressed protein is the same regardless of the splicing pattern (Bulun *et al.* 2005).

In mice, aromatase is also encoded by a single gene, *Cyp19a1*, located on chromosome 9. Similar to humans, the ATG translation start site lies in coding exon II and the coding region of aromatase protein is found in the downstream 29-kb portion of the gene and contains 9 exons (II–X). In contrast to the human gene, only 3 tissue-specific untranslated first exons of the mouse *Cyp19a1* gene have been reported, including an ovary-specific first exon (Eov), a testis-specific first exon (Etes), and a brain-specific first exon (Ebr). To generate mouse tissue-specific aromatase transcripts, a tissue-specific first exon is spliced onto a common coding region as a result of the activationof its upstream promoters, which regulate aromatase expression in the ovary, testis, or brain. The mouse ovary and brain-specific first exons share 100% and 93%homology with the human exons PII and I.f, respectively, whereas the recently discovered testis-specific first exon is unique to the mouse. According to a previous study, the entire mouse *Cyp19a1* locus is approximately 60 kb. The 3' untranslated first exons and their flanking promoter regions span more than 30 kb, whereas the 9 coding exons are restricted to about 29 kb. Promoters for Ebr and Etes are located about 31 kb and 10 kb upstream of the translation start site. As in other species, the promoter for Eov is the most proximal promoter, located 121 bp upstream of the translation start site (Zhao *et al.* 2009).

In the vertebrate brain, aromatase is primarily expressed in the hypothalamus, hippocampus and amygdala via a highly conserved promoter I.f (Lephart 1997; Honda *et al.* 1999). Aromatase expression in the hypothalamus is primarily localized in the medial preoptic area and the ventromedial nucleus of the hypothalamus, which are the centers that govern reproductive functions of both sexes of different species (Voig *et al.* 2007; Zhao *et al.* 2007).

The molecular mechanisms regarding regulation of brain-specific aromatase promoter I.f are not well understood. Recently, E_2 was found to alternately regulate hypothalamic aromatase mRNA expression and enzyme activity via estrogen receptors (*Esr1*) *in vitro* (Livonen *et al.* 2006; Yilmaz *et al.* 2009). These studies, however, did not establish an *in vivo* connection between aromatase expression and promoter I.f regulation in the brain. In this study, we focused on simultaneous mRNA expression profiles of *Cyp19a1* and *Esr1* in the fetal mouse brain at different stages of pregnancy and in the hypothalamus of estrogen receptor-alpha knockout female mice (*Esr1*KO).

MATERIAL AND METHODS

Animals were maintained and experiments were conducted in accordance with the accepted standards of humane animal care. Animal use procedures were approved by the Northwestern University and Cukurova University Animal Care and Use Committee. *Esr1*KO mice on a C57BL/6 background, and Swiss albino mice were maintained on a 14-hour light: 10-hour dark cycle with standard chow (7912; Harlan Teklad, Madison, WI) and water *ad libitum*. Female mice were mated with potential fertile male mice. Successful matings were confirmed by vaginal plug formation (the formation of vaginal plug=day 1 of gestation). Pregnant mice were sacrificed at gestational days 9, 11, 13, 15, 16, 19, 21 and the brain tissues of the fetuses were harvested along with five newborns at postnatal day 2. Fetal mouse brains of both sexes were pooled before any further procedures since their Cyp19a1 expression profiles are similar. Thirteen to fifteen week-old six *Esr1*KO (female) were sacrificed and their hypothalamus were excised out.

Total RNA from the brains of the littermates and hypothalamus of the adult mice were extracted with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). cDNA was synthesized from 5 µg RNA using oligo

(dT) primers with Superscript™ III first-strand kit as recommended by the supplier (Invitrogen, Carlsbad, California, USA). Real-time PCR was performed with 5 µl cDNA using Power SYBR® green PCR kit (Applied Biosystems, Foster City, California, USA) in an ABI 7900 HT fast real-time PCR system (Applied Biosystems). The primers used were as follows: *Cyp19a1*; forward 5'-ctgcagacactactactaca-3', reverse 5'-atccgagtcactgctctcag-3'; *Esr1*; forward 5'-atgaaaggcggcatacggaaag-3', reverse 5'-cacccatttcatttcggccttc-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); forward 5'-tgttaccaactgggacgaca-3', reverse 5'-ggggtgttgaaggtctcaaa-3'. Polymerase chain reactions were carried out in triplicates (SEMs were calculated according to PCR wells of each sample) in a 25 µl reaction volume with SYBR green PCR mix. The reactions were incubated at 50 °C for 2 min and 95 °C 10 min, followed by 40 cycles of 95 °C 15 sec and 60 °C 1 min. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. TaqMan C_T values were converted into absolute copy numbers using a standard curve. All RNA samples were normalized to the expression levels of a *GAPDH* gene endogenous control. "No template" and "no-RT" controls were used to ensure the absence of genomic DNA and reaction specificity. Three independent experiments were performed to demonstrate reproducibility. Real-time RT-PCR product specificity was confirmed by melt curve analysis, gel electrophoresis, and product sequencing.

Statistical analyses were performed by Welch paired t-test and one-way ANOVA followed by Tukey multiple comparisons test using the StatView 5.0 Statistical Software package (SAS Institute, Cary, NC, USA). Significance was determined at α=0.05 and β=0.20. The values for *Cyp19a1*and *Esr1* were provided as the mean ± SEM.

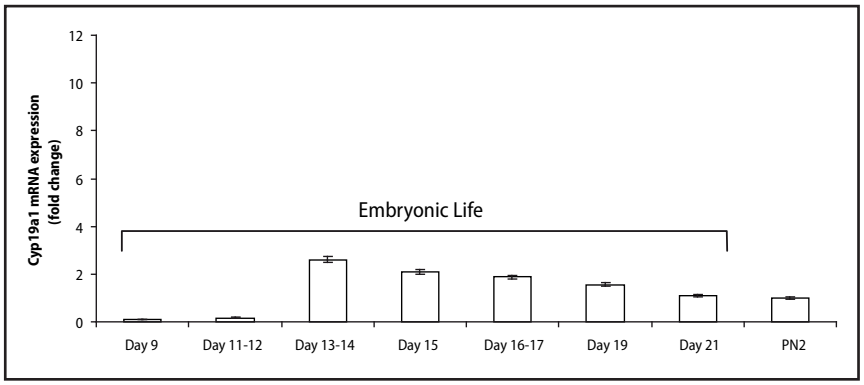

Fig. 1. Aromatase (*Cyp19a1*) mRNA expression in the fetal mouse brain. Fold change of *Cyp19a1* mRNA expression of various embryonic days and postnatal day 2 is shown. Results were normalized to the expression levels of *GAPDH* gene as an endogenous control. The results are expressed as mean ±S.E.M. from three independent experiments (p<0.01, paired t test).

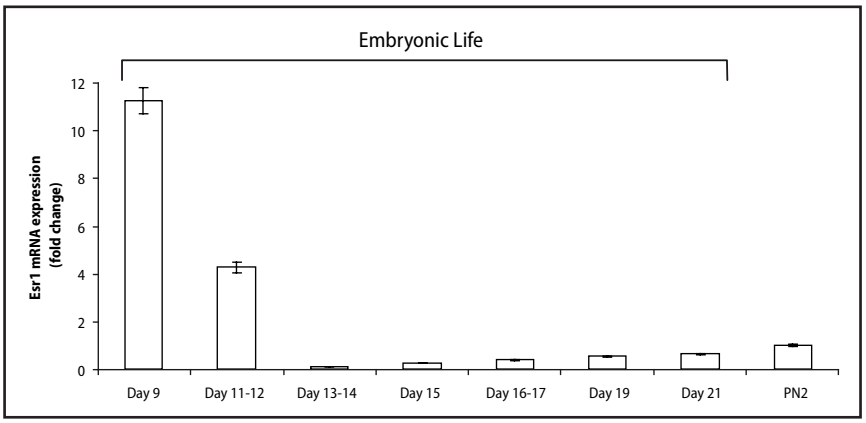

Fig. 2. Estrogen receptor (*Esr1*) mRNA expression in the fetal mouse brain. Fold changes of *Esr1* mRNA expression of various embryonic days and postnatal day 2 is shown. Results were normalized to the expression levels of *GAPDH* gene as an endogenous control. The results are expressed as mean ±S.E.M. from three independent experiments (p<0.01, paired t test).

RESULTS

To determine *Cyp19a1* and *Esr1* mRNA expressions in the fetal mouse brain, pregnant mice were sacrificed at gestational days 9, 11, 13, 15, 16, 19, 21 and RNAs were isolated from their brains for quantitative Real-time PCR.

Cyp19a1 mRNA expression levels for various embryonic days are shown in Figure 1. The highest *Cyp19a1* mRNA expression was found at days 13–15. Subsequently, *Cyp19a1* mRNA expression levels started to fall back to adult levels. In contrast to *Cyp19a1* mRNA expression, *Esr1* (Figure 2) mRNA expression peaked at day 9, and started to decline at days 11–12. By days 13–15, *Esr1* mRNA expression had dropped far below adult levels, followed by a gradual increase up to normal adult levels after day 17.

Estrogen receptor beta (*Esr2)* mRNA expression levels, however, remained at the similar levels compared to experimental controls (Data not shown).In the *Esr1*KO mice, hypothalamic *Cyp19a1* mRNA expression increased almost two-fold (Figure 3).

DISCUSSION

Aromatase is expressed primarily in gonads and the brain. Loss of local E_2 synthesis in the brain leads to the hormonal and behavioral phenotypes observed in

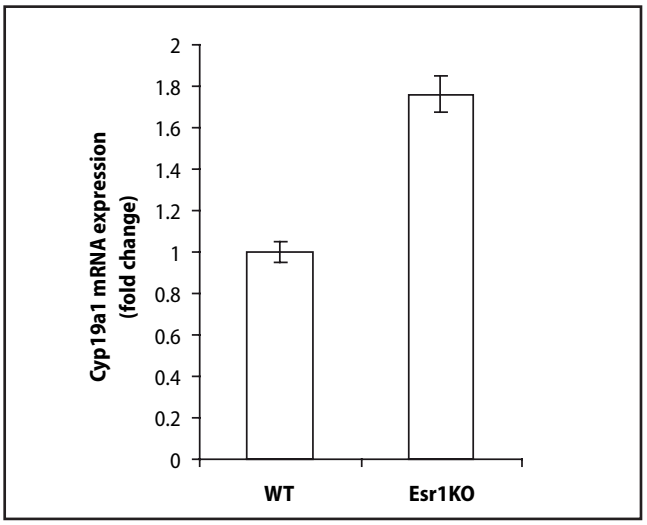

Fig. 3. *Cyp19a1* mRNA expression in the wild-type and *Esr1*KO mice. Fold changes of *Cyp19a1* mRNA were normalized to the expression levels of *GAPDH* gene as an endogenous control. The results are expressed as mean ±S.E.M. from three independent experiments ($p<0.01$, paired t test).

aromatase deficient humans and mice. In fact, alterations in brain aromatase expression have been directly linked to changes in libido and sexual behavior in birds, rodents, sheep, and humans (Bulun *et al.* 2005).

Aromatase expression in the fetal and adult brain differs from each other both in humans and mice. In both species aromatase mRNA expression and enzyme activity are higher in the prenatal hypothalamus than in the adult hypothalamus. In prenatal development, aromatase mRNA expression and enzyme activity increase simultaneously with the formation of hypothalamus. Aromatase mRNA expression and enzyme activity begins around embryonic day 9 in mice and embryonic day 90 in humans. In mice aromatase expression reaches to its maximum expression levels around embryonic days 15–16, when the expression level is almost three fold above adult aromatase levels. After embryonic day 19, the aromatase expression levels fall back to adult levels. Interestingly, Abe-Dohmae *et al.* (1996), cultured 10^{th}, 11^{th} and 13^{th} day mouse embryonic hypothalamic neuronal cells in order to reproduce *in vivo the* time-dependent increase of aromatase mRNA expression under *in vitro* settings. Embryonic day 13 hypothalamic neuronal cell culture *Cyp19a1* expression increased with a steep peak after 2–3 days after culturing similar to that of *in vivo* studies. However, time-dependent increase of *Cyp19a1* mRNA expression was not observed in hypothalamic cells cultured from gestational days 10 and 11 unlike its *in vivo* expression pattern. These findings suggest that the developmental increase of *Cyp19a1*mRNA in hypothalamic neurons is an endogenous characteristic and is possibly genetically determined before gestational day 12 (AbeDohme *et al.* 1996). Interestingly at this stage of the development the circulating sex steroids are either very low or below detectable limits in rodents suggesting different intrinsic mechanisms are responsible for governing aromatase expression in the brain during the gestational period (Habert & Picon 1984; Rhoda *et al.* 1987).

Endogenous estradiol synthesis is specifically critical for neuronal growth, gonadotropin secretion, sexual behavior and libido (Bulun *et al.* 2005; Kretz *et al.* 2004). Therefore the regulatory mechanisms of aromatase expression in the brain have been the focus of interest in recent years and are not well understood. Several groups of investigators found that protein kinases A and C and cAMP regulate aromatase expression and activity in the brain. Other groups showed that testosterone also upregulates aromatase mRNA and enzyme activity in the brain. Moreover, E_2 was found to upregulate or downregulate hypothalamic aromatase mRNA and enzyme activity (Lephart 1997; Livonen *et al.* 2006).

Recently our group showed that E_2 regulates *Cyp19a1* mRNA expression and enzyme activity through *Esr1in vitro* in mouse hypothalamic neuronal cell lines (Yilmaz et. al. 2009). Since aromatase and Esr1 are colocalized in the mouse hypothalamus (Dellovade et. al. 1995), we wanted to determine if there is also an *in vivo* link between *Cyp19a1* and *Esr1*. In this regard, we evaluated *Cyp19a1* and *Esr1 m*RNA expressions in the fetal mouse brain simultaneously. The highest *Cyp19a1* mRNA expression was at days 13–15. Afterwards, *Cyp19a1* mRNA expression levels started to fall back to adult levels. In contrast to *Cyp19a1* mRNA expression, *Esr1* mRNA expression was highest at day 9, and started to decline at days 11–12. By days 13–15, *Esr1* mRNA expression had dropped far below adult levels, followed by a gradual increase up to adult levels after day 17 (Figure 2), we also detected a similar expression pattern for Progesterone receptor (*Pgr*) but not estrogen receptor beta (*Esr2*) (data not shown). In the *Esr1*KO mice, hypothalamic *Cyp19a1* mRNA expression increased almost two-fold compared to age- and sex-matched wild types (Figure 3).

In summary, we found that *Esr1* mRNA expression levels drop far below their normal levels concurrently with a steep increase in *Cyp19a1* mRNA expression in the fetal mouse brain. We also found that *Cyp19a1* mRNA expression increased almost two-fold in the hypothalamus of *Esr1*KO mice further suggesting that *Esr1* and *Cyp19a1* mRNA expression levels are inversely correlated with each other *in vivo.* Together with previous studies, these findings might imply that *Esr1* mediates *Cyp19a1* expression both in the fetal and adult mouse brain.

In our study, we could not evaluate the aromatase protein levels in the mouse brain, which would confirm the changes in aromatase mRNA expression, due to lack of appropriate mouse aromatase antibody. Another important point is that, we were not able show direct interaction between aromatase and *Esr1* in vivo. Our main goal here was to determine if aromatase and Esr1 are by some means related under *in vivo* conditions,

which could be regarded as a ground work for future *in vivo* research in this area.

Taken together, our findings, which indicate that *in vivo* estrogen receptor and aromatase expression patterns are linked to each other in the brain, might pave the way for gaining detailed understanding of physiologic and pathologic functions of aromatase in the brain.

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