Ligands of Estrogen Receptor β in Prostate and Brain

A Dissertation submitted to
the Faculty of the Department of Biology and Biochemistry
University of Houston.

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

By
Selvaraj Muthusamy
May 2014

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ABSTRACT

Estrogen receptor β is a nuclear receptor expressed in various tissues in the body including prostate and brain. In addition to 17β-estradiol, other steroids like 5α-androstane-3β, 17β-diol (3β-Adiol) and 5-androstene-3β, 17β-diol have been reported to be endogenous ligands of ERβ. The concentration of these ligands in the tissue and the metabolism of these ligands by different enzymes regulate the transcriptional activity of ERβ.

In human prostate, 3β-Adiol acts on ERβ to exert an anti-proliferative effect to counteract the proliferative activity of 5α-dihydrotestosterone (DHT) mediated through androgen receptor. 3β-Adiol is produced from 5α-dihydrotestosterone. In chapter 2, we show that 17β-HSD6, a predominant enzyme expressed in human prostate, converts DHT to 3β-Adiol. This conversion of DHT to 3β-Adiol is capable of activating ERβ at physiological concentrations of DHT. Immunohistochemical analysis revealed that 17β-HSD6 is expressed in ERβ-positive epithelial cells of the human prostate and that, both ERβ and 17β-HSD6 were present in benign prostatic hyperplasia (BPH) samples and were undetectable in prostate cancers of Gleason grade higher than 3. These observations reveal that formation of 3β-Adiol via 17β-HSD6 from DHT is an important growth

regulatory pathway that is lost in prostate cancer.

ERβ plays an important role in development and homeostasis of brain. In chapter 3, using liquid extraction, solid phase extraction and LC-MS/MS, we show that estrone is the only detectable endogenous ligand of ERβ in mouse brain tissue. We also show that CYP7B1 knockout mice have significantly higher level of estrone compared to the sex matched wild type controls. We show that only 25% of estrone is converted to estradiol by brain tissue after 24 h of incubation, which indicates that the conversion of estrone to estradiol by brain tissue is very slow. Using cell based transactivation assays we show that estrone is capable of activating ERβ with an EC50 of 3.5 nM. Since the concentration of estrone in mouse brain is 25 nM to 35 nM, estrone could be a physiological ligand of ERβ in mouse brain.

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ABBREVIATIONS

17β-HSD 17β-hydroxysteroid dehydrogenase

3α-Adiol 5α-Androstane-3α,17β-diol

3β-Adiol 5α-Androstane-3β,17β-diol

3β-HSD 3β-hydroxysteroid dehydrogenase

AF-1 Activation function 1

AF-2 Activation function 2

AKR1C3 Aldoketo reductase 1C3

AP-1 Activating protein – 1

AR Androgen receptor

BPH Benign prostatic hyperplasia

cAMP Cyclic adenosine monophosphate

CAR Constitutive androstane receptor

CREB cAMP response element binding protein

CtBP C-terminal binding protein

DBD DNA binding domain

DHEA Dehydroepiandrosterone

DHEA-S Dehydroepiandrosterone Sulphate

DHT 5α-Dihydrotestosterone

DMEM Dulbecco's modified eagle medium

DNA Deoxyribonucleic acid

E1 Estrone, 1,3,5(10)-estratriene-3-ol-17-one

E2 Estradiol, 1,3,5(10)-estratriene-3,17β-diol

E3 Estriol, 1,3,5(10)-estratriene-3,16α,17β-triol

EDTA Ethylenediaminetetraacetic acid

EMT Epithelial to mesenchymal transition

ERα Estrogen receptor α

ERβ Estrogen receptor β

FOXO1 Forkhead box protein O1

FSH Follicle stimulating hormone

FXR Farnesoid X receptor

GC-MS/MS Gas chromatography – tandem mass spectrometry

HEK293 Human embryonic kidney 293 cells

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1α Hypoxia inducible factor - 1α

IGF-1 Insulin like growth factor-1

LBD Ligand binding domain

LC-MS/MS Liquid chromatography-tandem mass spectrometry

LH Luteinizing hormone

LRH-1 Liver receptor homologue – 1

LXRα Liver X receptor α

MAPK Mitogen Activated Protein kinase

MR Mineralocorticoid receptor

mRNA messenger Ribonucleic acid

NAD Nicotinamide adenine dinucleotide

NADP Nicotinamide adenine dinucleotide phosphate

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

NRE Nuclear receptor response element

NRF2 Nuclear respiratory factor 2

P450scc Cholesterol side chain cleavage enzyme

PBS Phosphate buffered saline

PGE2 Prostaglandin E2

PPARγ Peroxisome proliferator activated receptor

PTFE Polytetrafluoroethylene

PXR Pregnane X receptor

RNA Ribonucleic acid

RXR Retinoid X receptor

SF-1 Steroidogenic factor -1

SP-1 Specificity protein-1

SRC-1 Steroid receptor coactivator -1

SREBP Sterol Regulatory Element-Binding Protein

SULT Sulfotransferase

SUMO Small ubiquitin modification

T Testosterone

TIM Triosephosphate isomerase

TNF-α Tumor necrosis factor - α

Tris Tris(hydroxymethyl)aminomethane

UTR Untranslated region

VEGF-A Vascular endothelial growth factor-A

Chapter 1

INTRODUCTION

1.1 Nuclear Receptors: An Overview

Estrogen receptor β (ERβ) is a member of the nuclear receptor family of transcription factors (Kuiper et al., 1996). The nuclear receptors are classified into three categories. The class 1 nuclear receptors, also known as steroid hormone receptors, are normally present in the cytoplasm and bound to heat shock proteins. Upon ligand binding, the receptors are released from the heat shock proteins and enter the nucleus where they generally form homodimers, bind to target sequences (response elements) in DNA and recruit coactivators or corepressors to regulate transcription (for a review see Mangelsdorf et al., 1995). The second class is the thyroid/retinoid family of receptors. These proteins generally form heterodimers with retinoid X receptor (RXR). In the absence of ligands these receptors occupy the DNA response elements bound to corepressors. Upon ligand binding the corepressors are released and the receptor heterodimer recruits coactivators and activates transcription. Thyroid hormone, vitamin D and retinoic acid receptors belong to class 2 of nuclear receptors. The third class are orphan receptors. These receptors do not have known endogenous ligands, however possess powerful functions of transcriptional regulation (Bain et al.,2007).

The general structure of a nuclear receptor is shown in Fig 1. It

contains four domains, namely, A/B, C, D and E/ F. The A/B domain is also known as the N-terminal domain, which includes the transcriptional activation function domain 1 or AF-1 domain. This domain is the least conserved domain of the nuclear receptors and, unlike the other domains, its structure has not been solved (Lavery & McEwan, 2005). Interactions between the N-terminal domain and coactivators like CBP/P300 cause changes in the secondary structure of the AF-1 domain facilitating recruitment of other coactivators (Benecke et al., 2000; Kumar et al., 1999). The AF-1 domain can synergize with the ligand dependent coactivator recruitment and transcriptional activation mediated by the AF-2 domain (Kumar et al., 2004). Post- translational modifications such as phosphorylation of serine and threonine residues in the AF-1 domain can cause ligand independent activation (Tremblay et al., 1999). Alternatively, small ubiquitin modification or SUMOylation of the AF-1 domain can lead to repression of transcription (Poukka et al., 2000).

The DNA binding domain (DBD) is the most conserved of all domains. It has two zinc fingers stabilized by eight conserved cysteine residues (Shaffer et.al., 2004). The hinge region connects the DBD to the ligand-binding domain (LBD) of the receptor, which confers specificity of the receptor for its cognate ligand. The LBD consists of 12 α–helices.

Binding of ligand to the LBD causes a conformational change of the receptor which results in movement of helix 12, whereupon coactivators like SRC-1 are recruited through binding of their NR boxes to the LBD. The coactivators have histone acetyl transferase domains that can initiate histone modifications (Nettles et al., 2004). In addition, the coactivators can facilitate the recruitment of histone modifying enzymes like methyl transferases and mediator complex leading to the recruitment of RNA polymerase and transcriptional activation (McKenna et al., 1999). Alternatively, corepressors bind to the LBD to facilitate the recruitment of histone deacetylases, which leads to repression of transcription (Perissi & Rosenfeld, 2005)

Figure 1.1: Schematic diagram of functional domains of a nuclear receptor:

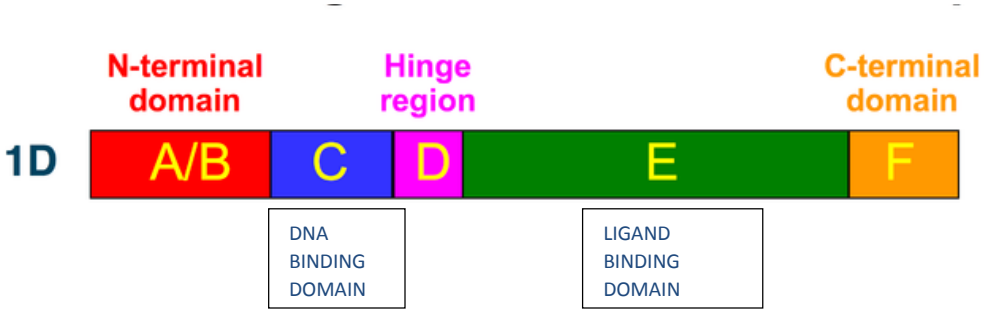
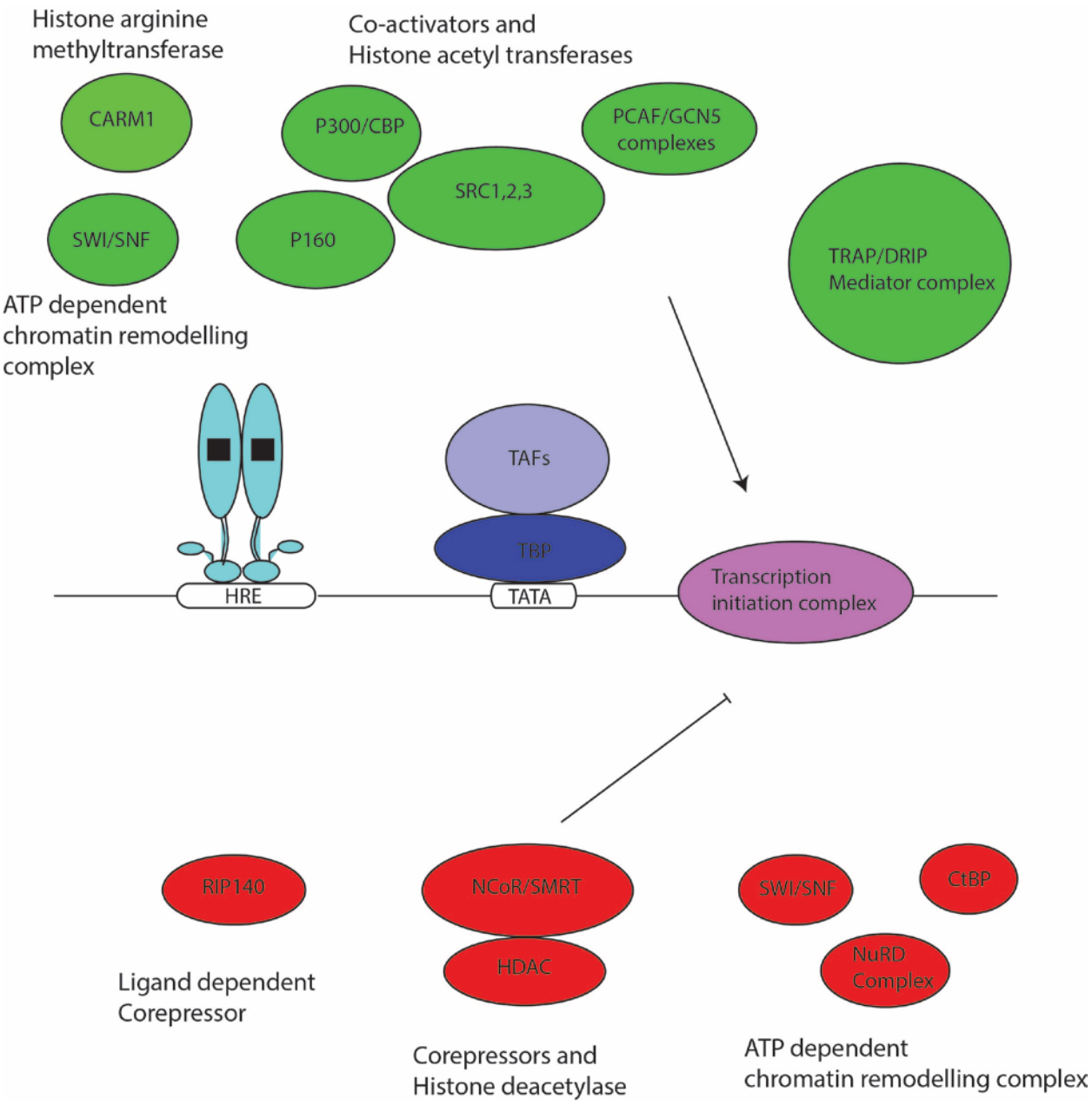


Figure 1.2: General mechanism of transcriptional activation and repression by nuclear receptors



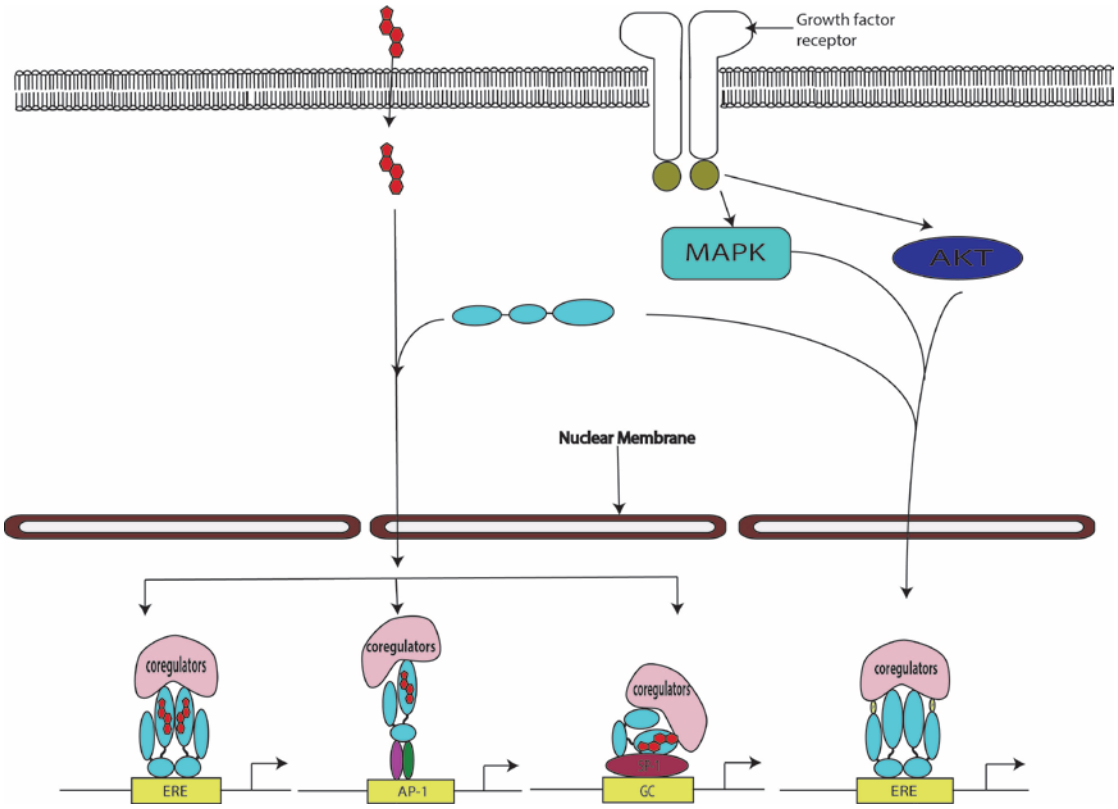
1.2 Mechanisms of Transcriptional Regulation by Estrogen Receptor β:

Estrogen receptor β can regulate gene transcription by two mechanisms (Fig 3). Upon ligand binding, the estrogen receptors are released from the heat shock proteins and are transported to the nucleus. Here, the receptors form homodimers or heterodimers with ERα to occupy estrogen response elements characterized by canonical sequence of GGTCA-nnn-TGACC. The ligand activated ERβ dimerizes, and recruits co-activators, which in turn recruit RNA polymerase to activate transcription (Heldring et al., 2007).

The second mechanism is tethering of ERβ dimers to DNA through adaptor proteins. Upon ligand binding, ERβ can become tethered to the AP-1 response elements through c-Fos and Jun heterodimers. Depending upon the properties of ligand, this complex then recruits coactivators or corepressors to activate or repress transcription (Paech et al., 1997). 4-Hydroxytamoxifen can activate the AP-1 mediated transcriptional activity of ERβ (Paech et al., 1997) whereas 5-androstene-3β,17β–diol (5-androstenediol) represses transcription in immune cells (Saijo et al., 2011). ERβ can also regulate transcription by binding to GC rich regions through the Sp1 protein (Safe & Kim, 2008).

In addition, ERβ can be activated through a ligand independent mechanism. The serine residues in the AF-1 domain of ERβ can be phosphorylated by MAP kinase and Akt signaling pathways and this phosphorylation has been shown to activate transcription (Tremblay et al., 1999).

Figure 1.3: Mechanisms of transcriptional regulation by estrogen receptor β.



1.3 Physiological Role of Estrogen Receptor β:

ERβ is expressed in various tissues and plays an important role in the development and differentiation of diverse organ systems. In the female reproductive system ERβ is highly expressed in the granulosa cells of the ovary (Krege et al., 1998) . ERβ knockout mice have defective follicular development and fewer corpora lutea, which leads to decreased fertility (Krege et al., 1998). In the male reproductive tract, ERβ is expressed at high levels in prostate and plays an important role in cellular differentiation (Imamov et al., 2004). The ERβ knockout mice develop hyperplasia and dysplastic lesions in the prostate (Imamov et al., 2004; Krege et al., 1998). Furthermore, ERβ is expressed at high levels in the pneumocytes and bronchiolar epithelial cells of the lung, where it plays an important role in the maintenance of extracellular matrix and gas exchange (Morani et al., 2006).

ERβ is also expressed at high levels in immune cells and ERβ knockout mice develop a myelo-proliferative phenotype (Shim et al., 2003). In embryonic mouse brain, ERβ is highly expressed between E12.5 to E18.5 and has been shown to play an important role in the migration of neurons into different layers of the cortex (Fan et al., 2006; Wang et al., 2003). In

adult mouse brain, ERβ is also expressed in the microglia (Wu et al., 2013). ERβ specific ligands reduced inflammation and alleviated the severity of experimental autoimmune encephalitis of a mouse model (Wu et al., 2013).

ERβ is also expressed in the mammary gland epithelium (Forster et al., 2002) and colon (Wada-Hiraike et al., 2006). In white adipose tissue, ERβ antagonizes PPARγ to reduce adiposity (Foryst-Ludwig et al., 2008). ERβ expression is frequently lost in advanced prostate cancer (Zhu et al., 2004), breast cancer (Hartman et al., 2009) and colorectal cancer (Rudolph et al., 2012), emphasizing the role of ERβ as a tumor suppressor.

In conclusion ERβ is expressed in a wide variety of tissues and it plays important roles in tissue differentiation and homeostasis.

1.4 Regulation of Estrogen Receptor β Expression:

Transcription of the ERβ gene is regulated by three different promoters, 0N, 0K and E1.The 5'UTR of ERβ mRNA varies according to the promoter used for transcription initiation. Variations in the 5'-UTR alter the efficiency of translation of ERβ (Zhao et al., 2003) . The availability of different promoters may confer with flexibility on ERβ transcription by different transcription factors depending on developmental stage and tissue type. Silencing of the promoter region by CpG methylation is one of the common mechanisms by which ERβ expression is suppressed in cancer and normal tissues over the course of development (Zhu et al., 2004).

1.5 Endogenous Steroid Ligands of Estrogen Receptor β:

1.5.1 Estradiol:

Estradiol-17β is the most potent endogenous ligand of ERβ. It binds to both ERα and β with similar affinity (Kuiper et al., 1997).

1.5.2 Estrone:

Estrone has a strong affinity for both ERα and β, but is a weak transcriptional activator (Kuiper et al., 1997).

1.5.3 Estriol:

Estriol is predominantly synthesized during pregnancy in humans. Estriol binds to ERβ with lower affinity than estradiol. It has a slightly higher affinity for ERβ compared to ERα (Kuiper et al., 1997).

1.5.4 Catechol estrogens:

2-Hydroxy and 4-hydroxy estradiol, also known as catechol-estrogens, have an affinity for ERβ that is about 10% lower than that of estradiol.

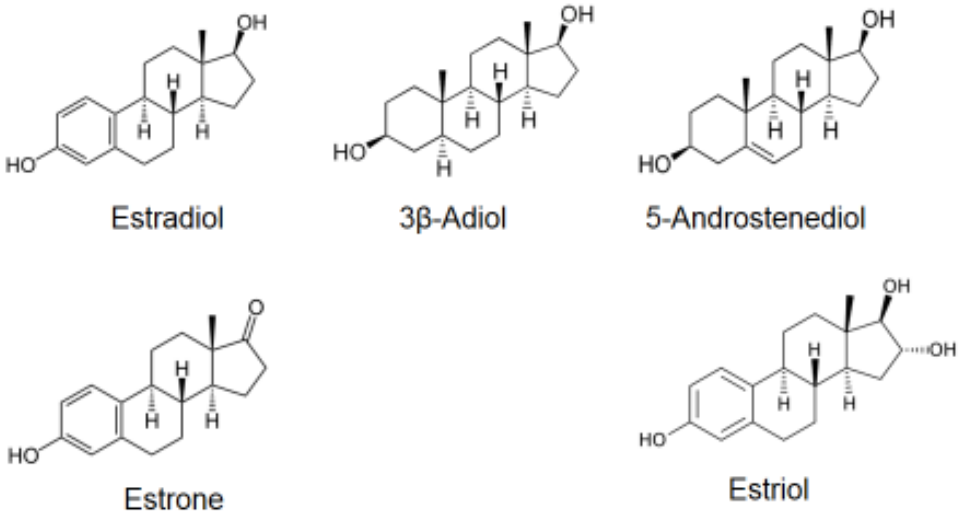
1.5.5 5α-Androstane-3β, 17β-diol:

5α-Androstane-3β, 17β-diol (3β-Adiol) is a potent endogenous ligand of ERβ. It has been shown to be present in rodent prostate (Weihua et al., 2002).

1.5.6 5-Androstene-3β, 17β-diol:

5-Androstenediol is a metabolite of DHEA. It is also a ligand of ERβ (Saijo et al., 2011), androgen receptor (Chang et al., 1999) and ERα (Adams, 1985). 5-Androstenediol can repress the transcription of pro-inflammatory genes in microglia cells by recruitment of the transcriptional corepressor C-terminal binding protein (CtBP) (Saijo et al., 2011).

Figure 1.4: Endogenous ligands of estrogen receptor β.



1.6 Biosynthesis and Metabolism of Endogenous Ligands of Estrogen Receptor β:

1.6.1 Biosynthesis of the endogenous steroid ligands of estrogen receptor β:

The enzymes involved in the biosynthesis of steroid hormones belong to either cytochrome P450 family of enzymes and hydroxysteroid dehydrogenases. The cytochrome P450 enzymes contain a heme group and use NADPH as cofactor. They catalyze hydroxylation reactions and cleavage of carbon-carbon bonds. The cytochrome P450 enzymes are divided into two different classes. Class 1 enzymes are located in the mitochondria and use ferredoxin reductase / ferredoxin as electron transfer system; class 2 enzymes are located in the endoplasmic reticulum and use P450 oxidoreductase as electron transfer system. The hydroxysteroid dehydrogenase enzymes use NADP/NADPH or NAD/NADH as cofactors and they function as either reductases or dehydrogenases (Miller & Auchus, 2011).

The first rate-limiting step of steroid hormone biosynthesis is catalyzed by the mitochondrial enzyme called cholesterol side chain cleavage enzyme (P450scc), which leads to the formation of pregnenolone from cholesterol. A tissue is considered to be steroidogenic if it expresses

P450scc. Pregnenolone is converted to 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) by the enzyme 17α- hydroxylase (CYP17A1).This enzyme possesses both 17α-hydroxylase and 17,20 lyase activities. In the adrenals, DHEA is conjugated with sulfate group to form DHEA-S and released into circulation. DHEA-S is deconjugated to form DHEA in the peripheral tissues and later converted to 5-androstenediol by 17β-HSD 1, 7 and 14 enzymes (Miller & Auchus, 2011).

In the Leydig cells of testis, DHEA is converted to androstenedione by the enzyme 3β-HSD2. Androstenedione is further converted to testosterone by the enzyme 17β-HSD3. Leydig cells expressing aromatase are capable of synthesizing estradiol from testosterone. In addition to Leydig cells, Sertoli cells in the seminiferous tubules also express aromatase enzyme (Carreau et al., 2006).

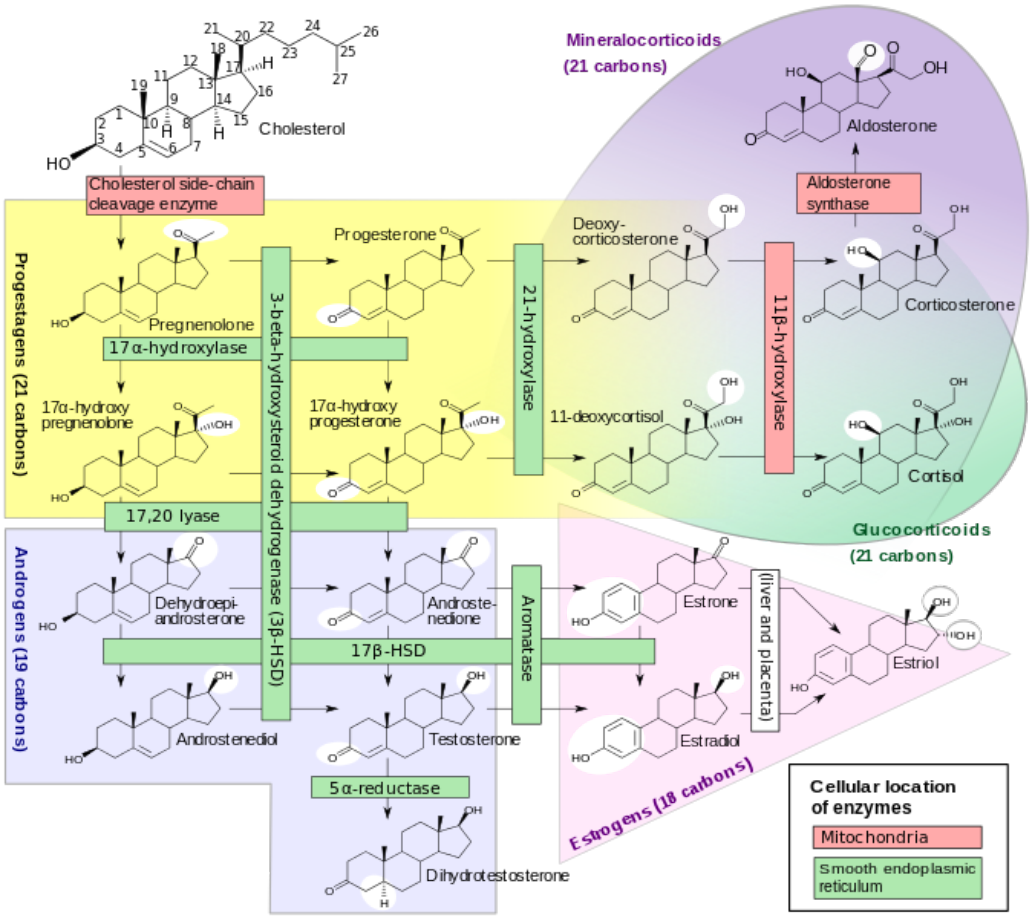
In females, estradiol is primarily synthesized by the granulosa cells of the ovary. Since the granulosa cells do not express 17α-hydroxylase, they depend on theca cells for androgens for estrogen synthesis. The ovarian theca cells, upon stimulation by luteinizing hormone, express P450scc and 17α-hydroxylase to produce DHEA which is converted to androstenedione by 3β-HSD2. Androstenedione released from theca cells is then aromatized

to estrone in the granulosa cells. Estrone is subsequently converted to estradiol by the enzyme 17β-HSD1 (Miller & Auchus, 2011).

During pregnancy, the human placenta expresses steroidogenic enzymes like P450scc, 3β-HSD and aromatase. In addition, starting from the seventh week of gestation, the fetal adrenals produce DHEA-S which is converted to 16α-hydroxy-DHEA-S by the enzyme CYP3A7 in the fetal liver. The 16α-hydroxy-DHEA-S is then desulfated and converted to 16α-hydroxy-androstenedione by the placenta. The placental aromatase and 17β-HSD1 convert 16α-hydroxy-androstenedione into estriol (Payne & Hales, 2004).

Interestingly, the steroidogenic pathways in rodent have some significant differences compared to human. The rodent adrenal does not express 17α-hydroxylase. So, DHEA is not produced by the rodent adrenal. Another difference is that the rodent placenta does not express aromatase so rodents are dependent on their ovaries to produce estrogens during pregnancy (Miller & Auchus, 2011).

Figure 1.5: Steroidogenic pathways:



1.6.2 Enzymes involved in the metabolism of the endogenous ligands of estrogen receptor β:

Aromatase:

Aromatase is a microsomal cytochrome P450 enzyme responsible for the conversion of C19 androgens to C18 estrogens. The reaction uses three oxygen equivalents and NADPH. The first step of the reaction is the oxidation of the C19 carbon, followed by a second hydroxylation of C19. Then enzyme assisted tautomerization causes the aromatization of the A ring. Addition of the third oxygen molecule results in cleavage of the bond between carbons 10 and 19 followed by formation of formic acid (Hong et al., 2007). Androstenedione, testosterone, 16α-hydroxyandrostenedione and epitestosterone (4-androsten-17α-ol-3-one) are the known substrates of aromatase (Santen et.al., 2009).

In human beings, aromatase gene is located on chromosome 15. The gene has 10 exons and encodes a 503 aa protein. In rodent, the gene is located on chromosome 9. The mRNA undergoes alternative splicing upstream of exon 2, which leads to differences in the 5' UTR of the aromatase mRNA. So, the 5'UTR of the aromatase gene varies in different tissues. The promoter has regulatory sequences like CREB like site (CLE), NRE that provides binding site for SF-1 and probably LRH-1, GATA-4 and

AP-3 (Payne & Hales, 2004).

Aromatase is expressed in a wide range of tissues. In female, aromatase is expressed in the granulosa cells of the preovulatory follicle, and luteal cells of the corpus luteum. In male, aromatase is expressed in the Leydig cells and Sertoli cells of the testis. It is also highly expressed in human placenta. Furthermore, aromatase is expressed in the adipose tissue, epiphyseal plates of bone, skin and brain. In adult human and rodent brain, aromatase is mainly expressed in the median preoptic and paraventricular nuclei of hypothalamus, amygdala, hippocampus, bed nucleus of stria terminalis and temporal cortex. In addition to neurons, aromatase expression has also been found in astrocytes (Simpson et al., 2002).

In the preovulatory follicle of the ovary, FSH upregulates aromatase expression in granulosa cells. During the secretory phase of the menstrual cycle, LH stimulates the expression of aromatase in the luteinized granulosa cells. LH also upregulates the expression of aromatase in Leydig cells of testis. FSH and LH activate cAMP production and protein kinase A which upregulates the transcription of aromatase. Estradiol and testosterone potentiates the effect of FSH on aromatase gene transcription. Steroidogenic factor-1 (SF-1) is an orphan nuclear receptor that plays a very important role in the development of steroidogenic tissues like the adrenal gland and the

gonads. SF-1 can bind to the aromatase promoter and increase the transcription of aromatase. FSH and IGF-1 can stimulate the Akt signaling pathway that leads to phosphorylation of FOXO1 and de-repression of the aromatase gene transcription. Activation of the protein kinase C signaling cascade on the other hand can inhibit the FSH induced up-regulation of aromatase expression (Belgorosky et al., 2009)

In addition to the gonadotropins, growth factors like IGF (Chabrolle et al., 2009), inflammatory mediators like PGE2 (Zhao et al., 1999), TNF-alpha and interleukins (To et al., 2013), can regulate the expression levels of aromatase.

Aromatase deficiency is a rare genetic disorder. The patients are unable to convert androgens to estrogens. Female patients have ambiguous genitalia at birth with virilization and delayed bone age at puberty. Male patients are normal at birth, but after puberty they manifest with tall stature due to delayed closure of epiphyses, and insulin resistance, however they respond very well to estrogen replacement therapy (Belgorosky et al., 2009).

Figure 1.6: Biosynthesis of Estradiol:

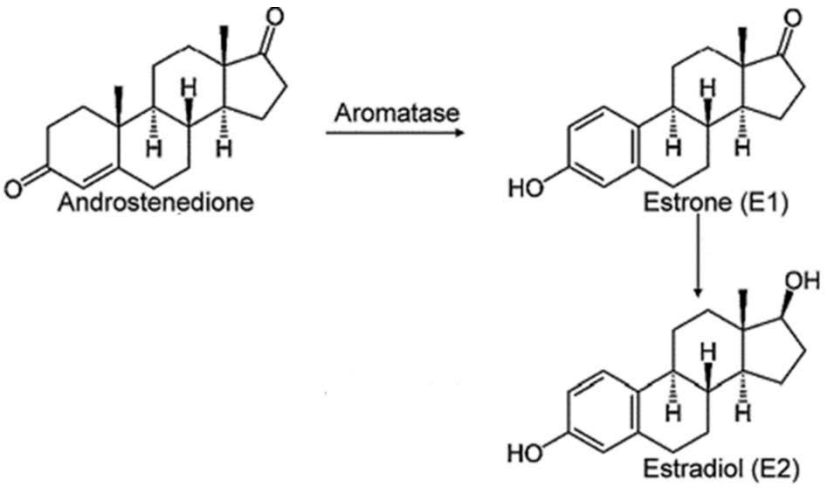
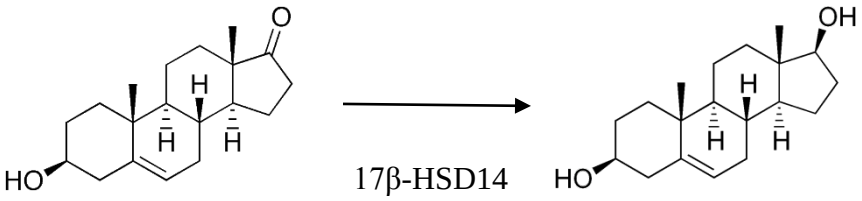


Figure 1.7: Biosynthesis of 5-androstene-3β,17β-diol:



CYP7B1:

CYP7B1 is a cytochrome P450 enzyme that functions as 6α- and 7α-hydroxylase for various substrates like 25-hydroxycholesterol and 27-hydroxycholesterol, 3β-Adiol, 5-androstenediol, DHEA, pregnenolone and estradiol. Since CYP7B1 metabolizes the ligands of nuclear receptors like oxysterols and steroids, it plays an important role in the pre-receptor regulation of nuclear receptors like liver X receptors and ERα and β. CYP7B1 preferentially hydroxylates carbon C-6 of 3β-Adiol, but C7 is the preferred position for hydroxylation of pregnenolone, DHEA, and oxysterols (Rose et al., 1997). CYP7B1 is expressed in brain, kidney and prostate and it has a sexually dimorphic pattern of expression that is regulated by the androgen receptor (Stiles et al., 2009). The enzyme is also a direct transcriptional target of the nuclear receptor RORα that upregulates the expression of CYP7B1 and the liver X receptor α (LXRα) negatively regulates the expression of CYP7B1 (Wada et al., 2008). CYP7B1 knockout mice show early puberty and premature ovarian failure, and abnormally large brain during early stages of development (Omoto et.al., 2005). The prostates of CYP7B1 knockout mice have lower proliferation rates compared to wild type mice (Weihua et al., 2002). Mutations of human CYP7B1 enzyme have been associated with a progressive motor neuron

disease, namely the hereditary spastic paraplegia (Stiles et al., 2009).

17β-hydroxysteroid dehydrogenases:

17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes function as dehydrogenases or reductases. There are 14 different isozymes of 17β-HSD in human tissues. Though the enzymatic actions of 17β-HSDs are reversible in vitro, the enzyme activity is unidirectional in vivo depending on substrate concentration and availability of cofactors. The reductases use NADPH as a cofactor and dehydrogenases use NAD+. 17β-HSDs belong to two different classes. The first group is the short chain dehydrogenase/reductase family. They contain characteristic beta-alpha-beta domains called Rossmann folds. The second group of the enzymes is the aldo-keto reductases (AKRs). They are cytosolic enzymes with characteristic of triosephosphateisomerase or TIM barrels, containing alternating eight α helices and eight parallel β strands (Payne & Hales, 2004).

17β-HSD 1:

17β-HSD 1 is a cytosolic enzyme and acts as a reductase in vivo. 17β-HSD1 converts estrone to estradiol. It can also reduce androstenedione to testosterone. The affinity of the human enzyme for estrone is 100-fold higher compared to C19 steroids. The enzyme is active only as a homodimer. The mouse ortholog has a similar affinity for androgens and

estrogens. 17β-HSD1 is expressed in the granulosa cells of ovary, syncytiotrophoblast cells of the placenta, ductal epithelium of the breast, in the endometrium and in selective regions of the brain such as the hypothalamus (Payne & Hales, 2004)

Expression of 17β-HSD1 in gonadal cells is regulated by the gonadotropins (FSH & LH). The promoter of 17β-HSD1 has CREB binding elements, retinoic acid response elements, SP1 and AP1 binding regions. Polymorphisms of the 17β-HSD1 gene are associated with breast cancer, endometriosis, endometrial cancer, colon cancer and depression (Payne & Hales, 2004).

17β-HSD 2:

17β-HSD2 is a microsomal oxidative enzyme responsible for conversions of estradiol to estrone, testosterone to androstenedione and 5α-dihydrotestosterone to 5α-androstanedione. The enzyme is expressed in most peripheral tissues such as breast, prostate, small intestine, placenta, and endometrium (Wu et al., 1993). In human endometrium, the expression of 17β-HSD2 is increased during the secretory phase of the menstrual cycle. The progesterone receptors in the stroma regulate the expression of 17β-HSD2 through epithelial-stromal interactions (Payne & Hales, 2004). Retinoic acid receptor-α upregulates the transcription of 17β-HSD2 in

human endometrial cell lines (Ito et al., 2001). Besides estrogen metabolism, the enzyme seems to play an important role in cholesterol and retinoic acid metabolism. The physiological significance of the enzyme is demonstrated in 17β-HSD2 knockout mice, which exhibit placental abnormalities and embryonic lethality. The embryos also have defects in brain and kidney (Rantakari et al., 2008). Polymorphisms in the 17β-HSD2 gene are associated with cancers of breast, colon and ovary (Payne & Hales, 2004).

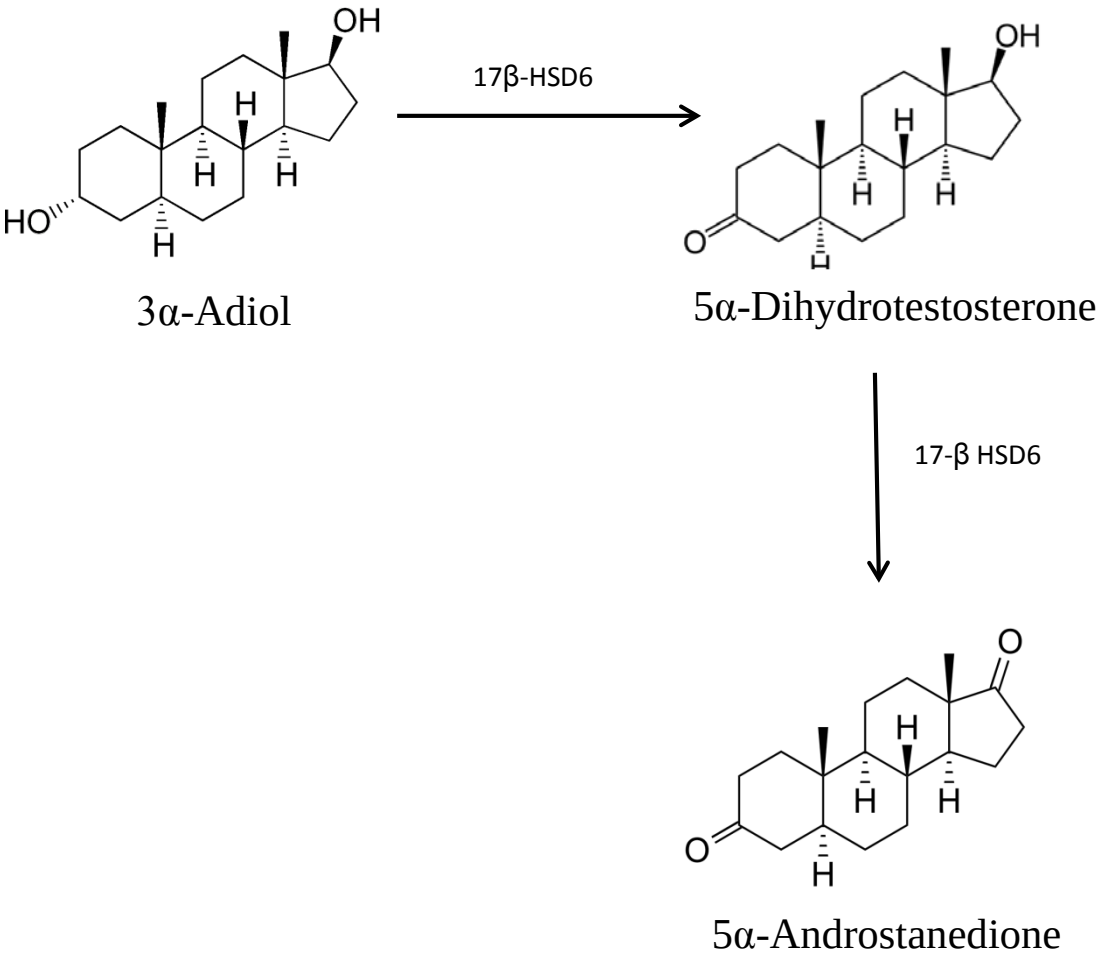
17β-HSD5:

17β-HSD5 is a cytosolic enzyme that belongs to the aldoketoreductase family. It is also known as AKR1C3. The enzyme is a reductase, that catalyzes conversion of DHEA to androstenediol and DHT to mainly 3α- and 3β-Adiol, but the efficiency of the enzyme to reduce 20-keto steroids like progesterone and deoxycorticosterone is far higher than the reduction of the 17-keto group in the androgens (Penning et al., 2000). AKR1C3 is considered to be an important enzyme in the “backdoor” pathway for synthesis of testosterone from DHEA in castration resistant prostate cancer (Miller & Auchus, 2011). In human prostate, activin A upregulates the expression of 17β-HSD 5 (Hofland et al., 2012). The enzyme is expressed ubiquitously in brain, prostate, kidney, liver, endometrium, and fetal adrenal gland (Payne & Hales, 2004).

17β-HSD6:

Also known as retinol dehydrogenase, 17β-HSD6 was first cloned from rat prostate and it has the ability to oxidize 3α-Adiol to DHT and DHT to androstanedione (Biswas & Russell, 1997). The human ortholog has also 3(α - β) epimerase activity. In addition to prostate the mRNA of this enzyme is highly expressed in liver, brain and lungs (Huang & Luu-The, 2000). The transcription of 17β-HSD6 is negatively regulated by the androgen receptor. In addition, the promoter sequence 2kb upstream of the transcriptional start site has potential binding sites for transcription factors such as ERs, SREBP, AP-1 and NF-kB (Huang & Luu-The, 2000).

Figure 1.8. Enzymatic action of 17β-HSD6:



17β-HSD7:

17β-HSD7 is expressed in the corpus luteum of the ovary and in the spongiotrophoblasts of the placenta in mice at E12.5 to E14.5. During the luteal phase of the menstrual cycle, the expression of 17β-HSD1 in granulosa cells declines, but the expression of 17β-HSD7 maintains the production of estradiol by the corpus luteum. In addition to reducing estrone to estradiol, the enzyme plays an important role in cholesterol biosynthesis by catalyzing the reduction of zymosterone to zymosterol (Payne & Hales, 2004). Transcription of 17β-HSD7 is regulated by luteinizing hormone. The promoter region of the enzyme has CREB binding sequences, SF-1 response elements, and GC rich regions available for Sp1 proteins. The 17β-HSD7 knockout is embryonically lethal because of alterations in cholesterol metabolism (Shehu et al., 2008).

17β-HSD14:

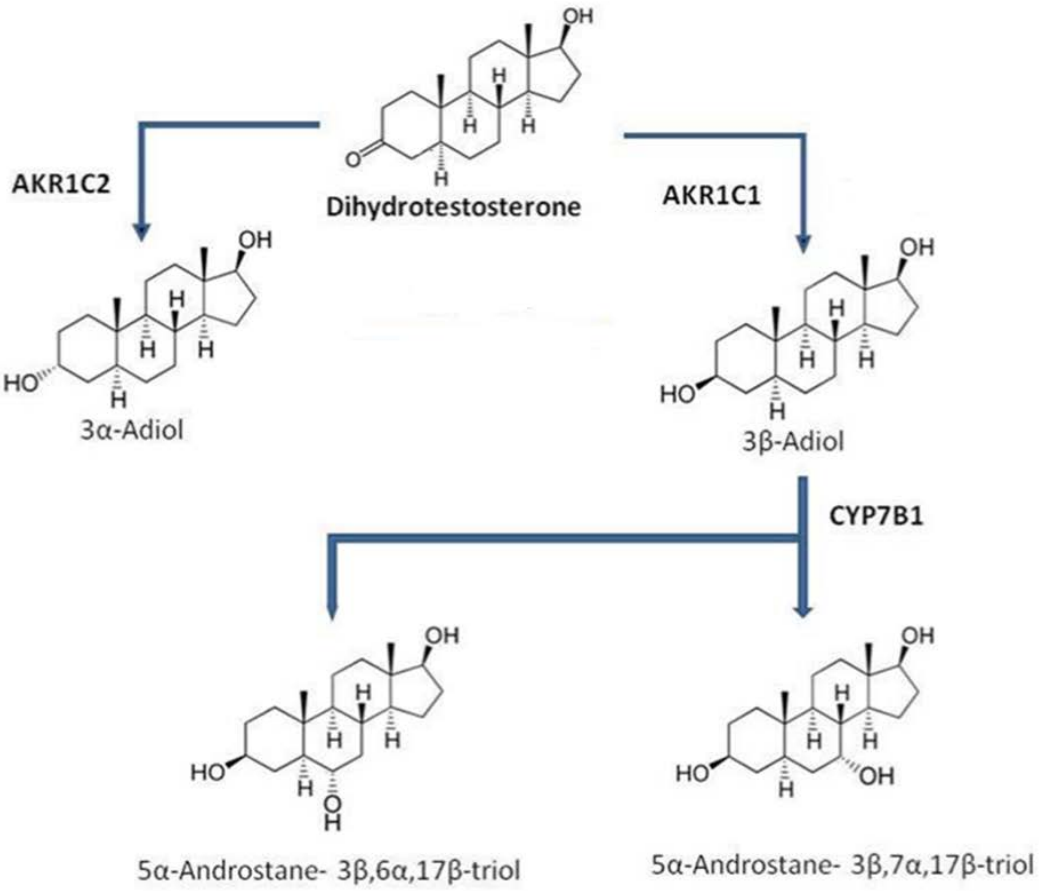
17β-HSD14 is a cytosolic enzyme, expressed at high levels in brain, liver and placenta and has been proposed to be the major 17β-HSD responsible for the conversion of DHEA to 5-androstenediol in microglial cells (Saijo et al., 2011).

Aldo-keto reductases:

Aldo-keto reductase 1C1:

Aldo-keto reductase 1C1 (AKR1C1) is a cytosolic enzyme that functions as a 20α-reductase for progestins and 3α/β-HSD for 5α-reduced androgens. It produces 3β-Adiol from dihydrotestosterone in the presence of NADPH in vitro (Steckelbroeck et al., 2004). Transcripts of AKR1C1 have been found in prostate, brain and liver, and AKR1C1 transcription is regulated by transcription factors Nrf2 and NF-Y (Selga et al., 2008).

Figure 1.9. Biosynthesis and metabolism of 5α-Androstane-3β,17β-diol.



Steroid sulfotransferase (SULT) and sulfatase:

The sulfatation reaction starts from the sulfate being conjugated with ATP to form adenosine phosphosulfate. Then another molecule of ATP reacts with adenosine phosphosulfate to produce phosphoadenosine phosphosulfate (PAPS). PAPS is the donor of the sulfate groups for the sulfatation reactions. There are several different forms of SULT enzymes in the body. SULT2E1 catalyzes sulfatation of estrogens and SULT2A1 catalyzes the sulfatation of Δ5 steroids like pregnenolone, DHEA and 5-androstenediol. The sulfated steroids are water soluble and released into circulation to be taken up by the target tissues expressing organic anion transporting peptide B. Deficiencies in steroid sulfotransferase are associated with symptoms of androgen excess, because the unconjugated DHEA is metabolized to high quantities of androstenedione (Payne & Hales, 2004).

In the target tissues, steroid sulfatase is responsible for cleaving the sulfate group off to release the active steroid molecule. Steroid sulfatase is encoded by the X chromosome. It is expressed in various peripheral tissues such as brain, placenta, and breast. Steroid sulfatase deficiency causes a disorder called X linked ichthyosis. This is because of accumulation of cholesterol sulfate in the keratinocytes (Elias et al., 2004). Since the majority of the circulating DHEA is DHEA-S, steroid sulfatase is essential

for the production of 5-androstenediol in peripheral tissues.

UDP-Glucuronosyl transferase:

Steroids are conjugated with glucuronic acid on the hydroxyl groups at third or seventeenth positions by the liver microsomal UDP-glucuronosyl transferase (UGT). There are several classes of UGT. UGT1A is the enzyme responsible for glucuronidation of C18 estrogens. UGT2A1 and UGT2B enzymes act on androgens. Steroid glucuronides are water soluble and are excreted by the kidney. UGTs are transcriptionally regulated by transcription factors aryl hydrocarbon receptor (AhR), Nrf2 and the nuclear receptors CAR, PXR, PPARα and FXR (Zhou et al., 2011).

Chapter 2: Identification of a 3β-Adiol synthase in human prostate

2.1 Introduction:

ERβ plays an important role in prostate growth and development. The receptor is expressed predominantly in the epithelial cells of the ventral prostate where it is activated by the endogenous ligands 5α-androstane-3β,17β-diol (3β-Adiol) and 17β-estradiol (E2) (Weihua et al., 2002). 3β-Adiol is a ligand of ERs and has slightly higher affinity for ERβ than α (Kuiper et al., 1997), however, 3β-Adiol has lower affinity for ERβ (K_i = 2 nM) than E2 (K_i = 0.12 nM) (Kuiper et al., 1997). Since the concentration of 3β-Adiol in the prostate (10 nM) is 100 times higher than the levels of E2 (0.1 nM) (Belanger et al., 1990), it has been proposed that 3β-Adiol is a physiological ligand of ERβ in the prostate.

The major circulating androgen, testosterone (T), is secreted by the Leydig cells of testis. T is converted to 5α-dihydrotestosterone (DHT) in androgen target tissues like prostate and brain by the enzymes 5α-reductase type 1 and 2 (Russell & Wilson, 1994). Even though both DHT and T bind to androgen receptor (AR) with high affinity, the ability of DHT to recruit coactivators and activate transcription is significantly higher than that of T (Heinlein et al., 2004). Hence, DHT is the more potent endogenous ligand of AR (Askew et al., 2007). In target androgen tissues, DHT can be reduced to 5α-androstane-3α,17β-diol (3α-Adiol) or 3β-Adiol. 3α-Adiol can be

reconverted to DHT, and it is a source of DHT in prostate. Unlike 3α-Adiol, 3β-Adiol is converted to 6α- and 7α-triols by CYP7B1. Hence, the conversion of DHT to 3β-Adiol is irreversible (Nacusi & Tindall, 2011).

As the predominant endogenous ligand of ERβ, 3β-Adiol plays an important role in regulating the growth and differentiation of the prostate. In adult prostate epithelium, 3β-Adiol acts through ERβ and has antiproliferative and proapoptotic effects (Weihua et al., 2002). In prostate cancer cell lines, PC3 and LNCap cells, 3β-Adiol decreases the ability of the cancer cells to migrate by facilitating the degradation of hypoxia-inducible factor-1α (HIF-1α) (Mak et al., 2010). The degradation of HIF-1α prevents epithelial to mesenchymal transition (EMT) by inhibiting VEGF-A mediated nuclear translocation of the transcription factor Snail1. The antimigratory effect of 3β-Adiol is mediated by ERβ (Mak et al., 2010).

DHT can be converted to 3β-Adiol by two different mechanisms. The first mechanism is through direct reduction of the 3-keto group. The second mechanism consists of two steps, reduction of DHT to 3α-Adiol followed by epimerization of 3α-Adiol to 3β-Adiol. It has been proposed that the enzyme responsible for the 3–keto reduction of DHT to 3β-Adiol is the aldo-keto reductase 1C1 (AKR1C1). AKR1C1 is a cytosolic enzyme which belongs to the aldo-keto reductase family of enzymes. The enzyme is

a low affinity, high capacity enzyme (Penning et al., 2000) which catalyzes the formation of 3β-Adiol from DHT *in vitro* and in intact cells at concentrations in the low μM range (Jin et al., 2009). AKR1C1 transcripts are found in the prostate (Bauman et al., 2006), however the cell-type specific expression of AKR1C1 protein in human prostate is not known. Rodent prostate does not express steroid metabolizing aldo-keto reductases (Velica et al., 2009).

Another candidate for the prostatic 3β-Adiol synthase is 17β-HSD7. This enzyme is able to catalyze the conversion of DHT to 3β-Adiol in vitro. However, the mRNA of 17β-HSD7 is expressed at very low levels in the human prostate (Liu et al., 2005) and the presence of the protein in the prostate has not been demonstrated.

17β-HSD6 or retinol-like 3α-hydroxysteroid dehydrogenase (RL-HSD) is a microsomal short chain dehydrogenase (Biswas & Russell, 1997). In addition to its oxidative 17β-HSD activity, the enzyme possesses epimerase activity (Huang & Luu-The, 2000). In the human prostate DHT can be converted to 3α-Adiol by AKR1C2 (Rizner et al., 2003) and 3α-Adiol can be further converted to 3β-Adiol by the epimerase activity of 17β-HSD6. The mRNA of 17β-HSD6 has been demonstrated in human prostate (Biswas

& Russell, 1997), but the cell-type specific expression of the protein has not been reported.

In the present study, we demonstrate that 17β-HSD6 catalyzes the conversion of physiological concentrations of DHT to 3β-Adiol in intact cells in quantities sufficient for activation of transcription by ERβ. Immunohistochemical studies revealed that 17β-HSD6 colocalizes with ERβ in prostate epithelium and that expression is lost along with ERβ in prostate cancer of Gleason grade 4 and 5. These findings emphasize the physiological significance of 17β-HSD6 in the pre-receptor regulation of ERβ in the prostate.

2.2 Materials and Methods:

2.2.1 Steroids.

Tritiated DHT (112 Ci/mmol) was purchased from Perkin-Elmer. Estradiol-17β (E2),17β-hydroxy-5α-androstan-3-one (DHT), 5α-androstane-3α,17β-diol (3α-Adiol), and 5α-androstane-3β,17β-diol (3β-Adiol) were obtained from Steraloids.

2.2.2 Human Samples.

Prostate biopsies were obtained from the Department of Urology at Danderyd Hospital, Stockholm, Sweden. Samples were fixed in buffered paraformaldehyde, dehydrated and imbedded in wax. The samples were categorized for their Gleason grade (Gleason & Mellinger, 1974) at Danderyd Hospital. Samples from seven different patients with benign prostatic hyperplasia (BPH) (Lee & Peehl, 2004), two patients with prostate cancer Gleason grade 3, three patients with prostate cancer Gleason grade 4, and two patients with prostate cancer Gleason grade 5 were used for this study.

2.2.3. Cell culture:

Human embryonic kidney 293 cells (HEK 293) (ATCC CRL no. 1573) were used for transactivation and enzyme activity assays. HEK 293

cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, CA) supplemented with 10% fetal bovine serum (Sigma), 50 µg/mL Kanamycin and 10 mM HEPES (pH 7) in a 75 cm^2 sterile cell culture flask (BD Falcon). On day 0 the cells were plated on a 24 well costar (Corning) plate containing 0.5 mL of phenol red free DMEM supplemented with 10% dextran treated/ charcoal stripped fetal bovine serum (Hyclone), 2% glutamine (Invitrogen), 50 µg/mL Kanamycin and 10 mM HEPES (pH 7). On day 1 the cells were transfected with expression plasmids to be used for transactivation or enzyme activity assays as described below.

2.2.4 Transactivation assays:

On day 1 the HEK 293 cells in 24 well costar plates were transfected with expression plasmids of pCMV human ERβ1 (60 ng/well), luciferase reporter plasmid whose transcription is driven by 3X estrogen response elements and a TATA box (75 ng/well), pRSV-β-galactosidase (30 ng/well) and either pCMV-17β HSD6 expression plasmids or pCMV vector alone (135 ng/well). The transfection was done using Fugene 6 transfection reagent (Roche diagnostics) at a ratio of 4 µl of transfection reagent per µg of plasmids. Forty-eight hours after the transfection, DHT, 3α-Adiol, 3β-Adiol and E2 were added to each well at a final concentration of 20 nM. The steroids were dissolved in dimethyl sulfoxide (DMSO) at 1000X the

concentration to be used in the experiment and then diluted in the medium to the desired final concentration. After 16 more hours of incubation with the steroids the cells were lysed, and assayed for luciferase activity using luciferase assay kit (Biovision). About 50µL of the cell lysate per well was loaded on a white 96 well plate (Corning), and luciferase activity was measured using Perkin Elmer Victor X4 plate reader according to manufacturer's instructions. β-Galactosidase level was measured using β-galactosidase assay buffer containing o-nitro phenyl β-galactoside (ONPG) as substrate. About 20µL of clear cell lysate was added in a transparent 96 well plate (BD Falcon) to which 100 µL of β-galactosidase assay buffer was added and incubated for 30 minutes at room temperature. The reaction was terminated by adding 100 µL of 1 M sodium carbonate solution. The optical density was measured with a 405 nm filter using Perkin Elmer Victor X4 plate reader. The relative transactivation was calculated by dividing the luciferase values by optical density values from β-galactosidase assay. The fold activation was calculated by normalizing the relative transactivation values of cells treated with steroids to those of cells treated with vehicle. For dose response curves with DHT and 3α-Adiol the cells were incubated with steroids of final concentrations starting from 1 nM to 10 µM. The transactivation assay was done as described above. The EC50 values were

calculated by fitting the values to a sigmoidal dose response curve using Graphpad prism 6 (Graphpad) software.

2.2.5 Enzyme activity assay;

To measure the enzyme activity of 17β-HSD6 in intact cells, HEK 293 cells were plated in 24 well costar plates on day 0. On day 1 the cells were transfected with expression plasmids of pCMV-17β-HSD 6 or pCMV vector alone, 270 ng/well along with β-galactosidase 30 ng/well using Fugene 6 as described above. Forty-eight hours after transfection, tritiated DHT was added at a final concentration of 20 nM/well. About 100 µL of medium was collected at 1, 2, 4 and 8 hours post incubation. The medium was then mixed thoroughly with 400 µL of Folch solvent mixture (chloroform:methanol 2:1) and then vortexed at 14000 X g on a microcentrifuge for five minutes. Then the lower organic phase was dried down under vacuum using speed-vac. The dried lipid extract from the medium was dissolved in 40 µL of Folch and spotted onto Partisil LK5D thin layer chromatography plates (Whatman). The plates were developed twice using chloroform:ethyl acetate (4:1). The steroid metabolites on the TLC plate were quantified using Bioscan AR 2000 scanner. After collecting the medium at the final time point the cells were lysed and β-galactosidase

values were measured. The β-galactosidase activity was used to normalize for the transfection efficiency.

2.2.6 Immunohistochemistry:

Paraffin fixed tissue sections were first deparaffinized using xylene and rehydrated using graded ethanol. To stain for 17β-HSD6 antigen retrieval was done using buffer containing Tris base 10 mM, EDTA 1 mM, 0.05% Tween 20, pH 9. The rehydrated slides were boiled for 20 minutes in the buffer in a microwave oven. For retrieving ERβ antigen, the slides were incubated with citrate buffer (10 mM, pH 6) in a pretreatment module (ThermoScientific) at 97°C for 15 minutes. Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 in 50% methanol for 30 minutes. The slides were then incubated with 3% bovine serum albumin in PBS for 30 minutes to block non-specific binding. To stain for the 17β-HSD6 two antibodies were used. The first one is a rabbit polyclonal antibody (cat. no. ab62221; Abcam) raised against N-terminal amino acids 1–50 of human 17β-HSD6 and the second antibody is a mouse polyclonal antibody (cat no. H8630-B01P; Novus Biologicals) raised against the full length 17β-HSD6 human protein. The primary antibody used for staining estrogen receptor β was raised in chickens (ERβ 503 IgY)(Saji et al., 2000). The primary antibodies were diluted in 3% bovine serum albumin in

phosphate buffered saline at a dilution of 1 in 300 for 17β-HSD6 and 1 in 200 for ERβ. The slides were incubated with the primary antibodies overnight at room temperature. The next day the slides were washed using 0.1% Nonidet P-40 (NP-40) in PBS for 30 minutes. For 17β-HSD6, the slides were incubated with rabbit on rodent HRP polymer (Biocare) for 30 minutes and then washed with 0.1 % NP-40 in PBS for 30 minutes. For ERβ the slides were incubated with biotinylated goat anti-chicken antibody (Abcam) diluted 1:200 in 3% BSA in PBS for one hour. After washing the slides with 0.1% NP-40 in PBS for 30 minutes, the slides were incubated with vectastain ABC (Vector labs) for one hour. Subsequently, the slides were incubated with diaminobenzidine (Dako) for 30 seconds and counterstained with hematoxylin. The slides were dehydrated using graded ethanol and mounted.

2.3 Results:

2.3.1 Effect of 17β-HSD6 on activation of ERβ in the presence of 5α-reduced C19 steroids:

Since 17β-HSD6 has epimerase activity, we investigated whether the enzyme is able to catalyze the conversion of 3α-Adiol and DHT to 3β-Adiol to activate transcription by ERβ. HEK 293 cells transfected with 17β-HSD6 were able to activate ERβ at significantly higher levels compared to vector transfected cells in the presence of 20 nM DHT or 3α-Adiol (Fig 8). Activation of ERβ by 3β-Adiol or E2 was not altered by the presence of the enzyme.

In HEK 293 cells transfected with 17β-HSD6 expression plasmids, DHT and 3α-Adiol were able to activate transcription through ERβ with EC50 values of 20-30 nM (Fig 9). Interestingly, the reported physiological prostatic concentrations of DHT, 3α-Adiol and 3β-Adiol are 20 nM, 2 nM and 10 nM, respectively (Belanger et al., 1990). HEK 293 cells transfected with empty vector also demonstrated increased activation of ERβ in the presence of DHT and 3α-Adiol. This is most probably due to the endogenous epimerase activity present in HEK 293 cells, which has been reported previously (Belyaeva et al., 2007) .

Fig 2.1: Transactivation of ERβ in the presence of 17β-HSD6 with 5α-reduced C19 steroids:

HEK 293 cells transfected with 17β-HSD6 (black) or empty vector (white) together with ERβ and ERE-luciferase and β-galactosidase were incubated with DHT, 3α-Adiol, 3β-Adiol or E2 (20 nM each). Fold transactivation was obtained by normalizing the relative transactivation (luciferase/β-galactosidase) of steroid treated cells i with to the relative transactivation of DMSO treated cells. Values are expressed as mean ± SEM of triplicate wells. Data are representative of three independent experiments.

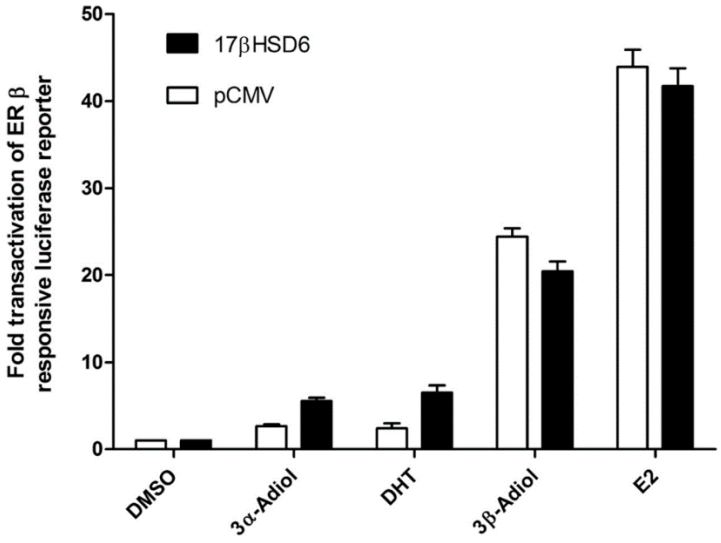
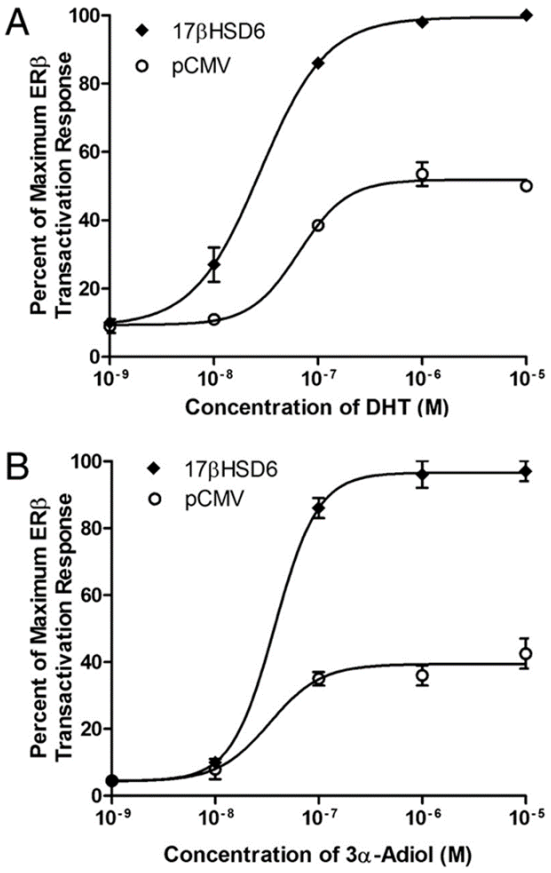


Fig 2.2: Dose response curves of ERβ transactivation in the presence of DHT (A) or 3α-Adiol (B) as substrates:

DHT in the presence of 17β-HSD 6 was able to transactivate ERβ with EC50 of ~ 20 nM and a maximum fold activation of 12.5. 3α-Adiol activated ERβ with EC50 of ~30 nM and a maximum fold activation of 21. Data are presented as mean ± SEM of triplicate wells. The cells were incubated with steroids for 8 hours. Data are representative of three independent experiments.



2.3.2: Enzyme activity of 17β-HSD6 in intact cells:

To confirm that 17β-HSD6 in fact catalyzes the conversion of DHT to 3β-Adiol in intact cells, we conducted a time course experiment using tritiated DHT. Forty-eight hours after transfection with 17β-HSD6 or empty vector, HEK 293 cells were incubated with tritiated DHT at a physiological concentration of 20 nM. After incubation, media were collected at 1, 2, 4 and 8 hour time points, steroids were extracted and analyzed using thin-layer chromatography. The cells transfected with 17β-HSD6 were able to convert ~13% of the total DHT to 3β-Adiol at the 4 hour time point (Fig 3 and 4). Over the time course, 17-keto metabolites accumulated in the medium in agreement with the previous reports (Biswas & Russell, 1997). HEK 293 cells transfected with pCMV vector alone demonstrated significantly lower level of DHT metabolism at the 4 hour time point. At 8 hours only 15-20% of DHT remained in the wells transfected with 17β-HSD6 compared to the 65% of DHT remaining in the wells transfected with vector.

Figure 2.3: Conversion of DHT to 3β-Adiol by 17β-HSD6 in intact HEK 293 cells:

Expression plasmids encoding 17β-HSD6 or empty vector were transfected into HEK-293 cells on day 1. Conversion of DHT to 3β-Adiol was assessed on day 3 using tritiated DHT (20 nM) as substrate. Product quantitation was performed by TLC and radioactivity scanning. Data represent the mean ± SEM of three independent experiments.

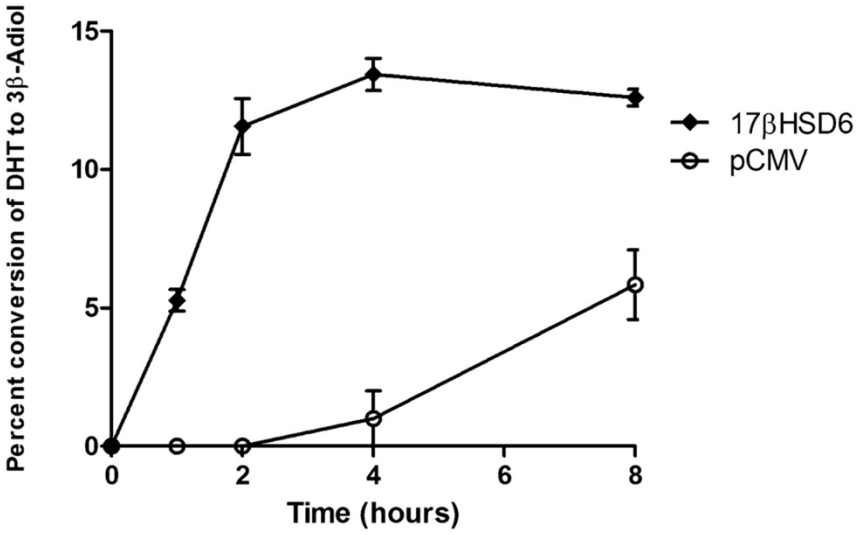
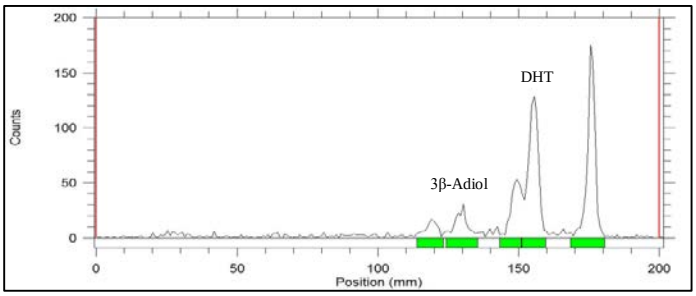


Figure 2.4: Thin layer chromatography analysis of metabolites of radioactive DHT by 17β-HSD6 in intact cells. Extracted steroids were resolved on Partisil LK5D plates and the radioactivity was measured using Bioscan AR2000.

(A) Thin layer chromatogram showing the conversion of DHT to 3β-Adiol by 17β-HSD6 in HEK 293 cells at 4 hours incubation



(B) Thin layer chromatogram showing insignificant DHT metabolism in vector transfected HEK 293 cells at 4 hours incubation.

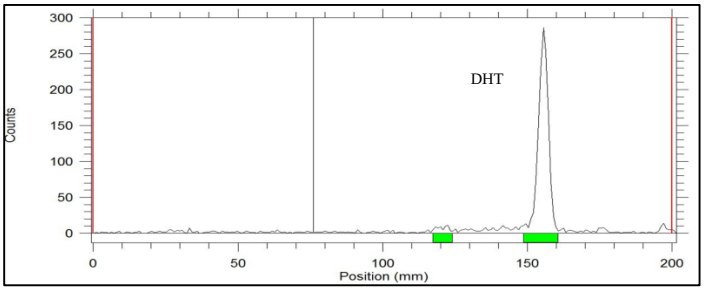
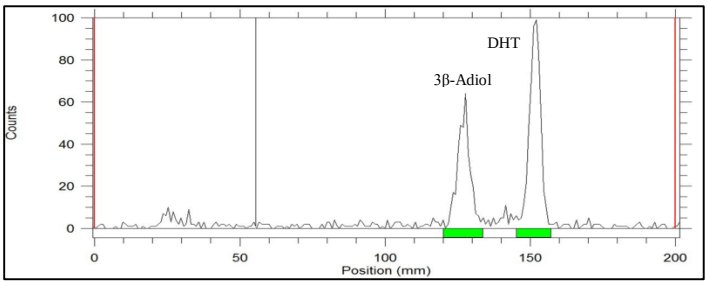


Fig 2.4 (cont.)

(C) Thin layer chromatogram of DHT and 3β-Adiol standards:



2.3.3. Immunohistochemical analysis of 17β-HSD6 in human prostate:

To investigate the physiological role of 17β-HSD6 in the regulation of the metabolism of ligands of ERβ in human prostate, we evaluated the cell-type specific expression of 17β-HSD6 and ERβ in benign prostatic hyperplasia (BPH) (Lee & Peehl, 2004) and prostate cancers of Gleason grades 3, 4 and 5 using immunohistochemistry. The expression of 17β-HSD6 was assessed using an antibody raised against the N-terminus of 17β-HSD6 (Abcam) and the results were verified using another antibody raised against the full length protein (Novus). Immunohistochemical analysis of BPH demonstrated perinuclear staining of 17β-HSD6 in the epithelial cells, especially in the basal epithelial cells (Fig 5). This result suggests that the enzyme is localized to the cytoplasm, which is in agreement with the previous reports (Biswas & Russell, 1997). ERβ was found in the epithelial cells (Fig 2.5). The expression of ERβ was lower in Gleason grade 3 prostate cancer compared to BPH, and ERβ expression was lost in Gleason grade 4 and 5(Figure 2.6). Interestingly, expression of 17β-HSD6 showed a similar trend (2.7). Gleason grade 3 prostate cancer samples demonstrated a significant decrease in the expression of 17β-HSD6 and the expression was lost in prostate cancers of Gleason grades 4 and 5.

Figure 2.5: Glandular epithelium of benign prostatic hyperplasia.

(A and B) Positive perinuclear staining for 17β-HSD6 (arrows). (C and D) Positive nuclear staining or ERβ (arrows). (Scale bars: A and C, 50 μm; B and D, 20 μm.)

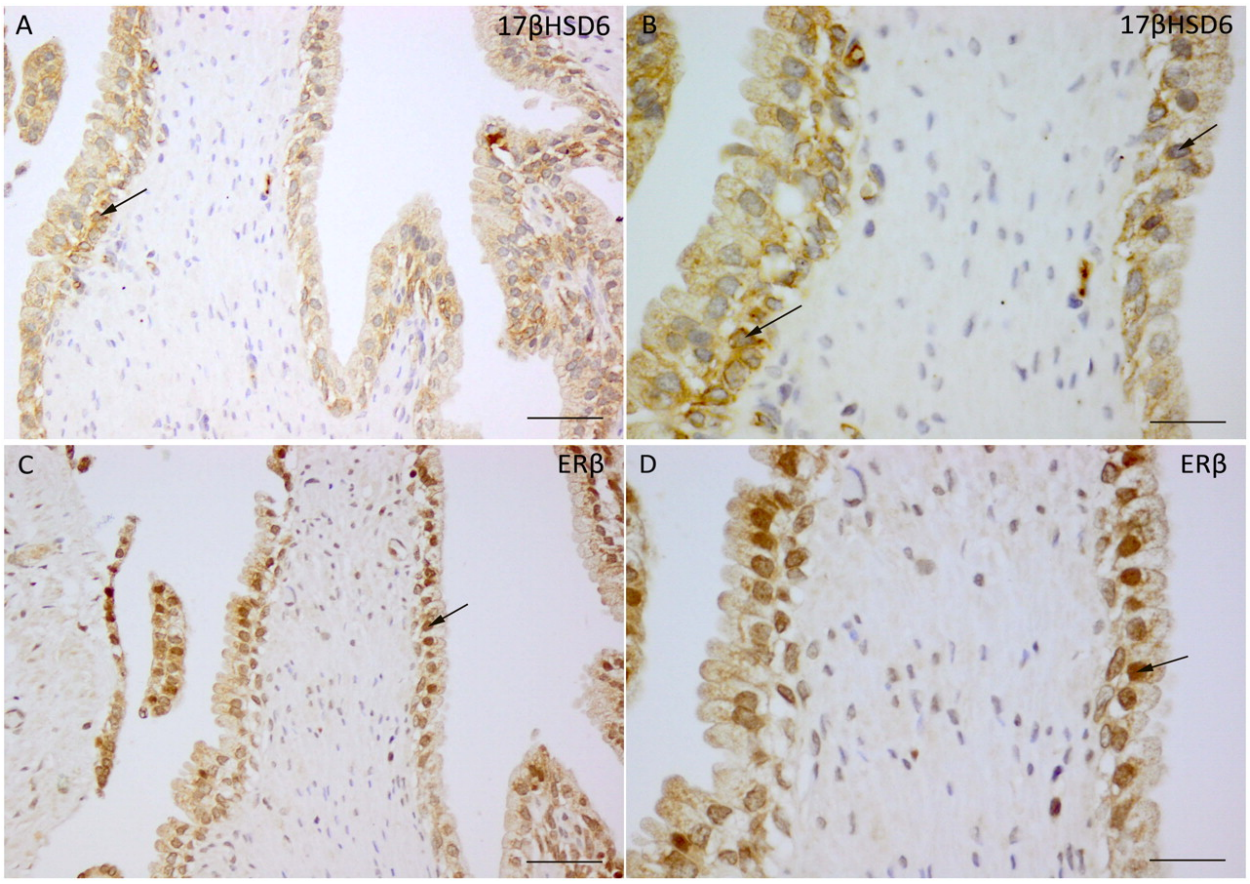


Figure 2.6: ERβ in prostate cancer.

(A) Gleason grade 3 Positive immunostaining in the epithelial cells. (B and C) Gleason grade 4. (D) Gleason grade 5. Loss of ERβ immunostaining.

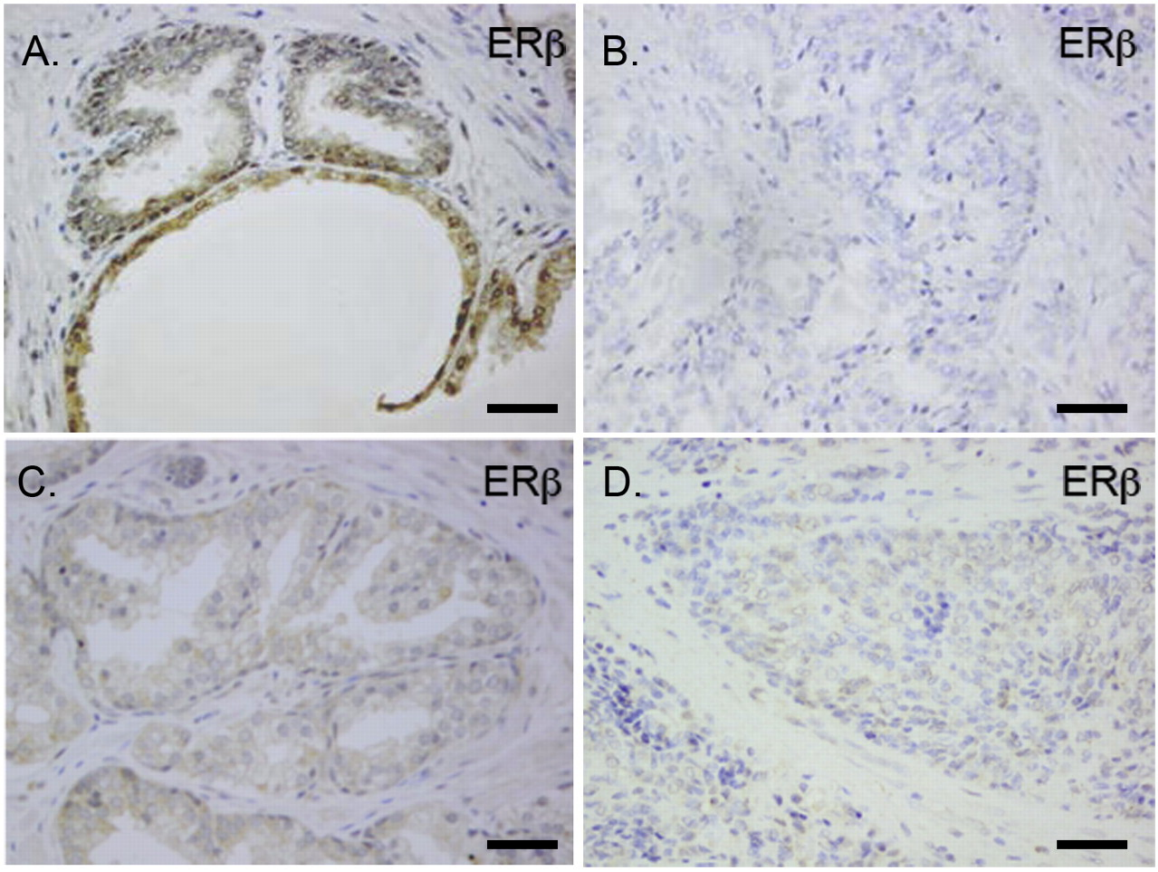
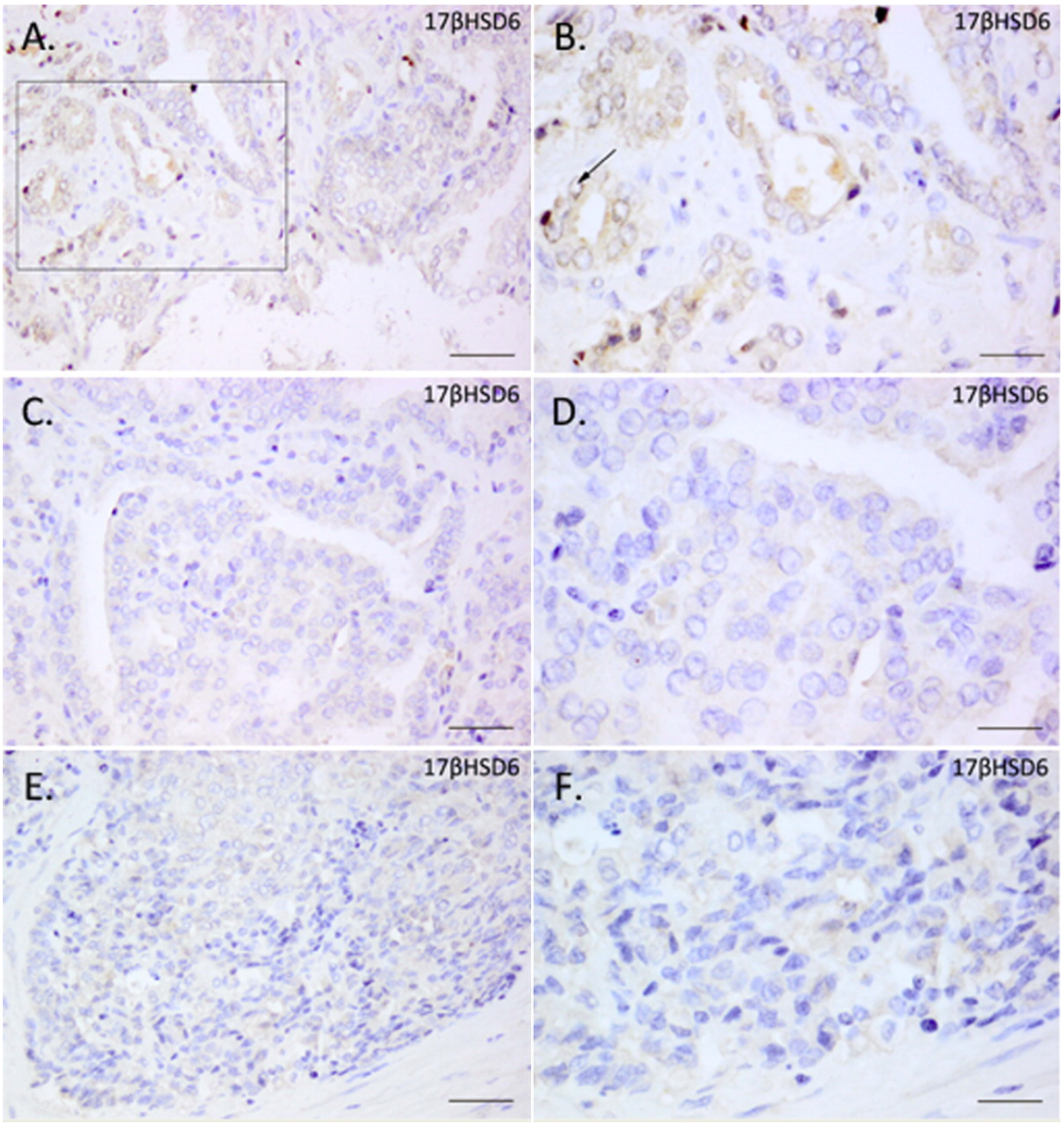


Figure 2.7: 17β-HSD6 in prostate cancer.

(A and B) Gleason grade 3. The number of cells expressing the enzyme is decreased in the epithelium compared with BPH. (C and D) Gleason grade 4. (E and F) Gleason grade 5. No positive immunostaining is seen in the epithelial cells. (Scale bars: A, C, and E, 50 μm; B, D, and F 20 μm.)



2.4 Discussion

Local metabolism of ligands in target tissues plays an important role in the pre-receptor regulation - regulation of the transcriptional activity of nuclear receptors by regulating the intracellular concentration of their cognate ligands. Circulating hormones can be converted into more potent ligands by intracellular enzymes to activate their cognate receptors as in the case of AR (Russell & Wilson, 1994) and thyroid hormone receptor (TR) (Bianco & Kim, 2006). Alternatively, hormones can be inactivated in the cells to prevent aberrant or prolonged activation of a receptor, like the inactivation of cortisol in tissues expressing mineralocorticoid receptor (MR) (Funder et al., 1988).

In prostate, AR and ERβ both play important roles in growth and differentiation (Leav et al., 2001). AR is expressed in the epithelium and the stroma of prostate. AR is pro-proliferative in prostatic epithelium and is a popular therapeutic target for prostate cancer (Saylor, 2013). ERβ on the other hand, is anti-proliferative and pro-apoptotic in the epithelial cells of prostate. The clinical significance of 3β-Adiol and ERβ in prostate cancer is further supported by the finding that subjects treated with 5α-reductase inhibitors presented increased incidence of high Gleason grade prostate cancer compared to subjects treated with placebo (Thompson et al., 2003).

The increased incidence of high grade prostate cancer might be due to the decrease in DHT and the resultant decrease in 3β-Adiol.

Knowledge about the enzymes involved in the metabolism of DHT and 3β-Adiol is necessary to better understand the intricate functional balance of AR and ERβ in their target tissues. In the prostate, testosterone secreted by the testis is converted to DHT by 5α-reductase 2 (Russell & Wilson, 1994). Here we show that 17β-HSD6 can convert DHT to 3β-Adiol in intact cells. Using cell based transactivation assays, we show that co-expression of 17β-HSD6 with ERβ is able to activate ERβ in presence of DHT and 3α-Adiol. The epimerase activity of 17β-HSD6 on C19 and C21 steroid substrates has previously been demonstrated in vitro and in intact cells (Huang & Luu-The, 2000), (Belyaeva et al., 2007). Alternatively, 17β-HSD6 can oxidize 3α-Adiol to DHT and then reduce DHT to 3β-Adiol. The role of 17β-HSD6 as a 3α-HSD to oxidize 3α-Adiol to DHT has been reported (Bauman et al., 2006). Interestingly, the concentration of 3α-Adiol in prostate is ten times lower than 3β-Adiol (Belanger et al., 1990). These findings indicate a pivotal role of 17β-HSD6 in maintaining the balance between DHT, 3α-Adiol and 3β-Adiol in prostate.

The perinuclear localization of 17β-HSD6 in immunohistochemical analysis supports the previous report of 17β-HSD6 is a microsomal enzyme

(Biswas & Russell, 1997). The proximity of the enzyme to the nucleus may provide efficient transfer and high local concentration of 3β-Adiol to the nucleus. The presence of 17β-HSD6 in the epithelial cells and its colocalization with ERβ implicates a physiological significance of the enzyme in the pre-receptor regulation of ERβ. The downregulation of 17β-HSD6 along with ERβ in Gleason grade 4 and 5 of prostate cancer further supports this notion. Interestingly, the promoter region of 17β-HSD6 2kb upstream of the transcriptional start site has several ER/Ap1 binding sites, suggesting that ERβ may regulate 17β-HSD6 at the level of transcription (Huang & Luu-The, 2001). In addition, the localization of 17β-HSD6 in the epithelial cells and the possibility of the enzyme being a transcriptional target of ERβ implies another mechanism by which ERβ can antagonize the proliferative effects of AR in prostate epithelium - by decreasing the intracellular levels of DHT.

The presence of CYP7B1, a cytochrome P450 enzyme that inactivates 3β-Adiol by converting it to 6α and 7α triols, adds another level of complexity to the pre-receptor regulation of ERβ and AR in prostate. High levels of CYP7B1 are found in proliferating cells with low levels of ERβ and cells expressing ERβ show low levels of CYP7B1 (Weihua et al., 2002). In the future, it will be interesting to study the expression of AR,

ERβ, 17β-HSD6 and CYP7B1 together and elucidate the role of these enzymes in the pre-receptor regulation of AR and ERβ.

CHAPTER 3: Endogenous ligands of ERβ in mouse brain

3.1 Introduction:

ERβ is expressed in the mouse brain during various stages of development - from embryo to adult, and plays important roles in the development and the function of brain.

In the adult mouse brain, ERβ is expressed in hypothalamus, bed nucleus of stria terminalis, and amygdala. ERβ is also expressed in locus coeruleus. Adrenergic fibers from locus coeruleus project to the forebrain regions such as cortex, hippocampus, striatum and hypothalamus (Mitra et al., 2003). In addition, ERβ has been shown to transcriptionally regulate tyrosine hydroxylase, the rate limiting enzyme in the synthesis of catecholamines (Maharjan et al., 2005). The adrenergic system plays an important role in regulation of stress responses in the brain. ERβ is also expressed in the in the ventral tegmental region of PONS (Mitra et al., 2003). The dopaminergic neurons of the ventral tegmental region are involved in the regulation of stress response. ERβ is also expressed in the serotonergic neurons of dorsal raphe (Suzuki et al., 2013), which is involved in mood regulation. Hence, by regulating transcription in the monoaminergic system of the brain, ERβ plays a very important role in mood, anxiety and depression.

In addition to neurons, ERβ is expressed in microglial cells where it modulates inflammation. Dysfunction of microglia is involved in the development of multiple sclerosis and amyotrophic lateral sclerosis. Selective activation of ERβ suppresses activation of microglia and improves symptoms in experimental autoimmune encephalitis (Wu et al., 2013).

The expression of ERβ mRNA in embryonic rodent brain starts at E10.5 (Lemmen et al., 1999). The expression of ERβ protein in rodent brain starts at the age of E12.5 and reaches a maximum at E18.5 (Fan et al., 2006). ERβ is important in the migration and layering of neurons in the cortex (Wang et al., 2003). ERβ knockout mice have significantly lower number of cells in layer ii, iii and iv of the cerebral cortex (Wang et al., 2001). At E18.5, ERβ regulates the expression of calretinin (Fan et al., 2006).

Estradiol (E2) is the most potent endogenous ligand of ERβ. E2 is synthesized from testosterone (T) by aromatase. In embryonic rodent brain, aromatase activity is absent until E19 (Colciago et al., 2005). In addition, the fetus is protected from E2 exposure by α-fetoprotein, a major protein in the fetal circulation. α-Fetoprotein is secreted by the fetal liver and yolk sac during pregnancy and binds to estradiol with high affinity (Kd = 1nM) (Uriel et al., 1976). The physiological significance of α-fetoprotein is emphasized by the finding that α-fetoprotein knock-out female mice show behaviors of

masculinization (Bakker et al., 2006). Since ERβ is expressed and functional before the maturation of aromatase activity in the embryonic rodent brain, and the developing brain is protected from maternal estradiol by α-fetoprotein, it is possible that some ligand other than estradiol with a lower affinity for α-fetoprotein is present and activates ERβ during embryonic development.

In adult female mice, the most probable endogenous ligand of ERβ is E2. The major source of estradiol is the gonads. It can also be synthesized locally in the brain from circulating T or estrone (E1) by aromatase and 17β-HSD1 respectively. In the adult mouse brain, aromatase is expressed in regions like the bed nucleus of stria terminalis, hypothalamus, amygdala and temporal cortex (Roselli et al., 2009). Even though the presence of E2 has been demonstrated in mouse brain by immunoassays, recent experiments with gas chromatography-tandem mass spectrometry (GC-MS/MS) (Meffre et al., 2007) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Konkle & McCarthy, 2011) failed to demonstrate the presence of E2 in rodent brain extracts. Interestingly, aromatase knockout mice have higher number of parvalbumin- positive interneurons in the layers ii, iii and iv of the neocortex which is in contrast to the decrease in the neurons in the layers ii, iii and iv/v seen in ERβ knockout mice (Anthoni et al., 2012).

Estrone (E1) is a weak estrogen compared to E2. It is found in the circulation at levels of 3 to 36 pg/mL whereas E2 is present at a concentration of 2 -42 pg/mL (Kushnir et al., 2008). E1 is derived from oxidation of E2, a reaction catalysed by 17β-HSD2 or it can be synthesized through aromatization of androstenedione (Miller & Auchus, 2011). The presence of E1 in mouse brain has been demonstrated by LC-MS/MS (Toran-Allerand et al., 2005). But the physiological significance of E1 in the brain is not known.

Recently, 5-androstene-3β,17β-diol (5-androstenediol) has been demonstrated to have a significant role in the immunosuppression mediated by ERβ (Saijo et al., 2011). So, 5-androstenediol could be a physiologically valid ligand of ERβ in the human central nervous system. 17β-HSD14, the enzyme responsible for the conversion of DHEA to 5-androstenediol, is expressed in the human brain (Sivik et al., 2012). In addition, cell free homogenates prepared from human temporal lobe biopsies have the ability to transform DHEA to 5-androstenediol in the presence of NADPH in vitro (Steckelbroeck et al., 2010). The physiological relevance of 5-androstenediol in the mouse is not clear since DHEA, the precursor of 5-androstenediol, is not produced by the rodent adrenal (Miller & Auchus, 2011).

5α-Androstane-3β,17β-diol (3β-Adiol) is another potential endogenous ligand of ERβ in brain. 3β-Adiol is synthesized from DHT by 3β-HSD (Steckelbroeck et al., 2004). 3β-HSD activity has been found in the adult brain cytosolic fractions (Steckelbroeck et al., 2010). 3β-Adiol is inactivated by the enzyme CYP7B1, which is abundantly expressed in the brain especially in the regions of hippocampus and cerebellum (Rose et al., 2001). Interestingly, the CYP7B1 knockout mouse brains are transiently enlarged in early postnatal life before P14 and this phenotype is corrected at around 3 months of age (Sugiyama et al., 2009). In addition to 3β-Adiol, CYP7B1 can also metabolize estradiol (Rose et al., 1997).

3.2 Materials and Methods:

3.2.1 Materials:

Estradiol, estrone and 3β-Adiol were obtained from Steraloids (Newport, RI). 2, 6-Bis (1,1-dimethylethyl)-4-methylphenol (BHT) was obtained from Sigma (St. Louis, MO). Dibasic potassium phosphate was obtained from Mallinkrodt chemicals. LC-MS grade hexane, methanol and acetonitrile were obtained from Sigma. Dichloromethane and ethyl acetate were purchased from Honeywell Burdick & Jackson (Morristown,NJ) and n-butanol was obtained from Fisher (Waltham, MA). Tritiated E1, E2 and 3β-Adiol were purchased from Perkin-Elmer (Waltham, MA).

3.2.2 Animals:

For this study we used adult wild type, aromatase knockout (ARKO), and CYP7B1 knockout mice of ages 3 to 12 months. The wild type, aromatase and CYP7B1 knockout mice were obtained from Jackson Labs, Maine. The animals were maintained in the University of Houston animal care facility under normal conditions. To isolate the brains, the animals were anaesthetized deeply using CO2 followed by perfusion using PBS. For steroid extraction, the brain tissue was isolated and snap-frozen using liquid nitrogen. Isolated brains were stored at -80°C for further experiments. Tails

of the mice were also collected for genotyping. All procedures were approved by the Institutional animal care and use committee of the University of Houston.

3.2.3 Steroid extraction from frozen mouse brain tissue:

To extract steroids from the mouse brain we used a two-step procedure. The total lipids from the mouse brain were first extracted using Folch extraction. About half of the brain tissue (~200 mg) was homogenized in a borosilicate glass culture tube (Pyrex) in 4 mL of Folch solvent, a mixture of chloroform and methanol at a ratio of 2:1, using a polytron hand-held homogenizer. BHT was added before homogenization at a final concentration of 1 μM to prevent oxidation. In addition, 50 pmols each of d4-E1 and d5-E2 was added as internal standards for quantification using LC-MS/MS. The other half of the brain was homogenized as described above without the internal standards to be used for the transactivation assay. The homogenate was then transferred to a glass culture tube with PTFE caps (Wheaton, NJ). The homogenate was sonicated with 120W for 2 minutes to increase the steroid recovery. The homogenate was left on a shaker at 4˚C overnight.

After 12 hours the homogenate was centrifuged in a Beckman Coulter Allegra 25R centrifuge. The supernatant was collected, dried down and redissolved in 2 mL of hexane:ethyl acetate mixed at a ratio of 4:1. The lipid extract was further purified by solid phase extraction using chromobond silica columns (cat.no 730075G, Macherey-Nagel). The silica columns were preconditioned using 4 mL of hexane: ethyl acetate (4:1).The lipid extract from mouse brain dissolved in hexane: ethyl acetate (4:1) was then applied to the preconditioned silica column. The column was then washed first with hexane: ethyl acetate 4:1 and hexane: ethyl acetate 2:1. The steroids were then eluted with ethyl acetate: methanol 4:1.The eluates were dried down and dissolved in ethanol. The extracts were further purified by removing the phospholipids using Phree solid phase extraction plates (Phenomenex). The extracts were dried down and redissolved in water:acetonitrile 50% and analysed using LC-MS/MS.

3.2.4 Liquid chromatography-Tandem Mass spectrometry (LC-MS/MS analysis of mouse brain extracts:

Targeted analysis and quantification of E1, E2 and 3β-Adiol in mouse brain extracts was performed using a Waters Acquity UPLC system coupled with Waters Xevo triple quadripole mass spectrometer. Data acquisition was done using Masslynx software (Waters) and the data

analysis and quantification was done using the TargetLynx software (Waters). Chromatographic separation of 17α-E2, 17β-E2 and E1 was performed on a Waters UPLC BEH Phenyl column 2.1 mm X 50 mm (catalog no. 186002884). The mobile phase A was water with 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. 17α-E2, 17β-E2 and E1 were resolved using an isocratic gradient of 53% of mobile phase B at a flow rate of 0.5 ml/min at 40˚C. The samples were resolved again on a BEH C18 column 2.1 mm X 50 mm (Waters catalog no: 176000861) for E1 and 3β-Adiol with an isocratic gradient of 55% solvent B at 40˚C.

Multiple reaction monitoring for E1, 17β-E2, 17α-E2, E1 and 3β-Adiol was performed using Waters Xevo triple quadripole mass spectrometer in positive ion mode. The capillary voltage was 1000 V and the desolvation gas flow was 1000 L/min and the desolvation temperature was 500˚C. Nitrogen was used as collision gas in Q2. The transitions monitored for quantification were - E1 m/z 271.13 > 133.03, E2 – 255.14> 133.02, 3β-Adiol – 275.2>175.13, d4-E1 – 275.14>135.10 and d5-E2 – 259.15> 135.05. Calibration curves for E1, E2, 3β-Adiol, d4-E1 and d5-E2 were calculated using 5, 10, 50, 100, 500, 1000 and 5000 fmols on column for each analyte.

3.2.5 Cell Culture:

HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen,CA) supplemented with 10% fetal bovine serum (Sigma), 50 µg/mL Kanamycin and 10 mM HEPES (pH 7) in a 75 cm2 sterile cell culture flask (BD Falcon). On day 0 the cells were plated on a 24 well costar (Corning) plate containing 0.5 mL of phenol red free DMEM supplemented with 10% dextran treated/ charcoal stripped fetal bovine serum (Hyclone), 2% glutamine (Invitrogen), 50 µg/mL Kanamycin and HEPES buffer. On day 1 the cells were transfected with expression plasmids to be used for transactivation assay as described below.

3.2.6 Transactivation assay:

On day 1 the HEK 293 cells in 24 well costar plates were transfected with expression plasmids of Gal4 responsive UAS-luciferase reporter plasmid (170 ng/well), pRSV-β galactosidase (30 ng/well) and pCMV-ERβLBD-Gal4DBD fusion construct (100 ng/well). The transfection was done using Fugene 6 transfection reagent (Roche diagnostics) at a ratio of 4 µl of transfection reagent per µg of plasmids. Twenty-four hours after the transfection the HPLC fractions of the mouse brain extracts dissolved in DMSO as described above was diluted in the medium at concentration of 1

μL per mL. E2, 3β-Adiol and E1 were used as positive controls. After 16 additional hours of incubation with the extracts the cells were lysed and assayed for luciferase activity using luciferase assay kit (Biovision). About 50μL of the cell lysate per well was loaded on a white 96 well plate (Corning), and luciferase activity was measured using Perkin Elmer Victor X4 plate reader according to manufacturer's instructions. β-Galactosidase level was measured using β-galactosidase assay buffer containing o-nitro-phenyl β- galactoside (ONPG) as substrate. About 20μl of clear cell lysate was added in a transparent 96 well plate (BD Falcon) to which 100 μL of β-galactosidase assay buffer was added and incubated for 30 minutes at room temperature. The reaction was terminated by adding 100 μl of 1M sodium carbonate solution. The optical density was measured with a 405 nm filter using Perkin Elmer Victor X4 plate reader. The relative transactivation was calculated by dividing the luciferase values by optical density values from β-galactosidase assay. The fold activation was calculated by normalizing the relative transactivation values of cells treated with extracts to those of cells treated with vehicle.

To evaluate the transcriptional activation of ERβ on ERE and AP-1 sites HEK 293 cells were transfected with 100 ng of pcDNA3- ERβ full length plasmid, 170 ng of ERE or AP-1 luciferase reporter plasmid, 30

ng, and pRSV- β-galactosidase plasmid using calcium phosphate transfection method. Briefly, the plasmids were mixed and diluted with water to a final volume of 20 µl/well. Then about 2.7 µl of 2.0 calcium chloride was added to the mixture. Then the whole mixture was added to 2X HEPES buffered saline at a volume of 22 µl/well carefully while bubbling the saline to prevent formation of clumps. The mixture was incubated for 30 minutes and 45µL of the final mixture was added per well. Six hours after transfection ligands were added to the cells. After additional 12 hours of incubation, the cells were lysed and the luciferase and β-galactosidase assays done as described above.

3.2.7 Evaluation of metabolism of E1 and E2 in HEK 293 cells:

To measure the conversion of E1 to E2, HEK 293 cells were plated in 24 well costar plates. Tritiated E1 and E2 were added to cells at concentrations of 1, 5, 10 and 25 nM and incubated for another 16 hours. About 100 µl of the collected medium was mixed thoroughly with 400 µL of Folch solvent mixture (chloroform: methanol 2:1) and centrifuged at 14000 X g for five minutes. Then the lower organic phase was dried down under vacuum using speed-vac. The dried lipid extract from the medium was dissolved in 40 µL of Folch and spotted onto Partisil LK5D thin-layer chromatography plates (Whatman). The plates were developed using

dichloromethane:ethyl acetate 5:1 twice. The steroid metabolites on the thin-layer chromatography plate were quantified using Bioscan AR 2000 scanner.

3.2.8 Evaluation of metabolism of E1 and E2 in mouse brain tissue

To evaluate the metabolism of estrone and estradiol in mouse brain, fresh mouse brain tissue was divided into two halves in the sagittal plane. One half of the brain tissue was minced and incubated with 5 ml of serum free DMEM containing tritiated E1 and the other half with tritiated E2 at a final concentration of 10 nM. 1 ml medium was collected at 1, 3, 6 and 24 hour time points after the start of incubation. 100 microliters of medium from each time point was extracted with 400 μl of Folch and analyzed using thin-layer chromatography as described above.

3.3 Results:

3.3.1 Transactivation assay for screening of ligands of ERβ:

We used Gal4 DBD-ERβ LBD constructs and Gal4 responsive luciferase reporter constructs to screen for the ligands of ERβ in mouse brain extracts. Preliminary experiments indicated that the lower limit of detection of the assay for E2 was 1 nM, which is equivalent to 1 picomole of E2 per well of volume 1 ml. The lower limit of detection for E1 was 5 nM equivalent to 5 picomoles of E1 per well of volume 1 ml (Fig 3.1). To assess whether E1 is reduced to E2 to transactivate the receptor, we incubated the HEK293 cells overnight with tritiated E1 or E2 at concentrations of 0.5,1,5,10 and 25 nM. Thin-layer chromatography analysis of the Folch extracts of the cell culture medium (collected after overnight of cell culture did not show any significant conversion of E1 to E2 (Fig 3.2).

Figure 3.1: Transactivation assay: Lower limit of detection for E1 and E2. Data presented as Mean ± SD of triplicate wells.

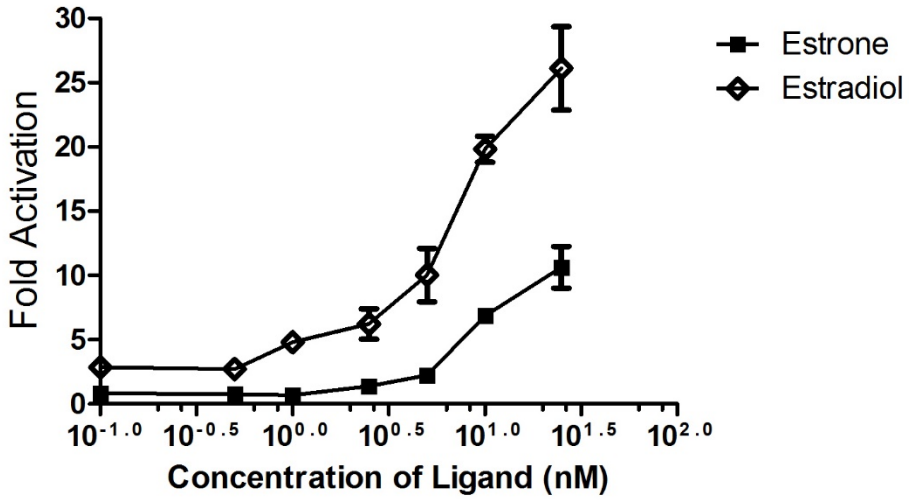
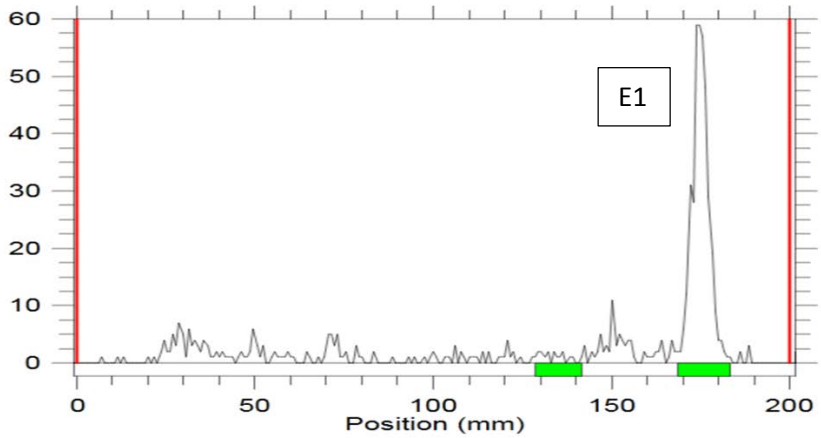
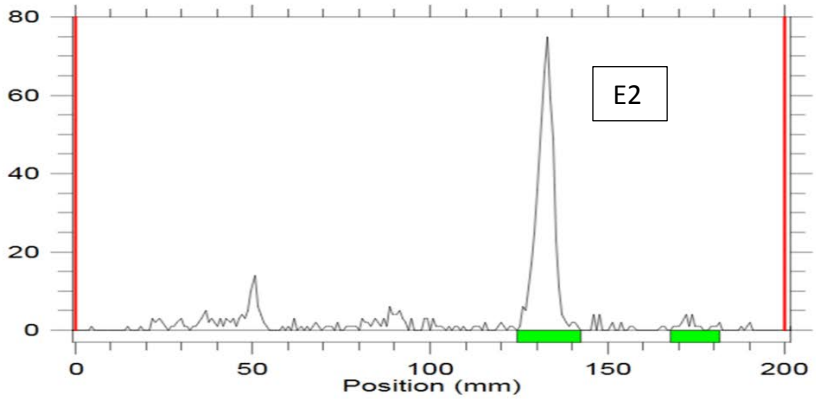


Figure 3.2: Thin-layer chromatography analysis of metabolism of E1 and E2 by HEK293 cells

A. Metabolism of tritiated E1 by HEK 293 cells. HEK 293 cells were incubated with tritiated E1 at 0.1,0.5,1,5,10 and 25 nM concentrations. Data is representative for metabolism of E1 at 10 nM.



B. Metabolism of tritiated E2 (25 nM) by HEK 293 cells. HEK 293 cells were incubated with tritiated E2 at 0.1,0.5,1,5,10 and 25 nM concentrations. Data is representative for metabolism of E2 at 25 nM.



3.3.2 Steroid extraction from mouse brain:

Total lipids from mouse brain were extracted using the classic Folch extraction method, homogenizing the brain tissue in a mixture of chloroform and methanol at a ratio of 2:1 (Folch, Lees, & Sloane Stanley, 1957). Additional sonication step after homogenization was added to improve steroid recovery. The total lipid extract was purified using solid phase extraction to concentrate the steroids and to remove the other lipids that could potentially interfere with the subsequent LC-MS/MS analysis of the steroids. For solid phase extraction we used both reverse phase (C18) and normal phase (silica) columns. Normal phase solid phase extraction using silica provided high extraction efficiency of steroids with minimum lipid mass in the steroid fraction. So, we used silica columns for solid phase extraction of the total lipids from mouse brain tissue. Using cell-based transactivation assay with Gal4 reporter construct, we found that most of the ERβ transactivation activity was extracted in the ethyl acetate: methanol (4:1) fraction (Fig 3.3) with only 10% of the total lipid mass eluting in the fraction. The average extraction efficiency of this method was 95%, as assessed by measuring the recovery of internal standards using LC-MS/MS.

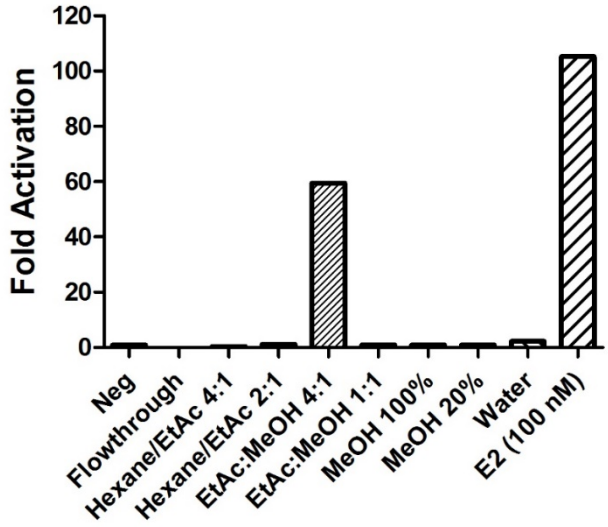
We used aromatase knockout mice to investigate ERβ transactivation in the brain extracts in the absence of E2. The aromatase

knockout mouse brain extracts did not show any transcriptional activation of ERβ (Fig 3.4).

We also investigated the ability of brain extracts from CYP7B1 knockout mice to activate ERβ in the transactivation assay. CYP7B1catalyzes the hydroxylation and inactivation of 3β-Adiol, and in its absence, 3β-Adiol content in the brain should be elevated. As anticipated, ERβ transactivation in extracts from CYP7B1 knockout mice was significantly higher than wild-type sex-matched controls (Fig 3.4).

Figure 3.3.Solid phase extraction of mouse brain extracts.

A.ERβ transactivation of solid phase extracts from wild type male mouse brain.



B. Mass distribution of the lipids in solid phase extraction fractions from wild type male mouse brain

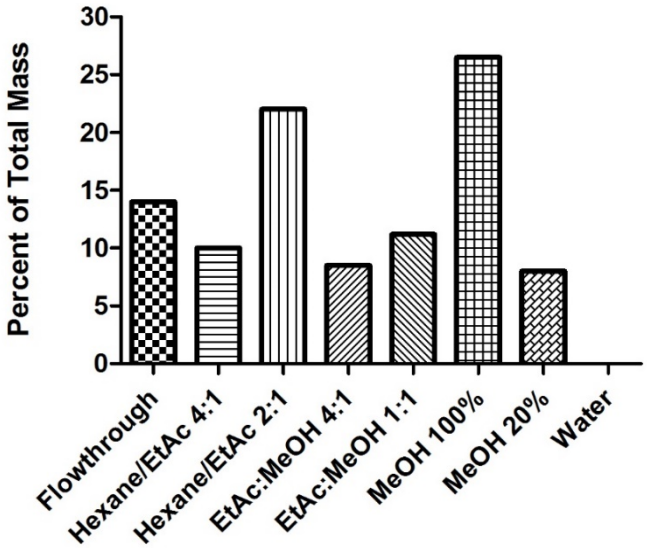


Figure 3.3 (cont.)

C. Recovery of radioactive E1 in solid phase extraction fractions from wild type male mouse brain

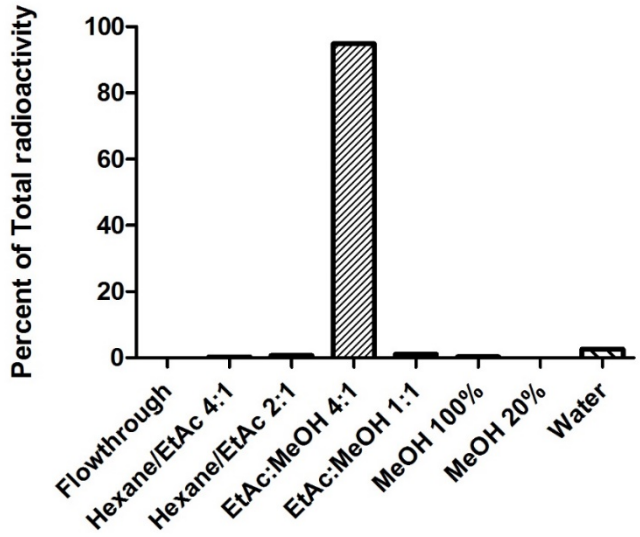
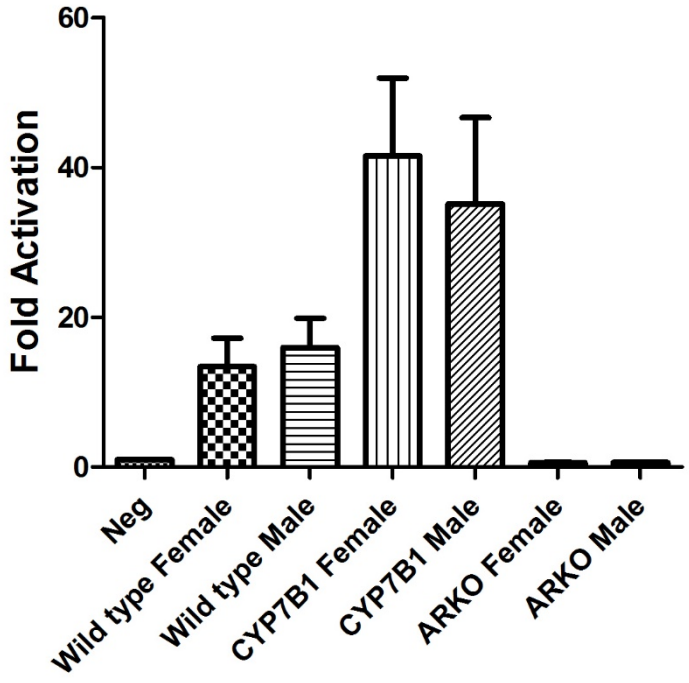


Figure 3.4: ERβ transactivation of the brain extracts of wild type, CYP7B1 and aromatase knockout mice. Data is presented as mean and standard error of mean of three independent experiments.



3.3.3 LC-MS/MS analysis of mouse brain extracts:

The brain extracts from wild type, aromatase knockout and CYP7B1 knockout mice were analyzed using LC-MS/MS. The mouse brain extracts were screened for E1, 17β-E2, 17α-E2 and 3β-Adiol. The lower limit of quantification for E1 and 17β-E2 was 10 fmol on column and for 3β-Adiol was 100 fmol on the column. In wild type, aromatase and CYP7B1 knockout mice 17β-E2, 17α-E2 and 3β-Adiol were below the limit of detection. E1 was the only steroid detected in both wild type and CYP7B1 knockout mice (Fig 3.5). Interestingly, E1 levels in the brain tissue of CYP7B1 knockout mice were significantly higher than that in wild type mouse brain tissue (Table 1)

Figure 3.5: LC-MS/MS analysis of mouse brain extracts:

A. Chromatogram of wild type female mouse brain extracts on BEH Phenyl columns.

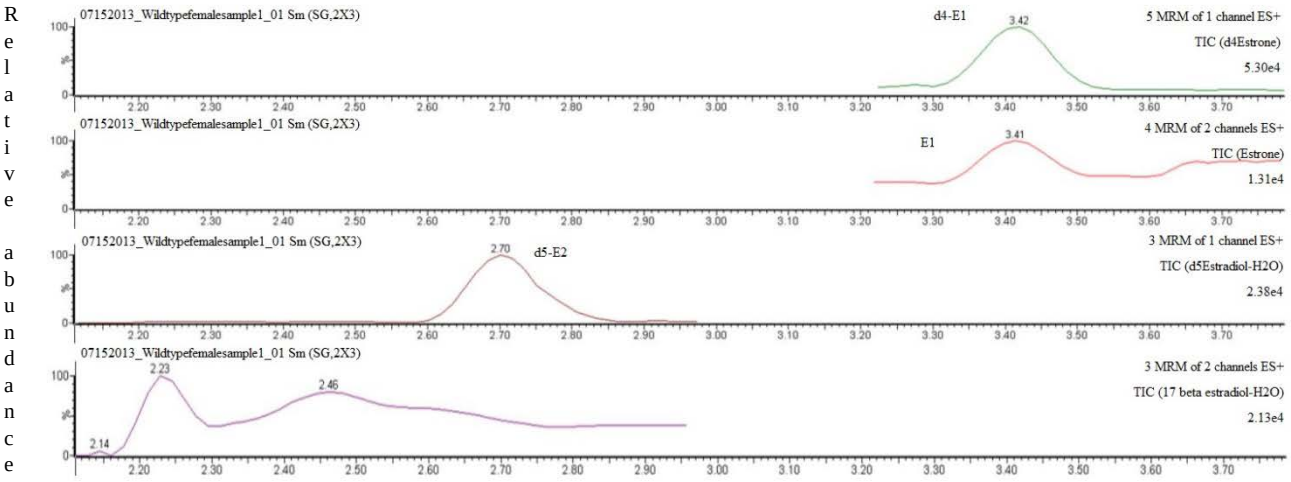


Figure 3.5 (cont.)

B. LC-MS/MS analysis mouse brain extracts: Chromatogram of CYP7B1 female mouse brain extracts on BEH C18 columns.

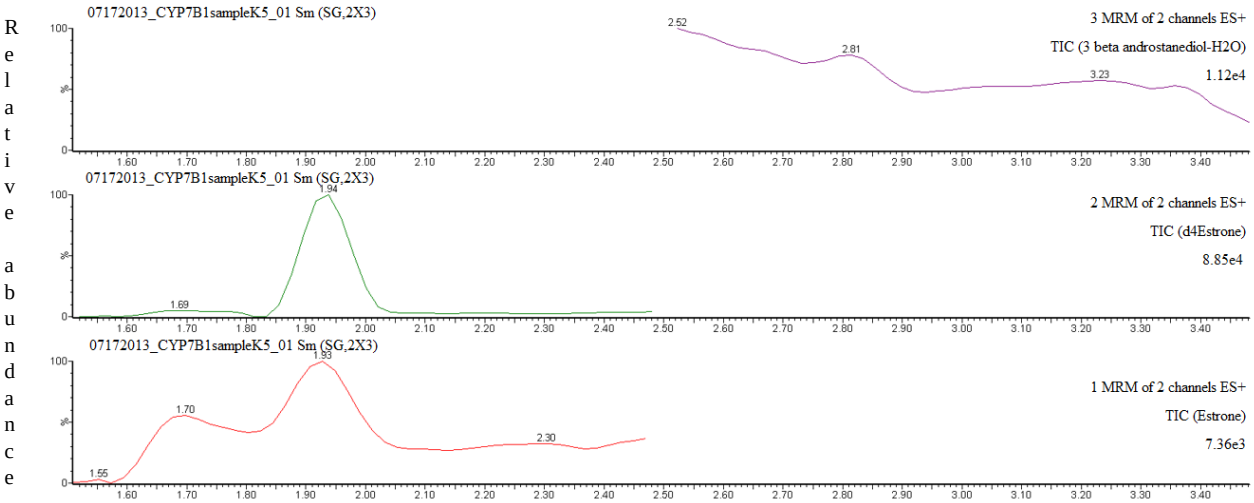


Table 1: LC-MS/MS analysis mouse brain extracts. Concentration of E1 in mouse brain extracts.

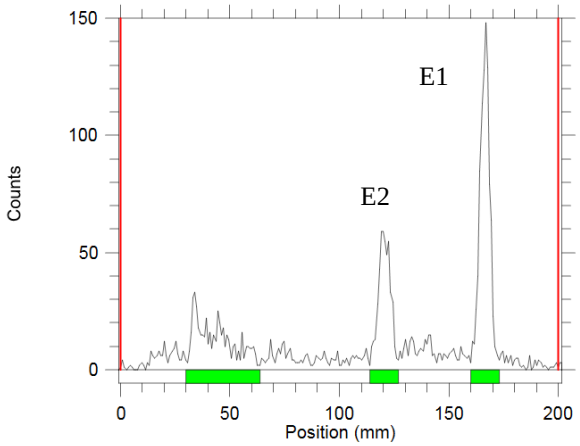
	Genotype	Mean concentration of E1 pmol/g	S.D	
1	Wild-type female	25.39	2.37	
2	CYP7B1 knockout female	35.77	2.19	p=0.0169
3	Wild-type male	35.77	3.85	
4	CYP7B1 knockout male	48.3	12.82	p=0.0386

3.3.4. Metabolism of E1 and E2 in mouse brain:

Metabolism of E1 and E2 in mouse brain tissue was evaluated by incubating tritiated E1 and E2 as substrates with fresh brain tissue. Until 6 hours both E1and E2 were not metabolized significantly to any other metabolite. At the end of 24 hours, 25% of E1 was converted to E2 and 27% of E1 was converted to a polar metabolite. But about only 10% of E2 was converted to E1 at the end of 24 hours.

Figure 3.6: Thin-layer chromatography analysis of metabolism of E1 and E2 by mouse brain tissue.

A. Metabolism of tritiated E1 (10 nM) by 200 mg of brain tissue after 24 hours.



B. Metabolism of tritiated E2 (10 nM) by 200 mg of brain tissue after 24 hours.

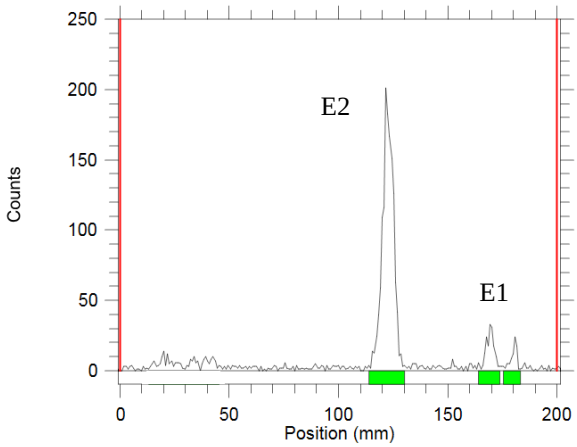
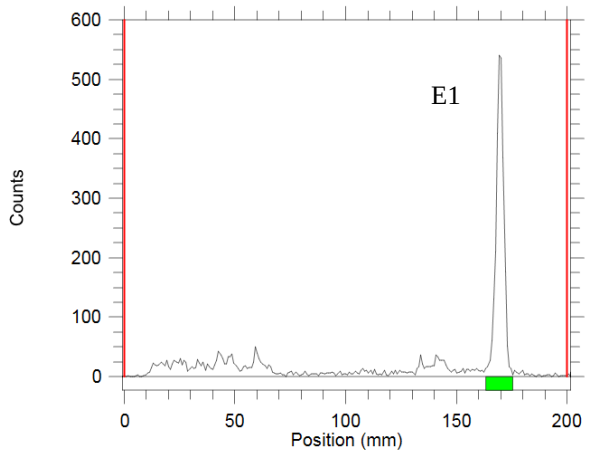
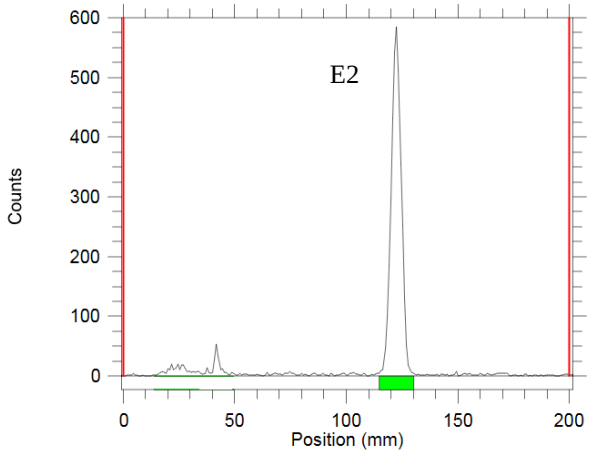


Figure 3.6 (cont.)

C. E1 and E2 standards.



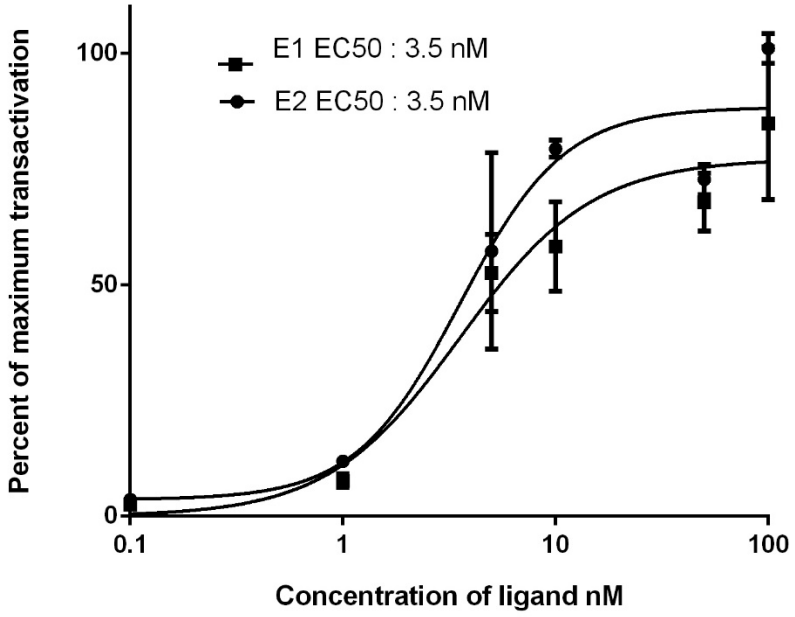


3.3.5. Transcriptional activation of ERβ by E1 and E2:

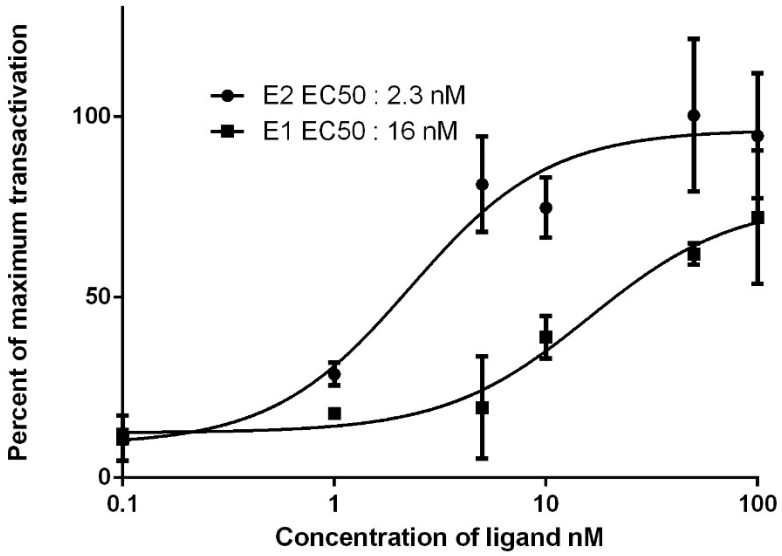
Transactivation assays, using full length ERβ and ERE luciferase reporter indicate that both E1 and E2 are capable of activating ERβ mediated transcription. The EC50 value was around 3.5 nM for both E1 and E2, but the maximum transactivation by E1 was about 85% of the maximum transactivation of ERβ by E2. Interestingly, the EC50 value of E1 for ERα mediated transactivation was about 16 nM (Fig 3.7). Both E1 and E2 did not activate ERβ mediated transcription on AP-1 or SP-1 sites. On the contrary, both E1 and E2, at 10 nM and 100 nM concentration, activated ERα on AP-1 and SP1 sites.

Figure 3.7: Transactivation of ERα and ERβ by E1 and E2 on ERE

A. Transactivation of ERβ on ERE – Dose response curve



B. Transactivation of ERα on ERE – Dose response curve



C. Comparison of E1 on ERα and ERβ – Dose response curve

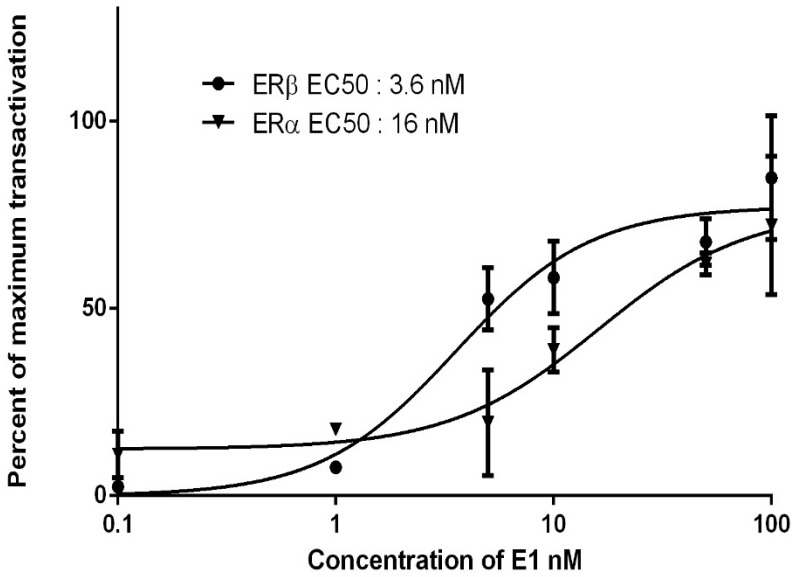
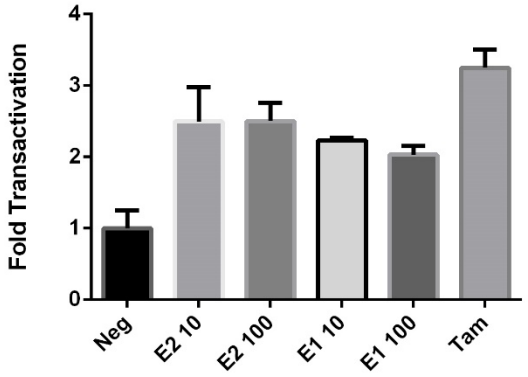


Figure 3.8. Transactivation of ERα and ERβ by E1 and E2 on AP-1 and SP-1 sites

A. Ligand dependent transactivation of ERα on AP-1 sites



B. Ligand dependent transactivation of ERβ on AP-1 sites

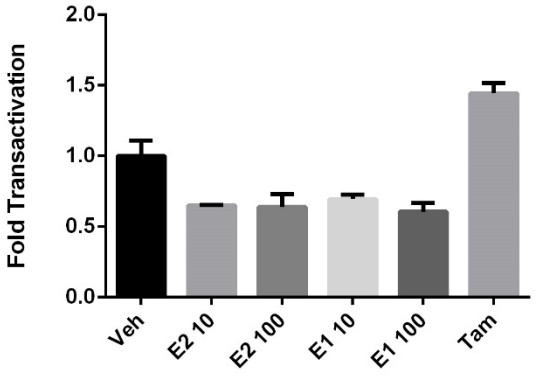
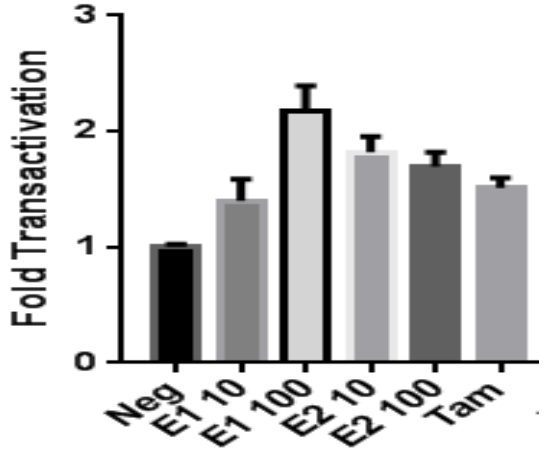
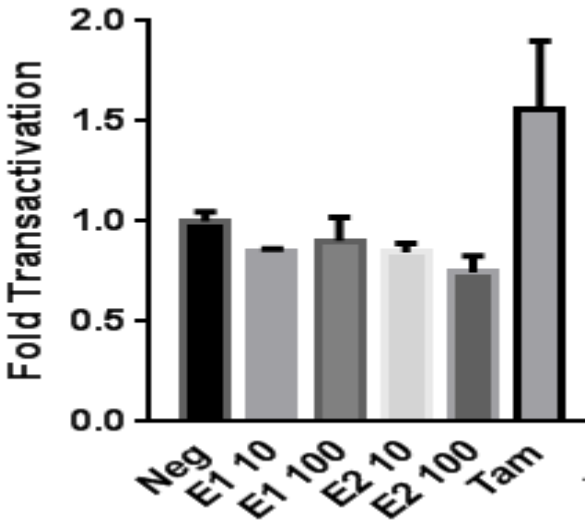


Figure 3.8 (cont.)

C. Ligand dependent transactivation of ERα on SP-1 sites



D. Ligand dependent transactivation of ERβ on SP-1 sites



3.4 Discussion:

Identification of novel endogenous ligands of estrogen receptors has become necessary because of the previous studies reporting that the deficiency of aromatase enzyme does not exactly reflect the phenotype of the ERα and ERβ knockout mice (Anthoni et al., 2012; Robertson et al., 2002). Our studies using wild type and CYP7B1 knockout mice show that the major endogenous estrogen present in the mouse brain is E1. The E1 extracted from the brain was capable of activating ERβ in cell based transactivation assay.

Liquid chromatography and mass spectrometry analyses indicated that the estrone was approximately 36 picomoles/gram tissue in the male mouse brain and 25 picomoles/gram tissue in the female mouse brain. In the adult mice the circulating E1 is around 10 pg/mL (Toran-Allerand et al., 2005). So, the concentration of E1 is much higher in the brain compared to the concentration of E1 in the circulation, which is in agreement with previous studies (Toran-Allerand et al., 2005). According to these studies, the source of E1 appears to be the gonads, because E1 was absent in the brain of the gonadectomized animals (Toran-Allerand et al., 2005).

In our studies, extracts from aromatase knockout mice brains did not have any ERβ transcriptional activity indicating that the ligand of ERβ in adult mouse brain requires aromatase for its synthesis. This finding validates the presence of E1 in the active fractions from wild type adult mouse brain extracts.

E1 is a weak estrogen synthesized from androstenedione by the enzyme aromatase in the gonads and released into circulation. The circulating androstenedione can be aromatized in the brain to provide E1. E1 can be converted to E2 by 17β-HSD1 (Miller & Auchus, 2011). In rodent brain, 17β-HSD1 is expressed in thalamus, hypothalamus, hippocampus, basal ganglia and the microglia (Saloniemi et al., 2012). So, it is possible that E1 is converted to E2 in small regions of brain to activate ERα and β. Our results also indicate that there is a conversion of E1 to E2 in mouse brain. We could not detect the conversion of E1 to E2 until the end of 6 hours after the start of the experiment, and only about 25% of E1 was converted to E2 by 200 mg of brain tissue. This indicates that an enzyme with high catalytic efficiency for conversion of E1 to E2 is expressed in very small regions of mouse brain, or if it is expressed in large regions of the brain, the efficiency of the enzyme is not high.

Surprisingly we could not detect E2 in male or female adult mouse brain extracts in either wild type or CYP7B1 knockout mice. This was unexpected because adult mice synthesize and release E2 into the bloodstream to be taken up by the brain. However, previous studies using GC-MS (Meffre et al., 2007) and LC-MS (Konkle & McCarthy, 2011) with or without derivatization could not detect E2 in the rodent brain. Since estrogen receptors are expressed in very limited regions of the mouse brain, the amount of E2 in the whole brain extracts may be below the limit of detection. But also previous studies analyzing estradiol content in small defined regions of the brain failed to detect the presence of E2 using LC-MS (Toran-Allerand et al., 2005).

Another possibility is that E2 taken up by the brain is rapidly oxidized to E1 or inactivated by conjugation with glucuronide or sulfate groups (Miller & Auchus, 2011). However, previous studies using LC-MS after deconjugation failed to detect the presence of E2 in brain extracts (Konkle & McCarthy, 2011). 17β-HSD2, one of the major the enzymes responsible for oxidation of E2, is not present in the brain (L. Wu et al., 1993). However, our results indicate that there is a low level of oxidation of E2 happening in the mouse brain tissue. Enzymes capable of oxidizing E2, like 17β-HSD4 (Normand et al., 1995), 17β-HSD8 (Pelletier et al., 2005) and 17β-HSD10

(He et al., 2002) are expressed in small regions of the brain. In addition, microglial cells, which are ERβ positive, express only the enzymes capable of oxidizing E2 to E1, namely 17β-HSD2, 17β-HSD4, 17β-HSD10 and 17β-HSD14 (Saijo et al., 2011). And microglial cells do not express 17β-HSD1, which is capable of reducing E1 to E2. So, even if there is small amount of E2 present the brain, it is most probably converted to E1 in the microglial cells.

Brain extracts from CYP7B1 knockout mice, as expected, were more effective at activating ERβ transcription than did those from wild type mice. LC-MS/MS analysis revealed that the concentration of E1 in the brain tissue of CYP7B1 knockout mice was approximately 45 pmol/g tissue in the male mice and 36 pmol/g tissue in the female mice. So the CYP7B1 mice have significantly higher E1 concentration in the brain tissue compared to the wild type mice. CYP7B1 has multiple substrates including DHEA, 3β-Adiol, 5-androstenediol, E2 (Rose et al., 1997) as well as E1 (Kim et al, 2004).

Challenges in analyzing E2 in mouse serum, brain tissue and human tissues have been realized in many recent studies (Haisenleder et al., 2011; Rosner et al., 2013). So far most of the studies quantifying E2 in mouse

brain used immunoassays. Recent investigations comparing immunoassays with GC-MS demonstrated the high incidence of false positive results and disproportionately high concentrations of E2 by immunoassays (Haisenleder et al., 2011).

Our inability to detect E2 and 3β-Adiol in the mouse brain extracts might be due to the technical difficulties in measuring the steroids using mass spectrometry. The lower the limit of quantification of E2 and 3β-Adiol using our LC-MS system was 10 femtomoles and 100 femtomoles, respectively. Reducing matrix interferences by solid phase extraction and removing phospholipids from the brain extracts did not improve the detection of E2 or 3β-Adiol. Furthermore E2 and 3β-Adiol could not be detected after pooling multiple brain extracts and concentrating the extracts by four folds. Previously published results indicate that methods using derivatization of brain extracts with dansyl chloride for LC-MS/MS (Konkle & McCarthy, 2011) or heptaflurobutyrate for GC-MS/MS failed to detect of E2 (Meffre et al., 2007).

The important question raised by our observations is whether E1 could be a physiological ligand of ERβ in brain. E1 is considered to be the inactive form of E2 which can be readily converted to E2 in the target tissues. The importance of E2 as a ligand of ERs in human beings is

demonstrated in postmenopausal women. After menopause the deficiency of E2 leads to symptoms like hot flushes, depression and osteoporosis. Even though after menopause the concentration of E1 also decreases by two thirds from about 112 pg/mL to 32 pg/mL, this decrease is lower compared to the decrease of E2 from about 264 pg/mL to 21 pg/mL (Kushnir et al., 2008).

However, E1 is a weak transcriptional activator. *In vitro* experiments have demonstrated that ERβ has stronger affinity for certain coactivator peptides in the presence of E1 compared to E2 (Ozers et al., 2005). This indicates the possibility of E1 initiating a completely different set of biological effects compared to E2 due to differential recruitment of coactivators. Such response has been demonstrated with 5-androstenediol acting on ERβ to suppress inflammation, an effect not produced by E2 (Saijo et al., 2011). Even though E1 binds to ERα and ERβ with similar affinity, it has almost 10 fold higher potency in terms of coactivator recruitment to ERβ compared to ERα (Jeyakumar et al., 2011). Our transactivation experiments using full length ERβ and ERE luciferase indicate that the EC50 value for E1 is 3.5 nM, which is almost similar to the EC50 of E2. According to our results, the concentration of E1 in brain tissue is about 25 to 35 nM, and E1 should therefore function as an effective endogenous ligand of ERβ in the brain tissue. Interestingly, E1 has been shown to be neuroprotective in rats

after traumatic brain injury (Gatson et al., 2012). E1 also protected male rats from kainite induced seizures whereas E2 did not (Budziszewska et al., 2001).

In summary, we have identified E1 in the mouse brain extracts is an endogenous ligand of ERβ. CYP7B1 knockout mice were found to have significantly higher E1 concentration than wild type mice in the brain. E1 is a substrate of CYP7B1 and the increase in E1 concentration is mostly due to the lack of the enzymatic action of CYP7B1 on E1.

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