LOCAL ESTROGEN PRODUCTION IN THE BRAIN AND ITS REGULATION UNDER PATHOLOGICAL CONDITIONS

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Estrogens have important roles in regulating brain structure and function. Estrogens are produced by conversion of androgens via the enzyme aromatase (ARO). ARO is expressed in specific regions of the brain, and recent studies suggest that local estrogen may have a greater impact on neuronal survival and plasticity than systemic one. In this dissertation study, I developed UPLC-MS/MS based assays to measure ARO activity and estradiol (E2) in the rat brain tissues. I also detected the E2 levels in the brain and compared with that in the serum in rats given different E2 and testosterone treatments. Moreover, I tested the effect of manipulating cholinergic systems and the effect of traumatic brain injury (TBI) on the expression of different isoforms of ARO and estrogen receptors (ER) in different brain regions. Results demonstrate that 1) the microsomal-based assay to detect ARO activity is highly sensitive, specific and reliable. By applying this method, I demonstrated a correlation between ARO activity and its long-form mRNA, and that their distribution was in accord with previous studies; 2) that E2 levels in brain regions with ARO are higher than that in serum in ovariectomized rats treated with different doses of estradiol benzoate and testosterone propionate. Letrozole (an ARO inhibitor) treatment reversed the differences between brain and serum estradiol levels, demonstrating that the differences were due to local estradiol production. Moreover, male rats had substantial levels of E2 in specific regions of the brain even though levels in the systemic circulation were not detectable; 3) Selective cholinergic lesions in the medial septum and daily cholinesterase inhibitor (ChEIs) injections, did not regulate ARO and ERs in the cortex and hippocampus; 4) In

the cortex, there was a significant increase of long-form ARO in females and a significant increase of ER alpha in both sexes, indicating an induction of estrogen signaling in the cortex after CCI. In the hippocampus, the long-form ARO expression was significantly decreased in both sexes with an increase of ER beta in males after CCI. Overall, this dissertation provides novel methods and important information about brain estrogen production within the field of neuroendocrinology.

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Preface

My journey in pursuing the Doctor degree of Pharmaceutical Science in the School of Pharmacy, University of Pittsburgh is wonderful and memorable. It allows me to explore and develop my personality and discover my interest in career. These cannot be achieved without the help and support from my excellent mentor, colleagues and peers in the program. I would like to thank everyone that I met and had pleasure of work with and special acknowledgement as below.

I would like to give my deeply special thanks to my beloved advisor: Dr. Robert B Gibbs. He is not only a mentor, but more like a father in my life, encourages me not only to be valuable researchers, but to be worthy citizens of the world. He provides me with a very friendly, supportive and flexible working environment, which allows me to enjoy the fun part of research and explore the beauty of science. His advice in response to my tribulations and struggles is always wise and logical and, though I may sometimes not listen to him, nearly always correct! During the Ph.D. training, he not only gave me the knowledge, but moreover, trained me to be a person who is capable of learning new things and taking challenges. Without him, I can never be the person I am.

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1.0 Introduction

1.1 Effects of systemic estrogen on brain function and behavior

Estrogens are present in both in females and males. Estrogens play important roles in maintaining the physical health of many organs, such as the gonads, heart, bone, breasts, and brain. Estrogens are mainly generated in the gonads for systemic circulation, but they can also be produced locally. There are three major types of endogenous estrogens, namely estrone, estriol, and estradiol (E2), with E2 being the most potent and prevalent in humans. Since puberty, estrogen levels fluctuate during the menstrual cycle and peak at approximate age of 35. Later on, estrogen levels decrease during menopause and become very low during post-menopausal years (Elmlinger et al., 2002). Interestingly, although significantly lower than that in females, males also have substantial amounts of estrogens which have important physiological roles in males. In fact, studies suggested that during the perinatal period, in which systemic E2 circulation is absent, local estrogen production in the brain is higher in males than in females, which in turn masculinize the male brain (Gladue and Clemens, 1980b; Konkle and McCarthy, 2011; Roselli and Klosterman, 1998).

In addition to the regulation of sexual dimorphisms in the brain during development and to affect sexual behavior in adults, estrogens also are associated with mood regulation. Specifically, changes in estrogen levels are linked to premenstrual syndrome, postmenopausal dysphoric disorder, and postpartum depression (Bebbington et al., 1981; Bloch et al., 2003;

Rubinow and Schmidt, 1995). Clinical studies report that depression and anxiety symptoms can be reduced after estrogen levels are stabilized or restored (Bebbington et al., 2003; Jenkins, 1987). Several studies illustrate that E2 has beneficial effects on performance on cognitive tests in rodents as well as primates including humans (Bimonte and Denenberg, 1999; Daniel et al., 1997; Frye et al., 2007; Gresack and Frick, 2006; Luine et al., 2003; Sherwin and Henry, 2008). Moreover, estrogen is suggested to prevent the progression of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (AD) and to exert neuroprotective effects. Studies have illustrated that estrogens influence the synthesis, release, and metabolism of dopamine and modulate dopamine receptor expression and function (Shulman, 2002). Several papers also report that estrogens increase cerebral blood flow (Greene, 2000; Maki and Resnick, 2001) and reduce atrophy in the hippocampus and cortex associated with aging and AD (Hua et al., 2007; Kramer and Erickson, 2007). Although most studies have reported beneficial effects of estrogen on the brain, debate has persisted due to the diversity of study designs. Moreover, estrogens are not currently used in the treatment of brain diseases due to the severe side effects reported for hormone replacement therapy.

1.2 Estrogen production and elimination

Estrogens are derived from cholesterol, which is converted in the mitochondria to 7α-hydroxy pregnenolone, and transferred to smooth endoplasmic reticulum, where it is converted to dehydroepiandrosterone and ultimately androstenedione. Androstenedione, a substance with weak androgenic activity, serves predominantly as a precursor for more potent androgens such as testosterone as well as estrogens. Androstenedione crosses the basal membrane into the

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surrounding granulosa cells, where it is immediately converted to estrone or to testosterone and subsequently to E2 in an additional step. The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD), whereas aromatase (ARO) catalyzes the conversion of androstenedione and testosterone into estrone and E2, respectively. Estrogens are metabolized via hydroxylation by cytochrome P450s such as CYP1A1 and CYP3A4 and via conjugation by estrogen sulfotransferases (sulfation) and UDP-glucuronosyltransferases (glucuronidation). In addition, E2 is dehydrogenated by 17β-HSD into the much less potent estrone.

1.3 Classes of Estrogen receptors

E2 executes regulatory functions in the brain by binding to its receptors. Three estrogen receptors (ERs) that mediate estrogenic functions have been reported. ER alpha and ER beta are nuclear receptors, and they mainly regulate gene transcription upon activation (Manavathi and Kumar, 2006; McEwen, 2002). Recently, ER alpha and ER beta also were found to be membrane-associated and to mediate rapid effects of estrogen signaling. GPR30 is a recently identified membrane-associated ER. Activation of these membrane receptors will trigger rapid second-messenger cascades (Brailoiu et al., 2007; Coleman and Smith, 2001; Fu and Simoncini, 2008; Funakoshi et al., 2006; Moriarty et al., 2006). The distribution of ERs varies among brain regions. In particular, ER alpha is distributed broadly, including the hippocampus, hypothalamus, amygdala, and brain stem nuclei. Conversely, the distribution of ER beta is relatively restricted and is found in particulare abundance in the hippocampus and some hypothalamic nuclei (Gillies and McArthur, 2010). GPR30 exists both intracellularly and on the plasma membrane, and it promotes rapid estrogen signaling in a variety of cell types. In the rat brain, GPR30 exists in many brain regions, including the cortex, hippocampus, and hypothalamus (Brailoiu et al., 2007; Hazell et al., 2009).

Studies by our group and others illustrated the important roles of GPR30 in mediating the effects of estrogen in the brain (Hammond and Gibbs, 2011; Hammond et al., 2012).

1.4 Sources of brain estrogen

Estrogen in the brain is primarily produced through two sources. Historically, estrogen synthesis has been believed to be restricted to the gonads. After release from the ovaries, partial estrogen binds to sex hormone-binding globulin, corticosteroid-binding globulin, and albumin in the peripheral circulation (Hammond, 2016; Pasquali et al., 1997). Only the free portion of E2 (~5% in the adult) crosses the blood–brain barrier via transmembrane diffusion through a non-saturable, bidirectional process, permitting its brain level to rapidly reach pseudo-equilibrium with the blood levels (Banks, 2012). Meanwhile, no efflux transporter has been reported to extrude E2 (Tsuji et al., 1992).

In 1971, ARO, the primary enzyme for E2 production, was discovered in the brain by Zoltan Petro and Frederick Naftolin (Weisz and Gibbs, 1974). Subsequently, all enzymes necessary for estrogen synthesis have been found in the central nervous system, indicating the possibility of local estrogen production (Rossetti et al., 2016). In fact, in postmenopausal women, and men, E2 is mainly produced extragonadally. Because of the difficulty of directly measuring E2 levels in tissue, the levels of the rate-limiting enzyme ARO are often utilized used to imply the level of local estrogen production.

ARO irreversibly converts androgens (both androstenedione and testosterone) into estrogens (estrone and E2) (Biegon et al., 2012). It was initially discovered in the hypothalamus,

which is important for regulating sexual dimorphism and behavior (Roselli and Resko, 1991; Roselli et al., 1998). ARO mRNA expression and activity have since been observed in many brain regions, with the highest levels detected in the preoptic area, medial amygdala, and bed nucleus of the stria terminalis, followed by hippocampus, cortex, and other brain regions (Li et al., 2016a; Roselli et al., 1997; Tabatadze et al., 2014). As a cytoplasmic cytochrome P450, ARO is mainly found in microsomes, whereas several papers using immunohistochemistry also detected ARO immunoreactivity in the presynaptic axon vesicles of the vertebrate brain (Balthazart and Ball, 2006). In addition, ARO was reported to be co-localized in brain regions that contain ERs, indicating the possibility of local estrogenic effects in the brain (Balthazart and Ball, 1998).

1.5 Local estrogen production: functions and regulations

1.5.1 ARO participates in behavior regulation

As mentioned previously, brain ARO has attracted increasing attention due to its essential roles in sexual differentiation and dimorphism. In fact, during the perinatal period, in which systemic E2 circulation is absent, ARO activity was transiently higher in the brain, and the circulating levels of testosterone and androstenedione were higher in males than in females, indicating that males are exposed to higher local estrogen levels during this period (Lephart, 1996). E2 and testosterone levels gradually decline after birth, reaching low levels in the hippocampus and frontal cortex in adulthood. ARO activity also declines after birth, but its levels remain substantial in the hypothalamic neurons and several regions in the limbic ring (olfactory

bulbs, hippocampus, hypothalamus, amygdala) (Konkle and McCarthy, 2011). The hypothalamus and preoptic area have particular importance in the regulation of sexual behavior, and ARO activity is higher in these regions than in others. Studies have revealed that the aromatization of testosterone to E2 in the hypothalamus contributes to luteinizing hormone-negative feedback regulation in male humans, non-human primates, sheep, and mice (Couse and Korach, 1999; Hayes et al., 2000; Roselli and Resko, 2001; Sharma et al., 1999). Moreover, treatment with E2 or testosterone, but not dihydrotestosterone, a non-aromatizable metabolite of testosterone, can completely restore sexual behaviors in adult castrated male rats (Baum and Vreeburg, 1973; McDonald et al., 1970; Putnam et al., 2003; Putnam et al., 2005). In fact, many male-typical sexual behaviors, such as sexual partner preference and copulatory behaviors, result from the organizational and activational effects of locally synthesized E2 (Gladue and Clemens, 1980a; Gladue and Clemens, 1980b; Roselli and Resko, 1993).

In addition to studies exploring the important roles of ARO in regulating sex differentiation in the brain, ARO has also been suggested to regulate emotional and cognitive functions. In prior research, ARO knockout (ArKO) ovariectomized (OVX) female mice exhibited increased depression-like behavior compared with wild-type OVX female mice in the forced swim test (Dalla et al., 2004). Moreover, specific deletion of ARO in the medial amygdala also increased aggression in both males and females (Unger et al., 2015). ARO inhibition also induced depression-like symptoms in women with breast cancer (Gallicchio et al., 2012). Meanwhile, the effects of ARO on cognitive functions are more controversial and appear to be sex-specific. Some clinical and preclinical studies revealed the beneficial effects of ARO. Implantation of E2 and testosterone into castrated male songbirds led to significant improvements in learning ability in a spatial learning task, whereas dihydrotestosterone had no

effect (Moradpour et al., 2006; Oberlander et al., 2004). One clinical study of healthy elderly men revealed that ARO inhibition prevented the improvement in verbal memory induced by testosterone (Cherrier et al., 2005). Another study of postmenopausal women with breast cancer illustrated that 3 months of anastrozole, an ARO inhibitor treatment resulted in worsened verbal and visual learning (Bender et al., 2007). On the contrary, other studies found that ARO had no beneficial effects on, or conversely impaired, cognition. Two randomized controlled trials conducted by International Breast Intervention Study II and the Tamoxifen and Exemestane (ARO inihibitor) Adjuvant Multinational study found no significant effects of anastrozole and exemestane on the performance of cognitive tasks in postmenopausal women with breast cancer (Jenkins et al., 2008; Schilder et al., 2010). One preclinical study observed that the systemic administration of letrozole (2.5 kg/ml) facilitated working memory acquisition in male rats (Alejandre-Gomez et al., 2007). These conflicting results may due to differences in the models, treatment dose, and treatment interval, as E2 exerts dose-, time-, and region-specific effects on cognition.

1.5.2 ARO regulates neuroplasticity and neuroprotection

Despite the complex effects of ARO on behavior, at the molecular level, ARO regulates plasticity in different brain regions, which may account for its divergent effects on behavior. Indirect evidence indicates that both testosterone propionate and dehydroepiandrosterone increase the density of spine synapses in pyramidal neurons in the CA1 area of the hippocampus of OVX adult female rats. This effect can be completely blocked by letrozole, a powerful non-steroidal ARO inhibitor (Leranth et al., 2004). In addition, ARO inhibition reduced axon outgrowth in neonatal hippocampal cell cultures (von Schassen et al., 2006). Systemic letrozole

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administration also reduced the spin synapse density in the basolateral nucleus of the amygdala and abolished long-term potentiation in acute brain slices of adult females but not males (Bender et al., 2017). Evidence has also revealed that active ARO is expressed by radial glial cells, one of the neural progenitor cell types in several species including rodents and birds (Alvarez-Buylla et al., 1990; Malatesta et al., 2000; Noctor et al., 2001), and by intermediate progenitor cells during cortical neurogenesis in vitro. The same finding was also noted in adult teleost and zebra finch brains (Forlano et al., 2001; Menuet et al., 2003; Pellegrini et al., 2005; Peterson et al., 2004). These findings suggest that brain ARO plays a role in regulating neuronal plasticity.

Under pathological conditions, ARO plays important roles in neuroprotection, possibly by increasing E2 production at the site of injury (Roselli, 2007). Prior research indicated that ARO expression can be induced in astrocytes, which do not express ARO under physiological conditions, in different disease models, such as models of brain injury (Peterson et al., 2007), experimental stroke (Carswell et al., 2005), global ischemia-reperfusion, spontaneous hypertension (Pietranera et al., 2011), and neuroinflammation (Duncan and Saldanha, 2011). Moreover, ArKO models or animals treated with ARO inhibitors display increased neuronal loss after excitotoxic injuries and increased tissue damage after ischemia for both males and females (Azcoitia et al., 2001a; Garcia-Segura et al., 2003; Veiga et al., 2005). Interestingly, the induction of ARO expression was also accompanied by the upregulation of other steroidogenic molecules, such as steroidogenic acute regulatory protein and cytochrome P450 side chain cleavage (Lavaque et al., 2006a), which are involved in the first step of steroidogenesis (Lavaque et al., 2006b; Sierra, 2004). In addition, in various experimental models, the neurodegenerative effect of ARO deficiency was counterbalanced by E2 administration (Azcoitia et al., 2001b; McCullough et al., 2003; Saldanha et al., 2009a; Sierra et al., 2003), indicating that the

neuroprotective effect of ARO is due to the production of E2 rather than reduction of E2 precursors. ARO also participates in neurogenesis after brain injury, as indicated by decreased cell proliferation and apoptosis in letrozole-treated hippocampal cultures (Fester et al., 2006). A recent in vivo study illustrated that ArKO mice have reduced stroke-induced neurogenesis (Li et al., 2011), in accordance with the results in zebra finch (Peterson et al., 2007; Walters et al., 2011).

Several studies also suggest that changes in brain ARO levels are relevant to AD. Carbo et al. identified a single-nucleotide polymorphism (SNP) in CYP19 that was associated with an earlier onset of AD (Corbo et al., 2009). Additionally, a SNP in the ARO gene was linked to a 60% increased risk of AD (Iivonen et al., 2004). Another group found that two haplotype blocks in CYP19 increased the risk of AD by 2-fold in APOE4 carriers (Huang and Poduslo, 2006). In addition, decreased ARO expression was reported in the hippocampus of patients with AD (Ishunina et al., 2007b). Inducing ARO and local E2 production protects hippocampal neurons against beta amyloid toxicity (Cui et al., 2011). This evidence suggests that ARO is important for protecting brain function and preventing neurodegeneration.

1.5.3 Regulation of ARO mRNA expression and activity

The regulation of ARO in the brain has been studied in both mammals and birds, and different mechanisms have been suggested. Typically, brain ARO can be regulated through two mechanisms. One mechanism involves the regulation of CYP19A1, the gene encoding ARO. It is located on the long arm of chromosome 9, and it spans 2863 bp in length. The coding region spans bp 613–2124 and contains nine exons (2–10). Upstream of the coding region are several alternative 5′-untranslated first exons resulting from the use of different promoters that are

tissue-specific (Azcoitia et al., 2011). The If promoter was found specifically to regulate the brain production of ARO, and certain substances, such as progesterone and the glucocorticoid dexamethasone, regulate brain ARO expression by binding to this promoter (Brooks et al., 2012; Yilmaz et al., 2011). A number of studies have demonstrated regulation of the If promoter by gonadal steroids including E2 and testosterone (Abdelgadir et al., 1994) (Iivonen et al., 2006) (Negri-Cesi et al., 2001b; Roselli et al., 1997), but the regulatory effect of gonadal hormones appears region-specific. In fact, one study illustrated that ARO in hypothalamic neurons was gonadectomy-sensitive, whereas that in the "limbic ring" including the amygdala and hippocampus was unresponsive to steroids (Jakab et al., 1993), suggesting at least two populations of ARO-positive cells in the brain. Using a highly sensitive ribonuclease protection assay, Roselli and co-workers identified two ARO fragments of different lengths in rat brain regions. The 430-nucleotide fragment was associated with enzyme activity in the brain, whereas the function of the shorter 300-nucleotide fragment was unknown and apparently region-specific (Roselli et al., 1997). A primer pair that specifically detects long-form ARO was also developed by Tabatadze, and by applying this primer pair, we identified a correlation between the distribution of long-form ARO mRNA expression and ARO activity across different brain regions (Li et al., 2016a; Tabatadze et al., 2014). Different brain regions have different distributions of these two ARO isoforms, making studies of ARO more complex.

Regarding the regulation of ARO gene transcription and translation, studies performed by Balthazart and co-workers demonstrated that ARO activity can be regulated rapidly. Using neural tissue from male Japanese quail, research uncovered that Mg2+, Ca2+, and ATP in hypothalamic homogenates and exposure to high Ca2+ levels following K+-induced depolarization in the brain can rapidly downregulate local ARO activity. This was achieved by

increasing the intercellular Ca2+ concentration to enhance ARO phosphorylation (Balthazart et al., 2001a; Balthazart et al., 2001b; Balthazart et al., 2005; Balthazart et al., 2003a). Several neurotransmitters, such as glutamate and catecholamines (including dopamine and norepinephrine), were proposed to regulate ARO activity in the same manner as they regulate adenylyl cyclase activity and cyclic AMP concentrations (Balthazart et al., 2006). In addition, this rapid non-genomic regulation was a reversible process that may occur at presynaptic terminals, leading to the hypothesis that estrogens can function as neuromodulators or neurotransmitters (Balthazart and Ball, 2006).

Although many studies explored the regulation of ARO mRNA expression and activity, some questions remain. One question is the discordance between the distribution of ARO protein expression detected by immunohistochemistry and ARO activity in the brain, especially in mammals. For example, several studies reported substantial staining of ARO in the cortex region, whereas ARO activity was hardly detected (Azcoitia et al., 2003; Li et al., 2016a; Roselli et al., 1997; Tabatadze et al., 2014; Zhang et al., 2014). Moreover, using DNA sequencing, one study found that cortical ARO mRNA did not contain the If promoter and ovarian-specific exon II, and the translation starting sequence "ATG" was not found in this mRNA type, indicating that ARO mRNA in cortical regions was not translatable (Yamada-Mouri et al., 1997). This discordance called into question the specificity of ARO antibodies. However, whether the findings are related to the quality of the antibody or other possible explanation requires further investigation.

1.6 Overview of the thesis

We have investigated the levels and sources of E2 in different brain regions and explored the regulation of ARO and ERs under different pathological conditions. Several major themes emerged. 1) We developed and validated a microsome-based assay for measuring ARO activity using a UPLC-MS/MS method. We then applied this method to detect the distribution of ARO activity and correlated this finding with its mRNA expression in different brain regions. This provided a sensitive, selective, and reliable method for detecting ARO activity in the rat brain. 2) E2 levels in serum and different regions of the brain were measured and compared in (a) OVX rats treated with E2, testosterone, or vehicle and (b) male rats. This enabled a characterization of the relationship between systemic E2 levels and E2 levels in specific brain regions and comparisons of levels between females and males. Systemic and brain levels of testosterone were measured and compared in OVX rats treated with testosterone and gonadally intact males. This enabled direct comparisons of brain E2 levels between males and females relative to known concentrations of its precursor testosterone. In addition, the effects of an ARO inhibitor (letrozole) on local E2 levels in the brain of hormone-treated male and female rats were measured to confirm that the elevated E2 levels detected in the brain are, in fact, due to ARO activity. 3) The effects of selective cholinergic lesions in the medial septum and daily cholinesterase inhibitor injection on ARO and ERs in the brain were examined. This permitted clarification regarding whether the cholinergic system mediates the effects of E2 on cognition through the regulation of local estrogen production and ERs. 4) We detected changes in the mRNA expression of ARO and ERs in the hippocampus and cortex after traumatic brain injury. This pilot study provided preliminary information to hypothesize the neuroprotective role of

local estrogen production after brain injury. These themes will be described in greater detail in the following chapters.

2.0 Development of a Microsomal Incubation Assay for Detection of ARO Activity in the Brain

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2.1 Introduction

As mentioned in the introduction section, many studies show that estrogens can have beneficial effects on brain function and cognition in rats and mice, as well as in non-human primates and humans (Bimonte and Denenberg, 1999; Daniel et al., 1997; Frye et al., 2007; Gresack and Frick, 2006; Luine et al., 2003; Sherwin and Henry, 2008). Most of these studies have evaluated the effects of systemic estrogen treatments, assuming that brain levels of estradiol and estrone mirror levels in the systemic circulation. The brain, however, contains all of the enzymes necessary to synthesize estradiol locally, and recent studies suggest that local estrogen synthesis may have a far greater impact on neuronal function than systemic estrogen administration (De Nicola et al., 2012; Saldanha et al., 2009b).

Aromatase (CYP19A1; ARO) is the last step Cytochrome P450 enzyme responsible for the production of estradiol from testosterone, and estrone from androstenedione (Biegon et al.,

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2012). Several methods have been used to measure ARO activity. In clinical or in vivo studies, where substrate concentrations are not under strict control and in which formation rate cannot be measured directly, a metabolic ratio of 17beta-estradiol to testosterone is often used to indicate ARO activity. When studied in vitro, a more direct method of evaluating product formation is preferred. One commonly used method for measuring ARO activity in tissues is the tritiated water assay developed by Roselli and co-workers (Roselli et al., 1984). It has been used to detect ARO activity in ovarian, placental, avian brain and mammalian brain. The assay is relatively straight forward and simple to apply, however, it has limitations with respect to reproducibility and sensitivity. This poses challenges for measuring the very low levels of ARO activity in brain. The purpose of the current study was to develop a highly sensitive microsomal assay for measuring ARO activity and to show that this method can be used to measure activity in discrete regions of the brain in relation to expression of the long-form of CYP19A1.

2.2 Materials and methods

35 Young female gonadally-intact Sprague-Dawley rats were purchased from Hilltop Laboratories, Inc. Rats were individually housed for two weeks in our facility on a 12 hour: 12 hour light/dark schedule with unrestricted access to food and water. On the day of dissection, animals were anesthetized using a mixture of 0.6 mg xylazine and 3mg ketamine. Brains were removed and tissues from the hippocampus, frontal cortex, preoptic area, and amygdala were collected. Tissues were stored at -80°C until use.

2.2.1 Microsomal incubation assay

2.2.1.1 Microsomal extraction

Brain tissues were collected. Tissues from the same brain region (50-250 mg) were pooled, homogenized in 50 mM Tris buffer (pH 7.4) containing 150 mM KCl, 0.1 mM dithiothreitol (Fluka, USA), 1 mM EDTA and 20% glycerol (Fisher), and mixed with 0.113 mM butylated hydroxytoluene (BHT) and 0.100 mM phenylmethylsulfonylflouride (PMSF) at 4ºC. Samples were centrifuged at 20,000g for 33 min at 4ºC. The supernatant was collected and centrifuged at 140,000g to obtain a microsomal pellet. Microsomal pellets were then dissolved in 200 µl 0.02 M Tris buffer containing 0.25 M sucrose. For this experiment, hippocampi were pooled from two rats, amygdala from three rats, and preoptic area from three rats to generate one data point for each brain region. Protein levels were determined by Bio-rad protein assay (Bio-Rad Laboratories, Inc). Microsomes were stored at -80°C until used.

2.2.1.2 Microsomal incubation assay

To measure ARO activity, 100-200 µg microsomes prepared from different brain regions were incubated with testosterone, and the amount of estradiol produced was measured by LC-MS/MS. Microsomes were added to the microsomal incubation buffer, which is a sodium phosphate buffer pH 7.4 containing 5.0 mM $MgCl_2$. Testosterone was added to each sample tube to a final concentration of 400 nM. To start the reaction, 50 µl of 0.02 M Nicotinamide adenine dinucleotide phosphate (NADPH) was added to each tube, vortexed for 5 sec and incubated at 37ºC. After 30 min, the reaction was stopped by rapid cooling on wet ice. The total volume of the reaction was 1.0 ml.

2.2.1.3 Estradiol extraction and derivatization

Estradiol was collected from the sample by liquid-liquid extraction using 3ml *n*-Butyl chloride and then derivatized with dansyl chloride. Samples were first spiked with internal standard 25µl 2,4,16,16,17-d5-17 beta-estradiol (1 ng/ml in methanol). 3 ml *n*-Butyl chloride was then added and vortexed for 1 min. The tubes were then centrifuged at 4,770 xg at room temperature (RT) for 10 min and the organic layer was transferred to salinized culture tubes and dried down under a soft steam of nitrogen at 37ºC for 20 min. Residues were derivatized in 0.1 ml buffered dansyl chloride solution (a 1:1 mix of acetonitrile: water, pH 10.5), heated at 60ºC for 3 min, and then transferred to glass vials for LC-MS/MS analysis.

2.2.1.4 Estradiol detection

We used a modified version of a UPLC-MS/MS method described by Nelson et al. (Nelson et al., 2004). Liquid chromatography was performed using an Acquity ultra performance LC autosampler (Waters, Milford, MA). Analytes were separated on a UPLC BEH C-18, 1.7 µm (2.1 x 150 mm) reverse-phase column (Waters, Milford, MA). Column temperature was maintained at 55ºC. Mobile phases, delivered at a flow rate of 0.3 ml/min, consisted of (A) acetonitrile and (B) 0.1% formic acid in water, at an initial mixture of 50:50 A and B. Mobile phase B was maintained at 50% for 1 min and then increased to 85% in a linear gradient over 3 min, where it remained for 1 min. This was followed by a linear return to initial conditions over 1.5 min. Total run time per sample was 6.5 min and all injection volumes were 7.5 µl.

Mass spectrometric analysis of analyte formation was performed using a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA) triple quadrupole mass spectrometer coupled with heated electrospray ionization source (HESI) operated in positive selective reaction

monitoring (SRM) mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. Quantification by SRM analysis of estradiol was performed by monitoring the *m/z* transitions. The retention time for estradiol was 5.64 min for native estradiol and 5.62 min for the deuterated internal standard. Parameters were optimized to obtain the highest [M-H]$^-$ ion abundance and were as follows: capillary temperature, 350ºC, vaporizer temperature, 355ºC, spray voltage, 4000 kV, and a source collision-induced dissociation set at 0 V. Sheath gas and auxiliary gas were set at 20 and 34 respectively. Scan time was set at 0.01 s and collision gas pressure was set at 1.5 mTorr. Analytical data was acquired and analyzed using Xcalibur software version 2.0.6 (Thermo Finnigan, San Jose, CA).

2.2.1.5 Aromatase activity calculation

Calibration curves were prepared and extracted along with unknowns. Ideally calibration curves would be prepared in a matrix of inactivated microsomes; however, repeated preparation of large amounts of microsomes from brain is costly in both time and rats. We therefore tested the potential of 2-hydroxypropyl-β-cyclodextrin (HPCD) (Alorich, USA) to be used as an alternative matrix for the preparation of standards and quality control (QC) samples. Specifically, we compared the analytic recovery from the microsomal matrix versus HPCD and showed there is no difference in the ability to extract and detect estradiol from these two matrices (see Results section for details). Based on these results, subsequent standard curves were prepared using HPCD as the matrix.

An estradiol standard curve within concentrations ranging from 2.5 pg/ml to 200 pg/ml was prepared in a matrix of 0.2% HPCD, extracted and derivatized at the same time as the microsomal samples. A stock solution of estradiol was prepared in methanol to achieve a series

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of working solutions at 1 µg/ml, 10 ng/ml and 1 ng/ml concentrations. Calibration standards and QC samples were prepared from separate stock solutions by spiking appropriate amounts into 0.2% HPCD. Calibrators contained estradiol at concentrations of 2.5, 5, 10, 25, 37.5, 50, 100, 200 pg/ml, QCs were 3 pg/ml, 30 pg/ml and 50 pg/ml at a volume of 500 µl. The concentration of estradiol pg/ml in the unknowns was determined by measuring the ratio of E2 to internal standard area under the peak, then interpolating from the standard curve and adjusting for differences in volume of the unknowns vs. the standards. The ARO activity is presented as pmol estradiol/h/mg microsome. Negative controls included samples that received no microsome or no NADPH. Diluted microsomes from ovarian tissue (10 µg) were used as a positive control.

2.2.2 q –RT-PCR

2.2.2.1 mRNA isolation and reverse transcription

All tissue collection was carried out under RNase-free conditions and different brain regions: hippocampus, frontal cortex, amygdala and preoptic area were dissected as described above. Tissue samples were sonicated in 1.0 ml Trizol (Invitrogen, Inc.) at 4°C. The homogenate was mixed with 250 µl chloroform and centrifuged at 12,000xg. The supernatant was collected and an equal volume of isopropyl alcohol was added to precipitate the nucleic acids. Nucleic acids were centrifuged at 12,000xg in 4ºC, and the pellet was dissolved in DEPC water for 10 min at 42ºC. DNA residues were then digested by adding DNAse (10 U/µl) and incubating at 37ºC for 30 min. The remaining RNA was re-extracted with phenol-chloroform and precipitated with sodium acetate and ETOH. The concentration of RNA was determined by spectrophotometry using a Nanodrop 2000 (Thermo Scientific). mRNA was then reverse

transcribed to cDNA using the SuperScript III kit (Invitrogen, Inc.) as per manufacturer's recommendations. RNase H was used to denature any residual mRNA.

2.2.2.2 q-RT-PCR Method

2.0 µl of cDNA, 10 µl of SYBR Green, and 1.2 µl primer pair was pipetted into each well of a 96 plate (0.1 ml/well). The primer pairs used for detection of total CYP19A1 were: (F) 5'-CGTCATGTTGCTTCTCATCG-3' and (R) 5'-TACCGCAGGCTCTCGTTAAT-3'. The primer pairs used for long-form CYP19A1 were: (F) 5'-CTCCTCCTGATTCGGAATTGT-3' and (R) 5'-TCTGCCATGGGAAATGAGAG-3'. GAPDH was used as the control gene: (F) 5'-TGCCACTCAGAAGACTGTGG-3' and (R) 5'-GGATGCAGGGATGATGTTCT-3'. The cDNA product amplified using the total CYP19A1 primers is 150 bp in length and is located in the coding region of the CYP19A1 gene within exons 8 and 9. The cDNA product amplified using the long-form CYP19A1 primers is 90 bp in length and contains portions of exons 2 and 3. This primer has been described by Tabatadze et al. (Tabatadze et al., 2014) and targets only the long-form CYP19A1. The cDNA product produced using the GAPDH primers is 85bp in length and is located on exon 6 of the GAPDH gene. These primer pairs were validated by Genbank primer-BLAST.

PCR was conducted using the 7500 Sequence Detection System (ABI), with an initial step at 50°C for 2 min, 95°C for 10 min and 15 s, then 40 cycles of 60ºC for 1 min. At the end of the amplification, samples were set to 95°C for 15 s, 60ºC for 30 s, and 95ºC for 15 s to obtain the melt curve. Data were analyzed using Sequence Detection System (SDS) software (ABI, Inc.), and results were obtained as Ct (threshold cycle number) values. The sample and reference data points were normalized to the geometric mean of GAPDH (ΔCT). Relative gene

expression was normalized to the amygdala, and was determined using the $2^{-\Delta\Delta(CT)}$ method, where $\Delta\Delta CT = \Delta CT$ (other brain regions) $- \Delta CT$ (amygdala) (Pfaffl, 2001). Expression was normalized to the amygdala for ease of comparison and because the amygdala consistently contained the highest amounts of CYP19A1 gene expression among the regions analyzed. The efficiencies for the primers were as follows: total CYP19A1: 2.53, long-form CYP19A1: 2.57, GAPDH: 2.56. The product size was validated by agarose gel electrophoresis and the linear detection range of each primer pair was validated by amplifying serial dilutions of cDNA and plotting cycle number vs. dilution.

2.2.3 UPLC-MS/MS method validation

Calibration and linearity: Validation of the estradiol assay was performed by using 8 standard concentrations of estradiol prepared in a 2 ml volume. The amount of estradiol in the standards ranged from 2.5 to 200 pg/ml. Three separate duplicate standard curves were prepared and analyzed over three consecutive days. Curves were calculated based on the peak area ratios between estradiol to the internal standard. Calibration curves were generated by weighted (1/Y) linear regression.

Precision and accuracy: Precision and accuracy of the method was determined by the analysis of QC samples. Estradiol was spiked into 0.2% HPCD to yield low and high QCs, corresponding to 20 and 80 pg/ml, respectively. Six samples at each level were analyzed for two days, followed by 12 replicates of each on the final day of validation. The lower limit of detection (LLOD) was determined by the minimum value with a signal-to-noise (S/N) ratio of $\geq$ 1000:1. The lower limit of quantitation (LLOQ) was determined by the minimum value with

accuracy and precision within ±15% deviation of the nominal value. Accuracy was determined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage. This accuracy, or relative standard error (RE %), was calculated using the equation RE %= (E−T)/T×100. Precision was evaluated and expressed as relative standard deviation (RSD %) of the mean concentrations (M) using the equation RSD % = SD/M×100. The criteria for acceptability of data included accuracy and precision within ±15% deviation of the nominal value.

2.2.4 Validation of the Microsomal Incubation Method

To validate the assay, the effects of varying microsomal concentration, incubation time, substrate concentration, inter- and intra-day precision and accuracy, and specificity were evaluated.

Reproducibility: The reproducibility of the method was determined using replicates (N=6) of diluted ovarian samples (10 µg microsome per reaction). Intra-day precision was determined by analyzing six replicates in one day, and inter-day precision was calculated by analyzing six replicates over three consecutive days. The CV was used for characterizing precision.

ARO activity as a function of microsomal concentration: To determine the amount of microsome needed to reliably measure differences in ARO activity, a serial dilution curve of microsomal mix derived from several different brain regions (hippocampus, amygdala, hypothalamus, thalamus and septum) was prepared. A range of 75– 600 µg of microsomes were used per reaction. The amount of estradiol produced during 30 min incubation with testosterone (400 nmol/L) was measured by LC-MS/MS, and the relationship between ARO activity and microsomal concentration was determined.

ARO activity as a function of incubation time: Microsomal mix (as above) were incubated with 400 nmol/L testosterone for 10, 20, 30, 40, 50, 60, and 90 min and the amount of estradiol produced was measured by LC-MS/MS. Estradiol production was then normalized to 1 mg microsomes plotted as a function of incubation time, and analyzed for formation rate using PRISM (GraphPad Software, La Jolla, CA).

ARO activity as a function of substrate concentration: Microsomes (300 µg) from three brain regions: hippocampus, amygdala and preoptic area were prepared separately and incubated with different amounts of testosterone to determine the amount of substrate necessary to produce saturating conditions. Testosterone concentrations included 12.5, 25, 50, 100, 200, 400 and 800 nmol/L. After 30 min incubation, estradiol production was measured and ARO activity was calculated.

Inhibition of ARO activity with an ARO inhibitor: As a negative control, 0 nM, 50 nM or 100 nM anastrozole, an ARO inhibitor, was added to hippocampal and ovarian microsomal samples. These samples were pre-incubated at 37°C for 10 min without NADPH, after which NADPH was added to start the reaction. ARO activity was measured after 30 min incubation.

2.2.5 Data analysis

Descriptive statistics (mean, SD, CV) were calculated using Microsoft Excel. Data were first transformed using an Eadie-Hofstee plot to visualize the potential for multiple binding sites or cooperativity. Hill plots were used to further evaluate the enzyme kinetics. Enzyme kinetic parameter values for maximal velocities (V_{max}) and Michaelis-Menten constants (K_m) for each brain region were determined by non-weighted non-linear regression analysis (Win-Nonlin) using PRISM. Initial parameter estimates used for non-linear regression analyses in a one-

enzyme model consisted of the observed V_{max} and K_m values from the substrate saturation experiments (Bolcato et al., 2003). The R^2 for goodness of fit for different brain regions were: hippocampus: 0.96; amygdala: 0.99; and preoptic area 0.93.

2.2.6 Correlations between CYP19A1 total and long form mRNA and ARO activity

Distribution of ARO activity in different brain regions and its correlation with relative levels of CYP19A1 mRNA: ARO activity, total and long-form CYP19A1 mRNA were detected for different brain regions: hippocampus, frontal cortex, amygdala and preoptic area in the ovariectomized (OVX) rats (n= 5 for mRNA detection, n= 12 for activity detection). Relative levels of CYP19A1 mRNA were reported as $2^{-\Delta\Delta Ct}$ normalized to amygdala. ARO activity also was normalized to the amygdala. Data were normalized to the amygdala for ease of comparison, and because levels of CYP19A1 mRNA and ARO activity were highest in the amygdala.

2.3 Results

2.3.1 Validation of UPLC-MS/MS method for quantitation of estradiol

Extraction recovery: Table 1 compares estradiol extraction and detection using inactivated microsomes vs. HPCD as the two matrices. Estradiol calibration curves were prepared in brain microsomes (200 µg/ml) that were boiled to inactivated CYP enzymes, and diluted in potassium phosphate and Tris-sucrose buffer. Calibrators contained estradiol at concentrations of 2.5, 5, 10, 25, 50, 100 and 200 pg/ml. Estradiol QC samples were prepared in

0.2% HPCD at 3 and 30 pg/ml. Both the calibration curve and QCs were repeated three times on three different days. Values of QCs were calculated using the microsomal calibration curve. All calculated QC values fell within 10% RE using calibration curves prepared in microsomes (Table 1). This shows that the extraction and detection of estradiol from HPCD is the same as from the microsomal matrix. Hence HPCD was used as the matrix for all calibration curves.

Table 1: Evaluation of microsomes vs. HPCD as a matrix for the extraction and detection of estradiol.

	Target Concentration Added (pg/ml, N=3)	Observed Concentration (mean±SD) (pg/ml, N=3)	% RSD	% RE
Quality controls in 0.2% HPCD				
Estradiol	3	3.08±0.25	8.16%	2.49%
	30	29.20±0.61	2.17%	-6.01%
Standards in Microsome Matrix				
Estradiol	blank	Undetectable		
	2.5	2.47±0.26	10.47%	-1.09%
	5	4.75±0.17	3.66%	-4.99%
	10	10.30±0.24	2.38%	3.01%
	25	24.64±1.02	4.14%	-1.43%
	50	51.32±3.71	7.24%	2.63%
	100	102.48±6.44	6.29%	2.48%
	200	197.47±9.64	4.88%	-1.27%

Estradiol calibration standards were prepared in in boiled brain microsomes (200 µg/ml) in potassium phosphate and Tris-sucrose buffer. QCs were prepared in 0.2% HPCD.

Linearity, precision and accuracy: Linear calibration curves were obtained for estradiol over the range of 2.5-200 pg/ml. The mean regression equations($\pm$SD) were as follows :

Estradiol: $Y= [0.008 (\pm 0.002)]X +0.035 (\pm 0.017)$

The mean correlation coefficient (R^2) was >0.998. The lower limit of quantification (LOQ) was 2.5pg/ml and was demonstrated to be reproducible (%RSD<15%). The intra- and inter-run precision and accuracy were within $\pm$15% for estradiol (Table 2).

Table 2: Intra-day and inter-day accuracy and precision for the detection of estradiol in 0.2% HPCD solution.

	Amount estradiol Added (pg/ml, N=3)	Observed estradiol (mean±SD) (pg/ml, N=3)	% RSD	% RE
Intra-assay reproducibility				
Quality controls				
Estradiol	3	3.05±0.19	6.10	1.63
	20	20.7±1.30	6.30	3.50
	80	80.1±4.31	5.38	0.13
Inter-day reproducibility				
Quality controls				
Estradiol	3	3.13±0.23	7.42	4.49
	20	20.8±1.53	7.37	4.00
	80	84.0±4.89	5.83	5.00
Standards				
Estradiol	2.5	2.44±0.24	9.95	-2.40
	5	4.97±0.46	9.30	-0.52
	7.5	7.28±0.41	5.63	-2.90
	10	8.93±1.27	14.20	-10.70
	25	27.9±2.22	7.96	11.60
	50	51.3±3.72	7.25	2.60
	100	90±8.12	9.03	-10.00
	200	202±6.38	3.23	1.00

2.3.2 Validation of microsomal incubation assay

Reproducibility: Table 3 shows the accuracy and reproducibility of the MIB assay. ARO activity in the same ovarian microsomes was detected using six repeats per day for three independent days. The inter-day and intra-day CVs were below 10%, indicating that the method has sufficient reproducibility.

Table 3: Inter-day and intra-day accuracy and precision for the detection of estradiol in ovarian microsomes.

	Individual Days (pmol/h.mg microsome)						Mean	SD	CV
Day1	2.70	2.46	2.63	2.72	2.57	2.55	2.604	0.101	3.88%
Day2	2.38	2.41	2.44	2.48	2.54	2.37	2.438	0.065	2.67%
Day3	2.37	2.69	2.52	2.69	2.80	2.72	2.631	0.157	5.97%

Microsomal concentration curves: Figure 1 (A) summarizes estradiol production as a function of microsomal concentration. A linear relationship was observed for concentrations ranging from 75 to 600 µg/reaction with an R^2 of 0.99, indicating that this method is able to accurately measure differences in ARO activity within this range. This curve was repeated with different microsomal samples and a similar result.

Incubation time curve: Figure 1 (B) summarizes the relationship between estradiol production and time of incubation for periods ranging from 15 to 90 min. Result shows two linear phases for estradiol production during the 90 min reaction. The rate of the reaction was higher during the first 30 min with a value of 0.33 pmol/ h/mg microsome. After 30 min, the rate of the reaction decreased to 0.09 pmol/h/mg microsome, which suggests that enzyme activity had decreased. An incubation time of 30 min was used in all future studies. This same experiment

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was repeated using a pool of hippocampal microsomes extracted from older animals. Again, two linear phases were observed with an inflection point at 30 minutes (not shown). Rates were 0.135 pmol/mg microsome during the first 30 min and 0.029 pmol/mg microsome during the next 60 min.

Substrate concentration curve: Figures 1C-1E show the relationship between estradiol production and substrate concentration. Eadie-Hofstee plots showed linearity with R^2 =0.94, 0.96 and 0.84 for hippocampus, amygdala and preoptic area respectively. This indicates that the reaction involves only one binding site. Data were then analyzed using Michaelis-Menten plots. For all regions in which activity was detected, the reaction saturated at a testosterone concentration of 400 nmol/L. Since our goal was to develop a method for comparing total enzyme activity in discrete tissue samples, subsequent experiments were conducted using saturating levels of testosterone (400 nmol/L) so that the functional enzyme expression, rather than substrate, is the limiting factor. Based on the Michaelis-Menten curves, we calculated that for hippocampus: V_{max} = 0.57 pmol estradiol/h/mg microsome and the K_m = 48.58 nmol/L; for amygdala: V_{max} = 1.69 pmol estradiol/h/mg microsome and the K_m = 48.4 nmol/L; and for preoptic are: V_{max} = 0.96 pmol estradiol/h/mg microsome and the K_m = 44.31 nmol/L.
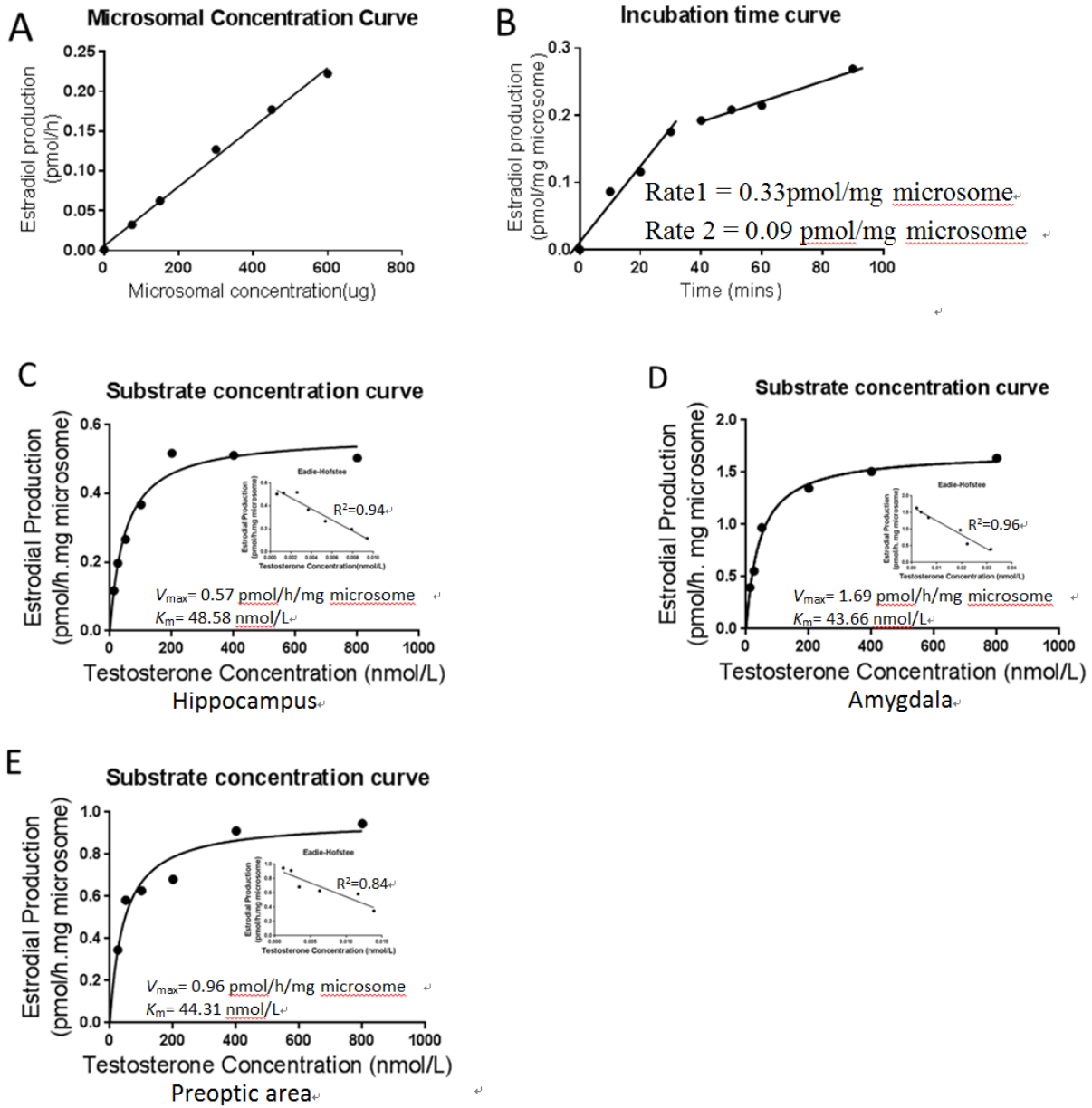
Figure 1: Optimization of MIB assay parameters. A) Graph showing a linear relationship between microsomal concentration and estradiol production (pmol/h). B) Plot showing that two linear phases of ARO activity were observed as a function of reaction time. C-E: Substrate saturation curves for C) hippocampus, D) amygdala and E) preoptic area. All curves were repeated twice.

Ability to inhibit ARO activity with anastrozole: Figures 2A and 2B show the effects of the ARO inhibitor anastrozole on estradiol production. Anastrozole is a well-characterized and highly selective aromatase inhibitor that is used in the treatment of breast cancer. Anastrozole inhibited estradiol production in hippocampal microsomes by approximately 80% at 50 nM, and nearly 100% at 100 nM. Estradiol produced by ovarian microsomes was decreased by 80% after treating with 100 nM anastrozole. This provides additional evidence that estradiol production detected in the brain by this assay is due to ARO activity since it is inhibited by a selective ARO antagonist.
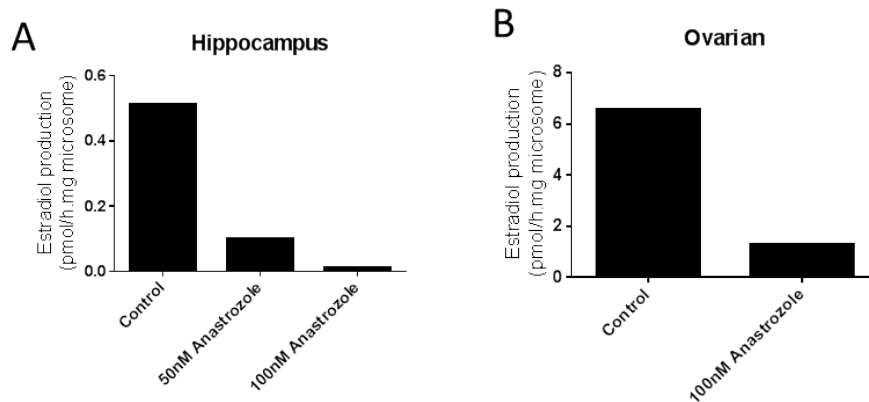
Figure 2: Effect of different concentrations of anastrozole on aromatase activity in ovarian and hippocampal microsomes.

2.3.3 Correlation between CYP19A1 Total and long form mRNA and ARO activity

Distribution of ARO in different brain regions: Table 4 shows the distribution of long-form CYP19A1 mRNA expression and ARO activity in different brain regions. Relative levels of CYP19A1 mRNA and activity were highest in the amygdala, followed by the preoptic area

and then the hippocampus. Despite the fact that total CYP19A1 mRNA in frontal cortex was similar to that in the hippocampus, the levels of long-form of the mRNA were approximately 1200 times lower and ARO activity in this region was below the limit of detection.

Table 4: Distribution of Total ARO mRNA, Long-form ARO mRNA expression and ARO activity in the different brain regions.

Regions	Total ARO mRNA	Long form ARO mRNA	ARO activity
HPC	17	16	31
FC	9	2^{-8}	0
AMG	100	100	100
POA	38	34	62

HPC: hippocampus, FC: frontal cortex, AMG: amygdala and POA: preoptic area. Both the ARO mRNA expressions and activity were normalized to the values in the Amygdala. Values represent % of Amygdala.

2.4 Discussion

Our goal was to develop a new reliable and sensitive assay for measuring ARO activity in discrete regions of the mammalian brain. Here we show that by isolating microsomes from discrete brain tissues and measuring conversion of testosterone to estradiol by UPLC-MS/MS, we can measure ARO activity at rates substantially less than 1 pmol/h/mg microsome during a 30 min incubation. This translates to the ability to measure ARO activity in as little as 50 – 150 mg of rat brain tissue (depending on the brain region). Sensitivity may be increased by using longer incubation times; however, our data show that the rate of product formation decreases

after 30 min, which would make measurements less reflective of total functional enzyme activity and potentially co-factor/substrate limited. Controls show that measurements are reproducible with a CV below 10%, that activity is linearly correlated with microsomal concentration and with incubation time up to 30 min, and that a testosterone concentration of 400 nmol/L is sufficient to achieve saturating conditions. In addition, matrix effects were not significant and product formation was blocked by a selective ARO inhibitor, suggesting that product formation is due solely to ARO activity. Based on these results, we conclude that the microsomal incubation (MIB) assay is sensitive and reliable, and enables us to measure ARO activity in small amounts of brain tissue.

For application, we showed that the regional distribution of ARO activity in the brain correlated with CYP19A1 mRNA expression detected by q-RT-PCR. In the frontal cortex, we detected significant quantities of total CYP19A1 mRNA, however, the long-form CYP19A1 mRNA was very low and the ARO activity was below the limit of detection. This is in accord with a previous study showing that only the long form CYP19A1 mRNA is associated with ARO activity (Roselli et al., 1997). In addition, our results are in agreement with previous reports showing that the highest ARO activity in the brain is in the amygdala, followed by the preoptic area and hippocampus (Roselli and Resko, 1991; Roselli et al., 1998; Sierra et al., 2003; Tabatadze et al., 2014; Takahashi et al., 2006). These results add confidence that the MIB assay accurately measures ARO activity and that activity corresponds with the functional CYP19A1 mRNA expression.

2.4.1 Comparison between Tritiated water assay and MIB assay

As mentioned above, a popular method for measuring ARO activity in tissues has been the tritiated water assay. This assay relies on the conversion of tritiated androstendione to estrone resulting in the production of $^{3}H_2O$. Tritiated androstenedione is then removed by passage through a hydrophobic column, and ARO activity is determined by measuring the rate of $^{3}H_2O$ production. This method has been used effectively to measure ARO activity in ovarian and placental tissues, as well as in avian and mammalian brain. Studies have shown that this method is quantitative and reproducible, particularly when ARO is relatively abundant. Our experience, however, is that the method is not able to reliably detect and quantify ARO activity in small pieces of rat brain tissue where ARO activity is relatively low. In such cases, many tissues need to be pooled (Roselli and Resko, 1993), incubation times can be very long (many hours) (Dean et al., 2012; Konkle and McCarthy, 2011) which raises concerns about the accuracy of the activity measure. Also, this assay methodology has added variability in the efficiency of the separation of the tritiated androstenedione from the $^{3}H_2O$. Instability of the substrate, which needs to be re-purified periodically (Roselli and Resko, 1991), also can add to variability and reduce sensitivity. Hence different labs have reported difficulty detecting and quantifying ARO activity in regions of the mammalian brain using the tritiated water assay.

In contrast, the MIB assay takes advantage of very sensitive UPLC-MS/MS methods to measure directly the conversion of testosterone to estradiol. Also, by focusing on the microsomal fraction, enzyme activity is concentrated and potential contamination by endogenous estradiol is reduced. In our hands, this method is more sensitive and reliable than the tritiated water assay for detecting and quantifying ARO activity in adult mammalian brain tissues.

Direct comparisons of kinetic parameters obtained using the two methods show differences, but these differences may be explained by methodological details. Using the MIB method, V_{max} and K_m values for the amygdala and preoptic area were amygdala: V_{max}= 1.69 pmol estradiol/h/mg microsome and the K_m= 48.4 nmol/L; preoptic are: V_{max}= 0.96 pmol estradiol/h/mg microsome and the K_m= 44.31 nmol/L. In comparison, values reported by Roselli et al. using the tritiated water assay are, Amygdala: V_{max}= 79.4 fmol/h/mg protein; K_m = 30 nmol/L, Preoptic area: V_{max}= 62.5 fmol/h/mg protein, K_m= 20nmol/L, (Roselli et al., 1984). Several factors may contribute to these differences. Firstly, the tritiated water assay is based on the conversion of adrostendione to estrone, whereas our method is based on the conversion of testosterone to estradiol. It is possible that ARO has different affinities and conversion efficacies for the two substrates. Secondly, values from the tritiated water assay are based on total protein concentration of the tissue homogenate, whereas values from the MIB assay are based on mg microsome. Thirdly, our values are based on a reaction time no greater than 30 min, whereas the studies by Roselli used a reaction time of 60 min. Our studies suggest that formation rate decreases after 30 min. These differences likely account for the differences in kinetic parameters obtained using the two assays.

A potential limitation of the MIB assay is that by focusing on the microsomal fraction, it is possible that ARO in other parts of the cell remains undetected. Two studies have reported immunohistochemical evidence for the presence of ARO in axon vesicles and dendrites (Balthazart and Ball, 2006; Peterson et al., 2005). Isolation of microsome excludes the detection of aromatase in axon and dendrites. Hence while the majority of ARO activity is associated with microsomes, it is possible that a small portion of the total ARO activity in cells is excluded from the microsomal fraction.

2.5 Summary

In summary, we have developed and validated a new microsomal incubation method that can sensitively and reliably detect ARO activity in rat brain tissues. As the importance of ARO in neuroplasticity and neuroprotection in the non-reproductive regions becomes increasingly recognized, the MIB assay will be a valuable tool for studying the role of ARO in the context of normal brain physiology and response to injury and disease.

3.0 Detection of Estradiol in Rat Brain Tissues: Contribution of Local Versus Systemic Production

3.1 Introduction

Since the discovery of ARO in the brain, the effect of local estrogen production on brain structure and function has received increasing attention. This raises important questions about the contributions of local vs systemic sources of estrogens to the brain, and about levels of E2 present in specific regions of the mammalian brain under different physiological and pathological conditions, particularly in females where ovariectomized (OVX) animals and E2 treatments are commonly used to model loss of ovarian function and E2 replacement.

In this study, we focused on measuring E2 levels in both brain and serum in OVX rats treated with different doses of estradiol benzoate (EB), using a novel and recently validated UPLC-MS/MS method (Li et al., 2016a). Results confirmed significantly higher levels of E2 in brain regions known to contain ARO activity than in serum, both in OVX controls and in rats treated with physiological doses of EB. Additional studies measured levels of E2 and T achieved in females treated with testosterone propionate (TP) for comparison with males, and also tested the ability to eliminate the differences between serum and brain levels of E2 by treating with the ARO inhibitor letrozole. Collectively the results provide a detailed analysis of brain region-specific E2 concentrations in OVX, E2-, and T-treated rats and demonstrate the degree to which these concentrations are ARO–dependent.

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3.2 Materials and methods

3.2.1 Animals and Treatments

All experiments were conducted in accordance with the NIH Guide for Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

66 Young female ovariectomized (OVX, ~250g) and sixteen gonadally intact male (~300g) Sprague-Dawley rats (3 months old) were purchased from Harlan Sprague Dawley, Inc. Rats were individually housed for at least two weeks in our facility on a 12 hour:12 hour light/dark schedule with unrestricted access to food and water prior to use. Three experiments were conducted (Fig. 3). In the first experiment, we compared the E2 levels in the brain and serum of OVX rats after treating with different doses of estradiol benzoate (EB). Females were divided into 4 groups, one group received daily sc injections of vehicle (sesame oil) and another three groups received different dose of EB (1.0, 2.5, or 5.0 µg/day, Sigma- Aldrich, Inc.). Injections were administered for 2 days. 20-24 h after the last injection, rats were anesthetized with a mixture of 6mg Xylazine: 30mg Ketamine, decapitated and the brain tissues and serum collected for analysis. In the second experiment, we characterized levels of E2 detected in the brain of OVX rats treated with T and compared this in levels detected in gonadally intact rats. OVX rats were treated with either vehicle (sesame oil) or testosterone propionate (TP, 0.5mg/day, Sigma- Aldrich, Inc.). Male rats received vehicle treatment. Brain tissues and serum from another set of OVX controls, OVX TP- treated females, and gonadally intact males (N=4 for each group) with the same treatment were collected to measure T levels. After 2 days, animals were euthanized and decapitated as in experiment 1. In the third experiment, we tested the degree

to which brain ARO activity contributes to the E2 levels detected in the brain. OVX female and gonadally intact male rats received daily injections of letrozole (4mg/kg, Sigma- Aldrich, dissolved in 10% Dimethyl sulfoxide (DMSO), 20% 2-hydroxypropyl-β-cyclodextrin (HPCD) for 7 days. On the last two days of treatment, OVX rats received either vehicle, 1µg EB, 2.5µg EB, or 0.5mg TP). Males received sesame oil. 20-24 hs after the last treatment rats were killed, trunk blood was collected, serum was harvested, and brains were processed for analysis.
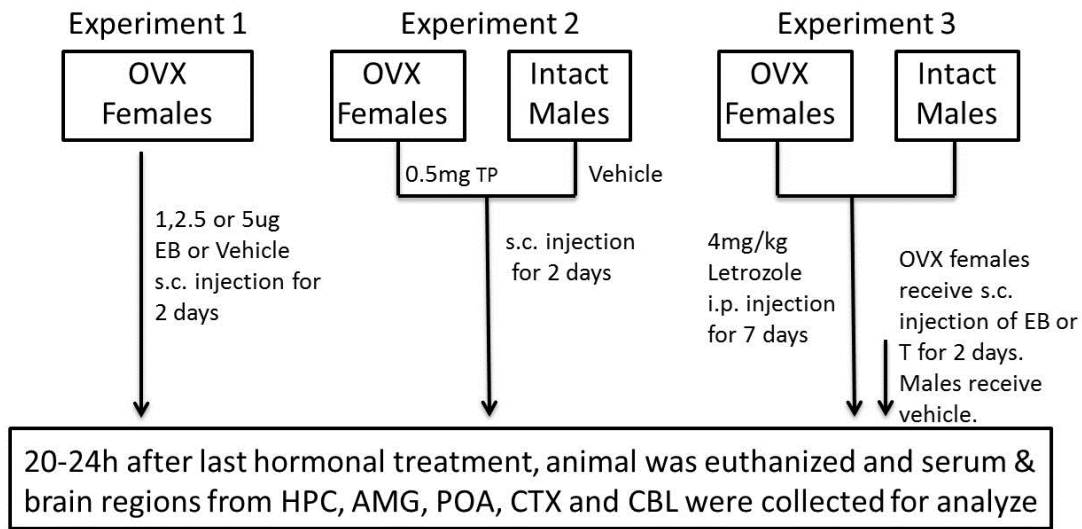
Figure 3: Flow diagram showing the experimental design. OVX: ovariectomized, HPC: hippocampus, AMG: amygdala, POA: preoptic area, CTX: frontal cortex and CBL: cerebellum.

The following brain regions were dissected and snap frozen on dry ice: frontal cortex (CTX), preoptic area (POA), hippocampus (HPC), amygdala (AMG), and cerebellum (CBL). Specifically, brains were sliced into 2 mm coronal slabs using a standard rat brain matrix (ASI, Inc.). POA (~ 50mg) corresponding to plates 18 through 25 of atlas of Paxinos and Watson (George Paxinos, 1997) was dissected by making a horizontal cut beneath the anterior commissure and a vertical cut through the olfactory tubercles on each side of the third ventricle. AMG (~80mg) corresponding to plates 25 to 33 was dissected by cutting from the optic tract

horizontally through the rhinal fissure, and then dissecting amygdala apart from surrounding cortex. CTX (~150mg) included tissues from frontal cortex areas 1-3. HPC (~180mg) from each side and cerebellum(~250mg) were collected whole. Serum also was collected and stored for analysis of circulating hormone levels. All tissues were stored at -80˚C until use.

3.2.2 Estradiol and Testosterone detection

UPLC-MS/MS was used to quantify E2 in the serum and brain tissue homogenates. Brain tissue was homogenized in potassium phosphate buffer (0.12M, pH 7.4) containing 4.0 mM $MgCl_2$, 4.0 mM Tris and 50 mM sucrose (100 mg tissue/ml). E2 was then measured in homogenates and serum as recently described (Li et al., 2016a). Briefly, samples were spiked with internal standard 25µl 2,4,16,16,17-d5-17 beta-estradiol (1 ng/ml in methanol, LC-grade). 3-4 ml n-Butyl chloride (Sigma-Aldrich, Inc.) was added and samples were vortexed for 1 min. Samples were then centrifuged at 4,770 x g at room temperature (RT) for 10 min, and the organic layer was transferred to salinized culture tubes and dried under a stream of nitrogen at 37ºC for 20 min. Residues were derivatized in 0.1 ml buffered dansyl chloride solution (0.5 mg/ml, a 1:1 mix of acetonitrile: water, pH 10.5, LC grade, Sigma-Aldrich, Inc.) in salinized tubes and heated to 60ºC for 3 min. Samples were centrifuged again for 1 min at 490 x g, and the supernatant (~0.1ml) was transferred into glass vials for UPLC-MS/MS analysis with an injection volume of 7.5 µl. Calibration curves were prepared at concentrations of 0.009, 0.018, 0.036, 0.09, 0.18, 0.36 and 0.72 pmol/ml and quality controls (QCs) at 0.01 and 0.10 pmol/ml in a matrix of 0.2% 2-hydroxypropyl-β-cyclodextrin (HPCD). Studies demonstrating the validity of using HPCD as the matrix for the standards and QCs were conducted and are described below.

Calibration curves and QCs were extracted and derivatized at the same time as the tissue and serum samples. E2 was then detected using UPLC-MS/MS as described above in section 2.2.3.

Testosterone (T) levels in serum and brain homogenates were quantified by a modification of the method described by Cawood (Cawood et al., 2005) and using UPLC-MS/MS similar to the E2 detection method described above. Briefly, samples were spiked with 0.25 ng/ml D3-testosterone (Sigma-Aldrich, Inc.) as the internal standard and then extracted with 3 ml n-butyl chloride. After centrifugation and evaporation, the residue was reconstituted in methanol and water (80 μl : 20 μl, LC grade), centrifuged again for 1 min at 490 x g and the supernatant was transferred into glass vials for UPLC-MS/MS analysis. T was eluted from the same column as E2, with a methanol: water (0.1% formic acid and 2 mM ammonium acetate) gradient from 50 to 85% methanol. Transitions used for analysis were 289 →97 for T and 292 → 97 for the deuterated T. The ratio of area under the peak between T and deuterated T was quantified and used to determine levels of T pmol/mL by comparison with standards ranging from 0.035 pmol/ml – 55 pmol/ml. The limit of detectability for this assay was 0.035 pmol/ml. The inter-day precision, accuracy and stability were within acceptable limits.

3.2.3 Validation of E2 detection in the brain tissue

Extraction recovery of brain tissue homogenate: Ideally, calibration curves would be prepared in a matrix of brain tissue; however, repeated preparation of brain tissue homogenate is costly in both time and rats. Previously we showed that HPCD is an acceptable matrix for comparing E2 levels in standards with E2 levels in microsomes and serum (Crago et al., 2015; Li et al., 2016a). We, therefore, tested HPCD as a matrix for comparing standards with brain homogenates. Whole rat brains were homogenized in buffer as above. 0.5 ml of tissue

homogenate was added to each tube. Samples were spiked with known concentrations of E2 for comparison with standards prepared at equivalent concentrations. E2 was then extracted and measured along with the calibration curve and QCs were prepared in the 0.2% HPCD. Both curves were repeated 3 times on three different days, and the mean concentrations (M) and the standard deviation (SD) were calculated. Accuracy is reported as a relative standard error (RE %), calculated as RE %= $(E - R)/R \times 100$, where E= calculated value and R= real value. Precision was evaluated and expressed as relative standard deviation (RSD %) of the mean concentrations using the equation RSD % = $SD/M \times 100$.

E2 level as a function of tissue volume: As part of our validation procedure, we tested the relationship between the amount of tissue homogenate used and the amount of E2 detected. Ideally, we should see a linear relationship between the amount of tissue homogenate and quantity of E2 detected, indicating that unknown factors in the homogenate were not interfering with E2 detection. A serial dilution curve of brain tissue homogenate was prepared. A volume range of 0.5 – 3 ml tissue homogenate was used per reaction. The amount of E2 was measured, and the relationship between E2 level and amount of tissue used was determined.

3.2.4 Statistical analyses

Data in Fig. 4,6,7 are presented as mean ± s.e.m. For experiment 1, effects of different EB doses on E2 levels in the different brain regions and serum were analyzed by two-way ANOVA using location (e.g., brain region, serum) as a within subject factor and dose as a between-subject factor. Non-linear least-squares regression (Hill equation: Y = Bmax*X/(Kd + X) was used to analyze the relationship between serum and brain E2 levels in rats treated with different doses of EB. This equation has been used as a general non-linear model for describing

data with a sigmoidal distribution (Goutelle et al., 2008). For experiment 2, comparisons of E2 among OVX, TP-treated OVX and gonadally intact male rats were analyzed by two-way ANOVA with location as a within-subject factor and treatment as a between-subject factor. T levels among these three groups were analyzed in the same way. For experiment 3, the effect of OVX, 1 µg and 2.5 µg EB in combination with letrozole on the levels of E2 in both brain and serum were analyzed by three-way ANOVA, with location (e.g., brain region, serum) as a within-subject factor, and dose of EB and letrozole treatments as two between-subject factors. The effect of TP treatment in comparison with males, as well as the effect of letrozole on E2 levels in the different brain regions and serum was likewise analyzed by three-way ANOVA. Statistically significant interactions were followed by simple effects analyses using Post-hoc Tukey tests. All statistical analyses were done using JMP (Pro12), with significance defined as p <0.05.

3.3 Results

3.3.1 Estradiol method validation

Extraction recovery: Table 5 compares estradiol (E2) extraction and detection using brain tissue homogenate vs. HPCD as the two matrices. E2 calibration curves were prepared in brain tissue homogenates (1mg tissue/ 10 µl potassium phosphate and Tris-sucrose buffer) as well as 0.2% HPCD. Calibrators contained E2 at concentrations of 0.009, 0.018, 0.036, 0.09, 0.18, 0.36 and 0.72 pmol/ml. Results show that RE% and RSD% were within 15% for all concentrations evaluated. These data indicate that the assay is accurate and reproducible and that extraction

from HPCD did not differ significantly when compared with extraction from brain tissue homogenate. Therefore, HPCD was used as the matrix for all subsequent calibration curves.

Table 5: Evaluation of brain tissue homogenate vs. HPCD as a matrix for the extraction and detection of E2.

	Amount E2 Added (pg/ml)	HPCD (pg/ml,N=3)	%RE	%RSD	Tissue homogenate (pg/ml, N=3)	%RE	%RSD
	2.5	2.41±0.08	-3.55194	3.28	2.44±0.28	-2.33	11.49
	5	5.53±0.69	10.60543	12.63	4.98±0.33	-0.46	6.68
Estradiol (E2)	10	9.91±0.62	-0.90148	6.21	10.85±0.50	8.51	4.59
	25	24.56±0.89	-1.76722	3.61	24.67±0.58	-1.31	2.36
	50	48.50±2.20	-3.00227	4.54	50.07±4.02	0.15	8.02
	100	99.39±1.61	-0.60787	1.62	98.52±2.75	-1.15	2.78
	200	202.51±2.30	1.255401	1.14	197.30±6.73	-1.35	3.41

E2 level as a function of tissue volume: Fig. 4 shows the relationship between quantity of tissue homogenate and E2 level detection. A linear relationship was observed for tissue homogenate volume ranging from 500 – 3000 μl with an R^2 of 0.99. This curve was repeated with different tissue homogenates with similar results. These results indicate that the method can accurately measure differences in E2 level across a wide range of concentrations and tissue volumes.

Tissue Volume Curve

$R^2 = 0.989$
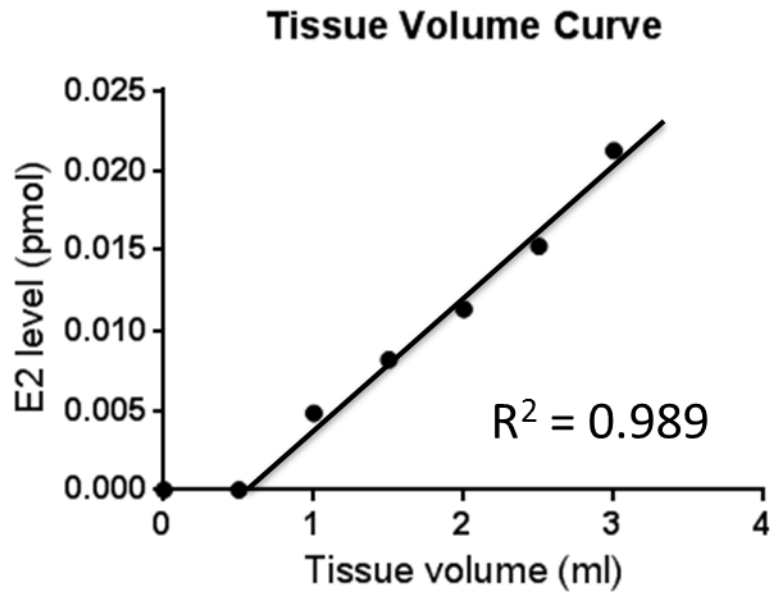
Figure 4: Plot illustrating linear detection of E2 as a function of volume of whole brain homogenate. Each point represents a measurement of sample pooled from three animals brain tissue.

3.3.2 Serum levels of estradiol

Serum levels of E2 in OVX controls were undetectable. Mean serum levels ± s.e.m. in rats from experiment 1 treated with 1.0, 2.5 and 5.0 µg estradiol benzoate (EB) were 0.076 ± 0.008, 0.168 ± 0.016 and 0.576 ± 0.089 pmol/ml. Serum E2 levels in TP treated OVX rats as well as serum E2 levels in male rats from experiment 2 were very low and in most samples were undetectable. In experiment 3, mean serum levels of E2 in OVX rats treated letrozole in addition to 1.0 and 2.5 µg EB were 0.079 ± 0.016, 0.176 ± 0.033, which were not significantly different from levels detected in non-letrozole treated rats (p=0.32).

3.3.3 Comparison of estradiol levels in select brain regions vs. serum after different dose of estradiol benzoate treatment

Fig. 5A illustrates the dissection of each brain region according to the atlas of Paxinos and Watson (George Paxinos, 1997). Fig. 5B summarizes the effects of different doses of EB on E2 levels detected in each brain region as well as in serum in OVX rats. Two-way ANOVA revealed a significant effect of treatment ($F[3,138]=64.32$, $P<0.001$), a significant effect of location ($F[5,138]=46.75$, $p<0.001$), and a significant treatment x location interaction ($F[15, 138]=15.19$, $p<0.001$). Post-hoc analyses revealed significant differences among different doses of EB treatments. E2 levels in EB treated rats were significantly higher than E2 levels in OVX rats. In addition, E2 levels significantly increased as a function of dose (OVX<1μg EB<2.5μg EB <5μg EB). Post-hoc analyses by brain region revealed that E2 levels in the HPC, AMG and POA were significantly higher than in serum in OVX rats treated with 1 μg or 2.5 μg EB. Note that in each of these brain regions significant levels of E2 were detected in OVX controls, and these levels were comparable to serum levels detected in rats treated with 1.0 μg E2. In contrast, E2 levels in the CTX and CBL of OVX controls were undetectable. In addition, levels of E2 detected in these brain regions in rats treated with 1.0 or 2.5 μg EB did not differ significantly from the levels detected in serum.

In rats that were treated with 5 μg EB, serum concentrations of E2 were well above the normal physiological range(>0.3 pmol/ml) (Butcher et al., 1974). Under this condition, E2 levels in all brain regions were lower than in serum. Moreover, E2 levels were similar across brain

regions (0.35 - 0.5 pmol/ml), in contrast to the differences observed when systemic E2 was in the physiological range.
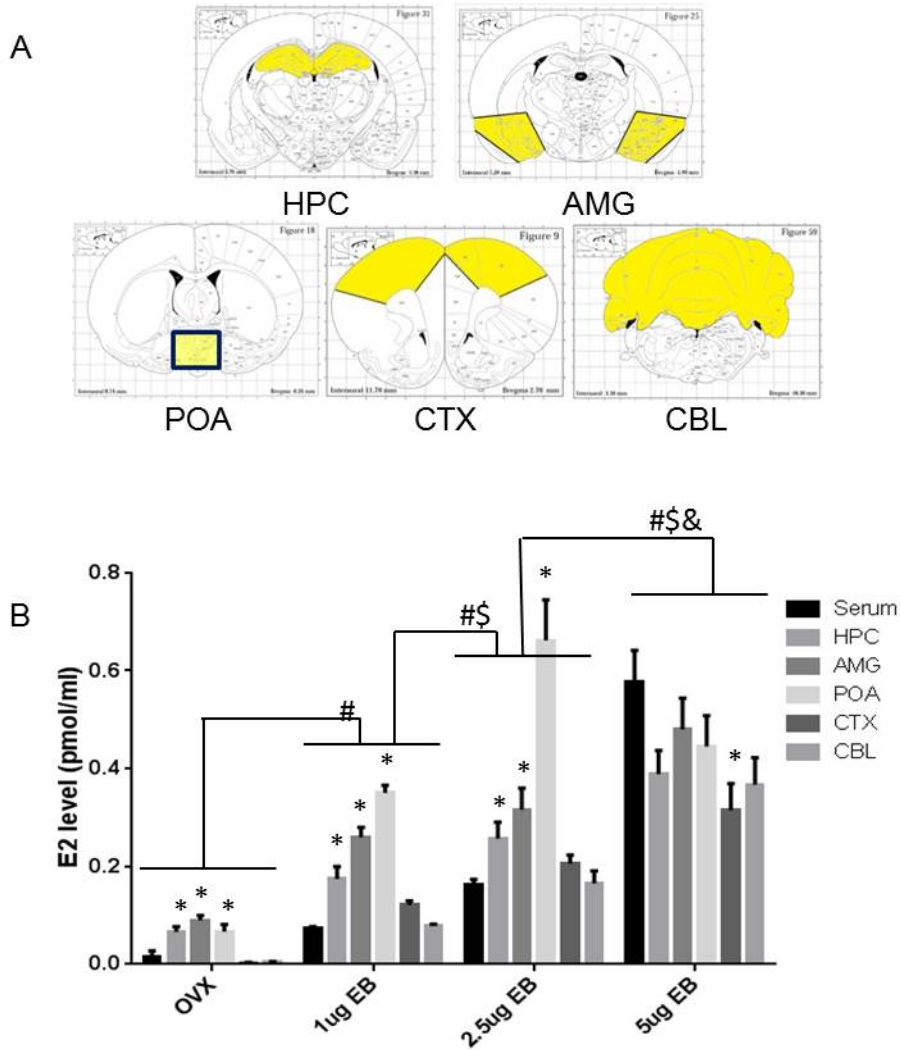
Figure 5: A) Illustrations of each brain region dissection (shown in yellow) corresponding to plates from Paxinos and Watson (1986). B) Comparison of E2 levels in the different brain regions and serum after different doses of EB treatment. Each bar represents the mean concentration of E2 $\pm$ s.e.m. [#] indicates significant difference ($p<0.05$) in E2 detected in rats treated with different doses of EB treatment compared with OVX animal.$ indicates significantly higher ($p<0.05$) levels of E2 compared with 1µg EB treatment group. & indicates significantly

higher (p<0.05) levels of E2 compared with 2.5µg EB treatment group. * indicates significant difference (p < 0.05) in E2 level in the brain region compared to serum under each EB treatment. N=12 for OVX: N=5 for each EB treatment.

Further analysis used a non-linear least squares regression to characterize the relationship between E2 levels in the serum and brain in the HPC, AMG, POA, CTX and CBL (Fig 6). In all brain regions, local levels of E2 increased in association with increasing levels of E2 in the serum, provided serum levels were within the physiological range. In the HPC, AMG and CTX, local levels appeared to approach a plateau as serum levels extended beyond 0.2 pmol/ml (Fig 6A, B & D). This also was apparent in the POA where brain E2 levels peaked at serum levels of approximately 0.2 pmol/mL and then decreased as serum levels further increased. Also note that in the HPC, AMG and POA, the Y-intercept was greater than zero indicative of local E2 production (Fig 6A, B & C). For CTX and CBL, the Y-intercept was 0, consistent with no local E2 production (Fig 6D & E).
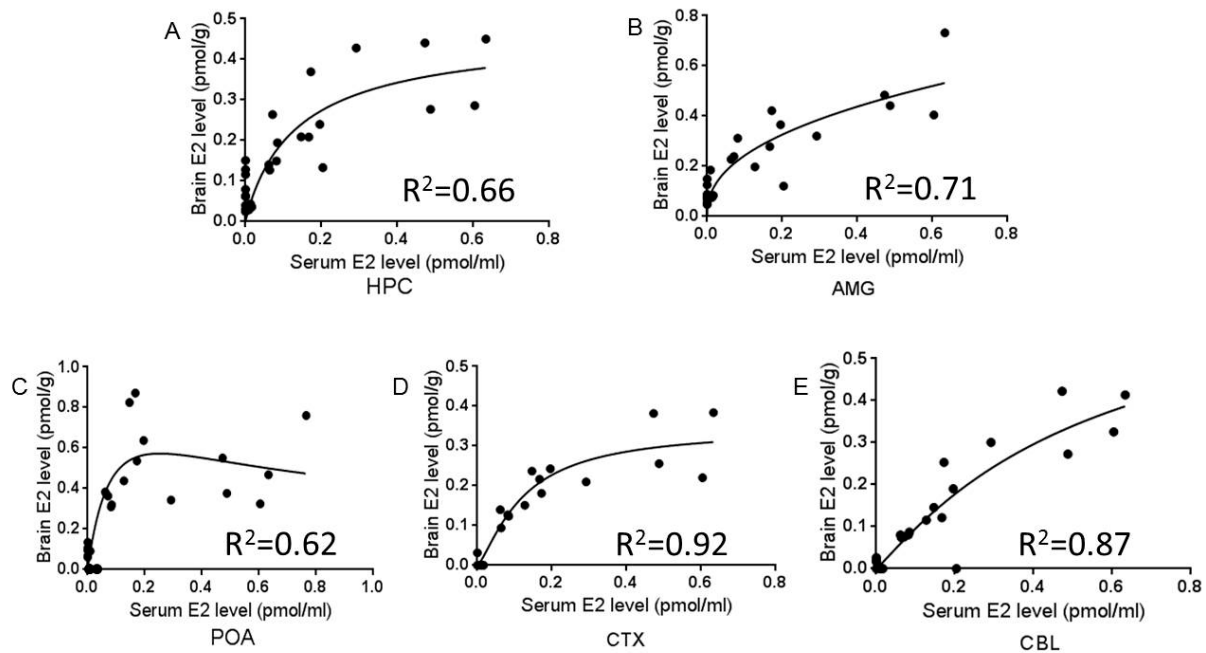
Figure 6: Correlations between E2 levels in serum vs brain in the different brain regions. Each data point represents data from one animal. Non-linear least-squares regression (Hill equation) was used to analyze the relationship between serum E2 and brain E2 levels in each region of the brain.

In summary, these results show that administering different doses of EB to OVX rats significantly increases E2 levels in the brain but that the proportional increase is greatly reduced or even reversed (i.e., POA) in some regions when systemic levels are well above the physiological range. Moreover, in the HPC, AMG and POA, the E2 levels were higher than that in the serum whereas in the CTX and CBL, brain levels were comparable to that in the serum. This is consistent with the local E2 production that has been described in specific brain regions.

3.3.4 Estradiol and testosterone levels in select brain regions vs. serum in OVX rats treated with testosterone-propionate in comparison with gonadally intact males

Testosterone (T) levels were measured in a separate set of OVX rats treated with testosterone-propionate (TP) and in gonadally intact male rats (Table 6). Two-way ANOVA showed a significant effect of treatment ($F[2,54]=11.69$, $p=0.0024$), a significant effect of location ($F[5,54]=16.46$, $p<0.001$), and a significant treatment*location interaction ($F[10,54]=6.07$, $p<0.001$). Post-hoc analyses showed that T levels in TP-treated OVX and gonadally intact male rats were not significantly different from each other, but were significantly higher than in OVX controls. In addition, serum levels of T in the TP-treated OVX group were significantly higher than levels detected in the brain regardless of brain region. In males, serum levels of T also were consistently higher then levels in the brain. This did not reach statistical significance, however, as the variance in serum T levels in gonadally intact males was quite high. Also, T levels across different brain regions in males did not differ significantly ($p=0.059$).

Table 6: Testosterone levels in the serum and in different brain regions in OVX, 0.5mgTP-treated OVX, and gonadally intact male rats.

	T level (pmol/ml)		
	OVX	0.5mg TP[*]	Male[*]
Serum	0.25 ± 0.04	16.92 ± 0.78[#]	13.01 ± 4.53
HPC	0.25 ± 0.07	9.27 ± 0.78	8.86 ± 2.70
AMG	0.33 ± 0.07	8.39 ± 0.87	7.40 ± 2.23
POA	0.34 ± 0.06	7.95 ± 0.44	8.23 ± 2.30
CTX	0.18 ± 0.01	11.01 ± 0.85	8.53 ± 2.65
CBL	0.18 ± 0.03	10.05 ± 1.20	7.66 ± 2.07

Values indicate the mean concentration of T ± s.e.m. * indicates significantly higher levels in TP-treated OVX and in males compared to the OVX controls. [#] indicates significantly higher T levels in serum compared to brain regions. N=4 for each group.

E2 levels in the TP-treated OVX rats, OVX controls, and vehicle-treated males are shown in Fig 7. Two-way ANOVA revealed a significant effect of treatment ($F[2, 210]=36.52$, $p<0.001$, a significant effect of location ($F[5, 210]=116.17$, $p<0.001$), and a significant treatment*location interaction ($F[10,210]=35.40$, $p<0.001$). Post-hoc analyses show that in the POA, AMG, and HPC, E2 levels were significantly higher than levels in the serum for all three groups. In males, E2 levels were detectable in the POA, AMG, and HPC even though levels were undetectable in serum. Moreover, male rats had significantly higher levels of E2 in the AMG ($p=0.002$) and POA ($p<0.001$) than TP-treated OVX rats. E2 levels in the AMG and POA of male rats also were similar to E2 levels in the AMG and POA of OVX rats treated with 2.5μg/day EB (compare Fig 5B with Fig 7). In contrast to levels in the POA and AMG, E2 levels in the HPC of TP treated OVX females and gonadally intact males were similar to E2 levels in the HPC of OVX controls. The E2 levels in CTX and CBL of TP treated OVX and male rats were similar to that in the serum and there was no statistically significant difference among these groups (Fig 7).

50
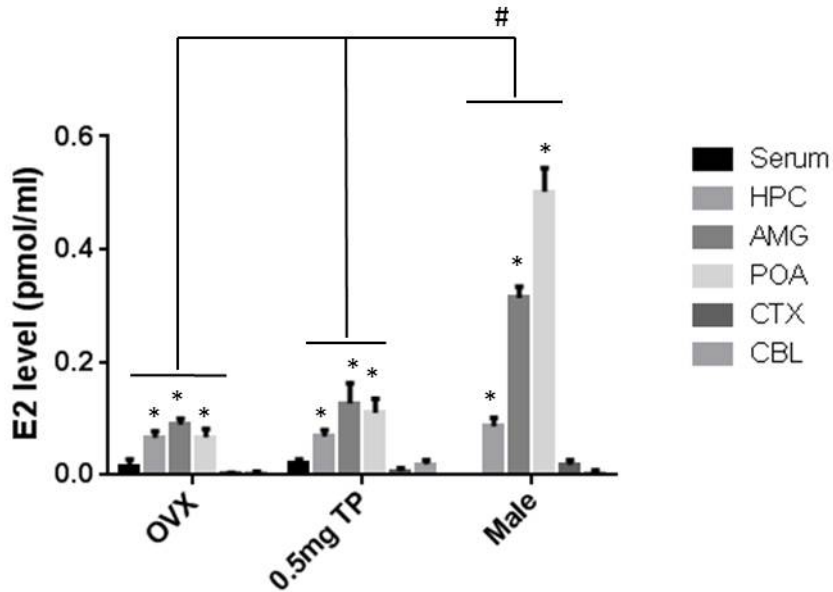
Figure 7: Effect of TP on E2 levels in the different brain regions vs serum and comparison with males.* indicates significant difference ($p < 0.05$) in E2 level in the brain region relative to serum. # indicates that E2 levels in males were significantly higher ($p<0.05$) than in TP treated OVX rats and OVX controls. N=12 for OVX; N=10 for 0.5mg TP; N=6 for Male.

In summary, these results show that administering TP to OVX rats significantly increased T levels both systemically and in the brain of OVX rats. However, the increases in T did not result in significantly elevated levels of E2 in the brain compared with vehicle-treated OVX controls. In comparison, males had significantly higher E2 levels in the AMG and POA compared with TP-treated rats and OVX controls. This is consistent with higher levels of ARO activity in the brains of males than females.

3.3.5 Effects of letrozole

3.3.5.1 Effects of letrozole on E2 levels detected in EB treated rats

Treatment with letrozole was used to test whether differences in E2 levels in brain vs serum were due to local ARO activity. Effects of letrozole on E2 levels detected in the brain and serum of OVX rats treated with EB are summarized in Fig. 8. Three-way ANOVA revealed a significant effect of EB treatment ($F_{[2,198]}= 206.60$, $p<0.001$), a significant effect of letrozole ($F_{[1,198]}=61.74$, $p<0.001$), and a significant effect of location ($F_{[5,198]}=80.00$, $p<0.001$) . The analysis also revealed significant interactions of EB*letrozole ($F_{[2,198]}=7.74$, $p=0.0017$), EB*location ($F_{[10,198]}=19.68$, $p<0.001$), letrozole*location ($F_{[5, 198]})=17.76$, $P<0.001$) and EB*letrozole*location ($F_{[10,198]}=4.13$, $P<0.001$). Post-hoc analyses showed that letrozole significantly reduced the differences between brain and serum E2 levels in the HPC, AMG and POA. Direct comparisons with non-letrozole-treated rats showed significant reductions in the HPC of OVX rats ($p=0.012$) and rats treated with 1.0 ($p<0.01$) and 2.5 ($p<0.05$) µg EB. Letrozole also significantly reduced E2 levels in the AMG of OVX rats ($p=0.015$) and in rats treated with 1.0 µg EB ($p<0.05$) (Fig 8A&B), and in the POA of rats treated with 2.5µg EB ($p<0.02$, Fig 8C). Letrozole also appeared to lower E2 levels in the POA of OVX, 1µg EB-treated rats, however this difference did not reach statistical significance, possibly due to the high variability at such low levels of detection.

Comparisons among different brain regions and serum showed that in OVX rats, the differences between brain and serum levels of E2 that were detected in the HPC, AMG and POA of non-letrozole treated rats were no longer apparent in letrozole-treated rats. There were three exceptions. One was the AMG where rats treated with 1µg EB + letrozole still had higher levels of E2 than in serum though lower than in rats that did not receive letrozole ($p=0.036$, Fig 8B).

The others were in the POA of rats treated with both 1.0 µg and 2.5 µg EB + letrozole where E2 levels were reduced in letrozole-treated rats compared to non-letrozole-treated rats, but were still higher than levels in serum (p=0.047, p=0.007, respectively, Fig 8C).
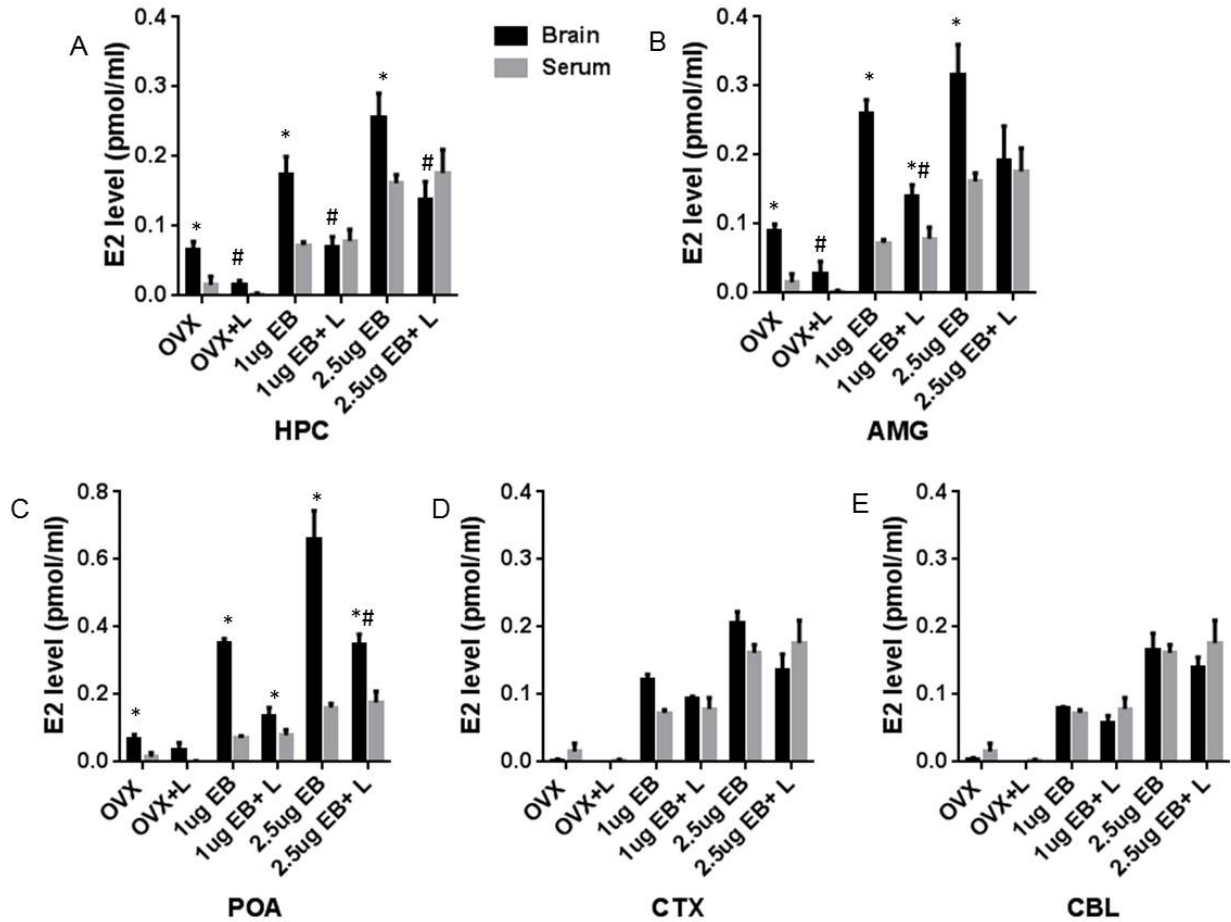
Figure 8: Comparison of E2 levels in brain vs. serum after different dose of E2 and letrozole in A) HPC, B) AMG, C) POA, D) CTX and E) CBL. * indicates significant difference $p < 0.05$ between brain and serum levels. [#] indicates significant difference p<0.05 in brain levels between letrozole and non-letrozole treated groups. N=12 for OVX; N=6 for OVX+ L: N=5 for EB treatment without L; N=5 for EB treatment with L.

These data show that letrozole significantly reversed the differences between brain and serum E2 levels in OVX and EB treated rats, suggesting that the higher E2 levels in the HPC, AMG and POA are due to local ARO activity.

3.3.5.2 Effects of letrozole on E2 levels detected in TP-treated OVX and male rats

Effects of letrozole on E2 levels detected in the brain and serum of male rats as well as OVX rats treated with TP are summarized in Fig. 9. Three-way ANOVA revealed a significant effect of TP ($F[2, 234]=18.62$, $p<0.001$), a significant effect of letrozole ($F[1, 234]=64.23$, $p<0.001$), and a significant effect of location ($F[5, 234]=57.75$, $p<0.001$). The analysis also revealed significant interactions between TP*letrozole ($F[2,234]=14.81$, $p<0.001$), TP*location ($F[10, 234]=18.60$, $p<0.001$), letrozole*location ($F[5, 32.81])=17.76$, $P<0.001$), and TP*letrozole*location ($F(10,234)=13.67$, $p<0.001$). In TP-treated OVX and gonadally intact male rats, letrozole reduced levels of E2 in the AMG and POA such that they were no longer different from levels in serum (Fig 9B &C). This was not the case, however, in the HPC where letrozole treatment did not appear to reduce E2 levels in TP-treated females, but did reduce E2 levels in gonadally intact males (Fig. 9A). No significant effect of letrozole on E2 levels was detected in the CTX or CBL (Fig 9D &E).
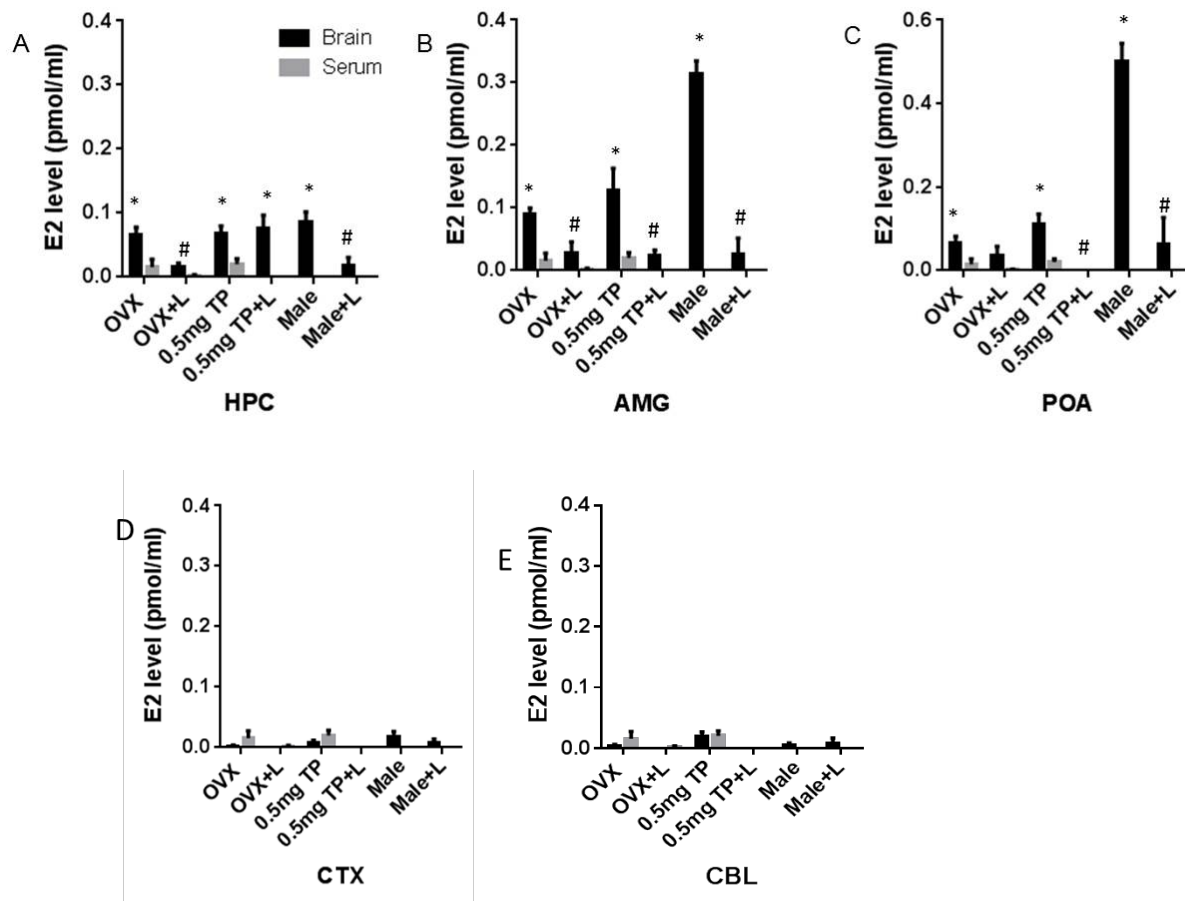
Figure 9: Comparison of E2 levels in brain vs. serum in the A) HPC, B) AMG, C) POA, D) CTX and E) CBL of OVX females, TP-treated females, and gonadally intact males treated with Letrozole. *indicates significant difference $p < 0.05$ between brain and serum levels. # indicates significant difference $p < 0.05$ in brain levels between letrozole and non-letrozole treated groups. N=12 for OVX; N=6 for OVX+ L; N=10 for 0.5mg TP; N=5 for TP+L; N= 6 for Males; N=6 for Male +L.

These results show that letrozole significantly decreased E2 levels in the HPC, AMG and POA in both TP-treated OVX and male rats, suggesting that the higher E2 levels in these regions are due to local ARO activity.

3.4 Discussion

One objective of this study was to investigate the relationship between circulating levels of E2 and levels in specific regions of the brain in adult OVX rats treated with different doses of EB, and to identify the contribution of local versus systemic sources of E2 in specific brain regions. Using a sensitive UPLC-MS/MS method, we showed that in the HPC, AMG and POA, which are brain regions known to contain significant levels of ARO activity (Li et al., 2016a), the levels of E2 significantly exceeded those present in the circulation. Moreover, letrozole treatment significantly reduced the E2 levels in these regions, indicating that the differences between brain and serum E2 levels are due, at least in part, to local E2 synthesis. We also showed significant levels of E2 in these same brain regions in male rats, confirming that local E2 synthesis plays an important role in determining E2 levels in the brain in both sexes.

Notably, we showed that E2 levels in the HPC, AMG and POA were significantly higher than in serum in OVX rats where circulating levels of E2 were undetectable, and also in rats treated with 1.0 and 2.5 µg EB. Note that levels detected in the HPC, AMG and POA of OVX-untreated rats were similar to serum levels detected in rats treated with 1µg EB. This is consistent with other reports showing higher levels of E2 in the brain than in the circulation in gonadally intact rats, in regions with significant ARO activity (Kato et al., 2013). In the HPC levels of E2 detected in rats treated with 1.0 or 2.5µg EB were comparable to values obtained by adding levels of E2 in the circulation with levels produced in the brain of OVX controls. This suggests that in the HPC the levels achieved reflect the simple addition of local production with systemic levels. In the AMG and POA, levels of E2 detected in rats treated with 1.0 and 2.5µg EB were even higher, suggesting that E2 treatment may actually increase local production in

these regions. In brain regions with relatively little ARO activity, local E2 levels were comparable to levels detected in the circulation.

Collectively, these data demonstrate that local E2 levels are brain region specific, and in some regions remain significantly elevated even following OVX when circulating levels are undetectable. The distribution of local E2 production is in agreement with the distribution of ARO activity in the brain reported in previous reports (Roselli and Resko, 1991; Roselli et al., 1998). Moreover, our results show that levels of E2 in the brain correlate with circulating levels provided those levels are within the physiological range (<0.2 pmol/ml). This is consistent with an earlier study showing that variations in systemic E2 levels during the estrous cycle correspond to varying levels of E2 in the brain (Kato et al., 2013).

Unexpectedly, we found that in some regions of the brain, the increases in E2 levels appeared to plateau as systemic E2 levels increased well above the physiological range (>0.3 pmol/ml). This suggests that in response to very high circulating levels of E2, local mechanisms are activated that prevent levels in specific regions of the brain from becoming too high. Also, under conditions of high circulating E2, local E2 production did not appear to add significantly to local levels. Studies show that ARO activity can be regulated by phosphorylation, with increased phosphorylation associated with reduced activity ((Balthazart et al., 2001a; Balthazart et al., 2001b; Balthazart et al., 2005; Balthazart et al., 2003a). It is possible that supraphysiological levels of E2 result in phosphorylation of local ARO in the brain, thus reducing enzyme activity and local E2 production (Cornil et al., 2006; Fester et al., 2016). This could be a mechanism for local regulation of ARO activity and needs to be investigated. Consequently, there may be a variety of mechanisms at play that serve to limit local levels of E2 in specific regions of the brain, particularly when circulating levels are very high. This requires

further study. This result also may help to explain why high (i.e., supraphysiological) doses of E2 can have lesser or even negative effects on brain endpoints compared with lower doses. For example, the results may help explain why lower doses of E2 produce a dose-related increase in ChAT immunoreactivity in the basal forebrain whereas high doses do not (Gibbs, 1997), or why low doses of E2 induce dendritic spine formation (Phan et al., 2012), while high doses do not (Kretz et al., 2004). Several studies indicate that local estrogen production has a significant impact on dendritic spines in the HPC (Bender et al., 2017; Leranth et al., 2004; von Schassen et al., 2006). These results highlight the importance of understanding the relationship between local and systemic E2 levels when interpreting effects of E2 in specific regions of the brain.

3.4.1 Effects of Letrozole

We hypothesized that the elevated E2 levels detected in the brain of OVX rats with low or physiological levels of circulating E2 were due to local E2 production. Alternatively, elevated levels could be due to sequestration of circulating E2 by binding to local estrogen receptors. To differentiate between these possibilities, we tested the ability to reduce or eliminate the differences between brain and serum levels of E2 by administering letrozole, a selective ARO inhibitor. Results show that E2 levels in brain regions with ARO activity were decreased significantly by letrozole treatment, whereas letrozole had little effect on E2 levels in brain regions which lack ARO. This indicates that, in these regions, the higher levels of E2 in the brain relative to serum are due, at least in part, to local estrogen production by ARO.

Three minor exceptions were the AMG of rats treated with 1µg EB + letrozole and the POA of rats treated with 1.0 and 2.5 µg EB + letrozole, where levels of E2 were still higher in the brain than in the serum though lower than in rats that did not receive letrozole. In these cases,

some residual E2 bound to a high density of estrogen receptors may contribute in part to elevated levels of E2 detected in these regions.

3.4.2 Effects of T on local E2 production in the brain

Another objective of this study was to test whether systemic administration of TP to OVX females would result in local E2 levels in specific regions of the brain comparable to levels seen in the brains of males. Assays confirmed that circulating levels of T in the TP-treated females were comparable to levels detected in the males. Using a separate set of rats we also confirmed that brain levels of T in TP-treated females are significantly elevated. In the AMG and POA, levels of E2 in TP-treated females were higher than in OVX controls, however these differences did not reach statistical significance. Moreover, there was no significant increase in E2 in the HPC of TP-treated females. The lack of effect of TP treatment was surprising given that significant levels of ARO activity have been detected in each of these regions (Li et al., 2016a; Roselli and Resko, 1993). The negative result was not due to a failure to provide adequate levels of T to the brain, as the levels of T in the brains of TP-treated females was comparable to the levels detected in males. It also is not likely that the ARO enzyme was saturated, as our previous studies indicate that microsomal ARO activity in these areas saturates at much higher levels of T (> 400 nmol/L, (Li et al., 2016a)). It is possible that the saturation concentration of T for the ARO enzyme in vivo is significantly different from that in vitro; however, there is no evidence that this is the case. A more likely explanation is that the increased levels of E2 are small relative to the high variability at the low end of the detectable range, thus making it difficult for the differences observed in the AMG and POA to reach statistical significance.

The E2 levels in these same brain regions in males were much higher than observed in the TP-treated females. This is consistent with the higher levels of ARO mRNA and activity that have been reported in these brain regions in males (Roselli et al., 1985; Tabatadze et al., 2014) and in accord with a study done in zebra finch showing significantly higher E2 levels in males (35pg/ml) than in females (10 pg/ml) during the subadult stage (Chao et al., 2015). In the current study, E2 levels in the HPC of males were similar to levels in the OVX and TP-treated females. This suggests that in this brain region, ARO activity may be similar between males and OVX females. Moreover, letrozole treatment eliminated the increased levels of E2 detected in brain vs. serum in both TP-treated females and gonadally intact males. This indicates that the higher levels of E2 were due to local E2 production in each of these cases.

As mentioned above, although ARO activity is interpreted to indicate the capability for local E2 production, actual E2 levels in the brain are less well studied. Konkle et al. (Konkle and McCarthy, 2011) reported E2 levels of 0.10, 0.50 and 5.0 pg/mg protein in the HPC, CTX and hypothalamus and 1.5 and 5 pg/mg protein for T in these three regions of intact young adult rat brain using LC-MS/MS methods. If we assume that 1g of tissue contains 100mg protein, then the concentrations of E2 and T reported by Konkle are similar to the concentrations reported here. However, significant differences in study design must also be considered: 1) here we used OVX rats as our model which eliminated native ovarian sources of E2 and other hormones. This may account for the slightly lower E2 levels detected in our study, 2) we detected a higher level of E2 in the HPC than in the CTX, which is opposite to that reported by Konkle et al (Konkle and McCarthy, 2011). This may due to differences in tissue dissection and local variances in ARO activity. 3) there were no sex differences in E2 levels and T levels measured in rats at 60 days of age in the prior study, whereas we detected significantly higher levels of E2 in intact male rats

compared to OVX female rats. This difference may due to the absence of systemic E2 input in the OVX female rats in our study.

Kato et al. (Kato et al., 2013) also measured E2 and T levels in the hippocampus and serum of intact males, normal cycling females and OVX Wistar rats (10 weeks old) using HPLC-MS/MS method. The serum E2 levels in both sexes measured in our study are similar to that reported in Kato's study. Moreover, the T levels of male rats measured in our study also matched with the results shown by Kato, demonstrating a lower T levels in the HPC than in serum (Hojo et al., 2004). However, the E2 levels measured in the HPC of males (~8 nM) and females (pro-estrous stage: ~4nM) were higher than levels measured in our study. Moreover, we did not see higher E2 levels in the HPC of males than in the HPC of OVX females. This may due to the different animal models or to differences in specific LC-MS/MS methods.

Other studies applied different methodologies and animal models to explore this question. Munetsuna et al (Munetsuna et al., 2009) reported approximately 19.0 fmol/mg protein E2 and 43.7 fmol/mg protein T in the hippocampal slice culture from 10 d-old male rats precultured in serum after 24h using radioimmunoassay. This also is similar to the levels of E2 and T reported in our study.

Studies also show that E2 levels in the brain can vary as a function of age or in association with different pathological conditions. For example, Konkle et al. (Konkle and McCarthy, 2011) reported much higher levels of E2 (5~50 pg/mg protein varying by brain region) and T (60~400 pg/mg) in prenatal rats than in adults. Several studies have reported induction of ARO following brain injury (Peterson et al., 2007), experimental stroke (Carswell et al., 2005), global ischemia-reperfusion, hypertension (Pietranera et al., 2011), and neuroinflammation (Duncan and Saldanha, 2011). Studies also have reported significant induction of ARO in

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astrocytes following brain injury (Peterson et al., 2007; Peterson et al., 2001; Pietranera et al., 2011) as well as an increase in ARO activity (Pedersen et al., 2017), consistent with induction of local E2 production. Sato et al. (Sato and Woolley, 2016) showed induction of E2 synthesis in the hippocampus of young adult rats after seizure in both males and females using hippocampal microdialysis followed by E2 measurement using enzyme immunoassay (EIA) methods. Further studies are needed to analyze the E2 levels in the brain under different physiological and pathological conditions as well as sex differences in response to injury.

3.4.3 Implications and Limitations

3.4.3.1 Functional Implications

The findings presented here, showing differences in E2 levels in specific regions of the brain under different treatment conditions, may have important functional and behavioral implications. Increasingly studies are showing that local estrogen production has an important impact on local neuronal function and behavior. The preoptic area plays a critical role in sex dimorphism and sex behavior regulation. In particular, accumulated evidence shows that estrogens of local and systemic origin cooperate to regulate the induction of the preovulatory (Gonadotropin-releasing hormone) GnRH/ luteinizing hormone (LH) surge and regulate receptivity and sexual motivation in females (Cornil, 2018; de Bournonville et al., 2016; Rissman, 1991; Terasawa and Kenealy, 2012). Aromatization of T to E2 in this region contributes to LH-negative feedback regulation in males (Hayes et al., 2000; Roselli and Resko, 2001; Sharma et al., 1999) and regulates male-typical sex behaviors (Gladue and Clemens, 1980a; Gladue and Clemens, 1980b; Roselli and Resko, 1993). Treatment with E2 or T, but not dihydrotestosterone, a non-aromatizable metabolite of T, has been shown to restore sex

behaviors in adult castrated male rats (Baum and Vreeburg, 1973; McDonald et al., 1970; Putnam et al., 2003; Putnam et al., 2005).

The hippocampus plays a critical role in learning and memory consolidation. As mentioned above, studies show significant effects of local estrogen production on synaptic plasticity and maintenance of long term potentiation (LTP) in the hippocampus of female, but not male animals (Fester and Rune, 2015; Vierk et al., 2014). Fewer studies have explored the effect of E2 on memory in male rodents and there were discrepant effects of E2 on the spatial memory and nonspatial tasks (reviewed by (Frick et al., 2015)). Work by Oberlander et al. and Bailey et al. (Bailey et al., 2013; Bailey et al., 2017; Oberlander et al., 2004) suggests an important role for local estrogen production on acquisition of spatial memory in song birds, and other evidence suggests that local E2 production may work through ERα signaling to impact memory after cessation of ovarian function in rats (Daniel et al., 2015).

The amygdala plays a critical role in fear and anxiety, and in social recognition behaviors. In prior research, ARO knockout (ArKO) in OVX female mice exhibited increased depression-like behavior compared with wild-type OVX female mice in the forced swim test (Dalla et al., 2004). Specific deletion of ARO in the medial amygdala also has been associated with increased aggression in both males and females (Unger et al., 2015). We recently showed that treating with cholinesterase inhibitors can significantly increase ARO activity in the amygdala (Li et al., 2018). These findings could have important implications for fear and anxiety-related behaviors, as well as for risk of anxiety-related disorders.

Further studies on the behavioral consequences of the local contribution of ARO to E2 levels in these regions both in the normal adult brain, following estrogen treatments, during critical periods of development and aging, and under pathological conditions is a high priority.

3.4.3.2 Limitations

One limitation of this study is that ARO expression and activity was not measured in the same rats where E2 levels were detected; however, other studies including one recent study conducted in our laboratory have reported regional differences in ARO expression and activity in brain (Balthazart et al., 2003b; Li et al., 2016a; Roselli et al., 1997). Nevertheless, potential effects of hormone treatments on brain ARO expression and activity were not evaluated and cannot be excluded. Several studies have reported effects of hormone treatment (primarily androgens) on ARO activity in brain. Androgens, such as T and dihydrotestosterone have been shown to bind to the ARO promoter and regulate its transcription and stability in sex-specific and region-specific ways (Abdelgadir et al., 1994; Negri-Cesi et al., 2001a; Roselli et al., 1997). In contrast, the effect of estrogen regulation of ARO mRNA expression and activity are more complex and may be region- sex- and species dependent (Roselli et al., 1997; Zhao et al., 2007 ; Abdelgadir, 1994 #93 ; Roselli, 1993 #637). More studies regarding the effects of different doses and durations of E2 treatment, estrous cycle regulation of ARO expression and activity need to be evaluated.

Another limitation of this study is the lack of information of pharmacokinetics (PK) and pharmacodynamics (PD) information of EB and TP in the rats. EB is an estradiol ester and a prodrug of E2, which can be cleavage by esterase into estradiol not only in liver, but also in the blood and in tissue (Kuhl, 2005). After Intramuscular injection, the half-life of EB is approximately 2 days in humans. TP is a prodrug of T, with a half-life of approximately 0.8 days in humans (Behre et al., 1999). There is little published regarding the PK of EB and TP in rats, which may be different from humans (Plowchalk and Teeguarden, 2002). As a small lipophilic molecule, E2 can rapidly diffuse across the blood brain barrier through a non-saturable,

bidirectional process (Banks, 2012). However, a big portion of E2 (~95%) in the plasma is bound to albumin (Plowchalk and Teeguarden, 2002), which will decrease the rate of E2 penetrate into the brain tissue. According to the principles of PK, a time period of 5 half-lives is sufficient for a drug to achieve pseudo-equilibrium between plasma and tissues, when the concentration in the tissue is more stable and parallels that in the plasma. For our study, we used a time point of 2 days after treatment, which may not be at the pseudo-equilibrium stage. It is possible that the E2 levels are still fluctuating between plasma and brain and may influence our detection. The final amount of E2 in the brain is decided by its affinity to tissue vs plasma binding protein. With letrozole treatment or in the brain regions without ARO activity (such as CTX and CBL), the levels of E2 in the brain regions were similar to the levels in the serum, This indicates that E2 may have similar binding affinity to tissue and the binding protein in the plasma, Further studies, such as PBPK modeling, that consider the characteristics of the chemicals and physical conditions may be helpful to explore how and how much of systemic E2 can get into the brain. Moreover, the elimination of E2 can also be different between brain and circulation, which may also influence E2 levels in the brain. One future direction would be to measure the PK of E2 in both plasma and brain to better characterize their relationships with time. This would also be helpful in interpreting the pharmacodynamics effects of E2 in the brain.

4.0 Effect of Cholinergic Lesions and Cholinesterase Inhibitors on Aromatase and Estrogen Receptor Expression in Different Regions of the Rat Brain

4.1 Introduction

The goal of this study was to explore potential effects of cholinergic manipulation on aromatase (ARO) activity and estrogen receptor (ERs) expression in different regions of the brain. Estrogens have been shown to have beneficial effects on learning, memory, and attention in multiple species including rats, mice, non-human primates, and in humans (Bimonte and Denenberg, 1999; Daniel et al., 1997; Frye et al., 2007; Gresack and Frick, 2006; Luine et al., 2003; Sherwin and Henry, 2008). Effects often are limited to females and are task specific. In rodents, estrogens (primarily estradiol (E2)) have been shown to enhance performance on a variety of spatial navigation tasks (Daniel et al., 1997; Fader et al., 1998; Gibbs and Johnson, 2008), and to enhance working memory (Bimonte and Denenberg, 1999; Bohacek and Daniel, 2007; Daniel et al., 2006), as well as novel object and object placement recognition (Fernandez et al., 2008; Frye et al., 2007; Luine et al., 2003). In humans beneficial effects have been observed on short-term and long-term verbal memory and logical reasoning (Krug et al., 2006;

Sherwin, 1988). Estrogens also have been shown to enhance synapse formation, connectivity, and NMDA receptor expression in the hippocampus, with corresponding effects on synaptic transmission and long-term potentiation (Jelks et al., 2007; McEwen et al., 2001; Mendez et al., 2011). These effects are thought to underlie some of the effects of estrogens on cognitive performance.

We and others also have demonstrated that cholinergic projections from the medial septum (MS) to the hippocampus are significantly affected by estrogens and that these projections can play an essential role in enabling estrogen-mediated effects on cognitive performance. For example, ovariectomy reduces and E2 treatment increases choline acetyltransferase (ChAT) mRNA in the MS and nucleus basalis magnocellularis (NBM), with corresponding effects on ChAT activity (Gibbs, 1996; Gibbs, 1997; Gibbs and Pfaff, 1992; Gibbs et al., 1994; Luine, 1985), high affinity choline uptake and acetylcholine (ACh) release in the frontal cortex and hippocampus (Gabor et al., 2003; Gibbs, 2000; Gibbs et al., 1997). E2 treatment has been shown to mitigate effects of scopolamine (a nonspecific antimuscarinic) on T-maze alternation in rats (Fader et al., 1998), and likewise to mitigate effects of both scopolamine and mecamillamine(a non-selective antagonist of the nicotinic receptor) on cognitive performance in post-menopausal women (Dumas et al., 2006). Notably, selective removal of cholinergic projections to the hippocampus prevents estrogen-mediated enhancement of a delayed-matching-to-position (DMP) spatial navigation task (Gibbs and Johnson, 2007; Johnson et al., 2002). Similar cholinergic lesions also have been shown to block estrogen-mediated increases in synaptic spines on CA1 neurons in the hippocampus (Lam and Leranth, 2003). More recent studies suggest that loss of ERs also contribute to loss of estrogen effects on cognitive function with age (Bean et al., 2014; Black et al., 2016; Foster, 2012), and that

increasing ERα expression can enhance cognitive performance (Foster et al., 2008). We have shown that beneficial effects of E2 on DMP acquisition can be restored by treating older rats and rats with partial cholinergic lesions with selective cholinesterase inhibitors (ChEIs) (Gibbs et al., 2011a; Gibbs et al., 2011b; Gibbs et al., 2009). Whether levels of ERs also were affected was not explored.

Collectively the findings demonstrate important interactions between basal forebrain cholinergic projections and estrogen effects on performance that impact brain aging and cognition. To date there has been little study of whether cholinergic projections significantly influence estrogen signaling, ER expression, or perhaps even local estrogen production, in the brain.

In this study, we proceeded to explore whether selective cholinergic lesions, as well as treatment with cholinesterase inhibitors, have effects on ARO expression, ARO activity, and ER expression in different regions of the brain.

4.2 Experimental Procedures

4.2.1 Animals

Ninety-four ovariectomized (OVX, 270-350g, 3 months old) Sprague-Dawley female rats were purchased from Harlan Sprague Dawley Inc. Rats were individually housed for two weeks in our facility on a 12 hour:12 hour light/dark schedule with unrestricted access to food and

water. All procedures were carried out in accordance with PHS policies and with the approval of the University of Pittsburgh's Institutional Animal Care and Use Committee.

In the first experiment, forty-six rats were used to test the effect of selective lesions of cholinergic neurons in the medial septum (MS) on aromatase (ARO) mRNA, activity and estrogen receptors (ERs) mRNA in the hippocampus and frontal cortex. Rats received intraseptal injections of 192IgG-Saporin (SAP) or vehicle as described below. Two weeks later, rats were anesthetized with an overdose of ketamine (3 mg) and xylazine (0.6 mg). Brains were removed; hippocampal and frontal cortex tissues were collected and stored at -80ºC until use. Of the 46 rats, tissues from 20 rats (10/grp) were analyzed for relative levels of ARO and ER mRNAs using qRT-PCR methods described below. Tissues from the other 26 rats were analyzed for ARO activity (13 rats/grp) in microsomes using a recently validated and highly sensitive UPLC-MS/MS assay (Li et al., 2016b). Immunohistochemical detection of ChAT–positive cells in the MS also was performed to confirm the loss of cholinergic neurons.

In the second experiment, a total of 48 rats were treated intraperitoneally with 3 mg/kg donepezil, 5 mg/kg galantamine or saline (as control) injected once daily for 7 days. Donepezil and galantamine are cholinesterase inhibitors (ChEIs) approved for the treatment of memory decline associated with Alzheimer's disease. Following treatment rats were anesthetized and brain tissues were dissected as above. In addition to collecting hippocampus and frontal cortex, tissues from the amygdala and preoptic area (POA) also were dissected and analyzed. Tissues from 12 rats (4 rats/grp) were analyzed for relative levels of ARO and ER mRNAs using qRT-PCR methods. Tissues from 36 rats (12 rats/grp) were analyzed for ARO activity.

4.2.2 Cholinergic lesions

Rats were anesthetized and placed on a standard stereotaxic apparatus. The skull was exposed and a hole was drilled at midline 0.3mm rostral to Bregma. A 28ga stainless steel cannula was lowered -5.6mm from dura into the medial septum. 2.0µl of 192IgG-SAP (0.2 mg/ml; Advanced Targeting Systems, Inc.) was injected at a rate of 0.2µl /min. Previous studies have shown that these injections cause a selective loss of cholinergic cells in the basal forebrain with little non-selective damage to GABAergic neurons and no damage to cholinergic neurons in the NBM (Baxter et al., 1995; Johnson et al., 2002; Schliebs et al., 1996).This is also accompanied by decreased activity of choline acetyltransferase (ChAT) and by reduced high-affinity uptake of [3H]choline into cholinergic nerve terminals in the hippocampus, but not the frontal cortex (Rossner et al., 1995b). Controls received intraseptal infusions of saline. The skin was sutured and rats were placed onto a heating pad during recovery. Following surgery, rats received ketofen (Fort Dodge, Inc.,3.0 mg/kg, i.p.) once per day for three days to relieve pain. After 14 days of recovery, rats were dissected and the hippocampus and frontal cortex were collected and analyzed. In addition, tissues containing the MS were fixed by immersion in 4% paraformaldehyde in 50mM phosphate buffered saline (PBS, pH7.2) at 4˚C overnight. These tissues were then transferred to 20% sucrose in PBS at 4 °C for several days prior to sectioning and immunostaining.

4.2.3 ChAT assay

ChAT activity in the hippocampus and frontal cortex were measured as previously described (Gibbs and Johnson, 2007). Briefly, 30mg tissues were sonicated in 300µl sonication

buffer (10mg tissue/mL) which contains 10mM EDTA and 0.5% Triton X-100. Samples were run in triplicate. 10µl of substrate solution, which contains 0.25mM [3H] acetyl-CoA (50,000-60,000 dpm/tube) was added to each reaction tube. 5µl aliquots of sample in sonication buffer were added to the tubes and incubated for 30 min at 37℃. The reaction was terminated with 4 mL sodium phosphate buffer (10mM) at 4℃. The production of [3H] acetylcholine was detected by adding scintillation LSC-cocktail (Packard Instruments, Meriden, CT) and counting cpm in the organic phase using an LKB beta-counter. ChAT activity was calculated for each sample as pmol acetylcholine manufactured/h/µg protein.

4.2.4 ChAT Immunohistochemistry (IHC)

To further confirm the loss of cholinergic neurons, 40 µm coronal sections through the MS (corresponding to plates 14–21 of The Rat Brain in Stereotaxic atlas (1986), separated from vertical and horizontal limbs of the diagonal band (vDB and hDB) by Medulam and co-workers(Mesulam et al., 1983)) were cut and stained for ChAT immunoreactivity (IR) as previously described(Johnson et al., 2002). Briefly, sections were placed in a solution containing primary antibody against ChAT (goat anti-ChAT 1:3500, EMD Millipore AB144P) for three days at 4℃. Sections were then rinsed with PBS and incubated with a biotinylated secondary antibody (horse anti-goat 1:220, Vector Laboratories, Inc.) for 1 hour at room temperature. Sections were then placed in an avidin/biotinyl-peroxidase solution (ABC Elite kit, Vector Laboratories, Inc.) for 1 h and then stained with a solution of 3-3'-diaminobenzidine, H2O2, and NiCl2. Sections were then rinsed with PBS, mounted onto glass slides, dehydrated, cover slipped and examined with a Leitz photomicroscope (Leica, Inc.).

4.2.5 qRT-PCR

The detailed method for qRT-PCR has been described in the Section 2.2.2. The primer pairs for ERs were : ERα: (F) 5′-TCCGGCACATGAGTAACAAA-3′ and (F) 5″-TGAAGACGATGAGCATCCAG-3′. ERβ: (F) 5′-AAAGTAGCCGGAAGCTGACA-3′; (R) 5′-ACTGCTGCTGGGAGGAGATA-3′ GPR30 (F) 5′-AGGAGGCCTGCTTCTGCTTT-3′; (R) 5′-ATAGCACAGGCCGATGATGG-3′. GAPDH was used as the control gene. The relative gene expression was normalized to sham animal left hemisphere using the $2^{-\Delta\Delta Ct}$ method.

4.2.6 Microsomal incubation assay

The detailed method for Microsomal incubation assay has been described in section 2.2.1.

4.2.7 Statistical analyses

In experiment 1, differences between the 192IgG-SAP-treated and the control group were analyzed by t-test. In experiment 2, effects of ChEI treatments were analyzed by one-way ANOVA followed by Tukey's post-hoc test. All statistical analyses were done using JMP (Pro12), with significance defined as $p < 0.05$.

4.3 Results

4.3.1 Effects of septal cholinergic lesions

4.3.1.1 Verification of the lesions

Lesions were evaluated by confirming loss of ChAT-positive cells in the septum, and by loss of ChAT activity in the hippocampus. Immunostaining confirmed that septal infusions of 192IgG-SAP eliminated most (>80%) of ChAT-IR cells in the MS (Figure10A and B). ChAT activity in the hippocampus also was significantly decreased (>80%) in 192IgG-SAP-treated rats relative to controls (t9=6.15, P<0.001; Figure C). In contrast, ChAT activity in the frontal cortex was not significantly affected (t9=0.52, P=0.62). This demonstrates a loss of cholinergic input to the hippocampus, but not the frontal cortex, consistent with previous reports (Gibbs et al., 2011b) (Gibbs and Johnson, 2007).
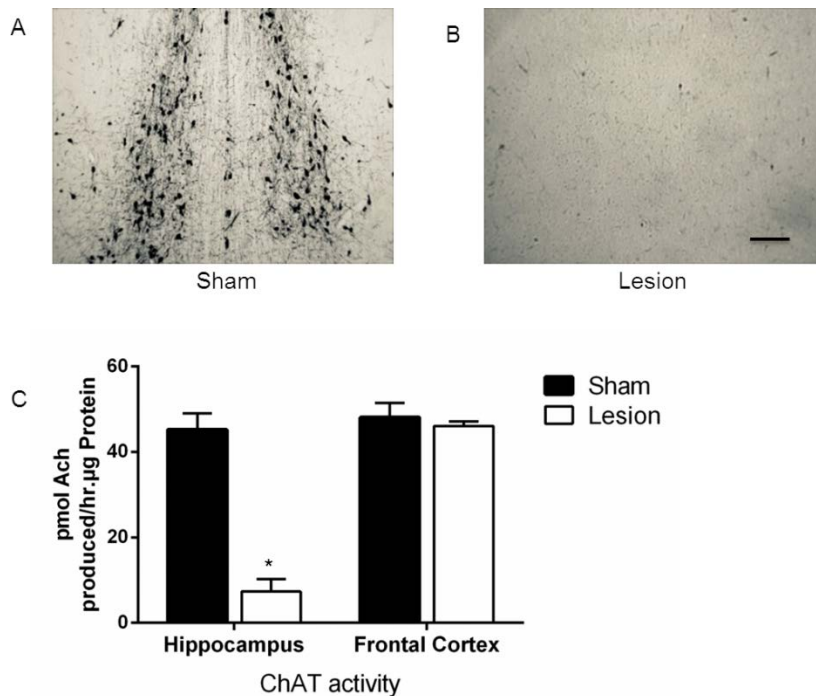
Figure 10: Effect of 192IgG- SAP-induced cholinergic lesions on ChAT immunoreactivity by immunohistochemistry (IHC) in the medial septum of rats treated with (A) Saline and (B) 192IgG- SAP as well as on (C) ChAT activity detection in the hippocampus and frontal cortex by radioactive assay. Two weeks following intracerebroventricular injection of 0.4µg 192IgG-SAP treatment, the number of positive ChAT stained cholinergic neurons was significantly decreased in the medial septum. No significant change of ChAT activity was measured in frontal cortex tissue while hippocampal tissue shows significant reduction in activity. In all panels, rostral is toward the top and midline extends top to bottom through the middle of the panel. Scale bar =0.1mm. * indicates the p ≤0.05 compared to control group. Animal number: N=23 for each group.

4.3.1.2 Effects of septal cholinergic lesions on ARO and ER mRNA levels and on ARO activity

Effects of cholinergic lesions on relative levels of ARO mRNA are summarized in Figures 11A and B. Levels of ARO_T mRNA were slightly higher (27.0%) and levels of ARO_L were slightly lower (18.1%) in the hippocampus of 192IgG-SAP-treated rats vs. controls; however, these differences were not statistically significant. Likewise, there was no significant difference in ARO activity detected in the hippocampus of 192IgG-SAP-treated rats vs. controls (Figure 11C). The frontal cortex, which was included as a negative control, also showed no significant effects of treatment on relative levels of total ARO mRNAs. As previously reported, levels of ARO mRNA were extremely low in the frontal cortex and ARO activity was not detected in this region (Li et al., 2016b).
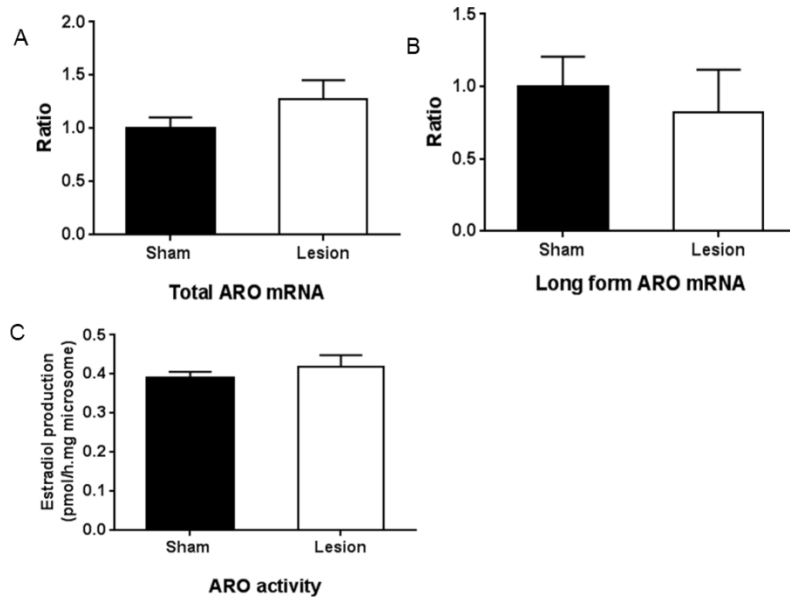
Figure 11: Effect of cholinergic lesion in the medial septum on (A) ARO_T mRNA, (B) ARO_L mRNA expression and (C) ARO activity in the hippocampus. No significant change was found in region-specific ARO mRNA and activity. Bars in (A) and (B) indicate the mean ratio of ARO mRNA relative to OVX controls ± s.e.m., after normalizing to GAPDH. Bars in (C) indicate the estradiol production (pmol/h.mg microsome) ± s.e.m, which represents the ARO activity. In the study of ARO mRNA, animal number: N= 10 for each group. For ARO activity study, animal number: N=13 for each group.

Relative levels of ER mRNAs detected in the hippocampus are summarized in Figure 12. No significant effects on the relative levels of ERα, ERß, or GPR30 were detected in 192IgG-SAP-treated rats vs. controls.
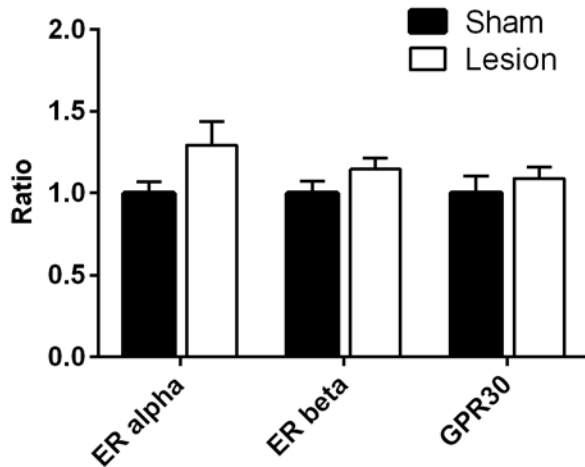
Figure 12: Effect of cholinergic lesions on ER alpha, ER beta and GPR30 expression in response to saline or 192IgG-SAP infusions. Bars represent ratio of ER mRNA levels that were normalized to control GAPDH mRNA ± s.e.m. Animal number: N=10 for each group.

Collectively these findings indicate no significant effects of selective cholinergic denervation on ARO or ER expression in the hippocampus.

4.3.2 Effect of ChEIs on ARO and ER mRNAs and on ARO activity

Effects of ChEIs on ARO mRNA and ARO activity are summarized in Figure 13. As previously reported, highest expression of both ARO_T and ARO_L was detected in the amygdala, followed by preoptic area, then hippocampus (Figures 13A & B) (Li et al., 2016b). Relative levels of ARO activity mirrored the relative levels of ARO_L mRNA expression in these regions. Levels of ARO_T and ARO_L mRNA in the frontal cortex were extremely low and no ARO activity was detected in this region.
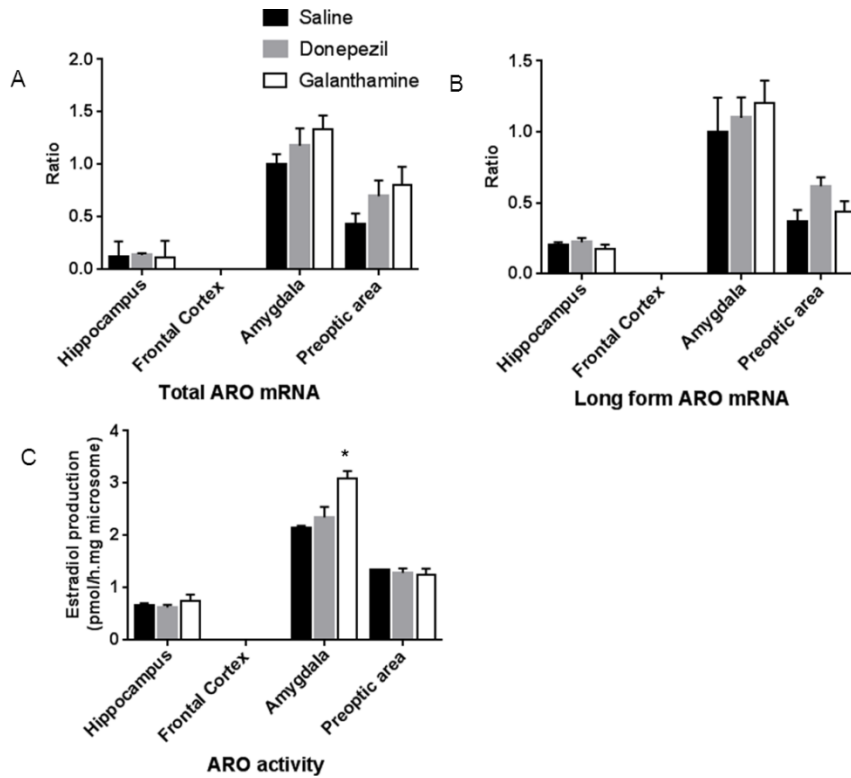
Figure 13: Effect of donepezil (3 mg/kg, daily injection for 7 days) and galantamine (5mg/kg/day for 7 days) on (A) ARO_T mRNA, (B) ARO_L mRNA expression and (C) ARO activity in hippocampus, frontal cortex, amygdala and preoptic regions. Bars in (A) and (B) indicate the mean ratio of total, long form ARO mRNA relative to Amygdala saline treatment controls $\pm$ s.e.m. after normalizing to GAPDH. Bars in (C) indicate the mean ARO activity $\pm$ s.e.m. * indicates the p $\leq$0.05 compared to control group. In the study of ARO mRNA, animal number: N=4 for each group. For ARO activity study, animal number: N=12 for each group.

No significant effects of donepezil or galantamine treatments were observed on relative levels of ARO mRNA in any of the brain regions examined. In the amygdala, mRNAs coding for both total and long form ARO showed slight increases in expression in galantamine (~30 $\pm$ 13 %) treated rats, but this increase was not statistically significant. Slightly larger increases in ARO_T mRNA were observed in the POA following treatment with galantamine (93 $\pm$ 17 %), and in

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ARO_L following treatment with donepezil (55 ± 6 %). These changes also were not statistically significant although the effect on ARO_L in the POA following donepezil treatment was close to significance (p=0.07).

With respect to ARO activity, no significant effects of ChEI treatments were detected in the hippocampus, preoptic area or frontal cortex (Figure 13C). In contrast, ARO activity in the amygdala was 45 ± 0.7 % greater in rats treated with galantamine, relative to controls (p<0.01). Effects of ChEI treatments on levels of ERα, ERß, or GPR30 mRNAs were also detected in the four brain regions (Figure 14).Although we saw an increase of ERα (56 ± 9%,p= 0.41) with donepezile and 50 ± 14% (p=0.33) decrease of GPR30 with galantamine treatment in the HPC, none of them reach statistical significant. Similarly, there were no significant effects of ChEIs treatment on ERα, ERß, or GPR30 mRNAs in any other brain regions.
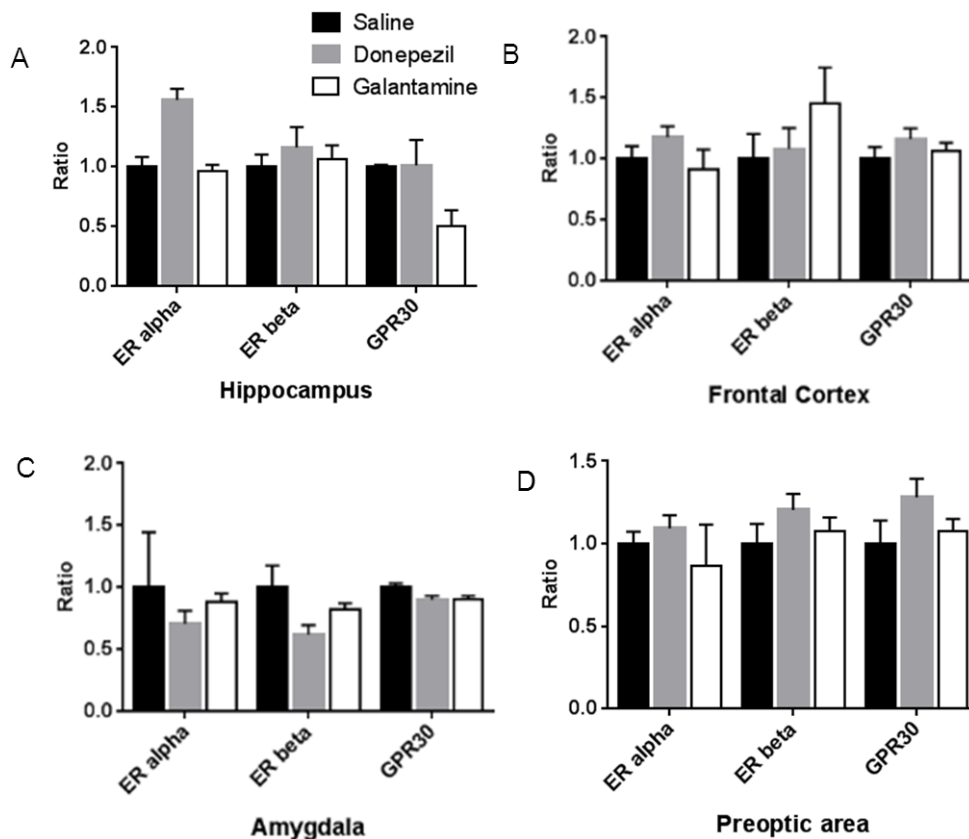
Figure 14: Effect of donepezil and galantamine on ER mRNA expression in (A) hippocampus, (B) frontal cortex, (C) amygdala and (D) preoptic area. Bars indicate the mean ratio of estrogen receptor mRNA relative to saline treatment ± s.e.m. after normalizing to GAPDH. Animal number:N=4 for each group.

4.4 Discussion

4.4.1 Cholinergic lesions

Our goal was to explore the role of cholinergic projections in regulating ARO mRNA, ARO activity, and the expression of ER mRNAs in specific regions of the rat brain. In the first experiment, we tested the effect of selectively removing cholinergic afferents to the hippocampus. Results show that removal of over 80% of the cholinergic afferents had no significant effect on relative levels of ARO_T, ARO_L mRNAs or ARO activity. This indicates that cholinergic inputs to the hippocampus do not play a significant role in regulating ARO and therefore are not likely to affect local estrogen production in this region. Likewise, loss of cholinergic inputs had no significant effect on relative levels of ER α, ERß, or GPR30 mRNAs in the hippocampus. This indicates that cholinergic inputs also do not play a significant role in regulating ER expression in this brain region. Collectively, these data suggest that cholinergic influences on estrogen effects in the hippocampus (e.g., spine density, NMDAR expression, synaptic plasticity) are not mediated by effects on either local estrogen production or by effects on ER expression.

The frontal cortex, which was used as a negative control, also showed no change in ARO or ER expression. As previously reported (Li et al., 2016b), levels of ARO_L in the frontal cortex were very low which is consistent with the inability to detect ARO activity in this region. Our findings show no induction of ARO activity in the frontal cortex as a result of the surgery. In addition, there was not any change in the levels of ARO or ER mRNAs in response to cholinergic denervation of the hippocampus. This shows that the surgical procedures associated with the intracranial injections of 192IgG-SAP or saline were not sufficient to significantly alter ARO or ER expression in the frontal cortex.

The fact that relative levels of ARO mRNA and activity in the hippocampus were not affected by the cholinergic lesions was unexpected. These lesions are robust, and our analysis of ChAT immunostaining and ChAT activity confirm that >80% of the cholinergic inputs to the hippocampus had been lost. Other studies have shown that ARO expression and activity increases in the brain in response to other types of injury such as trauma and stroke (Carswell et al., 2005; Duncan and Saldanha, 2011; Peterson et al., 2007; Pietranera et al., 2011), primarily due to induction of ARO in reactive astrocytes. 192IgG-SAP lesions have been shown to induce a strong activation of microglia and a moderate astrocytic reaction in the hippocampus (Rossner et al., 1995a; Rossner et al., 1995b), but in this case no significant effect on ARO was detected. This suggests that elevated ARO is not a universal response to brain injury, but rather is injury-, and perhaps brain region-specific. It may be that only lesions that produce a strong astrocytic reaction result in upregulation of ARO. It is also possible that the cholinergic lesions resulted in a reduction in ARO in neurons and an increase in ARO in astrocytes, resulting in no net change; however, this has not been observed in response to other lesions. Whether active astrocytes that

are present in the hippocampus following cholinergic lesions express ARO was not investigated and will need to be explored.

4.4.2 Cholinesterase inhibitors

In the second experiment, we tested the effect of two ChEIs, donepezil and galantamine, on ARO expression and activity in four regions of the brain. Donepezil is a piperidine-based mixed, non-competitive reversible inhibitor of acetylcholinesterase. It has an in vitro IC50 of approximately 6.7nM and an in vivo ID50 of approximately 2.6mg (6.8μMol)/kg brain tissue (Sugimoto et al., 2002). Galantamine is a less potent ChEI than donepezil (Bores et al., 1996), but unlike donepezil has been shown to act as an allosteric enhancer at nicotinic acetylcholine receptors (Samochocki et al., 2003; Schilstrom et al., 2007). Doses in the range of 1.5-5.0 mg/kg in rats have been reported to produce optimal brain concentrations for the allosteric potentiating ligand effect of galantamine (Geerts et al., 2005). Previously we showed that daily injections of these ChEIs at the same doses used in the current study were able to enhance estrogen effects on acquisition of a delay matching-to-position T-maze task in aged rats (Gibbs et al., 2011a; Gibbs et al., 2009), and in young adult rats with partial cholinergic lesions (Gibbs et al., 2011b).

In the current study, daily injections of donepezil or galantamine had no significant effects on ARO expression or activity in the hippocampus, frontal cortex, or POA of OVX rats. This suggests that the systemic up-regulation of cholinergic activity does not affect local estrogen production in these regions. Likewise, ChEI treatment had no significant effect on relative levels of ER mRNA in any of the regions examined. We conclude, therefore, that any ability of cholinergic inputs to alter estrogen effects in these regions is not mediated by effects on local estrogen production or ER expression.

One exception to these results was the amygdala where ARO activity was significantly increased in rats treated with galantamine, but not donepezil, despite the fact that relative levels of ARO mRNA were not significantly affected. The amygdala is a central structure in limbic circuitry, which plays an important role in regulating emotional expression, emotional experience, emotional memory, and fear (Gallagher and Chiba, 1996; Pessoa, 2010). It receives cholinergic afferents from basal forebrain cholinergic neurons. These inputs play an important role in regulating plasticity and coordinating different memory systems leading to the selection of appropriate behavioral strategies in conditioned-fear behaviors (Calandreau et al., 2006; Jiang et al., 2016; Nagai et al., 1982). Both muscarinic and nicotinic acetylcholine receptors are located in the amygdala (Muller et al., 2013; Pidoplichko et al., 2013). Human and animal studies show that exposure to nicotine can increase the rate of depression and anxiety in adolescent females (Biegon et al., 2012). ARO also is highly expressed in this region and studies have shown that ARO in the amygdala can influence aggression and mood in both males and females (Li et al., 2016b; Unger et al., 2015). ARO also has been shown to influence synaptic plasticity in the basolateral amygdala in females, but not in males (Bender et al., 2017). Moreover, studies by Balthazart and co-workers have shown that ARO activity can be regulated by phosphorylation in response to changes in intracellular calcium (Balthazart et al., 2001a; Balthazart et al., 2005). Hence the allosteric potentiation of nicotinic receptors by galantamine might account for the increase in ARO activity detected in the amygdala, in the absence of any effect on the levels of ARO mRNA. Further studies may apply different concentrations of galantamine or a selective nicotinic receptors agonist or antagonist to study the effect on ARO activity and perhaps, the effect on local estradiol production in the amygdala and its influence on behaviors.

Collectively, these results show that increasing cholinergic activity by treating with ChEIs has no significant effect on ARO or ER expression in several brain regions, including the hippocampus where cholinergic inputs have been shown to influence estrogen effects on synaptic plasticity and cognitive performance. One exception was the amygdala where galantamine increased ARO activity, possibly affecting synaptic plasticity in the basolateral amygdala with corresponding effects on fear-related behaviors.

4.4.3 Relevance to cognitive decline associated with aging and neurodegenerative disease

This study is the first to systematically evaluate the effects of hippocampal cholinergic denervation, and daily treatment with cholinesterase inhibitors (ChEIs), on ARO and ERs in different regions of the brain. Though many of the results are negative, these findings are important given the evidence for decreased central cholinergic function with aging and in neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Bohnen and Albin, 2011; Ferreira-Vieira et al., 2016; Mufson et al., 2008), as well as the frequent use of ChEIs in elderly individuals with memory impairment. Studies have shown a loss of ER and ER function with age (Foster, 2012), possibly due to methylation of the receptors and degradation by ubiquitin-proteasomal pathway (Pinzone et al., 2004; Tschugguel et al., 2003). There also is evidence for an association between ARO expression/polymorphism and risk for AD (Hiltunen et al., 2006). Decreased ARO expression has been reported in the hippocampus of AD patients (Ishunina et al., 2007a). Iivonen reported that a single SNP in the ARO gene was associated with a 60% increase in AD risk (Iivonen et al., 2004). Baker reported that ApoE4 carriers with SNPs in the ARO gene had a two-fold increased risk of AD (Baker et al., 2006). Given the evidence that ARO and ERs play a role in cognitive decline associated with aging and AD, and given the

frequent use of ChEIs in treating elderly patients with memory impairment, it is significant that we report relatively little acute effect of cholinergic manipulations on ARO or ER expression in several brain regions including the hippocampus.

4.5 Conclusions

Collectively, we showed that manipulating the cholinergic system by selectively destroying cholinergic projections to the hippocampus, or by treating with cholinesterase inhibitors, had little effect on ARO and ER expression in many regions of the rat brain. Galantamine increased ARO activity in the amygdala, possibly due to allosteric potentiation of nicotinic acetylcholine receptors. This raises the possibility that increasing cholinergic activity may increase local estrogen production in the amygdala and thereby affect amygdala function. To our knowledge, this is the first report of an effect of ChEI treatment on ARO activity in the brain. The clinical significance will require further examination.

5.0 TBI Influences the Expression of Aromatase and Estrogen Receptors in the Rat Brain

5.1 Introduction

Traumatic brain injury (TBI) occurs when an external force traumatically injures the brain. It is a major cause of death and disability worldwide, with annual incidence rate of 1.7 million in the USA and 10 million worldwide. It is not only the simple process of physical structural damage, but also secondary injury processes, which involve cell apoptosis and necrosis, inflammatory activity, oxidative stress and change of neurotransmitter release profile, that happen days to weeks after the event (Davis, 2000; Giza and Hovda, 2014; McAllister, 2011; McIntosh et al., 1996; Werner and Engelhard, 2007). Consequences after TBI can be long-lasting, with effects on motor ability, cognitive functions and emotional problems. Interestingly, there are reports showing the difference of recovery outcomes between females and males after TBI, with several well controlled animal studies and clinical studies showing less mortality, better functional outcomes, and histological improvement in females (Berry et al., 2009; Bramlett and Dietrich, 2001; O'Connor et al., 2003; Wagner et al., 2011; Wagner et al., 2004). One possible explanation for the sex difference may be the gonadal steroids and specifically estrogens.

As shown in Chapter 3, estrogens in the brain come from both the systemic circulation and local production in females, whereas it mainly comes from local production in males. Previous studies showed that estrogens have a neuroprotective effect after TBI. Specifically, E2 has been shown to reduce cortical contusion volumes, apoptosis, blood-brain barrier permeability,

edema, levels of pro-inflammatory cytokines, increase cerebral perfusion pressure(CPP), and improve neurological scores and mortality after brain injury (Bramlett and Dietrich; O'Connor et al.; Roof and Hall, 2000b; Sarkaki et al.; Shahrokhi et al.; Soustiel et al.). Recently, increasing literature reports also illustrate that local estrogens have neuroprotective effects against neurotoxicity, stresses, and inflammatory response. Studies showed that the expression and activity of aromatase (ARO) were significantly induced in the brain of both rodents and birds after brain injury (Arevalo et al., 2015). Moreover, the induction of ARO was not only in neurons, but also in the astrocytes, which do not contain ARO under physiological conditions (Garcia-Segura et al., 1999; Peterson et al., 2004). Inhibition of brain ARO increased excitotoxic neuronal death in the hippocampus of male rats and worsened gliosis and neuronal degeneration in songbirds (Azcoitia et al., 2001b; Wynne et al., 2008). In addition, inflammatory response without physical structural damage was reported to induce aromatase expression in astrocytes in a timeline following the induction of IL-6 and IL-1β in the songbirds (Duncan and Saldanha, 2011). Most of these studies, however, used male animals to develop the injury model. Little study has explored the expression of ARO and levels of E2 in TBI models and whether there are sexual dimorphisms.

Estrogens in the brain have neuroprotective effects via binding to estrogen receptors (ERs): ER alpha, ER beta, and GPR30 (Brailoiu et al., 2007; Funakoshi et al., 2006; Moriarty et al., 2006; Toran-Allerand, 2004). ERs are expressed in both neurons and glial cells (Dhandapani and Brann, 2007; Garcia-Ovejero et al., 2005) and can be induced in cell culture of male rat brain tissues after brain injury (Garcia-Ovejero et al., 2002). All three receptors were shown to mediate the neuroprotective function of estrogens through different mechanistic pathways and in a

region-specific manner (De Marinis et al., 2013; Ghisletti et al., 2005; Tang et al., 2014; Yang et al., 2010).

In order to study the heterogeneous nature of TBI, different TBI models in rat have been developed. There are four types of TBI models that are widely used cortical impact injury (CCI) (Dixon et al., 1991), weight drop impact acceleration injury (Marmarou et al., 1994), fluid percussion injury (FPI) (Dixon et al., 1987), and blast injury (Leung et al., 2008). The CCI model uses a pneumatic or electromagnetic impact device to drive a rapid impactor onto the exposed intact dura, and mimic cortical tissue loss, acute subdural hematoma, axonal injury, concussion, blood brain barrier (BBB) dysfunction and even coma. The mechanical factors, such as time, velocity and depth of the impact can be well controlled in this model. Compared with other models, it is highly reproducible and is easy to control the severity of the injury (reviewed by Xiong, et.al (Xiong et al., 2013)).

In this project, we hypothesized that TBI influences local estrogen production and the expression of ERs, which may impact neuronal survival and functional recovery and may account for sex differences in response to TBI. Here we report a pilot study evaluating the expression of ERs and ARO in the cortex (CTX) and hippocampus (HPC) of male and female rats 2 days following controlled cortical impact injury (CCI). Preliminary data reveal a substantial increase in ER alpha mRNA in the injured cortex in both males and females, an increase in long-form ARO (ARO_L) mRNA in the cortex of females but not males, and a substantial decrease in total ARO (ARO_T) mRNA in the cortex of both males and females. In the hippocampus there were no changes in ER alpha mRNA levels, an increase in ER beta mRNA in males but not females, and decreases in both ARO_T and ARO_L in both males and

females. These results suggest significant effects of TBI on estrogen production and signaling in the cortex and hippocampus, as well as sex differences in these effects.

5.2 Materials and methods

5.2.1 Animals

10 young female and 10 young male gonadally-intact Sprague-Dawley rats were housed in standard steel-wire mesh cages and maintained in a temperature ($21 \pm 1°C$) and light controlled (on 7:00 a.m. to 7:00 p.m.) environment with free access to food and water. Both female and male rats were divided into two groups. One group was subjected to a right hemisphere controlled cortical impact. Another group received sham injury. Two days after the injury, rats were anesthetized with Fatal-Plus (Henry Schein Animal Health, Columbus, OH 0.25 mL, intraperitoneally) and brain tissue from cortex (CTX), hippocampus (HPC), amygdala (AMG) and preoptic area (POA) were collected and stored in -80℃ until use.

5.2.2 Controlled cortical impact injury

The controlled cortical impact (CCI) model is a widely used TBI animal model, which is accurate, easy to control and produces traumatic brain injures similar to those seen in human(Romine et al., 2014). The procedure of CCI has been described in previous papers (Dixon et al., 1991; Kline et al., 2001, 2008, 2010, 2012; Bondi et al., 2014a,b). Briefly, isoflurane anesthesia was induced and maintained using a 2:1N2O/O2 gas mix at concentrations

of 4% and 2%, respectively. Rats were subsequently intubated endotracheally, placed on mechanical ventilation for anesthesia maintenance, and secured in a stereotaxic frame. A heating pad was used to maintain core temperature at 37 ℃, which was measured with a rectal probe throughout surgery. Using aseptic procedures, a craniectomy (6-mm in diameter) was performed in the right hemisphere with a dental drill. A TBI was then produced by impacting the exposed right parietal cortex to a depth of 2.8 mm tissue deformation at 4 m/sec to produce a brain injury of moderate severity. Immediately after the CCI, anesthesia is discontinued and the incision is promptly sutured. Sham rats undergo similar surgical procedures, but are not subjected to the impact.

5.2.3 q-RT-PCR

The procedure for detection of ARO_T, ARO_L and ERs has been described in sections 2.2.2 and 4.2.5. The primer pairs used for detection of IL-1β were : (F) 5'-CCTTGTGCAAGTGTCTGAAG-3', (R) 5'-GGGCTTGG AAGCAATCCTTA-3'; The primer pairs used for detection of TNF-α were: (F) 5'-AGCCCTGGTATGAGCCCATGTA-3', (R) 5'-CCGGACTCCGTGATGTCTAAGT-3'. GAPDH was used as the control gene. The relative gene expression was normalized to sham animal left hemisphere using the $2^{-\Delta\Delta Ct}$ method.

5.2.4 Statistical analysis

In this experiement, the effect of TBI on ARO and ER expression were analyzed by one-way ANOVA followed by Tukey's post-hoc test. For the analysis of inflammatory markers, three-way ANOVA was applied with injury side and gender as between factors and brain region

as a within factor. All statistical analyses were done using JMP (Pro12), with significance defined as $p < 0.05$.

5.3 Results

5.3.1 Effect of TBI on ARO mRNA expression in two sexes.

Effects of TBI on relative levels of ARO mRNA are summarized in Figure 15 for males and Figure 16 for females. In males, TBI significantly decreased the levels of ARO_T (0.56 ± 0.08 fold) with no change of the expression of ARO_L in the CTX. In the HPC, the levels of both ARO_T (0.48 ± 0.11 fold) and ARO_L (0.39 ± 0.16 fold) were decreased with TBI injury, with ARO_T showing a statistical significant decrease ($p < 0.05$), whereas the p-value for ARO_L was not quitesignificant ($p = 0.08$). In females, the levels of ARO_T also were decreased (0.45 ± 0.15 fold), whereas the levels of ARO_L were significantly increased (2.23 ± 0.47 fold) in the CTX after TBI on the injury side. TBI also significantly decreased the expression of both the ARO_T (0.38 ± 0.10 fold) and ARO_L (0.60 ± 0.16 fold) mRNAs in the HPC of females. No significant effect of TBI was observed on relative levels of ARO mRNA in the AMG or POA of either sex.
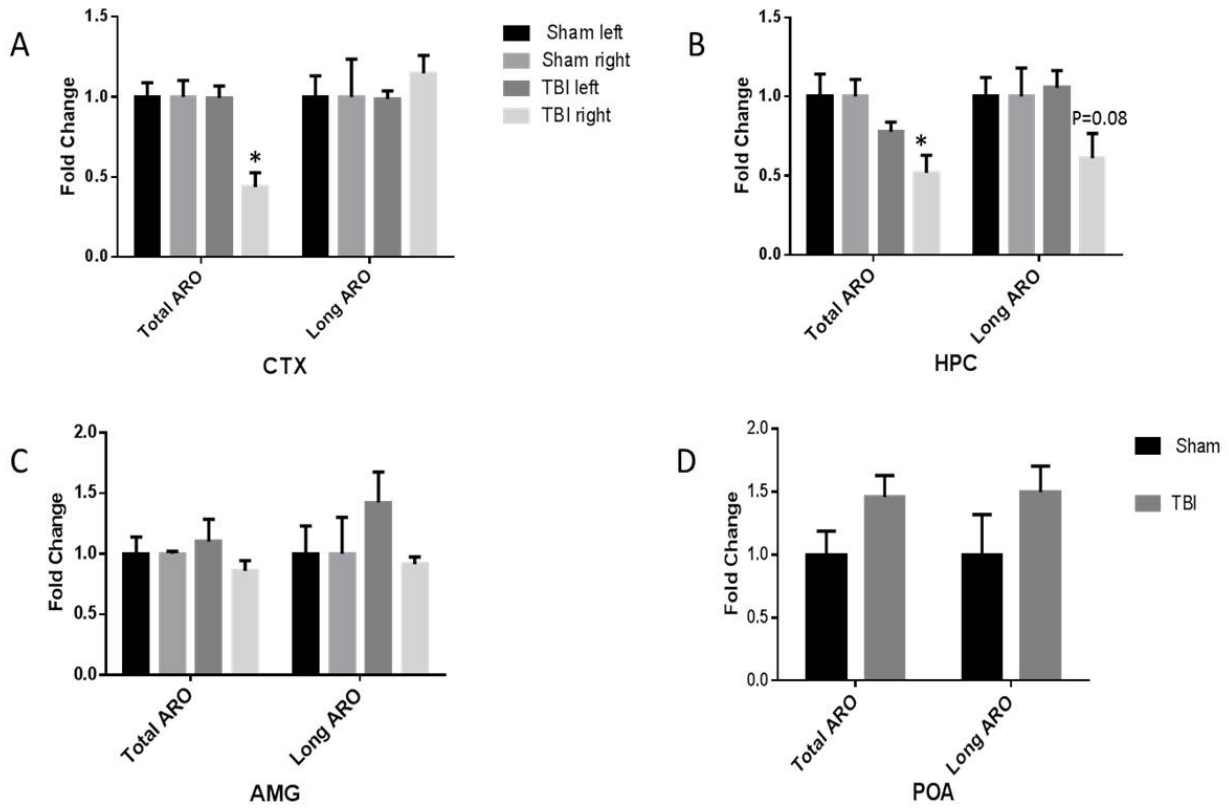
Figure 15: Effect of TBI on Total ARO and Long form ARO expression in the different brain regions of male rats. Bars represent fold change of ARO mRNA levels that were normalized to sham left side GAPDH mRNA ± s.e.m. * indicates significant difference between TBI right side and sham left side ($p \leq 0.05$).
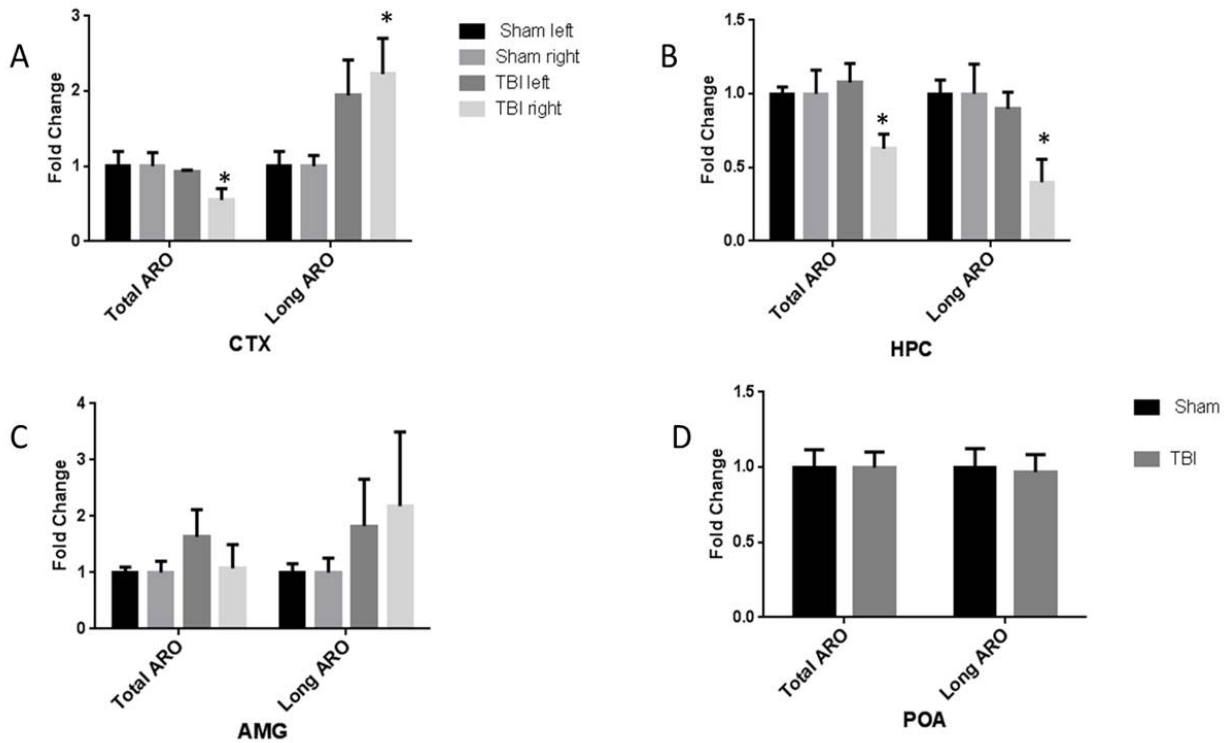
Figure 16: Effect of TBI on Total ARO and Long form ARO expression in the different brain regions of female rats.

5.3.2 Effect of TBI on ER mRNA expression in males and females.

Relative levels of ER mRNAs detected in the CTX and HPC of both sexes are summarized in Figure 17. TBI caused a significant induction of ER alpha expression in the CTX of both males (2.5 ± 0.3 fold) and females (1.6 ± 0.2 fold) compared to sham controls. In the HPC of TBI rats, a significant increase in the expression of ER beta was detected on the injury side compared to the non-injury side in males (1.87 ± 0.14 fold), with no change in females. No significant effects of TBI on relative levels of GPR30 were detected in any region of the brain in males or females.
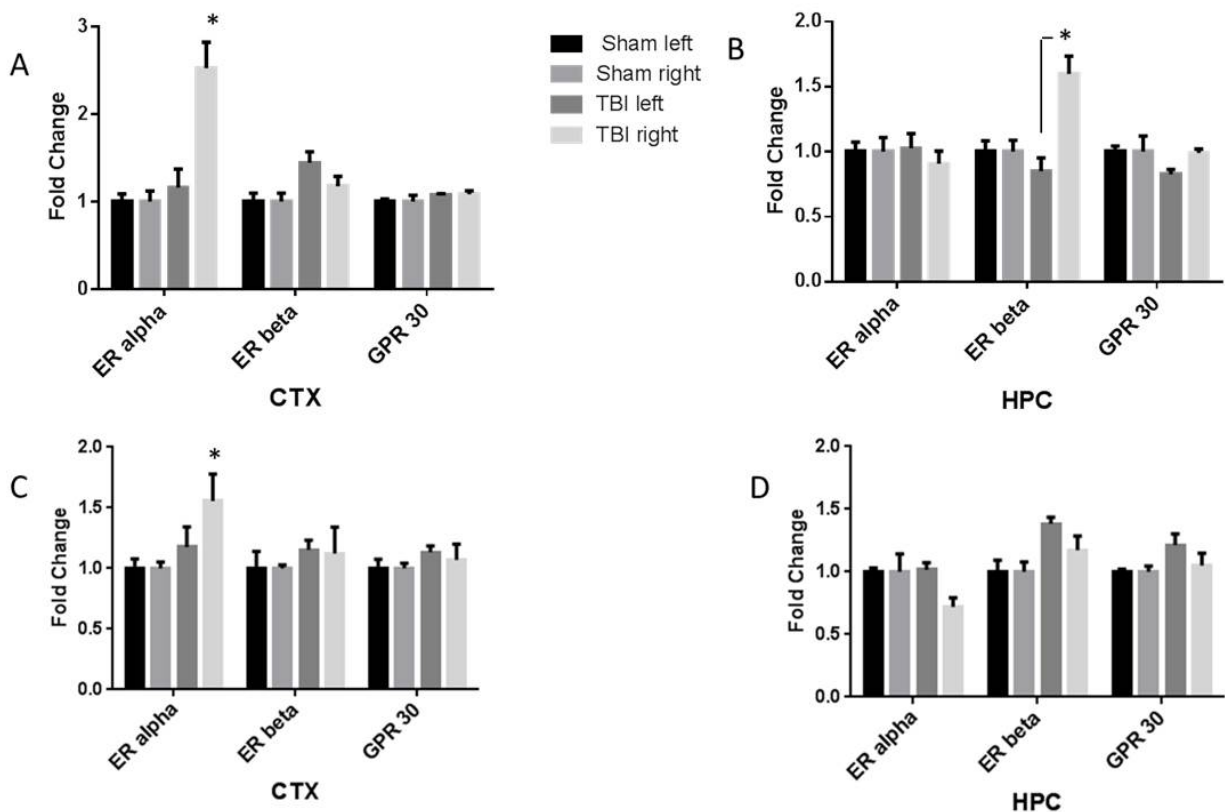
Figure 17: Effect of TBI on ER alpha, ER beta and GPR 30 in the CTX and HPC of male (A and B) and female (C and D) rats. Bars represent fold change of ERs mRNA levels that were normalized to sham left side GAPDH mRNA ± s.e.m. * indicates significant difference between TBI right side and sham left side or TBI left side as indicated in the figure ($p \leq 0.05$).

5.3.3 Effect of TBI on IL-1ß and TNF- α mRNAs in male and female rat brain.

Effects of TBI on relative levels of IL-1β and TNF-α mRNAs in both males and females are summarized in Figure 18. TBI injury significantly induced the expression of the two inflammatory markers in CTX and HPC in both sexes. Specifically, TBI caused a 33 ± 4.8 and 34 ± 5.9 fold increase of IL-1β in the CTX and HPC, respectively of male rats (Figure 18A and

B). Accordingly, the expression of TNF-α increased approximately 8 ± 1.7 fold and 4.7 ± 0.9 fold in these two brain regions in male rats. In females, the induction of IL-1β in the CTX (23.6 ± 4.5 fold) after TBI was less, whereas the increase of IL-1β in the HPC (44.4 ± 6.6 fold) was higher, than in males (Figure 18C and D). Statistical analysis showed a significant difference between regions (CTX < HPC, p=0.01), but no significant difference between sexes. In addition, the TBI injury showed a higher induction of TNF-α in the CTX (11 ± 1 fold) and HPC (9 ± 1 fold) in females. Statistical analysis showed a significantly higher level of TNF-α mRNA in the hippocampus in females. There were slight increases in the two markers in the TBI non-injury side for all regions in both sexes, but none were statistically significant.
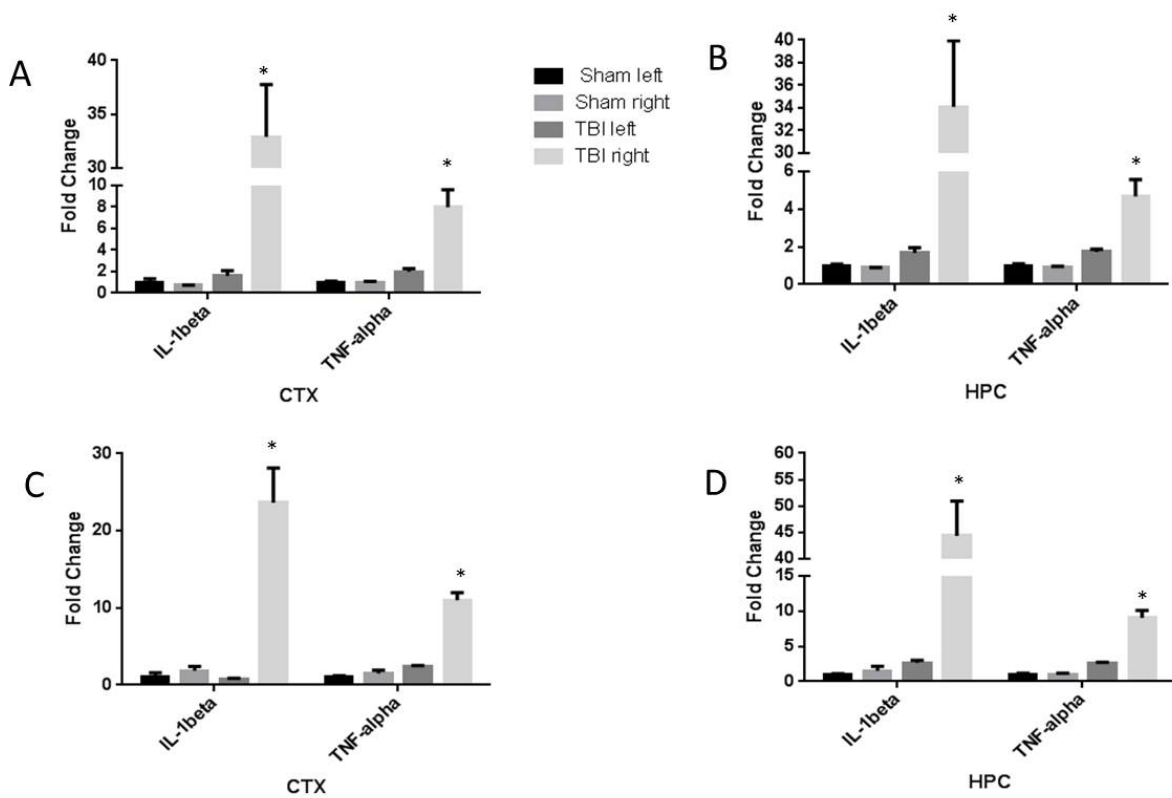
Figure 18: Alteration of IL-1beta and TNF-alpha in the CTX and HPC after TBI of male (A and B) and female (C and D) rats. Bars represent fold change of IL-1beta and TNF-alpha

mRNA levels that were normalized to sham left side GAPDH mRNA ± s.e.m. * indicates significant difference between TBI right side and other side or treatment ($p \leq 0.05$).

5.4 Discussion

Our goal was to explore the altered expression of ARO and ER mRNAs in the brain in response to a moderately severe traumatic brain injury (TBI). We showed that in the cortex, the expression of ARO_T was significantly decreased in both males and females, whereas the ARO_L mRNA was increased in females but not males. As shown in our previous studies, the expression of ARO_L is correlated with ARO activity; this result indicates that ARO activity likely increases in female rats after TBI, whereas no change occurs in males. Moreover, the down-regulation of ARO_T was accompanied by either no-change or an increase in ARO_L, suggesting that the decreased ARO_T mRNA level is due to a decrease in ARO short form, which has no known function. In the hippocampus, both the ARO_T and ARO_L were decreased in males and females, indicating that there may be a reduction in local estrogen production in this region. Further studies are needed to characterize the ARO activity and E2 level change in the brain after TBI.

A substantial increase in ER alpha mRNA in the injured cortex in both males and females, indicates that there may be an enhancement of estrogen signaling in cortex through ER alpha after TBI. In the hippocampus there were no changes in ER alpha mRNA levels, but an increase in ER beta mRNA in males but not females. This demonstrates that after TBI, the expressions of ERs are induced in a region-specific and sex-specific manner.

The roles that ERs play to mediate the neuroprotective functions of estrogens have been studied for decades. Three major pathways were discussed previously: 1) Activation of extracellular signal-regulated kinases 1(ERK1), ERK2 and PI3K to inhibit the pro-apoptotic Jun amino-terminal kinase (JNK) signals (Tang et al., 2014; Yang et al., 2010; Zhao and Brinton, 2007). 2) Upregulation of the expression of anti-apoptotic BCL-2 family members, such as BCL-X and BCL-W and down-regulation of the expression of pro-apoptotic BCL-2 families, such as BAD and BIM (Garcia-Segura et al., 1998; Yao et al., 2007). 3) Phosphorylation and inhibition of glycogen synthase kinase 3 beta (GSK3beta) and reduction in Tal phosphorylation and regulation of the activity of lymphocytes (Cardona-Gomez et al., 2004; D'Astous et al., 2006; Mendez and Garcia-Segura, 2006). Except for the common pathways, ER alpha also has been shown to have anti-inflammatory effects by blocking the activation and translocation of NF-kB to the cell nucleus (Ghisletti et al., 2005). ER beta has been shown to have anti-inflammatory effects by altering inflammasome activity and upregulating neuroglobin to protect neurons from hypoxia, glucose deprivation, oxidative stress and beta-amyloid (De Marinis et al., 2013). Different ERs were induced in the different brain regions of TBI animals. This indicates that the estrogen signaling was enhanced after the injury, which may contribute to neuroprotective effects of E2.

We also measured the alteration of inflammatory response after TBI in the cortex and hippocampus of both sexes. Two inflammatory markers: IL-1β and TNF-α were measured as these markers were prevalent in use to indicate the inflammation status in brain injury (Feuerstein et al., 1994; Longhi et al., 2013; Murray et al., 2015) and response to estrogen treatment (Johnson et al., 2006; Loram et al., 2012; Smith et al., 2011). Our results showed that both IL-1β and TNF-α mRNA expression were increased on the TBI injury side in both females

and males, indicating that there is an induction of inflammatory reaction after TBI. Interestingly, we also showed significantly higher levels of IL-1β mRNA in the HPC than CTX of females, but not males. Moreover, the level of TNF-α mRNA in the HPC of females was significantly higher than in males. These results suggest that the inflammatory reaction in the HPC of females was higher than that in other regions or in males. Along with the effects on ARO and ER mRNA expression, these results suggest that the induction of estrogen signaling may contribute to reduced inflammation after TBI. This result is in accord with our hypothesis that estrogen signaling plays a neuroprotective role in response to TBI.

In addition, studies showed that TBI would induce microglia activation after injury. Microglia serves as the main source of active immune defense in the CNS (Filiano et al., 2015). It has been shown that following TBI, microglia migrate to the site of injury and remove cellular and molecular debris (Faden et al., 2016). Activation of microglia also triggers the release of pro-inflammatory cytokines, reactive oxygen species (ROS), nitrogen species and excitatory neurotransmitters (Kreutzberg, 1996). Notably, cytokines have been shown to down-regulate the expression of cytochrome P-450 enzymes in hepatocytes (Abdel-Razzak et al., 1993). Both the mRNA levels and activities of CYPs (CYP1A2, CYP2C, CYP2E1, CYP3A) in hepatocyte primary cultures were decreased by more than 40% after 3 days treatment with IL-1β and TNF-α. In the brain, intracerebral injection of IL-1β and TNF-α was shown to induce astrogliosis in neonatal rats (Cai et al., 2004). Local induction of IL-1β and IL-6 by phytohemagglutinin (PHA, an inflammatory agent), was shown to induce ARO expression in astrocytes of song birds (Duncan and Saldanha, 2011). Although there was only few study done in the brain to test the effect of cytokines on brain CYP expression and activation, it is possible that the release of cytokines from microglia after TBI will influence the expression of ARO. Moreover, different

degrees of inflammatory responses may cause different degrees in the effects on ARO and ER

expression in males and females. These potential causal relationships need further study.

5.4.1 Sex differences in recovery after TBI

The sex differences in outcomes after TBI have been studied in both humans and animals.

However, the results are very complex and it is hard to conclude which sex has better recovery.

A study in rats using the Marmarou impact-acceleration head injury model showed a better

survival and recovery of cortical blood flow in females than males (Roof and Hall, 2000a).

Diffuse TBI induced in adult males and females showed no difference in mortality between the

sexes, but a better motor and cognitive outcome in females (O'Connor et al., 2003). Whereas in a

fluid percussion injury model study, E2 injection significantly improved the recovery of free

magnesium concentration and motor function in males, but not in female rats. Moreover, the

mortality in females was significantly worse than that in males (Emerson et al., 1993). Similar to

animal studies, the conclusions of sex effects on TBI outcomes in humans are controversial

(Farace and Alves, 2000; Slewa-Younan et al., 2004). The inconsistency of sex effects on TBI

recovery may be due to the different models, degree of the injury and outcome measurements.

Using the same CCI model as in our experiments, Wagner et al. showed better motor

function in females than in males, but no sex difference on the cognitive function tested by

Morris water maze (Wagner et al., 2004). Our results suggest a potentially higher induction of E2

signaling in the cortex (including motor cortex), whereas less E2 signaling in the hippocampus (a

region important for cognition) of female rats. This is in the same trend as the result shown by

Wagner et al., indicating that estrogens may contribute to sex differences in recovery after CCI.

5.4.2 Significance

The neuroprotective effects of estrogens after TBI have been studied for decades (Bramlett and Dietrich; O'Connor et al.; Roof and Hall, 2000b; Sarkaki et al.; Shahrokhi et al.; Soustiel et al.). Most studies have investigated the effects of systemic treatment with estrogens or with selective ER agonists and antagonists. Although systemic estrogen treatment was shown to be neuroprotective, there I much concern about adverse side effects. In addition to some common side effects, such as headaches, nausea, weight gain etc., estrogen replacement treatment (ERT) also increases the risk of stroke and specific cancers. Our primary interest, however, is in the therapeutic potential of local endogenous estrogen production. This is particularly important to menopausal women or men, as the main source of brain estrogen is from local production. Moreover, we would like to measure directly the level of E2 in the brain and explore its correlation with injury outcomes instead of using the circulating E2 level or ARO activity as indicators. This may help us better understand the effect of estrogen on the injury between sexes and provide us an alternative to ERT for the beneficial effect on brain disease.

5.4.3 Future direction

In this pilot experiment, we chose two days after TBI as our sample collection time point, as prior studies showed that the inflammation response would reach its peak approximately 2 days post-injury (Lagraoui et al., 2012). Our results were in accordance with other reports and showed a huge induction of IL-1β and TNF-α in response to the TBI injury. Studies also have shown that the up-regulated inflammatory response was sustained for 21 days following the injury and later returned to baseline (Lagraoui et al., 2012). Whether the expression of ARO and

ER also changes with time or maintains a sustained level following injury is unknown. It will be important to characterize the effect and to correlate with neuroprotective effects of local E2. This will require characterizing changes in inflammatory markers, ARO and ER expression at different time points after injury.

Although we showed alterations of ARO and ER mRNA levels after TBI, further experiments are needed to explore physiological significance of the results. Firstly, we showed a down-regulation of ARO_T in the CTX of both sexes, an up-regulation of ARO_L in the cortex of females and decrease of ARO_L in the HPC of both sexes. Whether these changes influence ARO activity and local estradiol production need to be tested. Moreover, the signaling of E2 mediated through ER needs to be further explored by measuring the ER protein levels, receptor-ligand binding affinities and functional ativation. The contribution of ERs in different brain regions to neuroprotective effects also needs to be further studied using selective ER agonists and antagonists.

We hypothesize that TBI influences the local estrogen production and the expression of ERs, which may impact inflammatory response, neuronal survival and functional recovery and may account for sex differences in response to TBI. In order to test the neuroprotective effects of local estradiol production, manipulations to down-regulate ARO or up-regulate E2 levels specifically in the brain need to be performed, along with effects on inflammation, cell-apoptosis, oxidative stress, etc. and motor, cognitive, and mental behaviors. In order to down-regulate ARO in the brain, a selective ARO inhibitor can be injected intracerebrally. Currently there is no way to pharmacologically up-regulate ARO in the brain. Such a tool would be very valuable for investigating the potential neuroprotective role of local estrogen production following brain injury. Recently, a prodrug 10beta,17beta-dihydroxyestra-1,4-dien-3-one (DHED), which was

reported to be converted by a short-chain NADPH-dependent dehydrogenase/reductase specifically expressed in the brain and to selectively deliver estradiol to the brain, has been described (Prokai et al., 2015). This may provide a novel tool and potential clinical approach in the treatment of estrogen-responsive brain disorders without producing elevated levels of E2 systemically.

We also showed sex differences in the expression of ARO, ERs and inflammatory markers. This may account for the variations of recovery outcomes between males and females. However, what accounts for the sex dimorphism: genomic or hormonal factors are still unknown. Moreover, what the mechanisms are for E2 signaling to influence the outcomes differently between sexes need to be further studied.

6.0 SUMMARY AND PERSPECTIVES

6.1 Key Research Findings

The purpose of this research was to investigate the production of local estradiol and the expression of estrogen receptor mRNAs in different brain regions, and to characterize their regulation up or down under different pathological conditions. In order to quantify the low levels of aromatase (ARO) activity in the adult rat brain, a microsomal based assay was developed and validated with high sensitivity, specificity and reliability. Using this method we demonstrated a correlation between ARO activity and its long form mRNA, and verified that their distribution is in accord with previous studies.

In addition to the measurement of the ARO enzyme, which is the final step in the conversion of testosterone to estradiol (E2), we also developed and validated a UPLC-MS/MS method to directly measure E2 levels in different regions of rat brain. In this study, we showed that E2 levels in brain regions with ARO are higher than that in serum in OVX rats treated with different doses of estradiol benzoate and testosterone propionate. Letrozole treatment eliminated the differences between brain and serum E2 levels, demonstrating that the differences were due to local E2 production. Moreover, we also showed that male rats have substantial levels of E2 in specific regions of the brain even though levels in the systemic circulation were barely detectable. This study, to our knowledge, is the first systematic study which characterizes the relationship

between circulating levels of E2 in relation to levels in specific regions of the adult mammalian brain.

In Chapter 4, we utilized the ARO assay that was developed in Chapter 2 to investigate the effect of manipulating the basal forebrain cholinergic system on levels of ARO and the expression of estrogen receptors in OVX rats. By using the two strategies, selective cholinergic lesions in the medial septum and daily injections of cholinesterase inhibitors (ChEIs), to manipulate the cholinergic system, our results showed that neither up-regulating or down-regulating the cholinergic system regulates ARO or ERs in the cortex and hippocampus. To our knowledge, this is the first report looking at cholinergic effects on ARO and ERs in these regions. We showed that galantamine did significantly increase ARO activity in the amygdala, which may suggest that cholinergic input regulates the function of the amygdala by the regulation of local estrogen production.

In Chapter 5, we explored the effects of traumatic brain injury (TBI) on ARO and ER expression and compared that between males and females. In the cortex, we saw a significant increase of ARO_L in females and a significant increase in ER alpha in both sexes, indicating an induction of estrogen signaling in the cortex after TBI. In the hippocampus, the ARO_L expression was significantly decreased in both sexes with an increase in ER beta mRNA in males. These results demonstrated that TBI changes ARO and ER expression in a region- and sex-specific manner, which indicates a change of local estrogen production and its signaling after TBI. These may impact neuronal survival and functional recovery, and contribute to sex differences in effects of TBI.

6.2 Clinical Significance and future directions

Our goal was to study the role and regulation of local estrogen production in the brain. This knowledge combined with the prior knowledge of systemic estrogen treatment provides us with a better understanding of the effects of estrogen on brain function, and hopefully help us develop novel and effective hormonal therapeutic strategies for brain diseases. Although estrogens have shown beneficial effects in many preclinical studies, so far no hormone replacement treatment is used clinically to treat brain disease. One gap is that in several clinical studies treatment groups showed non-positive effects compared to placebo groups as mentioned in Chapter 1. Note the patients in these clinical trials were mostly postmenopausal older women or breast cancer patients treated with ARO inhibitors, whereas in many preclinical studies young adult female or male animals were usually used as models. Better animal models to mimic the characteristic of clinical patients are needed to explore the role of E2 in the brain. Moreover, direct measurement of brain or CSF E2 levels rather than systemic levels as indicators in preclinical studies may be a better way to depute the relationship between E2 and outcomes.

Another main concern is the side effects of estrogen replacement therapy (ERT). In addition to some common side effects, such as headaches, nausea, weight gain etc., estrogen replacement treatment (ERT) also increases the risk of stroke and cancers. In 2002, a large study published by Women's Health Initiative (WHI), which involved more than 16,000 healthy women, showed that the combination of estrogen and progestin increased the risk of breast cancer, heart disease, stroke, and blood clots. Although the study concluded that using estrogen alone has no increasing risk of breast cancer or heart disease, it did appear to increase the risk of stroke.

Because of the previous disadvantages of systemic hormonal replacement treatment, studying the way to manipulate local estrogen production in the brain may provide a much safer and efficient way to achieve the beneficial effect of estrogen in brain disease, particularly for psychiatric disorders, such as autism spectrum disorders, attention-deficit/hyperactivity disorder, anxiety, depression, and Alzheimer's Disease (Cantwell, 1996; Mielke et al., 2014; Ramtekkar et al., 2010; Werling and Geschwind, 2013), which are sexually dimorphic in prevalence, Moreover, research in the roles of systemic and local E2 in female and male will help in determining whether the sex is significant factor in decision making for clinical study design.

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