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Detection of estradiol in rat brain tissues: Contribution of local versus systemic production

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ABSTRACT

Estrogens play important roles in regulating brain development, brain function, and behavior. Many studies have evaluated these effects using ovariectomized (OVX) rats or mice with different doses of estrogen replacement, assuming that estradiol levels in all regions of the brain are the same as levels achieved in the serum. It is well known, however, that the brain contains all the enzymes necessary to produce estrogens, and that estrogen levels in the brain are determined by both systemic and local production and are region-specific. The present study conducted a detailed analysis of the relationship between systemic levels of 17-β-estradiol (E2) achieved by estrogen replacement and levels achieved in specific regions of the brain. Levels of E2 were measured 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. Results confirmed significantly higher levels of E2 in the brain than in serum in brain regions known to contain aromatase (ARO) activity, both in OVX controls and in rats treated with physiological doses of EB. Additional studies compared the level of E2 and testosterone (T) in the brain and serum between testosterone propionate (TP) treated OVX and male. This demonstrated higher levels of E2 in certain brain regions of males than in TP treated OVX females even though T levels in the brain and serum were similar between the two groups. Studies also demonstrated that the differences between serum and brain levels of E2 can be eliminated by letrozole (ARO inhibitor) treatment, which indicates that the differences are due to local ARO activity. 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.

1. Introduction

Estrogens have important effects on brain structure, function and behavior. Estrogens influence not only reproductive behaviors, but also affect mood, cognitive performance, synaptic plasticity, neurodegenerative disease, and functional recovery following brain injury (Erickson et al., 2007; Garcia-Segura, 2008; Gibbs, 2010; Greene, 2000; Sherwin and Henry, 2008; Walf and Frye, 2006). Estrogens are derived from cholesterol, which is converted in the mitochondria to 17α-hydroxypregnenolone and then is transferred to smooth endoplasmic reticulum, converted to dehydroepiandrosterone and then to androgens (androstenedione and testosterone) (Boron and Boulpaep, 2003; Melmed, 2016). Estrogens are produced from androgens by the enzyme aromatase (ARO). 50 years ago it was believed that all estrogens in the brain were due to passive diffusion from the peripheral circulation (Banks, 2012). In 1974, ARO was detected in the brain (Naftolin et al., 1971, 1975; Weisz and Gibbs, 1974). More recent studies have shown that all of the enzymes necessary for estradiol (E2) production are present in the brain and support region-specific estrogen production (Fester et al., 2011; Rossetti et al., 2016).

ARO (CYP19A1) is the cytochrome P450 enzyme responsible for the ultimate conversion of testosterone (T) to E2, and androstenedione to estrone (Naftolin et al., 1971, 1975). ARO expression and activity have been studied in the brains of adult fish, birds, and mammals (Balthazart et al., 2009; Cheshenko et al., 2007; Roselli et al., 1997, 2009). The regulation of ARO activity has been particularly well studied in birds (Balthazart et al., 2001a,b, 2003a, 2005) in relation to its role in seasonal song production (Fusani et al., 2000; Riters et al., 2000; Wacker et al., 2010) and reproductive behaviors (Balthazart et al., 2009). ARO is present in specific regions of the brain. In adult mammals, ARO activity is highest in the amygdala (AMG), followed by the bed nucleus of the stria terminalis (BNST) and preoptic area (POA) (Roselli and Resko, 1991; Roselli et al., 1998). Several other brain regions including the hippocampus (HPC) and septum also contain ARO activity, but at lower levels (Li et al., 2016; Roselli et al., 1997; Tabatadze et al., 2014). Moreover, ARO is found in the brains of both males and females, and

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enzyme activity in males is reportedly higher than that in females (Roselli et al., 1985).

Since the discovery of ARO in the brain, the effect of local estrogen production on brain structure and function has received increasing attention. Previous studies showed that ARO in the preoptic area played essential roles in masculinization of the male brain during prenatal development and in regulating male sex behaviors in adults (Balthazart et al., 2009; Garcia-Segura, 2008; Lephart, 1996; Roselli et al., 2009; Simpson and Davis, 2001; Stocco, 2012). Studies also showed that ARO via local estrogen production can affect the GnRH/LH surge in females and can affect neuronal plasticity in the HPC and AMG with corresponding effects on behavior (Bender et al., 2017; Fester and Rune, 2015; Kretz et al., 2004; Terasawa and Kenealy, 2012; Unger et al., 2015; Vierk et al., 2014). Studies also demonstrated that local estrogen production can have a significant impact on neuronal function, not only under normal physiological conditions but also in response to injury and disease (Carswell et al., 2005; Duncan and Saldanha, 2011; Garcia-Segura, 2008; Peterson et al., 2007). 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., 2016). 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.

2. Materials and methods

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, ~250 g) and sixteen gonadally intact male (~300 g) 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 h:12 h light/dark schedule with unrestricted access to food and water prior to use. Three experiments were conducted (Fig. 1). 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 6 mg Xylazine:30 mg 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, 0.5 mg/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 (4 mg/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 vehicle, 1 μg EB, 2.5 μg EB, or 0.5 mg TP). Males received sesame oil. 20–24 h after the last treatment rats were killed, trunk blood was collected, serum was harvested, and brains were processed for analysis.

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 (~50 mg) 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 (~80 mg) corresponding to plates 25–33 was dissected by cutting from the optic tract horizontally through the rhinal fissure, and then dissecting amygdala apart from surrounding cortex. CTX (~150 mg) included tissues from frontal cortex areas 1–3. HPC (~180 mg) from each side and cerebellum (~250 mg) were collected whole. Serum also was collected and stored for analysis of circulating hormone levels. All tissues were stored at −80 °C until use.

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 (0.12 M, 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., 2016). Briefly, samples were spiked with internal standard 25 μl 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 4770 × *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 37 °C 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 60 °C for 3 min. Samples were centrifuged again for 1 min at 490 × *g*, and the supernatant (~0.1 ml) 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 eluted using a Waters Acquity UPLC BEH C18, 1.7 μm, 2.1 × 150 mm reversed-phase column, with an acetonitrile: water (0.1% formic acid) gradient. MS Detection and quantification were achieved in the positive mode. Transitions used for analysis were 506 → 171 for E2, and 511 → 171 for the deuterated internal standard. Tests show that this assay can reliably distinguish 17-β-estradiol (E2) from 17-α-estradiol and estrone, based on retention time. To calculate E2 level in the tissue and compare it with the serum level, we equated 1 g tissue to 1 ml water as described by Kato et al. (2013) and McIlwain (1985). The E2 concentration in both brain tissue and serum were calculated as pmol/ml. Inter-day and intra-day precision and accuracy of this assay have been described (Li et al., 2016) and are within acceptable limits. The limit of detectability for this assay is 0.009 pmol/ml.

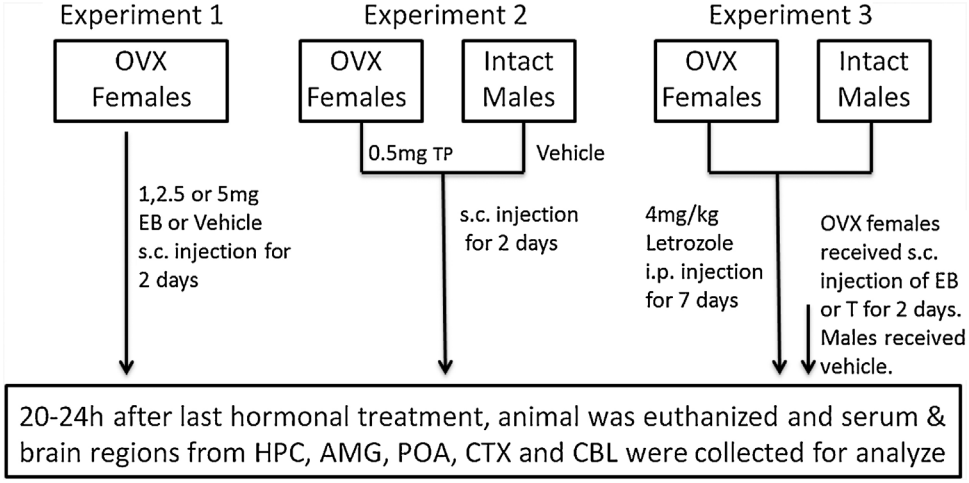

Fig. 1. Flow diagram showing the experimental design. OVX: ovariectomized, HPC: hippocampus, AMG: amygdala, POA: preoptic area, CTX: cortex and CBL: cerebellum.

Testosterone (T) levels in serum and brain homogenates were quantified by a modification of the method described by 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 × *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 to 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.

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., 2016). 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.

2.4. Statistical analyses

Data in Figs. 2, 4 and 5 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: $Y = \text{Bmax}*X/(Kd + X)$) 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

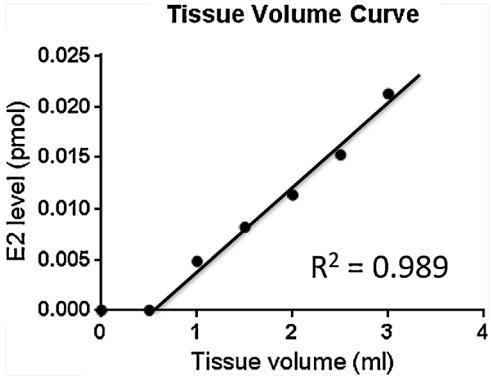

Fig. 2. Plot illustrating linear detection of E2 as a function of volume of whole brain homogenate. Each point represents a measurement of sample pooled from three animals brain tissue.

Table 1
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
Estradiol (E2)	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
	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

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. Results

3.1. Estradiol method validation

Extraction recovery: Table 1 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 (1 mg 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.

E2 level as a function of tissue volume: Fig. 2 shows the relationship between quantity of tissue homogenate and E2 level detection. A linear relationship was observed for tissue homogenate volume ranging from 500 to 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.

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

Fig. 3A illustrates the dissection of each brain region according to the atlas of Paxinos and Watson (George Paxinos, 1997). Fig. 3B 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 × 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 < 1 µg EB < 2.5 µg EB < 5 µg 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.

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. 4). 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. 4A, B and 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. 4A–C). For CTX and CBL, the *Y*-intercept was 0, consistent with no local E2 production (Fig. 4D and E).

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.

3.4. 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 2). 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

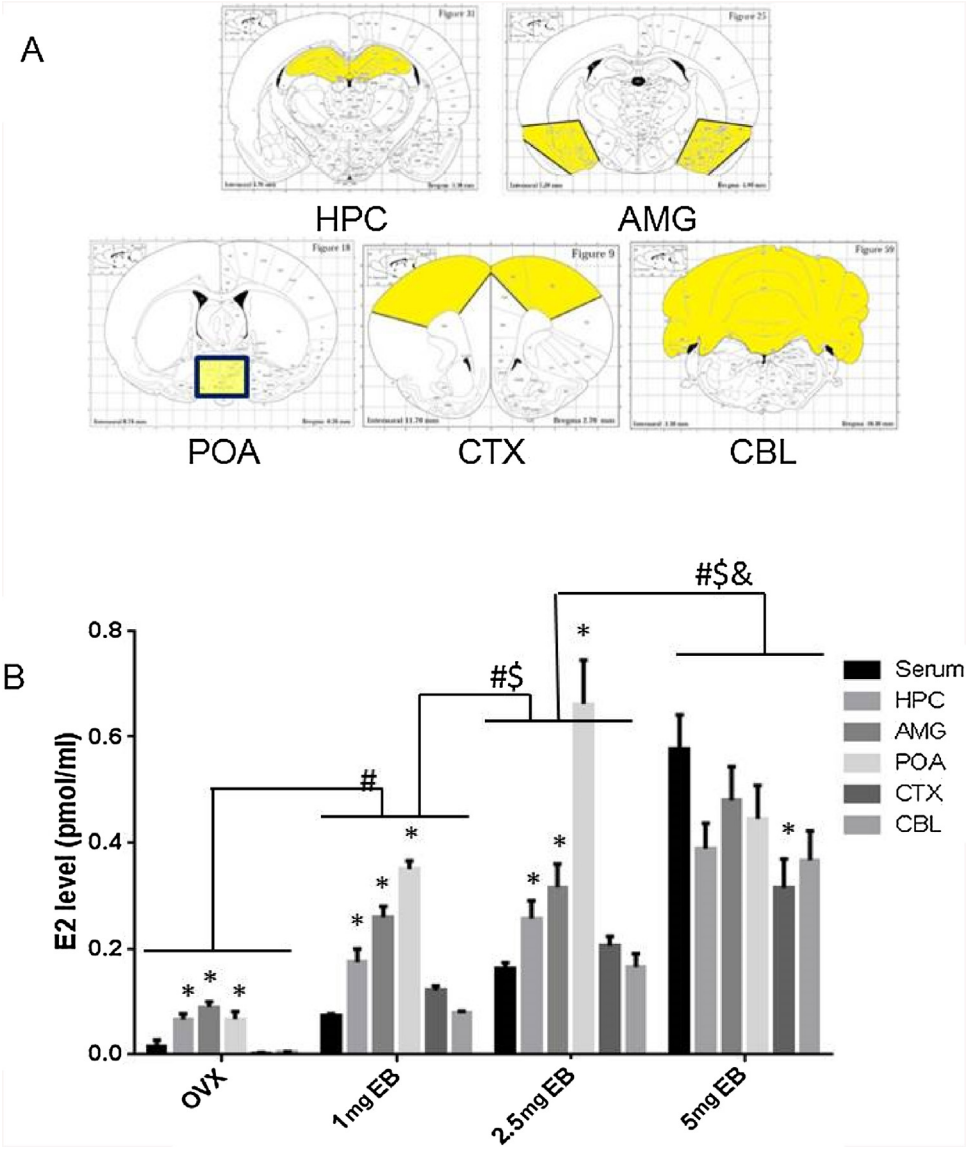

Fig. 3. (A) Illustrations of each brain region dissection (shown in yellow) corresponding to plates from Paxinos and Watson (1986). B) Comparison of E2 levels in the different brain regions and serum after different doses of EB treatment. Each bar represents the mean concentration of E2 ± s.e.m. # indicates significant difference ($p < 0.05$) in E2 detected in rats treated with different doses of EB treatment compared with OVX controls. $ indicates significantly higher ($p < 0.05$) levels of E2 compared with 1 μg EB treatment group. & indicates significantly higher ($p < 0.05$) levels of E2 compared with 2.5 μg 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 ($p = 0.059$).

E2 levels in the TP-treated OVX rats, OVX controls, and vehicle-treated males are shown in Fig. 5. 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 2.5 μg/day EB (compare Fig. 3B with Fig. 5). 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. 5).

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.

3.5. Effects of letrozole

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. 6. 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

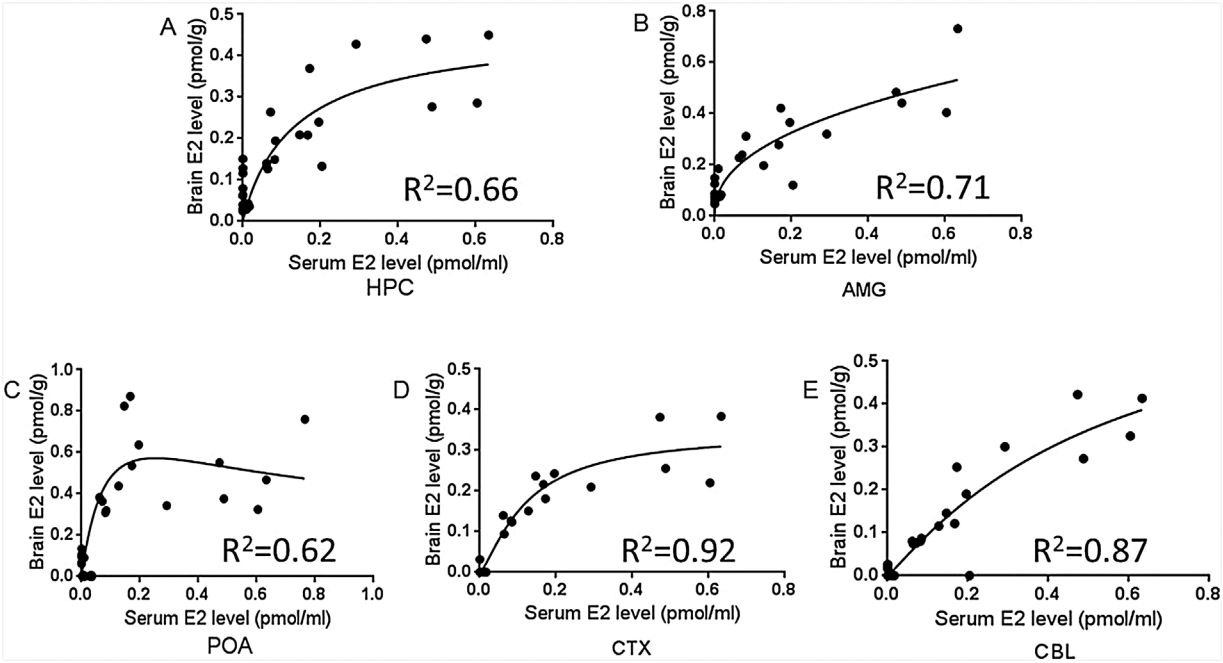

Fig. 4. Correlations between E2 levels in serum vs. brain in the different brain regions. Each data point represents data from one animal. Non-linear least-squares regression (Hill equation) was used to analyze the relationship between serum E2 and brain E2 levels in each region of the brain.

Table 2
Testosterone levels in the serum and in different brain regions in OVX, 0.5 mg TP-treated OVX, and gonadally intact male rats. Values indicate the mean concentration of T ± s.e.m.

	T level (pmol/ml)		
	OVX	0.5 mg 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

$N = 4$ for each group.
* 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.

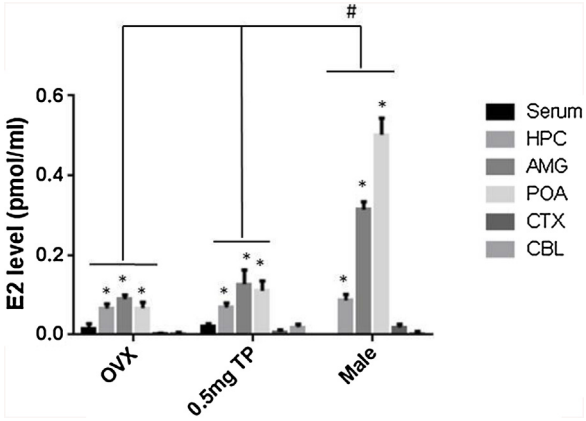

Fig. 5. Effect of TP on E2 levels in the different brain regions vs. serum and comparison with males. * indicates significant difference ($p < 0.05$) in E2 level in the brain region relative to serum. # indicates that E2 levels in males were significantly higher ($p < 0.05$) than in TP treated OVX rats and OVX controls. $N = 12$ for OVX; $N = 10$ for 0.5 mg TP; $N = 6$ for Males.

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. 6A and B), and in the POA of rats treated with 2.5 μg EB ($p < 0.02$, Fig. 6C). Letrozole also appeared to lower E2 levels in the POA of OVX, 1 μg 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 1 μg 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. 6B). 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. 6C).

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.

3.5.2. 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. 7. 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

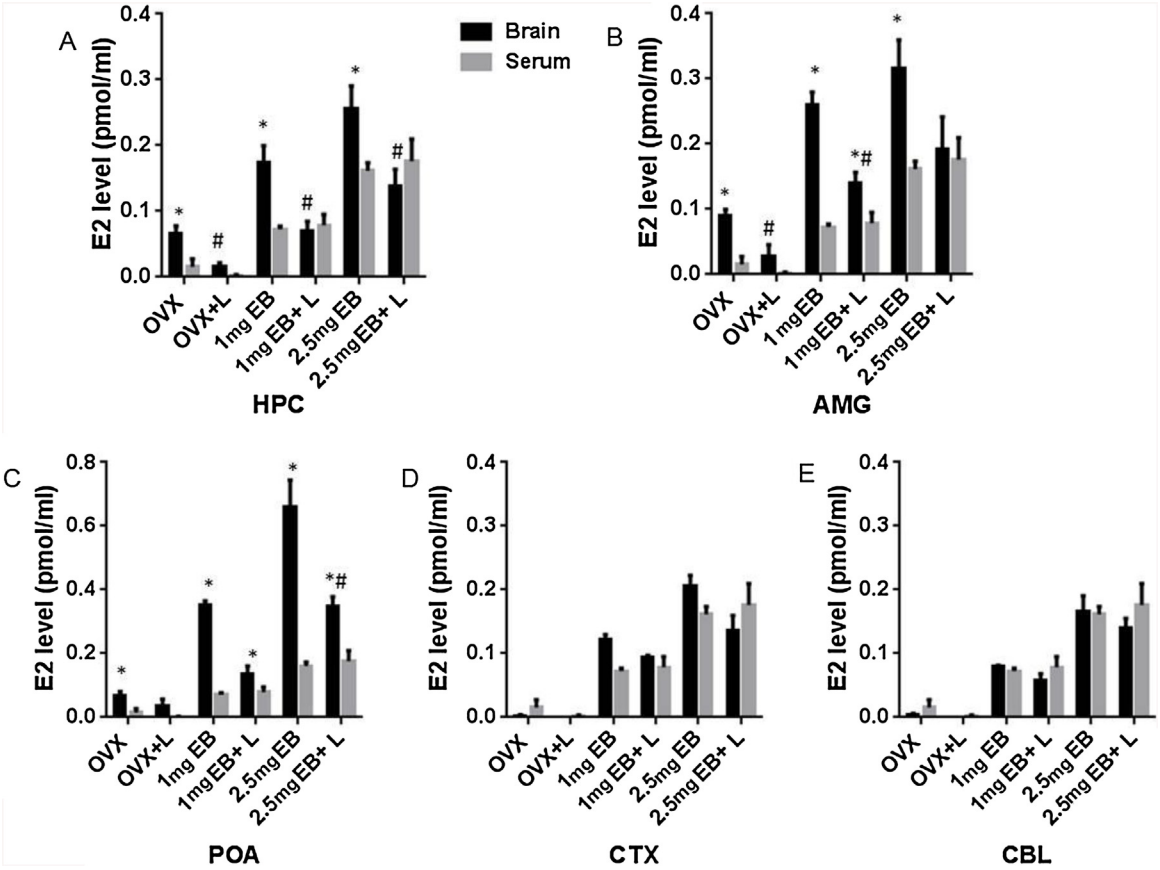

Fig. 6. Comparison of E2 levels in brain vs. serum after different dose of E2 and letrozole in (A) HPC, (B) AMG, (C) POA, (D) CTX and (E) CBL. * indicates significant difference $p < 0.05$ between brain and serum levels. # indicates significant difference $p < 0.05$ in brain levels between letrozole 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.

they were no longer different from levels in serum (Fig. 7B and 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. 7A). No significant effect of letrozole on E2 levels was detected in the CTX or CBL (data not shown).

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.

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., 2016), the levels of E2 significantly exceeded those present in the circulation. Moreover, letrozole treatment significantly reduced the E2 levels in these regions,

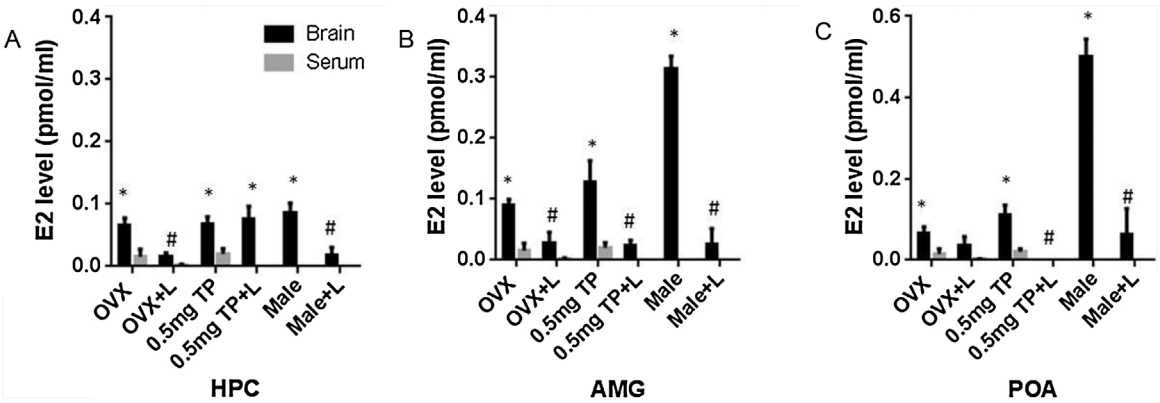

Fig. 7. Comparison of E2 levels in brain vs. serum in the (A) HPC, (B) AMG, and (C) POA of OVX females, TP-treated females, and gonadally intact males treated with Letrozole. * indicates significant difference $p < 0.05$ between brain and serum levels. # indicates significant difference $p < 0.05$ in brain levels between letrozole and non-letrozole treated groups. $N = 12$ for OVX; $N = 6$ for OVX + L; $N = 10$ for 0.5 mg TP; $N = 5$ for TP + L; $N = 6$ for Males; $N = 6$ for Male + L.

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 1 μg 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 2.5 μg 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 2.5 μg 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,b, 2003a, 2005). 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.

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 1 μg 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.

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., 2016; 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., 2016). 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 (35 pg/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 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 1 g of tissue contains 100 mg 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 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. (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: ~4 nM) 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. (2009) reported approximately 19.0 fmol/mg protein E2 and 43.7 fmol/mg protein T in the hippocampal slice culture from 10-day-old male rats precultured in serum after 24 h 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 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 astrocytes following brain injury (Peterson et al., 2001, 2007; Pietranera et al., 2011) as well as an increase in ARO activity (Pedersen et al., 2017), consistent with induction of local E2 production. 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.

4.3. Implications and limitations

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,b; 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, 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. (2004) and Bailey et al. (2013, 2017) 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.

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., 2016; 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., 2001; 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 et al., 1994, #93; Roselli and Resko, 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.

Conflict of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Contributor

In this paper, Miss Li participated in the hypothesis development, experimental plan, sample preparation and manuscript writing. Dr. Gibbs participated in the hypothesis development, experimental design

and grant support. All authors have approved the final article.

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