EFFECTS OF CHRONIC RESTRAINT STRESS ON AROMATASE, ESTROGEN RECEPTORS, INFLAMMATORY MARKERS AND LOCAL ESTROGEN PRODUCTION IN BRAIN

By

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Our goal is to identify factors that underlie sex dimorphisms in the risk of developing psychological disorders. Numerous studies demonstrate that stress is an important contributing factor. Chronic stress can induce an inflammatory response in the brain, presumably via the activation of microglial cells. Microglial cells are macrophage-like cells in the CNS and are the primary cells responsible for brain immune responses. Glucocorticoids have been reported to stimulate microglia to transform to a pro-inflammatory state and, hence, may be a primary mediator of microglial response to stress. There is evidence that microglia can respond to estrogens which may contribute to different responses in men vs. women. Specifically, estrogens have been shown to inhibit the microglial inflammatory response. Brain levels of estrogens are also determined by local conversion of androgens via the enzyme aromatase. Brain levels of aromatase are higher in males than in females which can result in higher local levels of estradiol in regions of the male brain than in the female brain. Here, we hypothesize that stress can influence the activity of aromatase in the brain in a sexually dimorphic way, affecting local levels of estrogens in specific brain regions and resulting in sex differences in microglial activation and

neuroinflammatory response. We first did a pilot study focusing on the effects of chronic restraint stress on aromatase in the brain in a sexually dimorphic way, affecting local levels of estrogens in specific brain regions and resulting in sex differences in neuroinflammatory response. We developed a method to detect CORT levels in rat serum. Sex differences occurred in serum corticosterone level and CORT levels in females that had undergone daily restraint stress responded more to a stressor than in males. Serum E2 levels were undetectable in males and very low. Serum E2 levels also were undetectable in many of the females. We also detected a pattern suggesting sex differences in the levels of ARO-L, estrogen receptors (ERα, ERβ and GPR30) and inflammatory markers (IL-1ß and TNF-α) mRNA under chronic restraint stress. We did not detect clear changes in local estrogen levels in cerebellum, hippocampus and amygdala. Larger sample sizes are needed to further examine the hypothesis.

List of Abbreviations

Corticosterone (CORT)

Estradiol (E2)

Testosterone (T)

Glucocorticoids (GCs)

Long-form aromatase (ARO-L)

Aromatase (ARO)

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1.0 INTRODUCTION

1.1 Stress is a contributing factor that underlies sex dimorphisms in the risk of developing psychological disorders

1.1.1 Stress increases risk for mental illness and induces an inflammatory response

Psychiatric illness, which includes depression, personality disorders, such as borderline personality disorder (BPD), anxiety disorders, schizophrenia, addictive behaviors and more [1], is a serious problem. In a given year, nearly one in five (19 percent) of U.S. adults ages 18-44 experience some form of psychiatric illness [2]. The effects of mental disorders continue to grow with significant influence on health and economic consequences all over the world. Over hundreds of billions of dollars is used annually on treating mental illness in the world and direct mental health care accounts for approximately 10% of medical costs, and 20% of state and local health care costs [3, 4]. About 80% of people in low- and middle-income countries and 40% of people in high-income countries with mental disorders receive no treatment [5]. More effort is needed to understand the factors that contribute to risk for psychiatric illness and to effective methods of prevention and treatment.

Sex and gender differences in mental disorders are among the most intriguing and stable findings in psychiatry. It is well known that certain types of psychological illnesses are associated with a strong sex difference in risk. For example, women have a higher lifetime prevalence of mood or anxiety disorders than men and a later onset of schizophrenic psychoses [6]. In contrast, some other disorders are much more common in men, such as alcohol dependence, which is twice

as high in men as in women, and antisocial personality disorder, which is three times more common in men than in women [7]. However, we still do not really understand the causes of these differences, and comparatively little research has been done to explain them. Therefore, we are interested in searching for the factors that underlie sex dimorphisms in the risk of developing psychological disorders.

Numerous studies demonstrate that stress is an important contributing factor. Many preclinical studies demonstrate that under acute or chronic stress exposure, the immune system becomes activated resulting in the expression of cytokines, chemokines, or other molecules known to invoke inflammatory processes in rats and mice, including in the brain. For example, interleukin-1β (IL-1) and tumor necrosis factor alpha (TNFα) levels in rat brain hippocampal microglia are increased after inescapable shock stress [8] and are proinflammatory cytokines. What's more, lots of stress models including restraint or immobilization stress, water avoidance, and forced swimming stress, have been shown in rats and mice to induce brain inflammatory responses and contribute to measures of mental illness [9]. Researchers in the field of psychoneuroimmunology (PNI) study relationships between the immune system and people's mental and emotional health. They have concluded that chronic stress can contribute to mental illness such as depression and anxiety. For example, a report in 2016 states that stressful experiences during childhood can increase levels of cytokines in brain which in turn increased risk of mental illness in adulthood [10]. It is also well known that some connections have been established between stress and neurological diseases such as neurodegenerative disorders, Alzheimer's and Parkinson's Disease [11]; Depressive Disorder [12] and Post-traumatic Stress Disorder [13]. Collectively, these studies show that stress can increase the risk for psychiatric illness and induce inflammatory response in the brain.

1.1.2 **Microglia play a major role in Inflammatory responses in brain and GCs are mediators of stress and can cause microglial activation.**

Microglial cells are macrophage-like cells in the CNS and are the primary cells responsible for brain immune responses [14]. In their resting state, microglia have a highly branched, ramified morphological phenotype [15] and are dispersed throughout the CNS [16]. These branches produce large surface area and have dynamic motility to sense and monitor the environment. In response to stress, microglia can undergo a dramatic morphological transformation [17] to an amoeboid shape with more branches and the release pro-inflammatory cytokines. This is called microglial activation. The innate CNS immune system acts differently through pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) [18]. To better classify the function of microglia, they are divided into two subtypes which include a pro-inflammatory M1 state and an M2 anti-inflammatory state [19]. Preclinical studies suggest that in response to injury or infection, microglia can be activated to an M1 (pro-inflammatory) state where they release inflammatory cytokines [18] including interleukin-1β (IL-1ß), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). There is compelling evidence that IL-1ß and TNFα are primarily synthesized by activated microglia levels ([20][21]) in rat brain. Microglia also are involved in neuroprotective responses to reduce tissue damage (M2 state) in the brain [22]. Studies have shown that the function of microglia can change from neuroprotection to pro-inflammatory when exposed to acute or chronic stress.

Chronic stress has also been shown to induce an inflammatory response in the brain, presumably via the activation of microglial cells. A study demonstrated that when animals were exposed to 10 days of variable stress and then received an intra-cortical injection of

lipopolysaccharide (LPS), there was significantly greater levels of MHCII expression which is expressed predominately on activated microglia. [23]. In human studies, microglia cells have been observed in the brain parenchyma from the early stage of development to the mature state and act as brain macrophages when programmed cell death occurs during brain development or when the CNS is injured or pathologically damaged. Increases in the expression of markers of microglia are widely reported in brains from patients with Alzheimer's disease [24]. Collectively, these studies show under stress exposure, microglial cells are the primary cells which are responsible for brain immune responses.

1.1.3 **Glucocorticoids have been reported to mediate microglial cell's pro-inflammatory responses to stress**

Notably, glucocorticoids (GCs) have been reported to be stress hormones which mediate and prime microglia cell pro-inflammatory responses [25]. In a series of animal studies, adrenalectomized rats were treated with CORT either in their drinking water [26] or through subcutaneously implanted osmotic pumps or pellets [27][8]for two weeks and were exposed to stress. The results show elevated CORT levels also increased the gene expression of NLRP3, Ibα-I and MHCII [28]in a concentration dependent manner [27] which shows that CORT can increase inflammatory responses in the brain. Other results show that elevated CORT levels exacerbate stress effects on NF-kB, MAP kinases, and pro-inflammatory gene expression [26]. In humans there is evidence for cause-effect relationships between prolonged stress, elevated GC levels, and

cognitive and mood disorders while the evidence for a link between chronic stress, GCs, and neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's (PD) diseases is growing [29]. These results suggest that chronic exposure to CORT mediates microglial transformation to a pro-inflammatory state and induces downstream signaling pathways in response to stress.

1.2 Estrogens are produced locally in the brain and are associated with sex differences in the risk for psychiatric illness

The sexual dimorphisms observed in certain types of psychiatric illness may be associated with different levels of sex hormones (estrogens) in the brains of females and males. *In vivo* and *in vitro* studies [30] have shown sexually dimorphic differences remain in rat brain microglial inflammatory responses when stimulated with an immune challenge. When male and female rat microglia cells were isolated and incubated with varying doses of E2, the E2 produced anti-inflammatory effects in the male microglia but a pro-inflammatory effect in female microglia in response to LPS [31][32]. Studies reported estrogen can activate nuclear estrogen receptors (ERs), ERα and ERβ [33] and regulate gene transcription and activate second messenger signaling pathways. A report also identified a novel membrane-associated estrogen receptor GPR30 [34], which is located both intracellularly and on the plasma membranes and plays a very important role in mediating estrogen effects in the brain [35, 36]. What's more, other studies also revealed that estrogens and estrogen receptor (ERα, ERβ and GPR30) agonists can reduce activation of microglia under stress and significantly decrease inflammatory response [37][38]. Therefore, estrogens can reduce inflammatory responses and cause sex differences in microglial responses under stress exposure.

Brain aromatase, composed of CYP19A1 and NADPH, is a local enzyme which irreversibly converts androgens (both androstenedione and testosterone) to estrogens (estrone and E2). There are two forms in the brain, a long form aromatase which is associated with ARO activity, and a short form (ARO-S) with no known function [39]. Therefore, we focused on detecting long form aromatase mRNA expression. In past years, research of ARO in the brain has mainly focused on its function to regulate sexual dimorphism and roles in neuroprotection [40, 41][42][43]. It is well known that males have more circulating levels of androgens than females, and aromatase will produce higher local levels of estrogens in the male brain than in the female brain. We hypothesize that the higher levels of estrogens produced locally in the brain can reduce microglial inflammatory responses and thus produce sex difference in females and males. Therefore, estrogens which are produced locally in the brain are factors which may contribute to sex differences in the risk for psychiatric illness.

1.2.1 **Evidence that stress can influence ARO expression and E2 production in the brain**

In quails, there are studies showing that ARO activity and E2 levels in the preoptic area (POA) of the brain were increased by acute stress in males [40, 44]; however, the circulating levels of testosterone and estradiol were unchanged in response to acute stress. The results suggested under stress exposure, sex differences occur due to ARO activity and local E2 levels in the quail brain.

However, in mammalian studies, less is known about the effects of ARO activity partly due to difficulty in measuring E2 in the brain. Recently, our lab has developed a reliable assay for

measuring E2 levels in different regions of the brain using UPLC-MS/MS methods [45]. Data show that in brain regions with significant ARO activity (e.g., hippocampus, amygdala), local levels of E2 can greatly exceed circulating levels in both males and females [46]. Data from our lab 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.

1.3 **Overview of thesis**

The purpose of this project is to investigate whether estrogens produced locally in the brain can be associated with sex differences in the risk for stress-related brain/ neuroinflammatory response. If that is true, it may provide a possible mechanism to explain sex dimorphisms in the risk of developing psychological disorders. Here we hypothesize that chronically elevated stress hormone (i.e., corticosterone), can influence the activity of aromatase in the brain in a sexually dimorphic way, affecting local levels of estrogens in specific brain regions and resulting in sex differences in microglial activation and neuroinflammatory response. To examine this hypothesis, our lab entered into a collaboration with Dr. Cheryl Conard at the Arizona State University to conduct a pilot study. The pilot study focused on effects of chronic restraint stress on aromatase, estrogen receptors, and inflammatory markers in brain. Here we hypothesize that chronic restraint stress can influence the activity of aromatase in the brain in a sexually dimorphic way, affecting local levels of estrogens in specific brain regions and resulting in sex differences in neuroinflammatory response. Specific goals were as follows: 1) to determine whether chronic restraint stress can influence brain neuroinflammatory response and aromatase expression in a sexually dimorphic way 2) test specifically whether local estrogen production is associated with

sexually dimorphic effects of chronic restraint stress on inflammatory response in the brain. These goals are described in greater detail in the following chapters.

2.0 METHOD DEVELOPMENT

In this project, we hypothesize that chronic restraint stress can influence the activity of aromatase in the brain in a sexually dimorphic way, affecting local levels of estrogen production in specific brain regions and resulting in sex differences in neuroinflammatory response. To measure changes in the levels of ARO-L, estrogen receptors (ERα, ERβ and GPR30) and inflammatory markers (IL-1ß and TNFα) mRNA, we performed quantitative real time reverse transcription PCR (q-RT-PCR) and checked RNA quality by gel electrophoresis. As part of our studies, this method was validated to have a wide detectable range, high specificity and reproducibility. To test the changes in E2, T and CORT levels in rat serum and local E2 production in different rat brain regions, we developed a method to measure CORT based on methods previously developed in our lab for measuring E2 and T using UPLC-MS/MS. This method was also validated to be sensitive and reproducible. Details of these methods are described below:

2.1 Q-RT-PCR method for comparing relative levels of ARO-L, estrogen receptors and Inflammatory markers mRNA

2.1.1 Description of q-RT-PCR method

Collection of tissues, extraction of mRNA and preparation of cDNA:

These series of methods have been developed in our lab [46]. Rats were anesthetized. Brains were removed and tissues from the hippocampus, cerebellum, medial prefrontal cortex and

amygdala were dissected. Each brain region was put into 1.5ml tube and weighed. Each piece of tissue was sonicated in 100mg/ml TRIzol (Invitrogen, Inc.) at 4˚C and then incubated for 5 min at room temperature. The homogenate was mixed with chloroform (250ul/1ml homogenate), incubated for 2 min, at room temperature and centrifuged at 4℃,12,000xg for 15min.The supernatant was collected, and an equal volume of isopropyl alcohol was added to precipitate nucleic acids. Nucleic acids were incubated for 10 min, washed with 70% ethanol and pelleted by centrifugation (4℃,12,000xg for 15min). The pellet was dissolved in DEPC water for 10 min at 42˚C. residues were then digested by adding DNAse enzyme (10U/µl), RNA enzyme inhibitor (40U/ul), 25mM MgCl2 and PCR buffer and incubating for 30 minutes in 37℃ water bath. 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.

qPCR Method: 2µl of cDNA, 10µl of SYBR Green, and 1.2µl primer pair was pipetted to each well of a 96-well plate (0.1mL/well). The primer pairs are summarized in **Table 1**.

Table 1 The sequence of target mRNA primer pairs

Primer Pair	sequence
ARO-L	F: 5'-CTCCTCCTGATTCGGAATTGT-3' R: 5'-TCTGCCATGGGAAATGAGAG-3'
ERα	F: 5'-TCCGGCACATGAGTAACAAA-3' R: 5'-TGAAGACGATGAGCATCCAG-3'
ERβ	F: 5'-AAAGTAGCCGGAAGCTGACA-3' R: 5'-ACTGCTGCTGGGAGGAGATA-3'
GPR30	F: 5'-AGGAGGCCTGCTTCTGCTTT-3' R:5'-ATAGCACAGGCCGATGATGG-3'
IL-1β	F:5'-CCTTGTGCAAGTGTCTGAAG-3' R:5'-GGGCTTGGAAGCAATCCTTA-3'
TNF-α	F: 5'-AGCCCTGGTATGAGCCCATGTA-3' R: 5'-CCGGACTCCGTGATGTCTAAGT-3'
GAPDH	F: 5'-TGCCACTCAGAAGACTGTGG-3' R: 5'-GGATGCAGGGATGATGTTCT-3'

These primer pairs were validated by Genebank primer-BLAST. The product of the GAPDH primer is 85bp length and is located on exon 6 of the GAPDH gene. PCR was conducted using the QuantStudio™ 3 - 96-Well 0.1 mL Block (ThermoFisher), with an initial step at 50 ˚C for 2 min ， 95 ˚C for 10 mins and 15 s, then 40 cycles of 60 ˚C for 1 min. At the end of the amplification, samples were set to 95˚C for 15s 60 ˚C for 30s, and 95˚C for 15s to obtain the melt curve. Data were analyzed using QuantStudio™ 3 software (ThermoFisher), and results were obtained as Ct (threshold cycle number) values. The effects of treatment on relative long-form aromatase (ARO-L), estrogen receptors (ERα, ERβ and GPR30) and inflammatory markers (IL-1ß and TNFα) mRNA levels were calculated using the following formula [47]:

$$\text{ratio} = \frac{E_{aro}^{\Delta C_t aro(\text{control-sample})}}{E_{GAPDH}^{\Delta C_t GAPDH(\text{control-sampl}}}$$

Where E = the efficiency of the PCR reaction defined as $10^{-1/\text{slope}}$ of a serial dilution curve, which showed a liner relation between cDNA concentration and Ct number. ΔC_t is the mean difference in Ct for treatment vs control (from Pfaffl)

[47]

Two-way ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression between stress and control groups in females and males.

2.1.2 **Validation of q-RT-PCR method**

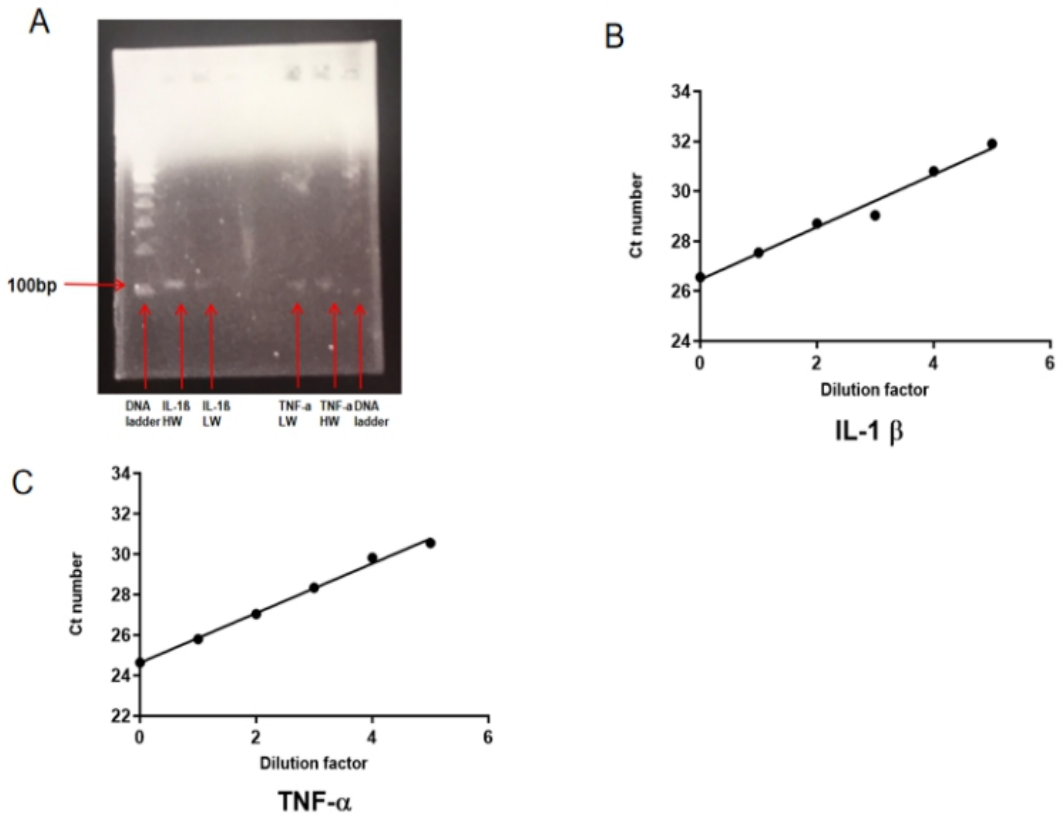

Figure 1 Validation of q-RT-PCR method: (A) Agarose gel illustrates the size of DNA products produced by PCR amplification using IL-1ß and TNFα primer pairs. (B) Dilution curve showing change in Ct for detection of IL-1ß mRNA. (C) Dilution curve showing the changes in Ct for detection of TNF α mRNA.

Product validation: Our lab had already validated ARO-L primer pairs, estrogen receptors primer pairs and control gene GAPDH primer pairs. To verify whether inflammatory markers (IL-

1ß and TNFα) primer pairs we amplified a product of the expected size, we ran the product on an 1.0% agarose gel. 1µl 100bp DNA ladder mixed with 2µl 6x Gel Loading buffer was loaded in one lane and was used to identify the size of the product. As the result shows in **Figure1 (A),** the product of IL-1ß primer pair is approximately 137bp length and the product of TNFα primer pair is approximately 109bp length as predicted. No other bands from these two primer pairs were detected, which suggests that these primer pairs for IL-1ß and TNFα specifically amplify IL-1ß and TNFα sequences as predicted.

Linear range: To find out the linear range of q-RT-PCR method, we did a serial dilution of the cDNA sample and used IL-1ß or TNFα primer pairs to test the cycle number change. **Figure 1(B) and (C)** show that each showed a linear relationship between dilution and cycle number in the range of 26 to 32 cycles ($Y = 1.051*X + 26.47$ $R=0.9787$, $F(1,4)=184.5$, $P=0.0002<0.05$) for IL-1ß and 24 to 32 cycles ($Y = 1.227*X + 24.64$,$R=0.995$, $F(1,4)=798.6$, $P<0.0001$) for TNFα, which suggests that q-RT PCR method is able to quantify changes in relative levels of IL-1ß and TNFα mRNA up to 32 cycles.

Reproducibility: We also tested the reproducibility of the q-RT-PCR method by calculating the coefficient of variation of both IL-1ß and TNFα. To do this, identical samples were analyzed in different wells of the plate. This experiment was repeated and the coefficient of variation (CV) was calculated for each run and each primer pair. Both the CV for IL-1ß and TNFα primer pairs were less than 2%, which suggests that the method has good reproducibility (data not shown).

2.1.3 **Validation of RNA quality ---- RNA Gel Electrophoresis**

RNA-based bioanalysis requiring high-quality, non-degraded RNA are a foundational element of many research studies. The integrity of experimental RNA is validated through an RNA Gel Electrophoresis [48] ("bleach gel") which is a modified method from DNA gel electrophoresis in 2.1.2. 1.0% v/v commercial chlorine bleach (6% sodium hypochlorite; Clorox®, Oakland, CA) was added to a standard TAE agarose gel and filled completely with 1x TAE buffer. Each gel was loaded with samples consisting of 1x DNA loading buffer (DNA Loading Dye), prepared from a 10x DNA loading buffer stock and 1μg of total RNA isolated from sample in both first and second shipment. The gels were run for 35 minutes with constant voltage (100 V) prior to imaging under UV transillumination.

2.2 **Method for detecting local E2 production in different brain regions and E2, T and CORT levels in serum**

2.2.1 **E2 and T detection**

Our lab has already developed and validated the assay for detecting T levels in the serum and E2 levels in both serum and brain tissue homogenates using UPLC-MS/MS, which have been proved to be extremely accurate [45, 46].

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 MgCl2, 4.0 mM Tris and 50 mM sucrose (100 mg tissue/ml). E2 was then measured in

homogenates and serum as recently described [45]. 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 4000 rpm at room temperature (RT) for 10 min, and the organic layer was transferred to sanitary 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 sanitary tubes and heated to 60 ˚C for 3 min. Samples were centrifuged again for 1 min at 490 x g, and the supernatant (∼0.1 ml) was transferred into glass vials for UPLC–MS/MS analysis with an injection volume of 7.5µl. 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, 1.7µm, 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. 500ul serum was used to measure serum E2 level. 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 [45] and are within acceptable limits. The limit of detectability for this assay is 0.009 pmol/ ml.

T levels in serum and brain homogenates were quantified by a modification of the method described by Cawood et al. and using UPLC–MS/MS similar to the E2 detection method described above. Briefly, 350ul serum 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 (80µl:20µl, 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.

2.2.2 **CORT detection**

CORT levels in serum were quantified by a modification of the methods described by Riffle, et al. [49] and by Yucel et al. [50], and using UPLC–MS/MS similar to the T detection method described above. 150ul to 200ul serum were spiked with 30µl D8- Corticosterone (5µg/ml in methanol, LC grade) as the internal standard and then extracted with 1.5 ml n-butyl chloride. After centrifugation and evaporation, the residue was reconstituted in acetonitrile: water

(30µl:30µl, LC grade), centrifuged again for 1 min at 490×g and the supernatant was transferred into glass vials for UPLC–MS/MS analysis. CORT was eluted from the same column as T, with an acetonitrile: water (0.1% formic acid) gradient (50%:50%). MS Detection and quantification were achieved in the positive mode. Transitions used for analysis were 347 → 121 for CORT, and 355 → 121 for the deuterated internal standard. The ratio of area under the peak between CORT and deuterated CORT was quantified and used to determine levels of CORT ng/ml by comparison with standards ranging from 25 ng/ ml to 500 ng/ml. The limit of detectability for this assay was 25 ng/ml.

Validation of UPLC-MS/MS method for quantitation of CORT: Extraction recovery. **Figure 2** evaluated the relationship between CORT extraction and detection using HPCD as matrix. CORT was prepared in rat serum (0, 25, 50, 75, 100 and 200ng/ml), and in 0.2% HPCD (0, 25, 50, 100, 250, 500 ng/ml). After UPLC–MS/MS analysis, values of CORT prepared in rat serum were calculated using CORT prepared in HPCD as calibration curve. Linear regression analysis revealed that observed CORT levels using HPCD as calibration curve had a significant linear regression with the amount CORT added into serum ($Y = 1.159*X + 176.6$, $R^2=0.9934$ $F(1,4) =601.5$, $P<0.0001$). This shows that the extraction and detection of CORT from HPCD is the same as from the rat serum. Hence HPCD was used as the matrix for all calibration curves

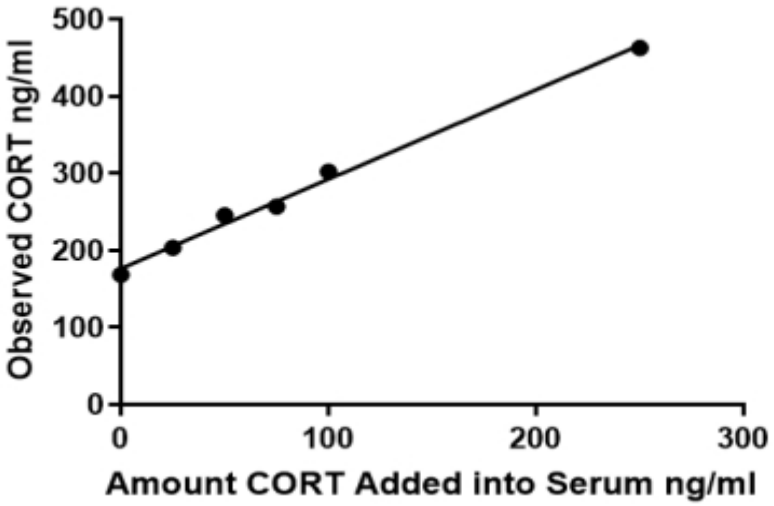

Figure 2 Evaluation of brain tissue homogenate vs. HPCD as a matrix for the extraction and detection of CORT

3.0 Effects of Chronic Restraint Stress on Aromatase, Estrogen Receptors, Inflammatory Markers and local Estrogen production in Brain

3.1 **Overview of study design**

Figure 4 contains a flow diagram showing the experimental design. 48 young female and male Sprague-Dawley rats (24 females, 24 males; n=12/Tx group) were purchased by the Conrad Lab, Arizona State University and were acclimated for one week after arrival in that facility. Rats were housed in pairs on a 12 hour:12-hour light/dark schedule with unrestricted access to food and water. Then half of each sex was restrained for 6 hours/day for 21 days. Restraint stress occurred during the rats' naturally active period. The timing of brain dissection took place the day following the end of chronic restraint stress (21 days). On day 22, Conrad Lab Team collected fresh brain tissues (bilaterally) of the hippocampus (HPC), mPFC, amygdala (AMG) and cerebellum (CB). The wet weight tissue for each sample was determined. Each sample was then frozen at -80°C. Additional measures included adrenal weights, thymus weights, uterine weights and body weights (the latter was collected weekly during restraint). Serum was also harvested from rat trunk blood. Samples were shipped to our lab in two shipments (half of each group) from Arizona. However, samples which were shipped first (half of each group) met a shipping problem and were delayed for several days during transit. We soon discovered that RNA in the first shipment sample were compromised and degraded because its target GAPDH cycle number increased from about 15 to 20 which was unusual. So we checked RNA quality of the samples by RNA gel electrophoresis as mentioned in 2.1.3.

In **Figure 3**, we show that sample from cerebellum 3 (CB3) and cerebellum 25 (CB25) which shipped first and sample from cerebellum 15 (CB15) which shipped second were run on an RNA agarose gel. When assessing the quality of RNA by gel electrophoresis, the presence of three distinct bands suggests high quality RNA. For CB15, the top band represents 28S ribosomal RNA (rRNA) and the second band represents 18S rRNA. Though the third band which represents 5.8S rRNA was invisible, the two bands still suggest RNA integrity in the sample from cerebellum 15 (CB15) which shipped second was high. But there were no bands in CB3 and CE25 which suggested degradation of RNA in samples which were shipped first. Taken together, this suggests that RNA of the samples which were shipped first were compromised and not suitable for q-RT-PCR analysis.

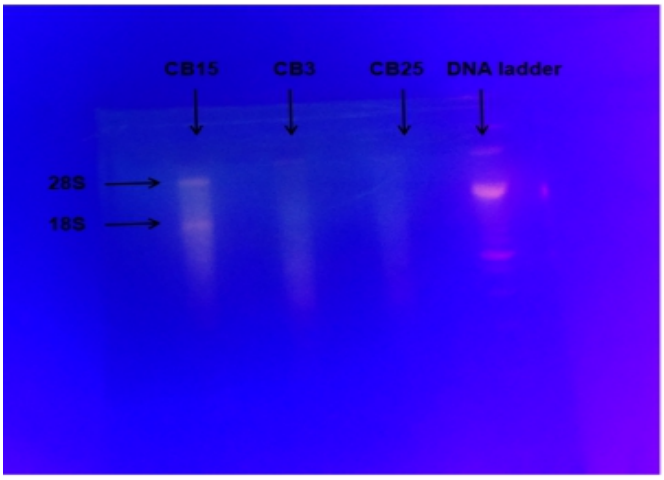

Figure 3 Cerebellum RNA gel electrophoresis. CB3: cerebellum3, CB25: cerebellum25, CB15: cerebellum15, 28S: 28S ribosomal RNA, 18S: 18S ribosomal RNA.

We had used 4 samples from each group in the first shipment from hippocampus, prefrontal cortex, amygdala, and preoptic area to detect ARO-L, estrogen receptors (ERα, ERβ and GPR30) and inflammatory markers (IL-1ß and TNFα) mRNA expression using the q-RT-PCR methods

described above in 2.1. But we quickly found the result using these samples from first shipment was inconclusive and had high variability (data not shown).

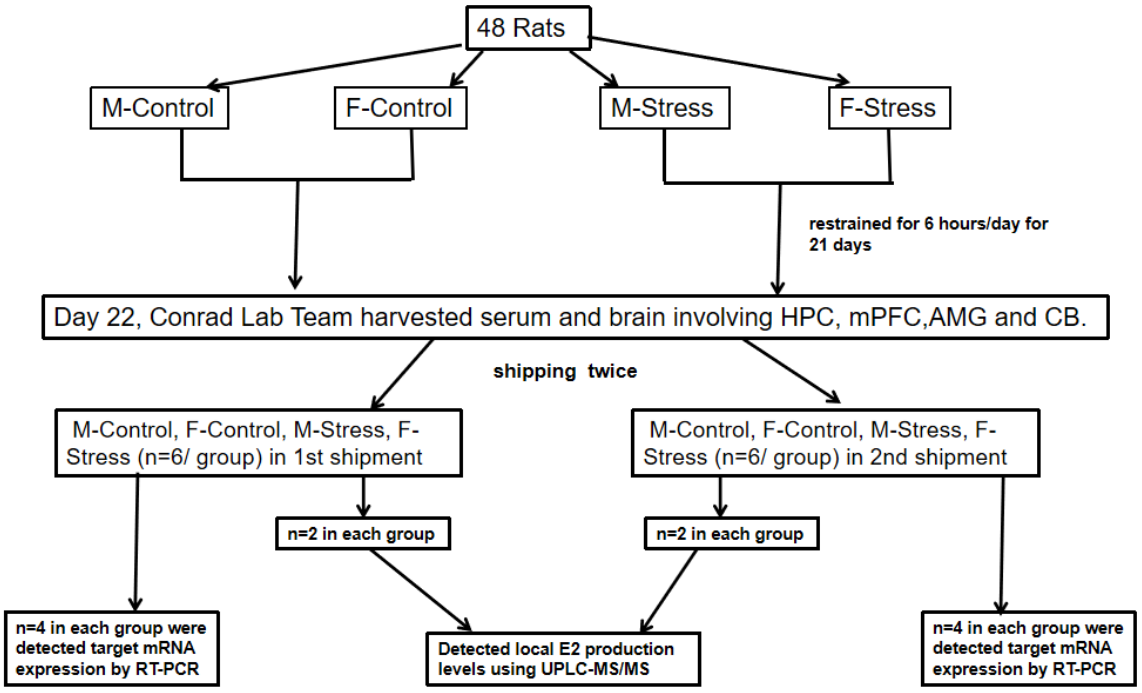

Figure 4 Flow diagram showing the experimental design. M-Control: male control group, F-Control: female control group, M-Stress: male restraint stress treated group, F-Stress: female restraint stress treated group, HPC: hippocampus, AMG: amygdala, cerebellum (CB)

We next proceeded to use 4 samples from each group in the second shipment from hippocampus, prefrontal cortex, amygdala, and preoptic area to test ARO-L, estrogen receptors (ERα, ERβ and GPR30) and inflammatory markers (IL-1ß and TNFα) mRNA expression using the q-RT-PCR methods described above. Ratios between all targets and GAPDH were calculated to present the relative mRNA level. All ratio values were normalized to the mean of male non-

stressed controls. ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression between sex in stress groups and control.

To detect local E2 production levels in different brain regions, we used the remaining 4 samples per group from cerebellum, hippocampus, prefrontal cortex, and amygdala from the first shipment to detect local E2 production levels using UPLC-MS/MS methods described above in 2.2. In each group, every two samples were combined and pooled to generate one point in hippocampus and amygdala. To detect E2, T and CORT levels in serum, 300µl serum were used to detect CORT level. 500µl serum were used to detect E2 level and 350µl serum were used to measure testosterone levels by UPLC-MS/MS assay in 2.2. Two-way ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression as a function of sex and stress vs. controls.

3.2 **Effects of Chronic Restraint Stress on E2, T and CORT levels in serum**

3.2.1 **Results**

Figure 5 illustrates serum CORT levels in the four groups of rats. Two-way ANOVA showed a significant effect of sex ($F [1,42] = 23.57$, $P<0.001$). CORT levels in females were higher than that in males. There was no significant effect of treatment ($F [1,42] = 1.32$, $P=0.2576$) or treatment*sex interaction ($F [11,42] = 3.89$, $P=0.0558$). Post hoc analyses showed that CORT levels between stress and control groups in females and males were not statistically significant.

But in females, serum CORT levels in the stress group was significantly higher than in the control group which was nearly significant (F [11,42] = 3.89, P=0.0558).

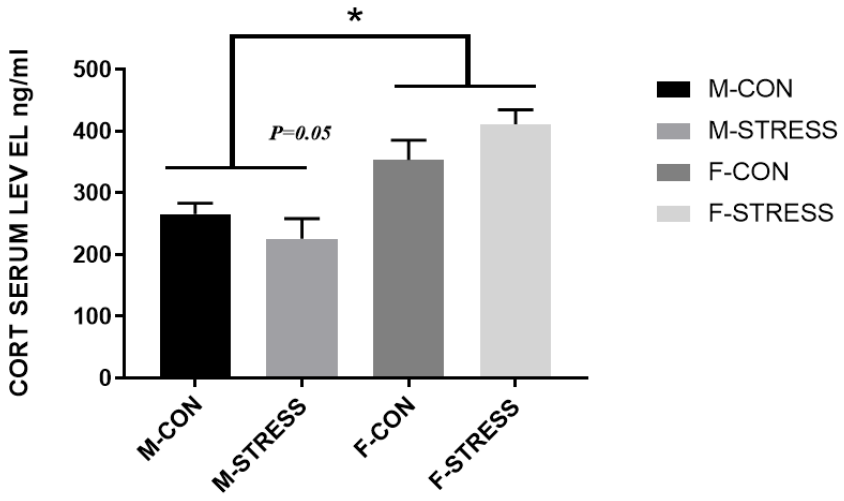

Figure 5 CORT levels in rats serum. Bars indicate the mean rats serum CORT levels ± s.e.m in M-CON: male control group, F-CON: female control group, M-STRESS: male restraint stress treated group, F-STRESS: female restraint stress treated group. N=4 for each group. * indicates the p ≤0.05 between sex. N=12 for each group.

Serum E2 levels in rats between stress and control groups in females and males are summarized in **Table 2**. In males, E2 levels were undetectable. In females, E2 levels were very low and in most samples were undetectable.

Table 2 Serum E2 levels. Data indicate animal name and E2 level in M-CON: male control group, F-CON: female control group, M-STRESS: male restraint stress treated group, F-STRESS: female restraint stress treated group. ND indicates not detectable and - indicate indicates not detectable and - indicates insufficient sample for analysis.

M-CON		M-STRESS		F-CON		F-STRESS	
Animal Number	Estradiol Level(pmol/mL)	Animal Number	Estradiol Level(pmol/mL)	Animal Number	Estradiol Level(pmol/mL)	Animal Number	Estradiol Level(pmol/mL)
s25	ND	s29	ND	s1	ND	s3	ND
s26	ND	s30	ND	s2	ND	s4	0.06
s27	ND	s31	ND	s7	-	s5	ND
s28	ND	s32	ND	s8	-	s6	0.09
s33	ND	s37	ND	s13	-	s9	-
s34	ND	s38	ND	s14	0.07	s10	ND
s35	ND	s41	ND	s15	0.02	s11	-
s36	ND	s42	ND	s16	ND	s12	-
s39	ND	s43	ND	s17	-	s21	0.08
s40	ND	s44	ND	s18	ND	s22	ND
s45	ND	s47	ND	s19	0.02	s23	ND
s46	ND	s48	-	s20	ND	s24	ND

3.2.2 **Discussion**

The goal of this analysis was to collect preliminary data on the effects of chronic restraint stress on E2, T and CORT levels in serum.

Results from CORT level study showed that after weeks of repeated restraint stress, sex differences occurred in serum CORT levels, and CORT levels in females responded more to stress than in males. Studies [51] have reported that under chronic restraint stress in adolescence male and female rats, results show that males and females exhibit different CORT responses and that

only adult female rats exposed to stress during adolescence show higher basal CORT levels compared to non-stressed controls. Our study replicated this result. Our results also reported that treatment had no effects on serum CORT level and the basal serum CORT level seems higher. But in Dr. Conrad's lab [52], they reported that chronic restraint stress did not elevate baseline CORT levels in serum. But CORT levels increased within 30-min from the start of restraint stress across all days measured and then they returned to baseline on all but the first day of restraint stress.

It is important to note that levels of CORT were elevated at sacrifice, indicating a stress response. The elevated levels of CORT in rat serum at sacrifice are due to the stress associated with the method of sacrifice. When the rats were sacrificed, the method which we use were regarded as a way of stressing and exposed to such stress, rats CORT levels were elevated.

Serum E2 levels were undetectable in males and very low, even undetectable in most females. We had verified our assay to be sensitive and reliable for measuring E2 levels by UPLC-MS/MS [45, 46]. Therefore, it is not likely our assay failed. As it has been reported [53] E2 is secreted across the estrous cycle in the female rats. E2 levels are low during diestrus (Day1) and at day2 begin to rise at the end of diestrus. It reaches the peak in late proestrus (P), and then drops to baseline levels during estrus (E). Behavioral changes associated with estrus occur during the dark phase. It is possible that female rats were sacrificed during diestrus; resulting in the low E2 levels.

We also attempted to measure serum T levels (data not shown); however, the method for measuring T levels failed. At first, we met a T contamination in the UPLC-MS/MS system. Later we solved this problem but found the background of T level was very high. We also have not enough amount of serum to retest T level again. Therefore, the data were not trustable and we were not able to measure T levels in the rat serum.

3.3 Effects of Chronic Restraint Stress on Aromatase, Estrogen Receptors, and Inflammatory Markers in different Brain regions between females and males

3.3.1 Effects of Chronic Restraint Stress on relative levels of ARO-L mRNA in different brain regions between females and males

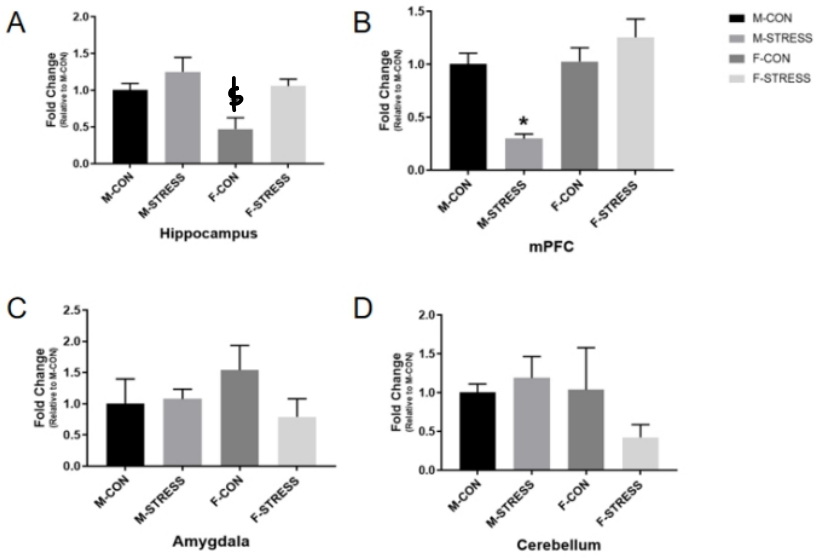

Figure 6 Effect of chronic restraint stress on long form ARO mRNA expression in (A) Hippocampus, (B) mPFC, (C) Amygdala,(D) Cerebellum. Bars indicate the mean fold changes of long form ARO mRNA relative to male control group ± s.e.m. after normalizing to GAPDH. M-CON: male control group, F-CON: female control group, M-STRESS: male restraint stress treated group, F-STRESS: female restraint stress treated group. * indicates significant difference ($p < 0.05$) in female control group ARO-L mRNA expression in HPC compared to male restraint stress treated group. $ indicates significant difference ($p < 0.05$) in to male restraint stress treated group ARO-L mRNA expression in HPC compared to female restraint stress treated group. N=4 for each group

Results:

The effect of chronic restraint stress on long-form ARO mRNA expression in different brain regions between females and males is summarized in **Figure 6.**

In hippocampus, two-way ANOVA revealed a significant effect of sex ($F[1,10] = 5.35$, $p<0.05$) and a significant effect of stress ($F[1,10] =7.22$, $p<0.05$), but no stress*sex interaction ($F[1,10]=1.18$, $p=0.3$). Post hoc analyses suggests that long-form ARO mRNA expression in female controls was not significantly different from female stress group or male control group but was significantly lower than male stress group.

In mPFC, two-way ANOVA revealed a significant effect of sex ($F[1,10]=6.12$, $p<0.05$) and a significant effect of stress*sex interaction ($F[1,10]=5.34$, $p<0.05$), but no effect of stress ($F[1,10]=0.78$, $p=0.4$). Post hoc analyses suggests that, long-form ARO mRNA expression in male controls was not significantly different from female controls and female stress group that levels were significantly lower in male stress group vs. all other groups.

In amygdala, two-way ANOVA revealed no significant effects of sex ($F[1,9]=0.12$, $p=0.74$), stress ($F[1,9]=0.82$, $p=0.39$) or stress*sex interaction ($F[1,9]=1.23$, $p=0.30$) on long-form ARO mRNA expression.

In cerebellum, two-way ANOVA revealed no significant effects of sex ($F[1,9]=0.85$, $p=0.38$), stress ($F[1,9]=0.28$, $p=0.60$) or stress*sex interaction ($F[1,9]=1.03$, $p=0.33$) on long-form ARO mRNA expression.

Discussion:

Figure 6 evaluated the effects of chronic restraint stress on relative levels of ARO mRNA between females and males in different regions of the brain. This was done to explore patterns about the effects of chronic restraint stress on aromatase mRNA expression in different regions of the brain in both males and females. It would give us the first step to examine our hypothesis that stress can influence the activity of aromatase in the brain in a sexually dimorphic way.

Our study is a pilot study and the sample sizes are small (four animals each group). As a result, the power analysis is limited and more numbers of animals in each group are needed to generate a meaningful conclusion in statistical analysis. Because we use delta-delta Ct method to quantify changes in relative levels of mRNA between groups, any changes in relative levels of mRNA more than 2-fold are believed to have some biological significance. Therefore, we can still get meaningful information by looking at patterns between groups though they were not statistically significant.

Our results suggest that in hippocampus, chronic restraint stress results in 30% and 50% elevated levels of ARO mRNA expression in stress groups than in control groups in both females and males. Females in mPFC and males in CB also had same patterns (30% elevation). In contrast, males in mPFC (70% decrease) and females in amygdala and cerebellum (50% decrease) have opposite patterns. Taken together, our results present a pattern indicating under chronic restraint stress, sex differences occurred on relative levels of ARO mRNA expression and treatment of stress also can alter ARO-L expression in different brain regions.

Only a few reports have been known to examine the effect of stress on aromatase in the brain using rodent models. Most researchers examine the effects of stress on aromatase expression using birds or fish models since E2 synthesized in the brain plays a critical role in the activation of sexual behavior in many vertebrate species and levels of E2 synthesized in the avian and amphibian brain are high [54]. Dickens et al. reported acute stress differentially affects aromatase activity in specific brain nuclei of adult male and female quail [40]. There is a paper which reported postmortem material from patients with Alzheimer's disease (AD) and in a mouse model for AD (5xFAD mice) [43] suggesting that hippocampal ARO expression may change in response to Alzheimer's disease in both humans and mice and supports the idea that brain-derived E2 has neuroprotective function. The sex differences found in the mouse model indicates that the neuroprotective role of brain-derived estradiol may be more important in females than in males and may help to explain why women are more prone to the disease than men [43]. Bender, RA [55] reported that the basolateral amygdala (BLA), a key structure of the fear circuit, has a sexually dimorphic regulation of synaptic plasticity involving neuronal aromatase, which produces the neurosteroid 17β-estradiol (E2). These findings could be relevant for the understanding of sex differences in mood disorders [55]. These findings appear to be in accordance with our finding that ARO expression can be altered by stress. But more data is needed.

3.3.2 Effects of Chronic Restraint Stress on relative levels of Estrogen Receptors in different brain regions between females and males

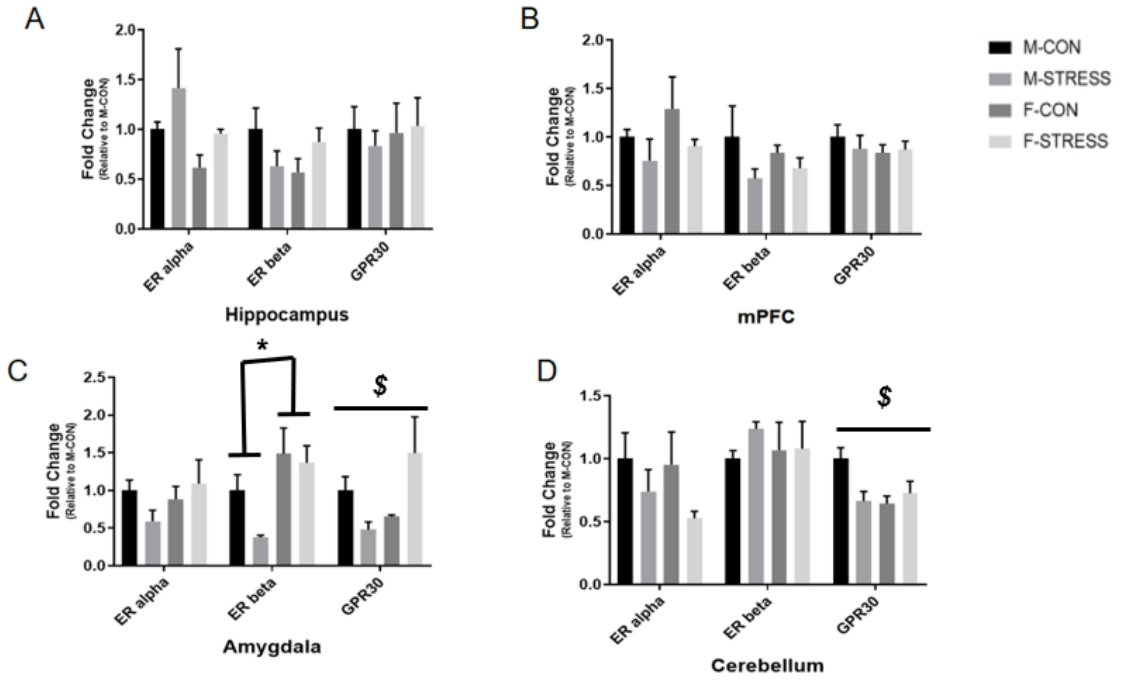

Figure 7 Effect of chronic restraint stress on estrogen receptors (ERα,ERβ and GPR30) mRNA expression in (A) Hippocampus, (B) mPFC, (C) Amygdala,(D) Cerebellum. Bars indicate the mean fold changes of estrogen receptors (ERα, ERβ and GPR30) mRNA relative to male control group ± s.e.m. after normalizing to GAPDH. M-CON: male control group, F-CON: female control group, M-STRESS: male restraint stress treated group, F-STRESS: female restraint stress treated group. . * indicates significant difference ($p < 0.05$) in AMG ERβ mRNA expression between sex. $ indicates a significant effect of treatment × sex interaction ($p < 0.05$) in AMG and CB GPR30 mRNA expression. N=4 for each group.

Results:

The effect of chronic restraint stress on estrogen receptors (ERα, ERβ and GPR30) mRNA expression in different brain regions between females and males is summarized in **Figure 7**.

In the amygdala, two-way ANOVA revealed no significant effects of sex ($F[1,11]=0.88$, $p=0.36$), stress ($F[1,11]=0.25$, $p=0.62$) or stress*sex interaction ($F[1,11]=2.34$, $p=0.15$) on ERα mRNA expression. Two-way ANOVA revealed a significant effect of sex ($F[1,8]=8.02$, $p<0.05$), no significant effects of stress ($F[1,8]=2.02$, $p=1.85$) or stress*sex interaction ($F[1,8]=0.92$, $p=0.35$) on ERβ mRNA expression. Two-way ANOVA revealed a significant effect of stress × sex interaction ($F[1,9]=7.89$, $p<0.05$), no significant effects of sex ($F[1,9]=1.89$, $p=0.19$) or stress ($F[1,9]=0.44$, $p=0.52$) on GPR30 mRNA expression.

In cerebellum, two-way ANOVA revealed no significant effects of sex ($F [1,10]=0.38$, $p=0.55$), stress ($F[1,10]=2.65$, $p=0.13$) or stress*sex interaction ($F[1,10]=0.15$, $p=0.70$) on ERα mRNA expression. Two-way ANOVA revealed no significant effects of sex ($F[1,11]=0.06$, $p=0.80$), stress ($F[1,11]=0.51$, $p=0.49$) or stress*sex interaction ($F[1,11]=0.41$, $p=0.53$) on ERβ mRNA expression. Two-way ANOVA revealed a significant effect of stress × sex interaction ($F[1,11]=6.32$, $p<0.05$), no significant effects of sex ($F[1,11]=3.12$, $p =0.10$) or stress ($F[1,11]=2.32$, $p=0.15$) on GPR30 mRNA expression.

In hippocampus, two-way ANOVA revealed no significant effects of sex ($F[1,12]=3.90$, $p=0.07$), stress ($F[1,12]=3.18$, $p=0.08$) or stress*sex interaction ($F[1,12]=0.03$, $p=0.86$) on ERα mRNA expression. Two-way ANOVA revealed no significant effects of sex ($F[1,10]=0.28$, $p=0.6$), stress ($F[1,10]=0.04$, $p=0.84$) or stress*sex interaction ($F[1,10]=3.72$, $p=0.08$) on ERβ mRNA expression. Two-way ANOVA revealed no significant effects of sex ($F[1,11]=0.10$,

p=0.75), stress (F[1,11]=0.03, p=0.85) or stress*sex interaction (F[1,11]=0.21, p=0.65) on GPR30 mRNA expression.

In mPFC, two-way ANOVA revealed no significant effects of sex (F[1,12]=1.14, p=0.30), stress (F[1,12]=2.31, p=0.15) or stress*sex interaction (F[1,12]=0.11, p=0.74) on ERα mRNA expression. Two-way ANOVA revealed no significant effects of sex (F[1,11]=0.02, p=0.88), stress (F[1,11]=2.31, p=0.15) or stress*sex interaction (F[1,11]=0.49, p=0.48) on ERβ mRNA expression. Two-way ANOVA revealed no significant effects of sex (F[1,12]=0.57 p=0.46), stress (F[1,12]=0.16, p=0.69) or stress*sex interaction (F[1,12]=0.46, p=0.50) on GPR30 mRNA expression.

Discussion:

The goal of this experiment was to collect data on the effects of chronic restraint stress on estrogen receptors (ERα, ERβ and GPR30) expression in different brain regions between females and males.

Our results indicate that in hippocampus, chronic restraint stress results in 40% and 30% elevated levels of ERα expression in stress groups than in control groups between both females and males. In contrast, males in amygdala (50% decrease) and males and females in both mPFC and cerebellum (25% decrease) have opposite patterns. Taken together, our results indicate a pattern suggesting under chronic restraint stress, sex differences occurred on relative levels of ERα mRNA expression and treatment of stress also can alter ERα expression in different brain regions.

For ERβ mRNA expression, the results indicated that males in hippocampus (50% decrease), mPFC (50% decrease) and amygdala (70% decrease), chronic restraint stress results in

decreased levels in stress groups than in control groups. In contrast, chronic restraint stress caused 30% elevated levels of ERβ expression in stress groups than in control groups in the female rats in hippocampus. What's more, in amygdala, the results suggested that chronic restraint stress resulted in 50% decreased levels in stress groups than in control groups, but at the same time, caused 90% elevation in GPR30 expression in stress groups than in control groups.

Reports from other laboratories about the effects of stress on estrogen receptor expression in the brain have been inconsistent. Some researchers used primary microglia, microglial cell lines, and vivo models to study the expression of ERs. But ER expression remains unclear. ERβ expression was shown to increase in monkey microglia during ischemia [56]. Sierra et al. demonstrated the presence of ERα in adult microglia, implying they are a direct target of steroid hormones, but not ERβ [57]. The expression of ERs in microglia is believed to depend on species and culture conditions and is thought to be regulated by a variety of stressful stimuli [37]. However, Byerly etal. [58] reported estrogen-related receptor β deficiency alters body composition and response to restraint stress. This result indicates that ERβ alters the function of the hypothalamic-pituitary-adrenocortical axis and indicates a role for ERβ in regulating stress response [58]. At this point, our data on ERβ expression is consistent with the report. But again, more data are needed to see the changes between each group.

3.3.3 Effects of Chronic Restraint Stress on relative levels of Inflammatory markers (IL-1ß and TNF α) in different brain regions between females and males

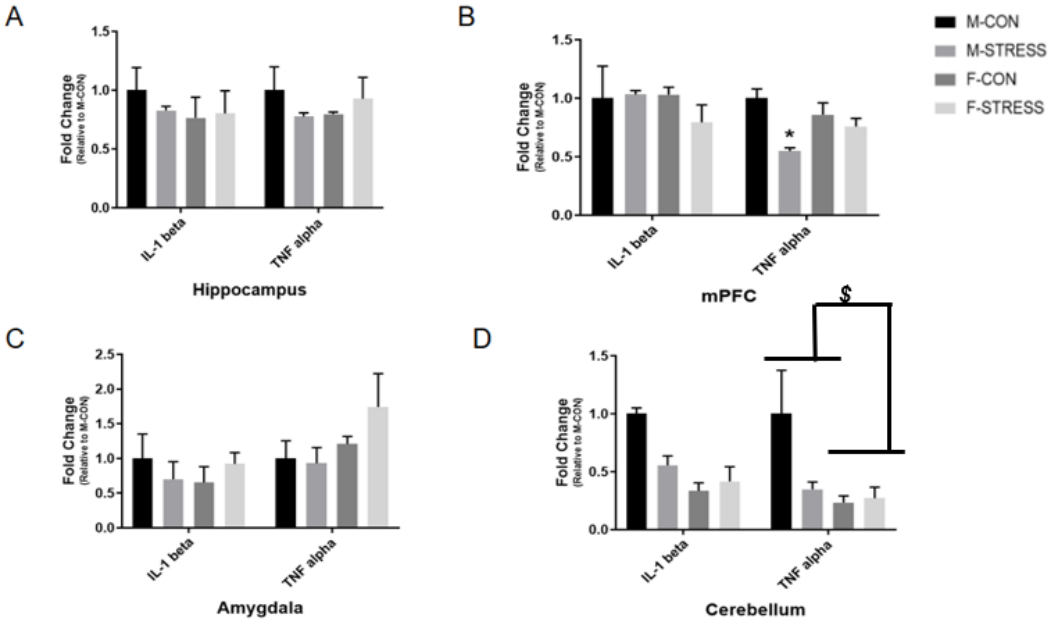

Figure 8 Effect of chronic restraint stress on Inflammatory markers (IL-1ß and TNFα) mRNA expression in (A) Hippocampus, (B) mPFC, (C) Amygdala, (D) Cerebellum. Bars indicate the mean fold changes of Inflammatory markers (IL-1ß and TNFα) mRNA relative to male co control group ± s.e.m. after normalizing to GAPDH. M-CON: male control group, F-CON: female control group, M-STRESS: male restraint stress treated group, F-STRESS: female restraint stress treated group. * indicates significant difference ($p < 0.05$) in male restraint stress treated group TNF-alpha mRNA expression in mPFC compared to male control group. $ indicates significant difference ($p < 0.05$) in CB TNF alpha mRNA expression between sex. N=4 for each group.

Results:

The effect of chronic restraint stress on Inflammatory markers (IL-1β and TNFα) expression in different brain regions between females and males is summarized in **Figure 8**. In mPFC, two-way ANOVA revealed no significant effects of sex ($F[1,11]=0.36$, $p=0.55$), stress ($F[1,11]=0.34$, $p=0.56$) or stress*sex interaction ($F[1,11]=0.61$, $p=0.45$) on IL-1β mRNA expression. Two-way ANOVA revealed a significant effect of stress ($F[1,11]=11.78$, $p<0.01$), no significant effects of sex ($F[1,11]=0.18$, $p=0.67$) or stress*sex interaction ($F[1,11]=4.79$, $p=0.05$) on TNFα mRNA expression. Post hoc analyses suggest that TNFα mRNA in male stress group was not significantly different from the female stress group and female control group but were significantly lower than in the male control group.

In cerebellum, two-way ANOVA revealed a significant effect of sex ($F[1,11]=18.7$, $p<0.005$) and stress*sex interaction ($F[1,11]=9.03$, $p<0.05$), no effect of stress ($F[1,11]=3.85$, $p=0.07$) on IL-1β mRNA expression. Two-way ANOVA revealed a significant effect of sex ($F[1,11]=5.41$, $p<0.05$), no significant effects of stress ($F[1,11]=2.88$, $p=0.15$) or stress*sex interaction ($F[1,11]=3.62$, $p=0.08$) on TNFα mRNA expression.

In the hippocampus, two-way ANOVA revealed no significant effects of sex ($F[1,11]=0.54$, $p=0.47$), stress ($F[1,11]=0.14$, $p=0.71$) or stress*sex interaction ($F[1,11]=0.35$, $p=0.56$) on IL-1β mRNA expression. Two-way ANOVA revealed no significant effects of sex ($F[1,11]=0.00$, $p=0.96$), stress ($F[1,11]=0.26$, $p=0.62$) or stress*sex interaction ($F[1,11]=0.90$, $p=0.36$) on TNFα mRNA expression.

In the amygdala, two-way ANOVA revealed no significant effects of sex ($F[1,12]=0.06$, $p=0.81$), stress ($F[1,12]=0.00$, $p=0.95$) or stress*sex interaction ($F[1,12]=1.19$, $p=0.29$) on IL-1β

mRNA expression. Two-way ANOVA revealed no significant effects of sex ($F[1,12]=2.93$, $p=0.11$), stress ($F[1,12]=0.59$, $p=0.45$) or stress*sex interaction ($F[1,12]=1.01$, $p=0.33$) on TNFα mRNA expression.

Discussion:

The goal of this experiment was to collect data on the effects of chronic restraint stress on Inflammatory markers (IL-1β and TNFα) expression in different brain regions between females and males. It will provide inference for the effects of chronic restraint stress and sex difference on microglial immune responses in the brain.

Our results indicate that only in cerebellum, chronic restraint stress reduced by 50% levels of IL-1β mRNA in stress groups than in control groups in male rats. Therefore, we can see a sex difference pattern on IL-1β expression under chronic restraint stress in cerebellum. IL-1β expression in males seems to be altered by chronic restraint stress; however, females did not. Barnard et. al. [59] showed that male and female rats underwent 4 days of stress exposure or served as non-stressed controls. Their findings revealed that stress exposure in male rats affects the regulation of brain IL-1β, while stress had no effect in the regulation of brain IL-1β in female rats. That is consistent with our data.

For TNFα expression, in mPFC and cerebellum, chronic restraint stress respectively reduced 50% and 75% levels of TNFα mRNA in stress groups than in control groups in male rats. But TNFα expression in female rats in amygdala was increased by 62% exposed to chronic restraint stress relative to controls. Overall, we can see that males tend to have a higher response

than females in TNFα expression exposure to chronic restraint stress; however, TNFα mRNA expression was inhibited by chronic restraint stress in cerebellum and mPFC.

There is a review reporting that after exposure to different stressors, cytokine mRNA expression patterns in the brain are influenced by sex [60]. Although, circulating cytokines can enter the brain through the blood brain barrier, in response to stress, microglia can also be activated and release pro-inflammatory cytokines [61]. Increases in pro-inflammatory cytokines exert neurotoxic effects on specific brain regions.

Our expectation for brain mRNA expression was that chronic restraint stress can differentially increase aromatase, estrogen receptors and inflammatory markers mRNA expression in females and males. The relative fold changes in mRNAs of inflammatory markers in females should be larger than that in males. Since more levels of estrogen could be produced in males brain, neuroinflammatory responses can be inhibited by estrogens and thus neurotoxic pro-inflammatory markers (IL-1β and TNFα) mRNA expression could be lower in males than that in females. In that way, the expectation is consistent with our hypothesis. However, taking results from 3.3, Our data provided patterns that under chronic restraint stress, aromatase mRNA expression were increased differently between sex. We saw some patterns indicating differences existing in sex and treatment in expression of estrogen receptors, but changes were not consistent. What's more, expression of inflammatory markers mRNA (IL-1β and TNFα) were inhibited somehow by chronic restraint stress which seems contrary to our expectation.

Here we only obtained data from mRNA expression to provide inferences which is not comprehensive. We haven't tested protein production levels of inflammatory markers. The results from protein levels might be different from mRNA levels. Therefore additional studies are needed to measure protein levels of inflammatory markers by ELISA or Western Blot. At the same time, larger sample sizes also are needed to adequately test the hypothesis.

3.4 **Effects of Chronic Restraint Stress on local E2 production in different brain regions**

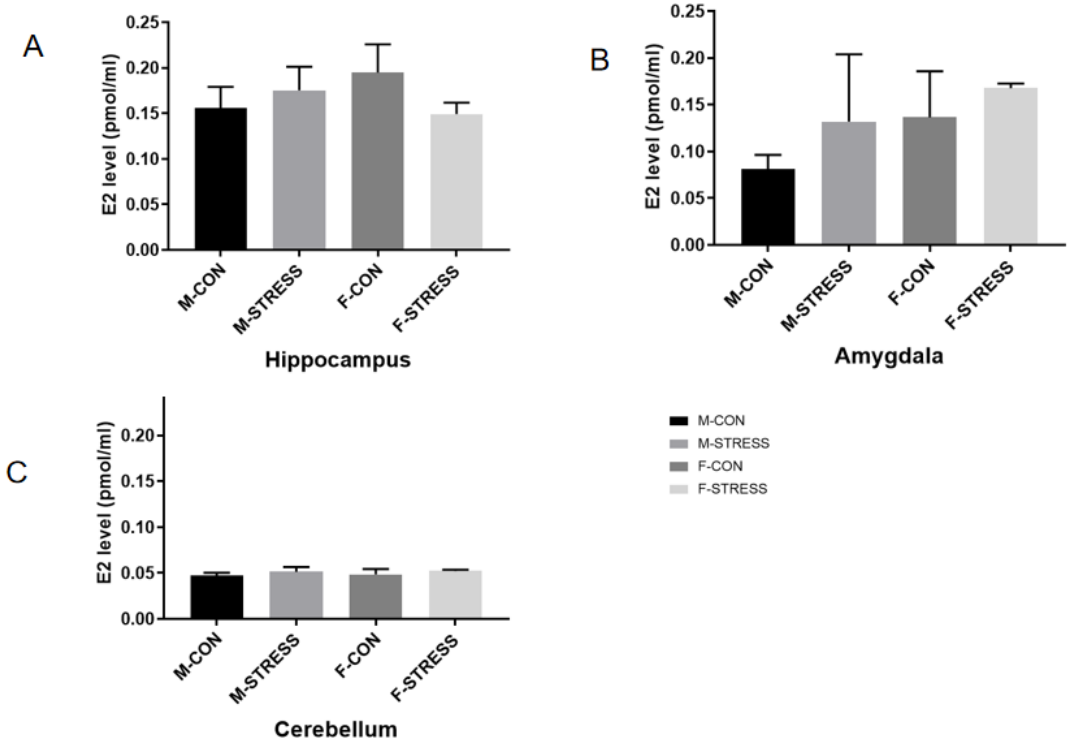

Figure 9 Effect of chronic restraint stress on local E2 production different brain regions, (A) Hippocampus, (B) Amygdala,(C) Cerebellum. Bars indicate the mean E2 levels ± s.e.m. M-CON: male control group, F-CON: female control group, M-STRESS: male restraint stress treated group, F-STRESS: female restraint stress treated group. N=4 for Cerebellum. Every 2 samples were combined and pooled to generate one point in Hippocampus and Amygdala (N=2) .

Results:

The effects of chronic restraint stress on levels of local E2 in cerebellum, hippocampus and amygdala are summarized in **Figure 9**. Every two samples were combined and pooled to generate

one point in hippocampus and amygdala. E2 levels were higher in the amygdala and hippocampus and low in cerebellum. E2 levels in the frontal cortex was below our ability to detect, even though we did detect ARO mRNA in this region and combined every two samples to generate one data point. In the hippocampus, two-way ANOVA revealed no significant effects of sex ($F[1,4]=0.06$, $p=0.80$), stress ($F[1,4]=0.28$, $p=0.62$) or stress*sex interaction ($F[1,4]=1.76$, $p=0.25$) on local E2 production. In amygdala, two-way ANOVA revealed no significant effects of sex ($F[1,4]=1.0$, $p=0.36$), stress ($F[1,4]=0.83$, $p=0.41$) or stress*sex interaction ($F[1,4]=0.04$, $p=0.83$) on local E2 production. In cerebellum, two-way ANOVA revealed no significant effects of sex ($F[1,12]=0.04$, $p=0.83$), stress ($F[1,12]=0.77$, $p=0.39$) or stress*sex interaction ($F[1,12]=0.0$, $p=0.96$) on local E2 production.

Discussion:

The goal of this experiment was to collect preliminary data and generate patterns on the effects of chronic restraint stress on local estradiol production in different brain regions. The sample size is very small (four or two animals each group), so we need more animals in each group to generate a meaningful conclusion in statistical analysis. But we still can get some inference through patterns between groups in this pilot study, though groups were not significant.

We did not see any large changes between the four groups. Although there are some changes in hippocampus and amygdala, no clear trend is observed. Our expectation is to see that sex differences in local E2 production level can be similar with long form ARO expression in the brain. If that is true, E2 produced locally in the brain can be associated with sex differences in the risk for stress-related brain neuroinflammatory response. Unfortunately, we do not get that result.

The sample size in this pilot study is very small and we need additional data to support and examine our hypothesis.

4.0 SUMMARY AND CONCLUSIONS

In this study, we developed and validated a method to detect CORT level in rat serum. We also tested E2 and T level in rat brain. Few studies reported sex differences in serum CORT levels and CORT levels in females respond more to a stressor than in males. Our data support this. We need to pay attention to this when developing additional studies. We could minimize the method for sacrificing rats. Rats serum E2 levels were undetectable in males and very low, even undetectable in females. We think the estrous cycle could account for low E2 levels. But we need more studies to confirm. Unfortunately, our analysis of serum T levels failed. This still needs to be investigated.

We have measured changes in the levels of ARO-L, estrogen receptors (ERα, ERβ and GPR30) and inflammatory markers (IL-1β and TNFα) mRNA under restraint stress. Our expectation for brain mRNA expression was that chronic restraint stress can differently increase aromatase, estrogen receptors and inflammatory markers expression in females and males. The changes of inflammatory markers in females could be larger than that in males. Our data present a pattern suggesting that under chronic restraint stress, aromatase mRNA expression was increased differently between sexes. We saw some patterns indicating differences existing in sex and stress in expression of estrogen receptors, but changes were not consistent. What's more, expression of inflammatory markers (IL-1ß and TNFα) were lowered in association with chronic restraint stress which seems contrary to our expectation. This means that our hypothesis might be wrong. But drawing conclusions only from mRNA expression is not complete, we need to test protein levels

of inflammatory markers. What's more, our study is a pilot study and the results are preliminary. Our ability to draw conclusions is very limited and more data are needed.

For local E2 levels in the brain, we expected to see sex differences in local E2 production consistent with long form ARO expression in the brain. But results did not support this. We only had two data points in each group in most brain regions which was not enough. By comparing serum E2 levels with that in brain regions, we can see that ARO activity in brain regions is significant and local levels of E2 can greatly exceed circulating levels in both males and females which is consistent with previous findings [46].

For future study, we have shown some evidence for sex differences in ARO, IL-1ß and TNFα mRNA expression as well as specific estrogen receptors. We need to increase sample size to further evaluate effects on ARO in the brain in a sexually dimorphic way, affecting local levels of estrogen in specific brain regions and resulting in sex differences in neuroinflammatory response.

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