Effect of Ovariectomy, Estrogen and Cholinergic Input on Aromatase in Different Brain Regions

by

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Our goal is to understand mechanisms by which estrogen can influence brain function and cognition. Cholinergic projections have a significant impact on neuronal plasticity and cognition in the brain. Estrogen has been shown to influence neuronal plasticity as well, and this effect can be mediated by the cholinergic system. Recent studies suggest that local estrogen synthesis, which is regulated by many neurotransmitters and hormones, can have a greater impact on neuronal survival and plasticity than systemic estrogen administration. One possible way for the cholinergic system to influence estrogen functions in the brain is through the regulation of local estrogen production. In this project, we hypothesize that cholinergic inputs can regulate aromatase (ARO) expression and activity in specific regions of the adult brain, leading to neuroprotection and increase synaptic plasticity. To test this hypothesis, a RT-PCR assay was developed to quantify ARO mRNA; and a microsomal incubation method was established to test ARO activity. First we tested the effects of ovariectomy and estrogen or G1 (a GPR30 agonist) treatments on ARO mRNA and activity in different brain regions. This was important because subsequent experiments would be conducted using ovariectomized rats. The second goal was to test the effects of removing cholinergic inputs on ARO in the hippocampus. Selective cholinergic lesions were performed, yet there was no effect of lesions on either ARO mRNA or activity in the hippocampus. The third goal was to test the effect of cholinesterase inhibitor (ChEI) treatments on ARO in different brain regions. Two ChEIs--Donepezil and Galantamine--which are used in treating Alzheimer's dementia were used. ChEI treatments changed neither ARO mRNA level nor activity in the hippocampus or frontal cortex. Hence, our results suggest that cholinergic system do not regulate ARO in these regions of the brain. This suggests that the regulation of local estrogen production is not a mechanism by which cholinergic inputs regulate neural plasticity in these regions. However, ChEIs did increase ARO activity in the amygdala, which is a region that is important for anxiety and emotion. Hence it is possible that cholinergic inputs may regulate emotional function in the amygdala through aromatase.

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1.0 INTRODUCTION

1.1 THE BENEFIT AND RISK OF ESTROGEN REPLACEMENT THERARY

Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, become an increasing concern for 21st century as the life span become longer than before. With age, there is a risk of cognitive function decline. We are interested in searching for the factors that cause the loss of learning and memory functions in old people. Particularly, post-menopausal women, accompanied by the decreasing estrogen level, are more vulnerable to cognitive decline. Many efforts have been made to prevent and protect women from loss of memory, wherein the effect of estrogen on cognitive function is considered to play a very important role.

Preclinical research suggests that estrogen has beneficial effects on cognitive functions. Systemic estrogen treatment significantly improved the performance of ovariectomized (OVX) rats in different cognitive tasks, such as: T-maze, radial maze task, visual object recognition and novel object placement task [1-5], which were used to test different types of memory. Human studies also showed beneficial effects of estrogen on specific cognitive tasks, that related to verbal memory and executive functions in younger surgically menopausal or premenopausal women [6].Estrogen was shown to help prevent the progression of Alzheimer's disease. Several papers reported that estrogen increased cerebral blood flow [7, 8] and reduced atrophy in the

hippocampus and cortex associated with aging and Alzheimer's disease (AD) [9, 10]. Clinical research has also reported that estrogen significantly reduce the memory decline in Benton Visual Retention Test (BVRT), a measure of short-term visual memory, visual perception, and constructional skills[11] and the risk of developing Alzheimer's disease (AD)-related dementia [12].

Estrogen affects brain functions by binding and activating estrogen receptors (ER). There are two nuclear estrogen receptors (ERs), ERα and ERβ [13], activation of which can regulate gene transcription but are also capable of activating second messenger signaling pathways such as mitogen-activated protein kinases (MAPK), calcium/calmodulin-dependent protein kinases (CamKII), and cAMP response element-binding proteins (CREB) [14, 15]. Recently, a novel membrane-associated estrogen receptor was identified, GPR30, which belongs to the seven-transmembrane-spanning G protein coupled receptor family, was identified [16-18]. It is located both intracellularly and on the plasma membranes and promotes a rapid estrogen signaling in a variety of cell types. In the rat brain, GPR30 exists in many regions, including the cortex, hippocampus and hypothalamus [17, 19]. Studies from our lab and others reported that GPR30 plays a very important role in mediating estrogen effects in the brain [20, 21].

Although systemic estrogen treatment was shown to be beneficial to cognition, people are concerned about its side effects. In addition to some common side effects, such as headaches, nausea, weight gain etc., estrogen replacement treatment (ERT) also increases the risk of stroke and cancers. In 2002, Women's Health Initiative (WHI) published a large study, which involved more than 16,000 healthy women, showed that the combination of estrogen and progestin increased the risk of breast cancer, heart disease, stroke, and blood clots. Although the study concluded that using estrogen alone has no increasing risk of breast cancer or heart disease, it did

appear to increase the risk of stroke. Since then, the use of HRT to relieve menopause symptoms has been sharply decreased. An alternative to ERT for the benefit of cognitive function is urgently needed.

1.2 BASAL FOREBRAIN CHOLINERGIC SYSTEM IS IMPORTANT FOR LEARNING AND MEMORY

Research in our lab suggests that the beneficial effect of estrogen on learning and memory may mediate through the cholinergic system pathway. Evidence showed that estrogen treatment enhances the cholinergic system function by increasing choline acetyltransferase (ChAT) [22-26], high affinity choline uptake in the hippocampus and frontal cortex [27], and potassium-stimulated acetylcholine (ACh) release [28, 29]. Selective cholinergic lesion in the medial septum, which reduced the ACh projections to the hippocampus, also blocked the effect of estrogen on spatial learning in a delayed matching-to-position (DMP) T-maze task [30]. On the contrary, giving acetylcholinesterase inhibitors partially restored estradiol (E2) effects on learning in aged rats [31, 32], and in young rats with partial cholinergic lesions [33]. These findings suggest that basal forebrain system is required for estrogen to influence cognition.

The basal forebrain cholinergic system is defined as the projection of cholinergic neurons from the medial septum (MS), diagonal band of Broca (DBB), and nucleus basalis magnocellularis (NBM) to the hippocampus and cerebral cortex. It was first recognized to play an important role in the regulation of learning and memory 40 years ago, where the cholinergic synapses were modified as a result of learning [34]. Administration of anti-muscarinic agents, such as atropine and scopolamine, as well as anti-nicotinic drugs, such as mecamylamine were

shown to impair the cognitive performance of rodents in many paradigms, such as spatial learning task, passive avoidance procedures and operant tasks [35-37]. Cholinergic lesions in the basal forebrain, which decreased cholinergic projections to the cortex and hippocampus, also showed the damage effect on those tasks [35]. In primates and humans, the damage of the basal forebrain system is also associated with memory deficits. These factors suggest that the basal forebrain cholinergic system is necessary for learning and memory.

Studies also indicate that the impairment of the basal forebrain cholinergic system is associated with the dementia symptom in Alzheimer's disease. The progressive loss of cholinergic neurons was shown in Alzheimer's disease patients and was accompanied by the reduction of cholinergic markers, such as choline acetyltransferase (ChAT) and high affinity choline transporter -- a rate limiting protein for acetylcholine production [38].A reduction of M1,M2-muscarinic receptors in the hippocampus and nicotinic cholinergic receptor in the cortex appeared in the transgenic Tg2576 mouse, a mouse model to mimic the production of β-amyloid, which is a pathological feature in AD patients [39]. β-amyloid can also inhibit choline uptake and decrease endogenous acetylcholine release without influence on ChAT activity under acute conditions in the hippocampus [40]. As a result, therapies to enhance cholinergic system functions, such as administration of acetylcholine precursor and the cholinesterase inhibitor (ChEI: Donepezil, Galantamine, Rivastigmine et al), which would decrease the degradation of acetylcholine, are employed to slow down the memory decline in AD patients.

Cholinergic projections are also important for regulating neuronal plasticity. For example, acetylcholine is involved in experience-dependent visual cortex plasticity at the level of synaptic transmission [41]. Muscarinic receptors are involved in, and specifically activation of the M1 receptor contributes to modifications of neural circuitry [42]. Evidence suggests that the

signal transduction cascade of the cholinergic pathway may act synergistically with the glutamate pathway through NMDA receptor , whose activation is required for cortical plasticity [41].In addition, several studies indicate that the cholinergic system mediates the effect of estrogen on cognition and plasticity. For example, administration of the acetylcholine muscarinic receptor antagonist, scopolamine, is able to block estrogenic enhancement of water maze task [43]. Destruction of cholinergic neurons in the medial septum prevented E2-mediated induction of dendritic spines on CA1 pyramidal cells [44] and reduced E2-mediated disinhibition of CA1 pyramidal cells [45]. Acetylcholine also mediates the E2 induced increase in NMDA binding in region CA1 of the hippocampus [43]. Since the manipulation of the cholinergic system influenced the effect of E2 on cognition and plasticity, we started to explore mechanisms to explain these results. The focus of my work is on one possible way for the cholinergic system to mediate estrogen function by regulating aromatase and local production of estrogen.

1.3THE FUNCTION AND REGULATION OF AROMATASE IN THE BRAIN

1.3.1 Aromatase is important for neuroprotection and the regulation of neural plasticity

As systemic estrogen treatment cannot improve learning and memory without severe side effects, local estrogen production in the brain has been increasingly drawing attention of scientists these days. Brain aromatase (ARO), which is composed of CYP19A1 and NADPH, is the enzyme that irreversibly converts androgen (both androstenedione and testosterone) to estrogen (estrone and estradiol) respectively [46]. In previous studies, the research of ARO

mainly focused on its function to regulate sexual dimorphism [47], as the highest ARO mRNA and activity were found in the preoptic area, medial amygdala and the bed nucleus of stria terminalis, which are important region for regulating sexual behavior. Recent evidence suggests that ARO also exists in other brain regions--such as the hippocampus, cortex--where it shows significant effects on neuronal survival, plasticity, and functionality in adult mammals. As a cytochrome P450 enzyme, the majority of ARO is in the microsome, whereas several papers showed that ARO immunoreactivity was also found in pre-synaptic axon vesicles in the zebra finch or other vertebrate brain, which made the local estradiol production work as neurotransmitter possible [48]. Also, ARO has been reported to be co-localized within brain regions that contain estrogen receptors (ERs). It is possible that the locally produced estrogen exerts its biological effects by binding to these intracellular ERs [49].

Several studies indicate that ARO plays important roles in neuroprotection. Evidence showed that different stress conditions, such as serum deprivation or addition of glutamate to the cultures, can enhance ARO expression in hippocampal astrocytes in vitro [50]. In vivo, ARO expression and activity also increased in several experimental disease models, such as brain injury [51], experimental stroke [52], spontaneously hypertension [53] and neuroinflammation [54]. In addition, ARO can protect the neuronal death induced by neurotoxin. Studies showed that ARO significantly protects neuronal loss in the hippocampus induced by domoic acid and kanic acid, which are well characterized neurotoxins for hilar neurons in rats. These effects can be blocked by an intracerebral injection of fadrozole, an ARO inhibitor, which indicates that the local cerebral ARO activity is involved in the neuroprotection [55]**.** Several studies also suggest that the change of ARO level in the brain is relevant to Alzheimer's disease (AD). Carbo et all showed that genetic variation of single-nucleotide polymorphism CYP19 was associated with the

earlier onset of AD [56]. Another group also found that two haplotype blocks in CYP19 increased the risk of developing Alzheimer's disease by two fold in APOE4 carriers [57]. In addition, decreased ARO expression was reported in the hippocampus of AD patients [58]. Inducing ARO and local E2 production was showed to protect hippocampal neurons from beta amyloid toxicity [59]. This evidence suggests that ARO is important to protect brain functions and prevent neurodegeneration.

Brain ARO also shows the effect on the regulation of brain plasticity, which is a possible mechanism to explain its role on cognition. Indirect evidence showed that both testosterone propionate and dehydroepiandrosterone (DHEA) increased the density of spine synapses on pyramidal neurons in the CA1 area of the hippocampus of ovariectomized (OVX) adult female rats. This can be completely blocked by letrozole, a powerful nonsteroidal ARO inhibitor [60]. In addition, ARO inhibition reduced axon outgrowth in the neonatal hippocampal cell cultures [61]. Evidence also showed that active ARO was expressed by radial glial cells, one of the neural progenitor cells in several species including rodents and birds [62-64] and IP cells during cortical neurogenesis in vitro. The same was also found in the adult teleost and zebra finch brains [65-68]. These findings suggest that brain ARO is important to regulate neuronal plasticity

This collectively suggests that like cholinergic projections, local E2 production in brain mediated by ARO can have powerful effects on neuronal protection. One possibility is that these events are connected, that the effects of cholinergic inputs in the brain are mediated via regulating the expression and activity of aromatase.

1.3.2 Regulation of aromatase

There are two ways to regulate brain aromatase (ARO). One way is through the regulation of ARO gene expression by multiple neurotransmitters and neurosteroids. The ARO gene locates on the long arm of chromosome 9. It spans 2863bp length, with 613-2124bp is the coding region contains 9 exons (2-10). Upstream are several alternative 5'-untranslated first exons resulting from the use of different promoters that are tissue specific [69].Promoter I.f has been found to regulate the production of specific brain ARO. Previous study showed that some substances, such as progesterone and the glucocorticoid, dexamethasone regulate the expression of brain ARO by binding to the specific region of promoter I.f [70, 71]. Some steroid hormones were also reported to regulate ARO through transcription level, although whether it was through the specific brain promoter I.f was still unknown. Androgens, such as testosterone and dihydrotestosterone (which was non-aromatasable) were shown to have a binding site on ARO gene to regulate its transcription and stability in a gender specific and region specific manner [72-74]. Except for the specific brain promoter I.f for ARO, other studies, which used a highly sensitive ribonuclease protection assay, demonstrated that there were two different length ARO fragments in the rat brain regions. One was 430nt length, which was associated with enzyme activity in the brain, the other was 300nt shorter, whose function was unknown and seemed to be brain specific. Different brain regions have different combinations of those two gene types, which made the study of ARO more complex [73].

Another possible way to influence ARO function is through the regulation of ARO activity. One possible mechanism is to rapidly change ARO activity though the regulation in post-transcription level. Studies done by Dr J Balthazart illustrated that in neural tissue of male Japanese quail, the presence of Mg^{2+}, Ca^{2+} and ATP in hypothalamic homogenates and the

exposure to high Ca^{2+} level following a K^{+}-induced depolarization in brain can rapidly down-regulate the local ARO activity. This was achieved by increasing the intercellular Ca^{2+} concentration to enhance the phosphorylation of ARO [75-78]. Several neurotransmitters, such as glutamatergic, catecholamine (including dopamine, noradrenaline) were proposed to regulate ARO activity in this way as they were known to regulate adenylyl cyclase activity and cyclic AMP concentration[79].

There are two kinds of acetylcholine receptor: muscarinic and nicotinic receptors. Muscarinic receptors are receptors belonging to G-protein-coupled receptors; the activation of them may trigger a second message cascade, which will deactivate adenylate cyclase and activate the K^{+} channel to hyperpolarize the cell. Nicotinic receptors are ligand-gated ion channels, binding with acetylcholine will trigger the diffusion of Na^{+} and K^{+} in to the cell and cause depolarization. Activation of both receptors will lead to the change of internal Ca^{2+} level, which makes the cholinergic system regulation of ARO possible.

1.4 OVERVIEW OF THESIS

The purpose of this project is to test whether the cholinergic system regulates aromatase (ARO) expression and activity in the brain, which if true, may provide a possible mechanism by which the cholinergic system mediates estrogen's function on learning and memory. We hypothesize that cholinergic inputs can regulate ARO expression and activity in specific regions of adult rat brain, leading to neuroprotection and increased synaptic plasticity. Specific goals were as follows: 1) test the effects of ovariectomy (OVX), E2 and G1 on ARO mRNA and activity in specific regions of the brain, 2) test the effects of selective septal cholinergic lesions

on ARO mRNA and activity in the hippocampus, 3) test the effects of systemic injection of cholinesterase inhibitor (ChEI) on ARO expression and activity in the hippocampus, frontal cortex, amygdala and preoptic area. These goals will be described in greater detail in the following chapters.

2.0 METHOD DEVELOPMENT

Overview: In this project, we hypothesize that cholinergic inputs can regulate aromatase (ARO) expression and activity in specific regions of the adult brain. To measure changes in the levels of ARO mRNA, we performed quantitative real time reverse transcription PCR (q-RT-PCR). As part of our studies, this method was validated to have a wide detectable range, high specificity and reproducibility. To test the changes in ARO activity, we first tried to use a radiometric assay described in the literature, which measures the production of tritiated water following aromatization of [^{3}H]androstenedione. This method was found to lack sufficient sensitivity to reliably detect ARO activity in adult brain tissues. Therefore, we developed a microsomal incubation method, which involves isolating microsomes and measuring the conversion of testosterone to estradiol using LC-MS-MS. This method was validated to be sensitive and reproducible. Details of these methods are described below:

2.1 qRT-PCR METHOD FOR COMPARING RELATIVE LEVELS OF AROMATASE mRNA:

2.1.1 Description of q-RT-PCR method

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

Animals were anesthetized using a mixture of 0.6mg xylazine and 3mg ketamine (overdose). Brains were removed and tissues from the hippocampus, frontal cortex, preoptic area, and amygdala were dissected. Each piece of tissue was sonicated in 1mL Trizol (Invitrogen, Inc.) at 4 ℃. The homogenate was mixed with an equal volume of chloroform and centrifuged at 12,000xg. The supernatant was collected and isopropyl alcohol was added to precipitate nucleic acids. Nucleic acids were pelleted by centrifugation, and the pellet was dissolved in DEPC water for 10 min at 42℃. DNA residues were then digested by adding DNA seI (10U/µl) and incubating for 10 minutes. The remaining RNA was re-extracted with phenol-chloroform and precipitated with sodium acetate and ETOH. The concentration of RNA was determined by spectrophotometry using a Nanodrop 2000 (Thermo Scientific). mRNA was then reverse transcribed to cDNA using the SuperScript III kit (Invitrogen, Inc.) as per manufacturer's recommendations. RNase H was used to denature any residual mRNA.

qPCR Method:

2µl of cDNA, 10µl of SYBR Green, and 1.2µl primer pair was pipetted to each well of a 96 plate (0.1mL/well). The primer pairs used for aromatase were: ARO primer paire 1: sense: 5'-GCTTCTCATCGCAGAGTATCCGG-3'; antisense: 5'-CAAGGGTAAATTCATTGGGCTTGG-3'. ARO primer pair 2: sense: 5'-CGTCATGTTGCTTCTCATCG-3'; anti-sense: 5'-TACCGCAGGCTCTCGTTAAT-3'. GAPDH was used as the control gene: GAPDH sense: 5'-TGCCACTCAGAAGACTGTGG-3'. GAPDH antisense: 5'-GGATGCAGGGATGATGTTCT-3'. The product of ARO primer 1 is 290bp length, whereas the product of ARO primer pair 2 is 150bp length. Both of the products are located in coding region of the ARO gene within exons 8 and 9. These primer pairs were

validated by Genebank primer-BLAST. Ultimately we chose to use ARO primer pair 2 since the resulting cycle number for amplification obtained with this primer pair was more consistent than with primer pair 1. The product of the GAPDH primer is 85bp length and is located on exon 6 of the GAPDH gene.

PCR was conducted using the 7300 Sequence Detection System (ABI), with an initial step at 50 ℃ for 2 min，95 ℃ for 10 mins and 15 s, then 40 cycles of 60 ℃ for 1 min. At the end of the amplification, samples were set to 95℃ for 15s， 60 ℃ for 30s, and 95℃ for15s to obtain the melt curve. Data were analyzed using Sequence Detection System (SDS) software (ABI, Inc.), and results were obtained as Ct (threshold cycle number) values. The effects of treatment on relative ARO mRNA levels were calculated using the following formula:

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

Where E = the efficiency of the PCR reaction defined as $10^{-1/slope}$ 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)

ANOVA evaluating the effects of treatment on Ct values for GAPDH did not detect any significant effects on Ct ($p=0.721>0.05$). This suggests that treatments had no significant effect on relative levels of GAPDH mRNA. All ratio values were normalized to the mean of vehicle treated controls. ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression between treatment groups and control.

2.1.2 Validation of q-RT-PCR method

Product validation: To verify whether the ARO primer pairs we designed amplify a product of the expected size, we ran the product on an 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 ARO primer pair 1 is approximately 250bp length and the product of ARO primer pair 2 is approximately 150bp length as predicted. No other bands from these two primer pairs were detected, which suggests that these primer pairs for ARO specifically amplify the ARO sequences as predicted.

Positive control: To test whether the ARO primer pair we designed was able to detect the change of ARO mRNA expression after drug treatment, we obtained the mRNA extracted from cultured rat ovarian granulosa cells (kindly provided by Dr. Anthony Zeleznik). Two groups of granulosa cells were used. One group was treated with follicle stimulating hormone (FSH), which is known to increase ARO mRNA expression in the granulosa cells. A second group was not treated with FSH and was used as a control. We used our PCR method to measure the change in ARO mRNA level between these two groups. Data were normalized to levels of GAPDH mRNA. Result in Table1 shows that with the FSH treatment, the ΔCt of ARO mRNA was 10 times less using cDNA obtained from the FSH-treated cells than for cDNA obtained from the un-treated cells. This suggests that levels of ARO mRNA were 2^{10} - fold higher in FSH-treated cells than in untreated cells, and indicates that our method is able to detect a large change in ARO mRNA expression in these cells.

Linear range: To find out the linear range of q-RT-PCR method, we did a serial dilution of the cDNA sample and used GAPDH or ARO primer pairs to test the cycle number change. Figure 1(B) and (C) show that there are linear relationship between dilution and cycle number in

the range of 17 to 34 cycles for GAPDH and 24 to 32 cycles for ARO, which suggest that q-RT-PCR method is able to quantify changes in relative levels of ARO mRNA under 32 cycle number.

Reproducibility: We also tested the reproducibility of q-RT-PCR method by calculating the coefficient of variation of both ARO and GAPDH. 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 GAPDH and ARO primer pairs were less than 2% for each time, which suggests that the method has good reproducibility.

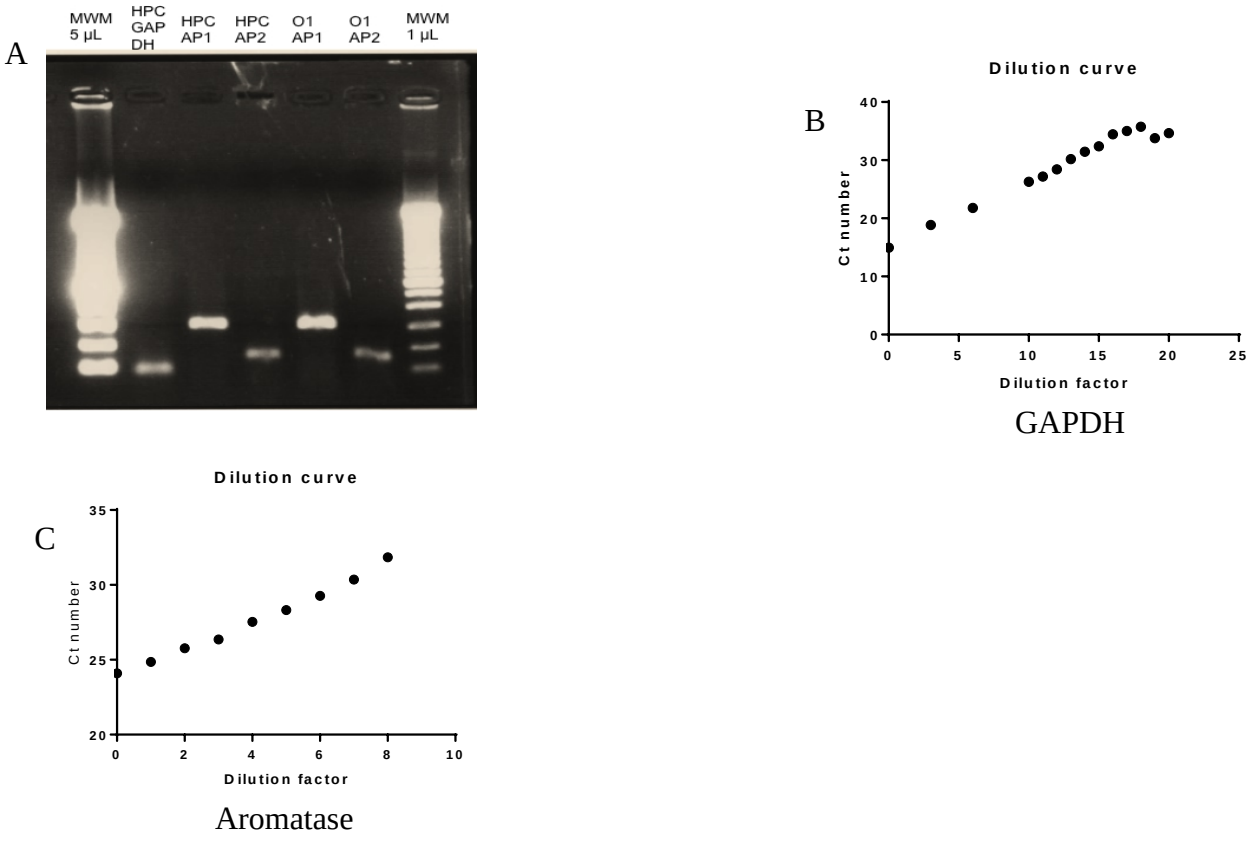


Figure 1: The validation of q-RT-PCR method: (A) Agarose gel illustrates the size of DNA products produced by PCR amplification using two ARO primer pairs and one primer pair for GAPDH. (B) Dilution curve showing change in Ct for detection of GAPDH mRNA. (C) Dilution curve showing the change in Ct for detection of ARO mRNA.

	-FSH	+FSH
GAPDH	17.8741	18.9585
Aromatase	29.3444	20.7128

Table 1: Ct number for aromatase and GAPDH obtained from granulosa cells with and without FSH treatment.

2.2METHODS FOR MEASURING AROMATASE ACTIVITY

Overview: Two methods for measuring aromatase (ARO) activity in brain tissues were developed and tested. One method is referred to as the tritiated water method. The other is referred to as the microsomal incubation method. Both methods are based on the ability of ARO to convert androgens (testosterone and androstenedione) to estrogens (estradiol and estrone). The tritiated water assay is well described and is the assay most commonly used in the literature for measuring ARO activity. In this assay, tritiated androstendione serves as the substrate, and activity is quantified by measuring the production of tritiated water. The reaction is as follow:

$$[^3H] \text{ androstendione} \xrightarrow{\text{Aromatase + NADPH}} \text{Estrone} + [^3H]\ H_2O$$

For the microsomal incubation method, a microsomal fraction is prepared from the tissues. Testosterone serves as the substrate and enzyme activity is quantified by measured the production of estradiol using LC-MS-MS. The reaction is as follow:

$$\text{Testosterone} \xrightarrow{\text{Aromatase + NADPH}} \text{Estradiol} + H_2O$$

2.2.1 Aromatase assay

i. ^{3}H-Water Method

This method was first described by Rossile (1991). Hippocampal tissue (approximately 80mg) was sonicated in buffer I (pH7.4), which contained 10mM Potassium phosphate, 100mM potassium chloride, 1mM DTT, and 1mM EDTA. 100µl of tissue sonicate was used as one point. 40pmol androstenediol (AD) mixture was prepared containing both [^{3}H]1β-AD (24.0 Ci/mmol)and cold AD (24 µM) to yield 700,000 cpm for each reaction. To begin the reaction, 20µl of 10mg/mL NADPH was added to each incubation tube. Tubes were capped, vortexed and placed into a water bath at 37℃ for one to four hours. For incubation time longer than1 hour, an additional 2.0 µl of 100mg/mL NADPH stock was added every 1 hour. To terminate the reaction, 400 µl ice cold 10% trichloroacetic acid containing 2% activated charcoal was added to each tube, vortexed and placed on ice. After that, the reaction tubes were centrifuged at 1200xg for 15min and the supernatants were filtered through AFDX-50 columns. These columns were then washed with 600µl ddH2O three times and effluents were collected in 200mL scintillation vials. Scintisafe scintillation fluid (Fisher Scientific) was added and the amount of tritium was determined using an LKB beta-counter. The total cpm included in each reaction also was tested without filtering through the column. Reference samples were included and included all components listed above with the exception of the tissue sample. The value of $^{3}H_{2}0$ was calculated by the average of triplicate samples subtracting the reference and normalized to the protein concentration. The protein concentration was determined by a Bovine Serum Albumin (BSA) standard curve (0.2-1.4mg/mL) using DC protein assay kit (Bio-Rad). Each standard and unknown was analyzed in triplicate. ARO activity is reported as cpm/h/µg protein.

ii. Limitations of this assay

Column: It was necessary to determine the appropriate length of the column for filtering each sample after completing the reaction. Specifically, the column length needed to be sufficient to remove the non-reacted [^{3}H]AD without retaining much of the ^3H_2O. As the column was hand-made, it was difficult to obtain a consistent column length. Also, the way in which samples were loaded onto the columns appeared to be critical. It appeared to be important that all of the samples went through the center of the column and through the resin so as not to pass between the resin and the side of the column. This would affect the efficiency with which [^{3}H]AD was retained. These technical issues added variability to the results which affected sensitivity (see below).

Sensitivity: Another limitation of the method was poor sensitivity and high background, resulting in the need to obtain large amounts of tissue to increase total activity. As part of our validation we compared ARO activity in ovarian tissue vs. hippocampal tissue. For ovarian tissue, ARO activity was high and activity was easy to detect. In contrast, we were not able to reliably detect ARO activity in hippocampal tissue, even when combining tissues from three animals (~500 mg). We concluded that this method would not be sensitive enough to measure changes in ARO activity in the adult brain and so proceeded to develop and validate an alternative method.

2.2.2 Microsomal incubation method

Due to the limitation of the tritiated water assay, we tried to develop a more sensitive and reproducible method to detect aromatase (ARO) activity. As estradiol is lipophilic, there is possibility that cytoplasmic membranes may sequester or trap some produced estradiol, which

makes the free estradiol undetectable. Based on that assumption, we extracted microsomes from tissues, incubated with testosterone, and used LC-MS-MS method to detect the production of estradiol. The LC-MS-MS method had been previous validated for measuring estradiol in serum was shown to be 1 pg/mL with reliable detection at 2.5 pg/mL.

i. Method

Microsome extraction: Tissues (200-300mg) were combined together and homogenized in 50mM Tris Buffer (pH 7.4) containing 150mM KCl, 0.1mM Dithiothreitol, 1mM EDTA and 20%Glycerol, and mixed with 0.113mM Butylated Hydroxytoluene (BHT) and 0.100mM Phenylmethylsulfonylflouride (PMSF) prior to the experiment at 4℃. After homogenization, we balanced the weight of the sample containing ultracentrifuge tubes and did the first spin at 90,000g for 33mins at 4℃ using the Ti70 rotor to get rid of the large cellular particles. After that, the supernatant was centrifuged at 240,000g to get the microsomal pellet. The microsomal pellets were then dissolved in 200µl 0.25M sucrose solution.

Protein assay: We used Bradford protein assay to measure the protein concentration of each sample. Briefly, Bio-Rad dye reagent was diluted 1:5 with water. 7µl microsomal solution was diluted 1:5 with 8% sucrose buffer. The protein standard solution was prepared by dissolving known concentrations of BSA into sucrose buffer. The range of the BSA standard curve was 0.1 to 0.5 mg/mL protein. 10µl of each standard solution was pipetted into each well of a 96-well plate. Each standard and unknown was analyzed in triplicate. 200µl of diluted dye was added to each well, mixed and incubated at room temperature for approximate 15 mins. Optical density at 595nm wavelength was determined using an E_{max} precesion microplate reader (Molecular Devices). The concentration of unknown was determined by interpolation of the average optical density for the unknown relative to the standard curve.

Microsomal incubation assay: To measure ARO activity, microsomes prepared from hippocampus, frontal cortex, amygdala, or preoptic area were incubated with testosterone, and the amount of estradiol produced was measured by LC-MS-MS. Microsomes were added in to the microsomal incubation buffer containing 0.12M Phosphate monobasic, 0.15M Phosphate dibasic and 5mM $MgCl_2$.10µl of 40nM testosterone was added to each sample tube to yield a final concentration of 400pM Testosterone. To start the reaction, 50µl of 0.02 M NADPH was added to each sample, vortexed for 5 sec and then placed at 37℃. After 30 mins, the reaction was stopped by rapid cooling on wet ice. 25µl Deuterated E_2 (0.5ng/mL) was added to each sample and served as an internal standard. 3mL n-butyl chloride was then added and vortexed for 1 min. The tubes were then centrifuged and the supernatant dried down under a soft steam of nitrogen at 37℃ for 20mins. 50µl 50mM Bicarb buffer and 50µl 1mg/ml Dansyl chloride were added to each tube and heated at 60℃ for 3 mins to derivatize the estradiol. E2 was eluted using a Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 X 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. Negative controls included samples that received no microsomes or no NADPH. Microsomes from ovarian tissue were used as a positive control. An estradiol standard curve also was produced and underwent extraction and derivatization at the same time as the unknowns. Known concentrations of E2 in methanol were also measured and compared with samples processed as above, to test for effects of the matrix. The amount of E2/mL in the unknowns was determined by measuring area under the curve and interpolation from the standard curve. As the volume for unknown sample was twice than the standard

solution, we adjusted the unknowns E2 value by dividing by 2. The aromatase activity is presented as pg estradiol/h/µg microsome.

ii. Validation of the Microsomal Incubation Method:

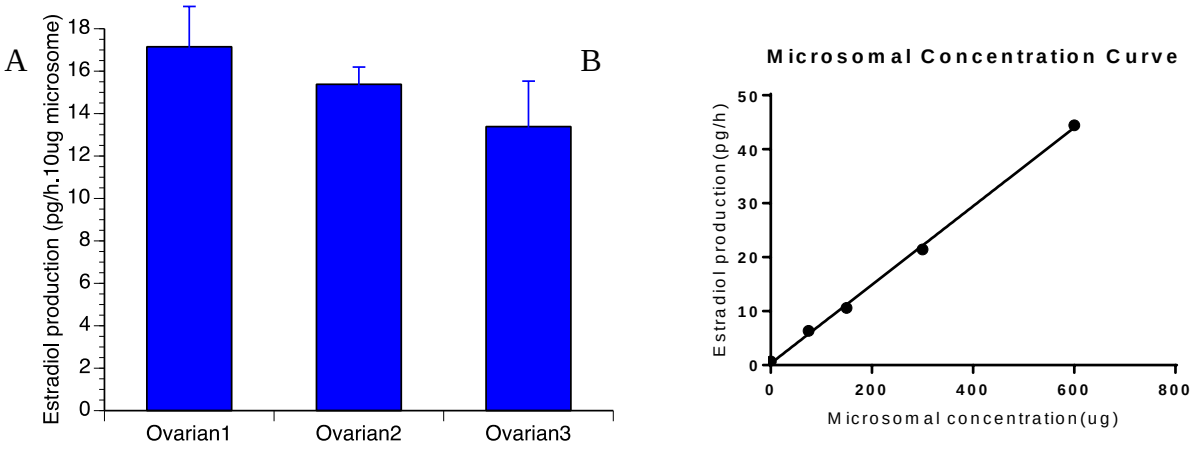
Reproducibility: To test the reproducibility of this method, we ran the same diluted ovarian samples six times each day on three different days and calculated the coefficient of variation (CV) for each run. According to the result shown in Figure 2(A), the CVs for all runs were lower than 10%, suggesting that the method has sufficient reproducibility. We do see a trend of decreased estradiol concentration over the three days. One possible explanation for this is that the microsomal activity was influenced by the repeated freeze-thaw of the tissue, leading to some decrease in enzyme activity.

Microsomal concentration curve: To verify whether this method can be used to accurately measure differences in ARO activity, a serial dilution curve of microsomes was prepared from hippocampal tissue. The range was 75µg - 600µg of microsome per reaction. Figure 2(B) shows that there is a linear relationship between microsomal concentration and estradiol production, which indicates that this method is able to accurately measure differences in ARO activity within this range.

Incubation time curve: Next we tested whether ARO activity changes with time during the incubation. Activity was measured after 10min, 20min, 30min, 45min, 60min, and 90min using microsomes prepared from the same hippocampal tissues. Figure 2(C) shows that there were two linear phases for ARO activity. The rate of the reaction was highest during the first 30 mins with a value of 0.15 pg/h/µg microsome. After that, the rate of the reaction decreased to 0.03 pg/h/µg microsome, which suggested that enzyme activity had decreased. This may be

caused by a limitation of substrate and/or co-factor, by an effect of product formation, or by changes to the enzyme itself. An incubation time of 30 mins was used in all future studies.

Testosterone concentration curve: Next we evaluated the relationship between substrate concentration and product formation, to determine the concentration of the substrate necessary to ensure saturating conditions. The substrate concentration curve is shown in Figure 2(D). From this curve we calculated that V_{max} = 73.7 pg estradiol/h/mg microsome and the Km is 94.71 nmol/L. One study reported aromatase activity in the amygdala (K_m=30nmol/L, V_{max}=79.4 fmol/h.mg protein) and preoptic area (Km=20nmol/L, V_{max}=62.5 fmol/h.mg protein) of adult male brain using the tritiated water assay [80]. Dr MaCarthy also used this method and reported the value of aromatase activity in the hippocampus（~15 fmol/h.mg protein）, cortex (~11 fmol/h.mg protein), and hypothalamus(~113fmol/h.mg protein) for new-born rats. In addition, she also saw a dramatic fall of brain estradiol level from new born to adult (1.27pg/mg protein to ~0.1pg/ mg protein), which mainly reflect the brain estradiol production [81]. The value we obtained from the microsomal incubation method is hard to compare with the values detected by tritiated water assay, as we did not test the total protein concentration we used before microsomal extraction. However, with a serial validation, we illustrated that our method is reliable and sensitive to detect aromatase activity in the female adult brain.



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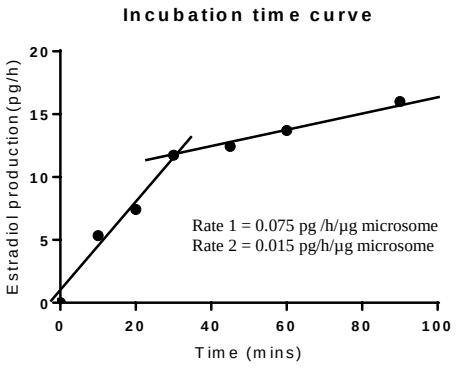

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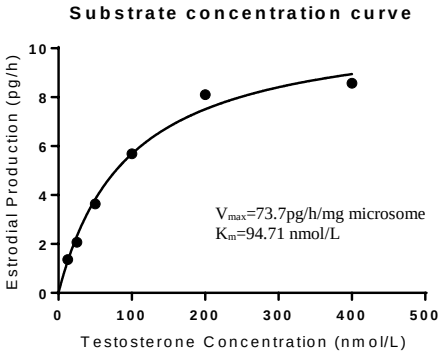


Figure 2: The validation of microsomal incubation method. (A) Graph indicates the reproducibility of this method. Bars represent for the estradiol production (pg/h*10**µ**g microsome) using diluted ovarian tissue. The coefficient of variation (CV) for each run was lower than 10%, suggesting that the method has sufficient reproducibility. (B) Microsomal concentration curve. (C) Incubation time curve. (D) Substrate concentration curve

3.0 EFFECT OF OVARIECTONMY, ESTRADIOL AND G1 ON BRAIN AROMATASE EXPRESSION AND ACTIVITY IN DIFFERENT BRAIN REGIONS.

3.1OVERVIEW

Previous studies showed that systemic injection of estradiol (E2) decreased aromatase (ARO) expression in the hippocampus, whereas continuous treatment brought about by subcutaneous implantation of E2 minipellets increased ARO expression [61]. In our first study, we tested the effects of ovariectomy (OVX), E2 and G1 (which is an agonist for GPR30) treatments on ARO expression and activity in different regions of the brain using the methods described above.

3.2 EFFECTS OF ESTRADIOL AND G1 ON RELATIVE LEVELS OF AROMATASE MRNA IN BRAIN

3.2.1 Method

40 young female Sprague-Dawley rats (10 intact, 30 ovariectomized: OVX) were purchased from Hilltop Laboratories, Inc. Rats were ovariectomized by the supplier, shipped, and then individually housed in our facility on a 12 hour:12 hour light/dark schedule with

unrestricted access to food and water. Rats were housed individually for two weeks before being used. Drugs were delivered at a dose of 5 µg/day using miniosmotic pumps (Alzet model 2002) implanted under the skin and containing either E2 or G1 dissolved in 20% ß-hydroxypropyl cyclodextran and 10% DMSO. These pumps delivered at a continuous rate of 0.5 µL/hour for 14 days. Controls received pumps containing vehicle alone.

On the day of surgery, rats were anesthetized with isoflurane and pumps were implanted under the skin on the back. Gonadally intact controls and OVX rats received pumps containing vehicle. Remaining OVX rats received pumps containing E2 or G1. After 7 days of treatment, rats were anesthetized with a mixture of 0.6mg xylazine and 3mg ketamine (overdose) and then decapitated. The brains were removed and hippocampus, frontal cortex, amygdala, and preoptic area were collected to test ARO mRNA expression using the qRT-PCR methods described above. Ratios between ARO and GAPDH were calculated to present the relative mRNA level. All ratio values were normalized to the mean of vehicle treated intact controls. ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression between treatment groups and control.

3.2.2 Result

∆ Ct values between Aromatase (ARO) and GAPDH for ovariectomized (OVX) rats are summarized in Table 1. Based on these values, ARO mRNA expression was highest in the amygdala, followed by the preoptic area, hippocampus and frontal cortex. This result is in accord with previous studies, which has reported that ARO is located in the different brain regions in the human, zebra finch and rodent [47, 69].

The effect of E2 and G1 on ARO mRNA expression in different brain regions is summarized in Figure 3. In OVX rats, mean levels of ARO mRNA were 38% greater in the hippocampus and 44% greater in the amygdala compared with intact controls. In the hippocampus, this effect was reversed by continuous E2 or G1 treatment. In contrast, in the amygdala, E2 further increased the level of ARO mRNA, whereas G1 had little effect. In the frontal cortex and preoptic area, OVX had little effect on mean levels of ARO mRNA expression compared with gonadally intact controls. E2 also showed little effect. In contrast, mean levels following G1 treatment were approximately 30% lower than gonadally intact controls in these two areas. Most of the differences that we observed were not statistically significant. ANOVA revealed no significant effect of treatment on ARO mRNA in the hippocampus ($F[7,31]=2.11$) , frontal cortex($F[7,31]=1.15$) and preoptic area($F[7,31]=0.807$). In the amygdala, post-hoc analysis revealed that E2 significantly increased the relative level of ARO mRNA ($p<0.05$) compared with the intact control.

3.2.3 Discussion

The goal of this experiment was to evaluate the effects of OVX, E2 and G1 treatment on relative levels of ARO mRNA in different regions of the brain. This was done to provide information about the effects of OVX and ER agonist treatments in advance of subsequent experiments which would be conducted in OVX rats.

Our results suggest that OVX results in elevated levels of ARO mRNA in the hippocampus and amygdala relative to gonadally intact controls. Differences were in the range of approximately 30-40%, but were not statistically significant using the current methods. In the hippocampus the effect appeared to be reversed by E2 and G1 treatment; whereas, in the

amygdala the effect was increased significantly by E2 treatment. This suggests that in the hippocampus, OVX increases and estrogen can suppress ARO expression and that the effects may be mediated via GPR30. In contrast, in the amygdala, it appears that both OVX and E2 can increase ARO mRNA expression, and activation of GPR30 appears to have little effect. The results suggest a role for GPR30 as well as other estrogen receptors in regulating ARO expression. The different effects observed in the hippocampus and amygdala may reflect differences in the expression of these receptors.

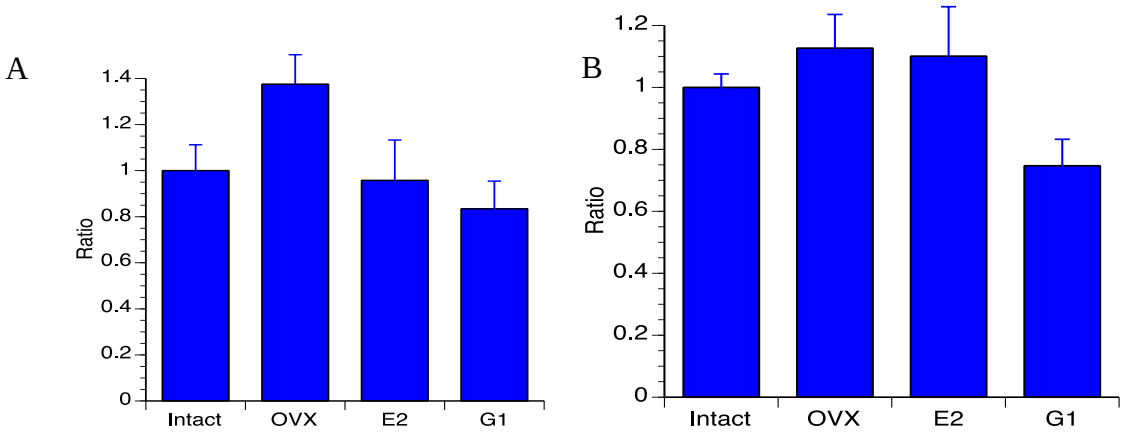
In the frontal cortex, G1 treatment produced a slight (~30%) decrease in ARO mRNA, whereas OVX and E2 treatment had little effect. Similar results were observed for the preoptic area. This suggests that in these regions estrogen has little influence over ARO mRNA expression, though there remains some evidence for regulation by GPR30. Note that in all of the regions studied, G-1 was associated with a decrease in mean levels of ARO mRNA relative to OVX rats, suggesting that activation of GPR30 may have a negative influence on ARO expression.

Reports from other laboratories about the effect of estrogen on aromatase in the brain have been inconsistent, and appear to depend on brain region, sex and species. Abdelgadir et al. reported that IP injection of 17β -estradiol down-regulates ARO expression, whereas tonic estrogen treatment up-regulates ARO expression in the hippocampus[82]. Balthazart reported that E2 had no effect on ARO mRNA in the preoptic area and amygdala of castrated male mice[83]. Analysis of the ARO gene shows that progesterone, glucocorticoid, and androgens, have specific binding sites on ARO gene [70-74], whereas there is no direct regulation site for estrogens. It is possible that E2 regulates ARO mRNA expression through the regulation of other steroids; however, our data suggest that E2 may influence ARO mRNA expression via GPR30.

It is important to note that most of the effects that we observed on relative levels of ARO mRNA were not statistically significant. This may be due to limitations in the qRT-PCR methods. The delta-delta C_t method is commonly used to quantify changes in relative levels of mRNA in tissues, but generally requires effects on the order of 2-fold or greater (i.e., one PCR cycle) to achieve statistical significance. However, many studies show that changes in relative levels of mRNA that are less than 2-fold can have some biological significance. Our findings provide preliminary data showing that OVX and E2 can influence ARO mRNA expression in a regionally specific way, and it is the first to provide some evidence that GPR30 may play a role. More sensitive methods, such as Northern blot or solution hybridization studies, could be used to further evaluate these findings.

Different brain regions	**Hippocampus**	**Frontal Cortex**	**Amygdala**	**Preoptic area**
ΔCt (ARO-GAPDH	9.40	10.61	8.14	9.58

Table 2: Relative level of ARO mRNA in the different brain regions for ovariactomized(OVX) rats. Numbers indicate theΔCt between ARO and GAPDH.



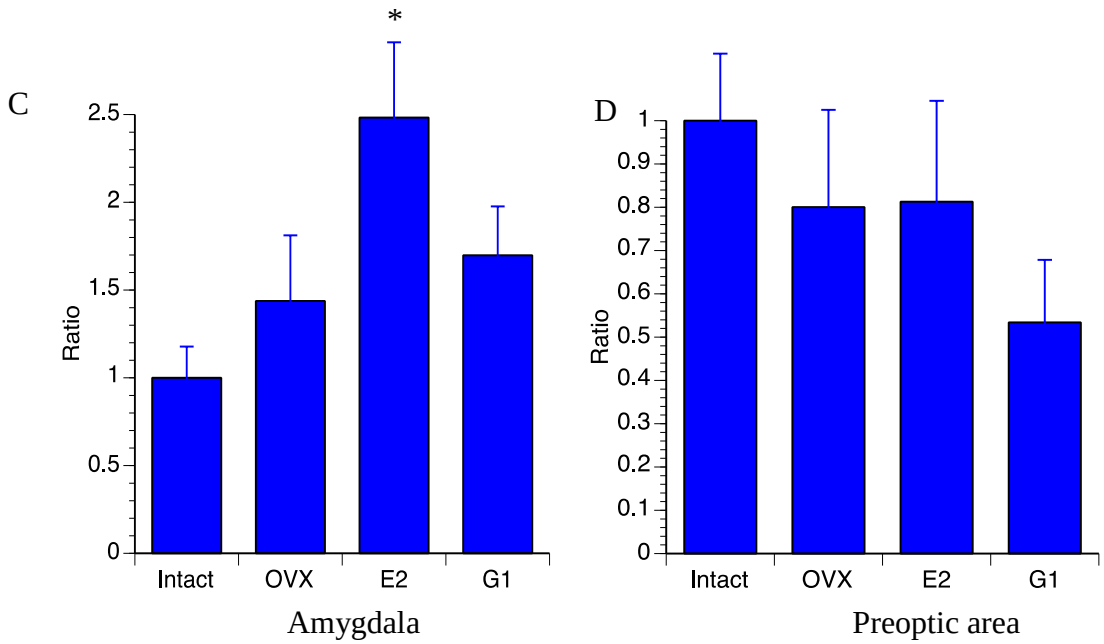


Figure 3: Effect of OVX, E2 and G1 on ARO mRNA expression in (A) Hippocampus, (B)Frontal cortex, (C) Amygdala and (D) Preoptic regions. Bars indicate the mean ratio of ARO mRNA relative to Intact controls ± s.e.m., after normalizing to GAPDH. * indicates the $p \leqslant 0.05$ compared to control group. N=8 for each group.

3.3EFFECT OF ESTRADIOL AND G1 ON AROMATASE ACTIVITY

3.3.1 Method

As a follow-up to our analysis of the effects of OVX, E2 and G-1 on relative levels of aromatase (ARO) mRNA, we conducted a preliminary experiment to test the effect of OVX, E2 and G1 on ARO activity using the microsomal assay described above. 20 OVX rats (n=4 for OVX- and G1-treated groups, n=6 for each E2-treated group) were used. These rats were treated with E2 at doses of 1µg/day and 2.5µg/day. Rats were housed in our facility for 1 week before

treatment. Different concentrations of E2 (1µg/day, 2.5µg/day) were used to mimic the physiological E2 levels of intact rats. 5µg/day of G1 was given to test the effect of activating GPR30 on ARO activity. The Alzet model 2002 miniosmotic pumps were used to deliver drugs continuously for 7 days. Controls received pumps containing vehicle. Hippocampus, frontal cortex, amygdala and preoptic area, were collected as described above. Serum was collected for measurement of circulating levels of E2. In order to extract 300µg microsomes from each region, we pooled 4 or 6 hippocampi, frontal cortex, amygdala or preoptic area together to get one data point for each region. Each bar in the result represented the one pooled sample for each treatment group for different brain regions.

3.3.2 Result

The distribution of aromatase (ARO) activity in the different brain regions is summarized in Figure 4(A). ARO activity was highest in the amygdala, followed by the preoptic area, hippocampus**.** ARO activity in the frontal cortex was below our ability to detect, despite the fact that we did detect ARO mRNA in this region.

The effect of E2 and G1 on ARO activity in different brain regions is also shown in Figure 4. In rats treated with 1µg E2/day ARO activity in the hippocampus was approximately 40% less than in controls. The effect was not as great in rats treated with 2.5 µg E2/day (~16%), but was greater in rats treated with G1 (~60%). In the amygdala, both low and high doses of E2 seemed to have little effect on ARO activity, whereas the ARO activity was 50% higher in the G1-treated group than in controls. In the preoptic area, rats treated with 1 and 2.5 µg E2/day had higher levels of ARO activity (>60% in each case) relative to controls, whereas G1 had less of an effect (~30%). No ARO activity was detected in the frontal cortex. These data were preliminary

as only one data point for each treatment group in each region; however, each data point represents a pooled sample, and hence the mean activity in 4-6 rats.

3.3.3 Discussion

The goal of this experiment was to collect preliminary data on the effects of OVX and of E2 or G1 treatment on aromatase (ARO) activity in different regions of the brain.

Results showed detection of ARO activity paralleled differences in relative levels of ARO mRNA, with highest levels of activity detected in the amygdala, and followed by preoptic area and hippocampus. Based on our initial estimates of activity in each region, tissues were pooled in order to obtain sufficient microsomes to reliably detect activity in each region. This required combining from 2-4 rats per region.

For the effect of estrogen on ARO activity, results showed that in the hippocampus, levels of ARO activity were less in E2 and G1-treated rats compared with OVX controls. This is consistent with the effects on relative levels of ARO mRNA shown above. Together, these results suggest that E2 may regulate ARO activity through the regulation of ARO mRNA and that this effect may be mediated through GPR30. However, this result must be treated as very preliminary since the data represented only one data point per treatment. In addition, with E2 at 1µg/day, ARO activity decreased more than with E2 treatment at 2.5µg/day, which may suggest that there is a dose-related effect of E2 on ARO activity. Further study is needed to confirm this effect.

In the amygdala, E2 had little effect on ARO activity, whereas G1 increased ARO activity by 50% compared with OVX control. The lack of effect of E2 on ARO activity was not in agreement with the increase in ARO mRNA shown above; however, the effect on ARO mRNA was observed using a higher dose of E2 (5 μg/day), which may account for the difference. The effect of G1 on ARO activity was, however, consistent with the increase in ARO mRNA shown above. This provides further evidence that activation of GPR30 can regulate ARO expression and activity.

In the preoptic area, rats treated with 1.0 and 2.5 μg E2/day had elevated levels of ARO activity (~60% greater) relative to controls. G1 had less of an effect (~26% greater). This is in contrast to the effects of E2 and G1 on mean level of ARO mRNA shown above.

As reported above, no ARO activity was detected in the frontal cortex, despite the fact that ARO mRNA was readily detectable. This suggests that local estrogen expression is very low in this region and may not be biologically relevant. One possibility is that the low level of ARO activity reflects the amount of expression for the short version ARO mRNA. The primers that we used for the RT-PCR analysis amplified a region located between exon 9 and 10, and cannot distinguish the longer active ARO RNA from the shorter inactive form. Therefore, it is possible that the ARO RNA in the frontal cortex is a non-active form the function of which is unknown.

Collectively, these data provide preliminary data suggesting that E2 and G1 can influence ARO activity in the brain, that the effects are region-specific, and that activation of GPR30 can play a role. To our knowledge, this is the first indication that activation of GPR30 can influence ARO expression and activity in the brain.

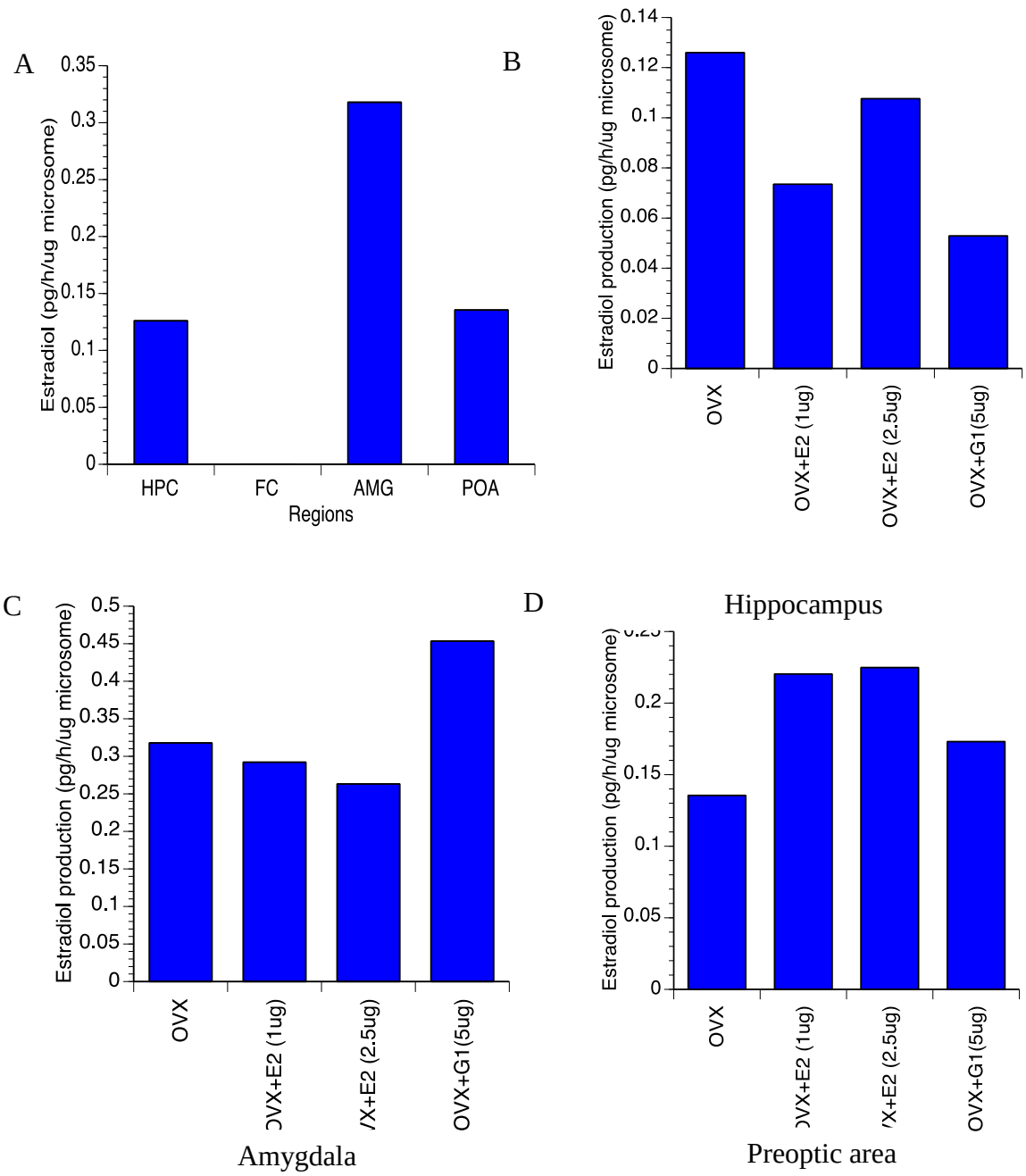


Figure 4: Distribution of ARO activity in the different brain regions (A) and effect of OVX, E2 and G1 on ARO activity in (B) hippocampus, (C) Amygdala and (D) Preoptic regions. Bars show ARO activity as the rate of estradiol production (pg/h/**µ**g microsome) in pooled samples. Each bar represents activity from a pool of 4-6 samples.

4.0 INVESTIGATION OF THE EFFECTS OF SELECTIVE CHOLINERGIC LESION ON AROMATASE EXPRESSION AND ACTIVITY IN THE HIPPOCAMPUS

4.1 OVERVIEW

Studies showed that estrogens can influence synaptic plasticity in the hippocampus as well as performance on specific cognitive tasks. We hypothesized that the cholinergic inputs may influence aromatase (ARO) activity in the hippocampus, with corresponding effects on local estrogen production. In this experiment, we selectively eliminated cholinergic inputs to the hippocampus, and looked for changes in ARO expression and activity.

4.2**METHOD**

4.2.1 Cholinergic lesion:

Forty-six, 3 month old Sprague-Dawley (SD) rats were purchased and housed in our animal facility for two weeks. 20 rats were used to test for effects on relative levels of ARO mRNA. The other 26 rats were used to test for effects on ARO activity. Rats were anesthetized using a mixture of ketamine (36mg/kg) and xylazine (7.2mg/kg), and then placed on a standard stereotaxic apparatus. The skull was exposed and a hole was drilled at midline 0.3mm rostral to

Bregma. A 28ga stainless steel cannula was lowered -5.6mm from dura to the medial septum based on the stereotaxic atlas of Paxinos and Watson (1986). 2.0µl of 192IgG-Saporin (SAP; Advanced Targeting Systems, Inc.), which is a selective cholinergic immunotoxin, was injected at a rate of 0.2µl /min. Previous studies showed that medial septal SAP injection caused a selective loss of cholinergic cells in the basal forebrain with little non-selective damage to GABAergic neurons [84].This is also accompanied by decreased activity of choline acetyltransferase (ChAT) and by reduced high-affinity uptake of [^{3}H]choline into cholinergic nerve terminals in the cerebral cortex and hippocampus [85]. Control group received the same amount of saline injection. The skin was sutured and rats were placed onto a heating patch until they woke up. Following surgery, rats received ketofen (3.0 mg/kg) for three days to relieve pain. After 14 days of recovery, rats were dissected and the hippocampus and frontal cortex was collected and analyzed for ChAT activity, qRT-PCR and ARO activity. Tissues containing the medial septum (MS) were fixed by immersion in 4% paraformaldehyde in 50mM phosphate buffered saline (PBS, pH7.2) at 4˚C overnight. These tissues were then transferred to 20% sucrose in PBS at 4 °C for several days. Sections through the MS were cut and stained for ChAT- immunoreactivity as described below.

4.2.2 ChAT assay

In order to verify the lesions, we measured ChAT activity in the hippocampus and frontal cortex. The expectation was to see a substantial reduction in ChAT activity in the hippocampus with little change in ChAT activity in frontal cortex in lesioned rats. Briefly, 30mg tissues were sonicated in 300µl medium (10mg tissue/mL) which contains 10mM EDTA and 0.5% Triton X-100. 10µl of substrate solution, which contains 0.25mM [^{3}H] acetyl-CoA (50,000-60,000

dpm/tube), 10.0mM choline chloride, 0.2mM physostigmine sulfate, 300mM NaCl, 50mM sodium phosphate buffer(pH 7.4) and 10mM EDTA was added to each reaction tube. 5µl (approximately 45mg) aliquots of sample were added to the tubes and incubated for 30 min at 37 ℃. Each sample was assayed in triplicate. The reaction was terminated with 4 mL sodium phosphate buffer (10mM) at 4℃ in the scintillation well. The production of [^{3}H] acetylcholine was detected by adding 1.6 mL of acetonitrile containing 5mg/mL tetrephenylboron followed by the addition of 8 mL EconoFluor scintillation cocktail (Packard Instruments, Meriden, CT) and counting cpm in the organic phase using an LKB beta-counter. The total cpm in the substrate solution was determined by aqueous scintillation cocktail. Background was determined using identical tubes with no sample added. Reference was incubated with no sample added. For each sample, triplicates were averaged and the difference between cpm in the sample and reference was calculated. The final amount of acetylcholine production was normalized to the total cpm in the substrate and protein concentration of each sample, which was detected as the average of aliquots using Bio-DC assay. ChAT activity was calculated for each sample as pmol acetylcholine manufactured/h/µg protein.

4.2.3 ChAT Immunocytochemistry (ICC)

To further confirm whether SAP treatment eliminated most of cholinergic neurons in the medial septum, we cut sections through the medial septum and stained them for ChAT using immunohistochemistry. Briefly, sections were rinsed in 50 mM PBS for one hour and then transferred to 0.3% H_2O_2 in 50 mM PBS for 10 mins. Sections were rinsed again in PBS, and then placed in a solution of 50 mM PBS, 5% normal horse serum and 0.05% Triton X-100

containing primary antibody against ChAT (goat anti-ChAT 1:3500). Sections were exposed to antibody for three days at 4℃. Then sections were rinsed with PBS for 30 mins and incubated with a Biotinylated secondary antibody (horse anti goat 1:220, Vector Laboratories) for 1 hour at room temperature. Sections were then rinsed with PBS for another 30 mins and placed in an A/B 'Elite' Horseradish peroxidase solution for 1 h at room temperature. Sections were then rinsed with Tris acetate solution (50mM, pH7.6) and transferred to the Tris/DAB solution, which contained 0.5 mg/ml 3,3'-diaminobenzidine, 0.01% H_2O_2 and 0.032% NiCl2 for 10 min. Sections were then rinsed with PBS, mounted onto glass slides, dehydrated, coverslipped and examined with a Leitz photomicroscope.

4.2.4 Tissue analysis

We used q-RT-PCR method to test ARO expression and microsomal incubation assay to test ARO activity, as described above. For ARO mRNA, ratios between ARO and GAPDH were calculated to present the relative mRNA level. All ratio values were normalized to the mean of saline treated OVX controls. ARO activity was presented as pg estradiol production per hour, per μg microsomes. ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression between treatment groups and control.

4.3RESULTS

Lesions were evaluated by confirming loss of ChAT-positive cells in the septum, and by loss of ChAT activity in the hippocampus. From Figure 5, SAP treatment eliminated most of ChAT-positive cells in the medial septum, which indicated a severe loss of cholinergic neurons in this region. Accordingly, ChAT activity in the hippocampus was significantly lower (<80%) in the lesion treatment group than in the control group ($P<0.05$). However, the ChAT activity in the frontal cortex did not decrease significantly, which was in agreement with results previously reported [86]. In the aromatase activity experiment, we did see several partial lesion rats with SAP treatment validated by immunohistochemistry, which may be caused by inappropriate injection site. As a result, we combined the partial lesion samples together as one group to detect aromatase activity. For the effect on aromatase (ARO), relative levels of ARO mRNA were 35% higher, and levels of ARO activity were 18.9% higher, in the hippocampus of rats with lesions vs. non-lesioned controls (Figure 6). Partial lesion also had little effect on ARO activity. ANOVA revealed no significant effect of the treatment on ARO mRNA ($F[5,4]=2.635$) and activity ($F[6,21]=0.287$) in the hippocampus. Since the ChAT activity did not change in the frontal cortex, this brain region was used as a negative control. The relative level of ARO mRNA did not change in this area and the ARO activity in this region was not detected.

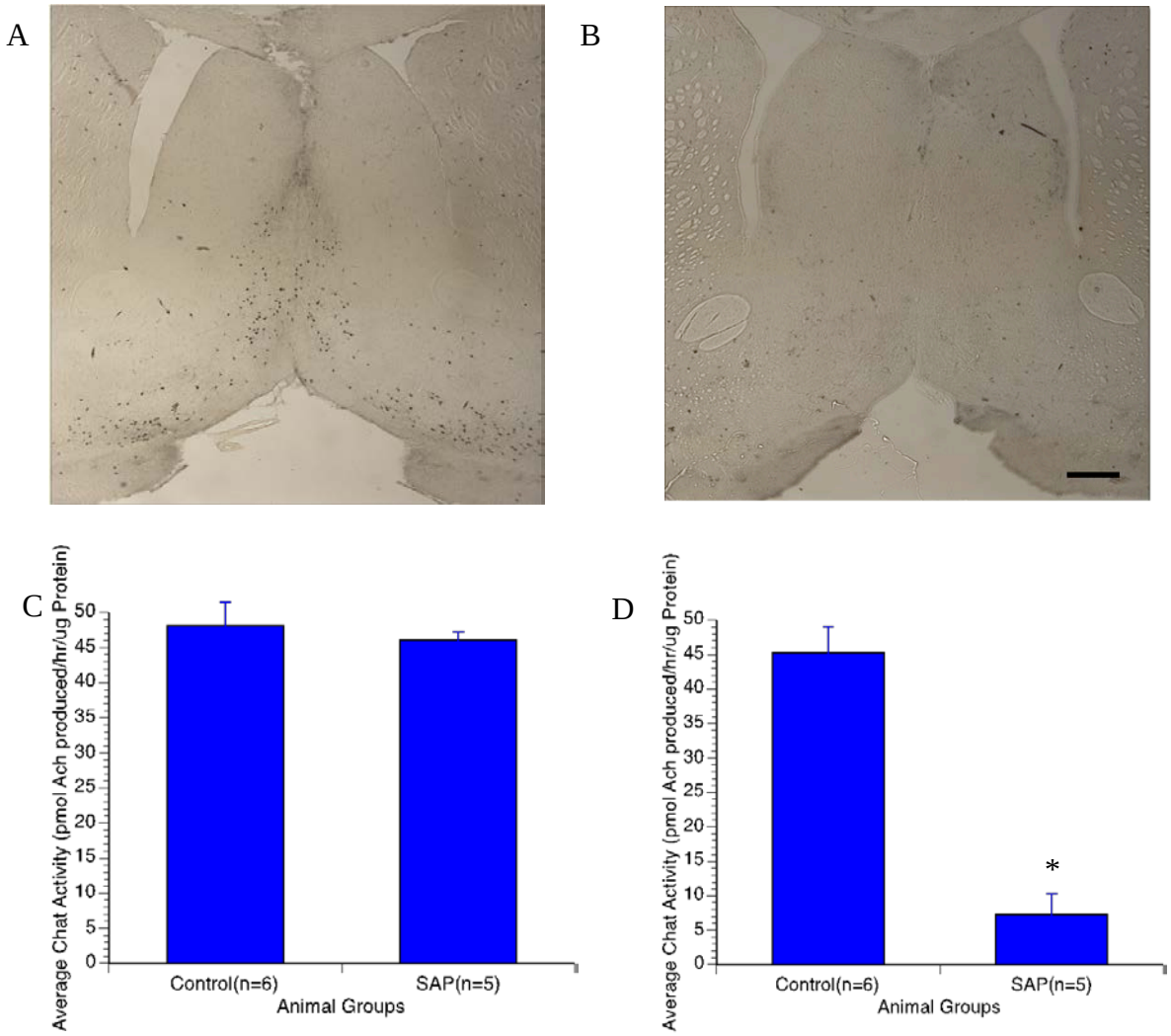


Figure 5: Effect of Saporin-induced cholinergic lesions in the medial septum on choline acetyltransferase (ChAT) activity. Panel A and B shows ChAT immunoreactivity in the medial septum of rats treated with (A) Saline and (B) 192-Saporin (SAP). Eleven days following SAP treatment, ChAT activity was evaluated in the (C) Frontal Cortex and (D) Hippocampus. After SAP treatment, no significant change in ChAT activity was measured in frontal cortex tissue while Hippocampal tissue shows significant reduction in activity. In all panels, rostral is toward the top and midline extends top to bottom through the middle of the panel. Scale bar =0.5mm.

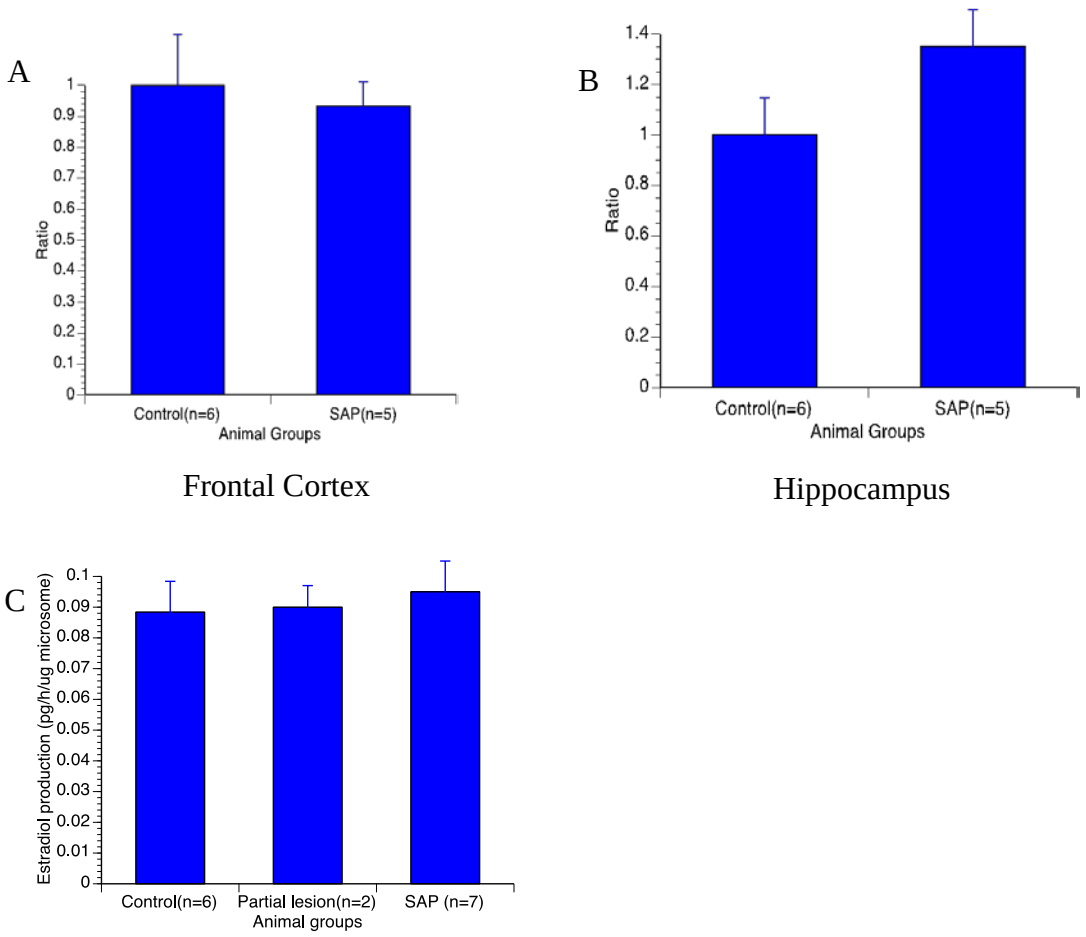


Figure 6: Effect of cholinergic lesion in the medial septum on ARO mRNA expression in (A) Frontal cortex, (B) Hippocampus and ARO activity in (C) Hippocampus. No significant change was found in region-specific ARO mRNA and activity. Bars in (A) and (B) indicate the mean ratio of ARO mRNA relative to OVX controls ± s.e.m., after normalizing to GAPDH. Bars in (C) indicate the estradiol production (pg/h*µg microsome) ± s.e.m, which represent the ARO activity.

4.4DISCUSSION

In this experiment, we tested the effect of removing cholinergic afferents on aromatase (ARO) mRNA and activity in the hippocampus. Our data suggest that removal of over 90% of the cholinergic afferents had a modest effect at best on relative levels of ARO mRNA and ARO activity in the hippocampus. Analysis of ChAT activity as well as inspection of the cholinergic neurons by immunohistochemistry confirm that the lesions were successful and, therefore, that the results were not due to a failure of the lesion surgeries. This suggests that cholinergic inputs to the hippocampus do not play a major role in regulating ARO in this region of the brain and therefore do not significantly regulate local estrogen production in this region. As a consequence, cholinergic regulation of local estrogen production is not likely to contribute to the effects on synaptic plasticity or hippocampal function.

This result was not our expectation as estrogen was shown to have many effects on neuroplasticity and cognition and these were mediated through the cholinergic system. In addition, studies have shown increasing the intercellular Ca^{2+} concentration can enhance the phosphorylation of ARO [75-78]. Both m1-m4 and α7 nicotinic receptors are located in the hippocampus, whereas α7 is expressed by GABAergic as well as glutamatergic synapses in the CA1 stratum radiatum [87], activation of which can change the intercellular Ca^{2+} level in these cells. We anticipated that cholinergic inputs may regulate ARO activity through the regulation of internal Ca^{2+} concentration. However, since our results showed relatively little effect of the cholinergic lesions on ARO, we suspect that effects on local estrogen production do not play a significant role in cholinergic effects on plasticity in this region.

There is another possible explanation for the little effect on ARO observed. Studies have shown that SAP lesions of cholinergic neurons are companied by a significant activation of microglia and a lesser extent of astrocyte cells in the hippocampus [85]. As has been shown before, ARO has neuroprotective function and different stress conditions can induce astrocyte ARO expression [51-54]. Study showed that cytokines released by microglia can also trigger the expression of ARO in astrocytes [54]. It is possible, therefore, that the cholinergic lesions induced ARO expression and activation in astrocytes, which compensated for a down-regulation of ARO in neurons of hippocampus. Since our methods do not distinguish ARO in neurons vs. astrocytes, we cannot rule out the possibility that cholinergic denervation had significant effects on ARO expression in specific cell populations which, collectively, are reflected by relatively little change in total ARO activity.

5.0 EFFECTS OF CHOLINEASTERASE INHIBITORS ON AROMATASE EXPRESSION AND ACTIVITY IN DIFFERENT BRAIN REGIONS.

5.1 OVERVIEW

After testing the effect of down-regulating cholinergic input to the hippocampus on aromatase (ARO) mRNA expression and activity, we tested the effect of increasing cholinergic activity by treating with cholinesterase inhibitors (ChEIs). Two ChEIs: galantamine and donepezil, which are employed to slow down memory decline in AD patients, were used to inhibit the activity of acetylcholinesterase, which is an enzyme that degrades acetylcholine. We then tested the effects of systemic administration of these drugs on ARO expression and activity in different brain regions.

5.2METHOD

A total of 48 rats (270-350g) were treated intraperitoneally with 3mg/kg donepezil, 5mg/kg galantamine or saline (as control) injected once daily for 7days. 12 rats were used to test the effect of ChEIs on relative levels of ARO mRNA (4 rats per group), 36 rats were used to test effects on ARO activity (12 rats per group). Animals were killed, the brains were removed and hippocampus, frontal cortex, amygdala and preoptic area were collected and evaluated for levels

of ARO mRNA and ARO activity as described above. For ARO mRNA, ratios between ARO and GAPDH were calculated to present the relative mRNA level. All ratio values were normalized to the mean of saline treated OVX controls. For ARO activity, tissues were pooled (n=2/hippocampus, n=3/frontal cortex, n=3/amygdala, n=4/preoptic area) to get one data point for each region. Microsomes were extracted as above, and then incubated with testosterone and NADPH for 30 mins. Then the estradiol was extracted as described above and the values were determined by comparison with the standard curve. ARO activity was reported as pg estradiol production per hour, per µg microsomes. ANOVA was used to analyze the differences between group means. Tukey HSD was used to determine significant differences in expression between treatment groups and control.

Donepezil is a piperidine-based highly potent mixed, non-competitive reversible inhibitor of acetylcholinesterase, with an in vitro IC50 of approximately 6.7nM and an in vivo ID50 of approximately 2.6mg (6.8µMol)/kg brain tissue [88]. Donepezil has a long half-life (~70 hours) and is highly bound to albumin [88, 89]. Donepezil is approved for treatment of memory decline associated with Alzheimer’ s disease.

Galantamine is a less potent ChEI than donepezil and has a shorter half-life [90], but has less binding to serum proteins and a better side-effect profile in humans. In addition, it has been shown to act as an allosteric enhancer at nicotinic receptors [91, 92]. A study by Geerts [93] suggests that doses in the range of 1.5-5.0 mg/kg in rats produce optimal brain concentrations for the allosteric potentiating ligand effect of galantamine. Like donepezil, galantamine is approved for treatment of memory decline associated with Alzheimer’s disease.

5.3RESULT

Effects of donepezil and galantamine on ARO mRNA and ARO activity are summarized in Figure 7. As shown in Figure 7 (A), no significant effects on relative levels of ARO mRNA were detected in the hippocampus, frontal cortex, or amygdala of rats treated with ChEIs vs. controls. In the POA, however, an increase in ARO mRNA was detected following treatment with donepezil (64%) and galantamine (90%, $p=0.08$). These effects were not statistically significant.

Figure 7 (B) summarizes effects on ARO activity. No significant effects of CHEIs treatment on ARO activity were detected in the hippocampus or preoptic area, and ARO activity in the frontal cortex was undetectable. In contrast, ARO activity in the amygdala was 30% greater in rats treated with donepezil, and 45% greater in rats treated with galantamine, relative to controls. The effect of galantamine was statistically significant ($p<0.05$).

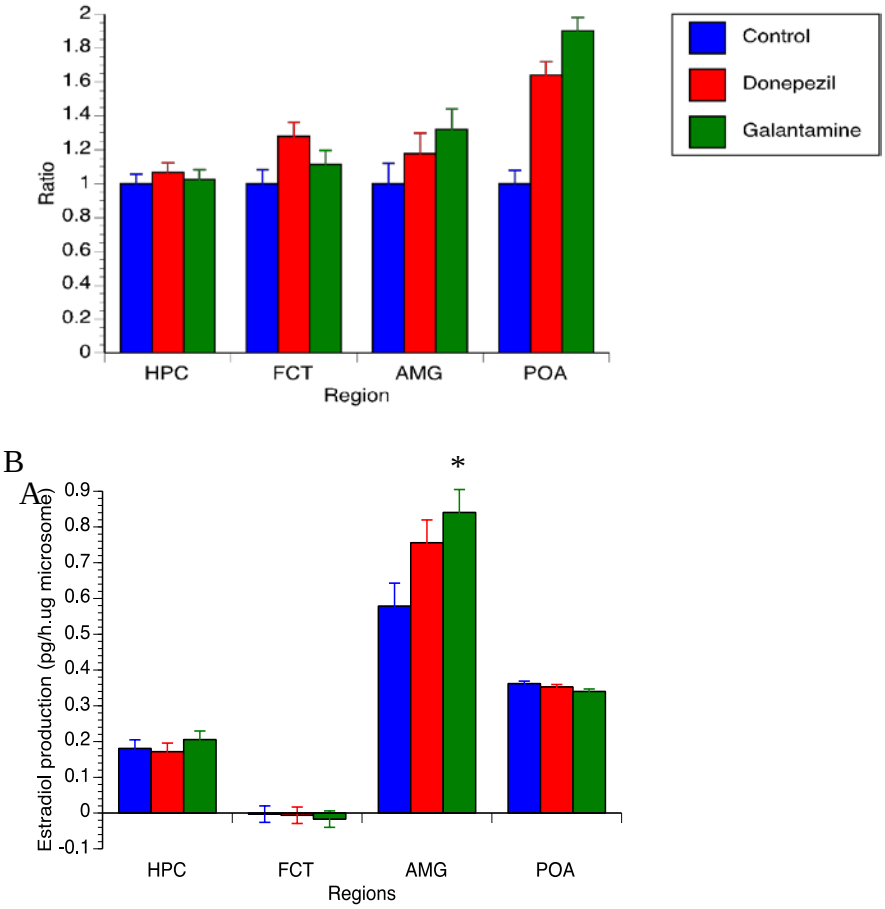


Figure 7: Effect of Donepezil and Galantamine on (A) ARO mRNA expression and (B) ARO activity in different brain regions of OVX rats. Bars in (A) indicate the mean ratio of ARO mRNA relative to controls ± s.e.m., after normalizing to GPADH. Bars in (B) indicate the mean of estradiol production (pg/h* **µ**g microsome) ± s.e.m., which represent the ARO activity. HPC: hippocampus. FCT: Frontal cortex. AMG: Amygdala. POA: preoptic area. * indicates the p $\leqslant$0.05 compared to control group.

5.4DISCUSSION

In this experiment, we tested the effect of two ChEIs on ARO expression and activity in different regions of the brain. We expected that systemic injection of ChEIs would increase ARO

mRNA expression and activity in the hippocampus and frontal cortex. Our result showed that the two ChEIs had little influence on ARO expression and activity in most of the brain regions examined. One exception is that both ChEIs appeared to increase levels of ARO mRNA in the preoptic area. Another is that both ChEIs appeared to increase ARO activity in the amygdala. These findings suggest that inhibition of cholinesterase, and hence increased cholinergic activity can influence ARO expression and activity in a region-specific way; however, there appear to be no major effects on ARO expression or activity in the hippocampus or frontal cortex. This suggests that the systemic up-regulation of cholinergic activity does not affect the local estrogen production in these two regions in OVX rats. This is consistent with the results of our previous study which showed that removal of cholinergic inputs did not influence ARO in the hippocampus.

It is unlikely that the negative effect is due to the fact that our drug treatments did not work. These two ChEIs have been used in our lab before and reported to significantly inhibit the cholinesterase in the brain and caused behavior change [88, 93]. Besides that, we did see an increase in chewing behavior for rats with both donepezil and galantamine treatments, indicating that our treatment works.

In contrast, ChEIs did increase ARO activity by approximately 50% in the amygdala without changing relative levels of ARO mRNA. This suggests that cholinergic system may regulate ARO in a post-transcriptional manner in this region. The amygdala is a central structure in limbic circuitry, which is an important system to regulate emotional expression, emotional experience and emotional memory, especially fear. It is important for emotional related memory formation, consolidation and the use of this memory to make correct response. It receives many cholinergic afferents, which contain large amounts of ACh from basal forebrain cholinergic

system as well as inputs from the hippocampus, and this is thought to play a critical role in the coordination among different memory systems leading to the selection of appropriate behavioral strategies [94]. Study showed that decreasing hippocampal cholinergic signal impaired contextual conditioning, whereas increasing cholinergic signal promoted contextual fear condition, which highlights the important regulation of cholinergic neurotransmission on amygdala functions [95]. Studies also showed that there were α7-nAChRs locating on somatic or somato-dendritic regions of basolateral amygdala (BLA) interneurons. These receptors were actived in the basal state to enhance GABAergic inhibition [96]. Estradiol is also involved in fear recognition and memory in amygdala [97, 98]. Previous study showed that exposure to nicotine can increase signs of depression and anxiety in adult female[46]. Based on our result, it is possible that cholinergic system from basal forebrain and hippocampus regulates fear learning and memory through the regulation of ARO activity in the amygdala. This will require further study.

In the preoptic area, increased levels of ARO mRNA were observed in rats treated with donepezil and galantamine; however, the effects were not large and did not reach statistical significance. As discussed above, this may due to limitations of the qRT-PCR method and will need to be verified using other more sensitive methods. Nevertheless, our data suggest that despite possible increases in ARO mRNA, levels of ARO activity did not change. There are several possible explanations. It is possible that some of the mRNA was degraded before being translated into the enzyme, or that the newly translated protein was not stable and was degraded, or that treatment increased transcription of the shorter non-active form of ARO. Further studies will be needed to determine which of these possibilities is correct.

Collectively, these data provide preliminary data suggesting that manipulation of the cholinergic system do not regulate local estrogen production in the hippocampus and frontal cortex, which indicates that effects of cholinergic afferents on cell survival and synaptic plasticity are not due to cholinergic regulation of local estrogen production. In the amygdala, we did see a significant increase in ARO activity after galantamine treatment. This raises the possibility that cholinergic inputs may influence local estrogen production in the amygdala, and thereby affect amygdala function. To our knowledge, this is the first report of the effect of manipulation of the cholinergic system on ARO expression and activity in the brain.

6.0 SUMMARY AND CONCLUSIONS

In this study, we developed and validated a new microsomal incubation method to detect aromatase (ARO) activity in rat brain. Before that, there were few studies testing the change of ARO activity in the brain using rat models, as the species' activity tends to be very low, and there is no reliable method to detect it. Because of this, Japanese quails, and zebra finches were used to study brain ARO due to the relative high levels of local estrogen production. However, there are limitations of using birds as study models, as birds cannot mimic humans well, and behavioral studies are not often performed on birds. We showed that the microsomal incubation assay was reliable and sensitive in detecting rat brain ARO activity, which gave us the chance to study the brain's local estrogen production in a rodent model.

We have shown that OVX and ER agonist have different effects on aromatases mRNA and activity in the different brain regions, although most of the effect were not statistically significant. Our findings provide preliminary data showing that OVX and E2 can influence ARO in a regionally specific way, and that activation of GPR30 can play a role. To our knowledge, this is the first indication that activation of GPR30 can influence ARO expression and activity in the brain. This study also raises the question of whether local estrogen production in the brain may change in surgically menopausal women, who have lower systemic estrogen levels, which could further influence brain functions.

For the effect of the cholinergic system on ARO, our results showed that selective cholinergic lesions in the medial septum had no effect on ARO mRNA and activity in the hippocampus. This suggests that elimination of the basal forebrain cholinergic inputs influence neither ARO mRNA level nor activity in this region. In the hippocampus and frontal cortex, donepezil and galantamine had no effects on ARO mRNA expression and activity. This result is in accord with the cholinergic lesions experiment and suggests that the manipulation of the cholinergic system does not regulate ARO in these two regions. To our knowledge, this is the first report of the cholinergic system's effect on regulating ARO in these regions. Based on these data, we concluded that unlike other neurotransmitters, such as glutamate and norepinephrine, manipulation of the cholinergic system seemed not to regulate ARO mRNA and activity in these regions. This suggests that the effects of cholinergic afferents on cell survival and synaptic plasticity in the hippocampus are not due to cholinergic regulation of local estrogen production.

We showed that galantamine did significantly increase ARO activity level in the amygdala, which may suggest that cholinergic input regulates the function of the amygdala, such as fear cognition and memory by the regulation of local estrogen production. This study raises the possibility that ChEI treatment for Alzheimer's disease in women might affect emotional changes in the patient.

For future study, since we have shown that GPR30 may mediate E2's effect on regulating ARO mRNA and activity in different brain regions, which provides a possible mechanism for systemic estrogen to influence local estrogen production, more study needs to be done to validate this effect. Also, the mechanism of why ER agonists act differently in the different brain regions need to be investigated. Also, we showed that ChEIs can regulate ARO activity in the amygdala,

which may influence emotional function. Further study is needed to test the role of local estrogen production on the function of amygdala.

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