Metabolomics Analysis of Brain Monoamines, Metabolites and Amino Acids in Two Models of Menopause with/without Estrogen Receptor Agonist Treatments

by

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Estrogens have many beneficial effects in the brain. Previous studies have evaluated effects of estrogens on specific neurotransmitter systems including dopaminergic, serotoninergic, noradrenergic as well as glutamatergic pathways. However, a comprehensive profile of the collection of neurochemical changes that occur following menopause and estrogen treatments within specific regions of the brain has not been available. Therefore, it is necessary to directly and systematically compare the effect of estrogens on these multiple interacting brain neurotransmitter (NT) pathways between the two clinically relevant menopauses: surgical menopause (OVX) and transitional menopause (VCD).

In the first part of my thesis research, I comprehensively characterized and compared the neurochemical changes associated with surgical and transitional menopausal rat models in three regions of the brain at two time points. Naturally cycling rats sacrificed on proestrus (high estrogen level) and diestrus (low estrogen level) phases were also added to the investigation. Results showed that while many of the effects of surgical vs. transitional menopause were the same, there also were some important differences that varied by brain region and by time following the menopause.

In the second part of this thesis, I further investigated and compared the effects of chronic treatments of estradiol (E2) and selective estrogen receptor (ER) agonists (PPT, DPN, G-1) on brain NT pathways between surgical and transitional menopause at two time points. The results demonstrated significant differences in the effects of ER agonist treatments on neurochemical endpoints in OVX vs. VCD-treated rats and the effects were also brain region-specific and time-

dependent. These results were the first to systematically and simultaneously evaluate the effects of ER agonist treatments on multiple neurochemical endpoints in multiple regions of the brain in two models of menopause. The fact that agonist treatments had lesser effects in VCD-treated rats than in OVX rats may help to explain reports of lesser effects of estrogen replacement on cognitive performance in women that have undergone transitional vs. surgical menopause.

Preface

I still vividly remember the scene when my friends drove me through Fort Pitt Tunnel for the first time. Once entering the tunnel, I felt overwhelmed by repressive surrounding: dirty and old ceiling, pale yellow lights and terrible traffic in such narrow space. The drive was quite long and boring. However, as we approached the exit, I could see the tunnel ending far away became bigger and nearer. Suddenly, the light shined in and it became too bright to see. When I reopened my eyes, welcomed me is the unexpected, inspiring and most breathtaking view of Pittsburgh skyline. Hello, Pittsburgh, my second hometown.

To embark on a new journey of pursuing a doctoral degree on another continent which I had never been to was one of the most crucial and critical decisions that I ever made in my life. This journey per se, though fairly challenging and difficult, is also filled with warm and sweet memories. My Ph.D. training is a process of cultivating my soul, accompanied by personal growth and filled with highs and lows, ups and downs. It allows me to hone my professional skills, discover my career interest and explore various of possibilities that life offers. These cannot be achieved without the help and support from my excellent mentor, faculty, and colleagues in the program. I would like to express my gratitude and appreciation to everyone that I met and had pleasure to work with.

Firstly, I would like to express my thanks to my late research advisor Dr. Jeffrey K. Yao. Dr.Yao encouraged me to collaborate with colleagues, offered me previous opportunities to get hands-on experience on various state of the art research instrumentations and urged me to complete each task in a conscientious and meticulous way. I greatly thank him for seeing the talent and potential in me and recruiting me to join his laboratory and Pitt family.

My most sincere gratitude goes to my current mentor Dr. Robert B. Gibbs. Dr. Gibbs offered me unconditional support and valuable mentorship in the most difficult time of my Ph.D. training. He provided me immense guidance, insightful expertise and endless support towards my research and dissertation work. He created a friendly and pleasant working environment, and gave me enormous opportunities to learn, develop and grow into an independent scientist. He is always very patient, caring and approachable to his students, and his love for research and teaching influenced me unconsciously yet deeply. I am so grateful that he is always there when I need help. I learn from him about his intelligence, diligence, patience and erudition through every conversation and discussion. His advice is always wise and reasonable, and most importantly, stems from the best interest of his students. What I have learned from Dr. Gibbs will have a long and lasting influence on my future career and research.

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Lastly, this dissertation is dedicated to my beloved family. They are the very foundation on which I build my future and with whom I share this accomplishment. I would like to first thank my parents Liuxia Zhang and Guangzhi Long for their unwavering support of my academic and professional pursuits. Every step of the way they showed strong belief in me and encouraged me to work hard and stay positive. I would like to express my deep gratitude to my dear wife Junwei Shen and our precious daughter Lingxi Long for their endless love, care and long-term support. I am also thankful for my parents-in-law Ping Chen and Ben Shen for their trust and understanding. They all have been constant motivations for me to achieve my goals and conquer every difficulty ahead of me. I am truly blessed to have them in my life.

Abbreviation

ANOVA	Analysis of Variance
AA	Amino Acid
ALA	Alanine
SER	Serine
GLU	Glutamate
GLN	Glutamine
AD	Alzheimer disease
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
GPCR	G protein coupled receptor
× g	Centrifugal force
GC	Gas Chromatography
HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
ml	Milliliter
mM	Millimolar
M	Molar
MW	Molecular weight
MWCO	Molecular weight cut-off
nM	Nanomolar
Phe	Phenylalanine
SD	Standard Deviation
S.E.M	Standard Error of Mean
SD rat	Sprague Dawley rat
µM	Micromolar
µg	Microgram
OVX	Ovariectomized
VCD	Vinylcyclohexene dioxide
ER	Estrogen Receptor
E2	17beta-estradiol
AD	Androstenedione
T	Testosterone
PPT	4,4',4''-(4-propyl-{1H}-pyrazole-1,3,5-triyl)trisphenol
DPN	Diarylpropionitrile
G-1	(1-[4-(6-bromobenzo[1,3] dioxol 5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone
HPβCD	Hydroxypropyl-β-cyclodextrin
NE	Norepinephrine
EPI	Epinephrine

DA	Dopamine
5-HT	Serotonin
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	Homovanillic Acid
5-HIAA	5-hydroxyindole acetic acid
TYR	Tyrosine
TRP	Tryptophan
FCX	Frontal Cortex
STR	Striatum
HIP	Hippocampus
HRT	Hormone Replacement Therapy
IHC	Immunohistochemical
i.p.	Intraperitoneal
vs.	Versus
ACN	Acetonitrile
MAO	Monoamine Oxidase
COMT	Catechol-O-methyltransferase
DBH	Dopamine beta-hydroxylase
AADC	Aromatic L-amino acid decarboxylase
TH	Tyrosine Hydroxylase
TPH	Tryptophan Hydroxylase
P	Proestrous
D	Diestrous
EAA	Excitatory amino acid
IAA	Inhibitory amino acid
TCA	Tricarboxylic acid cycle
BBB	Blood brain barrier
CYP450	Cytochrome P450

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1.0 CHAPTER 1: INTRODUCTION

1.1 EFFECTS OF ESTROGEN ON BRAIN FUNCTIONS

Ovarian hormones play critical roles in women's health throughout the lifespan. Thanks to the great improvement seen in the healthcare, life expectancy has increased significantly. Menopause in women occurs when all of the ovarian follicles have been lost due to ovulation or atresia. The average age of menopause is around 51 years, ranging from 45 to 55 years [4]. Since the age to menopause has not changed over the years, women are spending a longer time of their lives in the post-menopausal years. Menopause results in irregular fluctuations in ovarian hormones during the transition period, and culminates in a substantial decline in ovarian production of estrogens and progesterone [5]. This leads to the release of LH and FSH from the pituitary due to the loss of negative feedback regulation at the level of the hypothalamus. A large body of work has shown loss of estrogen associated with menopause is associated with an increased risk for many neurological conditions such as the development of Alzheimer's disease, cognitive dysfunction, depression [6-10]. For example, previous studies have demonstrated that declining ovarian function increases the risk of dementia and mild cognitive impairments in menopausal women[11].

Estrogens have many beneficial effects on the brain including neuroprotection from cardiac arrest and stroke [12], increasing neuronal connectivity [13], improving cognitive performance [14], and preventing or slowing age-related cognitive decline [15, 16]. A large amount of work has

been done to focus on estrogen's neuroprotective effects. For example, studies have shown that short-term estrogen replacement can improve measures of verbal learning and memory in perimenopausal women [17]. In addition, compared with hormone replacement therapy initiated in older women years after menopause, treatment started during perimenopause conferred greater cognitive benefit such as lowering the risk of dementia [18, 19]. Thus, the investigation of using transitional menopausal as a "Window of Opportunity" for estrogen treatment should be emphasized and studied in detail.

1.2 ESTROGEN RECEPTORS AND AGONISTS

1.2.1 ER α and ER β

Estrogens bind to two nuclear receptors, ERα and ERβ, which act largely as DNA transcription regulators [3, 20]. The classical estrogen receptor ERα was discovered in 1958 by Elwood Jensen and cloned in 1986 [21]. ERβ was then was then discovered and cloned in 1996 [22]. These receptors belong to a large superfamily of nuclear receptors that regulate gene transcription, but also have the abilities to interact with specific membrane compartments and activate second messenger signaling pathways such as MAPK, CamKII, and CREB [23]. Both receptors are expressed in a variety of isoforms, e.g. ERα1-3 and ERβ 1-5 [24, 25] and in different tissues, e.g. ERα expressed in uterus, testis, ovary and kidney while ERβ is expressed in prostate, ovary, lung and bladder [26]. They also have differential presence in the brain. In the brain, ERα is highly

expressed in areas of the hypothalamus that are responsible for reproduction, as well as in areas involved in learning, memory, emotionality, attention and motor functions such as the hippocampus and cortex. ERβ is widely distributed throughout the brain including hippocampus and cortex [27-29]. In addition to its role in reproduction, it has been demonstrated that ERα plays a significant role in certain learning and memory tasks [30], in the neuroprotective effects of estradiol in a model of ischemic injury [31], and is responsible for estrogen-mediated decreases in GABA release at CA1 synapses [32, 33]. On the other hand, ERβ has been implicated in certain learning and memory tasks, particularly those involving stress or anxiety [34-36], and recently has been shown to mediate estrogen effects on glutamate release at CA1 synapses [37] and on long term potentiation via a mechanism involving actin polymerization in postsynaptic cells [38].

The two receptors can be activated by estradiol as well as by selective agonists. 4,4',4''-(4-propyl-{1H}(-pyrazole-1,3,5-triyl))trisphenol (PPT) is an ERα-selective agonist that is approximately 410-fold more selective for ERα than ERβ [39, 40]. Diarylpropionitrile (DPN) is an ERβ-selective agonist with 70-fold higher binding affinity and 170-fold higher potency for ERβ than ERα [41].

1.2.2 GPR30

Recently, a membrane bound G protein-coupled receptor, GPR30, also has been identified. GPR30 is a novel membrane-associated estrogen receptor (ER) which is completely unrelated to ERα and ERβ [3, 42, 43]. It is localized in both intracellular and plasma membranes [44], and has been shown to promote rapid estrogen signaling in a variety of cell lines [45]. Previous immunohistochemical studies have confirmed the presence of GPR30 in brain, with significant

GPR30 expression in regions of the forebrain including hippocampus and hypothalamus [46]. The role that GPR30 plays in mediating estrogen effects in brain remains largely unknown. GPR30 can be activated by (1-[4-(6-bromobenzo[1,3] dioxol 5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone (G-1), which is a non-steroidal, high-affinity selective GPR30 agonist, with no binding to the classic ERα or ERβ [47]. Study showed that G-1 has similar effect of E2 in reducing 5-HT1A receptor signaling in the hypothalamus [48]. Also, G-1 treatment demonstrated E2-like effect in reducing freezing behavior in a mouse model of depression [49]. In non-human primates, GPR30 has been shown to play an important role in estrogen-mediated regulation of GnRH neurons [50]. Recent studies also show that activation of GPR30 with G-1 promotes neuronal survival following global ischemia in the brain [51], and have implicated GPR30 in the protective effects of E2 against glutamate-induced injury [52].

Previously our lab has shown intense GPR30 immunostaining in basal forebrain cholinergic neurons located in the multiple brain regions such as septum, diagonal band of Broca and striatum, as well as in the frontal cortex and hippocampus [53]. Additionally, our lab has also demonstrated that G-1 enhances potassium-stimulated acetylcholine release in the hippocampus, and enhances acquisition of a delayed matching-to-position T-maze task by ovariectomized (OVX) rats, analogous to E2 [54]. Contrast to GPR30 activation, receptor inhibition by G-15 impaired acquisition of the DMP task, similar to OVX.

1.3 NEUROTRANSMITTER PATHWAYS

The effects of E2 on neurotransmitter systems innervating the hippocampus, cortex and striatum have been widely studied during the past decades. The most studied NT pathways include cholinergic [55, 56], dopaminergic [57-59], and serotoninergic [60] pathways, as well as glutamate [37, 61, 62] and GABA signaling [33, 63-65].

1.3.1 Dopaminergic Pathway

Dopamine (DA) is a neurotransmitter that has two different pathways in the brain. The first pathway, which begins in the substantia nigra and projects to the basal ganglia, is implicated in movement. The other pathway originates at the ventral tegmental area and projects to other areas in the brain. It is commonly known as the reward or reinforcement pathway.

Previous study has reported that level of DA in HPC remained unchanged following OVX in mice [66]. In contrast to that, another study has showed a significant decrease in DA level in HPC at 20 days following OVX in mice [67]. Additionally, results reported by Bitar et al. [68] have shown markedly elevated concentrations of HVA in male and female rats following gonadectomy in STR. They also detected elevated DA and DOPAC levels following OVX [68]. Furthermore, a separate study using microdialysis approach has found that female rats had significantly higher extracellular striatal DA concentrations at proestrus than after OVX [69].

E2 and selective ER agonist treatments may have significant impacts on multiple endpoints in dopaminergic pathway. Previously, Inagaki et.al [70] reported that a single s.c. injection of E2 in OVX rats significantly increased levels of DA but had no effect on DOPAC in STR. Additionally,

levels of DA in HPC of OVX mice [66] and rats [71] were shown not to be affected by 4-6 weeks of chronic E2 treatment. Another study demonstrated that 4 days s.c. treatment of E2 did not alter levels of DA in the STR of OVX rats [72]. Jacome et al. [73] reported no effects on monoaminergic endpoints in OVX rat STR 2 days after a single s.c. injection of DPN.

1.3.2 Serotoninergic Pathway

Serotonin (5-HT) is another monoamine neurotransmitter in the brain, which is involved in the regulation of mood, perception of pain and gastrointestinal functions. The most important group of serotonergic neurons are present in the substantia nigra and raphe nuclei and project to the rest of the brain.

Previous studies have showed that decreased levels of 5-HIAA [66] in HPC were detected following OVX in mice. On the other hand, study by Bitar et al. [68] has showed that the levels of 5-HT and 5-HIAA remained unaltered in STR of the male and female rats following gonadectomy. Furthermore, reduced 5-HIAA/5-HT ratio in HPC was reported at 2 weeks but not 4 weeks after OVX in rats [74]. It has also been shown that levels of 5-HT in HPC remained unchanged following estrogen deprivation in mice [66].

E2 and selective ER agonist treatments also may have significant impacts on multiple endpoints in serotoninergic pathway. Study by Lubbers et al. [72] reported that increased levels of 5-HIAA were detected in the STR of OVX rats after 4 days s.c. treatment with E2 but not PPT. In addition, 4-6 weeks of chronic E2 treatment was reported to decrease levels of 5-HT in HPC of OVX mice [66] and in HPC and FCX of OVX rats [71]. Notably, Pestana-Olivera et al. [75] recently evaluated the effects of estrogen therapy on serotonin in VCD-treated rats and reported

that 3 weeks of E2 treatment significantly increased 5-HT levels in dorsal HPC of VCD-treated rats. In contract, no treatment effects were detected in several studies. For example, previous research found no change in 5-HIAA level in HPC following 4-6 weeks of chronic E2 treatment in OVX mice [66] and rats [71]. Inagaki et.al [70] reported that a single s.c. injection of E2 in OVX rats had no effect on 5-HIAA level in STR. Another study demonstrated that 4 days s.c. treatment of E2 did not alter levels of 5-HT in the STR of OVX rats [72].

1.3.3 Noradrenergic Pathway

Norepinephrine (NE) is a neurotransmitter involved in the sympathetic nervous system. Together with epinephrine (EPI), NE is involved in the "flight-or-flight" response that our body makes under stressed or dangerous situations. It is also involved in our sexual behavior and control of appetite. These cells originate in the locus coeruleus and project their axons through the rest of the brain.

Previous study has shown that decreased levels of NE were detected following OVX in mice [66, 67]. E2 and selective ER agonist treatments also may have significant impacts on multiple endpoints in noradrenergic pathway. It has also been reported that E2 and PPT treatments increased NE levels in the FCX of OVX rats[72]. On the other hand, a few previous studies indicated otherwise. For example, one study found no change in NE level in HPC following 4-6 weeks of chronic E2 treatment in OVX mice [66] and in HPC and FCX of OVX rats [71]. Another study by Inagaki et.al [70] reported that no effect on NE level in STR was detected after a single s.c. injection of E2 in OVX rats.

1.4 MENOPAUSAL RAT MODEL

1.4.1 Ovariectomy (OVX) model

Ovariectomy is to surgically remove all ovarian tissues. The OVX model is characterized by a sudden and complete loss of ovarian hormones, and most closely models the condition of surgical menopause. In the past decades, most of the preclinical studies have used ovariectomy followed by hormone treatment to study the effects of estrogens on the brain [76].

However, due to lack of a perimenopause phase, this surgically-induced menopausal rodent model fails to adequately recapitulate the phase of menopause transition. In addition, it should be noted that ovariectomy also removes all of the other ovarian hormones as well as androgen produced by residual ovarian tissues [77], which may also affect the brain.

1.4.2 VCD-induced model

The ova-toxin, industrial 4-Vinylcyclohexene dioxide (VCD), is a diepoxide metabolite of 4-vinylcyclohexene (VCH). Formation of the bioactive form (VCD) can occur in the liver through cytochrome P450. The ovary itself also expresses the relevant enzymes and is capable of this conversion[1]. The development of ovarian follicles in the ovary consists of several steps. Simply, the mammalian ovary has a finite number of follicles since birth. These follicles contain an oocyte

that is surrounded by somatic cells and are not regenerable or replaceable. The development of the follicles has several stages: starting from the most immature form termed primordial follicles which are surrounded by a single layer of squamous granulosa cells to primary follicles with a layer of cuboidal granulosa cells, then to primary follicles with theca interna cell and finally to preovulatory follicles responsible for 17β-estradiol production. After ovulation, the rest of the cells differentiate into corpus luteum, which produces progesterone. VCD specifically targets and destroys primordial and primary follicles, causing premature ovarian failure in rats, possibly due to accelerated atresia via apoptosis[1, 78]. No significant toxicity or inflammation are caused by VCD in other tissues when administered using recommended regimen.

The VCD-induced transitional menopausal rat model was first established and then further developed by Dr. Patricia Hoyer et.al at University of Arizona. The cellular effects of VCD-induced ovary toxicity have been well characterized. Repeated daily doses of VCD (80 mg/kg, i.p.) for 15 days leads to 50% of primordial and primary follicles being destroyed in the ovaries of immature rats [79, 80] Similar pattern of damage was seen in adult rats when the exposure of VCD was extended to 30 days [80].

In contrast to surgical menopause, natural menopause is characterized by a gradual loss of ovarian follicles in middle-age, a corresponding reduction in estrogen production but with continued production of androgens by the remaining ovarian cells. Therefore, unlike surgical menopause, natural menopause is associated with a shift in the ratio of estrogens to androgens. Recent study by Acosta et al. [81] showed that these differences are functionally important and result in differences in the effects of estrogen treatment on behavioral endpoints.

VCD-induced model replicates estrous acyclicity and low levels of estrogen observed in human perimenopause and postmenopause, thus successfully serves as a transitional menopausal

model. Another advantage of the model is that transitional menopause could be achieved in young adult animals via VCD-induced accelerated ovarian failure, thus allows for the dissociation of the effects of hormone levels from the effects of aging.

It should be noted that all of the previous studies investigating impact of estrogen deprivation and treatments on brain neurotransmitter endpoints have all used OVX surgical menopausal model. The first study of estrogen effects on brain neurotransmitter pathway was conducted by Pestana-Olivera et al. [75] using VCD-induced model. Level of 5-HT was the only neurotransmitter they measured in the brain following continuous administration of E2. Despite that study, the effects of estrogen deprivation and treatments on the neurochemical endpoints in multiple interacting NT pathways remained largely unknown in VCD-induced transitional menopause. Studies on how such effects differ in VCD-induced menopause from OVX surgical menopause are warranted.

1.5 OVERVIEW OF THE THESIS

Both basic and clinical studies suggest that the loss of estrogens following menopause has been associated with increased risk for a variety of neuropsychiatric and neurodegenerative diseases as well as age-related cognitive decline and dementia. Estrogen treatment has been shown to improve cognitive performance and slow age-related cognitive decline for menopausal women. However, studies are beginning to show that the differences between the surgical and transitional menopauses are functionally important and may result in differences in the effects of estrogen treatment on behavioral endpoints.

Our goal of the research is to understand mechanisms by which estrogens affect the brain. We hypothesize that many of the beneficial effects of estrogens on the brain are due in large part to effects on multiple interacting neurotransmitter (NT) pathways that occur within specific brain regions. In particular, we hypothesize that loss of ovarian function results in multiple and simultaneous alterations in specific monoaminergic pathways and amino acid levels in the brain, and that selective agonists acting at specific estrogen receptors can modify these deficits in NT levels, thus offering corresponding effects on cognitive performance. Also, we hypothesize that significant differences exist in terms of NT levels in specific region of the brain following estrogens deprivation and selective estrogen receptor agonist treatments between the models of surgical menopause and transitional menopause, contributing in part to the differences in the effects of estrogen treatments for these two types of menopause in the clinical settings.

In this thesis, we have directly and systematically conducted a metabolomics analysis of monoamines, precursors, metabolites and amino acids in three brain regions (hippocampus, frontal

cortex and striatum) at two time points, in two clinically relevant models of menopause with/without estrogen receptor agonists treatments. Two major specific aims are: Aim 1 is to investigate and compare the effects of two models of menopause on NT pathways of interest. Aim 2 is to investigate (a) whether specific estrogen receptor agonists produce distinct and simultaneous changes in NT pathways within specific regions of the brain; and (b) whether effects differ across two different models of menopause. These aims will be described in greater detail in the following chapters.

2.0 CHAPTER 2: COMPARISON OF TRANSITIONAL *VS* SURGICAL MENOPAUSE ON MONOAMINE AND AMINO ACID LEVELS IN THE RAT BRAIN

2.1 ABSTRACT

Loss of ovarian function has important effects on neurotransmitter production and release with corresponding effects on cognitive performance. To date, there has been little direct comparison of the effects of surgical and transitional menopause on neurotransmitter pathways in the brain. In this study, effects on monoamines, monoamine metabolites, and the amino acids tryptophan (TRP) and tyrosine (TYR) were evaluated in adult ovariectomized (OVX) rats and in rats that underwent selective and gradual ovarian follicle depletion by daily injection of 4-vinylcyclohexene-diepoxide (VCD). Tissues from the hippocampus (HPC), frontal cortex (FCX), and striatum (STR) were dissected and analyzed at 1- and 6-weeks following OVX or VCD treatments. Tissues from gonadally intact rats were collected at proestrus and diestrus to represent neurochemical levels during natural states of high and low estrogens. In gonadally intact rats, higher levels of serotonin (5-HT) were detected at proestrus than at diestrus in the FCX. In addition, the ratio of 5-hydroxyindoleacetic acid (5-HIAA)/5HT in the FCX and HPC was lower at proestrus than at diestrus, suggesting an effect on 5-HT turnover in these regions. No other significant differences

between proestrus and diestrus were observed. In OVX- and VCD-treated rats, changes were observed which were both brain region- and time point-dependent. In the HPC levels of norepinephrine, 5-HIAA, TRP and TYR were significantly reduced at 1 week, but not 6 weeks, in both OVX and VCD-treated rats relative to proestrus and diestrus. In the FCX, dopamine levels were elevated at 6 weeks after OVX relative to diestrus. A similar trend was observed at 1 week (but not 6 weeks) following VCD treatment. In the STR, norepinephrine levels were elevated at 1 week following OVX, and HVA levels were elevated at 1 week, but not 6 weeks, following VCD treatment, relative to proestrus and diestrus. Collectively, these data provide the first comprehensive analysis comparing the effects of two models of menopause on multiple neuroendocrine endpoints in the brain. These effects likely contribute to effects of surgical and transitional menopause on brain function and cognitive performance that have been reported.

Keywords: Loss of ovarian function; Ovariectomy; VCD; Hippocampus; Striatum; Frontal Cortex

2.2 INTRODUCTION

Estrogens have widespread effects in the brain, both during development and in adulthood. These include effects on neuronal organization and function, synaptic plasticity, and neuronal survival, with corresponding effects on cognitive function, neurodegenerative disease, and age-related cognitive decline. Studies also show that, in women, loss of estrogens following oophorectomy or menopause can contribute significantly to risk for neurodegenerative diseases like Alzheimer's disease and Parkinson's disease, as well as late-life mood disorders and age-related cognitive decline [76, 82, 83]. A few studies have documented effects on neurotransmitter systems that are known to play a role in mood and cognitive changes. These include effects on monoaminergic pathways [84, 85], as well as cholinergic [56], glutamatergic [37, 61, 62], and GABAergic [86, 87] transmission. Most studies have used ovariectomy models to study the effects of rapid hormone depletion followed by estrogen replacement to study effects on one or two neurotransmitter systems in a specific region of the brain. Few studies have focused specifically on the effects of menopause, and in particular on comparing effects associated with surgical vs. transitional menopause.

In primates, natural or transitional menopause is characterized by an accelerated loss of ovarian follicles, resulting in irregular fluctuations in ovarian hormones during the transition period, and culminating in a substantial decline in ovarian production of estrogens and progesterone [88]. As a consequence, the release of LH and FSH from the pituitary rises significantly due to the loss of negative feedback regulation. In addition, ovarian stromal cells continue to produce androgens post-menopause, resulting in a substantial increase in the ratio of

androgens:estrogens in the systemic circulation. In contrast, ovariectomy produces a surgical menopause characterized by a rapid and complete loss of all ovarian hormones. In the United States, most women (>80%) experience a transitional menopause. Far fewer women (~13%) experience a surgical menopause, and many of these surgeries are conducted in younger women (<40 years of age) to prevent ovarian and breast cancer associated with BRCA1 and BRCA2 mutations [89].

Unlike primates, rodents do not experience follicular atresia resulting in a natural menopause. Rodents enter a condition of reproductive senescence, characterized by long periods of elevated levels of circulating estradiol during middle-age, and followed by persistent diestrus in old age [90]. This change is related to a dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis that occurs as a result of normal aging [91]. Thus, until recently, rodents have not served as a good model of natural menopause in humans.

Notably, a rodent model of progressive ovarian failure has recently been characterized [78, 92-94]. This model involves daily injection of 4-vinylcyclohexene diepoxide (VCD), a chemical that selectively and over time destroys the majority of primordial and primary ovarian follicles [95]. Rodents treated with appropriate daily doses of VCD experience a progressive loss of ovarian follicles. Stromal cells remain intact and continue to produce androgens, thus mirroring conditions that occur in natural menopause. Using this model, studies have shown that transitional and surgical menopause produce differing effects on measures of brain function and cognitive performance [81]. The most recent evidence suggests that some of these differences may be due to elevated levels of androstenedione following transitional vs. surgical menopause, and the local conversion of androstenedione to estrone in the brain [96, 97].

To date, there has been little direct comparison of the effects of surgical vs. transitional menopause on neurotransmitter pathways in the brain. We hypothesize that surgical and transitional menopause produce different effects on cognitive functioning due to differential effects on multiple interacting neurotransmitter pathways. In this study, we utilized a high-pressure liquid chromatography-coulometric multi-electrode array system (HPLC-CMEAS) method to characterize neurochemical changes associated with surgical vs. VCD-induced transitional menopause in three regions of the rat brain. Results show that while many of the effects of surgical vs. transitional menopause are the same, there also are some important differences that vary by brain region and by time following the menopausal transition.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Female Sprague–Dawley rats (~11 weeks of age) were purchased from Hilltop Laboratories, Inc. Young adults were selected as opposed to middle-aged rats based on several considerations. Rodents do not experience a natural menopause. Instead, they experience what is known as reproductive senescence associated with dysregulation of the HPG axis. Characteristics of reproductive senescence include irregular cycles, a period of constant estrous where levels of circulating estradiol are chronically elevated, followed eventually by constant diestrus. The timing of these events varies by strain and typically begin in middle-age [98]. Humans do not experience reproductive senescence, but instead experience accelerated follicular atresia resulting in a natural menopause [99]. We therefore chose to use young adults as opposed to middle-aged rats in order to study the effects of chemically-induced follicular atresia in the absence of confounding factors associated with the process of reproductive senescence. Another reason to use young adult rats is that, with respect to surgical menopause, the majority of women who experience surgical menopause are young adults (e.g., early 30s) whose ovaries are removed to prevent ovarian and breast cancers associated with BRCA1 and BRCA2 mutations [89]. Hence, we concluded that young adult rats would be more appropriate for comparing models of surgical vs. natural menopause with respect to neurochemical endpoints.

All rats were individually-housed and maintained on a 12/12 h light–dark cycle (lights on at 7:00 a.m.) with free access to food and water and were allowed to acclimate to the housing environment for two weeks prior to use. All experiments were conducted in accordance with the NIH Guide for Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.3.2 Gonadally intact controls

Tissues were collected from rats at proestrus, and at diestrus, to represent neurochemical levels during natural states of high and low estrogen over the course of the estrous cycle. Daily vaginal smears were collected from these rats to determine stage of the estrous cycle. Cycle stage was characterized by vaginal cytology and the presence/appearance of three cell types, leukocytes, nucleated epithelial cells, and cornified epithelial cells [100]. See Figure 1. After collecting at least 2 weeks of cycle data, rats were killed either on proestrus or diestrus and serum as well as brain tissues were collected as described below.

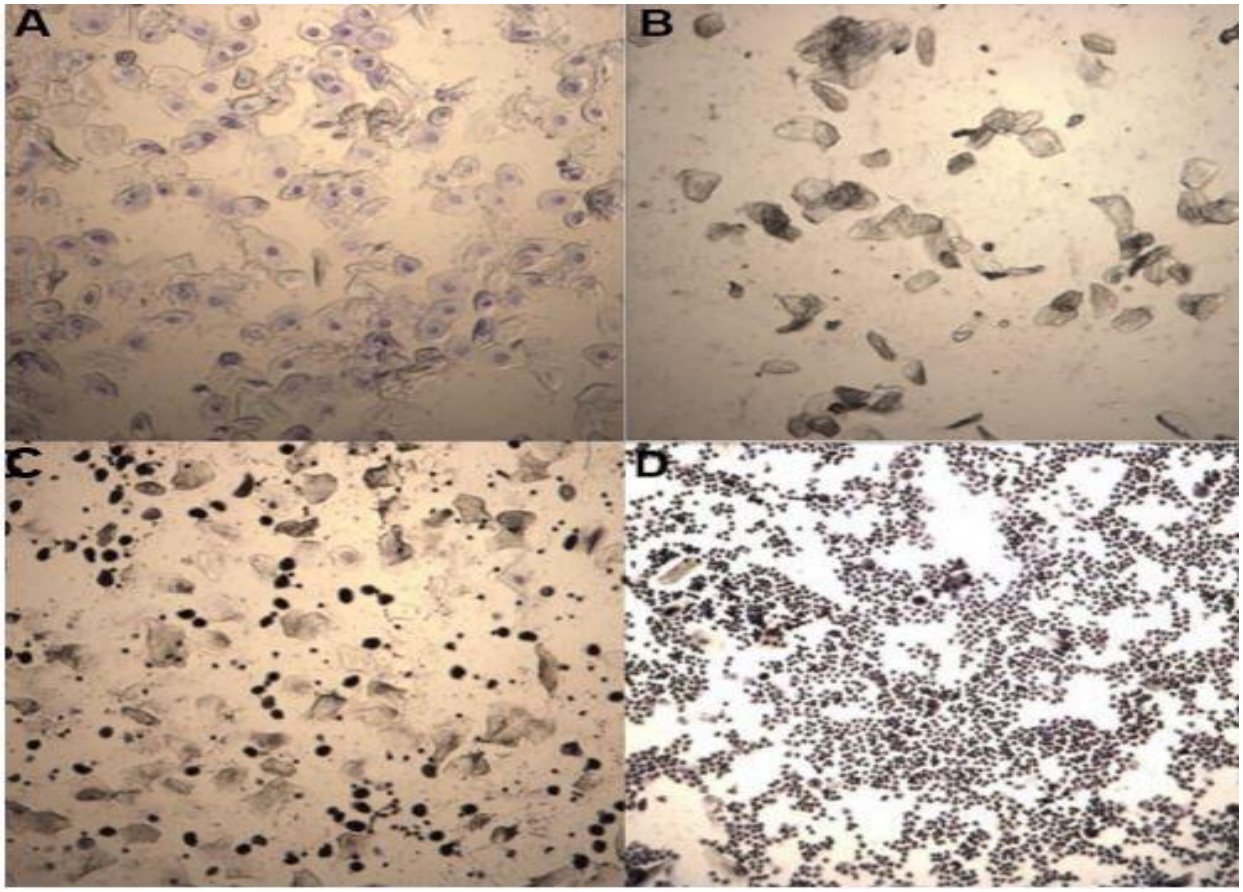


Figure 1. Photomicrographs (x20) of stained vaginal secretion from rats at (A) Proestrus: predominantly consisting of nucleated epithelial cells**; (B) Estrus:** consisting of anucleated cornified cells**; (C) Metestrus:** consisting of the three types of cell: leukocytes, cornified, and nucleated epithelial cells**;** and **(D) Diestrus:** consisting predominantly of leucocytes**.**

2.3.3 Menopausal models

Rats were randomly assigned to either the VCD or OVX group. Rats in the VCD group received 4-vinylcyclohexene diepoxide (VCD; Sigma Chemicals; St Louis, MO) at a dose of 80 mg/kg i.p. daily for a period of 30 days as previously described [101]. During this time, rats in the OVX group received daily injections of sesame oil (1ml/kg i.p.). At the end of 30 days, rats that

had received sesame oil underwent bilateral ovariectomy, and rats that received VCD received sham surgery. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (7 mg/kg) (0.1 cc/100 g.b.w.). Ovariectomy was performed using a lateral approach. The apical tips of the uterus were ligated and the ovaries removed. The peritoneal muscle was closed with 6.0 suture silk, and the overlying skin was closed with metal wound clips. Sham surgery, consisted of skin and muscle incisions and wound closure only. Antibiotic cream was applied to the wound to reduce the chance of infection. Rats received Ketofen (3 mg/kg, i.p.）every day for 3 days to reduce post-surgical pain.

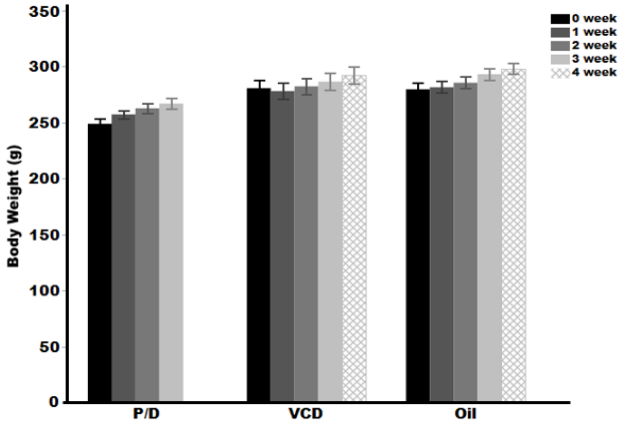


Figure 2. Rat body weight during period of VCD/Oil treatment. Neither of VCD or oil chronic treatment negatively affected rat body weight.

2.3.4 Tissue collection

At 1 week or 6 weeks following surgery, rats were anesthetized with an overdose of ketamine (3 mg) and xylazine (0.6 mg) and euthanized by decapitation. These time points were chosen to evaluate early and late effects of the two menopausal models and to evaluate changes in effects as

a function of time. Trunk blood was collected for determination of serum levels of estradiol, testosterone, and androstenedione. Dissections were performed according to plate designations in Paxinos and Watson (1998) [102] and were as follows: hippocampus (plates 21-41), frontal cortex (plates 11-21), and striatum (plates 11-21). An example of the brain matrix is shown in Figure 3. These brain regions were selected based on known monoaminergic innovation, their roles in learning, memory and attentional functions, and evidence that neuronal organization and functioning in these regions can be affected by gonadal hormones. Tissues were stored at -80 °C until processed.

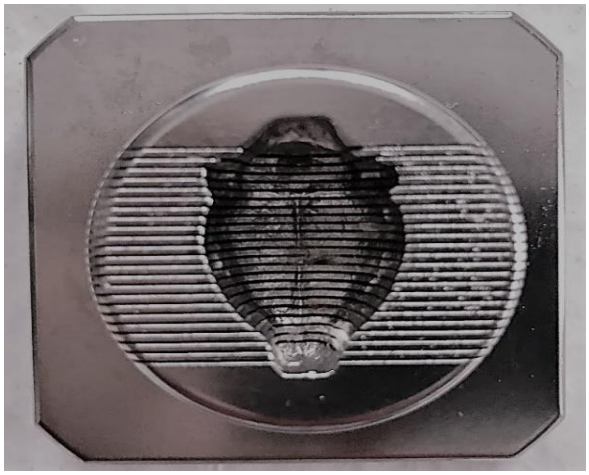


Figure 3. Brain matrix for dissection. Horizontal lines delineate where the brain is cut. Spacing between lines is 1 mm.

2.3.5 Brain sample preparation

500 µl 10 mM sodium acetate buffer (pH 6.5) were added to 50 mg tissue in a 1.5 ml microfuge tube. Tissue was sonicated until completely dissolved. After sonication, tissues were spun at

14,000 g for 10 min at 4˚C. Supernatant was collected and placed at 4 ˚C. This step was repeated three times and the three supernatants were combined. The cell free supernatants were spun at 15,000g for an additional 40 minutes. A 200 µl aliquot was taken for the determination of protein concentration using the Bradford method [103]. The remaining volume of each supernatant were filtered through a disposable membrane (0.22µm pore size) micropartition device (Millipore Ultrafree-MC) under centrifugation at 14,000g for 30 min at 4 °C to remove any compounds above 10,000 nominal molecular weight limit.

2.3.6 Protein assay

Protein levels were determined by Bio-rad protein assay (Bio-Rad Laboratories, Inc.). Seven dilutions of a protein standard were prepared to make a standard curve each time the assay was performed: 0.2,0.4,0.6,0.8,1.0,1.2,1.4mg/ml. 5 µl of standard and diluted the brain homogenate was pipetted into a clean dry microplate. To prepare the working reagent, 20 µl of reagent S was added to each ml of reagent A needed for the run. Then, 25 µl of reagent A was added into each well. The multichannel pipette was used to add 200 µl of reagent B into each well. The palate was then gently agitated to mix the reagent. After 15 minutes, absorbance was read at 650 nm. Each sample was tested by triplicate.

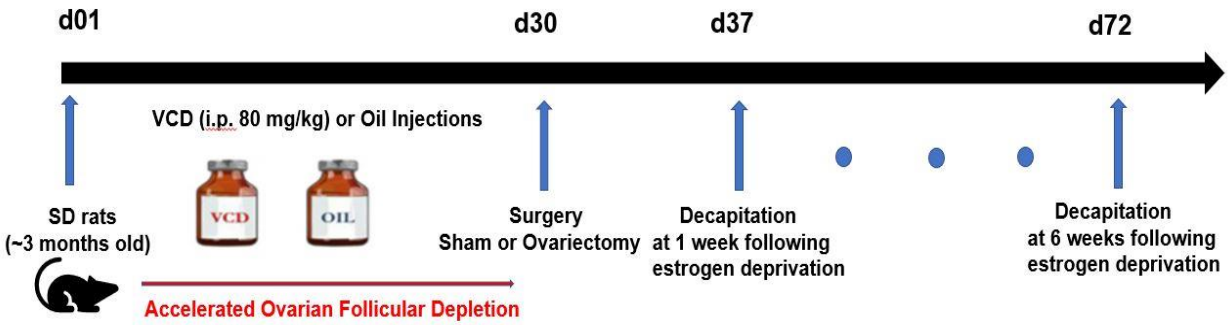


Figure 4. Schematic diagram showing the timeline of the experimental protocol 1.

2.3.7 Hormone assays

2.3.7.1 E2 assay

Serum levels of E2 were quantified as recently described [104, 105]. Briefly, samples were spiked with internal standard 25µl 2,4,16,16,17-d5-17 beta-estradiol (1 ng/ml in methanol). 3-4 ml n-Butyl chloride was added and samples were vortexed for 1 min. Samples were then centrifuged at 4,770 x g at room temperature for 10 min, and the organic layer was transferred to salinized culture tubes and dried under a steam of nitrogen at 37ºC for 20 min. Residues were derivatized in 0.1 ml buffered dansyl chloride solution (a 1:1 mix of acetonitrile: water, pH 10.5) and heated to 60ºC for 3 min. The reconstituted sample was then transferred into glass vials for LC-MS/MS analysis. 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 with a Thermo Fisher TSQ Quantum Ultra mass spectrometer interfaced via an electrospray ionization (ESI) probe with the Waters UPLC Acquity

solvent delivery system. Transitions used for analysis were 506 → 171 for E2, and 511 →171 for the deuterated internal standard. Area under the peak was quantified and used to determine absolute levels of E2/ml of sample by comparison with a series of standards. The limit of detectability for this assay is 2.5 pg/ml. Intra-assay statistics show relative standard errors below 8.1% and relative standard deviations below 10.4%. Inter-assay statistics show relative standard errors below 5.0% with relative standard deviations below 7.4%. The E2 serum concentration was calculated as pg/ml.

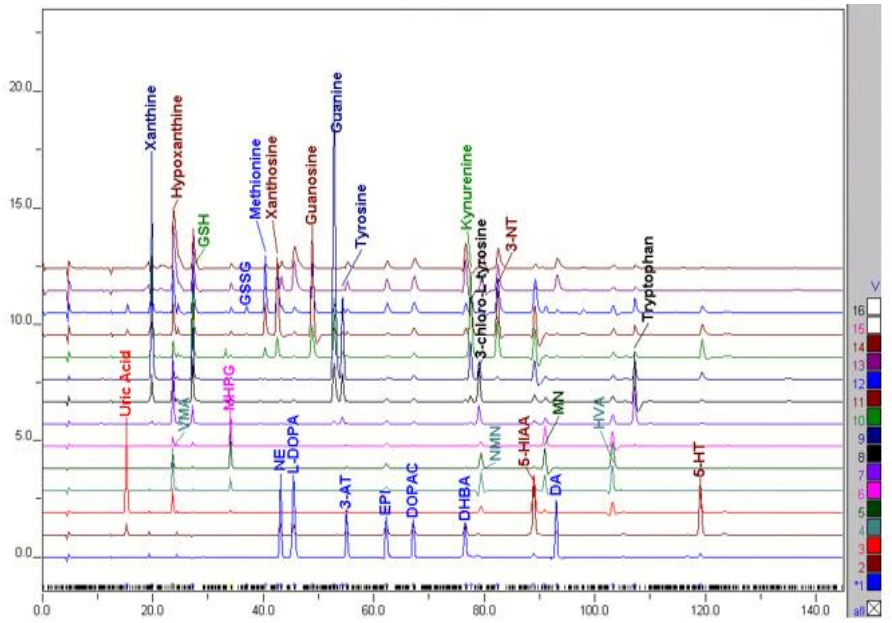
2.3.7.2 Testosterone and androstenedione assay

Testosterone (T) and Androstenedione (AD) levels in serum were quantified by a modification of the method described by Cawood [106] and using methods similar to the E2 detection method described above. Briefly, samples were spiked with 0.25 ng/ml D3-testosterone or D7-androstenedione as the internal standard and then extracted with 3 ml n-butyl chloride. After centrifugation and evaporation, the residue was reconstituted in methanol and water (80 µl: 20 µl), and was transferred into glass vials for UPLC-MS/MS analysis. T and AD 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 T analysis were 289 →97 for T and 292 →97 for the deuterated T; transitions used for AD analysis were 287→100 for AD and 294→ 100 for the deuterated AD. The limit of detectability for this assay is 25 pg/ml for both T and AD.

2.3.8 Monoamine analysis by HPLC-CMEAS

Monoamines and metabolites were measured with a modified version of a HPLC-ECD method described by Yao et al [107]. High-performance liquid chromatography (HPLC) with electrochemical detection was used to detect and quantify levels of amino acids, monoamines and metabolites, including tryptophan (TRP) and tyrosine (TYR); dopamine (DA) and its metabolites, 3-4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and epinephrine (EPI) and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). These neurochemical endpoints were selected not only because they are known to be involved in a variety of cognitive functions but also are reported to be regulated by estrogens. Within each sample, 100 µl was injected into an ESA CoulArray Model 5600 HPLC system, consisting of two Model 582 pumps, one dynamic gradient mixer, two PEEK pulse dampers, a Model 542 refrigerated autosampler injector, a CoulArray organizer module, and a serial array of 16 coulometric electrodes. The system was controlled, and chromatograms were analyzed using the ESA CoulArray for Windows-32 software program. Each sample was run on a single column (ESA Meta-250, 5 µm ODS, 250 x 4.6 mm ID) under a 68-minute gradient elution that ranged from 0% to 100% Mobile Phase B. Mobile Phase A consisted of 1.1% (w/v) of 1-pentane-sulfonic acid (Specrum, Inc.) pH was adjusted to 3.0 using acetic acid (Sigma-Aldrich, Inc.). Mobile Phase B consisted of 0.1 M lithium acetate (Sigma-Aldrich, Inc.) in a solvent mixture of methanol (Avantor Performance Materials, Inc.), acetonitrile (Honeywell, Inc.) and isopropanol (Mallinckrodt Baker, Inc.) at the ratio of 80/10/10 (v/v/v). pH was adjusted to 6.5 using acetic acid (Sigma-Aldrich, Inc.). The mobile phase was filtered through a 0.2 µm nylon filter (Sartorius Stedim Biotech,Inc.), and delivered at a fixed flow rate of 0.6 ml/min. The temperature of both

cells and the column was set at 25 ºC. The retention time and area of the peaks in tissue homogenates were measured and compared to an external calibrating standard solution containing TYR, TRP, DA, DOPAC, HVA, NE, EPI, 5-HT, and 5-HIAA (Sigma, St. Louis, MO, USA). Concentrations of these substances in the samples were calculated and expressed as ng/mg protein. Turnover ratios (metabolite/monoamine) were calculated as a measure of activity.



Standards	Peak ID
3-Amino-tyrosine	3-AT
Dopamine	DA
Dihydroxybenzylamine	DHBA
L-3-Hydroxy-tyrosine	L-DOPA
3,4-Dihydroxy-phenylacetic acid	DOPAC
Epinephrine	EPI
Glutathione	GSH
Oxidized glutathione	GSSG
5-Hydroxyindole-3-acetic acid	5-HIAA
Serotonin	5-HT
Homovanillic acid	HVA
Metanephrine	MN
Norepinephrine	NE
Normetanephrine	NMN
3-Nitro-tyrosine	3-NT
4-Hydroxy-3-methoxy mandelic acid	VMA

Figure 5. Separation of a standard mixture of redox-active compounds by HPLC-CMEAS in a single column using the modified gradient profile [107].

2.4 STATISTICAL ANALYSIS

Results are presented as mean ± standard error of the mean (SEM). A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed by three steps. First, comparisons of proestrus with diestrus were performed using Student's t-test. Second, all 6 groups were compared by one-way analysis of variance (ANOVA) followed by Tukey's test if overall $p < 0.05$. Third, a two-way analysis of variance (ANOVA) followed by Tukey's test was performed with ovarian status (OVX or VCD) as one factor and Time (1 week or 6 weeks) as a second factor to investigate to investigate interactions between menopausal model and time.

2.5 PRESENTATION OF THE DATA

Hormone levels by treatment group are shown in Figure 6. For each of the brain region, data for DA, 5-HT and NE are shown in Figure 7-9. Data for additional monoamines, metabolites, and metabolite/monoamine ratios also are included for those endpoints that were statistically significant. Table 1-4 summarize all of the data for monoamines and metabolites. Regional profile of monoamines, metabolite and precursor levels are shown in Figure 10.

2.6 RESULTS

2.6.1 Serum levels of hormones

Levels of E2, T, and AD in each of the treatment groups are summarized in Figure 6. Significantly higher levels of E2, T, and AD were detected on proestrus than on diestrus, ($t(14)=3.0$, $p<0.01$ for AD; $t(12)=3.1$, $p<0.01$ for T; $t(14)=4.5$, $p<0.001$ for E2). In proestrus rats, mean serum levels of circulating E2, AD and T were 46.8 ± 7.0 pg/ml, 196.5 ± 42.9 pg/ml and 210.5 ± 41.8 pg/ml. In diestrus rats, levels of E2, AD and T were 13.6 ± 2.4 pg/ml, 66.5 ± 6.3 pg/ml and 81.4 ± 6.0 pg/ml. None of these hormones were detectable in OVX rats. In rats treated with VCD, levels of E2, T, and AD were significantly lower than levels detected in proestrus rats ($p<0.05$ in all cases) and did not differ significantly from levels detected in diestrus rats. This was the case at both 1 week and at 6 weeks following the completion of VCD treatments.

2.6.2 Monoamines, monoamine metabolites, and amino acids levels

2.6.2.1 Comparison of proestrus vs. diestrus

Few differences were detected between brain tissues collected from gonadally intact cycling rats. In the frontal cortex, higher levels of 5-HT were detected at proestrus than at diestrus ($t(14) = 2.2$, $p<0.05$), and the ratio of 5-HIAA/5-HT was significantly lower at proestrus than at diestrus

(t (14) = 2.4, $p<0.05$). Likewise, in the hippocampus, the ratio of 5-HIAA/5-HT was significantly lower at proestrus than at diestrus (t (11) = 2.7, $p<0.05$). In the striatum, higher levels of TYR were detected at proestrus than at diestrus (t (13) = 2.2, $p<0.05$). No other significant differences were detected. There was, however, a trend for reduction in the ratio of 5-HIAA at proestrus vs. diestrus (t (11) = 1.8, p=0.1) in the HPC, and a strong trend for an increase in HVA (t (14) = 2.1, p=0.06) levels at proestrous vs. diestrous in STR.

2.6.2.2 Effects of OVX and VCD treatments

Significant effects of OVX and VCD treatments on monoamines, monoamine metabolites, and amino acid levels were detected.

HPC

One-way ANOVAs consistently revealed significant effects of OVX and VCD treatments on levels of NE ($F[5,35]=8.6$, $p<0.0001$), 5-HIAA ($F[5,35]=20.6$, $p<0.0001$), TYR and TRP ($p<0.0001$ in each case). Effects were observed primarily at 1 week following OVX and VCD treatment (Fig 7). Specifically, 1 week following OVX, levels of 5-HIAA were reduced relative to both diestrus ($p<0.0001$) and proestrus ($p<0.001$). There was a trend for reduction in 5-HIAA levels at proestrus vs. diestrus (p=0.07). Levels of NE ($p<0.05$) likewise were reduced relative to diestrus, with a trend towards reduced levels relative to proestrus (p=0.08). Similar effects were observed following VCD treatments. At 1 week following VCD treatments levels of both 5-HIAA and NE were reduced relative to both diestrus and proestrus ($p<0.0001$ for 5-HIAA; $p<0.01$ for NE in each case). These effects were not observed 6 weeks after OVX or 6 weeks following the

completion of VCD treatments. Levels of the amino acids (TYR and TRP) also were significantly reduced (up to 80%) at 1 week following OVX and VCD treatments relative to both proestrus and diestrus in gonadally intact controls (Figure 7; $p<0.0001$ in each case). By 6 weeks the levels of the amino acids had returned to levels comparable to normal cycling rats. Notably, the levels of DA and 5-HT were not affected. Levels of DOPAC, HVA and EPI were undetectable.

Significant effects on the ratios of DA/TYR ($F[5,35]=10.6$, $p<0.0001$) and 5-HIAA/5-HT ($F[5,35]=7.4$, $p<0.0001$) also were detected. The ratios of DA/TYR at 1 week following OVX or VCD treatments were significantly higher than the ratios detected at proestrus and diestrus ($p<0.05$ following OVX; $p<0.01$ following VCD). The ratios of 5-HT/TRP at 1 week following OVX or VCD treatments were significantly higher than the ratios detected at proestrus and diestrus ($p<0.001$ in each case). The ratio of 5-HIAA/5-HT was significantly lower at 1 week after OVX or VCD treatment relative to diestrus ($p<0.05$ in each case), but did not differ significantly from proestrus.

In no case did one-way or two-way ANOVAs reveal significant differences between the two menopausal models on any of the endpoints measured in the hippocampus. Results of the two-way ANOVAs on each of the measures revealed a significant overall effect ($F[3,24]>8.0$, $p<0.001$), a significant effect of time-point ($p<0.0001$ in each case), no main effect of model, and no interaction between model x time-point.

FCX

One-way ANOVA revealed significant effects on the levels of DA ($F[5,40]=3.8$, $p<0.01$). Elevated levels of DA were detected at 6 weeks following OVX relative to diestrus ($p<0.01$), as well as relative to VCD-treated rats at the 6-week time-point ($p<0.01$). Levels of DA at 1 week

following VCD treatments also were significantly higher than levels detected at 6 weeks following VCD treatments ($p<0.05$), with a trend towards being higher relative to diestrus ($p=0.07$) (Figure 8). One-way ANOVA also revealed a significant effect on the ratio of NE/DA ($F(5,40)=3.14$, $p<0.05$). This ratio was significantly lower 6 weeks following OVX than at 1 week following OVX. A trend for reduction in the ratio of NE/DA was detected at 6 weeks following OVX vs. VCD treatments ($p=0.07$). In no case did the ratio of NE/DA in OVX or VCD-treated rats differ significantly from the ratio detected in normal cycling rats.

Two-way ANOVA of DA levels revealed a significant overall effect ($F[3,26]=3.9$, $p<0.05$), no main effects of model or time-point, but a significant interaction between model x time-point ($p<0.01$) with the levels of DA higher at 6 weeks following OVX than at 6 weeks following VCD treatments ($p<0.05$). Likewise, two-way ANOVA of NE/DA ratio revealed a significant overall effect ($F[3,26]=4.6$, $p<0.01$), no main effects of model or time-point, but a significant interaction between model x time-point ($p<0.01$) with the ratio of NE/DA lower at 6 weeks following OVX than at 6 weeks following VCD treatments ($p<0.05$).

STR

One-way ANOVA revealed significant effects on the levels of NE ($F[5,40]=3.9$, $p<0.01$) and HVA ($F[5,40]=5.4$, $p<0.001$) in the STR. Elevated levels of NE were detected at 1 week following OVX relative to proesturs ($p<0.05$), diestrus ($p<0.01$), and at 1 week following VCD treatment ($p<0.01$). Elevated levels of HVA were detected at 1 week following OVX and VCD treatment relative to diestrus, and at 1 week following VCD treatment relative to proestrus ($p<0.05$ in each case) (Figure 9). No significant effects on the levels of TYR or TRP were detected in the STR.

Two-way ANOVA of NE levels revealed a significant overall effect ($F[3,26]=3.6$, $p<0.05$), a main effect of model ($p<0.05$), no main effect of time-point, and a significant interaction between model x time-point ($p<0.05$) with the levels of NE higher 1 week following OVX than 1 week following VCD treatments ($p<0.05$). Two-way ANOVA of HVA levels revealed no significant effects of model or time-point.

Table 1. Neurochemical Endpoints in HPC of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD.

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
HPC							
N		8	5	6	6	8	8
5HT (ng/mg)		1.3 ± 0.16	0.9 ± 0.28	1.4 ± 0.20	1.2 ± 0.26	1.1 ± 0.13	1.0 ± 0.21
5HIAA (ng/mg)	+++	16.2 ± 1.68^{A}	21.7 ± 2.76^{Ao}	8.1 ± 0.93^{B†}	16.7 ± 0.51^{A}	6.2 ± 0.28^{B}	17.0 ± 0.86^{A}
DA (ng/mg)		3.6 ± 0.15	3.8 ± 0.17	3.5 ± 0.35	3.4 ± 0.13	3.6 ± 0.52	3.4 ± 0.08
NE (ng/mg)	+++	6.0 ± 0.30^{AB}	6.4 ± 0.37^{A}	4.6 ± 0.21^{BC†}	6.8 ± 0.44^{A}	4.3 ± 0.20^{C}	6.0 ± 0.41^{AB}
TRP (ng/mg)	+++	255.6 ± 27.26^{A}	231.8 ± 31.14^{A}	101.5 ± 10.09^{B}	256.0 ± 29.25^{A}	99.1 ± 12.00^{B}	259.3 ± 21.67^{A}
TYR (ng/mg)	+++	475.8 ± 47.83^{A}	447.1 ± 65.5^{A}	142.5 ± 27.55^{B}	501.3 ± 62.87^{A}	118.0 ± 12.73^{B}	458.4 ± 37.53^{A}
5HIAA/5HT	+++	15.2 ± 3.33^{BC}	32.3 ± 6.11^{A*}	6.1 ± 0.38^{C}	18.1 ± 4.35^{ABC}	6.1 ± 0.75^{C}	22.9 ± 4.18^{AB}
5HT/TRP	+++	0.005 ± 0.001^{B}	0.004 ± 0.001^{B}	0.014 ± 0.001^{A}	0.005 ± 0.001^{B}	0.012 ± 0.002^{A}	0.004 ± 0.001^{B}
DA/TYR	+++	0.008 ± 0.001^{B}	0.009 ± 0.001^{B}	0.029 ± 0.006^{A}	0.007 ± 0.001^{B}	0.035 ± 0.007^{A}	0.008 ± 0.001^{B}
NE/DA		1.7 ± 0.08	1.7 ± 0.08	1.4 ± 0.19	2.0 ± 0.13	1.5 ± 0.30	1.8 ± 0.11

Table 2. Neurochemical Endpoints in FCX of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD.

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
FCX							
N		8	8	7	7	8	8
5HT (ng/mg)		2.9 ± 0.41	1.9 ± 0.22*	2.1 ± 0.27	2.6 ± 0.62	2.3 ± 0.24	2.5 ± 0.32
5HIAA (ng/mg)		7.1 ± 0.60	7.2 ± 0.44	7.3 ± 0.62	8.0 ± 0.88	7.9 ± 0.77	8.0 ± 0.67
DA (ng/mg)	++	2.7 ± 0.13^{ABC}	2.6 ± 0.11^{BC}	2.7 ± 0.08^{ABC}	3.3 ± 0.26^{A}	3.2 ± 0.12^{AB‡}	2.5 ± 0.21^{C}
NE (ng/mg)		4.3 ± 0.34	3.9 ± 0.31	4.8 ± 0.12	4.2 ± 0.32	4.6 ± 0.33	4.2 ± 0.36
TRP (ng/mg)		163.0 ± 23.07	139 ± 17.95	134.2 ± 13.97	146.9 ± 19.24	145.6 ± 13.76	145.4 ± 15.25
TYR (ng/mg)		296.5 ± 43.84	238.6 ± 47.82	285.2 ± 24.86	308.6 ± 32.18	296.0 ± 27.37	274.7 ± 25.24
5HIAA/5HT		2.8 ± 0.36	4.2 ± 0.48*	3.9 ± 0.64	4.1 ± 0.83	3.7 ± 0.53	3.5 ± 0.35
5HT/TRP		0.020 ± 0.004	0.014 ± 0.001	0.016 ± 0.002	0.017 ± 0.003	0.017 ± 0.002	0.017 ± 0.002
DA/TYR		0.011 ± 0.001	0.014 ± 0.003	0.010 ± 0.001	0.011 ± 0.000	0.011 ± 0.001	0.010 ± 0.001
NE/DA	+	1.6 ± 0.07^{AB}	1.5 ± 0.09^{AB}	1.8 ± 0.07^{A}	1.3 ± 0.11^{B}}$	1.4 ± 0.12^{AB}	1.7 ± 0.08^{AB}

Table 3. Neurochemical Endpoints in STR of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD.

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
STR							
N		8	8	7	7	8	8
5HT (ng/mg)		5.0 ± 0.78	5.9 ± 1.30	6.7 ± 0.81	5.6 ± 0.64	5.2 ± 0.80	5.3 ± 0.59
5HIAA (ng/mg)		19.1 ± 1.58	19.5 ± 2.02	20.5 ± 1.07	19.3 ± 1.47	18.4 ± 1.63	19.4 ± 1.61
DA (ng/mg)		106.6 ± 14.06	107.8 ± 22.46	111.9 ± 15.28	101.5 ± 12.32	104.9 ± 16.15	95.0 ± 17.41
DOPAC (ng/mg)		49.9 ± 5.89	42.3 ± 5.38	45.4 ± 3.97	55.3 ± 5.95	48.5 ± 3.30	47.9 ± 8.36
HVA (ng/mg)	+++	23.4 ± 0.72^{BC}	20.3 ± 1.27^{Co}	27.2 ± 2.04^{AB}	25.4 ± 1.69^{ABC}	29.1 ± 1.05^{A}	24.9 ± 1.06^{ABC}
NE (ng/mg)	++	3.8 ± 0.35^{B}	3.3 ± 0.52^{B}	8.0 ± 1.48^{A}	4.8 ± 0.95^{AB}	3.2 ± 0.62^{B}	4.6 ± 1.05^{AB}
EPI (ng/mg)		51.7 ± 7.90	52.4 ± 7.98	51.3 ± 9.71	66.0 ± 5.37	60.9 ± 3.11	56.5 ± 6.92
TRP (ng/mg)		214.7 ± 23.91	162.2 ± 16.50	181.5 ± 21.84	185.8 ± 18.86	187.1 ± 25.5	187.8 ± 11.93
TYR (ng/mg)		318.8 ± 32.72	233.0 ± 17.13*	288.5 ± 29.3	293.7 ± 21.71	295.7 ± 34.17	273.9 ± 18.6
5HIAA/5HT		4.6 ± 0.79	4.5 ± 0.97	3.3 ± 0.45	3.7 ± 0.35	4.0 ± 0.54	3.9 ± 0.54

Table 4. Neurochemical Endpoints in STR of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD (Cont'd).

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
STR							
N		8	8	7	7	8	8
DOPAC/DA		0.5 ± 0.11	0.5 ± 0.13	0.4 ± 0.07	0.6 ± 0.11	0.5 ± 0.06	0.5 ± 0.08
HVA/DA		0.3 ± 0.04	0.3 ± 0.06	0.3 ± 0.05	0.3 ± 0.03	0.3 ± 0.05	0.3 ± 0.05
EPI/NE		13.6 ± 1.84	20.4 ± 5.25	8.0 ± 1.81	26.3 ± 12.77	23.7 ± 3.95	24.7 ± 9.34
5HT/TRP		0.026 ± 0.005	0.037 ± 0.007	0.040 ± 0.006	0.033 ± 0.005	0.037 ± 0.012	0.029 ± 0.003
DA/TYR		0.373 ± 0.066	0.495 ± 0.102	0.393 ± 0.044	0.361 ± 0.051	0.407 ± 0.084	0.370 ± 0.081
NE/DA		0.039 ± 0.011	0.042 ± 0.012	0.080 ± 0.019	0.050 ± 0.013	0.039 ± 0.011	0.064 ± 0.019

Values are mean ± sem.

+		p<=0.05	ANOVA
++		p<0.01	ANOVA
+++		p<0.001	ANOVA
†	relative to P	0.05<p<0.1	ANOVA
‡	relative to D	0.05<p<0.1	ANOVA
$	relative to VCD-6w	0.05<p<0.1	ANOVA
*		p<0.05	t-test P vs D
o		0.05<p<0.01	t-test P vs D

For ANOVA results, groups not connect by the same letter (A, B or C) are significantly different by Tukey test

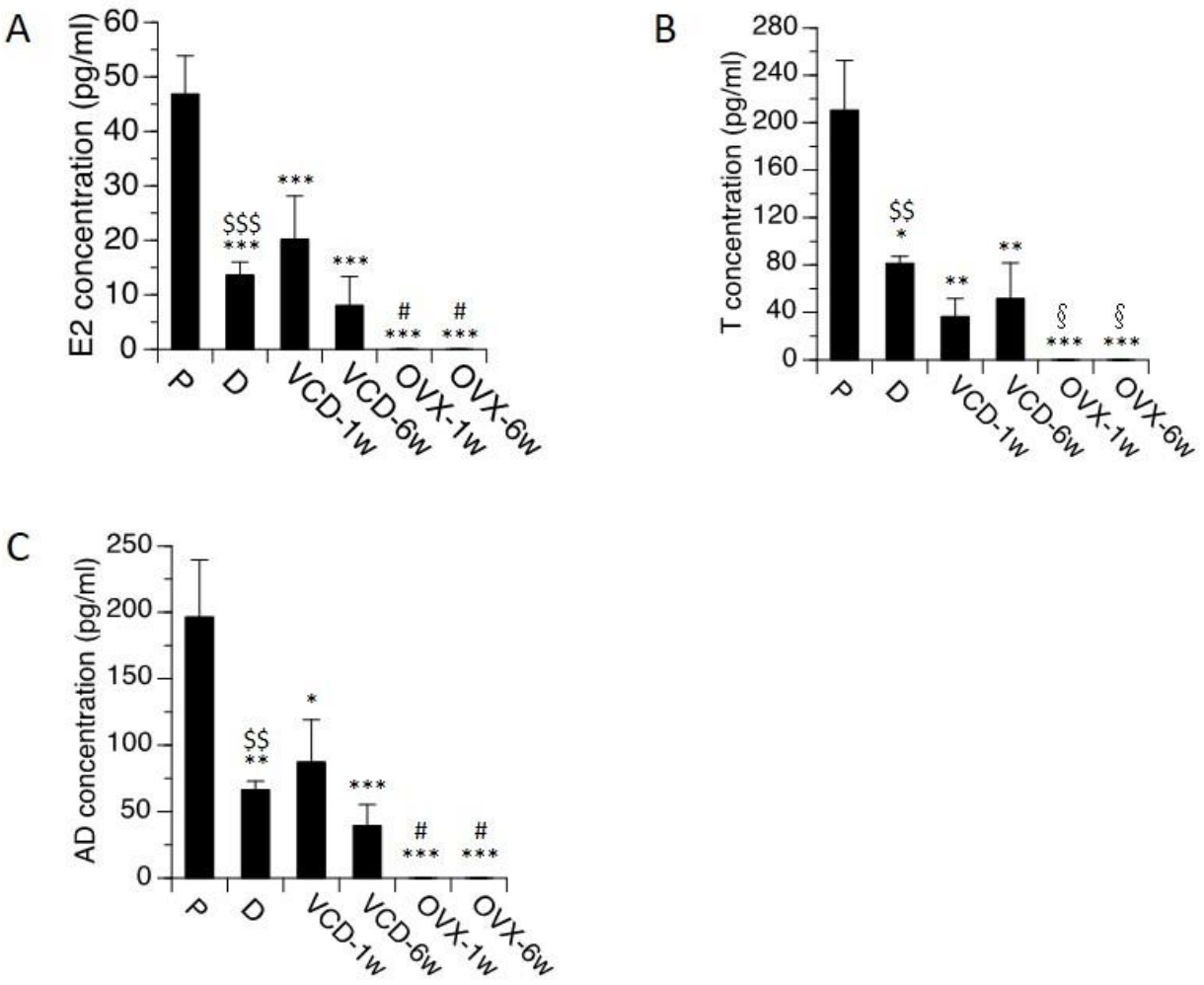
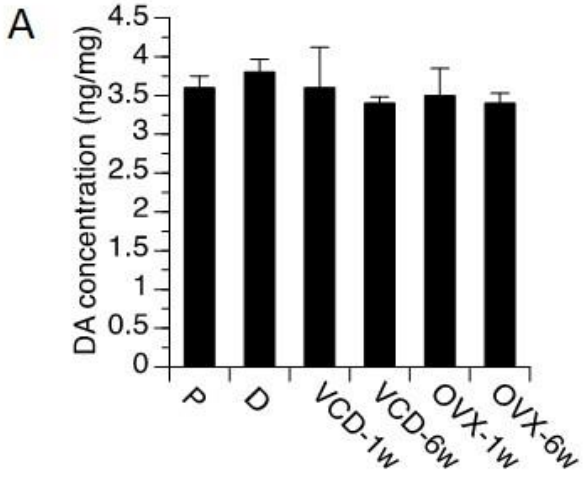
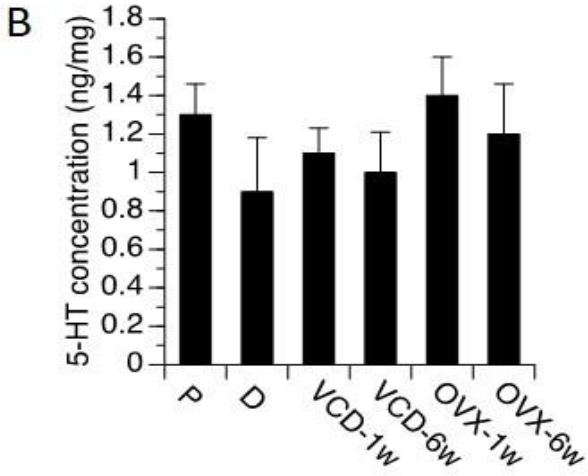
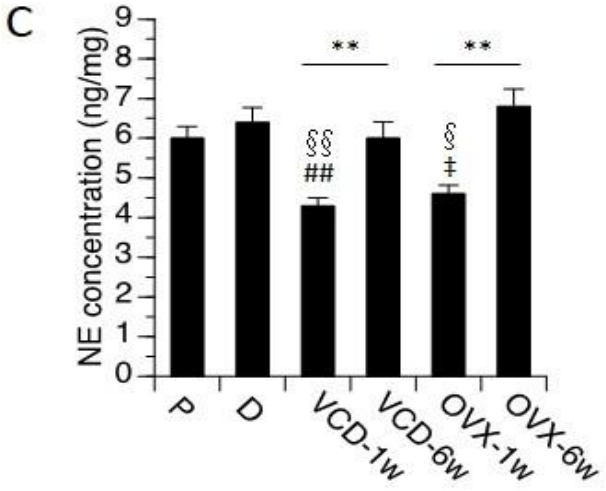
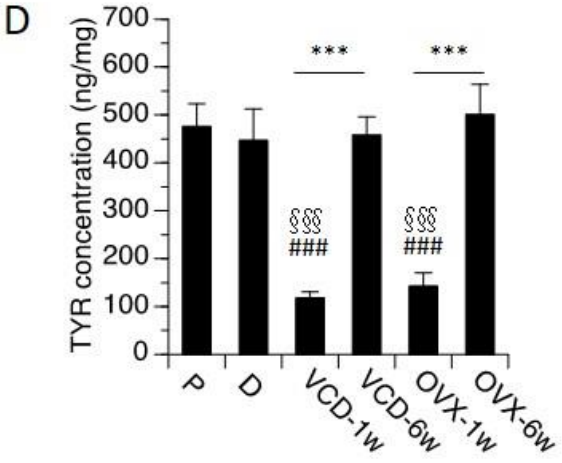


Figure 6. Serum (A) E2 (17β-estradiol), (B) T (testosterone) and (C) AD (androstenedione) levels by treatment groups. Rats at P (proestrous) and D (diestrous) are used as controls. Bars indicate Mean±SEM. One-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, relative to P; $^{\#}$p<0.05 relative to VCD-1w; §p<0.05 relative to VCD-6w. T-test: $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001, relative to P. None of these hormones were detectable in OVX rats.


A
DA concentration (ng/mg)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w


B
5-HT concentration (ng/mg)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w


C
NE concentration (ng/mg)
9
8
7
6
5
4
3
2
1
0
**
**
§§
##
§
‡
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w


D
TYR concentration (ng/mg)
700
600
500
400
300
200
100
0
***
***
§§§
###
§§§
###
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w

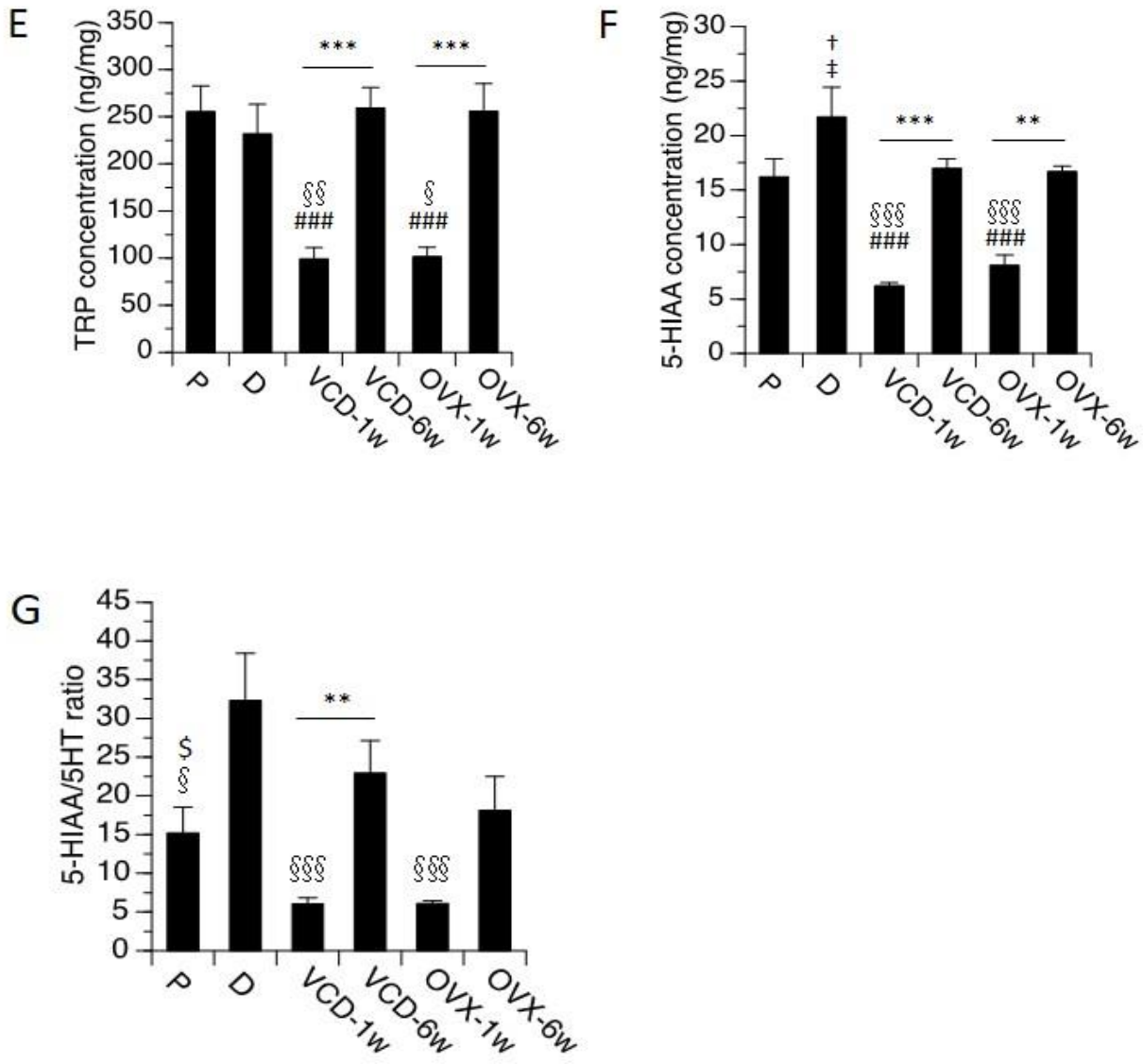


Figure 7. Monoamine, metabolite, amino acid levels and metabolite/monoamine ratio by treatment groups in HPC. Rats at P (proestrous) and D (diestrous) are used as controls. Bars indicate Mean ± SEM. One-way ANOVA: **p<0.01, ***p<0.001 relative to VCD/OVX-1w; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 relative to P; §p<0.05, §§p<0.01, §§§p<0.001 relative to D; ‡ 0.05<p<0.1, relative to P. T-test: $^{\$}$p<0.05, relative to D; †0.05<p<0.1, relative to P.

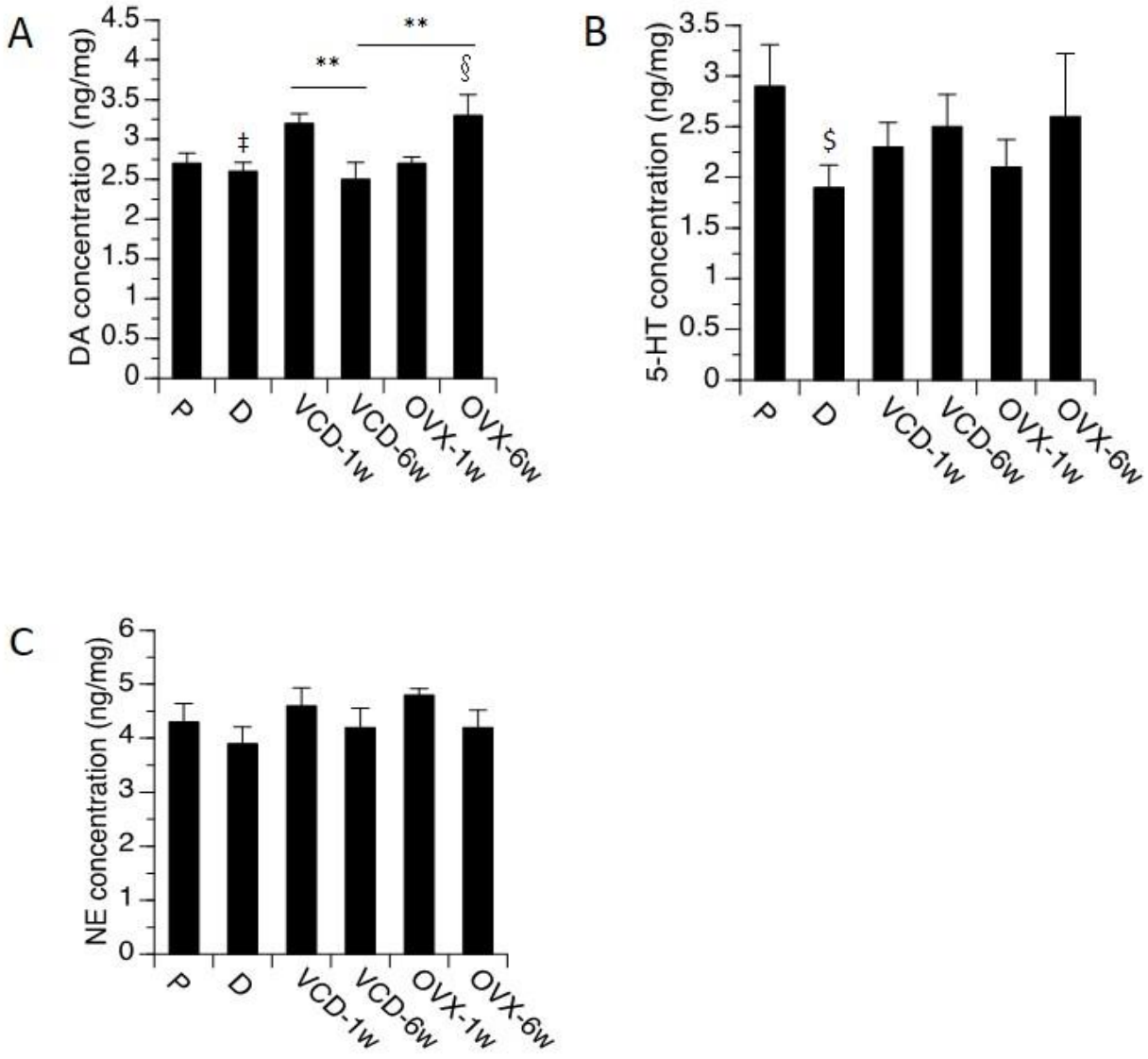


Figure 8. Monoamine levels by treatment groups in FCX. Rats at P (proestrous) and D (diestrous) are used as controls. Bars indicate Mean ± SEM. One-way ANOVA: **$p<0.01$ relative to VCD-6w; $^{\S}p<0.05$ relative to D; ‡ $0.05<p<0.1$, relative to VCD-1w. T-test: $^{\$}p<0.05$, relative to P.

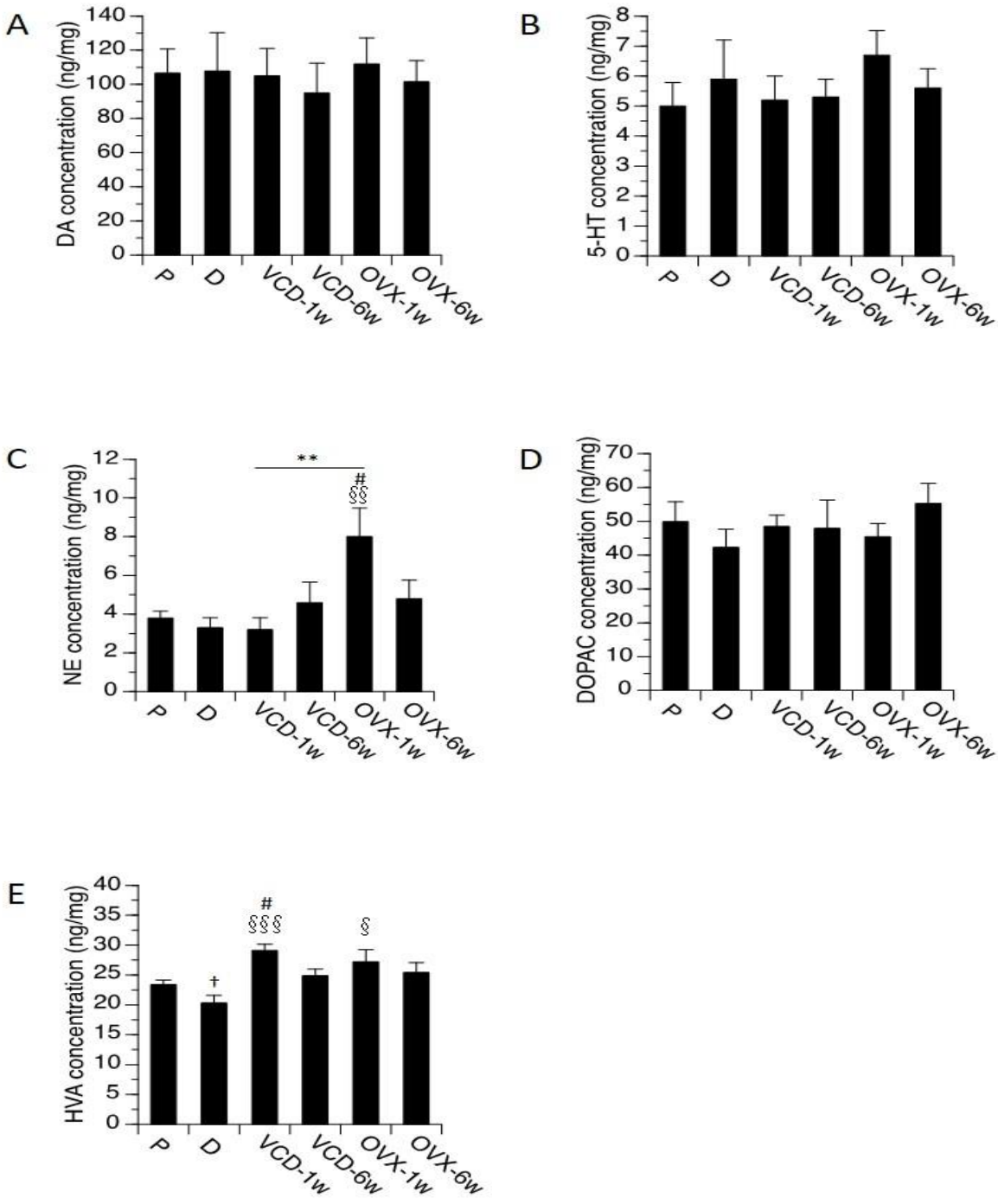
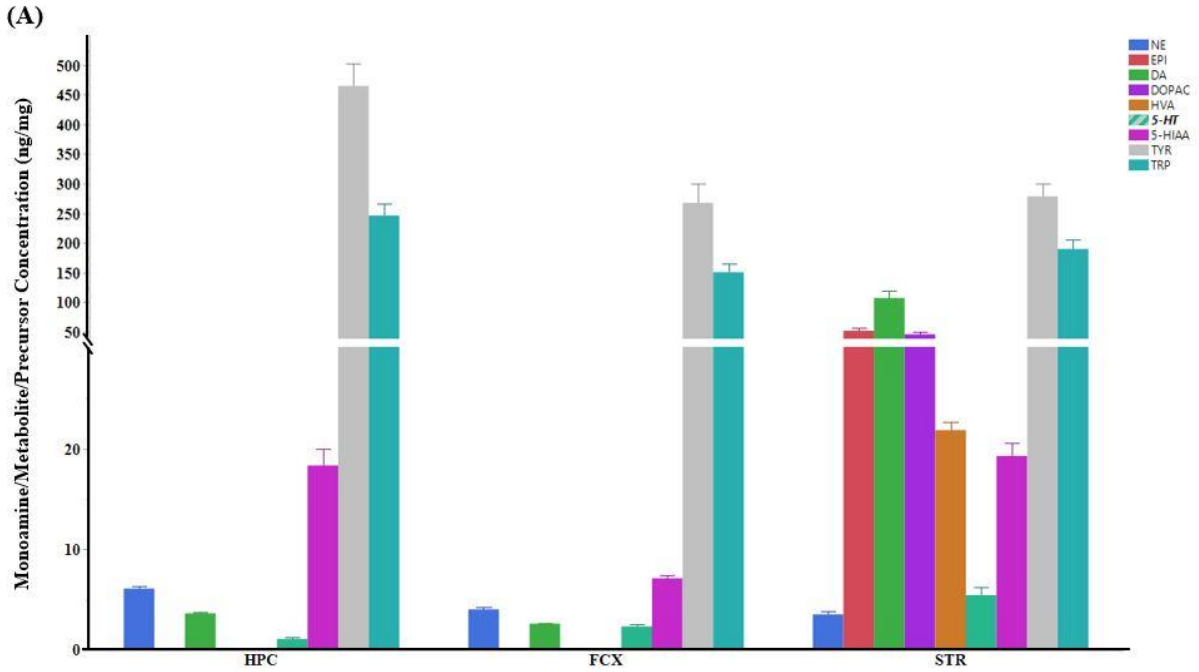
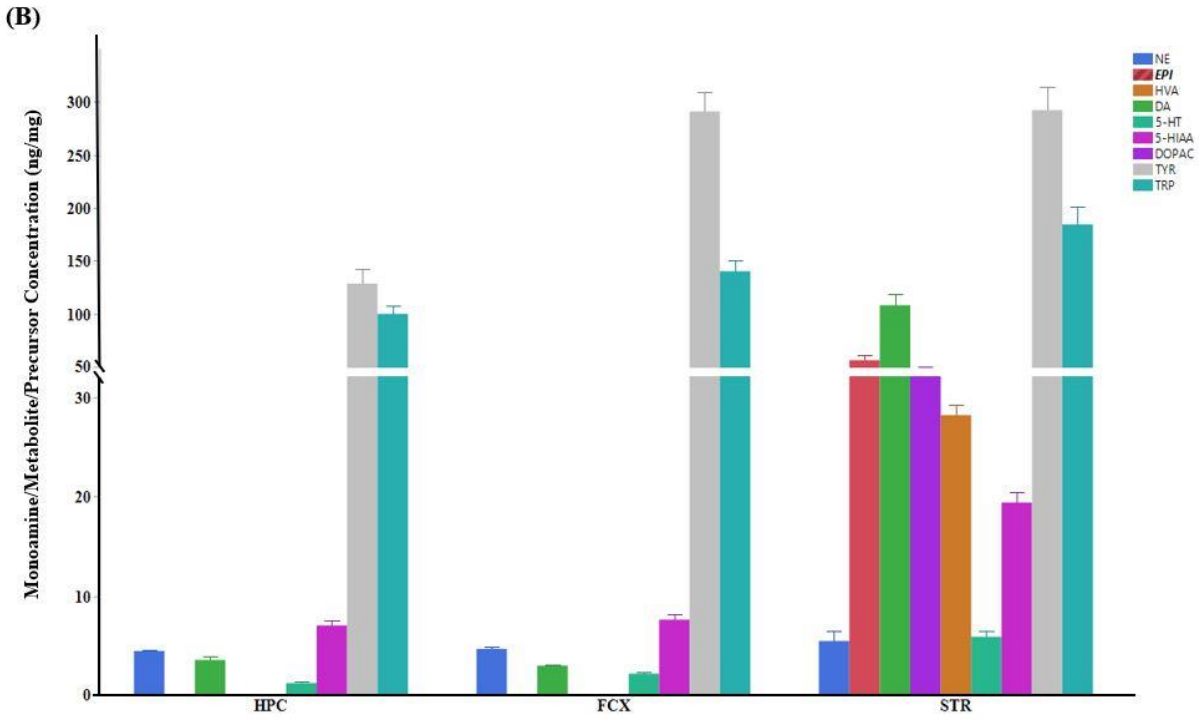


Figure 9. Monoamines and metabolite levels by treatment groups in STR. Rats at P (proestrous) and D (diestrous) are used as controls. Bars indicate Mean ± SEM. One-way ANOVA: **p<0.01 relative to VCD-1w; $^{\#}$p<0.05 relative to P; §p<0.05, §§p<0.01, §§§p<0.001 relative to D. T-test: † 0.05<p<0.1, relative to P.


(A)
Monoamine/Metabolite/Precursor Concentration (ng/mg)
500
450
400
350
300
250
200
150
100
50
20
10
0
HPC
FCX
STR
NE
EPI
DA
DOPAC
HVA
5-HT
5-HIAA
TYR
TRP


(B)
Monoamine/Metabolite/Precursor Concentration (ng/mg)
300
250
200
150
100
50
30
20
10
0
HPC
FCX
STR
NE
EPI
HVA
DA
5-HT
5-HIAA
DOPAC
TYR
TRP

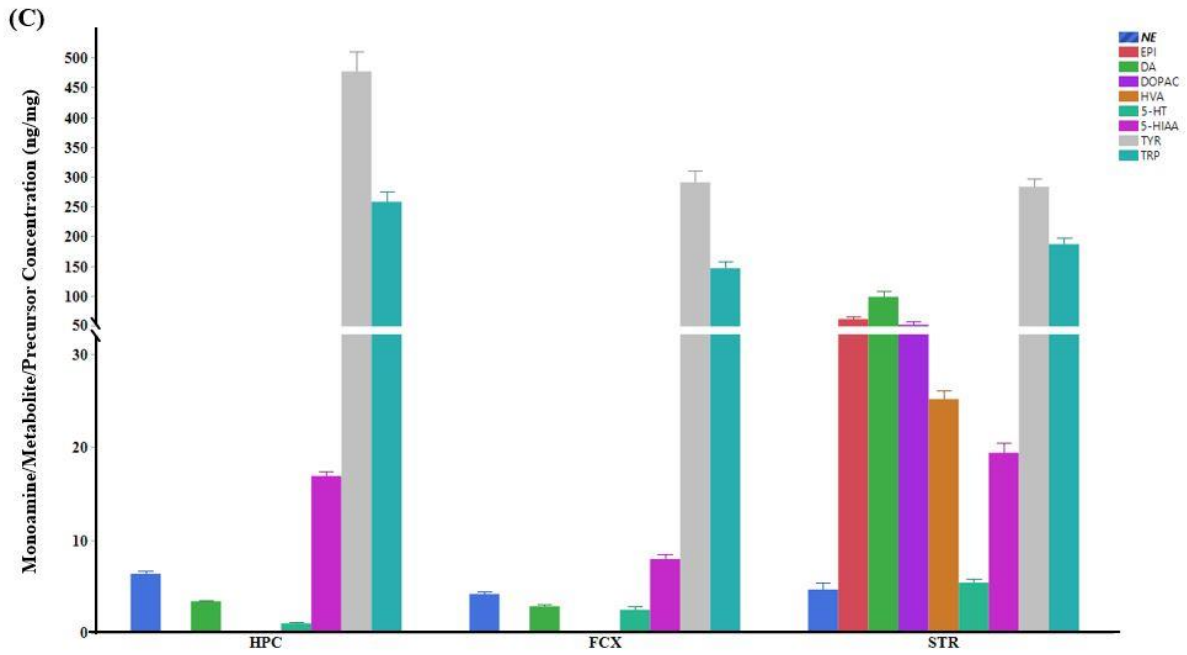


Figure 10. Profile of monoamines, metabolite and precursor levels by brain region. (A) Rats at P/D ;(B) Rats at 1W; (C) Rats at 6W. Since not many model differences on these endpoints were detected, data from OVX and VCD were combined. As expected, high levels of DA were detected in STR compared with HPC and FCX. Levels of EPI, DOPAC and HVA were not detectable in HPC and FCX, partially due to low levels of their precursors in the two regions.

2.7 DISCUSSION

2.7.1 Serum hormone measurements

Our goal was to characterize neurochemical changes in the brain associated with two models of menopause, surgical vs. transitional, and to compare effects with changes observed at two stages of the estrous cycle, proestrus and diestrus. Circulating levels of E2, T and AD confirm that representation of each of these conditions was successfully accomplished. Consistent with previous study using GC-MS/MS for hormone measurement [108], levels of E2, T and AD levels were significantly lower at diestrous than at proestrous. In addition, E2, T and AD were not detected 1 week and 6 weeks after OVX, as expected with complete removal of the ovaries. Unlike OVX, VCD treatments eliminate only the ovarian follicles, while the androgen-producing interstitial cells remain intact [77]. Consequently, in VCD-treated rats E2 levels were reduced, whereas androgens continued to be detected at levels comparable to diestrus. These findings are consistent with previous characterizations of this model [81]. In addition to AD levels, our study provides new information regarding T levels in two distinct estrous stages in comparison to VCD and OVX treatment.

2.7.2 Fluctuations in neurochemical endpoints detected in gonadally intact rats

Results show that significant effects are detected in the HPC and FCX associated with cycle stage and are limited primarily to the serotonin pathway. Specifically, levels of 5-HT in the FCX were significantly higher at proestrus than at diestrus, and the ratio of 5-HIAA/5-HT was significantly lower in the HPC and FCX in rats euthanized at proestrus vs. diestrus. Similar results were reported by Shimizu, where the ratio of 5-HIAA/5-HT was significantly reduced during proestrous in nucleus accumbens [109]. The increase in 5-HT coupled with a decrease in the ratio of 5-HIAA/5-HT in the FCX suggests a decrease in 5-HT release, resulting in elevated 5-HT and less 5-HIAA production. A similar reduction in 5-HT turnover may be occurring in the HPC, although levels of 5-HT were not elevated at proestrus in this region. There also was a trend for a reduction in the ratio of 5-HT/TRP in the HPC at proestrus vs. diestrus, though this did not reach statistical significance. This may reflect greater susceptibility of the HPC to effects on amino acid levels with corresponding effects on neurotransmitter endpoints. These effects appear minimal and difficult to detect in normal cycling rats, but were exacerbated in the two models of menopause (see below).

Some of our results differ from published studies. For example, we detected no significant differences in neurochemical endpoints as a function of cycle in the striatum. In contrast, Xiao et al. [69] reported that in female rats extracellular DA concentrations in the STR were significantly higher at proestrus than at diestrus. In contrast to the elevation in 5HT we detected in the FCX, Desan et al.[110] reported no significant differences in 5-HT levels in anterior cerebral cortex, hippocampus or cerebellum across the estrous cycle. Differences may be due in part to methodologies and to the absence of verification of hormone levels. Advantages of the current

study include the measurement of hormone levels, as well as multiple neurochemical endpoints, thus giving a more comprehensive characterization of the neurochemical signatures.

2.7.3 Effects of OVX and VCD treatments on neurochemical endpoints

A strength of the metabolomics approach is the ability to provide a comprehensive analysis of multiple endpoints for a variety of brain structures, all from the same set of tissues. Multiple effects were detected and most effects were both region-specific and time-dependent. One of the key findings of our analysis is the relatively few differences in the effects of the two models on neurochemical endpoints, despite the fact that the hormonal profiles, particularly androgen levels, were quite different. This suggests that surgical and transitional menopause produce similar effects on these endpoints, at least during the early weeks following menopause. This, in turn, suggests that any differences in cognitive performance between OVX and VCD-treated rats are not due to differential effects on monoaminergic pathways.

As we dissect the regional effects, we find that the greatest number of effects were detected in the HPC, suggesting that this region of the brain is particularly sensitive to loss of ovarian function. The hippocampus plays a critical role in memory consolidation as well as age-related cognitive decline and many studies have demonstrated significant effects of estrogens on synaptic plasticity and neuronal function in this region [111]. In the current study, effects of both surgical and VCD-induced loss of ovarian function in the HPC were detected at 1 week, but not at 6 weeks, suggesting that these are temporary effects. The results are in agreement with previous reports showing decreased levels of NE [66, 67] and 5-HIAA [66] following ovariectomy in mice. Furthermore,

our finding of reduced 5-HIAA/5-HT ratio observed at 1 week and not 6 weeks following OVX or VCD treatments agrees with previous results in rats where the ratio was reduced at 2 weeks, but not 4 weeks, after OVX [74]. The substantial reduction in TRP as well as elevated ratios of 5-HT/TRP and 5-HIAA/TRP suggest that at an early time-point following either OVX or VCD treatments serotonin turnover is significantly reduced, possibly due to reductions in the amino acid TRP. The fact that levels of DA and 5-HT in HPC remained unchanged following estrogen deprivation also concurs with earlier findings reported in mice [66].

The fact that 5-HIAA was reduced without a reduction in 5-HT suggests that less 5-HT was released, perhaps in response to a reduction in 5-HT, resulting from a reduction in TRP. A similar effect may be occurring in the DA and NE pathways. Levels of NE were reduced, possibly in response to a reduction in TYR. Since levels of DOPAC and HVA were not detectable in this region we were unable to evaluate potential effects on DA turnover; however, it may be that temporary reductions in TYR resulted in reduced DA release (thereby preserving DA levels) along with reduced metabolism of DA to NE.

Some of our findings were surprising and not entirely consistent with previous reports. For example, in contrast with the HPC, relatively few effects of OVX and VCD treatments were detected in the FCX. The FCX plays a critical role in attention as well as executive functions and significant effects of estrogen treatment on monoaminergic innervation of the FCX have been reported [112]. Nevertheless, in the present study none of the sizable decreases in 5-HIAA, NE and amino acids that were detected in the HPC at 1 week following OVX or VCD treatments were detected in the FCX. This was unexpected given the significantly higher levels of 5HT and the lower ratio of 5-HIAA/5-HT detected in the FCX at proestrus relative to diestrus. Hence, we anticipated lower levels of 5-HT in the FCX following OVX and VCD treatments compared with

proestrus in association with the lower levels of gonadal hormones. The unexpected result suggests that levels of 5-HT cease to fluctuate and are restored in non-cycling animals, even in a state of chronically low estrogen levels.

The significant increase in DA in the FCX of OVX-6W rats also was surprising, and contrasts with previous results showing a decrease in DA at 20 days following OVX treatment in mice [67]. This could reflect a species difference, or possibly differences associated with the time points when neurotransmitter levels were evaluated. In contrast to OVX rats, higher DA levels were detected in the FCX at 1 week relative to 6 weeks following VCD treatments. This was one of the few instances where effects of the two models differed. These increases in DA in OVX and VCD-treated rats may reflect compensatory changes in the expression or activity of metabolic enzymes involved in the production and degradation of the monoamines (see below).

In contrast with effects in the HPC and FCX, OVX and VCD treatments significantly affected the noradrenergic pathway in the STR. The striatum is part of the extrapyramidal motor system. Not only is it critically involved in extrapyramidal motor control but also plays a significant role in motor learning [113]. Notably, elevated estrogens have been shown to favor the use of hippocampal learning strategies whereas low estrogens have been shown to favor the use of striatal learning strategies [114].

Our data show an increase in NE in the STR at 1 week following OVX, but not following VCD-treatment. This again is one of the few model differences that were detected and may contribute to short-term differences in striatal function and motor learning following loss of ovarian function. We also detected increases in HVA at 1 week following OVX or VCD treatment. This agrees with results reported by Bitar et al. which showed markedly elevated concentrations of HVA in male and female rats following gonadectomy, whereas the level of 5-HT and 5-HIAA

remained unaltered in STR [68]. However, they also reported detected elevated DA and DOPAC levels after OVX whereas we did not. Using a microdialysis approach, a separate study found that female rats had significantly higher extracellular striatal dopamine concentrations at proestrus than after ovariectomy [69]. The reasons for these inconsistencies are unknown. Differences in analytical methodologies and in the length of time between ovariectomy and day of sacrifice could potentially account for differences.

In the present study, increases in NE and HVA following OVX suggests an increase in NE production and DA release at the 1-week time point in the surgical model. The increases in HVA detected in the OVX-1W and VCD-1W groups may indicate an increase in DA release at the early time-point in response to the loss of ovarian function. It was unexpected that elevated HVA levels were accompanied by unaltered DOPAC level in this brain structure. One possible explanation is that DA metabolism was shifted toward the DA-3MT (3- methoxytyramine)-HVA pathway (15% of DA turnover in normal condition), however additional study is required to elucidate the underlying mechanisms.

2.7.4 Potential mechanisms – anabolic and catabolic enzymes

Possible mechanism for some of the effects observed on the neurochemical endpoints could be alterations in synthetic and catabolic enzymes involved in monoamine regulation. Tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) are the rate limiting enzymes responsible for the conversion of tyrosine and tryptophan to dopamine and serotonin. Both of these enzymes are potently regulated by phosphorylation and are negatively regulated by monoamines [115, 116].

Numerous reports show that these enzymes also can be regulated by estrogens. Some reports are contradictory. For example, Beattie et al. [117] reported that ovariectomy caused a 2-3 fold increase in hypothalamic TH activity in the rat, whereas Krieger et al. [118] reported no significant increase in TH activity in the hypothalamus or medial preoptic area following ovariectomy. Ivanova and Beyer [119] reported that local estrogen production increases TH mRNA and protein expression in the ventral midbrain of mice during late prenatal and early postnatal development. Sabban et al. [120] reported differing effects of estradiol on TH expression in the nucleus of the solitary tract and in the locus coeruleus that differed depending on mode of delivery. Kritzer et al.[121] reported that the density of TH immunoreactive fibers decreased in the dorsolateral prefrontal cortex of rhesus monkeys following ovariectomy, which was reversed by combined treatment with estrogen + progesterone. These findings demonstrate the potential for TH expression to be regulated by estradiol in a region-specific way.

TPH expression also has been shown to be regulated by estrogen signaling. Smith et al. [122] reported that 1 month of estradiol treatment increased TPH mRNA in the dorsal raphe of rhesus monkeys. These same treatments also decreased expression of the serotonin transporter and decreased expression of monoamine oxidase (MAO)-A mRNAs. Hiroi et al. [123] showed similar increases in TPH-2 mRNA in the rat raphe in response to estradiol. More recently, Hiroi et al. [124] reported that treating with estradiol or with conjugated equine estrogens increased TPH-2 mRNA in the raphe with some differences in subregion-specific effects depending on which estrogens were administered. Collectively, these findings are consistent with estrogen enhancement of serotonin signaling.

Other enzymes that may contribute to the effects of menopause on neurochemical endpoints are dopamine beta hydroxylase (DBH), a key enzyme in NE synthesis, as well as MAO and

catechol-O-methyl transferase (COMT), key enzymes responsible for the inactivation of monoamines. Zhang et al. [125] reported a reduction in DBH in the locus coeruleus following OVX which was associated with a reduction NE levels, and Sabban et al. [120] reported that estradiol potently increased activation of the DBH promoter in PC12 cells. Studies also have reported significant increases in MAO activity in the hypothalamus [126] and COMT activity in the prefrontal cortex [127] following ovariectomy in rats. Hence, estrogen regulation of these enzymes is brain region specific and may differ as a function of menopause and length of time in a hypoestrogenic state. Further clarification of how these enzymes contribute to the effects of surgical and transitional menopause on neurochemical endpoints will require a detailed analysis of the anabolic and catabolic enzymes associated with the regulation of monoamines in each of the affected brain structures.

2.7.5 Effects on TRP and TYR levels

This study is also the first to analyze levels of TRP and TYR following loss of ovarian function in the same tissues in which multiple monoaminergic endpoints were measured. Most striking was the reduction in the levels of TRP and TYR detected in the HPC, but not in the FCX or STR, at 1 week following OVX or VCD treatments, which suggests loss of ovarian function has substantial acute effects on amino acid homeostasis in this region of the brain. This effect was only observed in the HPC, was not observed in normal cycling rats, and levels returned to normal within 6 weeks. To our knowledge this is the first report of a substantial reduction in amino acid levels detected in the HPC following the loss of ovarian function.

At present, the mechanisms responsible for the reductions in TRP and TYR at the 1-week time-point are unknown. TYR and TRP are large, neutral, aromatic amino acids. Their brain levels are controlled in part by their plasma concentrations, but are even more dependent on plasma concentrations of other large neutral amino acid (LNAA) and Branched Chain Amino Acids (BCAA). Previous research has demonstrated that certain LNAA (e.g. Phenlyalanine,PHE) as well as BCAAS (leucine,LEU; isoleucine ,ILE and valine ,VAL) [128] compete with TYR and TRP for shared transportation through the BBB by System L (e.g. LAT1), which is located on both luminal and abluminal sides of the BBB. In addition, it is known that LAT1 in brain capillary endothelial cells is typically saturated by amino acids in plasma under normal conditions because its Km value is smaller than the plasma concentrations of substrate amino acids. Therefore, an elevation in the plasma concentration of either LNAA or BCAA will influence transport activity of LAT1 and could reduce brain uptake of TRP and TYR. One study showed that OVX caused an array of metabolic changes in amino acid metabolism. Plasma levels of PHE, LEU, ILE and VAL, for example, all significantly increased in OVX treated rats compared with controls [129]. Another study reported similar effects (i.e. increases in PHE and decreases in ALA, GLN, TRP) in rat serum following OVX [130]. It is possible, therefore, that in our study OVX and VCD treatments resulted in increased levels of competing amino acids in plasma, which contributed to a reduction in TYR, TRP transportation through the BBB. This, however, would not account for the selective reduction of TRP and TYR in the HPC and not in other brain regions.

Other possibilities include effects on amino acid metabolism, and the rate of protein synthesis, all of which have been reported to be affected by estrogen deficiency. Estrogen plays an important role of sustaining glucose as the primary fuel source in the brain. One study previously showed OVX caused substantial reductions in plasma glucose levels compared with controls [129]. This

appears at first to be contrary to clinical observations that the prevalence of the metabolic syndrome such as hyperglycemia increases with menopause [131]. However, it should be noted that in rats hyperglycemia was observed at 13 weeks after OVX [132] while hypoglycemia was observed at 10 days after OVX [129], which is similar to the current study. Therefore, it is likely that estrogen deficiency leads to hypoglycemia at early time-points. Glucose transporter (GLUT-1) mediates glucose transport across the blood brain barrier (BBB) and is highly sensitive to changes in plasma glucose levels. Under normal conditions, GLUT-1 expression is closely regulated by glucose availability and is upregulated in the BBB during hypoglycemia [133]. However, it is found that OVX may induce a significant decline in expression of GLUT-1 in the hippocampus. Furthermore, the protein expression and activity of hexokinase, which irreversibly phosphorylates glucose to glucose-6-phosphate in the glycolysis process, also was significantly reduced following OVX [134]. Therefore, it is possible that by compromising these two rate-limiting steps, ovarian hormone loss further exacerbates decreased glucose availability and utilization in this brain structure. Previous research has reported that increased amino acid metabolism took place in rat neuron-enriched aggregate cultures when exposed to hypoglycaemic conditions [135]. Although not used primarily for cerebral energy production due to their low ATP production efficiency, glycogenic and ketogenic TYR and TRP might be degraded as alternative energy sources to form ketone bodies and TCA cycle intermediates (e.g. acetoacetate, fumarate and pyruvate) in response to short glucose supply.

2.8 CONCLUSIONS

Collectively, the results of our analysis describe significant changes in local levels of serotoninergic, dopaminergic, and noradrenergic endpoints in association with validated models of surgical and transitional menopause. Most notably, effects were brain-region specific and time-dependent. These changes are likely relevant to changes in neural function and cognitive performance associated with early and late time periods following menopause. However, interpretation of these findings needs to be carried out with caution, since the levels of these neurochemical endpoints do not necessarily represent a dynamic measure of neuronal activities. Similar studies that focus on the anabolic and catabolic enzymes involved in monoamine regulation will further add to our understanding of how monoaminergic regulation is affected by loss of ovarian function.

3.0 CHAPTER 3: COMPARISON OF TRANSITIONAL VS SURGICAL MENOPAUSE ON NONESSENTIAL AMINO ACID LEVELS IN THE RAT BRAIN

3.1 ABSTRACT

Estrogens play an important role in the central nervous systems in females. In our previous study, we compared the effects of two models of menopause on multiple monoaminergic and two aromatic amino acid endpoints in the brain. Emerging evidence suggests that inhibitory and excitatory amino acids may be involved in sex hormone effects on neuronal functions. In this study, we furthered our investigation by evaluating the impact of estrogen loss on levels of six nonessential amino acids (including excitatory and inhibitory amino acids) in the rat brain using a similar experimental design but with a different analytical method. Our results show that similar to the effects seen in the aromatic amino acids, significant reduction of each amino acid level was detected only in the HPC region and only at the 1-week time point following estrogen deprivation in both of the menopausal models. Such effects may potentially contribute to the changes in neural function and related cognitive performance after menopause.

Keywords: Menopause, Estrogen, Amino acid, VCD, hippocampus

3.2 INTRODUCTION

Estrogens play a fundamental role in the physiology of the reproductive and central nervous systems in females. In Chapter 2, we conducted the first comprehensive analysis comparing the effects of two models of menopause on neuroendocrine endpoints in the brain, which included multiple monoamine and metabolite endpoints as well as two aromatic amino acids TYR and TRP. One of the most interesting and surprising findings was the significant reduction of TYR and TRP levels only in HPC region, at 1-week time point following estrogen deprivation in both of the menopausal models. Given the critical roles that amino acids play in brain functions (e.g. used for neurotransmitter, neurotransmitter precursor, or protein synthesis), the significant reductions in amino acid levels in the brain are likely to contribute in part to the effects of surgical and transitional menopause on brain function and cognitive performance.

Nevertheless, to the best of our knowledge, previous studies investigating the effects of estrogen loss on brain amino acid levels are extremely limited. It is still unknown whether the loss of estrogen would have similar or different effects on brain amino acids other than TYR and TRP. This prompted us to further our investigation by evaluating six additional nonessential amino acids (alanine (ALA), serine (SER), glycine (GLY), aspartate (ASP), glutamate (GLU) and glutamine (GLN)) using similar experimental design but with a different analytical method in current study. These amino acids are chosen based on their chemical characteristics and unique roles in neurotransmission to represent a variety of amino acid classes. For example, based on the propensity of the side chain to be in contact with water, ALA and GLY are classified as hydrophobic, SER and GLN as polar and ASP and GLU as charged. GLU and ASP are major

excitatory amino acids (EAA) while GLY is considered an inhibitory amino acid (IAA). Results showed that similar to the effects seen in aromatic amino acids TYR and TRP, significant reduction of each amino acid level was detected only in HPC region and only at 1-week time point following estrogen deprivation in both of the menopausal models.

3.3 MATERIALS AND METHODS

3.3.1 Amino Acids Analysis by GC-FID

Multiple amino acid contents in rat brain homogenates were analyzed using a commercially available “EZ:faast GC–FID amino acid testing kit” by Phenomenex [136]. The modified assay procedure involves sample purification, extraction, derivatization of extracted amino acids, and GC analysis.

Sample Preparation by SPE and Derivatization: 50 µl of the brain homogenate and 50 µl of internal standard (the norvaline) were pipetted into each sample preparation vial. A sorbent tip was attached to a 1.5 mL syringe. Then the tip was immersed in the solution. The solution in the sample preparation vial was forced to pass through the sorbent tip by slowly pulling back the syringe piston. Following that, 100 µl of the wash solution was pipetted into the same sample preparation vial and let the solution pass slowly through the sorbent tip and into the syringe barrel. The liquid from the sorbent bed was drained by pulling air through the sorbent tip. Then the tip was detached and left in the sample preparation vial. After that, 100 µl of the freshly prepared elution medium was pipetted in the same sample preparation vial. Then the sorbent tip was attached to the 0.6 ml syringe with the piston pulled back halfway up the barrel. The sorbent in the tip was wet with the eluting medium and let the liquid rise through the sorbent particles and stop when the liquid reaches the filter plug in the sorbent tip. Then, the liquid and sorbent particles were ejected out of the tip and into the sample preparation vial. Repeat the ejection step until all of the sorbent particles in

the tip were expelled into the sample preparation vial. 25 µl of organic solution 1 was transferred using the adjustable microdispenser into the sample preparation vial. The liquid in the vial was emulsified by repeated vortexing. The emulsion would gradually separate into two layers after letting the reaction to proceed for at least 1 min. The liquid in the vial was re-emulsified and allowed to react for another minute. 50 µl of Reagent 5 was then transferred with the microdispenser and the liquid was med to react for additional 1 minute. Lastly, 50 µl of reagent 6 was pipetted into the vial. The emulsion was separated into two layers after quick vortexing. After sample cleanup procedure, the amino acids were quickly derivatized at room temperature with the addition of two reagents, which modify both the carboxyl and amino groups of the amino acids forming stable derivatives (Figure 11). The upper layer contains the derivatized AA to be analyzed by GC.

Gas Chromatography: A 10m× 0.25mm ZB-PAAC column from Phenomenex (Torrance, CA, USA) was used. Pressure of the carrier gas Heilium was kept constant during the run at 4psi. Nitrogen (30 mL/min), hydrogen (35 mL/min) and air (300 mL/min) were used as auxiliary gases. The oven temperature program was as follows: initial temperature is 110 ºC. Stage 1: a 30ºC /min ramp from 110 ºC to 200 ºC; Stage 2: 35 ºC /min ramp from 200 ºC to 305 ºC; Stage 3: a 25 ºC /min ramp from 305 ºC to 330 ºC, held for 2 min. The temperature of the injection port and detector was 250 ºC and 330 ºC. A 2 µL sample was injected in split mode with a split ratio of 5:1. The limit of quantification for this assay is 1.0 nmole/ml. Coefficients of Variation for amino acids were 1.5–6.2% (intra-assay) and 3.8–9.7% (interassay). Concentrations of amino acids in the samples were calculated and expressed as nmol/mg protein.

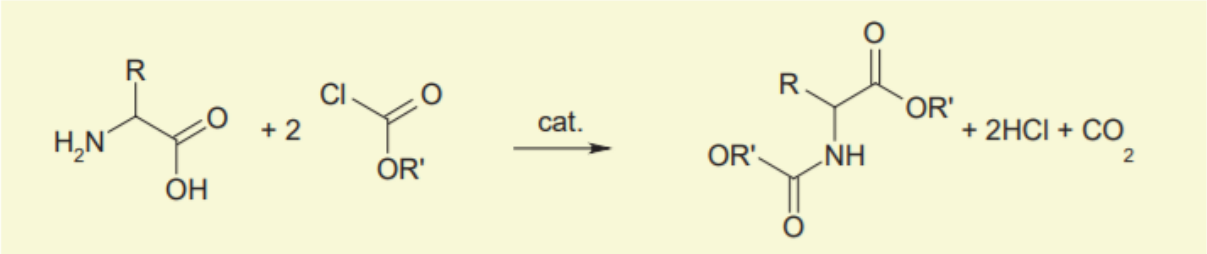


Figure 11. Simplified diagram illustrating EZ:faast derivatization reactions.

Other materials and methods have been described in detail section 2.3.

3.4 RESULTS

3.4.1 Amino acids levels

3.4.1.1 Comparison of proestrus vs. diestrus

Similar to monoamines and metabolites, few differences in levels of amino acids were detected in all of the brain tissues collected from gonadally intact cycling rats.

3.4.1.2 Effects of OVX and VCD treatments

Significant effects of OVX and VCD treatments on amino acid levels were detected only in HPC, not in FCX or STR.

HPC

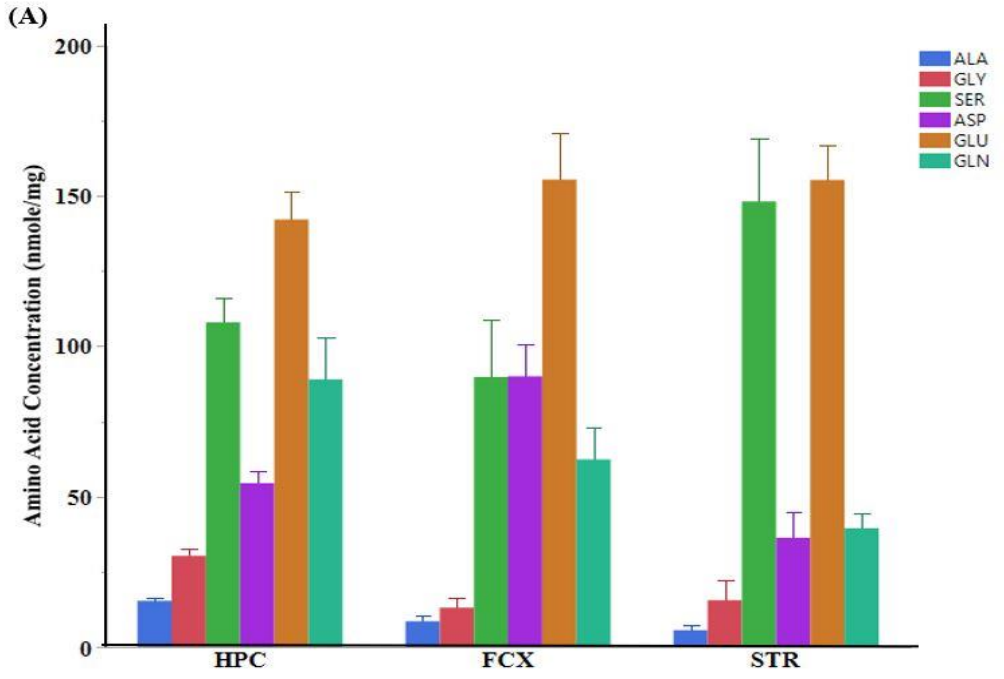
One-way ANOVAs consistently revealed significant effects of OVX and VCD treatments on all of the amino acids measured: ALA ($F[5,35]=18.1$, $p<0.0001$), SER ($F[5,35]=8.2$, $p<0.0001$), GLY ($F[5,35]=10.6$, $p<0.0001$) , ASP($F[5,35]=18.0$, $p<0.0001$), GLU ($F[5,35]=16.5$, $p<0.0001$) and GLN ($F[5,35]=6.0$, $p<0.0001$). Similar to changes in neurochemical endpoints reported in Chapter 2, effects were observed primarily at 1 week following OVX and VCD treatment (Figure 13). Specifically, 1 week following OVX and VCD treatments, levels of each amino acid were reduced relative to both diestrus ($p<0.05$) and proestrus ($p<0.05$). In contrast, these effects were not observed 6 weeks after OVX or 6 weeks following the completion of VCD treatments. Similar

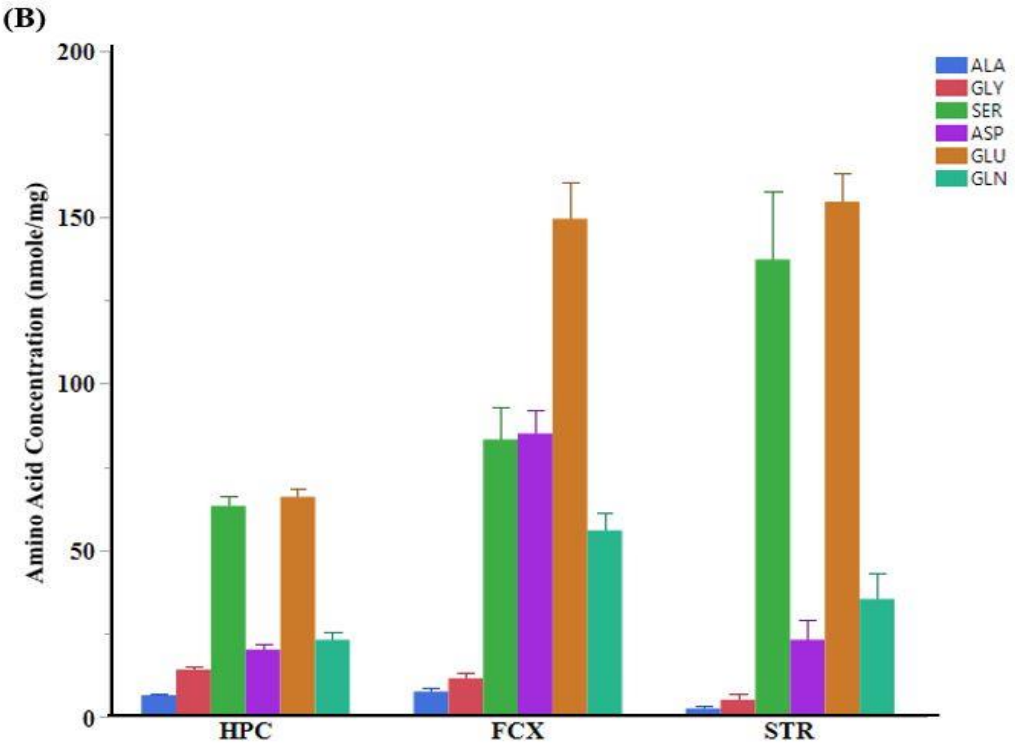
to previously studied aromatic amino acids TYR and TRP, by 6 weeks the levels of these amino acids had returned to levels comparable to normal cycling rats.

One-way or two-way ANOVAs did not reveal significant differences between the two menopausal models on any of the amino acid endpoints measured in the hippocampus. Results of the two-way ANOVAs on each of the measures revealed a significant overall effect ($F[3,23]>19.4$, $p<0.0001$), a significant effect of time-point ($p<0.0001$ in each case), no main effect of model, and no interaction between model x time-point.

FCX and STR

In contrast to HPC, one-way ANOVAs did not detect any significant effects of OVX and VCD treatments on amino acid levels in FCX and STR. Similarly, results of two-way ANOVAs did not reveal any significant overall effect, main effect of menopausal model, time-point as well as interaction between model x time-point.


(A)
Amino Acid Concentration (nmole/mg)
200
150
100
50
0
HPC
FCX
STR
ALA
GLY
SER
ASP
GLU
GLN


(B)
Amino Acid Concentration (nmole/mg)
200
150
100
50
0
HPC
FCX
STR
ALA
GLY
SER
ASP
GLU
GLN

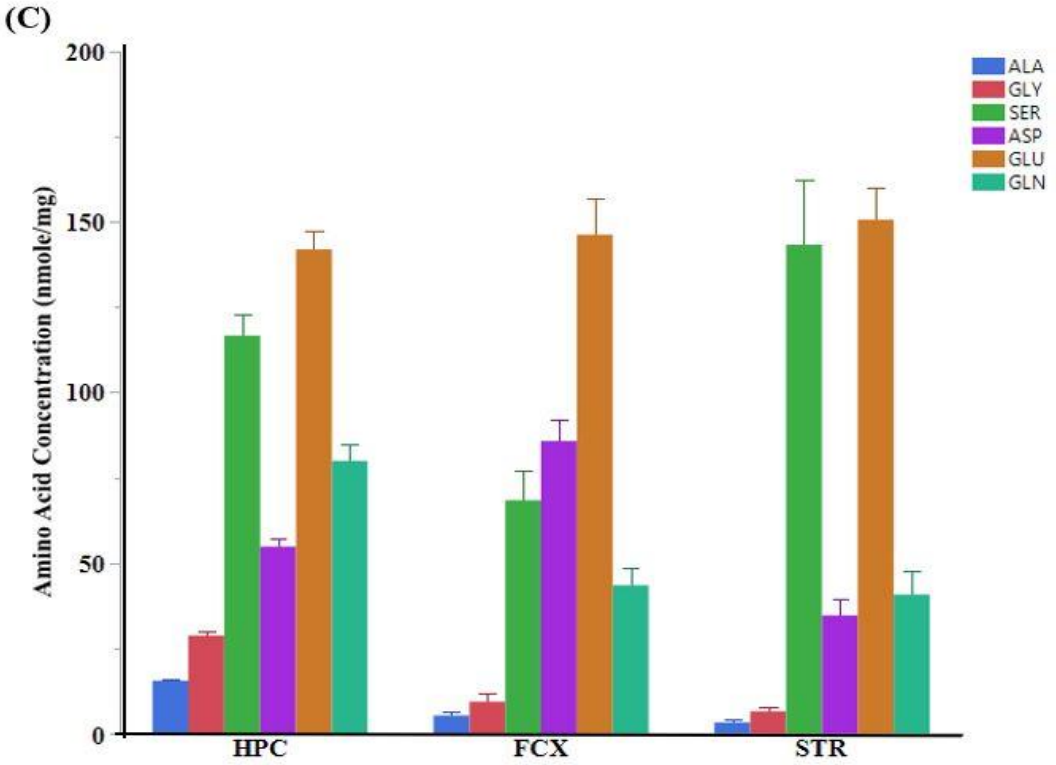
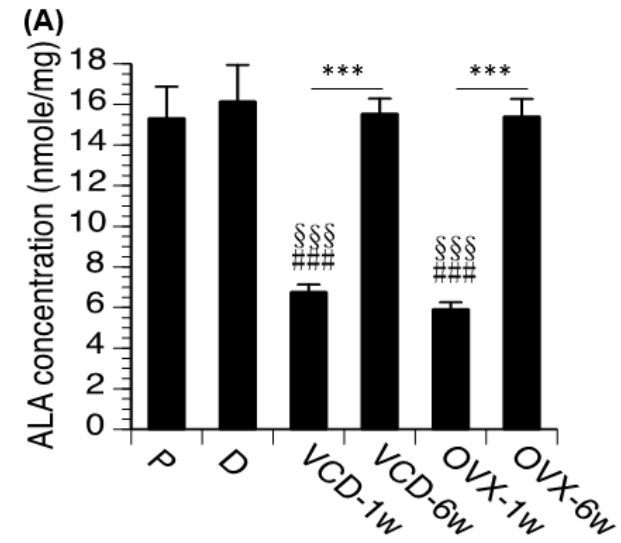
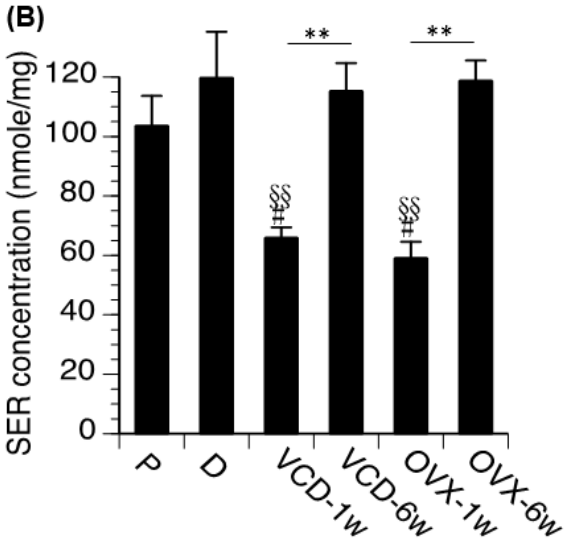
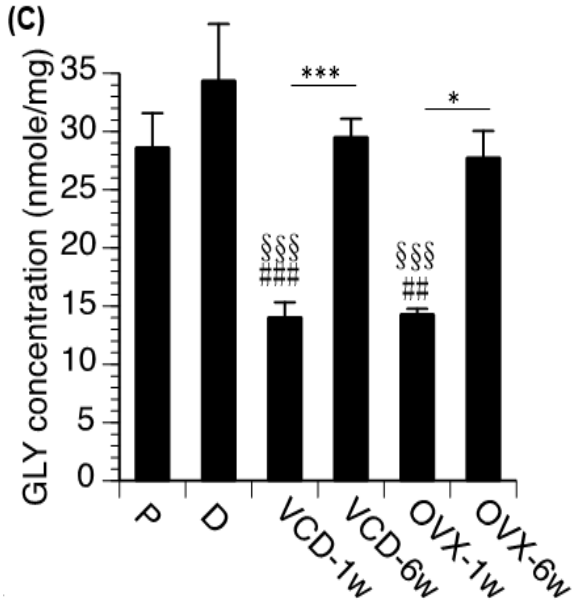
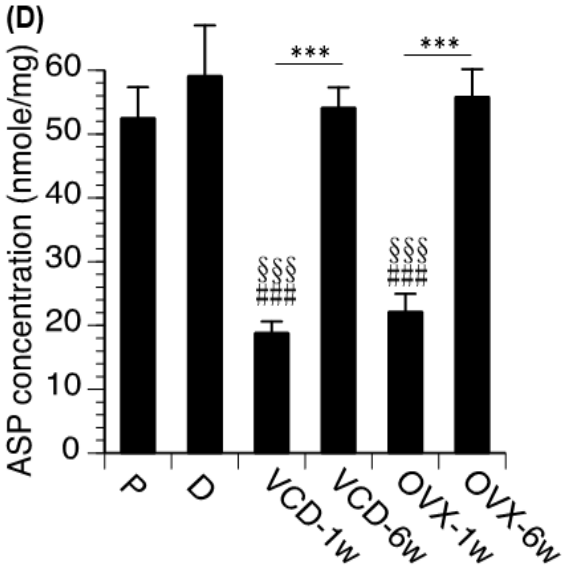


Figure 12. Profile of amino acid levels by brain region. (A) Rats at P/D ;(B) Rats at 1W; (C) Rats at 6W. Since not many model differences on these endpoints were detected, data from OVX and VCD were combined.


(A)
ALA concentration (nmole/mg)
0
2
4
6
8
10
12
14
16
18
***
***
§§§§
####
§§§§
####
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w


(B)
SER concentration (nmole/mg)
0
20
40
60
80
100
120
**
**
§§
##
§§
##
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w


(C)
GLY concentration (nmole/mg)
0
5
10
15
20
25
30
35
***
*
§§§§
####
§§§
##
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w


(D)
ASP concentration (nmole/mg)
0
10
20
30
40
50
60
***
***
§§§§
####
§§§§
###
P
D
VCD-1w
VCD-6w
OVX-1w
OVX-6w

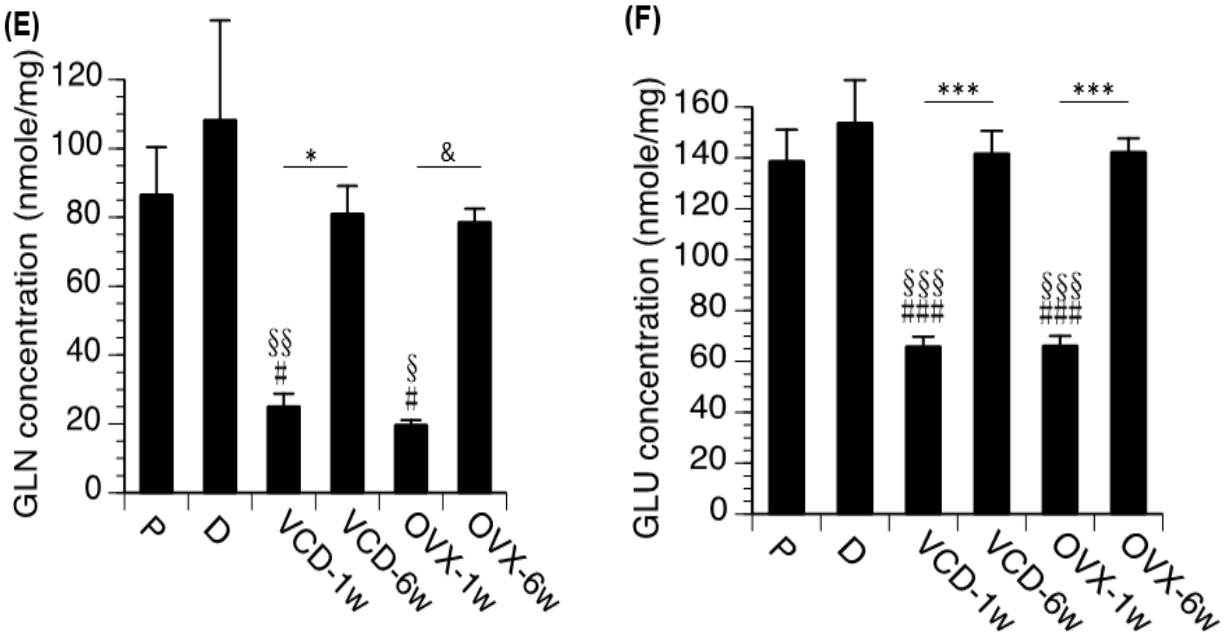


Figure 13. Amino acid levels by treatment groups in HPC. Rats at P (proestrous) and D (diestrous) are used as controls. Bars indicate Mean ± SEM. One-way ANOVA (followed by post-hoc Tukey's test if $p<0.05$): *$p<0.05$, **$p<0.01$, ***$p<0.001$ relative to VCD/OVX-1w; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ relative to P; $^{\S}p<0.05$,$^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$ relative to D; $0.05<p^{\&}<0.01$ relative to OVX-1w.

Table 5. Nonessential Amino Acid Endpoints in HPC of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD.

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
N		8	6	5	6	8	8
HPC (nmole/mg)							
ALA	+++	15.32±1.56^{A}	15.47±1.62^{A}	5.91±0.33^{B}	15.4±0.85^{A}	6.77±0.36^{B}	15.55±0.74^{A}
GLY	+++	28.63±2.93^{A}	32.58±4.36^{A}	14.31±0.46^{B}	27.75±2.28^{A}	14.05±1.28^{B}	29.51±1.55^{A}
SER	+++	103.65±9.99^{A}	113.76±13.99^{A}	59.08±5.5^{B}	118.76±6.85^{A}	65.95±3.49^{B}	115.29±9.42^{A}
ASP	+++	52.52±4.82^{A}	57.07±6.79^{A}	22.18±2.79^{B}	55.83±4.34^{A}	18.83±1.78^{B}	54.13±3.17^{A}
GLU	+++	138.77±12.36^{A}	146.93±15.32^{A}	66.17±3.86^{B}	142.3±5.44^{A}	65.94±3.82^{B}	141.76±8.83^{A}
GLN	+++	86.6±13.81^{A}	92.23±28.53^{A}	19.73±1.42^{B}	78.66±3.84^{AB}	25.11±3.72^{B}	81.03±8.02^{A}
Note:							
Values are mean ± sem.							
+		p<=0.05	ANOVA				
++		p<0.01	ANOVA				
+++		p<0.001	ANOVA				
For ANOVA results, groups not connect by the same letter (A, B or C) are significantly different by Tukey test							

Table 6. Nonessential Amino Acid Endpoints in FCX of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD.

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
N		7	7	6	7	8	8
FCX (nmole/mg)							
ALA		7.23±1.6	9.96±3.88	9.07±1.27	6.74±1.82	6.36±1.45	3.95±0.9
GLY		11.16±2.39	14.96±6.47	14.8±2.39	10.38±3.04	9.05±2.13	8.44±4.06
SER		78.39±13.43	101.26±37.02	97.49±11.47	79.53±15.05	72.71±13.65	58.73±8.55
ASP		91.25±8.52	89±20.08	91.59±8.81	100.14±10.73	80.29±10.65	73.24±3.99
GLU		158.5±19.52	152.49±25.64	150.88±18.41	160.26±19.63	148.65±14.3	134.16±9.96
GLN		56.86±11.58	67.91±18.09	61.88±7.74	47.66±9.37	51.57±7.58	39.76±4.4

Table 7. Nonessential Amino Acid Endpoints in STR of Female Rats Killed at Proestrus or Diestrus in Comparison with Rats that were Ovariectomized or Treated with VCD.

	1-WAY ANOVA	P	D	OVX-1W	OVX-6W	VCD-1W	VCD-6W
N		7	8	6	7	8	8
STR (nmole/mg)							
ALA		5.81±2.71	5.46±1.68	3±1.93	2.71±1.15	2.04±0.75	3.65±1
GLY		8.57±2.85	21.82±12.3	6.99±3.95	4.45±1.47	3.61±0.9	8.34±1.72
SER		169.7±30.79	129.55±28.67	157.1±34.47	119.76±32.82	122.63±25.41	163.96±20.68
ASP		31.76±6.29	40.27±15.13	28.17±9.16	25.94±6.5	19.29±7.81	42.33±5.34
GLU		148.13±10.64	161.66±19.94	142.63±12.02	137.49±13.48	163.66±11.57	162.37±12.16
GLN		39.62±6.42	39.36±7.93	39.18±16.99	45.44±13.58	32.39±6.99	36.76±4.66

3.5 DISCUSSION

3.5.1 Amino acid endpoints remained unchanged in gonadally intact rats

Similar to TYR and TRP reported in previous study, our results showed that no significant changes of amino acid levels were detected in all of the three brain regions associated with cycle stage. This is in agreement with the study reported by Löscher et.al, where concentrations of 11 amino acids (including all of the six amino acids investigated in current study) were determined in 12 brain regions (including 3 brain regions investigated in current study) of female rats during four different stages of the estrous cycle. Similarly, regional amino acid levels were found to remain unchanged during estrous cycle [137]. Proestrous and diestrous were chosen in our study with respect to their high and low estrogen levels in order to determine the effects of estrogen fluctuations in estrous cycle on brain amino acid levels. Our results suggested that brain amino acid levels are relatively stable and not sensitive to the estrogen fluctuations naturally. It may be due to the change of estrogen level in estrous cycle is transient and not persistent, so that the magnitude of effect is not big enough to incur alterations in amino acid levels.

3.5.2 Effects of OVX and VCD treatments on amino acid endpoints

Multiple effects were detected, and most effects were both region-specific and time-dependent. Interestingly, the effect pattern is quite similar among all of the six amino acids. Similar to

monoamine and metabolites, our analysis shows no significant differences in the effects of the two models on these amino acid endpoints, given the different sex hormone levels between the two models. This indicates that surgical and transitional menopause produce similar effects on these endpoints and further suggests that any differences in cognitive performance between OVX and VCD-treated rats are not due to differential effects on these nonessential amino acids.

One of the key findings is that alterations in amino acid levels were only detected in the HPC, suggesting that this region of the brain is particularly sensitive to loss of ovarian function. It also suggests that whatever mechanisms are responsible for the decreases in AA levels in the HPC do not appear to be at play in the FCX and STR. The results were also in agreement with our previous findings with aromatic amino acids TYR and TRP [138]. Similar to our previous study, effects of both surgical and VCD-induced loss of ovarian function in the HPC were detected only at 1 week, but not at 6 weeks, suggesting either the effects are transient or there may be some unknown self-compensatory processes in the brain that restored the levels of affected amino acids back to normal. This needs to be further investigated.

3.5.3 Potential mechanism of amino acid reduction

This study is also the first to analyze levels of major excitatory amino acids (GLU and ASP), inhibitory amino acid (GLY) as well as other amino acids (ALA, SER, GLN) following loss of ovarian function in the same tissues in which multiple amino acid endpoints were measured. The most surprising result was the reduction in levels of each amino acid in the HPC at 1 week following OVX or VCD treatments. This further confirms our previous finding that loss of ovarian function has substantial acute effects on amino acid homeostasis in this region of the brain [138].

The underlying mechanisms are not fully understood yet. Possible explanations may include decreased amino acid synthesis, increased amino acid degradation or decreased amino acid passage from blood to the brain following loss of ovarian function.

Firstly, all of these six amino acids are non-essential amino acids, which means that they can be synthesized in the body. These amino acids' synthesis requires carbon skeletons, which mainly come from intermediates of the glycolytic pathway and from intermediates in the Tricarboxylic Acid (TCA) cycle, where multiple transaminations, catalyszed by transaminases, take place to transfer alpha-amino groups to preexisting amino acids to form new ones. For example, SER and GLY can be formed from glycerate-3-phosphate; ALA can be formed from pyruvate; ASP can be formed from oxaloacetate and GLU, GLN can be formed from α-ketoglutarate. Therefore, it is necessary to conduct future studies to evaluate the levels of the above-mentioned intermediates as well as the expression and activities of related enzymes in HPC following loss of ovarian function.

Secondly, due to the important role that estrogen plays in energy balance and glucose homeostasis in the brain [139], it also is possible that increased amino acids have been degraded to products that can enter the TCA cycle and then be converted to intermediates or glucose, which can be utilized for energy production. For example, the six amino acids are all glucogenic amino acids, which can be converted into glucose and ketogenic amino acids through gluconeogenesis. Then they could be used for direct energy production or ketone body formation (acetoacetate, β-hydroxybutyrate, and acetone). Previous studies have reported a substantial reduction in plasma glucose level at 10 days after ovariectomy [5]. Further study is required to investigate if the degradation of amino acids for energy production has increased in HPC after loss of ovarian function. In addition, it is also necessary to evaluate the expression and activities of enzymes

involved in the TCA cycle, such as the pyruvate dehydrogenase complex, citrate synthase, aconitase, fumarase and malate dehydrogenase etc.

Lastly, the amino acids in the brain can either come from local synthesis in the brain or from plasma amino acids that pass the blood brain barrier. Since its major source is not fully understood, a decrease in plasma amino acid levels following loss of estrogen could potentially contribute to a decrease in their levels in the brain. Previous studies have reported reductions in amino acids (e.g. ALA, GLN) in rat serum following OVX [130]. Therefore, it is likely that in our study OVX and VCD treatments resulted in reduction of these non-essential amino acid levels in plasma, followed by reduced transportation to the brain through the BBB. Future study is warranted to evaluate the impact of estrogen deprivation on levels of these amino acids in plasma as well as the expression and function of related facilitative transporters in BBB.

3.6 CONCLUSIONS

Collectively, the results of our analysis, for the first time, described significant, region-specific and time-dependent changes in local levels of six non-essential amino acid endpoints, using validated models of surgical and transitional menopause. Results showed that amino acids in all of the three brain regions were surprisingly stable throughout the estrous cycle in female rats. In addition, the FCX and STR exhibited the least vulnerability in terms of amino acid concentrations to loss of estrogen. With respect to the possible functional meaning of the significant reductions in amino acid levels observed in the HPC shortly after loss of ovarian function, the most interesting alterations are certainly those of amino acids which act as excitatory or inhibitory neurotransmitters in the brain. In this regard, the drastic reductions in levels of GLU, ASP and GLY are of particular interest and are likely contributing to changes in synaptic neurotransmission, brain excitability and cognitive performance after menopause.

4.0 ESTRADIOL AND SELECTIVE ESTROGEN RECEPTOR AGONISTS DIFFERENTIALLY AFFECT BRAIN MONOAMINES AND AMINO ACIDS LEVELS IN TRANSITIONAL AND SURGICAL MENOPAUSAL RAT MODELS

4.1 ABSTRACT

Estrogens have many beneficial effects in the brain. Previously, we compared the effects of two models of menopause (surgical vs. transitional) on multiple monoaminergic endpoints in different regions of the adult rat brain. Although estrogen replacement has been shown to affect neurotransmitter (NT) production and release with corresponding effects on cognitive performance, to date there has been little direct comparison of the effects of estrogens and selective estrogen receptor (ER) agonist on brain NT pathways between surgical and transitional menopause. The objective of current study was to evaluate the effects of ER agonist treatments. Neurochemical endpoints were evaluated in the hippocampus (HPC), frontal cortex (FCX), and striatum (STR) of adult ovariectomized (OVX) rats and in rats that underwent selective and gradual ovarian follicle depletion by daily injection of 4-vinylcyclohexene-diepoxide (VCD), after 1- and 6-weeks treatment with 17β -estradiol (E2), or with selective ERα (PPT), ERß (DPN), or GPR30 (G-1) agonists. Endpoints included serotonin (5-HT) and 5-Hydroxyindoleacetic acid, dopamine (DA), 3,4-Dihydroxyphenylacetic acid and homovanillic acid, norepinephrine (NE) and epinephrine, as well as the amino acids tryptophan (TRP) and tyrosine (TYR). Significant differences between the models were detected. OVX rats were much more sensitive to ER agonist

treatments than VCD-treated rats. Significant differences between brain regions also were detected. Within OVX rats, more agonist effects were detected in the HPC than in any other region. One interesting finding was the substantial decrease in TRP and TYR detected in the HPC and FCX in response to agonist treatments, particularly in OVX rats. Other interesting findings included increases in the levels of 5-HT, DA, and NE in the HPC of OVX rats treated with DPN, increases in DA detected in the FCX of OVX rats treated with any of the ER agonists, and increases in 5-HT and DA detected in the STR of OVX rats treated with E2. Many effects that were observed at 1-week were no longer observed at 6-weeks, demonstrating that effects were temporary despite continued agonist treatment. Collectively, the results demonstrate significant differences in the effects of ER agonist treatments on monoaminergic endpoints in OVX vs. VCD-treated rats that also were brain region-specific and time-dependent. These results are the first to systematically and simultaneously evaluate the effects of ER agonist treatments on multiple monoaminergic endpoints in multiple regions of the brain in two models of menopause. The fact that agonist treatments had lesser effects in VCD-treated rats than in OVX rats may help to explain reports of lesser effects of estrogen replacement on cognitive performance in women that have undergone transitional vs. surgical menopause.

Keywords: Estrogen; Selective Estrogen Receptor Agonists; Menopause; VCD; Monoamine; Amino Acid; Hippocampus; Striatum; Frontal Cortex

4.2 INTRODUCTION

Loss of ovarian function has been associated with increased risk for a variety of neuropsychiatric and neurodegenerative diseases, including anxiety and depressive disorders as well as Alzheimer's and Parkinson's disease [76, 82]. Some women experience loss of ovarian function relatively early in adult life, due either to premature ovarian failure or to surgical removal of the ovaries for prevention of cancer or other medical conditions. Studies suggest that these women have significantly increased long-term risk of cognitive impairment and dementia, as well as depressive and anxiety symptoms, compared to women with normal ovarian function [140-144]. Most women, however, undergo a natural menopause due to accelerated loss of ovarian follicles occurring at approximately 51 years of age [145]. Studies suggest that loss of ovarian function due to natural menopause also is associated with increased risks for cognitive impairments and neurodegenerative disease in women [146, 147]. This includes evidence of frontal lobe dysfunction [148], as well as evidence of impaired performance on verbal learning, verbal memory, and working memory tasks [83, 149, 150]. The mechanisms responsible for the effects of surgical and natural menopause on cognitive performance are still largely unclear.

It is well known that estrogens can significantly affect neuronal connectivity and function in the brain, with corresponding effects on cognitive performance. Examples include increased neuronal connectivity and synaptic function in areas of the brain critical for learning and memory [145, 151, 152], improved performance on specific cognitive tasks, including spatial learning tasks in rodents [56, 153], and verbal learning and memory tasks in humans [154], and reductions in risk of age-related cognitive decline [143, 146, 147]. Consequently, it is assumed that loss of estrogens

contributes significantly to the cognitive impairments that have been associated with surgical and natural menopause. Estrogens exert their effects by binding to specific estrogen receptors (ERs) located throughout the brain. Three receptors have been identified. ERα and ERβ are ligand-dependent transcription factors acting in the nucleus to enhance nuclear transcription [22]. There is evidence that these receptors also can exist in membrane compartments and can participate in rapid effects of estrogens on specific transduction pathways [155]. Recently, a novel membrane bound G protein-coupled receptor, GPR30, was identified which is genetically and structurally unrelated to ERa and ERß, and can initiate estrogen responses at cellular membranes. [156]. All three receptors are expressed in many tissues, and within the brain that are well known to affect neuronal function, plasticity, and cognitive performance [25, 157].

Estrogens also have been shown to significantly affect monoaminergic neurotransmitter (NT) pathways in the brain, which are well known to be critically involved in neuropsychiatric and neurodegenerative diseases. The alteration of NT levels by estrogens and selective ER agonists is complicated and dependent on multiple factors such as brain region, dose, duration of treatment, type of treatment, as well as age and species of the animal. Many studies have been conducted by different laboratories demonstrating effects on specific NT pathways, however data are not always consistent [66, 70-73]. A comprehensive analysis of simultaneous changes that occur both within and across pathways in multiple brain regions has not yet been conducted. Also it is not clear what role the individual estrogen receptors play, or what effects selective estrogen receptor modulators have on these NT pathways.

Recently we compared the effects of two models of menopause on monoaminergic endpoints in different regions of the adult rat brain [158]. Ovariectomy, a model of surgical

menopause, was compared with the effects of daily injections of 4-vinylcyclohexene diepoxide (VCD), which has been well studied and validated for use as a model of natural menopause in women [92, 95, 101]. In contrast to surgical menopause which produces a rapid and complete loss of ovarian hormones, the VCD model produces a gradual loss of ovarian follicles, leading to a corresponding reduction in estrogen production but with continued production of androgens by the remaining ovarian cells [77]. Hence the VCD model more accurately reflects the shift in the ratio of estrogens to androgens that occurs with natural menopause. The study measured multiple endpoints in three different brain regions from each rat at two time points, thus enabling a comprehensive characterization of simultaneous effects on different NT pathways [158]. Results showed significant effects on serotoninergic, dopaminergic, and noradrenergic endpoints that were both model and brain region specific, and that varied by time following loss of ovarian function. In the current paper we used similar methods to investigate the effects of treating rats with selective ER agonists after undergoing either ovariectomy or VCD-induced follicular atresia. Results show that ER agonists produce effects on serotoninergic, dopaminergic, and noradrenergic endpoints that are agonist dependent, and that also vary by model, brain region, and duration of treatment. This is the first study to directly and systematically compare the effects of ER agonists on NT pathways in the brain in these two models of menopause.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Female Sprague–Dawley rats (~11 weeks of age) were purchased from Hilltop Laboratories, Inc. All rats were individually-housed and maintained on a 12/12 h light–dark cycle (lights on at 7:00 AM) with free access to food and water. Rats were allowed to acclimate to the housing environment for two weeks prior to use. All experiments were conducted in accordance with the NIH Guide for Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

4.3.2 Menopausal Models

Rats were randomly assigned either to the VCD or OVX group. Rats in the VCD group received 4-vinylcyclohexene diepoxide (VCD； Sigma Chemicals; St Louis, MO) at a dose of 80 mg/kg i.p. daily for a period of 30 days as previously described [101]. During this time, rats in the OVX group received daily injections of sesame oil (1ml/kg i.p.). At the end of 30 days, rats that had received sesame oil underwent bilateral ovariectomy, and rats that received VCD received sham surgery. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (7 mg/kg) (0.1 cc/100 g.b.w.). Ovariectomy was performed using a lateral approach. The apical tips of the uterus

were ligated, and the ovaries were removed. The peritoneal muscle was closed with 6.0 suture silk, and the overlying skin was closed with metal wound clips. Sham surgery consisted of skin and muscle incisions and wound closure only. Antibiotic cream was applied on the wound to reduce chance of infection. Rats received Ketofen (3 mg/kg, i.p.）every day for 3 days to reduce post-surgical pain. One week of time was allocated for the rats to recover from surgeries before beginning agonist or vehicle treatment.

4.3.3 Estrogen receptor agonist treatments

Rats received continuous administration of E2 (Sigma Chemicals; St Louis, MO), propylpyrazole triol (PPT; Tocris Cookson; Ellisville, MO), diarylpropiolnitrile (DPN; Tocris Cookson; Ellisville, MO), G-1 (1-[4-(6-bromobenzo[1,3] dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone; Cayman; Ann Arbor, MI), or vehicle (10% DMSO and 20% hydroxypropyl-β-cyclodextrin (HPβCD; Sigma Chemicals; St Louis, MO), beginning one week following ovariectomy or sham surgery. PPT is an ERα-selective agonist that is approximately 410-fold more selective for ERα than ERβ [39, 40]. DPN is an ERβ-selective agonist with 70-fold higher binding affinity and 170-fold higher potency for ERβ than ERα [41]. G-1 is non-steroidal, high-affinity selective GPR30 agonist, with no binding to the classic ERα or ERβ [47]. All drugs were administered by miniosmotic pump (Alzet model 2006; Durect Corp., Inc.) implanted subcutaneously in the dorsal neck region. These pumps deliver at a constant rate of 0.15 μl/hr for up to 6 weeks. E2 was administered at a rate of 3 μg/day (0.125 μg/hr). PPT, DPN

and G-1 treatments each were administered at a rate of 5 μg/day (0.208 μg/hr). This dose and route of administration was selected based on previous published data from our lab showing that these treatments significantly enhance acquisition of a spatial learning task [54], as well as studies showing that E2 and G-1 significantly enhance potassium-stimulated ACh release in the hippocampus [53]. Treatments continued for either 1 week or 6 weeks.

4.3.4 Tissue collection

After 1 week or 6 weeks of treatment rats were anesthetized with an overdose of ketamine (3 mg) and xylazine (0.6 mg) and killed by decapitation. Trunk blood was collected for determination of serum levels of estradiol, testosterone, and androstenedione. Dissections were performed according to plate designations in Paxinos & Watson (1998) [102] and were as follows: hippocampus (HPC; plates 21-41), frontal cortex (FCX; plates 11-21), and striatum (STR; plates 11-21). These regions were selected because of the large body of work showing that each region plays an important role in learning and memory processes as well as important roles in extrapyramidal motor function (e.g., the STR) [159]. In addition, each of these regions have been shown to be influenced by ovariectomy and estrogen treatment [56, 160]. Tissues were stored at -80 °C until processed.

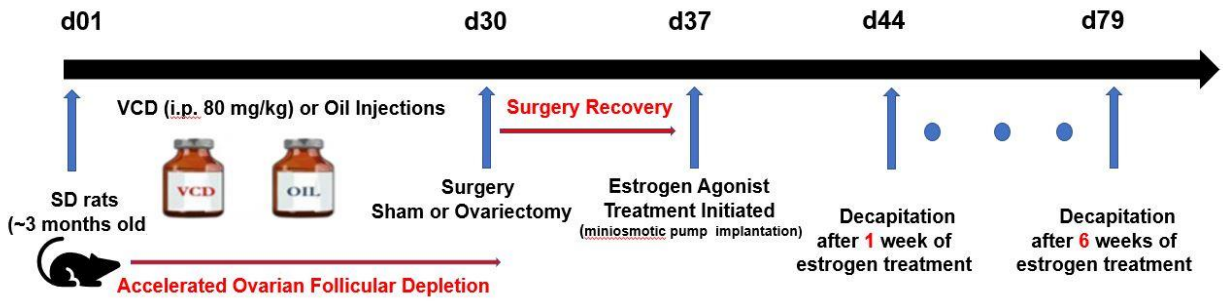


Figure 14. Schematic diagram showing the timeline of the experimental protocol 2.

4.3.5 Brain sample preparation

500 µl 10 mM sodium acetate buffer (pH 6.5) was added to 50 mg tissue in a 1.5 ml microfuge tube. Tissues were sonicated at 4 ˚C until completely dissolved. After sonication, tissues were spun at 14,000 g at 4˚C for 10 min. Supernatant was collected and placed at 4 ˚C. This step was repeated three times and the three supernatants were combined. The cell free supernatants were spun at 15,000g for an additional 40 minutes. A 200 µl aliquot was taken for the determination of protein concentration using the Bradford method [103]. The remaining volume of each supernatant was filtered through a disposable membrane (0.22µm pore size) micropartition device (Millipore Ultrafree-MC) under centrifugation at 14,000g for 30 min at 4 °C to remove any compounds above 10,000 nominal molecular weight limit.

4.3.6 Hormone assays

Serum levels of E2, T, and AD were quantified as recently described [104, 105, 158, 161]. For E2, samples were spiked with internal standard 25µl 2,4,16,16,17-d5-17 beta-estradiol (1 ng/ml in methanol) and extracted with 3-4 ml n-Butyl chloride. Samples were then centrifuged, and the organic layer was transferred to salinized culture tubes and dried under a steam of nitrogen. Residues were derivatized with dansyl chloride. Samples were centrifuged, and the supernatant was transferred into glass vials for LC-MS/MS analysis. 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.

T and AD levels in serum were quantified using methods similar to the detection of E2. Briefly, samples were spiked with 0.25 ng/ml D3-testosterone or D7-androstenedione as the internal standard and then extracted with n-butyl chloride. After centrifugation and evaporation, the residue was reconstituted in methanol and water (80 µl: 20 µl), centrifuged again and the supernatant transferred into glass vials for UPLC-MS/MS analysis. T and AD were 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.

MS detection and quantification of all hormones was achieved in the positive mode. Transitions used for the analysis of estradiol were 506 → 171 for E2, and 511 →171 for the deuterated internal standard. Transitions used for T analysis were 289 →97 for T and 292 →97 for the deuterated T. Transitions used for AD analysis were 287→100 for AD and 294→ 100 for the deuterated AD. Area under the peak was quantified and used to determine absolute levels of E2, T, or AD per ml by comparison with corresponding standards. The limit of detectability for the E2

assay is 2.5 pg/ml. The limit of detectability for the T and AD assays is 25 pg/ml. Intra-assay statistics show relative standard errors below 8.1% and relative standard deviations below 10.4%. Inter-assay statistics show relative standard errors below 5.0% with relative standard deviations below 7.4%.

4.3.7 Monoamine analysis by HPLC-CMEAS：

Monoamines and metabolites were measured with a modified version of a HPLC-ECD method and as described in our recent paper [158]. The goal was to characterize levels of targets associated with dopaminergic, noradrenergic, and serotoninergic pathways. Targets that were quantified included the amino acids tryptophan (TRP) and tyrosine (TYR); dopamine (DA) and its metabolites, 3-4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and epinephrine (EPI); and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). These endpoints were selected because these monoaminergic pathways are known to be affected by ovariectomy and estrogen treatment with corresponding effects on cognitive and motor performance [73, 162].

The HPLC methods have recently been described in detail [158]. Briefly, 100 µl of sample was injected into an ESA CoulArray Model 5600 HPLC system, consisting of two Model 582 pumps, one dynamic gradient mixer, two PEEK pulse dampers, a Model 542 refrigerated autosampler injector, a CoulArray organizer module, and a serial array of 16 coulometric electrodes. Mobile Phase A consisted of 1.1% (w/v) of 1-pentane-sulfonic acid (Specrum, Inc.) at

pH 3.0. Mobile Phase B consisted of 0.1 M lithium acetate (Sigma-Aldrich, Inc.) in a solvent mixture of methanol (Avantor Performance Materials, Inc.), acetonitrile (Honeywell, Inc.) and isopropanol (Mallinckrodt Baker,Inc.) at the ratio of 80/10/10 (v/v/v) at pH 6.5. Each sample was run on a single column (ESA Meta-250, 5 μm ODS, 250 x 4.6 mm ID) under a 68-minute gradient elution that ranged from 0% to 100% Mobile Phase B and delivered at a fixed flow rate of 0.6 ml/min. The retention time and area of the peaks in tissue homogenates were measured and compared to an external calibrating standard solution containing TYR, TRP, DA, DOPAC, HVA, NE, EPI, 5-HT, and 5-HIAA (Sigma, St. Louis, MO, USA). Concentrations of these substances in the samples were calculated and expressed as ng/mg protein. Turnover ratios (metabolite/monoamine) were calculated as a measure of activity.

4.4 STATISTICAL ANALYSIS

Results are presented as means ± standard error of the mean (SEM). Statistical analysis was conducted in several stages. An omnibus 3-way ANOVA was performed for each brain region with Model, Time, and Agonist as independent variables to investigate their main effects and interactions on NT endpoints. A 2-way ANOVA was conducted to explore the main effects and interactions between Model and Time in hormonal analysis. Specific effects of agonist treatments for a given Model and Time Point were further explored by 1-way ANOVA. Differences between groups were analyzed by Tukey's test and also by Dunnett's test comparing agonist treatments with corresponding controls. To efficiently present the data, Cohen's d index was calculated and plotted to demonstrate the effect size of agonist treatments on NT in each of the brain region. Concentrations below limit of detection was marked as ND (not detectable) and value of limit of detection was used for statistical analysis. Statistical significance was defined a $p \leq 0.05$.

4.5 RESULTS

4.5.1 Serum levels of hormones

Levels of E2, T, and AD in each of the treatment groups are summarized in Table 8-9. 3-way ANOVAs revealed a significant overall effect for E2 ($F[19,72]=8.0$, $p<0.0001$), T $F[19,68]=3.3$, $p<0.001$), and AD $F[19,70]=4.8$, $p<0.0001$) levels. For E2 levels, significant main effects of Time ($F[1,72]=8.1$, $p<0.01$) and Agonist Treatment ($F[4,72]=28.4$, $p<0.0001$) were detected, as well as a significant Time x Agonist interaction ($F[4,72]=5.7$, $p<0.001$). In OVX rats, levels of E2, as well as AD and T were undetectable or extremely low in rats treated with vehicle, PPT, DPN, or G-1 regardless of Time-Point. As expected, levels of E2 were significantly higher in E2-treated rats compared with all other groups ($p<0.05$). In addition, levels of E2 detected after 6 weeks of continuous treatment were significantly higher than levels detected after 1 week of treatment. In VCD-treated rats, levels of E2 were significantly higher in E2-treated rats than in all other groups. A 2-way ANOVA of Time and Agonist Treatment on E2 levels in VCD-treated rats revealed a significant main effect of Agonist ($F[4, 37]=11.7$, $p<0.0001$), a significant effect of Time ($F[1, 37]=5.2$, $p<0.05$) and a significant Time x Agonist interaction ($F[4,37]=3.0$, $p<0.05$). Post-hoc analyses revealed that E2 levels were higher in E2-treated rats relative to non-E2-treated rats (irrespective of time-point), and that rats treated with E2 for 6 weeks had significantly higher E2 levels than all other groups ($p<0.05$).

For AD levels, significant main effects of Model ($F[1,70]=45.4$, $p<0.0001$), Time ($F[1,70]=6.9$, $p<0.05$), and Agonist Treatment ($F[4,70]=3.5$, $p<0.05$) were detected, as well as significant Model x Agonist ($F[4,70]=3.1$, $p<0.05$) and Model x Time ($F[1,70]=6.0$, $p<0.05$)

interactions. Specifically, AD levels were much higher in VCD-treated than in OVX rats, were higher after 1 week than after 6 weeks of treatment, and were substantially lower in E2-treated rats than in all other groups when collapsed across Model and Time Point. Levels of AD in VCD-treated rats were quite variable. There was some indication that E2 treatment produced a decrease and DPN an increase in AD. A 2-way ANOVA of Time and Agonist Treatment on AD levels in VCD-treated rats revealed a significant main effect of Agonist ($F[4, 35]=3.4$, $p<0.05$), main effect of Time ($F[1, 35]=6.4$, $p<0.05$) but no Time x Agonist interaction ($F[4,35]=0.7$, $p=0.60$). Post-hoc analysis revealed that AD levels in DPN-treated rats were significantly higher than in E2-treated rats (irrespective of time-point), although neither group differed significantly from controls. This is likely due to high variability, and to the fact that AD levels in the controls were much lower at 6 weeks than at 1 week. 1-way ANOVA of AD levels in VCD-1W rats show that AD levels were significantly lower in E2-treated rats than in controls or DPN-treated rats. 1-way ANOVA of VCD levels in VCD-6W rats shows that AD levels were significantly higher in DPN-treated rats than in E2-treated rats or controls.

For T levels, a significant main effect of Model ($F[1,68]=34.1$, $p<0.0001$) was detected as well as a significant main effect of Agonist ($F[4,68]=2.7$, $p<0.05$) interaction. Like AD, levels of T were significantly higher in VCD-treated than in OVX rats. Among the VCD-treated rats, levels also were significantly higher in DPN-treated than in E2-treated rats when collapsed across Time. Like AD, levels of T in VCD-treated rats were quite variable. A 2-way ANOVA of Time and Agonist Treatment on T levels in VCD-treated rats produced a nearly significant effect of Agonist ($F[4,33]=2.5$, $p=0.06$), no significant effect of Time ($F[1,33]=0.4$, $p=0.54$), and no Time x Agonist interaction ($F[4,33]=1.3$, $p=0.3$). 1-way ANOVA of T levels in VCD-6W rats showed that T levels were significantly higher in DPN-treated rats than in controls by Dunnett test. No other significant

differences in T-levels were detected, again likely due to the very high variance in these values in the VCD-treated rats.

4.5.2 Effects of ER agonists on monoamine, monoamine metabolite and monoamine precursor levels in OVX-and VCD-treated rats

Detailed analysis consistently revealed significant effects of E2 as well as ER subtype-selective agonists on endpoints associated with noradrenergic, dopaminergic and serotoninergic pathways, in both VCD- and OVX-treated rats (refer to Table 10-15). Effects were model- and region-specific as well as time-dependent. For clarity, results are organized by brain region and by neurochemical pathway.

HPC

Serotoninergic Pathway:

3-way ANOVAs detected significant overall effects on 5-HT ($F[19,74]=6.0$, $p<0.0001$), 5-HIAA ($F[19,74]=12.1$, $p<0.0001$), the ratio of 5-HIAA/5-HT ($F[19,74]=8.1$, $p<0.0001$), and the amino acid TRP ($F[19,74]=61.5$, $p<0.0001$). Main effects of Model ($F[1,74]=6.8$, $p<0.05$) and Agonist ($F[1,74]=4.8$, $p<0.005$) were detected for the ratio of 5-HIAA/5-HT. Specifically, the ratio was higher in OVX than in VCD-treated rats when collapsed across Agonist and Time, and was greater in controls than in E2, DPN, or G-1 treated rats when collapsed across Model and Time. A main effect of Time was detected for the ratio of 5-HIAA/5-HT ($F[1,74]=73.9$, $p<0.0001$), as well as for 5-HT ($F[1,74]=89.8$, $p<0.0001$), 5-HIAA ($F[1,74]=207.1$, $p<0.0001$), and TRP ($F[1,74]=1144.8$, $p<0.0001$). Specifically, the levels of 5-HT, 5-HIAA, and TRP were significantly

lower, and the ratio of 5-HIAA/5-HT was significantly greater, at 1 week than at 6 weeks when collapsed across Model and Agonist Treatment. A Model x Agonist interaction was detected for the ratio of 5-HIAA/5-HT ($F[4,74]=3.0$, $p<0.05$) and for TRP ($F[1,74]=3.4$, $p<0.05$). Post-hoc analysis showed that the ratio of 5-HIAA/5-HT in OVX controls was significantly higher than in all E2, DPN, and G-1-treated rats and in VCD-treated rats treated with PPT, when collapsed across Time. Similar analysis showed that levels of TRP were significantly higher in OVX controls than in OVX rats treated with E or PPT, when collapsed across Time. A Model x Time interaction was detected for the ratio of 5-HIAA/5-HT ($F[1,74]=7.1$, $p<0.001$). Post-hoc analysis showed that the ratio of 5-HIAA/5-HT was significantly greater in OVX-1W rats than in all other groups when collapsed across Agonist Treatment, followed by VCD-1W rats, and then by both OVX-6W and VCD-6W rats. An interaction between Time x Agonist ($F[4,74]=4.2$, $p<0.005$) as well as a full 3-way interaction ($F[4,74]=4.1$, $p<0.005$) was detected for the ratio of 5-HIAA/5-HT. Post-hoc analyses showed that the ratio of 5-HIAA/5-HT was greatest in 1W controls compared to all other groups when collapsed across Model. This was followed by PPT-1W and G-1-1W, both of which differed significantly from E2-6W, PPT-6W, and G-1-6W when collapsed across Model.

1-way ANOVAs on serotoninergic endpoints in the HPC of OVX rats produced significant overall effects on 5-HIAA, TRP, and the ratio of 5-HIAA/5-HT at 1 week. Post-hoc analyses showed that levels of 5-HIAA were significantly lower in G-1-treated rats than in controls. Levels of TRP were significantly lower in E2-, PPT-, DPN-, and G-1-treated rats than in controls, and the ratios of 5-HIAA/5-HT were significantly lower in E2-, DPN-, and G-1-treated rats than in controls. No significant effects on levels of 5-HT, 5-HIAA, TRP, or the ratio of 5-HIAA/5-HT were detected in OVX rats after 6 weeks of treatment.

1-way ANOVAs on serotoninergic endpoints in the HPC of VCD-treated rats showed no significant overall effects after 1 week of agonist treatment. After 6 weeks of treatment a strong trend was observed for the ratio of 5-HIAA/5-HT ($F[4,19]=$, $p=0.06$). Slight reductions in E2- and G-1-treated rats relative to controls were detected using Dunnett's test.

Dopaminergic Pathway:

3-way ANOVAs detected significant overall effects on DA ($F[19,74]=40.4$, $p<0.0001$) and TYR ($F[19,74]=50.0$, $p<0.0001$). Levels of DOPAC and HVA were below the limit of detection in the HPC. Significant main effects of Model, Agonist Treatment, and Time Point were detected for both DA and TYR (DA: Model $F[1,74]=12.5$, $p<0.001$; Agonist $F[4,74]=2.7$, $p<0.05$; Time $F[1,74]=689.7$, $p<0.0001$; TYR: Model $F[1,74]=6.8$, $p<0.02$; Agonist $F[4,74]=9.1$, $p<0.0001$; Time $F[1,74]=909.9$, $p<0.0001$). Significant interactions also were detected. For DA these included interactions of Model x Time ($F[1,74]=12.6$, $p<0.001$), and Agonist Treatment x Time $F[4,74]=3.4$, $p<0.02$). Post-hoc analyses revealed that levels of DA were significantly higher in VCD-treated than in OVX rats when collapsed across Agonist and Time, that levels were significantly higher at 1W than at 6W when collapsed across Model and Agonist, and that levels of DA were significantly lower in OVX-PPT-treated rats than in VCD-E2, VCD-PPT, VCD-DPN, VCD-G-1 or OVX-DPN-treated rats, when collapsed across time. Analysis also revealed that levels in 1W-DPN treated rats were higher than in 1W-controls, 1W-PPT, and 1W-G-1-treated rats, when collapsed across time.

Interaction effects for levels of TYR included Model x Agonist Treatment ($F[4,74]=3.5$, $p<0.02$) and a 3-way interaction of Model x Agonist Treatment x Time ($F[4,74]=3.3$, $p<0.02$). Post-hoc analysis revealed that levels of TYR were significantly higher in OVX than in VCD-

treated rats when collapsed across Agonist Treatment and Time, were much higher at 6 weeks than at 1 week when collapsed across Model and Agonist Treatment, and were significantly lower in all treatment groups compared with OVX-controls with the exception of OVX-DPN-treated rats, when collapsed across time.

1-way ANOVAs on levels of DA and TYR in the HPC of OVX rats produced significant overall effects after both 1 week and 6 weeks of treatment. Post-hoc analyses showed that levels of DA were significantly higher in DPN-treated rats than in controls and PPT-treated rats after 1 week of treatment, and were significantly lower in E2-, PPT- and G-1-treated rats than in controls after 6 weeks of treatment. In contrast, levels of TYR were significantly lower in all agonist-treated rats relative to controls after 1 week of treatment and remained lower than controls in E2- and PPT-treated rats after 6 weeks of treatment.

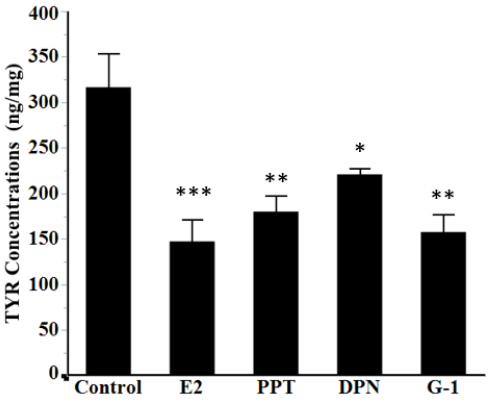
1-way ANOVAs on levels of DA and TYR in the HPC of VCD-treated rats showed no significant overall effects after 1 week of agonist treatment and no effect on DA levels after 6 weeks of treatment. An effect on TYR levels was detected after 6 weeks of treatment at which point levels in E2-treated rats were significantly lower than in controls.

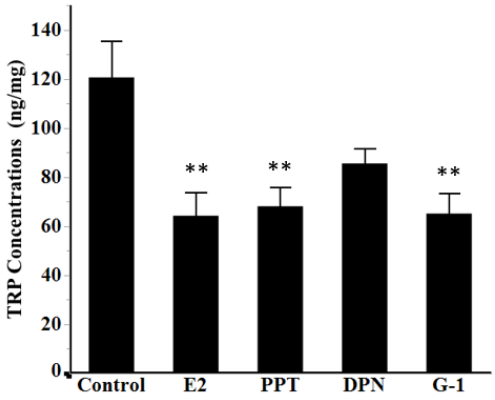
Noradrenergic Pathway:

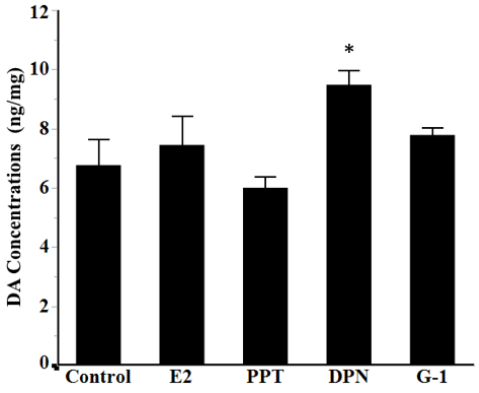
3-way ANOVA detected a significant overall effect on NE ($F[19,74]=3.1$, $p<0.0005$). Levels of EPI were below the limit of detection in the HPC. A significant main effects Time Point was detected ($F[1,74]=21.7$, $p<0.0001$) as well as a Model x Time interaction ($F[1,74]=12.0$, $p<0.001$). No significant main effects of Model or Agonist Treatment on NE levels were detected. Post-hoc analyses showed that NE levels were significantly higher at 6 weeks than at 1 week when collapsed across Model and Agonist Treatment, and that NE levels in OVX-1W rats were significantly lower

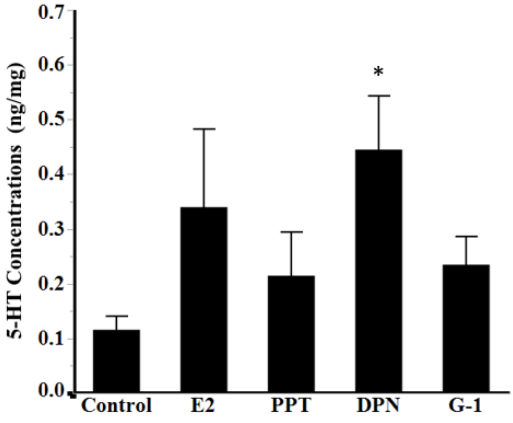
than in VCD-1W rats, which in turn were significantly lower than in OVX-6W rats, when collapsed across Agonist Treatment.

1-way ANOVAs on levels of NE in the HPC of OVX and VCD-treated rats revealed no significant effects of treatment when compared at each of the 2 time points.









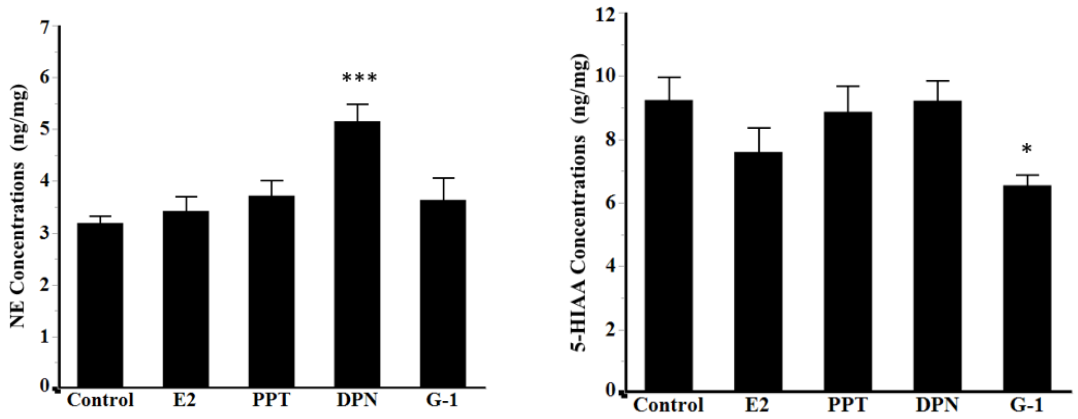


Figure 15. Levels of TYR, TRP, NE, DA, 5HT,5-HIAA in HPC changed following E2 and ER agonists treatments at 1-W in OVX model. Bars indicate Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.

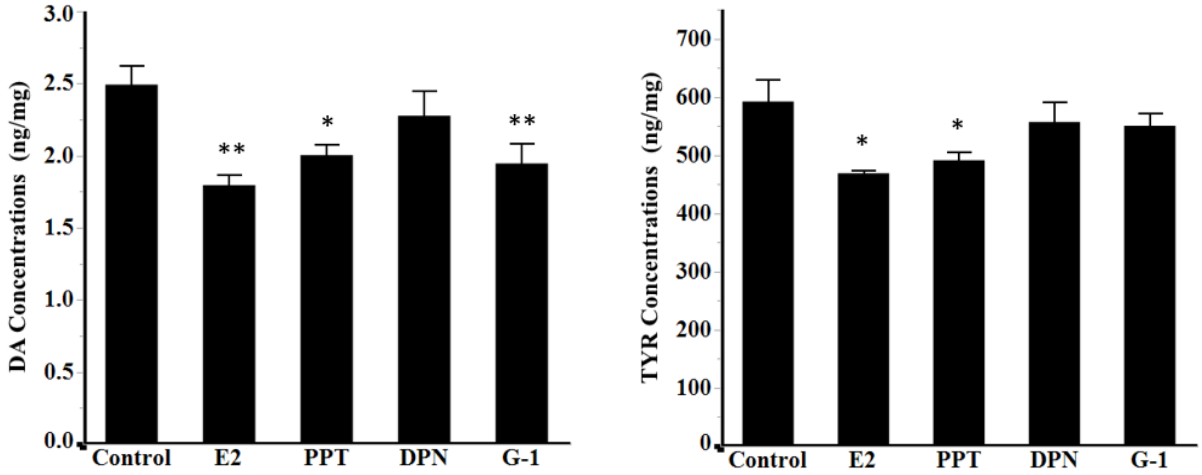


Figure 16. Levels of DA and TYR in HPC changed following E2 and ER agonists treatments at 6-W in OVX model. Bars indicate Mean ± SEM. *p<0.05, **p<0.01, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.

FCX

Serotoninergic Pathway:

3-way ANOVAs detected significant overall effects on 5-HT ($F[19,72]=3.8$, $p<0.0001$), 5-HIAA ($F[19,72]=5.3$, $p<0.0001$) and the amino acid TRP ($F[19,72]=7.2$, $p<0.0001$). Main effects of Model were detected for 5-HIAA ($F[1,72]=13.1$, $p<0.001$) and TRP ($F[1,72]=8.6$, $p<0.01$). Specifically, levels of both 5-HIAA and TRP was higher in OVX than in VCD-treated rats when collapsed across Agonist and Time. A main effect of Time was detected for ratio of 5-HIAA/5-HT ($F[1,72]=16.6$, $p<0.0001$) as well as 5-HT ($F[1,72]=61.5$, $p<0.0001$) , 5-HIAA ($F[1,72]=69.3$, $p<0.0001$), and TRP ($F[1,72]=100.6$, $p<0.0001$). Specifically, ratio of 5-HIAA/5-HT as well as the levels of 5-HT, 5-HIAA, and TRP were significantly lower at 1 week than at 6 weeks when collapsed across Model and Agonist Treatment. No interaction was detected for the endpoints in the serotoninergic pathway.

1-way ANOVAs on serotoninergic endpoints in the FCX of OVX rats produced significant overall effects only on TRP at 1 week. Post-hoc analyses showed that levels of TRP were significantly lower in E2-, PPT-, DPN-, and G-1-treated rats than in controls. No significant effects on levels of 5-HT, 5-HIAA, TRP, or the ratio of 5-HIAA/5-HT were detected in OVX rats after 6 weeks of treatment.

1-way ANOVAs on serotoninergic endpoints in the FCX of VCD-treated rats showed no significant overall effects after 1 week or 6 weeks of agonist treatment.

Dopaminergic Pathway:

3-way ANOVAs detected significant overall effects on DA ($F[19,72]=13.3$, $p<0.0001$) and TYR ($F[19,72]=5.6$, $p<0.0001$). Levels of DOPAC and HVA were below the limit of detection in

the FCX. Significant main effects of Model, Agonist Treatment, and Time Point were detected for TYR (Model $F[1,72]=11.5$, $p<0.01$; Agonist $F[4,72]=3.3$, $p<0.05$; Time $F[1,72]=62.3$, $p<0.0001$) while significant main effect of Time Point was detected for DA ($F[1,72]=210.1$, $p<0.0001$). Significant interactions were detected for DA, which included interactions of Model x Agonist Treatment ($F[4,72]=2.6$, $p<0.05$), Agonist Treatment x Time ($F[4,72]=2.8$, $p<0.05$) as well as a full 3-way interaction ($F[4,72]=2.5$, $p<0.05$).

Post-hoc analysis revealed that levels of TYR were significantly higher in OVX than in VCD-treated rats when collapsed across Agonist Treatment and Time; levels of TYR were much higher at 6 weeks than at 1 week while levels of DA were much higher at 1 week than at 6 weeks when collapsed across Model and Agonist Treatment.

1-way ANOVAs on levels of DA and TYR in the FCX of OVX rats produced significant overall effects after 1 week of treatment. Post-hoc analyses showed that levels of DA were significantly higher in all agonist-treated rats relative to controls after 1 week of treatment. In contrast, levels of TYR were significantly lower in all agonist-treated rats relative to controls after 1 week of treatment.

1-way ANOVAs on levels of DA and TYR in the FCX of VCD-treated rats showed no significant overall effects after 1 week or 6 weeks of agonist treatment.

Noradrenergic Pathway:

No significant effect on NE was detected through 3-way ANOVA or 1-way ANOVA. Levels of EPI were below the limit of detection in the FCX.

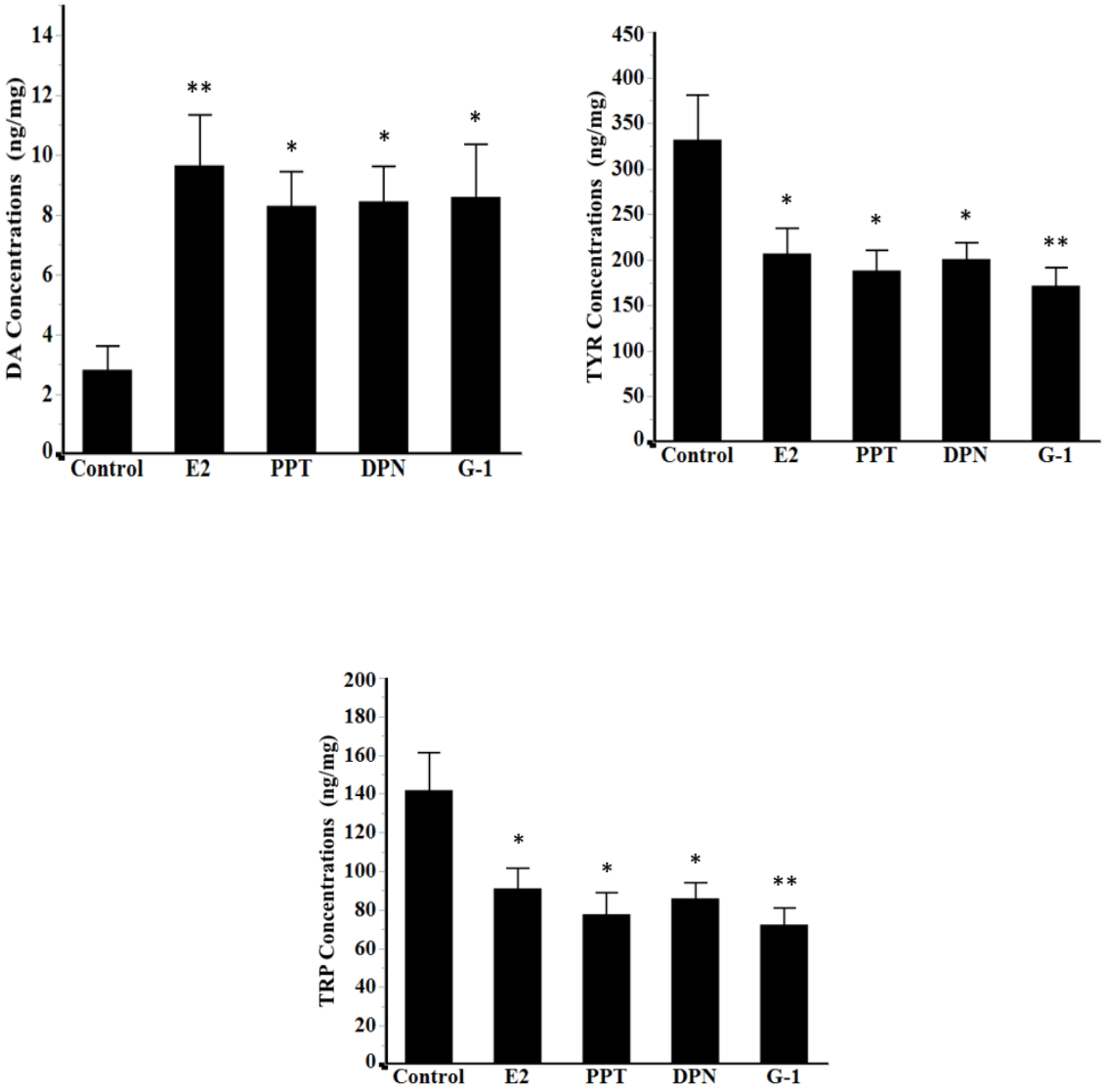


Figure 17. Levels of DA, TYR and TRP in FCX changed following E2 and ER agonists treatments at 1-W in OVX model. Bars indicate Mean ± SEM. *p<0.05, **p<0.01, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.

STR

Serotoninergic Pathway:

3-way ANOVAs detected significant overall effects on 5-HT ($F[19,72]=9.4$, $p<0.0001$), 5-HIAA ($F[19,72]=3.5$, $p<0.0001$) and ratio of 5-HT/5-HIAA ($F[19,72]=6.7$, $p<0.0001$) . Main effects of Time Point were detected for 5-HT ($F[1,72]=9.4$, $p<0.0001$), 5-HIAA ($F[1,72]=36.4$, $p<0.0001$) , ratio of 5-HT/5-HIAA ($F[1,72]=106.3$, $p<0.0001$) and TRP ($F[1,72]=20.8$, $p<0.0001$). Specifically, ratio of 5-HIAA/5-HT was significantly lower at 6 weeks than at 1 week while the levels of 5-HT, 5-HIAA, and TRP were significantly higher at 6 weeks than at a week when collapsed across Model and Agonist Treatment. The only interaction detected in the serotoninergic pathway was a full 3-way interaction for 5-HIAA ($F[4,72] =3.7, p<0.01$).

1-way ANOVAs on serotoninergic endpoints in the STR of OVX rats produced significant overall effects only on 5-HT and 5-HIAA at 1 week. Post-hoc analyses showed that level of 5-HT was significantly higher in E2-treated rats than in controls while level of 5-HIAA was significantly higher in DPN-treated rats than in controls. No significant effects on levels of 5-HT, 5-HIAA, TRP, or the ratio of 5-HIAA/5-HT were detected in OVX rats after 6 weeks of treatment.

1-way ANOVAs on serotoninergic endpoints in the STR of VCD-treated rats showed significant higher level of 5-HIAA in PPT-treated rats in relative to controls after 6 weeks of agonist treatment.

Dopaminergic Pathway:

3-way ANOVAs detected significant overall effects on DA ($F[19,72]=11.8$, $p<0.0001$) , HVA ($F[19,72]=8.7$, $p<0.0001$) and ratio of DOPAC/DA ($F[19,72]=8.1$, $p<0.0001$). Main effects of Time Point were detected for all of the endpoints in the dopaminergic pathway: DA

(F[1,72]=199.1, p<0.0001), DOPAC (F[1,72]=13.7, p<0.0001) , HVA (F[1,72]=115, p<0.0001) ratio of DOPAC/DA (F[1,72]=128.1, p<0.0001) and TYR (F[1,72]=4.1, p<0.05). Specifically, ratio of DOPAC/DA and level of DOPAC was significantly lower at 6 weeks than at 1 week while the levels of DA, HVA, and TYR were significantly higher at 6 weeks than at a week when collapsed across Model and Agonist Treatment. Interaction of Time x Agonist Treatment (F[4,72] =2.7,p<0.05) was detected for DA and interaction of Model x Time(F[1,72]=9,9,p<0.01), Model x Agonist Treatment (F[4,72]=3.3,p<0.05) and Time x Agonist Treatment (F[4,72]=3.5,p<0.05) were detected for HVA.

1-way ANOVAs on levels of DA and HVA in the STR of OVX rats produced significant overall effects after 1 week of treatment. Post-hoc analyses showed that level of DA was significantly higher in E2-treated rats relative to controls after 1 week of treatment. Levels of HVA were significantly lower in E2-, PPT-, DPN-treated rats relative to controls after 6 weeks of treatment.

1-way ANOVAs on levels of HVA in the STR of VCD-treated rats showed significant higher levels of HVA in all agonists-treated rats than controls only after 1 week of agonist treatment.

Noradrenergic Pathway:

3-way ANOVAs detected significant overall effects on NE (F[19,72]=1.9, p<0.05), EPI (F[19,72]=7.8, p<0.0001) and ratio of EPI/NE (F[19,72]=2.0, p<0.05) . Main effects of Time Point were also detected for NE (F[1,72]=19.6, p<0.0001), EPI (F[1,72]=137, p<0.0001) and ratio of EPI/NE (F[1,72]=21.7, p<0.0001) . Specifically, levels of NE, EPI as well as ratio of EPI/NE was significantly higher at 6 weeks than at 1 week when collapsed across Model and Agonist Treatment. No interaction detected in the noradrenergic pathway in STR.

1-way ANOVAs followed by post-hoc test in the STR of OVX rats revealed significant lower ratio of EPI/NE in PPT-treated rats relative to controls after 1 week of treatment while for the VCD-treated rats, higher level of EPI was detected in G-1-treated rats compared with controls after 1 week of treatment.

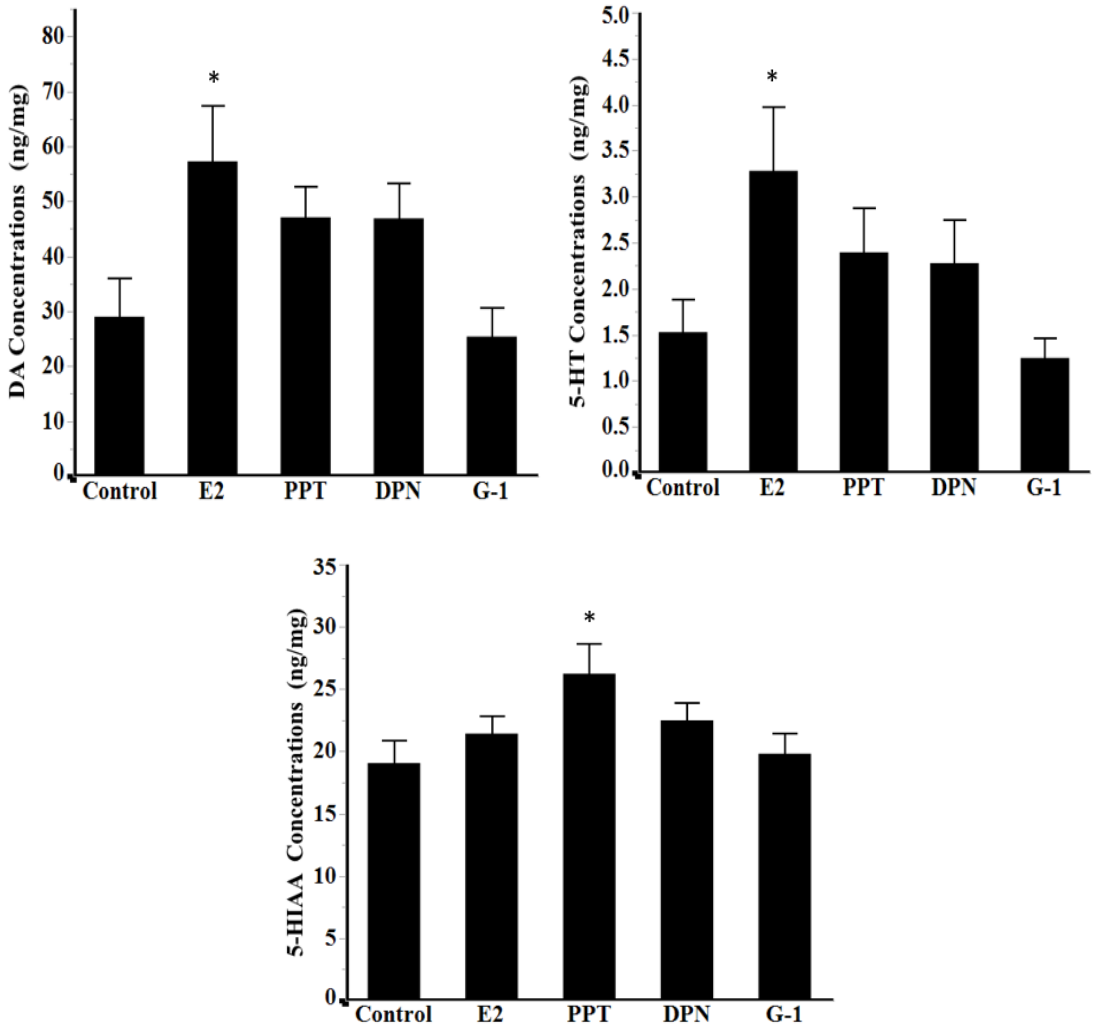


Figure 18. Levels of DA ,5-HT and 5-HIAA in STR changed following E2 and ER agonists treatments at 1-W in OVX model. Bars indicate Mean ± SEM. *p<0.05, **p<0.01, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.

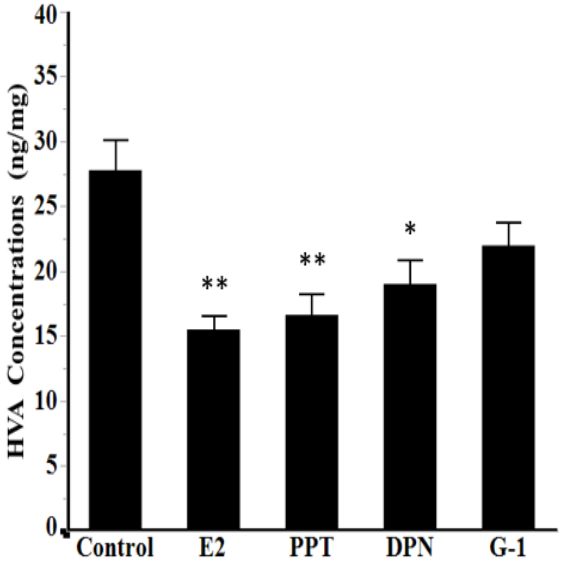
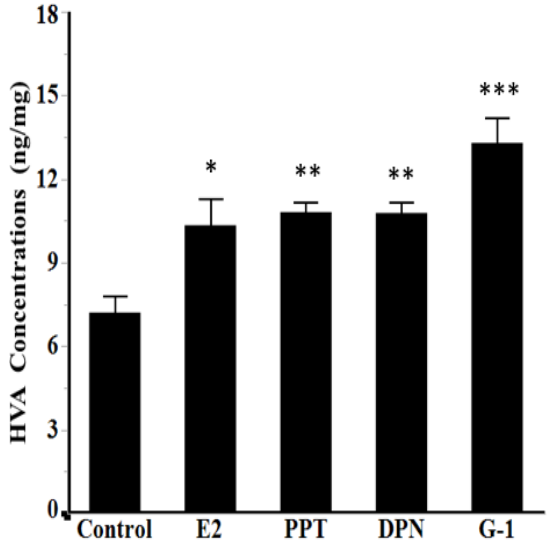


Figure 19. Levels of HVA in STR changed following E2 and ER agonists treatments at 6-W in OVX model. Bars indicate Mean ± SEM. *p<0.05, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.



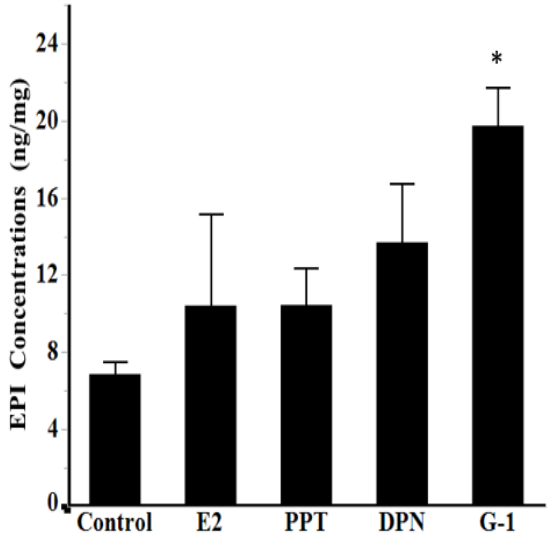


Figure 20. Levels of HVA and EPI in STR changed following E2 and ER agonists treatments at 1-W in VCD model. Bars indicate Mean ± SEM. *p<0.05, **p<0.01, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.

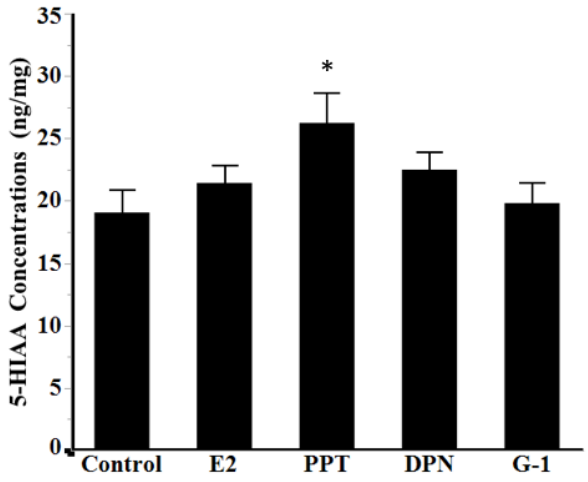


Figure 21. Levels of 5-HIAA in STR changed following E2 and ER agonists treatments at 6-W in VCD model. Bars indicate Mean ± SEM. *p<0.05, relative to control. 1-way ANOVA followed by Dunnett's test. Data of NT levels that remained unchanged were not shown.

Table 8. One-way ANOVA analysis for hormone levels in VCD-treated rats.

Ovarian Status	Agonist Treatment-Time	F Test		F Test		F Test	
		E2: F(4,19)=23.67,p<0.0001	**E2±SEM**	AD:F(4,17)=1.7,p=0.2	**AD±SEM**	T:F(4,16)=1.17,p=0.36	**T±SEM**
VCD	Control-1w		11.26±5.49		140.33±56.47		214.18±149.56
	E2-1w		**75.50±7.31*****†††		31.82±6.11		49.99±24.63
	G1-1w		6.40±1.82		132.62±51.55		127.46±59.82
	PPT-1w		14.66±6.34		99.53±22.49		92.13±28.52
	DPN-1w		17.46±5.85		155.16±30.75		173.28±27.51
		E2: F(4,19)=7.67,p<0.001	**E2±SEM**	AD:F(4,18)=3.1,p<0.05	**AD±SEM**	T:F(4,17)=2.73,p=0.06	**T±SEM**
VCD	Control-6w		14.65±5.69		30.07±11.53		53.36±19.33
	E2-6w		**212.27±65.16*****††		ND		52.944±26.78
	G1-6w		21.59±5.58		75.04±16.02		98.79±27.14
	PPT-6w		19.65±10.73		76.20±32.59		106.84±55.41
	DPN-6w		24.94±5.58		**109.88±28.31**$^{\wedge}$		**242.83±93.86***

Table 9. One-way ANOVA analysis for hormone levels in OVX rats.

Ovarian Status	Agonist Treatment-Time	F Test		F Test		F Test	
OVX		E2: F(4,16)=22.33,p<0.0001	E2±SEM	AD:F(4,16)=1.08,p=0.4	AD±SEM	T:F(4,16)=1.08,p=0.4	T±SEM
	Control-1w		ND		ND		ND
	E2-1w		104.45±22.46***†††		ND		ND
	G1-1w		ND		ND		ND
	PPT-1w		ND		ND		ND
	DPN-1w		ND		33.50±8.50		37.81±12.81
OVX		E2: F(4,19)=9.59,p<0.001	E2±SEM	NA	AD±SEM	T:F(4,19)=0.94,p=0.46	T±SEM
	Control-6w		ND		ND		ND
	E2-6w		242.65±88.68***†††		ND		ND
	G1-6w		3.80±1.16		ND		ND
	PPT-6w		5.04±2.12		ND		32.00±7.01
	DPN-6w		ND		ND		ND

Note:

Dunnetts test:

***p<0.001	in relative to Control
*p<0.05	in relative to Control

Tukey test:

††† p<0.001	in relative to all
†† p<0.01	in relative to all
^ p<0.05	in relative to E

ND	Not Detectable
Limit of Detection was used for statistical analysis in case of ND	

Table 10. Effects of ER Agonists on Neurochemical Endpoints in HPC of OVX Rats.

			OVX-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	4	4	4	4
5HT (ng/mg)	F(4,16)=2.3	p>0.05	0.12 ± 0.03	0.34 ± 0.14	0.22 ± 0.08	0.45 ± 0.10§	0.24 ± 0.05
5HIAA (ng/mg)	F(4,16)=2.9	p=0.054	9.2 ± 0.73	7.6 ± 0.78	8.9 ± 0.81	9.2 ± 0.63	6.6 ± 0.35§
DA (ng/mg)	F(4,16)=3.5	p<0.05	6.8 ± 0.87	7.4 ± 0.98	6.0 ± 0.37	9.5 ± 0.49†§	7.8 ± 0.26
NE (ng/mg)	F(4,16)=6.7	p<0.01	3.2 ± 0.14	3.4 ± 0.28	3.7 ± 0.30	5.2 ± 0.34$^{\#\S\S\S}$	3.6 ± 0.42
TRP (ng/mg)	F(4,16)=5.7	p<0.01	120.6 ± 14.95	64.3 ± 9.63*§§	68.1 ± 7.78*§§	85.5 ± 5.94	65.1 ± 8.15*§§
TYR (ng/mg)	F(4,16)=8.2	p<0.001	316.8 ± 37.14	147.6 ± 23.40**§§§	180.2 ± 17.03**§§	221.2 ± 6.02§	157.8 ± 19.49**§§
5HIAA/5HT	F(4,16)=4.5	p<0.05	91.4 ± 17.08	32.1 ± 7.72*§	59.8 ± 20.71	24.3 ± 5.67*§§	31.7 ± 6.59*§

			OVX-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	4	5	5	5
5HT (ng/mg)	F(4,19)=1.4	p>0.05	2.5 ± 0.87	1.3 ± 0.17	1.5 ± 0.31	1.2 ± 0.09	1.7 ± 0.22
5HIAA (ng/mg)	F(4,19)=1.6	p>0.05	15.8 ± 1.42	13.1 ± 0.78	14.4 ± 0.55	13.1 ± 0.79	14.1 ± 0.44
DA (ng/mg)	F(4,19)=4.6	p<0.01	2.5 ± 0.13	1.8 ± 0.07*§§	2.0 ± 0.07§	2.3 ± 0.17	1.9 ± 0.14*§
NE (ng/mg)	F(4,19)=1.2	p>0.05	5.5 ± 0.51	5.8 ± 0.61	5.0 ± 0.21	5.2 ± 0.46	6.2 ± 0.41
TRP (ng/mg)	F(4,19)=0.9	p>0.05	260.4 ± 10.13	239.2 ± 7.69	232.6 ± 3.00	240.2 ± 14.14	247.3 ± 16.05
TYR (ng/mg)	F(4,19)=3.4	p<0.05	592.1 ± 37.60	468.9 ± 4.74*§	491.2 ± 14.82§	557.0 ± 34.39	550.6 ± 22.31
5HIAA/5HT	F(4,19)=1.4	p>0.05	7.8 ± 1.27	10.1 ± 0.93	11.2 ± 1.63	11.2 ± 0.75	9.1 ± 1.24

Table 11. Effects of ER Agonists on Neurochemical Endpoints in FCX of OVX Rats.

			OVX-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			5	4	4	4	4
5HT (ng/mg)	F(4,16)=0.1	p>0.05	1.4 ± 0.30	1.6 ± 0.34	1.3 ± 0.34	1.3 ± 0.43	1.5 ± 0.35
5HIAA (ng/mg)	F(4,16)=2.2	p>0.05	6.9 ± 0.48	6.5 ± 0.66	5.6 ± 0.43	5.8 ± 0.95	4.7 ± 0.30
DA (ng/mg)	F(4,16)=4.7	p<0.05	2.8 ± 0.81	9.6 ± 1.70*§§	8.3 ± 1.15§	8.4 ± 1.19*§	8.6 ± 1.76*§
NE (ng/mg)	F(4,16)=0.6	p>0.05	5.1 ± 0.50	5.3 ± 0.34	4.3 ± 0.66	5.1 ± 0.68	4.8 ± 0.32
TRP (ng/mg)	F(4,16)=4.6	p<0.05	141.9 ± 19.58	91.1 ± 10.52§	77.8 ± 11.47*§	86.0 ± 8.03§	72.3 ± 8.81*§§
TYR (ng/mg)	F(4,16)=4.3	p<0.05	331.9 ± 49.67	207.1 ± 27.39§	188.4 ± 22.29*§	201 ± 17.75§	171.7 ± 20.06*§§
5HIAA/5HT	F(4,16)=0.6	p>0.05	5.8 ± 1.23	4.6 ± 0.83	5.1 ± 0.99	6.7 ± 2.8	3.7 ± 0.74

			OVX-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			5	4	5	5	5
5HT (ng/mg)	F(4,19)=0.2	p>0.05	2.9 ± 0.18	3.2 ± 0.39	2.8 ± 0.27	2.8 ± 0.15	2.9 ± 0.40
5HIAA (ng/mg)	F(4,19)=1.2	p>0.05	8.8 ± 0.48	8.8 ± 0.54	7.6 ± 0.53	8.2 ± 0.63	9.2 ± 0.62
DA (ng/mg)	F(4,19)=0.6	p>0.05	2.7 ± 0.16	2.3 ± 0.51	2.4 ± 0.11	2.7 ± 0.19	2.4 ± 0.30
NE (ng/mg)	F(4,19)=0.5	p>0.05	4.8 ± 0.17	4.8 ± 0.39	4.7 ± 0.41	4.8 ± 0.43	5.5 ± 0.86
TRP (ng/mg)	F(4,19)=1.0	p>0.05	196.4 ± 14.28	174.3 ± 19.09	162.6 ± 19.48	176.1 ± 24.10	211.6 ± 18.23
TYR (ng/mg)	F(4,19)=1.8	p>0.05	422.2 ± 32.29	335.3 ± 49.34	320.5 ± 66.91	378.2 ± 54.52	495.1 ± 51.04
5HIAA/5HT	F(4,19)=0.7	p>0.05	3.0 ± 0.16	2.9 ± 0.27	2.8 ± 0.24	2.9 ± 0.19	3.4 ± 0.45

Table 12. Effects of ER Agonists on Neurochemical Endpoints in STR of OVX Rats.

			OVX-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			5	4	4	4	4
5HT (ng/mg)	F(4,16)=2.9	p=0.055	1.5 ± 0.36	3.3 ± 0.7[§]	2.4 ± 0.48	2.3 ± 0.47	1.2 ± 0.22
5HIAA (ng/mg)	F(4,16)=3.0	p=0.052	13.6 ± 2.04	18.3 ± 4.14	19.3 ± 2.11	22.8 ± 1.18[§]	13.5 ± 0.65
DA (ng/mg)	F(4,16)=3.5	p<0.05	29.0 ± 6.98	57.3 ± 10.12[§]	47.1 ± 5.71	46.9 ± 6.51	25.4 ± 5.24[@]
DOPAC (ng/mg)	F(4,16)=0.9	p>0.05	51.1 ± 13.85	72.3 ± 18.85	58 ± 9.75	60.3 ± 13.16	37.5 ± 3.91
HVA (ng/mg)	F(4,16)=1.3	p>0.05	14.5 ± 2.54	15.9 ± 3.36	13.6 ± 1.69	13.6 ± 0.68	9.1 ± 1.19
NE (ng/mg)	F(4,16)=0.7	p>0.05	3.7 ± 0.47	6.0 ± 2.47	6.1 ± 0.40	5.3 ± 0.99	4.7 ± 0.74
EPI (ng/mg)	F(4,16)=2.2	p>0.05	23.7 ± 6.04	16.8 ± 4.25	6.8 ± 1.67	12.1 ± 3.44	16 ± 3.53
TRP (ng/mg)	F(4,16)=0.5	p>0.05	121.3 ± 27.29	112.5 ± 26.19	107.4 ± 10.8	119.4 ± 22.45	84.1 ± 5.90
TYR (ng/mg)	F(4,16)=0.5	p>0.05	254.3 ± 51.12	223.3 ± 54.33	231.4 ± 20.76	233.5 ± 34.54	178.3 ± 20.54
5HIAA/5HT	F(4,16)=3.3	p<0.05	9.5 ± 0.72	5.6 ± 0.3	8.7 ± 1.17	10.9 ± 1.55	12 ± 2.28[@]
DOPAC/DA	F(4,16)=0.9	p>0.05	1.7 ± 0.23	1.2 ± 0.17	1.2 ± 0.13	1.3 ± 0.26	1.8 ± 0.5
HVA/DA	F(4,16)=2.4	p=0.094	0.6 ± 0.08	0.3 ± 0.03	0.3 ± 0.06	0.3 ± 0.06	0.4 ± 0.13
EPI/NE	F(4,16)=2.9	p=0.057	6.6 ± 1.62	3.8 ± 1.55	1.2 ± 0.29[§]	2.6 ± 0.83	3.7 ± 0.92

	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			5	4	5	5	5
5HT (ng/mg)	F(4,19)=0.5	p>0.05	7.4 ± 0.47	6.9 ± 1.24	7.1 ± 0.73	7.4 ± 0.44	8.2 ± 0.66
5HIAA (ng/mg)	F(4,19)=0.3	p>0.05	23.3 ± 1.34	22.7 ± 2.52	21.2 ± 1.85	20.9 ± 1.9	22.2 ± 2.16
DA (ng/mg)	F(4,19)=2.1	p>0.05	134.1 ± 8.89	106.6 ± 17.63	117.4 ± 9.85	134.6 ± 10.22	154.4 ± 15.71
DOPAC (ng/mg)	F(4,19)=1.5	p>0.05	44.3 ± 2.64	35.6 ± 7.59	29.9 ± 3.16	35.5 ± 4.08	40.1 ± 5.36
HVA (ng/mg)	F(4,19)=6.9	p<0.01	27.8 ± 2.35	15.5 ± 1.06[**§§]	16.7 ± 1.64[**§§]	19.0 ± 1.88[*§]	22.0 ± 1.82
NE (ng/mg)	F(4,19)=0.8	p>0.05	7.7 ± 1.36	8.7 ± 1.15	6.6 ± 1.17	9.4 ± 0.52	7.9 ± 1.42
EPI (ng/mg)	F(4,19)=1.0	p>0.05	50.6 ± 3.97	44.4 ± 8.88	37.3 ± 3.03	44.3 ± 6.06	51.6 ± 6.53
TRP (ng/mg)	F(4,19)=0.3	p>0.05	179.0 ± 29.66	175.7 ± 47.15	136.4 ± 32.73	146.8 ± 18.68	162.5 ± 42.21
TYR (ng/mg)	F(4,19)=0.3	p>0.05	278.4 ± 44.98	242.6 ± 74.03	212.2 ± 67.03	223.8 ± 28.96	284.3 ± 68.88
5HIAA/5HT	F(4,19)=0.7	p>0.05	3.2 ± 0.14	3.6 ± 0.72	3.0 ± 0.28	2.8 ± 0.16	2.8 ± 0.41
DOPAC/DA	F(4,19)=1.0	p>0.05	0.3 ± 0.01	0.4 ± 0.13	0.3 ± 0.03	0.3 ± 0.03	0.3 ± 0.03
HVA/DA	F(4,19)=2.7	p=0.06	0.21 ± 0.03	0.16 ± 0.03	0.14 ± 0.01[§]	0.14 ± 0.01[§]	0.15 ± 0.01
EPI/NE	F(4,19)=0.6	p>0.05	7.4 ± 1.19	5.6 ± 1.42	6.9 ± 1.88	4.8 ± 0.65	7.3 ± 1.47

Table 13. Effects of ER Agonists on Neurochemical Endpoints in HPC of VCD-treated Rats.

			VCD-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	5	5	5	5
5HT (ng/mg)	F(4,20)=0.9	p>0.05	0.2 ± 0.03	0.4 ± 0.09	0.7 ± 0.20	0.7 ± 0.27	0.3 ± 0.12
5HIAA (ng/mg)	F(4,20)=0.5	p>0.05	8.0 ± 0.56	8.5 ± 0.72	8.8 ± 0.76	8.9 ± 0.39	9.2 ± 0.52
DA (ng/mg)	F(4,20)=1.1	p>0.05	8.5 ± 0.72	9.3 ± 0.61	9.5 ± 0.61	10.3 ± 1.22	8.3 ± 0.44
NE (ng/mg)	F(4,20)=0.8	p>0.05	4.0 ± 0.19	5.1 ± 0.61	5.3 ± 0.80	4.9 ± 0.68	4.5 ± 0.38
TRP (ng/mg)	F(4,20)=0.1	p>0.05	77.9 ± 7.52	84.2 ± 9.10	74.1 ± 4.52	78.2 ± 7.84	82.9 ± 6.97
TYR (ng/mg)	F(4,20)=0.1	p>0.05	193.6 ± 30.55	175.6 ± 24.07	156.4 ± 8.99	165.2 ± 19.08	197.8 ± 17.15
5HIAA/5HT	F(4,20)=0.9	p>0.05	39.2 ± 5.35	25.8 ± 6.18	17.0 ± 4.40	28.8 ± 13.39	39.4 ± 7.69

			VCD-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	5	4	5	5
5HT (ng/mg)	F(4,19)=1.1	p>0.05	1.17 ± 0.33	1.7 ± 0.29	1.66 ± 0.13	1.29 ± 0.23	1.77 ± 0.23
5HIAA (ng/mg)	F(4,19)=0.4	p>0.05	13.6 ± 0.79	12.6 ± 0.87	13.9 ± 1.30	13.3 ± 0.47	12.6 ± 1.2
DA (ng/mg)	F(4,19)=1.8	p>0.05	1.9 ± 0.26	2.3 ± 0.28	2.1 ± 0.08	1.8 ± 0.14	2.5 ± 0.23
NE (ng/mg)	F(4,19)=1.6	p>0.05	4.4 ± 0.58	5.8 ± 0.36	5.6 ± 0.32	4.8 ± 0.58	4.7 ± 0.46
TRP (ng/mg)	F(4,19)=0.4	p>0.05	239.0 ± 14.31	249.1 ± 13.77	259.4 ± 21.59	240.6 ± 8.04	242.3 ± 7.38
TYR (ng/mg)	F(4,19)=3.8	p<0.05	533.4 ± 19.67	452.6 ± 17.89†	555.7 ± 33.27	510.3 ± 12.07	460.8 ± 28.74
5HIAA/5HT	F(4,19)=2.7	p=0.06	14.6 ± 2.78	8.2 ± 1.41	8.5 ± 1.06	11.3 ± 1.6	7.7 ± 1.28§

Table 14. Effects of ER Agonists on Neurochemical Endpoints in FCX of VCD-treated Rats.

			VCD-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			4	5	5	5	5
5HT (ng/mg)	F(4,19)=1.1	p>0.05	2.3 ± 0.59	1.8 ± 0.39	1.4 ± 0.34	1.6 ± 0.42	1.3 ± 0.27
5HIAA (ng/mg)	F(4,19)=0.4	p>0.05	5.5 ± 0.39	4.8 ± 0.55	5.4 ± 0.56	5.2 ± 0.47	5.0 ± 0.41
DA (ng/mg)	F(4,19)=0.2	p<0.01	8.7 ± 0.78	8.2 ± 1.02	9.0 ± 0.78	9.4 ± 1.40	8.2 ± 1.52
NE (ng/mg)	F(4,19)=1.8	p>0.05	3.9 ± 0.17	5.8 ± 0.51	5.2 ± 0.43	5.2 ± 0.30	4.9 ± 0.73
TRP (ng/mg)	F(4,19)=0.4	p>0.05	77.9 ± 11.05	82.8 ± 10.87	77.1 ± 6.66	80.8 ± 6.13	75.4 ± 7.37
TYR (ng/mg)	F(4,19)=3.8	p<0.05	180.8 ± 28.13	163.7 ± 24.04	168.0 ± 13.79	174.3 ± 14.88	172.0 ± 12.52
5HIAA/5HT	F(4,19)=2.7	p>0.05	2.7 ± 0.40	3.3 ± 0.74	5.4 ± 1.59	4.7 ± 1.49	4.4 ± 0.74

			VCD-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			5	5	4	5	4
5HT (ng/mg)	F(4,18)=0.5	p>0.05	2.8 ± 0.22	2.4 ± 0.25	2.6 ± 0.47	3.1 ± 0.56	2.9 ± 0.48
5HIAA (ng/mg)	F(4,18)=0.3	p>0.05	7.1 ± 0.79	7.7 ± 0.66	6.7 ± 0.50	7.6 ± 1.04	7.0 ± 0.75
DA (ng/mg)	F(4,18)=0.7	p>0.05	2.5 ± 0.25	2.1 ± 0.31	1.9 ± 0.27	2.5 ± 0.34	2.2 ± 0.46
NE (ng/mg)	F(4,18)=2.3	p=0.097	4.1 ± 0.44	4.0 ± 0.37	3.1 ± 0.46	5.1 ± 0.63	4.8 ± 0.43
TRP (ng/mg)	F(4,18)=1.1	p>0.05	157.9 ± 23.14	131.6 ± 20.79	115.5 ± 17.11	166.7 ± 38.87	186.1 ± 12.90
TYR (ng/mg)	F(4,18)=1.3	p>0.05	334.7 ± 66.46	241.6 ± 44.29	234.5 ± 33.53	354.8 ± 70.73	369.8 ± 35.96
5HIAA/5HT	F(4,18)=0.7	p>0.05	2.6 ± 0.31	3.4 ± 0.46	2.9 ± 0.75	2.6 ± 0.33	2.6 ± 0.41

Table 15. Effects of ER Agonists on Neurochemical Endpoints in STR of VCD-treated Rats.

			VCD-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			4	4	5	5	5
5HT (ng/mg)	F(4,18)=0.8	p>0.05	2.7 ± 0.47	4.3 ± 2.84	2 ± 0.35	1.9 ± 0.46	1.8 ± 0.39
5HIAA (ng/mg)	F(4,18)=0.4	p>0.05	16.9 ± 1.98	17 ± 4.01	15.3 ± 1.15	15.1 ± 0.91	14.1 ± 0.77
DA (ng/mg)	F(4,18)=0.2	p>0.05	38.1 ± 9.76	50.5 ± 17.05	40.3 ± 8.3	42 ± 8.8	42.4 ± 11.72
DOPAC (ng/mg)	F(4,18)=0.9	p>0.05	37.6 ± 6.1	42.1 ± 5.67	44.9 ± 3.42	56.5 ± 12.05	51.9 ± 8.12
HVA (ng/mg)	F(4,18)=10.1	p<0.001	7.2 ± 0.6	10.3 ± 0.96*§	10.8 ± 0.36*§§	10.8 ± 0.37*§§	13.3 ± 0.9***@§§§
NE (ng/mg)	F(4,18)=0.7	p>0.05	7.3 ± 1.68	4.5 ± 1.28	5.2 ± 1.08	5.0 ± 0.62	5.2 ± 1.47
EPI (ng/mg)	F(4,18)=3.2	p<0.05	6.9 ± 0.67	10.4 ± 4.75	10.4 ± 1.92	13.7 ± 3.03	19.7 ± 2.02*§
TRP (ng/mg)	F(4,18)=0.8	p>0.05	113.3 ± 18.6	116.6 ± 34.73	89.8 ± 7.97	97.5 ± 9.95	82.1 ± 7.06
TYR (ng/mg)	F(4,18)=0.1	p>0.05	180.8 ± 41.04	207.6 ± 57	184.1 ± 12.54	192.3 ± 21.82	177.9 ± 14.94
5HIAA/5HT	F(4,18)=0.3	p>0.05	7.3 ± 2.22	8.5 ± 2.92	8.4 ± 1.15	10.3 ± 2.57	9.3 ± 1.63
DOPAC/DA	F(4,18)=0.3	p>0.05	1.1 ± 0.12	1.3 ± 0.45	1.3 ± 0.26	1.5 ± 0.29	1.5 ± 0.3
HVA/DA	F(4,18)=0.6	p>0.05	0.2 ± 0.04	0.3 ± 0.11	0.3 ± 0.06	0.3 ± 0.07	0.4 ± 0.08
EPI/NE	F(4,18)=1.2	p>0.05	1.1 ± 0.19	4.4 ± 3.27	2.4 ± 0.71	2.7 ± 0.37	5.5 ± 1.79

			VCD-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			5	5	4	5	5
5HT (ng/mg)	F(4,19)=1.0	p>0.05	7.2 ± 0.95	6.5 ± 0.6	7.3 ± 0.78	8.5 ± 1.16	6.1 ± 1.02
5HIAA (ng/mg)	F(4,19)=2.4	p=0.083	19.1 ± 1.84	21.4 ± 1.39	26.2 ± 2.41§	22.5 ± 1.42	19.8 ± 1.66
DA (ng/mg)	F(4,19)=0.5	p>0.05	132.8 ± 17.93	111 ± 25.06	115.4 ± 16.88	106.2 ± 8.05	129.1 ± 13.93
DOPAC (ng/mg)	F(4,19)=0.8	p>0.05	34.4 ± 3.91	33.4 ± 5.79	43.3 ± 7.42	35.9 ± 6.04	44.7 ± 5.98
HVA (ng/mg)	F(4,19)=0.4	p>0.05	22.9 ± 2.94	20.8 ± 1.1	21.9 ± 2.84	24.9 ± 3.25	24.7 ± 2.81
NE (ng/mg)	F(4,19)=1.4	p>0.05	6.3 ± 1.16	8.4 ± 0.97	6.7 ± 1.63	8.8 ± 1.14	5.9 ± 0.62
EPI (ng/mg)	F(4,19)=0.3	p>0.05	42.8 ± 8.02	45.4 ± 8.6	55.8 ± 9.79	54.7 ± 12.87	49.2 ± 9.01
TRP (ng/mg)	F(4,19)=1.2	p>0.05	175.5 ± 36	109.8 ± 13.77	210.1 ± 48.00	152.8 ± 6.23	167.5 ± 42.83
TYR (ng/mg)	F(4,19)=1.4	p>0.05	261.9 ± 49.73	160.4 ± 33.93	340.5 ± 85.32	227.4 ± 17.3	267.0 ± 69.43
5HIAA/5HT	F(4,19)=0.8	p>0.05	2.7 ± 0.3	3.5 ± 0.54	3.8 ± 0.66	2.8 ± 0.36	3.6 ± 0.64
DOPAC/DA	F(4,19)=0.4	p>0.05	0.3 ± 0.06	0.3 ± 0.04	0.4 ± 0.08	0.3 ± 0.08	0.4 ± 0.05
HVA/DA	F(4,19)=0.4	p>0.05	0.2 ± 0.03	0.3 ± 0.09	0.2 ± 0.03	0.2 ± 0.05	0.2 ± 0.03
EPI/NE	F(4,19)=0.6	p>0.05	7.9 ± 2.03	5.5 ± 0.90	11.3 ± 5.14	7.5 ± 2.61	9.1 ± 2.59

			Values are mean ± sem.			
Symbol			Alpha	Test		
§			p<0.05	Dunnetts	Differs from Controls	
§§			p<0.01	Dunnetts	Differs from Controls	
§§§			p<0.001	Dunnetts	Differs from Controls	
*			p<0.05	Tukey	Differs from Controls	
**			p<0.01	Tukey	Differs from Controls	
†			p<0.05	Tukey	Differs from PPT	
#			p<0.05	Tukey	Differs from all other groups	
##			p<0.01	Tukey	Differs from all other groups	
@			p<0.05	Tukey	Differs from E	

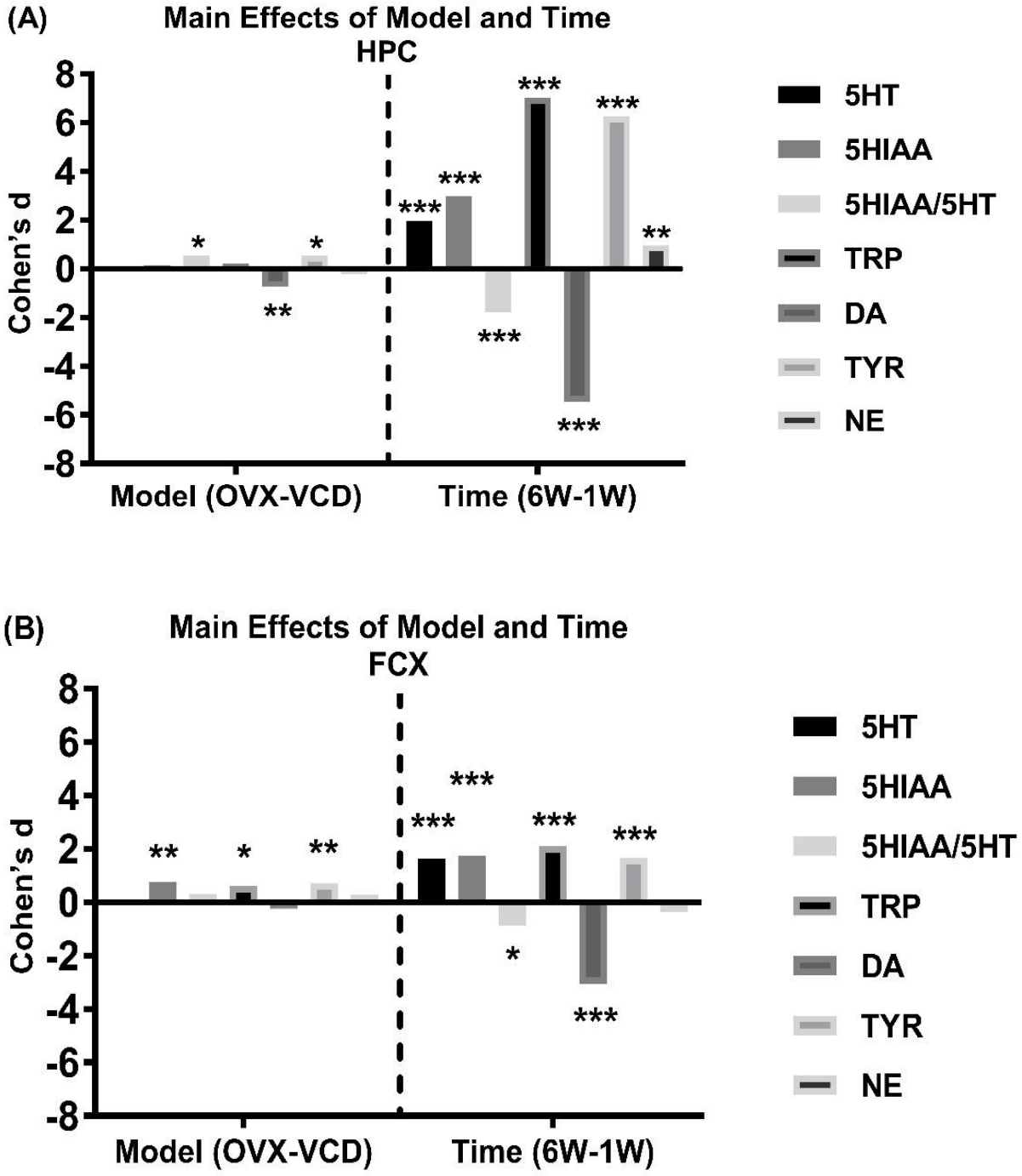


Figure 22. Main effects of Model and Time on monoamines, metabolites, amino acids and metabolite/monoamine ratios in (A) HPC and (B) FCX. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

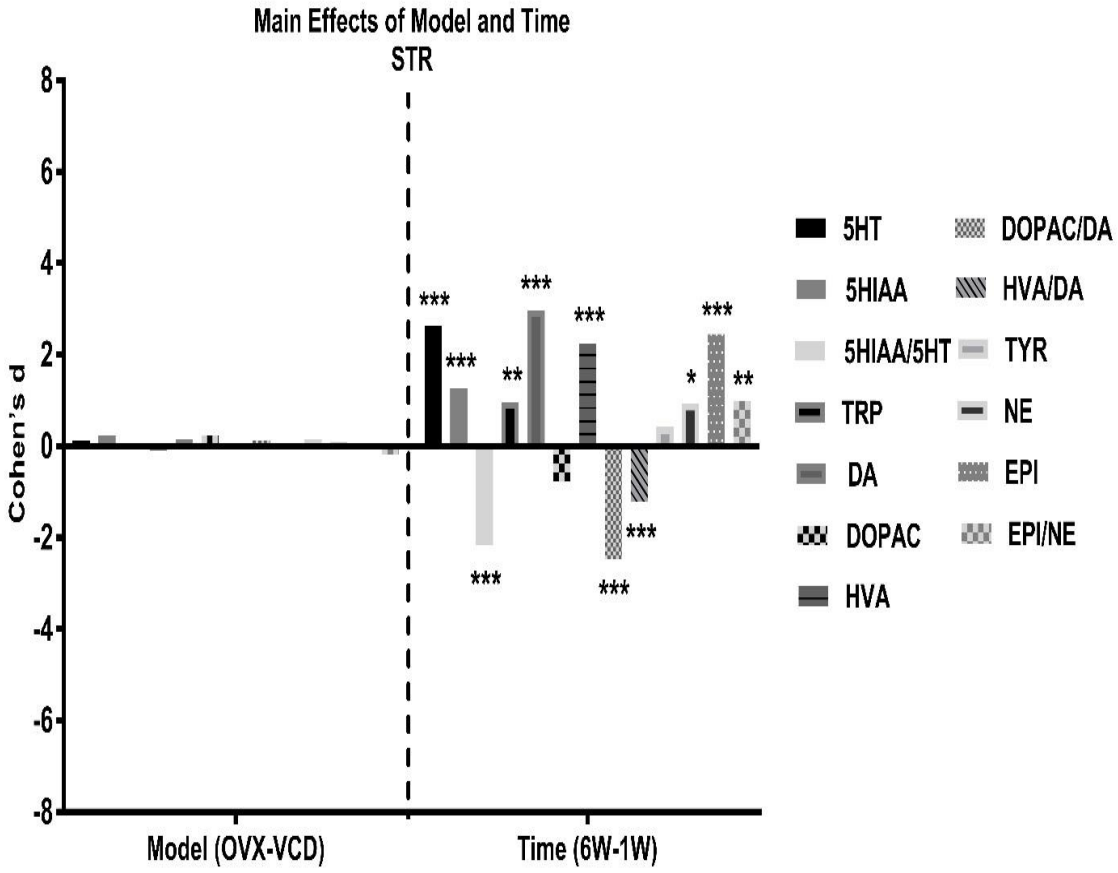


Figure 23. Main effects of Model and Time on monoamines, metabolites, amino acids and metabolite/monoamine ratios in STR. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

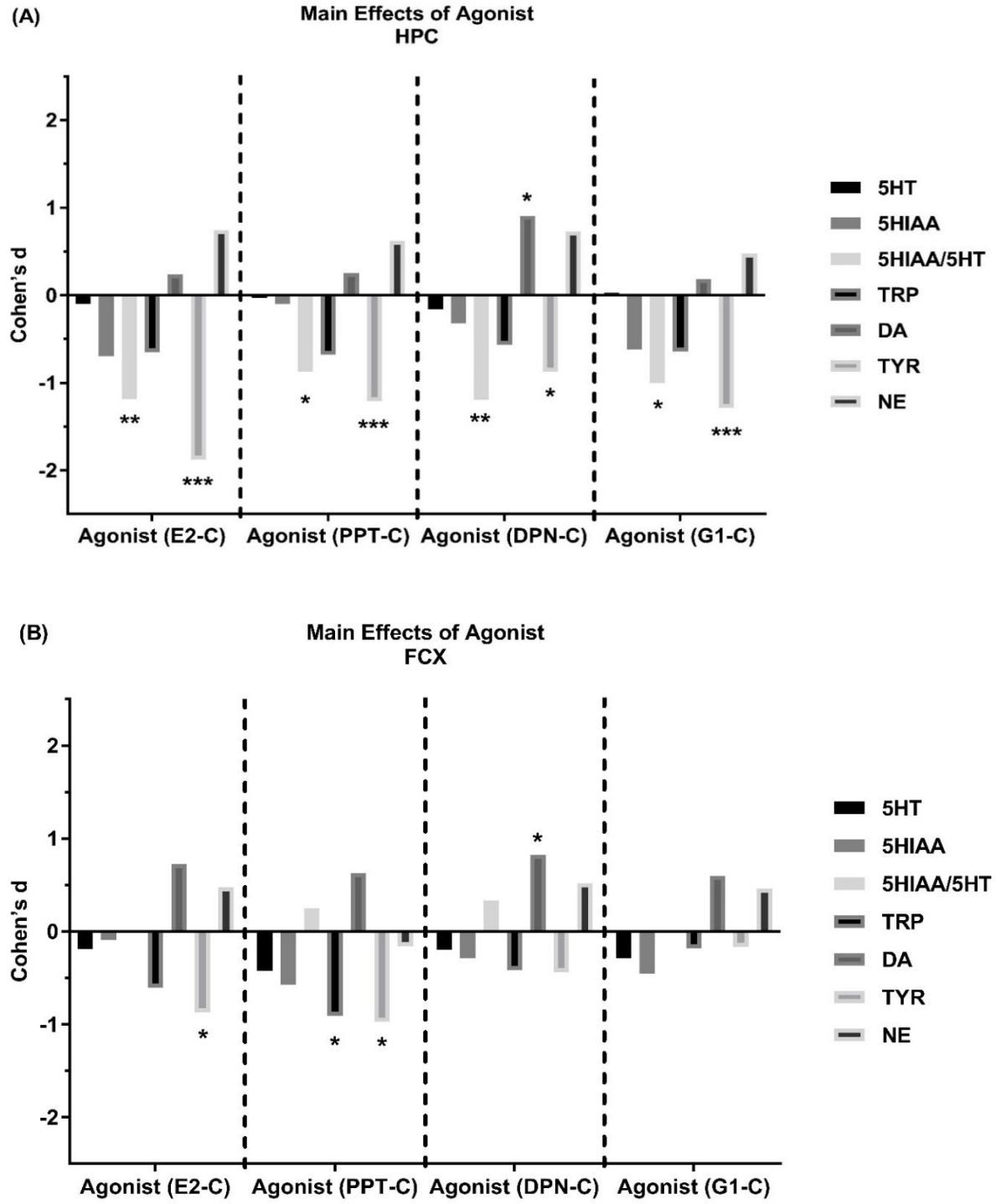


Figure 24. Main effects of Agonist on monoamines, metabolites, amino acids and metabolite/monoamine ratios in (A) HPC and (B) FCX. Bars indicate Cohen's d: value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

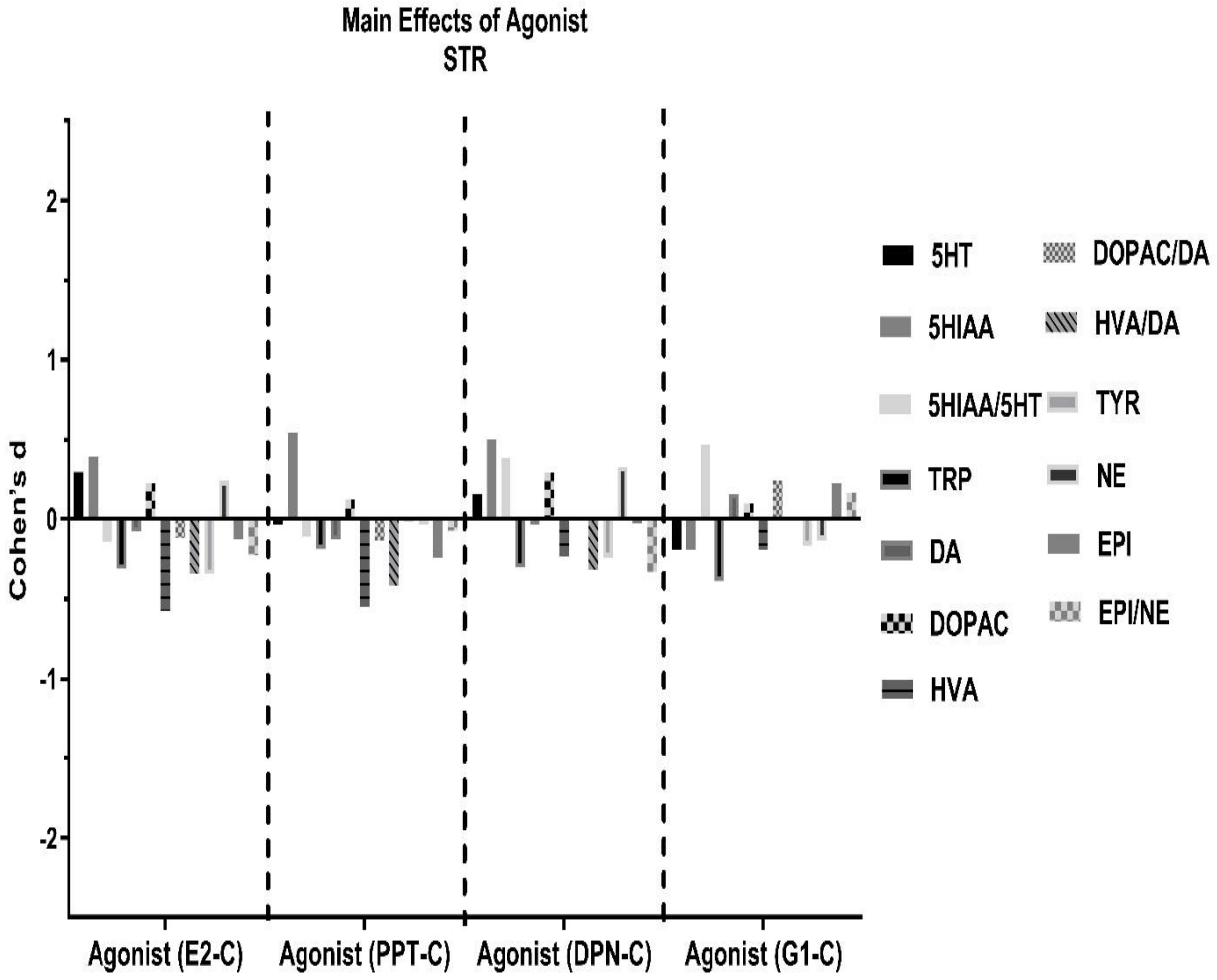


Figure 25. Main effects of Agonist on monoamines, metabolites, amino acids and metabolite/monoamine ratios in STR. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

4.6 DISCUSSIONS

4.6.1 Serum hormone measurements

The goal of current study was to characterize changes in relevant neurochemical endpoints in the brain associated with surgical and transitional menopause, in response to chronic treatments of estradiol and selective ER agonists. Circulating levels of E2, T and AD confirm that representation of the two menopausal models was successfully achieved. AD and T were readily detectable in most rats receiving VCD treatments. This is in agreement with prior studies [81, 158] and is due to the preservation of androgen-producing ovarian tissues. In contrast, levels of T, AD, and E2 were all below detectability in OVX controls.

Results show that 6 weeks of DPN treatments led to significant elevations in T and a strong trend for elevation of AD in VCD-treated rats. The exact mechanism of this is currently unknown. ERβ is expressed by both thecal and granulosa cells [163]. It may be that activation of ERβ by DPN stimulates thecal cells to increase biosynthesis of androgens, or alternatively inhibits the transformation of androgens to estrogens in the granulosa cells via an effect on aromatase. This needs to be investigated. As expected, rats receiving E2 demonstrated significantly higher plasma E2 levels relative to other groups. It is interesting that continuous administration of E2 at a dose of 3 μg/day resulted in physiological levels after 1 week of treatment and supra-physiological levels after 6 weeks of treatment. This indicates that long-term chronic treatment with even modest doses of E2 can exceed the capacity for clearance and result in accumulation beyond the

physiological range. This highlights the importance of verifying plasma hormone levels in any studies involving chronic E2 treatments. In this study we were concerned primarily with effects in the brain. The relationship between serum and brain levels of E2 in OVX rats treated with different doses of estradiol was recently characterized by our laboratory [164]. Results of this study showed that levels in the brain increase in accordance with levels in the serum when serum levels are in the physiological range, but that levels in several brain regions plateau when serum levels are well beyond the physiological range. This suggests that even at supra-physiological levels in serum, brain levels of E2 can remain within physiological limits.

4.6.2 Main effects of Model, Time-Point and Agonist Treatment

The current data demonstrate significant effects of Model and Time-Point on multiple measures of serotoninergic, dopaminergic and noradrenergic endpoints. These effects were region specific. For example, main effects of Model were detected in the HPC and FCX, but not in the STR. In the HPC levels of TYR were higher and levels of DA were lower in OVX vs VCD-treated rats. In the FCX levels of 5-HIAA, TRP and TYR were higher in OVX vs VCD-treated rats. These effects, while statistically significant, were relatively small. Indeed, many of the effects of 'Model' were not detected in our previous study which used fewer rats and had less statistical power [158]. Several effects were more striking, however, when separated by Time-Point. For example, the ratio of 5-HIAA/5-HT in the HPC was much higher in OVX vs VCD-treated rats at 1W, but not at 6W. This could indicate a strong effect of OVX on 5-HT turnover in this region at early time-points. In contrast, levels of DA in the HPC were much lower in OVX vs. VCD-treated rats at

1W, but not at 6W. This corresponds with significantly reduced levels of TYR in the HPC at the 1W time-point, and may indicate a significant reduction in DA activity in this region at early time-points following OVX. In the FCX, levels of 5-HIAA, TRP and TYR all were significantly higher in OVX vs. VCD-treated rats at 6W, but not at 1W. This may indicate significant compensatory responses resulting in superior normalization of amino acid levels and 5-HT turnover over time in the FCX following OVX than occur following VCD-induced loss of ovarian function. Collectively these findings demonstrate significant differences in the effects of OVX vs. VCD models of menopause on NT endpoints, and that the effects vary depending on brain region and time.

Main effects of 'Time-Point' (i.e., collapsed across model and agonist treatment) were much more robust than the main effects of 'Model' for all regions examined. In many cases endpoints were greater after 6W vs. 1W of treatment. Effects on several endpoints were consistent across brain regions. For example, levels of 5-HT, 5-HIAA, TRP and the ratio of 5-HIAA/5-HT were higher in each brain region at 6W vs 1W. This suggests significant recovery of endpoints associated with serotonin production and signaling over a period of weeks following OVX or VCD treatment. This is consistent with our prior report showing significant increases in TRP, TYR, 5-HIAA and the ratio of 5-HIAA/5-HT in the HPC at 6W vs 1W following OVX or VCD treatment. This also suggests that these effects reflect increases over time independent of ER agonist treatment. Effects of Time-Point on other endpoints differed across brain regions. In the HPC, levels of DA were lower, whereas levels of NE and TYR were higher, at 6W vs 1W. In the FCX levels of DA were lower, levels of TYR were higher, and levels of NE were unaffected at 6W vs 1W. In the STR levels of DA and NE were higher and levels of TYR were unaffected at 6W vs 1W. These findings indicate time- and region-specific effects on dopaminergic and noradrenergic

endpoints. Notably these effects were not observed in our prior study which did not include agonist treatments.

In addition to the main effects of Model and Time-Point, the data also show significant main effects of agonist treatment on multiple NT endpoints that differed by brain region. For example, in the HPC, E2, PPT, DPN and G-1 each were associated with decreases in the ratio of 5-HIAA/5-HT and in levels of TYR compared with untreated controls. This suggests that in the HPC these effects can be achieved by activation of any of the three ER subtypes. In contrast DPN, an ERβ selective agonist, was associated with an increase in DA whereas PPT and G-1 had no effect. In the FCX, E2 was associated with a decrease in TYR. PPT was associated with decreases in TRP and TYR, and DPN was associated with an increase in DA. This suggests that in the FCX activation of ERα is associated with decreases in TRP and TYR whereas activation of ERß is associated with increases in DA. No significant main effects of agonist treatment were detected in the STR. Collectively, these data indicate that estrogens can influence NT endpoints following loss of ovarian function, that endpoints are differentially affected by activation of specific ER subtypes, and that these effects are brain region-dependent.

4.6.3 Specific effects of Agonist Treatment as a Function of Model and Time-Point

This study is the first to analyze levels of TRP and TYR following ER agonist treatments in the same tissues in which multiple monoaminergic endpoints also were measured. Results indicate that the effects of agonist treatments are model- and time-point-dependent. This is clear from the interaction effects detected in the 3-way ANOVAs, and from the data presented in Table 10-15.

One general observation is that the majority of ER agonist effects were detected in OVX rats as opposed to VCD-treated rats and were detected after 1W vs. 6W of agonist treatment. This suggests that these NT endpoints are particularly sensitive to ER agonist treatments beginning soon following the complete and sudden loss of ovarian function, and that differences between treated rats vs. controls decline over time. In addition, the majority of ER agonist effects were detected in the HPC, suggesting that this region of the brain is particularly sensitive to ER agonist treatments. For clarity, the discussion of specific effects is organized below by brain region.

HPC

One surprising result was the low levels of TRP and TYR detected in the hippocampus of OVX rats after 1W of agonist treatment. Previously we reported that levels of TRP and TYR are significantly reduced in the HPC 1W after OVX or VCD treatment and that levels recover by 6W [158]. We predicted that agonist treatment would help to restore the amino acid levels. In contrast, OVX rats treated for 1W with E2 had much lower levels of TRP and TYR than vehicle-treated controls. This was also the case for rats treated with PPT and G-1, and to a lesser extent with DPN, suggesting that activation of any of the three ERs suppresses the amino acid levels even further at this early time point. Rats treated for 1W with E2 also had a significantly lower ratio of 5-HIAA/5-HT than controls, suggesting a reduction in 5-HT turnover. This also was the case for rats treated with DPN and G-1, but not PPT, suggesting that activation of ERß and GPR30 are sufficient to produce this effect. Rats treated for 1W with G1 resulted only in reductions in 5-HIAA, suggesting that activation of GPR30 is sufficient to reduce 5-HT metabolism. After 6 weeks of treatment the effects on TRP, 5-HIAA and on the ratio of 5-HIAA/5-HT were no longer evident. The effects on

TYR were still evident but were reduced in magnitude, corresponding to the reduction in DA. This indicates that the effects mitigate with time, even with continued ER agonist treatment.

Another interesting finding was that only activation of ERβ by DPN treatment at 1W increased levels of all three major neurotransmitters DA, 5-HT and NE simultaneously in HPC. Furthermore, DPN treatment reinstated the significant reduction in NE levels observed following ovariectomy in our previous study [158]. The differential effects between activation of ERα and ERβ on levels of DA, 5-HT and NE may possibly due to their relative expression in HPC after estrogen deprivation. This, however, would not account for the reductions in TRP and TYR observed in the HPC. Note that none of these effects of agonist treatment were observed in VCD-treated rats despite the fact that levels of TRP and TYR also are significantly reduced at 1W and recover by 6W following treatment with VCD [158]. This demonstrates that the HPC of VCD-treated rats is much less responsive to ER agonist treatment with respect to these endpoints than the HPC of OVX rats. This suggests a fundamental difference in the responsiveness of the HPC to estrogen therapy following transitional vs surgical menopause.

FCX

Agonist treatments produced fewer effects on neurochemical endpoints in FCX relative to HPC. As in the HPC, OVX rats treated for 1W with E2 had significantly lower levels of TRP and TYR than vehicle-treated controls. This also was the case for rats treated with PPT, DPN, and G-1. This suggests that, as in the HPC, activation of either ERα, ERβ, or GPR30, is sufficient to suppress these amino acid levels during the first week after OVX. The reduced levels of TYR may be due to its increased utilization to synthesize DA in the dopaminergic pathway, which may be caused by increased tryptophan hydrolase expression or activity following estrogen receptor

activation. Effects were no longer observed after 6 weeks of treatment, demonstrating that, as in the HPC, the effects mitigate with time even with continued treatment. Despite the reduced levels of TYR, no effect on NE levels were detected suggesting no adverse effect on NE signaling. Likewise, despite the reductions in TRP, no significant effects on 5-HT, 5-HIAA or the ratio of 5-HIAA/5-HT were detected, suggesting no significant adverse effects on 5-HT signaling. Elevated levels of DA were detected in OVX rats treated for 1W with E2, PPT, DPN, and G-1, suggesting either an increase in DA production or a reduction in DA release and degradation at this time point. As in the HPC, the effects of agonist treatments detected at 1W in OVX rats were not detected in VCD-treated rats, suggesting a fundamental difference in the responsiveness of the FCX to estrogen therapy following transitional vs. surgical menopause.

STR

Effects of agonist treatments on NT endpoints in the striatum were different in several ways from effects detected in the HPC and FCX. First, no effects of E2 or the other ER agonists on the levels of TRP or TYR were detected. Following OVX, rats treated for 1 week with E2 had significantly higher levels of 5-HT and DA than vehicle-treated controls. This was not observed in rats treated with any of the selective ER agonists. Since E2 will act at all three ERs this suggests that activation of two or more ER receptors is necessary to produce these effects. These results are consistent with either an increase in 5-HT and DA production, or with decreases in 5-HT and DA release and degradation in response to E2.

Treatment with PPT was associated with a significant increase in 5-HIAA relative to vehicle-treated controls, which would be consistent with an increase in 5-HT release. This effect was not observed at 6W despite continuous treatment. In contrast, OVX rats treated for 6W with E2 showed

a significant reduction in levels of HVA relative to vehicle-treated controls. Similar effects were observed in rats treated with PPT or DPN but not G-1. This suggests that activation of ERα or ERß, but not GPR30, is sufficient to elicit this effect and that the reductions in HVA manifest after longer-term continuous treatment. In contrast with the reductions in HVA detected in OVX rats after 6 weeks of treatment, significant increases in HVA were detected in VCD-treated rats after 1W of treatment with E2, PPT, DPN, or G-1. This is consistent with an increase in DA turnover, but notably was not accompanied by any increases in DOPAC or the DOPAC/DA or HVA/DA ratios. An increase in the level of EPI also was detected after 1W treatment with G-1, but not with any other agonist. This suggests that activation of GPR30 may have an effect on the NE pathway associated with increased NE turnover. The fact that a similar effect was not observed in rats treated with E2 suggests that this effect is unique to GPR30 activation and may be negated by activation of the other ERs. The effects on HVA and EPI levels in VCD-treated rats were not detected after 6W of treatment despite continuous treatment. These data again demonstrate a fundamental difference in the responsiveness to estrogen therapy following transitional vs. surgical menopause.

In summary, in contrast with surgical menopause, the VCD model of transitional menopause was associated with far less responsiveness to ER agonists treatment as reflected by fewer effects on NT endpoints.

4.6.4 Comparisons with existing literature

To our knowledge, this report is the first to provide a comprehensive description of changes in NT endpoints for dopaminergic, serotonergic and noradrenergic pathways in two models of menopause and following E2 and selective ER agonist treatments. Most prior studies have focused on OVX rats and mice and on the effects of ER agonists on small numbers of NT endpoints. Several of our current findings are consistent with previous results. For example, previous studies found no change in NE and 5-HIAA levels in HPC following 4-6 weeks of chronic E2 treatment in OVX mice [66] and rats [71]. This is consistent with the lack of effect of E2 treatment on these NT endpoints in HPC detected in our study. Inagaki et.al [70] reported that a single s.c. injection of E2 in OVX rats significantly increased levels of DA but had no effect on NE, DOPAC or 5-HIAA in STR. This is consistent with our results in OVX rats treated for 1W with E2. Lubbers et al. [72] reported that 4 days s.c. treatment with E2 but not PPT increased levels of 5-HIAA in the STR of OVX rats. This is consistent with our results suggesting that increased levels of 5-HIAA may be mediated by ERβ activation.

Several inconsistencies with our current findings also have been reported. For example, 4-6 weeks of chronic E2 treatment was reported to decrease levels of 5-HT, but not DA, in HPC of OVX mice [66] and rats [71]. In addition, it has been reported that E2 and PPT treatments increased NE levels [72] while E2 treatment reduced 5-HT levels in the FCX of OVX rats [71]. In another study, 4 days s.c. treatment of E2 was shown not to alter levels of DA and 5-HT in the STR of OVX rats [72]. Jacome et al. [73] reported no effects on monoaminergic endpoints in OVX rat STR 2 days after a single s.c. injection of DPN. Some of these differences with our current results likely are due to differences in dose, timing and duration of treatments.

Note that all of the above studies used OVX rats or mice when studying effects on NT endpoints. Pestana-Olivera et al. [75] recently evaluated the effects of estrogen therapy on serotonin in VCD-treated rats. They reported measurements of 5-HT, as well as mRNA levels of ERβ and progesterone receptor in several brain regions following continuous subcutaneous administration of E2. Specifically, 3W of E2 treatment significantly increased 5-HT levels in dorsal HPC of VCD-treated rats. This differs from our current finding in which no effect on 5-HT level in HPC was reported after 1W or 6W of E2 treatment in VCD-treated rats. Differences in E2 treatment duration (3W vs 1W/6W), circulating E2 levels after E2 supplementation (~40 vs ~70/210 pg/ml), specific brain region analyzed (dorsal HPC vs HPC), VCD dosing regimen (160 mg/kg daily for 15 days vs 80 mg/kg daily for 30 days) could potentially account for the differences in results. This suggests that effects of chronic ER agonist treatment after transitional menopause may vary considerably as a function of dose and time of treatment, as well as how quickly ovarian function is lost. This requires further study.

4.6.5 Potential mechanism of model differences in responsiveness to ER agonist treatments

One of the interesting findings was that OVX rats were much more responsive to ER agonist treatments than VCD-treated rats. The underlying mechanism remains largely unknown. One possibility could be due to the differences in terms of estrogen receptor expressions and functions following estrogen deprivation and treatments in the two menopausal models. It has been widely demonstrated that differential ER expressions could affect the sensitivity to steroid hormones and

consequently alter the effects of estrogens on the brain. In rodent brain, both up- and down-regulation of ER mRNA and protein levels by E2 has been reported. The effects are often brain region- and age-specific and are not always consistent, in part due to various treatment regimens and physiological state of the animals. Previous studies have reported that treatment of the hypothalamic neurons with E2 caused a rapid and transient increase of the ERα proteins in the plasma membrane. Furthermore, exposure of the neurons to E2 was found to significantly increase internalization of ERα membrane proteins, which is considered as a measure of receptor activation [165].

For OVX surgical menopausal model, previous study has demonstrated that following long-term OVX, the levels of ERα mRNA were significantly increased in the cerebral cortex but were not changed in the cerebellum and brainstem. It was also shown that ERα mRNA levels remained unchanged in all of the three brain regions following 12-week of E2 treatments [166]. Additionally, Scott et al. showed long-term estrogen deprivation caused tissue-specific reduction in ERα levels in the HPC, altering hippocampal sensitivity to E2, similar to the ERα reduction occurring naturally during the aging process [167]. Study has also shown that the expression of ERα and ERβ protein was differentially influenced by age and gonadal steroids in the mouse cerebral cortex. Specifically, it was shown that E2 supplementation increased ERα protein level in old OVX female mice and increased ERβ protein level in adult OVX female mice [168]. Additionally , OVX rats and mice receiving either an acute [169] or chronic [170] treatment of estradiol benzoate were found to have decreased levels of ERα mRNA in the hypothalamus. Since the VCD-induced transitional menopausal model is relatively new, studies on the effects of estrogens on ERs are quite limited. Previous study by Pestana-Oliveira et al.[75] demonstrated that the dorsal raphe

nucleus (DRN) of transitional menopausal rats exhibited lower expression of ERβ mRNA. In addition, they also showed that chronic E2 treatment restored the ERβ mRNA levels and number of serotonergic cells in the DRN caudal subregion and increased ERβ expression in HPC.

Androgens could also affect ER expression in females. Specifically, ER expressions were found to be downregulated by circulating androgens such as T. Previous study has shown that T treatment attenuates ERα mRNA expression in the hypothalamus[171]. Another study demonstrated that T supplementation decreased ERα protein level in old OVX female mice and decreased ERβ protein level in adult OVX female mice [168] in the cerebral cortex.

One important but understudied area of research is that whether systemic circulating hormones or locally synthesized hormones in the brain play a more significant role affecting brain NT levels. One mechanism for controlling local estrogen synthesis is regulation of the aromatase enzyme, which is a member of the cytochrome P450 superfamily and is widely expressed in the brain. For example, E2 and estrone (E1, predominant circulating estrogen after menopause) can be synthesized from T and AD through aromatase while E2 and T can be synthesized from E1 and AD through 17β-Hydroxysteroid dehydrogenase (17β-HSD) in the brain. Therefore, the levels of steroid hormones in the brain are determined by both systemic and local production and are usually region-specific. Previously, our lab conducted a detailed analysis of brain region-specific E2 concentrations in OVX, E2-, and T-treated rats and demonstrate the degree to which these concentrations are aromatase–dependent [164]. Similar studies in the future using VCD model could provide additional important information regarding how the two models would differ in terms of local hormonal synthesis.

In summary, both estrogens and androgens could affect ER expressions and functions in the brain. Two potential explanations to the model differences in responsiveness to ER agonist treatments are: 1) it is possible that 30 days of VCD treatment impaired ERs responsivity to agonist treatments; and 2) the residual androgens in VCD model may diminish agonist effects due to downregulation of ER expressions in specific brain region. Due to the massive degree of complexity of CNS, further investigations that simultaneously evaluate the ER expressions and steroid hormone synthesis enzymes (e.g. aromatase and 17β-HSD) in the same set of brain tissues are warranted.

4.6.6 Cognitive significance of NT changes after ER agonist treatments

Many of the effects on NT endpoints could have important implications for effects on cognitive performance. The HPC is critical to memory consolidation, which is adversely affected by Alzheimer's disease as well as by normal aging. Studies show that gene expression in the HPC is significantly altered following OVX [172]. E2 as well as specific ER agonists have been shown to affect hippocampal structure and function in several important ways, including effects on spine density in CA1 [13], synaptic formation and plasticity [173], and activation of PI3K/AKT and ERK/MARK neuronal signaling pathways [174].

The FCX also plays an important role in learning and memory, in addition to effects of sensory stimuli on mood and emotion. Effects on mesocortical dopaminergic pathways have been associated with cognitive processes such as short-term memory, anxiety and impulsivity. Previous studies have shown that increasing DA levels in FCX reduces impulsivity in humans [175]. In our

current study, DA is the only monoamine affected in the FCX and upregulation of DA levels were reported following E2 and all of the ER agonists treatments. Although 5-HT levels remained unchanged, estrogens also have been shown to affect serotoninergic endpoints through other mechanisms. For example, in the FCX of OVX rats, estrogens can produce rapid receptor-mediated decreases in 5-HT_{1A} receptor function [176]. Chronic exposure to E2 also induces a significant increase in the density of 5-HT_{2A} receptors [177]. Further study is required to provide a better understanding of the potential effects of estrogen replacement on cognitive and emotional functions in menopausal women.

The STR is part of the extrapyramidal motor system. In addition to modulating motor control, it also plays a critical role in motor (i.e., procedural) learning and memory [178]. Studies in rats show that OVX rats have a predisposition for using a response (i.e., procedure) strategy when solving a spatial task whereas E2-treated rats preferentially use an allocentric strategy [179-181]. This is consistent with our data showing effects of E2 on 5-HT, DA and metabolites in the STR of OVX rats, and suggests that E2 treatment can significantly affect extrapyrmidal function. However, the finding that effects differed in VCD-treated rats and varied as a function of time and ER agonist indicate that the effects are complex and may not be readily observed in women who undergo transitional menopause. Further study is needed to better understand the potential effects of ER agonists on extrapyramidal function and motor learning in women.

In the current study the HPC was more responsive to ER agonist treatments than other regions. In addition, OVX rats were much more responsive to ER agonist treatments than VCD-treated rats. Similar effects in humans would be consistent with observations that estrogen treatments have less of a positive effect in older women that have undergone transitional menopause than in younger

women that have experienced oophorectomy [76]. This may help account for the fewer benefits of estrogen replacement that have been reported in postmenopausal women [182].

Age also is a factor, and previous research suggests a window of opportunity for realizing beneficial effects of estrogen on cognitive performance in women [160, 183]. Note that in such studies it is sometimes difficult to distinguish effects of age from effects of menopause. In female rodents aging results in reproductive senescence which is characterized by a loss of cyclicity and an extended period of estrus. The current study used young adult rats to avoid confounding factors associated with reproductive senescence. The fact that ER agonists had less effect in VCD-treated rats than in OVX rats suggests a fundamental difference between surgical vs. transitional menopause in the responsiveness to ER agonist treatment independent of age. This could contribute to the significant differences that have been reported in the effects of estrogen treatments on cognitive performance in older women that have experienced transitional menopause vs. younger women that experience rapid and sudden loss of ovarian function due to oophorectomy [143, 184].

Different estrogen receptors also may contribute to different effects on cognitive performance. Both ERα and ERβ have been implicated in mediating effects on different types of learning and memory [185], as well as on anxiety [186]. In our study, the ERβ agonist DPN simultaneously upregulated levels of all three major NTs (DA, 5-HT and NE) in HPC of OVX rats following 1W of treatment. Jacome et al. [73] previously reported that ERβ mediates effects of estrogens on recognition memory and that memory enhancements by DPN may occur through alterations in multiple monoaminergic systems primarily in HPC. Walf and Frye [35] showed that administration of an ERβ selective agonist to the HPC decreased anxiety and depressive behavior in OVX rats. If

true in humans, this would provide support for selectively targeting ERβ in estrogen therapy to achieve specific beneficial effects while avoiding unfavorable side effects of ERα activation.

4.7 CONCLUSIONS

Collectively, our study is the first to directly and systematically compare the effects of ER agonists on NT pathways in the brain in these two models of menopause. The results of our analysis described significant changes in local levels of serotoninergic, dopaminergic, and noradrenergic endpoints in association with validated models of surgical and transitional menopause following E2 and selective agonist treatments. Notably, effects were brain-region specific and agonist-dependent. The alternations in neurotransmitter levels following estrogen deprivation are likely relevant to changes in neural function and cognitive performance, which are partially reversed by either estrogen or selective agonists treatments. Furthermore, the transitional menopausal animals seem to be much less sensitive to ER agonist treatments than the OVX model with minimal effects on NT endpoints observed in striatum. This could contribute to the significant differences that have been reported in the effects of estrogen treatments on cognitive performance in older women that have experienced transitional menopause vs. younger women that experience rapid and sudden loss of ovarian function due to oophorectomy. Future studies that focus specifically on the anabolic and catabolic enzymes involved in monoamine regulation, regional expression and activity of monoamine transporters, ER receptors as well as relevant behavioral tests will further add to our understanding of the way that hormonal replacement and selective estrogen receptor activation regulates monoamine levels in different parts of brain during surgical and transitional menopauses.

5.0 CHAPTER 5: ESTRADIOL AND SELECTIVE ESTROGEN RECEPTOR AGONISTS DIFFERENTIALLY AFFECT BRAIN NONESSENTIAL AMINO ACIDS LEVELS IN TRANSITIONAL AND SURGICAL MENOPAUSAL RAT MODELS

5.1 ABSTRACT

Estrogens have many beneficial effects on the brain that extend beyond their role in the control of the reproductive function. Previously, we conducted a comprehensive analysis of the effects of estrogens and selective ER agonists on brain monoamines and metabolites between surgical and transitional menopause. Although amino acids TYR and TRP were included in previous investigation as neurotransmitter precursors, the information of ER agonist effects on brain amino acid levels following loss of ovarian function is still scanty. Using similar experimental design, the objective of current study was to further evaluate the effects of ER agonist treatments on additional amino acid endpoints in the brain. Endpoints included ALA, GLY, SER, ASP, GLU and GLN. Our results showed that most of the amino acids in the brain remained surprisingly stable and were not significantly affected by E2 and selective agonist treatments. However, limited but significant changes were detected in local levels of amino acids e.g. ASP and GLU following agonist treatments. These effects were region- and model- specific as well as time-and agonist-

dependent. The reductions in excitatory amino acid levels following either E2 or selective agonist treatments are of great interest and may contribute in part to the balance of brain excitability and amino acid homeostasis.

Keywords: Estrogen; Selective Estrogen Receptor Agonists; Menopause; VCD; Amino Acid

5.2 INTRODUCTION

Amino acids are critically important for proper neurodevelopment. In addition to their basic role as protein building blocks, they also function as the precursors for a variety of neurotransmitters, nucleotides, sphingolipids, polyamines, or as the donors for nitric oxide. Some of them can also work as potent antioxidants, regulators of endocrine function, and signaling modulators [187, 188]. In addition, as amino acids (e.g. glutamate, aspartate, proline, GABA, glycine, serine,β-alanine, and taurine etc.) comprise the most abundant neurotransmitters in the CNS, a large body of work has been conducted to investigate the important roles that amino acids play in neurotransmission, especially for the amino acids that are excitatory (ASP and GLU) and inhibitory (gamma-aminobutyric acid (GABA) and glycine) [189][190].

GLU, for example, is the most abundant amino acid and also one of the major EAAs in the CNS. It is stored in synaptic vesicles and is released from presynaptic terminals by depolarization in a Ca2+ dependent manner. Once released into the synaptic cleft, GLU binds to specific postsynaptic neuronal receptors (e.g. N-methyl-n-aspartate (NMDA) receptor) and excite the postsynaptic neuron. NMDA activation requires the binding of two molecules of GLU or ASP as well as GLY as co-agonist [191]. Previous studies have shown that alterations in the concentration of GLY can dramatically alter NMDA-receptor-mediated responses [192-194]. After the receptor is activated, it directly leads to the opening of a group of ion channels that are typified by their different permeabilities to Na+, K+, and Ca2+. The receptor activation results in rapid glutamate-mediated excitatory synaptic transmission in the CNS and are critically involved in long-term potentiation (LTP) and synaptic plasticity underlying learning and memory storage. [195, 196].

A growing body of studies have suggested estrogens can affect EAA-induced neuronal activity, possibly due to activation of ERs [197, 198]. In Chapter 4, we compared the effects of E2 and selective ER agonist treatments on monoaminergic endpoints in different regions of the adult rat brain, using two models of menopause. Results showed significant effects on serotoninergic, dopaminergic, and noradrenergic endpoints that were both model and brain region specific, and that varied by time following loss of ovarian function. Although we also included evaluation of TYR and TRP levels in our previous study, only two amino acids (aromatic amino acids) were investigated in this respect. Despite that comprehensive metabolomics study on effects of estrogen treatments on neurochemical endpoints, there is still a paucity of information regarding whether significant changes in other amino acid levels in the brain would be induced by similar estrogen treatment regimen. Therefore, in the current paper we used similar methods to investigate the effects of treating rats with selective ER agonists after undergoing either ovariectomy or VCD-induced follicular atresia on brain amino acid levels, especially those which play important roles in neurotransmission. Like the previous study, we still chose to focus on three brain regions, hippocampus, frontal cortex and striatum, which are thought to be critically involved in the regulation of cognitive functions, motor functions and excitability. Results show that most of the amino acids in the brain remained stable and were not significantly altered following E2 and selective agonist treatments. A few but significant changes were detected in local levels of amino acids following agonist treatments. Notably, these effects were region- and model- specific as well as time-and agonist-dependent. This is the first study to directly and systematically compare the effects of ER agonists on multiple amino acid endpoints in the brain in these two clinically relevant models of menopause.

5.3 MATERIALS AND METHODS

The materials and methods have been described in detail in section 3.3 and section 4.3.

5.4 RESULTS

5.4.1 Effects of ER agonists on amino acid levels in OVX-and VCD-treated rats

Detailed analysis revealed significant effects of E2 as well as ER subtype-selective agonists on amino acid endpoints associated, in both VCD- and OVX-treated rats (refer to Figure 26, Table 16-21). Effects were also model- and region-specific as well as time-dependent. Results show that most of the effects of agonist treatments resulted in reductions in levels of amino acids. However, most of the amino acids seem to be quite stable and remained unchanged following E2 and selective agonist treatments. The most significant change in amino acid levels were seen between two time-points, as demonstrated in the main effect of time point analysis (shown in Figure 27-28). For clarity, results are organized by brain region.

HPC

3-way ANOVAs detected significant overall effects on each of the amino acids: ALA ($F[19,69]=4.36$, $p<0.0001$); GLY ($F[19,69]=3.84$, $p<0.0001$); SER ($F[19,69]=3.26$, $p<0.0001$);

ASP ($F[19,69]=2.69$, $p<0.01$); GLU ($F[19,69]=7.04$, $p<0.0001$) and GLN ($F[19,69]=2.54$, $p<0.01$). Also, a main effect of Time was detected for each of the amino acids: ALA ($F[1,69]=71.71$, $p<0.0001$); GLY ($F[1,69]=60.66$, $p<0.0001$); SER ($F[1,69]=47.38$, $p<0.0001$); ASP ($F[1,69]=37.86$, $p<0.0001$); GLU ($F[1,69]=122.65$, $p<0.0001$) as well as GLN ($F[1,69]=30.05$, $p<0.0001$). Specifically, the levels of each amino acid were significantly lower at 1 week than at 6 weeks when collapsed across Model and Agonist Treatment. No main effect of Model or Agonist Treatment was detected. No significant interaction effects of Model x Time Point, Agonist Treatment x Time Point, Model x Agonist Treatment or Model x Time Point x Agonist Treatment were detected in the HPC.

1-way ANOVAs on levels of amino acids in the HPC of OVX and VCD-treated rats revealed no significant effects of treatments when compared at the 1-week time point. Also, no significant effects of treatments were seen in OVX rats at 6 weeks. After 6 weeks of treatment in VCD-treated rats, a strong trend of reduction was observed for levels of GLY and SER in PPT-treated rats while trend of reduction for level of ASP was also detected in E2-treated rats. Furthermore, significant reduction in ASP level was detected following 6-week PPT treatment using Dunnett's test (Figure 26 A).

FCX

3-way ANOVAs detected significant overall effects on ALA ($F[19,73]=2.6$, $p<0.01$), SER ($F[19,73]=3.1$, $p<0.001$) , ASP ($F[19,73]=2.2$, $p<0.01$) and GLU ($F[19,73]=5.5$, $p<0.0001$). Main effects of Model were detected for ALA ($F[1,73]=10.6$, $p<0.01$), GLY ($F[1,73]=9.7$, $p<0.01$), SER ($F[1,73]=8.1$, $p<0.01$) and ASP ($F[1,73]=6.3$, $p<0.05$). Specifically, these amino acid levels were higher in OVX than in VCD-treated rats when collapsed across Agonist and Time. Also, a

main effect of Time was detected for these amino acids except GLY: ALA ($F[1,73]=11.9$, $p<0.001$); SER ($F[1,73]=20.3$, $p<0.0001$); ASP ($F[1,73]=14.4$, $p<0.001$); GLU ($F[1,73]=78.6$, $p<0.0001$) as well as GLN ($F[1,73]=14.8$, $p<0.001$). Specifically, ALA, ASP, GLU and GLN were significantly lower at 1 week than at 6 weeks when collapsed across Model and Agonist Treatment. On the contrary, SER was significantly higher at 1 week than at 6 weeks. Main effect of Agonist Treatment was not detected in FCX.

A few interactions were also detected in FCX. For example, interactions of Model x Time Point were detected on ALA ($F[1,73]=7.1$, $p<0.01$), ASP($F[1,73]=5.7$, $p<0.05$) and GLU ($F[1,73]=8.0$, $p<0.01$). Interactions of Time Point x Agonist Treatment were detected on ALA ($F[4,73]=2.9$, $p<0.05$) and GLY ($F[4,73]=2.7$, $p<0.05$). Interaction of Model x Agonist Treatment was detected on SER ($F[4,73]=3.6$, $p<0.01$). In addition, interaction of Model x Time Point x Agonist Treatment was detected on SER ($F[4,73]=2.9$, $p<0.05$).

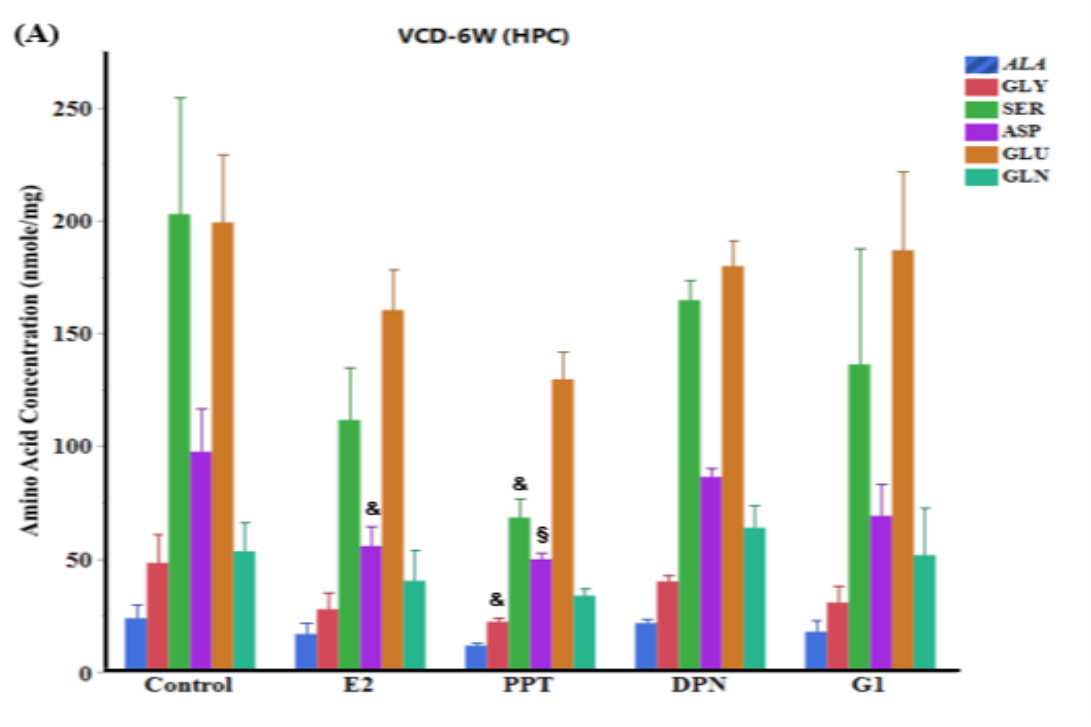
1-way ANOVAs on levels of amino acids in the FCX of OVX and VCD-treated rats revealed no significant effects of treatments when compared at 6-week time point. In addition, no significant effects of treatments were seen in VCD-treated rats at 1 week. Furthermore, 1-way ANOVAs on amino acids in the FCX of OVX rats produced significant overall effects only on SER at 1 week. Post-hoc analyses showed that levels of SER were significantly lower in G-1-treated rats than in controls by Dunnett's test. Post-hoc Tukey's test also suggested that SER level in G-1 treated rats were significantly lower than E2-treated rats as well as than controls (Figure 26 B).

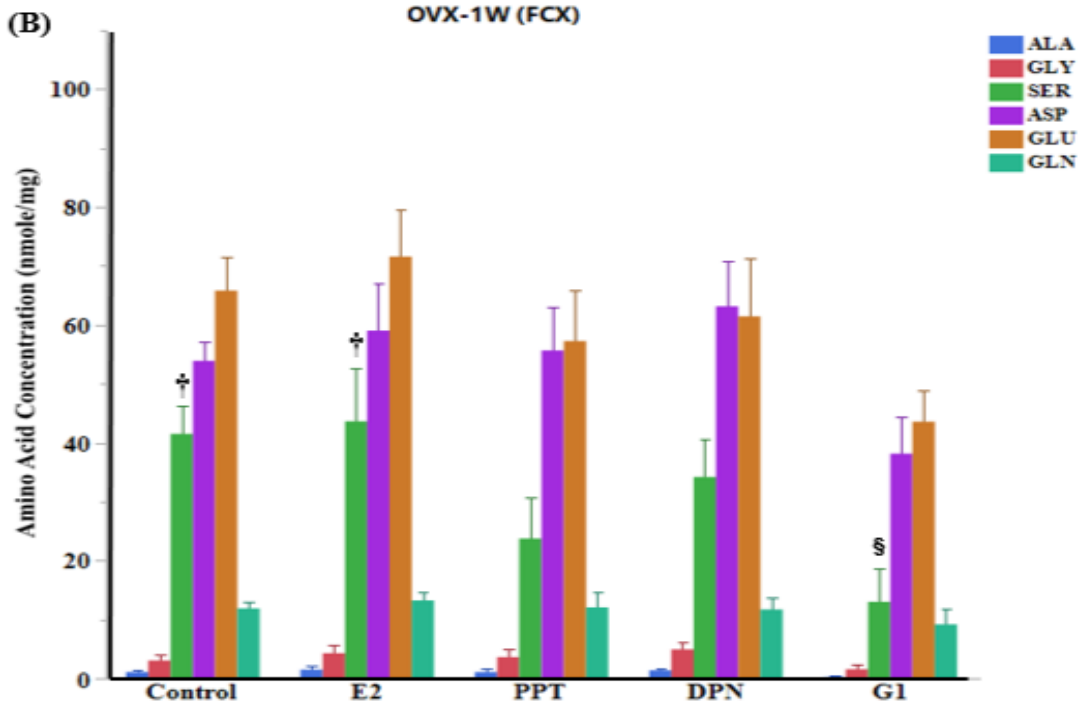
STR

3-way ANOVAs detected significant overall effects on ALA ($F[19,73]=3.7$, $p<0.0001$), GLU ($F[19,73]=36.5$, $p<0.0001$) and GLN ($F[19,73]=2.5$, $p<0.01$). Main effects of Time Point were

detected for ALA ($F[1,73]=26.3$, $p<0.0001$), GLY ($F[1,73]=11.2$, $p<0.01$) and GLU ($F[1,73]=650.3$, $p<0.0001$). Specifically, ALA and GLY were significantly lower at 6 weeks than at 1 week while the levels of GLU were significantly higher at 6 weeks than at a week when collapsed across Model and Agonist Treatment. Main effect of Agonist Treatment was found in ALA ($F[4,73]=3.4$, $p<0.05$). Main effect of Model was not detected in STR. Additionally, only two interactions were detected in STR: one was the interaction of Model x Treatment Agonist for GLU ($F[4,73]=3.02$, $p<0.05$) and the other was a full 3-way interaction for ALA ($F[4,73]=4.1$, $p<0.01$).

1-way ANOVAs followed by post-hoc test in the STR of OVX and VCD-treated rats revealed no significant change in amino acid levels following 6 weeks of agonist treatments. However, significant or strong trend of changes in several amino acids were detected in both of the models when compared at the 1-week time point. Specifically, in VCD model, significant reductions in levels of GLU were observed in PPT, DPN, G-1-treated rats as shown by post-hoc Dunnett's or Tukey's test (Figure 26 C). Also, a strong trend of reductions in levels of GLU and ASP compared with controls were observed after 1 week of E2 and G-1 treatments, respectively. In OVX model, post-hoc Tukey's test revealed that levels of ALA were significantly higher in PPT- than E2-treated rats. Dunnett's also showed higher levels of GLN were detected in PPT-treated rats in relative to controls. Most of the ALA and GLY levels were below the limit of detection (ND) in the STR at 6-week time point (Figure 26 D).


(A)
VCD-6W (HPC)
ALA
GLY
SER
ASP
GLU
GLN
Amino Acid Concentration (nmole/mg)
250
200
150
100
50
0
&
&
&
§
Control
E2
PPT
DPN
G1


(B)
OVX-1W (FCX)
ALA
GLY
SER
ASP
GLU
GLN
Amino Acid Concentration (nmole/mg)
100
80
60
40
20
0
ǂ
ǂ
§
Control
E2
PPT
DPN
G1

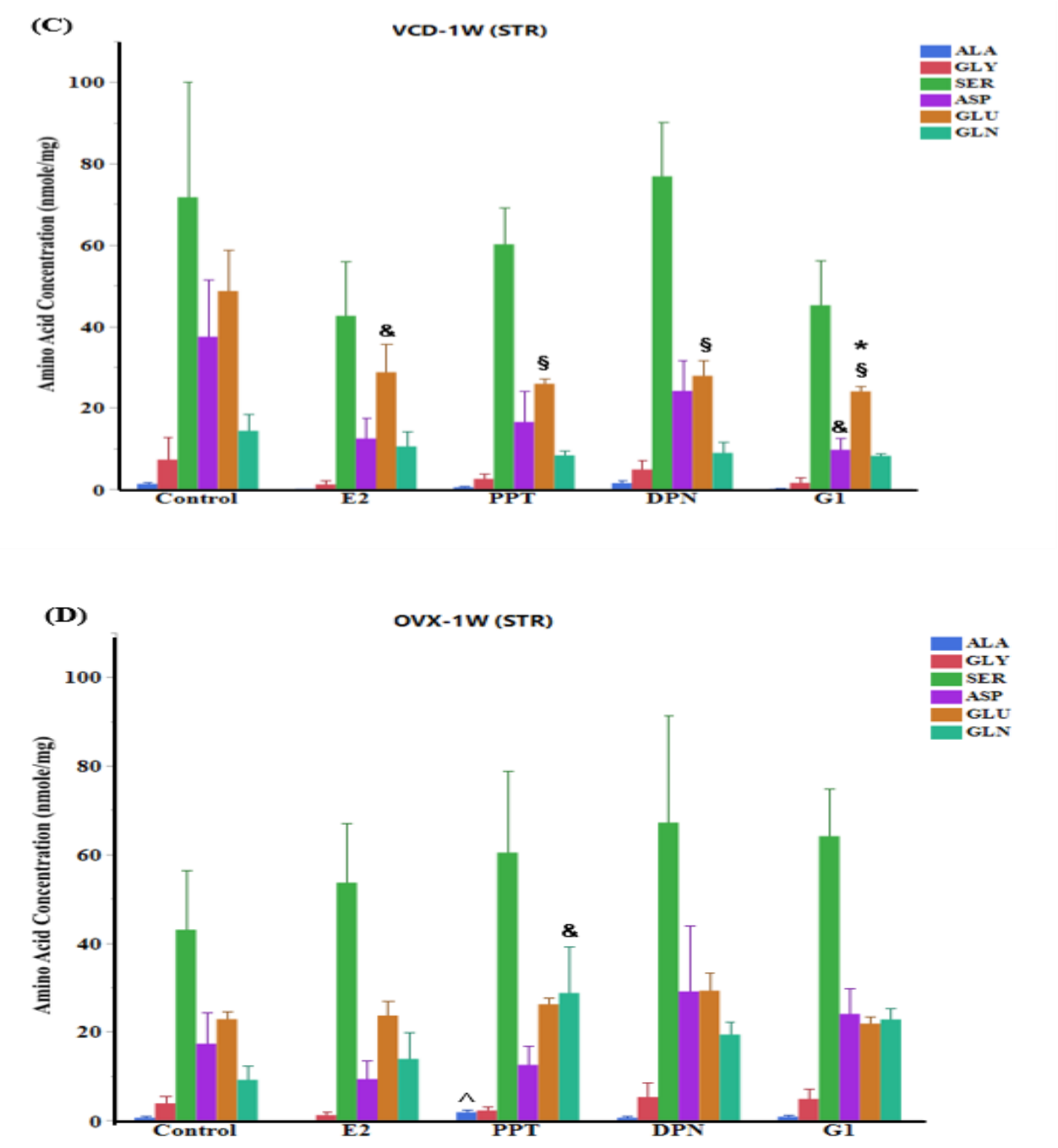


Figure 26. Nonessential amino acids levels in (A) VCD-6W (HPC), (B) OVX-1W (FCX) , (C) VCD-1W (STR) and (D) OVX-1W (STR) by treatment groups. Rats at vehicle treatment are used as controls. Bars indicate Mean±SEM. One-way ANOVA: *p<0.05 (post-hoc Tukey's test), relative to control; †p<0.05 (post-hoc Tukey's test), relative to G-1; ^p<0.05 (post-hoc Tukey's test), relative to E2. §p<0.05 (post-hoc Dunett's test) relative to control; & 0.05<p<0.01 (post-hoc Dunett's test) relative to control (trend).

Table 16. Effects of ER agonists on nonessential amino acid endpoints in HPC of OVX rats.

			OVX-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	4	4	4	4
ALA	F(4,16)=0.58	p>0.05	6.44±0.25	4.93±0.8	5.38±1.69	5.73±0.63	4.74±0.86
GLY	F(4,16)=1.02	p>0.05	16.63±1.13	12.23±1.18	13.67±3.49	13.07±1.44	12.16±1.64
SER	F(4,16)=0.39	p>0.05	44.97±2.15	34.9±4.48	42.22±13.51	36.82±5.59	44.01±7.12
ASP	F(4,16)=0.57	p>0.05	44.72±3.24	34.92±3.42	41.57±8.56	41.72±6.19	36.76±4.55
GLU	F(4,16)=0.13	p>0.05	47.32±3.28	43.54±3.74	50.19±10.46	46.71±5.97	45.17±3.89
GLN	F(4,16)=1.24	p>0.05	27.16±2.97	17.26±0.8	26.16±6.8	27.15±3.87	20.8±3.51

			OVX-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	4	5	5	5
ALA	F(4,19)=0.30	p>0.05	15.76±3.22	19.25±7.8	23.12±4.47	21.66±3.68	20.25±6.51
GLY	F(4,19)=0.14	p>0.05	30.65±4.87	36.41±15.66	38.69±6.49	38.97±5.51	36.66±11.64
SER	F(4,19)=0.42	p>0.05	98.46±23.19	123.1±68.91	171.75±40.11	161.05±37.31	143.61±57.09
ASP	F(4,19)=0.07	p>0.05	79.58±16.05	75.18±25.06	85.35±15.14	82.1±11.21	74±23.16
GLU	F(4,19)=0.15	p>0.05	187.19±41.94	162.79±53.04	206.4±34.8	190.62±31.59	182.02±38.91
GLN	F(4,19)=0.63	p>0.05	53.68±16.53	52.82±13.2	65.45±13.9	63.69±7.01	40.87±10.66

Table 17. Effects of ER agonists on nonessential amino acid endpoints in FCX of OVX rats.

			OVX-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			5	4	4	4	4
ALA	F(4,16)=1.29	p>0.05	1.12±0.25	1.49±0.64	1.11±0.5	1.39±0.36	0.29±0.13
GLY	F(4,16)=1.30	p>0.05	3.02±0.99	4.29±1.4	3.64±1.3	4.95±1.1	1.53±0.74
SER	F(4,16)=3.81	p<0.05	41.53±4.72†	43.66±8.92†	23.72±7.02	34.22±6.34	13.01±5.72§
ASP	F(4,16)=2.05	p>0.05	53.89±3.33	59.03±8.09	55.69±7.39	63.15±7.72	38.17±6.17
GLU	F(4,16)=1.92	p>0.05	65.85±5.7	71.58±8.01	57.3±8.6	61.46±9.83	43.6±5.21
GLN	F(4,16)=0.58	p>0.05	11.89±1.02	13.26±1.49	12.04±2.49	11.69±2.03	9.17±2.52

			OVX-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			5	4	5	5	5
ALA	F(4,19)=1.89	p>0.05	2.07±0.45	2.57±0.52	1.68±0.38	1.94±0.57	3.71±0.88
GLY	F(4,19)=1.18	p>0.05	4.6±0.7	3.9±1.15	3.16±0.74	2.37±1.12	5.94±2.12
SER	F(4,19)=0.70	p>0.05	23.5±3.33	22.07±6.51	16.98±2.19	14.37±4.62	22.61±6.69
ASP	F(4,19)=0.88	p>0.05	82.27±5.21	85.98±5.1	70.46±4.83	78.69±5.63	76.68±8.54
GLU	F(4,19)=0.75	p>0.05	107.89±4.01	117.63±9.74	103.44±4.64	106.88±6.22	115.94±9.27
GLN	F(4,19)=0.45	p>0.05	19.3±4.52	17.54±4.21	27.72±10.68	18.82±3.8	17.36±5.75

			Values are mean ± sem.			
Symbol			Alpha	Test		
§			p<0.05	Dunnetts	Differs from Controls	
†			p<0.05	Tukey	Differs from G-1	
Unit: nmole/mg						

Table 18. Effects of ER agonists on nonessential amino acid endpoints in STR of OVX rats.

			OVX-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			5	4	4	4	4
ALA	F(4,16)=2.61	p=0.07	0.55±0.41	0.05±0.05	1.8±0.61^	0.58±0.34	0.79±0.28
GLY	F(4,16)=0.71	p>0.05	3.78±1.65	1.15±0.84	2.24±0.81	5.23±3.29	4.79±2.37
SER	F(4,16)=0.37	p>0.05	42.99±13.38	53.59±13.52	60.37±18.56	67.17±24.26	64.1±10.72
ASP	F(4,16)=0.95	p>0.05	17.24±7.15	9.27±4.17	12.48±4.23	29.07±14.83	24.01±5.8
GLU	F(4,16)=1.37	p>0.05	22.82±1.63	23.66±3.26	26.16±1.48	29.22±3.97	21.83±1.54
GLN	F(4,16)=1.93	p>0.05	9.14±3.04	13.82±6.05	28.7±10.48&	19.36±2.74	22.78±2.47

			OVX-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			5	4	5	5	5
ALA	NA	NA	0.04±0.04	ND	ND	ND	ND
GLY	F(4,19)=0.22	p>0.05	1.09±1.09	0.8±0.61	0.89±0.89	0.98±0.98	1.95±1.2
SER	F(4,19)=2.19	p>0.05	51.77±9.98	71.8±15.67	59.3±11.54	69.63±11.75	30.57±7.6
ASP	F(4,19)=0.16	p>0.05	15.64±6.56	15.83±3.77	14.35±3.01	16.7±2.76	12.08±5.37
GLU	F(4,19)=0.90	p>0.05	86.23±4.98	90.93±4.16	93.71±8.9	99.93±4.93	87.89±3.85
GLN	F(4,19)=1.88	p>0.05	14.53±2.15	10.93±2.29	14.13±1.67	18.21±1.65	12.97±1.8

			Values are mean ± sem.			
Symbol			Alpha	Test		
&			0.05<p<0.1	Dunnetts	Differs from Controls (trend)	
^			p<0.05	Tukey	Differs from E2	
			0.05<p<0.1	Tukey	Not Shown	
Unit: nmole/mg						

Table 19. Effects of ER agonists on nonessential amino acid endpoints in HPC of VCD-treated rats.

			VCD-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			3	5	5	4	5
ALA	F(4,17)=0.63	p>0.05	4.86±1.01	5.13±1.31	3.55±0.74	5.67±1.05	5.05±0.83
GLY	F(4,17)=0.41	p>0.05	11.84±1.98	13.1±1.9	9.98±1.02	11.1±4.03	13.18±1.84
SER	F(4,17)=0.18	p>0.05	39.88±6.62	43.02±9.8	37.56±3.59	47.01±14.9	38.97±6.33
ASP	F(4,17)=0.22	p>0.05	44.57±7.33	44.79±5.86	41.95±2.21	47.05±6.64	41.14±4.25
GLU	F(4,17)=1.5	p>0.05	42.51±6.23	55.71±5.33	62.88±7.12	73.57±13.99	53.69±7.99
GLN	F(4,17)=0.54	p>0.05	26.12±4.96	25.15±5.3	19.28±2.31	26.83±5.28	22.35±3.22

			VCD-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
HPC/N			5	4	4	4	4
ALA	F(4,17)=0.58	p>0.05	23.8±6	16.79±4.93	11.59±1.44	21.65±1.87	17.88±5.27
GLY	F(4,17)=0.59	p>0.05	48.3±12.47	27.74±7.29	22.24±2.05[&]	40.1±2.65	30.75±7.54
SER	F(4,17)=0.60	p>0.05	202.77±51.95	111.57±23.08	68.43±8.1[&]	164.63±9.03	136.21±51.56
ASP	F(4,17)=2.74	p=0.06	97.54±19.4	55.83±8.92[&]	49.9±3.16[§]	86.42±4.12	69.15±14.09
GLU	F(4,17)=0.62	p>0.05	199.14±30.31	160.23±17.74	129.64±12.45	179.86±11	186.8±34.74
GLN	F(4,17)=0.63	p>0.05	53.36±13.14	40.32±13.34	33.79±3	63.86±9.94	51.68±21.24

			Values are mean ± sem.			
Symbol			Alpha	Test		
§			p<0.05	Dunnetts	Differs from Controls	
&			0.05<p<0.1	Dunnetts	Differs from Controls (trend)	
			0.05<p<0.1	Tukey	Not Shown	
Unit: nmole/mg						

Table 20. Effects of ER agonists on nonessential amino acid endpoints in FCX of VCD-treated rats.

			VCD-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			4	5	5	5	5
ALA	F(4,19)=0.54	p>0.05	0.61±0.23	1.4±0.65	1.14±0.52	0.84±0.24	0.77±0.17
GLY	F(4,19)=0.63	p>0.05	1.61±0.94	3.04±1.43	3.8±1.66	1.8±0.73	1.98±0.69
SER	F(4,19)=1.06	p>0.05	10.59±5.1	24.94±10.64	32.09±8.15	24.35±5.34	29.57±6.46
ASP	F(4,19)=1.15	p>0.05	38.69±12.18	58.95±11.98	64.42±9.53	57.26±4.44	48.29±5.76
GLU	F(4,19)=1.33	p>0.05	55.57±4.43	70.76±10.83	74.69±6.49	63±6.66	57.81±3.36
GLN	F(4,19)=0.83	p>0.05	10.82±2.24	15.74±4.3	9.73±1.19	10.48±2.94	11.29±0.82

			VCD-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
FCX/N			5	5	4	5	5
ALA	F(4,19)=0.88	p>0.05	1.6±0.64	0.97±0.36	0.49±0.16	1.11±0.5	1.44±0.32
GLY	F(4,19)=1.01	p>0.05	2.85±1.23	0.83±0.37	0.74±0.39	2.15±1.44	2.56±0.67
SER	F(4,19)=1.63	p>0.05	10.65±3.25	6.23±1.83	12.28±4.18	19.82±6.24	9.79±3.21
ASP	F(4,19)=0.79	p>0.05	65.14±6.26	52.72±6.15	49.29±2.88	75.25±21.36	53.62±11.17
GLU	F(4,19)=0.77	p>0.05	83.64±3.09	81.72±9.44	82.33±6.36	110.85±21.46	93.68±18.47
GLN	F(4,19)=0.90	p>0.05	15.76±3.4	14.04±2.3	17.44±2.83	24.76±6.9	19.19±4.44

Table 21. Effects of ER agonists on nonessential amino acid endpoints in STR of VCD-treated rats.

			VCD-1W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			4	5	5	5	5
ALA	F(4,19)=3.09	p<0.05	1.28±0.46	0.06±0.04	0.41±0.28	1.42±0.64	0.08±0.08
GLY	F(4,19)=0.95	p>0.05	7.26±5.6	1.17±1.01	2.53±1.37	4.8±2.34	1.5±1.31
SER	F(4,19)=1.04	p>0.05	71.7±28.49	42.55±13.34	60.18±8.97	76.84±13.32	45.19±10.94
ASP	F(4,19)=1.96	p>0.05	37.46±13.92	12.45±4.95	16.49±7.54	24.11±7.62	9.65±2.85[&]
GLU	F(4,19)=3.12	p<0.05	48.63±10.22	28.7±6.96[&]	25.86±1.25[§]	27.81±3.77[§]	23.98±1.29*[§]
GLN	F(4,19)=0.86	p>0.05	14.34±4.02	10.5±3.65	8.27±1.23	8.88±2.6	8.16±0.58

			VCD-6W				
	One-WAY ANOVA		C	E2	PPT	DPN	G-1
	F test	P value					
STR/N			5	5	4	5	5
ALA	F(4,19)=1.23	p>0.05	0.06±0.06	ND	0.53±0.53	ND	ND
GLY	F(4,19)=0.70	p>0.05	ND	1.28±1.28	0.69±0.66	1.11±0.74	ND
SER	F(4,19)=0.27	p>0.05	58.16±3.78	58.8±12.8	61.83±11.61	73.11±17	56.69±15.37
ASP	F(4,19)=0.23	p>0.05	14.94±3.94	14.96±6.29	16.82±5.23	21.44±5.6	21.63±11.34
GLU	F(4,19)=2.03	p>0.05	93.88±4.4	75.61±2.94	95.32±13.64	88.68±4.33	101.23±7.63
GLN	F(4,19)=1.42	p>0.05	19.57±3.43	11.3±1.27	18.75±2.23	19.39±3.22	16.48±3.61

			Values are mean ± sem.			
Symbol			Alpha	Test		
§			p<0.05	Dunnetts	Differs from Controls	
&			0.05<p<0.1	Dunnetts	Differs from Controls (trend)	
*			p<0.05	Tukey	Differs from Controls	
			0.05<p<0.1	Tukey	Not Shown	
Unit: nmole/mg						

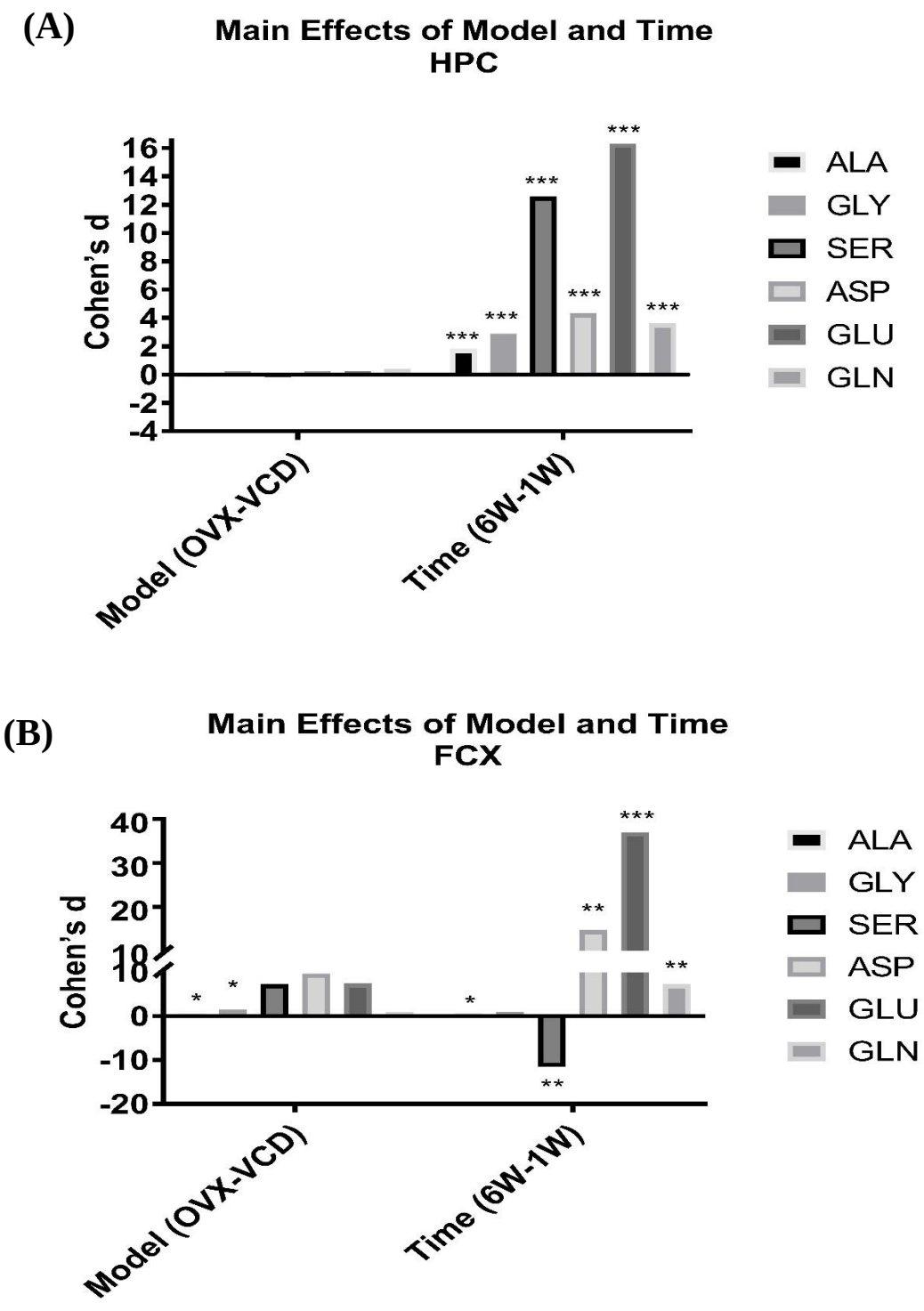


Figure 27. Main effects of Model and Time on nonessential amino acids in (A) HPC and (B) FCX. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

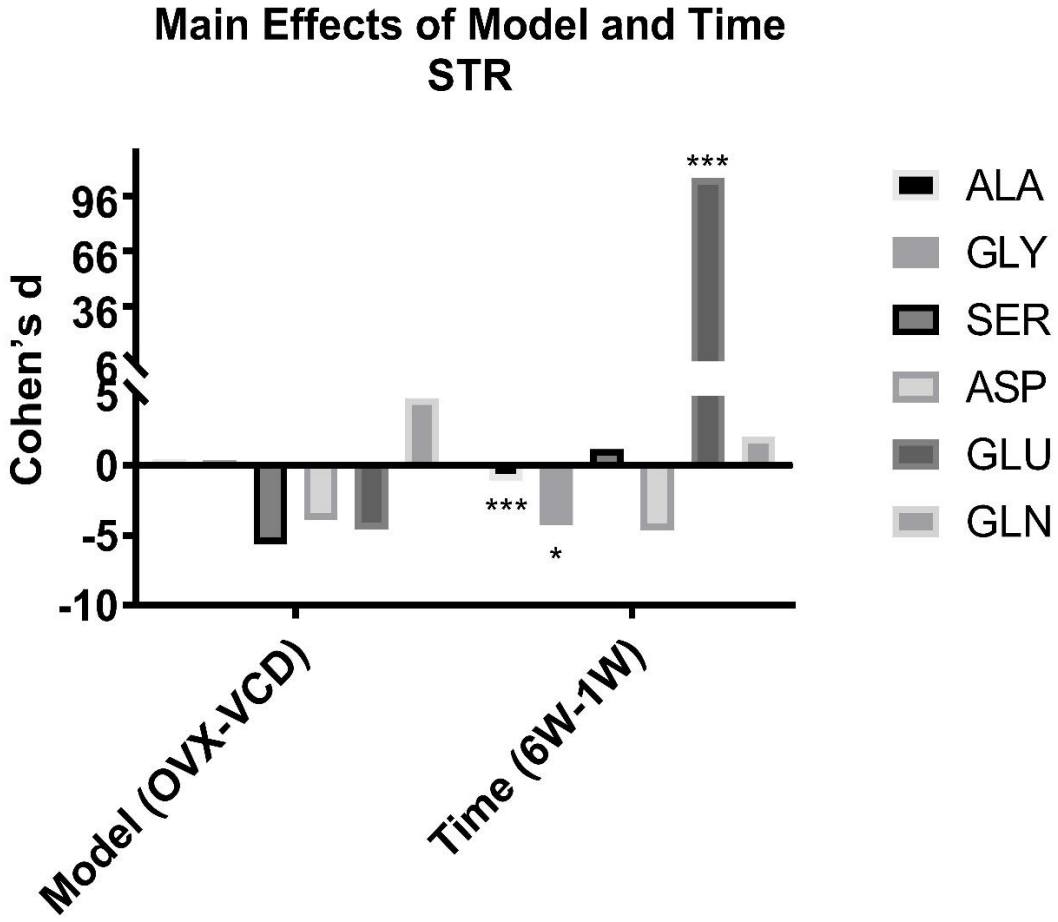


Figure 28. Main effects of Model and Time on nonessential amino acids in STR. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

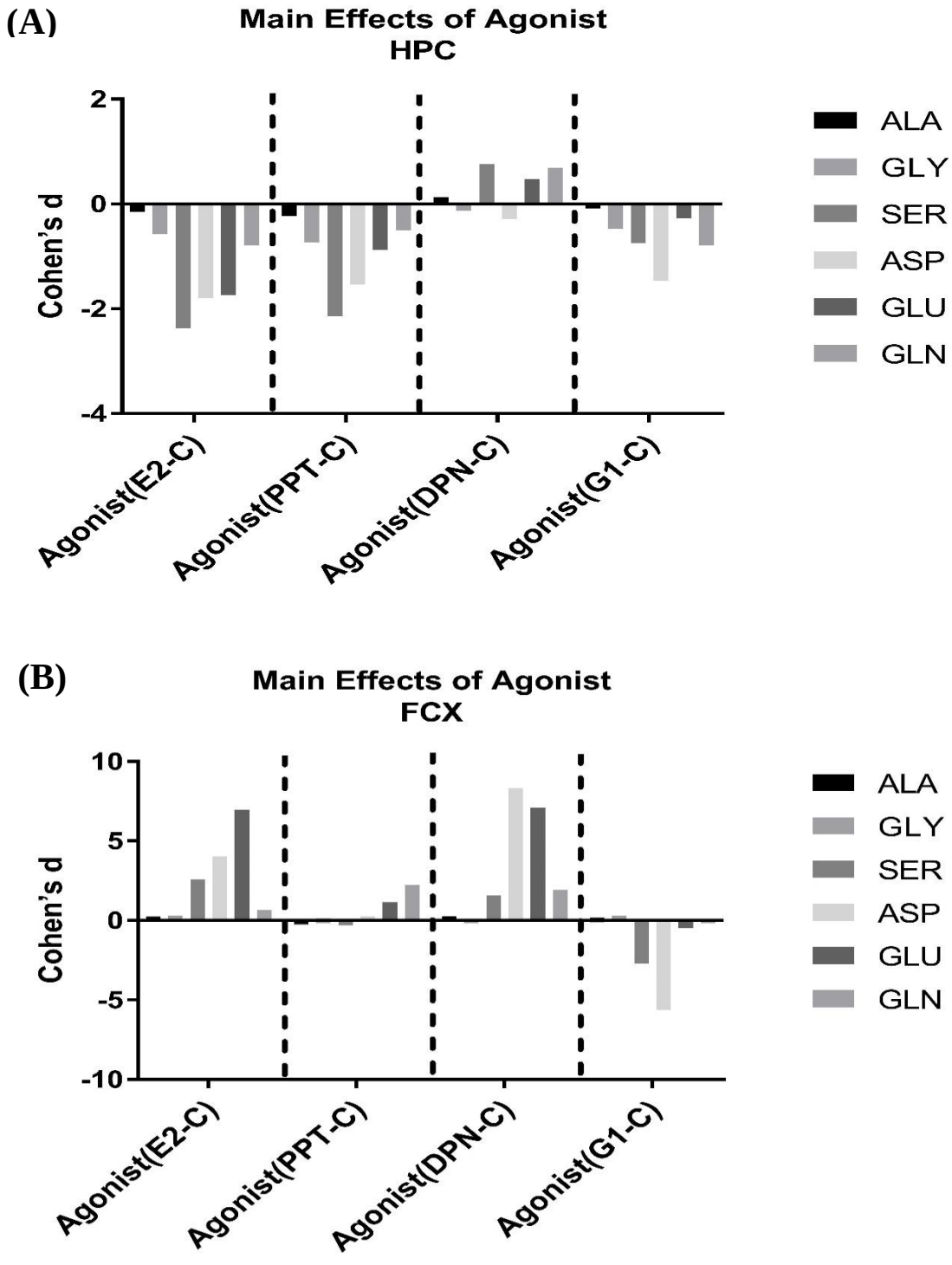


Figure 29. Main effects of Agonist on nonessential amino acids in (A) HPC and (B) FCX. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

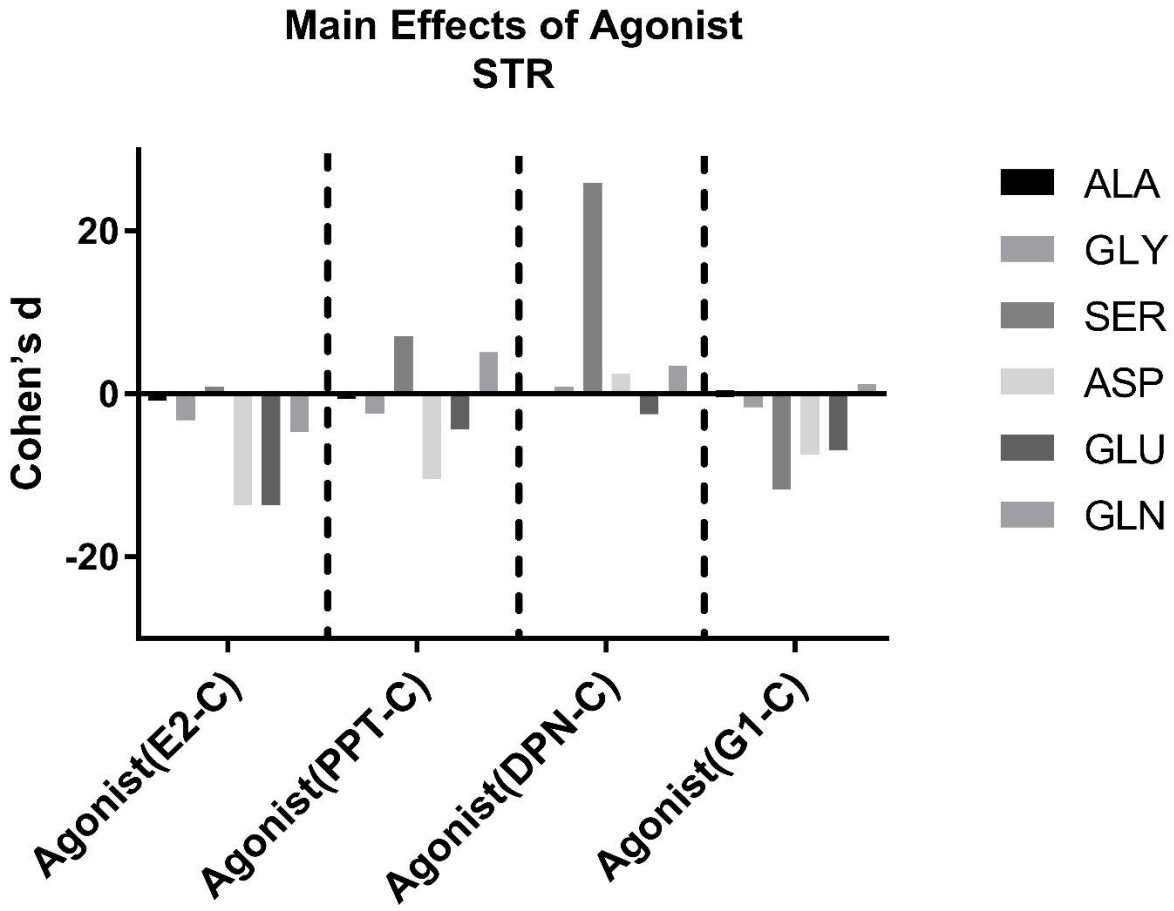


Figure 30. Main effects of Agonist on nonessential amino acids in STR. Bars indicate Cohen's d value. p value adjusted: *p<0.05, **p<0.01, ***p<0.001.

5.5 DISCUSSIONS

5.5.1 Main effects of Model, Time-Point and Agonist Treatment

The results of this analysis demonstrate significant effects of Model, Time-Point and Agonist Treatment on multiple amino acid endpoints. These effects were region specific. For example, main effects of Model were detected only in the FCX, but not in the HPC and STR. In the FCX, levels of ALA, GLY, SER and ASP were all higher in OVX vs VCD-treated rats. This is consistent with our previous analysis with TRP and TYR, where higher levels were detected in OVX vs. VCD-treated rats in FCX. Noted these effects, while statistically significant, were relatively small.

Main effects of Time-Point (i.e., collapsed across model and agonist treatment) were the most robust main effects for all regions examined. In many cases endpoints were greater after 6W vs. 1W of agonist treatments. Effects on several endpoints were consistent across two or three brain regions. For example, levels of ASP and GLN were higher in HPC and FCX at 6W vs. 1W. Levels of GLU were higher in HPC and STR at 6W vs 1W. This is in agreement with the prior reports for main effects of Time Point for TYR and TRP in Chapter 4, where higher amino acids levels were observed at 6W vs. 1W. This suggests significant increases in certain amino acids including major excitatory amino acids GLU and ASP over a period of weeks following OVX or VCD treatment. Effects on ALA were not entirely consistent across brain regions. Specifically, levels of ALA were higher in HPC and FCX while were lower in STR at 6W vs. 1W. On the other hand, effects on two endpoints differed between brain regions. Levels of GLY were higher in HPC but were lower in

STR at 6W vs. 1W. Similarly, levels of SER were higher in the HPC, but were lower in the FCX at 6W vs.1W. These findings indicate time- and region-specific effects on amino acid endpoints.

In addition to the main effects of Model and Time-Point, the data also showed significant main effects of Agonist Treatment but only on ALA and only in the STR. This may be due to relatively limited effects of Agonist Treatment on these endpoints especially analyzed with 3-way ANOVA with Model and Time Point collapsed. It suggests that the levels of these amino acids may not be as sensitive as the levels of monoamine/metabolites measured in Chapter 4 in response to estrogen and selective agonist treatments. Nevertheless, specific effects of agonist treatments were detected in all of the three brain regions. These effects were statistically significant and are discussed in further detail below

5.5.2 Specific effects of Agonist Treatment as a function of Model and Time-Point

This study is the first to analyze levels of excitatory amino acids GLU and ASP, inhibitory amino acid GLY as well as other amino acids following ER agonist treatments. Results indicated that, although not many, the effects of agonist treatments were model- and time-point-dependent. Two general observations were made from the results. One is that the majority of ER agonist treatments resulted in reductions in amino acid levels relative to controls, which included reductions in ASP in HPC and GLU in STR. This may indicate ER agonist treatments can affect neurotransmission or brain excitability through down-regulation of excitatory amino acid levels. Notably, it is consistent with the findings in Chapter 4 that ER agonist treatments significantly reduced levels of two aromatic amino acids TYR and TRP in both HPC and FCX. The other observation is that most of the effects were detected after 1W vs. 6W of agonist treatments. This

is also in agreement with our previous findings for monoamines, metabolites, TYR and TRP. This suggests that similar to NT endpoints reported in Chapter 4, the amino acid endpoints are also responsive to ER agonist treatments at an early stage following loss of ovarian function.

In our current study, VCD-treated rats treated with PPT for 6W had significantly lower ASP levels in the HPC than controls. Similarly, VCD-treated rats receiving E2 treatment for 6W also showed a strong trend for reductions in ASP levels relative to controls. This suggests that in the HPC the reduction of ASP can be achieved by activation of ERα. In FCX, OVX rats treated with G-1 for 1W had a significantly lower SER level relative to controls, suggesting that this effect may be mediated through activation of GPR30. Multiple effects of agonist treatments were detected in STR. Treatment with E2, PPT, DPN and G-1 for 1W all significantly reduced GLU levels compare with controls in VCD-treated rats, suggesting these effects can be achieved by activation of any of the three ER subtypes. Previous studies have reported that in regions with high GABA levels, such as the hypothalamus and substantia nigra, a significant percentage of GLU is used as a precursor for GABA synthesis [199]. In addition, studies also have reported that a large number of neurons and terminals in STR contain GABA and GABAergic inhibition has been thought to play a major role in regulating neuronal activity in the STR [200]. Thus, it is possible that a reduction in GLU levels in STR reflect increased synthesis of GABA, via decarboxylation in response to ER activation. Future investigation into changes in GABA levels and glutamic acid decarboxylase expression in STR following ER agonist treatments is warranted. A strong trend of ASP reduction also was detected following 1W of G-1 treatment. These findings further support that activation of ERs may decrease levels of excitatory amino acids in specific brain region. Collectively, these data indicated that estrogens can influence amino acid endpoints after loss of ovarian function.

Similar to monoamine and metabolites reported in previous study, amino acids are differentially affected by activation of specific estrogen receptors and the effects are brain region-dependent.

Another interesting finding was that the treatment effects seen in one model were not observed in the other model at same time point and at the same brain region. This clearly suggests a fundamental difference in the responsiveness to estrogen therapy between transitional vs surgical menopause. One of the possible explanations may be the different hormonal profiles inherent in the two models.

5.5.3 Potential mechanisms of estrogen induced neuroprotection

Although estrogen has well-described neuroprotective effects, the exact mechanism of estrogen induced neuroprotection has yet to be fully determined. One theory is that estrogens may mediate their neuroprotection via excitatory neurotransmitter systems. As major EAAs, GLU and ASP cause depolarization and excitation of neurons in the mammalian CNS [201]. Since excessive concentrations of GLU and ASP have been found to exert neurotoxic properties, the significant reductions in such amino acids following E2 and agonist treatments in discrete brain regions further supports this theory. Additionally, previous literatures have demonstrated multiple effects of estrogen on EAA-related neurotransmission. The effects appear to be region-specific and were not always consistent. For example, estrogens have been shown to attenuate glutamatergic receptor activation [202] . It has also been shown that the size of a glutamate-induced lesion was significantly reduced in rats given pretreatment with E2 prior to local glutamate application [203]. In contrast, potentiating actions of E2 on glutamate-induced excitation has also been previously

reported [204]. Also, it has been demonstrated that E2 plus progesterone causes a significant enhancement of veratridine-induced release of glutamate from preoptic-area synaptosomes of OVX rats [205]. In a recent study, it was shown that in the arcuate nucleus, E2 significantly increased protein levels of the two enzymes in the glutamate-glutamine cycle, glutamine synthetase and glutaminase, which underly functional changes in neurotransmitter availability [206].

In summary, this additional analysis provides a detailed description of changes in amino acid endpoints including excitatory and inhibitory amino acids in two models of menopause after E2 and selective ER agonist treatments. To the best of our knowledge, no similar study has been conducted before. However, cautions should be exercised when interpreting current data as the steady state amino acid concentrations observed in the present experiments may not correctly reflect the dynamic changes (e.g. release, turnover) for these endpoints. For example, no change observed among the endpoints could either mean no effects of ER agonist treatment or that a new dynamic balance of synthesis and catabolism is reached. Future studies on synthesizing and degrading enzymes of these amino acids and the corresponding behavioral testing are warranted to discern further mechanistic insights.

5.6 CONCLUSIONS

Collectively, our study is the first to provide a detailed and in-depth comparison of the effects of ER agonists on the levels of six nonessential amino acids in three brain regions, at two-time points following loss of ovarian function. The results of our analysis showed that most of the amino acids in the brain remained relatively stable and were not significantly altered following E2 and selective agonist treatments in models of surgical and transitional menopause. Despite that, we also demonstrated limited but significant changes in local levels of amino acids (including excitatory and inhibitory amino acids) following agonist treatments. Notably, these effects were region- and model- specific as well as time-and agonist-dependent. The reductions in excitatory amino acid levels following either estrogen or selective agonist treatments are of great interest and may contribute in part to the balance of brain excitability and amino acid homeostasis.

6.0 SUMMARY AND PERSPECTIVES

6.1 SUMMARY OF KEY RESEARCH FINDINGS

Estrogens have many beneficial effects in the brain. One of the mechanisms for estrogens to deliver the effects is through the regulation of multiple neurotransmitter levels involved in a variety of cognitive functions, in specific regions of the brain. Previously, different laboratories demonstrated changes in individual NT pathways under limited conditions. However, a comprehensive analysis of simultaneous changes both within and across pathways has not yet been available. Therefore, it is not clear what role the individual estrogen receptors play, or what effects selective estrogen receptor modulators have on these NT pathways. Given the fact that these complex NT pathways are constantly interacting with each other, results from analysis of individual NT pathways would be difficult and insufficient to explain the effects of estrogens. Additionally, recent studies suggest that the effects may differ depending on how and when loss of ovarian function occurs (e.g., surgical vs. natural menopause).

To address these issues, in this thesis, we have for the first time comprehensively and systematically conducted a metabolomics analysis of monoamines, precursors, metabolites and amino acids in three brain regions (hippocampus, frontal cortex and striatum) at two time points

(1 week vs. 6 weeks), in two clinically relevant models of menopause (surgical vs. transitional) with/without estrogen receptor agonist treatments.

In Chapters 2 and 3, we utilized both high-pressure liquid chromatography-coulometric multi-electrode array system (HPLC-CMEAS) and gas chromatography coupled to flame ionization detector (GC-FID) to investigate and compare the effects of two models of menopause on NT pathways of interest. Results show that (a) significant effects are detected in the HPC and FCX associated with cycle stage and are limited primarily to the serotonin pathway; (b) multiple effects were detected following OVX and VCD treatments and most effects were both region-specific and time-dependent. Relatively few differences were present in the effects of the two models on neurochemical endpoints, despite the fact that the hormonal profiles were quite different. This suggests that the two types of menopause produce similar effects on these endpoints, at least during the early weeks following menopause. This, in turn, suggests that any differences in cognitive performance between OVX and VCD-treated rats are not due to differential effects on monoaminergic pathways and amino acid signalings; (c) the greatest number of effects were detected in the HPC, suggesting that this region of the brain is particularly sensitive to loss of ovarian function; (d) significant reduction in the levels of amino acids detected in the HPC, but not in the FCX or STR, at 1 week following OVX or VCD treatments, suggesting loss of ovarian function has substantial acute effects on amino acid homeostasis in this region of the brain.

In Chapters 4 and 5, using similar analytical methods, we further evaluated the ability to modify the deficits on the NT pathways induced by OVX and VCD treatment by treating with different estrogen receptor agonists. Results show that (a) significant region-specific effects of "Model" and "Time-Point" were detected on multiple measures of serotoninergic, dopaminergic, noradrenergic and amino acid endpoints; (b) main effects of 'Time-Point' were much more robust

than the main effects of 'Model' for all regions examined. In many cases endpoints were higher at 6W vs. 1W after loss of estrogens. For example, effects on several serotoninergic endpoints were consistently higher across brain regions at 6W vs. 1W, suggesting significant recovery of endpoints associated with serotonin production and signaling over a period of weeks following OVX or VCD treatment; (c) significant main effects of "Agonist Treatment" were detected on multiple NT endpoints that differed by brain region; (d) specific effects of "Agonist Treatments" are model- and time-point-dependent. For monoamines, the majority of ER agonist effects were detected in OVX rats as opposed to VCD-treated rats and were detected after 1W vs. 6W of agonist treatments. In addition, the majority of ER agonist effects were detected in the HPC, suggesting that this region of the brain is particularly sensitive to ER agonist treatments. For amino acids, the majority of ER agonist treatments resulted in reduction of AA levels in relative to controls. In addition, most of the effects were detected after 1W vs. 6W of agonist treatments; (e) many of the effects on NT endpoints could have important implications for effects on cognitive performance.

Collectively, this dissertation has provided novel and rich information :1) describing and comparing neurochemical changes associated with two different and clinically relevant models of menopause in three regions of the brain; 2) describing and comparing neurochemical changes produced by selective ER agonists in each of the menopausal models; 3) first comprehensively evaluating the effects of a selective GPR30 agonist (G-1) on brain neurochemistry. These data will be extremely useful for understanding the effects of menopause and hormone treatment on cognitive processes, and for developing and designing better hormone replacement strategies for use in perimenopausal and postmenopausal women.

6.2 LIMITATIONS AND FUTURE DIRECTIONS

The studies presented in this thesis are metabolomics studies. The overall goal was to characterize neurochemical changes associated with surgical vs. VCD-induced transitional menopause as well as treatments of 17β-estradiol and selective estrogen receptor agonists in the rat brain. Like most '-omics' studies, the focus was descriptive, not mechanistic. Therefore, the possible mechanisms involved in the effects observed on neurotransmitter levels were not investigated in depth in current studies. However, based on our understanding of the topic and previous published literatures, serious efforts have been made to discuss the potential mechanisms as well as their related cognitive significance in specific sections of the chapters, thus providing more mechanistic underpinning of the results reported in our study. The interpretation of our findings needs to be carried out with caution, since the levels of these neurochemical endpoints do not necessarily represent a dynamic measure of neuronal activities. Utilization of techniques such as microdialysis in future studies may add valuable data and information to current understanding of this field. Also, since the behavioral studies are not included in the current studies, whether and how would such NT levels alternations translate to cognitive function remains unknown.

The neurochemical changes reported in the studies may be due to multiple factors e.g. regional differences in sensitivity to estrogen deprivation as well as estrogen-regulated alterations of expression and activities of anabolic and catabolic enzymes, specific estrogen receptors, autoreceptors, neurotransmitter reuptake transporters, etc. Furthermore, the mechanisms contributing to the differences in responsiveness of ER agonist treatments between surgical and transitional menopauses, between different brain regions have yet to be fully characterized. These

research areas would be valuable focuses for future mechanistic studies, building on the descriptive information reported here.

More studies are needed to further understand the underlying mechanisms. Future research plans are to expand the studies to evaluate (1) mechanisms that underlie the different effects of surgical vs. 'natural' menopause on the neurotransmitter signatures, such as expression and activities of anabolic and catabolic enzymes (e.g. monoamine oxidase, catechol-O-methyltransferase, dopamine beta-hydroxylase, aromatic L-amino acid decarboxylase, tyrosine hydroxylase, tryptophan hydroxylase ,etc.) ; (2) how the effects of surgical vs. natural menopause differentially affect cognitive performance by conducting a battery of behavioral tests (e.g. fear conditioning test, water maze test, object recognition task etc.) and (3) to determine if the effects differ as a function of age as well as the timing of hormone treatment relative to the loss of ovarian function.

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