LOW DOSE CHRONIC ESTRADIOL-17β EXPOSURE INDUCES OVARIAN PATHOLOGY AND INHIBITS TUBEROINFUNDIBULAR DOPAMINERGIC NEURONAL FUNCTION BY INDUCING A PROINFLAMMATORY STATE WITHIN THE ARCUATE NUCLEUS

By

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ABSTRACT

LOW DOSE CHRONIC ESTRADIOL-17β EXPOSURE INDUCES OVARIAN PATHOLOGY AND INHIBITS TUBEROINFUNDIBULAR DOPAMINERGIC NEURONAL FUNCTION BY INDUCING A PROINFLAMMATORY STATE WITHIN THE ARCUATE NUCLEUS

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Estrogen exposure is known to inhibit tuberoinfundibular dopaminergic (TIDA) neuronal function and cause hyperprolactinemia which can lead to the development of mammary and pituitary tumors. Dopamine produced by TIDA neurons acts on lactotrophs in the anterior pituitary gland to provide tonic inhibition of prolactin release. Therefore, if TIDA neuronal function is inhibited by estrogen, there is a decrease in dopamine (DA) release into the median eminence (ME), loss of inhibition to prolactin, and a resulting state of hyperprolactinemia. The mechanism by which estrogen exerts this effect has not been elucidated. We hypothesized that chronic exposure to a low dose of estradiol-17β (E2) would cause an increase in IL-1β and nitrate levels in the arcuate nucleus (AN), where TIDA neuronal cell bodies are located, and cause direct damage to TIDA function through nitration of tyrosine hydroxylase (TH), which is the rate-limiting enzyme in DA synthesis. To test this hypothesis, we exposed intact and ovariectomized Sprague-Dawley rats to E2 by subcutaneously implanting them with slow-release E2 pellets (20ng/day) for 90 days. After exposure, animals were sacrificed and serum was analyzed for prolactin, the ME for dopamine and nitrated tyrosine hydroxylase (nTH), and the AN was assessed for IL-1β and nitrate levels. Our results demonstrate that E2 exposure increased serum prolactin, decreased DA levels in the ME, increased IL-1β and nitrate levels in the AN and increased the ratio of nTH to TH in the ME. These findings provide strong evidence that chronic

exposure to a low level of E2 induces a proinflammatory state within the AN and this may be a mechanism by which E2 inhibits TIDA activity to cause hyperprolactinemia.

Additionally, estrogen is known to induce ovarian follicular cysts. Exposure to estrogen has been implicated as a model for polycystic ovary syndrome (PCOS) in women. However, most studies claiming this association, have primarily focused on the ovarian morphologic phenotype and have not thoroughly assessed other clinical parameters observed with PCOS. We explored whether our paradigm of E2 exposure could cause changes in ovarian morphology and hormonal profiles similar to that of PCOS. Adult female rats were implanted with slow-release E2 pellets for 30, 60 (, or 90 days. At the end of treatment, testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured, ovaries were analyzed histomorphologically and immunohistochemistry for CD68 (a macrophage marker) and Müllerian inhibiting substance (MIS) was assessed.. We found that follicular size increased with E2 exposure and number of corpora lutea (CL) decreased in an exposure-dependent manner indicating failure of ovulation. There was an E2 associated increase in follicular degeneration and CD68 positive cells, and a decrease in MIS. Testosterone levels decreased in a duration dependent manner and the ratio of LH to FSH remained unaffected. While ovarian changes observed in this model are similar to that seen in PCOS, the hormonal profiles arenot, and we therefore determined that this may not be a true model for this condition.

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I thank God for His grace and for blessing me through the kind actions of wonderful people.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS OR ABBREVIATIONS

AN	Arcuate nucleus
ANOVA	Analysis of variance
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL2	Monocyte chemoattractant protein
cDNA	Complementary deoxyribonucleic acid
Cl^-	Chloride
CL	Corpora luteum (lutea)
CNS	Central nervous system
CP	Caudate putamen
DA	Dopamine
DHBA	3,4-Dihydroxybenzylamine
E2	Estradiol-17β
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosrbant assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
FSH	Follicle stimulating hormone

GC	Granulosa cell
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
$HClO_4^-$	Perchloric acid
HPG	Hypothalamic pituitary gonadal
HPLC-EC	High performance liquid chromatography-electrochemical detection
IL-1	Interleukin 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
Jak-Stat	Janus kinase signal transducer and activator of transcription
k/o	Knock out
K^+	Potassium
L-DOPA	Levo-dihydroxyphenylalanine
LH	Luteinizing hormone
LPS	Lipopolyssacharide
Lta	Lymphotoxin A
MAPK	Mitogen activated protein kinase
ME	Median eminence
Micro BSA	Micro Bicinchoninic acid assay
mRNA	Messenger ribonucleic acid
NA	Nucleus accumbens

Na^+	Sodium
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NO	Nitric oxide
nT	Nitrotyrosine
nTH	Nitrated tyrosine hydroxylase
O_2^-	Superoxide
OCE	Old constant estrus
$ONOO^-$	Peroxynitrite
Ovx	Ovariectomized
P_4	Progesterone
PBS	Phosphate buffer saline
PCOS	Polycystic ovary syndrome
PMSF	Phenylmethanesulfonyl fluoride
PRL-R	Prolactin receptor
Ptgs2	Cyclooxygenase 2
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT-PCR	Reverse transcription polymerase chain reaction
SN	Substantia nigra
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopaminergic
TNF-α	Tumor necrosis factor alpha
VTA	Ventral tegmental area
WB	Western blot
WT	Wild type

CHAPTER 1

INTRODUCTION

Statement of Purpose

Humans are exposed to estrogens and estrogenic compounds on a daily basis. For example, estrogenic exposures occur by way of endogenous estrogens, as well as exogenous estrogens such as xenoestrogens (i.e., bisphenol A in plastic), phytoestrogens (i.e., genistein in soy), and pharmaceutical estrogens (i.e., ethinyl estradiol in oral contraceptives) [1, 2]. Additionally, women are exposed to high levels of endogenous estrogen during the follicular phase of the menstrual cycle [3], and during the 5-6 year perimenopausal period, a woman is exposed to high and erratic levels of estradiol [3, 4]. It is known that estrogen exposure causes a reduction in dopamine (DA) production by the hypothalamic tuberoinfundibular dopaminergic (TIDA) neurons which are the key inhibitory regulator of prolactin secretion by the pituitary gland. Loss of this dopaminergic inhibition subsequently leads to hyperprolactinemia [5-9]. This is significant because hyperprolactinemia can be associated with deleterious effects such as breast cancer and pituitary tumors [5, 7, 10].

Many of the experiments evaluating the effects of estrogen on the body and on the tuberoinfundibular dopaminergic pathway have studied the effects of high levels of estrogen and/or for short periods of time [8, 9, 11]. In the current experiment, however, we have evaluated the effects of a physiological dose of estrogen for a prolonged period of time (90 days *in vivo*). This experimental paradigm potentially allows us to make reliable comparisons to the effects of chronic estrogen exposure as most often occurs in humans. Furthermore, we have exposed the

animals to the most abundant and most potent biologically produced estrogen in the body—estradiol-17β (E2).

The exact mechanism behind the aforementioned estrogenic effect on the TIDA system is not known. It has been demonstrated that an increase in proinflammatory cytokines in the arcuate nucleus (AN) results in a reduction in TH activity in the ME, followed by an increase in serum prolactin levels [12]. Furthermore, nitric oxide (NO) has been shown to be produced alone or in conjunction with cytokines in neural tissues, and has been implicated to play a role in pathological processes in the brain [13]. We have proposed a series of experiments which will test our hypotheses that low dose, chronic estrogen exposure inhibits TIDA neuronal function by facilitating the production of inflammatory cytokines and NO in the AN. This local inflammatory state will cause nitration of tyrosine hydroxylase (TH)—the rate limiting enzyme in the synthesis of DA—in the TIDA neuronal terminals. Nitration of the tyrosine residues will render the TH enzyme dysfunctional, thereby resulting in decreased DA levels, with subsequent elevations in the concentration of serum prolactin. This will help to provide a mechanistic explanation behind the effect of estrogen on the TIDA system and on prolactin secretion. Based on these findings treatment strategies to ameliorate this unfavorable effect of estrogen can be developed and employed.

We also have expounded upon the effects of low dose, chronic E2 on the ovaries. It is known that exposure to estrogen causes cystic ovarian morphology [14], and it has been implicated as a model for polycystic ovary syndrome (PCOS) in women [15, 16]. However, the potential for estrogen exposure serving as a model for this disease syndrome has not been clearly defined. We sought to determine the histomorphologic effects as well as examine the effects of E2 on the

hormones that are known to be affected in women with PCOS, and to determine if E2 exposure is a true model of this disease.

Dopamine

Dopamine is a catecholaminergic monoamine neurotransmitter that serves various neurologic functions and is synthesized by neurons in various areas of the brain. A catecholamine is a neurologic chemical that is composed of a catechol (1,2-dihydroxyphenyl) moiety carrying an alkyl side chain with an amine group on the side chain [17]. A monoamine is a compound with 1 amine group [18] which is derived from an aromatic amino acid [19]. Dopamine is synthesized from the aromatic amino acid, tyrosine which is naturally supplied in the diet. The rate limiting enzyme in the synthesis of dopamine is tyrosine hydroxylase (TH) and TH is responsible for the conversion of tyrosine to Levo-dihydroxyphenylalanine (L-DOPA). L-DOPA is then converted to dopamine by the enzyme DOPA decarboxylase which is also known as aromatic amino acid decarboxylase [20]. Though I will not be focusing on the neurotransmitters norepinephrine and epinephrine, for completeness-sake it is worth noting that dopamine is a precursor for these neurotransmitters as dopamine can be converted to norepinephrine by the enzyme dopamine-β-hydroxylase, and norepinephrine can in turn be converted to epinephrine by the enzyme phenylethanolamine-*N*-methyl transferase [21] **(Fig. 1)**. By definition, a neurotransmitter is a chemical that modifies or results in the transmission of nerve impulses between synapses [17]. A hormone is a substance produced by one tissue and conveyed by the bloodstream to another to effect physiological activity [18]. Though most dopamine functions as a neurotransmitter in terms of its release and destination, the axon terminals of the TIDA neurons release dopamine into the hypophyseal portal circulation [22]. Therefore, the dopamine produced by TIDA neurons is referred to as a neurohormone by some.

Figure 1

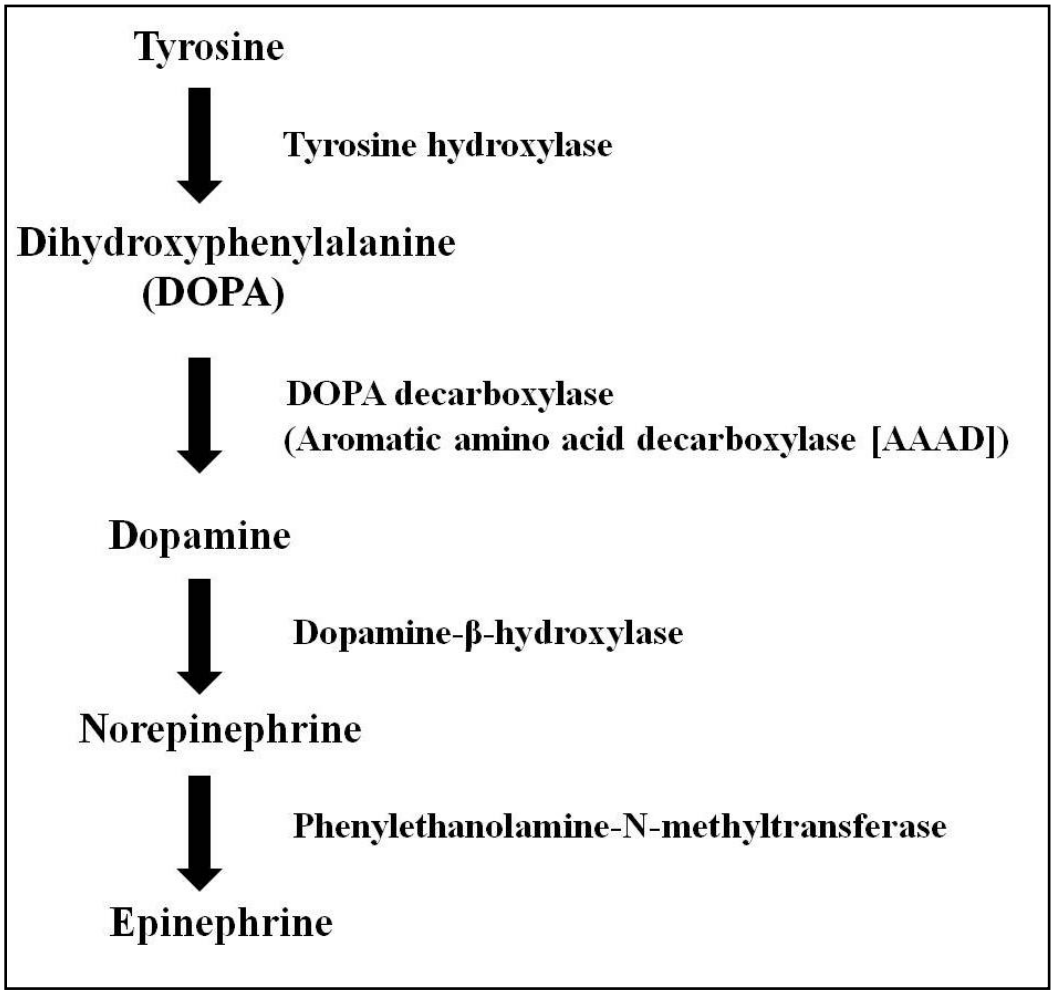


Figure 1. Enzymatic cascade depicting catecholamine synthesis.

Dopaminergic systems in the brain

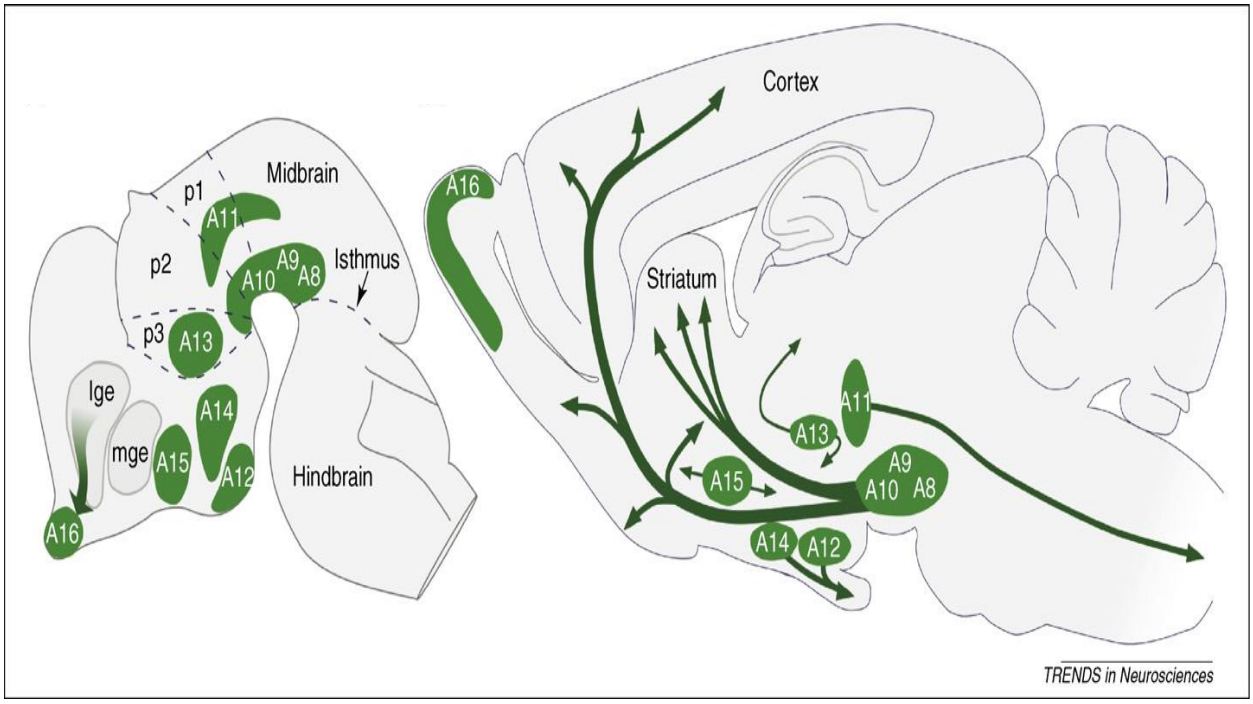
There are 9 dopamine-containing neuronal cell groups in the mammalian brain. They are identified as A8 – A16 cell groups **(Fig. 2)**. The dopaminergic neuronal groups of the mesencephalon include A8 – A10. The dopamine cell groups of the diencephalon are A11 – A15 [23]. The numerically last subset of dopaminergc neurons, A16, are located in the glomerular layer of the olfactory bulb [24]. There is also a subset of dopaminergic cells in the inner nuclear layer of the retina that are denominated as group A17 [25]. These dopaminergic cell groups are in multiple systems throughout the brain as follows:

The mesostriatal system consists of the retrorubral nucleus (A8), substantia nigra (SN) (A9) and ventral tegmental area (VTA) (A10). The mesolimbocortical system consists of the same dopaminergic cell groups as the mesostriatal system; however, the sites of their projections differ. In the mesostriatal system, the projections of the dopaminergic neurons are the caudate-putamen, ventral striatum (nucleus accumbens), olfactory tubercle, bed nucleus striae terminalis, globus pallidus, Island of Calleja, and subthalamic nucleus; whereas the projections of the dopaminergic neurons that constitute the mesolimbocortical pathway extend to the olfactory bulb, anterior olfactory nuclei, lateral septal nucleus, piriform cortex, amygdale, ventral entorhinal cortex, suprarhinal cortex, pregenual anteromedial cortex, supragenual anteromedial cortex, perirhinal cortex and temporal association cortex, lateral habenular nucleus, and locus coeruleus. The mesothalamic system consists of the VTA (A10) with projections to the thalamus. The diencephalospinal system consists of dopaminergic neurons in the dorsal and posterior hypothalamus, zona incerta, and caudal thalamus (A11) which project to the spinal cord. The incertohypothalamic system consists of the zona incerta and periventricular hypothalamic nuclei (A11, and A13 and A14, respectively) which project to the zona incerta, anterior medial preoptic

and periventricular hypothalamus, and the septum. The tuberoinfundibular system consists of neurons in the arcuate nucleus (A12) which extends to the median eminence. The tuberohypophyseal system consists of dopaminergic neurons in the periventricular hypothalamic nucleus (A14) and extends to the pars intermedia and nervosa of the pituitary. The periventricular system consists of the mesencejphalic periaqueductal gray and periventricular gray of caudal thalamus (A11) and extends to the periaqueductal gray and the medial thalamus and hypothalamus. The periglomerular system consists of the dopaminergic neurons in the olfactory bulb (A16) which project to dendritic processes into olfactory glomeruli. Finally, the retinal dopaminergic systm consists mainly of dopaminergic cells in the amacrine cells of the inner nuclear layer of the retina (A17) which project to local dendritic projections [26].

Of these dopaminergic cell groups, there are 4 major dopaminergic producing systems in the brain, which can consist of multiple dopaminergic groups. The 4 major dopaminergic pathways are: mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular [27]. I will briefly discuss the former three, and will more extensively discuss the tuberoinfundibular dopaminergic (TIDA) system, as this system is the primary focus of my thesis. It is worth noting that the mesocortical and mesolimbic systems are sometimes collectively referred to as the mesocorticolimbic dopaminergic system [26, 28-31] .

Figure 2



Bjorklund, A. and S.B. Dunnett, Dopamine neuron systems in the brain: an update.Trends Neurosci, 2007. 30(5): p. 194-202.

Figure 2. Dopamingergic neuronal systems within the rodent brain. For interepretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Mesocortical Dopaminergic System

The perikarya of the neurons involved in mesocortical dopaminergic pathway originate primarily from the VTA (A10), with minor projections arising from the SN (A9), and retrobulbar nucleus (A8). The axons from these neurons primarily extend to the prefrontal cortex [32, 33], which include the supragenual and pregenual anteromedial cortex [26], where dopamine is released and plays a role in regulation of memory, memory-guided behavior and reward [27, 33-35].

Mesolimbic Dopaminergic System

The neurons of the mesolimbic dopaminergic system, as with the mesocortical system, originate in the VTA as well as the SN, and retrobulbar nucleus [26]; however, for the mesolimbic pathway, the axon terminals extend primarily to the amygdala and hippocampus, which are components of the limbic system [27], and to the nucleus accumbens (NA) which is a component of the ventral striatum [36]. This dopaminergic system is involved in motivation, emotional response, and the sensations of reward and desire [27] and is activated during affective aggression, which is considered to be a reactive, hostile, emotional, or expressive behavior that is initiated in response to a perceived imminent threat [37]. However, the most prominent and well-studied effect of activation of the mesolimbic dopaminergic pathway is addiction and addiction-associated behaviors [28, 38, 39].

Nigrostriatal Dopaminergic System

The A9 DA cell groups in the zona compacta of the SN are the primary cells that give rise to the nigrostriatal pathway. Other minor components of this system include cells of the retrorubral nucleus (A8) and the VTA (A10). The main output of these neuronal extensions is to the caudate putamen (CP) [26], which makes up the dorsal striatum [40, 41]. This dopaminergic pathway is

primarily involved in motor control; loss of the dopaminergic neurons in the SN result in the progressive, neurodegenerative disease, Parkinson's disease [27].

Tuberoinfundibular Dopaminergic System

The TIDA system is composed of three distinct cell groups: A12 (Arcuate nucleus), A13 (Dorsolateral part of the nucleus dorsomedialis hypothalami pars medialis and A14 (Ventral part of the periventricular nucleus). Of these, the major dopaminergic contribution to the median eminence comes from the A12 cell group. The TIDA system is primarily involved in the control of prolactin release from the anterior pituitary gland. The perikarya of TIDA neurons are dopaminergic neurons that are localized in the dorsomedial aspect of the arcuate nucleus. The axon terminals of these neurons extend to the median eminence where dopamine is released into the hypophyseal portal circulation [23]. The hypophyseal portal circulation is the vascular system that resides in the infundibulum, or the pituitary stalk, and provides a circulatory pathway for the transport of regulatory neurohormones between the hypothalamus and the anterior pituitary gland (aka adenohypophysis) [17, 42, 43]. The secreted dopamine acts on prolactin-producing lactotrophs of the anterior pituitary gland, and is the key regulator of prolactin secretion, providing tonic inhibition of its release [6, 23].

TIDA system's regulation of prolactin

Dopamine released by TIDA neuron axon terminals exerts an inhibitory effect on prolactin secretion by lactotrophs by binding to dopamine 2 (D2) receptors on the membrane of lactotrophs. Activation of these receptors by dopamine results in a reduction of prolactin gene expression and prolactin exocytosis [44]. Dopamine receptors are G-protein coupled receptors. There are 2 classes of dopamine receptors: D1 and D2. The D1 class of receptors include receptors D1 and D5, and the D2 class of receptors include D2, D3, and D4. The D1 and D2

class of receptors molecularly and mechanistically function to stimulate and inhibit adenylyl cylcase, respectively. D2 receptors are also coupled to different types of G proteins which decrease cyclic adenosine monophosphate (cAMP) levels and alter the permeability of potassium channels, which is responsible for the reduction of prolactin exocytosis [45]. Unlike most hormones, prolactin does not have target-gland hormones which act to inhibit the production of prolactin via a classical negative feedback mechanism. Rather, prolactin regulates itself by means of a short feedback loop whereby increases in prolactin production stimulate increased TIDA activity through activation of prolactin receptors (PRL-R) that are present on TIDA neurons [44, 46]. There are short and long isoforms of PRL-R in the hypothalamus, and the long isoform is most prominent on the dopaminergic neurons in the AN and periventricular nucleus. Activation of the PRL-R on TIDA neurons employs the Jak-Stat (Janus kinase signal transducer and activator of transcription) second messenger pathway and results in nuclear transcription and an end-product of increased TH **(Fig. 3)**. Though the mechanism is not clear, there is also some evidence to suggest that activation of the TIDA system by prolactin is partly mediated through the tachykinin, substance P, as increased prolactin levels are associated with increases in hypothalamic substance P. In addition to regulation of TIDA activity by prolactin, there are other compounds known to mediate dopamine production by TIDA neurons. For example, estrogens and opioids have been shown to increase prolactin levels through decreased activity of TIDA neurons [44].

Figure 3

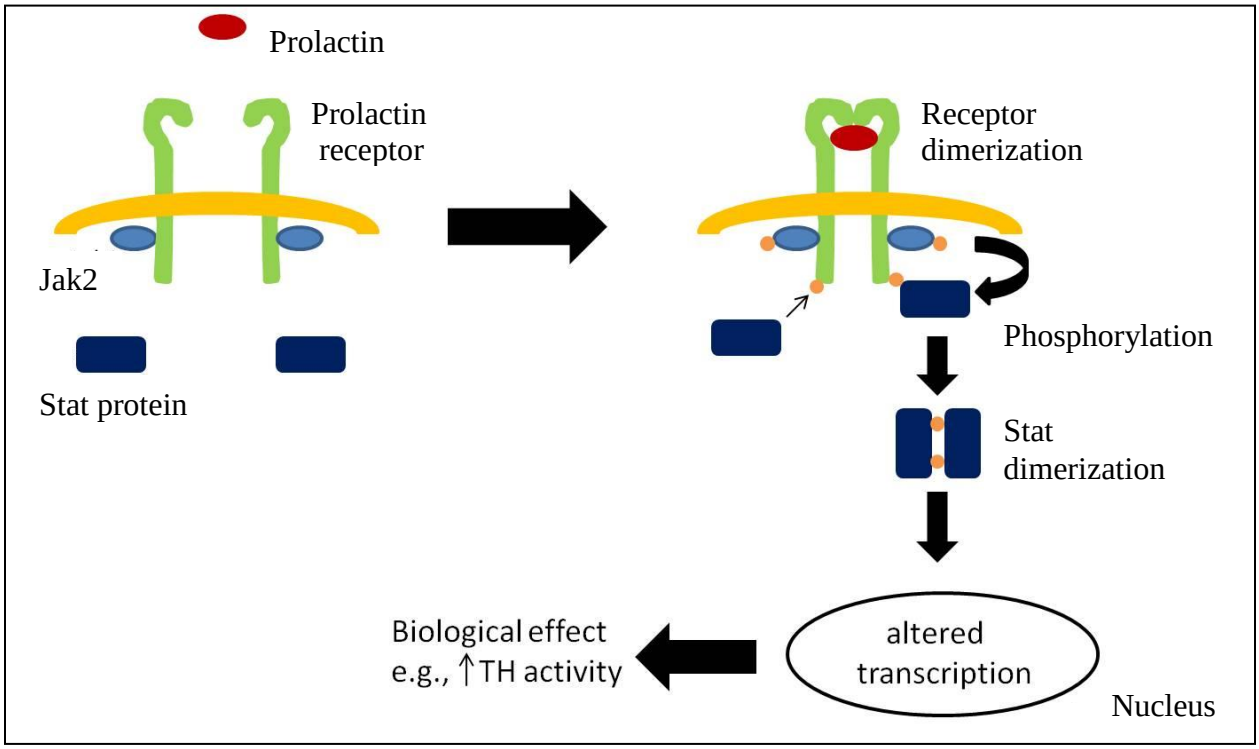


Figure 3. Schematic diagram of prolactin's interaction with its receptor and resultant post-translational gene transcription.

Prolactin

Prolactin is a polypeptide hormone that serves a variety of functions. It is primarily secreted by lactotrophs (a.k.a. mammotrophs), which are a subset of cells in the anterior pituitary gland. Secretion of prolactin is stimulated by prolactin secretagogues which are primarily growth hormone and thyrotropin hormone [47], as well as vasoactive intestinal peptide [44]. This is not surprising, as lactotrophs are derived from the same precursor cells as somatotrophs and thyrotrophs [48]. Additionally, lactotrophs secrete high basal levels of prolactin (82-90 ng/min in female rats) [49] as compared to basal secretory levels of other endocrine cells [44] (i.e., thyroxine [T_4] @ 39-69ng/min and insulin @ <1ng/hr in female rats) [50, 51]. Prolactin is not only produced by the anterior pituitary gland; it is also synthesized and secreted in low quantities by other regions of the central nervous system—including the telencephalic portion of the cerebral cortex, hippocampus, amygdala, septum, caudate putamen, brain stem, cerebellum, spinal cord, choroid plexi, and circumventricular organs. Likewise, many hypothalamic areas express prolactin immunoreactivity including the dorsomedial, ventromedial, supraoptic, and paraventricular nuclei. Similarly, peripheral tissues produce prolactin, including placenta, amnion, decidua, uterus, mammary gland, and lymphocytes [48].

Prolactin was originally characterized and was named after its ability to stimulate mammary development and lactation [48, 52]. It is best known for its necessity in mammogenesis, lactogenesis, and galactopoiesis. However, it has been shown that prolactin has a variety of biological functions. It has been shown to play an important role in to the initiation of maternal behavior, whereas suppression of endogenous prolactin with bromocriptine, a dopamine agonist, prevents the onset of maternal behavior. The role of prolactin in female reproductive behavior is contradictory; however, it has been clearly shown to diminish sexual behavior in male rats and

sheep [48]. In fact, hyperprolactinemia has been associated with impotence, galactorrhea, and amenorrhea. Not only does prolactin play a role in reproductive activities, but other non-reproductive physiological functions of prolactin have also been described. For example, it has been shown to play a role in osmoregulation by reducing renal excretion of sodium (Na^+) and potassium (K^+), decreasing Na^+ and chloride (Cl^-) in sweat and increasing water and salt absorption in the intestine [53, 54]. Additionally, prolactin is immunostimulatory with some immunostimulatory effects being macrophage activation and superoxide anion production [55-57]. Another effect of prolactin exposure is cellular proliferation and growth induction. Examples of such an effect include the stimulation of growth of skin melanocytes and keratinocytes, and an association with the turnover of hepatocytes [58-60]. Further, prolactin can contribute to the formation and continual proliferation of neoplastic cells; prolactin exposure has been particularly associated with mammary tumors, prostatic hyperplasia and prostatic neoplasia, lymphoma, and colorectal tumor growth [52].

In short, prolactin is not a functionally homogenous hormone and serves various roles; therefore, alterations in the synthesis of this hormone can have a multitude of aberrant physiological effects on many body systems.

Estrogen

Functions of estrogen

Estradiol has multiple physiological functions within the body. For example, it is necessary for the development of secondary sex characteristics in females [61], and is critical for several reproductive functions in males and females, such as ovulation, erection and ejaculation, and appropriate mating behaviors [62-66]. It has also been shown to modulate psychological well-

being by reducing anxiety and depression compared to individuals with insufficient estradiol production, and there has been a positive association of estradiol levels with memory function [67, 68]. Estrogen exposure has also been demonstrated to have neuroprotective effects after brain injury [69], and has a clear role in the maintenance of adequate bone density [70]. There have also been studies to demonstrate that estrogen has a positive influence on cardiovascular health [70] and the reduction of blood pressure [71]. In postmenopausal females, there is a higher incidence of osteoporosis, cardiovascular disease, mood swings and memory loss that are attributed to decreased E2 production. Nonetheless, recent studies have provided controversial evidence to some of the claims of hormone replacement therapy-associated benefits—particularly in regards to the cardiovascular system. It has been demonstrated that prolonged estrogen therapy increases the risk of coronary heart disease in women when given >10 years after the onset of menopause (>60 years of age) [72-75]. Further, our laboratory has recently demonstrated that prolonged exposure to a low level of E2 in rats, resulted in an increase in mean arterial blood pressure [76]. Besides causing hypertension, prolonged E2 exposure is also known to induce a variety of reproductive dysfunctions [77-81]. Additionally, it has been demonstrated that acute E2 exposure is associated with an anti-inflammatory biological state, whereas, chronic exposure causes a proinflammatory response [82]. The effects of E2 reported so far, appear to be dependent on the dose and the duration of exposure with acute exposures providing a beneficial effect, while chronic exposures produce a deleterious effect.

Within the body, E2 is normally secreted in a cyclic, pulsatile manner [83]. However, when exogenous estrogenic compounds—such as hormone replacement therapy, oral contraceptives, and environmental xenoestrogens—are introduced, the levels of estrogen in circulation increase and may persist throughout the duration of exposure. Furthermore, it has been shown that

xenoestrogens can induce a cumulative effect [84, 85]. Therefore, it is plausible that when extraneous exposures to estrogenic compounds occur, it may result in pathophysiological effects.

Because of the presence of ERs in various tissues, estrogen is known to be associated with a number of neoplasias: especially, estrogen-responsive breast cancer, endometrial proliferations, prostate tumors, colorectal tumors [86-88].

Types of estrogens

Major classifications of estrogens include biological, industrial, pharmaceutical, and phytogenic [1]. Biological estrogens are produced naturally by the body. There are 4 biological estrogens: estrone, estradiol, estriol and estetrone [89]. They are numerically characterized as E1, E2, E3 and E4, respectively, based on their relative time of synthesis in the steroid cascade of estrogen synthesis **(Fig. 4)**. All of the sex steroids—estrogens and androgens—are derived from cholesterol; actions of varying enzymes dictate the hormonal end-product [90, 91]. The most potent and abundantly produced biological estrogen in pre-menopausal women is estradiol-17β [91, 92]. There is also an optical isomer of estradiol-17β: estradiol-17α. However, estradiol-17α has traditionally been considered to be hormonally inactive [93, 94], as its binding affinity for estrogen receptors is 40-fold less than that of estradiol-17β [92]. Therefore, when the term estradiol is used, it almost inexplicably refers to estradiol 17β, which is also referred to as E2 or E_2. The granulosa cells of ovarian follicles are the predominant site of E2 production [95]. Estrone is the predominant estrogen produced in post-menopausal women, and adipose tissue is the most significant site of estrone production [95-97]. Estriol is considered to be the weakest estrogen. It is primarily synthesized in the liver and is present at lower levels than both estradiol and estrone [91]. Estriol is considered to be the "estrogen of pregnancy", as it is primarily

synthesized by the primate fetoplacental unit and is at its highest concentration in the peripheral circulation and urine of pregnant women [98, 99]. Estriol levels are often used as an indicator of fetal well-being [100-102]. Estetrol is exclusively synthesized by the fetal liver and is also elevated in the maternal circulation. However, the function of this hormone is yet to be elucidated [103] and it is sometimes excluded when the biological estrogens are being referenced.

Figure 4

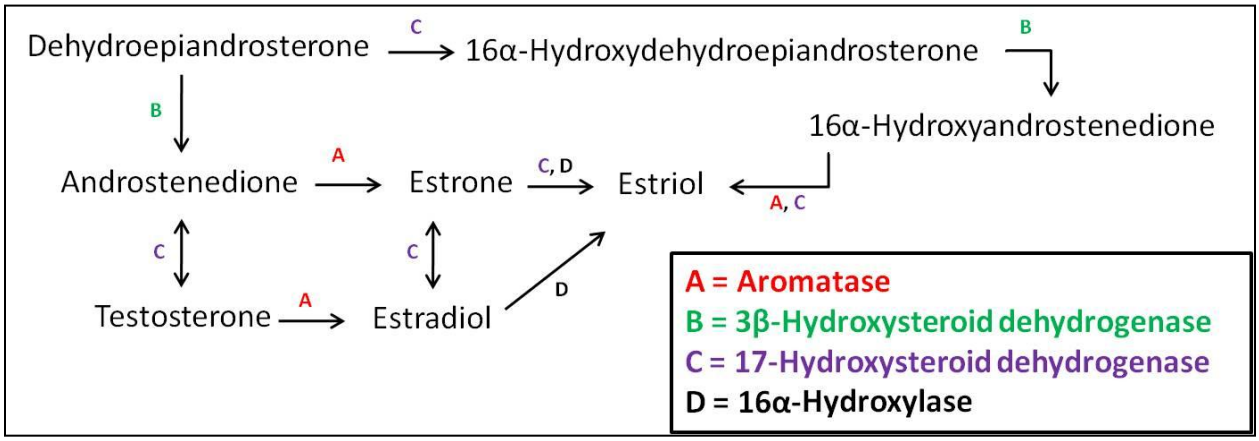


Figure 4. Cascade of estrogen synthesis from dehydroepiandrosterone (DHEA).

In addition to the endogenous estrogens that individuals are exposed to, there are also a variety of exogenous xenoestrogens that are present in the environment. Xenoestrogens are chemicals that mimic physiological estrogen and may therefore bind to estrogen receptors and either act agonistically as an estrogen, or may antagonistically interfere with the activity of endogenous estrogen [104].

Xenoestrogens are typically industrial by-products; some examples include bisphenol A [in plastics and lacquer coated food cans, etc.], nonylphenol [in lacquer coated food cans, industrial surfactants and emulsifiers, etc.], polychlorinated biphenyls [in lacquers and adhesives, etc.], and methoxychlor and hexachlorocyclohexane [in insecticides, etc.]. Individuals can also be exposed to synthetic pharmaceutical estrogens (i.e., ethinyl estradiol in oral contraceptives and hormone replacement therapy) voluntarily or inadvertently, as they are voluntarily consumed and are also released into the environment as waste [84].

Phytoestrogens are estrogenic compounds that are produced by plants. The principal classes of phytoestrogens in food are isoflavones, coumestans, flavonoids and lignans. Some common isoflavones include genistein and daidzein and are present in soybeans and lentils. Coumestrol is classified as a coumestan, which are structurally similar to isoflavones, and is present in young sprouting legumes, i.e., sweet clover and alfalfa. A flavonoid example is xanthohumol, and is present in some beer (hops). Lignans include matairesinol which is present in small amounts in cereal grains, fruits and vegetables. Linseed contains the highest known content of lignans [105].

In short, there are a plethora of circumstances and settings that potentiate exposure to estrogenic substances.

Estrogen receptors

The currenly known estrogen receptors are estrogen receptor alpha (ER-α), ER beta (ER-β), and G-protein coupled receptor 30 (GPR30) [64, 106, 107]. Steroid hormones, such as estrogen, are traditionally known to act through nuclear receptors, such as ER-α and ER-β which act as transcription factors to regulate expression of target genes. [106, 108]. We now know that there are also a small number of ER-α and -β that are located in the membranes and cytoplasm of some cells [106, 109]. Additionally, it has recently been identified that another way by which E2 can exert rapid effects is through interaction with extranuclear membrane receptors such as G-protein coupled receptors (GPCRs) [106, 107, 110, 111] and the cytosolic tyrosine kinase protein c-Src [111, 112]. G-protein coupled receptors are the largest class of signaling molecules [113] and are involved in cell function, growth, and differentiation [114]. The most studied GPCR that is facilitates E2 action is GPR30. c-Src is a tyrosine kinase cytosolic protein that is involved in signal transduction pathways, such as mitogen activated protein kinase (MAPK) pathways [115, 116] which are essential for cellular function and regulation of gene expression in response to external stimuli [117]. Src is activated by GPCRs [118], and their activation is required for GPCR-mediated activation of Ras, which is a GTPase that plays a key role in initiating the extracellular signal-regulated kinases (ERK)/MAPK pathway [116]. These extranuclear receptor interactions with E2 result in rapid effects, which are in contrast to the nuclear receptor-mediated effects which take a longer time to occur because they require genomic effects and post-translational modifications [117].

Estrogen receptors and their role in TIDA neuronal function

Estrogen receptors are present throughout the brain, including the arcuate nucleus of the hypothalamus, where TIDA neuronal cell bodies are localized. The prevalent ER in this location is ER-α. Estrogen is known to act on these neuronal cell bodies, yielding inhibition of TIDA neuronal function and an associated decrease in hypothalamic dopamine secretion in the median eminence [119, 120], hyperprolactinemia and formation of pituitary prolactinomas [5, 7, 9, 120, 121]. Additionally, it has been demonstrated that an increase in proinflammatory cytokines in the arcuate nucleus (AN) result in a reduction of TH activity in the ME, followed by an increase in serum prolactin levels [12]. Whether an increase in cytokines within the AN can be induced by estrogen administration had not been previously explored, to my knowledge.

Effects of estrogen on ovarian function and morphology

Under normal physiologic conditions, E2 is a key hormonal regulator of the menstrual cycle in females, stimulating the uterus and breasts to prepare for pregnancy, and playing a major role in ovulation, which is under the control of the hypothalamic-pituitary-gonadal (HPG) axis. Briefly, gonadotropin releasing hormone (GnRH) is produced by neurons which are predominantly located in the preoptico suprachiasmatic tuberoinfundibular area of the hypothalamus [122]. There are two subsets of GnRH neurons, one that is involved in the pulsatile secretion of GnRH and another that is involved in the generation of the luteinizing hormone (LH) surge that is critical for ovulation. At the time of the LH surge, GnRH from the hypothalamus acts upon gonadotrophs of the anterior pituitary gland to regulate secretion of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). In humans, following menstruation, FSH stimulates maturation of ovarian follicles. As ovarian follicles mature, granulosa cells produce E2. About midway through the menstrual cycle, E2 levels reach a peak concentration

which stimulates the LH surge, which is termed the preovulatory LH surge. This then induces ovulation of dominant ovulatory follicles, and the resultant corpora luteum (CL) which develops at the site of the ovulated follicle, produces progesterone (P_4) which would aid in the maintenance of pregnancy if the ova are fertilized. In the absence of pregnancy, the CL would regress, the uterine lining would shed, and the cycle would resume [123]. Unlike the average 28 days menstrual cycle in humans, rodents have a 4-5 day estrous cycle. It consists of four distinct stages: proestrus, estrus, diestrus I (aka metestrus) and diestrus II. During the proestrus stage, E2 levels peak and stimulate ovulation which occurs in the stage of estrus. Estrus is the stage in which the female is in "heat" and becomes receptive to mating. Diestrus I and diestrus II are characterized by low HPG hormones, with a progressive increase in hormonal activity during the transition between diestrus II and proestrus [124] (**Figure 5**).

Because E2 is such a major player in the normal functioning of the female reproductive axis, any change in the levels of estrogen has the potential to have adverse effects on ovarian physiology. Prolonged exposure to estrogenic compounds is known to cause functional and morphological ovarian alterations, including reduced fertility [125], advanced puberty, altered estrous cycles [126], premature reproductive aging [80], and cystic ovarian morphology [14]. It also results in a reduction of hypothalamic norepinephrine and abolishment of the luteinizing hormone (LH) surge [80], indicating it has a disruptive effect on the HPG axis.

Figure 5

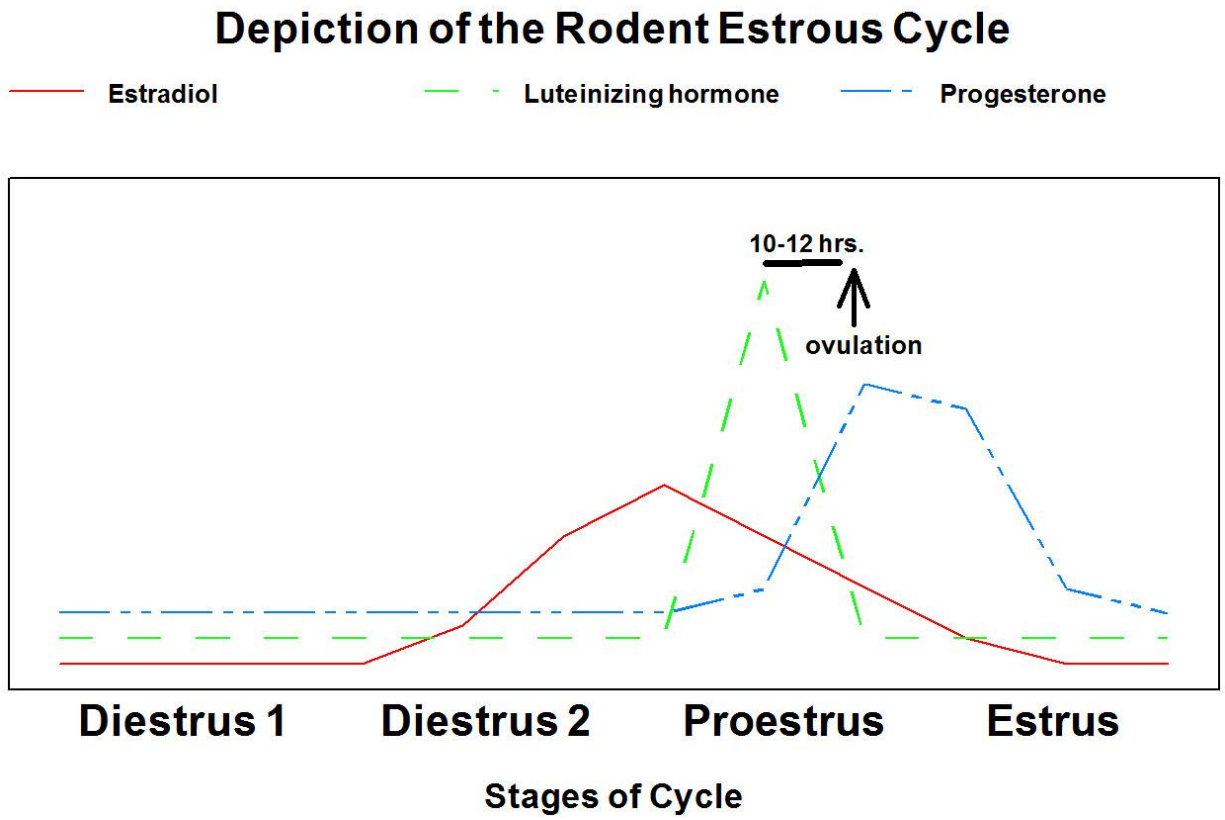


Figure 5. A diagrammatic representation of the rodent estrous cycle with indications of the stage of ovulation and the relative cyclicity of hormones E2, progesterone and LH.

Normal Ovarian Morphology

The female ovary contains follicles which consist of an oocyte surrounded by GCs with peripheral thecal cells. Thecal cells provide androgens required for E2 synthesis by GCs [127]. Most follicles undergo atresia [127, 128], as only select follicles progress to ovulation through dominant selection [129]. After ovulation, the follicle becomes a CL which then secrets P_4 to aid in maintenance of pregnancy if fertilization of the ovum occurs [127, 130]. The follicular stages are characterized as primordial (an oocyte surrounded by a single layer of flattened granulosa cells), primary (an oocyte surrounded by a single layer of cuboidal granulosa cells), secondary (an oocyte surrounded by greater than one layer of granulosa cells and no antral space formation), and tertiary (antral space formation) [127] **(Figure 5)**. Tertiary follicles >350μm in diameter are characterized as Graafian follicles [131]. Corpora lutea (CL) are characterized as "fresh" or "early" and "old". Fresh CL (or corpora hemorrhagica) contain hemorrhage and/or edema, and indicate recent ovulation; whereas old CL consist of a dense aggregate of granulosa lutein cells with no hemorrhage or edema [132] **(Figure 6)**.

Figure 6

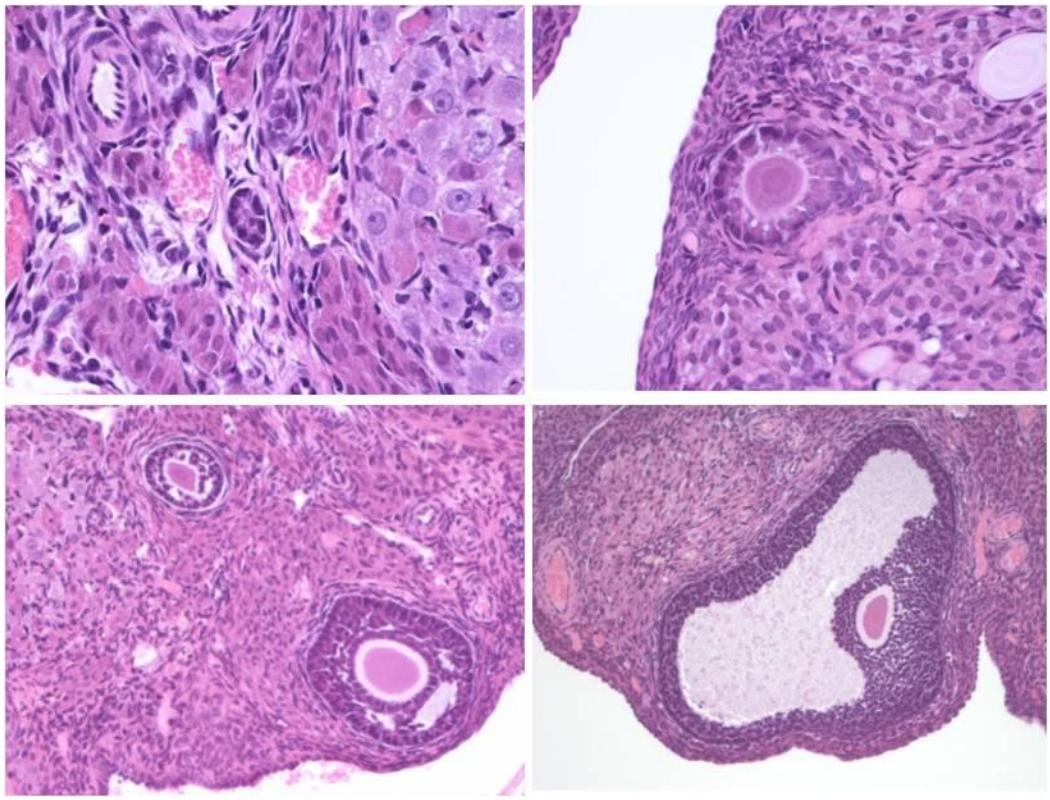


Fig. 6. A. An example of a primordial follicle characterized by a single layer of flattened cells surrounding an oocyte (oocyte not present in section photographed). **B.** An example of a primary follicle characterized by a single layer of cuboidal granulosa cells (GC). **C.** Examples of secondary follicles. The upper follicle is a classic secondary follicle with 2 or more layers of GCs surrounding an oocyte. The bottom follicle is a late secondary follicle that is in the process of progressing to a tertiary follicle as there is early antral space formation. **D.** A tertiary follicle with a well-defined antral space.

Figure 7

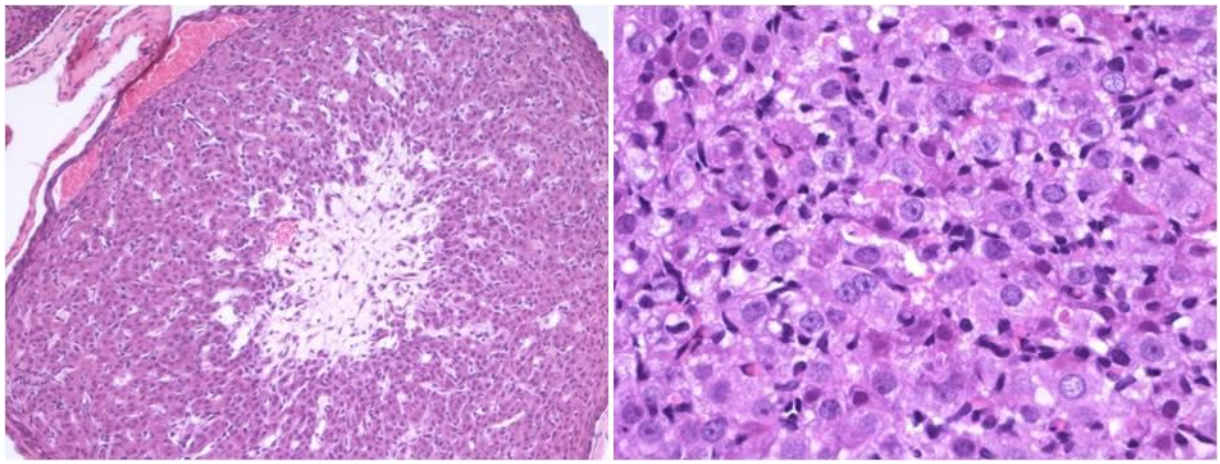


Fig. 7. A. A "fresh" corpora luteum (CL) with a central area of edema. **B.** High magnification of granulosa lutein cells that comprise CL.

CHAPTER 2

MATERIALS AND METHODS

Animals

Sprague Dawley rats

Female Sprague Dawley rats (n=8 per group) were used for the *in vivo* experiments. Rats were bred in-house and young (3-4 months) and middle age (10-12 months) Sprague-Dawley rats were used. Retired breeders (approximately 10 months of age) were obtained from Harlan Inc. (Indianapolis, IN). Retired breeders were maintained until they were in a state of persistent estrus and were classified as old constant estrus (OCE) rats. Animals were housed in light-controlled (lights on from 0700-1900h), air-conditioned (23±2°C) animal quarters and were fed rat chow and water ad libitum. Animals were used in accordance with the NIH guide for the Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Wild-type and Knockout mice

Breeding pairs of Wild type, inducible nitric oxide synthase (iNOS) knockout mice, and interleukin-1 receptor (IL-1 receptor) knockout mice were obtained from Harlan Inc. (Indianapolis, IN). Mice were bred in our colony and used in experiments. The absence of corresponding genes (iNOS and IL-1 receptor) were verified using a simple PCR using tail snip samples as described earlier [133].

Treatment of Animals

Young (3-4 months) and middle aged (10-12 months) Sprague-Dawley rats were reproductively intact or were ovariectomized (ovx), and were subcutaneously implanted with slow-release E2 pellets (Innovative Research America, Sarasota, FL) or were sham implanted (controls). The slow release pellets were capable of releasing 20ng/day of estradiol-17β (E2) for 90 days. Wild type and knockout mice were implanted with pellets capable of releasing 1µg of E2 per day. The animals were implanted with E2 pellets or sham implanted under isoflurane anesthesia as follows. Isoflurane was administered at the rate of 0.75L/min at a concentration of 3%v/v using a isoflurane Ohio vaporizer using a nose cone. The area over the dorsal aspect of the neck was shaved and wiped thrice alternatively with alcohol and betadine. Sterile instruments were used; scissors were used to make a small incision on the neck. Blunt dissection with curved forceps was used to create a "pocket" in the subcutaneous tissue extending down the left shoulder area. The pellet was placed in the subcutaneous pocket with separate sterile curved forceps. The skin was then lifted and apposed with a sterile staple. Five (5)mg/kg of ketoprofen was injected subcutaneously for pain control. The staple was removed after 7 days.

Daily/weekly observations: Estrous cyclicity in the animals was monitored periodically throughout the exposure period. Vaginal cytology was used to monitor cyclicity as described previously [124]. Briefly, warm water was used to obtain vaginal washes that were applied to a glass slide, dried and stained with a methylene blue solution as described previously [124]. The stained smears were examined under a light microscope and the stage of the estrous cycle was determined based on the predominant presence of epithelial cells, cornified cells or leukocytes.

Collection of Animal Tissues

Collection of Blood, Brain and Ovaries

At the end of the treatment period, all animals were sacrificed by rapid decapitation on the day of estrus. Brains and trunk blood were collected. When collecting the brains, all instruments and items that would come in contact with the brain were wiped with RNAse ZAP (Ambion Inc. Austin, TX), to minimize the risk of RNAse contamination. Plasma was obtained by collecting blood in heparinized tubes. Blood samples were centrifuged at 3,000 rpm for 5 minutes and plasma was collected. Brains and plasma were stored at -80°C.

Ovaries were collected at the time of sacrifice and a majority of the surrounding fat was removed. The ovaries were fixed in 10% neutral buffered formalin for at least 24 hours and for a maximum of 7 days prior to histological processing and paraffin embedding.

Brain microdissection

Brains were sectioned at 300µm using a cryostat (Slee, London, UK) maintained at -10°C and placed on glass slides. The slides were transferred to a cold stage maintained at -10°C and the arcuate nucleus (AN), median eminence (ME, substantia nigra (SN) and caudate putamen (CP) were dissected with a 500 µm micropunch, using Palkovit's microdissection technique [134] using the rat brain atlas [135] as a reference. Care was taken to include all subdivisions of individual nuclei for analysis. Tissue samples were collected in eppendorf tubes and stored at -80°C prior to analysis.

High Performance Liquid Chromatography-Electrochemical Detection (HPLC-EC)

The ME and CP tissue punches from frozen brains were homogenized in 100µl of 0.5M of perchloric acid ($HClO_4^-$). A micro-BSA protein assay was performed using 10µl of the sample.

The remaining homogenates were centrifuged at 13000rpm for 5 min. at 4°C. From each sample, 60µl of supernatant, 30µl of DHBA standard, and 30µl of $HClO_4^-$ was injected into the HPLC-EC system. The HPLC-EC system consisted of an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN), a glassy carbon working electrode, a phase II 5 µm ODS reverse phase 250 mm×4.6 mm C-18 column and C-R6A Chromatopac integrator (Shimadzu, Columbia, MD). The mobile phase consisted of monochloroacetic acid (14.5 g/l), sodium hydroxide (4.675 g/l), octanesulfonic acid disodium salt (0.3 g/l), ethylenediaminetetraacetic acid (0.25 g/l), acetonitrile (35 ml/l) and tetrahydrofuran (14 ml/l). The mobile phase was made with pyrogen-free water and filtered and degassed through a Milli-Q purification system (Millipore Co., Bedford, MA). The final pH of the mobile phase was adjusted to 3.1 using NaOH. The flow rate of the HPLC pump (Shimadzu LC-6A) was 1.7 ml/min. The column and the working electrode were kept in a Shimadzu CTO-6A oven at a temperature of 37 °C. The sensitivity of the detector was 1.0 nA full scale, and the potential of the working electrode was 0.65 V.

IL-1β ELISA

Brain Tissue Samples

A commercially available rat IL-1β enzyme immunometric assay kit from Assay Designs, Ann Arbor, MI was used. A cell homogenizer was used to homogenize the AN and SN samples in 130µl of ice cold phosphate buffer saline (PBS) [4.73g monobasic sodium phosphate; 9.74g dibasic sodium phosphate; 9g sodium chloride; autoclaved nanopure water to 1000ml], pH 7.5. Twenty (20) µl of the homogenate was extracted for protein analysis of 10µl in duplicates (Micro BCA protein assay, ThermoScientific, Rockford, IL). One hundred (100) µl of additional PBS was added to the remaining 110µl of sample homogenate. The samples were used for both the IL-1β and NO assays. Of the 210µl of homogenate, 100µl was used in the IL-1β assay as

50µl of sample was needed in duplicate. The samples were analyzed following the manufacturer's protocol. To determine nitrate and nitrite content, collectively, the commercially available total nitric oxide assay kit, from Assay Designs, Ann Arbor, MI was used.

Total NO detection kit (modified Griess assay)

Brain Tissue Samples

A commercially available total nitric oxide (NO) assay kit from Assay Designs, Ann Arbor, MI was used. Samples were homogenized as described above. The supernatant that was obtained from the above mentioned procedure was used for the total nitric oxide assay. The assay was done according to the manufacturer's protocol.

Western blot

Brain Tissue Samples

Sixty (60)µl of lysis buffer was added to the ME and CP punches and homogenized with a cell homogenizer. Ten (10)µl was removed to run a protein assay in duplicate. Tyrosine hydroxylase (TH) was immunoprecipitated using the following protocol: The volume of homogenate needed to obtain 50µg of protein was determined and the total volume was adjusted to 43.4 µl using lysis buffer (20mM Tris, 150mM sodium chloride [NaCl], 1mM EDTA [ethylenediaminetetraacetic acid], 10% glycerol, 1% TritonX, dissolved in autoclaved nanopure water). To this, 0.5µl PMSF (100mM PMSF dissolved in isopropanol), 6µl anti-TH antibody (Millipore, Temecula, CA), and 0.1µl sodium orthovanadate (Na_3VO_4) was added. The samples were vortexed and kept at 4°C overnight. The following day, 100 µl of Protein A agarose slurry (KPL, Gaithersburg, MD) was removed from the tube, was vortexed and centrifuged briefly. The supernatant was discarded and replaced with binding buffer (KPL, Gaithersburg, MD) at a volume of 100µl per sample. One hundred (100)µl of this agarose slurry was added to the

samples and this was kept at 4°C for 1.5 hours to facilitate the binding of the TH antibody to the agarose beads. The agarose slurry was vortexed between addition to each sample. The sample tubes containing the slurry were centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded (the agarose beads had the antigen antibody complex bound to them). The pellets containing agarose beads bound to the TH-antibody: TH complex were washed with 40 µl of Elution buffer (KPL, Gaithersburg, MD) and mixed briefly with a pipette tip. This was left on ice for 5 minutes and centrifuged again at 10000 rpm for 10 minutes. This resulted in elution of TH from the complex. The supernatant containing TH was divided into 2 equal fractions of 20µl each. The supernatant was mixed with 6.6µl of 4X Laemlli's loading buffer (1.0M Tris pH 8, sodium dodecyl sulfate [SDS], 40% glycerol, 4% of 0.5% bromophenolblue, 10% β-mercaptoethanol). The samples were then placed in a 90-100°C water bath for 4-5 minutes for denaturing the protein and then placed on ice. Each sample (20µl), and 5µl of fluorescent marker (Invitrogen, Carlsbad, CA)was loaded on two 4-20% gels (NuSep, Australia) and run at 60V until the dye reached the lower end of the gel. The proteins were transferred to nitrocellulose membrane using semi-dry blotting with the following protocol: Three mm thick filter paper was cut to match the size of the gel. Two papers were placed in anode buffer 1 (18.17g Tris, 100ml methanol, autoclaved nanopure water up to 500ml, pH 10.4) and then placed on the Semi Dry Blotting apparatus. The papers were rolled to remove air bubbles. Two additional papers were placed in anode buffer 2 (1.51g Tris, 100ml methanol, autoclaved nanopure water up to 500ml, pH10.4. These papers were placed on top of the first 2 papers and bubbles were rolled out. The nitrocellulose membrane was then soaked in anode buffer 2 and then placed on top of the stack of papers and air bubbles were rolled out. The gel was placed on top of the membrane and 2 papers soaked in cathode buffer (1.51g Tris, 2.62g 6-aminohexanoic acid, 100ml methanol,

autoclaved nanopure water up to 500ml, pH 9.4) were placed on top of the gel and air bubbles were rolled out. The lid was then closed and the apparatus was run at 25V for 30 minutes. The nitrocellulose membranes were placed in blocking solution overnight at 4°C with shaking, or it were soaked in blocking solution for 1 hour at room temperature with shaking. Following blocking, the membrane was rinsed once with tris buffered saline tween (TBST) (10X TBS [3.025g Tris, 20.455g NaCl, autoclaved nanopure water 250ml, pH 7.9], 0.05% tween 20, water to dilute 10X TBS to 1X), the membranes were then washed in TBST for 5 minutes, in TBST for 15 minutes, then in TBST 2 more times at 5 minutes each. The final rinse was in TBS for 5 minutes. The blot from one gel was probed for TH and the blot from the other gel was probed for nitrotyrosine (nT)). The TH antibody was used at a dilution of 1:1000 (30µl:30ml) + 30µl of 1% merthiolate, and the nT antibody (Abcam, Cambridge, MA) was used at a dilution of 2µg/ml. The membranes were incubated overnight at 4°C with shaking. The following day, the membranes were rinsed as described previously and incubated with fluorescent secondary antibody (anti-rabbit IgG IRDye800 conjugated fluorescent secondary antibody, Rockland Immunochemicals) at a dilution of 1:10,000 diluted in 1:1 blocking buffer:TBST for 1 hour, protected from light, with shaking. The membranes were then washed as described previously and then scanned with an Odyssey scanner at a channel of 700/800.

Protein assay

Protein concentrations in the tissue homogenates were determined using a micro Bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer's protocol. Absorbance values were obtained with a spectrophotometer at a wavelength of 562nm. Samples were assayed in duplicate and each 96 well plate had a set of standards in duplicate. BSA (2mg/ml) was used as the standard.

Radioimmunoassay

Serum E2 and prolactin levels were analyzed by double antibody radioimmunoassay using established protocols that have been described previously ([80]). The samples were analyzed by Dr. A.F. Parlow at Harbor-UCLA Medical Center, Torrance, CA.

RNA extraction from brain tissue samples

The RNA was extracted from the brainstem punches using MELT Total Nucleic Acid Isolation System (Ambion Inc, Austin, TX) according to the manufacturer's protocol. Using the Multi-Enzymatic Liquefaction of Tissue (MELT) mix provided in the kit, the tissue was digested. After on-bead Turbo DNAse digestion (Ambion), the RNA was eluted in a volume of 500 µl. First strand cDNA was synthesized by reverse transcribing 250ng of total RNA using Superscript III First strand synthesis Supermix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

qPCR array

Quantitative mRNA expression analysis of 24 genes was studied using a custom qPCR array that was developed by SABiosciences (Frederick, MD). The custom array focused on the following genes: interleukin 1 beta (Il1b), estrogen receptor 1 (Esr1), angiotensinogen (Agt), interleukin 6 (Il6), estrogen receptor 2 (ER beta) (Esr2), angiotensin II receptor, type 1b (Agtr1b), tumor necrosis factor (Tnf), prostaglandin-endoperoxide synthase 2 (Ptgs2), endothelin receptor type A (Ednra), lymphotoxin alpha (Lta), chemokine ligand 2 (Ccl2), endothelin 1 (Edn1), heat shock 70kD protein 1A (Hspa1a), leptin receptor (Lepr), endothelin 2 (Edn2), nitric oxide synthase 2 (Nos2), neuropeptide Y receptor Y1 (Npy1r), tyrosine hydroxylase (Th), brain derived neurotrophic factor (Bdnf), proopiomelanocortin (Pomc), agouti related protein homolog (Agrp), beta actin (Actb), ribosomal protein L13A (Rpl13a), lactate dehydrogenase A (Ldha). Aside

from the latter 3 housekeeping genes, the genes were subclassified into genes related to inflammation, genes related to feeding and energy metabolism, and genes related to hypertension. Estrogen receptor genes and genes for TH and brain derived neurotrophic factor (BDNF) were also analyzed. The genes related to inflammation were: IL1b, IL6, Tnf, Ptgs2, Lta, and Nos2. The genes related to feeding and energy metabolism were: Lepr, Npy1r, Agrp, and Pomc. The genes related to hypertension were: Agt, Agtr1b, Edn1, Ednra. Total RNA was isolated from brainstem areas of individual rats from the vehicle and IL-1β-treated groups (n=6) using the MELT Total Nucleic Acid Isolation System (Ambion Inc, Austin, TX). RNA from each sample (400ng) was converted to cDNA using the RT^2 First strand kit (SuperArray Bioscience Corporation). PCR was performed using RT^2 profiler PCR array PARN-018A (rat TLR) on the Applied Biosystems 7500 system according to manufacturer's specifications. The following 2-step cycling program was used: 1 cycle at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. qRT-PCR and gene products quantification were performed with the 7500 Real Time PCR machine (Applied Biosystems, Foster City, CA). cDNA from two rats was pooled and three of these sets were hybridized per treatment. The expression of each target gene was normalized using the expression of multiple house-keeping genes: β-actin, Lactate dehydrogenase and Ribosomal protein L13A and compared with the data obtained from the control group according to the $2^{-\Delta\Delta CT}$ method. The data analysis was performed using the Excel macro provided with the kit and Ingenuity Pathway analysis software (Ingenuity Systems Inc, Redwood City, CA).

Statistics

Changes in all the parameters assessed were analyzed using one-way ANOVA followed by posthoc Fisher's LSD test, unless otherwise specified. Significant mean difference between groups was set at $p<0.05$.

CHAPTER 3

THE EFFECTS OF E2 ON THE OVARY AND EXPLORING THE POTENTIAL FOR LOW DOSE CHRONIC E2 EXPOSURE TO SERVE AS A MODEL FOR POLYCYSTIC OVARY SYNDROME

Introduction

Humans are chronically exposed to low levels of estrogen in various forms, including oral contraceptives, hormone replacement therapy and estrogenic endocrine disruptors. Women of reproductive age are also exposed to endogenous estrogens for approximately 30 years during the pre- and peri-menopausal stages of their lives. Exposure to estrogenic compounds for prolonged periods of time is known to cause alterations in reproductive functions such as reduced fertility [125], advanced puberty, altered estrous cycles, increased susceptibility to breast cancer, [126], ovarian changes, premature reproductive aging [80], and cystic ovarian morphology [14]. Polycystic ovarian syndrome (PCOS) is a condition that affects 5-10% of females between the ages of 18 and 44 years [136]. This disease is often clinically associated with obesity, insulin resistance, hyperandrogenism, hirsutism, oligomenorrhea or amenorrhea, infertility and elevated circulating Mullerian inhibiting substance (MIS) [136-138]. The etiology of PCOS is not known. Several rodent models have been proposed in an attempt to understand the mechanism by which PCOS is induced [15, 16, 139-141]. However, true models of PCOS can't be based primarily on ovarian histomorphology alone [15, 16, 141-143]. They also have to exhibit other clinical parameters and hormonal changes associated with PCOS.

We demonstrated that exposure of adult, cycling Sprague-Dawley female rats to 20 ng/day of estradiol-17β (E2) for 60 or 90 days results in cystic ovaries, increases serum estrogen levels and

suppresses the hypothalamo-pituitary-gonadal axis [80]. We hypothesized that E2 exposure would result in a PCOS-like condition. To test this, we performed a detailed histomorphological examination of the ovaries. We compared the expression of Mullerian inhibiting substance (MIS) expression in the ovary because MIS levels are known to increase in PCOS patients [137]. We also examined the levels of hormones such as testosterone, LH, and the ratio of LH to FSH, which are known to be affected in PCOS.

Experimental Design

Animals

Adult, 4-6 months old, female Sprague-Dawley rats and retired breeders (approximately 10 months of age) were obtained from Harlan Inc. (Indianapolis, IN, USA). The rats were housed in light-controlled (lights on from 0700-1900h) and air-conditioned (23±2°C) animal rooms and were fed rat chow and water *ad libitum*. Animals were used in accordance with the NIH guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Treatment

Animals (n=8) were sham-implanted (controls) or implanted with slow-release E2 pellets (20ng/day; Innovative Research America, Sarasota, FL) for 30 (E-30 group), 60 (E-60 group), or 90 (E-90 group) days. Retired breeders were aged in our facility and used as old constant estrous (OCE) rats for comparison. They were 12-16 months of age at the time of experimentation. Estrous cyclicity was monitored by vaginal cytology periodically as described previously [80]. At the end of the experiment, control and E-30 animals that had regular estrous cycles were sacrificed by rapid decapitation on the day of estrous at 1200 h. Animals in the E-60, E-90 and OCE groups were in a state of constant estrous. Trunk blood was collected and serum and

plasma were stored at -80°C. Ovaries were collected and placed in 10% neutral buffered formalin and stored at 4°C.

Ovaries were processed for routine histology, and immunohistochemical labeling for CD68 and MIS. The sections were histomorphometrically and histopathologically evaluated. Serum was analyzed for testosterone by ELISA and for estradiol, prolactin, LH, and FSH by RIA.

Results

Estrous cyclicity

Effects of exposure to E2 on estrous cyclicity is shown in **Figure 8**. Ninety four percent of animals in the sham-implanted control group exhibited regular estrous cycles. In the E-30 group, 81% of the animals showed regular estrous cycles. In contrast, only 32% of the animals in E-60, 18% of the animals in E-90 and none of the animals in OCE group showed regular estrous cycles. These findings are comparable to that observed in a previous study in our lab [80].

Figure 8

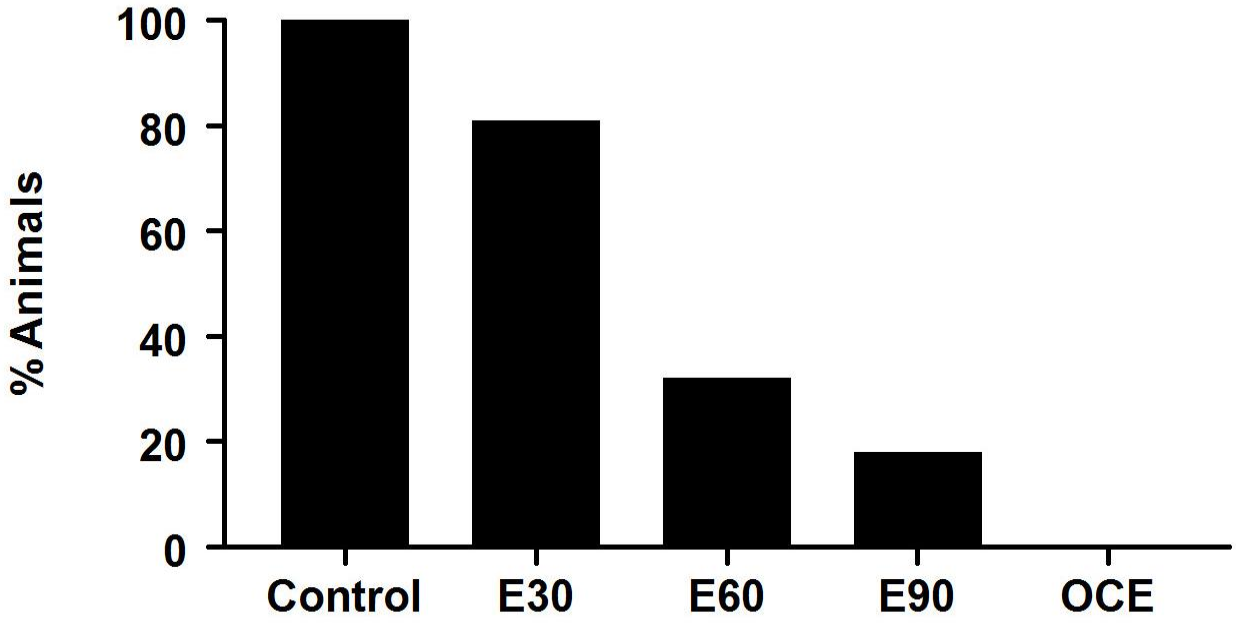


Fig. 8. Estrous cyclicity in control and E2-treated animals**.** Estrous cycles were determined by daily vaginal cytology. Graph represents percentage of animals demonstrating regular estrous cycles.

Ovarian Histology

Status of ovulation and follicular development

The total number of follicles was not different between groups (**Fig. 9a**). Primordial follicles were only increased in the E-30 group (3.8±1.0) compared to the control (0.7±0.3) ($p<0.05$). There were no differences in the numbers of primary, secondary, or tertiary follicles between the different treatment groups (**Table 1**).

Chronic exposure to E2 resulted in decreased ovulation in a duration-dependent manner. There was a marked decrease in the number of fresh corpora lutea (CL; mean±SE) in the E-30 group (0.2±0.2) and a complete absence in the other E2-treated groups and the OCE group, compared to the controls (1.3±0.4) ($p<0.0001$). Furthermore, the number of old CLs were also significantly decreased in the E-90 group (1.7±1.5) and old CLs were completely absent in the OCE group compared to the control (6.5±0.9) rats ($p<0.0001$), suggesting that this was a chronic condition (**Fig. 9b**).

Figure 9

A

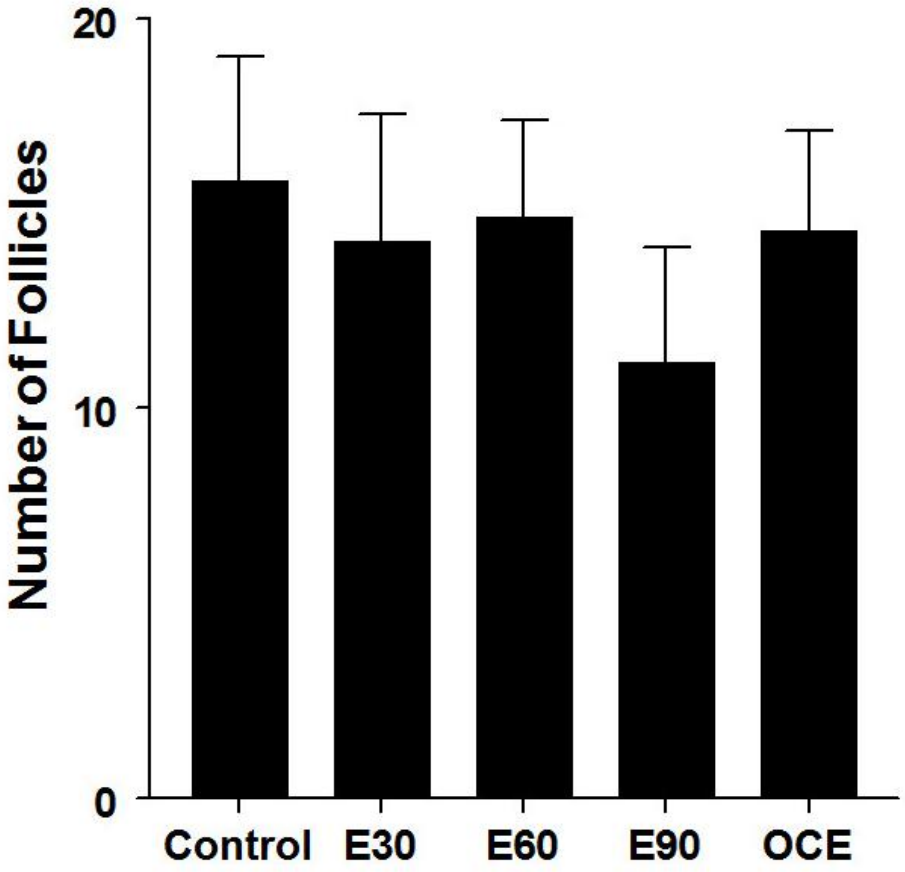


Fig 9. A. Total number of follicles in the different treatment groups. * indicates significant difference from all other groups, $p<0.0001$. In contrast, the number of old CLs was markedly reduced in the E-90 and OCE groups were markedly reduced compared to the control group (a indicates $p<0.0001$).

Figure 9 (cont'd)

B

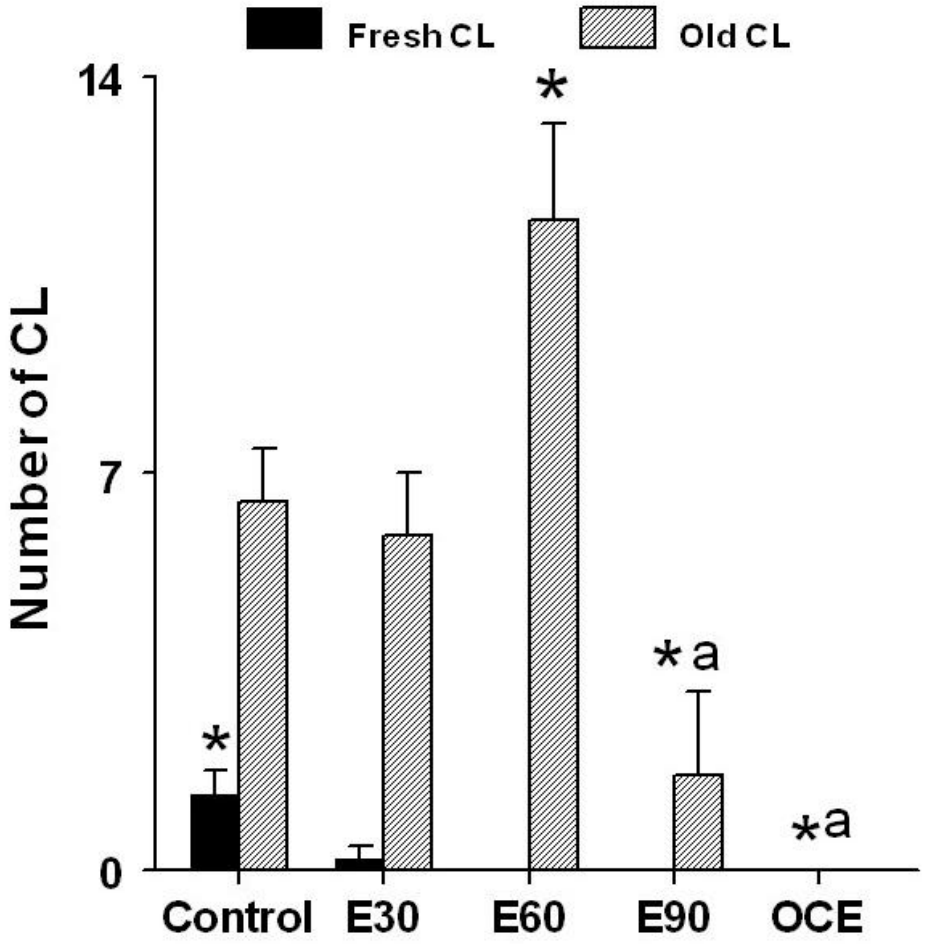


Fig 9. B. Total number of fresh and old CLs in the ovaries of different treatment groups. Note the reduction in the number of fresh CLs in the E-30 group and complete absence of fresh CLs in other E2-treated groups and the OCE group. * indicates significant difference from all other groups, $p<0.0001$. In contrast, the number of old CLs was markedly reduced in the E-90 and OCE groups were markedly reduced compared to the control group (a indicates $p<0.0001$).

Table 1

Type of Follicle	**Treatment groups**					**Significance**
	Control	**E-30**	**E-60**	**E-90**	**OCE**	
Primordial	0.67±0.3	3.8±0.9*****	1.18±0.4	1.3±0.8	1.2±0.7	$p<0.05$
Primary	4±1.4	2.9±1.0	4.27±0.8	2.67±1.4	3±1.1	NS
Secondary	2.5±0.9	2.5±0.6	2.64±0.7	1±0.6	0.57±0.4	NS
Tertiary	8.67±1.8	5.1±1.4	6.81±1.4	6.17±1.0	9.43±1.0	NS

Table 1. Effects of estradiol-17β exposure on follicular development. * indicates significant difference from control ($p<0.05$).

Presence of cystic follicles

Photomicrographs of representative ovarian sections from control, E2 treated and OCE animals are provided in **Fig. 10**. Histological examination revealed that there was a time-dependent increase in the size of mature follicles**.** The granulosa cell epithelium was often attenuated in these cystic follicles (arrow). The presence of cystic follicles was associated with a decrease in the number of secondary follicles **(Table 1)**. Cystic follicles were particularly evident in the E90 (panel D) and OCE (panel E) groups compared to the control (panel A) group. The percentage of tertiary follicles increased in an E2 exposure dependent manner **(Table 2)**. The percentage of tertiary follicles (mean±SE; %) was 69.1±11.5 and 71.8±7.0 in the E-90 and OCE groups. These were significantly high when compared to the percentage of tertiary follicles in the ovaries of E-30 (39.0±6.7) and E-60 (44.8±8.2) animals ($p<0.05$), but not from control rats. Morphometric analysis of the follicles indicated that there was a gradual increase in follicular size with increasing exposure to E2 **(Table 2)**. The size of the Graafian follicle (mean±SE; μm) in the E-90 (621.6±47.6) and OCE ovaries (614.0±30.0) were significantly larger when compared to that in the control group (492.4±15.3) ($p<0.05$). However, there were marked changes in the granulosa cell layer of follicles from E2- treated animals compared to control rats as explained below.

Figure 10

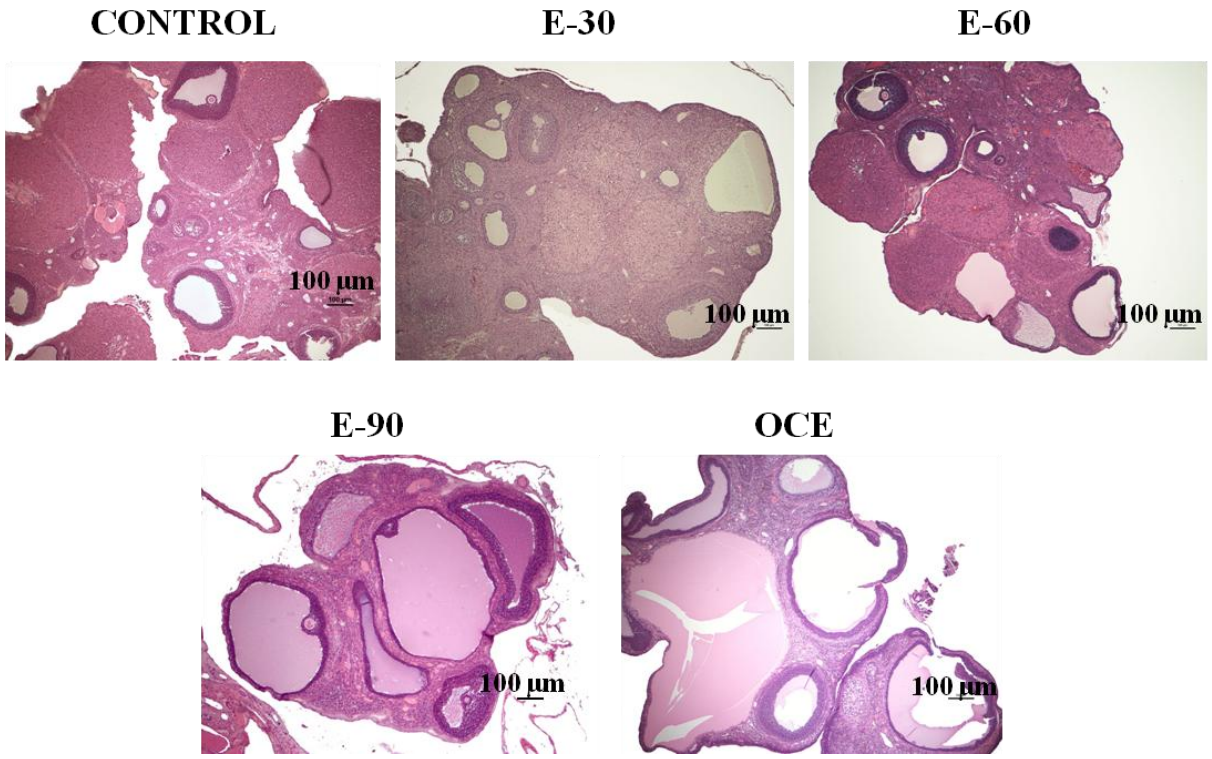


Fig 10. Representative histological sections (H&E staining) of ovaries from different groups**.** Control (panel 1); E-30 (panel 2), E-60 (panel 3), E-90 (panel 4) and OCE (panel 5). * represents fresh CL and # represents old CL.

Table 2

Treatment	% of Tertiary follicles (mean±S.E.)	Graafian follicle size (µm) (mean±S.E)
Control	51.2±7.9	492.4±15.3
E-30	39±6.7	479.4±20.0
E-60	44.8±8.2	512.0±53.2
E-90	69.1±11.5*	621.6±47.6*****
OCE	71.8±7*	614.0±30.0*****

Table 2. Effects of estradiol-17β exposure on Tertiary and Graafian follicles. * indicates significant difference from control ($p<0.05$).

Presence of degenerating follicles and pathological changes in the ovaries

Histological evidence of follicular degeneration and necrosis was present in the ovaries of E2-treated animals. As can be seen in **Fig.11 (panels A-D)**, the degree of degeneration/necrosis increased in severity with the duration of E2 exposure. Panel A has a representative section containing a Graafian follicle from a control animal. E2 exposure caused histopathological alterations including nuclear pyknosis of granulosa cells (GC), GC dispersion with loss of polarity, multifocal to focally transmural accumulations of neutrophils in the GC layers (Panels B and C), oocyte degeneration with loss of cell architecture, antral fluid basophilia with globular formations and accumulations of cells and cellular debris (panel D), mineralization, hydropic degeneration of GCs, and replacement of GCs with foamy macrophages (panel C). Aggregates of foamy macrophages were also present in the interstitium (Panel B). There were atretic follicles that had not yet formed a scar. These atretic follicles were present in ovaries from all treatment groups and controls, with OCE ovaries having significantly less atretic follicles than control and E60 ovaries. There were also mild to moderate degenerative changes in OCE follicles. However, these changes were not as extensive as that observed in the ovaries of E2-treated animals (Panel E).

Figure 11

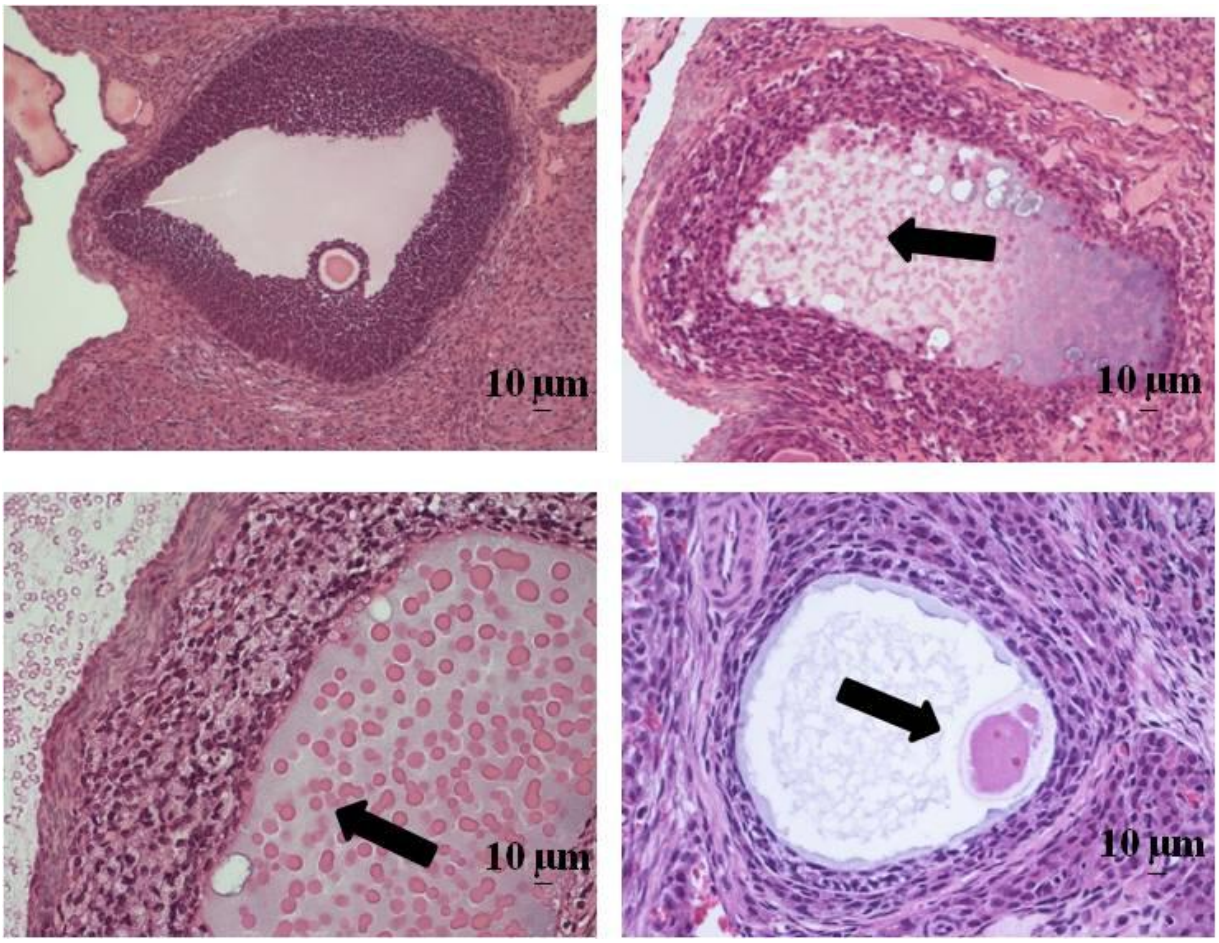


Fig 11. Degenerating follicular elements with E2 treatment. Graafian follicle in a Control ovary (**panel A**). Follicle from E90 ovary (**Panel B**): Note GC nuclear pyknosis, cell dispersion with loss of polarity, antral fluid basophilia (block arrow) and mineralization with globular formations and accumulation of cellular debris, and follicular wall neutrophilic infiltration. Follicle from E90 ovary (**Panel C**): Note marked accumulation of foamy macrophages. The antral fluid is degenerate and characterized by basophilia and accumulation of homogenous eosinophilic, globular debris (protein) (block arrow). (**Panel D)**: E90 follicle undergoing degeneration and a degenerate oocyte (block arrow) can be observed, along with fibrillar antral fluid, GC nuclear pyknosis, and GC dispersion.

CD68 Expression

Immunohistochemical labeling of anti-CD68 antibody, which is a marker that is specific for monocytes and macrophages, revealed the presence of macrophages infiltrating the granulosa cells in follicles that had histomorphologic characteristics of degeneration (**Fig. 12)**. The number of follicles infiltrated with CD68 positive cells were increased in a time of E2 exposure-dependent manner **(Fig. 13)**.

Figure 12

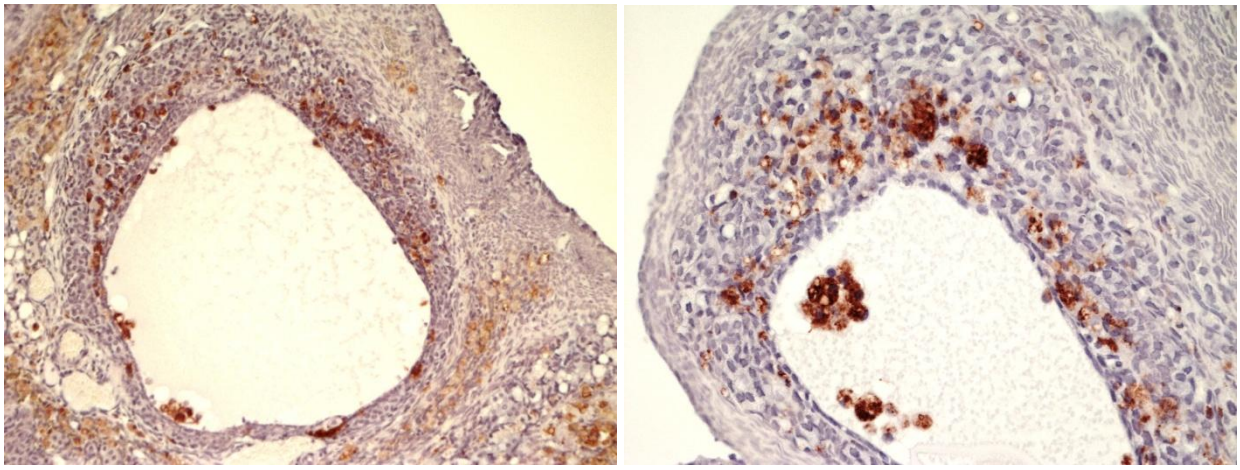


Figure 12. Follicles with infiltrated macrophages positively immunolabeled with anti-CD68 antibody. There is strong intracytoplasmic labeling of macrophages.

Figure 13

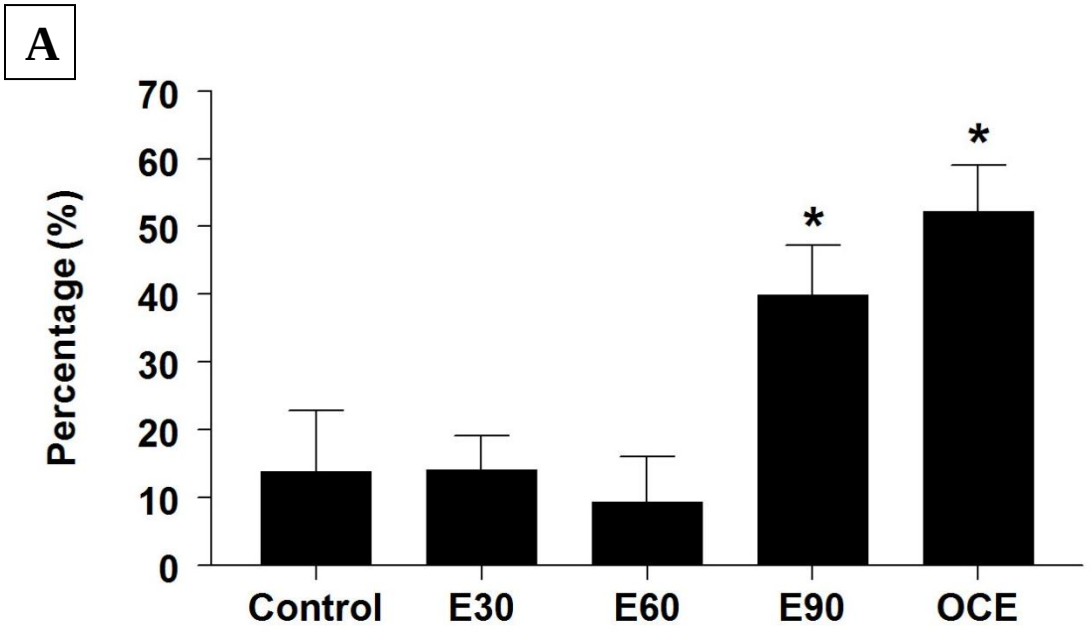


Figure 13. A. The percentage of follicles within ovary sections that contained CD68 positive macrophages. There was a significant increase in follicles that
had macrophage infiltration in the E90 and OCE groups. * indicates significant difference from control group ($p<0.05$).

Figure 13 (cont'd)

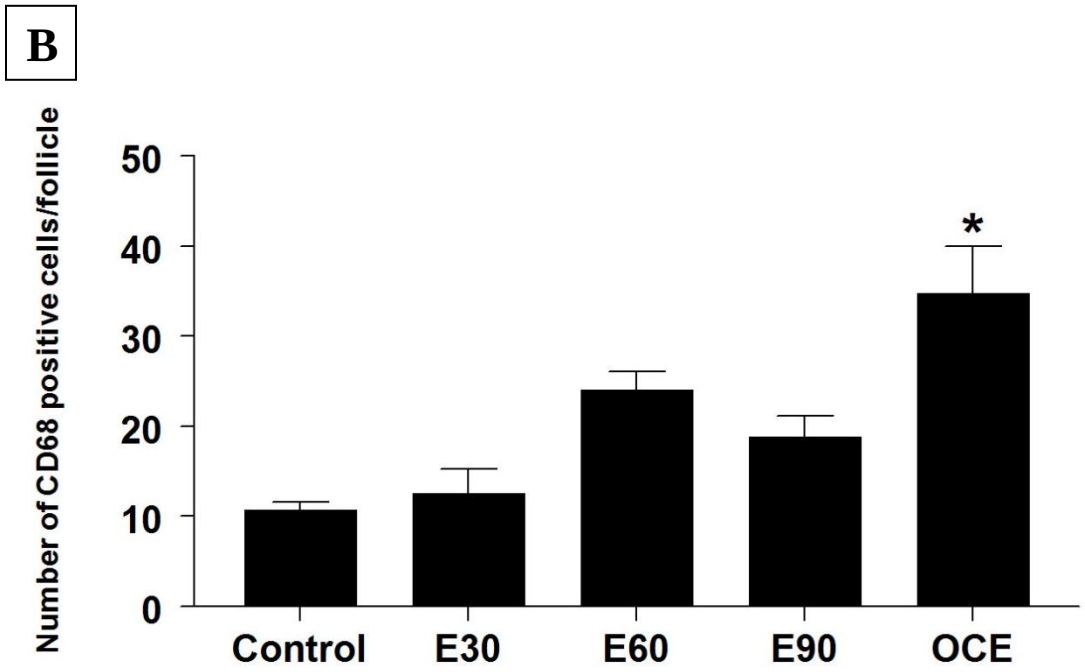


Figure 13. B. The number of CD68 positive cells per follicle that had macrophagic infiltrates. There was no significant difference between treatment groups and control animals. However, OCE animals did have a significant increase in the number of macrophages present in affected follicles. * indicates significant difference from control group ($p<0.05$).

MIS expression

Immunohistochemical labeling of anti-MIS antibody revealed intracytoplasmic expression of MIS in the GCs of primary, secondary and tertiary follicles of control ovaries **(Fig. 14a, panel A)**. The majority of the superficial epithelial cells of the ovary also expressed mild to moderate labeling. In contrast, primordial and large tertiary (Graafian/cystic) follicles did not express MIS. MIS expression was subjectively observed to be down-regulated in tertiary follicles as they increased in size. In the E-90 group, there was virtually no expression of MIS in any of the GCs regardless of follicular maturity **(Panel B)**. In the control ovaries, there was a large percentage of MIS expressing primary follicles (mean±SE; %; 95.0±5.0) compared to E-90 ovaries (9.0±5.6). None of the secondary follicles in the E-90 group had MIS expression while a relatively large percentage of secondary follicles in the control ovaries (90.0±10.0) were positive for MIS staining ($p<0.0001$) **(Fig 14b)**.

Figure 14

A

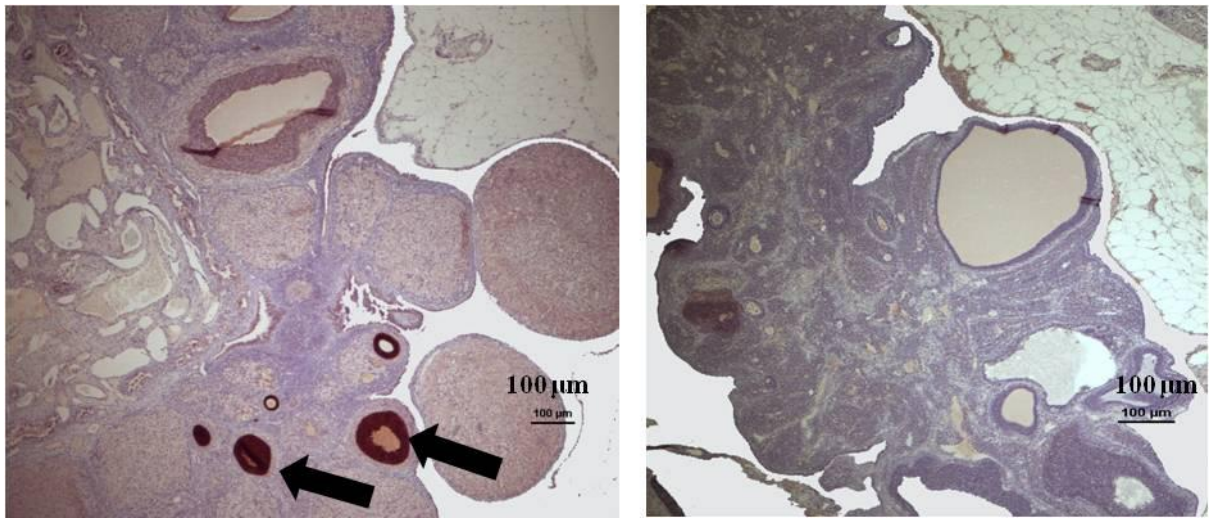


Fig. 14. A. MIS immunohistochemistry in ovaries from control and E2 treated animals. Panel 1: Section from a control ovary. There is strong MIS immunoreactivity (block arrow) in primary, secondary, and small tertiary follicles. Mild immunoreactivity is present in the larger tertiary follicle. Panel 2: Section from a E90 ovary. There is no MIS immunolabeling present in any of the follicles, regardless of the stage of development.

Figure 14 (cont'd)

B

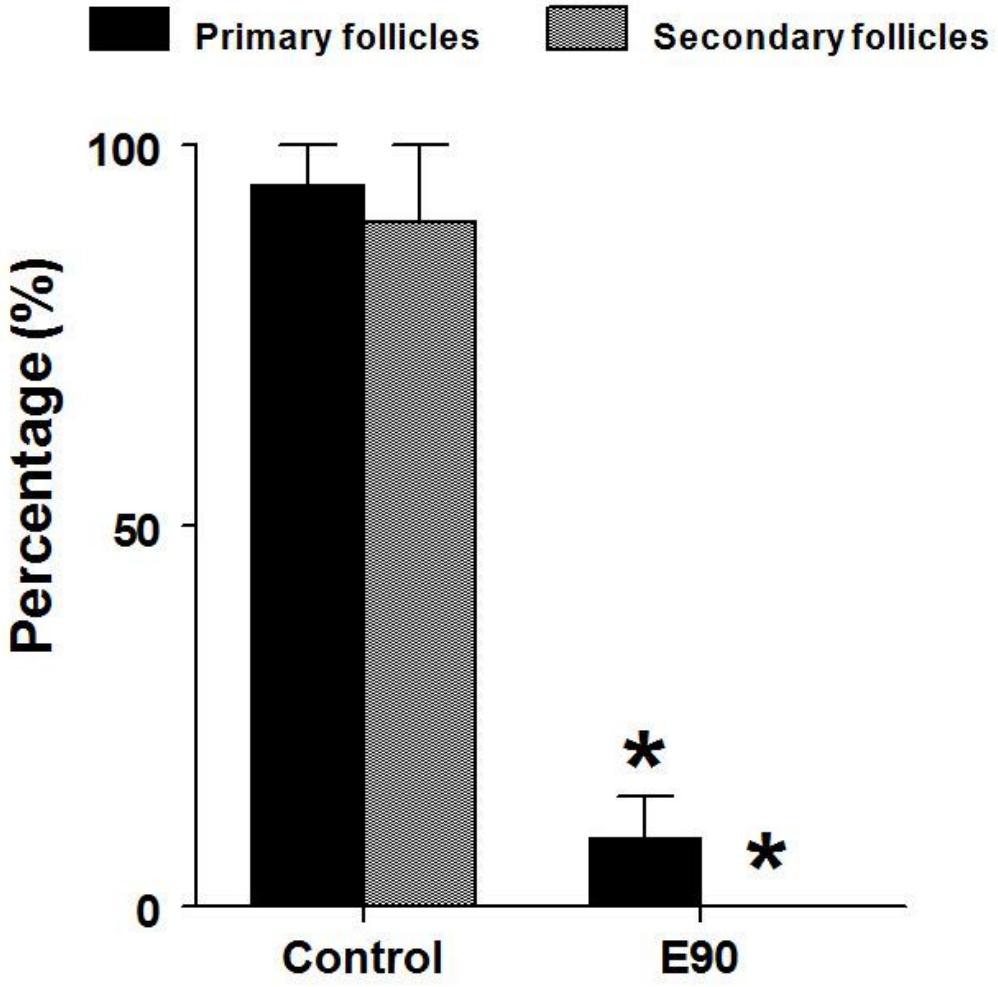


Fig. 14. B. Effect of E2 treatment on the percentage of follicles expressing MIS immunoreactivity. There was a significant reduction in the percentage (mean±SE; %) of immunohistochemical labeling of MIS in both primary and secondary follicles in E90 ovaries compared to control. * indicates significant decrease from control. $p<0.0001$.

Serum Testosterone and LH to FSH ratio

Serum testosterone levels (mean±SE; pg/ml) are shown in **Fig. 15a**. Serum testosterone levels decreased significantly in E-90 animals (64.1±6.7) compared to control rats (193.7±28.8; $p<0.05$). In contrast, serum testosterone levels increased significantly in OCE animals (335.7±112.5) compared to the control group ($p<0.05$; **Fig. 15a**). There was no difference in the LH to FSH ratio among the different groups (**Fig. 15b**).

Figure 15

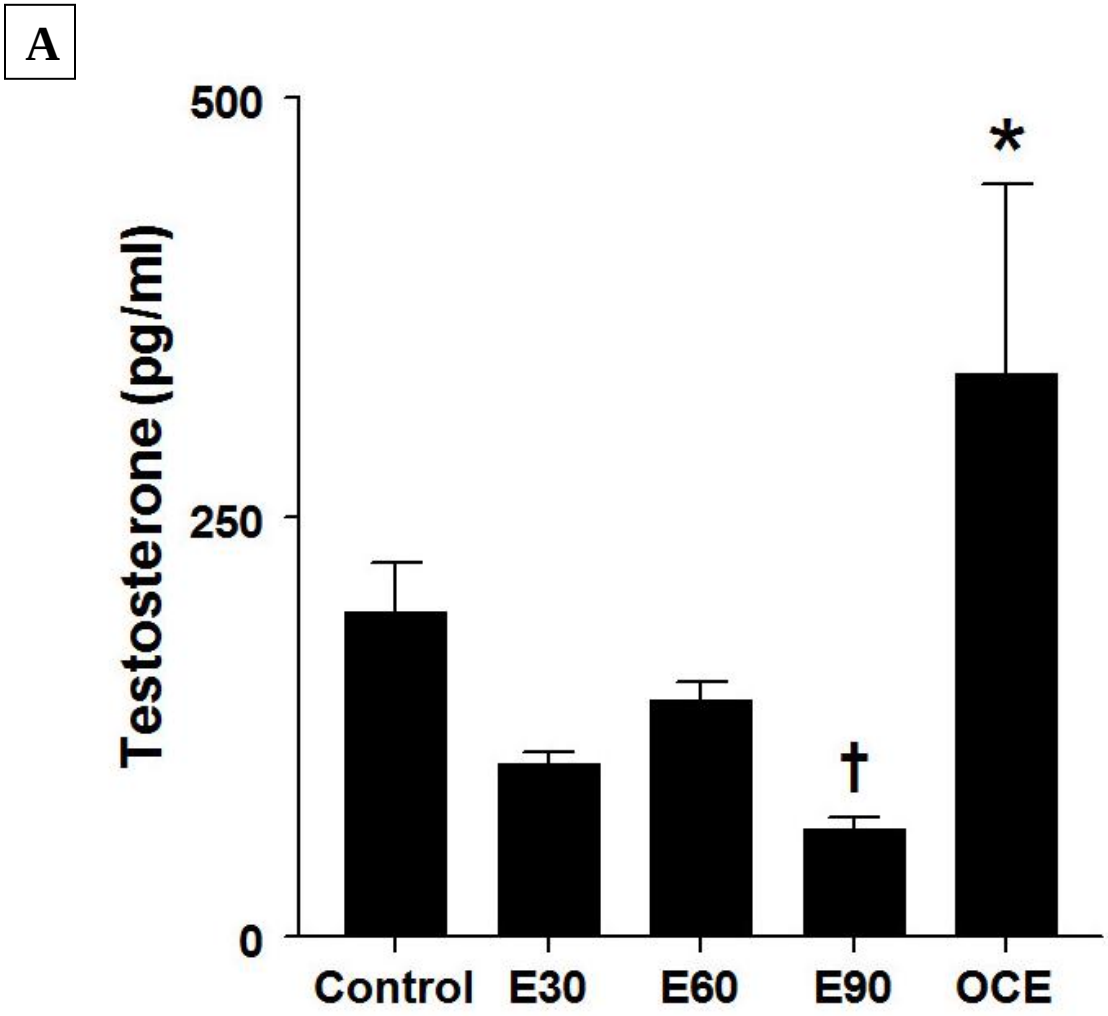


Fig. 15. A. Effect of E2 exposure on serum testosterone levels. Note the significant increase in testosterone levels in OCE animals, significant reduction in testosterone in E-90 animals compared to control. * indicates significant difference from the rest of the groups. † indicates significant decrease from control and OCE groups ($p<0.05$).

Figure 15 (cont'd)

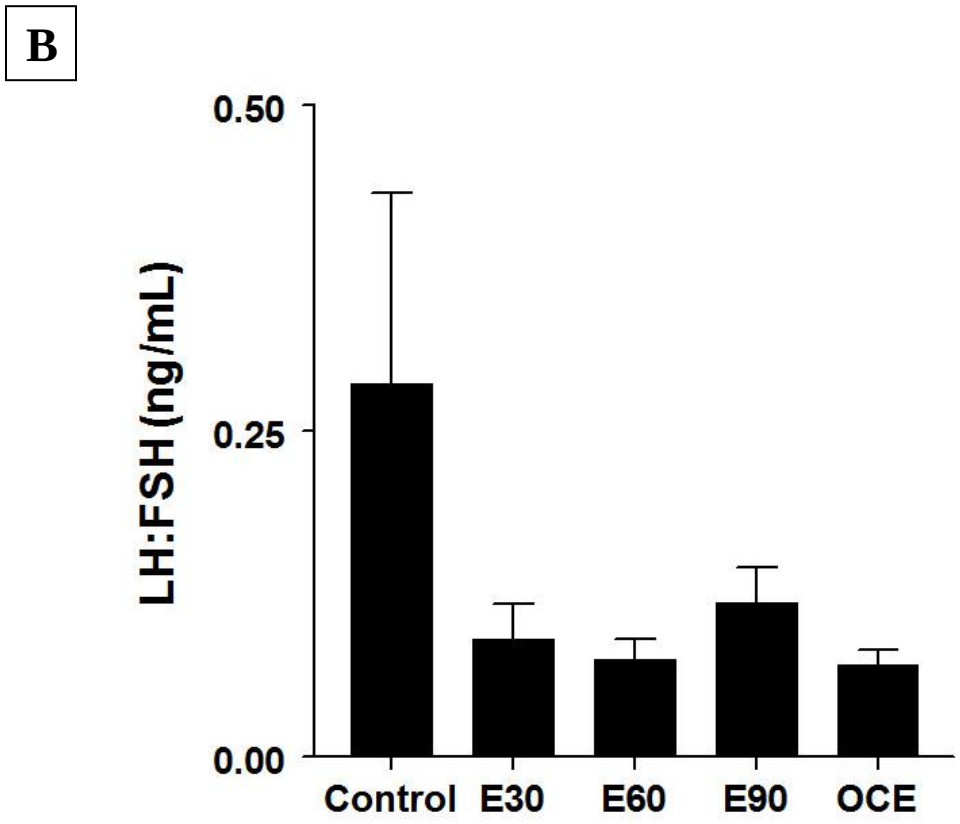


Fig. 15. B. Effect of E2 exposure on the ratio of serum LH to FSH. The LH:FSH ratio was not significantly different between the groups.

Discussion and Summary

The results from this study provide evidence that chronic exposure to low levels of E2 results in cystic ovarian morphology which is similar to that observed in aging female rats that are in the state of persistent estrus. However, there are more degenerative changes induced by E2 exposure in the follicles compared to that observed with aging. Chronic exposure to E2 also results in a significant reduction in the expression of MIS in the ovary. This was accompanied by a significant reduction in serum testosterone levels without any change in the ratio between gonadotropins. While the presence of cystic follicles resembles the histological characteristics observed in PCOS, E2 exposure produced several changes in the ovaries and hormonal profile that were very different from what is seen in PCOS leading us to conclude that this paradigm may not be a useful model for PCOS.

Chronic E2 exposure caused polycystic ovarian morphology that was dependent on the duration of exposure. A variety of estrogenic compounds have been shown to cause cystic ovarian morphology. The mechanism by which chronic E2 exposure causes polycystic ovaries is not clear. There is evidence that this may be due to a direct toxic effect of E2, which has been previously demonstrated in reproductive organs including the ovary [144, 145] and the epididymis [146]. On the other hand, this could be due to the effects of E2 on the hypothalamo pituitary gonadal axis that regulates reproductive function. In fact, similar exposure to E2, in terms of dose and duration, has been shown to inhibit hypothalamic monoamines, LH and cause acyclicity [80]. Thus, it is physiologically plausible that follicular recruitment and ovulation will cease with chronic E2 exposure due to insufficient gonadotropin production by the anterior pituitary. In addition to a cystic follicular ovarian phenotype, the present study demonstrates that E2-treated animals also exhibited time-dependent ovarian follicular degeneration.

Histomorphological analysis of ovarian sections revealed that there were some similarities between ovaries from E-90 and OCE rats. Both had cystic Graafian/tertiary follicles with a larger ratio of these follicles compared to primary, secondary, and small tertiary follicles. However, the main histological difference between ovaries of E-90-treated and OCE rats was that the E-90 ovaries had histological evidence of degeneration, macrophagic infiltration and necrosis. As in the present study, follicular degeneration is also a feature of PCOS [137]. However, in contrast to our findings, PCOS patients actually have an increase in the total number of follicles, and a 2-6 fold increase in the number of primary and secondary follicles [147, 148] as opposed to the present study where we did not observe a difference in the total number of follicles or in the number of primary and secondary follicles in E2-treated rats compared to control.

The other ovarian parameter that was assessed in this study was the follicular expression of MIS. MIS (also known as anti-Mullerian hormone) is produced by testicular Sertoli cells of male fetuses and causes regression of the Müllerian ducts. In female rodents, expression begins after birth and has been seen as early as 4 days post-gestation [149, 150]. It is predominantly expressed in granulosa cells (GC) of primary, secondary, and tertiary follicles. It is down-regulated in tertiary follicles as the follicle matures and is minimally or not expressed at all in preovulatory follicles [131, 151, 152]. The exact function and mechanism of action of MIS in the ovary is not fully understood, but it has been indicated as a key regulator of folliculogenesis and dominant follicle selection [131, 151-155]. MIS is known to be increased in the serum of women with PCOS, and this is due to increased production by the ovaries resulting from increased numbers of primary and secondary ovarian follicles [137, 152]. Ovarian expression of MIS in rats chronically treated with a low-dose of E2 had not been previously examined by others. In the present study, we provide evidence for the first time that chronic exposure to E2

for 90 days, results in the complete loss of MIS expression in the ovaries. Similarly, there was a complete loss of expression of MIS in the ovaries of OCE rats. This suggests that chronic E2 exposure not only causes failure of ovulation and follicular cysts, but also inhibits MIS expression. This is important because MIS is known to be involved in protecting primordial follicles. In fact, in MIS-deficient mice, primordial follicles are prematurely recruited and depleted followed by subsequent premature cessation of the estrous cycle [150]. Therefore, it is possible that E2-induced down-regulation of ovarian MIS may result in primordial follicle depletion, thereby preventing further estrous cyclicity.

We also studied degenerative changes in the ovaries after chronic E2 exposure. Although degenerative changes in ovarian follicles have not been reported in PCOS, these changes have been described in rats treated with pregnant mare serum gonadotropin [156]. These changes include blebbing of the cytoplasm and alterations in the shape of granulosa cells followed by a reduction in estradiol and testosterone levels in the ovaries. This is followed by the development of autophagic vacuoles, and infiltration of macrophages into the granulosa and thecal cell layers. Finally atresia is accompanied by pyknosis. In this study, we observed infiltration of macrophages in the granulosa cell layer with E2 treatment which probably contributes to the development of cystic follicles.

In addition to ovarian characteristics, serum hormones were also measured in this study. Though, our ovarian morphological characteristics in E2-treated animals were similar to those of PCOS patients, hormonal changes observed in the present study were inconsistent with findings most often associated with PCOS. Testosterone levels are characteristically increased in women with PCOS as hyperandrogenism is a key feature of this disease [157]. In contrast, there was a significant decrease in the testosterone level of the E-90 animals compared to controls. It is

suspected that estradiol causes suppression of steroidogenic enzymes in male rats [158] and a similar phenomenon may be operational in female rats as well. However, there was a marked increase in testosterone in OCE animals, which is in agreement with previous reports [159, 160]. The exact cause of this hyperandrogenemia in OCE animals is still unclear.

Additionally, PCOS is often associated with higher levels of LH and an increased LH to FSH ratio. However, in the present study the LH to FSH ratio was not different among various groups. This is in agreement with our previous study in which E2 exposure for similar durations resulted in lower levels of LH [80]. The main reason for this reduction was the decrease in hypothalamic norepinephrine. While moderate levels of E2 are essential for stimulating the LH surge during estrous cycles, the study by Kasturi et al demonstrated that exogenous administration of E2, even at low doses, can cause suppression of LH levels by acting at higher centers. In PCOS however, it appears that the ovary fails to respond properly to the higher levels of LH. Therefore the chronic E2 exposure paradigm may not be a suitable model for PCOS.

Overall, our study demonstrates that chronic exposure to low-doses of E2 can be detrimental to the ovaries. It induces cystic follicles, causes follicular degeneration and down-regulates MIS expression. Chronic E2 exposure also produces marked decreases in serum testosterone levels without altering the LH to FSH ratio. While some of the changes observed in the present study are similar to PCOS patients, there are still significant differences leading us to conclude that chronic exposure of cycling rats to E2 may not serve as a true model for PCOS.

CHAPTER 4

LOW DOSE CHRONIC E2 EXPOSURE REDUCES DOPAMINE IN THE MEDIAN EMINENCE, INCREASES PITUITARY GLAND WEIGHT AND CAUSES HYPERPROLACTINEMIA

Introduction

Estrogen is known to decrease dopamine secretion from tuberoinfundibular dopaminergic (TIDA) neurons [6-8], that results in hyperprolactinemia [5, 7, 9]. The increase in circulating prolactin levels can predispose individuals to the onset of mammary and pituitary gland adenomas. Many such studies reporting these effects of estrogens, involved short-term exposure and/or high doses of various estrogenic compounds [8, 9]. The exact mechanism by which estrogens cause suppression of TIDA function is not clear.

TIDA neurons are highly sensitive to estrogens. They have estrogen receptors [161]. Their cells bodies are located in the arcuate nucleus and their terminals extend to the median eminence[162]. Estrogen treatment has been shown to decrease mRNA levels of the rate limiting enzyme in dopamine synthesis, viz. tyrosine hydroxylase[11, 161]. Although this may be a mechanism by which estrogen decreases dopamine synthesis, other mechanisms are also likely to play a role. Tyrosine hydroxylase activity in TIDA neurons may decrease because of a reduction in enzyme levels or due to a decrease in enzyme activity.

In this study, we wanted to explore the second possibility. We hypothesized that chronic exposures to low levels of E2 is likely to cause a pro-inflammatory state in the arcuate nucleus leading to a reduction in dopamine synthesis. This is because, previous studies have shown that acute injections of estradiol valerate can cause degenerative lesions in the arcuate nucleus [163,

164]. These changes included an increase in glial activity in this area. Since changes in glial cell function can result in alterations in neurotransmitter levels [165], we hypothesized that E2 exposure would result in similar changes in TIDA neurons.

Since older animals are exposed to endogenous estrogens for a longer period of time compared to younger animals, we hypothesized that older animals will have an exaggerated inflammatory response in the arcuate compared to young animals when they are exposed to E2. To test this hypothesis, we administered a physiologically low dose of E2 in young and middle-aged rats. The dose of E2 that we used (20ng/day) results in E2 levels consistent with the physiological levels as seen in proestrus, in female rats which is about 50pg/ml. We also used separate groups of reproductively intact and ovariectomized, female Sprague Dawley rats, to determine the role of endogenous estrogens.

Experimental Design

Animals

Young female Sprague-Dawley rats (3-4 months), middle-aged (10-12 months), and retired breeders (>12 months) were obtained from Harlan Inc. (Indianapolis, IN). Old constant estrous (OCE) rats were used for comparison. The OCE rats were from the retired breeders which were aged in our facility. They were approximately 18-20 months of age and had greater than 10 consecutive days of vaginal smears indicative of estrus, supporting a state of acyclicity. Housing was light-controlled (lights on from 0700-1900h) and air-conditioned (23±2°C) and the rats were fed rat chow and water ad libitum. Animals were used in accordance with the NIH guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Treatment

Young and middle-aged animals (n=8) were each randomly assigned to 1 of 4 treatment groups: reproductively intact (sham-implanted); intact and implanted with a slow-release E2 pellet (20ng/day) (Innovative Research America, Sarasota, FL, USA) for 90 days; ovariectomized (OVX); or ovariectomized and implanted with an E2 pellet. We have previously established that E2-treated animals enter a state of constant estrous by 60 days of exposure. At the end of the treatment period, all animals were sacrificed by rapid decapitation on the day of estrus at noon. Estrus was determined by cytological examination of vaginal smears obtained on the morning of sacrifice. Brains, serum, and plasma were collected and stored at -80°C.

Pituitary Weight Measurement

After removal of the brain, the pituitary gland was carefully removed in its entirety, from the sella turcica, placed on pre-weighed filter paper and was weighed. The pituitary gland was examined for gross pathological changes.

Estradiol and Prolactin Analysis

Serum samples were analyzed for estradiol and prolactin concentrations by double antibody radioimmunoassay according to standard protocols. The assay was performed by Dr. A.F. Parlow at Harbor-UCLA Medical Center, Torrance, CA California. Estradiol levels were expressed as pg/ml and prolactin levels as ng/ml.

Microdissection of Brains

Brains were sectioned at 300μm in a cryostat (Slee Mainz, London, UK) and mounted on room temperature glass slides. The slides were transferred to a cold stage maintained at -10°C and the

median eminence (ME) and caudate putamen (CP) were dissected with a 500 µm micropunch, using Palkovits' microdissection technique [135].

Dopamine HPLC-EC Analysis in the Median Eminence and Caudate Putamen

A cell homogenizer was used to homogenize the ME samples in 100µl of 0.5M of perchloric acid ($HClO_4^-$). Twenty (20) ul of the homogenate was removed for protein analysis (Micro BCA protein assay, Pierce, Rockford, IL). The remaining homogenate was centrifuged at 13000rpm for 5 min. at 4°C. From each sample, 60µl of supernatant was mixed with 30µl of DHBA standard, and 30µl of $HClO_4^-$ and was injected into the high performance liquid chromatography electrochemical detection (HPLC-EC) system. The HPLC-EC sytem and the details of the mobile phase have been previously described [80]. Briefly, it consisted of an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN), a glassy carbon working electrode, a phase II 5 µm ODS reverse phase 250 mm×4.6 mm C-18 column and C-R6A Chromatopac integrator (Shimadzu, Columbia, MD). The mobile phase consisted of monochloroacetic acid (14.5 g/l), sodium hydroxide (4.675 g/l), octanesulfonic acid disodium salt (0.3 g/l), ethylenediaminetetraacetic acid (0.25 g/l), acetonitrile (35 ml/l) and tetrahydrofuran (14 ml/l). The mobile phase was made with pyrogen-free water and filtered and degassed through a Milli-Q purification system (Millipore Co., Bedford, MA). The final pH of the mobile phase was adjusted to 3.1 using NaOH. The flow rate of the HPLC pump (Shimadzu LC-6A) was 1.7 ml/min. The column and the working electrode were kept in a Shimadzu CTO-6A oven at a temperature of 37 °C. The sensitivity of the detector was 1.0 nA full scale, and the potential of the working electrode was 0.65 V.

Results

Serum Estradiol and Prolactin

Serum E2 levels (pg/ml; Mean±SE) were increased in intact (52.8±4.4 and 58.6±5.1 in young and middle aged animals respectively) and ovariectomized rats (42.7±3.22 and 41.87±4.2 in young and middle aged animals respectively) after 90 days of exposure to E2 at 20ng/day compared to intact sham-implanted rats (32.12±2.2 and 37.6±3.4 in young and middle aged rats respectively) and ovariectomized, sham-implanted rats (29.4±2.1 and 32.04±1.9 in young and middle aged rats respectively; $p<0.05$). There was also an increase in E2 levels in OCE rats (59.2±8.2) **(Fig.16).** There was a significant increase in prolactin levels (Mean±SE; ng/ml) in intact young (23.4±4.5) and middle-aged (33.7±3.3) , and OCE animals (41.3±8.4) compared to sham-implanted rats (9.8±2.7; $p<0.05$). The E2 treated ovariectomized (OVX) animals did not have a significant change in prolactin levels(5.66±1.12 and 7.7±1.7 in young and middle-aged animals respectively) compared to sham-implanted rats (4.37±.95 and8.1±1.2 in young and middle-aged animals respectively **(Fig. 17).**

Figure 16

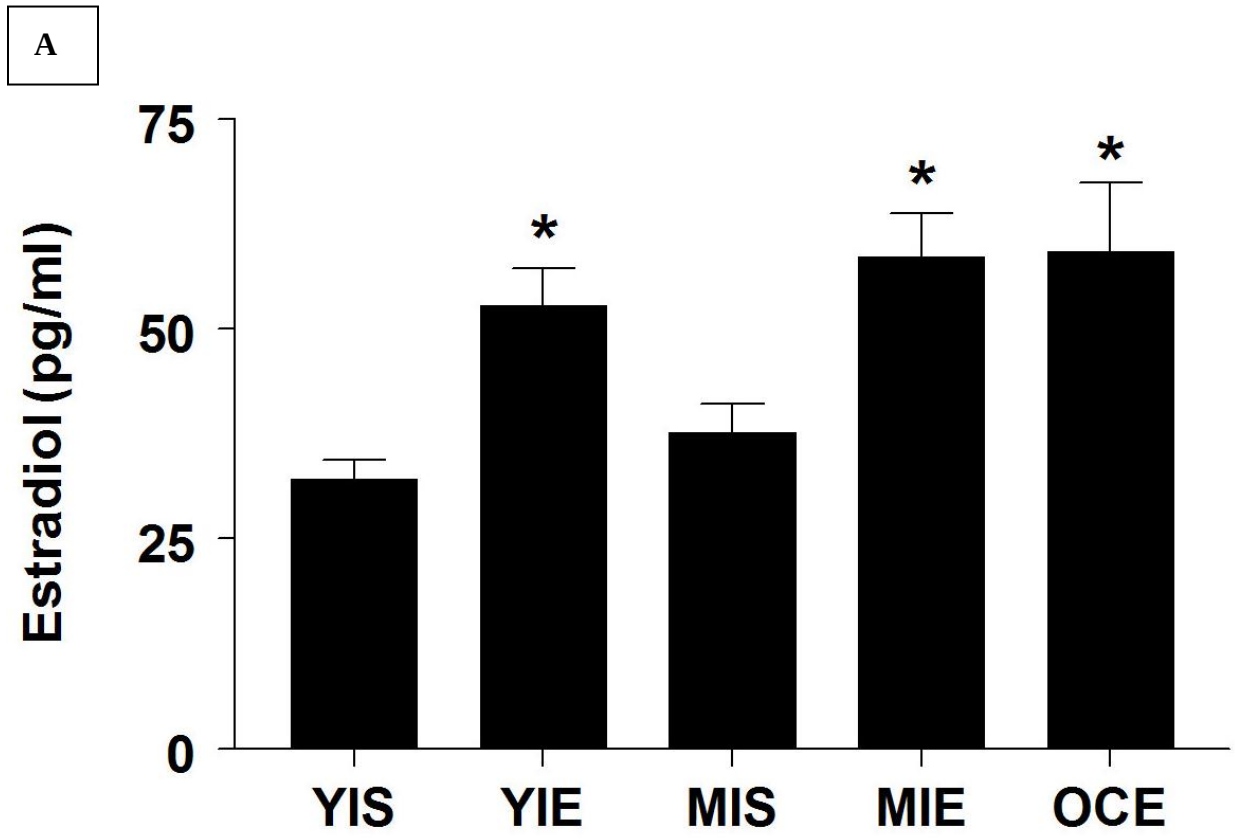


Fig. 16. A. Serum E2 levels (mean±SE; pg/ml) in reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, rats treated with slow-release E2 pellets for 90 days (YIE and MIE) and old constant estrus (OCE) rats (18-20 mos.). * indicates significant difference from YIS and MIS ($p<0.05$).

Figure 16 (cont'd)

B

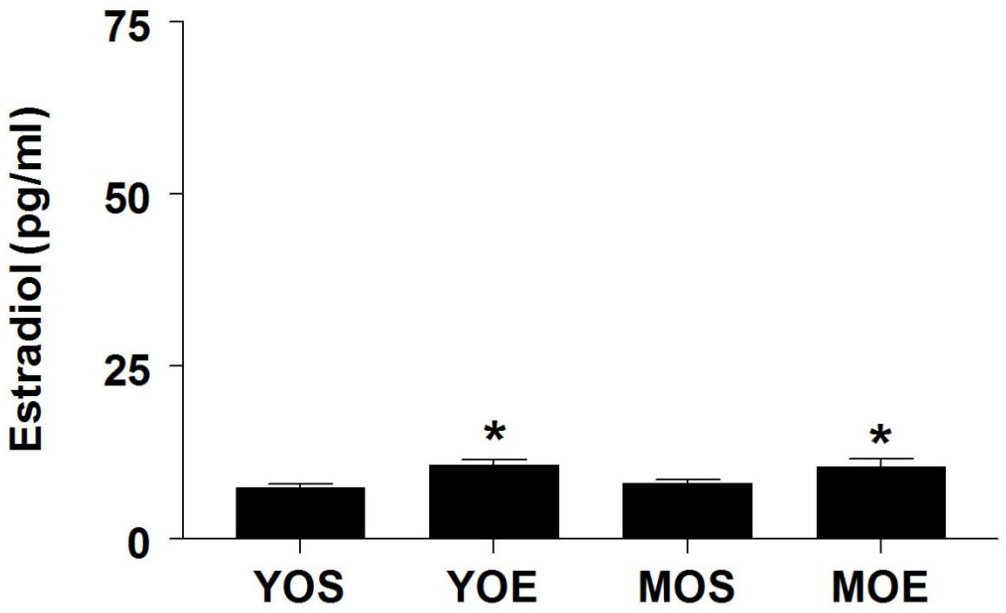


Fig. 16. B. Serum E2 levels (mean±SE; pg/ml) in ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YOE and MOE). * indicates significant difference from YOS and MOS ($p<0.05$).

Figure 17

A

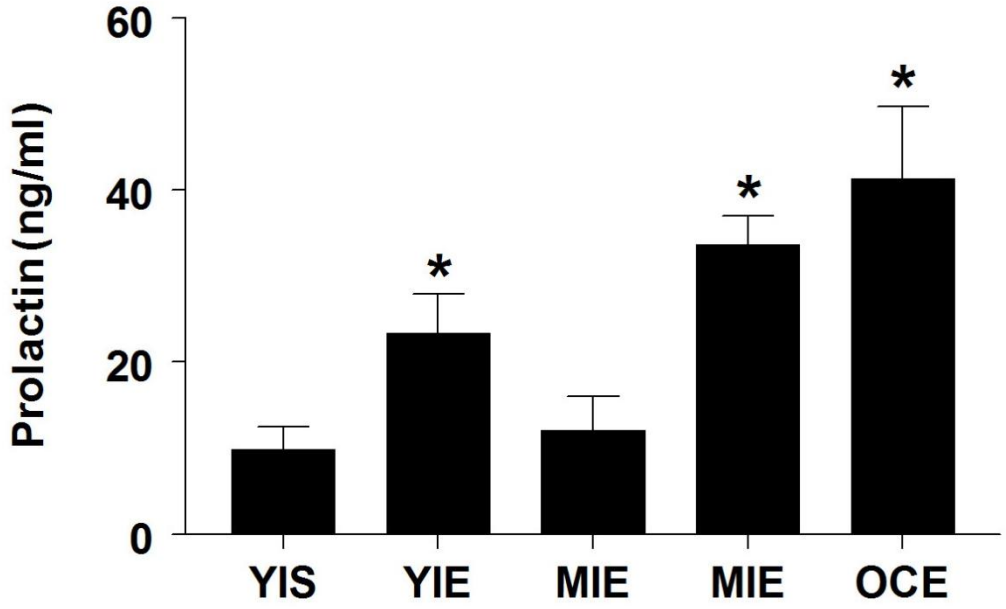


Fig. 17. A. Serum prolactin levels (mean±SE; ng/ml) in reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, rats treated with slow-release E2 pellets for 90 days (YIE and MIE), and old constant estrus (OCE) rats (18-20 mos.). * indicates significant difference from YIS ($p<0.05$).

Figure 17 (cont'd)

B

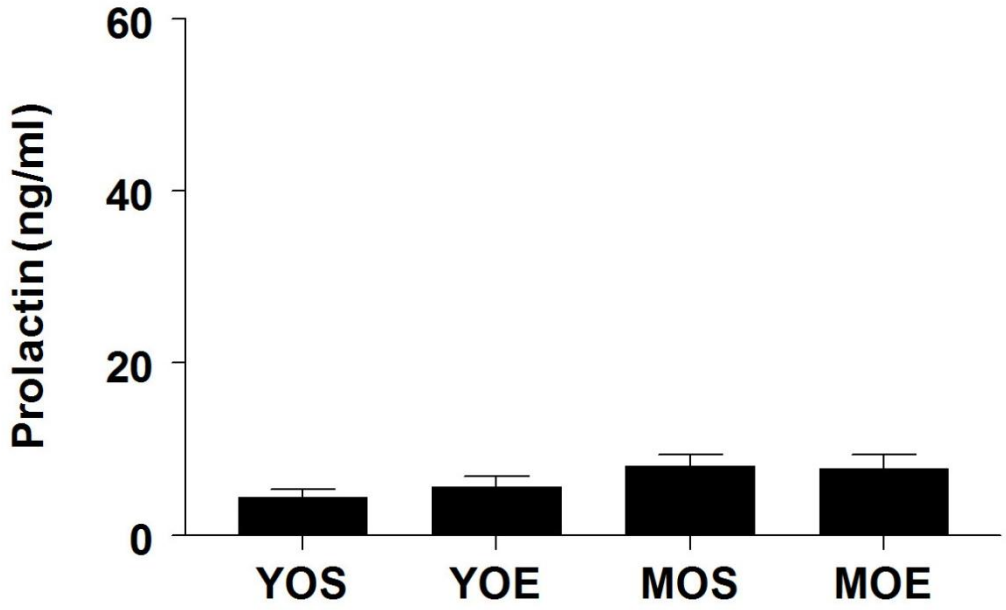


Fig 17. B. Serum prolactin levels (mean±SE; ng/ml) in ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YOE and MOE).

Median Eminence and Caudate Putamen Dopamine

Dopamine in the ME (mean±SE; pg/µg protein) of young and middle-aged intact E2 treated rats was significantly ($p<0.05$) lower (26.34±9.14 and 75.34±14.86 respectively) compared to YIS animals (11.1±4.4) **(Fig. 18a).** Dopamine levels in MIS animals (222.56±87.8) were not significantly different from that of YIS animals (99.5±19.5). However, dopamine in the ME of MOE rats (22.46±2.1) was significantly lower than that in YOS (150.6±22.3) **(Fig. 18b).** However, dopamine levels in MOS rats (145.9±67.5) and YOE rats (201.32±44.2) were not different from that of YOS rats (150.58±22.3). In the caudate putamen (CP), there was no significant difference in dopamine levels between the treatment groups **(Fig. 19)**.

Figure 18

A

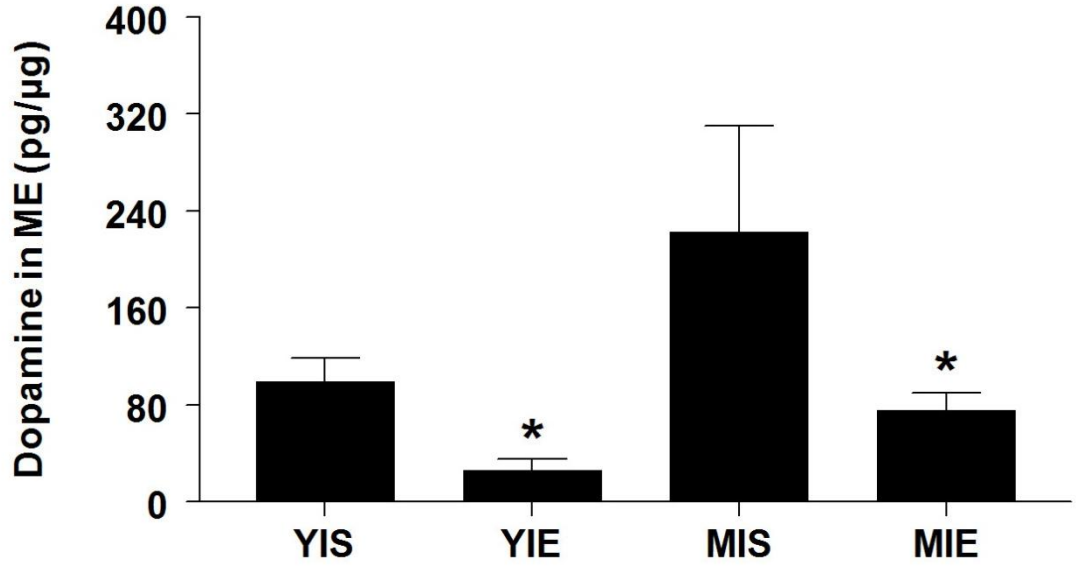


Fig. 18. A. Dopamine levels (mean±SE; pg/µg protein) in the median eminence (ME) of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YIE and MIE). * indicates significant difference from MIS ($p<0.05$).

Figure 18 (cont'd)

B

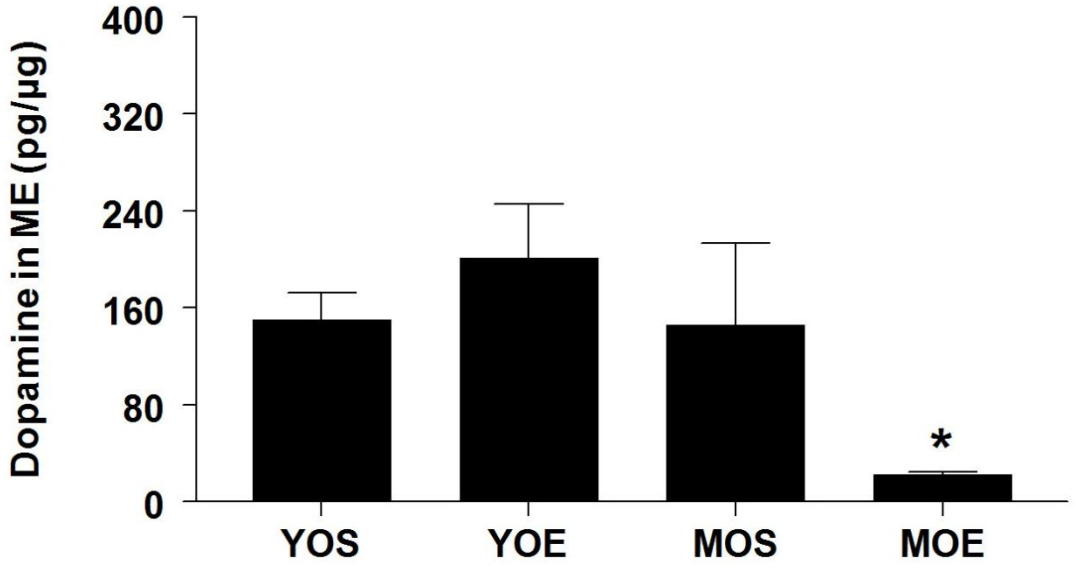


Fig 18. B. Dopamine levels (mean±SE; pg/µg protein) in the ME of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YOE and MOE). * indicates significant difference from YOS ($p<0.05$).

Figure 19

A

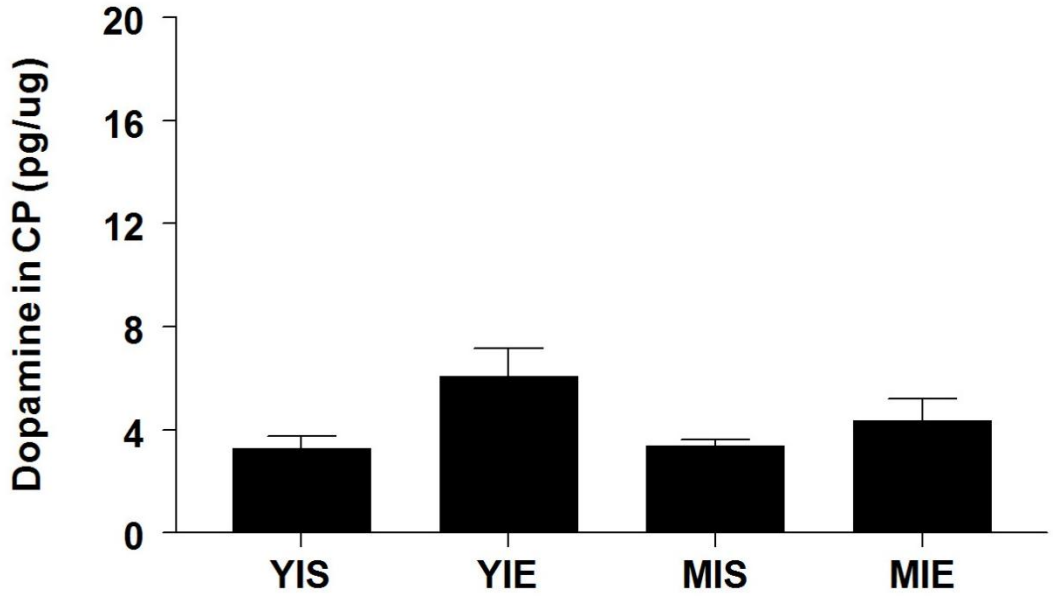


Fig. 19. A. Dopamine levels (mean±SE; pg/µg protein) in the caudate putamen (CP) of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YIE and MIE). There was no significant difference between groups.

Figure 19 (cont'd)

B

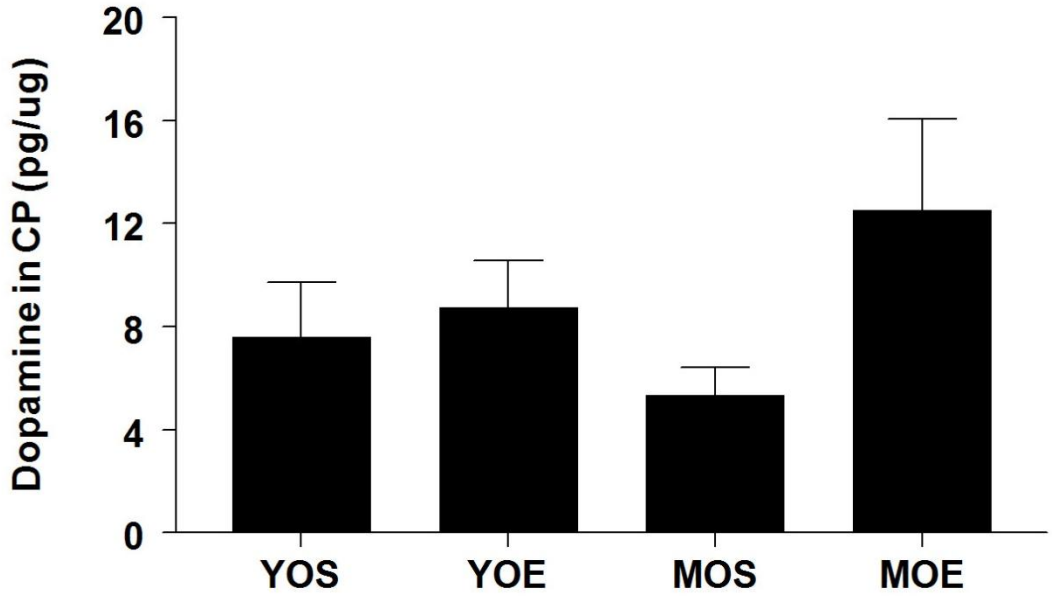


Fig 19 B. Dopamine levels (mean±SE; pg/µg protein) in the CP of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YOE and MOE). There was no significant difference between groups.

Pituitary Gland Weights

The pituitary gland weight (Mean±SE; g) was increased ($p<0.05$) in middle-aged animals, irrespective of exogenous E2 exposure (0.035±0.005 and 0.065±0.01, MIS and MIE respectively) when compared to YIS animals (0.014±0.001). However, the pituitary weight in the MIE group was higher ($p<0.001$) than that in the MIS group. **(Fig. 20a)**. The pituitary weight of MOE animals (0.028±0.004) was greater ($p<0.0001$) than that of YOS and YOE (0.015±0.001 and 0.013±0.001, respectively). Also, similar to the MIE animals, mean pituitary weight in the MOE group was greater ($p<0.05$) than that in the MOS group (0.02±0.003) **(Fig. 20b)**.

Figure 20

A

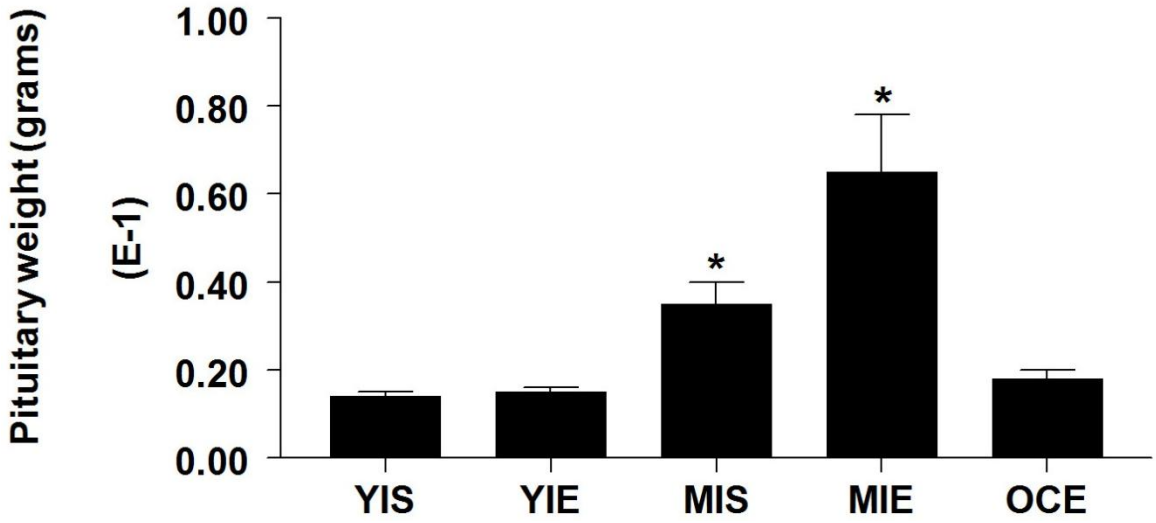


Fig. 20. A. Pituitary weights (mean±SE; g) of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, rats treated with slow-release E2 pellets for 90 days (YIE and MIE)), and old constant estrus (OCE) rats (18-20 mos.). There was no significant difference between groups. * indicates significant difference from YIS ($p<0.05$).

Figure 19 (cont'd)

B

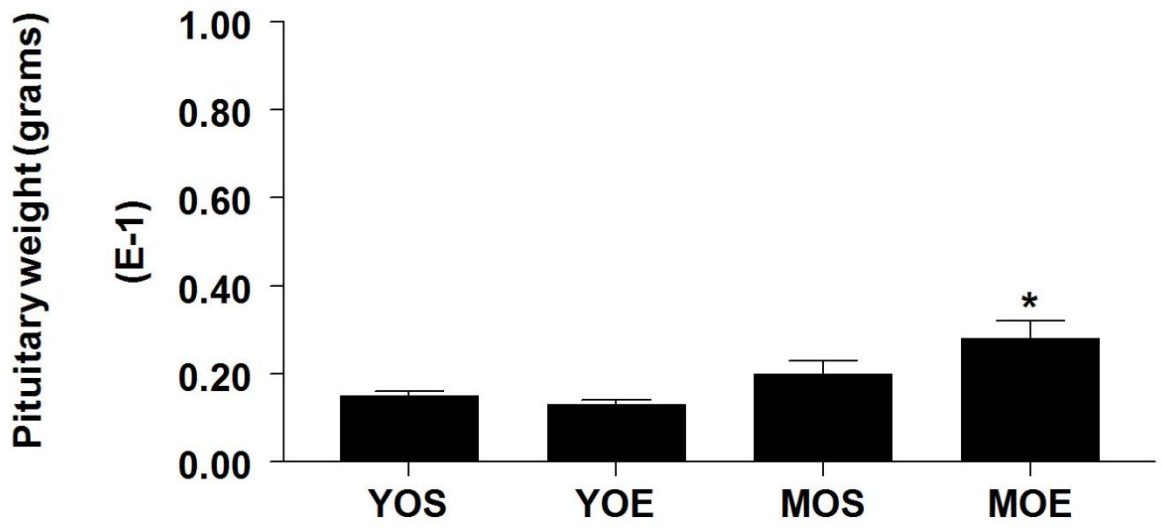


Fig 20. B. Pituitary weights (mean±SE; g) in ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YOE and MOE).

Discussion and Summary

Different forms of estrogen has been demonstrated to inhibit TIDA neuronal function in rats after varying durations of exposure [6-8]. This decrease in TIDA neuronal function is associated with estrogen-induced hyperprolactinemia and pituitary prolactinomas in rodents [5, 7, 9]. Furthermore, there is an association between hyperprolactinemia, as well as hypophyseal prolactinomas in women that use oral contraceptives for a prolonged period of time [166]. This estrogen-induced prolactin production is likely due to a disruption in TIDA activity. Though it is relatively well-established that estrogen induces hyperprolactinemia through reduced TIDA activity, this is the first study that examines the effects of such low concentrations of E2 on TIDA activity. This is important because people in today's society, are often exposed to estrogenic substances on a daily basis in some form or fashion. The rate of exposure, is often at a low level and for a prolonged period of time. [167, 168] Therefore, it is important to examine this paradigm of exposure and the effect it has on body systems.

We have previously demonstrated that exposing young intact Sprague-Dawley rats, to a physiologically low level of E2 (20ng/day) for a prolonged period of time (30, 60 or 90 days) induces hyperprolactinemia gradually and decreased dopamine concentrations in the median eminence (ME) of young intact animals in a duration-dependant fashion [169]. In the present study, we have taken this further by examining the effects of E2 on the TIDA system in middle-aged, irregularly cycling rats, ovariectomized rats and old constant estrus rats to examine the effect of aging, and the combined effect of E2-exposure and aging. We hypothesized that the E2-associated decline in the function of TIDA neurons would be exacerbated with age.

Our findings demonstrate that there does appear to be an estrogen as well as an age-associated decline in TIDA neuronal function. This is supported by our results which demonstrate that there is an age and E2–associated decline in dopamine concentrations in the arcuate nucleus, increase in serum prolactin levels, and an increase in pituitary weight, which suggests the presence of lactotroph hyperplasia or neoplasia. There was a positive association between serum prolactin and serum E2 levels, and a negative association between ME dopamine levels in young and middle-aged intact animals. Also, in ovariectomized animals, there was a similar association between prolactin and E2. Though dopamine was decreased in the ME of middle aged ovariectomized animals exposed to E2 (MOE), it was not changed in YOE animals. This is likely due to the fact that at the age of exposure (3-4 mos.) young animals had been cycling for only 1-2 months, and had therefore only been exposed to cycling endogenous E2 for 6-14 cycles before their ovaries were removed; whereas, the middle-aged animals had been exposed to E2 for approximately 56 cycles prior to being ovariectomized. Therefore, the cumulative exposure of endogenous E2 prior to ovariectomy, in addition to the exogenous exposure administered for 90 days likely had a greater impact on TIDA activity in middle aged animals than the effect of exogenous E2 exposure in young animals. We demonstrated that exposing both middle-aged and young intact rats to E2 resulted in their E2 and prolactin hormonal profiles being similar to that seen in aged OCE animals.

Besides acting through TIDA neurons, estrogens may also have a direct effect on pituitary lactotrophs to increase prolactin secretion. The increase in pituitary weight with E2 exposure indicates that there is increased cellular growth. Pituitary lactotroph hyperplasia and/or neoplasia are known to occur with E2 exposure [5]. Recent studies have reported increases in Wnt4 expression in the pituitary with estrogen exposure [170] and other studies have suggested that

estrogens might affect lactotrophs by acting through insulin-like growth factor [171], and epidermal growth factor [172]. The increase in pituitary weight in our experiment is suggestive of such a direct action of estrogen and was seen in MIS and MIE, as well as the MOE groups. This supports that there is an age-associated increase in pituitary weight, which is exacerbated upon exposure to E2 even at low levels. In contrast to middle aged animals exposed to E2, pituitary weight was not elevated in OCE animals. The reason for this unexpected result is not understood, as an increase in pituitary weight was expected to be associated with increased concentrations of serum E2 and prolactin. Furthermore, it has been previously shown that there is an increase in lacotroph cell number in OCE rats compared to mature rats of 6-7 mos of age [173]. One possibility is that there was atrophy of other subsets of cells in OCE rats which resulted in a net weight that was not significantly different from young intact sham animals. Recent studies indicate that estrogens bound to estrogen receptors can act as negative regulators of lactotrophs and may actually play a role not only in cell proliferation, but also in cell death [174]. Since OCE animals have been exposed to higher levels of endogenous estrogens for a much longer duration (~6 months) compared to E2-treated animals, there is a likelihood, that this could have contributed to a certain degree of apoptosis, and reduction in pituitary weight.

In summary, our findings indicate that chronic exposure to low levels of E2 increase serum E2 levels, decreases median eminence dopamine, increases serum prolactin, and increases pituitary weight. The effects were additive in aged animals and were greater in intact animals compared to ovariectomized rats. These findings can lead to inferences to the effect of prolonged exposure to low levels of estrogenic compounds in young as well as older individuals.

CHAPTER 5

LOW DOSE CHRONIC E2 EXPOSURE INCREASES IL-1β AND NITRATE LEVELS IN THE ARCUATE NUCLEUS AND INCREASES NITRATION OF TYROSINE HYDROXYLASE

Introduction

It is known that estrogen promotes hyperprolactinemia by inhibiting the release of dopamine from the tuberoinfundibular dopaminergic (TIDA) terminals [6-8]. However the mechanism of this effect has not been clearly elucidated. The perikarya of TIDA neurons are located in the hypothalamic arcuate nucleus (AN) and the terminals extend to the median eminence (ME) where dopamine is released [175]. Acute treatment with high doses of estrogenic compounds has been shown to incite an inflammatory response in the arcuate nucleus [163, 164]. This was marked by an increase in glial activity. Recently, our laboratory demonstrated that chronic exposures to low levels of E2 can induced gliosis as marked by an increase in glial fibrillary acid protein expression. This was accompanied by an increase in the levels of the proinflammatory cytokine, interleukin-1β (IL-1β) in the AN resulting in the generation of nitric-oxide related free radicals. This led to a reduction of tyrosine hydroxylase (TH) activity in the ME, followed by an increase in serum prolactin levels [12, 169].

Although our recent study demonstrated the various aspects of this novel hypothesis in young animals after estrogen exposure, it is not clear if a similar mechanism may play a role in aging animals. During the course of aging, rats are exposed to higher levels of endogenous estradiol once they enter the state of constant estrous and this occurs when they are about 12-14 months of age. They remain in this state for about 6 months during which time, prolactin levels increase

gradually to reach significantly higher levels during the later stages of constant estrous when the rats are about 18-20 months old[176]. This is believed to be one of the hall-marks of aging and is thought to play a key role in predisposing animals to spontaneous mammary and pituitary tumors [176]. The gradual increase in prolactin levels from early to late constant estrous signifies a gradual loss in TIDA function. However, the reason for this phenomenon and the mechanism by which this transpires has not been clearly understood.

Nitric oxide (NO) has been shown to be produced alone or in conjunction with cytokines in neural tissues, and has been implicated to play a role in pathological processes in the brain [13]. We hypothesized that E2 exposure and aging would be associated with increased proinflammatory cytokines and nitrate levels in the AN, leading to subsequent nitration of TH—the rate limiting enzyme in dopamine synthesis. Nitration of tyrosine residues in this protein would be expected to cause dysfunctional production of dopamine by TIDA neurons.

To test this, IL-1β and NO levels were assessed in the AN, and were also assessed in the substantia nigra (SN). The SN houses another set of dopaminergic neurons which extend to the caudate putamen (CP), making up the nigrostriatal dopmainergic system. This system was used to compare the findings with the dopaminergic system of our interest—the TIDA system, with another dopaminergic pathway in the brain.

Experimental Design

Animals

Young (3-4 months), middle-aged (10-12 months), and retired breeders (>12 months) female Sprague-Dawley rats were obtained from Harlan Inc. (Indianapolis, IN). Old constant estrous (OCE) rats were used for comparison. The OCE rats were from the retired breeders which were

aged in our facility. They were approximately 18-20 months of age and had greater than 10 consecutive days of vaginal smears indicative of estrus, supporting a state of acyclicity. Housing was light-controlled (lights on from 0700-1900h) and air-conditioned (23±2°C) and the rats were fed rat chow and water ad libitum. Animals were used in accordance with the NIH guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Treatment

Young and middle-aged animals (n=8) were each randomly assigned to 1 of 4 treatment groups: reproductively intact (sham-implanted); intact and implanted with a slow-release E2 pellet (20ng/day) (Innovative Research America, Sarasota, FL, USA) for 90 days; ovariectomized (OVX); or ovariectomized and implanted with an E2 pellet. Animals were sham-implanted or implanted with E2 pellets subcutaneously under light isoflurane anesthesia. A week before the end of treatment, animals were subjected to daily vaginal cytology to ensure that treated animals were in a state of constant estrous. Control animals that were cycling regulary were sacrificed along with treated animals when they were in estrous. All animals were sacrificed by rapid decapitation on the day of estrus at noon. Brains were collected and stored at -80°C.

Microdissection of Brains

Brains were sectioned at 300µm using a cryostat (Slee-Mainz, London, UK) and mounted on glass slides. The slides were transferred to a cold stage maintained at -10°C and the arcuate nucleus (AN), median eminence (ME), substantia nigra (SN), and caudate putamen (CP) were dissected with a 500 µm micropunch, using Palkovits' microdissection technique [135]. Care was taken to include individual subdivisions of all the nuclei. Tissue samples were stored at -80ºC until further analysis.

IL-1β and Nitrate Analysis in the Arcuate Nucleus and Substantia Nigra

A cell homogenizer (Kontes, Vineland, NJ) was used to homogenize the AN and SN samples in 130ul of ice cold phosphate buffer saline (PBS) [4.73g monobasic sodium phosphate; 9.74g dibasic sodium phosphate; 9g sodium chloride; autoclaved nanopure water to 1000ml], pH 7.5. Twenty (20) ul of the homogenate was saved for protein analysis using the Micro BCA protein assay (Pierce, Rockford, IL). One hundred (100) ul of additional PBS was added to the remaining 110ul of sample homogenate. The samples were used to perform both the IL-1β and NO assays. Of the 210ul of homogenate, 100ul was used in each assay as 50ul of sample was needed in duplicate. The samples were assayed for IL-1β and nitrate levels according to the manufacturer's protocol. To determine IL-1β levels, we used a commercially available rat IL-1β enzyme immunometric assay kit from Assay Designs, Ann Arbor, MI.. To determine nitrate and nitrite content, collectively, the commercially available total nitric oxide assay kit, from Assay Designs, Ann Arbor, MI was used.

Immunoprecipitation of Tyrosine Hydroxylase

We followed a previously published protocol by Ara et al for immunoprecipitating TH from brain tissue [177]. The volume of homogenate needed to obtain 50µg of protein was calculated from the protein assay, and the total volume was adjusted to 43.4 µl using lysis buffer [20mM Tris, 150mM sodium chloride (NaCl), 1mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1% TritonX, dissolved in autoclaved nanopure water]. To this, 0.5µl PMSF (100mM PMSF dissolved in isopropanol), 6µl anti-TH antibody (Millipore, Billerica, MA), and 0.1µl sodium orthovanadate (Na_3VO_4) was added. The samples were vortexed and kept at 4°C overnight. The following day, 100 µl of Protein A agarose slurry (KPL, Gaithersburg, MD) was vortexed and centrifuged briefly. The supernatant was discarded and replaced with binding

buffer (KPL, Gaithersburg, MD) at a volume of 100µl per sample. One hundred (100)µl of this agarose slurry was added to each sample and this was kept at 4°C for 1.5 hours. The agarose slurry was vortexed between addition to each sample. The tubes were centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded (the agarose beads had the antigen antibody complex bound to them). The pellets were washed with 40 µl of Elution buffer (KPL, Gaithersburg, MD) and mixed briefly with a pipette tip. This was left on ice for 5 minutes and centrifuged again at 10000 rpm for 10 minutes. The supernatant contained the immunoprecipitated TH and was divided into 2 equal fractions of 20µl each for western blot analysis.

Tyrosine hydroxylase and Nitrotyrosine Western Blot Analysis in the Median Eminence and Caudate Putamen

A cell homogenizer was used to homogenize the ME and CP samples in 60µl of lysis buffer, as mentioned above. Ten (10)µl of the homogenate was used to run a protein assay using 5µl of homogenate in duplicate. Tyrosine hydroxylase (TH) was immunoprecipitated as described above. The supernatant was mixed with 6.6µl of 4X Laemlli's loading buffer [1.0M Tris pH 8, sodium dodecyl sulfate (SDS), 40% glycerol, 4% of 0.5% bromophenolblue, 10% β-mercaptoethanol]. The samples were then left in a 90-100°C waterbath for 4-5 minutes and then placed on ice. Each sample (20µl), and 5µl of fluorescent marker (Invitrogen, Carlsbad, CA) was loaded on two 4-20% gels (NuSep, Athens, GA) and run at 60V. The proteins were transferred to nitrocellulose membrane using semi-dry blotting. The membranes were probed with either TH or nT primary antibody in TBST. The TH antibody was at a dilution of 1:1000 (30µl:30ml) + 30µl of 1% merthiolate, and the nT antibody (Sigma, St. Louis, MO) was at a dilution of 2µg/ml. The membranes were incubated with fluorescent secondary antibody (anti-rabbit IgG IRDye800

conjugated fluorescent secondary antibody, Rockland Immunochemicals, Gilbertsville, PA) at a dilution of 1:10,000 diluted in 1:1 blocking buffer:TBST. The membranes were scanned with an Odyssey scanner at a channel of 700/800nm. The blot image pixels were analyzed for quantification with Image J software version 1.44 (NIH, Bethesda, MD).

Protein Analysis

Protein concentrations in the AN, ME, SN, and CP homogenates were determined using a micro Bicinchoninic acid assay (Pierce, Rockford, IL). IL-1β concentrations were expressed as pg per µg protein and Nitrate concentrations were expressed as µM per µg protein.

Statistical Analysis

Differences between treatment and control groups were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. $P<0.05$ was considered significant.

Results

IL-1β Levels in the Arcuate Nucleus and Substantia Nigra

In both young and middle-aged, reproductively intact and E2 exposed rats, there was a significant increase in IL-1β **(Fig. 21a)** levels in the AN compared to young intact sham animals, suggesting the combined effect of endogenous and exogenous E2 is inducing a proinflammatory response in the AN. IL-1β levels (Mean±SE; pg/µg protein) in the AN of YIE (32.11±8.03) animals were significantly higher ($p<0.05$) when compared to YIS (9.78±2.6). Similar changes were observed in middle aged animals. E2 treatment also produced a significant increase in IL-1β levels in young OVX rats (29.73±6.88) compared to sham-implanted animals (15.03±4.96) **(Fig 21b)**. Although there was a trend toward an increase in middle aged OVX E2 treated rats,

the levels were not significantly different from the MOS group. In contrast to what we observed in the AN, there were no significant differences in IL-1β levels in the SN **(Fig 22)**.

Nitrate levels in the Arcuate nucleus and Substantia Nigra

Effects of E2 exposure on nitrate levels in the Arcuate nucleus of intact rats is shown in **Fig 23a**. E2 treatment increased nitrate levels (Mean±SE; μM/μg protein) in both young (3.81±0.9) and middle aged animals (4.97±1.2) compared to their sham-implanted counterparts (1.93±0.4 and 1.59±0.3 in young and middle aged animals respectively). However, there were no significant differences in the levels of nitrate in the arcuate nucleus of OVX animals **(Fig. 23b)**. In the SN, there were no differences in nitrate concentrations between any of the treatment groups **(Fig. 24)**.

Figure 21

A

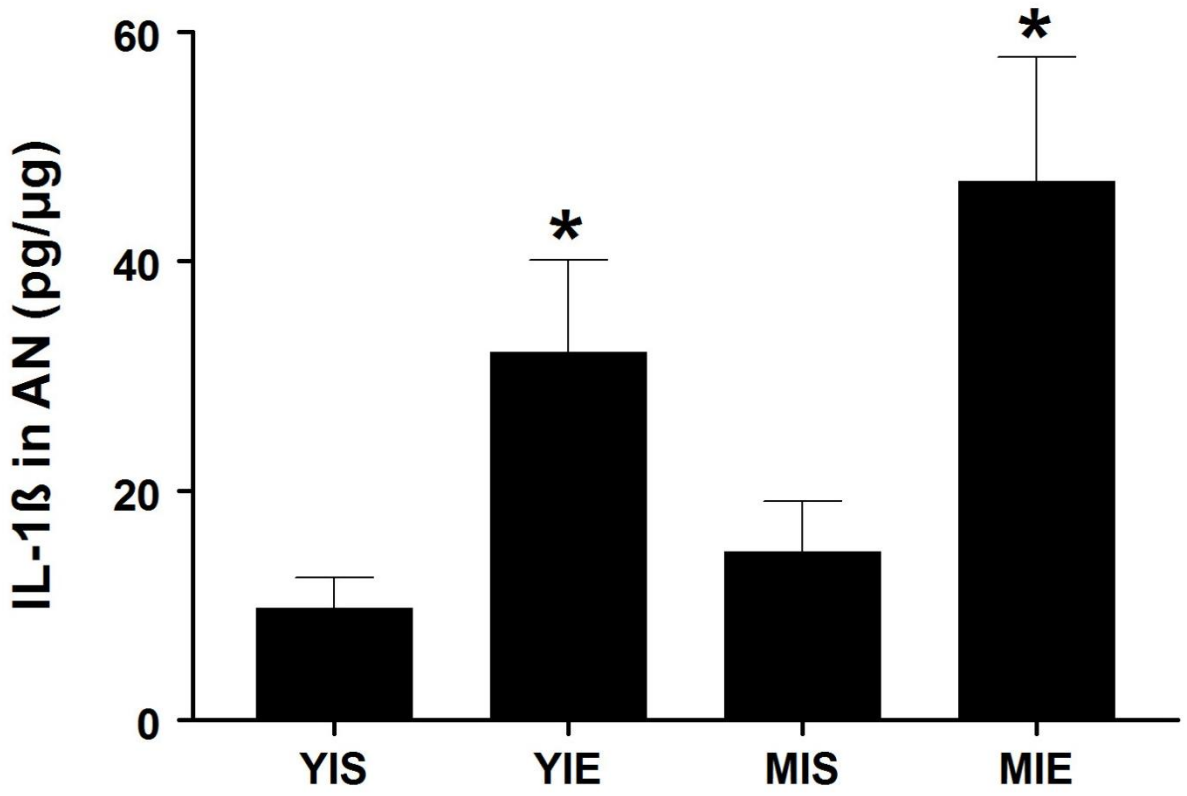


Figure 21. A. Interleukin 1-beta (IL-1β) concentrations (pg/µg±SE) in the arcuate nucleus of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YIE and MIE). * indicates significant difference from YIS ($p<0.05$).

Figure 21 (cont'd)

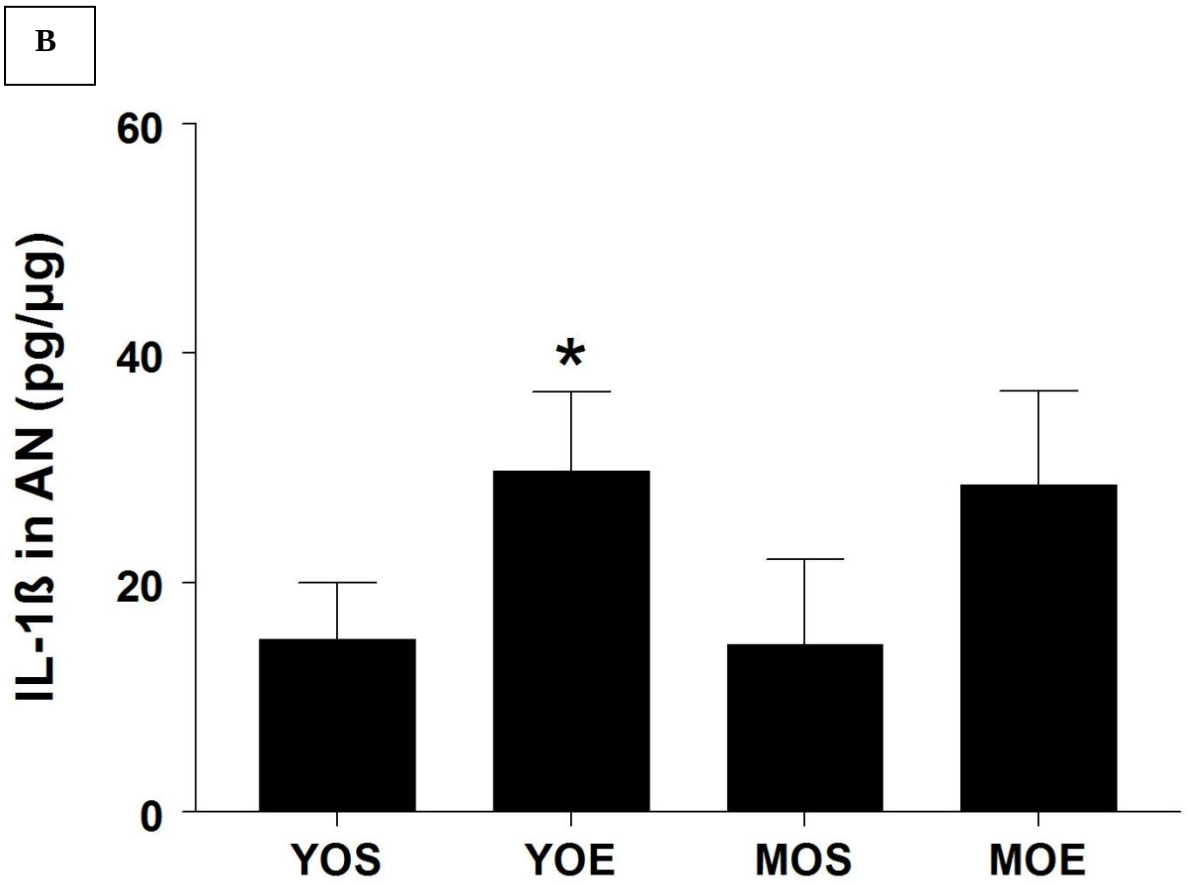


Fig. 21. B. Interleukin 1-beta (IL-1β) concentrations (pg/µg±SE) in the arcuate nucleus of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YOE and MOE). * indicates significant difference from YOS ($p<0.05$).

Figure 22

A

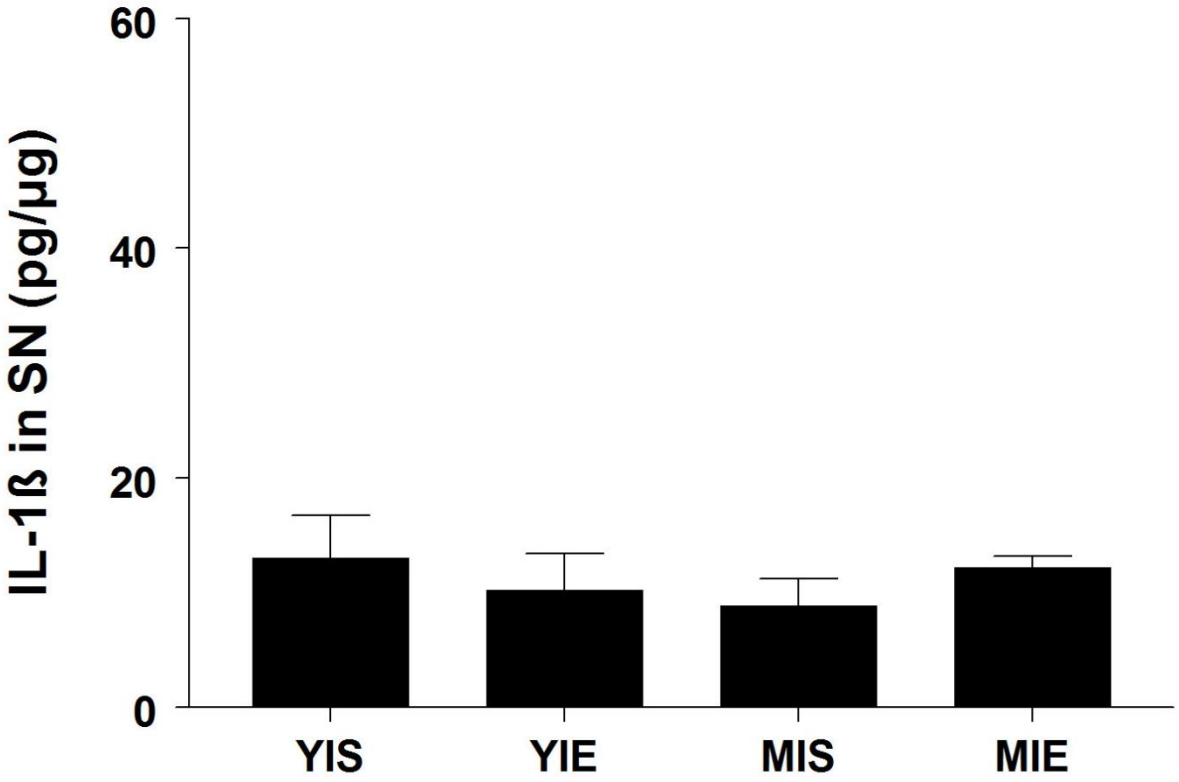


Figure 22. A. Interleukin 1-beta (IL-1β) concentrations (pg/µg±SE) in the substantia nigra of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YIE and MIE).

Figure 22 (cont'd)

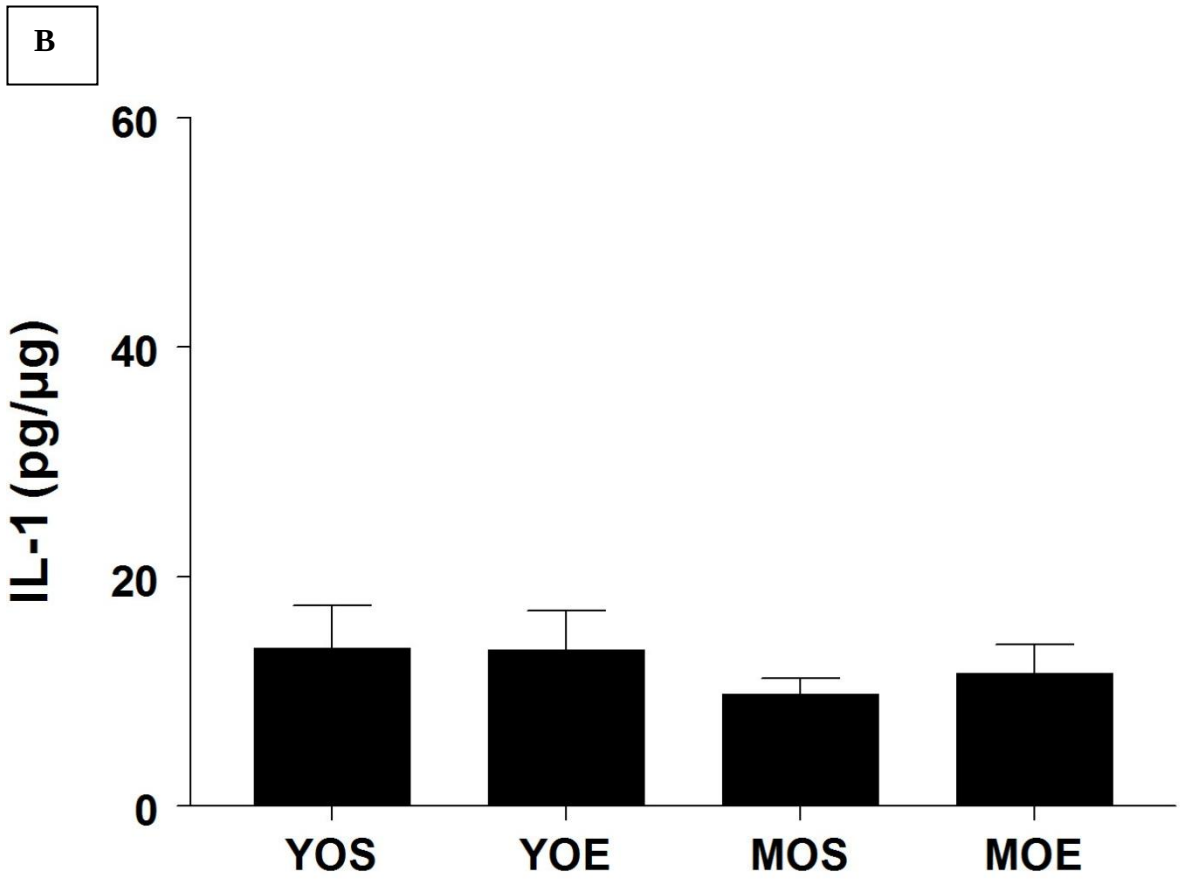


Figure 22. B. Interleukin 1-beta (IL-1β) concentrations (pg/µg±SE) in the substantia nigra of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YOE and MOE).

Figure 23

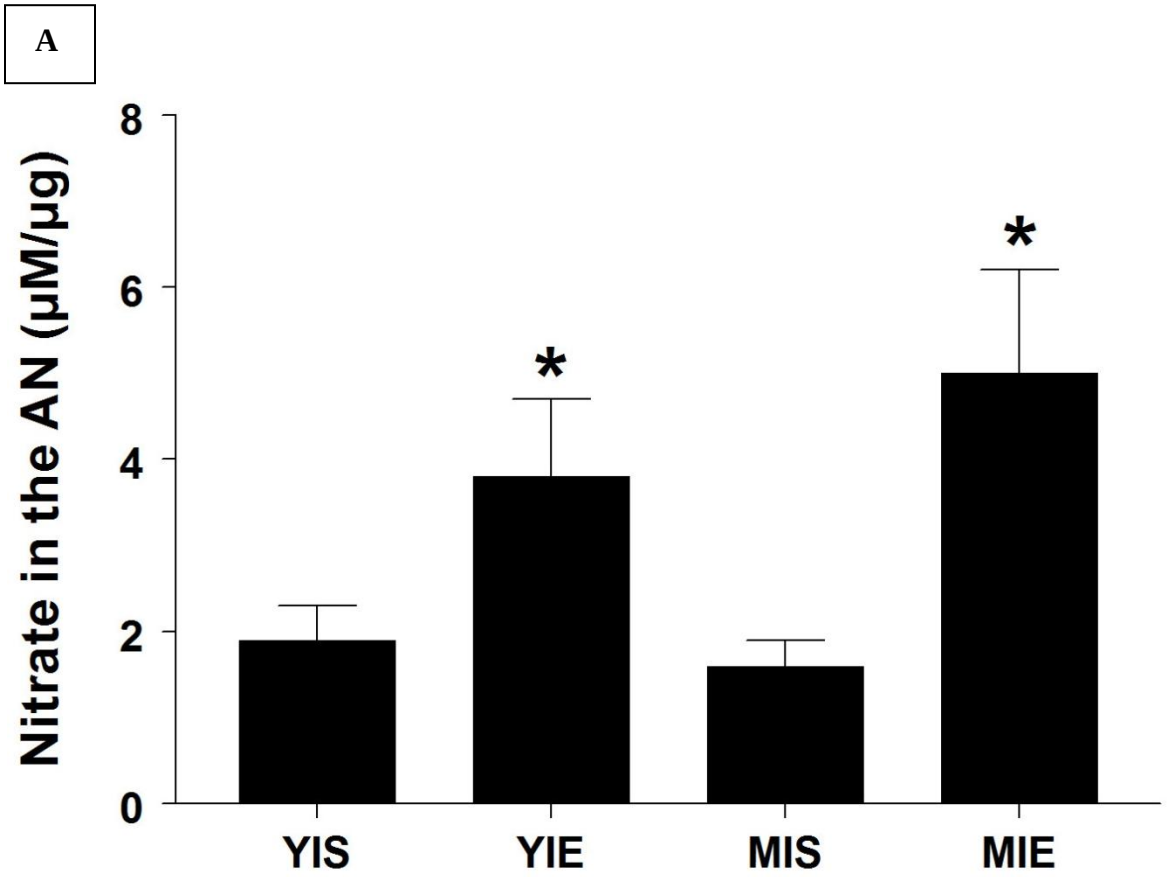


Figure 23. A. Nitrate concentrations (µM/µg±SE) in the arcuate nucleus of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YIE and MIE). * indicates significant difference from YIS ($p<0.05$).

Figure 23 (cont'd)

B

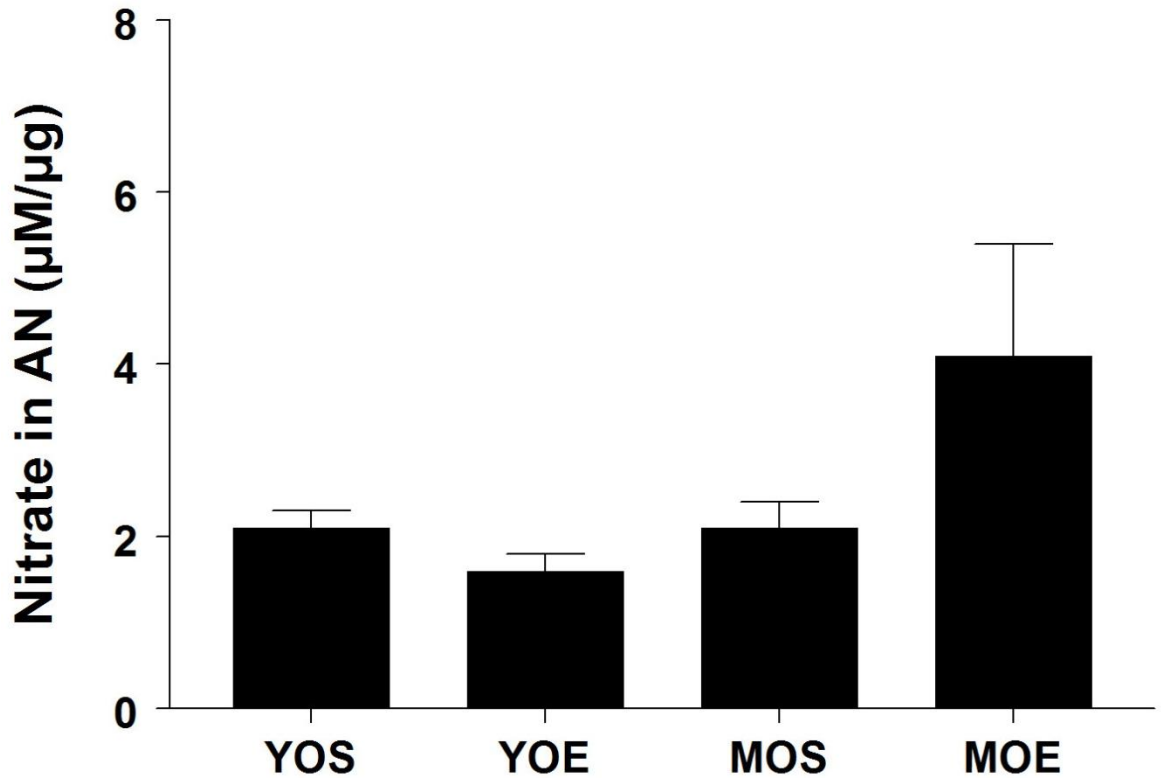


Fig. 23. B. Nitrate concentrations (µM/µg±SE) in the arcuate nucleus of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YOE and MOE).

Figure 24

A

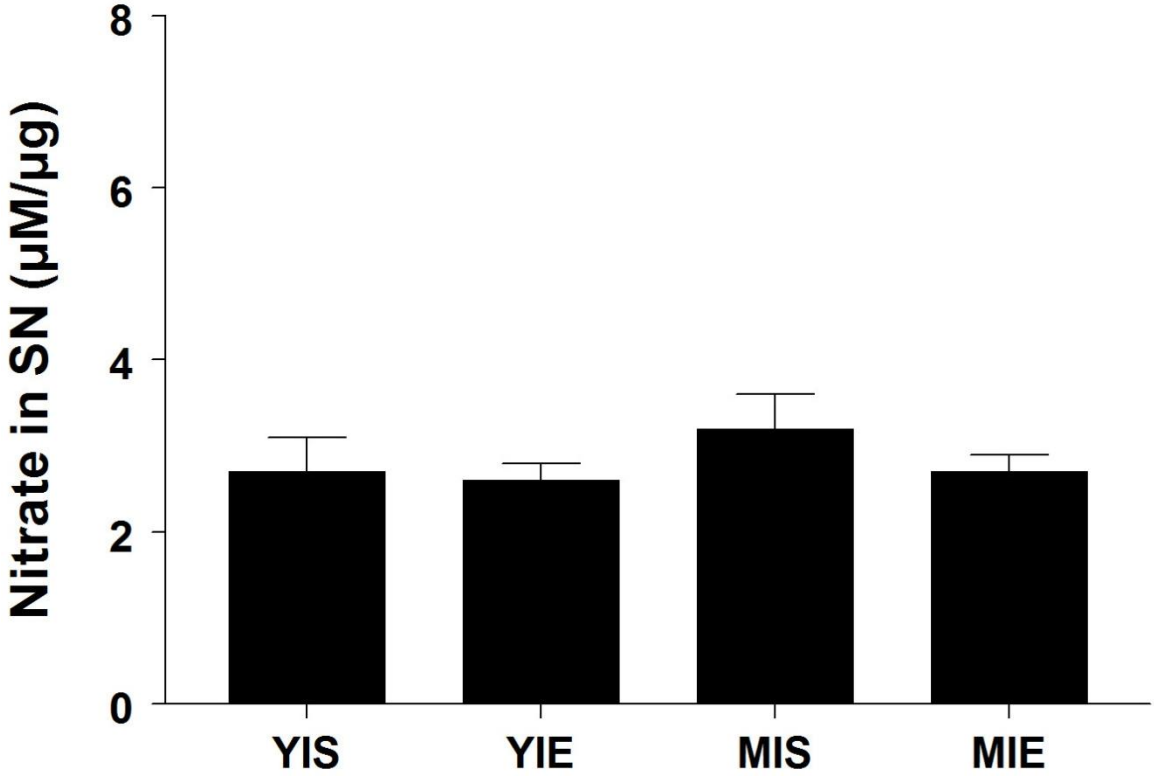


Figure 24. A. Nitrate concentrations (µM/µg±SE) in the substantia nigra of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YIE and MIE).

Figure 24 (cont'd)

B

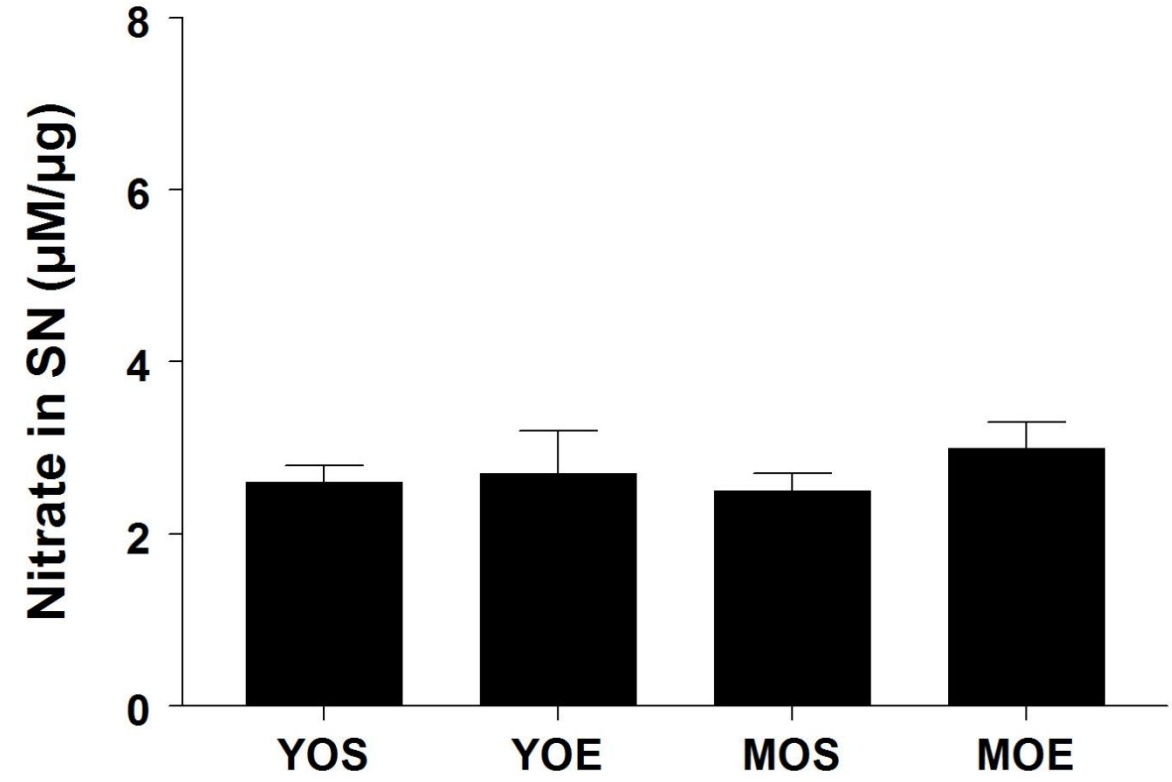


Fig 24. B. Nitrate concentrations (µM/µg±SE) in the substantia nigra of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YOE and MOE).

Nitrotyrosine to Tyrosine Hydroxylase Ratio in the Median Eminence and Caudate Putamen

In both young and middle-aged, intact and ovariectomized rats, there was an E2-associated increase in tyrosine hydroxylase nitration, characterized by an increased ratio of nitrotyrosine to tyrosine hydroxylase in the ME of E2-exposed rats **(Fig. 25A and 5B)**. There was also an increase in nitrotyrosine in the ME of middle-aged intact sham rats compared to young intact sham. There was also an increase in nitrotyrosine in the ME of middle-aged intact sham rats as well as OCE rats as compared to young intact sham **(Fig. 25A)** suggesting there is also an age-associated increase in nitration of tyrosine residues in the ME. There was no appreciable nitrotyrosine visualized on the immunoblots of samples from CP in any of the treatment groups.

Figure 25

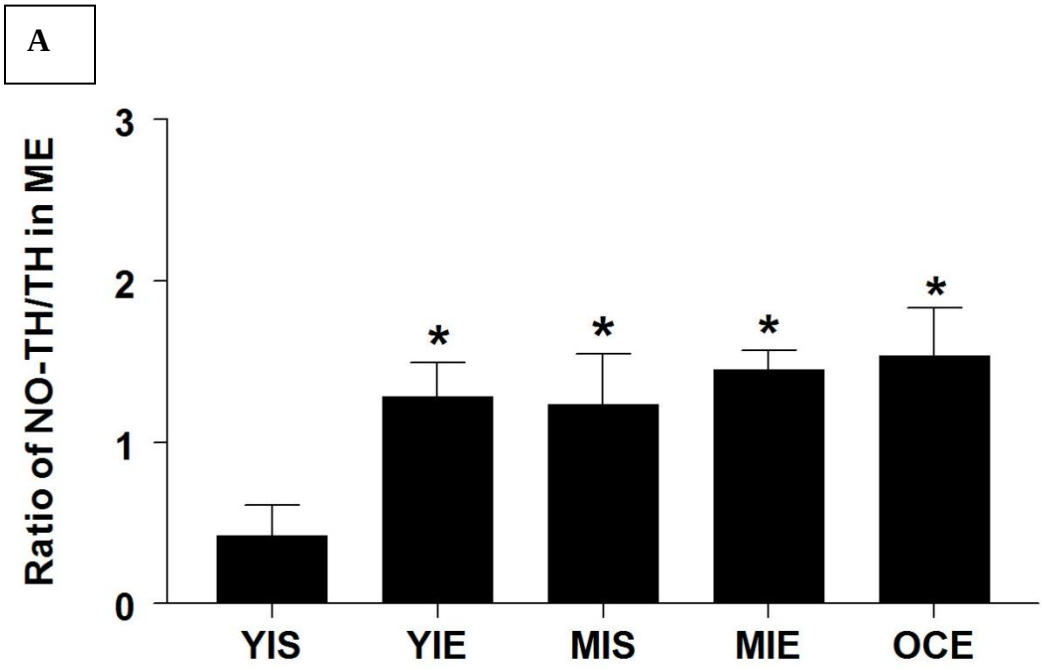


Fig. 25. A. The ratio of nitrotyrosine to tyrosine hydroxylase in the median eminence of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) sham (YIS and MIS, respectively) rats, rats treated with slow-release E2 pellets for 90 days (YIE and MIE), and old constant estrus (OCE rats (18-20 mos.). * indicates significant difference from YIS ($p<0.05$).

Figure 25 (cont'd)

B

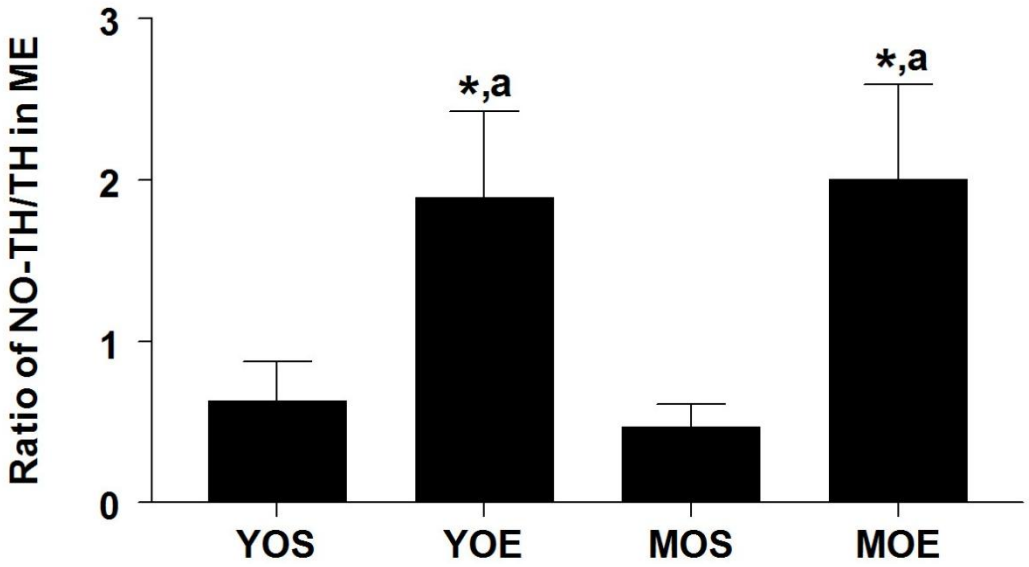


Fig 25. B. The ratio of nitrotyrosine to tyrosine hydroxylase in the median eminence of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) sham (YOS and MOS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YOE and MOE). * indicates significant difference from YOS and 'a' indicates significant difference from MOS ($p<0.05$).

Discussion and Summary

In the previous chapter, we demonstrated that exposure to low levels of E2 (20ng/day) for 90 days produced a significant decrease in dopamine levels in the median eminence, that was associated with a marked increase in prolactin secretion. In this chapter, we take these results a step further in our novel hypothesis and demonstrate that the reduction in dopamine is associated with an increase in pro-inflammatory reactions in the arcuate nucleus that is associated with an increase in nitric-oxide related free radical synthesis that leads to the nitration of tyrosine hydroxylase. These results are in accordance with our recently published study in young intact rats. We have previously demonstrated that in young intact Sprague-Dawley rats, E2-induced hyperprolactinemia and decreased dopamine release in the median eminence (ME) is associated with glial activation—characterized by an increase in the astrocyte-specific marker glial acidic fibrillary protein (GFAP), increased production of the proinflammatory cytokine IL-1β, and increased production of nitric oxide in the arcuate nucleus (AN) [169]. We propose that this may be a mechanism by which estrogen disrupts TIDA function.

In the present study, we have further examined the effects of E2 on the TIDA system and have incorporated middle-aged, irregularly cycling rats and old constant estrus rats to examine the effect of aging, and the combined effect of E2-exposure and aging, on the function of this dopaminergic system. We proposed that there would be an E2 dependant decline in the function of TIDA neurons and that this would be exacerbated with age. We hypothesized that low dose, chronic E2 would inhibit TIDA dopamine production and induce hyperprolactinemia through increased production of the proinflammatory cytokine, IL-1β, from E2-induced glial activation in the AN.

Cytokines and nitrates are known to play a role in neurodegenerative processes [178-180]. Major cytokines that are produced in the central nervous system include IL-1β, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [181]. We have focused on IL-1β, as it is one of the primary cytokines shown to be induced in neural tissue *in vitro* [181] and *in vivo* [182, 183]. In one study, intracerebroventricular (icv) administration of LPS induced transient IL-1β immunoreactivity in microglial cells within the AN with a subsequent reduction in TH activity in the ME, followed by an increase in serum prolactin levels [12]. Similar to the implication of cytokines in neurodegeneration, NO production has also been implicated to play a role in pathological processes in the brain—alone or in conjuction with cytokines. It has been shown that upon exposure to a pro-inflammatory stimulus (LPS and interferon-gamma (IFN-γ) stimulated microglia produced NO [184]. Additionaly, activation of cultured microglial cells with LPS, caused production of cytokines as well as NO [181]. Furthermore, direct stimulation of distinct areas of the brain, *in vivo*, with inflammatory cytokines, have been shown to produce iNOS derived NO [13]. There has been controversy as to the source of the free radical NO production. Some authors have demonstrated that astrocytes are the source [185]; whereas, others have demonstrated that microglia, and neither astrocytes nor neurons are the source [184]. We have demonstrated that in association with increased NO production in the AN, there is an associated increase in GFAP [169], suggesting astrocytes may be a source of the NO production, as GFAP is an astrocyte-specific marker.

A byproduct of NO production is peroxynitrite ($ONOO^-$). It is a product of a reaction between NO and superoxide (O_2^-). Peroxynitrite is a powerful oxidant that can induce nitration in protein residues. Tyrosine residues are highly susceptible to nitration. [186-189] Therefore, the NO produced in the AN, in the presence of O_2^-, could result in the formation of $ONOO^-$ and

subsequent nitration of tyrosine residues within the enzyme tyrosine hydroxylase (TH). TH is the rate-limiting enzyme in dopamine biosynthesis and it utlilizes molecular oxygen and a co-factor, tetrahydrobiopterin to convert L-tyrosine to L-DOPA. The activity of this enzyme is regulated by a domain located at its amino terminal that is made up of 150 aminoacids. There are 4 serine residues: Ser 8, Ser 19, Ser 31 and Ser 40 that are important for its activity[190]. Activation of this enzyme requires phosphorylation of these serine residues and dephosphorylation by two phosphatases. Nitration of tyrosine residues especially at the active site of the enzyme is believed to cause steric hindrance and interfere with ligand binding, thereby limiting the activity of the enzyme. NO can also cause glutathionylation of cysteine residues further interfering with the activity of this enzyme [191].

We demonstrated that there was a significant increase in the ratio of nitrotyrosine to tyrosine hydroxylase in the AN of young and middle-aged E2-exposed animals whether they were intact or ovariectomized. There was also an age-associated increase in nitrotyrosine in the intact animals irrespective of exogenous E2 exposure, as nitrotyrosine expression was increased in middle-aged intact sham and OCE rats compared to young intact sham animals. The presence of nitrotyrosine suggests that the tyrosine moieties within the tyrosine hydroxylase protein were nitrated and are highly dependent on E2 exposure, regardless of whether the E2 was endogenous or exogenous. The increase in nitration observed with aging suggests that there could be ongoing oxidative changes in the arcuate nucleus with age that may or may not be E2 dependant. When combined with the additive effect of exogenous E2, the effect on nitration of TH is amplified. Our findings demonstrate that there does appear to be an estrogen and age-associated increase in nitrate production which is further characterized by an increase in the nitrotyrosine to

tyrosine hydroxylase ratio in OCE rats, in concert with their increased E2 and prolactin concentrations.

In summary, IL-1β and NO were increased in the AN of animals exposed to a low dose of E2 for 90 days, as compared with controls. This was associated with an increase in tyrosine nitration in the ME of young and middle-aged E2-exposed animals. This free radical and cytokine increase was also positively associated with circulating prolactin, and inversely associated with ME levels of DA. These findings corroborate our hypothesis and suggest that chronic exposure to low-dose estrogen may affect TIDA activity by initiating a proinflammatory response in this brain region leading to nitration of TH and subsequent dysfunction and reduced dopamine production. This E2-associated effect may be exacerbated with age.

CHAPTER 6

LOW DOSE CHRONIC ESTRADIOL EXPOSURE ALTERS PROINFLAMMATORY GENE EXPRESSION IN THE ARCUATE NUCLEUS

Introduction

Estrogen is generally considered to be neuroprotective and anti-inflammatory [69, 192, 193]. However, this conclusion has been primarily based on *in vitro* studies [193-195] and studies looking at the effects of acute exposure to estrogen [193, 195]. Furthermore, the effect of E2 on the hypothalamus has not been widely explored, and most conclusions of neurogenic responses to E2 have been based on studies examining non-hypothalamic regions. Prolonged exposure to estrogen can cause a proinflammatory response in macrophages [82]. In the previous two chapters we have demonstrated that E2 exposure can increase IL-1β and NO levels in the arcuate nucleus, but not in the SN. Moreover, this effect is exacerbated with age. This is corroborated by a recent study from our lab where we demonstrated this phenomenon in young cycling rats [169]. This further supports the proinflammatory effect of long term E2 exposure.

IL-1β is only one of the many cytokines that are involved in an inflammatory reaction. Inflammation involves several other cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), interferons etc. Moreover, the increase in NO levels is probably due to an increase in the activity of inducible nitric oxide synthase that plays an important role in the generation of nitric-oxide related free radicals [196]

In this experiment, we wanted to examine the possibility that E2 causes up regulation of proinflammatory genes that may incite local hazardous cellular conditions in the AN which could alter the function of TIDA neurons. We used RNA extraction, RT-PCR, and qPCR to

determine the expression of iNOS and IL-1β mRNA, in addition to the mRNA expression of other proinflammatory genes, which included interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), cyclooxygenase 2 (Ptgs2), monocyte chemoattractant protein (CCL2) and lymphotoxin A (Lta).

.Experimental Design

Animals

Young (3-4 months of age) and middle-aged (10-12 months of age) female Sprague-Dawley rats were obtained from Harlan Inc. (Indianapolis, IN). Housing was light-controlled (lights on from 0700-1900h) and air-conditioned (23±2°C) and the rats were fed rat chow and water ad libitum. Animals were used in accordance with the NIH guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Treatment

Young and middle-aged animals (n=5) were each randomly assigned to 1 of 4 treatment groups: reproductively intact (sham-implanted) (YIS and MIS, respectively); intact and implanted with a slow-release E2 pellet (20ng/day) (YIE and MIE, respectively) (Innovative Research America, Sarasota, FL) for 90 days; ovariectomized (YOS and MOS, respectively); or ovariectomized and implanted with E2 pellets (YOE and MOE, respectively). All animals were sacrificed by rapid decapitation on the day of estrus at noon as described in earlier chapters. Estrus was determined by cytological examination of vaginal smears obtained on the morning of sacrifice. Brains, serum, and plasma were collected and stored at -80°C.

mRNA Extraction

Brains were sectioned at 300µm using a cryostat (Slee-Mainz, London, UK) after pretreating the blades, glass slides etc. with RNAse Zap (Ambion Inc., Austin, TX). The arcuate nucleus (AN) and substantia nigra (SN) were dissected with a 500 µm micropunch, using the Palkovits' microdissection technique [135]. Messenger RNA (mRNA) was isolated using the MELT^{TM} Total Nucleic Acid Isolation System (Ambion, Austin TX) and its established protocol. The concentration of the isolated mRNA (ng/µL) was quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

RT and qRT-PCR

The RT^2 First Strand Kit (SABiosciences, Frederick, MD) was used to perform reverse transcription (RT) PCR to isolate complementary DNA (cDNA) from the mRNA. The volume of mRNA required to obtain 400 ng of mRNA was calculated and used for the cDNA conversion. The cDNA samples were then used to perform quantitative real-time PCR (qRT-PCR). RT^2 Real-Time PCR SYBR Green/ROX Master Mix (SABiosciences, Frederick, MD), cDNA samples, and the appropriate amount of RNA nuclease-free water were combined. The volumes/well equated to 12.5 µL of PCR master mix, 2 µL of cDNA, and 10.5 µL of water. Twenty-five (25) µL of sample were added to each well.

A toll-like receptor gene array was used (SABiosciences, Frederick, MD) and samples from a YIS animal and a YIE animal were analyzed. Subsequently, a customized gene array was established using known pro-inflammatory genes, with gene selections partially based on the results of the toll-like receptor array (SABiosciences, Frederick, MD). The following genes were included in the array: interleukin 1 beta, interleukin 6, tumor necrosis factor alpha, lymphotoxin A, chemokine ligand 2 (aka: monocyte chemoattractant protein), nitric oxide synthase 2 (aka:

inducible nitric oxide synthase), beta actin, ribosomal protein L13A, lactate dehydrogenase A. After addition of the cDNA samples to the array plate, the PCR plates were centrifuged at 1500 rpm for 1 minute and PCR was performed using the following 2-step cycling program: 1 cycle at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. qRT-PCR and gene products quantification were performed with the 7500 Real Time PCR machine (Applied Biosystems, Foster City, CA).

Statistical Analysis

Fold change in gene expression was determined using the 2^-ΔΔCT method. The fold change in expression of each gene was determined based on comparison with the respective control groups (i.e., among intact animals, estradiol treated groups were normalized to the young intact sham group. Similarly, among ovariectomized animals, the estradiol treated groups were normalized to the young ovariectomized sham group). The difference in fold change between groups was compared using a one way ANOVA and post hoc Fisher's Least Square Difference analysis. To examine the possible interaction between age and treatment, a two way ANOVA was also performed.

Results

The expression pattern of proinflammatory genes in response to chronic exposure to E2 in the AN of intact animals are shown in **Figure 26**. Fold change for all the genes in the intact animals were normalized to the young intact group. Estradiol treatment produced a significant upregulation in iNOS mRNA expression in young animals (116 fold, $p<0.05$) while no change was seen in middle-aged (MA) animals. In contrast, IL-1β expression was significantly upregulated (2 fold, $p<0.05$) in MA E2 exposedanimals but not in young animals when compared to their sham counterparts. Chronic E2 exposure also produced a marked upregulation

of IL-6 in MA animals (16 fold, $p<0.05$), which is significantly different from young E2 treated animals. No changes were observed in the expression of TNF-α, CCL2, Ptgs2 and Lta with E2 treatment. In order to determine the individual effects of E2 treatment and aging, Two-way ANOVA was performed. Aging produced a significant upregulation of IL-6 expression ($p\leq0.05$). Estrogen treatment alone had a significant effect on iNOS expression ($p\leq0.05$).

Figure 26

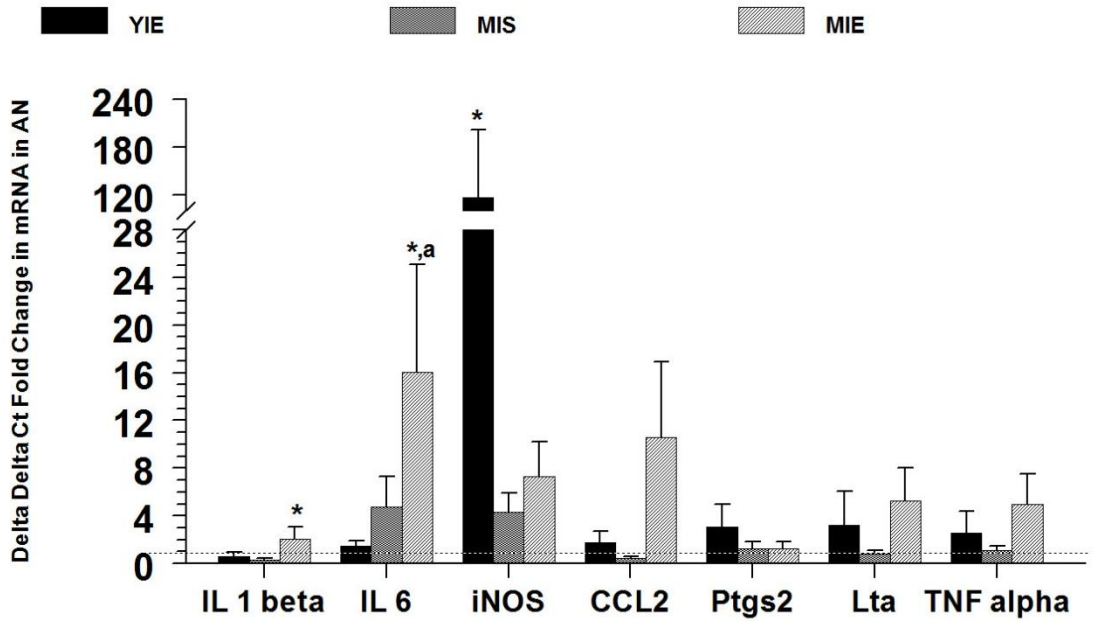


Figure 26. Expression of inflammatory genes in the AN of reproductively intact young (3-4 mos.) and middle-aged (10-12 mos.) rats treated with slow-release E2 pellets for 90 days (YIE and MIE) and middle-aged sham rats (MIS). The expression of these genes were normalized to young intact sham rats and the results were expressed as delta delta Ct fold change. * indicates significant difference from YIS, and a indicates significant difference from YIE ($p<0.05$).

Changes in the expression of inflammatory genes in the arcuate nucleus of ovariectomized (ovx) animals exposed to E2 are shown in **Figure 27**. Fold change for all the examined genes in the ovx animals were normalized to young ovx sham group. Interleukin-6, iNOS and TNFα were significantly upregulated (3 fold, 2 fold and 7 fold respectively, $p<0.05$) in the MOS group compared to the YOS group. Interestingly E2 treatment significantly ($p<0.05$) decreased the expression of iNOS, CCL2 and TNFα in MOE animals compared to their controls. Two-way ANOVA analysis revealed significant aging x estrogen treatment interaction in the downregulation of CCL2 and iNOS ($p<0.05$). There were no significant differences between treatment groups in the expression of the examined genes within the SN (**Figure 28**).

Figure 27

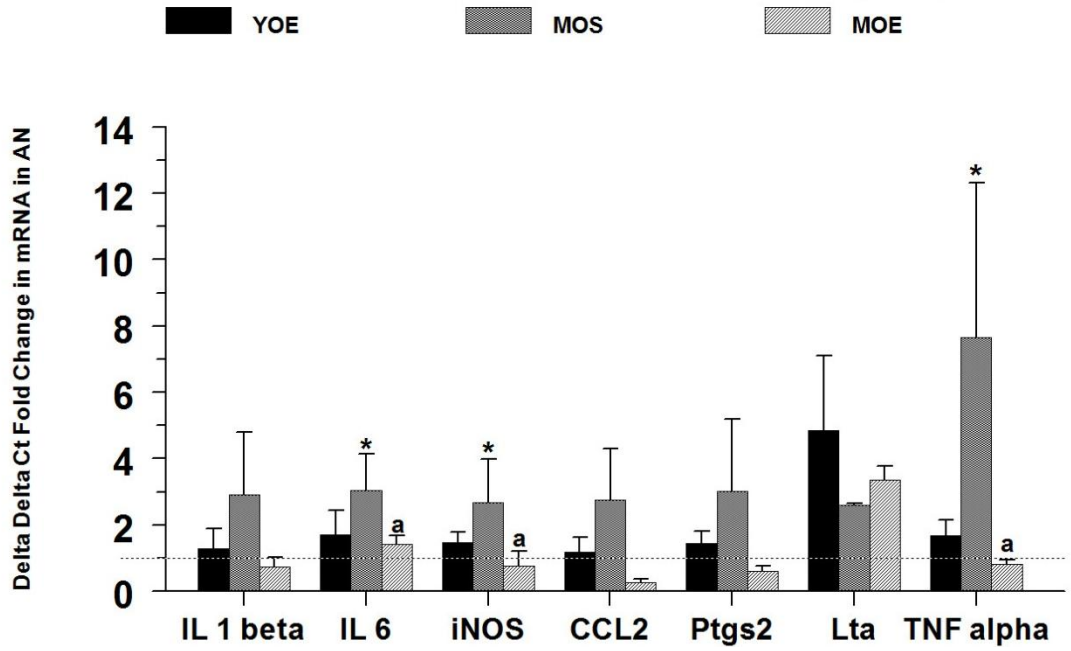


Figure 27. Expression of inflammatory genes in the AN of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) rats treated with slow-release E2 pellets for 90 days (YOE and MOE) and middle-aged sham rats (MOS). The expression of these genes were normalized to young ovariectomized sham rats and the results were expressed as delta delta Ct fold change. * indicates significant difference from YOS, and a indicates significant difference from MOS ($p<0.05$).

Figure 28

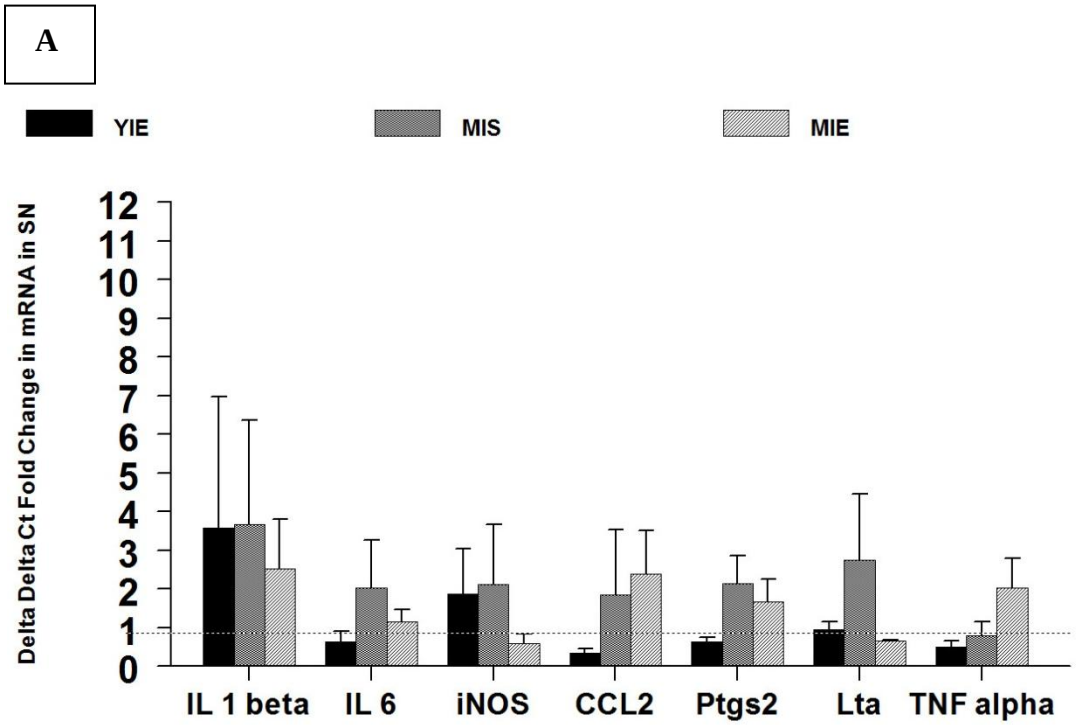


Figure 28. A. Expression of inflammatory genes in the SN of intact young (3-4 mos.) and middle-aged (10-12 mos.) rats treated with slow-release E2 pellets for 90 days (YIE and MIE) and middle-aged sham rats (MIS). The expression of these genes were normalized to young intact sham rats and the results were expressed as delta delta Ct fold change.

Figure 28 (cont'd)

B

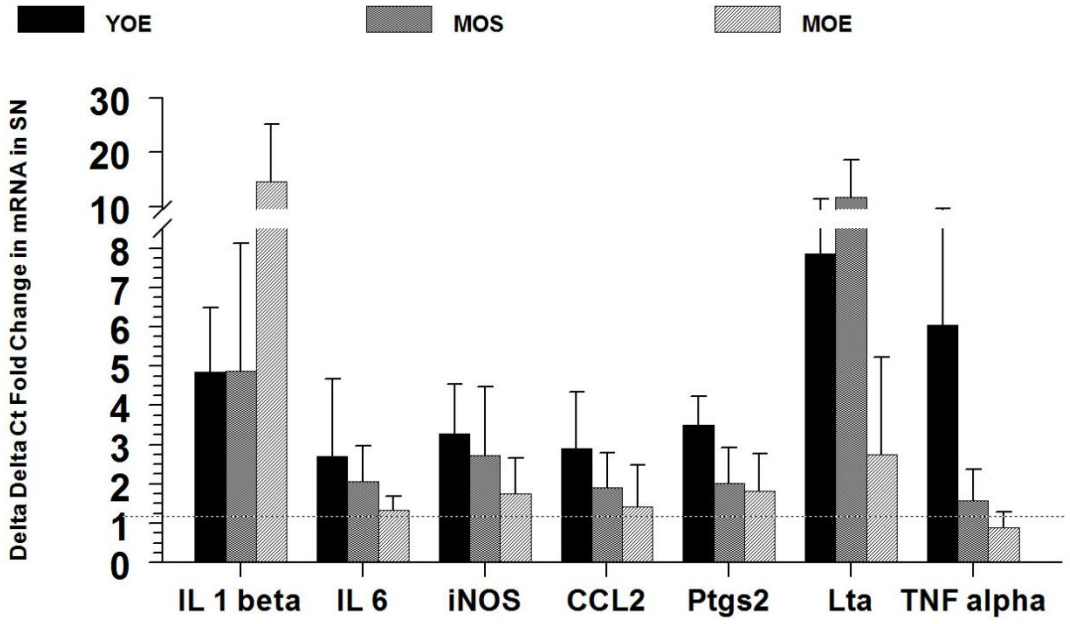


Fig 28. B. Expression of inflammatory genes in the SN of ovariectomized young (3-4 mos.) and middle-aged (10-12 mos.) rats treated with slow-release E2 pellets for 90 days (YOE and MOE) and middle-aged sham rats (MOS). The expression of these genes were normalized to young ovariectomized sham rats and the results were expressed as delta delta Ct fold change.

Discussion and Summary

Both chronic E2 treatment and aging per se were capable of producing significant changes in the expression of multiple genes in the AN. In contrast, profound transcriptional effects were not noticed in the SN. This is not unexpected, as the AN is known to be more sensitive to the effects of E2 [197]. In young animals iNOS was significantly increased in intact animals exposed to E2. However, chronic E2 treatment produced upregulation in more cytokine genes in middle aged animals compared to the young animals. Whether or not the animal was ovx also determined the effect of E2. While IL-1 and IL-6 were upregulated in the middle aged intact estradiol group; IL-6, iNOS and TNF-alpha were upregulated in the middle aged ovariectomized estradiol group. The general trend appears to be that in the middle aged animals whether intact or ovariectomized, estradiol treatment produced a significant increase in inflammatory gene expression.

A wide range of studies support the anti-inflammatory effects of E2 on the brain [198, 199]. However, it appears that the effect of E2 on inflammatory mediators in the brain also depends on age. One study reported a reduced anti-inflammatory effect of estradiol on cerebrovascular inflammation in the brains of 30-month old female rats when compared to 4 month old counterparts[200]. In isolated microglial cultures from young female rats estrogen produced an anti-inflammatory effect, which was absent in microglial cultures from 13 month old female rats. In fact, some inflammatory markers were upregulated in response to E2 treatment [201]. Moreover, basal inflammatory responses were higher in the aged animals when compared to young in the above studies, which was also observed in our study. Thus our findings are in concert with similar studies that consider the age factor in evaluating the effects of E2.

The fact that E2 exposure produced a very region specific effect on inflammatory gene expression adds strength and consistency to the findings from the previous chapters. The effect is specific to the arcuate nucleus and not to the SN. Although it is unclear if E2 can increase inflammatory gene expression in other parts of the brain, it definitely appears to do so in the AN. Many studies have reported over and over again that E2 may have an anti-inflammatory effect and is actually beneficial in preventing re-perfusion injury after stroke or traumatic brain injury [202]. Estrogens are believed to regulate remyelination, control edema formation, and modulate the inflammatory response after injury. Moreover, estrogens stimulate the release of certain growth factors and promote neuronal survival [203]. Although it is likely that E2 could have beneficial effects, we wanted to investigate the mechanisms by which E2 suppresses TIDA function. Our results indicate that E2 does indeed increases the levels of specific cytokines and enzymes that promote inflammation in the brain.

The increase in IL-1β gene expression and iNOS expression with E2 exposure in young animals correlates well with the results from the previous chapter that indicate an increase in both IL-1β and iNOS levels in the arcuate nucleus.. In contrast to what was observed in young animals, cytokine expression increased in middle aged sham-implanted animals indicating an age-effect. However, when these animals were treated with estrogen, it produced a down-regulation of most cytokine genes corroborating with earlier studies that assign a protective role for estrogens. However, this difference in gene expression does not correlate with cytokine protein levels or nitrate levels observed in previous chapters. The reason for the reduction in gene expression with estrogen treatment in middle-aged rats is not clear. It could reflect an automatic down-regulation due to increased levels of the protein. Further studies are needed to clearly understand the reason why this is happening.

In conclusion, chronic exposure to physiological levels of E2 produced predominantly inflammatory changes in the AN of female rats and this effect was compounded by proinflammatory changes seen in the brain as a result of aging.

CHAPTER 7

LOW DOSE CHRONIC E2 EFFECTS ARE MEDIATED THROUGH ER-α AND AFFECTS iNOS KNOCK-OUT MICE LESS THAN WILD TYPE MICE

Introduction

Results from the previous chapters indicate that chronic E2 exposure increases NO concentrations and increases iNOS mRNA in the AN. We have demonstrated that the increased NO production is associated with nitration of TH.. This may be one of the mechanisms behind E2 associated dysfunction of TIDA neurons. To further support our findings and corroborate our hypothesis, we wanted to determine if chronic E2 exposure would produce the same effect in iNOS knockout mice. We hypothesized that chronic E2 exposure would fail to suppress TIDA function in iNOS knockout mice when compared to wild type mice. The dose of E2 to be used in mice is slightly higher than that used in rats because mice have a higher metabolic rate. We therefore looked at the effects of low dose (0.18µg per day) E2 exposure for 90 days on the TIDA function in NOS2 (iNOS) knockout mice as compared to their wild-type C57BL6/J counterparts. Because the principal estrogen receptor (ER) in the arcuate nucleus (AN) is ER-α [204], it is expected that the E2-associated effects in the AN are mediated through this receptor isoform. Therefore, we also looked at the effect of E2 in ER-α k/o mice. The function of TIDA neurons was assessed by utilizing HPLC to measure dopamine concentrations in the ME of these genetically engineered mice.

Experimental Design

Animals

Breeding pairs of C57BL6/J (wild type), inducible nitric oxide synthase (iNOS) knockout mice and estrogen receptor alpha (ER-α) knockout mice were obtained from Harlan Inc. (Indianapolis, IN). Mice were bred in our colony and used in experiments. The absence of corresponding genes (iNOS and ER-α) were verified using a simple PCR using tail snip samples as described earlier [133] .

Animals were housed in light-controlled (lights on from 0700-1900h), air-conditioned (23±2°C) animal quarters and were fed mouse chow and water ad libitum. Animals were used in accordance with the NIH guide for the Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Treatment of Animals

Adult reproductively mature mice (2-5 months) were subcutaneously implanted with slow-release E2 pellets (Innovative Research America, Sarasota, FL) or were sham implanted (controls). The slow release pellets were capable of releasing 2µg of E2 per day. The animals were implanted with E2 pellets or sham implanted under isoflurane anesthesia as follows: Isoflurane was administered at the rate of 0.75L/min at a concentration of 3%v/v using a isoflurane Ohio vaporizer using a nose cone. The area over the dorsal aspect of the neck was shaved and wiped thrice alternatively with alcohol and betadine. Sterile instruments were used; scissors were used to make a small incision on the neck. Blunt dissection with curved forceps was used to create a "pocket" in the subcutaneous tissue extending down the left shoulder area. The pellet was placed in the subcutaneous pocket with separate sterile curved forceps. The

skin was then lifted and apposed with a sterile staple. Five (5) mg/kg of ketoprofen was injected subcutaneously for pain control. The staple was removed after 7 days.

Determination of Estrous Cyclicity

Estrous cyclicity in the animals was monitored periodically throughout the exposure period. Vaginal cytology was used to monitor cyclicity as described previously [80]. Briefly, warm water was used to obtain vaginal washes that were applied to a glass slide, dried and stained with a methylene blue solution as described previously [80]. The stained smears were examined under a light microscope and the stage of the estrous cycle was determined based on the predominant presence of epithelial cells, cornified cells or leukocytes.

Collection of Brains

At the end of the treatment period, all animals were sacrificed by rapid decapitation on the day of estrus. Brains and trunk blood were collected. When collecting the brains, all instruments and items that would come in contact with the brain were wiped with RNAse ZAP (Ambion Inc. Austin, TX), to minimize the risk of RNAse contamination. Serum was obtained by collecting blood in sterile Ependorff tubes. Blood samples were centrifuged at 3,000 rpm for 15 minutes and serum was collected. Brains and serum were stored at -80°C. Pituitary weights were also obtained.

Brain microdissection

Brains were sectioned at 120µm using a cryostat (Slee, London, UK) maintained at -10°C and placed on glass slides. The slides were transferred to a cold stage maintained at -10°C and the median eminence (ME) and arcuate nucleus (AN) were dissected with a 200µm micropunch, using Palkovit's microdissection technique [134] using the mouse brain atlas [135] as a

reference. Care was taken to include all subdivisions of individual nuclei for analysis. Tissue samples were collected in eppendorf tubes and stored at -80°C prior to analysis.

Dopamine HPLC-EC Analysis in the Median Eminence

A cell homogenizer was used to homogenize the ME samples in 100µl of 0.5M of perchloric acid ($HClO_4^-$). Twenty (20) ul of the homogenate was extracted for protein analysis of 10ul in duplicates (Micro BCA protein assay, ThermoScientific, Rockford, IL). The remaining homogenate was centrifuged @13000rpm for 5 min. at 4°C. From each sample, 60µl of supernatant, 30µl of DHBA standard, and 30µl of $HClO_4^-$ was injected into the high performance liquid chromatography electrochemical detection (HPLC-EC) system. The HPLC-EC sytem and the details of the mobile phase have been previously described [ ]. Briefly, it consisted of an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN), a glassy carbon working electrode, a phase II 5 µm ODS reverse phase 250 mm×4.6 mm C-18 column and C-R6A Chromatopac integrator (Shimadzu, Columbia, MD). The mobile phase consisted of monochloroacetic acid (14.5 g/l), sodium hydroxide (4.675 g/l), octanesulfonic acid disodium salt (0.3 g/l), ethylenediaminetetraacetic acid (0.25 g/l), acetonitrile (35 ml/l) and tetrahydrofuran (14 ml/l). The mobile phase was made with pyrogen-free water and filtered and degassed through a Milli-Q purification system (Millipore Co., Bedford, MA). The final pH of the mobile phase was adjusted to 3.1 using NaOH. The flow rate of the HPLC pump (Shimadzu LC-6A) was 1.7 ml/min. The column and the working electrode were kept in a Shimadzu CTO-6A oven at a temperature of 37 °C. The sensitivity of the detector was 1.0 nA full scale, and the potential of the working electrode was 0.65 V.

Results

Estrous Cyclicity

Estrous cyclicity was decreased (Mean±SE; %) in WT mice exposed to E2 (26.7±11.8) compared to their sham counterparts (100±0); however, in iNOS k/o mice exposed to E2, though their estrous cyclicity was significantly decreased (53.8±14.4) as compared to sham iNOS k/o mice (81.8±12.2), it was still greater than that of WT E2 treated animals.

There was a loss of responsiveness to E2 in the ER-α k/o mice indicated by the complete absence of E2-associated persistent estrus **(Fig. 29)**.

Figure 29

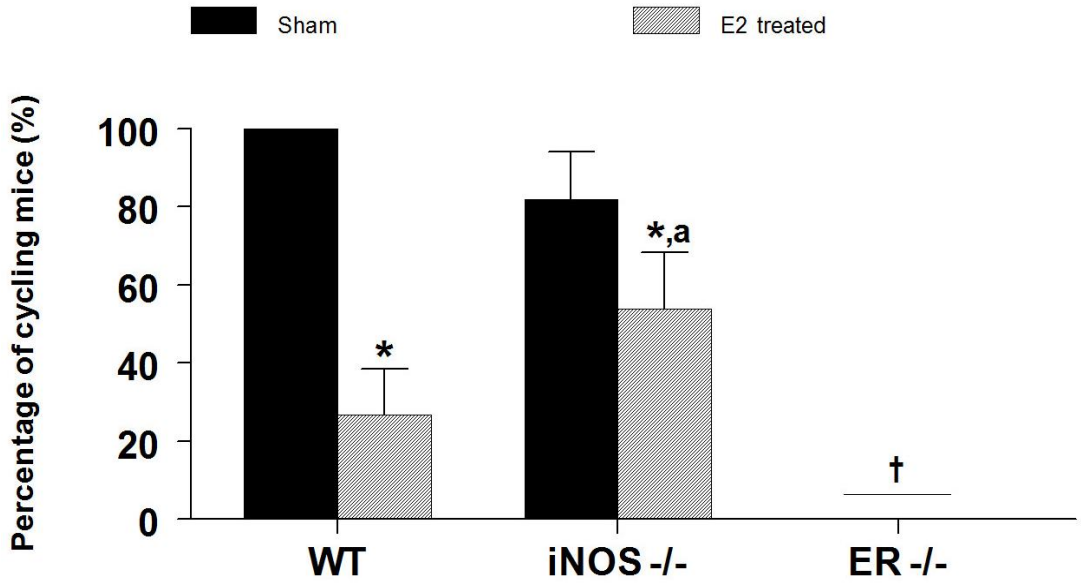


Fig. 29. Percentage of mice cycling with (E2 treated) or without (sham) E2 exposure. * indicates significantly less than WT and iNOS k/o sham)$p<0.01$). 'a' indicates significantly greater than WT E2 treated ($p<0.05$). † indicates significantly less than all other groups ($p<0.01$).

Pituitary Weight

The pituitary weights (mean±SE; g) of sham animals were significantly different between groups. Among the sham animals, the ER-α k/o mice had a significantly lower ($p<0.01$) pituitary weight ($0.0012\pm1.3x10^{-4}$) than WT ($0.0024\pm2.2x10^{-4}$) and iNOS k/o mice ($0.0026\pm1.0x10^{-4}$) **(Figure 30)**. Likewise in the E2 treated animals, the ER-α k/o mice had a significantly lower pituitary weight ($0.0015\pm2.8x10^{-4}$) than WT ($0.0036\pm2.3x10^{-4}$) ($p<0.0001$) and iNOS k/o mice ($0.0028\pm2.7x10^{-4}$) ($p<0.05$). Moreover, in the E2 exposed animals, the pituitary weight of the iNOS k/o mice was significantly lower than that of the WT mice ($p<0.05$) **(Figure 31)**. Furthermore, there was a significant increase in pituitary weights of E2 treated WT mice as compared to their sham counterparts; however, there was no difference between the pituitary weights of sham and E2 treated iNOS or ER-α k/o mice **(Figure 32 a-c)**.

Figure 30

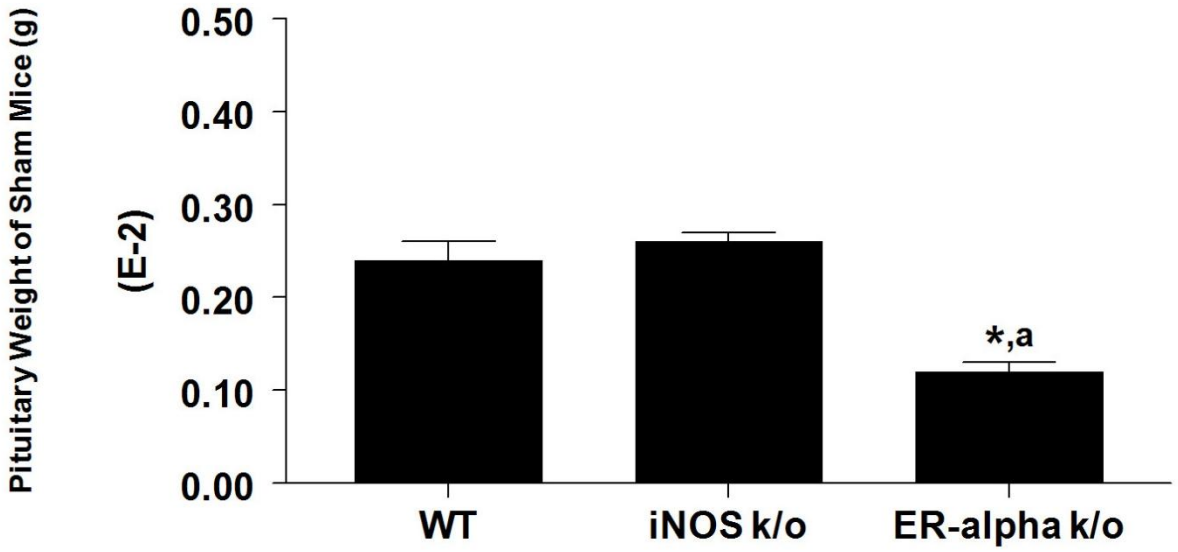


Fig. 30. Comparison between pituitary weights from WT and knockout mice that were sham-implanted. Pituitary weights of the different mouse strains [WT=wild type (C57BL6/J); iNOS k/o=inducible nitric oxide synthase knock out; and ER-alpha k/o=estrogen receptor alpha knock out] that underwent sham surgery for E2 pellet implantation. * indicates significant difference from control and 'a' indicates significant difference from iNOS k/o ($p<0.01$).

Figure 31

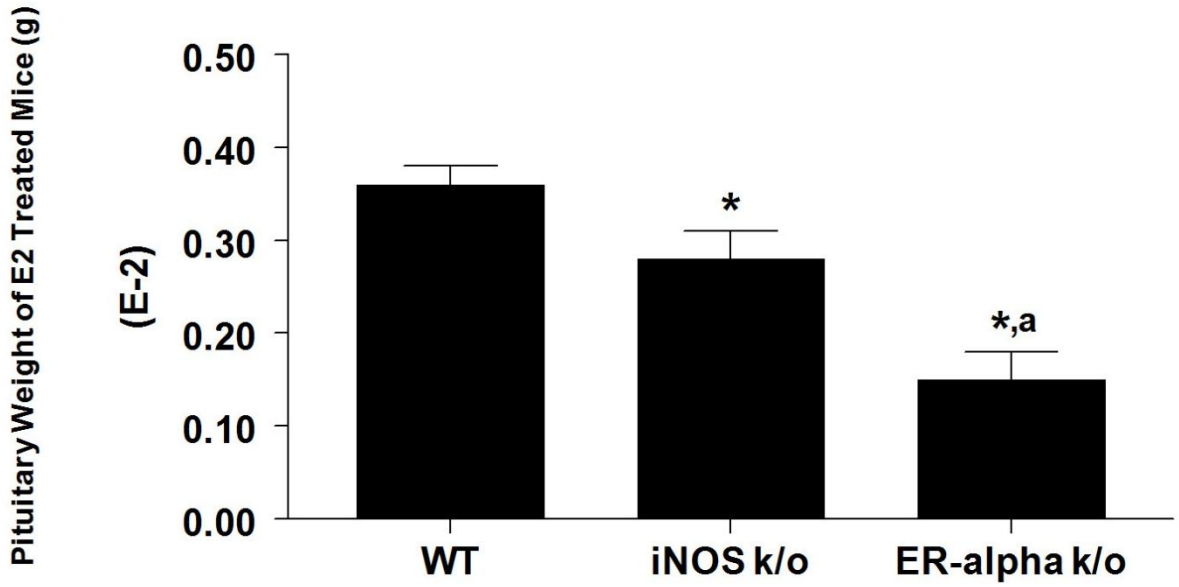


Fig. 31. Comparison between pituitary weights of WT and Knockout mice treated with E2. Pituitary weights of the different mouse strains [WT=wild type (C57BL6/J); iNOS k/o=inducible nitric oxide synthase knock out; and ER-alpha k/o=estrogen receptor alpha knock out] that were implanted with slow release E2 pellets (2µg/day) for 90 days. '*' indicates significant difference from control and 'a' indicates significant difference from iNOS k/o ($p<0.05$).

Figure 32

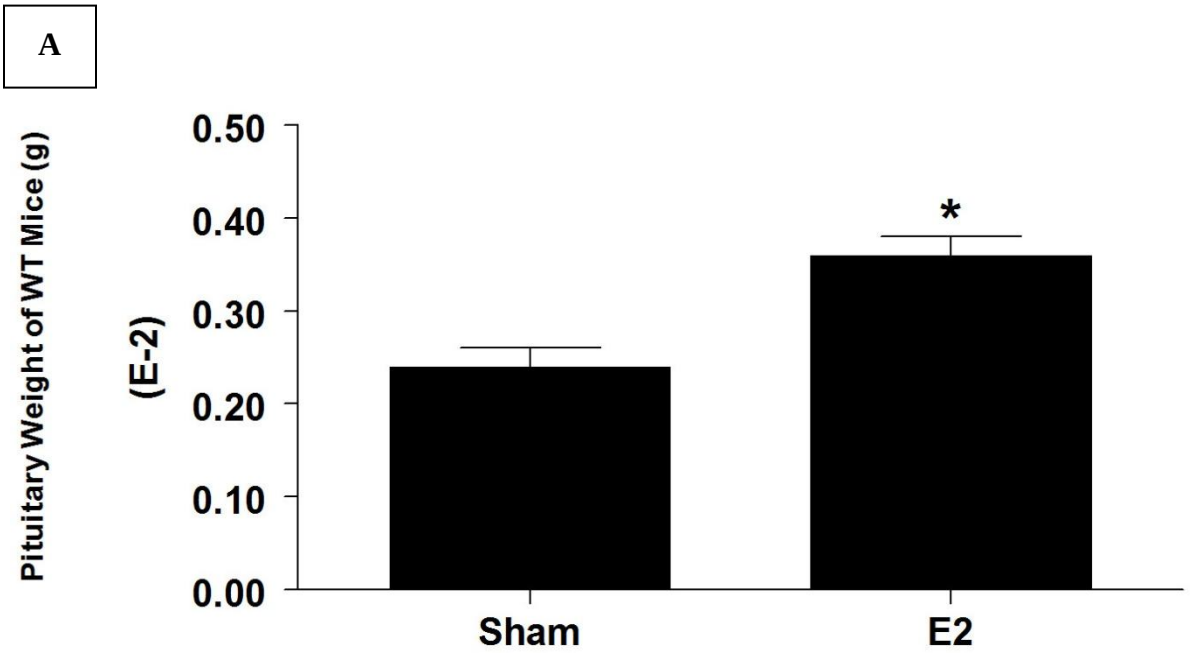


Fig. 32. A. Pituitary weights of the sham and E2 treated counterparts of WT mice. * indicates significant difference from sham ($p<0.01$).

Figure 32 (cont'd)

B

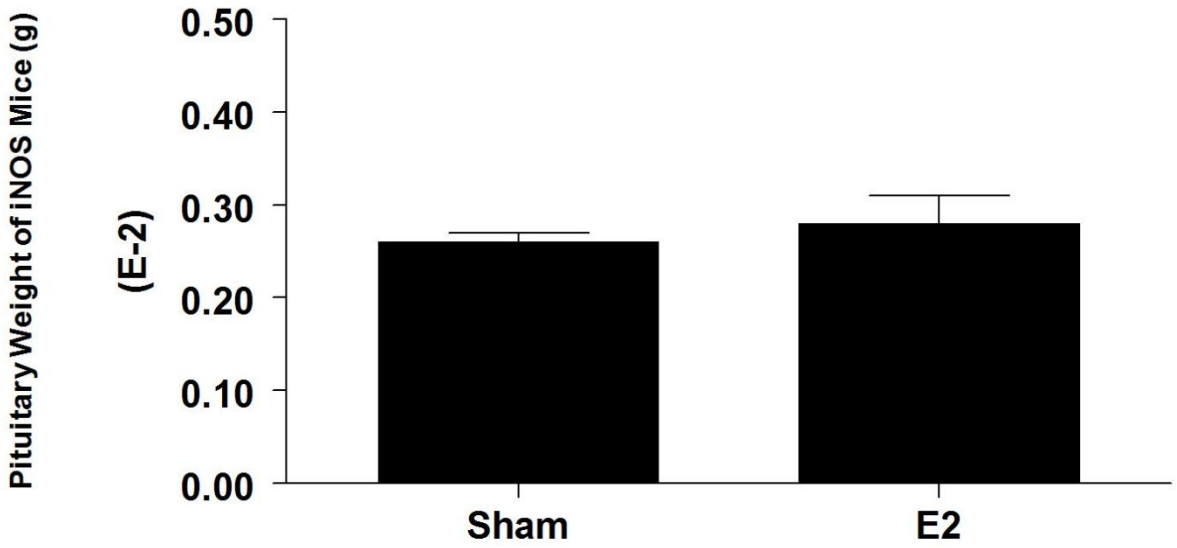


Fig 32. B. Pituitary weight in iNOS knockout mice that were sham-implanted or implanted with slow release E2 pellets.

Figure 32 (cont'd)

C

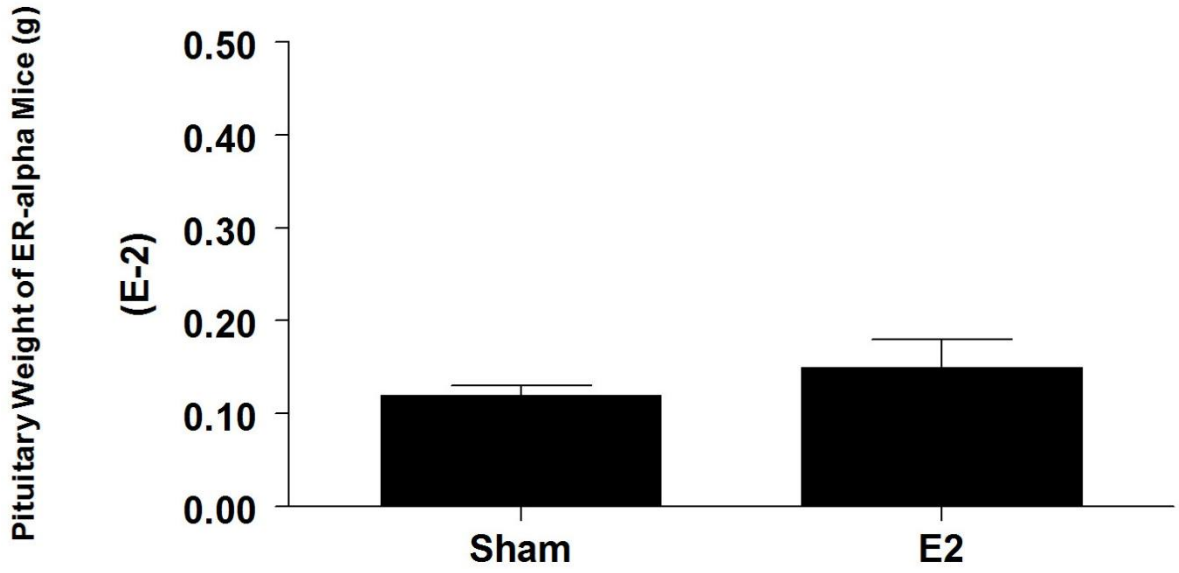


Fig 32. C. Pituitary weight of sham-implanted and E2-treated ER-alpha knockout mice.

Serum Prolactin

Serum prolactin levels (Mean($x10^{-3}$)±SE($x10^{-3}$); pg/ml) were significantly increased ($p<0.001$) in the WT E2 treated mice (25.38±7.3) compared to WT sham mice (4.29±1.3) as well as iNOS k/o and ER-α k/o mice, regardless of exposure to E2 (1.96±0.6 and 4.45±1.5, iNOS k/o and ER-α k/o sham mice, respectively; and 0.56±0.2 and 0.58±0.2, iNOS k/o and ER-α k/o E2-treated mice, respectively) **(Fig. 33)**.

Figure 33

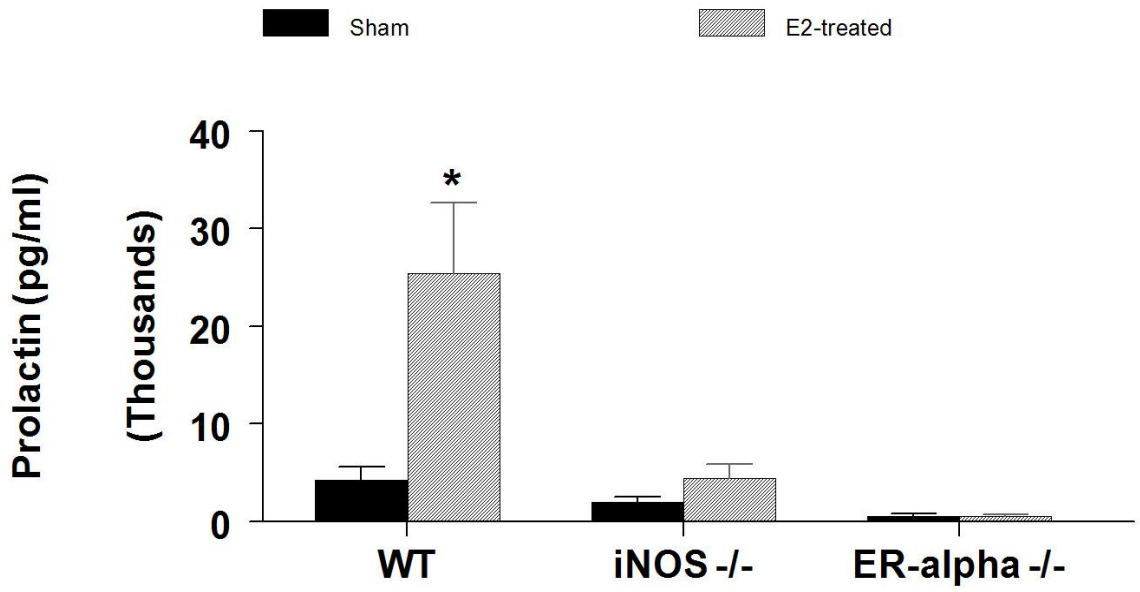


Fig. 33. Serum prolactin levels in WT, iNOS k/o and ER-α k/o mice that were treated with E2 or were sham controls. * indicates significant difference from all other groups ($p<0.001$).

Discussion and Summary

We have utilized genetically engineered knock-out (k/o) mice to further explore our hypothesis that the inhibition of TIDA function is mediated through proinflammatory conditions in the AN. E2-induced effects on TIDA neurons are probably mediated through estrogen receptor alpha. Using the background strain of the k/o mice (C57BL6/J)—characterized as wild-type (WT), iNOS k/o mice, and ER-α k/o mice, we have compared the effects of E2 on the hypothalamic-pituitary-gonadal (HPG) axis by assessing estrous cyclicity. We have assessed the functionality of the hypothalamic-pituitary TIDA system by measuring dopamine in the median eminence (ME). We have further provided an indirect measure of dopamine's presence and action on the anterior pituitary by weighing the pituitary glands which serves as an indicator of pituitary cell hyperplastic or hypertrophic processes as would be seen with lactotroph adenomas or prolactinomas.

During the estrous cycle, there is a milieu of hormonal changes taking place. Following the surge of E2 there is not only a preovulatory LH surge, but there is also a prolactin surge as well. This prolactin surge which occurs in the afternoon of proestrus is associated with decreases in the levels of ME tyrosine hydroxylase (TH) activity and dopamine levels [205] [206]. Therefore, the estrous cycle can be an indirect determinant of normal TIDA activity.

We demonstrated that in the ER-α k/o mice, there was complete loss of estrous cycle regularity and lack of response to E2 characterized by the absence of persistent estrus with prolonged E2 exposure. There was also no change in the pituitary weight of E2 treated ER-α k/o mice compared to sham. This indicates that ER-α mediates the E2–mediated loss of TIDA function that is associated with proliferation of anterior pituitary lactotrophs. Likewise, in sham and E2

treated ER-α k/o mice, the pituitary weight was less than both WT and iNOS mice that underwent the same treatment. ER-α plays a clear role in the regulation of the HPG axis [207, 208] and its presence has been demonstrated in the hypothalamus—particularly TH neurons in the AN, the pituitary, and the ovaries [161, 204, 209, 210]. ER-α is a nuclear receptor that plays an important role in many of estrogen's effects. Binding of estrogens or estrogenic compounds to this receptor can result in the transcription of a variety of genes leading to the effects of these compounds. Since this receptor was completely absent in the ER-α knockout mice, this animal model serves as an excellent tool to determine if E2's effects are indeed mediated through ER-α. Estrogen also has another receptor, ER-β, however its distribution in the brain is limited. In the present study, since we did not observe any increase in pituitary weight, it is safe to assume that E2's effects on TIDA neuronal function are most likely mediated through ER-α.

In the iNOS k/o mice, the loss of estrous cyclicity as seen in WT E2 exposed mice was ameliorated. Furthermore, there was a decrease in the pituitary weight of E2 treated iNOS k/o mice compared to WT, and the difference in pituitary weights of E2 exposed iNOS k/o mice was not different from that of their sham counterparts. This suggests that inducible NO production may play a role in the E2-induced loss of estrous cyclicity. It has been previously shown by our lab that E2 exposure in Sprague Dawley rats results in increased nitrate levels in the hypothalamus [169] and is associated with a decrease in LH and subsequent anovulation [80]. These previous findings in conjunction with the current results, strongly suggests thath E2 leads to the production of iNOS which may act at the level of the HPG axis to alter normal reproductive functions. Furthermore, the lack of increase in pituitary weight and absence of increased prolactin levels after E2 exposure provides strong evidence that iNOS induced NO is a key regulator in E2–induced lactotroph proliferation associated with a decrease in dopamine due

to inhibition of TIDA activity. Although the current data provides reasonable information about the role of iNOS and ER-α in E2-induced effects, they have to be even more definitively confirmed by measuring dopamine in the median eminence.

CHAPTER 8

SUMMARY AND CONCLUSIONS

We have provided strong evidence that supports a mechanism by which E2 inhibits TIDA activity. This is important because when TIDA activity is inhibited, the key regulation that this dopaminergic pathway provides to prolactin secretion from the anterior pituitary gland is attenuated. Thereby, inhibition of TIDA activity can lead to hyperprolactinemia which is associated with detrimental effects such as breast cancer [211], prostate cancer [10], pituitary tumors [7], osteoporosis [212] and immunosupression [213, 214]. It is established that E2 inhibits the activity of TIDA neurons [119, 120]. However, the mechanism behind this effect has not been elucidated. Based on results that our laboratory identified, demonstrating gliosis—characterized by increased GFAP—in the arcuate nucleus of E2 exposed rats [169], we hypothesized that chronic E2 exposure will increase proinflammatory cytokines and reactive oxygen species produced by glial cells, thereby damaging TIDA neurons and diminishing their activity.

We established that the dose (20ng/day) and duration (90 days) of E2 exposure leads to an increase in serum E2 levels and results in hyperprolactinemia. We demonstrated that in intact and ovariectomized E2 treated young and middle-aged rats, as well as in OCE rats, there was an increase in serum E2 when compared to sham animals. This increase in serum E2 was associated with a corresponding increase in serum prolactin levels in intact rats. Furthermore, there was an associated decrease in ME DA concentrations in young and middle-aged intact E2 treated rats as well as in middle aged ovariectomized animals that were treated with E2. There was also

evidence suggestive of lactotroph cell proliferation characterized by increased pituitary weights in middle-aged, intact and ovx, E2 treated rats as well as in middle aged sham implanted rats, suggesting an age-associated effect as well. These results indicate that the paradigm of E2 exposure utilized in this research leads to inhibition of TIDA activity.

The results that we demonstrated supports the hypothesis that low dose chronic E2 increases the proinflammatory cytokine IL-1β in the AN. By performing IL-1β ELISA with microdissected AN brain samples, we demonstrated that IL-1β was significantly increased in young and middle-aged intact and ovx E2 treated rats compared to sham animals. Also, nitrate levels were increased in intact young and middle-aged E2 treated rats. This indicates that chronic exposure to physiologic levels of E2 does induce a proinflammatory state within the AN characterized by increased IL-1β and nitrate. These results were further supported by the fact that we were able to observe increases in proinflammatory genes in the AN after E2 treatment and with age. However, in ovariectomized middle aged animals, E2 treatment appeared to suppress the expression of genes associated with the inflammatory response. This was an interesting observation that prompts the question of what role the other ovarian hormones (i.e., P_4) may play in inciting or inhibiting proinflammatory gene expression in the AN. Further investigation of this phenomenon is warranted. Also, considering that there was an effect of age and E2 treatment on proinflammatory gene expression, perhaps there is an age-associated increase in glial cells as well. Exploration of this may be beneficial in further elucidating the mechanism of potential pathologies in the aged brain with or without the presence of E2.

We further hypothesized that the increased nitrate production in the AN would lead to nitration of tyrosine moieties in the enzyme tyrosine hydroxylase, which is the rate limiting enzyme in DA

synthesis. A powerful nitrating oxidant that can be a byproduct of NO production is peroxynitrite ($ONOO^-$). Peroxynitrite is a product of the reaction between NO and the reactive oxygen species (ROS), superoxide (O_2^-) and is the most common mediator of protein nitration [187, 189]. If NO levels are increased, as we demonstrated occurs in the AN in the presence of E2, then $ONOO^-$ levels will also be increased in the presence of O_2^-. Western blot analysis demonstrated that there was an increase in the ratio of nitrated TH to TH in the ME of young and middle-aged intact and ovx animals exposed to E2 and there was also an increased in the nTH:TH ratio in the ME of MIS animals. This demonstrates that there is E2 induced, as well as an age-associated increase in nitration of tyrosine in the ME. Nitration of tyrosine in the TH enzyme will inhibit the function of this protein, thereby reducing the production of DA. The reason for this increase in nTH in MIS animals is not clear. There was no corresponding increase in nitrate levels in this group. This suggests that there may be an age associated increase in O_2^- levels which may have resulted in excessive production of $ONOO^-$ in the absence of increased levels of nitrate. Our findings indicate that low dose, chronic E2 induces a proinflammatory state in the AN and leads to nitration of tyrosine hydroxylase in the ME. This is a likely mechanism behind the E2 – associated disruption of TIDA activity and resultant hyperprolactinemia.

To determine the specificity of the observed effects to the TIDA system, we compared our results with identical parameter assessments in another major dopaminergic pathway in the CNS—the nigrostriatal dopaminergic system. We found that E2 did not have a similar effect on this system. There were no differences in the levels of IL-1β and nitrate in the substantia nigra (SN), the levels of dopamine or the nTH:TH in the caudate putamen (CP), between intact and ovx or sham and E2 treated rats. This suggests that the E2–associated effects that we

demonstrated may be specific to the TIDA system. The reason for the observed preferential effect of E2 on the TIDA system is not entirely clear. As the dopaminergic neurons of the SN contain ER-β, and the TIDA neurons contain ER-α, this may play a role in the differential effect of E2 on these 2 dopaminergic systems. It would be interesting to explore if the proinflammatory effect observed in our model, would be observed in other cells that solely express ER-α, but not in cells that express ER-β. This would provide insights into the mechanism behind the proinflammatory effect of chronic exposure to low dose E2 as we observed.

In addition to the above rat model of E2 exposure, we wanted to mechanistically explore the effects of E2 on the TIDA system by exposing iNOS and ER-α knock-out mice, and WT mice to physiologic levels of E2 for 90 days. We analyzed estrous cyclicity, pituitary weights and serum prolactin levels. iNOS and ER-α k/o mice did not have E2 associated increases in pituitary weights as did WT mice when compared to sham mice. This suggests that E2 may not stimulate proliferation of lactotroph cells and result in prolactinemia in these k/o mice. Additionally, there was a significant decrease in the pituitary weight in iNOS k/o mice ER-α k/o mice compared to WT when exposed to E2. Our results further support the role of iNOS in the E2–induced inhibition of TIDA activity and indicates that ER-α is a mediator of this effect.

The other system that we assessed in our model of low dose, chronic E2 exposure is the female gonadal system. Estrogen induces polycystic ovarian morphology and has been speculated to serve as a model for polycystic ovary syndrome (PCOS) in women. However, various estrogenic compounds have been used to induce polycystic ovaries in rodents, and the phenotypic cystic ovarian morphology has been the major feature to lead to the conclusion that estrogen exposure may serve as a model for PCOS. We have exposed rats to a physiologic dose of E2, the most common estrogen produced biologically, for 30, 60, and 90 days (E30, E60, E90, respectively)

and assessed multiple clinical parameters that are known to be affected in women with this disease syndrome. We also compared results with findings in OCE rats. We demonstrated that a low dose of E2 induced cystic ovarian morphology with the size of Graafian follicles being significantly increased in the E90 and OCE rats. There was a reduction in ovulation characterized by the loss of corpora lutea (CL) in a time of exposure-dependent manner. There was also histomorphologic evidence of degeneration that increased in severity in an exposure-dependent manner. The degenerative changes were accompanied by increased infiltration of macrophages in the follicular granulosa cell layer of E90 and OCE ovaries, characterized by increased follicles with CD68 immunopositive cells. The aforementioned findings are similar to those seen in women with PCOS as prominent clinical signs include cystic ovarian morphology, anovulation, ovarian degenerative changes, and increased ovarian macrophages [137, 215]. Nonetheless, other features of PCOS, which we explored in our model, include increased primary and secondary ovarian follicles [147], increased expression of Mullerian inhibiting substance (MIS) in granulosa cells [137, 152], increased serum testosterone [157], and an increased ratio of luteinizing hormone (LH) to follicle stimulating hormone (FSH) [216]. We did not identify any of the latter features in our rats exposed to E2 for 30, 60 or 90 days. Therefore, we concluded that our paradigm of low dose, chronic exposure to E2 in the Sprague Dawley rat is not a good model for PCOS in women. Nonetheless, since our paradigm of exposure does cause some ovarian features that are consistent with PCOS, other exposures may enhance the potential for low dose, chronic E2 exposure serving as a model for PCOS. For example, addition of testosterone in addition to the E2, may induce some of the features that would be exhibited by an individual with PCOS, rendering a more suitable animal model for this disease syndrome. Though we did not conclusively identify a good animal model for PCOS, the ovarian findings

that we identified in our model have implications for potential detrimental effects that long term exposure to low levels of E2 can have on the reproductive axis.

In summary, we identified that low dose, chronic exposure to E2 can have detrimental ramifications. It induced morphological and functional alterations in the ovary. It elevated IL-1β and nitrate levels in the arcuate nucleus and increased nitration of tyrosine hydroxylase in the median eminence. This was associated with decreased dopamine in the ME and hyperprolactinemia. Aging sometimes had an additive effect. These findings provide support for a mechanistic pathway through which E2 mediates inhibition of TIDA activity and causes prolactinomas and hyperprolactinemia. The recent studies that are reporting adverse effects of estrogen exposure, as well as the results from the research presented in this dissertation, warrant concern for individuals in today's society who are continuously exposed to various estrogens and xenoestrogens which are ubiquitous in the environment.

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