THESIS

ESTRADIOL EXPOSURE ALTERS GONADOTHROPIN-RELEASING HORMONE (GNRH) INDUCED GONADOTROPE PLASTICIY

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY CHERYL HARTSHORN ENTITLED "ESTRADIOL EXPOSURE ALTERS GONADOTROPIN-RELEASING HORMONE (GNRH) INDUCED GONADOTROPE PLASTICITY" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

ESTRADIOL EXPOSURE ALTERS GONADOTROPIN-RELEASING HORMONE (GNRH) INDUCED GONADOTROPE PLASTICITY

The reproductive axis is dependent upon communication among the hypothalamus, pituitary and gonads. For successful ovulation, a large increase in circulating estradiol provides positive feedback at both the hypothalamic and pituitary levels to promote an luteinizing hormone (LH) surge. An LH surge is necessary for the final maturation of the pre-ovulatory follicle and ovulation. The cellular and molecular events underlying estradiol's action(s) upon the anterior pituitary gland, specifically gonadotropes, remain elusive. Recent video microscopy experiments showed that pituitary cells *in vitro* in slice culture move in response to GnRH [Navratil, et al., 2007]; presumably these cells were gonadotropes. The current study utilized a novel transgenic animal model that has gonadotrope specific fluorescence provided by yellow fluorescent protein (YFP) [Wen et al., 2008]. I sought to determine if 17β-estradiol (E2) working through either a genomic or non-genomic mechanism affected gonadotrope specific movements in response to GnRH. Consistent with earlier studies [Navratil et al., 2007], application of GnRH [100nM] altered the cytoarchitecture of gonadotropes with observable cell process extensions. Using live video-

microscopy, exposure to 10nM E2 for fourteen hours significantly enhanced the ability of gonadotropes to extend processes in response to GnRH compared to short-term exposure of E2 (1.5 hours) or vehicle. There was no demonstrable effect of 1.5 hours of E2 exposure on GnRH-induced process extensions. I hypothesize that the differential effect of short-term versus long-term E2 exposure is due to a genomic mechanism that may underlie the ability of E2 to enhance GnRH induced cellular plasticity. Thus, E2 and GnRH may cooperate to maximize the secretory interface between gonadotropes and the adjacent vasculature during the pre-ovulatory LH surge.

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CHAPTER ONE

INTRODUCTION

Classic steroid hormones include glucocorticoids, androgens, mineralocorticoids, progestins and estrogens. Steroids are lipids that may be characterized by their four ring carbon structure with activity dependent upon functional groups and oxidation state of the rings. Steroid hormones are a critical aspect of the field of endocrinology, which developed in the late 1800s and early 1900s. With the identification of hormones, increased sensitivity of assays, receptor structure identification, chemical characterization, and the expanding field of membrane-initiated hormone signaling cascades, the understanding of hormone actions and signaling continues to grow rapidly.

1. Estrogens as a class of hormones

A. Steroidogenesis Pathway

Steroid hormones act as hormones synthetically derived from cholesterol. Cholesterol has 27 carbons derived from acetate by an array of enzymes. The synthesis of steroidal hormones from cholesterol originates with the conversion of cholesterol to pregnenolone that occurs within the adrenals, ovaries, testes, and placenta [Miller, 1988]. Recent studies also provide strong evidence for steroid hormone synthesis in particular brain regions [Do Rego et al., 2009]. Pregnenolone may choose one of two different conversion pathways; either progesterone or 17OH-pregnenolone. Progesterone is a critical steroid hormone

produced by the placenta and corpus luteum and is essential for maintenance of pregnancy and an active, cyclic hormone in the reproductive cycle.

At this divergent point in the steroidogenesis pathway, the following major hormones may be produced and converted into a plethora of hormones based upon enzyme(s) availability and location of synthesis: corticosterone, aldosterone, 17OH-pregnenolone, cortisol, dehydroepiandrosterone (DHEA), 17OH-progesterone, androstenedione, testosterone, estrone, and estradiol. For the purpose of this thesis, we are most interested in the synthesis of estradiol, and will discuss it in detail. Historically, testosterone may be converted to estradiol within the ovaries, but aromatase - the enzyme responsible for the conversion of testosterone to estradiol - has also been localized in adipose tissue and the brain, indicating that estrogens are synthesized outside of their primary site, the ovary. Both 4-androstenedione and testosterone are aromatized to active estrogens, estrone and estradiol, respectively. Not only can estradiol be converted into estrone, it is the precursor for another active form of estrogen, estriol [reviewed in Hormones chapter 2].

B. Characterization of Estrogens

Within the class of steroid hormones, estrogen signaling is of particular interest, as estrogens modulate numerous biological processes including, but not limited to the central nervous system, disease development, and reproduction. Derived from the aromatization of androgens, estrogens are characterized by the loss of carbon-19, the formation of an aromatic A ring, along with the formation of two functional oxygen groups: one on carbon-3 and one on carbon-17 [Norman

and Litwick, 1997]. Estrone, estriol and estradiol are three forms of estrogens commonly found in the human body, with 17β-estradiol (E2) being the most potent form for activating estrogen receptors. While its isoform, 17α-estradiol has generally been thought to have little biological activity. However, recent data suggests that this might not always be the case for brain [Toran-Allerand et al., 2005].

C. Estrogen Receptors

Historically, steriod hormone receptors are characterized as transcription factors active within cell nuclei that contain three major functional domains: trans-activating, DNA-binding, and ligand-binding. The ligand binding domain recognizes a specific hormone or ligand and once bound may interact with other transcription factors. Clever studies demonstrating the active region for DNA binding was demonstrated by replacing the putative estrogen receptor DNA binding domain with the putative glucocorticoid receptor DNA binding domain. They reported that the resultant receptor product bound estradiol as the ligand binding domain was intact, yet the receptor bound the glucocorticoid receptor DNA binding region [Chambon, 2005]. Many, but certainly not all, unliganded hormone receptors are bound to heat shock proteins within the cytoplasm. Once a ligand binds, receptors undergo a conformational change and translocate into the nucleus. It is notable that estrogen receptors are thought to reside within nuclei even in the absence of a ligand along with other compartments of the cell (see below). Liganded steroid hormone receptors homodimerize with hormone response elements (HREs) on target genes [Ribeiro, et al., 1995] and act as

transcriptional activators, or inhibitors, depending upon target tissue. Steroid hormone receptors may also be located at the cell membrane affecting second messenger signaling cascades in addition to residing within the cytoplasm and nucleus. Emerging evidence and understanding of membrane-bound steroid hormone receptors in conjunction with the classical view of steroid hormone signaling through nuclear receptors to modulate transcription complicates our understanding of steroid hormone signaling. A deeper understanding of the mechanisms mediating steroid hormone(s) actions is needed.

As technology and science progress, novel estrogen receptors are being identified. The first estrogen receptor identified, estrogen receptor α (ERα), was isolated in the 1950s by Dr. Elwood Jenson at the University of Chicago [Jensen and Jacobsen, 1960]. Starting in the 1990s with the discovery of a second estrogen receptor, estrogen receptor β (ERβ), [Kuiper et al., 1996] the number of putative estrogen receptors continues to rise complicating the current understanding of estrogen signaling [Wadas and Tobet, 2008]. The classical view of estrogen receptors is as nuclear steroid receptors, ERα and ERβ, that homo- or heterodimerize and bind to estrogen response elements modulating transcription, activating or inhibiting it. Unbound receptors were thought to reside in the cytoplasm and only translocating from the cytoplasm to nucleus upon ligand binding [Jensen et al., 1968].

Nearly twenty years later in 1984, Welshons et al., demonstrated that some unliganded ERs were located within the nucleus followed by Blaustein in 1992 suggesting ERs were located throughout the cell. In recent years,

technological advances in method and sensitivity of detection have enabled the classical receptors, ERα and ERβ, to be localized to the plasma membrane [Vasudayen and Pfaff, 2007]. In addition, novel proteins have been proposed to act as estrogen receptors at the plasma membrane or in the cytoplasm; namely, G-protein coupled receptor 30 (GPR30) [Prossnitz et al., 2007] and ER(x) [Toran-Allerand CD et al., 2002]. GPR30 belongs to the orphan G-protein coupled receptor family and may be activated by several estrogens. It is located on the endoplasmic reticulum instead of the plasma membrane [Revankar et al., 2005]. There are reports of GPR30 localization by immunohistochemistry in several regions of the brain and the anterior pituitary gland of rats, [Brailoiu et al., 2007] as well as mice, by immunohistochemistry and in situ hybridization [Hazell et al., 2009]; however, little or no functional studies of GPR30 in the pituitary gland have been conducted. A putative membrane ER with signaling characteristics of a G-protein coupled receptor (GPCR) was reported by Qui et al., [2006] by activation of a compound (STX) known to activate Galpha-q yet had estrogenic effects. They report that in hypoestrogenic female guinea pigs treatment with STX decreased weight gain associated with low levels of estrogens.

2. Estrogen Signaling

A. Hormone-dependent Breast Cancer

Estrogens play important roles in disease development, particularly with hormone-dependent breast cancers. A better understanding of estrogen signaling is crucial for treating breast cancer development, as two-thirds of breast

cancers are estrogen responsive [Henderson and Patek, 1998]. The transcriptional activity of estrogens, either in modulating activation or when inhibiting transcription, is of particular interest for treatment of ER positive breast cancers [reviewed in Vasudevan and Pfaff, 2007]. Additionally, treatment with E2 in ER positive forms of breast cancer increase proliferation, but treatment with pharmacological agents that inhibit ERα signaling decreases cell proliferation, [Robertson, 2002] and is frequently used to treat ER positive breast cancers. Breast cancers that were first diagnosed as an estrogen responsive form may switch from estrogen responsive to estrogen independent, usually coinciding with an increase in malignant progression. Interestingly, this shift also coincides with an increase in nuclear factor kappa activated B Cells, (NF-kB) activity [Kalaitzidis and Gilmore, 2005].

There may also be non-genomic actions of E2 in breast cancer development and formation. The breast carcinoma cell line, MCF-7, responds rapidly to E2 to increase second messenger cascades. Estrogens can activate the mitogen activated protein kinase (MAPK)/ extracellular regulated kinase (ERK) pathway in a rapid time course, suggesting a non-genomic site of action [Song et al., 2002]. Protein kinase C (PKC) activity has also been shown to increase with exposure to E2, and a membrane-limited form of E2 conjugated to bovine serum albumin (E2-BSA), in MCF-7 cells within 90 min. This suggests a membrane-initiated site of action [Boyan et al., 2003]. Tamoxifen, an estrogen receptor antagonist, blocked the E2 induced increase in PKC in MCF-7 cells indicating the importance of estrogen signaling in the mediation of the second

messenger signaling cascade within these cells. These second messenger-signaling cascades may be an interesting target for breast cancer treatment moving forward.

B. As a Neuroprotective Agent

Estrogen-mediated neuroprotection has been demonstrated against ischemia, neural insults, degenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke and amyotrophic lateral sclerosis [Marin et al., 2005; Brinton 2004; 2005]. While the exact mechinsm underlying estrogen signaling in many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke and amyotrophic lateral sclerosis are yet to be elucidated, estrogens potentially decrease symptoms or incidences by altering or inhibiting the inflammatory response [Czonkowska et al., 2006].

Estradiol levels may also affect mood disorders as depression and anxiety increase in women premenstrually and perimenopausally [Osterlund et al., 2005]. Kulkarini et al., [2008], conducted a randomized study examining the effects of estradiol treatment on women of childbearing age with active or chronic schizoprenia in conjunction with their antipsychotic medication. Women receiving 100μg E2 by a transdermal patch along with their antipsychotic medication for 28 days significantly reduced their psychotic episodes compared to those taking a placebo transdermal patch alone. This implicates E2's importance in neuronal signaling [Kulkarini et al, 2002; 2008]. Estrogen may also play a role as a neuroprotective agent fighting or delaying the onset of

Alzheimer's Disease (AD), as perimenopausal women who take estrogen replacement therapy may decrease their risk for AD later in life [Wharton et al., 2009].

Estrogen signaling may increase neuronal cell survival. Using animal models to represent strokes, Suzuki et al., [2009] presented a model in which the middle cerebral artery is permanently occluded, inducing stroke-like conditions. Treatment with low-levels of E2 (within the physiological levels) significantly decreased amount of damaged tissue in this stroke model. Within the same study, they found that E2 also decreases number of TUNEL-positive cells (apoptosis indicator), suggesting that E2 increases cell survival [Suzuki et al., 2009]. Estrogen receptor alpha knock out mice are more susceptible to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinson's Disease (PD) than wild type litter mates, implicating that ER alpha may act as a neuroprotective agent in PD development [Morissette et al., 2008].

C. Modulates Neuronal Activity

Not only does estrogen act as a neuroprotective agent within the central nervous system, it also contributes to neuronal activity. Reviewed by Kelly and Ronnekleiv [2009], there are numerous studies that implicate E2 as a modulator of neuronal activity at a membrane-initiated site of action. For example, E2 activity in hippocampal CA3-CA1 neuronal cultures demonstrated an increase in mitogen activated protein kinase (MAPK)-dependent cAMP-responsive element binding protein (CREB) phosphorylation, which was mimicked with E2-BSA, indicating a non-genomic site of action. Along these lines, E2 has also been

implicated in activating numerous second-messenger signaling cascades including in a rapid time course including MAPK, phosphoinositide 3-kinase (PI3K), cAMP-protein kinase (PKA), and protein kinase C (PKC) pathways that may affect gene transcription downstream [Kelly and Ronnekleiv, 2008].

One population of neurons that act as a critical trigger for the reproductive axis, the gonadotropin-releasing hormone (GnRH) neurons, are of particular interest as a target for E2 activity, either directly or indirectly. E2 signaling to GnRH neurons via ERα is believed to be indirect, while some evidence stands that ERβ is located in some GnRH neurons [Hrabovszky et al., 2000]. In spite of this, ERβ's role in GnRH neurons is yet to be defined. Within the hypothalamus, E2 modulates GnRH neurons ability to synthesize and secrete GnRH [Roy, et al., 1999; Christian, et al., 2008]. The mechanism of E2 signaling to activate or suppress GnRH secretion remains unclear. However, recent work by Christian et al., [2008] suggests genomic signaling as a mechanism necessary for both circumstances using their mouse model that disrupts ERα interactions with estrogen response elements (ERE). Studies examining GnRH neuronal signaling is confusing due to the diverse population of GnRH neurons along with their distribution throughout the forebrain and few number of GnRH neurons within a given brain. Another study suggests that an E2 membrane-initiated signaling cascade may increase intracellular concentrations of calcium in GnRH neurons due to its rapid time course [Abe et al., 2008].

Although there are numerous studies examining E2's potential roles and affects on GnRH neurons, there is poor evidence for estrogen receptor alpha to

be localized within or on GnRH neurons. Therefore, GnRH neurons along with several other neuronal populations may be affected by E2 indirectly by acting upon an upstream neuron. In the GnRH neuronal signaling pathway, kisspeptin neurons are believed to be the "upstream" actor. Kisspeptin neurons are localized in two regions of the brain. One population of kisspeptin neurons, residing in the rostral periventricular region of the third ventricle (RP3V), is sensitive to increases in E2 [Clarkson et al., 2009]. Kisspeptin's receptor, GPR54, is expressed in adult rodent GnRH neurons and activation of this receptor may initiate the signaling cascade responsible for increasing GnRH synthesis and secretion in response to raising levels of E2 [reviewed in Clarkson et al., 2010].

D. Reproduction: Estrogen in the Hypothalamic-Pituitary-Gonadal (HPG) Axis

Estrogen signaling, at both the genomic and non-genomic levels, plays important roles in the nervous system, disease development, and critical roles in reproduction. The hypothalamic-pituitary-gonadal (HPG) axis participates in a complex interplay of positive and negative feedback of gonadal hormones to regulate reproduction. As described above, estrogen signaling with kisspeptin and GnRH neurons is critical for reproduction. Gonadal hormones characteristically provide inhibitory actions (i.e., negative feedback) on the HPG axis. Reproduction in females is characterized by a cyclic fluctuation in hormones coordinating events within the HPG axis. Of the cyclic hormones, I find E2's actions particularly important and intriguing as its necessary for the

luteinizing hormone (LH) surge, which causes ovulation along with the vast physiological affects on other systems.

Primarily in females, E2 switches from inhibitory to stimulatory during the preovulatory period acting upon the hypothalamus and the anterior pituitary gland. A few studies suggest that in castrated male humans, treatment with estrogens can induce a positive feedback response [Goh et al., 1985] whereas intact males do not exhibit a positive feedback. Nonetheless, the mechanism(s) remains unclear how E2 signaling changes from inhibitory to stimulatory. During the preovulatory rise of estrogen levels, E2 stimulates the release of the decapeptide GnRH from terminals of hypothalamic neurons at the median eminence into the portal blood system (Brinkley, 1981).

In conjunction, E2 also primes the anterior pituitary gland for the LH surge. E2 acts upon the anterior pituitary gland to sensitize gonadotropes to GnRH by increasing GnRH receptor number that has been demonstrated in several species in primary culture including sheep [Gregg and Nett, 1989], rat [Tang and Spies, 1975; Drouin and Labrie, 1981], and mouse [Naik et al., 1985]. E2 also causes an acute suppression of basal LH release [Arrequin-Arevalo et al., 2005] potentially preparing it for the surge of LH. The fundamental mechanisms underlying this 'priming' of the pituitary by E2 remain elusive.

GnRH acts upon gonadotropes within the anterior pituitary gland to release gonadotropins, follicle stimulating hormone (FSH) and LH, into the vasculature. FSH and LH in turn act upon the ovaries and testes to promote further synthesis of gonadal hormones. The rise in circulating E2 signals GnRH

neurons to increase secretion of GnRH, which then causes the rise in LH released from gonadotropes within the anterior pituitary gland. In basal conditions, GnRH is released in a pulsatile fashion from the hypothalamus, but increases in amplitude and frequency with the rise in E2 in the preovulatory phase. In females, FSH stimulates growth and maturation of immature follicles. In humans, once a follicle matures it begins secreting estradiol, which initiates a change from negative to positive feedback upon the anterior pituitary gland and the hypothalamus.

Both of the classic estrogen receptors, ERα and ERβ, are implicated in reproduction; however, there are phenotypic differences between estrogen receptor subtype knockout (KO) animals. ERαKO females are infertile while ERβKO females are sub-fertile. ERαKO animals compared to wild type have increased basal levels of LH and irregular cycles portraying the importance of ERα for regulation of the hypothalamic-pituitary-gonadal axis [Couse and Korach, 1999].

E. Regulators of gonadotropes: estrogens, activin, inhibin, follistatin

While estrogen signaling and GnRH are critical signaling molecules for the HPG axis, its interpretation by the anterior pituitary gland, specifically gonadotropes, controls reproduction. With the improvement in technology and growing understanding and complexity of estrogen signaling cascades, estradiol's effect(s) at the level of the pituitary gland may provide insight to both genomic and non-genomic signaling mechanism(s). E2 treatment in ovariectomized ewes reduced LH levels within 10 minutes of exposure, but had a

latent response to E2. LH levels increased 14 hours after E2 exposure mimicking an LH surge. This latent response suggests a genomic site of action for an LH-surge level of hormone secretion to occur. Application of a membrane-limited estrogenic compound, E2 conjugated to bovine serum albumin (E2-BSA), caused an immediate reduction of LH within 10 minutes of exposure [Arrequin-Arevalo et al., 2006]. This study suggests that there are non-genomic actions of E2 that reduce LH levels, but is not the likely signaling event for an LH surge. Potentially, the membrane-initiated sites of estrogen action primes the anterior pituitary gland by diminishing the release of LH, so that LH granules can be stored for the large increase in LH secretion characteristic of the surge.

Additional studies have also implicated genomic and non-genomic sites of estrogen action in gonadotropes. In primary cultures, the transcriptional inhibitor, actinomycin D, inhibited E2 stimulatory effects on GnRH receptor expression suggesting a genomic action [Gregg and Nett, 1989; Gregg et al., 1990]. Estrogen responsiveness of ovine GnRH receptor gene promoter in transgenic mouse models has been reported [Duval et al., 2000; McCue et al., 1997]. Albeit an ERE has not been identified on any GnRH receptor gene promoter to date. Studies have also reported changes in second messenger signaling cascades with increased levels of phosphorylated ERK-1/2-immunoreactivity in gonadotropes within 15 minutes of E2 exposure [Iqbal, et al., 2007]. The fast time course is congruent with a non-genomic action of E2 on gonadotropes.

These are not the only hormones effecting gonadotrope activity. Produced and stored within gonadotropes, activin stimulates FSH gonadotropes

production and release [Childs, 1997]. Interestingly, unlike estrogens and estrogen receptors, neither activin nor its receptor are changed during the estrous cycle [Halvorson et al., 1994]. An inhibitory peptide, appropriately named inhibin, also regulates FSH secretion and synthesis. Inhibin is produced by the gonads and can decrease the number of GnRH receptors, yet it does not overrule the stimulatory affects of activin on GnRH receptor [Childs, 1997]. Another hormone critical to gonadotrope regulation and signaling is follistatin. It inhibits FSH release, and may bind activin, thus, regulating activin's stimulatory actions on FSH synthesis.

F. Pituitary as a Model for E2 signaling

Due to the pituitary's sensitivity to E2 and critical function in reproduction, the anterior pituitary gland presents a particularly useful model to study estrogen signaling and potentially fundamental intracellular signaling cascades. Gonadotropes are not the only cell affected by levels of circulating E2. Estrogens also effect many of the four cell types of the anterior pituitary gland: lactotropes, thyrotropes, corticotropes, and somatotropes. One additional cell type in the anterior pituitary gland that is also sensitive to estradiol is folliculostellate cells, [Allen et al., 1997]. A recent study conducted by Zarate et al., [2009] showed E2 induced apoptosis of lactotropes and somatotropes by a membrane estrogen receptor using pharmacological agents that were membrane limited ER agonists (E2-BSA) with treatment for 60 minutes. Estradiol has also been implicated in effecting glutamate signaling in thyrotropes and gonadotropes by significantly increasing type-2 vesicular glutamate transporter (VGLUT2)

localization by in situ hybridization methods in the adenohypophysis with E2 treatment to adult ovariectomized rats [Hrabovsky et al., 2006].

My research has focused on the hypothalamic-pituitary-gonadal axis and the role estrogen plays in communicating with the pituitary gland in timing the LH surge. While E2 is an important and necessary component leading to the significant gonadotrope secretion of LH as a surge, GnRH is the critical trigger. Recent experiments in our lab demonstrated that GnRH alters cell morphology and evokes motion in pituitary cells [Navratil et al., 2007]. Induced movement in response to GnRH exposure may enable cells to move closer to their site of release - blood vessels. In vitro studies revealed morphological changes may be a result of actin cytoskeletal reorganization when exposed to GnRH. The anterior pituitary gland of mice with fluorescent gonadotropes [Wen et al., 2008] provides a means to quantify E2 effects that alter the ability of GnRH to induce cellular plasticity and a subsequent LH surge.

CHAPTER TWO

ESTRADIOL EXPOSURE ALTERS GONADOTROPIN-RELEASING HORMONE (GnRH) INDUCED GONADOTROPE PLASTICITY

Introduction

The reproductive axis is dependent upon communication among the hypothalamus, the pituitary gland and gonads (the HPG axis). Cyclic fluctuations of hormones coordinate events within the HPG axis of females. While normally inhibitory or negative feedback of gonadal hormones control the HPG axis, in females, preovulatory E2 changes from providing negative feedback to positive feedback. GnRH released from the median eminence of the hypothalamus in a pulsatile manner acts upon gonadotropes in the anterior pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the pituitary vasculature. For successful ovulation, a large increase in circulating estradiol provides positive feedback at both the hypothalamic and pituitary levels to promote the LH surge. The cellular and molecular events underlying estradiol's action(s) upon the anterior pituitary gland, specifically gonadotropes, remain elusive.

Studies suggest both a genomic and non-genomic site of E2 action for priming the anterior pituitary gland in response to rising levels of circulating E2. E2 has been demonstrated in several species to increase GnRH receptor number in primary culture including sheep [Gregg and Nett, 1989], rat [Tang and Spies, 1975; Drouin and Labrie, 1981], and mouse [Naik et al., 1985]. However, these results have been difficult to replicate *in vitro*, it has been demonstrated in transgenic mouse models with ovine GnRH receptor promoter driving luciferase expression [Duval et al., 2000]. In ovarectomized ewes, E2 treatment also produces acute and latent effects on LH levels suggesting a non-genomic and

genomic site of action [Arrequin-Arevalo et al., 2006]. Other studies have suggested a membrane-initiated site of E2 action in gonadotropes by activating second-messenger signaling cascades and intracellular calcium levels. Iqbal et al., [2007] reported an increase in intracellular levels of phosphorylated ERK-1/2-immunoreactivity in gonadotropes within 15 min of E2 treatment.

As positive feedback from rising E2 levels is important for priming the anterior pituitary gland for the LH surge, experiments within sought to gain a greater understanding of E2 affects on GnRH signaling in gonadotropes. Recent video microscopy studies have shown pituitary cells in *in vitro* slice culture are able to move and develop process extensions in response to GnRH [Navratil, et al., 2007], presumably gonadotropes. The current study utilized a novel transgenic animal model that has yellow fluorescent protein selectively expressed in gonadotropes [Wen et al., 2008]. We sought to determine if 17β-estradiol (E2) works through genomic or non-genomic mechanisms to affect the morphological plasticity of gonadotropes in response to GnRH.

Methods and Materials

Animals

Mice were maintained on a 12 h light, 12 h dark cycle with access to rodent chow (Harlan 2918) and water *ad libitum*. For all experiments, a transgenic mouse model was used in which gonadotropes selectively express YFP [Wen et al., 2008]. This model is based on utilizing a cross between 2 lines of mice: homozygous mice with the GnRH receptor promoter driving Cre

recombinase expression and homozygous mice with the ROSA26 gene promoter with a floxed stop codon driving YFP expression. When crossed, cre recombinase excises the stop codon resulting in constitutive expression of YFP in GnRH receptor and ROSA26 expressing cells. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Colorado State University Animal Care and Use Committee.

Organotypic Slice

Adult female mice 2-8 months of age were sacrificed in diestrous 1 phase of the estrus cycle as assessed by vaginal cytology. Trunk blood was collected at time of tissue harvest, allowed to clot for several hours and then centrifuged for 2 min to separate serum. Serum was then collected and stored at -80° C until quantified by radioimmunoassay (RIA) for estradiol levels. Murine pituitaries were dissected in cold Krebs' solution (126mM NaCl; 2.5mM KCl; 2.5mM $CaCl_2$; 1.2mM $MgCl_2$; 1.2mM NaH_2PO_4; 11mM glucose; 25mM $NaHCO_2$) was embedded in 8% agarose (type VII-A; Sigma; maintained as liguid at 39° C) for sectioning at 200μm in the sagittal plane. Slices were then placed in a post-cutting sterile filtered Kreb's cutting buffer containing 0.01M HEPES, 100units/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml gentamicin for a minimum of 15 min, but no longer than 2 h. Slices chosen for video were then plated on glass bottom 35mm culture dishes (Mat Tek) that were coated with poly-d-lysine and collagen (Advanced BioMatrix PureCol, purified bovine colagen solution #5005-

B). Slices were then covered with 40μl collagen solution containing 1 ml collagen, 125μl 10X MEM, 23μl pen-strep (10,000 units penicillin and 10 mg/ml streptomycin) and 33μl of 1M sodium carbonate. This was allowed to polymerize for up to 1 h in a high humidity incubator. 1ml of serum-free media was added to the slices [Adult Neurobasal Medium (GIBCO BRL Laboratories) with B27 supplement and supplemented further with 25μM glutamate,1.34 mM glutamine, 0.5% glucose, 134U/ml penicillin and 0.13mg/ml streptomyocin]. Slices were kept at 36° C in an incubator with 5% CO_2. For estradiol treatment studies, slices were treated with one of three hormone treatment durations: long-term 10nM E2 (14 h+ before time of video acquistion), short-term 10nM E2 (treated at time of video acquisition, therefore, before GnRH treatment exposure for 1.5 h) or vehicle treated (1μl/1ml of 100% ethanol.) For microfilament studies, long-term 10nM E2 treated slices (14 h+) at time of video acquisition were either treated with vehicle (5μl/1ml DMSO) or the actin depolymerizing drug 5μM latrunculin A; thus, they were exposed to inhibitor or vehicle for 1.5 h before 100nM GnRH treatment.

Time-lapse Video

Time-lapse video microscopy was conducted 1-2 days after pituitaries were harvested. Slices were maintained at 36-37° C for the duration of the video. Images were captured using a Hamamatsu C10600 ORCA camera interfaced with a Dell Precision T3500 computer utilizing Metamorph software (version 7.0, Universal Imaging Corp., Downingtown, PA). Images were acquired

every 5 min for a total video time of 3 h with each frame exposed for 30 msec using a YFP filterset. The first 21 frames (1 h 30 min) were either vehicle, long-term 10nM E2 or short-term 10nM E2. The last 21 frames (1 h 30 min) were in the presence of 100nM GnRH. For latrunculin A treatment groups, 5uM latrunculin A or 10μl DMSO vehicle was given at the start of video acquisition. All slices were treated with 1 μl ethidium bromide for 10 minutes after video acquisition and images captured as an indicator of cell death. Compromised cells were revealed by nuclear localization of fluorescence when cell membranes were compromised and ethidium bromide was taken up and bound to DNA. Media was collected before and after video acquisition to quantify LH levels.

Video Analysis

Images were analyzed for movement during baseline hormone treatment (1.5 h) and during GnRH treatment (1.5 h). The percentage of cells with process extensions was quantified throughout the video duration (3 h) along with the time dependence for the first, second and third 30 min segments after GnRH treatment (note, some cells were responding in each segment of the video or possibly 2 out of 3 time periods.) Five cells were analyzed from each video with prominent process extensions after GnRH treatment to quantify average process length from edge of cell to end of process extension using ImageJ software and reported in microns.

Dextran Texas Red Injected Dye

For blood vessel visualization, Dextran Texas Red (tetramethylrhodamine, 70,000 MW, lysine fixable from Invitrogen #622085) was diluted in PBS for a working solution of 10mg/1ml. Adult female animals were deeply anesthetized with 80mg/kg Ketamine and 8mg/kg Xylazine before an intracardial injection of 200μl of Dextran Texas Red. Brains and pituitaries were harvested and submersion fixed in 4% paraformaldehyde overnight and then rinsed in 0.1M phosphate buffer (PB). Pituitaries were sectioned at 50μm on a vibrating microtome (Leica VT1000s) and then sections were mounted on gelatin-coated slides, dried, and coverslipped with Aqua Poly/Mount (Polysciences, Inc. Cat#18606 Lot#609292).

Immunocytochemistry

Immunocytochemistry (ICC) was performed as previously reported [Bisenius et al., 2006]. Briefly free-floating sections were incubated in 0.05 M PBS with 0.1 M glycine for 30 min, washed in PBS, incubated in 0.5% sodium borohydride for 15 min, and washed in PBS again. Sections were blocked for at least 30 min in 5% normal goat serum (NGS; Sigma-Aldrich, St. Louis, MO) with 1% hydrogen peroxide in 0.5% Triton-X-100 in PBS (TxPBS). Following block, sections were incubated at 4° C for 48 h in an anti-luteinizing hormone beta subunit guinea pig polyclonal primary antibody (generously provided by Colin Clay). All washes were done at 4° C in 0.05M PBS.

After primary antiserum incubation, sections were rinsed in 0.05M PBS with 1% NGS and 0.02% TxPBS at room temperature (RT), then incubated in secondary antibody for 2 h at RT (anti-guinea pig conjugated to CY3 diluted 1:2500 Jackson ImmunoResearch, West Grove, PA). Sections were mounted on gelatin-coated slides, dried, and coverslipped with Aqua Poly/Mount (Polysciences, Inc. Cat#18606 Lot#609292).

Figures

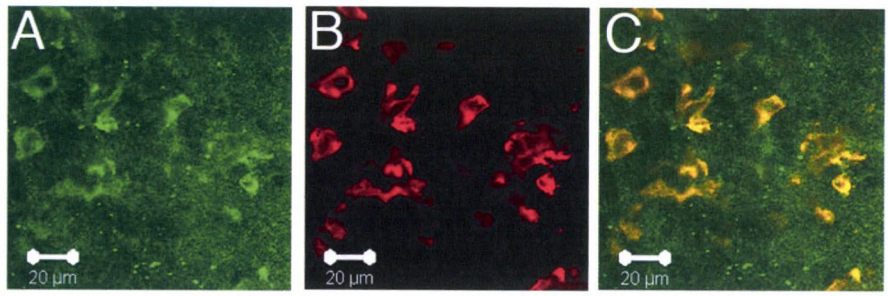

Fig 1: Gonadotrope colocalization of YFP and LH-beta. A section (50µm) through the pituitary of a transgenic mouse shows gonadotropes expressing yellow fluorescent protein (A). Immunoreactive LH-beta subunit in red (B) with merged photo (C) indicates that YFP is in gonadotropes.

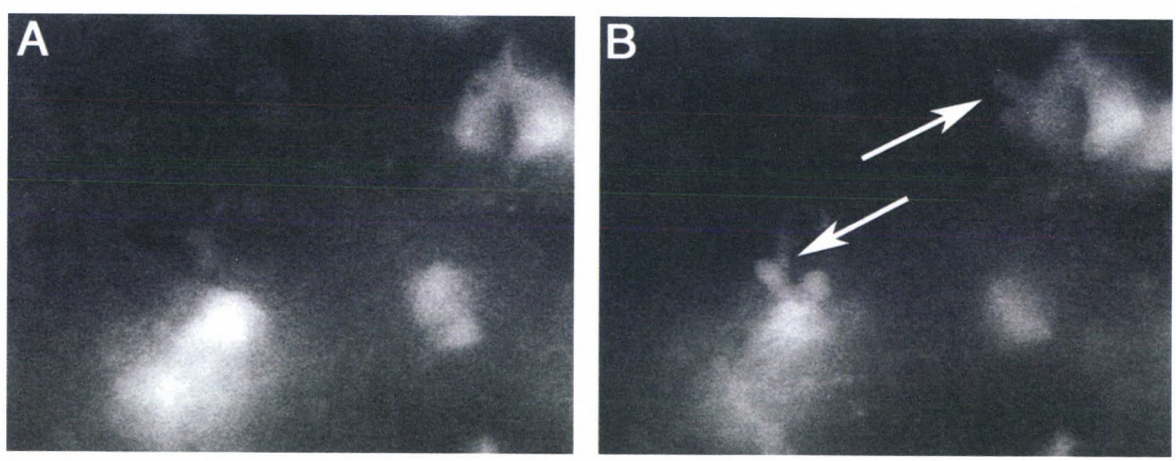

Fig. 2: Gonadotrope process extension with long-term E2 and GnRH exposure. In panel A, YFP + gonadotropes with 14 h E2 exposure are portrayed. After 100nM GnRH, processes extend from several of the gonadotropes shown (B; white arrows).

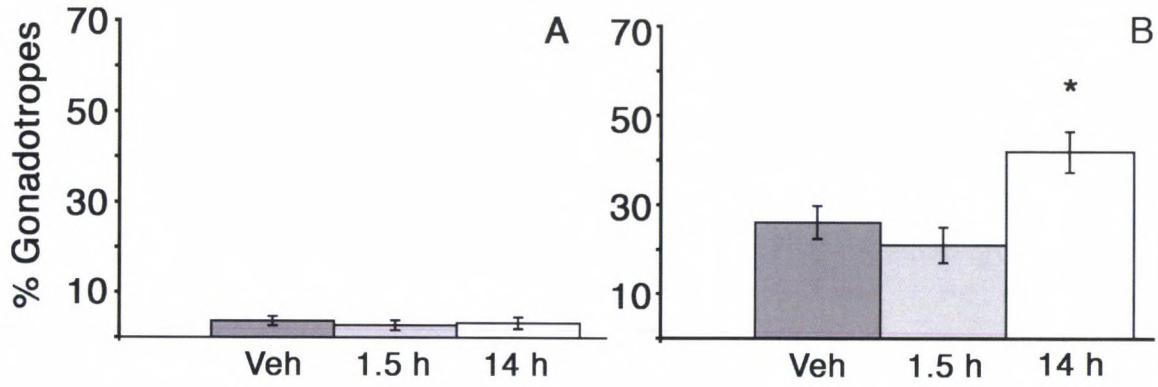

Fig. 3: Long-term estradiol treatment increased GnRH-induced plasticity in live pituitary slices. Adult transgenic female mice in diestrous 1 with yellow fluorescent protein expression in gonadotropes were used for live time-lapse video microscopy. In panel A, the graph depicts estradiol or vehicle exposure (no GnRH treatment). In panel B, the graph portrays the same pituitary slices as in A, but with GnRH treatment. There was no effect of estradiol treatment alone on the percentage of gonadotropes with a process extension in a given region of interest (A). Long-term estradiol treatment (14 h+) significantly increased GnRH-induced cell process extension compared to vehicle and 1.5 h E2 (B). There was no effect of short-term estradiol treatment (1.5 h) on GnRH-induced process extension. Veh: vehicle (n=8); 1.5 h: 1.5 h of E2 treatment exposure started at start of video acquisition (n=9); 14 h: 14 hours plus of estradiol treatment before start of video acquisition (n=11); * denotes $p < 0.01$.

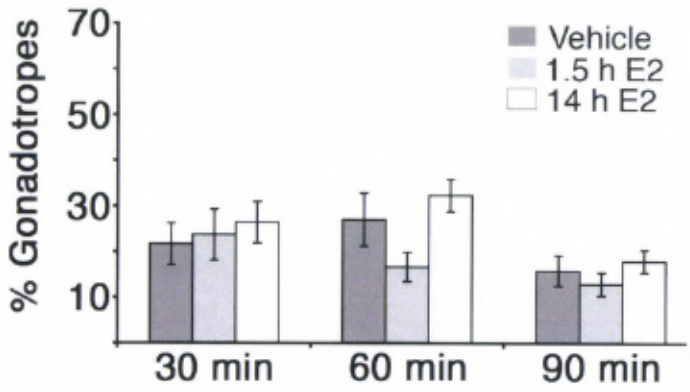

Fig. 4: There was no differential effect of estradiol on process extensions over time. Time-lapse video microscopy of live murine pituitary slices treated with GnRH for 90 min. Video acquisition began at start of GnRH treatment and was analyzed in thirty minute segments to quantify time-dependent cell process extension (note: some cells had process extension for more than one 30 min segment and were counted in each time period they had a process extension.) Estradiol treatment for short-term (1.5 h) and long-term (14 h+) was analyzed compared to vehicle (1µl/1ml of 100% ethanol).

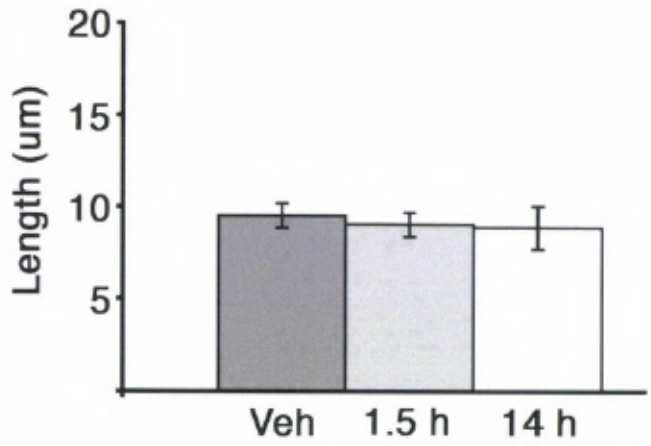

Fig. 5: There was no effect of estradiol pretreatment on the length of cell process extensions following GnRH treatment. Lengths of prominent process extensions from five gonadotropes per video after GnRH treatment were analyzed to quantify average length of process extension. There was no difference between estradiol treatment groups and vehicle. Length is reported in microns from edge of cell to edge of process extension.

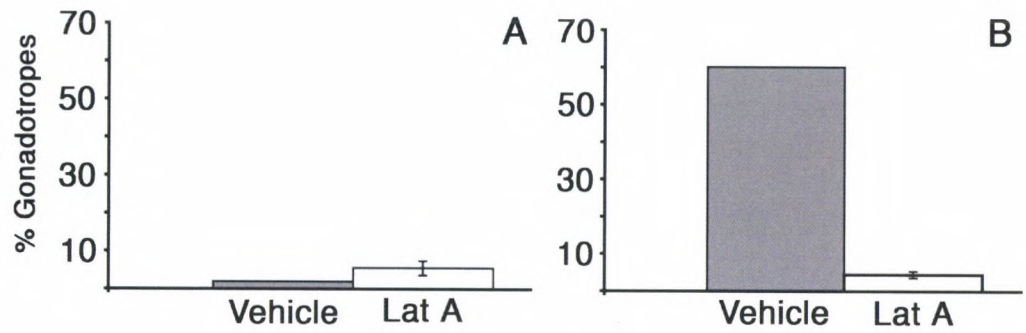

Fig. 6: Actin cytoskeletal inhibitor, Latrunculin A, disrupted GnRH-induced process extension. Live pituitary slices treated with latrunculin A at the start of video acquisition had a large decrease in GnRH-induced process extension. Both vehicle and latrunculin A treated videos were exposed to estradiol for 14 h+ before start of video acquisition and either vehicle (DMSO) or Latrunculin A at start of video acquisition. The graph in panel A represents percentage of gonadotrophs with process extension with vehicle or latrunculin A treatment over 1.5 h period. The graph in panel B depicts GnRH treatment and percent of cells with process extension over 1.5 h period. Veh: vehicle (n=2) and Lat A: latrunculin A (n=4).

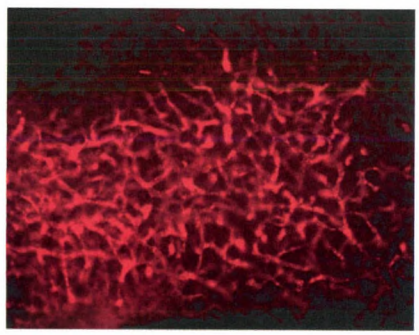

Fig. 7: Vasculature of anterior pituitary gland. Blood vessels in an adult mouse anterior pituitary gland were labeled with an injection of Dextran Texas Red dye.

Results

Transgenic Mouse Model

Immunocytochemical experiments verified transgenic animals in our facility had YFP in cells that were selectively gonadotropes based on col-localization with LHβ. In perfusion fixed pituitary sections from adult mice immunoreactive YFP was found in the same cells as immunoreactive LHβ (Fig. 1). 100% of cells immunoreactive for LHβ were also expressing YFP in areas that were examined.

Live Pituitary Slice Video Microscopy

Long-term exposure to estradiol increases responsiveness of gonadotropes to GnRH

Time-lapse video microscopy of live murine pituitary slices was analyzed for movement and process extension induced by E2 treatment of different durations along with GnRH treatment. In figure 2, before and after GnRH exposure, gonadotrope morphology is portrayed with slices treated long-term with E2 (14+ h). Images depict that process extensions form after GnRH exposure. There was no impact on percentage of gonadotropes with process extensions with only 10nM estradiol treatment, short 1.5 h or long 14 h+, compared to vehicle at baseline video (fig. 3A). However, following GnRH treatment, gonadotropes in slices exposed longer-term to 10nM E2 (n=11) had a significantly greater percentage of gonadotropes with process extensions with an average of 41.8% gonadotropes compared to short-term estradiol treatment

(n=9) with 21.1% and vehicle (n=8) with 26.1% gonadotropes in a given video field (fig. 3B) [$F(2,25)=5.96$; $p<0.01$].

No change in time-dependent process extension activation

Gonadotropes responding with a process extension were often visualized within five minutes of GnRH treatment, consistent with prior results [Navratil et al., 2007]. However, there was no time-dependence observed in gonadotropes that responded to GnRH between treatment groups divided into three 30 min periods (fig. 4). Some cells were seen responding in 1, 2, or 3 of the three 30 min segments and were included in the count in each segment if responding. Although, there appears to be a difference between 30-60 min of video acquisition in short-term and long-term estradiol treatment (fig. 4) [$F(4,50)=2.33$; $p<0.07$], the difference did not reach statistical significance. It appeared that more gonadotropes responded within the first thirty minutes than those responding in the last thirty minutes; however, it was also not statistically significant.

No difference observed in process extension length with estradiol treatment

Time-lapse video microscopy was analyzed for process extension length quantified by measuring from the edge of a cell to end of process extension using ImageJ (version 1.37v) software. Reported in microns, the average length between all three treatment groups (vehicle, 1.5 h E2, 14 h+ E2) was similar (fig.

5) suggesting an all or none response of process extensions. If a gonadotrope responded to GnRH, a process extension was approximately 10 microns in length and did not vary significantly with estradiol treatment.

Actin cytoskeleton is required for process extension

Latrunculin A, which disrupts actin-mediated processes, diminished GnRH-induced gonadotrope plasticity (fig. 6). In figure 6, all groups were treated with E2 for 14 h + before video acquisition to determine mechanisms of E2 gonadotrope action. As expected, disrupting the cytoskeleton, specifically actin filaments, reduced GnRH-induced cytoskeleton changes. However, data presented for vehicle is from n=2, preventing a strict statistical comparison. Nonetheless, given the similarity of the control values to other control experiments conducted at different times and the difference in the presence of latrunculin (n=4) it is likely that process extensions were strongly inhibited.

Vasculature of Anterior Pituitary Gland

Visualization of adult mouse pituitary gland blood vessel network is portrayed in figure 7 showing a dense network of vasculature comprising the anterior pituitary gland. Unfortunately, variability in the success of the labeling method precluded detailed analyses of the relationship of blood vessels to gonadotropes.

Discussion

The experiments presented provide new insight into a potential stimulatory affect of estradiol on the anterior pituitary gland, specifically gonadotropes. The stimulatory affect of E2 to prime gonadotropes for GnRH signaling is portrayed as an increase in GnRH-induced morphological plasticity. Gonadotropes within the anterior pituitary gland are a heterogeneous population of cells. In a prior study using the same line of mice only 50% of gonadotropes expressing YFP responded to GnRH by releasing LH quantified by reverse hemolytic plaque assay [Wen et al., 2008]. The studies presented herein are congruent with a heterogeneous population, as only a subpopulation responded to GnRH and E2. Only 25% of gonadotropes responded with process extension(s) to GnRH. Long-term E2 treatment increased the percentage of cells responding to GnRH with a process extension to 41%.

Long-term E2 treatment increased the responsiveness of gonadotropes to GnRH, but short-term E2 did not, suggesting an effect for which time is essential; perhaps protein synthesis. With 14 h+ E2 treatment gonadotropes sensitive to GnRH-induced plasticity increased almost 2 fold from 25% to 41%. However, it was still not a majority of the gonadotrope population. Experiments were conducted using pituitaries harvested from females in diestrous I, which is characterized by low estradiol levels. Neither E2 nor E2 exposure duration had an observable affect on percentage of cells with process extensions over time or the length of process extensions. This suggests that GnRH-induced formation of process extensions is an all or none response. The percentage of cells

responding to GnRH to secrete LH and GnRH-induced plasticity of gonadotropes brings to question the heterogeneity of this population of cells and what potential function(s) might be mediated by such heterogeneity.

Choice of animals in the disetrous I stage may have impacted the percentage of gonadotropes with process extensions as Funabashi et al., [1994] correlated levels of GnRH receptor mRNA levels with the stages of the estrous cycle. They concluded that the highest levels of GnRH receptor mRNA was observed during the end of diestrous II and the beginning of proestrous. These findings are congruent with studies that have demonstrated estradiol levels impact gonadotropes and that estradiol increases GnRH receptor synthesis [Conn et al., 1995; Lloyd and Childs, 1988a,b.] As the E2 effect presented in these studies is an increase in responsiveness to GnRH signaling, long-term E2 may mediate these effects by increasing levels of GnRH receptor gene expression. To address this, studies are still in progress using using real-time PCR to quantify mRNA levels of GnRH receptor after exposure to long-term E2. The long-term E2 exposure of 14 h + is consistent with a genomic affect of E2; however, the GnRH receptor gene promoter does not contain an ERE sequence. As short-term E2 exposure had no effect on the percentage of cells responding to GnRH-induced plasticity, it suggests there is not a short-term membrane-initiated site of action.

While GnRH signaling has numerous downstream targets, one intracellular locus is imperative to the experiments conducted; cytoskeleton. As GnRH receptors have been localized to lipid rafts, communication between

cytoskeletal elements and GnRH receptors may be through lipid raft localization [Navratil et al., 2003]. Several studies have also implicated cytoskeletal reorganization in response to GnRH signaling with formation of process extensions [Lloyd and Childs, 1988]. The experiments presented suggest that actin filaments are also critical for cell process extensions and for GnRH induced plasticity as the actin filament inhibitor, Latrunculin A, diminished GnRH-induced process extension. GnRH induced cytoskeletal reorganization was shown in studies using HEK293 cells [Davidson et al., 2004] along with cultured ovine pituitary cells and *ex vivo* live pituitary slices [Navratil et al., 2007]. Interestingly, Navratil et al., [2007] also observed cell movements that occurred in response to GnRH. For visualization of cells in the *ex vivo* live murine pituitary slices, slices were infected with an adenovirus-containing Rous sarcoma virus green fluroescent protein. Cells responding with spatial repositioning may not have been gonadotropes. The cell movements were GnRH sensitive, but Childs et al., [1994] reports that GnRH receptor binding has been localized to somatotropes and gonadotropes, thus cells moving may be a different population of pituitary cells than gonadotropes. Gonadotropes in this transgenic model do respond to GnRH with process extensions. However, using the gonadotrope selective YFP+ model, gonadotrope cell bodies were not observed moving.

Unfortunately, the directionality of process extensions is unknown. We hypothesize that processes extend in the direction of pituitary vasculature to increase the secretory impact of gonadotropes. The pituitary gland is highly

vascularized and reports have indicated that hormone secreting endocrine cells in rats show a close spatial relationship with blood vessels [Itoh et al., 2003].

CHAPTER THREE

CONCLUSIONS

While estradiol is essential to increase GnRH secretion and pulsatile release of GnRH at the level of the hypothalamus during the preovulatory period, estradiol effects at the level of the anterior pituitary gland have been less clear. Studies have suggested both genomic and non-genomic actions at the level of the anterior pituitary gland. The studies herein provide new insight into a potential stimulatory affect of E2 on gonadotropes mediated under the umbrella of long-term influences. Based on the time-course of E2 treatment of the studies presented, I hypothesize a nuclear (genomic) site of E2 action that leads to an increase in GnRH-induced gonadotrope plasticity.

Several studies have characterized the heterogeneity of gonadotropes. There are differences in size, physical density, morphology, responsiveness, and morphology [reviewed in Evans, 2002]. Potentially, the different populations of gonadotropes may serve different functions. Only 25% of gonadotropes respond with process extension(s) to GnRH. Long-term E2 treatment increased the responsiveness of gonadotropes to GnRH to 41%, but short-term E2 did not affect the percentage of cells indicating that a population of cells is sensitive to E2.

Studies suggest gonadotropes undergo cyclic fluctuations in preparation for ovulation in synthesis of gonadotropins [Childs et al., 1997]. These cyclic modifications may be characterized by the amount of gonadotropins within the

cell, with lowest levels during diestrous I and highest levels during proestrous leading to the LH surge. The cyclical modification of gonadotropes is believed to be controlled by estrogens in particular [Clarke, 2002], but the mechanism is unknown. Pituitary cells, including gonadotropes, express estrogen receptors and estrogen receptor numbers change throughout the cycle in dispersed rat pituitary cells. Gonadotropes, identified by co-localization of ER (α or β) and gonadotropins (LH or FSH), doubled from 5% in estrus to 10% in proestrous of total pituitary cells [Childs et al., 2001]. This indicates that there are significant fluctuations of estrogen receptor expression in pituitary cells including gonadotropes over the reproductive cycle.

Actinomycin D Preliminary Data

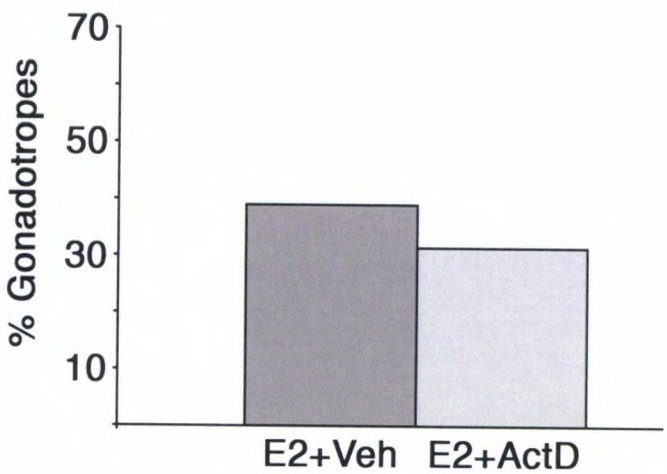

Fig. 1: Actinomycin D did not inhibit E2 effects on GnRH-induced plasticity. Preliminary experiments using the transcriptional inhibitor, Actinomycin D, on live pituitary slices suggests E2 is not acting through transcription to increase the percentage of gonadotropes with process extensions. The slices were treated with 8μM Actinomycin D for 6 h along with 10nM E2 or vehicle + 10nM E2. After 6 h exposure, media was replaced and video was acquired 8 h later to mimic the 14 h time course of long-term E2. The data are preliminary as only n=2 for each group. In E2+Veh group, 38% gonadotropes had a process extension while E2+ActD group had 31%. E2+Veh: E2 + vehicle (1μl/1ml of 100% ethanol); E2 +ActD: E2 + Actinomycin D.

With long-term E2, but not short-term E2, demonstrating an effect on GnRH-induced gonadotrope plasticity, a genomic site of action is a reasonable hypothesis for mediating the effect. However, preliminary data suggests that GnRH may not be acting through a transcriptional mechanism. Further studies are needed with different protocols using the transcriptional inhibitor, Actinomycin D, for 14 h+ and treatment with E2 or vehicle to more completely tease apart the mechanism of long-term E2 increasing GnRH-induced cell process extension. Preliminary data (n=2 for each group) did not indicate a difference between long-term E2 and long-term E2 + Actinomycin D treatment, 38% and 31% gonadotropes with process extensions with GnRH exposure respectively (fig. 1; see above). However, the preliminary experiments did not parallel the length of E2 long-term treatment due to fear of Actinomycin D inducing apoptosis and was truncated to 6 h treatment of E2 with either Actinomycin D or vehicle then removed and replaced with supplemented media for 8 h before video acquisition. This timeline was originally planned to minimize potential lethal effects of Actinomycin D by limiting exposure. More recent preliminary experiments testing whether slices are viable after 14 h+ Actinomycin D exposure indicate that the slices remain healthy and viable. Moving forward, in additional experiments examining transcriptional involvement, slices will be treated with E2 and Actinomycin D for 14 h+ before video acquisition along with a method to ensure that the treatment of Actinomycin D at 8μM concentration is an effective

treatment. GnRH alone acts in a short response time period suggesting that its signaling does not need transcription to induce process extensions.

GnRH receptor is within the G-protein coupled receptor family and is a membrane-bound receptor. Once activated by the decapeptide, GnRH, it activates a G-protein coupled second messenger signaling cascade activating $G\alpha_{q/11}$ that in turn dissociates from $G\beta/\gamma$, activating phospholipase $C\beta$ ($PLC\beta$). This increases the production of inositol 1,4,5 bisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate, which activates the release of Ca^{++} from intracellular stores along with activation of protein kinase C (PKC) [reviewed in Naor, 2009]. A rise in intracellular calcium levels is involved in the secretion of LH [Stojilkovic et al., 1989]. Also, an increase of calcium from extracellular sources via L-type voltage-gated calcium channels is critical for the mitogen-activated protein kinase (MAPK) family activation specifically the extracellular-signal regulated kinase (ERK) in the GnRH signaling pathway [Roberson et al., 2005]. Several MAPK pathways are activated by GnRH including ERK, jun-N-terminal kinase (JNK) and p38MAPK [reviewed in Naor, 2009]. These signaling pathways of GnRH may lead to activation of early response genes such as *c-fos* [Roberson et al., 1999]. G-protein signaling cascades may influence morphological plasticity through one or several of these signaling cascades.

As secretory cells in the anterior pituitary gland, the question arises as to why gonadotropes need process extensions and formation of process extensions. A few studies conducted by Childs et al., [1998] demonstrate that

gonadotrope process formation and extension is in the direction of blood vessels and correlates with the LH-surge. The anterior pituitary gland is highly vascularized with a dense network of capillaries. A few studies associate hormone secreting endocrine cells with rat pituitary blood vessels and show the close relationship between them [Itoh et al., 2003]. Another anterior pituitary cell population, the corticotropes, have shown process extensions in close proximity of blood vessels [Itoh et al., 2000]. Therefore, the formation of process extensions may be an avenue to increase secretory impact of cells into the anterior pituitary vasculature system.

To further our understanding of E2 influence on GnRH-induced plasticity, additional studies addressing the physiological impact of gonadotrope motions must be conducted. We plan to quantify LH levels using radioimmunoassy (RIA) to ask whether E2 treatment increased gonadotropes ability to increase secretion of LH. However, our experiment model, a pituitary slice, has its limitations. By quantifying LH levels in the media, it does not address whether this is a change in pituitary vasculature levels or simply an undirected release of LH. Another limitation of the studies presented is the inability to conclude direction of where the process extension is developing or moving. Our hypothesis is that the process extensions are sensing the surrounding area to maximize the secretory surface of the gonadotropes. Albeit, to date, we have no evidence of gonadotropes secreting from process extensions. I attempted to dual label blood vessels and gonadotropes by filling the blood vessels of transgenic mice with dextran Texas red dye. Unfortunately, the dye was not

retained sufficiently in vitro to allow the dual label to work. To address this issue another way, time-lapse video microscopy with an animal model containing a fluorescent marker labeling blood vessels crossed with the YFP+ gonadotrope model would be ideal. Such a mouse exists in which the Tie2 promoter drives fluorescent protein expression [Coveney et al., 2008]. This may allow us to ask whether there is directionality of the gonadotrope process extensions observed.

Nonetheless, the studies presented and discussed present information that there is a differential affect of the duration of E2 exposure on gonadotrope plasticity. The data extends previous work [Navratil et al., 2007], by confirming that it is gonadotropes that develop process extensions in response to GnRH in a short time period. However, it further suggests that prior visualization of cell body movements may not be gonadotropes, but rather another as yet unidentified cell type. This experimental model examining E2 affects on cell morphology and movement will help further our understanding of E2 signaling and may present a new signaling cascade to be elucidated on how GnRH signaling through the GnRH receptor modulates actin filament dynamics.

CHAPTER FOUR

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