

Estrogen in Female Reproductive Axis: Positive Estrogen Feedback to Pituitary

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I. Introduction

Estrogen plays pivotal roles in the regulation of reproduction, cancer development, cardiovascular system, and food intake and energy expenditure.^{1,2} Accordingly, it is well documented that a disruption of estrogen signaling leads to the complete loss of fertility in both sexes, altered response to estrogen in various tumors, increased chance of heart problems, and a metabolic syndrome marked by obesity, impaired tolerance to glucose, and reduced energy expenditure.^{2,3}

In particular, estrogens play a central role in regulating female reproduction throughout the hypothalamic-pituitary-ovarian (HPO) axis, each tissue of which expresses ER α (Figure 1). The HPO axis is central to female reproduction. Gonadotropin releasing hormone (GnRH) secreted from the hypothalamus stimulates pituitary gonadotrophs to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) which in turn regulate folliculogenesis and ovulation in the ovary. Upon gonadotropin stimulation, the ovarian cells produce steroid hormones, particularly estrogen, which feed back

to both hypothalamus and pituitary to regulate gonadotropin release.⁴⁻⁶ The importance of estrogen in the LH surge in this feedback regulation is evident. Rats and mice which are ovariectomized, thereby deficient of the major source of estrogen, do not show an LH surge and are anovulatory (Figure 2).^{7,8} Aromatase knockout (ArKO) mice, which are incapable of producing estrogen, do not have an LH surge and are infertile.^{9,10} Furthermore ER α KO mice are anovulatory and thus infertile. Despite the clear significance of estrogen in the LH surge and ovulation, the mechanism of how estrogen regulates intracellular events in regulating LH release is not yet clear.

Estrogen actions are mediated by estrogen receptors (ERs), ER α and ER β . ERs are nuclear receptor transcription factors that mediate estrogen action by targeting transcription of genes whose products will ultimately alter the physiology of the cell. Both ER α and ER β are expressed in the pituitary gonadotroph,¹¹ yet diverse lines of evidence indicate that ER α is the major mediator of estrogen action in the pituitary. Agonists for ER α but not ER β are capable of inducing LH secretion in estrogen primed pituitaries *in vitro*.^{12,13} ER α KO female mice are completely infertile, have elevated basal levels of LH, and do not ovulate, while ER β KO mice are fertile.^{14,15}

As estrogen gives feedback effects to both hypothalamus and pituitary, determining the tissue-specific

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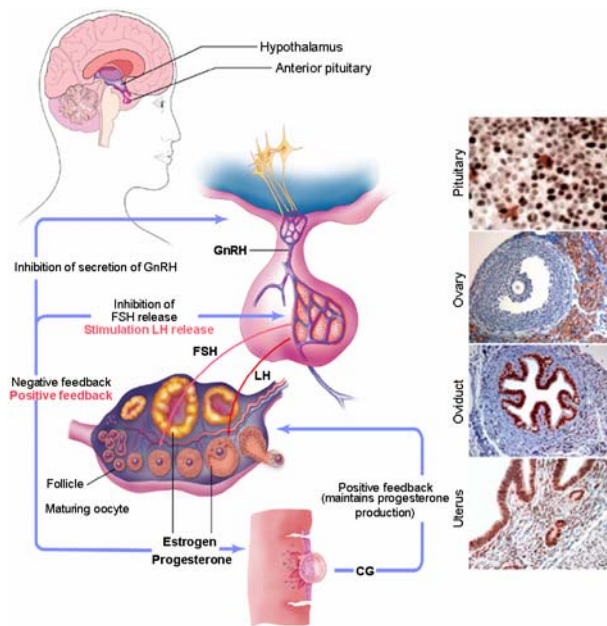


Figure 1. Estrogen feedback network and ER α expression in the female reproductive tissues. Illustration of the HPO feedback system that governs female reproduction (left). Immunostaining for ER α (brown nuclear stain) in primary reproductive tissues. Ovary, oviduct, and uterus counterstained with hematoxylin.

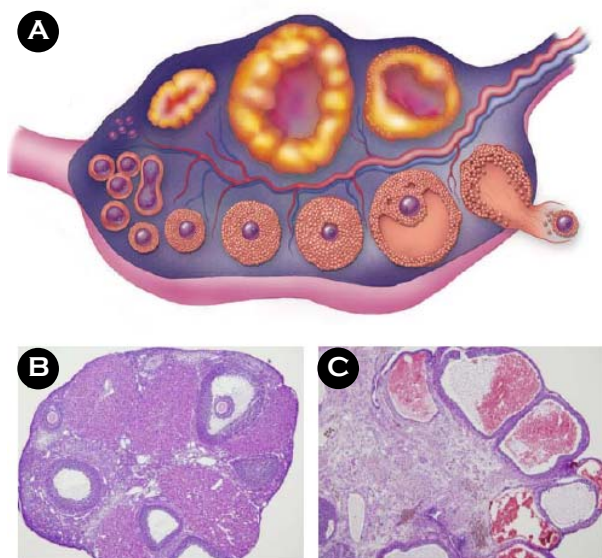


Figure 2. Follicular growth, ovulation, and the ER α knockout effect on hemorrhagic cyst formation. **(A)** Diagram of normal ovary with growing follicles, follicle rupture, and corpus lutea formation. **(B)** Wild-type ovary displaying healthy follicles with oocyte and corpus lutea. **(C)** Ovary from ER α KO forms hemorrhagic cyst and no corpus lutea.

role of estrogen in regulating LH secretion has been challenging. Either pharmacological treatment or ER knockout should compromise estrogen action in both tissues. Due to the challenge of isolating effects of estrogen in the pituitary, most of the studies in LH secretion were performed with cultured cells *in vitro*.^{12,13,16,17} A much anticipated approach of the manipulation of genes in a spatio-temporally controlled manner is being employed in studying estrogen/ER α in female reproduction. This 'conditional gene knockout' approach allows investigators to delete ERs selectively in either pituitary or hypothalamus, and thus will eventually lead to the determination of downstream molecular pathways of ER α activation in regulating LH secretion.

II. Selective Deletion of ER α in the Pituitary and Hypothalamus

1. Pituitary-specific ER α knockout (ER $\alpha^{\text{fllox/fllox}}$ α GSU $^{\text{cre}}$)

Recently, our laboratory generated pituitary-specific ER α knockout (ER α KO) mice, in which ER α was selectively deleted in the pituitary cells that express glycoprotein hormone α subunit (α GSU)-gonadotroph and thyrotroph. We crossed ER $\alpha^{\text{fllox/fllox}}$ mice that possess two loxP sites in the introns flanking exon 3 of the ER α gene (Figure 3) with α GSU $^{\text{cre}}$ mice in which the 4.6 kb promoter of the α GSU gene drives the expression of Cre recombinase in the α GSU expressing cells.^{14,18} The resulting heterozygote (ER $\alpha^{\text{fllox/+}}$ α GSU $^{\text{cre}}$) mice were then further bred with ER $\alpha^{\text{fllox/fllox}}$ mice to produce ER $\alpha^{\text{fllox/fllox}}$ α GSU $^{\text{cre}}$ mice. In these mice, the Cre excises DNA sequence spanning the two loxP sites including exon 3 of the ER α gene in the gonadotrophs and thyrotrophs. The excision of exon 3 results in the production of a fragmented ER α protein that lacks the DNA binding and hormone binding domains as well as the AF-2 trans-activation domain,¹⁴ which makes it completely non-

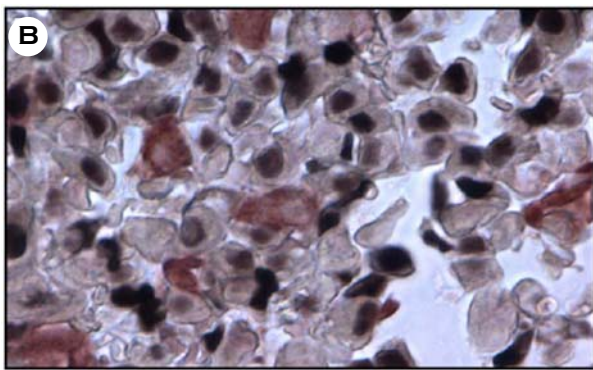
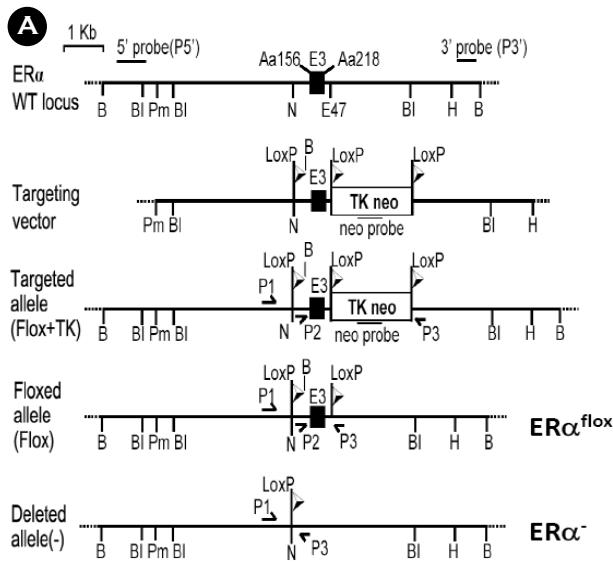


Figure 3. ER α is not expressed in gonadotroph of piER α KO mice. **(A)** Scheme for targeting of exon 3 of ER α which results in deletion of exon 3 upon cre excision. **(B)** Pituitary from ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ double immunostained for ER α (brown nuclear stain) and LH β (red cytoplasmic stain).

functional. The successful deletion of ER α gene was confirmed by the lack of ER α immunopositive signal in both gonadotrophs (Figure 3) and thyrotrophs (data not shown) of the ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ mouse.

Most notably, ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ female mice exhibit signs of estrus and mate with proven males, but none of them show normal pattern of cycling or any sign of pregnancy, indicating that they are infertile. Interestingly, the ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ female mice retain intact machineries for gonadotropin secretion and ovulation as was

determined by the normal ranges of basal serum levels of LH and FSH and ovulatory response to exogenous gonadotropin treatment. Furthermore, corpus lutea are occasionally seen in the adult ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ ovary,¹⁹ an evidence of natural ovulation, without exogenous gonadotropin stimulation. These phenotypes are in contrast with those of ER α KO mice in which ER α is globally deleted: ER α KO mice (1) do not show any sign of estrus, (2) maintain constitutively elevated serum LH level, (3) do not ovulate, and (4) form hemorrhagic cysts in the ovary.^{14,20,21}

It is very intriguing that even though ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ female mice present multiple signs of fertility such as normal level of serum gonadotropins, ability to ovulate and formation of corpora lutea, they are infertile. It is speculated from this unique feature that the gonadotroph of ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ female mice may have secretory machinery fully equipped with components necessary for basal secretion of gonadotropins, but without key regulatory components that may be required for a massive LH release in response to the GnRH stimulation for the ovulatory LH surge. In other words, the preovulatory rise of estrogen may induce the expression of the key regulatory components that are specifically required for an unknown but unique intracellular process that prepares the gonadotroph to discharge a large amount of LH at the time of the LH surge. This assumption precludes that the deficiency of ER α in the gonadotroph of ER $\alpha^{\text{flox/flox}}$ α GSU $^{\text{cre}}$ female mice would hamper the unique process, which would be represented by the loss of LH surge and thus abnormal pattern of estrous cycles, eventually causing infertility.

Taken together, this novel finding/speculation lead us to hypothesize that ER α may mediate estrogen action in the pituitary by modulating the expression of the unique regulatory components of the secretory machinery specifically required for gonadotropin secretion at the time of LH surge. A hypothetical molecular mechanism gover-

ning this process will be discussed in the later part of this review.

2. Neuron-specific ER α knockout (ER $\alpha^{\text{flox/flox}}$ CamKII α^{iCre})

ER α expressing neurons which innervate GnRH (gonadotropin-releasing hormone) neurons are located in the rostral periventricular regions of the hypothalamus. Estrogen actions in brain regions other than the pituitary are considered important as they are believed to generate the GnRH surge which precedes the LH surge. To study the mechanisms through which estrogen acts on GnRH neurons, neuron-specific ER α knockout mice, ER $\alpha^{\text{flox/flox}}$ CamKII α^{iCre} , were generated by Dr. Gunther Schutz's Laboratory.²² The neuron-specific ER α knockout mice were found to be infertile and have many abnormalities in their reproductive organs. The uterus in the mutant model was found to be grossly enlarged and filled with liquid, the endometrium atrophic and lacking glandular structures. The endometrium stroma was condensed and showed granulocyte infiltration.²² Mutant ovaries contained no corpora lutea, an indication of failure to ovulate, and contained a large number of antral follicles and theca cells that appeared hypertropic and luteinized, indicative of improper ovarian stimulation. ER $\alpha^{\text{flox/flox}}$ CamKII α^{iCre} mice exhibit a wild type range of basal serum LH level.

Using these ER $\alpha^{\text{flox/flox}}$ CamKII α^{iCre} mice, the stimulatory effects of the estrogen positive feedback were tested. Ovariectomized wild type, ER β KO and ER $\alpha^{\text{flox/flox}}$ CamKII α^{iCre} mice were given estrogen treatment, an estradiol capsule followed by an injection of estradiol benzoate. As a result, ER $\alpha^{\text{flox/flox}}$ CamKII α^{iCre} mice showed no sign of an LH surge whereas wild type and ER β mutant mice exhibited an LH surge at the time of lights out around 19:00 hr, a clear result that neuronal ER α is necessary for estrogen positive feedback. Next it was determined whether or not neuronal ER α pathways

were sufficient to induce an LH surge. For this experiment, WT mice were given the ER α -selective compound 16 α -LE2 as a second estradiol injection. Wild type mice treated with the ER α -selective compound exhibited an LH surge providing conclusive evidence that cells expressing ER α are necessary and sufficient for estrogen positive feedback actions on GnRH neurons.²²

The neuronal-specific ER α knockout model demonstrated that although other cell types expressing ER α might play a role in positive feedback leading to the LH surge and ovulation, ER α expressing neurons are critical for estrogen positive feedback. The absence of this positive feedback in the ER α mutant mice is consistent with the infertile reproductive phenotype they exhibit. The mechanism of estrogen action via ER α in the neuron bears further investigation.

III. Downstream Pathway of ER α Activation in Gonadotroph

It has been well established that estrogens prime the pituitary for the LH surge, yet by a mechanism yet to be delineated. This 'priming' theory has been well supported by the findings from both *in vivo* and *in vitro* experiments that the pituitary cells need to be exposed to high levels of estradiol for an extended period to release surge level of LH in response to GnRH treatment.⁴

ER α is a transcription factor, and its functional role is manifested by its control over the expression of its downstream genes. Therefore, it would be reasonable to speculate that ER α may regulate a cohort of genes involved either in GnRH receptor signaling, hormone production, hormone secretion, or all of these. Our result from the pituitary-specific ER α knockout experiment supports the priming theory in that ER α mediates estrogen action in the pituitary gonadotroph (Figure 4).

In fact, previous studies have provided evidence that estrogens regulate the expression of GnRH receptor

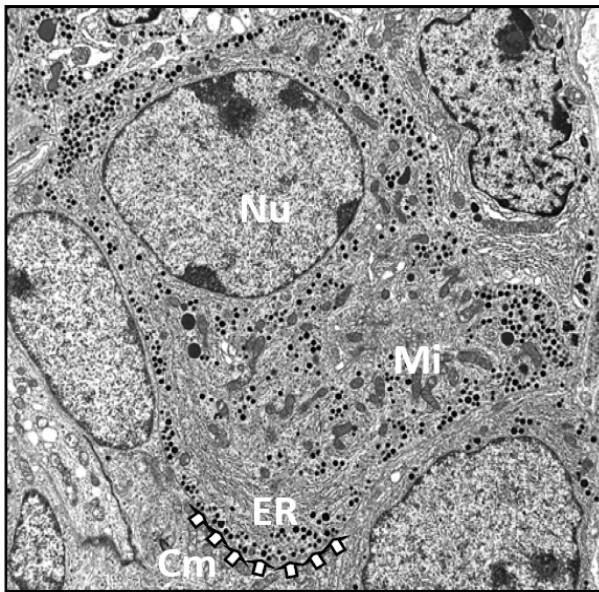


Figure 4. Electron microscopic images of a gonadotroph. Control pituitaries were fixed in glutaraldehyde, infiltrated with osmium, and embedded in resin for thin sectioning. Nu, nucleus; Mi, mitochondria; ER, endoplasmic reticulum; Cm cytoplasmic membrane.

(GnRH-R), ion channel proteins, and components of secretory machinery.^{4,23,24} Regarding the role of estrogens in GnRH-R expression, it is generally agreed that while estrogen increases GnRH-R expression in the pituitary, an increase in receptor number alone is not sufficient to account for the GnRH-induced surge of LH release.^{23,25} For the possibility of increasing gonadotropin gene expression and production as a mechanism of estrogen priming of the pituitary, a variety of laboratories published contrasting reports,^{26,27} indicating that estrogens may influence gonadotropin production while this may not be a critical factor in priming pituitary. The third possibility is that estrogens may establish the 'priming' status by positioning secretory LH vesicles at the secretory machinery of the plasma membrane, which would be made possible by inducing the expression of molecules involved with this process. Supporting the third possibility, a study done in sheep shows estrogen helps to mobilize LH-containing vesicles to the plasma

membrane of gonadotrophs.²⁸ Underscoring the role of secretory vesicles in the LH surge, double knockout mice of IA-2 and IA-2 β , both transmembrane structural proteins found in dense core secretory vesicles, were incapable of producing an LH surge and infertile.²⁹

IV. ER α on Hormone Subunit Expression

Based on our evidence that ER α is indeed the mediator of estrogen action in the gonadotroph, we examined whether ER α plays a role in regulating the expression of gonadotropin subunits. First, we tested whether 17 β -estradiol induced the transcription of α GSU, LH β , FSH β , and TSH β . In neither regularly cycling nor the ovariectomized wild type mice did 17 β -estradiol increase the transcription of α GSU, LH β , or TSH β . Given the fact that an estrogen-responsive element has been identified in the rat LH β gene³⁰ and estrogen directly increases transcription of the rat gene *in vitro*,²⁶ it was interesting to find that 17 β -estradiol did not increase transcription of LH β . One possibility is that a small immediate increase in transcription after 17 β -estradiol treatment was not detected *in vivo*. There is evidence that LH β mRNA expression increases before the LH surge in cycling rats.³¹ The loss of ER α in the total ER α KO also results in increases in transcript of gonadotropin subunits,²¹ suggesting that ER α regulates this transcription. The total ER α KO does not isolate effects in the pituitary as ER α is absent in all tissues. Thus, this increase may be due to disruption of estrogen's regulation of GnRH, and consequently GnRH's control of gonadotropin transcription, and may explain the difference in the ER $\alpha^{\text{fllox/fllox}}$ α GSU^{cre} which did not show an increase and are presumed to have normal GnRH pulsatility. Supporting this speculation, no difference in LH β protein expression was observed on the days of metestrus and diestrus compared to the day of proestrus as determined by immunostaining in regularly cycling mice.¹⁹

V. ER α in Regulating Genes Involved 'Uniquely' in LH Surge

LH is packaged into electron dense secretory vesicles, transported to the cell membrane, and then discharged to the extracellular space or the blood stream upon GnRH stimulation. In general, the very last process of vesicle release (exocytosis) is carried out by a SNARE complex which is composed of a host of proteins including VAMP, syntaxin, and SNAP25 subfamily proteins; Rab3, Munc13-1, complexin, and synaptotagmins.³² In the sheep pituitary, expression of a variety of SNARE complex proteins were detected, which includes SNAP-25, VAMP2, rab3A, Munc18-1, and synaptotagmin I, indicating that the SNARE complex may participate in LH release.³³ Microarray analysis performed in our laboratory confirmed that the basic components of this secretory machinery are constitutively expressed in the mouse pituitary, independent of estrogen/ER α regulation (unpublished data). These findings suggest that ER α may 'prime' the gonadotroph for LH secretion not by regulating the expression of the components of the general exocytosis machinery but by modulating the expression of the genes that are critically involved in 'controlling' the exocytosis machinery.

We recently reported a finding that cholecystinin type A receptor (CCK-AR), a molecule associated with protein secretion, is an ER α -downstream gene in the mouse anterior pituitary. In the cycling mouse pituitary, the expression of CCK-AR mRNA is markedly higher in the afternoon of proestrus compared to metestrus. Both ovariectomy and null mutation of the ER α gene completely abolished CCK-AR mRNA expression in the gonadotroph. Injection of 17 β -estradiol to ovariectomized wild type mice recovers CCK-AR mRNA expression to levels observed at proestrus, but no such recovery was induced in ovariectomized ER α KO mice, showing the

ER α mediates the estrogen action in inducing CCK-AR expression. The same pattern of estrogen-dependency in inducing CCK-AR mRNA expression was seen in cultured primary anterior pituitary cells, indicating that estrogen directly acts on pituitary cells to induce CCK-AR expression. The functional significance of the ER α -mediated CCK-AR induction in LH release was tested by using primary pituitary cells. When the cells were primed with 17 β -estradiol and then treated with GnRH in the presence or absence of lorglumide, a CCK-AR antagonist, the lorglumide-treated cells released significantly reduced amount of LH (30% reduction) into the culture media (34). Therefore, this study illustrates an example that ER α 'primes' the gonadotroph by equipping it with a 'secretion enhancer' that is not an essential component of secretory machinery, but enhances hormone secretion in response to GnRH at the time of LH surge.

To further identify ER α -mediators in the pituitary, we employed a genome-wide gene expression profiling approach using DNA microarray technique using pituitaries prepared from different estrogen environments (unpublished). Briefly, four groups of mice were used for this purpose. In the first group, naturally cycling ER $\alpha^{\text{flox/flox}}$ mice were sacrificed on either the day of metestrus, when estrogen level was low, or the day of proestrus, when estrogen level was highest. Groups two, three, and four consisted of ovariectomized ER $\alpha^{\text{flox/flox}}$, ER α KO and ER $\alpha^{\text{flox/flox}}$ α GSU^{cre} mice, respectively. Three weeks later, each group was injected with 10 μ g of 17 β -estradiol or vehicle (sesame oil) at 0900 h of day 22, and again at 0900 hour of day 23. At 1500 h on day 23, the mice were sacrificed and pituitaries harvested. RNA was isolated from whole pituitary and used for microarray analysis. The microarray data was compiled into a mouse Pituitary Gene Expression Database (mPiGED) for facile analysis (Figure 5). This database also contains gene expression data from male pituitary and three brain

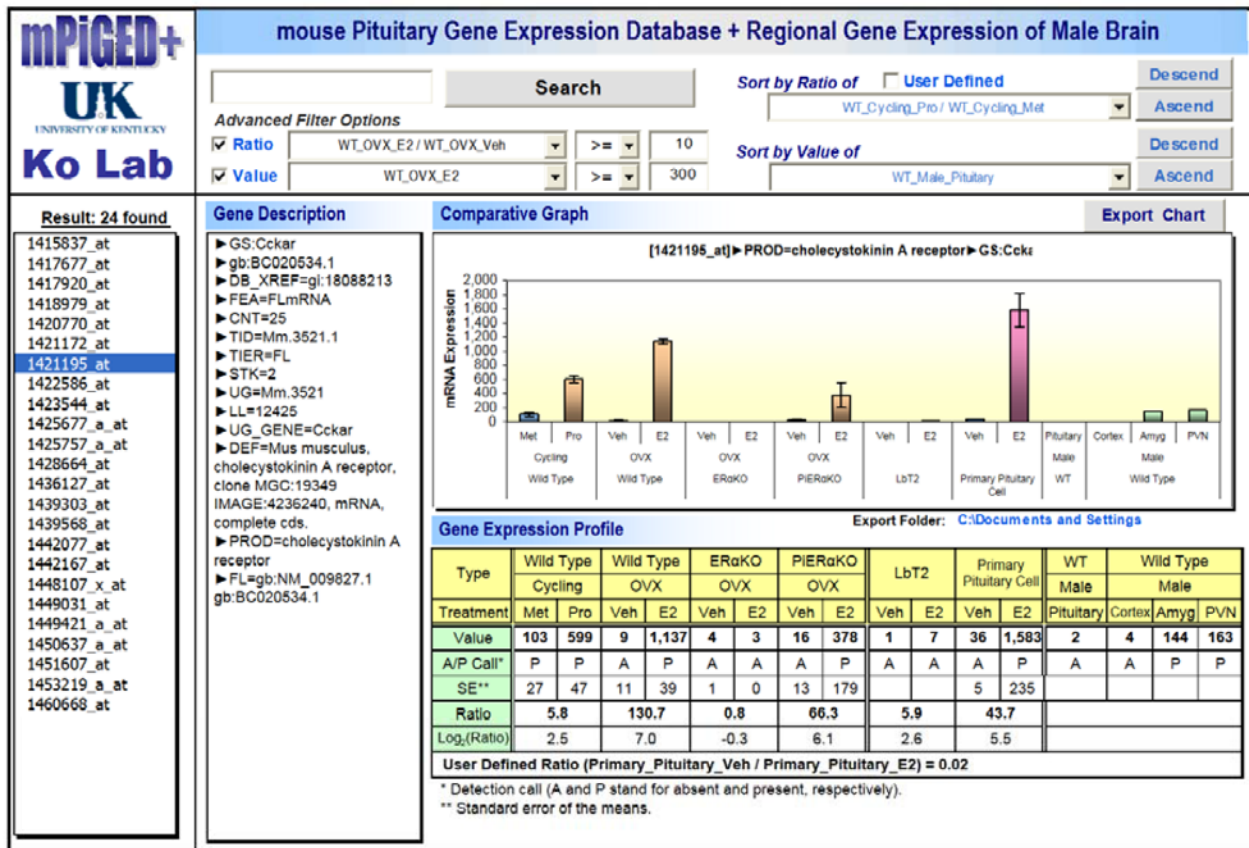


Figure 5. The mPiGED profiles of gonadotropin gene mRNA expressions. For WT Met, WT Pro, mice which showed a 4~5 day cycle were used and sacrificed at 1700 h on the day of metestrus or proestrus. For WT OVX + vehicle, WT OVX + E2, ERα^{-/-} OVX + vehicle, ERα^{-/-} OVX + E2, ERα^{flx/flx} αGSU^{cre} OVX + vehicle, ERα^{flx/flx} αGSU^{cre} OVX + E2, mice were ovariectomized at 1.5 to 4 months of age. Three weeks later, mice were injected with either 10 μg of 17-β-estradiol or oil (vehicle) at 0900 h of day 1 and 0900 h of day 2. Pituitaries were collected at 1500 h on day 2. The total RNA used for DNA microarray was pooled from at least five mice for WT Met, WT Pro, WT OVX, and at least two mice for ERα^{-/-} OVX and ERα^{flx/flx} αGSU^{cre} OVX. The array was done in duplicate. Data are represented by mean plus standard error of the mean from two independent array results.

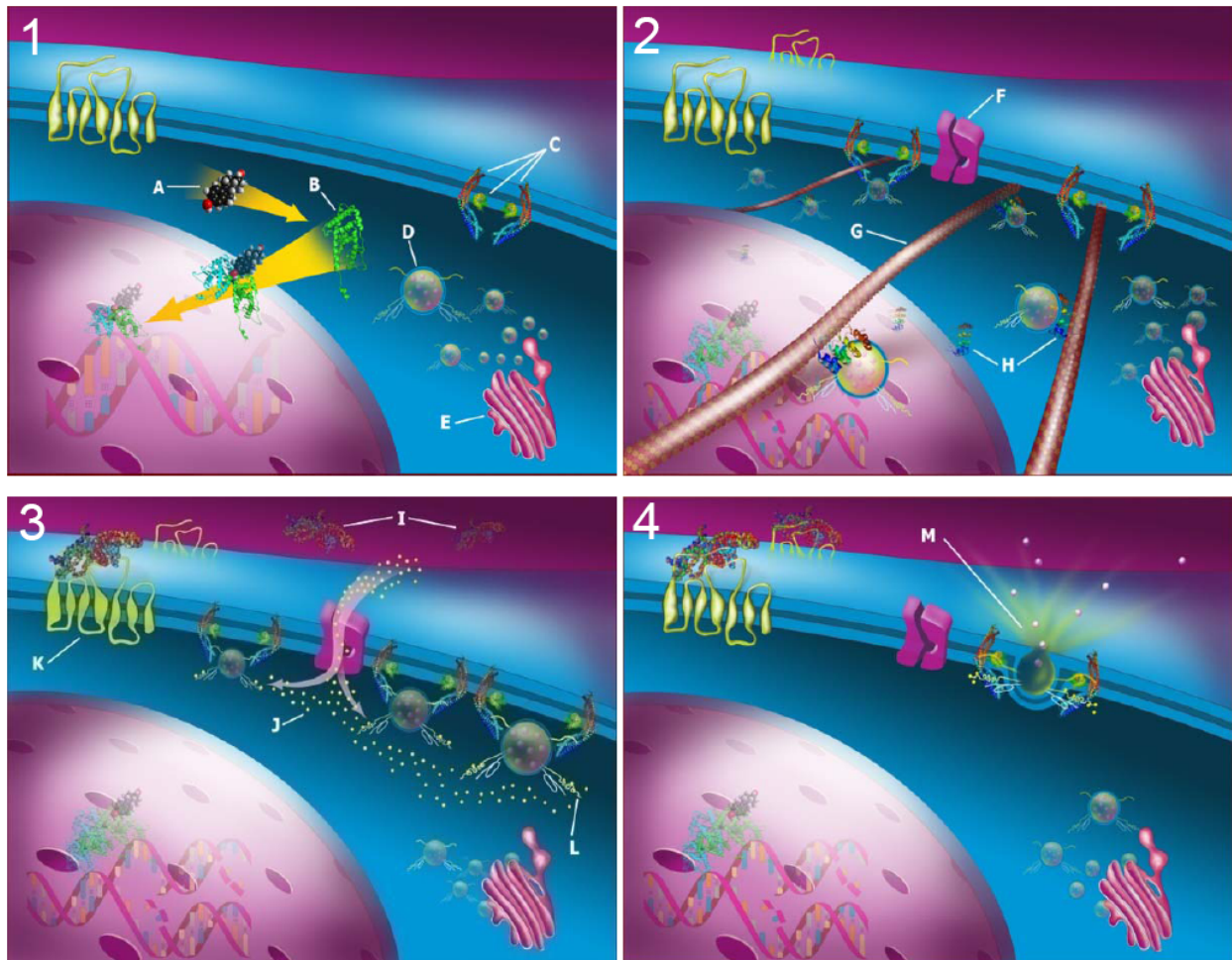
regions (cortex, amygdala, and paraventricular nucleus), which can serve as controls.

Through an extensive analysis of the mPiGED, we have identified a cohort of ERα-induced genes that are either involved mostly in intracellular vesicle transport, ion channel formation (unpublished), or regulation of gene expression. This on-going unpublished analysis provides us with an ample opportunity to envision a molecular mechanism of ERα-mediated estrogen priming of the pituitary. Our current working hypothesis that 'estrogen via ERα changes the status of gonado-

troph from basal to massive secretion mode by mobilizing LH vesicles to secretory machinery and equipping the gonadotroph with regulatory molecules that elevate the responsiveness to the repetitive GnRH stimulation at the time of LH surge' is illustrated in the following section.

VI. Proposed Mechanism of Estrogen-Induced Pituitary Priming for LH Surge

The hypothetical priming mechanism postulates a cascade of intracellular molecular events prior to and at



A, Estrogen; B-ERα; C, t-SNASRE complex; D, LH vesicle; E, Golgi; F, Calcium Channel G, Microtubule; H, Ankyrin; I, GnRH; J, Calcium ions; K, GnRH receptor, L, Synaptotagmin; M, LH release

Figure 6. A hypothetical cascade of LH secretion upon GnRH stimulation. 1. As estradiol levels rise, the molecule binds to ER α , promoting transcription of specific genes involved in vesicle transport or exocytosis. 2. LH vesicles are transported to the membrane and prepared for exocytosis. 3. GnRH binds to the gonadotroph, triggering the influx of calcium which is the last signal for exocytosis. 4. LH hormone is released into the bloodstream.

the time of the GnRH-induced LH release as illustrated in Figure 6. When intracellular estrogen concentration rises, transcriptional activity of ER α increases, leading to the production of proteins that directly or indirectly contribute to the priming process (Indirect Via transcriptional regulation of other subsets of ER α downstream genes). The 'priming' is achieved by the three major biochemical/physical processes: (1) pre-docking LH vesicles to the exocytosis complex, (2) increasing responsiveness to GnRH and (3) pre-positioning of gonadotrophs for

fast delivery of secreted LH to the blood stream.

1. LH vesicles to exocytosis complex

The surge of LH is achieved by the massive release of LH within a short period of time. Therefore, it would be reasonable to speculate that the secretory LH vesicles should be ready for discharge prior to GnRH stimulation. Supporting this idea, the mPiGED shows that genes involved in vesicle transport (e.g. ankyrin) are induced by estrogen/ER α .

2. Increased responsiveness

Increased frequency as well as magnitude of GnRH release from the hypothalamus is the driving force of surge release of LH. Therefore, after each discharge of LH, the gonadotroph needs to prepare for the next GnRH stimulation. One of the most critical events in maintaining responsiveness is to establish polarization across the cell membrane.³⁵ The mPiGED shows that multiple kinds of ion channel proteins including Cav2.1 are induced by estrogen/ER α .

3. Pre-positioning of gonadotrophs

In addition to a massive discharge within a short time, to achieve high blood concentration of LH, it is critical to deliver the released LH to the blood stream as quickly as possible because the half life of LH is very short (20 minutes in circulation).³⁶ This can be achieved by positioning the secretory gonadotrophs close to blood vessels, loosening extracellular matrix, or/and directly discharging the hormone into blood stream. All of these changes require tissue remodeling accompanied by increased matrix metalloproteinase activity and/or cell migration. Interestingly, mPiGED shows that estrogen/ER α induces meltrin (ADAM12) and reelin, which are involved in tissue remodeling and cell migration, respectively.

VII. Summary

New tools such as conditional knockout mice are allowing a clearer picture of the role of estrogen in exerting positive feedback to the hypothalamus and pituitary to induce the LH surge. Current evidence indicates that both the hypothalamus and pituitary are critical in mediating the estrogen signal to induce the LH surge. In particular, ER α may have a role in preparing a unique set of players which equip the pituitary

gonadotroph to efficiently release a large amount of LH hormone over a short period. These players are speculated to be involved in positioning gonadotrophs close to blood vessels, localizing LH vesicles to the membrane, and increasing the responsiveness of gonadotrophs to GnRH.

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