UNIVERSITY OF CALIFORNIA, SAN DIEGO

Androgens Mediate Sex Differences in Gonadotropin Gene Expression in *Utero*

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Michael Joseph Kreisman

Committee in charge:

Professor Kellie Breen Church, Chair
Professor P.A. George Fortes, Co-Chair
Professor Michael David

2016

The Thesis of Michael Joseph Kreisman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2016

TABLE OF CONTENTS

Signature Page iii

Table of Contents iv

List of Figures v

List of Tables vi

Acknowledgements vii

Abstract of the Thesis viii

Introduction 1

Materials and Methods 8

Results 11

Discussion 15

Figures and Tables 20

References 27

LIST OF FIGURES

Figure 1: Females express gonadotrope related genes and prolactin significantly higher than males 20

Figure 2: Males and females have similar amounts of gonadotrope cells in the medial region of the anterior pituitary 21

Figure 3: Males and females have similar amounts of gonadotrope cells in the lateral region of the anterior pituitary 22

Figure 4: Gonadotrope cells in males and females contain androgen receptors 23

Figure 5: Androgens are required for differential expression of gonadotropin genes .. 24

Figure 6: GnRH signaling is necessary for sex difference 25

LIST OF TABLES

Table 1: Amount of cDNA loaded for each gene in quantitative-PCR, and the sequence of quantitative-PCR primer sequences. 26

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor Dr. Church for giving me the opportunity to join her lab. These past two years have been an incredible learning experience that I will always remember. I would also like to thank Dr. Church for always being available to help me with experiments, talk about data, and also help me with reviewing my thesis and practicing my thesis presentation. Lastly thank you for always being optimistic about this process, which gave me confidence that I would be able to finish this journey.

I would also like to thank members of the Breen Church lab. I would like to thank Elena Luo for training me in the techniques necessary to complete this project. In addition, I would like to thank Christopher Song and Sharon Chaing, who were always willing to help me with parts of my project. Without them, experiments would have taken twice as long. I would also like to thank Christopher for his contribution of figure two, and thank Kathleen Yip for her contribution of figure one.

Figure and tables, in full, is a reprint of the material currently being prepared for submission for publication. Kreisman, Michael J; Song, Christopher I; Yip, Kathleen; Breen, Kellie M. "Androgens Mediate Sex Differences in Gonadotropin Gene Expression *in Utero"*. Michael Joseph Kreisman was the primary investigator and author of this paper.

ABSTRACT OF THE THESIS

Androgens Mediate Sex Differences in Gonadotropin Gene Expression in *Utero*

by

Michael Joseph Kreisman

Master of Science in Biology

University of California, San Diego, 2016

Professor Kellie Breen Church, Chair
Professor P.A. George Fortes, Co-Chair

It is well known that the brain undergoes changes in a sexually dimorphic manner during fetal development. Here, we postulate that sex differences are present in another neuroendocrine tissue, the pituitary gland. We tested the hypothesis that androgens mediate sex differences in gonadotropin gene expression during fetal development of the anterior pituitary gland. We compared gonadotropin gene expression in fetal male and female mice treated with either an androgen receptor agonist or antagonist from embryonic day 15.5-17.5. Multiple gonadotrope-derived genes were expressed at

significantly higher levels in females compared to males, including luteinizing hormone beta (*Lhb*), follicle-stimulating hormone beta (*Fshb*) and gonadotropin-releasing hormone receptor (*GnRHr*). Treatment with flutamide, testosterone, or dihydrotestosterone eliminated the sex difference in gene expression. Specifically, flutamide relieved the suppression in male gene expression to the level observed in females. In contrast, testosterone and dihydrotestosterone attenuated female gene expression to male levels. Having determined that male and female fetal pituitary glands contain an equivalent number of gonadotrope cells, we focused on understanding the mechanism underlying sex-dependent transcriptional regulation within this cell type. Fetuses either lacking GnRH or GnRH receptor showed no sex difference in expression of *Lhb*, *Fshb*, or *GnRHr*, suggesting that GnRH signaling is important for expression in a sex-dependent manner. Collectively, our data demonstrate that there is a sex difference in gonadotropin gene expression during fetal development which is regulated by androgens. Furthermore, we postulate that hypothalamic drive to the pituitary is greater in female fetuses and blunted in males during fetal development.

INTRODUCTION

The Hypothalamic-Pituitary-Gonadal Axis

Communication between the brain, pituitary gland and gonad is essential for reproduction in rodents, domestic animals, primates and humans. These tissues communicate via neurohormone or hormone signals commonly referred to as the hypothalamic-pituitary-gonadal (HPG) axis which has been well characterized in postpubertal animals. In both males and females, the gonadotropin releasing hormone (GnRH) neurons release GnRH in a pulsatile manner into the hypophyseal portal system, allowing the hormones of the hypothalamus to reach the anterior pituitary gland in high concentrations. The primary target of GnRH is the gonadotrope cell population of the anterior pituitary gland. GnRH stimulates the gonadotrope to synthesize and release the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both of these hormones are released in a pulsatile manner as well.

In males, LH and FSH will stimulate the testis to produce testosterone and promote spermatogenesis. In females, the gonadotropins act on the ovaries to stimulate follicle growth, estradiol production, and ovulation. Importantly, ovulation is driven by another mode of secretion only present in females. The activity of the HPG axis differs in females compared to males. While females do have the same pulsatile release manner of GnRH and gonadotropins as males, the female HPG axis is also capable of eliciting a surge in hormone secretion. The surge in GnRH secretion results in a very large release of LH not seen in males, and results in ovulation of an oocyte from a mature follicle. Testosterone and estradiol, at low concentrations, act as negative regulators of gonadotropin release, while at high concentrations estradiol results in positive feedback

and leads to the GnRH and LH surge, which are required for ovulation.

Testosterone produced by the testis and estrogen produced by the ovaries both regulate hormone production within the HPG axis. Both these steroid hormones can act directly on the pituitary to regulate gonadotropin synthesis and secretion. Another site of action appears to be at the hypothalamic level to alter GnRH neuron activity. However, GnRH neurons do not have androgen receptors (AR) or estrogen receptor alpha, so this neuron population appears to not be directly regulated by these steroids.[1, 2] Instead, GnRH neurons are indirectly regulated by steroids through interneurons, such as kisspeptin neurons. There are two populations of kisspeptin neurons located in the hypothalamus that are important for HPG axis regulation. One population is located in the anteroventral periventricular nucleus (AVPV), while the other is located in the arcuate nucleus. Both these populations are capable of responding to steroids, but differ in their regulation of GnRH neurons. Kisspeptin neurons in the AVPV stimulate GnRH neurons responsible for the GnRH surge and LH surge, whereas arcuate kisspeptin neurons inhibit GnRH neuron activity to maintain the pulsatile release of GnRH.[3] While the function and the regulation of the HPG axis is well understood post-puberty, the functions and development of this axis is not well understood during fetal development.

Role of the Gonadotrope Cell

Gonadotrope cells are responsible for producing LH and FSH. LH and FSH are both heterodimers, and consist of an alpha and beta subunit. The alpha subunit, commonly called glycoprotein hormone alpha-subunit (aGSU), is the same for both LH

and FSH. Therefore, the defining characteristic of LH and FSH is the beta subunit. The beta subunit for LH is called LH beta-subunit (LHb) and for FSH is called FSH beta subunit (FSHb). Transcription of these beta subunits is what is considered the rate limiting step in the production of LH and FSH.[4, 5] Therefore, the amount of beta subunit will determine the amount of mature LH or FSH hormone synthesized within the gonadotrope cell.

Gonadotropes have been shown to be regulated by sex steroids in both gene expression and secretion of gonadotropins. For example, castration eliminates gonadal steroid negative feedback and allows for elevated levels of *Lhb* and *Fshb* gene expression. Estrogen and androgens have both been shown to suppress elevated *Lhb* gene expression in castrated male and female rats, demonstrating regulation of gene expression by gonadal steroids.[6] Although the actions of gonadal steroids may be mediated at the hypothalamic level, evidence from in vivo pituitary culture systems suggest that regulation can be via direct actions on pituitary cells. In rat pituitary cultures, treatment of dispersed pituitary cells with estradiol has been shown to increase the transcription rate of *Lhb*, while having no effect on *aGsu* or *Fshb*.[7] A similar response was observed in ovine pituitary cultures, where estradiol stimulated the release of LH, but inhibited the release of FSH.[8] Collectively these findings suggest that estradiol may produce different effects on the synthesis and secretion of LH as compared to FSH. Studies of primary pituitary cultures treated with testosterone showed testosterone increased *Fshb* expression, while decreasing *Lhb* and *aGsu* expression.[9] In vivo studies corroborated the effects of testosterone in pituitary cultures, where castrated rats treated with GnRH antagonist and testosterone increased *Fshb* transcription, while decreasing *Lhb*

transcription.[10] Demonstrating that testosterone also regulates LH and FSH in differential manners, and both estradiol and testosterone can regulate gonadotropin gene expression.

Gonadotrope cells synthesize and secrete LH and FSH due to the binding of GnRH to the GnRH receptors, a 7-transmembrane receptor located on the gonadotrope cell surface. The expression of GnRH receptors are highly regulated. Estradiol has been shown to increase mRNA expression of GnRH receptors (*GnRHr*) and the number of receptors on the plasma membrane, together increasing the responsiveness of the gonadotropes to GnRH.[11] While, blocking GnRH actions results in a decrease in *GnRHr* expression and the number of GnRH receptors, indicating that GnRH is a critical hormone regulating pituitary responsiveness as GnRH receptors will not be expressed without the presence of GnRH.[11]

GnRH Neuron and Pituitary Development *in Utero*

The pituitary gland and GnRH neurons both develop and begin to function *in utero*. In rats, GnRH neurons are found in the olfactory placode during prenatal development, yet by late gestation these cells have migrated to their proper location and have established initial synaptic coordination.[12] Also, it has been shown in mice that during late gestation, embryonic day (e.)18, GnRH neurons are properly organized within the hypothalamus and GnRH is being secreted.[13] As the GnRH neurons are migrating and developing *in utero*, the pituitary gland also begins to develop at this time.

In mice, with a gestational length of 18.5 days, the pituitary gland begins to develop as early as e.6 with the formation of the cell structure at the base of the

hypothalamus. The presence or absence of various transcription factors have been shown to play a vital role in causing differentiation of the various anterior pituitary cells. In mice, differentiation of anterior pituitary cells occurs from e.10 to e.17. The gonadotropes were seen to begin differentiating at e.14 and to be finished by e.17 as visualized by the presence of *Lhb* and *Fshb* expression.[14]

A sex difference has been observed in gene expression and secretion of gonadotropins *in utero*. During human gestation, females have significantly higher levels of circulating levels of LH compared to males.[15] In addition, a sex difference was observed in *Fshb* and *Lhb* gene expression on the day of birth in mice. Female mice expressed significantly higher levels of *Fshb* and *Lhb* than male mice.[16] While there is a clear sex difference in the HPG axis causing a sex difference in gonadotropin expression and secretion, what causes this difference and what part of the HPG axis is being acted on has not been determined.

Role of Androgen Action in Gonadotrope Development

A possible cause for the sex difference seen in gonadotrope cells during *in utero* development may be through the actions of androgens. During late gestation in rodents, blood serum testosterone in male rodents is significantly higher than testosterone levels in female rodents.[17] Moreover, during perinatal development many sex differences begin to develop and high levels of androgens have been shown to be a main cause of these differences. A sex difference has been observed in one of the regulators of the HPG axis, kisspeptin neurons. Kisspeptin cells in the AVPV and the amount of gene expression were shown to be significantly higher in adult female rats compared to males. Exposing

female rats on the day of birth to testosterone eliminated the sex difference by reducing the amount of kisspeptin cells in the AVPV and gene expression in these cells to levels observed in males.[18] The experiment showed that sex differences begin developing perinatally and that androgens play a role in creating the sex difference.

Furthermore, elevated androgens during late gestation in female mice have been shown to alter GnRH activity. Treating female mice with dihydrotestosterone, a non-aromatizable androgen, during late gestation resulted in an increase firing rate in the GnRH neurons of these female mice.[19] Androgens have been shown to alter a key component of the HPG axis in the GnRH neurons and has been shown to be responsible for creating sex differences in an HPG axis regulator. However, not much is known regarding how androgens affect gonadotrope cells *in utero*.

Isolating Pituitary Function within the Context of the HPG Axis

Testing whether androgens act directly on the pituitary or not is a difficult task due to the fact that the upstream regulators of the gonadotropes, GnRH and kisspeptin, are also regulated by androgens, and that males and females may be receiving different levels of GnRH stimulation of the gonadotropes. In an attempt to isolate the pituitary from the system, hypogondal (hpg) mice will be used in this experiment. Hpg mice lack GnRH, and utilizing these mice therefore eliminates the potential difference in the amount of GnRH input onto gonadotrope cells received between males and females.

Rationale for Overall Hypothesis

Multiple pieces of evidence point to the overall hypothesis tested in this thesis. First, females have higher levels of circulating LH during gestation and right after birth have higher levels in *Lhb* and *Fshb* gene expression. Second, testosterone has been shown to be elevated prenatally in male rodents compared to female rodents, and has been shown to be capable of suppressing *Lhb* and *aGsu* gene expression. Third, androgen action has been shown to alter prenatal development of HPG neurons and interneurons. Collectively, we hypothesize that a sex difference exists between males and females during *in utero* development of gonadotropes, and that this sex difference is due to androgens acting directly on the pituitary. We postulate androgens act directly on the pituitary gland as opposed to other parts of the HPG axis or its regulators because it has been shown that fetal and newborn males have high frequency of GnRH release, which should result in high levels of gonadotropin expression. Therefore, we hypothesize that androgens are acting directly on gonadotrope cells to attenuate the responsiveness to GnRH.

Materials and Methods

Animals

C57BL/6J males and females were mated, and the presence of a copulatory plug determined to be e.0.5. In experiments using mutant models, mutant offspring were generated using two well characterized models that lack GnRH signaling and are both infertile. The hpg knockout mice lack the ability to translate GnRH, while GnRHr mutant mice lack functional GnRH receptors. C57BL/6J and GnRHr dams were euthanized under isoflurane at e.18.5, while hpg dams were euthanized at e.17.5, and fetuses were collected. All animal procedures were performed in accordance with the University of California, San Diego (UCSD) Institutional Animal Care and Use Committee regulations. All mice were housed under standard conditions and a 12-h light, 12-h dark cycle with *ad libitum* chow and water.

Immunohistochemistry

Fetal mouse heads collected at e.18.5 were fixed in formaldehyde acetic acid, and ethanol and blocked in paraffin wax. Tissue was sectioned sagittally at 5um onto slides and heated for 48h at 37^{o}C. Three consecutive slides were selected every 100 μm and stained with hematoxylin and eosin. Two comparable regions were chosen in each animal, a lateral region characterized by the presence of a thin region of the lumen and the lack of posterior pituitary. A second region, the medial region was chosen by the flattened structure of the anterior pituitary, a greater lumen region, and a large posterior pituitary. A slide of three consecutive sections was chosen from each animal at each region and stained for LHB containing cells or LHB containing cells that co-express AR

as reported.[20] Sections were antigen retrieved using an antigen retriever, heated for 30 minutes with 1x Citra, then endogenous peroxidase activity was quenched in 0.3% H_20_2 in H_20. Sections were blocked with 5% goat serum/0.3% Triton X for 20 minutes and incubated in goat anti-rat LHB primary antibody (1:500) for 24h at 4°C. Next, sections were treated with rabbit anti-goat antibody (1:300) for 30 minutes, and after treated with Vectastain ABC according to directions for 30 minutes. Sections were treated with peroxidase enzyme, counterstained with hematoxylin, and visualized as reported. Negative controls were prepared by incubating slides in block solution rather than primary antibody.[16]

For LHB and AR double staining, slides were prepared in the same fashion. Sections were antigen retrieved using an antigen retriever, heated for 30 minutes with 1x Citra, then endogenous peroxidase activity was quenched in 0.3% H_20_2 in H_20. Sections were blocked with 5% goat serum/0.3% Triton X/ 1% BSA for 20 minutes and incubated in goat anti-rat AR (1:100) for 1h at room temperature then 24h at 4°C. Next, sections were treated with rabbit anti-goat antibody (1:300) for 30 minutes, and after treated with Vectastain ABC according to directions for 30 minutes. Sections were treated with peroxidase enzyme. Sections were then blocked with 5% goat serum/0.3% Triton X for 20 minutes and incubated in goat anti-rat LHB primary antibody (1:1000) for 24h at 4°C. Next, sections were treated with rabbit anti-goat antibody (1:300) for 30 minutes, and after treated with Vectastain ABC according to directions for 30 minutes. Sections were treated with peroxidase enzyme, counterstained with nuclear fast red, and visualized as reported.

Hormone Injections

From e.15.5-17.5, dams were injected subcutaneously, once daily, with flutamide (5mg), testosterone (5 mg), dihydrotestosterone (2.5 mg), or sesame oil. Hormone treatments were deemed to be effective due to the elimination of the difference seen in anogenital distance between fetal males and females.

Gene Expression analysis of fetal pituitaries

Fetal pituitaries were collected under a dissecting microscope, frozen in liquid nitrogen, and stored at -80°C. Tail DNA was genotyped to distinguish females from males, and wildtypes from mutants among littermates. RNA was extracted from fetal pituitaries using RNA Mini Kit (Ambion) as directed, and DNA contamination was removed using DNA-free kit (Ambion) as directed. RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-RAD) to synthesize 650-1000 ug of cDNA. For quantitative-PCR, cDNA and primers were loaded according to table 1, along with Sybr green. Data was analyzed using the ΔΔCt method.[21]

Statistical analysis

For quantitative-PCR, statistical significance was determined by one-way ANOVA and Student's T Test or by two-way ANOVA and Tukey's HSD of the relative quantity. For cell counting, statistical significance was determined by one-way ANOVA and Student's T Test.

RESULTS

Multiple gonadotropin genes exhibit robust differences in gene expression in females compared to males

Gene expression was assessed in individual male and female pituitary glands from fetal mice at e.18.5. We observed a robust sex difference in the expression of multiple genes encoding anterior pituitary hormones. In each case in which a sex difference was detected, the expression was significantly higher in females compared to males (Fig. 1). Specifically, the gonadotrope-derived genes *Lhb*, *Fshb*, and *GnRHr* were expressed significantly higher in females compared to males. In addition, the gonadotrope and thyrotrope-derived gene, *aGsu*, and lactotrope-derived prolactin (*Prl*) were expressed in the same sex dependent manner. In contrast, thyrotropin stimulating hormone beta (*Tshb*), proopiomelanocortin (*Pomc*), and growth hormone (*Gh*) were not expressed differently between males and females at e.18.5. Our finding that a sex difference in gene expression was not a global pituitary effect, but rather more prevalent from gonadotrope derived genes suggests a physiologic difference between male and female gonadotropes during prenatal development

Neither the gonadotrope population nor expression of androgen receptor display a difference between males and females

We tested the hypothesis that the sex difference observed in gonadotropin gene expression, was due to a larger gonadotrope population in females. To test the hypothesis that females have a larger population of gonadotrope cells, we conducted immunohistochemistry on sagittal pituitary sections from male and female e.18.5 embryos (Fig. 2, 3). Two individual sections, one from the lateral and one from the

medial region of the anterior pituitary, were analyzed (Fig. 2A, 2B. 3A, 3B). Our analysis of the total number of cells in each region revealed that the pituitary of the male and female contain a similar amount of gonadotrope cells in either the medial or lateral region of the pituitary (Fig. 2C, 3C). LHB containing gonadotropes were then quantified in the two pituitary regions, and the percent of positively stained cells for males or females was equivalent, 2.5%-3.5%, showing no significant difference in the amount of LHB containing gonadotropes in either region (Fig. 2D, 3D). Since both males and females have similar amounts of LHB containing gonadotrope cells, but *Lhb* gene expression is significantly higher in females than males suggests that the number of gonadotropes is not responsible for the sex difference seen in gonadotropin gene expression, and that there is another cause upregulating or downregulating transcription.

Due to the observation that gonadotropin gene expression was being regulated, we hypothesized that androgens were down regulating gonadotropin gene expression in males. We identified the percentage of LHB containing gonadotropes that contained androgen receptors via immunohistochemistry to determine if gonadotropes in males and females differed in their ability to respond to androgens (Fig. 4). We observed that both male and female gonadotropes contained androgen receptors (Fig. 4A, B), and that there was no significant difference in the amount of gonadotropes containing androgen receptor (Fig. 4C). Therefore, since both sexes have equivalent levels of androgen receptors, the sex difference may be caused by the amount of circulating androgens, which is greater in males than females at this time point.

Gonadotropin Gene Expression is Responsive to Androgens

Having found no difference in gonadotrope population between males and females, we tested whether a difference in gonadotropes responsiveness to androgens in males and females is responsible for the sex difference seen in gonadotropin gene expression. Androgens have previously been shown to play critical role in masculinizing prenatal neuronal development. To investigate the role of androgens, we administered one of four prenatal treatments, vehicle, flutamide, testosterone, or dihydrotestosterone to dams, from e.15.5 to e.17.5. Gene expression at e.18.5 in male and female fetuses exposed to vehicle daily late in gestation exhibited a robust sex difference in *Lhb*, *Fshb*, and *GnRHr*, but no sex difference was observed in the expression of *aGsu* (Fig. 5). Treatment with flutamide, relieved the suppression of *Lhb*, *Fshb*, and *GnRHr* in males, and increased the level of expression to be not significantly different from flutamide treated females. In contrast, treatment with testosterone blunted expression of *Lhb*, *Fshb*, and *GnRHr* in females to levels observed in males. Treatment with DHT recapitulated the response to testosterone. The sex difference in gene expression was eliminated by either treatment with flutamide, testosterone, or DHT indicating that androgens are a critical factor in controlling the sex difference seen in gonadotropin gene expression by suppressing the male gonadotropin gene expression.

Determining the Role of Hypothalamic GnRH for Gonadotropin Expression

We investigated whether the sex difference seen in gonadotropin gene expression depends on hypothalamic signaling, by investigating if the sex difference is present in the absence of GnRH input to the pituitary. We used two different genetically modified mouse models that both lack GnRH signaling, hpg and GnRHr mutant lines. The hpg

knockout mice lack the ability to translate GnRH, while GnRHr mutant mice lack functional GnRH receptors. Gonadotropin gene expression in wildtype hpg and GnRHr littermates exhibited the same robust sex difference in males and females as seen before in *Lhb*, *Fshb*, and *GnRHr* (Fig. 6; GnRHr WT and hpg WT). The elimination of GnRH action, in GnRH and hpg mutants, attenuated female expression in *Lhb*, *Fshb*, and *GnRHr*, and gene expression in females was no longer significantly different from males (Fig. 6; GnRHr KO and hpg KO). Removing GnRH signaling eliminated the sex difference in gonadotropin gene expression demonstrating that the actions of GnRH are necessary to express the sex difference in gonadotropin gene expression, and these actions may be responsible for the sex difference

DISCUSSION

In these experiments, we sought to investigate the sex difference in gonadotropin gene expression that arises during prenatal pituitary gland development in the mouse. We were interested in determining the neuroendocrine site and molecular mechanism responsible for creating this sex difference in gene expression within the gonadotrope. We hypothesized that this sex difference is a result of direct regulation of the gonadotrope cells of the pituitary gland by androgens.

We demonstrate that a sex difference does indeed exist during prenatal development of the gonadotrope cell, in which female mice express *Lhb*, *Fshb*, and *GnRHr* at significantly higher levels than male mice. We first tested the hypothesis that this sex difference may be due to higher number of gonadotrope cells in female pituitary glands compared to males. However, both male and female pituitaries have similar number of gonadotrope cells showing that the sex difference was not due to a difference in cell number. Since there was no difference in gonadotrope cell number, we hypothesized that the sex difference was due to a difference in gene regulation. We hypothesized that differential regulation by androgens results in suppressed male gene expression because testosterone levels are elevated in males compared to females during prenatal development. We show that both female and male gonadotrope cells have androgen receptors, and therefore can be regulated by androgens. Our results reveal that the actions of androgens are necessary for creating the sex difference, as blocking androgen action with flutamide, an androgen receptor antagonist, relieved suppression of *Lhb*, *Fshb*, and *GnRHr* expression in males. In addition, both prenatal treatments with androgens, testosterone and DHT, attenuated female *Lhb*, *Fshb*, and *GnRHr* expression

and eliminated the sex difference. These experiments demonstrate that the actions of androgens are not only sufficient, but necessary for the sex difference in gonadotropin gene expression in males and females. Furthermore, our studies with mice lacking GnRH or the receptor for GnRH confirm the necessity of GnRH signaling for enhanced gonadotropin secretion in females.

Although our data support a role of androgens in the observed sex difference in gonadotropin gene expression in males and females during prenatal development, we cannot exclude the action of estradiol as a potential mediator of this effect. We have shown that testosterone and DHT are both able to eliminate the sex difference by attenuating female gonadotropin gene expression levels to that of males. DHT is a non-aromatizable androgen, meaning it cannot be converted into estradiol, which shows that activating androgen receptors is sufficient to cause the suppression seen in male gonadotropin levels. However, during prenatal development testosterone is what is elevated in males compared to females. Testosterone is an aromatizable androgen meaning it is free to be converted by aromatase to estradiol. Therefore, it is possible that the actions of estradiol contribute to gonadotropin gene expression levels in addition to the role of androgens.

A key question arising from our findings that androgens do play a role in regulating the sex difference is where within the reproductive neuroendocrine axis are androgens acting to mediate this effect? Both the hypothalamus and pituitary gland contain androgen receptors. Indeed, our data suggest that both male and female fetuses express androgen receptors within gonadotrope cells. Therefore, we sought to determine whether androgens are acting directly upon the pituitary or at the hypothalamic level to

reduce gonadotropin gene expression in the male. We utilized two mutant mouse models that both lack GnRH action on the pituitary to assess whether GnRH signaling or reduced pituitary responsiveness to GnRH were required for the sex difference. The assessment regarding the requirement of GnRH was rather straight forward. In both of these models the sex difference was eliminated due to a decrease in female gonadotropin gene expression suggesting that GnRH is either required in creating the sex difference or required to visualize the sex difference. Although our findings suggest GnRH signaling is indispensable for the sex difference in gene expression, the question regarding the role of androgens acting directly upon the pituitary remained an open question.

We hypothesized that androgens were acting within the gonadotrope to suppress GnRH-induced *Lhb* and *Fshb* expression in the male. To test this hypothesis, we used the hpg model, in which knockouts lacks endogenous GnRH and are themselves hypogonadal, and prenatally exposed dams to exogenous GnRH to test the ability of male and female fetuses to respond to GnRH. Male hpg fetuses still express high levels of testosterone, so we postulated that circulating androgens in this male would blunt gene expression induced by exogenous GnRH. Unfortunately, we were unable to determine the ability of male or female pituitary to respond to GnRH exposure as we were unable to see an increase in gonadotropin gene expression in any group. Since we were unable to see an increase in gonadotropin gene expression, while exposing fetuses to GnRH, we believed that GnRH was unable to travel through the placenta to reach the fetuses.

Although we were unable to determine where androgens acted to create the observed sex difference, other research allows us to speculate about a mechanism. Research has shown that males have high frequency of GnRH release during prenatal

development. Knowing that males release high frequency of GnRH suggest to us that GnRH release is not being regulated by androgens to cause the sex difference in gonadotropin gene expression as we would of expected low frequency of GnRH release resulting in low level of gonadotropin gene expression. Therefore, we believe this could suggest that androgens act directly on gonadotrope cells. That study though only focused on male GnRH release, so it would be interesting to know how the GnRH release frequency in prenatal females compares to males as it could provide some insight into regulation of HPG activity. As our model of exposing mice that lacked endogenous GnRH (hpg knock out mice) with exogenous GnRH failed to determine where androgens act, a model using mice that have androgen receptors knocked out in gonadotrope cells could help answer this question by testing whether the absence of androgen receptors within gonadotrope cells in males relieves the suppression of gonadotropin gene expression during prenatal development.

Sex differences in steroid regulation of the HPG axis post-puberty has been well characterized, and has been shown to be essential for proper reproduction. We have identified that hypothalamic-pituitary activity is sex dependent in mice during late gestation. Furthermore, we have characterized androgen mediated regulation of gonadotropin gene expression that exists in males, but not females. We propose alterations in this sex dependent expression may explain reproductive issues originating from fetal exposure. For example, perturbations in gonadal steroid activity during this critical period can elicit reproductive disturbances in male and female offspring as adults, by environmental toxicants or pathophysiologic conditions such as congenital adrenal

hyperplasia. While we have not shown that high gonadotropin gene expression corresponds to high circulating protein levels, we do know that in humans, females have higher circulating levels of LH than males. Therefore, we believe that these female mice with high expression will also have high circulating LH. Therefore, it may be possible that LH may be necessary for to feminize certain characteristics during development, while low LH in males during development may play a role in masculinization of certain characteristics.In summary, our findings support the conclusion that androgens regulate a sex difference in gonadotropin gene expression by suppressing male gonadotropin expression, but we do not where these androgens act to create this sex difference.

FIGURES AND TABLES

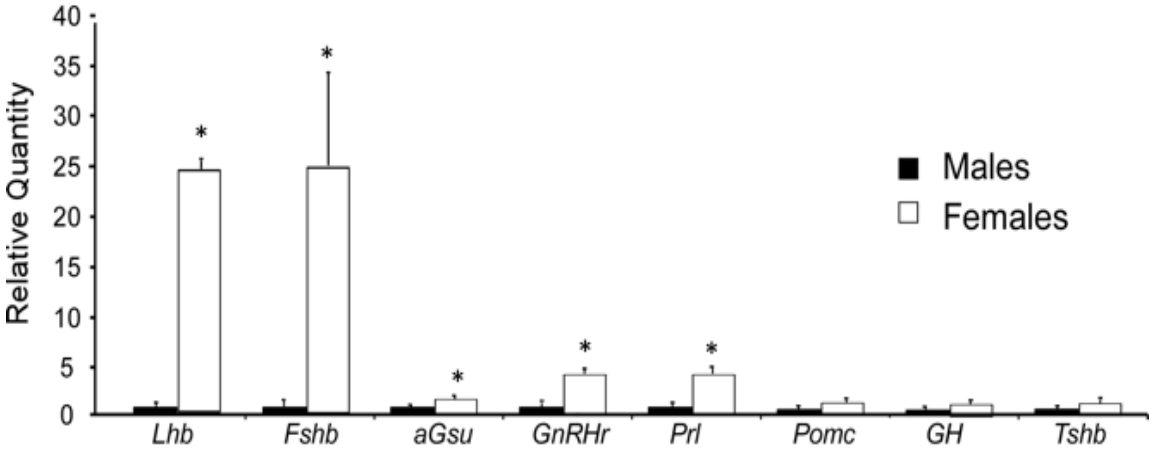

Figure 1: Females express gonadotrope related genes and prolactin significantly higher than males- Quantitative RT-PCR of genes of the anterior pituitary gland in individual fetal mice at e. 18.5. A sex difference was detected in *Lhb, Fshb, aGsu, GnRHr,* and *Prl,* in which females express these genes at higher levels compared to males. There was no difference between males and females in expression of, *Pomc, Gh,* and *Tshb.* Relative quantity was determined by comparing the gene of interest to *Gapdh* and utilizing the delta delta Ct method. Data was analyzed by one-way ANOVA and student's T test (*, $p<.05$)

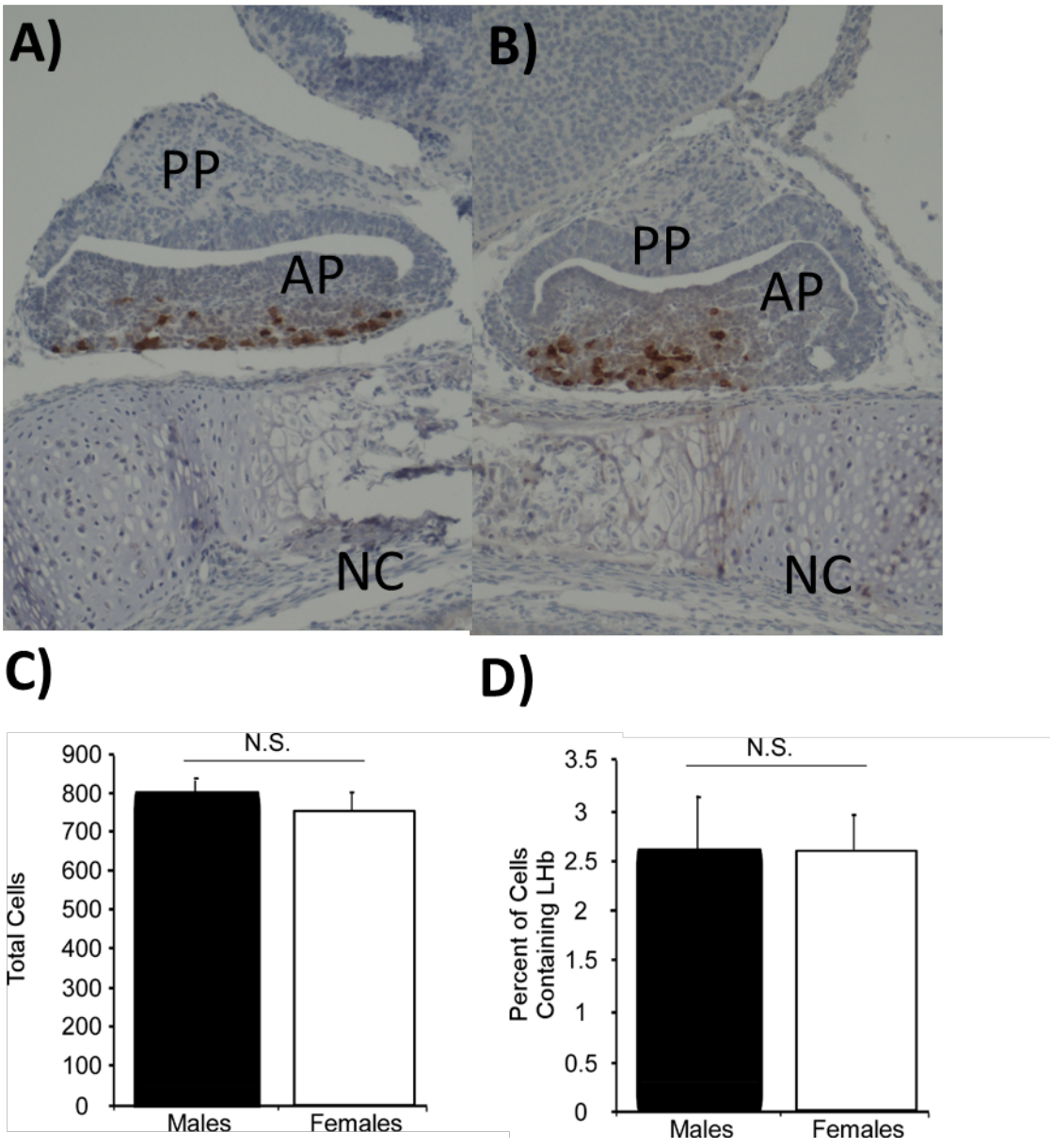

Figure 2: Males and females have similar amounts of gonadotrope cells in the medial region of the anterior pituitary- Sagittal sections from the lateral region of individual male (A) and female (B) pituitaries were stained for *Lhb* and counterstained with hematoxylin. *Lhb* cells are identified by brown precipitate. There was no difference between males and females in the amount of total cells (C) or the percent of pituitary cells identified to contain *Lhb* (D). Key PP = posterior pituitary, AP = anterior pituitary, NC = nasal cartilage. Data was analyzed by one-way ANOVA and student's T test (N.S., not significant).

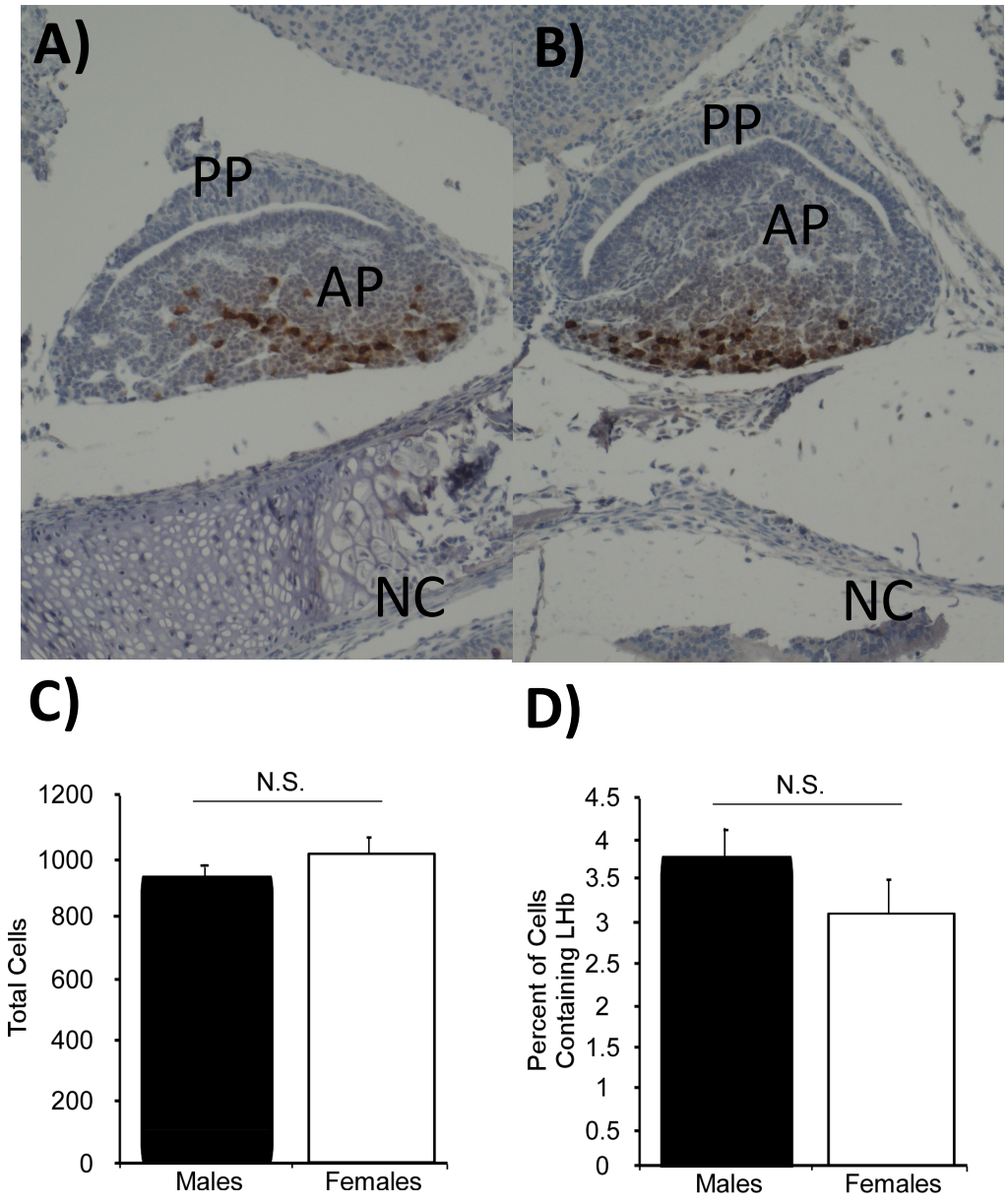

Figure 3: Males and females have similar amounts of gonadotrope cells in the lateral region of the anterior pituitary- Sagittal sections from the lateral region of individual male (A) and female (B) pituitaries were stained for *Lhb* and counterstained with hematoxylin. *Lhb* cells are identified by brown precipitate. There was no difference between males and females in the amount of total cells (C) or the percent of pituitary cells identified to contain *Lhb* (D). Key PP = posterior pituitary, AP = anterior pituitary, NC = nasal cartilage. Data was analyzed by one-way ANOVA and student's T test (N.S., not significant).

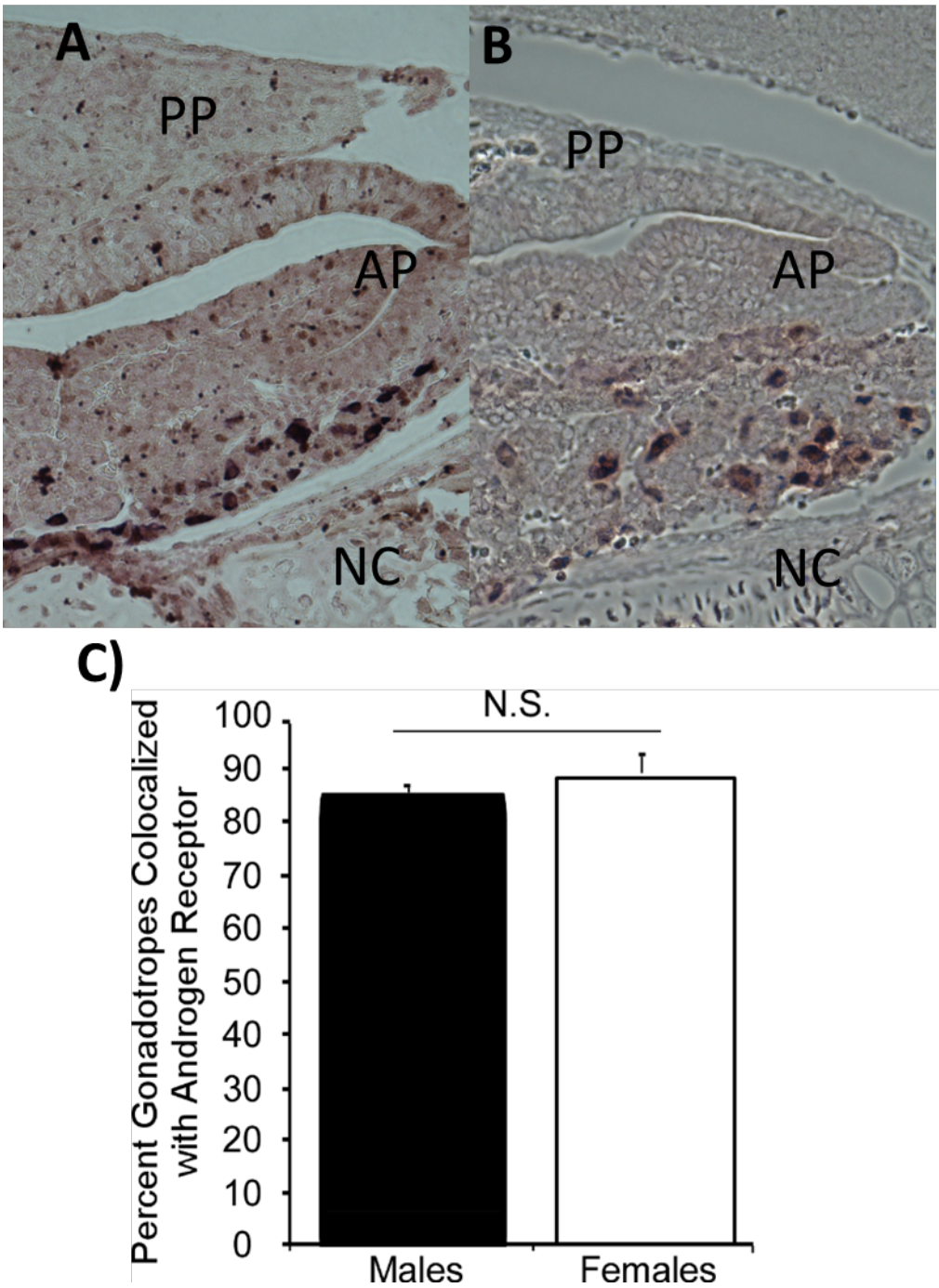

Figure 4: Gonadotrope cells in males and females contain androgen receptors- Sagittal sections from individual male (A) and female (B) pituitaries were stained for *Lhb*, androgen receptor, and counterstained with nuclear fast red. *Lhb* cells are identified by purples precipitate, and androgen receptors were identified by brown precipitate. Both males and females had similar number of *Lhb* cells that contained androgen receptor (C). Key PP = posterior pituitary, AP = anterior pituitary, NC = nasal cartilage. Data was analyzed by one-way ANOVA and student's T test (N.S., not significant).

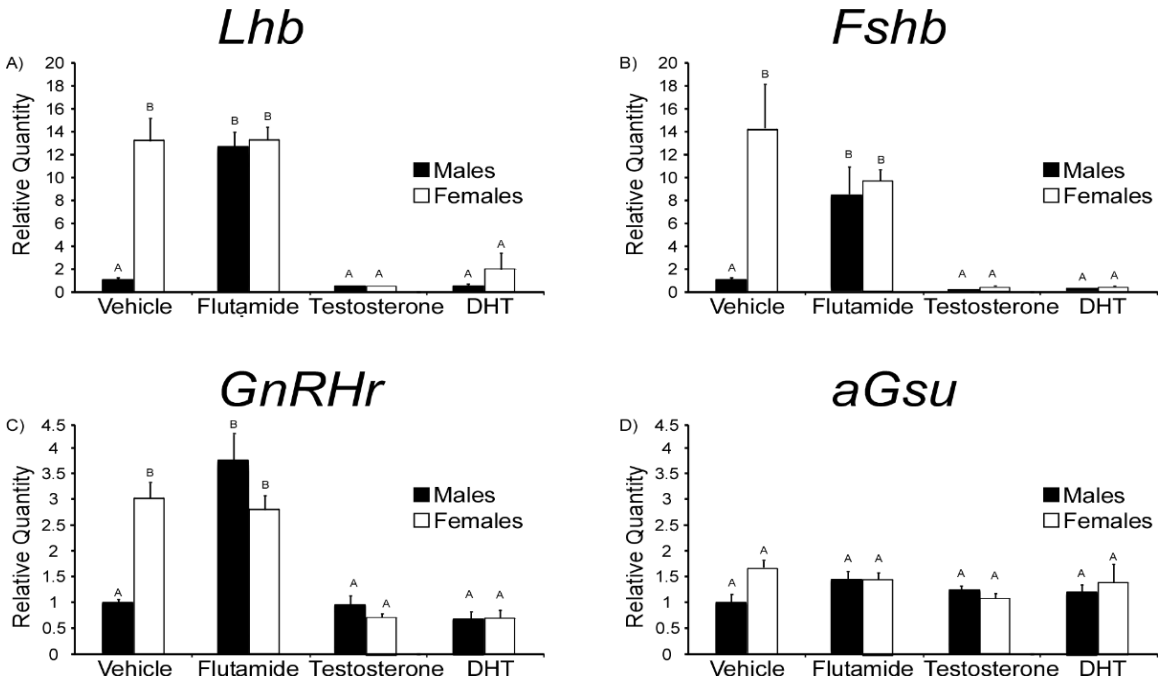

Figure 5: Androgens are required for differential expression of gonadotropin genes- Quantitative RT-PCR analysis of *Lhb* (A), *Fshb* (B), GnRHr (C), and *aGsu* (D) expression in individual fetal mouse pituitaries. Pituitaries were collected from fetuses exposed to vehicle, flutamide (androgen receptor antagonist), testosterone, or dihydrotestosterone (DHT; non-aromatizable androgen) during late gestation via daily dam injection. A sex difference in expression of *Lhb*, *Fshb*, and *GnRHr* was observed in vehicle animals, while the three other treatments eliminated the sex difference seen in vehicle treated fetuses. No sex difference was observed in *aGsu* expression in any treatment group. Relative quantity was determined by comparing the gene of interest to Gapdh by the delta delta Ct method and analyzed by two-way ANOVA and Tukey's HSD. Different letters signify statistical difference between comparisons ($p<.05$).

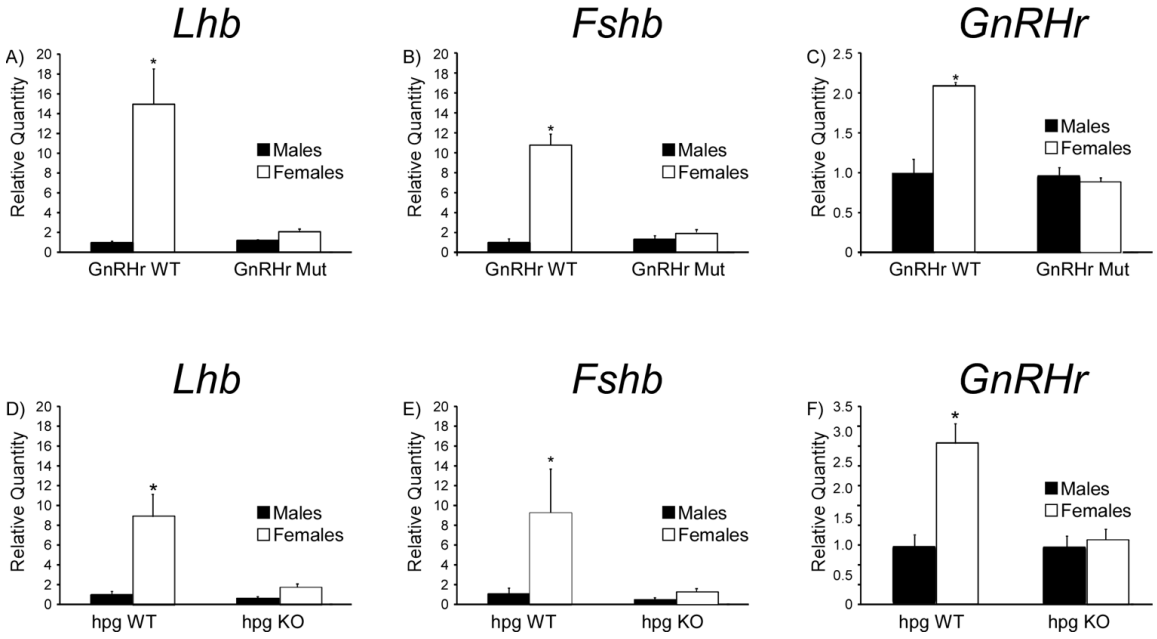

Figure 6: GnRH signaling is necessary for sex difference- Quantitative RT-PCR of genes *Lhb* (A, D), *Fshb* (B, E), and *GnRHr* (C, F) in individual fetal mouse pituitaries. In mouse models lacking GnRH receptor (A-C) or GnRH itself (D-F) individual fetal wildtype (WT), or mutant pituitaries, either lacking functional GnRH receptor (GnRHr MUT) or GnRH (hpg KO), were collected and quantified. The absence of GnRH receptor or GnRH eliminated the sex difference seen in these genes. Relative quantity was determined by comparing the gene of interest to *Gapdh* and utilizing the delta delta Ct method. Data was analyzed by two-way ANOVA and Tukey's HSD (*, $p<.05$)

Table 1: Amount of cDNA loaded for each gene in quantitative-PCR, and the sequence of quantitative-PCR primer sequences.

Gene of Interest	cDNA (ug)	forward sequence	reverse sequence
Pomc	0.4	CAGTGCCAGGACCTCACC	CAGCGAGAGGTCGAGTTTG
Tshb	0.4	AAGCAGCATCCTTTTGTATTCCC	CCTGGTATTTCCACCGTTCTG
aGsu	0.4	ATTCTGGTCATGCTGTCCATGT	CAGCCCATACACTGGTAGATGG
Gh	0.4	CCTCAGCAGGATTTTCACCA	CTTGAGGATCTGCCCAACAC
Gapdh	20	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
GnRHr	40	GCCCCTTGCTGTACAAAGC	CCGTCTGCTAGGTAGATCATCC
Lhb	40	CTGTCAACGCAACTCTGG	ACAGGAGGCAAAGCAGC
Prl	40	CTGCCAATCTGTTCCGCTG	GAGGGACTTTCAGGGCTTG
Fshb	150	GCCGTTTCTGCATAAGC	CAATCTTACGGTCTCGTATACC

Figure and tables, in full, is a reprint of the material currently being prepared for submission for publication. Kreisman, Michael J; Song, Christopher I; Yip, Kathleen; Breen, Kellie M. "Androgens Mediate Sex Differences in Gonadotropin Gene Expression *in Utero"*. Michael Joseph Kreisman was the primary investigator and author of this paper.

REFERENCES

1. Huang X, Harlan RE. Absence of androgen receptors in LHRH immunoreactive neurons. Brain Res 1993; 624:309-311

2. Herbison AE, Theodosis DT. Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience 1992; 50:283-298

3. d'Anglemont de Tassigny X, Colledge WH. The role of kisspeptin signaling in reproduction. Physiology (Bethesda) 2010; 25:207-217

4. Kaiser UB, Conn PM, Chin WW. Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. Endocr Rev 1997; 18:46-70

5. Pierce JG, Parsons TF. Glycoprotein hormones: structure and function. Annu Rev Biochem 1981; 50:465-495

6. Burger LL, Haisenleder DJ, Dalkin AC, Marshall JC. Regulation of gonadotropin subunit gene transcription. J Mol Endocrinol 2004; 33:559-584

7. Shupnik MA, Gharib SD, Chin WW. Divergent effects of estradiol on gonadotropin gene transcription in pituitary fragments. Mol Endocrinol 1989; 3:474-480

8. Baratta M, West LA, Turzillo AM, Nett TM. Activin modulates differential effects of estradiol on synthesis and secretion of follicle-stimulating hormone in ovine pituitary cells. Biol Reprod 2001; 64:714-719

9. Winters SJ, Ishizaka K, Kitahara S, Troen P, Attardi B. Effects of testosterone on gonadotropin subunit messenger ribonucleic acids in the presence or absence of gonadotropin-releasing hormone. Endocrinology 1992; 130:726-734

10. Burger LL, Haisenleder DJ, Aylor KW, Dalkin AC, Prendergast KA, Marshall JC. Regulation of luteinizing hormone-beta and follicle-stimulating hormone (FSH)-beta gene transcription by androgens: testosterone directly stimulates FSH-beta transcription independent from its role on follistatin gene expression. Endocrinology 2004; 145:71-78

11. Rispoli LA, Nett TM. Pituitary gonadotropin-releasing hormone (GnRH) receptor: structure, distribution and regulation of expression. Anim Reprod Sci 2005; 88:57-74

12. Jennes L. Prenatal development of the gonadotropin-releasing hormone-containing systems in rat brain. Brain Res 1989; 482:97-108

13. Glanowska KM, Burger LL, Moenter SM. Development of gonadotropin-releasing hormone secretion and pituitary response. J Neurosci 2014; 34:15060-15069

14. Cheung CC, Lustig RH. Pituitary development and physiology. Pituitary 2007; 10:335-350

15. Choi J, Smitz J. Luteinizing hormone and human chorionic gonadotropin: distinguishing unique physiologic roles. Gynecol Endocrinol 2014; 30:174-181

16. Ishikawa M, Murai E, Hashiguchi Y, Iguchi T, Sato T. Effects of diethylstilbestrol on luteinizing hormone-producing cells in the mouse anterior pituitary. Exp Biol Med (Maywood) 2014; 239:311-319

17. Weisz J, Ward IL. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology 1980; 106:306-316

18. Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M. Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 2007; 148:1774-1783

19. Roland AV, Moenter SM. Prenatal androgenization of female mice programs an increase in firing activity of gonadotropin-releasing hormone (GnRH) neurons that is reversed by metformin treatment in adulthood. Endocrinology 2011; 152:618-628

20. Ozaydin T, Sur E, Oznurlu Y, Celik I, Uluisik D. Immunohistochemical distribution of heat shock protein 70 and proliferating cell nuclear antigen in mouse placenta at different gestational stages. Microsc Res Tech 2016;

21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-408