Title
Androgen receptor and kisspeptin receptor : roles in reproduction

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Androgen Receptor and Kisspeptin Receptor: Roles in Reproduction

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Emily A. Witham

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor R. Jeffrey Chang
Professor Alexander S. Kauffman
Professor Albert La Spada
Professor Mark A. Lawson
Professor Nicholas J. G. Webster

2012
The Dissertation of Emily A. Witham is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

[Signature]

Chair

University of California, San Diego

2012
DEDICATION

I dedicate this dissertation to my parents Deborah and James Witham, and my late grandparents Esther and Sidney Brookmeyer, all of who have instilled in me the value of education and constant learning, and who have always supported and encouraged me.
# TABLE OF CONTENTS

SIGNATURE PAGE .................................................................................................................................. iii
DEDICATION .......................................................................................................................................... iv
TABLE OF CONTENTS ......................................................................................................................... v
LIST OF ABBREVIATIONS .................................................................................................................... vi
LIST OF FIGURES .................................................................................................................................. viii
LIST OF TABLES ...................................................................................................................................... x
ACKNOWLEDGEMENTS ....................................................................................................................... xi
VITA ......................................................................................................................................................... xiii
ABSTRACT OF THE DISSERTATION ................................................................................................. xiv
INTRODUCTION ....................................................................................................................................... 1

<table>
<thead>
<tr>
<th>CHAPTER 1: Prenatal Exposure to Low Levels of Androgen Accelerates Female Puberty Onset and Reproductive Senescence in Mice</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ...........................................................................</td>
<td>10</td>
</tr>
<tr>
<td>Introduction .....................................................................</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods ..................................................</td>
<td>13</td>
</tr>
<tr>
<td>Results ............................................................................</td>
<td>17</td>
</tr>
<tr>
<td>Discussion .......................................................................</td>
<td>23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2: Functional Roles of Kisspeptin Receptor (Kiss1R) in Pituitary Gonadotropes: Regulation of Gonadotropin Gene Expression</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ..................................................................................................................................................</td>
<td>39</td>
</tr>
<tr>
<td>Introduction ......................................................................................................................................</td>
<td>40</td>
</tr>
<tr>
<td>Materials and Methods ..................................................................................................................................................</td>
<td>42</td>
</tr>
<tr>
<td>Results ..................................................................................................................................................</td>
<td>47</td>
</tr>
<tr>
<td>Discussion .........................................................................................................................................</td>
<td>53</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3: Mechanisms of androgen-mediated gonadotropin-releasing hormone receptor regulation</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ..................................................................................................................................................</td>
<td>68</td>
</tr>
<tr>
<td>Introduction ......................................................................................................................................</td>
<td>68</td>
</tr>
<tr>
<td>Materials and Methods ...............................................................................................................................................</td>
<td>70</td>
</tr>
<tr>
<td>Results ..................................................................................................................................................</td>
<td>73</td>
</tr>
<tr>
<td>Discussion and Future Directions ..................................................................................................................</td>
<td>77</td>
</tr>
</tbody>
</table>

CONCLUSION ......................................................................................................................................... 91
REFERENCES ......................................................................................................................................... 96
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIS</td>
<td>Androgen insensitivity syndrome</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator-protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>AVPV</td>
<td>Anteroventral periventricular</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively active</td>
</tr>
<tr>
<td>CL</td>
<td>Corpora lutea</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP-response element</td>
</tr>
<tr>
<td>CX</td>
<td>Castration</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EGR-1</td>
<td>Early growth response</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>ETS</td>
<td>E twenty-six</td>
</tr>
<tr>
<td>FAH</td>
<td>Functional adrenal hyperandrogenism</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-Stimulating Hormone</td>
</tr>
<tr>
<td>GF</td>
<td>Graffian follicle</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
</tr>
<tr>
<td>GnRHR</td>
<td>Gonadotropin-Releasing Hormone receptor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamic-Pituitary-Gonadal</td>
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<tr>
<td>HRE</td>
<td>Hormone response element</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHH</td>
<td>Idiopathic hypogonadotropic hypogonadism</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
</tr>
<tr>
<td>Pitx-1</td>
<td>Paired-like homeodomain 1</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PNA</td>
<td>Prenatal androgen</td>
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<tr>
<td>PND</td>
<td>Postnatal day</td>
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<tr>
<td>PPA</td>
<td>Prepubertal androgen</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PVC</td>
<td>Persistent vaginal cornification</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor 1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>VO</td>
<td>Vaginal opening</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>VO was advanced in PNA and PPA, but not in PNA progeny</td>
<td>29</td>
</tr>
<tr>
<td>1.2</td>
<td>PNA treatment did not result in changes in body weight</td>
<td>30</td>
</tr>
<tr>
<td>1.3</td>
<td>VO was advanced in PPA AR/Syn-Cre and Kiss1r-null, and in PNA Kiss1r-null mice</td>
<td>31</td>
</tr>
<tr>
<td>1.4</td>
<td>PNA females had no sexually dimorphic Kiss1 expression in the AVPV</td>
<td>32</td>
</tr>
<tr>
<td>1.5</td>
<td>PNA exposure resulted in irregular estrus cycles and increased T in females</td>
<td>33</td>
</tr>
<tr>
<td>1.6</td>
<td>Testosterone implantation resulted in disrupted estrous cycling in wild-type females</td>
<td>34</td>
</tr>
<tr>
<td>1.7</td>
<td>PNA females had an initial delay in fertility</td>
<td>35</td>
</tr>
<tr>
<td>1.8</td>
<td>PNA females exhibited ovarian morphology consistent with the ability to produce litters</td>
<td>36</td>
</tr>
<tr>
<td>1.9</td>
<td>PNA treatment did not affect glucose tolerance</td>
<td>37</td>
</tr>
<tr>
<td>1.10</td>
<td>Advanced reproductive senescence in aging PNA females</td>
<td>38</td>
</tr>
<tr>
<td>2.1</td>
<td>Kiss1R is expressed in mouse pituitary and LβT2 cells</td>
<td>58</td>
</tr>
<tr>
<td>2.2</td>
<td>Kisspeptin induced LHβ and FSHβ but not GnRHR</td>
<td>59</td>
</tr>
<tr>
<td>2.3</td>
<td>Kisspeptin induced LHβ via the proximal -50 bp Egr-1 site</td>
<td>60</td>
</tr>
<tr>
<td>2.4</td>
<td>Kisspeptin induced FSHβ via cFos-dependent AP-1 activation</td>
<td>61</td>
</tr>
<tr>
<td>2.5</td>
<td>Kisspeptin induction of LHβ, FSHβ, Egr-1, and cFos was PKC-dependent</td>
<td>62</td>
</tr>
<tr>
<td>2.6</td>
<td>Hox and STAT regulatory regions on the cFos promoter are required for kisspeptin induction</td>
<td>63</td>
</tr>
<tr>
<td>2.7</td>
<td>The -5 kb Kiss1R promoter is not regulated by sex steroids, GnRH, kiss, or activin in LβT2 cells</td>
<td>64</td>
</tr>
<tr>
<td>2.8</td>
<td>Kisspeptin induction of gonadotrope genes and regulation of pituitary Kiss1R expression in vivo</td>
<td>65</td>
</tr>
<tr>
<td>2.9</td>
<td>Hypothalamic Kiss1R expression is up-regulated during the LH surge in females</td>
<td>66</td>
</tr>
<tr>
<td>3.1</td>
<td>AR induction of GnRHR maps downstream of -600 bp</td>
<td>83</td>
</tr>
<tr>
<td>3.2</td>
<td>GnRHR responds more strongly to R1881 and DHT than T</td>
<td>84</td>
</tr>
<tr>
<td>3.3</td>
<td>HRE half-sites at -159 bp and -499 bp are required for AR induction of GnRHR</td>
<td>85</td>
</tr>
<tr>
<td>3.4</td>
<td>The DNA-binding domain and the ligand-binding domain are required for AR induction of GnRHR</td>
<td>86</td>
</tr>
</tbody>
</table>
Figure 3.5. AR binds the -159 bp HRE.......................................................... 87
Figure 3.6. Pubertal onset in AR/αGSU-iCRE.................................................... 88
Figure 3.7 Fertility assessment in AR/αGSU-iCRE........................................... 89
LIST OF TABLES

Table 2.1. Oligonucleotide sequences used for PCR, qPCR and mutagenesis

Table 3.1. Oligonucleotide sequences for EMSA and mutagenesis of the GnRHR promoter
I would like to thank my advisor and mentor, Professor Pamela Mellon, for her support and guidance throughout my thesis project. I would like to thank the entire Mellon lab for their encouragement and for training me in important techniques and skills, especially Rachel Larder, Anita Iyer, Christine Glidewell-Kenney, Jason Meadows, and Kellie Church for providing me with a strong foundation early in my graduate career which has helped me to gain independence over time. I would also like to thank Shadi Shojaei, who has contributed to my research and whose Master’s project formed the basis for part of this dissertation. I would like to thank our collaborators Varykina Thackray, Djurdjica Coss, Mark Lawson, and Sasha Kauffman for their assistance and helpful scientific discussions. I would particularly like to thank Sasha Kauffman, who has always shown an interest in my research and has been an invaluable resource. To that end, I would also like to thank the members of the Kauffman lab for their assistance and support, especially Matthew Poling and Sheila Semaan. I would also like to thank Polly Huang, Courtney Benson, and Danalea Skarra for their support and friendship. I would like to thank Hanne Hoffmann for her guidance and helpful scientific discussions, and particularly for fostering a collaborative environment in the lab from which my research has greatly benefited. I would like to thank the UCSD Biomedical Sciences program for providing me with the opportunity to conduct my doctoral research in a high-quality scientific environment. Lastly, I would like to thank my thesis committee members Jeffrey Chang, Albert La Spada, Mark Lawson, Nicholas Webster, and Sasha Kauffman for their valuable insights and guidance.

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Chapter 1 has been published in *Endocrinology* (September 2012). The dissertation author was the primary investigator and author of this material. Shadi Shojaei and Jason Meadows assisted with the various *in vivo* experiments. Sasha Kauffman provided technical support for the *in situ* hybridization studies and also provided guidance and advice. Pamela Mellon supervised the project and provided advice.

Chapter 2, in part, is currently being prepared for submission for publication of this material. The dissertation author was the primary investigator and author of this material. Hanne Hoffmann and Jason Meadows provided assistance with ovariectomies and tissue preparation. Jason Meadows also assisted with the study design and tissue culture experiments. Djurdjica Coss provided various reagents and advice. Sasha Kauffman provided helpful guidance and assisted with the study design. Pamela Mellon supervised the project and provided advice.

Chapter 3, in part, is currently being prepared for submission for publication of this material. The dissertation author was the author of this material and is the co-primary investigator with Shadi Shojaei. Pamela Mellon supervised the project and provided advice.
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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

Androgen Receptor and Kisspeptin Receptor: Roles in Reproduction

by

Emily A. Witham

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2012

Professor Pamela L. Mellon, Chair

Reproductive function in mammals is primarily regulated by the production, release, and feedback actions of hormones in the hypothalamus and pituitary. Sex steroids such as androgens regulate the kisspeptin neurons, which activate the gonadotropin-releasing hormone (GnRH) neurons, stimulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release from the pituitary. The work presented herein investigates the molecular mechanisms of the effects of kisspeptin and androgens on gene expression in the pituitary, as well as the reproductive consequences of early androgen exposure in female mice. Two models of androgen exposure in females are described: prenatal androgen (PNA) and prepubertal androgen (PPA). PNA caused advanced puberty, delayed fertility with concomitant cycling irregularities, and advanced reproductive senescence, while PPA caused early puberty with no lasting effects. Thus, both the late prenatal and prepubertal time frames represent windows of androgen sensitivity in
females, but the PNA treatment has developmental influences that alter reproductive function throughout adulthood, while PPA only acts transiently to induce early puberty. The neuropeptide kisspeptin (and its receptor, Kiss1R) are required for puberty onset through their roles in the hypothalamus, but whether kisspeptin acts on the pituitary to regulate reproduction has not been extensively studied. In the pituitary gonadotrope cell line, LβT2, ligand-activated Kiss1R increased promoter activity of LHβ and FSHβ. This induction required the activation of the immediate early genes Egr-1 and cFos, and was dependent on protein kinase C (PKC). Furthermore, pituitary Kiss1R expression was induced in ovariectomized female mice undergoing the LH surge. This increase in pituitary Kiss1R, coupled with the kisspeptin-mediated gonadotropin induction, is strong evidence of a physiological role of Kiss1R in the pituitary to promote the reproductively important LH surge. Lastly, studies of androgen action in the pituitary showed that androgen receptor (AR) up-regulates the GnRH receptor (GnRHR) promoter through two proximal hormone response element (HRE) sites and direct binding of AR. Furthermore, a novel, pituitary-specific, AR-null mouse will yield valuable information on the reproductive role of AR in the pituitary. Overall, the research presented will further our understanding of the mechanisms by which the reproductive axis is regulated, under both normal and pathological conditions.
INTRODUCTION

The Reproductive Neuroendocrine Axis

Mammalian reproductive function, including puberty, menses, fertility, and menopause, originates in the brain. The brain regulates reproduction through the hypothalamic-pituitary-gonadal (HPG) axis. A subset of neurons in the hypothalamus known as the gonadotropin-releasing hormone (GnRH) neurons, integrates a wide variety of signals from other neural pathways. These inputs to the GnRH neurons represent physiological states such as sex steroid feedback, stress, metabolic and energy status, and circadian rhythms, and ultimately regulate the release of GnRH into the median eminence, where it then travels to the anterior pituitary gland. GnRH acts on the anterior pituitary gonadotropes, where it, in addition to other hormones such as sex steroids, regulates the transcription and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH exert their effects on the gonads (the ovaries and testes), and regulate follicle growth and release in females and spermatogenesis and sperm maturation in males.

The gonadotropins also induce the release of the sex steroids, estradiol, progesterone, and testosterone. The sex steroids have feedback effects on the kisspeptin neurons of the hypothalamus, which are potent activators of GnRH neurons, as well as on pituitary gonadotropes. Fluctuation in sex steroid levels occurs throughout the reproductive cycle in females. The feedback by sex steroids is sexually dimorphic, with females (but not males) undergoing a rise in estradiol levels (representing a switch from negative to positive feedback), which precedes a GnRH-induced LH surge, as well as a secondary FSH surge. This rise in gonadotropin levels promotes ovulation. Sexual dimorphism of the kisspeptin system in the hypothalamus underlies the specificity of the LH surge for females. Females have higher numbers of kisspeptin neurons than males, and therefore respond more strongly to fluctuations in estradiol levels than males (1).
Tools with which to study the reproductive axis

The complexities of the neuroendocrine reproductive axis necessitate that the study of its molecular mechanisms of regulation be accomplished with the use of homogenous cell lines. The GnRH neurons are widely dispersed throughout the hypothalamus and exist in low numbers (approximately 800 per mouse brain), making the study of the molecular mechanisms regulating them difficult to achieve in vivo. The anterior pituitary contains other hormone-producing cell types in addition to the gonadotropes, so studies of gonadotropes in vivo may be influenced by the affects of the other cell types. The heterogeneous populations of neurons in the hypothalamus and hormone-secreting cells in the pituitary can be circumvented with the use of cell lines developed by targeted oncogenesis. The GT1-7 cell line is an immortalized GnRH neuronal line, and the LβT2 line represents pituitary gonadotropes. The GN11 and αT3-1 cell lines are immature GnRH neurons and pituitary gonadotropes, respectively, and allow for the study of the activation of various gene programs during development. The GT1-7 cells and, importantly for this dissertation, the LβT2 gonadotropes, make it possible to study the regulation of GnRH and the gonadotropins on a molecular level.

The molecular mechanisms of sex steroid feedback have been partly elucidated in the LβT2 cell line, but the physiological effects must be studied in animal models. Rodents (particularly rats and mice) are useful species in which to study sex steroid feedback in vivo. Gonadectomy of mice with or without sex steroid hormone replacement allows investigators to control the amount of circulating sex steroids, as well as study the effects of their absence or presence under various experimental conditions. Male mice may be castrated and supplemented with testosterone (T), while female mice may be ovariectomized (OVX) and supplemented with either low or high levels of estradiol, to represent negative or positive feedback. Furthermore, a wide variety of genetic knockout mice pertaining to the reproductive axis have been described, including but not limited to: hpg (GnRH-null), GPR54−/− (Kiss1 receptor-null), and GnRH
The Kisspeptin receptor is critical for reproductive development and function

The kisspeptin receptor (Kiss1R, formerly termed GPR54) is a G\(\alpha_{q/11}\)-coupled receptor present in a variety of tissues, including the brain, pituitary, placenta, testis, and kidney (10). Activation of Kiss1R by its ligand kisspeptin induces the phospholipase-C signaling cascade, leading to second messenger-mediated Ca\(^{2+}\) release followed by protein kinase C (PKC) and ERK1/2 activation (10). Kisspeptin neurons synapse directly on GnRH neurons, which express Kiss1R (11, 12). In GnRH neurons, kisspeptinergic inputs from Kiss1 neurons strongly stimulate GnRH release (13). The Kiss1 neurons relay information to the GnRH neurons about sex steroid feedback status, and are thought to represent an upstream “link” that connects sex steroid levels to GnRH output (14). Both testosterone and estradiol are potent regulators of Kiss1 expression, and most hypothalamic Kiss1 neurons express AR and ER\(\alpha\), neither of which is expressed in GnRH neurons (14).

Three key studies from 2003 describing mutations in the Kiss1R gene in humans, as well as Kiss1R-null mice, have underlined its importance in normal reproduction (7, 15, 16). A study of human subjects with isolated hypogonadotropic hypogonadism (IHH) found a deletion within the Kiss1R gene resulting in a truncated protein lacking transmembrane domains 6 and 7 (15). As a result of this mutation, these subjects had impaired puberty and fertility, low sex steroid levels, and low gonadotropin levels stemming from deficient GnRH production (15). A second study in both mice and humans found various mutations in Kiss1R in IHH patients (16). This study also characterized Kiss1R-null mice, which had low sex steroid and gonadotropin levels, a lack of estrous cycles in females, and deficient spermatogenesis in males (16). A third study further
characterized the Kiss1R-deficient mice, and found evidence of hypogonadism in both males and females and infertility (7). Interestingly, this study also reported that Kiss1R exerts its main effects during the postnatal time frame prior to puberty, as Kiss1R-null embryos were histologically normal (7). These studies were the first to describe the necessity of intact kisspeptin/Kiss1R signaling for pubertal development and normal reproductive function. Conversely, an activating mutation in the Kiss1R gene was reported in a human patient with central precocious puberty, further supporting the role of Kiss1R in normal puberty (17).

While the regulation of Kiss1 in the brain by sex steroids has been extensively characterized (18), the way in which Kiss1R is regulated is less well understood. One study reported that hypothalamic Kiss1R expression was lowest during the prepubertal time frame, highest during the initiation of puberty, and fluctuated throughout the estrous cycle in female rats, suggesting a contribution of sex steroids in the regulation of Kiss1R (19). However, another study in OVX female rats showed no changes in hypothalamic Kiss1R expression regardless of sex steroid status (20). The mechanisms of Kiss1R regulation in the hypothalamus remain to be fully elucidated. A study in OVX rats showed that OVX without steroid replacement resulted in an increase in Kiss1R levels in the pituitary, which was decreased by 17β-estradiol replacement (21). Lastly, recent evidence suggests that Kiss1R may be regulated by its own ligand, kisspeptin; in female OVX ewes, prolonged exposure to kisspeptin resulted in a down-regulation of Kiss1R expression in GnRH neurons (22). More extensive in vivo studies are required to determine the extent to which Kiss1R is regulated by sex steroids in both the hypothalamus and pituitary. There is also a lack of studies of the molecular mechanisms of Kiss1R regulation in vitro. One study has reported the presence of several specificity protein-1 (Sp-1) sites as well as a partial estrogen-responsive element on the Kiss1R promoter, but no follow-up studies have been conducted (23). The regulatory region of the Kiss1R promoter remains to be confirmed and extensively characterized in vitro, which is necessary to fully understand its mechanisms of regulation.
The role of androgen receptor (AR) in females

AR is a member of the nuclear receptor superfamily, and ligand-bound AR exerts its effects through both classical and non-classical signaling mechanisms (24). Testosterone metabolism involves the production by 5-α-reductase of dihydrotestosterone (DHT), an androgen with a high affinity for AR (24). AR is essential for normal reproductive development and function in males, as evidenced by AR-null mice, as well as humans who are genetically male but have androgen-insensitivity syndrome (AIS). In males, AR is required for secondary sex characteristics, sexual differentiation, and spermatogenesis (25). Male AR-null mice and humans with AIS have profound deficits in sexual maturation and reproductive function, to the extent that they are phenotypically female (26). However, AR is also important for female reproduction. The generation of female AR-null mice showed deficits in cyclicity, fertility, progesterone levels, and ovarian morphology (27). Despite a normal appearance, these female AR-null mice had longer estrous cycles as well as fewer numbers of oocytes and corpora lutea (27). This demonstrates that despite the obvious role of AR in male reproduction, it is also required for normal female reproduction.

The female AR-null mice display a full-body knockout, but the use of tissue-specific strategies to generate conditional AR knockout mice presents a powerful approach with which to tease apart the effects of AR on reproduction. The cre-loxP system is a common way to generate cell type-specific knockout mice, requiring a cross between a promoter-specific cre and a gene (or exon) of interest flanked by loxP sites (flox). A granulosa cell-specific AR knockout mouse was described in 2002, and showed similar reproductive phenotypes to that of the full-body AR-null females, suggesting that ovarian expression of AR is required for normal female reproduction (24). AR was also found to be necessary for preantral follicle growth in these mice, which is consistent with the reduced fertility phenotype found in both the full-body and granulosa cell AR-
null females (24). By contrast, an oocyte-specific AR knockout female displayed normal fertility, suggesting that AR specifically in the granulosa cells of the ovary is required for female reproductive function (24).

While studies from AR-null female mice show that AR plays a role in the ovaries, it is also involved in androgen feedback in the hypothalamus and the pituitary. AR is expressed in Kiss1 neurons in the hypothalamus, which are regulated in part by androgens. In monkeys, testosterone has been shown to decrease Kiss1 expression, leading to a reduction in LH (28).

Transcriptional studies in LβT2 pituitary gonadotropes have shown that AR differentially regulates LHβ and FSHβ. The distal region of the rat LHβ promoter contains two Sp-1 binding sites, and AR has been shown to decrease Sp-1 binding to these sites, thereby suppressing promoter expression, which was relieved when the Sp-1 sites were mutated (29). Additionally, in the bovine LHβ promoter, AR was shown to interact with the transcription factors SF-1, Egr-1, and Pitx-1 to suppress promoter activity (30). By contrast, FSHβ is induced by AR. Androgens act in synergy with the peptide hormone activin (through interaction with Smad proteins) to activate the FSHβ promoter (31). AR can also stimulate the FSHβ independently of activin, via direct DNA binding to a hormone responsive element in the proximal region of the promoter (32).

In summary, in vivo studies have shown that AR is required in the ovary for normal female reproduction, while in vitro studies suggest an additional role of AR at the level of the pituitary gonadotrope to regulate LH and FSH levels.

AR in female reproductive disorders

While androgen action through AR is required for normal female reproduction, pathological states of excess androgen can disrupt the organization and activity of the HPG axis and its feedback pathways. A common female reproductive disorder in which this occurs is polycystic ovary syndrome (PCOS). PCOS is a complex disorder affecting approximately 5-10%
of women, and is characterized by but not limited to insulin resistance, anovulation, hirsutism, abnormal LH secretion, and polycystic ovaries with abnormal folliculogenesis (33). The mechanisms of the development of PCOS are not fully understood but it is clear that environmental factors (i.e. the intrauterine milieu) and genetic predisposition play significant roles in its etiology (34). High levels of circulating androgens in PCOS women who are pregnant may be a source of androgen exposure to the fetus, potentially leading to androgen-mediated effects on development (35). Both hyperandrogenemia and hyperinsulinemia (which can lead to PCOS) have a degree of heritability, and several PCOS candidate genes have been identified that are involved in folliculogenesis, as well as the synthesis and secretion of androgen and insulin (33).

Premature puberty is a significant risk factor for PCOS, affecting 1 in 5000 children (being 10 times more common in girls) (36, 37). Hyperandrogenemia has been found in both women with PCOS and girls with premature puberty, and studies have linked prenatal effects of increased androgens to these reproductive disorders (38). Metabolic dysfunction is commonly found in cases of hyperandrogenemia and premature puberty. PCOS and premature puberty are both associated with dyslipidemia, including increased total body fat, LDL:HDL ratios, and triglycerides (39). A clinical study of normal weight and obese girls in the early stages of puberty showed a strong association between obesity and increased levels of free testosterone and insulin (with a concomitant decrease in sex hormone binding globulin) (38). Girls diagnosed with premature puberty have an increased occurrence of functional ovarian hyperandrogenism (FAH, considered to be an adolescent form of PCOS), which is associated with hyperinsulinemia (40). A study of girls with premature pubarche, hyperinsulinemia, and hyperandrogenemia found that insulin sensitization therapy with metformin lowered the risk of progression to PCOS and improved the metabolic profiles of these girls (39). The exact way in which metabolic and androgenic dynamics influence each other to promote premature puberty and PCOS remains to be
unraveled.

Several lines of evidence suggest that low-level exposure to androgens during prenatal development can “preprogram” females for reproductive abnormalities later in life. Prenatal androgen treatment in fetal sheep resulted in disrupted hormonal dynamics around the time of ovulation including a delayed and increased estrogen surge as well as increased LH, suggesting desensitization of estrogen-mediated positive feedback in the hypothalamus and pituitary (41). This decrease in responsiveness to estrogen positive feedback has also been seen in female rats with early androgen exposure (42). This alteration prevents the LH surge responsible for triggering ovulation, which could explain the anovulation and lack of cyclicity seen in PCOS women and in vivo models (34). Another study of prenatally androgenized sheep found an increased LH response to exogenous GnRH, as well as decreased estrogen-mediated feedback inhibition of LH, leading to the LH rise; this LH increase continued into adulthood (35). A similar study conducted in rhesus monkeys found that females exposed to prenatal testosterone had irregular or absent cycles, metabolic dysfunction, increased LH levels and sensitivity to GnRH, and diminished negative feedback in the hypothalamus and pituitary; high levels of LH and circulating androgens persisted into infancy, again showing that the prenatal androgen treatment has more than just a transient effect (41). Furthermore, another study of prenatal androgenization in female sheep demonstrated virilized genitalia, masculinized reproductive behavior, increased aggression towards males, and fewer “behavioral estrous cycles”, showing that the preprogramming effects of prenatal androgens can extend into the phenotypic and behavioral realm (43).

As described above, the role of androgens and AR signaling under both normal and pathological conditions, and the role of Kiss1R in reproduction have all been studied in various capacities. Important questions remain, however, that will be the foundation of the work contained herein. Most animal models of prenatal androgen exposure use testosterone, the effects
of which may stem from either ER or AR. The specific role of AR in such animal models has not been extensively studied. Furthermore, AR action in pituitary gonadotropes under normal conditions is not fully understood, particularly with regard to GnRHR expression and overall reproductive function \textit{in vivo}. Lastly, while Kiss1R is critical for reproductive function, whether it plays a regulatory role in the pituitary with regard to gene expression has not been investigated.

This dissertation will address three primary research questions: the effect of early androgen exposure on female reproduction, the transcriptional role of Kiss1R in pituitary gonadotropes, and mechanisms of AR-mediated GnRHR regulation in pituitary gonadotropes.
CHAPTER 1: Prenatal Exposure to Low Levels of Androgen Accelerates Female Puberty Onset and Reproductive Senescence in Mice

Abstract

Sex steroid hormone production and feedback mechanisms are critical components of the hypothalamic-pituitary-gonadal (HPG) axis, and regulate fetal development, puberty, fertility, and menopause. In female mammals, developmental exposure to excess androgens alters the development of the HPG axis and has pathophysiological effects on adult reproductive function. This study presents an in-depth reproductive analysis of a murine model of prenatal androgenization (PNA) in which females are exposed to a low dose of dihydrotestosterone (DHT) during late prenatal development on embryonic days 16.5-18.5. We determined that PNA females had advanced pubertal onset and a delay in the time to first litter, compared to vehicle-treated controls. The PNA mice also had elevated testosterone (T), irregular estrous cyclicity, and advanced reproductive senescence. To assess the importance of the window of androgen exposure, DHT was administered to a separate cohort of female mice on postnatal days 21-23 (prepubertal androgenization; PPA). PPA significantly advanced the timing of pubertal onset, as observed by age of vaginal opening, yet had no effects on T or estrous cycling in adulthood. The absence of kisspeptin receptor in Kiss1r-null mice did not change the acceleration of puberty by the PNA and PPA paradigms, indicating that kisspeptin signaling is not required for androgens to advance puberty. Thus, prenatal, but not prepubertal, exposure to low levels of androgens disrupts normal reproductive function throughout life from puberty to reproductive senescence.
Introduction

The hypothalamic-pituitary-gonadal (HPG) axis controls all stages of the reproductive lifespan and is active from puberty through menopause. Hypothalamic gonadotropin-releasing hormone (GnRH) neurons activate pituitary gonadotropes to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then stimulate gonadal steroid hormone production. In females, the ovaries produce testosterone (T), which is aromatized by follicle-derived aromatase into estradiol (E$_2$) (44). E$_2$ and T exert feedback effects (E$_2$, positive and negative; T, negative) at the level of the hypothalamus and pituitary to control reproductive hormone production (45). The tight regulation of a complex interplay of positive and negative feedback loops is required for many reproductive functions including follicle maturation, ovulation, and estrous cyclicity.

GnRH neurons are the central regulators of the HPG axis. During the juvenile stage, the HPG axis is quiescent, with the subsequent onset of puberty reflecting an increase in GnRH secretion, leading to secondary sex characteristics, estrous or menstrual cycling, and ovulation in females (46). A key hallmark of puberty is the activation of the GnRH neurons, which occurs, in part, through the upstream kisspeptin neurons. Kisspeptin, a neuropeptide encoded by the Kiss1 gene, potently stimulates GnRH neurons by signaling through the kisspeptin receptor (encoded by Kiss1r, formerly termed GPR54) (46, 47). Puberty is impaired in the absence of kisspeptin signaling, such as in Kiss1 or Kiss1r null mice (48), or in humans lacking functional kisspeptin or its receptor (16, 49).

The release of GnRH drives not only puberty, but has also been suggested to play a role in reproductive senescence (50). In aging rodents, LH pulsatility is altered, the preovulatory LH surge (caused by increased GnRH) is attenuated, and functional activity of the GnRH neurons is decreased (50). This reduction in GnRH neuronal activity during senescence is associated with
decreased ovarian sex steroid levels and a concomitant loss of sex steroid negative feedback in
the brain (50).

The negative feedback by sex steroids, such as androgens, is necessary for normal
functioning of the reproductive neuroendocrine axis. The importance of androgens and their
receptor (AR) in reproductive development and function has long been known in males, but is
less well understood in females. Evidence from female AR-null mice has demonstrated that AR is
necessary for normal estrous cyclicity, ovarian morphology, progesterone production, and fertility
(27). Exposure of females to exogenous, supraphysiological levels of androgens, however, may
disrupt the normal role of androgens and have negative impacts on female reproductive outcomes
(33).

The reproductive neuroendocrine system is particularly vulnerable to environmental and
hormonal disturbances. Exposure to endocrine disrupting chemicals (EDC’s, such as
steroidogenic compounds) during critical periods of development can have profound effects on
reproduction and fertility later in life (51). The mechanisms by which EDC’s alter neuroendocrine
function is not fully understood, but it has been suggested that prenatal sex steroid exposure can
increase the risk of reproductive disorders, such as precocious puberty and polycystic ovarian
syndrome (PCOS), the leading cause of infertility in women (33, 39). The study of prenatal
androgenization in animal models may therefore lead to a better understanding of the endocrine
abnormalities and underlying mechanisms resulting from EDC exposure and PCOS in females.
Indeed, studies in rats, sheep, and non-human primates suggest that low-level exposure to
androgens during prenatal development can preprogram females for reproductive abnormalities
later in life, disrupting hormonal dynamics that influence cyclicity and sensitivity to sex steroid
feedback (34, 35, 41, 42). A prenatal androgen model has been described in female rats in which
pregnant females are treated with T on embryonic days e16-e19 (52). This paradigm results in a
disruption in neuroendocrine feedback in the prenatally androgenized female rats, but because T
can be aromatized to E₂, the hormonal mechanisms and pathways underlying these effects may be mediated by either estrogen receptor (ER) or androgen receptor (AR) (52).

Using dihydrotestosterone (DHT) instead of T avoids potential effects resulting from aromatization of T to E₂. In a similar prenatal androgenization model (termed PNA) in mice (53), pregnant dams are treated with low-dose DHT on days e16.5-e18.5. PNA female offspring have normal genitalia but do not cycle regularly in adulthood, and have increased serum levels of T (53), mirroring oligomenorrhea and increased androgen levels observed in women with PCOS (33). While the extent to which the PNA model fully recapitulates the clinical characteristics of PCOS is not fully established, it still offers important insights into the mechanisms and pathophysiology of female reproductive disorders caused by early androgen exposure.

The overall goal of this study was to determine the effects of developmental DHT exposure on female reproductive outcomes. To accomplish this, our study had two main objectives. First, we compared the reproductive phenotype resulting from prenatal and peripubertal exposure to DHT in females. Secondly, we utilized neuron-specific AR knockout and Kiss1r-null mice to investigate the role of neuroendocrine mechanisms in response to developmental DHT exposure.

Materials and Methods

Animals

All animal procedures were performed in accordance with the UCSD Institutional Animal Care and Use Committee regulations. All mice were on a C57BL/6J background and were group-housed on a 12-h light:12-h dark cycle with ad libitum chow (11% of calories fat, 17% of calories protein) and water. Kiss1r-null animals were from Omeros, Inc. Seattle, WA (54, 55). Heterozygous Kiss1r animals bred to generate homozygous Kiss1r knockout (KO) and wildtype (WT) littermates for study. To generate mice lacking AR exclusively in neurons
(AR/Syn-Cre), AR “flox” males (26) in which the second exon of the AR gene is flanked by loxP sites) were bred to Synapsin-Cre females [in which Cre is expressed exclusively in neurons (26, 56)]. Offspring, termed AR/Syn-Cre, have glial but not neuronal AR, as this is a neuron-specific knockout (data not shown). Littermate homozygous AR flox with and without Syn-Cre were compared.

**PNA and PPA paradigms**

To generate PNA females, pregnant dams were injected (s.c.) with 100 µL of vehicle (sesame oil, Sigma) or 250 µg of DHT (5α-androstan-17β-ol-3-one, Sigma) dissolved in 100 µL of sesame oil. Injections were given once daily on gestational days e16.5-18.5. This low dose of prenatal DHT can influence the functioning of the HPG axis of the female offspring without secondary effects resulting from virilized female genitalia (data not shown) (53). To generate PPA females, mice were injected once daily on postnatal days (PND) 21-23 with 100 µL of vehicle (sesame oil) or 250 µg of DHT dissolved in 100 µL of sesame oil. The dose of 250 µg of DHT was based on a half-log dose response study and calculation of equivalent dose per gram weight as the PNA treatments (weight of the pregnant dam).

**Pubertal assessment and estrous cycles**

All experimental animals were monitored for vaginal opening status, an external marker of puberty onset beginning after weaning on PND 21 (Fig. 1A). Of note, Kiss1r-null females normally have either severely delayed or absent vaginal opening; therefore, PND 35 was used as the upper limit of vaginal opening assessment for statistical purposes.

To assess estrous cyclicity, adult PNA and PPA females were housed 1-2 per cage approximately one week before analysis (single housing did not affect cyclicity). Vaginal smears were taken once a day for 15-25 consecutive days, and vaginal cytology determined the cycle
stage (diestrus, proestrus, estrus, metestrus). To evaluate the onset of reproductive senescence, mice were classified as having persistent vaginal cornification (PVC) if their vaginal smears contained cornified cells on 75% or more of days checked.

**Testosterone implantation**

The estrous cycles of sixteen 2-month-old wild-type females were monitored for fifteen days, followed by a subcutaneous implantation of a silastic capsule (Dow Corning Corp., Midland, MI; inner diameter = 1.47 mm; outer diameter = 1.95 mm) filled with testosterone (17β-Hydroxy-3-oxo-4-androstene, Sigma) diluted 1:40 in cholesterol (Sigma). Pilot studies showed that this dose resulted in serum T levels comparable to PNA females when measured at one week after implantation. Eight females each were implanted with T capsules, or cholesterol vehicle capsules. Animals were allowed to recover for one week, and then estrous cycles were measured again for fifteen days.

**Single-label *in-situ* hybridization (ISH)**

Brains from 4 month-old control diestrus PNA females and male mice were harvested between 10:30 am and 12:30 pm, fresh-frozen on dry ice, cryosectioned, and probed for *Kiss1* via ISH as described (1). Briefly, slide-mounted sections were fixed in 4% PFA, treated with acetic anhydride, washed in 2x sodium citrate, sodium chloride (SSC), delipidated in chloroform, dehydrated in ethanol, and air dried. The *Kiss1* radioactive probe was denatured and added to hybridization buffer at 0.05 pmol/ml. 100 µL of the probe-buffer mix was added to each slide, and allowed to hybridize at 55°C for 16 hours. Slides were washed, then treated with RNase. After several more washes, the slides were dipped in Kodak NTB emulsion and stored at 4°C for 9 days before developing. For quantification, slides were analyzed with custom grain-counting
software (Don Clifton, University of Washington). The number of Kiss1-expressing neurons and silver grains per cell (a semi-quantitative measure of mRNA levels per cell) were counted.

**Serum collection and hormone assays**

Blood was collected at the time of sacrifice from 4-month-old PNA and PPA females in diestrus. Briefly, mice were anesthetized with isoflurane (Abbott) and blood was collected through the abdominal aorta by syringe prior to 13:00 h. After coagulation, blood samples were centrifuged at 2655 × g for 15 minutes, sera were transferred to a fresh tube, and stored at -20°C. Sera were analyzed by RIA at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

**Ovarian morphology**

Ovaries were dissected from 2-, 3-, 7-, and 10-month-old PNA females, fixed in 4% paraformaldehyde, washed in PBS, and stored in 70% EtOH. One ovary per animal was embedded in paraffin (UCSD histology core facility). 10 µm sections were stained with hematoxylin and eosin (UCSD histology core facility). Three separated sections per ovary were quantified for corpora lutea, preantral follicles, and antral follicles.

**Glucose and insulin tolerance tests**

Oral glucose tolerance tests (GTT) were performed on 1 and 6 month-old PNA females and oil-treated controls. Mice were administered 1 g/kg of an oral gavage of 50% glucose following 7 hours of fasting. Insulin tolerance tests (ITT) were performed on 1 month-old PNA females, using 6 U/kg of insulin-BSA (0.5 U/mL). For both the GTT and ITT, blood glucose levels were measured from tail blood samples using a glucose meter. Animals were then
transferred to a high-fat diet (60% calories from fat, 20% calories from protein) for 6 months, and subjected to another GTT.

**Statistical analyses**

All results are presented as mean±SEM. Student’s t-test, one-way ANOVA, two-way ANOVA, chi-square, and survival analysis were used where indicated. P<0.05 was considered statistically significant. Because Kiss1r-null females do not normally display VO within the normal time frame of wild-type mice, VO was not recorded past PND 35. We therefore determined statistical significance using “survival analysis”, followed by a log-rank chi-square test.

**Results**

**Pubertal onset is advanced in PNA and PPA females**

Prior studies of androgenization during critical developmental periods have focused on adult reproductive parameters, such as sex steroid feedback (34, 35, 41, 42), but have not investigated the effects of developmental androgen exposure on puberty. To determine whether there are distinct sensitive periods of development when androgens act to alter sexual maturation and induce reproductive abnormalities, we assessed the onset of puberty in females exposed to DHT. Female mice were subjected to one of two DHT treatment paradigms: prenatal (PNA: DHT exposure during embryonic days e16.5-e18.5) or peripubertal (PPA: DHT exposure from PND 21-23) and were compared with oil-treated (control) females (12-15 animals/group) (Fig. 1A). VO age was significantly advanced in PNA females compared to vehicle-treated controls (p<0.001, Fig. 1B). As body weight can play a significant role in the timing of pubertal onset (40), we measured body weight in PNA at PND 20, 24, and 4 months of age and found no differences (Fig. 2). Therefore, prenatal DHT does not advance VO due to increased body weight.
Surprisingly, peripubertal DHT exposure significantly advanced pubertal onset as well (p<0.001, Fig. 1C). The PPA mice also showed no differences in overall weight at 4 months of age (data not shown).

Some studies have shown transgenerational effects of endocrine disruption (51). We therefore next investigated whether early VO also occurred in the progeny of PNA females. The female offspring of PNA females displayed VO comparable to controls (Fig. 1D), suggesting that this pubertal effect of DHT exposure does not have a heritable component.

**The role of neuronal AR and Kiss1r in DHT-mediated advanced pubertal onset**

The initiation of puberty involves the activation of kisspeptin and GnRH signaling, as well as an increase in sex steroids, which feed back on kisspeptin neurons via ERα and AR (46). Whether prenatal DHT exposure affects reproductive parameters by altering kisspeptin circuits is currently unknown. To answer this question, we tested whether developmental DHT exposure of Kiss1r-null mice, as well as of mice conditionally lacking androgen receptor (AR) in neurons, would also impact pubertal and reproductive parameters. This allowed discrimination between effects of DHT on the hypothalamic component of the HPG axis versus the pituitary or ovaries.

To test whether peripubertal DHT exposure regulates pubertal onset via neuronal AR, we subjected female mice with a neuron-specific AR knockout (AR/Syn-Cre) to the PPA paradigm. Vaginal opening was monitored in PPA AR/Syn-Cre and AR flox (control) females given peripubertal DHT or oil (6-8 animals/group). As DHT is non-aromatizable, we hypothesized that any effects of DHT in the brain to induce early pubertal onset would be prevented by the absence of neuronal AR. However, PPA caused a significant advancement in VO in the AR/Syn-Cre mice, as well as in the AR flox controls (p<0.001 compared to vehicle-treated mice, Fig. 3A), indicating that peripubertal DHT is not acting through neuronal AR to induce early puberty.
To further dissect the mechanism and site of action of DHT in PNA, we utilized a genetic model of HPG axis inactivation: Kiss1r-null mice (15, 57, 58). These mice display hypogonadotropic hypogonadism and fail to progress through normal sexual maturation (15). Study of PNA in the Kiss1r-null mice should yield more precise mechanistic results than the much broader AR/Syn-Cre elimination of AR in neurons. We first confirmed that vehicle-treated Kiss1r-null mice had absent VO compared to vehicle-treated wild-type females (p<0.001, Fig. 3C). Interestingly, we found that PPA treatment caused VO to occur early in Kiss1r-null mice (KO; p<0.001 versus vehicle-treated mice, Fig. 3B). We next evaluated the effect of prenatal DHT on VO in Kiss1r-null mice. As in PPA females, PNA treatment resulted in early VO in Kiss1r-null mice (p<0.05, Fig. 3C).

To further investigate the role of the kisspeptin system in mediating the effects of PNA, we examined Kiss1 expression in the AVPV of adult PNA and control mice by ISH. Kiss1 is expressed in the AVPV in a sexually dimorphic manner, at much higher levels in females than males (1). We found no differences in Kiss1 expression in the AVPV between PNA and control females, and confirmed that the control males had low levels (p<0.01, Fig. 4). Thus, the development and sexual dimorphism of the Kiss1 neurons is not affected by PNA treatment. Furthermore, Kiss1 expression in the arcuate nucleus was also unchanged in these females, suggesting that PNA treatment does not alter sex steroid negative feedback on kisspeptin neurons (data not shown).

**PNA, but not PPA, causes irregular estrous cycling in adults**

Having established that both PNA and PPA advanced pubertal onset, we next determined whether cyclicity was similarly affected in the two models. Estrous cycling is initiated in female rodents following VO, and previous evidence has suggested that PNA can cause irregular estrous cycling between 4-6 months of age (53). We investigated whether PNA can cause irregular
cycling at an earlier age when fertility is robust (3 months). To evaluate whether DHT acts to influence reproductive parameters in both developmental androgen exposure paradigms, we compared PNA to age-matched PPA females. The cycles of DHT- and oil-treated PNA and PPA females were followed. To quantify the regularity of cycling, the proportion of time spent in each cycle stage was compared between DHT- and oil-treated groups (7-8 animals/group). Figure 5A shows that 3 month-old PNA females had irregular cycling compared to oil-treated controls. In contrast, 3 month-old PPA females showed normal estrous cycling, and were therefore not further studied. Quantification of the proportion of time spent in each cycle stage (diestrus, proestrus, estrus, and metestrus) showed significant differences in that PNA mice spent less time in diestrus and more time in metestrus than control females (p<0.001, Fig. 5B). This suggests a mechanistic divergence of DHT action between these two time frames: PNA has a lasting effect by adversely influencing estrous cycling in adulthood, while PPA does not. To further investigate this idea, testosterone levels were measured in 4 month-old PNA and PPA females (this time point was chosen as the animals have sufficiently progressed into adulthood but have not yet reached reproductive senescence). We found a modest increase of T in the serum of PNA mice as compared to control females, but no change in T levels in PPA females (Fig.5C).

The role of testosterone in the irregularity of estrous cycling in PNA mice

Given that cycling irregularities, as well as increased T, were found in PNA females but not PPA females, we hypothesized that the higher T maintained by the PNA females adversely affects estrous cycling. To test this, T implants, at a dose resulting in levels of T similar to those measured in PNA females, were administered to adult, wild-type females and estrous cycling was monitored both before and after the implantation. The appropriate dose of T was established by extensive pilot studies measuring T levels one week after the implantation (data not shown). Vaginal cytology was quantified and the T-implanted females spent less time in proestrus and
more time in metestrus compared to controls (p<0.05, Fig. 6) mirroring the effects of the elevated endogenous T in the PNA mice.

**PNA delays fertility in early adulthood**

Since PNA females have irregular cycling at 3 months of age, developmental DHT exposure could have significant adverse effects on reproductive output. A 6-month-long fertility assessment was therefore conducted in PNA females compared to oil-treated controls, beginning at 2 months of age. DHT and oil females (2 months old, 6 animals/group) were each housed with a single C57BL6 male continuously for 6 months. The time to first litter and the number of litters produced were determined. After the first month of the fertility assessment, more control mice had given birth to their first litter than PNA females (p<0.05, Fig. 7A). As the fertility assessment progressed, the PNA females produced numbers of litters, as well as litter sizes (data not shown), similar to the controls (Fig. 7B), suggesting that PNA only temporarily affects fertility.

**Ovarian morphology and metabolic factors in PNA mice**

Polycystic ovaries and impaired insulin sensitivity are two common hallmarks of PCOS (33). To complement our findings from the estrous cycling and fertility assessments and to further evaluate the extent to which PNA represents PCOS in a murine model, ovarian morphology was examined in 2-, 3- 7-and 10 month-old PNA mice by observing the presence or absence of ovarian cysts, corpora lutea, and follicles.

Ovaries from 2-, 3- and 7-, and 10-month-old PNA and control mice were analyzed for morphological features. The numbers of CL’s, preantral follicles, and antral follicles were comparable between PNA and control mice at each age (Fig. 8). Furthermore, there were no detectable ovarian cysts, suggesting that PNA did not result in a persistent ovarian phenotype. The presence of follicles and corpora lutea is consistent with the overall ability of the PNA
females to produce litters after prolonged exposure to a male, as was seen in the fertility assessment.

Despite the absence of ovarian cysts, the PNA mice displayed precocious puberty, irregular cycling accompanied by a decreased fertility, and elevated levels of testosterone, all of which are associated with PCOS (38). Because PCOS patients also commonly present with metabolic changes such as increased body weight and insulin resistance (33), we further tested whether PNA mice exhibited changes in these metabolic endpoints. We found no group differences in body weight in adulthood at 4 or 6 months of age (Fig. 2B) or at PND 20 or 24 (Fig. 2A) or in adult PPA females (data not shown). As indicators of metabolic function, an oral glucose tolerance (GTT) test and an insulin tolerance test (ITT) were performed in PNA females versus controls to test whether PNA adversely affected insulin sensitivity and blood glucose regulation in PNA females at 1 month of age. However, no differences were detected between PNA and controls in glucose clearance or insulin sensitivity (Fig. 9). The PNA animals were then transferred to a high-fat diet for 6 months. GTT following the high-fat diet revealed a greater change in glucose metabolism in the controls compared to PNA animals. GTT was also performed on 6 month-old PNA females that were not subjected to a high-fat diet, and no differences were detected (Fig. 9).

**PNA advances reproductive aging**

To our knowledge, studies of prenatal androgenization in experimental animals have not yet investigated the effect of early androgen exposure on reproductive senescence in females. We hypothesized that prenatal DHT would have negative effects on estrous cycling not only in early adulthood, but continuing as the animals aged. Therefore, because puberty onset was advanced and cycling was irregular in PNA females compared to controls, we investigated whether the onset of reproductive aging occurred earlier in PNA mice. In female mice, reproductive aging
with regard to estrous cycling is characterized by persistent cornification of the vaginal epithelial cells as determined by cytology (59). Cytology was monitored in PNA mice at 8 and 10 months of age, comparing oil- and DHT-treated females (20-23 animals/group). Estrous cyclicity was measured in the female mice for 25 days determining that the incidence of persistent vaginal cornification (PVC) occurred more frequently in PNA mice compared to controls at both ages (p<0.01, Fig. 10). This higher incidence of aberrant cycling in older PNA mice indicates that PNA may advance the onset of reproductive aging.

**Discussion**

Exposure to EDC’s, including androgens, during critical periods of development in females can dramatically alter HPG axis function and reproductive physiology later in life (51). The present work has characterized the reproductive system of a prenatal androgenization mouse model (53) by studying the effects of PNA on puberty onset, fertility, and reproductive senescence. Exposure to low-level DHT prenatally accelerates onset of puberty, delays fertility, causes irregular estrous cycling, increases T, and advances reproductive senescence, while exposure prepubertally only advances puberty. Further, the advanced pubertal onset induced by DHT administered prenatally and prepubertally occurs independently of the kisspeptin system and AR in neurons.

**Advanced pubertal onset in PNA and PPA mice**

Increased levels of androgens are associated with premature puberty in girls (38). Exposure to androgens during critical developmental periods can predispose females to reproductive disorders, but the mechanisms by which this occurs are not fully understood. The PNA mouse represents a useful model (53) for studying the effects and mechanisms of prenatal DHT exposure and reflects many aspects of reproductive disorders in women (53).
The use of DHT in the PNA and PPA models is advantageous, as opposed to T, in that DHT is not aromatized, and therefore acts through AR only and not ERα. A caveat of this is that the DHT metabolite 5α-androstane-3β,17β-diol acts through estrogen receptor β (ERβ) (60), and therefore a possible contribution of ERβ signaling should be considered. Indeed, administration of an ERβ agonist to female rats resulted in advanced pubertal onset (61). Future studies using the PNA and PPA models could avoid the possibility of DHT having secondary effects through ERβ signaling by additionally treating with an ERβ antagonist such as raloxifene or an AR antagonist such as flutamide. However, we chose not to use these antagonists, which have extensive activities of their own, so as to ensure that our models best reflect androgen exposure in women.

The studies presented herein show that pubertal onset is advanced in PNA female mice, as previously noted (62). However, whether other time frames exist during which the female reproductive axis is sensitive to sex steroid modulation has not been studied (to our knowledge). We therefore compared pubertal onset in PNA to a novel model of DHT exposure, PPA. That VO is advanced in both PNA and PPA indicates that it can act during both the late prenatal and prepubertal stages to influence the timing of pubertal onset. Because VO in PNA mice occurs 3-4 weeks after DHT treatment, while VO in PPA mice occurs immediately after treatment, it is possible that these effects are occurring through different mechanisms. PNA may have permanent, organizational effects on the neuronal circuitry driving the HPG axis and/or sex steroid feedback, though sexual dimorphism of the kisspeptin system is not affected, while PPA may only act transiently and peripherally to induce puberty onset. Given the critical importance of Kiss1r in puberty, we sought to determine whether kisspeptin signaling (which responds to sex steroid feedback and drives GnRH and the rest of the reproductive axis) was involved in either or both of the PNA and PPA paradigms.
Advanced puberty onset in PNA is Kiss1r-independent

Kiss1r is critical for the onset of puberty both in humans and in mice (15, 16, 48), but its role in mediating the advanced VO in PNA was unknown. Our finding that VO occurred early after PNA regardless of the absence of Kiss1r indicates an alternative pathway influencing pubertal onset, possibly through a peripheral mechanism. Furthermore, the effects of PNA are not mediated by masculinization of the sexual dimorphism of the Kiss1 system, suggesting the kisspeptin system is not playing a major role in the actions of PNA. This lack of Kiss1 sexual dimorphism was not surprising given that our animals were treated with non-aromatizable DHT, thus avoiding the effects of testosterone-derived estradiol. While the effects of PNA appear to be independent of kisspeptin signaling, it is possible that GnRH may still be involved in the early puberty onset, as it can be regulated by factors other than kisspeptin, such as neurokinin B and steroid-responsive glial inputs (63, 64).

Advanced puberty onset in PPA is independent of neuronal AR and Kiss1r

We used the PPA paradigm to determine whether this model also did not require Kiss1r, as well as whether PPA was exerting a broad effect through neuronal AR to induce early puberty. The acceleration of VO by PPA in the absence of neuronal AR and Kiss1r indicated that the advancement of puberty in this model may be occurring through a non-neuronal mechanism. Because the Kiss1r-null animals have little or no hypothalamic activation of the reproductive axis, it appears that PPA is bypassing the kisspeptin neuronal circuitry to cause early VO. Since Kiss1r-null mice do not have VO or puberty onset, induction of VO by PPA and PNA in these mice indicates that DHT is capable of activating VO in the absence of normal puberty. Whether there is a contribution from the pituitary and ovaries remains to be determined.

PNA resulted in long-term effects on fertility, cycling, and aging
Studies of the PNA mouse had not previously investigated the effects on fertility, so we conducted a fertility assessment to determine if PNA treatment could influence reproductive function. Despite an early delay, the PNA females were capable of producing litters as the fertility assessment progressed, suggesting that the PNA paradigm was not sufficient to render females infertile. This is consistent with our investigation of ovarian morphology, which provided evidence of ovulation. Interestingly, the time frame during which the early decrease in fertility was observed (when the females were 3 months old) coincided with the age at which they showed irregular cycling. The reduction in litters produced by PNA females at this age could be explained by abnormalities in estrous cycling.

Estrous cycling of PNA and PPA females showed irregularities at 3 months of age in PNA but not PPA. Thus, despite a similar advancement in pubertal onset between the two paradigms, the effects of PNA persist into adulthood to influence estrous cycling, while PPA effects do not. As a complex interplay of sex steroid feedback effects is necessary to promote progression through the estrous cycle, we therefore propose that PNA (but not PPA) results in organizational changes during HPG axis development (52), possibly affecting sex steroid production, which persist throughout the reproductive lifespan. The increased T in PNA mice is consistent with PNA, but not PPA, affecting reproductive outcomes in adulthood (53). We directly tested the role of increased T in estrous cyclicity and found that even low-level exposure to T, comparable to the levels in PNA, caused irregularities in estrous cycling. Sex steroids can increase cornification of the vaginal epithelium (65), which may explain the increased incidence of metestrus (which is partly characterized by cornified epithelial cells) in both PNA and T-implanted females. We therefore conclude that an increase in T resulting from PNA treatment is at least partly responsible for the alterations in estrous cycling.

These effects on reproductive parameters were not associated with increases in body weight or changes in glucose metabolism. A high-fat diet in 7 month-old PNA animals also did
not adversely affect glucose metabolism as strongly as controls. This is in contrast with a previous study (62) that found impaired glucose tolerance in PNA females. While the cause of these dissimilar results has not been fully determined, there may be a possible contribution of mouse strain and background differences. Other studies have found strain differences to be the cause of variation in metabolic parameters (66). Roland et al. (62), studied animals that were originally bred from CBB6/F1 transgenic mice, while the mice in the current study were 100% C57Bl/6J. The difference in strains may partly account for the differences seen in glucose tolerance.

Because PNA influenced the timing of pubertal onset and fertility in early adulthood, we asked whether PNA could also influence reproduction later in life by altering the timing of reproductive aging. PNA females displayed evidence of reproductive senescence earlier than controls, demonstrating that PNA can influence reproductive parameters even 8-10 months after exposure. This finding, combined with the irregular cycling in PNA but not PPA, suggests a mechanistic difference in DHT action between the PNA and PPA models, where PNA has organizational, lasting effects while PPA acts only transiently to induce VO. The HPG axis is more sensitive to DHT during the late prenatal period than the peripubertal period, and changes that occur in utero affect reproductive function late into adulthood.

In conclusion, we have studied the PNA mouse model in depth to show a kisspeptin-independent advancement of pubertal onset, irregular cycling in early adulthood, with a concomitant delay in fertility, and early reproductive senescence. We have compared PNA to a novel model of precocious puberty, PPA. The advanced pubertal onset in PPA occurs independently of neuronal AR and Kiss1r. PPA has only a transient effect to accelerate the onset of puberty, while PNA has lasting effects throughout the reproductive lifespan, influencing, not only puberty but also fertility, cycling, and reproductive senescence. Thus, prenatal androgen exposure alters reproductive function throughout life, indicating that androgen exposure during
this stage of development may be important for the etiology of female reproductive disorders.

Chapter 1 has been published in *Endocrinology* (September 2012). The dissertation author was the primary investigator and author of this material. Shadi Shojaei and Jason Meadows assisted with the various *in vivo* experiments. Sasha Kauffman provided technical support for the *in situ* hybridization studies and also provided guidance and advice. Pamela Mellon supervised the project and provided advice.
Figure 1.1. VO was advanced in PNA and PPA, but not in PNA progeny. A, Diagram illustrates the timeline for DHT administration in the prenatal androgen (PNA) and peripubertal androgen (PPA) paradigms and the time frame for monitoring pubertal onset by vaginal opening (VO). B, Wild-type females were subjected to the PNA paradigm and were monitored daily for VO; *** p<0.001 by Student’s t-test. C, Wild-type females were subjected to the PPA paradigm and were monitored daily for VO; *** p<0.001 by Student’s t-test. D, Adult PNA females were mated to wild-type males and the female progeny were monitored daily for VO; p>0.05 by Student’s t-test.
Figure 1.2. PNA treatment did not result in changes in body weight. Body weight was measured in PNA females at A, PND 20 and PND 24, and B, 4 months and 6 months.
Figure 1.3. VO was advanced in PPA AR/Syn-Cre and Kiss1r-null, and in PNA Kiss1r-null mice. A, AR/Syn-Cre females were mated to AR flox males. Female AR flox (Cre- ) and AR/Syn-Cre (Cre+ ) offspring were subjected to the PPA paradigm and were monitored daily for VO; *** p<0.001 versus control by two-way ANOVA. B, Heterozygous Kiss1r animals were mated. Female homozygous WT and KO Kiss1r-null offspring were subjected to the PPA paradigm and were monitored daily for VO; *** p<0.001 versus control by log-rank chi-square. VO was not recorded after postnatal day 35. C, Heterozygous Kiss1r dams were administered PNA. Female homozygous WT and Kiss1r-null (KO) offspring were subjected to the PNA paradigm and were monitored daily for VO; * p<0.05 versus control by log-rank chi-square. *** p<0.001 versus oil-treated knockout by log-rank chi-square. VO was not recorded after postnatal day 35.
Figure 1.4. PNA females had no sexually dimorphic Kiss1 expression in the AVPV. A, In situ hybridization for Kiss1 in the AVPV of control female (left), PNA female (center), and male (right). B, Quantification of in situ hybridization: left, number of Kiss1-positive cells; right, Kiss1 mRNA per Kiss1 cell as measured by silver grains per Kiss1 cell. N = 7 oil, 7 DHT, and 3 males; ** p<0.01 by one-way ANOVA.
Figure 1.5. PNA exposure resulted in irregular estrus cycles and increased T in females.
A, Estrous stages for 3-month-old PNA and PPA females were tested daily for 15-20 days. Representative cycle traces are shown; M: metestrus, E: estrus, P: proestrus, D: diestrus. B, The proportion of time spent in each cycle stage was quantified for the PNA females. N=8 oil and 7 DHT; *** p<0.001 by Student’s t-test. C, Four-month-old PNA and PPA females were sacrificed in diestrus and sera were analyzed for T levels; * p=0.05 by Student’s t-test.
Figure 1.6. Testosterone implantation resulted in disrupted estrous cycling in wild-type females. Estrous stages were tested in wild-type females (2-3 months old) daily for 15 days both before (A) and after (B) T or cholesterol implantation, and the proportion of time spent in each cycle stage was quantified. N=8 cholesterol and 8 T; * p<0.05 by Student’s t-test.
Figure 1.7. PNA females had an initial delay in fertility. PNA females were pair-housed continuously with single wild-type males for 6 months. A, The number of days until birth of the first litter was measured; * p<0.05 by Student’s t-test. B, The total number of litters born during each month of the fertility assessment was quantified; * p<0.05 by Student’s t-test.
Figure 1.8. PNA females exhibited ovarian morphology consistent with the ability to produce litters. PNA females were sacrificed in diestrus at 2 (A), 3 (B), 7 (C), and 10 (D) months of age. Ovaries were fixed, sectioned, and examined for normal follicles, Graaffian follicles (GF), and corpora lutea (CL). N = 3 for 2, 3 and 10 months, n = 2 for 7 months. CL’s, preantral follicles, and antral follicles were quantified and no differences were found.
Figure 1.9. PNA treatment did not affect glucose tolerance. A, 1 month old PNA females were subjected to an insulin tolerance test (ITT) and glucose levels were measured at the indicated time points. N=9 oil and 12 DHT. B, 1 month old PNA females were subjected to an oral glucose tolerance test (GTT) and glucose levels were measured at the indicated time points. N=13 oil and 13 DHT. C, 6 month old PNA females were subjected to an oral GTT and glucose levels were measured at the indicated time points. N=5 oil and 5 DHT. D, An oral GTT was performed on 7 month old PNA females after 6 months of exposure to a high-fat diet. * p<0.001 by Student’s t-test.
Figure 1.10. Advanced reproductive senescence in aging PNA females. PNA females were cycled daily for 25 days at 8 and 10 months of age, and the incidence of persistent vaginal cornification (PVC) was quantified; ** p<0.01 by chi square.
CHAPTER 2: Functional Roles of Kisspeptin Receptor (Kiss1R) in Pituitary Gonadotropes: Regulation of Gonadotropin Gene Expression

Abstract

Kisspeptin signaling through its receptor, Kiss1R, is crucial for many reproductive functions including puberty, sex steroid feedback, and overall fertility. While the importance of Kiss1R in the brain is firmly established, its role in regulating reproduction at the level of the pituitary is not well understood. This study presents molecular analysis of the role of kisspeptin and Kiss1R signaling in the transcriptional regulation of the gonadotropin gene beta subunits, LHβ and FSHβ, utilizing LβT2 gonadotrope cells and murine primary pituitary cells. We show that kisspeptin induces LHβ and FSHβ gene expression, and this induction is PKC-dependent and mediated by the immediate early genes, Egr-1 and cFos, respectively. Furthermore, kisspeptin induces transcription of the Egr-1 and cFos promoters in LβT2 cells. Kisspeptin also increases gonadotropin gene expression in mouse primary pituitary cells in culture. Furthermore, we find that Kiss1r expression is enhanced in the pituitary of female mice during the estradiol-induced LH surge, a critical component of the reproductive cycle. Overall, our findings indicate that kisspeptin regulates gonadotropin gene expression through the activation of Kiss1R signaling through PKC, inducing immediate early genes in vitro, and responds to physiologically relevant cues in vivo, suggesting that kisspeptin affects pituitary gene expression to regulate reproductive function.
Introduction

The reproductive neuroendocrine axis is centrally regulated by inputs from kisspeptin neurons to gonadotropin-releasing hormone (GnRH) neurons, which in turn regulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gonadotropes. LH and FSH exert their actions on the gonads, regulating estradiol and testosterone production, which have feedback effects on the pituitary and hypothalamus (including kisspeptin neurons). Kisspeptin, the product of the Kiss1 gene, and its Gq/11-coupled receptor Kiss1R (formerly termed GPR54) were first discovered to be important for reproduction in 2003, when three studies reported profound deficits in fertility, puberty, cyclicity, and sex steroid and gonadotropin levels in humans and mice carrying mutations in the Kiss1R gene (7, 15, 16). The role of the kisspeptin system in reproductive function has since been further characterized with the use of Kiss1R- and Kiss1-null mice (54, 67-70). It is now well established that kisspeptin signaling through Kiss1R is necessary for normal functioning of the reproductive axis.

Abundant evidence in a variety of mammals demonstrate the direct and potent actions of hypothalamic kisspeptins acting at the level of the GnRH neurons: kisspeptin induces LH and FSH secretion which is prevented by a GnRH receptor antagonist, kisspeptin induces GnRH neuronal firing and release of GnRH in hypothalamic explants, and GnRH neurons express Kiss1R (12, 13, 46). While hypothalamic kisspeptin signaling has been firmly substantiated, less is known of the role of kisspeptin and Kiss1R in the pituitary. In a number of mammalian species, Kiss1r mRNA is expressed in the pituitary, along with several other peripheral tissues, including kidney, testis, ovary, pancreas, adipocytes, and placenta (46, 71).

Previous studies demonstrated that kisspeptin treatment can increase LH in numerous mammalian species, presumably due to kisspeptin acting on GnRH neurons (46, 72). Importantly, several studies have shown that treatment of primary pituitary cultures from rats and baboons
with the bioactive form of kisspeptin, kisspeptin-10, can induce LH secretion (73, 74). These findings that kisspeptin can stimulate pituitary cultures to secrete LH suggest that kisspeptin may act at various levels of the hypothalamic-pituitary-gonadal axis, as illustrated by its capacity to increase both GnRH and LH release. Moreover, the findings that kisspeptin is present in the portal bloodstream of the median eminence in sheep and primates, and that Kiss1R colocalizes with LHβ in rat primary pituitary cultures (71, 75), further supports a possible role for kisspeptin signaling outside of the hypothalamus.

While it is well known that Kiss1 expression is strongly regulated by sex steroid feedback (46, 76, 77), hormonal regulation of Kiss1r is less well understood. In ovariectomized rats, pituitary Kiss1r was decreased under negative feedback by 17β-estradiol (21). This suggests a physiologically relevant role of pituitary Kiss1R, but this has not been investigated in other animal models or under other sex steroid feedback conditions, such as the estradiol-induced LH surge. Further clarification of sex steroid-dependent regulation of KissrR expression will provide a useful context for interpreting the studies of kisspeptin-induced LH secretion.

Although the studies of kisspeptin-induced LH secretion from primary pituitary cultures have been informative, it is currently unknown whether kisspeptin signaling can regulate gonadotropins at the transcriptional level. The observation that kisspeptin can act directly on the pituitary to cause LH secretion does not necessarily provide information about whether kisspeptin activation of Kiss1R is influencing LH (or FSH) gene expression, and if so, by what mechanism. The LβT2 pituitary gonadotrope cell line is a useful and appropriate in vitro model with which to test the transcriptional effects of Kiss1R signaling. LβT2 gonadotropes express the gonadotropin subunits LHβ and FSHβ, as well as the common αGSU subunit, and can respond to GnRH, activin, and sex steroid treatments (45). The mechanisms of the transcriptional regulation of LHβ and FSHβ in LβT2 gonadotropes have been extensively studied (45), but whether Kiss1R signaling modulates expression of these genes is unknown.
Materials and Methods

Hormones and treatments

Human kisspeptin-10 (Metastin [45-54] amide), 17β-estradiol, and GnRH were purchased from Sigma-Aldrich (St. Louis, MO). A protein kinase C inhibitor (PKCi), Go 6983, was purchased from Tocris Bioscience (Minneapolis, MN). Activin was purchased from Calbiochem (La Jolla, CA). R1881 (methyltrienolone) was purchased from NEN Life Sciences (Boston, MA).

Plasmid Constructs

The -1.7 kb rat LHβ, -1 kb murine FSHb, and -1.1 kb GnRH receptor luciferase (luc) reporters were described previously (45, 78, 79). The -1 kb cFos and FSHb containing an AP-1 mutation have been previously described (80). The 1 kb murine Egr-1 promoter was cloned by PCR from genomic DNA and linked to the luciferase reporter in pGL3 basic plasmid using KpnI and HindIII sites. Mutagenesis of the c-Fos and Egr1 promoter luciferase plasmids was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions and mutations were confirmed by dideoxyribonucleotide sequencing. The Egr-1 multimer, consisting of 4 tandem copies of the consensus Egr-1 site with 2-bp spacers, was cloned in the multiple cloning site in front of the thymidine kinase (tk) minimal promoter in the pGL3 basic luciferase reporter. LHβ containing SF-1 and Egr-1 mutations were provided by Dr. Varykina Thackray (81). The SF-1 mutations were at -128 bp and -59 bp, and the Egr-1 mutations were at -111 bp and -50 bp, all of which have been previously described (82-84). The AP-1 multimer was purchased from Agilent (Santa Clara, CA) and the AFos expression vector
has been previously described (80). The Kiss1R expression vector was purchased from Thermo Scientific (Logan, UT). The following mutations in the Egr-1 promoter (serum response factor, SRF; E-twenty six, Ets; cAMP response element, CRE) were designed using the primers listed in Table 1: -83 bp SRF, -105 bp SRF, -353 bp SRF, -368 bp SRF, -91 bp ETS, and -138 bp CRE.

Mutations in two Hox sites on the cFos promoter at -313 bp and -59 bp were designed using the primers listed in Table 1. The AP-1 mutation at -295 bp, the STAT mutation at -345 bp, and the SRF mutation at -310 bp in the cFos promoter have been previously described (80). The -5 kb Kiss1R promoter was provided by Dr. Daniel Clark.

**Cell Culture and Transient Transfections**

Transient transfections were performed in the LβT2 pituitary gonadotrope cell line (85). Cells were maintained on 10-cm dishes (at 37°C and 5% CO2) in DMEM (Mediatech Inc., Herndon, VA) containing 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA) and penicillin/streptomycin antibiotics (Life Technologies, Inc./Invitrogen, Grand Island, NY) as previously described (86). 1x Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) was used to dissociate the cells. Cells were plated in 12-well plates at 4.5 x 10^5 cells per well and were transfected approximately 24 h later with PolyJet transfection reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer’s instructions.

For all experiments, cells were transfected with 300 ng of the indicated reporter plasmid, 100 ng of the Kiss1R expression vector to ensure consistent and adequate expression, the AFos expression vector where indicated, and 100 ng of a of a β-galactosidase reporter plasmid driven by the herpes virus thymidine kinase promoter to control for transfection efficiency (86). After 6 h of transfection, cells were switched to serum-free DMEM containing 0.1% BSA and 5 mg/L transferrin. Cells were treated for 12 h (overnight) with 100 nM kisspeptin-10 (kiss) and/or 10 nM GnRH as indicated in serum-free media. For experiments using PKCi (Go 6983), serum-free
media with or without 5 µM PKCi replaced the previous media for 30 min, after which kisspeptin and control treatments were added directly to the wells. The dose and duration of the kisspeptin treatment and PKCi were determined by dose response and time course studies (data not shown). Vehicle control for kisspeptin and PKCi was 1:1000 DMSO and 0.1% BSA for GnRH.

Luciferase and β-galactosidase assays

After hormone treatment, luc reporter and β-galactosidase activity were assayed as previously described (86). Briefly, cells were washed with PBS and lysed with 0.1 M potassium phosphate buffer (pH 7.8) containing 0.2% Triton X-100. Luciferase activity was measured using a buffer containing 100 mM Tris-HCl (pH 7.8), 15 mM MgSO₄, 10 mM ATP, and 65 µM luciferin. β-Galactosidase activity was assayed using the Tropix Galacto-light assay (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Both assays were measured using a Veritas Microplate Luminometer (Promega, Madison, WI).

Animals

All animal procedures were performed in accordance with the UCSD Institutional Animal Care and Use Committee regulations. All mice were on a C57BL/6J background and were group-housed on a 12 h light:12 h dark cycle with ad libitum chow (11% of calories fat, 17% of calories protein) and water.

Ovariectomy and estrogen replacement

Estradiol feedback paradigms for animals sacrificed in the morning (a.m.) and the evening (p.m.) were tested in female mice, as previously described (87). Briefly, C57BL/6J female mice between 2-3 months of age were anesthetized by isoflurane inhalation and were
ovariectomized. At the time of surgery, animals were subcutaneously implanted with capsules containing either vehicle (OVX) or 2.5 µg 17β-estradiol (OVX+E). One week after surgery, OVX+E females were injected subcutaneously with 1 µg estradiol benzoate (E8515; Sigma Aldrich, St. Louis, MO) in 100 µL of sesame oil. OVX females were injected with 100 µL of sesame oil. All injections occurred between 09:00 and 10:00 h. The a.m. animals were sacrificed the following morning, prior to 11:00 h, by CO₂ inhalation followed by exsanguination. The p.m. animals were sacrificed that evening at 18:30 h (30 min after lights out). Serum LH was measured from OVX and OVX+E animals by RIA (University of Virginia, Ligand and Assay Core).

To test androgen regulation of pituitary Kiss1R, male C57BL/6J mice were castrated (CX) and subcutaneously implanted with 10 mm Silastic capsules containing either cholesterol as a control, testosterone (17β-Hydroxy-3-oxo-4-androstene, Sigma), or dihydrotestosterone (DHT; 5α-androstan-17β-ol-3-one, Sigma). One week after surgery, all CX males were sacrificed prior to 11:00 h by CO₂ inhalation followed by exsanguination.

Primary Pituitary Isolation

Two month-old C57BL/6J male mice were sacrificed and pituitaries were harvested (n=10 animals/group) according to published methods (80). Isolated pituitaries were rinsed in cold PBS and were then minced with sharp scissors. Individual pituitaries were added to 10 mL 0.25% Trypsin-EDTA (Invitrogen Life Technologies, Grand Island, NY) containing 0.25% collagenase (Invitrogen Life Technologies, Grand Island, NY) in a 50 mL conical tube. Tubes were then agitated for 30 min at 80 rpm at 37°C at a 45° angle. Next, 10 mL of 10% fetal bovine serum in DMEM was added and the solution was mixed. 500 µL of DNase I (Sigma Aldrich, St. Louis, MO) was added to the solution at a final concentration of 25 µg/mL, and the tube was agitated as described above for 15 min. After mixing, the solution containing the suspended cells was removed from the cellular debris, and was centrifuged at room temperature for 8 min at 1000
rpm. The supernatant was discarded and the pelleted cells were resuspended in 2 mL of 10% fetal bovine serum in DMEM. Cells were plated at 5 x 10^5 cells/well in a 24-well plate, and were allowed to attach for 7 h, following which the media was changed to serum-free DMEM. The next day, cells were treated with 30 nM GnRH, 100 nM kisspeptin, or vehicle (1:1000 DMSO for kisspeptin and 0.1% BSA for GnRH) for 6 hrs. RNA was then harvested using TRIzol reagent (Invitrogen, Grand Island, NY). Genomic DNA was removed from all RNA samples using a DNA-free kit (Ambion/Invitrogen, Grand Island, NY). RT-PCR was performed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCR for LHβ, FSHβ, and TATA-binding protein (TBP, as an internal control) was performed using the primer sequences listed in Supplemental Table 1.

**RT-PCR and qPCR**

Pituitaries from C57BL/6J male and diestrus female mice (2-3 months old) were harvested and frozen. Total RNA was extracted from pituitaries and skin biopsies using TRIzol reagent (Invitrogen, Grand Island, NY). RNA was also extracted from pituitaries from the OVX and OVX+E female mice. RNA was isolated from LβT2 cells using a QiaShredder and an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Genomic DNA was removed from all RNA samples using a DNA-free kit (Ambion/Invitrogen, Grand Island, NY). RT-PCR was performed using either an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) or a SuperScript III First-Strand Synthesis kit (Invitrogen, Grand Island, NY), according to the manufacturer’s instructions. qPCR for Kiss1R and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as an internal control) was performed using the primer sequences listed in Table 1.
**Statistical Analysis**

All transient transfections were repeated independently at least three times. Data were normalized for luciferase activity relative to β-galactosidase to control for transfection efficiency. Data were also normalized to the pGL3 plasmid, as well as to vehicle control for all hormone treatments. Results are presented as mean±SEM of the fold induction relative to vehicle control. Statistical tests were performed on luciferase activity normalized to β-galactosidase. Student’s t-tests, one-way ANOVA followed by Tukey post-hoc tests, and two-way ANOVA were used as indicated, and p<0.05 was considered statistically significant.

**Results**

*Kiss1R* is expressed in pituitary gonadotropes and regulates gonadotropin gene expression

Kisspeptin has been shown to induce LH secretion by direct actions on pituitary cells in primary culture (73, 74), but the role of Kiss1R in mediating transcription of the gonadotropin genes, LHβ and FSHβ, has not been investigated. We performed RT-PCR for *Kiss1r* on pituitaries harvested from adult C57BL/6J male and female mice (2-3 months of age) and found that it was expressed in both sexes (Fig. 1A), supporting the *in vivo* relevance of kisspeptin at the level of the pituitary. Because the pituitary contains a heterogeneous cell population, we also determined that the LβT2 gonadotrope cell line expresses *Kiss1r*, indicating that it is an appropriate *in vitro* system with which to test the transcriptional role of Kiss1R (Fig. 1A). These results were in agreement with a previous study in rats (73), and further confirm the presence of *Kiss1r* in pituitary gonadotropes. As expected, *Kiss1* (encoding kisspeptin) was not expressed in the LβT2 cells (data not shown). The relative levels of *Kiss1r* expression in the pituitaries and LβT2 cells were quantified by qPCR (Fig. 1B). We also confirmed by *in situ* hybridization that
Kiss1r mRNA is present in a population of LHβ-expressing gonadotropes in mouse pituitaries (data not shown).

To initially determine whether Kiss1R can influence gene transcription in the gonadotrope, we tested whether kisspeptin could induce LHβ and FSHβ expression in mouse primary pituitary cultures. Kisspeptin treatment increased LHβ and FSHβ relative to vehicle (p<0.05 and p<0.01 respectively, Fig. 6A, B). In addition, expression of Kiss1R in mouse primary pituitary cells was not influenced by GnRH treatment (data not shown). To determine the mechanism of kisspeptin action in gonadotropes, we first performed transient transfections in LβT2 cells with -1.7 kb LHβ, -1 kb FSHβ, and -1.1 kb GnRHR promoter luciferase constructs with kisspeptin treatment. We found that a 12 h treatment with 100 nM kisspeptin induced the LHβ and FSHβ promoters (p<0.01 compared to control) but not GnRHR (Fig. 2A). As GnRH has been shown to act in synergy with other hormones to regulate FSHβ (45), we tested whether a combination of 10 nM GnRH and 100 nM kisspeptin treatment (for 12 h) could synergistically induce LHβ and FSHβ. Co-treatment with both GnRH and kisspeptin did not induce LHβ or FSHβ more than the individual treatments, suggesting a lack of synergy between these two hormones under these experimental conditions (Fig. 2B, C).

**Kisspeptin induces LHβ through an Egr-1-dependent pathway**

It has previously been shown that early growth response factor 1 (Egr-1), an immediate early gene, is necessary for LHβ induction by GnRH (32). Another transcription factor, steroidogenic factor 1 (SF1), is also involved in LHβ transcriptional regulation (30). Our finding that kisspeptin induced LHβ promoter activity led us to investigate the mechanism of this regulation, and whether it occurred through Egr-1 or SF1. After previously determining the optimal time and dose, all subsequent experiments were treated for 12 h, using the above
concentrations for kisspeptin and GnRH. We first performed a truncation analysis using LHβ promoter constructs of decreasing lengths (-1.7 kb, -1.5 kb, -500 bp, -300 bp, and -150 bp), and found that kisspeptin induction of LHβ is downstream of -150 bp, as all promoter truncations, including the -150 bp, were activated by kisspeptin treatment (p<0.05, Fig. 3A). It has been shown that the proximal region of the LHβ promoter contains two sites each for SF1 (-128 bp and -59 bp) and Egr-1 (-111 bp and -50 bp), all downstream of -150 bp (Fig. 3B) (82-84).

To determine whether any of the SF1 or Egr-1 sites were required for kisspeptin induction of LHβ, we transfected LHβ promoter constructs containing mutations in either of the Egr-1 sites and both of the SF1 sites (82-84) into LβT2 cells. Reporter activation by kisspeptin was absent with the -50 bp Egr-1 site mutation, demonstrating the necessity of this site in LHβ induction by kisspeptin (Fig. 3B). Conversely, we then tested whether the Egr-1 site alone was sufficient for kisspeptin activation. We transfected with an Egr-1 multimer construct containing tandem repeats of the Egr-1 binding sequence on a heterologous promoter, and found a significant induction by kisspeptin (p<0.05, Fig. 3C). In addition, we showed that the -1 kb Egr-1 promoter was robustly induced by kisspeptin (p<0.01, Fig. 3D), further supporting that Kiss1R stimulates LHβ in an Egr-1-dependent manner. GnRH-dependent activation of Egr-1 is critical for LHβ transcription (88), so we co-treated with GnRH and kisspeptin, but found no synergistic interaction (Fig. 3E).

**Kiss1R induces FSHβ via AP-1**

Having determined that Kiss1R-mediated activation of LHβ occurs through Egr-1, we next explored the mechanism of FSHβ induction, as both β gonadotropin subunits are differentially regulated at the transcriptional level (45). We approached this question in the same manner as for LHβ, with a truncation analysis using FSHβ promoter truncations of -1 kb, -398 bp, -304 bp, -230 bp, and -64 bp in length. All reporters were significantly induced by kisspeptin
except -64 bp (Fig. 4A), suggesting that Kiss1R signaling is acting downstream of -230 bp. FSHβ is in part regulated by activator protein-1 (AP-1), which is comprised of dimers of Fos and Jun isoforms (45), acting through an AP-1 site located -72/-69 bp (89). We transfected a reporter with a mutation in this AP-1 site within the -398 bp FSHβ promoter, and found that the induction by kisspeptin was significantly lower than in the wild-type -398 bp FSHβ promoter (p<0.01, Fig. 4B).

The requirement of the AP-1 site for FSHβ promoter induction by kisspeptin led us to test its sufficiency by transfecting an AP-1 multimer containing tandem repeats of the AP-1 sequence on a luciferase reporter. The AP-1 multimer was highly activated by kisspeptin, reaching approximately 700-fold that of the control treatment (p<0.05, Fig. 4C). We further confirmed the necessity of the functional c-Fos/c-Jun heterodimer in mediating kisspeptin induction by co-transfecting AFos (a dominant negative cFos) along with the AP-1 multimer and found that AFos reduced the induction by kisspeptin (p<0.01, Fig. 4D). Lastly, as cFos is an important component of AP-1, we transfected with -1 kb of the cFos promoter driving luciferase and found an increase with kisspeptin treatment (p<0.01, Fig. 4E). GnRH is also known to induce FSHβ through AP-1, but co-treatment with kisspeptin and GnRH did not synergistically induce the cFos promoter (Fig. 4E). Taken together, these results demonstrate that Kiss1R mediates FSHβ induction through cFos-dependent AP-1 activation.

**Kiss1R induction of the LHβ, FSHβ, cFos, and Egr-1 promoters is PKC-dependent**

As a Gq/11-coupled receptor, Kiss1R exerts its transcriptional effects by the activation of intracellular signaling pathways including phospholipase-C (PLC)/calcium mobilization, protein kinase C (PKC), and mitogen activated protein kinase (MAPK) (90, 91). In GnRH neurons, kisspeptin is thought to stimulate GnRH release via PKC (92) but this has not been investigated in pituitary gonadotropes. We therefore tested whether PKC signaling was required for kiss-
mediated induction of the LHβ, FSHβ, Egr-1, and cFos promoters by treating with a PKC inhibitor (PKCi). We found that 12 h treatment with the PKC inhibitor Go 6983 at 5 µM significantly decreased the effect of kisspeptin on each of these promoters (p<0.05, Fig. 5).

**Known regulatory sites on the cFos promoter are required for Kiss1R induction**

We have shown through studies of the cFos and Egr-1 promoters and multimers that these genes are induced by kisspeptin in LβT2 cells. We next sought to explore the transcriptional mechanism by which these promoters are activated by kisspeptin. Transfections with mutations in four SRF sites as well as an ETS and a CRE site within the Egr-1 promoter showed that none of these sites are required for kisspeptin induction of Egr-1, as none showed a significant repression of the induction by kisspeptin (Fig. 6A). It is possible that mutations in multiple regulatory sites are needed to determine the mechanism of kiss-mediated Egr-1 induction. However, transfections with mutations in AP-1, SRF, STAT, and two Hox sites within the cFos promoter showed that the -313 Hox site, the -59 Hox site, and the -345 STAT site were all required for kisspeptin induction of the cFos promoter (p<0.01, Fig. 6B).

**The Kiss1R promoter is not regulated by sex steroids, GnRH, or activin in LβT2 cells**

Having determined that Kiss1R regulates the transcription of LHβ and FSHβ in LβT2 cells, we next investigated whether Kiss1R itself was regulated on the transcriptional level *in vitro*. A 5 kb construct of the Kiss1R promoter has been generated in our group (Lara Kose and Daniel Clark, unpublished data). We found that the -5 kb Kiss1R promoter was not regulated (either positively or negatively) by 17β-estradiol, R1881, or GnRH (Fig. 7A-C). The -5 kb Kiss1R promoter was also not self-regulated by kisspeptin treatment (Fig. 7D). Furthermore, GnRH did not act synergistically with either activin or R1881 to regulate the -5 kb Kiss1R promoter (Fig. 7E-F).
Kisspeptin regulates LHβ gene expression in mouse pituitary cells

To demonstrate the relevance of our finding that kisspeptin can induce gonadotropin promoter activity in transfected LβT2 gonadotropes, we next tested whether kisspeptin could induce LHβ and FSHβ expression in mouse primary pituitary cultures. Kisspeptin treatment increased LHβ and FSHβ relative to vehicle (p<0.05 and p<0.01 respectively, Fig. 8A, B). In addition, expression of Kiss1r in mouse primary pituitary cells was not influenced by GnRH treatment (data not shown).

Pituitary Kiss1r expression increases during the E-induced LH surge

Our results thus far demonstrate that kisspeptin/Kiss1R signaling acts through specific transcriptional mechanisms in LβT2 gonadotropes to induce LHβ and FSHβ via the activation of immediate early genes Egr-1 and cFos, respectively. This increase in kisspeptin-mediation transcription also occurs primary mouse pituitary cells. To extend our study of the transcriptional role of Kiss1R, we tested whether Kiss1r itself was regulated in the mouse pituitary. A previous study suggested that pituitary Kiss1r is responsive to regulation by estradiol in female OVX rats (21), but it is unknown whether this occurs in mice, and whether Kiss1r expression is influenced by either circadian factors or high estradiol levels during the LH surge.

We performed qPCR on pituitaries from OVX mice with or without estradiol replacement that were sacrificed in the morning (a.m., representing low LH levels due to negative feedback) or the evening (p.m., representing the LH surge due to positive feedback). Pituitary Kiss1r was unchanged in the OVX animals between the a.m. and p.m. groups, but was significantly higher in the OVX+E p.m. animals compared to OVX+E a.m. mice and both groups of OVX females (p<0.001, Fig. 8C). Serum LH from all animals was measured to confirm the effectiveness of the surgeries and the hormonal paradigm (data not shown).
For comparative purposes, we also performed qPCR for Kiss1R on the hypothalami of the OVX females as well as the CX males. No significant differences were found for the males, although the females did show higher Kiss1R in the p.m. animals compared to a.m., consistent with our findings in the pituitary (p=0.05, Fig. 9).

**Discussion**

Many lines of evidence have demonstrated the importance of kisspeptin signaling in the hypothalamus, including animal and clinical studies showing the necessity of the kisspeptin system in promoting reproductive function (46). Only recently, however, has a role for kisspeptin signaling begun to be described in the pituitary. While a limited number of studies have shown that kisspeptin can stimulate LH secretion at the level of the pituitary, it is unknown whether Kiss1R can also regulate transcription of gonadotrope-specific genes, and if so, by what mechanism.

**Kiss1R signaling induces gonadotropin transcription via immediate early gene activation**

We initially tested whether kisspeptin can activate the biologically specific subunits of the gonadotropin genes, LHβ and FSHβ, as well as GnRHR. We found that the effect of kisspeptin treatment was specific to the gonadotropin beta subunits. Truncation analysis of the LHβ promoter showed that the induction by kisspeptin mapped to a proximal area of the promoter containing two Egr-1 sites. The more proximal of these two sites was found to be required for kisspeptin induction of LHβ, and is also important for GnRH-mediated activation of LHβ (83). Egr-1 is both necessary and sufficient for kisspeptin-mediated LHβ induction, as indicated by Egr-1 mutation and multimer studies.
Our studies of Kiss1R-mediated LH\textsubscript{b} induction have implications for our understanding of the LH surge, a critical event in the female reproductive cycle. A sharp rise in GnRH triggers the increase in LH transcription, but our findings open the possibility that Kiss1R may also be influencing the fluctuations in LH levels. In addition to LH, FSH also increases at the time of the surge. We therefore next investigated the mechanism for FSH\textsubscript{b} induction, which is regulated differently by GnRH than LH\textsubscript{b}. Truncation analysis of the FSH\textsubscript{b} promoter mapped the effect of kisspeptin to a region between -64 bp and -230 bp, which contains an AP-1 half-site required for GnRH induction of FSH\textsubscript{b} (89). An AP-1 multimer responded robustly to kisspeptin in a cFos-dependent manner, demonstrating that AP-1 is both necessary and sufficient to activate FSH\textsubscript{b} via Kiss1R-mediated signaling.

There was no synergistic interaction between kisspeptin and GnRH to induce the LH\textsubscript{b}, FSH\textsubscript{b}, Egr-1, or cFos promoters, which leads to the possibility that both of these hormones may be activating the same signaling pathway. This is supported by our finding that a PKC inhibitor prevented the induction of these promoters by kisspeptin, which is consistent with previous reports that GnRH also signals through PKC and the fact that the GnRH receptor is also coupled to Gq/11 (93). However, there are many forms of PKC (most of which are expressed in L\textbeta{}T2 cells), thus it is possible that kisspeptin and GnRH are activating different isoforms. PKC signaling has been shown to activate the mitogen activated the protein kinase (MAPK)-dependent ERK1/2 phosphorylation cascade, and ERK1/2 induces both cFos and Egr-1 (94-96). While the LH surge results from the rapid secretion of LH, it is plausible that kisspeptin may augment GnRH to stimulate gonadotropin transcription as a means to replace the LH and FSH that are released. As immediate early genes, cFos and Egr-1 are rapidly activated; this is consistent with their induction by kisspeptin to replenish the gonadotropins that are secreted during the surge, which also occurs over a very short period of time. We therefore propose that in pituitary
gonadotropes, similar to GnRH, Kiss1R activates PKC signaling which leads to both cFos and Egr-1 induction, and ultimately expression of FSHβ and LHβ, respectively.

In addition to transfected LbT2 gonadotropes, we have also shown that kisspeptin induces gonadotropin gene expression in mouse primary pituitary cells. The endogenous Kiss1R in mouse pituitaries was sufficient for this effect, providing physiologically relevant evidence that this occurs in vivo as well as in vitro.

**The -5 kb Kiss1R promoter is not responsive to hormone regulation in vitro**

The -5 kb Kiss1R promoter did not respond to hormone treatment by 17β-estradiol, R1881, GnRH, kisspeptin or co-treatment with GnRH and activin or R1881. This may be partly explained by a currently inadequate characterization of the Kiss1R promoter. It is possible that there are regulatory and hormone-responsive sites not contained within this particular promoter construct, and extensive studies of the entire region in and around the gene must be performed to better predict its mechanisms of regulation. The study of hormonal regulation of Kiss1R in vivo (as described below) will provide a more meaningful understanding of the way in which the Kiss1R gene is regulated.

**Pituitary Kiss1R is up-regulated at the time of the LH surge**

Pituitary Kiss1r has previously been shown to respond to estradiol status in OVX female rats (21), but it is unknown whether pituitary Kiss1R responds to circadian regulation and/or positive feedback by estradiol, as occurs at the time of the LH surge (87). Our finding that pituitary Kiss1R was increased during the LH surge in OVX+E females provides a physiological context for furthering the understanding of its role in the pituitary. Circadian cues and GnRH are ultimately responsible for triggering the LH surge, and because the levels of estradiol were consistent between the two OVX+E groups (the only difference being the time of day at which
the animals were sacrificed), it is likely that estradiol was not solely causing the increased Kiss1r expression in the pituitary. Kiss1r expression in the OVX+E a.m. group was similar to that of the OVX a.m. group, further suggesting a lack of a direct effect of estradiol. However, estradiol is required for the GnRH surge, thus, it is possible that a combination of circadian factors and GnRH are responsible for the increased Kiss1r expression in the p.m. group. The increase in pituitary Kiss1r required both the presence of estradiol and the p.m. time point, suggesting that any influence of circadian regulation in the absence of estradiol was not sufficient. Furthermore, primary pituitary cells treated with GnRH did not show an increase in Kiss1r expression, suggesting that GnRH alone is also not sufficient. We therefore propose that both circadian factors, as well as an estradiol-mediated GnRH surge, are required for the increase in pituitary Kiss1r.

The LH surge in humans, as well as female mice, is a crucial component of the reproductive cycle in that it promotes ovulation. The increase in pituitary Kiss1r coupled with the kisspeptin-mediated gonadotropin induction from our in vitro studies is strong evidence that Kiss1R in the pituitary is involved in the reproductively important LH surge. Overall, increased Kiss1R in the pituitary (likely resulting from circadian and GnRH regulation) may augment the actions of GnRH to stimulate transcription to replenish the gonadotropins released during the surge. The generation of gonadotrope-specific Kiss1R-null mice would allow for more extensive studies of the in vivo role of pituitary Kiss1R.

In conclusion, we have shown for the first time that Kiss1R signaling can regulate the gonadotropins at the transcriptional level both in vivo and in vitro. We have shown that in LβT2 gonadotropes, kisspeptin acts similarly to GnRH, by inducing PKC-dependent expression of LHb and FSHb via Egr-1 and AP-1, respectively. While the integration of various physiological factors that regulate the LH surge cannot be fully recapitulated in vitro, our transcriptional studies do suggest that kisspeptin/Kiss1R may be involved in regulating the increase in gonadotropin
gene expression during this important reproductive stage. To that end, we also report a novel finding that pituitary Kiss1r expression is regulated in accordance with the circadian- and GnRH-mediated LH surge in female mice. The transcriptional mechanisms activated by kisspeptin to influence gonadotropin expression, as well as its own up-regulation during the LH surge in females, shows that Kiss1R has functional roles in the pituitary, which will further our understanding of how the reproductive axis is regulated.

Chapter 2, in part, is currently being prepared for submission for publication of this material. The dissertation author was the primary investigator and author of this material. Hanne Hoffmann and Jason Meadows provided assistance with ovariectomies and tissue preparation. Jason Meadows also assisted with the study design and tissue culture experiments. Djurdjica Coss provided various reagents and advice. Sasha Kauffman provided helpful guidance and assisted with the study design. Pamela Mellon supervised the project and provided advice.
Figure 2.1. Kiss1R is expressed in LβT2 gonadotropes. A) RT-PCR was performed on cDNA from female and male mouse pituitaries, mouse skin biopsies, and LβT2 cells, +/- reverse transcriptase (RT). Mouse pituitaries served as positive controls, and skin served as a negative control. The primers yielded a 195 bp product indicated by the arrow. B) qPCR was performed for Kiss1R on male pituitary, female pituitary, and LβT2 cells (n=3 animals/group and n=3 different passages of cells).
Figure 2.2. Kisspeptin induces LHβ and FSHβ but not GnRHR. LβT2 cells were transiently transfected with a Kiss1R expression vector and the indicated luciferase reporter. Cells were treated for 12 h with vehicle (DMSO for kiss and 0.1% BSA for GnRH), 100 nM kisspeptin, 10 nM GnRH, or co-treatment as indicated and subjected to luciferase assay. Data were normalized to vehicle-treated control. A) The -1.7 kb LHβ, -1 kb FSHβ, and -1.1 kb GnRHR promoters were transfected, cells were treated with vehicle or kisspeptin, and activity was compared between vehicle and kisspeptin. ** p<0.01 by Student’s t-test. B) The -1.7 kb LHβ promoter was transfected into LβT2 cells, which were treated with vehicle, 100 nM kisspeptin, 10 nM GnRH, or co-treatment, and activity was compared across treatment conditions. ** p<0.01 and * p<0.05 by one-way ANOVA. C) The -1 kb FSHβ promoter was transfected into LβT2 cells, which were treated with vehicle, 100 nM kisspeptin, 10 nM GnRH, or co-treatment, and activity was compared across treatment conditions. * p<0.05 by one-way ANOVA.
Figure 2.3. **Kisspeptin induces LHβ via the proximal -50 bp Egr-1 site.** LβT2 cells were transiently transfected with a Kiss1R expression vector and the indicated luciferase reporter. Cells were treated for 12 h with vehicle (DMSO for kisspeptin and 0.1% BSA for GnRH), 100 nM kisspeptin, 10 nM GnRH, or co-treatment as indicated and subjected to luciferase assay. Data were normalized to vehicle-treated control. A) 5' truncations of the LHβ promoter were transfected into LβT2 cells, which were treated with vehicle or 100 nM kisspeptin, and activity was compared between vehicle and kisspeptin treatment. * p<0.05 by Student’s t-test. B) Mutations in either of the two Egr-1 sites (-111 bp or -50 bp) in the proximal region of the LHβ promoter (as indicated by the diagram) were transfected into LβT2 cells, which were treated with vehicle or 100 nM kisspeptin, and activity was compared between vehicle and kisspeptin treatment. * p<0.05 by Student’s t-test. C) An Egr-1 multimer was transfected into LβT2 cells, which were treated with vehicle or 100 nM kisspeptin, and activity was compared between vehicle and kisspeptin treatment. * p<0.05 by Student’s t-test. D) -1 kb of the Egr-1 promoter was transfected into LβT2 cells, which were treated with vehicle, 100 nM kisspeptin, 10 nM GnRH, or co-treatment, and activity was compared across treatment conditions. ** p<0.01 and * p<0.05 by one-way ANOVA.
Figure 2.4. Kisspeptin induced FSHβ via cFos-dependent AP-1 activation. LβT2 cells were transiently transfected with a Kiss1R expression vector and the indicated luciferase reporter. Cells were treated for 12 h with vehicle (DMSO for kisspeptin and 0.1% BSA for GnRH), 100 nM kisspeptin, 10 nM GnRH, or co-treatment as indicated and subjected to luciferase assay. Data were normalized to vehicle-treated control. A) 5' truncations of the FSHβ promoter were transfected into LβT2 cells, which were treated with vehicle or kisspeptin, and activity was compared between vehicle and 100 nM kisspeptin treatment. * p<0.05 by Student’s t-test. B) The wild-type -398 bp FSHβ promoter and the -398 bp promoter containing a mutation in the -72/-69 bp AP-1 half-site were transfected into LβT2 cells, which were treated with vehicle or 100 nM kisspeptin, and activity was compared between kisspeptin treatment for the two promoters and between kisspeptin and vehicle. ** p<0.01 by two-way ANOVA. C) An AP-1 multimer on a luciferase reporter was transfected into LβT2 cells, which were treated with vehicle or 100 nM kisspeptin, and activity was compared between vehicle and kisspeptin treatment. * p<0.05 by Student’s t-test. D) The AP-1 multimer was transfected into LβT2 cells with or without a dominant negative cFos, AFos. Cells were treated with vehicle or 100 nM kisspeptin, and activity was compared for the kisspeptin treatment in the presence or absence of AFos. ** p<0.01 by one-way ANOVA. E) -1 kb of the cFos promoter was transfected into LβT2 cells, which were treated with vehicle, 100 nM kisspeptin, 10 nM GnRH, or co-treatment, and activity was compared across treatment conditions. ** p<0.01 by one-way ANOVA.
Figure 2.5. Kisspeptin induction of LHβ, FSHβ, Egr-1, and cFos was PKC-dependent. LHβ, FSHβ, Egr-1, and cFos induction by Kiss1R was PKC-dependent. LβT2 cells were transiently transfected with a Kiss1R expression vector and the indicated luciferase reporter. The -1.7 kb LHβ (A), -1 kb FSHβ (B), -1 kb Egr-1 (C), and -1 kb cFos (D) promoters were transfected into LβT2 cells, and the cells were treated for 30 min with 5 µM Go 6983 or vehicle followed by a 12 h treatment with DMSO vehicle or 100 nM kisspeptin. Cells were then subjected to luciferase assay and data were normalized to vehicle-treated control. Promoter activity was compared across the treatment conditions. # p<0.05 compared to kisspeptin treatment by two-way ANOVA.
Figure 2.6. Hox and STAT regulatory regions on the cFos promoter are required for kisspeptin induction. LβT2 cells were transiently transfected with a Kiss1R expression vector and the indicated mutations on the Egr-1 promoter (A) and the cFos promoter (B). Cells were treated for 12 h with DMSO vehicle or 100 nM kisspeptin and subjected to luciferase assay. Data were normalized to vehicle-treated control. Kisspeptin induction was compared across all reporters. * p<0.05, ** p<0.01, and *** p<0.001 vs -1 kb cFos by one-way ANOVA.
Figure 2.7. The -5 kb Kiss1R promoter is not regulated by sex steroids, GnRH, kiss, or activin in LβT2 cells. LβT2 cells were transiently transfected with the -5 kb Kiss1R luciferase reporter or the estrogen responsive element (ERE) luciferase reporter where indicated, and the ERα or AR expression vectors were indicated. Cells were treated for 12 h with 100 nM kiss, 6 h with 10 nM GnRH, and 24 h with 10 nM 17β-estradiol, 10 ng/mL activin, and 100 nM R1881 as indicated and subjected to luciferase assay. Data were normalized to vehicle-treated control. A) Cells were transfected with either the -5 kb Kiss1R or the ERE on tk-luc with or without ERα. Cells were treated with vehicle, 17β-estradiol, or a media change (no treatment). Activity was compared to the “no treatment” condition with and without ERα. p<0.05 by one-way ANOVA. B) Cells were transfected with the -5 kb Kiss1R with or without AR. Cells were treated with vehicle or R1881, and activity was compared to control with or without AR. C) Cells were transfected with the -5 kb Kiss1R and treated with vehicle or GnRH. Activity was compared to control. D) Cells were transfected with the -5 kb Kiss1R and treated with vehicle or kisspeptin. Activity was compared to control. E) Cells were transfected with the -5 kb Kiss1R and treated with vehicle, activin, GnRH or co-treatment. Activity was compared to control. F) Cells were transfected with the -5 kb Kiss1R with or without AR and treated with vehicle, R1881, GnRH, or co-treatment. Activity was compared to control with or without AR.
Figure 2.8. Kisspeptin induction of gonadotrope genes and regulation of pituitary Kiss1R expression in vivo. Primary pituitary cells from male mice (10 animals/group) were harvested and treated for 6 h with 100 nM kisspeptin or vehicle. qPCR was performed for LHβ (A) and FSHβ (B). Data are normalized to TBP. * p<0.05 and ** p<0.01 compared to control by Student’s t-test. C) Pituitaries were harvested from OVX and OVX+E females for both a.m. and p.m groups (4-9 animals/group). qPCR was performed for Kiss1R, and data are normalized to GAPDH. *** p<0.001 by two-way ANOVA.
Figure 2.9. Hypothalamic Kiss1R expression is up-regulated during the LH surge in females.
A) Hypothalami were harvested from OVX+E females for both negative and positive estradiol feedback. Kiss1R and GAPDH were measured, and data are shown as Kiss1R/GAPDH. The negative and positive feedback groups were compared. * p=0.05 by Student’s t-test.
B) Hypothalami were harvested from all CX groups of males (blank, DHT, and T). Kiss1R and GAPDH were measured, and data are shown as Kiss1R/GAPDH. The blank, DHT, and T groups were compared.
**Table 2.1. Oligonucleotide sequences used for PCR, qPCR and mutagenesis.** Only forward sequences are listed for mutagenesis primers, which are indicated by “mut”.

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CHAPTER 3: Mechanisms of androgen-mediated gonadotropin-releasing hormone receptor regulation

Abstract

Feedback by sex steroids such as androgens is a critical component of the hypothalamic-pituitary-gonadal axis. Androgen feedback in the pituitary gonadotropes differentially regulates transcription of the beta gonadotropin genes, but its effects on gonadotropin-releasing hormone receptor (GnRHR) expression have not been extensively studied. GnRHR plays an important role in reproduction in that it mediates GnRH signaling in the gonadotropes, regulating gonadotropin expression. In this study, we present two approaches to understanding the effect of androgens and androgen receptor (AR) on GnRHR. First, we show that the synthetic androgen R1881 increases transcription of GnRHR through two hormone-response element (HRE) half-sites on the GnRHR promoter. This induction requires the DNA-binding and ligand-binding domains of AR. Second, we describe a novel pituitary-specific AR-null mouse termed AR/αGSU-iCRE. We are evaluating pubertal onset, GnRHR gene expression in the pituitary, blood hormone levels, and fertility in male AR/αGSU-iCRE mice. The results presented herein will further our understanding of the role of AR in regulating reproductive function through its effects on GnRHR.

Introduction

A fundamental component of the reproductive neuroendocrine axis is the sex steroid feedback loop, which modulates both the hypothalamic and pituitary control of reproductive function. Sex steroid feedback influences many aspects of reproduction, including puberty, ovulation, and overall fertility. In the hypothalamus, this feedback occurs primarily on the kisspeptin neurons (46, 76) and to a lesser extent the gonadotropin-releasing hormone (GnRH) neurons (25). However, sex steroids such as androgens can also directly act on the pituitary, as
has been demonstrated by *in vivo* studies in castrated male rats as well as studies in primary pituitary cultures (97, 98).

Androgens act through the androgen receptor (AR), a member of the nuclear receptor superfamily. Ligand-bound AR exhibits classical signaling, whereby it homodimerizes and regulates gene expression through direct DNA binding, but can also act indirectly through the recruitment of other cofactors (32). AR is closely related to the glucocorticoid receptor (GR) and progesterone receptor (PR), all of which recognize response elements containing TGTTC[TG] half-sites organized as 15-bp inverted repeats with 3-bp spacers (32). Three domains are contained within AR that are important for its function: an amino-terminal domain, a DNA-binding domain (DBD), and a ligand-binding domain (LBD), the latter two of which are connected by a hinge region (99).

AR is critical for many aspects of male reproductive function such as sexual differentiation, secondary sex characteristics, and spermatogenesis (25, 27). The loss of AR results in testicular feminization in male mice, and androgen-insensitivity syndrome in humans, both of which produce males that are phenotypically female (26). AR also plays a role in female reproductive function, as evidenced by subfertility in female AR-null mice (26). These AR-null females have defects in estrous cyclicity, ovarian morphology, and progesterone production (27). Even though AR is important for both male and female reproduction (its absence producing a profound reproductive phenotype in males), its molecular mechanisms of action are not fully understood.

The LβT2 immortalized gonadotrope cell line is a useful model system with which to study the effects of AR *in vitro*. LβT2 cells endogenously express the specific gonadotropin subunits LHβ and FSHβ, the common αGSU subunit, and importantly for this study, GnRH receptor (GnRHR). These cells also express AR (100). Previous studies have shown that androgens can differentially regulate LHβ and FSHβ in LβT2 cells (32), but less is known about
the effects of AR on GnRHR. GnRHR is highly important in regulating the reproductive axis in that it mediates the effect of GnRH on gonadotropin production and release (101). In LβT2 cells, the GnRHR promoter is positively regulated by dihydrotestosterone (DHT; an androgen with a high affinity for AR), suggesting that AR can indirectly regulate the response of the gonadotropins to GnRH (78). The mechanism by which AR regulates GnRHR, however, has not been extensively investigated. In this study, we sought to address two main objectives: 1) map and characterize the stimulatory effect of AR on GnRHR expression, and 2) determine the physiological role of pituitary AR with the generation and study of a novel gonadotrope-specific AR-knockout mouse.

Materials and Methods

Plasmids and cloning

The following expression plasmids were used: human AR in a pSG5 expression vector (hAR) (99), constitutively active AR (mut AR CA) (99), and AR containing a mutation in the DNA-binding domain (mut AR DBD) (102). The pGL3-MMTV reporter plasmid was obtained from Ligand Pharmaceuticals (La Jolla, CA). The -1.1 kb GnRHR reporter plasmid has been previously described (78). The -600 bp GnRHR reporter plasmid was provided by Dr. Huimin Xie. The truncated GnRHR promoter constructs of -838 bp, -738 bp, and -413 bp, as well as mutations in two hormone response element (HRE) sites at -499 bp and -159 bp were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All primer sequences are provided in Table 1.

Cell culture and transient transfections
All transient transfections were performed in the LβT2 gonadotrope cell line, using the methods described in Chapter 2. To ensure adequate and consistent AR expression, human AR (or its empty vector, pSG5) was transfected in all experiments as indicated. For the dose response experiments, cells were treated 24 hr after transfection with the synthetic androgen R1881 (methyltrienolone; NEN Life Sciences, Boston, MA), testosterone (T; 17β-Hydroxy-3-oxo-4-androstene, Sigma), or dihydrotestosterone (DHT; 5α-androstan-17β-ol-3-one, Sigma) at concentrations ranging from 10^{-5}M to 10^{-9}M as indicated. Ethanol was used as vehicle treatment for all hormones. All subsequent transfections used 100 nM R1881 or ethanol vehicle. Cells were lysed 24 hr after treatment and assayed for luciferase and β-galactosidase activity as previously described (25).

**Electrophoretic Mobility Shift Assays (EMSA)**

Full-length, human AR was over-expressed in Sf9 insect cells via a baculovirus system, and whole cell extracts were prepared as previously described (32). The whole cell extracts were incubated with 1 fmol of ^{32}P-labeled oligonucleotide at 4 C for 30 min in a DNA-binding buffer [10 mM HEPES (pH 7.8), 50 mM KCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 1 mM dithiothreitol, 2 µg polydeoxyinosinic deoxycytidylic acid, and 10% glycerol]. The oligonucleotides were end-labeled with T4 DNA polymerase and [γ^{32}P]ATP. After 30 min, the DNA binding reactions were run on a 5% polyacrylamide gel (30:1 acrylamide-bisacrylamide) containing 2.5% glycerol in a 0.5× Tris-acetate-EDTA buffer. A rabbit polyclonal AR antibody (Santa Cruz) was used to supershift AR. Mouse IgG was used as a control for nonspecific binding, and a 100-fold excess of the relevant oligonucleotide was used for competition. The oligonucleotide sequences are listed in Table 1.
Animals

All animal procedures were performed in accordance with the UCSD Institutional Animal Care and Use Committee regulations. All mice were on a C57BL/6J background and were group-housed on a 12-h light:12-h dark cycle with ad libitum chow (11% of calories fat, 17% of calories protein) and water. To generate mice lacking AR specifically in pituitary gonadotropes, homozygous AR “flox” females (in which the second exon of the AR gene is flanked by loxP sites) (26) were bred to male αGSU-iCRE mice (obtained from Dr. Sally Camper, University of Michigan). The male offspring, termed AR/αGSU, were studied and compared to littermate AR flox males without αGSU-iCRE.

Pubertal assessment

AR/αGSU males and AR flox controls were monitored for preputial separation as previously described (103). Animals were weaned on postnatal day (PND) 21, and were checked for preputial separation as a measure of pubertal onset beginning on PND 28. Separation was recorded as the day on which the foreskin could be separated from the penile gland by gentle manual pressure on the abdomen.

Fertility assessment

AR/αGSU males and AR flox controls were monitored for their ability to produce a copulatory plug when paired with a wild-type female as previously described (104). Two month-old males were mated continuously for 10 d with age-matched females, and the females were checked daily for the presence of a plug. Matings were separated after a plug was observed.
Statistical analysis

All transient transfections were repeated independently at least three times. Data were normalized for luciferase activity relative to β-galactosidase to control for transfection efficiency. Data were also normalized to the pGL3 plasmid, as well as to vehicle control for all hormone treatments. Results are presented as mean±SEM. Student’s t-tests, one-way ANOVA followed by Tukey post-hoc tests, and two-way ANOVA were used as indicated, and p<0.05 was considered statistically significant.

Results

Mapping the GnRHR induction by AR

We initially sought to map the GnRHR induction by AR with a truncation analysis with GnRHR promoter constructs of 1.1 kb, 838 bp, 738 bp, 600 bp, and 413 bp in length. Cells were treated with 100 nM R1881, as described in a previous study in LβT2 cells (32). We found significant GnRHR induction by R1881 in the presence of AR only in the -738 bp and -600 bp promoter constructs (p<0.05 and p<0.01, respectively, Fig. 1). None of the promoter constructs were induced by R1881 in the absence of AR, indicating that the endogenous AR in the LβT2 cells is not sufficient to mediate this effect. The -1.1 kb and -838 bp promoters were not significantly induced, the former being in disagreement with the previous study of GnRHR induction by androgens, although that study used the more rapidly metabolized DHT, not the more stable, synthetic R1881 (78). The -413 bp promoter was also not induced by AR, suggesting that the induction occurred between -600/-413, with the possible presence of a repressive element upstream of -738 bp. Furthermore, the induction of the -600 bp GnRHR was significantly higher
than the -1.1 kb promoter in the presence of R1881 and AR (p<0.01, Figure 1). We therefore chose to use the -600 bp GnRHR promoter construct for all subsequent experiments.

Even though we found a significant effect of R1881 on the -600 bp promoter, the use of DHT or T in our transfections would add physiological relevance and would better represent the feedback by androgens on pituitary gonadotropes *in vivo*. We therefore conducted an extensive evaluation of the effects of R1881, DHT, and T at doses ranging from $10^{-5}$M to $10^{-9}$M. The results of treatment with R1881, DHT, and T are shown in Fig. 2A, B, and C, respectively. In addition, we treated wells containing no AR (instead its empty vector) with the highest concentration ($10^{-5}$M) of each hormone to further determine whether exogenous AR is necessary to achieve GnRHR induction in LβT2 cells. We found that for R1881, the highest and lowest treatment concentrations did not induce the -600 bp promoter, while the $10^{-6}$, $10^{-7}$, and $10^{-8}$M did cause significant induction (p<0.05, p<0.01, p<0.05, respectively, Fig. 2A). All doses of the DHT treatment significantly induced the -600 bp GnRHR promoter (Fig. 2B). For T treatment, none of the concentrations resulted in significant -600 bp GnRHR promoter induction (Fig. 2C). This discrepancy between the T and DHT treatments may be due to the higher affinity of DHT for AR, and possibly points towards an opposing effect of estradiol, a metabolite of T. While both DHT and R1881 induced GnRHR, we chose to use 100 nM R1881 for all subsequent transfections because its effectiveness in LβT2 cells has previously been described, as well as its greater metabolic stability compared to DHT (25, 32, 105).

**AR induction of GnRHR maps to HRE sites at -499 bp and -159 bp**

As shown in Figure 1, the induction of GnRHR maps to a region downstream of -600 bp. This region contains an HRE half-site (AGAACA) at -499 bp. Further examination of the proximal region of the GnRHR also revealed a second HRE half-site (TGTTCT) at -159 bp. We transfected with mutations in either of these HRE’s, as well as a double mutation all within
the -600 bp promoter. The wild-type GnRHR promoter was significantly induced by R1881 in the presence of AR as expected (p<0.01), but none of the -499 mutant, the -159 mutant, and the double -499/-159 mutant were induced, (Fig. 3A-C). Deletion of the -499 bp HRE also abolished the AR-mediated induction (data not shown). Interestingly, in the case of the double -499/-159 mutant, R1881 treatment in the presence of AR caused a significant decrease in promoter activity compared to the absence of AR (p<0.05, Fig. 3C). These results suggest that both -499 and -159 HRE sites are required for AR-mediated GnRHR induction, and that AR regulation of GnRHR may be necessary to maintain basal promoter activity.

Both the DNA-binding domain and the ligand-binding domain are required for AR induction of GnRHR

Having determined above that both of the HRE sites on the GnRHR promoter are necessary for AR-mediated induction, we then sought to test whether the DNA-binding domain (DBD) of AR is also required for this effect. We transfected with the GnRHR promoter and either empty vector, wild-type AR, or AR µDBD. We found that R1881 treatment only induced the GnRHR promoter in the presence of wild-type AR compared to vehicle, and not in the absence of AR or in the presence of AR µDBD (p<0.05, Fig. 4A). After determining that the DBD is necessary for AR induction of GnRHR, we next asked whether the ligand-binding domain (LBD) was also necessary. A mutation in the hinge region of AR rendered it constitutively active (99), which we confirmed by co-transfecting AR µCA with the androgen-responsive mouse mammary tumor virus (MMTV) promoter. In the presence of wild-type AR, the MMTV promoter was induced by R1881 compared to vehicle (p<0.05, Fig. 4B). By contrast, in the presence of AR µCA, the MMTV promoter was induced regardless of whether the cells were treated with vehicle or R1881 (Fig. 4B), thus confirming the constitutive activity of AR µCA. We then co-transfected
AR µCA with the GnRHR promoter and found no effect of R1881 (Fig. 4C), suggesting that an intact LBD is necessary for AR induction of the GnRHR promoter.

**AR binds the -159 bp HRE site**

Electrophoretic mobility shift assays (EMSA) were used to determine whether AR directly binds the HRE sites at -159 bp and -499 bp on the GnRHR promoter. For both the consensus HRE and the -159 bp HRE, the complex was supershifted by the AR antibody (Fig. 5, lanes 3 and 7) but not by IgG (Fig. 5, lanes 2 and 6). Both complexes showed self-competition (Fig. 5, lanes 4 and 8), and the -159 bp HRE also showed competition by the consensus HRE (Fig. 5, lane 9). The -499 bp HRE site has a lower affinity for binding AR, and thus no complexes were observed (data not shown).

**Pubertal onset in AR/αGSU-iCRE**

We have initiated experiments to monitor the onset of puberty in male AR/αGSU-iCRE mice by determining the date of preputial separation. Preliminary data indicate no significant differences in pubertal onset between AR flox controls and AR/αGSU-iCRE males (Fig. 6). Further studies are needed to confirm whether pituitary AR is necessary for normal pubertal onset in male mice.

**Fertility assessment in AR/αGSU-iCRE**

Preliminary results from a 10 d mating assessment in male AR flox controls and AR/αGSU-iCRE mice shows that 100% of the females paired with a control male had evidence of a copulatory plug by day 7, whereas this was seen in only 67% of the females paired with an αGSU-iCRE male (Fig. 7).
Discussion and Future Directions

AR is critical for male reproduction, and also plays an important role in female reproduction. The mechanisms by which AR regulates reproductive function at the level of the pituitary gonadotrope are not fully understood. Ligand-bound AR can induce the GnRHR promoter in LβT2 gonadotropes, but the mechanisms by which this occurs have not been characterized. The effect of AR on GnRHR in vivo has also not been investigated. We aimed to describe the molecular mechanism of AR induction of GnRHR in vitro, as well as to characterize the reproductive phenotype of a novel pituitary-specific AR-null mouse termed AR/αGSU-iCRE.

Two HRE half-sites are required for AR induction of GnRHR

A 5' truncation analysis of AR induction of the GnRHR promoter revealed the strongest induction at -600 bp, which was diminished upstream of -738 bp. This suggests a possible repressive element in this upstream region, which may warrant further investigation. While Spady et al. (2004) found a significant increase in the -1.1 kb promoter activity and we did not, their experiments were performed by treating the cells with DHT instead of R1881; these compounds differ in their metabolic stability as well as physiological relevance, thus direct comparisons must be considered with caution (78). We have consistently found a reliable and significant effect of ligand-bound AR on the -600 bp GnRHR promoter, which we chose to use as a positive control in all subsequent experiments.

The truncation analysis also showed a loss of the AR-mediated induction at -413 bp, suggesting a required element between -600 bp and -413 bp. This region contains an HRE half-site at -499 bp, the mutation of which abolished the induction. A second HRE half-site at -159 bp was also found to be required for the induction by AR. Not only were these two individual sites
necessary for AR induction of GnRHR, but a double mutation caused significantly lower promoter activity in the presence of ligand-bound AR compared to the absence of AR. This suggests that AR may also be involved in maintaining basal promoter activity.

Ongoing and future studies will address whether the two HRE sites are sufficient to mediate AR induction of GnRHR, and whether AR directly binds the GnRHR promoter. An EMSA suggested that AR is directly binding the -159 bp HRE, while the -499 bp HRE had a lower affinity for AR and thus did not reveal a complex. HRE multimers on a heterologous promoter will also be used to investigate these questions.

**High-affinity AR ligands are necessary to induce GnRHR**

While the synthetic R1881 is highly stable and has been previously used in similar *in vitro* experiments (25, 32), we sought to demonstrate the physiological relevance of our study of AR-mediated GnRHR induction by additionally treating the LβT2 cells with DHT and T. DHT induced GnRHR at a range of doses, while there was no significant effect of T on GnRHR promoter activity. DHT (a metabolite of T) has a ten-fold higher affinity for AR than T itself, thus its effects on GnRHR were not surprising (106). Even though T is an androgen, it can be aromatized to estradiol, which acts through ERα and ERβ, not AR. While ligand-bound AR has a stimulatory effect on GnRHR as evidenced by our dose-response study with R1881 and DHT, the finding that T did not induce GnRHR suggests that estradiol may be playing an opposing role to androgens. We therefore propose two possible explanations for why the effect of T on GnRHR is much weaker than R1881 or DHT: the latter two have a much higher affinity for AR resulting in a more pronounced effect, or the T is converted to estradiol which may have an inhibitory effect on GnRHR promoter activity. Future studies may investigate this possibility by treating LβT2 cells with R1881, estradiol, or both in the presence of the appropriate steroid receptor. DHT mimicked the effect of R1881 on GnRHR activation, suggesting that R1881 is acting in a physiological
manner. Given that R1881 is more stable than DHT and has been described in many other publications using similar experimental approaches, we chose to use R1881 for all experiments.

One caveat to our findings is that the co-transfection of exogenous AR is necessary to achieve the induction of GnRHR by R1881. The LβT2 cells have previously been shown to express endogenous AR, and we thus included no-AR controls in all experiments. However, GnRHR was only induced in the presence of AR. Even high doses of androgen treatment (10^{-5}M DHT and R1881) did not stimulate GnRHR activity in the absence of AR, suggesting that the endogenous AR in the LβT2 cells is not sufficient to mediate the effects of the ligand. This is consistent with other studies of the effects of androgens in LβT2 cells, however, and the necessity to co-transfect exogenous AR should not detract from the relevance of our findings.

**GnRHR induction requires the DNA- and ligand-binding domain of AR**

As a member of the nuclear receptor family, AR is capable of regulating gene transcription through classical (direct DNA binding) or nonclassical (interaction with other cofactors) signaling (78). The loss of AR-mediated induction of GnRHR when the -499 and -159 HRE half-sites were mutated showed that AR may be binding directly to the GnRHR promoter at these sites to regulate activity. We therefore tested a mutation within the DNA-binding domain (DBD) of AR, and found a complete loss of the induction of GnRHR with R1881 treatment. This is strong evidence of classical AR regulation of the GnRHR promoter. As AR never independently induced GnRHR in the absence of ligand, we sought to confirm that an intact ligand-binding domain (LBD) was also necessary for the induction. An AR construct lacking the LBD did not induce GnRHR, demonstrating the requirement of the LBD for this effect. Of note, the deletion of the LBD conferred constitutive activity in the mutated AR protein via a conformation change, which we confirmed by its induction of the androgen-responsive MMTV
promoter, regardless of the presence or absence of the R1881 ligand. In summary, ligand-bound AR with an intact DBD is necessary to activate GnRHR.

Androgen signaling through AR is inhibitory with regard to the hypothalamic control of reproduction, but is stimulatory at the level of the pituitary gonadotrope for GnRHR (as well as FSH). The physiological relevance of our findings must be considered in the context of GnRH status. As GnRHR mediates GnRH signaling, which ultimately regulates gonadotropin expression, the up-regulation of GnRHR by androgen feedback may confer enhanced sensitivity to GnRH in the gonadotropes. In women, androgen levels rise starting from menstruation, peaking at the time of ovulation, and coinciding with a sharp rise in GnRH that triggers the LH surge (107). It is plausible that the increase in androgens preceding ovulation may increase GnRHR expression in the gonadotropes, thereby rendering these cells more responsive to GnRH, and ultimately promoting the LH surge.

**Generation and characterization of a gonadotrope-specific AR-null mouse**

While *in vitro* studies of the mechanisms of AR action in pituitary gonadotropes with regard to GnRHR are useful and informative, they cannot fully reflect the physiological complexity of an animal model. To further understand the role of AR in gonadotropes *in vivo*, we have generated a gonadotrope-specific AR-null mouse using the cre-loxP approach. We bred αGSU-iCRE animals to animals expressing “floxed” AR, resulting in AR/αGSU-iCRE offspring. While full-body and brain-restricted AR-null mice have been previously described (26, 108), to our knowledge a gonadotrope-specific AR-null mouse has not yet been developed. A caveat of using the αGSU promoter to drive cre expression is that αGSU is also expressed in the thyrotrope cells of the anterior pituitary, which will presumably result in an additional excision of functional AR in thyrotropes as well. We do not anticipate profound or direct effects of thyrotropin-
stimulating hormone on reproductive function, but the lack of AR in the thyrotrope cells must be
considered when interpreting results.

We aim to investigate many reproductive parameters in male αGSU-iCRE mice, including pubertal onset, blood hormone levels, pituitary gene expression, and fertility. We have initiated studies of pubertal onset, as determined by the date of preputial separation. No differences in pubertal onset have been detected as of yet, but increasing the sample size will provide stronger evidence of whether pubertal onset in males is dependent on pituitary AR.

Preliminary results assessing fertility using a copulatory plug paradigm suggest that the iCRE males may have a fertility defect, as they plugged a lower percentage of females compared to AR flox controls. These animals may subsequently be subjected to a longer and more extensive fertility assessment, where the number and frequency of litters would be determined over the course of six months. Male sexual behavior may also be assessed as an alternative to a prolonged fertility assessment. Young adult males will also be sacrificed, and serum levels of LH and T will be measured via RIA (University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core). At the time of sacrifice, pituitaries will be harvested and cDNA will be prepared. Quantitative PCR will be performed for such genes as AR, GnRHR, LHβ, and FSHβ comparing AR/αGSU-iCRE with AR flox controls. We will also quantify sperm content and perform morphological analyses of testicular structures. This study may also be extended to females, but because AR is X-linked, the generation of homozygous flox AR/αGSU-iCRE females requires a second breeding step. Overall, an extensive study of the reproductive function of the AR/αGSU-iCRE animals will be the first to investigate the role of AR in the pituitary in regulating the reproductive axis.

In conclusion, we have shown that AR induces GnRHR promoter activity in LβT2 cells through two HRE half-sites. In this way, AR exhibits a classical mechanism of action to stimulate GnRHR, requiring both the DBD and LBD of the AR protein. This adds to our knowledge of the
way in which AR regulates gonadotrope-specific genes on the transcriptional level. We have also introduced a novel pituitary-restricted AR-null mouse termed AR/αGSU-iCRE, the characterization of which will allow us to further understand the role of pituitary AR in driving reproductive function.

Chapter 3, in part, is currently being prepared for submission for publication of this material. The dissertation author was the author of this material and is the co-primary investigator with Shadi Shojaei. Pamela Mellon supervised the project and provided advice.
Figure 3.1. AR induction of GnRHR maps downstream of -600 bp. LβT2 cells were transiently transfected with an AR expression vector, empty vector control, and the indicated luciferase reporter. Cells were treated for 24 h with vehicle or 100 nM R1881 and were subjected to luciferase assay. Data were normalized to vehicle-treated control. 5’ truncations of the GnRHR promoter were transfected, and luciferase activity was compared to vehicle control without AR. * p<0.05 by one-way ANOVA.
Figure 3.2. GnRHR responds more strongly to R1881 and DHT than T. LβT2 cells were transiently transfected with an AR expression vector, empty vector control, and the -600 bp GnRHR. Cells were treated for 24 h with vehicle or the indicated concentration of R1881 (A), DHT (B), and T (C) and were subjected to luciferase assay. Data were normalized to vehicle-treated control. Luciferase activity was compared between treatment and vehicle. * p<0.01 by Student’s t-test.
Figure 3.3. HRE half-sites at -159 bp and -499 bp are required for AR induction of GnRHR. LβT2 cells were transiently transfected with an AR expression vector, empty vector control, the -600 bp GnRHR promoter, and the indicated HRE mutation. Cells were treated for 24 h with vehicle or 100 nM R1881 and were subjected to luciferase assay. Data were normalized to vehicle-treated control without AR. A) A mutation in the -159 bp HRE half-site in the GnRHR promoter was transfected along with the -600 bp GnRHR promoter, cells were treated with vehicle or R1881, and luciferase activity was compared between treatment and vehicle control without AR. * p<0.05 by one-way ANOVA. B) A mutation in the -499 bp HRE half-site in the GnRHR promoter was transfected along with the -600 bp GnRHR promoter, cells were treated with vehicle or R1881, and luciferase activity was compared between treatment and vehicle control without AR. * p<0.05 by one-way ANOVA. C) A double mutation in the -159 bp and -499 bp HRE half-sites in the GnRHR promoter was transfected along with the -600 bp GnRHR promoter, cells were treated with vehicle or R1881, and luciferase activity was compared between treatment and vehicle control without AR. * p<0.05 by one-way ANOVA.
Figure 3.4. The DNA-binding domain and the ligand-binding domain are required for AR induction of GnRHR. LβT2 cells were transiently transfected with the -600 bp GnRHR promoter, the MMTV reporter, empty vector, wild-type AR, AR with a mutation in the DNA-binding domain (mut AR DBD), or AR with a mutation in the ligand-binding domain (mut AR CA) as indicated. Cells were treated for 24 h with vehicle or 100 nM R1881 and were subjected to luciferase assay. A) The -600 bp GnRHR promoter was transfected with empty vector, AR, or mut AR DBD and cells were treated with either vehicle or R1881. Luciferase activity was compared between vehicle and R1881 for each expression vector. * p<0.05 by Student’s t-test. B) The MMTV promoter was transfected with empty vector, AR, or mut AR CA and cells were treated with either vehicle or R1881. Luciferase activity was normalized to β-galactosidase, and compared between vehicle and R1881. * p<0.05 by Student’s t-test. C) The 600 bp GnRHR promoter was transfected with empty vector, AR, or mut AR CA and cells were treated with either vehicle or R1881. Data were normalized to vehicle control without AR. * p<0.01 by two-way ANOVA.
Figure 3.5. AR binds the -159 bp HRE. Whole cell extracts containing over-expressed AR from baculovirus-infected cells were incubated with either the consensus HRE or the -159 probe and tested for complex formation in EMSA. The relevant AR-DNA complexes are shown in lanes 1 and 5, whereas the AR antibody supershifts are shown in lanes 3 and 7. IgG control is shown in lanes 2 and 6, self competition is shown in lanes 4 and 8, and competition of the -159 with the consensus HRE is shown in lane 9.
Figure 3.6. Pubertal onset in AR/αGSU-iCRE. The postnatal day (PND) of preputial separation was monitored in AR/αGSU-iCRE males and AR flox controls. N=3 AR/αGSU-iCRE and 6 AR flox. These preliminary results have not reached statistical significance.
Figure 3.7. Fertility assessment in AR/αGSU-iCRE. Two month-old αGSU-iCRE and AR Flox controls were mated continuously for 10 d with wild-type females. The percentage of females displaying a copulatory plug by each day of the assessment is shown. 100% of females mated to control males were plugged by the end of the study, whereas only 67% of females mated to iCRE males had plugged. N=3 AR/αGSU-iCRE and 4 AR Flox.
Table 3.2. Oligonucleotide sequences for EMSA and mutagenesis of the GnRHR promoter. Only forward sequences are listed for mutagenesis primers.

<table>
<thead>
<tr>
<th>Sequence Type</th>
<th>Sequence</th>
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<tr>
<td>-838 bp GnRHR</td>
<td>GTGCCAGAACAATTTCTCTATCGATAATGAAGTCATACC AATATTG</td>
</tr>
<tr>
<td>-738 bp GnRHR</td>
<td>GTGCCAGAACAATTTCTCTATCGATAAGGATTATAAATTA TAAATTAG</td>
</tr>
<tr>
<td>-413 bp GnRHR</td>
<td>GTTGGAGAGACATAACTTCTCAATATCGATAGAGAAATGT TCTGGCAC</td>
</tr>
<tr>
<td>-499 bp HRE mutation</td>
<td>TAGATAAATTGGTATAATAATAGGCTGCTTAAAAACAGT AAAGTACTAGC</td>
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<tr>
<td>-159 bp HRE mutation</td>
<td>CTTCAAGGAGGCGTCATATTATGTTAGCACTCTTTTA GA</td>
</tr>
<tr>
<td>EMSA; consensus HRE</td>
<td>ACGGGTTGGAACGCCTGTCTTTTTGCGACG</td>
</tr>
<tr>
<td>EMSA; -159 HRE</td>
<td>GAGGGCTTGGCAGTCTCTGGTTCCTGACTCTTT</td>
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CONCLUSION

The research described herein has investigated the following questions: the effect of early androgen exposure on female reproduction, the transcriptional role of Kiss1R in pituitary gonadotropes, and mechanisms of AR-mediated GnRHR regulation. These three studies, while being distinct from one another, all have added to the understanding of the mechanisms by which the neuroendocrine reproductive axis functions at different levels, under either physiological or pathological conditions. The research contained in this dissertation provides an in-depth characterization of the reproductive phenotype of female mice exposed to androgens during critical periods of development, describes novel transcriptional and functional roles for Kiss1R in pituitary gonadotropes, and explores the role of pituitary AR in GnRHR regulation and in male reproductive function in vivo.

We have conducted an extensive study of the previously described PNA mouse model, investigating the effects of PNA on puberty, cycling, fertility, and reproductive aging. We have also introduced a novel mouse model of premature puberty resulting from DHT exposure during the prepubertal time frame (PPA). Comparisons between the PNA and PPA models indicated that PNA treatment has organizational and developmental effects, leading to alterations in the timing of puberty, fertility, and reproductive senescence. PPA, however, acts only transiently to induce early puberty, with no lasting effects on reproductive function. The time frame of DHT exposure in these two models is highly significant: females are sensitive to exogenous androgens at both time frames, but while PNA ultimately influences many aspects of reproduction that are only detectable long after exposure, PPA immediately affects the onset of puberty with no long-term consequences. The possibility of an effect of the DHT metabolite 5α-androstane-3β,17β-diol cannot be discounted when considering the mechanisms of DHT action in the PNA and PPA models. The use of ERβ and AR inhibitors in future studies would confirm that the effects of DHT are indeed specific to AR.
Of note, the early puberty seen in both models occurs independently of reproductively important Kiss1R. Given the importance of the kisspeptin system in the initiation of puberty, this surprising finding strongly suggests that DHT in both PNA and PPA is acting at a different level of the reproductive axis to influence puberty. The newly generated AR/αGSU-iCRE mouse described in Chapter 3 will provide a way to test whether androgen signaling in the pituitary gonadotropes is driving the early puberty in these models. It is also possible that DHT is acting directly at the level of the ovaries to induce puberty onset, without any influence from the hypothalamic or pituitary components of the reproductive axis. A granulosa cell-restricted AR-null mouse would also be useful in finding the source of this phenotype. Besides kisspeptin, GnRH is also critical for puberty. Pilot studies implementing the PNA paradigm in GnRH-null mice (hpg−/−) were inconclusive with regard to puberty onset (data not shown), but a more extensive study of these mice may help determine whether the effects of PNA and PPA on puberty are GnRH-dependent. The same holds true for GnRHR-null mice, which would also yield information on whether GnRH signaling is involved in the early puberty with PNA and PPA.

The importance of Kiss1R in reproduction is well known, but whether it plays a role at the level of the pituitary in reproductive function has not been extensively studied. We have introduced novel findings that Kisspeptin can regulate gene expression of LHβ and FSHβ in LβT2 gonadotropes, and that Kiss1R itself is up-regulated during the LH surge in female mice. Luciferase reporter assays in LβT2 cells demonstrated that Kisspeptin can induce LHβ and FSHβ promoter activity in a PKC-dependent manner. This activation of LHβ and FSHβ occurs through the immediate early factors Egr-1 and AP-1/cFos, respectively. Interestingly, the involvement of the PKC pathway, Egr-1 with regard to LHβ, and AP-1 with regard to FSHβ, suggests that Kiss1R is signaling in a similar manner to GnRH. In further support of this observation, co-treatment with kiss and GnRH did not synergistically induce any of the LHβ, FSHβ, cFos, or Egr-1 promoters, suggesting that the same pathway is involved for both kiss and GnRH signaling. As
GnRH-null mice have profound reproductive defects, it is likely that Kiss1R does not compensate for the lack of GnRH to promote a functional reproductive axis. It is possible that kisspeptin may have a more subtle, complementary effect to the primary actions of GnRH in the regulation of gonadotropin expression.

Determining the molecular mechanisms of the regulation of pituitary Kiss1R provides an almost entirely uninvestigated avenue for future studies. Our preliminary studies of the Kiss1R promoter in LβT2 cells did not reveal any evidence of hormonal regulation, but the promoter is as yet uncharacterized and it is possible that the construct cloned in our lab did not contain all of the necessary regulatory elements. BLAST® analysis shows that our -5 kb Kiss1R promoter construct is indeed contained within the mouse Kiss1R sequence, but whether this contains the entire regulatory region of the promoter (including potential upstream enhancers as exist on the GnRH gene) has not been independently confirmed. A thorough bioinformatic analysis and characterization of the accurate promoter sequence must be undertaken before conclusions can be drawn regarding the way in which Kiss1R is regulated in gonadotropes on the transcriptional level. Until the regulatory sites on the Kiss1R promoter are more defined, the mechanisms of its regulation in the pituitary must be studied in vivo.

Our finding that Kiss1R expression in the pituitary is robustly increased during the LH surge in the evening (as compared to animals sacrificed in the morning, representative of diestrus) is a strong indicator of a functional role of pituitary Kiss1R in vivo. The LH surge is of great physiological importance in females (without which ovulation would not occur), and the observation that Kiss1R is up-regulated at this time is consistent with our studies in LβT2 gonadotropes of Kiss1R-mediated induction of the gonadotropins. Further studies are needed to determine whether this increase in Kiss1R is a direct result of estradiol signaling in the gonadotropes, or whether it is responsive to other regulators, such as GnRH or circadian cues. A sharp increase in GnRH triggers the LH surge, and it is plausible that GnRH may be
simultaneously up-regulating *Kiss1R* in the gonadotropes to facilitate the rise in LH. Ultimately, the generation of a pituitary-specific Kiss1R-null mouse would provide a direct means to understand the full role of Kiss1R in the pituitary.

While the PNA study demonstrated the physiological effects of exogenous androgens, the work described in Chapter 3 investigated the role of AR on the molecular level. While it is known that AR can induce GnRHR promoter activity, the mechanism by which this occurs has not been studied. We have shown that AR activates the GnRHR through two HRE sites in the proximal region of the promoter. Both sites must be intact for the induction to occur, and mutation in both sites resulted in lower activity, suggesting that not only does ligand-bound AR induce GnRHR, but it may also be required to maintain baseline activity. The DNA-binding domain of AR was also necessary for this induction, suggesting that this effect occurs through classical signaling (which may be confirmed with EMSA). A broader physiological explanation for the induction of GnRHR by AR is that in females, androgens rise in conjunction with the LH surge, which may increase GnRHR expression, thus rendering the gonadotropes more responsive to GnRH and facilitating the rise in LH.

We have also described the generation of a novel pituitary-specific AR-null mouse termed AR/αGSU-iCRE. Myriad reproductive parameters will be studied in the male mice, including puberty, blood hormone levels, pituitary gene expression, and fertility. Behavioral studies to quantify stereotypical male sexual behavior may also be performed, depending on the results from the preliminary assessments of fertility. To our knowledge, the studies in these mice will be the first to provide information about the reproductive role of AR in the pituitary *in vivo*. Male sexual behavior in rodents is quite robust, and even male mice lacking neuronal AR can still exhibit all aspects of mating (our unpublished observations). Therefore, if a fertility defect is not found in the male AR/αGSU-iCRE mice, females will also be generated and tested for fertility.
Females are more sensitive than males to factors that may influence fertility, and it is possible that a subtle or partial effect on fertility in these mice would only be revealed in females.
REFERENCES


hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264-272


32. Thackray VG, McGillivray SM, Mellon PL 2006 Androgens, progestins and glucocorticoids induce follicle-stimulating hormone β-subunit gene expression at the level of the gonadotrope. Molecular endocrinology (Baltimore, Md 20:2062-2079


43. Thackray VG, Mellon PL, Coss D 2010 Hormones in synergy: Regulation of the pituitary gonadotropin genes. Molecular and cellular endocrinology 314:192-203

44. Kauffman AS 2010 Coming of age in the kisspeptin era: sex differences, development, and puberty. Molecular and cellular endocrinology 324:51-63


50. Kermath BA, Gore AC 2012 Neuroendocrine control of the transition to reproductive senescence: Lessons learned from the female rodent model. Neuroendocrinology in press


60. Handa RJ, Pak TR, Kudwa AE, Lund TD, Hinds L 2008 An alternate pathway for androgen regulation of brain function: activation of estrogen receptor beta by the
metabolite of dihydrotestosterone, 5alpha-androstane-3beta,17beta-diol. Horm Behav 53:741-752


75. Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ 2008 Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin in vivo. Endocrinology 149:1951-1959

76. Smith JT, Clay CM, Caraty A, Clarke IJ 2007 KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148:1150-1157

77. Smith JT, Li Q, Pereira A, Clarke IJ 2009 Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology 150:5530-5538


84. Quirk CC, Lozada KL, Keri RA, Nilson JH 2001 A single Pitx1 binding site is essential for activity of the LHbeta promoter in transgenic mice. Molecular endocrinology (Baltimore, Md 15:734-746


98. Gharib SD, Leung PC, Carroll RS, Chin WW 1990 Androgens positively regulate follicle-stimulating hormone beta-subunit mRNA levels in rat pituitary cells. Mol Endocrinol 4:1620-1626


