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Androgen Receptor's Role in GnRH Receptor Gene Regulation and Mouse
Fertility

A Thesis submitted in partial satisfaction of the requirements

For the degree Master of Science

in

Biology

by

Shadi Shojaei

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor Nicholas C. Spitzer, Co chair
Professor Michael David

2013

The Thesis of Shadi Shojaei is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2013

DEDICATION

I would like to dedicate this thesis to my corky, wonderful, loving family, Javad Shojaei, Sharareh Bikae, Palmira Yosefi, and Shahriar Shojaei. I'd especially like to recognize my parents Javad and Sharareh for their innumerable sacrifices, strength, and support, without which my achievements and pursuit of happiness would not have been possible. I would also like to dedicate this thesis to my aunt Soheila and uncle Shahram for reaching above and beyond to facilitate my family's move to the United States. Thank you for giving me a shot at the American Dream. Finally, I am dedicating this project to my grandparents and especially to my grandfather Babagol, may his soul rest in peace for all eternity.

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Chapter 1, in part, is currently being prepared for submission of publication. The thesis author was the primary investigator and author of this material. Emily Witham assisted with planning and analysis of transient transfections as well as designing plasmid constructs. Jason Meadows assisted with generating the Hormone Response Element multimer constructs. Varykina

Thackray has provided guidance for the Electrophoretic Mobility Shift Assay technique. Pamela Mellon supervised the project.

Chapter 2, in part, is currently being prepared for submission of publication. The thesis author was the primary investigator and author of this material. Emily Witham and Jason Meadows assisted in designing the AR Flox/ α GSU-iCRE mouse line. Emily Witham assisted in designing and carrying out the puberty and fertility assessments, as well as Quantitative PCR. Jason Meadows assisted in tissue extraction from the AR Flox/ α GSU-iCRE male mice. Pamela Mellon supervised the project.

ABSTRACT OF THE THESIS

Androgen Receptor's Role in GnRH Receptor Gene Regulation and
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by

Shadi Shojaei

Master of Science in Biology

University of California, San Diego, 2013

Professor Pamela L. Mellon, Chair

Gonad-derived sex steroids have been known to play an active role in the regulatory hormone feedback loop within the hypothalamic-pituitary-gonad (HPG)

Axis. At the pituitary level, sex steroids regulate gonadotropin expression through regulation of the gonadotropin-releasing hormone (GnRH) receptor. Androgens in particular have been shown to suppress Luteinizing Hormone (LH) release while increasing Follicle-Stimulating Hormone (FSH) release. However, the mechanism of gonadotropin regulation by androgens remains largely unknown. Androgens are known to affect change in target cells through binding androgen receptor (AR), which has been shown to induce expression of GnRHR in pituitary gonadotropes *in vivo*. AR is expressed in various tissues and affects many reproductive functions. While the physiological role of AR has been partially studied through generation of whole-body and gonad-specific AR knockout (ARKO) mice, the role of pituitary-specific AR has not been addressed. My project therefore has centered on extrapolating a more detailed mechanism of AR action in regulating androgen-induced GnRHR expression, as well as its pituitary-specific role in mouse reproductive physiology. To that end, the immortalized gonadotrope L β T2 cell line has proven an effective context for our transcriptional studies. The generation of a novel pituitary-specific AR knockout mouse line labeled ARF/ α GSUiCRE also served as an instrumental model for the analysis of the physiological impact of pituitary derived AR.

INTRODUCTION

HPG Axis

The Hypothalamic-Pituitary-Gonad (HPG) Axis is the collaborative team of organs responsible for the coordination and execution of mammalian reproduction. The HPG axis consists of the hypothalamic kisspeptin and gonadotropin releasing hormone (GnRH) neurons, the anterior pituitary gonadotropes, and the gonads; testes in males and ovaries in females. At the onset of puberty, the mechanism of which remains unclear, kisspeptin neurons begin signaling the release of Gonadotropin Releasing Hormone (GnRH) from the GnRH neurons (1). GnRH in turn travels through the hypophyseal portal system to the anterior pituitary, stimulating gonadotrope secretion of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). LH and FSH circulate to reach the gonads (testes and ovaries) where they perform their respective reproductive functions, among them being sex steroid production. In males for example, LH and FSH stimulate testosterone secretion and spermatogenesis, while in females they stimulate follicular maturation, ovulation, and secretion of estrogen and progesterone. Sex steroids play diverse roles in reproductive orchestration, feedback regulation being one such. Self-moderation plays an important role in maintaining optimal functioning of the HPG Axis. One type of self-moderation is negative feedback whereby sex steroids travel up to the pituitary to decrease gonadotropin secretion, thereby decreasing the stimulatory

signal for their own production. Sex steroids can also positively feedback to the pituitary by further stimulate gonadotropin production, thereby further stimulating sex steroid production (2). One example of positive feedback within the HPG that is the basis of my project is the androgen induction of FSH.

Anterior Pituitary

The anterior pituitary, also known as adenohypophysis occupies the anterior lobe of the pituitary gland and is composed of cell types according to the trophic hormones they produce: adrenocorticotrophic hormone (ACTH) produced by corticotropes, Thyroid stimulating hormone produced by thyrotropes; Luteinizing hormone (LH) and follicle stimulating hormone (FSH), collectively known as gonadotropins and produced by gonadotropes; Prolactin produced by lactotropes; growth hormone produced by somatotropes. Hormone secretion of each cell type is controlled by hormonal stimulation from the hypothalamus, and can be conducted in either a pulsatile or episodic manner. Each cell type expresses G protein-coupled receptors specific to their hypothalamic regulatory hormone delivered through the portal vasculature. Their responsiveness is modulated by multiple inputs in the form of GnRH, neurotransmitters, melatonin, pituitary hormones, growth factors, and gonadal steroid and peptide hormones (3). The regulatory hypothalamic hormones exert their influence through transcriptional activation leading to hormone synthesis, as well as stimulation of cell surface receptors to induce release of the synthesized hormones (4).

Androgens

Androgens are the male sex steroids derived from cholesterol in the Leydig cells of the testes upon stimulation by LH. Cholesterol is initially converted to Pregnenolone, which is converted to Progesterone and Dehydroepiandrosterone, both of which are converted to Androstenedione, which is then converted to Testosterone. Testosterone is then either converted to Estradiol by Aromatase, or to Dihydrotestosterone (DHT) via 5 α -Reductase (5). DHT can further be metabolized into Diols, implying that some of Testosterone's actions are through DHT metabolites. Interestingly, some DHT metabolites have been reported to preferentially bind Estrogen Receptor (ER) (5).

During development Testosterone promotes differentiation of the wolffian duct into male internal genitalia (Epididymes, Vas Deferens, and Seminal Vesicles). In humans, starting at 8 weeks of gestation, DHT guides the differentiation of male internal and external genitalia (Penis, Scrotum, and Prostate). Testosterone is then responsible for continued growth of the penis in later stages of development (6). After birth androgen levels decrease and for the most part remain low, until puberty. With the onset of puberty, LH secretion stimulates Leydig cell production of Testosterone, which then stimulates the FSH-aided Sertoli cell Spermatogenesis in the testes (7).

In females also, testosterone level rises with the onset of puberty, though substantially lower than that of males. In females testosterone is produced in the ovaries, and while it mainly serves as a precursor to Estrogen, it itself plays physiologically important roles. While those roles remain unclear, androgens do

certainly play a role as evidenced by the presence of AR in many female tissues. Androgen therapy in sexually dysfunctional women showed increases in both sexual function and mood (8).

While much of testosterone's actions within the HPG axis are executed through its aromatization to Estradiol, testosterone also influences sexual behaviors and hormonal regulation both by itself (9) and through metabolism to DHT. In fact, one of its non-aromatizable effects has been shown to increase AR levels in gonadotropes in order to increase DHT binding to AR (10).

Aside from their direct effects on metabolism, gender differentiation, and reproduction, androgens also instigate their autoregulation mechanism. Their feedback to the hypothalamus and the pituitary gonadotropes has been shown to differentially regulate gonadotropin levels, increasing FSH while decreasing LH. This effect was shown to be androgen-specific, as FSH decreased with Estrogen (11).

Androgens exert their effect by diffusing through the plasma membrane and binding Androgen Receptor (AR), which then translocate to the nucleus to regulate gene transcription via binding to Hormone Response Elements (HREs) (12).

Androgen Receptor: Mechanism

AR is a ligand-dependent nuclear receptor, consisting of a N-terminal transactivating domain (NTD), a conserved central DNA binding domain (DBD), and a C-terminal Ligand Binding Domain (LBD). It is expressed in areas that

undergo sexual differentiation, such as the medial amygdala, arcuate nucleus, ventromedial hypothalamus, anteroventral periventricular nucleus, and the bed nucleus of stria terminalis (9). Like other nuclear receptors, it regulates transcription by binding cis-acting positive DNA elements to activate it, binding to cis-acting negative DNA elements to repress it, and transcriptional interference through interaction with other trans-acting elements (13). Upon ligand binding, cytosolic AR is released from its multi-protein complex and heat shock proteins, free to undergo the commonly known N/C interaction.

Ligand binding causes repositioning of helix 12 in the C-terminal ligand-binding domain, resulting in the formation of a hydrophobic pocket/groove. While this groove is open for cofactor binding, in the AR specifically it preferentially interacts with FxxLF motifs. One such motif is the AR NTD FQNLF motif, which upon binding the C-terminal pocket in the commonly known N/C interaction yields an extra layer of stability to the ligand-bound groove. This interaction can be either intermolecular or intramolecular. This N/C interaction occurs mainly while ligand-bound AR is in its mobile phase, before entering into the transient DNA-binding immobility phase. It has been strongly suggested that when not associated to DNA, AR uses its N-terminal FQNLF motif to block its C-terminal groove, and only allow access by coregulators after DNA binding. In this way self-blocking allows AR to transiently regulate the spatiotemporal nature of coregulator interaction (14).

Like all nuclear receptors, AR DBD is arranged into three alpha helices containing two cysteine-rich zinc finger-like motifs, coordination of which

facilitates the structure and function of DBD. One zinc finger contains a P Box characterized by a five amino acid stretch, interacting with the major groove of DNA and facilitating DNA sequence recognition and binding. The second zinc finger contains a D Box also consisting of a five amino acid stretch, and is required for stable receptor-DNA interaction. The D Box contains the major residues involved in AR homodimerization and half-site spacing once in the nucleus (13). AR dimerization through the D-Box transitions intramolecular interactions to intermolecular ones. The AR dimer is then able to stably bind DNA promoter/enhancer sequences at hexameric half-sites of the consensus sequence 5'-TGTTCT-3', arranged as inverted repeats separated by three nucleotides important in maintaining spatial orientation. The first zinc finger of each monomer in the AR dimer binds to one half site, while the second zinc finger of each monomer binds to the other monomer (14).

Androgen dependence of AR is mediated by the sex-limited protein SIp gene. AR response to Androgen involves the DNA-binding factor Oct-1, which interacts strongly with the AR FxxLF motif when both are bound to DNA. AR binding to this gene induces conformational changes in AR-LBD, increasing its interaction with Oct-1. The non-consensus Oct-1 binding site overlaps with a HRE half site (HRE-1), such that binding of Oct-1 to this region induces a conformational change that in turn facilitates the cooperative recruitment of SRC-1 to the transcription-activating complex. It is believed the enhanced AR-Oct-1 interaction is due to conformational changes in the LBD (N/C interaction) imposed by DNA rather than DNA-directed presentation of other proteins. This in

turn enhances recruitment of co-activators such as SRC-1. SRC-1 enhances AR activation by facilitating the N/C interaction. The sequence and arrangement of binding sites in the SIp enhancer promote the selective interaction of Oct-1 that is specific only to AR. AP-1 and NF- κ B also recruit this coactivator. (15)

Androgen Receptor: Physiological Importance

As previously mentioned, AR is expressed in various regions and mediates the transcriptional effects of androgens in those tissues. The significance of its action has been shown through mutagenesis studies.

Androgen Insensitivity Syndrome

In humans, AR gene mutation results in the X-linked Androgen Insensitivity Syndrome (AIS), which can be either complete (CAIS), partial (PAIS), or mild (MAIS). AR mutations that severely affect the amount, structure or function of the receptor lead to CAIS. Genetically male individuals with this syndrome are born with female external genitalia, raised as females, and identify with the female gender in adulthood (16), (17). They are also infertile.

The phenotype of partial AIS however varies widely, ranging from female external genitalia along with clitoromegaly/posterior labial fusion, to normal but small morphological genitalia. Similar to CAIS individuals, all PAIS individuals are also infertile (17).

MAIS is characterized by either infertility and impaired spermatogenesis or normal to sufficient spermatogenesis, in which case mutant AR dysfunction is compensated for by collaboration with coregulators in sertoli cells (17). Variable

expression of the mutations has thus far made it difficult to link any specific ones to infertility. This variable expression has been attributed in some cases to somatic mosaicism (17).

Effects of various AR mutations

Characteristic of the different AR mutations is an expansion of the polyglutamine repeat in the receptor's N-terminal region (18). Mutations occur in both the LBD and DBD, and have different debilitating effects on AR function and phenotype. Since coactivators and corepressors mostly interact with AR LBD, mutations in the LBD may conserve AR's androgen binding ability while compromising its cofactor binding ability. Similarly, mutations in AR DBD can endow AR with greater ability to recognize auxiliary proteins involved in transcription activation while disabling its DNA recognition and binding capacities. Other mutations reduce AR's DNA-binding activity at only one androgen response element (ARE). AREs are a type of hormone response element (HRE), located on the promoter of the regulated gene. The elements serve as binding/action sites for ligand-bound nuclear receptors to affect transcription upon the receptor's translocation to the nucleus. Such mutations fail to interfere with AR interaction with promoters containing multiple ARE's however. This was explained by the demonstration by Liu et al. that such destabilization disrupts binding and activity at a single response element while increasing receptor's synergistic activity on reporter genes containing multiple elements (19). Yet another mutation causes only PAIS with male phenotype and occasional incidence of breast cancer. Additionally, AR mutations can also

modulate the activity of other nuclear receptors, which can be attributed to the wide range of phenotypes seen in AIS (13).

Whole-body AR Knock out

The effects of complete absence of AR has also been studied through full androgen receptor knock out (ARKO). These mice lack androgen receptor in all tissues that normally express it. ARKO males show female-like appearance in their genito-anal distance, external genitalia and body weight. They have smaller testes that are also less cellular and have thinner seminiferous tubules, sometimes lacking leydig cells and possessing fewer germ cells. Where germ cells did exist, the spermatogonia were hypoplastic. No round/elongated spermatids or mature spermatozoa were found in the entire testes, suggesting that spermatogenesis may have been arrested. They also had lower testosterone concentrations and decreased bone volume. ARKO females remained fertile but produced smaller litters. (20)

Gonad-Specific AR Knockout

Sertolli-specific ARKO studies have demonstrated the importance of androgen action on sertolli cells for spermatogenesis, steroidogenesis, and the development of Leydig cells (21), (22). Furthermore, normal Leydig cell function has been partially attributed to AR-mediated androgen action on vascular smooth muscle (23).

Nervous system-specific ARKO studies have implicated AR in sexual motivation to initiate mating and aggressive/territorial behavior (23).

In females, Granulosa cell-specific ARKO mice showed phenotypic abnormalities similar to general ARKO females: reduced fertility, fewer and smaller litters, premature ovarian failure, longer estrous cycles, less frequent ovulation.

Therefore the normal reproductive functioning of female mice is attributed to AR-mediated androgen actions specifically in Granulosa cells (23).

GnRH Receptor: Activation

The GnRH receptor is a seven transmembrane G-protein coupled receptor. It possesses the prototypical N-terminal domain, the seven alpha helical transmembrane domains connected by three extracellular loop domains, and three intracellular loop domains. Unlike other G-proteins however, GnRH receptor lacks the C-terminal, suggesting an evolved role of the receptor that is specific to mammals. The extracellular domains and surface regions of the transmembrane domains are involved in hormone binding. GnRH mechanism of action and its regulation involve the complex interaction of many factors.

GnRH binding induces transmembrane domain-mediated conformational changes in the intracellular loop domains, activating and coupling them to the G_q subunit, which leads to the activation of Phospholipase C (PLC). PLC then proceeds to cleave PIP_2 into IP_3 and DAG resulting in a Ca^{2+} surge as well as PKC activation (24).

GnRH binding also activates phospholipase D (PLD) and phospholipase A2 (PLA2). PLD acts mainly on the membrane phospholipid phosphatidylcholine. The PA formed by PLD can be converted to DAG by a specific

PAphosphohydrolase. PLA2 activation provides long chain unsaturated fatty acids such as arachidonic (AA), oleic, linoleic, and linolenic acids, which have been implicated in the activation of selected PKC isoforms as well as GnRH-induced gonadotropin synthesis and release (3).

As mentioned before, GnRH binding to its receptor leads to activation of PKC. PKC then phosphorylates ERK1/2, which has been shown to play a role in the expression of such immediate early genes as c-fos, ATF-3, and Per-1. The ERK pathway has also been tied to the expression of glycoprotein hormone alpha-subunit, the LH beta subunit, and the regulatory MAPK phosphatase MKP2. Additionally, it may play an inhibitory role in regulatory GnRHR transcription, as inhibition of PKC showed stimulation of the receptor promoter activity (24). The ERK pathway is one of the four MAPK pathways known in mammals: ERK1-2(p42 and p44), JNK 1–3, p38a-d, and ERK5 (big MAPK; BMK). The activation of MAPK by GnRH seems to be 'cell context-dependent' (3).

GnRH and activin display synergistic activation of GnRHR-induced intracellular pathway. This synergy is achieved through convergence at the crosstalk point between SMAD and AP-1 proteins, each of which have their own binding site on the GnRH receptor promoter. SMADs exert their transcriptional effects upon binding the Activin-Responsive-Factor (ARF) complex, which then binds -327/-322 region of the promoter, facilitated by AP-1. AP-1 and SMADs therefore cooperate at GRAS. In Figure i.1, the overlapping consensus SBE and GRAS are shown in the nucleus, along with the cis-regulatory sequences binding

SMAD3/4 and the Fos/Jun complex. GnRH and activin increase SMAD and AP-1 binding to this region. These transcriptional factors have been assumed to act either directly through protein interactions or alongside co-repressors/-activators to affect both basal and stimulated levels of GnRH receptor transcription (25).

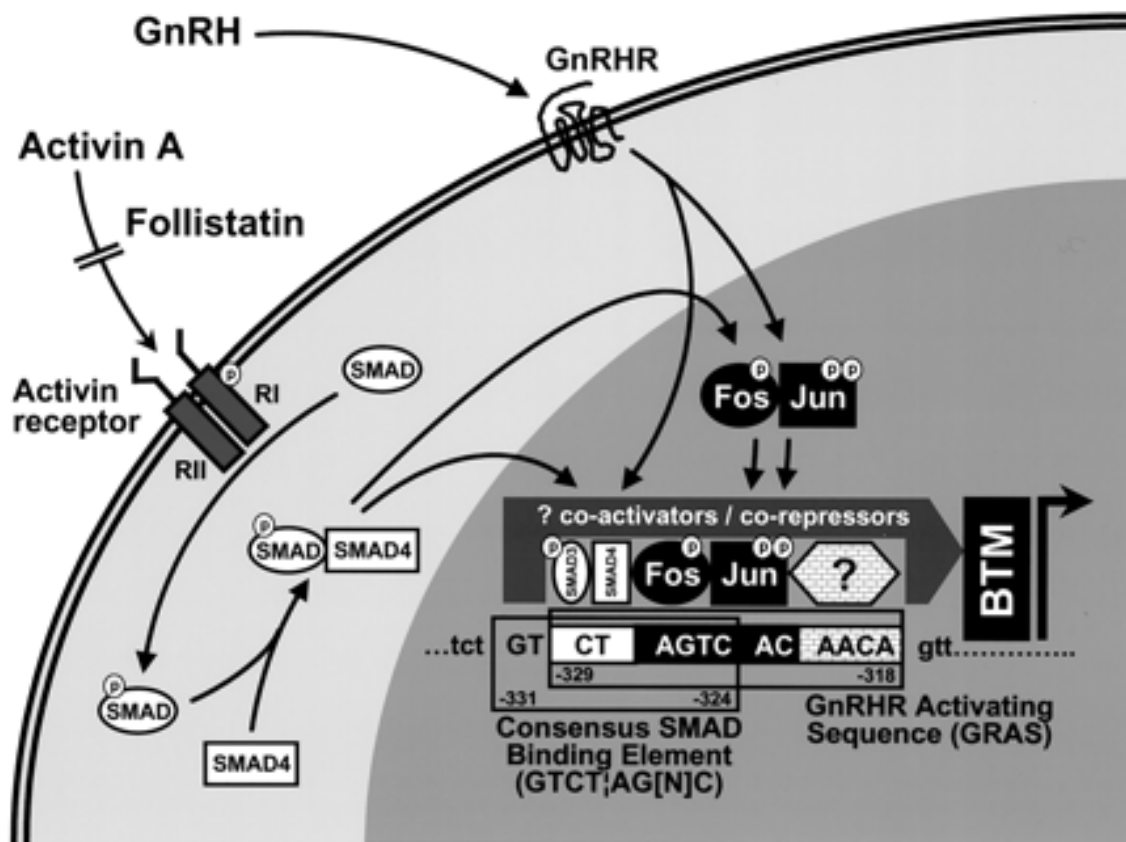


Figure I.1: Proposed interaction and collaboration among intracellular signal transducers in GnRH- and Activin-mediated GnRHR gene transcription (26)

GnRH Receptor: Regulation

Transcriptional Stimulation

While GnRH binding to GnRHR activates downstream signaling pathways, it's also been shown to downregulate the expression of the receptor itself (24).

At the transcriptional level, GnRH receptor expression is controlled by two cAMP response elements (CREs) and a number of cis-acting elements working together, such as LIM (lin-11, isl-1, mec-3) homeodomain protein, seven E-boxes, the alpha ACT element that binds members of the GATA binding factor family, and the gonadotrope-specific element (GSE) that binds steroidogenic factor-1 (SF-1), which then binds the receptor promoter at -181/-173 (26).

The receptor promoter contains seven E-boxes, of which E-Box 3 has been shown to play a role during pituitary development. E-box 3 binds and is activated by group A bHLH transcription factors NeuroD1 in aT3-1 and Mash1 in LBT2 cells to activate GnRH receptor expression. This binding is facilitated through heterodimerization with E47 (27).

LIM homeodomain transcription factors are known to interact with a variety of proteins through the LIM domain, and such cofactors could be interacting with LHX3 (28). The LIM proteins contain a homeodomain that serves as a DNA binding domain and two amino-terminal LIM domains used for protein interactions. LHX3 (LIM3/P-LIM) is expressed throughout the developing and adult anterior pituitary and is required for normal pituitary development and differentiation, and also for maintaining the pituitary during adulthood. It activates

the alpha-glycoprotein subunit (α GSU), prolactin (Prl), TSHbeta, FSHbeta, and the POU homeodomain transcription factor Pit-1.

Two additional cis-elements also play a role: SURG (sequence underlying responsiveness to GnRH)-1 at -292/-285, and SURG-2 at -276/-269 (AP-1 site-basal). SURG-2 overlaps with AP-1 consensus binding site. SURG-1 has been shown to bind transcription factors NF-Y and Oct-1. In complexes or in conjunction with other co-activators, NF-Y mediates both basal and GnRH-induced levels of receptor expression. Oct-1's activity is tissue-specific. Overlap between the two transcription factors' binding sites within the SURG-1 element is indicative of an interaction between the two, possibly a competitive one. Evidence shows cooperation between Oct-1 and AP-1. Oct-1 and NF-Y may be regulating receptor activity both independently and through possible interaction with AP-1 (29).

Basal Transcriptional Regulation

The upstream region of the promoter has been shown to be important for basal level of GnRHR transcription, particularly the regions -1300/-1080 and -2197/-1900. This region contains transcription factor binding sites for Oct-1, AP-1, C/EBP, and GATA (26).

Basal GnRHR activity regulation has also been mapped to the regions -482, -475, and -393/-386 bp. The -393/-386 bp region is yet another GRAS, acting specifically in gonadotrope cells. Furthermore, the necessary contribution of a canonical AP-1 site located at -336/-330 has also been demonstrated. It is

believed that GRAS, AP-1 and SF-1 work in combination, as mutation of any of the three elements significantly reduces promoter activity (26).

GnRH-induced sensitization of the receptor involves the GRAS element at -329/-318, which binds AP-1, SMAD, and FoxL2, mediating response to both activin and GnRH. The -387/-308 region contains the overlapping GnRHR activating sequence (GRAS) and SMAD binding element (SBE) at -329/-318 and -331/-324 respectively, and is also a mediator for GnRH-induced receptor expression (29).

GnRHR Importance

Mutations in the GnRH receptor have been implicated in Idiopathic Hypogonadotropic Hypogonadism (IHH), an autosomal recessive disease with either partial or complete phenotypic range. In humans, Complete IHH results in delayed puberty, lack of secondary sexual characteristics, low gonadotrophin, and low sex steroid levels in both sexes. Partial IHH results in partial ovarian steroid synthesis results in primary amenorrhea with normal breast development in females. Males had small testes and low testosterone levels, while LH and FSH levels were normal (30).

The mouse model of hypogonadotropic hypogonadism has also been studied, characterized by a recessive mutation in the third transmembrane domain of the receptor gene, decoupling it from intracellular signal transduction pathways. HH male mice possess several abnormalities in their internal and external genitalia, and are infertile due to arrested spermatogenesis. HH female

mice are also affected, with abnormalities in internal genitalia, delayed vaginal opening, and arrested oogenesis (31).

The mouse and human phenotypes of HH greatly parallel those of full body ARKO mice. While AR is expressed in a variety of tissues, GnRH receptor is expressed mainly in the pituitary. In the immortalized pituitary L β T2 gonadotropes, AR has been shown to induce GnRHR (32) and FSH β (33) promoters in the presence of androgen. It's been suggested that AR-mediated androgen induction of FSH β is indirect, through increasing stimulation of GnRHR by GnRH. Therefore, it is possible that the phenotypes caused by full body AR deficiency are at least in part attributed to AR's actions in the pituitary, actions that GnRHR also plays a role in.

CHAPTER 1: Androgen Receptor Regulates GnRH Receptor expression through downstream HRE sites

Abstract

As part of their characterized role in HPG feedback regulation, androgens have been shown to differentially regulate gonadotropin release at the anterior pituitary level, decreasing LHB and increasing FSHB expression. Additionally, androgens have been shown to upregulate pituitary GnRH receptor expression, through binding AR. There has been interest in understanding whether androgens act directly and differentially on the gonadotropin gene to regulate their expression, or whether their effect is indirect, through upregulation of GnRH, thus increasing gonadotrope response to GnRH. Our experiments show that androgen-bound AR induces GnRHR expression through binding to two Hormone Response Elements (HRE) half-sites on the GnRH receptor promoter, and that this binding is mediated through AR's DNA-binding and Ligand binding domains.

Introduction

Sex Steroids regulate the amount of their production via negative feedback at the hypothalamic and anterior pituitary level. This negative feedback stops the production of GnRH and differentially regulates gonadotropin levels. At the hypothalamic level this feedback is achieved by changing GnRH gene

expression (34), while at anterior pituitary level steroids change GnRH receptor gene expression (35). These regulations are mediated by AR.

Androgen-bound AR suppress gonadotropin expression via the alpha subunit promoter by binding the junctional regulatory element (JRE) (36). AR also suppresses GnRH stimulation of LH β promoter via interaction with Sp1, CArG, SF-1. This in turn eliminates the stimulatory signal for gonadal androgen production.

In contrast, androgens increase FSH β expression in both males and females (32). This increase is synergized in the presence of GnRH or activin. Spady et al showed that androgens actually increase GnRH Receptor levels, presumably through AR. This increase in receptor level expression is assumed to play a role in androgen-induced FSH β expression, as Thackray et al 2006 showed that AR is necessary for the increase in FSH β (33). Moreover, Spady has suggested that the androgen-induced expression of FSH β is indirect and mediated by GnRH (32).

Our research followed from the work done by Spady et. al. Thus far AR has been shown to play a role in the androgen-induced upregulation of GnRHR which in turn increases GnRH induction of FSH β expression. The mechanism of AR action however remains unclear. What is known is that androgen-bound AR translocates to the nucleus and binds the receptor promoter at Androgen Response Element half sites, thereby recruiting an array of cofactors to form a complex that activates gene transcription. Our objective therefore was to map the exact region of AR binding on the GnRH receptor promoter, and to determine

whether binding to that region alone would be sufficient for the receptor induction, or if the aid of other factors would be necessary.

All transcriptional analysis were carried out in the L β T2 immortalized gonadotrope cell line, which has proven to be a useful model for studying in vitro effects of AR. These cells endogenously express LH β , FSH β , the common α GSU subunit, AR, and importantly, GnRHR. Though AR is endogenously expressed, we transfected the cells with exogenous AR to ensure a robust response, as demonstrated by Thackray et al 2006.

Materials and Methods

Plasmids and cloning

The following expression plasmids were used: human AR in a pSG5 expression vector (hAR) (37), constitutively active AR (mut AR CA) (37), and AR containing a mutation in the DNA-binding domain (mut AR DBD) (38). The pGL3-MMTV reporter plasmid was obtained from Ligand Pharmaceuticals (La Jolla, CA). The -1.1 kb GnRHR reporter plasmid has been previously described (32). 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 of the GnRHR promoter were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All primer sequences are provided in Table 1. We created multimer constructs of the -499 and -413 bp HRE sites

containing four repeats separated by two nucleotides, driven by the TK-Luc promoter on a pGL3 backbone.

Cell culture and transient transfections

Transient transfections were performed in the L β T2 pituitary gonadotrope cell line (39). 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 (40). 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 10⁵ 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 AR expression vector to ensure consistent and adequate expression, and 100 ng of a β -galactosidase reporter plasmid driven by the herpes virus thymidine kinase promoter to control for transfection efficiency (40). After 6 h of transfection, cells were switched to serum-free DMEM containing 0.1% BSA and 5 mg/L transferrin. 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; 17 β -Hydroxy-3-oxo-4-

androstene, Sigma), or dihydrotestosterone (DHT; 5 α -androstan-17 β -ol-3-one, Sigma) at concentrations ranging from 10⁻⁵M to 10⁻⁹M 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 b-galactosidase activity as previously described (34).

Luciferase and β -galactosidase assays

After hormone treatment, luc reporter and β -galactosidase activity were assayed as previously described (40). 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 μ M 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).

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 (33). The whole cell extracts were incubated with 1 fmol of ³²P-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 μ g 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 \times 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.

Results

GnRH Receptor Promoter Truncation Analysis

In order to narrow down the site of AR action to a specific region, we generated four truncations of the full-length 1.2 kb promoter, at 838 bp, 738 bp, 600 bp, and 413 bp. Each construct was transfected into the L β T2 cells, along with human AR, and then treated with 100nM R1881. In the presence of AR, only the -738 bp and -600 bp promoter constructs were induced by R1881 ($p < 0.05$ and $p < 0.01$, respectively, Fig. 1). The -1.1 kb, -838 bp, and -413 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 (32). Together these data point to the AR binding site in the -600/-413 region. That no induction was observed with the -1.1 kb and -838 bp promoter constructs suggests the possible presence of a repressive element upstream of -738 bp. Because the androgen-bound AR induction of the -600 bp promoter construct

was significantly higher than that of the -1.1 kb promoter ($p < 0.01$, Figure 1), we chose the -600 bp construct as our positive control in subsequent experiments. Before proceeding further with the mapping experiments, we sought to confirm the physiological relevance of using R1881. We therefore performed a series of transfections using each of the androgens R1881, T, and DHT, at a range of doses from 10^{-5} M to 10^{-9} M. Figures 2A, B, and C show the results for each of the respective androgens. The highest and lowest treatment concentrations of R1881 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 DHT on the other hand did lead to significant induction (Fig. 2B). T however did not significantly induce the promoter construct at any concentrations (Fig. 2C), presumably because of its lower affinity for AR, as compared with DHT. Furthermore, this lack of induction by T points to a possible opposing effect of the T metabolite estradiol.

HRE Mutagenesis on the GnRH Receptor Promoter

Using a bioinformatics search we found two hormone response elements on the GnRHR promoter located at -499 bp and -159 bp. The HRE at -499 bp is characterized by the AGAACA half site while the HRE at -159 bp is characterized by the TGTTCT half site. In order to elucidate their potential involvement in AR regulation of GnRHR, we mutated each of these half sites on the -600 bp promoter. We also generated a double mutation in order to explore a possible collaboration between the two sites. Compared to the significant induction seen

with the transfected -600 bp promoter ($p < 0.01$), neither the individual mutations nor the double mutations showed induction (Fig. 3A-C). Furthermore, the double mutation actually caused a significant decrease in the promoter activity in the presence of AR compared with its absence ($p < 0.05$, Fig. 3C). Collectively our data imply that both HRE half sites are required for AR-mediated GnRHR induction, and that AR may also be involved in the regulation of basal receptor promoter activity.

AR directly binds the HRE sites at -499 bp and -159 bp

In order to determine whether AR interacts directly with the two promoter HRE sites, or if indirectly through other coactivators, we performed Electrophoretic Mobility Shift Assays (EMSA). We also used the consensus HRE probe as our positive control. We were able to detect a supershifted complex in both the positive control and the two HRE sites in the presence of AR antibody (Fig. 4, lanes 3 and 7). This supershift was not present in the presence of IgG. All probes showed self-competition when mixed with their cold competitors (Fig. 4, lanes 4 and 8), since the cold competitors were added at higher concentrations in order to facilitate the potential out-competition of the radioactive sites. The sites were also out-competed by the more concentrated cold consensus HRE (Fig. 4, lane 9).

As our AR source we used nuclear whole cell extract from bees, which conveniently also contains the androgen DHT. We used the AR consensus binding site as our positive control. We used the antibody IgG as our negative

control antibody. We used non-radioactive (cold) versions of each oligonucleotide (consensus, -499 HRE half site, -159 HRE half site) as our positive control antibody. The consensus HRE oligo displayed very strong binding to AR, resulting in the darkening of lanes 1-3, such that the bands were indistinguishable. The image of lanes 1-4 therefore was exposed at a higher level than that of the rest of lanes. The two differently exposed image sections were pasted together at the vertical interface indicated by the dotted line.

Each HRE site is necessary but not sufficient for GnRHR induction by AR

Having discovered two HRE sites in the mechanism of AR induction of the GnRHR, we sought to find out whether each or any of these two HRE sites serve as the sole accomplice or whether other co-activators would also be necessary. Figure 5 shows the results of our L β T2 co-transfection of the individual multimer constructs along with AR. Neither GnRH receptor HRE site alone was induced in the presence of androgen-bound AR.

GnRHR induction requires both the DNA-binding and Ligand-Binding

Domains of AR

Having determined the AR sites of action on the GnRH receptor promoter, we proceeded to test whether AR requires its DNA-binding and ligand-binding domains in interaction with these HRE sites. We therefore mutated the AR DBD as described in the methods and transfected it along side our positive control and R1881. As expected, R1881 was unable to induce the DBD-mutant AR's, as it

did the wild-type AR ($p < 0.05$, Fig. 6A). We then proceeded to study the effects of LBD mutation in AR, using a constitutively active AR containing a mutation in its hinge region. We confirmed its constitutive activity by co-transfecting 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. 6B). By contrast, in the presence of AR μ CA, the MMTV promoter was induced regardless of whether the cells were treated with vehicle or R1881 (Fig. 6B), thus confirming the constitutive activity of AR μ CA. When we proceeded to transfect the AR μ CA with the GnRHR promoter, we found androgen once again unable to induce AR-mediated GnRHR promoter activation (Fig. 6C). Together these data confirm that AR requires both its DNA- and ligand-binding domains in GnRH receptor expression induction.

Discussion

Androgens have been shown to differentially regulate the gonadotropin levels, feeding back negatively to LH, and positively to FSH. Furthermore, Spady et al 2004 has demonstrated androgen induction of the GnRH receptor in the L β T2 immortalized gonadotrope cells. Androgen's role in GnRH receptor induction and differential regulation of the gonadotropins is presumably mediated by AR. However, little is known about the underlying mechanism of androgen and AR action in this respect. We therefore sought to characterize in greater detail the mechanism of AR-mediated induction of GnRHR by androgen.

Regional Mapping of AR Action on GnRH Receptor Promoter

We began our investigation by asking where on the GnRH receptor promoter does androgen-induced AR act to activate its transcription. To find out, we generated a series of promoter truncations, at -838 bp, -738 bp, -600 bp, and -413 bp. We co-transfected each of these constructs, along with AR into L β T2 cells in order to see which regions were unresponsive to androgen treatment. We also separately transfected AR with the full-length 1.1 kb promoter as our positive control. To our surprise, the -1.1 kb promoter was not significantly induced as demonstrated by Spady et al (2004). However, their study used DHT treatment instead of R1881, which has different metabolic stability and physiological relevance. In our case, the most significant induction was seen with the -600 bp construct and to a lesser but still significant extent the -738 bp construct. Furthermore, the induction was lost with the -838 and -413 bp constructs. We therefore concluded that androgen-induced AR's site of action falls within the -600/-413 bp region, and that there was a possible inhibitory site upstream of -738 bp. Since the -600 bp construct was most dramatically induced, we chose to use it as our positive control in later experiments. We've supported our use of R1881 by showing its ability to induce our positive control as effectively as DHT (Fig. 1.2), in addition to its advantages of greater metabolic stability.

AR action maps specifically to HRE sites at -499 bp and -159 bp

We found that mutation of each HRE individually obliterated induction by AR. More surprising however was the significant signal reduction of the double mutant in the presence of AR compared to its absence. We therefore concluded that AR not only requires both HRE sites in induction of GnRH receptor promoter,

but that it may also play a role in the regulation of basal receptor activity, through the two sites.

To determine whether AR interacts with the two HRE's via direct binding or indirectly through binding a complex or some other protein that would then bind the sites we performed Electrophoretic Motility Shift Assays. If AR binds any of these sites directly, then we would see a supershifted complex in lanes containing AR was mixed with AR antibody and each of the HRE oligos. Indeed that is exactly what we found. Not only did the hot oligos form a supershifted complex with AR in the presence of AR antibody, but they were also outcompeted by their cold competitors, further confirming AR's direct binding to both HRE sites. Since the cold oligos were added at a much higher concentration than the radiolabeled oligo, AR showed preferentially binding to the cold version, creating a non-radiolabeled and undetectable complex. This is signified by the lack of bands for both the supershifted complex and the non-complexed oligos.

HRE's at -499 bp and -159 bp are necessary but not sufficient for GnRHR induction by AR

Having demonstrated the involvement of these two HRE sites, we generated multimer constructs of each site, consisting of four half site repeats separated by two nucleotides, inserted into a TK-Luc vector held on a PGL3 backbone. In individually transfecting these multimer constructs into L β T2 cells along with AR, we wanted to find out whether each of these sites alone would be sufficient for induction by AR and R1881. Induction of either or both of these

constructs would imply direct and unaided interaction induction by AR. In contrast to our positive control, we found no induction of any of the HRE multimers by AR. This suggests AR's need for either the participation of other coactivators or the necessity for the presence of both sites together in its induction of GnRHR promoter. The construction of a double multimer construct or co-transfection of the two multimer constructs will be necessary to determine whether AR needs both HRE sites and no other factors to induce the promoter, or whether AR needs both sites and the aid of other factors.

AR requires its DNA-Binding and Ligand-Binding Domains for GnRHR Induction

To confirm that AR action on GnRHR is stimulated by direct androgen binding, we mutated the AR ligand-binding domain, rendering it constitutively active. Co-transfection of this mutant AR with the GnRH receptor promoter failed to elicit an induction, suggesting that a normal functioning LBD is necessary for AR's induction of GnRHR. Mutation of the AR DNA binding domain also rendered AR unable to stimulate GnRHR.

Taken altogether, we've been able to shed more light on the interplay between AR and GnRHR in mediating the androgen/GnRH stimulation of FSH. We've demonstrated that Androgen binding to AR-LBD induces AR's direct binding to two Hormone Response Element half sites on the GnRH receptor promoter, through its DBD. (presumably through the inter-and intra-molecular interactions with AR domains) However, while our studies have identified some of the factors involved, they've also paved the path for future studies. For instance, our experiments indicated the necessity of both HREs on the GnRH

receptor promoter for AR action, but that individually they are not sufficient to carry out the induction. It remains to be seen however whether the two HREs together (as opposed to individually) would be sufficient, or whether the participation of other co-activators is also a requirement.

Chapter 1, in part, is currently being prepared for submission of publication. The thesis author was the primary investigator and author of this material. Emily Witham assisted with planning and analysis of transient transfections as well as designing plasmid constructs. Jason Meadows assisted with generating the Hormone Response Element multimer constructs. Varykina Thackray has provided guidance for the Electrophoretic Mobility Shift Assay technique. Pamela Mellon supervised the project.

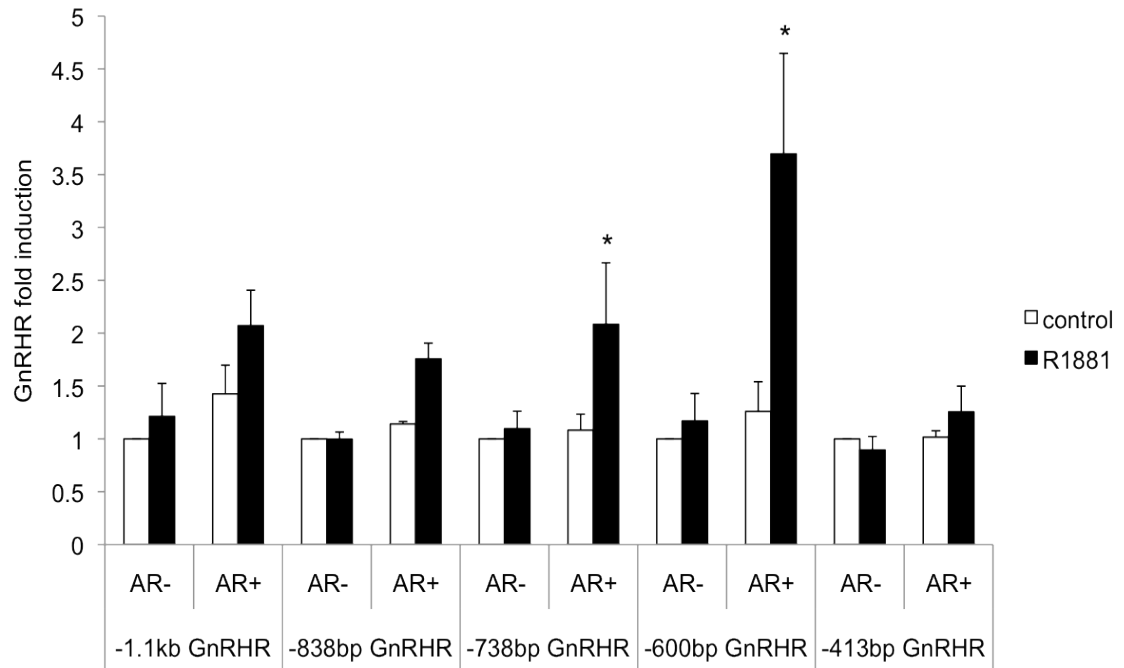


Figure 1.1: AR induction of GnRHR maps downstream of -600 bp

Each of the 5' truncations of the GnRHR promoter was transiently co-transfected in L β T2 cells with the indicated luciferase reporter and either an AR expression vector or empty vector control. 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. Luciferase activity of each truncation was compared to vehicle control without AR. * $p < 0.05$ by one-way ANOVA.

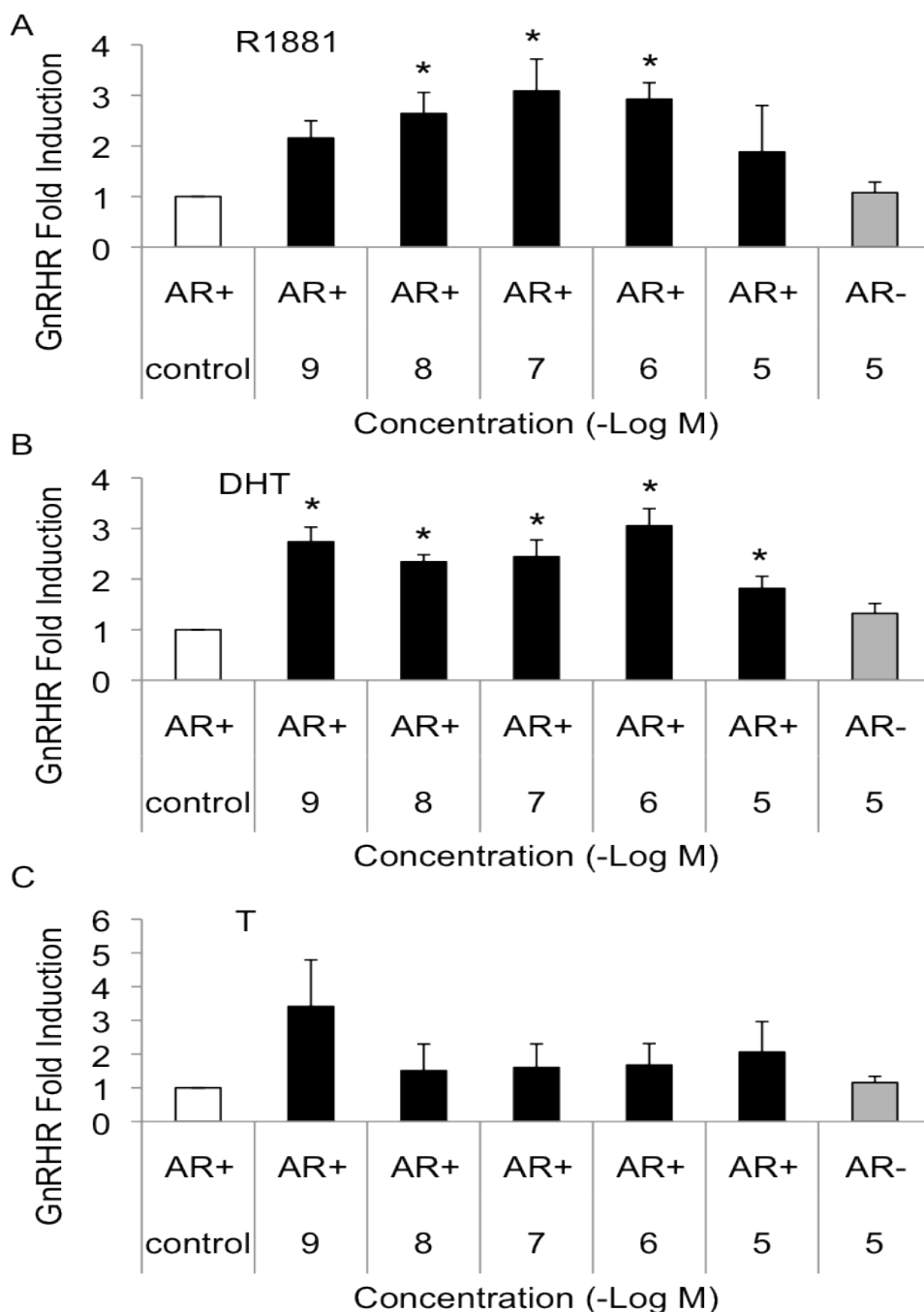


Figure 1.2: GnRHR responds more strongly to R1881 and DHT than T

L β T2 cells were transiently co-transfected with the -600 bp GnRHR promoter and either an AR expression vector or the empty vector control. 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 t-test.

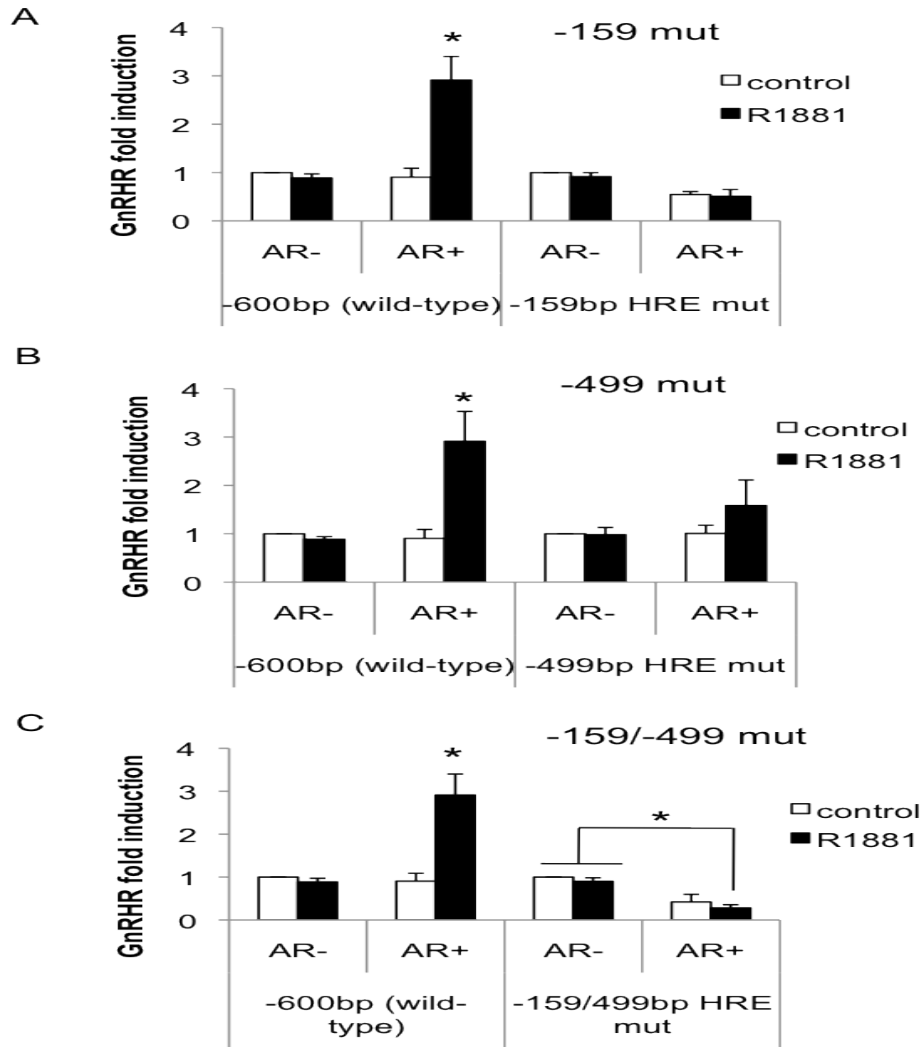


Figure 1.3: HRE half-sites at -159 bp and -499 bp are required for AR induction of GnRHR

L β T2 cells were transiently co-transfected with either the wild-type -600 bp GnRHR promoter or the indicated HRE mutant promoter, along with either an AR expression vector or its empty vector control. Cells were treated for 24 h with vehicle or 100 nM R1881 and were subjected to luciferase assay. Luciferase activity was compared between treatment and vehicle control without AR. Data were normalized to vehicle-treated control without AR. A) A mutation in the -159 bp HRE half-site in the GnRHR promoter rendered it unable to activate in the presence of AR and R1881. * $p < 0.05$ by one-way ANOVA. B) A mutation in the -499 bp HRE half-site in the GnRHR promoter rendered it unable to activate in the presence of AR and R1881. * $p < 0.05$ by one-way ANOVA. C) A double mutation in both -159 bp and -499 bp HRE half-sites in the GnRHR promoter not only eliminated induction in the presence of androgen-bound AR, but also reduced below basal level. * $p < 0.05$ by one-way ANOVA.

AR binds directly to -159 and -499 HRE

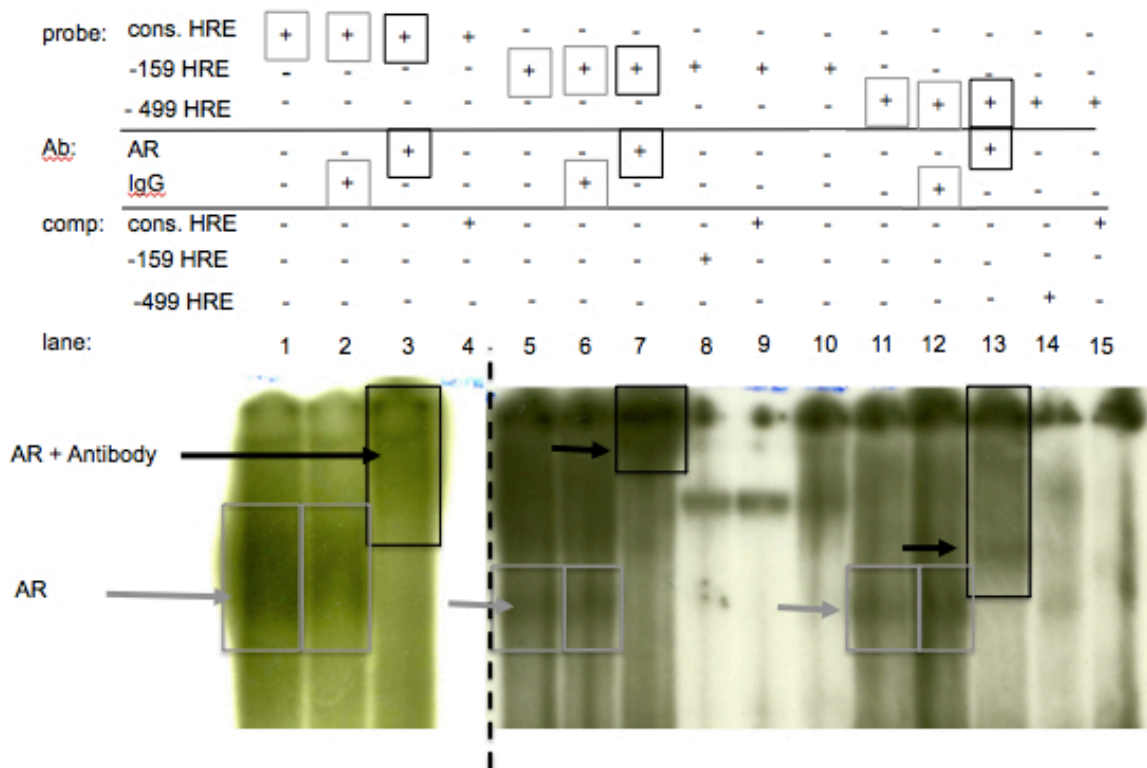


Figure 1.4: AR directly binds the -499 and -159 bp HREs

Whole cell extracts containing over-expressed AR from baculovirus-infected cells were incubated with either the consensus HRE, -499 HRE half site probe or the -159 HRE half site probe and tested for complex formation in EMSA. The relevant AR-DNA complexes are shown in lanes 1, 5, and 11. Lanes 3, 7, and 13 show the AR-DNA complex supershifted in the presence of the AR antibody. IgG control is shown in lanes 2, 6, and 12. Self-competition of each radiolabeled HRE oligo by its respective non-radiolabeled competitor is shown in lanes 4, 8, and 14. Competition of the -159 HRE probe with the consensus HRE is shown in lane 9. Competition of the -499 HRE probe with the consensus HRE is shown in lane 15. Grey boxes and arrows indicate AR-DNA complexes, while black boxes and arrows indicate supershifted AR-DNA complexes in the presence of AR antibody. Dotted line separating lanes 4 and 5 indicates differently-exposed sections of the same image pasted together.

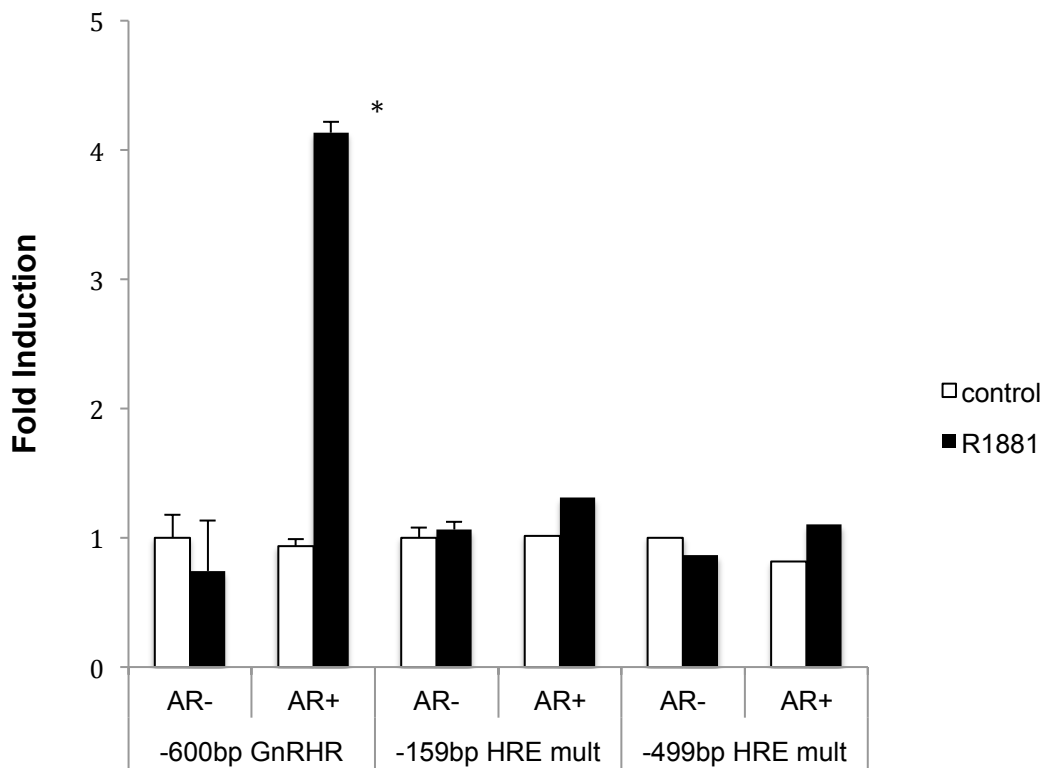


Figure 1.5: HRE insufficient to induce GnRHR promoter by AR

Each of the 5' truncations of the GnRHR promoter was transiently co-transfected in LbT2 cells with the indicated luciferase reporter and either an AR expression vector or empty vector control. 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. Luciferase activity of each truncation was compared to vehicle control without AR.

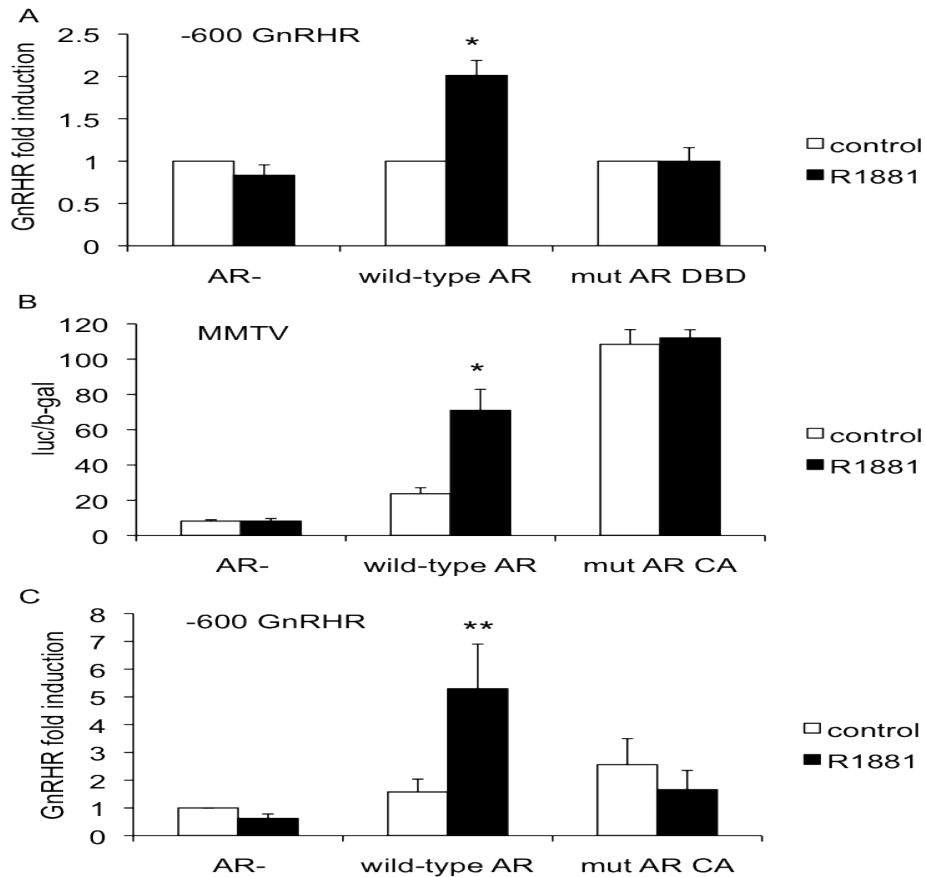


Figure 1.6: AR DNA-binding and ligand-binding domains are required for induction of GnRHR

L β T2 cells were transiently transfected with either the -600 bp GnRHR promoter, the MMTV reporter, or empty vector, along with either wild-type AR, DBD mutant AR (mut AR DBD), or LBD-mutant AR (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 b-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.

Table 1.1: Oligonucleotide sequences for EMSA and transfections of the GnRHR promoter.

Only forward sequences are listed.

-838 bp GnRHR	GTGCCAGAACATTTCTCTATCGATAATGGAAGTCA TACCAATATTTG
-738 bp GnRHR	GTGCCAGAACATTTCTCTATCGATAAGGATTATAA ATTATAAATTAG
-413 bp GnRHR	GTTTGAAGACCATACTTCTCAATATCGATAGAGAA ATGTTCTGGCAC
-499 bp HRE mutation	TAGAATAATTGGTATTATAATAGGCTGCTTAAACA GTTAAAGTACTAGC
-159 bp HRE mutation	CTTCAGGAGGGCTTGGCATATTATGTTAGCACTCT TTAGA
EMSA; consensus HRE	ACGGGTGGAACGCGGTGTTCTTTTGGCACG
EMSA; -159 HRE	GAGGGCTTGGCATGTTCTGTTAGCACTCTT
EMSA; -499 HRE	ATAATTGGTATTAGAACAGGCTGCTTAAAA
-159 bp HRE multimer	CTAGCTGTTCTAATGTTCTAATGTTCTAATGTTCTA ATGC
-499 bp HRE multimer	CTAGCAGAACATTAGAACATTAGAACATTAGAACA TTAGAACAC

CHAPTER 2: Importance of AR in mouse reproductive physiology

Abstract

In the *in vivo* part of our study, we set out to determine which of the many known functions of AR is mechanistically linked to its pituitary-specific role, given that AR is expressed in other tissues as well. In our pursuit we generated a novel pituitary-specific AR Knockout mouse line, ARF/ α GSU α CRE. Though still in progress, our work thus far demonstrates a role for pituitary-specific AR in timing of pubertal onset, and also confirms our previous finding of its induction of pituitary GnRH receptor promoter.

Introduction

Thus far whole-body AR knockout mice have been studied. Whole-body ARKO male mice have female-like external genitalia, and incomplete internal genitalia. They have low T, low bone volume, and seem to have arrested spermatogenesis. Whole-body ARKO females maintain fertility but produce smaller litters. As discussed before, AR is expressed in a variety of tissues, including the pituitary, so that individual tissue-specific knockouts are necessary to delineate AR's specific role in its various functions.

Since AR is X-linked, and since Tfm males are infertile, it had been impossible to generate homozygous ARKO females by conventional gene mutation in the parent mice. In this respect, the Cre-loxP system (41) provides a

detour, so that parents with wild-type AR can give rise to tissue-specific AR knock out offspring. Therefore we've been able to study both male and female tissue-specific AR knockout mice. Following our exploration of the mechanistic role of AR in the GnRH receptor-mediated increase in FSHB expression in the pituitary, we sought to study the broader physiological impact of AR absence from the pituitary in vivo.

Our generation of this offspring- and pituitary-specific AR knockout mouse line holds scientific significance because of the difficulty to do so in the past. Conventional ARKO methods used AR gene mutagenesis in the parent, which rendered the male parent externally feminized and infertile, thereby unable to mate and breed homozygous female offspring. The Cre-loxP system circumvents this issue by utilizing parents as carriers of the knockout machinery, while leaving their fertility intact.

The Cre-loxP system can be used to incur deletions, duplications, inversions and translocations in the genome, both in vitro and in vivo. The in vivo approach utilized in our study involves crossing an AR-floxed female with a Cre-expressing male. The expression of the Cre protein in the parent and offspring is placed under the control of a ubiquitous or specific promoter. In our case, it was placed under the control of the gonadotrope-specific α GSU promoter, hence the name of the parent mouse lines α GSUiCRE and AR Flox (ARF). The offspring mouse line was thus termed ARF/ α GSUiCRE; the i in iCre stands for improved Cre. Males were hemizygous AR knockouts, and female offspring were heterozygous AR knockouts, since they only received one AR floxed X

chromosome from their ARF mother, while the other X chromosome was inherited from their non-AR floxed father.

In order to study the effects of pituitary-specific ARKO in females, we needed to knock out the AR gene in both alleles, since it is X-linked dominant. We therefore crossed the heterozygous ARF females from our first cross with ARF males, which generated, again, hemizygous ARKO males and homozygous ARKO females.

Having generated pituitary-specific ARKO males and females, we sought to test the physiological impact of tissue-specific absence AR on pubertal onset, fertility, gonad morphology, blood hormone levels, pituitary GnRH and gonadotropin levels, and hypothalamic GnRH levels.

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. 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) (20) were bred to male α GSU-iCRE mice (obtained from Dr. Sally Camper, University of Michigan). The male offspring, termed ARF/ α GSU, were studied and compared to littermate AR flox males without α GSU-iCRE.

To study the effects of pituitary-specific AR Knockout in females, ARF/ α GSU females were crossed with AR flox males, breeding homozygous ARF/ α GSU females.

Pubertal assessment

ARF/ α GSU males and AR flox controls were monitored for preputial separation as previously described (42). 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.

Homozygous ARF/ α GSU females were assessed for PND pubertal onset by checking for vaginal opening upon starting on weaning day (PND 21). PND of pubertal onset was recorded when a full vaginal opening was observed.

Female Estrous Cycling

Vaginal smears were taken from homozygous ARF/ α GSU females once a day for 20 consecutive days, within the same 2-hour window each day. Vaginal cytology determined the cycle stage. While the conventional terminology of the estrous cycle stages proceeds from diestrus to proestrus to estrus and finally to metestrus, stage labeling is often subjective due to diversion of cell morphology from the conventional norm. In this study, neither wild type nor knockout mice experienced estrus or metestrus, and instead consistently experienced

incomplete transition between stages. Therefore it was more fitting to label the stages as D1 (early diestrus), D (mid diestrus), D2 (late diestrus), D2/P (combination of late diestrus and proestrus), P (proestrus), and P/E (transition stage between proestrus and estrus).

Fertility assessment

ARF/ α GSU males and AR floxed controls were monitored for their ability to produce a copulatory plug when paired with a wild-type female as previously described (43). 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.

Tissue Processing

Animals were sacrificed at approximately 3 to 4 months of age. Both testes, the hypothalamus, and the pituitary were taken. One of the testes was fixed in formaldehyde overnight at 4°C and stored in 70% EtOH at -20°C. The other testis along with the hypothalamus and pituitary were stored at -80°C while awaiting RNA extraction. The tissues were later homogenized using either the Qiagen RNeasy kit or trizol, their cellular debris pelleted and their aqueous solution containing their extracted RNA isolated and stored at -80°C. A fraction of the isolated RNA was then treated with Ambion DNA-free DNA-removal kit, and then reverse transcribed to cDNA using the biorad iScript RT kit.

Quantitative PCR

The cDNA described above was first assayed for GAPDH level as an indication of basal metabolic activity in the isolated tissue. Quantified GAPDH levels served as control/standard against which to compare GnRHR gene levels.

Statistical analysis

Results are presented as mean \pm 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

Delayed Pubertal onset in AR/ α GSU-iCRE males

Starting at PND 21, male pups were weaned and checked for date of full preputial separation, which is an indicative parameter of pubertal onset. Pituitary-specific ARKO males experienced full preputial separation 3 days later than ARF controls, and this difference was significant ($p < 0.05$) (Fig. 2.1).

Fertility assessment in AR/ α GSU-iCRE males

At 2-3 months old, ARF controls and ARF/ α GSU-iCRE males were mated to wild type females of approximately the same age. They were allowed to mate for 10 days, on each of which females were checked for the presence of a vaginal plug, indicative of penetration by male mate. Results are displayed as percent of males from each group that penetrated their female mate on each of

the ten days. There was no significant difference observed in wild type versus knockout copulatory behavior (Fig. 2.2), suggesting either that the androgen-bound androgen receptor-mediated GnRHR induction in the pituitary does not play the only role or any role in male fertility.

GnRHR level in knockout vs. wild type pituitary

We sought to compare wild type versus knockout pituitary GnRHR levels to confirm that androgen induction of FSHB expression at the pituitary level was indirectly through increased GnRHR stimulation. Data is presented as GnRHR divided by GAPDH levels. Thus far preliminary results indicate 10-fold reduction in ARF/ α GSU*i*CRE pituitary GnRHR levels relative to ARF male pituitaries (Fig. 2.3).

Assessment of pubertal onset in homozygous AR/ α GSU-*i*CRE females

As done with our males, females were weaned and checked for date of full vaginal opening starting on PND 21. Vaginal opening in females is indicative of pubertal onset. While I am still in the process of breeding and data collection, preliminary data shows an approximately 3-day delay between wild type and ARF/ α GSU*i*CRE ($P < 0.05$) (Fig. 2.4)

Assessment of homozygous AR/ α GSU-*i*CRE female estrous cycling

At approximately 2 months old, we began collecting vaginal cells every day for 20 days. By observing the morphology of the cells we determined the

stage of the estrous cycle for each mouse for each day. Knockouts and wild types were compared on the order of stages cycled throughout the 20 days (Fig. 2.5A and B) as well as the percent of days spent in each stage (Fig. 2.5C). I found no significant difference in cycling pattern between wild type and ARF/ α GSUiCRE.

Discussion

Androgens and their receptor AR have been implicated in numerous physiological functions and have been found to act in various tissues. One such tissue is the anterior pituitary, where androgens were shown to increase both FSH β and GnRHR expression. The second part of our study therefore focused on exploring *in vivo* the physiological relevance of AR's pituitary-specific role in mediating androgen stimulation of the GnRH receptor.

With our pituitary AR knockout mice, we were able to study AR's role in the pituitary on a variety of reproductive-related parameters, including timeliness of pubertal onset, pattern of estrous cycling in females, fertility, blood hormone levels, pituitary hormone levels, and testes morphology.

In males, we detected a significant 3-day delay in pubertal onset of ARF/ α GSUiCRE males relative to ARF controls. Though still in progress, quantification of pituitary GnRHR levels thus far shows a 10-fold reduction in the pituitary of ARF/ α GSUiCRE males relative to controls. We detected no difference in male fertility, specifically in their ability to penetrate their female mate, relative to wild type males. Because the ARF/ α GSUiCRE did eventually

show preputial separation despite the 3-day delay, these findings demonstrate that Androgen-bound AR's action in the pituitary is at least in part responsible for coordinating the timing of puberty. In the case of male fertility, AR's action in the pituitary is either not at all involved, or its involvement is so small that it can be compensated for by other players in the HPG axis. Furthermore, the finding that pituitary-specific AR knockouts showed a reduction in pituitary GnRH receptor levels provides *in vivo* confirmation of the importance of AR in mediating androgen up-regulation of the receptor.

In females, we detected an approximately 3-day delay in ARF/ α GSU*i*CRE female pubertal onset, as indicated by full vaginal opening, relative to ARF controls. No significant difference in estrous cycling patterns was detected between the two experimental groups. This is not surprising, as majority of female reproductive function has been attributed to AR's role in the granulosa cells. However, the 3-day delay in vaginal opening does partially implicate pituitary AR in coordination of the timing of puberty in females. The fact that vaginal opening was eventually observed implies that pituitary AR is not the only factor controlling timing and onset of puberty.

Chapter 2, in part, is currently being prepared for submission of publication. The thesis author was the primary investigator and author of this material. Emily Witham and Jason Meadows assisted in designing the AR Flox/ α GSU-*i*CRE mouse line. Emily Witham assisted in designing and carrying out the puberty and fertility assessments, as well as Quantitative PCR. Jason

Meadows assisted in tissue extraction from the AR Flox/ α GSU-iCRE male mice.

Pamela Mellon supervised the project.

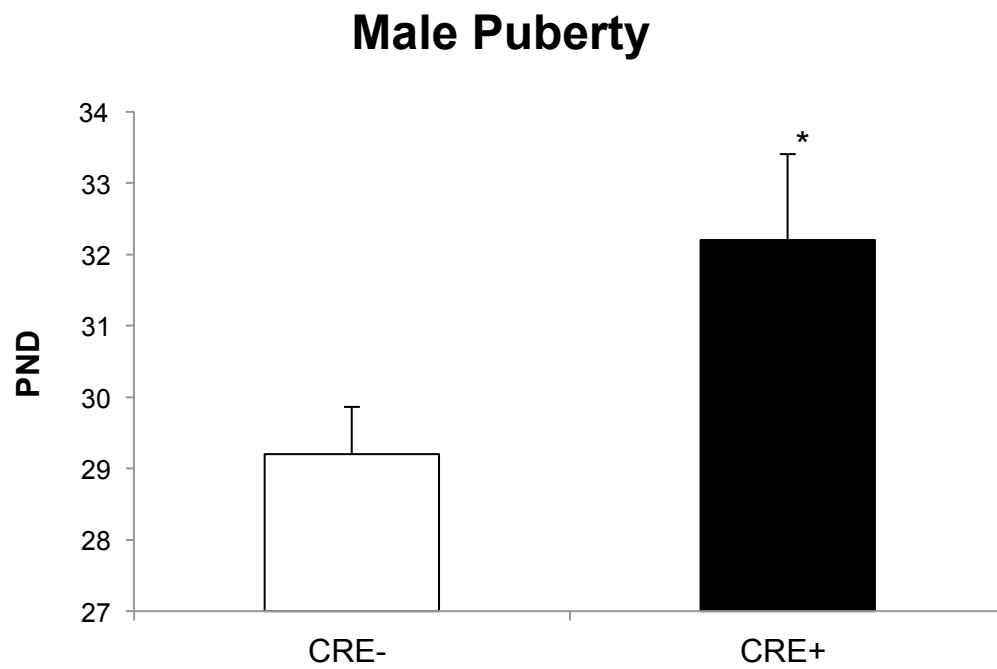


Figure 2.1: Pubertal onset in ARF/ α GSU-iCRE males.

The postnatal day (PND) of preputial separation was monitored in ARF/ α GSU-iCRE males and AR flox controls. Values are presented as averages. N=10 ARF/ α GSU-iCRE and 12 AR flox. * $p < 0.05$

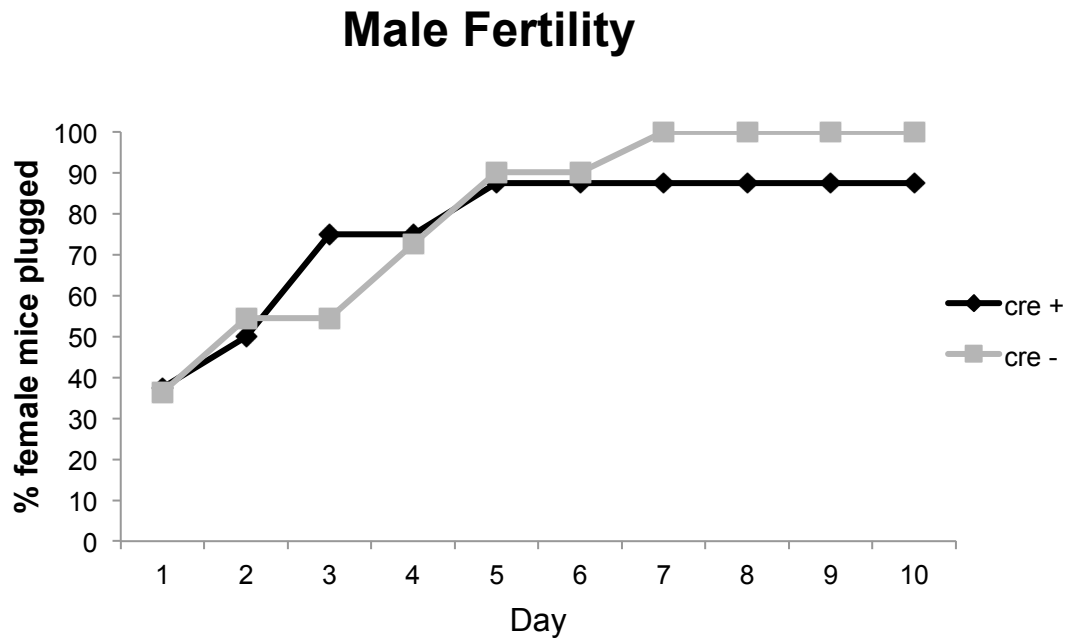


Figure 2.2: Fertility assessment in AR/aGSU-iCRE.

Two month-old aGSU-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. No significant difference detected. N=8 AR/aGSU-iCRE and 8 AR Flox.

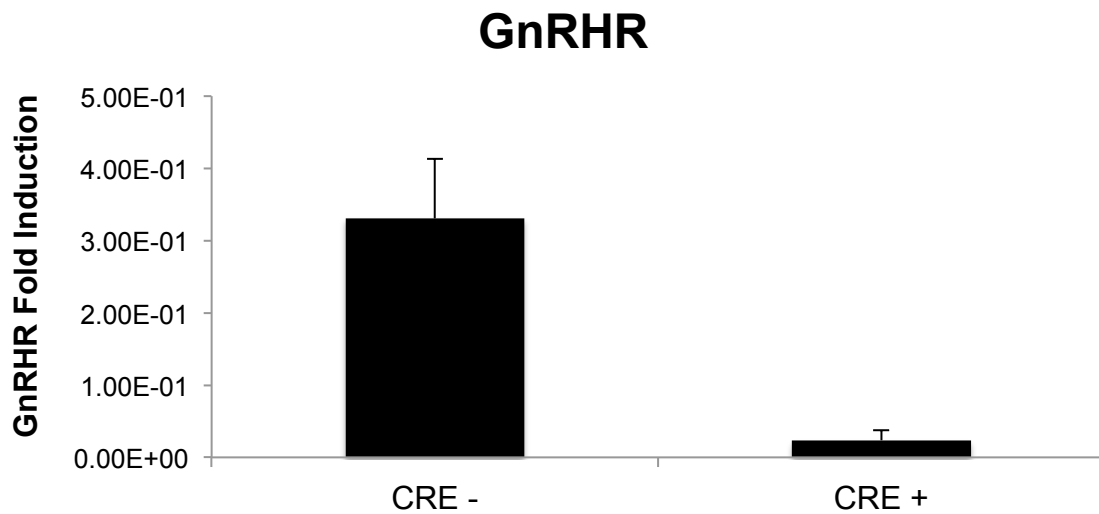


Figure 2.3: GnRHR quantification in ARF/ α GSUiCRE male pituitary

At 3-4 months old, mouse pituitaries were surgically removed, homogenized, and their RNA extracted. qPCR was performed for GnRHR.

Data were normalized to GAPDH. Values are presented as averages. N= 4 ARF/ α GSUiCRE and 4 ARF * $p < 0.05$ compared to control by Student's t-test.

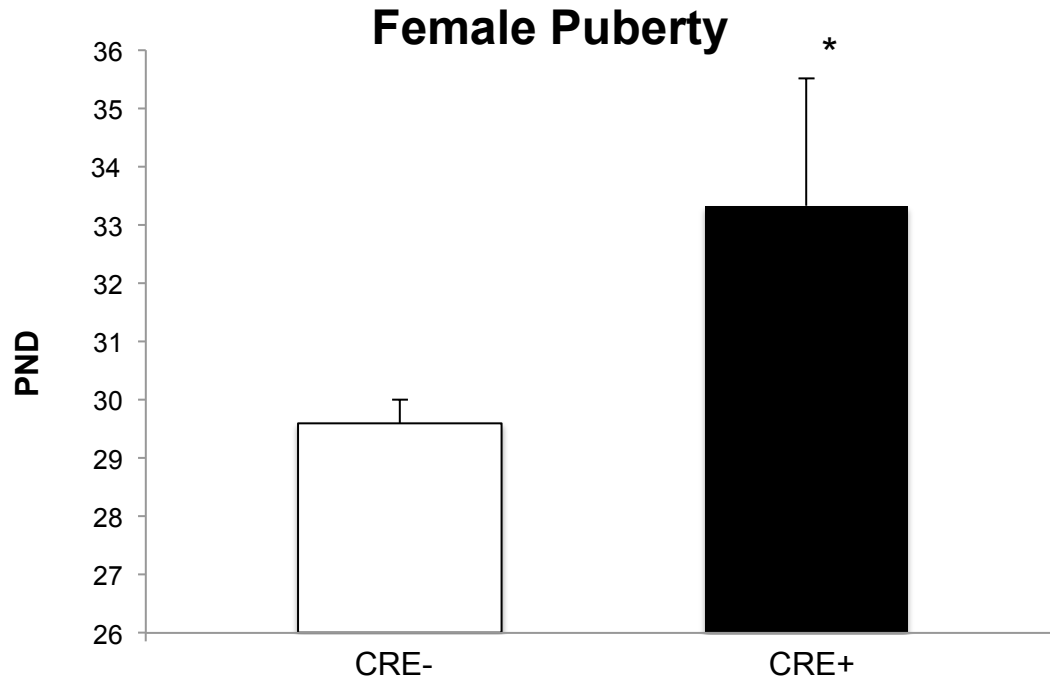
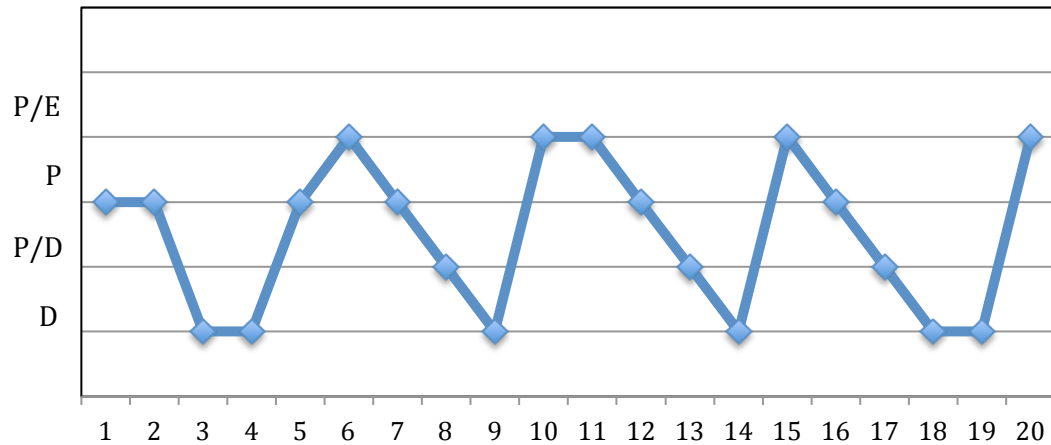


Figure 2.4: Pubertal onset in ARF/ α GSU-iCRE females

The postnatal day (PND) of full vaginal opening was monitored in ARF/ α GSU-iCRE females and AR flox controls. Though still in the process of breeding females with the CRE+ genotype, there does seem to be statistically significant delay in the vaginal opening of ARF/ α GSU-iCRE females. Values are presented as averages. N=3 ARF/ α GSU-iCRE and 12 ARF. * p<0.05

A) ARF/ α GSUiCRE



B) ARF

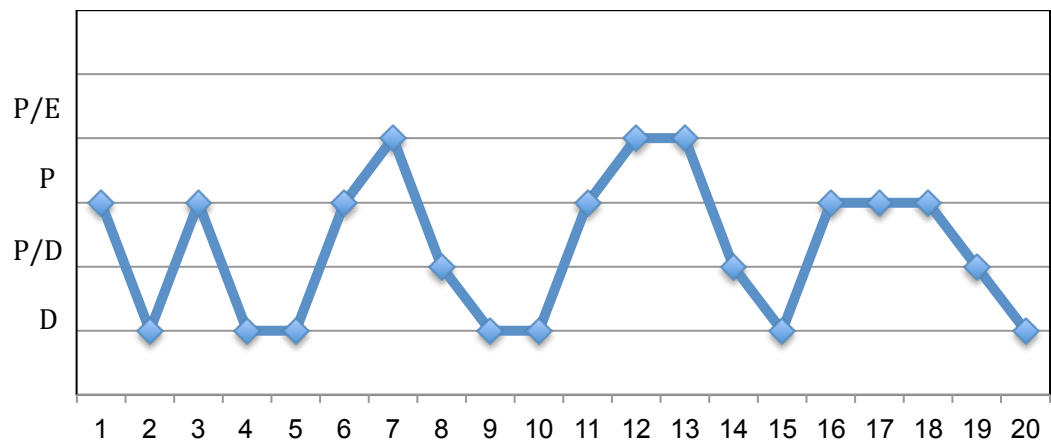


Figure 2.5: Female Estrous Cycling

Beginning at 2 months old, females were tested for 20 consecutive days to determine the Estrous stage on each day. Represented stages are as follows: D1: early diestrus, D: mid diestrus, D2: late diestrus, D2/P: diestrus and proestrus combined, P: proestrus, P/E: transitioning to estrus. A) Cycling pattern for ARF/ α GSUiCRE females. B) Cycling pattern for ARF control females. C) Average percentage of days spent in each stage by ARF/ α GSUiCRE (CRE+) and ARF (CRE-) females. N= 3 for each group. No significant difference detected.

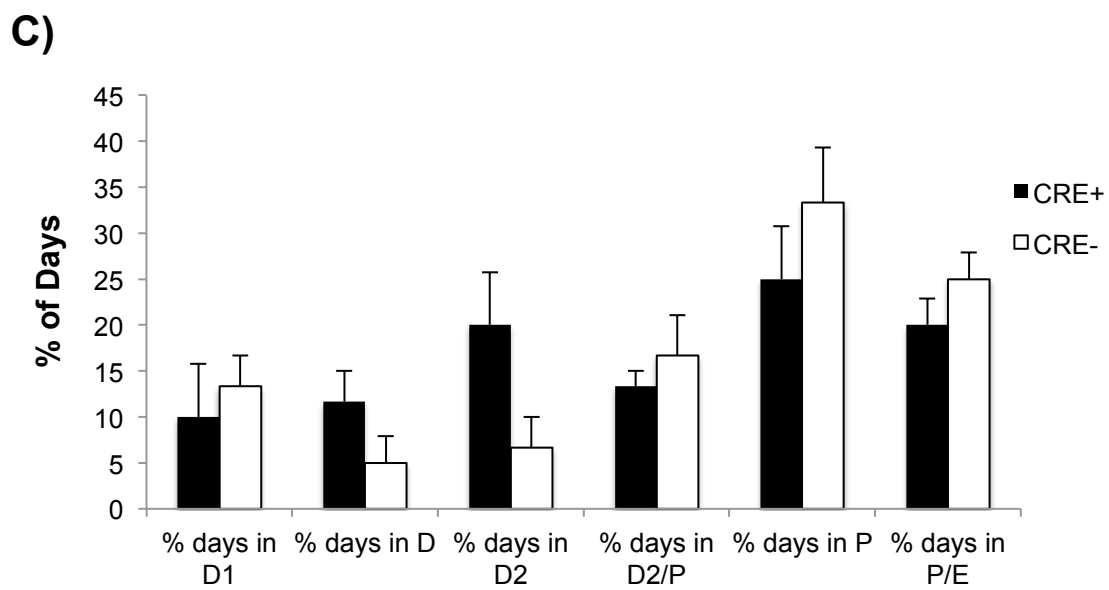


Figure 2.5: Female Estrous Cycling continued.

Table 2.1: Quantitative PCR primer sequences

Only forward sequences are listed.

GAPDH	TGCACCACCAACTGCTTAG
GnRHR	GCCCCTTGCTGTACAAAGC

CONCLUSION

The work presented in this paper encompasses our efforts to more precisely characterize the mechanism of AR-mediated androgen stimulation of the GnRH receptor in the pituitary, as well as the physiological relevance of this stimulation. Using the immortalized gonadotrope L β T2 cell line as the *in vitro* context for our studies, we confirmed the steroid-receptor-typical activation mechanism of AR, in which androgen binding to AR LBD is a requirement for its activation (i.e. release from heat shock proteins and stabilization through N/C interactions). Upon subsequent translocation to the nucleus AR dimerizes and binds to the receptor promoter through its DBD, which we also demonstrated the necessity of. We further demonstrated that leads to demonstrated that AR's induction of the GnRH receptor promoter AR binding occurs downstream of -600 bp, specifically at two hormone response elements. Each of the HREs on its own was not sufficient to carry out induction by AR. However, future studies are needed to determine whether the two HREs present together would be sufficient or whether other co-activators are also necessary.

To better understand the physiological role of AR action specifically in the pituitary we generated the novel pituitary-specific AR knockout mouse line ARF/ α GSUiCRE. As mentioned before, these mice were bred from female parents with 'floxed' AR alleles crossed with males expressing the CRE protein on the pituitary-specific α GSU promoter. In order to generate complete pituitary AR knockout females, we crossed the heterozygous ARF/ α GSUiCRE females

with hemizygous ARF males, and selected for the homozygous ARF/ α GSUiCRE females. Of note was our finding of a statistically significant 3-day delay in the pubertal onset of both males and female knockouts relative to controls. In addition, the 10-fold reduction in the GnRH receptor levels of the ARF/ α GSUiCRE male pituitaries further strengthens the hypothesis that the pituitary-specific AR-mediated actions of androgens are conveyed indirectly, through increasing gonadotrope sensitivity to GnRH. This *in vivo* reduction in pituitary GnRH levels in the absence of AR also confirmed the physiological relevance of our *in vitro* finding of AR-mediated androgen induction of the GnRH receptor promoter. While we were unable to detect a link between male fertility and/or female estrous cycling and pituitary-AR, further studies are needed to determine whether AR plays a partial role through its actions in the pituitary in coordinating those reproductive aspects, or whether it plays no role at all. Furthermore, while our study of male fertility showed no difference in sexual motivation and copulatory behavior, it is worthwhile to also explore potential differences in number of penetrated females who actually become pregnant, as well as differences in litter size and viability.

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